quentional monitoring in the pre-eclamptic and diabetic patient throughout the third trimester of pregnancy. These two conditions require rapid daily estimates of the urinary estrogen (4). Because concomitant renal insufficiency is so frequent, an estrogen/creatinine ratio (E/C ratio) is far more meaningful to the clinician. Daily fluctuations in total estrogen values are minimized by this method. Likewise, when values are changing rapidly and 2-h specimens are requested to evaluate the state of the pregnancy, variations in data are less dramatic when the E/C ratio is used (5, 6). In this study, the contrary is true. In Table 2, one will note that a false decrease in urinary estrogen values is accompanied also by a marked increase in creatinine excretion, owing to the diuretic, thus resulting in even a further false decrease in the E/C ratio. An additional advantage of using the salting-out approach is that glucosuria does not have any effect on the extraction and subsequent quantitation of total placental estrogens. Here again, the E/C ratio is a far more stable value than the estrogen value alone.

These results illustrate a major technical problem to the laboratory that uses the method of Rourke et al. (2), at present widely used by those that do this assay. Although the procedure of Rourke et al. does not involve hydrolysis with hot acid, which causes a false decrease in estrogen levels (1), a remarkable apparent decrease in total urinary estrogens is encountered. These results indicate a direct inhibition of or interference with the Kober chromogens that are formed by reaction of estrogen and the hydroquinone and sulfuric acid reagent. Calculated concentrations, which are admittedly high because of the assumption of total absorption of the hydrochlorothiazide, would indicate that a level of only 0.015 μg (2.0 × 10⁻⁷ moles) of drug per milliliter of urine results in a 40% artifactual decrease in the apparent endogenous secretion of estrogens.

When the recorded spectral scans are carefully examined, after use of pure estriol standards plus 0.01 to 0.5 mg of hydrochlorothiazide per standard tube, one also notes a 5- to 7.5-nm bathochromic shift in addition to the obvious decrease in color intensity. By itself, this small bathochromic shift would not account for the observed decrease in calculated concentration.

The Kober chromogens have not been structurally characterized, and so we cannot specify the chromophores participating in the reaction. It would involve protonation of the phenolic ring A, oxonium ion formation by the carbonyl group, and carbonium ion structures of the hydroxyl groups. Thus, the exact nature of the mechanism of the inhibition we report here must remain obscure at present.

This raises the question as to how one might circumvent these analytical problems. One solution would be to use only chlorothiazide in the pregnant patient. This is not always practicable, especially when a value is needed after the medication is given. We know of no short, obviating analytical maneuver.

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References


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Some Drugs and Other Substances That Interfere with Protein Determination

D. H. Pashley, G. S. Schuster, P. Palmer, and M. M. Sharawy

We show that a variety of substances may interfere positively, to different extents, with the determination of protein by either the Lowry procedure or absorbance at 280 nm, or both. Some of these substances may interfere in concentrations that could be present in clinical laboratory specimens or in research samples.

Additional Keyphrases: Folin–Ciocalteu reagent for proteins • Lowry method for proteins • ultraviolet measurement of proteins

The Lowry procedure (1) for colorimetry of proteins is widely used. Several variations or modifications of the technique have been developed, all based on use of the Folin phenol reagent, as is the original technique. The reaction is not specific, and many compounds, of several different chemical types, interfere with it. The degree of interference may vary with the particular modification from the Departments of Oral Biology, Physiology, and Cell and Molecular Biology, Medical College of Georgia, Schools of Dentistry and Medicine, Augusta, Ga. 30902.
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used. Substances that have been shown to interfere include: purines, phenols, and hydrazines (1); buffers such as tris(hydroxymethyl)aminomethane (2); N-(2-hydroxyethyl)piperazine-N-2-ethanesulfonic acid ("HEPES"), N,N-bis-(hydroxyethyl)glycine ("Bicine"), N-tris(hydroxymethyl)glycine ("Tricine"), and other analogous compounds (3–5); sodium salicylate, acetylsalicylic acid, and some antibacterial agents (6, 7); sugars, including hexosamines (8–11); sulfhydryl and disulfide reagents such as dithioerythritol and 2-mercaptoethanol (12); oxidized lipids (13); and catecholamines and their analogs (7). Studies in which these methods are used for protein estimation generally do not point out possibly substantive interferences with the reaction.

While conducting studies of various pharmacologic agents, we noted some interference with the Lowry color reaction. Because the urinary concentration of many of the interfering substances may become relatively high, we measured the interference by these compounds and some of their derivatives. The results are reported here.

