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STANDARDIZED METHOD FOR THE ESTIMATION OF β_2-MICROGLOBULIN, RETINOL-BINDING PROTEIN AND ALBUMIN IN URINE

(Technical Report)

Prepared for publication by

ROBERT F. M. HERBER[1], ALFRED BERNARD[2] and KARLHEINZ SCHALLER[3]

[1]Coronel Laboratory for Occupational & Environmental Health, University of Amsterdam, Meibergdreef 15 NL, 1105-AZ Amsterdam, Netherlands
[2]Unité de Toxicologie Industrielle et Médecine du Travail, Université Catholique de Louvain, Clos Chapelle-aux-champs 30, B-1200 Bruxelles, Belgium
[3]Institut für Arbeits- Sozialmedizin und Poliklinik für Berufskrankheiten der Universität Erlangen-Nürnberg, Schillerstrasse 25–29, D-8520 Erlangen, Germany

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Standardized method for the estimation of β_2-microglobulin, retinol-binding protein and albumin in urine (Technical Report)

Abstract

Increased excretion of proteins in urine are early signs of exposure of subjects of the general population and in occupation to xenobiotics. These signs are parameters of an early effect on the kidney and may be used as biological effect parameter. Xenobiotics who may cause such effects are metals such as cadmium and lead, solvents as toluene and styrene, and some pesticides. Effects on the kidney tubuli are responsible for e.g. enhanced beta-2 microglobulin and retinol binding protein concentrations in urine; effects on the glomeruli for albumin in urine. Standardization of methods to determine physiological concentrations will be necessary to compare the results of epidemiological studies. A protocol for such methods is described in which the proteins beta-2 microglobulin retinol binding protein and albumin are determined routinely. Also, a calibration curve program is presented. The results of several comparison studies show that the methods described in this report are reasonably to well comparable between different laboratories.

INTRODUCTION

Proteinuria is an early sign in the development of kidney toxicity due to chemical exposure (1,2). Increase in urinary concentrations of beta-2 microglobulin , retinol binding protein and albumin may be due to exposure to metals such as cadmium, lead, mercury or chromium, or to solvents (3,4). Recent literature about effects of cadmium on the kidney has been reviewed (5). The requirements for the determination of parameters of function should be based on the fact that dose-effect relation-ships start already at physiological concentrations of the parameters of effect. So, determinations of parameters of effect have to be sensitive and specific for that particular parameter. For the determination of proteinuria, many methods can be used, but only a few can determine physiological concentrations reliably.

Typical protein in urine levels to be determined routinely are: up to 200-300 µg/L β_2M, up to 150-300 µg/L RBP, up to 12 mg/L Alb and up to 200-250 mg/L TP (5).

The method for the determination of β_2M in urine used to be radioimmunoassay (6). Later on, an enzymeimmunoassay (7) and a latex immunoassay (8) were developed. All three methods have in common that (I) a plastic tube, plate or sphere is coated with anti-β_2M antibody raised in animals and (II) test antigen is added, which binds to the antibody (9).

In RIA, anti-β_2M antibodies bound to sephadex particles are incubated with the test antigen. Subsequently (III) [^{125}I] ligand is added. (IV) The unbound ligand is washed away. (V) The radioactivity of the particles is counted on a gamma-

