Simple Method for Quantification of Bence Jones Proteins

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Background: Quantification of free monoclonal light chains in urine [Bence Jones proteins (BJPs)] is used to diagnose multiple myeloma and to evaluate response to treatment. We have developed and evaluated an optimized approach for quantification of BJPs.

Methods: High-resolution gel electrophoresis of unconcentrated urine and albumin calibrators was carried out on Sebia’s Hydrasys instrument with Hydragel HR agarose gels. After staining with acid violet, the gels were scanned densitometrically. The staining intensities of BJP bands relative to the staining intensities of albumin solutions were used to determine the BJP concentrations. Results for patient samples were compared with conventional agarose gel electrophoresis on concentrated samples.

Results: The relationships between staining intensity and the protein concentrations of albumin and BJPs were linear up to protein concentrations of ~2000 mg/L. The detection limit was ~20 mg/L. The interassay imprecision (CV) was ~8% (n = 23, duplicate analysis), and the results (y) showed a close positive relationship to the comparison method: slope = 0.82 (confidence interval, 0.75-0.88); y-intercept = 34 (−14 to 81) mg/L; n = 29; r² = 0.96.

Conclusions: Agarose gel electrophoresis of unconcentrated urine samples together with a series of albumin calibrators followed by acid violet staining and densitometric scanning is sufficiently reproducible and sensitive to quantify clinically relevant BJPs.

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Approximately 50% of patients with multiple myeloma excrete monoclonal free immunoglobulin light chains [Bence Jones proteins (BJPs)]3 in the urine (1). The most sensitive method to detect BJPs is immunofixation electrophoresis (IFE) of concentrated urine (2), but it provides only qualitative results. Quantification of BJPs provides important information as it is strongly correlated with myeloma tumor burden (3). Accordingly, the rate of BJP excretion has been incorporated in several clinical scores of multiple myeloma, e.g., the classic Myeloma Staging System (4), and more recently, in the criteria for disease response and progression (5). Thus the clinical use of BJP quantification in diagnosis, staging, and treatment monitoring, including the documentation of complete remission, demand analytical methods that are specific, accurate, and sensitive.

Several methods are used for quantification of BJPs, the most common of which is agarose gel electrophoresis of concentrated urine followed by densitometry (in the following referred to as “comparison method”); the BJP concentration is calculated by multiplication of the fraction of the total urinary protein in the BJP band by the total urinary protein concentration. This method has two well-known problems. The first problem is that proteins may be lost during the concentration procedure, depending on their size and charge (6). As a result, the relative amount of a BJP compared with other urinary proteins may be altered by the concentration procedure. The second problem is that laboratories use different methods (e.g., precipitation methods, protein binding dyes, or Biuret) to quantify total urine protein concentrations. The ability of most total urine protein quantification methods to accurately measure light chains is uncertain (7). Accordingly, even when the same method is used to concentrate the proteins, the measured BJP concentrations can vary considerably depending on which method is used for quantification of total proteins in the urine. This variation is illustrated by the results of analyses of four different BJP-containing samples by ~170 different laboratories in the United Kingdom External Quality Assessment program. These analyses produced CVs in BJP

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1 Nonstandard abbreviations: BJP, Bence Jones protein; IFE, immunofixation electrophoresis; PBS, phosphate-buffered saline; HRAGE, high-resolution agarose gel electrophoresis; and AUC, area under the curve.
measurements of 30%, 65%, 68%, and 76%, respectively (United Kingdom National External Quality Assessment distributed the samples in January, March, May, and June 2001, respectively).

In this study we developed and evaluated a simple method for quantification of BJPs designed to eliminate these pitfalls.

