Quantitation of Sputum Protein by Use of the Biuret Reaction

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A simple, accurate method is described for quantitatively determining the protein content of sputum by use of the biuret reaction. Fifteen mechanically homogenized specimens were examined with the biuret reagent described by Rosenthal and Cundiff, and the results compared with those obtained by the microKjeldahl technique. Over a range of 542 to 1917 mg of protein per 100 ml, the techniques correlated significantly. Difficulties with deproteinization of the sputum before nonprotein nitrogen was determined were overcome by the use of zinc sulfate and pH adjustment. The proposed method is fast and reproducible.

Additional Keyphrases microKjeldahl results compared • nonprotein nitrogen content • EDTA stabilizer • variation among patients

Increasing interest in the various chronic bronchopulmonary diseases has stimulated investigation relating to the chemical, physical, and cellular characteristics of sputum. Workers recently investigating protein composition of the sputum generally have used electrophoretic and immuno-electrophoretic techniques, which provide only qualitative data.

We sought a simple method for the quantitative determination of the sputum protein, one that could also be used to check the protein concentration at various stages of preparing the sputum. We encountered difficulties with the most commonly used Kjeldahl method, which prompted us to search for an easier and more reliable method. Since Autenrieth (1) first used the biuret reaction in 1917, the basic reagent has been subjected to many modifications (2, 3). The biuret reagent described by Rosenthal and Cundiff (4) best suited our purposes.

Materials and Methods

Patients

To evaluate this new technique, the concentration of protein in sputum specimens collected from patients with chronic productive cough was measured by both the biuret and Kjeldahl methods. Fifteen specimens were selected from clinically stable out-patients of the Lung Station (Tufts) at the Boston City Hospital who had sufficient volume for the necessary tests. The patients' diagnoses were either chronic bronchitis or chronic asthmatic bronchitis. These specimens varied widely with respect to degree of purulence, mucoid nature, and thickness. No visible hemoptysis was present.

In applying the biuret (or Kjeldahl) method to sputum, we usually found it helpful to first homogenize the viscous secretions. Fresh, unfrozen, 24-h collections of sputum were used.

Procedure

The specimen is placed in a VirTis homogenizing flask (The Virtis Company, Inc., Gardiner, N. Y.) for use with the VirTis Model 23 homogenizer and homogenized at 3660 rpm for 30 s, the speed is then reduced to 2400 rpm for 10 min, then to 1800 rpm for 10 min. The homogenized sputum is allowed to remain undisturbed at about 4°C. The liquefied layer that separates is aspirated with a pipet, and provides the material for subsequent analysis. The amount of original sputum volume required depends on the amount needed to provide a minimum of 1 ml of this liquid phase. Generally, a minimum of 5 to 10 ml of starting specimen is required.

Since the biuret method depends on the use of a clear solution, we needed to know if the liquid phase is representative of the whole sample; this was checked by comparing the nitrogen content of this liquid layer with that of the remaining plug of homogenized material. They were found to be equivalent, so we assumed that the protein concentration in the liquefied layer was indeed representative.

One milliliter of this liquid phase is diluted and mixed with 4 ml of distilled water. In secretions having a high protein content, a greater dilution with distilled water may be required. Distilled water is used rather than a balanced salt solution.

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because there are sufficient salts present in sputum to provide an adequate electrolyte solvent in which the proteins are soluble. The mixture is then centrifuged at 3000 rpm for 20 min. The supernatant fluid is removed for the biuret analysis.

Place 2.4 ml of this supernatant fluid in a test tube, add 3.6 ml of the biuret reagent, and mix by inversion. The biuret reagent (4) is prepared by dissolving 1.50 g of copper sulfate (CuSO₄·5H₂O) in about 500 ml of water. Add 6.0 g of disodium EDTA and 1.0 g of KI and dissolve. Add 300 ml of 2.5N sodium hydroxide, mix well, and dilute to 1 liter with distilled water. This reagent is stable when stored at room temperature in polyethylene containers. The mixture of supernatant fluid and biuret reagent is kept at room temperature for 24 h, then filtered through a 12-μ Preflex membrane (Carl Schleicher & Schuell Co., Keene, N.H.). Place this entire filtrate in a round 19 × 105-mm cuvet. In a separate cuvet prepare the reagent blank control, with 0.9% saline in place of the supernatant fluid. Determine the transmittance (%T) of the filtrate at 555 nm in a suitable spectrophotometer (a Coleman Junior was used in this study) adjusted to 100% T with the reagent blank. The protein concentration, determined by reading the corresponding %T as obtained from a standard protein curve, is multiplied by the dilution factor used.

