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Determination of picogram levels of heptylphysostigmine in human plasma using high-performance liquid chromatography with fluorescence detection

M. L. Herold, M. L. Constanzer and B. K. Matuszewski

Merck Sharp and Dohme Research Laboratories, West Point, PA 19486 (USA)

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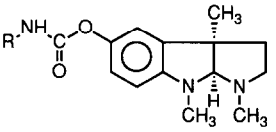
ABSTRACT

A sensitive (50 pg/ml) method for the determination of heptylphysostigmine in human plasma is described. The procedure is based on liquid–liquid extraction of the drug from buffered plasma, and analysis of the concentrated organic extract using high-performance liquid chromatography on a silica column, under normal-phase chromatographic conditions, with fluorescence detection. Physostigmine was used as an internal standard. The assay has been fully validated in the concentration range 50–2000 pg/ml and utilized for the analysis of clinical samples from subjects dosed with heptylphysostigmine.

INTRODUCTION

Heptylphysostigmine (**1**) is a derivative of physostigmine (**2**), in which a carbamoylmethyl group has been substituted with a heptyl group (Fig. 1). Similarly as **2**, the heptyl analogue inhibits acetylcholinesterase activity [1–4] and is being evaluated as an alternative treatment for Alzheimer's disease. The potential advantages of **1** include an increased bioavailability and lower toxicity. In order to support clinical pharmacokinetic program with **1**, a very sensitive assay (< 1 ng/ml) in plasma was required. The development of this assay with a limit of reliable quantification (LOQ) of 50 pg/ml is the subject of this paper.

The determination of physostigmine (**2**) in biological fluids was the subject of numerous papers [5–10]. The two most successful approaches were based on electrochemical detection (ED) and fluorescence detection (FD) after separation using high-performance liquid chromatography (HPLC) under normal-phase (NP) conditions [11,12]. In the ED method, a dual-electrode electrochemical detector in the redox mode, with reduction (−0.3 V) in the first electrode followed by oxidation (+0.42 V) in the second electrode, was utilized [11]. Using this technique the LOQ, defined as the lowest point on the standard curve for which the assay precision is lower than 10%, was 200 pg/ml when up to 4 ml of plasma were



1, R = $(CH_2)_6CH_3$

2, R = CH_3

Fig. 1. Structures of heptylphysostigmine (**1**) and the internal standard (physostigmine, **2**).

Correspondence to: Dr. B. K. Matuszewski, Merck Sharp and Dohme Research Laboratories, West Point, PA 19486, USA.

used. The HPLC method with FD [12] utilized 100% methanol containing 0.013% perchloric acid as mobile phase and separation on a silica column. With this method, the LOQ was 200 pg/ml when 0.5 ml of plasma was utilized.

All of these methods for **2** constituted a good starting point for the development of an assay for **1** and were all initially evaluated. However, none of them was found to be applicable for the measurements of **1** with the high precision (coefficient of variation, C.V. $< 10\%$) and accuracy (10%) required for assaying human plasma samples from clinical studies. In addition, all of these methods suffered from poor and variable recoveries of **1** from biological fluids, especially at low plasma drug concentrations. Therefore, a new methodology for assaying **1** was required and is the subject of this paper. The method is based on a double liquid–liquid extraction of the drug from the pH-adjusted and pretreated plasma (2 ml), evaporation of the extract to dryness, reconstitution of the residue in the NP mobile phase and analysis by NP-HPLC with FD. The method was shown to give a remarkably low LOQ of 50 pg/ml, with both precision and accuracy better than 10%, and was successfully applied to the analysis of plasma samples from various clinical, preclinical, and safety assessment studies with **1**.

Recently, an assay method for **1** in human plasma based on an HPLC–ED approach similar to the one described for **2** [8] has been published [13]. The method is based on NP chromatography and ED in the dual-electrode detection mode, with two electrodes operating at oxidation potentials of $+0.25$ and $+0.95$ V. The precision of the method (intra-day C.V.), at all concentrations within the reported concentration range 250–2500 pg/ml, was higher than 10%, making it not useful for our applications requiring a C.V. $< 10\%$ at all concentrations within the standard curve range. Although the recovery data at individual drug concentrations were not reported, the overall recovery of the drug from plasma was poor and highly variable ($53 \pm 8\%$, $n = 20$), probably contributing to the poor method precision. The advantages of the method described in this paper over the published method [13] are: at least five times better sensitivity (50 pg/ml), using the same volume of plasma (2 ml), with better assay precision (C.V. $< 10\%$); utilization of an internal standard; high recovery ($\geq 87\%$) at all concentrations within the standard curve range 50–2000 pg/ml; excellent stability of **1** stored for three months in pretreated plasma at $-20°C$; and utilization of more reliable FD *versus* ED for multi-sample routine analyses. The long-term performance and ruggedness of the assay described here have also been demonstrated.

