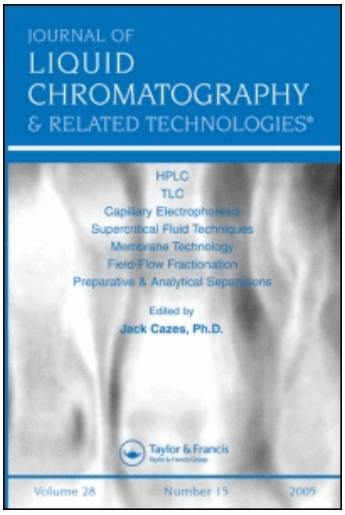

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High Performance Liquid Chromatographic Method for Detection of Physostigmine and Eseroline in Plasma Using a Silica Gel Column and a Perchloric Acid Mobile Phase

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR DETECTION OF PHYSOSTIGMINE AND ESEROLINE IN PLASMA USING A SILICA GEL COLUMN AND A PERCHLORIC ACID MOBILE PHASE

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ABSTRACT

A high-performance liquid chromatographic (HPLC) procedure that employs a silica gel column, a perchloric acid in methanol mobile phase and fluorescence detection has been developed for the determination of the concentrations of physostigmine and its metabolite, eseroline, in plasma. The method requires plasma samples to contain an ascorbic acid anti-oxidation solution. Plasma samples are extracted with 5 ml of methyl-t-butyl ether under alkaline conditions. The organic phase is evaporated to dryness and reconstituted with mobile phase. The quantitation range is 0.1 to 5.0 ng/ml (base) for both compounds. Mean ± S.D. recoveries for 4 concentrations within this range were 80.7 ± 4.3% for physostigmine and 84.1 ± 3.6% for eseroline. Interday and intraday (n = 6) coefficients of variation, respectively, for 4 concentrations in the 0.2 to 3.0 ng/ml range were 2.82 - 6.99% and 1.59 - 6.48% for physostigmine and 3.59 - 6.82% and 4.05 - 8.78% for eseroline. Bias for blind samples at 4 concentrations from 0.13

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to 5.39 ng/ml ranged -12.5 to 3.46% for physostigmine and at 4 concentrations from 0.12 to 4.98 ng/ml ranged -16.6 to 43.1% for eseroline. Both compounds were found to be stable in prepared plasma when stored at -20°C for up to 4 months. The method was found to be reliable, fast, simple, accurate, precise, and capable of being used for routine analysis.

INTRODUCTION

Physostigmine (Figure 1) is an alkaloid extracted from the calabar bean that has long been used in ophthalmology.[1] Today physostigmine is still mainly used as a miotic, but also as an antidote for poisoning by anticholinergic drugs and for reversing anticholinergic effects.[2] Recently, its inhibiting effect on acetylcholinesterase has been investigated for the treatment of senile dementia in Alzheimer's disease[3] and for its possible prophylactic function in the defense against organofluorophosphate intoxication.[4] In aqueous solution, physostigmine is hydrolyzed to form a colorless phenolic compound, eseroline, (Figure 1) which is subsequently oxidized to rubreserine[5] (Figure 1) and other colored compounds. Its short duration of action suggests that it has a short half-life. Low doses (typically 0.5-4 mg) coupled with rapid metabolism mean that an assay for human and animal studies must be capable of measuring nanogram or sub-nanogram amounts.[6,7]

Methods developed in the past few years for the determination of physostigmine in biological fluids include thin layer chromatography,[5] gas chromatography[8] and high performance liquid chromatography (HPLC) with ultraviolet,[7,9-12] electrochemical (ECD)[7,13,14] and fluorescence detection.[15,16] The most successful results in terms of detection limit have been reported by Whelpton and Moore,[13] who used HPLC-ECD and 2 ml of plasma to achieve a limit of sensitivity of 100 pg/ml, although lower levels were attainable using larger sample volumes (up to 4 ml).

