Micro Sampling Techniques for Human Blood Cholinesterase Analysis with the pH Stat[1]

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INTRODUCTION

Commercially available, heparin-coated, disposable glass micropipets of constant bore and accurate calibration were investigated for drawing blood for the determination of cholinesterase activity. Their ease in handling fractionated blood and the use of a correction factor for plasma enzyme activity allowed accurate calculation of the activity in any fraction.

STUBBS and FALES (1960) used capillary tubes to draw blood for the MICHEL (1949) method of determining cholinesterase activity. Sahli pipets were used to take aliquots from the blood sample for analysis. Recently, ALDRICH et al. (1969) used capillary tubes to draw blood from finger tips for analysis with the pH Stat. Accurate sample volume measurements, however, proved to be very difficult.

Our use of accurate constant bore micropipets, instead of ordinary capillary tubes, allows the measurements of sample volumes directly within the tubes. Blood from finger or earlobe punctures, or in syringes or heparinized vacutainers, can be sampled simply and advantageously with these versatile instruments.

MATERIALS

Equipment and Glassware

1. Hematocrit centrifuge: i.e. International Model M.B. or equivalent.
2. pH Stat - Sargent Welch Scientific Company or equivalent.
3. Vortex mixer.

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4. Disposable glass 100 µl pipets containing sufficient heparin to keep blood from coagulating for at least 24 hours--Dade Div. of American Hospital Supply Co. or equivalent.
6. Opticlear vials, 16 ml capacity (o.d. = 25 mm, hgt = 52 mm).
7. Tungsten carbide-tipped glass marking pencil
8. Ruler with millimeter markings.
9. Calibrated glass syringe; 1.0 ml with a piece of rubber tubing attached to its tip to accomodate the ends of the micropipets.
10. Calibrated glass syringe; 5.0 ml with a piece of rubber tubing attached to its tip to accomodate the ends of the micropipets.
11. Teflon-coated stirring magnets (15 mm X 1.5 mm dia.
12. Micropipeter--Clinac Micro Model Pipeter or equivalent.
13. B - D, Microlance #419 blood lancets for earlobe punctures.
14. Heparinized vacutainers with needles and holders.

Solutions and Reagents

1. Physiological saline, 0.9% solution.
2. Substrate solution, 0.11N acetycholine iodide. 0.7510 gm diluted to 25.0 ml with distilled water.
3. Titrant solution, 0.01N NaOH, standardized against phthalic acid daily.
4. Standard potasium acid phthalate, 0.018M solution.
5. Buffers-pH 4.0 and 8.0 for standardization of pH Stat daily.

METHODS

Arm Venipuncture Blood Collection

Blood samples (5 - 10 ml) were collected in heparinized vacutainer tubes. After gentle mixing, 75-100 µl were drawn by the pipeter into the micropipets The ends were then sealed with hematocrit clay for storage or centrifugation and hematocrit measurements. Since most hematocrit centrifuges accomodate tubes only up to 80 mm in length, micropipets before use were cut back to 78 ml, a length completely adequate to encompass a volume of 100 µl.

The remaining blood in the vacutainer was retained for enzyme analysis by standard procedures using the pH Stat (NABB & WHITFIELD 1967).

Earlobe Blood Collection

After swabbing with 70% alcohol the earlobe was held against a piece of sterile gauze over a small cork and punctured three or four times with a disposable blood lancet. Blood (75 - 100 µl) was drawn directly into micropipets whose ends were then sealed with hematocrit clay.

