Estimates of Variability in a Comparative Standardized Cholinesterase Assay

John A. Burkart, Dennis Y. Takade, and Randol Potter
Utah Biomedical Test Laboratory
520 Wakara Way
Salt Lake City, Utah 84108

The sensitivity of acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) to inhibition by insecticidally active organophosphorus compounds has resulted in the use of blood cholinesterase activity as an indicator of pesticide exposure. Indeed, several investigators have used the measurement of blood cholinesterase, both AChE and BuChE, in estimating worker hazard upon reentry into pesticide treated fields (WARE et al. 1972; WARE et al. 1974; SPEAR et al. in press).

The cholinesterase assay method as an indicator of human exposure to insecticides is made difficult by individual variability in normal subjects such as that described by YAGER et al. (1976) and GAGE (1967). The problem of within subject variability is compounded by the variability in analysis procedures among laboratories (SERAT and MENGLE 1973) and day-to-day variability within laboratories. The running of known laboratory standard enzyme samples would seem appropriate to reduce day-to-day variability as a result of laboratory procedures. But YAGER et al. (1976) showed that two known control sera, Hyland Scan I and Hyland Scan II, did not correlate well with daily means of blood cholinesterase measurements and hence were not satisfactory for controlling variability. These results were also confirmed in this laboratory in an analogous study using the same protocol.

This communication gives the results of a statistical study to ascertain day-to-day variability in two laboratories using five selected commercially available cholinesterase controls, thereby eliminating the within subject variability arising from repeated sampling of a subject.

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Materials and Methods

Experimental Design

Two laboratories, the Utah Biomedical Test Laboratory (UBTL) and Intermountain Laboratories (IL) conducted a comparative study of cholinesterase determinations. Five control samples were divided into 20 lots and each laboratory performed cholinesterase determinations for the five samples on each of ten different days. Identical protocols, procedures, and reagents were used.

Preparation of Standard Enzyme Solutions

Bovine erythrocyte acetylcholinesterase (BChE) was obtained as a lyophilized gelatin stabilized powder (Sigma Chemical, St. Louis, Missouri) and was diluted in Sorensen phosphate buffer (pH 8.0), made according to HENRY (1974). The final concentrations of BChE were 4.0 mg/ml and 1.0 mg/ml. These concentrations constituted samples A and B respectively.

Hyland Scans I and II (Standard Scientific, Piscataway, New Jersey) were diluted with 10 ml glass distilled water. Scan I constitutes sample D and Scan II constitutes samples C and E.

Each sample was then divided into 20 lots which were divided in half and frozen. Ten lots of each sample were retained by UBTL and 10 lots of each sample were given to Intermountain Laboratories.

Preparation of Reagents

Propionylthiocholine iodide (PSCh)(Aldrich Chemical, Milwaukee, Wisconsin) was used as the substrate and was dissolved in glass distilled water to give a final concentration of 1.5 mg/ml. The substrate solution was made fresh for each experiment.

To a 0.9% aqueous sodium chloride solution was added 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) (Aldrich Chemical Milwaukee, Wisconsin) to give a final concentration 0.2 mg DTNB per ml sodium chloride solution. This solution was then added to an equivalent volume of Sorensen phosphate buffer (pH 8.0) to give the final working solution which was stable for one week when kept refrigerated.

Tetram oxalate* (O,O diethyl S-2(diethyl ammonium)-ethyl phosphorothioate hydrogen oxalate) was dissolved in distilled water to a final concentration of 0.00359 mg/ml.

Enzyme Assay Procedure

The procedure used for the assay of acetylcholinesterase is essentially the method described by VOSS and SACHSSE (1970) which is a modification of the Ellman technique (ELLMAN 1961). The Ellman method is a colorimetric method which follows the formation of the thiolate anion at 412 nm.

The following modifications were made in the original Voss and Sachsse procedure:

1. Tetram oxalate was used to stop the reaction in place of eserine sulfate.

2. A reagent blank was used in place of a serum blank.

3. Ten (10) µl of enzyme was used in each measurement.

4. The cholinesterase was added to the PSCh-DTNB-buffer solution rather than addition of PSCh to cholinesterase-DTNB-buffer solution.

