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Note

Analysis of plasma physostigmine concentrations by liquid chromatography

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Physostigmine, an alkaloid from the Calabar bean, is a potent inhibitor of cholinesterase. Unlike other carbamate anticholinesterases, such as neostigmine and pyridostigmine which are quaternary ammonium compounds, physostigmine is a tertiary amine and is rapidly absorbed after oral or subcutaneous administration. It readily penetrates the central nervous system. It may be used to treat poisoning by anticholinergic compounds (e.g. atropine or tricyclic antidepressants) and certain neurological disorders or to investigate central cholinergic mechanisms.

Low doses (typically 0.5—2 mg) coupled with rapid metabolism mean that a plasma assay must be capable of measuring nanogram or even sub-nanogram amounts. The aim of the present investigation was to measure plasma concentrations after a single subcutaneous injection of 1 mg physostigmine salicylate (equivalent to 0.67 mg of the base). If this amount were distributed instantaneously through total body water then the plasma concentration would be about 15 ng ml^{-1} in a 70-kg individual (i.e. 9.5 $\mu g\ kg^{-1}$). A combination of an absorptive phase following subcutaneous injection, an apparent volume of distribution greater than total body water (which is likely, considering the lipophilic nature of the compound) and rapid metabolism will produce plasma concentrations considerably less than this. The existing enzymatic method [1] with a sensitivity of ca. 7 ng ml^{-1} in blood was considered unsuitable. A liquid chromatographic assay has been applied to measuring physostigmine in cat brains after intravenous injection of 270 $\mu g\ kg^{-1}$ [2]. The sensitivity was 100 ng g^{-1} of tissue. Other analytical methods have only been applied to assaying pharmaceutical preparations [3—5].

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EXPERIMENTAL

Reagents and stock solutions

All reagents were of analytical grade apart from the methanol used for preparing the eluent, which was HPLC grade (Fisons Scientific Apparatus, Loughborough, Great Britain). Stock solutions of physostigmine and neostigmine bromide (Sigma, Poole, Great Britain) were prepared at 1 mg ml^{-1} in methanol and water, respectively. Eseroline and rubreserine were synthesized as described by Ellis [6].

Plasma samples

A male volunteer, aged 34 years and weighing 72 kg, was injected subcutaneously with 1 ml physostigmine salicylate solution B.P. (equivalent to 0.67 mg base). Venous blood (10 ml) was withdrawn into heparinised tubes and neostigmine bromide solution (1 mg ml^{-1}, 10 μl) added. The blood was mixed and centrifuged at 4°C to separate the plasma, after which it was stored at 4°C until assay later in the day. Samples were taken before and at 15, 30, 60, 90 and 120 min after injection. The protocol was approved by the Tower Hamlets District Ethics Committee.

Extraction procedure

Plasma (3 ml) and ammonium hydroxide solution (1 *M*, 1 ml) were pipetted into a screw-cap tube. Diethyl ether (5 ml) was added and the capped tube shaken mechanically for 20 min. After centrifugation the ether layer (4 ml) was transferred to a pointed tube and evaporated at 40°C under a gentle stream of nitrogen. The residue was dissolved in methanol (60 μl) and 50 μl injected into the chromatograph.

Standard solutions were prepared at 20, 10, 5, 2, 1, 0.5 and 0 ng ml^{-1} in plasma containing neostigmine bromide (10 μg ml^{-1}) and taken through the extraction procedure along with the unknown samples.

Chromatographic system

The stainless-steel column, 250 × 4.5 mm I.D., was slurry packed with 5-μm silica particles (Spherisorb, Phase Separations, Queensferry, Great Britain) in methanol. The eluent was methanol—1 *M* ammonium nitrate buffer, pH 8.6 (9:1) and degassed to remove dissolved oxygen before use. The flow-rate was maintained at 1 ml min^{-1} using a Laboratory Data Control Constametric pump. Samples were introduced via a Rheodyne valve fitted with a 50-μl loop. Detection was by either a fixed-wavelength (254 nm) UV detector or a Bioanalytical Systems electrochemical detector. The Type 8A glassy-carbon cell was operated at a potential of 0.8 V relative to the silver—silver chloride electrode (SSCE).

