A System of Clinical Chemical Analysis

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In recent years, clinical chemists have turned their attention to the use of submicro procedures for the determination of normal and pathologic constituents of body fluids. Several papers employing these technics have been published (1, 2, 3) and a text containing a collection of ultramicro clinical chemical procedures is available (4).

Despite this trend, there still exists a need for a combined analytic procedure adaptable to the rapid and accurate processing of many specimens—on the scale frequently encountered in the laboratory of a large hospital, yet employing minute amounts of sample. This procedure should be able to determine several important biochemical constituents without resort to intricate collection procedures, expensive and specialized equipment, nor should extensive retraining of the analyst performing these determinations be required. This report presents a system of analysis, developed in our laboratory, whereby glucose, urea N, chloride, sodium, and potassium can be determined using but 1.0 ml. of serum. The system is based on the use of the Somogyi barium hydroxide-zinc sulfate centrifugate.

METHODS

PREPARATION OF PROTEIN-FREE FILTRATE (5)

1. Reagents
   a. Glycerol-jack bean meal extract (6): 15 Gm. of Permutit are thoroughly shaken with 200 ml. 2% acetic acid, allowed to settle and the supernatant decanted. The sediment is washed three times with 200 ml. portions of water. Each

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portion is removed by decantation as before. To the sediment 50 ml. 0.001N sulfuric acid and 30 Gm. jack bean meal are added and the mixture is gently shaken for 30 minutes. 150 milliliters glycerin (ACS Grade) are added and the suspension thoroughly mixed and allowed to stand in the refrigerator overnight. The suspension is filtered through several thicknesses of gauze and then centrifuged at high speed for about 30 minutes. The supernatant is poured off and stored in a refrigerator. This solution is remarkably stable and gives low blank values which should be checked from time to time.

Its potency should be checked against solutions of urea. 0.1 ml. good-quality urease should convert 1 mg. of urea N to ammonia at room temperature in about 20 minutes.

b. Zinc sulfate: 5%
c. Barium hydroxide: 0.086N (7). Prepared by diluting 2 volumes 0.3N, which has been standardized against the zinc sulfate solution, with 5 volumes of water (7).

2. Procedure: To 1.0 clear unhemolyzed serum in a 16- by 150-mm. test tube, 1 drop of urease extract is added, the tube is stoppered and allowed to stand at room temperature (20–25°) for 5 minutes. 7 ml. of barium hydroxide solution and 2.0 ml. of zinc sulfate solution are added, the mixture is well shaken and centrifuged at 2500 rpm for 10 minutes. The centrifugate is poured off into another test tube. Yield: Approximately 8.5 ml.

DETERMINATION OF GLUCOSE (8)

1. Reagents
   a. Alkaline copper reagent (8): Anhydrous disodium hydrogen phosphate, 28 Gm., and 40 Gm. Rochelle salt are dissolved in 700 ml. distilled water, 100 ml. 1N NaOH is added followed by 80 ml. of 10% copper sulfate solution, which is added slowly with constant shaking until clear. Finally, 180 Gm. anhydrous sodium sulfate is added and when completely dissolved, the solution is diluted to 1 liter and allowed to stand 24 hours.

   Any sediment is filtered off and the reagent is ready for use.

   b. Nelson's arsenomolybdate reagent (9): Ammonium molybdate, 50 Gm. is dissolved in 900 ml. distilled water. With
cooling, 42 ml. concentrated reagent sulfuric acid is slowly
added, followed by a solution of 6 Gm. sodium arsenate in
50 ml. distilled water. The solution is incubated at 37° for
48 hours, after which it is ready for use. Note: This solu-
tion should be kept out of contact with rubber stoppers or
other organic matter and stored in an amber, glass-stop-
pered bottle.
c. Glucose standards: The following standards are prepared
from a stock 1.00% glucose solution in saturated benzoic
acid: 1 ml. = 0.1 mg.; 1 ml. = 0.2 mg.
2. Procedure: To 0.5 ml. centrifugate in a 19- by 150-mm. photome-
ter cuvet calibrated at 10.0 ml., add 1.0 ml. of alkaline copper reagent,
mix well, and heat in a boiling water bath for 10 minutes. Cool.
Tubes containing 0.5 ml. standard glucose solutions and a blank tube
containing 0.5 ml. distilled water are similarly treated. To all tubes
1.0 ml. arsenomolybdate reagent is added with mixing. The tubes are
diluted to the calibration mark, mixed, and the absorbance measured
at 540 mμ after setting the zero absorbance reading with the blank
tube.
3. Calculation (for 1 ml. = 0.1 mg. glucose):
\[
\frac{\text{Absorbance of unknown}}{\text{Absorbance of standard}} \times 100 = \text{mg. glucose per 100 ml. serum}
\]

