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High-performance liquid chromatographic method for the detection and quantitation of haloperidol and seven of its metabolites in microsomal preparations

Jian Fang☆ and John W. Gorrod*

Chelsea Department of Pharmacy, King's College, University of London, Manresa Road, London SW3 6LX (UK)

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ABSTRACT

An isocratic high-performance liquid chromatographic (HPLC) system was developed to analyze haloperidol and its potential metabolites. These compounds included 4-(4-chlorophenyl)-4-hydroxypiperidine (CPHP), haloperidol N-oxide (HNO), reduced haloperidol (RHAL), the 1,2,3,6-tetrahydropyridine analogue and its N-oxide, and the pyridinium ion from haloperidol (HP^+). The HPLC system comprised a Hypersil CPS5 column with a mobile phase of acetonitrile (67%) and ammonium acetate (final concentration 10 m*M*) which was adjusted to pH 5.4 by acetic acid. The solvent was delivered at 1 ml/min. RHAL and CPHP were determined by an ultraviolet detector at 220 nm with a detection limit of 1 nmol/ml. All other compounds were determined at 245 nm and had a detection limit of 0.3 nmol/ml. This system was used to analyze a microsomal metabolic mixture of haloperidol. It was found that all above compounds except HNO were metabolites of haloperidol. In addition, two other metabolites were also well separated in this HPLC system which are proposed to be oxygenated haloperidol and the pyridone analogue of haloperidol. The HPLC system was used to carry out quantitative metabolic studies of haloperidol. It was found that the metabolism of haloperidol exhibits large inter-species differences. The apparent enzyme kinetic parameters were also determined using mice microsomes.

INTRODUCTION

The metabolism of haloperidol {4-(4-chlorophenyl)-1-[4-(4-fluorophenyl)-4-oxobutyl]-4-piperidinol; HAL} is currently a field of active research. Until recently, it was generally believed that oxidative N-dealkylation and ketone reduction were the two major metabolic pathways of HAL [1,2]. It was subsequently shown [3–8] that, concurrent with these two metabolic pathways, HAL is also metabolised to its pyridinium analogue (haloperidol pyridinium, HP^+). We have demonstrated that HAL is firstly dehydrated to its 1,2,3,6-tetrahydropyridine derivative (HTP). HTP is then further converted in a similar manner to the activation of a dopaminergic neurotoxin, N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), to produce HP^+ [5]. We have also detected 4-(4-chlorophenyl)-4-hydroxypiperidine (CPHP), haloperidol-1,2,3,6-tetrahydropyridine N-oxide (HTPNO) and two unknown compounds (designated M3 and M5) as metabolites of HAL [5–8]. These researchers have demonstrated that HAL undergoes a complex metabolic process similar to that of the neurotoxin MPTP in biological systems [9]. This may have important pharmacological and toxicological consequences.

A number of analytical methods have previ-

* Corresponding author.
☆ Present address: Neuropsychiatric Research Unit, A114 Medical Research Building, University of Saskatchewan, Saskatoon, Saskatchewan S7N 0W0, Canada.

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ously been published for metabolic and pharmacokinetic studies of HAL [10–17]. In these methods, UV [10–13] or electrochemical [14–17] detectors were employed. Greatest sensitivities were achieved using a coulometric-type electrochemical detector [16]. Recently Igarashi and Castagnoli [18] developed an HPLC method for the analysis of HP^+ with fluorescence detection. But in all these HPLC methods, either reduced HAL or HP^+ was the only metabolite measured. Therefore an HPLC method was urgently required for the analysis of the above metabolites in order to carry out detailed studies on these metabolic pathways.

In this report, a new isocratic HPLC procedure for the analysis of HAL and its metabolites is described. This analytical procedure detects most of the important phase I metabolites of HAL, that is CPHP, HTPNO, reduced haloperidol (RHAL), HTP, HP^+ and two as yet uncharacterised metabolites, and thus represent the most comprehensive method for the analysis of HAL and its metabolites. This highly specific system separates eleven compounds in 15 min and proves suitable for direct application using a thermospray LC–MS detector. *p*-Fluorobenzoylpropionic acid (FBPA) can also be separated by this system, but as it usually runs close to the solvent front it is difficult to be seen in metabolic extracts.

