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Simple liquid chromatographic method for the determination of physostigmine and its metabolite eseroline in rat plasma: application to a pharmacokinetic study

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Abstract

Physostigmine, an anticholinergic drug, and its metabolite eseroline can be quantitated by high-performance liquid chromatography (HPLC) with photodiode-array detection. After addition of the structurally related internal standard (−)-*N*-methylphysostigmine, rat plasma samples were extracted and cleaned using a Varian Bond Elut C_{18} column. The methanol–ammonia (98:2) eluate was evaporated to dryness and reconstituted with 0.01 *M* sodium dihydrogenphosphate (pH 3). Physostigmine and eseroline were separated on an Alltech Ultrasphere Silica column (250×4.6 mm I.D.; particle size 5 μm) at a flow-rate of 1 ml/min, with a mobile phase of 0.01 *M* sodium dihydrogenphosphate (pH 3)–acetonitrile (85:15). The limits of detection were 10 and 25 ng/ml for physostigmine and eseroline, respectively; the signal-to-noise ratio for this concentration was approximately 3:1. Spiked rat plasma containing 0.1–2.5 μg/ml of physostigmine and eseroline gives good linearity. The average percentage recovery from five spiked plasma samples was 88.0±2.9 and 61.1±5.6% for physostigmine and eseroline, respectively. Within the concentration range 0.1–2.5 μg/ml, the within-day precision was 1.9–8.3 and 3.0–7.7% for physostigmine and eseroline, respectively, and the between-day precision was 4.1–9.3 and 3.7–11% for physostigmine and eseroline, respectively. The method is rapid, simple and reliable, thus it is suitable for pharmacokinetic studies in the rat.

Keywords: Physostigmine; Eseroline

1. Introduction

Physostigmine is a powerful and reversible acetylcholine esterase inhibitor which effectively increases the concentration of acetylcholine at the sites of cholinergic transmission. It is an alkaloid found in the seeds of the fabaceous plant *Physostigma venenosum*. This West African plant has been used as a ''trial-by-ordeal'' plant in Africa, but today serves as the source of physostigmine used to treat glaucoma [1]. Clinically, physostigmine is also used to reverse the effect upon the central nervous system caused by clinical or toxic dosages of drugs capable of producing the anticholinergic syndrome [2,3]. It has recent-

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ly been shown that physostigmine is more effective than pyridostigmine for protection against the detrimental effects of organophosphorus nerve agents in humans [4]. More recent studies have shown that physostigmine may aid and improve the performance of everyday working memory when administered to people by infusion in laboratory tests [5].

The major route for the metabolism of physostigmine identified to date is hydrolysis to eseroline [6]. Eseroline possesses morphine-like effects and produces untoward stimulation of the CNS [7]. The hydrolysis product is comparatively more toxic than the parent compound, physostigmine. It seems that eseroline causes neuronal cell death by a mechanism involving loss of cell ATP [8]. Thus, the formation of eseroline may contribute to the toxic effect of physostigmine.

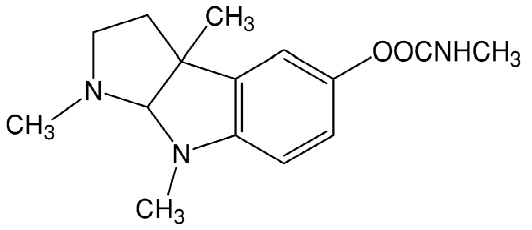
Several methods for the identification and quantification of physostigmine and its metabolite eseroline in biological fluids make use of high-performance liquid chromatography (HPLC) coupled with UV [9], electrochemical [10], and dual-electrode amperometric [6] detection techniques. A HPLC post-column fluorescent ion-pair extraction system was also reported for the determination of physostigmine and eseroline in human serum, using sodium α-(3,4-dimethyloxyphenyl)cinnamoniyrile-2′-sulfonate (D-PS) as a fluorescent ion-pair reagent [11]. Most of these methods employ liquid–liquid extraction, and are generally more complex than the procedure described here.

