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High-performance liquid chromatographic analysis of (*S*)-α-amino-5-phosphonomethyl[1,1′-biphenyl]-3-propanoic acid (EAB 515) in brain and blood microdialysate (on-line) and in plasma ultrafiltrate of freely moving rats

B.K. Malhotra[1,a], M. Lemaire[b], J.F. Brouillard[b], R.J. Sawchuk[a,*]

[a]*Department of Pharmaceutics, University of Minnesota, Minneapolis, MN 55455, USA*
[b]*Biopharmaceutical Department, Sandoz Pharma Ltd., CH-4002, Basel, Switzerland*

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Abstract

(*S*)-α-Amino-5-phosphonomethyl[1,1′-biphenyl]-3-propanoic acid (EAB 515, I), a competitive antagonist of the N-methyl-D-aspartate receptor, has significant pharmacological activity in the central nervous system (CNS). An extremely sensitive and selective analytical method was developed for the simultaneous analysis of I and its hydroxylated analog (RDC, II) in the microdialysate (MD) and plasma ultrafiltrate (UF) of rats. Microdialysis was used for in vivo sampling of unbound drug in the CSF, cortical extracellular fluid and in the blood of freely moving rats. Compound II was used for retrodialysis-based in vivo calibration of microdialysis probes to estimate the recovery of I. Compound I, being extremely hydrophilic with a high degree of ionization at the physiological pH of 7.4, has limited access to the brain regions. This, combined with its low microdialysis recovery, made the estimation of low brain concentrations of I a challenge. The analytes in MD and UF were separated (within 5 min) by reversed-phase HPLC on a 250×4.6 mm I.D. Maxsil 5 μm RP-2 column, and fluorescence of the eluent was monitored at 255 nm (λ_{ex}) and 320 nm (λ_{em}). A 0.09% (v/v) aqueous solution of trifluoroacetic acid (1 ml/min) was used as the mobile phase. The response for I in MD and UF samples was linear from 5 to 2000 ng/ml and from 20 to 10 000 ng/ml, respectively. The between-run (n=6) and within-run (n=3) variability of the assay was <15%. Plasma-protein binding of I (f_u=0.68) was determined to be linear from 0.1 to 10 μg/ml. The analytical sensitivity, precision and accuracy of this method was suitable for the characterization of the pharmacokinetics and the CNS distribution of I, following administration of intravenous (i.v.) infusion, single i.v. bolus and multiple i.v. bolus doses of I to freely moving rats, with continuous microdialysate sampling of multiple tissues and simultaneous on-line HPLC analysis. Pharmacokinetic parameters for I, as determined from concentrations in blood MD samples with on-line analysis, were in good agreement with those estimated from concentrations in the UF of plasma samples obtained by conventional sampling.

Keywords: (*S*)-α-Amino-5-phosphonomethyl[1,1′-biphenyl]-3-propanoic acid; N-Methyl-D-aspartate

1. Introduction

(*S*)-α-Amino-5-phosphonomethyl[1,1′-biphenyl]-3-propanoic acid (EAB 515, I) is a competitive

*Corresponding author.
[1]Present address: Bristol-Myers Squibb, Princeton, NJ 08543, USA.

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antagonist of the N-methyl-D-aspartate (NMDA) receptor which has been shown to have significant pharmacological activity in the central nervous system. It is a potent neuroprotector in models of cerebral ischemia, and also demonstrates social and non-social behavioral alteration following systemic administration in animals [1–4]. Since the NMDA receptors are located on the neuronal cell-membrane, quantification of unbound I in the extracellular fluid (ECF) is pharmacologically relevant.

Over the past several years, microdialysis has been increasingly used for in vivo sampling of unbound drug in the ECF of different tissue types such as brain, muscle, blood, etc. in various animal models [5–12]. Sampling by this technique involves continuous perfusion of fluid through microdialysis probes implanted in the tissue space of interest. Dialysis is a non-equilibrium situation under these conditions and the concentration of the solute measured in the dialysate is only a fraction of its unbound concentration in the extracellular space being dialyzed. This fraction, calculated as the ratio of dialysate to extracellular space concentrations, is termed relative recovery and its knowledge is required for calculating unbound concentrations in the dialyzed space from the respective dialysate concentrations. Retrodialysis [13,14] is one of several methods that may be used to estimate the probe recovery in vivo. This method involves monitoring the loss of a "suitable" calibrator from probes during simultaneous microdialysis of the analyte of interest. RDC (II), a hydroxylated analog of I, has been proposed as a prospective in vivo retrodialysis calibrator of I.

