Clinica Chimica Act (2012) 413(1-2):175-181.

Preparation of highlypurifiedmonomeric human serum albumin as secondary reference material for standardization of urinary albumin immunoassays

Itoh, Yoshihisa ; Ichihara, Kiyoshi ; Kishi, Kouji ; Hosogaya, Shigemi ; Yamada, Toshiyuki

Preparation of highly purified monomeric human serum albumin as secondary reference material for standardization of urinary albumin immunoassays

Yoshihisa Itoh[a*]

[1]Department of laboratory Medicine, Asahikawa Medical University,

Asahikawa,

Kiyoshi Ichihara[b]

[b]Department of Laboratory Sciences,

Yamaguchi University Graduate School of Medicine,

Ube

Kouji Kishi[c]

[c]Diagnostic Reagent Development, Sysmex Corporation

Kobe,

Shigemi Hosogaya[d]

[d]Department of Medical Technology,

Kagawa Prefectural College of Health Sciences

Takamatsu,

Toshiyuki Yamada[e]

[e]Clinical Laboratory Medicine, Jichi Medical University

Tochigi, Japan

.

Acknowledgement: The present work was supported by the Joint Project of the Japan Society of Clinical Chemistry (JCCLS) and the New Energy and Industrial Technology Development Organization (NEDO).
Part of this study was carried out in a project of JSCC. We are grateful to professor Greg Miller, Virginia Commonwealth University, for kindly reviewing our paper, to Mr. Itsuo Sawada, Clinical Laboratory, Jichi Medical University for her excellent technical assistance, and to Dr Roy Uemura and Dr Y Watatsu at Sysmex Research Institute for their valuable scientific and technical advices.

Correspondence: *Yoshihisa Itoh

Midorigaoka Higashi 2-1-1-1, Asahikawa City, Hokkaido, Japan

TEL: 81-166-68-2745 FAX: 81-166-68-2744

E-mail: yoshiito@asahikawa-med.ac.jp

*Keywords***:**Albumin;Urine;Reference material;Reference measurement system;ERM-DA470

Abstract

Background: International external quality assessments have shown variation in results of urinary albumin among various immunoassays. A well-defined candidate reference material for urine albumin (cRM-UA) was prepared to improve standardization.

Methods: cRM-UA was prepared from a commercially available preparation of human serum albumin by using gel-filtration HPLC. The value was assigned by transfer from ERM-DA470 using immunoassay systems qualified based on the linearity and variability observed in dilution tests of pooled urine and the calibrators. Effectiveness of recalibration using the cRM-UA was evaluated by measuring 129 urine specimens.

Results: The cRM-UA had a monomeric albumin peak which accounted for 98.9% of the total area by gel filtration HPLC. The lyophilized preparation of the cRM-UA had suitable homogeneity, and short- and long-term stability. Nine of 14 immunoassays met the criteria were used for value assignment. The assigned concentration was 225.1 ± 9.11 mg/L [mean ±*U*: expanded uncertainty with k=2] when reconstituted with 3.00 mL of purified wateron weight basis. Recalibration of 7 qualified immunoassays using the cRM-UA resulted in between-method CV of 6.6%.

*Conclusions:*The cRM-UA was successful in achieving standardization of urine albumin results among 7 immunoassays which possess performance attributes representing uniform reactivity to both cRM-UA and clinical urine samples.

1. Introduction

Albuminuria is a well-established diagnostic and prognostic indicator for diabetic nephropathy[1].It is also an important biomarker in cardiovascular diseases, chronic kidney diseases, and hypertension [2, 3]. Reducing albuminuria, proteinuria, or both, proved effective in preventing progression of these diseases [4,5]. Currently, 17 immunoassays for urine albumin are commercially available in Japan. In a joint survey carried out by the Japan Diabetic Association and the Japan Society of Nephrology, pooled urines of 3 different concentrations were sent to 86 clinical laboratories throughout Japan. Test results reported by these laboratories varied from 12.5% to 33.0% for the 3 concentrations measured reflecting a poor standardization [6]. Similar discrepancies have been reported in EQAS surveys in North America, Australia and Europe [7-9].

Preparation of a reference material (RM) with well-defined properties and a properly assigned concentration is essential in establishing the traceability chain of urine albumin immunoassays. A certified RM is available for serum proteins including albumin: ERM-DA470 [10-12] and its replacement lot ERM-DA470k/IFCC [13]. It has played an important role for standardization of immunoassays for the major serum proteins[11, 12, 14].However, only modest attempts have been made to produce RM for urine albumin, for which an increasing number of automated immunoassays are being developed in response to increasing clinical need. An IFCC working group conducted an explorative investigation to assess the feasibility of developing RM for several urine proteins, including albumin [15].