An HPLC method to determine concentrations of physostigmine and its metabolite, eseroline, in plasma is reported here that has advantages over other currently available procedures. The major

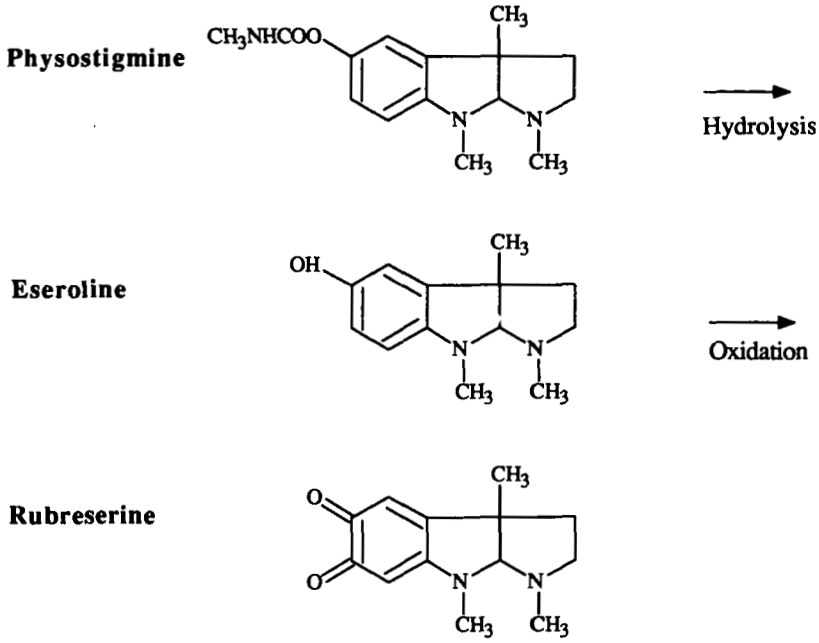

Figure 1. Degradation of physostigmine.

advantages of the method are simplicity, selectivity and the ability to quantify physostigmine and its metabolite in the same sample to 100 pg/ml using only 0.5 ml of sample.

MATERIALS

Instrumentation

The instruments used were a Beckman 110A Solvent Delivery system (Beckman Instruments, Inc., Fullerton, CA), a Waters Intelligent Sample Processor (WISP) Model 710 B equipped with refrigeration unit (Waters Associates, Milford, MA), a Shimadzu RF-535 Fluorescence detector (Shimadzu Corp., Tokyo, Japan), and an HP 3392A Integrator (Hewlett-Packard, Santa Clara, CA). The detector was fitted with a Xenon source lamp and operated at an excitation wavelength of 254 nm and emission wavelength of 355 nm.

Separations were carried out on a Beckman Ultrasphere Si column (5 µm particle size, 4.6 x 250 mm, Beckman Instruments, Inc., Berkeley, CA). The mobile phase consisted of 100% methanol containing 0.013% $HClO_4$. The flow rate was 1.0 ml/min.

Reagents and Stock Solutions

Methanol (HPLC grade) was purchased from Fisher Scientific Co. (Fair Lawn, NJ). Physostigmine and eseroline were obtained from the Walter Reed Army Institute of Research (Washington, D.C.). Neostigmine bromide was purchased from Aldrich Chemical Co. Desipramine (internal standard) was supplied by Alltech Associates, Inc. All other reagents were analytical or reagent grade. Stock solutions of physostigmine and eseroline were prepared at 50 µg/ml in methanol containing 0.2% 6 N HCl. The internal standard stock solution was prepared at 2 µg/ml in methanol. The antioxidant solution was prepared by dissolving 20 g of ascorbic acid in 94 ml of H_2O and adding 6 ml of H_3PO_4. Alkaline borate buffer solution was prepared by dissolving 40 g of boric acid and 48 g of KCl in 1 liter of H_2O and adjusting to pH 8.5-9.0 with 5% NaOH.

