Microtransferrinuria and Microalbuminuria: Enhanced Immunoassay

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ABSTRACT

An enhanced-sensitivity immunoassay for urinary microtransferrin and microalbumin was devised based on protein precipitation with cold trichloroacetic acid followed by dissolution of the precipitate in a small volume of phosphate buffer. Samples can be concentrated 10-fold by this method while at the same time removing many of the chromogens present in urine. Concentrated samples were assayed by immunoturbidity and radial immunodiffusion. The average recovery for urinary microtransferrin was 82 percent and for microalbumin 91 percent. The reference range for 80 normal adults for microtransferrinuria and microalbuminuria is 0 to 0.9 and 5 to 32 mg per g creatinine, respectively. The same method can be used for the assay of other proteins such as B₂-microglobulin in the urine or the cerebrospinal fluid.

Introduction

Microalbuminuria¹² and microtransferrinuria are terms which indicate increased levels of these respective proteins when the total urinary protein is in the normal reference range. These proteins serve as an early sensitive indicator of nephropathy in systemic lupus, hypertension and especially in diabetes.²,³,⁸,¹⁰,¹¹ Since transferrin has a higher pI relative to albumin, it is expected to be filtered more readily than albumin and thus it may be a more sensitive indicator of early renal nephropathy than microalbuminuria.² The proportion of serum transferrin is about five percent of serum albumin levels. Thus, the urinary transferrin level in the normal subject is expected to be 20 times lower than that of albumin, i.e., about one mg per g creatinine. This level is below the detection level of immunonephelometric/turbidimetric instruments and radial immunodiffusion (RID) plates. In order to increase the sensitivity of the assay 10-fold, a simple concentration step was devised. This step can be applied for the immunoassay in general (nephelometric, turbidimetric, and radial immunodiffusion) of transferrin as well as several other pro-
teins such as albumin and B$_2$-microglobulin, while at the same time removing many of the interfering chromogens present in urine.

**Materials and Methods**

**Sample Concentration**

Fifty µL of a carrier protein, ovalbumin or bovine albumin 10 mg per mL saline (nine g per L), to decrease transferrin adsorption to the test tubes, was added to 2.5 mL urine cooled to 4°C. Ice cold 20 percent trichloroacetic acid, 0.5 mL was added to the tubes and mixed well to precipitate the proteins. After five min, the tubes were centrifuged for five min at 5,000 rpm. The supernatant was decanted removing carefully the last drop of fluid to avoid sample dilution. The precipitate was dissolved in 250 µL of 0.4 mol per L phosphate buffer at pH 7.8.

**Immunoturbidity/ Immunonephelometry**

*Antibody:* Anti-albumin and antitransferrin raised in goats were obtained.*

*PEG reagent:* Polyethylene glycol (PEG) 6,000 (four percent) prepared in phosphate buffer, 10 mmol per L, pH 7.4 containing nine g per L of sodium chloride.

**Instrumentation**

*The Express*†: The instrument was programmed to add 30 µL of the concentrated sample for transferrin or (four µL for albumin) to 220 µL of four percent PEG. After 20 seconds, the absorbance difference between 340 nm and 600 nm was recorded, and a 50 µL aliquot of the diluted antibody (100 µL antibody in 1,000 µL of PEG) was added to the cuvet. After five minutes of incubation at 37°C, the absorbance difference at 340 nm and 600 nm was recorded again, and the difference between the two intervals used to calculate the results. The absorbance was plotted against the concentration on semi-log paper, and the final answer was read from the chart. Standards were included in each run.

*The RA 1000*‡: The instrument was programmed to add 25 µL of the concentrated sample for transferrin (or five µL for albumin) to 360 µL test reagent (200 µL antisera in 10 mL PEG). This mixture is read against a blank (the same sample volume in 360 µL PEG reagent) after 300 seconds at 340 nm. The final answer is calculated by the instrument.

**Radial Immunodiffusion:**

Agarose (Type I),§ one percent in a 40 mmol per L phosphate buffer, pH 7.4 containing nine g per L of saline and 50 mg per L of sodium azide, was melted at 100°C. After cooling to 44°C, 75 µL of the antisera were mixed in 10 ml of agarose. The agarose was poured rapidly into the plates (two mm thick) which have small metal rods (5 mm length x 3 mm i.d.) as templates for the wells. After the agar solidified, the metal rods were removed. The wells were filled with samples (15 µL for transferrin and 10 µL for albumin), and the plates were incubated for 24 hours at 37°C.

**Standards**

Human pure albumin (500 mg per L) and transferrin (30 mg per L) were dis-

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* Cappel, West Chester, PA.
† Ciba Corning, Oberlin, OH.
‡ Technicon Instruments, Tarrytown, NY.
§ Sigma, St. Louis, MO.
solved in saline (nine g per L) containing 50 mg per L sodium azide, aliquoted in small tubes and frozen. The standards were diluted 10-fold before use with saline.

**NORMAL SUBJECTS**

Eighty adult patients excreting normal amounts of protein and glucose who were visiting an outpatient clinic were used for the reference range.

