Albumin-based or albumin-linked calibrators cause a positive bias in serum proteins assayed by the biuret method

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Abstract

Background: Assay of total serum protein by the biuret method calibrated with albumin standards according to the reference method provides results with a positive bias approximately 3%–5% exceeding the total error of 3.4% allowable for total protein in serum analysis made by analysers using two-part reagents and short-term procedures.

Methods: We used two types of two-part biuret reagents utilised in a short-term measurement in analysers with albumin or serum calibrators, in which protein was attested by the Kjeldahl method.

Results: Tests with potentially interfering substances proved that serum blanking used in a short-term biuret procedure is not capable of sufficiently eliminating effects of serum interferents. A short-term blanking is evidently capable of suppressing only an absorbance caused by serum-present coloured and turbid interferents, but its capacity to transform them (oxidise, hydrolyse, saponify, etc.) to some other not-interfering substances is very low compared with a long-term procedure. Lipids and bilirubin are responsible for significant positive bias of total protein in normal serum samples (approximately 3%) and even a greater positive offset in lipaemic and icteric sera (approximately 5%). We verified that interference tests based on a normal serum spiked with endogenous lipids and bilirubin give quite false and misleading results in the biuret reaction. A pure albumin, not depending on its bovine/human origin, gives absorbance responding only to its copper complexes with protein with a biuret reagent, while its absorbance with a serum also includes the absorbance of interferents present in serum. The simplest way to improve current short-term biuret procedures is the use of a human serum calibrator with total protein attested by the Kjeldahl method. A serum calibrator, behaving analogously to serum samples, compensates for a positive bias in most normal sera. Reagents with a greater concentration of active biuret components (copper and alkali, reference method included) seem to be unnecessarily aggressive to proteins and are responsible for a lower accuracy when used in short-term measurements.

Conclusions: Standard Reference Material 927c based on pure bovine albumin is still recommended and used as the primary standard for assays of total protein by colourimetric methods. The albumin calibrator is responsible for a positive bias of approximately 3%–5% in serum total protein assayed by the biuret reaction both in the reference and in current methods. Its substitution by a serum calibrator attested by the Kjeldahl method could solve this drawback.


Keywords: albumin calibrators cause systematic protein bias; positive bias in serum protein; serum calibrator for total protein.

Introduction

The determination of total protein (TP) in human serum is mostly based on the biuret method, i.e., on the reaction of proteins with copper ions in an alkaline region. The reference method for measuring TP in serum using the biuret reaction was developed in 1981 by the working group (WG) of Doumas et al. (1, 2) who included previously described copper-tartrate complex, 12 mmol/L, in NaOH, 0.6 mol/L, as their biuret reagent (BR) (3). They used and recommended the Standard Reference Material SRM 927c bovine albumin as the calibrator, even though bovine albumin behaved differently from both human albumin and human serum in their end-point measurement, probably because it was the only SRM protein available at this time.

The WG described the composition and stability of their BR and accepted a long reaction time for colour development (1), because “the complete colour development requires 60 min at 25°C or 10 min at 37°C; when shortened to 30 min, the colour yield was about 1.5% less; with short incubation times (tens of seconds) and bovine solution as standards, protein in
serum albumin (BSA), reaction yield were nearly the same (67% of theoretical). With proteins made by stopped-flow spectrophotometry, we calculated first order rate constants with proteins made by stopped-flow spectrophotometry, knowing the reaction mechanism with proteins. The biuret method (1–3) was developed without any real protein recovery. Unfortunately, even the reference biuret method (1–3) was developed without any real knowledge of the reaction mechanism with proteins.

In our study (6) on the biuret reaction mechanism with proteins made by stopped-flow spectrophotometry, we calculated first order rate constants $k$ (s$^{-1}$) at 25°C with our EDTA-chelated BR as follows: bovine serum albumin (BSA), $k = 0.069$; human serum albumin (HSA), $k = 0.077$ and a human serum, $k = 0.080$. Calculated theoretical times necessary to reach a 99% reaction were about 20 min at 37°C constant or shows even a negligible decrease, the absorbance of copper complexes with HSA or with a human serum increases slightly but consistently all the time”. After all, similar differences in BSA and HSA rate curves were also registered by the WG [cf. (1)]. Unfortunately, they preferred the use of a commercially available BSA calibrator.

In our preliminary experiments (7, 8), we observed that the reference method calibrated with BSA standards (or with secondary standards linked to BSA) falsely increases results of serum TP. Moreover, all standards based on a pure fat-free protein differ fundamentally from human sera containing interfering endogenous lipids which are known to be the main source of errors in the determination of serum TP by the biuret reaction (5, 9, 10).

Like most of the reference methods, even this method (1, 2) is not suitable for daily use owing to complicated and long-term measurements. To enable an automation of TP analysis, a BR divided into two parts is now used (11, 12): BR1 containing alkaline tartrate (AT) for serum blanking and BR2 containing concentrated AT with copper-tartrate. Serum blank is usually measured 1 or 4 min after mixing a sample with BR1, then BR2 is added and resulting absorbance is measured after another 3 or 5 min, the exact timing depending on the analyser setting. An analyser recalculates all measurements to the same volume, subtracts the absorbance of serum blanks and blanks of both reagents BR1 and BR2 and calculates TP in a sample against the used calibrator. Contrary to explicit WG warnings (1) “not to use BSA standards in short-time measurements”, BSA calibrators are still used as primary standards in all automated procedures.