METHODS

Sample Preparation

Human or animal blood (approximately 6 ml, when possible) was withdrawn into heparinized tubes containing neostigmine bromide (10 mg/ml, 20 µl). The blood was mixed and centrifuged at 4°C to separate the plasma, which was put into tubes containing antioxidant solution (200 µl per 3 ml of plasma), then stored at -20°C until assayed.

Plasma (0.5 ml) containing antioxidant solution was pipetted into a screw-capped tube and 20 µl of internal standard solution was added. This solution was adjusted to pH 8.5-10 with 0.5% NaOH before addition of 200 µl of the alkaline borate buffer solution and 5 ml of methyl-*t*-butyl ether. The samples were vortexed for 30 seconds, and following centrifugation at 3000 *g* for 6 minutes, the organic phase was transferred to another tube and evaporated

to dryness under N_2. Finally, 200 µl of mobile phase was added, samples were vortexed for 15 seconds and 100-150 µl aliquots were injected onto the HPLC column. Standard curve samples were prepared at 0.1, 0.2, 0.4, 0.8, 1.0, 1.5, 3.0 and 5.0 ng/ml physostigmine and eseroline concentrations in plasma containing antioxidant solution and taken through the extraction procedure along with the unknown samples.

RESULTS

Separation

A silica gel column and a mobile phase that consisted of methanol with 0.013% $HClO_4$ were used to separate physostigmine from its metabolite and from interfering endogenous substances. Figure 2 (and Figure 3) illustrates typical chromatograms for a blank sample and for a sample spiked with physostigmine, eseroline and the internal standard, desipramine. Note that the three peaks are baseline separated and are separated from other components of

Table 1: Standard Curves for Physostigmine and Eseroline in Human Plasma

Spiked Concentration (ng/ml)	Physostigmine Calculated Concentration (ng/ml)	Eseroline Calculated Concentration (ng/ml)
0	0	0
0.1	0.072	0.110
0.2	0.180	0.205
0.4	0.389	0.316
0.8	0.831	0.821
1.0	1.02	1.04
1.5	1.52	1.59
3.0	3.09	2.83
5.0	4.93	5.07

