Biochemical Pharmacology, Vol. 26, pp. 1263–1266. Pergamon Press, 1977. Printed in Great Britain.

A COMPARISON OF METHODS FOR MEASURING ACETYL CHOLINESTERASE ACTIVITY IN BLOOD SAMPLES INHIBITED BY CARBAMATES

MARY C. FRENCH, DAVID J. SELLERS and RODNEY G. WILKINSON*
Chemical Defence Establishment, Porton Down, Salisbury, Wiltshire, SP4 0JQ, England

(*Received* 20 *October* 1976; *accepted* 31 *December* 1976)

Abstract—An unambiguous method based on the measurement of the acetylcholinesterase activity resulting from complete decarbamoylation of the acetylcholinesterase in blood samples inhibited by a carbamate is described and compared with a spectrophotometric modification of Ellman's method.

Several methods are available for measuring the acetylcholinesterase activity of whole blood. These include manometric methods [1], electrometric methods such as that of Michel [2], titrimetric methods [3] and colorimetric methods such as that of Sabine [4] or Ellman [5]. These methods are readily applicable to studies involving the interaction of organophosphates with the acetylcholinesterase of whole blood, since the phosphonylated enzyme formed is usually only slowly reactivated and thus the free acetylcholinesterase level remains constant during the assay. When acetylcholinesterase is inhibited by a carbamate, the carbamoylated enzyme formed undergoes spontaneous reactivation to regenerate acetylcholinesterase. The activity of the acetylcholinesterase will therefore increase during the time taken to complete the assay. Several workers have examined methods of measuring cholinesterase activity in the presence of a carbamate [7, 8], but these have not given unambiguous results.

As part of a study of the mode of action of carbamates *in vivo*, we required a rapid analytical method for assaying whole blood acetylcholinesterase activity so that time–acetylcholinesterase activity profiles could be obtained, and thus the effect and duration of action of the carbamate measured. An unambiguous, indirect method of analysis of whole blood acetylcholinesterase activity in the presence of a carbamate has been developed. The basis of the method is to remove the non-carbamoylated enzyme by irreversible phosphonylation and to measure the acetylcholinesterase activity resulting from complete decarbamoylation of that enzyme which had been carbamoylated. The unambiguous titrimetric method of analysis has been compared with a direct spectrophotometric method [5].

MATERIALS AND METHODS

Materials

The enzymes used in the calibration work were purified bovine erythrocyte acetylcholinesterase (E.C. 3.1.1.7) and purified horse serum cholinesterase (E.C. 3.1.1.8)(ex Sigma Chemical Co.). The substrates used were acetylcholine iodide, acetylthiolcholine iodide and butyrylthiocholine iodide (ex British Drug Houses). 5,5′-dithio (2-nitro benzoic acid) was obtained from Sigma Chemical Co., and pyridostigmine bromide was prepared using a published procedure [9]. Soman (pinacolyl methyl phosphonofluoridate) was synthesised with the establishment and was more than 95% pure.

Methods

1. *Apparatus.* Potentiometric titrations were carried out using a Radiometer TTT2 Autotitrator and SBR3 Titrigraph fitted to an ABU 11 Autoburette using a GK 2320C combination electrode. The spectrophotometers used were a Pye Unicam SP 1800 coupled to a Weyfringe ADCP-2 digital printer and a SP 700a spectrophotometer.

2. *Measurement of acetylcholinesterase activity.*

(i) Direct titration—A red cell membrane acetylcholinesterase preparation was used in this method. Heparinised whole blood (25 ml) was centrifuged at 2,000 *g* for 10 min and the plasma removed and discarded. The red blood cells were resuspended in the plasma volume of 100 mM sodium chloride and recentrifuged, repeating the washing twice. The red blood cells were resuspended in 250 ml 20 m OsM phosphate buffer, pH 7.4 at 4° and allowed to hemolyse overnight. The red cell membranes were removed by centrifugation at 18,000 *g* for 30 min, washed twice and then freeze dried. An Enzyme solution (5 ml) containing *ca.* 2 units/ml (where 1 unit of enzyme will hydrolyse 1 μmole acetylcholine per min at pH 8.0, 25°) was prepared in 5 mM phosphate buffer, pH 7.4 containing 100 mM sodium chloride, and incubated at 37° in a water bath.

