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# Determination of 6-chloro-3-(3-cyclopropyl 1,2,4-oxadiazol-5-yl)-5-methyl-imidazo<1,5-*a*>-quinoxalin-4(5*h*)-one in rat serum, urine and brain by solid-phase extraction and liquid chromatography

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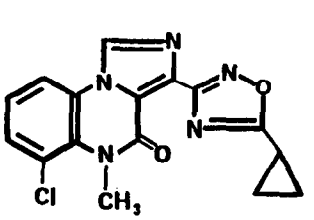
**Abstract**: A simple, rapid, and accurate liquid chromatographic method with ultraviolet detection and solid-phase extraction is described for the quantitation of 6-chloro-3-(3-cyclopropyl 1,2,4-oxadiazol-5-yl)-5-methyl-imidazo<1,5-*a*>-quinoxalin-4(5*h*)-one (**I**, U-80447) in rat serum, urine and brain. Linear calibration curves were obtained in the concentration ranges of 5 ng $ml^{-1}$–20 μg $ml^{-1}$ (serum), 20 ng $ml^{-1}$–20 μg $ml^{-1}$ (urine), and 50 ng $g^{-1}$–200 μg $g^{-1}$ (brain). Intra- and inter-assay precision and accuracy were all found to be <10% at the three concentrations evaluated. The absolute extraction recovery each from serum, urine and brain was ≥90%. Application of this method to the quantitation of the title compound in rat serum and brain for a pharmacokinetic study is reported.

**Keywords**: *LC; solid-phase extraction; serum; urine; brain; rats; brain/serum ratio; pharmacokinetics.*

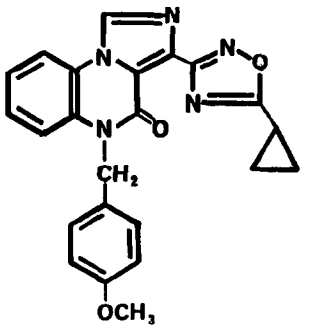
## Introduction

6-Chloro-3-(3-cyclopropyl 1,2,4-oxadiazol-5-yl)-5-methyl-imidazo<1,5-*a*>-quinoxalin-4(5*h*)-one (**I**, U-80447) (Fig. 1) is under evaluation as an hypnotic agent. **I** is an oxadiazole substituted benzodiazepine which showed reduced physical dependence inducing properties compared with benzodiazepine agonists, minimal amnesia inducing effects and high potency on locomotor based hypnotic test.

To determine the oral bioavailability, pharmacokinetics and initial toxicology of **I** in animals, it was necessary to have a sensitive and selective analytical method to determine the concentrations of **I** in serum and urine. Being a potential hypnotic it was also particularly important to know the brain concentrations of **I** in rats. Several liquid chromatographic (LC) [1–3] and gas chromatographic (GC) [4, 5] methods have been reported for the quantitation of other imidazobenzodiazepines in biological samples. However, most of those methods were for plasma samples and have used a liquid–liquid extraction procedure. The present work was concerned with the development of an LC method with ultraviolet (UV) detection using a solid-phase




I




II (IS)

**Figure 1**
Chemical structures of compounds **I** and **II** (IS).

extraction (SPE) for the determination of **I** in rat serum, urine and brain.

## Experimental

*Reagents and materials*

Compounds **I** and **II** (internal standard) (Fig. 1) were provided by The Upjohn Company (Kalamazoo, MI, USA). HPLC grade acetonitrile was obtained from Burdick and Jackson (Muskegon, MI, USA). Acetic acid, ammonium hydroxide and potassium phosphate (dibasic) were of analytical reagent grade and purchased from Mallinckrodt Inc. (Paris, KY, USA). Purified water was produced by a Milli-Q reagent water system (Millipore Corporation, Bedford, MA, USA).

