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SENSITIVE LIQUID CHROMATOGRAPHIC METHOD FOR PHYSOSTIGMINE IN BIOLOGICAL FLUIDS USING DUAL-ELECTRODE ELECTROCHEMICAL DETECTION

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SUMMARY

A liquid chromatographic method using dual-electrode detection has been developed for determination of physostigmine in biological fluids. The limit of detection is in the order of 25—50 pg mol^{-1} of plasma. A high sample throughput is obtained by a single solvent extraction step and autoinjection into the chromatograph. Data following oral doses of physostigmine are presented.

INTRODUCTION

Physostigmine, an alkaloid of the Calabar bean, is a potent inhibitor of cholinesterase. It is a lipophilic tertiary amine which is absorbed from the gastrointestinal tract and enters the central nervous system. Its short duration of action suggests that it has a short elimination half-time but pharmacokinetic analysis has not been possible due to the lack of a suitable plasma assay. Liquid chromatography (LC) with ultraviolet (UV) detection [1, 2] is too insensitive for determining plasma concentrations in man. Using electrochemical detection an LC method sensitive to approximately 0.5 ng ml^{-1} has been reported [3] and, although plasma concentrations following a single subcutaneous dose of 1 mg physostigmine salicylate were determined, it was obvious that greater sensitivity was required. The electrochemical method has been modified to give approximately a ten-fold improvement in sensitivity.

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EXPERIMENTAL

Materials and apparatus

HPLC-grade methanol and acetonitrile were purchased from Fisons Scientific (Loughborough, U.K.). Spherisorb 3-μm columns, 150 × 4.6 mm I.D., were from Phase Separations (Queensferry, U.K.). Physostigmine, physostigmine sulphate and neostigmine bromide were from Sigma (Poole, U.K.). Other reagents were analytical-reagent grade.

Chromatographic eluents were prepared by mixing 1 vol. of 0.1 *M* ammonium nitrate buffer with either 9 vols. of methanol or 9 vols. of methanol—acetonitrile (1:1). The eluents were pumped with an Applied Chromatography Systems Series 300 pump and samples introduced manually via a Rheodyne valve fitted with a 50-μl sample loop. Alternatively, a Kontron MSI 660 autosampler was used. Detection was either by a Laboratory Data Control variable-wavelength detector Model 1204A, or an Environmental Science Association (ESA) Model 5100A Coulochem detector.

Effect of pH on electrochemistry of physostigmine

Solutions of physostigmine (1 μg ml^{-1}) in 50% methanol: Britton—Robinson buffers (0.1 *M*) were pumped through the electrochemical detector cell at a flow-rate of 1 ml min^{-1}. Current—voltage curves were obtained using the scanning facility on the detector. Subtraction of the signals obtained using physostigmine-free solvents gave typical sigmoidal polarographic waves. The experiment was performed using buffers at pH 3, 5, 7 and 9.

Preparation and evaluation of compounds as internal standards

Eseroline was prepared by hydrolysing physostigmine sulphate as described by Ellis [4]. Crystallization from benzene–light petroleum gave pale buff needles, m.p. 128—129°C.

Ethyl and propyl carbamate homologues of physostigmine were prepared by treating eseroline with the appropriate alkyl isocyanate. Eseroline was dissolved in diethyl ether in the presence of a speck of sodium and the alkyl isocyanate added [5]. The N,N-dimethylcarbamate analogue was prepared by dissolving eseroline in ethyl acetate to which a few drops of pyridine had been added and treating with N,N-dimethylcarbamyl chloride. The required products were separated by thin-layer chromatography (SiO_2; chloroform—ethanol—0.88 SG ammonium hydroxide, 80:10:1). The spots were located as shadows under UV light, removed from the plates and the compounds eluted with methanol. The solutions were examined by UV spectroscopy, LC and mass spectroscopy.

pH-Controlled solvent extraction

Britton—Robinson buffers (0.1 *M*) were prepared to cover the range pH 4 to pH 12. Solutions of physostigmine and its analogues were prepared in diethyl ether at approximately 2 μg ml^{-1}. Aliquots of the ether solutions (2 ml) were shaken with buffer solutions (1 ml) for 15 min. After centrifugation, 1 ml of the ether layer was transferred to a clean tube, evaporated under nitrogen and the residue dissolved in methanol (1 ml). Samples were assayed by LC at 254 nm. The results were plotted as percentage present in ether layer versus pH.