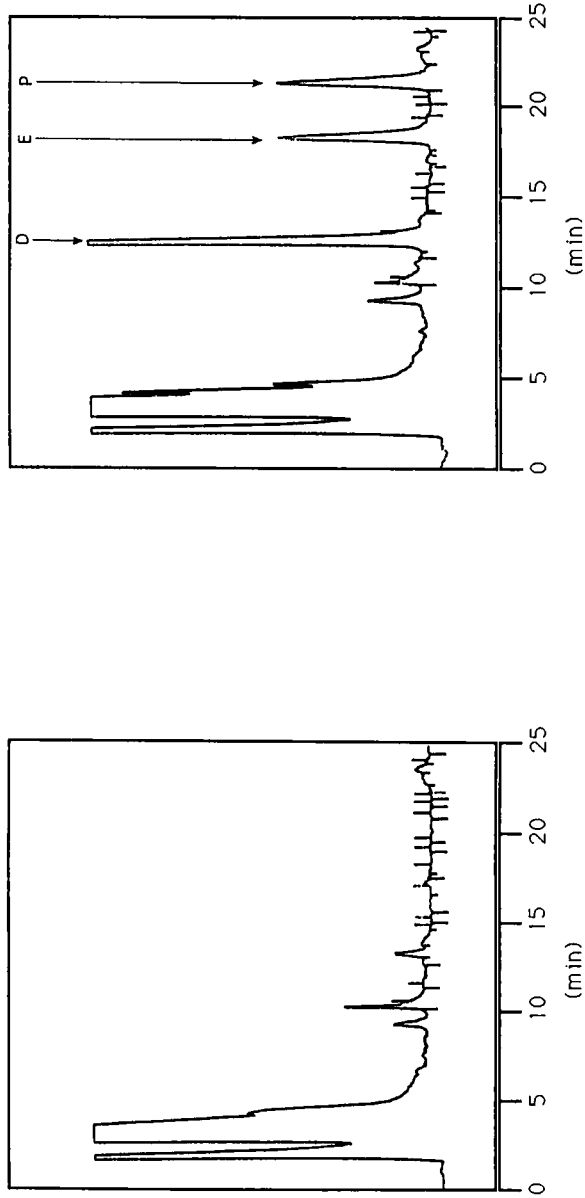

Figure 2. Blank and Spiked Human Plasma Chromatograms.

I. Blank human plasma.

II. Human plasma (0.5 ml sample) spiked with physostigmine (P) (0.8 ng/ml), eseroline (E) (0.8 ng/ml), and internal standard (D).

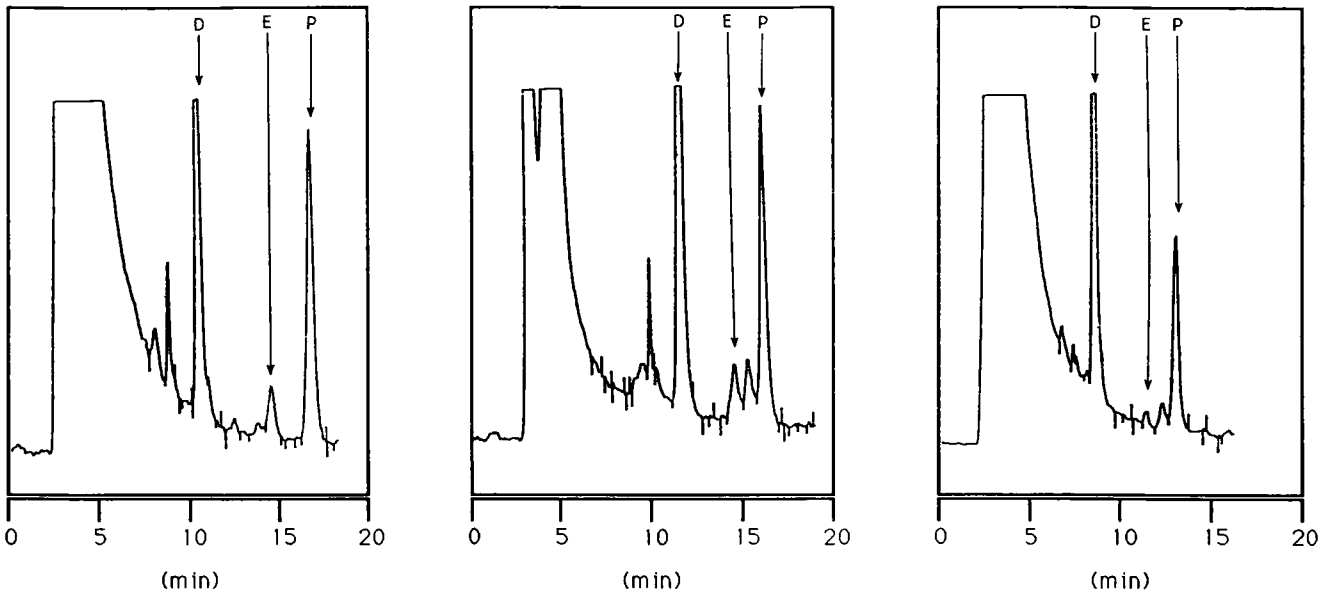

Figure 3. Animal Plasma Chromatograms.

I. Monkey plasma (250 µl sample) 5 hr after 0.1 mg/kg physostigmine dose showing 3.94 ng/ml physostigmine (P), 0.629 ng/ml eseroline (E) and internal standard (D) peaks.

