Quantitation of Serum Monoclonal Proteins: Relationship between Agarose Gel Electrophoresis and Immunonephelometry

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BACKGROUND: Previous comparisons of monoclonal protein quantification identified a bias between serum protein electrophoresis (SPEP) and immunonephelometry (NEPH).

METHODS: We reviewed data from 2845 patients in whom a single sample provided a y fraction M-spike by SPEP, a heavy chain isotype by immunofixation electrophoresis (IFE), and an Ig quantification by NEPH. We examined the relationship between SPEP and NEPH. Selected sera with high monoclonal protein concentrations were diluted and reassessed.

RESULTS: For all isotypes, the relationship between SPEP and NEPH was best fitted with cubic curves. We determined the concentrations of each isotype that fitted a linear relationship. IgA had the best correspondence (slope 0.92, 95% CI 0.87–1.02), whereas IgM demonstrated a systematic bias of higher values by NEPH (slope 1.80, 95% CI 1.68–1.92). IgG demonstrated a nonlinear relationship between SPEP and NEPH, with a linear region <19.2 g/L having a slope of 0.83 (95% CI 0.79–0.89) and a second linear region having a slope of 1.47 (95% CI 1.39–1.53) at higher concentrations. Dilutions of high-concentration IgG monoclonal proteins were linear by NEPH and nonlinear by SPEP.

CONCLUSIONS: There are systematic differences in the quantification of monoclonal IgM and IgG by SPEP and NEPH. The bias in IgM is from NEPH overestimation. The nonlinearity of SPEP at high monoclonal IgG concentrations may obscure changes in plasma cell populations. Clinicians should be made aware of the biases and nonlinearity in these tests to make proper conclusions regarding treatment response.

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The identification of a monoclonal gammopathy depends on serum and urine protein electrophoresis and immunoelectrophoresis (IFE) and/or quantitative free light chain assays. Once a monoclonal gammopathy has been identified and a particular plasma cell proliferative disorder has been diagnosed, the quantification of the monoclonal protein is a good surrogate marker for monitoring the population size of clonal plasma cells. The recommendation has been to use the serum or urine M-spike if it is available (1–3). This recommendation is based on the serum M-spike fractionation being more specific for the monoclonal protein than Ig quantification, which includes polyclonal as well as monoclonal immunoglobulins. This specificity is especially apparent with low-concentration monoclonal proteins in the presence of polyclonal immunoglobulins. Many physicians, however, use Ig quantification to monitor monoclonal proteins, and some use both the M-spike and Ig quantification to monitor treatment.

The quantification of monoclonal proteins by serum protein electrophoresis (SPEP) relies on separating the proteins by electrophoresis, staining the gel with a protein dye, scanning the gel to determine the percent of protein in the various fractions, and determining the serum total protein concentration by a separate assay to convert the percentage to a concentration. The quantification of immunoglobulins by immunonephelometry (NEPH) depends on the light scatter generated by the binding of heavy chain–specific antisera. The results of these 2 assays do not always agree. Some of these differences may be due to protein stains and immune reagents reacting differently to specific monoclonal protein amino acid sequences, and would therefore result in clone-specific variation in staining and antibody binding. There have been some observations, however, of systematic differences in the quantification of monoclonal proteins by...
the 2 methods (4–7). Sinclair et al. (6) suggested that at low concentrations of monoclonal proteins, SPEP overestimates concentrations but is reliable at high concentrations. They also noted that NEPH overestimates IgM at high concentrations. Riches et al. (4) reported that immunonephelometry overestimates all 3 isotypes and that IgM was the most divergent. In addition to these studies, comparisons of agarose gel electrophoresis and capillary zone electrophoresis have reported divergence of M-spike quantification at high IgG values (8, 9). This has been interpreted as saturation of staining of the agarose gel, with capillary electrophoresis therefore yielding higher values than SPEP. This suspected gel saturation has also been invoked as the reason for artifactually high albumin results from agarose gels containing large M-spikes (10–12).

The aim of this study was to describe the relationship between SPEP M-spike and NEPH Ig quantification for monitoring monoclonal proteins as well as identifying any concentrations at which the methods do not compare in a linear manner. We compared protein concentrations on diluted sera to assess the linearity of the methods and to investigate the underlying cause of bias between the techniques.