Loss of physostigmine in vitro

Samples of blood bank plasma (19.9 ml) either with or without neostigmine bromide (50 μg ml^{-1}) were brought to temperature (4°C, 22°C or 37°C) and physostigmine solution (0.1 ml) was added to give an initial concentration of 5 ng ml^{-1}. Aliquots (2 ml) were withdrawn at intervals over 4 h for physostigmine assay as described below. The percentage remaining at each time was calculated.

Extraction from plasma, blood and urine

Calibration curves for plasma or blood assays were prepared using blood bank plasma containing neostigmine bromide (50 μg ml^{-1}). Samples (2 ml) were pipetted into screw-cap extraction tubes and 0.1 ml internal standard solution, the N,N-dimethylcarbamyl ester of eseroline (approximately 40 ng ml^{-1} in methanol) was added. Ammonium hydroxide solution (1 ml, 0.1 *M*) and freshly distilled diethyl ether (5 ml) were added. The tubes were shaken mechanically for 15 min, centrifuged to separate the layers and the organic layers (4 ml) transferred to clean pointed tubes. The diethyl ether was evaporated under a gentle stream of nitrogen and the residue dissolved in methanol (0.1 ml). Aliquots of methanol were either injected into the liquid chromatograph or transferred to glass autosampler vials. Routinely, calibration curves were prepared between 10 and 0.1 ng ml^{-1}. For low concentrations, e.g. those expected after oral dosing, larger samples (up to 4 ml) were taken and the final volume of methanol was 0.06 ml. Calibration standards were prepared between 2 and 0.025 ng ml^{-1}.

Urine samples (2 ml) were extracted as for blood or plasma using benzene in place of diethyl ether. The calibration range and amount of internal standard were adjusted to suit the samples being analyzed.

An estimate of precision was obtained by assaying replicate samples. Inter-assay precision was estimated by assaying six blood and plasma samples containing 3 ng ml^{-1} physostigmine. Samples were stored at −20°C and assayed on separate days over a three-week period.

Chromatographic conditions

For the pH partition studies methanol—pH 8.6 buffer was used as eluent which was pumped at a flow-rate of 0.5 ml min^{-1}. For biological extracts the pH of the ammonium nitrate buffer was increased to 8.9 and a mixture of methanol—acetonitrile used rather than methanol. Two columns were connected in series. The flow-rate was 1 ml min^{-1}.

The guard cell of the Coulochem detector was placed between the column exit and the analytical cell. The guard cell voltage was + 0.4 V. The analytical cell electrode potentials were + 0.7 V and − 0.2 V.

Biological samples

The data presented are from a healthy female volunteer, age 38 years, weight 59 kg, who received oral doses of physostigmine on three separate occasions. Physostigmine salicylate (1, 2 or 4 mg) was given with 100 ml of water to drink. Blood samples (10 ml) were drawn into heparinized tubes and neostigmine bromide solution (500 μg in 0.05 ml water) was added and mixed

immediately. Samples were taken 15, 30, 45, 60, 75, 90, 120, 150 and 180 min after the dose. Urine was collected at approximately 30-min intervals. The protocol was approved by the Tower Hamlets District Ethics Committee.

Red cell partitioning

Physostigmine was added to heparinized blood samples collected from four male volunteers (22—36 years) to give a final concentration of 1 ng ml^{-1}. Blood samples were divided and centrifuged to separate the plasma. Plasma (2 ml) was transferred to an extraction tube containing 100 μg neostigmine bromide. The contents of the other centrifuge tube were shaken to reconstitute blood and a sample (2 ml) was transferred as described for plasma. The blood and plasma samples were extracted and assayed along with calibration standards as described above.

RESULTS AND DISCUSSION

The influence of pH on the polarographic properties of physostigmine is shown in Fig. 1. The curves at pH 9 and 7 were almost superimposed but at lower pH values the responses were lower. The use of alkaline eluent was compatible with this finding. Using acidic eluents with modified silicas [1, 2, 6] was considered inappropriate and there appeared to be no advantage in using acid eluents and changing the pH value post-column before electrochemical detection. The areas of chromatographic peaks obtained from repeat injections of physostigmine (10 ng) increased as the flow-rate was reduced and the detector temperature was raised. The maximum area obtained suggested that the oxidation processes involved a three-electron transfer. The chromatographic conditions were optimised to give maximum sensitivity in terms of peak height.