EXPERIMENTAL

Reagents

Ammonium hydroxide, ammonium acetate, glacial acetic acid, hydrochloric acid, sodium dihydrogenphosphate, sodium carbonate, sodium bicarbonate (all reagent grade) were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Ascorbic acid, physostigmine (**2**), triethylamine, and pyridostigmine bromide were from Sigma (St. Louis, MO, USA). Methanol, methyl *tert.*-butyl ether (MTBE), and acetonitrile (all Omni-Solv HPLC grade) originated from EM Science (Gibbstown, NJ, USA). 1-Methylnaphthalene (1-MN) was obtained from Aldrich (St. Louis, MO, USA). Heptylphysostigmine (**1**) was obtained as a tartrate salt from Merck Sharp and Dohme Research Labs. (Rahway, NJ, USA). Drug-free human control plasma was purchased from Sera-Tec Biologicals (North Brunswick, NJ, USA). Deionized water was prepared using a Milli-Q reagent water system (Millipore, Milford, MA, USA).

Instrumentation

A Waters Assoc. HPLC system equipped with a 600E chromatographic pump and a WISP 715 automatic injector from Waters-Millipore (Milford, MA USA) was used for all analyses. Two fluorescence detectors were evaluated: McPherson detector (Model FL-750A, $\lambda_{exc} = 250$ nm, $\lambda_{em} > 300$ nm) equipped with deuterium lamp, high-sensitivity attachment (HSA), R928 photomultiplier tube and autozero (McPherson, Acton, MA, USA); and Perkin-Elmer (P-E) detec-

tor (Model LC-240, λ_{exc} = 259 nm, λ_{em} > 310 nm) equipped with a pulsed xenon lamp, total emission mirror accessory, emission filter wheel and autozero (Perkin-Elmer, Norwalk, CT, USA). The detector output was interfaced to a Hewlett-Packard laboratory automation system (HP 3357 LAS, Palo Alto, CA, USA) for data collection, peak integration and analyses. Absorption and fluorescence spectra were obtained using a diode-array spectrophotometer (HP 8452), and a P-E 650-10S spectrofluorometer.

The cyclic voltammograms were recorded with a Bioanalytical Systems (West Lafayette, IN, USA) Model CV-1B cyclic voltammograph, equipped with a Model VC-2 cell, a glassy carbon electrode, and an Ag/AgCl reference electrode. As an electrochemical detector, the LC-17A EC flow cell with dual glassy carbon working electrode, RE-4 Ag/AgCl reference electrode and two LC-4B amperometric controllers were utilized.

A Beckman GPKR refrigerated centrifuge with prechilled GH-3.7 rotor operated at 10°C and at 3000 rpm was used during plasma sample preparation.

The analytical columns for NP chromatography consisted of a Brownlee Labs. silica New-Guard column (15 mm × 3.2 mm I.D., Rainin, Woburn, MA, USA) and two Hypersil silica columns (3 μm, 100 Å, 150 mm × 4.6 mm I.D.) in tandem (Alltech Assoc., Chicago, IL, USA). A hand-packed silica saturator column was placed between the pump and autosampler. For optimum chromatographic performance, the guard column was replaced after 100–110 injections of plasma extracts.

Several other HPLC columns for reversed-phase (RP) chromatography were utilized in the exploratory part of this work: among them, a Zorbax RX-C_8 base-deactivated column (Mac-Mod Analytical, Chadds Ford, PA, USA) and a Bakerbond cyanopropyl column (5 μm, 250 nm × 4.6 mm I.D., Baker, Phillipsburg, NJ, USA).

Chromatographic conditions

The mobile phase consisted of a mixture of acetonitrile–methanol–0.1 *M* ammonium acetate (pH adjusted to 4.3 with glacial acetic acid) (80:15:5, v/v/v) delivered at a flow-rate of 1 ml/min. All mobile phase components were measured separately, mixed, and filtered through a 0.2-μm nylon 66 filter (Rainin).

In the exploratory work under RP chromatographic conditions the mobile phase consisted of a 40:60 (v/v) mixture of acetonitrile–0.02 *M* sodium dihydrogenphosphate containing 4 ml/l triethylamine and adjusted to pH 3.5 with concentrated phosphoric acid. NP mobile phases containing varying amounts of methanol, acetonitrile, and up to 10% (v/v) of different aqueous salts (for ED) were also evaluated. Sodium acetate (0.01 *M*), ammonium nitrate (0.1 *M*), and ammonium acetate (0.1 *M*) solutions in water, adjusted to pH 4.3–8.9, were utilized as the minor components of the NP mobile phase system.