II. Dog plasma (0.5 ml sample) 1.5 hr after 0.1 mg/kg physostigmine dose showing 7.62 ng/ml P, 1.11 ng/ml E, and D (internal standard) peaks.

III. Rat Plasma (30 µl sample) 1 hr after physostigmine dose showing 27.6 ng/ml P, 1.92 ng/ml E, and D (internal standard) peaks.

the sample. The retention times (subject to change depending on temperature and column performance) of physostigmine, eseroline and desipramine were 14.6, 12.8 and 11.5 min, respectively.

Linearity, Precision and Recovery

The calibration curves were obtained for the concentration range of 0.1 to 5.0 ng/ml in plasma. Linear regression of concentration versus peak height ratio gave coefficients of determination of 0.9992 and 0.9977 for typical physostigmine and eseroline standard curves (see Table 1).

The precision of the method was estimated by analysis of spiked plasma samples. The estimate of the precision of this method over the entire working range was determined by the analysis of replicate samples (n = 6) spiked to four concentrations (0.2, 0.8, 1.5 and 3.0 ng/ml) of physostigmine and eseroline. The coefficients of variation (CV) of results ranged from 1.59% to 6.48% for the interday analysis and from 2.82% to 6.99% for the interday analysis for physostigmine. The CV of results ranged from 4.05% to 8.78% for the interday analysis and from 3.59% to 6.82% for the interday analysis for eseroline.

Recoveries of physostigmine and eseroline from plasma were measured by comparing the results of analysis of blank plasma samples spiked with physostigmine and eseroline to extraction solvent samples spiked with proportionate amounts of the two compounds. The recoveries obtained ranged from 74.9% to 85.2% for physostigmine and 80.9% to 89.0% for eseroline.

DISCUSSION

The use of an unmodified silica column and a non-aqueous mobile phase is a sensitive and selective method that causes negligible column deterioration. Other assays using simple solvent extraction, unmodified silica columns and non-aqueous mobile phases have been developed successfully in our laboratory. It was found that methanol containing an ionic modifier, such as perchloric acid, is an efficient mobile phase for a variety of

basic drugs, since perchloric acid is adequately soluble in methanol and is strongly resistant to oxidation.[17] This kind of mobile phase is easy to prepare and seems to lengthen column life.[18] It is clear that this system has many advantages over bonded-phase systems that use aqueous eluents in the HPLC analysis of basic drugs.

Pharmacological investigations of physostigmine and eseroline have been marked by difficulties due to instability and low analyte concentrations in clinical samples. The stability of physostigmine and eseroline poses great concern since their quantitative recovery from plasma is critically important. Physostigmine is hydrolized, enzymatically or in alkali, to eseroline which, in the presence of air, is rapidly oxidized to rubreserine. Figure 4 indicates that physostigmine concentration (20 ng/ml) falls dramatically at room temperature; about 80% of physostigmine initially present in the plasma was lost within 5 hours. Addition of neostigmine to the plasma inhibits this decomposition by competing for the enzymatic reaction sites; no significant physostigmine loss occurred in the presence of excess neostigmine (30 μg/ml of plasma) for 50 hours.[13] Temperature also affected the speed of hydrolysis during centrifugation, as shown in Table 2. Neostigmine must be added immediately after blood is sampled and before centrifugation to prevent enzyme hydrolysis of physostigmine.

The metabolite of physostigmine, eseroline, is more readily decomposed than physostigmine. It was found that eseroline is stable in acidic solution (pH 2.8), but unstable under more alkaline conditions (pH > 5), as the metabolite oxidizes to rubreserine (the solution turns red) within several minutes. A stock solution of eseroline (1 μg/ml) with pH below 3 is stable for several months at 4°C. In order to protect the eseroline from oxidation during an alkaline extraction, several antioxidants were tested including EDTA, sodium metabisulfite, sodium bisulfite and ascorbic acid. Ascorbic acid was the most effective. Figure 5 shows that when ascorbic acid was not present, only 40% of