DETERMINATION OF UREA N

1. Reagents
a. Nessler’s solution (10): Mercuric iodide, 100 Gm. and 70
   Gm. potassium iodide are dissolved in about 400 ml. of wa-
ter. 150 ml. of saturated sodium hydroxide are diluted with
cooling to 400 ml. When cool, the alkali is slowly added to
the double iodide solution and the entire solution is diluted
to 1 liter. The solution is allowed to stand 24 hours before
use. The Vanselow reagent can also be used (11).
b. Nitrogen standard: 1.0 ml. = 0.015 mg. N

2. Procedure: 2 ml. centrifugate in a 19- by 150-mm. photometer
tube calibrated at 10.0 ml. are diluted with water to the calibration
mark. 1.0 ml. Nessler’s reagent is added, the contents of the tube
are mixed, allowed to stand 5 minutes, and the absorbance measured
at 490-520 mμ against a distilled water blank. The absorbance of 2.0
ml. of standard similarly treated is also measured.
3. Calculation:
\[
\frac{\text{Absorbance of unknown}}{\text{Absorbance of standard}} \times 15 = \text{mg. urea N per 100 ml. serum}
\]

DETERMINATION OF CHLORIDE (7)

1. Reagents
   a. Silver nitrate: 0.01N solution and standardized against 0.01N NaCl solution.
   b. Dichlorofluorescein indicator solution: 0.05% in 70% ethanol.

2. Procedure:
   To 1.0 ml. centrifugate, 1 drop of indicator solution is added and the greenish solution is titrated with 0.01N silver nitrate until a pink color is seen throughout the solution.

3. Calculation: \( \text{AgNO}_3 \) used \( \times F = \text{mM. chloride per liter} \)

\[
F = \frac{100}{\text{ml. AgNO}_3 \text{ used in standardization}}
\]

DETERMINATION OF SODIUM AND POTASSIUM

1. Reagents
   a. Li\(^+\) solution for internal standardization: Stock solution: 3 mM./ml. 127.2 Gm. of LiCl or 191.0 Gm. Li\(_2\)SO\(_4\)\cdot H\(_2\)O is dissolved in water and diluted to 1 liter. The working solution 0.3 mm./ml. is prepared by diluting 10 ml. of stock to 100 ml. with distilled water.
   b. Na and K standards:
      1. Concentrated stock solutions: 100 mM. Na per liter. 5.845 Gm. NaCl in 1 liter of solution. 10 mM. K per liter: 0.7455 Gm. KCl in a liter.
      2. Combined working standards:
         (a) 150 mM. Na and 5 mM. K per liter. 15.0 ml. of stock Na standard and 5.0 ml. of stock K standard are mixed and diluted to 100 ml.
         (b) 120 mM. Na and 3.0 mM. K per liter. 12.0 ml. of stock Na standard and 3.0 ml. of stock K standard are diluted to 100 ml.
         (c) 100 mM. Na and 2.0 mM. K per liter. 10.0 ml. of stock Na standard and 2.0 ml. of stock K standard are diluted to 100 ml. Other standard concentrations
can be prepared by suitable dilution of the stock standards.

2. Procedure: To 1.0 ml. of centrifugate in a test tube calibrated at 10 ml., 1.0 ml. of dilute Li solution is added, the solution is diluted to the mark and mixed. About 2–3 ml. of this solution are atomized in a flame photometer set for internal standard operation, first with the sodium filter in place and then with the potassium filter in position. 1 ml. of each of the combined Na and K standards is similarly treated with diluted Li solution, diluted to mark, and atomized.

3. Calculation: The concentration of Na and K in the unknown is obtained by the bracketing method (16).

DISCUSSION

The first system of clinical chemical analysis was presented in 1919 by Folin and Wu (12) and was based on the use of the tungstic acid filtrate they introduced. From the filtrate of 2.0 ml. whole blood, glucose, nonprotein N, urea N, creatinine, chloride, and uric acid could be determined. The system of analysis presented here was also developed for the needs of a busy laboratory where many glucose, urea N, chloride, sodium, and potassium determinations are performed each working laboratory day. A uniform system of sample preparation for these determinations is therefore time saving, eliminates repetitive identical operations by several individuals concerned with these determinations, and effects an over-all reduction in the amount of serum sample required.

