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Determination of the pyridinium metabolite derived from haloperidol in brain tissue, plasma and urine by high-performance liquid chromatography with fluorescence detection

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ABSTRACT

A sensitive and selective method for the determination of the pyridinium metabolite (HPP^+) derived from the antipsychotic drug haloperidol (HP) in brain tissue, plasma and urine using high-performance liquid chromatography with fluorescence detection is described. The HPP^+ present in biological samples was extracted using a Sep-Pak C_{18} cartridge. Recoveries of HPP^+ ranged from 78 to 90%. Final separation and quantitative estimations of HPP^+ were achieved on a C_{18} reversed-phase column employing a mobile phase of acetonitrile–30 m*M* ammonium acetate (40:60, v/v) containing 10 m*M* triethylamine and adjusted to pH 3 with trifluoroacetic acid. The fluorescence detection utilized an excitation wavelength of 304 nm and an emission wavelength of 374 nm. Standard curves were linear in the range of 2.5–100 ng/ml for brain tissue homogenate and plasma samples and 10–500 ng/ml for urine samples. The detection limit of HPP^+ was about 1 ng/ml in all biological samples. The concentrations of HPP^+ in brain tissue, plasma and urine from HP-treated rats were determined using this method.

INTRODUCTION

Haloperidol, [4-(4-chlorophenyl)-4-hydroxy-1-piperidinyl]-1-(4-fluorophenyl)-1-butanone (**1**, HP, Fig. 1), is a clinically useful antipsychotic drug which can cause severe extrapyramidal side-effects including parkinsonism and, following chronic exposure, tardive dyskinesias [1]. Extensive studies have established that, in addition to conjugation of the C-4 hydroxy group, oxidative N-dealkylation to yield 4-(4-chlorophenyl)pyridine (**2**) and reduction of the butyrophenone carbonyl group to yield the reduced haloperidol derivative RHP (**3**) are the principal routes of HP metabolism in humans [2–6]. Recently we have

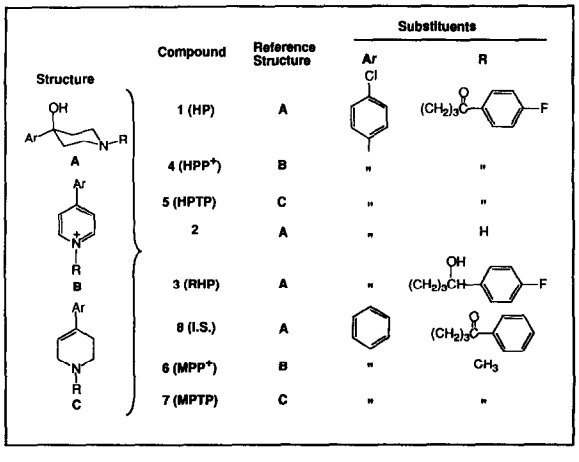

Fig. 1. Structures of compounds discussed in the text.

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reported that HP also is metabolized to the corresponding pyridinium metabolite HPP^+ (**4**) in rodents [7]. This metabolite also has been detected in the urine of HP-treated patients [8]. The enzymatic pathway responsible for the conversion of HP to HPP^+ has not been identified but may involve dehydration of the tertiary carbinol to generate the corresponding haloperidol tetrahydropyridine derivative HPTP (**5**) followed by a two-step oxidation to yield the final pyridinium product. These observations are of considerable toxicological interest since HPP^+ is structurally similar to the 1-methyl-4-phenylpyridinium species (MPP^+) (**6**), the ultimate neurotoxic metabolite produced from the parkinsonian inducing agent 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (**7**) in a reaction catalyzed by monoamine oxidase B [9,10]. Furthermore, intracerebral microdialysis studies have established that HPP^+ displays neurotoxic properties analogous to those of MPP^+ in the rat [7].

This report describes the development of a sensitive and specific assay for HPP^+ in biological samples using a liquid–solid extraction step to remove components derived from the biological matrices followed by high-performance liquid chromatographic (HPLC) analysis with fluorescence detection. Previously reported HPLC separations of MPP^+ employing cation-exchange chromatography [7] and reversed-phase chromatography [11] could not be applied to the estimation of HPP^+ directly because of the lipophilic character of this pyridinium compound. The ability of this assay to provide quantitative information on the concentrations of HPP^+ in brain tissue, plasma and urine samples isolated from rats treated with HP is demonstrated.