Standard solutions

A stock standard solution of **1** (10 μg/ml) was prepared in the mixture of acetonitrile–0.01 *M* HCl (30:70, v/v), by weighing 1.414 mg of the tartrate salt of **1** and diluting with solvent to 100 ml. The solution was further diluted in the same solvent to give a series of working standards with concentrations of 40, 20, 16, 12, 8, 4, 2, and 1 ng/ml. The working standard solution of the internal standard **2** (160 ng/ml) was prepared by the dilution of a similarly prepared standard solution (10 μg/ml) of **2**. A 25-μl volume of this internal standard solution was used for spiking all plasma samples (equivalent to 4 ng per sample). All standard solutions were stored at −5°C and kept for a period of up to one week.

Sample preparation

The standard curve was constructed daily by analyzing pretreated blank human plasma (2 ml) spiked with 100 μl of working standard **1** and 25 μl of the standard solution of **2**. In order to prevent enzymatic hydrolysis of **1** and **2** in plasma, a second cholinesterase inhibitor (pyridostigmine bromide) was added (~50 μg/ml), as reported for stabilization of **2** [6,7,11]. In addition, ascorbic acid (20 mg/ml) was added to plasma to prevent the potential oxidation of eseroline, a metabolite

of **1**, as suggested earlier during the studies with **2** [12]. Human plasma was pretreated by mixing 20 g of ascorbic acid with 1000 ml of plasma, followed by the addition of 2.5 ml of the stock solution of 20 mg/ml pyridostigmine bromide in water, and thoroughly mixed. Each solution of standards in plasma, placed in 30-ml screw-capped polypropylene centrifuge tubes, was vortex-mixed, followed by the addition of 400 μl of a 3% ammonium hydroxide solution, 5 ml of 1 *M* carbonate buffer (pH 9.3) and 15 ml of MTBE. The samples were rotated on a mixer for 15 min, centrifuged for 5 min, and 13.5 ml of the organic layer, after transferring to a conical evaporation tube, were evaporated at room temperature under a stream of nitrogen. The remaining solution containing plasma was extracted again with 15 ml of MTBE, and 13.5 ml of the organic extract were transferred to the appropriate evaporation tube and evaporated to dryness. The residue was reconstituted in 400 μl of the mobile phase, and after sonication and vortexing at room temperature, 200 μl were injected directly onto the HPLC system.

In the case of analysis of plasma samples from patients, instead of 100 μl of the working standard of **1**, 100 μl of solvent (acetonitrile–0.01 *M* HCl, 3:7, v/v) were used for spiking plasma samples.

Collection of plasma samples from patients

Whole blood (10 ml) was collected in chilled, heparinized Vacutainers containing 25 μl of a 20 mg/ml solution of pyridostigmine bromide in water. Plasma was obtained by spinning immediately in a refrigerated (4°C) centrifuge. If separation of plasma had to be delayed, samples were kept on wet ice before centrifugation. Plasma was transferred to chilled (4°C) polypropylene cryotubes containing 100 mg of L-ascorbic acid and after vortexing samples were frozen at −20°C until the time of analysis.

Precision, accuracy, linearity, recovery and selectivity

The precision of the method was determined by the replicate analyses ($n = 5$) of human plasma containing **1** at all concentrations utilized for constructing calibration curves. A series of quality control (QC) standards were prepared at the start of clinical studies to monitor the stability of **1** in plasma stored at −20°C, and to assess assay performance and accuracy on the day-to-day basis. These QC samples were assayed daily with unknown samples using a daily constructed standard line. The accuracy of the assay was expressed by (mean observed concentration)/(expected concentration) × 100.

The linearity of each standard curve was confirmed by plotting the peak-height ratios of **1** to internal standard **2** *versus* drug concentration. Unknown sample concentrations were calculated from the equation $y = mx + b$, as determined by the weighted $(1/y)$ linear regression of the standard line.

The recovery was assessed at each concentration within the standard curve range by comparing the peak height of **1** extracted from plasma to that of directly injected standards.

The assay selectivity was assessed by analyzing blank control plasma and various patient's predose plasma samples. No endogenous interference was encountered. In addition, the retention time of eseroline, the potential metabolite of **1**, was much longer (27.9 min in method C, *vide infra*) than that of **1** (17.7 min) or **2** (23.0 min).

