A Rapid Semiautomatic System of Chemical Analysis using True Microspecimens

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This paper describes some biochemical applications of a self-filling, self-measuring, disposable, dilution micropipet. The device consists of a precision capillary in a plastic adapter and an attachable plastic bottle containing a premeasured volume of diluent or reagent. The human error in measuring microvolumes of liquid is eliminated by the self-filling, self-measuring action of the capillary. A quantitative transfer of sample to reagent is made by introducing the capillary into the bottle and rinsing the capillary by applying and releasing pressure on the walls of the container. The volume and composition of the reagent in the bottle can be varied depending on the test to be performed. An interesting application is its use as a disposable centrifuge tube containing a premeasured volume of stable deproteinizing agent. Other examples are determination of protein, bilirubin, serum sodium, and potassium, and predilution of serum or plasma for the microglucose determination using automatic chemical analysis.

Several papers have been published which present rapid or semiautomatic systems of microchemical analysis (1, 2). Inquiry into these systems almost invariably reveals that the quantity of specimen required for analysis is 0.1 or 0.2 ml.

Several instrument manufacturers have offered “systems” of microchemical analysis which, because of the special equipment needed to handle microquantities of material, have been expensive. Results of microanalysis using these systems in the workaday clinical laboratory milieu have proved spotty 3–5.

Without embarking on the long and usually fruitless discussion of what a “microsystem” is, let us merely state that in the area of clinical
chemistry there are frequent occasions when the total amount of specimen available for analysis is much less than 0.1 ml.—frequently on the order of 20 or 30 μl.

There is a need, then, for a system of clinical chemical analysis which is reliable in relatively unskilled hands, which can be used with specimens whose total volume is not in excess of 60 μl, and which does not require special "micro" equipment or extraordinary adaptations of equipment commonly available in a clinical laboratory.

This paper presents such a system, the heart of which is the self-filling, self-measuring, disposable micropipet invented and developed by H. W. Gerarde.

The pipet-reservoir unit* (Fig. 1) consists of a straight, thin-walled, uniform-bore, glass capillary tube of 20 μl ± 1% capacity fitted into a plastic holder having an overflow rinse chamber and an attachable plastic reservoir containing a premeasured volume (± 1%) of diluent or reagent and closed with a tightly fitting, solid plastic plug. The reservoir is opened by pushing the plug into the reservoir with the thumb. The tip of the capillary is touched to the specimen, which is

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*Unopette, Becton-Dickinson & Co., Inc., Rutherford, N. J.
immediately drawn into the tube. The flow into the tube stops automatically when the liquid has reached the end of the capillary. Since the tube collects liquid automatically and stops when filled, there is no need to measure the volume of specimen collected in the capillary. The human error of drawing the specimen up to a scribe mark on the pipet is thus eliminated. Serum, plasma, spinal fluid, urine, or whole blood may be used (Fig. 2).

After the specimen of serum or plasma is collected in the capillary tube, a dilution may be made by introducing the capillary into the reservoir. The walls of the reservoir are squeezed slightly (Fig. 2) before fitting the capillary holder into the reservoir, so that a negative pressure is created when the walls are released and the specimen is drawn from the capillary into a suitable diluent or reagent. The capillary tube may then be rinsed by gently squeezing the reservoir, forcing fluid into the capillary and overflow chamber. When the pressure is released, the diluent or reagent is again drawn back into the reservoir (Fig. 2). This action may be repeated as often as desired, but usually a single rinse of the capillary is adequate.

Fig. 2. A. Venous blood collection with capillary from open Vacutainer tube. B. Venous blood collection with capillary from Vacutainer tube needle. C. Addition of blood to reagent or diluent in Unopette reservoir. D. Rinsing capillary with reagent or diluent in reservoir.

After the dilution is made and the capillary rinsed, the pipet assembly is shaken to insure uniform distribution of the specimen in the diluent or reagent. Because the reservoir is cylindrical and not completely filled with liquid, uniform distribution of the specimen is readily effected by vigorous shaking for a few seconds. Thus, in two
simple operations, neither of which requires any volume measurement on the part of a technician, an amount of specimen is accurately measured out and a suitable initial dilution or chemical treatment may be made.

The description, performance, and accuracy of the micropipet has been previously reported (6–8).

The systems of microspecimen analysis presented here depend upon two things; the well-known principle of Goldenberg (9) that allows decantation for making quantitative transfers of supernatant from a precipitate, and the equally well-known work of Saifer and Seligson (10) in popularizing the use of automatic syringe pipets for constant delivery of reagents.

