Studies of Bence Jones Proteins by Immunonephelometry*†

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ABSTRACT

Plasma cell dyscrasia is a disease caused by a monoclonal population of plasma cells. Most often the workup for this disease is prompted by the appearance of a paraprotein in serum, but a significant number of cases exhibit only a Bence Jones protein. Immunofixation electrophoresis is the most sensitive method presently used for identifying Bence Jones proteinuria. This methodology is laborious, expensive, and difficult to interpret. As a result, quantitative immunochemical methodologies have been suggested for measuring Bence Jones proteinuria.

In this manuscript, it is demonstrated by rate nephelometry that: (1) although serum polyclonal immunoglobulins are accurately measured as compared to the calibration material, serum monoclonal immunoglobulins are not; (2) measurement of immunoglobulins in the urine of patients with generalized proteinuria is biased towards an increased number of light chains or a decreased number of heavy chains; and (3) Bence Jones proteins react with the assay antibodies differently from the calibration material and from one another.

It is concluded that, although there is a qualitative relationship between concentrations of Bence Jones proteins and immunoglobulins used for calibration, measurement of absolute levels of Bence Jones proteins using currently available methods will lead to inaccuracies resulting from peculiarities between the antibody-antigen reaction and because of the spill-over of polyclonal free light chains into urine. Nevertheless, the data provides credence for studies suggesting that measurement of the k/A ratio in serum and urine may be a reliable way to identify Bence Jones proteins by automated assays.

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Introduction

Plasma cell dyscrasia is a process whereby a monoclonal population of plasma cells either derived from a single malignant clone or from a nonprolifera-
tive population causes disease. Classically, this is manifested as multiple myeloma, a malignant process, which may exhibit lytic bone lesions, bone marrow suppression, hypercalcemia, renal failure, and recurrent infections. Multiple myeloma is characterized by >10 percent plasma cells in bone marrow in the presence of other evidence, such as significant monoclonal component in serum, lytic bone lesions with hypogammaglobulinemia, greater than one g of free light chain in urine (Bence Jones protein) per 24 hours, or more than 30 percent plasma cells in the absence of a monoclonal immunoglobulin.

Alternatively, plasma cell dyscrasia is manifested as primary amyloidosis (AL) or light chain deposition disease (LCDD), in which a deposition of free light chains in the form of fibrils without apparent plasma cell proliferation causes disease in heart, gastrointestinal tract, tongue, nerves, skin, and kidney. In the absence of myeloma, the median plasma cell level in patients with AL is about five percent, with only about 30 percent showing a positive diagnosis upon bone marrow examination. Biopsy of other tissues may yield positive findings of amyloidosis in up to 76 percent of patients. One quarter of the patients with amyloidosis have myeloma. In the absence of myeloma, plasma cell dyscrasia is very difficult to detect early. No available radiologic or blood test will unequivocally establish the diagnosis. Although not as well studied, LCDD appears to be equally difficult to identify.

Early diagnosis of these diseases is important for prevention and control of complications, and for treatment. An increased number of plasma cells in the bone marrow or a tissue biopsy are the major diagnostic criteria for diagnosis. Yet, because these are invasive procedures, unless a monoclonal immunoglobulin has been identified, they are often not attempted until late in the disease.

Although in most myeloma patients, additional workup is prompted by the observation of a significant spike representing an intact immunoglobulin upon serum protein electrophoresis (SPE), about 20 percent of patients exhibit only a free light chain in urine. In AL, and presumably LCDD, about which less is known, a monoclonal spike in serum is seen infrequently, and is often small (<10 g per L), with a still larger number of patients exhibiting only a free light chain in urine. As a result, the identification of a Bence Jones protein is an important ingredient for the early diagnosis and treatment of these diseases.