Compound I is an extremely hydrophilic compound with a high degree of ionization at the physiological pH of 7.4 and upon systemic administration, it has a limited access to the brain regions. This, combined with its low microdialysis recovery, makes the estimation of low brain ECF concentrations of I a challenge. Thus, it became necessary to develop an analytical method that is extremely sensitive and selective for the simultaneous analysis of I and II in the microdialysate of cortical ECF and cerebrospinal fluid (CSF). The importance of analytical considerations in the use of microdialysis as a sampling tool have been addressed by Kissinger [15] and by Pettit and Justice [16].

Since microdialysis of the CSF and brain ECF provides samples that are devoid of macromolecules, they do not require any processing prior to injection onto an HPLC column. However, the plasma samples need to be treated before they can be analyzed by HPLC, in order to avoid precipitation of plasma proteins which may increase back-pressure in the system and also reduce the performance and/or the life expectancy of the column [17,18]. Ultrafiltration [19–22], a process that produces a protein-free matrix, was used to measure the concentrations of unbound I in the plasma samples.

The technique of microdialysis may also be applied in the determination of unbound analyte concentration in the plasma in vivo. Surgical implantation of microdialysis probes in the jugular vein allows microdialysis sampling of unbound drug in the systemic circulation after dosing. Furthermore, the simultaneous analysis of drug in the portal blood can be used for estimation of intestinal elimination of drugs.

2. Experimental

2.1. Conditions

Compound I and its hydroxylated analog, II, were provided by Sandoz Pharma (Basel, Switzerland). Simulated CSF solution was prepared to give a final concentration of 1.1 m*M* Mg^{2+}, 144.2 m*M* Na^{+}, 1.35 m*M* Ca^{2+}, 129.5 m*M* Cl^{-}, 3.0 m*M* K^{+}, and 0.242 m*M* PO_4^{3-} at a pH of 7.6 [23]. Compound I was dissolved in Sorensen's pH 7.4 buffer for intravenous administration. Solutions of II (1000 ng/ml), to be perfused through the probes for retrodialysis, were prepared in simulated CSF for brain microdialysis and in normal saline for blood microdialysis. Standard solutions of I were prepared in artificial CSF and normal saline, for brain and blood microdialysis, respectively. The concentrations of I were measured over a wide range, in both microdialysate as well as ultrafiltrate samples (5–2000 ng/ml and 20–10 000 ng/ml, respectively). All solvents used for HPLC and for perfusion through the probes were degassed by sonication for 15 min and filtered through a polycarbonate membrane of 0.2 μm pore size (Costar, Pleasanton, CA, USA). Trifluoroacetic acid (99% pure) was obtained from

Janssen (Geel, Belgium). Male Sprague–Dawley (Harlan Sprague–Dawley, Indianapolis, IN, USA) rats, weighing approximately 300 g, were used for this study.

Chromatographic separation of the peaks of interest, viz., I and II, in the microdialysate and ultrafiltrate samples was achieved on a 250×4.6 mm I.D. Maxsil 5 μm RP-2 column (Phenomenex, Torrance, CA, USA). The two analyte peaks were monitored at wavelength settings of 255 nm for excitation and 320 nm for emission. For microdialysate samples from the rat brain and ultrafiltrate samples of rat plasma, resolution of the peak for I from the endogenous peaks was attained by using a 0.09% (v/v) solution of TFA in distilled water as the mobile phase, at a flow-rate of 1 ml/min. The mobile phase composition was changed to 2.5% (v/v) acetonitrile and 0.09% (v/v) TFA in distilled water, for adequate resolution of peaks in the analysis of blood microdialysate samples.

2.2. Microdialysis and ultrafiltration

Microdialysis probes (Bio Analytical Systems, West Lafayette, IN, USA) of CMA-12 and CMA-20 types were used as the sampling devices for measuring the concentrations of I in the brain and blood, respectively. These microdialysis probes had a dialysis membrane length of 2 mm for the cortex, 1 mm for the lateral ventricle and 10 mm for the jugular vein. The dialysis membrane had a M_r 20 000 molecular mass cut-off. The detailed operation and set-up for microdialysis with on-line HPLC analysis is described elsewhere [16,24]. Two similar on-line systems were used in this study, and on both of these, a digital valve sequence programmer (Valco; Model DVSP2, VICI, Houston, TX, USA) was used for the time programming to control the collection intervals, loop switching, sample injection and to start the integrator. The injection loops were fitted into a 10-port valve body (Valco; Model E 36, VICI). For chromatography, one of the on-line systems used a Waters-510 (Waters, Milford, MA, USA) HPLC pump, a Jasco FP-821 (Jasco, Easton, MD, USA) fluorescence detector (attenuation 1; gain×100) and a Spectra Physics-4290 (Spectra Physics, San Jose, CA, USA) integrator. The second on-line system employed a Shimadzu LC-10AD (Shimadzu, Columbia, MD, USA) HPLC pump, a Shimadzu RF-535 (Shimadzu) fluorescence detector (high sensitivity setting) and a HP-3396 integrator (Hewlett-Packard, Wilmington, DE, USA).

