Evaluation of Blood-Brain Barrier Passage of a Muscarine M1 Agonist and a Series of Analogous Tetrahydropyridines Measured by *In Vivo* Microdialysis

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Purpose. To investigate the blood-brain barrier (BBB) passage of the M1 muscarine agonist Lu 25-109 (5-(2-Ethyl-2H-tetrazol-5-yl)-1,2,3,6-tetrahydro-methylpyridine) and potential metabolites using in vivo microdialysis.

Methods. Anesthetized rats were administered an intravenous infusion of one of seven analogs with a Log $D_{7.4}$ ranging from 0.35 to −2.4. Microdialysis probes were implanted in the brain and the jugular vein. The integrity of the BBB was evaluated using 2-amino-3-(3-hydroxy-5-phenylisoxazol-4-yl)propionic acid (APPA), a compound not expected to penetrate the BBB. The data was corrected for in vitro recovery.

Results. Lu 25-109, Lu 24-165 (demethylated Lu 25-109) and Lu 25-077 (N-demethylated Lu 25-109) entered the brain in a 1:1 ratio with the blood. Although Lu 29-081 (hydroxylated Lu 25-109) presented a similar Log $D_{7.4}$ to Lu 25-109 and Lu 24-164, it entered the brain with a lower brain:blood ratio of 0.5. Lu 32-181 (Lu 25-109 N-oxide), Lu 35-026 (deethylated and oxidized Lu 25-109) and Lu 31-126 (deethylated Lu 25-109) were not detected in the brain samples, indicating no penetration. Infusion of Lu 25-109 resulted in a time perspective of the formation and distribution of the two metabolites Lu 25-077 and Lu 32-181. Although the hydroxylated compound (Lu 29-081) had a Log $D_{7.4}$ of −0.6, within the range 0.35 to −0.83 of the compounds penetrating the BBB, it showed a brain: blood ratio of 0.5. Lu 35-026 showed an unusual infusion profile with a t_{max} of 100–150 min and a subsequent decrease in blood concentration.

Conclusions. Compounds with Log $D_{7.4}$ above −0.83 penetrated the BBB, whereas compounds below −1.5 did not. Knowledge of Log $D_{7.4}$ values is not sufficient to evaluate BBB passage because the value does not predict the influence of active transport processes.

KEY WORDS: *in vivo* microdialysis; blood-brain barrier; rat; log $D_{7.4}$; metabolism; Lu 25-109.

INTRODUCTION

The unique characteristics of brain capillaries with tight junctions and multiple carrier systems is referred to as the Blood-brain barrier (BBB) (1). A compound's passage of this barrier is restricted by both physical and chemical characteristics such as lipophilicity, ionization, and molecular size. Molecular size limits have been studied by Levin *et al.*, who has concluded molecules should weigh less than M_w 400–600 to be able to cross the BBB (2–4). The influence of the lipid solubility has been studied by Brodie *et al.* and others and the optimal Log $D_{7.4}$ for BBB passage seems to be higher than −1 (5,2). The BBB contains several active transport mechanisms intended for transport of nutrients and transmitter substances in and out of the brain. Drugs with similar characteristics can be transported across the BBB by that route (6). This barrier plays a very important role in the development of drugs intended for treatment of diseases in the central nervous system. Primarily, the active compound must penetrate the BBB to serve its pharmacological purpose. Secondly, the distribution of active and/or toxic metabolites to the brain may affect the pharmacokinetics and dynamics of the drug. Consequently it is necessary to have methods for evaluation of the BBB passage of a compound and there are several described in the literature (for review see 6–8).

In vivo microdialysis is a method that allows sampling from a selected position in the brain without disturbing the fluid balance in the area. Many samples can be taken from one animal at various locations in the body, which gives a time-concentration profile without interindividual variation. The microdialysis probe has a semipermeable membrane, thus producing protein free samples of the unbound fraction of the drug, which can be analyzed directly without sample preparation, and enzymatic degradation of the sample is avoided (6,7). Microdialysis makes it possible to measure free tissue concentrations directly, which may be more comparable to *in vitro* receptor binding data than indexes and permeability-surface area products obtained by other methods. As any other technique microdialysis also has its drawbacks. An essential issue in the investigation of BBB passage is the integrity of the BBB, and as microdialysis is an invasive technique this constitutes a potential problem. Another important object of discussion is determination of *in vivo* recovery of the drug.

