Determination of Urine Albumin by New Simple High-Performance Liquid Chromatography Method

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Background: A simple high-performance liquid chromatography (HPLC) method was developed for the determination of albumin in patients' urine samples without coeluting proteins and was compared with the immunoturbidimetric determination of albumin. Urine albumin is important biomarker in diabetic patients, but part of it is immuno-nonreactive. **Methods:** Albumin was determined by high-performance liquid chromatography (HPLC), UV detection at 280 nm, Zorbax 300SB-C3 column. Immunoturbidimetric analysis was performed using commercial kit on automatic biochemistry analyzer COBAS INTEGRA® 400, Roche Diagnostics GmbH, Mannheim, Germany. **Results:** The HPLC method was

fully validated. No significant interference with other proteins (transferrin, α -1-acid glycoprotein, α -1-antichymotrypsin, antitrypsin, hemopexin) was found. The results from 301 urine samples were compared with immunochemical determination. We found a statistically significant difference between these methods ($P = 0.0001$, Mann-Whitney test). **Conclusion:** New simple HPLC method was developed for the determination of urine albumin without coeluting proteins. Our data indicate that the HPLC method is highly specific and more sensitive than immunoturbidimetry. J. Clin. Lab. Anal. 30:1226–1231, 2016. © 2016 Wiley Periodicals, Inc.

Key words: high-performance liquid chromatography; microalbuminuria; proteinuria; urine albumin

INTRODUCTION

The measurement of urine albumin is an early prognostic indicator of outcome in most forms of renal disease in persons with diabetes mellitus, and it also correlates with cardiovascular diseases. Normal urinary excretion of albumin has been established to be <30 mg of albumin/day. Excretion of 30–300 mg of albumin/day is defined as microalbuminuria and is considered as pathologic increase. Angiotensin-converting enzyme (ACE) inhibitors and angiotensin receptor blockers (ARB) have been proved to have renoprotective properties. Early treatment with these agents can prevent progression to overt proteinuria and end-stage renal failure in these patients (1–7).

The concentration of urine albumin has traditionally been measured by various immunochemical methods, such as immunoturbidimetry (IT), immunonephelometry (IN), radioimmunoassay (RIA), radial immunodiffusion, and enzyme-linked immunosorbent assay (ELISA) (8). Measurement of urine albumin is

potentially complicated by the fact that it exists in several forms (9). Recent studies have demonstrated that the nature of urine albumin is complex. Albumin filtered by the kidney is biochemically modified by lysosomal enzymes, resulting in the excretion of intact albumin and albumin-derived fragments (8). In tubular fluid, these albumin fragments influence the intact albumin in the way it loses its immunoreactivity.

The disulfide bridges are destroyed, followed by conformational changes resulting in immuno-unreactive “nicked albumin” (10–12). This albumin is undetectable by conventional immunoassays (immuno-unreactive albumin), but it is detectable by high-performance liquid chromatography (HPLC). HPLC analysis measures

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both immunoreactive albumin and immuno-unreactive albumin (total intact albumin = immunoreactive albumin + immuno-unreactive albumin) (13). Recent progress in urine albumin analysis is the application of size-exclusion HPLC. This method is commercially available as a kit (Accumin; AusAm Biotechnologies, Santa Monica, CA) to quantify total intact urine albumin (10–12, 14–16). Measurement of total intact urine albumin may have diagnostic value. It can predict the onset of persistent albuminuria 2.4–3.9 years earlier than that determined by the measurement of immuno-unreactive albumin alone by conventional immunoassays (13).

Immuno-unreactive albumin has unique albumin-like properties. It coelutes with native albumin on size-exclusion HPLC and comigrates exactly with native albumin in native polyacrylamide gel electrophoresis (PAGE). No other protein normally found in urine shows these properties. The major difference with native serum albumin is that immuno-unreactive albumin is not detected by antibodies to native albumin, together with the fact that the molecule dissociates in reducing sodium dodecyl sulfate (SDS)-PAGE (13). It has been hypothesized that the higher values of urine albumin with the HPLC assay may result from modified forms of albumin of the same size as albumin. More recently, size-exclusion HPLC has been proved as a more sensitive method of measuring urine albumin. In the low range (<100 mg/l), HPLC gives higher values than conventional immunoassays.