Our standard protein spectrophotometric curve was determined with the Coleman spectrophotometer and round 19 × 105-mm cuvets. Normal human serum of known protein concentration was diluted through multiples of 10 to a dilution of 1:80. This provides a linear graph with 40 to 80% T at a wavelength of 555 nm, a range suitable for protein concentrations of about 80–350 mg/100 ml, if low protein samples are used undiluted; or 400–1750 mg/100 ml, when samples containing more protein are diluted fivefold. Greater dilutions are used for samples with protein concentrations greater than 1750 mg/100 ml.

The control method used in this study was the classical quantitative microKjeldahl distillation technique according to the Pregl method. The homogenized sputum sample was diluted 10-fold and a 1.0-ml sample taken for acid digestion. The ammonia that distills over was subsequently titrated with 0.01N hydrochloric acid, which is equivalent to 0.14 mg of nitrogen per ml.

The procedure for the nonprotein nitrogen analysis is based on the precipitation of the proteins in the homogenized sputum with ZnSO₄ (6). To 1.0 ml of homogenized sputum add 8.0 ml of distilled water and mix. To this mixture enough 0.5N sodium hydroxide or 0.67N sulfuric acid is added if necessary to bring the pH to 4.5, since it was noted that this pH gives consistently clear filtrates. After mixing, 0.5 ml of ZnSO₄ (10 g/100 ml) is added and stirred, followed by 0.5 ml of 0.5N NaOH. The solution is thoroughly stirred and allowed to stand 10 to 20 min before centrifuging. An aliquot of the filtrate is digested and assayed by the above Kjeldahl method. The nonprotein nitrogen concentration is deducted from the total nitrogen, and the protein, in mg/100 ml, is calculated by multiplying by the accepted constant, 6.25, for expressing nitrogen in terms of protein.

**Results**

The results of the 15 sputum protein determinations are tabulated in Table 1. Only fresh sputum samples were used, to avoid the complication introduced by the variable degree of proteolysis that occurs on standing, which affects the results by the biuret method. The pH of the fresh sputum samples was about 5.0 (range, 4.3 to 7.0).

There was considerable variation in the sputum protein concentration within this small group of patients (Table 1), but the results of microKjeldahl and biuret methods agreed closely; in 13 of the 15 specimens, the results differed by less than 5%. There was an insignificant tendency for results of the biuret method to be higher: the mean of the percentage differences was 0.8%, the standard deviation ±4.4%. Because different samples varied so much in protein concentration, the standard deviations for the actual determinations are expectedly high, although almost identical, by both methods. The mean values by each method are also almost identical.

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*Subsequent observations have prompted decreasing the time to an hour.*

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<table>
<thead>
<tr>
<th>Kjeldahl</th>
<th>Biuret</th>
<th>Diff. of protein, biuret vs. Kjeldahl, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>946.9</td>
<td>968.5</td>
<td>+ 2.30</td>
</tr>
<tr>
<td>1000.0</td>
<td>1021.0</td>
<td>+ 2.10</td>
</tr>
<tr>
<td>892.5</td>
<td>875.0</td>
<td>(-) 2.00</td>
</tr>
<tr>
<td>1050.0</td>
<td>1000.0</td>
<td>(-) 4.80</td>
</tr>
<tr>
<td>1251.3</td>
<td>1271.0</td>
<td>+ 1.60</td>
</tr>
<tr>
<td>828.1</td>
<td>792.0</td>
<td>(-) 4.40</td>
</tr>
<tr>
<td>937.5</td>
<td>1021.0</td>
<td>+ 8.90</td>
</tr>
<tr>
<td>581.3</td>
<td>589.0</td>
<td>- 1.30</td>
</tr>
<tr>
<td>987.5</td>
<td>990.0</td>
<td>+ 0.25</td>
</tr>
<tr>
<td>990.6</td>
<td>1000.0</td>
<td>+ 0.95</td>
</tr>
<tr>
<td>803.8</td>
<td>814.0</td>
<td>+ 1.30</td>
</tr>
<tr>
<td>802.5</td>
<td>770.0</td>
<td>(-) 4.00</td>
</tr>
<tr>
<td>556.3</td>
<td>542.0</td>
<td>(-) 2.60</td>
</tr>
<tr>
<td>1921.9</td>
<td>1917.0</td>
<td>(-) 0.25</td>
</tr>
<tr>
<td>597.5</td>
<td>667.0</td>
<td>+ 11.60</td>
</tr>
<tr>
<td>943.2 ± 319.1</td>
<td>949 ± 318.0</td>
<td>0.82 ± 4.39</td>
</tr>
</tbody>
</table>