The subject of the present work was an M_1 muscarine agonist, Lu 25-109, with CNS effects (for chemical structure see Table I). During the development of Lu 25-109, standard substances of potential metabolites were synthesized. Some of these compounds were used in this study, representing a Log $D_{7.4}$ range of 0.35 to −2.4. Of the selected compounds, two (Lu 29-081 and Lu 24-165) proved not to be metabolites *in vivo* (9), but they are included in this study as they compliment the Log $D_{7.4}$ span.

The N-oxide of Lu 25-109 (Lu 32-181) was the major metabolite and the de-methylated metabolite (Lu 25-077) was pharmacologically active, which makes the distribution of these two metabolites interesting to investigate (9). To our knowledge only few *in vivo* microdialysis studies have been performed on a series of analogous compounds with regard to BBB passage (10,11). Microdialysis studies of the formation and distribution of metabolites have been done on other compounds such as phenol in liver (12) and recently a 5 HT_{2a} receptor antagonist (MDL 100,907) in brain (13).

The purpose of the study was to investigate the BBB passage of Lu 25-109 and potential metabolites by *in vivo* microdialysis and compare the results to Log $D_{7.4}$.

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ABBREVIATIONS: BBB, blood-brain barrier; aCSF, artificial cerebrospinal fluid, APPA, 2-amino-3-(3-hydroxy-5-phenylisoxazol-4-yl)propionic acid; t_{max}, time for maximum concentration; AUC, area under the time-concentration curve; RMI(E)SQC, Rat and Mouse No. 1 Maintenance Diet, Expanded Diet, Special Quality Control, Special Diet Services (SDS) Limited, Essex, England.

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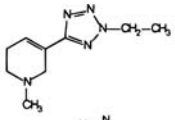
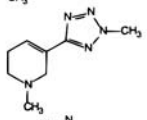
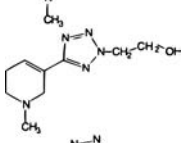
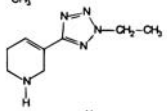
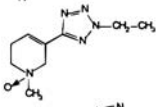
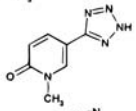
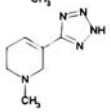
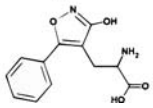
Table I. Structure and Log $D_{7.4}$ of the Test Compounds

Compound	Structure	Log $D_{7.4}$
Lu 25-109		0.35
Lu 24-165		−0.01
Lu 29-081		−0.6
Lu 25-077		−0.83
Lu 32-181		−1.5
Lu 35-026		−2.3
Lu 31-126		−2.4
APPA		−2.2

MATERIALS AND METHODS

Standard Substances

The test compounds shown in Table I were all synthesized at H. Lundbeck A/S, Copenhagen, Denmark. Table I shows both structure and Log $D_{7.4}$.

Chemicals

Halothan (2-brom-2-Chlor-1,1,1-Fluorethan) was from Halocarbon Laboratories (North Augusta, SC, USA). KH_2PO_4, CH_3CN, H_3PO_4, KOH, NaCl, $CaCl_2(H_2O)_2$, $Na_2HPO_4(H_2O)_2$ were purchased from Merck KGaA (Darmstadt, Germany), KCl, $MgCl_2(H_2O)_6$ from Sigma (St. Louis, MO), and Na-octanesulphonic acid from Fluka Chemie AG (Buchs, Switzerland). All chemicals were of analytical grade. All solutions were made with filtered, deionized water.