*Instrumental parameters*

The HPLC system consisted of a Waters M-6000A pump (Milford, MA, USA) coupled with a Kratos Spectroflow 783 variable wavelength ultraviolet (UV) detector (Ramsey, NJ, USA), and a Perkin–Elmer ISS-100 Autosampler (Norwalk, CT, USA). The analytical column contained an ODS phase (250 × 4.6 mm i.d., 5 μm, Jones Chromatography, Littleton, CO, USA) and was protected by a guard column (Pelliguard ODS, 50 × 2.1 mm i.d., 32 μm, Whatman, Clifton, NJ, USA). The mobile phase for the isocratic reverse phase chromatography was acetonitrile–water–acetic acid (45:53.8:1.2, v/v/v), with a final apparent pH of 6.0 ± 0.1 adjusted with ammonium hydroxide solution, and was filtered and helium degassed prior to use. The chromatographic system was operated at ambient (21–23°C) temperature with a flow rate of 1.0 ml $min^{-1}$. The UV absorbance of column effluent was monitored at 325 nm with 0.005 a.u.f.s. sensitivity.

Quantitation was accomplished by measurement of the peak height ratio of drug to internal standard (IS), which was integrated by a Harris Computer System. The standard curve, along with a statistical evaluation of standards linear fit, were computed by the linear regression program. The unknown concentration was determined by inverse prediction against the standard curve.

*Procedures*

*Preparation of standards.* Stock solutions of **I** and IS were prepared by dissolving 20 mg accurately weighed compound in 50 ml acetonitrile and diluting to volume with water in a 100-ml volumetric flask to give a concentration of 200 μg $ml^{-1}$ **I** or IS. The stock solutions were diluted with acetonitrile–water (30:70, v/v) to 10, 1 and 0.1 μg $ml^{-1}$ working solutions. Stock and working solutions were stored at 4°C. Serum, urine, or brain standards were prepared by aliquoting appropriate volumes of stock and working solutions to 1 ml of control rat serum, urine, or brain homogenate (drug free) to produce a concentration series ranging from 5 ng $ml^{-1}$ to 20 μg $ml^{-1}$, 20 ng $ml^{-1}$ to 20 μg $ml^{-1}$, or 50 ng $g^{-1}$ to 200 μg $g^{-1}$ for serum, urine and brain, respectively.

*Sample preparation.*

*Serum.* Twelve $C_{18}$ SPE columns (100 mg per 1.0 ml, Analytichem International Inc., Harbor City, CA, USA) placed on the Vacuum Extraction Manifold (Supelco Inc., Bellefonte, PA, USA) were prewashed with one column volume of acetonitrile followed by one column volume of 0.1 M $K_2HPO_4$ solution. Unknown serum samples (1 ml) were mixed with 50 μl of IS working solution (10 μg $ml^{-1}$) and loaded onto each SPE column with a negative pressure of 86 kPa. After the SPE columns were vacuum-aspirated (approximately 27 kPa) for 5 min, 100 μl of acetonitrile–water (30:70, v/v) followed by 2 ml of 0.1 M $K_2HPO_4$ solution was applied to rinse each column (86 kPa). The columns were dried with vacuum aspiration (approximately 27 kPa) for 10 min. Compounds **I** and IS were eluted from the column with 300 μl of acetonitrile by applying a slow uniform pressure to the top of the column using nitrogen gas (about 0.2 kg $cm^{-2}$). Each eluate was collected into a 2 ml autosampler vial and mixed with 200 μl of purified water, and 50 μl of the mixture was injected onto the LC system for analysis.

*Urine.* One millilitre of urine sample was mixed with 100 μl of IS working solution (10 μg $ml^{-1}$) and transferred to the prewashed SPE column. Except for the column rinse step, which used 150 μl acetonitrile–water (30:70, v/v) under gravity flow (without vacuum) followed by 2 ml of 0.1 M $K_2HPO_4$ solution with vacuum aspiration (86 kPa) to rinse the column, and for the volume of water (700 μl instead of 200 μl) used to mix with the acetonitrile extract for final LC analysis, the urine extraction procedure was the same as that described for serum.