In this paper, we report a simple, sensitive and reliable isocratic HPLC assay for the simultaneous determination of physostigmine and its metabolite eseroline in rat plasma using a one-step solid-phase extraction (SPE) procedure and photodiode-array detection. This method has been successfully employed on a routine basis for our study of the metabolism and pharmacokinetics of physostigmine.

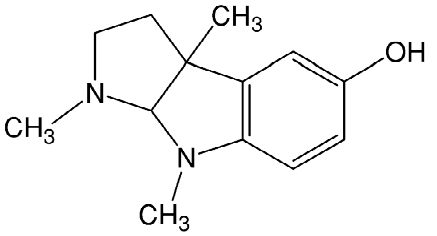
2. Experimental

2.1. Chemicals and materials

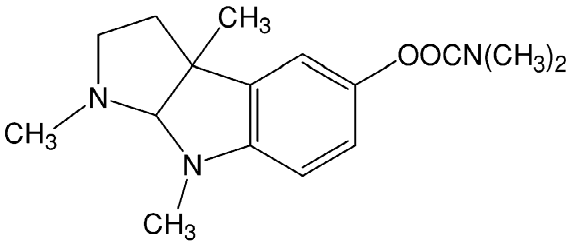
Physostigmine (purity 99%) and eseroline fumarate (purity 97%) were obtained from ICN Biomedicals (Costa Mesa, CA, USA). (−)-*N*-Methylphysostigmine (purity 98%) was obtained from Sigma (St. Louis, MO, USA). The chemical structures are shown in Fig. 1. Methanol, ammonia, sodium dihydrogenphosphate and acetonitrile were obtained from Merk (Darmstadt, Germany). All solvents were HPLC grade and chemicals were reagent grade. All the reagents were used without further purification. Deionized water was used throughout the study and purified by a Milli Q system (Millipore, Milford, MA, USA). Aqueous stock solutions of physostigmine, eseroline and (−)-



Physostigmine



Eseroline



(-)-N-methylphysostigmine

Fig. 1. Chemical structures of physostigmine, eseroline and (−)-*N*-methylphysostigmine (I.S.).

N-methylphysostigmine were prepared at 1 mg/ml and stored at −20 °C. The working solutions for spiking blank rat plasma samples were prepared fresh daily. Bond Elut C_{18} SPE columns (100 mg) were obtained from Varian (Middelburg, The Netherlands).

2.2. Instrumentation

The HPLC system (Waters 2690 Separation Module) consisted of a Waters 600E multisolvent delivery system pump, a Waters Ultra WISP 715 autoinjector, and a Waters 996 diode-array detection system set at 246 nm (all from Waters, Milford, MA, USA). Physostigmine and its metabolite eseroline were separated on an Alltech Ultrasphere Silica Column (250×4.6 mm I.D., particle size 5 μm) from Alltech at a flow-rate of 1 ml/min, with a mobile phase of 0.01 *M* sodium dihydrogenphosphate (pH 3)–acetonitrile (85:15, v/v).

2.3. Sample preparation

Rat plasma (0.1 ml) samples were spiked with concentrations ranging between 0.1 and 2.5 μg/ml each of physostigmine and eseroline. 20 μl of 50 μg/ml (−)-*N*-methylphysostigmine solution was added to spiked and treated samples as internal standard (I.S.) and denatured with 0.1 ml acetonitrile. Disposable Bond Elut C_{18} (100 mg) columns were conditioned with 1 ml methanol and then equilibrated using 1 ml water before use. The spiked plasma samples were vortexed for 10 s, centrifuged for 3 min at 1000 *g*, the supernatant was loaded into disposable C_{18} SPE columns, then washed with 1 ml water and 1 ml 10 m*M* HCl, eluted with 1 ml methanol–ammonia solution (98:2), and evaporated to dryness under nitrogen. The resultant residue was reconstituted with 0.1 ml 0.01 *M* sodium dihydrogenphosphate (pH 3) and 80 μl was injected into the HPLC system. All chromatography was performed at room temperature (20 °C).

