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(54)【発明の名称】分析法および分析に使用する試薬

(57)【要約】
本発明は、試験サンプルまたは試験サンプルのアリコート中の１つまたは複数の分析物の定量法および該方法で使用する試薬に関する。本発明の試薬は、定量すべき各分析物の少なくとも１つの型の特異的結合分子およびサンプルと試薬との混合の結果としてシグナルが変化する蛍光物質を含む。さらに、このシグナル変化を使用して、異なる凝集状態に分離することなく分析物の濃度を計算することができる。

【特許請求の範囲】
【請求項１】
複雑な生物流体の試験サンプルまたは複合体の試験サンプルのアリコート中の１つまたは複数の分析物の濃度を定量する方法において、
ａ）該サンプルまたは該サンプルのアリコートと固体、溶液、プレミックス溶液などの１つの試薬とを混合し、該試薬は１つのコンテナまたはコンテナの区画中に入れられ、該方法の実行中に他の試薬を添加せず、該試薬は１つまたは複数の該分析物と特異的親和性を有する少なくとも１つの型の結合分子を含み、さらに該試薬は該結合分子と共有結合する蛍光部分または該分析物（単数または複数）の蛍光アナログ、蛍光フラグメント、もしくは蛍光誘導体のいずれかを含み、
ｂ）該混合によって得られた混合物に偏光を照射して該蛍光分子を励起させ、
ｃ）放射光の偏光を測定し、
ｄ）該分析物（単数または複数）の濃度（単数または複数）を計算することを特徴とする定量法。
【請求項２】
ａ）前記分析物（単数または複数）に特異的親和性を示す抗体または抗体の免疫活性フラグメントと、
ｂ）前記分析物（単数または複数）の蛍光アナログもしくは蛍光フラグメントまたは蛍光誘導体との間に、
免疫複合体を含む各分析物用の試薬を使用することを特徴とする請求項１に記載の方法。
【請求項３】
ａ）前記分析物に特異的親和性を示すアプタマーまたは別の合成結合剤と、
ｂ）前記分析物（単数または複数）の蛍光アナログもしくは蛍光フラグメントまたは蛍光誘導体との間に、
複合体を含む各分析物用の試薬を使用することを特徴とする請求項１に記載の方法。
【請求項４】
１つまたは複数の前記分析物に特異的親和性を示す結合分子および前記結合分子に共有結合した６００ｎｍと１０００ｎｍとの間、好ましくは６２０ｎｍを超える吸収極大の蛍光部分を含む試薬を使用し、前記結合分子が任意選択的に組み合わせ化学技術、ファージディスプレイ、または核酸選択技術によって同定されるペプチドまたは合成結合剤であることを特徴とする請求項１に記載の方法。
【請求項５】
１つの分析物に特異的親和性を示す蛍光結合分子または１つの分析物のみの蛍光アナログ、蛍光フラグメント、もしくは蛍光誘導体を含む試薬を使用することを特徴とする請求項１乃至４のいずれか１項に記載の方法。
【請求項６】
異なる特異的親和性を示す異なる結合分子に共有結合した、異なる蛍光部分を含む試薬を使用することを特徴とする請求項１乃至５のいずれか１項に記載の方法。
【請求項７】
分析物に特異的結合親和性を示す１つまたは複数のペプチドまたはペプチド誘導体を含む試薬を使用し、該結合ペプチドが、共有結合した蛍光性残基を有し３０個未満のアミノ酸で構成されていることを特徴とする請求項１乃至６のいずれか１項に記載の方法。
【請求項８】
前記結合ペプチドが、２０個未満のアミノ酸で構成されていることを特徴とする請求項７に記載の方法。
【請求項９】
前記結合ペプチドが、１５個未満のアミノ酸で構成されていることを特徴とする請求項８に記載の方法。
【請求項１０】
Ｃ反応性タンパク質の定量のために、アミノ酸配列Ａｌａ－Ａｒｇ－Ａｓｎ－Ａｒｇ－Ａ

ｓｎまたはＡｌａ－Ａｒｇ－Ａｓｎ－Ｇｌｙ－Ａｓｎを含むペプチドまたはペプチド誘導体を含む試薬を使用することを特徴とする請求項１乃至９のいずれか１項に記載の方法。
【請求項１１】
最大吸光係数が、６４０ｎｍを超える波長の蛍光性残基を含む試薬を使用することを特徴とする請求項１乃至１０のいずれか１項に記載の方法。
【請求項１２】
細胞溶解物質または抗凝固薬または界面活性剤を含む試薬を使用することを特徴とする請求項１乃至１１のいずれか１項に記載の方法。
【請求項１３】
フルオレセイン、テキサスレッド、Ｃｙ５、他のＣｙ　Ｄｙｅ　ＦｌｕｏｒＬｉｎｋ物質、他のシアニン誘導体、ローダミン、メチルローダミン、Ｂｉｏｄｙｐｉ　６３０／６５０－Ｘ／ＭｅＯＨ、Ｂｉｏｄｙｐｉ　６５０／６５５－Ｘ／ＭｅＯＨ、Ｂｉｏｄｙｐｉ　ＦＬ／ＭｅＯＨ、Ｂｉｏｄｙｐｉ　Ｒ６Ｇ／ＭｅＯＨ、Ｂｉｏｄｙｐｉ　ＴＭＲ－Ｘ／ＭｅＯＨ　Ｂｉｏｄｙｐｉ　ＴＲ－Ｘ／ＭｅＯＨ、またはＢｉｏｄｙｐｙ群物質由来の他の物質、波長の異なるＡｌｅｘａ　Ｆｌｕｏｒ　Ｄｙｅ、ルテニウムリガンド複合体、ＤＴＰＡ、ＥＤＴＡ、またはＮ１などのキレートリガンドに結合させたユーロピウム、サマリウム、またはテルビウム複合体などのランタノイド元素からなる群から選択される１つまたは複数の蛍光部分を含む試薬を使用することを特徴とする請求項１乃至１２のいずれか１項に記載の方法。
【請求項１４】
放射光の偏光が、連続的な速度の読み取りもしくは１つまたは複数の測定点の間の放射光の偏光の変化の読み取りとしての時間の関数、または規定の測定点後の放射光の偏光の測定によって測定されることを特徴とする請求項１乃至１３のいずれか１項に記載の方法。
【請求項１５】
サンプル物質またはサンプル物質のアリコートが、生体物質または該生体物質の希釈物もしくは抽出物から構成されているか、該生体物質から溶解されているか濾過されていることを特徴とする請求項１乃至１４のいずれか１項に記載の方法。
【請求項１６】
サンプル物質または該サンプル物質のアリコートが、血液、血清、血漿、血球、または血液もしくは血球由来の溶解物、尿、脳脊髄液、涙液、痰、精液、原形質、胃腸管もしくは糞便から吸引した精液または物質、糞尿の懸濁液からの抽出物または濾過物、植物物質もしくはその抽出物、またはその植物溶解物もしくは濾過物から構成されていることを特徴とする請求項１乃至１５のいずれか１項に記載の方法。
【請求項１７】
既知濃度の分析物（単数または複数）を含む標準物質またはキャリブレータを使用し、さらに、未知のサンプル中の該分析物（単数または複数）の濃度（単数または複数）が、未知のサンプルから得た値を既知の標準物質またはキャリブレータから得た検量線に代入して計算されることを特徴とする請求項１乃至１６のいずれか１項に記載の方法。
【請求項１８】
任意選択的に蛍光偏光装置使用時に接続された人工メモリに格納された検量線を使用することを特徴とする請求項１乃至１７のいずれか１項に記載の方法。
【請求項１９】
標準物質および未知のサンプル、標準物質間もしくは未知のサンプル間の異なる測定時間での温度差による蛍光偏光の差異を補正するために、経験的または理論的に作成された温度修正アルゴリズムを使用することを特徴とする請求項１乃至１８のいずれか１項に記載の方法。
【請求項２０】
使用前に希釈または再構成される濃縮形態または乾燥形態の試薬であって、該試薬が、使用前に１つの試薬に組み合わされるために異なる区画に分割されていることを特徴とする請求項１乃至１９のいずれか１項に記載の方法。

【請求項２１】
１つまたは複数の分析物に特異的親和性を示す少なくとも１つの型の結合分子を含み、結合分子に共有結合した蛍光部分、分析物（単数または複数）の蛍光アナログ、蛍光フラグメント、または蛍光誘導体をさらに含むことを特徴とする、請求項１乃至２０のいずれか１項に記載の方法の実行に用いられる試薬。
【請求項２２】
ａ）少なくとも１つの分析物に特異的親和性を示す抗体、抗体の免疫活性フラグメント、アプタマー、または合成結合剤と、
ｂ）前記分析物（単数または複数）の蛍光アナログ、蛍光フラグメント、または蛍光誘導体との間に複合体を含むことを特徴とする請求項２１に記載の試薬。
【請求項２３】
１つまたは複数の前記分析物に特異的親和性を示す結合分子および任意選択的に前記結合分子に共有結合した６００ｎｍと１０００ｎｍとの間、好ましくは６２０ｎｍを超え、より好ましくは６４０ｎｍを超える吸収極大の蛍光部分を含む試薬であり、前記結合分子が任意選択的に組み合わせ化学技術、ファージディスプレイ、または核酸選択技術によって同定されるペプチド、アプタマー組成物、または合成結合剤であることを特徴とする請求項２１又は２２に記載の試薬。
【請求項２４】
アミノ酸配列が、Ａｌａ－Ａｒｇ－Ａｓｎ－Ａｒｇ－Ａｓｎおよび／またはＡｌａ－Ａｒｇ－Ａｓｎ－Ｇｌｙ－Ａｓｎを含む、前記結合剤の蛍光誘導体が含まれるペプチド結合剤またはペプチド誘導体の結合剤を含むアッセイ試薬であることを特徴とする請求項２１乃至２３のいずれか１項に記載の試薬。
【請求項２５】
必要とされる生きた生物由来の生体物質のサンプル中の臨床関連物質の濃度を測定するための請求項１乃至２０のいずれか１項に記載の方法の使用。
【請求項２６】
複雑な生物流体の試験サンプルまたは試験サンプルのアリコート中の１つまたは複数の分析物の濃度の定量用キットにおいて、１つまたは複数のコンテナを含み、該コンテナ（単数または複数）またはコンテナの区画（単数または複数）は、好ましくは液状の請求項２１乃至２４のいずれか１項に記載の１つの試薬を含み、該試薬は測定すべき分析物（単数または複数）に対する１つまたは複数の蛍光標識した特異的結合分子、または該分析物の蛍光標識アナログ、蛍光フラグメントもしくは蛍光誘導体を含み、試験すべき複雑な生物流体の正確な体積（単数または複数）を得るため該方法を適切に行うために必要なデバイスを同様にを含むことを特徴とするキット。
【請求項２７】
コンテナまたはコンテナの区画に含まれる試薬が、分析前、分析直前、または分析と同時に異なるコンテナの内容物と混合されることによって利用可能な状態の試薬に形成することを特徴とする請求項２６に記載のキット。
【発明の詳細な説明】
【０００１】
（技術分野）
本発明は、試験サンプルまたは試験サンプルのアリコート中の１つまたは複数の分析物の定量法および該方法で使用する試薬に関する。
【０００２】
（背景技術）
「分析物（単数または複数）」は、試験サンプル材料中での定性または定量分析が望ましい物質の総称である。分析物は、一般に、十分に定義された分子であるが、互いに類似しているか同一の機能を有する分子の集団であってもよい。特定の材料（例えば、脂肪酸結合タンパク質のクラスターまたは血球のクラス）もまた、分析物ということができる。
【０００３】

試験材料中の分析物の化学的定量分析もまた、しばしば濃度定量という。化学的定性分析を使用して濃度定量を行うことにより、濃度定量から試験材料中の分析物の濃度が所与の値より高いか低いかという情報を得ることもできる。

【０００４】

複雑な材料中の非常に低濃度の分析物の化学的濃度定量法は、Ｒ．Ｓ．Ｙａｌｏｗ、Ｓ．Ａ．Ｂｅｒｓｏｎ ａｎｄ ａｓｓｏｃｉａｔｅｓが免疫化学的測定法として後に知られる方法を開発後、非常に急速に開発された。これらの方法は、所与の動物の免疫系が、その動物に対する外来物として認知する材料への暴露に対する特異的な免疫応答としてほとんどの場合、脊椎動物の個体が免疫グロブリンの形態の抗体を産生するという原理を使用している。この認識に到達するまでの古典的で確固とした研究は、Ｓ．Ａ．Ｂｅｒｓｏｎ、Ｒ．Ｓ．Ｙａｌｏｗ、Ａ．Ｂａｕｍａｎ、Ｍ．Ａ．Ｒｏｔｈｓｃｈｉｌｄ、ａｎｄ Ｋ．Ｎｅｗｅｒｌｙによる文献「Ｉｎｓｕｌｉｎ－Ｉ１３１ Ｍｅｔａｂｏｌｉｓｍ ｉｎ Ｈｕｍａｎ Ｓｕｂｊｅｃｔｓ：Ｄｅｍｏｎｓｔｒａｔｉｏｎ ｏｆ Ｉｎｓｕｌｉｎ Ｂｉｎｄｉｎｇ Ｇｌｏｂｕｌｉｎ ｉｎ ｔｈｅ Ｃｉｒｃｕｌａｔｉｏｎ ｏｆ Ｉｎｓｕｌｉｎ－Ｔｒｅａｔｅｄ Ｓｕｂｊｅｃｔｓ（ヒト被験体におけるインスリンＩ１３１代謝：インスリン処理被験体の代謝におけるインスリン結合グロブリンの証明）」、Ｊ．Ｃｌｉｎ．Ｉｎｖｅｓｔ．３５巻、１９５６、１７０～１９０頁に公開されている。精製形態または他の物質との混合物の形態（例えば、血清の形態）のこれらのグロブリン（後に抗体とする）の使用によって、多数の分析物の特異的同定法または定量法が開発された。これら多数の分析物についての実用的な測定法は以前には存在していなかった。したがって、血液タンパク質ならびに体液および組織由来のタンパク質の範囲の特異定測定法を開発し、その後非タンパク質物質についての測定法も開発した。早い段階でホルモン分析を開発した。その一般的方法は「免疫アッセイ」と名づけられ、分析の特異性が免疫応答の結果であることを基本とする試薬に基づく。これは多数のテキスト（例えば、「Ｐｒｉｎｃｉｐｌｅｓ ａｎｄ Ｐｒａｃｔｉｃｅ ｏｆ Ｉｍｍｕｎｏａｓｓａｙ（免疫アッセイの原理と実践）」、２版、Ｃｈｒｉｓｔｏｐｈｅｒ Ｐ．Ｐｒｉｃｅ ａｎｄ Ｄａｖｉｄ Ｊ．Ｎｅｗｍａｎ編、ＩＳＢＮ：１５６１５９１７５０、Ｇｒｏｖｅｓ Ｄｉｃｔｉｏｎａｒｉｅｓ，Ｉｎｃ．、８月１９９８）で十分に説明されている。

【０００５】

これらの試薬を使用して定量分析される試験材料を、複雑な生体材料と説明することができる。典型的な試験材料は、血液、血清、血漿、尿、糞便、糞便抽出物、および脳脊髄液である。分析物は、通常、試験材料の非常に小さな部分から構成される。

【０００６】

化学分析を行う試験材料は、異なる凝集状態（例えば、気体、液体、固体、または異なる凝集状態の混合物）であり得る。定量分析を行う試験材料（すなわち分析物）と混合する試薬も同様に異なる凝集状態で存在し得るが、典型的には、溶液または固体物質および／または固体物質と流体との組み合わせからなる。試薬を試験材料と混合すると、ほとんどが（しかし、全てではない）流動体における物質の溶液を形成する。この溶液は、「アッセイ溶液」という総称を使用し、これは、試験材料および定量分析に必要な化学薬品を含む。

【０００７】

免疫アッセイの発明の初期は、抗体の混合に使用された供給源は免疫化動物由来の血清である抗血清であり、これは他の血清タンパク質も含まれ、抗体は凝集血清タンパク質のより小さな部分から構成されている。今日まで、大量の抗血清形態の抗体が販売されている（例えば、Ｃｈｅｍｉｃｏｎ Ｉｎｃ．，Ｃａｌｉｆｏｒｎｉａなどの業者のカタログを参照のこと）。しかし、精製免疫グロブリンクラスの形態の抗体（最も一般的には免疫グロブリンＧ型）は早い時期に日常的に導入されており、これは今日最も一般的に使用されている抗体試薬である（例えば、Ｄａｋｏ ＡＳ，Ｄｅｎｍａｒｋの製品カタログを参照のこと）。

【０００８】

動物が外来物質（抗原と呼ばれる）に暴露されたとき（いわゆるワクチン接種または免疫化と呼ばれる形態での活性な標的化された暴露がしばしば使用される）に引き起こされる抗体の産生は、身体が産生する異なる構造の抗体におけるすべての範囲の異なる細胞として特徴づけられるが、これらは目的の抗原に結合する（親和性を示す）という共通の特徴を有する。しかし、これらの同一の抗原に結合する異なる抗体は、結合強度および結合速度が異なり、長期間多数の動物を使用して抗体を産生させた場合一定の質に保つのは困難である。

【０００９】

したがって、Ｋｏｈｌｅｒ ａｎｄ Ｍｉｌｓｔｅｉｎが１９７４年に再生可能な化学構造および均一で一定の結合性を有する標準化抗体を作製可能な、モノクローナル抗体作製法を開発し、この方法は非常に進歩した。これらの方法は、書籍（「Ｍｏｎｏｃｌｏｎａｌ Ａｎｔｉｂｏｄｉｅｓ：Ｐｒｏｄｕｃｔｉｏｎ，Ｅｎｇｉｎｅｅｒｉｎｇ ａｎｄ Ｃｌｉｎｉｃａｌ Ａｐｐｌｉｃａｔｉｏｎ（モノクローナル抗体：産生、操作、および臨床への応用）」、Ｍａｒｙ Ａ．Ｒｉｔｔｅｒ ａｎｄ Ｈｅａｔｈｅｒ Ｍ．Ｌａｄｙｍａｎ編、ＩＳＢＮ：０５２１４２５０３４、Ｃａｍｂｒｉｄｇｅ Ｕｎｉｖｅｒｓｉｔｙ Ｐｒｅｓｓ、２月１９９５で十分に説明されている。この書籍はまた、どのようにして抗体を断片化し、これらの結合特性を有する抗体のフラグメントを、全抗体を使用する代わりに使用するのかということを説明している。

【００１０】

モノクローナル抗体の発明から数年後、現代生物工学を使用して、所望の結合特性を有する結合タンパク質およびタンパク質フラグメント（ペプチドとも呼ばれる）の新規の作製法が開発された。特異的結合特性を有する試薬の作製のためのペプチド構造の作製、暴露、および選択にウイルス技術を使用するファージディスプレイ法の開発によって実質的に進歩した。

【００１１】

Ｃｏｌｌｉｎｓ Ｊ．ａｎｄ Ｒｏｔｔｇｅｎ Ｐ．による、１９９４；「Ｈｙｐｅｒｖａｒｉａｂｌｅ ｐｈａｇｅｍｉｄ ｄｉｓｐｌａｙ ｇｅｎｅ ｂａｎｋｓ ｆｏｒ ｔｈｅ ｓｅｌｅｃｔｉｏｎ ｏｆ ｓｔｒｏｎｇｌｙ ｂｉｎｄｉｎｇ ｌｉｇａｎｄｓ，ｉｎｃｌｕｄｉｎｇ ｔｈｅｉｒ ｕｓｅ ｆｏｒ ｔｈｅ ｉｓｏｌａｔｉｏｎ ｏｆ ｓｅｒｉｎｅ ｐｒｏｔｅａｓｅ ｉｎｈｉｂｉｔｏｒｓ（セリンプロテアーゼインヒビターの単離での使用を含む、強力な結合リガンドの選択用の超可変ファージミドディスプレイ遺伝子バンク）」；１９９９年７月２０日に付与された米国特許第５９２５５５９号「Ｐｈａｇｅｍｉｄｓ ａｎｄ ｐｒｏｃｅｓｓ ｏｆ ｐｒｅｐａｒａｔｉｏｎ（ファージミドおよびその作製法）」を取得した欧州特許出願１９９４ ０００ １０８ ６８９（４月１９９４）、およびＣｏｌｌｉｎｓ，Ｊ．、Ｒｏｔｔｇｅｎによる、１９９７；「Ｃｏｓｍｉｘ－ｐｌｅｘｉｎｇ ａ ｍｅｔｈｏｄ ｆｏｒ ｒｅｃｏｍｂｉｎａｔｉｏｎ．．．（・・・組換え法のコスミックスプレキシング）」、Ｃｏｓｍｉｘ ＧｍｂＨがＰＣＴ／ＥＰ９８／００５３３（２月２日、１９９８）およびＷＯ９８ ３３９０１（８月６日、１９９８）として提出したＥＰ９７ １０１ ５３９．１（１月３１日、１９９７）に記載のように、抗体産生細胞の一部の遺伝子配列を組み込み、ファージディスプレイ粒子中で意図的に変異または配列を変更して、ファージディスプレイ法をさらに発展させた。

【００１２】

さらに、免疫アッセイ様方法における特異的結合分子として核酸配列（アプタマー）が使用され始めていたが、おそらく用語的にこれらを免疫アッセイとすることはあまり正確ではないであろう。米国特許ＵＳ０５５６７５８８「Ｓｙｓｔｅｍａｔｉｃ ｅｖｏｌｕｔｉｏｎ ｏｆ ｌｉｇａｎｄｓ ｂｙ ｅｘｐｏｎｅｎｔｉａｌ ｅｎｒｉｃｈｍｅｎｔ：Ｓｏｌｕｔｉｏｎ ＳＥＬＥＸ（指数関数的富化によるリガンドの体系的発展：ソリューションＳＥＬＥＸ）」は、このアプタマー技術を記載している。最終的に、より小さなエレメントの体系的組み合わせ（いわゆる組み合わせライブラリー）を使用した分子の巨

大なライブラリーの合成を開始した時、これらの結合アッセイ周辺の生物学的枠組みの範囲を完全に超えた。これらのエレメントは、部分的または全体的に、アミノ酸鎖（ペプチドと呼ばれる）からなるが、他の結合ブロックからも構成され得る。この主題の概要は、Ｊ．Ｂｕｒｂａｕｍらによる文献「Ａ ｐａｒａｄｉｇｍ ｆｏｒ ｄｒｕｇ ｄｉｓｃｏｖｅｒｙ ｅｍｐｌｏｙｉｎｇ ｃｏｍｂｉｎａｔｏｒｉａｌ ｌｉｂｒａｒｉｅｓ（組み合わせライブラリーを使用した薬物発見のパラダイム）」、Ｐｒｏｃ．Ｎａｔｌ．Ａｃａｄ．Ｓｃｉｅｎｔ．ＵＳＡ、９９巻、６０２７～６０３１頁、１９９５に見出される。

【００１３】

したがって、今日、分子量が高いまたは低く、化学的性質が異なり（結合強度が異なる）、均一または不均一な構造の結合分子の作製法の非常に複雑な生物学的および合成による回収が存在する。この特許出願では、これらの分子を総称して「結合分子」というが、これらは特定の構造に結合するので、特異的結合分子と呼ばれる。結合分子は、上記のようにペプチドの性質、核酸の性質、または別の化学的性質の構造を変化させることができ、勿論これらは異なる結合特異性について異なる構造を有する。さらに、これらは特定の構造に結合する異なる型のシグナル発生残基（例えば、異なる型の蛍光性残基）を有し得る。本特許出願では、異なる型の結合分子は異なる型の構造（ペプチド、核酸、または他の構造）、または１つの構造型のうちの異なる組成物（例えば、異なる型の結合特性が得られるペプチド構造内の異なるアミノ酸配列）を意味する異なる型ならびにシグナル発生残基の異なる型（例えば、異なる型の蛍光性、つまり、異なる励起または放射波長などを有する蛍光性残基）の異なる型を使用することができる。

【００１４】

複雑なサンプル溶液中の物質の定量に使用する上記の特異的結合分子は、一般に、サンプルに存在し得る他の物質よりも、定量すべき物質、前記物質のアナログ、フラグメント、または誘導体により高い親和性を示す。典型的には、モノクローナル抗体は、抗体を産生する場合、試験溶液中の他の物質よりも免疫化および選択に使用する物質に高い親和性を示すであろう。特異的結合分子として抗体を使用する場合、抗体が高い親和性を示す物質を抗原またはハプテン（後者は、より大きい分子におけるより小さな構造である場合にしばしば使用される）と呼ぶ。特異的結合分子が抗体ではない場合、用語「抗原」よりも用語「リガンド」を使用する。本特許出願では、特異的結合反応において互いに結合する分子の総称として用語「結合対」を使用し、結合対を構成する各分子を結合パートナーという。

【００１５】

一般に、本発明者らは、形成可能な２つの結合パートナーおよび結合対との間の反応について以下の式を定めることができる。

Ｂ１＋Ｂ２＝ＢＢ

（式中、Ｂ１は結合パートナー１を記号化したものであり、Ｂ２は結合パートナー２を記号化したものであり、ＢＢは形成された結合対を記号化したものである）。

【００１６】

さらに、形成された遊離の結合パートナーと結合対との間のこの式は、Ｓｔｅｖｅｎ Ｓ．Ｚｕｍｄａｈｌによる書籍「Ｃｈｅｍｉｃａｌ Ｐｒｉｎｃｉｐｌｅｓ（化学的原理）」、３版、１９２～１９３頁、ＩＳＢＮ ０－３９５－８３９９５－５に記載の質量作用の法則（Ｇｕｌｄｂｅｒｇ ａｎｄ Ｗａａｇｅ、１８６４）に関連する平衡定数ｋ（結合平衡定数と呼ばれる場合もある）によって特徴づけられる。

$$k = \frac{[BB]}{[B1]*[B2]}$$

このように示した定数はしばしば結合平衡定数と呼ばれ、この逆の形態は解離平衡定数と呼ばれる。

【００１７】

これらの結合分子の分析物への化学結合の形成を測定するために、結合パートナーの１つの放射性標識を最初に使用した。最初に、分析物と化学的に同一であるかほぼ同一の既知の量の放射性標識結合パートナーＢ１またはＢ２を添加し、この放射性標識物質の、使用した結合分子（初めは完全な免疫グログリン）における結合について、分析物と競合する能力を測定する「Ｉｍｍｕｎｏａｓｓａｙ ｏｆ Ｐｒｏｔｅｉｎ Ｈｏｒｍｏｎｅｓ（タンパク質ホルモンの免疫アッセイ） Ｔｈｅ Ｈｏｒｍｏｎｅｓ：Ｐｈｙｓｉｏｌｏｇｙ，Ｃｈｅｍｉｓｔｒｙ，ａｎｄ Ａｐｐｌｉｃａｔｉｏｎｓ、４巻、５５７～６３０頁、（Ｇ．Ｐｉｎｃｕｓら編、Ａｃａｄｅｍｉｃ Ｐｒｅｓｓ、１９６４）を参照のこと。

【００１８】

このいわゆる競合アッセイ法では、低濃度の結合分子について分析物分子をこのように標識アナログ分子と競合させた。その後、一方は分析物を単離する（例えば、分析物分子を固相に結合する）ための抗体であり、一方は測定用のシグナルを発生させるための放射性標識抗体という、最もよく使用される異なる２つの抗体を使用する、過剰量の抗体を用いた非競合法が開発された。一般的な概要は、Ｌａｗ Ｂｒｉａｎによる書籍「Ｉｍｍｕｎｏａｓｓａｙ：Ａ Ｐｒａｃｔｉｃａｌ Ｇｕｉｄｅ（免疫アッセイ：解説書）」、ＩＳＢＮ：０７４８４０５６０７に記載されている。

【００１９】

競合法と免疫測定法との共通点は、測定したシグナルを分析物濃度に対し較正および相関させるための既知量の標準化試験溶液の使用および、いわゆる検量線または較正曲線の作成であった。未知の濃度の分析物を使用した試験にはこの検量線への代入によって定量した。

【００２０】

放射性標識法は、未だに最も頻繁に使用されているが、最初の意図は分析物アナログまたは結合分子の存在を測定する非放射性法を見出すことであった。シグナル発生分子としての酵素の使用によって、放射能を回避し、試薬をより持続させ、より簡単な測定装置（典型的には、光吸収分光光度計）を使用することができるなどの大きな進歩を遂げていた。これに関する概要は、Ｐ．Ｔｉｊｓｓｅｎによる「Ｐｒａｃｔｉｃｅ ａｎｄ ｔｈｅｏｒｙ ｏｆ ｅｎｚｙｍｅ ｉｍｍｕｎｏａｓｓａｙｓ（酵素免疫アッセイの実践と理論）」、ＩＳＢＮ ０ ４４４ ８０６ ３３４に記載されている。蛍光分子をシグナル発生分子として使用することにより別の大きな進歩を遂げた。これにより、純粋な吸光度測定法と比較して感度が改良された。書籍「Ｅｎｚｙｍｅ ａｎｄ Ｆｌｕｏｒｅｓｃｅｎｃｅ Ｉｍｍｕｎｏａｓｓａｙｓ：Ｔｅｎｔａｔｉｖｅ Ｇｕｉｄｅｌｉｎｅ（酵素および蛍光免疫アッセイ：試験的ガイドライン）」、６巻、Ｎａｔｉｏｎａｌ Ｃｏｍｍｉｔｔｅｅ Ｆｏｒ Ｃｌｉｎｉｃａｌ Ｌａｂｏｒａｔｏｒｙ Ｓｔａｎｄａｒｄｓ、ＩＳＢＮ：１５６２３８０６７２、７月、１９８６に、これに関する適切な概要が記載されている。

【００２１】

Ｋｎｏｘ ａｎｄ Ｒｉｃｈａｒｄ ｖａｎ Ｄｙｋｅによる「Ｌｕｍｉｎｅｓｃｅｎｃｅ Ｉｍｍｕｎｏａｓｓａｙ ａｎｄ Ｍｏｌｅｃｕｌｅｓ Ａｐｐｌｉｃａｔｉｏｎｓ（発光免疫アッセイおよび分子の応用）」、ＣＲＣ Ｐｒｅｓｓ、１月、１９９０、ＩＳ

ＢＮ　０８４９３５８６５５に記載のように、化学発光法では感度がさらに高まった。

【００２２】

ほとんどの型のシグナル発生残基（放射性、蛍光、および酵素物質）の使用には、結合分子に結合していない残基から、特異的結合分子に結合している分子に結合しているシグナル発生残基を分離する必要がある。典型的には、これは、より大きな結合分子に結合している分析物分子から、非結合の小さな分子を持つ分析物分子を分離することができる、固定した結合分子を含むプラスチック表面、ガラス表面、多孔質フィルター、もしくは粒子ベースの基質、またはより非特異的な媒体、（例えば、活性石炭など）であり得る。この分野の一般的で適切な概要を、書籍「Ｐｒｉｎｃｉｐｌｅｓ　ａｎｄ　Ｐｒａｃｔｉｃｅ　ｏｆ　Ｉｍｍｕｎｏａｓｓａｙ（免疫アッセイの原理と実践）」、Ｃｈｒｉｓｔｏｐｈｅｒ　Ｐ．　Ｐｒｉｃｅ　ａｎｄ　Ｄａｖｉｄ　Ｊ．Ｎｅｗｍａｎ編、２版、Ｇｏｖｅｓ　Ｄｉｃｔｉｏｎａｒｉｅｓ　Ｉｎｃ．、８月１９９８、ＩＳＢＮ１５６１５９１７５０に見出すことができる。

【００２３】

抗体自体が分析物を沈殿させることができ、これをいかなるシグナル発生物質結合残基を使用することなく、分析物の定量に使用することができるということが初期に認められていた。抗体沈殿を補助的に使用した濃度定量は感度が低いが、洗浄工程を伴う固体分離相を使用する必要が無いので非常に実用的であり、これにより実行が簡素化できる。ゲル中および直接に液体中での沈殿分析（Ｋｉｌｌｉｎｇｓｗｏｒｔｈら、「Ｎｅｐｈｅｌｍｅｔｒｉｃ　Ｓｔｕｄｉｅｓ　ｏｆ　ｔｈｅ　Ｐｒｅｃｉｐｉｔｉｎ　Ｒｅａｃｔｉｏｎ：　Ａ　Ｍｏｄｅｌ　Ｓｙｓｔｅｍ　ｆｏｒ　Ｓｐｅｃｉｆｉｃ　Ｐｒｏｔｅｉｎ　Ｍｅａｓｕｒｅｍｅｎｔｓ（沈殿反応の比濁分析：特異的タンパク質測定のモデルシステム）」、Ｃｌｉｎ．Ｃｈｅｍ．、１９（４）巻、４０３～４０７頁、１９７３）により、免疫アッセイがさらに広く使用され、Ｈｉｔａｔｃｈｉ　ｉｎｓｔｒｕｍｅｎｔｓなどの自動分光光度測定装置および、例えばＤａｄｅ　Ｂｅｈｒｉｎｇ　ｃｏｍｐａｎｙから供給されている自動化比濁計などで高度に自動化された。感度の制限に加えて、沈殿分析法でモノクローナル抗体を使用することは困難である。モノクローナル抗体はしばしば分析分子の１つの領域上のみで結合するが、ポリクローナル抗体は通常分析物分子上のいくつかの領域で結合するので、容易に沈殿する抗体および分析物分子が容易により広範に凝集すると推測される。したがって、本発明と対照的に、上記の方法は免疫複合体を含まず、定量のために蛍光を使用せず、蛍光偏光アッセイではなく、感度が低い。

【００２４】

いわゆるＢｉａＣｏｒｅ　ｉｎｓｔｒｕｍｅｎｔｓおよびプラスモン共鳴（Ｊ．Ｍｅｌｅｎｄｅｚ、Ｒ．Ｃａｒｒ、Ｄ．Ｕ．Ｂａｒｔｈｏｌｏｍｅｗ、Ｋ．Ｋｕｋａｎｓｋｉｓ、Ｊ．Ｅｌｋｉｎｄ、Ｓ．Ｙｅｅ、Ｃ．Ｆｕｒｌｏｎｇ、Ｒ．Ｗｏｏｄｂｕｒｙ、Ａ　ｃｏｍｍｅｒｃｉａｌ　ｓｏｌｕｔｉｏｎ　ｆｏｒ　ｓｕｒｆａｃｅ　ｐｌａｓｍｏｎ　ｓｅｎｓｉｎｇ，Ｓｅｎｓｏｒｓ　ａｎｄ　Ａｃｔｕａｔｏｒｓ－（表面プラスモン検出のための市販の溶液、感知器および作動装置）Ｂ３５、１９９６年、１～５）に基づく他の技術は、シグナル発生残基を使用しない分析物の直接測定が可能であることをさらに示した。しかし、これらの方法は、さらに感度の低い比濁法と同一の単純な技術的方法である。その上プラスモン共鳴装置は、ほとんどが非常に高い装置である。

【００２５】

分離デバイスおよび固相を使用せず、基質および洗浄液も使用しないで結合分子への分析物の結合を測定する必要のため、いわゆるプロキミティアッセイが得られた。

【００２６】

ＳＹＶＡという企業は１９７４年に抗体への結合により酵素活性に対して直接的な効果が得られ、分離および洗浄溶液を使用することなく測定することができる、抗体への結合用の分析物分子と酵素標識分析物アナログとの間の競合に基づく、小分子用の免疫アッセイ技術に着手した。（Ｋｅｎｎｅｔｈ　Ｅ．Ｒｕｂｅｎｓｔｅｉｎ　ａｎｄ　Ｅｄｗｉｎ　Ｆ．Ｕｌｌｍａｎに付与された米国特許ＵＳ３８５２１５７「Ｃｏｍｐｏｕｎｄｓ　ｆｏ

ｒ ｅｎｚｙｍｅ ａｍｐｌｉｆｉｃａｔｉｏｎ ａｓｓａｙ（酵素増幅アッセイ用化合物）」）。蛍光偏光免疫アッセイ（ＦＰＩＡｓ）が１９７０年代の終わりには導入されていた（概要文献「Ｆｌｕｏｒｅｓｃｅｎｃｅ ｐｏｌａｒｉｚａｔｉｏｎ ｉｎ ｉｍｍｕｎｏｃｈｅｍｉｓｔｒｙ（免疫化学における蛍光偏光）」、Ｄａｎｄｌｉｋｅｒ ａｎｄ Ｓａｕｓｓｕｒｅ ｉｎ Ｉｍｍｕｎｏｃｈｅｍｉｓｔｒｙ、７巻、７９９～８２８頁、１９７０を参照のこと）。この方法はまた、小分子分析物を使用すると最もうまくいくが、小分子蛍光標識分析物アナログ競合の使用により、タンパク質などの大分子分析物の定量法の使用が可能となった（Ａｉ－Ｐｅｎｇ Ｗｅｉ ａｎｄ Ｊａｍｅｓ Ｈｅｒｏｎ：Ｕｓｅ ｏｆ ｓｙｎｔｈｅｔｉｃ ｐｅｐｔｉｄｅｓ ａｓ ｔｒａｃｅｒ ａｎｔｉｇｅｎｓ ｉｎ ｆｌｕｏｒｅｓｃｅｎｃｅ ｐｏｌａｒｉｚａｔｉｏｎ ｉｍｍｕｎｏａｓｓａｙｓ ｏｆ ｈｉｇｈ ｍｏｌｅｃｕｌａｒ ｗｅｉｇｈｔ ａｎａｌｙｔｅｓ（高分子量分析物の蛍光偏光免疫アッセイにおけるトレーサー抗原としての合成ペプチドの使用）、Ａｎａｌ．Ｃｈｅｍ．、１９９３、６５巻、３３７２～３３７７頁）。いくつかのタンパク質もまた、比較的可動性を示すサブユニットを有し、直接蛍光偏光免疫アッセイ測定が可能となる（１９９０年にＢｅｎｎｅｔ ａｎｄ Ｃｈｉａｐｅｔｔａに付与された米国特許第４，９０２，６３０号「Ｆｌｕｏｒｅｓｃｅｎｃｅ ｐｏｌａｒｉｚａｔｉｏｎ ｉｍｍｕｎｏａｓｓａｙ ａｎｄ ｒｅａｇｅｎｔ ｆｏｒ ｍｅａｓｕｒｅｍｅｎｔ ｏｆ Ｃ－ｒｅａｃｔｉｖｅ ｐｒｏｔｅｉｎ（蛍光偏光免疫アッセイおよびＣ反応性タンパク質の測定用試薬）」）。Ｔｅｒｐｅｔｓｃｈｎｉｇ，Ｅ．らの「Ｆｌｕｏｒｅｓｃｅｎｃｅ ｐｏｌａｒｉｓａｔｉｏｎ ｉｍｍｕｎｏａｓｓａｙ ｏｆ ａ ｈｉｇｈ－ｍｏｌｅｃｕｌａｒ－ｗｅｉｇｈｔ ａｎｔｉｇｅｎ ｂａｓｅｄ ｏｎ ａ ｌｏｎｇ－ｌｉｆｅｔｉｍｅ Ｒｕ－ｌｉｇａｎｄ ｃｏｍｐｌｅｘ（寿命の長いＲｕ－リガンド複合体ベースの高分子量抗原の蛍光偏光免疫アッセイ）」、Ａｎａｌ．Ｂｉｏｃｈｅｍ．、２２７巻、１４０～４７頁、１９９５および「Ｍｅｔａｌ ｌｉｇａｎｄ ｃｏｍｐｌｅｘｅｓ ａｓ ａ ｎｅｗ ｃｌａｓｓ ｏｆ ｌｏｎｇ－ｌｉｖｅｄ ｆｌｕｏｒｏｐｈｏｒｅｓ ｆｏｒ ｐｒｏｔｅｉｎ ｈｙｄｒｏｄｙｎａｍｉｃｓ（タンパク質水力学用の新規のクラスの寿命の長い蛍光物質としての金属リガンド複合体）」、Ｂｉｏｐｈｙｓ．Ｊ．、６８巻、３４２頁、１９９５は、さらにどのようにして非対称ルテニウム複合体を高分子量の分析物の蛍光偏光測定に使用することができるのかについて説明している。しかし、これらをヘモグロビンまたは高濃度のビリルビンの存在下で使用することはできない。

【００２７】

ほとんどの蛍光偏光免疫アッセイは、分析物の蛍光標識アナログを添加し、典型的には抗体の形態の特異的結合分子について試験溶液中で分析物分子と競合させる競合法に基づいている。このようなアッセイではしばしば比較的高い凝集体濃度の抗体を使用する必要あり、競合が予想される濃度でさえも競合は起こらない。したがって、この種の市販のアッセイのほとんどは、抗体の凝集体濃度が非常に高く、比較的効果の低い結合分子濃度で競合することが明らかである。これについての体系的論文は存在しないが、Ｍｏｈａｍｍｅｄ Ｔ．Ｓｈｉｐｃｈａｎｄｌｅｒ ａｎｄ Ｅｄｗｉｎ Ｇ．Ｍｏｏｒｅによる文献「Ｒａｐｉｄ，ｆｕｌｌｙ ａｕｔｏｍａｔｅｄ ｍｅａｓｕｒｅｍｅｎｔ ｏｆ ｐｌａｓｍａ ｈｏｍｏｃｙｓｔｅｉｎｅ ｗｉｔｈ ｔｈｅ Ａｂｂｏｔｔ Ｉｍｘ Ａｎａｌｙｚｅｒ（Ａｂｂｏｔｔ Ｉｍｘ分析器を使用した迅速で完全に自動化された血漿ホモシステインの測定）」、を参考にすることができ、類似の分析により他の市販の競合蛍光偏光免疫アッセイが比較的高い凝集体抗体濃度に基づくことを示すこともできる。

【００２８】

１９８０年代半ばにはＡｍｅｒｓｈａｍという企業が、結合パートナーから放出された放射性β線により問題の結合対における他の結合パートナーに結合した蛍光粒子から蛍光が放射されるｓｃｉｎｔｉｌｌａｔｉｏｎ ｐｒｏｘｉｍｉｔｙ ａｓｓａｙｓ（シンチレーションプロキシミティアッセイ）（米国特許第４，５６８，６４９号、欧州特許第０，１５４，７３４号）を既に導入していた。この技術は、研究室環境での治療および他の特

異的結合剤の研究で広範に使用されているが、実践的な臨床診断には使用されていない。

【００２９】

１９７６年には、Ｕｌｌｍａｎ ａｎｄ Ｓｃｈｗａｒｚｂｅｒｇに付与された米国特許第３，９９６，３４５号、「Ｆｌｕｏｒｅｓｃｅｎｃｅ ｑｕｅｎｃｈｉｎｇ ｗｉｔｈ ｉｍｍｕｎｏｌｏｇｉｃａｌ ｐａｉｒｓ ｉｎ ｉｍｍｕｎｏａｓｓａｙｓ（免疫アッセイにおける免疫対を用いた蛍光消光法）」が既に公開されていた。これにより、分離および洗浄工程を経ずに蛍光プロキシミティーアッセイを行うことができるが、最新式の蛍光光度計が依然として必要であり、消光技術は日常的に臨床で使用される技術に対する競争力は無い。１９９８年に、Ｂｕｅｃｈｌｅｒらは、米国特許第５，７６３，１８９号で主に結合分子および異なる波長の光と相互作用する異なる分子を含む進歩した粒子を基本とした「Ｆｌｕｏｒｅｓｃｅｎｃｅ ｅｎｅｒｇｙ ｔｒａｎｓｆｅｒ ａｎｄ ｉｎｔｒａｍｏｌｅｃｕｌａｒ ｅｎｅｒｇｙ ｔｒａｎｓｆｅｒ ｉｎ ｐａｒｔｉｃｌｅｓ ｕｓｉｎｇ ｎｏｖｅｌ ｃｏｍｐｏｕｎｄｓ（新規の化合物を使用した粒子内の蛍光エネルギー移動および分子内エネルギー移動）」を公開した。そのような粒子に関連して、かなりの開発コストまたは作製コストが必要である。これは均一系免疫アッセイではなく、このようなアッセイの作製、輸送、および実行において固相で処理する必要がある。

【００３０】

上記の全ての技術の共通点は、これらの使用試薬を、通常ピペッティング装置を使用していくつかの試薬コンテナから添加することであり、これには特別に訓練された者が必要であることである。公共医療においては、いくつかの試薬コンテナおよび専門的なピペッティング装置および専門家に相当な費用が必要である。

【００３１】

さらに、いくつかの発明は、試験サンプル中の有機化合物の検出または定量法およびアッセイに関する。

【００３２】

ＷＯ ００／１６０９９（Ｗｏｌｆ）には、サンプル中の炭水化物の検出または定量に使用することができる結合価減少炭水化物結合リガンド（ＣＢＬｓ）が記載されている。ＣＢＬを蛍光共鳴エネルギー移動（ＦＲＥＴ）とともに使用し、例えばプロキシミティーベースのシグナル発生標識部分の使用によって、遊離の炭水化物または化合物を含む炭水化物内の遊離の炭水化物を評価することができる。本発明と対照的に、この方法は、蛍光偏光アッセイではない。

【００３３】

ＥＰ０ ５６１ ６５３ Ａ１（Ｌａｋｏｗｉｃｚら）は、ドナー－受容体対中の受容体を分析物と競合的に置換することができる、試験サンプルとドナー－受容体対との接触によるサンプル中のグルコースの定量を記載している。ドナーは、発光性または蛍光であり得る。この方法は、より高濃度の分析物に適切であり、この方法は蛍光偏光アッセイではない。

【００３４】

ＷＯ ００／２５１３４（Ｂｌａｎｃｈａｒｄら）は、シンチレーションプロキシミティーおよびＦＲＥＴを使用した、核受容体のリガンドの同定アッセイを記載している。本発明と対照的に、このアッセイは蛍光偏光アッセイではない。これは、ヘテロ二量体パートナーを使用しており、蛍光偏光アッセイには適切ではない。

【００３５】

ＵＳ５８１４４４９（Ｓｃｈｕｌｔｚ ａｎｄ Ｂａｌｌｅｒｓｔａｄｔ）は、目的の分析物のための少なくとも２つの結合部位を有する受容体保有分子を使用した、ガラクトースおよびグルコースの検出法を記載している。さらに、２つの分子群（１つの群（蛍光色素）は他の分子の近傍で検出可能に反応することができる）が存在する。分子群は、目的の分析物のアナログに結合する。分析物が存在する場合、アナログの分析物への競合的置換により結合複合体は解離する。複雑な感知器を用いて検出を行い、本発明と対照的にこ

の方法は蛍光偏光アッセイではない。
【００３６】
ＥＰ０９８４２８１　Ａ２（Ｕｌｌｍａｎら）は、特異的結合対（ｓｂｐ）中の分子に会合した光増感剤およびｓｂｐ分子に会合している化学発光成分を記載している。光増感剤の活性化による化学発光化合物から放たれた光量は、サンプル中の分析物の量に比例する。この方法には、試薬のいくつかのピペッティングおよび試薬の添加工程が必要であり、さらに、蛍光偏光アッセイではない。
【００３７】
今日の技術の限界およびそれに関連する必要な改良点
生物流体（血液、血清、血漿、尿、および髄液など）試験の日常的分析の大部分はいわゆる「救急」状態で行われ、試験材料を専門研究機関へに送付して分析結果が主治医に返送されていては遅い。さらに、保険医療制度では相当な費用がかかることが問題であり、有効であると同時に作製が安価で使用が簡単な感度の高い試薬である必要がある。したがって、（粒子の懸濁液と対照的に）作製、保存、および安定性に問題のない理想溶液に溶解した均一系試薬は、明らかに有利である。さらに、地理的条件によっては、専門家および専門機関が不足しているので良質のピペットおよび操作、適切な操作装置の必要な洗浄工程および維持を行うことが困難である。
【００３８】
したがって、本発明の目的は、非常に少ないか好ましくはたった１つの試薬コンテナに入れられた好ましくは理想溶液（懸濁液ではない）中に溶解した安定した耐久性のある試薬に基づき、いかなる有意なピペット操作も必要なく、洗浄すべき固相も分離すべき２つの異なる相も使用せず、好ましくはヘモグロビンおよび血球の存在下でおそらく血球の溶解後または溶解と同時に血液試験を行うことができる、感度の高い特異的測定法を提供することにある。
【００３９】
上記目的は、添付の特許請求の範囲によって特徴づけられる本発明によって得られる。
【００４０】
（発明の開示）
本発明は、本発明によって特徴づけられる試薬を試験溶液と混合し、その後前記試薬中に含まれる蛍光物質から発生したシグナルを測定してこれに基づいて分析物の濃度を計算するという事実によって特徴づけられる、試験における１つまたは複数の分析物の濃度定量法を提供する。この該シグナルの変化をいわゆる終点測定（新規の化学平衡の確立後およびほぼ確立後）および速度（単位時間あたりまたは時間間隔あたりのシグナルの変化の測定による）の両方で測定することができる。
【００４１】
本発明は、さらに、前記試薬の成分が個別に保持されずに１つのコンテナまたは区画に入っており、前記試薬は各分析物についての少なくとも１つの型の特異的結合分子（濃度は定量されているべきである）をさらに含み、前記試薬は試験サンプルと試薬との混合の結果としてシグナルが変化する蛍光物質をさらに含み、このシグナルの変化がサンプル中の分析物（単数または複数）の濃度の関数であり、このシグナルの変化を使用して異なる状態の凝集を分離することなく分析物の濃度（単数または複数）を計算することができる方法に関する。
【００４２】
本発明はさらに、１つまたは複数の試験分析物の濃度定量用試薬を、結合パートナーが濃度を定量すべき各分析物について互いに可逆的に結合する、結合対の前記試薬中に存在することによってさらに特徴づけられ、蛍光残基を含む各結合対中の少なくとも１つの結合パートナーによってさらに特徴づけられる方法に関する。本発明は、さらに、定量すべき１つの分析物に親和性を示す各結合対中の少なくとも１つの結合パートナーによって特徴づけられ、試薬を前記試験に混合した場合、各分析物は少なくとも１つの結合対中の結合パートナー間で結合するため競合し、試験における分析物（単数または複数）の濃度の変

化により前記結合パートナー間の結合対の形成についての平衡に含まれる他の分子の濃度が変化する。

【００４３】

本発明は、さらに、試薬は各分析物についてのいくつかの型の結合パートナーまたは結合対を含み得るという事実によって特徴づけられる。

【００４４】

本発明は、さらに、たった１つの分析物を定量するために試薬をデザインすることができるという事実によって特徴づけられ、さらに、たった１つの型の特異的結合分子を含むことによって特徴づけられる。

【００４５】

本発明は、さらに、蛍光残基は１つまたは複数の特異的結合分子に結合することができ、該特異的結合分子が分析物分子に結合した結果発生し得る蛍光シグナルが変化するという事実によって特徴づけられる。

【００４６】

本発明は、さらに、試験において異なる分析物を定量するための異なる蛍光波長を得るために、前記蛍光残基が異なる蛍光物質であり得るという事実によって特徴づけられる。

【００４７】

本発明は、さらに、ヘモグロビンを用いた分析において蛍光残基の最大吸光係数は、６００ｎｍと１０００ｎｍとの間の波長が好ましく、６２０ｎｍを超えることがより好ましく、６４０ｎｍを超えることが最も好ましいという事実によって特徴づけられる。

【００４８】

成分は個別に維持されないが１つのコンテナに存在し、低分子量で最大吸光係数の波長が６００ｎｍと１０００ｎｍとの間、より好ましくは６２０ｎｍを超える波長、さらにより好ましくは６４０ｎｍを超える波長の特異的結合分子に結合した蛍光残基を含む試薬が特に好ましい。

【００４９】

本発明は、さらに、モノクローナルもしくはポリクローナル抗体またはこれらの免疫反応性フラグメント（例えば、ＦＡＢフラグメント、一本鎖フラグメント、または一本鎖抗体）、ファージディスプレイまたは他の生物学的組み合わせ技術によって産生されるペプチドまたは他のポリマー、核酸ポリマーまたはそのアナログまたは誘導体、ライブラリー技術または合成組み合わせ化学に基づいて産生されるポリマーからなる特異的結合分子を含む試薬によって特徴づけられる。本発明は、さらに、前記結合対中の他の結合パートナーが少なくとも１つの定量すべき分析物を特徴づける構造の誘導体、アナログ、フラグメント、一部、または模倣物という事実によって特徴づけることができる。

【００５０】

本発明は、さらに、試薬は１つまたは複数の型の特異的結合分子および１つまたは複数の前記特異的結合分子の結合パートナーを含み得るという事実および、この前記結合パートナーが分析物の蛍光誘導体、分析物蛍光アナログ、分析物の蛍光フラグメント、分析物の蛍光部分、または前記試薬を使用して定量した濃度であるべき少なくとも１つの分析物によって特徴づけられる構造の蛍光模倣物から構成されるという事実によって特徴づけられる。

【００５１】

本発明は、さらに、前記試薬は溶解物質、凝集抑制剤、界面活性剤、沈殿物質、または分離物質を含み得るという事実によって特徴づけられる。

【００５２】

本発明は、さらに、前記蛍光残基がシアニン色素であり得るという事実によって特徴づけられる。本発明は、さらに、Ａｌｅｘａ　Ｆｌｕｏｒ　ＤｙｅｓまたはＭｏｌｅｃｕｌａｒ　Ｐｒｏｂｅｓから販売のＢｏｄｉｐｙ群の物質を使用することができるという事実によって特徴づけられる。

【００５３】

本発明は、さらに、個別のコンテナ中に試薬の一部を維持することが可能であるかそれが望ましいという事実、および前記試薬が試薬使用前に使用者によって調合されるという事実によって特徴づけられる。

【００５４】

この方法は、さらに、測定すべきシグナルを蛍光偏光測定を使用して測定するという事実によって特徴づけられる。蛍光シグナルは、一定時間内の連続的読み取りの形態での時間の関数として、２つまたはそれ以上の測定点の間の単位時間あたりの変化として、または２つまたはそれ以上の測定点の間の絶対変化として読み取られる。このような測定を、しばしば速度測定または速度読み取りと呼ぶ。本発明はまた、記載の蛍光測定法の残りの形態を使用した速度読み取り法を使用することができる。

【００５５】

本方法は、さらに、前記試薬の異なる成分を、所望するならば同時に添加するかわりに工程に分けて添加することができるという事実によって特徴づけられる。本方法は、さらに、本発明の試薬の添加後にさらなる試薬または他の試薬を添加することができるという事実によって特徴づけられる。本発明の試薬を分割して複数の工程で添加する場合、所望するならば他の試薬を本発明の試薬の段階的添加の間に添加することができる。

【００５６】

本方法は、さらに、試験サンプルは生体材料、その抽出物、希釈物、または濃縮物、濾過物であり得るという事実によって特徴づけられる。本方法は、さらに、生体溶液が、血液、血清、血漿、血液由来の細胞、血液溶解物、尿、脳脊髄液、涙液、唾液、胃腸管由来の吸引液、精液もしくは精漿、糞便、糞便抽出物、糞便希釈物もしくは懸濁液であり得るという事実によって特徴づけられる。生体溶液は、さらに、植物界由来の溶液、抽出物、誘導体、または濾過物であり得る。

【００５７】

本発明の濃度定量法は、分析物（単数または複数）の既知の濃度の標準液およびキャリブレータを使用することができ、蛍光残基から測定したシグナルをキャリブレータまたは標準液を使用した検量線に代入して分析物（単数または複数）の濃度を定量するという事実によって特徴づけられる。

【００５８】

本発明の濃度定量法は、関連するユーザの状況においてキャリブレータまたは標準液の分析を行う必要がないように、前記検量線を分析システムに接続された人工メモリに格納することができるという事実によって特徴づけられる。

【００５９】

本発明の濃度定量法は、一定の温度で行われ、既知濃度の分析物（単数または複数）の試験溶液に対する温度の影響についての研究によって経験的に作成された修正アルゴリズムの使用によって本方法を行うことができるという事実によって特徴づけられる。

【００６０】

本発明によれば、本方法を使用して、必要な生きた生物由来の生体材料のサンプル中の、臨床的に関連する物質の濃度を定量することができる。このような生物には、植物、昆虫、鳥類、哺乳動物などの動物（好ましくは霊長類、より好ましくはヒト）を含み得る。

【００６１】

さらに、本発明は、測定すべき特定の分析物に対して１つのコンテナと、特定体積の目的の生体材料を得るのためのコンテナとを組み合わせるべき予め決定した体積の成分を含むコンテナを含むキットに関する。別の実施形態では、キットは、測定すべき分析物に特異的な試薬を含む１つのコンテナおよび特定体積の目的の生体サンプルを得るためのコンテナを含む。

【００６２】

本発明を、図面および実施例を参照してさらに詳述する。

【００６３】

材料の化学分析のほとんどは、材料からサンプルを採取し（すなわち、試験材料／試験サ

ンプル）、その後の所望の化学分析を行う。試験サンプルは、例えば、液体、気体、固体、または前記状態の凝集した混合物であり得る。したがって、サンプルは均一であっても不均一であってもよい。例えば、サンプルが液相である場合、液相は特定の材料を含みことができるので、不均一であるだろう。十分に調製された血清は、均一な生体サンプル材料の例であるが、血液自体が大量の血球を含むので、均一な材料ではない。

【００６４】

試験サンプル中の１つまたは複数の分析物の濃度定量のほとんどは、サンプルへの他の化学物質の混合によって行われ、その後、混合の結果として得られるシグナルまたはシグナルの変化を観察するか測定する。これらのシグナルは、電磁放射線、放射線、温度、または物理的影響に対する反応（蛍光または光の吸収など）の形態の化学的または物理的シグナルであり得る。典型的には免疫アッセイ系の形態の蛍光測定を使用した既に公知の定量分析用の市販の製品では、典型的には抗体の形態の結合対中に含まれる結合パートナーおよび蛍光残基で標識した分析物アナログは、互いに単離して維持されている（典型的には異なる試薬コンテナに分離されている）。これにより、いくつかの工程では正確な装置（例えば、ピペット）の使用によって異なる試薬を添加する必要がある。

【００６５】

現在使用されている技術に関連する異なる問題を回避するために、本発明は、物質の１つの集合物（通常液体）中に蛍光物質および結合パートナーを含み且つ１つのコンテナに合わされている、全ての必要な化学物質を提供する。この化学物質の集合物を試薬と呼ぶ。１つの試薬中に化学物質の１つの準備された混合物を含むことが利点である理由は、これにより正確な成分濃度が予め測定されている点である。これは、特別な化学訓練を受けていない者が試薬と試験材料の混合を行うことができることを意味する。典型的には、試験材料を、予め較正したキャピラリまたは別の試験デバイス（例えば、Ｓａｍｃｏ　Ｓｃｉｅｎｔｉｆｉｃ、ＵＳＡの一定体積に自己較正するピペット）に集め、その後、コンテナ中の試薬に注ぐかそれと合わせる（例えば、滴下する）。試薬を含むコンテナは、異なる材料（例えば、ガラスバイアル、ガラス製またはプラスチック製試験管、プラスチックコンテナ、ホイルポケット、プラスチックパッド、または試薬注入に使用することができる他のデバイスなど）からなり得る。

【００６６】

伝統的には、結合対を使用した分析系中の結合パートナーと蛍光測定物との間の結合はできるだけ強力であることが望まれている。結合平衡定数が高いと濃度定量の感度が高くなり、他の物質からの干渉および物理的／化学的環境下でのばらつきが抑えられる。以前の技術では、同一の試薬および試薬コンテナ中に結合パートナーを維持すると結合平衡定数が高くなるので、凝集するか、沈殿するか、不可逆的に化学変化を起こし、溶液が不均一になった。したがって、本発明の場合のようにこれらを１つの試薬に維持することは避けられ、試薬をいくつかに分けることが好ましかった。その代わり、定量を実行している間に２つまたはそれ以上の結合パートナーの混合を行った。正確な結果を得るために、手動（いわゆるピペッティング）または化学自動装置を使用して正確な試薬の量を移す必要があった。手動ピペットの使用は非常に難しく、研究者および化学技術者は基本的訓練が必須である。自動装置は高価であり、通常サンプルを試験するまたは取扱う研究所または巨大な施設でのみ利用可能である。このような問題を回避するために、本発明の方法は、サンプル中の１つまたは複数の分析物の濃度定量用であって、個別に維持されていないが１つのコンテナに存在するたった１つの試薬のみを使用する。さらに、本発明の方法で使用される試薬は、濃度定量すべき各分析物についての少なくとも１つの特異的結合分子およびサンプルと試薬との混合の結果によって蛍光が変化する蛍光物質を含む。この蛍光シグナルの変化は、サンプル中の分析物（単数または複数）の濃度の関数であり、これを異なる凝集状態の分離を行わずに分析物濃度の計算に使用することができる。

【００６７】

したがって、総称「試薬」を、本明細書中で１つまたは複数の分析物の測定のためにサンプル溶液と混合した物質の集団の総称として使用する。通常、試薬は、緩衝物質、塩、界

面活性剤、および試薬中の微生物の増殖を回避するために添加される抗生物質を容易に含む流動体（いくつかの物質の溶液）である。しかしいくつかの試薬の使用の場合には、試薬の使用直前に流動物を添加するか、使用前に例えばサンプル材料中に固体試薬を溶解することにより試薬を固体で使用すると有利であり得る。試薬を、スタンドまたはデバイス上でさらに乾燥するか、カプセルまたは錠剤中に封入することができる。

【００６８】

本発明の試薬の好ましい実施形態は、サンプルの分析に使用される一定量の試薬を使用前にピペッティングすることも、割り当てることも、他の試薬と混合することも必要ない１つのコンテナで試薬が使用できる状態であることである。

【００６９】

しかし、その代わりに、分析前または分析中に適切な体積を割り当てることができる。したがって、通常あまり好ましくないが、既製の試薬が分析前もしくは分析直前または分析中に混合されるように、１つまたは複数の個別のコンテナ中に試薬の一部を維持することも全く不可能ではない。これを望むならば、試薬の一部をコンテナ、デバイス、またはフィルターなどに入れ、溶液（例えば、アッセイ溶液）と接触させるために移すことができる。これを望むならば、試薬の全部または一部は、乾燥または粉末状態であってよく、所望ならば、定量分析前、分析直前、または分析中に流動物を試薬に添加することができる。次いで、巨大で高度な研究所でしばしば使用されるような分析自動装置によって、使用できるようにさらに混合することができる。しかし、複雑な材料のサンプル中の分析物の分析のための、１つの試薬と蛍光偏光アッセイとの組み合わせによる本発明の方法自体が、先行技術（例えば、さらに感度の高い方法である濁り測定および比濁分析、非放射性物質のみの使用によるシンチレーションプロキシミティー、およびあまり高価ではなく且つ作成があまり複雑ではない理想溶液の使用によるＦＲＥＴ）と異なることに留意することが重要である。