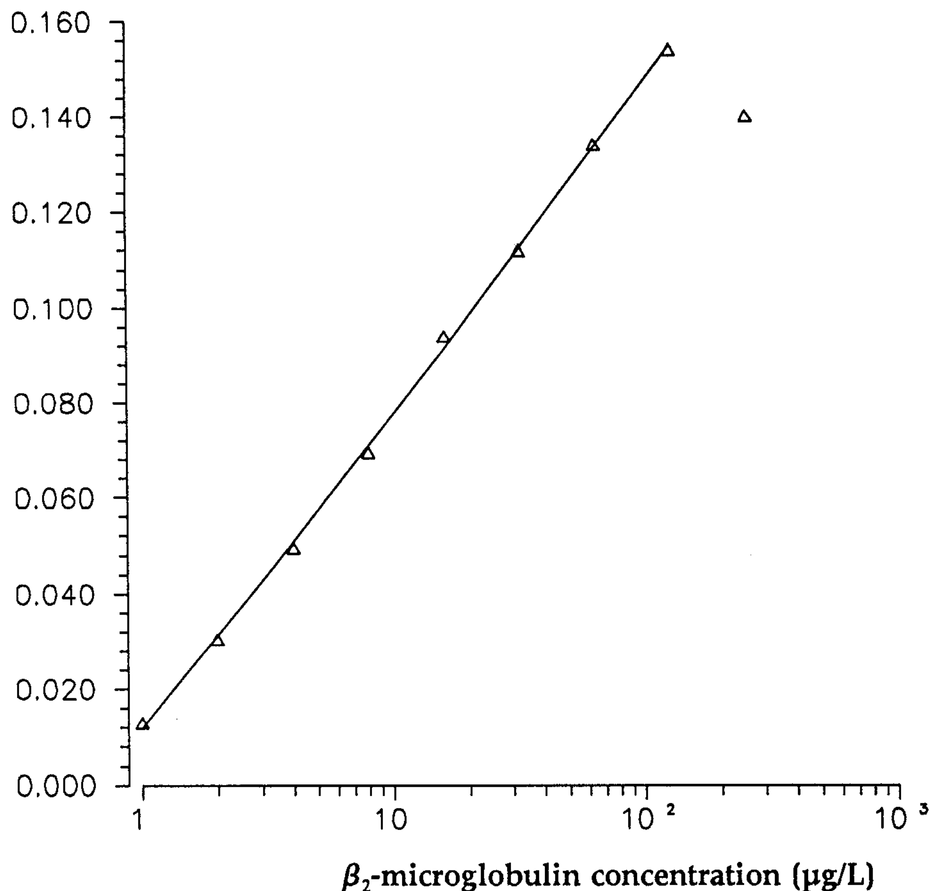

Figure 1 Typical calibration curve for the determination of beta-2 microglobulin in urine.

counter. With increasing amounts of test antigen, the decrease in radioactivity assumes a sigmoidal curve.

In EIA, test antigen (in urine) is also incubated on antibody-coated sephadex particles. Subsequently (III) an antigen coupled to an enzyme such as peroxidase as ligand is added, (IV) the unbound ligand is washed away, (V) a chromogen is added to form a coloured complex and (VI) the absorbance of the solution is measured with a spectrometer. With increasing amounts of test antigen, the decrease in absorbance assumes a sigmoidal curve.

In LIA, urine is incubated on antibody-coated polystyrene, called latex particles. Subsequently (II) the ligand is added and the particles agglutinate. The number concentration of the suspended particles is measured either with a (blood)cell counter or with a spectrometer. As agglutination is proportional to the concentration of test antigen, the curve rises from a background level through a linear stage to a plateau.

For all types of assay only the linear part of the curves can be used for measurement when accurate determinations are required including quality control using linear regression statistics.

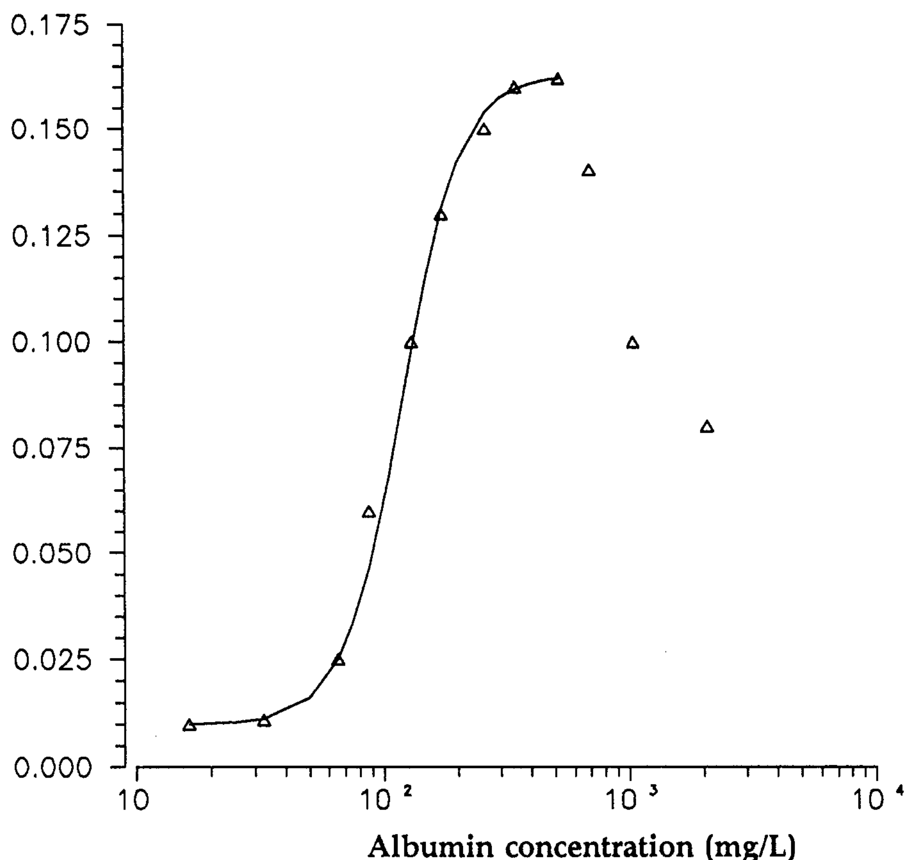

Figure 2 Typical calibration curve for the determination of albumin in urine

For β_2M, RBP and Alb in urine, other immunological methods have been published (10-13) too, capable of determination of physiological levels in urine.