Bence Jones proteins are preferentially identified in urine, where they are concentrated, rather than serum. Immunofixation electrophoresis (IFE) is presently the most sensitive method commonly used in clinical laboratories to identify monoclonal immunoglobulins. Although IFE is very sensitive when the antigen and antibody are in the correct ratio, as is usually the case when analyzing serum, when analyzing urine samples, false negative results may occur when the antigen or antibody is in excess, and the patterns may be difficult to interpret.

As a result of these difficulties, highly sensitive alternative immunochemical capture techniques for measuring concentrations of immunoglobulins, such as radioimmunoassay (RIA) and enzyme linked immunosorbent assay (ELISA), have been suggested for replacing IFE. These techniques are presently beyond the reach of most clinical laboratories. Automated immunonephelometry is the immunochemical technique most commonly used in clinical laboratories for identifying monoclonal immunoglobulins in serum. Studies indicate that, when used in conjunction with SPE, measure-
ment of the κ/λ ratio in serum can reduce the need for IFE in nearly 90 percent of cases. Yet, this technique has not been widely applied in clinical laboratories for identifying Bence Jones proteins in urine.

Measurement of the κ/λ ratio in conjunction with other proteins in urine by immunonephelometry has been used as a screen to reduce the need for IFE. In our laboratory, it was found that a comparison of the κ/λ ratios measured by immunonephelometry from urine and serum (κ/λ index) was specific for discriminating between patients with proteinuria without Bence Jones proteins and patients with Bence Jones proteins. In order to lay a foundation for this approach, the performance of a widely used immunonephelometric system for measuring light chains in urine was studied. This was accomplished by comparing the sums of the heavy and light chains from selected samples with one another and by parallelism studies. The results of these studies are presented which indicate that antigen–antibody peculiarities cause inaccuracies in the quantitative measurement of absolute levels of immunoglobulins in urine.

Based on the present and other studies, problems associated with the use of IFE, immunonephelometry and other immunochromemical techniques for identifying Bence Jones proteins are reviewed. The evidence indicates that, although attempts to identify Bence Jones proteins by measuring absolute levels of free light chains with currently available systems will lead to inaccurate results, the identification of Bence Jones proteins by measuring κ/λ ratios, which are relative relationships, may be reliable.

Materials and Method

Serum and urine samples were obtained in the usual way without the use of preservatives. All urine samples were 24 hour specimens. Specimens were kept either refrigerated and assayed within one week, or frozen at −70°C and thawed just before being assayed. Total protein in urine was assayed using a precipitation method with benzethonium chloride.*

Prior to protein electrophoresis and IFE, urine samples were concentrated approximately 80 to 100 fold using minicon-B15 concentrators.† Protein electrophoresis was performed with universal II Agarose Film/12, Ciba Corning Protein System‡ according to the kit directions. Staining was with amido black. Densitometry was performed with an edc densitometer.§ Immunofixation electrophoresis on serum was performed using the Titan Gel Immunofix procedure.§ Antisera supplied with the kit are specific for IgG, IgA, IgM, free and bound kappa and lambda chains. Immunofixation electrophoresis on urine samples was performed in the same way, except antisera for free lambda, and kappa light chain (catalog #9413 and 9412)$ was substituted for the free and bound.

Kappa and lambda chains were measured by rate immunonephelometry using the Array according to the instructions with the IgG, IgA, IgM, and the kappa and lambda light chain kits. The antisera reacts with both free and bound light chains. The serum and urine samples were assayed without being concentrated.

In order to compare the relationship between the sums of the heavy and light chains in the urines of patients with generalized proteinuria with low concentrations of intact immunoglobulins, two

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sera were diluted between 1:16 and 1:128 with urine. One of these was diluted with a hypertonic urine (800 mOsm per L) and the other with a hypotonic urine (241 mOsm per L). For comparison purposes, a normal serum was diluted into the same low range with 0.05 mol per L phosphate buffered saline, pH adjusted to 7.4 ± 0.1 (PBS).

