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Note

Determination of physostigmine in plasma by high-performance liquid chromatography

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The alkaloid physostigmine is an anticholinesterase drug used in the treatment of glaucoma, but more recently in the treatment of Alzheimer's disease [1]. The drug is hydrolysed enzymatically or in the presence of alkali to eseroline, which is rapidly oxidised to rubreserine (Fig. 1).

Until recently, few analytical methods were available of sufficient specificity or sensitivity to measure physostigmine in biological fluids. Furthermore, few methods document conditions necessary to stabilise physostigmine during sample collection or storage, which is of utmost importance as physostigmine rapidly degrades in blood and plasma.

Quantitation of physostigmine in biological fluids has been achieved by spectrofluorimetry [2], high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection [3] and HPLC with electrochemical detection (ED) [4]. All these methods are too insensitive to accurately measure drug levels circulating after single-dose oral administration.

More recently, a sensitive and specific method for physostigmine in biological fluid by HPLC using a dual-electrode electrochemical detector has been published [5]. Using a 2-ml sample volume, a limit of detection of 100 pg/ml was achieved, although lower levels were attainable using larger sample volumes (up to 4 ml).

This paper describes an alternative HPLC procedure for the measurement of physostigmine in plasma incorporating a high-sensitivity fluorimetric detector which utilises the powerful natural fluorescence of physostigmine. The method is also applicable to the measurement of physostigmine in whole-blood samples.

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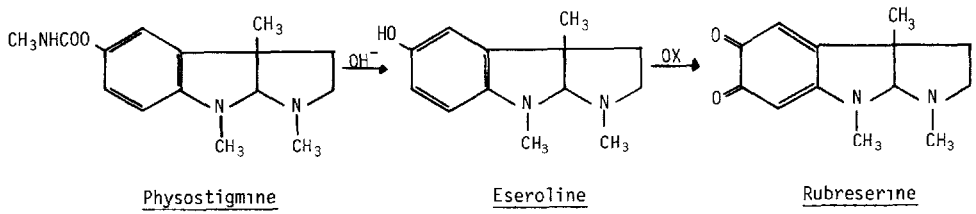

Fig. 1. Chemical structures of physostigmine and its degradation products.

Conditions necessary for the stabilisation of the biological fluid during sample collection are also described.

EXPERIMENTAL

All reagents were of analytical-reagent grade and all inorganic reagents were prepared in freshly glass-distilled water. Acetonitrile (Koch-Light, Colnbrook, U.K.) and methyl *tert.*-butyl ether (Fisons Scientific Apparatus, Loughborough, U.K.) were HPLC grade. Physostigmine base, neostigmine bromide and acetylsalicylic acid were obtained from Sigma (Poole, U.K.). Pyridostigmine bromide was supplied by Roche Products (Welwyn Garden City, U.K.). Eseroline, rubreserine and the N,N-dimethylcarbamate analogue of physostigmine, used as the internal standard (Fig. 2), were supplied by the Chemical Defence Establishment (Porton Down, U.K.).

Stock standard solutions of physostigmine were prepared at a concentration of 0.1 μg/ml in methanol, internal standard at a concentration of 2 μg/ml in acetonitrile and pyridostigmine bromide at a concentration of 10 mg/ml in distilled water.

Instrumentation

The liquid chromatograph consisted of a Spectra-Physics pump Model SP 8770 (Spectra-Physics, St. Albans, U.K.) and WISP™ 710B autosampler (Millipore, Harrow, U.K.), coupled to an LS 4 fluorescence detector (Perkin-Elmer, Beaconsfield, U.K.), fitted with a Xenon source lamp and operated at an excitation wavelength of 254 nm (slit width 10 nm) and emission wavelength 346 nm (slit width 20 nm). Sensitivity was set at fixed scale ×5 and off-set by 10%. Chromatograms were recorded on a Trilab II computing integrator (Trivector Scientific, Sandy, U.K.). Peak-height measurements and peak-height ratios were automatically calculated by the integrator. The column used for the analysis was

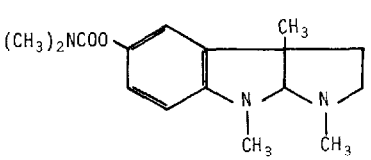

Fig. 2. Chemical structure of internal standard.