Fluorescence quantum efficiency

The fluorescence quantum efficiency (Φ_f) for **1** and eseroline, the potential decarbamoylated hydroxy metabolite of **1**, was determined relative to 1-MN as standard ($\Phi_f = 0.25$ in cyclohexane) [14], according to the general procedure described previously [15]. A series of relative Φ_f values for **1** and eseroline in cyclohexane and RP mobile phase as solvents *versus* 1-MN were measured. The fluorescence intensity measurements were done at the same λ_{exc} using solutions with the same absorbances at λ_{exc} for various solvent–analyte combinations. The relative Φ_f values were corrected next for the difference in the refractive indexes of solvents. The uncorrected spectra were utilized for the measurements of relative fluorescence intensities, since the emission

maxima and the half-width of the fluorescence bands for **1**, eseroline, and 1-MN were very similar to each other.

RESULTS

Absorption and fluorescence spectra of **1** *and its electrochemical properties*

In order to select the most sensitive method of detection after HPLC separation, the spectroscopic properties of **1** were evaluated. Since the initial chromatography was performed under RP conditions, all spectroscopic properties were studied in the RP mobile phase as a solvent (see Experimental).

Ultraviolet (UV) absorption spectra indicated the presence of two absorption bands in the region above 220 nm: at 248 nm and 304 nm. The corresponding molar absorption coefficients (ε) were 12 800 and 2700 M^{-1} cm^{-1}, respectively. The two absorption maxima were only slightly shifted to 246 nm (ε = 13 600) and 302 nm (ε = 2800) in methanol containing 0.013% perchloric acid. These absorption characteristics indicated that the development of an assay at levels below 5 ng/ml **1** per ml of plasma using UV detection was highly unlikely.

Compound **1** was found to be fluorescent, with the fluorescence maximum at 340–350 nm. The intensity of fluorescence was similar to the intensity observed for **2** and was practically the same in different solvents, including NP mobile phases, and at different pH. The fluorescence quantum efficiency (Φ_f) of **1** and eseroline, the product of its enzymatic or base-catalyzed hydrolysis, was determined in the RP mobile phase utilized initially in the assay. The Φ_f values were high (0.38 for **1** and 0.48 for eseroline), indicating that both compounds could be detected with high sensitivity using FD.

The ED properties of **1** were also evaluated. Cyclic voltammetry measurements indicated that, similarly to **2**, **1** was ED-active, with a half-wave potential of +0.95 V. This high oxidation potential makes sensitive and selective ED, using a single-electrode system, highly unlikely. However, after the initial oxidation, it was observed that a new ED-active product was formed, which oxidized at about +0.5 V, a much more favorable potential for the development of a selective assay. An HPLC system with dual amperometric detection was set-up, in which the eluent from the analytical column was oxidized at the first electrode at +0.95 V and the product of the oxidation was detected at the second detector set a potential of +0.5 V. An ED peak was observed and compared with the peak obtained using FD. The sensitivity of FD (at the same signal-to-noise ratio) was much better than that of ED in the described electrode potential combination. A similar conclusion was reached after comparing FD with ED under NP conditions using a dual-electrode system set at potentials +0.25 and +0.95 V (see below). Therefore, the method based on fluorescence detection was selected for the development.

RP chromatography and fluorescence detection

Initially, attempts were made to develop an assay based on RP-HPLC with FD. Using a Zorbax RX C_8 column and RP mobile phase (see Experimental for details), an efficient separation of **1** from the endogenous plasma components was achieved. The retention time of **1** was 8.4 min at a flow-rate of 1 ml/min. The drug was isolated from buffered plasma (pH 9.1) using single liquid–liquid extraction with MTBE (8 ml), followed by back-extraction from the partially evaporated organic phase with 200 μl of 0.01 *M* HCl and injection of the acidic layer onto HPLC system. A validation of this method was attempted, but the recoveries of the drug at various concentrations were variable leading to a poor assay precision (C.V. > 10%). In addition, day-to-day assay reproducibility was inadequate, and the slopes of the daily standard lines were variable.

In order to eliminate irreproducibility of the daily standard line slopes, detailed recovery experiments from plasma were performed. The recovery studies indicated that the liquid–liquid extraction step was highly efficient (>90%), whereas back-extraction with 0.01 *M* HCl gave only 50–60% recovery. An attempt was made to eliminate the inefficient back-extraction step by evap-

oration of the MTBE extract to dryness followed by reconstitution in the mobile phase, or by solid-phase extraction (SPE) of **1** from the MTBE extract. The first approach gave chromatograms containing many plasma interferences, whereas the SPE required relatively large volumes of eluting solvents (2 ml methanol–0.1% HCl) to displace the drug from a silica, cyano, or diol phase on which **1** was effectively retained when applied from MTBE. In addition, the variability in reconstitution of the residue after evaporation to dryness using an RP mobile phase and lack of an internal standard (**2** eluted in the solvent front) made the SPE technique highly unlikely to succeed. Therefore, it was decided to pursue a different approach based on NP chromatography on a silica column and FD or ED.