Most of the biologically/clinically important analytes undergo natural changes/variabilities (13, 14). Biological variations (BVs) of laboratory analytes are crucial for setting limits for their acceptable/allowable analytical performance imprecision expressed as a total error (TE). According to the latest data, fixed limit for TP TE is 3.4% (15). A TE, which is usually below one-third of the respective BV, includes all errors and uncertainties of the whole analytical process from sampling through calibrators and standards to used methods, reagents, equipment, personnel, etc. (14).

We previously reported (7, 8) that BSA used as a primary calibrator in the reference method causes positive TP bias (approximately 2%–3%). In this study, we tested the ability of two-part reagents and short-term measurements used in analysers to suppress an effect of serum interfering substances, such as bilirubin, HB and lipids. We found that such measurements, when combined with albumin calibrators, are responsible for an even greater positive bias at approximately 3%–5%.
cates the clinician’s ability to identify important changes in test results of their patients (13). However, we found a simple method to resolve the situation (16) using human serum based calibrators attested by the Kjeldahl reference method (17).

Materials and methods

Equipment

A Shimadzu UV-160 spectrophotometer (Shimadzu, Tokyo, Japan), with a tungsten and deuterium lamp, 10±0.01 mm light-path quartz cells and 6-position cell-rack changer heated to 37±0.1°C, was used. A Shimadzu spectrophotometer was used in all manual quantitative procedures A/B and for absorbance measurements presented in Figures 1 and 2.

A Hitachi 911 automatic analyser (Hitachi Ltd., Tokyo, Japan), with concave diffraction grating, was used. All analyses were made in 10-mm reaction cells heated to 37±0.2°C with bichromatic reading of the absorbance at 546/700 nm. Analyser software for TP was preset according to the scheme in Table 1.

A Konelab 30i automatic analyser (Thermo Clinical Lab-systems, Vantaa, Finland), with one-channel photometer with interference filters within 340–800 nm, was used. All analyses were made in 10-mm reaction cells heated to 37±0.2°C with bichromatic reading of the absorbance at 540/700 nm, see the analysis scheme in Table 1.

A Kjeldahl micro-apparatus for steam distillation was made in our Institute by our glass blower according to previously described methods (17). Distillation apparatus consisted of a 150-mL round-bottom two-necked flask; a smaller side neck served as an outlet to the vertical pipe cooler which was ~20 cm long; a wide central neck with stopcock combined a ~25 mL funnel with a ~20 cm long stem reaching to the flask bottom as a sampler and steam input, respectively. A steam riser was realised by a 500-mL distillation bottle with electric heating mantle and was flexibly connected to the steam input. For wet ashing, we used Kjeldahl flasks ~20 cm long with a ~5 mL bulb and sand bath with electrical heating. All pipettes, burettes and volumetric flasks used throughout the Kjeldahl method were recalibrated by weighing with distilled water and the real volumes of all vessels were always recalculated to the given nominal value used in our calculations.

Materials

BSA was from Sigma-Aldrich (Munich, Germany; A0281, 99% by agaro gel electrophoresis, fatty acids and globulin-free, lyophilized powder, M, ~66,000 g/mol). HSA was from Sigma-Aldrich (A3782, 99% by agaro gel electrophoresis, fatty acids free, lyophilized powder, M, ~68,000 g/mol). Bilirubin was from Sigma-Aldrich (B4126, 98% by photometry, powder, M, ~584.0 g/mol). Human Hb was from Sigma-Aldrich (H7379, lyophilised powder). Intralipid™ 20%, fat emulsion for intravenous use was from Fresenius Kabi (Uppsala, Sweden; Intralipid contains in 1000 mL: 200 g of purified soybean oil, 12 g of egg lecithin, 22 g of glycerol and water). Intralipid™ was diluted with physiological solution (PS) before its use for serum spiking.

Four groups of human sera used for the estimation of our reference interval were collected from clear normal sera of “ambulatory healthy patients”; all analytical tests determined in a hospital laboratory were situated within reference intervals. Each testing laboratory collected its own group of 60 samples containing sera of 20 children (aged 2–10 years), 20 women (aged 16–60 years) and 20 men (aged 18–80 years), see Table 2.

Two groups of human sera used for interference tests of endogenous lipids/bilirubin, were collected from patients’ sera with known concentration of lipids (as TGs) and total bilirubin, respectively. All icteric sera were clear, but a group of lipaemic sera combined clear, slightly turbid and some milky turbid samples, see Figures 3–6.

Interference tests based on a serum spiked with exogenous interfering substances were performed with a pool of “normal, clear sera”.

Figure 1 Absorbance of sample blanks in AT (7).

Absorbance BSA, HSA and lipaemic sera in AT was registered at 37°C against AT with PS at 540 nm.

