Glycylglycylglycine as a Synthetic Standard for Serum Protein Determination

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Glycylglycylglycine was investigated as a reference standard for use in serum protein measurement by the biuret reaction. The tripeptide-biuret solution has a molar absorptivity of 96 at 565 nm, and absorbances at both 550 nm and 565 nm are proportional to concentration. By a manual reference procedure, the 550-nm absorbance of 1.0 g of tripeptide was equivalent to that given by 1.72 ± 0.03 g of human serum albumin or 1.43 ± 0.03 g of bovine serum albumin. By the Technicon N14b automated procedure, the absorbance of 1.0 g of tripeptide at 550 nm was equivalent to that of 1.81 ± 0.02 g of human serum albumin or 1.89 ± 0.03 g of bovine serum albumin.

Results for serum protein analyses over the range 4.0 to 9.0 g/dl, when tripeptide or serum albumin was used to prepare calibration curves, showed mean differences of 0.15 g/dl in the manual mode and 0.08 g/dl in the automated mode.

Peters (1) proposed that bovine serum albumin be used as a reference material for standardizing serum protein measurements by the biuret reaction but suggested that an ideal standard would be a synthetic polypeptide of high molecular weight and known composition. Rappaport and Loew (2) had used a peptone solution to standardize protein analyses, but found considerable variation in biuret response from batch to batch. Malonamide, H₂NCOCH₂CONH₂, was recommended by Haffkenscheid and Jansen (3), who used dilutions of an 0.78 mol/liter solution as standards for automated protein analyses. This report describes an investigation of the use of glycylglycylglycine, the simplest tripeptide, as a reference compound for protein analyses. Results of serum protein analyses by both manual and automated biuret techniques with the tripeptide as reference agree well with those obtained when human or bovine serum albumin is used for standardization.

Materials and Methods

Glycylglycylglycine. Many experiments were performed with the tripeptide, Lots T1635 and S4747, purchased from Schwarz/Mann, Orangeburg, N. Y. 10962 as reference standard. Both preparations, mp 220–230 °C (dec.), were 96.5 ± 0.2% and 97.0 ± 0.2% pure by phase-solubility measurements (ethanol-water, 1:1) and peptide analysis, respectively. I also used tripeptide, mp 237–240 °C (dec.), that was synthesized by Dr. Arthur M. Felix, Chemical Research Department, and Dr. John Maricq, Technical Development Department, and was 99.1 ± 0.2% and 99.7 ± 0.2% pure, as determined by phase-solubility studies and peptide analysis, respectively. Samples from all these preparations exhibited a single spot (R₁ = 0.36) with ninhydrin on silica-gel thin-layer chromatography (developer: n-butanol-acetic acid-ethyl acetate-water, 1:1:1:1 by vol). The data presented here were obtained with the latter preparation, which had been dried under reduced pressure at 60 °C.

Standard solutions were prepared by dissolving 1, 2, 3, 4, and 5 g of tripeptide in 100 ml of de-ionized water with heating. The more concentrated solutions contained traces of insoluble noncrystalline material which were removed by passage through a 0.22-μm (av pore size) filter (Millipore Filter Corp., Bedford, Mass. 01730).

Bovine serum albumin. Protein reference standard, with a protein content of 10.9 ± 0.1 g/dl, was purchased (Item 81-016, Lot 14) from Pentex, Research Products Division, Miles Laboratories, Inc., Kankakee, Ill. 60901.

Human serum albumin, Fraction V. Protein reference standard, with a protein content of 12.0 ± 0.1 g/dl, was purchased (Item 81-017, Lot 15) from the same source.

Biuret reagent. The reagent, prepared by diluting the stock solution 1:5 with NaOH (0.2 mol/liter) as described in Standard Methods of Clinical Chemistry (4), was used for both manual and automated analyses.

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Table 1. Determinations of Protein-Tripeptide Ratio

<table>
<thead>
<tr>
<th>Protein</th>
<th>Tripeptide</th>
<th>Protein/tripeptide</th>
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<tr>
<td>0.10</td>
<td>1.40</td>
<td>0.80</td>
</tr>
<tr>
<td>0.20</td>
<td>2.80</td>
<td>1.60</td>
</tr>
<tr>
<td>0.30</td>
<td>4.20</td>
<td>2.40</td>
</tr>
<tr>
<td>0.40</td>
<td>5.50</td>
<td>3.20</td>
</tr>
<tr>
<td>0.50</td>
<td>6.85</td>
<td>4.00</td>
</tr>
<tr>
<td>0.60</td>
<td>8.20</td>
<td>4.75</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td></td>
</tr>
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</table>

Procedure

For manual analyses, the procedure described for total protein in ref. 4 was used. For automated analyses, the Technicon N-14b procedure (5) was used.

Results

Figure 1 shows spectrophotometric absorption curves of the product of the biuret reaction with human serum albumin (5.5 g/dl), bovine serum albumin (6.0 g/dl), whole human serum (5.0 g/dl), and glycylglycylglycine (3.0 g/dl). For the first three substances, there was an absorbance maximum between 540–555 nm; for the tripeptide the maximum was between 560–570 nm (molar absorptivity, 96). Absorbances of human serum albumin, bovine serum albumin, and glycylglycylglycine at these respective absorbance maxima were linearly related to concentration. When the 550-nm absorbances of the tripeptide-biuret and the human serum albumin-biuret reaction products were plotted vs. concentration, typical results were as shown in Figure 2. From this plot, the protein/tripeptide (g/g) ratio for each 0.10 absorbance unit was calculated. Table 1 shows such a calculation for the manual biuret procedure, the mean ratio being 1.73. Daily comparisons over an extended period by the manual biuret method gave a mean absorbance for 1.0 g of tripeptide at 550 nm that was equivalent to that given by 1.72 ± 0.03 (SD) g of human serum albumin or by 1.43 ± 0.03 (SD) g of bovine serum albumin. By the automated procedure, the corresponding figures were, respectively, 1.81 ± 0.02 and 1.89 ± 0.03. Thus, a calibration curve prepared by plotting absorbances (at 550 nm) of the tripeptide concentration (multiplied by the appropriate correlation factor so that the results are expressed as protein) and the curve produced by the protein are almost superimposable (Figure 3).

