Brain Penetration and *In Vivo* Recovery of NMDA Receptor Antagonists Amantadine and Memantine: A Quantitative Microdialysis Study

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Purpose: To determine free brain concentrations of the clinically used uncompetitive NMDA antagonists memantine and amantadine using microdialysis corrected for *in vivo* recovery in relations to serum, CSF and brain tissue levels and their *in vitro* potency at NMDA receptors.
Methods: Microdialysis corrected for *in vivo* recovery was used to determine brain ECF concentrations after steady-state administration of either memantine or amantadine. Additionally CSF, serum, and brain tissue were analyzed.
Results: Following 7 days of infusion of memantine or amantadine (20 and 100 mg/kg/day respectively) whole brain concentrations were 44-and 16-fold higher than free concentrations in serum respectively. The free brain ECF concentration of memantine (0.83 ± 0.05 μM) was comparable to free serum and CSF concentrations. In case of amantadine, it was lower. A higher *in vivo* than *in vitro* recovery was found for memantine.
Conclusions: At clinically relevant doses memantine reaches a brain ECF concentration in range of its affinity for the NMDA receptor and close to its free serum concentration. This is not the case for amantadine and different mechanisms of action may be operational.

KEY WORDS: aminoadamantanes; *in vivo* recovery; microdialysis.

INTRODUCTION

Microdialysis has been extensively used in neuroscience, initially to determine changes in neurotransmitter concentrations in the brain (1). Recently, however, it has also been adopted to measure brain penetration of drugs acting within the CNS (2). Microdialysis can estimate drug levels in the extracellular space of the brain which is crucial to verify the mechanism of action-based on comparison with *in vitro* potency at various targets. For a number of agents it has been shown that brain ECF concentrations are below plasma concentrations (3,4) hence, plasma levels do not give reliable estimates of 'active' brain concentrations for drugs acting at extracellular targets (e.g. neurotransmitter receptors). Also, the use of brain homogenates can be misleading since whole brain tissue concentrations do not necessarily equal free brain ECF concentrations. In fact, some drugs accumulate in the acidic cellular compartments like lysosomes or in lipophilic membrane constituents (5). Even CSF concentrations do not necessarily reflect ECF levels (6). Therefore, sampling the extracellular space of the brain using microdialysis should give the most correct concentration estimate of the drug acting extracellularly.

The major challenge to tackle when using microdialysis for pharmacokinetic studies, is the issue of recovery i.e. relating dialysate to brain ECF concentrations. The recovery depends on the perfusion flow rate, the dialysis membrane geometry, and composition, the diffusion coefficient of the solute of interest and the medium surrounding the probe (7). Initially, the recovery was determined *in vitro* ('water-recovery' method). However, the assumption that diffusion through a water-solution and brain tissue is the same, does not often hold true (8). For hydrophilic compounds it has been shown that this method overestimates the recovery and hence, underestimates the *in vivo* concentration (9).

To overcome this pitfall several methods for estimation of the *in vivo* recovery have been developed. The purely empirical ZNF method (zero-net flux), developed by Lönnroth *et al.* (10), is based on the assumption that if the compound of interest is included in the perfusate in varying concentrations, the diffusion flux is directed either into or out of the probe depending on the direction of the concentration gradient. At the point of ZNF there is no concentration gradient and $C_{in} = C_{out} = C_{ECF}$.

A more mathematical approach is the MT (mass transfer) method, which is based on the fact that the relation between recovery and perfusion rate depends on the diffusion characteristics of the medium surrounding the probe (11,12). By reducing the perfusion rate to approach zero, the recovery reaches 100%.

In the studies presented, the free brain ECF concentration of two aminoadamantanes: memantine (1-amino-3,5-dimethyladamantane) and amantadine (1-aminoadamantane) was determined. These uncompetitive antagonists of the NMDA receptor are cationic amphiphilic drugs that are known to be accumulated in the lysosomal fraction (5). In patients, the aminoadamantane concentration in total brain tissue is found to be 20 to 30 times higher than in serum and CSF (13,14), similarly to experimental animals (14,15). The concentrations of memantine and amantadine in serum and CSF are within range of their *in vitro* potency as uncompetitive NMDA receptor antagonists, while brain tissue levels are considerably higher (14).