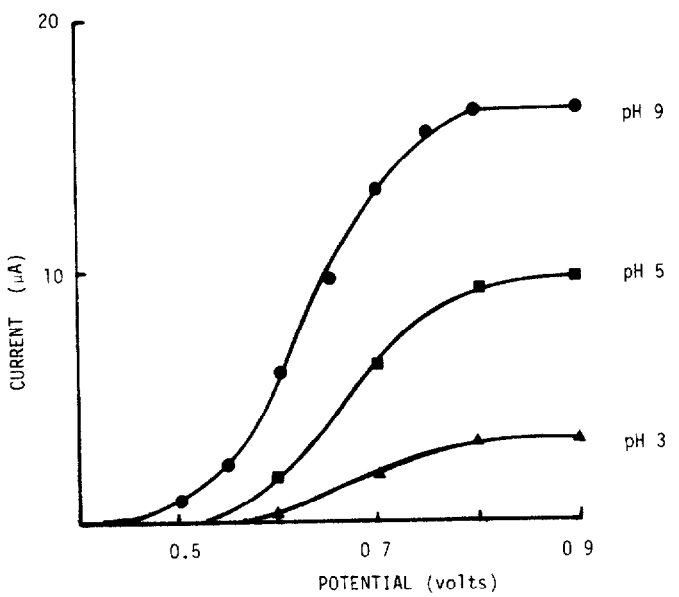

Fig. 1. Effect of buffer pH on current—voltage of physostigmine.

Under these conditions the efficiency of the oxidation process was approximately 66%.

The N,N-dimethylcarbamate analogue of physostigmine was chosen as the internal standard. This compound was suitable because of its extraction characteristics and its chromatographic properties, Figs. 2 and 3.

The N-ethyl homologue, eluting between physostigmine (N-methyl) and the propyl homologue, was insufficiently resolved from physostigmine to be used as the internal standard (Fig. 2). The extraction characteristics of the N-propyl carbamate were the least like those of physostigmine whereas the extraction of the dimethyl compound was very similar (Fig. 3). All the compounds gave the expected sigmoidal pH—extraction curves up to about pH 10 beyond which the concentrations in the ether layers declined. This was assumed to be due to decomposition by alkaline hydrolysis [7]. Between pH 8.5 and 11 the ratio of the amount of physostigmine to the amount of internal standard extracted varied by less than 2%. The ratio was unaffected by the nature of the sample — plasma, blood or urine — being extracted.

A potential problem when analysing esters in blood or plasma is hydrolysis

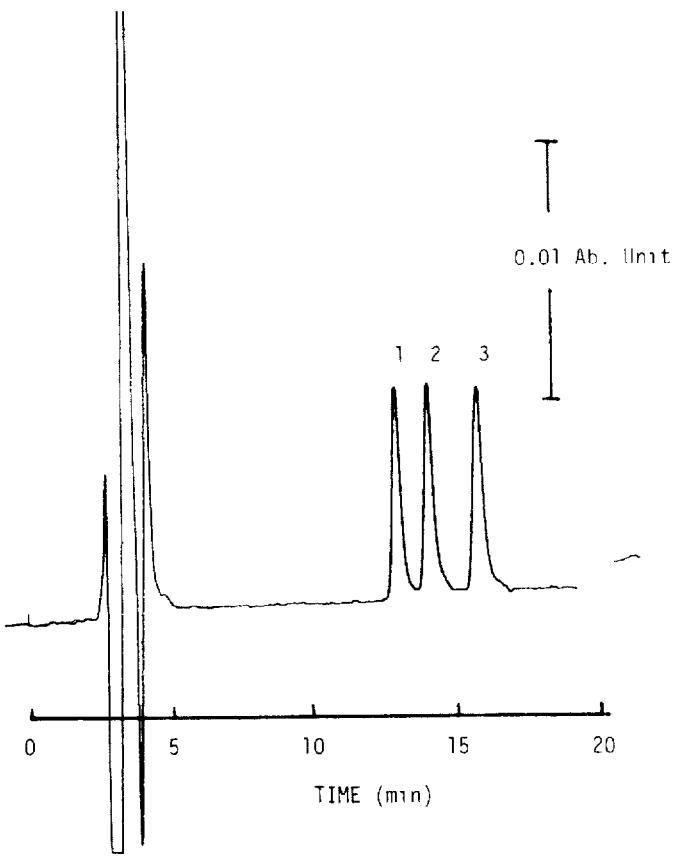

Fig. 2. Chromatogram of physostigmine (2) and its N-propyl (1) and N,N-dimethyl (3) analogues. The N-ethyl homologue, chromatographing between peaks 1 and 2, has been omitted for clarity.