2.4. Assay validation

Samples were quantified using the peak area ratio of physostigmine or eseroline to I.S. Standard calibration curves were constructed by spiking drug-free pooled plasma with a known amount of physostigmine and eseroline in the range 0.1–2.5 μg/ml. These plasma standards were also used to determine the extraction recovery, within-day and between-day precision and accuracy ($n=5$) of the method. The recovery of the extraction procedure was determined by comparing the peak area obtained after extraction with that of an aqueous drug solution of corresponding concentration without extraction. Limits of detection and quantitation were determined from the ratio of baseline noise to calibration point (i.e. 1:3 and 1:10, respectively). The limit of quantitation was determined five times for confirmation.

2.5. Example application

One male Sprague–Dawley rat (National University Animal Holding Unit, Singapore), weighing 250 g was used for the experiments. Left jugular vein cannulation was performed under sterile conditions. The proximal end of the silastic catheter was inserted into the jugular vein and the distal end of the catheter was threaded s.c. and exited through a small incision in the back of the animal. The catheter was flushed with 0.9% saline with 50 U of heparin and sealed with fishing line when not in use. The rat was left for 1 day after the surgery to recover from the anesthesia. At 0, 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3 and 4 h after a single oral dose of physostigmine (1000 μg/kg), 0.5 ml of blood was taken from the jugular catheter. The plasma was obtained immediately by centrifugation at 2000 *g* for 5 min and stored at −20 °C until analysis by HPLC. Pharmacokinetic parameters were calculated using the pharmacokinetic program packages WinNonlin, version 3.2 (Pharsight, Mountain View, CA, USA).

3. Results and discussion

Regression analysis was performed on the plasma calibration curve. The calibration curves for the analytes obtained over five independent runs were linear within the calibration range (0.1–2.5 μg/ml). The regression equations describing the calibration runs were: physostigmine, $y = 0.1013(\pm 0.0061)x - 0.0058(\pm 0.0031)$ ($r^2 = 0.9998$); and eseroline, $y = 0.3343(\pm 0.0234)x - 0.0208(\pm 0.0098)$ ($r^2 = 0.9994$),

where y is the peak area ratio of physostigmine or eseroline to I.S. and x is the concentration (μg/ml). Representative chromatograms of blank and spiked plasma samples are shown in Fig. 2. Under the described conditions, the retention times were 6.1, 4.1 and 8.9 min for physostigmine, eseroline and the I.S., respectively. The total run time was 12 min. The clean chromatogram shows no interference from endogenous substances in the plasma sample. Limits of detection of the assay were 10 ng/ml for physostigmine and 25 ng/ml for eseroline. Limits of quantitation were 75 and 100 ng/ml for physostigmine and eseroline, respectively. Taking into account the differences in peak shapes (e.g., the physostigmine peaks were sharper), all analytes, including the I.S., showed comparable UV absorbances (Fig. 3). This is consistent with their chemical structures, differing only by the absence or presence of methyl groups on the side chain.

In this study, the solid-phase method was used for the extraction procedure. Initially, a HCX mixed-mode extraction column was used in order to obtain clean chromatograms. However, the method was not reproducible and the extraction efficiency was less than 40%. The clean-up procedure using the Bond Elut C_{18} (100 mg) column was found to yield reproducible results and clean chromatograms. For the extraction from plasma, the recoveries (mean±SD) of physostigmine were found to be 88.0±2.9% at a concentration of 0.25 μg/ml, whereas the recoveries (mean±SD) of eseroline for the same concentration were reduced (61.1±5.6%). Spiked plasma samples were extracted and analyzed for each concentration in five replicates. Recovery of the I.S. was also good (81.2±2.8%) at 50 μg/ml. In the C_{18} clean-up procedure, 0.1 ml of acetonitrile was used to denature plasma proteins. Denaturation with acids or more than 0.1 ml acetonitrile reduced the extraction recoveries of the analytes.