An appropriate concentration of pyridostigmine was added and a 0.5 ml sample removed and added to a titration vessel containing 100 mM sodium chloride (10 ml) and acetylcholine iodide (0.5 mM). The solution was assayed at pH 7.4 and 37°, titrating the acid produced with sodium hydroxide (10 mM). The substrate concentration was maintained constant by using a second syringe containing acetylcholine iodide (10 mM) driven synchronously with the syringe containing sodium hydroxide. The procedure was repeated for samples removed at various time intervals until a constant enzyme activity was reached. At the concentration of carbamate used, further inhibition during the assay was minimal. This technique

was not applicable to whole blood due to hemolysis of the blood.

(ii) Indirect titration—whole blood (10 ml) in a test tube was placed in a water bath at 37°. Pyridostigmine was added and at appropriate time intervals a sample (1 ml) was removed. The sample was immediately treated with sufficient soman (pinacolyl methyl phosphonofluoridate) to phosphonylate the free acetylcholinesterase in the sample, and then washed three times with sodium chloride (100 mM) to remove plasma and any excess organophosphate. The red blood cells were suspended in 20 m OsM phosphate buffer, pH 7.4 and kept at 4° overnight. The sample was centrifuged to bring down the red cell membranes, these were suspended in 100 mM sodium chloride (2.5 ml) and titrated as above using acetylcholine (0.2 mM) as substrate, to measure the activity of acetylcholinesterase which had been carbamoylated. The method was also used for the red cell membrane preparation. Experiments were carried out to ensure no dephosphonylation of a phosphonylated blood sample occurred during the method and to ensure that no activity was lost from decarbamoylated samples because of unremoved organophosphate.

(iii) Spectrophotometric technique—An acetylcholinesterase solution containing 1 unit/ml was prepared in 5 mM phosphate buffer (pH 8.0, 100 mM sodium chloride), and a sample (50 μl) injected into a 5 ml graduated flask containing acetylthiocholine iodide (0.5 mM), 5,5′-dithio bis (2-nitro benzoic acid)(0.33 mM), (DTNB), in 100 mM phosphate buffer, pH 8.0 at 25°. The solution was mixed and transferred to a 1 cm cuvette and placed in the sample holder of the Unicam SP 700a spectrophotometer which was thermostatted at 25°. One min after the enzyme was added, the chart recorder was switched on and the absorbance change at 412 nm recorded for 3 min against a DTNB/substrate blank. This procedure was repeated for samples from 5–50 μl of the enzyme solution. It was shown that there was a linear correlation between the absorbance change per minute and the enzyme concentration.

This technique was then modified so that whole blood samples could be assayed. Heparinised whole blood (5 μl) was added to a 5 ml graduated flask, containing acetylthiocholine iodide (0.5 mM) and DTNB (0.33 mM) in 100 mM pH 8.0 phosphate buffer, solution was transferred to a 1 cm cuvette, placed in the spectrophotometer and the absorbance readings at 412 nm were printed automatically for 3 min by the Weyfringe ADCP-2 digital printer coupled to a Unicam SP 1800 spectrophotometer. Each assay was carried out in duplicate and was corrected for the non-enzymatic acetylthiocholine hydrolysis and a DTNB/tissue/thiol blank.

