A Simple Radial Immunodiffusion Method for Assay of $\beta_2$-Microglobulin in Serum

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In this method for clinical measurement of $\beta_2$-microglobulin, a 10 g/L agarose gel containing anti-$\beta_2$-microglobulin is used, with subsequent staining with Coomassie Blue. Although the method is slow (requiring 30 h), it is inexpensive, reliable, and accurate over the range of 1 to 10 mg/L, and is useful in the determination of $\beta_2$-microglobulin in serum and cerebrospinal fluid. A modified procedure in which staining with silver is used may be sufficiently sensitive for the clinical assay of urine.

Additional Keyphrases: urine • cerebrospinal fluid • agarose gel • cancer • AIDS • reference interval

$\beta_2$-Microglobulin is a small protein ($M_r$ 11,818) that is associated with human leukocyte antigen (HLA) and other cell-membrane antigens, and is present on the surface of all normal nucleated human cells. As recently reviewed by Messner (1), $\beta_2$-microglobulin concentrations in serum are increased in various disorders, including renal disease (proportional to the decrease in glomerular filtration rate) and many diseases that are associated with increased cell turnover, inflammatory conditions such as viral infections, acquired immunodeficiency syndrome, autoimmune diseases, and transplant rejection. Increased concentrations are also noted in some malignancies, particularly leukemias and non-Hodgkin’s lymphomas. Low values for serum are not associated with any known human disease.

Although accurate radioimmunoassay (RIA) methods are available for $\beta_2$-microglobulin measurement, these are relatively expensive. Furthermore, $^{125}$I-labeling is used in the kits and therefore they have a short shelf-life. Because this test is ordered relatively infrequently, the short shelf-life compounds the problem of high cost for all but the largest medical centers. A simpler, less expensive test would be desirable. We have therefore developed a simple, inexpensive radial immunodiffusion (RID) method (2) for measuring $\beta_2$-microglobulin in serum that is sufficiently sensitive and accurate for clinical use.

Materials and Methods

Materials

"Seacem" agarose and "Gelbond™" film were obtained from Marine Colloids Division, FMC, Rockland, ME 04841. Anti-$\beta_2$-microglobulin was obtained from DAKO, Santa Barbara, CA 93103. This polyclonal antibody was prepared in rabbits, and was an immunoglobulin fraction purified by ion-exchange chromatography. It was certified by DAKO as being of sufficient strength to precipitate 120 $\mu$g of $\beta_2$-microglobulin in 1 mL of undiluted antibody, leaving nei-

ther antibody nor antigen in the resulting supernate. Plastic bags were used to store the gels until use. "Phadebas" $\beta_2$-microglobulin RIA kits were obtained from Pharmacia Fine Chemicals, Piscataway, NJ 08854.

Preparation of the Gels

The RID assay was performed in 10 g/L agarose gels bound to Gelbond film, prepared by the method described by Marine Colloids Division, in sodium phosphate buffer (10 mmol/L, pH 6.8). Shortly before casting the gels, at 42 °C, we added various amounts of anti-$\beta_2$-microglobulin to 30 mL of agarose solution. (Adding the antibody at a higher temperature showed a substantial loss of $\beta_2$-microglobulin binding.) We then cast the solution as a slab, generally 1.2 mm thick, and allowed it to gel. Wells measuring 3 mm in diameter were cut in the agar at least 1 cm apart and 0.5 cm from the edges of the gel. The gels were then stored at 4 °C in airtight plastic bags until use. The gels were stable (in the plastic bags) at 4 °C for at least six months.

To alter the sensitivity of the assay, we added various amounts of antibody, to produce final dilutions of the antibody from 1:150 to 1:600 in the agarose gel; slight adjustments in antibody concentration may be necessary with each new batch of antibody. At a 1:300 or less dilution of antibody in the gel, Coomassie Blue staining was feasible, but at lower antibody concentrations (1:600), staining with silver was required. We also varied gel thickness from 1 to 2 mm, to vary the amount of sample delivered and thus assess the effect of a change in sensitivity.

RIA Procedure

Pharmacia’s assay procedure for $\beta_2$-microglobulin in serum or urine was followed in all details.

RID Procedure

We used a method similar to that of Mancini et al. (2) to prepare RID gels, which we then stained by either of two methods. An agarose gel film of appropriate size was cut and placed in a humid chamber. Eight microliters of the standards, controls, or patients’ samples (all in duplicate) were placed in each well. The gel was allowed to incubate for 24 h at room temperature (shorter incubations resulted in incomplete equilibration and less reproducibility). The gel was then washed in two changes of 10 mmol/L phosphate-buffered isotonic saline for 5 h in an agitator to wash out unbound proteins. It was then press-dried and stained with either Coomassie Blue or silver reagent.

