Quantitative Assay of Physostigmine in Human Whole Blood

WILLIAM A. GROFF ˣ, ROBERT I. ELLIN, and RICKIE L. SKALSKY

Abstract □ A quantitative assay for measuring physostigmine in human whole blood is described. Blood samples are maintained at 37°, and cholinesterase activity is measured periodically. The time required for enzyme reactivation is related to physostigmine concentration. The precision of the method is within ±4% over a physostigmine concentration range of 0.5–5.0 × 10^{-7} M.

Keyphrases □ Physostigmine—quantitative analysis by measurement of duration of cholinesterase inhibition, human blood □ Cholinesterase—duration of inhibition by physostigmine measured, related to physostigmine concentration, human blood □ Cholinergics, ophthalmic—physostigmine, quantitative analysis by measurement of duration of cholinesterase inhibition, human blood

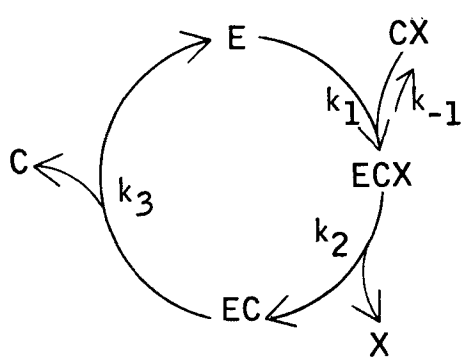
Physostigmine has been used to treat various clinical conditions: to reduce intraocular pressure in glaucoma (1), to treat muscular weakness in myasthenia gravis (2), to counteract atropine coma (3) and scopolamine delirium (4), and as an antidote against certain hallucinogenic agents (5). The primary physiological effect of physostigmine results from the inhibition or inactivation of cholinesterase at sites of cholinergic transmission.

BACKGROUND

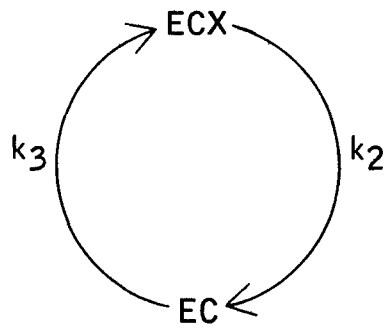
The inhibition of cholinesterase by carbamates occurs by a mechanism analogous to that of organophosphorus inhibition. With both, a reversible complex is formed first, followed by acylation of the enzyme and, finally, deacylation to yield the original enzyme. However, since the rate of decarbamylation is relatively fast as compared with dephosphorylation (6), progressive carbamate inhibition levels off before complete inhibition is attained.

The cyclic reaction of carbamate inhibition is shown in Scheme I. In the presence of excess inhibitor, an apparent steady-state condition ensues, since the free enzyme, E, resulting from decarbamylation is promptly recomplexed. Since k_1 is extremely fast with relation to k_{-1}, one can simplify the cyclic reaction as shown in Scheme II. Because the rate of carbamylation (k_2 = ~10 min^{-1}) is slower than the rate of complex formation ($K_a = k_{-1}/k_1 = 3.3 \times 10^{-6}$ mole/liter) and the rate of decarbamylation ($k_3 = 1.8 \times 10^{-2}$ min^{-1}) is much slower than the rate of carbamylation, there is a small accumulation of the complex, ECX. Almost all inhibited enzyme is the carbamylated enzyme, EC. The ECX complex is readily reversible (6–9) by high concentration of substrate, dilution, and dialysis. Due to this reversibility and the rate of decarbamylation, it is not possible to demonstrate complete enzyme inhibition even with high concentrations of carbamate (7).

The degree of inhibition of blood cholinesterase is generally accepted as a reflection of the intensity and duration of action of the drug in the body. The purpose of this work was to develop a method that would quantitatively relate the duration of enzyme inhibition to the concentration of physostigmine in blood. Since the drug is given to patients in low doses (2 mg) and its metabolism in the body is quite rapid, only nanogram levels of the drug are found in the blood. Existing colorimetric (10), spectrophotofluorometric (11), phosphorescent (12), and TLC (13) methods are not sufficiently sensitive to measure these low levels.



Scheme I



Scheme II

The problem is further complicated by the fact that inhibition of the enzyme by physostigmine is reversible. However, by taking these parameters into consideration, along with temperature and time control of a sample obtained under clinical conditions, an assay for physostigmine sensitive to 2.5 × 10^{-8} M or 7 ng of drug/ml of blood was developed.