Calculation of results. The calculations were performed on the data using the expression: Total number of μmoles of ASCh hydrolysed = $(\Delta A/\text{min})/\epsilon \times 1 \times V/v$ where $\Delta A/\text{min} \equiv$ change in absorbance per min

ϵ = molar extinction coefficient of the DTNB anion at 412 nm, $1.34 \times 10^4\ M^{-1}\ min^{-1}$
1 = path length of cell, 1 cm
V = cuvette volume
v = sample volume

Using whole blood, the change in absorbance is due to the hydrolysis of acetylthiocholine by both acetylcholinesterase and butyrylcholinesterase. Measurement of the contribution of butyrylcholinesterase was therefore necessary. A standard solution containing 1 unit/ml of horse serum butyrylcholinesterase (E.C. 3.1.1.8)(ex Sigma) was prepared (where 1 unit of cholinesterase is defined as that enzyme which will hydrolyse 1 μmole of butyrylcholine per min at pH 8.0 and 25°) and used to obtain the calibrations between the absorbance change per min and the enzyme concentration for the hydrolysis of both acetylthiocholine and butyrylthiocholine, using the technique described above. It was found that acetylcholinesterase hydrolysed acetylthiocholine (0.5 mM) and butyrylthiocholine (0.5 mM) at the rates 1.4×10^{-3} and 1.1×10^{-5} μmoles per min respectively. For cholinesterase, acetylthiocholine and butyrylthiocholine were hydrolysed at the rates 2.0×10^{-4} and 3.4×10^{-4} μmoles per min respectively at a substrate concentration of 0.5 mM. Thus acetylthiocholine is hydrolysed by acetylcholinesterase at a rate seven times higher than that for cholinesterase, and butyrylthiocholine is hydrolysed by cholinesterase at a rate thirty times higher that that for acetylcholinesterase. This means that the presence of 1 unit of cholinesterase in blood containing 1 unit of acetylcholinesterase will result in the hydrolysis of acetylthiocholine being increased by *ca.* 14%.

A sample of whole blood was then used to hydrolyse butyrylthiocholine and from the absorbance change measured, the concentration of butyrylcholinesterase could be obtained from the calibration obtained above. The contribution that this concentration of butyrylcholinesterase made to the hydrolysis of acetylthiocholine could then be obtained from the butyrylcholinesterase/acetylthiocholine calibration.

For both rat and rabbit whole blood, the butyrylcholinesterase contribution to the absorbance change measured was less than 5 per cent. For guinea pig whole blood the contribution was very variable, ranging from 10–20 per cent of the change in absorbance per min measured, depending on the animal used.

3. *Carbamoylation studies. In vitro* carbamoylation rates were measured using the incubation methods described in 2(i) and 2(ii). Blood samples were analysed by the methods described above.

Observed first order rate coefficients were obtained by plotting log $(E_t - E_e)$ against time (E_t = enzyme activity at time, t. E_e = equilibrium enzyme activity).

Rate coefficients obtained by regression analysis were reproducible to $\pm$ 5%, and the maximum degree of carbamoylation reproducible to $\pm$ 3%. Values quoted are the average of three experiments.

For the *in vivo* studies, two blood samples were taken from the rabbit before starting an experiment to establish a base line acetylcholinesterase level (*ca.* 1 ml for the indirect titrimetric analysis, *ca.* 0.2 ml for spectrophotometric analysis).

The animal was then injected with the carbamate (pyridostigmine in isotonic saline) and blood samples removed at various times for analysis. In the rat experiments, the animal was anaesthetised with nembutal to facilitate the removal of blood samples. Blank experiments with nembutal showed that the anaes-

Table 1. A comparison of methods for measuring the inhibition of red cell membrane acetylcholinesterase by pyridostigmine

Species	10^7 [pyridostigmine] (M)	Direct Method		Indirect Method		Spectrophotometric Method	
		$10^3 k_{obs}(s^{-1})$	% EC	$10^3 k_{obs}(s^{-1})$	% EC*	$10^3 k_{obs}(s^{-1})$	% EC
Rabbit	2.5	1.2	63	1.3	68	1.2	66
Rat	2.5	1.0	62	1.0	64	—	—

* EC refers to carbamoylated acetylcholinesterase.