Apart from the aminoadamantanes, accumulation in brain tissue has been described for (+)MK-801 (16,17), dextrorphan, dextromethorphan (18), and ketamine (19). Brain ECF concentrations were not determined in any of these reports. It has always been assumed, but never shown experimentally, that the free 'active' concentration in the brain is comparable to plasma rather than brain tissue levels.

The aim of the present study was to determine, using microdialysis corrected for *in vivo* recovery, free ECF levels of amantadine and memantine at plasma concentrations comparable to those seen in the clinic; in order to determine whether NMDA receptor antagonism can be considered their major mechanism of action. For the sake of comparison, CSF, serum, and brain homogenate levels were also determined.

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ABBREVIATIONS: aCSF, artificial CSF; CSF, cerebrospinal fluid; ECF, extracellular fluid; IS, internal standard; MT, mass-transfer; ZNF, zero-net flux; NMDA, N-methyl-D-aspartate; BBB, blood-brain barrier.

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METHODS

Subjects

Adult male Sprague Dawley rats (body weight 235–275 g, Charles River, Germany) were kept on a diet of 15 g/day laboratory rat food with free access to water under standard laboratory conditions (12/12 hr dark/light cycle and 20°C).

In Vitro Microdialysis Experiments

Water Recovery Method

A microdialysis probe (CMA 10, membrane length 3 mm) was placed in a stirred 37°C aCSF solution (in mM: NaCl 145, KCl 0.6, $MgCl_2$ 1.0, $CaCl_2$ 1.2, ascorbic acid 0.2 in a 2 mM potassium phosphate buffer pH 7.4) containing memantine or amantadine (1 and 2.5 μM respectively.). The probe was perfused (CMA/100, syringe pump) with aCSF at a speed of 3 μl/min for 2 hr to allow establishment of steady-state before three 20 min samples were collected (CMA/140, microfraction collector).

Surgical Procedure

ALZET Minipump Implantation

ALZET osmotic minipumps (ALZA, Palo Alto, California, USA, model 2ML2) were filled with a memantine or amantadine solution in water ascertaining a dose of 20 and 100 mg/kg/day respectively. The animals were anesthetized (Hypnorm, 0.25 ml/kg im, Janssen Pharmaceuticals), a 1.5 cm long incision was made in the neck of the animal, and an ALZET pump was introduced and the incision was closed using wound clamps.

Microdialysis Surgery

Seven days after the ALZET pump implantation a microdialysis probe was implanted. The animals were anesthetized with Hypnorm, placed in a stereotaxic frame, and the skull was exposed. A small hole was drilled to allow the implantation of a microdialysis guide canulla (CMA/10) in the anterior striatum relative to bregma (AP: 1.0; L: 2.5, V:−3.0). Then a screw was secured into the skull and cemented together with the guide canulla on to the skull using dental cement (Paladur, Heraeus, Germany). A microdialysis probe (CMA/10) was inserted into the guide canulla immediately after the surgery and the animals were allowed to recover for 22 to 26 hr.

In Vivo Microdialysis Experiment

At the start of the experiment the inflow line of the microdialysis probe was connected to a syringe pump by means of a dual channel swivel and the probe was perfused with aCSF (3 μl/min). The outlet line was connected to a microfraction collector and 20 min fractions were collected.

ZNF Method

The microdialysis probe was continuously perfused with aCSF containing different concentrations of memantine (n = 5; 0, 1, 2, 10, and 50 μM) or amantadine (n = 4; 0, 2.5, 5, and 25 μM). Every inflow-concentration was perfused for 2 hr before three 20 min samples were collected. At the end of the experiment the animals were anesthetized with nembutal (1.5 ml/kg), a blood sample was taken by heart punction and the brain was removed. The blood samples were centrifuged at 4000 rpm. Half of the brain was homogenated in aCSF and centrifuged. Subsequently, the supernatant and half of the serum were centrifuged in ultrafiltration tubes (MW cut-off = 10.000, Centrisart, Sartorius, Germany) in order to estimate the protein binding. Microdialysate, serum, and brain tissue samples (whole tissue, supernatant, and ultrafiltrate) were frozen at −20°C until analysis.