*Brain.* Unknown brain samples were prepared by homogenizing accurately weighed rat brain sample (approximate 200 mg) in a 5 ml grinding chamber, in which, 1 ml of acetonitrile–water (50:50, v/v) was added. The homogenate was combined with 1 ml of water, which was used for rinsing the grinder piston, and then vortexed for 30 s. One millilitre of the brain homogenate was transferred to a 1.5 ml microcentrifuge tube, mixed with 50 μl of IS working solution (10 μg $ml^{-1}$), and centrifuged at 3000 rpm for 1 min by a Brinkmann 5415 Micro Centrifuge (Westbury, NY, USA). The supernatant of brain homogenate was then transferred to a $C_{18}$ SPE column. The rest of the SPE procedure was the same as that for the serum method.

*Validation.* To determine the linear range of the method, freshly prepared standard curves for **I** were analysed on 4 different days [6]. the limit of quantitation (LOQ) was estimated by analysing fortified serum samples at the presumed LOQ in five replicates to determine if it had acceptable precision and accuracy (<15%) [7]. The precision and accuracy of the method were evaluated at three concentrations. The intra-assay precision was determined by analysing five fortified serum samples at each concentration on the same day, and the inter-assay precision was obtained by analysing one fortified serum sample at each concentration on 4 different days [8]. The absolute extraction recovery was determined at three concentrations for **I** and IS. Samples at each concentration were extracted as outlined for unknown samples and analysed in four replicates. The peak heights of the extracted samples were compared with those of unextracted external reference standards containing the corresponding concentrations. In all cases, the means, standard deviations (SD), and relative standard deviation (RSD) were calculated. A *P*-value of $<0.05$ was considered as significant.

*Animal study.* The pharmacokinetic study of compound **I** in rats was conducted using 66 male Sprague–Dawley rats with approximate weight of 250 g. The vehicle group consisting of three rats received a single oral dose of control article (vehicle: 98.35% purified water, 1.25% avicel 591, 0.2% sorbic acid, and 0.2% polysorbate) and were killed for blood and brain samples at 1 h after dosing. The remaining three groups each containing 21 rats received a single oral dose of **I** by gavage at the dose levels of 10, 30 and 100 mg $kg^{-1}$, respectively. Three rats were killed for blood and brain samples at each time point of 0.5, 1, 2, 4, 8, 16 and 24 h after dosing. Blood samples were collected in clean vials and allowed to clot at room temperature for 20 min. The serum samples were harvested by centrifugation and stored at −20°C until analysis. Brains were immediately removed from rats and frozen on dry ice and stored at −20°C until analysis.

*Pharmacokinetic analysis.* Model-independent pharmacokinetics [9] were evaluated from the mean ($n = 3$) serum or brain concentration-time profiles of **I** utilizing a computer program PKCALC [10]. The maximum plasma concentration ($C_{max}$) and the time to reach $C_{max}$ ($T_{max}$) were determined directly from the serum or brain concentration–time profiles. The area under the serum/brain concentration–time curve from time zero up to the time of the last sample with an analyte concentration above LOQ ($AUC_t$) was calculated using the linear trapezoidal rule. Dose normalized $AUC_t$ was calculated by dividing $AUC_t$ by dose.

## Results and Discussion

### *LC method development*

Isocratic elution was preferred to gradient for the LC system. The composition of the mobile phase where the two compounds (**I** and IS) were eluted in a capacity factor ($k'$) range of 2–5 was selected by applying the strategies of DeSmet *et al.* [11] and Costanzo [12]. Acetonitrile–water–acetic acid (30:69:1, v/v/v) with a final pH of 4 (adjusted with ammonium hydroxide) was used as the starting point for optimization. An increase of acetonitrile and acetic acid percentage and pH of the mobile phase decreased the retention times of **I** and IS. The mobile phase eventually selected was acetonitrile–water–acetic acid (45:53.8:1.2, v/v/v) with a final pH of 6.0 ± 0.1, based on optimal $k'$ and peak shape of these two compounds. The chromatographic conditions gave sharp, symmetrical, and well-resolved peaks for **I** and IS with retention times ranging from 8.6 to 9.0 and 10.8 to 11.2 min, respectively ($k' = 2.5$ and 3.0, respectively). The total chromatographic run time was 14 min. Because the UV maximum of **I** is at 325 nm, a

relatively clean baseline was achieved, therefore the detector could be operated at 0.005 a.u.f.s. for higher sensitivity.

