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(54) **METHOD FOR KIDNEY DISEASE DETECTION**

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Related U.S. Application Data

(60) Division of application No. 10/391,202, filed on Mar. 19, 2003, which is a continuation-in-part of application No. 09/892,797, filed on Jun. 28, 2001, now Pat. No. 6,589,748, which is a continuation-in-part of application No. 09/415,217, filed on Oct. 12, 1999, now Pat. No. 6,447,989.

(30) **Foreign Application Priority Data**

Dec. 21, 1998 (AU) PP7843

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(52) **U.S. Cl.** **435/7.2**; 435/69.1; 435/320.1; 435/325; 530/350; 536/23.5

(57) **ABSTRACT**

A method for diagnosing early stage renal disease and/or renal complications of a disease in which intact albumin is an indicator of the renal disease and/or complications. The method includes an isolated intact protein, an anti-intact protein antibody thereto, and methods for preparing the same.

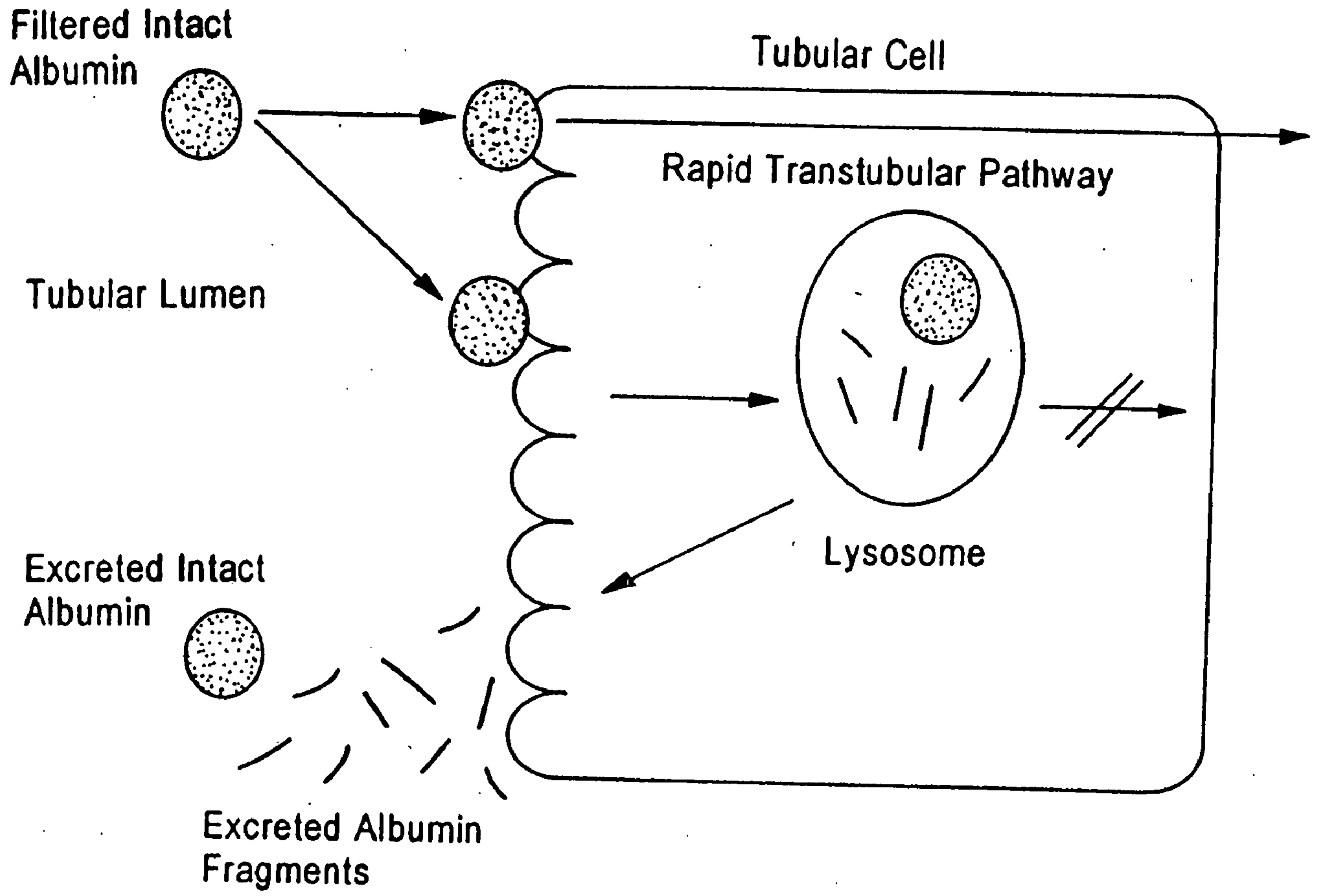

Filtered Intact
Albumin
Tubular Cell
Rapid Transtubular Pathway
Tubular Lumen
Lysosome
Excreted Intact
Albumin
Excreted Albumin
Fragments

FIG. 1

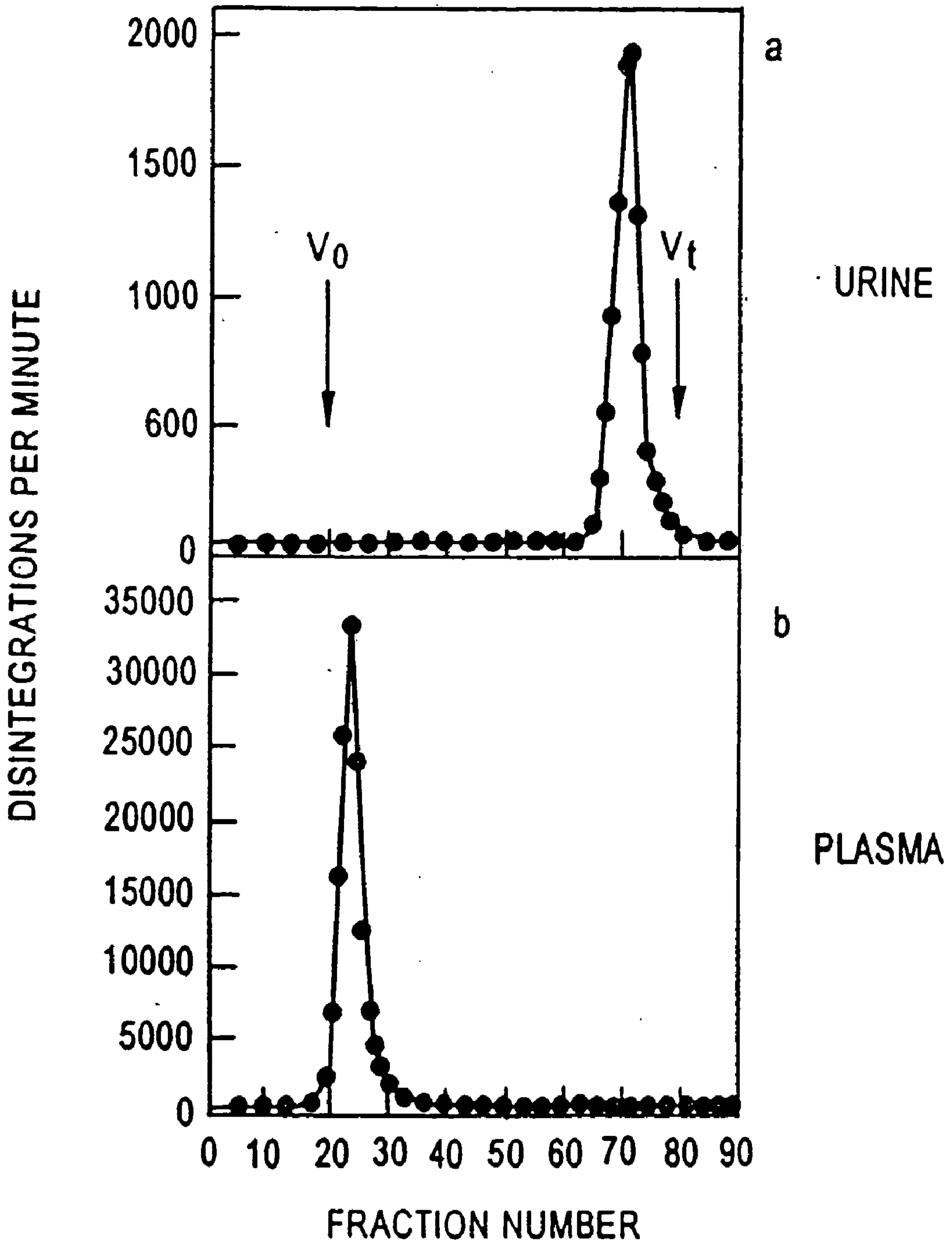

a
URINE
b
PLASMA
DISINTEGRATIONS PER MINUTE
2000
1500
1000
600
0
35000
30000
25000
20000
15000
10000
5000
0
V_0
V_t
0 10 20 30 40 50 60 70 80 90
FRACTION NUMBER

FIG. 2

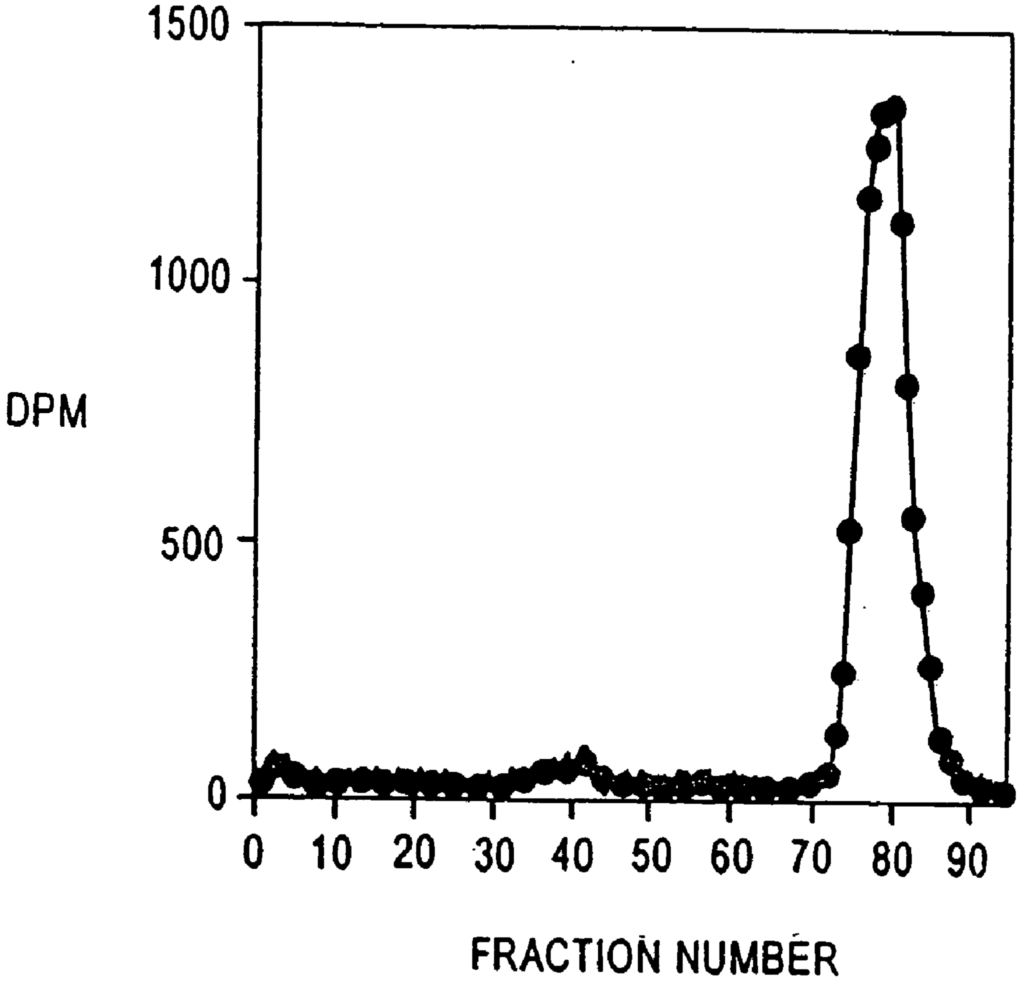


FIG. 3

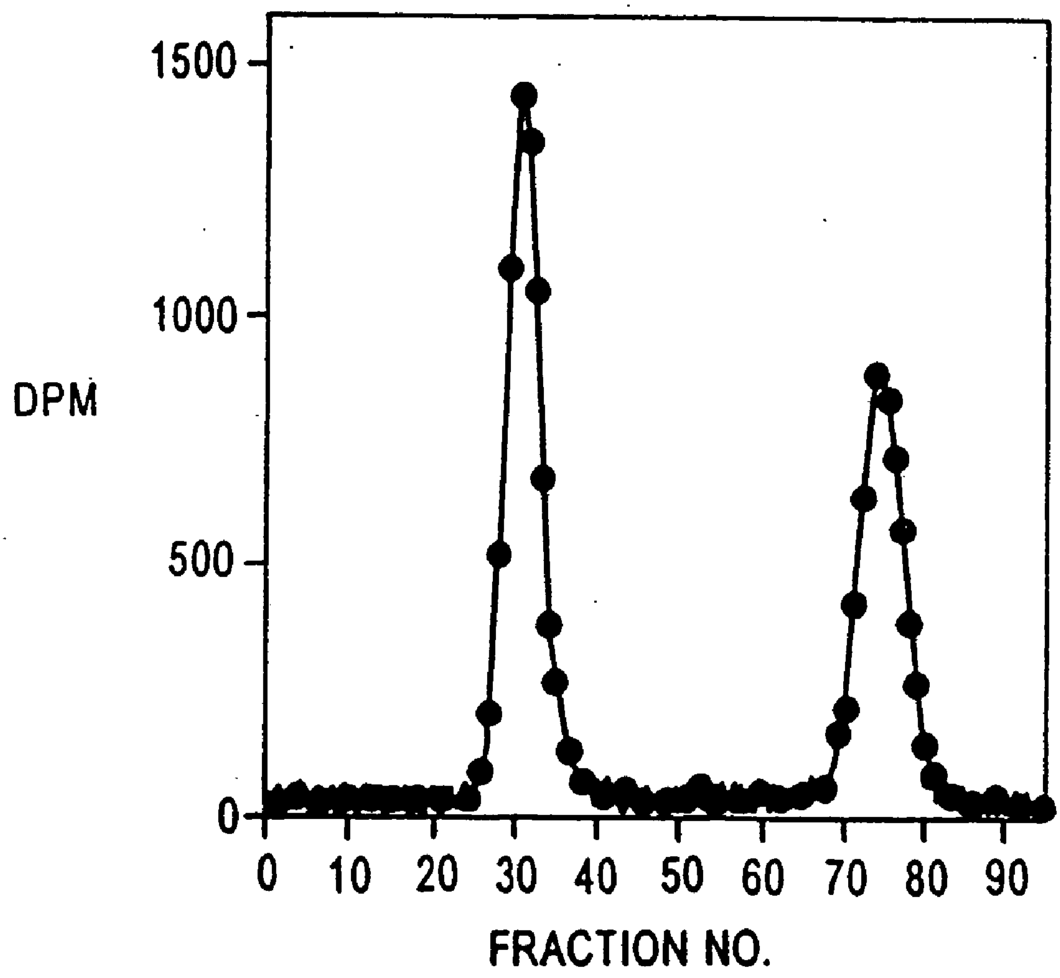
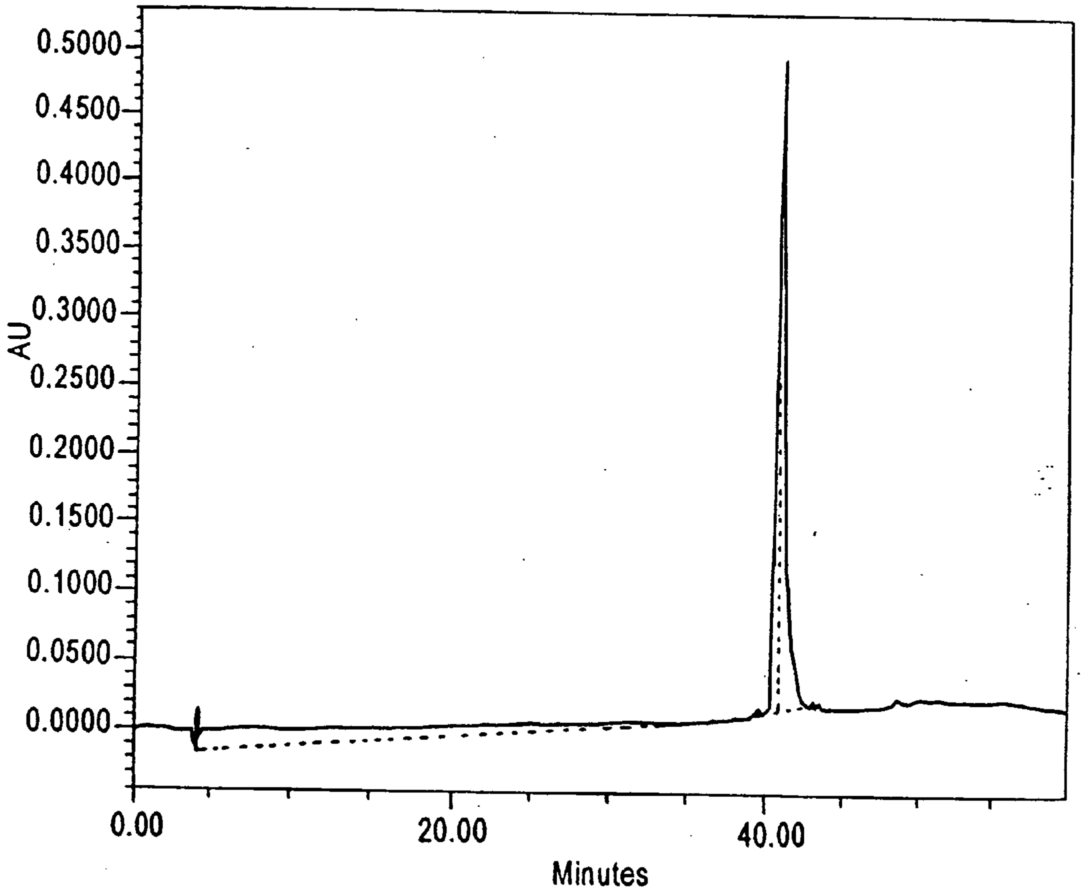


FIG. 4

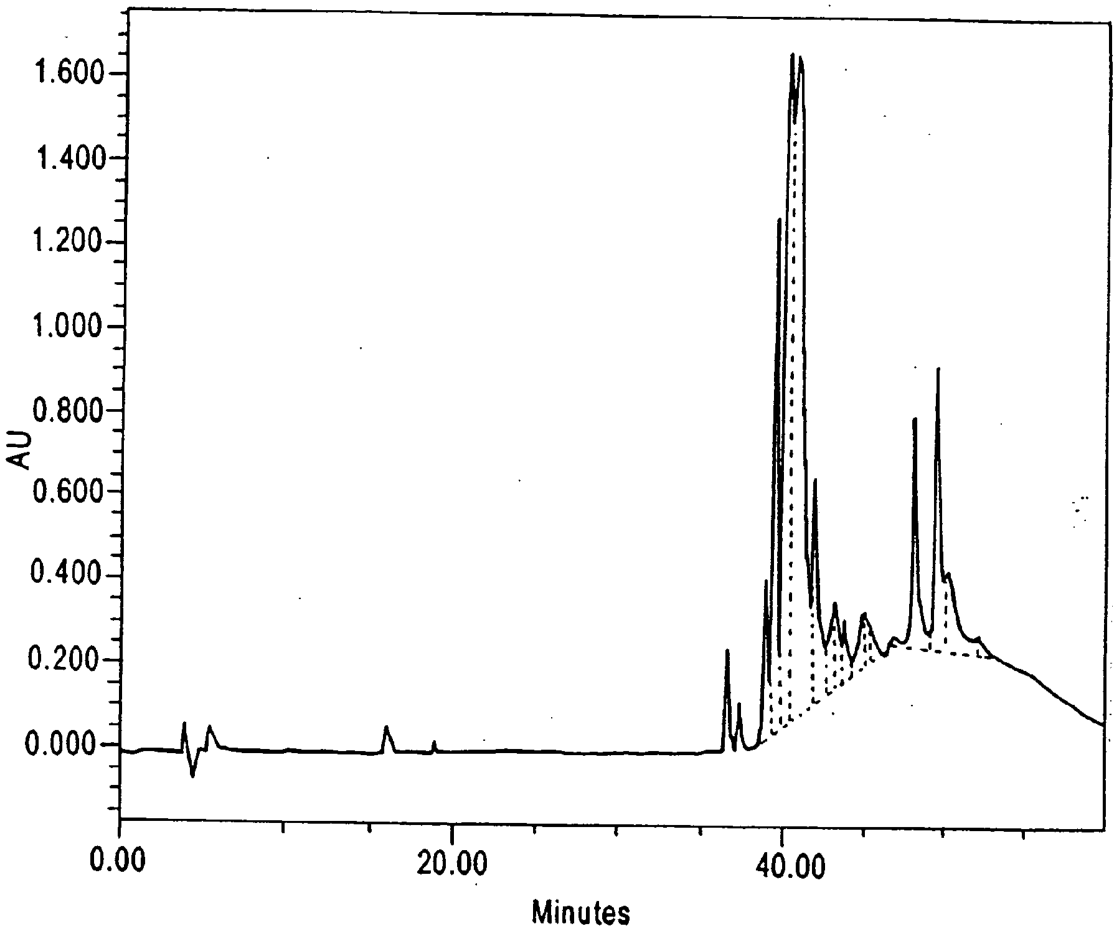
FIG. 5



Peak Results

#	Name	Ret Time (min)	Area (uV*sec)	Height (uV)	Amount	Int Type
1		4.117	329140	40683		BB
2		4.883	15767507	16240		BB
3		39.467	101020	6703		BB
4		40.000	46216	3171		BB
5		40.733	3138458	242228		BV
6		41.017	13063671	486192		VB
7		43.233	62005	5618		BV
8		43.700	33150	2909		VB
9		48.683	76368	3554		BB

