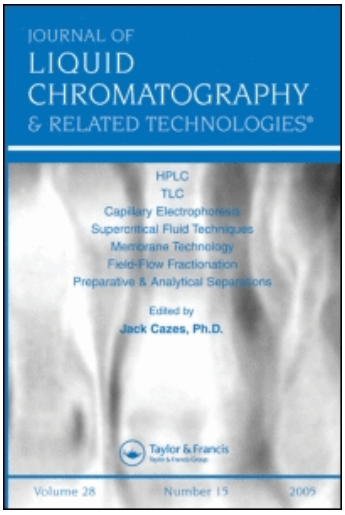

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ABSTRACT

A chromatographic method for quantifying the potential antipsychotic compound BIMG 80 (I) was developed and employed for plasma and brain measurements in rats given pharmacologically effective (ED_{50}) doses in the dexamphetamine-antagonism test. The analytical procedure is based on isolation of the compound and the internal standard (haloperidol) from plasma and brain constituents using the LC-18 SPE cartridge, with satisfactory recovery and specificity, isocratic separation on a C18 reversed-phase column coupled with a UV detector set to 225 nm.

Pharmacokinetic studies showed that mean plasma and brain concentration-times curves of I were parallel but concentrations in brain were 3-5 times of those in plasma. Maximum brain concentrations were of similar magnitude after equiactive doses given by different routes, ranging from 0.30 (± 0.04) µg/g after s.c. injection (1 mg/kg) to 0.98 (± O.68) µg/g but with broad inter-animal variability when given i.p. (11 mg/kg). These results suggest that I brain concentrations must be between 0.3-1 µg/g in the rat in order to inhibit hypermotility induced by dexamphetamine in 50% of the animals.

INTRODUCTION

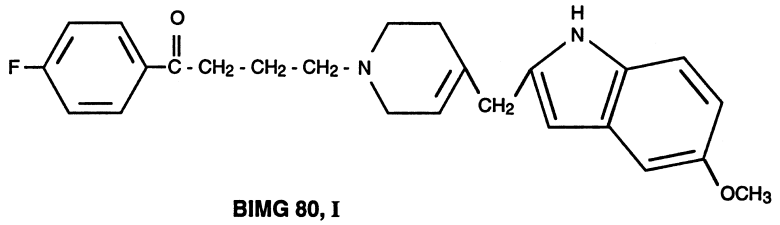
BIMG 80 (I), 5-methoxy-3-[N-(4-(4-fluoro-phenyl)-4-oxobutyl)-1,2,5,6-tetrahydropyridin-3-yl-methyl]-1H-indole is a butyrophenone derivative (see Figure 1) with potential antipsychotic activity.[1] In *in vitro* and *in vivo* neurochemical studies it shows a multireceptorial mechanism of action that leads to increased dopamine release preferentially in the medial pre-frontal cortex.[1] In behavioural tests it presents an atypical profile showing activity in animal models predictive of efficacy on positive symptoms of schizophrenia (i.e antagonism toward dexamphetamine-induced hypermotility in the rat) and inactivity in those predictive of induction of extrapyramidal side effects (antagonism toward dexamphetamine-induced stereotypy and catalepsy).[2] Until now, however, no information is available on whether the compound crosses the blood-brain barrier (BBB) and the pharmacologically effective concentrations achieved at the site of action.

The development of the present high performance liquid chromatographic (HPLC) procedure for the quantification of I in plasma and brain tissue made it possible to investigate the concentrations of I achieved in brain tissue after doses inhibiting hypermotility in rats (ED_{50} in the dexamphetamine-antagonism test) and their relationship with plasma concentrations.

