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Sensitive analysis of plasma physostigmine levels using dual-cell electrochemistry in the redox mode

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SUMMARY

A column liquid chromatographic method using dual-electrode, redox electrochemical detection has been developed for measuring plasma and cerebrospinal fluid physostigmine levels. The method is suitable for detecting drug levels in a geriatric population following oral ingestion of sustained-release physostigmine preparations and for determining the pharmacokinetics of these preparations in biological fluids.

INTRODUCTION

Physostigmine, a carbamate anticholinesterase and a tertiary amine, is rapidly absorbed after oral or subcutaneous administration. It readily penetrates the central nervous system and may be used to treat poisoning by anticholinesterase compounds such as atropine or tricyclic antidepressants. One of its other clinical uses is for the treatment of glaucoma. Recently, it has been used for the experimental treatment of memory loss in Alzheimer's disease [1]. Pharmacokinetic studies have indicated that physostigmine has a rather short half-life [2,3]. Recently, longer-acting controlled-release preparations have been formulated [4].

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Sensitive measures of physostigmine in human plasma have been achieved by high-performance liquid chromatography (HPLC) using a dual-electrode electrochemical detector in the differential mode utilizing the following three outputs: channel 1, oxidation at +0.7 V; channel 2, reduction at −0.2 V; channel 3, the sum of the signals from channels 1 and 2 [2]. Most recently sensitive detection of physostigmine has been reported using HPLC combined with fluorimetric detection [5].

This paper describes our method (using a modification of Whelpton and Moore's detection technique [2]) combining HPLC with electrochemical detection (ED) using dual electrodes in the redox instead of differential mode. Our technique combines reduction (−0.3 V) in the first electrode followed by oxidation (+0.42 V) in the second one. The redox mode provides for enhanced detector selectivity since only those compounds that will oxidize between −0.3 and +0.42 V will be detected at detector 2. This methodology was developed to enable us to carry out pharmacokinetic studies on reasonably small plasma sample volumes and to monitor plasma physostigmine levels in a geriatric population during clinical trials. Using 1.0–4.0 ml sample volumes, a limit of quantitation of 75 pg/ml was achieved. A simplified modification of this technique can also be used to detect physostigmine in cerebrospinal fluid (CSF).

EXPERIMENTAL

Materials and apparatus

All reagents were of analytic grade except for methanol and acetonitrile which were of HPLC grade (Fisher, Pittsburgh, PA, U.S.A.). Water used in preparation of solutions or in the HPLC mobile phase was of Milli-Q quality (Millipore, Bedford, MA, U.S.A.). Borate buffer, 0.5 *M* was prepared by dissolving 10 g sodium hydroxide with 15.5 g boric acid (Sigma, St. Louis, MO, U.S.A.) in water. The pH was adjusted to 10.5 with concentrated hydrochloric acid (Fisher) and the solution brought to a final volume of 500 ml with water. Stock solutions of physostigmine and neostigmine (Sigma) were prepared at 1.0 mg/ml in methanol and water, respectively.

Preparation of plasma and CSF calibrators

Physostigmine plasma standards were prepared from discarded blood bank plasma, tested for the presence of interfering peaks prior to its use. Standard samples were prepared in multiple sets containing six standards by first adding 55 μg neostigmine to each of six 27-ml aliquots of plasma. Fig. 1 demonstrates the structures of physostigmine and neostigmine, a second cholinesterase inhibitor used in our biological sample preparations to prevent enzymatic degradation of the physostigmine in plasma. To each of the six 27-ml standards, a different concentration of physostigmine bromide was added. Each 27-ml sample was thoroughly mixed and divided into five 5.0-ml aliquots resulting in