The influence of mobile phase composition on the retention times and separation of physostigmine, eseroline and the I.S. was studied by varying the ratio of acetonitrile and 0.01 *M* sodium dihydrogenphosphate (pH 3). A bare silica column was used in place of the C_{18} column as the peaks of the analytes had a narrower base and a better resolution was achieved. The acetonitrile–0.01 *M* sodium dihydrogenphosphate volume composition (v/v) was varied as follows: 5:95, 10:90, 15:85, 20:80, 25:75, 30:70, 35:65, 45:55 and 50:50. The study revealed that varying the composition of the mobile phase changed the retention times of the analytes. Increasing the organic component of the solvent system retarded the retention times. The physostigmine and eseroline peaks interfered with other co-eluting peaks when solvent systems acetonitrile–0.01 *M* sodium dihydrogenphosphate (30:70–50:50, v/v) were used. Stability of the retention times of the analytes was only observed when acetonitrile–phosphate buffer (15:85, v/v) was used. The detection of physostig-

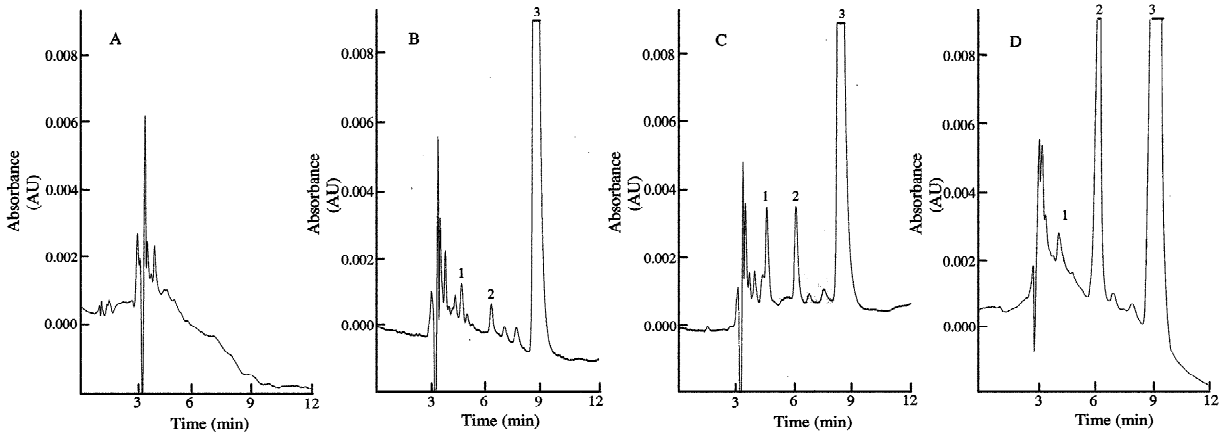


Fig. 2. HPLC chromatograms of blank rat plasma (A), rat plasma spiked with physostigmine and eseroline standards (B, limit of quantitation; C, 0.5 μg/ml) and a plasma sample from a rat administered a single oral dose of physostigmine (1000 μg/kg) in the first hour (D). Peaks: 1=eseroline, 2=physostigmine, 3=I.S. [(−)-*N*-methylphysostigmine].