Figure 2 Reaction rate: formation of copper complexes with BSA and HSA (7).

Procedure A. Absorbance of copper complexes with BSA/HSA was measured at 37°C against reagent blank with PS at 540 nm. Protein concentration in a sample (g/L): 67.5 BSA, 74.5 HSA. The increase of copper(II)-HSA absorbance ∆A between 20 and 40 min was approximately 2.3 g/L. Dotted vertical lines indicate the final reading time used by analysers.
Table 1  Analysis scheme: dosed volumes (μL), measurement timing (s) and resulting concentration (mmol/L) of NaOH/CuSO₄ in the incubation mixture.

<table>
<thead>
<tr>
<th>Analyser</th>
<th>Procedure</th>
<th>Dosed volumes: sample/BR1/BR2</th>
<th>Measurement: blank*finalab</th>
<th>Incubation mixture: NaOH/CuSO₄</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hitachi 911</td>
<td>P</td>
<td>6/200/100</td>
<td>72/180</td>
<td>588/11.8</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>7/250*/<em>250</em></td>
<td>72/180</td>
<td>197/6.0</td>
</tr>
<tr>
<td>Konelab 30i</td>
<td>P</td>
<td>4/134/67</td>
<td>90/210</td>
<td>588/11.8</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>3/50/54</td>
<td>90/210</td>
<td>197/6.0</td>
</tr>
<tr>
<td>Advia 1650</td>
<td>P</td>
<td>2.4/90/40</td>
<td>277/290</td>
<td>596/11.8</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>2.8/100/100</td>
<td>277/290</td>
<td>197/6.0</td>
</tr>
</tbody>
</table>

*Blank reading (s) after mixing a sample with BR1. **Final reading (s) after adding BR2. ¹Analyzer dilutes both Roche reagents dispensed into an incubation mixture with the same volume of water: 125 μL.

Table 2  Serum TP (g/L) found in four independent groups of sera by four participants/analysers using two different short-term procedures.

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Calibrator</th>
<th>P</th>
<th>R</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>BSA</td>
<td>HSA</td>
</tr>
<tr>
<td>Analyzer Hitachi I</td>
<td>BSA</td>
<td>71.5/0.7</td>
<td>64.7–81.4</td>
</tr>
<tr>
<td>Median/OR</td>
<td>69.2/0.6</td>
<td>62.9–79.2</td>
<td></td>
</tr>
<tr>
<td>RI</td>
<td>73.0/0.3</td>
<td>65.9–82.6</td>
<td></td>
</tr>
<tr>
<td>Analyzer Hitachi II</td>
<td>BSA</td>
<td>73.4/0.9</td>
<td>59.7–84.1</td>
</tr>
<tr>
<td>Median/OR</td>
<td>70.3/0.8</td>
<td>57.7–80.1</td>
<td></td>
</tr>
<tr>
<td>RI</td>
<td>75.6/0.6</td>
<td>67.7–83.3</td>
<td></td>
</tr>
<tr>
<td>Analyzer Konelab</td>
<td>BSA</td>
<td>72.4/1.0</td>
<td>61.6–83.0</td>
</tr>
<tr>
<td>Median/OR</td>
<td>71.5/1.0</td>
<td>60.3–78.9</td>
<td></td>
</tr>
<tr>
<td>RI</td>
<td>72.4/0.9</td>
<td>62.5–80.4</td>
<td></td>
</tr>
<tr>
<td>Analyzer Advia</td>
<td>BSA</td>
<td>73.1/1.3</td>
<td>57.3–81.2</td>
</tr>
<tr>
<td>Median/OR</td>
<td>69.5/1.2</td>
<td>54.8–77.1</td>
<td></td>
</tr>
<tr>
<td>RI</td>
<td>72.0/1.0</td>
<td>57.1–80.9</td>
<td></td>
</tr>
<tr>
<td>Combined 240 results found by the same procedure</td>
<td>BSA</td>
<td>72.6/1.0</td>
<td>62.6–81.4</td>
</tr>
<tr>
<td>Median/OR</td>
<td>69.9/0.9</td>
<td>60.3–78.2</td>
<td></td>
</tr>
<tr>
<td>RI</td>
<td>73.0/0.7</td>
<td>63.3–82.4</td>
<td></td>
</tr>
</tbody>
</table>

Four sample groups, each group with 60 sera, were analysed in duplicate. Calibrators: our Kjeldahl-assayed standards. Correlation data for combined results (as Y vs. X). Procedure P, HSA vs. BSA: r = 0.970, y = 0.979x + 1.5; SI-II vs. BSA: r = 0.978, y = 0.930x + 2.4. Procedure R, BSA vs. HSA: r = 0.945, y = 0.934x + 5.6; SI-SII vs. BSA: r = 0.970; y = 0.902x + 4.2.

Figure 3  Effect of exogenous lipids added to a serum. Konelab analyser: blanking with reagent R (black triangles), procedure A without any serum blanking (black circles). As a 100% recovery, we took TP concentration found in serum prior to the addition of fat emulsion.