*The diagnosis of the first 10 patients listed was chronic bronchitis, that of the last five, chronic asthmatic bronchitis.*
(943.2 mg/100 ml by the Kjeldahl method and 949 mg/100 ml by biuret) and correlated significantly \((p, 0.95 \text{ to } 0.98; \text{correlation coefficient, } 0.994)\). Analysis of the paired data by the Wilcoxin signed rank test also indicated a high correlation.

The amount of nonprotein nitrogen varied considerably from specimen to specimen and the proportion of nonprotein nitrogen to total nitrogen determined by the Kjeldahl method varied from 11.6 to 40.2\%. The amount of nonprotein nitrogen in a given specimen is unpredictable.

The copper salt in the reagent was reproducibly reduced by occasional sputum samples, usually within 5 to 30 min.

During the development of the method, replicates of samples were assayed on the same day of preparation by both methods and were highly reproducible. After refrigeration for as long as a week, however, reproducibility was poorer. Changes during storage under various conditions are not fully understood and are currently being investigated.

**Discussion**

Sputum can now be added to the list of biologic fluids for which the biuret reagent can be used to determine quantitatively the protein concentration. Biuret reagent was previously attempted for sputum by Rusca (6) in 1911, but he was unsuccessful in applying it to sputum from patients with tuberculosis. It has been successfully applied to another similar biologic fluid (nasal secretions) by Rossen et al. (7). They reported good correlation with the microKjeldahl method, but found that salt intervention provided higher apparent concentrations than either of these. They did not specify whether the nonprotein nitrogen was considered in their calculation of protein by the microKjeldahl method, so the degree of correlation with biuret method is unclear. Possibly our success with the biuret reagent, as modified by Rosenthal and Cundiff, is attributable to the use of the chelating agent, EDTA, to stabilize the copper. This reagent helps to prevent turbidity in the homogenized sputum specimen, which is due in part to the precipitation of otherwise available calcium and magnesium in alkaline solution. The occasional reduction of the copper salt in the reagent that we occasionally saw in sputum specimens was noted by others with serum and cerebrospinal fluid (3, 4).

The biuret method has several advantages over the microKjeldahl method for determining sputum protein: it is easier and faster, its results are entirely comparable, and it more directly measures protein since it depends on co-ordination of copper atoms to peptide bond nitrogen. The Kjeldahl method requires determination of the total nitrogen concentration and the nonprotein concentration of each sample, and one must assume that the difference between these represents the protein nitrogen. In using the Kjeldahl method, we found it necessary to modify the usual procedures for separating the protein from the nonprotein nitrogen because of problems with pH adjustment when the usual deproteinizing agents were used. Results for nonprotein nitrogen were not reproducible with the Kjeldahl method until \(\text{ZnSO}_4\) was used to deproteinize and the final pH was adjusted to 4.5. These modifications produced a clearer filtrate and reproducible nonprotein nitrogen determinations.

The interest in the chemical composition of sputum in various bronchopulmonary diseases has been hampered by the difficulty in handling sputum without seriously changing the nature of the organic and inorganic constituents. Proteins, being readily denatured, are no exception. Von Hoesslin (8) reviewed the literature prior to 1926 concerning sputum protein. The variability of results for quantitative protein determinations probably reflects inadequate techniques. Nevertheless, the sense of diagnostic and prognostic importance of the quantitative and qualitative proteins in sputum is clear, even in these early investigations. Recent evidence on the importance of immunoglobulins in secretions strengthens these earlier impressions. This simple means of quantifying the protein content of sputum should contribute an additional dimension to such studies.

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**References**