For ultrafiltration of the plasma samples, an ultrafiltration device (Centrifree model 4104, Amicon, Beverly, MA, USA) was used. Centrifugation was done at room temperature in a centrifuge (Triac; Model 0200, Clay Adams, Parsippany, NJ, USA) for 10 min on the blood setting. The protein-free ultrafiltrate containing unbound drug was collected in a receptacle cup and analyzed for the unbound drug concentration. Approximately 100 μl of the plasma sample produced about 30 μl of ultrafiltrate, 10 μl of which was directly injected for HPLC analysis of unbound I. The pump and injector modules of an integrated HP-1050 (Hewlett-Packard) HPLC system were used for solvent delivery and sample injection, respectively. Peaks were monitored using the Shimadzu RF-535 fluorescence detector on its low sensitivity setting. Integration of the peaks in terms of height was achieved on the HP-3396 integrator.

2.3. Calculations

The absolute peak-height (PH) values of I and II were used in all data analyses. Weighted least squares linear regression of the peak heights of I on the concentration in standard solutions of I was applied to each standard curve. Equal weighting was applied to the peak heights measured for different concentrations. The lower limit of detection was determined from a signal-to-noise (S/N) ratio of 2, from the regression of S/N on the concentration of I in a series of standard solutions.

The respective slopes of regression for the spiked aqueous and plasma ultrafiltrate standards were compared for estimation of the unbound fraction of I in plasma (f_u). The linearity of plasma-protein binding for I was verified by the examination of the ratio of the peak height of I in spiked plasma over that in aqueous ultrafiltrate, at several concentrations ranging from 50–10 000 ng/ml. The unbound concentrations of I in plasma ultrafiltrate samples were determined from the regression equation for aqueous standards.

The standards for microdialysis were analyzed in by-pass mode on the on-line HPLC system. The

concentrations of I in the microdialysate samples were determined from regression of peak heights generated from analysis of the aqueous standard curve. The in vivo microdialysis recovery of I was determined from the retrodialysis loss of II, calculated as follows:

$$\text{Loss}_{\text{II}} = 1 - \left[\frac{\text{PH}_{\text{II, through probe}}}{\text{PH}_{\text{II, by-pass}}} \right] \quad (1)$$

The loss of II has been shown to be a good estimator of the recovery of I [24], i.e.,

$$\text{Loss}_{\text{II}} = \text{Recovery}_{\text{I}} \quad (2)$$

The dialysate concentrations of I were corrected for in vivo probe recovery to yield the unbound concentration in the extracellular space of the dialyzed tissue, as follows:

$$C_{\text{I, unbound, tissue}} = \frac{C_{\text{I, dialysate}}}{\text{Loss}_{\text{II}}} \quad (3)$$

2.4. Pharmacokinetic studies in the rat

Freely moving male Sprague–Dawley rats (~300 g body weight) were used in all pharmacokinetic studies presented here. Compound I was administered via intravenous (i.v.) route as a bolus or as a constant-rate infusion.

A group of twelve rats received an i.v. infusion of I (21–69 μg/min for 900 min). Detailed results of this study are published elsewhere [24]. Following intravenous infusion of I in the femoral vein, blood samples (~200 μl) were collected every 2–3 h towards the end of the infusion and every 40–50 min post-infusion. Plasma was harvested and frozen at −20°C until analysis by HPLC following ultrafiltration. The total body clearance (CL) of I was computed as follows:

$$\text{CL}_{\text{total}} = \frac{\text{infusion rate}}{C_{\text{total, steady state}}} \quad (4)$$

where

$$C_{\text{total, steady state}} = \frac{C_{\text{unbound, steady state}}}{f_{\text{u}}} \quad (5)$$

In two other rats, microdialysis probes (CMA-20, Bioanalytical Systems, Indianapolis, IN, USA) were implanted in the portal and jugular veins for the determination of I concentrations in the portal and systemic blood, respectively, following the administration of an i.v. bolus (1.75 mg) in the femoral vein. The unbound concentrations observed in the systemic blood were used to fit the parameters of a two-compartment open model described by the following equation:

$$C_{\text{unbound}} = A \cdot \exp^{(-\alpha \cdot t)} + B \cdot \exp^{(-\beta \cdot t)} \quad (6)$$

The compartmental estimates of A, α, B, β and the value of f_{u} for I were used to compute its total body clearance following intravenous bolus dosing as follows:

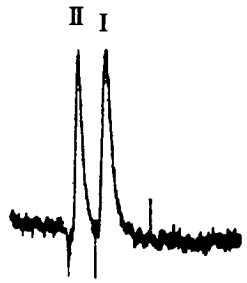
$$\text{CL}_{\text{total}} = \frac{f_{\text{u}} \cdot \text{dose}}{\left[\frac{A}{\alpha}\right] + \left[\frac{B}{\beta}\right]} \quad (7)$$

Another group of four rats received an i.v. bolus of I (4 mg each, dissolved in 0.8 ml of Sorensen's pH 7.4 buffer) at 0, 3, 6 and 9 h, successively. The drug was administered into the femoral vein, and the CSF and cortical ECF were monitored for I concentrations by microdialysis.