Comparison of the three methods showed that LIA had the best characteristics and had no bias (14). LIA may be used for the determination of RBP (15) and Alb (16) and in fact for any protein, provided that specific antibodies are available. However endorsement of LIA does not mean that it can reach the standard of the more common quantitative methods, e.g. for trace metals. Firstly, the assays have a limited calibration range. Secondly, subtraction is necessary between two large quantities leading to greater imprecision. Thirdly, the function is linear for log concentration and absorbance[1] (or radioactivity), leading again to more imprecision than for a linear function of concentration and absorbance.

[1] absorbance: in this case absorbance ought strictly to be called attenuation of radiation. The measurement includes also the effect of scatter.

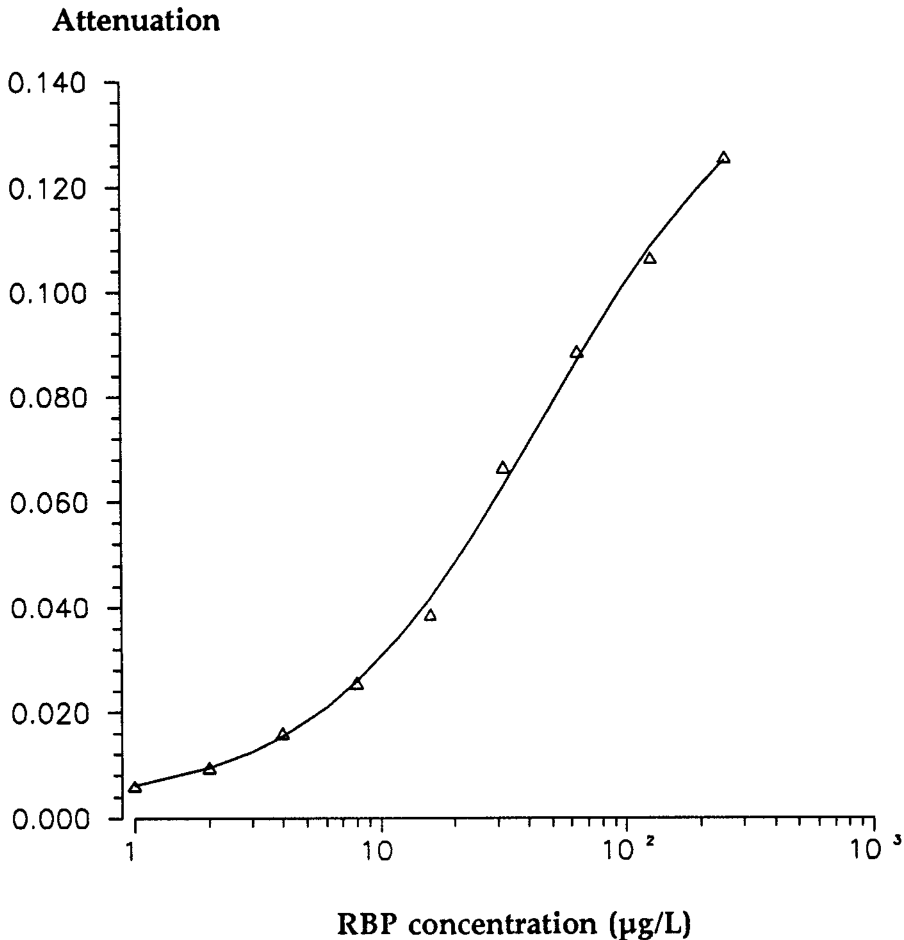

Figure 3 Typical calibration curve for the determination of RBP in urine

In an earlier study [8], the performance of the determination of β_2M with LIA was compared with the determination with RIA. For a range of 10-10000 µg/L, a regression equation was found of LIA = 0.21 + 0.89 x RIA, with an explained variance of r^2 = 0.94. These are good to fair results for the whole range.

