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Short Communication

Determination of heptylphysostigmine in plasma by high-performance liquid chromatography with electrochemical detection

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ABSTRACT

Heptylphysostigmine is a new and very promising cholinergic drug for the treatment of Alzheimer disease. A method has been developed for its determination in plasma with a detection limit of 50 pg/ml. The drug was extracted in *n*-hexane by a simple one-step procedure, after buffering with sodium bicarbonate. Samples were analysed on a 25 cm × 4.6 mm I.D. silica column (5 μm particle size) using a mixture of acetonitrile, methanol and ammonium nitrate as mobile phase. Since this molecule is quite unstable in plasma, pyridostigmine bromide was added to samples to limit the decomposition. Physostigmine was employed as internal standard. The molecule was electrochemically detected by oxidizing potential (+0.75 V). The method was applied to the analysis of blood samples taken from one healthy volunteer administered this drug. In the same subject the inhibition rate of acetylcholinesterase in plasma and red cells was also measured.

INTRODUCTION

Patients affected by Alzheimer disease are usually treated with drugs that inhibit the enzyme acetylcholinesterase (ChE). This restores the levels of acetylchloline, which are typically reduced in this disease. To achieve this goal, drugs such as tetrahydroaminoacridine and physostigmine have been used [1,2]. However, both those molecules have considerable side-effects and a short duration of action. Recently the heptyl derivative of physostigmine, heptylphysostigmine (HPS) [3,4], was developed. It was claimed that this drug, in comparison with any previous agent inhibiting ChE [5,6], has a greater duration of action, a greater margin of safety and other advantages.

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Because of the narrow therapeutic range and the clinical and kinetics studies to be performed, a method of measuring this drug in body fluids is needed. A high-performance liquid chromatographic assay with electrochemical detection for measurement of HPS in plasma was recently reported [7]. However, this method requires a time-consuming double-step extraction, and in our hands is not sufficiently sensitive for samples from kinetic or clinical studies.

In this paper we report a simple, rapid and sensitive method for the analysis of HPS in plasma, suitable for clinical studies.

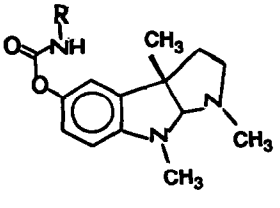
EXPERIMENTAL

Materials

Heptastigmine tartrate and physostigmine (PS) (Fig. 1) were kindly supplied by Mediolanum Farmaceutici (Milan, Italy). Pyridostigmine bromide was purchased from Sigma (St. Louis, MO, USA). All chemicals and reagents used were of analytical grade (Carlo Erba, Milan, Italy). Stock solution of HPS and physostigmine (0.5 mg/ml) in methanol were prepared weekly, stored at −40°C and protected from light. Dilutions up to 0.05 μg/ml were prepared daily in 0.1 *M* hydrochloric acid.

Equipment and chromatographic conditions

A Series 10 Perkin Elmer (Norwalk, CT, USA) liquid chromatograph and an LC-4B/17 AT electrochemical detector (Bioanalytical System, West Lafayette, IN, USA) with a glassy carbon electrode were used. A Violet (Milan, Italy) 5 μm particle size silica column (25 cm × 4.6 mm I.D.) was operated at room temperature. A potential of +0.75 V was applied *versus* the reference electrode. The mobile phase contained acetonitrile–methanol–ammonium nitrate buffer (50:40:10, v/v/v) and was pumped at 1.0 ml/min. The buffer was 0.08 *M* ammonium nitrate adjusted with 1.5 *M* ammonium hydroxide to pH 8.90. A Shimadzu CR4A system was employed as recorder and integrator of peaks.



R = - CH_3 Physostigmine
R = - C_7H_{15} Heptylphysostigmine

Fig. 1. Structure of heptylphysostigmine and its internal standard, physostigmine.