EXPERIMENTAL

Chemicals

HAL and pirenzepine were obtained from Sigma (Pool, UK); CPHP was from Aldrich (Gillingham, UK); HPLC-grade acetonitrile (far UV grade) was purchased from FSA Laboratory Supplies (Loughborough, UK). RHAL was synthesised by the method of Odia *et al.* [19]. Haloperidol N-oxide (HNO), HTP, HTPNO and HP^+ were synthesised in this laboratory. Details of these syntheses will be described in a separate report [8].

Distilled water was further purified by passing through an Elgastat Spectrum water purification system (Elga, Bucks, UK) fitted with SC1 and SC6 cartridges before use for preparation of HPLC solvents. All solvents were filtered through a Sartorius 40-μm filter (Sartorius Instruments, Belmont, UK) then degassed by passing a gentle stream of helium gas for 10 min followed by sonication for 5 min prior to use. Standard stock solutions of authentic compounds (10 m*M*) were prepared in methanol.

High-performance liquid chromatography

The HPLC system comprised a ConstaMetric 3000 solvent delivery system (Milton Roy, Riviera Beach, FL, USA), a Rheodyne 7125 injector with a 100-μl loop and a Rapiscan SA6508 detector (Severn Analytical, Shefford, UK) set to measure at 220- and 245-nm wavelengths. A Tandon TM7002 computer was used to record, store and analyse chromatograms. The HPLC system used a 5-μm Hypersil CPS5 column (silica gel with bonded cyanopropyl groups, 250 mm × 4.6 mm I.D.) (Thames Chromatography, Berkshire, UK) coupled with an Upright C-130B guard column (30 mm × 2 mm I.D.) (Upchurch Scientific, Oak Harbor, WA, USA) packed with 5-μm Hypersil CPS column material.

The mobile phase was a combination of acetonitrile (67%) and ammonium acetate buffer (final concentration 10 m*M*) adjusted to pH 5.4 with acetic acid. The solvent was delivered at a flow-rate of 1 ml/min. During the development of the HPLC system, solvent systems of different pH were investigated. These solvent systems consisted of acetonitrile (60%) and sodium phosphate (20 m*M*) which were adjusted to different pH values by phosphoric acid.

Calibration curves were prepared as follows: known quantities of CPHP, HTPNO, RHAL, HAL, HTP and HP^+ (in 0.5 ml of water) were added to phosphate buffer (0.2 *M*, pH 7.4; 2 ml) and heat-inactivated (100°C for 10 min) microsomes (1 ml) equivalent to 0.5 g of original liver tissue in Sovirel tubes. Sample preparation and HPLC analysis were as described below. The preparation of the calibration curves was carried out twice in duplicate. Intra- and inter-assay variation of the method was examined by injecting a standard mixture containing 8 nmol per incubate

of each of the standard compounds in the same day and on three different days.

Metabolic studies

Male LACA albino mice (weight 30–40 g), New Zealand White rabbits (weight 2–3 kg), Syrian hamsters (weight 80–100 g) and Albino Dunkin Hartley guinea-pigs (weight 400–600 g) were obtained from the Animal Supply Unit, King's College, University of London (London, UK). Animals were fasted overnight before sacrifice. Hepatic microsomal preparations were prepared using the centrifugation method [20]. Protein concentrations were determined by the method of Lowry *et al.* [21] as modified by Miller [22].

Incubation procedures were as follows: a cofactor generating system consisting of $NADP^+$ (2 μmol), glucose 6-phosphate (10 μmol), glucose-6-phosphate dehydrogenase (1 U) and $MgCl_2$ (2 mg) all in 2 ml of phosphate buffer (0.2 *M*, pH 7.4) was preincubated at 37°C for 5 min. Enzymatic reactions were initiated by the addition of HAL and microsomal preparations equivalent to 0.25 g of original tissue. Controls consisted of incubates containing either no cofactor, or no substrate, or no microsomes, or inactivated microsomes. Incubations were carried out for 10 min. Biological reactions were terminated by addition of acetonitrile (3 ml) followed by addition of an internal standard solution (pirenzepine, 0.05 m*M*, 200 μl). The mixtures were left at room temperature for 30 min and the denatured proteins were removed by centrifugation (MSE Centaur 2, 4000 g, 20 min). The clear supernatants were subjected to HPLC analysis (50 μl). Loss of metabolites due to protein precipitation was negligible.