Preparation of Blood Samples

Whole blood in sealed micropipets was centrifuged for five minutes at 11,000 RPM in an hematocrit centrifuge. The lengths of the columns representing the RBC and plasma fractions were then measured with a millimeter ruler. From these measurements, hematocrit calculations were made. Volumes of packed red cells, plasma and of whole blood were determined by comparisons of the lengths of their respective columns to the lengths of the constant bore tubes that represented 100 microliters (the length from the tip to the 100 µl mark). To remove fractions for analysis, a micropipet was first scratched in three places with a tungsten carbide-tipped pencil--at the interface of the clay plug and the packed red cells, between the cells and the plasma and between the clay plug and the plasma. The tube was then broken at the RBC-plasma interface. Over an open opticlear vial, the clay plug was broken away and the packed RBC rinsed with 0.5 ml of distilled water from a syringe. The resulting hemolysate was mixed thoroughly by a vortex mixer. From another syringe, the tube was then rinsed with 4.2 ml of physiological saline into the vial containing the hemolysate. Similarly, the plasma fraction was rinsed into another opticlear vial but with 4.2 ml of physiological saline only. For whole blood cholinesterase analysis the content of a micropipet was rinsed into another opticlear vial--after removal of the clay plugs--with 0.5 ml of distilled water. The resulting hemolysate was mixed thoroughly and the pipet was rinsed into the vial with 4.2 ml of physiological saline.

The cholinesterase activity of the whole blood or the fractions was measured by the pH Stat at 37°C. For RBC and whole blood cholinesterase analyses 0.1 ml of 0.11N acetycholine iodide was injected into the sample mixture and the titration volume was recorded against time. For plasma cholinesterase analysis 0.6 ml of 0.11N acetylcholine iodide was injected into the mixture and the titration volume recorded. Measurement of enzyme activity was allowed to proceed for at least three minutes for each analysis.

CALCULATIONS

A calculated value for whole blood, plasma or RBC cholinesterase activity could be made if two of the values were measured. The relationships of the whole blood (ChE_{WB}), plasma (ChE_{PL}) and RBC (ChE_{RBC}) cholinesterase activities are as follows, with all the cholinesterase values $\mu m\ ml^{-1}\ min^{-1}$:

Calculated ChE_{RBC} =

$$\left[(ChE_{WB}) - (ChE_{PL})\ (0.6)\ (1 - hct)\right] / (hct)$$

Calculated ChE_{WB} =

$$\left[(ChE_{RBC})(hct)\right] + \left[(ChE_{PL})(0.6)(1- hct)\right]$$

Calculated ChE_{PL} =

$$\left[(ChE_{WB}) - (ChE_{RBC})(hct)\right] / (1 - hct)(0.6)$$

where hct is the hematocrit value.

The factor 0.6 was used in the calculations to account for rate differences in measuring plasma enzyme activity alone, where the optimum substrate concentration is 0.0132M, and in the presence of red cells whose cholinesterase activity estimation is carried out at 0.0022M substrate. The contribution of plasma cholinesterase activity when measured in whole blood cholinesterase determinations was only about 60% of the value measured separately in plasma cholinesterase determinations.

RESULTS

Using a single venous blood sample, a series of determinations incorporating both the micropipet modification as well as the standard pH Stat procedure were made to compare the precision and variability of the former procedure with that of the latter. The results are shown in Table I. Variations in activities for comparable fractions measured by the two procedures are not considered since such paired determinations were not made in sequence on the same day. As noted on the table the coefficients of variation are all within 5 percent.

Measured and calculated cholinesterase activity values on a series of blood samples, handled by the modified procedure or that of the standard pH Stat technique, are shown in Table II together with regressic expressions, a comparison of means of paired values and

TABLE I

PRECISION IN THE MEASUREMENT OF CHOLINESTERASE ACTIVITY. COMPARISONS BETWEEN THE MICROPIPET MODIFICATION AND STANDARD pH STAT PROCEDURE

MEASUREMENT	NUMBER OF DETER-MINATIONS	VALUE[1]	STANDARD DEVIATION	COEFFICIENT OF VARIATION[2]
Micropipet Modification				
Hematocrit	11	0.440	± 0.003	0.7
Plasma ChE	6	3.47	± 0.13	3.8
WB ChE	5	7.07	± 0.22	3.1
WB ChE[3]	6	7.51	± 0.16	2.1
RBC ChE	6	14.35	± 0.32	2.2
RBC ChE[3]	6	13.39	± 0.49	3.7
Standard pH Stat Procedure				
Plasma ChE	6	3.12	± 0.15	4.6
WB ChE	6	7.25	± 0.15	2.0
RBC ChE[3]	6	14.01	± 0.33	2.4

[1] Values of cholinesterase activity in $\mu m\ min^{-1}\ ml^{-1}$.
[2] Standard deviation as percent of the mean.
[3] Calculated activity.

correlation coefficients.