Although albumin is the most abundant plasma protein, there are many other plasma proteins of approximately the same molecular size, for example, transferrin, α_1 -acid glycoprotein, α_1 -antichymotrypsin, and hemopexin, which are major urinary components. Some authors suggest that HPLC methods and size-exclusion HPLC methods may overestimate albumin concentration, as it has been demonstrated that proteins of similar molecular mass may coelute with albumin (9, 14, 17, 18).

Our aim was to develop an HPLC method for determination of albumin in urine and confirm or reject the hypothesis stated earlier.

MATERIAL AND METHODS

Purified proteins (albumin, transferrin, α_1 -acid glycoprotein, α_1 -antichymotrypsin, antitrypsin, hemopexin), acetonitrile, and water (HPLC grade) were obtained from Sigma-Aldrich (Prague, Czech Republic). Trifluoroacetic acid and NaH_2PO_4 were purchased from Penta (Prague, Czech Republic). Microalbumin controls were obtained from Bio-Rad (Munich, Germany).

HPLC analysis was performed using an Agilent 1200 HPLC system (Agilent Technologies, Santa

Clara, CA) equipped with a photodiode array detector. The separation was carried out on a reversed phase column Zorbax 300SB-C3 (4.6 × 50 mm, particle size 3.5 μm ; Agilent Technologies). The column was operated at 22°C. The mobile phase components A, B, C, and D consisted of water (A), acetonitrile (B), 0.1 M sodium hydrogen phosphate (C), and 1% trifluoroacetic acid (D). Separations were accomplished using the gradient conditions stated in Table 1. The flow rate of the mobile phase was 2.0 ml/min. Ultraviolet detection was carried out at 280 nm, and the injected volume of centrifuged urine samples was 15 μl . The chromatograms were processed by Chemstation (Chem 32; Agilent Technologies). The total run time per sample was 8 min.

Stock albumin solution was prepared by diluting of certified material with distilled water to a concentration of 2000 mg/l. Standard concentration used was 10, 30, 50, 100, 300, 600, and 800 mg/l. Control samples were prepared at concentrations of 70 and 350 mg/l.

Transferrin, α_1 -acid glycoprotein, α_1 -antichymotrypsin, and antitrypsin were dissolved in water to make 2000 mg/l stock solutions, and the concentration of hemopexin was 1000 mg/l.

Urine random samples from 301 hospitalized patients were analyzed by HPLC, and the immunoturbidimetric method (COBAS INTEGRA[®] 400; Roche Diagnostics GmbH) was used to confirm or reject the hypothesis about the existence of coeluting proteins during HPLC analysis.

Urine samples from 301 diabetic patients from University Hospital Motol were obtained in urine collection tubes (Vacurette; Sarstedt, Nümbrecht, Germany), centrifuged 10 min at 3,727 g, and kept at 2–8°C.

Statistical analysis

Differences in the data obtained by HPLC and immunoturbidimetric methods were tested by Mann–Whitney test. The data have nonparametrical distribution, as was shown by the D'Agostino–Pearson normality test. A value of $P < 0.0001$ was considered significant. The data were analyzed by the GraphPad Prism 5.0 (San Diego, CA) statistical software package.

TABLE 1. The Gradient of Mobile Phases

Time (min)	Flow (ml/min)	%A	%B	%C	%D
0	2	44.0	36.0	0	20
2	2	42.0	38.0	0	20
4	2	51.0	39.0	10	0
6	2	35.0	60.0	0	5
8	2	44.0	36.0	0	20

RESULTS

Retention time of albumin was 3.0 min. The assay was linear (r^2 was 0.99941) across the whole range of concentrations. Quantification was based on peak area. In the HPLC assay, samples with no proteins in patients' urine showed no peaks interfering with albumin.

