

(19)



(11)

**EP 1 644 743 B1**

(12)

**EUROPEAN PATENT SPECIFICATION**

(45) Date of publication and mention of the grant of the patent:

**12.11.2014 Bulletin 2014/46**

(21) Application number: **04727197.8**

(22) Date of filing: **14.04.2004**

(51) Int Cl.:

**G01N 33/78 (2006.01)**

(86) International application number:

**PCT/CA2004/000555**

(87) International publication number:

**WO 2004/090553 (21.10.2004 Gazette 2004/43)**

**(54) THYROID HORMONE ANALYSIS BY MASS SPECTROMETRY**

BESTIMMUNG VON THYROIDHORMONEN MITTELS MASSENSPEKTROMETRIE

ANALYSE D'HORMONES THYROIDIENNES PAR SPECTROMETRIE DE MASSE

(84) Designated Contracting States:  
**AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HU IE IT LI LU MC NL PL PT RO SE SI SK TR**

(30) Priority: **14.04.2003 US 462745 P**

(43) Date of publication of application:  
**12.04.2006 Bulletin 2006/15**

(73) Proprietor: **GEORGETOWN UNIVERSITY Washington, DC 20057 (US)**

(72) Inventor: **SOLDIN, Steven, J. Bethesda, Maryland 20816 (US)**

(74) Representative: **Roberts, Mark Peter J A Kemp 14 South Square Gray's Inn London WC1R 5JJ (GB)**

(56) References cited:  
**WO-A-02/46772**

- **SIEKMANN L: "Measurement of thyroxine in human serum by isotope dilution mass spectrometry. Definitive methods in clinical chemistry, V" BIOMEDICAL AND ENVIRONMENTAL MASS SPECTROMETRY, vol. 14, no. 11, November 1987 (1987-11), pages 683-688, XP009032600**

- **THIENPONT LM ET AL: "Development of a new method for the determination of thyroxine in serum based on isotope dilution gas chromatography mass spectrometry" BIOL MASS SPECTROM, vol. 23, no. 8, 1994, pages 475-482, XP009032585 cited in the application**
- **DE BRABANDERE VI ET AL: "Isotope dilution-liquid chromatography/electrospray ionization-tandem mass spectrometry for the determination of serum thyroxine as a potential reference method" RAPID COMMUN MASS SPECTROM, vol. 12, no. 16, 1998, pages 1099-1103, XP009032587**
- **KOSAKA TAEKO ET AL: "Analysis of thyroid hormones in health foods by LC/MS" SHOKUJIN EISEIGAKU ZASSHI, vol. 43, no. 4, August 2002 (2002-08), pages 225-229, XP009032605**
- **TAI, SSC ET AL: "Candidate reference method for total thyroxine in human serum: use of isotope dilution liquid chromatography-mass spectrometry with electrospray ionization" CLINICAL CHEMISTRY, vol. 48, no. 4, 2002, pages 637-642, XP001182083**
- **CHANG Y-C ET AL: "Quantitative measurement of male steroid hormones using automated on line solid phase extraction liquid chromatography tandem mass spectrometry and comparison with radioimmunoassay" ANALYST, vol. 128, no. 4, 1 April 2003 (2003-04-01), pages 363-368, XP009032863 UK**

**EP 1 644 743 B1**

Note: Within nine months of the publication of the mention of the grant of the European patent in the European Patent Bulletin, any person may give notice to the European Patent Office of opposition to that patent, in accordance with the Implementing Regulations. Notice of opposition shall not be deemed to have been filed until the opposition fee has been paid. (Art. 99(1) European Patent Convention).

**Description****Field of the Invention**

5 **[0001]** The present invention combines the fields of hormone analysis and mass spectrometry. In particular the invention relates to analyzing thyroid hormones using mass spectrometry.

**Background of the Invention**

10 **[0002]** Hormones are biological messengers. They are synthesized by specific tissues (glands) and are secreted into the blood. The blood carries them to target cells where they act to alter the activities of the target cells.

**[0003]** Hormones are chemically diverse, and are generally categorized into three main groups: (1) small molecules derived from amino acids, for example thyroxine, (2) polypeptides or proteins, for example insulin and thyroid-stimulating hormone, and (3) molecules derived from cholesterol, for example steroids.

15 **[0004]** An important class of hormone is the thyroid hormones. Examples of thyroid hormones are thyroxine (T4), free thyroxine (FT4) and triiodothyronine (T3). T4 and T3 enter cells and bind to intracellular receptors where they increase the metabolic capabilities of the cell by increasing mitochondria and mitochondrial enzymes. T4 and T3 are important in regulating a number of biological processes, including growth and development, carbohydrate metabolism, oxygen consumption, protein synthesis and fetal neurodevelopment. Synthesis of all circulating T4 and a small percentage of circulating T3 occurs on thyroglobulin molecules located within the thyroid. The bulk of the T3 present in the blood is produced enzymatically via monodeiodination of T4 by specific intracellular deiodinases - enzymes present in the follicular cells and the cells of target tissues [1]. In serum drawn from healthy human subjects, total T4 is present at about 60-fold higher concentration than total T3. T4 acts as a prohormone, as the reservoir for the production of T3, the active hormone. The metabolic activity associated with thyroid hormone (TH) is initiated by T3 binding to specific nuclear receptors within target cells. Thyroid hormone concentrations in blood are essential tests for the assessment of thyroid function.

20 **[0005]** Steroids make up another important class of hormones. Examples of steroid hormones include estrogens, progesterone and testosterone. Estrogen is the name of a group of hormones of which there are three principle forms, estrone, estradiol and estriol. Estrogens and progesterone cause the development of the female secondary sexual characteristics and develop and maintain the reproductive function. Testosterone develops and maintains the male secondary sex characteristics, promotes growth and formation of sperm. Steroids enter target cells and bind to intracellular receptors and then cause the production of mRNA coding for proteins that manifest the changes induced by steroids.

30 **[0006]** The accurate analysis and quantification of hormones is becoming more important. For example, estrogen and estrogen like compounds are playing an ever-increasing role in today's society through hormone replacement therapy. Also, the analysis and quantification of estrogen and estrogen-like compounds helps in the management of estrogen-related diseases, like breast cancer. In addition, the accurate analysis and quantification of T4 and T3 is an issue recognized by those skilled in the art. The presence of circulating iodothyronine-binding autoantibodies that interfere with total T4 and T3 radioimmunoassays ("RIAs") is a known phenomenon [2], [3], [4]. These autoantibodies may give falsely high, or falsely low values of thyroid hormone measurements depending on the assay separation method used, and are often in discordance with the clinical features [2], [3], [4]. Direct serum free T4 and T3 (FT4 and FT3) measurements are a way to compensate for such abnormal binding. However, technically, it is difficult to measure the free hormone concentrations since these are so low. It is easier to measure the total (free and protein-bound) thyroid hormone concentrations; total hormone concentrations are measured at nanomolar levels whereas free hormone concentrations are measured in the picomole range and to be valid, must be free from interference by the much higher total hormone concentrations.

45 **[0007]** Presently, the common methods of hormone analysis use immunoassay techniques. Table 1 lists the common hormones and the current methods for their analysis.

**[0008]** For example, estriol is analyzed by a radioimmunoassay utilizing radiolabelled antigen (iodine 125) in competition with unlabelled estriol in the sample, for a known amount of antibody. The assay is read using a gamma counter.

50 **[0009]** Androstenedione is analyzed using an enzyme immunoassay comprising horseradish peroxidase. Unlabeled antigen in the sample is in competition with enzyme labeled antigen for a fixed number of antibody binding sites. The assay is read using a microtitre plate enzyme immunoassay reader.

**[0010]** Several hormones are currently analyzed using a chemiluminescent immunoassay. For example, progesterone, testosterone, cortisol and T3 are analyzed using this method. The assay utilizes an assay-specific antibody-coated bead.

55 The assay is read using a photon counter.

**[0011]** However, the current immunoassays are disadvantageous for the following reasons:

- (1) Immunoassays are specific to one hormone, therefore every hormone must be analyzed separately.

## EP 1 644 743 B1

(2) Numerous kits must be purchased and procedures must be learned for each hormone being analyzed.

(3) Various instruments to read the results from the immunoassays must be purchased. For example, the analysis of estriol and progesterone from a sample requires both a gamma counter and a photon counter.

(4) The kits for the assays can be expensive.

(5) The current immunoassays lack specificity and may show approximately 15 fold difference in results using kits from different manufacturers [5].

(6) The procedures involve many steps and can take a significant amount of time.

(7) In the case of a radioimmunoassay, precautions are necessary because of the radioisotopes involved.

**[0012]** Immunoassays are notoriously unreliable with more and more literature published supporting their lack of specificity [6-13]. Table 2 shows the major differences reported by the College of American Pathologists program for proficiency testing of thyroid hormones that clearly illustrates the difference in specificity of the various antibodies used. For example, Table 2 shows mean results between different methods reported in the College of American Pathologists Proficiency Testing (CAP PT) Program can vary by a factor of approximately 2. Some factors such as pregnancy, estrogen therapy or genetic abnormalities in protein binding have also reportedly made Immunoassay methods for T4 and T3 diagnostically unreliable [2], [3], [14], [15]. Currently serum total T4 (TT4) and total serum T3 (TT3) concentrations are most commonly measured by immunoassay methods. Recently some reports of quantitative measurement of T4 and T3 by high performance liquid chromatography (HPLC), gas chromatography mass spectrometry (GC-MS), liquid chromatography mass spectrometry (LC-MS) or tandem mass spectrometry (LC-MS/MS) were published [16-20]. All those methods required extraction, derivatization and even prior chromatographic separation that are very time-consuming [21], [22].

**[0013]** More recently, hormones have been analysed and quantified by mass spectrometry. However, there are several disadvantages to these methods.

**[0014]** For example, a method of analyzing urinary testosterone and dihydrotestosterone glucuronides using electrospray tandem mass spectrometry has been described [23]. The method involves a complex system employing high performance liquid chromatography (HPLC) and a three-column two-switching valve. The shortcomings include the following: (i) the hormone glucuronides were analyzed, not the hormones, (ii) the method is applicable to urine only and (iii) only two analytes were analysed simultaneously, (iv) the limit of detection (LOD) was 200 pg ml<sup>-1</sup> for testosterone and the limit of quantification was 10 ug L<sup>-1</sup> for dihydrotestosterone and (v) the method is complex.

**[0015]** Another publication discloses a method for the determination of estradiol in bovine plasma by an ion trap gas chromatography-tandem mass spectrometry technique [24]. The shortcomings include the following: (i) only one analyte was analyzed, (ii) 4 ml of plasma was required for the analysis of one analyte, (iii) the limit of detection was 5 pg ml<sup>-1</sup>, and (iv) derivatization was required because the method employs gas chromatography.

**[0016]** A method for analysis of 17-hydroxyprogesterone by HPLC electrospray ionization tandem mass spectrometry from dried blood spots has also been described [25]. However, this method analyses only one analyte at a time, and requires liquid-liquid extraction, which is laborious and time consuming, with sample extraction alone taking 50 minutes to complete.

**[0017]** Finally, a gas chromatography mass spectrometry method to analyze the production rates of testosterone and dihydrosterone has been disclosed [26].

**TABLE 1: METHODS AND INSTRUMENTS FOR STEROID AND THYROID HORMONES [1].**

ANALYTE	Percentage of Use	Instrument	METHOD
Androstenedione	35%	DSL solid	EIA
11-Deoxycortisol	50%	ICN Immuchem DA	RIA
DHEA Sulfate	39%	DPC Immulite	ECIA
Estradiol	16%	Bayer ADVIA Centaur	FIA
Estriol, unconjugated	25%	DSL liquid	RIA
Estriol, Total	50%	DPC Coat-a-Count	RIA
17-Hydroxyprogesterone	51%	DPC Coat-a-Count	RIA

**EP 1 644 743 B1**

(continued)

5  
10  
15  
20

<b>ANALYTE</b>	<b>Percentage of Use</b>	<b>Instrument</b>	<b>METHOD</b>
Progesterone	23%	Bayer ADVIA Centaur	CIA
Testosterone	29%	Bayer ADVIA Centaur	CIA
Testosterone, Free	65%	DPC Coat-a-Count	RIA
Aldosterone	76%	DPC Coat-a-Count	RIA
Cortisol	25%	Bayer ADVIA Centaur	CIA
T3	29%	Abbott AxSYM	FPIA
T3, Free	31%	Bayer ADVIA Centaur	CIA
T4	30%	Abbott AxSYM	FPIA
T4, Free	34%	Abbott AxSYM	FPIA
RIA: Radioimmunoassay EIA: Enzyme Linked Immunoassay FPIA: Fluorescence Polarization Immunoassay			

**Table 2: Problems with Immunoassays: Data acquired from CAP PT Program 2003**

25  
30  
35

Analyte	Mean CAP Result for Method Giving Lowest Value	Mean CAP Result for Method Giving Highest Value
Triiodothyronine (ng/dL)	108.5	190.2
	364.8	610.1
Thyroxine (ug/dL)	5.64	10.09
	1.64	3.65
	8.73	13.12
<b>Table 2: Problems with Immunoassays: Data acquired for samples from the CAP PT Program 2003.</b>		

**Summary of the Invention**

**[0018]** The invention provides a fast and accurate method of hormone analysis and quantification using a mass spectrometer.

