Comparison of conventional methods of albumin, transferrin, and ceruloplasmin determination in serum with immunological reference methods

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Comparison of Conventional Methods of Albumin, Transferrin, and Ceruloplasmin Determination in Serum with Immunological Reference Methods

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Abstract

Radial immunodiffusion, which is a presently accepted reference method, was compared with the mechanized immunoprecipitation. Each of these specific methods was compared with two normal clinical-chemical methods for the determination of albumin and transferrin, and an enzymic method for the determination of ceruloplasmin. The correlation coefficients, the mean slope for regression, and the percentage variation of the average values from those of the average values of the reference methods are presented. In the determination of albumin and ceruloplasmin, radial immunodiffusion and mechanized immunoprecipitation give the same results. Surprisingly, these two specific methods give different results in the determination of transferrin. In as far as there is no doubt concerning the accuracy of the mechanized immunoprecipitation, this method is recommended, since it also has the advantage of being less demanding in cost and time.
With the introduction of specific immunological methods in clinical chemistry, the question arises as to the "accuracy" of conventional clinical-chemical analysis methods.

Using albumin, transferrin or total iron-binding capacity, and ceruloplasmin as examples, methods are being compared and related to so-called reference methods.

The single radial immunodiffusion method (ref. 1) has so far been considered as the reference method of choice for serum protein determinations. As a new reference method, the mechanized specific-immunological determination employing a nephelometric measuring technique (ref. 2) is compared with the radial immunodiffusion method.

Material and Methods

From sera which were stored for approximately three months at -20°C and which originated from chronically hemodialyzed patients, analyses were carried out according to the following methods:

Using the mechanized immunoprecipitation method, quantitative determinations of albumin, transferrin, and
ceruloplasmin were carried out nephelometrically according to the procedure described by Ebeling (ref. 3). Monospecific antihuman sera supplied by BERINGWERKE AG, HYLAND, and DAKOPATTS were used.

2. Quantitative determinations of albumin, transferrin, and ceruloplasmin were also carried out on M-Partigen immunodiffusion plates (BEHRINGWERKE AG) using the single radial immunodiffusion method described by Mancini et al. (ref. 1).

3. The total protein content of the serum was measured with biuret reagent according to the method described by Richterich (ref. 4) modified according to Hoffmann et al. (ref. 5).

The protein electrophoreses were carried out on a microscale on cellulose acetate film (SARTORIUS) in a BOS-KAMP chamber, and analyzed after ponceau S coloration in an Elphormat* (BENDER and HOBEIN). The albumin concentration was calculated from the relative percentages of the albumin fraction and the total protein content of the serum.

4. In addition, albumin was quantitatively determined using the bromocresol green micro method according to Schirardin et al. (ref. 6) as a colour test.

*Translator's note: Presumably a commercial designation.
5. The serum iron concentration (Fe) was determined with bathophenanthroline and the TECHNICON AutoAnalyzer in accordance with the method described by Zak et al. (ref. 7).

6. The latent iron-binding capacity was determined using the Irosorb-59 test (ABBOTT) based on ion exchange. The radioactivity was measured in a well-type scintillation counter (FRIESICKE and HOEPFNER).

7. The total iron-binding capacity of the serum was determined manually using a micro method according to Führ (refs. 8, 9).

8. As a clinical-chemical test for the ceruloplasmin determination, we used the enzyme test (DR. HAURY) which measures the oxidoreductase property of the ceruloplasmin via the conversion of paraphenylenediamine.

For the immunological methods, protein standards and control sera obtained from BERINGWERKE AG were used for calibration and quality control respectively. Control sera obtained from BEHRINGWERKE AG in addition to sera obtained from the DADE Company were also used for quality control of the colorimetric methods and for the Irosorb method.
Results

The individual methods used were evaluated by determining, among other things, their precision. The different methods were compared and their accuracy estimated by determining the mean regression lines and the percentage deviations of the mean values from the mean values of the reference methods.