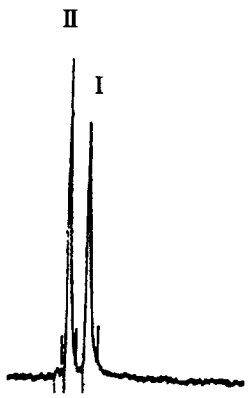
3. Results and discussion

HPLC of the extremely polar compounds I and II on silica-based C_{18} columns resulted in poor peak shape and drift in retention time, possibly caused by interaction of the compounds with surface silanol groups of the bonded phase. Limited analyte partitioning in the C_{18} phase also resulted in very fast elution of these polar compounds, sometimes with the solvent front in reversed-phase chromatography. However, when a reversed-phase C_2 column was used, a significant improvement was observed in the chromatographic separation of these two compounds. Fig. 1 shows the peak shapes of I and II following chromatographic separation on a Supelcosil® LC-ABZ column (9% acetonitrile at 1 ml/min), and on the Maxsil® RP-2 column (0.09% TFA at 1 ml/min). The latter column resulted in much narrower and more symmetrical peaks, which would optimize the performance of an HPLC method based upon peak height measurements.

Fig. 2 and Fig. 3 show representative chromato-



A: Supelcosil LC-ABZ® Column
(II: 4.36 min; I: 6.03 min)



B: Maxsil RP-2® Column
(II: 3.82 min; I: 4.99 min)

Fig. 1. Separation of I and II using HPLC under two separate sets of conditions. (A) Supelcosil LC-ABZ column (250×4.6 mm) with 9% acetonitrile in phosphate buffer (pH 6.1) at 1 ml/min. (B) Hypersil RP-2 column (250×4.6 mm) with 0% acetonitrile in pH 2.3 trifluoroacetic acid buffer (0.09%, v/v) at 1 ml/min. In both panels, the first peak corresponds to II and the second to I.

grams of blank microdialysate and ultrafiltrate samples, and of those corresponding to the respective samples obtained after dosing a typical rat with I.

During microdialysis with on-line HPLC, each chromatogram (Fig. 2) represented a composite of two sequential chromatographic analyses from each of the two sites being dialyzed simultaneously (cortical ECF and CSF in brain microdialysis, or portal and jugular vein in blood microdialysis). While the dialysate sample which had been collected from a given site was being chromatographed, the dialysate sample from that site was directed to waste and that from the other site was being collected in the alternate injection loop, and vice versa. Thus, only alternate samples from each site were analyzed during on-line HPLC with two sites being dialyzed. For example, if the duration of the collection interval is 20 min, the frequency of sample analysis from a particular site was only 40 min.

Retention times for II and I were reproducible at 3.8 and 5.0 min, respectively. The peak for II was well resolved from that for I. However, the II peak could be separated from endogenous peaks in dialysate but not in the plasma ultrafiltrate. This permitted the use of II as a retrodialysis calibrator in brain and blood microdialysis but not as an internal standard in the analysis of plasma ultrafiltrate. Using the auto-injector, variability in injection volume at 10 μl, as calculated from peak heights upon repeated injection ($n=4$) of several standard solutions of I, was found to be less than 5%. Also, since the analysis of plasma samples did not involve extraction, the need for an internal/external standard was obviated.

The peak heights of I, measured in both microdialysate and ultrafiltrate standard curves, were linear over a wide range of concentrations (5–2000 ng/ml and 20–10 000 ng/ml, respectively). Three sets of standard solutions were analyzed for estimation of within-day variability in the microdialysate and plasma ultrafiltrate assay for I. Day-to-day variability was assessed by analyzing freshly prepared standard solutions, for both microdialysate and ultrafiltrate analytical procedures, over a period of six months. Table 1 shows that the within-run variability was 0.82–16% ($n=3$) for microdialysate and 0.2–15% ($n=3$) for ultrafiltrate analysis. The between-run variability was found to be 3.4–16% ($n=6$) for microdialysate and 1–15% ($n=5$) for ultrafiltrate analysis. For I, a S/N ratio of 2 was obtained at a concentration of 4 ng/ml in a microdialysate volume of 5 μl. This corresponds to 20 pg injected on-column. For a 10-μl plasma ultrafiltrate sample the S/N ratio was 2 at 10 ng/ml. The accuracy of the assay for microdialysate and ultrafiltrate samples is demonstrated in Fig. 4. For the analytical method for microdialysate samples, the mean±S.D. of the calculated concentrations were statistically the same as the nominal concentrations, as evidenced by the proximity of the data to the line of identity.