**[0019]** In accordance with a first aspect, there is provided a method for mass spectrometric analysis of a 100µl sample possibly containing one or more thyroid hormones, consisting of the steps:

- (a) deproteinating a sample containing one or more thyroid hormones;
- (b) separating by liquid chromatography the one or more thyroid hormones from the sample; and
- (c) analyzing the one or more thyroid hormones using a mass spectrometer.

**[0020]** In accordance with a second aspect, there is provided a method for mass spectrometric analysis of a 700µl sample possibly containing one or more thyroid hormones and one or more steroid hormones, consisting of the steps:

- (a) deproteinating a sample containing one or more thyroid hormones;
- (b) separating by liquid chromatography the one or more thyroid hormones and the one or more steroid hormones from the sample; and
- (c) analyzing the one or more thyroid hormones and the one or more steroid hormones using a mass spectrometer.

**[0021]** A plurality of hormones can be analyzed simultaneously or sequentially. The procedure allows for as little as 100 µL of a sample to be analyzed. In addition, minimal sample preparation time is required.

**[0022]** The invention permits the analysis of hormones in a number of complex matrices as they might be found in

nature, e.g. the human body. For example, hormone analysis can be performed on samples of blood, saliva, serum, plasma and urine.

**[0023]** There are several advantages to this invention:

- 5 (1) It provides a total and specific analysis for hormones in a sample. The present method allows for the analysis of many hormones simultaneously or sequentially.
- (2) The procedure does not require an immunoprecipitation reaction. The majority of other methods for hormone analysis required an immunoassay. Immunoassays are expensive, specific to a particular analyte and involve several  
10 steps.
- (3) The present invention requires minimal sample preparation time. For example, preparing a sample for hormone analysis can be done within 6 minutes.
- 15 (4) The procedure does not require a large sample size. A plasma or serum sample can be as small as 100  $\mu$ L for thyroid hormones. The current methods for hormone analysis that utilize mass spectrometry require 4-15 mL of plasma.
- (5) The invention uses simple preparation techniques that are easy to use and highly reproducible.  
20
- (6) The invention permits analysis to be performed on a variety of sample types.
- (7) The invention permits the analysis of hormones in a sample of saliva or urine which permits simple sample acquisition and the remote submission of samples to a clinic for analysis. In previous other clinical methods, samples  
25 are taken by invasive means directly from the patient in a clinic.
- (8) The analysis by mass spectrometry is highly accurate. In addition, the procedure of the present invention is highly reproducible.
- 30 (9) The invention permits the analysis of a wide range of hormone concentrations. In addition, the limit of detection can be fairly low.

**[0024]** Accordingly, we describe a use for a mass spectrometer for simultaneously or sequentially analyzing a sample for a plurality of hormones in a fast, simple and accurate way. The sample may be, for example, serum, plasma, urine  
35 or saliva.

**[0025]** We also describe a system for the fast, simple and accurate analysis of a plurality of hormones comprising: reagents for the preparation of the sample, reagents to perform the analysis on a mass spectrometer, and a mass spectrometer to perform the analysis.

**[0026]** We also describe a kit, comprising the various reagents required for simultaneously or sequentially analyzing, within a sample, a plurality of hormones, including steroid hormones, thyroid hormones and other hormones. The kit  
40 may include a standard solution of the hormones of interest, compounds as internal standards, mobile phase solutions, methods and tools for separating hormones from samples, for example HPLC columns, and quality control specimens.

**[0027]** We also describe a method for the simultaneous or sequential analysis of one or more hormones comprising ionizing the hormones and analyzing the hormones by mass spectrometry.

**[0028]** We also describe a method for the simultaneous or sequential analysis of one or more hormones comprising: obtaining a sample containing or suspected of containing one or more hormones removing proteins from the sample separating the hormones from the sample, ionizing the hormones and analyzing the hormones in a mass spectrometer.  
45

**[0029]** We also describe a method for the analysis of one or more thyroid hormones comprising: obtaining a sample containing or suspected of containing one or more thyroid hormones, removing proteins from the sample, separating  
50 the thyroid hormone from the sample, ionizing the thyroid hormones, for example by electrospray ionization, and analyzing the hormone in a mass spectrometer, preferably in the negative mode.

**[0030]** We also describe a method for the simultaneous or sequential analysis of one or more thyroid hormones comprising: obtaining a sample containing or suspected of containing one or more thyroid hormones, removing proteins from the sample, separating the thyroid hormones from the sample, ionizing the thyroid hormone, for example by elec-  
55 tro-spray ionization, and analyzing the hormones in a mass spectrometer, preferably in the negative mode.

**[0031]** We also describe a method for the simultaneous or sequential analysis of a plurality of thyroid hormones and a plurality of steroid hormones comprising: obtaining a sample containing or suspected of containing a plurality of hormones, removing proteins from the sample, separating the hormones from the sample, ionizing the thyroid hormones,

for example, by electrospray ionization, ionizing the steroid hormones by photoionization, and analyzing the hormones in a mass spectrometer, in the negative or positive modes.

**Brief Description of the Drawings**

5

**[0032]** The invention, including the best approaches known to the inventors, can be better understood with reference to the following detailed description taken in combination with the following drawings, in which:

10

Figure 1 is a mass spectrum of a sample of plasma containing T4 and T3.

Figure 2 is a mass spectrum of a globulin standard containing T4 and T3.

Figure 3 is a typical tandem mass spectrometric chromatogram obtained for T4 and T3.

15

Figure 4 is a graph showing T3 measured by Isotope Dilution Tandem Mass Spectrometry vs. Immunoassay.

Figure 5 is a graph showing T4 measured by Isotope Dilution Tandem Mass Spectrometry vs. Immunoassay.

**Detailed Description of the Exemplified Embodiment**

20

**[0033]** The invention provides methods of analysis for hormones. The hormones may include:

25

- Dehydroepiandrosterone (DHEA)
- Dehydroepiandrosterone sulphate (DHEAS)
- Aldosterone
- Cortisol
- 11-Deoxycortisol
- Androstenedione
- Testosterone
- 30 Estradiol
- 17-OH Progesterone
- Progesterone
- Allopregnanolone
- 16-OH Estrone
- 35 2-OH Estrone
- Estrone
- Estriol
- Vitamin D
- thyroxine
- 40 free thyroxine
- triiodothyronine
- catecholamines
- metanephrines
- other steroid hormones
- 45 other thyroid hormones
- other small peptide hormones
- other amines

50

**Sample**

**[0034]** Any sample containing or suspected of containing a hormone can be used, including a sample of blood, plasma, serum, urine or saliva. The sample may contain both free and conjugated or bound hormones. A sample size of at least about 100  $\mu$ L for hormones generally, or at least about 700  $\mu$ L for steroid hormones, is presently preferred.

55

**Deproteinization**

**[0035]** The sample is de-proteinated. This can be done by conventional techniques known to those skilled in the art. For example, a sample can be de-proteinated with acetonitrile, containing internal standard, followed by vortexing and

centrifugation. The internal standard may be, for example, the deuterated hormone.

Separation of hormones from the sample

5 **[0036]** The hormones are separated by methods known to those skilled in the art. For example, the hormones may be separated by liquid chromatography through a column. The column may be a C-18 column. The hormones are subsequently eluted from the column.

Introduction of hormones into a mass spectrometer

10 **[0037]** The hormones are then introduced into a mass spectrometer. Optionally, the separation step and step of introducing the hormones into a mass spectrometer can be combined using a combined liquid chromatography spectrometry apparatus (LC/MS). This procedure is based on an online extraction of the injected sample with subsequent introduction into the mass spectrometer using a built-in switching valve.

Isotope Dilution Tandem Mass Spectrometry

15 **[0038]** Isotope dilution tandem mass spectrometry incorporates additional dilution steps that act as an internal calibration so that an independent isotopic reference material is not required. It avoids the need to measure the isotope ratio of the highly enriched spike directly, and enables the final results to be arranged as a combination of measurements that are largely insensitive to instrumental bias and drift. Consequently, it has the potential to extend the scope of application of isotope dilution tandem mass spectrometry to include analysis for which reference materials with certified isotope ratios are not available or where contamination of the instrument by the highly-enriched spike causes difficulty.

Instrumentation and ionization techniques

25 **[0039]** The hormones are subjected to ionization. Various ionization techniques can be used. For example, photoionization, electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), and electron capture ionization may be used. Preferably, electrospray ionization is utilized when analyzing thyroid hormones.

30 **[0040]** The following mass spectrometers can be used: any tandem-mass spectrometer, including hybrid quadrupole-linear ion trap mass spectrometers and liquid chromatography-tandem mass spectrometers such as the API 2000™ mass spectrometer, the API 3000™ mass spectrometer, and the API 4000™ mass spectrometer, described in U.S. patents 4,121,099; 4,137,750; 4,328,420; 4,963,736; 5,179,278; 5,248,875; 5,412,208; and 5,847,386 (Applied Biosystems/MDS SCIEX, Foster City, Calif./Concord Ontario, Canada). When analyzing thyroid hormones, a spectrometer with a turbo spray ion source, such as the API 2000™ and API 3000™ mass spectrometers, is presently preferred. When analyzing FT4, the API 4000™ mass spectrometer is presently preferred.

35 **[0041]** Ionization may be performed by utilizing the mass spectrometer in the negative or the positive mode, depending on a particular analyte's tendency to give rise to a particular ion form, as is known to those skilled in the art. Typically, for thyroid hormones, the spectrometer is employed in the negative mode.

40 **[0042]** Hormones are identified on the basis of the mass to charge ratio of their molecular ions and fragment ions, as is known to those skilled in the art. When the hormones are purified by liquid chromatography, they can also be identified by their retention times.

**[0043]** Hormones are quantified by their intensity as determined in the mass spectrometer in counts per second. Calibration curves for known concentrations of the hormones are established for comparison.

45 **EXAMPLES**

**[0044]** The invention will now be demonstrated using the following examples, provided to demonstrate but not limit the embodiments of the present invention:

50 *1. Analysis of a sample for thyroid hormones*

**[0045]** A sample of 100  $\mu\text{L}$  of plasma was used. Proteins were precipitated with 150  $\mu\text{L}$  of acetonitrile, capped and vortexed. The sample was then centrifuged, and 200  $\mu\text{L}$  of the supernatant was injected onto a Supelco LC-18-DB™ chromatographic column equipped with Supelco Discovery C-18™ guard column, coupled to a tandem mass spectrometer (LC/MS/MS). The column was washed with 20% methanol in 5mM ammonium acetate for 3 minutes. The valve was switched and the sample was eluted in 75% to 95% methanol. The total run time was 6 minutes. Slight adjustments to the volumes, concentrations and times described can be made, as is known to those skilled in the art.

## EP 1 644 743 B1

[0046] The eluant was introduced into an ion-spray ionization chamber and analyzed by API 2000™ mass spectrometer using the negative mode. The mass/charge ratios for T4 and T3 ions is 775.8 and 650 respectively. The ionization may be by electrospray using a turboionspray chamber.

[0047] This demonstrates a simple method of preparing a complex biological matrix for analysis of hormone content, and a sensitive analytical method that permits the simultaneous analysis of two hormones, T3 and T4.

### 2. Analysis of thyroid hormones using a methanol gradient to elute the hormones

[0048] A sample of 100  $\mu\text{L}$  of plasma was used. Proteins were precipitated with 150  $\mu\text{L}$  of acetonitrile, containing an internal standard of deuterated T<sub>4</sub>, and vortexed. The sample was centrifuged, and 200  $\mu\text{L}$  of the supernatant was injected onto a C-18 column coupled to a tandem mass spectrometer (LC/MS/MS). The column was washed with 20% methanol in 5mM ammonium acetate for 3 minutes. The valve on the column was switched and the sample was eluted in a methanol gradient of 20 to 100%. The total run time was 7 minutes. Slight adjustments to the volumes, concentrations and times described can be made by those skilled in the art.