Materials and Methods

Protein was measured by the Oyama and Eagle (14) modification of the method of Lowry et al. (1). This involves use of an alkaline copper solution made from Na₂CO₃, NaOH, sodium potassium tartrate, and CuSO₄·5H₂O, and of a second solution, the Folin–Ciocalteu reagent. A standard curve (0–100 μg of bovine serum albumin in 60 ml total volume) was prepared. The zero-protein tube served as a blank.

For the Lowry method, test substances were freshly prepared in distilled water in a final concentration of 4.6 × 10⁻⁶ mol/liter, except for albumin, which was in a concentration of 2.2 × 10⁻⁷ mol/liter (based on a molecular weight of 69,000). Color was measured after 30 min at 660 nm in a Model 240 Spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio 44074), with a 1-cm light path.

Ultraviolet absorption was measured at 280 nm in quartz cuvets (1-cm light path). Concentrations of test substances measured at 280 nm were 1 × 10⁻³ mol/liter, except for sodium p-aminobenzoate and p-aminobenzoic acid, which were at 1 × 10⁻⁴ mol/liter, and albumin, which was at a concentration of 1.4 × 10⁻³ mol/liter.

Results

As Table 1 shows, numerous substances interfere with the Oyama and Eagle (14) modification of the Lowry method for protein determination. Here, they are divided into four groups: those that interfere severely (equivalent to 100 μg of protein or greater in the 6.6 ml volume), moderately (equivalent to 25 to 100 μg of protein), slightly (up to 25 g of protein), and those that do not interfere.

The greater sensitivity of the modified Lowry method as compared with the measurement of absorbance at 280 nm is shown in Table 1 when one compares the extinction coefficients of bovine serum albumin estimated by the two methods. Absorptivity by the modified Lowry method is twenty-eight times that of the ultraviolet method.

The fact that the absorptivity for many of the interfering substances is high at both 660 nm (with the Lowry reagents) and at 280 nm (in the absence of these reagents) indicates that both methods of estimating protein are subject to serious interferences.

Discussion

Our results are consistent with the numerous previously published reports of the nonspecificity of the Lowry method (2–13). Despite these reports, many laboratorians are

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Table 1. Interference with Protein Determinations

<table>
<thead>
<tr>
<th>Substance</th>
<th>Absorptivity, (modified Lowry method)</th>
<th>Protein equivalenta</th>
<th>Absorptivity, 280 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine serum albumin</td>
<td>1.19 × 10⁴</td>
<td>. . .</td>
<td>4.19 × 10⁴</td>
</tr>
<tr>
<td>Tyramine-HCl</td>
<td>1.19 × 10⁴</td>
<td>210</td>
<td>2.47 × 10³</td>
</tr>
<tr>
<td>Dopamine</td>
<td>1.11 × 10⁴</td>
<td>206</td>
<td>2.20 × 10³</td>
</tr>
<tr>
<td>Levarterenol bitartrate</td>
<td>9.39 × 10⁴</td>
<td>165</td>
<td>2.47 × 10³</td>
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<tr>
<td>D-Norepinephrine bitartrate</td>
<td>8.37 × 10⁴</td>
<td>147</td>
<td>2.53 × 10³</td>
</tr>
<tr>
<td>Epinephrine-HCl</td>
<td>7.20 × 10⁴</td>
<td>127</td>
<td>2.95 × 10³</td>
</tr>
<tr>
<td>Adrenochrome</td>
<td>6.89 × 10⁴</td>
<td>121</td>
<td>2.14 × 10³</td>
</tr>
<tr>
<td>L-DOPA</td>
<td>6.26 × 10⁴</td>
<td>110</td>
<td>2.50 × 10³</td>
</tr>
<tr>
<td>L-Epinephrine bitartrate</td>
<td>4.76 × 10³</td>
<td>84</td>
<td>2.65 × 10³</td>
</tr>
<tr>
<td>Sodium iodohiippurate</td>
<td>4.43 × 10³</td>
<td>78</td>
<td>. . .</td>
</tr>
<tr>
<td>Isoproterenol</td>
<td>3.48 × 10³</td>
<td>61</td>
<td>2.62 × 10³</td>
</tr>
<tr>
<td>p-Aminobenzoic acid</td>
<td>3.46 × 10³</td>
<td>61</td>
<td>2.89 × 10³</td>
</tr>
<tr>
<td>Iodopyractef (&quot;Diodrast&quot;)</td>
<td>3.33 × 10³</td>
<td>59</td>
<td>3.06 × 10³</td>
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<tr>
<td>Ephedrine sulfate</td>
<td>2.63 × 10³</td>
<td>46</td>
<td>. . .</td>
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<tr>
<td>Dextran 75</td>
<td>1.96 × 10³</td>
<td>36</td>
<td>2.47 × 10²</td>
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<td>Dextran 40</td>
<td>1.41 × 10³</td>
<td>26</td>
<td>4.38 × 10²</td>
</tr>
<tr>
<td>Sodium p-aminobenzoate</td>
<td>1.24 × 10³</td>
<td>22</td>
<td>5.84 × 10³</td>
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<tr>
<td>p-Aminobenzoic acid</td>
<td>8.70 × 10²</td>
<td>15</td>
<td>2.52 × 10³</td>
</tr>
<tr>
<td>Pilocarpine</td>
<td>3.04 × 10²</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>&quot;Renografin&quot;b</td>
<td>2.61 × 10²</td>
<td>4</td>
<td>2.24 × 10³</td>
</tr>
<tr>
<td>Tuaminoheptane sulfate</td>
<td>1.52 × 10²</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Creatinine</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Inulin</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