Log $D_{7.4}$ Determination

Distribution coefficients for the compounds were determined as follows: A stock solution of each compound was prepared in phosphate buffer (pH 7.4). Two ml of the stock solution was added to 20 ml of octanol and the mixture was vortexed. The mixture was allowed to equilibrate for 5 minutes and vortexed again. The two phases were allowed to separate and a sample of the buffer-phase was quantified by HPLC as well as the stock solution. The measurements were made in duplicate. The Log $D_{7.4}$ values were calculated from the obtained areas, correcting for the large octanol volume. The procedure was performed at 25°C (14).

Perfusion Fluid

An artificial cerebrospinal fluid (aCSF) was used as perfusion fluid in all microdialysis experiments in both blood and brain. The aCSF chosen is a modified Mock-CSF solution containing 135 mM NaCl, 3 mM KCl, 1.2 mM $CaCl_2$, 1 mM $MgCl_2$, and 1 mM Na_2HPO_4 (pH 7.4) (15,16).

Test Animals

30 Male Wistar rats (200–350 g) from M&B (Denmark) were used. The rats had free access to food (RMI(E)SQC) and water ad libitum. The experiments were conducted in accordance with Council Directive 86/609/EEC of 24 November 1986 on the approximation of laws, regulations and administrative provisions of the Member States regarding the protection of animals used for experimental and other scientific purposes.

Microdialysis Probes

For intracerebral microdialysis the rigid cannula style probe, CMA/12 (4 mm membrane) was implanted in Striatum. The CMA/20 (10 mm membrane) flexible cannula style probe was used for microdialysis blood sampling in the jugular vein. All microdialysis was performed with CMA FEP-tubing. Both probes and tubing were purchased from CMA Microdialysis, Solna, Sweden.

Recovery

The definition of the recovery of a drug over the dialysis membrane is as follows:

$$\text{Recovery} = C_{dialysate}/C_{media} * 100\%$$

where C represents the drug concentration in dialysate and media, respectively.

The recovery used to correct the *in vivo* data in this study was determined *in vitro*. The experiment was made by microdialyzing an unstirred, thermostatted media (37°C) containing the drug of interest in three concentrations, 0.1, 0.5, and 1 μg/ml (n = 6). The recovery used for further calculations was determined as the slope of $C_{dialysate}$ plotted against C_{media} (n = 18), calculated by linear regression. The recovery experiments were performed for each drug and for each probe, to account for differences between probes. Additionally *in vivo* recovery, determined by the no net flux method (7) described in the *in vivo* microdialysis section, was calculated for the compounds that entered the brain in order to confirm the brain/blood ratio calculated with the *in vitro* recoveries. The *in vivo* data was not corrected for these in vivo recoveries as they were obtained with other probes and the recovery consequently not directly applicable to the data.

Each compound was tested for carry over. This was done by dialyzing a blank media, shifting to a media of high concentration, and back to a blank. Dead volume of the system was calculated and taken into consideration in the experimental set-up. For all *in vitro* determinations, the drug was dissolved in aCSF.

Surgical Procedure

The rats were kept anestesized during the experiment by inhalation of a Halothan, N_2O, and O_2 (1.5:70:30%) mixture. The femoral vein was cannulated with PE-10 polyethylene tubing, to allow infusion of the selected compound. A CMA/20 microdialysis probe was implanted in the jugular vein for sampling from the blood. The rat was placed in a Kopf stereotaxic frame and a CMA/12 microdialysis probe was implanted in Striatum (stereotaxic coordinates: 2.7 mm lateral, 0.4 anterior to the Bregma and 7.0 mm ventral to the scull surface), to obtain samples from the brain. The body temperature was maintained at 37°C by a heating pad controlled by a thermo rectal probe (CMA/150). The rats were sacrificed by an overdose of halothan at the end of the experiment.

In Vivo Microdialysis

Microdialysis samples from blood and brain were collected in parallel for 340 minutes. The flow used through the microdialysis probes was 5 μl/min with a collection time of 10 minutes.

The drug was administered by intravenous infusion at a rate of 1 or 2 mg/kg/h ~ 0.3–0.7 ml/h. The infusion was turned on from t = 50 minutes until approximately t = 270 minutes. The dosing solution of the drug was prepared in an isotonic, isohydric, and filtered (0.45 μm) NaCl solution (1 or 2 mg/ml).