[0049] A sample of the eluant was introduced into an ion-spray ionization chamber and analyzed by an AP 2000™ mass spectrometer using the negative mode. The ionization may be by electrospray using a turboionspray chamber. See Figure 1 and Figure 2 for mass spectrums generated for T3 and T4.

[0050] This demonstrates a simple method of preparing a complex biological matrix for analysis of thyroid hormone content, and a sensitive analytical method that permits the simultaneous analysis of multiple hormones.

### 3. Analysis of thyroid hormones using isotope dilution tandem mass spectrometry

[0051] This example describes an isotope dilution tandem mass spectrometry method for the simultaneous determination of T4 and T3 in serum. The method is accurate, specific, precise (%CVs between 3.5 and 9.0), simple - requiring no extraction and only protein precipitation and fast (<7 min).

#### Chemicals and reagents

[0052] Standards of T4 and T3 were purchased from Sigma (St. Louis, MO, USA). A stable deuterium-labeled internal standard, L-thyroxin-d<sub>2</sub> was synthesized according to procedures described in the literature [16], [17] by Dr Tomas Class from the Chemistry Department at Georgetown University. HPLC grade methanol was purchased from VWR Scientific. All other chemicals were of analytical grade and purchased from Sigma.

#### Solutions and standards

[0053] Stock solutions of T3, T4 and internal standard (IS) were prepared separately to obtain concentration of 1mg/mL for each. 40% ammonium hydroxide (v/v) in methanol was used as a solvent. The analyte stock solutions were diluted with methanol to obtain the spiking solutions. The solutions were stored at 4°C and could be used for several months. Standards for the calibration curve in the range of 0.325 to 5 ng/mL for T3 and 12.5 to 200 ng/mL for T4 were prepared by adding the analyses to 3 % human  $\gamma$ -globulin (volume of spiking solution < 2% of final volume). Quality control (QC) samples (Diagnostic Product Corp., Los Angeles, USA) at low, medium and high levels were used. A solution of 50-ng/mL d<sub>2</sub>-T4 in methanol was used as the internal standard.

#### Sample preparation

[0054] Serum/plasma samples were thawed at room temperature. 150  $\mu\text{L}$  of IS solution was added to aliquots of 100  $\mu\text{L}$  of the serum or plasma sample. After 30 seconds of vortex mixing, the samples were stored for 10 min at room temperature to allow complete protein precipitation. The samples were centrifuged for 10 min at 15,000 rpm and 100  $\mu\text{L}$  of supernatant was injected into the LC-MS-MS system.

#### LC/MS/MS conditions.

[0055] An API 3000™ tandem mass-spectrometer (SCIEX, Toronto, Canada) equipped with TurbolonSpray and Shimadzu HPLC system was used to perform the analysis. Negative ion multiple reaction-monitoring (MRM) mode was used. The transitions to monitor were selected at m/z 650→127 for T3, m/z 776→127 for T4, m/z 778→127 for d<sub>2</sub>-T4. Nitrogen served as auxiliary, curtain and collision gas. Gas flow rates, source temperature, Ion Spray voltages and collision energies were optimized for every compound by infusion of 1 $\mu\text{g/mL}$  of the standard solutions in methanol at 20  $\mu\text{L/min}$  and by flow-injection analysis (FIA) at LC flow rate. The main working parameters for the mass spectrometer

are summarized in Table 3. Data processing was performed on Analyst 1.2 software package.

#### LC-MS-MS procedure

5 **[0056]** The procedure is based on an online extraction/cleaning of the injected samples with subsequent introduction into the mass-spectrometer by using a built-in Valco switching valve. 100  $\mu$ l of the sample were injected onto a Supelco LC-18-DB (3.3 cm x 3.0 mm, 3.0  $\mu$ m ID) chromatographic column equipped with a Supelco Discovery C-18 (3.0 mm) Guard column, where it underwent cleaning with 20% (v/v) methanol in 5 mM ammonium acetate pH=4.0 at flow rate 0.8 mL/min. After 3.5 min of cleaning the switching valve was activated, the column was flushed with water/ methanol  
10 gradient at flow rate 0.5 mL/min and the samples were introduced into the mass-spectrometer. The gradient parameters are shown in Table 4.

#### Immunoassays for T4 and T3

15 **[0057]** T4 was measured by the Dade RxL Dimension (Dade-Behring Diagnostics, Glasgow, DE) and T3 by the DPC Immulite (Diagnostic Product Corporation, Los Angeles, CA) according to the manufacturer's specifications.

#### Results

20 **[0058]** The optimal mass spectrometer working parameters are shown in Tables 3 and 4.  
**[0059]** Replicate sera were assayed both within-day and between-day at several concentrations. The within-day and between-day precision data is provided in Tables 5 and 6.  
**[0060]** Recovery studies for T4 and T3 are shown in Tables 7 and 8. All results shown are the means of 8 replicates.  
**[0061]** Figure 3 shows a typical tandem mass spectrometric chromatogram obtained for T3 and T4.  
25 **[0062]** Specimens were tested for T3 and T4 by both immunoassay (T3 DPC Immulite, T4 Dade Behring Dimension RxL) and by tandem mass spectrometry. Linear regression correlations (Prism) are shown in Figures 4 and 5.  
**[0063]** The lower limit of quantitation of the mass spectrometry method was found to be 0.15 ng/mL for both T3 and T4. Detection limit was around 0.062 ng/mL.

#### 30 Discussion

**[0064]** Evidence initially gleaned from both the CAP PT Program and pediatric reference ranges employing different immunoassays indicated the probability of lack of specificity for T4 and T3 immunoassay tests. To adequately assess this phenomenon we developed the isotope dilution tandem mass spectrometric method described in this example.  
35 Serum T4 and T3 detection methods have evolved through a variety of technologies since the 1950s. Radioimmunoassay (RIA) methods to detect THs were developed in the 1970s. Serum T4 and T3 concentrations are currently measured by competitive immunoassay methods (IAs) that are mostly non-isotopic and use enzymes, fluorescence or chemiluminescence molecules as signals [27]. Table 2 clearly indicates that current IAs for T4 and T3 lack specificity and give mean results differing by a factor of approximately 2 in CAP PT programs. Total hormone assays necessitate the inclusion  
40 of a displacing agent (such as salicylate) to release the hormone from its binding proteins [28]. The displacement of hormone binding from serum proteins by such agents, together with the large sample dilution employed in modern assays, facilitates the binding of hormone to the antibody reagent.

**[0065]** Since T3 is ten-fold lower in concentration compared with T4 in blood it therefore presents both a technical sensitivity and precision challenge despite the use of a higher specimen volume. Although a reliable high-range T3  
45 measurement is critical for diagnosing hyperthyroidism, a reliable normal-range measurement is also important for adjusting antithyroid drug dosage and detecting hyperthyroidism in sick hospitalized patients, in whom a paradoxically normal T3 value may indicate hyperthyroidism.

**[0066]** The correlation coefficient for the T4 comparisons (0.931) is significantly better than for the T3 comparisons (0.848) (Figures 4 and 5). T3 by tandem mass spectrometry gave slightly higher results than those obtained by the DPC Immulite (Figure 4). While this is true for children, our preliminary data for non-pregnant and pregnant women indicates  
50 a very poor correlation for T3 in both groups ( $r$  between 0.407-0.574).

**[0067]** The reasons for this are not clear but could include standardization issues, heterophilic antibodies etc. Of importance, we determined that reverse T3, which lacks a daughter ion of 127  $m/z$ , therefore does not interfere in our tandem mass spectrometry method. Applying the tandem mass spectrometric method to CAP PT samples in the K/KN  
55 general ligand program again revealed that around 85% of the immunoassay methods gave means on samples which were lower than the means obtained by our tandem mass spectrometry method while 15% had higher means.

**[0068]** In conclusion, correlations between immunoassays and tandem mass spectrometry for T4 proved to be adequate except for the pregnant population, while the data for T3 was far less impressive especially during pregnancy.

## EP 1 644 743 B1

Recovery studies from several different sera using deuterated T4 as internal standard showed consistent (90-109%) recoveries for both T4 and T3 (Tables 7 and 8). The recovery differences found between samples were surprisingly larger for T4 than for T3. This indicates a lack of need to use deuterated T3 as the T3 internal standard. The isotope dilution tandem mass spectrometric method we developed is rapid (<7 min), accurate (provides the true result as has been assessed by recovery studies), specific (measures only the analyte it purports to measure), precise (low %CV) and easy to perform.

**Table 3: Tandem mass-spectrometer working parameters**

Parameter	Value
Nebulizer gas (NEB)	8
Curtain gas (CUR)	10
Collision gas (CAD)	6
Turbolon Spray Heater gas	7 L/min
Turbolon Spray (IS) voltage	4500 V
Entrance Potential (EP)	7.5 V
Collision cell Exit Potential (CXP)	5 V
Source temperature	450°
Dwell time	250 msec

**Table 4: Gradient parameters**

Time (min)	Methanol (%)
3.50	75
5.25	76
5.50	100
7.00	End

**Table 5: Within day precision (n=10)**

Analyte	CONTROL 1			CONTROL 2		
	Mean (ng/mL)	SD	CV (%)	Mean (ng/mL)	SD	CV (%)
<b>T3</b>	1.04	0.014	1.36	2.44	0.077	3.19
<b>T4</b>	24.1	0.437	1.81	81.2	1.502	1.85

**Table 6: Between day precision (n=20, 1 run per day for 20 days)**

Analyte	CONTROL 1			CONTROL 2			CONTROL 3		
	Mean (ng/mL)	SD	CV (%)	Mean (ng/mL)	SD	CV (%)	Mean (ng/mL)	SD	CV (%)
T3	1.08	0.05	4.47	2.39	0.22	9.21	3.49	0.31	9.00
T4	24.4	1.39	5.69	76.6	3.11	4.06	116.3	4.15	3.57

**Table 7: Recovery of added thyroxine (T4)**

Sample #	Added (ng/mL)	Detected mean	Added amount recovered	Recovery, %
1 (n=8)	0	85.9	NA*	NA

EP 1 644 743 B1

(continued)

Sample #	Added (ng/mL)	Detected mean	Added amount recovered	Recovery, %
	10	96.7	10.8	108.0
	40	127.5	41.6	104.0
2 (n=5)	0	62.6	NA	NA
	10	72.1	9.5	95.0
	40	98.0	35.4	90.0
3 (n=5)	0	73.8	NA	NA
	10	84.7	10.9	109.0
	40	116	42.2	105.0
4 (n=5)	0	58.3	NA	NA
	10	68.0	9.7	97.0
	40	95.0	36.7	92.0
*NA - not applicable				

Table 8: Recovery of added triiodothyronine (T3)

Sample #	Added (ng/mL)	Detected mean	Added amount recovered	Recovery, %
1 (n=8)	0	1.88	NA	NA
	0.25	2.12	0.24	96.0
	1.00	2.85	0.97	97.0
2 (n=5)	0	1.70	NA	NA
	0.25	1.96	0.26	104.0
	1.00	2.76	1.06	106.0
3 (n=5)	0	1.56	NA	NA
	0.25	1.81	0.25	100.0
	1.00	2.62	1.06	106.0
4 (n=5)	0	0.49	NA	NA
	0.25	0.74	0.25	100.0
	1.00	1.50	1.01	101.0
*NA - not applicable				

4. Analysis of thyroid hormones and steroid hormones

**[0069]** A sample of 100  $\mu$ L of plasma is used. Proteins are precipitated with 150  $\mu$ L of acetonitrile and vortexed. The sample is centrifuged, and 200  $\mu$ L of the supernatant is injected onto a C-18 column coupled to a tandem mass spectrometer (LC/MS/MS). The column is washed with 20% methanol in 5mM ammonium acetate for 3 minutes. The valve on the column is switched and the sample is eluted in a methanol gradient of 20 to 100%. The total run time is 10 minutes. Slight adjustments to the volumes, concentrations and times described can be made, as is known to those skilled in the art.

