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

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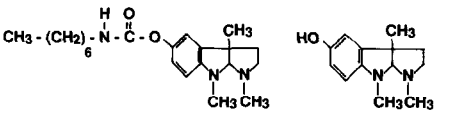
ABSTRACT

An analytical method was developed with sensitivity to detect clinically significant concentrations of heptylphysostigmine (HP), a new physostigmine derivative with potent and long-lasting inhibitory activity on cholinesterase. HP, an experimental drug for Alzheimer disease, was measured in human plasma by high-performance liquid chromatography with electrochemical detection with use of a normal-phase column and acetonitrile buffer containing tetrahydrofuran and sodium acetate, pH 4.6. The limit of detection of the method was 0.125 ng/ml using a 2-ml sample of plasma. Analytical recovery of HP was 53.04 ± 7.75% for plasma in the range 0.25–2.5 ng/ml. Stability studies conducted at 37°C indicated that the drug was stable in 1 *M* hydrochloric acid, 1 *M* hydrogen peroxide and sodium acetate-buffered solution at pH 4 for at least 6 h but at pH 7 it degraded slightly to 79% at 6 h and was unstable in 1 *M* sodium hydroxide with only 9% of the parent compound remaining at 30 s. HP was stable when exposed to ultraviolet light at 22°C or 100% relative humidity at 37°C, with almost 80 and 75% of the parent compound remaining after 4 and 28 days, respectively. HP was stable in plasma at 4°C for 0.25 h, and it slowly degraded to 56 and 28% of the original concentration by 1 and 2 h, respectively. HP was highly unstable in plasma at higher temperatures; at 22 and 37°C it degraded immediately to 48 and 36% of the original concentration and was not detected after 0.5 and 0.25 h, respectively.

INTRODUCTION

Heptylphysostigmine (HP, MF 201, eptastigmine, heptastigmine) is a new physostigmine (Phy) derivative with potent and long-lasting inhibitory activity on cholinesterase (ChE, EC 3.1.1.8) [1–5]. The structures of HP and its metabolite, eseroline (Es), are given in Fig. 1. A drug that achieves adequate levels of ChE inhibition in the central nervous system (CNS) of humans with Alzheimer disease (AD) would be potentially useful for improving mental and social functioning. Preliminary studies have shown that HP unlike its parent compound Phy produces higher and long-lasting levels of ChE inhibition with minimal or no side-effects after oral administration in humans [6,7].

In order to better understand the pharmacokinetic and pharmacodynamic profile of drugs it is necessary to have sensitive analytical methods available to measure drug levels after dosing. There are no analytical methods reported for HP determination. The high-performance liquid



Heptylphysostigmine **Eseroline**

Fig. 1. Structures of heptylphysostigmine (HP) and eseroline (Es).

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chromatographic (HPLC) method using electrochemical detection (ED) we developed for Phy [8] was utilized with modifications to develop a sensitive method for HP determination in human plasma.

Here, we report for the first time, an HPLC–ED method to detect clinically significant concentrations of HP in plasma after drug administration in humans. The stability of the drug under various conditions was also studied. Also, the method was successfully used to measure plasma drug levels in healthy volunteers after oral dosing [7].

EXPERIMENTAL

Drugs and chemicals

Heptylphysostigmine tartrate was obtained from Mediolanum Farmaceutici (Milan, Italy). Eseroline fumarate was from Research Biochemical (Natick, MA, USA). Acetylcholine (ACh) chloride was from Sigma (St. Louis, MO, USA) and [^{3}H]ACh was from New England Nuclear (Boston, MA, USA). Acetic acid of HPLC grade was obtained form J. T. Baker (Phillipsburg, NJ, USA). Sodium acetate, acetonitrile, tetrahydrofuran (THF) and diethyl ether of HPLC grade and all other chemicals of reagent grade were from Fisher Scientific (St. Louis, MO, USA).

Equipment

The HPLC system consisted of a Beckman 110 pump (Beckman Instruments, Palo Alto, CA, USA), and two LC-4B amperometric controllers, a dual glassy carbon working electrode and an Ag/AgCl reference electrode (all from Bioanalytical Systems, West Lafayette, IN, USA). Samples were injected via a Rheodyne 7125 injector valve with a 100-μl loop and the chromatograms were plotted with a Model RYT recorder (all from Bioanalytical Systems).