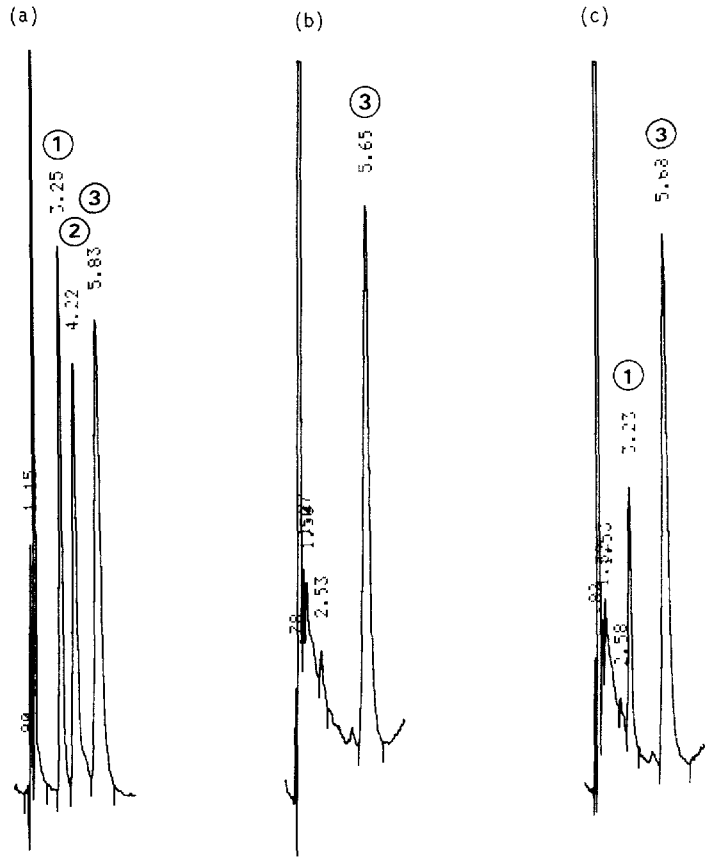

Fig. 3. Chromatograms of (a) reference standards, (b) control plasma and (c) plasma containing physostigmine at a concentration of 1 ng/ml. Chromatographic conditions: column, Spherisorb 5ODS1 (12.5 cm×0.49 cm I.D.); mobile phase, acetonitrile–0.01 *M* sodium acetate (95:5, v/v); flow-rate, 2 ml/min; detection, fluorescence, excitation wavelength 254 nm (slit width 10 nm), emission wavelength 346 nm (slit width 20 nm). Peaks: 1 = physostigmine; 2 = eseroline; 3 = internal standard.

constructed of stainless steel (12.5 cm×0.49 cm I.D.) and packed with Spherisorb 5ODS1 (mean particle diameter 5 μm, Hichrom, Reading, U.K.). A precolumn (5 cm×0.2 cm I.D.) dry-packed with pellicular ODS (particle range 37–53 μm, Whatman, Maidstone, U.K.) was installed in-front of the analytical column to protect it from contamination. The pump was set to deliver a flow-rate of 2 ml/min. Under these conditions, physostigmine eluted in ca. 3 min and the internal standard in ca. 6 min (Fig. 3). New columns required equilibration with mobile phase overnight until consistent retention times were obtained. As the column aged, retention times tended to shorten. Columns were regenerated by eluting with methanol followed by equilibration with mobile phase. The autosampler was set for a run time of 10 min to allow for a late-eluting component present in some plasma samples.

Sample preparation procedure

Samples of plasma or whole blood (2 ml) were mixed with 500 μl of 3.5% (v/v) ammonium hydroxide solution and 20 μl of internal standard solution (ca. 40 ng), and extracted with 8 ml of freshly redistilled methyl *tert.*-butyl ether using a rotary mixer for 10 min. The organic layer was transferred to a conical centrifuge tube and evaporated to an approximate volume of 0.5 ml at ca. 37 °C under a stream of nitrogen. The organic phase was mixed with 100 μl of 0.01 *M* hydrochloric acid on a vortex mixer for 10 s. After centrifugation, an aliquot (70–80 μl) of the aqueous layer was removed and injected on column.