RESULTS AND DISCUSSION

Physostigmine is hydrolysed, enzymatically or in alkali, to the phenol eseroline which, in the presence of air, is rapidly oxidised to the orthoquinone, rubreserine. Under the conditions described the retention volumes were: rubreserine, 4.1 ml; physostigmine, 5.6 ml and eseroline, 6.1 ml. Eseroline was

not completely resolved from physostigmine but the retention times were sufficiently different for the two not to be confused. Furthermore, the current—voltage curves were so different that the compounds could be distinguished by changing the oxidation potential (Fig. 1). Eseroline was more readily oxidised than physostigmine, having a half-wave potential of 0.21 V relative to the SSCE compared with 0.70 V relative to the SSCE for physostigmine. Rubreserine showed no signs of electrochemical oxidation up to a potential of 1 V.

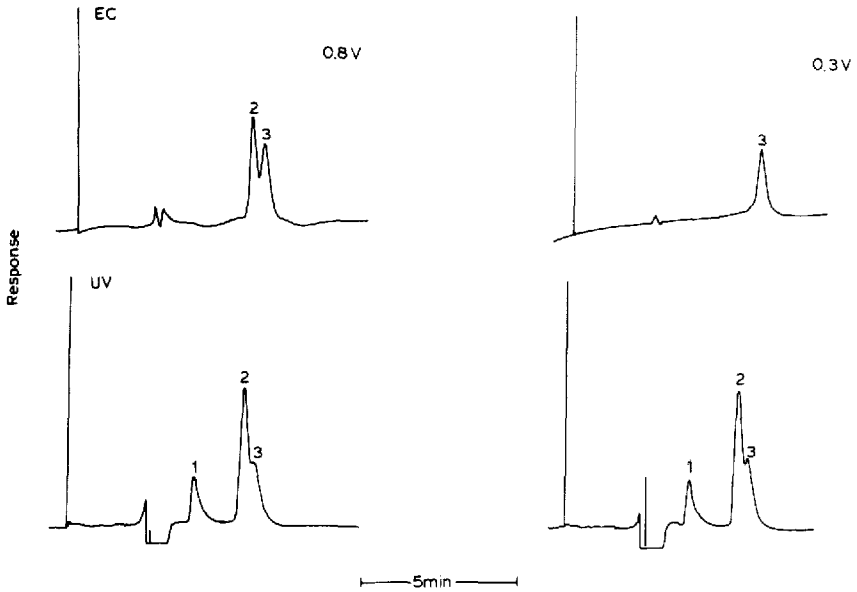

Fig. 1. Chromatograms showing simultaneous recording of UV (254 nm) and electrochemical responses to illustrate the effect of oxidation potential on the responses to reference compounds. Left: electrochemical detector at 0.8 V; right: electrochemical detector at 0.3 V. Compounds (50 ng of each): (1) rubreserine, (2) physostigmine and (3) eseroline.

Using eluent containing ammonium nitrate buffer, pH 8.6, physostigmine was resolved from an electro-active co-extractant which has been present in all the plasma samples tested to date. At pH 9.0, the retention volumes of physostigmine was reduced and the compound was no longer resolved from the contaminant. UV detection was unsuitable for plasma extracts because of a second contaminant which chromatographed at almost the same retention time as physostigmine and absorbed light at 254 nm. Fortunately, this compound was devoid of electro-activity at 0.8 V and it was for this reason that electrochemical detection was chosen.

Physostigmine contains two basic nitrogen atoms with pK_a values of 1.8 and 7.9 [7]. Consequently, solvent extraction is from alkaline aqueous solutions. If the pH of the aqueous medium is high, too much physostigmine may be hydrolysed during the extraction procedure. One millilitre of 1 *M* ammonium hydroxide in 3 ml of plasma gave pH 10 (approximately two units greater than the higher pK_a value). Assuming hydrolysis in dilute solution to

be pseudo-first order, an estimate of the decomposition during extraction can be made using the data of Christenson [8]. The decomposition at 25°C and pH 10 is approximately 5%, whereas at pH 11 it is 38%/h and by pH 12, less than 5% of the original concentration would be present after 1 h. Extractions were completed in less than 1 h, and standard plasma solutions extracted at the same time as the unknown samples to minimise the effects of decomposition. Once extracted, the residues can be stored overnight at 4°C without noticeable losses.