【００７０】

本発明で使用される試薬は、低分子量の（同一または異なる）特異的結合分子／結合パートナーに結合した蛍光残基を含む。放出された光中の励起光の偏光を保存する能力は、分子の回転速度の関数であり、言い換えると、小分子がより大きな分子よりも早く回転するような分子サイズの関数であることが当業者に公知である。したがって、本発明の試薬の特に好ましい実施形態は、低分子量、好ましくは５０００以下の分子量、より好ましくは３０００以下、さらにより好ましくは１５００以下の１つまたは複数の結合パートナーに蛍光シグナル発生物質が結合することである。蛍光標識結合パートナーは、その結合パートナーから解離するかその結合パートナーに結合し、蛍光残基とともに回転する分子の全分子サイズが変化し、これを蛍光偏光の変化として検出することができる。

【００７１】

しかし、本発明の別の実施形態によれば、蛍光結合パートナー（すなわち、結合パートナーに結合した蛍光残基を含む結合パートナー）は上記よりも高分子量である試薬を使用することができ、これを蛍光偏光アッセイに使用して分析物（単数または複数）を検出することもできる（実施例１５）。しかし、この場合、蛍光残基は低分子量の特異的結合分子に結合した蛍光残基と比較して、より長い減衰時間を示さなければならない。

【００７２】

本発明の好ましい実施形態で使用される試薬中の蛍光標識結合分子は（すなわち、低分子量の結合パートナー（単数または複数）の使用）、本発明の競合実施形態によれば定量すべき分析物（単数または複数）のアナログ、フラグメント、または誘導体のいずれかであるが、本発明の非競合実施形態では１つまたは複数の前記分析物に特異的親和性を示すペプチド（単数または複数）または合成結合剤（任意選択的に組み合わせ化学技術、ファージディスプレイ、または核酸選択技術によって同定される）などの結合分子である。さらに、本発明の非競合実施形態での試薬で使用される特異的結合分子に結合した蛍光残基は、好ましくは６００ｎｍと１０００ｎｍとの間の波長、さらに好ましくは６２０ｎｍを超える波長、さらにより好ましくは６４０ｎｍを超える波長の最大吸光係数を有する。

【００７３】
本発明によれば、試験サンプル溶液は、生体材料またはその抽出物、例えば血液、血清、血漿、涙液、糞便抽出物、植物抽出物、胃腸管または精液または精漿からの吸引液、（希釈液または保存溶液中に希釈されていても、凝固、微生物増殖、酸化、還元を防ぎ、または酸性度を制御するために加えられた他の試薬、あるいは代わりに誘導体、または濾過物を含んでもよい）などであり得る。
【００７４】
本発明の異なる実施形態は、使用する周囲温度、試験サンプル溶液、試薬、混合物、装置もしくは装置の測定区画の変化に影響を受け得る。このような温度の影響を、試薬および／または装置の温度制御、周知の濃度の分析物を含むキャリブレータの使用によって相殺することができる。しかし、本発明の１つの目的は、キャリブレータを使用しない測定法を提供することにある。しかし、本発明によって提供される試薬を使用した測定に基づいた経験的測定および理論的計算は、温度変化の結果としての推定される偏差の基礎を形成する。したがって、本発明によれば、所望ならば濃度定量における温度による偏差の全てまたは一部を修正する測定装置に接続した温度感知器及びソフトウェアとの組み合わせまたは測定装置の一部として使用することができる。
【００７５】
本発明の使用により、多数の分析物濃度を定量することが可能になる。以下の分析物を列挙することができるが、この表に含まれていない多数の他の分析物が存在することは明らかである。
ヘモグロビン
アルブミン
Ｃ反応性タンパク質
尿中アルブミン
糖化アルブミン
糖化ヘモグロビン
フェリチン
ＡＳＡＴ
ＡＬＡＴ
ＬＤＨ
ミオグロビン
トロポニンＩ
脂肪酸結合タンパク質
アミラーゼ
グルコース
ＨＣＧ
Ｕ－ＨＣＧ
ＴＡ試験物
インスリン
抗インスリン抗体
ヘリコバクター抗体
チロキシン
遊離リオキシン
前立腺特異的抗原
遊離前立腺特異的抗原
チロイド刺激ホルモン
クレアチンキナーゼＭＢ型
【００７６】
これらおよび多数の他の分析物は参考図書、例えば、「Ｔｉｅｔｚ　Ｔｅｘｔｂｏｏｋ　ｏｆ　Ｃｌｉｎｉｃａｌ　Ｃｈｅｍｉｓｔｒｙ（ティッツェの臨床化学）」、Ｓａｕｎｄ

ｅｒｓ　Ｃｏｍｐａｎｙ、ＩＳＢＮ　０－７２１６－４４７２－４、１９９４などや、その後のテキスト、臨床化学および病理学の一般検査に記載されている。

【００７７】

本発明の競合実施形態によれば、各結合対中の結合パートナー１つのコンテナに存在し、試薬が結合対の間の業種、沈殿、および不可逆的結合を回避するような方法で構成される１つまたは複数の結合対を含む、すぐに使用可能な試薬を使用する。試験材料が試薬と混合されると、分析物は少なくとも１つの結合対において試薬中に既に存在する結合パートナーとの間で結合を競合する。

【００７８】

本発明の競合実施形態のために提供された試薬を１つまたは複数の濃度定量に使用することができ、これは、各分析物用の試薬は結合パートナーが可逆的に互いに結合する少なくとも１つの結合対を含むという事実により特徴づけることができる。さらに、本発明のこの実施例に関する試薬を、化学蛍光残基を含む、またはそれに結合した少なくとも１つの結合対中の少なくとも１つの結合パートナーによって特徴づけることができる。

【００７９】

「可逆的に結合した」は、本明細書中では、結合パートナー間の結合と競合する物質の添加により適度な時間内（典型的には添加から１時間後）に前記結合パートナー間の結合対形成の平衡に含まれる他の分子の濃度が変化を導くような結合を意味する。本発明は、さらに、蛍光残基から発生することができる蛍光シグナルの変化を導く結合パートナー間の結合対の形成平衡に含まれる分子濃度の変化によって特徴づけられる。本発明によって特徴づけられる試薬において、残基が結合パートナー分子に結合していない結合パートナー分子に結合した場合に発生するシグナルと比較して蛍光残基が関連する結合対の一部である場合に変化するシグナルを発生することができるような蛍光残基を使用するので、このような変化が起こる。

【００８０】

本発明の競合実施形態で使用する試薬は、結合パートナーが互いに結合していない遊離条件及び結合対が互いに結合した結合条件との間の平衡がさらに存在する１つまたは複数の結合対を含むことによって特徴づけられる。この平衡を一般的な化学の法則（質量作用の法則など）に当てはめる。化学平衡に含まれる１つの分子濃度が変化すると、平衡に含まれる残りの分子濃度も変化する。さらに、対応する結合パートナーへの結合に対する競合に適切なように構造が類似する結合パートナーのアナログまたは結合パートナーの誘導体またはアナログの添加によって平衡が変化する。蛍光残基に結合した物質の構造を劇的に変化させない蛍光残基の結合に修飾が使用される場合でもこのような構造の類似性はほとんど保存される。異なる蛍光を含む蛍光残基または標識を使用する場合、同一の溶液中で異なる化学反応を同時に測定することが可能であることがさらに当業者に一般的に公知である。したがって、本発明の試薬は、異なる蛍光を含む残基または標識を使用により、同一サンプル中の異なる分析物を同時に定量することができるという事実によってさらに特徴づけられる。

【００８１】

伝統的には、抗体などの生体特異的結合剤の使用には可能な限り結合定数が高いことが望ましかった。結合定数は、会合速度定数を解離速度定数で除した複素量であり、伝統的な結合定数は、望ましくは１０Ｅ７を超え、好ましくは１０Ｅ８を超え、より好ましくは１０Ｅ９を超える。特に低濃度では、結合剤への分析物の許容可能な共有部分への結合が可能であるように１０Ｅ１０を超える結合定数が望ましい。特に、高解離速度は避けられなければならないと主張されている（「Ｉｍｍｏｎｏａｓｓａｙ（免疫アッセイ）」、Ｅ．Ｐ．Ｄｉａｍａｎｄｉｓ、Ｔ．Ｅ．Ｃｈｒｉｓｔｏｐｏｕｌｏｓ編、Ｓａｎ　Ｄｉｅｇｏ、ＣＡ、Ａｃａｄｅｍｉｃ　Ｐｒｅｓｓ、１９９６）。ポリクローナル抗体はポリクローナルであるので、同一の調製物中での結合定数が非常に変化するが、モノクローナル抗体（発明の背景で説明されている生体または合成結合剤）は、同一の調製物中で同様または同一の結合定数を有し、異なる調製によってもより一定の結合性を示す。本発明の競合実

施形態では、以前に望まれていたこととは対照的に、特に高い結合定数は望ましくなく、且つ特に低すぎる解離定数も望ましくない。

【００８２】

本発明の背景の節で説明したように、当業者は異なる型の特異的結合分子を使用する。本発明の競合実施形態の試薬は、全ての型および変形形態の結合対を形成する結合パートナーを含み得る。一方では伝統的なポリクローナル抗体および他方では抗原を特異的結合分子として使用することができる。ポリクローナル抗体の代わりに、モノクローナル抗体を使用することができる。抗体は完全抗体であるか反応フラグメントの形態であり得る。ＦＡＢフラグメント、一本鎖抗体、一本鎖抗体フラグメントなどのより小さなフラグメントが特に好ましい。真核細胞中で産生される抗体または抗体フラグメントの代わりに、ポリペプチド、他のポリマー型、またはポリ核酸の形態のファージディスプレイ技術またはさらに進歩したファージディスプレイ技術によって得られる結合剤または様々な物質から構成される単位から構成され、且つライブラリー技術を使用して選択される結合剤を使用することが可能である。合成組み合わせ化学が急速に発達しており、これを使用して特異的結合分子を産生することができ、ＲＮＡまたはＤＮＡまたアナログモノマーを使用したポリマーの生成の頻度が増加しており、勿論本発明でも特異的結合分子として使用することができる。

【００８３】

上述の抗原は、免疫アッセイ技術では伝統的な結合パートナーであるが、近年、抗原、抗原フラグメント（いわゆるハプテン）、および抗原またはハプテンの誘導体が広く使用されている。定量すべき分析物（単数または複数）に非常に構造が類似した合成または生物学的に産生された分子を使用することもできる。本発明は、適切な結合パートナーが見出された場合、結合対中のこれら全ての構造を使用することができるという事実によって特徴づけられる。

【００８４】

本発明は、さらに、結合パートナーの結合が分析物（単数または複数）の濃度に影響を受ける予め調製された試薬中での結合対の使用によって特徴づけられる。分析物濃度が増加すると低濃度の分析物の場合と比較して１つまたは複数の結合対中の１つの結合パートナーへの結合についての競合が増加する。本発明は、さらに、これにより、サンプル材料と混合する前に試薬中に存在する別の濃度の１つまたは複数の非結合パートナーおよび／または結合対が得られるという事実によって特徴付けられる。本発明の試薬は、さらに、１つまたは複数の結合パートナーのこの濃度変化を１つまたは複数の上記方法を使用して検出することができるような方法から構成されるという事実によって特徴づけられる。

【００８５】

競合結合アッセイにおいて分析物を測定するためには、競合する結合パートナー（例えば、特異的抗体）は、分析物濃度に対して比較的不足させるべきである（競合を惹起するため）。しかし、蛍光残基を含む分析物分子が結合パートナーに結合して変化するシグナルを測定することが望まれるので、結合パートナー（例えば、抗体）の濃度は、分析物分子のほとんどの部分が結合パートナーに結合することができるほど高くなければならない。ここでは、溶液中の分析物濃度ならびに適切な結合定数および解離定数の特異的結合分子の選択によって制御される実用的な均衡を見出すことが必要である。さらに、本発明の競合実施形態によれば、結合分子および分析分子の蛍光誘導体またはアナログを、同一の試薬コンテナ中に結合対として維持する。したがって、典型的には試薬またはアッセイ溶液中で沈殿し得るので、巨大分子分析物と組み合わせた特異的結合分子としてのポリクローナル抗体の使用は回避すべきである。たいていポリクローナル抗体よりモノクローナル抗体が好ましく、より小さな分子サイズの一価の結合分子（例えば、抗体のＦＡＢフラグメント、ポリペプチド、またはアプタマー）がさらに好ましい。

【００８６】

本発明の競合実施形態の試薬を濃度を定量すべき分析物を含むサンプルと混合した場合、サンプル材料中の分析物の濃度により結合パートナー間の結合対の形成についての平衡に

含まれる他の分子の濃度が変化する。本発明は、さらに、結合パートナー間の結合対の形成についての平衡に含まれる分子濃度の変化により蛍光残基から発生し得るシグナルが変化し、これらの変化を分析物の定量に使用することができるという事実によって特徴づけられる。

【００８７】

蛍光シグナルの変化は、言い換えれば、分析物濃度の一次関数であり、これらのシグナルの変化を、分析物濃度の定量に使用することができる。したがって、本発明の蛍光残基は、蛍光残基に結合した１つまたは複数の特異的結合分子および分析物分子に結合する特異的結合分子の結果として変化し得る蛍光シグナルを有し得る。

【００８８】

濃度が約１．０＊１０Ｅ－９モルの分析物がアッセイ溶液（本発明の試薬を試験材料と混合して作製した溶液）に存在する場合、所望するならば、有効濃度が０．５＊１０Ｅ－９モルの特異的結合分子の使用によって無限に高い結合定数が得られ、平衡での結合パートナーの分析物の結合が５０％となる。結合定数が低い場合（例えば、１０＊１０Ｅ－９）、平衡状態は幾らか異なる。

【００８９】

分析物分子が分析物分子に結合する蛍光残基を有するかどうか結合分子（例えば、抗体）と同一の親和性を示す場合、

ａ＝結合定数、

ｂ＝結合パートナー分子の凝集体濃度（結合および遊離）、

ｃ＝分析物分子の凝集体濃度（全てが蛍光残基を含む分析物分子を含む本発明のサンプルおよび試薬）、

質量作用の法則によれば、結合対（例えば、抗体－抗原複合体）の凝集体濃度＝ｘは以下の平衡である。

ｘ＝ａ（ｂ－ｘ）（ｃ－ｘ）

【００９０】

ａ＝１．０＊１０Ｅ９、ｂ＝１．０＊１０Ｅ－１０、およびｃ＝２．０＊１０Ｅ－９である場合、計算により、平衡

ｘ＝０．７＊１０Ｅ－１０モルであり、遊離の特異的結合分子（例えば、抗体）の有効濃度は０．３＊１０Ｅ－１０モルであり、さらに非結合分析物分子（蛍光残基が結合しているか結合していない）の濃度は、１９．３＊１０Ｅ－１０モルであることが示される。

【００９１】

これにより結合パートナーへの分析物分子の結合の結果としてシグナルがほんのわずか変化することが認められる。この場合、５％未満の分析物分子が結合パートナーに結合可能である。

【００９２】

結合定数ａが１．０＊１０Ｅ８であるが、ｂおよびｃは上記と変わらない場合、結合した分析物分子のフラクションは非常に小さく、もはや競合は起こらないことが示される。

【００９３】

蛍光分子が結合した修飾分析物分子に対する特異的結合分子（例えば、抗体）の親和性が非修飾分析物分子の親和性よりも高いような状況の場合、有効に競合することができるようにサンプル中の分析物分子にはより低濃度の修飾分析物分子を使用する必要がある。これは、特異的結合分子の有効濃度が低い状況に対応し、質量作用の法則にしたがって幾らか高い結合定数の特異的結合分子が必要である。

【００９４】

蛍光残基が結合した修飾分析物分子に対する特異的結合分子（例えば、抗体）の親和性が非修飾分析物分子の親和性平衡よりも低いような状況の場合、より高い濃度の修飾分析分子を使用することが望ましい。しかし、この競合状態を達成するためには、特異的結合分子の有効濃度はサンプル中の分析物分子に対するモル濃度が低くなければならず、この低濃度の特異的結合分子は、結合パートナーと分析物分子との間の結合定数が高いことがさ

らに必要である。しかし、結合分子の有効濃度はいわゆる結合分子の全量よりも有意に低くても良いことは特記に値する。実際の結合分子（例えば、モノクローナル抗体）のフラクションのみが実際にその結合パートナーと結合することがある。これは、例えば分子の合成における構造の変化（例えば、翻訳後修飾、標識化学または単なる未知の起源によって導入された修飾）に起因し得る。

【００９５】

したがって、本発明の競合実施形態によれば、結合定数は、シグナル発生残基が結合した修飾分析物分子濃度の逆数の少なくとも１／３の値でなければならないと結論付けることができる。修飾分析物分子の濃度の逆数を超える結合定数がより好ましく、修飾分析物分子の濃度の逆数の２倍を超える結合定数がさらに好ましい。したがって、本発明の競合実施形態で得た試薬では、互いに結合する結合パートナーと非結合形態の遊離の結合パートナーとの間の平衡が確立する。試薬をサンプルに添加すると、濃度が変化して新規の平衡が確立する。以前の技術では、結合分子（例えば、抗体）の添加直後に平衡が確立するような高い結合定数及び、特に高い解離定数が望ましかった。本発明の競合実施形態では、サンプルの添加前に試薬中で結合対が形成されており、その後、より高い解離定数により、サンプル添加後から適切な時間の後に新規の平衡を達成することができる。

【００９６】

結合定数が、

ａ＝１．０＊１０Ｅ１０／モル、

ｂ＝結合剤（例えば、抗体）の凝集体濃度＝１．０＊１０Ｅ－９モル、

ｃ＝分析物分子の凝集体濃度（シグナル発生残基を含むか含まない）＝１．０＊１０Ｅ－９モル

である場合、質量平衡および質量作用の法則を使用して、結合パートナー（シグナル発生残基を含むか含まない分析物分子および結合剤（例えば、抗体など））間の複合体の濃度平衡＝０．７３＊１０Ｅ－９モル、結合パートナーに結合していない分析物分子の濃度＝０．２７＊１０Ｅ－９モル、および非結合特異的結合分子（例えば、抗体）の濃度＝０．２７＊１０Ｅ－９モルであることを示すことができる。次いで１．０＊１０Ｅ－９モルの分析物分子を添加する試験サンプルを添加する場合、アッセイ溶液中の分析物分子の全量は２．０＊１０Ｅ－９モルになり、しばらくしてから新規の平衡が確立される。

【００９７】

これらの例では、サンプルの添加によりアッセイ溶液の全体積は大幅に変化しないと想定される。計算により、アッセイ溶液がわずかに変化したとしても、上記の計算例は記載の条件と非常に近いことを示すことができる。

【００９８】

結合定数が１０倍（１累乗）高い場合、状況は以下のようになる。

【００９９】

結合定数が

ａ＝１．０＊１０Ｅ１１／モル、

ｂ＝結合剤（例えば、抗体）の凝集体濃度＝１．０＊１０Ｅ－９モル、

ｃ＝分析物分子の凝集体濃度（シグナル発生残基を含むか含まない）＝１．０＊１０Ｅ－９モル

である場合、上記に対応する計算を使用して、本発明の競合実施形態では、主にサンプル添加後に平衡またはほぼ平衡を達成するために必要な時間を決定するのは、解離定数であることを示すことができる。やや簡単に述べると、図１～図９は、結合定数の値は達成することができるシグナルの変化の範囲を示す最も重要な因子である一方、この値は分析物分子を含むサンプル中での混合後にどれぐらいの速さで新規の平衡またはほぼ平衡を達成することができるか（したがって、どのぐらいの速さで新規の安定な蛍光シグナルが達成されるか）を同定する解離定数の値であることを示すことができる。したがって、本発明の競合実施形態での終点測定に使用する試薬は、０．００３／秒の解離定数の結合対が好ましい。０．０１／秒を超える解離定数の結合対がより好ましく、０．０２／秒を超える

解離定数の結合対がさらに好ましい。

【０１００】

速度読み取り、すなわち、時間単位あたりまたは規定の時間間隔内での蛍光シグナルの変化の測定を適用した場合、新規の平衡が確立する前に測定することができ、さらに、シグナルの変化を使用して分析物濃度（単数または複数）を計算することができる。したがって、本発明の競合実施形態では、速度読み取りによって終点読み取りで使用したものよりも大幅に低い解離定数の結合対が使用可能である。０．０００１／秒ほどの低さの解離定数の結合対を使用することができるが、０．００１／秒を超える解離定数がより好ましく、０．００５／秒を超える解離定数がさらに好ましい。

【０１０１】

本発明の方法の特定の実施形態は、本発明の試薬を使用してサンプル材料中での混合後の時間単位あたり、時間の関数として、または所与の時間間隔内の蛍光シグナルの変更の程度の変化を測定することである。この実施形態では、蛍光シグナル（単数または複数）は、時間の関数として、一定時間内の連続的読み取りとして、２つまたはそれ以上の測定点の間の時間単位あたりの変化として、または、２つまたはそれ以上の測定点の間の絶対変化として読み取ることができる。このような測定を、しばしば速度測定または読み取りと呼ばれる。本発明の方法はまた、記載の蛍光測定法の他の形態を使用した速度読み取り法を使用することができる。上記の方法は、本発明の方法の競合および非競合実施形態の両方に適用される。

【０１０２】

本発明の適用可能な実施形態はさらに、蛍光残基の最大吸光係数の波長が６００ｎｍより高く、好ましくは６２０ｎｍ、より好ましくは６４０ｎｍを超える本発明の試薬と組み合わせた上記の蛍光偏光速度測定を使用することである。ヘモグロビンを含むサンプルの分析には、ヘモグロビンが分析物（単数または複数）の濃度定量を実質的に妨害するので、最大吸光係数は６２０ｎｍを超えるべきである。このような妨害は、例えば血液を使用した蛍光偏光アッセイを使用する場合に主な問題である。

【０１０３】

本発明の非競合実施形態では、蛍光残基は、通常（しかし、常にではない）、特異的結合分子に結合している。特異的結合分子は、添加される試験サンプル／分析物分子と比較して過剰に存在し、分析物分子が特異的結合分子に結合している場合に発生し得る蛍光シグナルが変化する。このような適切な特異的結合分子の好例は、Ｂｏｃｋ　＆　ａｌ．による「Ｓｅｌｅｃｔｉｏｎ　ｏｆ　ｓｉｎｇｌｅｓｔｒａｎｄｅｄ　ＤＮＡ　ｍｏｌｅｃｕｌｅｓ　ｔｈａｔ　ｂｉｎｄ　ａｎｄ　ｉｎｈｉｂｉｔ　ｈｕｍａｎ　ｔｈｒｏｍｂｉｎ（ヒトトロンビンに結合して阻害する一本鎖ＤＮＡ分子の選択）」、Ｂｏｃｋ　＆　ａｌ．、Ｎａｔｕｒｅ、３５５巻、５６４～５６頁、１９９２に記載のアプタマーである。この文献は、特異的結合分子の産生の一般的な基本としての一般的技術について言及しており、ヌクレオチド配列ＧＧＴＴＧＧＴＧＴＧＧＴＴＧＧまたはＧＧＴＴＧＧを含むアプタマーはヒトトロンビンに特異的に結合することを示している。

【０１０４】

本発明の競合実施形態ならびに非競合実施形態でアプタマーを使用することができる。Ｇｏｌｄ　＆　ａｌ．、「Ｄｉｖｅｒｓｉｔｙ　ｏｆ　ｏｌｉｇｏｎｕｃｌｅｏｔｉｄｅ　ｆｕｎｃｔｉｏｎｓ（オリゴヌクレオチド機能の多様性）」、Ａｎｎｅｌ．Ｒｅｖ．Ｂｉｃｈｅｍ．、６４巻、７６３～９７頁、１９９５は、本発明の試薬で使用することもできる特異的結合剤の産生用の一般的なアプタマー技術の多数の使用例を記載している。テオフィリンに属する特異的結合剤（Ｓｃｉｅｎｃｅ、２６３巻、１９９４年3月１１日に記載）は、競合実施形態に特に適切な例であるが、Ｔ４　ＤＮＡポリメラーゼを結合する同一の論文記載のＲＮＡ－アプタマーは、非競合実施形態に適切である。

【０１０５】

Ｒｉｃｋ　Ｋｏｎｒａｄら、「Ｉｓｏｅｎｚｙｍｅ－ｓｐｅｃｉｆｉｃ　ｉｎｈｉｂｉｔｉｏｎ　ｏｆ　Ｐｒｏｔｅｉｎ　Ｋｉｎａｓｅ　ｂｙ　ＲＮＡ　ａｐｔａｍｅｒｓ（ＲＮ

Ａアプタマーによるプロテインキナーゼのアイソザイム特異的阻害）」、Ｊ．Ｂｉｏｌ．Ｃｈｅｍ．２６９巻、３２０５１～５４頁、１９９４は、本発明の試薬で使用される特異的結合分子の例として使用することができるプロテインキナーゼＣの特異的結合分子として適切な２つの異なるアプタマーを記載している。

【０１０６】

本発明の競合および非競合実施形態では、ペプチド配列を含む特異的結合分子を使用することができる。非競合実施形態で特に適切なペプチド配列の例は、Ｃｈａｋｒａｖａｒｔｈｙ　＆　ａｌ．、Ａｎａｌ．Ｂｉｏｃｈｅｍ．、１９６巻、１４４～１５０頁、１９９１に記載のペプチド配列である。特異的結合剤として有用なペプチド配列の他の例は、Ｙｕｅら、Ｔｈｅ　Ｊｏｕｒｎａｌ　ｏｆ　Ｂｉｏｌｏｇｉｃａｌ　Ｃｈｅｍｉｓｔｒｙ、２７１巻、２２２４５～２２２５０頁、１９９６に記載されている。これらは、多数の試薬、放射性物質、およびオートラジオグラフィー（時間がかかり且つ高価な方法）を使用したドットブロットアッセイにおいてＣ反応性タンパク質に結合するペプチドを同定することができた。

【０１０７】

本発明の試薬は、本方法の競合および非競合実施形態において、分析物の濃度定量のみのために調製されるという事実によって特徴づけることができる。それにもかかわらず、所望するならば試薬は、１つを超える特異的結合分子を含むことができ、所望ならば、１つを超える特異的結合分子は、蛍光物質（単数または複数）を含むことができる。しかし、試薬はまた、いくつかの分析物の所望するならば同時、所望するならば各分析物についてのいくつかの異なる特異的結合分子の濃度定量用に調製されるという事実によって特徴づけることができる。所望するならば、蛍光残基を含む結合パートナーまたは異なる結合対中の異なる結合パートナーに結合するいくつかのさらに異なる蛍光残基（単数または複数）を含む１つを超えるこれらの結合対を使用することが可能である。シグナル発生系の可能性はほぼ無限である。いくつかのシグナル発生系は、濃度定量の精度が潜在的に増すが、それと同時に測定システムの複雑さも増す。

【０１０８】

フルオレセイン、テキサスレッド、オレゴングリーン、ローダミン、テトラメチルローダミン、アミノメチルクマリンなどの伝統的な蛍光物質ならびに広範な他の物質を蛍光残基として使用することができる。例えば、Ｒｉｃｈａｒｄ　Ｐ．Ｈａｕｇｌａｎｄによる「Ｈａｎｄｂｏｏｋ　ｏｆ　Ｆｌｕｏｒｅｓｃｅｎｔ　Ｐｒｏｂｅｓ　ａｎｄ　ｒｅａｓｅａｒｃｈ　ｃｈｅｍｉｃａｌｓ（蛍光プローブおよび研究試薬ハンドブック）」、第６版、ＩＳＢＮ　０－９６５２２４０－０－７を参照のこと。このハンドブックはまた、蛍光物質のアミノ基、チオール基、アルコール基、ケトン、ジオール、およびカルボン酸への結合法を記載している。さらに、蛍光物質のペプチド、タンパク質、抗体、核酸ポリマー（アプタマーなど）、および他のポリマーへの結合法を記載しているが、これは当業者に周知である。

【０１０９】

しばしば、本発明の医学的および生物学的使用において大量のヘモグロビンまたは胆汁色素がサンプル中に存在する。ヘモグロビンまたはビリルビンによって吸収される励起または発光波長を有する蛍光物質を含む試薬は、しばしば蛍光物質を特徴づける光の吸収に影響を受ける。したがって、既に記載のように、全血または血液溶解物を分析する場合の本発明の特に好ましい実施形態は、蛍光残基の最大吸光係数が６００ｎｍを超えるか、より好ましくは６２０ｎｍを超えるか、さらに好ましくは６４０ｎｍを超える蛍光分子を含む試薬に基づく。ヘモグロビンを含むサンプルの分析には、ヘモグロビンが分析物（単数または複数）の濃度定量を実質的に妨害するため、最大吸光係数は６２０ｎｍを超えるべきである。このような物質は、Ａｍｅｒｓｈａｍ　Ｐｈａｒｍａｃｉａ　Ｂｉｏｔｅｃｈからさまざまな励起波長（化学構造のアナログによって変化する）および標識すべき物質への結合用の様々な活性化官能基の数を有するＣｙＤｙｅ　ＦｌｕｏｒｏＬｉｎｋ　Ｒｅａｃｔｉｖｅ　Ｄｙｅなどの商標で販売されている。典型的には、結合点が多いほど分子の

回転自由度は減少し、蛍光偏光測定法の利用可能性は減少する。

【０１１０】

適切なシアニン色素は、１９９７年５月６日、Ｗａｇｇｏｎｅｒ；Ａｌａｎ Ｓに付与された米国特許第５，６２７，０２７号：「Ｃｙａｎｉｎｅ ｄｙｅｓ ａｓ ｌａｂｅｌｉｎｇ ｒｅａｇｅｎｔｓ ｆｏｒ ｄｅｔｅｃｔｉｏｎ ｏｆ ｂｉｐｌｏｇｉｃａｌ ａｎｄ ｏｔｈｅｒ ｍａｔｅｒｉａｌｓ ｂｙ ｌｕｍｉｎｅｓｃｅｎｅ ｍｅｔｈｏｄｓ（発光法による生体材料および他の材料の検出用の標識試薬としてのシアニン色素）」にさらに記載されている。さらに、適切な物質は、Ｍｕｊｕｍｄａｒ、Ｌａｕｒｅｎ、Ｍｕｊｕｍｄａｒ ａｎｄ Ｗａｇｇｏｎｅｒ、Ｃｙｔｏｍｅｔｒｙ、１０：１１～１９、１９８９またはＳｏｕｔｈｗｉｃｋ ＆ ａｌ．、Ｃｙｔｏｍｅｔｒｙ、１１、４１８～４３０、１９９０またはＬａｕｒｅｎ ＆ ａｌ．、Ｃｙｔｏｍｅｔｒｙ、１０；３～１０、１９９０またはＷａｇｇｏｎｅｒら、米国特許第６，００８，３７３号またはＢｒｕｓｈ ａｎｄ Ｒｅｉｍｅｒ、米国特許第５，９８６，０８６号またはＫｒａｎｄｉｋａｒ ＆ ａｌ．、米国特許第５，８５２，１９１号またはＫｕｓａｋａｔａ ＆ ａｌ．、米国特許第４，８４７，３８５号またはＷａｇｇｏｎｅｒの米国特許第５，５６９，５８７号に記載されている。

【０１１１】

「Ｈａｎｄｂｏｏｋ ｏｆ Ｆｌｕｏｒｅｓｃｅｎｔ Ｐｒｏｂｅｓ ａｎｄ ｒｅｓｅａｒｃｈ ｃｈｅｍｉｃａｌｓ（蛍光プローブおよび研究試薬ハンドブック）」、Ｒｉｃｈａｒｄ Ｐ．Ｈａｕｇｌａｎｄ、第６版、ＩＳＢＮ ０－９６５２２４０－０－７はまた、それぞれ６００ｎｍ、６２０ｎｍ、および６４０ｎｍを超える良好な蛍光シグナルを発生する蛍光物質群Ｂｉｏｄｙｐｉの使用を説明している。現在、Ｂｉｏｄｙｐｉ ６３０／６５０－Ｘ／ＭｅＯＨ、Ｂｉｏｄｙｐｉ ６５０／６５５－Ｘ／ＭｅＯＨ、Ｂｉｏｄｙｐｉ ＦＬ／ＭｅＯＨ、Ｂｉｏｄｙｐｉ Ｒ６Ｇ／ＭｅＯＨ、Ｂｉｏｄｙｐｉ ＴＭＲ－Ｘ／ＭｅＯＨ、およびＢｉｏｄｙｐｉ ＴＲ－Ｘ／ＭｅＯＨならびにＡｌｅｘａ Ｆｌｕｏｒ ＳｕｂｓｔａｎｃｅｓはＭｏｌｅｃｕｌａｒ Ｐｒｏｂｅｓ Ｃｏｍｐａｎｙから販売されており、より類似の物質を予測することができる。これらの物質は、シアニン色素の良好な代替物であり、本発明の好ましい試薬である。

【０１１２】

適切な蛍光残基もユーロピウム、サマリウム、またはテルビウムなどの複合体結合ランタノイド元素を含むＤＴＰＡおよびＮ１などの複合体形成物に結合することができる。これらの蛍光残基中の有利な質は、ストークスシフトが非常に長く（励起波長と発光波長との間の波長が非常に異なる）、励起と発光との間の時間間隔が比較的長い（時間解離蛍光測定を使用する場合に好ましい）ことである。これらの蛍光残基は、インターネット上のＰｅｒｋｉｎ Ｅｌｍｅｒ Ｌｉｆｅ Ｓｃｉｅｎｃｅのホームページおよび製品カタログで十分説明されている。

【０１１３】

同様に、本発明の試薬は、蛍光シグナル発生物質が部分的にポリペプチド鎖からなり、このポリペプチド鎖は好ましくは３０個未満のアミノ酸、より好ましくは２０個未満のアミノ酸、さらに好ましくは１２個未満のアミノ酸を含むという事実によって特徴づけることができる。

【０１１４】

実施例１７および１８を参照して、異なる波長での間欠的励起の使用を、異なる励起波長を使用したいくつかの分析物の分析のために記載し、例えば異なる波長の異なるダイオードレーザーの間欠的使用による異なる波長の間欠的光を使用することが好ましい。異なる光源の使用の中断は、少なくとも実際の蛍光物質の励起条件についての寿命に対応すべきであることを留意すべきである。

【０１１５】

（発明を実施するための最良の形態）

最良の形態を実施例、好ましくは本発明の競合実施形態についての実施例９および本発明

の非競合実施形態についての実施例１４に例示する。

【０１１６】

（実施例）

実施例１：全血分析用の蛍光リガンド結合

従来技術によってペプチドＴｙｒ－Ｔｒｐ－Ａｌａ－Ａｓｎ－Ｐｈｅ－Ａｌａ－Ａｒｇ－Ａｓｎ－Ａｒｇ－Ａｓｎを合成し、これを純水中に２ｍｇ／ｍｌの濃度に溶解する。５０μｌのペプチド溶液と５０μｌの重炭酸ナトリウム緩衝液（ｐＨ９．２）と混合する。Ａｍｅｒｈａｍ　Ｐｈａｒｍａｃｉａ　Ｂｉｏｔｅｃｈから販売されており、任意選択的に無水ＤＭＳＯに溶解した０．１μｍｏｌのＣｙ５　Ｆｌｕｏｒｏｌｉｎｋ活性化シアニン色素と前記溶液とを混合し、暗所の室温で一晩静置し、その後溶液を暗所の冷蔵庫で保存する。所望ならば、５μｌの１０ｍｍｏｌエタノールアミン溶液を添加して、全ての残存する活性化蛍光色素をブロックするか、溶液（以後ストック溶液と呼ぶ）を放置して保存中に未反応色素を加水分解させることができる。

【０１１７】

ストック溶液由来の純粋なＣｙ５標識Ｔｙｒ－Ｔｒｐ－Ａｌａ－Ａｓｎ－Ｐｈｅ－Ａｌａ－Ａｒｇ－Ａｓｎ－Ａｒｇ－Ａｓｎを、溶出液として０．１％トリフルオロ酢酸を使用し、０％～６０％のアセトニトリルの勾配の０．１％トリフルオロ酢酸を使用したＣ４カラム（Ｗａｔｅｒｓ、Ｕ．Ｓ．を含む複数の業者が販売）の逆相クロマトグラフィーによって原液から単離する。フローセルに接続した光検出器を使用して、３４０ｎｍでの透過測定によりペプチド含有量をモニターし、６５０ｎｍでの透過測定によりＣｙ－５をモニターし、Ｃｙ－５抱合ペプチドを単離する。凍結乾燥によってトリフルオロ酢酸、アセトニトリル、および水を除去する。

【０１１８】

ストック溶液の純粋なＣｙ５標識Ｔｙｒ－Ｔｒｐ－Ａｌａ－Ａｓｎ－Ｐｈｅ－Ａｌａ－Ａｒｇ－Ａｓｎ－Ａｒｇ－Ａｓｎを、薄層クロマトグラフィーによって単離することも可能である。シリカゲルプレート上にストック溶液のアリコートを加え、ｎ－ブタノール：酢酸：水混合物で溶出する。シリカゲルの質に依存して、ｎ－ブタノール：酢酸：水の相対含有量を調整して理想的な分離を得ることができる。従来技術での溶出後、シリカゲルプレートを乾燥し、目視およびＵＶランプ（任意選択的に並行実験でニンヒドリンスプレーを使用する）によって検査し、非標識ペプチドおよび遊離のＣｙ－５色素分子から分離したＣｙ５標識Ｔｙｒ－Ｔｒｐ－Ａｌａ－Ａｓｎ－Ｐｈｅ－Ａｌａ－Ａｒｇ－Ａｓｎ－Ａｒｇ－Ａｓｎスポットを同定する。鋏またはスパチュラでＣｙ５標識Ｔｙｒ－Ｔｒｐ－Ａｌａ－Ａｓｎ－Ｐｈｅ－Ａｌａ－Ａｒｇ－Ａｓｎ－Ａｒｇ－Ａｓｎを含むシリカゲルを単離する。１０ｍＭ　ＴＲＩＳ緩衝液（ｐＨ＝８．０）中に単離したシリカゲルを懸濁し、溶液中にＣｙ５標識Ｔｙｒ－Ｔｒｐ－Ａｌａ－Ａｓｎ－Ｐｈｅ－Ａｌａ－Ａｒｇ－Ａｓｎ－Ａｒｇ－Ａｓｎを溶出させる。チューブの底にシリカゲルを沈殿させる。精製Ｃｙ５標識Ｔｙｒ－Ｔｒｐ－Ａｌａ－Ａｓｎ－Ｐｈｅ－Ａｌａ－Ａｒｇ－Ａｓｎ－Ａｒｇ－Ａｓｎを含むＴＲＩＳ緩衝液をデカントする。

【０１１９】

所望ならば、スケールアップのために薄層クロマトグラフィーの代わりに当業者に周知の他の従来のＨＰＬＣ分離技術を使用することができる。

【０１２０】

実施例２：全血分析用の傾向リガンド結合

Ｔｙｒ－Ｔｒｐ－Ａｌａ－Ａｓｎ－Ｐｈｅ－Ａｌａ－Ａｒｇ－Ａｓｎ－Ａｒｇ－Ａｓｎの代わりにＴｙｒ－Ｔｒｐ－Ａｌａ－Ａｓｎ－Ｐｈｅ－Ａｌａ－Ａｒｇ－Ａｓｎ－Ｇｌｙ－Ａｓｎを使用する以外は実施例１に記載の本発明の方法を行い、Ｃｙ５標識Ｔｙｒ－Ｔｒｐ－Ａｌａ－Ａｓｎ－Ｐｈｅ－Ａｌａ－Ａｒｇ－Ａｓｎ－Ｇｌｙ－Ａｓｎを得る。

【０１２１】

実施例３：全血サンプル中のＣ反応性タンパク質の測定法

１５０ｍＭ塩化ナトリウムを含む１００ｍＭリン酸緩衝液（ｐＨ＝７．４）の作製によっ

てアッセイ試薬を作製し、Ｓｉｇｍａのウシγグロブミンを２ｍｇ／ｍｌの濃度で、及びＰｉｅｒｃｅ Ｃｈｅｍｉｃａｌ Ｃｏｍｐａｎｙ、ＵＳのＴｒｉｔｏｎ Ｘ－１００を最終濃度０．１％ｖ／ｖで添加する。非常に低いバックグラウンド蛍光を有する高純度の試薬を選択すべきである。１．０＊１０Ｅ－１１ｍｏｌ／ｍｌのＣｙ５標識Ｔｙｒ－Ｔｒｐ－Ａｌａ－Ａｓｎ－Ｐｈｅ－Ａｌａ－Ａｒｇ－Ａｓｎ－Ａｒｇ－Ａｓｎを上記の実施例１に従って添加し、任意選択的に長期保存には０．０１％アジ化ナトリウムなどの適切な静菌薬を添加する。未知の血液サンプルまたは血液サンプルのアリコートと組み合わせるための個別のコンテナ中にこの混合物のアリコート（例えば、１ｍｌ）を維持する。

【０１２２】

Ｃ反応性タンパク質の定量時に、例えば予め測定したキャピラリの使用によってアリコート（例えば、２０μｌの全血サンプル）を取り出し、この予め測定したキャピラリと上記個別コンテナ中のアッセイ試薬とを合わせ、個別のコンテナを温度を３２℃に調節した場所で維持する。

【０１２３】

典型的には、蛍光偏光測定用に構築された装置で測定される４つの研磨した透明の側面を有するキュベットの形態のコンテナを使用するが、このコンテナは取り外し可能なストッパまたはキャピラリの単純な落とし入れまたはシールを介したキャピラリの導入によってキャピラリをコンテナに挿入するシールを含む。さらに、キャピラリ／コンテナは、キャピラリがコンテナの底に落とし込まれて励起光または発光を妨害しないようにデザインする（以下を参照のこと）。その後コンテナを震盪し、キャピラリからの血流を流れ出させ、細胞をアッセイ試薬で溶解する。試験サンプルアリコートのＣ反応性タンパク質は、アッセイ試薬のＣｙ５標識Ｔｙｒ－Ｔｒｐ－Ａｌａ－Ａｓｎ－Ｐｈｅ－Ａｌａ－Ａｒｇ－Ａｓｎ－Ａｒｇ－Ａｓｎとの反応を開始する。

【０１２４】

震盪直後、コンテナを３２℃の蛍光偏光測定装置中に入れる。発光蛍光の偏光の測定のために、典型的には装置中の小さな偏光レーザダイオードを使用してコンテナ中の混合物に６５０ｎｍの波長の偏光を照射し、発光の偏光が一定である場合に装置で測定可能ならば狭いほうのバンド幅の偏光（６７０ｎｍ波長）を測定する。周知のＣ反応性タンパク質濃度の測定によって得た検量線への測定した発光の偏光値の代入によって未知のサンプルのＣ反応性タンパク質の濃度を計算する。このような検量線を、しばしば測定装置のコンピュータに保存して、装置上のＣ反応性タンパク質濃度を直接読み取ることができる。

【０１２５】

フローセルを具備する測定装置を使用する場合、装置の製造者の指示に従って、測定のために混合物をフローセル測定に通過させる。

【０１２６】

典型的には、軽度の細菌感染では、２０μｌサンプルのＣ反応性タンパク質は、１０～１００ｍｇ／ｌであるが、重篤な臨床状態ではさらに高い値も認められる。それに対して、特に心臓病および軽度の炎症リスクのスクリーニングでは、所定の１ｍｇ／ｌ未満の測定値である。さらに、各Ｃ反応性タンパク質分子は、Ｃｙ５標識Ｔｙｒ－Ｔｒｐ－Ａｌａ－Ａｓｎ－Ｐｈｅ－Ａｌａ－Ａｒｇ－Ａｓｎ－Ａｒｇ－Ａｓｎの５つの分子と反応することができる。したがって、Ｃ反応性タンパク質の目的の濃度は非常に変化するので、Ｃｙ５標識Ｔｙｒ－Ｔｒｐ－Ａｌａ－Ａｓｎ－Ｐｈｅ－Ａｌａ－Ａｒｇ－Ａｓｎ－Ａｒｇ－Ａｓｎ濃度を、アッセイ試薬と合わせるべき血液体積と比較して調整しなければならない。

【０１２７】

実施例４：全血サンプル中のＣ反応性タンパク質の測定法

Ｃｙ５標識Ｔｙｒ－Ｔｒｐ－Ａｌａ－Ａｓｎ－Ｐｈｅ－Ａｌａ－Ａｒｇ－Ａｓｎ－Ａｒｇ－Ａｓｎの代わりにＣｙ５標識Ｔｙｒ－Ｔｒｐ－Ａｌａ－Ａｓｎ－Ｐｈｅ－Ａｌａ－Ａｒｇ－Ａｓｎ－Ｇｌｙ－Ａｓｎを使用する以外は実施例３に記載の本発明の方法を行う。この方法の例は、Ｃｙ５標識Ｔｙｒ－Ｔｒｐ－Ａｌａ－Ａｓｎ－Ｐｈｅ－Ａｌａ－Ａｒｇ－Ａｓｎ－Ｇｌｙ－ＡｓｎがＣ反応性タンパク質に対する親和性が低いため、実施例３より

も非常に高濃度のＣ反応性タンパク質に適切である。同様にこの方法の例は、低濃度のＣ反応性タンパク質に使用するにはあまり適切ではない。

【０１２８】

実施例５：全血サンプル中のＣ反応性タンパク質の測定法

偏光値の安定後に偏光を読み取る代わりに速度様式での時間の関数として偏光値を測定する以外は実施例３に記載の本発明の方法を行う。利用可能ならば、測定装置を任意選択的にソフトウェアで定義したシグナルを連続的または異なる測定点で読み取るコンピュータに接続する。次いで、保存した値と時間の関数として既知のＣ反応性タンパク質濃度の標準物質の測定によって得られた値とを比較する。任意選択的に、偏光値、異なる時間、およびＣ反応性タンパク質濃度値を使用して３次元検量線を読み取り、得られた値と未知のサンプルとを比較して、任意選択的に標準的な統計学のテキストに記載の最適な適合のための最小二乗法の使用し、任意選択的に測定装置に接続されたコンピュータまたは別の人工メモリによってＣ反応性タンパク質濃度を計算する。

【０１２９】

実施例６：全血サンプル中のＣ反応性タンパク質の測定法

前記個別のコンテナ（未知の血液サンプルまたは血液サンプルのアリコートと合わせる）を室温に維持し、蛍光偏光装置の中またはこの装置上の電子温度測定デバイスを使用し、偏光値、異なる時間、およびＣ反応性タンパク質濃度値に加えて４次元の検量線を使用し（第４次元は温度である）、得られた値とを未知のサンプルから得た値と比較して任意選択的に標準的な統計学のテキストに記載の最適な適合のための最小二乗法を使用し、任意選択的に測定装置に接続したコンピュータまたは別の人工メモリによってＣ反応性タンパク質濃度を計算すること以外は、実施例５に記載の本発明の方法を行う。

【０１３０】

実施例７：テオフィリンのシナニン－５アナログの合成

Ｒｅｓｅａｒｃｈ Ｃｏｍｍｕｎｉｃａｔｉｏｎｓ ｉｎ Ｃｈｅｍｉｃａｌ Ｐａｔｈｏｌｏｇｙ ａｎｄ Ｐｈａｒｍａｃｏｌｏｇｙ、１３巻、４９７～５０５頁、１９７６およびＣｌｉｎｉｃａｌ Ｃｈｅｍｉｓｔｒｙ、２７巻、２２～２２６頁、１９８１に記載のように８－（３－カルボキシプロピル）－１，３－ジメチルキサンチン無水物を合成する。ジアミノプロパノールを無水テトラヒドロフランに溶解する。別のフラスコ中に、半量の８－（３－カルボキシプロピル）－１，３－ジメチルキサンチン無水物をテトラヒドロフラン溶液に溶解する。ジアミノプロパノール溶液を撹拌しながら８－（３－カルボキシプロピル）－１，３－ジメチルキサンチン無水物溶液を添加し、得られた溶液を室温で一晩静置する。任意選択的に、活性化シアニン色素をあまり消費したくない場合、当業者に周知の従来技術を使用したＨＰＬＣクロマトグラフィーによって得られた付加物を精製する（以下を参照のこと）。

【０１３１】

その後、Ａｍｅｒｓｈａｍ Ｐｈａｒｍａｃｉａ Ｂｉｏｔｅｃｈ、ＵＫから販売のＣｙ５ Ｆｌｕｏｒｏｌｉｎｋ活性化シアニンのジアミノプロパノールに使用するモル量を無水テトラヒドロフラン溶液中に６回溶解し、これを上記の溶液に撹拌しながら添加する。得られた混合物を放置して室温で一晩暗所にて反応させる。この方法では、水溶性ジアミノプロパノールスペーサーを含むＣｙ５ Ｆｌｕｏｒｏｌｉｎｋ活性化シアニン色素を含む純粋ではない８－（３－カルボキシプロピル）－１，３－ジメチルキサンチン付加物のストック溶液が得られる。

【０１３２】

実施例１に記載の薄層クロマトグラフィーによって、水溶性ジアミノプロパノールスペーサーを含むＣｙ５ Ｆｌｕｏｒｏｌｉｎｋ活性化シアニン色素を含む得られた８－（３－カルボキシプロピル）－１，３－ジメチルキサンチン付加物を精製し、シリカゲルプレートの質に依存して溶出混合物中のｎ－ブタノール、酢酸、および水の体積を調整して良好に分離させる。従来技術での溶出後、シリカゲルプレートを乾燥させ、目視およびＵＶランプによって検査して、Ｃｙ５ Ｆｌｕｏｒｏｌｉｎｋスポットを含む８－（３－カルボ

キシプロピル）－１，３－ジメチルキサンチン付加物のスポットを同定する。Ｃｙ５　Ｆｌｕｏｒｏｌｉｎｋスポットを含む８－（３－カルボキシプロピル）－１，３－ジメチルキサンチン付加物を含むシリカゲルを鋏またはスパチュラによって単離する。単離したシリカゲルを１０ｍＭ　ＴＲＩＳ緩衝液（ｐＨ＝８．０）に懸濁し、Ｃｙ５　Ｆｌｕｏｒｏｌｉｎｋを含む８－（３－カルボキシプロピル）－１，３－ジメチルキサンチン付加物を溶液に溶出する。チューブの底にシリカゲルを沈殿させる。Ｃｙ５　Ｆｌｕｏｒｏｌｉｎｋを含む８－（３－カルボキシプロピル）－１，３－ジメチルキサンチン付加物を含むＴＲＩＳ緩衝液をデカントする。

【０１３３】

所望ならば、スケールアップのために薄層クロマトグラフィーの代わりに当業者に周知の従来のＨＰＬＣ分離技術を使用することができる。例えば、実施例１に記載のＨＰＬＣ法を使用するが、Ｃ４またはＣ６逆相カラムを使用する。

【０１３４】

実施例８：テオフィリンに親和性を示す抗体のＦａｂフラグメント

Ｒｅｓｅａｒｃｈ　Ｃｏｍｍｕｎｉｃａｔｉｏｎｓ　ｉｎ　Ｃｈｅｍｉｃａｌ　Ｐａｔｈｏｌｏｇｙ　ａｎｄ　Ｐｈａｒｍａｃｏｌｏｇｙ、１３巻、４９７～５０５頁、１９７６に記載のように、８－（３－カルボキシプロピル）－１，３－ジメチルキサンチン無水物を合成し、アルブミン抱合体抗原を調製する。この抗原を使用して、マウスハイブリドーマを作製する。実施例７のテオフィリンのシアニン－５アナログに特異的反応性を示クローンを選択する。先行技術は、いくつかのハイブリドーマスクリーニング法を、例えば、Ｃｈａｍｐｅｌｌによる書籍「Ｍｏｎｏｃｌｏｎａｌ　ａｎｔｉｂｏｄｙ　ｔｅｃｈｎｏｌｏｇｙ（モノクローナル抗体技術）」、ＩＳＢＮ　０－４４４－８０５７５－３またはモノクローナル抗体技術に関する他のテキストで教示している。しかし本発明の発明者は、１５０ｍＭの塩化ナトリウムを含む１００ｍＭリン酸緩衝液（ｐＨ＝７．４）の作製を好んでいる。この緩衝液に、Ｓｉｇｍａのウシγグロブリンを２ｍｇ／ｍｌの濃度で、およびＰｉｅｒｃｅ　Ｃｈｅｍｉｃａｌ　Ｃｏｍｐａｎｙ、ＵＳのＴｒｉｔｏｎ　Ｘ－１００を０．１％ｖ／ｖの最終濃度で添加する。

【０１３５】

次いで、実施例７に記載のように作製した２ｍｇ／ｌの最終濃度のテオフィリンのシアニン－５アナログを添加する。コンテナ中の混合物を典型的には、装置中の小さな偏光レーザダイオードの使用によって６５０ｎｍの波長の偏光で照射したときに発光する蛍光の偏光を測定し、また、発光の偏光が一定である場合に装置で測定可能ならば狭いほうのバンド幅の偏光（６７０ｎｍ波長）を測定する。このような蛍光偏光測定による監視下で、調査すべきハイブリドーマ細胞から単離した抗体を添加する。各添加ごとに、更なる抗体の添加前に蛍光偏光シグナルが安定化するまで待つ。混合物の蛍光偏光が特異的に増加する１つまたは複数のハイブリドーマから抗体を選択するが、蛍光偏光技術に対する効果がテオフィリンで処理されていないヒト血清の区画またはヒト個体由来の血清によってブロックされるか有意に減少するハイブリドーマ由来の抗体を選択しない（しばしば対抗選択と呼ばれる）。目的のハイブリドーマから単離した抗体がカフェイン、他の薬物、またはテオフィリンに類似の食物成分と反応しないかを特にチェックする。

【０１３６】

選択したハイブリドーマ由来の抗体が単離された場合、Ｐｉｅｃｅ　Ｃｈｅｍｉｃａｌ　Ｃｏｍｐａｎｙから販売のＩｍｍｎｕＰｕｒｅ　Ｆａｂ調製キットを使用し、キットの説明に従ってＦＡＢフラグメントを調製する。

【０１３７】

実施例９：蛍光免疫複合体の使用による全血中のテオフィリン濃度の定量法

実施例８に記載の抗体のＦＡＢフラグメントおよび実施例７に記載のテオフィリンのシナニン－５アナログを作製する。

【０１３８】

その後、以下のプロトコールにしたがって、３２℃で一時的アッセイ試薬を作製する：１

５０ｍＭ塩化ナトリウムを含む１００ｍＭリン酸緩衝液（ｐＨ＝７．４）。この緩衝液にＳｉｇｍａのウシγグロブリンを２ｍｇ／ｍｌの濃度で添加し、最終濃度０．１％ｖ／ｖのＰｉｅｒｃｅ Ｃｈｅｍｉｃａｌ Ｃｏｍｐａｎｙ、ＵＳのＴｒｉｔｏｎ Ｘ－１００を最終純度０．１％ｖ／ｖ添加する。非常にバックグラウンドの低い蛍光を有する高純度の試薬を選択すべきである。

【０１３９】

次いで、実施例７に記載のように作製した２ｍｇ／ｌの最終濃度のテオフィリンのシナニン－５アナログを添加する。コンテナ中の混合物を典型的には、装置中の小さな偏光レーザダイオードの使用によって６５０ｎｍの波長の偏光で照射したときに発光する蛍光の偏光を測定し、また、発光の偏光が一定である場合に装置で測定可能ならば狭いほうのバンド幅の偏光（６７０ｎｍ波長）を測定する。このような蛍光偏光測定による監視下で、抗テオフィリンＦＡＢフラグメントを添加する。各添加ごとに、更なるＦＡＢフラグメントの添加前に蛍光偏光シグナルが安定化するまで待つ。ＦＡＢフラグメントの増加に伴って偏光値が有意に増加しなくなるまでＦＡＢフラグメントを添加しつづける。この蛍光偏光値を記録する。初期傾向偏光値を差し引き、値の間の差、（以下「偏光値の差」と称す）を計算する。

【０１４０】

最初からやり直し、一時的アッセイ試薬と同一の最終アッセイ試薬を作成するが、蛍光偏光値が初期偏光値＋８０％の「偏光値の差」と等しくなったときにＦＡＢフラグメントの添加を終了する。

【０１４１】

最終アッセイ試薬の用意は完了したが、任意選択的に長期保存には０．０１％アジ化ナトリウムなどの適切な静菌薬を添加する。未知の血液サンプルまたは血液サンプルのアリコートと組み合わせるために、個別のコンテナ中にこの混合物のアリコート（例えば、１ｍｌ）を維持する。

【０１４２】

血液サンプル中のテオフィリン濃度の定量時に、例えば予め測定したキャピラリの使用によってアリコート（例えば、体積２０μｌの全血サンプル）を取り出し、その後この予め測定したキャピラリと個別のコンテナ中のアッセイ試薬とを合わせ、個別のコンテナは温度を３２℃に制御した場所に維持する。典型的には、実施例３に記載のコンテナを使用する。その後、コンテナを震盪し、キャピラリから血液を流れ出させ、細胞をアッセイ試薬で溶解する。試験サンプルアリコートのテオフィリンは、アッセイ試薬の免疫複合体の蛍光テオフィリンと置換し始める。

【０１４３】

震盪直後、コンテナを３２℃の蛍光偏光測定装置に入れる。発光蛍光の偏光の測定のために、典型的には小さな偏光レーザダイオードの使用によってコンテナ中の混合物に６５０ｎｍの波長の偏光を照射し、また、発光の偏光が一定である場合に装置で測定可能ならば狭いほうのバンド幅の偏光（６７０ｎｍ波長）を測定する。周知の濃度のテオフィリン濃度の基準物質の測定によって得た検量線への測定した発光の偏光値の代入によって未知のサンプルのテオフィリン濃度を計算する。このような検量線を、しばしば測定装置のコンピュータに保存して、この装置でテオフィリン濃度を直接読み取ることができる。

【０１４４】

フローセルを具備する測定装置を使用する場合、装置の製造者の指示に従って、測定のために混合物をフローセル測定に通過させる。

【０１４５】

測定装置が可能であるならば、偏光値が安定後に偏光を読み取る代わりに速度様式での時間の関数としての偏光値を測定した場合に、さらに良好な精度を得られる。可能ならば、測定装置を任意選択的にソフトウェアで定義されたシグナルを連続的または異なる測定点で記録するコンピュータに接続する。次いで、保存した値と時間の関数としての既知のテオフィリン濃度の標準物質の測定によって得られた値とを比較する。任意選択的に、偏光

値、異なる時間、およびテオフィリン濃度値を使用した３次元検量線を読み取り、得られた値と未知のサンプルとを比較して、任意選択的に標準的な統計学のテキストに記載の最適な適合のための最小二乗法を使用し、任意選択的に測定装置に接続したコンピュータまたは別の人工メモリによってテオフィリン濃度を計算する。さらに、温度記録装置にアクセスする場合、前記個別のコンテナ（未知の血液サンプルまたは血液サンプルのアリコートと合わせる）を室温に維持し、蛍光偏光装置の中またはこの装置上の電子温度測定デバイスを使用し、偏光値、異なる時間、およびテオフィリン濃度値に加えて４次元の検量線を使用し（第４次元は温度である）、得られた値と未知のサンプルとを比較して任意選択的に標準的な統計学のテキストに記載の最適な適合のための最小二乗法を使用し、任意選択的に測定装置に接続したコンピュータまたは別の人工メモリによってテオフィリン濃度を計算する。

【０１４６】

血液サンプル中のテオフィリン濃度は、有意に変化する。主に治療濃度値の測定に関心が寄せられている。しかし、より高い毒素濃度は法医学で関心が寄せられており、より低い濃度はスポーツ医学で関心が寄せられている。本発明の方法の実施例の実施では、テオフィリンのシナニン－５アナログ濃度を調整し、ＦＡＢフラグメント濃度を調整する。高濃度範囲では、高い解離定数のＦＡＢフラグメントを選択すべきである。低濃度範囲では、高解離定数で且つ高結合定数のＦＡＢフラグメントを使用する。

【０１４７】

実施例１０：尿中アルブミン濃度定量用の蛍光ペプチド

従来技術によってペプチドＡｓｐ－Ａｌａ－Ｈｉｓ－Ｌｙｓ－Ｓｅｒ－Ｇｌｕ－Ｖａｌ－Ａｌａ（ヒトアルブミンのＮ末端ペプチド）を合成し、２ｍｇ／ｍｌの濃度で純水に溶解する。５０μｌの前記ペプチド溶液と５０μｌの重炭酸ナトリウム緩衝液（ｐＨ＝９．２）と混合する。任意選択的に前記溶液を含む無水ＤＭＳＯに溶解した０．１μｍｏｌの６－カルボキシフルオロセイン－Ｎ－ヒドロキシスクシニミドを混合し、暗所の室温で一晩静置し、その後溶液を暗所の冷蔵庫で保存する。所望ならば、５μｌの１０ｍｍｏｌエタノールアミン溶液を添加して、全ての残存する活性化蛍光色素をブロックするか、溶液（以下ストック溶液と称す）を放置して保存中に未反応色素を加水分解させることができる。

【０１４８】

薄層クロマトグラフィーによってストック溶液から蛍光標識ペプチドを精製する。シリカゲルプレート上にストック溶液のアリコートを加え、ｎ－ブタノール：酢酸：水混合物で溶出する。シリカゲルの質に依存して、ｎ－ブタノール：酢酸：水の相対含有量を調整して理想的な分離を得ることができる。従来技術での溶出後、シリカゲルプレートを乾燥し、目視およびＵＶランプ（任意選択的に並行実験でニンヒドリンスプレーを使用する）によって検査して、非標識ペプチドおよび遊離のフルオレセイン色素分子から分離した標識ペプチドスポットを同定する。鋏またはスパチュラでフルオレセイン標識ペプチドを含むシリカゲルを単離する。５０ｍＭ　ＢＩＳ－ＴＲＩＳ緩衝液（ｐＨ＝８．０）中に単離したシリカゲルを懸濁し、溶液中にフルオレセイン標識ペプチドを溶出させる。チューブの底にシリカゲルを沈殿させる。精製フルオレセイン標識ペプチドを含むＴＲＩＳ緩衝液をデカントする。

【０１４９】

所望ならば、スケールアップのために薄層クロマトグラフィーの代わりに当業者に周知の他の従来のＨＰＬＣ分離技術を使用することができる。例えば、実施例１に記載のＨＰＬＣ法を使用する。

【０１５０】

実施例１１：蛍光免疫複合体の使用による尿中アルブミン濃度の定量法

当業者に周知の従来技術を使用して（例えば、キーホールリンペットシアニンへのペプチドの結合によって形成された抗原の使用）、抗Ａｓｐ－Ａｌａ－Ｈｉｓ－Ｌｙｓ－Ｓｅｒ－Ｇｌｕ－Ｖａｌ－Ａｌａ抗体を作製する。あるいは、ペプチド合成物を購入し、例えば