In order to study the relationships between Bence Jones proteins and intact immunoglobulins with dilution, three urine samples from patients with lambda Bence Jones proteinuria and serum from a patient with a polyclonal gammopathy were diluted with PBS. The undiluted urine samples contained Bence Jones protein, according to the densitometric scan, in amounts of 82 percent, 71 percent, and 60 percent of the total protein. An additional normal urine, without proteinuria, was augmented with immunoglobulin by adding γ-globulins (Human:Cohn Fraction II, III, Lot 106F9315), and the solution was diluted with PBS.

Statistics were calculated using the software Statview SE plus graphics** on a Macintosh.

Results

In figure 1 is illustrated the relationship between the sum of the light chains and the sum of the heavy chains in serum from persons with kidney disease and generalized proteinuria but without a monoclonal immunoglobulin. For 19 samples, a great degree of correspondence between the concentrations of IgG + IgA + IgM and that of the kappa + lambda chains is seen (r^2 = 0.98), with a regression line (slope 1.012 and intercept −0.82) nearly identical to the ideal line (slope of 1.0 and intercept of 0). This indicates that the calibration curves have been set for the sum of the light chains and the sum of the heavy chains to be equal on a mass per volume basis when polyclonal intact immunoglobulins in a serum base, which are similar to the calibration material, are assayed.

In figure 2 is shown the relationship between the sum of the light chains and the sum of the heavy chains (thin solid line) when immunoglobulins were measured in the urines of the same 19 patients indicated previously, with generalized proteinuria but without a monoclonal immunoglobulin. Although the regression line (slope = 0.84 with 95 percent confidence limits of 0.58 to 1.1), is somewhat parallel to the ideal line (thick solid line), it is apparent that there is a constant bias from ideal correspondence indicated by the y-intercept of 0.34 g per L. Furthermore, there is a large

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Figure 2. Sum of the light chains from the urine of patients with generalized proteinuria without Bence Jones proteins. Line derived from the urines of patients is the thin solid line (■): \(y = 0.863x + 0.34, \ r = 0.844, \ n = 19, \ SEM = 0.22, \ p = 0.001.\) The thick solid line is the ideal line. The dotted line (○) is derived from diluting serum immunoglobulins with PBS: \(y = 0.99x + 0.02, \ r = 0.998, \ n = 5.\) The dashed lines were derived from diluting intact immunoglobulins with urine: (□), line given by: \(y = 0.97x - 0.03, \ r = 0.998, \ n = 5\) (urine = 241 mOsm/L), and (△), line given by: \(y = 1.0x - 0.05, \ r = 0.996, \ n = 5\) (urine = 800 mOsm/L).

The degree of scatter about the regression line, indicating much poorer correspondence \(r^2 = 0.71\) than seen with the sera of the same patients (figure 1).

Three mechanisms could be postulated to be responsible for this bias, the first two are directly related to the low concentrations of immunoglobulins in these urine samples, and, hence, the need for smaller sample dilutions (1:36 to neat) when assaying urine as compared to greater dilutions when assaying serum (>1:36). These mechanisms are: (1) The assay may perform poorly in the low range for analytical reasons. (2) The concentrated matrix of the minimally diluted urine samples may effect the assay. (3) Samples from patients with renal disease have an increased spill over of oligoclonal free kappa and lambda light chains and this may be responsible for the discrepancy from ideal.

These possibilities were tested by diluting serum samples with urine and with phosphate-buffered saline (PBS) into the same assay range as used for testing the urines of the patients. The results of these experiments are illustrated in figure 2. The line generated by diluting normal serum with PBS (dotted line given by triangles) is nearly identical to the ideal line indicating that the discrepancy was not due to poor performance of the assay. The dashed lines reflects the results of diluting samples with urine. These lines are also very similar to the ideal line, indicating that a matrix effect was not responsible for the bias. Thus, it appears that the best explanation for the discrepancy is increased spill over of oligoclonal light chains into the urine of these patients, a phenomenon that has been well documented \(11,21,24,25,27\).