Figure 4  Effect of lipids in native lipaemic sera. Konelab analyser: duplicate measurement, 27 samples, SRM bovine albumin calibrator. TP found (g/L) as an average/range/OR. Procedure A: 75.4/65.3–85.6/1.0, B: 71.1/60.4–81.1/1.0, P: 73.1/59.1–82.4/0.8, R: 73.8/63.0–81.7/0.8. Blanking with reagent P (black squares), with R (black triangles) and procedure A without any blanking (black circles). As a 100% recovery were taken TP values found by check measurement B (horizontal line, white circles).
Reagents

Reagent grade chemicals were used throughout this study. PS was prepared by dissolving 9 g of NaCl in distilled water in a 1000-mL volumetric flask. Bilirubin stock solution ~600 µmol/L was prepared by dissolving 5.9 mg of unconjugated bilirubin in 100 mL of N-methyl-D-glucamine 0.1 mol/L (used instead of NaOH) with 10 mg of Na₂EDTA as a stabiliser and was further diluted with N-methyl-D-glucamine. Hb stock solution in a 25-mL volumetric flask and was further diluted with PS.

A serum spiked with interfering substances was prepared by mixing one part of properly diluted solution of an interferent with nine parts of pooled “normal clear serum”. Native human sera with known concentration of lipids/bilirubin were prepared as described in the Materials section.

Bilirubin stock solution a stabiliser and was further diluted with N-methyl-D-glucamine 98% and was standardised against anhydrous disodium carbonate used as primary standard; disodium carbonate was, before its use, heated for 6 h in an oven at 280°C and then kept in a refrigerator over the silica gel. Prior to protein analysis, we used glycine as a standard to assess the overall Kjeldahl procedure (from wet ashing through steam distillation of ammonia to its titration with sulphuric acid). Glycine was from Loba Chemie (Vienna, Austria; guaranteed as at least 99%, actual batch value attested by the producer was 99.9%). We repeatedly found glycine content of 99.7±0.1% via the Kjeldahl method using our reagents.

Calibrators

We used the National Institute of Standards and Technology SRM 927c as primary standard for TP (18), but only as the calibration in interference tests: ~7% solution of BSA with certified protein concentration of 71.57±0.74 g/L.

Our two calibrators based on pure BSA/HSA proteins were prepared by dissolving approximately 4.2 g of an albumin in 50-mL of PS. Solution was dispensed into glass bottles and...
frozen below −60°C. We found the following TP using the Kjeldahl procedure in our albumin calibrators: HSA 74.13 ± 0.08 and BSA 79.32 ± 0.06 g/L.

Our two serum-based calibrators were prepared from two pools of clear sera from “ambulatory healthy patients” with a lower (SI) and a higher level of TP (SII). Serum pools were dispensed into glass bottles, frozen and stored below −60°C. We found the following TP using the Kjeldahl procedure in our serum calibrators: SI 59.90 ± 0.06 and SII 70.66 ± 0.06 g/L.

Procedures

Concentration of TP in calibrators using the Kjeldahl method was determined 10 times and was statistically evaluated. All other quantitative photometric measurements were carried out in duplicate at 37°C either manually (procedures A/B) or using analysers with a preset software program (procedures P/R, see Table 1). All analysers were two-point calibrated with PS-HSA and PS-BSA, respectively, and three-point calibrated with PS-SI-SII. All reagents used manually were pre-heated to 37°C. TP results listed in all Figures/Tables are the means of duplicate analyses. We used duplicate measurements to calculate an “overall reproducibility (OR)”, see the statistics section.

Sample blanks in AT. In a cell preheated to 37°C, mix 2.50 mL of AT with 0.05 mL of a sample (serum, standard) and register the absorbance for 60 min at 540 nm against AT with PS. Sample blanking in Figure 1 was used to show the difference in the absorbance found during long/short-term blanking.

BSA/HSA rate curves. In a cell preheated to 37°C, mix 2.50 mL of reagent I with 0.05 mL of a sample and register the absorbance for 60 min at 540 nm against reagent I with PS. Rate curves in Figure 2 were used to show differences between a long-term measurement used in the reference procedure and in a short-term measurement used in analysers.

TP by manual procedure A. In a cell preheated to 37°C, mix 2.50 mL of reagent I with 0.05 mL of a sample (serum, standard) and read the absorbance at 540 nm against reagent I with PS. Rate curves in Figure 2 were used to show differences between a long-term measurement used in the reference procedure and in a short-term measurement used in analysers.

TP by manual check measurement B with acetone extraction of lipids. In a test tube, mix 0.05 mL of a sample with 0.30 mL of water, add 5.0 mL of acetone, shake or mix for approximately 1 min and centrifuge. Pour off the supernate fluid, invert the test tube, and let it stand for approximately 5 min. Mix the sediment with 2.50 mL of reagent I preheated to 37°C. Stop the tube and shake for approximately 5 min and pour into a cell preheated to 37°C. Read the absorbance at 540 nm exactly 20 min after mixing the protein sediment with reagent I against reagent blank with PS. We used procedure B (5) as a standard method proved to eliminate lipid interferences (1) and also as a check measurement capable of eliminating interfering serum bilirubin (7, 8).