Chromatographic conditions

Chromatography was carried out at room temperature using a 5-μm Spherisorb silica column (150 mm × 4.6 mm I.D.), a guard column packed with pellicular silica (both from Alltech Assoc., Deerfield, IL, USA) and a mobile phase consisting of 10 m*M* sodium acetate in a mixture of acetonitrile–glacial acetic acid–water–THF (92:2:5:1) (pH 4.6). The mobile phase was filtered under reduced pressure through a 0.45 μm pore size Nylon 66 filter (Rainin Instrument, Woburn, MA, USA) and sonicated to remove the air bubbles. The flow-rate was 1.5 ml/min. The dual glassy carbon electrode was used in series and operated at oxidation potentials +0.25 V (W1) and +0.95 V (W2), respectively. The detector sensitivity range was set at 2 nA full scale deflection.

Source of plasma

Human plasma (pH 7.0) from normal volunteers was obtained from the blood bank at St. Johns Hospital (Springfield, IL, USA).

Baseline control blood from AD patients (n = 4) [9] and blood from normal volunteers prior to and 1 h after HP dosing (40 mg, oral) [6,7] were collected in heparinized tubes and immediately placed on ice. Samples were immediately centrifuged (800 g, 10 min, 4°C) and placed on ice. Plasma and red blood cells were separated and stored at −70°C until analysis.

Prior to extraction plasma was thawed on ice and brought to 4°C.

Extraction procedure

Plasma and reagents were kept on ice prior to and during the first extraction step. In a screw-cap test tube containing 2 ml of human plasma, 3.5% ammonium hydroxide (500 μl) followed immediately by diethyl ether were added and the tube was vortex-mixed for about 10 s. Samples were then centrifuged at 800 g for 10 min at 4°C in an IEC Centra-7R centrifuge (from Fisher Scientific). After centrifugation, 4 ml of the ether (upper) layer was transferred to a 5-ml conical-bottom glass test tube and evaporated under a gentle stream of nitrogen at room temperature. The residue was re-extracted with 200 μl of water, centrifuged (800 g for 15 min at 4°C) and transferred to an Eppendorf tube; diethyl ether (800 μl) was added to the tube, and the mixture vortex-mixed for 20 s and centrifuged (11 000 g for 5 min at 4°C) in a microcentrifuge. The organic layer (about 700 μl) was pipetted into an Eppendorf tube and evaporated to dryness under a gentle

stream of nitrogen at room temperature. The residue was reconstituted with 120 μl of acetonitrile, vortex-mixed and centrifuged (11 000 *g* for 5 min at 4°C), and a 100-μl aliquot of the supernatant was injected into the chromatographic column.

Calibration curves

Standard HP solution in water. Aliquots of HP solution in water (50 ng/ml) corresponding to 0.25–5 ng were injected into the chromatographic column, and the peak heights for HP were measured.

Plasma spiked with HP solution. To 2-ml plasma samples, HP was added to obtain final concentrations ranging from 0.25 to 2.5 ng/ml. These were extracted as described above.

Other studies

Chromatographic separation of HP from its metabolite Es. A solution of HP and Es (10 ng/ml each) or Es alone (10 ng/ml) was prepared in sterile water. The retention times of HP and Es were recorded after injecting 100 μl of the solution into HPLC column.

Solubility characteristics of HP at 22°C. Water, acetonitrile or ethanol were added in steps of 5–20 μl increments to a known weight of HP powder until no visible solute remained. The solubility was confirmed by quantitating HP present in the solution by analysis of a known volume by HPLC.

Stability of HP

In water at various temperatures. HP was dissolved in sterile water (10 μg/ml) and maintained up to 6 days at 4, 22 and 37°C. At regular intervals known volumes of solution were analyzed by HPLC.

At 37°C in pH 4 and 7. HP (10 ng/ml) in sodium acetate buffer at pH 4 and 7 was incubated at 37°C, an aliquot removed at various time intervals up to 6 h and analyzed by HPLC.

At 37°C in NaOH, HCl and H_2O_2 solution. HP (10 mg/ml) was digested at 37°C in 1 *M* NaOH (or 1 *M* HCl or 1 *M* H_2O_2) and aliquots were removed at various intervals up to 7 days and analyzed by HPLC.

In acetonitrile at various temperatures. Aliquots of solutions of HP in acetonitrile (1 μg/ml) stored at 22, −20 and −70°C were analyzed by HPLC at regular intervals during one year.