Table 1 shows the coefficients of variation of the precision measurements using the various methods of determination. In the case of composite determinations, the coefficient of variation contains the relative error of propagation.

The concentrations of the precision controls give an indication of the sensitivity of the particular method, and, indirectly; allow one to infer the method-dependent serum requirements.

In order to be able to compare total iron-binding capacities with the results of the mechanized immunoprecipitation and the radial immunodiffusion, we converted the transferrin concentrations into transferrin iron-binding capacities. In doing so, we assumed that at iron saturation each transferrin molecule binds two iron atoms (=Fe^{+++}) and that the molecular weight of transferrin was 89000 (ref. 10).
Table 1: Precision of the methods of determination of a control serum.

<table>
<thead>
<tr>
<th>1.</th>
<th>2.</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>Konzentration der Präzisionskontrolle</td>
</tr>
<tr>
<td>---</td>
<td>-----------------------------------</td>
</tr>
<tr>
<td>Albumin</td>
<td></td>
</tr>
<tr>
<td>3. mechanisierte Immunpräzipitation</td>
<td>12</td>
</tr>
<tr>
<td>4. radiale Immundiffusion</td>
<td>60</td>
</tr>
<tr>
<td>5. Farbstest</td>
<td>20</td>
</tr>
<tr>
<td>6. Elektrophorese</td>
<td>5</td>
</tr>
<tr>
<td>Eisenbindungskapazität</td>
<td>μmol/l</td>
</tr>
<tr>
<td>3. mechanisierte Immunpräzipitation</td>
<td>15</td>
</tr>
<tr>
<td>4. radiale Immundiffusion</td>
<td>28</td>
</tr>
<tr>
<td>8. latente Eisenbindungskapazität + Fe</td>
<td>8</td>
</tr>
<tr>
<td>9. totale Eisenbindungskapazität</td>
<td>20</td>
</tr>
<tr>
<td>Coeruloplasmin</td>
<td>mg/l or U/l</td>
</tr>
<tr>
<td>3. mechanisierte Immunpräzipitation</td>
<td>10</td>
</tr>
<tr>
<td>4. radiale Immundiffusion</td>
<td>8</td>
</tr>
<tr>
<td>11. enzymatisch</td>
<td>8</td>
</tr>
</tbody>
</table>

+ in series  
++ per series  
+++ relative propagated error per series

Translation of Numbered Items:

1. concentration of precision control  
2. coefficient of variation  
3. mechanized immunoprecipitation  
4. radial immunodiffusion  
5. colour test  
6. electrophoresis  
7. iron-binding capacity  
8. latent iron-binding capacity + Fe  
9. total iron-binding capacity  
10. ceruloplasmin  
11. enzymatically

Translator's note: Note that German "comma" = English decimal point.
The results of the comparison of the methods with one another and with the reference methods are shown in Table 2.

The graphs show only the results of the comparisons of the specific immunological methods. Apart from the experimentally measured values and the mean regression lines, the graphs also show the required or expected ideal relationships between the methods.

Table 3 shows, among other things, the percentage deviation of the mean value of a particular method from the mean value of the corresponding reference method.

Discussion

In order for the reference methods used here to be regarded as accurate, the antigen-antibody specificity and quality of the analysis methods should be optimal. Moreover, stable, qualitatively sufficiently concentrated antisera should be available, as well as protein calibration standards of extremely high quality, stability, and weight precision, especially in the case of high-precision quantitative analyses. The accuracy of the methods is determined by the extent to which these conditions are satisfied.
Table 2: Comparison of methods by means of the correlation coefficient and the mean regression line.