In figure 3 is illustrated the relationship between the sum of the light chains and the sum of the heavy chains in serum from persons with an M-component and Bence Jones proteinuria. Because some of these patients may have an excess of light chain, it would be expected that the concentration of light chains would be equal to or greater than the concentration of IgG + IgA + IgM. Yet, some of the sera show a kappa + lambda concentration that is less than the IgG + IgA + IgM concentration (Solid circles). This finding indicates inaccuracy in the measurement of at least three of 17 samples (17.6 percent), significantly different from an expected frequency of 17 (\(p = 0.02, \ Chi^2\) with correction = 6.6). This effect appears to be due to peculiarity of the antibody–antigen reaction.

In figure 4 is demonstrated the relationship between dilution and measured concentration of lambda chain from poly-
clonal intact immunoglobulins in serum which are similar to the calibrator, polyclonal immunoglobulins in urine (augmented with intact serum immunoglobulins), and lambda Bence Jones proteins in urine. It can be seen that the lines derived from measuring intact immunoglobulins from urine and serum (dotted lines) are highly linear (linear correlation, $r > 0.99$) with dilution, indicating good parallelism with the calibration line. On the other hand, although the Bence Jones proteins show a degree of linearity ($r$ between 0.9–0.94), the lines derived from measuring Bence Jones proteins are not sufficiently linear for quantitative measurement. Furthermore, the curves are not parallel with the serum calibration line nor with one another. These results indicate that while polyclonal intact immunoglobulins in serum and urine are reacting with the antibody in a manner that is similar to the calibrator, the Bence Jones proteins are reacting with the antibody in a manner that is quantitatively different from both the calibrator and from one another. Similar non-linear behavior was found when kappa Bence Jones protein was compared with polyclonal serum immunoglobulins (not shown).

BENCE JONES PROTEINS

**FIGURE 3.** Sum of the light chains from the serum of patients with Bence Jones proteinuria compared with the sum of the heavy chains. Solid line is the linear regression line: $y = 0.89x + 5.33$, $r = 0.78$, $n = 17$, SEM = 19.8, $p = 0.0002$. Dotted line indicates ideal line with a one to one correspondence. $\bullet$, indicates points that are underestimated.

**FIGURE 4.** Measurement with dilution of light chain concentrations in endogenous lambda Bence Jones proteins from urine. The points represent the means of duplicate measurements. The lines were optimized to generate a correlation coefficient of >0.99 (○), a urine, which undiluted was estimated to have 1.5 g per L of Bence Jones protein (82% of total), solid polynomial curve: $y = 5 - 18x + 29x^2 - 10x^3$, $r^p = 0.999$, $n = 10$, (the linear correlation coefficient ($r$) was $r = 0.94$); (□), a urine, which undiluted was estimated to have 0.43 g per L of Bence Jones protein (72 percent of total), solid polynomial curve: $y = 0.2 + 9.0x + 33x^2 - 31x^3$, $r^p = 0.99$, $n = 10$, ($r = 0.91$); (△), a urine, which undiluted was estimated to have 0.720 g per L of Bence Jones protein (60 percent of total), solid polynomial curve: $y = 2 - 17x + 112x^2 - 84x^3$, $r^p = 0.997$, $n = 10$, ($r = 0.9$). For comparison purposes, two lines generated from intact serum immunoglobulins (dotted lines), diluted with PBS are shown: (●), urine augmented with intact immunoglobulin: straight line, $y = 14.5x + 0.21$, $r = 0.993$, $n = 5$, and (○), serum diluted with PBS: straight line, $y = 19.9x - 0.34$, $r = 0.998$, $n = 5$. The correlation coefficients for the polynomials are represented by $r^p$. 
Identification of a free light chain in urine is often important for obtaining early and correct diagnosis of plasma cell dyscrasias. It also has prognostic value because, in myeloma, Bence Jones proteins tend to be associated with more aggressive disease. Because of its tedious nature, most laboratories screen samples prior to IFE by urine protein electrophoresis (UPE) which is self complicated, requiring a concentrating step. Immunofixation electrophoresis magnifies small bands as compared to UPE, as a result samples with small monoclonal components may be misclassified after the screen without undergoing IFE. Screening with UPE is particularly perilous when there is generalized proteinuria because Bence Jones proteins may be masked by the other proteins. Furthermore, plasma cell dyscrasias are commonly associated with kidney disease and generalized proteinuria.