Physostigmine stability

The in vitro rates of decomposition of physostigmine in plasma at ambient temperature (ca. 22 °C), with and without the addition of stabilising agents, were determined as follows. Aqueous physostigmine stock solution (100 μl, 1 μg/ml) was added to samples (20 ml) of (1) distilled water (control), (2) fresh human plasma, (3) human plasma containing 50 mg acetylsalicylic acid, (4) human plasma containing dilute ammonium hydroxide solution (1 ml), (5) dog plasma, (6) human plasma containing neostigmine bromide (2 mg) and (7) human plasma containing pyridostigmine bromide (2.5 mg), to produce a final concentration of 5 ng/ml. Samples were thoroughly mixed and aliquots (2 ml) removed immediately (zero time) and at 10, 20, 40 (samples 2 and 5 only), 60, 90, 195 and 300 min after preparation. Samples were mixed with internal standard solution and analysed for physostigmine. The resultant peak-height ratio measurements of physostigmine to internal standard were then plotted as a function of time and the half-life of physostigmine under each set of conditions was determined.

Physostigmine blood cell/plasma distribution

The distribution of physostigmine between plasma and erythrocytes and the effect of pyridostigmine on this distribution was determined at physostigmine concentrations of 5 and 1 ng/ml.

At each concentration, replicate ($n=8$) samples of whole blood (20 ml, containing lithium–heparin anticoagulant) were mixed with aqueous physostigmine standard solution. Two whole-blood samples were centrifuged to obtain plasma and triplicate aliquots (2 ml) from these plasma samples, together with triplicate aliquots (2 ml) of two whole-blood samples, were subjected to the previously described preparation procedure.

The four remaining samples of whole blood were immediately mixed with pyridostigmine bromide solution (1000 μg, to produce a concentration of 50 μg/ml). This ensured that decomposition in all the samples had occurred at the same rate. These samples were thoroughly mixed and two samples were centrifuged to obtain plasma. Triplicate aliquots (2 ml) of each plasma and whole-blood sample were then subjected to the preparation procedure in a similar manner to the samples prepared in the absence of pyridostigmine.

TABLE I

INTRA-ASSAY PRECISION MEASUREMENTS OF PHYSOSTIGMINE IN PLASMA

Concentration present (ng/ml)	Mean measured concentration (ng/ml)	Number of replicates	Coefficient of variation (%)	Relative error (%)
0.10	0.102	8	4.9	+2.0
1.00	0.973	5	3.5	−2.7
3.00	2.813	5	0.8	−6.2

Plasma calibration procedure

Calibration standards of physostigmine in plasma were prepared over the concentration range 0.1–3.0 ng/ml.

Control human plasma was mixed with aqueous pyridostigmine bromide solution (10 mg/ml) to produce a final concentration of 50 μg/ml. Aliquots (2 ml) of this plasma were then spiked with a methanolic solution (2–60 μl) of physostigmine (0.1 μg/ml) to produce calibration standards containing physostigmine at concentrations of 0.1, 0.2, 0.5, 0.8, 1, 2 and 3 ng/ml. Samples of plasma containing no physostigmine were also taken to act as blank samples. The samples were mixed thoroughly and subjected to the sample preparation procedure previously described.

Calibration procedure, precision and accuracy of measurement

Calibration measurements of peak-height ratios of physostigmine to internal standard against concentration of physostigmine present in the sample were subject to least-squares regression analysis. The plots of peak-height ratio against concentration were linear over the range 0.1–3.0 ng/ml ($r=0.9995$); linearity was also good up to 5.0 ng/ml. The mean of replicate measurements at 0.1, 1.0 and 3.0 ng/ml gave measured concentrations corresponding to 102.0, 97.3 and 93.8% of the true concentration respectively (Table I).

The precision of the assay determined from these samples, as indicated by the coefficient of variation of the measured concentration, was ±4.9% at 0.1 ng/ml, ±3.5% at 1.0 ng/ml and ±0.8% at 3.0 ng/ml (Table I).

Recovery (extraction efficiency) of physostigmine and internal standard from plasma

The recovery of physostigmine and internal standard from plasma was determined by comparing the responses of standards taken through the extraction procedure with those of standard solutions at the same concentration injected directly into the chromatograph without extraction. The mean recovery of physostigmine from plasma was 94.2% at physostigmine concentrations of 2.0 ng/ml and 90.4% at concentrations of 5.0 ng/ml. The mean recovery of internal standard was 77.8%.

Selectivity and limit of accurate measurement

Some chromatograms of control human plasma samples taken through the analytical procedure contained a small peak with a retention time similar to phy-

sostigmine. The metabolites, eseroline and rubreserine, did not interfere with the assay. Eseroline had a retention time of ca. 4 min (Fig. 3) and was resolved from physostigmine, and rubreserine was not detected under the described conditions.