The advantage of this approach was the potential of avoiding the highly inefficient back-extraction step by increasing the efficiency and reproducibility of reconstitution of the plasma residue in a neat organic (NP) *versus* RP (mobile phase) solvent, elimination of the potential adsorption of **1** at low concentrations to glass or plastic tubes during work-up and the renewed possibility of using **2** as an internal standard.

NP chromatography and comparison of FD with ED

NP mobile phases containing varying amounts of methanol, acetonitrile, and up to 10% (v/v) of different aqueous salt solutions (0.01 *M* sodium acetate, 0.1 *M* ammonium nitrite, and 0.1 *M* ammonium acetate adjusted to pH 4.3–8.9) for ED were evaluated. A baseline separation of **1** from **2** was observed under a variety of conditions. The on-line comparison of detection sensitivity using ED in the dual amperometric mode (oxidation potential of +0.25 V at the first electrode, followed by +0.95 V at the second electrode from which the signal was monitored) with FD (McPherson) revealed that the limit of detection (amount of standard injected) of **1**, at a signal-to-noise ratio of 3:1, was about 30 pg for FD and 120 pg for ED. In addition, when plasma extracts were analyzed using ED, many interfering compounds coeluting with **1** and **2** were observed. Therefore, it was decided to develop an assay based on NP-HPLC with FD.

Validation of the NP-HPLC–FD assay

Method A. A successful validation of the assay in plasma in the concentration range 0.2–2.0 ng/ml was achieved with a single liquid–liquid extraction with MTBE, chromatography on a single silica column, and detection using McPherson FD. The initial single liquid–liquid extraction step was similar to the extraction of **2** [5,6,8,11] and **1** [13] reported earlier. Remarkably clean chromatograms from plasma were obtained with retention times of 8.7 and 11.3 min for **1** and **2**, respectively. However, when QC samples were prepared from one lot of control plasma and assayed against the standard curve obtained in plasma from other source, the QC values were 20–30% lower than the nominal values. It was found that the efficiency of extraction of **2** was different from these two lots of plasma. Several experiments were performed to eliminate this difference, including extraction from plasma saturated with sodium chloride, ion-pairing extraction in the presence of sodium dodecyl sulfate, use of PTFE instead of plastic or glass tubes for extraction, and sonication of the residue during final reconstitution of the extract. However, under all these conditions the slopes of the standard lines from the two lots of plasma were still different. This problem was solved by double extraction of the plasma with a second portion (15 ml) of MTBE and evaporation of the organic extracts to dryness at room temperature instead of at 40°C. The assay has been revalidated under these new conditions producing a standard line ($y = 0.465x + 0.00156$, unweighted) with a correlation coefficient of >0.999 and accuracy of 99.6–98.2%. The C.V. of replicate analyses at all concentrations within the standard curve range (0.2, 0.4, 0.6, 0.8, 1.0, and 2.0 ng/ml) were less than 3.2% and ranged from 0.8 to 3.1%, indicating an excellent assay precision. The QC samples analyzed under these double-extraction conditions gave mean values ($n = 5$) of 0.50 ± 0.03 and 1.53 ± 0.01 ng/ml for the spiked standards at 0.5 and 1.5 ng/ml, respectively.

Method B. When subjects' post-dose plasma samples from the first single-dose study in healthy volunteers were analyzed using method A, several small peaks of the potential metabolites of **1** partially coeluting with **1** and **2** were observed. Therefore, method A had to be modified to separate these interferences from the compounds of interest.

Several chromatographic parameters have been initially changed including mobile phase composition, flow-rate and column length. The best separation was achieved when two analytical silica columns (150 mm × 4.6 mm I.D., 3 μm) in series were utilized. Under these new conditions, the retention times for **1** and **2** were 17.7 and 23.0 min, respectively, (Fig. 2).

When pooled subjects' plasma extracts were analyzed under these new conditions, the interfering peaks were baseline-separated from the peaks of **1** and **2**. The new double-column method has been fully validated in the concentration range 0.2–2.0 ng/ml. The correlation coefficient of the standard line was >0.998 and the C.V. values of replicate analyses (n = 5) were 6.3–1.2% over the assay concentration range. Selected clinical samples from three studies were assayed using this double-column method (*vide infra*).

Method C. In order to increase further sensitivity of the assay and to improve the LOQ from plasma, several HPLC fluorescence detectors were evaluated. A considerable improvement in the detectability of **1** and **2** was achieved when a McPherson fluorescence detection utilized in methods A and B, was replaced with a P-E LC-240 detector set at λ_{exc} = 259 nm and λ_{em} > 310 nm. About three to four times better sensitivity of detection (at the same signal-to-noise ratio) was observed using the latter detector (Fig. 3).