MT Method

The microdialysis probe was continuously perfused with aCSF at different perfusion speeds (7, 5, 3, 2, and 1 μl/min) for both memantine (n = 4) and amantadine (n = 4) treated animals. The aCSF did not contain any memantine or amantadine throughout this experiment. Every perfusion speed was used for 2 hr before three 20 min samples were taken. The relationship between the flow-rate and dialysate concentration was linearised by plotting the inverse of the dialysate concentration versus the flow-rate (12).

CSF Sampling

Seven days after the ALZET pump implantation the animals were anesthetized with Hypnorm (1.0 ml/kg i.m.), placed in a stereotaxic frame and the skull was exposed (n = 3, for both memantine and amantadine). A small hole was drilled on the sagittal midline, immediately rostral to the interparietal-occipital bone structure. Using a 27 gauge needle and a 1 ml syringe a 150 μl sample was taken from the cisterna magna. Blood (5 ml) was removed by heart punction and the brain was removed. The blood samples were centrifuged at 4000 rpm. The supernatant was collected and CSF, serum, and brain tissue samples were frozen at −20°C until analysis.

Analytical Methods

Brains (0.5 g of tissue) were treated with 2 ml of 2.5 M H_2SO_4 and 100 μl internal standard (IS, amantadine hydrochloride, or memantine hydrochloride was added) at 90°C for 60 minutes; 1 ml of serum was pipetted into a culture tube and then; 1 ml of 2 M hydrochloric acid and 2 ml of IS were added. The sample was treated at 70°C for 15 min. Extractions were then the same for brain, CSF, and serum samples. After cooling to room temperature, 0.6 ml hexane and 0.6 ml 10 M NaOH were added. This mixture was extracted on a cooling mixer for 30 min, and afterwards the organic phase was transferred into a GC-vial. The samples were then processed by a gas-chromatography system (5970/5971 Hewlett Packard) coupled to a mass selective detector. The analytical column (Restek Stabilwax DB L = 30 m, ID = 0.25 mm.) was used with an injection mode splitless 1–3 μl and gas 10 psi Helium 1 ml/min. The injection temperature was 220°C and the detection temperature was 240°C. The ionization mode was positive electrical ionization.

RESULTS

In Vitro Recovery Determination

Comparable *in vitro* recovery's were obtained for memantine and amantadine by the use of the water method (31 ± 1

and 37 ± 4% respectively). This is not surprising since their chemical structures are comparable and they differ only in two methyl groups.

In Vivo Recovery Determination

The determination of the *in vivo* recovery using either the ZNF or the MT method required steady-state conditions and were determined 7 days after implantation of ALZET osmotic minipumps (zero-order infusion of amantadine or memantine 100 mg/kg/day and 20 mg/kg/day respectively). For memantine, it has been previously shown that steady-state levels are reached within 2 days in brain tissue and serum (15).

Since it is known that both memantine and amantadine strongly accumulate in brain tissue, whereas free ECF concentrations are unknown, a relatively wide range of ingoing concentrations (C_{in}) were used in the ZNF experiments *in vivo* (Fig. 1). Using this method, a recovery of 39.4 ± 2.3% and 24.3 ± 6.4% was found for memantine and amantadine respectively. The correlation coefficients of the regression lines were 0.999 ± 0.0005 for memantine and 0.90 ± 0.04 for amantadine.