**Materials and Methods**

**SAMPLES**

Aliquots of 24-h urine collections from 29 patients attending the myeloma clinic of Rigshospitalet (Copenhagen) were selected for the presence of BJPs. Because the BJP concentration was measured as part of our usual clinical evaluation, we did not seek approval from the local ethics committee system. One part of the aliquot was used for measurement of BJPs in concentrated urine by a comparison method used by another laboratory. Another part of the aliquot was centrifuged and stored at −20 °C for a maximum of 6 month until used for BJP quantification in unconcentrated urine. Additional urine samples with variable background protein content or osmolality were randomly acquired from the routine laboratory. Human albumin (200 g/L; human plasma albumin; SS), bovine serum albumin (2000 mg/L; Pierce or Sigma), and >96% pure monoclonal κ and λ light chains, isolated from BJP urine by SCIPAC were diluted in phosphate-buffered saline (PBS; 8 g/L NaCl, 0.2 g/L KCl, 1.44 g/L Na2HPO4, 0.2 g/L KH2PO4, pH 7.5) and stored at 4 °C. Concentrations of the dilution series were adjusted by measurement on a Hitachi 917 automated analyzer [turbidimetric quantification of benzethonium chloride-precipitated proteins (Roche A/S)].

**QUANTIFICATION OF BJP IN UNCONCENTRATED URINE**

High-resolution agarose gel electrophoresis (HRAGE) was carried out on the semiautomated Hydrasys instrument (SEBIA) with Hydragel 15 HR electrophoresis gels (SEBIA) according to the manufacturer’s instructions. Unconcentrated urine (10 μL) was loaded on the applicator, which was placed in the instrument together with buffer strips and a gel plate. After electrophoresis (Program 15 HR 3 for 30 min), the gel was removed and stained with acid violet (SEBIA) in the gel-processing module of the instrument. Stained gels were scanned at 570 nm with a Hyrys Densitometer (SEBIA), and the area under the curve (AUC) corresponding to the BJP or albumin peak was determined in arbitrary units by the software of the densitometer. Analysis of a series of human albumin dilutions was used to generate a calibration curve of AUC vs protein concentration. The AUC of a BJP band was converted into protein concentration by use of the albumin calibration curve. Of note, bovine albumin yielded ~20% higher dye uptake than human albumin and correspondingly lower BJP concentration estimates.

**QUANTIFICATION OF BJP IN CONCENTRATED URINE WITH A COMPARISON METHOD**

BJP was also measured by Nova Medical Medi-Lab A/S (Copenhagen, Denmark) according to a widely used protocol. Briefly, urine specimens were concentrated ~100-fold with a Minicon-B concentrator (Amicon Corp.), subjected to standard agarose gel electrophoresis (SPE-agarose gel on a Paragon electrophoresis instrument; Beckman Coulter), and stained with the Paragon Blue stain (Beckman). Gels were scanned densitometrically (Appraise Densitometer; Beckman) to assess the BJP fraction of the total urine proteins. The BJP concentration was calculated by multiplication of the BJP fraction by the total urine protein concentration. The concentration of total urine proteins was measured at the Department of Clinical Biochemistry, Rigshospitalet, on a Hitachi 917 automated analyzer (Roche) as described above.

IFE

Electrophoresis and immunofixation were done on the Hydrasys instrument with IFE gels according to the manufacturer’s instructions and with specific antisera against free λ or free κ light chains (SEBIA).

**Results**

**PROTEIN QUANTIFICATION BY DENSITOMETRIC SCANNING OF HRAGE GELS**

Dilution series of purified albumin were subjected to HRAGE on Sebia’s semiautomated electrophoresis instrument and stained with acid violet (Fig. 1A). The gels were scanned densitometrically (Fig. 1B), and the AUC corresponding to the albumin band was plotted as a function of the protein concentration (Fig. 1C). There was a close linear relationship between the AUC and the protein concentration up to ~2000 mg/L. We were always able to visibly detect and densitometrically quantify samples with an albumin concentration down to 20 mg/L (Fig. 1A). The between-gel imprecision (CV) for the AUCs for protein quantification by densitometric scanning of hrage gels was carried out on the semiautomated Hydrasys instrument (SEBIA) with Hydragel 15 HR electrophoresis gels (SEBIA) according to the manufacturer’s instructions and with specific antisera against free λ or free κ light chains (SEBIA).

To assess whether BJPs could likewise be detected and quantified by densitometric scanning of acid violet-stained HRAGE gels, we analyzed purified κ and λ chains (Fig. 2, A and B). The apparent protein concentration of the κ light chain solution was 921 ± 18 mg/L (mean ± SE; n = 7) as determined on the Hitachi 917 automated analyzer and 875 ± 43 mg/L (n = 4) as determined with HRAGE and acid violet staining using human albumin as the calibrator. The corresponding concentrations of the λ light chain solution were 533 ± 17 mg/L (n = 3) and 616 ± 43 mg/L (n = 3), respectively. The dilution curves of the κ and λ light chain solutions (Fig. 2, A and B) were similar to the albumin dilution curve (Fig. 1B).