EXPERIMENTAL

Chemicals and reagents

HP was purchased from Sigma (St. Louis, MO, USA) while RHP was a gift from Research Biochemicals (Nitack, MA, USA). The hydrochloride salt of HPTP, the perchlorate salt of HPP^+, and the perchlorate salt of the 4-phenyl-1-(4-phenyl-4-oxobutyl)pyridinium species (**8**), used as an internal standard (I.S.), were synthesized in our laboratory [12]. HPLC-grade water, acetonitrile and methanol were obtained from Fisher Scientific Products (Fair Lawn, NJ, USA). All other solvents and reagents used were of analytical-reagent grade.

HPLC apparatus

The chromatographic system consisted of a Model 110A constant-flow pump (Beckman Instruments, Fullerton, CA, USA) and a Model LS-40 fluorescence detector (Perkin Elmer, UK). A Model LCI-100 computing integrator (Perkin Elmer) was utilized to monitor chromatographic performance. A Model spectromonitor-D variable-wavelength UV detector (LDC Analytical, Riviera Beach, FL, USA) was also used to detect the non-fluorescent HP during assay development.

HPLC conditions

A reversed-phase μBondapak C_{18} column (150 mm × 3.9 mm I.D., 10 μm particle size, Waters, Assoc., Milford, MA USA) was used for the analysis. The mobile phase consisted of acetonitrile–30 m*M* ammonium acetate (40:60, v/v) containing 10 m*M* triethylamine and adjusted to pH 3 with trifluoroacetic acid. The solvent flow-rate was 1 ml/min. The mobile phase was degassed in an ultrasonic bath prior to use. The HPP^+ was detected by spectrofluorimetry employing an excitation wavelength of 304 nm and an emission wavelength of 374 nm. The variable-wavelength UV detector was set at 245 nm. All analyses were carried out at ambient temperature.

Animal studies

Male Sprague–Dawley rats weighing about 310 g received two injections of HP in saline (10 mg/kg, intraperitoneally) per day for three days. Animals were housed in individual metabolic cages permitting the separate collection of urine and feces. Food and drinking water were supplied *ad libitum*. Urine samples were collected every 24 h after the first administration of HP. Blood samples were obtained by cardiac puncture using heparinized syringes from animals

anaesthesized with diethyl ether at 1 and 24 h after the last injection. After centrifugation at 1000 *g* for 5 min, each plasma fraction was transferred to a separate tube. Some animals were sacrificed at 1 h after the last injection and the whole brains (from which the striatum was dissected in one case) were excised and the tissues were weighed. These samples were stored at −20°C prior to analysis.

Extraction and recovery studies

The recoveries of HPP^+ employing a liquid–liquid extraction step or a liquid–solid extraction step were examined under a variety of conditions. In the liquid–liquid extraction studies, a 1-ml aliquot of buffered solution (pH 4.0–10.0) containing 50 ng of HPP^+ was combined with 2 ml of ethyl acetate or chloroform. The resulting mixture was vortex-mixed for 30 s following which the organic layer was transferred to a second tube and the solvent removed under reduced pressure with the aid of a rotary evaporator. The residue obtained was dissolved in 1 ml of the HPLC mobile phase and a 20-μl aliquot of the solution was injected onto the HPLC system. In the liquid–solid extraction procedure, sample clean-up employed Sep-Pak C_{18} cartridges (Waters Assoc.) which were preconditioned by washing with 10 ml of methanol followed by 10 ml of 30 m*M* ammonium acetate. A 0.1-ml aliquot of the standard solution (20 ng/ml in water) was mixed with 1.0 ml of 30 m*M* ammonium acetate and the resulting mixture was loaded onto a preconditioned Sep-Pak C_{18} cartridge. After a 1-min equilibration period, the solvent was pulled through under gentle suction. The cartridge was washed with 7 ml of 30 m*M* ammonium acetate followed by 4 ml of methanol–water (50:50, v/v). HPP^+ then was eluted with 3 ml of methanol or a solution of 0.5% glacial acetic acid in methanol. The eluent was evaporated to dryness *in vacuo*, the residue was dissolved in 0.1 ml of the mobile phase and a 30-μl aliquot was injected onto the HPLC system. Recoveries of HPP^+ from the biological samples were determined using the same procedures except that spiked biological samples were used in place of the standard solutions. Recoveries were calculated by comparing the peak height of the extracted HPP^+ *versus* the peak height obtained with a standard solution of HPP^+.

