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16. Abstract

Among aerial applicator personnel, the primary value of periodic blood cholinesterase (ChE) assays is the detection of pesticide poisoning indicated by a decrease in enzyme activity since the previous (or pre-season) assay. Comparison of these values is difficult if they are based on different methodologies and expressed in different units, which is frequently the case. This report provides an evaluation of four serum or plasma ChE methods which are currently in use and establishes the relationships for interconversion among their respective units. This was accomplished by performing simultaneous assays by each method on a series of samples whose activities covered the range from normal levels down to the very low level found in organophosphate-poisoned individuals. The resulting conversion data (regression equations) are also compatible with those described in Report No. AM-70-13, thereby providing direct interconversion capability among the units of seven commonly-used serum ChE methods.

17. Key Words

Aerial applicators
Organophosphate pesticides
Methodology
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A COMPARISON OF SERUM CHOLINESTERASE METHODS: II

. Introduction.

The utility of measurement of the activity of he acetylcholine-hydrolyzing enzyme present in lood plasma as a diagnostic tool in organophos- hate poisonings and for monitoring the health- ıfety aspects of aerial applicator operations has een previously discussed.[1]

The activity of this enzyme in unexposed in- ividuals varies widely, with the normal range ctending above and below the population mean y as much as 30%. Therefore, any such value otained after potential contact with an anti- ıolinesterase should be compared to the in- ividual's normal, pre-exposure value to deter- ine whether inhibition is present. Such a mparison can be made only if both values are pressed in the same units. Unfortunately for e purpose of comparison, the various methods r cholinesterase (ChE) assay produce dis- milar units, and no single "standard" method ists; consequently, a means of converting tivity units obtained by one analytical method those of another is required.

This paper represents the second in a series ıich will provide conversion data to transform ıE activity units obtained by one method into e corresponding units of other methods. Most the methods developed primarily to measure e activity of the pseudocholinesterase present in e fluid portion of blood, including those com- red in this study, customarily utilize serum for e determination. However, plasma can be used r the measurement, with identical results, pro- led that the presence of fibrinogen does not roduce interference. There is a distinct ad- ntage in performing the assay on plasma if e method permits, since it allows the measure- nt of erythrocyte enzyme activity on the same od specimen. Therefore, plasma was utilized this study.

The procedure used to develop these compari- ı data was to perform simultaneous assays by h method on aliquots of the same sample of plasma, using the pH-Stat method as a primary reference. Heparinized, whole blood was pur- chased from a local blood bank. Part of the separated plasma was heated in a water bath at 60° C. until pH-Stat assay indicated a loss of about 95% activity (10–20 minutes). The in- activated plasma was then mixed in varying proportions with fresh plasma to obtain a gradient of enzyme activity.

II. pH-Stat Reference Method.

Materials and Method. Butyrylcholine iodide (Mann Research Laboratories, New York, N.Y.) was dried overnight in a vacuum desiccator and stored therein until used. An aqueous solution, 0.163M (49 mg/ml), was prepared weekly and stored at 4° C. The sodium hydroxide titrant was prepared at a concentration of approximately 0.005N using CO_2-free distilled water. The solu- tion was stored in the titrant reservoir of the pH-Stat and protected from CO_2 absorption by a drying tube filled with Mallcosorb, 30–50 mesh, indicating type CO_2 absorbent (Mallinckrodt). This solution was standardized daily by titration of a potassium hydrogen phthalate standard. Ringer's salts solution for diluting plasma samples was prepared as needed using 9.00 g NaCl, 0.20 g KCl, 0.26 g $CaCl_2$, and 0.20 g $NaHCO_3$ brought to a 1 liter final volume with distilled water.

A Radiometer pH-Stat (Radiometer A/S; Copenhagen, N.V., Denmark) was used for all constant-pH titrations, and consisted of the fol- lowing units:

PHM26—expanded scale pH meter
TTT11b—electronic titration control unit
ABU1b—semi-micro automatic buret for titrant delivery, 2.5 ml capacity
TTA3—glass-jacketed reaction vessel, 3 ml, with plastic-coated magnetic strring disc
VTS13c—constant-temperature water bath and circulator, set to maintain 25° C. in reaction vessel
SBR2c—recorder for indicating volume of titrant delivered per unit time