A series of standardization investigations and trials have been performed in the joint project by the Japan Society of Clinical Chemistry (JSCC) and New Energy and Industrial Technology Development Organization (NEDO) [16]. We prepared working RMs for urine albumin, designated Prototypes I and II (pRM-UAI, pRM-UAII), purified from a commercial human serum albumin (HSA) preparation between 2002 and 2005. Based on results from these investigations, we developed a new well-defined RM, designated as candidate reference material for urine albumin (cRM-UA), which was intended to be used for global standardization of urine albumin assays. With collaboration of 14 manufacturers of urine albumin immunoassays in Japan, we report evaluation of the feasibility of standardization using cRM-UA.

2. Materials and Methods

The source material for purification was high-purity HSA in lyophilized powder (Sigma-Aldrich, catalogue number A3782). This material was fatty acidsdeleted from the material (≤ 0.02% in content) and had essentially globulin free (≤1% of total peak on agarose electrophoresis). ERM-DA470/CRM470 [10] was purchased from JCCLS, Tokyo. pRM-UAII, used as a control, was essentially the same as cRM-UA in structure and properties. It was prepared in 2005 by 3 steps of chromatography including anion-exchange, cation-exchange chromatography, and gel-filtration [16, 17].

2.1 Preparation of cRM-UA

High-purity HSA was dissolved into purified water and dialyzed in cellulose membrane (5 nm, Sanko Junyaku) against 0.1 mol/L sodium phosphate (pH 7.1) containing 0.15 mol/L sodium chloride (PBS). After passing it through membrane filter (0.2 μm, Millipore Japan), 20 mL of HSA solution was subjected to gel-filtration (Sephacryl S200, 5 cm × 108 cm, GE Health Care). The protein was fractionated at 25°C at the flow rate of 3.6 mL/minute with the same PBS and determined by measuring absorbance at 280 nm (UV-8020, Tosoh).

The concentration of albumin in eachpreparation step was determined by a turbidimetric immunoassay (TIA) on an automated analyzer (Hitachi 7170, Hitachi Technologies Co.) using a commercial albumin reagent (Sysmex Corporation). Total protein concentration was measured bya Biuret method (TP reagents Kokusai, Sysmex).

The purity of the monomeric albumin fraction was confirmed by gel filtration high performance liquid chromatography (HPLC; TSK G3000SW column, 7.8 mm × 30.0 cm, Tosoh) using the same PBS fractionation carried out at 25 °C at a flow rate of 1.0 mL/minute. Protein peaks weredetectedby thesame UV detector.

In a manner similar to that previously reported [17], a total of 3 μg of the fraction containing the monomeric albumin was subjected to native (non-denaturing) PAGE and SDS-PAGE (Bio-Rad) at 100 V for 1 hour. The polyacrylamide gel concentration was 5% to 10%. The gel was stained with Coomassie Brilliant Blue G-250 (Bio-safe Coomassie G-250Stain,

Bio-Rad). Prestained SDS-PAGE Molecular Weight Standardsand Precision Protein Standard (Bio-Rad)were used for a molecular mass marker.

2.2 Bottling and lyophilization

cRM-UA was bottled and lyophilized at the Sysmex Research Institute, Kobe. After dialysis against a 0.02 mol/L phosphate buffer (pH 7.1) containing 0.15 mol/L sodium chloride, 10 g/L sucrose, and 0.5 g/L sodium azide, 1.00 mL of the albumin solution was dispensed into transparent silicated borosilicate glass vials, frozen at −70°C, and lyophilized under vacuum for 25 hours at the sequential temperature change −70 to 15°C. Vials were sealed under nitrogen gas with polyisoprenestoppers.

2.3Evaluation of the cRM-UA

Serologic tests for pathogenic microorganisms were performed at a private clinical laboratory (SRL, Tokyo). These included Hepatitis B antigen, Hepatitis C antibody, and HIV I and II antibody tests. To check bacteria contamination reconstituted solution was inoculated in trypt-soy agar (Eiken) at 37 °C for 7days.