Under otherwise the same assay conditions as in method B, and using the P-E detector, the 50 pg/ml LOQ of **1** from plasma was achieved with adequate assay precision and accuracy (Table I).

Stability of **1** *in plasma*

Long-term stability of **1** in plasma (pretreated with pyridostigmine bromide and ascorbic acid)

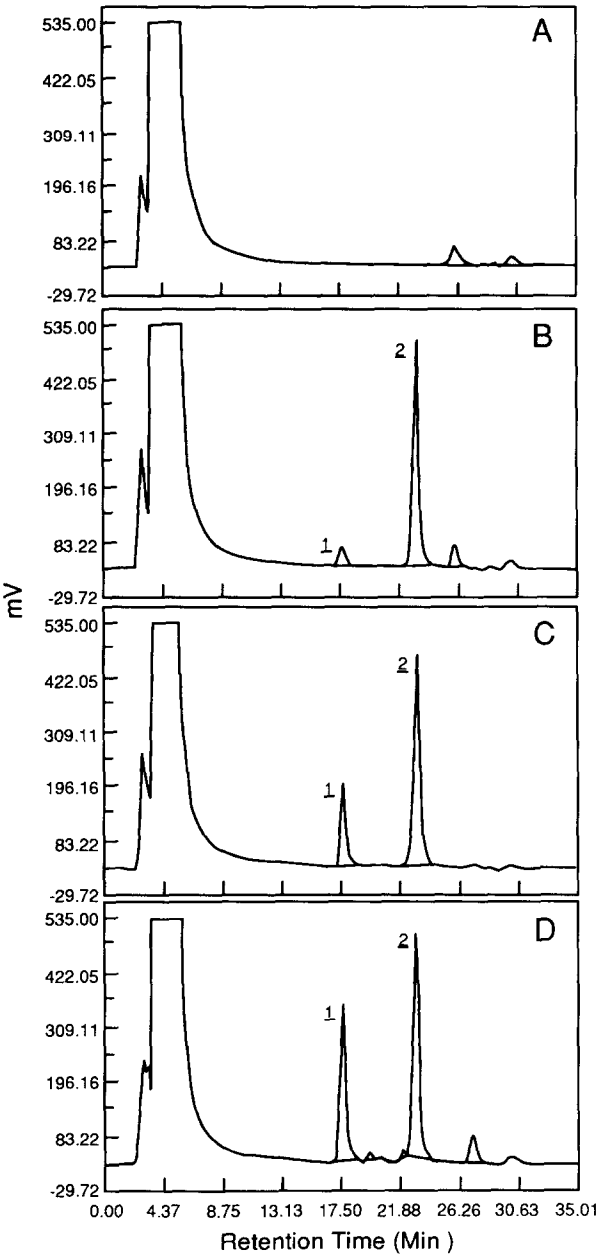


Fig. 2. Representative chromatograms of human plasma spiked with **1** and an internal standard, **2**. (A) Blank control plasma; (B) plasma spiked with 200 pg/ml **1** and 2 ng/ml **2**; (C) subject 14 plasma (after 1:16 dilution with control plasma), 1 h after oral administration of 40 mg of **1** (concentration equivalent to 1.0 ng/ml **1** and 2 ng/ml **2**); (D) subject 19 plasma (after 1:2 dilution with control plasma), 1 h after oral administration of 40 mg of **1** (concentration equivalent to 1.7 ng/ml **1** and 2.0 ng/ml **2**).

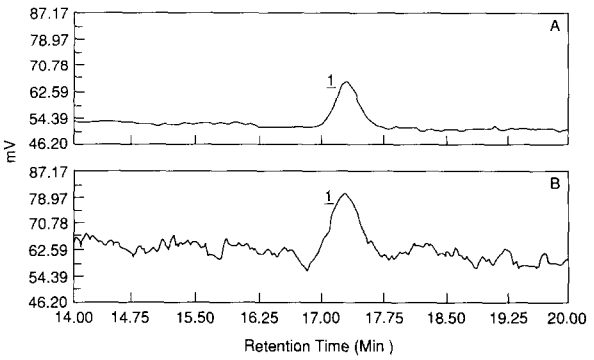


Fig. 3. Comparison of the HPLC fluorescence peaks after extraction of 100 pg/ml **1** from plasma, using two fluorescence detectors (see Experimental for details). (A) P-E LC-240 fluorescence detector; (B) McPherson detector.

at −20°C, under the conditions utilized for collection and storage of clinical specimens, was assessed by analyzing QC samples that have been prepared and stored for 90 days (Table II). The assayed concentrations of the QC standards at two concentrations immediately after preparation (0.50 and 1.47 ng/ml) and after 90 days of storage (0.46 and 1.42 ng/ml, $n = 19$) were the same, within experimental error, indicating the adequate stability of **1** under storage conditions.

