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PSEUDOCHOLINESTERASES

I. A Quick Screening Test for Determination of Serum Pseudocholinesterase Activity.

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A rapid and dependable screening test is needed for plasma cholinesterase activity determination. In fact, the Department of Anesthesia at Hacettepe Medical Center has recently had experience with individuals who were deficient or defective in pseudocholinesterase and who required resuscitation after succinyl-dicholine injection. Numerous screening tests have been reported in the literature for rapid determination of plasma pseudocholinesterase activity. However, none of them is completely satisfactory. We have recently developed a rapid spot test which is now in routine use in our Department of Anesthesia and in the Blood Bank. This test has the following advantages : (1) Although it is a semiquantitative test, it correlates closely with spectrophotometric determinations. (2) It easily detects pseudocholinesterase anenzymia and low plasma cholinesterase caused by liver disease. (3) The test is designed in such a manner that it detects the heterozygote pseudocholinesterase defective individual with mild dibucaine insensitivity. As we encountered no fluoride insensitive defective pseudocholinesterase individuals, we do not yet know whether the test will detect those or not. (4) The

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test is not complicated by erythrocytes or hemolysis even if these components were present in frank amounts in sera. (5) No colorimeter is required, making this test ideal for field work. (6) The reagents used are fairly stable. (7) The test can be done by any technician and requires no special training. (8) Its cost is very little and does not require more than a few minutes to run.

We are reporting here the preliminary results of our test. Detailed biochemical investigations will appear elsewhere.

Pseudocholinesterase is an esterase that catalyzes the hydrolysis of a choline-type alcohol linked ester.

$$R_1 - O - \overset{O}{\overset{\|}{C}} - R_2 + H_2O \longrightarrow R_1 - OH + R_2 - \overset{O}{\overset{\|}{C}} - OH$$

R_1 = choline $\qquad$ R_2 = aliphatic, aromatic

This enzyme is not specific for $R_1 - OH$, but its activity increases as the R_2 group becomes lipophilic.[1] The activity is assessed either by measuring the amount of the ester before and after the enzymic assay (Hestrin's method)[2], or manometrically, following the liberation of acid,[1] or spectrophotometrically, following the esterase activity by the decrease of the ester bond absorption in the ultraviolet region of the spectrum (at 229 milimicrons).[3] Of these methods, the manometric and the spectrophotometric are certainly much more sensitive than Hestrin's method. However, they have their disadvantages : the manometric method requires expensive equipment and is time-consuming; the spectrophotometric method requires an ultraviolet spectrophotometer.

Recently, Ellman described a new method for determining cholinesterase activity.[4] This method depends on estimation of the amount of thiocholine liberated by the cholinesterase from a thiocholine ester.

$$(CH_3)_3 - \overset{+}{N} - CH - S - \overset{O}{\overset{\|}{C}} - R_1 + H_2O \longrightarrow$$

$$(CH_3)_3 - \overset{+}{N} - CH_2 - SH + R_1 - \overset{O}{\overset{\|}{C}} - OH$$

R_1 = acetic acid, butyric acid.

The liberated thiol reacts readily with 5-5' dithiobis 2-nitrobenzoate ion.

Whereas 5-5' dithiobis 2-nitrobenzoate has an ultraviolet absorption maximum, the product of the reaction, 5-thio-2-nitrobenzoate has an absorption band in the visible spectrum, between 410 and 420 milimicrons (Figure 1). Hence, the end result of the enzymic reac-