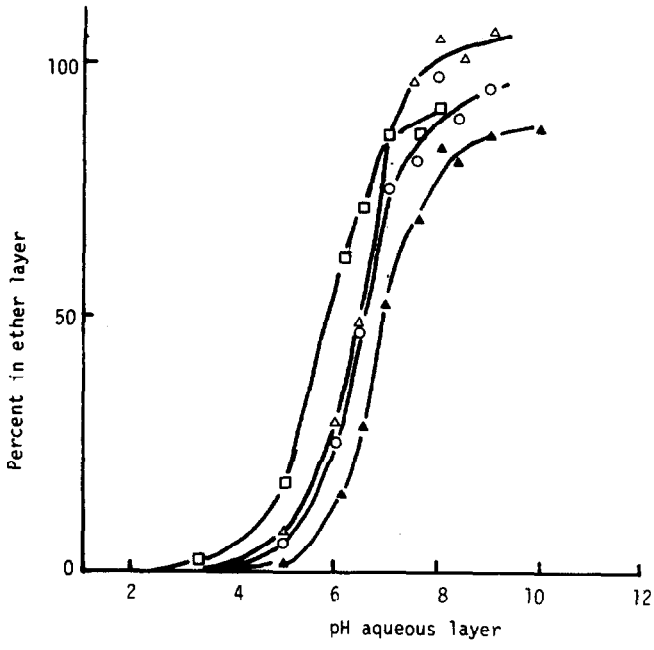

Fig. 3. Extractions of physostigmine and its analogues from various pH buffers into diethyl ether. (▲) N-Methyl carbamate (physostigmine); (○) N,N-dimethyl carbamate; (△) N-ethyl carbamate; (□) N-propyl carbamate.

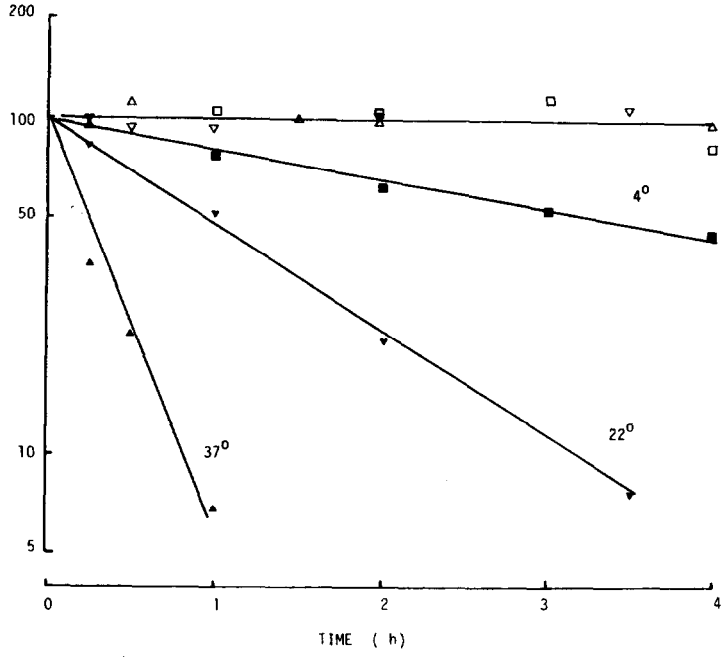

Fig. 4. Percentage physostigmine remaining in plasma as a function of time and temperature. Closed symbols: without added neostigmine; open symbols: with neostigmine (50 μg ml^{-1}).

of the compound by plasma esterases after blood samples have been taken. An indication of the magnitude of the problem with physostigmine is shown by Fig. 4. Physostigmine concentrations declined from an initial value of 5 ng ml^{-1}, with apparent first-order kinetics. At 37°C the apparent first-order half-time was approximately 15 min. At room temperature (22°C) 52% physostigmine was lost in 1 h. Even at 4°C the loss was not negligible. Physostigmine hydrolysis was enzymic. No loss occurred in the presence of excess neostigmine (Fig. 4, open symbols). Loss due to adsorption was discounted as adding neostigmine to tubes at the end of the incubation period failed to restore the physostigmine concentrations. When physostigmine was incubated in urine at 37°C for up to 4 h there was no measurable decrease in concentration. Neostigmine was chosen for its similarity to physostigmine, but being a quaternary ammonium compound its extraction into diethyl ether or benzene is negligible. Calibration standards were prepared containing neostigmine bromide (50 μg ml^{-1}) or venous blood was drawn into tubes containing sufficient neostigmine to give a final concentration of 50 μg ml^{-1}. To date, there has been no indication that neostigmine interferes with the assay of physostigmine.