As the data and analyses indicate there is generally a good agreement between values for cholinesterase activities, measured or calculated, as determined by either the standard procedure or the micropipet modification. Correlation coefficients were high for plasma and the whole blood enzyme activities but were lower for the RBC activities. Such differences, however, may reflect greater relative variances in the analytical procedure for measuring RBC cholinesterase activities than in manipulatory techniques employed prior to determining enzyme activities.

Micropipets can be used to collect blood directly from a small incision without jeopardizing the accuracy of subsequent enzyme activity measurements. The incision must produce blood that flows unassisted, as any mechanical manipulation to produce flow may result in blood diluted with lymph. To illustrate such usage comparisons were made of ChE activities in blood obtained from earlobes directly into micropipets, and from arm

venipunctures drawn first into vacutainer tubes and hence into pipets. Comparable enzyme activity measurements were noted for plasma or whole blood from either source, but again the largest variations were found with the RBC fractions. Data are given in Table III.

TABLE II

COMPARISONS OF CHOLINESTERASE ACTIVITIES MEASURED AND CALCULATED

SUBJECT AND DATA	PLASMA[1]		WHOLE BLOOD[1]		RBC		WHOLE BLOOD		CALCULATED RBC	
	Y	X	Y	X	Y[1]	X[2]	Y[3]	X[1]	Y	X
Mean	4.3	4.5	7.9	8.2	14.9	15.1	8.1	8.2	14.4	15.1
W.S. 1/29	3.1	3.9	7.3	7.7	13.2	13.8	8.1	8.2	14.4	15.1
W.S. 1/31	3.3	3.6	7.3	7.3	14.0	13.2	7.7	7.3	13.2	13.2
C.M. 1/29	5.6	6.5	8.9	9.8	15.4	16.3	9.1	9.8	14.7	16.3
D.M. 1/31	5.5	5.8	8.6	9.5	14.4	16.3	8.6	9.5	14.3	16.3
L.S. 1/29	3.8	3.7	6.4	6.7	14.3	14.4	6.6	6.7	13.2	14.4
L.S. 1/31	3.4	3.3	6.1	6.9	14.6	15.4	6.6	6.9	13.3	15.4
L.J. 2/ 8	3.9	3.4	8.8	8.4	16.9	15.7	9.1	8.4	16.3	15.7
M.D. 2/ 8	4.4	4.3	8.2	8.1	15.2	15.0	8.3	8.1	15.1	15.0
B.B. 2/ 8	5.9	6.0	9.6	9.7	16.2	16.2	9.8	9.7	15.8	16.2

For Plasma[1]: $Y = 0.81X+0.68$; $\overline{Y} = 0.96\overline{X}$; $r = 0.93$
For Whole Blood[1]: $Y = 0.92X+0.32$; $\overline{Y} = 0.96\overline{X}$; $r = 0.92$
For RBC $Y = 0.66X+4.87$; $\overline{Y} = 0.99\overline{X}$; $r = 0.66$
For Whole Blood[3,1]: $Y = 0.86X+0.99$; $\overline{Y} = 0.99\overline{X}$; $r = 0.90$
For Calculated RBC: $Y = 0.68X+3.96$; $\overline{Y} = 0.95\overline{X}$; $r = 0.65$

1 Measured activity.
2 Calculated from measured whole blood and measured plasma cholinesterase activities.
3 Calculated activity from measured activities of plasma and RBC.

Note: ChE activity in units of $\mu m\ min^{-1}\ ml^{-1}$.
Y Designates ChE activity using micropipet modification and X using the standard pH Stat technique.
r Is the correlation coefficient.