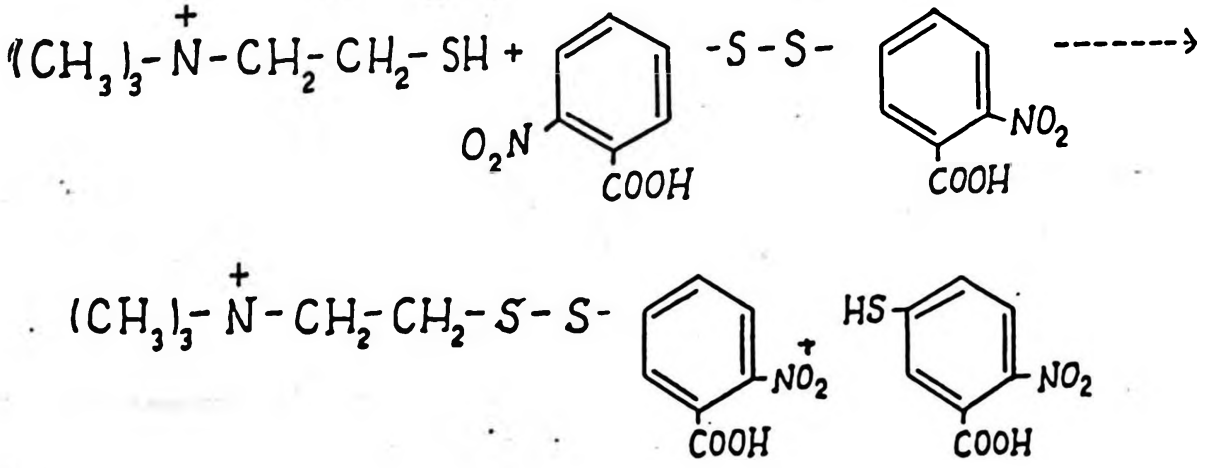

Figure 1. The reaction of thiocholine with 5-5'-dithiobisnitrobenzate.

tion is yellow. This yellow color is intense, in fact twice as intense as the color produced by the spectrophotometric ultraviolet method for cholinesterase estimation, which is the most sensitive method in current use. Also pseudocholinesterase reacts readily with either acetylthiocholine or butyrylthiocholine.[4, 5] The ultraviolet estimation depends on the decrease in optical density of the assay system, whereas this new method depends on an increase in optical density and that enables very accurate measurement of the initial rates. It is also superior to Hestrin's method or other manometric methods in that initial rates can be estimated before one per cent of the hydrolysis has proceeded. This last point is important but is often overlooked. As the $\triangle F^\circ$ value of the cholinesterase reaction is not highly negative, reversion of the reaction will obscure the kinetics.[6] Under the usual assay conditions, at 23 C, the equilibrium constant, K, is 0.25, and the $\triangle F^\circ$ that equals $RT \ln \frac{55.5}{K}$ will give a value of —3200 calories. So, if rates are calculated, for example, after the usual ten cent per cent hydrolysis with Hestrin's method, a significant reversion will occur and the results will not allow kinetic calculations. Thus, it is essential to measure the rate before the hydrolytic reaction has proceeded far. The Ellman method is highly suited to this purpose,[6] because the color produced is intense before the hydrolysis has proceeded to the critical limit. For the above reasons, we selected

the Ellman method as the basis from which we developed our screening test.

Simple methods of pseudocholinesterase screening, based on the change in color of suitable indicators, have been reported.[7 - 11] However, none of the reported techniques is really suitable as a quick spot test because they depend on the liberation of acid and the end point determination is not adequately defined because it depends on rather insensitive indicators. On the other hand, the brilliant yellow color produced by the Ellman method can easily be detected visually and this point is extremely valuable for screening purposes. By matching appropriate color standards at a predetermined optical density, serum pseudocholinesterase activity can be measured without reference to a spectrophotometer.

Materials and Method

Material : Butyrylthiocholine iodide and acetylthiocholine iodide were purchased from Sigma Chemical Co., St. Louis, Missouri, U.S.A. Dithiobisnitrobenzoic acid was purchased from the Aldrich Chemical Company, Milwaukee, Wisconsin, U.S.A. The other chemicals were all of C.P. grade and obtained on the market. Pyrex tubes, 750 by 12 mm, were used without further selection for exact inner diameter.

Preparation of the Color Standard : The color standard is not stable and has to be prepared every two days. The solution consists of 1:800 diluted saturated picric acid and 10^{-5} M bromophenol blue in tenth-normal HCl. This solution has an optical density of 0.600 at 412 milimicrons in a one cm. light pathway. It is prepared by diluting a stock of saturated picric acid solution in water and 10^{-3} M bromophenol blue in absolute ethanol with tenth-normal HCl. The stock solutions of picric and bromophenol blue are stable at room temperature for a long time. Three mililiters of the color standard are pipetted into the 750 by 12 mm test tubes and covered with a cork.

Preparation of the Mixture for Enzyme Determination : This mixture can be prepared in two ways : (a) a solution of potassium phosphate, 2×10^{-1} M at pH 8, dithiobisnitrobenzoic acid, 7×10^{-5} M, and butyrylthiocholine, 8.5×10^{-3} M is prepared. The solution is then distributed in three mililiter amounts to 750 by 12 mm test tubes. No special precaution is necessary to choose test tubes of exactly the same inner diameter. The mouth of each tube is then closed with

a piece of parafilm and the tube is kept frozen at —20 C. This solution is stable for at least one month. (b) For field surveys, 50 mililiter test tubes are used and marked at 30 cc. Into each test tube, 2.1 micro Moles of dithiobisnitrobenzoic acid and 2.45 micro Moles of butyrylthiocholine are placed, and the tubes covered with parafilm. This mixture is stable at room temperature for at least 15 days, if kept dry. When enzyme activity is to be determined, 30 ml of 200 mM KPO_4 at pH 8 must be added up to the 30 cc mark, and the chemicals dissolved. Next, three millimeter portions are transferred to 750 by 12 mm test tubes, which are then used for the enzyme determination. Either of the mixtures (a) or (b) must be brought to room temperature for enzyme determination and may be kept at this temperature for four hours at the most.