**[0070]** A sample of the eluant is introduced into an ion-spray ionization chamber and analyzed by API 3000™ mass spectrometer using the negative mode for thyroid hormones in the sample. Steroid hormones in the sample are ionized by photoionization, with the spectrometer in the negative or positive mode. Analysis in the positive mode is typically made for DHEA, Aldosterone, Cortisol, 11-Deoxycortisol, Androstenedione, Testosterone, Estradiol, 17-OH Progesterone, Progesterone, Allopregalone, and Vitamin D, whereas analysis in the negative mode is typically made for 16-OH

Estrone, 2-OH Estrone, Estriol and DHEAS. However, it is possible to analyze any of the hormones in either positive or negative mode.

**[0071]** This demonstrates a simple method of preparing a complex biological matrix for analysis of possible steroid and thyroid hormone content, and a sensitive analytical method that permits the simultaneous analysis of steroid and thyroid hormones.

**[0072]** The results indicate that this technique, allows for the identification and characterization of low levels of thyroid hormone in human plasma and saliva.

## REFERENCES

### **[0073]**

1. Lum SM, Nicoloff JT, Spencer CA, Kaptein EM. Peripheral tissue mechanism for maintenance of serum triiodothyronine values in a thyroxine-deficient state in man. *J Clin Invest* 1984; 73(2):570-575.

2. Sakata S, Nakamura S, Miura K. Autoantibodies against thyroid hormones or iodothyronine. Implications in diagnosis, thyroid function, treatment, and pathogenesis. *Ann Intern Med* 1985; 103(4):579-589.

3. Beck-Peccoz P, Romelli PB, Cattaneo MG, Faglia G, White EL, Barlow JW, Stockigt JR. Evaluation of free thyroxine methods in the presence of iodothyronine-binding autoantibodies. *J Clin Endocrinol Metab* 1984; 58(4):736-739.

4. Klee GG. Human anti-mouse antibodies. *Arch Pathol Lab Med* 2000; 124(6): 921-923.

5. College of American Pathologists Proficiency Survey Report on Y-03, RAP-03 and K-06 specimens for 2003.

6. Soldin SJ. Digoxin--issues and controversies. *Clin Chem* 1986; 32(1 Pt 1):5-12.

7. Soldin SJ, Papanastasiou-Diamandi A, Heyes J, Lingwood C, Olley P. Are immunoassays for digoxin reliable? *Clin Biochem* 1984; 17(5):317-320.

8. Thong B, Soldin SJ, Lingwood CA. Lack of specificity of current anti-digoxin antibodies, and preparation of a new, specific polyclonal antibody that recognizes the carbohydrate moiety of digoxin. *Clin Chem* 1985; 31(10):1625-1631.

9. Murthy JN, Davis DL, Yatscoff RW, Soldin SJ. Tacrolimus metabolite cross-reactivity in different tacrolimus assays. *Clin Biochem* 1998; 31(8):613-617.

10. Murthy JN, Yatscoff RW, Soldin SJ. Cyclosporine metabolite cross-reactivity in different cyclosporine assays. *Clin Biochem* 1998; 31(3):159-163.

11. Shen S, Elin RJ, Soldin SJ. Characterization of cross reactivity by carbamazepine 10,11-epoxide with carbamazepine assays. *Clin Biochem* 2001; 4(2):157-158.

12. Ghoshal AK, Soldin SJ. tacrolimus II assay: is it reliable at low blood concentrations? A comparison with tandem MS/MS. *Clin Biochem* 2002; 35(5): 389-392.

13. Soldin SJ, Steele BW, Witte DL, Wang E, Elin RJ. Lack of specificity of cyclosporine immunoassays. Results of a College of American Pathologists Study. *Arch Pathol Lab Med* 2003; 127(1):19-22.

14. Despres N, Grant AM. Antibody interference in thyroid assays: a potential for clinical misinformation. *Clin Chem* 1998; 44(3):440-454.

15. Same DH, Refetoff S, Nelson JC, Linarelli LG. A new inherited abnormality of thyroxine-binding globulin (TBG-San Diego) with decreased affinity for thyroxine and triiodothyronine. *J Clin Endocrinol Metab* 1989; 68(1):114-119.

16. Burman KD, Bongiovanni R, Garis RK, Wartofsky L, Boehm TM. Measurement of serum T4 concentration by high performance liquid chromatography. *J Clin Endocrinol Metab*, 1981; 53(5): 909-912.

17. Tai SS, Sniegoski LT, Welch MJ. Candidate reference method for total thyroxine in human serum: use of isotope-dilution liquid chromatography-mass spectrometry with electrospray ionization. *Clin Chem* 2002; 48(4):637-642.

18. Thienpont LM, De Brabandere VI, Stockl D, De Leenheer AP. Development of a new method for the determination of thyroxine in serum based on isotope dilution gas chromatography mass spectrometry. *Biol Mass Spectrom* 1994; 23(8): 475-482.

19. Thienpont LM, Fierens C, De Leenheer AP, Przywara L. Isotope dilution-gas chromatography/mass spectrometry and liquid chromatography/electrospray ionization-tandem mass spectrometry for the determination of triiodo-L-thyronine in serum. *Rapid Commun Mass Spectrom* 1999; 13(19):1924-1931.

20. De Brabandere VI, Hou P, Stockl D, Thienpont LM, De Leenheer AP. Isotope dilution-liquid chromatography/electrospray ionization-tandem mass spectrometry for the determination of serum thyroxine as a potential reference method. *Rapid Commun Mass Spectrom* 1998; 12(16): 1099-1103.

21. Ramsden, D.B. and M.J. Farmer, Development of a gas chromatographic selected ion monitoring assay for thyroxine (T4) in human serum. *Biomed Mass Spectrom* 1984; 11(8):421-427.

22. Nishinaga A, Cahnmann HJ, Kon H, Matsuura T. Model reactions for the biosynthesis of thyroxine. XII. The nature of a thyroxine precursor formed in the synthesis of thyroxine from diiodotyrosine and its keto acid analog. *Biochemistry* 1968; 7(1):388-397.

23. Choi MH, Kim, JN, Chung BC. Rapid HPLC-Electrospray Tandem Mass Spectrometric Assay for Urinary Testosterone and Dihydrosterone Glucuronides from Patients with Benign Prostate Hyperplasia. *Clin Chem* 2003;49(2): 22-325.

24. Biancotto G, Angeletti R, Traldi P, Silvestri MS, Guidugli F. Determination of 17 $\beta$ -Estradiol in Bovine Plasma: Development of a Highly Sensitive Technique by Ion Trap Gas Chromatography-Tandem Mass Spectrometry Using Negative Ion Chemical Ionization. *J Mass Spectrom* 2002;37: 1226-1271.

25. Lai CC, Tsai CH, Tsai FJ, Wu JY, Lin WD, Lee CC. Rapid Screening Assay of Congenital Adrenal Hyperplasia by Measuring 17 $\alpha$ -Hydroxy- progesterone with High-Performance Liquid Chromatography/ Electrospray Ionization Tandem Mass Spectrometry From Dried Blood Spots. *J Clin Lab Anal* 2002;16: 20-25.

26. Vierhapper H, Nowotny P, Waldausl W. Reduced Production Rates of Testosterone and Dihydrosterone in Healthy Men Treated with Rosiglitazone. *Metabolism* 2000;52(2): 230-232.

27. Nelson JC, Wilcox RB. Analytical performance of free and total thyroxine assays. *Clin Chem* 1996; 42(1):146-154.

28. Evans SE, Burr WA, Hogan TC. A reassessment of 8-anilino-1-naphthalene sulphonic acid as a thyroxine binding inhibitor in the radioimmunoassay of thyroxine. *Ann Clin Biochem* 1977; 14(6):330-334.

**Claims**

1. A method for mass spectrometric analysis of a 100 $\mu$ l sample possibly containing one or more thyroid hormones, consisting of the steps:

- (a) deproteinating a sample containing one or more thyroid hormones;
- (b) separating by liquid chromatography the one or more thyroid hormones from the sample; and
- (c) analyzing the one or more thyroid hormones using a mass spectrometer.

2. The method according to claim 1, wherein the one or more thyroid hormones are selected from T3, T4 and FT4.

3. The method according to claim 1 or 2, wherein the sample possibly containing one or more thyroid hormones is obtained from a biological sample selected from blood, plasma, serum, urine and saliva.

4. The method of claim 3, wherein the biological sample is blood.

**EP 1 644 743 B1**

5. The method of claim 3, wherein the biological sample is plasma.
6. The method of claim 3, wherein the biological sample is serum.
- 5 7. The method of claim 3, wherein the biological sample is urine.
8. The method of claim 3, wherein the biological sample is saliva.
9. The method according to any one of the preceding claims, wherein said step of deproteinating the sample comprises:  
10 (a) adding acetonitrile, containing internal standards;  
(b) vortexing the sample; and  
(c) subjecting the sample to centrifugation.
- 15 10. The method according to any one of claims 1 to 8, wherein said step of deproteinating the sample comprises subjecting the sample to precipitation with an agent containing internal standards, said agent selected from methanol, ethanol and salt.
11. The method according to any one of the preceding claims, wherein said step of separating the one or more thyroid  
20 hormones from the sample comprises introducing the sample to a liquid chromatography apparatus and subsequently eluting the thyroid hormones from the column.
12. The method according to claim 11, wherein said step of separating the one or more thyroid hormones from the  
25 sample comprises the use of a C-18 column.
13. The method according to any one of claims 1 to 10, wherein said step of separating the one or more thyroid hormones  
from the sample comprises the use of a combined liquid chromatography spectrometry apparatus.
14. The method according to claim 13, wherein the one or more thyroid hormones are introduced into a mass spectrometer  
30 directly after being separated from the sample by way of an on-line extraction and use of a built-in switch valve.
15. The method according to any one of the preceding claims, wherein the mass spectrometer is a liquid chromatography-  
tandem-mass spectrometer.
- 35 16. The method according to claim 15, wherein the liquid chromatography-tandem mass spectrometer is equipped with  
an electrospray ionization source.
17. The method according to any one of the preceding claims, wherein said step of analyzing the one or more thyroid  
40 hormones using a mass spectrometer comprises an ionization technique selected from photoionization, electrospray  
ionization, atmospheric pressure chemical ionization and electron capture ionization.
18. The method according to claim 17, wherein said ionization technique is electrospray ionization.
19. The method according to claim 17, wherein said ionization is performed in positive mode.
- 45 20. The method according to claim 17, wherein said ionization is performed in negative mode.
21. The method according to any one of claims 1 to 16, wherein said step of analyzing the one or more thyroid hormones  
using a mass spectrometer comprises multiple reaction monitoring.
- 50 22. The method according to any one of the preceding claims, wherein said step of analyzing the one or more thyroid  
hormones using a mass spectrometer comprises selected ion monitoring.
23. The method according to any one of the preceding claims, wherein the sample comprises a plurality of thyroid  
55 hormones and they are analyzed simultaneously.
24. The method according to any one of claims 1 to 22, wherein the sample comprises a plurality of thyroid hormones  
and they are analyzed sequentially.

## EP 1 644 743 B1

25. The method according to any one of claims 1 to 14, wherein the sample is analyzed by isotope dilution tandem mass spectrometry.
- 5 26. A method for mass spectrometric analysis of a 700 $\mu$ l sample possibly containing one or more thyroid hormones and one or more steroid hormones, consisting of the steps:
- (a) deproteinating a sample containing one or more thyroid hormones;
  - (b) separating by liquid chromatography the one or more thyroid hormones and the one or more steroid hormones from the sample; and
  - 10 (c) analyzing the one or more thyroid hormones and the one or more steroid hormones using a mass spectrometer.
27. The method according to claim 26, wherein the thyroid hormones are selected from T3, T4 and FT4.
- 15 28. The method according to claim 26, wherein the steroid hormones are selected from Dehydroepiandrosterone (DHEA), Dehydroepiandrosterone sulphate (DHEAS), Aldosterone, Cortisol, 11-Deoxycortisol, Androstenedione, Testosterone, Estradiol, 17-OH Progesterone, Progesterone, Allopregnanolone, 16-OH Estrone, 2-OH Estrone, Estrone and Estriol. 1.
- 20 29. The method according to claim 26, wherein the sample is obtained from a biological sample selected from blood, plasma, serum, urine and saliva.
- 30 30. The method of claim 29, wherein the biological sample is blood.
- 25 31. The method of claim 29, wherein the biological sample is plasma.
32. The method of claim 29, wherein the biological sample is serum.
33. The method of claim 29, wherein the biological sample is urine.
- 30 34. The method of claim 29, wherein the biological sample is saliva.
- 35 35. The method according to claim 26, wherein said step of deproteinating the sample comprises:
- (a) adding acetonitrile, containing internal standards;
  - (b) vortexing the sample; and
  - (c) subjecting the sample to centrifugation.
- 40 36. The method according to any one of claims 26 to 34, wherein said step of deproteinating the sample comprises subjecting the sample to precipitation with an agent containing internal standards, said agent selected from methanol, ethanol and salt.
- 45 37. The method according to any one of claims 26 to 36, wherein said step of separating the one or more thyroid hormones and the one or more steroid hormones from the sample comprises introducing the sample to a liquid chromatography apparatus and subsequently eluting the hormones from the column.
38. The method according to claim 37, wherein said step of separating the one or more thyroid hormones and the one or more steroid hormones from the sample comprises the use of C-18 column.
- 50 39. The method according to any one of claims 26 to 36, wherein said step of separating the one or more thyroid hormones and the one or more steroid hormones from the sample comprises the use of a combined liquid chromatography spectrometry apparatus.
- 55 40. The method according to claim 39, wherein the one or more thyroid hormones and the one or more steroid hormones are introduced into the mass spectrometer directly after being separated from the sample by way of an on-line extraction and use of a built-in switch valve.
41. The method according to any one of claims 26 to 40, wherein the mass spectrometer is a liquid chromatography-

tandem-mass spectrometer.