Results and Discussion

Mean cholinesterase values reported as optical density (O.D.) units are given by laboratory, day, and sample in Table 1. Table 2 presents the analysis of variance table. The data were analyzed as a three factor factorial design with two replicates. Laboratories and samples were specifically selected and are fixed factors in the analysis, while days and replicates are considered random factors.

*Extremely toxic material.

TABLE 1. Cholinesterase Mean Values (O.D. Units)

	UBTL	IL
Overall Mean	0.438	0.468
Day		
1	0.432	0.453
2	0.437	0.460
3	0.443	0.458
4	0.416	0.463
5	0.446	0.493
6	0.465	0.497
7	0.437	0.470
8	0.456	0.472
9	0.425	0.475
10	0.427	0.447
Sample		
A	0.811	0.962
B	0.197	0.247
C	0.379	0.368
D	0.432	0.409
E	0.372	0.359

TABLE 2. Analysis of Variance of Cholinesterase Values Obtained from UBTL and IL

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F-Ratio	Signif-icance
Laboratories	1	0.04654	0.04654	51.71	p<.001
Days	9	0.03529	0.00392	30.15	p<.001
Samples	4	10.26772	2.56693	5580.28	p<.001
Labs x Days	9	0.00813	0.00090	6.92	p<.001
Labs x Samples	4	0.21376	0.05344	71.25	p<.001
Days x Samples	36	0.01652	0.00046	3.54	p<.001
Labs x Days x Samples	36	0.02702	0.00075	5.77	p<.001
Within Replicates	100	0.01338	0.00013		
Total	199	10.62836			

Estimates of variance components:

Days	Days x Labs	Days x Samples	Days x Labs x Samples	Replicates
.00019	.00008	.00008	.00031	.00013

Standard error for comparing the same sample on 2 different days in the same lab = 0.040

Standard error for comparing 2 different samples on the same day in the same lab = 0.032.

All effects and interactions in the study were highly statistically significant. The two laboratories were not comparable between each other and each was not consistent within itself with regard to cholinesterase determinations on a variety of control samples over ten days of repeated analyses. The differences are illustrated in Figures 1a to 1e. The largest differences between laboratories occurred in the analysis of the largest and the smallest samples, while differences were smaller in the middle range of cholinesterase levels. For each laboratory, the coefficient of variation averaged 5% over 10 days of repeated analyses on each sample.

Estimates of variance components show that the largest source of variation is the laboratory x days x samples interaction, or rather the general inconsistencies between laboratories on several samples on several days. The day-to-day variation is slightly larger than the within-day variation. This analysis did not estimate variability due to laboratories since only two selected laboratories were utilized; however, the data suggests that among laboratory variation would be considerable if an experiment using a larger number of randomly selected laboratories was done.

The standard deviation among replicates is 0.011 O.D. units giving a 95% confidence interval for the repeatability of a particular observation to be ± 0.022 O.D. units, or about 5%. The standard error for comparing the same sample on different days in the same laboratory is 0.040 O.D. units. The difference between daily measurements would have to be more than 0.080 O.D. units (about 20% on the average) for that difference to be statistically significant at the 5% level. On a particular day in a particular laboratory, the standard error for comparing two different samples is 0.032 O.D. units. The corresponding 95% confidence limits (± 0.064 O.D. units) would allow for discrimination between sample A and the others, sample B and the others, but would not distinguish among samples C, D, and E.

This analysis has demonstrated that, even when following a common protocol controlling for many experimental factors, cholinesterase determinations on control sera are very imprecise. The use of control sera to reduce daily or laboratory variation is unacceptable and attempts should be made to find the factors responsible for unexplained variability. The presence of significant among and within laboratory differences on control sera make only large real differences in cholinesterase determinations detectable.

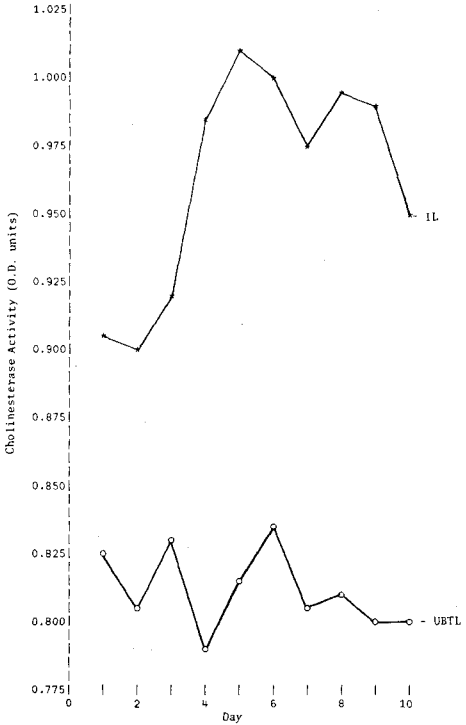

Figure 1a. Sample A.