a"Renografin" consists of (per liter) 0.72 moles of meglumine diatrizoate and 0.13 moles of sodium diatrizoate.

bProtein equivalents were calculated for the Lowry data by using the albumin standard curve, and are expressed as μg/6.6 ml, the final volume in the analytical mixture.
not aware of the number and variety of substances that give positive reactions with this assay method. Color development is due to the presence of tyrosine and tryptophan as well as interaction between copper and the amino nitrogen of peptide bonds (15). The color development seen with dextran suggests that an additional mechanism is involved.

Many of the interfering substances listed in the references are not normally present in blood at concentrations that would produce a significant color (16). The urinary concentrations of substances that are excreted by the kidneys, however, are often one or two orders of magnitude higher than their plasma concentrations (17). Such is the case for penicillin, a substance reported by Zondag and Van Boetzeela (6) to interfere with the Lowry method. p-Aminohippurate (PAH) is another interfering substance (Table 1) that is secreted into the urine and achieves a high urinary concentration. Although plasma concentrations of PAH, as it is used in renal function tests, are only 1-2 mg/100 ml (0.05-0.1 mmol/liter), the urinary concentrations may be 400-800 mg/100 ml (20-40 mmol/liter). The extinction coefficients listed for PAH in Table 1 indicate that high urinary PAH concentrations would interfere significantly with either method. This emphasizes the risks involved in attempting to measure protein in the urine by these procedures.

Clinically, glomerular filtration rate is estimated by measuring the renal clearance of endogenous creatinine or exogenous insulin (17). Fortunately, neither of these substances interferes with protein determination by either method (Table 1).

The therapeutic use of L-DOPA in parkinsonism is accompanied by relatively high urinary concentrations of dopamine (18). Patients receiving 1-g oral doses of L-DOPA excrete urine containing up to 2 μg of DOPA per milliliter, while the dopamine concentration in the same urine may be as great as 25 μg per milliliter (18). It is not unusual for patients to receive 3-10 g of DOPA per day, which would provide even higher amounts in urine. Even 25 μg of dopamine per milliliter in urine would give significant interference with the Lowry method (Table 1).

Dextran, used as plasma expanders, are excreted by the kidneys. The urinary concentration of dextran in human urine after intravenous administration of 500 ml of 10 g/100 ml dextran with an average molecular weight of 40,000 varies between 1-11 g/100 ml (19). Such urinary dextran concentrations would cause significant interferences with the Lowry method. Moore and Sax (20) recently reported that serum containing dextrans became turbid when biuret reagent was added, thereby causing interference. The extinction coefficient for dextran listed in Table 1 was the results of actual color, not just turbidity.

Many laboratories estimate protein concentration by measuring the absorbance of these solutions at 280 nm. Most of the absorbance of proteins at this wavelength is due to the tyrosine and tryptophan content. The data in Table 1 indicate that many of the compounds that give high extinction coefficients at 660 nm with the Lowry reagents also give high extinction coefficients at 280 nm. Although the extinction of the drugs at 280 nm is an order of magnitude lower than the extinction of albumin at 280 nm, if these drugs are present at equal or greater molar concentrations, compared to protein, serious errors in protein estimation might result.

Drugs that are used in vitro are frequently used at higher concentrations than in vivo. This fact, coupled with the high sensitivity but low specificity (Table 1) of the Lowry method, precludes the use of this technique for estimating protein in situations where the difference in the extinction between drug and protein is small.

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References