ベルギーのＥｕｒｏｇｅｎｅｎｔｅｃｈ Ｃｏｍｐａｎｙなどのサービス提供企業からの抗体を惹起する。Ｐｉｅｒｃｅ Ｃｈｅｍｉｃａｌ Ｃｏｍｐａｎｙから販売のＩｍｍｎｕＰｕｒｅ Ｆａｂ調製キットを使用し、キットの説明に従って抗体からＦＡＢフラグメントを調製する。実施例１０に記載のようにフルオレセイン標識ペプチドを作製する。モノクローナル抗体の選択については、以下を参照のこと。

【０１５１】

その後、以下のプロトコールにしたがって、３２℃で一時的アッセイ試薬を作製する。１５０ｍＭ塩化ナトリウムを含む１００ｍＭリン酸緩衝液（ｐＨ＝７．４）を製作する。この緩衝液にＳｉｇｍａのウシγグロブリンを２ｍｇ／ｍｌの濃度で添加し、最終濃度０．１％ｖ／ｖのＰｉｅｒｃｅ Ｃｈｅｍｉｃａｌ Ｃｏｍｐａｎｙ、ＵＳのＴｒｉｔｏｎ Ｘ－１００を最終濃度０．１％ｖ／ｖで添加する。使用されるモノクローナル抗体に反応性を示すタンパク質を除去する必要がある場合には（以下を参照のこと）、当業者に周知の従来の免疫免疫吸着技術の使用によって、溶液を抗ヒトアルブミンモノクローナル抗体を含む免疫吸着剤に通す。

【０１５２】

非常に低いバックグラウンドの蛍光を有する試薬を選択すべきである。

【０１５３】

次いで、１．０＊１０Ｅ－９ｍｏｌ／ｌの最終濃度のフルオレセイン標識ペプチドを添加する。コンテナ中の混合物を４７５ｎｍの波長の偏光で照射したときに発光する蛍光の偏光を測定し、発光の偏光が一定である場合に装置で測定可能ならば狭いほうのバンド幅（典型的には１０ｎｍ）の偏光（５２５ｎｍ波長）を測定する。このような蛍光偏光測定による監視下で、前記抗アルブミンＦＡＢフラグメントを添加する。各添加のために、より多数のＦＡＢフラグメントの添加前に蛍光偏光シグナルが安定化するまで待つ。ＦＡＢフラグメントの増加に伴って偏光値が有意に増加しなくなるまでＦＡＢフラグメントを添加しつづける。この蛍光偏光値を記録する。初期傾向偏光値を差し引き、値の間の差（以下「偏光値の差」と称す）を計算する。

【０１５４】

最初からやり直し、一時的アッセイ試薬と同一の最終アッセイ試薬を作成するが、蛍光偏光値が初期偏光値＋８０％の「偏光値の差」と等しくなったときにＦＡＢ抗アルブミンフラグメントの添加を終了する。

【０１５５】

最終アッセイ試薬を今用意するが、任意選択的に長期保存には０．０１％アジ化ナトリウムなどの適切な静菌薬を添加する。未知の尿サンプルまたは尿サンプルのアリコートと組み合わせるために、個別のコンテナ中にこの混合物のアリコート（例えば、２ｍｌ）を維持する。

【０１５６】

尿サンプル中のアルブミン濃度の定量時に、例えば予め測定したキャピラリの使用によってアリコート（体積２０μｌ）の尿サンプルを取り、その後この予め測定したキャピラリと個別のコンテナ（個別のコンテナは温度を３２℃に制御した場所に維持されている）中のアッセイ試薬とを合わせる。典型的には、実施例３に記載のコンテナを使用する。その後、コンテナを震盪し、キャピラリから尿を流れ出させる。試験サンプルアリコートのアルブミンは、アッセイ試薬の免疫複合体のフルオレセイン標識ペプチドと置換し始める。

【０１５７】

震盪直後、コンテナを３２℃の蛍光偏光測定装置に入れる。発光蛍光の偏光の測定のために、コンテナ中の混合物に４７５ｎｍの波長の偏光を照射し、発光の偏光が一定である場合に装置で測定可能ならば狭いほうのバンド幅（典型的には１０ｎｍ）の偏光（５２５ｎｍ波長）を測定する。既知のヒトアルブミン濃度の基準物質の測定によって得た検量線への測定した発光の偏光値の代入によって未知のサンプルのヒトアルブミン濃度を計算する。このような検量線を、しばしば測定装置のコンピュータに保存して、この装置でアルブミン濃度を直接読み取ることができる。

【０１５８】
フローセルを具備する測定装置を使用する場合、装置の製造者の指示に従って、測定のために混合物をフローセル測定に通過させる。
【０１５９】
測定装置が可能であるならば、偏光値が安定になった後の偏光の読み取りの代わりに時間の関数としての偏光値を速度様式で測定した場合に、さらに良好な精度を得られる。可能ならば、シグナルを任意選択的にソフトウェアで定義された連続的または異なる測定点で記録するコンピュータに測定装置を接続する。次いで、記録した値と時間の関数としての既知のヒトアルブミン濃度の標準物質の測定によって得た値とを比較する。任意選択的に、偏光値、異なる時間、およびヒトアルブミン濃度値を使用した３次元検量線を記録し、未知のサンプルから得た値と比較して任意選択的に標準的な統計学のテキストに記載の最適な適合のための最小二乗法を使用し、任意選択的に測定装置に接続したコンピュータまたは別の人工メモリによってヒトアルブミン濃度を計算する。さらに、温度記録装置にアクセスする場合、前記個別のコンテナ（未知の血液サンプルまたは血液サンプルのアリコートと合わせる）を室温に維持し、蛍光偏光装置の中またはこの装置上の温度測定デバイスを使用し、偏光値、異なる時間、およびヒトアルブミン濃度値に加えて４次元の検量線を使用し（第４次元は温度である）、未知のサンプルから得た値と比較して任意選択的に標準的な統計学のテキストに記載の最適な適合のための最小二乗法を使用し、任意選択的に測定装置に接続したコンピュータまたは別の人工メモリによってヒトアルブミン濃度を計算する。
【０１６０】
尿サンプル中のアルブミン濃度は、有意に変化する。基準体積は２０ｍｇ／ｌの範囲である。不確かな供給源のわずかな値の上昇が何人かの個体で認められ、この個体は老年まで健康を維持し得る。糖尿病性腎臓損傷の初期徴候としてアルブミン値の中程度の上昇が認められている。重篤な腎臓病では、尿中アルブミン濃度は、数百ｍｇ／ｌまで上昇し得る。本発明の方法の実施例の実施では、フルオレセイン標識ペプチド濃度を調整し、ＦＡＢフラグメント濃度を調整する。高濃度範囲では、高い解離定数のＦＡＢフラグメントを選択すべきである。低濃度範囲では、高解離定数で且つ高結合定数のＦＡＢフラグメントを使用する。
【０１６１】
実施例１２：全血中のトブラマイシンの測定法
トブラマイシンに結合するＲＮＡ分子を、Ｗａｎｇ　＆　ａｌ．による論文「ＲＮＡ　ｍｏｌｅｃｕｌｅｓ　ｔｈａｔ　ｓｐｅｃｉｆｉｃａｌｌｙ　ａｎｄ　ｓｔｏｉｃｈｉｏｍｔｅｒｉｃａｌｌｙ　ｂｉｎｄ　ａｍｉｎｏｇｌｙｃｏｓｉｄｅ　ａｎｔｉｂｉｏｔｉｃｓ　ｗｉｔｈ　ｈｉｇｈ　ａｆｆｉｎｉｔｉｅｓ（高親和性のアミノグリコシド抗生物質に特異的且つ化学量論的に結合するＲＮＡ分子）」、「Ｂｉｏｃｈｅｍｉｓｔｒｙ」、１９９６、３５巻、１２３３８～１２３４６頁にしたがって合成する。
【０１６２】
Ｆｌｕｋａからトブラマイシンを購入する。２００μｍｏｌのトブラマイシンを５００μｌの水に溶解する。５００μｌのジメチルホルムアミドを添加する。溶液を５℃に冷却する。等モル濃度のＣｙ５　Ｆｌｕｏｒｏ　Ｌｉｎｋ分子のＤＭＦ溶液を添加し、混合物を５℃で２時間撹拌する。
【０１６３】
混合物を、０～５００ｍＭの水酸化アンモニウム勾配のＣＧ５０陽イオン交換クロマトグラフィーで溶出する。
【０１６４】
所望の抱合体を単離し、凍結乾燥して水酸化アンモニウムを除去する。
【０１６５】
１４０ｍＭ　ＮａＣｌ、５ｍＭ　ＫＣｌ、１ｍＭ　塩化マンガン、１ｍＭ塩化カルシウムおよび２０ｍＭ　Ｈｅｐｅｓ、３ｍｇウシγグロブリン／ｍｌ（ｐＨ＝７．４）からなる

アッセイ緩衝液を作製する。３０ｎｇのトブラマイシン－Ｃｙ－５－抱合体／ｍｌアッセイ溶液を添加し、その後等モル量の合成ＲＮＡ分子を添加する。

【０１６６】

最終アッセイ試薬を今用意するが、任意選択的に長期保存には０．０１％アジ化ナトリウムなどの適切な静菌薬を添加する。未知の血液サンプルまたは血液サンプルのアリコートと組み合わせるために、個別のコンテナ中にこの混合物のアリコート（例えば、１ｍｌ）を維持する。

【０１６７】

血液サンプル中のトブラマイシン濃度の定量時に、例えば予め測定したキャピラリの使用によってアリコート（体積２０μｌ）の血液サンプルを取り、その後この予め測定したキャピラリと個別のコンテナ（個別のコンテナは温度を３２℃に制御した場所に維持されている）中のアッセイ試薬とを合わせる。典型的には、実施例３に記載のコンテナを使用する。その後、コンテナを震盪し、キャピラリから血液を流れ出させる。試験サンプルアリコートのトブラマイシンは、アッセイ試薬のＲＮＡ／Ｃｙ－５－トブラマイシン複合体のＣｙ－５抱合トブラマイシンと置換し始める。

【０１６８】

震盪直後、コンテナを３２℃の蛍光偏光測定装置に入れる。発光蛍光の偏光の測定のために、コンテナ中の混合物に６４９ｎｍの波長の偏光を照射し、発光の偏光が一定である場合に装置で測定可能ならば狭いほうのバンド幅（典型的には１０ｎｍ）の偏光（６７０ｎｍ波長）を測定する。既知のヒトアルブミン濃度の基準物質の測定によって得た検量線への測定した発光の偏光値の代入によって未知のサンプルのトブラマイシン濃度を計算する。このような検量線を、しばしば測定装置のコンピュータに保存して、この装置でアルブミン濃度を直接読み取ることができる。

【０１６９】

フローセルを具備する測定装置を使用する場合、装置の製造者の指示に従って、測定のために混合物をフローセル測定に通過させる。

【０１７０】

測定装置が可能であるならば、偏光値が安定になった後の偏光の読み取りの代わりに時間の関数としての偏光値を速度様式で測定した場合に、さらに良好な精度を得られる。可能ならば、シグナルを任意選択的にソフトウェアで定義された連続的または異なる測定点で記録するコンピュータに測定装置を接続する。次いで、記録した値と時間の関数としての既知のトブラマイシン濃度の標準物質の測定によって得た値とを比較する。任意選択的に、偏光値、異なる時間、およびトブラマイシン濃度値を使用した３次元検量線を記録し、未知のサンプルから得た値と比較して任意選択的に標準的な統計学のテキストに記載の最適な適合のための最小二乗法を使用し、任意選択的に測定装置に接続したコンピュータまたは別の人工メモリによってトブラマイシン濃度を計算する。さらに、温度記録装置にアクセスする場合、前記個別のコンテナ（未知の血液サンプルまたは血液サンプルのアリコートと合わせる）を室温に維持し、蛍光偏光装置の中またはこの装置上の温度測定デバイスを使用し、偏光値、異なる時間、およびトブラマイシン濃度値に加えて４次元の検量線を使用し（第４次元は温度である）、未知のサンプルから得た値と比較して任意選択的に標準的な統計学のテキストに記載の最適な適合のための最小二乗法を使用し、任意選択的に測定装置に接続したコンピュータまたは別の人工メモリによってトブラマイシン濃度を計算する。

【０１７１】

血液サンプル中のトブラマイシン濃度は、使用するアッセイに依存して有意に変化する。治療濃度の測定は、臨床指標によって変化し、薬物動態学研究における血液濃度の測定と異なる。したがって、本発明の方法の実施例の実施では、トブラマイシン－Ｃｙ－５－抱合体およびＲＮＡ分子濃度を適切なレベルに調整する。「Ｂｉｏｃｈｅｍｉｓｔｒｙ」、１９９６、３５巻、１２３３８～１２３４６頁で公開されたＪ６ＲＮＡ　ＲＮＡ分子は適切に低濃度であり、他の低親和性のＲＮＡ分子を、Ｓｅｌｅｘ技術および当業者に周知の

合成法によって同定することができる。
【０１７２】
実施例１３：ヒト自己抗インスリン抗体濃度定量用の蛍光インスリン
従来技術によってヒトインスリンを合成し、２ｍｇ／ｍｌの濃度で純水に溶解する。５０μｌの前記ペプチド溶液と５０μｌの重炭酸ナトリウム緩衝液（ｐＨ＝９．２）と混合する。任意選択的に前記溶液を含む無水ＤＭＳＯに溶解した０．２のＣｙ５　Ｆｌｕｏｒｏｌｉｎｋ活性化シアニン色素（Ａｍｅｒｓｈａｍ　Ｐｈａｒｍａｃｉａ　Ｂｉｏｔｅｃｈ）を混合し、暗所の室温で一晩静置し、その後溶液を暗所の冷蔵庫で保存する。所望ならば、５μｌの１０ｍｍｏｌエタノールアミン溶液を添加して、全ての残存する活性化蛍光色素をブロックするか、溶液（以後ストック溶液と称す）を放置して保存中に未反応色素を加水分解させることができる。
【０１７３】
ストック溶液由来の純粋なＣｙ５標識インスリンを、溶出液として０．１％トリフルオロ酢酸を使用し、０％～６０％のアセトニトリルの勾配の０．１％トリフルオロ酢酸を使用したＣ４カラム（Ｗａｔｅｒｓ，ＵＳを含め、多くの業者により販売されている。）の逆相クロマトグラフィーによって精製する。フローセルに接続した光検出器を使用して、３４０ｎｍでの透過測定によりペプチド含有量をモニターし、６５０ｎｍでの透過測定によりＣｙ－５をモニターし、Ｃｙ－５－抱合インスリンを単離する。凍結乾燥によってアセトニトリルおよびトリフルオロ酢酸を除去する。
【０１７４】
あるいは、当業者に周知のペプチド抱合体単離用の他のＨＰＬＣ法を選択することができる。
【０１７５】
実施例１４：全血サンプル中の抗インスリン抗体の測定法
１５０ｍＭ塩化ナトリウムを含む１００ｍＭリン酸緩衝液（ｐＨ＝７．４）の作製によってアッセイ試薬を作製し、Ｓｉｇｍａの無インスリンウシγグロブリンを濃度２ｍｇ／ｍｌで、およびＰｉｅｒｃｅ　Ｃｈｅｍｉｃａｌ　Ｃｏｍｐａｎｙ，ＵＳのＴｒｉｔｏｎ　Ｘ－１００を最終濃度０．１％ｖ／ｖで添加する。非常に低いバックグラウンド蛍光を有する高純度の試薬を選択すべきである。１．０＊１０Ｅ－１２ｍｏｌ／ｍｌ（濃度の選択用、以下を参照のこと）の上記の実施例１３のＣｙ５－標識ヒトインスリンを添加し、任意選択的に長期保存には０．０１％アジ化ナトリウムなどの適切な静菌薬を添加する。未知の血液サンプルまたは血液サンプルのアリコートと組み合わせるための個別のコンテナ中にこの混合物のアリコート（例えば、１ｍｌ）を維持する。
【０１７６】
血液サンプル中の抗インスリン抗体濃度の定量時に、例えば予め測定したキャピラリの使用によってアリコート（例えば、２０μｌ）の全血サンプルを取り出し、その後この予め測定したキャピラリと個別コンテナ（個別のコンテナは温度を３２℃に調節した場所で維持されている）中のアッセイ試薬とを合わせる。典型的には、蛍光偏光測定用に構築された装置で測定される４つの研磨した透明の側面を有するキュベットの形態のコンテナを使用するが、このコンテナは取り外し可能なストッパまたはキャピラリの単純な落とし入れまたはシールを介したキャピラリの導入によってキャピラリをコンテナに挿入するシールを含む。さらに、キャピラリ／コンテナを、キャピラリがコンテナの底に落とし込まれて励起光または発光を妨害しないようにデザインする（以下を参照のこと）。その後コンテナを震盪し、キャピラリからの血液を流れ出させ、細胞をアッセイ試薬で溶解する。試験サンプルアリコートの抗インスリン抗体は、アッセイ試薬のＣｙ５－標識インスリンとの反応を開始する。
【０１７７】
震盪直後、コンテナを３２℃の蛍光偏光測定装置に入れる。発光蛍光の偏光の測定のために、典型的には装置中の小さな偏光レーザダイオードを使用して、コンテナ中の混合物に６５０ｎｍの波長の偏光を照射し、発光の偏光が一定である場合に狭いほうのバンド幅（

好ましくは５ｎｍのバンド）の偏光（６７０ｎｍ波長）を測定する。既知のインスリン濃度の基準物質の測定によって得た検量線への測定した発光の偏光値の代入によって未知のサンプルのインスリン濃度を計算する。このような検量線を、しばしば測定装置のコンピュータに保存して、この装置でインスリン濃度を直接読み取ることができる。

【０１７８】

フローセルを具備する測定装置を使用する場合、装置の製造者の指示に従って、測定のために混合物をフローセル測定に通過させる。インスリンは分子半径が幾らか長いので、光の偏光度の測定には、精度の高い蛍光偏光装置が好ましい。

【０１７９】

測定装置が可能であるならば、偏光値が安定になった後の偏光の読み取りの代わりに時間の関数としての偏光値を速度様式で測定した場合に、さらに良好な精度を得られる。可能ならば、シグナルを任意選択的にソフトウェアで定義された連続的または異なる測定点で記録するコンピュータに測定装置を接続する。次いで、記録した値と時間の関数としての既知の抗インスリン抗体濃度の標準物質の測定によって得た値とを比較する。任意選択的に、偏光値、異なる時間、および抗インスリン抗体濃度値を使用した３次元検量線を記録し、未知のサンプルから得た値と比較して任意選択的に標準的な統計学のテキストに記載の最適な適合のための最小二乗法を使用し、任意選択的に測定装置に接続したコンピュータまたは別の人工メモリによって抗インスリン抗体濃度を計算する。さらに、温度記録装置にアクセスする場合、前記個別のコンテナ（未知の血液サンプルまたは血液サンプルのアリコートと合わせる）を室温に維持し、蛍光偏光装置の中またはこの装置上の温度測定デバイスを使用し、偏光値、異なる時間、および抗インスリン抗体濃度値に加えて４次元の検量線を使用し（第４次元は温度である）、未知のサンプルから得た値と比較して任意選択的に標準的な統計学のテキストに記載の最適な適合のための最小二乗法を使用し、任意選択的に測定装置に接続したコンピュータまたは別の人工メモリによって抗インスリン抗体濃度を計算する。

【０１８０】

抗インスリン抗体は、典型的には、健常な被験体では非常に低濃度で存在する。初期段階の軽度の糖尿病は、典型的にはインスリンに対する抗体の濃度は低いが、長年インスリン治療を受けている患者は、典型的にはインスリンに対する抗体の濃度は高い。したがって、Ｃｙ－５－抱合インスリン濃度および全サンプル体積を、サンプルを採取した患者の種類にしたがって選択しなければならない。測定した蛍光偏光が選択したＣｙ－５－インスリン抱合体濃度およびサンプル体積から得た検量線から外れている場合、Ｃｙ－５－インスリン抱合体濃度およびサンプル体積を選択しなければならない。

【０１８１】

実施例１５：Ｒｕ－リガンド免疫複合体による尿濃度の定量

本実施例によれば、分析物（単数または複数）の濃度を定量するために、蛍光残基が結合している結合パートナーの分子量を増加させることが可能であることが示される。しかし、先に記載のように、これには、減衰期間がより長い蛍光残基を使用する必要がある。

【０１８２】

Ｐｉｅｒｃｅ　Ｃｈｅｍｉｃａｌ　Ｃｏｍｐａｎｙから購入したＩｍｍｕｎｏＰｕｒｅ　Ｆａｂ調製キットを使用し、キットの説明書にしたがって、ＤａｋｏＡＳ、Ｄｅｎｍａｒｋから購入したウサギ抗ヒトアルブミン抗体からＦＡＢフラグメントを作製する。

【０１８３】

さらに、Ｔｅｒｐｅｔｓｃｈｉｎｇ　＆　ａｌ．による、「Ａｎａｌｙｔｉｃａｌ　Ｂｉｏｃｈｅｍｉｓｔｒｙ」、２２７巻、１４０～１４７頁、１９９５に記載のように、Ｒｕ（ｂｐｙ）ｄｃｂｐｙとのヒト血清アルブミン抱合体を作製する。

【０１８４】

その後、以下のプロトコールにしたがって、３２℃で一時的アッセイ試薬を作製する：１５０ｍＭ塩化ナトリウムを含む１００ｍＭリン酸緩衝液（ｐＨ＝７．４）を作製する。この緩衝液にＳｉｇｍａのウシγグロブリンを濃度２ｍｇ／ｍｌで添加し、Ｐｉｅｒｃｅ

Ｃｈｅｍｉｃａｌ Ｃｏｍｐａｎｙ，ＵＳのＴｒｉｔｏｎ Ｘ－１００を最終濃度０．０１％ｖ／ｖで添加する。非常に低いバックグラウンドの蛍光を有する高純度の試薬を選択すべきである。

【０１８５】

次いで、１．０＊１０Ｅ－９ｍｏｌ／ｌの最終濃度のＲｕ（ｂｐｙ）ｄｃｂｐｙとの前記ヒト血清アルブミン抱合体を添加する。コンテナ中の混合物を４８５（あるいは３６０、Ｔｅｒｐｅｔｓｃｈｉｎｇの前記論文を参照のこと）ｎｍの波長の偏光で照射したときに発光する蛍光の偏光を測定し、発光の偏光が一定である場合に装置で測定可能ならば狭いほうのバンド幅（典型的には１０ｎｍ）の偏光（６６０ｎｍ波長）を測定する。このような蛍光偏光測定による監視下で、抗アルブミンＦＡＢフラグメントを添加する。各添加のために、より多数のＦＡＢフラグメントの添加前に蛍光偏光シグナルが安定化するまで待つ。ＦＡＢフラグメントの増加に伴って偏光値が著しく増加しなくなるまでＦＡＢフラグメントを添加しつづける。この蛍光偏光値を記録する。初期傾向偏光値を差し引き、値の間の差（以下「偏光値の差」と称す）を計算する。

【０１８６】

最初からやり直し、一時的アッセイ試薬と同一の最終アッセイ試薬を作成するが、蛍光偏光値が初期偏光値＋８０％の「偏光値の差」と等しくなったときにＦＡＢ抗アルブミンフラグメントの添加を終了する。

【０１８７】

最終アッセイ試薬を今用意するが、任意選択的に長期保存には０．０１％アジ化ナトリウムなどの適切な静菌薬を添加する。未知の尿サンプルまたは尿サンプルのアリコートと組み合わせるために、個別のコンテナ中にこの混合物のアリコート（例えば、２ｍｌ）を維持する。

【０１８８】

尿サンプル中のアルブミン濃度の定量時に、例えば予め測定したキャピラリの使用によってアリコート（体積２０μｌ）の尿サンプルを取り、その後この予め測定したキャピラリと個別のコンテナ（個別のコンテナは温度を３２℃に制御した場所に維持されている）中のアッセイ試薬とを合わせる。典型的には、実施例３に記載のコンテナを使用する。その後、コンテナを震盪し、キャピラリから尿を流れ出させる。試験サンプルアリコートのアルブミンは、アッセイ試薬の免疫複合体のＲｕ（ｂｐｙ）ｄｃｂｐｙのヒト血清アルブミン抱合体と置換し始める。

【０１８９】

震盪直後、コンテナを３２℃の蛍光偏光測定装置に入れる。発光蛍光の偏光の測定については、上記の実施例を参照のこと。既知のヒトアルブミン濃度の基準物質の測定によって得た検量線への測定した発光の偏光値の代入によって未知のサンプルのヒトアルブミン濃度を計算する。このような検量線を、しばしば測定装置のコンピュータに保存して、アルブミン濃度を直接読み取ることができる。

【０１９０】

フローセルを具備する測定装置を使用する場合、装置の製造者の指示に従って、測定のために混合物をフローセル測定に通過させる。

【０１９１】

測定装置が可能であるならば、偏光値が安定になった後の偏光の読み取りの代わりに時間の関数としての偏光値を速度様式で測定した場合に、さらに良好な精度を得られる。可能ならば、シグナルを任意選択的にソフトウェアで定義された連続的または異なる測定点で記録するコンピュータに測定装置を接続する。次いで、記録した値と時間の関数としての既知のヒトアルブミン濃度の標準物質の測定によって得た値とを比較する。任意選択的に、偏光値、異なる時間、およびヒトアルブミン濃度値を使用した３次元検量線を記録し、未知のサンプルから得た値と比較して任意選択的に標準的な統計学のテキストに記載の最適な適合のための最小二乗法を使用し、任意選択的に測定装置に接続したコンピュータまたは別の人工メモリによってヒトアルブミン濃度を計算する。さらに、温度記録装置にア

クセスする場合、前記個別のコンテナ（未知の血液サンプルまたは血液サンプルのアリコートと合わせる）を室温に維持し、蛍光偏光装置の中またはこの装置上の温度測定デバイスを使用し、偏光値、異なる時間、およびヒトアルブミン濃度値に加えて４次元の検量線を使用し（第４次元は温度である）、未知のサンプルから得た値と比較して任意選択的に標準的な統計学のテキストに記載の最適な適合のための最小二乗法を使用し、任意選択的に測定装置に接続したコンピュータまたは別の人工メモリによってヒトアルブミン濃度を計算する。

【０１９２】

尿サンプル中のアルブミン濃度は、有意に変化する。基準体積は２０ｍｇ／ｌの範囲である。不確かな供給源のわずかな値の上昇が何人かの個体で認められ、この個体は老年まで健康を維持し得る。糖尿病性腎臓損傷の初期徴候としてアルブミン値の中程度の上昇が認められている。重篤な腎臓病では、尿中アルブミン濃度は、数百ｍｇ／ｌまで上昇し得る。本発明の方法の実施例の実施では、Ｒｕ（ｂｐｙ）ｄｃｂｐｙとのヒト血清アルブミン抱合体濃度を調整し、ＦＡＢフラグメント濃度を調整する。高濃度範囲では、高い解離定数のＦＡＢフラグメントを選択すべきである。低濃度範囲では、高解離定数で且つ高結合定数のＦＡＢフラグメントを使用する。

【０１９３】

実施例１６：アプタマー複合体の使用による血液テオフィリンの測定法。

Ｒ．Ｄ．Ｊｅｎｉｓｏｎ　＆　ａｌ　ｉｎ　Ｓｃｉｅｎｃｅ、２６３巻、１９９４に記載のように、１．０＊１０Ｅ７／ｍｏｌａｒの結合定数でテオフィリンに結合するＲＮＡアプタマーを合成する。

【０１９４】

実施例７に記載のようにテオフィリンのシアニン－５アナログを合成する。

【０１９５】

１４０ｍＭ　ＮａＣｌ、５ｍＭ　ＫＣｌ、１ｍＭ　塩化マンガン、１ｍＭ塩化カルシウムおよび２０ｍＭ　Ｈｅｐｅｓ、３ｍｇウシγグロブリン／ｍｌ（ｐＨ＝７．４）からなるアッセイ緩衝液を作製する。２ｍｇのテオフィリンのシアニン－５アナログ／ｌアッセイ溶液を添加し、その後等モル量の合成ＲＮＡ分子を添加する。

【０１９６】

最終アッセイ試薬を今用意するが、任意選択的に長期保存には０．０１％アジ化ナトリウムなどの適切な静菌薬を添加する。未知の血液サンプルまたは血液サンプルのアリコートと組み合わせるために、個別のコンテナ中にこの混合物のアリコート（例えば、１ｍｌ）を維持する。

【０１９７】

血液サンプル中のテオフィリン濃度の定量時に、例えば予め測定したキャピラリの使用によってアリコート（体積２０μｌ）の血液サンプルを取り、その後この予め測定したキャピラリと個別のコンテナ（個別のコンテナは温度を３２℃に制御した場所に維持されている）中のアッセイ試薬とを合わせる。典型的には、実施例３に記載のコンテナを使用する。その後、コンテナを震盪し、キャピラリから血液を流れ出させる。試験サンプルアリコートのテオフィリンは、アッセイ試薬のテオフィリン複合体のＲＮＡ／シアニン－５アナログのテオフィリンのシアニン－５アナログと置換し始める。

【０１９８】

震盪直後、コンテナを３２℃の蛍光偏光測定装置に入れる。発光蛍光の偏光の測定のために、コンテナ中の混合物に６４９ｎｍの波長の偏光を照射し、発光の偏光が一定である場合に装置で測定可能ならば狭いほうのバンド幅（典型的には１０ｎｍ）の偏光（６７０ｎｍ波長）を測定する。既知のヒトアルブミン濃度の基準物質の測定によって得た検量線への測定した発光の偏光値の代入によって未知のサンプルのテオフィリン濃度を計算する。このような検量線を、しばしば測定装置のコンピュータに保存して、この装置でテオフィリン濃度を直接読み取ることができる。

【０１９９】

フローセルを具備する測定装置を使用する場合、装置の製造者の指示に従って、測定のために混合物をフローセル測定に通過させる。

【０２００】

測定装置が可能であるならば、偏光値が安定になった後の偏光の読み取りの代わりに時間の関数としての偏光値を速度様式で測定した場合に、さらに良好な精度を得られる。可能ならば、シグナルを任意選択的にソフトウェアで定義された連続的または異なる測定点で記録するコンピュータに測定装置を接続する。次いで、記録した値と時間の関数としての既知のテオフィリン濃度の標準物質の測定によって得た値とを比較する。任意選択的に、偏光値、異なる時間、およびテオフィリン濃度値を使用した３次元検量線を記録し、未知のサンプルから得た値と比較して任意選択的に標準的な統計学のテキストに記載の最適な適合のための最小二乗法を使用し、任意選択的に測定装置に接続したコンピュータまたは別の人工メモリによってテオフィリン濃度を計算する。さらに、温度記録装置にアクセスする場合、前記個別のコンテナ（未知の血液サンプルまたは血液サンプルのアリコートと合わせる）を室温に維持し、蛍光偏光装置の中またはこの装置上の温度測定デバイスを使用し、偏光値、異なる時間、およびテオフィリン濃度値に加えて４次元の検量線を使用し（第４次元は温度である）、未知のサンプルから得た値と比較して任意選択的に標準的な統計学のテキストに記載の最適な適合のための最小二乗法を使用し、任意選択的に測定装置に接続したコンピュータまたは別の人工メモリによってテオフィリン濃度を計算する。

【０２０１】

血液サンプル中のテオフィリンン濃度は、有意に変化する。主に治療濃度値の測定に関心が寄せられている。しかし、より高い毒素濃度は法医学で関心が寄せられており、より低い濃度はスポーツ医学で関心が寄せられている。本発明の方法の実施例の実施では、テオフィリンのシナニン－５アナログ濃度を調整するので、ＲＮＡアプタマー濃度が調整される。

【０２０２】

実施例１７：尿中のヒト絨毛膜ゴナドトロピンおよびアルブミンの同時測定

Ｓｉｇｍａからヒトβサブユニット絨毛膜ゴナドトロピン（ＢＨＣＧ）を購入し、当業者に周知の方法を使用して、溶出液として０．１５Ｍ塩化ナトリウムを含む１０ｍＭリン酸緩衝液（ｐＨ＝７．２）を使用したサイズ排除クロマトグラフィーによってさらに精製する。Ａｍｅｒｓｈａｍ Ｐｈａｒｍａｃｉａ Ｂｉｏｔｅｃｈのパッケージの説明にしたがって、ヒトβサブユニット絨毛膜ゴナドトロピンをＣｙ－５ Ｆｌｕｏｒｏｌｉｎｋで標識し、Ａｍｅｒｓｈａｍ Ｐｈａｒｍａｃｉａ Ｂｉｏｔｅｃｈが推奨するようにサイズ排除クロマトグラフィーによる精製後に同一の方法で分子ＢＨＣＧあたりのＣｙ－５の含有量を測定する。

【０２０３】

Ｃｈｅｍｉｃｏｎ Ｉｎｃ．、ＵＳからモノクローナル抗ＢＨＣＧを購入し、Ｐｉｅｒｃｅ Ｃｈｅｍｉｃａｌ ＣｏｍｐａｎｙのＩｍｍｕｎｏＰｕｒｅ ＦＡＢ調製キットによって抗体からＦＡＢフラグメントを調製する。

【０２０４】

当業者に周知の従来技術を使用して（例えば、キーホールリンペットシアニンへのペプチドの結合によって形成された抗原の使用）、抗Ａｓｐ－Ａｌａ－Ｈｉｓ－Ｌｙｓ－Ｓｅｒ－Ｇｌｕ－Ｖａｌ－Ａｌａ抗体を作製する。あるいは、ペプチド合成物を購入し、例えばベルギーのＥｕｒｏｇｅｎｅｎｔｅｃｈなどのサービス提供企業からの抗体を惹起する。Ｐｉｅｒｃｅ Ｃｈｅｍｉｃａｌ Ｃｏｍｐａｎｙから販売されているＩｍｍｎｕＰｕｒｅ Ｆａｂ調製キットを使用し、キットの説明に従って抗体からＦＡＢフラグメントを調製する。実施例１０に記載のようにフルオレセイン標識ペプチドを作製し、実施例１１に記載のように尿中アルブミン定量用試薬を調製する。その後、尿中アルブミン定量用試薬にＣｙ５－標識ＢＨＣＧ（尿中での測定にはＢＨＣＧレベルの１／１００の分子濃度）を添加する。（適切なレベルは３０００Ｕ／ｌに対応するレベルであり得る）。

【０２０５】

モノクローナル抗体ｆの選択については、以下を参照のこと。

【０２０６】

コンテナ中の混合物を５５０ｎｍの波長の偏光で照射したときに発光する蛍光の偏光を測定し、発光の偏光が一定である場合に装置で測定可能ならば狭いほうのバンド幅（典型的には１０ｎｍ）の偏光（５７０ｎｍ波長）を測定する。このような蛍光偏光測定による監視下で、前記抗ＢＨＣＧＦＡＢフラグメントを添加する。各添加のために、より多数のＦＡＢフラグメントの添加前に蛍光偏光シグナルが安定化するまで待つ。ＦＡＢフラグメントの増加に伴って偏光値が有意に増加しなくなるまでＦＡＢフラグメントを添加しつづける。この蛍光偏光値を記録する。初期傾向偏光値を差し引き、値の間の差（以下「偏光値の差」と称す）を計算する。

【０２０７】

最初からやり直し、一時的アッセイ試薬と同一の最終アッセイ試薬を作成するが、蛍光偏光値が初期偏光値＋８０％の「偏光値の差」と等しくなったときにＦＡＢ抗ＢＨＣＧフラグメントの添加を終了する。

【０２０８】

最終アッセイ試薬を今用意するが、任意選択的に長期保存には０．０１％アジ化ナトリウムなどの適切な静菌薬を添加する。未知の尿サンプルまたは尿サンプルのアリコートと組み合わせるために、個別のコンテナ中にこの混合物のアリコート（例えば、２ｍｌ）を維持する。

【０２０９】

尿サンプル中のアルブミンおよびＨＣＧ濃度の定量時に、例えば予め測定したキャピラリの使用によってアリコート（体積２０μｌ）の尿サンプルを取り、その後この予め測定したキャピラリと個別のコンテナ（個別のコンテナは温度を３２℃に制御した場所に維持されている）中のアッセイ試薬とを合わせる。典型的には、実施例３に記載のコンテナを使用する。その後、コンテナを震盪し、キャピラリから尿を流れ出させる。試験サンプルアリコートのアルブミンは、アッセイ試薬のフルオレセイン標識免疫複合体のフルオレセイン標識インスリン様ペプチドと置換し始める。ＨＣＧは、アッセイ試薬のＣｙ－５標識免疫複合体中のＣｙ－５標識ＢＨＣＧを置換し始める。

【０２１０】

震盪直後、コンテナを３２℃の蛍光偏光測定装置に入れる。発光蛍光の偏光の測定のために、コンテナ中の混合物に４７５ｎｍおよび６５０ｎｍの波長の偏光を照射し、発光の偏光が一定である場合に装置で測定可能ならば狭いほうのバンド幅（典型的には１０ｎｍ）の５２５ｎｍおよび６７０ｎｍの波長で発光する偏光を測定する。スペクトルの重複および比較的高分子量のＣｙ－５標識ＢＨＣＧのために、偏光測定精度が高いさらに優れた二重波長照射装置が必要である。４７５ｎｍおよび６５０ｎｍでの間欠的照射を使用して過剰なスペクトルの干渉を回避することが好ましい。既知濃度のヒトアルブミンおよびＨＣＧの基準物質の測定によって得た「検量線」への測定した発光（５２５ｎｍおよび６７０ｎｍ）の偏光値の代入によって未知のサンプルのヒトアルブミンおよびＨＣＧ濃度を計算する。両波長での偏光によりアルブミンおよびＨＣＧの濃度が変化するので、このような検量線は４次元である。この検量線を測定装置のコンピュータに保存して、アルブミンおよびＨＣＧ濃度を直接読み取ることができる。

【０２１１】

フローセルを具備する測定装置を使用する場合、測定のために混合物をフローセル測定に通過させる。

【０２１２】

測定装置が可能であるならば、偏光値が安定になった後の偏光の読み取りの代わりに時間の関数としての偏光値を速度様式で測定した場合に、さらに良好な精度を得られる。可能ならば、シグナルを任意選択的にソフトウェアで定義された連続的または異なる測定点で記録するコンピュータに測定装置を接続する。次いで、記録した値と時間の関数としての既知のヒトアルブミンおよびＨＣＧ濃度の標準物質の測定によって得た値とを比較する。

任意選択的に、２つの波長の偏光値、異なる時間、およびヒトアルブミンおよびＨＣＧ濃度値を使用したデータセットを記録し、未知のサンプルから得た値と比較して任意選択的に標準的な統計学のテキストに記載の最適な適合のための最小二乗法を使用し、任意選択的に測定装置に接続したコンピュータまたは別の人工メモリによってヒトアルブミン濃度を計算する。さらに、温度記録装置にアクセスする場合、前記個別のコンテナ（未知の血液サンプルまたは血液サンプルのアリコートと合わせる）を室温に維持し、蛍光偏光装置の中またはこの装置上の温度測定デバイスを使用し、任意選択的に標準的な統計学のテキストに記載の最適な適合のための最小二乗法を使用し、任意選択的に測定装置に接続したコンピュータまたは別の人工メモリによって異なる温度を使用したさらに多数のデータセットを使用する。

【０２１３】

尿サンプル中のアルブミン濃度は、有意に変化する。基準体積は２０ｍｇ／ｌの範囲である。不確かな供給源のわずかな値の上昇が何人かの個体で認められ、この個体は老年まで健康を維持し得る。糖尿病性腎臓損傷の初期徴候としてアルブミン値の中程度の上昇が認められている。重篤な腎臓病では、尿中アルブミン濃度は、数百ｍｇ／ｌまで上昇し得る。本発明の方法の実施例の実施では、フルオレセイン標識ペプチド濃度を調整し、ＦＡＢフラグメント濃度を調整する。高濃度範囲では、高い解離定数のＦＡＢフラグメントを選択すべきである。低濃度範囲では、高解離定数で且つ高結合定数のＦＡＢフラグメントを使用する。

【０２１４】

同様に、ＨＣＧ濃度は、一定の疾患、妊娠期によって異なり、性は明らかに異なる。ＦＡＢフラグメントを調製するために、抗ＢＨＣＧ抗体には抗ペプチド抗体フラグメントと類似の検討材料を使用しなければならない。

【０２１５】

妊婦の尿中ＨＣＧおよび尿中アルブミンの両方を定量することは興味深い。

【０２１６】

実施例１８：ヒト尿中のゴナドトロピン、アルブミン、および免疫グロブリンＧの同時測定

腎臓の主要な機能は（他の多数の機能に加えて）尿および他の小分子物質を排出することであるが、血中にアルブミンおよび他のタンパク質が残存している。蛋白尿の妊婦では、腎臓の小タンパク質および巨大タンパク質の選択性が向上することが興味深い。アルブミンおよび免疫グロブリンＧの同時測定を使用して、このような選択性を評価する。通常、尿は、アルブミンおよび免疫グロブリンを微量しか含まないが、免疫グロブリンよりも少なくとも１０倍のアルブミンを含む。重篤な腎機能障害を発症すると、通常このような選択性は失われる。

【０２１７】

ヒト尿中でのゴナドトロピン、アルブミン、および免疫グロブリンＧの同時測定に適切な試薬を得るために、以下のプロトコールを使用する。

【０２１８】

上記のＣＯＳＭＩＸプレキシングファージディスプレイ技術を使用して、免疫グロブリンクラスＧ分子に選択的に結合するペプチドを同定する。これは、ＣＯＳＭＩＸ　ＧｍｂＨ　ｃｏｍｐａｎｙから購入することができるサービスである。あるいは、プロテインＡをペプチドフラグメントに消化して、当該分野で周知の従来技術（例えば、Ｙｕｅら、Ｔｈｅ　Ｊｏｕｒｎａｌ　ｏｆ　Ｂｉｏｌｏｇｉｃａｌ　Ｃｈｅｍｉｓｔｒｙ、２７１巻、２２２４５～２２２５０頁、１９９６に記載）を使用して消化物からＩｇＧ結合ペプチドを同定および精製する。同定および精製したペプチドを、Ｍｏｌｅｃｕｌａｒ　Ｐｒｏｂｅｓ，Ｉｎｃ．から購入した５－カルボキシテトラメチルローダミンスクシジニミジルエステルでこの会社のパッケージの説明に記載の従来技術を使用して標識する。上記の他の実施例に記載の従来技術を使用した逆相クロマトグラフィーを使用してペプチドを標識したテトラメチルローダミンを精製する。

【０２１９】
実施例１７に記載の試薬に、ペプチドを標識したテトラメチルローダミンを添加する。試薬が例えば５０～５００ｍｇ／ｌの濃度範囲のアルブミン濃度の測定を意図する場合、ペプチドを標識したテトラメチルローダミンを０．０５μｍｏｌ／ｌの最終濃度で添加する。この方法では、テトラメチルローダミン－ペプチドへのＩｇＧ結合の増加として選択性の損失が認められる。
【０２２０】
最終アッセイ試薬を今用意するが、任意選択的に長期保存には０．０１％アジ化ナトリウムなどの適切な静菌薬を添加する。未知の尿サンプルまたは尿サンプルのアリコートと組み合わせるために、個別のコンテナ中にこの混合物のアリコート（例えば、２ｍｌ）を維持する。
【０２２１】
尿サンプル中のアルブミン、ＩｇＧ，およびＨＣＧ濃度の定量時に、例えば予め測定したキャピラリの使用によってアリコート（体積２０μｌ）の尿サンプルを取り、その後この予め測定したキャピラリと個別のコンテナ（個別のコンテナは温度を３２℃に制御した場所に維持されている）中のアッセイ試薬とを合わせる。典型的には、実施例３に記載のコンテナを使用する。その後、コンテナを震盪し、キャピラリから尿を流れ出させる。試験サンプルアリコートのアルブミンは、アッセイ試薬のフルオレセイン標識免疫複合体のフルオレセイン標識インスリン様ペプチドと置換し始める。ＨＣＧは、アッセイ試薬のＣｙ－５標識免疫複合体中のＣｙ－５標識ＢＨＣＧを置換し始める。ＩｇＧは、ローダミン標識ペプチドと結合し始める。
【０２２２】
震盪直後、コンテナを３２℃の蛍光偏光測定装置に入れる。発光蛍光の偏光の測定のために、コンテナ中の混合物に３つの異なる波長を間欠的に照射するために構築した蛍光偏光測定装置を使用して４７５ｎｍ、５５０ｎｍ、および６５０ｎｍの波長の偏光を照射する。発光の偏光が一定である場合に装置で測定可能ならば狭いほうのバンド幅（典型的には１０ｎｍ）の５２５ｎｍ、５８２ｎｍ、および６７０ｎｍの波長で発光する偏光を測定する。スペクトルの重複および比較的高分子量のＣｙ－５－標識ＢＨＣＧのために、偏光測定精度が高いさらに優れた照射装置が必要である。既知濃度のヒトアルブミン、ＩｇＧ、およびＨＣＧの基準物質の測定によって得た「検量線」への測定した発光（５２５ｎｍ、５８２ｎｍ、および６７０ｎｍ）の偏光値の代入によって未知のサンプルのヒトアルブミン、ＩｇＧ、およびＨＣＧ濃度を計算する。３つすべての波長での偏光によりアルブミン、ＩｇＧ、およびＨＣＧの濃度が変化するので、このような検量線は多次元である。この検量線を測定装置のコンピュータに保存して、アルブミンおよびＨＣＧ濃度を直接読み取ることができる。
【０２２３】
フローセルを具備する測定装置を使用する場合、測定のために混合物をフローセル測定に通過させる。
【０２２４】
測定装置が可能であるならば、偏光値が安定になった後の偏光の読み取りの代わりに時間の関数としての偏光値を速度様式で測定した場合に、さらに良好な精度を得られる。可能ならば、シグナルを任意選択的にソフトウェアで定義された連続的または異なる測定点で記録するコンピュータに測定装置を接続する。次いで、記録した値と時間の関数としての既知のヒトアルブミン、ＩｇＧ、およびＨＣＧ濃度の標準物質の測定によって得た値とを比較する。任意選択的に、３つの波長の偏光値、異なる時間、およびヒトアルブミン、ＩｇＧ、およびＨＣＧ濃度値を使用したデータセットを記録し、未知のサンプルから得た値と比較して任意選択的に標準的な統計学のテキストに記載の最適な適合のための最小二乗法を使用し、任意選択的に測定装置に接続したコンピュータまたは別の人工メモリによってヒトアルブミン濃度を計算する。さらに、温度記録装置にアクセスする場合、前記個別のコンテナ（未知の血液サンプルまたは血液サンプルのアリコートと合わせる）を室温に

維持し、蛍光偏光装置の中またはこの装置上の温度測定デバイスを使用し、任意選択的に標準的な統計学のテキストに記載の最適な適合のための最小二乗法を使用し、任意選択的に測定装置に接続したコンピュータまたは別の人工メモリによって異なる温度を使用したさらに多数のデータセットを使用する。記載の多分析物アッセイでは、演算容量を幾らか増加させる必要があるが、このような演算容量は今日では容易に入手可能であり、いくつかの未知のパラメータの分析および計算用のソフトウェアプログラムの使用（例えば、ＣＡＭＯ　ｃｏｍｐａｎｙ、Ｏｓｌｏ、Ｎｏｒｗａｙが販売しているＵｎｓｃｒａｍｂｌｅｒプログラムの使用）が好ましい。

【図面の簡単な説明】

【図１】

一方の分析物分子と他方の特異的結合分子との間の結合定数が１０＊Ｅ１０／モルであり、解離定数が０．１／秒である場合の時間の関数としての濃度の例。

Ａ＝分析物分子の濃度

Ｂ＝蛍光結合対（特異的結合分子と分析物分子の蛍光誘導体との複合体）の濃度

Ｃ＝分析物分子の蛍光誘導体の濃度

Ｄ＝特異的結合分子の濃度

Ｅ＝蛍光を含まない結合対（特異的結合分子と分析物分子との複合体）の濃度

反応式：

$k_{結合}$＝１．０＊１０Ｅ１０

分析物分子＋結合分子＝非蛍光結合対

$k_{解離}$＝１．０＊１０Ｅ－１

蛍光結合対＝特異的結合分子＋蛍光分析物分子誘導体

$k_{結合}$＝１．０＊１０Ｅ１０

蛍光分析物分子＋結合分子＝蛍光結合対

$k_{解離}$＝１．０＊１０Ｅ－１

非蛍光結合対＝特異的結合分子＋分析物分子

【図２】

一方の分析物分子と他方の特異的結合分子との間の結合定数が１０＊Ｅ１０／モルであり、解離定数が０．０１／秒である場合の時間の関数としての濃度の例。

Ａ＝分析物分子の濃度

Ｂ＝蛍光結合対（特異的結合分子と分析物分子の蛍光誘導体との複合体）の濃度

Ｃ＝分析物分子の蛍光誘導体の濃度

Ｄ＝特異的結合分子の濃度

Ｅ＝蛍光を含まない結合対（特異的結合分子と分析物分子との複合体）の濃度

反応式：

$k_{結合}$＝１．０＊１０Ｅ１０

分析物分子＋結合分子＝非蛍光結合対

$k_{解離}$＝１．０＊１０Ｅ－２

蛍光結合対＝特異的結合分子＋蛍光分析物分子誘導体

$k_{結合}$＝１．０＊１０Ｅ１０

蛍光分析物分子＋結合分子＝蛍光結合対

$k_{解離}$＝１．０＊１０Ｅ－２

非蛍光結合対＝特異的結合分子＋分析物分子

【図３】

一方の分析物分子と他方の特異的結合分子との間の結合定数が１０＊Ｅ１０／モルであり、解離定数が０．００１／秒である場合の時間の関数としての濃度の例。

Ａ＝分析物分子の濃度

Ｂ＝蛍光結合対（特異的結合分子と分析物分子の蛍光誘導体との複合体）の濃度

Ｃ＝分析物分子の蛍光誘導体の濃度

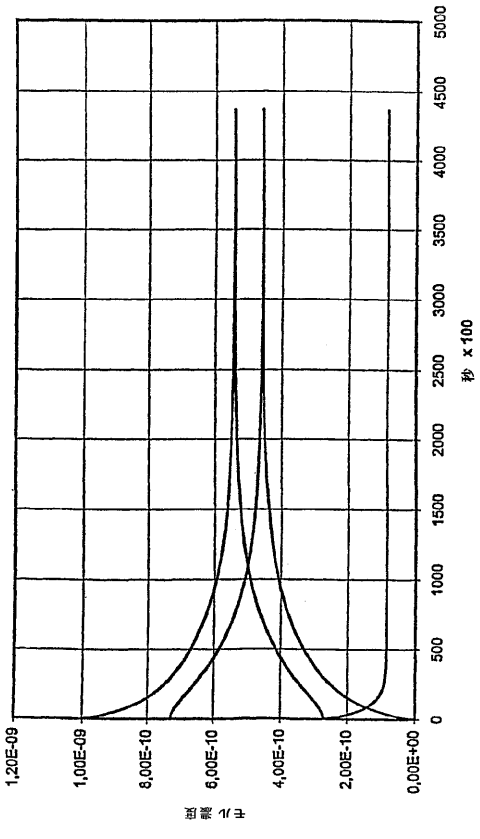
Ｄ＝特異的結合分子の濃度

Ｅ＝蛍光を含まない結合対（特異的結合分子と分析物分子との複合体）の濃度
反応式：
$k_{結合}$＝１．０＊１０Ｅ１０
分析物分子＋結合分子＝非蛍光結合対
$k_{解離}$＝１．０＊１０Ｅ－３
蛍光結合対＝特異的結合分子＋蛍光分析物分子誘導体
$k_{結合}$＝１．０＊１０Ｅ１０
蛍光分析物分子＋結合分子＝蛍光結合対
$k_{解離}$＝１．０＊１０Ｅ－３
非蛍光結合対＝特異的結合分子＋分析物分子
【図４】
一方の分析物分子と他方の特異的結合分子との間の結合定数が１０＊Ｅ１１／モルであり、解離定数が０．０１／秒である場合の時間の関数としての濃度の例。
Ａ＝分析物分子の濃度
Ｂ＝蛍光結合対（特異的結合分子と分析物分子の蛍光誘導体との複合体）の濃度
Ｃ＝分析物分子の蛍光誘導体の濃度
Ｄ＝特異的結合分子の濃度
Ｅ＝蛍光を含まない結合対（特異的結合分子と分析物分子との複合体）の濃度
反応式：
$k_{結合}$＝１．０＊１０Ｅ１１
分析物分子＋結合分子＝非蛍光結合対
$k_{解離}$＝１．０＊１０Ｅ－１
蛍光結合対＝特異的結合分子＋蛍光分析物分子誘導体
$k_{結合}$＝１．０＊１０Ｅ１１
蛍光分析物分子＋結合分子＝蛍光結合対
$k_{解離}$＝１．０＊１０Ｅ－１
非蛍光結合対＝特異的結合分子＋分析物分子
【図５】
一方の分析物分子と他方の特異的結合分子との間の結合定数が１０＊Ｅ１１／モルであり、解離定数が０．０１／秒である場合の時間の関数としての濃度の例。
Ａ＝分析物分子の濃度
Ｂ＝蛍光結合対（特異的結合分子と分析物分子の蛍光誘導体との複合体）の濃度
Ｃ＝分析物分子の蛍光誘導体の濃度
Ｄ＝特異的結合分子の濃度
Ｅ＝蛍光を含まない結合対（特異的結合分子と分析物分子との複合体）の濃度
反応式：
$k_{結合}$＝１．０＊１０Ｅ１１
分析物分子＋結合分子＝非蛍光結合対
$k_{解離}$＝１．０＊１０Ｅ－２
蛍光結合対＝特異的結合分子＋蛍光分析物分子誘導体
$k_{結合}$＝１．０＊１０Ｅ１１
蛍光分析物分子＋結合分子＝蛍光結合対
$k_{解離}$＝１．０＊１０Ｅ－２
非蛍光結合対＝特異的結合分子＋分析物分子
【図６】
一方の分析物分子と他方の特異的結合分子との間の結合定数が１０＊Ｅ１１／モルであり、解離定数が０．００１／秒である場合の時間の関数としての濃度の例。
Ａ＝分析物分子の濃度
Ｂ＝蛍光結合対（特異的結合分子と分析物分子の蛍光誘導体との複合体）の濃度
Ｃ＝分析物分子の蛍光誘導体の濃度

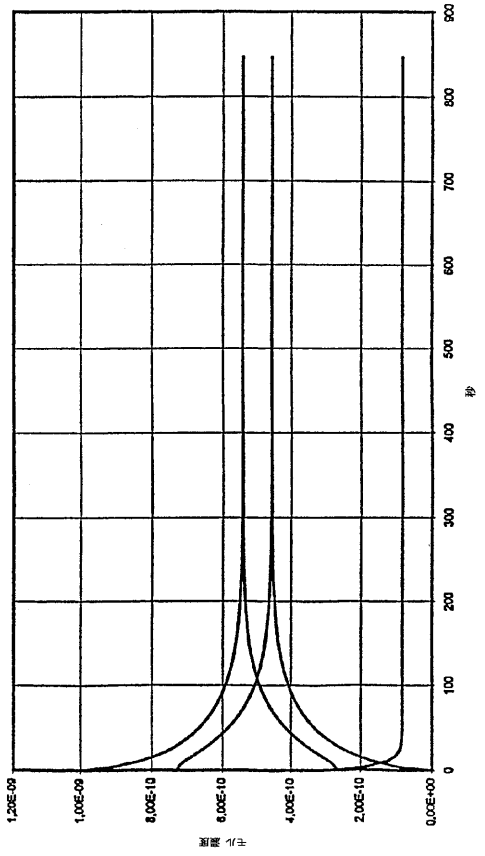
Ｄ＝特異的結合分子の濃度
Ｅ＝蛍光を含まない結合対（特異的結合分子と分析物分子との複合体）の濃度
反応式：
$k_{結合}$＝１．０＊１０Ｅ１１
分析物分子＋結合分子＝非蛍光結合対
$k_{解離}$＝１．０＊１０Ｅ－３
蛍光結合対＝特異的結合分子＋蛍光分析物分子誘導体
$k_{結合}$＝１．０＊１０Ｅ１１
蛍光分析物分子＋結合分子＝蛍光結合対
$k_{解離}$＝１．０＊１０Ｅ－３
非蛍光結合対＝特異的結合分子＋分析物分子
【図７】
一方の分析物分子と他方の特異的結合分子との間の結合定数が５＊１０Ｅ９／モルであり、解離定数が０．１／秒である場合の時間の関数としての濃度の例。
Ａ＝分析物分子の濃度
Ｂ＝蛍光結合対（特異的結合分子と分析物分子の蛍光誘導体との複合体）の濃度
Ｃ＝分析物分子の蛍光誘導体の濃度
Ｄ＝特異的結合分子の濃度
Ｅ＝蛍光を含まない結合対（特異的結合分子と分析物分子との複合体）の濃度
反応式：
$k_{結合}$＝５．０＊１０Ｅ９
分析物分子＋結合分子＝非蛍光結合対
$k_{解離}$＝１．０＊１０Ｅ－１
蛍光結合対＝特異的結合分子＋蛍光分析物分子誘導体
$k_{結合}$＝５．０＊１０Ｅ９
蛍光分析物分子＋結合分子＝蛍光結合対
$k_{解離}$＝１．０＊１０Ｅ－１
非蛍光結合対＝特異的結合分子＋分析物分子
【図８】
一方の分析物分子と他方の特異的結合分子との間の結合定数が５＊１０Ｅ９／モルであり、解離定数が０．０１／秒である場合の時間の関数としての濃度の例。
Ａ＝分析物分子の濃度
Ｂ＝蛍光結合対（特異的結合分子と分析物分子の蛍光誘導体との複合体）の濃度
Ｃ＝分析物分子の蛍光誘導体の濃度
Ｄ＝特異的結合分子の濃度
Ｅ＝蛍光を含まない結合対（特異的結合分子と分析物分子との複合体）の濃度
反応式：
$k_{結合}$＝５．０＊１０Ｅ９
分析物分子＋結合分子＝非蛍光結合対
$k_{解離}$＝１．０＊１０Ｅ－２
蛍光結合対＝特異的結合分子＋蛍光分析物分子誘導体
$k_{結合}$＝５．０＊１０Ｅ９
蛍光分析物分子＋結合分子＝蛍光結合対
$k_{解離}$＝１．０＊１０Ｅ－２
非蛍光結合対＝特異的結合分子＋分析物分子
【図９】
一方の分析物分子と他方の特異的結合分子との間の結合定数が５＊１０Ｅ９／モルであり、解離定数が０．００１／秒である場合の時間の関数としての濃度の例。
Ａ＝分析物分子の濃度
Ｂ＝蛍光結合対（特異的結合分子と分析物分子の蛍光誘導体との複合体）の濃度

Ｃ＝分析物分子の蛍光誘導体の濃度
Ｄ＝特異的結合分子の濃度
Ｅ＝蛍光を含まない結合対（特異的結合分子と分析物分子との複合体）の濃度
反応式：
$k_{結合}$＝５．０＊１０Ｅ９
分析物分子＋結合分子＝非蛍光結合対
$k_{解離}$＝１．０＊１０Ｅ－３
蛍光結合対＝特異的結合分子＋蛍光分析物分子誘導体
$k_{結合}$＝５．０＊１０Ｅ９
蛍光分析物分子＋結合分子＝蛍光結合対
$k_{解離}$＝１．０＊１０Ｅ－３
非蛍光結合対＝特異的結合分子＋分析物分子

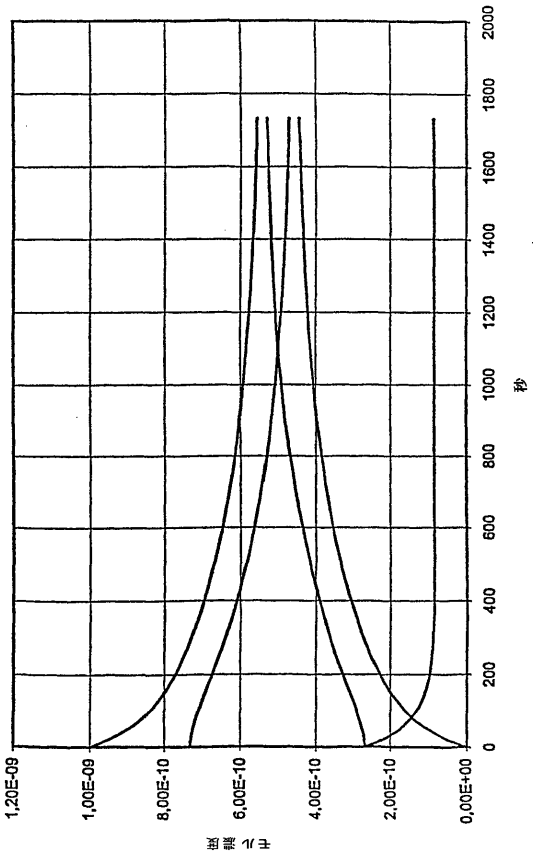
【図１】



【図２】



【図３】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1200
1400
1600
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2000
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8,00E-10
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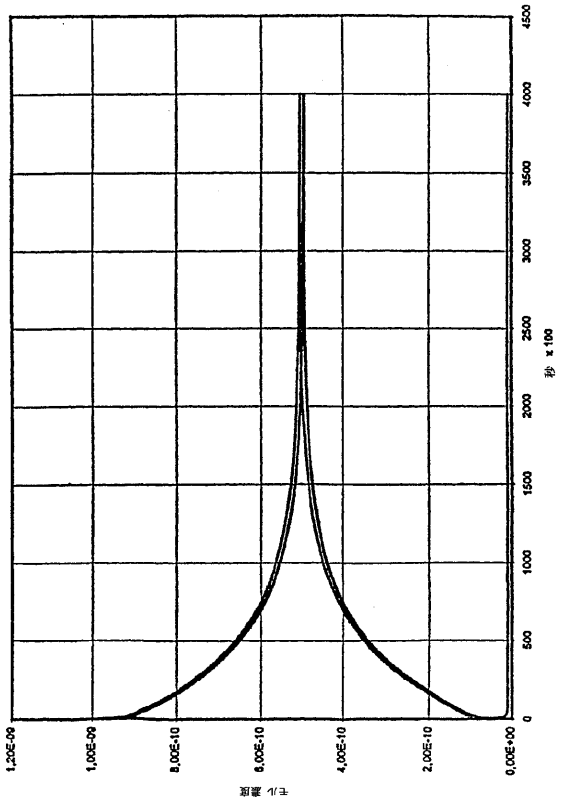
1,20E-09
モル濃度

【図４】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3500
4000
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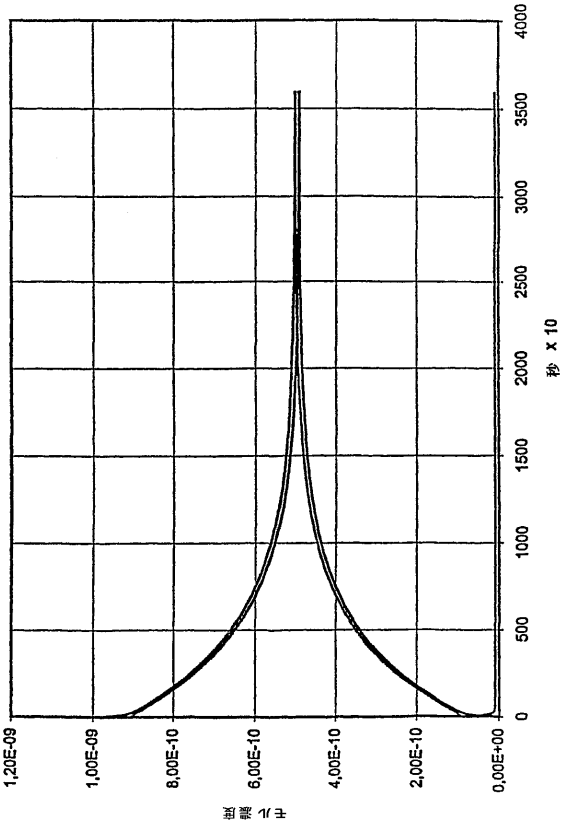
8,00E-10
1,00E-09
1,20E-09
モル濃度

【図５】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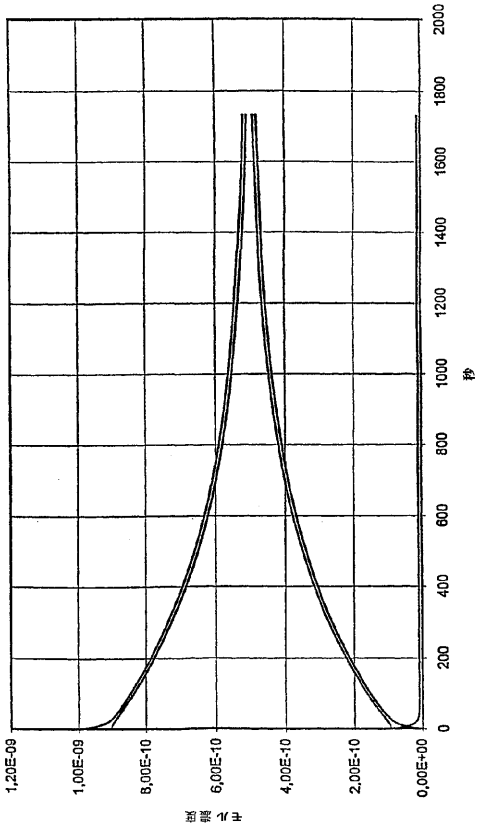
モル濃度

【図６】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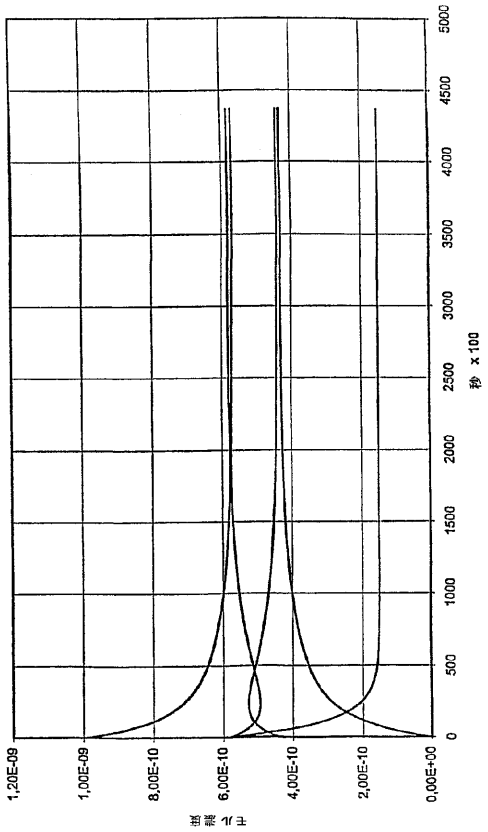
モル濃度

【図７】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2500
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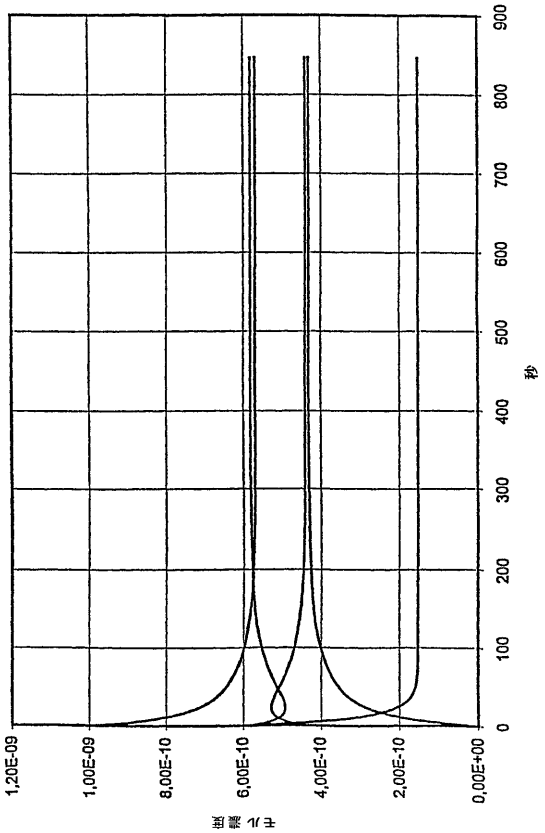
【図８】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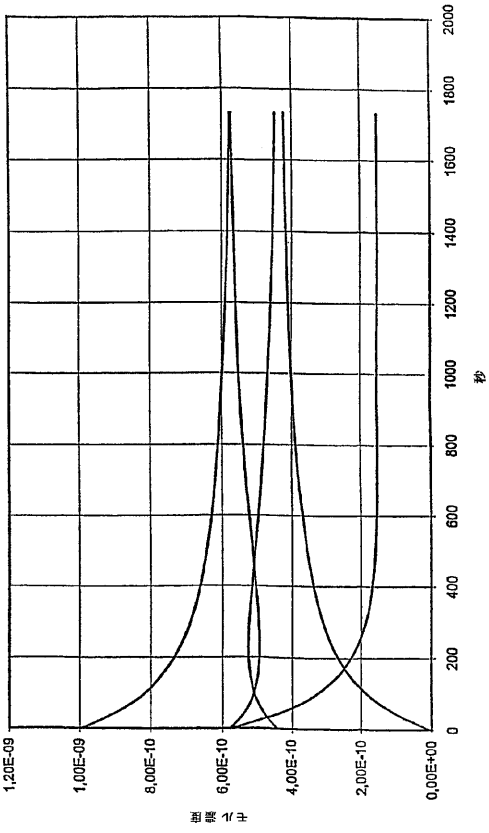
【図９】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(54) **Title:** ANALYTIC METHOD AND REAGENT FOR USE THEREOF

(57) **Abstract:** The present invention relates to a method for determination of one or more analytes in a test sample or an aliquot of a test sample, as well as a reagent for use in the metod. The reagent according to the present invention comprises at least one type of specific binding molecule for each analyte that is to be quantitated, as well as fluorescent substances whose signals change as a result of admixing a sample to the reagent. Furthermore, the signal change may be used to calculate the concentration or concentrations of analytes, without separating different states of aggregation.