Typical chromatograms of the extracts of control (drug-free) rat biological samples added with IS, control biological samples

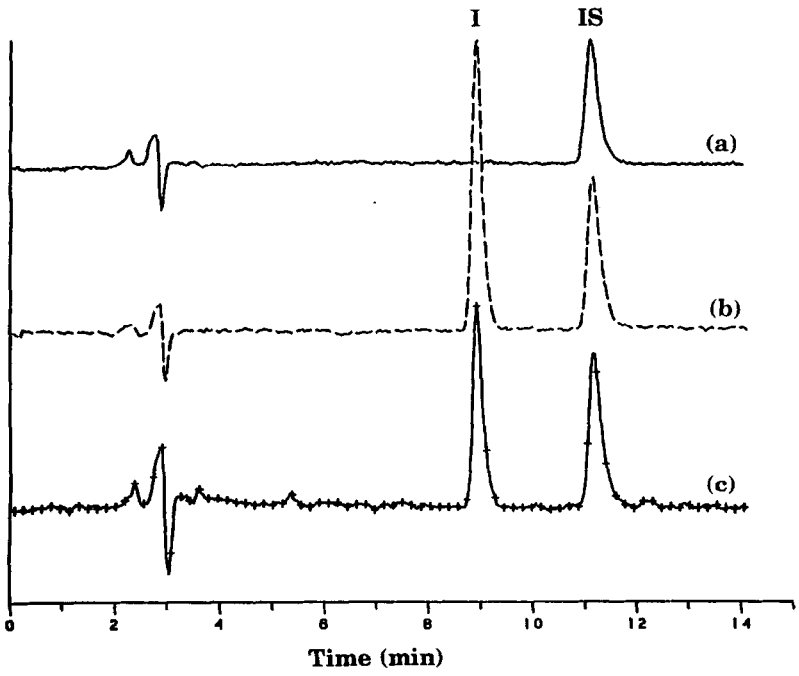



**Figure 2**
Chromatograms after extraction of (a) a rat serum blank added with 0.5 μg $ml^{-1}$ IS, (b) a serum blank fortified with 0.5 μg $ml^{-1}$ each of I and IS, and (c) a serum sample collected 1 h after a rat receiving 30 mg $kg^{-1}$ oral dose of **I**.

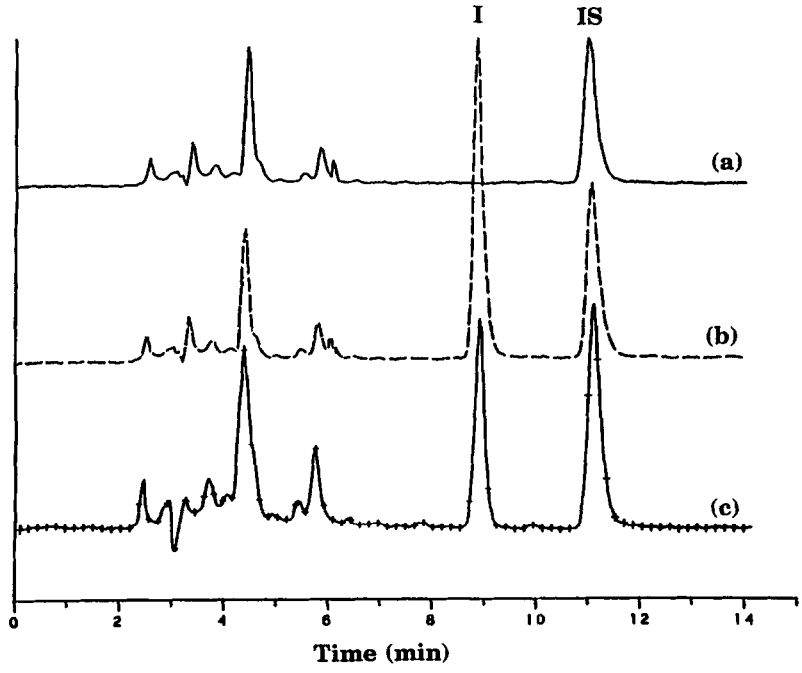



**Figure 3**
Chromatograms after extraction of (a) a rat urine blank added with 0.5 μg $ml^{-1}$ IS, (b) a urine blank fortified with 1 μg $ml^{-1}$ each of I and IS, and (c) a urine sample collected over a period of 15 h after the last dosing from a 14-day toxicity study with daily oral dosing of 30 mg $kg^{-1}$ **I**.