Interference tests. All automated tests based on a compensation of interfering substances by a short-term sample blanking were performed with the Konelab analyser using two-part reagents/procedures P and/or R, see analysis scheme in Table 1. Results given in Figures 3–6 are for the comparison complemented with direct determination of TP by manual procedure A (without any blanking) and by manual check measurement B involving extraction of lipids and bilirubin from serum samples. All interference tests given in Figures 3–6 were calibrated against SRM bovine albumin (18) and all analyses were carried out in duplicate.

Determination of TP made by analysers. Cooperating laboratories programmed their analysers according to the instructions given in used analytical kits for TP, see analysis scheme in Table 1. Each laboratory separately used either two albumin calibrators or a combination of two serum calibrators SI and SII, all attested by the Kjeldahl procedure. Results are given in Table 2.

The determination of protein nitrogen. Total nitrogen in our albumin/serum calibrators was determined by wet ashing: into a Kjeldahl flask, pipette exactly 0.50 mL of a sample, add 0.5 g of the catalyst and 2.0 mL of sulphuric acid (98%). Into the flask neck, insert a small funnel as a reverse cooler. Place the flask askew into a sand bath and heat it until the mixture turns clear (approximately 40 min). Let it cool, transfer and dilute clear solution with distilled water into a calibrated 25-mL volumetric flask (solution A). A 5.00-mL of solution A corresponds to exactly 0.10 mL of the original protein sample.

Non-protein nitrogen was determined as follows: into a centrifuge tube, pipette 2.50 mL of a sample and the same volumes of distilled water and trichloroacetic acid, mix and centrifuge. Use supernate fluid for the determination of non-protein nitrogen (solution B). A 3.00-mL of solution B corresponds to exactly 1.00 mL of the original protein sample.

Steam distillation procedure: into a distillation apparatus, pipette through a sampling funnel exactly 5.00 mL of solution A (total nitrogen) and/or exactly 3.00 of solution B (non-protein nitrogen), respectively. Wash the sampling funnel with five small portions of water, add approximately 0.20 g of zinc powder and 3.0 mL of NaOH solution. Submerge the cooler outlet into the solution of boric acid in a titration vessel (5.0 mL of boric acid with two drops of indicator, red-orange colour). Distil until the total volume of a solution to be titrated is approximately 25 mL (approximately 15 min, blue colour) and wash up inner walls and cooler outlet with water into the titration vessel.

Acidimetric titration: using a calibrated 10-mL burette with capillary tip, titrate distillate from solutions A and B, respectively, with a solution of 0.01 N sulphuric acid until the colour changes to orange-red. One millilitre of exactly 0.01 N H2SO4 corresponds to 0.140 mg of nitrogen.

Kjeldahl calculation: TP (in g/L) = (V1−V2) × 0.14 × 10 × F, where V1 is a utilisation of exactly 0.01 N sulphuric acid in mL for total nitrogen, V2 is one-tenth of a utilisation of sulphuric acid for non-protein nitrogen, F = 6.25 (100% divided by 16%) is the factor commonly used for recalculation of serum protein nitrogen (%) to protein (g/L).

Statistics and calculations used in this study. Standard robust summary statistics were used to describe the primary data (median, minimum, maximum, percentiles). Pearson product-limit correlation was applied for the validation of consistency of methods. Differences between procedures were analysed by means of the paired t-test. A probability level of α = 0.05 was considered as the critical level of statistical significance for all analyses. The analyses were performed using Statistica for Windows 8.0 (19). We also used a sum of absolute values of differences between duplicate measurements divided by a number of sera to calculate a participant OR as our simple measure of their analysis accuracy. Our reference intervals (RIs) of serum TP were calculated as 3rd–97th percentile (Table 2) using 60 results.
obtained by an individual participant and procedure, combining all 240 results from all participants together according to used procedures and/or combining all 480 results obtained by all participants and used procedures.

Results

The differences between a long-/short-term serum blanking and between long-/short-term measurements of the final absorbance of biuret complexes with serum proteins are given in Figures 1 and 2. Figure 1 shows processes running in AT with proteins during a long-term blanking. While an absorbance of a blank with both fat-free albumins remained nearly constant, the one with lipaemic sera exhibited at least two different phases: in the first phase, the absorbance decreases and more or less turbid blanking mixture is slowly cleared, while in the second phase the absorbance rises significantly. Figure 2 shows rate curves of copper complexes with HSA/BSA in a long-term measurement. While the final absorbance of a complex of copper-BSA remains practically unchanged after 20 min, the one with HSA or with a human serum rises slowly but constantly all the time, the absorbance rise \( \Delta A \) between 20 and 40 min being approximately 2.3% or \( \sim 1.6 \) g/L. A short-term final measurement is now usually done in the 3rd or in the 5th minute on a steeply rising part of the rate curve.

