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Short Communication

Determination of HP 749, a potential therapeutic agent for Alzheimer's disease, in plasma by high-performance liquid chromatography

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ABSTRACT

N-(*n*-Propyl)-N-(4-pyridinyl)-1H-indol-1-amine hydrochloride (HP 749, I), a non-receptor-dependent cholinomimetic agent with noradrenergic activity, is a potential agent for the treatment of Alzheimer's disease. Pharmacokinetic studies in animals and humans showed that I was well absorbed and metabolized primarily to the N-despropyl metabolite (P7480, II) after oral administration. To facilitate the kinetic studies, a sensitive and selective high-performance chromatographic assay was developed. I and II are extracted from plasma by a mixture of cyclohexane–ethyl acetate and chromatographed on an isocratic reversed-phase high-performance liquid chromatographic system employing an analytical phenyl column with acetonitrile–ammonium formate as mobile phase. The concentrations of these two compounds, quantitated by internal standardization, are monitored by ultraviolet detection. The method is linear in the plasma assay over a concentration range of 0.5–500 ng/ml for both compounds with a quantitation limit of 0.5 ng/ml. The precision and accuracy of the calibration curves and/or method are less than 10%. The recovery of I and II from plasma is 63–74 and 63–68%, respectively, over a concentration range of 0.5–500 ng/ml.

INTRODUCTION

N-(*n*-Propyl)-N-(4-pyridinyl)-1H-indol-1-amine hydrochloride (HP 749, I) (Fig. 1), an indole-substituted analogue of 4-aminopyridine, is a potential drug currently being studied for the treatment of Alzheimer's disease. Preclinical pharmacological testings have shown I to have cholinergic and noradrenergic activities both *in vitro* and *in vivo* [1]. Animal studies have shown that I was active in the reversal of scopolamine dementia and enhancement of dark avoidance behavior [2]. In addition, I was active in reversing the passive avoidance deficit produced by the double lesions of both the nucleus basalis of Meynert and ascending noradrenergic bundle in rats [2]. Pharmacokinetic studies in animals [3] and man

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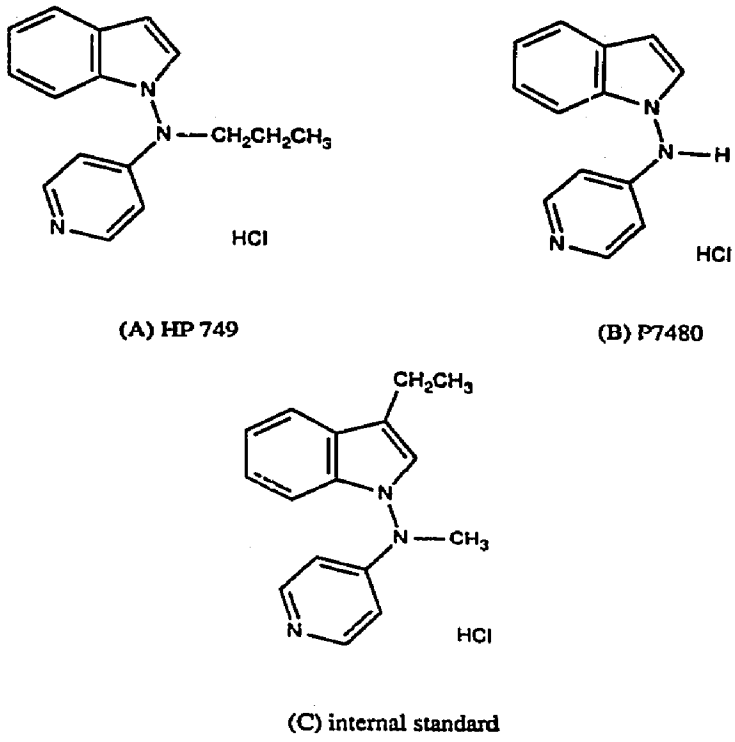

Fig. 1. Chemical structures of (A) HP 749, N-(*n*-propyl)-N-(4-pyridinyl)-1H-indol-1-amine hydrochloride (I), (B) P7480, N-(4-pyridinyl)-1H-indol-1-amine hydrochloride (II), and (C) internal standard, 3-ethyl-N-methyl-N-(4-pyridinyl)-1H-indol-1-amine hydrochloride.

showed that I was well absorbed and metabolized primarily to an active N-despropyl metabolite (P7480, II) (Fig. 1) after oral administration. To facilitate the kinetic studies and relate pharmacological effects to drug concentrations, a sensitive and selective high-performance liquid chromatographic (HPLC) assay was developed for the quantitation of I and II.