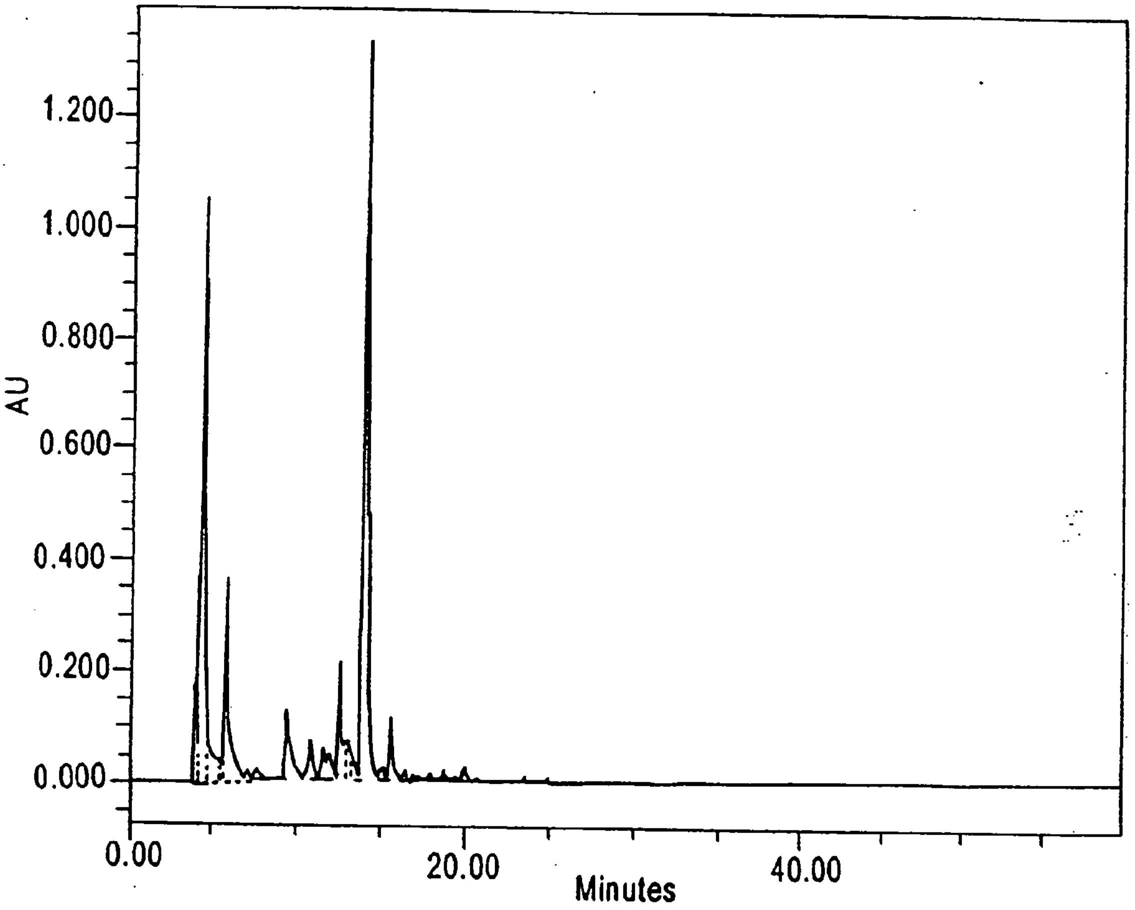
FIG. 6



Peak Results

#	Name	Ret Time (min)	Area (uV*sec)	Height (uV)	Amount	Int Type
1		4.133	1802825	122050		BB
2		4.933	1874787	51415		BV
3		5.550	2903127	94067		VB
4		14.633	31135	3556		BB
5		15.617	54780	5951		BV
6		16.117	1518301	71355		VB
7		18.817	354458	32687		BB
8		28.717	16597	2098		BB
9		31.967	41230	4687		BB
10		32.567	107938	2454		BB
11		36.633	3763801	256381		BV
12		37.383	2135643	127492		VV
13		37.933	3054	972		VB
14		38.233	16226	2010		BB
15		38.950	8424624	387355		BV

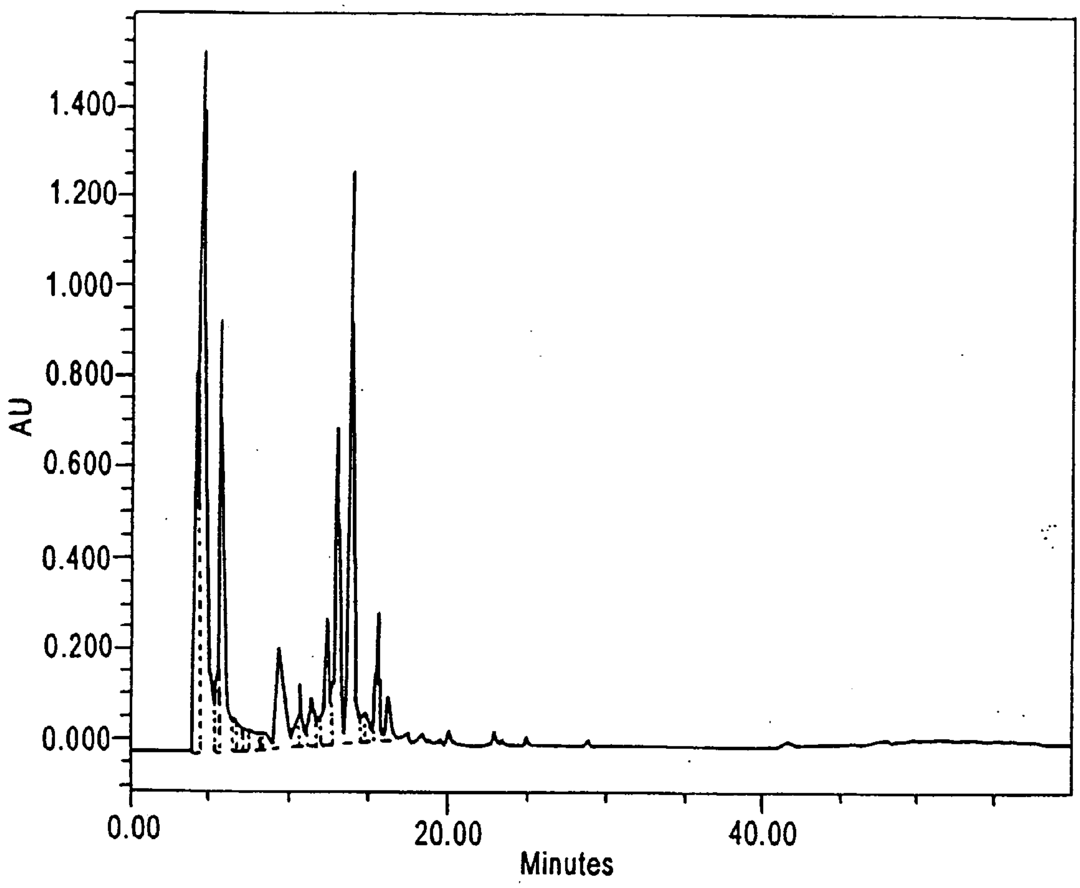
FIG. 7



Peak Results

#	Name	Ret Time (min)	Area (uV*sec)	Height (uV)	Amount	Int Type
1		4.067	2434486	185800		BV
2		4.483	16280311	1058547		VV
3		4.833	3864081	191585		VV
4		5.600	706286	46936		VV
5		5.983	6703449	371298		VV
6		7.133	451038	24299		VV
7		7.617	542790	22245		VB
8		9.600	3068571	128855		BV
9		10.333	151779	12552		VV
10		10.767	284984	25596		VV
11		11.017	919890	80160		VV
12		11.350	108494	10375		VV
13		11.650	738559	61334		VV
14		12.033	705972	48248		VV
15		12.200	429100	36385		VV

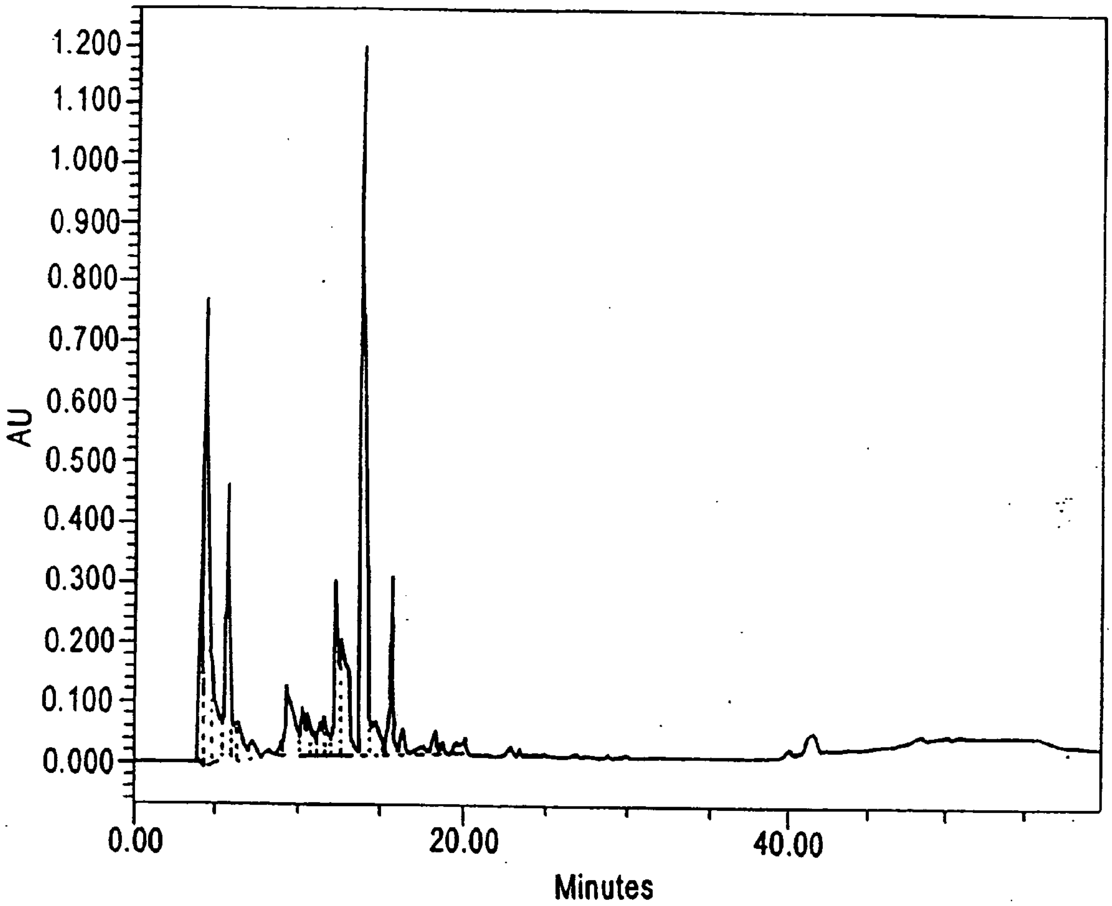
FIG. 8



Peak Results

#	Name	Ret Time (min)	Area (uV*sec)	Height (uV)	Amount	Int Type
1		4.067	11142185	841146		BV
2		4.467	42897717	1567808		VV
3		5.333	2771843	185077		VV
4		5.750	16970479	962668		VV
5		6.433	1506454	80128		VV
6		6.800	1187124	68430		VV
7		7.150	1105355	55641		VV
8		7.500	322563	40826		VV
9		7.633	1108215	42173		VV
10		8.183	522250	36801		VV
11		8.400	1110198	40131		VV
12		9.317	7318394	222305		VV
13		10.400	866046	57467		VV
14		10.667	2300590	148511		VV
15		11.050	399236	32665		VV

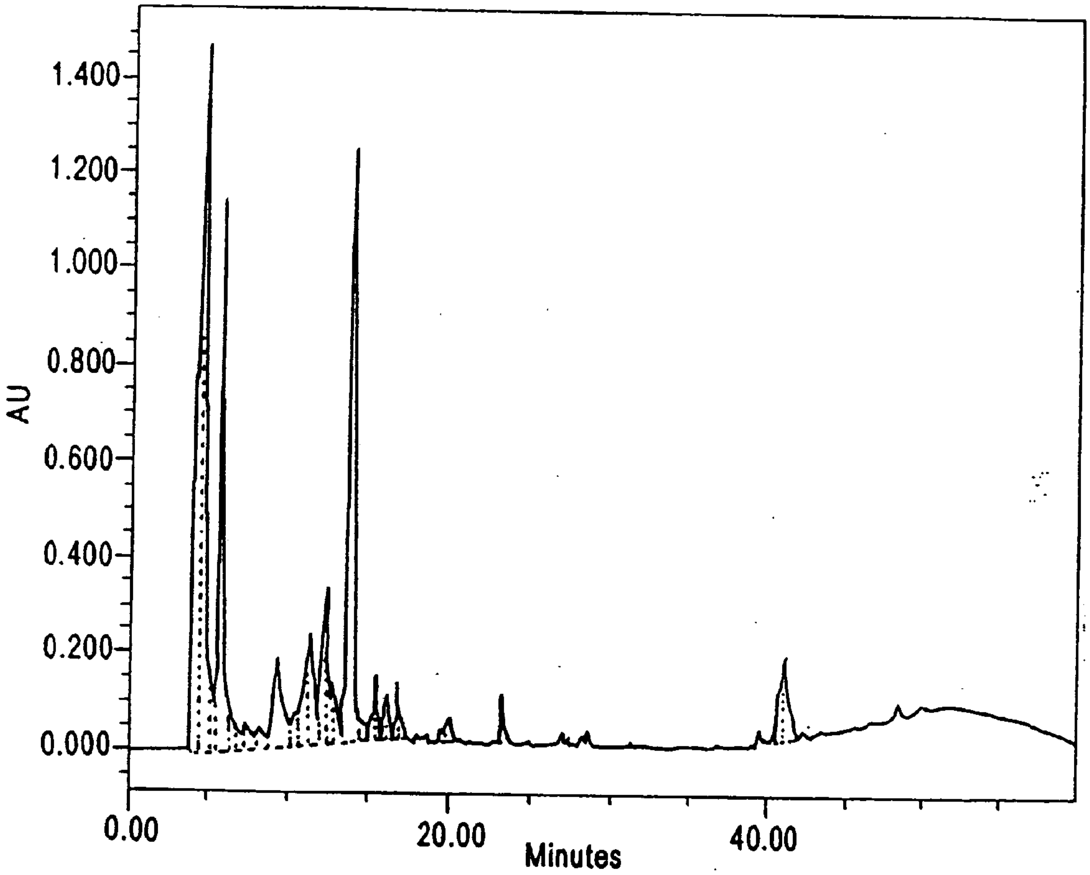
FIG. 9



Peak Results

#	Name	Ret Time (min)	Area (uV*sec)	Height (uV)	Amount	Int Type
1		4.083	3547997	287640		BV
2		4.417	13468709	786559		VV
3		4.767	4659330	247452		VV
4		5.750	8516445	470707		VV
5		6.317	2011722	70109		VV
6		7.200	883132	30217		VB
7		8.150	94619	7076		BB
8		9.017	439910	25556		BV
9		9.517	3872051	120729		VV
10		10.333	1190270	78799		VV
11		10.650	1054310	76753		VV
12		11.033	385789	28539		VV
13		11.333	761432	56564		VV
14		11.700	1033818	69004		VV
15		11.950	384784	35582		VV

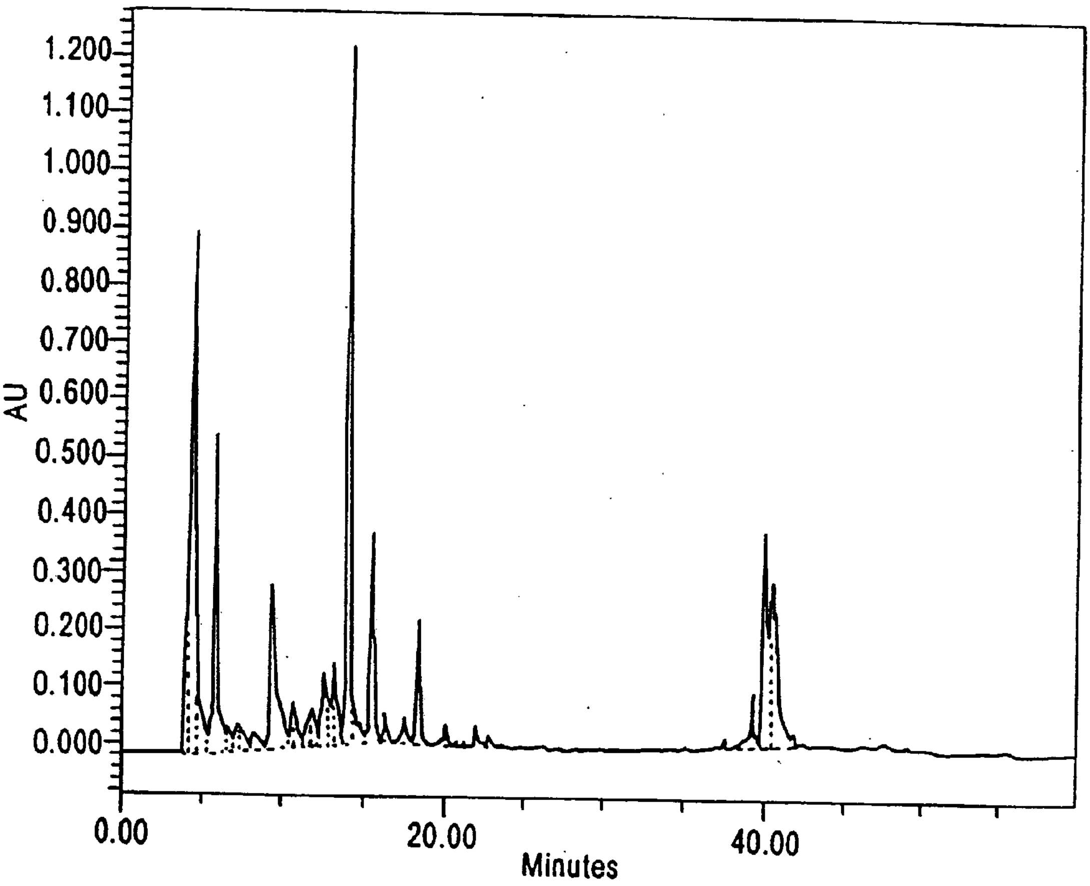
FIG. 10



Peak Results

#	Name	Ret Time (min)	Area (uV*sec)	Height (uV)	Amount	Int Type
1		4.083	10518737	792810		BV
2		4.367	14687929	1410889		VV
3		4.533	22637504	1494339		VV
4		5.163	3593396	219436		VV
5		5.717	22692299	1152174		VV
6		6.817	960777	60477		VV
7		7.150	682488	44596		VV
8		7.500	1795219	58004		VV
9		8.250	1518279	43057		VV
10		9.250	7251773	190341		VV
11		10.367	1000343	60821		VV
12		10.633	1192302	77226		VV
13		11.050	2788523	178468		VV
14		11.417	6160036	248690		VV
15		12.200	4008326	298350		VV

FIG. 11



Peak Results

#	Name	Ret Time (min)	Area (uV*sec)	Height (uV)	Amount	Int Type
1		4.100	2979946	236299		BV
2		4.450	15028113	921410		VV
3		4.817	4812815	249684		VV
4		5.550	814070	71678		VV
5		5.850	9688029	563550		VV
6		6.600	914291	48992		VV
7		7.000	272597	28204		VV
8		7.250	1918070	53043		VV
9		8.267	1161101	29288		VV
10		9.367	9258559	294071		VV
11		10.617	903487	62562		VV
12		10.833	1216206	83275		VV
13		11.250	178790	15563		VV
14		11.550	532841	42407		VV
15		11.867	1315340	70286		VV