RESULTS AND DISCUSSION

It was the object of this work to develop an HPLC system for the separation of CPHP, HNO, HTPNO, RHAL, HAL, HTP, HP^+ (Fig. 1) as well as two unknown metabolites M3 and M5. These compounds comprise a large range of lipophilicity, pK_a, and polarity. Problems arise as to how it is possible to analyse all these compounds in a single chromatogram. In preliminary experiments, gradient systems were tried in order to reduce the analysis time with a C_{18} column, but the systems gave very noisy baselines in the chromatogram when the UV detector was set at 220 nm which was necessary for the detection of CPHP and RHAL. Consequently, we moved to the use of isocratic systems with polar bonded columns. C_4 and phenyl columns were tried but failed to significantly reduce the retention time of lipophilic compounds. Cyano columns were found to give reasonable retention times for all the compounds. Thus the HPLC system was further optimised using a Hypersil CPS column. It is worth noticing that increasing the pH of the solvent system reduced the retention time of the two N-oxides (Fig. 2). This may be because the oxygens were protonated under basic conditions.

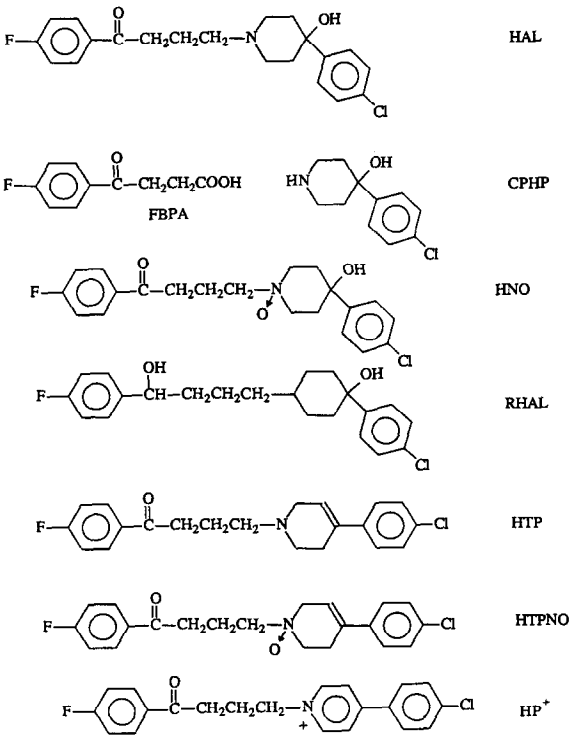

Fig. 1. Structures of haloperidol and its potential metabolites.

The optimised solvent system consisted of acetonitrile (67%)–ammonium acetate buffer (10 m*M*) at pH 5.4. Ammonium acetate was chosen as buffer because this would allow the HPLC sys-

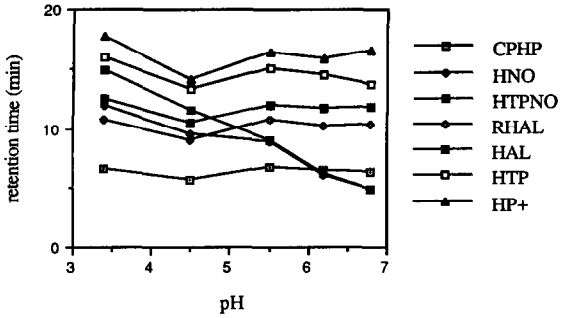

Fig. 2. Dependence of retention times of metabolites of haloperidol on pH values of the HPLC solvent system. See text for experimental details.

tem to be linked to a thermal spray detector without any modification of the solvent system [5,6]. With this system, the back-pressure for the analytical HPLC column was 4.2 MPa which increased to 8.4 MPa when the guard column was incorporated into the HPLC system.