Analytic method and reagent for use therof.

The present invention relates to a method for determination of one or more analytes in a test sample or an aliquot of a test sample and a reagent for use in the metod.

Background for the Invention

«Analyte» or «analytes» is the generic name of those substances for which qualitative or quantitative analysis is desired in a test sample material. An analyte is usually a well-defined molecule, but may be a collection of molecules resembling each other or carrying out the same function. Particulate materials, e.g. clusters of fatty proteins or classes of blood cells can also be referred to as analytes.

Quantitative chemical analysis of an analyte in a test material is also often referred to as determination of concentration. Concentration determination may also be carried out using a qualitative chemical analysis, whereby concentration determination provides information on whether the concentration of the analyte in the test material is higher than or lower than a given value.

The development of methods for chemical concentration determination of analytes in very low concentration in complex materials increased very rapidly after R.S. Yalow, S. A. Berson and associates developed what was later to be known as the immunochemical measurement methods. They made use of the principle that individuals of vertebrates create antibodies, most often in the form of immunoglobulins, as a specific immunological response to exposure to materials that the given animal's immune system perceives as alien to the animal. The classical and decisive work to reach this perception was published by S.A. Berson, R.S. Yalow, A. Bauman, M.A. Rothschild, and K. Newerly in the article "Insulin-I131 Metabolism in Human Subjects: Demonstration of Insulin Binding Globulin in the Circulation of Insulin-Treated Subjects" in J. Clin. Invest. 35 (1956): 170-190. By using these globulins, later referred to as antibodies, either in a purified form or in a mixture with other substances, e.g. in the form of blood serum, methods for specific identification or quantitation of a large number of analytes were developed. For many of these analytes there had previously not existed practically applicable measurement methods. Thus, specific measurement methods for a range of blood proteins and proteins from other body fluids and tissue were developed, and later on for non-proteinaceous substances as well. Hormone analyses were developed at an early stage. The general method was named «immunoassay», based on the reagent making the basis for the specificity of the analysis being the result of an immune response. This is well described in a large number of textbooks on the subject, e.g. in «Principles and Practice of Immunoassay», 2nd Edition, edited by Christopher P. Price and David J. Newman, ISBN: 1561591750, Groves Dictionaries, Inc., August 1998.

The test material undergoing quantitative analysis using these reagents can be described as complex biological materials. Typical test materials are blood, blood serum, plasma, urine, feces, feces extracts and cerebrospinal fluid. The analytes usually constitute a very small part of the test material.

Test material that is to undergo chemical analysis may be in different states of aggregation, e.g. gaseous state, fluid state, solid state or mixtures of different states of aggregation. The reagents that are mixed with the test material to perform the quantitative analysis or analyses, may correspondingly be present in different states of aggregation, but typically consist of solutions or solid substances and/or combinations of solid substances and fluids. When the reagents are mixed with the test material, most often (but not always) a solution of substances in fluid will be formed. This solution is often referred to using the generic name «the assay solution», and comprises both test material and those chemicals necessary for the quantitative analysis to take place.

In the early days after the invention of the immunoassays, the source used for admixture of antibodies was antiserum, which is blood serum from the immunized animal, which also included other serum proteins, and where the antibodies constitute a smaller portion of the aggregate serum proteins. To this day a substantial amount of antibodies in the form of antiserum are sold, see e.g. catalogues from suppliers such as Chemicon Inc., California. However, early on the routine use of antibodies in the form of purified immunoglobulin classes was introduced, most commonly immunoglobulin type G, which is the most commonly used antibody reagent today, see e.g. the product catalogue from the company Dako AS, Denmark.

The production of antibodies occurring when animals are exposed to alien substances (called antigens), often using active, targeted exposure in the form of so called vaccination or immunization, is characterized by a whole range of different cells in the body producing antibodies with different structures, but they have the common characteristic that they bind to (have affinity for) the antigen in question. These different antibodies, binding to the same antigen, have different strength and rates of binding, however, and are difficult to make with constant qualities when producing antibodies over a long time span and using many animals.

It was therefore a considerable progress when Köhler and Milstein in 1974 developed the method for creating monoclonal antibodies, which made possible standardized antibodies with a reproducible chemical structure and homogeneous constant binding qualities. These methods are well described in the book «Monoclonal Antibodies: Production, Engineering and Clinical Application», edited by Mary A. Ritter and Heather M. Ladyman, ISBN: 0521425034, Cambridge University Press, February 1995. The book also describes well how to fragment the antibodies and use those

«Monoclonal Antibodies: Production, Engineering and Clinical Application», edited by Mary A. Ritter and Heather M. Ladyman, ISBN: 0521425034, Cambridge University Press, February 1995. The book also describes well how to fragment the antibodies and use those fragments of the antibodies that have binding qualities, instead of using the entire antibodies.

In the years following the invention of the monoclonal antibodies, using modern biotechnology, new methods were developed for producing binding proteins and fragments of proteins (also called peptides) with the desired binding qualities. A substantial step forward was made with the development of the phage display method, in which virus technology was used for production, exposure and choice of peptide structures for the production of reagents with specific binding characteristics.

The phage display technique was developed further when the gene sequence for parts of the antibody producing cells was incorporated and systematically varied or permuted in the phage display particles, as described by Collins J. and Röttgen P. (1994); "Hypervariable phagemid display gene banks for the selection of strongly binding ligands, including their use for the isolation of serine protease inhibitors"; European patent application 1994 000 108 689 (April 1994) taken further as US 5925559 «Phagemids and process of preparation» issued 20 July 1999, and by Collins, J., Röttgen, (1997); «Cosmix-plexing a method for recombination....» EP 97 101 539.1 (31.01.1997), filed by Cosmix GmbH PCT/EP98/00533 (02.02.1998) and WO 98 33901 (6.08.1998).

Further on, one started to use nucleic acid sequences (aptamers) as such as specific binding molecules in an immunoassay-like way, even though terminologically it is probably not quite correct to call these immunoassays. US patent US05567588 «Systematic evolution of ligands by exponential enrichment: Solution SELEX» describes this aptamer technology. Finally, things went completely beyond the scope of the biological framework surrounding these binding assays when one started, synthetically, to produce large libraries of molecules, using systematic combination of smaller elements, so called combinatory libraries. These elements may partially or totally consist of amino acids in chains (called peptides), but could also consist of other building blocks. An overview on this subject is to be found in the article «A paradigm for drug discovery employing combinatorial libraries» by J. Burbaum et. al., in Proc. Natl. Acad. Scient. USA Vol 99, pp. 6027-6031, 1995.

So today there exists a very complex biological and synthetic collection of methods for creation of binding molecules of a higher or lower molecular weight, of different chemical nature, with different binding strength, and of homogeneous or heterogeneous structure. In this patent application, these molecules are referred to

specificities. In addition, they may have different types of signal-providing residues bound to them, e.g. different types of fluorescent residues. In the present patent application, different types of binding molecules may be used, both different types in the meaning different types of structures (peptides, nucleic acids or other structures) or different compositions within one type of structure (e.g. different amino acid sequences within a peptide structure, which would give different types of binding qualities) as well as different types of signal-providing residues, e.g. fluorescent residues with different types of fluorescent qualities, e.g. different excitation or emission wavelengths.

The above mentioned specific binding molecules, used for quantitation of substances in complex sample solutions, will as a rule have higher affinity for the substances to be quantified, or analogues or fragments or derivatives of said substances, than for other substances that may be present in the sample. Typically, a monoclonal antibody would have higher affinity for the substance used for immunization and selection when producing the antibodies, than for other substances in the test solution. When antibodies are used as a specific binding molecule, the substance for which the antibody has high affinity is called antigen, or hapten (the last term is often used if it is a smaller structure in a larger molecule). When the specific binding molecule is not an antibody, the term «ligand» is often used more than the term «antigen». In this patent application, the term «binding pair» is used as a generic name for the molecules that are bound to each other in the specific binding reaction, and the individual molecules that constitute the binding pairs will be referred to as binding partners.

Generally we can set up the following equation for the reaction between the two binding partners and the binding pairs they can form:

$$B1 + B2 = BB$$

where

B1 symbolizes binding partner 1 and B2 symbolizes binding partner 2, and BB can symbolize the binding pair they form.

Furthermore the equilibrium between the free binding partners and the binding pair they form, will be characterized by an equilibrium constant k (often called affinity equilibrium constant) in accordance with the law of mass action (Guldberg and Waage 1864, described in Steven S. Zumdahl's book »Chemical Principles», third edition, page 192-193, ISBN 0-395-83995-5):

$$[BB]$$

$$k = \frac{5}{[B1]*[B2]}$$

Expressed like this, the constant is often called the affinity equilibrium constant, while the inverse form is called the dissociation equilibrium constant.

To be able to measure these binding molecules' formation of chemical binding to the analytes, one originally used a radioactive labeling of one of the binding partners. Originally, one would add a known quantity of a radioactively labeled binding partner B1 or B2, chemically identical to or near identical to the analyte, and measure this radioactively labeled substance's ability to compete with the analyte for the binding on the binding molecules that are used (originally complete immunoglobulins), see »Immunoassay of Protein Hormones," in The Hormones: Physiology, Chemistry, and Applications, vol. 4, 557-630 (G. Pincus et al, eds. Academic Press, 1964).

In this so-called competitive assay method the analyte molecules thus competed with labeled analogue molecules for binding molecules, which were in concentration deficit. Later on, non-competitive methods, with a surplus of antibodies, were developed, where most often two different antibodies are used, one antibody to isolate the analyte (e.g. bind the analyte molecules to a solid phase), and one radioactively labeled antibody to generate a signal for measuring. This method was called the immunometric method. A general overview is given in the book »Immunoassay : A Practical Guide», by Law Brian, ISBN: 0748405607.

Common to the competitive and the immunometric method, was the use of standardized test solutions with a known quantity of analyte to calibrate and correlate the measured signal with the concentration of analyte, and the generation of a so-called standard curve or calibration curve. Tests with unknown concentrations of analyte were determined by interpolation on this curve.

The radioactive labeling methods are still the ones most frequently used, but early on attempts were made to find non-radioactive methods for determining the presence of analyte analogues or binding molecules. The use of enzymes as signal generating molecules constituted a big step forward, as radioactivity was avoided, longer durability of the reagents was achieved, and simpler measuring equipment, typically light absorption spectrophotometers, could be used. There is an overview on this in »Practice and theory of enzyme immunoassays» by P.Tijssen, ISBN 0 444 806 334. Another big step forward was achieved when fluorescent molecules were taken into use as signal generating molecules. This improved the sensitivity compared to the pure absorption photometric methods. The book «Enzyme and Fluorescence

Immunoassays: Tentative Guideline», Vol. 6, National Committee For Clinical Laboratory Standards ISBN: 1562380672, July 1986, provides a good overview of this.

Chemoluminescence methods have increased the sensitivity further, as described in «Luminescence Immunoassay and Molecules Applications» by Knox and Richard van Dyke, CRC Press January 1990, ISBN 0849358655.

With the use of most types of signal-generating residues, both radioactive, fluorescent and enzymatic substances, the need arose to separate the signal-generating residues bound to molecules that had been bounded to the specific binding molecules, from those residues that were not bound to said binding molecules. Typically, this could be plastic surfaces, glass surfaces, porous filters or particle based matrices with immobilized binding molecules or more unspecific media, such as e.g. active coal, which can separate unbound small-molecular analyte-molecules from analyte-molecules bound to larger binding molecules. A general good overview of this field can be found in the book «Principles and Practice of Immunoassay», 2nd Edition, edited by Christopher P. Price and David J. Newman, Goves Dictionaries Inc., Aug. 1998, ISBN 1561591750.

It was observed early on, that antibodies in themselves could precipitate analytes, and this could be used for quantification of the analytes without any of the signal-generating attached residues being involved. Concentration determination by the help of antibody precipitation has low sensitivity, but is very practical because it is not necessary to use a solid separation phase involving a washing step, and this simplifies the execution. Precipitation analysis in gels and directly in a fluid state (Killingsworth et al, "Nephelmetric Studies of the Precipitin Reaction: A Model System for Specific Protein Measurements," Clin. Chem. 19 (4): 403-407, (1973)) has lead to a more widespread use of immunoassays and made way for a high degree of automation in spectrophotometric automatons such as Hitatchi-instruments and automated nephelometres which are for instance delivered by the Dade Behring company. In addition to the limited sensitivity, it has been difficult to use monoclonal antibodies in the precipitation analytic methods. It is presumed that monoclonal antibodies often only bind onto one area of the analyte molecule, whereas polyclonal antibodies usually will bind onto several places on the analyte molecules, and thus more easily lead to larger aggregations of antibodies and analyte molecules that easily precipitate. Thus in contrast to the present invention the above mentioned method does not comprise immunocomplexes, does not use fluorescence for quantification, is not a fluorescence polarisation assay and has low sensitivity.

The so-called BiaCore instruments and other technology based on plasmon resonance (J. Melendez, R. Carr, D. U. Bartholomew, K. Kukanskis, J. Elkind, S. Yee, C.

Furlong, R. Woodbury, A commercial solution for surface plasmon sensing, Sensors and Actuators-B 35 (1996) 1-5) have further shown that direct measurement of an analyte without signal-providing residues is possible. These methods do not, however, have the same simple technical execution as the less sensitive turbidimetric methods. Plasmon resonance instruments are in addition most often very expensive instruments.

The need to measure the analytes' binding to the binding molecules without using separation devices and solid phases, and also without using substrate or washing solutions, led to the so-called proximity assays:

The company SYVA in 1974 launched an immunoassay technology for small molecules, based on competition between the analyte molecules and enzyme labeled analyte analogues for binding to antibodies, where binding onto the antibodies gave a direct effect on the enzyme activity, which could be measured without separation or washing solutions. (US patent US3852157 «Compounds for enzyme amplification assay» by Kenneth E. Rubenstein and Edwin F. Ullman.) Fluorescence polarization immunoassays (FPIAs) were introduced as early as the end of the 1970's; see the overview article »Fluorescence polarization in immunochemistry» by Dandliker and Saussure in Immunochemistry, Vol. 7, p. 799-828, 1970. This method has also been most successful with small-molecular analytes, but the use of competing small-molecular fluorescence labeled analyte analogues opened up for the use of the method for quantification of large-molecular analytes, such as proteins. (Ai-Peng Wei and James Herron: Use of synthetic peptides as tracer antigens in fluorescence polarization immunoassays of high molecular weight analytes. Anal. Chem. 1993, 65, 3372-3377.) Some proteins also have relatively mobile subunits, making possible direct fluorescence polarization immunoassay measurements, as in the US patent 4,902,630 «Fluorescence polarization immunoassay and reagent for measurement of C-reactive protein», by Bennet and Chiapetta, (1990). Terpetschnig,E. et al. in «Fluorescence polarisation immunoassay of a high-molecular-weight antigen based on a long-lifetime Ru-ligand complex», Anal.Biochem. 227, 140-47, 1995, and in «Metal ligand complexes as a new class of long-lived fluorophores for protein hydrodynamics» Biophys.J. 68, 342, 1995, have further described how asymmetric ruthenium-complexes can be used for fluorescence polarization measurements of analytes with higher molecular weight. These can, however, not be used under the presence of hemoglobin or high bilirubin concentrations.

Most fluorescence polarization immunoassays are based on competitive methods, where fluorescence labeled analogues of the analyte have been added and have competed with the analyte molecules in the test solution for the specific binding molecule, typically in the form of an antibody. It has often been necessary to use a relatively high aggregate concentration of antibodies in such assays, even concentrations where one would expect competition not to occur. So most

commercial assays of this kind have a quite high aggregate concentration of antibodies, and apparently competition takes place for a considerably lower efficient binding molecule concentration. No systematic literature on this is available, but reference can be made to the article «Rapid, fully automated measurement of plasma homocysteine with the Abbott Imx Analyzer» by Mohammed T. Shipchandler and Edwin G. Moore, and a closer analysis might show that also other commercial competitive fluorescence polarization immunoassays are based on a relatively high aggregate antibody concentration.

Already in the mid 1980's, the company Amersham introduced scintillation proximity assays (US patent number 4,568,649, European patent number 0,154,734) in which a radioactive beta ray emitted from a binding partner triggers a fluorescence radiation from a fluorescent particle that is bound to the other binding partner in the binding pair in question. This technology has found a wide range of use in the search for remedies and other specific binders in a laboratory environment, but has never found much use in practical clinical diagnostics.

Already in 1976 Ullman and Schwarzberg, in US patent 3,996,345, published a method for «Fluorescence quenching with immunological pairs in immunoassays». This made possible fluorescence proximity assays without the use of separation and washing steps, but advanced fluorimeters were still needed, the quenching technology was never found to be competitive in clinical routine use. In 1998 Buechler & al. published US patent 5,763,189 «Fluorescence energy transfer and intramolecular energy transfer in particles using novel compounds», mainly based on advanced particles comprising both binding molecules and different molecules that interact with light at different wavelengths. There are considerable development costs and production costs related to such particles. This is not a homogeneous immunoassay, and the handling of the solid phase is demanding, both in production, transportation and in execution of such assays.

Common to all the above described technologies is that those reagents used are added from several reagent containers, usually using pipetting equipment, and it requires trained specialized personnel to carry this out. The need for several reagent containers and specialized pipetting equipment and specialized personnel is a considerable cost in health service.

Furthermore, several inventions are related to methods and assays detecting or quantitating organic compounds in test samples.

WO 00/16099 (Wolf) describes reduced valency carbohydrate binding ligands (CBLs) that can be used to detect or quantitate carbohydrates in a sample. CBL can be used with fluorescence resonance energy transfer (FRET) to evaluate free carbohydrate or

those within a carbohydrate containing compound by using e.g. a proximity-based signal generating label moiety. Contrary to the present invention this method is not a fluorescence polarisation assay.

EP 0 561 653 A1 (Lakowicz et al.) describes determination of glucose in a sample by contacting the test sample with a donor- acceptor pair, wherein the acceptor in the donor-acceptor pair can be competitively replaced by the analyte. The donor can be photo luminizing or fluorescent. This method is suitable for higher concentrations of analyte and the method is not a fluorescence polarisation assay.

WO 00/25134 (Blanchard et al.) describes an assay for identifying ligands for nuclear receptors, utilizing scintillation proximity and FRET. Contrary to the present invention this assay is not a fluorescence polarisation assay. It is using a heterodimeric partner, and is not suitable for fluorescence polarisation assay.

US 5814449 (Schultz and Ballerstadt) describes a method for detection of galactose and glucose, using a receptor carrying molecule with at least two binding sites for the analyte of interest. In addition there are two groups of molecules wherein one group (fluorochrome) can produce a detectable response in the proximity of the other molecule. The group of molecules is bound to an analogue of the analyte of interest. When the analyte is present the binding complex will dissociate due to competitive replacement of the analogue with the analyte. The detection is performed with a complicated sensor and contrary to the present invention this method is not a fluorescence polarisation assay.

EP 0984281 A2 (Ullman et al.) describes a photosensitizer associated with a molecule in in a specific binding pair (sbp), and a chemo luminous component associated with a sbp molecule, and wherein the amount of light emitted from the chemo luminous compound due to the activation of the photosensitizer is related to the amont of analyte in the sample.This method requires several steps of pipetting and adding of reagents and is furthermore not a fluorescence polarisation assay.

Limitations in today's technology and related needs for improvements:

A considerable part of the routine analyses of tests of biological fluids, such as blood, serum, plasma, urine and spinal fluids, are carried out in so-called «emergency» situations, in which sending away test material to specialized laboratories causes delays before the analysis results are returned to the attending physician. Furthermore, a considerable cost problem is present in the health care system, and there is a need for efficient and at the same time sensitive reagents that are cheap to produce and simple to use. Homogeneous reagents in an ideal solution, where there are no production, storage and stability problems (contrary to with particle suspensions) are therefore a clear advantage. Furthermore, in some geographical areas there is a lack

of specialized personnel and specialized laboratories, which makes it difficult to perform quality pipette and handling work, as well as necessary washing procedures and maintenance of adequate handling equipment.

There is therefore an object to provide sensitive specific measurement methods based on stabile, durable reagents, preferably in ideal solution (not in suspension) supplied in very few and preferably just one single reagent container, not requiring any significant pipette work, with no solid phase to be washed or two different phases to be separated, and which preferably can be carried out on blood tests with the presence of hemoglobin and blood cells, possibly after or with simultaneous lysis of the blood cells.

The above mentioned object is obtained by the present invention characterised by the enclosed claims.

Brief description of the invention:

The present invention provides a method for concentration determination of one or more analytes in a test, which is characterized by the fact that the reagent characterised by this invention is mixed with the said test solution, after which the signals generated by the fluorescent substances included in the said reagent are measured, in order to calculate the concentration of the said analytes on this basis. The said signal changes may be measured both as so-called endpoint measurements (after the establishment or near establishment of new chemical equilibriums), and kinetically (by measuring the signals' change per time unit or within a time interval).

The present invention further relates to a method wherein the ingredients of the said reagent is not kept separately but supplied in a single container or compartment, and the said reagent furthermore comprises at least one type of specific binding molecule for each analyte, for which the concentration should be determined, and the reagent furthermore comprises fluorescent substances whose signals change as a result of admixing a test sample with the reagent, and that this signal change is a function of the concentrations of the analyte or analytes in the sample, and that this signal change may be used to calculate the concentration or concentrations of analytes, without using separation of different states of aggregation.

The present invention relates further to a method wherein the reagent for concentration determination of one or more analytes in a test may further be characterized by the presence in the said reagent of a binding pair where the binding partners are reversibly bound to each other for each analyte to be concentration determined, and further characterized by at least one of the binding partners in each of the binding pairs having a fluorescent residue. The invention is further characterized by at least one of the binding partners in each of the said binding pairs

having affinity for one of the analytes to be quantified, and that – when the reagent is mixed with the said test – each of the analytes compete for the binding between the binding partners in at least on of the binding pairs, and that varying concentration of the analyte or the analytes in the test leads to changes in the concentrations of the other molecules that are included in the equilibrium for creation of binding pairs between the said binding partners.

The invention is further characterized by the fact that the reagent may include several types of binding partners or binding pairs for each analyte.

The invention is further characterized by the fact that the reagent may be designed to quantify only one analyte and further be characterized by comprising only one type of specific binding molecule.

The invention is further characterized by the fact that the fluorescent residues may be bound to one or more of the specific binding molecules, and that the fluorescence signal that may be generated is changed as a consequence of the said specific binding molecules being bound to analyte molecules.

The invention is further characterized by the fact that the said fluorescent residues may be different fluorescent substances in order to achieve different fluorescence wavelengths to quantify different analytes in the test.

The invention is further characterized by the fact that for analyses with the presence of hemoglobin, fluorescent residues with a maximum absorption coefficient at a wavelength between 600 nm and 1000 nm are preferred, more preferred exceeding 620 nm, most preferred exceeding 640 nm.

Especially preferred are reagents which ingredients are not kept separately but are present in one single container and comprise fluorescent residues bound to specific binding molecules with a low molecular weight and with fluorescent residues with a maximum absorption coefficient at a wavelength between 600 nm and 1000 nm, more preferred exceeding 620 nm, and even more preferred exceeding 640 nm.

The invention is further characterized by the reagent including specific binding molecules consisting of monoclonal or polyclonal antibodies or immunoreactive fragments of these, e.g. FAB fragments or single chain fragments or single chain antibodies, or peptides or other polymers produced by Phage Display or other biological combinatory techniques, or nucleic acid polymers or analogues or derivatives of these, or polymers produced on the basis of library technologies or synthetic combinatory chemistry. The invention may furthermore be characterized by the fact that other binding partners in said binding pair might be a derivative or

analogue or fragment or part of or an imitation of the structure characterizing at least one of the analytes to be quantified.

The invention is further characterized by the fact that the reagent may include one or more types of specific binding molecules and one or more binding partners to the said specific binding molecules, and that this or these said binding partners are constituted by a fluorescent derivative of an analyte or a fluorescent analogue of an analyte or a fluorescent fragment of an analyte or a fluorescent part of an analyte or a fluorescent imitation of the structure characterized by at least one of the analytes that are to be concentration determined using the reagent.

The invention is further characterized by the fact that the said reagent may comprise lysing substances or coagulation restrainers or surface-active substances or precipitating substances or separating substances.

The invention is further characterized by the fact that the said fluorescent residues may be cyanine dyes. The invention is further characterized by the fact that Alexa Fluor Dyes or substances in the group Bodipy delivered by Molecular Probes may be used.

The invention is further characterized by the fact that it may be possible or desirable to keep parts of the reagent in separate containers, and that the reagent is ready-mixed by the user before using the reagent.

The said method is further characterized by the fact that the signals to be measured are measured using fluorescence polarization measurements. The fluorescence signals are read as a function of time, either in the form of continuous reading within a period of time or as change per time unit between 2 or more points of time or as an absolute change between 2 or more points of time. Such measurements are often called kinetic measurements or readings. The present invention may also use kinetic reading methods with the remaining forms of fluorescence measurement methods that are described.

The said method is further characterized by the fact that the different constituents of the said reagent may – if desirable – be added in steps instead of at the same time. The said method is further characterized by the fact that – if desired – more, or other, reagents may be added after the reagent characterized by this invention is added. If the reagent characterized by this invention is split up and added in steps, other reagents may - if desirable – be added in between the said stepwise adding of the reagent characterized by this invention.

The said method is further characterized by the fact that the test sample may be a biological material or extract or a dilution or concentrate, or a filtrate thereof. The

said method is further characterized by the fact that the biological solutions may be blood, serum, plasma, cells from blood, lysate of blood, urine, cerebrospinal fluid, lachrymal fluid, saliva, aspirate from the gastrointestinal tract, semen or seminal fluid or feces or fecal extract or fecal dilution or suspension. The biological solution may furthermore come from the plant kingdom in the form of solutions, extracts or derivatives or filtrates.

The method for concentration determination in compliance with the invention is further characterized by the fact that standard solutions or calibrators with known concentrations of the analyte or analytes may be used, and that the concentration of the analyte or analytes is determined when the signals measured from the fluorescent residues are interpolated on the standard curve achieved using the said calibrators or standard solutions.

The method for concentration determination in compliance with the invention is further characterized by the fact that the said standard curves may be stored in an artificial memory connected to the analysis system, so that in the relevant user situation it is not necessary to perform analyses of the said calibrators or standard solutions.

The method for concentration determination in compliance with the invention is further characterized by the fact that the method may be carried out at a constant temperature, or by the use of correction algorithms empirically generated by way of studies of the temperature's influence on test solutions with a known concentration of the analyte or analytes.

According to the present invention the method may be used to determine concentrations of clinically related substances in samples of biological material from living organisms in need thereof. Such organisms may constitute plants, insects, birds, animals such as mammals, preferably primates, more preferably humans.

Furthermore the present invention is related to a kit comprising containers containing predetermined volumes of ingredients to be combined in in one single container in relation to the specific analyte to be measured, and a container for drawing specific volumes of the sample of the biological material of interest. In another embodiment the kit comprises a single container containing the reagent specific for the analyte to be measured , and a container for drawing specific volumes of the biological sample of interest.

The present invention will now be described in more detail, with reference to figures and examples.

FIGURES

Figure 1:

An example of concentrations as a function of time when the affinity equilibrium constant between the analyte molecules on the one side and the specific binding molecule on the other side is 10*E10/Molar, and the dissociation rate constant is 0,1 per sec.

A= The concentration of analyte molecules
B= The concentration of fluorescent binding pairs (complexes between the specific binding molecules and fluorescent derivates of analyte molecules)..
C= The concentration of fluorescent derivates of analyte molecules.
D= The concentration of a specific binding molecule.
E= The concentration of binding pairs without fluorescence (complexes between specific binding molecules and analyte molecules).

Reaction diagram:

$k_{affinity\ equilibrium}$ = **1.0*10E10**

Analyte molecule + binding molecule = non-fluorescent binding pair

$k_{dissociation}$ = **1.0*10E-1**

Fluorescent binding pair = specific binding molecule + fluorescent analyte molecule derivate

$k_{affinity\ equilibrium}$ = **1.0*10E10**

Fluorescent analyte molecule + binding molecule = fluorescent binding pair

$k_{dissociation\ rate}$ = **1.0*10E-1**

Non-fluorescent binding pair = specific binding molecule + analyte molecule

Figure 2:

An example of concentrations as a function of time when the affinity equilibrium constant between the analyte molecules on the one side and the specific binding molecule on the other side is 10*E10/Molar, and the dissociation rate constant is 0,01 per sec.

A= The concentration of analyte molecules
B= The concentration of fluorescent binding pairs (complexes between the specific binding molecules and fluorescent derivates of analyte molecules).
C= The concentration of fluorescent derivates of analyte molecules.
D= The concentration of a specific binding molecule.
E= The concentration of binding pairs without fluorescence (complexes between specific binding molecules and analyte molecules).

Reaction diagram:

$k_{affinity\ equilibrium} = 1.0*10E10$

Analyte molecule + binding molecule = non-fluorescent binding pair

$k_{dissociation\ rate} = 1.0*10E-2$

Fluorescent binding pair = specific binding molecule +
fluorescent analyte molecule derivate

$k_{affinity\ equilibrium} = 1.0*10E10$

Fluorescent analyte molecule + binding molecule = fluorescent binding pair

$k_{dissociation\ rate} = 1.0*10E-2$

Non-fluorescent binding pair = specific binding molecule + analyte molecule

Figure 3:

An example of concentrations as a function of time when the affinity equilibrium constant between the analyte molecules on the one side and the specific binding molecule on the other side is 10*E10/Molar, and the dissociation rate constant is 0,001 per sec.

A= The concentration of analyte molecules
B= The concentration of fluorescent binding pairs (complexes between the specific binding molecules and fluorescent derivates of analyte molecules).
C= The concentration of fluorescent derivates of analyte molecules.
D= The concentration of a specific binding molecule.
E= The concentration of binding pairs without fluorescence (complexes between specific binding molecules and analyte molecules).

Reaction diagram:

$k_{affinity\ equilibrium} = 1.0*10E10$

Analyte molecule + binding molecule = non-fluorescent binding pair

$k_{dissociation\ rate} = 1.0*10E-3$

Fluorescent binding pair = specific binding molecule +
fluorescent analyte molecule derivate

$k_{affinity\ equilibrium} = 1.0*10E10$

Fluorescent analyte molecule + binding molecule = fluorescent binding pair

$k_{dissociation\ rate} = 1.0*10E-3$

Non-fluorescent binding pair = specific binding molecule + analyte molecule

Figure 4:

An example of concentrations as a function of time when the affinity equilibrium constant between the analyte molecules on the one side and the specific binding molecule on the other side is 10*E11/Molar, and the dissociation rate constant is 0,01 per sec.

A= The concentration of analyte molecules
B= The concentration of fluorescent binding pairs (complexes between the specific binding molecules and fluorescent derivates of analyte molecules).
C= The concentration of fluorescent derivates of analyte molecules.
D= The concentration of a specific binding molecule.
E= The concentration of binding pairs without fluorescence (complexes between specific binding molecules and analyte molecules).

Reaction diagram:

$k_{affinity\ equilibrium}$ **=1.0*10E11**

Analyte molecule + binding molecule = non-fluorescent binding pair

$k_{dissociation\ rate}$ **=1.0*10E-1**

Fluorescent binding pair = specific binding molecule + fluorescent analyte molecule derivate

$k_{affinity\ equilibrium}$ **=1.0*10E11**

Fluorescent analyte molecule + binding molecule = fluorescent binding pair

$k_{dissociation\ rate}$ **=1.0*10E-1**

Non-fluorescent binding pair = specific binding molecule + analyte molecule

Figure 5:

An example of concentrations as a function of time when the affinity equilibrium constant between the analyte molecules on the one side and the specific binding molecule on the other side is 10*E11/Molar, and the dissociation rate constant is 0,01 per sec.

A= The concentration of analyte molecules
B= The concentration of fluorescent binding pairs (complexes between the specific binding molecules and fluorescent derivates of analyte molecules).
C= The concentration of fluorescent derivates of analyte molecules.
D= The concentration of a specific binding molecule.
E= The concentration of binding pairs without fluorescence (complexes between specific binding molecules and analyte molecules).

Reaction diagram:

$k_{affinity\ equilibrium}$ =1.0*10E11
Analyte molecule + binding molecule = non-fluorescent binding pair

$k_{dissociation\ rate}$ =1.0*10E-2
Fluorescent binding pair = specific binding molecule +
fluorescent analyte molecule derivate

$k_{affinity\ equilibrium}$ =1.0*10E11
Fluorescent analyte molecule + binding molecule = fluorescent binding pair

$k_{dissociation\ rate}$ =1.0*10E-2
Non-fluorescent binding pair = specific binding molecule + analyte molecule

Figure 6:

An example of concentrations as a function of time when the affinity equilibrium constant between the analyte molecules on the one side and the specific binding molecule on the other side is 10*E11/Molar, and the dissociation rate constant is 0,001 per sec.

A= The concentration of analyte molecules
B= The concentration of fluorescent binding pairs (complexes between the specific binding molecules and fluorescent derivates of analyte molecules).
C= The concentration of fluorescent derivates of analyte molecules.
D= The concentration of a specific binding molecule.
E= The concentration of binding pairs without fluorescence (complexes between specific binding molecules and analyte molecules).

Reaction diagram:

$k_{affinity\ equilibrium}$ =1.0*10E11
Analyte molecule + binding molecule = non-fluorescent binding pair

$k_{dissociation\ rate}$ =1.0*10E-3
Fluorescent binding pair = specific binding molecule +
fluorescent analyte molecule derivate

$k_{affinity\ equilibrium}$ =1.0*10E11
Fluorescent analyte molecule + binding molecule = fluorescent binding pair

$k_{dissociation\ rate}$ =1.0*10E-3
Non-fluorescent binding pair = specific binding molecule + analyte molecule

Figure 7:

An example of concentrations as a function of time when the affinity equilibrium constant between the analyte molecules on the one side and the specific binding molecule on the other side is 5*10E9/Molar, and the dissociation rate constant is 0,1 per sec.

A= The concentration of analyte molecules
B= The concentration of fluorescent binding pairs (complexes between the specific binding molecules and fluorescent derivates of analyte molecules).
C= The concentration of fluorescent derivates of analyte molecules.
D= The concentration of a specific binding molecule.
E= The concentration of binding pairs without fluorescence (complexes between specific binding molecules and analyte molecules).

Reaction diagram:

$k_{affinity\ equilibrium}$ **=5.0*10E9**

Analyte molecule + binding molecule = non-fluorescent binding pair

$k_{dissociation\ rate}$ **=1.0*10E-1**

Fluorescent binding pair = specific binding molecule +
fluorescent analyte molecule derivate

$k_{affinity\ equilibrium}$ **=5.0*10E9**

Fluorescent analyte molecule + binding molecule = fluorescent binding pair

$k_{dissociation\ rate}$ **=1.0*10E-1**

Non-fluorescent binding pair = specific binding molecule + analyte molecule

Figure 8:

An example of concentrations as a function of time when the affinity equilibrium constant between the analyte molecules on the one side and the specific binding molecule on the other side is 5*10E9/Molar, and the dissociation rate constant is 0,01 per sec.

A= The concentration of analyte molecules
B= The concentration of fluorescent binding pairs (complexes between the specific binding molecules and fluorescent derivates of analyte molecules).
C= The concentration of fluorescent derivates of analyte molecules.
D= The concentration of a specific binding molecule.
E= The concentration of binding pairs without fluorescence (complexes between specific binding molecules and analyte molecules).

Reaction diagram:

$k_{affinity\ equilibrium}$ =5.0*10E9

Analyte molecule + binding molecule = non-fluorescent binding pair

$k_{dissociation\ rate}$ =1.0*10E-2

Fluorescent binding pair = specific binding molecule +
fluorescent analyte molecule derivate

$k_{affinity\ equilibrium}$ =5.0*10E9

Fluorescent analyte molecule + binding molecule = fluorescent binding pair

$k_{dissociation\ rate}$ =1.0*10E-2

Non-fluorescent binding pair = specific binding molecule + analyte molecule

Figure 9:

An example of concentrations as a function of time when the affinity equilibrium constant between the analyte molecules on the one side and the specific binding molecule on the other side is 5*10E9/Molar, and the dissociation rate constant is 0,001 per sec.

A= The concentration of analyte molecules
B= The concentration of fluorescent binding pairs (complexes between the specific binding molecules and fluorescent derivates of analyte molecules).
C= The concentration of fluorescent derivates of analyte molecules.
D= The concentration of a specific binding molecule.
E= The concentration of binding pairs without fluorescence (complexes between specific binding molecules and analyte molecules).

Reaction diagram:

$k_{affinity\ equilibrium}$ =5.0*10E9

Analyte molecule + binding molecule = non-fluorescent binding pair

$k_{dissociation\ rate}$ =1.0*10E-3

Fluorescent binding pair = specific binding molecule +
fluorescent analyte molecule derivate

$k_{affinity\ equilibrium}$ =5.0*10E9

Fluorescent analyte molecule + binding molecule = fluorescent binding pair

$k_{dissociation\ rate}$ =1.0*10E-3

Non-fluorescent binding pair = specific binding molecule + analyte molecule.

Chemical analyses of materials are most often performed by taking a sample of the material (i.e. test material/test sample), which subsequently undergoes the desired chemical analysis. The test sample may be e.g. in the fluid state, gaseous state, solid state or mixtures of the said states of aggregation. The sample can furthermore be homogeneous or inhomogeneous. If, for instance, the sample is in a fluid phase, the fluid phase could comprise particulate material, and would thus be inhomogeneous. Well-prepared blood serum is an example of a homogeneous biological sample material, whereas the blood as such comprises considerable amounts of blood cells, and thus is not a homogeneous material.

Concentration determination of one or more analytes in a test sample is most often achieved by admixing other chemical substances to the sample, after which the signals or signal changes appearing as a consequence of the mixing are observed or measured. These signals may be chemical or physical signals in the form of electromagnetic radiation, radioactive radiation, temperature or response to physical influence, such as fluorescence or absorption of light. In previously known commercial products for quantitative analysis using fluorescence measurements, typically in the form of immunoassay systems, the binding partners included in binding pairs, typically in the form of antibodies and analyte analogues labeled with fluorescent residues, have been kept isolated from each other, typically separated in different reagent containers. This have made it necessary to add different reagents in several steps by the use of accurate instruments, e.g. pipettes.

In order to avoid the different problems that are connected to the currently used technology, the present invention provides all the necessary chemical substances, including fluorescent substances and binding partners, in one single collection of substances, usually in a fluid state, and gathered in one single container. This collection of chemical substances is called a reagent. The reason why it is an advantage to have one single ready mixture of the chemical substances in one reagent is that this provides a pre-measured amount of correct concentrations of the ingredients. This means that persons without specialized chemical training can perform the mixing of the reagent with the test material. Typically, the test material will be collected in a pre-calibrated capillary or another testing device, e.g. a self-calibrating constant volume pipette e.g. from Samco Scientific (USA), which is subsequently emptied into or combined with (e.g. dropped into) the reagent in its container. The container with the reagent may consist of different materials, such as e.g. glass vials, glass or plastic test tubes, plastic containers, foil pockets, plastic pads or other devices that can be used to contain reagents.

Traditionally, the wish has been that the binding between the binding partners in analysis systems using binding pairs and fluorescence measurements should be as strong as possible. High affinity equilibrium constants have led to very high sensitivities for the concentration determinations, and ensured less interference from other substances and variations in the physical/chemical circumstances. In the earlier state of the art, keeping binding partners in the same reagent and reagent container has – due to the high affinity equilibrium constants – led to aggregations, precipitations and irreversible chemical changes, as well as inhomogeneous solutions. Thus, keeping them in one single reagent, as is the case according to the present invention, has therefore been avoided, and several separate reagents has been preferred. The mixing of two or more binding partners has instead been carried out during the performance of the quantitation method. To achieve a precise result, precise transfer of volumes of the reagents has therefore been necessary, either by manual so-called pipetting or using chemical automatons. Using manual pipettes is very demanding and is an essential part of the basic training for laboratory workers and chemical engineers. The automatons are expensive and usually only available in laboratories or larger centers for testing or handling of samples. To avoid such problems, the method according to the present invention uses only one reagent for concentration determination of one or more analytes in a sample, wherein the said reagent is not kept separately but is present in one single container. Furthermore, the reagent to be used in the method according to the present invention comprises at least one specific binding molecule for each analyte to be concentration determined, as well as fluorescent substances whose fluorescence changes as a result of mixing in a sample to the reagent. This change in the fluorescence signal is a function of the concentrations of the analyte or analytes in the sample, and can be used for calculation of the analyte concentration(s) without using separation of different states of aggregation.

Thus, the generic term «reagent» is used in this description as a generic name for the collection of substances that are mixed with a sample solution for measuring one or more analytes. The reagent will normally be in a fluidal state, in the form of a solution of several substances, readily with buffer substances, salts, surface-active agents and anti-biological substances added to avoid growth of microorganisms in the reagent. But for some uses of the reagent, it may be an advantage if the reagent is used in a solid state, possibly by adding fluid immediately prior to using the reagent, or possibly by dissolving the solid reagent, for instance in the sample material, prior to or during use. The reagent may further be dried on a stand or a device, or enclosed in capsules or tablets.

The preferred embodiment of the reagent according to the present invention, is that the reagent is present, ready for use, in one single container where the amount of

reagent that is to be used to analyze a sample will not need to be pipetted or meted out or mixed with other reagents before use.

As an alternative, however, the appropriate volume may be meted out prior to the analysis or in the course of the execution of the analysis. Thus, it is normally less preferred, but still absolutely possible, to keep parts of the reagent in one or more separate containers, so that the ready-for-use reagent is mixed prior to or immediately prior to or in connection with the execution of the analysis. If so desired, parts of the reagent may be impregnated onto or into containers or devices or filters etc. and be mobilized at contact with a solution, e.g. the assay solution. If so desired, the entirety or parts of the reagent may be in a dry or desiccated state, and if desired, it can be designed so that fluid can be added to it prior to, immediately prior to or in connection with the quantitative analysis. The reagent may then further be mixed ready-for-use by such analysis automatons that are often used by larger, more sophisticated laboratories. However it is important to note that the methode according to the present invention, by combining a single reagent and fluorescence polarisation assay for analysis of analytes in samples of complex materials, distinguishes itself from the previous state of the art; e.g. from turbidimetry and nephelometry by being a more sensitive method, from scintillation proximity by the use of non-radioactive substances only and from FRET by employing ideal solutions which are less expensive and less complicated to produce.

The reagent to be used according to the present invention comprises fluorescent residues bound to (same or different) specific binding molecules/binding partners with a low molecular weight. It is known to the skilled man of the art that the ability to conserve the polarization of the exciting light in the emitted light as well, is a function of the rotation speed of the molecules, which in turn is a function of the molecular size as small molecules rotate faster than larger molecules. Thus, an especially preferred embodiment of the reagent in accordance with the present invention, is therefore to bind the fluorescent signal-providing substance to one or more binding partners with a low molecular weight, preferably a molecular weight under 5000, more preferred under 3000 and even more preferred under 1500. When the fluorescence-labeled binding partner either dissociates from its binding partner or binds to its binding partner, the total molecular size for the molecule that is rotating with the fluorescent residue will change, and this can be detected as a change in fluorescence polarization.

It is however, and according to another embodiment of the present invention, possible to use a reagent wherein the fluorescent binding partner(s) (i.e. binding partner(s) with fluorescent residue(s) bound to them) has higher molecular weight than the above mentioned, and to still use fluorescence polarisation assay in order to detect an analyte/analytes (see example 15). However, in this case the fluorescent residue(s)

must display longer decay time when compared to the fluorescent residue(s) bound to spesific binding molecules with low molecular weight.

The fluorescence-labeled binding molecules in the reagent that are used according to the preferred embodiment of the present method (i.e. the use of binding partner(s) with low molecular weight) are, according to the competitive embodiment of the present invention, either analogues or fragments or derivatives of the analyte(s) to be determined, whereas they in the non-competitive embodiment of the present inventioin are binding molecules such as a peptide/peptides or synthetic binders (optionally being identified by combinatory chemistry techniques or phage display or nucleic acid selection technology) with specific affinity for one or more of the said analytes. Furthermore, the fluorescent residues that are bound to the spesific binding molecules that are used in the reagent according to the non-competitive embodiment of the present invention, preferably have a maximum absorption coefficient at a wavelength exceeding between 600 nm and 1000 nm, further preferred exceeding 620 nm, and even further preferred exceeding 640 nm.

According to the present invention the test sample solution may be a biological material or extracts thereof, such as e.g. blood, blood serum or blood plasma, lachrymal fluid, extracts of feces, plant extracts, aspirate from the gastrointestinal tract or semen or seminal fluids, possibly diluted in diluent solutions or depository solutions, possibly with other reagents added to prevent coagulation or microbiological growth or oxidation or reduction or to regulate the acidity, alternatively derivatives or filtrates.

The different embodiments of present invention may be influenced by changes in the surrounding temperature or in the test sample solution or the reagent or in the mixture or in the instrument or the measurement compartment of the instrument to be used. Such temperature influences can be counteracted by the reagents and/or the instrument being temperature regulated, or by using calibrators with known concentrations of the analyte. However, one of the aims of the present invention is to provide measurement methods where calibrators are not used. Empirical measurements and theoretical calculations based on measurements with the reagents provided by the present invention may, however, form the basis for estimated deviation as a result of varying temperatures. Thus, according to the present invention and if desired, it is possible to use combinations of temperature sensors and software connected to or as a part of the measurement instrument, providing total or partial correction for the temperature-provoked deviation in the concentration determinations.

With the use of the present invention a large number of analytes can be concentration determined. The following analytes can be listed tabularly, but there is obviously a large number of other analytes that have not been included in this listing:

Hemoglobin
Albumin
C-reactive protein
Albumin in urine
Glycated albumin
Glycated hemoglobin
Ferritin
ASAT
ALAT
LDH
Myoglobin
Troponin I
Fatty Acid Binding Protein
Amylase
Glucose
HCG
U-HCG
TA-tests
Insulin
Anti-insulin antibodies
Helicobacter antibodies
Thyroxin
Free ryoxin
Prostate specific antigen
Free Prostate specific antigen
Thyroid stimulating hormone
Creatine kinase type MB

These and a large number of other analytes are referred to in reference books, such as e.g. in Tietz Textbook of Clinical Chemistry, Saunders Company, ISBN 0-7216-4472-4, 1994, and others, and also later text books and general surveys in clinical chemistry and pathology.

According to the competing embodiment of the present invention, a ready-to-use preformed reagent, comprising one or more binding pairs where both binding partners in each binding pair is present in one single container, and where the reagent is composed in such a way that aggregations, precipitations and irreversible binding between the binding pairs are avoided, is used. When the test material is mixed with

the reagent, the analyte(s) compete with the binding between the binding partners already present in the reagent in at least one of the binding pairs.

The reagent provided for the competitive embodiment of the present invention, can be used for concentration determination of one or more analytes, and may be characterized by the fact that for each analyte the reagent comprises at least one binding pair in which the binding partners are reversibly bound to each other. Furthermore, the reagent related to this embodiment of the invention may be characterized by at least one of the binding partners in at least one of the said binding pairs comprising or having bound to it a fluorescent chemical residue.

By «reversibly bound» what is meant here is a binding that is such that addition of a substance that competes with the binding between the binding partners leads to a change in the concentrations of the other molecules included in the equilibrium for formation of binding pairs between said binding partners within a reasonable amount of time - typically within one hour after adding. The invention is further characterized by the said changed concentrations of the molecules included in the equilibrium for formation of binding pairs between the binding partners leading to a change in the fluorescence signals that can be generated from the said fluorescent residues. This can occur because in the reagent characterized by this invention such fluorescent residues are used which can generate signals that change when the fluorescent residue is part of a connected binding pair as compared to the signals generated when the residue is bound to a binding partner molecule that is not bound to its binding partner molecules.

The reagent used according to the competitive embodiment of the present invention is characterized by comprising one or more binding pairs for which there is, furthermore, an equilibrium between the free condition in which the binding partners are not bound to each other, and the bound condition in which the binding pairs are bound to each other. This equilibrium is subject to general chemical laws, such as the law of mass action. When the concentrations of one of the molecules included in the chemical equilibrium are changed, the concentrations of the remaining molecules included in the equilibrium will change as well. Furthermore, the equilibrium will shift by adding analogues of the binding partners or derivatives or analogues of binding partners, where the structural similarity is adequate to bring about competition over the binding to the corresponding binding partner. Such structural similarity is mostly conserved, also when modifications are used for attaching fluorescent residues that do not dramatically change the structure of the substance that is attached onto this fluorescent residue. It is further commonly known to the skilled man of the art that if fluorescent residues or labels with different fluorescence are used, it is possible to simultaneously measure different chemical reactions in the same solution. Thus, the reagent in accordance with the present invention is further characterized by the fact

that by using residues or labels with different fluorescence, it can be used to quantify different analytes simultaneously in the same sample.

Traditionally, as high affinity equilibrium constant as possible was desired for the use of bio specific binders such as antibodies. The affinity equilibrium constant is a complex quantity, constituted by the association velocity constant divided by the dissociation velocity constant, and traditionally affinity equilibrium constants exceeding 10E7 have been desired, preferably exceeding 10E8 and even more preferred exceeding 10E9. At especially low concentrations affinity equilibrium constants exceeding 10E10 have been desired in order to achieve that it should be possible to bind an acceptable share of the analyte to the binder. Especially, it has been claimed that high dissociation velocity must be avoided (Immonoassay (E.P.Diamandis, T.E. Christopoulos, eds.) San Diego, CA; Academic Press (1996). Polyclonal antibodies have – since they are polyclonal – very varying affinity equilibrium constants in the same preparation, whereas monoclonal antibodies, or the biological or synthetic binders accounted for in the background for this invention, have more uniform or identical affinity equilibrium constants within the same preparation, and also often a more constant affinity from preparation to preparation. In the competitive embodiment of the present invention, and contrary to what was previously desired, especially high affinity equilibrium constants are not desired, and in particular not too low dissociation velocity constants.

As accounted for in the paragraph on the background for the present invention, the average skilled man of the art is used to using different types of specific binding molecules. The reagent in accordance with the competitive embodiment of the present invention may comprise binding partners that form binding pairs of all types and varieties. Traditional polyclonal antibodies on the one hand and antigens on the other hand may be used as specific binding molecules. Instead of polyclonal antibodies monoclonal antibodies may be used. The antibodies may be complete or in the form of reactive fragments. Especially preferred are smaller fragments of antibodies such as FAB fragments or single chain antibodies or single chain antibody fragments. Instead of antibodies or antibody fragments produced in eucaryotic cells, it is possible to use binders provided through phage display or further advancements of phage display technology, in the form of polypeptides or other types of polymers, polynucleic acids, or binders composed of building blocks that are variedly composed and picked out using library technology. Synthetic combinatory chemistry is rapidly developing and can be used to produce specific binding molecules, and production of polymers with RNA or DNA or analogue monomers is used with increasing frequency, and may of course also be used as specific binding molecules in the present invention.

The antigens mentioned above are traditional binding partners in immunoassay technology, but in recent years parts of antigens, antigen fragments, so-called

haptens, and derivatives of antigens or haptens, have been used to a greater extent. Synthetically or biologically produced molecules with a high structural similarity to the analyte or analytes that are to be quantified, can also be used. The present invention is characterized by the fact that all these structures may be used in binding pairs, when a suitable binding partner is found.

The present invention may further be characterized by the use of binding pairs in the preformed reagent for which the binding of the binding partners is influenced by the concentration of the analyte or analytes. A high concentration of an analyte will lead to an increased competition for binding to one of the binding partners in one or more binding pairs, compared to what would be the case with a lower concentration of said analyte. The present invention is further characterized by the fact that this leads to another concentration of one or more of the unbound binding partners and/or the binding pairs that were present in the reagent before it was mixed with the sample material. The reagent in accordance with the present invention is further characterized by the fact that it is composed in such a way that this changed concentration of one or more of the binding partners can be detected using one or more of the methods that are described above.

In order to measure an analyte in a competitive binding assay, the binding partner that the competition is about, e.g. the specific antibody, must be in effective relative deficit in relation to the concentration of the analyte (in order for competition to arise). But since what is desired is to measure the signal that is changed as analyte molecules with fluorescent residue are bound to the binding partner, the concentration of the binding partner (e.g. antibody) must be so high that a considerable part of the analyte molecules can be bound to the binding partner. Here it is necessary to find a practical balance that is regulated by the analyte concentration in the solution and the choice of specific binding molecule with suitable affinity equilibrium constant and dissociation velocity constant. Furthermore, according to the competitive embodiment of the present invention, the binding molecules and fluorescent derivatives or analogues of the analyte molecules are kept as binding pairs in the same reagent container. Thus the use of polyclonal antibodies as specific binding molecules in combination with large molecular analytes should be avoided, since this typically could result in precipitation in the reagent or the assay solution. Monoclonal antibodies are most often to be preferred over polyclonal antibodies, and often further preferred are monovalent binding molecules readily of smaller molecular size, e.g. FAB-fragments of antibodies or polypeptides or aptamers.

When a reagent in accordance with the competitive embodiments of the present invention is mixed with a sample containing the analytes that are to be concentration determined, the concentration of the analytes in the sample material will lead to changes in the concentrations of the other molecules included in the equilibriums for

formation of said binding pairs between said binding partners. The invention is further characterized by the fact that the said changed concentrations of the molecules included in the equilibrium for formation of binding pairs between the binding partners lead to a change in the signals that can be generated from the said fluorescent residues, and that these signal changes can be used for quantification of said analytes.

The said changes in the fluorescence signals are, in other words, a direct function of the concentrations of the analytes, and these signal changes can be used for concentration determination of the said analytes. Thus, the fluorescent residues according to the present invention may have one or more of the specific binding molecules bound to them, and the fluorescence signal that can be generated changes as a consequence of the said specific binding molecules binding to the analyte molecules.

If a concentration of approximately 1.0*10E-9 molar analyte is present in the assay solution (the solution arrived at when the reagent related to the present invention is mixed with the test material), and we have an infinitely high affinity equilibrium constant, we will, by using an effective concentration of 0.5* 10E-9 molar specific binding molecule, achieve 50 % binding of the analyte to the binding partner at equilibrium. If the affinity equilibrium constant is lower, e.g. 1.0*10E-9, the situation at equilibrium is somewhat different:

Given that the analyte molecules have the same affinity for the binding molecules (e.g. antibodies) whether they have fluorescent residues bound to them or not, and

a = affinity equilibrium constant,

b = aggregate concentration of binding partner molecules (bound and free put together), and

c = aggregate concentration analyte molecules (totally from the sample and the reagent related to the present invention, including analyte molecules with fluorescent residues),

according to the law of mass action the aggregate concentration of binding pairs (e.g. antibody-antigen complexes) = x would be as follows at equilibrium:

$x = a(b-x)(c-x)$

In a situation where a = 1.0*10E9, b = 1.0*10E-10 and c = 2.0*10E-9, calculation shows that at equilibrium

x = 0.7*10E-10 molar, and further that the effective concentration of free specific binding molecule (e.g. antibody) is 0.3*10E-10 molar and further that the

concentration of unbound analyte molecules (with and without fluorescent residues bound to them) is 19.3*10E-10 molar.

We see that this will give a very small change in the signal as a consequence of the analyte molecules' binding to the binding partner. In this case it would be possible to bind less than 5 % of the analyte molecules to the binding partner.

If the affinity equilibrium constant a is 1.0*10E8 but b and c above are unchanged, it could accordingly be shown that the fraction of bound analyte molecule is infinitesimally small, and no competition what so ever occurs.