For staining with Coomassie Blue we placed the dried film in Coomassie Brilliant Blue solution for 10 min, followed by destaining solution until the background was relatively clear. The Coomassie Blue solution consisted of 0.5 g of Coomassie Brilliant Blue R in 90.8 mL of distilled water, plus 90.8 mL of methanol and 18.4 mL of glacial acetic acid. The destaining solution consisted of 200 mL of

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glacial acetic acid, 20 mL of glycerol, 1 L of distilled water, and 1 L of ethanol.

Much higher sensitivity could be obtained with a silver reagent instead of (or subsequent to) Coomassie Blue staining. We used a method similar to that of Willoughby and Lambert (3). After rinsing the gel film with distilled water for at least 10 min, we placed it into a freshly prepared equimolar mixture of solution A and solution B—solution A being 25 g of Na₂CO₃ in 500 mL of water, and solution B consisting of 1 g of NH₄NO₃, 1 g of AgNO₃, 5 g of tungstosalic acid, and 3.35 mL of 370 mL/L (concentrated) formaldehyde solution in 500 mL of water. The gels were placed in this mixture and agitated for 10 to 15 min or until stain development was adequately visible. When the staining was complete, we dipped the gels in dilute (50 mL/L) acetic acid. Scrupulous purity and cleanliness of all solutions and containers was maintained, as is required for staining with silver.

We measured the diameters of the stained areas in two directions, using calipers. The mean of these diameters for each patient was compared with that of the standard. The standards had been prepared from the pooled sera of patients in renal failure, assayed by the Pharmacia RIA for β₂-microglobulin and then adjusted to a β₂-microglobulin concentration of 8 to 10 mg/L and stored frozen in small aliquots. In addition to this undiluted standard, we used dilutions of the standard (with phosphate-buffered saline)—1:1, 1:2, 1:3, 1:4, 1:5, 1:7, and 1:9. A control was similarly prepared and adjusted to a β₂-microglobulin content of 3 to 4 mg/L. Similar but more dilute standards and controls were prepared for the more sensitive assays, with concentrations in the range of 0.1 to 0.8 mg of β₂-microglobulin per liter.

Sera from 14 individuals, principally patients with lymphoid malignancies, were studied by both methods. These cases were chosen as representative of the patient population for which this test is usually ordered at our institution.

**Results**

**Range and sensitivity.** Using serum with an antibody dilution of 1:300 in the agarose gel, 8 μL of sample in 1.2-mm thick gels, and Coomassie Blue staining, we plotted the concentrations of standards, ranging from 1.0 to 10.8 mg/L, against diameter squared (Figure 1B). Good linearity was observed over the entire range of β₂-microglobulin concentration, and the sensitivity was adequate to measure this range. RID assays performed under these conditions were considered adequate for analysis of serum or cerebrospinal fluid. Except for minor batch-to-batch adjustments in the antibody concentration, these conditions are those under which the clinical assay is performed. These conditions were also used in our assessment of accuracy and precision described below.

Using diluted serum with an antibody dilution of 1:600 in the agarose gel, silver staining, and either 20 μL of sample in 2-mm-thick gels or 8 μL of sample in 1-mm-thick gels, we plotted concentrations of standards from 0.15 to 1.18 mg/L against diameter squared (Figure 1A). The β₂-microglobulin concentration was linearly related to diameter squared at all but perhaps the greatest β₂-microglobulin concentration. Also, as anticipated, the thicker gels, containing more sample, had moderately increased sensitivity and were adequate to measure 0.15 mg of β₂-microglobulin per liter. Sensitivity could also be increased by adding sample to the wells more than once.

**Reference range.** We assayed normal plasma from 35 blood donors. Except for a single excluded outlier (3.5 mg/L), the β₂-microglobulin values ranged from 0.8 to 2.1 mg/L (mean 1.94 mg/L, SD 0.33 mg/L). On the basis of these data, our normal reference interval is 0.8 to 2.1 mg/L.

**Accuracy.** We compared the RID assay with the Pharmacia RIA for β₂-microglobulin, testing both methods simultaneously on the same 14 specimens from patients. We found 1.1 to 5.75 mg/L by the RIA (y) method and similar values by the RID method (x). The results (Figure 2) correlated well (r = 0.95) and are described by the linear regression equation y = 1.07x − 1.10 mg/L (standard error 0.47 mg/L). By both tests, 10 of the 14 specimens had increased values for β₂-microglobulin (≥2.1 mg/L), and three cases had normal values. In one case the value was slightly above normal by the RID method, 2.15 mg/L, but normal by RIA, 2.0 mg/L. The variation appeared to be normally distributed.