<table>
<thead>
<tr>
<th>Method</th>
<th>N</th>
<th>r</th>
<th>a</th>
<th>b</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Albumin</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RID/AIP</td>
<td>39</td>
<td>0.935</td>
<td>1.11</td>
<td>-3.64</td>
</tr>
<tr>
<td>Farbtest/AIP</td>
<td>42</td>
<td>0.937</td>
<td>1.08</td>
<td>-5.56</td>
</tr>
<tr>
<td>Elektrophoresis/AIP</td>
<td>39</td>
<td>0.913</td>
<td>1.11</td>
<td>-5.33</td>
</tr>
<tr>
<td>Farbtest/RID</td>
<td>42</td>
<td>0.975</td>
<td>0.98</td>
<td>-1.49</td>
</tr>
<tr>
<td>Elektrophoresis/RID</td>
<td>45</td>
<td>0.954</td>
<td>1.02</td>
<td>-1.98</td>
</tr>
<tr>
<td>Farbtest/Elektrophoresis</td>
<td>45</td>
<td>0.946</td>
<td>1.04</td>
<td>-0.30</td>
</tr>
<tr>
<td><strong>Eisenbindungskapazität</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AIP/RID</td>
<td>40</td>
<td>0.921</td>
<td>0.95</td>
<td>+8.75</td>
</tr>
<tr>
<td>latent EBK + Fe/AIP</td>
<td>37</td>
<td>0.907</td>
<td>1.12</td>
<td>-18.02</td>
</tr>
<tr>
<td>total EBK/AIP</td>
<td>35</td>
<td>0.938</td>
<td>0.84</td>
<td>-3.82</td>
</tr>
<tr>
<td>latent EBK + Fe/RID</td>
<td>34</td>
<td>0.933</td>
<td>1.30</td>
<td>-20.97</td>
</tr>
<tr>
<td>total EBK/RID</td>
<td>40</td>
<td>0.944</td>
<td>1.09</td>
<td>-13.29</td>
</tr>
<tr>
<td>latent EBK + Fe/total EBK</td>
<td>38</td>
<td>0.948</td>
<td>1.13</td>
<td>+0.63</td>
</tr>
<tr>
<td><strong>Ceruloplasmin</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AIP/RID</td>
<td>37</td>
<td>0.923</td>
<td>0.99</td>
<td>+9.96</td>
</tr>
<tr>
<td>enzymatisch/AIP</td>
<td>37</td>
<td>0.915</td>
<td>15.43</td>
<td>-90.30</td>
</tr>
<tr>
<td>enzymatisch/RID</td>
<td>37</td>
<td>0.882</td>
<td>13.14</td>
<td>-15.46</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.03</td>
<td>-0.20++++</td>
</tr>
</tbody>
</table>

+++ in this case, the measured conversion factor of 12.82 was used (see text).

RID = radial immunodiffusion
AIP = mechanized immunoprecipitation
latent EBK + Fe = latent iron-binding capacity + Fe
total EBK = total iron-binding capacity

Translation of Numbered Items:

1. mean regression line \( (y = ax + b) \)  
2. colour test/AIP  
3. electrophoresis/AIP  
4. colour test/RID  
5. electrophoresis/RID  
6. colour test/electrophoresis  
7. iron-binding capacity  
8. ceruloplasmin  
9. enzymatically/AIP  
10. enzymatically/RID
Table 3: Percentage deviations of the mean value from the mean value of the reference method.