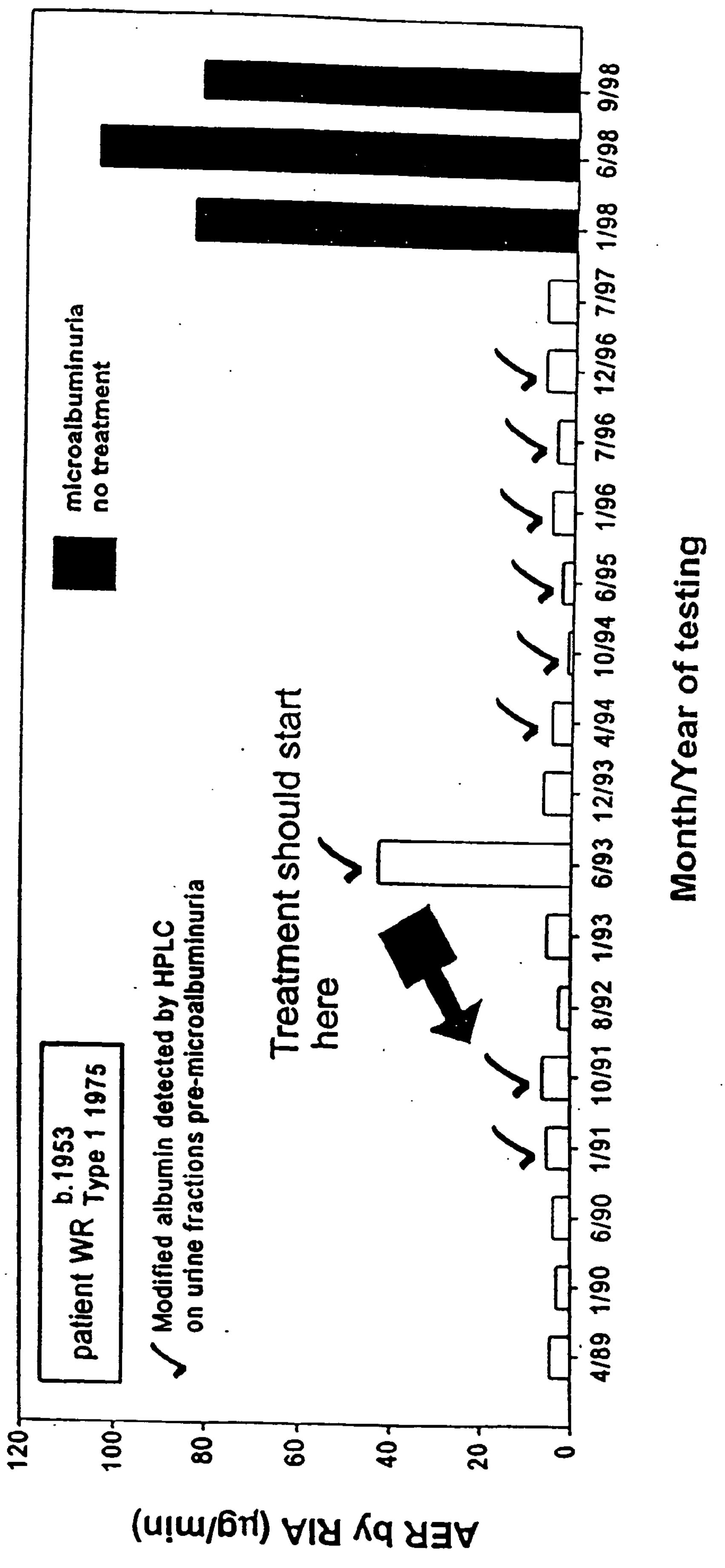

patient WR b.1953 Type 1 1975
✓ Modified albumin detected by HPLC on urine fractions pre-microalbuminuria
microalbuminuria no treatment
Treatment should start here
AER by RIA (μg/min)
120
100
80
60
40
20
0
4/89
1/90
6/90
1/91
10/91
8/92
1/93
6/93
12/93
4/94
10/94
6/95
1/96
7/96
12/96
7/97
1/98
6/98
9/98
Month/Year of testing

FIG. 12

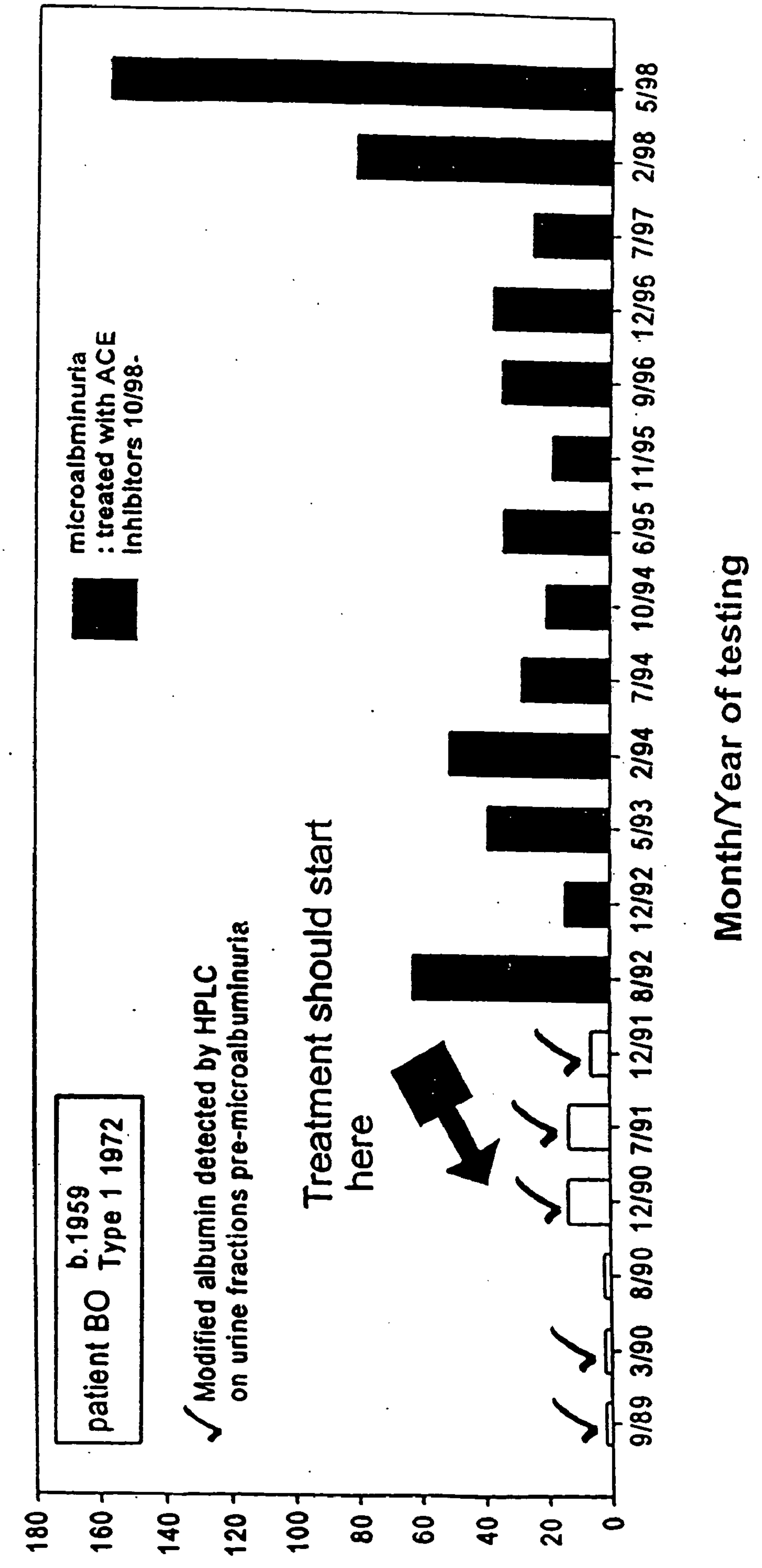

patient BO b.1959
Type 1 1972
✓ Modified albumin detected by HPLC on urine fractions pre-microalbuminuria
Treatment should start here
microalbminuria : treated with ACE inhibitors 10/98-
180
160
140
120
100
80
60
40
20
0
9/89
3/90
8/90
12/90
7/91
12/91
8/92
12/92
5/93
2/94
7/94
10/94
6/95
11/95
9/96
12/96
7/97
2/98
5/98
Month/Year of testing

FIG. 13

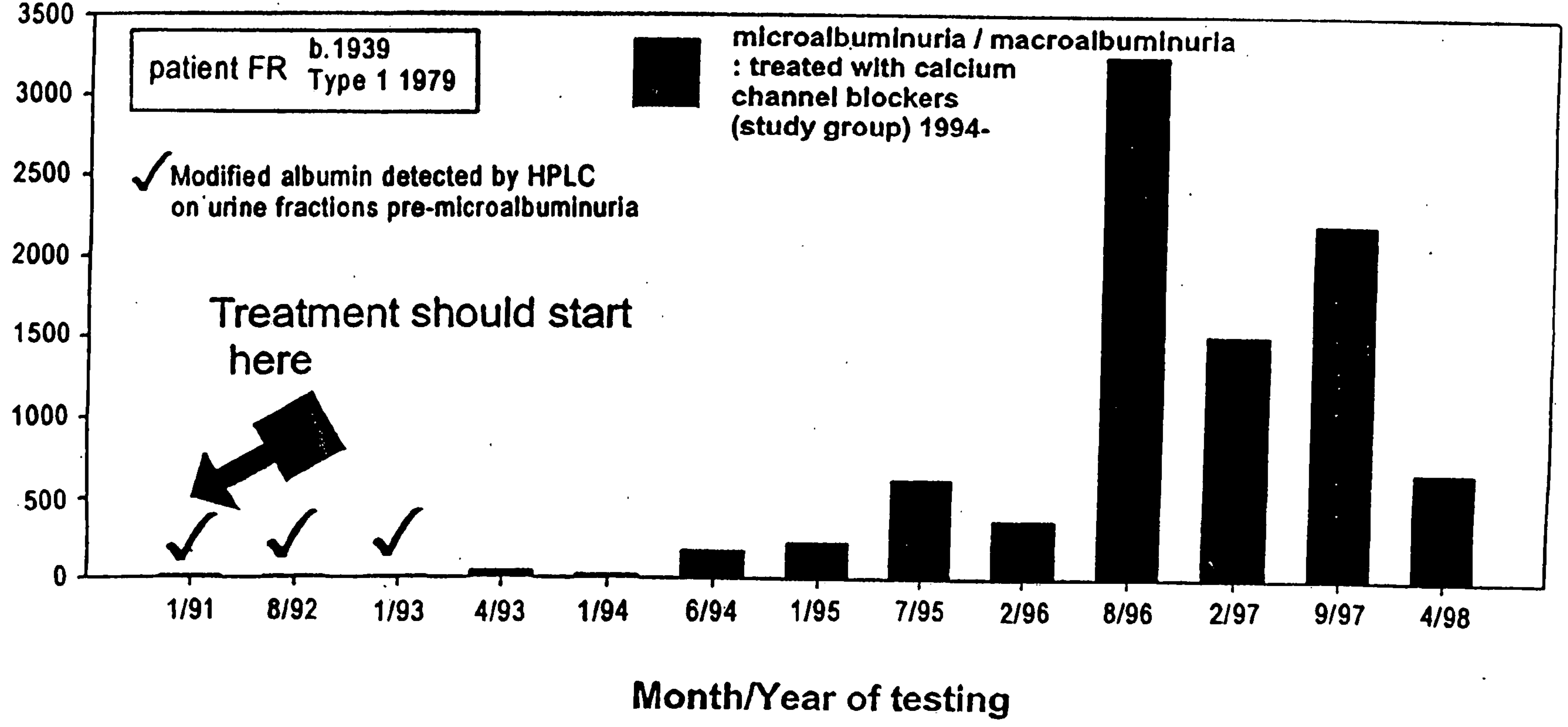

3500
3000
2500
2000
1500
1000
500
0
patient FR b.1939 Type 1 1979
✓Modified albumin detected by HPLC on urine fractions pre-microalbuminuria
Treatment should start here
microalbuminuria / macroalbuminuria : treated with calcium channel blockers (study group) 1994-
1/91
8/92
1/93
4/93
1/94
6/94
1/95
7/95
2/96
8/96
2/97
9/97
4/98
Month/Year of testing

FIG. 14

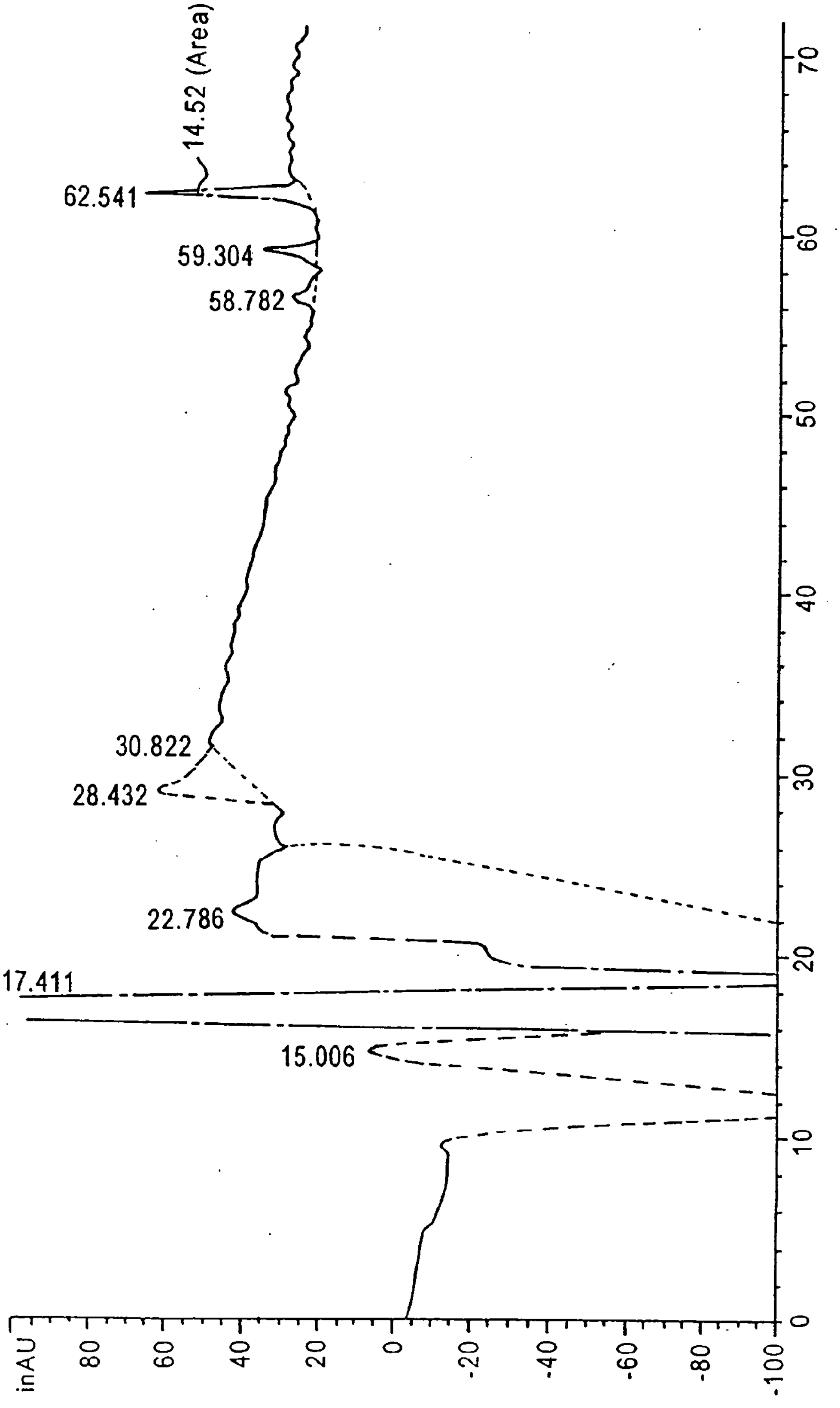

mAU
80
60
40
20
0
-20
-40
-60
-80
-100
0
10
20
30
40
50
60
70
14.52 (Area)
62.541
59.304
58.782
30.822
28.432
22.786
17.411
15.006

FIG. 15

Protein fragmentation array

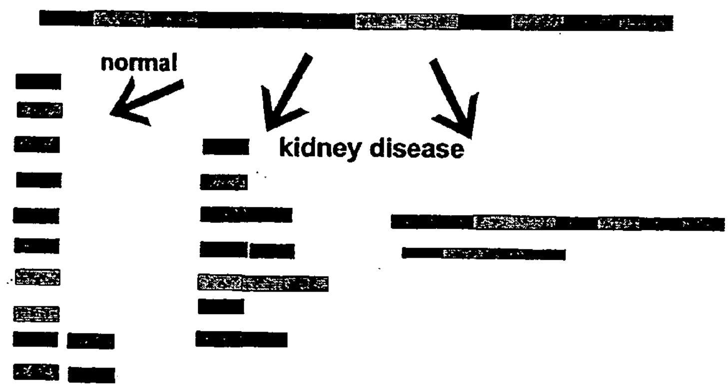


FIG. 16

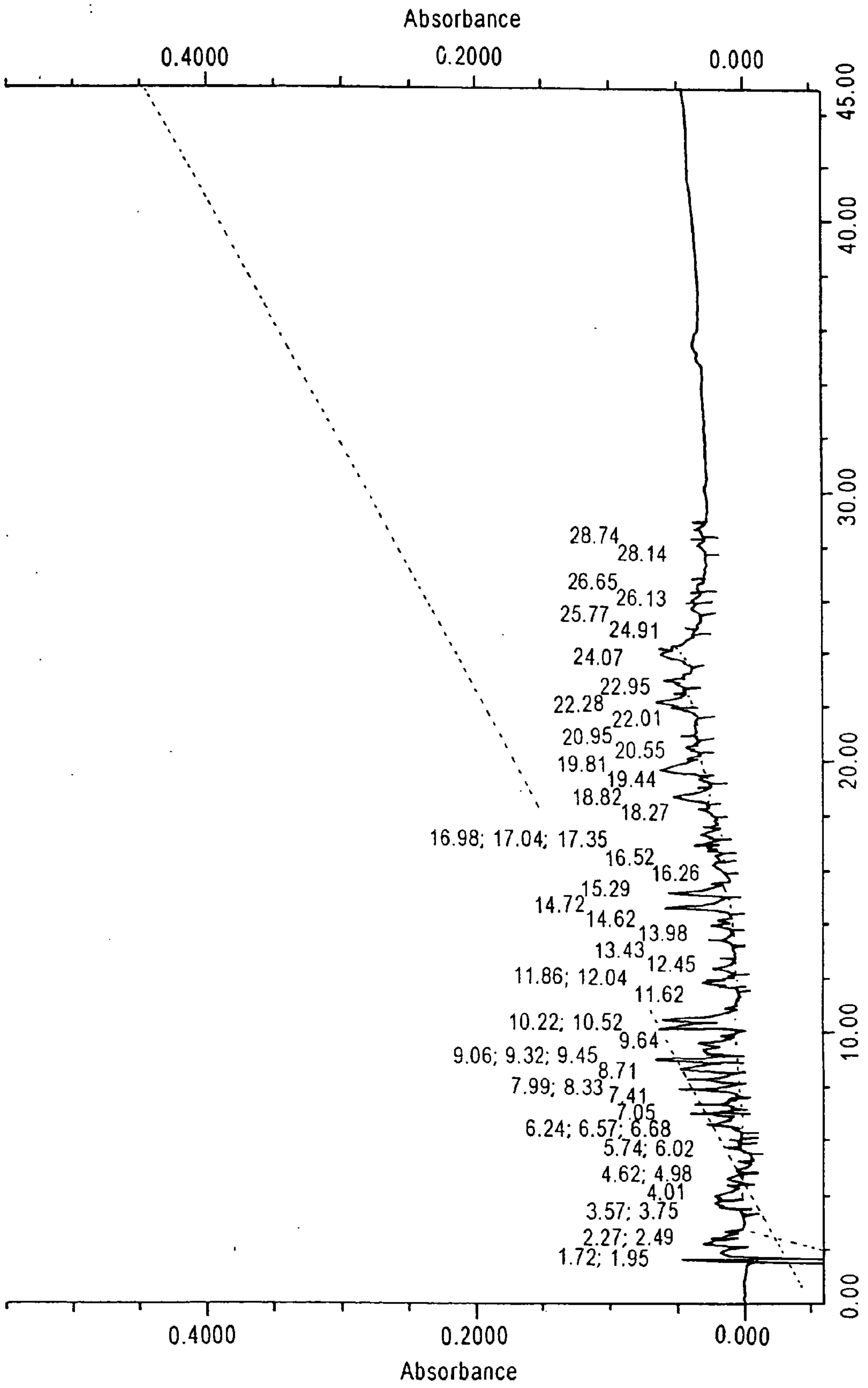

Absorbance
0.4000
0.2000
0.000
45.00
40.00
30.00
20.00
10.00
0.00
28.74
28.14
26.65
26.13
25.77
24.91
24.07
22.95
22.28
22.01
20.95
20.55
19.81
19.44
18.82
18.27
16.98; 17.04; 17.35
16.52
16.26
15.29
14.72
14.62
13.98
13.43
12.45
11.86; 12.04
11.62
10.22; 10.52
9.64
9.06; 9.32; 9.45
8.71
7.99; 8.33
7.41
7.05
6.24; 6.57; 6.68
5.74; 6.02
4.62; 4.98
4.01
3.57; 3.75
2.27; 2.49
1.72; 1.95
0.4000
0.2000
0.000
Absorbance