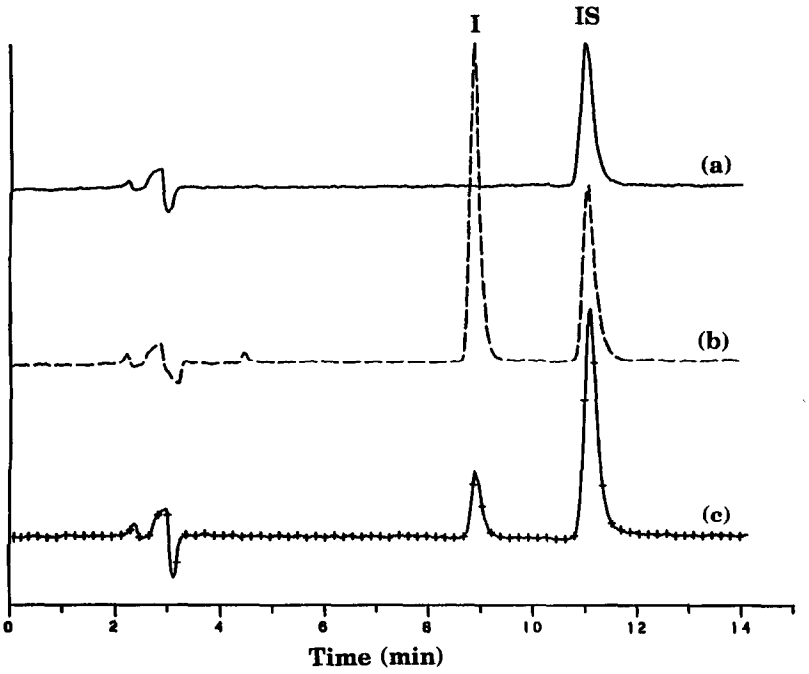



**Figure 4**
Chromatograms after extraction of (a) a rat brain blank added with 0.5 μg $ml^{-1}$ IS, (b) brain blank fortified with 5 μg $g^{-1}$ of I and 0.5 μg $ml^{-1}$ of IS, and (c) a brain sample collected 1 h after the rat receiving 30 mg $kg^{-1}$ oral dose of I.

**Table 1**
Absolute extraction recovery

| | Concentration | Extraction recovery (Mean ± SD, $n$ = 4) (%) Compound I | Compound II (IS) |
|---|---|---|---|
| Serum | 10 (ng $ml^{-1}$) | 97.1 ± 2.8 | 95.6 ± 2.1 |
| | 500 (ng $ml^{-1}$) | 95.4 ± 2.5 | 93.5 ± 3.0 |
| | 10 (μg $ml^{-1}$) | 94.5 ± 2.0 | 93.9 ± 2.3 |
| Urine | 20 (ng $ml^{-1}$) | 92.8 ± 3.2 | 94.0 ± 3.5 |
| | 500 (ng $ml^{-1}$) | 90.4 ± 1.6 | 91.6 ± 2.1 |
| | 10 (μg $ml^{-1}$) | 91.5 ± 3.1 | 91.2 ± 3.3 |
| Brain | 100 (ng $g^{-1}$) | 89.5 ± 3.0 | 88.7 ± 6.1 |
| | 5 (μg $g^{-1}$) | 87.8 ± 6.2 | 90.9 ± 1.2 |
| | 100 (μg $g^{-1}$) | 92.3 ± 3.4 | 95.4 ± 4.8 |

fortified with I and IS, and a post-dose biological sample collected from pharmacokinetic or toxicological studies, for serum, urine and brain, respectively, are shown in Figs 2–4. The control rat serum, urine, or brain chromatograms were free from endogenous matrix interferences at the retention times of I and IS. No potential metabolite peaks were observed in the extracted post-dose rat serum, urine, or brain samples.