Method: A dry hemoglobin pipet is used to place 20 λ of serum or plasma into the determination mixture at room temperature (22 to 25 C). The pipet must be clean and free of acetone as acetone is an inhibitor of pseudocholinesterase. The chronometer is then started and the mixture covered with parafilm and mixed by inverting. The tube is then placed in a rack between two color standard tubes and read against a white background. The tube containing the test material should not be held in the hand as the reaction is sensitive to temperature. When the color of the test material is equal to the color of the references the chronometer is stopped and the time is recorded. If the sample being tested contains a normal enzyme in normal amounts, the mixture will become the same color as the standards in not more than four minutes.

Results

Thirty-two randomly selected samples of sera were taken from the blood bank for enzyme investigation. Results of the test were obtained within 79 to 218 seconds, the mean time being 142 seconds, $\pm$ 6 seconds standard deviation. Samples from patients with liver disease (infectious hepatitis, liver cirrhosis, lymphoblastic invasion of the liver due to acute blastic leukemia, kala-azar, and hepatomegaly of unknown origin) gave results after 300 seconds or more. In testing plasma from a patient with fulminating infectious hepatitis, the elapsed time was 34 minutes, considerably longer than the upper limit of four minutes for the normal population. Sera from severely jaundiced patients will obscure the test results because of the yellow bilirubin color. However, there is a parallel between liver function tests dependent on liver protein synthesis and the spot test.

One of the patients referred to our service, N. K., was most probably a heterozygous dibucaine-insensitive individual (E_1^u E_1^a). This six year old boy had a mandibular tumor with primary hyperparathyroidism. The tumor was removed surgically and was diagnosed as Brown tumor of hyperparathyroidism with fibrous dysplasia and giant cells. The patient experienced a somewhat prolonged apnea after a single dose of succinyldicholine injected during surgery. Serum samples were collected 48 hours after surgery and subjected to investigation (Table 1). Mild dibucaine insensitivity was evident with both acetylthiocholine and butyrylthiocholine as substrates. The kinetic behavior of the serum towards butyrylthiocholine was

TABLE 1

DIBUCAINE, QUINIDINE SENSITIVITY OF NORMAL SUBJECTS AND THE DEFECTIVE PATIENT

Enzyme rate is measured at 30 C° in 1 cm. light pathway at 412 mM. The unit is expressed as millimoles of substrate hydrolyzed per hour per liter of serum.

Subject	Unit	Inhibition with Quinidine 4×10^{-7} M (Acetylthiocholine 10^{-4} M)	Inhibition with Quinidine 4×10^{-5} M (Acetylthiocholine 10^{-4} M)	Inhibition with Dibucaine 4×10^{-6} M (Acetylthiocholine 10^{-3} M)	Inhibition with Dibucaine 4×10^{-4} M (Acetylthiocholine 10^{-3} M)
Normals :					
Y. B.	120	85.3 %	96.8 %	74.8 %	96.5 %
T. T.	138	64.3 %	100.0 %	70.0 %	96.0 %
M. O.	151	58.6 %	97.8 %	71.7 %	96.7 %
S. S.	165	61.6 %	99.2 %	81.7 %	97.3 %
M. L.	198	60.8 %	99.0 %	60.0 %	98.9 %
A. K.	165	64.3 %	99.0 %	75.7 %	99.0 %
Mean	—	60.8 ± 1.4 %	98.8 ± 0.5 %	72.3 ± 1.9 %	97.4 ± 0.5 %
Purified pseudocholinesterase from pooled human plasma		68.4 %	99.0 %	67.2 %	99.0 %
Patient :					
N. K.	92	36.0 %	92.0 %	36.0 %	50.0 %

abnormal and had a higher Km value.[12] For that reason, at the butyrylthiocholine concentration used in the spot test, the length of time required to obtain the result was markedly prolonged (630 seconds).