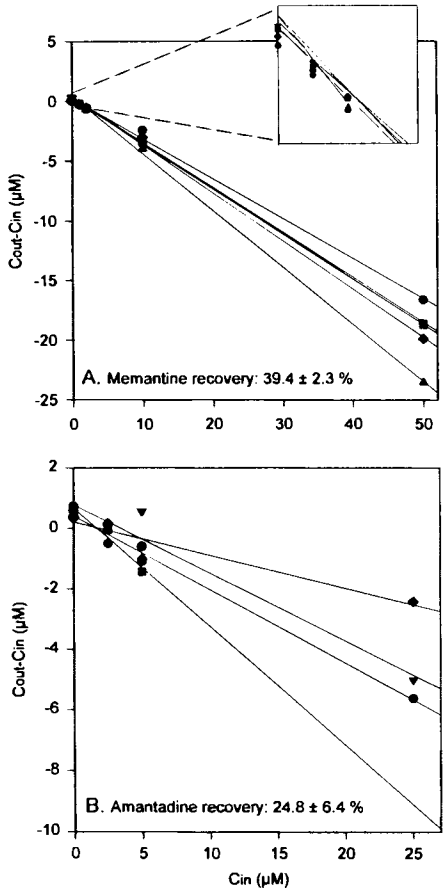

Fig. 1. *In vivo* recovery of memantine (A) and amantadine (B) using the ZNF method. Steady-state concentrations of memantine and amantadine were established by implantation of ALZET osmotic minipumps, ascertaining a dose of 20 and 100 mg/kg/day for memantine and amantadine respectively, 7 days before the experiment. The intercept with the X-axis is the estimate of the brain ECF concentration. The slope is the inverse *in vivo* recovery. Every regression line is derived from an experiment in a single animal.

By using the MT method *in vivo* (Fig. 2), lower values for the recovery of memantine and amantadine were obtained, i.e. 19.9 ± 5.3% and 19.2 ± 2.2% respectively. The linear regression lines of the inverse dialysate concentration versus the flow rate showed high correlation coefficients (0.983 ± 0.005 and 0.97 ± 0.02 for memantine and amantadine respectively).

Distribution Between Different Compartments

Estimates of the free brain ECF concentration were obtained using the two different methods of *in vivo* recovery. By using the ZNF method, a free ECF concentration of