In a study performed by the Coronel Laboratory (Lab C) and Nofer's Institute of Occupational Medicine in Lodz, Poland (Prof.M. Jakubowski, Lab D), the LIA method was compared between the two laboratories (collaborative study). 20 Urine test substances were prepared in Lodz and determined by both laboratories. Results for β_2M were as follows: range (Lab C): 15 - 26944 µg/L; regression equation: Lab D = 0.076 + 0.97 x Lab C (log values); explained variance: r^2 = 0.996. These results can be qualified as excellent, both for the regression and r^2. Results for RBP were as follows: range (Lab C): 23 - 21537

μg/L; regression equation Lab D = 0.046 + 0.92 x Lab C (log values); r^2 = 0.951. Hence results for lower values until 3000 μg/L were excellent to fair; at higher levels results were poor. Explained variance was on the borderline acceptable. Results for Alb were as follows: range (Lab C): 3.4 - 40.7 mg/L; Lab D = 4.23 + 0.807 x Lab C (linear values); r^2 = 0.93. These results were apparently not so good as those for β_2M or RBP, partly this is due to the much smaller concentration range of Alb (β_2M 1:1700, RBP 1:936, and Alb 1:12) leading to both a less good regression equation and explained variance. However, a paired t testing showed that for df = 19, t = 0.004 (not significant) with $x_{mean(Lab\ C)} = x_{mean(Lab\ D)}$ = 21.90 mg/L.
Thus, no differences could be found with this test.

By advocating a provisional standardized procedure it is hoped that results will become more reliable and comparable between investigators. The IUPAC Commission on Toxicology is sponsoring the development of standard methods for the determination of beta-2 microglobulin, retinol-binding protein and albumin in urine in order to promote international standardization of these early indicators of renal toxicity.

The present study was conducted as a cooperative effort between only three laboratories, which, however, represented the most extensive experience in these procedures. This means that each laboratory worked out more than 1000 determinations in triplicate at physiological or somewhat enhanced level for at least 5 years.

The text of this standard method does not mention commercial suppliers of reagents, apparatus or equipment. Sources of such items are cited in footnotes for the convenience of users. Such citations do not imply endorsement of the products by IUPAC or by the authors.

List of abbreviations

Alb: albumin
β_2M: beta-2 microglobulin
BSA: bovine serum albumin
EIA: enzyme immunoassay
GBS: glycine-buffered saline
LIA: latex immuno assay
RBP: retinol binding protein
RIA: radioimmunoassay

Principle of the method

The sample containing β_2M, RBP or Alb is incubated with latex particles on which an antibody is adsorbed. The reaction of, for instance, β_2M with the antibody results in agglutination of the latex particles proportional to the concentration of the protein. The number concentration of the suspended particles can be determined either with a particle counter or in terms of attenuation of radiation with a spectrometer. As the latter detection method is used in most laboratories, the protocol has been developed for spectrometer detection only.

Critical aspects

The agglutination reaction depends on many variables such as specificity of the antibody, titre of antibody, reaction temperature, reaction time, amounts of antibody and latex, and on disturbing reactions, such as mechanical disruption of the agglutinated particles and contamination with detergents. The most critical of these variables must be optimized. In particular, the optimal amount of antibody charged onto the latex particles must be found for each antibody-antigen system. In the 'Discussion', the critical aspects are addressed more comprehensively. It is necessary to assay all urines at 2 dilutions (see later). Other methods may not have this disadvantage.

Equipment

- Polystyrene tubes, 11 mm x 63 mm
- Micropipette[2], 50-200 µL
- Micropipette[2], 200-1000 µL
- Dispenser 2 mL
- Centrifuge 2.5 $km.s^{-2}$ (25 000 $\mathbf{g}_n$)
- Shaker[3]
- Water bath, 37 °C
- Sonifier[4]
- Filter membranes[5], pore size 0.45 µm
- Filtration equipment[5]
- Spectrometer with quartz flow-through cuvette, pathlength 10 mm
- pH meter
- Foil[6]

Reagents

- Latex polystyrene particles[7], diameter 0.79 µm
  These particles, supplied as a suspension of mass concentration 100 g/L, are used directly without washing.
- Antibody. Rabbit immunoglobulins directed against human β_2M, RBP and Alb[8]

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[2] E.g. Finn, Finland

[3] E.g. Vortex, Scientific Instruments Inc., U.S.A.

[4] E.g. Bransonic, F.R.G.

[5] E.g. Millipore, U.S.A.

[6] E.g. "M" foil, Parafilm, U.S.A.