Under UV light. A solution of HP in water (10 mg/ml) was exposed to UV light (0.882 W/ft^2) at 22°C. Aliquots were removed at various times up to 4 days and samples injected into the HPLC column.

At 37°C and 100% relative humidity. Known weights of HP powder (about 25 mg each) in 6-ml glass vials were kept open in a 37°C incubator at 100% relative humidity. At various times up to 28 days, a vial was removed and the content dissolved in water and analyzed by injecting a known volume into HPLC.

In plasma. To study the long-term storage of HP in plasma at −70°C, plasma was equilibrated at 4°C and HP added to a final concentration of 5 ng/ml. One aliquot of plasma was stored frozen at −70°C and analyzed after a year and another aliquot was extracted immediately as described above for determination of HP. To determine the stability of HP in plasma at various temperatures, plasma was allowed to equilibrate at 4, 22 or 37°C. HP was added to obtain a final concentration of 5 ng/ml. Aliquots (2 ml each) removed prior to the addition of HP and at various times ranging from 30 s to 2 h were frozen at −70°C until analysis. The plasma samples were thawed on ice and extracted as described above for the determination of HP.

ChE activity determination

The plasma samples from the above stability studies were thawed on ice and ChE activity was measured by slight modifications of a previously published method [10]. The reaction mixture (total volume, 100 μl) containing 0.52 m*M* ACh and 25 μl of diluted plasma (1:40) was incubated for 10 min at 28°C. Blank assays were carried out by substituting plasma with buffer and all analysis were done in triplicate.

RESULTS

The chromatogram obtained by injecting a mixture of standard HP and Es is shown in Fig. 2. HP (retention time, 12 min) was well separated from its metabolite Es (retention time, 13.3 min).

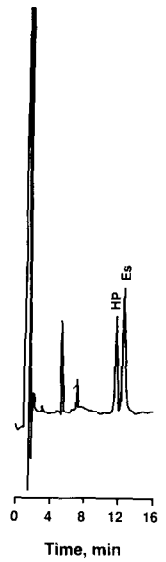

Fig. 2. Chromatogram of a mixture of standard HP and Es (0.1 ng each).

As seen in Fig. 2, the peak heights of HP and Es are almost the same indicating that the chromatographic sensitivity of the present method for Es should be similar to that of HP. We found peak heights were linear in the range from 0.25 to 5 ng indicating that they can be used as a measure of HP concentration. A plot of the amount of HP injected *versus* peak height gave a slope and *y*-intercept of 4.499 ng/cm and −0.413 cm, respectively (r = 0.999). Fig. 3a and b shows the chromatograms obtained after extraction of 2 ml of human plasma from normal volunteers prior to and after HP dosing [6,7]. The method was calibrated using extracted plasma standards. The plot of experimentally determined concentrations of plasma (2 ml) supplemented with HP in the range 0.25–2.5 ng/ml *versus* theoretical values was linear and gave slope and *y*-intercept values of 0.518 and −0.0038, respectively (r^2 = 0.99) indicating an expected recovery of 51.8%. The actual recovery of HP from plasma was 53.04 ± 7.75% (n = 20). The detection limit of the method was 0.125 ng/ml using 2 ml of plasma. The within-day (n = 8) and between-day (n = 8) coefficients of variation were less than 2.81 and 3.07% for standard HP and 11.10 and 11.52% for plasma spiked with HP in the range 0.25–2.5 ng/ml, respectively.

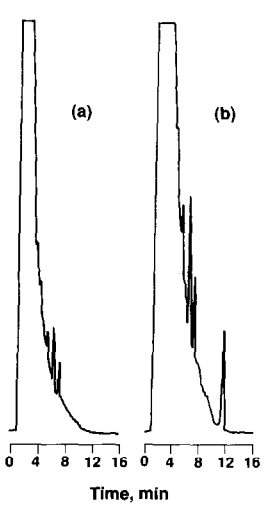

Fig. 3. Chromatograms of 2-ml samples of plasma from normal volunteers (a) prior to dosing and (b) 1 h after HP dosing (40 mg, oral).

Baseline control plasmas from AD patients (n = 4) were analyzed for the possible occurrence of endogenous interference peaks. Results showed the absence of any peak at the retention time of HP, and the chromatograms looked similar to that of a blank plasma extraction shown in Fig. 3a.