FIG. 17

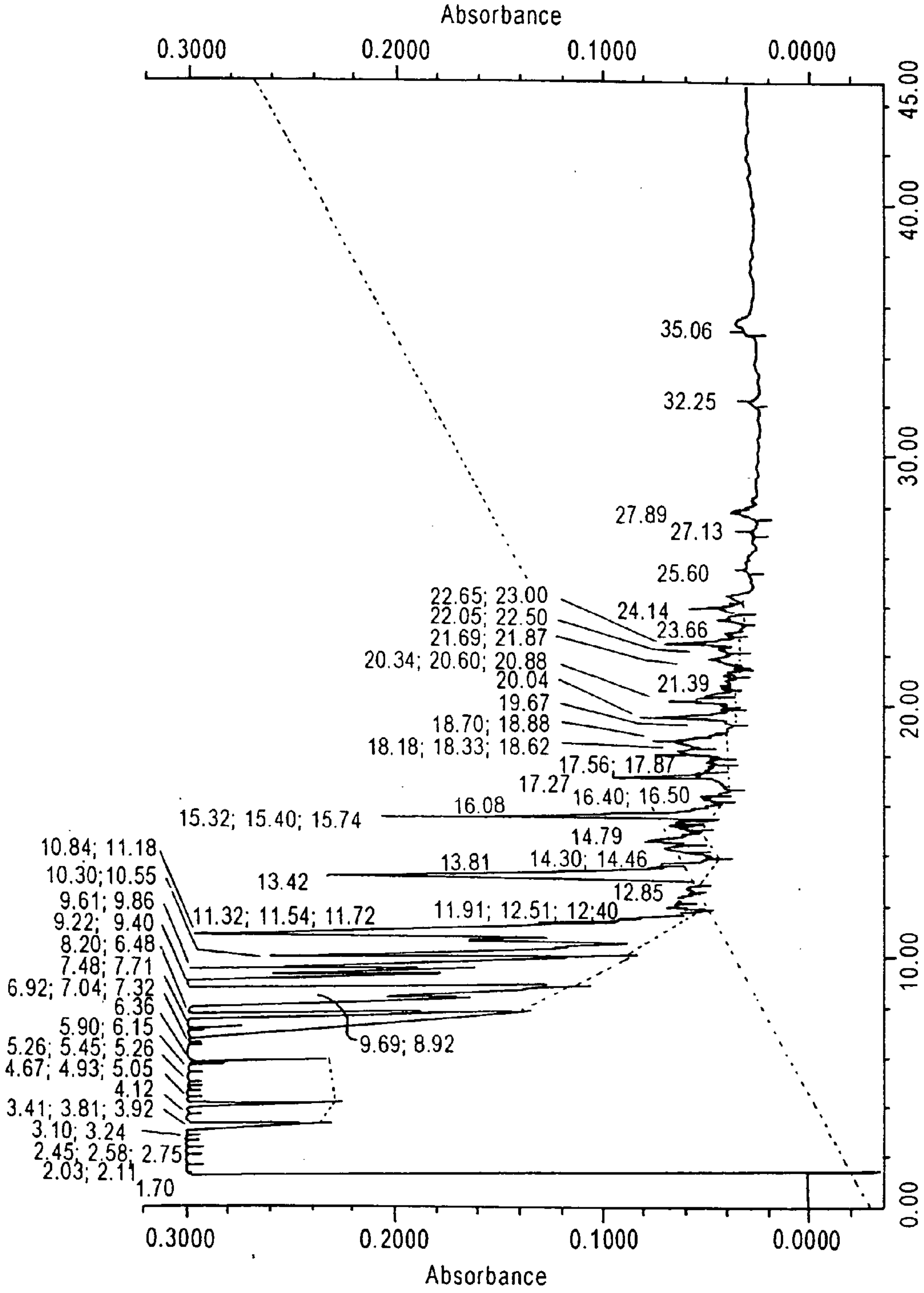

Absorbance
0.3000
0.2000
0.1000
0.0000
45.00
40.00
30.00
20.00
10.00
0.00
35.06
32.25
27.89
27.13
25.60
22.65; 23.00
22.05; 22.50
21.69; 21.87
20.34; 20.60; 20.88
20.04
19.67
18.70; 18.88
18.18; 18.33; 18.62
24.14
23.66
21.39
17.56; 17.87
17.27
16.40; 16.50
16.08
15.32; 15.40; 15.74
14.79
14.30; 14.46
13.81
13.42
12.85
11.91; 12.51; 12.40
11.32; 11.54; 11.72
10.84; 11.18
10.30;10.55
9.61; 9.86
9.22; 9.40
8.20; 6.48
7.48; 7.71
6.92; 7.04; 7.32
6.36
5.90; 6.15
5.26; 5.45; 5.26
4.67; 4.93; 5.05
4.12
3.41; 3.81; 3.92
3.10; 3.24
2.45; 2.58; 2.75
2.03; 2.11
1.70
9.69; 8.92
0.3000
0.2000
0.1000
0.0000
Absorbance

FIG. 18

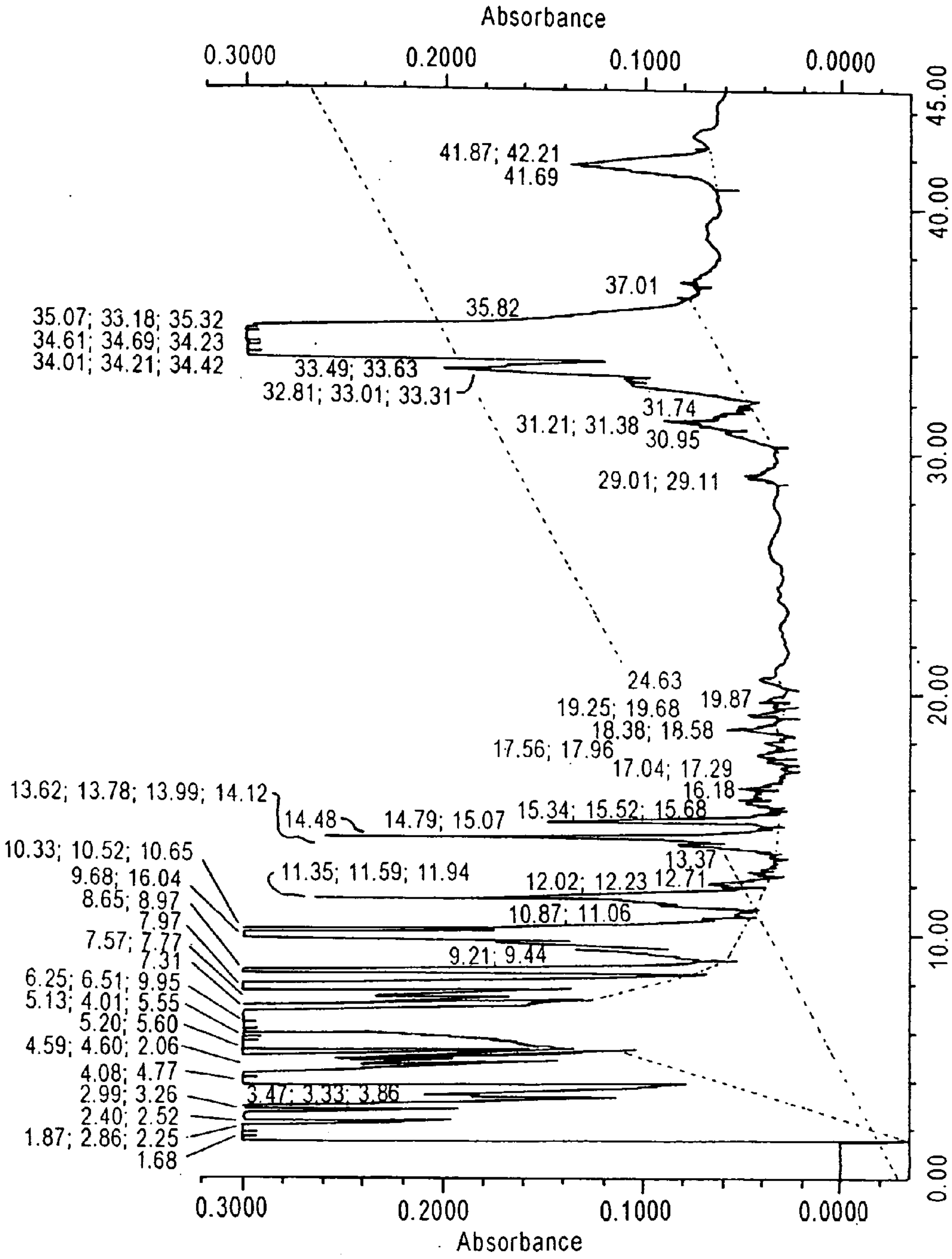

Absorbance
0.3000
0.2000
0.1000
0.0000
45.00
40.00
30.00
20.00
10.00
0.00
41.87; 42.21
41.69
37.01
35.82
35.07; 33.18; 35.32
34.61; 34.69; 34.23
34.01; 34.21; 34.42
33.49; 33.63
32.81; 33.01; 33.31
31.74
31.21; 31.38
30.95
29.01; 29.11
24.63
19.25; 19.68
19.87
18.38; 18.58
17.56; 17.96
17.04; 17.29
16.18
13.62; 13.78; 13.99; 14.12
14.48
14.79; 15.07
15.34; 15.52; 15.68
10.33; 10.52; 10.65
13.37
9.68; 16.04
11.35; 11.59; 11.94
12.02; 12.23
12.71
8.65; 8.97
7.97
10.87; 11.06
7.57; 7.77
7.31
9.21; 9.44
6.25; 6.51; 9.95
5.13; 4.01; 5.55
5.20; 5.60
4.59; 4.60; 2.06
4.08; 4.77
2.99; 3.26
3.47; 3.33; 3.86
2.40; 2.52
1.87; 2.86; 2.25
1.68
0.3000
0.2000
0.1000
0.0000
Absorbance

FIG. 19

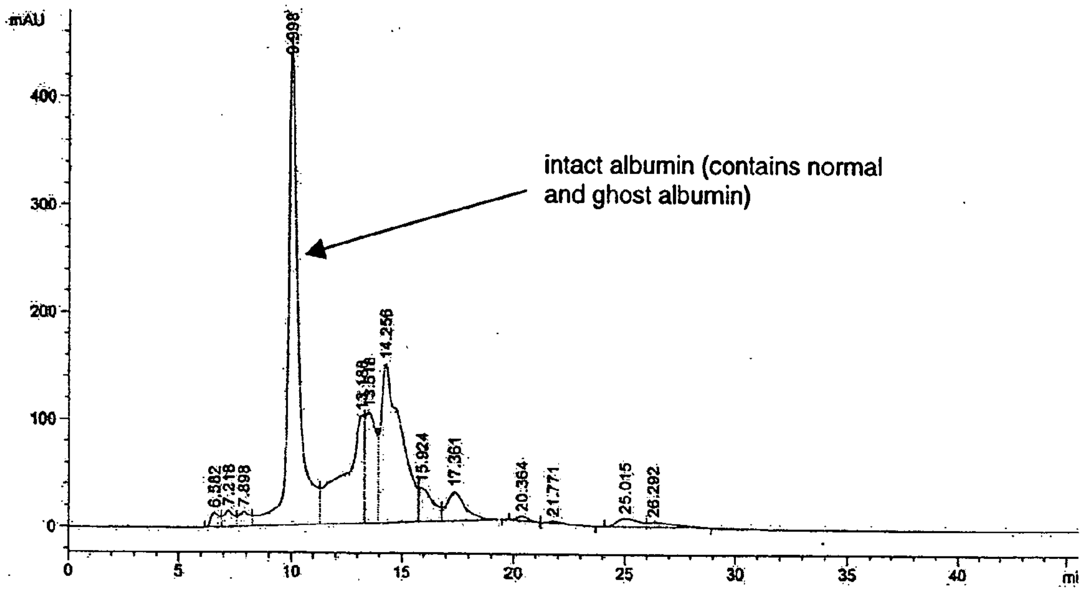

intact albumin (contains normal and ghost albumin)

FIG. 20

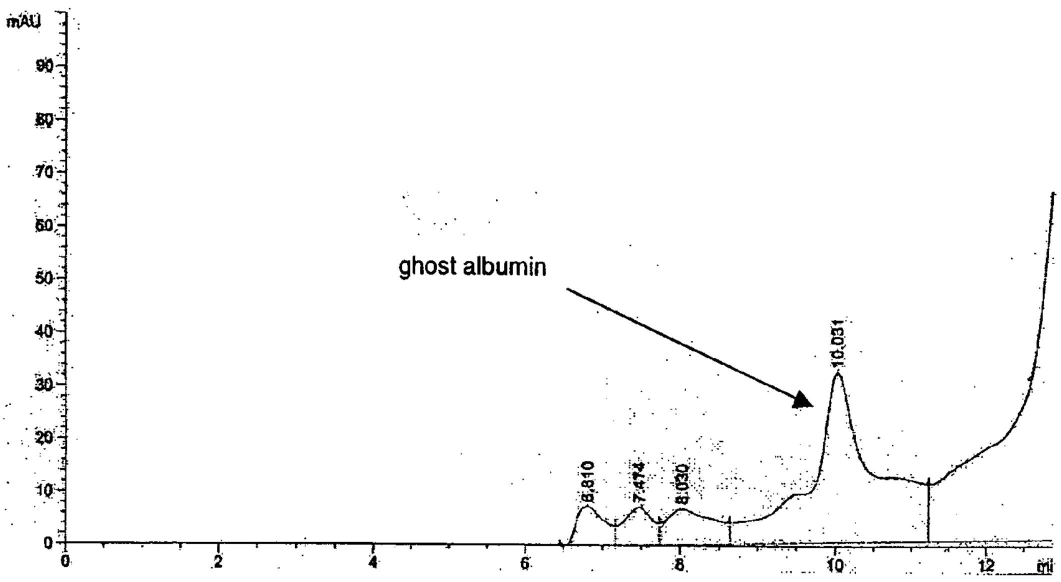

mAU
90
80
70
60
50
40
30
20
10
0
0
2
4
6
8
10
12
ml
6.810
7.474
8.030
10.031
ghost albumin

FIG. 21

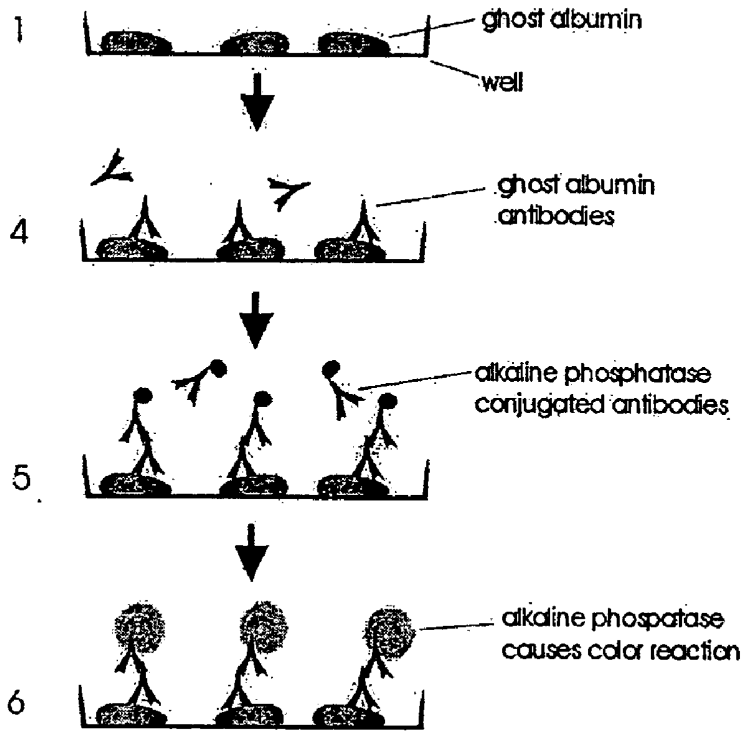

1
ghost albumin
well
4
ghost albumin
antibodies
alkaline phosphatase
conjugated antibodies
5
alkaline phospatase
causes color reaction
6

FIG. 22

METHOD FOR KIDNEY DISEASE DETECTION

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application is a division of U.S. patent application Ser. No. 10/391,202 filed on Mar. 19, 2003, which is a continuation-in-part of U.S. patent application Ser. No. 09/892,797 filed on Jun. 28, 2001, which is a continuation-in-part of U.S. patent application Ser. No. 09/415,217 filed on Oct. 12, 1999, which claims priority to Australian Patent Application Serial No. PP7843 filed on Dec. 21, 1998, the entire disclosures of which are incorporated herein by reference.

FIELD OF THE INVENTION

[0002] The invention relates to improved methods of detecting and treating an early stage of renal disease and/or renal complications of a disease, particularly diabetes.

BACKGROUND OF THE INVENTION

[0003] The appearance of excess protein such as albumin in the urine is indicative of kidney disease. Diabetic nephropathy is such a disease.

[0004] The applicant has found that proteins, including albumin, are normally excreted as a mixture of native protein and fragments that are specifically produced during renal passage Osicka T. M. et al., *Nephrology,* 2:199-212 (1996)). Proteins are heavily degraded during renal passage by post-glomerular (basement membrane) cells that may include tubular cells. Lysosomes in renal tubular cells may be responsible for the breakdown of proteins excreted during renal passage. **FIG. 1** illustrates the progress of filtered intact albumin into tubular cells and breakdown of albumin to provide excreted albumin fragments. The breakdown products are excreted into the tubular lumen. In normal individuals, most of the albumin in the urine is fragmented.

[0005] When lysosome activity or intracellular processes directing substrates to lysosomes is reduced, more of the high molecular weight, and substantially full length albumin appears in the urine. This reflects an imbalance in the cellular processes in the kidney tissue.

[0006] The applicant has discovered that when proteins, such as α_1 acid glycoprotein (orosomucoid), alpha-1-acid antitrypsin, α_1 glycoprotein, α_1 lipoprotein, alpha-1-beta-2-glycoprotein, beta-2-microglobin, ceruloplasmin, euglobulin, fibrinogen, globulin (α-globulin (α1-globulin, α2-globulin, β-globulin, γ-globulin), glucose oxidase, growth hormone, haptoglobin, horseradish peroxidase, insulin, lactate dehydrogenase, lysozyme, myoglobin, protein hormone, pseudoglobulin I and II, and parathyroid hormone, prealbumin, retinol binding protein, and tamm horsfall glycoprotein, and major plasma proteins such as albumin and immunoglobulins A, E, G and M, are filtered by the kidney, they are subsequently degraded by cells in the kidney prior to the material being excreted (see, PCT published application WO 00/37944). It is likely that tubular cells take up filtered proteins. Tubular cells lie beyond the kidney filter and come in direct contact with the primary filtrate. When the tubular cells internalize proteins, they are directed towards the lysosomes, where they are partially degraded to various size fragments, and then regurgitated to outside the cell. These regurgitated fragments, of which there may be at least 60 different fragments generated from any one particular type of protein, are then excreted into the urine.

[0007] The applicant has discovered that in renal disease fragmentation of proteins is inhibited. This means that substantially full-length filtered proteins are excreted in a person suffering from renal disease. This transition from fragmentation to inhibition of fragmentation of excreted proteins is a basis for the development of new drugs and diagnostic assays. For example, initial changes that occur with the onset of renal complications in diabetes are associated with a change in the fragmentation profile of excreted albumin. This leads to an apparent microalbuminuria that is synonymous with the development of diabetic nephropathy. It is likely that this is due to an inhibition in the lysosomal activity of tubular cells in diabetes. Thus, drugs can be formulated to turn on lysosomal activity in diabetes where renal complications are occurring. The drugs may also be useful in other renal diseases where lysosomal activities are affected, or in diabetes without renal complications in situations where lysosomal activity is turned off in non-renal tissues. Such drugs include antiproliferative drugs, such as anti cancer drugs.

[0008] Until now, it was thought that the conventional radioimmunoassay was suitable for detecting all of a specific protein in a sample (i.e., Total protein). But the total content of the protein may include more than those that are identifiable by known antibodies using conventional radioimmunoassay (RIA). Currently available radioimmunoassays rely on antibodies to detect proteins such as albumin. Antibody detection is very sensitive down to nanogram levels. However, the specificity of the antibodies influences detection of the protein. The antibody detects certain epitopes. If the specific epitope on the albumin is absent, altered or masked, or the albumin is modified in any other way so that the antibody fails to detect the albumin, conventional radioimmunoassays may not provide a true representation of the true amount of albumin present in a urine sample.