Determination of serum TP was performed with two types of two-part reagents now used in automated procedures: procedure/reagent P (12) following closely formulation of BR from the reference procedure (1) and procedure/reagent R (11) introduced around 1991 by Boehringer Mannheim Diagnostics for their Hitachi 717 automatic analyser and based on the modified Weichselbaum’s reagent (20). Reagent R contains 0.2 mol/L NaOH and 6.1 mmol/L copper sulphate, which correspond to only one-third of NaOH and one-half of copper sulphate used in the reference reagent I. Compared with R, reagent P gives approximately 1.4 times greater colour yield with proteins, which means that P is “more aggressive to protein, splits protein to a greater number of peptide chains acting as new biuret ligands” (8). We applied both reagents/procedures given above to the Konelab analyser and tested interferences caused by lipids, bilirubin and Hb. All analyses in interference tests were carried out in duplicate and were calibrated with SRM albumin (18).

Interference caused by lipids

First of all, we simulated lipids by a serum spiked with Intralipid™ emulsion. Added emulsion of fat uniformly increased turbidity in the incubation mixture which was easily compensated by sample blanks up to \( \sim 18 \) mmol/L TGs (Figure 3).

The effect of native endogenous serum lipids on TP and their compensation by serum blanking was tested on a group of lipaemic sera with known concentrations of TGs (Figure 4). Compared with check measurement B, a direct determination without any serum blanking (procedure A) yielded TP results generally shifting to higher values \((+2–4 \text{ g/L})\), the extreme differences in individual results being up to 6 g/L. Both blanking procedures P/R partly lowered interference of endogenous lipids but compared with B gave results \( \sim 3%–4\% \) higher, and mutual correlation P and/or R vs. B was poor. A reagent P with its greater concentration of both active biuret components gave results a little closer to procedure B.

Interference caused by bilirubin

The effect of bilirubin on TP and its correction by sample blanking was also tested in two experiments. The first one was again based on a serum pool spiked with a solution of unconjugated free bilirubin (Figure 5), the procedure which was also employed by the WG (1) and which is still frequently used in interference tests for bilirubin. Similar to fat emulsion, added bilirubin uniformly increased the final absorbance and thus also TP in a tested sample and was again easily compensated by blanking up to \( \sim 700 \) µmol/L.

The second experiment (Figure 6) was carried out with a group of native icteric sera with known concentrations of total bilirubin. In this experiment, we also utilized procedure B as a check measurement because we previously found that acetone also releases/solubilises bilirubin from its protein bonds (7, 8); acetone acts on bilirubin similar to methanol introduced as a bilirubin accelerator by Malloy and Evelyn in their total bilirubin method (21). Even though both P/R procedures lowered the effect of endogenous bilirubin a little (compared with A), resulting TP was always approximately 3%–6% greater (compared with B) and results were shifted mainly to a positive offset. Comparing P and R, the first one was a little closer to measurement B with its greater concentration of CuSO4/NaOH.

In the interference tests with Hb, we were forced to use only serum spiked with a solution of Hb, having no other simple reference/check measurement. We found that both P/R procedures gave approximately 5% lower but still acceptable TP results (cf. (4)), reagent R being a little closer to a 100% recovery (detailed data are not shown).

The estimation of TP reference interval

Analyses were made in four cooperating laboratories using three different types of automatic analysers. Each laboratory set up their analyser a little differently (Table 1) but within limits given in the instructions for used analytical kits (11, 12). Analysers were two-point calibrated (PS-HSA or PS-BSA) and three-point calibrated (PS-SI-SII) with our standards assayed by the Kjeldahl method. Results are given in Table 2 and a more detailed statistical evaluation is listed in Table 3.

First of all, using the Advia analyser, procedure P and a group of 21 sera, we tested the effects of two reaction times, 3 vs. 5 min, used in analysers. The difference found between medians was only 0.3 g/L and also correlation data \((y \text{ vs. } x)\) were excellent:
and Advia analysers (approximately 1.1 g/L or

Apparently, lower accuracy reached with the Konelab

R is obviously caused by its very different formula-

A better accuracy reached with reagent/procedure

samples and reagents dosed for analysis, see Table

with reagent R) is probably due to smaller volumes of

A practical point of view, the results can be considered

The paired t-test with \( p < 0.05 \) was significant. From

Both Hitachi analysers worked with a better accu-

and alkali is less aggressive to proteins than reagent/

procedure P (8).

Discussion

A sample blanking (a subtraction of the absorbance of

serum blank in an incomplete reagent) is the usual

method to eliminate or at least to suppress the effect

of some interfering substances in sample matrix.

Long/short-term blanking differ both in the time of

contact/reaction of serum components with AT, in

blanking sequences and thus also in blanking

efficiency.

The use of an artificial fat emulsion mixed with

pooled serum was originally introduced by some

manufacturers of diagnostics (Roche, Bayer, etc.) to

simulate lipid interference in their validation tests. We

proved that this procedure for the biuret method is

quite misleading. This is in agreement with a study

by Bornhost et al. (22) who observed “only minor

assay interference caused by Intralipid supplementa-

tion compared with endogenous lipids because of the

lack of higher-order lipid-lipoprotein complexes”.