TABLE I

INTRA-DAY ASSAY PRECISION, ACCURACY AND RECOVERY DATA FOR THE ANALYSIS OF **1** IN HUMAN PLASMA

Method C, $n = 5$.

Concentration (pg/ml)	C.V. (%)	Accuracy[a] (%)	Recovery (%)
50	5.1	108.0	87.2
100	8.5	96.1	102.9
200	2.6	95.9	100.5
400	5.9	100.3	99.0
600	2.2	101.2	98.7
800	3.3	99.5	99.8
1000	2.4	100.8	101.0
2000	1.2	99.8	98.7

[a] Expressed as (observed concentration)/(spiked concentration) × 100.

TABLE II

INTER-DAY VARIABILITY FOR THE ASSAY OF QUALITY CONTROL PLASMA SAMPLES SPIKED WITH **1**

Spiked concentration (ng/ml)	n[a]	Mean calculated concentration (ng/ml)	C.V. (%)
0.5	19	0.46	9.2
1.5	19	1.42	6.9

[a] Assayed over a period of three months.

Analysis of samples from clinical studies

The assay was used for the determination of **1** in human plasma from the single- and multiple-dose safety and tolerability studies. The representative concentration–time data in plasma for two subjects after oral administration of **1** are presented in Table III.

These data indicate that the peak plasma concentrations were highly variable and were below LOQ (0.2 ng/ml, method B) 6–10 h after dosing. The assay of the same plasma samples using method C (LOQ = 50 pg/ml) was not performed

TABLE III

REPRESENTATIVE CONCENTRATIONS OF **1** IN PLASMA AFTER ORAL ADMINISTRATION OF 40 mg OF **1** TO SELECTED HUMAN SUBJECTS

Time post-dose (h)	Concentration of **1** (ng/ml)	
	Subject 1	Subject 2
0	0.00	0.00
1	0.79	16.11
2	1.29	15.34
4	0.31	3.68
6	0.00[a]	1.18
8	0.00[a]	0.54
10	–[b]	0.23
24	0.00[a]	0.00[a]

[a] A "zero" value represents less than 0.2 ng of **1** per ml of plasma. These data were obtained using method B, and were not repeated using method C due to the insufficient sample volume for analysis.

[b] Insufficient sample volume for analysis.

due to the unavailability of sufficient volume (2 ml) of subjects' plasma for analyses.

DISCUSSION

Various approaches to the isolation of **1** from plasma (liquid–liquid extraction *versus* SPE), HPLC separation (RP *versus* NP chromatography), and sensitive and selective detection (ED *versus* FD) were evaluated. The combination of double liquid–liquid extraction, NP chromatography, and FD gave superior results, with very clean chromatograms from plasma (Fig. 2), remarkable assay sensitivity (LOQ = 50 pg/ml), and adequate assay ruggedness and long-term performance required for multi-sample analyses of samples from human pharmacokinetic studies.

The dual liquid–liquid extraction gave not only a very high and reproducible ($\geq$87%) recovery of **1** from plasma at low concentrations (50 pg/ml, Table I), but was also required to achieve the same recovery of an internal standard **2** from different lots of plasma. Poor recoveries (53 ± 8%), even at much higher concentrations of **1** in plasma (>250 pg/ml) than studied in this paper, lack of internal standard, and instability of **1** in untreated plasma used in sample collection and/or sample preparation probably contributed to the poor assay precision for **1** reported in the HPLC–ED method [13]. With double extraction, utilization of an internal standard, and an efficient stabilization of **1** and **2** by the addition of a second cholinesterase inhibitor to plasma to prevent their enzymatic degradation, an assay with high recovery (mean value of 98 ± 5%), high precision (C.V. = 1.2–8.5%), and excellent accuracy (100.2 ± 3.7%, Table I) was developed. More than 40 plasma samples a day were analyzed routinely using the assay described in this paper. Compound **1** was shown to be stable in pretreated plasma at −20°C for a period of at least three months.