The limit of accurate measurement was taken as 0.1 ng/ml. The response at this level was at least twice that associated with instrument noise or endogenous material. A series of representative chromatograms of calibration standards are shown in Fig. 3.

Stability of physostigmine in frozen plasma in the presence of pyridostigmine bromide

Two samples (50 ml) of control human plasma were mixed with pyridostigmine bromide to produce a final concentration of 50 μg/ml. Physostigmine was added to these samples to produce bulk plasma standards at concentrations of 1 and 0.2 ng/ml. The samples were thoroughly mixed and aliquots (2 ml) transferred to extraction tubes, stoppered and frozen at ca. −20°C until taken for analysis at intervals after storage.

RESULTS AND DISCUSSION

The described analytical procedure for the measurement of physostigmine has been validated in terms of precision, accuracy, specificity and linearity over the concentration range 0.1–3.0 ng/ml.

The mechanism of retention of physostigmine under the described conditions appears to be due to interaction of physostigmine molecules with residual silanol groups remaining on the surface of the Spherisorb ODS1 stationary phase. This stationary phase consists of a mono-molecular layer with a 7% (w/w, 0.3 mM/g) carbon coverage and a controlled number of residual silanol groups remaining after the capping of the major silanol groups. Using a column with identical dimensions and the same mobile phase, but packed with Spherisorb ODS2 stationary phase, physostigmine is virtually unretained. Spherisorb ODS2 has a higher carbon loading of 12% (w/w, 0.5 mM/g) but is fully capped with a very low level of residual silanol groups.

The resultant separation conditions allow the use of high organic modifier levels in the mobile phase, which results in the majority of coextracted endogenous material eluting immediately after the void volume of the column and thus shortening analysis times.

Physostigmine in vitro stability measurements

The in vitro rates of decomposition of physostigmine in plasma at ambient temperature (ca. 22°C) were determined with and without the presence of stabilising agents.

The half-life of physostigmine in fresh human plasma was approximately 30 min (Fig. 4). This compared to a value of 320 min in dog plasma. The addition of a large excess of acetylsalicylic acid or dilute ammonia solution reduced the rate of decomposition, but insufficiently to be of practical use. The excess addition of the quaternary ammonium compound neostigmine bromide was found as