Aliquots of samples were diluted 1:10 with Ringer's salts solution. One ml of the diluted plasma was added to the reaction vessel, the magnetic stirrer was started and the solution was allowed to come to temperature equilibrium (25° C.). The pH was adjusted with the automatic titrator to the pre-set value of 8.1. One-half ml of butyrylcholine iodide substrate was added to the reaction vessel using a 1.0 ml tuberculin syringe and the recorder chart drive was turned on. The reaction was allowed to continue until the recorder had plotted a straight line (titrant volume vs time) for a minimum of 3 minutes, or until a minimum titrant volume of 0.5 ml had been added.*

Calculations:

All activities were expressed as micromoles of substrate hydrolyzed/minute/ml of plasma. Calculations were made as follows:

$$\text{Activity} = \frac{\text{S.D.} \times (0.025) \times (\text{N}) \times (1000) \times (\text{D.F.})}{\text{t}}$$

Where S.D. = recorder scale divisions (linear portion)
t = time in minutes for linear portion of trace
1000 = μmoles/mmole
0.025 = ml of titrant per S.D.
N = normality of titrant
D.F. = dilution factor (i.e., 10)

III. Michel Method.

The Michel method is an electrometric determination based on the measurement of the acid produced by the action of ChE on acetylcholine.[2] The acetic acid produced is measured in terms of the change in pH produced in a buffer-substrate mixture during a specific incubation period. The decrease in ChE activity with decreasing pH is taken into consideration by selecting a buffer whose capacity closely parallels the activity loss over the range from pH 8 to pH 6; the buffering effect of plasma is minimized by sufficient dilution (1:111). As performed in this laboratory, the basic method of Michel[2] as modified by Larson[3] was used.

Materials and Method. Acetylcholine perchlorate (K & K Laboratories, Plainview, N.Y.) was dried overnight in a vacuum desiccator over Drierite and stored therein until used. The substrate was prepared by dissolving 0.405 g of the salt in a final volume of 10.0 ml of water This solution is stable for up to four weeks i refrigerated.

A buffer stock solution was prepared by dis solving 4.124 g sodium barbital, 0.545 g KH_2PO and 44.730 g KCl in distilled water and dilutin to a final volume of 200 ml. Immediately befor use, the plasma buffer was prepared by dilutin 6.4 ml of the stock solution with 75 ml o distilled water; the pH was adjusted to 8.00 witl 0.1N HCl and the solution adjusted to a fina volume of 100 ml with distilled water.

Plasma aliquots of 20 μl were withdrawn usin a Sahli pipet and expelled into 12-ml test tube containing 1.0 ml of distilled water. One ml o buffer solution was added and the mixture wa allowed to equilibrate in a 25° C. water bath fc 10 minutes. After recording the pH to th nearest 0.01 pH unit, 0.2 ml of substrate wa added and the tubes were allowed to incubate a 25° C. for one hour. The pH was read again an the change in pH/hr was calculated. Contr tubes containing only distilled water, buffer an substrate were treated similarly and the chang in pH in the control tubes was subtracted fro the values obtained from plasma to correct fc non-enzymic hydrolysis.

Calculated Michel units ($\triangle$pH/hr) wer plotted against pH-Stat units obtained for th same plasma samples assayed simultaneousl The linear regression equations and correlatic coefficients were calculated, each point represen ing the mean of duplicate assays of the sam sample by each method. The statistical regre sion of the pH-Stat units on Michel units graphically represented in Figure 1.

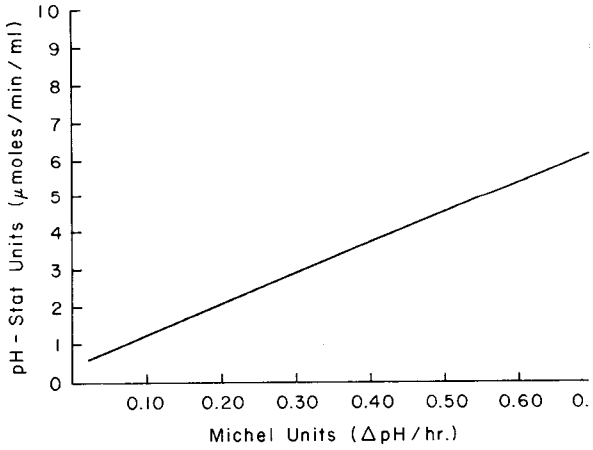


FIGURE 1. Correlation between pH-Stat units and Mich units. N=20 points; correlation coefficient =0.986

*Since full scale on the recorder (100 scale divisions) represents 2.5 ml of titrant, 0.5 ml is represented by 20 scale divisions. The recorder trace can be read to 0.2 scale division; therefore, the precision in reading titrant volume is 1%. With a chart speed of 2 cm per min, the precision in reading elapsed time of 3-min trace is also 1%.