We previously hypothesised (6) that “endogenous

lipids protected as micelles and bound in lipoproteins/

chylomicon are in an alkaline solution gradually
decomposed to unprotected fat droplets which are able
to increase the turbidity either alone or after their

alkaline saponification to sparingly soluble free fatty

acids”. Free acids could form insoluble soaps either

with serum-present cations or with copper ions pres-

ent in a great excess in a BR itself. While a long reac-
tion time used in long-term blanking can cause a deep

chemical change of fat micelles to unprotected fat

droplets and possibly also to saponify fat to free

acids, a short-term blanking most probably cannot.

Moreover, a serum blank is measured in the reference

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acids, a short-term blanking most probably cannot.

Moreover, a serum blank is measured in the reference

method quite separately and only in a solution of AT.

In all short-term procedures, a BR2 with copper-tar-

trate is always added into the same serum blank in

<table>
<thead>
<tr>
<th>Procedure</th>
<th>P</th>
<th>R</th>
</tr>
</thead>
<tbody>
<tr>
<td>Analysers Hitachi I</td>
<td>Median 0.49*</td>
<td>1.20*</td>
</tr>
<tr>
<td>Range -0.21/1.25</td>
<td>-0.80/1.83</td>
<td></td>
</tr>
<tr>
<td>Analysers Hitachi II</td>
<td>Median -0.65*</td>
<td>-1.84*</td>
</tr>
<tr>
<td>Range -2.10/0.97</td>
<td>-2.53/-1.42</td>
<td></td>
</tr>
<tr>
<td>Analysers Konelab</td>
<td>Median -0.50*</td>
<td>1.78*</td>
</tr>
<tr>
<td>Range -3.05/4.00</td>
<td>-0.83/4.70</td>
<td></td>
</tr>
<tr>
<td>Analysers Advia</td>
<td>Median 0.33</td>
<td>2.10*</td>
</tr>
<tr>
<td>Range -0.55/0.95</td>
<td>1.60/2.85</td>
<td></td>
</tr>
<tr>
<td>All analysers, combined group of 240 sera</td>
<td>Median 0.13</td>
<td>1.29*</td>
</tr>
<tr>
<td>Range -2.02/1.58</td>
<td>-2.20/3.05</td>
<td></td>
</tr>
</tbody>
</table>

The statistical difference was analysed using the paired t-test. *Significant differences at \( \alpha = 0.05 \) level.
BRS. Just as an AT, a BR also decomposes fat complexes, hydrolyses fats and forms insoluble soaps and evidently partly acts against sample blanking.

We also observed (Figure 4) that the behaviour of serum endogenous lipids in the biuret reaction differs from sample to sample depending on specific features of their lipid particles, probably on their size and stability. In this respect, all so-called normal nonlipaemic sera, i.e., clear sera with a low TG, could behave in the biuret reaction like strongly lipaemic and vice versa. In concordance with Twomey et al. (23), we verified a concentration of serum TG could not be explicitly used for predicting lipid interferences even in the biuret reaction. It is evident that a short-term blanking is not capable of sufficiently suppressing the effect of serum lipids. In this respect, a long-term blanking used in the reference method is apparently more efficient and is able to compensate endogenous serum lipids up to ~11.5 mmol/L TGs (see the WG data we recapitulated briefly in the introduction section).

Similar to the Jaffe reaction, an elimination of bilirubin interference in the biuret reaction is based on its oxidation to biliverdin. The oxidation (running in an alkaline media spontaneously and needing usually tens of minutes) can be catalysed by ferricyanide (24) or by copper complexes (25). Every BR acts as a catalyst, its catalysing efficiency being dependent on the concentration of its active biuret components, reaction time and temperature. During a long-term blanking (reference method), bilirubin can be oxidised in AT to biliverdin even without any catalyst, while during a short-term blanking, it apparently cannot: its oxidation proceeds mainly after adding the second part of a BR containing catalysing copper-tartrate. This probably also explains why reagent P with its doubled concentration of copper ions and three times greater concentration of NaOH is a little more efficient compared with R (Figure 6, Table 2). We also verified that the interference test for bilirubin based on a serum spiked with solution of free bilirubin is misleading (at least in the biuret reaction). Such a test cannot substitute for all serum-present bilirubin forms (free, conjugated, free bound to albumin, etc.).

Hb, being a haemoprotein, can interfere both by its own colour and as a protein reacting with a BR as well. Serum blanking can only correct/substract Hb colour, its protein interference being unavoidable. We confirmed an observation made by the WG (1) that “if uncorrected by a sample blank, Hb increases TP”.

A short-term serum blanking is evidently capable of subtracting an absorbance caused by serum-present coloured/turbid interfering substances, but its capacity to transform them into some other not-interfering products is low. It is important to note that increased TP found in lipaemic/icteric sera (Figures 4, 6) correlates well with increased TP (~2.4 g/L) found in serum samples from “normal healthy ambulatory patients”. Lipids and bilirubin are apparently the crucial serum interferents in the biuret method for TP.

Both P/R reagents have nearly the same performance data (see the section on reagents), even though P with a greater concentration of its active biuret components is more aggressive to proteins (8) and exhibits approximately 1.4-times greater colour yield. On the other hand, a less aggressive R operates with a slightly better accuracy compared with P (~0.7 g/L and ~0.9 g/L, respectively).