Blood samples

Venous blood samples (10 ml) were collected in heparinized Vacutainer tubes containing 25 μl of pyridostigmine bromide as aqueous solutions (20 mg/ml). Blood was immediately centrifuged at 4°C for 10 min. Then plasma was transferred into polypropylene tubes containing 100 mg of ascorbic acid and stored at −40°C until assay. One healthy volunteer received a 30-mg oral dose of HPS and subsequently underwent venepuncture at 0.5, 1, 1.5, 2, 3, 4, 6 and 24 h.

Plasma for acetylcholinesterase activity measurement was separated by collecting blood (5 ml) into tubes containing EDTA and analysed within seven days after sampling. Red cells for acetylcholinesterase activity determination were obtained by collecting blood (5 ml) in tubes containing citric acid–sodium citrate–glucose, which were stored at 4°C until analysis (within three days).

Extraction procedure

To 1 ml of plasma contained in a stoppered glass tube 40 μl of physostigmine solution (internal standard, 0.05 μg/ml) were added. After shaking, 5 ml of *n*-hexane were added, followed by 1 ml of 0.5 *M* sodium bicarbonate (pH 9.0). Tubes were shaken for 10 min and centrifuged at 1500 *g* for 5 min. A 4-ml volume of organic phase was transferred into a clean tube and evaporated under nitrogen flow at 30°C.

The residue was immediately reconstituted in 100 μl of a mixture of methanol and acetonitrile (50:50, v/v), then 70 μl were injected into the chromatograph. The tubes before reconstitution were stored in a freezer at −40°C until injection. Plasma calibration standards were prepared with

0.2, 0.5, 1.0, 2.0 ng/ml HPS and 2.0 ng/ml internal standard. Repeated analyses were carried out to evaluate the recovery, accuracy, precision and linearity of the method. Data were gathered to construct curves of current intensity against the applied potential, by injecting, at different potential values, the drug and internal standard.

Acetylcholinesterase activity measurement

The determinations were carried out on plasma and red cells by using a previously described potentiometric procedure [8] with minor modifications [9]. This method needs 20 μl of plasma for the determination of acetylcholinesterase activity and 10 μl of whole blood for the assay of red cell acetylcholinesterase. Each analysis is completed within 2 min.

The within-run imprecision (measured in terms of C.V.) is 4.0% relative to plasma acetylcholinesterase and 1.8% for red cell acetylcholinesterase.

RESULTS AND DISCUSSION

As shown in Fig. 2A the chromatogram of a plasma blank processed as described does not show evidence of any interfering peak at the elution times of HPS and internal standard.

Several drugs were tested for interference, particularly those used most often for the treatment of Alzheimer patients: diazepam, lorazepam, flunitrazepam, chlorpromazine and amitriptyline. None of these drugs showed any interference in our method. The use of a low-polarity solvent like *n*-hexane and mild buffering with sodium bicarbonate made the extraction very specific and provided good chromatograms. Extraction was also rapid, and thus our extraction procedure offers several advantages over that reported previously [7]. In fact, plasma samples as small as 1 ml could be analysed with our method and one single-step extraction was employed.

A particular problem in this work was the stability of HPS. Stock solutions of HPS and PS at 0.5 mg/ml in methanol were found to be stable at −40°C over sixteen days as shown in Fig. 3. In blood and plasma, because of the presence of esterase enzymes, the drug decomposed immediately. In practice, when 5–10 ng were added to plasma (1 ml) no more compound could be extracted and detected. In previous studies [7] plasma samples were shown to be stable for one year at −70°C, while an extensive decomposition (48% in 30 s) occurred at 22°C. Pyridostigmine bromide was added to blood and plasma samples to reduce this type of decomposition, as this drug is a potent esterase inhibitor. Furthermore, ascorbic acid was also added to protect against oxidation. After this treatment the final recovery was increased up to 60%.