Evaluations for short-term and long-term stability were performed in a similar manner as pRM-UAII (16). The preparations were consistently measured by use of the same TIA shown above. In short-term stability of cRM-UA, the preparation was stored at 4~10ºC between 0 and 10 hours after reconstituted with 2.0mL of pure water. Two vials were used for the study and the significance of time dependent changes in albumin concentration in triplicate measurements was judged by two-way ANOVA with repeated measures for detection of between-vial and between-time variations. If no between-vial difference was detected, the least-square linear regression analysis was conducted after pooling the data to test for a significant difference of the slope from zero. For the evaluation of long-term stability, randomly chosen three vials of cRM-UA stored at −80 ºC were measured in quintuple at 0, 4, 16 and 40 months. By plotting the time period (month) on the x-axis and the albumin concentration on the y-axis, the least square regression line was derived and the slope was tested whether or not it was regarded as zero**.**

2.4Dilution test of pooled urine

A dilution test was conducted as a simple and sensitive method for

validating the performance of the assay system/reagent.
Pooled urine was prepared from three fresh urine specimens with protein ++ positive by a dipstick test at Kishimoto Clinical Laboratory, Tomakomai, Japan. Its concentration was approximately 600 mg/L. They were frozen and stored at –80°C until use. The preparation was made anonymously in accordance with the company's ethic rule.
It was sent to the 14 manufacturers of albumin immunoassays in Japan: Kyokuto Pharmaceutical Industrial; Nittobo Medical; Sekisui Medical; Roche Diagnostics; Kanto Chemical; Denka Seiken; Eiken Chemical; ARKRAY Marketing; Beckman Coulter KK (all in Tokyo); Serotec, Sapporo; Medical & Biological Laboratories, Nagoya; Wako Pure Chemical Industries, Osaka; Sysmex. Method characteristics are summarized in Table 1.

The urine was measured in quintuplicate after serial dilutions (1:1, 1:2, 1:4~ 1:512)by use of the sample dilution buffer specific to each assay system. The relationship between theoretical albumin concentrations of the diluted preparations on x-axis and measured concentrations on y-axis was examined based on the least-square linear regression: y=a+bx. The precision or scatter around the regression line (Syx), linearity, and equivalence of the intercept to zero were evaluated [10, 18]. We regarded Syx around the regression line less than 3.0 (mg/L) and non-significance by χ^2 test for linearity at the level of P=0.05 as the acceptable performance of the assay system in the dilution test.
The approximate limit of quantitation (aLOQ), the lowest concentration above which within-assay CV stayed below a specified level, was determined from the result of the dilution test. The allowable level of CV was arbitrarily set at 12% in reference to similar analyses performed in immunoassays for CRP and TSH [19, 20].

2.5**Value assignment**

The value assignment work for cRM-UA and pRM-UAII from ERM-DA470 was conducted in the joint research project by JSCC and NEDO with participation of the manufacturers listed above. Procedures described in ISO Guide 35 and a report of the European Community Bureau of Reference (BCR) was followed [10, 18-22]. One vial of ERM-DA470, three vials each of cRM-UA, and pRM-UAII were sent to each manufacturer. ERM-DA470 was reconstituted on weight basis with 1.00 mL of purified water, allowed to stand 60 minutes at room temperature,

mixed by inversion 5 times and stored for 24 hours at 4°C before use [10]. The other two RMs were also reconstituted on the weight basis using 3.00 mL of purified water at room temperature, allowed to stand below 10 °C for 60 minutes. The mixing was performed gently to avoid non-specific aggregation of HSA by frothing.

The cRM-UA, pRM-UAII, ERM-DA470, and each immunoassay's calibrator at approximately 200 mg/L were serially diluted on a weight basis (1:1, 1:2, 1:4, 1:8, 1:16) with the buffer of each assay system. Each company was asked to measure all the diluted specimens using its own assay calibrator in a single day, each in quintuplicate (total of 3 vials ×5 test results, each for the cRM-UA and pRM-UAII, and 5 results for ERM-DA 470).
Test results of albumin for the four serially diluted preparations of ERM-DA 470 were plotted on x-axis (x_1~x_4) and those from cRM-UA or pRM-UAII were plotted on y–axis (y_1~y_4). The regression line y = bx, passing through the origin, was estimated by the least squares method. The average of the slope 'b' ($\bar{b}$) of qualified (see results) immunoassays was used as an estimate of the relative concentrations of cRM-UA and pRM-UAII to those of ERM-DA470. The value for cRM-UA was thus assigned as $\bar{b}$ multiplied by theoretical concentration of ERM-DA470 (198.5 mg/L).
The uncertainty of the assigned value consists of standard relative uncertainty in certified value of ERM-DA470 (u_{cal})[10], the standard relative uncertainty of assigning value to RMs (determining the relative concentration) (u_{char}), the standard relative uncertainty due to between-vial difference (u_{bvd}). The combined uncertainty U_c was thus computed as the square root of the sums of the 3 component of squared uncertainties:

$$u_c = \sqrt{u_{cal}^2 + u_{char}^2 + u_{bvd}^2}$$

[22]. For the computation of expanded uncertainty we used the coverage factor of 2.