Precision and sensitivity

The intra-assay precision was tested by replicate analyses of spiked samples. The intra-assay coefficients of variation (C.V.) obtained for assaying replicate 2-ml samples of plasma containing 10, 1 or 0.1 mg ml^{-1} physostigmine are shown in Table I. The results were calculated using data from the first electrode (oxidation, +0.7 V) and the second electrode (reduction, −0.2 V). The linearity of the method can be seen from the mean ratio (physostigmine response/internal standard response) as a function of concentration over two orders of magnitude (Table I). Intra-assay C.V. values ($n = 6$) for urine at 10 and 1 ng ml^{-1} were 3.8% and 3.9%, respectively. The mean concentrations and inter-assay C.V. values for the six stored plasma and blood samples were 2.86 ng ml^{-1} and 13.6%, and 2.96 ng ml^{-1} and 16.6%, respectively.

Sensitivity is not a fixed quantity but varies from sample to sample, run, laboratory, etc. It is also a function of the selectivity. Using 2 ml of plasma or blood, the limit of detection was in the order of 100 pg ml^{-1}. The sensitivity could be increased by taking a 4-ml sample for assay and using the sum of the oxidation and reduction signals. (The detector has a third output for this purpose, Fig. 5.) In this way, 50 pg ml^{-1} could be quantified with a C.V. of 14.5% ($n = 5$) and 25 pg ml^{-1} with a C.V. of 19.6% ($n = 5$).

TABLE I

INTRA-ASSAY COEFFICIENTS OF VARIATION USING OXIDATION OR REDUCTION SIGNALS ($n = 6$)

Concentration (ng ml^{-1})	Oxidation		Reduction	
	Mean ratio	C.V. (%)	Mean ratio	C.V. (%)
10	2.19	1.55	4.48	3.29
1.0	0.22	3.77	0.46	3.80
0.1	0.022	10.22	0.048	14.30

