Automated Immunochemical Method for Determination of Urinary Protein of Plasma Origin

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An automated continuous-flow procedure has been developed for the rapid determination of urinary proteins of plasma origin. Antiserum to whole human plasma was used as the reagent, and the antigen–antibody reactions were quantitated by nephelometry. By adding polyethylene glycol (mol wt 6000–7500) to the reaction medium, reaction time was decreased to <3 min; no sample blanks were required, and samples were analyzed at a rate of 70 per hour. Recovery studies yielded an average of 98.5% of the added protein. In-run replicate precision (CV) of the method was 1.45%; day-to-day precision was 2.58%.

Additional Keyphrases: nephelometry • immunochemistry • continuous-flow analysis • kidney disease • normal (reference) values

Clinical evaluation of patients with kidney disease usually includes estimation of urinary protein excretion. The wide range of protein concentrations and the many interfering substances in normal and pathological urine make determination of urinary protein a difficult analytical task. These factors have led to the development of numerous analytical techniques. The Folin–Lowry phenol reaction has been proposed, but is subject to interference from drugs and other substances (1, 2). Turbidimetric procedures that utilize trichloroacetic acid, sulfosalicylic acid, or β-naphthalene sulfonic acid lack sensitivity and precision (2). Biuret procedures can provide accurate results, but the required removal of interfering substances usually makes them quite laborious and not adaptable to automation (3-6). Over the past few years, we have investigated the use of immunochemical reagents to measure specific proteins in bodily fluids (7-10), and have shown that antiserum to whole human plasma can be used to determine plasma protein concentration (11). Here, we describe an automated immunochemical procedure for measuring the urinary proteins of plasma origin.

Materials and Methods

Apparatus

We used an automatic pipette (Model 25000; MicroMedic Systems, Inc., Philadelphia, Pa. 19104) equipped with a 200-μl sample pump and a 5-ml diluent pump to prepare an initial 10-fold dilution of all samples and standards, and to make additional dilutions of samples with markedly increased protein.

A continuous-flow system was assembled from modules of the AutoAnalyzer II (Technicon Instruments Corp., Tarrytown, N. Y. 10591) and included a sampler, proportioning pump, modified analytical cartridge, fluoronephelometer, and recorder. The manifold (Figure 1) included a 1.4-ml delay coil, which resulted in a reaction time of 140 s.

Reagents

Phosphate-buffered saline (PBS), 50 mmol/liter, pH 7.4. Dissolve 5.69 g of Na₂HPO₄, 1.32 g of KH₂PO₄, and 5.85 g of NaCl in 1 liter of distilled, deionized water and add 0.3 ml of sorbitan monolaurate polyoxyalkylene (“Tween 20”) surfactant (Technicon).


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Antiserum. Goat antiserum to whole human plasma was obtained commercially (Meloy Laboratories, Springfield, Va. 22151). The multireactivity of this reagent was confirmed by immunoelectrophoretic studies with human serum, concentrated urine, and purified solutions of albumin, transferrin, and IgG (Figure 2). Before use, the antiserum was diluted 25-fold with phosphate-buffered saline and filtered (with a filter of 0.22 μm av pore size; Millipore Corp., Bedford, Mass. 07130). The diluted antiserum was stable for one week when refrigerated, but daily filtration was required.

Albustix (Ames Co., Elkhart, Ind. 56415). These were used to screen for abnormal amounts of urinary protein.

Standards

Standards were prepared from commercial calibration serum (SMA Reference Serum, Technicon). The calibration serum was diluted to produce seven standards ranging in protein concentration from 10 to 1000 mg/liter. These values were chosen to reflect the urinary protein concentrations expected in normal and pathological urines. The nonlinear standard curve was well characterized by the seven working standards.

Procedure

Urine total volumes from 24-h collections were measured and aliquots frozen at −20 °C on the day of collection. On the day of the assay, the samples were thawed, mixed, filtered, and tested with Albustix as an estimate of protein concentration.

All standards, controls, and urine samples were diluted 10-fold with PBS by using the automatic pipette. The pipette settings were then changed, and additional dilutions were prepared on those samples that gave a 2+ or greater Albustix reading. Samples with a 2+ reading were subjected to subsequent 2-, 4-, and 10-fold dilutions, resulting in dilution factors of 20, 40, and 100, respectively. The total pre-instrumental dilution factors for samples with a 3+ reading were 100 and 200, and 4+ samples were diluted 200- and 500-fold. These subsequent dilutions were necessary to maintain and confirm the condition of antibody excess.