Besides being tedious and expensive to perform, IFE itself is associated with a number of problems related to interpretation. Bence Jones proteins in urine often migrate anomalously as multiple bands or appear as slurred patterns with the bands running together. Among other reasons, such peculiar patterns may occur because: (1) kappa and lambda light chains may occur as monomers or aggregates in various degrees of association, (2) degradation leading to fragments may occur owing to storage or biological modification, and (3) other urinary proteins may effect the migration causing anomalous patterns. To avoid these anomalous patterns, it has been suggested that the SPE be immunoblotted and that the blots be treated with mercaptoethanol and dodecyl sulfate prior to incubation with the antiserum. This is beyond the capacity of most clinical laboratories. Studies indicate that polyclonal kappa and lambda free light chains are preferentially excreted in the urine of patients with renal tubular damage as compared to normal, and that upon IFE these migrate as oligoclonal bands which may be confused with monoclonal Bence Jones proteins.

Because of these difficulties, quantification of immunoglobulins in urine by immunoassay methods has been suggested as a potential alternative to IFE. One potential roadblock to this approach is the assumption that peculiarities of the abnormal free light chain antigens and the antibody cause inaccurate measurement of Bence Jones proteins. In fact, this supposition has not been well documented in the literature. Studies using western blotting and ELISA indicate that anti-light chain antibodies react with normal light chains and monoclonal light chains with complete specificity. Immunonephelometry is the most common technique used for quantitating immunoglobulins in clinical laboratories. Studies indicate that this technique is substantially unaffected by the molecular size or other physicochemical properties of pathological immunoglobulins in serum, and, using immunonephelometry, the difference between the sums of the heavy and light chains has purportedly been used to identify paraproteins.

Reportedly, in cases of pure Bence Jones proteinuria, the amount of immunoglobulin light chain determined by immunonephelometry is in good agreement with the chemically determined total protein in urine. Furthermore, although it was shown that antibodies against free light chains react preferentially with some polymeric forms of free kappa chains, antibodies against free and bound kappa chains and antibodies against lambda reacted in a similar way with all forms.

Here, nephelometry was examined for measuring monoclonal and normal immunoglobulins in serum and urine.
Examination of the relationship between the sums of the heavy and light chains indicated that polyclonal serum immunoglobulins are accurately measured as compared to the calibration material (figure 1). Based on these data, if monoclonal immunoglobulins reacted similarly, the concentration of free light chains could be determined by subtracting the sums of the heavy and light chains, with the excess being the free. Alternatively, the light chains could be measured by interpolation from the light chain calibration curve.

Unfortunately, when monoclonal immunoglobulins in serum were measured, it was clear these relationships were not valid (figure 3). Furthermore, when urine was tested, it was found that the sums of the polyclonal immunoglobulins were biased towards an increase in light chains or a decrease in heavy chains as compared to measurement in serum (figure 2). This behavior did not appear to be due to matrix effects or poor performance of the assay in the low range, and is consistent with findings that showed there is an increased secretion of polyclonal light chains in patients with renal disease,11,21,24,25 which seems to be the best explanation. Finally, it was shown that, while polyclonal intact immunoglobulins in serum and urine reacted with the antibody in a fashion that was quantitatively similar to the calibrator, Bence Jones proteins reacted in a way that was quantitatively different from both the calibrator and from one another (figure 4). It is clear that Bence Jones proteins cannot be accurately measured.