[7] E.g. Estapor k 080, from Rhône-Poulenc, France

[8] E.g. Dakopatts, Denmark. Code β_2M: A 072; RBP: A 040; Alb: 001

- Stock Standard. Urine of patients with high concentrations of proteins, due to nephropathy or toxic exposure, may be used as well as commercial available urine concentrates[9]. For urine specimens of patients, a purification procedure must be used (17). In either case, the concentration of the different proteins in the concentrated standards should be checked by an alternative method, e.g. radial immunodiffusion.
- Albumin[10], bovine
- Pure albumin, bovine serum fraction V[11]
- Glycine-buffered saline (GBS) stock buffer. Glycine 1 mol/L, NaCl 1.7 mol/L (analytical grade), NaN_3 76 mmol/L (pure), bring to pH 9.0 with NaOH 10 mol/L (analytical grade)
- GBS buffer. Dilute GBS stock buffer 1 + 9 with demineralized water
- GBS bovine serum albumin (BSA) buffer. Add 1 g of pure albumin (bovine serum fraction V) to 1 L of GBS Buffer. Add NaOH 10 mol/L to pH 9.6 and filter using a 0.45 µm pore size filter. Store at 4 °C.
- Stop buffer. Adjust GBS buffer to pH 10.0 with NaOH 10 mol/L and filter using a 0.45 µm pore size filter. Store at 4 °C.
- Coating solution. Solution of 0.1 mol/L NaCl and NaN_3 15 mmol/L. Store at 4 °C.
- Stabilization buffer. Adjust GBS stock buffer to pH 10.1 with NaOH 10 mol/L. Filter through a filter of pore size 0.45 µm and store at 4 °C.

Coating of the latex particles with antibody

For β_2M and RBP, add 4 mL of freshly-made GBS buffer to 60 µL anti-β_2M immunoglobulin or 60 µL anti-RBP immunoglobulin. For Alb, add 4 mL of freshly-made GBS buffer to 200 µL anti-Alb immunoglobulin. Mix well.

Add, with continuously shaking, 0.5 mL of latex suspension (100 g/L) to the antibody GBS solution. This latex suspension must be sonicated just before use.

Incubate for 60 minutes at room temperature (20-25 °C) while gently mixing or shaking. Centrifuge the suspension at 2.5 $km.s^{-2}$ (25 000 $\mathbf{g}_n$) and 25 °C for 10 min. Decant the supernatant and resuspend the particles in 4 mL of coating solution. Centrifuge, decant and resuspend twice more. In the final resuspension step, use 10 mL coating solution to resuspend the particles. This preparation can be stored for 6 months at 4 °C.

The amount of latex to be added depends on the quality of the latex. When using latex several months after the date of manufacturing, the amount to be added is more than when using a new lot. The amount of antibody to be added depends on the titre and affinity of the antibody.

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[9] E.g. Behring, F.R.G. lot 011285

[10] E.g. Sigma, U.S.A. A 9647, lot nr 70 F-0546

[11] E.g. Calbiochem, U.S.A., fraction V, fatty acid free, 126609 lot nr 309229 purity 98%

Preparation of standards

Commercial standards with a high concentration of saline should be dissolved in 0.5 mL of demineralized water. Commercial standards with a low concentration of saline or standards derived from urine of patients should be dissolved in 0.5 mL of NaCl 154 mmol/L in demineralized water. In either case, the NaCl concentration should not sink below 154 mmol/L. The solution is diluted 100 times (working standard) with a solution of NaCl 154 mmol/L and NaN_3 76 mmol/L in demineralized water, divided into portions and frozen at -20 °C. This working standard solution is stable for 6 months.

Typical concentrations are: β_2M 8 mg/L, RBP 3 mg/L and Alb 800 mg/L. The solution may be diluted with demineralized water or NaCl 154 mmol/L, provided that the NaCl concentration be not less than 154 mmol/L. If thawed for use and restored at 4 °C, the working standard is stable for up to three days.

The working standard should be adequately diluted just before use with GBS-BSA buffer to concentrations in the range of 1-256 µg/L for β_2M and RBP and 15-2100 µg/L for Alb. The standards are made in duplicate and should be prepared at the same time as the samples, with the same batch of GBS-BSA buffer. For β_2M and RBP about 9 standards should be made, for Alb about 12 (Figures 1-3).

Preparation of samples

Determination of β_2M in urine with pH < 5.5 is unreliable because of the instability of the protein. If fresh urine samples are stored at -20 °C, they will be stable for at least 6 months. Another possibility is storage at 4 °C with the addition of NaN_3 until a final concentration of 15 mmol/L NaN_3 to prevent bacterial growth (8, 15, 16).

Clear urine can be analysed directly, other specimens should be centrifuged at 150 $m.s^{-2}$ (1 500 g_n) before determination. Urines should be diluted in duplicate with GBS-BSA buffer 1+19 and 1+49 for β_2M and RBP, 1+39 and 1+199 for Alb. The attenuance of the diluted urines should fall into the linear part of the calibration curve. The minimum dilution to achieve that attenuance is 1+19 for β_2M, and 1+4 for RBP and Alb. In case of a dilution of 1+4 for RBP, the colour of the urine can influence the attenuance. For such urines extra blanks have to be added to compensate for the absorption of the urine colour.

Preparation of the latex suspension and determination

Weigh in a polystyrene tube 25 mg pure albumin (bovine serum fraction V). Add 1.75 mL of demineralized water and add 0.5 mL of antibody-coated latex suspension. The latex suspension should be mixed thoroughly on a shaker and sonicated for 10 minutes just before use. The amount of 2.25 mL of albumin-latex suspension is sufficient for 9 standards in duplicate for β_2M or RBP, plus 2 unincubated blanks and 2 latex check solutions, together with 10 samples in duplicate and 4 incubated blanks. For Alb, besides the standards and the blanks, 8 samples can be determined with this amount.