The analytical method has been successfully validated. The intra- and inter-day accuracy and precision were evaluated on two QC samples by multiple analysis ($n = 20$). Intraday CV for albumin was 5.00% and 3.00%. Interday CV was 4.00% and 3.90%. Within-day accuracy expressed by the calculated bias between observed and theoretical concentrations for albumin was 4.23% and 2.48%. Limit of detection for albumin in urine was found at 2.72 mg/l, and the limit of quantification was found at 7.81 mg/l. Interference with other proteins was tested. Under the conditions described above, we achieved a fine enough separation of alpha-1-acid glycoprotein, transferrin, and hemopexin from albumin (Fig. 1). The elution of albumin takes place within the third minute of the analysis, and its retention time remains the same throughout the whole test, whereas its retention time shifted with the methods previously applied.

Alpha-1-acid glycoprotein is separated into two peaks and is eluted within the first minute of the analysis, that is, before elution of albumin. As a result, the albumin peak is clearly distinguishable from the peaks of other proteins. Due to this significant difference in

retention time of both proteins, the differentiation of the peaks of alpha-1-acid glycoprotein and albumin is quite evident.

The elution of transferrin takes place in the second minute of the analysis; the transferrin peak is easy to distinguish from that of albumin. Hemopexin is eluted within fourth minute of analysis. Its peak is easily traceable, and there is no coelution with albumin. The peaks of the tested proteins are clearly shown in Figure 2 to be quite distinct from the albumin peak. Antichymotrypsin and antitrypsin have not eluted under the conditions described above during the whole test, meaning that these two proteins do not interfere with the elution of the other proteins. Retention times of tested compounds are stated in Table 2.

During analysis of the patient urine samples, we observed the phenomenon of albumin splitting albumin into two peaks when present in a high concentration in urine. This phenomenon is also visible and reproducible with high concentrations of albumin standards, and this is also described in the literature (9). However, this splitting effect of the albumin peak has no influence on the results of analysis.

The statistical comparison of both methods shows significant differences in concentration of albumin ($P < 0.0001$, Mann-Whitney test). The results (expressed as median) are as follows: 30.4 mg/l measured by HPLC (minimum 8.4 mg/l, maximum 3465 mg/l) vs. 21.1 mg/l (minimum 3.0 mg/l, maximum 3124 mg/l) measured by immunoturbidimetry (Fig. 3).

Fig. 1. Analysis of (a) albumin, (b) alpha-1-acid glycoprotein, (c) transferrin, and (d) hemopexin.

Fig. 2. Analysis of tested proteins.

TABLE 2. Retention Times of Tested Compounds

Compound	Retention time (min)
Albumin	3.02
Transferrin	2.05
alpha-1-acid glycoprotein	0.72
Hemopexin	3.55
Antitrypsin	Not eluted
α_1 -antichymotrypsin	Not eluted

DISCUSSION

The biggest established advantage of our HPLC method is that there are no interfering peaks from other compounds tested in the chromatogram at the retention time of albumin. That is very important for the evaluation of such a complex matrix.

It is important that we did not find any study in the relevant bibliography describing the separation of albumin from other proteins present in the urine samples. Turpeinen et al. (19) imply in their study the successful separation of beta-2-microglobulin from 1-acid glycoprotein; however, we have not been able to reproduce their results; no proteins have been detected.

Shaikh et al. (9) compared the determination of concentration of albumin in mouse urine by

Fig. 3. Comparison of HPLC and immunoturbidimetry (expressed as median $\pm$ SEM).

immunoturbidimetry, HPLC-MS (liquid chromatography combined with mass spectrometry detection), and size-exclusion HPLC. They analyzed 150 samples of urine from patients with different concentrations of

albumin. The values in the samples were measured by a routine immunoturbidimetric test (Roche Hitachi 912), HPLC-MS, and size-exclusion HPLC, where a Accumin™ kit (AusAm Biotechnologies, Los Angeles, CA) was used for the preparation of samples and calibration. The results of size-exclusion HPLC analysis showed higher values compared with immunochemical analysis and with the HPLC-MS.