2.6 Recalibration study using cRM-UAA set of 129 individual patients' urine specimens which remained after routine test for urine albumin by immunoassay were collected at the Clinical Laboratory, Jichi Medical University Hospital (Tochigi-ken) to investigate effectiveness of cRM-UA, pRM-UAII, and ERM-DA470 on standardization of urine immunoassays. The samples were chosen only based on whether or not the albumin concentrations were within the target range between 10 and 200 mg/L. Each sample (1.00 mL) was a liquotted in 2.00 mL polypropylene cryovials and frozen within 1.5 hours at –80°C until use. The study was approved by the institutional review board. The frozen aliquots were measured in quadruplicate, within 1.5 hours of thawing, by use of reagents for immunoassays which were regarded as possessing acceptable performance: linearity of test results for serial dilution of the pooled urine and the reference materials, and intra-assay CVs within 2.0% which were determined from each manufacturer's quality control specimens with urine albumin concentrations between 10 and 200 mg/L. All the measurements were performed collectively using a single automated analyzer, Hitachi 7170. The assays were calibrated using each manufacturer's calibrators with or without recalibration based on cRM-UA or ERM-DA470.Two-way analysis of variance (two-way ANOVA) was used to compute between-method CV, which represents the level of agreement of test results across the reagents after the recalibration.

3. Results

3.1 Isolation of monomeric albumin on gel-filtration

The single passage of the high-purity HSA through Sephacryl S200 preparative gel-filtration chromatography produced a solitary peak of albumin. Of the total of 9.51 g of the starting material applied, 3.44 g (36.2%) was recovered. The monomeric nature of the peak was confirmed by analytical HPLC shown in Fig. 1. No polymerized or fragmented peaks were detected. The area under the monomeric albumin peak accounted for 98.9% of the total area.

The purity of the peak was further confirmed by both SDS-PAGE and native PAGE. A single band was observed at a position corresponding to 66,000 by both methods. In native PAGE some trailing can occur that was negligible (Fig. 1).

3.2 Evaluation of the reference material

3.2.1 Sterility

The results of serologic tests were all negative for HBs antigen, anti-HBc antibody, anti-HCV antibody, and anti-HIV I, II antibodies. No bacterial growth was detected seven days after inoculation on trypt-soy agar at 37 °C.

3.2.2Homogeneity test

The concentration of albumin in 10 randomly selected vials of cRM-UA, each reconstituted with 2.00 mL of pure water, was measured in triplicate on an automated analyzer. Albumin results for cRM-UA were 329.4 ± 4.3 mg/L (mean ± 1 SD), and between-vial CV was 1.31%.

3.2.3 Short-term stability

The concentrations of albumin in 2 vials of the cRM-UA reconstituted with 2.00 mL of pure water and stored at 4~10 ºC for up to 10 hours were measured in triplicate (Supplemental data, Table 1). The two-way ANOVA with repeated measured revealed neither any significant between-vial nor between-time variations. Thus, the results for the two vials were pooled and served for the linear regression analysis to test for the significant deviation of the slope from zero. The relationship of albumin test results with time (short-term variability) was thus denied over the period of period of 10 hours after reconstitution. Stability was also confirmed for pRM-UAII held under the same conditions.

3.2.4 Long-term stability

We studied the stability of cRM-UA stored at –80° C for 40 months. Dilution was performed on weight basis. Three vials were measured inquintuplicate using Sysmex’s assay after reconstitution with 2.00 mL of pure water and the value was corrected each time with ERM-DA470.The slope of the test results in function of time over the 40 month period was not significantly different from zero (Table 2). Thus, the albumin in cRM-UA was regarded as not unstable in the condition.

The long-term stability of albumin in pRM-UAII that had been stored in a refrigerator at 4~10 °C was also assessed by measuring the concentration. No significant time dependent change was detected for a period up to at least47months (data not shown). On SDS-PAGE of cRM-UA stored for 40 months and pRM-UAII stored for 70 months, a single band was confirmed for each RM at a molecular weight of 66,000 without any band representing degradation or polymer formation(Fig. 1, Supplemental Data).

3.2.5Linearity in dilution series of the pooled urines

Table 1(right) compares the linearity of the quintuplicate measurements of the serially diluted pooled urines by the 14 immunoassays. Some assay systems showed a slight deviation from the linearity in the concentration between 300 to 600 mg/L. The assays were regarded eligible for the value assignment work when there was linearity in the concentration range below 300 mg/L. A large scatter (Syx) around the regression line was observed in two immunoassays (Assays 9 and 10). $\chi 2$ test indicated lack of linearity in Assay 9 ($p<0.05$) and in Assay 13 ($p<0.01$).