Adsorption of the analyte(s) of interest onto the

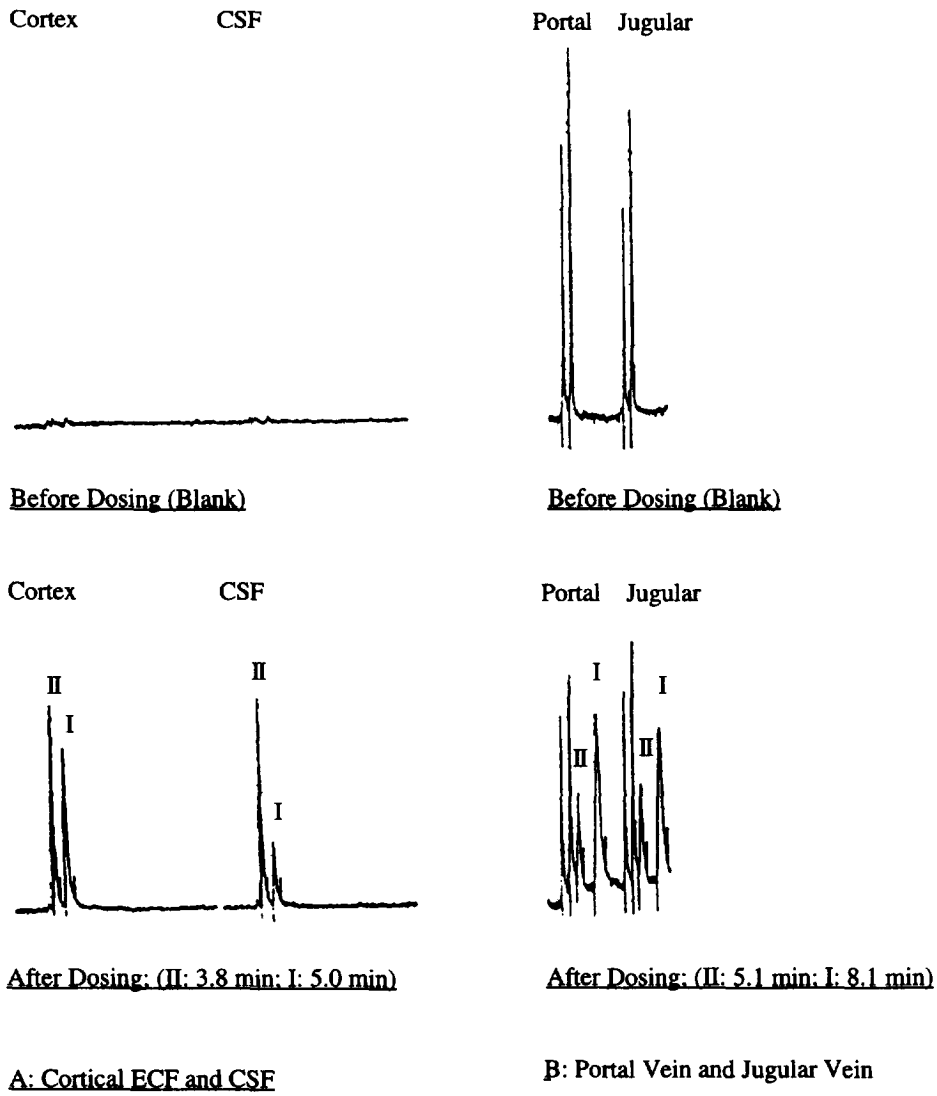


Fig. 2. Representative chromatograms of microdialysate samples before and after administration of an intravenous dose of I (with simultaneous in vivo retrodialysis using II). Each chromatogram represents samples from two sites analyzed consecutively. (A) Injections every 20 min from cortical ECF and CSF alternately in that order [II at 3.8 min (cortex) and 23.8 min (CSF); I at 4.9 min (cortex) and 24.9 min (CSF)]. (B) Injections every 10 min from portal vein and jugular vein alternately, in that order [II at 5.1 min (portal) and 15.1 min (jugular); I at 8.1 min (portal) and 18.1 min (jugular)].