- 5
42. The method according to claim 41, wherein the liquid chromatography-tandem mass spectrometer is equipped with an atmospheric pressure ionization source.
- 10
43. The method according to claim 41, wherein the liquid chromatography-tandem mass spectrometer is equipped with an electrospray ionization source.
44. The method according to any one of claims 26 to 43, wherein said step of analyzing the one or more thyroid hormones and the one or more steroid hormones using a mass spectrometer comprises an ionization technique selected from photoionization, electrospray ionization, atmospheric pressure chemical ionization and electron capture ionization.
- 15
45. The method according to claim 44, wherein said ionization is performed in positive mode.
46. The method according claim 44, wherein said ionization is performed in negative mode.
47. The method according to claim 44, wherein said ionization is performed in positive mode or negative mode.
- 20
48. The method according to any one of claims 26 to 47, wherein said step of analyzing the one or more thyroid hormones and the one or more steroid hormones using a mass spectrometer comprises multiple reaction monitoring.
49. The method according to any one of claims 26 to 47, wherein said step of analyzing the one or more thyroid hormones and the one or more steroid hormones using a mass spectrometer comprises selected ion monitoring.
- 25
50. The method according to any one of claims 26 to 49, wherein the sample comprises a plurality of thyroid hormones and a plurality of steroid hormones, and they are analyzed sequentially.
- 30
51. The method according to any one of claims 26 to 50, wherein the sample is analyzed by isotope dilution tandem mass spectrometry.

### Patentansprüche

- 35
1. Verfahren zur massenspektrometrischen Analyse einer 100- $\mu$ l-Probe, welche möglicherweise ein oder mehrere Thyroidhormon(e) enthält, welches aus den Schritten besteht:
- (a) Deproteinisieren einer Probe, welche ein oder mehrere Thyroidhormon(e) enthält,  
(b) Abtrennen des Thyroidhormons oder der Thyroidhormone von der Probe durch Flüssigkeitschromatographie, und  
40 (c) Analysieren des Thyroidhormons oder der Thyroidhormone mit Hilfe eines Massenspektrometers.
2. Verfahren gemäß Anspruch 1, wobei das eine Thyroidhormon oder die mehreren Thyroidhormone ausgewählt ist/sind aus T3, T4 und FT4.
- 45
3. Verfahren gemäß Anspruch 1 oder 2, wobei die Probe, welche möglicherweise ein oder mehrere Thyroidhormon(e) enthält, aus einer biologischen Probe, ausgewählt aus Blut, Plasma, Serum, Urin und Speichel, erhalten wird.
4. Verfahren gemäß Anspruch 3, wobei die biologische Probe Blut ist.
- 50
5. Verfahren gemäß Anspruch 3, wobei die biologische Probe Plasma ist.
6. Verfahren gemäß Anspruch 3, wobei die biologische Probe Serum ist.
7. Verfahren gemäß Anspruch 3, wobei die biologische Probe Urin ist.
- 55
8. Verfahren gemäß Anspruch 3, wobei die biologische Probe Speichel ist.
9. Verfahren gemäß einem der vorangehenden Ansprüche, wobei der Schritt der Deproteinisierung der Probe umfasst:

## EP 1 644 743 B1

- (a) Hinzufügen von Acetonitril, welches interne Standards enthält,
- (b) Vortexen der Probe, und
- (c) die Probe der Zentrifugation unterziehen.

- 5     **10.** Verfahren gemäß einem der Ansprüche 1 bis 8, wobei der Schritt der Deproteinisierung der Probe Unterziehen der Probe einer Fällung mit einem Agens umfasst, welches interne Standards enthält, wobei das Agens ausgewählt wird aus Methanol, Ethanol und Salz.
- 10    **11.** Verfahren gemäß einem der vorangehenden Ansprüche, wobei der Schritt des Abtrennens des einen Thyroidhormons oder der mehreren Thyroidhormone von der Probe das Einführen der Probe in eine Flüssigkeitschromatographie-Vorrichtung und das nachfolgende Eluieren der Thyroidhormone von der Säule umfasst.
- 15    **12.** Verfahren gemäß Anspruch 11, wobei der Schritt des Abtrennens des einen Thyroidhormons oder der mehreren Thyroidhormone von der Probe die Verwendung einer C-18-Säule umfasst.
- 20    **13.** Verfahren gemäß einem der Ansprüche 1 bis 10, wobei der Schritt des Abtrennens des einen Thyroidhormons oder der mehreren Thyroidhormone von der Probe die Verwendung einer kombinierten Flüssigkeitschromatographie-Spektrometrie-Vorrichtung umfasst.
- 25    **14.** Verfahren gemäß Anspruch 13, wobei das eine Thyroidhormon oder die mehreren Thyroidhormone direkt in ein Massenspektrometer eingeführt wird/werden, nachdem es/sie von der Probe durch eine On-line-Extraktion und die Verwendung eines eingebauten Schaltventils abgetrennt worden ist/sind.
- 30    **15.** Verfahren gemäß einem der vorangehenden Ansprüche, wobei das Massenspektrometer ein Flüssigkeitschromatographie-Tandem-Massenspektrometer ist.
- 35    **16.** Verfahren gemäß Anspruch 15, wobei das Flüssigkeitschromatographie-Tandem-Massenspektrometer mit einer Elektrospray-Ionierungsquelle ausgestattet ist.
- 40    **17.** Verfahren gemäß einem der vorangehenden Ansprüche, worin der Schritt des Analysierens des einen Thyroidhormons oder der mehreren Thyroidhormone mit Hilfe eines Massenspektrometers eine Ionisierungstechnik, ausgewählt aus Photoionisierung, Elektrospray-Ionisierung, chemischer Ionisierung unter atmosphärischem Druck und Elektronen-Einfang-Ionisierung umfasst.
- 45    **18.** Verfahren gemäß Anspruch 17, wobei die Ionisierungstechnik Elektrospray-Ionisierung ist.
- 50    **19.** Verfahren gemäß Anspruch 17, wobei die Ionisierung im positiven Modus durchgeführt wird.
- 55    **20.** Verfahren gemäß Anspruch 17, wobei die Ionisierung im negativen Modus durchgeführt wird.
- 21.** Verfahren gemäß einem der Ansprüche 1 bis 16, wobei der Schritt des Analysierens des einen Thyroidhormons oder der mehreren Thyroidhormone mit Hilfe eines Massenspektrometers Multiple-reaction-monitoring umfasst.
- 22.** Verfahren gemäß einem der vorangehenden Ansprüche, wobei der Schritt des Analysierens des einen Thyroidhormons oder der mehreren Thyroidhormone mit Hilfe eines Massenspektrometers Einzelmassenregistrierung umfasst.
- 23.** Verfahren gemäß einem der vorangehenden Ansprüche, wobei die Probe eine Vielzahl von Thyroidhormonen umfasst, und diese gleichzeitig analysiert werden.
- 24.** Verfahren gemäß einem der Ansprüche 1 bis 22, wobei die Probe eine Vielzahl von Thyroidhormonen umfasst, und diese nacheinander analysiert werden.
- 25.** Verfahren gemäß einem der Ansprüche 1 bis 14, wobei die Probe durch Isotopenverdünnungs-Tandem-Massenspektrometrie analysiert wird.
- 26.** Verfahren zur massenspektrometrischen Analyse einer 700- $\mu$ l-Probe, welche möglicherweise ein oder mehrere Thyroidhormon(e) und ein oder mehrere Steroidhormon(e) enthält, bestehend aus den Schritten:

## EP 1 644 743 B1

- (a) Deproteinisieren einer Probe, welche ein oder mehrere Thyroidhormon(e) enthält,  
(b) Abtrennen des einen Thyroidhormons oder der mehreren Thyroidhormone und des einen Steroidhormons oder der mehreren Steroidhormone von der Probe, und  
(c) Analysieren des einen Thyroidhormons oder der mehreren Thyroidhormone und des einen Steroidhormons oder der mehreren Steroidhormone mit Hilfe eines Massenspektrometers.

5

27. Verfahren gemäß Anspruch 26, wobei die Thyroidhormone ausgewählt werden aus T3, T4 und FT4.

10

28. Verfahren gemäß Anspruch 26, wobei die Steroidhormone ausgewählt werden aus Dehydroepiandrosteron (DHEA), Dehydroepiandrosteronsulfat (DHEAS), Aldosteron, Cortisol, 11-Deoxycortisol, Androstendion, Testosteron, Östadiol, 17-OH-Progesteron, Progesteron, Allopregnanolon, 16-OH-Östron, 2-OH-Östron, Östron und Östriol.

15

29. Verfahren gemäß Anspruch 26, wobei die Probe aus einer biologischen Probe, ausgewählt aus Blut, Plasma, Serum, Urin und Speichel, erhalten wird.

30. Verfahren gemäß Anspruch 29, wobei die biologische Probe Blut ist.

31. Verfahren gemäß Anspruch 29, wobei die biologische Probe Plasma ist.

20

32. Verfahren gemäß Anspruch 29, wobei die biologische Probe Serum ist.

33. Verfahren gemäß Anspruch 29, wobei die biologische Probe Urin ist.

25

34. Verfahren gemäß Anspruch 29, wobei die biologische Probe Speichel ist.

35. Verfahren gemäß Anspruch 26, wobei der Schritt des Deproteinisierens der Probe umfasst:

30

- (a) Hinzufügen von Acetonitril, welches interne Standards enthält,  
(b) Vortexen der Probe, und  
(c) Aussetzen der Probe einer Zentrifugation.

35

36. Verfahren gemäß einem der Ansprüche 26 bis 34, wobei der Schritt des Deproteinisierens der Probe Unterziehen der Probe einer Fällung mit einem Agens umfasst, welches interne Standards enthält, wobei das Agens ausgewählt wird aus Methanol, Ethanol und Salz.

40

37. Verfahren gemäß einem der Ansprüche 26 bis 36, wobei der Schritt des Abtrennens des einen Thyroidhormons oder der mehreren Thyroidhormone und des einen Steroidhormons oder der mehreren Steroidhormone von der Probe Einführen der Probe in eine Flüssigkeitschromatographie-Vorrichtung und anschließend das Eluieren der Hormone von der Säule umfasst.

45

38. Verfahren gemäß Anspruch 37, wobei der Schritt des Abtrennens des einen Thyroidhormons oder der mehreren Thyroidhormone und des einen Steroidhormons oder der mehreren Steroidhormone von der Probe die Verwendung einer C-18-Säule umfasst.

50

39. Verfahren gemäß einem der Ansprüche 26 bis 36, wobei der Schritt des Abtrennens des einen Thyroidhormons oder der mehreren Thyroidhormone und des einen Steroidhormons oder der mehreren Steroidhormone von der Probe die Verwendung einer kombinierten Flüssigkeitschromatographie-Spektrometrie-Vorrichtung umfasst.

40. Verfahren gemäß Anspruch 39, wobei das eine Thyroidhormon oder die mehreren Thyroidhormone und das eine Steroidhormon oder die mehreren Steroidhormone direkt in das Massenspektrometer eingeführt werden, nachdem sie von der Probe durch eine On-line-Extraktion und die Verwendung eines eingebauten Schaltventils abgetrennt worden ist/sind.