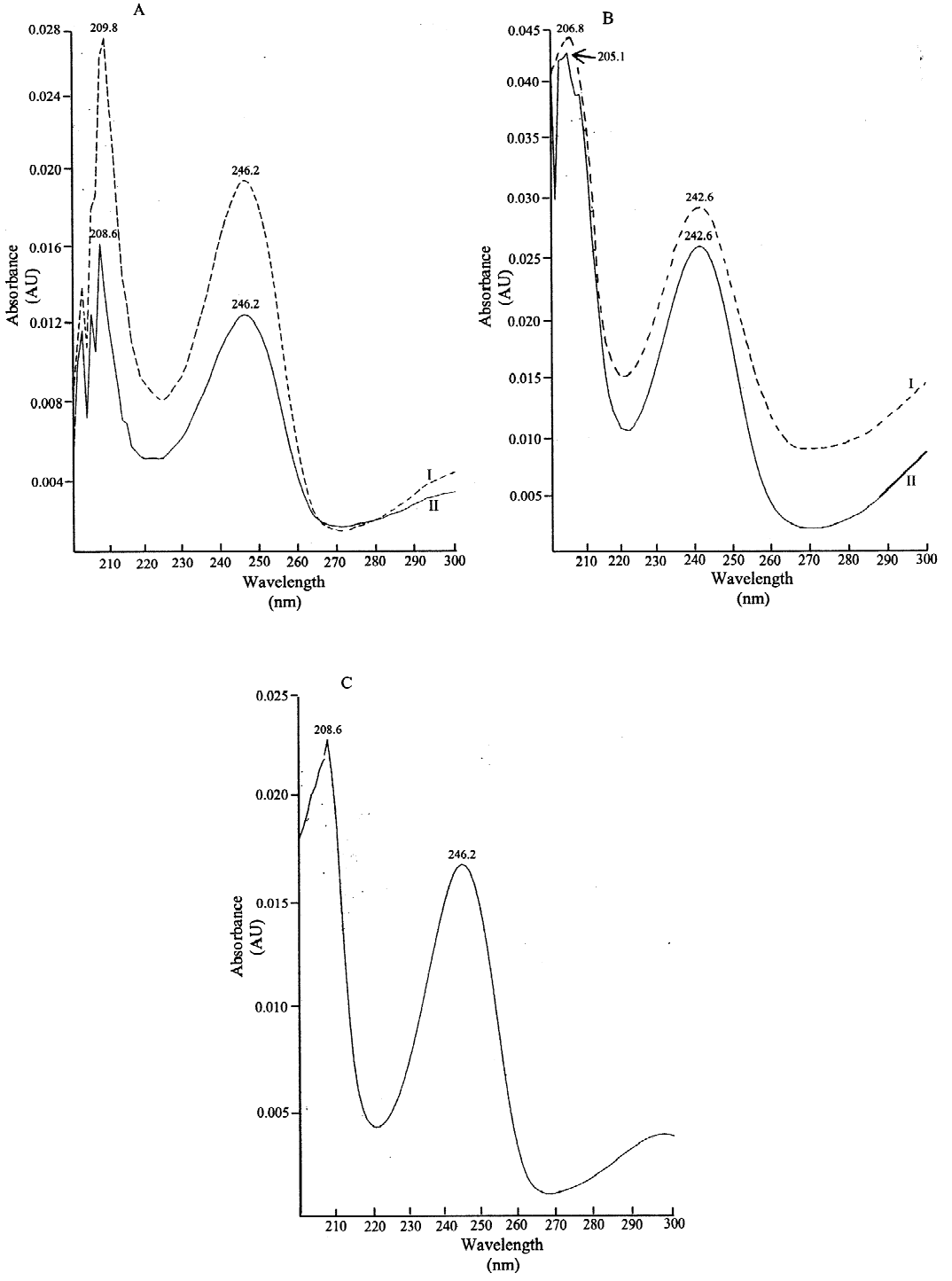


Fig. 3. UV absorbance spectra of the standard (I) and the rat plasma sample (II). (A) Physostigmine, (B) eseroline, (C) I.S. [(−)-*N*-methylphysostigmine].

mine and its metabolite at 12 min succeeded without any problems. Thus, optimal resolution and separation of the analytes were achieved with the use of the acetonitrile–0.01 *M* sodium dihydrogenphosphate (15:85, v/v) solvent system.

The within- and between-day variations ($n=5$) of the method are given in Table 1. For each level, the repeatability and reproducibility criteria were clearly satisfactory. For the two analytes, the within-day variability was less than 8.3%, while the between-day variability did not exceed 11%. The within-day variability averaged 5.5% for physostigmine and 5.0% for eseroline. The mean between-day variability was 6% for physostigmine and 7.6% for eseroline. The accuracy never deviated from 100% by more than 10%. Mean accuracies were 100.4 and 99.7% for physostigmine and eseroline, respectively (Table 1). To minimize analytical variability, analyte concentrations in rat plasma were always derived according to the same-day standard curve.