Mix the suspension of albumin, water and latex on the shaker and sonicate for 15 minutes. Depending on the number of tests, this procedure can be repeated at will. Subsequently add 250 µL of stabilization buffer to the solution prepared. Be

sure that this procedure is the same each time, e.g. use the same tubes and take care that the water level in the sonifier is the same each time and that the water temperature does not exceed 25 °C.

In the meantime, add 50 µL of the standards and 50 µL of the samples in polystyrene test-tubes. For the blanks, use 50 µL of GBS-BSA buffer. Prepare two blanks in the beginning of the standard series, two at the end of the standard series and two at regular intervals between the samples.

Subsequently add 50 µL albumin-latex suspension to the standards, blanks and samples in the following way (Table 1):
pipette 50 µL of albumin-latex suspension into the tubes, cover the tubes with a piece of foil and mix on a shaker for at least 5 seconds. Take care that no drop of the solution hangs on the upper side of the tube and foil. Place the tube in a water bath at 37 °C. Allow intervals of 20 seconds between tubes. Incubate for exactly 30 minutes; each tube must be withdrawn from the water bath at 30 minutes. Then immediately add 2 mL of stop buffer and shake gently by hand.

To check non-specific agglutination, prepare in two tubes 50 µL of GBS-BSA buffer, 50 µL of latex suspension and 2 mL of stop buffer. These unincubated blanks must be prepared just before incubation and should not be incubated in the water bath at 37 °C. The relative difference in attenuance between incubated and non-incubated blanks must be less than 0.1 for a reliable determination.

Prepare two other tubes with 50 µL of latex suspension and 2 mL of stop buffer to check for destruction of aggregates formed during sonication (destruction-aggregation blank).

Table 1 Pipetting scheme for the determination of $ß_2M$, RBP and Alb in urine.

	Volume (µL)	Volume of albumin latex suspension (µL)	Incubation time (min)	Volume of stopbuffer (mL)
Standards	50	50	30	2
Samples	50	50	30	2
Incubated blanks	50	50	30	2
Non-incubated blanks*	50 GBS-BSA Buffer	50	-	2
Destruction aggregation blank	-	50	-	2

* At the beginning of the standard series; subsequently every 10 samples/standards

Spectrometric determination

Wavelength :360 nm; half-width of wavelength interval not critical.
Before transferring the solution into the cuvette, turn the tube gently upside-down by hand. Introduction into the cuvette is by simple pouring. Zero should be set with the stop buffer. At first, the destruction-aggregation blank must be measured. The attenuance should be greater than 0.6. If the attenuance is below 0.6, incomplete aggregation is apparent, due to e.g. low-quality latex particles or incomplete coating of the antibodies and the analysis will be unreliable and unspecific.

Subsequently the blanks, standards and samples are measured.

Calculations

Subtract the attenuances (absorbances) of the standards or samples from the incubated blanks, i.e. for samples:
$\Delta A = A$ (incubated blank) - A (sample) ,
and for standards:
$\Delta A = A$ (incubated blank) - A (standard).

The standard values are plotted on linear-logarithmic paper against the corresponding mass concentrations (in µg/L). The linear part of the calibration curve is between 60 and 300 µg/L for β_2M, RBP and Alb. The appendix describes a computer programme for a logit-log function.

Remark

The calibration curve of albumin shows a phenomenon called prozone effect. Therefore, urine samples should be systematically determined at two widely different dilutions to avoid erroneous results, e.g. dilution factors of 40 and 200.

Evaluation and Discussion

The protocol methods were tested in an intralaboratory survey. Polystyrene tubes with pooled human urine samples 15 mL from patients suffering from different kidney diseases were sent to two laboratories. The urine was centrifuged at 200 m.s^{-2} and NaN_3 (final concentration 15 mmol.L^{-1}) was added to prevent bacterial growth. Laboratory A used the enzyme-immunoassay for β_2M (7) and the laser nephelometry method for Alb (19). Laboratory B used the protocol method, but with a flow-injection analysis system and particle counter detector (18). So, the methods in Laboratory B and the Coronel Laboratory (Laboratory C) should be comparable and the method in Laboratory A is different. The first run covered the whole range from physiologically 'normal' concentrations (β_2M and RBP $\leq$ 300 µg/L, Alb $\leq$ 20 mg/L) to clinically abnormal concentrations (β_2M and RBP up to 200 mg/L, Alb up to 1.5 g/L), whereas Runs 2 and 3 covered concentrations from 'normal' to somewhat enhanced (β_2M and RBP < mg/L, Alb < 60 mg/L). Tables 2-4 give the results for each concentration in each laboratory. Each test was performed in triplicate on three different days.