EXPERIMENTAL

Materials

Compounds I, II and 3-ethyl-N-methyl-N-(4-pyridinyl)-1H-indol-1-amine hydrochloride (internal standard) (Fig. 1) were synthesized in the Chemical Research Department of Hoechst-Roussel Pharmaceuticals. Acetonitrile, cyclohexane, ethyl acetate, sodium hydroxide, formic acid and ammonium formate were all analytical grade and purchased from Fisher Scientific (Springfield, NJ, USA). The ammonium formate buffer was prepared by mixing formic acid and ammonium formate solution until desired pH and concentration were obtained. Deionized water (Milli-Q water purification system, Millipore, Bedford, MA, USA) was used throughout the study. Human plasma was obtained from Biological Specialty (Landsdale, PA, USA).

Preparation of standard solutions

Stock solutions (1 mg/ml) of I and II were each prepared in methanol and stored at −20°C in borosilicate test tubes (Fisher Scientific). From the corresponding stock solutions, standard solutions of I and II at concentrations 0.5, 1, 2, 5, 10, 50, 100, 250 and 500 ng per 50 μl were prepared in methanol. Internal standard solution was prepared at a concentration of 150 ng per 50 μl of methanol. The standard solutions of I, II and internal standard were stored at room temperature and were stable for at least two weeks. Calibration and spiked standards were prepared each week.

Quantitation of calibration plasma sample

Calibration curves were constructed with nine different plasma standards covering a concentration range of 0.5–500 ng/ml. Calibration curves were established by analyzing six replicates at each concentration. Linearity of the calibration curves was determined by least-squares regression analysis.

To a series of calibration sample tubes were added 50 μl of I and II and 150 ng of internal standard. Subsequently, 1 ml of control plasma and 1 ml of 0.1 *M* sodium hydroxide were added. The tubes were vortexed briefly and then 5 ml of cyclohexane–ethyl acetate (1:1, v/v) were added. The tubes were then shaken for 10 min on an Eberbach shaker and centrifuged at 2000 *g* for 10 min in a Model J-6B centrifuge (Beckman Instruments, Fullerton, CA, USA). The organic phase was transferred to a tapered centrifuge tube and evaporated to dryness under a nitrogen stream in a water bath a 40°C. The residue was then reconstituted in 150 μl of mobile phase and an aliquot analyzed by HPLC.

Quantitation of dosed plasma sample

The dosed human plasma samples were collected from six healthy male volunteers. Venous blood from all volunteers was collected in heparinized vacutainer tubes (navy blue stopper, Becton Dickinson Cat. No. 6527) at specific time points after oral administration of 30 mg of I. Plasma was separated from erythrocytes by centrifugation, and then transferred into sample tubes and stored frozen until assay. An aliquot of plasma (1 ml) was added to test tubes containing 150 ng of internal standard. The quantitation of I and II was according to the procedures described above.

High-performance liquid chromatography

Plasma samples prepared according to the above procedures were analyzed by an HPLC system employing a Shandon Hypersil 3 μm phenyl column (100 mm × 4.6 mm I.D.) (ACE Scientific, East Brunswick, NJ, USA). The samples were injected by a Waters WISP 710B autosample injector (Milford, MA, USA) and eluted with acetonitrile–0.02 *M* ammonium formate buffer, pH 2.95 (80:20, v/v) at a flow-rate of 1.5 ml/min. The elution was carried out at ambient temperature and column effluent was quantitated using a Kratos Model 773 UV spectropho-

tometer (Ramsey, NJ, USA) with detection at 270 nm. The chromatograms were integrated using an ACCESS*CHROM Model 6000 from Perkin Elmer Nelson (Cupertino, CA, USA).