The recorder baseline was set with antiserum passing through the flow cell of the continuous-flow system. Seventy samples per hour were aspirated into the manifold, and the reactions quantitated by nephelometry. Recorder peak heights were then measured and protein concentrations computed from the standard curve (Figure 3). The marked dilution of the urine samples made blank corrections unnecessary.

Final results were calculated with reference to the total urine volume, and reported as protein excretion per day.

Results

Analytical Variables

Precipitin curve. Thirteen different dilutions of the reference serum, ranging in concentration from 10 to 6400 mg/liter, were aspirated into the automated manifold to characterize the total protein precipitin curve (Figure 4). No apparent region of antigen excess was reached at concentrations up to 6400 mg/liter. In experiments with solutions of purified albumin (150 to 2400 mg/liter) and purified gamma globulin (80 to 1650 mg/liter), the antibody reagent was in excess up to about 2000 and at least 1650 mg/liter, respectively (Figure 5). Reaction responses were essentially the same for equivalent concentrations of albumin or gamma globulin up to about 1200 mg/liter.

Steady-state and carryover. A 70-per-hour sample cam with a 1:1 sample/wash ratio provided 96% of the steady-state reaction response with 1.72% carryover
from the 800 mg/liter standard to the 80 mg/liter standard.

Reaction time. The light-scattering responses from a series of standards were analyzed by using six delay coils which produced reaction times ranging from 95 to 235 s. The results of this study (Figure 6) indicated that the maximum reaction response was obtained at 140 s. Proper mixing did not occur with shorter times; longer delays resulted in decreased response as a result of flocculation of precipitates.

Precision. In-run replicate analyses of a pooled urine sample (n = 30) gave a mean of 585 mg/liter, a standard deviation of 8.5 mg/liter, and a coefficient of variation of 1.45%. Analysis of a pooled urine sample during 30 days (n = 33) yielded a precision (CV) of 2.58%.

Correlation Studies

Urine samples from 45 hospital patients were assayed by both the present technique and an automated nephelometric method in which saline was the diluent. The method was also compared with the modified biuret procedure of Rice (6). The results of these investigations (Table 1) indicated excellent correlation between the two nephelometric procedures. The immunochemical technique gave generally lower results than the biuret method. This was expected, however, since the antiserum reagent is nonreactive against urinary tract proteins.

Recovery Studies

A series of recovery studies were done by adding a preassayed reference serum to 25 urine samples. The final concentration of protein in these samples ranged from 400 to 3250 mg/liter and the mean recovery was 98.5% (±5.6%, SD).

Reference Values

Plasma-derived urinary protein was determined on 24-hour specimens from 34 normal adults. Because the data were not gaussian distributed, and the population size was not large enough for the use of non-parametric statistics, we wish to report only the mean normal excretion rate of 15.8 mg/24 h.

Discussion

Standardization of the technique with human plasma and the use of antihuman plasma as reagent would be inappropriate if plasma proteins were significantly changed antigenically on passage through the glomerulus. Immunoelectrophoretic studies, however, have shown reactions of immunologic identity between plasma proteins and their urinary counterparts (12).

Also of interest in a discussion of the urinary proteins of plasma origin are the so-called Bence Jones proteins and other products resulting from plasma
cell dyscrasias. Reactivity of the antiserum with immunoglobulins was demonstrated in the immunoelctrophoretic studies (Figure 2), and reactivity against free kappa and free lambda light chains was observed on nephelometric analysis of commercially available Bence Jones protein controls.

Proteins derived from the urinary tract itself (13) are not detected by this procedure. Measurement of proteins derived from the urinary tract is of limited value at present, however, because the clinical significance of these proteins has not been extensively studied.

The precipitin curve for the multicomponent antigen–antibody reaction (Figure 4) was studied to assure antibody excess over a definite range for two major plasma protein components of proteinuria. The conditions of assay resulted in antibody excess for albumin and gamma globulin to well above the concentration of the highest working standard (Figure 3). Moreover, reaction response was the same for equivalent amounts of albumin and gamma globulin. This is not true for most turbidimetric procedures (2). Another precaution to assure antibody excess was to routinely include several additional dilutions of any samples giving a 2+ or greater Albustix reading.

Inhibition of the immunochemical reaction by urea or extremes in pH was minimized by the 110-fold dilution of all urine samples in phosphate-buffered saline. This dilution also eliminated the need for blank corrections and decreased absorption by highly colored samples.

This immunochemical procedure has been in routine use in our laboratory for the past nine months, and our results have correlated well with the clinical status of kidney-disease patients. We believe that it represents an improvement over the more widely used chemical methods, in terms of sensitivity, specificity, precision, and ease of analysis.

References