3.3 Value assignment from ERM DA470

In consideration of clinically relevant albumin concentration, both cRM-UA and pRM-UAII were reconstituted with 3.00 mL of purified water to make a presumptive concentration of 216.8 mg/L and 186.8 mg/L, respectively, based on the Sysmex immunoassay.
The assigned value of albumin in ERM-DA470 was 39.7 g/L±0.8 mg/L [mean± U] after reconstitution by adding 1.00 mL of purified water [10]. The solution was diluted 200 times using the sample dilution buffer belonging to each immunoassay to produce a final albumin concentration of 198.5 mg/L. Four solutions were prepared by two-fold serial dilution of each RM using the dilution buffer of each immunoassay. Each was measured in quintuplicate.

The linear relationships of test results for serially diluted preparations of cRM-UA (pRM-UAII) with those of ERM470 were investigated for the 14 immunoassays. In Assay 1, Assay 9 and Assay 13, the CV of slope ‘b’ CV(b) for cRM-UA and pRM-UA was larger than the others. In Assay 4,

there was a mix-up in specimen handling which led to invalid test results. Taking the performance in the dilution test of the pooled urine into consideration, total of 5 assays:1, 4, 9, 10, and 13 were excluded from the value assignment work.

The average of 'b' and its CV derived from the remaining 9 immunoassays which were qualified for value assignment was computed as 1.13 and 1.30%, respectively, for cRM-UA, and 0.970 and 1.51% for pRM-UAII (Table 3). Assigned values were thus determined as 225.1 (=1.13×198.5) mg/L for cRM-UA and 192.6 (=0.97×198.5) mg/L for pRM-UAII. The sources of standard uncertainties were 1.01% for the assigned value of ERM-DA470(u_{cal})[10], 1.29 % (1.51%)due to the value assignment procedure for cRM-UA (pRM-UAII) (u_{char}), and 1.18 % (1.10%) due to between-vial differences (u_{bvd})calculated for relative between-vial homogeneity. Thus, the combined standard uncertainty was 2.024 % (2.125 %). Consequently, the assigned values with uncertainty were determined as 225.1 ±9.11mg/L (mean ±*U*) for cRM-UA, and 192.6 ±7.76 mg/L for pRM-UAII, where U represents the expanded uncertainty with the coverage factor of 2.0.

3.4 Recalibration study

Of the 9immunoassays with acceptable performance both in the dilution test and in the value assignment work, additional two immunoassays were excluded from the recalibration study: one required a dedicated an alyzerand could not be adapted to the common analytical platform used for all reagents, and the other went out of supply. Manufacturers of the remaining 7 immunoassays agreed to participate in the investigation of the effectiveness of recalibration using the RMs for standardization of urine albumin results. The comparative measurements of 129 individual urine specimens were performed using a single autoanalyzer in Jichi Medical University Hospital employing the reagents and instrument settings provided by the 7 manufacturers. Intra-assay CV of urine albumin for the 7 reagents ranged from 1.0 to 2.0% for each of two controls belonging to each reagent which were measured in triplicate in one assay.

The comparability of the recalibrated test results among the seven reagents is shown in Figures2 in three ways according to the calibrators used for

recalibration. The scatter around the regression lines and the correlation coefficients were excellent irrespective of the calibrators used for the comparison. However, the regression lines best agreed with the concordance line, y=x (dashed line), when cRM-UA was used for recalibration (Figure 2A). Results obtained by use of two reagents, Assay 7 and Assay 14, showed a larger scatter around the regression line compared with the other reagents. The between-method CV was determined by the two-way ANOVA as 6.6% using calibrator value assigned by cRM-UA, 9.7% by ERM-DA470, and 8.8% by each manufacturer's original calibrators.

4. Discussion

Several attempts have been so far made for the global standardization of major serum proteins [23-25]. ERM-DA470 had the most decisive role to ward the establishment of standardization of clinically-useful serum protein assays [14, 26-27]. No definite standardization project for urine proteins has, however, been reported by preparing reference material. The cRM-UA was produced with the goal to use it to standardize urine albumin immunoassays. The material is highly-purified monomeric HSA in a buffer. It met the requirements as a reference material in terms of safety, short-term and long-term stability, and a value assigned by traceability to serum-matrix-based ERM-DA470 by well-defined qualified procedures. ERM-DA470 has been the de facto primary reference material [10, 28-29] used for calibration traceability for urine albumin assays after dilution into the assay buffer used by each immunoassay. Consequently cRM-UA is considered a secondary reference material positioned at a lower order under ERM-DA470 and its second lotERM-DA470/k IFCC in the hierarchy of the traceability scheme [13,14, 20].Characteristics of the cRM-UARM are summarized in Table 3.