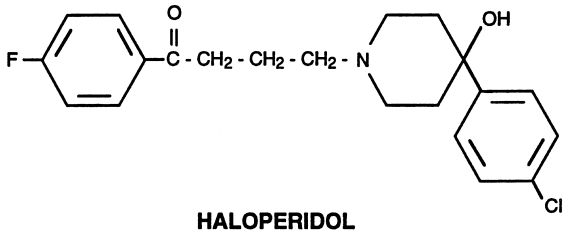
MATERIALS AND METHODS

Chemicals

Compound I hemisuccinate was supplied by Boehringer Ingelheim Italia (Milano, Italy). Haloperidol, the internal standard (I.S), was a kind gift from Lusofarmaco (Milano, Italy). Stock solutions of I and I.S were prepared by



BIMG 80, I



HALOPERIDOL

Figure 1. Chemical structures of compound I and haloperidol (the internal standard).

dissolving the compounds in methanol at a concentration of 1 mg/mL, and were stable for at least one month when stored at -20°C. Working standard solutions were prepared from the stock solutions by dilution with methanol and kept at -20°C.

Other chemicals and solvents, CH_3CN (C. Erba, Milano, Italy), methanol, H_3PO_4, KH_2PO_4, NH_4OH, triethylamine (E. Merck, Darmstadt, Germany), were of analytical-reagent grade and were used without further purification.

Chromatographic Apparatus and Conditions

HPLC analysis was done on a Waters system equipped with a Wisp-712 sample processor, a Model 510 solvent delivery system and a Model 486 UV detector set at 225 nm (Waters, Milford, MA, USA), coupled to a model C-R6A Chromatopac Shimadzu integrator (Shimadzu, Kyoto, Japan). Separation was carried out on a Hypersil ODS column (250 x 4.6 mm I.D., 5 µm particle size) (C.P.S. Analitica, Milano, Italy) at 30°C, protected by a MPLC New Guard precolumn (Applied Biosystems, Inc., CA, USA). The mobile phase was CH_3CN (solvent A) and an aqueous solution containing 20 mM KH_2PO_4 and 20

mM triethylamine buffered to pH 3.0 with H_3PO_4 (solvent B) in a ratio of 35:65 (v/v). The eluent was filtered through a 0.45-µm filter, degassed before use, and delivered isocratically at a flow-rate of 1.0 mL/min.

Extraction Procedure

Supelchem LC-18 SPE cartridges (Supelco, Milano, Italy) were used to clean up plasma and brain samples. The cartridges were pre-wetted with 4 mL of methanol, 4 mL of distilled water and 2 mL of KH_2PO_4. Then, after adding 50 µL of the I.S (2 or 10 µg/mL), 1 mL of plasma (diluted to 2 mL with 0.5 M KH_2PO_4) was added and the cartridges were washed with 2 mL KH_2PO_4, 4 mL distilled water, 4 mL of CH_3CN-water (60:40, v/v) and 0.6 mL of 1 M NH_4OH in methanol. The compound was removed by eluting the cartridges with 2 mL of 1 M NH_4OH in methanol, and evaporated to dryness in vacuo. The residue was dissolved in the mobile phase and analyzed by HPLC with UV detection (225 nm).

Brain tissue was homogenized (10 mL/g) in 0.5 M KH_2PO_4-CH_3CN (70:30, v/v) and 2 mL were centrifuged at 15000 g for 15 min. The supernatant was processed as described for plasma.

To verify the stability of BIMG 80 in rat plasma and brain homogenate before extraction, spiked samples were stored at room temperature for 2 h and at -20°C for one month before analysis. The analytical responses of these samples were compared with those of immediately analyzed samples. The stability of I in the mobile phase in the autosampler at room temperature was assessed by repeated injection of spiked plasma and brain samples for 36 h.

Accuracy and Precision

The precision and accuracy of the method were determined by replicate analyses of quality control samples (QC) containing small, medium, and large known amounts of I, stored at -20°C. On different days these QC were assayed with standard samples and the calculated concentrations were compared (inter-assay variance). Intra-assay variance was checked by replicate analysis of QC samples on the same day.

Daily standard curves with five concentrations over the working range were plotted in duplicate with QC samples injected between the two sets of standards. The relative response factor was computed as the I to the I.S peak-height ratio.

Calibration lines were constructed by least squares linear regression on the relative response factors against the nominal concentration of the compound. Concentrations of QC (and unknown samples) were obtained by back-solving the usual equation of the calibration line.

The lowest calibration standard corresponded to the limit of quantification (LOQ), i.e., the lowest concentration that could be measured with acceptable accuracy and precision (≤20%),[3] as determined in separate studies.