If the situation is such that the specific binding molecule's (e.g. the antibody's) affinity for modified analyte molecules with fluorescent residue bound to them is higher than for unmodified analyte molecules, a lower concentration of the modified analyte molecules needs to be used for the analyte molecules in the sample to be able to efficiently compete. This corresponds to a situation with a lower effective concentration of specific binding molecule, and will require a somewhat higher affinity equilibrium constant of the specific binding molecule according to the law of mass action.

If the situation is such that the specific binding molecule's (e.g. the antibody's) affinity equilibrium constant for modified analyte molecules with fluorescent residue bound to them is lower than for unmodified analyte molecules, it might be desirable to use a higher concentration of the modified analyte molecules. To achieve a situation with competition, however, the effective concentration of specific binding molecule must be in molar deficiency relative to the analyte molecules in the sample, and this low concentration of the specific binding molecule still requires that the affinity equilibrium constant between binding partner and analyte molecules is high. It is worth mentioning, however, that the effective concentration of binding molecules may be considerably less than the total amount of so-called binding molecules. It is not seldom than only a fraction of the actual binding molecules – e.g. monoclonal antibodies – really bind its binding partners. This can be due to structural alterations, e.g. in the synthesis of the molecules, e.g. post-translation alterations, or modifications introduced by labeling chemistries or simply of unknown origin.

Thus we can conclude that the affinity equilibrium constant, according to a competitive embodiment of the present invention, must have a value that at least equals one third of the inverse value of the concentration of the modified analyte molecules with signal providing residues bound to them. More preferred are affinity equilibrium constants that are higher than the inverse value of the concentration of the modified analyte molecules, and even more preferred are affinity equilibrium constants higher than twice the inverse value of the concentration of the modified analyte

molecules. Thus, in the reagent provided according to the competitive embodiment of the present invention, an equilibrium between the binding partners bound to each other and the binding partners in free, non-bound form will be established. When the said reagent is added to a sample, the concentrations change, and a new equilibrium is established. In the earlier technical state, a high affinity equilibrium constant and an especially high association constant was desired so that the equilibrium could be established soon after adding a binding molecule, e.g. a antibody. In the competitive embodiment of the present invention binding pairs have formed in the reagent before the sample is added, and then a higher dissociation velocity constant is sooner desired, so that a new equilibrium can be achieved within reasonable time after the sample has been added.

If affinity equilibrium constant

a = 1.0*10E10/mol,

b = aggregate concentration of binder (e.g. antibody) = 1.0*10E-9 molar

c = aggregate concentration analyte molecules (with and without signal providing residues) = 1.0*10E-9 molar

it can, by using mass balance and the law of mass action, be shown that at equilibrium the concentration of complex between the binding partners (analyte molecules with or without signal-providing residue and binder such as e.g. antibody) = 0.73*10E-9 molar, the concentration of analyte molecules unbound to binding partner = 0.27*10E-9 molar, and the concentration of unbound specific binding molecules (e.g. antibody) = 0.27*10E-9 molar. If then a test sample that adds 1.0*10E-9 molar analyte molecules is added, so that the total amount of analyte molecules in the assay solution becomes 2.0*10E-9 molar, a new equilibrium will be established after a while.

In these examples it is assumed that adding a sample does not change the total volume of the assay solution considerably. Calculations can be made showing that even though the assay solution is slightly changed, the above calculation examples will be very close to the conditions described.

If the affinity equilibrium constant is a power of 10 higher, the situation is as follows:

If affinity equilibrium constant

a = 1.0*10E11/Molar

b = aggregate concentration binder (e.g. antibody) = 1.0*10E-9 Molar

c = aggregate concentration analyte molecule (with and without signal providing residues) = 1.0*10E-9 molar

it can, using calculations corresponding to the above, be shown that in the competitive embodiment of the invention, it is predominantly the dissociation velocity constants that determines the time it takes to achieve equilibrium or near equilibrium after adding a sample. Slightly simplified we can say that figures 1 to 9 show that the value of the affinity equilibrium constant is the most important factor in deciding the range of signal change that can be achieved, whilst it is the value of the dissociation velocity constant that determines how quickly a new equilibrium or near equilibrium can be achieved after mixing in a sample comprising analyte molecules, and thus how quickly a new stabile fluorescence signal is achieved. In the reagent used for endpoint measurement in the competitive embodiment of the present invention, binding pairs with dissociation velocity constants 0.003 per second are therefore preferred. Binding pairs with dissociation velocity constants exceeding 0.01 per second are more preferred, and binding pairs with dissociation velocity constants exceeding 0.02 per second even more preferred.

If kinetic reading is applied, i.e. measuring the change in the fluorescence signal per time unit or within a defined time interval, measurements can be made before a new equilibrium is established, and still the signal change can be used to calculate the analyte concentration or analyte concentrations. Thus, in the competitive embodiment of the present invention kinetic readings therefore allow the use binding pairs with dissociation velocity constants that are considerably lower than those used for endpoint readings. Then binding pairs with dissociation velocity constants as low as 0.0001 per second can be used, but more preferred are dissociation velocity constants exceeding 0.001 per second, and still more preferred are dissociation velocity constants exceeding 0.005 per second.

A special embodiment of the method according to the present invention is to use a reagent in accordance with the present invention, and measure the change in degree of polarization of the fluorescence signal per time unit, or as a function of time, or within a given time interval after mixing in the sample material. In this embodiment the fluorescence signal or fluorescence signals can be read as a function of time, either as a continuous reading within a period of time, or as change per time unit between 2 or more points of time, or as an absolute change between 2 or more points of time. Such measurements are often called kinetic measurements or readings. The method according to present invention can also use kinetic reading methods with the other forms of fluorescence measurement methods described. The above mentioned applies to both the competitive and the non-competitive embodiments of the method according to the present invention.

An applicable embodiment of the present invention is further to use the above mentioned kinetic fluorescence polarization measurement, combined with the use of a reagent in accordance with the present invention, for which the wavelength of the maximum absorption coefficient for the fluorescent residues is higher than 600 nm, or further preferred 620 nm or even more preferred exceeding 640 nm. For analysis of samples comprising hemoglobin, the maximum absorption coefficient should exceed 620 nm, since the hemoglobin interfers substantially with the concentration determination of the analyte or analytes. Such interference have usually been a major problem when fluorescence polarisation assay has been used with e.g. blood.

In the non-competitive embodiment of the present invention, the fluorescent residues will usually, but not always, be bound to the specific binding molecule. The specific binding molecule may be present in excess compared to the test sample/analyte molecules to be added, and the fluorescence signal that may be generated will change if analyte molecules have bound to the specific binding molecules. A good example of such a suitable specific binding molecule is aptamers, described in «Selection of singlestranded DNA molecules that bind and inhibit human thrombin», by Bock & al., Nature vol. 355 pp 564-56, 1992. This article refers to a generic technology as general basis for production of specific binding molecules, and shows that aptamers comprising the nucleotide sequence GGTTGGTGTGGTTGG or GGTTGG are specifically bound to human thrombin.

Aptamers can also be used in the competitive embodiments of this invention, as well as in the non-competitive embodiments. Gold & al. in «Diversity of oligonucleotide functions» in Annel. Rev. Bichem. vol 64, pp 763-97, 1995, describe a large number of examples of use of the generic aptamer technology for production of specific binders, that can also be used in the reagent according to the present invention. The specific binder (described in Science, vol. 263, 11. March, 1994) that is assigned to theophyllins is an example that is especially well suited for the competitive embodiments, whereas the RNA-aptamer described in the same place, which binds T4 DNA polemerase, is suited for the non-competitive embodiments.

Rick Konrad et al. in «Isoenzyme-specific inhibition of Protein Kinase by RNA aptamers» in J. Biol. Chem. vol. 269, pp 32051-54, 1994, have described two different aptamer frequencies suitable as specific binding molecules for Protein Kinase C, which may serve as an example of specific binding molecules to be used in the reagent according to the present invention.

Both in the competitive and the non-competitive embodiments of the present invention, specific binding molecules that include peptide sequences may be used. An example of a peptide sequence that is especially suitable in the non-competitive embodiment is the peptide sequence described by Chakravarthy & al. in Anal.

Biochem. vol. 196, 144- 150, 1991. Other examples of peptide sequences usefull as spesific binders are described by Yue et al. in The Journal of Biological Chemistry, vol. 271, p. 22245-22250, 1996. They were able to identify peptides that bind C-reactive protein in a dot blot assay employing numerous reagents, radioactive substances and autoradiography, a time consuming and expensive procedure.

The reagent in accordance with the invention, and in both the competitive and the non-competitive embodiment of the method, may be characterized by the fact that it is prepared solely for concentration determination of an analyte. The reagent may nevertheless, if so desired, comprise more than one specific binding molecule, and if desired, more than one specific binding molecule may include said fluorescent substance or include several fluorescent substances. The reagent may however also be characterized by the fact that it is prepared for concentration determination of several analytes, if desired - simultaneously, and - if desired - with several different specific binding molecules for each analyte. If so desired it is possible to use more than one of these binding pairs which include a binding partner with a fluorescent residue, or even several different fluorescent residues bound to different binding partners in different binding pairs. An almost unlimited amount of possibilities for signal-providing systems exists here. Several signal-providing systems will potentially increase the precision of the concentration determinations, but will at the same time increase the complexity of the measurement systems.

Traditional fluorescent substances such as fluorescein and Texas Red, Oregon Green, Rhodamin, tetramethyl-rhodamin, amino methyl coumarin, as well as a wide range of other substances may be used as fluorescent residues, see e.g. «Handbook of Fluorescent Probes and research chemicals» by Richard P. Haugland, sixth edition, ISBN 0-9652240-0-7. This handbook also provides method description for binding of the fluorescent substances to amino groups, thiol groups, alcohol groups, ketones, dioles and carboxylic acids. Furthermore it provides methods for binding of fluorescent substances to peptides, proteins, antibodies, nucleic acid polymers such as aptamers and other polymers which, however, is well known for a person skilled in the art.

Often, substantial amounts of hemoglobin or bilious pigments are present in the sample material during medical and biological use of the present invention. Then reagents comprising fluorescent substances with excitation or emission wavelengths that are absorbed by hemoglobin or bilirubin will often be influenced by the light absorption that characterizes the said substances. As already mentioned, an especially preferred embodiment of the present invention when analysing whole blood or blood lysates, is therefore based on reagents with fluorescent molecules for which the wavelength for maximum absorption coefficient of the fluorescent residues exceeds 600 nm, or more preferred 620 nm or even more preferred exceeding 640 nm. For

fluorescent residue, or even several different fluorescent residues bound to different binding partners in different binding pairs. An almost unlimited amount of possibilities for signal-providing systems exists here. Several signal-providing systems will potentially increase the precision of the concentration determinations, but will at the same time increase the complexity of the measurement systems.

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Often, substantial amounts of hemoglobin or bilious pigments are present in the sample material during medical and biological use of the present invention. Then reagents comprising fluorescent substances with excitation or emission wavelengths that are absorbed by hemoglobin or bilirubin will often be influenced by the light absorption that characterizes the said substances. As already mentioned, an especially preferred embodiment of the present invention when analysing whole blood or blood lysates, is therefore based on reagents with fluorescent molecules for which the wavelength for maximum absorption coefficient of the fluorescent residues exceeds 600 nm, or more preferred 620 nm or even more preferred exceeding 640 nm. For analysis of samples comprising hemoglobin, the maximum absorption coefficient shoul exceed 620 nm since the hemoglobin interferes substantially with the concentration determination of the analyte or analytes. Such substances are sold by the company Amersham Pharmacia Biotech, under labels such as CyDye FluoroLink Reactive Dyes, with varying excitation wavelength (varying with analogues of the chemical structure), and with varying numbers of activated groups for binding to the substance that is to be labeled. More binding points will typically reduce the molecule's degree of freedom of rotation, and reduce the possibility for using fluorescence polarization measurement methods.

Suitable cyanine dyes are further described in US5627027: «Cyanine dyes as labeling reagents for detection of biological and other materials by luminescence methods» by Waggoner; Alan S, 6. May 1997. Furthermore, suitable substances are described in Mujumdar, Lauren, Mujumdar and Waggoner in Cytometry 10: 11-19, 1989 or Southwick & al in Cytometry 11, 418-430, 1990 or Lauren & al. in

less than 30 amino acids, and more preferred less than 20 amino acids, and even more preferred less than 12 amino acids.

Reffering to examples 17 and 18, where use of intermittent exitation at different wavelenghts is described for the analysis of several analytes using different exuitation wavelenghts, it is preferred to use intermittent light with different wavelengthts, e.g. by the intermittent use of different diode lasers with different wavelengths. It should be noted that the break between use of the different light sources should at least correspond to the lifetime for the exited condition of the actual fluorophore.

Best mode

Best mode is illustrated by the examples, preferably example 9 for the competitive embodiment of the invention, and by example 14 for the non-sompetitive embodiment of the invention.

EXAMPLES

Example 1: Fluorescent binding ligand for whole blood analysis.

Synthesise the peptide Tyr-Trp-Ala-Asn-Phe-Ala-Arg-Asn-Arg-Asn by conventional technique, and dissolve it in pure water to a concentration of 2 mg per ml. Mix 50 ul of the said peptide solution with 50 ul sodium bicarbonate buffer pH = 9.2. Mix 0.1 micromol Cy5 Fluorolink activated cyanin dye, supplied from Amerham Pharmacia Biotech, optionally dissolved in water-free DMSO with said solution and leav it to stand overnight at room temperature in darkness, and thereafter store the solution in a refrigerator in darkness. If wanted, 5 ul of 10 mmolar ethanolamine solution can be added to block any remaining activated fluorescent dye, or the solution - hereinafter called the stock solution - can be left to hydrolyse the non-reacted dye during storage.

Isolate pure Cy5-labelled Tyr-Trp-Ala-Asn-Phe-Ala-Arg-Asn-Arg-Asn from the stock solution by reverse phase chromatography in a C4 column (sold from many suppliers, including Waters, U.S.), using 0.1 % trifluoroacetic acid as eluant and employ a gradiant of 0 % to 60 % acetonitrile in 0.1 % trifluoroacetic acid. Employ a photodetector coupled to a flow cell to monitor content of peptides by transmission measurements at 340 nm and Cy-5 by transmisson measurements at 650 nm, and isolate the Cy-5 –conjugated peptide. Remove the trifluoroacetic acid and the acetonitrile and the water by lyophilisation.

Pure Cy5-labelled Tyr-Trp-Ala-Asn-Phe-Ala-Arg-Asn-Arg-Asn of the stock solution can also be isolated by thin layer chromatography: Apply aliquots of stock solution on silica gel plates and elute with n-butanol:acetic acid:water mixtures. Depending on the quality of the silica gel, the relative content of n-butanol:acetic acid:water can be adjusted to obtain ideal separation. After elution by conventional technique, dry the

silica gel plate and inspect visually and by UV lamp (and optionally using nihydrin spray in parallell experiments) to identify the Cy5-labelled Tyr-Trp-Ala-Asn-Phe-Ala-Arg-Asn-Arg-Asn spot, separated from non-labelled peptide and free Cy-5 dye molecules. Isolate the silicagel comprising Cy5-labelled Tyr-Trp-Ala-Asn-Phe-Ala-Arg-Asn-Arg-Asn by scissors or spatulum. Suspend the isolatedsilica gel in 10 mM TRIS-buffer pH = 8.0, whereby the Cy5-labelled Tyr-Trp-Ala-Asn-Phe-Ala-Arg-Asn-Arg-Asn is eluted into solution. The silica gel settles in the bottom of the tube. Decant off the TRIS-buffered solution with the purified Cy5-labelled Tyr-Trp-Ala-Asn-Phe-Ala-Arg-Asn-Arg-Asn.

If wanted, and for up-scaling, other conventional HPLC separation techniques well known to the skilled man of the art can be used in stead of thin layer chromatography.

Example 2: Fluorescent binding ligand for whole blood analysis.

Perform the method of the present invention according to example 1, except use Tyr-Trp-Ala-Asn-Phe-Ala-Arg-Asn-Gly-Asn instead of Tyr-Trp-Ala-Asn-Phe-Ala-Arg-Asn-Arg-Asn, to obtain Cy5-labelled Tyr-Trp-Ala-Asn-Phe-Ala-Arg-Asn-Gly-Asn.

Eksempel 3: Method for measurement of C-reactive protein in samples of whole blood.

Make an assay reagent by making a buffer of 150 mM sodium chloride 100 mM phosphate, pH = 7.4. To this buffer, add bovine gammaglobulin from Sigma to a concentration of 2 mg/ml, and Triton X-100 from Pierce Chemical Company, US, to a final concentration of 0.1 % v/v. Reagents of high purity with very low background fluorescence should be chosen. Add 1.0*10E-11 mol per ml of Cy5-labelled Tyr-Trp-Ala-Asn-Phe-Ala-Arg-Asn-Arg-Asn according to example 1 above, and optionally add a suitable bacterostatic agentlike 0.01 % sodium azide for prolonged storage. Keep aliquots of this mixture, e.g. 1 ml, in separate containers to be combined with unknown blood samples or aliquots of blood samples.

At the time for the determination of the C-reactive protein, take an aliquot, e.g. a volume of 20 ul of the whole blood sample e.g. by the use of a pre-measured capillary, and combine thereafter the content of this pre-measured capillary with the assay reagent in the said separate container, the said separate container being kept in a place where the temperature is regulated to 32 degrees of Celcius.

Typically, use a container which is in the form of a cuvette with 4 polished transparent sides to be measured in an instrument built for fluorescent polarisation measurements, but said container comprising a removable stopper or seal which allows the capillary to enter the container, either by simply dropping the capillary or

by introducing it through the seal. Furthermore, in its preferred that the capillary/container is designed so that the capillary falls to the bottom of the container and does not interfere with the excitation light or the emission light (see below). Thereafter, shake the container, and the blood flows out of the capillary and the cells are being lysed by the assay reagent. The C-reactive protein of the test sample aliquot starts to react with the Cy5-labelled Tyr-Trp-Ala-Asn-Phe-Ala-Arg-Asn-Arg-Asn of the assay reagent.

Immediately after the shaking, place the container in the fluorescence polarisation measurement instrument, having a temperature of 32 degrees of Celcius. For measurement of polarisation of the emitted fluorescence, the mixture in the container is irradiated with polarized light of 650 nm wavelength, typically by the use of a small polarized laser diode in the instrument, and when the polarisation of the emitted light constant, measure its polarisation a wavelength of 670 nm, with a rather narrow bandwidth, if the instruments allows for that. Calculate the concentration of C-reactive protein of the unknown sample by interpolation of the polarisation value of the emitted light measured on a standard curve obtained by measurement of standards of known C-reactive protein concentration. Such a standard curve can often be stored on the computer of the measurement instrument, enabling a direct reading of the concentration of C-reactive protein on the instrument.

If a measurement instrument with a flow cell is used, then bring the mixture to pass through the flow cell for measurement, according to the instructions given by the instrument manufacturer.

Typically, with a mild bacterial infection, the content of C-reactive protein of a 20 ul sample is between 10 and 100 mg per l, but event much higher values can be seen in severe clinical conditions. On the other side, especially in screening of risk for heart disease and low grade of inflammation, measurement of values below 1 mg per liter is of interest. Furthermore, each C-reactive protein molecule can react with five molecules of Cy5-labelled Tyr-Trp-Ala-Asn-Phe-Ala-Arg-Asn-Arg-Asn. The concentrations of interest for C-reactive protein therefore varies a lot, and the concentration of Cy5-labelled Tyr-Trp-Ala-Asn-Phe-Ala-Arg-Asn-Arg-Asn therefore may have to be adjusted compared to the blood volume to be combined with the assay reagent.

Eaxmple 4: Method for measurement of C-reactive protein in samples of whole blood.

Perform the method of the present invention according to example 3, except use Cy5-labelled Tyr-Trp-Ala-Asn-Phe-Ala-Arg-Asn-Gly-Asn in the place of Cy5-labelled Tyr-Trp-Ala-Asn-Phe-Ala-Arg-Asn-Arg-Asn. This example of the method is more

adequate for very high concentrations of C-reactive protein than example 3 because of Cy5-labelled Tyr-Trp-Ala-Asn-Phe-Ala-Arg-Asn-Gly-Asn lower affinity for C-reactive protein. Correspondingly, this example of the method is less appropriate to use for low concentrations of C-reactive protein.

Example 5: Method for measurement of C-reactive protein in samples of whole blood.

Perform the method of the present invention according to example 3, except however, instead of reading the polarisation after the polarisation value has become stable, measure the polarisation value as a function of time in a kinetic manner. If available, connect the measurement instrument to a computer recording the signal continuously or at different time points, optionally defined by a software. Compare then the recorded values to values obtained by measurement of standards of known C-reactive protein concentration as a function of time. Optionally, record a three-dimensional standard curve with polarisation values, different times and C-reactive protein concentration values, and compare the values obtained with the unknown sample to calculate the C-reactive protein concentration, optionally by the use of the least-square methods for best fit according to standard textbooks of statistics, optionally by means of a computer or another artificial memory connected to the measurement instrument.

Example 6: Method for measurement of C-reactive protein in samples of whole blood.

Perform the method of the present invention according to example 5, except keep the said separate containers (to be combined with unknown blood samples or aliquots of blood samples) at the room temperature, and use a electronic temperature measurement device in or at the fluorescence polarisation instrument, and use a four-dimensional standard curve, the forth dimension being temperature, in addition to polarisation values, different times and C-reactive protein concentration values, and compare the values obtained with the unknown sample to calculate the C-reactive protein concentration, optionally by the use of the least-square methods for best fit according to standard textbooks of statistics, optionally by means of a computer or another artificial memory connected to the measurement instrument.

Example 7: Synthesis of Cynanin-5 analogue of theophyllin.

Make a synthesis of 8-(3-carboxypropyl)-1,3-dimethylxanthin anhydrid as described in Research Communications in Chemical Pathology and Pharmacology , vol. 13, p. 497-505, 1976, and in Clinical Chemistry. vol. 27, page 22-226, 1981. Dissolve diaminopropanol in water-free tetrahydrofuran. In another flask, dissolve half of the equimolar amount of the said 8-(3-carboxypropyl)-1,3-dimethylxanthin anhydrid in

water-free tetrahydrofuran. Add the said 8-(3-carboxypropyl)-1,3-dimethylxanthin anhydrid solution drop-wise to the diaminopropanol solution while stirring, and let the resulting solution react over night at room temperature. Optionally purify the resulting adduct by HPLC chromatography using conventional techniques well known to the skilled man of the art, if less consumption of activated cyanin dye is wanted (see below).

Thereafter, dissolve 6 times the molar amount which was used for diaminopropanol, of Cy5 Fluorolink activated cyanin dye supplied from Amersham Pharmacia Biotech, U.K., in water-free tetrahydrofuran, and add it previously described solution while stirring. Leave the resulting mixture to react over night a room temperature in darkness. In this way, a stock solution of non-pure 8-(3-carboxypropyl)-1,3-dimethylxanthin adduct with Cy5 Fluorolink activated cyanin dye with a water-soluble diaminopropanol spacer is obtained.

Purify the resulting 8-(3-carboxypropyl)-1,3-dimethylxanthin adduct with Cy5 Fluorolink activated cyanin dye with a water-soluble diaminopropanol spacer by means of thin layer chromatography according to example 1, and adjust the volumes of n-butanol, acetic acid and water in the elution mixture depending on the quality of the silica gel plates to obtain good separation. After elution by conventional technique, dry the silica gel plate and inspect visually and by UV lamp to identifythe spot of 8-(3-carboxypropyl)-1,3-dimethylxanthin adduct with Cy5 Fluorolink spot. Isolate the silicagel 8-(3-carboxypropyl)-1,3-dimethylxanthin adduct with Cy5 Fluorolink by scissors or spatulum. Suspend the isolated silica gel in 10 mM TRIS-buffer pH = 8.0, whereby 8-(3-carboxypropyl)-1,3-dimethylxanthin adduct with Cy5 Fluorolink is eluted into solution. The silica gel settles in the bottom of the tube. Decant off the TRIS-buffered solution with the purified 8-(3-carboxypropyl)-1,3-dimethylxanthin adduct with Cy5 Fluorolink.

If wanted, and for upscaling, conventional HPLC separation techniques well known to the skilled man of the art can be used in stead of thin layer chromatography. E.g Use the HPLC method described in example 1, however using a C4 or a C6 reversed phase column.

Exampel 8: Fab-fragments of antibodies with affinity for theophyllin.

Synthesise 8-(3-carboxypropyl)-1,3-dimethylxanthin anhydrid and prepare an albumin conjugate antigen as described in Research Communications in Chemical Pathology and Pharmacology , vol. 13, p. 497-505, 1976. Using this antigen, make mouse hybridomas. Select a clone the is specifically reactive to the Cyanin-5-analogue of theophyllin in example 7. The prior art teaches several ways of screening hybrodimas, e.g. in Campell s book on «Monoclonal antibody technology», ISBN 0-444-80575-3

or other textbooks on monoclonal antibody technology. The inventor of the present invention, however, prefers to make 150 mM sodium chloride 100 mM phosphate, pH = 7.4 . To this buffer, add bovine gammaglobulin from Sigma to a concentration of 2 mg/ml, and Triton X-100 from Pierce Chemical Company, US, to a final concentration of 0.1 % v/v. Reagents of high purity with very low background fluorescence should be chosen.

Then add Cynanin-5 analogue of theophyllin, made according to example 7, to a final concentration of 2 mg per l. Measure the polarisation of the emitted fluorescence when the mixture in the container is irradiated with polarized light of 650 nm wavelength, typically by the use of a small polarized laser diode in the instrument, and when the polarisation of the emitted light constant, measure its polarisation a wavelength of 670 nm, with a rather narrow bandwidth, if the instruments allows for that. Under monitoration by such fluorescence polarisation measurements, add antibodies isolated from the hybridoma cells to be investigated. For each addition, wait until the fluorescence polarisation signal is stable, before adding more antibody. Select antibodies from one or more hybridomas which specicially increases the fluorescence polarisation of the mixture, and do not select (often called counterselect) antibodies from hybridomas whose effect on the fluorescence polarisation technology is blocked or very significantly reduced by components of human serum or serum from human individuals not treated with theophyllin. Especially check that the antibodies isolated from the hybridomas in question do not react with cafein or other drugs or food constituents that resembels theophyllin.

When antibodies from the selected hybridoma has been isolated, prepare FAB fragments by the use the ImmnuPure Fab preparation kit supplied by Pirece Chemical Company, and follow the instruction for the said kit.

Example 9: Method to determine the concentration of theophyllin in whole blood by the use of a fluorescent immunocomplex.

Make anti-theophyllin FAB fagments of antibodies according to example 8 and a Cynanin-5 analogue of theophyllin according to example 7.

Thereafter, make a tentative assay reagent at 32 degrees Celcius according to the following protocol: Make 150 mM sodium chloride 100 mM phosphate, pH = 7.4. To this buffer, add bovine gammaglobulin from Sigma to a concentration of 2 mg/ml, and Triton X-100 from Pierce Chemical Company, US, to a final concentration of 0.1 % v/v. Reagents of high purity with very low background fluorescence should be chosen.

Then add Cynanin-5 analogue of theophyllin, made according to example 7, to a final concentration of 2 mg per l. Measure the polarisation of the emitted fluorescence

when the mixture in the container is irradiated with polarized light of 650 nm wavelength, typically by the use of a small polarized laser diode in the instrument, and when the polarisation of the emitted light constant, measure its polarisation a wavelength of 670 nm, with a rather narrow bandwidth, if the instruments allows for that. Under monitoration by such fluorescence polarisation measurements, add said anti-theophyllin FAB fragments. For each addition, wait until the fluorescence polarisation signal is stable, before adding more FAB fragments. . Continue the addition of FAB fragments until noe more significant increase of polarisation value is measured with increased concentration of FAB fragments. Record this value of fluorescence polarisation. Subtract the initial fluorescence polarisation value, and calculate the difference between the values, hereinafter called «polarisation value difference».

Start over again and make a final assay reagent identical to the tentative assay reagent, however, end the addition of said FAB fragments when the fluorescence polarisation value is equal to the initial polarisation value + 80 % of the «polarisation value difference».

The final assay reagent is now ready, and optionally add a suitable bacterostatic agentlike 0.01 % sodium azide for prolonged storage. Keep aliquots of this mixture, e.g. 1 ml, in separate containers to be combined with unknown blood samples or aliquots of blood samples.

At the time for the determination of concentration of theophyllin in blood samples, take an aliquot, e.g. a volume of 20 ul of the whole blood sample e.g. by the use of a pre-measured capillary, and combine thereafter the content of this pre-measured capillary with the assay reagent in the said separate container, the said separate container being kept in a place where the temperature is regulated to 32 degrees of Celcius. Typically, use a container as described in example 3. Thereafter, shake the container, and the blood flows out of the capillary and the cells are being lysed by the assay reagent. theophyllin of the test sample aliquot starts to displace the fluorescent theophyllin of the immunocomplexes of the assay reagent.

Immediately after the shaking, place the container in the fluorescence polariasation measurement instrument, having a temperature of 32 degrees of Celcius. For measurement of polarisation of the emitted fluorescence, the mixture in the container is irradiated with polarized light of 650 nm wavelength, typically by the use of a small polarized laser diode in the instrument, and when the polarisation of the emitted light constant, measure its polarisation a wavelength of 670 nm, with a rather narrow bandwidth, if the instruments allows for that. Calculate the concentration of theophyllin of the unknown sample by interpolation of the polarisation value of the emitted light measured on a standard curve obtained by measurement of standards of

known theophyllin concentration. Such a standard curve can often be stored on the computer of the measurement instrument, enabling a direct reading of the concentration of theophyllin on the instrument.

If a measurement instrument with a flow cell is used, then bring the mixture to pass through the flow cell for measurement, according to the instructions given by the instrument manufacturer.

If the measurement instrument allows it, much better precision is obtained if - instead of reading the polarisation after the polarisation value has become stable - the polarisation value as a function of time is measured in a kinetic manner. If available, connect the measurement instrument to a computer recording the signal continuously or at different time points, optionally defined by a software. Compare then the recorded values to values obtained by measurement of standards of known theophyllin concentration as a function of time. Optionally, record a three-dimensional standard curve with polarisation values, different times and theophyllin concentration values, and compare the values obtained with the unknown sample to calculate the theophyllin concentration, optionally by the use of the least-square methods for best fit according to standard textbooks of statistics, optionally by means of a computer or another artificial memory connected to the measurement instrument. Furthermore, if you have access to a temperature recording instrument keep the said separate containers (to be combined with unknown blood samples or aliquots of blood samples) at the room temperature, and use the temperature measurement device in or at the fluorescence polarisation instrument, and use a four-dimensional standard curve, the forth dimension being temperature, in addition to polarisation values, different times and theophyllin concentration values, and compare the values obtained with the unknown sample to calculate the theophyllin concentration, optionally by the use of the least-square methods for best fit according to standard textbooks of statistics, optionally by means of a computer or another artificial memory connected to the measurement instrument.

The concentration of theophyllin in blood samples varies significantly. The main interest is measurements of therapeutic concentration values. However, higher and toxic values are of interest in forensic medicine, and lower concentrations are of interests in sports medicine. In the performance of this example of the method of the present invention, please therefore adjust the concentration of the Cynanin-5 analogue of theophyllin, and hence the FAB fragment concentration. At high concentration ranges, FAB fragments of high dissociation rate constant must be chosen. At low concenration ranges, use FAB fragments with both high association rate constant and high dossociation rate constant.

Example 10: Fluorescent peptide for determination of concentration of albumin in urine.

Synthesise the peptide Asp-Ala-His-Lys-Ser-Glu-Val-Ala (the N-terminal peptide of human albumin) by conventional technique, and dissolve it in pure water to a concentration of 2 mg per ml. Mix 50 ul of the said peptide solution with 50 ul sodium bicarbonate buffer pH = 9.2. Mix 0.1 micromol 6-carboxyfluorescein-N-hydroxysuccinimide, optionally dissolved in water-free DMSO with said solution and leave it to stand overnight at room temperature in darkness, and thereafter store the solution in a refrigerator in darkness. If wanted, 5 ul of 10 mmolar ethanolamine solution can be added to block any remaining activated fluorescent dye, or the solution – herinafter called the stock solution - can be left to hydrolyse the un-reacted dye during storage.

Purify fluorescein labelled peptide from the stock solution by thin layer chromatography: Apply aliquots of stock solution on silica gel plates and elute with n-butanol:acetic acid:water in a mixture. Depending on the quality of the silica gel, the relative content of n-butanol:acetic acid:water can be adjusted to obtain ideal separation. After elution by conventional technique, dry the silica gel plate and inspect visually and by UV lamp (and optionally using nihydrin spray in parallell experiments) to identify the fluorescein labelled peptide spot, separated from non-labelled peptide and free fluorescin dye molecules. Isolate the silicagel comprising fluorescein labelled peptide by scissors or spatulum. Suspend the isolatedsilica gel in 50 mM BIS-TRIS-buffer pH = 8.0, whereby fluorescein labelled peptide is eluted into solution. The silica gel settles in the bottom of the tube. Decant off the TRIS-buffered solution with the purified fluorescein labelled peptide.

If wanted, and for upscaling, conventional HPLC separation techniques well known to the skilled man of the art can be used in stead of thin layer chromatography. E.g. Use the HPLC method described in example 1.

Example 11: Method to determine the concentration of albumin in urine by the use of a fluorescent immunocomplex.

Make anti- Asp-Ala-His-Lys-Ser-Glu-Val-Ala antibodies using conventional techniques well known to the skilled man of the art, e.g. by the use of an antigen formed by coupling of the peptide to keyholde limpet cyanin. Alternatively, purchase the peptide synthesis and the raising of antibodies from a service providing company, e.g. by Eurogenentech of Belgium. Make FAB fragments from the antibodies antibodies by the use the ImmnuPure Fab preparation kit supplied by Pirece Chemical Company, and follow the instruction for the said kit.

Make fluorescein labelled peptide as described in example 10. On selection of monoclonal antibody, see below.

Thereafter, make a tentative assay reagent at 32 degrees Celcius according to the following protocol: Make 150 mM sodium chloride 100 mM phosphate, pH = 7.4 . To this buffer, add bovine gammaglobulin from Sigma to a concentration of 2 mg/ml, and Triton X-100 from Pierce Chemical Company, US, to a final concentration of 0.01 % v/v. If necessary to remove protein reactive to the monoclonal antibody to be used (see below), pass the solution over an immunosorbent with anti-human albumin monoclonal antibodies, by the use of conventional immunoadsorbtion techniques well known to the skilled man of the art.

Reagents of high purity with very low background fluorescence should be chosen.

Then add said fluorescein labelled peptide to a final concentration 1.0*10E-9 mol per l. Measure the polarisation of the emitted fluorescence when the mixture in the container is irradiated with polarized light of 475 nm wavelength., and when the polarisation of the emitted light constant, measure its polarisation a wavelength of 525 nm, with a rather narrow bandwidth, typical of 10 nm, if the instruments allows for that. Under monitoration by such fluorescence polarisation measurements, add said anti-albumin FAB fragments. For each addition, wait until the fluorescence polarisation signal is stable, before adding more FAB fragments. Continue the addition of FAB fragments until no more significant increase of polarisation value is measured with increased concentration of FAB fragments. Record this value of fluorescence polarisation. Subtract the initial fluorescence polarisation value, and calculate the difference between the values, hereinafter called «polarisation value difference».

Start over again and make a final assay reagent identical to the tentative assay reagent, however, end the addition of said FAB anti-albumin fragments when the fluorescence polarisation value is equal to the initial polarisation value + 80 % of the «polarisation value difference».

The final assay reagent is now ready, and optionally add a suitable bacterostatic agent like 0.01 % sodium azide for prolonged storage. Keep aliquots of this mixture, e.g. 2 ml, in separate containers to be combined with unknown urine samples or aliquots of urine samples.

At the time for the determination of concentration of albumin in a urine samples, take an aliquot, e.g. a volume of 20 ul of the urine sample e.g. by the use of a pre-measured capillary, and combine thereafter the content of this pre-measured capillary with the assay reagent in the said separate container, the said separate container being kept in a place where the temperature is regulated to 32 degrees of Celcius.

Typically, use a container as described in example 3. Thereafter, shake the container, and the urine flows out of the capillary. Albumin of the test sample aliquot starts to displace the fluorescein labelled peptide of the immuno-complexes of the assay reagent.

Immediately after the shaking, place the container in the fluorescence polarisation measurement instrument, having a temperature of 32 degrees of Celcius. For measurement of polarisation of the emitted fluorescence, the mixture in the container is irradiated with polarized light of 475 nm wavelength, and when the polarisation of the emitted light constant, measure its polarisation a wavelength of 525 nm, with a rather narrow bandwidth, typically 10 nm, if the instruments allows for that. Calculate the concentration of human albumin of the unknown sample by interpolation of the polarisation value of the emitted light measured on a standard curve obtained by measurement of standards of known human albumin concentration. Such a standard curve can often be stored on the computer of the measurement instrument, enabling a direct reading of the concentration of albumin.

If a measurement instrument with a flow cell is used, then bring the mixture to pass through the flow cell for measurement, according to the instructions given by the instrument manufacturer.

If the measurement instrument allows it, much better precision is obtained if - instead of reading the polarisation after the polarisation value has become stable - the polarisation value as a function of time is measured in a kinetic manner. If available, connect the measurement instrument to a computer recording the signal continuously or at different time points, optionally defined by a software. Compare then the recorded values to values obtained by measurement of standards of known human albumin concentration as a function of time. Optionally, record a three-dimensional standard curve with polarisation values, different times and human albumin concentration values, and compare the values obtained with the unknown sample to calculate the human albumin concentration, optionally by the use of the least-square methods for best fit according to standard textbooks of statistics, optionally by means of a computer or another artificial memory connected to the measurement instrument. Furthermore, if you have access to a temperature recording instrument keep the said separate containers (to be combined with unknown blood samples or aliquots of blood samples) at the room temperature, and use the temperature measurement device in or at the fluorescence polarisation instrument, and use a four-dimensional standard curve, the forth dimension being temperature, in addition to polarisation values, different times and human albumin concentration values, and compare the values obtained with the unknown sample to calculate the human albumin concentration, optionally by the use of the least-square methods for best fit according to standard

textbooks of statistics, optionally by means of a computer or another artificial memory connected to the measurement instrument.

The concentration of albumin in urine samples varies significantly. Reference values are in the range of up to 20 mg per liter. Slightly elevated values of uncertain origin are found in some healthy individuals, individuals who may stay healthy to old ages. Moderate elevation of the albumin values are seen as early signs of diabetic kidney damage. In severe kidney disease, urine albumin concentration may rise to many hundred mgs per liter. In the performance of this example of the method of the present invention, please therefore adjust the concentration of the fluorescein labelled peptide , and hence the FAB fragment concentration. At high concentration ranges, FAB fragments of high dissociation rate constant must be chosen. At low concentration ranges, use FAB fragments with both high association rate constant and high dissociation rate constant.

Example 12: Method for measurement of Tobramycin in whole blood.

Synthesise RNA molecules which bind tobramycin is synthesised according to the article «RNA molecules that specifically and stoichiomterically bind aminoglycoside antibiotics with high affinities» by Wang & al., published in «Biochemistry» 1996, 35, 12338-12346.

Buy Tobramycin from Fluka. Dissolve 200 umoles Tobramycin in 500 ul water. Add 500 ul dimethylformamide. Cool the solution to 5 degrees Celcius. Equimolar concentration Cy5 Fluoro Link molecules in DMF solution is added, and the mixture is stirred at 5 degrees Celcius for 2 hours.

Elute the mixture CG 50 cathion exchange chromatography, with a gradient of 0 to 500 mM ammonium hydroxyde.

The desired conjugated is thereby isolated and lyophilized to remove the ammonium hydroxyde.

Make an assay buffer consisting of 140 mM NaCl, 5 mM KCl, 1 mM manganes chloride. 1 mM calcium chloride and 20 mM Hepes , and 3 mg bovine gamma globulin per ml, and pH = 7.4. Add 30 ng Tobramycin-Cy-5-conjugate per ml assay solution, and thereafter add an equimolare amount of the synthesised RNA molecules.

The final assay reagent is now ready, and optionally add a suitable bacterostatic agentlike 0.01 % sodium azide for prolonged storage. Keep aliquots of this mixture, e.g. 1 ml, in separate containers to be combined with unknown blood samples or aliquots of blood samples.

At the time for the determination of concentration of Tobramycin in blood samples, take an aliquot, e.g. a volume of 20 ul of the blood sample sample e.g. by the use of a pre-measured capillary, and combine thereafter the content of this pre-measured capillary with the assay reagent in the said separate container, the said separate container being kept in a place where the temperature is regulated to 32 degrees of Celcius. Typically, use a container as described in example 3. Thereafter, shake the container, and the blood flows out of the capillary. Tobramycin of the test sample aliquot starts to displace the Cy-5 conjugated Tobramycin of the RNA/Cy-5-Tobramycin complex of the assay reagent.

Immediately after the shaking, place the container in the fluorescence polarisation measurement instrument, having a temperature of 32 degrees of Celcius. For measurement of polarisation of the emitted fluorescence, the mixture in the container is irradiated with polarized light of 649 nm wavelength, and when the polarisation of the emitted light constant, measure its polarisation a wavelength of 670 nm, with a rather narrow bandwidth, typically 10 nm, if the instruments allows for that. Calculate the concentration of Tobramycin of the unknown sample by interpolation of the polarisation value of the emitted light measured on a standard curve obtained by measurement of standards of known human albumin concentration. Such a standard curve can often be stored on the computer of the measurement instrument, enabling a direct reading of the concentration of albumin.

If a measurement instrument with a flow cell is used, then bring the mixture to pass through the flow cell for measurement, according to the instructions given by the instrument manufacturer.

If the measurement instrument allows it, much better precision is obtained if - instead of reading the polarisation after the polarisation value has become stable - the polarisation value as a function of time is measured in a kinetic manner. If available, connect the measurement instrument to a computer recording the signal continuously or at different time points, optionally defined by a software. Compare then the recorded values to values obtained by measurement of standards of known Tobramycin concentration as a function of time. Optionally, record a three-dimensional standard curve with polarisation values, different times and Tobramycin concentration values, and compare the values obtained with the unknown sample to calculate the Tobramycin concentration, optionally by the use of the least-square methods for best fit according to standard textbooks of statistics, optionally by means of a computer or another artificial memory connected to the measurement instrument. Furthermore, if you have access to a temperature recording instrument keep the said separate containers (to be combined with unknown blood samples or aliquots of blood samples) at the room temperature, and use the temperature measurement device in or at the fluorescence polarisation instrument, and use a four-dimensional standard

curve, the forth dimension being temperature, in addition to polarisation values, different times and Tobramycin concentration values, and compare the values obtained with the unknown sample to calculate the Tobramycin concentration, optionally by the use of the least-square methods for best fit according to standard textbooks of statistics, optionally by means of a computer or another artificial memory connected to the measurement instrument.

The concentration of Tobramycin in blood samples varies significantly, dependant on for what the assay is used. Measurements of therapeutic concentrations varies with clinical indications, and measurements of blood concentrations in pharmacokintetic studies will be different. In the performance of this example of the method of the present invention, please therefore adjust the concentration of the Tobramycin-Cy-5-conjugate and RNA molecules to the appropriate level. The J6RNA RNA molecule published in «Biochemistry» 1996, 35, 12338-12346, is appropriate at low concentrations, other RNA molecules with lower affinity can be identified by the Selex technology and by synthesis methods well known to the skilled man of the art.

Example 13: Fluorescent insulin for determination of concentration of human auto-anti-insulin antibodies.

Synthesise human insulin by conventional technique, and dissolve it in pure water to a concentration of 2 mg per ml. Mix 50 ul of the said peptide solution with 50 ul sodium bicarbonate buffer pH = 9.2. Mix 0.2 Cy5 Fluorolink activated cyanin dye, supplied from Amersham Pharmacia Biotech, optionally dissolved in water-free DMSO with said solution and leave it to stand overnight at room temperature in darkness, and thereafter store the solution in a refrigerator in darkness. If wanted, 5 ul of 10 mmolar ethanolamine solution can be added to block any remaining activated fluorescent dye, or the solution – herinafter called the stock solution - can be left to hydrolyse the un-reacted dye during storage.

Purify Cy-5 labelled insulin from the stock solution by reverse phase chromatography in C4 column (sold from many suppliers, including Waters, U.S.), using 0.1 % trifluoroacetic acid as eluant and employ a gradiant of 0 % to 60 % acetonitrile in 0.1 % trifluoroacetic acid. Employ a photodetector coupled to a flow cell to monitor content of peptides by transmission measurements at 340 nm and Cy-5 by transmisson measurements at 650 nm, and isolate the Cy-5 –conjugated insulin. Remove the acetonotrile and the trifluoracetic acid by lyophilization.

Alternatively, other HPLC methods for isolation of peptide conjugates well known to the skilled man of the art may be chosen.

Eksempel 14: Method for measurement of anti-insulin antibodies in samples of whole blood.

Make an assay reagent by making a buffer of 150 mM sodium chloride 100 mM phosphate, pH = 7.4. To this buffer, add insulin free bovine gammaglobulin from Sigma to a concentration of 2 mg/ml, and Triton X-100 from Pierce Chemical Company, US, to a final concentration of 0.1 % v/v. Reagents of high purity with very low background fluorescence should be chosen. Add e.g. 1.0*10E-12 mol per ml (for choice of concenration, see below) of Cy5-labelled human insulin according to example 13 above, and optionally add a suitable bacterostatic agentlike 0.01 % sodium azide for prolonged storage. Keep aliquots of this mixture, e.g. 1 ml, in separate containers to be combined with unknown blood samples or aliquots of blood samples.

At the time for the determination of concentration of anti-insulin antibodies in a blood sample, take an aliquot, e.g. a volume of 20 ul of the whole blood sample e.g. by the use of a pre-measured capillary, and combine thereafter the content of this pre-measured capillary with the assay reagent in the said separate container, the said separate container being kept in a place where the temperature is regulated to 32 degrees of Celcius. Typically, use a container is in the form of a cuvette with 4 polished transparent sides to be measured in an instrument built for fluorescent polarisation measurements, but said container comprising a removable stopper or seal which allows the capillary to enter the container, either by simply dropping the capillary or by introducing it through the seal. Furthermore, in its preferred that the capillary/container is designed so that the capillary falls to the bottom of the container and does not interfere with the excitation light or the emission light (see below). Thereafter, shake the container, and the blood flows out of the capillary and the cells are being lysed by the assay reagent. Anti-insulin antibodies of the test sample aliquot starts to react with the Cy5-labelled insulin of the assay reagent.

Immediately after the shaking, place the container in the fluorescence polarisation measurement instrument, having a temperature of 32 degrees of Celcius. For measurement of polarisation of the emitted fluorescence, the mixture in the container is irradiated with polarized light of 650 nm wavelength, typically by the use of a small polarized laser diode in the instrument, and when the polarisation of the emitted light constant, measure its polarisation a wavelength of 670 nm, with a rather narrow bandwidth, preferentially band withs of 5 nm. Calculate the concentration of insulin of the unknown sample by interpolation of the polarisation value of the emitted light measured on a standard curve obtained by measurement of standards of known insulin concentration. Such a standard curve can often be stored on the computer of the measurement instrument, enabling a direct reading of the concentration of insulin on the instrument.

If a measurement instrument with a flow cell is used, then bring the mixture to pass through the flow cell for measurement, according to the instructions given by the

instrument manufacturer. Since insulin has a rather high molecular radius, a fluorescent polarisation instrument with a high precision in the measurement of the degree of polarisasion of the light is prefered.

If the measurement instrument allows it, much better precision is obtained if - instead of reading the polarisation after the polarisation value has become stable - the polarisation value as a function of time is measured in a kinetic manner. If available, connect the measurement instrument to a computer recording the signal continuously or at different time points, optionally defined by a software. Compare then the recorded values to values obtained by measurement of standards of known anti-insulin antibody concentration as a function of time. Optionally, record a three-dimensional standard curve with polarisation values, different times and insulin antibody concentration values, and compare the values obtained with the unknown sample to calculate the concentration of anti-insulin antibodies, optionally by the use of the least-square methods for best fit according to standard textbooks of statistics, optionally by means of a computer or another artificial memory connected to the measurement instrument. Furthermore, if you have access to a temperature recording instrument keep the said separate containers (to be combined with unknown blood samples or aliquots of blood samples) at the room temperature, and use the temperature measurement device in or at the fluorescence polarisation instrument, and use a four-dimensional standard curve, the forth dimension being temperature, in addition to polarisation values, different times and anti-insuli antibody concentration values, and compare the values obtained with the unknown sample to calculate the anti-insulin antibody concentration, optionally by the use of the least-square methods for best fit according to standard textbooks of statistics, optionally by means of a computer or another artificial memory connected to the measurement instrument.

Anti-insulin antibodies are typically present in very low concentrations values in healthy subjects. Diabetics in mild er early phases of the condition will typically have low antibodies towards insulin, while patients having received insulin treatment for many years typically will have very high concenrations of antibodies towards insulin. The concentration of the Cy-5-conjugated insulin and the total sample volume must therefore be chosen according to which kind of patients the sample is taken from. If the fluorescence polarisation measured is outside the standad curve obtained with the chosen concentration of Cy-5-insulin conjugate and sample volume, another concentration of Cy-5-insulin conjugate and sample volume must be chosen.

Eksempel 15: Determination of concentration in urine by means of a Ru-ligand immunocomplex.

According to this example it is shown that it is possible to increase the molecular weight of the binding partner(s) with the fluorescent residue(s) attached, in order to

determine the concentration of an analyte/analytes. However, as mentioned earlier, this requires the use of fluorescent residue(s) with longer decay time.

Make FAB-fragments from rabbit anti-human albumin antibodies, purchased from DakoAS, Denmark, using the ImmunoPure Fab preparation kit supplied by Pirece Chemical Company, and follow the instruction for the said kit.

Furthermore, make human serum albumin conjugate with Ru(bpy)dcbpy as described by Terpetsching & al. in Analytical Biochemistry 227, 140-147, 1995.

Thereafter, make a tentative assay reagent at 32 degrees Celcius according to the following protocol: Make 150 mM sodium chloride 100 mM phosphate, pH = 7.4 . To this buffer, add bovine gammaglobulin from Sigma to a concentration of 2 mg/ml, and Triton X-100 from Pierce Chemical Company, US, to a final concentration of 0.01 % v/v. Reagents of high purity with very low background fluorescence should be chosen.

Then add said human serum albumin conjugate with Ru(bpy)dcbpy to a final concentration 1.0*10E-9 mol per l. Measure the polarisation of the emitted fluorescence when the mixture in the container is irradiated with polarized light of 485 (alternatively 360, see the said article of Terpetsching) nm wavelength., and when the polarisation of the emitted light constant, measure its polarisation a wavelength of 660 nm, with a rather narrow bandwidth, typical of 10 nm, if the instruments allows for that. Under monitoration by such fluorescence polarisation measurements, add said anti-albumin FAB fragments. For each addition, wait until the fluorescence polarisation signal is stable, before adding more FAB fragments. Continue the addition of FAB fragments until no more significant increase of polarisation value is measured with increased concentration of FAB fragments. Record this value of fluorescence polarisation. Subtract the initial fluorescence polarisation value, and calculate the difference between the values, hereinafter called «polarisation value difference».

Start over again and make a final assay reagent identical to the tentative assay reagent, however, end the addition of said FAB anti-albumin fragments when the fluorescence polarisation value is equal to the initial polarisation value + 80 % of the «polarisation value difference».

The final assay reagent is now ready, and optionally add a suitable bacterostatic agent like 0.01 % sodium azide for prolonged storage. Keep aliquots of this mixture, e.g. 2 ml, in separate containers to be combined with unknown urine samples or aliquots of urine samples.

At the time for the determination of concentration of albumin in a urine samples, take an aliquot, e.g. a volume of 20 ul of the urine sample e.g. by the use of a pre-measured capillary, and combine thereafter the content of this pre-measured capillary with the assay reagent in the said separate container, the said separate container being kept in a place where the temperature is regulated to 32 degrees of Celcius. Typically, use a container as described in example 3. Thereafter, shake the container, and the urine flows out of the capillary. Albumin of the test sample aliquot starts to displace the human serum albumin conjugate with Ru(bpy)dcbpy of the immuno-complexes of the assay reagent.

Immediately after the shaking, place the container in the fluorescence polarisation measurement instrument, having a temperature of 32 degrees of Celcius. For measurement of polarisation of the emitted fluorescence, see above in this xample. Calculate the concentration of human albumin of the unknown sample by interpolation of the polarisation value of the emitted light measured on a standard curve obtained by measurement of standards of known human albumin concentration. Such a standard curve can often be stored on the computer of the measurement instrument, enabling a direct reading of the concentration of albumin.

If a measurement instrument with a flow cell is used, then bring the mixture to pass through the flow cell for measurement, according to the instructions given by the instrument manufacturer.

If the measurement instrument allows it, much better precision is obtained if - instead of reading the polarisation after the polarisation value has become stable - the polarisation value as a function of time is measured in a kinetic manner. If available, connect the measurement instrument to a computer recording the signal continuously or at different time points, optionally defined by a software. Compare then the recorded values to values obtained by measurement of standards of known human albumin concentration as a function of time. Optionally, record a three-dimensional standard curve with polarisation values, different times and human albumin concentration values, and compare the values obtained with the unknown sample to calculate the human albumin concentration, optionally by the use of the least-square methods for best fit according to standard textbooks of statistics, optionally by means of a computer or another artificial memory connected to the measurement instrument. Furthermore, if you have access to a temperature recording instrument keep the said separate containers (to be combined with unknown blood samples or aliquots of blood samples) at the room temperature, and use the temperature measurement device in or at the fluorescence polarisation instrument, and use a four-dimensional standard curve, the forth dimension being temperature, in addition to polarisation values, different times and human albumin concentration values, and compare the values obtained with the unknown sample to calculate the human albumin concentration,

optionally by the use of the least-square methods for best fit according to standard textbooks of statistics, optionally by means of a computer or another artificial memory connected to the measurement instrument.

The concentration of albumin in urine samples varies significantly. Reference values are in the range of up to 20 mg per liter. Slightly elevated values of uncertain origin are found in some healthy individuals, individuals who may stay healthy to old ages. Moderate elevation of the albumin values are seen as early signs of diabetic kidney damage. In severe kidney disease, urine albumin concentration may rise to many hundred mgs per liter. In the performance of this example of the method of the present invention, please therefore adjust the concentration of the human serum albumin conjugate with Ru(bpy)dcbpy, and hence the FAB fragment concentration. At high concentration ranges, FAB fragments of high dissociation rate constant must be chosen. At low concentration ranges, use FAB fragments with both high association rate constant and high dissociation rate constant.

Example 16. Method for measurement of blood theophyllin by the use of an aptamer complex.

Synthesise the RNA aptamer which binds to theophyllin with an association afinity constant of 1.0 * 10E7 /molar, described by R.D. Jenison & al in Science, vol 263, 1994.

Synthesise Cyanin-5 analouge of theophyllin according to example 7.

Make an assay buffer consisting of 140 mM NaCl, 5 mM KCl, 1 mM manganes chloride. 1 mM calcium chloride and 20 mM Hepes , and 3 mg bovine gamma globulin per ml, and pH = 7.4. Add 2 mg Cyanin-5 analouge of theophyllin per l assay solution, and thereafter add an equimolare amount of the synthesised RNA molecules.

The final assay reagent is now ready, and optionally add a suitable bacterostatic agentlike 0.01 % sodium azide for prolonged storage. Keep aliquots of this mixture, e.g. 1 ml, in separate containers to be combined with unknown blood samples or aliquots of blood samples.

At the time for the determination of concentration of theophyllin in blood samples, take an aliquot, e.g. a volume of 20 ul of the blood sample sample e.g. by the use of a pre-measured capillary, and combine thereafter the content of this pre-measured capillary with the assay reagent in the said separate container, the said separate container being kept in a place where the temperature is regulated to 32 degrees of Celcius. Typically, use a container as described in example 3. Thereafter, shake the container, and the blood flows out of the capillary. Theophyllin of the test sample

aliquot starts to displace the Cyanin-5 analouge of theophyllin of the RNA/ Cyanin-5 analouge of theophyllin complex of the assay reagent.

Immediately after the shaking, place the container in the fluorescence polarisation measurement instrument, having a temperature of 32 degrees of Celcius. For measurement of polarisation of the emitted fluorescence, the mixture in the container is irradiated with polarized light of 649 nm wavelength, and when the polarisation of the emitted light constant, measure its polarisation a wavelength of 670 nm, with a rather narrow bandwidth, typically 10 nm, if the instruments allows for that. Calculate the concentration of Theophyllin of the unknown sample by interpolation of the polarisation value of the emitted light measured on a standard curve obtained by measurement of standards of known human albumin concentration. Such a standard curve can often be stored on the computer of the measurement instrument, enabling a direct reading of the concentration of Theophyllin.

If a measurement instrument with a flow cell is used, then bring the mixture to pass through the flow cell for measurement, according to the instructions given by the instrument manufacturer.

If the measurement instrument allows it, a much better precision is obtained if - instead of reading the polarisation after the polarisation value has become stable - the polarisation value as a function of time is measured in a kinetic manner. If available, connect the measurement instrument to a computer recording the signal continuously or at different time points, optionally defined by a software. Compare then the recorded values to values obtained by measurement of standards of known theophyllin concentration as a function of time. Optionally, record a three-dimensional standard curve with polarisation values, different times and theophyllin concentration values, and compare the values obtained with the unknown sample to calculate the theophyllin concentration, optionally by the use of the least-square methods for best fit according to standard textbooks of statistics, optionally by means of a computer or another artificial memory connected to the measurement instrument. Furthermore, if you have access to a temperature recording instrument keep the said separate containers (to be combined with unknown blood samples or aliquots of blood samples) at the room temperature, and use the temperature measurement device in or at the fluorescence polarisation instrument, and use a four-dimensional standard curve, the forth dimension being temperature, in addition to polarisation values, different times and theophyllin concentration values, and compare the values obtained with the unknown sample to calculate the theophyllin concentration, optionally by the use of the least-square methods for best fit according to standard textbooks of statistics, optionally by means of a computer or another artificial memory connected to the measurement instrument.

The concentration of theophyllin in blood samples varies significantly. The main interest is measurements of therapeutic concentration values. However, higher and toxic values are of interest in forensic medicine, and lower concentrations are of interests in sports medicine. In the performance of this example of the method of the present invention, please therefore adjust the concentration of the Cynanin-5 analogue of theophyllin, and hence the RNA aptamer concentration.

Example 17: Simultaneous measurement of human choriongonadotropin and albumin in urine.

Purchase human beta-subunit choriongonadotropin (BHCG) from Sigma, and further purify it by size exclusion chromatography with mehods well known to the skilled man of the art, using 10 mM phosphate buffer pH = 7.2 witm 0.15 M sodium chlorid as eluant. Label the human beta-subunit of choriongonadotropin with Cy-5 Fluorolink following the package insert of Amerham Pharmacia Biotech, and measure the content of Cy-5 per molecule BHCG according to the same method after purification by size exclution chromatography, as recommended by Amersham Pharmacia Biotech.

Purchase monoclonal anti-BHCG from Chemicon Inc, US, and prepare FAB fragments from the antibodies according to the ImmunoPure FAB preparation kit from Pierce Cemical Company.

Make anti- Asp-Ala-His-Lys-Ser-Glu-Val-Ala antibodies using conventional techniques well known to the skilled man of the art, e.g. by the use of an antigen formed by coupling of the peptide to keyholde limpet cyanin. Alternatively, purchase the peptide synthesis and the raising of antibodies from a service providing company, e.g. by Eurogenentech of Belgium. Make FAB fragments from the antibodies antibodies by the use the ImmnuPure Fab preparation kit supplied by Pirece Chemical Company, and follow the instruction for the said kit.

Make fluorescein labelled peptide as described in example 10, and the reagent for determination of albumin in urine according to example 11. Thereafter, add to the said reagent for determination of urine albumin, Cy5-labelled BHCG 1/100 of molecular concentration of the BHCG level intended to measure in urine. (A suitable level to could be levels corresponding to 3000 U per l).

On selection of monoclonal antibody f, see below.

Measure the polarisation of the emitted fluorescence when the mixture in the container is irradiated with polarized light of 550 nm wavelength., and when the polarisation of the emitted light constant, measure its polarisation a wavelength of 570 nm, with a rather narrow bandwidth, typical of 10 nm, if the instruments allows for that. Under monitoration by such fluorescence polarisation measurements, add

said anti-BHCG FAB fragments. For each addition, wait until the fluorescence polarisation signal is stable, before adding more FAB fragments. Continue the addition of FAB fragments until no more significant increase of polarisation value is measured with increased concentration of FAB fragments. Record this value of fluorescence polarisation. Subtract the initial fluorescence polarisation value, and calculate the difference between the values, hereinafter called «polarisation value difference».

Start over again and make a final assay reagent identical to the tentative assay reagent, however, end the addition of said FAB anti-BHCG fragments when the fluorescence polarisation value is equal to the initial polarisation value + 80 % of the «polarisation value difference».

The final assay reagent is now ready, and optionally add a suitable bacterostatic agent like 0.01 % sodium azide for prolonged storage. Keep aliquots of this mixture, e.g. 2 ml, in separate containers to be combined with unknown urine samples or aliquots of urine samples.

At the time for the determination of concentration of albumin and HCG in a urine samples, take an aliquot, e.g. a volume of 20 ul of the urine sample e.g. by the use of a pre-measured capillary, and combine thereafter the content of this pre-measured capillary with the assay reagent in the said separate container, the said separate container being kept in a place where the temperature is regulated to 32 degrees of Celcius. Typically, use a container as described in example 3. Thereafter, shake the container, and the urine flows out of the capillary. Albumin of the test sample aliquot starts to displace the fluorescein labelled insulin-like peptide of the fluorescein-labelled immuno-complexes of the assay reagent. HCG starts to displace Cy-5-labelled BHCG in the Cy-5 –labelled immunocomplexes of the assay reagent.

Immediately after the shaking, place the container in the fluorescence polarisation measurement instrument, having a temperature of 32 degrees of Celcius. For measurement of polarisation of the emitted fluorescence, the mixture in the container is irradiated with polarized light of 475 nm and 650 nm wavelengths, and when the polarisation of the emitted light constant, measure its polarisation of emitted light at the wavelengths of 525 nm and 670 nm, with a rather narrow bandwidth, typically 10 nm, if the instruments allows for that. A rather advances dual wavelength irradiation instrument with high precision of polarisation measurements is necessary, because of the overlap of the spectra and the relatively high molecular weight of Cy-5-labelled BHCG. It is preferred to use an instrument which intermittently irradiates at 475 nm and 650 nm, to avoid much spectral interference. Calculate the concentration of human albumin and HCG of the unknown sample by interpolation of the polarisation values of the emitted light at both 525 and 670 nm, measured on a «standard curve»

obtained by measurement of standards of known human albumin and HCG concentrations. Such a standard curve will be four dimensional since the polarization at both wavelengths will vary with concentrations of both albumin and of HCG. It should be stored stored on the computer of the measurement instrument, enabling a direct calculation of the concentration of albumin and HCG.