In the no net flux experiments performed to calculate *in vivo* recovery Lu 25-077 and Lu 29-081 were administered as a loading dose in supplement to the infusion to achieve a constant concentration level within the experiment because steady state is required by this method. In the steady state period the microdialysis probes were perfused with aCSF containing 0, 250, 500, 1000, and 2000 ng/ml of the compound. Four samples were collected at each level. One experiment was performed with each of the four compounds.

Integrity of the BBB

Having a Log $D_{7.4}$ of −2.2, APPA was not expected to enter the brain, when infused in the blood stream. Other studies evaluating the effect of APPA as a glutamate agonist *in vivo* confirm this, as APPA only shows effect when administered intracerebroventriculary and not after intravenous or subcutaneous administration (17). The APPA microdialysis experiment (n = 4) was essentially different from the others in two respects. The infusion (5 mg/kg/h) of APPA was started 20 minutes before implantation of the brain microdialysis probe to achieve a high blood concentration of APPA. BBB damage during the implantation of the probe would easily be detected by the presence of APPA in the brain dialysate. The blood concentrations in these experiments were determined from 6 whole blood samples (400 μl) collected from the carotic artery. The blood samples were subsequently microdialysed *in vitro* and analyzed by HPLC.

HPLC Analysis

The samples (injection volume 40 μl) were analyzed directly by a Merck-Hitachi HPLC system. The limit of detection was determined as three times the baseline noise in the chromatograms of blood samples. Lu 25-109, Lu 25-077, and Lu 32-181 were analyzed using a YMC basic C8 (5 μm, 4.6 × 150 mm) column and mobile phase consisting of 10:90 CH_3CN:NaH_2PO_4 (25 mM, pH 5.25) + 200 mg/l of Na-octanesulfonic acid at 35°C. Lu 24-165 was analyzed using the same system although the pH of the buffer was raised to 6.5. The limits of detection were approximately 10 ng/ml at 215 nm UV detection.

Lu 31-126 was analyzed using a Chrompack, Sperisorb, (5 μm, 4.6 × 150 mm) column and a mobile phase consisting of 35:65 Methanol:Ammonium Acetate (10 mM, pH 4.5) at 35°C. The limit of detection was approximately 5 ng/ml at 215 nm UV detection.

Lu 35-026 was analyzed using a YMC-pack ODS AQ (5 μm, 4.6 × 250 mm) column and a mobile phase consisting of 5:95 CH_3CN:Ammonium Acetate (0.2 M, pH 5) at 35°C. The limit of detection was approximately 2 ng/ml at 255 nm UV detection.

Lu 29-081 was analyzed using a Chrompack IonoSpher C (5 μm, 4.6 × 150 mm) column and a mobile phase consisting of 40:60 CH_3CN:KH_2PO_4 (100 mM) at 45°C. The limit of detection was approximately 15 ng/ml at 229 nm UV detection.

APPA was analyzed using a YMC basic C8 (5 μm, 2 × 100 mm) column and a mobile phase consisting of 10:990:0.25 CH_3CN:Ammoniumacetate (10 mM, pH 5): $(CH_3CH_2)_3N$ at a flow of 0.3 ml/min. The injection volume was 25 μl and APPA was detected by fluorescence (Ex = 250 nm, Em = 395 nm) resulting in a limit of detection of 2 ng/ml.

RESULTS

Recovery

The microdialysis probes and compounds were examined *in vitro* prior to *in vivo* experiments including investigation of carry over and recovery as previously described. *In vivo* recoveries were calculated from no net flux experiments in steady state of the compounds that enter the brain as confirmation of the *in vivo* results. The *in vivo* recoveries for the compounds that did not enter the brain were not critical to this study as the brain/blood ratio would be zero regardless of the recovery. *In vitro* and *in vivo* recoveries are shown in Table II.