**Results and Discussion**

Originally, detection of microalbuminuria was accomplished by radioimmunoassay\(^{10,12}\) because of the low concentration of albumin in urine. Later on, few methods have been described for the direct immunoturbidity/nephelometry of microalbuminuria.\(^{1,7,9}\) The immunoturbidity/nephelometric methods correlate well with the radioimmunoassay methods.\(^{13}\) An immunoturbidity method for direct assay of elevated transferrin values has been also described.\(^9\) The normal range for the latter method is quite high (0 to 1.9 mg per L) probably owing to the lack of sensitivity. A latex particle agglutination assay for urinary transferrin has also been described.

**Figure 1.** Reaction of transferrin with the antisera (middle well) before and after concentration with trichloracetic acid on Ouchterlony plate. Well A: 4 mg per L transferrin standard concentrated. Well B: 40 mg per L transferrin standard applied directly. Well C: urine applied with no concentration. Well D: same urine sample concentrated 10-fold.
TABLE I
Recovery of Added Albumin and Transferrin To a Urine Pool

<table>
<thead>
<tr>
<th>mg/L Added</th>
<th>Percent Recovery</th>
<th>Unconcentrated</th>
<th>Concentrated</th>
</tr>
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<tbody>
<tr>
<td>Albumin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>90</td>
<td>101</td>
<td></td>
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<tr>
<td>40</td>
<td>93</td>
<td>85</td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>95</td>
<td>87</td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td>93</td>
<td>91</td>
<td></td>
</tr>
<tr>
<td>Transferrin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.2</td>
<td>--</td>
<td>76</td>
<td></td>
</tr>
<tr>
<td>2.4</td>
<td>--</td>
<td>83</td>
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<tr>
<td>4.8</td>
<td>--</td>
<td>87</td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td>--</td>
<td>82</td>
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</tr>
</tbody>
</table>

which utilizes automated counting of the agglutinated particles. This method requires special instrumentation and skill that is not amenable to the clinical laboratory. In general, immunonephelometric/immunoturbidity assays have just enough sensitivity to measure urinary albumin (about five mg per L) but not transferrin levels in the normal individual.

It is believed that acid treatment denatures proteins with a loss in their antigenic reactivity. Using an Ouchterlony plate, (figure 1), it is evident that such treatment under the described conditions does not alter the antigenicity of the proteins studied: transferrin, albumin, or B2-microglobulin. However, acid treatment allowed 10-fold sample concentration. Since the concentration of albumin is relatively about 20 times higher than transferrin in urine, some instruments which can add a relatively large sample volume to the reagent, such as the Express 550, can perform microalbuminuria determination directly on urines without the need for such degree of concentration. However, for instruments which are limited by the volume of sample added, the concentration step is important to increase the sensitivity of the assay. In table I is illustrated the recovery for albumin and transferrin added to a urine pool. Concentration of the urine sample by the method described raises the albumin concentration to that of the cerebrospinal fluid, allowing the two tests to be performed with the same program on the analyzer.

The present method measures transferrin and albumin levels both in the normal as well as the elevated range. Urine concentration measured by this method has four advantages which are: (1) greatly increasing the sensitivity of the assay; (2) decreasing the background owing to interfering chromogens in the urine (table II); (3) removing substances which inhibit the antigen-antibody reaction, such as urea; and (4) improving the precision of the test. Urine has variable amounts of chromogens which absorb light at the wavelengths used most often.
for immunoturbidity, such as 405 nm or 340 nm. The excess chromogens increase the instrument noise, leading to poor precision and decreased linearity. In table II is an illustration of how sample concentration decreases the background quite considerably. It has also been demonstrated that the C.V. of assays for albuminuria is quite dependent on the concentration of albumin in the sample.\textsuperscript{1} The precision of our assays for albumin and transferrin is summarized in table III.

The histogram of 80 normal individ-
uals for albumin and transferrin is illustrated in figure 2. Based on this distribution, a reference range was used of 5 to 32 mg per g creatinine for albumin and of 0 to 0.9 mg per g creatinine for transferrin. These values are close to the ranged reported earlier for these two tests.\textsuperscript{2,4}

The assay for albumin is not affected by high levels of gamma globulins (500 mg per L) or transferrin (50 mg per L). In addition, the transferrin assay is not
affected by albumin (1,000 mg per L) or gamma globulins (500 mg per L).

Both assays produced reactions which were linear on semi-log paper between 10 to 100 mg per L for albumin and 0.4 to 4 mg per L for transferrin (figure 3).

For the radial immunodiffusion plates, the concentration was linear against the square of the diameter from 5 to 60 mg per L albumin and 0.5 to 4 mg per L for transferrin (figure 4).

An excess of antigen can give falsely
low values. Neither instrument used in this study was programmed to detect excess of antigen. In order to avoid this problem, total protein was run on every urine sample. Those samples which had elevated protein levels above the normal range (0 to 250 mg per g creatinine) were repeated after dilution.

A higher degree of urine concentration can be obtained by using larger urine volume and trichloroacetic acid. This method can be applicable to other urinary proteins, such as B2-microglobulin. In addition, it can be utilized with other fluids which have low protein concentration, such as cerebrospinal fluid and peritoneal fluids. For example, this method has been utilized successfully to measure IgA and IgM in the cerebrospinal fluid.

Acknowledgment

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References