cRM-UA is the first buffer-based reference material specifically made for urine protein. Considering the high physiologic and chemical heterogeneity of urine, and the related unpredictable urine matrix influences on immune reactions [30], preparation of a standardized form of reference material may be valuable as a reference for uniform calibration of routine immunoassays. To assure long-term stability in structure and immunochemical reactivity, we used PBS containing sucrose as a matrix

for cRM-UA. The merit of cRM-UA over diluted ERM-DA470 is its ability to minimize dilution errors and to provide a simplified matrix which appeared to have consistent reactivity with several different immunoassays. Furthermore, cRM-UA was designed to make concentrations that are clinically relevant for a variety of body fluids by adjusting the volume of purified water used for reconstitution, e.g. from 0.5 to 4.0mL. With an addition of 2 mL, the concentration becomes approximately 300 mg/L, close to the decision limit separating micro- from macro-albuminuria. JCCLS has recently defined the concentration of a urine protein positive (+) result in a dipstick test as 300 mg/L [31]. Use of the RM can lead to standardization of semi-quantitative urine albumin tests as well.

We have demonstrated the feasibility of improving standardization of the urine albumin results from 7 immunoassay reagents by recalibration using cRM-UA. A critical factor to achieve standardized results proved to be the performance attributes of the assay reagents. For both reference materials and biological specimens, an immunoassay must have measured results that have a linear relationship to the albumin concentrations over the measuring interval, and the dilution of reference materials calibrators and patient samples should have a proportional relationship that converges at the origin [2, 19]. When an appropriate reference material is used, the calibration of an immunoassay with these performance attributes can be adjusted to achieve traceability to the concentration of the reference material [7].

In a preliminary study in 1995 more than 88 urines were measured by the 14 similar assay systems used in this investigation. The between-method CV was 16.9% (unpublished data). In the present study, the 7 methods examined after recalibration with cRM-UA had between-method CV of 6.6%, and 9.7% when calibrated with ERM DA470 (Fig. 2A, B).
In the previous study all the assays were allowed indiscriminately for evaluation including those with poor performance and mismatched working range of calibrators. Appreciable change was brought about in alignment of test results by limiting reagents to those of 7 systems. This could be explained by each manufacturer’s efforts in preparing calibrators based on highly purified monomeric HSA and assigning value to its calibrators using either by the optical density method or the dry mass weight method.
Interestingly, the CV was 8.8 % when calibrated using the manufacturers' calibrators before value assignment (Fig. 2) suggesting these 7 manufacturers had already achieved a good compatibility in their calibration. With use of cRM-UA, further improvement in the status to an

ultimate degree was reached.

Preparation and selection of a qualified specific antibody is another important requirement to assure uniform results among different immunoassay systems. The ideal antibody will react to common major epitopes with good affinity and avidity in each assay, thus enabling formation of an optimized antigen-antibody reaction for proper value transfer. Most of these major preconditions were shared by the qualified assays/regents used in this investigation and likely contributed to the successful demonstration of standardization.

A small number of urines showed discrepancy among different immunoassays (Fig. 2). Such discrepant cases have been reported in all of the previous comparative studies [32]. Such outliers are encountered in clinical urine samples and likely reflect the structural and immunochemical heterogeneity of albumin that has been observed in both serum and urine. Heterogeneity includes fragmented forms, N-terminal or C-terminal truncated forms, aggregation and/or polymer forms, and modifications caused by ligand-binding [9, 23-24, 33-36]. While uniformity in calibration can be achieved using a purified monomeric albumin reference material, the immunochemical reactivity of heterogeneous forms of albumin in clinical urine samples remains a challenge in achieving completely standardized results. Preparation and selection of qualified antibodies that recognize only the clinically important forms of urine albumin is a crucial aspect in determining the results obtained. Since clinically relevant forms of urine albumin have not been fully characterized, it is difficult to address the requirements for antibody recognition at this time [9, 37].

In summary cRM-UA has attributes which make it a practical buffer-based secondary reference material for urine albumin to establish traceability of the immunoassays to the serum protein reference material ERM-DA470. cRM-UA may also be useful for various principles of assay systems, both qualitative and quantitative, when the sample matrix is closer to an aqueousfluid such as urine and cerebrospinal fluid.