The detection limit for the light chains on acid violet-stained gels was compared with that of immunofixation (Fig. 2C). Immunofixation detected both light chains...
down to a concentration of at least 10 mg/L, whereas the detection limits on acid violet-stained HRAGE gels were 20 mg/L for the κ chain and 10 mg/L for the λ chain. Urine osmolality (Fig. 3A) and urine total protein concentration (Fig. 3B) did not affect the measurement of BJPs with the HRAGE-based method.

**QUANTIFICATION OF BJP IN UNCONCENTRATED URINE BY DENSITOMETRIC SCANNING OF HRAGE GELS**

The presence of BJPs in 29 urine samples was initially ascertained by immunofixation. Unconcentrated urine was then subjected to HRAGE and acid violet staining (Fig. 4A). The AUC corresponding to a BJP was determined by densitometric scanning. The BJP concentration was determined with the AUCs of the albumin concentration calibration curve (Fig. 1C). This analysis was done twice for the 23 patient specimens (Fig. 4B). The between-day CV of these independent measurements was 8.5% for samples with a BJP concentration >100 mg/L (n = 13) and 7.0% for samples with a BJP concentration <100 mg/L (n = 10), indicating reproducible protein loading on the gels.

We compared the BJP concentrations obtained with HRAGE and acid violet staining, using on-gel human albumin calibrators with the BJP concentrations obtained with a comparison method, i.e., multiplication of the BJP fraction of the total urine proteins (determined by agarose gel electrophoresis of concentrated urine) by the total...
urine protein concentration (determined on the Hitachi 917 automated analyzer; Fig. 5A). There was a close correlation of results obtained with the two methods, but the HRAGE-based method yielded an ~18% lower BJP protein concentration than the comparison method. To explore whether this difference was attributable to a shift of the urine protein composition during the urine concentration step in the comparison method, we multiplied the BJP fraction obtained with the HRAGE-based method using unconcentrated urine by the total urine protein concentration and compared the results with those obtained with the comparison method (Fig. 5B). The results obtained with the two methods were similar, although the values based on the BJP fraction in unconcentrated urine tended to give higher BJP concentrations (Fig. 5B).

Discussion

We present a rapid, easy, and reliable method for quantification of BJPs. This method can quantify BJPs within a range of 20–2000 mg/L without concentration of the urine. Urine samples with a BJP concentration >2000 mg/L should be diluted in PBS. Importantly, the HRAGE-based method meets the requirements stated in clinical scoring systems. As examples, the criteria for the initial clinical staging of multiple myeloma patients require discrimination of <4 g or >12 g of BJP excretion per 24 h (4). The definitions of response, relapse, and progression of multiple myeloma use 200 mg/24 h as the cutoff limit for partial response and include relative changes of BJPs in other response definitions (5). Two major problems associated with other electrophoresis-based methods for quantification of BJPs are eliminated with the present method. For example, the need to concentrate urine specimens before analysis, which is not standardized for amount and method and possibly alters protein compo-
sition, is rendered superfluous. In addition, separate measurement of total urine protein concentration, which can vary considerably depending on the method used, is avoided. Instead we include serial dilutions of commercially available albumin calibrators in the electrophoresis and scanning procedure.

During the last decade, alternative methods for quantification of urinary monoclonal components have been developed. Nephelometric and turbidimetric immunoassays are commonly used, but previously, antibodies and calibrators were not optimized to detect the unique and monotypic antigenic determinants of the monoclonal component (8, 9). However, recently Bradwell et al. (10) have described an automated immunoassay for detection of free k and l light chains in serum and urine that solves these problems. On the basis of this assay, the measurement of immunoglobulin free light chains in serum not only allows the diagnosis of light chain myeloma, but might even become an alternative method to monitor disease activity in such patients (11). Capillary electrophoresis allows high sample throughput and provides a better sensitivity to detect monoclonal components in serum than does agarose gel electrophoresis (12, 13). Capillary electrophoresis could be useful to quantify BJPs, although, to the best of our knowledge, such an application has not been described. Sodium dodecyl sulfate–agarose gel electrophoresis of unconcentrated urine may be more sensitive than conventional nonreducing agarose gel electrophoresis of concentrated urine (14). A disadvantage of this method is that proteins are separated by their molecular weight. Thus, a BJP is not separated from polyclonal light chains, and the abundance of a BJP with a low concentration may be overestimated by this method.