EXPERIMENTAL

Materials—Physostigmine salicylate[1] USP was used as received. A stock solution (10^{-3} M) was prepared by dissolving 0.0413 g in 100 ml of distilled water. A working solution (10^{-5} M) was prepared fresh each week by diluting 1 ml of the stock solution to 100 ml with isotonic saline. These solutions were stored at 4°. An aliquot of the working solution was brought to room temperature each day before use. Human whole blood[2] was collected by vacuum tubes[3], with edetic acid as the anticoagulant.

Cholinesterase Measurement—Whole blood cholinesterase activity was determined by an automated procedure (14). Enzyme activity was expressed as micromoles of substrate hydrolyzed by 1 ml of sample per minute of incubation time. The substrate was acetylthiocholine iodide at 2 × 10^{-3} M. The reaction mixture, containing sample, substrate, and 5,5′-dithiobis(2-nitrobenzoic acid) (I), was buffered to pH 8.2 with 0.05 M tromethamine and incubated for approximately 6 min at 37°. Thiocholine, resulting from enzymatic hydrolysis of the substrate, reacts with I to form a colored anion. This colored anion is dialyzed into pH 8.2 buffer, and the absorbance was measured at 420 nm.

***In Vitro* Assay**—Microliter volumes of physostigmine solution were added, using micropipets[4], to 5-ml aliquots of whole blood. Concentrations of inhibitor were 0.25, 0.50, 1.00, 1.25, 2.50, and 5.00 × 10^{-7} M; 10 whole blood samples were prepared at each concentration. Each tube was immediately stoppered, shaken, and placed in a water bath at 37°. Zero time was recorded for each tube. At selected times, the blood samples were gently mixed; transfer pipets were used to remove aliquots to sample cups[5] for enzyme assay. Each aliquot was assayed within 1 min after removal from the incubation bath.

***In Vivo* Assay**—Physostigmine was administered intravenously to 10 subjects and intramuscularly to nine subjects. Blood samples for the physostigmine assay were collected from all subjects 30 min after dosing. These samples were placed immediately in a 37° incubation bath, and

[1] Control No. 932-LCA-11, S. B. Penick and Co., New York, N.Y.

[2] The volunteers in these tests were enlisted U.S. Army personnel. These tests were governed by the principles, policies, and rules for medical volunteers as established in AR 70-25 and the Declaration of Helsinki.

[3] Vacutainer, Scientific Products, Columbia, MD 21045.

[4] Eppendorf pipets, Arthur H. Thomas, Philadelphia, PA 19105.

[5] Technicon sample cups, Technicon Instruments Corp., Tarrytown, NY 10591.

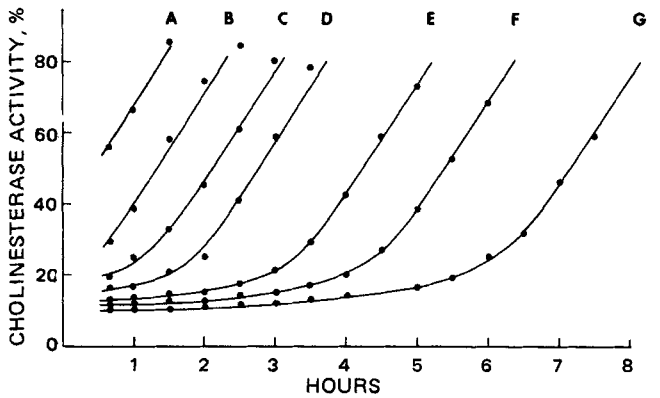


Figure 1—*Time course of whole blood cholinesterase activity after inhibition by physostigmine. Key: A, 0.5 × 10^{-7}; B, 1.0 × 10^{-7}; C, 1.5 × 10^{-7}; D, 2.0 × 10^{-7}; E, 3.0 × 10^{-7}; F, 4.0 × 10^{-7}; and G, 5.0 × 10^{-7}* M.

the time was recorded. Cholinesterase activity was measured periodically as described. The concentration obtained is a measure of the physostigmine present in the blood at the time the sample is withdrawn.

Precision—The precision of the method was determined by statistical analysis of the data obtained at the six concentrations of physostigmine that were added to blood *in vitro*.

Correlation—Concentration values measured in the precision study were correlated with concentrations added, and a regression curve was plotted.

RESULTS

The time course of human whole blood cholinesterase inhibition produced by physostigmine exhibits three phases: rapid inhibition; steady state, during which the cyclic reaction occurs; and reactivation (decarbamylation). A family of curves is shown in Fig. 1 for physostigmine concentrations ranging from 0.5 to 5.0 × 10^{-7} *M* at 37°. Percent cholinesterase activity is plotted *versus* time on Cartesian coordinate graph paper for each concentration of inhibitor until the enzyme activity has recovered to approximately 80% of the control level. The plot of activity *versus* time over the range of 30–70% activity is nearly linear, even though the decarbamylation rate is not zero order. This apparent linearity was used to determine the concentration of physostigmine present at the time of inhibition.