Materials and Methods

We queried our dysproteinemia database (which contains data on all patients with a plasma cell proliferative disease seen at Mayo Clinic Rochester) for results from patients in whom a single sample provided an M-spike from SPEP, a heavy chain isotype from IFE, and Ig quantification by NEPH. If a patient had multiple samples, only the first result was included in this analysis. The sample set was also restricted to M-spikes that migrated in the γ fraction, and biclonal gammopathies were excluded. The Mayo Institutional Review Board approved this data search.

All assays were performed according to protocols in the Clinical Immunology Laboratory. SPEP was performed on the SPIFE SPE system (Helena Laboratories), IFE on Sebia 9IF gels (Sebia), and NEPH with Dade Behring BNII and reagents for IgG, IgA, IgM, and albumin (Dade Behring). The total protein concentration was determined by colorimetric assay using biuret reagents from Roche and a Roche Hitachi 912 chemistry analyzer system (Roche).

To visualize agreement between SPEP and NEPH, we constructed Bland–Altman plots for IgA, IgG, and IgM, with 2.5th and 97.5th percentiles of the differences as a function of the mean. We also included the median bias (50th percentile of the differences) to capture the relationship between the differences and the size of measurement (13). For these 3 percentile functions, quantile regression methods (14) were performed using the library quantreg implemented in the R statistical software. Quantile regression method was also used to find a best relationship between SPEP and NEPH, allowing high degree of polynomials for SPEP to express the median function of NEPH. If a nonlinear relationship was identified, we found the optimal linear range by starting with the best degree of polynomials and repeatedly narrowing the boundaries until there was no significant second-order polynomial and the 95% CI of the second-order term included zero.

To estimate linearity of the techniques, specimens from patients meeting the above criteria for inclusion in the study were diluted and reassayed by SPEP and NEPH. We included 7 IgG, 1 IgA, and 3 IgM monoclonal protein samples and 2 normal controls in the dilution study. We assessed the linearity of the results by a polynomial regression and selected a parsimonious model for each case, thus allowing the simplest statistical fit.

Results

The database query yielded results from 2845 patients who had an M-spike in the SPEP γ fraction, monoclonal heavy and light chains defined by IFE, and quantitative immunoglobulins assessed by NEPH and in whom all results were from the same serum sample. The isotype distribution, defined by the IFE results, was 2095 IgG, 456 IgM, and 294 IgA monoclonal proteins. The relative paucity of IgA monoclonal proteins was due to the definition of the cohort requiring the M-spike to be in the γ fraction. We did not subdivide the proteins according to light chain isotype.

Fig. 1 shows scatter plots of SPEP M-spike values and the corresponding NEPH Ig concentration for each heavy chain isotype. Each of the plots has the line of identity (slope = 1) and the best-fit cubic curve superimposed for reference. Bland–Altman plots are presented in Fig. 2, with 2.5th and 97.5th percentiles of differences and a median bias as a function of mean values. The plots show fewer samples at high Ig concentrations, and all 3 plots of median bias (Fig. 2) show that the 95% CI for the difference between the methods increases as their mean concentration increases. In addition, the median bias for IgG and IgM increases as their mean concentrations increase. At low IgG concentrations, the Bland–Altman plot (Fig. 2B) demonstrates that the relationship between IgG by SPEP and NEPH does not go through the origin (Fig. 1B). This is presumably due to the presence of polyclonal IgG in most sera with small M-spikes. The normal reference ranges of polyclonal IgA and IgM are much smaller than IgG, and their effects on the IgA and IgM plots are not as pronounced.
We performed another quantile regression model to identify the ranges in which the median of Ig quantification by NEPH was a linear function of M-spike measured by SPEP. Because the best-fit curves for each Ig class were cubic, we identified a linear range by repeatedly narrowing the boundaries until we obtained the region in which there was a linear relationship between SPEP and NEPH. Table 1 shows the lower and upper cutoff values of these linear ranges, as well as the linear slopes and correlations from the observations in the linear regions. The slope for the monoclonal IgA proteins in the linear region (11.0 to 67.0 g/L) is 0.92 (95% CI 0.87–1.02). The 95% CI includes a slope of 1, illustrating a very good correspondence with the NEPH quantification. The slope for the IgM monoclonal proteins in the linear region (8.5 to 25.1 g/L) is 1.80 (95% CI 1.68–1.92), illustrating a systematic higher result using NEPH than SPEP. Although these portions of the IgA and IgM data are described by a linear model, the correlations are only 0.84. The spread around the fitted line is presumably due to clone-specific differences affecting SPEP and NEPH.