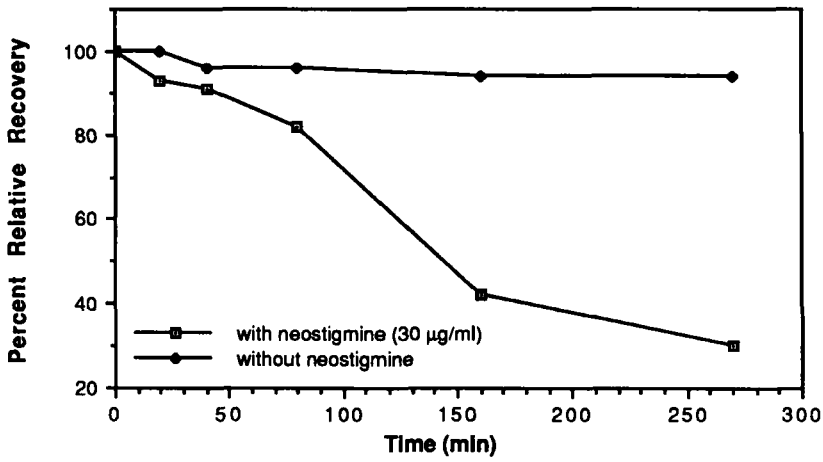

Figure 4. Effect of neostigmine on recovery of physostigmine in plasma.

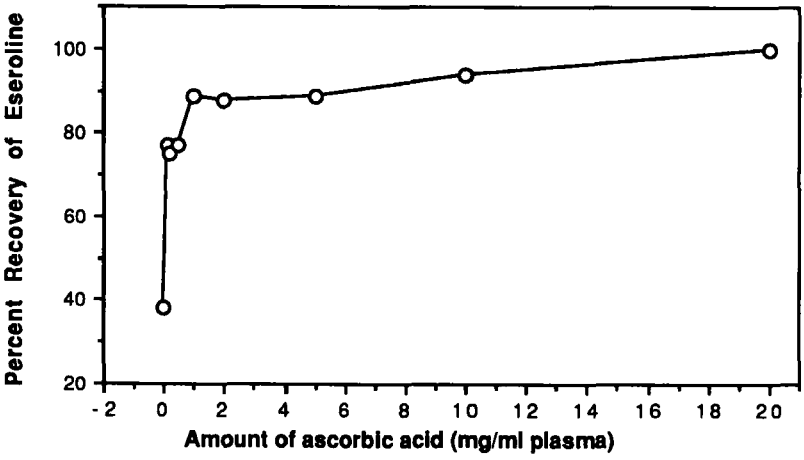

Figure 5. Effect of ascorbic acid on recovery of eseroline under extraction conditions.

Table 2. Effect of centrifugation (10 min), temperature, and presence of antioxidant when 5 ml of human plasma is spiked with 50 ng of physostigmine.

Temperature	Antioxidant	Peak Height Ratio for Physostigmine
Room Temp.	none	1.213
Room Temp.	ascorbic acid	1.435
Room Temp. neostigmine	ascorbic acid	1.739
0°C	none	1.455

eseroline initially present in plasma was recovered, while with the addition of 4 mg or more of ascorbic acid to 0.5 ml of plasma, more than 95% of the drug initially present in the plasma was stabilized. Plasma containing neostigmine (30 µg/ml) and ascorbic acid (14 mg/ml) adjusted to below pH 3 with H_3PO_4 showed no loss of physostigmine or eseroline after 4 months at -20°C.