If a measurement instrument with a flow cell is used, then bring the mixture to pass through the flow cell for measurement.

If the measurement instrument allows it, much better precision is obtained if - instead of reading the polarisation after the polarisation value has become stable - the polarisation value as a function of time is measured in a kinetic manner. If available, connect the measurement instrument to a computer recording the signal continuously or at different time points, optionally defined by a software. Compare then the recorded values to values obtained by measurement of standards of known human albumin and HCG concentration as a function of time. Optionally, record a data set with polarisation values at the two wave-lengths, different times and different human albumin and HCG concentration values, and compare the values obtained with the unknown sample to calculate the human albumin concentration, optionally by the use of the least-square methods for best fit according to standard textbooks of statistics, optionally by means of a computer or another artificial memory connected to the measurement instrument. Furthermore, if you have access to a temperature recording instrument keep the said separate containers (to be combined with unknown blood samples or aliquots of blood samples) at the room temperature, and use the temperature measurement device in or at the fluorescence polarisation instrument, and use an even larger data set with different temperatures included. optionally by the use of the least-square methods for best fit according to standard textbooks of statistics, optionally by means of a computer or another artificial memory connected to the measurement instrument.

The concentration of albumin in urine samples varies significantly. Reference values are in the range of up to 20 mg per liter. Slightly elevated values of uncertain origin are found in some healthy individuals, individuals who may stay healthy to old ages. Moderate elevation of the albumin values are seen as early signs of diabetic kidney damage. In severe kidney disease, urine albumin concentration may rise to many hundred mgs per liter. In the performance of this example of the method of the present invention, please therefore adjust the concentration of the fluorescein labelled peptide , and hence the FAB fragment concentration. At high concentration ranges, FAB fragments of high dissociation rate constant must be chosen. At low concentration ranges, use FAB fragments with both high association rate constant and high dissociation rate constant.

Correspondingly, concentration of HCG differ with certain diseases, stage of pregnancy, and obviously with sex. Similar consideration as with the anti-peptide antibody fragment must be used with the anti- BHCG antibodies from which to prepare FAB fragments.

There is an interest to determine both urine HCG and urine albumin in pregnant women.

Example 18: Simultaneous measurement of gonadotropin, albumin and immunoglobulin G in human urine.

One of the main functions of the kidneys is to excrete urea and other small molecular subtances but retain albumin and other proteins in blood (in addition to many other functions). In pregnant women with proteinuria, there is an interest in evaluating the kidneys selectivity between smaller and larger proteins. Simultaneous measurement of albumin and immunoglobulin G is used to assess such selectivity. Normally, the urine contains only trace amounts of albumin and immunoglobulins, but at least 10 timer more albumin than immunoglobulin. With severe impairment of the renal function, such selecively is usually lost.

To obtain a suitable reagent for Simultaneous measurement of gonodotropin, albumin and immunoglobulin G in human urine, use the following protocol:

Use the COSMIX plexing phage display technology referred to above to identify a peptide that binds selectively to Immunoglobulin class G molecules. This is a service that can be bought from the COSMIX GmbH company. Alternatively, digest Protein A into peptide fragments, and identify and purify a IgG binding peptide from the digest, using conventional techniques well known to the art, e.g. as described in Yue et al in The Journal of Biological Chemistry vol 271, p. 22245-22250, 1996. Label the identified and purified peptide with 5-caboxytetramethylrhodamine succidinimidyl ester purchased from Molecular Probes, Inc, using conventional techniques as described in the package insert from aid company. Purify the tetramethylrhodamine – labelled peptide by reversed phase chromatography using conventional techniques described in other examples above.

To the reagent described in example 17, add said tetramethylrhodamine –labelled peptide. If the reagent is intended to measure albumin concentrations e.g. in the concentration range of 50 to 500 mg per liter, add tetramethylrhodamine –labelled peptide to a final concentration of 0.05 micromoles per liter. In this way an impairment of selectivity will be shown as increased binding of IgG to tetramethylrhodamine-peptide.

The final assay reagent is now ready, and optionally add a suitable bacterostatic agent like 0.01 % sodium azide for prolonged storage. Keep aliquots of this mixture, e.g. 2 ml, in separate containers to be combined with unknown urine samples or aliquots of urine samples.

At the time for the determination of concentration of albumin, IgG and HCG in a urine samples, take an aliquot, e.g. a volume of 20 ul of the urine sample e.g. by the use of a pre-measured capillary, and combine thereafter the content of this pre-measured capillary with the assay reagent in the said separate container, the said separate container being kept in a place where the temperature is regulated to 32 degrees of Celcius. Typically, use a container as described in example 3. Thereafter, shake the container, and the urine flows out of the capillary. Albumin of the test sample aliquot starts to displace the fluorescein labelled insulin-like peptide of the fluorescein-labelled immuno-complexes of the assay reagent. HCG starts to displace Cy-5-labelled BHCG in the Cy-5 –labelled immunocomplexes of the assay reagent. IgG starts to bind to the rhodamine-labelled peptide.

Immediately after the shaking, place the container in the fluorescence polarisation measurement instrument, having a temperature of 32 degrees of Celcius. For measurement of polarisation of the emitted fluorescence, the mixture in the container is irradiated with polarized light of 475 nm, 550 nm and 650 nm wavelengths, using a fluorescence polarization measurement instrument constructed to irradiate the three different wavelengths intermittantly. When the polarisation of the emitted light for each of the different irradiation wavelengths is constant, measure the polarisation of emitted light at the wavelengths of 525 nm, 582 and 670 nm, with a rather narrow bandwidth, typically 10 nm, if the instruments allows for that. A rather advanced irradiation instrument with high precision of polarisation measurements is necessary, because of the overlap of the spectra and the relatively high molecular weight of Cy-5-labelled BHCG. Calculate the concentration of human albumin, IgG and HCG of the unknown sample by interpolation of the polarisation values of the emitted light at both 525, 582 and 670 nm, measured on a «standard curve» obtained by measurement of standards of known human albumin, IgG and HCG concentrations. Such a standard curve will be multi dimensional since the polarization at all three , wavelengths will vary with concentrations of both albumin, IgG and of HCG. It should be stored on the computer of the measurement instrument, enabling a direct calculation of the concentration of albumin and HCG.

If a measurement instrument with a flow cell is used, then bring the mixture to pass through the flow cell for measurement.

If the measurement instrument allows it, much better precision is obtained if - instead of reading the polarisation after the polarisation values have become stable - the

polarisation values as a function of time is measured in a kinetic manner. If available, connect the measurement instrument to a computer recording the signal continuously or at different time points, optionally defined by a software. Compare then the recorded values to values obtained by measurement of standards of known human albumin, IgG and HCG concentration as a function of time. Optionally, record a data set with polarisation values at the three wave-lengths, different times and different human albumin, IgG and HCG concentration values, and compare the values obtained with the unknown sample to calculate the human albumin concentration, optionally by the use of the least-square methods for best fit according to standard textbooks of statistics, optionally by means of a computer or another artificial memory connected to the measurement instrument. Furthermore, if you have access to a temperature recording instrument keep the said separate containers (to be combined with unknown blood samples or aliquots of blood samples) at the room temperature, and use the temperature measurement device in or at the fluorescence polarisation instrument, and use an even larger data set with different temperatures included, optionally by the use of the least-square methods for best fit according to standard textbooks of statistics, optionally by means of a computer or another artificial memory connected to the measurement instrument. In a multi-analyte assay as described, a rather high computing capacity is necessary, but such computing capacity is today easily available, and use soft-ware programs for analysis and calculations of several unknown parameters is preferred, e.g. the use of the Unscrambler prgram, delivered by the CAMO company, Oslo, Norway.

CLAIMS

1. A method for determination of concentration of one or more analytes in a test sample or an aliquot of a test sample of a complex biological a fluid, characterised by
a) mixing the said sample or aliquot of the said sample with one single reagent, such as a solid, a solution or premixed solution, wherein said reagent being provided in one single container or compartment of a container, and no other reagent is added during the performance of said method, and said reagent further comprises at least one type of binding molecule with specific affinity for one or more of the said analytes, and said reagent furthermore comprises either fluorescent moieties covalently linked to the said binding molecules or fluorescent analogues of or fluorescent fragments of or fluorescent derivatives of said analyte or analytes, and
b) said mixing resulting in a mixture which is being irradiated with polarized light which permits the exitation of said fluorescent molecules, and
c) measuring the polarisation of the emitted light, and
d) calculating the concentration or concentrations of said analyte or analytes.

2. A method according to claim 1,
characterised by using a reagent for each analyte comprising immmunocomplexes between
a) an antibody or an immunoactive fragment of an antibody with specific affinity for said analyte or analytes, and
b) fluorescent analogues or fluorescent fragments of or fluorescent derivatives of said analyte or analytes.

3. A method according to claim 1,
characterised by using a reagent for each analyte comprising complexes between
a) an aptamer or another synthetic binder with a specific affinity for said analyte, and
b) fluorescent analogues or fluorescent fragments of or fluorescent derivatives of said analyte or analytes.

4. A method according to claim 1,
characterised by using a reagent comprising binding molecules with specific affinity for one or more of the said analytes and with fluorescent moieties with absorption maximum between 600 nm and 1000 nm, preferably above 620 nm, covalently linked to the said binding molecules, and said binding molecules being either a peptide or being synthetic binders, optionally being identified by combinatory chemistry techniques or phage display or nucleic acid selection technology.

5. A method according to any of the claims 1 to 4,
characterised by using a reagent comprising fluorescent binding molecules with

specific affinity for one analyte, or comprising fluorescent analogues of, or fluorescent fragments of, or fluorescent derivatives of one analyte only.

6. A method according to any of the claims 1 to 5, characterised by the use of a reagent comprising different fluorescent moieties covalently bound to different binding molecules with different specific affinities.

7. A method according to any of the claims 1 to 6, characterised by the use of a reagent comprising one or more peptides or derivatives of peptides with specific binding affinity for an analyte, said binding peptides having a fluorescent residue covalently linked and being constituted by less than 30 amino acids.

8. A method according to claim 7, characterised in that binding peptide is constituted by less than 20 amino acids.

9. A method according to claim 8, characterised in that binding peptide is constituted by less than 15 amino acids.

10. A method according to any of the claims 1 to 9, characterised by the use of a reagent comprising peptids or derivatives of peptids containing amino acid sequence Ala-Arg-Asn-Arg-Asn or Ala-Arg-Asn-Gly-Asn for quantitation of C-reactive protein.

11. A method according to any of the claims 1 to 10, characterised by the use of a reagent with fluorescent residues with maximum coefficient of absorption at a wavelength above 640 nm.

12. A method according to any of the claims 1 to 11, characterised by the use of a reagent comprising cell lysing substances or anti-coagulants or detergents.

13. A method according to any of the claims 1 to 12, characterised by the use of a reagent comprising one or more fluorescent moieties selected from the group consisting of fluoresceine, Texas Red, Cy5, other Cy Dye FluorLink substances, other Cyanin derivatives, Rhodamin, Methyl Rhodamin, Biodypi 630/650-X/MeOH, Biodypi 650/655-X/MeOH, Biodypi FL/MeOH, Biodypi R6G/MeOH, Biodypi TMR-X/MeOH Biodypi TR-X/MeOH or other substances from the Biodipy group of substances, Alexa Fluor Dyes of different wavelengths, Ruthenium ligand complexes, lanthanoid elements such as Europium, Samarium or Terbium complex bound to a chelating ligand like DTPA, EDTA or NI.

14. A method according to any of the claims 1 to 13, characterised by that the polarisation of the emitted light is measured as a function of

time, either as a continuous kinetic reading or a reading of the change in polarisation of the emitted light between two or more time points, or as a measurement of the polarisation of the emitted light after a defined point of time.

15. A method according to any of the claims 1 to 14, characterised by that sample material or aliquot of the sample material is constituted by a biological material, or a dilution or an extract or being dissolved from or being filtrated from the said biological material.

16. A method according to any of the claims 1 to 15, characterised by that sample material or aliquot of the sample material is constituted by blood, or blood serum, or blood plasma, or blood cells, or lysate from blood or blood cells, or urine, or cerebrospinal fluid, or tear liquid, or sputum, or semen, or plasma, or semen or material aspirated from the gastro-intestinal tract or feces, or extract or filtrate of suspension of feces, or plant material or extracts thereof, or dissolved plant material or filtrate thereof.

17. A method according to any of the claims 1 to 16, characterised by the use of standards or calibrators comprising known concentrations of the analyte or the analytes, and furthermore wherein the concentration or concentrations of said analyte or analytes in unknown samples is calculated by interpolation of the values obtained from the unknown samples on the standard curve obtained from said known standards or calibrators.

18. A method according to any of the claims 1 to 17, characterised by the use of a standard curve stored in an artificial memory, optionally connected to the fluorescent polarisation instrument in use.

19. A method according to any of the claims 1 to 18, characterised by the use of temperature correction algorithms, either generated empirically or theoretically, to compensate for differences in fluorescence polarisation caused by differences in temperature at different time of measurements of standards and unknown samples, or between standards, or between unknown samples.

20. A method according to any of the claims 1 to 19, characterised by being provided in concentrated or dry form, to be diluted or reconstituted before use, the said reagent being provided divided between different compartments for combination into one reagent prior to use.

21. A reagent for the performance of the method according to any of the claims 1 to 20, characterised in that said reagent comprises at least one type of binding molecule with specific affinity for one or more of the said analytes, and said reagent

furthermore comprises fluorescent moities covalently linked to the said binding molecules or fluorescent analogues of or fluorescent fragments of or fluorescent derivatives of said analyte or analytes.

22. A reagent according to claim 21,
characterised in that the reagent comprises complexes between
a) an antibody or an immunoactive fragment of an antibody or an aptamer or a synthetic binder with specific affinity for at least one analyte and
b) fluorescent analogues or fluorescent fragments of or fluorescent derivatives of said analyte or analytes.

23. A reagent according to claims 21 to 22,
characterised in comprising binding molecules with specific affinity for one or more of the said analytes and optionally with fluorescent moities with absorption maximum between 600 nm and 1000 nm, preferably exceeding 620 nm, more preferably exceeding 640 nm, covalently linked to the said binding molecules, and said binding molecules being either of peptide or aptamer composition or being synthetic binders, optionally being identified by combinatory chemistry techniques or phage display or nucleic acid selection technology.

24. A reagent according to claims 21 to 23,
characterised in being an assay reagent comprising peptid binders or binders of derivatives of peptids, including fluorescent derivatives of said binders, containing the amino acid sequence Ala-Arg-Asn-Arg-Asn and/or Ala-Arg-Asn-Gly-Asn.

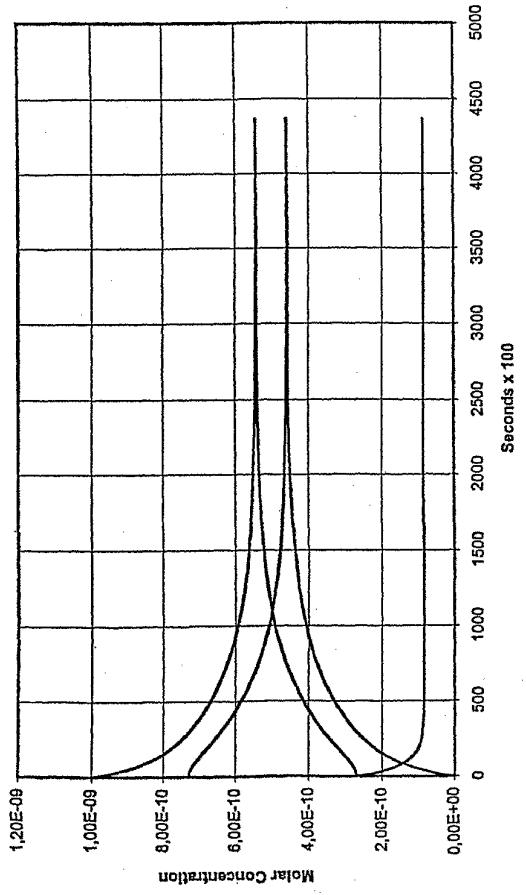
25. Use of the method according to claims 1 to 20 to determine concentrations of clinically related substances in samples of biological material from living organisms in need thereof.

26. Kit for the determination of concentration of one or more analytes in a test sample or an aliquot of a test sample of complex biological fluid,
characterized in comprising one or more containers, wherein the container(s) or compartment of the container(s) contains one single reagent, preferably in the fluidal state and according to any of the claims 21-24, and wherein the reagent comprises one or more fluorescense-labelled spesific binding molecules towards the analyte(s) to be measured, or a fluorescence-labelled analogue or a fluorescent fragment or a eluorescent derivative of said analyte(s), as well as device for obtaining the exact volume(s) of the complex biological fluid to be tested and that is needed in order to perform the method adequately.

27. Kit according to claim 26,
characterized in that the reagent which is contained in a container or a compartment of a container, is formed to a ready-for-use reagent by mixing the content from

different containers prior to or immediately prior to or in connection with the execution of the analysis.

Figure 1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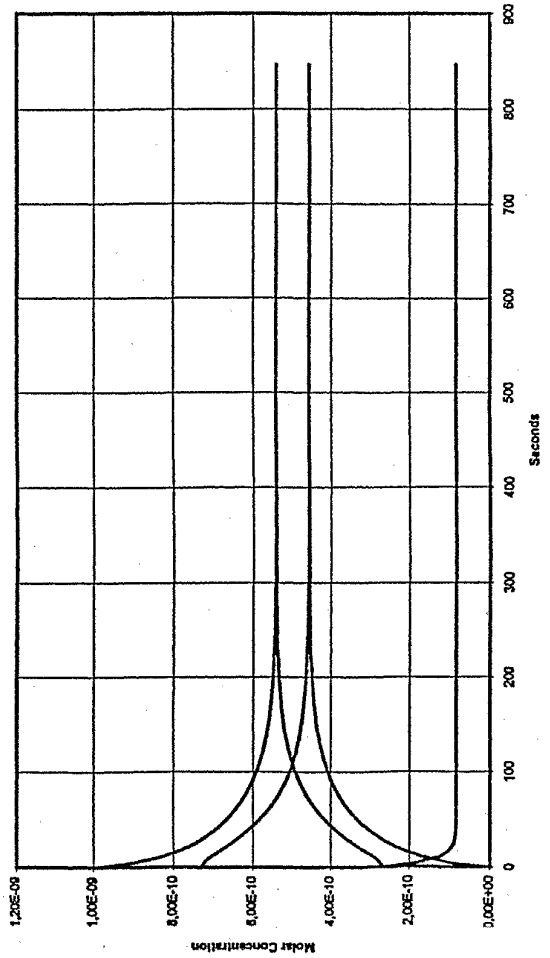
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Figure 2

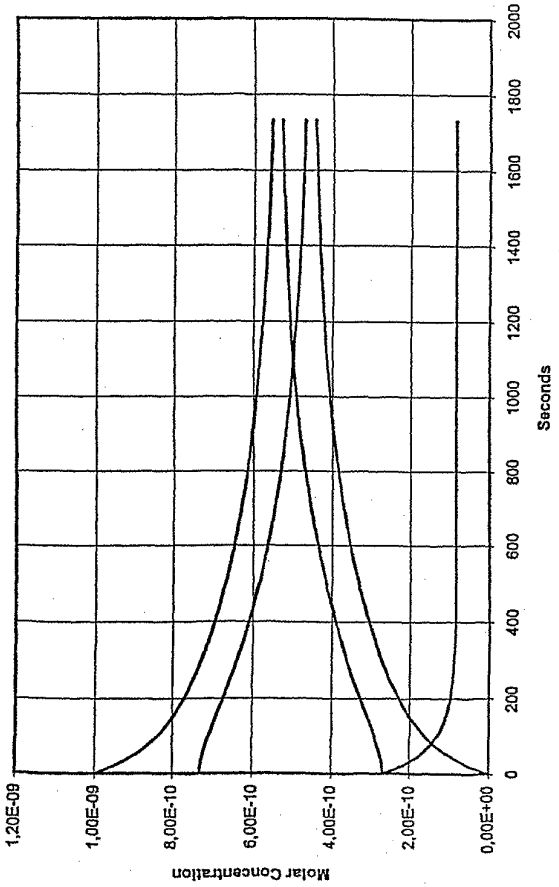
Molar Concentration
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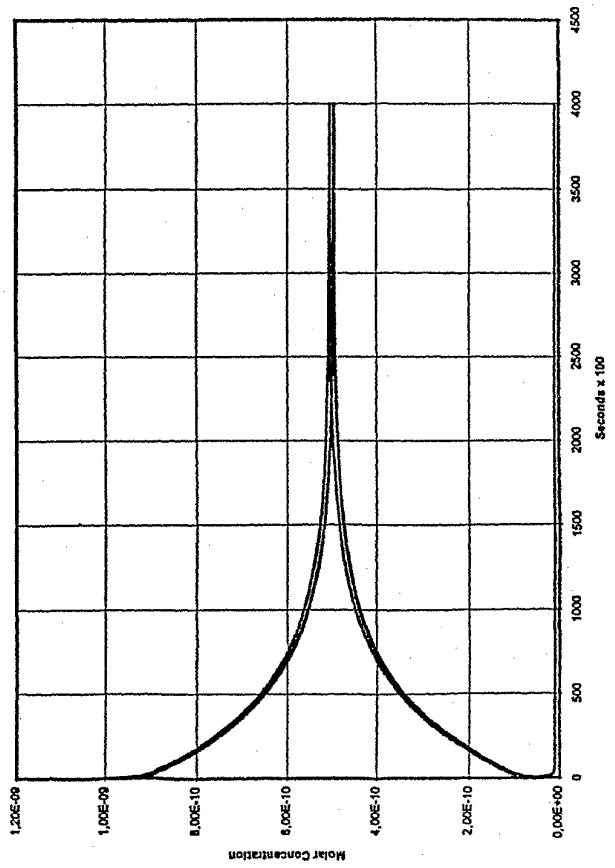
Figure 3

Figure 4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Molar Concentration
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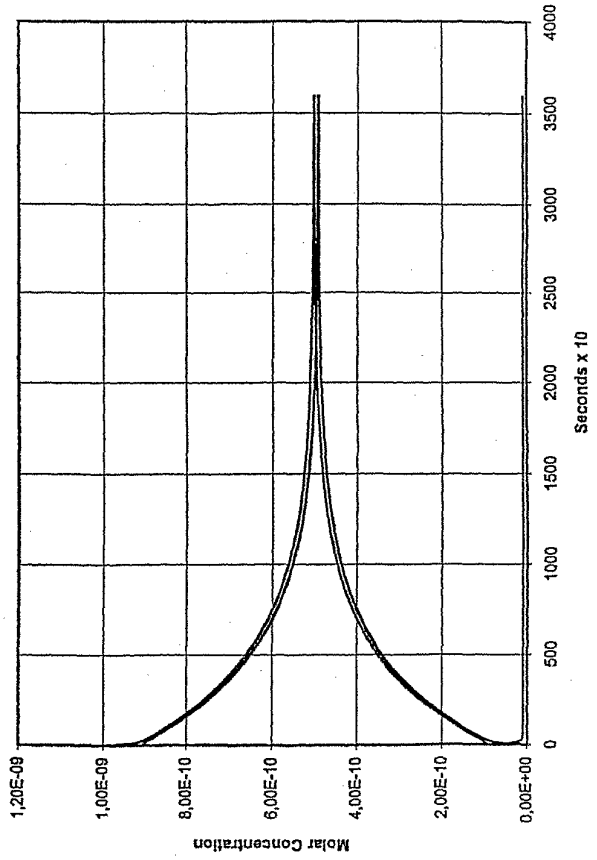
Figure 5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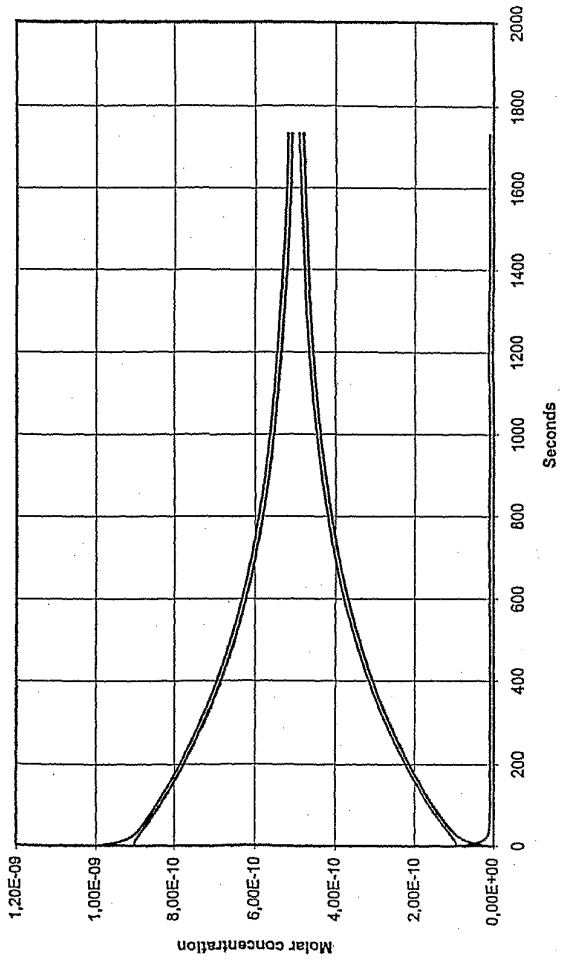
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Figure 6

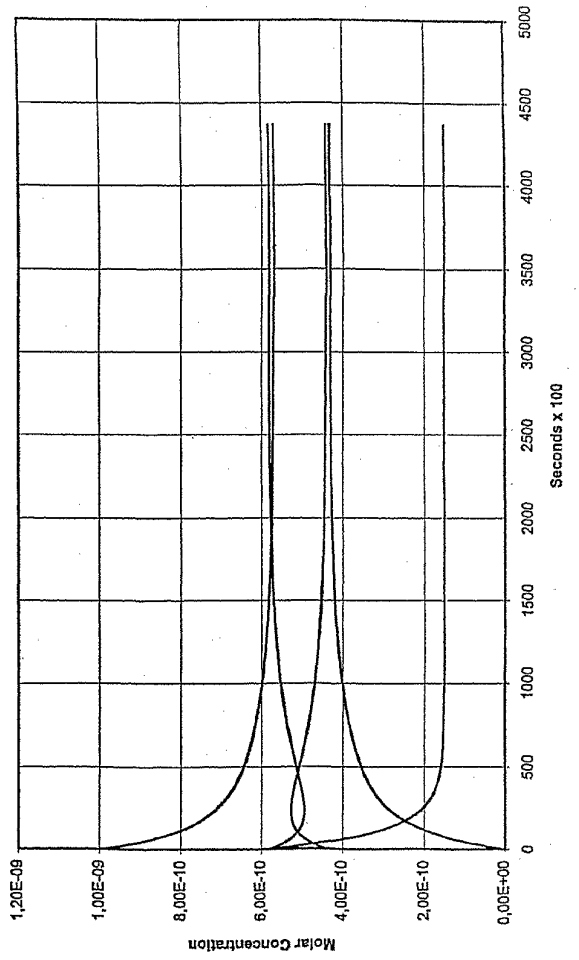
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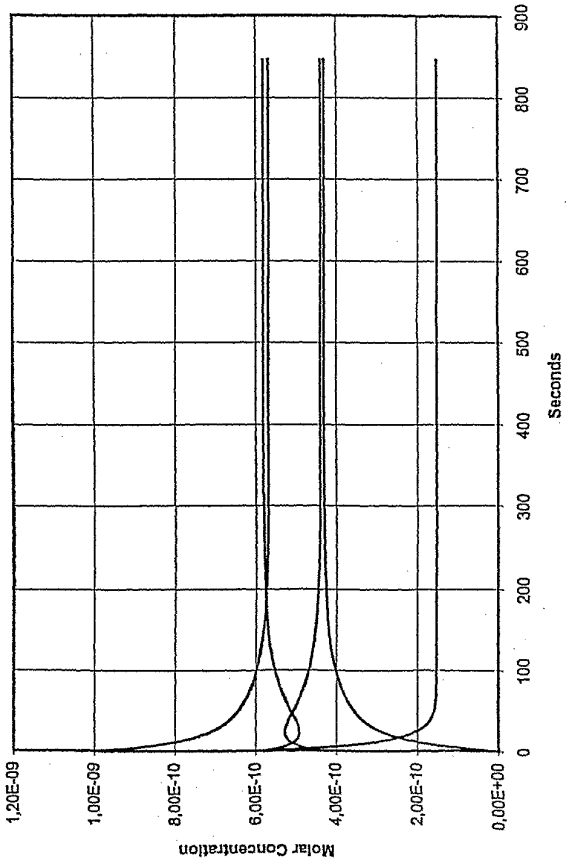
Figure 7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Molar Concentration
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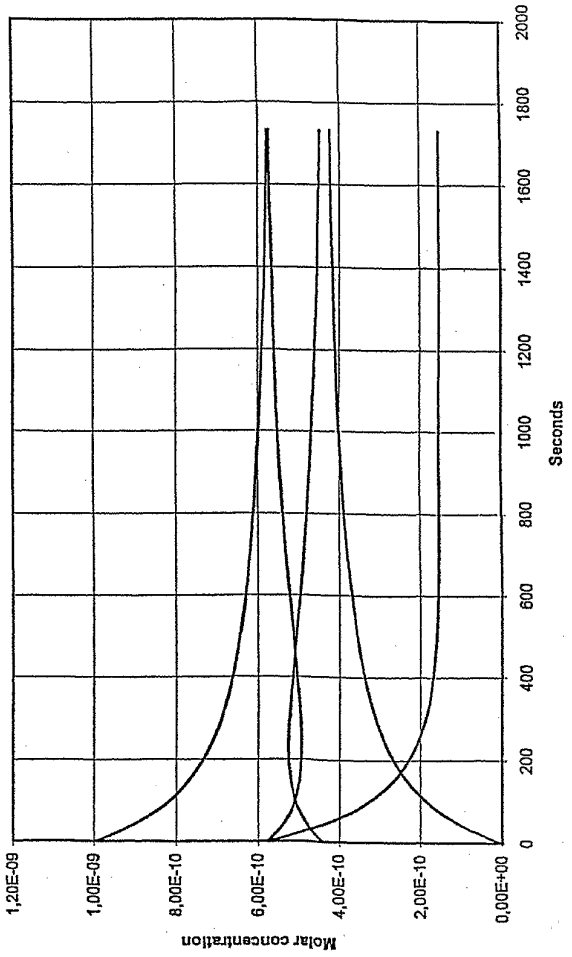
Figure 8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Molar concentration
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8,00E-10
6,00E-10
4,00E-10
2,00E-10
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Figure 9

【国際公開パンフレット（コレクトバージョン）】

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WO 02/044721 A1

(54) Title: ANALYTIC METHOD AND REAGENT FOR USE THEREOF

(57) Abstract: The present invention relates to a method for determination of one or more analytes in a test sample or an aliquot of a test sample, as well as a reagent for use in the metod. The reagent according to the present invention comprises at least one type of specific binding molecule for each analyte that is to be quantitated, as well as fluorescent substances whose signals change as a result of admixing a sample to the reagent. Furthermore, the signal change may be used to calculate the concentration or concentrations of analytes, without separating different states of aggregation.

Analytic method and reagent for use therof.

The present invention relates to a method for determination of one or more analytes in a test sample or an aliquot of a test sample and a reagent for use in the metod.

Background for the Invention

«Analyte» or «analytes» is the generic name of those substances for which qualitative or quantitative analysis is desired in a test sample material. An analyte is usually a well-defined molecule, but may be a collection of molecules resembling each other or carrying out the same function. Particulate materials, e.g. clusters of fatty proteins or classes of blood cells can also be referred to as analytes.

Quantitative chemical analysis of an analyte in a test material is also often referred to as determination of concentration. Concentration determination may also be carried out using a qualitative chemical analysis, whereby concentration determination provides information on whether the concentration of the analyte in the test material is higher than or lower than a given value.

The development of methods for chemical concentration determination of analytes in very low concentration in complex materials increased very rapidly after R.S. Yalow, S. A. Berson and associates developed what was later to be known as the immunochemical measurement methods. They made use of the principle that individuals of vertebrates create antibodies, most often in the form of immunoglobulins, as a specific immunological response to exposure to materials that the given animal's immune system perceives as alien to the animal. The classical and decisive work to reach this perception was published by S.A. Berson, R.S. Yalow, A. Bauman, M.A. Rothschild, and K. Newerly in the article "Insulin-I131 Metabolism in Human Subjects: Demonstration of Insulin Binding Globulin in the Circulation of Insulin-Treated Subjects" in J. Clin. Invest. 35 (1956): 170-190. By using these globulins, later referred to as antibodies, either in a purified form or in a mixture with other substances, e.g. in the form of blood serum, methods for specific identification or quantitation of a large number of analytes were developed. For many of these analytes there had previously not existed practically applicable measurement methods. Thus, specific measurement methods for a range of blood proteins and proteins from other body fluids and tissue were developed, and later on for non-proteinaceous substances as well. Hormone analyses were developed at an early stage. The general method was named «immunoassay», based on the reagent making the basis for the specificity of the analysis being the result of an immune response. This is well described in a large number of textbooks on the subject, e.g. in «Principles and Practice of Immunoassay», 2nd Edition, edited by Christopher P. Price and David J. Newman, ISBN: 1561591750, Groves Dictionaries, Inc., August 1998.

The test material undergoing quantitative analysis using these reagents can be described as complex biological materials. Typical test materials are blood, blood serum, plasma, urine, feces, feces extracts and cerebrospinal fluid. The analytes usually constitute a very small part of the test material.

Test material that is to undergo chemical analysis may be in different states of aggregation, e.g. gaseous state, fluid state, solid state or mixtures of different states of aggregation. The reagents that are mixed with the test material to perform the quantitative analysis or analyses, may correspondingly be present in different states of aggregation, but typically consist of solutions or solid substances and/or combinations of solid substances and fluids. When the reagents are mixed with the test material, most often (but not always) a solution of substances in fluid will be formed. This solution is often referred to using the generic name «the assay solution», and comprises both test material and those chemicals necessary for the quantitative analysis to take place.

In the early days after the invention of the immunoassays, the source used for admixture of antibodies was antiserum, which is blood serum from the immunized animal, which also included other serum proteins, and where the antibodies constitute a smaller portion of the aggregate serum proteins. To this day a substantial amount of antibodies in the form of antiserum are sold, see e.g. catalogues from suppliers such as Chemicon Inc., California. However, early on the routine use of antibodies in the form of purified immunoglobulin classes was introduced, most commonly immunoglobulin type G, which is the most commonly used antibody reagent today, see e.g. the product catalogue from the company Dako AS, Denmark.

The production of antibodies occurring when animals are exposed to alien substances (called antigens), often using active, targeted exposure in the form of so called vaccination or immunization, is characterized by a whole range of different cells in the body producing antibodies with different structures, but they have the common characteristic that they bind to (have affinity for) the antigen in question. These different antibodies, binding to the same antigen, have different strength and rates of binding, however, and are difficult to make with constant qualities when producing antibodies over a long time span and using many animals.

It was therefore a considerable progress when Köhler and Milstein in 1974 developed the method for creating monoclonal antibodies, which made possible standardized antibodies with a reproducible chemical structure and homogeneous constant binding qualities. These methods are well described in the book «Monoclonal Antibodies: Production, Engineering and Clinical Application», edited by Mary A. Ritter and Heather M. Ladyman, ISBN: 0521425034, Cambridge University Press, February 1995. The book also describes well how to fragment the antibodies and use those

fragments of the antibodies that have binding qualities, instead of using the entire antibodies.

In the years following the invention of the monoclonal antibodies, using modern biotechnology, new methods were developed for producing binding proteins and fragments of proteins (also called peptides) with the desired binding qualities. A substantial step forward was made with the development of the phage display method, in which virus technology was used for production, exposure and choice of peptide structures for the production of reagents with specific binding characteristics.

The phage display technique was developed further when the gene sequence for parts of the antibody producing cells was incorporated and systematically varied or permuted in the phage display particles, as described by Collins J. and Röttgen P. (1994); "Hypervariable phagemid display gene banks for the selection of strongly binding ligands, including their use for the isolation of serine protease inhibitors"; European patent application 1994 000 108 689 (April 1994) taken further as US 5925559 «Phagemids and process of preparation» issued 20 July 1999, and by Collins, J., Röttgen, (1997); «Cosmix-plexing a method for recombination....» EP 97 101 539.1 (31.01.1997), filed by Cosmix GmbH PCT/EP98/00533 (02.02.1998) and WO 98 33901 (6.08.1998).

Further on, one started to use nucleic acid sequences (aptamers) as such as specific binding molecules in an immunoassay-like way, even though terminologically it is probably not quite correct to call these immunoassays. US patent US05567588 «Systematic evolution of ligands by exponential enrichment: Solution SELEX» describes this aptamer technology. Finally, things went completely beyond the scope of the biological framework surrounding these binding assays when one started, synthetically, to produce large libraries of molecules, using systematic combination of smaller elements, so called combinatory libraries. These elements may partially or totally consist of amino acids in chains (called peptides), but could also consist of other building blocks. An overview on this subject is to be found in the article «A paradigm for drug discovery employing combinatorial libraries» by J. Burbaum et. al., in Proc. Natl. Acad. Scient. USA Vol 99, pp. 6027-6031, 1995.

So today there exists a very complex biological and synthetic collection of methods for creation of binding molecules of a higher or lower molecular weight, of different chemical nature, with different binding strength, and of homogeneous or heterogeneous structure. In this patent application, these molecules are referred to by the generic name binding molecules, and since they bind a specific structure, they are called specific binding molecules. The binding molecules can be of varying structure as described above, of a peptide nature, nucleic acid nature or of another chemical nature, and they will of course have different structures for different binding

specificities. In addition, they may have different types of signal-providing residues bound to them, e.g. different types of fluorescent residues. In the present patent application, different types of binding molecules may be used, both different types in the meaning different types of structures (peptides, nucleic acids or other structures) or different compositions within one type of structure (e.g. different amino acid sequences within a peptide structure, which would give different types of binding qualities) as well as different types of signal-providing residues, e.g. fluorescent residues with different types of fluorescent qualities, e.g. different excitation or emission wavelengths.

The above mentioned specific binding molecules, used for quantitation of substances in complex sample solutions, will as a rule have higher affinity for the substances to be quantified, or analogues or fragments or derivatives of said substances, than for other substances that may be present in the sample. Typically, a monoclonal antibody would have higher affinity for the substance used for immunization and selection when producing the antibodies, than for other substances in the test solution. When antibodies are used as a specific binding molecule, the substance for which the antibody has high affinity is called antigen, or hapten (the last term is often used if it is a smaller structure in a larger molecule). When the specific binding molecule is not an antibody, the term «ligand» is often used more than the term «antigen». In this patent application, the term «binding pair» is used as a generic name for the molecules that are bound to each other in the specific binding reaction, and the individual molecules that constitute the binding pairs will be referred to as binding partners.

Generally we can set up the following equation for the reaction between the two binding partners and the binding pairs they can form:

B1 + B2 = BB

where

B1 symbolizes binding partner 1 and B2 symbolizes binding partner 2, and BB can symbolize the binding pair they form.

Furthermore the equilibrium between the free binding partners and the binding pair they form, will be characterized by an equilibrium constant k (often called affinity equilibrium constant) in accordance with the law of mass action (Guldberg and Waage 1864, described in Steven S. Zumdahl's book »Chemical Principles», third edition, page 192-193, ISBN 0-395-83995-5):

[BB]

$$k = \frac{}{[B1]*[B2]}$$

Expressed like this, the constant is often called the affinity equilibrium constant, while the inverse form is called the dissociation equilibrium constant.

To be able to measure these binding molecules' formation of chemical binding to the analytes, one originally used a radioactive labeling of one of the binding partners. Originally, one would add a known quantity of a radioactively labeled binding partner B1 or B2, chemically identical to or near identical to the analyte, and measure this radioactively labeled substance's ability to compete with the analyte for the binding on the binding molecules that are used (originally complete immunoglobulins), see »Immunoassay of Protein Hormones," in The Hormones: Physiology, Chemistry, and Applications, vol. 4, 557-630 (G. Pincus et al, eds. Academic Press, 1964).

In this so-called competitive assay method the analyte molecules thus competed with labeled analogue molecules for binding molecules, which were in concentration deficit. Later on, non-competitive methods, with a surplus of antibodies, were developed, where most often two different antibodies are used, one antibody to isolate the analyte (e.g. bind the analyte molecules to a solid phase), and one radioactively labeled antibody to generate a signal for measuring. This method was called the immunometric method. A general overview is given in the book »Immunoassay : A Practical Guide», by Law Brian, ISBN: 0748405607.

Common to the competitive and the immunometric method, was the use of standardized test solutions with a known quantity of analyte to calibrate and correlate the measured signal with the concentration of analyte, and the generation of a so-called standard curve or calibration curve. Tests with unknown concentrations of analyte were determined by interpolation on this curve.

The radioactive labeling methods are still the ones most frequently used, but early on attempts were made to find non-radioactive methods for determining the presence of analyte analogues or binding molecules. The use of enzymes as signal generating molecules constituted a big step forward, as radioactivity was avoided, longer durability of the reagents was achieved, and simpler measuring equipment, typically light absorption spectrophotometers, could be used. There is an overview on this in »Practice and theory of enzyme immunoassays» by P.Tijssen, ISBN 0 444 806 334. Another big step forward was achieved when fluorescent molecules were taken into use as signal generating molecules. This improved the sensitivity compared to the pure absorption photometric methods. The book «Enzyme and Fluorescence

Immunoassays: Tentative Guideline», Vol. 6, National Committee For Clinical Laboratory Standards ISBN: 1562380672, July 1986, provides a good overview of this.

Chemoluminescence methods have increased the sensitivity further, as described in «Luminescence Immunoassay and Molecules Applications» by Knox and Richard van Dyke, CRC Press January 1990, ISBN 0849358655.

With the use of most types of signal-generating residues, both radioactive, fluorescent and enzymatic substances, the need arose to separate the signal-generating residues bound to molecules that had been bounded to the specific binding molecules, from those residues that were not bound to said binding molecules. Typically, this could be plastic surfaces, glass surfaces, porous filters or particle based matrices with immobilized binding molecules or more unspecific media, such as e.g. active coal, which can separate unbound small-molecular analyte-molecules from analyte-molecules bound to larger binding molecules. A general good overview of this field can be found in the book «Principles and Practice of Immunoassay», 2nd Edition, edited by Christopher P. Price and David J. Newman, Goves Dictionaries Inc., Aug. 1998, ISBN 1561591750.

It was observed early on, that antibodies in themselves could precipitate analytes, and this could be used for quantification of the analytes without any of the signal-generating attached residues being involved. Concentration determination by the help of antibody precipitation has low sensitivity, but is very practical because it is not necessary to use a solid separation phase involving a washing step, and this simplifies the execution. Precipitation analysis in gels and directly in a fluid state (Killingsworth et al, "Nephelmetric Studies of the Precipitin Reaction: A Model System for Specific Protein Measurements," Clin. Chem. 19 (4): 403-407, (1973)) has lead to a more widespread use of immunoassays and made way for a high degree of automation in spectrophotometric automatons such as Hitatchi-instruments and automated nephelometres which are for instance delivered by the Dade Behring company. In addition to the limited sensitivity, it has been difficult to use monoclonal antibodies in the precipitation analytic methods. It is presumed that monoclonal antibodies often only bind onto one area of the analyte molecule, whereas polyclonal antibodies usually will bind onto several places on the analyte molecules, and thus more easily lead to larger aggregations of antibodies and analyte molecules that easily precipitate. Thus in contrast to the present invention the above mentioned method does not comprise immunocomplexes, does not use fluorescence for quantification, is not a fluorescence polarisation assay and has low sensitivity.

The so-called BiaCore instruments and other technology based on plasmon resonance (J. Melendez, R. Carr, D. U. Bartholomew, K. Kukanskis, J. Elkind, S. Yee, C.

Furlong, R. Woodbury, A commercial solution for surface plasmon sensing, Sensors and Actuators-B 35 (1996) 1-5) have further shown that direct measurement of an analyte without signal-providing residues is possible. These methods do not, however, have the same simple technical execution as the less sensitive turbidimetric methods. Plasmon resonance instruments are in addition most often very expensive instruments.

The need to measure the analytes' binding to the binding molecules without using separation devices and solid phases, and also without using substrate or washing solutions, led to the so-called proximity assays:

The company SYVA in 1974 launched an immunoassay technology for small molecules, based on competition between the analyte molecules and enzyme labeled analyte analogues for binding to antibodies, where binding onto the antibodies gave a direct effect on the enzyme activity, which could be measured without separation or washing solutions. (US patent US3852157 «Compounds for enzyme amplification assay» by Kenneth E. Rubenstein and Edwin F. Ullman.) Fluorescence polarization immunoassays (FPIAs) were introduced as early as the end of the 1970's; see the overview article »Fluorescence polarization in immunochemistry» by Dandliker and Saussure in Immunochemistry, Vol. 7, p. 799-828, 1970. This method has also been most successful with small-molecular analytes, but the use of competing small-molecular fluorescence labeled analyte analogues opened up for the use of the method for quantification of large-molecular analytes, such as proteins. (Ai-Peng Wei and James Herron: Use of synthetic peptides as tracer antigens in fluorescence polarization immunoassays of high molecular weight analytes. Anal. Chem. 1993, 65, 3372-3377.) Some proteins also have relatively mobile subunits, making possible direct fluorescence polarization immunoassay measurements, as in the US patent 4,902,630 «Fluorescence polarization immunoassay and reagent for measurement of C-reactive protein», by Bennet and Chiapetta, (1990). Terpetschnig,E. et al. in «Fluorescence polarisation immunoassay of a high-molecular-weight antigen based on a long-lifetime Ru-ligand complex», Anal.Biochem. 227, 140-47, 1995, and in «Metal ligand complexes as a new class of long-lived fluorophores for protein hydrodynamics» Biophys.J. 68, 342, 1995, have further described how asymmetric ruthenium-complexes can be used for fluorescence polarization measurements of analytes with higher molecular weight. These can, however, not be used under the presence of hemoglobin or high bilirubin concentrations.

Most fluorescence polarization immunoassays are based on competitive methods, where fluorescence labeled analogues of the analyte have been added and have competed with the analyte molecules in the test solution for the specific binding molecule, typically in the form of an antibody. It has often been necessary to use a relatively high aggregate concentration of antibodies in such assays, even concentrations where one would expect competition not to occur. So most

commercial assays of this kind have a quite high aggregate concentration of antibodies, and apparently competition takes place for a considerably lower efficient binding molecule concentration. No systematic literature on this is available, but reference can be made to the article «Rapid, fully automated measurement of plasma homocysteine with the Abbott Imx Analyzer» by Mohammed T. Shipchandler and Edwin G. Moore, and a closer analysis might show that also other commercial competitive fluorescence polarization immunoassays are based on a relatively high aggregate antibody concentration.

Already in the mid 1980's, the company Amersham introduced scintillation proximity assays (US patent number 4,568,649, European patent number 0,154,734) in which a radioactive beta ray emitted from a binding partner triggers a fluorescence radiation from a fluorescent particle that is bound to the other binding partner in the binding pair in question. This technology has found a wide range of use in the search for remedies and other specific binders in a laboratory environment, but has never found much use in practical clinical diagnostics.

Already in 1976 Ullman and Schwarzberg, in US patent 3,996,345, published a method for «Fluorescence quenching with immunological pairs in immunoassays». This made possible fluorescence proximity assays without the use of separation and washing steps, but advanced fluorimeters were still needed, the quenching technology was never found to be competitive in clinical routine use. In 1998 Buechler & al. published US patent 5,763,189 «Fluorescence energy transfer and intramolecular energy transfer in particles using novel compounds», mainly based on advanced particles comprising both binding molecules and different molecules that interact with light at different wavelengths. There are considerable development costs and production costs related to such particles. This is not a homogeneous immunoassay, and the handling of the solid phase is demanding, both in production, transportation and in execution of such assays.

Common to all the above described technologies is that those reagents used are added from several reagent containers, usually using pipetting equipment, and it requires trained specialized personnel to carry this out. The need for several reagent containers and specialized pipetting equipment and specialized personnel is a considerable cost in health service.

Furthermore, several inventions are related to methods and assays detecting or quantitating organic compounds in test samples.

WO 00/16099 (Wolf) describes reduced valency carbohydrate binding ligands (CBLs) that can be used to detect or quantitate carbohydrates in a sample. CBL can be used with fluorescence resonance energy transfer (FRET) to evaluate free carbohydrate or

those within a carbohydrate containing compound by using e.g. a proximity-based signal generating label moiety. Contrary to the present invention this method is not a fluorescence polarisation assay.

EP 0 561 653 A1 (Lakowicz et al.) describes determination of glucose in a sample by contacting the test sample with a donor- acceptor pair, wherein the acceptor in the donor-acceptor pair can be competitively replaced by the analyte. The donor can be photo luminizing or fluorescent. This method is suitable for higher concentrations of analyte and the method is not a fluorescence polarisation assay.

WO 00/25134 (Blanchard et al.) describes an assay for identifying ligands for nuclear receptors, utilizing scintillation proximity and FRET. Contrary to the present invention this assay is not a fluorescence polarisation assay. It is using a heterodimeric partner, and is not suitable for fluorescence polarisation assay.

US 5814449 (Schultz and Ballerstadt) describes a method for detection of galactose and glucose, using a receptor carrying molecule with at least two binding sites for the analyte of interest. In addition there are two groups of molecules wherein one group (fluorochrome) can produce a detectable response in the proximity of the other molecule. The group of molecules is bound to an analogue of the analyte of interest. When the analyte is present the binding complex will dissociate due to competitive replacement of the analogue with the analyte. The detection is performed with a complicated sensor and contrary to the present invention this method is not a fluorescence polarisation assay.

EP 0984281 A2 (Ullman et al.) describes a photosensitizer associated with a molecule in in a specific binding pair (sbp), and a chemo luminous component associated with a sbp molecule, and wherein the amount of light emitted from the chemo luminous compound due to the activation of the photosensitizer is related to the amont of analyte in the sample.This method requires several steps of pipetting and adding of reagents and is furthermore not a fluorescence polarisation assay.

Limitations in today's technology and related needs for improvements:

A considerable part of the routine analyses of tests of biological fluids, such as blood, serum, plasma, urine and spinal fluids, are carried out in so-called «emergency» situations, in which sending away test material to specialized laboratories causes delays before the analysis results are returned to the attending physician. Furthermore, a considerable cost problem is present in the health care system, and there is a need for efficient and at the same time sensitive reagents that are cheap to produce and simple to use. Homogeneous reagents in an ideal solution, where there are no production, storage and stability problems (contrary to with particle suspensions) are therefore a clear advantage. Furthermore, in some geographical areas there is a lack

of specialized personnel and specialized laboratories, which makes it difficult to perform quality pipette and handling work, as well as necessary washing procedures and maintenance of adequate handling equipment.

There is therefore an object to provide sensitive specific measurement methods based on stabile, durable reagents, preferably in ideal solution (not in suspension) supplied in very few and preferably just one single reagent container, not requiring any significant pipette work, with no solid phase to be washed or two different phases to be separated, and which preferably can be carried out on blood tests with the presence of hemoglobin and blood cells, possibly after or with simultaneous lysis of the blood cells.

The above mentioned object is obtained by the present invention characterised by the enclosed claims.

Brief description of the invention:

The present invention provides a method for concentration determination of one or more analytes in a test, which is characterized by the fact that the reagent characterised by this invention is mixed with the said test solution, after which the signals generated by the fluorescent substances included in the said reagent are measured, in order to calculate the concentration of the said analytes on this basis. The said signal changes may be measured both as so-called endpoint measurements (after the establishment or near establishment of new chemical equilibriums), and kinetically (by measuring the signals' change per time unit or within a time interval).

The present invention further relates to a method wherein the ingredients of the said reagent is not kept separately but supplied in a single container or compartment, and the said reagent furthermore comprises at least one type of specific binding molecule for each analyte, for which the concentration should be determined, and the reagent furthermore comprises fluorescent substances whose signals change as a result of admixing a test sample with the reagent, and that this signal change is a function of the concentrations of the analyte or analytes in the sample, and that this signal change may be used to calculate the concentration or concentrations of analytes, without using separation of different states of aggregation.

The present invention relates further to a method wherein the reagent for concentration determination of one or more analytes in a test may further be characterized by the presence in the said reagent of a binding pair where the binding partners are reversibly bound to each other for each analyte to be concentration determined, and further characterized by at least one of the binding partners in each of the binding pairs having a fluorescent residue. The invention is further characterized by at least one of the binding partners in each of the said binding pairs

having affinity for one of the analytes to be quantified, and that – when the reagent is mixed with the said test – each of the analytes compete for the binding between the binding partners in at least on of the binding pairs, and that varying concentration of the analyte or the analytes in the test leads to changes in the concentrations of the other molecules that are included in the equilibrium for creation of binding pairs between the said binding partners.

The invention is further characterized by the fact that the reagent may include several types of binding partners or binding pairs for each analyte.

The invention is further characterized by the fact that the reagent may be designed to quantify only one analyte and further be characterized by comprising only one type of specific binding molecule.

The invention is further characterized by the fact that the fluorescent residues may be bound to one or more of the specific binding molecules, and that the fluorescence signal that may be generated is changed as a consequence of the said specific binding molecules being bound to analyte molecules.

The invention is further characterized by the fact that the said fluorescent residues may be different fluorescent substances in order to achieve different fluorescence wavelengths to quantify different analytes in the test.

The invention is further characterized by the fact that for analyses with the presence of hemoglobin, fluorescent residues with a maximum absorption coefficient at a wavelength between 600 nm and 1000 nm are preferred, more preferred exceeding 620 nm, most preferred exceeding 640 nm.

Especially preferred are reagents which ingredients are not kept separately but are present in one single container and comprise fluorescent residues bound to specific binding molecules with a low molecular weight and with fluorescent residues with a maximum absorption coefficient at a wavelength between 600 nm and 1000 nm, more preferred exceeding 620 nm, and even more preferred exceeding 640 nm.

The invention is further characterized by the reagent including specific binding molecules consisting of monoclonal or polyclonal antibodies or immunoreactive fragments of these, e.g. FAB fragments or single chain fragments or single chain antibodies, or peptides or other polymers produced by Phage Display or other biological combinatory techniques, or nucleic acid polymers or analogues or derivatives of these, or polymers produced on the basis of library technologies or synthetic combinatory chemistry. The invention may furthermore be characterized by the fact that other binding partners in said binding pair might be a derivative or

analogue or fragment or part of or an imitation of the structure characterizing at least one of the analytes to be quantified.

The invention is further characterized by the fact that the reagent may include one or more types of specific binding molecules and one or more binding partners to the said specific binding molecules, and that this or these said binding partners are constituted by a fluorescent derivative of an analyte or a fluorescent analogue of an analyte or a fluorescent fragment of an analyte or a fluorescent part of an analyte or a fluorescent imitation of the structure characterized by at least one of the analytes that are to be concentration determined using the reagent.

The invention is further characterized by the fact that the said reagent may comprise lysing substances or coagulation restrainers or surface-active substances or precipitating substances or separating substances.

The invention is further characterized by the fact that the said fluorescent residues may be cyanine dyes. The invention is further characterized by the fact that Alexa Fluor Dyes or substances in the group Bodipy delivered by Molecular Probes may be used.

The invention is further characterized by the fact that it may be possible or desirable to keep parts of the reagent in separate containers, and that the reagent is ready-mixed by the user before using the reagent.

The said method is further characterized by the fact that the signals to be measured are measured using fluorescence polarization measurements. The fluorescence signals are read as a function of time, either in the form of continuous reading within a period of time or as change per time unit between 2 or more points of time or as an absolute change between 2 or more points of time. Such measurements are often called kinetic measurements or readings. The present invention may also use kinetic reading methods with the remaining forms of fluorescence measurement methods that are described.

The said method is further characterized by the fact that the different constituents of the said reagent may – if desirable – be added in steps instead of at the same time. The said method is further characterized by the fact that – if desired – more, or other, reagents may be added after the reagent characterized by this invention is added. If the reagent characterized by this invention is split up and added in steps, other reagents may - if desirable – be added in between the said stepwise adding of the reagent characterized by this invention.

The said method is further characterized by the fact that the test sample may be a biological material or extract or a dilution or concentrate, or a filtrate thereof. The

said method is further characterized by the fact that the biological solutions may be blood, serum, plasma, cells from blood, lysate of blood, urine, cerebrospinal fluid, lachrymal fluid, saliva, aspirate from the gastrointestinal tract, semen or seminal fluid or feces or fecal extract or fecal dilution or suspension. The biological solution may furthermore come from the plant kingdom in the form of solutions, extracts or derivatives or filtrates.

The method for concentration determination in compliance with the invention is further characterized by the fact that standard solutions or calibrators with known concentrations of the analyte or analytes may be used, and that the concentration of the analyte or analytes is determined when the signals measured from the fluorescent residues are interpolated on the standard curve achieved using the said calibrators or standard solutions.

The method for concentration determination in compliance with the invention is further characterized by the fact that the said standard curves may be stored in an artificial memory connected to the analysis system, so that in the relevant user situation it is not necessary to perform analyses of the said calibrators or standard solutions.

The method for concentration determination in compliance with the invention is further characterized by the fact that the method may be carried out at a constant temperature, or by the use of correction algorithms empirically generated by way of studies of the temperature's influence on test solutions with a known concentration of the analyte or analytes.

According to the present invention the method may be used to determine concentrations of clinically related substances in samples of biological material from living organisms in need thereof. Such organisms may constitute plants, insects, birds, animals such as mammals, preferably primates, more preferably humans.

Furthermore the present invention is related to a kit comprising containers containing predetermined volumes of ingredients to be combined in in one single container in relation to the specific analyte to be measured, and a container for drawing specific volumes of the sample of the biological material of interest. In another embodiment the kit comprises a single container containing the reagent specific for the analyte to be measured , and a container for drawing specific volumes of the biological sample of interest.

The present invention will now be described in more detail, with reference to figures and examples.

FIGURES

Figure 1:

An example of concentrations as a function of time when the affinity equilibrium constant between the analyte molecules on the one side and the specific binding molecule on the other side is 10*E10/Molar, and the dissociation rate constant is 0,1 per sec.

A= The concentration of analyte molecules
B= The concentration of fluorescent binding pairs (complexes between the specific binding molecules and fluorescent derivates of analyte molecules)..
C= The concentration of fluorescent derivates of analyte molecules.
D= The concentration of a specific binding molecule.
E= The concentration of binding pairs without fluorescence (complexes between specific binding molecules and analyte molecules).

Reaction diagram:

$k_{affinity\ equilibrium}$ **=1.0*10E10**
Analyte molecule + binding molecule = non-fluorescent binding pair

$k_{dissociation}$ **=1.0*10E-1**
Fluorescent binding pair = specific binding molecule +
fluorescent analyte molecule derivate

$k_{affinity\ equilibrium}$ **=1.0*10E10**
Fluorescent analyte molecule + binding molecule = fluorescent binding pair

$k_{dissociation\ rate}$ **=1.0*10E-1**
Non-fluorescent binding pair = specific binding molecule + analyte molecule

Figure 2:

An example of concentrations as a function of time when the affinity equilibrium constant between the analyte molecules on the one side and the specific binding molecule on the other side is 10*E10/Molar, and the dissociation rate constant is 0,01 per sec.

A= The concentration of analyte molecules
B= The concentration of fluorescent binding pairs (complexes between the specific binding molecules and fluorescent derivates of analyte molecules).
C= The concentration of fluorescent derivates of analyte molecules.
D= The concentration of a specific binding molecule.
E= The concentration of binding pairs without fluorescence (complexes between specific binding molecules and analyte molecules).

Reaction diagram:

$k_{affinity\ equilibrium}$ **=1.0*10E10**
Analyte molecule + binding molecule = non-fluorescent binding pair

$k_{dissociation\ rate}$ **=1.0*10E-2**
Fluorescent binding pair = specific binding molecule +
fluorescent analyte molecule derivate

$k_{affinity\ equilibrium}$ **=1.0*10E10**
Fluorescent analyte molecule + binding molecule = fluorescent binding pair

$k_{dissociation\ rate}$ **=1.0*10E-2**
Non-fluorescent binding pair = specific binding molecule + analyte molecule

Figure 3:

An example of concentrations as a function of time when the affinity equilibrium constant between the analyte molecules on the one side and the specific binding molecule on the other side is 10*E10/Molar, and the dissociation rate constant is 0,001 per sec.

A= The concentration of analyte molecules
B= The concentration of fluorescent binding pairs (complexes between the specific binding molecules and fluorescent derivates of analyte molecules).
C= The concentration of fluorescent derivates of analyte molecules.
D= The concentration of a specific binding molecule.
E= The concentration of binding pairs without fluorescence (complexes between specific binding molecules and analyte molecules).

Reaction diagram:

$k_{affinity\ equilibrium}$ **=1.0*10E10**
Analyte molecule + binding molecule = non-fluorescent binding pair

$k_{dissociation\ rate}$ **=1.0*10E-3**
Fluorescent binding pair = specific binding molecule +
fluorescent analyte molecule derivate

$k_{affinity\ equilibrium}$ **=1.0*10E10**
Fluorescent analyte molecule + binding molecule = fluorescent binding pair

$k_{dissociation\ rate}$ **=1.0*10E-3**
Non-fluorescent binding pair = specific binding molecule + analyte molecule

Figure 4:

An example of concentrations as a function of time when the affinity equilibrium constant between the analyte molecules on the one side and the specific binding molecule on the other side is 10*E11/Molar, and the dissociation rate constant is 0,01 per sec.

A= The concentration of analyte molecules
B= The concentration of fluorescent binding pairs (complexes between the specific binding molecules and fluorescent derivates of analyte molecules).
C= The concentration of fluorescent derivates of analyte molecules.
D= The concentration of a specific binding molecule.
E= The concentration of binding pairs without fluorescence (complexes between specific binding molecules and analyte molecules).