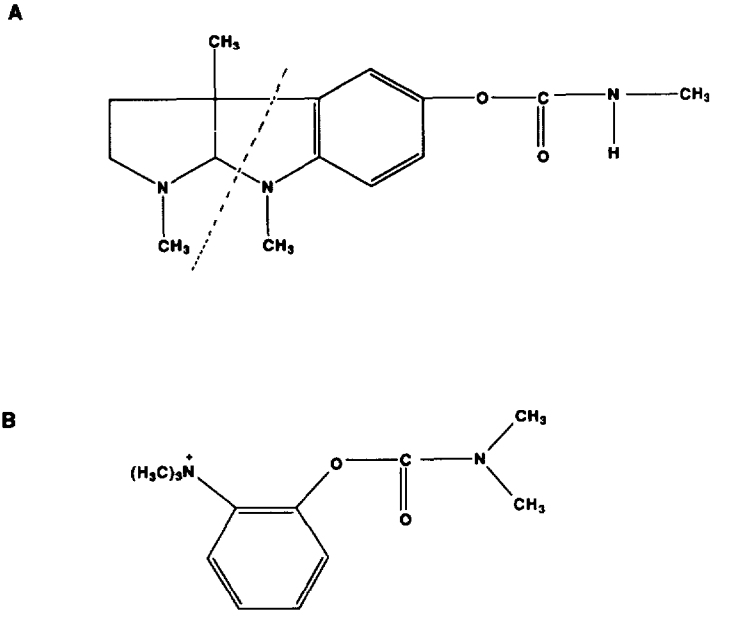

Fig. 1. Structures of physostigmine (A) and neostigmine (B), the cholinesterase inhibitors used in our biological sample preparations.

five sets of six tubes of serum calibrators containing 0, 0.5, 2.5, 5.0, 7.5 and 10.0 ng per 5.0-ml sample.

CSF standards were prepared using artificial CSF in place of pretested plasma and adjusting concentrations and volumes of neostigmine and physostigmine such that the final CSF aliquots of 0.6 ml each contained the same range of physostigmine concentrations per sample as described above for plasma standards. All preparations were stored at −70°C until used and were stable for at least two months.

Extraction of physostigmine from plasma and CSF

EDTA (1.5–2.0 mg/ml of blood) and neostigmine (5 μg/ml of blood) were added to polypropylene tubes prior to use. The addition of neostigmine was necessary to maintain the stability of physostigmine until assay. Blood was drawn from volunteer subjects prior to and following ingestion of 9, 12 or 15 mg controlled-release physostigmine. The total number of samples per patient included three or four baseline samples in addition to eight to eleven samples obtained at indicated intervals over the 12 h following drug (see summary curves in Fig. 5). Immediately following blood collection, the contents of the tubes were thoroughly mixed, centrifuged at 4°C, separated and stored at −70°C until quantitative determinations were performed. Just prior to assay, plasma samples were thawed and centrifuged at 900 *g* at 4°C for 10 min. Plasma

was transferred to polypropylene tubes containing 50 μl of 0.01 *M* hydrochloric acid and brought to 5.0 ml with blood bank plasma which we had previously tested for the absence of interfering peaks. Samples were deproteinized with 170 μl of 70% perchloric acid and centrifuged at 5000 *g* for 10 min. A 3-ml volume of clear supernatant was transferred to acid-cleaned glass tubes, to which 1.0 ml of 0.5 *M* borate buffer, pH 10.5, was added. The pH was then adjusted to between 9.5 and 10 with 10 *M* sodium hydroxide solution. Diethyl ether (5.0 ml) was added and the tubes were capped and shaken mechanically for 30 min. After centrifugation at 1000 *g* at 4°C for 10 min, 4.0 ml of the organic layer were transferred to disposable sodium borate tubes. The contents were evaporated to dryness under nitrogen and the tubes were capped and stored at 0°C in the dark until assayed.

CSF physostigmine was extracted in the same manner, but it was unnecessary to deproteinize these samples because of their extremely low content of protein. Therefore, the extraction procedure began with the transfer of 600–800 μl of CSF to acid-cleaned tubes followed by the addition of 0.5 *M* borate buffer, pH 10.5, and 10 *M* sodium hydroxide solution to adjust the pH as described above. Volumes were adjusted to accommodate the smaller available volumes of CSF.

Chromatographic conditions

Chromatography was performed using a Bio-Rad Model 1330 HPLC pump (Richmond, CA, U.S.A.) with a 100 mm × 4.5 mm I.D. Spherisphorb 5-μm silicon column, (Phase Separations, Norwalk, CT, U.S.A.). The eluent was methanol–acetonitrile–0.1 *M* ammonium nitrate pH 8.9–water (450:450:80:20, v/v), pumped at a flow-rate of 0.9 ml/min. Prior to chromatography, extracted samples were resuspended in 150–250 μl methanol and injected via a Rheodyne valve fitted with a 50-μl sample loop. Detection of peaks was by an ESA Coulochem Model 5100A dual-electrode electrochemical detector (Bedford, MA, U.S.A.) using the redox mode with a potential of +0.8 V on the conditioning cell followed by a reductive analytical first cell with a potential of −0.3 V and an oxidative potential of +0.42 V in the second cell. Readings were made on the second electrode of the analytical cell with the signals being registered on a Hewlett-Packard Model 3392A integrator (San Jose, CA, U.S.A.). Measurement of peak heights (in mm) of detected physostigmine was used for all subsequent calculations.