None of the compounds showed carry over. The recoveries for each compound were calculated and the probe and drug specific in vitro recoveries were used to correct the *in vivo* results.

Integrity of the BBB

APPA was used for evaluation of the damage induced by the brain microdialysis probe during implantation. The object of the experiments was to determine a suitable time period to let the BBB recover before starting infusion of a drug. Other studies have shown recovery periods from 30 minutes to 48 hours (15,18,19,20). The results of the experiments are shown in Fig. 1. APPA was only detectable in the 10 and 20 min brain samples, whereas the blood level was relatively constant at app. 2 μg/ml, which was about 100 fold higher than measured in the brain samples. The limit of detection (LOD) was 2 ng/ml. Corrected for an *in vitro* recovery of 18.9%, LOD is approximately 11 ng/ml. The study was performed on four rats, which all showed the same pattern. Figure 1 represents a mean of the

Table II. *In Vivo* and *In Vitro* Recoveries and Brain/Blood Ratios

Compound	Probe	Recovery *In vitro* (%)	Recovery *In vivo* (%)	Brain Blood *in vitro*[a]	Brain Blood *in vivo*[a]
Lu 24-165	Blood	19.8 ± 0.4	21.7 ± 0.8		
	Brain	15.1 ± 0.5	6.8 ± 0.5	0.69	0.31
		12.4 ± 0.2			
Lu 25-109	Blood	34.5 ± 1.4	28.5 ± 2.2		
		35.6 ± 2.9		0.54	0.39
	Brain	18.8 ± 1.7	11.0 ± 1.0		
Lu 25-077	Blood	34.4 ± 0.8	28.6 ± 3.1		
		34.3 ± 1.3			
		40.0 ± 1.7		0.52	0.33
	Brain	14.6 ± 0.4	9.41 ± 1.4		
		23.4 ± 1.7			
Lu 29-081	Blood	30.2 ± 0.3	9.65 ± 2.8		
		32.5 ± 0.7	6.87 ± 1.6	0.52	0.43
	Brain	14.1 ± 0.1	3.86 ± 0.4		
		18.5 ± 0.2	3.21 ± 0.9		

[a] Brain/Blood ratios were calculated from mean recovery values.

four datasets. Based on this study the rats were allowed at least 40 minutes to recover after implantation of the brain probes in the following studies.

BBB Distribution of Test Compounds

The results are summarized in Table III and examples of time-concentration curves for each compound are shown in Fig. 2. The AUC ratios were calculated using the trapezoid rule for the infusion period. The data showed that Lu 35-026, Lu 32-181, and Lu 31-126 did not cross the blood-brain-barrier. The AUC ratio values in Table III for these three compounds represents the highest possible values calculated from the limits of detection.

Lu 25-109, Lu 25-077, and Lu 24-165 passed the BBB in an approximately 1:1 ratio, when the results were corrected for *in vitro* recovery. Lu 29-081 with Log $D_{7.4}$ between Lu 25-077 and Lu 24-165 had an AUC ratio of 0.48 with a relatively low standard deviation and thus behaved atypical. Correction for *in vivo* recoveries, obtained from later experiments with other probes resulted in higher brain:blood ratios as presented in Table III.

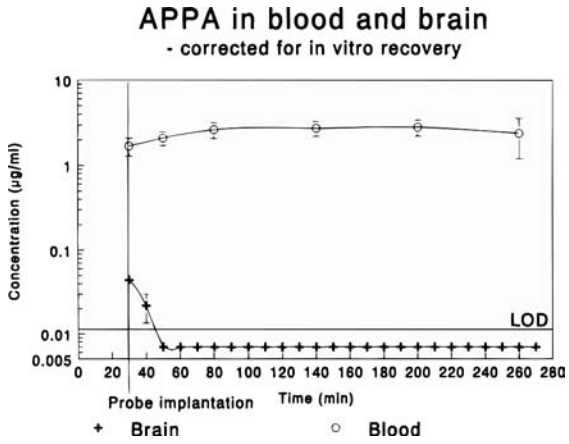


Fig. 1. Evaluation of the integrity of the BBB. Infusion of APPA starts at 0 min and the probe is implanted at 30 min. APPA is present in the brain samples 10 and 20 min after implantation. In the rest of the brain samples APPA is not detected or below the limit of detection (LOD). This graph represents a mean ± SD of four rats.