*Extraction efficiency*

The mean absolute extraction recoveries (mean ±SD, $n$ = 4) at the three concentrations of I or IS for the serum, urine and brain assays are listed in Table 1. The overall mean ($n$ = 4) extraction recovery ranged from 94 to 97% and 93 to 96% (serum), 90 to 93% and 91 to 94% (urine), and 88 to 92% and 89 to 94%, for I and IS, respectively.

Various SPE columns including CN, PH, $C_8$ and $C_{18}$, were evaluated to determine their extraction efficiencies. Among these, the $C_{18}$ column proved to be the most satisfactory phase. Three hundred microlitres of acetonitrile was selected for the elution of I and IS after an investigation in which various solvents and volumes of solvent were tested. The

recovery was further improved to >90% when the SPE column was conditioned with 2 ml of 0.1 M $K_2HPO_4$ prior to the elution step. A large impurity front from endogenous urine components was observed following the extraction procedure developed for serum samples. When the SPE column was rinsed by 150 μl acetonitrile–water (30:70, v/v) under gravity flow, most of the endogenous urine components were eliminated without reducing the recovery. The solvent used for extracting compound **I** from brain was acetonitrile–water (50:50, v/v), 1 ml of which was sufficient to obtain an extraction recovery greater than 90%. The extract was further purified through the SPE, yielding a clean chromatogram with no interference peaks at the retention volumes of **I** and IS.

*Validation*

*Linearity and sensitivity.* Linear calibration curves were obtained in the concentration range of 5 ng $ml^{-1}$ to 20 μg $ml^{-1}$, 20 ng $ml^{-1}$ to 20 μg $ml^{-1}$, and 50 ng $g^{-1}$ to 200 μg $g^{-1}$, for serum, urine and brain, respectively, with correlation coefficients greater than 0.999 and intercepts being not significantly ($P > 0.05$) different from zero (Table 2). The slopes generated from the four calibration curves were not significantly ($P > 0.05$) different. Based on a signal-to-noise ratio of 3, the detection limits were 3 ng $ml^{-1}$ for serum, 10 ng $ml^{-1}$ for urine, and 30 ng $g^{-1}$ for brain. However, the LOQ at which the precision (RSD) and accuracy (bias) were acceptable, were 5 and 20 ng $ml^{-1}$ for serum and urine, respectively, and 50 ng $g^{-1}$ for brain based on using 1 ml serum or urine and 200 mg brain for extraction.

**Table 2**
Linear regression of fortified serum standard curves

| | Day | Slope | Intercept ($\times 10^{-1}$) | Correlation coefficient |
|---|---|---|---|---|
| Serum | 1 | 3.757 | −0.1975 | 0.9999 |
| | 2 | 3.813 | −0.5275 | 0.9999 |
| | 3 | 3.823 | −0.3824 | 0.9998 |
| | 4 | 3.739 | 0.1044 | 1.0000 |
| Urine | 1 | 4.097 | 0.1282 | 0.9998 |
| | 2 | 3.914 | −0.8299 | 0.9996 |
| | 3 | 3.972 | 0.1788 | 0.9999 |
| | 4 | 3.834 | −0.2354 | 0.9995 |
| Brain | 1 | 3.704 | 0.1151 | 0.9997 |
| | 2 | 3.566 | 0.2341 | 1.0000 |
| | 3 | 3.813 | 0.1172 | 0.9998 |
| | 4 | 3.893 | −0.2796 | 0.9994 |

*Precision and accuracy.* Excellent intra-assay ($n = 15$) and inter-assay ($n = 12$) precision (RSD) ranging from 1.0 to 5.8% and 0.9 to 7.5%, 1.5 to 6.7% and 1.6 to 9.8%, and 3.3 to 6.0% and 1.9 to 7.9%, for serum, urine and brain, respectively, was obtained at the three concentrations studied (Table 3). The accuracy was ≤6.0% in all cases. The system precision, determined by injecting a prepared sample five times, was found to be no greater than 0.6% in most cases.