**Precision.** Within-run variation for normal, moderately above-normal, and markedly above-normal values for the
pooled sera was measured by three runs each of 20, 10, and 10 determinations (Figure 3). The standard deviation was ≤0.26 mg/L, the CV < 6.7% in all cases (Table 1).

Five sera with normal to markedly increased concentrations of β₂-microglobulin were analyzed on four consecutive weeks. Between-run variations were <0.24 mg/L, for a CV of <5.4% (Table 2).

Stability. Aliquots from two sera were stored for four weeks at 4 and −20 °C and at −20 °C after lyophilization. No difference in β₂-microglobulin activity was noted in any of the aliquots of the two sera, regardless of the method of storage.

Fig. 3. Gel stained with Coomassie Blue, showing reproducibility
The gel was prepared as for Fig. 1b, with 8 μL of control serum (2.2 mg/L) applied to each well. Notice the uniform size and sharp borders of the stained areas.

Table 1. Within-Run Variation, Radial Immunodiffusion Method

<table>
<thead>
<tr>
<th>Sample</th>
<th>No. of determinations</th>
<th>β₂-Microglobulin mean, mg/L</th>
<th>SD</th>
<th>CV, %</th>
</tr>
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<tbody>
<tr>
<td>Normal</td>
<td>20</td>
<td>2.00</td>
<td>0.125</td>
<td>6.2</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>2.06</td>
<td>0.136</td>
<td>6.6</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>1.95</td>
<td>0.101</td>
<td>5.2</td>
</tr>
<tr>
<td>Borderline</td>
<td>20</td>
<td>2.65</td>
<td>0.141</td>
<td>5.3</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>2.72</td>
<td>0.150</td>
<td>5.5</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>2.61</td>
<td>0.120</td>
<td>4.6</td>
</tr>
<tr>
<td>Elevated</td>
<td>20</td>
<td>4.37</td>
<td>0.260</td>
<td>5.9</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>4.13</td>
<td>0.093</td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>4.07</td>
<td>0.120</td>
<td>2.5</td>
</tr>
</tbody>
</table>

* Pooled sera from normal healthy subjects. b "Elevated" sera were pooled from patients with renal impairment. The "borderline" sera consisted of "elevated" sera mixed with normal sera.

Table 2. Between-Run Variation, Radial Immunodiffusion Method

<table>
<thead>
<tr>
<th>β₂-Microglobulin concn, mg/L</th>
<th>Mean</th>
<th>SD</th>
<th>CV, %</th>
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<tbody>
<tr>
<td></td>
<td>2.37</td>
<td>0.126</td>
<td>5.3</td>
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<td></td>
<td>4.13</td>
<td>0.186</td>
<td>4.5</td>
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<td></td>
<td>4.40</td>
<td>0.159</td>
<td>3.6</td>
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<td></td>
<td>4.57</td>
<td>0.171</td>
<td>3.7</td>
</tr>
<tr>
<td></td>
<td>6.50</td>
<td>0.234</td>
<td>3.7</td>
</tr>
</tbody>
</table>

Results of determining the β₂-microglobulin concentration of sera four times at weekly intervals.

Discussion
The RID results for β₂-microglobulin compare well with those by the Pharmacia RIA, and the range of sensitivity is similar to the concentrations found in serum and cerebrospinal fluid samples. Although abnormally low concentrations in serum would not be detected, low serum values of β₂-microglobulin are not known to be associated with any human disease. The RID serum assay has been in use in our laboratory for more than one year, and we have encountered no problems with it.

The β₂-microglobulin concentration is approximately proportional to the area stained (2), which in turn is proportional to the square of the diameter. This results in a linear plot of concentration vs diameter squared, which we observed over nearly the entire range assayed, although slightly smaller than predicted diameters were seen at high β₂-microglobulin concentrations. Plotting the standard curve with the diameter instead of square of the diameter (not shown) produced significantly less linear curves, particularly at large diameters. Although it is slightly easier to plot the diameter rather than diameter squared, we believe that the greater accuracy of the diameter-squared plots justifies their use.

The RID test has several advantages over RIA methods for measuring serum β₂-microglobulin, including substantial savings in reagent costs and technician time. For assaying a single sample with all required controls and standards, current reagent cost for the Pharmacia RIA is $50, in contrast to $1 for the RID method. Furthermore, the 125I RID kits have a shelf life of only four months, so that the cost of expired kits could drive the price higher. The RID requires approximately 90 min of labor, as opposed to 30 min for the RID method. In addition, a gamma-radiation counter is required for the RIA.