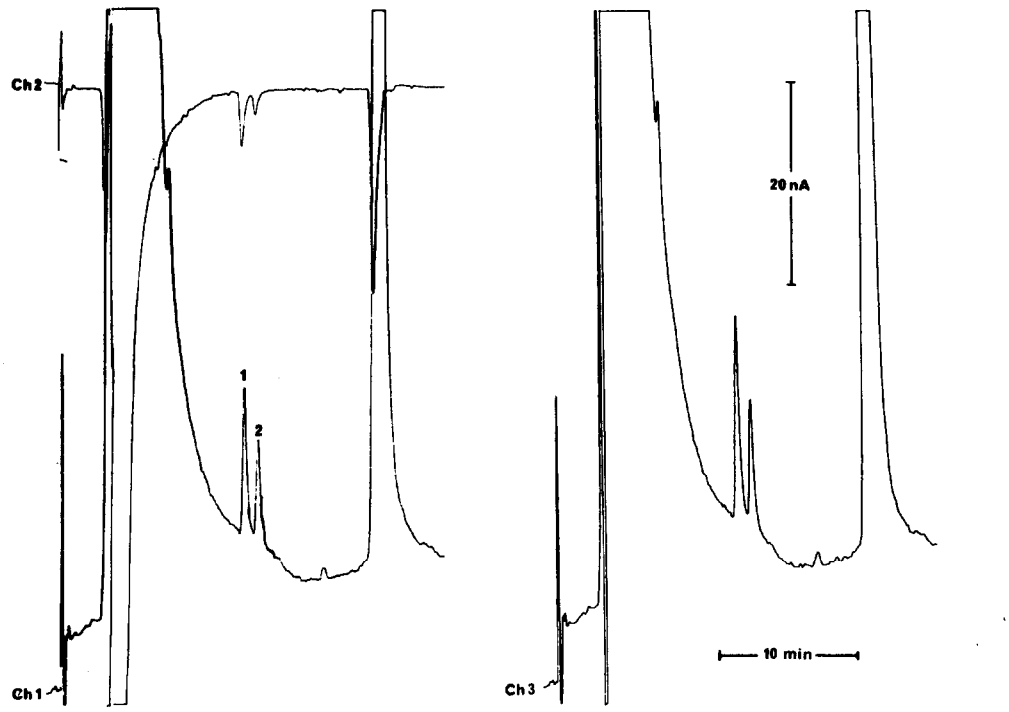

Fig. 5. Chromatogram of a plasma extract containing physostigmine (1) and internal standard (2) showing the three outputs of the ESA detector. Channel (Ch) 1, oxidation at + 0.7 V; channel 2, reduction at − 0.2 V and channel 3, the sum of the signals from channels 1 and 2. The plasma contained 0.5 ng ml^{-1} physostigmine and a 4-ml sample was extracted.

The eluent buffer pH was important in achieving the required selectivity and sensitivity. Although pH 8.6 buffer gave better resolution of physostigmine and internal standard, biological samples were assayed using buffer at pH 8.9. At the lower pH, a compound found in some biological extracts co-eluted with physostigmine. At pH 8.9 the chromatogram was free of interfering peaks in the region of physostigmine and internal standard.

Diethyl ether extraction of urine was unsuitable because it resulted in too many interfering peaks. When benzene was used the number and size of interfering peaks were considerably reduced, but the recovery of physostigmine was not affected. Benzene can be substituted for diethyl ether for extraction of blood and plasma. Benzene is more convenient as analytical grade reagent can be used without prior pretreatment. Diethyl ether has to be freshly distilled to remove electroactive anti-oxidants.

Red cell partitioning

The results of the erythrocyte-binding experiments illustrate the problem of determining plasma concentrations. When the partitioning was carried out in the presence of neostigmine bromide (50 $\mu g\ ml^{-1}$) the mean (n = 4) blood physostigmine concentration was 1.04 ng ml^{-1} and the range of red cell/plasma ratios 0.95—1.30. When plasma was separated before neostigmine was added, the mean blood concentration was 0.28 ng ml^{-1} and the red cell/plasma ratios

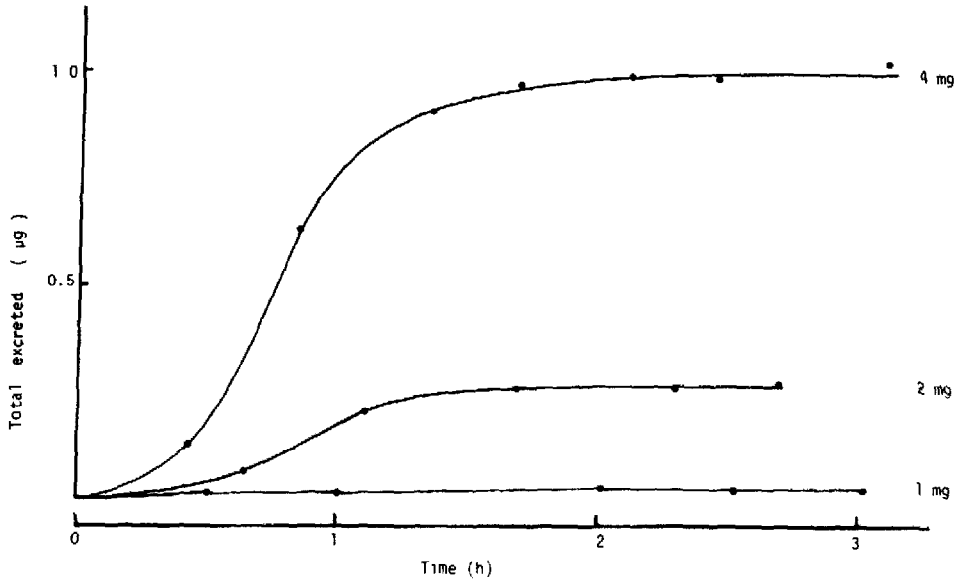

Fig. 6. Cumulative physostigmine urinary excretion curves in a volunteer who received 1, 2 and 4 mg physostigmine salicylate on separate occasions.