The upper limit of quantitation was arbitrarily defined as 0.5 µg/mL for plasma and 2.5 µg/g for brain tissue, these concentrations exceeding those likely to be encountered in brain-to-blood distribution studies, as determined in pilot studies.

Animals and Treatment

Male CD-COBS rats weighing 175-200 g (Charles River, Italy) were used. Procedures involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance with national (D.L. n. 116, G.U., suppl. 40, 18 Febbraio 1992, Circolare No. 8, G.U., 14 Luglio 1994) and international laws and policies (EEC Council Directive 86/609, OJ L 358,1, Dec. 12, 1987; Guide for the Care and Use of Laboratory Animals, U.S. National Research Council, 1996).

On three different occasions rats received compound I (dissolved in 0.0085 M phosphoric acid containing 4.5 g mannitol), orally, intraperitoneally (i.p.) or subcutaneously (s.c.) at doses of 65, 11, and 1 mg/kg (as free base) in volumes of 10, 5, and 2 mL/kg body weight, respectively.

The doses and vehicle of I were derived from pharmacological studies carried out at the Boehringer Ingelheim Laboratories (Milan, Italy). Water and food were freely available throughout the study.

Rats were killed by decapitation under deep anesthesia, 15, 45, and 75 min after dosing. Blood samples were collected in heparinized tubes, centrifuged and the plasma was stored at -20°C. Brains were rapidly removed, blotted with paper to remove excess surface blood and stored at -20°C until analysis.

The area under the plasma and brain concentration-time curves over the 75-min test period (AUC_t = $AUC_{0\text{-}75min}$) was determined by the linear trapezoidal rule. The maximum concentration (C_{max}) and the time of its occurrence (t_{max}) were read directly from the plasma and brain concentration-time data.

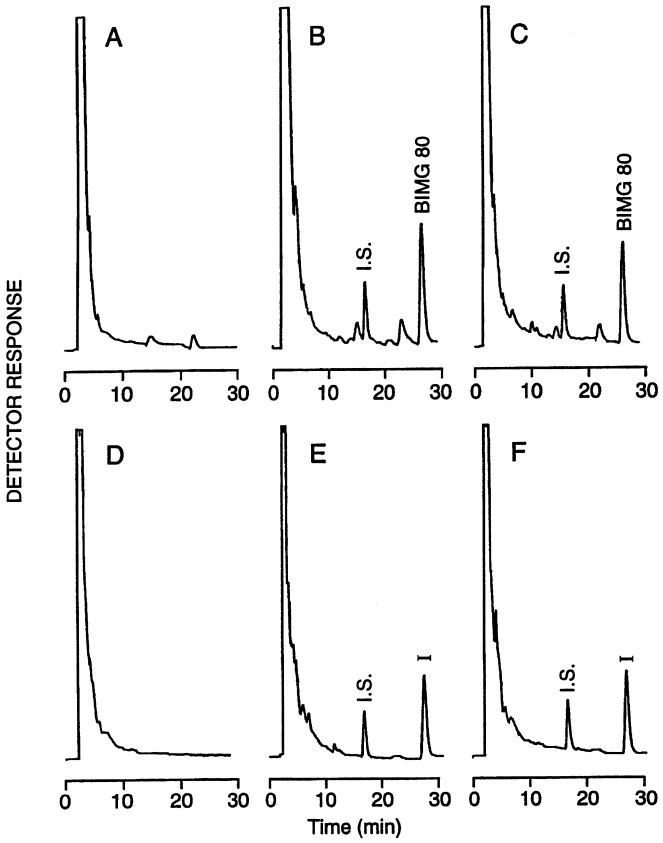


Figure 2. Chromatograms of extracts from drug-free plasma (A) and brain (D), spiked plasma (B) and brain (E) and samples from an intraperitoneally treated rat (10 mg/kg I, 30 min after dosing). I.S = internal standard (0.1 µg/mL or 0.5 µg/g). Column: Hypersil ODS 5 µm (250 cm x 4.6 mm I.D). Mobile phase: CH_3CN (solvent A) and 20 mM KH_2PO_4 containing 20 mM triethylamine buffered to pH 3.0 (A:B = 35:65, v/v). UV detection 225 nm.