The time required for activity to reach a given percent activity level (between 30 and 70% on each curve) is proportional to the initial physostigmine concentration. For example, at the 50% cholinesterase activity level in Fig. 1, time values are 1.8 hr at 1.50 × 10^{-7} *M*, 2.4 hr at 2.00 × 10^{-7} *M*, 3.9 hr at 3.00 × 10^{-7} *M*, 5.0 hr at 4.00 × 10^{-7} *M*, and 6.8 hr at 5.00 × 10^{-7} *M* physostigmine. These values and values for other activity levels are plotted *versus* concentration on Cartesian coordinate graph paper in Fig. 2. This set of standard curves is used to convert time/percent activity values of an unknown blood sample to physostigmine concentration.

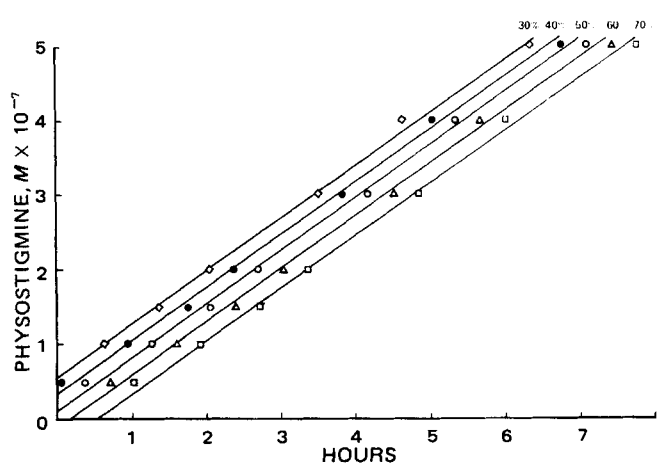


Figure 2—*Standard curves to convert time/percent activity values to physostigmine concentration.*

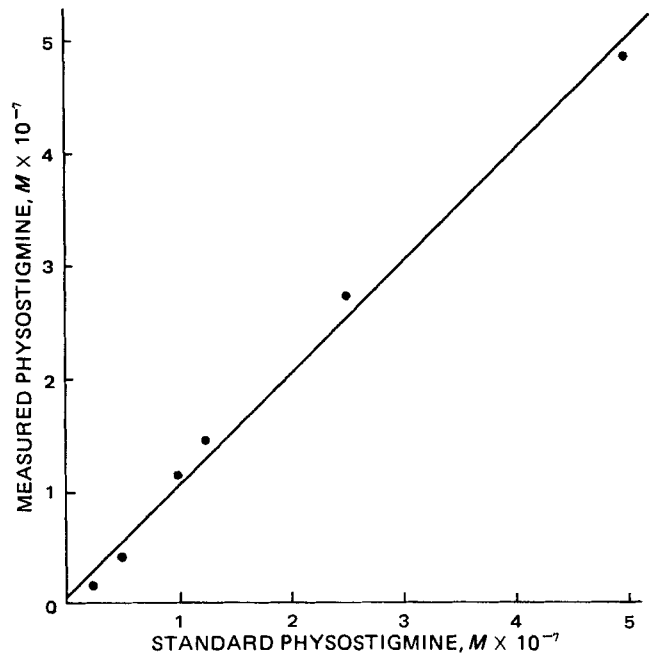


Figure 3—*Comparison of measured physostigmine concentrations with theoretical concentrations added to whole blood samples* (y = 0.975x + 0.079; r = 0.996_6).

A blood sample containing an unknown concentration of physostigmine is incubated at 37° and periodically assayed to determine cholinesterase activity until several data points are obtained that fall within the 30–70% activity range. These points are plotted on Cartesian coordinate graph paper, and a line is drawn through the points. A time value is taken from this line at any activity level for which a line is graphed in Fig. 2. The time value is then converted to concentration by means of this figure. The resulting concentration value must be corrected for any difference between the control (uninhibited) activity (in micromoles per milliliter per minute) of the unknown blood sample and the control activity of the standard blood sample. This correction is necessary because the time required for decarbamylation is proportional to the enzyme concentration in the blood.

The physostigmine concentration in the unknown can be calculated from:

$$\text{physostigmine concentration } [\times 10^{-7} \text{ mole/liter}] = \frac{\text{unknown control activity}}{\text{standard control activity}} \times \frac{\text{concentration}}{\text{(from Fig. 2)}} \quad \text{(Eq. 1)}$$

A micromole per milliliter per minute activity unit is a fixed unit for the conditions of assay expressed in this report. A percent activity unit is variable and is dependent upon the relative activity of each blood sample.

If the control activity level of the unknown blood sample cannot be determined in advance, it must be obtained by assaying the sample until no further increase in activity is obtained.