[0009] As such, by the time the excess albumin is detected, kidney disease has progressed, possibly to a stage where it is irreversible and treatment has little effect. Therefore there is a continuing need in the art to provide a test that is more sensitive than the currently known radioimmunoassay to detect such a disease as early as possible so that the disease can be either prevented or a treatment protocol commenced early on in the disease.

[0010] However, previous attempts to use urinary protein profiles for diagnostic purposes have been rather disappointing with respect to their clinical validity, in part because of the insufficient reproducibility, sensitivity, and rapidity of available techniques. Thus, there exists a continuing need for an improvement in methods for of detecting an early stage of renal disease and/or renal complications of a disease, particularly the renal complications of diabetes.

SUMMARY OF THE INVENTION

[0011] In one aspect, the invention provides improved methods of detecting an early stage of renal disease and/or renal complications of a disease, particularly diabetes. A fragmentation profile is determined in terms of the size, and sequence of particular fragments derived from intact filtered proteins together with the position where enzyme scission occurs along the protein polypeptide chain. The fragmentation profile is characteristic of the diseased state of the kidney. Accordingly, methods of detecting early signs of a disease, including kidney disease, determining a patient's propensity for the disease, preventing the onset of the disease, and treating the disease at the earliest stage possible are some of the aspects of the invention.

[0012] The method involves taking urine from a subject, and separating all the protein fragments therein. In a preferred embodiment, the separation is accomplished by HPLC (single dimensional or two dimensional or three dimensional electrophoresis and/or chromatography), optionally followed by sizing the fragments by mass spectrometry and using amino acid sequencing to determine the peptide sequence and where enzyme scission has occurred.

[0013] Although not limited to any particular disease, according to the method of the invention, the disease sought to be diagnosed includes nephropathy, diabetes insipidus, diabetes type **1**, diabetes II, renal disease (glomerulonephritis, bacterial and viral glomerulonephritides, IgA nephropathy and Henoch-Schönlein Purpura, membranoproliferative glomerulonephritis, membranous nephropathy, Sjögren's syndrome, nephrotic syndrome (minimal change disease, focal glomerulosclerosis and related disorders), acute renal failure, acute tubulointerstitial nephritis, pyelonephritis, GU tract inflammatory disease, Pre-clampsia, renal graft rejection, leprosy, reflux nephropathy, nephrolithiasis), genetic renal disease (medullary cystic, medullar sponge, polycystic kidney disease (autosomal dominant polycystic kidney disease, autosomal recessive polycystic kidney disease, tuborous sclerosis), von Hippel-Lindau disease, familial thin-glomerular basement membrane disease, collagen III glomerulopathy, fibronectin glomerulopathy, Alport's syndrome, Fabry's disease, Nail-Patella Syndrome, congenital urologic anomalies), monoclonal gammopathies (multiple myeloma, amyloidosis and related disorders), febrile illness (familial Mediterranean fever, HIV infection—AIDS), inflammatory disease (systemic vasculitides (polyarteritis nodosa, Wegener's granulomatosis, polyarteritis, necrotizing and crescentic glomerulonephritis), polymyositis-dermatomyositis, pancreatitis, rheumatoid arthritis, systemic lupus erythematosus, gout), blood disorders (sickle cell disease, thrombotic thrombocytopenia purpura, hemolytic-uremic syndrome, acute cortical necrosis, renal thromboembolism), trauma and surgery (extensive injury, burns, abdominal and vascular surgery, induction of anesthesia), drugs (penicillamine, steroids) and drug abuse, malignant disease (epithelial (lung, breast), adenocarcinoma (renal), melanoma, lymphoreticular, multiple myeloma), circulatory disease (myocardial infarction, cardiac failure, peripheral vascular disease, hypertension, coronary heart disease, non-atherosclerotic cardiovascular disease, atherosclerotic cardiovascular disease), skin disease (psoriasis, systemic sclerosis), respiratory disease (COPD, obstructive sleep apnoea, hypoia at high altitude) and endocrine disease (acromegaly, diabetes mellitus, diabetes insipidus). Specific proteinuria, and in particular, albuminuria (micro- and macro-), is a marker of these disease.

[0014] In another embodiment, the invention provides improved methods of detecting non-renal diseases. With the recognition that filtered proteins are degraded during renal passage, the methods described in this application can also be used to detect protein fragments derived from proteins generated by non-renal disease. Non-renal diseases, such as cancers, generate increased levels of proteins into the circulation. Urinary analysis of filtered proteins currently does not detect the intact form of these proteins. Therefore a method as described below to detect and analyze fragments resulting from degradation during renal passage that will be able to detect the seriousness of the disease.

[0015] In another aspect of the present invention there is a method of measuring intact modified albumin useful for the detection of disease, by concentrating a urine sample, denaturing the concentrated sample by enzymic or chemical breakdown and analyzing the products, for example, by electrophoresis.

[0016] Both embodiments can use non-antibody technology as well, by separating a desired protein and its fragments from urine samples in a three-dimensional fashion; isolating the fragments; and determining the sequence of the protein and its fragments. This assay is repeated over a period of time. A change in the fragmentation profile over time indicates early stage of a particular disease. A change in the size of the fragments, as determined by sequence analysis, can indicate which type of renal disease the subject has a propensity to develop.

[0017] In still another aspect of the invention, antibody technology is used to detect intact albumin in urine. The invention provides a specific method for preparing purified or substantially purified intact albumin from a urine sample. From such prepared and purified or substantially purified intact albumin, specific anti-intact albumin antibodies are developed. Such anti-intact albumin antibodies are useful for the development of diagnostic immunoassays for intact albumin that can be used to predict the onset and/or progress of disease.

[0018] These and other objects of the invention will be more fully understood from the following description of the invention, the referenced drawings attached hereto and the claims appended hereto.

BRIEF DESCRIPTION OF THE DRAWINGS

[0019] **FIG. 1** illustrates the progress of filtered intact albumin into tubular cells and breakdown of albumin to provide excreted albumin fragments.

[0020] **FIG. 2** (**2***a* and **2***b*) illustrate a representative profile of (3H)HSA in (a) urine and (b) plasma collected from normal, healthy volunteers by size exclusion chromatography. Urine contains mostly fragmented albumin. And plasma contains mostly intact albumin.

[0021] **FIG. 3** illustrates urine from normal, healthy volunteer showing a fragmented albumin peak, but no intact albumin peak from size exclusion chromatography.

[0022] **FIG. 4** illustrates urine from a diabetic patient showing both intact and fragmented albumin peaks from size exclusion chromatography.

[0023] **FIG. 5** illustrates a HPLC profile of albumin alone.

[0024] **FIG. 6** illustrates the HPLC profile of plasma from normal, healthy volunteer showing albumin peaks.

[0025] **FIG. 7** shows the HPLC profile of urine from normal, healthy volunteer with fragmented products of albumin but no intact albumin peak.

[0026] **FIG. 8** shows the HPLC profile of a urine sample from a normoalbuminuric diabetic patient showing albumin breakdown products and a small-modified albumin peak at approximately 39-44 minutes retention time.

[0027] **FIG. 9** shows the HPLC profile of urine from a normoalbuminuric diabetic patient showing signs of kidney failure and the presence of the characteristic spiked albumin peak at approximately 39-44 minutes retention time.

[0028] **FIG. 10** illustrates a HPLC profile of a normoalbuminuric diabetic patient showing signs of kidney failure and the presence of the characteristic spiked modified albumin peak at approximately 39-44 minutes retention time.

[0029] **FIG. 11** illustrates a HPLC of a macroalbuminuric diabetic patient showing high levels of the native albumin as well as the characteristic spiked appearance at approximately 39-44 minutes retention time.

[0030] **FIG. 12** illustrates a longitudinal study of a patient in which the modified protein was detected at a time prior to onset of diabetic nephropathy, indicating predisposition to diabetic nephropathy, and the delay in treatment caused by relying on conventional RIA methods.

[0031] **FIG. 13** illustrates a longitudinal study of a patient in which the modified protein was detected at a time prior to onset of diabetic nephropathy, indicating predisposition to diabetic nephropathy, and the delay in treatment caused by relying on conventional RIA methods.

[0032] **FIG. 14** illustrates a longitudinal study of a patient in which the modified protein was detected at a time prior to onset of diabetic nephropathy, indicating predisposition to diabetic nephropathy, and the delay in treatment caused by relying on conventional RIA methods.

[0033] **FIG. 15** shows the HPLC chromatogram used as a criterion of purity of the modified albumin of Example 4.

[0034] **FIG. 16** is a schematic diagram illustrating the manner in which an intact filtered protein may be degraded by normal functioning kidneys and diseased kidneys.

[0035] **FIG. 17** illustrates the HPLC profile of a trypsin digested sample of albumin that has been filtered through a 30,000 molecular weight cut-off membrane. The filtrate yields many peaks eluting between 2 to 30 minutes.

[0036] **FIG. 18** illustrates the HPLC profile of a control, normal subject showing many fragments in the eluting range of 10 to 30 minutes. The HPLC profile of a diabetic patient with macroalbuminuria (1457 microgram per minute) shows a significantly different fragment profile in the range of 10-30 minutes.

[0037] **FIG. 19** illustrates the HPLC profile of a subject with renal disease. As compared with **FIG. 18**, the fragmentation process of filtered proteins is inhibited. The number of fragments is decreased and the size of the fragments is increased.

[0038] **FIG. 20** illustrates the HPLC profile of urine from a diabetic patient with kidney disease after concentration showing intact albumin, including both native albumin and intact albumin.

[0039] **FIG. 21** illustrates the HPLC profile of urine from a diabetic patient with kidney disease after affinity purification showing intact albumin.

[0040] **FIG. 22** illustrates a schematic diagram showing the steps involved in performing an ELISA to detect intact albumin.

DETAILED DESCRIPTION OF THE INVENTION

[0041] The applicant has discovered that when proteins, including α1 acid glycoprotein (orosomucoid), α1 acid antitrypsin, α1 glycoprotein, α1 lipoprotein, alpha-1-microglobumin, α2 19S glycoprotein, bence-jones proteins, β1 lipoprotein, β1 transferrin, β2 glycoprotein, β2 microglobin, ceruloplasmin, euglobulin, fibrinogen, globulin (α-globulin (α1-globulin, α2-globulin) β-globulin, γ-globulin), glucose oxidase, growth hormone, haptoglobin, horseradish peroxidase, insulin, lactate dehydrogenase, lysozyme, myoglobin, protein hormone, pseudoglobulin I and II, and parathyroid hormone, prealbumin, retinol binding protein, and tamm horsfall glycoprotein and major plasma proteins such as albumin and immunoglobulins A, E, G and M, are filtered by the kidney they are subsequently degraded by cells in the kidney prior to the material being excreted. Tubular cells likely take up the filtered proteins. Tubular cells lie beyond the kidney filter and come in direct contact with the primary filtrate. When the tubular cells internalize proteins, they are directed towards the lysosomes, where they are partially degraded to various size fragments, and then regurgitated to outside the cell. These regurgitated fragments, of which there may be at least 60 different fragments generated from any one particular type of protein, are then excreted into the urine.

[0042] The applicant has discovered that in renal disease fragmentation of proteins is inhibited. This means that substantially full-length filtered proteins will be excreted in a person suffering from renal disease. This transition from fragmentation to inhibition of fragmentation of excreted proteins is a basis for the development of new drugs and diagnostic assays. For example, initial changes that occur with the onset of renal complications in diabetes are associated with a change in the fragmentation profile of excreted albumin. This leads to an apparent microalbuminuria, which is synonymous with the development of diabetic nephropathy. It is likely that this is due to an inhibition in the lysosomal activity of tubular cells in diabetes.

[0043] Thus, drugs can be formulated to turn on lysosomal activity in diabetes where renal complications are occurring. The drugs may also be useful in other renal diseases where lysosomal activities are affected, or in diabetes without renal complications in situations where lysosomal activity is turned off in non-renal tissues. Such drugs include antiproliferative drugs, such as anti cancer drugs or antibodies to neutralize TGF-beta.

[0044] The applicant has discovered a unique assay for detecting protein fragment arrays of specific proteins, which are detected in the urine of subjects. Detection of the protein fragment array and changes to the protein fragment array are predictive of a predisposition to renal disease.

[0045] The principle of the protein fragment array is shown in **FIG. 16**. The intact protein is represented by a series of regions representing specific amino acid sequences within the protein. All proteins have these specific primary structures. When such a protein from plasma, like albumin or immunoglobulin is filtered it is filtered intact. However, after the protein is filtered it may be taken up by renal cells, such as early proximal tubular cells, and be degraded, by enzymes within lysosomes, to many fragments (**FIG. 16**). These fragments are excreted in urine. For normal functioning kidneys, the fragmentation process is maximal with small fragments derived from many individual filtered proteins being produced and ultimately excreted. **FIG. 17** illustrates a fragmentation profile from the trypsin digest of albumin. A similar profile is seen in the urine of a control, normal volunteer (**FIG. 18**). In terms of the number of fragments produced from each protein and the nature of the peptide splitting (i.e., the position along the protein where scission occurs), the fragmentation profile is specific. The size and sequence characteristic of the individual fragments will be characteristic of the specificity and activity of lysosomal enzymes acting on the protein.

[0046] Proteases such as V-8, trypsin and Lys-C can be used to produce a peptide map of a purified protein. Other proteases can be used, preferably proteases that cause limited proteolysis ("enzyme scission"), in which a protease cleaves only one or a limited number of peptide bonds of a target protein. The protease can be from any group of proteases, such as the serine proteinases (chymotrypsin, trypsin, elastase, kallikrein, and the substilisin family), the cysteine proteinases (the plant proteases such as papain, actinidin or bromelain, some cathepsins, the cytosolic calpains, and parasitic proteases (e.g., from Trypanosoma, Schistosoma), the aspartic proteinases. (pepsin family members such as pepsin, chymosin, some cathepsins D, and renin; certain fungal proteases (penicillopepsin, rhizopuspepsin, endothiapepsin); and viral proteinases such as retropepsin); and the metalloproteinases (including thermolysin, neprilysin, alanyl aminopeptidase, and astacin).

[0047] In renal disease, the fragmentation process of filtered proteins is inhibited. The number of fragments is decreased and the size of the fragments is increased (**FIG. 19**). This is due to the fact that there are less points of scission by lysosmal enzymes. Therefore, in terms of the size and amino acid sequence, the fragment profile is considerably different from that obtained in normal kidneys for any particular filtered protein, such as albumin or immunoglobulin. The degree of inhibition of fragmentation will depend on the severity of the disease. As disease progresses the degree of fragmentation will become less as demonstrated in FIG A.

[0048] U.S. Pat. No. 5,246,835 discloses a method of diagnosing renal diseases by detecting fragments of albumin in human urine. The '835 patent discloses that the fragments are derived from the plasma and are filtered by the kidney, unaltered, and are ultimately excreted. The method of detection of the urinary fragments in the '835 patent preferably involves the use of affinity binding to conventional albumin antibodies. In contrast to the method of present invention, there is an increased detection of albumin fragments in diabetes in the method of the '835 patent. In the present invention, the diagnosis of diabetic nephropathy can occur when there is a decrease in the number of fragments. The albumin fragments examined in the present invention are not necessarily detected by albumin antibodies.

[0049] In contrast to the method of the '835 patent, one embodiment of the invention is the taking urine from a patient, and separating all the fragments by HPLC (single dimensional or two dimensional or three dimensional electrophoresis and/or chromatography) and then sizing the fragments by mass spectrometry and then using amino acid sequencing to determine the peptide sequence and where peptide scission occurred.

[0050] The protein fragments can be detected and separated by a variety of methods that are well-known in the art, including, but not limited to chromatography, electrophoresis and sedimentation, or a combination of these, which are described in Karger B L, Hancock W S (eds.) High Resolution Separation and Analysis of biological Macromolecules. Part A Fundamentals in Methods in Enzymology, Vol. 270, 1996, Academic Press, San Diego, Calif., USA; Karger B L, Hancock W S (eds.) High Resolution Separation and Analysis of biological Macromolecules. Part B Applications in Methods in Enzymology, Vol. 271, 1996, Academic Press, San Diego, Calif., USA; or Harding S E, Rowe, A J, Horton J C (eds.) Analytical Ultracentrifugation in Biochemistry and Polymer Science. 1992, Royal Soc. Chemistry, Cambridge, UK, which references are incorporated herein by reference in their entirety.

[0051] The electrophoresis method includes, but is not limited to, moving-boundary electrophoresis, zone electrophoresis, and isoelectric focusing.

[0052] The chromatography method includes, but is not limited to, partition chromatography, adsorption chromatography, paper chromatography, thin-layer chromatography, gas-liquid chromatography, gel chromatography, ion-exchange chromatography, affinity chromatography, and hydrophobic interaction chromatography. Preferably, the method is a sizing gel chromatography and hydrophobic interaction chromatography. More preferably, the method is hydrophobic interaction chromatography using a HPLC column.

[0053] HPLC is preferred for generating a fragmentation profile. A fragmentation profile on HPLC is characterized by a series of peaks representing a number of fragment species.

[0054] A HPLC column for detecting modified albumin or unmodified albumin may be a hydrophobicity column, such as Zorbax **300** SB-CB (4.6 mm×150 mm). A 50 μl sample loop may be used. Elution solvents suitable for HPLC in detecting albumin and its breakdown products may include standard elution solvents such as acetonitrile solvents. Preferably a buffer of water/1% trifluoro acetic acid (TFA) followed by a buffer of 60% acetonitrile/0.09% TFA may be used. A gradient of 0 to 100% of a 60% acetonitrile/0.09% TFA has been found to be suitable.

[0055] Suitable HPLC conditions for a hydrophobicity column may be as follows:

[0056] Solvent A H2O, 1% trifluoro acetic acid

[0057] Solvent B 60% acetonitrile, 0.09% TFA

[0058] Solvent A2 99.96>00.00:49.58 min

[0059] Pressure 9.014 Mpascalls (~1100 psi)

[0060] Solvent B2 0.04>100.0:49.58 min

[0061] Pressure 7.154 Mpascalls

[0062] The wavelength used in HPLC may be approximately 214 nm. For albumin, modified albumin may elute between 39-44 minutes (**FIG. 5**). Albumin fragments may elute much earlier, mainly at less than 20 minutes.

[0063] The applicant has developed a unique method for the preparation and isolation of purified or substantially purified intact albumin. Such purified or substantially purified intact albumin is useful for the preparation of anti-intact albumin antibodies, which are useful for developing diagnostic immunoassays for intact albumin that can be used as a predictor of the early onset of, or progression toward renal disease and/or kidney complications of disease. The assay is preferably repeated to detect intact albumin over a period of time. An increase in the level of intact albumin in the urine over time indicates early stage of a renal disease and/or renal complications of a particular disease.

[0064] Definitions

[0065] "Anti-intact albumin antibody" refers to a defense protein, like an antibody or immunogen, that possesses antigen-binding sites to, and/or binds specifically to, intact albumin. "Anti-intact protein antibody" refers to a defense protein, like an antibody or immunogen, that possesses antigen-binding sites to, and/or binds specifically to, an intact protein.

[0066] "Fragmented protein or fragment albumin" includes post-glomerular breakdown products after chemical, enzymatic or physical breakdown that occurs during renal passage. These components have a reduced size and/or may have changed hydrophobicity.

[0067] "Intact albumin, modified albumin, or modified form of albumin" as used herein means a compound having similar size and structural characteristics to native albumin, wherein the amino acid sequence is substantially the same as the native albumin. It is preferably a filtered intact protein. It elutes at or near the same position as native albumin on high-pressure liquid chromatography (HPLC) (**FIG. 5**). However, the structure has been modified biochemically either by minor enzyme mediated modification or addition to its basic structure and/or physically through a change in its three dimensional structure so that it escapes detection by conventionally used anti-albumin antibodies. Biochemical modification may be made by enzymes such as endo- or exo-peptidases. The 3D structure of albumin may have been altered in some way. Ligands may have bound to the albumin, or it may be any combination of these. The modified albumin detected in the method of the invention is not detectable by current and conventional radioimmunoassays using available antibodies and is not a fragment.

[0068] Conventional anti-albumin antibodies can be purchased from any purveyor of immunochemicals. For example, monoclonal antibody catalog numbers A6684 (clone no. HSA-I **1**), and A2672 (clone no. HSA-9), as well as liquid whole serum, lyophilized fractionates, liquid IgG fraction, and the monoclonal antibodies in liquid ascites fluids form, can be obtained from Sigma, St. Louis, Mo., as found in the Immunochemicals section at pages 1151-1152 in the 1994 Sigma-Biochemicals Organic Compounds for Research and Diagnostic Reagents catalog.

[0069] As used herein, intact/modified albumin includes albumin that is substantially full-length, fragmented, chemically modified, or physically modified. As used herein, intact/modified albumin is meant to indicate albumin that is less than, equal to, or greater in molecular weight than the full-length albumin, and elutes at or near the native albumin position in a separation medium, such as chromatography, preferably HPLC, and most preferably hydrophobicity HPLC. As used herein, fragmented albumin is meant to refer to the fragment of albumin that is not detected by conventional anti-albumin antibody, and its presence is detected in diagnosing an early stage of renal disease and/or renal complications of a disease. The detection of the presence of intact/modified albumin is an indication of a predisposition to renal disease.