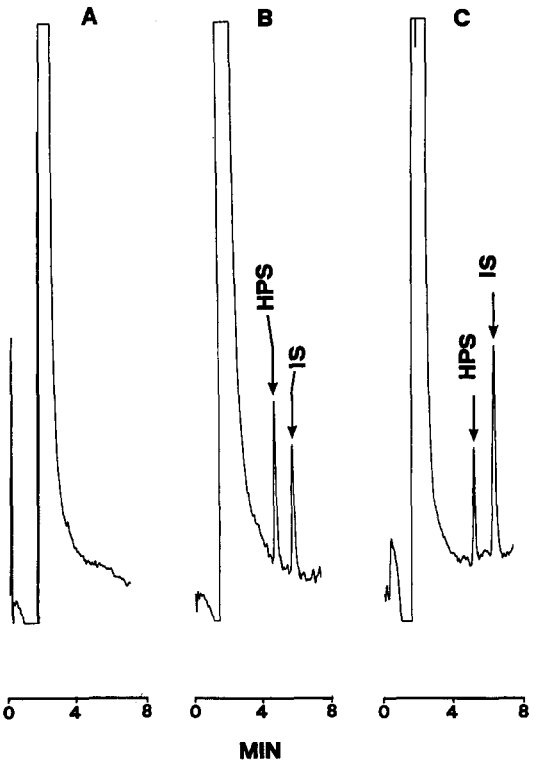


Fig. 2. Chromatograms of plasma extracts obtained with the procedure described in the text. (A) Blank; (B) standard spiked with 1.0 ng/ml heptylphysostigmine (HPS) and 2.0 ng/ml physostigmine, internal standard (IS); (C) sample obtained from one subject 1 h after receiving a 30-mg oral dose of HPS and spiked with 2.0 ng/ml internal standard.

Several homologous molecules were tested as internal standards, for example butyl, cyclohexyl, hexyl and nonyl derivatives, however, the best separation was obtained with PS.

Owing to the instability of HPS, it was in fact

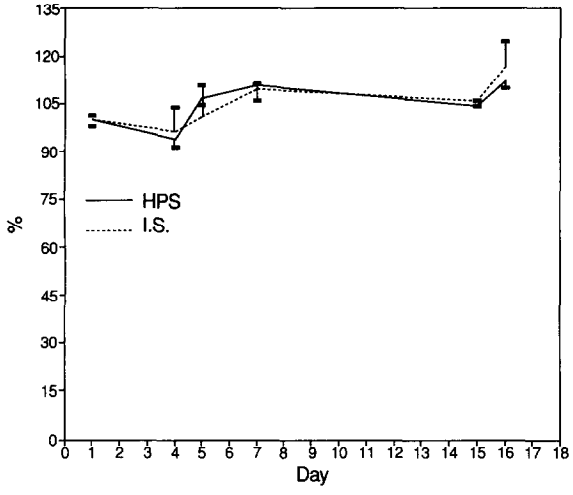


Fig. 3. Curves of stability of HPS and PS (I.S.) at 0.5 ng/ml in methanol. Values are expressed as percentage of the concentration at the first day. Solutions were stored at −40°C protected from light in glass tubes.

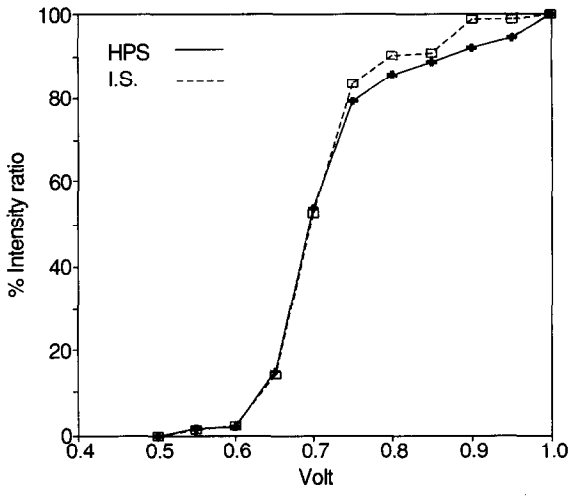


Fig. 4. Graph representing the current intensity of HPS and PS (I.S.) *versus* the potential applied to the glassy carbon electrode. The response values reported were calculated as percentage of peak areas relative to the maximum value obtained with the highest voltage.