55

41. Verfahren gemäß einem der Ansprüche 26 bis 40, wobei das Massenspektrometer ein Flüssigkeitschromatographie-Tandem-Massenspektrometer ist.

42. Verfahren gemäß Anspruch 41, wobei das Flüssigkeitschromatographie-Tandem-Massenspektrometer mit einer Atmosphärendruck-Ionisierungsquelle ausgestattet ist.

## EP 1 644 743 B1

43. Verfahren gemäß Anspruch 41, wobei das Flüssigkeitschromatographie-Tandem-Massenspektrometer mit einer Elektrospray-Ionisierungsquelle ausgestattet ist.
- 5 44. Verfahren gemäß einem der Ansprüche 26 bis 43, wobei der Schritt des Analysierens des einen Thyroidhormons oder der mehreren Thyroidhormone und des einen Steroidhormons oder der mehreren Steroidhormone mit Hilfe eines Massenspektrometers eine Ionisierungstechnik, ausgewählt aus Photoionisierung, Elektrospray-Ionisierung, chemischer Ionisierung bei Atmosphärendruck und Elektronen-Einfang-Ionisierung umfasst.
- 10 45. Verfahren gemäß Anspruch 44, wobei die Ionisierung im positiven Modus durchgeführt wird.
46. Verfahren gemäß Anspruch 44, wobei die Ionisierung im negativen Modus durchgeführt wird.
47. Verfahren gemäß Anspruch 44, wobei die Ionisierung im positiven oder im negativen Modus durchgeführt wird.
- 15 48. Verfahren gemäß einem der Ansprüche 26 bis 47, wobei der Schritt des Analysierens des einen Thyroidhormons oder der mehreren Thyroidhormone und des einen Steroidhormons oder der mehreren Steroidhormone mit Hilfe eines Massenspektrometers Multiple-reaction-monitoring umfasst.
- 20 49. Verfahren gemäß einem der Ansprüche 26 bis 47, wobei der Schritt des Analysierens des einen Thyroidhormons oder der mehreren Thyroidhormone oder des einen Steroidhormons oder der mehreren Steroidhormone mit Hilfe eines Massenspektrometers Einzelmassenregistrierung umfasst.
- 25 50. Verfahren gemäß einem der Ansprüche 26 bis 49, wobei die Probe eine Vielzahl von Thyroidhormonen und eine Vielzahl von Steroidhormonen umfasst, und diese nacheinander analysiert werden.
51. Verfahren gemäß einem der Ansprüche 26 bis 50, wobei die Probe durch Isotopenverdünnungs-Tandem-Massenspektrometrie analysiert wird.

### 30 **Revendications**

1. Procédé pour l'analyse par spectrométrie de masse d'un échantillon de 100 µl susceptible de contenir une ou plusieurs hormones thyroïdiennes, consistant en les étapes :
- 35 (a) déprotéination d'un échantillon contenant une ou plusieurs hormones thyroïdiennes ;  
(b) séparation par chromatographie liquide desdites une ou plusieurs hormones thyroïdiennes d'avec l'échantillon ; et  
(c) analyse desdites une ou plusieurs hormones thyroïdiennes à l'aide d'un spectromètre de masse.
- 40 2. Procédé selon la revendication 1, dans lequel lesdites une ou plusieurs hormones thyroïdiennes sont choisies parmi T3, T4 et FT4.
3. Procédé selon la revendication 1 ou 2, dans lequel l'échantillon susceptible de contenir une ou plusieurs hormones thyroïdiennes est obtenu à partir d'un échantillon biologique choisi parmi le sang, le plasma, le sérum, l'urine et la salive.
- 45 4. Procédé selon la revendication 3, dans lequel l'échantillon biologique est du sang.
5. Procédé selon la revendication 3, dans lequel l'échantillon biologique est du plasma.
- 50 6. Procédé selon la revendication 3, dans lequel l'échantillon biologique est du sérum.
7. Procédé selon la revendication 3, dans lequel l'échantillon biologique est de l'urine.
- 55 8. Procédé selon la revendication 3, dans lequel l'échantillon biologique est de la salive.
9. Procédé selon l'une quelconque des revendications précédentes, dans lequel ladite étape de déprotéination de l'échantillon comprend :

## EP 1 644 743 B1

- (a) l'addition d'acétonitrile, contenant des étalons internes ;
- (b) l'agitation de l'échantillon sur un agitateur à vortex ; et
- (c) la soumission de l'échantillon à une centrifugation.

- 5 **10.** Procédé selon l'une quelconque des revendications 1 à 10, dans lequel ladite étape de déprotéination de l'échantillon comprend la soumission de l'échantillon à une précipitation avec un agent contenant des étalons internes, ledit agent étant choisi parmi le méthanol, l'éthanol et un sel.
- 10 **11.** Procédé selon l'une quelconque des revendications précédentes, dans lequel ladite étape de séparation desdites une ou plusieurs hormones thyroïdiennes d'avec l'échantillon comprend l'introduction de l'échantillon dans un appareil de chromatographie liquide et ensuite l'élution des hormones thyroïdiennes à partir de la colonne.
- 12.** Procédé selon la revendication 11, dans lequel ladite étape de séparation desdites une ou plusieurs hormones thyroïdiennes d'avec l'échantillon comprend l'utilisation d'une colonne de C-18.
- 15 **13.** Procédé selon l'une quelconque des revendications 1 à 10, dans lequel ladite étape de séparation desdites une ou plusieurs hormones thyroïdiennes d'avec l'échantillon comprend l'utilisation d'un appareil de chromatographie liquide-spectrométrie combinées.
- 20 **14.** Procédé selon la revendication 13, dans lequel lesdites une ou plusieurs hormones thyroïdiennes sont introduites dans un spectromètre de masse directement après avoir été séparées de l'échantillon au moyen d'une extraction en ligne et de l'utilisation d'une vanne de commutation intégrée.
- 25 **15.** Procédé selon l'une quelconque des revendications précédentes, dans lequel le spectromètre de masse est un spectromètre de masse en tandem-chromatographie liquide.
- 16.** Procédé selon la revendication 15, dans lequel le spectromètre de masse en tandem-chromatographie liquide est muni d'une source d'ionisation par électronébulisation.
- 30 **17.** Procédé selon l'une quelconque des revendications précédentes, dans lequel ladite étape d'analyse desdites une ou plusieurs hormones thyroïdiennes à l'aide d'un spectromètre de masse comprend une technique d'ionisation choisie parmi la photoionisation, l'ionisation par électronébulisation, l'ionisation chimique sous la pression atmosphérique et l'ionisation par capture d'électrons.
- 35 **18.** Procédé selon la revendication 17, dans lequel ladite technique d'ionisation est l'ionisation par électronébulisation.
- 19.** Procédé selon la revendication 17, dans lequel ladite ionisation est effectuée en mode positif.
- 20.** Procédé selon la revendication 17, dans lequel ladite ionisation est effectuée en mode négatif.
- 40 **21.** Procédé selon l'une quelconque des revendications 1 à 16, dans lequel ladite étape d'analyse desdites une ou plusieurs hormones thyroïdiennes à l'aide d'un spectromètre de masse comprend une détection de réactions multiples.
- 45 **22.** Procédé selon l'une quelconque des revendications précédentes, dans lequel ladite étape d'analyse desdites une ou plusieurs hormones thyroïdiennes à l'aide d'un spectromètre de masse comprend une détection d'ions sélectionnés.
- 50 **23.** Procédé selon l'une quelconque des revendications précédentes, dans lequel l'échantillon comprend une pluralité d'hormones thyroïdiennes et ces dernières sont analysées simultanément.
- 24.** Procédé selon l'une quelconque des revendications 1 à 22, dans lequel l'échantillon comprend une pluralité d'hormones thyroïdiennes et ces dernières sont analysées séquentiellement.
- 55 **25.** Procédé selon l'une quelconque des revendications 1 à 14, dans lequel l'échantillon est analysé par spectrométrie de masse en tandem avec dilution d'isotopes.
- 26.** Procédé pour l'analyse par spectrométrie de masse d'un échantillon de 700 µl susceptible de contenir une ou

## EP 1 644 743 B1

plusieurs hormones thyroïdiennes et une ou plusieurs hormones stéroïdiennes, consistant en les étapes :

- 5 (a) déprotéination d'un échantillon contenant une ou plusieurs hormones thyroïdiennes ;  
(b) séparation par chromatographie liquide desdites une ou plusieurs hormones thyroïdiennes et desdites une ou plusieurs hormones stéroïdiennes d'avec l'échantillon ; et  
(c) analyse desdites une ou plusieurs hormones thyroïdiennes et desdites une ou plusieurs hormones stéroïdiennes à l'aide d'un spectromètre de masse.
- 10 **27.** Procédé selon la revendication 26, dans lequel les hormones thyroïdiennes sont choisies parmi T3, T4 et FT4.
- 15 **28.** Procédé selon la revendication 26, dans lequel les hormones stéroïdiennes sont choisies parmi la déhydroépiandrosterone (DHEA), la déhydroépiandrosterone sulfate (DHEAS), l'aldostérone, le cortisol, le 11-désoxycortisol, l'androstènedione, la testostérone, l'estradiol, la 17-OH-progestérone, la progestérone, l'alloprégnanone, la 16-OH-estrone, la 2-OH-estrone, l'estrone et l'estriol.
- 20 **29.** Procédé selon la revendication 26, dans lequel l'échantillon est obtenu à partir d'un échantillon biologique choisi parmi le sang, le plasma, le sérum, l'urine et la salive.
- 30.** Procédé selon la revendication 29, dans lequel l'échantillon biologique est du sang.
- 31.** Procédé selon la revendication 29, dans lequel l'échantillon biologique est du plasma.
- 32.** Procédé selon la revendication 29, dans lequel l'échantillon biologique est du sérum.
- 25 **33.** Procédé selon la revendication 29, dans lequel l'échantillon biologique est de l'urine.
- 34.** Procédé selon la revendication 29, dans lequel l'échantillon biologique est de la salive.
- 30 **35.** Procédé selon la revendication 26, dans lequel ladite étape de déprotéination de l'échantillon comprend :
- (a) l'addition d'acétonitrile, contenant des étalons internes ;  
(b) l'agitation de l'échantillon sur un agitateur à vortex ; et  
(c) la soumission de l'échantillon à une centrifugation.
- 35 **36.** Procédé selon l'une quelconque des revendications 26 à 34, dans lequel ladite étape de déprotéination de l'échantillon comprend la soumission de l'échantillon à une précipitation avec un agent contenant des étalons internes, ledit agent étant choisi parmi le méthanol, l'éthanol et un sel.
- 40 **37.** Procédé selon l'une quelconque des revendications 26 à 36, dans lequel ladite étape de séparation desdites une ou plusieurs hormones thyroïdiennes et desdites une ou plusieurs hormones stéroïdiennes d'avec l'échantillon comprend l'introduction de l'échantillon dans un appareil de chromatographie liquide et ensuite l'élution des hormones thyroïdiennes à partir de la colonne.
- 45 **38.** Procédé selon la revendication 37, dans lequel ladite étape de séparation desdites une ou plusieurs hormones thyroïdiennes et desdites une ou plusieurs hormones stéroïdiennes d'avec l'échantillon comprend l'utilisation d'une colonne de C-18.
- 50 **39.** Procédé selon l'une quelconque des revendications 26 à 36, dans lequel ladite étape de séparation desdites une ou plusieurs hormones thyroïdiennes et desdites une ou plusieurs hormones stéroïdiennes d'avec l'échantillon comprend l'utilisation d'un appareil de chromatographie liquide-spectrométrie combinées.
- 40.** Procédé selon la revendication 39, dans lequel lesdites une ou plusieurs hormones thyroïdiennes et lesdites une ou plusieurs hormones stéroïdiennes sont introduites dans le spectromètre de masse directement après avoir été séparées de l'échantillon au moyen d'une extraction en ligne et de l'utilisation d'une vanne de commutation intégrée.
- 55 **41.** Procédé selon l'une quelconque des revendications 26 à 40, dans lequel le spectromètre de masse est un spectromètre de masse en tandem-chromatographie liquide.