IV. Pfizer ChE-tel Method.

The Pfizer method is a colorimetric determination based on Garry and Routh's[4] modification of the earlier work of Ellman.[5] In this reaction, the artificial substrate, propionylthiocholine, is hydrolyzed by cholinesterase and the released thiocholine displaces the yellow anion of 5-thio-2-nitrobenzoic acid from 5,5′-dithiobis-2-nitrobenzoate (DTNB). The rate of color production is measured at 410 nm. Quinidine sulfate, an inhibitor, is used to stop the reaction at the end of the incubation period.

Materials and Method. All materials used for the assay were supplied in the Pfizer #5360 ChE-tel Test Set (Pfizer diagnostics, 300 West 43rd St., New York, N.Y. 10036) and the method outline supplied with the kit was followed. Spectrophotometric measurements were performed using a Beckman DU monochromator attached to a Gilford Model 222 photometer and power supply.

Individual assays were performed by adding 0μl of plasma to a tube containing 4.0 ml of buffered DTNB (0.1 mg/ml DTNB in 0.05M Tris buffer, pH 7.40, with ionic strength increased to .153 with NaCl) previously equilibrated at 37° C. With the tube immersed in the incubation bath, 0.50 ml of substrate (propionylthiocholine iodide, 0.028M) was added and mixed. After exactly 3 minutes incubation, 1.0 ml of quinidine sulfate (0.5% w/v) was added to stop the reaction and the absorbance was read at 410 nm. Zero absorbance calibration was provided by a blank prepared exactly as described for the sample except that the quinidine sulfate was added prior to the substrate.

ChE-tel units were calculated by multiplying the absorbance by 120, the conversion factor for 10-mm cuvettes. Calculated ChE-tel units over the range of 9.5 to 90 were plotted against pH-Stat units obtained for the same plasma samples assayed simultaneously. The linear regression equations and correlation coefficients were calculated, each point representing the mean of triplicate assays of the same sample by each method. The regression of pH-Stat units on ChE-tel units is shown in Figure 2.

Caraway Method.

The Caraway method is a colorimetric procedure utilizing the acid-base indicator, phenol red, to measure the reduction in pH which accompanies the hydrolysis of acetylcholine.[6] The phenol red absorbance is decreased as the acetic acid produced lowers the pH of the buffered reaction mixture. The ratio of the final to initial absorbance in each sample is compared to a standard curve, similarly prepared by substituting known quantities of acetic acid for the substrate. The method requires equipment ordinarily possessed by a clinical laboratory and the reagents are relatively stable.

Materials and Method. Acetylcholine perchlorate (K & K Laboratories, Plainview, N.Y.) was dried overnight in a vacuum desiccator; a 0.663M solution was prepared by dissolving 4.074 g of the reagent in distilled water and diluting to 25 ml. The buffer-indicator mixture was prepared by grinding 100 mg phenol red with 28 ml of 0.01N NaOH until dissolved; then 10 ml of the phenol red solution was added to a solution containing 6.65 g of anhydrous Na_2HPO_4 and 0.43 g of anhydrous KH_2PO_4, and diluted to a final volume of 1 liter with distilled water.

For each assay, duplicate tubes containing 0.5 ml of plasma and 5.0 ml of the buffer-indicator solution were allowed to come to temperature equilibrium for 10 minutes in a 25° C. water bath. The substrate was added (0.5 ml), mixed,