Abbreviation

RM, reference material; HSA, human serum albumin; HPLC, IRMM, Institute of Reference Materials and Methods, JCCLS, Japan Committee of Clinical Laboratory Standards; high performance liquid chromatography; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; EQAS, External quality assurance schemes; ERM, European reference material; BCR, European Community Bureau of Reference; CAP, College of American Pathologists; RPPHS, Reference Preparation for Proteins in Human Serum, JSCC, Japan Society of Clinical Chemistry; TIA, turbidimetric immunoassay. cRM-UA; candidate reference material for urine albumin; pRM-I, prototype I reference material; pRM-II, prototype II reference material; LOQ, Limit of quantitation

References

1. Mogensen CE, Christensen CK. Predicting diabetic nephropathy in insulin-dependent patients. N Engl J Med. 1984; 311: 89-93.
2. Parving HH, Lewis JB, Ravid M, Remuzzi G, Hunsicker LG.Prevalence and risk factors for microalbuminuria in a referred cohort of type II diabetic patients: a global perspective. Kidney Int. 2006; 69: 2057-63.
3. Hillege HL, Fidler V, Diercks GF, et al. Prevention of Renal and Vascular End Stage Disease (PREVEND) Study Group. Urinary albumin excretion predicts cardiovascular and noncardiovascular mortality in general population. Circulation. 2002; 106: 1777-82.
4. Araki S, Haneda M, Koya D, Kashiwagi A, Uzu T, Kikkawa R. Clinical impact of reducing microalbuminuria in patients with type 2 diabetes mellitus.Diabetes Res Clin Pract. 2008; 13; 82 Suppl 1: S54-8.
5. Rossing K, Christensen PK, Hovind P, Parving HH. Remission of nephrotic-range albuminuria reduces risk of end-stage renal disease and improves survival in type 2 diabetic patients. Diabetologia 2005; 48: 2241-7.
6. Sasaki H, Yoshikawa R, Akanuma Y, Kanazawa A, et al. Reference

intervals in healthy Japanese populations (in Japanese). J Jpn Diabetic Society 2001; 44: 467-72.

7. Harwell TS, Nelson RG, Little RR,McDowall JM, Helgerson SD, Gohdes D. Testing for microalbuminuria in 2002: barriers to implementing current guidelines. Am J Kidney. 2003; 42(2): 245-8.
8. Von Chenck H. Validation of albumin determined in urine with HemoCue point of care analyzer. Scand J Lab Invest. 2003; 63: 119-23.
9. Miller WG, Bruns DE, Hortin GL, Sandberg S, Aakre KM, McQueen MJ, et al. National Kidney Disease Education Program-IFCC Working Group on Standardization of Albumin in Urine. Current issues in measurement and reporting of urinary albumin excretion. Clin Chem. 2009; 55: 24-38.
10. Baudner S, Bienvenu J, Blirup-Jensen S, Carlstrom A, Petersen PH, Johnson AM, et al. Certification of a matrix reference material for immunochemical measurement of 14 human serum proteins. CRM470. Brussels: Community Bureau of Reference, Commission of the European Communities, BCR information, reference material. Report EUR 15243 EN 1993: 1-172.
11. Johnson AM,Whicher JT. Effect of Certified Reference Material 470 (CRM 470) on National Quality Assurance Programs for Serum

Proteins in Europe. Clin Chem Lab Med. 2001; 39: 1123–8.

12. Itoh Y, Ichihara K. Standardization of immunoassay for CRM-related proteins in Japan: from evaluating CRM 470 to setting reference intervals. Clin Chem Lab Med. 2001; 39: 1154-61.

13. Zegers I, Schreiber W, LinsteadS, Lammers M, McCusker M, MuṅozAet al. Development and preparation of a new serum protein reference material: feasibility studies and processing. Clin Chem Lab Med. 2010; 48: 805-13.

14. Merlini G, Blirup-Jensen S, Johnson AM, Sheldon J, Zegers I; IFCC Committee on Plasma Proteins (C-PP). Standardizing plasma protein measurements worldwide: a challenging enterprise. Clin Chem Lab Med. 2010; 48(11): 1567-75.

15. Price CP, Newman DJ, Blirup-Jensen S, Guder WG, Grubb A, Itoh Y. First international reference preparation for individual proteins in urine. IFCC working group on urine proteins. Clin Biochem. 1998; 31:467-74.

16. Itoh Y, Hosogaya S, Kishi K, Suzuki H. Urine albumin immunoassay standardization. Jpn J Clin Chemistry 2008; 37: 6-14.

17. Okutani R, Itoh Y, Hirata H, Kasahara T, Mukaida N, Kawai T. Simple and high-yield purification of urine protein 1 using immunoaffinity

chromatography: evidence for the identity of urine protein 1 and human Clara cell 10-kilodalton protein. J Chromatogr. 1992; 20; 577: 25-35.