Table 2. A comparison of different analytical methods for measuring the *in vitro* inhibition of whole blood acetylcholinesterase by pyridostigmine

Species	10^7 [pyridostigmine] (M)	Indirect Method		Spectrophotometric Method	
		$10^3 k_{obs}(s^{-1})$	% EC	$10^3 k_{obs}(s^{-1})$	% EC
Rabbit	2.5	1.6	68	1.6	72
Guinea pig	2.5	2.2	67	2.1	67
Rat	2.5	2.3	64	2.2	68

thetic had no effect on the measured acetylcholinesterase activity during the course of the experiment. Samples were analysed in duplicate and the values obtained were always in close agreement (within ± 3%).

RESULTS AND DISCUSSION

Using red cell membrane acetylcholinesterase it was possible to titrate directly the free acetylcholinesterase in a sample inhibited by carbamate. It can be shown that neither further inhibition by carbamate in the assay solution, nor significant decarbamoylation occurs during the assay. Experiments were carried out to ensure that the indirect method of assay, where the acetylcholinesterase measured is that originally carbamoylated by the carbamate and which has fully decarbamoylated, gave identical results to the direct titrimetric and spectrophotometric methods, where the free acetylcholinesterase is measured directly. The data are presented in Table 1.

The results from the direct methods are in excellent agreement with those from the indirect method. The indirect method thus provided the basis for an unambiguous method of assaying whole blood samples.

The inhibition of whole blood acetylcholinesterase from the rabbit, guinea pig and rat by pyridostigmine was measured using the analytical methods described. The data obtained is given in Table 2.

It can be seen that there is excellent agreement between the methods used, and therefore the methods were used in whole animal experiments. The data obtained from a typical *in vivo* experiment where these methods of analysis were used are recorded in Table 3.

Once again agreement between the methods of analysis is excellent and either of the methods can be used to study the effect of pyridostigmine administered to an animal.

The indirect method was used originally for measuring the degree of carbamoylation of whole blood because it provided a completely unambiguous means of assaying the acetylcholinesterase produced by decarbamoylation of the carbamoylated acetylcholinesterase. The disadvantage of the method lies in the long time required to obtain the data (the sample must be washed free of organophosphate, allowed to decarbamoylate and the red cells hemolyse before the analysis can be carried out), and in the relatively large blood samples required—this is not possible for small animals.

The spectrophotometric method has been shown to be capable of giving results in agreement with the indirect method and has the advantage that the method is rapid, blood samples can be analysed within 5 min of taking the sample from the animal, and is very suitable for use in small animal experiments where only small volumes of blood can be removed.

Table 3. Measurement of a time–acetylcholinesterase activity profile for a rabbit* given pyridostigmine (100 μg per kg i.v.) using different analytical methods to assay the acetylcholinesterase activity

Time (min)	% Carbamolyated acetylcholinesterase	
	Indirect method	Spectrophotometric method
5	26	28
4	33	37
7		39
15	45	46
30	42	40
45		37
60	28	32
90	22	25
120		16
150	10	8

* Female, Old English, body weight 2.2 kg.

REFERENCES

1. W. Kalow and H. A. Lindsay, *Can. J. Biochem. Physiol.* **33,** 568 (1955).
2. H. O. Michel, *J. Lab. clin. Med.* **34,** 1564 (1949).
3. F. D. Aldrich, G. F. Walker and C. A. Patnoe, *Arch. Environ. Hlth* **19,** 617 (1969).
4. J. C. Sabine, *Blood* **10,** 1132 (1955).
5. G. L. Ellman, K. D. Courtenay, V. Andres and H. M. Featherstone, *Biochem. Pharmac.* **7,** 88 (1961).
6. I. B. Wilson, M. A. Harrison and S. Ginsburg, *J. biol. Chem.* **236,** 1498 (1961).
7. C. E. Pickering and R. G. Pickering, *Arch. Toxicol.* **27,** 292 (1971).
8. K. Wilholm, M. Vandekar and E. Reiner, *Bull. Wid. Hlth. Org.* **48,** 41 (1973).
9. F. Hoffman, La Roche and Co., A. G., Brit. Pat. 613, 168.