The criteria for acceptance of individual results for β_2M and RBP were that the

Table 2 and Table 3 Performance of the individual participants for the β_2M and RBP determination. Results of each laboratory are means of triplicate determination on three different days. Criterium of acceptance: ± 40% of mean. +: accepted ; -: not accepted. Lab B and C standardized method. TS: test specimen.

Table 2 β_2M
Mass concentrations of β_2M in µg/L

TS	LABORATORY			
RUN 1				
	A	B	C	Mean
1	43713 +	45000 +	58633 +	49115
2	26680 +	32917 +	28633 +	29410
3	28331 +	33167 +	28500 +	29999
4	123 +	144 +	102 +	123
5	81080 +	111833 +	117000 +	103304
6	181 +	278 +	151 +	203
7	216 +	211 +	161 +	196
RUN 2				
1	284 +	269 +	231 +	261
2	1508 +	1683 +	1759 +	1650
3	1035 +	940 +	1091 +	1022
4	513 +	474 +	478 +	488
5	1694 +	1824 +	2037 +	1852
6	358 +	359 +	314 +	343
RUN 3				
1	-	1604 +	985 +	1295
2	-	720 +	323 +	522
3	-	3719 +	3037 +	3378
4	-	2376 +	1471 +	1924
5	-	1627 +	884 +	1256
6	-	357 +	172 +	265

Table 3 RBP
Mass concentrations of RBP in µg/L

TS	LABORATORY		
RUN 1			
	B	C	Mean
1	54067 +	63933 +	59000
2	23933 +	27000 +	25467
3	44083 +	56867 +	50475
4	160 +	126 +	143
5	107067 +	136000 +	121534
6	1148 +	709 +	929
7	50 +	129 +	90
RUN 2			
11	243 +	129 +	186
12	2139 +	2318 +	2229
13	1557 +	1134 +	1346
RUN 3			
17	1473 +	1961 +	1717
18	633 +	532 +	583
19	2033 +	3024 +	2529
20	1232 +	1426 +	1329
21	799 +	1005 +	902
22	339 +	173 +	259

Table 4 Performance of the individual participants for the Alb determination. Results of each laboratory are means of triplicate determination on three different days. Criterium of acceptance: ± 20% of the mean. + : accepted; - : not accepted. Mass concentrations of Alb in mg/L. Lab B and C standardized method. TS: test specimen

TS	LABORATORY				TS	LABORATORY				TS	LABORATORY			
	RUN 1					RUN 2					RUN 3			
	A	B	C	Mean		A	B	C	Mean		A	B	C	Mean
1	183 +	174 +	192 +	183	1	-	6.4 +	5.9 +	6.2	1	50.0 -	37.0 +	35.9 +	41.0
2	96 +	107 +	107 +	103	2	49.0 +	72.8 -	53.9 +	58.6	2	19.8 +	16.2 +	13.5 +	16.5
3	1210 +	969 +	998 +	1059	3	28.4 -	120.8 -	27.8 -	59.0	3	20.0 +	17.2 +	13.6 -	16.9
4	8 +	9 +	8 +	8	4	14.0 +	16.4 +	15.9 +	15.4	4	12.0 +	12.3 +	9.5 +	11.3
5	56 -	41 +	36 +	44	5	57.6 +	66.5 +	55.9 +	60.0	5	10.2 +	9.6 +	8.9 +	9.6
6	-	1351 +	1153 +	1252	6	10.8 +	11.9 +	12.4 +	11.7	6	3.3 +	6.4 +	6.3 +	5.3
7	15 +	16 +	16 +	16										

mean of each Laboratory Run should be 40% of the mean of the laboratories, and for Alb ± 20%, as established at a WHO meeting (20). These criteria were arbitrary. However, it has been assessed that variability between individuals for β_2M and RBP was about 10-150% relative standard deviation at physiological levels of 20-300 µg/L, and for Alb 30-75% RSD at physiological levels of 3-50 mg/L in a group of 13 apparently healthy subjects. In a recent study variability of RBP within and between individuals were assessed in a group of 16 apparently healthy people who collected first morning urine for 5 days. The analytical relative standard deviation was 7%, the variation within individuals 81% and between individuals 161% as relative standard deviation at a level of RBP of 80 µg/L (22). Therefore, the chosen criteria of 20% and 40% seem reasonable.