Application of the Method

This method has been applied to animal studies for the determination of eseroline and physostigmine concentrations in monkey, rat and dog plasma. The results obtained for chromatographic separation, recovery and sensitivity correlated well with those obtained for human plasma. Chromatograms of monkey, rat and dog plasma extracts (Figure 3) show that physostigmine, eseroline and the internal standard peaks were separated from other endogenous peaks. An example of the mean time-course of physostigmine and eseroline plasma levels after intravenous bolus administration of 100 µg/kg of physostigmine to 5 rats is given in Figure 6. Figures 7 and 8 show the time-course of physostigmine and eseroline concentrations in monkey and dog plasma after IV and oral administration of 100 µg/kg. The present method is simple, has high degrees of accuracy and sensitivity and should be useful for basic and clinical pharmacological investigations.

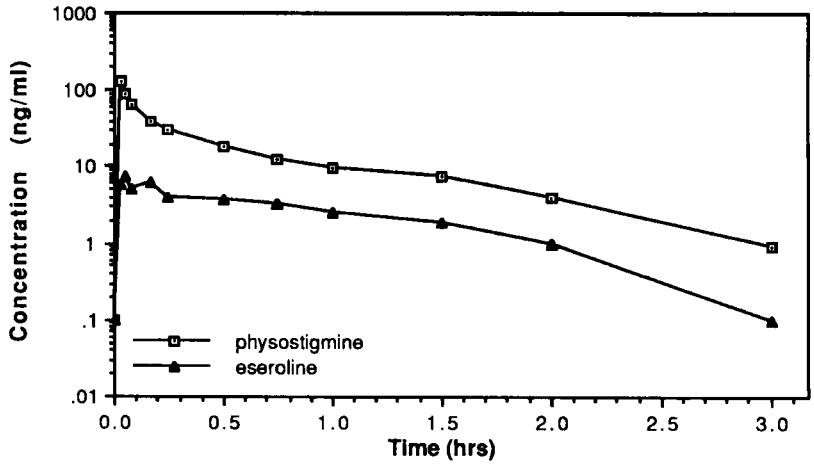

Figure 6. Mean physostigmine and eseroline concentrations in rat plasma after IV administration of physostigmine (100 µg/kg).

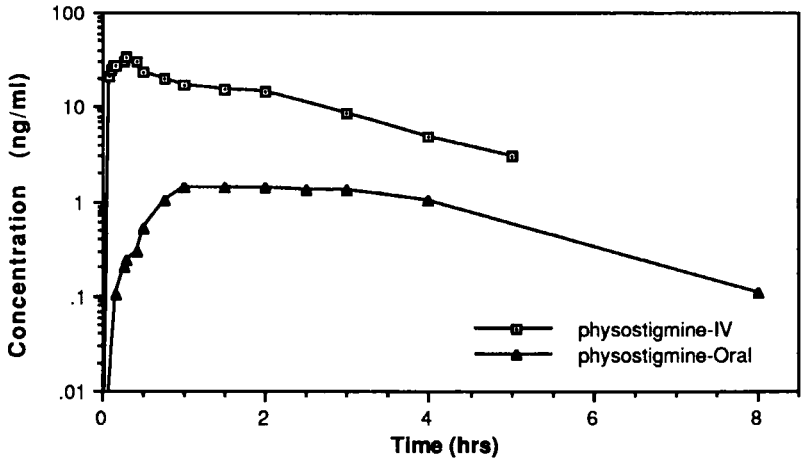

Figure 7a Monkey plasma concentrations of physostigmine after IV and oral administration of physostigmine (100 µg/kg).