Among the advantages of the system under discussion is first, the five most frequently determined serum constituents are analyzed using only 1.0 ml. of specimen. Second, this entire sample is taken in only one pipetting. Third, after deproteinization all aliquots are taken with 0.5, 1.0, and 2.0 ml. pipets, stock items of equipment in all laboratories. Fourth, no new skills need be developed and precision is still of a high order. Fifth, in effect, 0.05-, 0.1-, and 0.2-ml. amounts of serum are used.

In the following paragraphs the system will be examined in detail.

PREPARATION OF THE PROTEIN-FREE FILTRATE

Isolated procedures based on the use of the Somogyi barium hydroxide–zinc sulfate filtrate have been consolidated and modified to enable the analyst to use the centrifugate prepared by the deproteinization of 1.0 ml. of serum for all the determinations outlined above.
In *Standard Methods of Clinical Chemistry* (5) it is indicated that this filtrate can be used for both glucose and urea N. In 1951 a report from this laboratory described its use for the estimation of serum chloride (7).

The modifications introduced here include first, the use of a diluted barium hydroxide solution. This solution now includes enough water to effect the final 1:10 dilution and eliminates the dilution step of the original deproteinization. Multiliter amounts of this solution can be prepared, depending on the work load of the laboratory and, when stored in an automatic buret equipped with a soda-lime trap as protection against atmospheric carbon dioxide, will keep indefinitely.

The second feature is the high-speed centrifugation of the precipitated protein mixture. The deproteinization is carried out in 16-× 150-mm. test tubes. In this laboratory, the tubes are centrifuged in a Size 2 International centrifuge carrying a 16 place head fitted with 3 tube trunnion carriers, so that 48 tubes can be processed in one centrifuge cycle. With 3 such centrifuges in operation, 144 deproteinized specimens are prepared. At 2000 to 2500 rpm for 8 to 10 minutes, as much as 8.5 ml. of clear centrifugate can be recovered. This is four times faster than a similar number of specimens can be processed by filtration and requires one-fourth the bench space.

In the early trials, the centrifugate was poured off into test tubes with one rapid motion thus avoiding the specks of protein material which had not been thrown down. With additional experience 1- and 2-ml amounts of supernatant can be pipetted consecutively, without disturbing the sediment and thereby avoid the necessity to transfer or recentrifuge the tube.

As approximately 8.5 ml. of centrifugate is realized compared to the 5.5 to 6 ml. of filtrate obtained by the usual means of separation, enough material is available for duplication of analyses if required.

Another time-saving device is adaptation of procedures to and the conduct of the photometric procedures in selected 19-× 150-mm. spectrophotometer tubes calibrated at 10.0 ml. Dilutions to mark are easily and rapidly made and tubes can be read in any of the three types of photometers available in our laboratory, e.g., the Coleman 6A, Coleman 14, or the Spectronic "20" using the ¾-inch adapter. The spring clip in the latter is removed as it scratches the tubes after a time. No detriment to the operation or performance of the instrument was experienced.
DETERMINATION OF GLUCOSE

The high alkalinity copper reagent used here has been found adequate for glucose values ranging from 35 to more than 400 mg. per 100 ml. On the other hand, if prior knowledge is available that the specimen contains abnormally high or abnormally low concentrations of glucose, then the aliquots taken can be adjusted accordingly. We have had no experience with the most recent Somogyi carbonate-copper reagent (13).

DETERMINATION OF UREA N

The experience in this laboratory has been that the use of the glycerol-urease extract and a Nessler's reagent prepared by the Bock and Benedict or the Vanselow formula gives a final solution that is sparkling clear, stable, and does not cloud even in solutions containing the equivalent of 160 mg. urea N per 100 ml. of serum. No protective colloids are necessary. Here, too, adjustment of the aliquot taken for analysis is advantageous when prior knowledge is available of an abnormally high urea N. By adjusting the wavelength (490-520 m\(\mu\)) so that the standard absorbance reads 0.150, the absorbance of the unknown \(\times 100\) gives mg./100 ml. urea N. directly.