membrane and/or other device materials may be a possible reason for the unsuitability of the ultrafiltration devices in protein-binding studies. Unpublished results from this laboratory have shown that there is measureable uptake of I by plastic HPLC vials, only at concentrations of less than 10 ng/ml, as indicated by the lower peak heights of samples stored in plastic vials vs. those stored in glass vials. However, over a wide range of concentrations, the peak heights determined for I were similar in solutions analyzed with and without ultrafiltration through the Amicon Centrifree apparatus, indicating that no significant binding of I, to any part of this assembly including the YMT membrane, took place. Fig. 5 shows a plot of the peak height vs. concentration for the ultrafiltrates of spiked aqueous and plasma standards, over

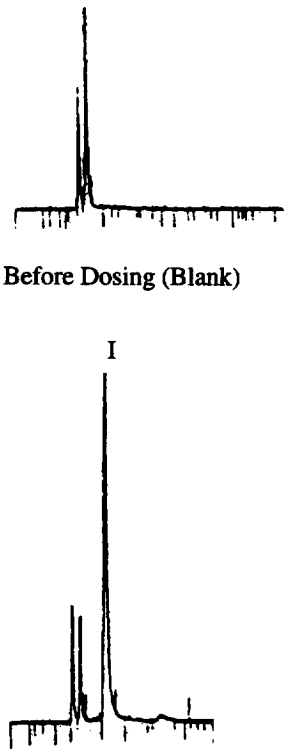


Fig. 3. Representative chromatograms of rat-plasma ultrafiltrate before and after administration of an intravenous dose of I. (A) Before dosing. (B) After dosing with I (retention time for I peak is 5 min).

Table 1
Analytical precision for the assay of I

Concentration (ng/ml)	Peak height			
	Within run ($n=3$)		Between runs ($n=5$)	
	Mean$\times 10^{-3}$	C.V. (%)	Mean$\times 10^{-3}$	C.V. (%)
Spiked aqueous standard solutions for ultrafiltrate analysis				
5	–[a]	–[a]	–[a]	–[a]
20	1.64	14.91	1.97	14.52
50	3.89	3.18	4.68	13.50
200	15.4	4.14	19.6	9.96
500	39.4	0.23	50.9	9.58
700	51.2	1.60	79.0	1.02
1000	80.0	1.00	106	7.80
2000	165	0.94	210	5.76
10 000	884	1.83	1220	7.63
Slope	88.7	1.87	123.2	6.80
R^2	0.9999		0.9991	
Spiked aqueous standard solutions for microdialysate analysis				
5	0.250	16.13	0.206	15.86
20	0.832	7.18	0.723	6.99
50	2.10	3.03	1.79	7.95
200	8.53	0.91	7.57	6.12
500	21.9	0.82	19.4	4.98
700	–[a]	–[a]	–[a]	–[a]
1000	45.0	1.13	40.0	5.93
2000	91.4	1.19	85.2	3.43
10 000	–[a]	–[a]	–[a]	–[a]
Slope	45.7	1.10	41.7	4.60
R^2	0.9999		0.9997	

[a] No measurements at these concentrations.

two separate runs. In comparison of the ultrafiltrate from spiked plasma standards to the spiked aqueous standards, it was assumed that the two matrices (aqueous and plasma ultrafiltrate) have no effect on the peak height of I, in an exact aliquot at a given concentration. The linearity of protein binding of I in plasma was verified by the peak-height ratios (plasma over aqueous ultrafiltrates), which did not change significantly over a wide range of concentrations (as shown in Table 2). The peak-height ratio analysis and the slope ratio analysis estimated f_u to be 0.69 ± 0.03 ($n=3$) and 0.68 ± 0.04 ($n=6$), respectively.

The analytical methods developed and described in this article were applied to the pharmacokinetic characterization and central nervous system distribution of I in rats. Fig. 6 shows the CSF, cortical ECF and plasma concentrations of unbound I observed during i.v. infusion of I at 24 μg/min for 900 min in a typical rat. The CSF and ECF concentrations were determined from their microdialysate concentrations, corrected for in vivo probe recovery, according to Eqs. 1–3. The plasma concentrations in this study were determined from ultrafiltrate of plasma harvested from the blood samples collected from the femoral artery.