Another possibility for acceptance criteria is the comparison of precision suggested by Horwitz (23). There an inverse-log function between concentration and precision is found with residual standard deviation of 2 exp $(1 - 0.5 \log_{10} c)$ (RSD_R), with RSD_R: relative standard deviation and c the substance concentra tion. These precision criteria were found for compounds of low molar mass and are based on many intercomparisons. The performance of the β_2M determination was acceptable (Table 2). None of the results were unacceptable. When the precision of the β_2M determination was compared to the criteria of Horwitz (23) the performance was acceptable for the concentration range from 0.1-10 mg/L. However by these criteria, the performance at 100 mg/L was unacceptable.

The performance of the RBP determination was acceptable too (Table 3). Not a single test result was rejected. However according to the criteria of Horwitz, the results were acceptable only at the 10 mg/L level (Table 5).

For the Alb determination (Table 4) Lab A encountered a problem five times (29%), Lab B twice (11%) and Lab C also twice (11%). Laboratories B and C used the standardized method and performed better. By the criteria of Horwitz, the precision for the whole range of albumin concentrations from 10 mg/L to 1 g/L was acceptable (Table 5).

Table 5 Performance of the standardized method if compared with the Horwitz criteria (15) and compared with the WHO criteria (14).

Mass concentration level	Horwitz precision criteria (17) RSD_R (%)	Precision found (%)			Acceptance criteria (16)	
		ß$_2$M	RBP	Alb		
1 g/l	5.6	-	-	12		
100 mg/l	8	19	17	6		20[b]
10 mg/l	11	9	9	9	40[a]	20[b]
1 mg/l	16	7	22	-	40[a]	20[b]
100 µg/l	23	17	45	-	40[a]	

$RSD_R = 2 \exp (1 - 0.5 \log c)$ where c: mass concentration

[a]: for ß$_2$M and RBP

[b]: for Alb

Conclusions

Although no 'definite' method is available, determination of β_2M in urine with the standardized method can be unreservedly recommended. The criteria of Horwitz and WHO were met for this method with the exception of the very high clinical range up to 100 mg/L. Also for the earlier performed collaborative and intercomaprison studies (cf. Introduction) support the recommendation of the standardized method.

Determination of RBP in urine by the standardized method can be were not met for the very high clinical range of 100 mg/L and the lower ranges from 100 µg/L to 1 mg/L, one might question whether these criteria, originally developed for the components of low molar mass in products like food, drugs and pesticides, should be used without adjustment on less well-defined proteins. The earlier performed collaborative study supports the recommendation of the standardized method for low values until 3 mg/L (cf. Introduction).

Determination of Alb in urine gave some minor problems. However the standardized method performed better than the nephelometric method and the criteria of Horwitz were met for the whole range from 10 mg/L to 1 g/L. The collaborative study supports the recommendation of the standard method as no differences could by found between the two participants (cf. Introduction).

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Appendix

Calibration curve fitting

Anton J.W. Verplanke

The simplest way of curve fitting of a non-linear shaped curve is plotting by hand on grid paper. However as this method is unfavourable because of the subjective drawing of the line, other methods are to be preferred. When there is a (nearly) linear part on the sigmoidal curve,one can calculate a linear equation by the least- square methods in the form:

ATT = A + B*CONC with

A: intercept with ordinate
B: slope
ATT: measured attenuance
CONC: concentration of the standards

The linearity should be tested with an F test. This linear-curve fitting has the disadvantages that only a part of the curve is used for the calculation and that the linear part is often limited. The best method is use of a non-linear curve-fitting program, employing a personal computer. This program is based on the transformation of the symmetric sigmoidal curves followed by statistical analysis. A 4-component logit transformation (24) is used to tranform the sigmoidal curve into a linear function. The formula was originally designed for fitting competitive radio immunoassay (RIA), but it can easily be applied for latex immunoassay

(LIA). For LIA, estimation of all 4 parameters of the logit transformation is needed. A transformed equation, as suggested by Winkler and coworkers (25), is used:

ATT = 1n [(ATT* - *A*)/(*B* - ATT* - *A*)] = C + d*1n (CONC) (a)

with
ATT*: measured attenuation
ATT: transformed attenuation
A: lower asymptotic value
B: upper asymptotic value
C: intercept with the ordinate
D: slope of the straight line

CONC: concentration of the standard solution

Equation (a) transforms the ordinate values ATT of the sigmoidal curve to new ordinate values ATT*. ATT* is a linear function of 1n (CONC). The position of this line is determined by linear regression. The values for *A* and *B*, resulting in the highest correlation coefficient for the straight line, are determined by an iteration procedure. The final values of *A*, *B*, *C*, and *D* are used to transform the straight line back to the sigmoidal curve. The resulting calibration line is used to calculate the unknown concentrations of Alb, β_2M and RBP in urine or blood. The program was written in MACRO language of a commonly used spreadsheet computer program. The spreadsheet facilitates data input, graphic illustration and communication with other statistical programs for further evaluation of the data.

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