Fig. 3 shows a chromatogram of a mixture of authentic compounds and a metabolic mixture obtained from HAL and its control incubate; CPHP, M3, HTPNO, RHAL, HTP, HP^+ and M5 were detected in the incubation mixture free of interference from endogenous substances.

CPHP and RHAL were detected at 220 nm. Due to the high background absorption caused by ammonium acetate the sensitivities for RHAL and CPHP were lower than those detected at 245 nm and was 3 nmol per incubate; however, this sensitivity was sufficient for *in vitro* metabolic studies. All the other compounds were detected at 245 nm with lower detection limits at about 1 nmol per incubate.

For each sample, the ratio of the peak height of the sample to that of the internal standard was calculated (Table I). Peak-height ratios of HTP, HTPNO, HP^+, RHAL and CPHP to internal standard showed a linear relationship to their concentration over a range of at least 1 to 200 nmol per tube (Table I). For HAL the linear range was at least 10–2000 nmol per tube. Intra-assay (within-day) and inter-assay (between-day) coefficients of variation for each compound are shown in Table II. The excellent linear relationship in the calibration curves and the reasonably

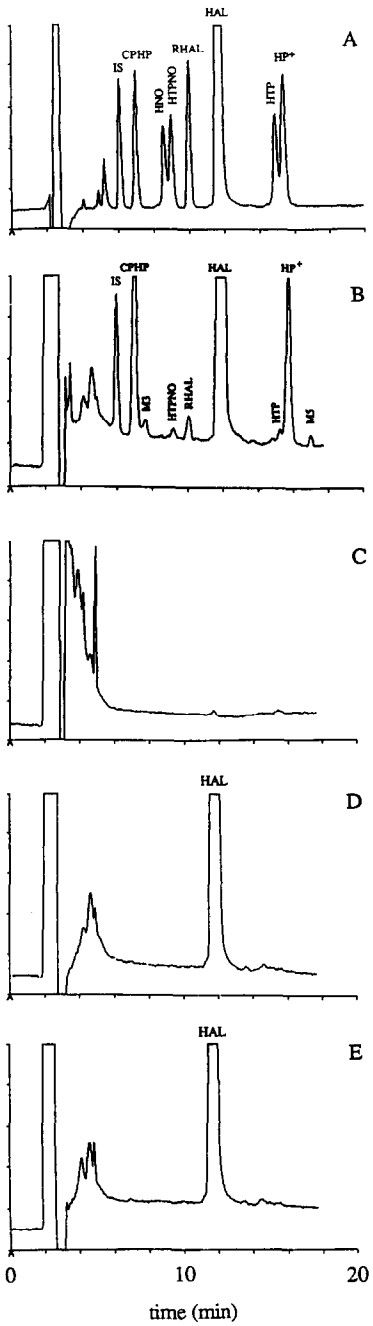

Fig. 3. HPLC of (A) a mixture of authentic compounds (10 nmol), (B) haloperidol metabolic mixture from mouse hepatic microsomes and (C–E) their control incubates. The control incubates are those without substrate (C), without cofactor (D) and with inactivated microsomes (E). Metabolic incubations were caried out for 30 min with 1 μmol of haliperidol per incubate. Monitored at 220 nm.

TABLE I

CALIBRATION CURVE OF HALOPERIDOL AND ITS METABOLITES FOR HPLC ANALYSIS

Results were from two duplicate experiments.Linear regression equations were: CPHP: $y = -0.019 + 0.0908x$ ($r = 0.999$); RHAL: $y = -0.001 + 0.0787x$ ($r = 1.000$); HTPNO: $y = -0.115 + 0.448x$ ($r = 1.000$); HTP: $y = 0.097 + 0.319x$ ($r = 1.000$);HP^+: $y = 0.041 + 0.150x$ ($r = 1.000$); HAL: $y = 1.063 + 0.168x$ ($r = 1.000$).