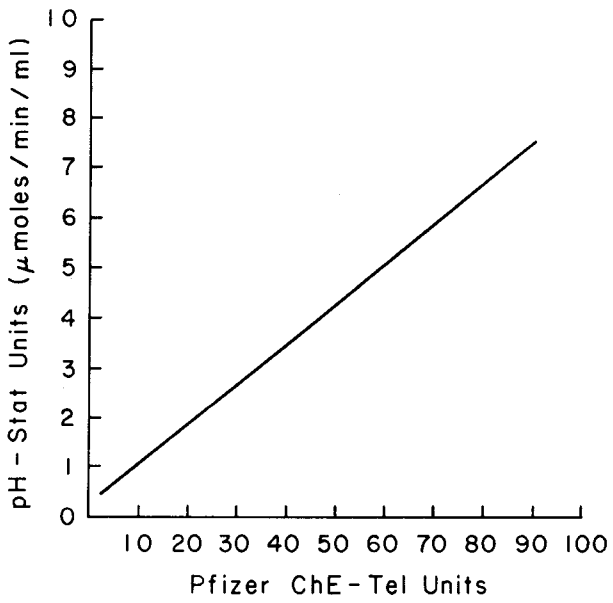


FIGURE 2. Correlation between pH-Stat units and Pfizer ChE-Tel units. N=25 points; correlation coefficient = 0.980

and the absorbance was determined at 540 nm on an instrument zeroed with a distilled water blank. After 30 minutes of incubation, the absorbance was determined a second time and the ratio of the final absorbance (A_2) to the initial absorbance (A_1) was determined (i.e. A_2/A_1). Two control tubes, with 0.5 ml of distilled water substituted for the plasma, were prepared and incubated, then the A_2/A_1 ratio was obtained as before. The average value of A_2/A_1 for the control tubes was subtracted from the ratio for each test replicate to correct for non-enzymic hydrolysis.

A standard curve was prepared by reading the absorbances of a series of eight tubes, each containing 5.0 ml of buffer-indicator solution, 0.5 ml of pooled plasma, and 0.5 ml of 0.01N–0.15N acetic acid (corresponding to 10–150 Caraway activity units/ml) and plotting the ratios of each of these absorbances to the absorbance of a tube similarly prepared but containing no acetic acid. These ratios were plotted on the log axis of semi-log paper against the corresponding Caraway units on the linear axis and the sample activity was read directly from the curve.

Calculated Caraway units were plotted against the pH-Stat units obtained for the same plasma samples assayed simultaneously. Regression equations and correlation coefficients were calculated, with each point representing the mean of duplicate assays by each method. The regression of pH-Stat units on Caraway units is depicted in Figure 3.

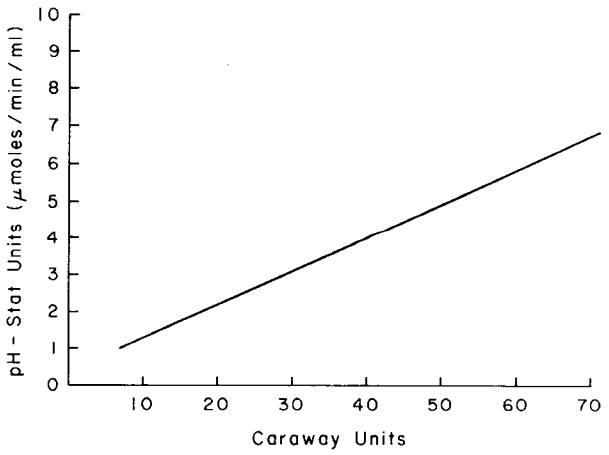


FIGURE 3. Correlation between pH-Stat units and Caraway units. N=20 points; correlation coefficient = 0.987

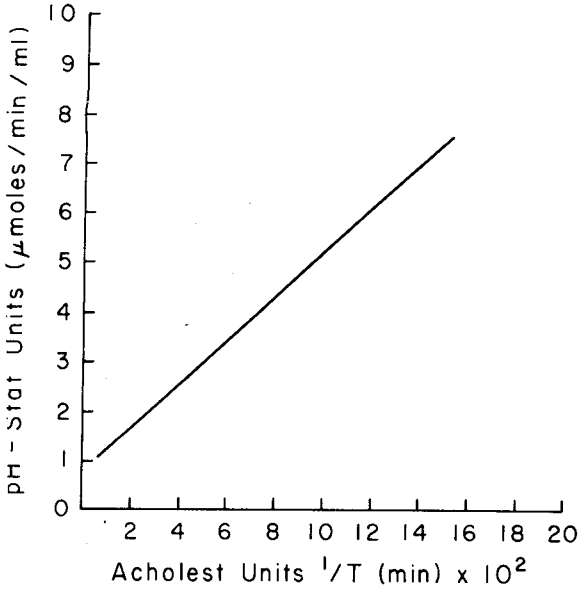
VI. Acholest Method.