Thus, this system of chemical analysis on microspecimens consists of four basic steps:

1. Initial collection of blood (or other) specimen in heparinized capillary tubes (This step is not necessary where whole blood may be used.)
2. The use of the pipetting device described to sample and measure the specimen automatically
3. The use of Goldenberg’s principle of quantitative decantation of “filtrate”
4. The use of automatic syringe pipets to add reagents as necessary in each individual procedure

It should be noted that there is essentially nothing new in any method presented here. What is new is the Gerarde Unopette, which allows the accurate automatic sampling routinely of quantities which heretofore were the domain of chemists and technologists with better-than-average manual dexterity. No scribe mark need be read in measuring a specimen, thus eliminating the major source of error in ultramicro-sample analysis.

The procedures included here have not been chosen necessarily because they are the best, but because they are probably the most familiar. It is essential to understand that the system will be applicable to virtually any procedure which may be chosen. Thus, arguments concerning which is the best procedure for a blood “sugar” determination are specious; the system can be applied to any procedure.

**Automatic Analysis Using Microspecimen Samples**

The advent of automation in the clinical laboratory has been universally hailed for the resultant increase in productivity, speed, and
usually accuracy. The usual procedure advocated for microspecimen analysis with the AutoAnalyzer* involves an initial dilution, usually 10- or 20-fold, of the specimen before its presentation to the machine. Doubling the area of dialysis usually allows recovery of sufficient sample into the reagent line for analysis to be performed. This initial dilution can be most conveniently handled by means of the system presented. For instance, 40 μl of specimen diluted with 1.2 ml of isotonic saline (giving a 1/30 dilution) will give good results for sugar determinations with the manifold modifications shown in Table 1.

### Table 1. Auto-Analyzer Manifold Modifications for Use of Unopette in Micro Blood Sugar Determinations

<table>
<thead>
<tr>
<th>Sample line</th>
<th>Standard macro (I.D. of tube in inches)</th>
<th>1/80 micro (I.D. of tube in inches)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Air line</td>
<td>0.056</td>
<td>0.035</td>
</tr>
<tr>
<td>0.9% NaCl + 0.5% KCN line</td>
<td>0.081</td>
<td>0.035</td>
</tr>
<tr>
<td>0.075% potassium ferricyanide line</td>
<td>0.100</td>
<td>0.045</td>
</tr>
<tr>
<td>Air line</td>
<td>0.056</td>
<td>0.035</td>
</tr>
</tbody>
</table>

### Total Protein (Biuret)

**Principle**

A solution of alkaline copper sulfate reacts with peptide linkages to produce a violet color which may be measured colorimetrically.

**Reagents**

*Biuret reagent* (Gornall) (11)

**Procedure**

1. Obtain 0.020 ml serum from capillary or venous blood. (A few drops of blood from a finger puncture is collected in an unheparinized Natelson tube, allowed to clot, sealed with clay or plastic, and spun down. The resulting serum is used for this test.)
2. Wash into a pipet-reservoir unit containing 2.0 ml biuret reagent.
3. Allow to stand for 10 min.
4. Squeeze into a 12-mm. cuvet and read at 550 μ against the contents of an unused biuret Unopette as a blank.

*Technicon Instruments Co., Chauncey, N. Y.*
5. Prepare control.* Reconstitute and use as serum.
6. Determine unknown from absorbance curve (Fig. 3).

**Blood “Sugar”**

**Principle**

The principle is the well-known copper-reduction method of Folin (12–14).

![Graph](attachment:image.png)

*Fig. 3. Standard curve for total protein using Coleman Jr. spectrophotometer with 12-mm. round cuvets at 550 mμ.*

**Reagents**

The reagents are those of the classic Folin sugar procedure (15).

**Procedure**

1. Sample 0.020 ml. of capillary or venous blood, serum, or plasma with the pipet capillary. This is the unknown.
2. Deliver sample into a pipet reservoir unit containing 2.0 ml. of stable tungstic acid-deproteinizing reagent.
3. Shake to mix, then remove the capillary from reservoir and plug with the shield (to act as handle).
4. Centrifuge at approximately 1500 rpm for about 2 min.
5. Invert reservoir over a 10-ml. test tube and, by squeezing a few times, decant entire centrifugate into test tube.
6. Add 1.0 ml. alkaline copper reagent and mix well.
7. Prepare blank by adding contents of an unused tungstic acid reservoir to a 10-ml. test tube and treat as for centrifugate.
8. Prepare controls. Use control sera at two concentration levels. Reconstitute as per instructions, and treat as for unknown.