Amount (nmol per tube)	Peak-height ratio to internal standard					
	CPHP	RHAL	HTPNO	HTP	HP^+	HAL[a]
1.0			0.490	0.420	0.211	2.079
3.0	0.342	0.256	1.387	1.061	0.487	5.730
8.0	0.739	0.646	3.634	2.877	1.342	15.418
20.0	1.681	1.549	8.250	6.479	3.063	34.724
50.0	4.399	3.889	21.825	15.943	7.488	85.440
100.0	9.143	7.900	44.970	31.970	15.093	168.957
200.0	18.535	16.453	92.051	65.289	31.564	335.001

[a] The concentrations of haloperiodol were ten times the amount indicated.

good reproducibility of the HPLC system are probably owed to the simple treatment of the samples prior to analysis. The sample preparation procedures used in the present experiment were virtually, from a precision point of view, a series of dilutions. This provides maximum accuracy and minimum artifacts introduced during sample preparation. Thus this HPLC system was deemed suitable for quantitative purposes.

Table III shows the amount of metabolites produced by microsomal preparations from different animal species. The amount of M3 was estimated by assuming the UV absorption of M3 is similar to that of HAL and using the same calibration curve as that used for HAL. This assumption was made because the UV spectrum of M3 is very similar to that of its parent compound, HAL [6].

It can be seen (Table III) that the metabolism of HAL exhibits large species differences. The or-

TABLE II

REPRODUCIBILITY OF THE HPLC SYSTEM

Compound	Added (nmol per tube)	Intra-assay[a]		Inter-assay[b]	
		Found (mean ± S.D.) (nmol per tube)	R.S.D. (%)	Found (mean ± S.D.) (nmol per tube)	R.S.D. (%)
HTPNO	8	8.37 ± 0.02	0.3	8.51 ± 0.41	4.8
HTP	8	8.71 ± 0.09	1.0	8.48 ± 0.71	8.4
HP^+	8	8.67 ± 0.35	4.0	8.78 ± 0.54	6.2
CPHP	8	8.86 ± 0.46	5.2	8.91 ± 0.95	10.7
RHAL	8	8.23 ± 0.26	3.2	8.12 ± 1.13	13.9
HAL	80	82.10 ± 2.36	2.9	85.30 ± 6.70	7.9

[a] Results were from four determinations.

[b] Results were from three duplicate experiments.

TABLE III

SPECIES DIFFERENCES IN THE METABOLISM OF HALOPERIDOL

Incubation time was 10 min; haliperidol concentration was 100 μmol per incubate. Data are presented as nmol/mg of protein (mean ± S.D., n = 4).

Metabolite	Mice	Hamster	Rabbit	Guinea-pig
CPHP	4.73 ± 0.39	15.57 ± 0.44	6.15 ± 0.66	3.98 ± 0.44
HTPNO	0.18 ± 0.01	0.84 ± 0.12	0.12 ± 0.04	0.44 ± 0.08
RH	0.75 ± 0.12	0.99 ± 0.16	3.40 ± 0.47	3.30 ± 0.39
HTP	0.66 ± 0.12	0.63 ± 0.05	0.34 ± 0.12	0.04 ± 0.03
HP^+	2.63 ± 0.30	4.37 ± 0.39	1.94 ± 0.48	1.77 ± 0.23
M3	0.42 ± 0.06	1.90 ± 0.21	0.37 ± 0.12	35.27 ± 6.25

der of the amount of metabolites formed for CPHP is hamster > rabbit > mouse > guinea-pig; RHAL: rabbit = guinea-pig ≫ hamster = mouse; HP^+: hamster > mouse > rabbit > guinea-pig; M3: guinea-pig > hamster > mouse = rabbit. HTP and HTPNO were formed in relatively smaller amounts. The formation of HTP was in the order: mouse = hamster > rabbit > guinea-pig; for HTPNO: hamster > guinea-pig > mouse = rabbit. It is worth noticing that guinea-pig microsomes produce a large amount of M3 from HAL. This should assist the purification of M3 and allow the unambiguous characterisation of its structure. It can be calculated, from the amount of remaining HAL, that 61, 44, 21 and 6% of HAL was converted to metabolites by microsomal preparations from guinea-pig, hamster, mouse and rabbit, respectively. Therefore, the order of overall potency to metabolise HAL is guinea-pig > hamster > mouse > rabbit.