In order to determine the optimal conditions for handling plasma samples containing HP and for analysis by HPLC, stability studies were conducted under various temperatures. We found HP was stable in human plasma for at least one year when stored at −70°C. Fig. 4 shows the stability of HP in plasma at 4, 22 and 37°C. All of HP was recovered from plasma at 4°C for 15 min whereas plasma kept at 22 and 37°C rapidly degraded with only 48 and 36% of the original amount remaining by 30 s. A plot of the time

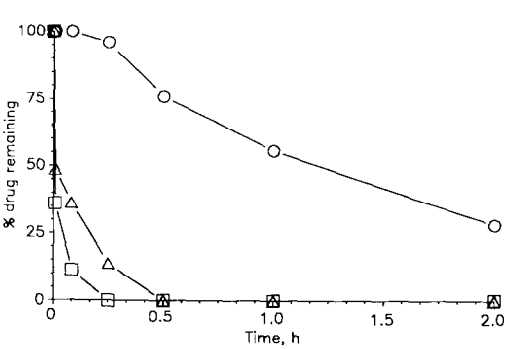

Fig. 4. Time course of HP stability in plasma at 4°C (○), 22°C (△) and 37°C (□).

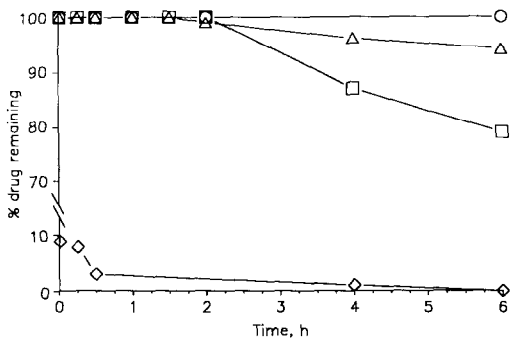

Fig. 5. Time course of HP stability at 37°C in 1 *M* HCl (○), 1 *M* NaOH (◇) and sodium acetate buffer at pH 4 (△) and pH 7 (□).

course of logarithmic drug concentration in plasma at 4, 22 and 37°C resulted in drug half-lives of 64.05 min (r^2 = 0.99), 6.21 min (r^2 = 0.94) and 1.90 min (r^2 = 0.92), respectively. As we expected no enzyme inhibition was seen in any of the plasma samples on analysis for ChE activity since *in vitro* studies in our laboratory [11] showed that a minimum HP concentration of 8–10 ng/ml in plasma is needed to obtain detectable levels of enzyme inhibition.

The solubility of HP in water, acetonitrile and ethanol was 720, 5 and 50 mg/ml, respectively. 100% HP was stable in doubly distilled water at 4 and 22°C for at least 6 days and for 1 day at 37°C and only degraded to 87% of the original activity by 6 days. Stability studies have indicated that solutions of HP in acetonitrile can be stored at 22°C for at least 6 days and at −20° or −70°C for at least one year without any degradation taking place. Stability data (6 h) of HP at 37°C in 1 *M* HCl, 1 *M* NaOH and sodium acetate-buffered solutions at pH 4 and 7 are shown in Fig. 5. The stability of HP increased with decreased pH. HP was stable in 1 *M* HCl for up to 7 days (up to 6 h data shown in the figure) and at pH 4 and 7 for 2 h but degraded gradually with 95 and 80% of the original activity remaining, respectively, by 6 h. HP was unstable in 1 *M* NaOH solution with only 9% of the original activity remaining by 30 s and was completely degraded by 6 h. HP was relatively less stable in H_2O_2 at 37°C and gradually declined to 95 and 58% of the original amount by 6 and 24 h, respectively. Exposure to UV light at room temperature showed that almost 83% of the parent compound was present at 4 h and stayed at that level for at least 4 days whereas exposure to 100% relative humidity at 37°C resulted in almost 75% of the original activity being present after 28 days. In no case Es was seen in levels that can be quantified in any of the studies.

DISCUSSION

An analytical method was developed for the first time in our laboratory for the isolation and quantitation of HP. HPLC–ED was utilized for drug determination in human plasma. The chromatographic conditions enabled the separation of HP from the metabolite Es (Fig. 2). The chromatographic conditions and extraction procedures used in the Phy method [8] were modified to develop a sensitive method suitable for the determination of HP in human plasma. Due to the unstability of HP in plasma at room temperature, it was essential to conduct the extraction step on ice. Also, diethyl ether should be added immediately after the additon of ammonium hydroxide to plasma to minimize the degradation of HP under basic conditions. The time after ammonium hydroxide and prior to diethyl ether addition took no longer than 3–5 s.