Reaction diagram:

$k_{affinity\ equilibrium}$ **=1.0*10E11**
Analyte molecule + binding molecule = non-fluorescent binding pair

$k_{dissociation\ rate}$ **=1.0*10E-1**
Fluorescent binding pair = specific binding molecule +
fluorescent analyte molecule derivate

$k_{affinity\ equilibrium}$ **=1.0*10E11**
Fluorescent analyte molecule + binding molecule = fluorescent binding pair

$k_{dissociation\ rate}$ **=1.0*10E-1**
Non-fluorescent binding pair = specific binding molecule + analyte molecule

Figure 5:

An example of concentrations as a function of time when the affinity equilibrium constant between the analyte molecules on the one side and the specific binding molecule on the other side is 10*E11/Molar, and the dissociation rate constant is 0,01 per sec.

A= The concentration of analyte molecules
B= The concentration of fluorescent binding pairs (complexes between the specific binding molecules and fluorescent derivates of analyte molecules).
C= The concentration of fluorescent derivates of analyte molecules.
D= The concentration of a specific binding molecule.
E= The concentration of binding pairs without fluorescence (complexes between specific binding molecules and analyte molecules).

Reaction diagram:

$k_{affinity\ equilibrium}$ =1.0*10E11
Analyte molecule + binding molecule = non-fluorescent binding pair

$k_{dissociation\ rate}$ =1.0*10E-2
Fluorescent binding pair = specific binding molecule +
fluorescent analyte molecule derivate

$k_{affinity\ equilibrium}$ =1.0*10E11
Fluorescent analyte molecule + binding molecule = fluorescent binding pair

$k_{dissociation\ rate}$ =1.0*10E-2
Non-fluorescent binding pair = specific binding molecule + analyte molecule

Figure 6:

An example of concentrations as a function of time when the affinity equilibrium constant between the analyte molecules on the one side and the specific binding molecule on the other side is 10*E11/Molar, and the dissociation rate constant is 0,001 per sec.

A= The concentration of analyte molecules
B= The concentration of fluorescent binding pairs (complexes between the specific binding molecules and fluorescent derivates of analyte molecules).
C= The concentration of fluorescent derivates of analyte molecules.
D= The concentration of a specific binding molecule.
E= The concentration of binding pairs without fluorescence (complexes between specific binding molecules and analyte molecules).

Reaction diagram:

$k_{affinity\ equilibrium}$ =1.0*10E11
Analyte molecule + binding molecule = non-fluorescent binding pair

$k_{dissociation\ rate}$ =1.0*10E-3
Fluorescent binding pair = specific binding molecule +
fluorescent analyte molecule derivate

$k_{affinity\ equilibrium}$ =1.0*10E11
Fluorescent analyte molecule + binding molecule = fluorescent binding pair

$k_{dissociation\ rate}$ =1.0*10E-3
Non-fluorescent binding pair = specific binding molecule + analyte molecule

Figure 7:

An example of concentrations as a function of time when the affinity equilibrium constant between the analyte molecules on the one side and the specific binding molecule on the other side is 5*10E9/Molar, and the dissociation rate constant is 0,1 per sec.

A= The concentration of analyte molecules
B= The concentration of fluorescent binding pairs (complexes between the specific binding molecules and fluorescent derivates of analyte molecules).
C= The concentration of fluorescent derivates of analyte molecules.
D= The concentration of a specific binding molecule.
E= The concentration of binding pairs without fluorescence (complexes between specific binding molecules and analyte molecules).

Reaction diagram:

$$k_{\text{affinity equilibrium}} = \mathbf{5.0*10E9}$$

Analyte molecule + binding molecule = non-fluorescent binding pair

$$k_{\text{dissociation rate}} = \mathbf{1.0*10E-1}$$

Fluorescent binding pair = specific binding molecule +
fluorescent analyte molecule derivate

$$k_{\text{affinity equilibrium}} = \mathbf{5.0*10E9}$$

Fluorescent analyte molecule + binding molecule = fluorescent binding pair

$$k_{\text{dissociation rate}} = \mathbf{1.0*10E-1}$$

Non-fluorescent binding pair = specific binding molecule + analyte molecule

Figure 8:

An example of concentrations as a function of time when the affinity equilibrium constant between the analyte molecules on the one side and the specific binding molecule on the other side is 5*10E9/Molar, and the dissociation rate constant is 0,01 per sec.

A= The concentration of analyte molecules
B= The concentration of fluorescent binding pairs (complexes between the specific binding molecules and fluorescent derivates of analyte molecules).
C= The concentration of fluorescent derivates of analyte molecules.
D= The concentration of a specific binding molecule.
E= The concentration of binding pairs without fluorescence (complexes between specific binding molecules and analyte molecules).

Reaction diagram:

$k_{affinity\ equilibrium}$ =5.0*10E9
Analyte molecule + binding molecule = non-fluorescent binding pair

$k_{dissociation\ rate}$ =1.0*10E-2
Fluorescent binding pair = specific binding molecule +
fluorescent analyte molecule derivate

$k_{affinity\ equilibrium}$ =5.0*10E9
Fluorescent analyte molecule + binding molecule = fluorescent binding pair

$k_{dissociation\ rate}$ =1.0*10E-2
Non-fluorescent binding pair = specific binding molecule + analyte molecule

Figure 9:

An example of concentrations as a function of time when the affinity equilibrium constant between the analyte molecules on the one side and the specific binding molecule on the other side is 5*10E9/Molar, and the dissociation rate constant is 0,001 per sec.

A= The concentration of analyte molecules
B= The concentration of fluorescent binding pairs (complexes between the specific binding molecules and fluorescent derivates of analyte molecules).
C= The concentration of fluorescent derivates of analyte molecules.
D= The concentration of a specific binding molecule.
E= The concentration of binding pairs without fluorescence (complexes between specific binding molecules and analyte molecules).

Reaction diagram:

$k_{affinity\ equilibrium}$ =5.0*10E9
Analyte molecule + binding molecule = non-fluorescent binding pair

$k_{dissociation\ rate}$ =1.0*10E-3
Fluorescent binding pair = specific binding molecule +
fluorescent analyte molecule derivate

$k_{affinity\ equilibrium}$ =5.0*10E9
Fluorescent analyte molecule + binding molecule = fluorescent binding pair

$k_{dissociation\ rate}$ =1.0*10E-3
Non-fluorescent binding pair = specific binding molecule + analyte molecule.

Chemical analyses of materials are most often performed by taking a sample of the material (i.e. test material/test sample), which subsequently undergoes the desired chemical analysis. The test sample may be e.g. in the fluid state, gaseous state, solid state or mixtures of the said states of aggregation. The sample can furthermore be homogeneous or inhomogeneous. If, for instance, the sample is in a fluid phase, the fluid phase could comprise particulate material, and would thus be inhomogeneous. Well-prepared blood serum is an example of a homogeneous biological sample material, whereas the blood as such comprises considerable amounts of blood cells, and thus is not a homogeneous material.

Concentration determination of one or more analytes in a test sample is most often achieved by admixing other chemical substances to the sample, after which the signals or signal changes appearing as a consequence of the mixing are observed or measured. These signals may be chemical or physical signals in the form of electromagnetic radiation, radioactive radiation, temperature or response to physical influence, such as fluorescence or absorption of light. In previously known commercial products for quantitative analysis using fluorescence measurements, typically in the form of immunoassay systems, the binding partners included in binding pairs, typically in the form of antibodies and analyte analogues labeled with fluorescent residues, have been kept isolated from each other, typically separated in different reagent containers. This have made it necessary to add different reagents in several steps by the use of accurate instruments, e.g. pipettes.

In order to avoid the different problems that are connected to the currently used technology, the present invention provides all the necessary chemical substances, including fluorescent substances and binding partners, in one single collection of substances, usually in a fluid state, and gathered in one single container. This collection of chemical substances is called a reagent. The reason why it is an advantage to have one single ready mixture of the chemical substances in one reagent is that this provides a pre-measured amount of correct concentrations of the ingredients. This means that persons without specialized chemical training can perform the mixing of the reagent with the test material. Typically, the test material will be collected in a pre-calibrated capillary or another testing device, e.g. a self-calibrating constant volume pipette e.g. from Samco Scientific (USA), which is subsequently emptied into or combined with (e.g. dropped into) the reagent in its container. The container with the reagent may consist of different materials, such as e.g. glass vials, glass or plastic test tubes, plastic containers, foil pockets, plastic pads or other devices that can be used to contain reagents.

Traditionally, the wish has been that the binding between the binding partners in analysis systems using binding pairs and fluorescence measurements should be as strong as possible. High affinity equilibrium constants have led to very high sensitivities for the concentration determinations, and ensured less interference from other substances and variations in the physical/chemical circumstances. In the earlier state of the art, keeping binding partners in the same reagent and reagent container has – due to the high affinity equilibrium constants – led to aggregations, precipitations and irreversible chemical changes, as well as inhomogeneous solutions. Thus, keeping them in one single reagent, as is the case according to the present invention, has therefore been avoided, and several separate reagents has been preferred. The mixing of two or more binding partners has instead been carried out during the performance of the quantitation method. To achieve a precise result, precise transfer of volumes of the reagents has therefore been necessary, either by manual so-called pipetting or using chemical automatons. Using manual pipettes is very demanding and is an essential part of the basic training for laboratory workers and chemical engineers. The automatons are expensive and usually only available in laboratories or larger centers for testing or handling of samples. To avoid such problems, the method according to the present invention uses only one reagent for concentration determination of one or more analytes in a sample, wherein the said reagent is not kept separately but is present in one single container. Furthermore, the reagent to be used in the method according to the present invention comprises at least one specific binding molecule for each analyte to be concentration determined, as well as fluorescent substances whose fluorescence changes as a result of mixing in a sample to the reagent. This change in the fluorescence signal is a function of the concentrations of the analyte or analytes in the sample, and can be used for calculation of the analyte concentration(s) without using separation of different states of aggregation.

Thus, the generic term «reagent» is used in this description as a generic name for the collection of substances that are mixed with a sample solution for measuring one or more analytes. The reagent will normally be in a fluidal state, in the form of a solution of several substances, readily with buffer substances, salts, surface-active agents and anti-biological substances added to avoid growth of microorganisms in the reagent. But for some uses of the reagent, it may be an advantage if the reagent is used in a solid state, possibly by adding fluid immediately prior to using the reagent, or possibly by dissolving the solid reagent, for instance in the sample material, prior to or during use. The reagent may further be dried on a stand or a device, or enclosed in capsules or tablets.

The preferred embodiment of the reagent according to the present invention, is that the reagent is present, ready for use, in one single container where the amount of

reagent that is to be used to analyze a sample will not need to be pipetted or meted out or mixed with other reagents before use.

As an alternative, however, the appropriate volume may be meted out prior to the analysis or in the course of the execution of the analysis. Thus, it is normally less preferred, but still absolutely possible, to keep parts of the reagent in one or more separate containers, so that the ready-for-use reagent is mixed prior to or immediately prior to or in connection with the execution of the analysis. If so desired, parts of the reagent may be impregnated onto or into containers or devices or filters etc. and be mobilized at contact with a solution, e.g. the assay solution. If so desired, the entirety or parts of the reagent may be in a dry or desiccated state, and if desired, it can be designed so that fluid can be added to it prior to, immediately prior to or in connection with the quantitative analysis. The reagent may then further be mixed ready-for-use by such analysis automatons that are often used by larger, more sophisticated laboratories. However it is important to note that the methode according to the present invention, by combining a single reagent and fluorescence polarisation assay for analysis of analytes in samples of complex materials, distinguishes itself from the previous state of the art; e.g. from turbidimetry and nephelometry by being a more sensitive method, from scintillation proximity by the use of non-radioactive substances only and from FRET by employing ideal solutions which are less expensive and less complicated to produce.

The reagent to be used according to the present invention comprises fluorescent residues bound to (same or different) specific binding molecules/binding partners with a low molecular weight. It is known to the skilled man of the art that the ability to conserve the polarization of the exciting light in the emitted light as well, is a function of the rotation speed of the molecules, which in turn is a function of the molecular size as small molecules rotate faster than larger molecules. Thus, an especially preferred embodiment of the reagent in accordance with the present invention, is therefore to bind the fluorescent signal-providing substance to one or more binding partners with a low molecular weight, preferably a molecular weight under 5000, more preferred under 3000 and even more preferred under 1500. When the fluorescence-labeled binding partner either dissociates from its binding partner or binds to its binding partner, the total molecular size for the molecule that is rotating with the fluorescent residue will change, and this can be detected as a change in fluorescence polarization.

It is however, and according to another embodiment of the present invention, possible to use a reagent wherein the fluorescent binding partner(s) (i.e. binding partner(s) with fluorescent residue(s) bound to them) has higher molecular weight than the above mentioned, and to still use fluorescence polarisation assay in order to detect an analyte/analytes (see example 15). However, in this case the fluorescent residue(s)

must display longer decay time when compared to the fluorescent residue(s) bound to spesific binding molecules with low molecular weight.

The fluorescence-labeled binding molecules in the reagent that are used according to the preferred embodiment of the present method (i.e. the use of binding partner(s) with low molecular weight) are, according to the competitive embodiment of the present invention, either analogues or fragments or derivatives of the analyte(s) to be determined, whereas they in the non-competitive embodiment of the present inventioin are binding molecules such as a peptide/peptides or synthetic binders (optionally being identified by combinatory chemistry techniques or phage display or nucleic acid selection technology) with specific affinity for one or more of the said analytes. Furthermore, the fluorescent residues that are bound to the spesific binding molecules that are used in the reagent according to the non-competitive embodiment of the present invention, preferably have a maximum absorption coefficient at a wavelength exceeding between 600 nm and 1000 nm, further preferred exceeding 620 nm, and even further preferred exceeding 640 nm.

According to the present invention the test sample solution may be a biological material or extracts thereof, such as e.g. blood, blood serum or blood plasma, lachrymal fluid, extracts of feces, plant extracts, aspirate from the gastrointestinal tract or semen or seminal fluids, possibly diluted in diluent solutions or depository solutions, possibly with other reagents added to prevent coagulation or microbiological growth or oxidation or reduction or to regulate the acidity, alternatively derivatives or filtrates.

The different embodiments of present invention may be influenced by changes in the surrounding temperature or in the test sample solution or the reagent or in the mixture or in the instrument or the measurement compartment of the instrument to be used. Such temperature influences can be counteracted by the reagents and/or the instrument being temperature regulated, or by using calibrators with known concentrations of the analyte. However, one of the aims of the present invention is to provide measurement methods where calibrators are not used. Empirical measurements and theoretical calculations based on measurements with the reagents provided by the present invention may, however, form the basis for estimated deviation as a result of varying temperatures. Thus, according to the present invention and if desired, it is possible to use combinations of temperature sensors and software connected to or as a part of the measurement instrument, providing total or partial correction for the temperature-provoked deviation in the concentration determinations.

With the use of the present invention a large number of analytes can be concentration determined. The following analytes can be listed tabularly, but there is obviously a large number of other analytes that have not been included in this listing:

Hemoglobin
Albumin
C-reactive protein
Albumin in urine
Glycated albumin
Glycated hemoglobin
Ferritin
ASAT
ALAT
LDH
Myoglobin
Troponin I
Fatty Acid Binding Protein
Amylase
Glucose
HCG
U-HCG
TA-tests
Insulin
Anti-insulin antibodies
Helicobacter antibodies
Thyroxin
Free ryoxin
Prostate specific antigen
Free Prostate specific antigen
Thyroid stimulating hormone
Creatine kinase type MB

These and a large number of other analytes are referred to in reference books, such as e.g. in Tietz Textbook of Clinical Chemistry, Saunders Company, ISBN 0-7216-4472-4, 1994, and others, and also later text books and general surveys in clinical chemistry and pathology.

According to the competing embodiment of the present invention, a ready-to-use preformed reagent, comprising one or more binding pairs where both binding partners in each binding pair is present in one single container, and where the reagent is composed in such a way that aggregations, precipitations and irreversible binding between the binding pairs are avoided, is used. When the test material is mixed with

the reagent, the analyte(s) compete with the binding between the binding partners already present in the reagent in at least one of the binding pairs.

The reagent provided for the competitive embodiment of the present invention, can be used for concentration determination of one or more analytes, and may be characterized by the fact that for each analyte the reagent comprises at least one binding pair in which the binding partners are reversibly bound to each other. Furthermore, the reagent related to this embodiment of the invention may be characterized by at least one of the binding partners in at least one of the said binding pairs comprising or having bound to it a fluorescent chemical residue.

By «reversibly bound» what is meant here is a binding that is such that addition of a substance that competes with the binding between the binding partners leads to a change in the concentrations of the other molecules included in the equilibrium for formation of binding pairs between said binding partners within a reasonable amount of time - typically within one hour after adding. The invention is further characterized by the said changed concentrations of the molecules included in the equilibrium for formation of binding pairs between the binding partners leading to a change in the fluorescence signals that can be generated from the said fluorescent residues. This can occur because in the reagent characterized by this invention such fluorescent residues are used which can generate signals that change when the fluorescent residue is part of a connected binding pair as compared to the signals generated when the residue is bound to a binding partner molecule that is not bound to its binding partner molecules.

The reagent used according to the competitive embodiment of the present invention is characterized by comprising one or more binding pairs for which there is, furthermore, an equilibrium between the free condition in which the binding partners are not bound to each other, and the bound condition in which the binding pairs are bound to each other. This equilibrium is subject to general chemical laws, such as the law of mass action. When the concentrations of one of the molecules included in the chemical equilibrium are changed, the concentrations of the remaining molecules included in the equilibrium will change as well. Furthermore, the equilibrium will shift by adding analogues of the binding partners or derivatives or analogues of binding partners, where the structural similarity is adequate to bring about competition over the binding to the corresponding binding partner. Such structural similarity is mostly conserved, also when modifications are used for attaching fluorescent residues that do not dramatically change the structure of the substance that is attached onto this fluorescent residue. It is further commonly known to the skilled man of the art that if fluorescent residues or labels with different fluorescence are used, it is possible to simultaneously measure different chemical reactions in the same solution. Thus, the reagent in accordance with the present invention is further characterized by the fact

that by using residues or labels with different fluorescence, it can be used to quantify different analytes simultaneously in the same sample.

Traditionally, as high affinity equilibrium constant as possible was desired for the use of bio specific binders such as antibodies. The affinity equilibrium constant is a complex quantity, constituted by the association velocity constant divided by the dissociation velocity constant, and traditionally affinity equilibrium constants exceeding 10E7 have been desired, preferably exceeding 10E8 and even more preferred exceeding 10E9. At especially low concentrations affinity equilibrium constants exceeding 10E10 have been desired in order to achieve that it should be possible to bind an acceptable share of the analyte to the binder. Especially, it has been claimed that high dissociation velocity must be avoided (Immonoassay (E.P.Diamandis, T.E. Christopoulos, eds.) San Diego, CA; Academic Press (1996). Polyclonal antibodies have – since they are polyclonal – very varying affinity equilibrium constants in the same preparation, whereas monoclonal antibodies, or the biological or synthetic binders accounted for in the background for this invention, have more uniform or identical affinity equilibrium constants within the same preparation, and also often a more constant affinity from preparation to preparation. In the competitive embodiment of the present invention, and contrary to what was previously desired, especially high affinity equilibrium constants are not desired, and in particular not too low dissociation velocity constants.

As accounted for in the paragraph on the background for the present invention, the average skilled man of the art is used to using different types of specific binding molecules. The reagent in accordance with the competitive embodiment of the present invention may comprise binding partners that form binding pairs of all types and varieties. Traditional polyclonal antibodies on the one hand and antigens on the other hand may be used as specific binding molecules. Instead of polyclonal antibodies monoclonal antibodies may be used. The antibodies may be complete or in the form of reactive fragments. Especially preferred are smaller fragments of antibodies such as FAB fragments or single chain antibodies or single chain antibody fragments. Instead of antibodies or antibody fragments produced in eucaryotic cells, it is possible to use binders provided through phage display or further advancements of phage display technology, in the form of polypeptides or other types of polymers, polynucleic acids, or binders composed of building blocks that are variedly composed and picked out using library technology. Synthetic combinatory chemistry is rapidly developing and can be used to produce specific binding molecules, and production of polymers with RNA or DNA or analogue monomers is used with increasing frequency, and may of course also be used as specific binding molecules in the present invention.

The antigens mentioned above are traditional binding partners in immunoassay technology, but in recent years parts of antigens, antigen fragments, so-called

haptens, and derivatives of antigens or haptens, have been used to a greater extent. Synthetically or biologically produced molecules with a high structural similarity to the analyte or analytes that are to be quantified, can also be used. The present invention is characterized by the fact that all these structures may be used in binding pairs, when a suitable binding partner is found.

The present invention may further be characterized by the use of binding pairs in the preformed reagent for which the binding of the binding partners is influenced by the concentration of the analyte or analytes. A high concentration of an analyte will lead to an increased competition for binding to one of the binding partners in one or more binding pairs, compared to what would be the case with a lower concentration of said analyte. The present invention is further characterized by the fact that this leads to another concentration of one or more of the unbound binding partners and/or the binding pairs that were present in the reagent before it was mixed with the sample material. The reagent in accordance with the present invention is further characterized by the fact that it is composed in such a way that this changed concentration of one or more of the binding partners can be detected using one or more of the methods that are described above.

In order to measure an analyte in a competitive binding assay, the binding partner that the competition is about, e.g. the specific antibody, must be in effective relative deficit in relation to the concentration of the analyte (in order for competition to arise). But since what is desired is to measure the signal that is changed as analyte molecules with fluorescent residue are bound to the binding partner, the concentration of the binding partner (e.g. antibody) must be so high that a considerable part of the analyte molecules can be bound to the binding partner. Here it is necessary to find a practical balance that is regulated by the analyte concentration in the solution and the choice of specific binding molecule with suitable affinity equilibrium constant and dissociation velocity constant. Furthermore, according to the competitive embodiment of the present invention, the binding molecules and fluorescent derivatives or analogues of the analyte molecules are kept as binding pairs in the same reagent container. Thus the use of polyclonal antibodies as specific binding molecules in combination with large molecular analytes should be avoided, since this typically could result in precipitation in the reagent or the assay solution. Monoclonal antibodies are most often to be preferred over polyclonal antibodies, and often further preferred are monovalent binding molecules readily of smaller molecular size, e.g. FAB-fragments of antibodies or polypeptides or aptamers.

When a reagent in accordance with the competitive embodiments of the present invention is mixed with a sample containing the analytes that are to be concentration determined, the concentration of the analytes in the sample material will lead to changes in the concentrations of the other molecules included in the equilibriums for

formation of said binding pairs between said binding partners. The invention is further characterized by the fact that the said changed concentrations of the molecules included in the equilibrium for formation of binding pairs between the binding partners lead to a change in the signals that can be generated from the said fluorescent residues, and that these signal changes can be used for quantification of said analytes.

The said changes in the fluorescence signals are, in other words, a direct function of the concentrations of the analytes, and these signal changes can be used for concentration determination of the said analytes. Thus, the fluorescent residues according to the present invention may have one or more of the specific binding molecules bound to them, and the fluorescence signal that can be generated changes as a consequence of the said specific binding molecules binding to the analyte molecules.

If a concentration of approximately 1.0*10E-9 molar analyte is present in the assay solution (the solution arrived at when the reagent related to the present invention is mixed with the test material), and we have an infinitely high affinity equilibrium constant, we will, by using an effective concentration of 0.5* 10E-9 molar specific binding molecule, achieve 50 % binding of the analyte to the binding partner at equilibrium. If the affinity equilibrium constant is lower, e.g. 1.0*10E-9, the situation at equilibrium is somewhat different:

Given that the analyte molecules have the same affinity for the binding molecules (e.g. antibodies) whether they have fluorescent residues bound to them or not, and

a = affinity equilibrium constant,

b = aggregate concentration of binding partner molecules (bound and free put together), and

c = aggregate concentration analyte molecules (totally from the sample and the reagent related to the present invention, including analyte molecules with fluorescent residues),

according to the law of mass action the aggregate concentration of binding pairs (e.g. antibody-antigen complexes) = x would be as follows at equilibrium:

$x = a(b-x)(c-x)$

In a situation where a = 1.0*10E9, b = 1.0*10E-10 and c = 2.0*10E-9, calculation shows that at equilibrium

x = 0.7*10E-10 molar, and further that the effective concentration of free specific binding molecule (e.g. antibody) is 0.3*10E-10 molar and further that the

concentration of unbound analyte molecules (with and without fluorescent residues bound to them) is 19.3*10E-10 molar.

We see that this will give a very small change in the signal as a consequence of the analyte molecules' binding to the binding partner. In this case it would be possible to bind less than 5 % of the analyte molecules to the binding partner.

If the affinity equilibrium constant a is 1.0*10E8 but b and c above are unchanged, it could accordingly be shown that the fraction of bound analyte molecule is infinitesimally small, and no competition what so ever occurs.

If the situation is such that the specific binding molecule's (e.g. the antibody's) affinity for modified analyte molecules with fluorescent residue bound to them is higher than for unmodified analyte molecules, a lower concentration of the modified analyte molecules needs to be used for the analyte molecules in the sample to be able to efficiently compete. This corresponds to a situation with a lower effective concentration of specific binding molecule, and will require a somewhat higher affinity equilibrium constant of the specific binding molecule according to the law of mass action.

If the situation is such that the specific binding molecule's (e.g. the antibody's) affinity equilibrium constant for modified analyte molecules with fluorescent residue bound to them is lower than for unmodified analyte molecules, it might be desirable to use a higher concentration of the modified analyte molecules. To achieve a situation with competition, however, the effective concentration of specific binding molecule must be in molar deficiency relative to the analyte molecules in the sample, and this low concentration of the specific binding molecule still requires that the affinity equilibrium constant between binding partner and analyte molecules is high. It is worth mentioning, however, that the effective concentration of binding molecules may be considerably less than the total amount of so-called binding molecules. It is not seldom than only a fraction of the actual binding molecules – e.g. monoclonal antibodies – really bind its binding partners. This can be due to structural alterations, e.g. in the synthesis of the molecules, e.g. post-translation alterations, or modifications introduced by labeling chemistries or simply of unknown origin.

Thus we can conclude that the affinity equilibrium constant, according to a competitive embodiment of the present invention, must have a value that at least equals one third of the inverse value of the concentration of the modified analyte molecules with signal providing residues bound to them. More preferred are affinity equilibrium constants that are higher than the inverse value of the concentration of the modified analyte molecules, and even more preferred are affinity equilibrium constants higher than twice the inverse value of the concentration of the modified analyte

molecules. Thus, in the reagent provided according to the competitive embodiment of the present invention, an equilibrium between the binding partners bound to each other and the binding partners in free, non-bound form will be established. When the said reagent is added to a sample, the concentrations change, and a new equilibrium is established. In the earlier technical state, a high affinity equilibrium constant and an especially high association constant was desired so that the equilibrium could be established soon after adding a binding molecule, e.g. a antibody. In the competitive embodiment of the present invention binding pairs have formed in the reagent before the sample is added, and then a higher dissociation velocity constant is sooner desired, so that a new equilibrium can be achieved within reasonable time after the sample has been added.

If affinity equilibrium constant

a = 1.0*10E10/mol,

b = aggregate concentration of binder (e.g. antibody) = 1.0*10E-9 molar

c = aggregate concentration analyte molecules (with and without signal providing residues) = 1.0*10E-9 molar

it can, by using mass balance and the law of mass action, be shown that at equilibrium the concentration of complex between the binding partners (analyte molecules with or without signal-providing residue and binder such as e.g. antibody) = 0.73*10E-9 molar, the concentration of analyte molecules unbound to binding partner = 0.27*10E-9 molar, and the concentration of unbound specific binding molecules (e.g. antibody) = 0.27*10E-9 molar. If then a test sample that adds 1.0*10E-9 molar analyte molecules is added, so that the total amount of analyte molecules in the assay solution becomes 2.0*10E-9 molar, a new equilibrium will be established after a while.

In these examples it is assumed that adding a sample does not change the total volume of the assay solution considerably. Calculations can be made showing that even though the assay solution is slightly changed, the above calculation examples will be very close to the conditions described.

If the affinity equilibrium constant is a power of 10 higher, the situation is as follows:

If affinity equilibrium constant

a = 1.0*10E11/Molar

b = aggregate concentration binder (e.g. antibody) = 1.0*10E-9 Molar

c = aggregate concentration analyte molecule (with and without signal providing residues) = 1.0*10E-9 molar

it can, using calculations corresponding to the above, be shown that in the competitive embodiment of the invention, it is predominantly the dissociation velocity constants that determines the time it takes to achieve equilibrium or near equilibrium after adding a sample. Slightly simplified we can say that figures 1 to 9 show that the value of the affinity equilibrium constant is the most important factor in deciding the range of signal change that can be achieved, whilst it is the value of the dissociation velocity constant that determines how quickly a new equilibrium or near equilibrium can be achieved after mixing in a sample comprising analyte molecules, and thus how quickly a new stabile fluorescence signal is achieved. In the reagent used for endpoint measurement in the competitive embodiment of the present invention, binding pairs with dissociation velocity constants 0.003 per second are therefore preferred. Binding pairs with dissociation velocity constants exceeding 0.01 per second are more preferred, and binding pairs with dissociation velocity constants exceeding 0.02 per second even more preferred.

If kinetic reading is applied, i.e. measuring the change in the fluorescence signal per time unit or within a defined time interval, measurements can be made before a new equilibrium is established, and still the signal change can be used to calculate the analyte concentration or analyte concentrations. Thus, in the competitive embodiment of the present invention kinetic readings therefore allow the use binding pairs with dissociation velocity constants that are considerably lower than those used for endpoint readings. Then binding pairs with dissociation velocity constants as low as 0.0001 per second can be used, but more preferred are dissociation velocity constants exceeding 0.001 per second, and still more preferred are dissociation velocity constants exceeding 0.005 per second.

A special embodiment of the method according to the present invention is to use a reagent in accordance with the present invention, and measure the change in degree of polarization of the fluorescence signal per time unit, or as a function of time, or within a given time interval after mixing in the sample material. In this embodiment the fluorescence signal or fluorescence signals can be read as a function of time, either as a continuous reading within a period of time, or as change per time unit between 2 or more points of time, or as an absolute change between 2 or more points of time. Such measurements are often called kinetic measurements or readings. The method according to present invention can also use kinetic reading methods with the other forms of fluorescence measurement methods described. The above mentioned applies to both the competitive and the non-competitive embodiments of the method according to the present invention.

An applicable embodiment of the present invention is further to use the above mentioned kinetic fluorescence polarization measurement, combined with the use of a reagent in accordance with the present invention, for which the wavelength of the maximum absorption coefficient for the fluorescent residues is higher than 600 nm, or further preferred 620 nm or even more preferred exceeding 640 nm. For analysis of samples comprising hemoglobin, the maximum absorption coefficient should exceed 620 nm, since the hemoglobin interfers substantially with the concentration determination of the analyte or analytes. Such interference have usually been a major problem when fluorescence polarisation assay has been used with e.g. blood.

In the non-competitive embodiment of the present invention, the fluorescent residues will usually, but not always, be bound to the specific binding molecule. The specific binding molecule may be present in excess compared to the test sample/analyte molecules to be added, and the fluorescence signal that may be generated will change if analyte molecules have bound to the specific binding molecules. A good example of such a suitable specific binding molecule is aptamers, described in «Selection of singlestranded DNA molecules that bind and inhibit human thrombin», by Bock & al., Nature vol. 355 pp 564-56, 1992. This article refers to a generic technology as general basis for production of specific binding molecules, and shows that aptamers comprising the nucleotide sequence GGTTGGTGTGGTTGG or GGTTGG are specifically bound to human thrombin.

Aptamers can also be used in the competitive embodiments of this invention, as well as in the non-competitive embodiments. Gold & al. in «Diversity of oligonucleotide functions» in Annel. Rev. Bichem. vol 64, pp 763-97, 1995, describe a large number of examples of use of the generic aptamer technology for production of specific binders, that can also be used in the reagent according to the present invention. The specific binder (described in Science, vol. 263, 11. March, 1994) that is assigned to theophyllins is an example that is especially well suited for the competitive embodiments, whereas the RNA-aptamer described in the same place, which binds T4 DNA polemerase, is suited for the non-competitive embodiments.

Rick Konrad et al. in «Isoenzyme-specific inhibition of Protein Kinase by RNA aptamers» in J. Biol. Chem. vol. 269, pp 32051-54, 1994, have described two different aptamer frequencies suitable as specific binding molecules for Protein Kinase C, which may serve as an example of specific binding molecules to be used in the reagent according to the present invention.

Both in the competitive and the non-competitive embodiments of the present invention, specific binding molecules that include peptide sequences may be used. An example of a peptide sequence that is especially suitable in the non-competitive embodiment is the peptide sequence described by Chakravarthy & al. in Anal.

Biochem. vol. 196, 144- 150, 1991. Other examples of peptide sequences usefull as spesific binders are described by Yue et al. in The Journal of Biological Chemistry, vol. 271, p. 22245-22250, 1996. They were able to identify peptides that bind C-reactive protein in a dot blot assay employing numerous reagents, radioactive substances and autoradiography, a time consuming and expensive procedure.

The reagent in accordance with the invention, and in both the competitive and the non-competitive embodiment of the method, may be characterized by the fact that it is prepared solely for concentration determination of an analyte. The reagent may nevertheless, if so desired, comprise more than one specific binding molecule, and if desired, more than one specific binding molecule may include said fluorescent substance or include several fluorescent substances. The reagent may however also be characterized by the fact that it is prepared for concentration determination of several analytes, if desired - simultaneously, and - if desired - with several different specific binding molecules for each analyte. If so desired it is possible to use more than one of these binding pairs which include a binding partner with a fluorescent residue, or even several different fluorescent residues bound to different binding partners in different binding pairs. An almost unlimited amount of possibilities for signal-providing systems exists here. Several signal-providing systems will potentially increase the precision of the concentration determinations, but will at the same time increase the complexity of the measurement systems.

Traditional fluorescent substances such as fluorescein and Texas Red, Oregon Green, Rhodamin, tetramethyl-rhodamin, amino methyl coumarin, as well as a wide range of other substances may be used as fluorescent residues, see e.g. «Handbook of Fluorescent Probes and research chemicals» by Richard P. Haugland, sixth edition, ISBN 0-9652240-0-7. This handbook also provides method description for binding of the fluorescent substances to amino groups, thiol groups, alcohol groups, ketones, dioles and carboxylic acids. Furthermore it provides methods for binding of fluorescent substances to peptides, proteins, antibodies, nucleic acid polymers such as aptamers and other polymers which, however, is well known for a person skilled in the art.

Often, substantial amounts of hemoglobin or bilious pigments are present in the sample material during medical and biological use of the present invention. Then reagents comprising fluorescent substances with excitation or emission wavelengths that are absorbed by hemoglobin or bilirubin will often be influenced by the light absorption that characterizes the said substances. As already mentioned, an especially preferred embodiment of the present invention when analysing whole blood or blood lysates, is therefore based on reagents with fluorescent molecules for which the wavelength for maximum absorption coefficient of the fluorescent residues exceeds 600 nm, or more preferred 620 nm or even more preferred exceeding 640 nm. For

analysis of samples comprising hemoglobin, the maximum absorption coefficient shoul exceed 620 nm since the hemoglobin interferes substantially with the concentration determination of the analyte or analytes. Such substances are sold by the company Amersham Pharmacia Biotech, under labels such as CyDye FluoroLink Reactive Dyes, with varying excitation wavelength (varying with analogues of the chemical structure), and with varying numbers of activated groups for binding to the substance that is to be labeled. More binding points will typically reduce the molecule's degree of freedom of rotation, and reduce the possibility for using fluorescence polarization measurement methods.

Suitable cyanine dyes are further described in US5627027: «Cyanine dyes as labeling reagents for detection of biological and other materials by luminescence methods» by Waggoner; Alan S, 6. May 1997. Furthermore, suitable substances are described in Mujumdar, Lauren, Mujumdar and Waggoner in Cytometry 10: 11-19, 1989 or Southwick & al in Cytometry 11, 418-430, 1990 or Lauren & al. in Cytometry 10: 3-10, 1990, or in Waggoner et al US patent 6008373 or Brush and Reimer US patent 5986086 or Krandikar & al. US patent 5852191 or Kusakata & al. US patent 4847385 or Waggoner's US Patent 5569587.

«Handbook of Fluorescent Probes and research chemicals» by Richard P. Haugland, sixth edition, ISBN 0-9652240-0-7, also provides instructions for using the fluorescence substances group Biodypi, which provides good fluorescence signals exceeding 600, 620 and 640 nm, respectively. At present the substances Biodypi 630/650-X/MeOH, Biodypi 650/655-X/MeOH, Biodypi FL/MeOH, Biodypi R6G/MeOH, Biodypi TMR-X/MeOH and Biodypi TR-X/MeOH, as well as the Alexa Fluor Substances, are sold from the company Molecular Probes, and more similar substances can be expected. These substances are good alternatives to the cyanin dyes, and are among the preferred reagents in accordance with the present invention.

Suitable fluorescence residues can also have bound to them complex formers such as DTPA and N1 comprising complex-bound Lanthanide elements such as Europium, Samarium or Terbium. Advantageous qualities in these fluorescence residues are that they have very long Stokes-shifts (large difference in wavelength between excitation wavelength and emission wavelength), as well as a relatively long time interval between excitation and emission, which is preferred when using time dissolution fluorescence measurements. These fluorescent residues are well explained in Perkin Elmer Life Sciences' homepage on the Internet, and in their product catalogues.

Correspondingly the reagent in accordance with the present invention may be characterized by the fact that the fluorescent signal-providing substance partially consists of a polypeptide chain, and that this polypeptide chain preferably comprises

less than 30 amino acids, and more preferred less than 20 amino acids, and even more preferred less than 12 amino acids.

Reffering to examples 17 and 18, where use of intermittent exitation at different wavelenghts is described for the analysis of several analytes using different exuitation wavelenghts, it is preferred to use intermittent light with different wavelengthts, e.g. by the intermittent use of different diode lasers with different wavelengths. It should be noted that the break between use of the different light sources should at least correspond to the lifetime for the exited condition of the actual fluorophore.

Best mode

Best mode is illustrated by the examples, preferably example 9 for the competitive embodiment of the invention, and by example 14 for the non-sompetitive embodiment of the invention.

EXAMPLES

Example 1: Fluorescent binding ligand for whole blood analysis.

Synthesise the peptide Tyr-Trp-Ala-Asn-Phe-Ala-Arg-Asn-Arg-Asn by conventional technique, and dissolve it in pure water to a concentration of 2 mg per ml. Mix 50 ul of the said peptide solution with 50 ul sodium bicarbonate buffer pH = 9.2. Mix 0.1 micromol Cy5 Fluorolink activated cyanin dye, supplied from Amerham Pharmacia Biotech, optionally dissolved in water-free DMSO with said solution and leav it to stand overnight at room temperature in darkness, and thereafter store the solution in a refrigerator in darkness. If wanted, 5 ul of 10 mmolar ethanolamine solution can be added to block any remaining activated fluorescent dye, or the solution - hereinafter called the stock solution - can be left to hydrolyse the non-reacted dye during storage.

Isolate pure Cy5-labelled Tyr-Trp-Ala-Asn-Phe-Ala-Arg-Asn-Arg-Asn from the stock solution by reverse phase chromatography in a C4 column (sold from many suppliers, including Waters, U.S.), using 0.1 % trifluoroacetic acid as eluant and employ a gradiant of 0 % to 60 % acetonitrile in 0.1 % trifluoroacetic acid. Employ a photodetector coupled to a flow cell to monitor content of peptides by transmission measurements at 340 nm and Cy-5 by transmisson measurements at 650 nm, and isolate the Cy-5 –conjugated peptide. Remove the trifluoroacetic acid and the acetonitrile and the water by lyophilisation.

Pure Cy5-labelled Tyr-Trp-Ala-Asn-Phe-Ala-Arg-Asn-Arg-Asn of the stock solution can also be isolated by thin layer chromatography: Apply aliquots of stock solution on silica gel plates and elute with n-butanol:acetic acid:water mixtures. Depending on the quality of the silica gel, the relative content of n-butanol:acetic acid:water can be adjusted to obtain ideal separation. After elution by conventional technique, dry the

silica gel plate and inspect visually and by UV lamp (and optionally using nihydrin spray in parallell experiments) to identify the Cy5-labelled Tyr-Trp-Ala-Asn-Phe-Ala-Arg-Asn-Arg-Asn spot, separated from non-labelled peptide and free Cy-5 dye molecules. Isolate the silicagel comprising Cy5-labelled Tyr-Trp-Ala-Asn-Phe-Ala-Arg-Asn-Arg-Asn by scissors or spatulum. Suspend the isolatedsilica gel in 10 mM TRIS-buffer pH = 8.0, whereby the Cy5-labelled Tyr-Trp-Ala-Asn-Phe-Ala-Arg-Asn-Arg-Asn is eluted into solution. The silica gel settles in the bottom of the tube. Decant off the TRIS-buffered solution with the purified Cy5-labelled Tyr-Trp-Ala-Asn-Phe-Ala-Arg-Asn-Arg-Asn.

If wanted, and for up-scaling, other conventional HPLC separation techniques well known to the skilled man of the art can be used in stead of thin layer chromatography.

Example 2: Fluorescent binding ligand for whole blood analysis.

Perform the method of the present invention according to example 1, except use Tyr-Trp-Ala-Asn-Phe-Ala-Arg-Asn-Gly-Asn instead of Tyr-Trp-Ala-Asn-Phe-Ala-Arg-Asn-Arg-Asn, to obtain Cy5-labelled Tyr-Trp-Ala-Asn-Phe-Ala-Arg-Asn-Gly-Asn.

Eksempel 3: Method for measurement of C-reactive protein in samples of whole blood.

Make an assay reagent by making a buffer of 150 mM sodium chloride 100 mM phosphate, pH = 7.4. To this buffer, add bovine gammaglobulin from Sigma to a concentration of 2 mg/ml, and Triton X-100 from Pierce Chemical Company, US, to a final concentration of 0.1 % v/v. Reagents of high purity with very low background fluorescence should be chosen. Add 1.0*10E-11 mol per ml of Cy5-labelled Tyr-Trp-Ala-Asn-Phe-Ala-Arg-Asn-Arg-Asn according to example 1 above, and optionally add a suitable bacterostatic agentlike 0.01 % sodium azide for prolonged storage. Keep aliquots of this mixture, e.g. 1 ml, in separate containers to be combined with unknown blood samples or aliquots of blood samples.

At the time for the determination of the C-reactive protein, take an aliquot, e.g. a volume of 20 ul of the whole blood sample e.g. by the use of a pre-measured capillary, and combine thereafter the content of this pre-measured capillary with the assay reagent in the said separate container, the said separate container being kept in a place where the temperature is regulated to 32 degrees of Celcius.

Typically, use a container which is in the form of a cuvette with 4 polished transparent sides to be measured in an instrument built for fluorescent polarisation measurements, but said container comprising a removable stopper or seal which allows the capillary to enter the container, either by simply dropping the capillary or

by introducing it through the seal. Furthermore, in its preferred that the capillary/container is designed so that the capillary falls to the bottom of the container and does not interfere with the excitation light or the emission light (see below). Thereafter, shake the container, and the blood flows out of the capillary and the cells are being lysed by the assay reagent. The C-reactive protein of the test sample aliquot starts to react with the Cy5-labelled Tyr-Trp-Ala-Asn-Phe-Ala-Arg-Asn-Arg-Asn of the assay reagent.

Immediately after the shaking, place the container in the fluorescence polarisation measurement instrument, having a temperature of 32 degrees of Celcius. For measurement of polarisation of the emitted fluorescence, the mixture in the container is irradiated with polarized light of 650 nm wavelength, typically by the use of a small polarized laser diode in the instrument, and when the polarisation of the emitted light constant, measure its polarisation a wavelength of 670 nm, with a rather narrow bandwidth, if the instruments allows for that. Calculate the concentration of C-reactive protein of the unknown sample by interpolation of the polarisation value of the emitted light measured on a standard curve obtained by measurement of standards of known C-reactive protein concentration. Such a standard curve can often be stored on the computer of the measurement instrument, enabling a direct reading of the concentration of C-reactive protein on the instrument.

If a measurement instrument with a flow cell is used, then bring the mixture to pass through the flow cell for measurement, according to the instructions given by the instrument manufacturer.

Typically, with a mild bacterial infection, the content of C-reactive protein of a 20 ul sample is between 10 and 100 mg per l, but event much higher values can be seen in severe clinical conditions. On the other side, especially in screening of risk for heart disease and low grade of inflammation, measurement of values below 1 mg per liter is of interest. Furthermore, each C-reactive protein molecule can react with five molecules of Cy5-labelled Tyr-Trp-Ala-Asn-Phe-Ala-Arg-Asn-Arg-Asn. The concentrations of interest for C-reactive protein therefore varies a lot, and the concentration of Cy5-labelled Tyr-Trp-Ala-Asn-Phe-Ala-Arg-Asn-Arg-Asn therefore may have to be adjusted compared to the blood volume to be combined with the assay reagent.

Eaxmple 4: Method for measurement of C-reactive protein in samples of whole blood.

Perform the method of the present invention according to example 3, except use Cy5-labelled Tyr-Trp-Ala-Asn-Phe-Ala-Arg-Asn-Gly-Asn in the place of Cy5-labelled Tyr-Trp-Ala-Asn-Phe-Ala-Arg-Asn-Arg-Asn. This example of the method is more

adequate for very high concentrations of C-reactive protein than example 3 because of Cy5-labelled Tyr-Trp-Ala-Asn-Phe-Ala-Arg-Asn-Gly-Asn lower affinity for C-reactive protein. Correspondingly, this example of the method is less appropriate to use for low concentrations of C-reactive protein.

Example 5: Method for measurement of C-reactive protein in samples of whole blood.

Perform the method of the present invention according to example 3, except however, instead of reading the polarisation after the polarisation value has become stable, measure the polarisation value as a function of time in a kinetic manner. If available, connect the measurement instrument to a computer recording the signal continuously or at different time points, optionally defined by a software. Compare then the recorded values to values obtained by measurement of standards of known C-reactive protein concentration as a function of time. Optionally, record a three-dimensional standard curve with polarisation values, different times and C-reactive protein concentration values, and compare the values obtained with the unknown sample to calculate the C-reactive protein concentration, optionally by the use of the least-square methods for best fit according to standard textbooks of statistics, optionally by means of a computer or another artificial memory connected to the measurement instrument.

Example 6: Method for measurement of C-reactive protein in samples of whole blood.

Perform the method of the present invention according to example 5, except keep the said separate containers (to be combined with unknown blood samples or aliquots of blood samples) at the room temperature, and use a electronic temperature measurement device in or at the fluorescence polarisation instrument, and use a four-dimensional standard curve, the forth dimension being temperature, in addition to polarisation values, different times and C-reactive protein concentration values, and compare the values obtained with the unknown sample to calculate the C-reactive protein concentration, optionally by the use of the least-square methods for best fit according to standard textbooks of statistics, optionally by means of a computer or another artificial memory connected to the measurement instrument.

Example 7: Synthesis of Cynanin-5 analogue of theophyllin.

Make a synthesis of 8-(3-carboxypropyl)-1,3-dimethylxanthin anhydrid as described in Research Communications in Chemical Pathology and Pharmacology , vol. 13, p. 497-505, 1976, and in Clinical Chemistry. vol. 27, page 22-226, 1981. Dissolve diaminopropanol in water-free tetrahydrofuran. In another flask, dissolve half of the equimolar amount of the said 8-(3-carboxypropyl)-1,3-dimethylxanthin anhydrid in

water-free tetrahydrofuran. Add the said 8-(3-carboxypropyl)-1,3-dimethylxanthin anhydrid solution drop-wise to the diaminopropanol solution while stirring, and let the resulting solution react over night at room temperature. Optionally purify the resulting adduct by HPLC chromatography using conventional techniques well known to the skilled man of the art, if less consumption of activated cyanin dye is wanted (see below).

Thereafter, dissolve 6 times the molar amount which was used for diaminopropanol, of Cy5 Fluorolink activated cyanin dye supplied from Amersham Pharmacia Biotech, U.K., in water-free tetrahydrofuran, and add it previously described solution while stirring. Leave the resulting mixture to react over night a room temperature in darkness. In this way, a stock solution of non-pure 8-(3-carboxypropyl)-1,3-dimethylxanthin adduct with Cy5 Fluorolink activated cyanin dye with a water-soluble diaminopropanol spacer is obtained.

Purify the resulting 8-(3-carboxypropyl)-1,3-dimethylxanthin adduct with Cy5 Fluorolink activated cyanin dye with a water-soluble diaminopropanol spacer by means of thin layer chromatography according to example 1, and adjust the volumes of n-butanol, acetic acid and water in the elution mixture depending on the quality of the silica gel plates to obtain good separation. After elution by conventional technique, dry the silica gel plate and inspect visually and by UV lamp to identifythe spot of 8-(3-carboxypropyl)-1,3-dimethylxanthin adduct with Cy5 Fluorolink spot. Isolate the silicagel 8-(3-carboxypropyl)-1,3-dimethylxanthin adduct with Cy5 Fluorolink by scissors or spatulum. Suspend the isolated silica gel in 10 mM TRIS-buffer pH = 8.0, whereby 8-(3-carboxypropyl)-1,3-dimethylxanthin adduct with Cy5 Fluorolink is eluted into solution. The silica gel settles in the bottom of the tube. Decant off the TRIS-buffered solution with the purified 8-(3-carboxypropyl)-1,3-dimethylxanthin adduct with Cy5 Fluorolink.

If wanted, and for upscaling, conventional HPLC separation techniques well known to the skilled man of the art can be used in stead of thin layer chromatography. E.g Use the HPLC method described in example 1, however using a C4 or a C6 reversed phase column.

Exampel 8: Fab-fragments of antibodies with affinity for theophyllin.

Synthesise 8-(3-carboxypropyl)-1,3-dimethylxanthin anhydrid and prepare an albumin conjugate antigen as described in Research Communications in Chemical Pathology and Pharmacology , vol. 13, p. 497-505, 1976. Using this antigen, make mouse hybridomas. Select a clone the is specifically reactive to the Cyanin-5-analogue of theophyllin in example 7. The prior art teaches several ways of screening hybrodimas, e.g. in Campell s book on «Monoclonal antibody technology», ISBN 0-444-80575-3

or other textbooks on monoclonal antibody technology. The inventor of the present invention, however, prefers to make 150 mM sodium chloride 100 mM phosphate, pH = 7.4 . To this buffer, add bovine gammaglobulin from Sigma to a concentration of 2 mg/ml, and Triton X-100 from Pierce Chemical Company, US, to a final concentration of 0.1 % v/v. Reagents of high purity with very low background fluorescence should be chosen.

Then add Cynanin-5 analogue of theophyllin, made according to example 7, to a final concentration of 2 mg per l. Measure the polarisation of the emitted fluorescence when the mixture in the container is irradiated with polarized light of 650 nm wavelength, typically by the use of a small polarized laser diode in the instrument, and when the polarisation of the emitted light constant, measure its polarisation a wavelength of 670 nm, with a rather narrow bandwidth, if the instruments allows for that. Under monitoration by such fluorescence polarisation measurements, add antibodies isolated from the hybridoma cells to be investigated. For each addition, wait until the fluorescence polarisation signal is stable, before adding more antibody. Select antibodies from one or more hybridomas which specicially increases the fluorescence polarisation of the mixture, and do not select (often called counterselect) antibodies from hybridomas whose effect on the fluorescence polarisation technology is blocked or very significantly reduced by components of human serum or serum from human individuals not treated with theophyllin. Especially check that the antibodies isolated from the hybridomas in question do not react with cafein or other drugs or food constituents that resembels theophyllin.

When antibodies from the selected hybridoma has been isolated, prepare FAB fragments by the use the ImmnuPure Fab preparation kit supplied by Pirece Chemical Company, and follow the instruction for the said kit.

Example 9: Method to determine the concentration of theophyllin in whole blood by the use of a fluorescent immunocomplex.

Make anti-theophyllin FAB fagments of antibodies according to example 8 and a Cynanin-5 analogue of theophyllin according to example 7.

Thereafter, make a tentative assay reagent at 32 degrees Celcius according to the following protocol: Make 150 mM sodium chloride 100 mM phosphate, pH = 7.4. To this buffer, add bovine gammaglobulin from Sigma to a concentration of 2 mg/ml, and Triton X-100 from Pierce Chemical Company, US, to a final concentration of 0.1 % v/v. Reagents of high purity with very low background fluorescence should be chosen.

Then add Cynanin-5 analogue of theophyllin, made according to example 7, to a final concentration of 2 mg per l. Measure the polarisation of the emitted fluorescence

when the mixture in the container is irradiated with polarized light of 650 nm wavelength, typically by the use of a small polarized laser diode in the instrument, and when the polarisation of the emitted light constant, measure its polarisation a wavelength of 670 nm, with a rather narrow bandwidth, if the instruments allows for that. Under monitoration by such fluorescence polarisation measurements, add said anti-theophyllin FAB fragments. For each addition, wait until the fluorescence polarisation signal is stable, before adding more FAB fragments. . Continue the addition of FAB fragments until noe more significant increase of polarisation value is measured with increased concentration of FAB fragments. Record this value of fluorescence polarisation. Subtract the initial fluorescence polarisation value, and calculate the difference between the values, hereinafter called «polarisation value difference».

Start over again and make a final assay reagent identical to the tentative assay reagent, however, end the addition of said FAB fragments when the fluorescence polarisation value is equal to the initial polarisation value + 80 % of the «polarisation value difference».

The final assay reagent is now ready, and optionally add a suitable bacterostatic agentlike 0.01 % sodium azide for prolonged storage. Keep aliquots of this mixture, e.g. 1 ml, in separate containers to be combined with unknown blood samples or aliquots of blood samples.

At the time for the determination of concentration of theophyllin in blood samples, take an aliquot, e.g. a volume of 20 ul of the whole blood sample e.g. by the use of a pre-measured capillary, and combine thereafter the content of this pre-measured capillary with the assay reagent in the said separate container, the said separate container being kept in a place where the temperature is regulated to 32 degrees of Celcius. Typically, use a container as described in example 3. Thereafter, shake the container, and the blood flows out of the capillary and the cells are being lysed by the assay reagent. theophyllin of the test sample aliquot starts to displace the fluorescent theophyllin of the immunocomplexes of the assay reagent.

Immediately after the shaking, place the container in the fluorescence polariasation measurement instrument, having a temperature of 32 degrees of Celcius. For measurement of polarisation of the emitted fluorescence, the mixture in the container is irradiated with polarized light of 650 nm wavelength, typically by the use of a small polarized laser diode in the instrument, and when the polarisation of the emitted light constant, measure its polarisation a wavelength of 670 nm, with a rather narrow bandwidth, if the instruments allows for that. Calculate the concentration of theophyllin of the unknown sample by interpolation of the polarisation value of the emitted light measured on a standard curve obtained by measurement of standards of

known theophyllin concentration. Such a standard curve can often be stored on the computer of the measurement instrument, enabling a direct reading of the concentration of theophyllin on the instrument.

If a measurement instrument with a flow cell is used, then bring the mixture to pass through the flow cell for measurement, according to the instructions given by the instrument manufacturer.

If the measurement instrument allows it, much better precision is obtained if - instead of reading the polarisation after the polarisation value has become stable - the polarisation value as a function of time is measured in a kinetic manner. If available, connect the measurement instrument to a computer recording the signal continuously or at different time points, optionally defined by a software. Compare then the recorded values to values obtained by measurement of standards of known theophyllin concentration as a function of time. Optionally, record a three-dimensional standard curve with polarisation values, different times and theophyllin concentration values, and compare the values obtained with the unknown sample to calculate the theophyllin concentration, optionally by the use of the least-square methods for best fit according to standard textbooks of statistics, optionally by means of a computer or another artificial memory connected to the measurement instrument. Furthermore, if you have access to a temperature recording instrument keep the said separate containers (to be combined with unknown blood samples or aliquots of blood samples) at the room temperature, and use the temperature measurement device in or at the fluorescence polarisation instrument, and use a four-dimensional standard curve, the forth dimension being temperature, in addition to polarisation values, different times and theophyllin concentration values, and compare the values obtained with the unknown sample to calculate the theophyllin concentration, optionally by the use of the least-square methods for best fit according to standard textbooks of statistics, optionally by means of a computer or another artificial memory connected to the measurement instrument.

The concentration of theophyllin in blood samples varies significantly. The main interest is measurements of therapeutic concentration values. However, higher and toxic values are of interest in forensic medicine, and lower concentrations are of interests in sports medicine. In the performance of this example of the method of the present invention, please therefore adjust the concentration of the Cynanin-5 analogue of theophyllin, and hence the FAB fragment concentration. At high concentration ranges, FAB fragments of high dissociation rate constant must be chosen. At low concenration ranges, use FAB fragments with both high association rate constant and high dossociation rate constant.

Example 10: Fluorescent peptide for determination of concentration of albumin in urine.

Synthesise the peptide Asp-Ala-His-Lys-Ser-Glu-Val-Ala (the N-terminal peptide of human albumin) by conventional technique, and dissolve it in pure water to a concentration of 2 mg per ml. Mix 50 ul of the said peptide solution with 50 ul sodium bicarbonate buffer pH = 9.2. Mix 0.1 micromol 6-carboxyfluorescein-N-hydroxysuccinimide, optionally dissolved in water-free DMSO with said solution and leave it to stand overnight at room temperature in darkness, and thereafter store the solution in a refrigerator in darkness. If wanted, 5 ul of 10 mmolar ethanolamine solution can be added to block any remaining activated fluorescent dye, or the solution – herinafter called the stock solution - can be left to hydrolyse the un-reacted dye during storage.

Purify fluorescein labelled peptide from the stock solution by thin layer chromatography: Apply aliquots of stock solution on silica gel plates and elute with n-butanol:acetic acid:water in a mixture. Depending on the quality of the silica gel, the relative content of n-butanol:acetic acid:water can be adjusted to obtain ideal separation. After elution by conventional technique, dry the silica gel plate and inspect visually and by UV lamp (and optionally using nihydrin spray in parallell experiments) to identify the fluorescein labelled peptide spot, separated from non-labelled peptide and free fluorescin dye molecules. Isolate the silicagel comprising fluorescein labelled peptide by scissors or spatulum. Suspend the isolatedsilica gel in 50 mM BIS-TRIS-buffer pH = 8.0, whereby fluorescein labelled peptide is eluted into solution. The silica gel settles in the bottom of the tube. Decant off the TRIS-buffered solution with the purified fluorescein labelled peptide.

If wanted, and for upscaling, conventional HPLC separation techniques well known to the skilled man of the art can be used in stead of thin layer chromatography. E.g. Use the HPLC method described in example 1.

Example 11: Method to determine the concentration of albumin in urine by the use of a fluorescent immunocomplex.

Make anti- Asp-Ala-His-Lys-Ser-Glu-Val-Ala antibodies using conventional techniques well known to the skilled man of the art, e.g. by the use of an antigen formed by coupling of the peptide to keyholde limpet cyanin. Alternatively, purchase the peptide synthesis and the raising of antibodies from a service providing company, e.g. by Eurogenentech of Belgium. Make FAB fragments from the antibodies antibodies by the use the ImmnuPure Fab preparation kit supplied by Pirece Chemical Company, and follow the instruction for the said kit.

Make fluorescein labelled peptide as described in example 10. On selection of monoclonal antibody, see below.

Thereafter, make a tentative assay reagent at 32 degrees Celcius according to the following protocol: Make 150 mM sodium chloride 100 mM phosphate, pH = 7.4 . To this buffer, add bovine gammaglobulin from Sigma to a concentration of 2 mg/ml, and Triton X-100 from Pierce Chemical Company, US, to a final concentration of 0.01 % v/v. If necessary to remove protein reactive to the monoclonal antibody to be used (see below), pass the solution over an immunosorbent with anti-human albumin monoclonal antibodies, by the use of conventional immunoadsorbtion techniques well known to the skilled man of the art.

Reagents of high purity with very low background fluorescence should be chosen.

Then add said fluorescein labelled peptide to a final concentration 1.0*10E-9 mol per l. Measure the polarisation of the emitted fluorescence when the mixture in the container is irradiated with polarized light of 475 nm wavelength., and when the polarisation of the emitted light constant, measure its polarisation a wavelength of 525 nm, with a rather narrow bandwidth, typical of 10 nm, if the instruments allows for that. Under monitoration by such fluorescence polarisation measurements, add said anti-albumin FAB fragments. For each addition, wait until the fluorescence polarisation signal is stable, before adding more FAB fragments. Continue the addition of FAB fragments until no more significant increase of polarisation value is measured with increased concentration of FAB fragments. Record this value of fluorescence polarisation. Subtract the initial fluorescence polarisation value, and calculate the difference between the values, hereinafter called «polarisation value difference».

Start over again and make a final assay reagent identical to the tentative assay reagent, however, end the addition of said FAB anti-albumin fragments when the fluorescence polarisation value is equal to the initial polarisation value + 80 % of the «polarisation value difference».

The final assay reagent is now ready, and optionally add a suitable bacterostatic agent like 0.01 % sodium azide for prolonged storage. Keep aliquots of this mixture, e.g. 2 ml, in separate containers to be combined with unknown urine samples or aliquots of urine samples.

At the time for the determination of concentration of albumin in a urine samples, take an aliquot, e.g. a volume of 20 ul of the urine sample e.g. by the use of a pre-measured capillary, and combine thereafter the content of this pre-measured capillary with the assay reagent in the said separate container, the said separate container being kept in a place where the temperature is regulated to 32 degrees of Celcius.

Typically, use a container as described in example 3. Thereafter, shake the container, and the urine flows out of the capillary. Albumin of the test sample aliquot starts to displace the fluorescein labelled peptide of the immuno-complexes of the assay reagent.

Immediately after the shaking, place the container in the fluorescence polarisation measurement instrument, having a temperature of 32 degrees of Celcius. For measurement of polarisation of the emitted fluorescence, the mixture in the container is irradiated with polarized light of 475 nm wavelength, and when the polarisation of the emitted light constant, measure its polarisation a wavelength of 525 nm, with a rather narrow bandwidth, typically 10 nm, if the instruments allows for that. Calculate the concentration of human albumin of the unknown sample by interpolation of the polarisation value of the emitted light measured on a standard curve obtained by measurement of standards of known human albumin concentration. Such a standard curve can often be stored on the computer of the measurement instrument, enabling a direct reading of the concentration of albumin.

If a measurement instrument with a flow cell is used, then bring the mixture to pass through the flow cell for measurement, according to the instructions given by the instrument manufacturer.