The shape of the IgG curve (Figs. 1B and 2B) suggests the possibility of 2 distinct linear relationships between SPEP and NEPH quantification. One linear region from 19.2 to 56 g/L was identified by iteratively narrowing the analysis region. The data in the lower concentration region between 0 and 19.2 g/L was best

Fig. 1. Scatter plots of M-spike from protein electrophoresis and quantitation by immunonephelometry for 294 IgA monoclonal proteins (A), 2095 IgG monoclonal proteins (B), and 456 IgM monoclonal proteins (C). Each plot also contains the line of equality and line of best fit.
fitted by a nonlinear curve, but the difference between the best nonlinear fit and a linear fit was small. Interestingly, the slope at lower concentration is lower than unity (slope 0.83, 95% CI 0.79–0.89), whereas the slope at higher concentrations is 1.47 (95% CI 1.39–1.53). This nonlinearity has been previously noted and hypothesized to be due to saturation of the dense, narrow IgG monoclonal protein gel migration (15).

To further investigate the reasons for the nonlinearity between the methods for IgG quantification, we performed serial dilutions of patient specimens meeting defined standards for inclusion in the study and

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**Table 1. Relationship between SPEP M-spike and NEPH quantitation in the linear regions from patient database.**

<table>
<thead>
<tr>
<th>Isotype</th>
<th>Linear region, g/L</th>
<th>Slope (95% CI)</th>
<th>Correlation</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgA</td>
<td>11.0–67.0</td>
<td>0.92 (0.87–1.02)</td>
<td>0.84</td>
</tr>
<tr>
<td>IgG</td>
<td>0.0–19.2</td>
<td>0.83 (0.79–0.89)</td>
<td>0.78</td>
</tr>
<tr>
<td></td>
<td>19.2–56.0</td>
<td>1.47 (1.39–1.53)</td>
<td>0.89</td>
</tr>
<tr>
<td>IgM</td>
<td>8.5–25.1</td>
<td>1.80 (1.68–1.92)</td>
<td>0.84</td>
</tr>
</tbody>
</table>

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**Fig. 2.** Bland–Altman plots of M-spike from protein electrophoresis and quantitation by immunonephelometry for 294 IgA monoclonal proteins (A), 2095 IgG monoclonal proteins (B), and 456 IgM monoclonal proteins (C). Each plot has the 95% and 2.5% data range of the differences (dotted line) as a function of the mean. Solid line represents zero bias, and dashed lines represent median bias.
examined the results for linearity. Except for sera containing large IgG M-spikes, the SPEP and NEPH data for immunoglobulins and albumin was linear (Table 2). The IgG dilution data by SPEP was nonlinear, however, and deviated from linearity by 7.5% of the median percentage change between predicted values from a best quadratic function and those from a linear function. The dilution curves for IgG by NEPH (Fig. 3A) and SPEP (Fig. 3B) illustrate this point. The comparison of the dilution data for SPEP and NEPH (Fig. 3C) has the same shape as the comparison of the patient data (Fig. 1B). The only apparent difference in this curve is the absence of polyclonal IgG in these sera and the intersection through the origin. The relationship of the albumin quantification by SPEP and NEPH showed an opposite effect with dilution (Fig. 3D). Quantification of total protein, monoclonal protein by NEPH and SPEP, and albumin by NEPH and SPEP for 3 samples with large M-spikes is listed in Table 3. These 3 patients all had M-spikes of approximately 60 g/L. The sum of the NEPH IgM and albumin add up to more than the serum total protein (109%) compared with all the other measurements that are between 68% and 78%. This suggests that the bias in the relationship of IgM quantification with NEPH and SPEP is due to overestimation of IgM by NEPH. The sum of the IgG and albumin by SPEP is slightly lower than the other sums, suggesting that not all the underestimation of IgG is compensated for by an increase in albumin quantification.