DETERMINATION OF CHLORIDE

The original procedure has been modified insofar as a smaller aliquot is taken and the concentration of the titrant has been adjusted accordingly. Some recent experience has indicated that 0.5 ml. of centrifugate can be titrated with 0.005N AgNO\(_3\) without loss of accuracy.

DETERMINATION OF SODIUM AND POTASSIUM

Here the system has its novel feature. Most procedures call for direct dilution of the serum. A few procedures are available for flame photometric determination of sodium and potassium on deproteinized samples. Several years ago Beckman Application Data Sheet DU-

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1 A recent publication [Pitner, G., and Barr, W. W., \textit{Am. J. Clin. Path.} 29, 553 (1958)] describes interference by reducing materials in unspecified urease preparations in the determination of glucose on a single deproteinized filtrate. This has not been observed in the procedure reported here. The possibility of such interference had been considered earlier during a study on the use of serum for glucose analysis [Weissman, M., and Klein, B., \textit{Clin. Chem.} 4, 420 (1958)]. The variations as reported in the latter paper were randomly distributed over specimens which had been treated with glycerol-urease extract, as well as specimens which had not been exposed to enzyme action. Even so, the variation found in this laboratory never reached the 50 to 60 mg. per 100 ml. difference reported by Pitner and Barr. Furthermore, the increases would have been found in the nonfluoride-treated specimens. Actually this was not so.
12-B pointed out the desirability of removing proteins prior to flame photometry. This would aid most materially in keeping aspirator nozzles clean and free from encrusted material. Earlier, Overman and Davis (17) and later Hunter (18) determined Na and K on trichloracetic acid filtrates by flame photometry. Hunter found that trichloracetic acid protein precipitates carried down traces of Na but no K or Li. Willebrands (19) reported lower values by direct flame photometry when trichloracetic acid was used to precipitate proteins in more dilute solutions. Deproteinized filtrates or ashed samples have long been used for flame photometric determination of calcium (20, 21). The advantage of the Somogyi filtrate is that no additional cations or anions are contributed to the solution, in amounts to interfere with the determination of sodium and potassium (7).

Several procedures have been described in which 1:100 dilutions of serum are used for the analysis of both sodium and potassium (14, 15). This dilution is taken as a compromise but without any loss of accuracy, provided the internal standard solution is accurately measured. This is the only critical measurement of the entire system. Initially, 1.0 ml centrifugate was measured into a 10.0-ml volumetric flask containing a few milliliters of distilled water to which 0.1 ml of lithium solution was added with a 0.1 ml. Normax grade washout pipet and then diluted to the mark. The precision of replicate analyses was acceptable but not outstanding. The precision improved when the procedure was changed to the one described. Large volumes of diluent containing the requisite amount of internal standard solution can be prepared accurately. The required volume of internal standard solution is now added to the aliquot of centrifugate in one step and thereby insures the success of the most critical measurement.

The standard solutions combine both sodium and potassium in about the same ratio they exist physiologically and thus minimize

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Manufacturer's value</th>
<th>Found</th>
<th>St. Dev.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>89.0</td>
<td>91.0</td>
<td>3.4</td>
</tr>
<tr>
<td>Urea N</td>
<td>12.0</td>
<td>13.0</td>
<td>1.8</td>
</tr>
<tr>
<td>Chloride</td>
<td>103.0</td>
<td>103.0</td>
<td>1.5</td>
</tr>
<tr>
<td>Sodium</td>
<td>141.0</td>
<td>140.0</td>
<td>2.9</td>
</tr>
<tr>
<td>Potassium</td>
<td>5.1</td>
<td>5.1</td>
<td>0.005</td>
</tr>
</tbody>
</table>

Values found are the averages of five determinations for each constituent.

*Versatol, lot #16516 (Warner-Chilcott).
enhanced radiation effects, or expressed differently, produce these effects in about the same ratio as the actual sample.

Obviously, the serum used for analysis must be unhemolyzed, else the centrifugate cannot be used for potassium analysis.

In Table I are listed the results obtained by the system described, using a commercial control serum (VERSATOL—Warner-Chilcott) as the analytic sample. The values given are the mean of replicate analyses.

SUMMARY

A system of analysis has been presented for the determination of glucose, urea N, chloride, sodium, and potassium, using 1.0 ml. of serum. The centrifugate of the barium hydroxide–zinc sulfate deproteinization is utilized for all analyses. The system is readily adaptable to large-scale operations.

REFERENCES