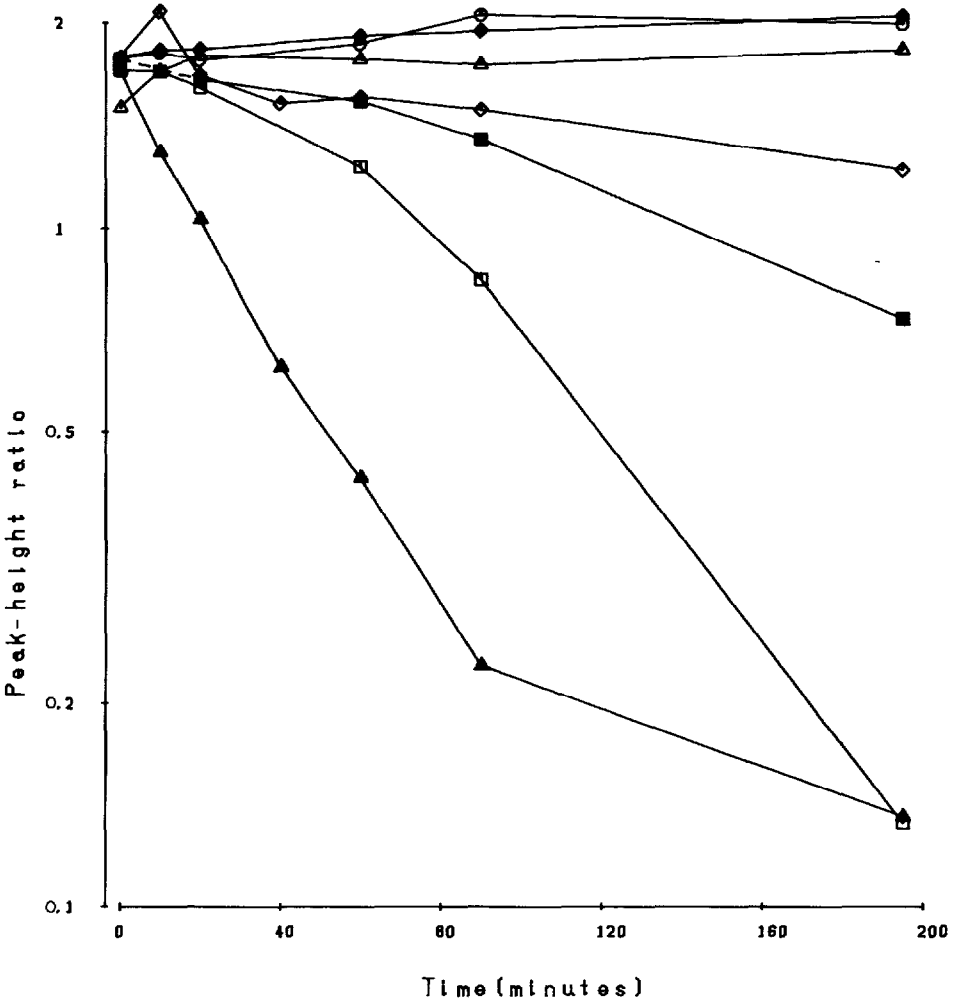

Fig. 4. Decomposition of physostigmine in water (△), fresh human plasma (▲), human plasma containing acetylsalicylic acid (□), human plasma containing ammonium hydroxide (■), dog plasma (◇), human plasma containing neostigmine (♦) and human plasma containing pyridostigmine (○).

previously shown [4], to prevent the decomposition of physostigmine. Unfortunately, under the described chromatographic conditions, neostigmine produced an interfering component in plasma extracts which co-eluted with physostigmine making low level measurements inaccurate.

The alternative quaternary ammonium compound, pyridostigmine bromide, was also found to prevent the decomposition of physostigmine in plasma for at least 195 min after its addition. A concentration of pyridostigmine bromide of 50 μg/ml was sufficient to inhibit physostigmine decomposition and chromatograms of control plasma, containing pyridostigmine bromide, at this concentration, taken through the analytical procedure showed no interfering peaks; for this reason, pyridostigmine bromide was selected as the most suitable stabilising agent.

TABLE II

PHYSOSTIGMINE WHOLE BLOOD/PLASMA DISTRIBUTION RATIO MEASUREMENTS IN THE PRESENCE AND ABSENCE OF PYRIDOSTIGMINE BROMIDE AT A CONCENTRATION OF 50 μg/ml

PHR = Peak-height ratio of physostigmine to internal standard ($n=6$).

Concentration of physostigmine in whole blood (ng/ml)	PHR (mean ± S.D.)		Distribution of physostigmine (whole blood/plasma)
	Whole blood	Plasma	
In the absence of pyridostigmine bromide			
5	0.636 ± 0.020	0.684 ± 0.015	0.930
1	0.136 ± 0.007	0.133 ± 0.005	1.023
Mean			0.977
In the presence of pyridostigmine bromide			
5	0.698 ± 0.019	0.701 ± 0.019	0.996
1	0.141 ± 0.005	0.146 ± 0.003	0.966
Mean			0.981

Physostigmine blood cell/plasma distribution

The distribution of physostigmine between plasma and erythrocytes, and the effect of pyridostigmine bromide upon this distribution, was studied (Table II).

The overall mean whole blood/plasma ratio for physostigmine at concentrations of 1 and 5 ng/ml in the absence of pyridostigmine bromide was 0.977 ($n=12$), and that in the presence of pyridostigmine bromide (at a concentration of 50 μg/ml) was 0.981 ($n=12$). Pyridostigmine bromide had no apparent effect on the blood cell uptake of physostigmine.

Once stabilised, the whole blood or separated plasma can be analysed directly for physostigmine, but as plasma has better storage properties than whole blood, is less likely to be subject to analytical interferences and gives a measure of bioavailable physostigmine, analysis of plasma is more convenient.

Storage stability of physostigmine in plasma at ca. −20° C

Samples of control human plasma stabilised with pyridostigmine bromide (50 μg/ml) and containing physostigmine at concentrations of 1 and 0.2 ng/ml have shown no appreciable loss of physostigmine content after nine months storage at ca. −20° C prior to thawing and analysis.

The described method is currently being evaluated using samples taken from human volunteers administered oral doses of physostigmine.

ACKNOWLEDGEMENT

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