*Application*

The analytical method described above was applied to the determinaton of **I** concentrations in serum and brain samples collected from a pilot pharmacokinetic study with compound **I**

**Table 3**
Assay precision* and accuracy†

| | | Intra-assay ($n = 5$) | | | Inter-assay ($n = 4$) | | |
|---|---|---|---|---|---|---|---|
| | Theoretical conc. | Mean ± SD ($n = 5$) | Precision (%) | Accuracy (%) | Mean ± SD ($n = 4$) | Precision (%) | Accuracy (%) |
| Serum | 10 (ng $ml^{-1}$) | 10.3 ± 0.6 | 5.8 | 3.0 | 10.6 ± 0.8 | 7.5 | 6.0 |
| | 500 (ng $ml^{-1}$) | 506.5 ± 15.3 | 3.0 | 1.3 | 511.7 ± 18.4 | 3.6 | 2.3 |
| | 10 (μg $ml^{-1}$) | 10.4 ± 0.1 | 1.0 | 4.0 | 10.2 ± 0.1 | 0.9 | 2.0 |
| Urine | 20 (ng $ml^{-1}$) | 20.9 ± 1.4 | 6.7 | 4.5 | 21.5 ± 2.1 | 9.8 | 6.0 |
| | 500 (ng $ml^{-1}$) | 492.3 ± 7.6 | 1.5 | −1.5 | 512.3 ± 8.6 | 1.6 | 2.5 |
| | 10 (μg $ml^{-1}$) | 10.2 ± 0.2 | 2.0 | 2.0 | 10.1 ± 0.2 | 2.9 | 1.0 |
| Brain | 100 (ng $g^{-1}$) | 97.7 ± 5.6 | 5.7 | −2.4 | 98.1 ± 7.9 | 8.0 | −1.9 |
| | 5 (μg $g^{-1}$) | 5.2 ± 0.3 | 6.0 | 4.0 | 4.9 ± 0.2 | 4.1 | 2.0 |
| | 100 (μg $g^{-1}$) | 99.1 ± 3.3 | 3.3 | −0.9 | 103 ± 4.1 | 4.0 | 3.1 |

*Precision = RSD = SD/mean × 100.
†Accuracy = per cent deviation = (measured–theoretical)/theoretical × 100.

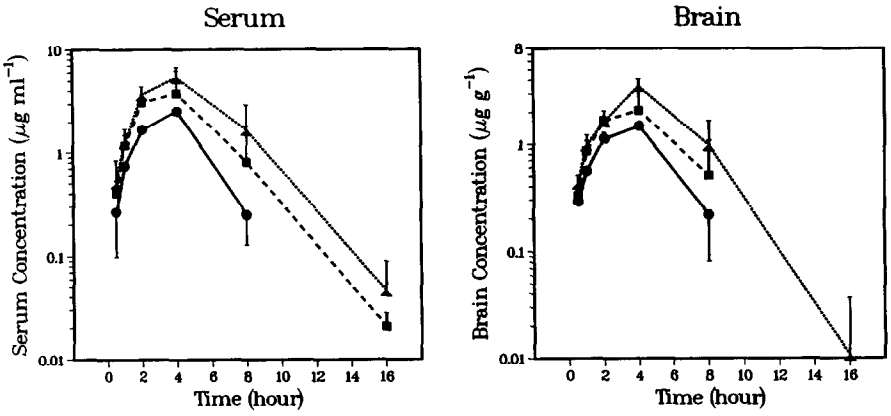



**Figure 5**
Average serum and brain concentration–time profiles of **I** in rats after oral doses of 10 mg $kg^{-1}$ (——), 30 mg $kg^{-1}$ (– – –) and 100 mg $kg^{-1}$ (· · ·) of **I**.