Serum Analyses

When sera were analyzed by the manual biuret procedure and the protein content was calculated by reference to a bovine serum albumin calibration curve or by reference to the tripeptide calibration curve multiplied by the appropriate correlation factor, a mean difference of 0.15 ± 0.025 g (SD) (range: 0.09–0.18 g) was found.
When another group of sera was analyzed by the automated protein procedure and the protein content was calculated by reference to a human serum albumin calibration curve and to a tripeptide calibration curve multiplied by the appropriate correlation factor, a mean difference of 0.08 ± 0.02 g (SD) (range: 0.05–0.11 g) was found.

**Analytical Variables**

*Precision and reproducibility.* Within-run, the coefficients of variation for the automated tripeptide procedure and for human serum albumin were 0.2% and 2.06%, respectively. The reproducibility (coefficient of variation) of the manual biuret reaction with increasing concentrations of the tripeptide standards over a twice-daily, 10-day interval was 1.1% (range: 0.5–1.7%); for human serum albumin the corresponding figure was 1.9% (range: 0.9–3.8%).

*Results for sera of abnormal protein content.* Two such studies were conducted. In the first, sera with normal and subnormal protein content were analyzed (Table 2). Aliquots of each serum were mixed with an equal volume of serum taken from a serum pool containing 8.8 g of protein per deciliter and reanalyzed, 100.3% (mean) of the protein was accounted for. In the second study (Table 3) 1.0-ml aliquots of the same sera used in the first study were mixed with 1.0-ml aliquots of the 2.0 g tripeptide per deciliter solution (equivalent to 3.62 g protein per deciliter) and analyzed; an average of 97.7% of the protein was analytically accounted for.

**Discussion**

The simplest tripeptide, glycylglycylglycine, fulfills almost all of Peters’ suggested criteria (1) for a reference compound for serum protein analysis: it is a stable substance of known composition, which can be readily synthesized and purified to primary standard quality; structurally, the tripeptide possesses the minimal two amido groups required for coordination with Cu²⁺ in the biuret reaction; and it is water soluble, and in solution the tripeptide is stable, especially if suitable preservatives are added. (Extensive stability studies were not undertaken.)

Hafkenscheid and Jansen (3) found, in their automated method, that the Gornall biuret reagent (6) had to be augmented with additional cupric sulfate and sodium potassium tartrate to linearize the biuret reaction with malonamide. In a limited study, I also found that the undiluted Gornall formulation contained about one-third the optimum Cu²⁺ concentration when used for tripeptide standardization exactly as described in a manual reference method (4) or in the automated protein analysis. On the other hand, tripeptide solutions give optimum biuret response with the diluted Weichselbaum formulation (4). The already-familiar reagent needed no modification, because it contained experimentally re-determined optimal concentrations of both alkali and Cu²⁺.
The only criterion not fully satisfied is molecular weight. The molecular weight of glycyglycylglycine is 189; that for bovine serum albumin is about 64,000. This in no way diminishes the usefulness of the compound.

In the automated analysis both human and bovine serum albumin had very similar biuret absorbance ratios vs. that of the tripeptide. By contrast, in the manual procedure, the slopes of human and bovine serum albumin biuret absorbances plotted vs. tripeptide absorbances were markedly different. The reason for this is not immediately obvious. The spectral absorption curve is the same for the product derived manually and by the automated procedure.

The present work describes our experience with and the feasibility of the tripeptide as a reference material. It is essential, of course, that each analyst redetermine the correlation factor relating tripeptide to bovine or human serum albumin, whichever is the preferred reference material. This is particularly important if a biuret reagent other than the one used in this study is used. I have illustrated how to do this simply. At the same time, the protein content of the albumin reference solutions, if purchased, should be verified independently. Our own standardization preference has been a pool of normal human serum whose protein nitrogen content had been accurately determined (microKjeldahl) with correction for nonprotein nitrogen. This laborious procedure stimulated our search for a synthetic substitute.

The two recovery experiments presented were performed to answer two questions. Firstly, are there differences in tripeptide-biuret ratios between sera with normal protein content and those with pathologically low protein content? It will be recalled that this was one of the pitfalls in attempts to use the Folin–Ciocalteau reagent for serum protein analysis. It had been observed that tyrosine/protein ratios were different in normal serum from that in serum with pathologically low protein (tyrosine?) content. With the present procedure, the peptide/protein ratios are similar, if not identical.

Secondly, are biuret responses the same whether the peptide sequences are the natural ones as found in serum protein, or are they altered by the introduction of an artificial sequence? The data demonstrate that the responses are not altered.

This investigation originated following a discussion of protein standardization with Dr. Roland Richterich, Inselspital, Berne, Switzerland, and I am grateful to him for his suggestions and interest. I am grateful also to Drs. Arthur M. Felix and John Maricq for generous quantities of highest-quality tripeptide and to Dr. John Donohue, Analytical Research Department, for data concerning the purity of tripeptide samples. I am indebted to my associates for fine technical support: James A. Foreman and Norman B. Kleinman (preliminary experiments and spectral measurements), Lois B. Lucas and Fontaine Standaert (manual analyses), and Joan E. Sheehan (automated analyses).

References