Neostigmine was added to the samples to prevent enzymatic hydrolysis before extraction. Non-enzymatic hydrolysis was not considered important for the few hours that plasma samples were stored at 4°C as at 25°C and pH 7.8 (the lowest value for which data were available) the rate of decomposition is $< 0.1\%/h$.

Precision and sensitivity

Intra-assay coefficients of variation, determined by assaying six samples containing 10 or 1 ng ml^{-1}, were 6.3% and 7.3%, respectively. Recovery did not appear to be concentration dependent: the mean value was 93%, after correction for aliquot losses, at both concentrations.

The sensitivity of the method was judged to be in the order of 0.5 ng ml^{-1}, using 3-ml plasma samples. At this concentration peaks were (typically) 3—5 mm high and distinguishable from the background. The calibration line between 0.5 and 20 ng ml^{-1} was linear (e.g. $r = 0.9999$, $n = 5$) with a slightly negative, but insignificant, intercept (e.g. -0.0231 ± 0.0146 cm). From this it was concluded that adsorptive or other non-exponential losses, either in the extraction or chromatography were absent or unimportant over the range of concentrations studied.

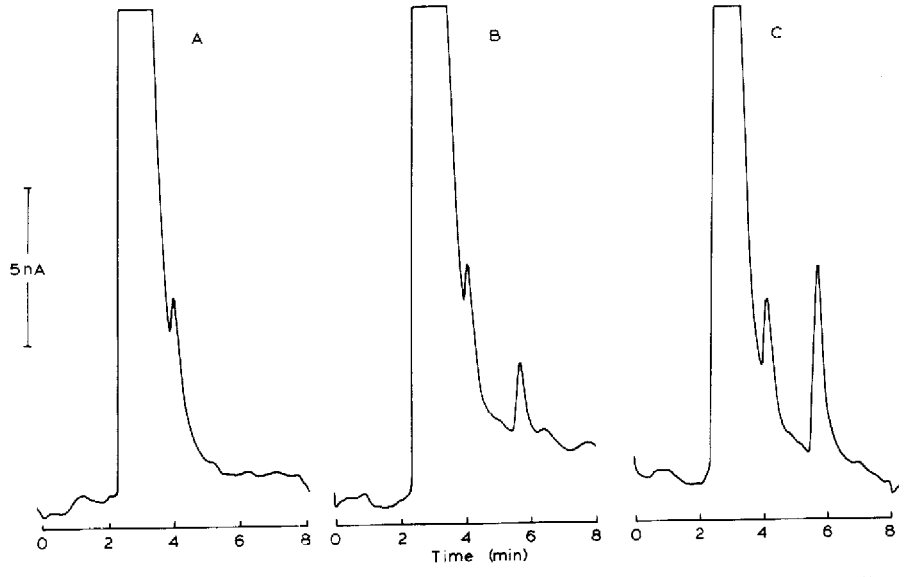

Fig. 2. Chromatograms of physostigmine. (A) Pre-dose plasma; (B) plasma collected 15 min after 1 mg physostigmine salicylate was administered subcutaneously; (C) plasma spiked with physostigmine at 10 ng ml^{-1}. Electrochemical detection at 0.8 V.

Plasma concentrations

Physostigmine concentrations in the samples from the volunteer were 3.6, 1.3 and 0.5 ng ml^{-1} at 15, 30 and 60 min respectively after the dose. By 90 min the concentration had fallen below the limit of detection. Plasma collected before the dose was free of interfering peaks at the retention volume of physostigmine (Fig. 2). The absence of a rising phase probably reflects partly the speed with which a subcutaneous dose is absorbed, and partly the difficulty of ensuring that the injection is purely subcutaneous. The rate of decline from plasma suggests an elimination half-time in the order of 15—20 min. This is in keeping with the idea that a subcutaneous dose is largely destroyed in about 2 h [9].

CONCLUSION

The described method is selective and sensitive enough for monitoring physostigmine concentrations after single doses in the therapeutic range.

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