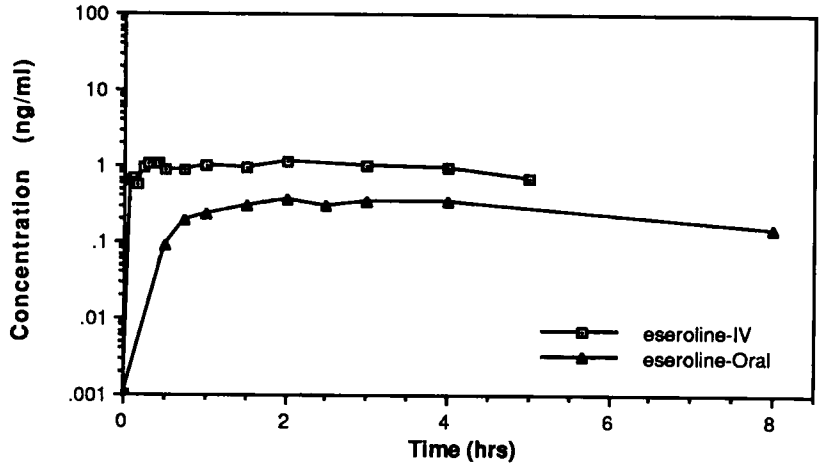

Figure 7b Monkey plasma concentrations of eseroline after IV and oral administration of physostigmine (100 µg/kg).

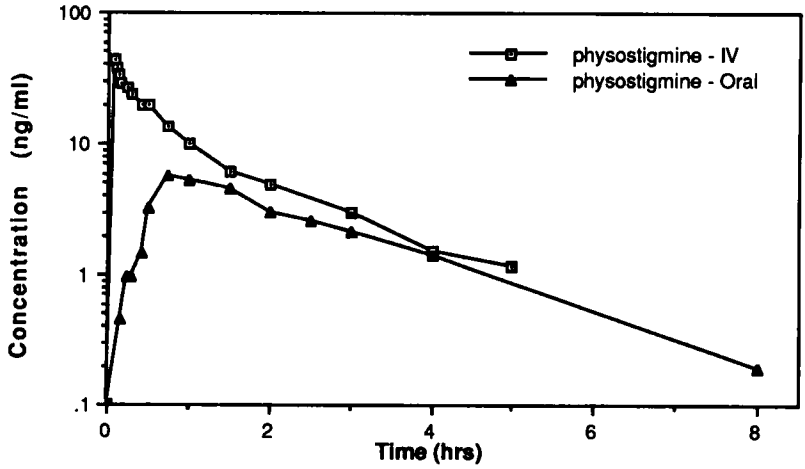

Figure 8a Dog plasma concentrations of physostigmine after IV and oral administration of physostigmine (100 µg/kg).

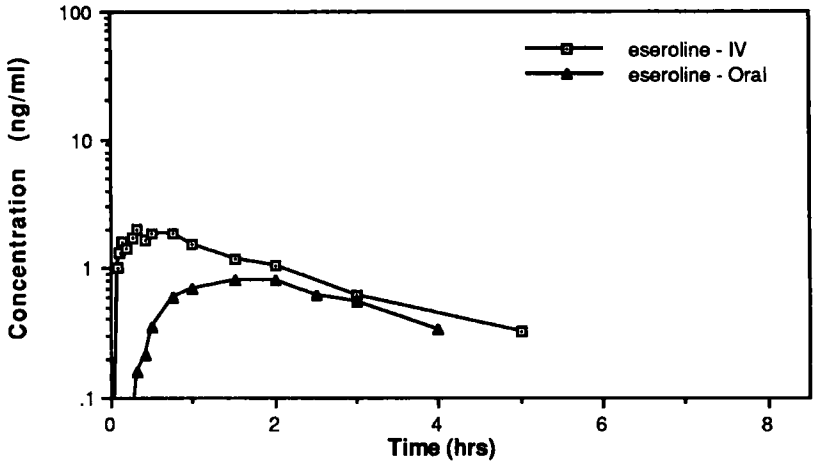

Figure 8b Dog plasma concentrations of eseroline after IV and oral administration of physostigmine (100 µg/kg).

CONCLUSION

This assay optimizes chromatographic resolution of both the parent drug and metabolite and combines sensitive quantitation of these compounds with a short run time. It can be adapted directly for the determination of physostigmine and eseroline in biological fluids at concentrations likely to be encountered in pharmacokinetic studies.

ACKNOWLEDGEMENTS

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