*Versatol and Versatol A control serums, manufactured by General Diagnostics Div., Warner-Chilcott Laboratories, Morris Plains, N. J., have been used as control specimens throughout this study.*
9. Place blank, controls, and unknowns in boiling-water bath for 10 min.
10. Cool in water bath to room temperature for 5 min.
11. Add 1.0 of phosphomolybdate reagent to each tube.
12. Mix well. Allow to stand for 10 min. for maximum color development. Do not allow to stand longer than 40 min., after which color will begin to fade.
13. Read at 420 mµ against the blank set at zero absorbance.
14. Calculate as follows.

**Control check**

\[
\frac{\text{Absorbance of control}}{\text{Absorbance of control}} \times \text{value control} = \text{value control A (mg./100 ml.)}
\]

**Unknowns**

\[
\frac{\text{Absorbance of unknown}}{\text{Absorbance of control}} \times \text{assay control} = \text{value of unknown (mg./100 ml.)}
\]

See Fig. 4 for absorbance curve.

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**Fig. 4. Standard curve for blood “sugar” using Coleman Jr. spectrophotometer with 12-mm. round cuvets at 420 mµ.**

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**Serum Sodium and Potassium**

**Principle**

Since preparation of the specimen is determined by the physical makeup of the individual flame photometer, which, in turn, dictates what the carrier reagent shall be, only the direct method will be discussed here. The microsample procedure will be the same, only the carrier reagent will differ.

**Reagents**

\[\text{Sterox,* } 0.02\% \text{ in } H_2O\]

Procedure

1. Draw off a 0.02-ml. sample of capillary or venous serum with a Unopette capillary.
2. Deliver sample into a pipet reservoir unit containing 2.0 ml of Sterox 0.02% in H₂O.
4. For blank. Use a reservoir with 2.0 ml Sterox with no serum added.
5. Use control serums at two concentration levels. Reconstitute as per directions and treat as serum. Set instrument to read at the appropriate assay for sodium or potassium for each of these controls.
6. For a direct-reading flame photometer, read concentration from calibrated galvanometer scale.

Since most flame photometers require, at most, 0.5 ml to read, 2.0 ml has proven more than sufficient. With flame photometers which use the principle of aspiration, a polyethylene tube of suitable diameter may be attached to the aspirator tube to lead to the diluted test specimens which are held in a rack (16). Thus, by merely placing the tip of this aspirator extension in each reservoir, multiple analysis may be done quickly and easily with no sample transfer. It then becomes an easy matter to wash, blank, or check calibration at any point in the series.

Total Serum Bilirubin

Reagents

 Sodium nitrite, 5.0 gm.  In 1 L. distilled water (Solution 1).
 Sulfanilic acid, 3.0 gm.  Add to 1 L. water containing 15 ml. concentrated HCl (Solution 2).
 Diazo reagent 0.3 ml. 0.5% sodium nitrite (Solution 1) plus 10 ml of sulfanilic acid reagent (Solution 2). Make fresh each day. Do not use when solution is tinged with yellow.

Procedure

Collect a few drops of blood from a finger puncture in an unheparinized Natelson tube, allow to clot, seal with clay or plastic, and spin down. Using two 20-µl capillaries, draw off 0.04 ml of the resulting serum for a sample.

2. Deliver sample into a pipet reservoir unit containing 1.0 ml of a mixture of 1:1 methanol and 0.025% w/v caffeine and sodium benzoate in water. Mix well. The mixture will be cloudy, but this does not matter. This is the unknown.
3. Prepare a second pipet unit exactly as for sample. This will be the blank.
4. Use control serums at two concentration levels. Reconstitute as per instructions, and treat as unknown.
5. To the unit containing the unknown, add 0.10 ml. freshly made double-strength diazo reagent. The cloudiness will disappear upon mixing.
6. To the unit containing the blank, add 0.10 ml. 0.1N HCl. The cloudiness will disappear upon mixing.
7. Wait 5 min. and transfer entire contents of each unit to separate 10-mm. round cuvets.
8. Read unknown at 540 mμ against the blank set at zero absorbance.
9. Calculate as follows.

Control

\[
\frac{\text{Absorbance of control A}}{\text{Absorbance of control}} \times \text{value of control} = \text{value of control A in mg./100 ml.}
\]

Unknowns

\[
\frac{\text{Absorbance of unknown}}{\text{Absorbance of control}} \times \text{value of control} = \text{value of unknown in mg./100 ml.}
\]

See Fig. 5 for absorbance curve.

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Fig. 5. Standard curve for total bilirubin using Coleman Jr. spectrophotometer with 10-mm. round cuvets at 540 mμ.

References