Sviridov et al. (14) used samples of urine from healthy individuals to determine the concentration of albumin. As the first step in the method, size-exclusion chromatography with a Zorbax Bio Series GF-250 column and a phosphate buffer was used. For the next step, the samples of fractions of proteins gained by size-exclusion chromatography were analyzed on the classic HPLC. After the final evaluation of results, the authors came to conclusion that the final value of albumin in urine determined by HPLC represents not only albumin but also the globulins similar to albumin in their size which were not separated by size-exclusion chromatography. Our method has not used size-exclusion chromatography to “clean” the sample, but has shown an excellent resolution from other proteins in urine.

Comper et al. (8) compared the difference in detection and separation of albumin in urine by radioimmunoanalysis, immunonephelometry, immunoturbidimetry, and HPLC. By comparing these applied methods, we concluded the following: when using immunochemical methods, only intact immune reactive albumin and fragments of albumin >12 kDa and polymer aggregates of albumin can be detected. Using HPLC, even nonreactive fragments of albumin can be detected. Fragments of albumin of less than 10 kDa in size cannot be detected using any of these methods.

Turpeinen et al. (19) developed an HPLC method to determine beta-2-microglobulin, alfa-1-acid glycoprotein, and albumin contained in urine. The results acquired were compared with results from quantitative immune chemical tests. The aim of this study was to develop a quick method for detecting beta-2-microglobulin, alfa-1-acid glycoprotein, and albumin in urine. By comparing the data with the results from immune chemical tests, the authors found that this method to be convenient for the detection of the respective proteins and their quantification in urine.

Kushnir et al. (20) used a MARS column (Agilent Technologies) to determine proteins in urine. This column is based on the immune chemical principle and retains albumin, transferrin, haptoglobin, IgG, IgA, and alfa-1-antitrypsin. The efficiency of retention of the mentioned proteins was confirmed by SDS-PAGE. The samples were subsequently analyzed on an Agilent

1200 nano-HPLC with detection on an Agilent 6510 Q-TOF (Agilent Technologies). These authors stated that HPLC methods can detect a lower concentration of proteins compared to immunochemistry.

Bachmann et al. (21) compared results from 17 commercially available urine albumin measurement procedures with an isotope dilution mass spectrometry (IDMS). The comparison of results from COBAS c501 and IDMS showed a discrepancy, with bias varied from –20% to –15%, for low concentrations of albumin (up to 30 mg/l). This is similar to our results. These authors also showed that most used procedures exhibited substantial biases that varied with concentration. An explanation could be linked to the calibration procedures of the different analytical systems; the producers should improve the calibration strategies as part of their contribution to the standardization process. Similar results were also published by Graziani and Plebani (22).

CONCLUSION

Currently available immunochemical methods are not able to detect the full amount of urinary albumin. HPLC methods or LC-MS are quite sufficient for quantification of the real concentration of urinary albumin. Requests for allowable turnaround time (TAT) of analysis in clinical laboratories lead to a preference for immunochemical methods because they are not very time consuming and they are quite simple procedures, even though there are many possible cross-reactions which can falsely affect the result of the determination. The advantages of the HPLC method presented here are not only the ability to separate albumin from other urine proteins but also the possibility to inject the patient urine into the HPLC system without any time-consuming procedures such as liquid–liquid extraction or solid-phase extraction. These advantages facilitate the use of this method in routine laboratory practice at special medical departments for a specific group of patients (diabetics and patients with renal diseases), and thus the earlier diagnosis of albuminuria.

Our results prove that the HPLC method for albumin detection in urine is more sensitive than immunoturbidimetry and sufficiently specific with no need of pretreatment of the sample. There are also no significant interferences with other urine proteins.

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