Sample preparation for HPLC analysis

The plasma samples (0.2 ml) were mixed with 1.0 ml of 30 m*M* ammonium acetate. Each sample then was loaded onto a preconditioned Sep-Pak C_{18} cartridge and treated as described above. After elution of HPP^+, the eluent was evaporated to dryness *in vacuo* and the residue was dissolved in 0.1 ml of the mobile phase. The I.S. solution (0.1 ml containing 500 ng I.S. per ml mobile phase) was added to the sample and a 30-μl aliquot of the resulting mixture was injected onto the HPLC system. Each brain tissue sample was homogenized in four volumes of 1.15% KCl solution using a Potter–Elvejheim homogenizer. The homogenate (0.2 ml) was mixed with 0.2 ml of cold methanol to precipitate protein, and, following sonication for 30 s, this mixture was centrifuged at 10 000 *g* for 5 min. The supernatant (0.2 ml) was treated according to the same procedure described for the plasma samples. Urine samples (0.1 ml) also were treated according to the procedure described for the plasma samples except that, after evaporation of the eluent from the cartridge, the residue was dissolved in 0.5 ml of the mobile phase to which was added 0.5 ml of the I.S. solution.

RESULTS AND DISCUSSION

Chromatographic conditions

The fluorescence characteristics of HPP^+, I.S. and HP were determined in the HPLC mobile phase. Based on the resulting spectra, the excitation wavelength of 304 nm and emission wavelength of 374 nm, the λ_{max} values for HPP^+, were selected for maximum sensitivity. The excitation and emission λ_{max} values of the I.S. were 275 and 330 nm, respectivley. Its fluorescence intensity under the assay conditions was about 1/15th that of HPP^+. Analysis of a 10 μg/ml solution each of HP, RHP and HPTP, *i.e.* about 1000 times the concentration used to establish the fluorescence

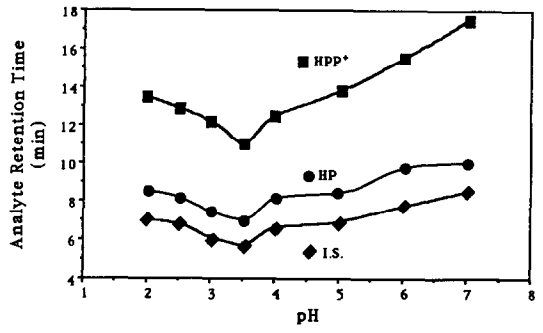

Fig. 2. Variations in retention time of HPP^+, HP and I.S. *versus* pH. The mobile phase consisted of acetonitrile–30 m*M* ammonium acetate (40:60, v/v) containing 10 m*M* triethylamine.

properties of HPP^+, did not yield detectable peaks under assay conditions.

In a previous study [7] we used an Alltech (25 cm × 4.6 mm I.D., 5 μm particle size) amino column to detect HPP^+ in the urine of HP-treated rats. Under these conditions the HPP^+ and I.S. coeluted. Naoi *et al.* [11] have reported the determination of MPP^+ by HPLC using an ODS column. We therefore investigated the chromatographic behavior of HPP^+, HP and I.S. on an ODS column using several mobile phases and UV detection at 245 nm. The retention times of HPP^+, HP and I.S. in methanol–water (40:60, v/v) and acetonitrile–water (40:60, v/v) were greater than 45 min and the peak shapes had considerable tailing. Changing the mobile phase to

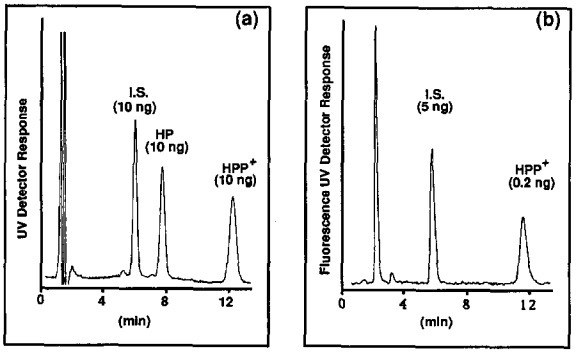

Fig. 3. Typical chromatograms obtained with (a) UV (245 nm) and (b) fluorescence (excitation at 304 nm, emission at 374 nm) detection of 20-μl injections of standards solutions. For UV (a) the concentration of each analyte was 500 ng/ml; for fluorescence (b) the concentration of I.S. was 250 ng/ml and of HPP^+ was 10 ng/ml.

acetonitrile–30 m*M* ammonium acetate (40:60, v/v) containing 10 m*M* triethylamine and adjusted to pH 3 with trifluoroacetic acid resulted in excellent resolution and peak shapes and retention times of 21.5 min or less for the three analytes. The effects of pH (2–7) on the retention times of the analytes also were investigated. As shown in Fig. 2, the retention time for HPP^+ was longest at pH 7.0 and shortest at pH 3.5. Comparable data (not shown) were obtained for HP and I.S. The fluorescence intensity of HPP^+ proved to be essentially independent of pH. This result contrasts with that of Naoi *et al.* [11] who reported that the fluorescence intensity of MPP^+ is dependent on the pH of the mobile phase. Based on these results, we selected the mobile phase described in the Experimental section. Under these conditions excellent resolution and peak shapes were observed for HPP^+, HP and I.S. (retention times of 12.2, 7.5 and 6.1 min, respectively, Fig. 3).