[0070] "Intact protein, modified protein or modified form of a protein" as used herein includes those forms of substantially full-length protein which are undetectable by conventional radioimmunoassay. The protein includes, but is not limited to, albumin, α1 acid glycoprotein (orosomucoid), α1 acid antitrypsin, α1 glycoprotein, α1 lipoprotein, alpha-1-microglobumin, α2 19S glycoprotein, bence-jones proteins, β1 lipoprotein, β1 transferrin, β2 glycoprotein, β2 microglobin, ceruloplasmin, euglobulin, fibrinogen, globulin α-globulin (α1-globulin, α2-globulin) β3-globulin, γ-globulin), glucose oxidase, growth hormone, haptoglobin, horseradish peroxidase, immunoglobulins A, E, G and M, insulin, lactate dehydrogenase, lysozyme, myoglobin, protein hormone, pseudoglobulin I and II, and parathyroid hormone, prealbumin, retinol binding protein, and tamm horsfall glycoprotein.

[0071] "Kidney disease" as used herein includes any malfunction of the kidney. Kidney disease may be identified by the presence of intact or modified albumin in the urine. Preferably, an early diagnosis of the kidney disease may be made by detecting the presence of modified protein in the urine, or an increase in the modified protein in the urine over time.

[0072] "Low lysosome activity" as used herein is compared against normal levels of lysosome activity and/or lysosome machinery that traffics protein to the lysosome in a normal individual. The activity is insufficient for the lysosome to fragment proteins so that intact protein is excreted at a greater amount than at normally low levels.

[0073] "Lysosome-activating compound" as used herein refers to a compound that is beneficial to reactivation of the lysosome. The compound may work directly or indirectly on the lysosome resulting in activation of lysosomal function. These compounds may be selected from the group including, but not limited to, anticancer compounds, antiproliferation compounds, paracetamol, vitamin A (retinoic acid) or derivatives of retinol, or compounds, including antibodies, to neutralize TGF beta.

[0074] "Macroalbuminuria" is a condition where an individual excretes greater than 200 μg albumin/min in the urine as measured by conventional radioimmunoassay (RIA).

[0075] "Microalbuminuria" is a condition where an individual excretes at least 20 μg albumin/min in the urine as measured by conventional radioimmunoassay (RIA). RIA measures down to 15.6 ng/ml and is able to measure albumin in urine of normal subjects who have clearance of less than 6 μg/min. However, when albumin excretion exceeds 20 μg/min, treatment of the kidney disease is limited and full recovery is difficult from this point.

[0076] "Microalbuminuric" as used herein is a condition when albumin is detected in the urine at an excretion rate of at least 20 μg/min as measured by conventional RIA.

[0077] As used herein, "native" and "unmodified" are used interchangeably to describe a protein that is naturally found in an organism, preferably a human, which has not been modified by the filtering process of the renal glomeruli. Native albumin as defined herein is detectable by conventional immunoassays using conventional albumin antibodies.

[0078] "Normal individual" as used herein is an individual who does not have a disease in which intact protein found in urine is an indicator of the disease. Preferably, the disease is kidney disease.

[0079] "Normal levels of lysosome activity" are levels of lysosome activity found in undiseased kidney of a normal individual.

[0080] "Normoalbuminuric" as used herein means a condition where albumin is excreted in the urine and is not detectable by RIA, or less than 20 μg/min (as measured by RIA) is excreted.

[0081] "Propensity for a disease" as used herein means that a disease may result in an individual as judged by a determination of the presence and excretion rate of a modified protein such as modified albumin.

[0082] "Proteinuria" as used herein is the existence of protein in the urine, usually in the form of albumin, a protein that is soluble in water and can be coagulated by heat. Related to this, "specific proteinuria" refers to the existence of a particular protein in the urine.

[0083] "Purified or substantially purified" refers to a substance, for example a protein, that is substantially free from contaminants, including, without limitation, native protein.

[0084] "Radioimmunoassay" as used herein is a method for detection and measurement of substances using radioactively labeled specific antibodies or antigens.

[0085] "Reactivation of the lysosome" as used herein includes an activation of lysosome activity preferably so that breakdown of proteins, particularly albumin, is increased compared with an inactivated state of the lysosome.

[0086] "Restore" as used herein means to restore in full or in part so that the component being restored has an improved function compared with its previous function.

[0087] The "sum of intact and intact modified protein" as used herein refers to the total amount of intact protein, and intact modified protein present in a biological sample.

[0088] "Total protein" as used herein refers to a particular filtered protein present in native, unmodified, modified or fragmented form that is excreted in urine. It includes protein that is not detected by conventional radioimmunoassay or conventional methods, which are currently available to detect the protein. Preferably the protein is albumin.

[0089] Methods of Detection

[0090] Urinary protein profiles can be created and examined using the methods of Hampel D J et al., J. Am. Soc. Nephrol. 12(5): 1026-35 (2001), who have developed a sensitive, high-throughput technique, namely surface-enhanced laser desorption/ionization (SELDI) ProteinChip® array-time of flight mass spectrometry. Hampel et al. tested the applicability of the technique for protein profiling of urine and to exemplify its use for patients receiving radiocontrast medium. Assessment of the accuracy, sensitivity, and reproducibility of SELDI in test urinary protein profiling was performed in rats before and after intravenous administration of either ioxilan or hypertonic saline solution as a control. Administration of ioxilan to rats resulted in changes in the abundance of proteins of varying weights. Then, urine samples from patients undergoing cardiac catheterization were obtained. For patients, even in uncomplicated cases of radiocontrast medium infusion during cardiac catheterization, perturbations in the protein composition occurred but returned to baseline values after 6 to 12 hours. Proteins with certain defined molecular masses changed in abundance. For patients with impaired renal function, these changes were not reversible within 6 to 12 hours. As a proof of principle, one of the proteins was identified as β2-microglobulin. Even for patients without renal complications, proteins with a broad range of molecular masses either appear in or disappear from the urine.

[0091] Urinary protein profiles can also be created and examined using the commercially available ProteinChip® System (Ciphergen Biosystems, Fremont, Calif., USA), which uses SELDI (Surface-Enhanced Laser Desorption/Ionization) technology to rapidly perform the separation, detection and analysis of proteins at the femtomole level directly from biological samples. Each aluminum chip contains eight individual, chemically treated spots for sample application; this set-up facilitates simultaneous analysis of multiple samples. A colored, hydrophobic coating retains samples on the spots and simultaneously allows for quick identification of chip type. Typically, a few microliters of sample applied on the ProteinChip® Array yield sufficient protein for analysis with the ProteinChip® Reader.

[0092] For more dilute samples, a ProteinChip® Bioprocessor can be used to apply up to 500 μl. The mass determination of protein samples is accomplished by sample crystallization, sample ionization, flight through a vacuum tube, and detection of the ionized proteins. After washing off non-specifically bound proteins and other contaminants from the ProteinChip® Array, a chemical Energy Absorbing Molecule (EAM) solution is applied and allowed to dry, during which time minute crystals form on the chip. These crystals contain the EAM and the protein(s) of interest. After inserting the ProteinChip Array into the ProteinChip Reader, a laser beam is focused upon the sample, which causes the proteins embedded in the EAM crystals to desorb and ionize. Released ions then experience an accelerating electrical field that causes them to "fly" through a vacuum tube, towards the ion detector. Finally, the ionized proteins are detected and an accurate mass is determined based on the time of flight (TOF).

[0093] Proteases such as V-8, trypsin and Lys-C can be used to produce a peptide map of a purified protein bound to the ProteinChip® Array by on-chip protease digestion as shown in the figure to the right. The molecular weights of the resulting fragments can be compared to a peptide database for identification. The process takes less than an hour.

[0094] Additionally, twelve ProteinChip Arrays aligned side-by-side create a 96-well plate footprint. A typical experiment using ProteinChip Array technology requires one to three hours of work at the bench followed by automated sample analysis with the ProteinChip Reader. The entire process thus can be completed in a single afternoon.

[0095] Other Methods

[0096] According to the present invention, the diseases to be treated include, but are not limited to renal disease (glomerulonephritis, bacterial and viral glomerulonephritides, IgA nephropathy and Henoch-Schönlein Purpura, membranoproliferative glomerulonephritis, membranous nephropathy, Sjögren's syndrome, diabetic nephropathy, nephrotic syndrome (minimal change disease, focal glomerulosclerosis, and related disorders), acute renal failure, acute tubulointerstitial nephritis, pyelonephritis, GU tract inflammatory disease, Pre-clampsia, renal graft rejection, leprosy, reflux nephropathy, nephrolithiasis), genetic renal disease (medullary cystic, medullar sponge, polycystic kidney disease (autosomal dominant polycystic kidney disease, autosomal recessive polycystic kidney disease, tuborous sclerosis), von Hippel-Lindau disease, familial thin-glomerular basement membrane disease, collagen III glomerulopathy, fibronectin glomerulopathy, Alport's syndrome, Fabry's disease, Nail-Patella Syndrome, congenital urologic anomalies).

[0097] In one aspect of the invention, there is provided a method for determining a propensity for or early diagnosis of renal disease and/or renal complications of a disease. The method includes determining a change in the albumin content in a urine sample. The disease may be a kidney disease, although not necessarily limited to a kidney disease.

[0098] In the method of the invention, albumin is used herein only as an example of a protein to be detected in urine. When the albumin in a patient is analyzed by conventional RIA, it is expected that a normoalbuminuric patient or normal individual would have albumin in the urine in the range of 3-10 μg/min in young people and greater in older people. However, normoalbuminuric patients also show levels of albumin in the urine if measured by HPLC. Applicant has found that these levels may be in the order of 5 μg/min. As kidney disease progresses, the level of intact/modified albumin will increase to microalbuminuria levels in the order of 20 to 200 μg/min as determined by RIA. This will be much higher when determined by HPLC or a method that determines the sum of intact albumin and intact modified albumin. By monitoring the increase in intact/modified albumin, early signs of kidney disease may be detected. However, these levels are not detectable by the methods currently available such as radioimmunoassay using antibodies currently commercially in use, possibly for the reason that antibodies detect certain epitopes. If the albumin is modified in any way as described above, the epitope may be destroyed thereby leaving the modified albumin undetectable.

[0099] A patient suspected of having diabetic kidney disease will not show signs of kidney degeneration until well after 10 to 15 years when albumin is detected by currently available methods such as RIA methods. Urinary excretion rates of at least 20 μg/min may be detected by RIA when an individual enters a microalbuminuric state. Again, by observing the excretion of modified albumin, a change in the kidney and possibly onset of a kidney disease may be detected.

[0100] A normoalbuminuric subject, or normoalbuminuric diabetic patient may continue to have a low albumin excretion rate of less than 20 μg/min as determined by RIA, for many years. The presence of albumin in the urine is a sign that functions of the kidney may be impaired. Once this level begins to change, treatment may be initiated.

[0101] In a normal individual a small amount of albumin is detectable in the urine. Total filtered albumin appears mainly as fragmented albumin in urine. Some albumin may be detected in normoalbuminuric individuals. However, the excretion rate of albumin in urine in a normoalbuminuric individual may be as low as 5 μg/min. This level is generally detectable by RIA.

[0102] The modified protein of the invention can be detected by a variety of methods that are well-known in the art, including, but not limited to chromatography, electrophoresis and sedimentation, or a combination of these, which are described in Karger B L, Hancock W S (eds.) High Resolution Separation and Analysis of biological Macromolecules. Part A Fundamentals in Methods in Enzymology, Vol. 270, 1996, Academic Press, San Diego, Calif., USA; Karger B L, Hancock W S (eds.) High Resolution Separation and Analysis of biological Macromolecules. Part B Applications in Methods in Enzymology, Vol. 271, 1996, Academic Press, San Diego, Calif., USA; or Harding S E, Rowe, A J, Horton J C (eds.) Analytical Ultracentrifugation in Biochemistry and Polymer Science. 1992, Royal Soc. Chemistry, Cambridge, UK, which references are incorporated herein by reference in their entirety.

[0103] The electrophoresis method includes, but is not limited to, moving-boundary electrophoresis, zone electrophoresis, and isoelectric focusing.

[0104] The chromatography method includes, but is not limited to, partition chromatography, adsorption chromatography, paper chromatography, thin-layer chromatography, gas-liquid chromatography, gel chromatography, ion-exchange chromatography, affinity chromatography, and hydrophobic interaction chromatography. Preferably, the method is a sizing gel chromatography and hydrophobic interaction chromatography. More preferably, the method is hydrophobic interaction chromatography using a HPLC column.

[0105] The modified protein can also be detected by the use of specific albumin dyes. Such methods are described by Pegoraro et al., American Journal of Kidney Diseases 35(4): 739-744 (April 2000), the entire disclosure of which is hereby incorporated by reference. The modified albumin, as well as the whole albumin, is detectable by this dye method to provide the sum of modified albumin and whole or intact albumin. This detection method may be used with or without an initial separation of the albumin components from urine. Such dyes normally do not detect fragments <10,000 in molecular weight, but will detect the modified albumin.

[0106] In this dye method of detection, a dye such as Albumin Blue **580** is used. Such dyes are naturally non-fluorescent, but fluoresce on binding to intact albumin as well as the modified albumin, but do not bind to globulins. Therefore, globulins do not interfere with the assay so that measurements can be made in unfractionated urine.

[0107] Applicant has found that among diabetics, a normoalbuminuric diabetic patient has almost undetectable levels of modified or fragments of albumin when analyzed by conventional RIA. They appear to be normal. However, when the urine is tested by HPLC, the levels of modified albumin are much greater than found in a normal individual.

This difference in albumin may be attributed to the inability of conventional RIA's to adequately detect all albumin (total albumin) in intact or modified forms. Thus, HPLC is preferred for generating a fragmentation profile. A fragmentation profile on HPLC is characterized by a series of peaks representing a number of species of albumin as fragments or in intact or modified forms.

[0108] In a preferred aspect of the present invention, the method of determining a propensity for or early diagnosis of a kidney disease in a subject is determined before the subject becomes microalbuminuric.

[0109] Measuring albumin content in a sample by an HPLC method of the present invention may provide different results from its measurement by conventional RIA. In the HPLC technique, a low level of albumin is observed in normal individuals. When the level of modified albumin begins to be detected and its level increases, and progresses toward microalbuminuria then a patient can be determined to have a propensity for kidney disease.

[0110] In a normal individual, the HPLC generated fragmentation profile is characterized by the absence of a peak in a region where full-length native albumin elutes. Instead, multiple fragmented albumin is detectable. A pure protein product (unmodified) produces essentially a single peak. For example, using a hydrophobicity HPLC, albumin was observed to elute in the range of 39-44 minutes (**FIG. 5**). Thus, a normal individual would provide a distinct fragmentation profile indicative of an absence of kidney disease or no propensity for a kidney disease. However, as kidney disease progresses, an increasing amount of modified albumin first, and then native form later are detectable. The fragmentation profile begins to change and more products in the region of full-length albumin manifests as additional spikes or an enlarged peak indicative of more intact/modified albumin in the urine.

[0111] In a HPLC generated fragmentation profile of a urine sample, the modified albumin may appear in a region where native albumin elutes but may be manifest as multiple peaks indicating the presence of multiple forms of modified albumin.

[0112] In a further preferred embodiment, the propensity for kidney disease may be measured by determining the presence of or identifying at least one species of modified albumin. This may be determined or identified by the presence of a specific peak on a HPLC profile, preferably the peak is within the range of position that corresponds to the elution position of the native albumin.

[0113] The method for determining the propensity for kidney disease is applicable to any individual. Kidney disease may be caused by a number of factors including bacterial infection, allergic, congenital defects, stones, tumors, and chemicals, or from diabetes. Preferably, the method is applicable for determining a propensity for kidney disease in diabetic patients that may progress to a kidney disease. Preferably, the individual is a normoalbuminuric diabetic. However, normal individuals may be monitored for propensity for the disease by determining increased levels of intact or modified albumin in the urine.

[0114] The method of the invention can be carried out using non-antibody separation procedures as described above. However, antibody specific for modified protein may also be used to detect the presence of the modified protein.

[0115] The antibody to the modified protein may be obtained using the following method. The procedure is described specifically for albumin by way of example only, and can be readily applied to antibody production against any other protein in the urine. The method seeks to determine which modified albumin molecule is the most sensitive marker to identify diabetic patients, for example, who will progress to kidney complications.

[0116] The modified albumin is characterized by carrying out a quantitative separation of the modified albumin molecules, such as by preparative HPLC. The modified proteins are analyzed for ligand binding, such as glycation. Subsequently, amino acid sequence of the individual modified protein is determined, preferably by mass spectrometry using methods described in Karger B L, Hancock W S (eds.) High Resolution Separation and Analysis of biological Macromolecules. Part A Fundamentals in Methods in Enzymology, Vol. 270, 1996, Academic Press, San Diego, Calif., USA; or Karger B L, Hancock W S (eds.) High Resolution Separation and Analysis of biological Macromolecules. Part B Applications in Methods in Enzymology, Vol. 271, 1996, Academic Press, San Diego, Calif., USA, for example, which references are incorporated herein by reference in their entirety. In a preferred embodiment, there may be about 3 to 4 modified albumin species.

[0117] The method of generating antibody against the modified albumin seeks to develop a diagnostic immunoassay for the modified albumin that predicts those diabetic patients, for example, that progress to kidney complications. To accomplish this, sufficient quantities of modified albumin is prepared by HPLC. Antibodies are made by sequential injection of the modified albumin in an animal such as a rabbit, to generate good titer, and the antibodies are isolated using conventional techniques using methods described in Goding J W, Monoclonal Antibodies: Principles and Practice. Production and Application of Monoclonal Antibodies in Cell Biology, Biochemistry and Immunology, 2nd Edition **1986**, Academic Press, London, UK; or Johnstone A, Thorpe R, Immunochemistry in Practice, 3rd edition **1996**, Blackwell Science Ltd, Oxford, UK, for example, which references are incorporated herein by reference in their entirety. The obtained antibodies may be polyclonal antibodies or monoclonal antibodies.

[0118] Preferably, at least one species of a modified albumin is isolated and identified for use in determining a propensity for kidney disease. The isolated species may be used to generate antibodies for use in immunoassays. The antibodies may be tagged with an enzymatic, radioactive, fluorescent or chemiluminescent label. The detection method may include, but is not limited to radioimmuoassay, immunoradiometric assay, fluorescent immunoassay, enzyme linked immunoassay, and protein A immunoassay. The assays may be carried out in the manner described in Goding J W, Monoclonal Antibodies: Principles and Practice. Production and Application of Monoclonal Antibodies in Cell Biology, Biochemistry and Immunology. 2nd Edition **1986**, Academic Press, London, UK; Johnstone A, Thorpe R, Immunochemistry in Practice, 3rd edition **1996**, Blackwell Science Ltd, Oxford, UK; or Price C P, Newman D J (eds.) Principles and Practice of Immunoassay, 2nd Edition, 1997

Stockton Press, New York, N.Y., USA, for example, which references are incorporated herein by reference in their entirety.

[0119] In another aspect of the present invention there is a method of measuring intact modified albumin useful for the detection of disease. The present invention recognizes that there are two types of intact protein fragments that are distinguished by their source. As mentioned above, filtered proteins are degraded during renal passage and the fragments so generated appear in the urine (i.e., the first source). A second source of intact protein fragments is the outcome of a methodology of measuring intact protein. We have observed that under denaturing conditions during electrophoresis, the protein may dissociate into large fragments. Such dissociation during electrophoresis does not occur under non-denaturing. Therefore the present invention provides a method to measure and analyze fragments resulting from denaturation that will be able to detect the disease. Preferably, the propensity for renal disease and/or renal complications of a disease may be measured by determining the presence of intact protein, like albumin, in a urine sample or samples by concentrating the urine, denaturing the sample by enzymic or chemical breakdown and analyzing the sample for intact protein. Analyses for intact protein include applying the urine sample on a chromatography, electrophoresis or sedimentation apparatus. Non-limiting exemplary methods of analysis include partition chromatography, thin layer chromatography, gas-liquid chromatography, gel chromatography, ion-exchange chromatography, affinity chromatography, or hydrophobic interaction chromatography, moving-boundary electrophoresis, zone electrophoresis, or isoelectric focusing.

[0120] In still another aspect of the invention, the propensity for renal disease and/or renal complications of a disease may be measured by determining the presence of intact albumin in a urine sample or samples with an antibody prepared from or with purified or substantially purified form of intact albumin. As such, in another method of the invention, intact albumin is purified or substantially purified using the following separation/purification procedure.

[0121] Preferably, urine is collected from a diabetic patient. The urine is concentrated through a filter containing small pores allowing water and small molecules to be removed from the urine (less than 50 kDa in size) while retaining any intact albumin (69 kDa in size). Native albumin is removed from the concentrated urine using affinity chromatography, for example. Such chromatography involves coupling a commercially available antibody that detects native albumin (but not intact albumin) to a special matrix (cyanogen bromide activated sepharose) under mild conditions to form a bond between the antibody and the agarose matrix. The urine sample is then applied to the antibody-agarose matrix and all the native albumin in the sample binds to the antibody. The unbound intact albumin is then eluted from the matrix. Preferably, affinity purified intact albumin is further purified to remove any remaining contaminants using HPLC, for example. The time taken for native albumin to elute on the HPLC column can be determined to be used as a standard control. Samples of the affinity purified urine are then applied to the HPLC and only material eluting at the same times as the albumin standard are collected. HPLC purified intact albumin is further concentrated to remove water as described above using a filter containing small pores allowing water and small molecules to be removed from the urine (less than 50 kDa in size).