The chromatographic conditions developed for HP can also be applied to the quantitation of Phy and are superior in sensitivity (about a two-fold increase) than our previously published method [8].

Similar to Phy, HP metabolized to Es and rubreserine under alkaline conditions. The stability of HP in ammonium hydroxide was similar to what we reported for Phy [8]. Therefore it appears that similar to Phy, alkaline precipitation of plasma apparently will not produce a loss of Phy recovery through hydrolysis to the metabolites. Absence of any peak at the retention time of HP after alkaline hydrolysis at 37°C in 1 *M* NaOH indicated that rubreserine or any other metabolite does not co-elute with HP. In all the studies no Es was present in any of the samples in levels that can be quantified. Therefore it appears that either Es is highly unstable and degrades immediately in plasma or the extraction method for HP is not suitable for Es determination.

Unlike Phy, HP was relatively unaffected on

exposure to UV light and, therefore, it was not necessary to light-shield the samples during analysis. Also, in stability studies conducted in water at 4, 22 and 37°C HP shows superior stability to its parent compound Phy.

A half-life of 30 min has been reported for Phy in plasma at 22°C [12]. The present study suggests that HP is more susceptible to degradation by plasma enzymes than its parent compound, Phy. In the present method plasma extraction was carried out at 4°C and the extraction step took a maximum of 15 min and therefore stability of HP in plasma was not a problem during this process. However, it is important to note that heat loss will not be instantaneous when blood samples from patients are cooled on ice to 4°C. Therefore, to minimize drug degradation, if any, blood collection tubes should be cooled on ice prior to blood draw. The solubility of HP tartrate in water at 22°C was 720 mg/ml (1.415 *M* free base) compared to 13.33 mg/ml at 25°C (0.032 *M* free base) for Phy salicylate [13] resulting in a 44-fold increase in the solubility in water of HP compared to Phy. The relatively low absolute recovery of 51.8% for the method can be attributed to the high solubility of HP in water similar to what we observed for Phy [8]. Unfortunately, it was not possible to quantitate HP remaining in aqueous phase after extraction with diethyl ether, due to contaminant peaks co-eluting with HP, on analysis by HPLC.

We have reported elsewhere a preliminary study on three healthy volunteers after an oral administration of 40 mg HP [6,7]. At this dose HP produced no detected side-effects and relatively high, long-lasting inhibition of plasma ChE (46.4 ± 3.7) and red blood cell ChE (41.6 ± 1.4) [6,7] compared to Phy [14]. Mean plasma drug level in these subjects peaked at 1 h (0.78 ± 0.49 ng/ml) and was below the detection limit by 3 h [7]. We [7] suggested that the absence of correlation between plasma drug level and plasma and red blood cell ChE inhibition values may be due to the extensive first-pass effect of the drug and/or a very high volume of distribution with extensive tissue uptake as occurs with Phy.

Urso *et al.* [15] reported the pharmacokinetics of HP in humans. The authors extracted the drug from plasma, incubated the extract with the enzyme ChE and used the anti-ChE activity as an indirect measure of drug level. They reported detection of a minimum concentration of 3–5 ng/ml.

Our results show that HP is extremely unstable in plasma at 22 and 37°C. A minimum plasma HP concentration of 8–10 ng/ml is necessary to obtain detectable levels of plasma ChE inhibition [10]. Therefore, we found no plasma ChE inhibition on incubation with 5 ng/ml HP. In the absence of any description of technical details by Urso *et al.* [15] it is impossible to locate possible sources of discrepancy in the two studies. We feel that plasma ChE inhibition should not be used as a measure of HP concentration.

We are working to find a drug which can be administered orally to AD patients with minimal side-effects and long-lasting brain ChE inhibiting effect. The drug should not accumulate in the body which increases the risk of long-term toxicity. De Sarno *et al.* [2] has shown a prolonged inhibition of rat brain AChE after intramuscular administration of 5 mg/kg HP to rats. In humans we have observed significant red blood cell ChE inhibition after HP administration in our preliminary study. Since the ChE found in both brain and red blood cells is primarily AChE, our findings suggest that HP can produce long-lasting inhibition in human brain. HP has potential clinical advantages due to its short half-life and long-lasting effect on ChE. The method described here would be useful for monitoring this drug in AD therapy.

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