Table III. Results of *In Vivo* BBB Penetration Studies

Compound	Log $D_{7.4}$	*In vivo* recovery AUC_{brain}/AUC_{blood} (infusion period)[a] (Mean ± SD)	*In vitro* recovery AUC_{brain}/AUC_{blood} (infusion period)[a] (Mean ± SD)
Lu 25-109	0.35	1.90 ± 0.70	1.35 ± 0.50
Lu 24-165	−0.01	2.20 ± 0.61	1.02 ± 0.44
Lu 29-081	−0.60	0.59 ± 0.05	0.48 ± 0.04
Lu 25-077	−0.83	1.68 ± 0.12	0.96 ± 0.07
Lu 32-181	−1.5	ND	ND i.e. < 0.10
Lu 35-026	−2.3	ND	ND i.e. < 0.05
Lu 31-126	−2.4	ND	ND i.e. < 0.03

[a] n = 3 for all compounds.
Note: ND, not detected.

Formation and Distribution of Two Lu 25-109 Metabolites

Infusion of Lu 25-109 additionally resulted in formation of the two metabolites Lu 25-077 and Lu 32-181 which were quantified by HPLC. Distribution of these metabolites mirrored the results obtained with direct infusion of the metabolites, i.e., Lu 32-181 was only detected in blood whereas Lu 25-077 was detected in both brain and blood. This experiment provided a time perspective for the formation and distribution of the metabolites, which showed that Lu 25-109 was rapidly metabolized. Lu 32-181 reached free blood concentrations comparable to the parent compound within the infusion period. Lu 25-077 was formed in considerably lower concentrations. The result of this experiment is shown in Fig. 3.

DISCUSSION

Microdialysis has in this study proven to be a suitable method to determine whether or not a compound enters the brain to a detectable degree. The detection limit is an important issue in microdialysis as the perfusion fluid dilutes the samples and only the unbound fraction of the compound is sampled, thus challenging the analytical method. On the other hand microdialysis also facilitates the analytical procedure by producing protein-free, aqueous samples that can be injected directly into an HPLC without sample preparation. The sensitivity of the analytical method and the minimum sample volume of the autosampler limits the selection of perfusate flow. A flow of 5 µl/min meets both these demands in this experimental set-up, yielding 50 µl samples. The concentration of the analyte depends on the recovery, which was determined *in vitro* in this study. This was done for practical reasons as the purpose was to evaluate qualitatively which compounds entered the brain and compare these data to the Log $D_{7.4}$ values. To confirm the brain/blood ratios of the compounds entering the brain, *in vivo* recoveries were determined by the no net flux method (7).