RESULTS AND DISCUSSION

Typical chromatograms after the injection of 50 pg of physostigmine revealed a peak with a retention time of 11.5 min; mobile phase without physostigmine demonstrated the absence of a peak (Fig. 2). Repeated injections ($n=10$) of the sample yielded a coefficient of variation (C.V.) of 3.4% with

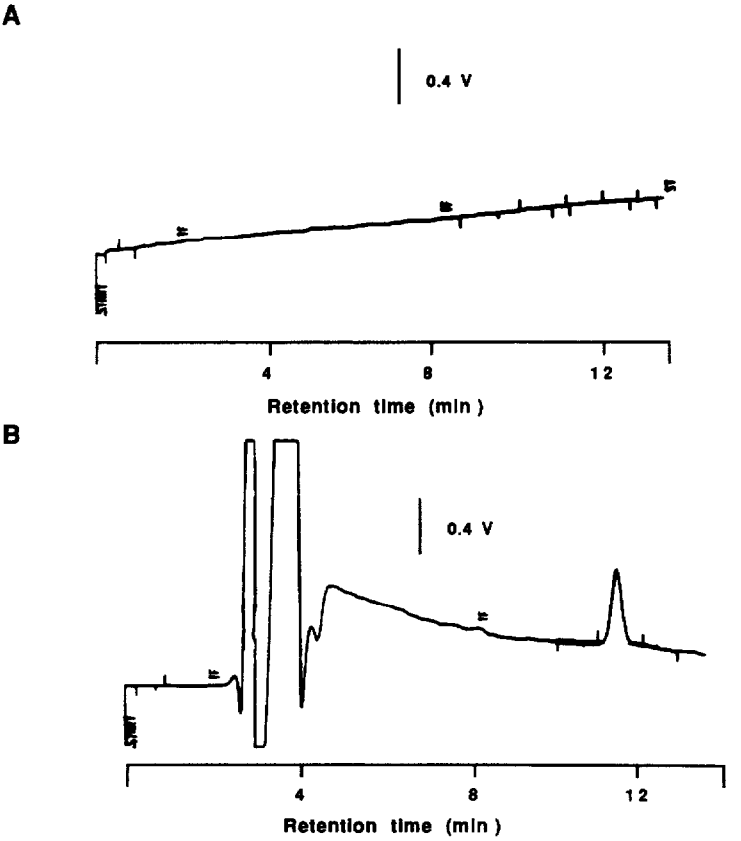

Fig. 2. (A) Chromatogram of a mobile phase blank (methanol–acetonitrile–0.1 *M* ammonium nitrate pH 8.9–water (450·450:80·20, v/v). (B) Chromatogram showing the elution of a physostigmine standard (50 pg) in methanol.

regard to peak height, demonstrating the reliability of the chromatographic system in use.

Although all participating subjects were drug-free, we tested for the possible interference of neostigmine, the second anticholinesterase used in our assay. Chromatographs of physostigmine obtained in the absence (Fig. 3A) and presence of 1×, 10× and 100× the concentration of neostigmine (Fig. 3B, 3C and 3D, respectively) show no detectable neostigmine peak.

To be certain that our incubation/extraction procedures were not altering physostigmine potentials and therefore affecting its chromatographic properties, we compared the shapes of current–voltage curves for non-extracted physostigmine and of physostigmine extracted from plasma in the presence of neostigmine, respectively (Fig. 4A and 4B). The similar curve shapes for the