The total parent HAL plus metabolites detected are 104, 87, 89 and 103 nmol for guinea-pig, hamster, mouse and rabbit, respectively. Although, as the amount of M3 was calculated using the calibration curve for HAL, these figures should not be taken as absolute. Nevertheless, it can be seen that whereas nearly all the substrate can be accounted for using guinea-pig and rabbit preparations, it seems that a small portion of metabolites of hamster and mouse were not detected with the current analytical method.

Incubation with varying HAL concentrations was carried out and apparent Michaelis constants (K_m) and maximum velocity (V_{max}) values were obtained using the Lineweaver–Burk plot. The Lineweaver–Burk plot of the rate of formation of CPHP, HTPNO, HTP, HP^+ and M3 are shown in Fig. 4. Apparent V_{max} and K_m values calculated are listed in Table IV. M3 was quantified using the same calibration curve as HAL.

Clearly one must be cautious in interpreting these kinetic data, because at lower HAL concentrations, the rate measured during 10 min incubation may already be starting to deviate from line-

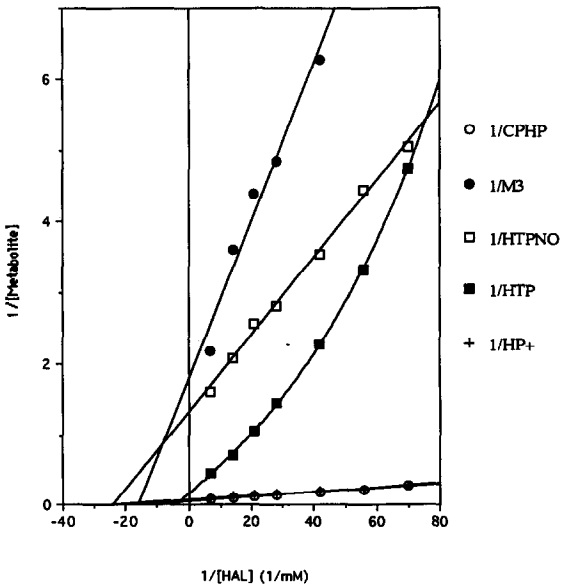

Fig. 4. Lineweaver–Burk plots of the rate of formation of CPHP, HTPNO, HTP, HP^+ and M3. Values for y-axis were expressed as 1/(nmol/g protein/min). The amounts of M3 were estimated using the calibration curve of HAL.

TABLE IV

APPARENT KINETIC PARAMETERS FOR THE FORMATION OF SOME METABOLITES OF HALOPERIDOL BY MICE HEPATIC MICROSOMAL PREPARATIONS

Compound	K_m (μM)	V_{max} (nmol/g protein/min)	R
CPHP	63.6	20.0	0.997
HTPNO	41.6	0.76	0.996
HP^+	35.9	13.1	0.999
M3	62.3	0.562	0.964

arity because of a falling substrate concentration; which would decrease the apparent V_{max}. On the other hand, lipophilic drugs like butyrophenones are readily absorbed into cell membrane lipid bilayers and thus may escape any action of the enzymes studied [23], which would increase the apparent K_m of the reduction. Furthermore, HP^+ and HTPNO are probably formed from a metabolic intermediate, HTP. This would further reduce the values of the kinetic data obtained. Nevertheless, the reaction rates of formation of CPHP, HTPNO, HP^+ and M3 gave a straight line against HAL concentration in the Lineweaver–Burk plot. The Lineweaver–Burk plot of HTP formation shows curvature. This is probably because HTP is further converted to HP^+ and HTPNO as well as its dealkylation product CPTP [6]. The further metabolism of a primary metabolite would result in upward curvature at low substrate concentrations in a Lineweaver–Burk plot [24].

It was shown that the formation of the metabolites of HAL were dependent on incubation time, microsome concentration and was saturable with increased substrate concentration. No metabolic products were found in control experiments. All these are characteristics of enzymatic reactions.

In conclusion, the HPLC system selectively analysed most Phase I metabolites of HAL with good selectivity and reproducibility. This provides a useful tool for metabolic studies of HAL. Further studies are currently being carried out on the enzymology of HAL metabolism and its clinical relevance.

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