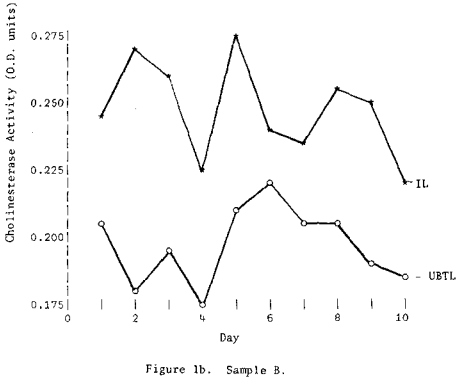

Figure 1b. Sample B.

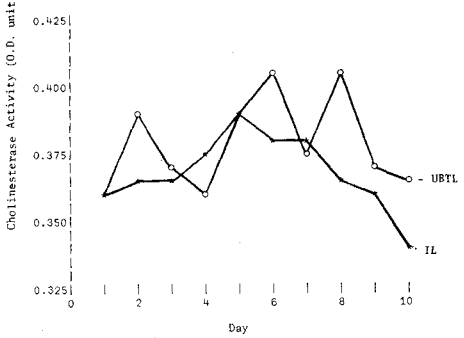

Figure 1c. Sample C.

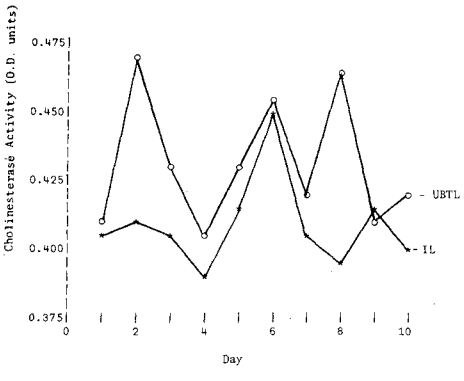

Figure 1d. Sample D.

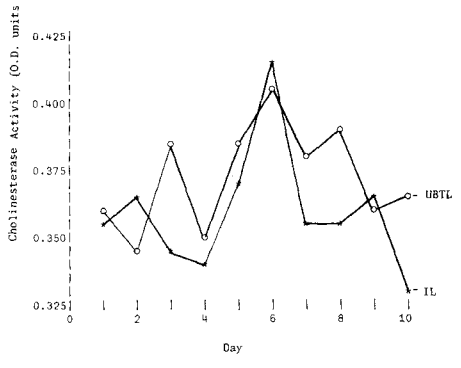

Figure 1e. Sample E.

Figure 1. Comparison of Cholinesterase Activity by Laboratory and Day for 5 Control Samples.

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References

ELLMAN, G. K., K. D. COURTNEY, V. ANDRES and R. M. FEATHERSTONE: Biochem. Pharmacol. 7, 88 (1961).

GAGE, J. C.: Res. Rev. 18, 159 (1967).

HENRY, R. J., D. C. CANNON, and J. W. WINKELMAN: Clinical Chemistry: Principles and Techniques. 2nd Ed. Harper and Row, New York (1974).

SERAT, W. F., and D. C. MENGLE: Bull. Environ. Contam. Toxicol. 9, 24 (1973).

SPEAR, R. C., W. J. POPENDORF, J. T. LEFFINGWELL, T. H. MILBY, J. E. DAVIES, and W. F. SPENCER: (in press).

VOSS, G., and K. SACHSSE: Toxicol. Appl. Pharmacol. 16, 764 (1970).

WARE, G. W., B. ESTESEN, and W. P. CAHILL: Bull. Environ. Contam. Toxicol. 8, 36 (1972).

WARE, G. W., D. P. MORGAN, B. J. ESTESEN, and W. P. CAHILL: Arch. Environ. Contam. Toxicol. 2, 117 (1974).

YAGER, J., H. McLEAN, M. HUDES, and R. C. SPEAR: J. Occup. Med. 18, 242 (1976).