in rats. The mean serum and brain concentration–time curves at each dose level for **I** are shown in Fig. 5. The serum and brain concentration–time profiles were quite similar, therefore, similar distribution and disposition profiles of **I** can be anticipated. The high correlation between serum and brain concentrations was also demonstrated by their linear relationship, which yielded a significantly positive slope of 1.7 ($P < 0.05$) with a correlation coefficient of 0.97, and an interecept not being significantly different ($P > 0.05$) to zero (Fig. 6). The average brain–serum concentration ratio was 0.72 ± 0.23 μg $g^{-1}$/μg $ml^{-1}$ ($n$ = 82). These observations suggested that rapid equilibrium of **I** between serum and brain was reached after the rat received compound **I** orally, therefore, the brain does not represent a pharmacokinetic compartment distinct from serum.

Selected pharmacokinetic parameters calculated from the average concentration profile for serum and brain are summarized in Table 4. The mean concentration–time profiles of **I**

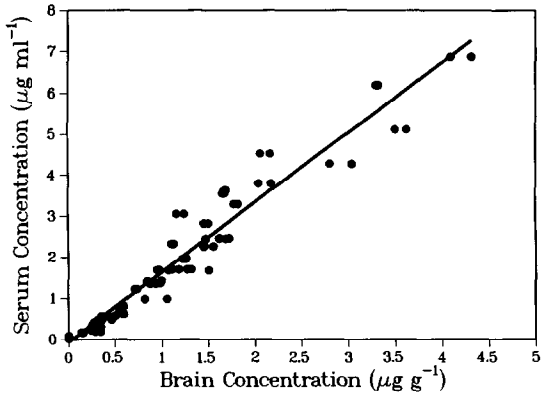



**Figure 6**
Correlation of brain and serum concentrations of **I** in rats.

were calculated from separate groups of three rats at each time point, and were evaluated using non-compartmental pharmacokinetic methods. The mean $T_{max}$ value was 4 h for both serum and brain in all doses. Although $C_{max}$ and $AUC_t$ increased with increasing dose, a 10-fold increase in the dose only

**Table 4**
Selected mean ($n$ = 3) pharmacokinetic parameters

| | Dose (mg $kg^{-1}$) | $C_{max}$ (μg $ml^{-1}$) (μg $g^{-1}$) | $T_{max}$ (h) | $AUC_t$ (μg h $ml^{-1}$) (μg h $g^{-1}$) | $AUC_t$/Dose |
|---|---|---|---|---|---|
| Serum | 10 | 2.550 | 4.0 | 12.004 | 0.833 |
| | 30 | 3.775 | 4.0 | 21.882 | 1.372 |
| | 100 | 5.419 | 4.0 | 33.023 | 3.030 |
| Brain | 10 | 1.497 | 4.0 | 6.779 | 1.475 |
| | 30 | 2.105 | 4.0 | 10.686 | 2.809 |
| | 100 | 3.563 | 4.0 | 16.086 | 6.211 |

produced a two- to three-fold increase in $AUC_t$ and $C_{max}$. The disproportionality was also indicated by the increase of dose-normalized $AUC_t$ ($AUC_t$/Dose) from 0.83 to 3.03 and 1.48 to 6.21 for serum and brain, respectively, when the dose increased from 10 to 100 mg $kg^{-1}$. Poor water solubility of **I** (<2.5 μg $ml^{-1}$) appeared to be a likely factor that contributed to the dose-dependent drug absorption.

## Conclusions

The HPLC method described in this report is rapid, sensitive, and allows the accurate and precise determination of **I** in serum, urine and brain using a simple solid-phase extraction technique with a minimum of organic solvent volume (300 μl). The assay proved useful in performing a preclinical pharmacokinetic study in rats, which indicated a rapid equilibrium of **I** between serum and brain and a dose-dependent drug absorption after oral administration. Furthermore, the method should be a practical procedure for the determination of a number of analogues of **I**, including the compound **II** (IS), in serum, urine and brain. Therefore, it could be useful in the selection of hypnotic lead compounds based on their *in vivo* pharmacokinetic characteristics.

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