A calibration with BSA/HSA gave practically the same TP with the medians differing only within usual OR limits (Table 2). Also, BSA/HSA correlation data, listed in the footnote in Table 2, are quite promising. This, together with their very similar rate constants (6) valid for a rapid stage of the biuret reaction, indicates that BSA/HSA might be theoretically used as calibrators in all short-term procedures. A warning by the WG “not to use bovine solutions as standards with short incubation times because human albumin reacts slower than bovine albumin and protein in sera is underestimated” is apparently inaccurate. In fact, according to its rate constants (6), HSA reacts really quicker, but its absorbance (compared with BSA) rises slightly/constantly in the final reaction phase used for end-point measurement in the reference method and not in the rapid reaction phase used in analysers. We repeatedly confirmed that a different behaviour of BSA from HSA and/or from a human serum during a long-term measurement is their typical and inherent feature (8).

To demonstrate a current/real situation in the determination of serum TP in patients, we also combined all 480 results together. Combined medians/RIs found with our BSA/HSA calibrators were 72.8/73.1, respectively, and 62.8–81.6/62.0–82.0, respectively. They are nearly the same and very similar to data published by the WG (1, 2) for their combined results calibrated with SRM bovine albumin (see Introduction section), which is further proof that the biuret reaction provides results depending primarily on used calibrator and not on a BR, reaction procedure/scheme and analyser.

We also expected that very close rate constants found with proteins in our EDTA-chelated BR (6) will also be similarly and mutually close in a tartrate-chelated BR. Our expectation was proved correct but only by results obtained with the same BR: compare nearly the same means obtained with BSA/HSA calibrators lying within OR reached by individual procedures P/R, respectively. Each of the P/R reagents with their very different concentration of NaOH/CuSO₄ has to react with proteins in the rapid reaction phase with a different velocity, because each reagent’s formulation is responsible for a considerably distinct amount of new peptide ligands formed/splitted from proteins during the slow reaction phase (8).

Both groups of results listed under procedures P/R and all 480 results combined together clearly show the positive TP bias (approximately 5%) caused by calibrators based on pure proteins. Contrary to both pure albumins, calibration with the Kjeldahl-assayed serum standards gave a distintively lower TP result. The explanation results from a generally poor capacity of both short-term procedures to compensate for interfering serum substances, such as lipids and bilirubin. A pure albumin, not depending on its bovine/
human origin, gives a final absorbance corresponding only to its copper-protein complexes with a BR, while also includes the absorbance of serum interferents.

A calibration with BSA standards still used in all biuret procedures leads to a positive offset of serum TP in both long-/short-term measurements, even though for different reasons. The positive bias we observed in the reference method (7, 8) consists mainly of a different behaviour of BSA (compared with human serum) during the final reaction phase (8), while for the positive bias in all short-term measurements serum-present interferents are responsible, which are poorly uncompensated by a short-term blanking (16).

A positive bias of serum TP caused by albumin calibrators is approximately 3% in healthy men and a positive bias of approximately 5% can be expected in patients with increased lipids and bilirubin. Such a systematic bias alone exceeds a 3.4% limit of TE fixed for serum protein (15), leaves no room for other performance imprecisions and eradicates the clinicians’ ability to distinguish a real TP change in patients (14).

Conclusions

1. Rate curves illustrating the formation of biuret complexes with proteins show three reaction stages (7, 8): an abrupt rise of absorbance (rapid phase, several seconds, formation of copper-protein complexes) gradually decelerates (slow phase, several minutes, alkaline proteolysis of protein ester/amide bonds to shorter fragments bound immediately into further copper complexes) and passes into a final phase (20–60 min). In the final phase, the absorbance of copper complexes with BSA remains stable or even slightly diminishes while the absorbance of complexes with HSA and/or with serum proteins rises slowly but permanently all the time.

2. A Biuret reagent, procedure and calibration with SRM bovine albumin as a primary standard (18) developed in 1981 are still valid (1) as the reference method for TP in human sera. Moreover, SRM bovine albumin is generally recommended as a reference material for all TP assays by colorimetric methods.

3. Similar to most of the reference methods even this one is not suitable for automation. Biuret automated procedures now use two-part reagents and short-term measurements starting in a rapid reaction phase.

4. A capacity of a short-term serum blanking to compensate for the main serum interferents, such as lipids and bilirubin, is poor. Each calibration based on a pure protein shall be responsible for a significant TP bias in serum samples.

5. The simplest method to improve both reference/current short-term biuret procedures is the use of a human serum standard with TP assayed by the Kjeldahl method. A calibrator, behaving analogously to serum samples, will compensate for positive bias in most normal sera.

6. A formulation of BR used in the reference method (1, 2) seems to be unnecessarily aggressive to proteins and is responsible for worse reproducibility compared with procedures using a BR with lower concentration of active biuret components. A reference method is supposed to yield results close to the true value. This method (1, 2) provides unacceptable positive bias which must not be tolerated intentionally. Therefore, it is recommended to revise the whole reference method.

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