The above method was applied to a pharmacokinetic study of physostigmine in the rat. After SPE extraction, the HPLC chromatogram shown in Fig. 2D was obtained. Plasma concentration–time curves for physostigmine and its metabolite eseroline are shown in Fig. 4. A peak concentration of physostigmine of 1.81 μg/ml was achieved within 1 h of administration. Plasma levels then declined with half-lives of 0.93 h. The area under the plasma concentration–time curve (AUC) was 2.78 μg/ml/h. Prior to the start of our work, there was little information available on the pharmacokinetic behavior of the metabolite eseroline. In this study, the metabolite peak was observed within 1.5 h of administration. The peak concentration of eseroline was 0.25 μg/ml and its level declined with a half-life of 2.51 h and an AUC of 0.62 μg/ml/h.

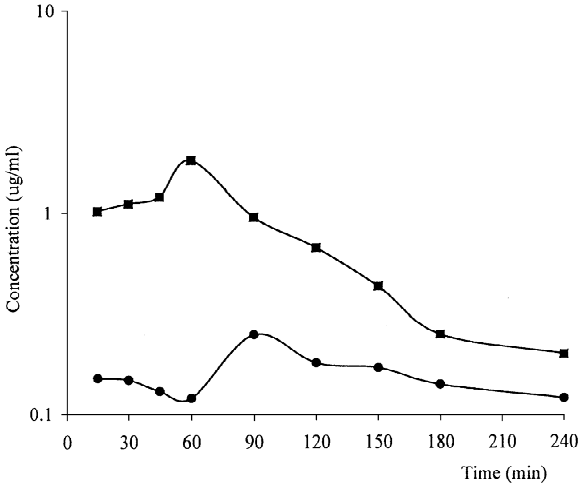


Fig. 4. Plasma concentration–time curves for physostigmine (■) and its metabolite eseroline (●) in a rat following oral administration of 1000 μg/kg physostigmine.

Physostigmine has a high first pass metabolism

Table 1
Within- and between-day assay variability

Added concentration (μg/ml)	Measured concentration (μg/ml)					
	Within-day			Between-day		
	Mean±SD	C.V. (%)	Accuracy (%)	Mean±SD	C.V. (%)	Accuracy (%)
Physostigmine						
0.10	0.102±0.007	6.9	102.0	0.11±0.008	7.3	110.0
0.25	0.24±0.02	8.3	96.0	0.24±0.01	4.2	96.0
0.50	0.53±0.03	5.7	106.0	0.49±0.02	4.1	98.0
1.00	1.05±0.02	1.9	105.0	0.99±0.05	5.1	99.0
2.50	2.62±0.12	4.6	104.8	2.47±0.23	9.3	98.8
Eseroline						
0.10	0.099±0.003	3.0	99.0	0.092±0.007	7.6	92.0
0.25	0.26±0.02	7.7	104.0	0.27±0.01	3.7	108.0
0.50	0.52±0.03	5.8	104.0	0.49±0.04	8.2	98.0
1.00	1.05±0.05	4.8	105.0	1.03±0.08	7.8	103.0
2.50	2.56±0.10	3.9	102.4	2.44±0.26	11	97.6

and a short elimination half-life and is a good candidate for controlled release studies. A new formulation of physostigmine poly(lactide-co-glycolide) (PLGA) co-polymer submicron spheres has been developed in our laboratory and its pharmacokinetic behaviour has been compared with that of physostigmine solution using the above HPLC method.

4. Conclusions

The described photodiode-array HPLC method is specific, precise, accurate and sufficiently sensitive for the analysis of physostigmine and its metabolite eseroline in plasma. The relatively simple Bond Elut C_{18} clean-up procedure for sample preparation and only one isocratic chromatographic elution that separates and quantitates the two analytes enhance the efficiency of the procedure. This assay is used for pharmacokinetic studies of physostigmine in a controlled-release therapeutic system formulation in the rat, and is almost certainly applicable to the analysis of human samples.

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