Sample extraction and recovery

Previous results had established that the lipophilic characteristics of HPP^+ made it possible to extract this pyridinium species into organic solvents such as chloroform [7]. More quantitative experiments established that HPP^+ may be extracted from aqueous solutions buffered to pH 4–10 with overall recoveries of 36–45% using ethyl acetate and 53–55% using chloroform. In addition to these rather marginal recoveries, the corresponding extracts from biological samples spiked with the analytes showed many background peaks rendering this approach unacceptable. In an effort to improve the efficiency and selectivity of the initial concentration step in the assay, the plasma and brain tissue protein fractions first were precipitated with trichloroacetic acid, acetonitrile or methanol. Although good recoveries were achieved (about 96%), the chromatograms continued to display significant biological background peaks. An alternative liquid–solid clean-up and extraction method using Sep-Pak C_{18} cartridges was investigated. When aqueous solutions containing HPP^+ (20 ng/ml) were loaded onto the cartridges, elution with methanol

TABLE I

ACCURACY AND REPRODUCIBILITY FOR HPP^+ ASSAY

Sample	Spiked concentration (ng/ml)	Intra-day (n = 5)		Inter-day (n = 5)	
		Concentration found (mean) (ng/ml)	C.V. (%)	Concentration found (mean) (ng/ml)	C.V. (%)
Plasma	5	4.8	6.3	4.7	6.8
	20	19.7	5.7	19.5	5.9
Urine	50	49.7	4.3	50.1	4.6
	200	199.3	4.1	199.7	5.0

gave recoveries of 77%. Elution with 0.5% glacial acetic acid in methanol improved the recoveries to 94%. Moreover, washing the cartridge with 30 mM ammonium acetate and 50% aqueous methanol removed many endogenous components from the biological samples. Addition of the I.S. to blank brain tissue homogenates which were subsequently extracted gave variable results. It is possible that the I.S. binds to proteins as the variability correlated to protein content. Efforts to obtain a more suitable I.S. which could be added prior to the protein precipitation step were unsuccessful. The good recoveries of HPP^+ from spiked biological samples, however, argue that the addition of the I.S. to the evaporated residues of the eluent from the cartridges, while less than ideal, is acceptable. Under these conditions recoveries of known concentrations of HPP^+ (5, 20 and 50 ng/g or ng/ml) from spiked blank biological samples compared to standard solutions of HPP^+ injected directly were 75–80% (brain tissue), 79–85% (plasma) and 91–95% (urine).

Assay linearity, detection limit, accuracy and reproducibility

The standard curves for HPP^+ were linear in the concentration range examined (2.5–100 ng/ml for spiked brain tissue homogenates and plasma, and 10–500 ng/ml for spiked urine). The re-

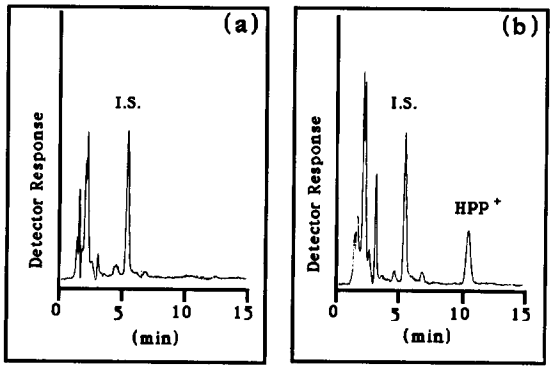

Fig. 4. Chromatograms of the brain tissue sample from (a) a control rat and (b) a rat treated intraperitoneally with 10 mg/kg HP twice a day for three days and then sacrificed 1 h after the last injection. The peak for HPP^+ shown in (b) corresponds to 34.2 ng HPP^+ per g wet tissue.