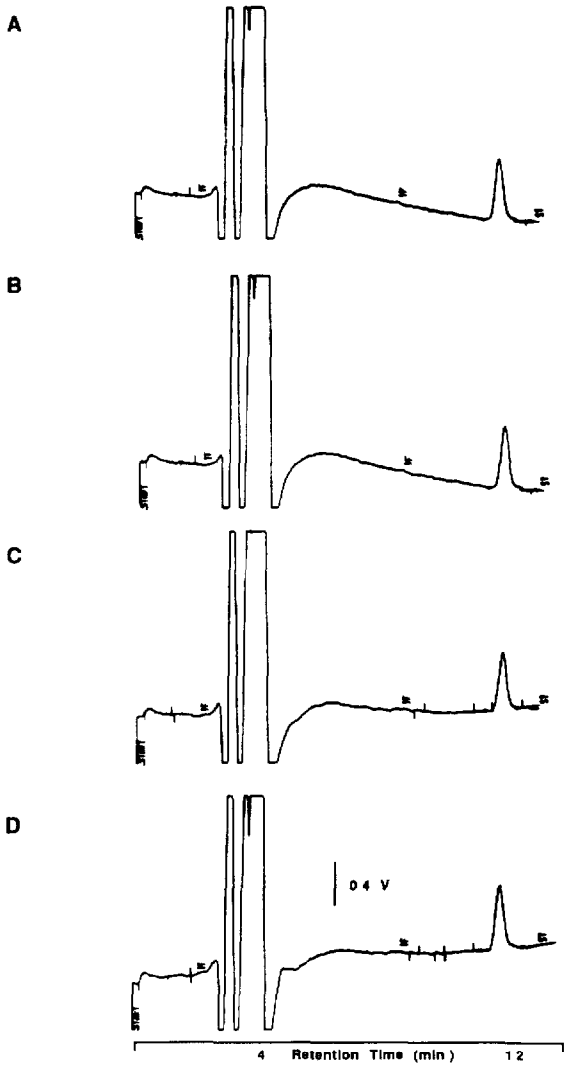

Fig. 3. Comparison of 50 pg injected physostigmine in the absence (A) and in the presence of 50 (B), 500 (C) and 5000 (D) pg of neostigmine.

two different sources of physostigmine standards assured the identity of our extracted substance.

Following extraction of physostigmine at low, medium and high concentrations, peak height was linearly related to the quantity of injected physostigmine (Table I; $r=0.99$). A typical standard curve of six singleton points yielded a similar coefficient of correlation ($r=0.9904$) and was included each time a

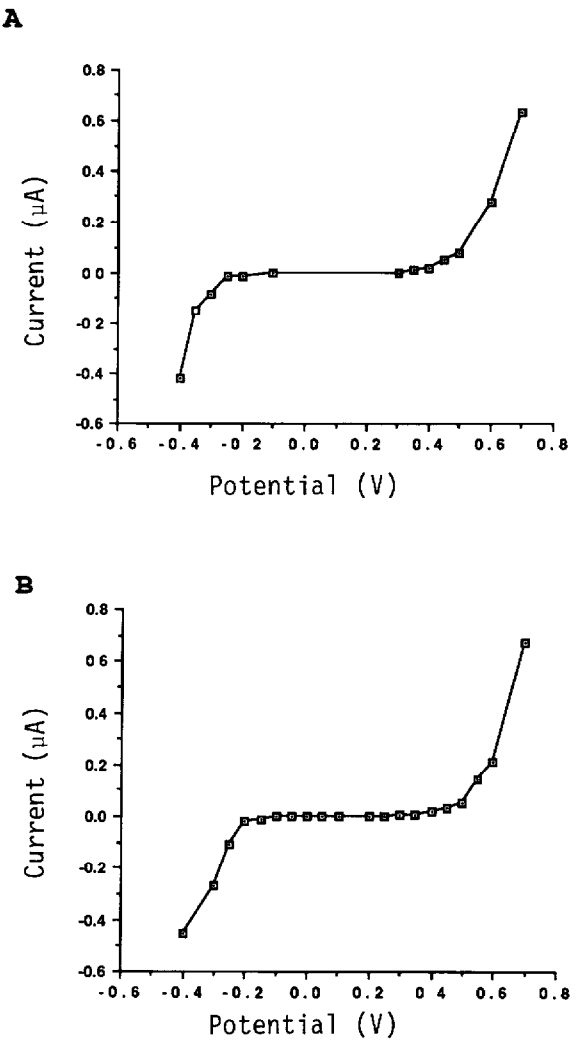

Fig. 4. Current voltammogram of two preparations of physostigmine. (A) 50 pg per 50 μl per data point of standard, non-extracted physostigmine in methanol; (B) 100 pg of extracted physostigmine, solubilized in 50 μl methanol per data point.

physostigmine time course was performed to insure accuracy of drug levels since recovery varied from 66 to 92%.

The coefficient of correlation varied as a function of pH (Table II). Although the data obtained from extractions performed at pH 7.0–7.5 through 9.5–10.0 are similar, the highest coefficient of correlation values were most consistently achieved at the higher end of the range (pH 9.5–10.0) although at extremely high pHs of 12.5–15 physostigmine was not recoverable. Physo-

TABLE I

VALIDATION OF PHYSOSTIGMINE CHROMATOGRAPHIC METHODS USING PLASMA SAMPLES

$r=0.9993$.