Discussion

The results from this study are similar to those of other reports (4–7). Several studies comparing the quantification of monoclonal proteins by various techniques have demonstrated significant correlation with bias. Smith and Thompson (16) compared radial immunodiffusion and SPEP. They observed comparable results for IgG but higher radial immunodiffusion values for IgA and IgM. Riches et al. (4) showed overestimation of all subtypes of monoclonal protein using NEPH in comparison to SPEP. This was especially true for IgM. Our data, in contrast, suggest that for IgA monoclonal proteins, SPEP and NEPH correlate with a slope near unity. For IgM >8.5 g/L, SPEP and NEPH were linearly correlated, although the slope was 1.80 (95% CI 1.68–1.92). Riches et al. demonstrated the bias to be dependent on the species and source of antibodies, but that this is only part of the bias (4). Sinclair et al. (6) reported an increased concentration of IgM when the sample was treated with 2-mercaptoethanol.
This suggested interference from low molecular weight forms of the IgM antibodies. Our observation of nephelometric quantification of IgM plus albumin being greater than the total protein suggests that NEPH overestimates the IgM concentration. This is presumably due to the pentameric form of IgM and the resulting increase in the rate of formation of antigen–antibody complexes and the associated increased rate of formation of light scatter.

The data for IgG quantification by SPEP shows nonlinearity with an apparent break in the slope at 20 g/L. This nonlinearity means that changes in plasma cell populations may not be accurately reflected by changes in M-spike values. If a 70 g/L monoclonal protein is reduced to 50 g/L at 1 month postchemotherapy, for example, the M-spike may only change from 55 g/L to 50 g/L, and this could be interpreted as a nonresponse and influence treatment decisions. We hypothesize that this nonlinearity is partially due to a dye-saturation effect of the gel, limiting its accuracy at higher monoclonal IgG protein concentrations that migrate in a narrow, dense band. Data from the patient database (Fig. 1B and Table 1) and the dilution correlation (Fig. 3) demonstrate a change in slope when the IgG M-spike protein concentrations are >20–30 g/L, adding evidence for the dye saturation of the SPEP IgG.

Fig. 3. Sera from 7 patients with large IgG M-spikes were serially diluted and IgG and albumin were quantitated by NEPH and SPEP.

(A), Dilution curves for IgG quantitation by immunonephelometry. (B), Dilution curves for IgG M-spike by SPEP. (C), Relationship of the IgG dilution data by NEPH and SPEP. (D), Relationship of the albumin dilution data by NEPH and SPEP.
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Table 3. Comparison of serum total protein to the summation of monoclonal Ig and albumin in 3 patients with large M-sipes and hypogammaglobulinemic background.

<table>
<thead>
<tr>
<th>Monoclonal protein</th>
<th>NEPH Serum TP,a g/L</th>
<th>IgG, g/L</th>
<th>Albumin, g/L</th>
<th>IgG + albumin/TP, %</th>
<th>M-spike, g/L</th>
<th>SPEP Albumin, g/L</th>
<th>Monoclonal + albumin/TP, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgA</td>
<td>123</td>
<td>64</td>
<td>25</td>
<td>72</td>
<td>61</td>
<td>34</td>
<td>77</td>
</tr>
<tr>
<td>IgG</td>
<td>126</td>
<td>76</td>
<td>21</td>
<td>77</td>
<td>56</td>
<td>30</td>
<td>68</td>
</tr>
<tr>
<td>IgM</td>
<td>149</td>
<td>122</td>
<td>41</td>
<td>109</td>
<td>68</td>
<td>48</td>
<td>78</td>
</tr>
</tbody>
</table>

*TP, total protein.

peaks. Values of NEPH more accurately reflect the actual serum concentration for IgG paraproteins >20 g/L, and those of SPEP more accurately reflect serum concentrations for IgM.

The SPEP bias for albumin quantification in samples with large M-sipes (Fig. 3D) is in accordance with data published by Snozek et al. (12). These authors observed a positive bias of 0.8 g/L with bromcresol green (BCG) and NEPH compared with SPEP albumin quantification in samples with no monoclonal protein. With increasing M-sipe values, however, SPEP overestimated albumin in comparison to BCG and NEPH. Those findings and the current data predict that the SPEP albumin concentration is directly related to the underestimation of the IgG M-sipe, causing the total protein to be distributed proportionally elsewhere in the electropherogram.

Because of the systematic biases between IgM and IgG quantification by SPEP and NEPH, we suggest that clinicians do not alternate between methods when monitoring patients with paraproteins. Clinicians should also be aware that at high IgG paraprotein concentrations, the SPEP IgG concentrations will not linearly reflect changes in plasma cell populations.

Author Contributions: All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.

Authors’ Disclosures of Potential Conflicts of Interest: Upon manuscript submission, all authors completed the Disclosures of Potential Conflict of Interest form. Potential conflicts of interest:

Employment or Leadership: None declared.
Consultant or Advisory Role: None declared.
Stock Ownership: None declared.

References