The precision of the method was determined by statistical evaluation

Table I—Precision of Physostigmine Assay

Concentration Added, × 10^{-7} *M*	Concentration Measured: Mean, × 10^{-7} *M*	*SD*, × 10^{-7} *M*	Coefficient of Variation, %
0.25	0.19	0.019	±10.0
0.50	0.42	0.016	±3.8
1.00	1.13	0.021	±1.9
1.25	1.43	0.029	±2.0
2.50	2.71	0.049	±1.8
5.00	4.83	0.159	±3.3

Table II—Blood Physostigmine Levels ($\times 10^{-7}$ *M*) in Humans 30 min after Administration

Route	Dose Administered, μg/kg				
	15	20	25	30	45
Intravenous	—[a]			0.40	0.90
	—[a]			0.29	0.59
	—[a]				0.90
	—[a]				0.78
Intramuscular	—[a]	0.35	0.25		
	—[a]	—[a]	0.31		
		—[a]	0.27		
			—[a]		

[a] $<0.25 \times 10^{-7}$ *M*.

of data obtained from a reproducibility study. Ten whole blood samples were prepared at each of six different concentrations of physostigmine, ranging from 0.25 to 5.00 $\times 10^{-7}$ *M*. Means, standard deviations, and coefficients of variation for each set of 10 assays are presented in Table I. Precision was within ±3.8% for physostigmine concentrations ranging from 0.50 to 5.00 $\times 10^{-7}$ *M*. At 0.25 $\times 10^{-7}$ *M*, precision was ±10.0%.

The mean physostigmine concentrations obtained in the precision study were correlated with the concentrations added to the blood samples. The plot of data points and the equation of the regression line are given in Fig. 3.

Blood levels at 30 min after intravenous and intramuscular injections of physostigmine are presented in Table II. The relationship of dose administered to blood levels was approximately the same for both routes of administration.

DISCUSSION

To relate physostigmine concentration to cholinesterase inhibition, it is necessary to measure enzyme activity after inhibition has progressed until none of the enzyme remains in the complexed form. At this time, when the enzyme activity is between 30 and 70% of the control level, only decarbamylation occurs. The time course at 37°, from inhibition until a near linear reactivation rate is attained, together with the enzyme activity level at this time, can be used to determine the concentration of physostigmine in the blood at the time the *in vitro* sample was inhibited. With an *in vivo* blood sample, the physostigmine concentration can be determined for the time the blood was withdrawn from the patient.

This assay method assumes that decarbamylation occurs over the entire period from the time of inhibition. The error in the assumption is quite small, since an initial time period of less than 2 min is required for formation of an appreciable concentration of carbamylated enzyme. The time required for enzyme reactivation should be, and has been shown to be, a function of the inhibitor concentration. The time required to complete an assay varies directly with the physostigmine concentration, from approximately 1 hr for 0.50 $\times 10^{-7}$ *M* to approximately 7.5 hr for 5.00 $\times 10^{-7}$ *M*. Considering the inherent errors, the precision of this method is excellent.

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Influence of Zinc–Ligand Mixtures on Serum Zinc Levels in Rats

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Abstract □ The influence of various salts, chelates, and other complexes of zinc given by gavage on serum zinc levels in rats was studied. Serum zinc concentrations were determined over 6 hr after administration of zinc sulfate at doses of 5, 10, 20, 50, and 100 mg of zinc/kg. Serum zinc levels following doses of zinc salts and complexes were compared with those after zinc sulfate. Phytic acid, aminopolycarboxylic acids (including edetate disodium), and penicillamine significantly suppressed increases in serum zinc concentration. Some natural amino acid–zinc sulfate mixtures (those with lysine, cysteine, glycine, and histidine) produced greater levels than the equivalent dose of zinc sulfate alone. Several thiocarboxylic acids, such as mercaptoacetic acid and thiosalicylic acid, also increased serum zinc concentrations. These observations form a basis for attempted modification of zinc absorption in other species.

Keyphrases □ Zinc—serum levels, effect of various salts, chelates, and other complexes, rats □ Salts, zinc—effect on serum zinc levels, rats □ Chelates, various—with zinc, effect on serum zinc levels, rats □ Complexes, various—with zinc, effect on serum zinc levels, rats □ Metals—zinc, various salts, chelates, and other complexes, effect on serum zinc levels, rats

Treatment of humans with zinc usually involves administration of zinc sulfate heptahydrate, taken with meals to avoid gastric irritation. However, Schelling *et al.* (1) reported that when it is given simultaneously with meals, the apparent absorption of zinc may be nil. Poor absorption may partly account for equivocal clinical results of zinc therapy (2–7).

GI absorption of zinc in several species has been studied