Four rats each received four i.v. bolus doses of I, as 4 mg of I every 180 min. During this multiple dosing regimen, the CSF and cortical ECF were monitored by microdialysis for concentrations of I. Fig. 7 shows the brain concentrations observed for a typical rat in this part of the study. The accumulation of drug in the brain can be noted from the continual increase in cortical peak concentrations following subsequent doses. However, because of alternate sampling from the two sites, the peak concentration

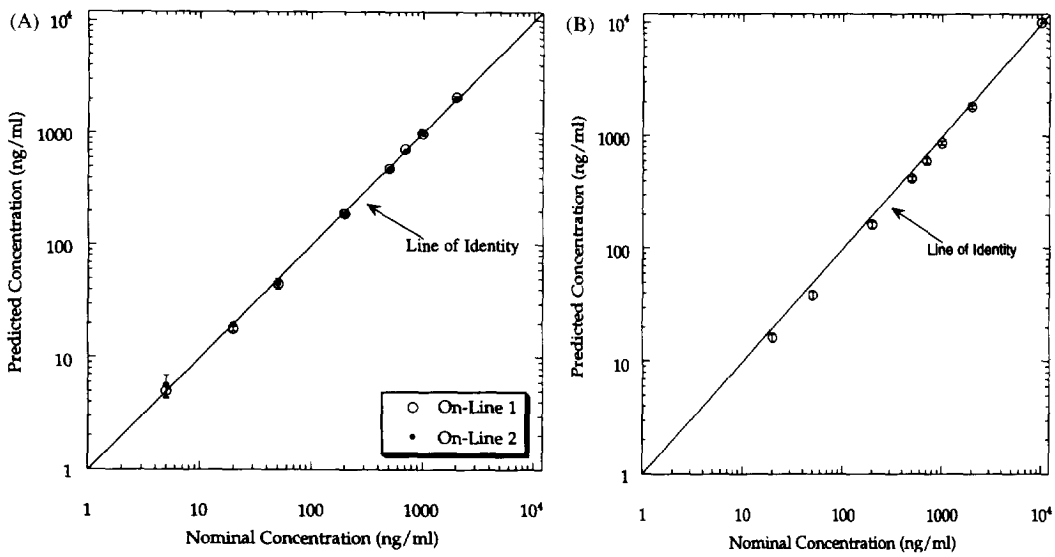


Fig. 4. (A) Nominal vs. predicted concentrations of I in microdialysate samples by on-line HPLC analysis. The line of identity is shown on the plot to indicate the accuracy of analysis. (B) Nominal vs. predicted concentrations of I in plasma ultrafiltrate samples by HPLC analysis. The line of identity is shown on the plot to indicate the accuracy of analysis.

was not characterized in the CSF of this animal. In animals that were dosed in the collection interval for the other site, the peak for the cortical site was missed.

Fig. 8 shows the concentration vs. time profiles of unbound I in the portal and systemic blood of one of the two freely moving rats that received an i.v. bolus of I (1.75 mg each). The profiles for both these sites of measurement were superimposable on each other, indicating that no significan first-pass intestinal elimination of I occurred [25]. Therefore, data from the two sites were analyzed together to fit the parameters of an open two-compartment model [26], based upon Eq. 6. The values of α (0.048 min^{-1} and 0.049 min^{-1}) and β (0.0043 min^{-1} and 0.0054 min^{-1}), after i.v. bolus administration to two rats

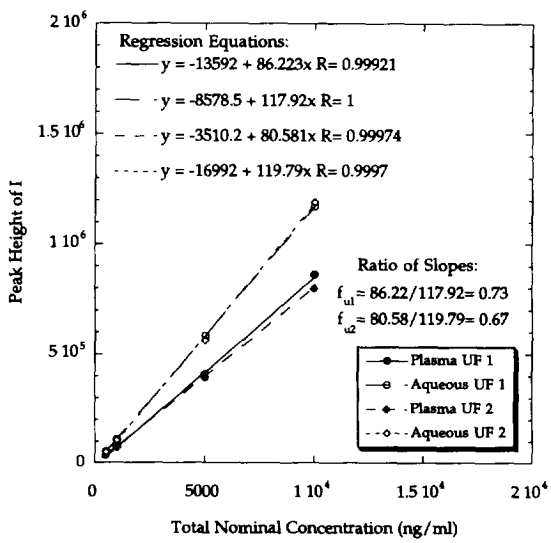


Fig. 5. Calculation of the fraction of unbound I in spiked rat plasma by ultrafiltration.

Table 2
Calculation of the fraction of I unbound in rat plasma based upon ratios of peak heights and slopes

Concentration (ng/ml)	n	Peak-height ratio	
		Mean	C.V. (%)
100	4	0.68	2.87
200	3	0.72	5.14
500	3	0.71	0.72
700	3	0.65	2.31
1000	6	0.67	6.25
2000	3	0.72	0.26
5000	5	0.71	2.88
10 000	6	0.68	7.06
Peak-height ratio[a]	33	0.69	3.62
Slope ratio[a]	6	0.68	6.57

[a] Plasma ultrafiltrate/aqueous ultrafiltrate.