[0122] In another preferred embodiment, provided is a method of the invention to generate antibody against the purified or substantially purified intact albumin to develop a diagnostic immunoassay for intact albumin. The antibody may be polyclonal or monoclonal. Detection of intact albumin in a sample is indicative of the onset or presence of renal disease and/or kidney complications of disease.

[0123] Preferably, urine is collected from a patient, such as a diabetic patient. The urine is concentrated through a filter containing small pores to allow water and small molecules to be removed from the urine (less than 30 kDa in sized) while retaining any intact albumin (69 kDa in size). The concentrated urine is dialyzed to remove any small contaminants less than 15 kDa in size. The dialyzed sample (antigen) is mixed with an adjuvant, more preferably with an equal amount of an adjuvant. Animals such as rabbits are injected with the antigen-adjuvant mixture, and preferably injected under the skin at multiple sites along the back. The animals are repeatedly injected with antigen-adjuvant mixture periodically to increase the blood concentration of antibody. A sample of blood from the animal is removed, preferably removed from the ear vein, and tested by ELISA.

[0124] More preferably, monoclonal antibodies are prepared against purified or substantially purified intact albumin to develop a diagnostic immunoassay for intact albumin. Mice are immunized with an antigen, in this case intact albumin, and are given an intravenous booster immunization three days before they are killed in order to produce a large population of spleen cells secreting specific antibody. Spleen cells are harvested and are fused with immortal myeloma cells using polyethylene glycol. The fused cells are known as a hybrid cell line called a hybridoma and are cultured/grown in hypoxanthine-aminopterin-thymidine (HAT) medium. Only immortal hybridomas proliferate and the unfused cells die. Individual hybridomas are screened by known methods in the art, such as using an enzyme linked immunosorbent assay or ELISA, for antibody production and cells that make antibody of the desired specificity are cloned by growing them up from a single antibody producing cell. The cloned hybridoma cells are grown in bulk culture to produce large amounts of antibody. As each hybridoma is descended from a single cell, all the cells of a hybridoma cell line make the same antibody molecule (i.e., a monoclonal antibody).

[0125] It is to be understood that the methods described herein for generating intact albumin antibodies from purified or substantially purified intact albumin can also be used to generate antibodies to other intact proteins that are not detected by conventional antibodies. For example, the present methods can be used to generate a purified or substantially purified form of modified protein in the urine that are not detected by conventional antibodies, presumably as a result of the modification(s). For example, it is known that in patients with proteinuria, there is an increase of protein in the urine, such as for example, albumin, $\alpha 1$ acid glycoprotein (orosomucoid), $\alpha 1$ acid antitrypsin, $\alpha 1$ glycoprotein, $\alpha 1$ lipoprotein, alpha-1-microglobumin, $\alpha 2$ 19S glycoprotein, bence-jones proteins, $\beta 1$ lipoprotein, $\beta 1$ transferrin, $\beta 2$ glycoprotein, $\beta 2$ microglobin, ceruloplasmin, euglobulin, fibrinogen, globulin (α-globulin ($\alpha 1$-globulin, α2-globulin) β-globulin, γ-globulin), glucose oxidase, growth hormone, haptoglobin, horseradish peroxidase, immunoglobulins A, E, G and M, insulin, lactate dehydrogenase, lysozyme, myoglobin, protein hormone, pseudoglobulin I and II, and parathyroid hormone, prealbumin, retinol binding protein, and tamm horsfall glycoprotein. These proteins can be treated as described herein for albumin to remove native protein, and any intact protein can be used to generate anti-intact protein antibodies. The anti-intact protein antibody can then be used to diagnose pathologic conditions, such as proteinuria or kidney disease.

[0126] The invention also provides an article of matter or a kit for rapidly and accurately determining the presence or absence of modified protein such as modified native albumin or intact albumin, in a sample quantitatively or non-quantitatively as desired. Each component of the kit(s) may be individually packaged in its own suitable container. The individual container may also be labeled in a manner, which identifies the contents. Moreover, the individually packaged components may be placed in a larger container capable of holding all desired components. Associated with the kit may be instructions, which explain how to use the kit. These instructions may be written on or attached to the kit.

[0127] The invention is also directed to a method of determining a treatment agent for renal disease and/or renal complications of a disease, comprising:

[0128] (a) administering to a person an agent that is suspected of being able to treat the disease;

[0129] (b) obtaining a urine sample from the person; and

[0130] (c) assaying for the modified form of the protein in the sample, wherein either the presence of or lack of presence of a modified form of the protein in the urine or decreasing amount of the modified form of the protein over time indicates that the agent is a treatment agent for the disease. The treatment agent may be a lysosome activating agent that may act directly or indirectly to activate lysosome, and thereby cause the lysosome to digest post-glomerular filtered proteins, which is a sign of a healthy kidney.

[0131] The process of trafficking of proteins to the lysosomes plays a role in the mechanism of albuminuria in diabetes. An intracellular molecule that is involved in trafficking is protein kinase C (PKC). It is contemplated that a drug or agent can be formulated that will activate lysosomal trafficking or inhibit PKC.

[0132] Accordingly, in one aspect of the present invention, there is provided a lysosome-activating compound for use in reactivating lysosomes or processes that direct substrates to the lysosome or products away from the lysosome.

[0133] In another aspect of the present invention, there is provided a composition comprising a lysosome-activating compound and a carrier.

[0134] In yet another aspect of the invention there is provided a method of preventing or treating kidney disease, said method including administering an effective amount of a lysosome-activating compound to a subject.

[0135] In yet another aspect of the present invention, there is provided a method of screening a multiplicity of compounds to identify a compound capable of activating lysosomes or processes that direct substrates to the lysosome or products away from the lysosome, said method including the steps of:

[0136] (a) exposing said compound to a lysosome and assaying said compound for the ability to activate a lysosome wherein said lysosome when activated has a changed activity;

[0137] (b) assaying for the ability to restore a cellular process to substantially normal levels in kidney tissue, wherein said kidney tissue has a low lysosome activity; and/or

[0138] (c) assaying for the ability to restore tissue turnover to substantially normal levels in kidney tissue, wherein said kidney tissue has low lysosome activity.

[0139] Lysosomes may be associated with the breakdown of proteins, particularly albumin, in the kidney. In cases of microalbuminuria, substantial amounts of albumin escape lysosomal breakdown possibly due to a deactivated lysosome. Restoration of lysosomal breakdown may restore the balance in the kidney of cellular processes and tissue turnover.

[0140] A lysosome-activating compound may be a compound that acts directly or indirectly on the lysosome. By acting indirectly, the compound may act on a component, which influences the activity of the lysosome. Nevertheless, the outcome results in an activation of the lysosome, thereby providing enhanced protein breakdown.

[0141] In another aspect of the present invention, there is provided a composition comprising a lysosome-activating compound and a carrier.

[0142] The composition may be a physiologically acceptable or pharmaceutically acceptable composition. However, it will be a composition which allows for stable storage of the lysosome activating compound. Where the composition is a pharmaceutically acceptable composition, it may be suitable for use in a method of preventing or treating kidney disease.

[0143] In yet another aspect of the invention there is provided a method of preventing or treating kidney disease, said method including administering an effective amount of a lysosome-activating compound to a subject.

[0144] As described above, the lysosome-activating compound may act by reactivating the lysosome so that cellular processes and tissue turnover are restored fully or in part, thereby resulting in the kidney being restored partially or fully. In any case, administering a lysosome activating compound to an animal having kidney disease may restore lysosome activity fully or in part.

[0145] Methods of administering may be oral or parenteral. Oral may include administering with tablets, capsules, powders, syrups, etc. Parenteral administration may include intravenous, intramuscular, subcutaneous or intraperitoneal routes.

[0146] The changed activity of the lysosome is preferably a change which enhances the activity of the lysosome so that albumin breakdown is improved. The ability to not only activate lysosome but also improve cellular processes and/or tissue turnover is a characteristic of the most desirable lysosome activating compound. Preferably, it is desired to use the lysosome activating compound to restore kidney function.

[**0147**] In another aspect of the present invention there is provided a method for preventing kidney disease in a subject, said method including:

[**0148**] (a) measuring the amount of intact and modified intact albumin content in a urine sample;

[**0149**] (b) determining a change in the amount of intact albumin in the urine that has been modified so as to be not detectable by conventional RIA methods wherein the change is indicative of a propensity for kidney disease; and

[**0150**] (c) treating the animal for a kidney disease when a change is determined.

[**0151**] The following examples are offered by way of illustration of the present invention, and not by way of limitation.

EXAMPLES

Example 1

[**0152**] Size Exclusion Chromatography of Human Serum Albumin (HSA)

[**0153**] Normal, healthy volunteers were used to provide urine for analyzing the distribution of albumin in their urine.

[**0154**] ^{3}H[HSA] (Human Serum Albumin) was injected into healthy volunteers and urine and plasma were collected and analyzed by size exclusion chromatography using a G-100 column. The column was eluted with PBS (pH=7.4) at 20 ml/hr at 4° C. The void volume (V_0) of the column was determined with blue dextran T2000 and the total volume with tritiated water.

[**0155**] Tritium radioactivity was determined in 1 ml aqueous samples with 3 ml scintillant and measured on a Wallac **1410** liquid scintillation counter (Wallac Turku, Finland).

[**0156**] **FIG. 2** illustrates the distribution of albumin in urine and in plasma.

Example 2

[**0157**] Albumin Excretion in a Normal, Healthy Volunteer and Diabetic Patient ^{3}H[HSA] as used in Example I was injected into a normal, healthy volunteer and a diabetic patient. Samples of urine were collected and ^{3}H[HSA] was determined as in Example 1.

[**0158**] The normal, healthy volunteer (**FIG. 3**) shows the excretion of fragments of albumin on a size exclusion chromatography as performed in Example 1.

[**0159**] The diabetic patient (**FIG. 4**) shows the presence of substantially full-length and fragmented albumin on size exclusion chromatography. However, excretion rates of albumin detectable by these methods were in the order of 5 μg/min (control) and 1457 μg/min (diabetic).

Example 3

[**0160**] Determination of Intact Albumin, and Intact/Modified Albumin on HPLC

[**0161**] Urine samples were collected from normal, healthy volunteer, normoalbuminuric diabetic patients and from macroalbuminuric patients. Urine was collected midstream in 50 ml urine specimen containers. The urine was frozen until further use. Prior to HPLC analysis the urine was centrifuged at 5000 g.

[**0162**] Samples were analyzed on HPLC using a hydrophobicity column Zorbax **300** SB-CB (4.6 mmx 150 mm). A 50 μl sample loop was used.

[**0163**] Samples were eluted from the columns using the following conditions.

[**0164**] Solvent A H2O, 1% trifluoro acetic acid

[**0165**] Solvent B 60% acetonitrile, 0.09% TFA

[**0166**] Solvent A2 99.96>00.00:49.58 min Pressure 9.014 Mpascalls (~110 psi)

[**0167**] Solvent B2 0.04>100.0:49.58 min Pressure 7.154 Mpascalls

[**0168**] A wavelength of 214 nm was used.

Example 4

[**0169**] Purification of Modified Albumin for Antibody Production by Standard Techniques

[**0170**] Urine from microalbuminuric patient which had an intact albumin concentration of 43.5 mg/L as determined by turbitimer (involving conventional immunochemical assay) was initially filtered through a 30 kDa membrane to separate the modified albumin from low molecular weight (<30,000) protein fragments in urine. The material that was retained by the filter gave a yield of intact albumin of 27.4 mg/L as determined by turbitimer assay. This retained material was then subjected to size exclusion chromatography on Sephadex G100. The material collected was the peak fraction that coelutes with intact albumin. This material gave a yield of 15.2 ml/L of albumin as determined by the turbitimer method. This material was then subjected to affinity chromatography on an intact albumin antibody column. This column will only bind albumin that has conventional epitopes. The yield of material that eluted from the column was <6 mg/L (lowest sensitivity of the turbitimer). This is expected as the immunoreactive albumin would have bound to the affinity column. The eluate was then subject to reverse phase HPLC chromatography (as described above) to determine the amount of immuno-unreactive albumin in the sample. A 1452 unit area corresponding to 30.91 mg/L of purified modified albumin was noted as shown in **FIG. 5**. This purified modified albumin can then be used for antibody production by standard means.

[**0171**] Results

[**0172**] **FIG. 5** illustrates a HPLC profile of albumin alone. Essentially a single peak which elutes at approximately 39-44 minutes retention time was obtained.

[**0173**] **FIG. 6** illustrates a HPLC profile of plasma showing a distinct albumin peak at approximately 39-44 minutes as well as other peaks corresponding to other plasma proteins.

[**0174**] **FIG. 7** illustrates a HPLC profile of a normal, healthy volunteer showing no albumin peak in the urine sample. This individual breaks down the albumin excreted into the urine possibly via an active lysosome. Substantial fragmented products were evident showing prominence of some species, particularly of a species at approximately less than 14.5 minutes retention time.

[0175] When urine from a normoalbuminuric diabetic patient (with an albumin excretion rate of 8.07 μg/min, as measured by RIA) is analyzed (**FIG. 8**), small amounts of modified albumin eluting at approximately 39-44 minutes retention time is evident. Whereas conventional test indicates the presence of <6 mg/l of albumin in the urine sample, the method of the invention showed that the true albumin content in the urine sample was 26.7 mg/l. Treatment for the disease should have begun on this individual. Albumin by-products or fragmented albumin is present as in the normal, healthy volunteer.

[0176] Another urine sample from normoalbuminuric diabetic patient (with albumin excretion rate of 17.04 μg/min) was analyzed (**FIG. 9**). RIA tests show albumin excreted in the urine for this patient. However, on HPLC (**FIG. 9**) an albumin or modified albumin peak is evident at approximately 39-44 minutes retention time. Whereas conventional test indicates the presence of <6 mg/l of albumin in the urine sample, the method of the invention showed that the true albumin content in the urine sample was 81.3 mg/l. Treatment for the disease should have begun on this individual. This peak begins to show a multiple peaked appearance. A smaller peak corresponding to intact albumin shows that modified albumin may represent the peak at 39-44 minutes. The presence of this albumin peak compared with the profile of a normal, healthy volunteer having no albumin peak shows a change in the detectable levels of the amount of intact/modified albumin. This may signal a propensity for a kidney disease.

[0177] A further urine sample from a normoalbuminuric diabetic patient (with an albumin excretion rate of 4.37 μg/min) was analyzed, and the HPLC profile is illustrated in **FIG. 10**. Again, modified albumin was detected at approximately 39-44 minutes retention time showing multiple peaks. This patient again did register normal albumin by RIA. Whereas conventional test indicates the presence of <6 mg/l of albumin in the urine sample, the method of the invention showed that the true albumin content in the urine sample was 491 mg/l. Treatment for the disease should have begun on this individual. It is clear that modified albumin assessment is necessary to identify these changes. This patient would be determined to have a propensity for kidney disease. As kidney disease progresses, the modified albumin peak will continue to increase.

[0178] This is shown in **FIG. 11** where a urine sample of a macroalbuminuric patient was analyzed. A quite significant albumin peak at approximately 39-44 minutes retention time showing multiple peaks was evident. The patient's albumin content was 1796 mg/l. Treatment for this individual is in progress.

[0179] The method of the invention results in early detection of a propensity for a renal disease as illustrated by the longitudinal studies in **FIGS. 12-14**. **FIGS. 12-14** show situations in which the ACE inhibitor treatment for diabetes was begun later than it should have had the modified albumin detection method of the invention been used. Detecting modified protein using the method according to the invention is a more effective method for predicting the onset of a renal disease than using conventional RIA.

Example 5

[0180] **FIG. 16** is a schematic diagram illustrating the manner in which an intact filtered protein may be degraded by normal functioning kidneys and diseased kidneys.

[0181] **FIG. 17** illustrates the HPLC profile of a trypsin digested sample of albumin that has been filtered through a 30,000 molecular weight cut-off membrane. The filtrate yields many peaks eluting between 2 to 30 minutes.

[0182] **FIG. 18** illustrates the HPLC profile of a control, normal subject showing many fragments in the eluting range of 10 to 30 minutes. The HPLC profile of a diabetic patient with macroalbuminuria (1457 microgram per minute) shows a significantly different fragment profile in the range of 10-30 minutes.

[0183] **FIG. 19** illustrates the HPLC profile of a subject with renal disease. As compared with **FIG. 18**, the fragmentation process of filtered proteins is inhibited. The number of fragments is decreased and the size of the fragments is increased.

Example 6

[0184] Preparation of Purified or Substantially Purified Intact Albumin for Antibody Production

[0185] Purified or substantially purified intact protein (in this case albumin) was prepared for antibody production for the detection of disease, in this case kidney disease.

[0186] Urine was collected from a diabetic patient who had kidney disease. The amount of intact albumin in the urine was found to be 231 mg/L as measured by a conventional immunoassay (immunoturbidimetry) and 326 mg/L as measured by HPLC. The urine was concentrated through a filter containing small pores allowing water and small molecules to be removed from the urine (<50 kDa in size), while retaining any intact albumin (69 kDa in size). The final concentration of native albumin in the urine was now 464 mg/L as measured by immunoturbidimetry and 945 mg/L as measured by HPLC as shown in **FIG. 20**.

[0187] Native albumin was removed from the concentrated urine using affinity chromatography. This involves coupling a commercially available antibody that detects native albumin (but not intact albumin) to a special matrix (cyanogen bromide activated sepharose) under mild conditions to form a bond between the antibody and the agarose matrix. The urine sample was then applied to the antibody-agarose matrix and all the native albumin in the sample binds to the antibody. The unbound intact albumin was then eluted from the matrix. The concentration of intact albumin eluted from the matrix was <6 mg/L as measured by immunoturbidimetry and 103 mg/L as measured by HPLC as shown in **FIG. 21**.

[0188] Affinity purified intact albumin was further purified to remove any remaining contaminants using HPLC. The time taken for native albumin to elute on the HPLC column was determined. Samples of the affinity purified urine were then applied to the HPLC and only material eluting at the same time as the albumin standard was collected. The final concentration of intact albumin eluted from the HPLC was ~7.6 mg/L as measured by HPLC. Finally, HPLC purified intact albumin was further concentrated to remove water as described above (point 1) to give a final concentration of 30.8 mg/L as measured by HPLC.

Example 7

[0189] Preparation of Anti-intact Albumin Antibodies

[0190] To obtain anti-intact protein antibodies (in this case albumin), animal, in this case rabbits, were repeatedly exposed to a foreign antigen (in this case intact albumin). As their immune system recognizes the antigen to be foreign to the body, it elicits an immune response to produce antibodies, thereby allowing the body to eliminate the foreign molecule. It is these antibodies that are harvested.

[0191] Urine was collected from a diabetic patient who had kidney disease. The amount of intact albumin in the urine was found to be 231 mg/L as measured by a conventional immunoassay (immunoturbidimetry) and 326 mg/L as measured by HPLC. The urine was concentrated through a filter containing small pores to allow water and small molecules to be removed from the urine (<30 kDa in size) while retaining any intact albumin (69 kDa in size). The final concentration of native albumin in the urine was now 786 mg/L as measured by immunoturbidimetry. The concentrated urine was placed in dialysis tubing containing small pores and allowing any small contaminants (<15 kDa in size) to be removed. The dialyzed sample (antigen) was mixed with an equal amount of adjuvant (a solution which helps elicit an antibody response) and the rabbits were injected under the skin at multiple sites along the back. Rabbits were repeatedly injected with the antigen-adjuvant mixture periodically to increase the blood concentration of antibody. A sample of blood was removed from the ear vein and tested by ELISA as described below.

Example 8

[0192] Assay to Test Intact Albumin Antibodies

[0193] An ELISA (enzyme-linked immunosorbent assay was performed to quantitate the antigen, in this case, intact albumin. The steps involved in performing an ELISA for intact albumin are as follows. **FIG. 22** is a schematic diagram showing the first, fourth, fifth and last steps involved in performing an ELISA for intact albumin.

[0194] First, a 96-well ELISA plate was prepared as set forth in Table 1.

TABLE 1

	1	2	3	4	5	6	7	8	9	10	11	12
Blank	B	B	B	B	HSA	HSA	HSA	HSA	gAlb	gAlb	gAlb	gAlb
αHSA	B	B	B	B	HSA	HSA	HSA	HSA	gAlb	gAlb	gAlb	gAlb
A751	B	B	B	B	HSA	HSA	HSA	HSA	gAlb	gAlb	gAlb	gAlb
A752	B	B	B	B	HSA	HSA	HSA	HSA	gAlb	gAlb	gAlb	gAlb
241	B	B	B	B	HSA	HSA	HSA	HSA	gAlb	gAlb	gAlb	gAlb
242	B	B	B	B	HSA	HSA	HSA	HSA	gAlb	gAlb	gAlb	gAlb
244	B	B	B	B	HSA	HSA	HSA	HSA	gAlb	gAlb	gAlb	gAlb
Blank	B	B	B	B	B	B	B	B	B	B	B	B

[0195] Wells marked ‘HSA’ were coated (bound) with native albumin. Wells marked ‘gAlb’ were coated with the purified intact albumin (described above) and wells marked ‘B’ were left blank. The plate was incubated overnight at 4° C.