Physostigmine (pg/ml)	n	Mean peak height (mm)	C V. (%)	S.D. (mm)
0	2	0	–	–
75	4	3.6	0.132	0.48
200	4	12.6	0.049	0.63
500	4	24.3	0.062	1.50
2000	4	92.0	0.035	0.82

TABLE II

EXTRACTION pH VERSUS COEFFICIENT OF CORRELATION

pH	n	r^a
3.0–4.0	1	0.3996
5.0–6.0	1	0.7827
7.0–7.5	2	0.9845
8.0–8.5	2	0.9741
9.5–10.0	3	0.9996
11.5–12.0	1	0.9603
12.5–13.0	1	N.D.[b]

[a]Six-point standard curves.
[b]Not detectable.

stigmine is most stable at acidic pH; its extraction from aqueous phase into diethyl ether did not occur uniformly below pH 7.0–7.5

A commonly encountered biological fluid in addition to plasma is CSF. Multiple extractions of artificial CSF spiked with physostigmine concentrations identical to those used for plasma again revealed that peak height was linearly related to the quantity of injected physostigmine (Table III; $r=0.99$). Better recovery was achieved with CSF (83–92%) which may be due to the absence of significant amounts of protein in CSF in contrast to plasma.

We examined the pharmacokinetics obtained from the repeated ingestions of 2.0 mg physostigmine by one individual in the fasting state (Table IV). In this single subject, peak concentration (T_{max}) always occurred at 1 h. Although the maximal concentration (C_{max}) in plasma varied by 2–3 fold, the area under the curve (AUC) varied by only 1.7-fold suggesting relatively uniform absorption of the preparation within a single individual. In contrast, the pharmacokinetics obtained from five different individuals, each of whom ingested 2.0 mg

TABLE III

VALIDATION OF PHYSOSTIGMINE CHROMATOGRAPHIC METHODS USING CSF SAMPLES

$r = 0.9998$.

Physostigmine (ng/ml)	n	Mean peak height (mm)	C.V. (%)	S.D. (mm)
0.00	3	0.00	–	–
0.83	3	5.50	0.73	2.18
4.17	3	25.00	3.18	9.54
8.33	3	50.00	6.64	19.92
12.50	3	76.67	10.01	30.02
16.67	3	104.00	13.32	39.95

TABLE IV

PHARMACOKINETICS OF REPLICATE PHYSOSTIGMINE DOSES (2.0 mg) IN THE SAME SUBJECT

Dose	AUC[a] (cm^2)	C_{max}[b] (ng/ml)	T_{max}[c] (h)	$t_{1/2}$[d] (h)
1	2.39	1.50	1.00	0.50
2	1.35	1.16	1.00	0.50
3	1.34	0.64	1.00	0 50
Mean ± S.D.	1.69 ± 0.60	1.10 ± 0.43	1.00 ± 0.00	0.50 ± 0.00

[a]AUC = area under the curve.
[b]C_{max} = maximum concentration obtained following ingestion.
[c]T_{max} = the time at which the peak plasma level was reached.
[d]Calculated as time from maximal to half maximal peak height.

physostigmine in the fasting state, showed that the AUC varied by 4.8-fold despite a similar T_{max} and half-life ($t_{1/2}$). This demonstrates more variable absorption when multiple persons ingested the same dose compared to repeated ingestions of a fixed dose by one person (Table V).

Mean plasma physostigmine curves from volunteers (five per group) receiving 9, 12 or 15 mg of controlled-release physostigmine demonstrate peak plasma levels of approximately 0.51, 0.70 and 1.04 ng/ml occurring 3.0 h following drug ingestion (Fig. 5). Plasma half-life for the various doses was 1.16–1.55 h (Table VI). Most importantly, plasma levels above 0.25 ng/ml were detectable for up to 7 h following ingestion (Fig. 5) suggesting that twice a day dosing will be sufficient to produce long-lasting daytime inhibition of plasma cholinesterase. The very close estimates of dose proportionality suggests that absorption is uniform across the three different dose preparations.