There are two disadvantages, however, to the RID method (using staining with Coomassie Blue) relative to the RIA. The RID method requires 30 h to complete (including an overnight incubation), compared with 4.5 h for the RIA. The RIA is more sensitive and is accurate for a wider range of values. It can detect as little as 0.01 mg/L or, as the Pharmacia urine test is usually run, 0.05 mg/L. This is more than sufficient for β₂-microglobulin analysis of urine, which is a useful marker of renal tubular function (4); however, β₂-microglobulin is quite unstable in acidic urine (5), and the RIA and RID methods would both be unreliable if precautions are not taken to minimize urine acidity.

The modified RID method, with use of lower antibody concentrations and silver staining, although not thoroughly tested in this study, is sufficiently sensitive to detect normal concentrations of β₂-microglobulin in urine. However, silver staining is relatively time consuming and more costly, and urine concentrations of β₂-microglobulin are infrequently ordered at our institution.

In summary, the RID method is a slow but inexpensive, simple, and reliable test for β₂-microglobulin in serum and cerebrospinal fluid. A modification of this procedure appears to be adequate for determining β₂-microglobulin in urine, although further testing of the procedure is urged before clinical use.

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References
Simple Spectrophotometric Quantification of Urinary Excretion of Glycosaminoglycan Sulfates

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We describe a simple, rapid, precise, and sensitive spectrophotometric method for measuring urinary glycosaminoglycan (GAG) sulfate excretion. The GAG sulfates are precipitated with cetylpyridinium chloride, resuspended in water, and mixed with the basic dye 1,9-dimethylmethylene blue to produce a complex with the polyanionic molecule of sulfated 3AGs. Absorbance is read at 535 nm. The standard curve for reaction was linear up to 12 μg of the different GAGs: dermatan sulfate, heparan sulfate, keratan sulfate, chondroitin 4-sulfate, and chondroitin 6-sulfate. Within- and between-assay precision (CV), measured at three different GAG concentrations (normal and pathological), varied from 1.6% to 2.5% and from 1.8% to 4.5%, respectively. Analytical recovery ranged from 71% to 107%. Urinary GAG excretion, measured by this procedure, correlates (r = 0.837; p < 0.001) with the values obtained with the borate–carbazole reaction. (Anal Biochem 1962;4:330–4).

Additional Keyphrases: 1,9-dimethylmethylene blue · mucopolysaccharidosis · heritable disorders · enzyme deficiencies · Morquio’s syndrome · reference interval

The mucopolysaccharidoses result from deficiency of specific lysosomal enzymes involved in the degradative pathway of the sulfated glycosaminoglycans (GAGs): dermatan sulfate, heparan sulfate, and keratan sulfate (1). The clinical diagnosis is first confirmed by the finding of an excessive urinary GAG excretion, and is definitively established by demonstrating the lack of a specific hydrolase.

Quantitative determinations of urinary GAGs are commonly performed by measuring the hexuronic acid residues of GAG molecules with the borate–carbazole procedure of Bitter and Muir (2). This method, however, does not detect the keratan sulfatulia of Morquio’s syndrome (mucopolysaccharidosis IV), the hexuronic acid residues being replaced with galactose in keratan sulfate. Furthermore, the assay is potentially dangerous, owing to the use of concentrated sulfuric acid.

Other procedures involve the use of basic metachromatic dyes, that complex with the polyanionic sulfated GAGs, giving rise to a shift of the absorption spectrum of the dye. Whiteman (3) developed a test by which urinary GAGs are precipitated when complexed with Alcian blue; the dye eluted from the precipitate is then measured colorimetrically. Gold (4) reported a simplified technique for determining total urinary GAG content by mixing untreated urine samples with freshly prepared Alcian Blue solutions.

To the best of our knowledge, there has been no report as to the clinical usefulness of either of these methods. Whiteman’s procedure, though reportedly able to precipitate keratan sulfate, yields finely dispersed precipitates that are often difficult to harvest (5). Gold’s method is subject to interference by negatively charged molecules other than sulfated GAGs, because of the absence of isolation steps during the assay.

The basic dye 1,9-dimethylmethylene blue (DMB), first studied by Taylor and Jeffree (6), has been used to assay sulfated GAGs in cartilage tissue cultures (7). We have adapted this procedure for the quantitative determination of total sulfated GAGs in urinary samples, as a diagnostic step in the laboratory assessment of mucopolysaccharidoses.

Materials and Methods

Apparatus

We used a Coleman 55 spectrophotometer (Perkin-Elmer Corp., Norwalk, CT 06856), a desktop centrifuge equipped