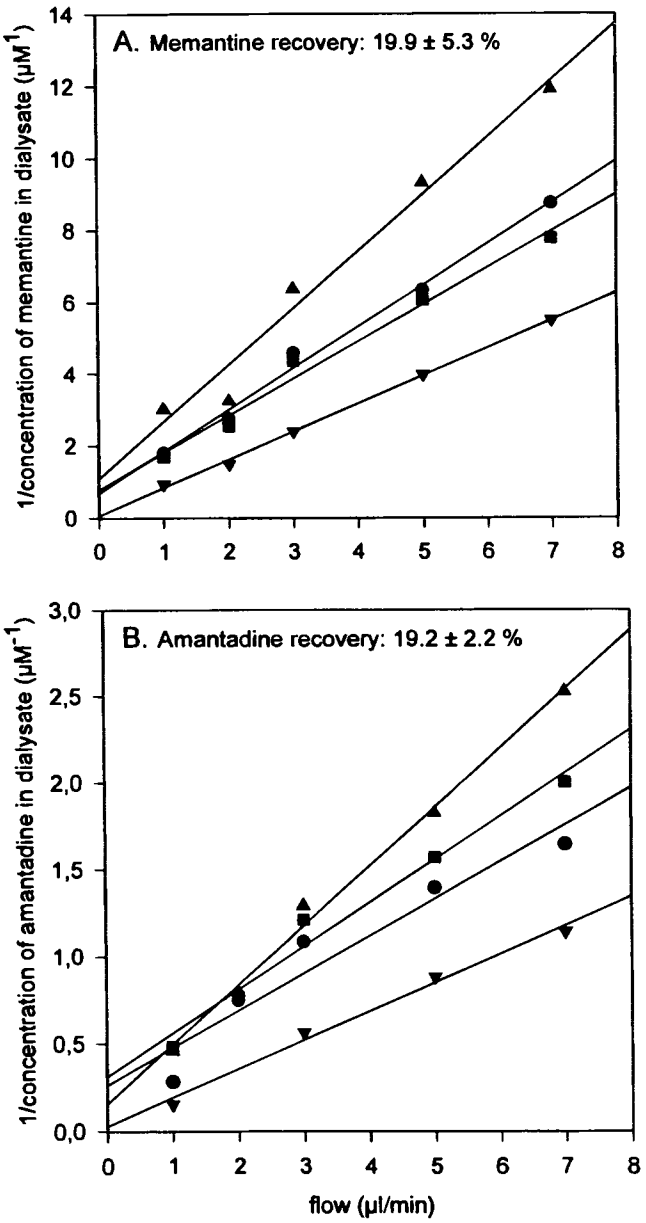

Fig. 2. *In vivo* recovery of memantine (A) and amantadine (B) using an adaptation of the MT method. The dialysis probe was perfused at different flow rates (1, 2, 3, 5, and 7 µl/min) and the outgoing dialysate concentrations were measured. (see Fig. 1 for further description).

0.83 ± 0.05 μM and 2.23 ± 0. 4 μM was found for memantine and amantadine, respectively. Significantly higher ECF concentrations were estimated when the MT method was used i.e. 1.42 ± 0.21 μM and 6.4 ± 1.7 μM. The third method of estimating brain ECF concentrations, the ultrafiltrate of brain homogenates (of the animals used in the ZNF experiment), yielded even higher values i.e. 3.00 ± 0.38 and 12.6 ± 2.1 μM, for memantine and amantadine, respectively.

In CSF samples taken from the cisterna magna memantine reached a concentration of 0.59 ± 0.07 μM, while amantadine attained a levels of 4.63 ± 0.96 μM. Serum levels of both compounds in this experiment were comparable to the serum levels determined in the ZNF experiment i.e., between 1.01 and 1.35 μM (results obtained from these two separate experiments) for memantine and between 7.5 and 8.7 μM for amantadine (Table 1). The free fraction of memantine and amantadine in serum was comparable (59 ± 3% and 51 ± 7% respectively).

Brain tissue concentrations of the aminoadamantanes were considerably higher than the concentrations reached in any other compartment studied (25.9–31.3 μM for memantine and 64.9–72.5 μM for amantadine). Thus both accumulated c.a. 30-fold in brain tissue as compared to free ECF concentration (Table I).

DISCUSSION

Using the ZNF method of microdialysis, an ECF concentration of 0.83 μM and 2.23 μM was obtained for memantine and amantadine respectively. When the memantine ECF concentration was compared to the free serum (0.58 μM), slightly higher (1.4 times) levels were observed in the former compartment. However, for amantadine, only a 54% penetration from serum (free = 4.1 μM) to brain ECF was found. This would mean the BBB possesses different barrier properties for these two drugs, even though they only differ by two methyl groups. The plausible explanation would be that amantadine is a substrate for an unidirectional transport system located at the BBB that does not transport memantine, such as the multidrug resistance transport system, P-glycoprotein, but no support for this hypothesis exists.

Even though the aminoadamantanes, memantine and amantadine, are accumulated in whole brain tissue, their free brain ECF concentrations are comparable to serum and CSF concentration. This means, in the case of memantine, that at therapeutically relevant doses in rats (20 mg/kg/day sc, based on serum levels in humans of up to 1 μM) the ECF concentration (0.83 μM) is within the range shown to affect NMDA receptor function *in vitro* (14,21,22). Moreover, the memantine dose used in these experiments (20 mg/kg/day) has previously been shown to be effective against NMDA receptor mediated neurotoxicity (15,23). Thus, NMDA receptor antagonism is likely the major, if not only, mechanism of action of memantine at therapeutic doses.

For amantadine this is less clear. Depending on experimental procedure, its potency at the NMDA receptor ranges from 12.4 to 71 μM in electrophysiological studies to 20–80 μM in binding experiments (14). The free ECF concentration found in this study (2.23 μM), was clearly below the concentration needed to block NMDA receptor function. Serum concentrations (8.7 μM) were however within range of the concentrations found to be effective in humans (3–13.5 μM) as well as in laboratory animals (4.5–21 μM). This would indicate NMDA receptor antagonism is not the major mechanism of action of amantadine, and other mechanisms e.g. at nicotinic, sigma receptors etc. have to be considered (14).