<table>
<thead>
<tr>
<th>Method</th>
<th>N</th>
<th>( \bar{x} )</th>
<th>Mittelwertsabweichung in %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Albumin</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. mechanisierte Immunpräzipitation</td>
<td>40</td>
<td>36,3</td>
<td></td>
</tr>
<tr>
<td>3. radiale Immundiffusion</td>
<td>42</td>
<td>36,2</td>
<td>- 0,25</td>
</tr>
<tr>
<td>4. Farbtest</td>
<td>43</td>
<td>38,8</td>
<td>+ 7,00</td>
</tr>
<tr>
<td>5. Elektrophorese</td>
<td>43</td>
<td>37,9</td>
<td>+ 4,50</td>
</tr>
<tr>
<td>6. Eisenbindungskapazität</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. mechanisierte</td>
<td>38</td>
<td>54,8</td>
<td></td>
</tr>
<tr>
<td>3. radiale Immundiffusion latente</td>
<td>38</td>
<td>62,7</td>
<td>+ 14,40</td>
</tr>
<tr>
<td>7. Eisenbindungskapazität + Fe</td>
<td>37</td>
<td>63,6</td>
<td>+ 16,10</td>
</tr>
<tr>
<td>8. totale Eisenbindungskapazität</td>
<td>38</td>
<td>70,9</td>
<td>+ 29,40</td>
</tr>
<tr>
<td>3. radiale Immundiffusion</td>
<td>38</td>
<td>62,7</td>
<td></td>
</tr>
<tr>
<td>2. mechanisierte</td>
<td>38</td>
<td>54,8</td>
<td>- 12,60</td>
</tr>
<tr>
<td>7. Eisenbindungskapazität + Fe</td>
<td>37</td>
<td>63,6</td>
<td>+ 1,40</td>
</tr>
<tr>
<td>8. totale Eisenbindungskapazität</td>
<td>38</td>
<td>70,9</td>
<td>+ 13,10</td>
</tr>
<tr>
<td>9. Coeruloplasmin</td>
<td></td>
<td>ng/l or mg/l</td>
<td></td>
</tr>
<tr>
<td>2. mechanisierte</td>
<td>37</td>
<td>349,80</td>
<td></td>
</tr>
<tr>
<td>10. enzymatisch</td>
<td>37</td>
<td>354,96</td>
<td>+ 1,48</td>
</tr>
<tr>
<td>2. mechanisierte</td>
<td>37</td>
<td>354,96</td>
<td></td>
</tr>
<tr>
<td>10. enzymatisch</td>
<td>37</td>
<td>27,73</td>
<td>+ 0,14</td>
</tr>
</tbody>
</table>

+++ in this case, the measured conversion factor of 12.82 was used (see text).

(for translation of numbered items, see next page)
Translation of Numbered Items in Table 3:

1. mean value deviation in %
2. mechanized immunoprecipitation
3. radial immunodiffusion
4. colour test
5. electrophoresis
6. iron-binding capacity
7. latent iron-binding capacity + Fe
8. total iron-binding capacity
9. ceruloplasmin
10. enzymatically
It is evident from the comparison of the albumin methods, that the single radial immunodiffusion method and the mechanized nephelometric immunoprecipitation method yield the same results. Thus, at equal precision, the two specific methods of albumin determination can be regarded as equivalent reference methods. However, since the mechanized immunoprecipitation method is less demanding in cost and time, we consider it today as the preferable method (refs. 3, 11). By comparison, the two unspecific methods of albumin determination, the colour test with bromocresol green, and the electrophoretic separation and quantification of the relative percentages through total protein determinations yield values which are too high. Evidently, the dyeability of albumin after the serum electrophoresis with ponceau S is greater than that of other protein fractions. This is no doubt due, among other things, to the fact that the so-called albumin band does not represent a protein-chemically homogeneous fraction, which means that the measurement includes also other proteins, apart from the difference in the protein-dye affinity (ref. 12). Nor is the bromocresol green method only specific to albumin, because other proteins evidently also take part in the reaction and thus give rise to higher apparent albumin concentrations (ref. 11). However, the two latter methods correlate well, as was confirmed by Schirardin et al. (ref. 6) and Booij (ref. 12).
In order to be able to compare the two specific immunological methods of transferrin determination with the unspecific methods, i.e. the serum iron-binding capacity methods, the transferrin concentration was expressed as transferrin iron-binding capacity in accordance with Haeckel et al. (ref. 13). As was reconfirmed by our results, the concentrations obtained with the unspecific total serum iron-binding capacity methods, the Irosorb-59 plus serum iron method, and, above all, the method according to Führ, are higher than the concentrations obtained with the specific methods. The fact that there exists a discrepancy between the total and the transferrin iron-binding capacity was observed by Stojceski et al. (ref. 14) and Scuro et al. (ref. 15), among others. Van der Heul et al. (ref. 16) were able to show that other iron-binding serum components also enter into the total serum iron-binding capacity apart from transferrin. For this reason alone, a determination of the total iron-binding capacity can no longer be equated with a transferrin determination (refs. 13, 17).