RESULTS AND DISCUSSION

Chromatography

Initial efforts to isolate I from plasma and brain homogenized with available liquid-liquid extraction procedures for butyrophenone derivatives[4-6] were not satisfactory under our experimental conditions in terms of specificity.

It was then found that the compound could be recovered selectively and efficiently using C18 disposable extraction cartridges. Various solvents were tested to clean up the samples and the one described was found to extract only a few impurities from plasma and no interfering substances from plasma and brain, at the same time yielding acceptable recoveries of I from both tissues. Haloperidol was chosen as I.S. because it was well separated from I and the endogenous substances and its extraction recovery from plasma and brain was similar to that of I.

Examples of chromatograms of extracts from spiked plasma (B) and brain (E) and plasma (C) and brain (F) of a rat treated with I i.p. are shown in Figure 2. Approximate retention times were 26 min for BIMG 80 and 16 min for the I.S., and no interferences was observed from drug-free plasma (A) and brain (D).

The overall mean recovery, determined by comparing the peak height of I obtained from plasma and brain plasma spiked at three levels (low, medium and high) with those resulting from direct injection of the compound dissolved in the mobile phase, averaged 89 ± 10% for plasma and 68 ± 5% for brain tissue, with no significant dependence on concentration over the range investigated. Mean recovery of the I.S at the concentration of 100 ng/mL or 500 ng/g averaged 93 ± 3% in plasma and 63 ± 4% in brain.

The relationships between the peak-height ratios of I to the I.S and the amount of the compound added to plasma and brain homogenate were always linear, with the r^2 invariably exceeding 0.99. The slope of these curves ranged from 0.0183 to 0.0196 for plasma (average regression equation y = 0.0187 + 0.1190) and from 0.01666 to 0.01774 for brain (y = 0.0169x + 0.0844). The QC results obtained in these studies are summarized in Table 1. Mean coefficients of variation (C.V) ranged from 4% to 13% in plasma and from 6% to 17% in brain for the different experiments with two QC at each compound level and experiment.

The overall mean accuracy (R.E), calculated from the deviation of the mean concentration from the nominal value indicated an inter-assay variation from -7% to 0% for plasma and from -5% to 1% for brain. The limit of quantification was approximately 25 ng/mL or 125 µg/g using 1 mL of plasma or approximately 200 mg of brain tissue.

Compound I was stable in plasma and brain homogenates for at least 2 h at room temperature and up to one month at -20°C (response 97-104% of freshly prepared samples). Replicate injection of QC samples confirmed that the compound was stable in the automatic sampler for at least 36 h, without the need for refrigeration.

Table 1

Summary of Quality Control Results for I Analysis in Plasma and Brain

Conc. Added (ng/mL or g)	Conc. Found (ng/mL or g)	Coefficient of Variation (%)	Accuracy (%)
Plasma[a]			
25	23 ± 3	13	-7
100	100 ± 4	4	0
500	491 ± 39	8	-2
Brain[a]			
125	119 ± 20	17	-5
500	507 ± 45	9	1
2500	2482 ± 137	6	-1

[a] 1 mL of plasma or approximately 0.2 g of brain tissue.

Animal Studies

After effective doses (ED_{50}) in the dexamphetamine-antagonism test I was rapidly absorbed, as evidenced by the plasma t_{max} which averaged 15 min, regardless of the route of administration. Although mean C_{max} and AUC_t within the 75-min test period were slightly lower after s.c. (0.10 ± 0.04 µg/mL and 4 µg/mL.min, respectively) than i.p. (0.18 ± 0.12 µg/mL and 7 µg/mL.min) or oral dosing (0.11 ± 0.13 µg/mL and 5 µg/mL.min), plasma concentrations varied. Similar variability has been observed in initial toxicokinetic studies of oral I in rats and dogs. This may be due to differences between animals in the rate and extent of absorption and first-pass metabolism. However, the fact that it was almost comparable after i.p. and oral doses suggests that differences in first-pass metabolism rather than absorption from the gastro-intestinal tract or intraperitoneal cavity account for the large variability of I plasma concentrations in the rat.