TABLE III

COMPARISON IN CHOLINESTERASE MEASUREMENTS FOR BLOOD DRAWN BY ARM VENIPUNCTURE OR FROM THE EARLOBE AND HANDLED BY MICROPIPETS

CHOLINESTERASE ACTIVITIES[1] AND ACTIVITY RATIOS: EARLOBE BLOOD/ARM BLOOD

SUBJECT AND DATE	Plasma			RBC			WHOLE BLOOD		
	Arm	Ear-lobe	Ratio	Arm	Ear-lobe	Ratio	Arm	Ear lobe	Ratio
W.S. 1/17	3.7	3.9	1.05	12.1	11.6	0.96	6.7[a]	6.6[a]	0.99
W.S. 1/22	3.7	3.9	1.05	13.7	15.4	1.12	7.3[a]	7.9[a]	1.08
W.S. 1/29	3.1	3.2	1.03	13.2	11.7	0.89	7.2[a]	6.8[a]	0.94
D.M. 1/29	5.6	5.7	1.02	15.4	15.8	1.03	9.1[a]	9.2[a]	1.01
L.S. 1/29	3.8	4.0	1.05	14.3	14.4	1.01	6.6[a]	6.7[a]	1.02
L.J. 2/ 7	3.9	3.7	0.95	16.9	17.1	1.01	8.8[b]	9.2[b]	1.05
M.D. 2/ 7	4.4	4.6	1.05	15.3	15.2	0.99	8.1[b]	8.3[b]	1.02
B.B. 2/ 8	5.9	6.1	1.03	16.2	14.5	0.90	9.6[b]	10.0[b]	1.04

[1] $\mu m\ min^{-1}\ ml^{-1}$.
[a] Calculated whole blood activity from plasma and RBC activities.
[b] Measured whole blood activity.

DISCUSSION

The micropipet and its potential for improving the analytical capabilities of routine pH Stat techniques was considered from several points of view. With constant bore micropipets available, whose accuracy was stated at $\pm$ 0.2%, it is possible to determine contained volumes by simple measurements of lengths. Blood samples, drawn into heparinized micropipets can be shipped to a location of analytical capability with considerably less demand on shipping volume and weight than in the shipment of test tube quantities. Additionally, the micropipets serve easily as a device for the hematocrit determinations and for the separation of blood fractions. Each blood sample can be considered relative to its own hematocrit. But most important, blood fractions can be expressed from their respective portions of the separated pipets into the reaction vessels without the necessity of intervening measurements.

The capacity, then, to analyze whole blood as well as the fractions as obtained from the micropipets means

that only two of the three measurements need be made from which the third can be calculated by expressions given previously. As a verification one measurement of enzyme activity in each of the three fractions allows calculations to be made for the activity in each other fraction, a technique not feasible by the standard pH Stat procedure. In the measurement of RBC enzyme activity by the latter method, it is necessary to withdraw aliquots of packed red cells, a procedure which has proven difficult to repeat quantitatively.

Manipulations of small volumes with the micropipets do not sacrifice precision in the determination of cholinesterase activity, as can be noted in Table I. Comparisons of cholinesterase activities, involving the micropipet or standard procedures for values either measured or calculated, showed a high degree of correlation for plasma or whole blood activities (Table II). That the values for RBC activities were less in agreement reflects on portions of the total RBC cholinesterase determination other than the use of micropipets.

The earlobe appears to offer another convenient source of blood, particularly when there are objections to venipuncture. Cholinesterase activities are comparable for blood from either source though again correlations are less with the RBC activities (Table III). The micropipet procedure is particularly convenient when small amounts of blood are available.

Micropipets have been employed successfully in determinations of cholinesterase activity by a simple technique using an expanded scale pH meter to measure changes in hydrogen ion concentrations near the pK_2 for carbonic acid in the presence of the reacting enzyme (LEE & SERAT in press).

Laboratories performing few cholinesterase analyses may find savings in the use of micropipets relative to vacutainers, particularly when blood shipments are involved.

Studies on the effects of blood storage on cholinesterase activities showed that blood can be retained for 1½ to 2 times longer in micropipets than in vacutainer tubes under both refrigerated and room temperature conditions without activity loss. Blood samples kept well in micropipets for almost five days at room temperature with very little change in plasma or RBC cholinesterase activity.

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