necessary to use as internal standard a molecule with the same structure but differing in the side-chain. Because of the high sensitivity needed for this analysis the operating potential of the detector was only chosen after constructing the voltammogram reported in Fig. 4. A C_{18} column with an acetonitrile–acetate buffer was also tested and provided a good separation, but the sensitivity was insufficient for pharmacokinetic work because of the high noise generated by this mobile phase at the lower sensitivity range of the detector. The limit of detection of the present method was 0.05 ng per ml of plasma with a signal-to-noise ratio of 2. In a previous work [7] the limit of detection was 0.125 ng/ml for plasma. In Table I values for the intra-day and inter-day imprecision are given. The method was linear in the range of interest for clinical studies (0.2–2.0 ng/ml of plasma). The current (y) *versus* concentration (x) regression equation is: $y = 0.442x + 0.131$ ($r^2 = 0.9977$).

The recovery was measured in plasma samples

TABLE I

ACCURACY AND PRECISION OF HPS DETERMINATIONS IN PLASMA

Intra-day ($n = 7$)				Inter-day ($n = 5$)			
Amount added (ng/ml)	Amount found (ng/ml)	S.D. ($n-1$)	C.V. (%)	Amount added (ng/ml)	Amount found (ng/ml)	S.D. ($n-1$)	C.V. (%)
0.2	0.22	0.02	8.9	0.2[a]	0.23	0.03	10.9
0.5	0.58	0.03	4.5	0.5[a]	0.50	0.04	6.9
1.0	1.15	0.05	4.3	1.0	0.99	0.08	7.6
2.0[a]	2.34	0.05	2.1	2.0[a]	1.81	0.10	5.4

[a] $n = 4$.

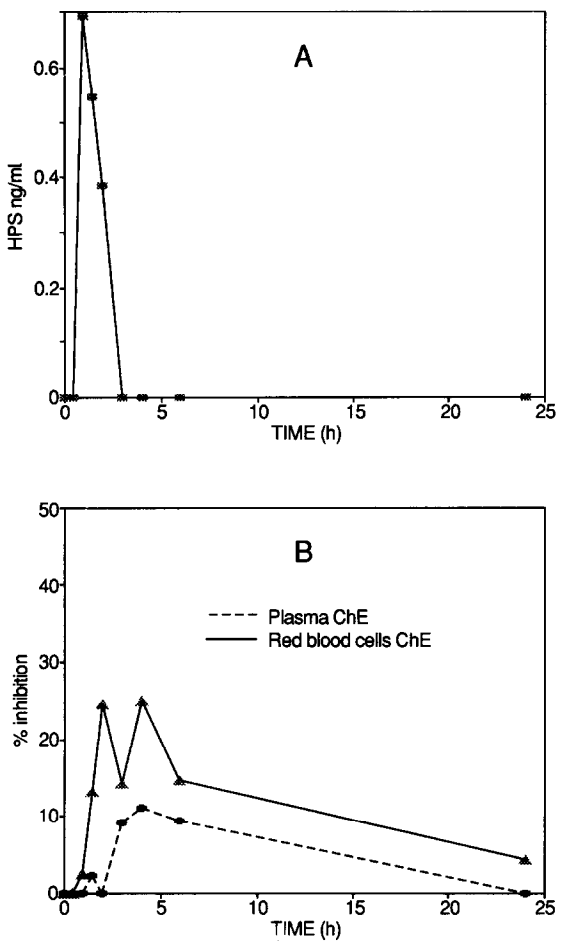


Fig. 5. (A) Plasma concentrations of HPS in one healthy volunteer after one single oral dose of the drug (30 mg). (B) Curves of relative cholinesterase inhibition in plasma and red cells of the same subject.

spiked with 1.0 ng/ml (five repetitions) and 2.0 ng/ml (five repetitions) HPS and was found to be 57.1 ± 12.8 and 54.5 ± 11.2%, respectively.

In Fig. 5A the drug profile in plasma of one healthy volunteer administered one single dose (30 mg) of HPS is reported. An early concentration peak at 1 h is followed by a rapid decay. In Fig. 5B the time course of cholinesterase activity in plasma and red blood cells, expressed as inhibition rate of the basal value measured just before drug dosing, are reported.

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