Nevertheless, the Bence Jones proteins did exhibit qualitative, linear behavior relative to the calibrator ($r = 0.9$ to 0.94). This qualitative relationship may explain why other investigators concluded that Bence Jones proteins react with normal light chain antibodies with a large degree of specificity.13,19,26 Although, the relationships were not sufficiently linear for accurate quantitative measurement, our data support use of the $\kappa/\lambda$ ratio as a qualitative indication of increased kappa or lambda chains for circumventing problems related to absolute standards. This ratio has been widely used in clinical laboratories in conjunction with SPE for typing paraproteins,16,30 but it has not been widely applied for testing with urine.

Boege and associates showed that a screen consisting of the measurement of albumin, transferrin, $\alpha_1$-microglobulin, and IgG in urine in conjunction with the $\kappa/\lambda$ ratio could reduce the need for IFE, by assuming that samples with $\kappa/\lambda$ ratios between 1.0 and 5.2 do not contain a Bence Jones protein.24 Data from our laboratory suggest that the $\kappa/\lambda$ ratio in serum compared with the $\kappa/\lambda$ ratio in urine ($\kappa/\lambda$ Index) provides a more definitive estimate of the presence or absence of Bence Jones protein.20 The $\kappa/\lambda$ index is calculated by dividing by one another the $\kappa/\lambda$ ratios from serum and urine from a patient with the lower ratio being the divisor and the higher ratio being the numerator to give a quotient in multiples of the lower ratio.20 Seven patients with generalized proteinuria and Bence-Jones proteinuria showed a $\kappa/\lambda$ Index that was many multiples greater than the respective serum, while the serum and urine from seven reference samples with generalized proteinuria but without Bence Jones proteins exhibited $\kappa/\lambda$ Indexes of less than two. These results could have happened by chance less than one time in 100 times. To date, the $\kappa/\lambda$ index has been determined by us on 12 patients with Bence Jones proteinuria and 20 with generalized proteinuria, respectively, and successfully discriminated between the two groups in all 32 cases.

The difficulty with the rate nephelometric technique is that the lower limit of detection is 50 mg per L for lambda
chains and 18.5 mg per L for kappa chains, which is substantially above the 1.8 to 0.75 mg per L that has been reported for free light chains in normal urine.\textsuperscript{2,25} As a result, use of the present technique is limited to urine samples containing elevated levels of both kappa and lambda chains, usually samples from patients with generalized proteinuria.\textsuperscript{10,11,21} Nevertheless, this index offers the potential to be a definitive prognosticator for patients with generalized proteinuria.\textsuperscript{10,11,21}

Sensitive immunoassays for measuring kappa and lambda components have been described using competitive protein binding and two site immunometric assays.\textsuperscript{4,24,25,27} It might be possible to eliminate peculiar antibody–antigen effects of Bence Jones proteins using these sensitive assays with monoclonal antibodies directed against common light chain loci along with free light chain calibrators, but these assays would presumably suffer from the same peculiarities of Bence Jones proteins as the immunonephelometric assay if polyclonal antibodies are used for measuring the absolute concentrations of free light chains. Besides, because of the increased spill over of oligoclonal free light chains associated with renal tubular damage,\textsuperscript{11,21,24,25} very sensitive assays would be prone towards giving false positive absolute results in patients with renal disease.

A mean $\kappa/\lambda$ ratio has been found by us from the serum of 19 patients with generalized proteinuria without Bence Jones proteinuria of 2.1 ± 0.5 (standard deviation) and a mean from urine of 2.4 ± 0.8 which is similar to that of 2.4 to 2.5 for normal persons assayed by enzyme- and radioimmuno-assay.\textsuperscript{2,23,25} The $\kappa/\lambda$ ratio in urine is only slightly increased over that of serum because the spill over of both kappa and lambda chain appear to be increased.\textsuperscript{11,21,24,25,27} Thus, the $\kappa/\lambda$ index, described here, used with automated non isotopic immunoassays could potentially reduce the need for IFE in clinical laboratories.

References