If the measurement instrument allows it, much better precision is obtained if - instead of reading the polarisation after the polarisation value has become stable - the polarisation value as a function of time is measured in a kinetic manner. If available, connect the measurement instrument to a computer recording the signal continuously or at different time points, optionally defined by a software. Compare then the recorded values to values obtained by measurement of standards of known human albumin concentration as a function of time. Optionally, record a three-dimensional standard curve with polarisation values, different times and human albumin concentration values, and compare the values obtained with the unknown sample to calculate the human albumin concentration, optionally by the use of the least-square methods for best fit according to standard textbooks of statistics, optionally by means of a computer or another artificial memory connected to the measurement instrument. Furthermore, if you have access to a temperature recording instrument keep the said separate containers (to be combined with unknown blood samples or aliquots of blood samples) at the room temperature, and use the temperature measurement device in or at the fluorescence polarisation instrument, and use a four-dimensional standard curve, the forth dimension being temperature, in addition to polarisation values, different times and human albumin concentration values, and compare the values obtained with the unknown sample to calculate the human albumin concentration, optionally by the use of the least-square methods for best fit according to standard

textbooks of statistics, optionally by means of a computer or another artificial memory connected to the measurement instrument.

The concentration of albumin in urine samples varies significantly. Reference values are in the range of up to 20 mg per liter. Slightly elevated values of uncertain origin are found in some healthy individuals, individuals who may stay healthy to old ages. Moderate elevation of the albumin values are seen as early signs of diabetic kidney damage. In severe kidney disease, urine albumin concentration may rise to many hundred mgs per liter. In the performance of this example of the method of the present invention, please therefore adjust the concentration of the fluorescein labelled peptide , and hence the FAB fragment concentration. At high concentration ranges, FAB fragments of high dissociation rate constant must be chosen. At low concentration ranges, use FAB fragments with both high association rate constant and high dissociation rate constant.

Example 12: Method for measurement of Tobramycin in whole blood.

Synthesise RNA molecules which bind tobramycin is synthesised according to the article «RNA molecules that specifically and stoichiomterically bind aminoglycoside antibiotics with high affinities» by Wang & al., published in «Biochemistry» 1996, 35, 12338-12346.

Buy Tobramycin from Fluka. Dissolve 200 umoles Tobramycin in 500 ul water. Add 500 ul dimethylformamide. Cool the solution to 5 degrees Celcius. Equimolar concentration Cy5 Fluoro Link molecules in DMF solution is added, and the mixture is stirred at 5 degrees Celcius for 2 hours.

Elute the mixture CG 50 cathion exchange chromatography, with a gradient of 0 to 500 mM ammonium hydroxyde.

The desired conjugated is thereby isolated and lyophilized to remove the ammonium hydroxyde.

Make an assay buffer consisting of 140 mM NaCl, 5 mM KCl, 1 mM manganes chloride. 1 mM calcium chloride and 20 mM Hepes , and 3 mg bovine gamma globulin per ml, and pH = 7.4. Add 30 ng Tobramycin-Cy-5-conjugate per ml assay solution, and thereafter add an equimolare amount of the synthesised RNA molecules.

The final assay reagent is now ready, and optionally add a suitable bacterostatic agentlike 0.01 % sodium azide for prolonged storage. Keep aliquots of this mixture, e.g. 1 ml, in separate containers to be combined with unknown blood samples or aliquots of blood samples.

At the time for the determination of concentration of Tobramycin in blood samples, take an aliquot, e.g. a volume of 20 ul of the blood sample sample e.g. by the use of a pre-measured capillary, and combine thereafter the content of this pre-measured capillary with the assay reagent in the said separate container, the said separate container being kept in a place where the temperature is regulated to 32 degrees of Celcius. Typically, use a container as described in example 3. Thereafter, shake the container, and the blood flows out of the capillary. Tobramycin of the test sample aliquot starts to displace the Cy-5 conjugated Tobramycin of the RNA/Cy-5-Tobramycin complex of the assay reagent.

Immediately after the shaking, place the container in the fluorescence polarisation measurement instrument, having a temperature of 32 degrees of Celcius. For measurement of polarisation of the emitted fluorescence, the mixture in the container is irradiated with polarized light of 649 nm wavelength, and when the polarisation of the emitted light constant, measure its polarisation a wavelength of 670 nm, with a rather narrow bandwidth, typically 10 nm, if the instruments allows for that. Calculate the concentration of Tobramycin of the unknown sample by interpolation of the polarisation value of the emitted light measured on a standard curve obtained by measurement of standards of known human albumin concentration. Such a standard curve can often be stored on the computer of the measurement instrument, enabling a direct reading of the concentration of albumin.

If a measurement instrument with a flow cell is used, then bring the mixture to pass through the flow cell for measurement, according to the instructions given by the instrument manufacturer.

If the measurement instrument allows it, much better precision is obtained if - instead of reading the polarisation after the polarisation value has become stable - the polarisation value as a function of time is measured in a kinetic manner. If available, connect the measurement instrument to a computer recording the signal continuously or at different time points, optionally defined by a software. Compare then the recorded values to values obtained by measurement of standards of known Tobramycin concentration as a function of time. Optionally, record a three-dimensional standard curve with polarisation values, different times and Tobramycin concentration values, and compare the values obtained with the unknown sample to calculate the Tobramycin concentration, optionally by the use of the least-square methods for best fit according to standard textbooks of statistics, optionally by means of a computer or another artificial memory connected to the measurement instrument. Furthermore, if you have access to a temperature recording instrument keep the said separate containers (to be combined with unknown blood samples or aliquots of blood samples) at the room temperature, and use the temperature measurement device in or at the fluorescence polarisation instrument, and use a four-dimensional standard

curve, the forth dimension being temperature, in addition to polarisation values, different times and Tobramycin concentration values, and compare the values obtained with the unknown sample to calculate the Tobramycin concentration, optionally by the use of the least-square methods for best fit according to standard textbooks of statistics, optionally by means of a computer or another artificial memory connected to the measurement instrument.

The concentration of Tobramycin in blood samples varies significantly, dependant on for what the assay is used. Measurements of therapeutic concentrations varies with clinical indications, and measurements of blood concentrations in pharmacokintetic studies will be different. In the performance of this example of the method of the present invention, please therefore adjust the concentration of the Tobramycin-Cy-5-conjugate and RNA molecules to the appropriate level. The J6RNA RNA molecule published in «Biochemistry» 1996, 35, 12338-12346, is appropriate at low concentrations, other RNA molecules with lower affinity can be identified by the Selex technology and by synthesis methods well known to the skilled man of the art.

Example 13: Fluorescent insulin for determination of concentration of human auto-anti-insulin antibodies.

Synthesise human insulin by conventional technique, and dissolve it in pure water to a concentration of 2 mg per ml. Mix 50 ul of the said peptide solution with 50 ul sodium bicarbonate buffer pH = 9.2. Mix 0.2 Cy5 Fluorolink activated cyanin dye, supplied from Amersham Pharmacia Biotech, optionally dissolved in water-free DMSO with said solution and leave it to stand overnight at room temperature in darkness, and thereafter store the solution in a refrigerator in darkness. If wanted, 5 ul of 10 mmolar ethanolamine solution can be added to block any remaining activated fluorescent dye, or the solution – herinafter called the stock solution - can be left to hydrolyse the un-reacted dye during storage.

Purify Cy-5 labelled insulin from the stock solution by reverse phase chromatography in C4 column (sold from many suppliers, including Waters, U.S.), using 0.1 % trifluoroacetic acid as eluant and employ a gradiant of 0 % to 60 % acetonitrile in 0.1 % trifluoroacetic acid. Employ a photodetector coupled to a flow cell to monitor content of peptides by transmission measurements at 340 nm and Cy-5 by transmisson measurements at 650 nm, and isolate the Cy-5 –conjugated insulin. Remove the acetonotrile and the trifluoracetic acid by lyophilization.

Alternatively, other HPLC methods for isolation of peptide conjugates well known to the skilled man of the art may be chosen.

Eksempel 14: Method for measurement of anti-insulin antibodies in samples of whole blood.

Make an assay reagent by making a buffer of 150 mM sodium chloride 100 mM phosphate, pH = 7.4. To this buffer, add insulin free bovine gammaglobulin from Sigma to a concentration of 2 mg/ml, and Triton X-100 from Pierce Chemical Company, US, to a final concentration of 0.1 % v/v. Reagents of high purity with very low background fluorescence should be chosen. Add e.g. 1.0*10E-12 mol per ml (for choice of concenration, see below) of Cy5-labelled human insulin according to example 13 above, and optionally add a suitable bacterostatic agentlike 0.01 % sodium azide for prolonged storage. Keep aliquots of this mixture, e.g. 1 ml, in separate containers to be combined with unknown blood samples or aliquots of blood samples.

At the time for the determination of concentration of anti-insulin antibodies in a blood sample, take an aliquot, e.g. a volume of 20 ul of the whole blood sample e.g. by the use of a pre-measured capillary, and combine thereafter the content of this pre-measured capillary with the assay reagent in the said separate container, the said separate container being kept in a place where the temperature is regulated to 32 degrees of Celcius. Typically, use a container is in the form of a cuvette with 4 polished transparent sides to be measured in an instrument built for fluorescent polarisation measurements, but said container comprising a removable stopper or seal which allows the capillary to enter the container, either by simply dropping the capillary or by introducing it through the seal. Furthermore, in its preferred that the capillary/container is designed so that the capillary falls to the bottom of the container and does not interfere with the excitation light or the emission light (see below). Thereafter, shake the container, and the blood flows out of the capillary and the cells are being lysed by the assay reagent. Anti-insulin antibodies of the test sample aliquot starts to react with the Cy5-labelled insulin of the assay reagent.

Immediately after the shaking, place the container in the fluorescence polarisation measurement instrument, having a temperature of 32 degrees of Celcius. For measurement of polarisation of the emitted fluorescence, the mixture in the container is irradiated with polarized light of 650 nm wavelength, typically by the use of a small polarized laser diode in the instrument, and when the polarisation of the emitted light constant, measure its polarisation a wavelength of 670 nm, with a rather narrow bandwidth, preferentially band withs of 5 nm. Calculate the concentration of insulin of the unknown sample by interpolation of the polarisation value of the emitted light measured on a standard curve obtained by measurement of standards of known insulin concentration. Such a standard curve can often be stored on the computer of the measurement instrument, enabling a direct reading of the concentration of insulin on the instrument.

If a measurement instrument with a flow cell is used, then bring the mixture to pass through the flow cell for measurement, according to the instructions given by the

instrument manufacturer. Since insulin has a rather high molecular radius, a fluorescent polarisation instrument with a high precision in the measurement of the degree of polarisasion of the light is prefered.

If the measurement instrument allows it, much better precision is obtained if - instead of reading the polarisation after the polarisation value has become stable - the polarisation value as a function of time is measured in a kinetic manner. If available, connect the measurement instrument to a computer recording the signal continuously or at different time points, optionally defined by a software. Compare then the recorded values to values obtained by measurement of standards of known anti-insulin antibody concentration as a function of time. Optionally, record a three-dimensional standard curve with polarisation values, different times and insulin antibody concentration values, and compare the values obtained with the unknown sample to calculate the concentration of anti-insulin antibodies, optionally by the use of the least-square methods for best fit according to standard textbooks of statistics, optionally by means of a computer or another artificial memory connected to the measurement instrument. Furthermore, if you have access to a temperature recording instrument keep the said separate containers (to be combined with unknown blood samples or aliquots of blood samples) at the room temperature, and use the temperature measurement device in or at the fluorescence polarisation instrument, and use a four-dimensional standard curve, the forth dimension being temperature, in addition to polarisation values, different times and anti-insuli antibody concentration values, and compare the values obtained with the unknown sample to calculate the anti-insulin antibody concentration, optionally by the use of the least-square methods for best fit according to standard textbooks of statistics, optionally by means of a computer or another artificial memory connected to the measurement instrument.

Anti-insulin antibodies are typically present in very low concentrations values in healthy subjects. Diabetics in mild er early phases of the condition will typically have low antibodies towards insulin, while patients having received insulin treatment for many years typically will have very high concenrations of antibodies towards insulin. The concentration of the Cy-5-conjugated insulin and the total sample volume must therefore be chosen according to which kind of patients the sample is taken from. If the fluorescence polarisation measured is outside the standad curve obtained with the chosen concentration of Cy-5-insulin conjugate and sample volume, another concentration of Cy-5-insulin conjugate and sample volume must be chosen.

Eksempel 15: Determination of concentration in urine by means of a Ru-ligand immunocomplex.

According to this example it is shown that it is possible to increase the molecular weight of the binding partner(s) with the fluorescent residue(s) attached, in order to

determine the concentration of an analyte/analytes. However, as mentioned earlier, this requires the use of fluorescent residue(s) with longer decay time.

Make FAB-fragments from rabbit anti-human albumin antibodies, purchased from DakoAS, Denmark, using the ImmunoPure Fab preparation kit supplied by Pirece Chemical Company, and follow the instruction for the said kit.

Furthermore, make human serum albumin conjugate with Ru(bpy)dcbpy as described by Terpetsching & al. in Analytical Biochemistry 227, 140-147, 1995.

Thereafter, make a tentative assay reagent at 32 degrees Celcius according to the following protocol: Make 150 mM sodium chloride 100 mM phosphate, pH = 7.4 . To this buffer, add bovine gammaglobulin from Sigma to a concentration of 2 mg/ml, and Triton X-100 from Pierce Chemical Company, US, to a final concentration of 0.01 % v/v. Reagents of high purity with very low background fluorescence should be chosen.

Then add said human serum albumin conjugate with Ru(bpy)dcbpy to a final concentration 1.0*10E-9 mol per l. Measure the polarisation of the emitted fluorescence when the mixture in the container is irradiated with polarized light of 485 (alternatively 360, see the said article of Terpetsching) nm wavelength., and when the polarisation of the emitted light constant, measure its polarisation a wavelength of 660 nm, with a rather narrow bandwidth, typical of 10 nm, if the instruments allows for that. Under monitoration by such fluorescence polarisation measurements, add said anti-albumin FAB fragments. For each addition, wait until the fluorescence polarisation signal is stable, before adding more FAB fragments. Continue the addition of FAB fragments until no more significant increase of polarisation value is measured with increased concentration of FAB fragments. Record this value of fluorescence polarisation. Subtract the initial fluorescence polarisation value, and calculate the difference between the values, hereinafter called «polarisation value difference».

Start over again and make a final assay reagent identical to the tentative assay reagent, however, end the addition of said FAB anti-albumin fragments when the fluorescence polarisation value is equal to the initial polarisation value + 80 % of the «polarisation value difference».

The final assay reagent is now ready, and optionally add a suitable bacterostatic agent like 0.01 % sodium azide for prolonged storage. Keep aliquots of this mixture, e.g. 2 ml, in separate containers to be combined with unknown urine samples or aliquots of urine samples.

At the time for the determination of concentration of albumin in a urine samples, take an aliquot, e.g. a volume of 20 ul of the urine sample e.g. by the use of a pre-measured capillary, and combine thereafter the content of this pre-measured capillary with the assay reagent in the said separate container, the said separate container being kept in a place where the temperature is regulated to 32 degrees of Celcius. Typically, use a container as described in example 3. Thereafter, shake the container, and the urine flows out of the capillary. Albumin of the test sample aliquot starts to displace the human serum albumin conjugate with Ru(bpy)dcbpy of the immuno-complexes of the assay reagent.

Immediately after the shaking, place the container in the fluorescence polarisation measurement instrument, having a temperature of 32 degrees of Celcius. For measurement of polarisation of the emitted fluorescence, see above in this xample. Calculate the concentration of human albumin of the unknown sample by interpolation of the polarisation value of the emitted light measured on a standard curve obtained by measurement of standards of known human albumin concentration. Such a standard curve can often be stored on the computer of the measurement instrument, enabling a direct reading of the concentration of albumin.

If a measurement instrument with a flow cell is used, then bring the mixture to pass through the flow cell for measurement, according to the instructions given by the instrument manufacturer.

If the measurement instrument allows it, much better precision is obtained if - instead of reading the polarisation after the polarisation value has become stable - the polarisation value as a function of time is measured in a kinetic manner. If available, connect the measurement instrument to a computer recording the signal continuously or at different time points, optionally defined by a software. Compare then the recorded values to values obtained by measurement of standards of known human albumin concentration as a function of time. Optionally, record a three-dimensional standard curve with polarisation values, different times and human albumin concentration values, and compare the values obtained with the unknown sample to calculate the human albumin concentration, optionally by the use of the least-square methods for best fit according to standard textbooks of statistics, optionally by means of a computer or another artificial memory connected to the measurement instrument. Furthermore, if you have access to a temperature recording instrument keep the said separate containers (to be combined with unknown blood samples or aliquots of blood samples) at the room temperature, and use the temperature measurement device in or at the fluorescence polarisation instrument, and use a four-dimensional standard curve, the forth dimension being temperature, in addition to polarisation values, different times and human albumin concentration values, and compare the values obtained with the unknown sample to calculate the human albumin concentration,

optionally by the use of the least-square methods for best fit according to standard textbooks of statistics, optionally by means of a computer or another artificial memory connected to the measurement instrument.

The concentration of albumin in urine samples varies significantly. Reference values are in the range of up to 20 mg per liter. Slightly elevated values of uncertain origin are found in some healthy individuals, individuals who may stay healthy to old ages. Moderate elevation of the albumin values are seen as early signs of diabetic kidney damage. In severe kidney disease, urine albumin concentration may rise to many hundred mgs per liter. In the performance of this example of the method of the present invention, please therefore adjust the concentration of the human serum albumin conjugate with Ru(bpy)dcbpy, and hence the FAB fragment concentration. At high concentration ranges, FAB fragments of high dissociation rate constant must be chosen. At low concentration ranges, use FAB fragments with both high association rate constant and high dissociation rate constant.

Example 16. Method for measurement of blood theophyllin by the use of an aptamer complex.

Synthesise the RNA aptamer which binds to theophyllin with an association afinity constant of 1.0 * 10E7 /molar, described by R.D. Jenison & al in Science, vol 263, 1994.

Synthesise Cyanin-5 analouge of theophyllin according to example 7.

Make an assay buffer consisting of 140 mM NaCl, 5 mM KCl, 1 mM manganes chloride. 1 mM calcium chloride and 20 mM Hepes , and 3 mg bovine gamma globulin per ml, and pH = 7.4. Add 2 mg Cyanin-5 analouge of theophyllin per l assay solution, and thereafter add an equimolare amount of the synthesised RNA molecules.

The final assay reagent is now ready, and optionally add a suitable bacterostatic agentlike 0.01 % sodium azide for prolonged storage. Keep aliquots of this mixture, e.g. 1 ml, in separate containers to be combined with unknown blood samples or aliquots of blood samples.

At the time for the determination of concentration of theophyllin in blood samples, take an aliquot, e.g. a volume of 20 ul of the blood sample sample e.g. by the use of a pre-measured capillary, and combine thereafter the content of this pre-measured capillary with the assay reagent in the said separate container, the said separate container being kept in a place where the temperature is regulated to 32 degrees of Celcius. Typically, use a container as described in example 3. Thereafter, shake the container, and the blood flows out of the capillary. Theophyllin of the test sample

aliquot starts to displace the Cyanin-5 analouge of theophyilin of the RNA/ Cyanin-5 analouge of theophyllin complex of the assay reagent.

Immediately after the shaking, place the container in the fluorescence polarisation measurement instrument, having a temperature of 32 degrees of Celcius. For measurement of polarisation of the emitted fluorescence, the mixture in the container is irradiated with polarized light of 649 nm wavelength, and when the polarisation of the emitted light constant, measure its polarisation a wavelength of 670 nm, with a rather narrow bandwidth, typically 10 nm, if the instruments allows for that. Calculate the concentration of Theophyllin of the unknown sample by interpolation of the polarisation value of the emitted light measured on a standard curve obtained by measurement of standards of known human albumin concentration. Such a standard curve can often be stored on the computer of the measurement instrument, enabling a direct reading of the concentration of Theophyllin.

If a measurement instrument with a flow cell is used, then bring the mixture to pass through the flow cell for measurement, according to the instructions given by the instrument manufacturer.

If the measurement instrument allows it, a much better precision is obtained if - instead of reading the polarisation after the polarisation value has become stable - the polarisation value as a function of time is measured in a kinetic manner. If available, connect the measurement instrument to a computer recording the signal continuously or at different time points, optionally defined by a software. Compare then the recorded values to values obtained by measurement of standards of known theophyllin concentration as a function of time. Optionally, record a three-dimensional standard curve with polarisation values, different times and theophyllin concentration values, and compare the values obtained with the unknown sample to calculate the theophyllin concentration, optionally by the use of the least-square methods for best fit according to standard textbooks of statistics, optionally by means of a computer or another artificial memory connected to the measurement instrument. Furthermore, if you have access to a temperature recording instrument keep the said separate containers (to be combined with unknown blood samples or aliquots of blood samples) at the room temperature, and use the temperature measurement device in or at the fluorescence polarisation instrument, and use a four-dimensional standard curve, the forth dimension being temperature, in addition to polarisation values, different times and theophyllin concentration values, and compare the values obtained with the unknown sample to calculate the theophyllin concentration, optionally by the use of the least-square methods for best fit according to standard textbooks of statistics, optionally by means of a computer or another artificial memory connected to the measurement instrument.

The concentration of theophyllin in blood samples varies significantly. The main interest is measurements of therapeutic concentration values. However, higher and toxic values are of interest in forensic medicine, and lower concentrations are of interests in sports medicine. In the performance of this example of the method of the present invention, please therefore adjust the concentration of the Cynanin-5 analogue of theophyllin, and hence the RNA aptamer concentration.

Example 17: Simultaneous measurement of human choriongonadotropin and albumin in urine.

Purchase human beta-subunit choriongonadotropin (BHCG) from Sigma, and further purify it by size exclusion chromatography with mehods well known to the skilled man of the art, using 10 mM phosphate buffer pH = 7.2 witm 0.15 M sodium chlorid as eluant. Label the human beta-subunit of choriongonadotropin with Cy-5 Fluorolink following the package insert of Amerham Pharmacia Biotech, and measure the content of Cy-5 per molecule BHCG according to the same method after purification by size exclution chromatography, as recommended by Amersham Pharmacia Biotech.

Purchase monoclonal anti-BHCG from Chemicon Inc, US, and prepare FAB fragments from the antibodies according to the ImmunoPure FAB preparation kit from Pierce Cemical Company.

Make anti- Asp-Ala-His-Lys-Ser-Glu-Val-Ala antibodies using conventional techniques well known to the skilled man of the art, e.g. by the use of an antigen formed by coupling of the peptide to keyholde limpet cyanin. Alternatively, purchase the peptide synthesis and the raising of antibodies from a service providing company, e.g. by Eurogenentech of Belgium. Make FAB fragments from the antibodies antibodies by the use the ImmnuPure Fab preparation kit supplied by Pirece Chemical Company, and follow the instruction for the said kit.

Make fluorescein labelled peptide as described in example 10, and the reagent for determination of albumin in urine according to example 11. Thereafter, add to the said reagent for determination of urine albumin, Cy5-labelled BHCG 1/100 of molecular concentration of the BHCG level intended to measure in urine. (A suitable level to could be levels corresponding to 3000 U per l).

On selection of monoclonal antibody f, see below.

Measure the polarisation of the emitted fluorescence when the mixture in the container is irradiated with polarized light of 550 nm wavelength., and when the polarisation of the emitted light constant, measure its polarisation a wavelength of 570 nm, with a rather narrow bandwidth, typical of 10 nm, if the instruments allows for that. Under monitoration by such fluorescence polarisation measurements, add

said anti-BHCG FAB fragments. For each addition, wait until the fluorescence polarisation signal is stable, before adding more FAB fragments. Continue the addition of FAB fragments until no more significant increase of polarisation value is measured with increased concentration of FAB fragments. Record this value of fluorescence polarisation. Subtract the initial fluorescence polarisation value, and calculate the difference between the values, hereinafter called «polarisation value difference».

Start over again and make a final assay reagent identical to the tentative assay reagent, however, end the addition of said FAB anti-BHCG fragments when the fluorescence polarisation value is equal to the initial polarisation value + 80 % of the «polarisation value difference».

The final assay reagent is now ready, and optionally add a suitable bacterostatic agent like 0.01 % sodium azide for prolonged storage. Keep aliquots of this mixture, e.g. 2 ml, in separate containers to be combined with unknown urine samples or aliquots of urine samples.

At the time for the determination of concentration of albumin and HCG in a urine samples, take an aliquot, e.g. a volume of 20 ul of the urine sample e.g. by the use of a pre-measured capillary, and combine thereafter the content of this pre-measured capillary with the assay reagent in the said separate container, the said separate container being kept in a place where the temperature is regulated to 32 degrees of Celcius. Typically, use a container as described in example 3. Thereafter, shake the container, and the urine flows out of the capillary. Albumin of the test sample aliquot starts to displace the fluorescein labelled insulin-like peptide of the fluorescein-labelled immuno-complexes of the assay reagent. HCG starts to displace Cy-5-labelled BHCG in the Cy-5 –labelled immunocomplexes of the assay reagent.

Immediately after the shaking, place the container in the fluorescence polarisation measurement instrument, having a temperature of 32 degrees of Celcius. For measurement of polarisation of the emitted fluorescence, the mixture in the container is irradiated with polarized light of 475 nm and 650 nm wavelengths, and when the polarisation of the emitted light constant, measure its polarisation of emitted light at the wavelengths of 525 nm and 670 nm, with a rather narrow bandwidth, typically 10 nm, if the instruments allows for that. A rather advances dual wavelength irradiation instrument with high precision of polarisation measurements is necessary, because of the overlap of the spectra and the relatively high molecular weight of Cy-5-labelled BHCG. It is preferred to use an instrument which intermittently irradiates at 475 nm and 650 nm, to avoid much spectral interference. Calculate the concentration of human albumin and HCG of the unknown sample by interpolation of the polarisation values of the emitted light at both 525 and 670 nm, measured on a «standard curve»

obtained by measurement of standards of known human albumin and HCG concentrations. Such a standard curve will be four dimensional since the polarization at both wavelengths will vary with concentrations of both albumin and of HCG. It should be stored stored on the computer of the measurement instrument, enabling a direct calculation of the concentration of albumin and HCG.

If a measurement instrument with a flow cell is used, then bring the mixture to pass through the flow cell for measurement.

If the measurement instrument allows it, much better precision is obtained if - instead of reading the polarisation after the polarisation value has become stable - the polarisation value as a function of time is measured in a kinetic manner. If available, connect the measurement instrument to a computer recording the signal continuously or at different time points, optionally defined by a software. Compare then the recorded values to values obtained by measurement of standards of known human albumin and HCG concentration as a function of time. Optionally, record a data set with polarisation values at the two wave-lengths, different times and different human albumin and HCG concentration values, and compare the values obtained with the unknown sample to calculate the human albumin concentration, optionally by the use of the least-square methods for best fit according to standard textbooks of statistics, optionally by means of a computer or another artificial memory connected to the measurement instrument. Furthermore, if you have access to a temperature recording instrument keep the said separate containers (to be combined with unknown blood samples or aliquots of blood samples) at the room temperature, and use the temperature measurement device in or at the fluorescence polarisation instrument, and use an even larger data set with different temperatures included. optionally by the use of the least-square methods for best fit according to standard textbooks of statistics, optionally by means of a computer or another artificial memory connected to the measurement instrument.

The concentration of albumin in urine samples varies significantly. Reference values are in the range of up to 20 mg per liter. Slightly elevated values of uncertain origin are found in some healthy individuals, individuals who may stay healthy to old ages. Moderate elevation of the albumin values are seen as early signs of diabetic kidney damage. In severe kidney disease, urine albumin concentration may rise to many hundred mgs per liter. In the performance of this example of the method of the present invention, please therefore adjust the concentration of the fluorescein labelled peptide , and hence the FAB fragment concentration. At high concentration ranges, FAB fragments of high dissociation rate constant must be chosen. At low concentration ranges, use FAB fragments with both high association rate constant and high dissociation rate constant.

Correspondingly, concentration of HCG differ with certain diseases, stage of pregnancy, and obviously with sex. Similar consideration as with the anti-peptide antibody fragment must be used with the anti- BHCG antibodies from which to prepare FAB fragments.

There is an interest to determine both urine HCG and urine albumin in pregnant women.

Example 18: Simultaneous measurement of gonadotropin, albumin and immunoglobulin G in human urine.

One of the main functions of the kidneys is to excrete urea and other small molecular subtances but retain albumin and other proteins in blood (in addition to many other functions). In pregnant women with proteinuria, there is an interest in evaluating the kidneys selectivity between smaller and larger proteins. Simultaneous measurement of albumin and immunoglobulin G is used to assess such selectivity. Normally, the urine contains only trace amounts of albumin and immunoglobulins, but at least 10 timer more albumin than immunoglobulin. With severe impairment of the renal function, such selecively is usually lost.

To obtain a suitable reagent for Simultaneous measurement of gonodotropin, albumin and immunoglobulin G in human urine, use the following protocol:

Use the COSMIX plexing phage display technology referred to above to identify a peptide that binds selectively to Immunoglobulin class G molecules. This is a service that can be bought from the COSMIX GmbH company. Alternatively, digest Protein A into peptide fragments, and identify and purify a IgG binding peptide from the digest, using conventional techniques well known to the art, e.g. as described in Yue et al in The Journal of Biological Chemistry vol 271, p. 22245-22250, 1996. Label the identified and purified peptide with 5-caboxytetramethylrhodamine succidinimidyl ester purchased from Molecular Probes, Inc, using conventional techniques as described in the package insert from aid company. Purify the tetramethylrhodamine – labelled peptide by reversed phase chromatography using conventional techniques described in other examples above.

To the reagent described in example 17, add said tetramethylrhodamine –labelled peptide. If the reagent is intended to measure albumin concentrations e.g. in the concentration range of 50 to 500 mg per liter, add tetramethylrhodamine –labelled peptide to a final concentration of 0.05 micromoles per liter. In this way an impairment of selectivity will be shown as increased binding of IgG to tetramethylrhodamine-peptide.

The final assay reagent is now ready, and optionally add a suitable bacterostatic agent like 0.01 % sodium azide for prolonged storage. Keep aliquots of this mixture, e.g. 2 ml, in separate containers to be combined with unknown urine samples or aliquots of urine samples.

At the time for the determination of concentration of albumin, IgG and HCG in a urine samples, take an aliquot, e.g. a volume of 20 ul of the urine sample e.g. by the use of a pre-measured capillary, and combine thereafter the content of this pre-measured capillary with the assay reagent in the said separate container, the said separate container being kept in a place where the temperature is regulated to 32 degrees of Celcius. Typically, use a container as described in example 3. Thereafter, shake the container, and the urine flows out of the capillary. Albumin of the test sample aliquot starts to displace the fluorescein labelled insulin-like peptide of the fluorescein-labelled immuno-complexes of the assay reagent. HCG starts to displace Cy-5-labelled BHCG in the Cy-5 –labelled immunocomplexes of the assay reagent. IgG starts to bind to the rhodamine-labelled peptide.

Immediately after the shaking, place the container in the fluorescence polarisation measurement instrument, having a temperature of 32 degrees of Celcius. For measurement of polarisation of the emitted fluorescence, the mixture in the container is irradiated with polarized light of 475 nm, 550 nm and 650 nm wavelengths, using a fluorescence polarization measurement instrument constructed to irradiate the three different wavelengths intermittantly. When the polarisation of the emitted light for each of the different irradiation wavelengths is constant, measure the polarisation of emitted light at the wavelengths of 525 nm, 582 and 670 nm, with a rather narrow bandwidth, typically 10 nm, if the instruments allows for that. A rather advanced irradiation instrument with high precision of polarisation measurements is necessary, because of the overlap of the spectra and the relatively high molecular weight of Cy-5-labelled BHCG. Calculate the concentration of human albumin, IgG and HCG of the unknown sample by interpolation of the polarisation values of the emitted light at both 525, 582 and 670 nm, measured on a «standard curve» obtained by measurement of standards of known human albumin, IgG and HCG concentrations. Such a standard curve will be multi dimensional since the polarization at all three , wavelengths will vary with concentrations of both albumin, IgG and of HCG. It should be stored on the computer of the measurement instrument, enabling a direct calculation of the concentration of albumin and HCG.

If a measurement instrument with a flow cell is used, then bring the mixture to pass through the flow cell for measurement.

If the measurement instrument allows it, much better precision is obtained if - instead of reading the polarisation after the polarisation values have become stable - the

polarisation values as a function of time is measured in a kinetic manner. If available, connect the measurement instrument to a computer recording the signal continuously or at different time points, optionally defined by a software. Compare then the recorded values to values obtained by measurement of standards of known human albumin, IgG and HCG concentration as a function of time. Optionally, record a data set with polarisation values at the three wave-lengths, different times and different human albumin, IgG and HCG concentration values, and compare the values obtained with the unknown sample to calculate the human albumin concentration, optionally by the use of the least-square methods for best fit according to standard textbooks of statistics, optionally by means of a computer or another artificial memory connected to the measurement instrument. Furthermore, if you have access to a temperature recording instrument keep the said separate containers (to be combined with unknown blood samples or aliquots of blood samples) at the room temperature, and use the temperature measurement device in or at the fluorescence polarisation instrument, and use an even larger data set with different temperatures included, optionally by the use of the least-square methods for best fit according to standard textbooks of statistics, optionally by means of a computer or another artificial memory connected to the measurement instrument. In a multi-analyte assay as described, a rather high computing capacity is necessary, but such computing capacity is today easily available, and use soft-ware programs for analysis and calculations of several unknown parameters is preferred, e.g. the use of the Unscrambler prgram, delivered by the CAMO company, Oslo, Norway.

CLAIMS

1. A method for determination of concentration of one or more analytes in a test sample or an aliquot of a test sample of a complex biological a fluid, characterised by
a) mixing the said sample or aliquot of the said sample with one single reagent, such as a solid, a solution or premixed solution, wherein said reagent being provided in one single container or compartment of a container, and no other reagent is added during the performance of said method, and said reagent further comprises at least one type of binding molecule with specific affinity for one or more of the said analytes, and said reagent furthermore comprises either fluorescent moieties covalently linked to the said binding molecules or fluorescent analogues of or fluorescent fragments of or fluorescent derivatives of said analyte or analytes, and
b) said mixing resulting in a mixture which is being irradiated with polarized light which permits the exitation of said fluorescent molecules, and
c) measuring the polarisation of the emitted light, and
d) calculating the concentration or concentrations of said analyte or analytes.

2. A method according to claim 1,
characterised by using a reagent for each analyte comprising immmunocomplexes between
a) an antibody or an immunoactive fragment of an antibody with specific affinity for said analyte or analytes, and
b) fluorescent analogues or fluorescent fragments of or fluorescent derivatives of said analyte or analytes.

3. A method according to claim 1,
characterised by using a reagent for each analyte comprising complexes between
a) an aptamer or another synthetic binder with a specific affinity for said analyte, and
b) fluorescent analogues or fluorescent fragments of or fluorescent derivatives of said analyte or analytes.

4. A method according to claim 1,
characterised by using a reagent comprising binding molecules with specific affinity for one or more of the said analytes and with fluorescent moieties with absorption maximum between 600 nm and 1000 nm, preferably above 620 nm, covalently linked to the said binding molecules, and said binding molecules being either a peptide or being synthetic binders, optionally being identified by combinatory chemistry techniques or phage display or nucleic acid selection technology.

5. A method according to any of the claims 1 to 4,
characterised by using a reagent comprising fluorescent binding molecules with

specific affinity for one analyte, or comprising fluorescent analogues of, or fluorescent fragments of, or fluorescent derivatives of one analyte only.

6. A method according to any of the claims 1 to 5,
characterised by the use of a reagent comprising different fluorescent moieties covalently bound to different binding molecules with different specific affinities.

7. A method according to any of the claims 1 to 6,
characterised by the use of a reagent comprising one or more peptides or derivatives of peptides with specific binding affinity for an analyte, said binding peptides having a fluorescent residue covalently linked and being constituted by less than 30 amino acids.

8. A method according to claim 7,
characterised in that binding peptide is constituted by less than 20 amino acids.

9. A method according to claim 8,
characterised in that binding peptide is constituted by less than 15 amino acids.

10. A method according to any of the claims 1 to 9,
characterised by the use of a reagent comprising peptids or derivatives of peptids containing amino acid sequence Ala-Arg-Asn-Arg-Asn or Ala-Arg-Asn-Gly-Asn for quantitation of C-reactive protein.

11. A method according to any of the claims 1 to 10,
characterised by the use of a reagent with fluorescent residues with maximum coefficient of absorption at a wavelength above 640 nm.

12. A method according to any of the claims 1 to 11,
characterised by the use of a reagent comprising cell lysing substances or anti-coagulants or detergents.

13. A method according to any of the claims 1 to 12,
characterised by the use of a reagent comprising one or more fluorescent moieties selected from the group consisting of fluoresceine, Texas Red, Cy5, other Cy Dye FluorLink substances, other Cyanin derivatives, Rhodamin, Methyl Rhodamin, Biodypi 630/650-X/MeOH, Biodypi 650/655-X/MeOH, Biodypi FL/MeOH, Biodypi R6G/MeOH, Biodypi TMR-X/MeOH Biodypi TR-X/MeOH or other substances from the Biodipy group of substances, Alexa Fluor Dyes of different wavelengths, Ruthenium ligand complexes, lanthanoid elements such as Europium, Samarium or Terbium complex bound to a chelating ligand like DTPA, EDTA or N1.

14. A method according to any of the claims 1 to 13,
characterised by that the polarisation of the emitted light is measured as a function of

time, either as a continuous kinetic reading or a reading of the change in polarisation of the emitted light between two or more time points, or as a measurement of the polarisation of the emitted light after a defined point of time.

15. A method according to any of the claims 1 to 14,
characterised by that sample material or aliquot of the sample material is constituted by a biological material, or a dilution or an extract or being dissolved from or being filtrated from the said biological material.

16. A method according to any of the claims 1 to 15,
characterised by that sample material or aliquot of the sample material is constituted by blood, or blood serum, or blood plasma, or blood cells, or lysate from blood or blood cells, or urine, or cerebrospinal fluid, or tear liquid, or sputum, or semen, or plasma, or semen or material aspirated from the gastro-intestinal tract or feces, or extract or filtrate of suspension of feces, or plant material or extracts thereof, or dissolved plant material or filtrate thereof.

17. A method according to any of the claims 1 to 16,
characterised by the use of standards or calibrators comprising known concentrations of the analyte or the analytes, and furthermore wherein the concentration or concentrations of said analyte or analytes in unknown samples is calculated by interpolation of the values obtained from the unknown samples on the standard curve obtained from said known standards or calibrators.

18. A method according to any of the claims 1 to 17,
characterised by the use of a standard curve stored in an artificial memory, optionally connected to the fluorescent polarisation instrument in use.

19. A method according to any of the claims 1 to 18,
characterised by the use of temperature correction algorithms, either generated empirically or theoretically, to compensate for differences in fluorescence polarisation caused by differences in temperature at different time of measurements of standards and unknown samples, or between standards, or between unknown samples.

20. A method according to any of the claims 1 to 19,
characterised by being provided in concentrated or dry form, to be diluted or reconstituted before use, the said reagent being provided divided between different compartments for combination into one reagent prior to use.

21. A reagent for the performance of the method according to any of the claims 1 to 20,
characterised in that said reagent comprises at least one type of binding molecule with specific affinity for one or more of the said analytes, and said reagent

furthermore comprises fluorescent moities covalently linked to the said binding molecules or fluorescent analogues of or fluorescent fragments of or fluorescent derivatives of said analyte or analytes.

22. A reagent according to claim 21,
characterised in that the reagent comprises complexes between
a) an antibody or an immunoactive fragment of an antibody or an aptamer or a synthetic binder with specific affinity for at least one analyte and
b) fluorescent analogues or fluorescent fragments of or fluorescent derivatives of said analyte or analytes.

23. A reagent according to claims 21 to 22,
characterised in comprising binding molecules with specific affinity for one or more of the said analytes and optionally with fluorescent moities with absorption maximum between 600 nm and 1000 nm, preferably exceeding 620 nm, more preferably exceeding 640 nm, covalently linked to the said binding molecules, and said binding molecules being either of peptide or aptamer composition or being synthetic binders, optionally being identified by combinatory chemistry techniques or phage display or nucleic acid selection technology.

24. A reagent according to claims 21 to 23,
characterised in being an assay reagent comprising peptid binders or binders of derivatives of peptids, including fluorescent derivatives of said binders, containing the amino acid sequence Ala-Arg-Asn-Arg-Asn and/or Ala-Arg-Asn-Gly-Asn.

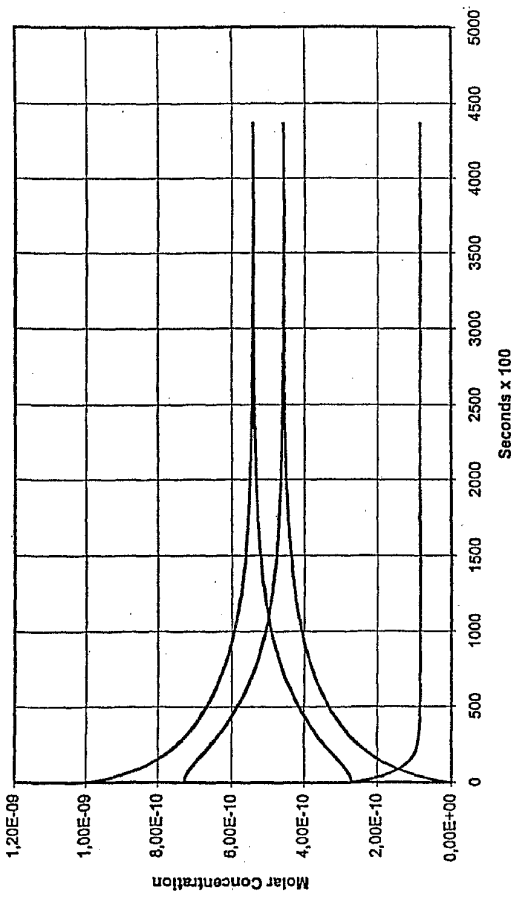
25. Use of the method according to claims 1 to 20 to determine concentrations of clinically related substances in samples of biological material from living organisms in need thereof.

26. Kit for the determination of concentration of one or more analytes in a test sample or an aliquot of a test sample of complex biological fluid,
characterized in comprising one or more containers, wherein the container(s) or compartment of the container(s) contains one single reagent, preferably in the fluidal state and according to any of the claims 21-24, and wherein the reagent comprises one or more fluorescense-labelled spesific binding molecules towards the analyte(s) to be measured, or a fluorescence-labelled analogue or a fluorescent fragment or a eluorescent derivative of said analyte(s), as well as device for obtaining the exact volume(s) of the complex biological fluid to be tested and that is needed in order to perform the method adequately.

27. Kit according to claim 26,
characterized in that the reagent which is contained in a container or a compartment of a container, is formed to a ready-for-use reagent by mixing the content from

different containers prior to or immediately prior to or in connection with the execution of the analysis.

Figure 1


Molar Concentration
1,20E-09
1,00E-09
8,00E-10
6,00E-10
4,00E-10
2,00E-10
0,00E+00
Seconds x 100
0
500
1000
1500
2000
2500
3000
3500
4000
4500
5000

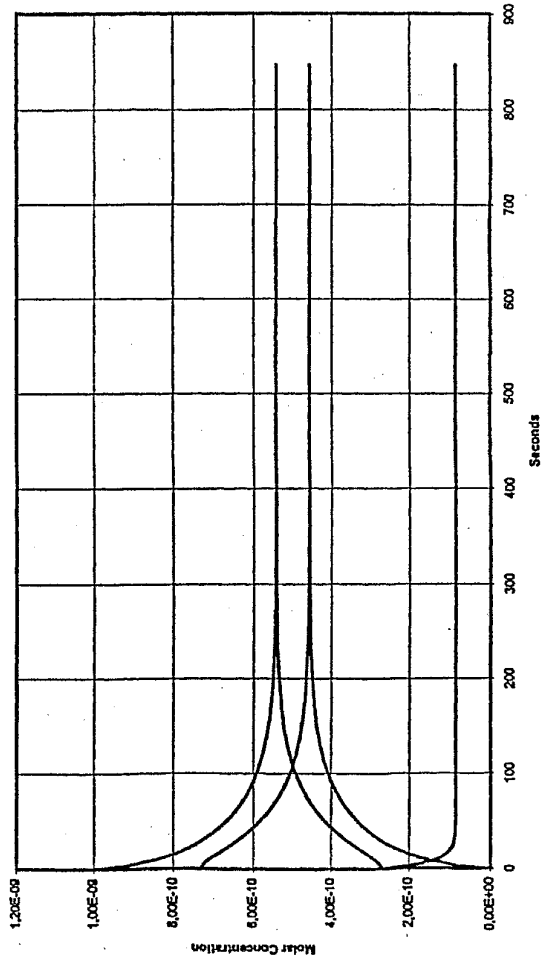

1,20E-09
1,00E-09
8,00E-10
6,00E-10
4,00E-10
2,00E-10
0,00E+00
Molar Concentration
0
100
200
300
400
500
600
700
800
900
Seconds

Figure 2

Figure 3

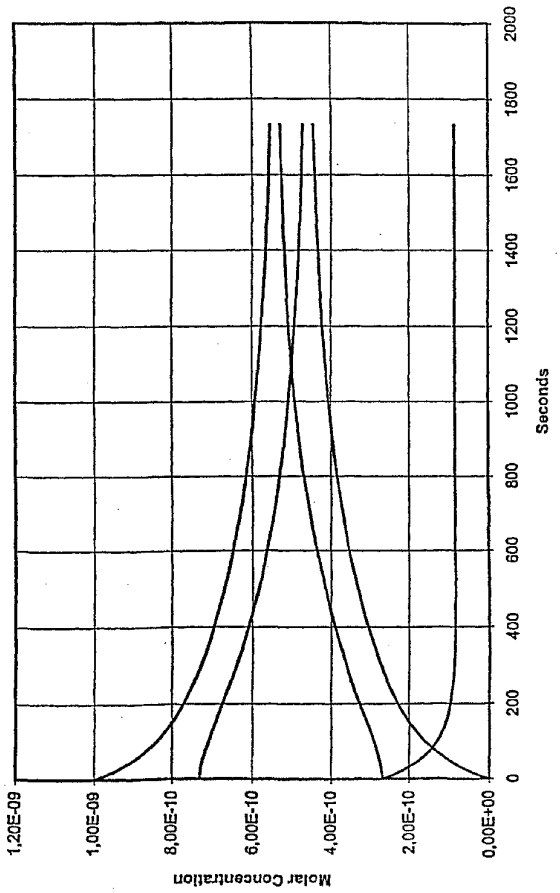

Molar Concentration
1,20E-09
1,00E-09
8,00E-10
6,00E-10
4,00E-10
2,00E-10
0,00E+00
Seconds
0
200
400
600
800
1000
1200
1400
1600
1800
2000

Figure 4

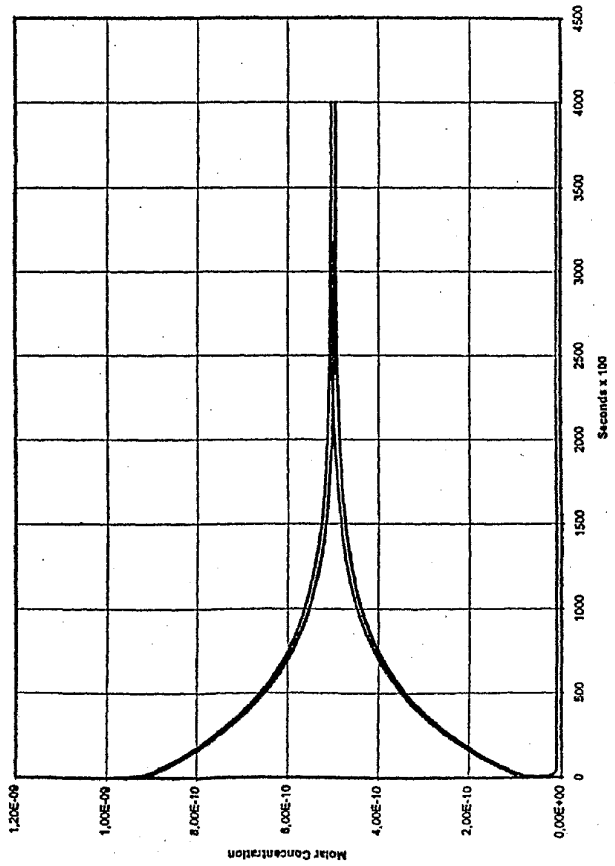
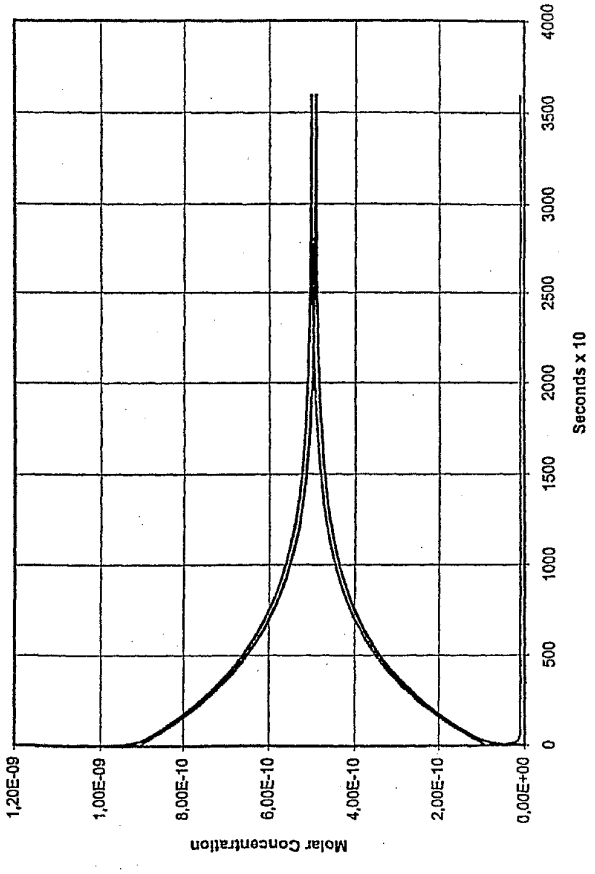

Seconds x 100
0
500
1000
1500
2000
2500
3000
3500
4000
4500
Molar Concentration
1,20E-09
1,00E-09
8,00E-10
6,00E-10
4,00E-10
2,00E-10
0,00E+00

Figure 5


Molar Concentration
1,20E-09
1,00E-09
8,00E-10
6,00E-10
4,00E-10
2,00E-10
0,00E+00
Seconds x 10
0
500
1000
1500
2000
2500
3000
3500
4000

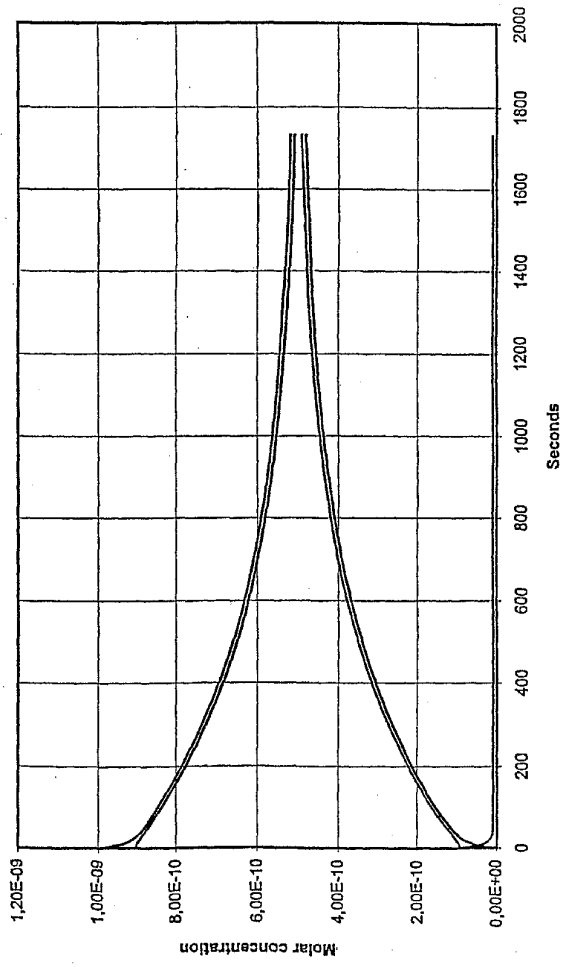

Seconds
0
200
400
600
800
1000
1200
1400
1600
1800
2000
Molar concentration
1,20E-09
1,00E-09
8,00E-10
6,00E-10
4,00E-10
2,00E-10
0,00E+00

Figure 6

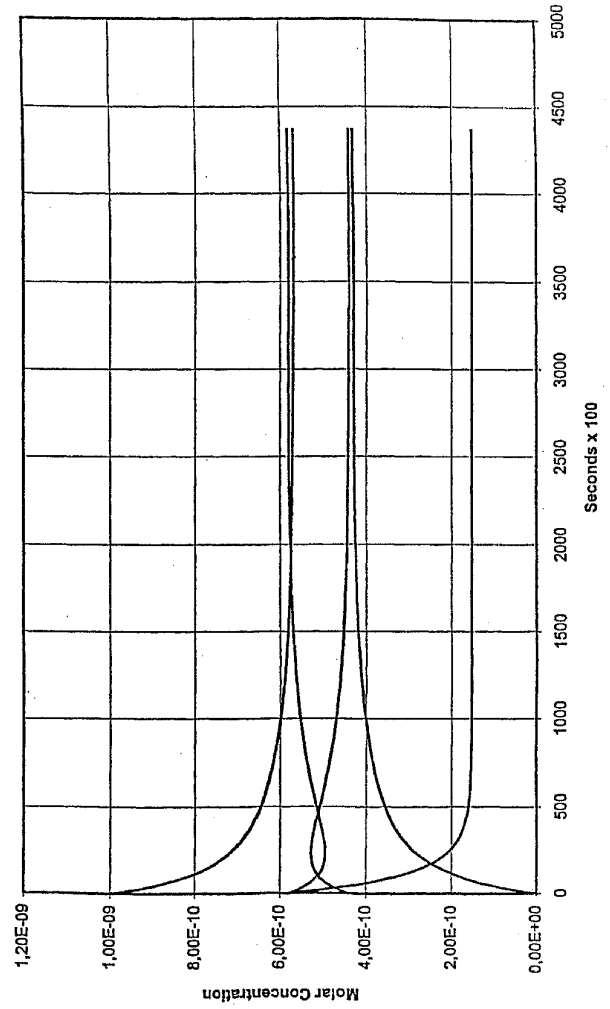

Seconds x 100
0
500
1000
1500
2000
2500
3000
3500
4000
4500
5000
Molar Concentration
1,20E-09
1,00E-09
8,00E-10
6,00E-10
4,00E-10
2,00E-10
0,00E+00

Figure 7

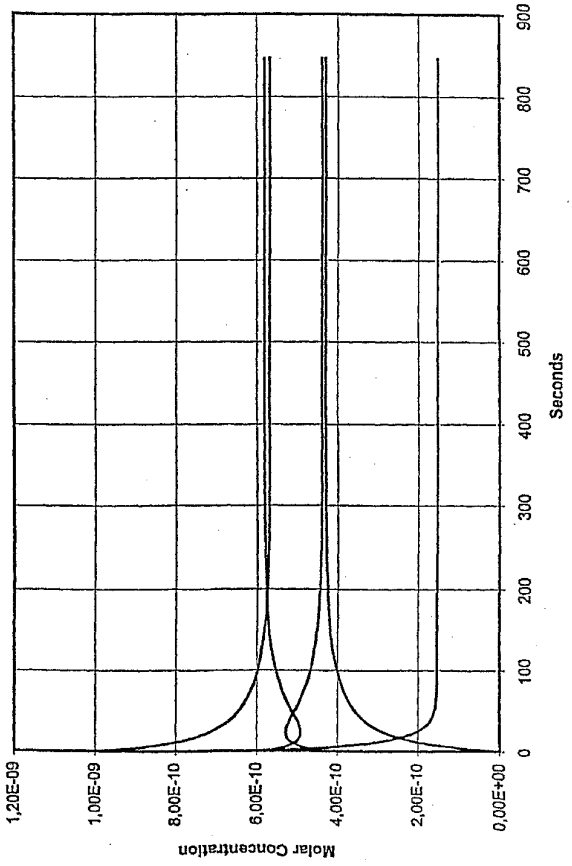

Molar Concentration
1,20E-09
1,00E-09
8,00E-10
6,00E-10
4,00E-10
2,00E-10
0,00E+00
Seconds
0
100
200
300
400
500
600
700
800
900

Figure 8

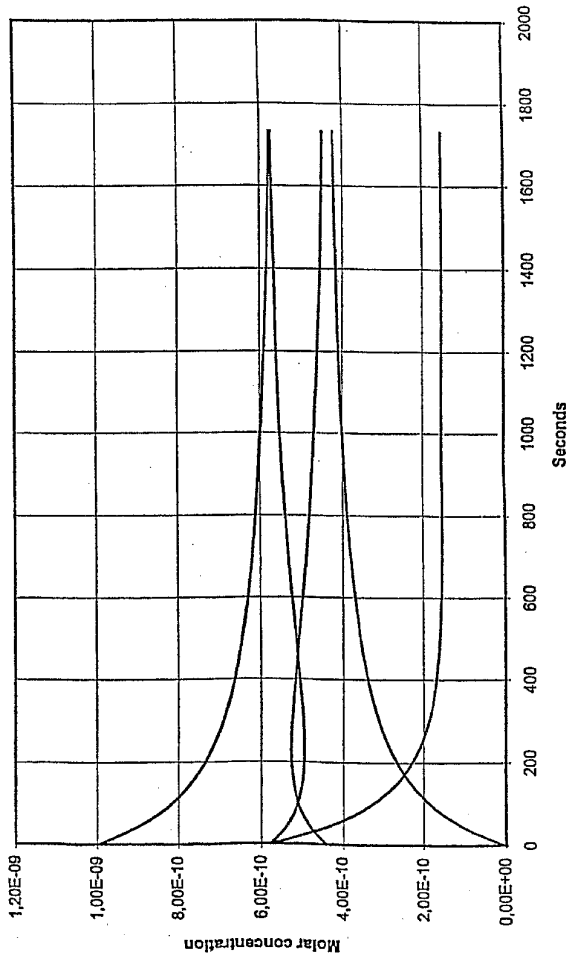

Seconds
0
200
400
600
800
1000
1200
1400
1600
1800
2000
Molar concentration
1,20E-09
1,00E-09
8,00E-10
6,00E-10
4,00E-10
2,00E-10
0,00E+00

Figure 9

【国際調査報告】

INTERNATIONAL SEARCH REPORT

International application No.
PCT/NO 01/00480

A. CLASSIFICATION OF SUBJECT MATTER

IPC7: G01N 33/53, G01N 33/542 // G01N 33/533, G01N 33/68
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7: G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, EPO INTERNAL, PAJ, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5976820 A (MICHAEL E. JOLLEY ET AL), 2 November 1999 (02.11.99), column 2, line 52 - line 63, abstract ---	1-27
X	WO 0017649 A1 (DIACHEMIX CORPORATION), 30 March 2000 (30.03.00), page 25, line 1 - line 12, claims 1,7,16 ---	1-27
X	US 6110750 A (EDWARD A. SUGDEN ET AL), 29 August 2000 (29.08.00), claims 1-4 ---	1-27

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 March 2002	25 -03- 2002
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Lars Wallentin/Els Telephone No. +46 8 782 25 00

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/NO 01/00480

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 6171807 B1 (THOMAS J. NOVITSKY ET AL), 9 January 2001 (09.01.01), column 7, line 62 - column 8, line 5, claims 3,4 --	1-27
A	US 5066426 A (CHAO-HUEI J. WANG ET AL), 19 November 1991 (19.11.91), column 2, line 3 - line 39, abstract --	1-27
A	US 5070025 A (CHRISTIAN KLEIN ET AL), 3 December 1991 (03.12.91), abstract --	1-27
A	US 6159750 A (DAN M. EDMONDS), 12 December 2000 (12.12.00), column 2, line 20 - line 44 --	1-27

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INTERNATIONAL SEARCH REPORT
Information on patent family members

28/01/02

International application No.
PCT/NO 01/00480

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5976820 A	02/11/99	EP 0788605 A	13/08/97
		WO 9708559 A	06/03/97
WO 0017649 A1	30/03/00	AU 1094800 A	10/04/00
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(51)Int.Cl.[7]	FI	テーマコード（参考）
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(81)指定国 AP(GH,GM,KE,LS,MW,MZ,SD,SL,SZ,TZ,UG,ZM,ZW),EA(AM,AZ,BY,KG,KZ,MD,RU,TJ,TM),EP(AT,BE,CH,CY,DE,DK,ES,FI,FR,GB,GR,IE,IT,LU,MC,NL,PT,SE,TR),OA(BF,BJ,CF,CG,CI,CM,GA,GN,GQ,GW,ML,MR,NE,SN,TD,TG),AE,AG,AL,AM,AT,AU,AZ,BA,BB,BG,BR,BY,BZ,CA,CH,CN,CO,CR,CU,CZ,DE,DK,DM,DZ,EC,EE,ES,FI,GB,GD,GE,GH,GM,HR,HU,ID,IL,IN,IS,JP,KE,KG,KP,KR,KZ,LC,LK,LR,LS,LT,LU,LV,MA,MD,MG,MK,MN,MW,MX,MZ,NO,NZ,OM,PH,PL,PT,RO,RU,SD,SE,SG,SI,SK,SL,TJ,TM,TR,TT,TZ,UA,UG,US,UZ,VN,YU,ZA,ZM,ZW

Fターム(参考) 2G054 AA06 AA07 AB07 CA23 CE02 EA03 JA08

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申请号	JP2002546214	申请日	2001-11-30
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申请(专利权)人(译)	阮晋勇多尔哈根，风环		
[标]发明人	ズンドレハーゲンエアリング		
发明人	ズンドレハーゲン,エアリング		
IPC分类号	G01N21/64 G01N21/78 G01N33/53 G01N33/533 G01N33/542		
CPC分类号	G01N33/542		
FI分类号	G01N33/533 G01N21/64.A G01N21/64.F G01N21/78.C G01N33/53.D G01N33/542.A		
F-TERM分类号	2G043/AA01 2G043/BA16 2G043/CA03 2G043/DA02 2G043/DA08 2G043/EA01 2G043/FA03 2G043/FA06 2G043/FA07 2G043/HA07 2G043/KA01 2G043/KA02 2G043/KA03 2G043/KA05 2G043/KA07 2G043/KA09 2G043/NA01 2G043/NA11 2G054/AA06 2G054/AA07 2G054/AB07 2G054/CA23 2G054/CE02 2G054/EA03 2G054/JA08		
优先权	20006130 2000-12-01 NO		
其他公开文献	JP2004514906A		

摘要(译)

本发明涉及一种定量测试样品或测试样品的等分试样中的一种或多种分析物和该方法中使用的试剂的方法。本发明的试剂包括荧光材料信号被改变为待定量的特异性结合分子和样品和至少一种类型的每种分析物的试剂的混合的结果。此外，使用该信号的变化，所以能够计算出分析物的浓度，而不分离不同聚集状态。