## EP 1 644 743 B1

42. Procédé selon la revendication 41, dans lequel le spectromètre de masse en tandem-chromatographie liquide est muni d'une source d'ionisation sous la pression atmosphérique.
- 5 43. Procédé selon la revendication 41, dans lequel le spectromètre de masse en tandem-chromatographie liquide est muni d'une source d'ionisation par électronébulisation.
- 10 44. Procédé selon l'une quelconque des revendications 26 à 43, dans lequel ladite étape d'analyse desdites une ou plusieurs hormones thyroïdiennes et desdites une ou plusieurs hormones stéroïdiennes à l'aide d'un spectromètre de masse comprend une technique d'ionisation choisie parmi la photoionisation, l'ionisation par électronébulisation, l'ionisation chimique sous la pression atmosphérique et l'ionisation par capture d'électrons.
- 15 45. Procédé selon la revendication 44, dans lequel ladite ionisation est effectuée en mode positif.
46. Procédé selon la revendication 44, dans lequel ladite ionisation est effectuée en mode négatif.
- 20 47. Procédé selon la revendication 44, dans lequel ladite ionisation est effectuée en mode positif ou en mode négatif.
48. Procédé selon l'une quelconque des revendications 26 à 47, dans lequel ladite étape d'analyse desdites une ou plusieurs hormones thyroïdiennes et desdites une ou plusieurs hormones stéroïdiennes à l'aide d'un spectromètre de masse comprend une détection de réactions multiples.
- 25 49. Procédé selon l'une quelconque des revendications 26 à 47, dans lequel ladite étape d'analyse desdites une ou plusieurs hormones thyroïdiennes et desdites une ou plusieurs hormones stéroïdiennes à l'aide d'un spectromètre de masse comprend une détection d'ions sélectionnés.
- 30 50. Procédé selon l'une quelconque des revendications 26 à 49, dans lequel l'échantillon comprend une pluralité d'hormones thyroïdiennes et une pluralité d'hormones stéroïdiennes et elles sont analysées séquentiellement.
- 35 51. Procédé selon l'une quelconque des revendications 26 à 50, dans lequel l'échantillon est analysé par spectrométrie de masse en tandem avec dilution d'isotopes.
- 40
- 45
- 50
- 55

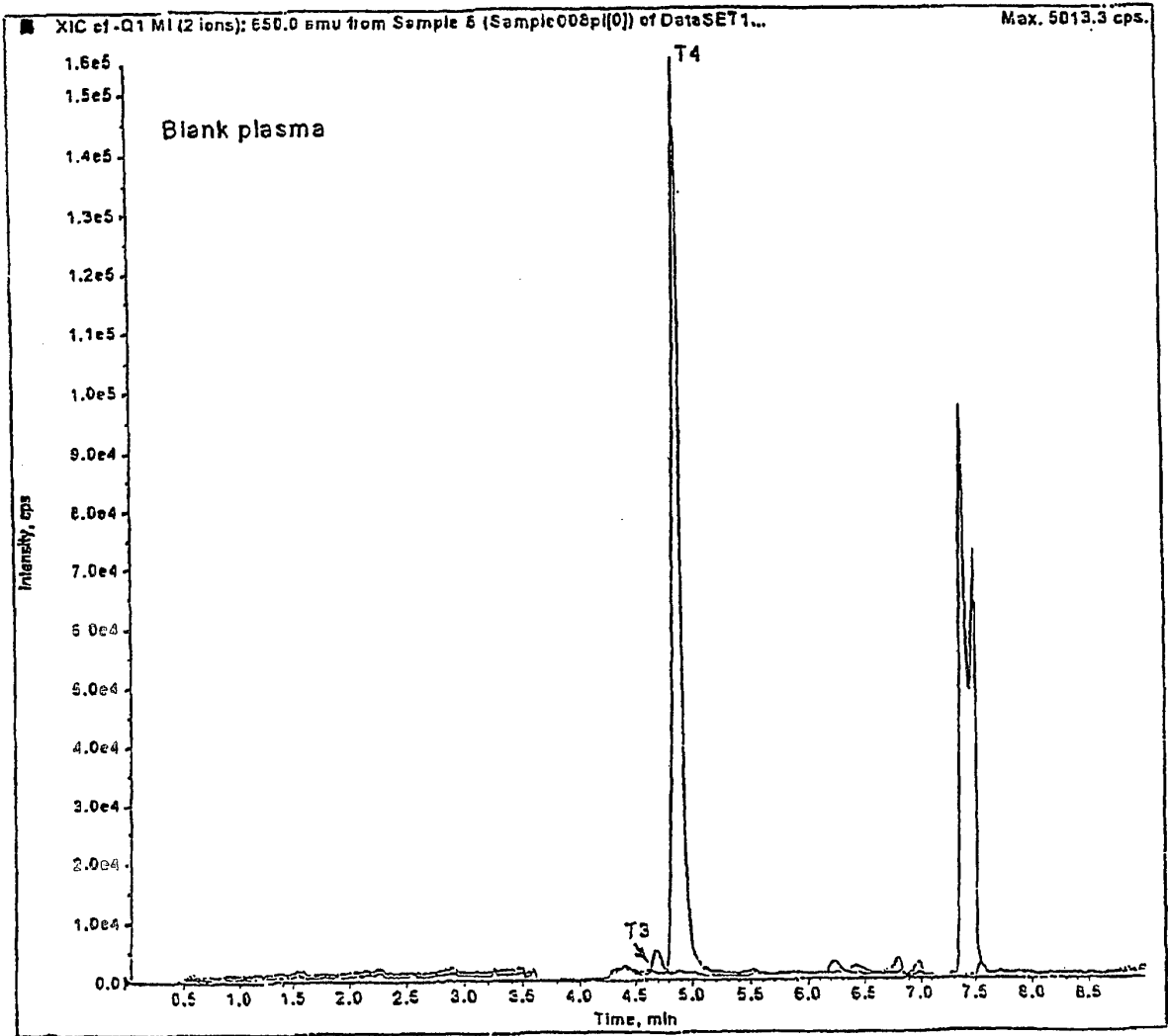


Fig. 1

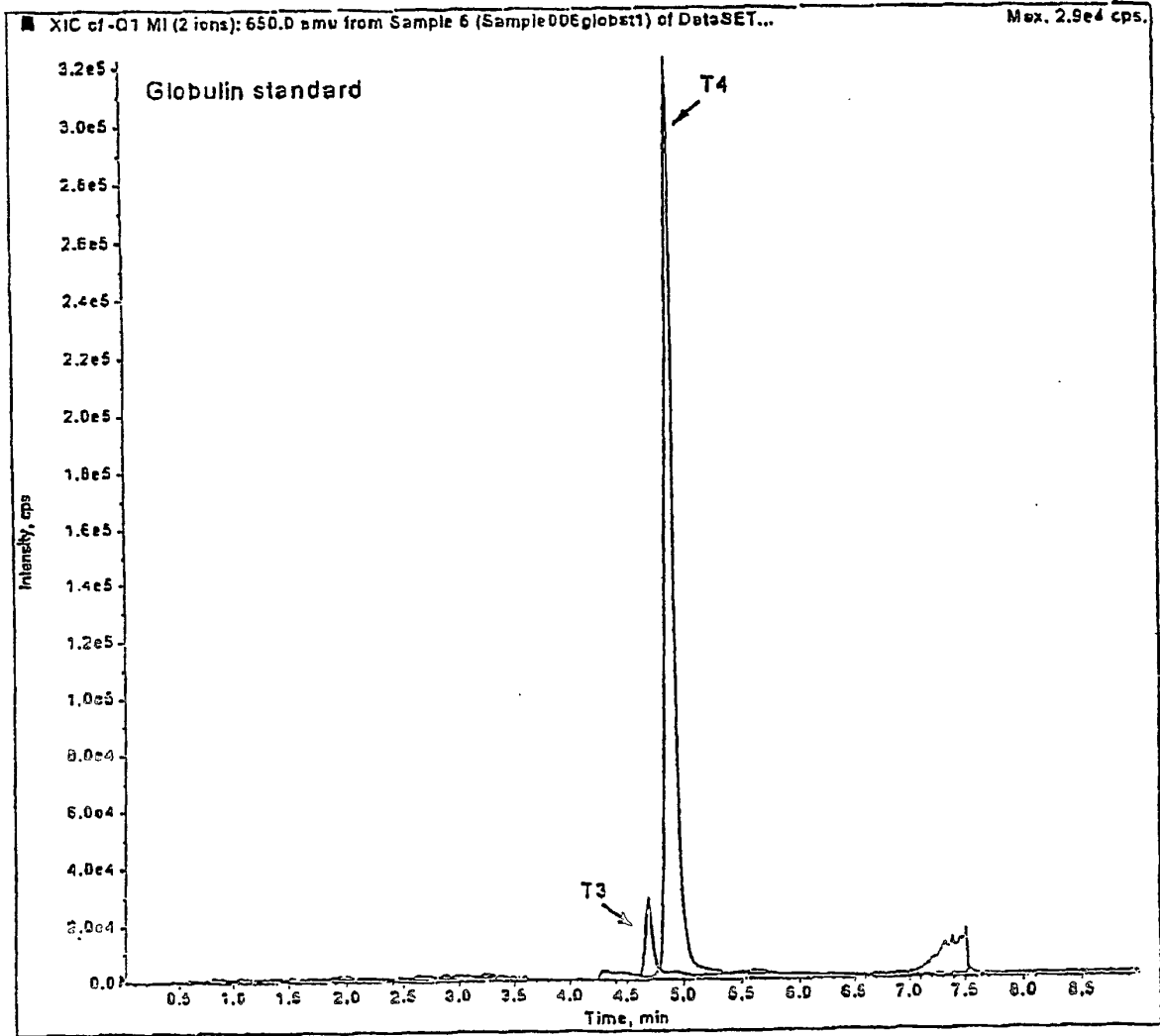


Fig. 2

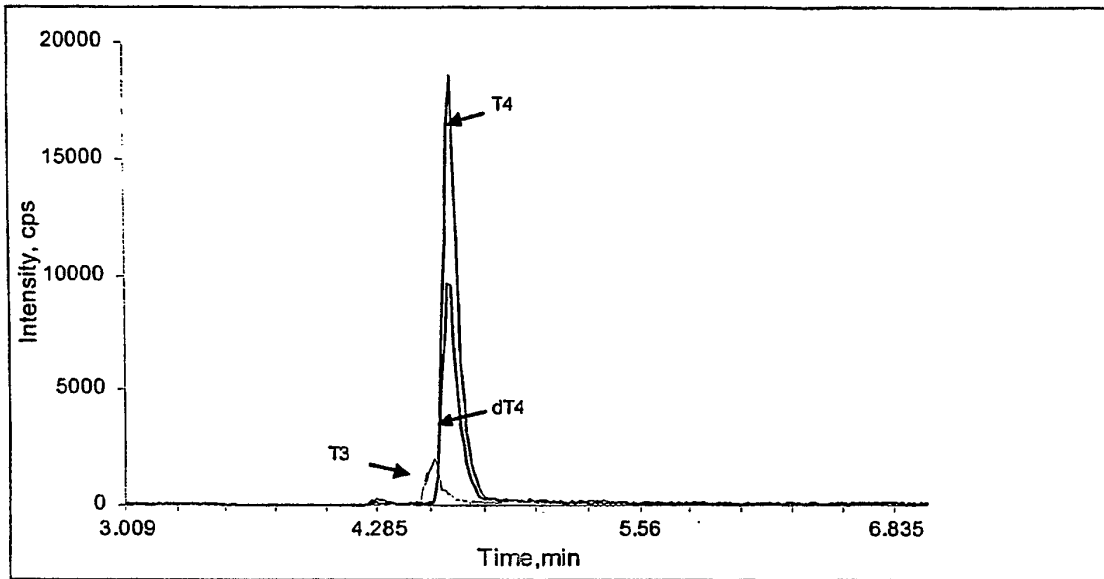


Fig. 3

Tandem mass spectrometric chromatogram for a plasma sample.  
T4 m/z (776/127); D<sub>2</sub>T4 m/z (778/127); T3 m/z (650/127).