Validation procedures

The recovery of I, II and internal standard from plasma was assessed by adding known amounts to blank human plasma to give final concentrations of 1, 5, 10, 50, 100 and 250 ng/ml. The samples (four replicates at each concentration) were extracted and analyzed by HPLC. Extraction efficiencies of I, II and internal standard were calculated by comparing the peak areas obtained from spiked plasma with the mean peak areas ($n = 4$) after injected standards in mobile phase.

Assay precision was evaluated at concentrations of 5, 50 and 500 ng/ml. Plasma samples were prepared by spiking blank human plasma with I and II and stored at −20°C until analyzed. Intra-assay variation was examined by analyzing six replicates at each concentration, and inter-assay precision was determined in triplicates on six occasions over a period of four weeks.

RESULTS AND DISCUSSION

Selectivity

Plasma samples were analyzed by an isocratic reversed-phase HPLC system employing a Shandon analytical phenyl column and UV detection. Concentrations of I and II were determined by internal standardization. Typical chromatograms for control blank, spiked and dosed human plasma are shown in Fig. 2. Using this chromatographic system, no endogenous components, extracted from human plasma, interfered with I, II or the internal standard in the sample assay. Interfering substances were either eluted at the solvent front under the HPLC conditions or did not absorb UV at 270 nm under these conditions. The small peak with a retention time of 6.5 min, shown in Fig. 2, was a polar metabolite. The structure of this metabolite was recently identified as 5-hydroxy-HP 749 by mass spectrometry [4].

Linearity and detection limit

Plasma standards were prepared from blank human plasma spiked with I and II in the range 0.5–500 ng/ml. Calibration curves showed good linearity between peak-area ratios and concentrations from 0.5 to 250 ng/ml for I and II in plasma (I: $y = 0.00252x + 0.00117$, $r = 0.99988$; II: $y = 0.00385x + 0.00229$, $r = 0.99976$; y, x and r are peak-area ratio, concentration and correlation coefficient, respectively). The observed coefficient of variation (C.V.) and relative error (R.E.) for the standard curves, calculated from the least-squares regression equations, was less than 10% (Tables I and II). The quantitation limit for the method, defined as the smallest quantity of analyte which can be determined with acceptable accuracy and precision (less than 20%), was 0.5 ng/ml for I and II in plasma (Tables I and II).