Reflecting the time profile in blood, I achieved higher mean concentrations at the site of action at approximately the same time as in plasma. Mean brain C_{max} were 3-5 times the plasma C_{max} but again comparable in the three different experimental conditions (0.30 ± 0.04, 0.98 ± 0.68 and 0.41 ± 0.20 µg/g after s.c.., i.p.., and oral dosing). After the peak mean brain concentrations remained consistently 3-5 times the plasma concentrations, the mean AUC_t was, respectively, 18.1, 36.4, and 22.7 µg/g x min after s.c., i.p., and oral dosing.

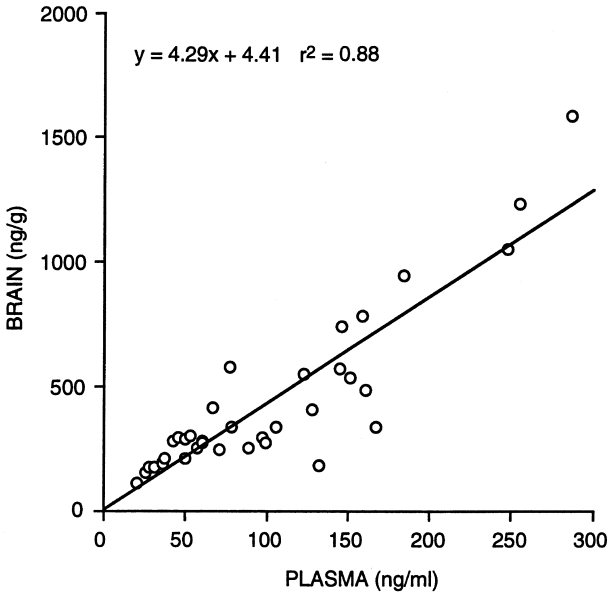


Figure 3. The relationship between the brain and plasma concentrations of I within the antagonism test period after subcutaneous (1 mg/kg), intraperitoneal (11 mg/kg) and oral ED_{50} (65 mg/kg).

Although it could not be established that pseudoequilibrium was really achieved within the 75-min dosing interval there was a linear relationship between the brain and plasma concentrations (brain concentration = 4.3 plasma concentration + 4.4; $r^2 = 0.88$) (Figure 3).

CONCLUSIONS

For centrally acting drugs it is essential to know the brain-to-blood partition and brain concentrations reached at the site of action so as to clarify their pharmacokinetic behavior, interpret their neurochemical actions and any relationships with the pharmacological responses.[7] The method proposed here for quantification of the potential antipsychotic compound I in plasma and brain is relatively simple and selective. Sensitivity appears adequate and linearity and precision acceptable within the range of concentrations likely to be encountered in plasma and brain after pharmacologically effective doses. The method can be applied to measure I in biological specimens of other species in toxicokinetic studies (unpublished results).

It has also been recently employed to determine brain concentrations of haloperidol after doses affecting dopaminergic mechanisms in the rat,[8] and may thus have potential for other pharmacological applications.

The preliminary pharmacokinetic study in the rat provides evidence that I rapidly crosses the BBB, achieving mean concentrations at the site of action higher than in blood, as does haloperidol and pharmacologically related drugs.[7,9-10] Mean brain C_{max} of I were variable, reflecting the variability in plasma, but of similar magnitude after equiactive s.c., i.p. and oral doses in the dexamphetamine-induced hypermotility test. While these data suggest that the bioavailability of the compound is low in the rat they indicate that mean brain concentrations must be between 0.3-1 µg/g (about 0.7-2.5 µM, assuming 1 g of brain tissue equivalent to 1 mL of water) in the rat brain in order to inhibit dexamphetamine-induced hypermotility in 50% of the animals. Interestingly, these *in vivo* concentrations are close to those *in vitro* affecting the release of [^{3}H]-dopamine in superfused synaptosomes (M. Gobbi, personal communication), thus an interaction with the dopaminergic system is very probably involved in the mechanisms underliyng the pharmacological activity in rats.

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