With nonnaturating agarose gel electrophoresis, identification of the monoclonal band maybe problematic because not all BJPs are found as monomers, but in several other conformations (15). This may eventually give rise to ladder-like structures in the electropherogram. Because multiple myeloma often is associated with renal disease, additional proteins may be excreted in the urine. Comigration of such proteins with the BJP may hinder proper identification and quantification of the monoclonal peak. These problems have been discussed elsewhere (16). On the other hand, our results suggest that the currently applied HRAGE-based method has several features that render it suitable for routine evaluations of patients with BJPs. The first feature is that, on duplicate analysis, the results were highly reproducible (Fig. 4B). The second is that the AUC-vs-protein concentration dilution curves of k and l light chains and albumin were linear. The third feature is that the BJP concentration measurements were not affected by large variations in total urine protein concentration or urine osmolality (Fig. 3).

To assess the accuracy of the present HRAGE-based method, we compared it with a widely used method. The results obtained with the HRAGE-based method were ~18% lower than those obtained with the comparison method. There are several potential explanations for this difference. We assessed whether the difference was caused by selective loss of non-BJP proteins during the urine concentration step in the comparison method. When we calculated BJP concentrations from the BJP fractions of total urine proteins that were obtained either with the HRAGE-based method or with analysis of concentrated urine in the comparison method, the results were closely correlated. This suggests that the divergent results did not reflect loss of non-BJP proteins during the concentration step. Interestingly, this result also implies that HRAGE-based determination of the BJP fraction can replace the electrophoresis of concentrated urine in the comparison method, thus rendering the urine concentration procedure superfluous.

We also assessed whether the divergent results of the HRAGE-based and the comparison method could reflect a difference in dye uptake between the human albumin
isolated light chain solutions, as determined with the HRAGE-based method or an automated total urine protein quantification method, we found that the HRAGE-based method yielded a 5% lower concentration than the total urine protein analysis for the isolated $\kappa$ light chain solution and a 16% higher concentration for the isolated $\lambda$ light chain solution. This might reflect differences in dye uptake among different BJPs. However, it might also, at least to some extent, reflect problems in determining the total protein concentration in purified light chain solutions (e.g., because of light chain instability when resuspended in PBS) with the automated total urine protein analysis. Finally, it is possible that the different results obtained with the HRAGE-based method and the comparison method reflect an overestimation of the comparison method because of inaccuracy of total urine protein concentration determinations in the presence of BJPs. Indeed, the United Kingdom National External Quality Assessment program for measurement of BJPs found 31–53% variations among the total urine protein concentrations in the presence of a BJP with different methods. Nevertheless, because there is no BJP standard for BJP quantification available at this stage and because the HRAGE-based method with on-gel human albumin calibrators presumably would yield similar results in different laboratories, we suggest that the use of a human albumin calibrator is the best choice for routine BJP quantification.

Advancement in the treatment of multiple myeloma is often based on multicenter clinical studies. Therefore, method-dependent variations in quantification of M-components in plasma and BJPs in urine is a major challenge [Gimsing P, Tureson I, Carlsson J, Dahl I-MS, Forsberg P-O, Grubb A, et al. Implications of interlaboratory imprecision in quantification of M-components in plasma and urine for multicenter clinical studies of multiple myeloma. VI International Workshop on Multiple Myeloma, June 14–18, 1997, Boston, MA, Poster Session (poster no. 4-5)]. The presented method for quantification of BJPs could presumably meet this challenge because it uses commercially available and widely used materials and equipment and reduces potential artifacts that are associated with older methods. One would therefore expect a low interlaboratory variation, which is mandatory in multicenter trials, although this must be demonstrated in another study.

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