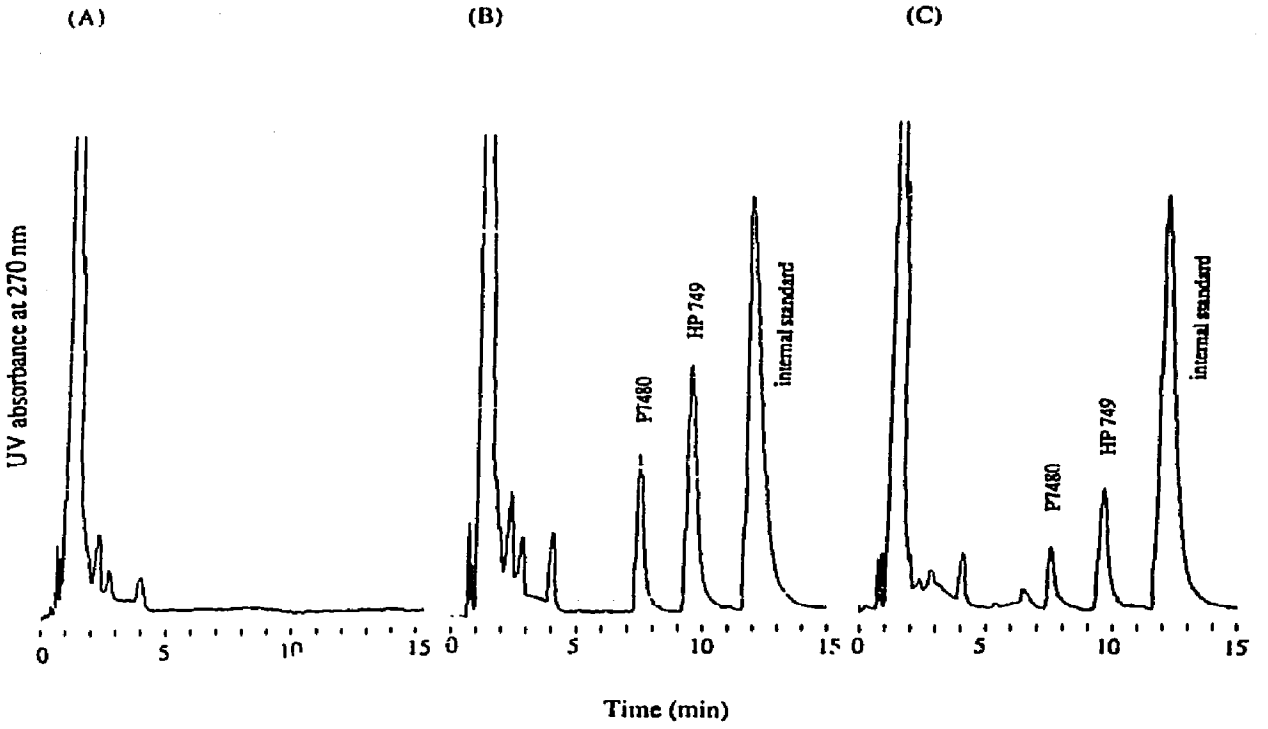

Fig. 2. Representative HPLC profiles of (A) blank human plasma, (B) blank human plasma containing 50 ng/ml I and II and 150 ng/ml internal standard and (C) human plasma 2.5 h after oral administration of 30 mg of I. Plasma samples were chromatographed on a Shandon Hypersil 3 μm phenyl column at laboratory temperature using acetonitrile–0.02 *M* ammonium formate buffer, pH 2.95 (80:20, v/v) as mobile phase. Column eluate was monitored by UV detection at 270 nm.

TABLE I

HPLC CALIBRATION DATA OF I IN PLASMA

Concentration added (ng/ml)	Peak-area ratio (mean ± S.D., n = 6)	Concentration measured[a] (mean ± S.D.) (ng/ml)	C.V.[b] (%)	Bias	R.E.[b] (%)
0.5	0.0017 ± 0.0001	0.46 ± 0.028	6.1	−0.04	−8.3
1	0.0039 ± 0.0002	1.01 ± 0.038	3.7	0.01	1.0
2	0.0078 ± 0.0005	2.03 ± 0.128	6.3	0.02	1.3
5	0.0189 ± 0.0008	4.91 ± 0.203	4.1	−0.09	−1.8
10	0.0388 ± 0.0011	10.1 ± 0.25	2.5	0.11	1.1
50	0.1990 ± 0.0068	51.8 ± 1.62	3.1	1.82	3.6
100	0.4046 ± 0.0083	105.4 ± 1.97	1.9	5.36	5.4
250	0.9576 ± 0.0295	249.4 ± 7.02	2.8	−0.63	−0.3
500	1.8868 ± 0.0627	491.4 ± 14.91	3.0	−8.64	−1.7

[a] Measured concentration from the least-squares regression equation $y = 0.00252x + 0.00117$, $r = 0.99988$, where y, x and r are peak-area ratio, concentration and correlation coefficient, respectively.

[b] Coefficient of variation (C.V.) and relative error (R.E.), calculated from standard deviation and bias, are used to assess the precision and accuracy of the calibration curve, respectively.

TABLE II

HPLC CALIBRATION DATA OF II IN PLASMA

Concentration added (ng/ml)	Peak-area ratio (mean ± S.D., n = 6)	Concentration measured[a] (mean ± S.D.) (ng/ml)	C.V.[b] (%)	Bias	R.E.[b] (%)
0.5	0.0014 ± 0.0001	0.53 ± 0.028	5.3	0.03	6.0
1	0.0026 ± 0.0002	1.01 ± 0.068	6.7	0.01	1.2
2	0.0048 ± 0.0005	1.91 ± 0.161	8.5	0.02	−4.8
5	0.0128 ± 0.0010	5.03 ± 0.369	7.3	0.03	0.6
10	0.0255 ± 0.0007	10.02 ± 0.256	2.6	0.02	0.2
50	0.1294 ± 0.0141	50.95 ± 1.49	2.9	0.95	1.9
100	0.2602 ± 0.0079	102.5 ± 2.84	2.8	2.45	2.5
250	0.6266 ± 0.0177	246.7 ± 6.32	2.6	−3.30	−1.3
500	1.1884 ± 0.0306	467.9 ± 10.99	2.4	−32.11	−6.4

[a] Measured concentration from the least-squares regression equation $y = 0.00385x + 0.00229$, $r = 0.99976$, where y, x and r are peak-area ratio, concentration and correlation coefficient, respectively.