[0196] Second, the plate was washed to remove any unbound material.

[0197] Third, all unreacted sites in the wells were blocked with skim milk powder, incubated at 37° C. for 1.5 hours, followed by a wash phase.

[0198] Fourth, the following antibodies were then applied to the wells of the plate as shown in Table 1.

[0199] αHSA native albumin antibody (Dako)

[0200] A751, A752 intact albumin antibody (BioSource)

[0201] **241, 242, 244** intact albumin antibody (Biodesign)

[0202] Blank rows, indicated as such by “B”, had assay buffer added. The plate was incubated for 1 hour at 37° C., followed by a wash phase.

[0203] Fifth, to determine the amount of intact albumin antibody bound to the intact albumin, the wells were reacted with a detection antibody (sheep anti-rabbit IgG), which was conjugated to alkaline phosphatase to allow for a color reaction. This was applied to each well and incubated for 1 hour at 37° C., followed by a wash phase.

[0204] Lastly, to enable the color reaction to occur, each well was reacted with an enzyme substrate (p-nitrophenyl phosphate) and the intensity of the color reaction was measured by a plate reader.

[0205] Results of ELISA for Intact Albumin

TABLE 2

		1	2	3	4	5	6	7	8	9	10	11	12
		Antigen											
		Blank Coat				Serum Albumin Coat				gAlb Coat			
Antiserum	Blank0	.161	0.160	0.158	0.160	0.158	0.158	0.160	0.160	0.170	0.162	0.165	0.165
	αHSA	0.181	0.189	0.182	0.189	1.371	1.459	1.178	1.191	0.627	0.601	0.557	0.534
	A751	0.178	0.176	0.175	0.174	1.080	1.030	1.046	1.012	1.148	1.188	1.143	1.183
	A752	0.179	0.187	0.173	0.320	0.731	0.826	0.805	0.590	1.129	1.149	1.129	1.040
	241	0.171	0.169	0.165	0.166	0.842	1.003	0.811	0.803	0.747	0.773	0.764	0.740
	242	0.187	0.183	0.162	0.177	0.831	0.873	0.794	0.835	0.963	1.093	1.108	1.085
	244	0.175	0.176	0.176	0.175	0.990	1.042	0.943	0.896	0.996	0.925	0.938	0.875
	Blank0	.164	0.162	0.164	0.159	0.162	0.158	0.397	0.159	0.161	0.162	0.162	0.163

Averages

		Antigen		
		Blank	HSAg	Alb
Antiserum	Blank0	.160	0.159	0.166
	αHSA	0.185	1.300	0.580
	A751	0.176	1.042	1.166
	A752	0.214	0.738	1.112
	241	0.168	0.865	0.756
	242	0.182	0.833	1.062
	244	0.176	0.968	0.934
	Blank0	.162	0.219	0.162

[0206] The plate reader gives a value for the color intensity in each well for the ELISA and the results are shown above. The higher the number, the greater the binding between the antigen and antibody. The results for the blank wells indicate the background color intensity for each well. The results for the wells incubated with the various antibodies indicate that blood obtained from all rabbits maintained by BioSource and Biodesign have significant and similar binding activity towards both native albumin and intact albumin. The relatively high reactivity of the commercial native albumin antibody for the intact albumin could be due to the fact that it was used at 1 part in 1,000 dilution; a much higher concentration than that used normally for assay (1 part in 20,000).

[0207] All of the references cited herein are incorporated by reference in their entirety.

[0208] Finally, it is to be understood that various other modifications and/or alterations may be made without departing from the spirit of the present invention as outlined herein.

1. An isolated intact protein.

2. The intact protein according to claim 1, wherein the intact protein is obtained by a process comprising:

a. collecting a urine sample;

b. concentrating the sample by removing water and small molecules from the sample; and

c. removing native protein from the sample.

3. The intact protein according to claim 2, wherein the step of concentrating the sample comprises filtering the sample through a filter having pores sufficiently small to allow water and molecules to pass while retaining any intact protein.

4. The intact protein according to claim 2, wherein the step of removing native protein comprises:

a. coupling an antibody that detects native protein to a matrix to form an antibody-matrix bond;

b. applying the sample to the antibody-matrix, wherein the native protein binds to the antibody; and

c. eluting intact protein from the matrix.

5. The intact protein according to claim 4, wherein the matrix is a cyanogen bromide activated sepharose matrix.

6. The intact protein according to claim 1, wherein the intact protein is selected from the group consisting of albumin, α_1 acid glycoprotein, α_1 acid antitrypsin, α_1 glycoprotein, α_1 lipoprotein, alpha-1-microglobumin, α_2 19S glycoprotein, bence-jones proteins, β_1 lipoprotein, β_1 transferrin, β2 glycoprotein, β2 microglobin, ceruloplasmin, euglobulin, fibrinogen, globulin, glucose oxidase, growth hormone, haptoglobin, horseradish peroxidase, immunoglobulins A, E, G and M, insulin, lactate dehydrogenase, lysozyme, myoglobin, protein hormone, pseudoglobulin I and II, and parathyroid hormone, prealbumin, retinol binding protein, and tamm horsfall glycoprotein.

7. The method according to claim 6, wherein the intact protein is albumin.

8. A method for preparing intact protein from a body sample comprising:

a. collecting a urine sample;

b. concentrating the sample by removing water and small molecules from the sample; and

c. removing native protein from the sample.

9. The method according to claim 8, wherein the step of concentrating the sample comprises filtering the sample through a filter having pores sufficiently small to allow water and molecules to pass while retaining any intact protein.

10. The method according to claim 8, wherein the step of removing native protein comprises:

a. coupling an antibody that detects native protein to a matrix to form an antibody-matrix bond;

b. applying the sample to the antibody-matrix, wherein the native protein binds to the antibody; and

c. eluting intact protein from the matrix.

11. The method according to claim 10, wherein the matrix is a cyanogen bromide activated sepharose matrix.

12. The method according to claim 8, wherein the intact protein is selected from the group consisting of albumin, α_1 acid glycoprotein, α_1 acid antitrypsin, α_1 glycoprotein, α_1 lipoprotein, alpha-1-microglobumin, α_2 19S glycoprotein, bence-jones proteins, β_1 lipoprotein, β_1 transferrin, β_2 glycoprotein, β_2 microglobin, ceruloplasmin, euglobulin, fibrinogen, globulin, glucose oxidase, growth hormone, haptoglobin, horseradish peroxidase, immunoglobulins A, E, G and M, insulin, lactate dehydrogenase, lysozyme, myoglobin, protein hormone, pseudoglobulin I and II, and parathyroid hormone, prealbumin, retinol binding protein, and tamm horsfall glycoprotein.

13. The method according to claim 12, wherein the intact protein is albumin.

14-21. (Cancelled)

22. A method for preparing anti-intact protein antibody, said method comprising:

a. collecting a urine sample from a subject;

b. concentrating the sample by removing water and small molecules;

c. removing contaminants from the concentrated sample;

d. mixing the sample of step c. with an adjuvant;

e. injecting the sample into an animal to elicit an antibody response;

f. collecting a blood sample from the animal; and

g. isolating anti-intact protein antibody from at least one blood sample.

23. The method according to claim 22, wherein the step for concentrating the sample comprises filtering the sample through a filter containing pores sufficiently small to allow water and molecules to be removed from the sample while retaining any intact modified protein.

24. The method according to claim 22, wherein the step for removing contaminants comprises dialyzing the sample.

25. The method according to claim 24, wherein the dialysis removes contaminants of less than about 15 kDa.

26. The method according to claim 22, wherein the step for mixing the sample with an adjuvant comprises mixing the sample and adjuvant in equal parts.

27. The method according to claim 22, wherein the intact protein is selected from the group consisting of albumin, α_1 acid glycoprotein, α_1 acid antitrypsin, α_1 glycoprotein, α_1 lipoprotein, alpha-1-microglobumin, α_2 19S glycoprotein, bence-jones proteins, β_1 lipoprotein, β_1 transferrin, β_2 glycoprotein, β_2 microglobin, ceruloplasmin, euglobulin, fibrinogen, globulin, glucose oxidase, growth hormone, haptoglobin, horseradish peroxidase, immunoglobulins A, E, G and M, insulin, lactate dehydrogenase, lysozyme, myoglobin, protein hormone, pseudoglobulin I and II, and parathyroid hormone, prealbumin, retinol binding protein, and tamm horsfall glycoprotein.

28. The method according to claim 27, wherein the intact protein is albumin.

29. An isolated monoclonal anti-intact protein antibody.

30. The anti-intact protein antibody according to claim 29, wherein the antibody is obtained by a process comprising:

a. collecting a urine sample from a subject;

b. concentrating the sample by removing water and small molecules;

c. removing contaminants from the concentrated sample;

d. mixing the sample of step c. with an adjuvant;

e. injecting the sample into an animal to elicit an antibody response;

f. collecting a spleen cell sample from the animal;

g. fusing the spleen cell sample with immortal myeloma cells to form hybridoma(s);

h. growing the hybridomas;

i. screening individual hybridomas for antibody production of a desired specificity;

j. cloning cells from a hybridoma that makes an antibody of the desired specificity; and

k. isolating monoclonal anti-intact protein antibody from the cloned cells.

31. The monoclonal anti-intact protein antibody according to claim 30, wherein the step for concentrating the sample comprises filtering the sample through a filter having pores sufficiently small to allow water and molecules to be removed while retaining any intact modified protein.

32. The monoclonal anti-intact protein antibody according to claim 30, wherein the step for removing contaminants comprises dialyzing the sample.

33. The monoclonal anti-intact protein antibody according to claim 32, wherein the dialysis removes contaminants of less than about 15 kDa.

34. The monoclonal anti-intact protein antibody according to claim 30, wherein the step for mixing the sample with an adjuvant comprises mixing the sample and adjuvant in equal parts.

35. The monoclonal anti-intact protein antibody according to claim 30, wherein the step for fusing spleen cells further comprises polyethylene glycol to fuse spleen cells with immortal myeloma cells.

36. The monoclonal anti-intact protein antibody according to claim 30, wherein the step for growing hybridomas further comprises a hypoxanthine-aminopterin-thymidine medium.

37. The monoclonal anti-intact protein antibody according to claim 30, wherein the step for screening individual hybridomas further comprises screening by an enzyme linked immunosorbent assay.

38. The antibody according to claim 29, wherein the intact protein is selected from the group consisting of albumin, α_1 acid glycoprotein, α_1 acid antitrypsin, α_1 glycoprotein, α_1 lipoprotein, alpha-1-microglobumin, α_2 19S glycoprotein, bence-jones proteins, β_1 lipoprotein, β_1 transferrin, β_2 glycoprotein, β_2 microglobin, ceruloplasmin, euglobulin, fibrinogen, globulin, glucose oxidase, growth hormone, haptoglobin, horseradish peroxidase, immunoglobulins A, E, G and M, insulin, lactate dehydrogenase, lysozyme, myoglobin, protein hormone, pseudoglobulin I and II, and parathyroid hormone, prealbumin, retinol binding protein, and tamm horsfall glycoprotein.

39. The antibody according to claim 38, wherein the intact protein is albumin.

40. A method for preparing a monoclonal anti-intact protein antibody, the method comprising:

a. collecting a urine sample from a subject;

b. concentrating the sample by removing water and small molecules;

c. removing contaminants from the concentrated sample;

d. mixing the sample of step c. with an adjuvant;

e. injecting the sample into an animal to elicit an antibody response;

f. collecting a spleen cell sample from the animal;

g. fusing the spleen cell sample with immortal myeloma cells to form hybridomas;

h. growing the hybridomas;

i. screening the hybridomas for antibody production of a desired specificity;

j. cloning cells that make an antibody of the desired specificity; and

k. isolating monoclonal anti-intact protein antibody from the cloned cells.

41. The method according to claim 40, wherein the step for concentrating the sample comprises filtering the sample through a filter containing pores sufficiently small to allow water and molecules to be removed from the sample while retaining any intact modified protein.

42. The method according to claim 40, wherein the step for removing contaminants comprises dialyzing the sample.

43. The method according to claim 40, wherein the dialysis removes contaminants of less than about 15 kDa.

44. The method according to claim 40, wherein the step for mixing the sample with an adjuvant comprises mixing the sample and adjuvant in equal parts.

45. The monoclonal anti-intact protein antibody according to claim 40, wherein the step for fusing spleen cells further comprises polyethylene glycol to fuse spleen cells with immortal myeloma cells.

46. The monoclonal anti-intact protein antibody according to claim 40, wherein the step for growing hybridomas further comprises a hypoxanthine-aminopterin-thymidine medium.

47. The monoclonal anti-intact protein antibody according to claim 40, wherein the step for screening individual hybridomas further comprises screening by an enzyme linked immunosorbent assay.

48. The method according to claim 29, wherein the intact protein is selected from the group consisting of albumin, α_1 acid glycoprotein, α_1 acid antitrypsin, α_1 glycoprotein, α_1 lipoprotein, alpha-1-microglobumin, α_2 19S glycoprotein, bence-jones proteins, β_1 lipoprotein, β_1 transferrin, β_2 glycoprotein, β_2 microglobin, ceruloplasmin, euglobulin, fibrinogen, globulin, glucose oxidase, growth hormone, haptoglobin, horseradish peroxidase, immunoglobulins A, E, G and M, insulin, lactate dehydrogenase, lysozyme, myoglobin, protein hormone, pseudoglobulin I and II, and parathyroid hormone, prealbumin, retinol binding protein, and tamm horsfall glycoprotein.

49. The method according to claim 48, wherein the intact protein is albumin.

50. An assay for detecting the presence of intact protein in a urine sample, comprising introducing an antibody that binds selectively to intact protein and determining whether the antibody binds to a component of the sample.

51. The assay according to claim 50, wherein the antibody is labeled with a detectable label.

52. The assay according to claim 50, wherein the intact protein is selected from the group consisting of albumin, α_1 acid glycoprotein, α_1 acid antitrypsin, α_1 glycoprotein, α_1 lipoprotein, alpha-1-microglobumin, α_2 19S glycoprotein, bence-jones proteins, β_1 lipoprotein, β_1 transferrin, β_2 glycoprotein, β_2 microglobin, ceruloplasmin, euglobulin, fibrinogen, globulin, glucose oxidase, growth hormone, haptoglobin, horseradish peroxidase, immunoglobulins A, E, G and M, insulin, lactate dehydrogenase, lysozyme, myoglobin, protein hormone, pseudoglobulin I and II, and parathyroid hormone, prealbumin, retinol binding protein, and tamm horsfall glycoprotein.

53. The method of claim 52, wherein the intact protein is albumin.

54. A method for diagnosing a renal disease and/or renal complications of a disease in a subject, comprising:

a. collecting a urine sample from the subject;

b. introducing an antibody that binds selectively to intact albumin;

c. determining whether the antibody binds to a component of the sample; and

d. correlating detection of intact albumin with the presence of renal disease and/or complications of a disease.

55. The method according to claim 54, wherein renal disease and/or renal complications of a disease cause an increase in the level of intact albumin in the urine of a subject.

56. The method according to claim 54, wherein the antibody is labeled with a detectable label.

57. A method for detecting an intact protein from a body sample comprising:

a. collecting a urine sample;

b. concentrating the sample by removing water and small molecules from the sample;

c. denaturing the sample; and

d. analyzing the sample for intact protein.

58. The method according to claim 57, wherein the step of concentrating the sample comprises filtering the sample through a filter having pores sufficiently small to allow water and molecules to pass while retaining any protein.

59. The method according to claim 57, wherein the step of denaturing the sample comprises enzymic or chemical breakdown of the protein in the sample.

60. The method according to claim 57, wherein the step of analyzing the sample comprises applying the sample on a chromatography, electrophoresis or sedimentation apparatus to test for intact protein.

61. The method according to claim 57, wherein the intact protein is selected from the group consisting of albumin, α_1 acid glycoprotein, α_1 acid antitrypsin, α_1 glycoprotein, α_1 lipoprotein, alpha-1-microglobumin, α_2 19S glycoprotein, bence-jones proteins, β_1 lipoprotein, β1 transferrin, β_2 glycoprotein, β_2 microglobin, ceruloplasmin, euglobulin, fibrinogen, globulin, glucose oxidase, growth hormone, haptoglobin, horseradish peroxidase, immunoglobulins A, E, G and M, insulin, lactate dehydrogenase, lysozyme, myoglobin, protein hormone, pseudoglobulin I and II, and parathyroid hormone, prealbumin, retinol binding protein, and tamm horsfall glycoprotein.

62. The method according to claim 61, wherein the intact protein is albumin.

63. A method of diagnosing a renal disease and/or renal complications of a disease in a subject comprising, detecting the presence of intact protein in a urine sample comprising the steps of:

a. collecting a urine sample from a the subject;

b. concentrating the sample by removing water and small molecules from the sample;

c. denaturing the sample; and

d. analyzing the sample for intact protein, where the presence of intact protein is indicative of renal disease and/or renal complications of a disease.

64. The method according to claim 63, wherein the step of concentrating the sample comprises filtering the sample through a filter having pores sufficiently small to allow water and molecules to pass while retaining any protein.

65. The method according to claim 63, wherein the step of denaturing the sample comprises enzymic or chemical breakdown of the protein in the sample.

66. The method according to claim 63, wherein the step of analyzing the step comprises applying the sample on a chromatography, electrophoresis or sedimentation apparatus to test for intact protein.

67. The method according to claim 63, wherein the intact protein is selected from the group consisting of albumin, α_1 acid glycoprotein, α_1 acid antitrypsin, α_1 glycoprotein, α_1 lipoprotein, alpha-1-microglobumin, α_2 19S glycoprotein, bence-jones proteins, β_1 lipoprotein, β_1 transferrin, β_2 glycoprotein, β_2 microglobin, ceruloplasmin, euglobulin, fibrinogen, globulin, glucose oxidase, growth hormone, haptoglobin, horseradish peroxidase, immunoglobulins A, E, G and M, insulin, lactate dehydrogenase, lysozyme, myoglobin, protein hormone, pseudoglobulin I and II, and parathyroid hormone, prealbumin, retinol binding protein, and tamm horsfall glycoprotein.

68. The method according to claim 67, wherein the intact protein is albumin.

* * * * *

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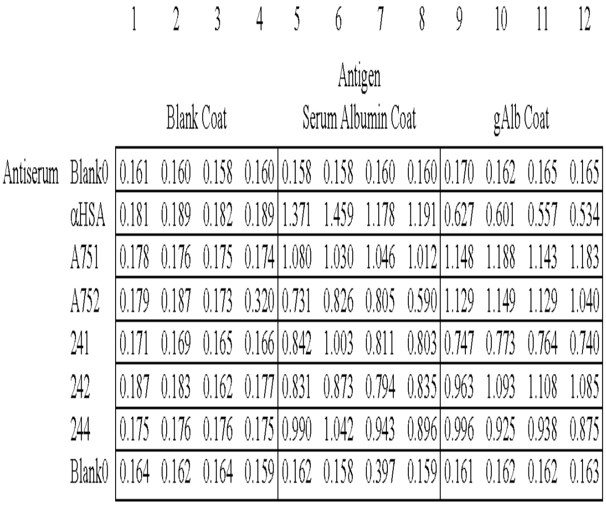
摘要(译)

一种诊断疾病的早期肾病和/或肾并发症的方法，其中完整白蛋白是肾病和/或并发症的指标。该方法包括分离的完整蛋白质，其抗完整蛋白质抗体，及其制备方法。



		1	2	3	4	5	6	7	8	9	10	11	12
		Antigen											
		Blank Coat				Serum Albumin Coat				gAlb Coat			
Antiserum	Blank0	0.161	0.160	0.158	0.160	0.158	0.158	0.160	0.160	0.170	0.162	0.165	0.165
	αHSA	0.181	0.189	0.182	0.189	1.371	1.459	1.178	1.191	0.627	0.601	0.557	0.534
	A751	0.178	0.176	0.175	0.174	1.080	1.030	1.046	1.012	1.148	1.188	1.143	1.183
	A752	0.179	0.187	0.173	0.320	0.731	0.826	0.805	0.590	1.129	1.149	1.129	1.040
	241	0.171	0.169	0.165	0.166	0.842	1.003	0.811	0.803	0.747	0.773	0.764	0.740
	242	0.187	0.183	0.162	0.177	0.831	0.873	0.794	0.835	0.963	1.093	1.108	1.085
	244	0.175	0.176	0.176	0.175	0.990	1.042	0.943	0.896	0.996	0.925	0.938	0.875
	Blank0	0.164	0.162	0.164	0.159	0.162	0.158	0.397	0.159	0.161	0.162	0.162	0.163