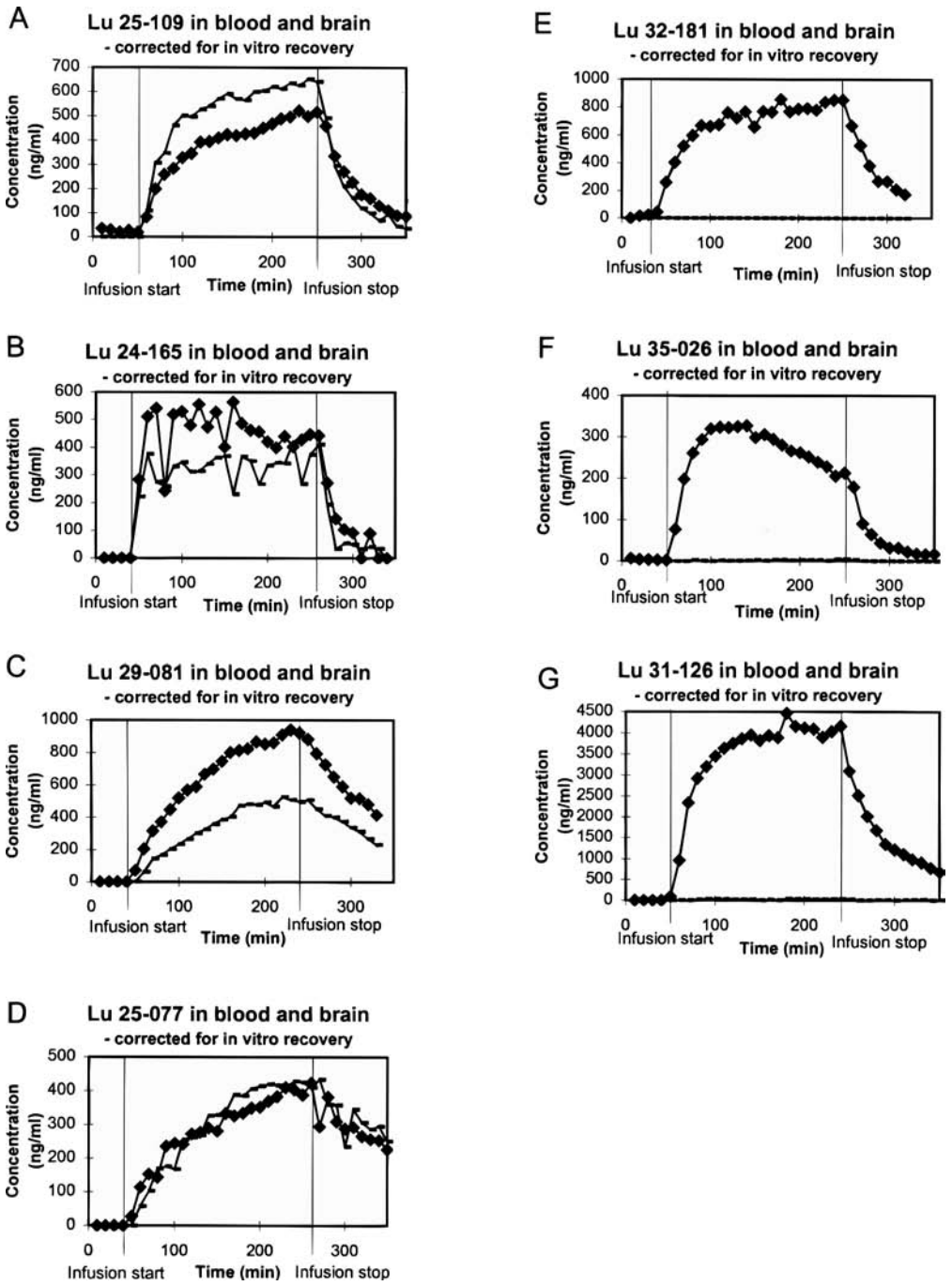


Fig. 2. Examples of infusion profiles of the seven selected compounds. (A) Lu 25-109, (B) Lu 24-165, (C) Lu 29-081, (D) Lu 25-077, (E) Lu 32-181, (F) Lu 35-026 and (G) Lu 31-126. The compounds were sampled from both blood (♦) and brain (–). Each data point represents a 10 minute microdialysis sample collected from an anaesthetised rat. Blood and brain samples were collected from the same rat and corrected for the appropriate *in vitro* recovery.

These values showed that the *in vitro* method generally overestimated the brain recovery relative to the blood recovery, consequently underestimating the brain blood ratio but it does not change the conclusions of this study. This observation have also been made by others and may be explained by the tortuosity of the extracellular channels and the relatively small extracellular space in the brain compared to blood (7,15). An element of uncertainty in this study is the use of animals in anesthesia because it interferes with numerous physiological processes such as absorption and elimination and results in longer half-lives for both processes (19). With this in mind a qualitative observation of the kinetics can still be made assuming the interference with the half-lives was comparable for all the investigated compounds. Lu 29-081 and Lu 25-077 seem to have longer half-lives than the rest of the compounds because the concentration levels never reach steady state within the infusion