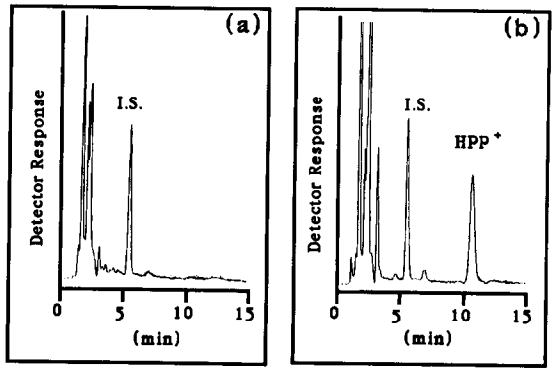

Fig. 5. Chromatograms of the plasma sample extracts obtained from (a) a control rat and (b) a rat treated intraperitoneally with 10 mg/kg HP twice a day for three days and then sacrificed 1 h after the last injection. The peak for HPP^+ shown in (b) corresponds to 14.3 ng HPP^+ per ml plasma.

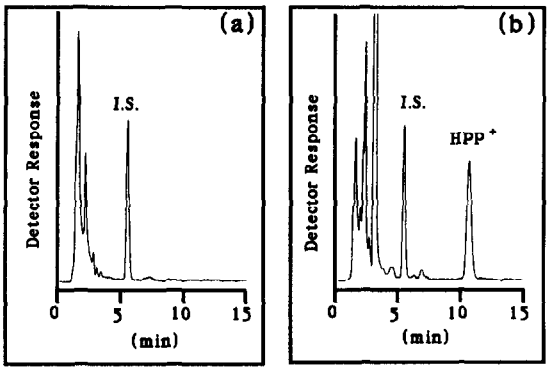

Fig. 6. Chromatograms of (a) a control urine sample and (b) a urine sample collected during the 24-h period following the first 10 mg/kg HP injection. The peak for HPP^+ in (b) corresponds to 211.2 ng HPP^+ per ml urine.

gression equations were $y = 0.008 + 0.048x$ ($r = 0.999$) for plasma, $y = 0.001 + 0.045x$ ($r = 0.999$) for brain tissue and $y = 0.002 + 0.052x$ ($r = 0.999$) for urine. The standard curves showed little day-to-day variability in slopes and intercepts [coefficient of variation (C.V.), $<5\%$]. The minimum detectable amounts of HPP^+ in the biological samples were calculated to be about 1 ng/ml with a signal-to-noise ratio of 4:1. The fluorescence detector offered a sensitivity enhancement over the UV detector (set at 300 nm, λ_{max} for HPP^+) in the detection of HPP^+ of at least a factor of 20.

The intra- and inter-day reproducibility and accuracy data are summarized in Table I. In the intra-day study the average C.V. values for the assay of HPP^+ in spiked plasma (5 and 20 ng/ml) and urine (50 and 200 ng/ml) were 6.0 and 4.2%, respectively. Moreover, the inter-day data indicated good reproducibility (average C.V. = 6.3% for plasma and 4.8% for urine). The accuracy of the assay ranged from 94.0% (plasma) to 100.2% (urine, 50 ng/ml).

Application to biological samples

The method was applied to the quantitative estimation of HPP^+ in brain tissue, plasma and urine samples obtained from rats treated with HP. Figs. 4a–6a illustrate typical chromatograms obtained from the extracts of control brain tissue, plasma and urine samples. Figs. 4b–6b show typical chromatograms of HPP^+ derived from extracts of brain tissue, plasma and urine samples following HP treatment. The chromatograms from control samples were free of interference at the retention times of interest. HPP^+ was detected in all samples prepared from HP-treated rats. Table II shows the concentrations of HPP^+ after intraperitoneal administration of HP (10 mg/kg, two times per day for three days). It is especially interesting that HPP^+ was detected in the brain since intracerebral microdialysis studies in the rat have established that HPP^+ possesses neurotoxic properties similar to those of MPP^+. This method also was found to be sufficiently sensitive to detect HPP^+ in plasma of treated rats 24 h after the last dose. Currently, we are designing studies to evaluate the pharmacokinetic parameters of HPP^+ in humans treated with HP.

TABLE II

CONCENTRATION OF HPP^+ IN BRAIN, PLASMA AND URINE OF RAT RECEIVING HALOPERIDOL

The samples were obtained from two rats administered haloperidol (10 mg/kg) intraperitoneally twice a day for three days.

Sample	HPP^+ level (ng/g or ng/ml)	
	Rat 1	Rat 2
(1) Brain tissue 1 h after last injection		
Striatum	343.6	205.3
Whole brain (minus striatum)	58.8	34.2
(2) Plasma 1 h after last injection	14.3	12.3
Plasma 24 h after last injection	2.5	2.4
(3) Urine, 24-h collection after first injection	211.2	155.6

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