TABLE V

PHARMACOKINETICS OF REPLICATE PHYSOSTIGMINE DOSES (2.0 mg) IN DIFFERENT SUBJECTS

Subject	AUC[a] (cm^2)	C_{max}[b] (ng/ml)	T_{max}[c] (h)	$t_{1/2}$[d] (h)
1	1.30	0.85	1.00	0.50[e]
2	3.19	0.60	1.00	0 75
3	0.66	0.30	1.00	0 50
4	1.27	0.22	0.50	1.25
5	1.36	0.60	1.00	0 25
Mean ± S.D.	1.56 ± 0 96	0.51 ± 0.25	0.90 ± 0.22	0.65 ± 0.38

[a]AUC = area under the curve.
[b]C_{max} = maximum concentration obtained following ingestion.
[c]T_{max} = the time at which the peak plasma level was reached.
[d]Calculated as time from maximal to half maximal peak height.
[e]Calculated on basis of major peak.

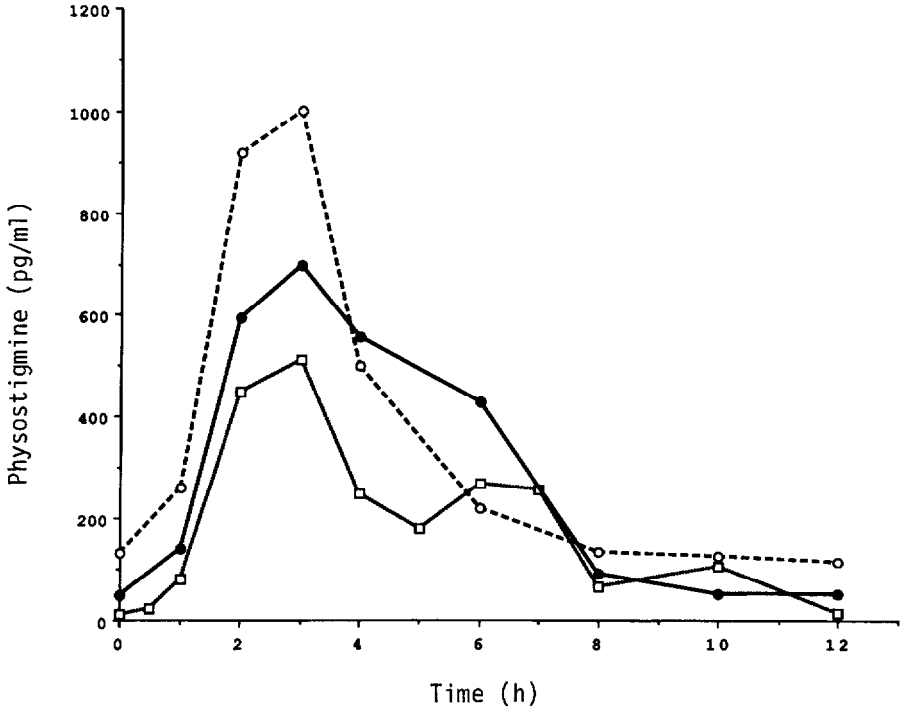

Fig. 5. Plasma physostigmine profiles following ingestion of three doses of slow-release physostigmine: (□) 9 mg; (●) 12 mg; (○) 15 mg. Pharmacokinetic indices of these data are presented in Table VI.

TABLE VI

PHARMACOKINETIC INDICES FOR CONTROLLED-RELEASE PHYSOSTIGMINE IN PLASMA

Dosage (mg/kg)	AUC[a] (cm^2)	C_{max}[b] (ng/ml)	T_{max}[c] (h)	$t_{1/2}$[d] (h)	Dose proportionality (dose/AUC)
9	5.625	0.51 ± 0.62	3.00 ± 0.63	1.36 ± 1.05[e]	1.60
12	8.000	0.70 ± 0.387	3.00 ± 1.37	1.55 ± 0.78	1.50
15	10.250	1.04 ± 0.54	3.00 ± 0.63	1.16 ± 0.37	1 46

[a]AUC = area under the curve.
[b]C_{max} = maximum concentration obtained following ingestion.
[c]T_{max} = the time at which the peak plasma level was reached.
[d]Calculated as time from maximal to half maximal peak height.
[e]Calculated on basis of major peak.

CONCLUSIONS

HPLC–ED of physostigmine using two electrodes in the redox mode as we have described provides sensitive and reliable measures of drug levels in biological fluids. Our technique is suitable for determining plasma physostigmine levels likely to be encountered in a geriatric population after oral drug ingestion. Additionally, the method can be used to detect CSF physostigmine levels.

This assay will be useful in clinical drug trials to determine if there is a systematic relationship between absorption of physostigmine and entry into the central nervous system as reflected by its appearance in CSF and improvement in cognition.

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