The dibucaine number determined according to the procedure of Kalow [13] was 78 for the pooled human plasma pseudocholinesterase, and 55 for the patient (N. K.). This finding is in accordance with present knowledge, and definitely proves that this individual is in fact a heterozygote.

Sera from two patients with pseudocholinesterase anenzymia showed no reaction and, in each case, results were obtained only after 13 hours.[13]

Conclusion

It is important for the clinician to be aware of the plasma pseudocholinesterase activity in various hepatic disorders, in clinical conditions where there is a negative protein balance, or in patients exposed to nerve gases and insecticides.

Above all it is important to detect the apparently healthy subject who has a genetical disorder at the pseudocholinesterase *operon*. In the latter cases, where pseudocholinesterase is either lacking or defective, injection of succinyldicholine for surgical purposes will lead to serious trouble for the patient and the anesthetist.[13] Also detecting such individuals with pseudocholinesterase abnormality will be of value for the statistician and the geneticist. Especially if the detection test is a simple device, as described here, a mass screening will be a most helpful genetic marker.

The advantages of this screening test are as follows :

1. The end point is easily visible and errors of determination and individual variations in reading are not more than ten per cent.
2. It is well suited for screening of an entire population and can be used in remote areas as it requires no complicated equipment.
3. It detects defective heterozygotes (E_1^u E_1^a) and individuals with pseudocholinesterase anenzymia.
4. Its cost is very low.
5. The reagents are stable and for that reason suitable for field work.
6. Use of butyrylthiocholine instead of acetylthiocholine makes the test specific for serum cholinesterase and the results are reliable even if the sera are frankly hemolytic.

7. The simplicity of the test makes it a valuable tool for the clinical pathologist as a liver function test.

We hope to use this test for large scale surveys of various ethnic populations in Turkey, as it is most simple to carry out and can be easily applied in remote parts of the country where there are no clinical biochemical facilities available. Secondly, we hope that we will detect the abnormal individuals before they go to surgery as the test is now routinely carried out on sera that is sent to the Blood Bank for typing and cross-matching by the Department of Surgery.

Summary

A quick screening test for serum pseudocholinesterase is reported.

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REFERENCES

1. Augustinsson, K. B.: Assay methods for cholinesterases, in Methods of Biochemical Analysis, London, Interscience Publishers Limited, volume 5, 1957, pp. 1 - 64.
2. Hestrin, S.: The reaction of acetylcholine and other carboxylic acid derivatives with hydroxylamine and its analytical application, J. Biol. Chem. 18 : 249, 1949.
3. Gal, E. M., and Roth, E.: Spectrophotometric methods for determination of cholinesterase activity, Clin. Chim. Acta 2 : 316, 1957.
4. Ellman, G. C., Courtney, K. P., Andres, V. Jr., and Featherstone, R. M.: A new and rapid colorimetric determination of acetylcholinesterase activity, Biochem. Pharmacol. 7 : 88, 1961.
5. Karahasanoğlu, A. M., and Özand, P.: Manuscript in preparation.
6. Nachmansohn, D., and Wilson, I. B.: The kinetic enzymic hydrolysis and synthesis of acetylcholine, in Advances in Enzymology, edited by Nord, F. F., volume 12, 1951, pp. 259 - 340.
7. Limperos, G., and Ranta, K. E.: Rapid screening test for determination of approximate cholinesterase activity in human blood, Science 117 : 453, 1955.
8. Fleischer, J. H., Woodson, G. S., and Simet, L.: Visual method for estimating blood cholinesterase, Arch. Indust. Health 14 : 510, 1956.
9. Wolfise, J. H., and Winter, G. D.: Bromothymol blue screening test, Arch. Indust. Hyg. Occup. Med. 91 : 396, 1954.

10. Herzeld, E., and Stumpf, C.: Ein neuer kurztest zur bestimmung der serum cholinesterase aktivitat, Wien. Klin. Wschr. 67 : 874, 1953.

11. Wang, R. I. H.: Determining cholinesterase activity in human plasma, J. A. M. A. 183 : 792, 1963.

12. Karahasanoğlu, A. M., and Özand P.: Manuscript in preparation.

13. Kalow, W.: Heredity and the Response to Drugs, London, W. B. Saunders Company, 1962.

14. Harris, H.: Enzymes and drug sensitivity; the genetics of serum cholinesterase deficiency in relation to suxamethonium apnoea, Proc. Roy. Soc. Med. 57 : 503, 1964.

15. Karahasanoğlu, A. M., and Özand, P.: Pseudocholinesterases II. Two cases of pseudocholinesterase deficiency, Turkish J. Pediat. 8 : 10, 1966.