Contrary to our own expectations and those of other authors (ref. 13), a comparison of the two specific methods shows that the reference method for transferrin commonly used so far, i.e. single radial immunodiffusion, yields higher concentrations than the mechanized precipitation method. The
mean value obtained by immunodiffusion was approximately 14% higher than the value obtained by nephelometry. This difference is statistically significant at the \( p < 0.025 \) level using the \( t \)-test.

So far, we have been unable to resolve the question as to the causes of this astonishing discrepancy between the two methods. It would be interesting to investigate whether this discrepancy in the results is due to physicochemical differences in the principles of measurement or whether, as a result of different, higher concentration ratios than in the nephelometric method, the measurement by the radial immunodiffusion method includes an "unspecific" component in the antigen-antibody reaction and thus leads to higher values. It should be pointed out that the same antigen standards were used in both methods, and that the quality and specificity of the Partigen-plate antibodies can probably be assumed to correspond to the quality and specificity of the antisera in the bottles from the same Company.

As long as the question as to which one of the two "specific" methods yields the correct result remains unresolved, it is difficult to accord one of the two methods the rating of "reference" method.
It is evident from a comparison of the ceruloplasmin methods that the concentrations determined via radial immunodiffusion and nephelometric immunoprecipitation differ only slightly. Thus, it can be concluded that these two methods are equivalent reference methods, as in the case of albumin.

The values obtained in the present study by oxidoreductase activity determination of ceruloplasmin correlated well with the values obtained with the reference methods; however, in contrast to comparisons of mg/1 to U/1 such as 10.4/1 and 10/1 reported in the literature (refs. 18, 19), we obtained values of mg to U such as 12.82 to 1. As in the study by Haralambie (ref. 19), our ceruloplasmin-protein standard also yielded lower enzyme-kinetically determined values than immunologically determined values, assuming a ratio of 10/1 mg/1 immunologically determined to U/1 enzyme-kinetically determined. The good agreement of 10/1 of immunologically and enzyme-kinetically determined values was obtained by Haralambie only if fresh sera were used for the enzyme-kinetic method (ref. 19). Despite the enzyme activity losses described (cf. loc. cit. [ref. 19]), the immunological activity of the ceruloplasmin remains preserved (ref. 19). The sera used in our determination were all frozen for approximately three months at -20°C, and yielded approximately the same activity difference immunologically to enzymatically as the lyophilized
Fig. 1: Comparison of methods: albumin

--- mean regression line
--- ideal curve.
Fig. 2: Comparison of methods: transferrin. The concentrations are expressed as transferrin iron-binding capacities.

--- mean regression line

------- ideal curve
Fig. 3: Comparison of methods: ceruloplasmin

--- mean regression line

--- ideal curve
ceruloplasmin standard of BEHRINGWERKE AG with 300 mg/l to 23.4 U/l (see Table 3).

The ratio, which was obtained from the ceruloplasmin standardized on a weight basis to the enzyme activity measured by us, was used in Table 3 as conversion factor for the percentage deviation of the mean values of the enzymatic method from the mean values of the reference methods.

Since there is evidently no immunological activity loss to be expected in the two specific immunological methods, in contrast to the enzyme-kinetic ceruloplasmin determination, immunological values are more reliable and reproducible.

Measurements obtained by specific quantitative analysis methods provide physicians with reliable information. Therefore, the unspecific methods still widely used in clinical chemistry should be replaced by more specific methods.

Provided that there is no doubt about the "accuracy" of the methods, we recommend the mechanized immunoprecipitation for serum protein determinations. If it may be assumed that, as part of the trend towards general automation, clinical laboratories have at their disposal the necessary facilities, including continuous-flow fluoronephelometers (TURNER or
Technicon, the method of mechanized immunoprecipitation is superior to immunodiffusion for reasons of reagent costs and speed, and, as far as the reproducibility of the results is concerned, it is not inferior to immunodiffusion.

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Bibliography

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