18. Reference materials - General and statistical principles for certification. ISO Guide 35: 2006.
19. DenhamE, Mohn B, Tucker L, Lun A, Cleave P, Boswell DR. Evaluation of immunoturbidimetric specific protein methods using the Architect ci8200: comparison with immunonephelometry. Ann Clin Biochem. 2007; 44: 529-36.
20. Armbruster DA, Pry T. Limit of blank, limit of detection and limit of quantitation. Clin Biochem Rev. 2008; 29: S49–S52.
21. Blirup-Jensen S, Johnson AM, Larsen M; IFCC Committee on Plasma Proteins. Protein standardization V: value transfer. A practical protocol for the assignment of serum protein values from a Reference Material to a Target Material. Clin Chem Lab Med. 2008; 46: 1470-9.
22. Zegers I, Keller T, Schreiber W, Sheldon J, Albertini R, Blirup-Jensen S, et. al. Characterization of the new serum protein reference material ERM-DA470k/IFCC: value assignment by immunoassay. Clin Chem. 2010; 56: 1880-8.
23. Ishibashi M. Standardization of Prostate-Specific Antigen (PSA) Assays: Can Interchangeability of PSA Measurements Be Improved? Clin Chem. 2006; 52: 1-2.
24. Meany D, Chan D. Comparability of tumor marker immunoassays.: still

an important issue for clinical diagnostics? Clin Chem Lab Med. 2008; 46: 575–6.

25. Miller WG, Thienpont LM, Van Uytfanghe K, et al. Toward standardization of insulin immunoassays. Clin Chem. 2009; 55: 1011-8.
26. Johnson A , Whicher JT. Effect of certified material 470 (CRM470) on national quality assurance programs for serum proteins in Europe. Clin Chem Lab Med. 2001; 39: 1123-1128.
27. Itoh Y, Ichihara K. Standardization of immunoassay for CRM-related proteins in Japan: from evaluating CRM 470 to setting reference intervals. Clin Chem Lab Med. 2001; 39: 1154-61.
28. Joint Committee for Traceability in Laboratory Medicine (JCTLM). Database of higher-order reference materials, measurement methods/procedures and services. http://www.bipm.org/jctlm/ (Accessed November 2008).
29. Morris HA. Traceability and standardization of immunoassays: A major challenge. Clin Biochem. 2009; 42: 241-245.
30. ISO 17511: 2003 In vitro diagnostic medical devices-Measurement of quantities in biological samples-Metrological traceability of values assigned to calibrators and control materials.
31. Aoki Y, Takahashi K, Ito K, Guideline for urine dipstick test. JCCLS

Journal 2004; 19: 53-55.

32. Seegmiller JC, Sviridov D, Larson TS, Borland TM,Hortin GL, Lieske JC. Comparison of urinary albumin quantification by immunoturbidimetry, competitive Immunoassay, and protein-cleavage liquid chromatography–tandem mass spectrometry. Clin Chem. 2009; 55:1991-94

33. Sviridov D, Drake SK, Hortin GL. Reactivity of urinary albumin (microalbumin) assays with fragmented or modified albumin. Clin Chem. 2008; 54: 61– 8.

34. Wiggins RC, Kshrisagar B, Kelsch RC, Wilson BS.Fragmentation and polymeric complexes of albumin in human urine. Clin Chim Acta. 1985; 15: 149: 155-63.

35. Brennan SO, George PM. Three truncated forms of serum albumin associated with pancreatic pseudocyst. Biochim Biophys Acta. 2000; 1481: 337-43.

36. Tejler L, Grubb A. A complex-forming glycoprotein heterogeneous in charge and present in human plasma, urine, and cerebrospinal fluid. Biochim Biophys Acta. 1976; 439: 82-94.

37. Comper WD, Osicka TM, Jerums G. High prevalence of immuno-unreactive intact albumin in urine of diabetic patients. Am J Kidney Dis. 2003; 41: 336-42.

Legends to Figures

Fig. 1 Elution profile of separated monomeric albumin on HPLC

Peak analysis indicated that purity exceeded 98.9%.
On SDS-PAGE and native PAGE a single band was observed at a molecular weight of 66,000.
Lane 1-4: cRM-U

Fig.2 Effect of recalibration on commutability of test results byseven turbidimetric immunoassay reagents

All combination cross-check test results across the seven reagents are shown. The regression lines were obtained based on the major-axis linear regression method. The 95% confidence bands around the regression lines were depicted to show the degree of scatter around the regression line.

(A) The upper inverted triangular matrix zone represents all combination cross check test results after recalibrated on the new cRM-UA RM, while the lower triangular zone represents results recalibrated on each manufacturer's calibrator; (B) The upper inverted triangle of the matrix represents cross-check results after recalibration on ERM-DA470, while the lower half zone represents the results based on each manufacturer's calibrator.

Fig.1 Supplemental
Stability of cRM-UA and pRM-UAII analyzed on SDS-PAGE and native PAGE
Lane 1: marker proteins, Lane 2: cRM-UA stored at −80ºC for 40 months, Lane 4: pRM-UA at 4~10 ºC for 70 months