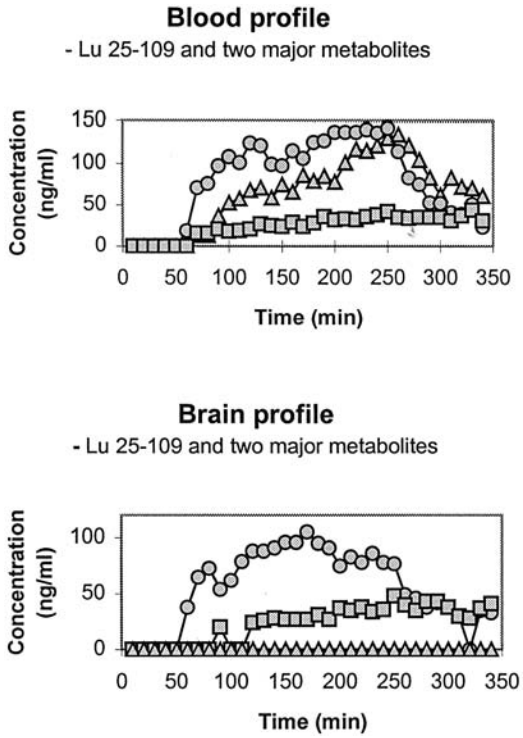


Fig. 3. Infusion of Lu 25-109 (○). The parent compound and its two major metabolites Lu 32-181 (△) and Lu 25-077 (□) were measured in blood and brain microdialysis samples. The infusion of Lu 25-109 was started at 50 minutes and stopped at 250 minutes after implantation of the microdialysis probe (n = 1).

period. This observation fits the conclusion of other studies concluding that a slower equilibration over the BBB by a hydrophilic compound is to be expected (5). In the graphs of Lu 29-081 it was obvious that the brain curve was lower than the blood curve. This was unexpected as it implied that Lu 29-081 was distributed between brain and blood in a ratio of about 1:2. The two compounds, with the closest log $D_{7.4}$ on each side, both passed the blood-brain-barrier in a ratio of at least 1:1. Theoretically the difference could come from incorrect recoveries, but this is not likely to be the case, since both *in vitro* and *in vivo* recoveries result in similar brain/blood ratios for this compound. Another explanation could be that the presence of a hydroxyl group on Lu 29-081 makes it a better target for hydrogen bonding than the other compounds, which might influence the passage of the BBB (6,21). The hydrogen bonding could theoretically be accounted for by using Δ log $D_{7.4}$ (hexane/water-octanol/water) instead of log $D_{7.4}$. Unfortunately Δ log $D_{7.4}$ values are not available for these compounds. The distribution indicates that the compound undergoes active transport out of the brain, consequently making the brain concentration lower than the free blood concentration (22). The free blood concentration of Lu 35-026 seems to reach a maximum at 100–150 minutes after which the concentration decreases slowly in spite of the ongoing infusion. This phenomenon was observed in all the experiments with this compound. This implies that Lu 35-026 or a metabolite of this compound activates the elimination process. This could hypothetically happen by increased blood flow of the eliminating organs, increased secretion, decreased reabsorption or induced metabolism. Usually it takes more than a few hours to induce an enzyme, thus the latter suggestion is improbable.

In conclusion, Log $D_{7.4}$ values are not sufficient to evaluate the BBB passage of a compound. The Log $D_{7.4}$ did not predict the 1:2 brain:blood distribution of Lu 29-081. Log $D_{7.4}$ is a purely physio-chemical parameter from which it is impossible to predict the influence of physiological processes such as active transport mechanisms. The results are in agreement with previous work (2,5) in which it was reported that Log $D_{7.4}$ should be in excess of -1 to pass the BBB. The microdialysis technique permits continuous sampling from both blood and brain of the same animal over a time span, allowing investigation of BBB passage as well as a qualitative pharmacokinetic evaluation. The elimination of Lu 35-026 and the possible transport mechanisms of Lu 29-081 need further investigation before an understanding of these observations can be achieved.

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