Both RP and NP chromatography were evaluated. The poor efficiency of the back-extraction step from the organic phase plasma extract with the acid, and/or inadequate reproducibility of reconstitution of the residue after evaporation of the organic phase extract to dryness, led to unacceptable variability of the assay based on RP chromatography. Instead, the assay in NP mode has proven to be superior in terms of assay precision, recovery, and the ease and reproducibility of reconstitution of organic phase residue after extraction from plasma in NP mobile phase before injection on column. Similar observations about the advantages of NP over RP chromatography for the determination of various amines were reported earlier [16–18]. Utilization of two silica columns (method B) was necessary to achieve the required assay specificity and to separate a number of potential human metabolites of **1** from both **1** and the internal standard **2** (Fig. 1).

Finally, FD over ED was chosen for the sensitive assay of **1**. This choice was based on at least four times better sensitivity of FD *versus* dual-electrode ED, much cleaner chromatograms obtained from plasma, and long-term reliability and ruggedness of the assay based on FD. The high fluorescence quantum efficiency (Φ_f = 0.38) for **1** makes FD highly favorable for low-sensitivity detection. For the maximum assay sensitivity, a number of fluorescence detectors have been tested. The best sensitivity was achieved when the P-E LC-240 fluorescence detector with xenon discharge lamp and pulsed excitation equivalent to 20 kW for 8 μs duration was used. Almost four times better sensitivity and LOQ was achieved using this detector (method C) in comparison with a fluorescence detector (McPherson) equipped with deuterium lamp and constant cell illumination (method B).

The assay for **1** described here can also be applied to the quantitation of **2** in plasma with similar or better recovery, sensitivity, and precision than the one described in the literature [12]. The assay was found to be fully applicable for multi-sample analyses of plasma samples from various human and animal pharmacokinetic studies.

CONCLUSION

A sensitive (50 pg/ml) and selective assay for the determination of **1** in human plasma, based

on NP chromatography and fluorescence detection, has been developed. The method has been fully validated in the concentration range 0.05–2.00 ng/ml, and was utilized for the analyses of plasma samples from subjects dosed with **1**. The assay can also be applied to the analyses of **1** in dog plasma. Heptylphysostigmine stored in plasma pretreated with pyridostigmine bromide at −20°C was shown to be stable for a period of at least three months.

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REFERENCES

1 M. Brufani, M. Marta and M. Pomponi, *Eur. J. Biochem.*, 157 (1986) 115.
2 M. Brufani, C. Castellano, M. Marta, A. Oliverio, P. G. Pagella, F. Pavone, M. Pomponi and P. L. Rugarli, *Pharmacol. Biochem. Behav.*, 26 (1987) 625.
3 M. Marta, C. Castellano, A. Oliverio, F. Pavone, P. G. Pagella, M. Brufani and M. Pomponi, *Life Sci.*, 43 (1988) 1921.
4 P. De Sarno, M. Pomponi, E. Giacobini, X. C. Tang and E. Williams, *Neurochem. Res.*, 14 (1989) 971.
5 R. Whelpton and T. Moore, *J. Chromatogr.*, 341 (1985) 361.
6 R. R. Brodie, L. F. Chasseaud and A. D. Robins, *J. Chromatogr.*, 415 (1987) 423.
7 P. R. Hurst and R. Whelpton, *Biomed. Chromatogr.*, 3 (1989) 226.
8 L. K. Unni, M. E. Hannant, R. E. Becker and E. Giacobini, *Clin. Chem.*, 35 (1989) 292.
9 N. M. Elsayed, J. R. G. Ryabik, S. Ferraris, C. R. Wheeler and D. W. Korte, *Anal. Biochem.*, 177 (1989) 207.
10 G. D. Lawrence and N. Yatim, *J. Pharmacol. Methods*, 24 (1990) 137.
11 S. Knapp, M. L. Wardlow and L. J. Thal, *J. Chromatogr.*, 526 (1990) 97.
12 Y. Q. Wu, E. Reinecke, E. T. Lin, A. D. Theoharides and L. Fleckenstein, *J. Liq. Chromatogr.*, 13 (1990) 275.
13 L. K. Unni and R. E. Becker, *J. Chromatogr.*, 573 (1992) 275.
14 J. B. Berlman, *Handbook of Fluorescence Spectra of Aromatic Molecules*, Academic Press, New York, 2nd ed., 1971.
15 B. K. Matuszewski, R. S. Givens, K. Srinivasachar, R. G. Carlson and T. Higuchi, *Anal. Chem.*, 59 (1987) 1105.
16 B. A. Bidlingmeyer, J. K. Del Rios and J. Korpl, *Anal. Chem.*, 54 (1982) 442.
17 R. J. Flanagan, R. K. Bhamra, S. Walker, S. C. Monkman and D. W. Holt, *J. Liq. Chromatogr.*, 11 (1988) 1015.
18 J. R. Cashman and Z.-Ch. Yang, *J. Chromatogr.*, 532 (1990) 405.