[b] Coefficient of variation (C.V.) and relative error (R.E.), calculated from standard deviation and bias, are used to assess the precision and accuracy of the calibration curve, respectively.

Method precision and plasma standard stability

The reproducibility of the plasma assay was determined by analyzing the plasma standards prepared at levels of 5, 50 and 500 ng/ml. The results of intra-assay and inter-assay precision are expressed as C.V. The assay variations at levels of 5, 50 and 500 ng/ml are less than 10% for I and II (Table III).

TABLE III

INTRA-ASSAY AND INTER-ASSAY PRECISION FOR I AND II

Compound	Intra-assay (n = 6)		Inter-assay (n = 6)	
	Concentration (ng/ml)	C.V. (%)	Concentration (ng/ml)	C.V. (%)
I	5.0 ± 0.2	4.0	5.0 ± 0.2	0.4
	45.8 ± 0.6	1.3	47.7 ± 2.0	4.2
	503.5 ± 4.6	0.9	503.0 ± 2.3	0.5
II	5.1 ± 0.3	5.9	5.1 ± 0.1	2.0
	47.9 ± 2.4	5.0	48.0 ± 1.4	2.9
	504.2 ± 11.2	2.2	503.2 ± 2.2	0.4

TABLE IV

ASSAY RECOVERY OF I AND II FROM PLASMA

Concentration added (ng/ml)	Recovery (mean ± S.D., n = 4) (%)	
	I	II
0.5	62.5 ± 6.4	65.5 ± 7.5
5	73.7 ± 7.8	68.3 ± 6.8
50	62.8 ± 1.6	63.2 ± 1.4
500	65.1 ± 2.8	65.0 ± 2.9

Recovery

Extraction efficiency was determined by comparing the peak areas from spiked samples with those obtained from injection in mobile phase (Table IV). The recovery of I and II from plasma by the sample preparation procedure was about 63–74% and 63–68%, respectively, over a concentration range of 0.5–500 ng/ml. The recovery of internal standard (150 ng) was 65%.

Assay application

Human plasma concentration–time profiles of I and II after a single oral dose of 30 mg of I are shown in Fig. 3. Compound I was rapidly absorbed and metabolized primarily to the N-despropyl metabolite (II) after oral dosing. I and II reached maximal concentrations at about 1.5–2.5 h followed by a biphasic decline with half-lives of 1.5–2 and 6–9 h for the rapid and slower elimination processes.

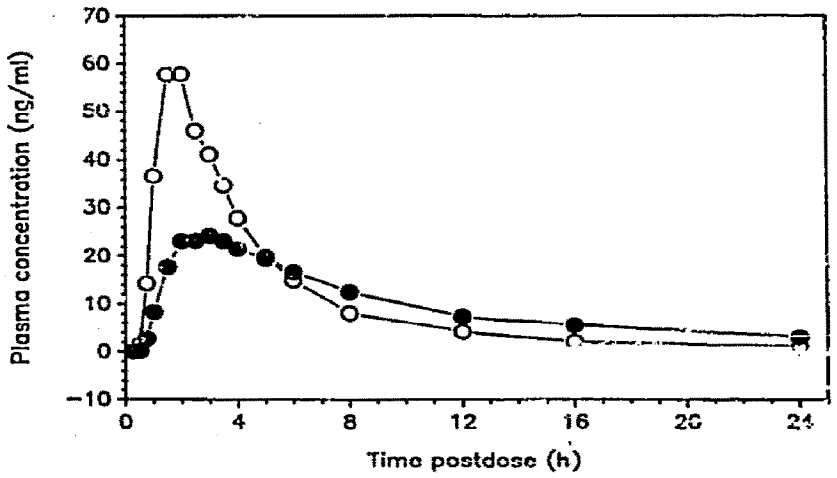

Fig. 3. Mean plasma concentrations of I (○) and II (●) observed in six healthy volunteers following a single oral administration of 30 mg of I.

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