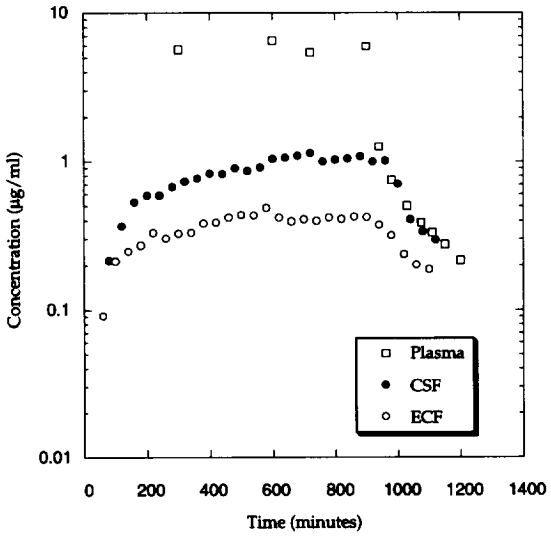


Fig. 6. Analysis of I in plasma, CSF and cortical ECF of a typical freely-moving rat during and following intravenous infusion of I (24 μg/min for 900 min). Unbound plasma concentrations were determined from analysis of the ultrafiltrate of plasma harvested from blood samples. CSF and cortical ECF concentrations were determined by microdialysis with on-line HPLC analysis.

with microdialysis of blood, were not statistically different ($P=0.17$ and 0.18 for α and β, respectively) from those obtained in the i.v. infusion studies, where blood was collected by phlebotomy

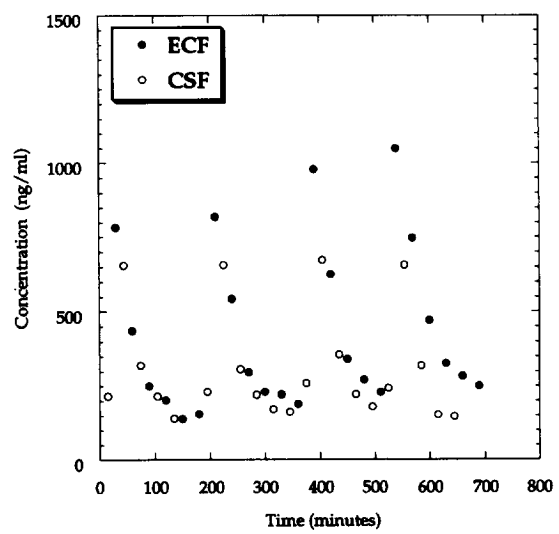


Fig. 7. Microdialysis sampling for analysis of unbound I in CSF and cortical ECF of a freely-moving rat during multiple intravenous bolus dosing of I (4 mg every 180 min).

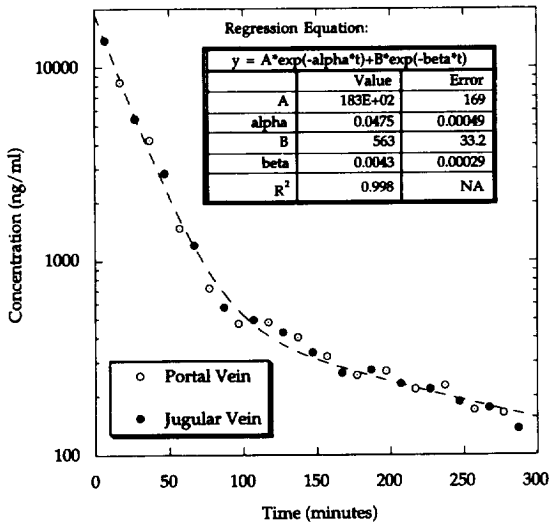


Fig. 8. Microdialysis sampling to determine unbound concentration of I in the portal and systemic blood of a freely moving rat following a single intravenous bolus of I (1.75 mg). The best fit of the data, based upon a two-compartment model analysis, is shown.

(0.037±0.010 and 0.0057±0.0007, respectively; $n=9$). Using Eq. 7, the total body clearance of I was estimated to be 7.4 and 9.3 ml/min.kg for these two rats and it was statistically not different ($P=0.90$) from the value of 8.5±1.6 ml/min per kg, as estimated from Eq. 4 and Eq. 5, using direct sampling in an i.v. infusion study conducted in 12 rats.

4. Conclusions

A sensitive chromatographic method utilizing fluorescent detection was developed for the analysis of I in blood microdialysate, brain microdialysate and in plasma ultrafiltrate samples. The sensitivity of the method met the needs of a microdialysis method for sampling brain ECF, where low concentrations of I are encountered. Simultaneous analysis of II permitted its use as a calibrator of probe recovery in vivo. The analytical precision and accuracy of this method was found to be suitable for the pharmacokinetic characterization of I in the rat. Pharmacokinetic parameters for I, as determined from concentrations in blood microdialysate samples with on-line analysis, were in good agreement with those

estimated from concentrations in the ultrafiltrate of plasma samples obtained by conventional sampling.

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