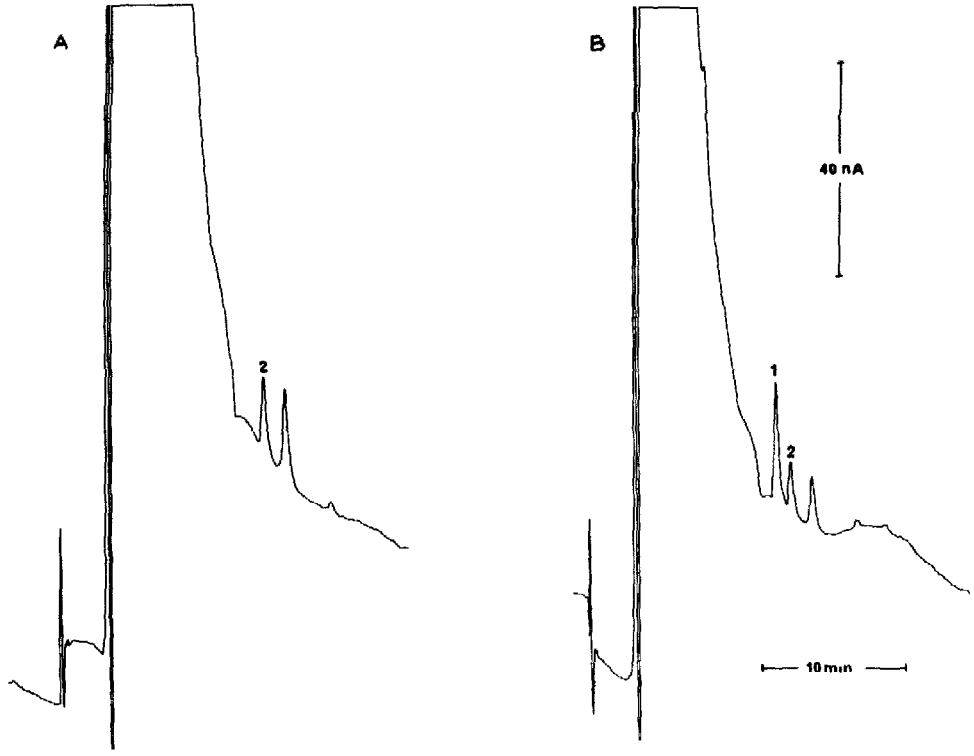

Fig. 7. Chromatograms, recorded from channel 3, of extracts from (A) pre-dose blood (4 ml) and (B) blood sampled 45 min after physostigmine salicylate (4 mg, orally). Peaks: 1 = physostigmine, 2 = internal standard.

were in the range 1.2—1.8. Neostigmine bromide must be added immediately after blood is sampled to prevent enzyme hydrolysis of physostigmine. However, as this perturbs the red cell binding, plasma assays will not give the true plasma concentration of the sample when the blood was taken. For this reason it is suggested that blood, rather than plasma, be used for analysis.

Physostigmine concentrations after oral dosing

After oral doses of 1 mg and 2 mg physostigmine salicylate, equivalent to 0.67 and 1.34 mg of physostigmine base, no drug could be detected in blood samples collected for up to 3 h after the dose. Although the concentrations were very low, physostigmine was detectable in urine. The maximum excretion rates (Fig. 6) occurred during the first hour indicating that the blood concentrations had reached maximum values, but that these were below the detection limit of the assay, < 50 pg ml^{-1}. The total excreted in the urine, in 3 h amounted to 0.005% and 0.02% of the dose after 1 mg and 2 mg, respectively. After 4 mg, orally, the total excreted into the urine accounted for approximately 0.04% of the dose.

Blood collected 45 min after the 4-mg dose contained the highest physostigmine concentration, 0.78 ng ml^{-1} (Fig. 7). Concentrations after the peak declined rapidly, suggesting a half-time of approximately 25—30 min (Table II). Physostigmine was undetectable in the 3-h blood sample.

TABLE II

BLOOD CONCENTRATIONS OF PHYSOSTIGMINE AFTER 4 mg PHYSOSTIGMINE SALICYLATE, ORALLY

Time (min)	Concentration (ng ml^{-1})
0	N.D.*
15.5	0.13
31	0.30
45	0.78
60	0.48
75.5	0.30
90	0.16
120	0.08
150	0.04
180	N.D.

*N.D. = Not detected.

CONCLUSION

LC with electrochemical detection is suitable for assaying physostigmine in biological fluids at concentrations likely to be encountered in pharmacokinetic studies. Blood and urine concentrations after oral doses are reported here. The assay is currently being applied to samples collected after intravenous infusions (0.6 mg physostigmine salicylate over 10 min) and the data will be reported when the study is complete.

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