The Acholest method is a simple "test-paper" procedure of the type used for field screening where multiple cases of suspected pesticide exposure have occurred. The method is based on the time required for the enzyme activity of a plasma sample to change the color of a piece of filter paper impregnated with buffer, substrate, and pH indicator to match a filter paper similarly treated but containing no substrate. Under stable temperature conditions, we have found the results from this method to correlate well with those obtained by more sophisticated techniques. The principal advantages are inexpensive materials with long storage life and very simple equipment requirements. These factors might induce some clinical laboratories having few requests for ChE assays to maintain this capability.

Materials and Method. All materials used for the assay were supplied in the Acholest Test Paper Kit (donated by E. Fougera & Co., Inc., Hicksville, New York, Distributor) and the modified procedure described by Braid and Nix was followed. These modifications consisted of cutting both test and control strips into ¼-inch discs with a paper punch prior to use and utilizing an incandescent light source for viewing the color change against a dull-black background.

Individual plasma samples were assayed by placing six 7-μl drops on a clean microscope slide; three test discs and three control discs were lowered onto the drops with tweezers and the timer was activated. A second slide was lowered onto the first and pressed gently to assure saturation of the discs with the plasma. The time required for the color of the test discs to match the color of the control discs was recorded. All tests performed during the unit conversion study were at 25° C.

Reciprocal Acholest units (i.e., 1/minutes to color match) were plotted against pH-Stat units obtained by simultaneous assays of the same samples. Regression equations and correlation coefficients were calculated, each point representing the mean of duplicate assays of the same sample by each method. The regression of pH-Stat units on Acholest reciprocal units is shown in Figure 4.



IGURE 4. Correlation between pH-Stat units and Acholest units. N=13 points; correlation coefficient = 0.991

II. Conclusions.

The relative precision of the methods in this laboratory as represented by the coefficient of variation (standard deviation/mean) for replicate samples was 1.70%, 1.45%, 2.0%, 2.79% for the pH-Stat, Michel, Caraway, and Pfizer ChE-tel methods respectively. A precision study was not performed for the Acholest method, however Ward advises that the procedure produces a coefficient of variation of approximately 8%. Any of the methods discussed would be satisfactory for confirming cases of acute organophosphate poisoning. The more precise methods would obviously be more satisfactory for the routine monitoring of low-level chronic exposure.

Regression equations were calculated from the experimental data for the following conversions:

a. To convert Michel units to pH-Stat units:
pH-Stat units = (8.32) (Michel units) + 0.41

b. To convert pH-Stat units to Michel units:
Michel units = (0.117) (pH-Stat units) − 0.039

c. To convert Pfizer (ChE-tel) units to pH-Stat units:
pH-Stat units = (0.08) (ChE-tel units) + 0.30

d. To convert pH-Stat units to Pfizer (ChE-tel) units:
ChE-tel units = (12.07) (pH-Stat units) − 1.83

e. To convert Caraway units to pH-Stat units:
pH-Stat units = (0.092) (Caraway units) + 0.366

f. To convert pH-Stat units to Caraway units:
Caraway units = (10.67) (pH-Stat units) − 3.15

g. To convert Acholest units (minutes to color match at 25° C.) to pH-Stat units:
pH-Stat units = (44.9/T) + 0.827 where T = minutes to color match by the Acholest method

h. To convert pH-Stat units to Acholest units:
Acholest units = (81.4/pH-Stat Units) − 5.19

Conversion equations for the relationships between the units of the Michel, Pfizer, Caraway, and Acholest methods may be readily derived from the preceding equations. Also, since the same pH-Stat primary method was used in an earlier paper to interconvert the units of the Sigma and Boehringer methods,[1] these two papers provide the clinical worker with the necessary data to effect a conversion of units between any of seven commonly used serum (or plasma) ChE methods.

Thus, it is possible to directly compare pre-exposure ChE units with post-exposure units, even when these values were determined by different techniques. Such a comparison can frequently identify a case of low-level chronic ChE inhibition which would be undetectable by comparing a single ChE value with the broad normal range of values for a specific method. With direct interconvertibility of units, any one of these methods best suited to the resources and facilities of the laboratory can be used for the medical monitoring of aerial applicator personnel or for confirmatory diagnosis in acute organophosphate poisoning. Regardless of the method selected, however, each aerial applicator should have a pre-exposure test from which any subsequent depression of ChE activity can be measured.

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