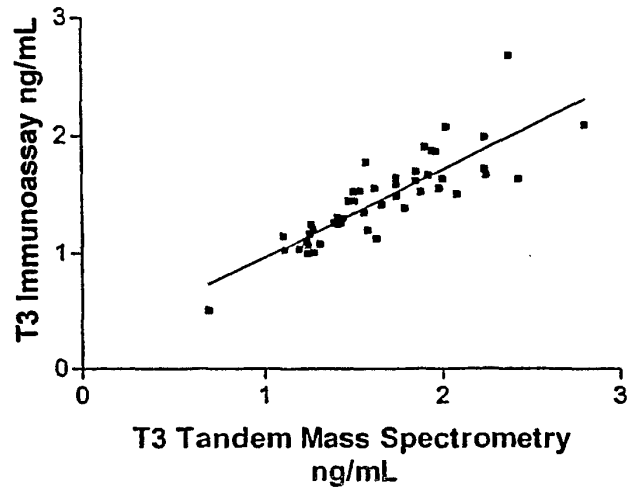


Fig. 4

T3 measured by Isotope dilution tandem mass spectrometry vs. immunoassay.  $IA = 0.75 MS + 0.21$ ;  $r = 0.848$ ;  $S_{y,x} = 0.1956$ ;  $n = 49$

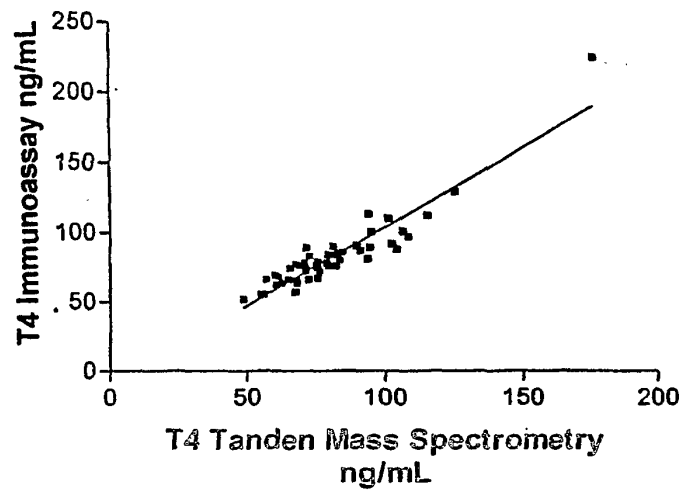


Fig. 5

T4 measured by isotope dilution tandem mass spectrometry vs. immunoassay.  $IA = 1.13 MS - 8.99$ ;  $r = 0.931$ ;  $S_{y,x} = 9.54$ ;  $n=50$

## REFERENCES CITED IN THE DESCRIPTION

This list of references cited by the applicant is for the reader's convenience only. It does not form part of the European patent document. Even though great care has been taken in compiling the references, errors or omissions cannot be excluded and the EPO disclaims all liability in this regard.

## Patent documents cited in the description

- US 4121099 A [0040]
- US 4137750 A [0040]
- US 4328420 A [0040]
- US 4963736 A [0040]
- US 5179278 A [0040]
- US 5248875 A [0040]
- US 5412208 A [0040]
- US 5847386 A [0040]

## Non-patent literature cited in the description

- **LUM SM ; NICOLOFF JT ; SPENCER CA ; KAPTEIN EM.** Peripheral tissue mechanism for maintenance of serum triiodothyronine values in a thyroxine-deficient state in man. *J Clin Invest*, 1984, vol. 73 (2), 570-575 [0073]
- **SAKATA S ; NAKAMURA S ; MIURA K.** Autoantibodies against thyroid hormones or iodothyronine. Implications in diagnosis, thyroid function, treatment, and pathogenesis. *Ann Intern Med*, 1985, vol. 103 (4), 579-589 [0073]
- **BECK-PECCOZ P ; ROMELLI PB ; CATTANEO MG ; FAGLIA G ; WHITE EL ; BARLOW JW ; STOCKIGT JR.** Evaluation of free thyroxine methods in the presence of iodothyronine-binding autoantibodies. *J Clin Endocrinol Metab*, 1984, vol. 58 (4), 736-739 [0073]
- **KLEE GG.** Human anti-mouse antibodies. *Arch Pathol Lab Med*, 2000, vol. 124 (6), 921-923 [0073]
- *College of American Pathologists Proficiency Survey Report on Y-03, RAP-03 and K-06 specimens*, 2003 [0073]
- **SOLDIN SJ.** Digoxin--issues and controversies. *Clin Chem*, 1986, vol. 32, 5-12 [0073]
- **SOLDIN SJ ; PAPANASTASIOU-DIAMANDI A ; HEYES J ; LINGWOOD C ; OLLEY P.** Are immunoassays for digoxin reliable?. *Clin Biochem*, 1984, vol. 17 (5), 317-320 [0073]
- **THONG B ; SOLDIN SJ ; LINGWOOD CA.** Lack of specificity of current anti-digoxin antibodies, and preparation of a new, specific polyclonal antibody that recognizes the carbohydrate moiety of digoxin. *Clin Chem*, 1985, vol. 31 (10), 1625-1631 [0073]
- **MURTHY JN ; DAVIS DL ; YATSCOFF RW ; SOLDIN SJ.** Tacrolimus metabolite cross-reactivity in different tacrolimus assays. *Clin Biochem*, 1998, vol. 31 (8), 613-617 [0073]
- **MURTHY JN ; YATSCOFF RW ; SOLDIN SJ.** Cyclosporine metabolite cross-reactivity in different cyclosporine assays. *Clin Biochem*, 1998, vol. 31 (3), 159-163 [0073]
- **SHEN S ; ELIN RJ ; SOLDIN SJ.** Characterization of cross reactivity by carbamazepine 10,11-epoxide with carbamazepine assays. *Clin Biochem*, 2001, vol. 4 (2), 157-158 [0073]
- **GHOSHAL AK ; SOLDIN SJ.** tacrolimus II assay: is it reliable at low blood concentrations? A comparison with tandem MS/MS. *Clin Biochem*, 2002, vol. 35 (5), 389-392 [0073]
- **SOLDIN SJ ; STEELE BW ; WITTE DL ; WANG E ; ELIN RJ.** Lack of specificity of cyclosporine immunoassays. Results of a College of American Pathologists Study. *Arch Pathol Lab Med*, 2003, vol. 127 (1), 19-22 [0073]
- **DESPRES N ; GRANT AM.** Antibody interference in thyroid assays: a potential for clinical misinformation. *Clin Chem*, 1998, vol. 44 (3), 440-454 [0073]
- **SAME DH ; REFETTOFF S ; NELSON JC ; LINARELLI LG.** A new inherited abnormality of thyroxine-binding globulin (TBG-San Diego) with decreased affinity for thyroxine and triiodothyronine. *J Clin Endocrinol Metab*, 1989, vol. 68 (1), 114-119 [0073]
- **BURMAN KD ; BONGIOVANNI R ; GARIS RK ; WARTOFSKY L. ; BOEHM TM.** Measurement of serum T4 concentration by high performance liquid chromatography. *J Clin Endocrinol Metab*, 1981, vol. 53 (5), 909-912 [0073]
- **TAI SS ; SNIEGOSKI LT ; WELCH MJ.** Candidate reference method for total thyroxine in human serum: use of isotope-dilution liquid chromatography-mass spectrometry with electrospray ionization. *Clin Chem*, 2002, vol. 48 (4), 637-642 [0073]
- **THIENPONT LM ; DE BRABANDERE VI ; STOCKL D ; DE LEENHEER AP.** Development of a new method for the determination of thyroxine in serum based on isotope dilution gas chromatography mass spectrometry. *Biol Mass Spectrom*, 1994, vol. 23 (8), 475-482 [0073]

- **THIENPONT LM ; FIERENS C ; DE LEENHEER AP ; PRZYWARAL.** Isotope dilution-gas chromatography/mass spectrometry and liquid chromatography/electrospray ionization-tandem mass spectrometry for the determination of triiodo-L-thyronine in serum. *Rapid Commun Mass Spectrom*, 1999, vol. 13 (19), 1924-1931 [0073]
- **DE BRABANDERE VI ; HOU P ; STOCKL D ; THIENPONT LM ; DE LEENHEER AP.** Isotope dilution-liquid chromatography/ electrospray ionization-tandem mass spectrometry for the determination of serum thyroxine as a potential reference method. *Rapid Commun Mass Spectrom*, 1998, vol. 12 (16), 1099-1103 [0073]
- **RAMSDEN, D.B. ; M.J. FARMER.** Development of a gas chromatographic selected ion monitoring assay for thyroxine (T4) in human serum. *Biomed Mass Spectrom*, 1984, vol. 11 (8), 421-427 [0073]
- **NISHINAGA A ; CAHNMANN HJ ; KON H ; MATSUURA T.** Model reactions for the biosynthesis of thyroxine. XII. The nature of a thyroxine precursor formed in the synthesis of thyroxine from diiodotyrosine and its keto acid analog. *Biochemistry*, 1968, vol. 7 (1), 388-397 [0073]
- **CHOIMH ; KIM, JN ; CHUNG BC.** Rapid HPLC-Electrospray Tandem Mass Spectrometric Assay for Urinary Testosterone and Dihydrosterone Glucuronides from Patients with Benign Prostate Hyperplasia. *Clin Chem*, 2003, vol. 49 (2), 22-325 [0073]
- **BIANCOTTO G ; ANGELETTI R ; TRALDI P ; SILVESTRIMS ; GUIDUGLIF.** Determination of 17 $\beta$ -Estradiol in Bovine Plasma: Development of a Highly Sensitive Technique by Ion Trap Gas Chromatography-Tandem Mass Spectrometry Using Negative Ion Chemical Ionization. *J Mass Spectrom*, 2002, vol. 37, 1226-1271 [0073]
- **LAI CC ; TSAI CH ; TSAI FJ ; WU JY ; LIN WD ; LEE CC.** Rapid Screening Assay of Congenital Adrenal Hyperplasia by Measuring 17 $\alpha$ -Hydroxyprogesterone with High-Performance Liquid Chromatography/ Electrospray Ionization Tandem Mass Spectrometry From Dried Blood Spots. *J Clin Lab Anal*, 2002, vol. 16, 20-25 [0073]
- **VIERHAPPER H ; NOWOTNY P ; WALDAUSL W.** Reduced Production Rates of Testosterone and Dihydrosterone in Healthy Men Treated with Rosiglitazone. *Metabolism*, 2000, vol. 52 (2), 230-232 [0073]
- **NELSON JC ; WILCOX RB.** Analytical performance of free and total thyroxine assays. *Clin Chem*, 1996, vol. 42 (1), 146-154 [0073]
- **EVANS SE ; BURR WA ; HOGAN TC.** A reassessment of 8-anilino-1-naphthalene sulphonic acid as a thyroxine binding inhibitor in the radioimmunoassay of thyroxine. *Ann Clin Biochem*, 1977, vol. 14 (6), 330-334 [0073]

专利名称(译)	通过质谱分析甲状腺激素		
公开(公告)号	<a href="#">EP1644743B1</a>	公开(公告)日	2014-11-12
申请号	EP2004727197	申请日	2004-04-14
[标]申请(专利权)人(译)	乔治敦大学		
申请(专利权)人(译)	乔治城大学		
当前申请(专利权)人(译)	乔治城大学		
[标]发明人	SOLDIN STEVEN J		
发明人	SOLDIN, STEVEN, J.		
IPC分类号	G01N33/78 C12N9/10 G01N33/53 G01N33/68		
CPC分类号	G01N33/6848 G01N33/78 Y10T436/24		
优先权	60/462745 2003-04-14 US		
其他公开文献	EP1644743A1		
外部链接	<a href="#">Espacenet</a>		

摘要(译)

公开了通过质谱法同时或顺序分析一种或多种激素的方法，系统和试剂盒。该方法需要最小的样本量和最短的准备时间。该方法包括电离激素和通过质谱分析激素。此外，公开了用于同时或顺序分析甲状腺激素的方法，系统和试剂盒，包括使用电喷雾源以负模式电离甲状腺激素。

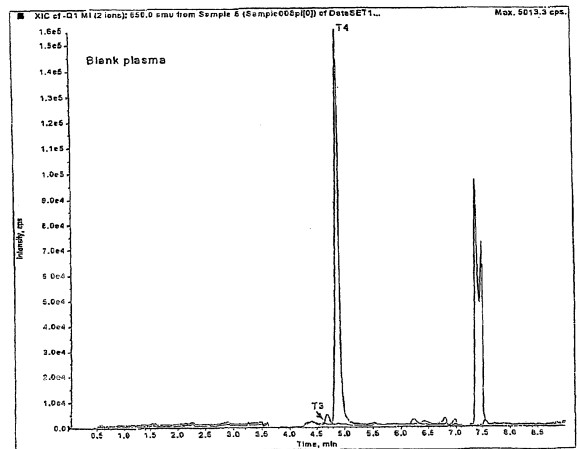


Fig. 1