The values obtained using the ZNF method are the closest possible estimates of the 'true' ECF concentration since no assumptions or extrapolations are made at the point of ZNF. C_{out} equals C_{in} and this concentration equals the extracellular concentration (C_{ECF}). In the simplified adaptation of the MT method it is assumed that a linear relationship exists between flow rate and the inverse of the concentration in the dialysate in this area of the curve (24,25). This is only partly true as can be seen from the too high estimates of the ECF concentration obtained with this method. In fact, it has been previously shown that microdialysate concentrations equal brain ECF concentrations even before the flow-rate approaches zero (20). This means the ECF concentration is overestimated by this linear extrapolation.

Table I. Concentrations Memantine and Amantadine in Serum and Different Compartments of the Brain as Determined by Microdialysis or Direct Tissue Sampling

	Memantine (μM)			Amantadine (μM)		
Compartment	ZNF experiment	MT experiment	CSF experiment	ZNF experiment	MT experiment	CSF experiment
Brain	25.9 ± 4.2		31.3 ± 4.8	64.9 ± 8.8		72.5 ± 13.9
Supernatant	7.10 ± 0.83			23.8 ± 3.8		
Ultrafiltrate	3.00 ± 0.38			12.6 ± 2.1		
ECF	0.83 ± 0.05	1.42 ± 0.21		2.23 ± 0.41	6.4 ± 1.1	
CSF			0.59 ± 0.07			4.63 ± 0.96
Serum-total	1.01 ± 0.10		1.35 ± 0.17	8.7 ± 2.0		7.5 ± 1.8
Serum free	0.59 ± 0.10			4.1 ± 0.6		

Note: Memantine and amantadine were administered for 7 days (20 and 100 mg/kg/day respectively), and the ECF, CSF, serum, and brain tissue concentrations were determined in three separate experiments (ZNF-microdialysis, MT-microdialysis, and CSF sampling). Serum was removed using heart punction and subsequently the brain was removed. The brain tissue was homogenated, centrifuged and ultrafiltrated. The whole tissue, supernatant and the ultrafiltrate were analyzed for memantine and amantadine concentration. Results are average ± SEM, n = 4–6 from separate groups of animals.

Surprisingly the *in vivo* recovery for memantine (39%) obtained with the ZNF method is higher than the *in vitro* recovery (31%). Usually, the *in vivo* recovery is lower, as is the case with amantadine (25% *in vivo* versus 37% *in vitro*) (9). However, in case of cocaine, Menacherry and co-workers (26) previously have reported a higher *in vivo* recovery. This was explained as an effect of microvasculature transport i.e., BBB-transport. This phenomenon would disturb the concentration gradient of solute in close vicinity of the probe which builds up by removal of solute from the tissue. The occurrence of a depletion-zone in close vicinity of the probe, was first described by Bungay and colleagues (20). In case of the memantine, high intracellular accumulation in and the possible subsequent release from brain tissue could cause this disturbance (5).

However, this would not explain the difference between memantine ($Rec._{in\ vivo\ ZNF} > Rec._{in\ vitro}$) and amantadine ($Rec._{in\ vivo\ ZNF} < Rec._{in\ vitro}$). The difference between amantadine and memantine is especially remarkable since the *in vitro* recovery, logP (2.44 and 3.28 respectively) and pKa (10.63 and 10.42 respectively) values are comparable for both compounds (27). A possible explanation for this difference could be the accumulation into brain tissue. Compared to ECF this accumulation is equal for these two aminoadamantanes (c.a 30-fold), however a major difference exists in their brain tissue to free serum ratios. In the case of amantadine, whole brain tissue concentrations are 15.8-times higher than free serum concentrations, while in case of memantine a 43.9-fold difference exists.

This is the first study to show (using *in vivo* recovery) that even though the aminoadamantanes, memantine and amantadine are accumulated in brain tissue, their free ECF concentrations are comparable or even below their free serum concentration. In case of memantine, clinically relevant doses reach a brain ECF concentration within range of its affinity for the channel site of the NMDA receptor. However, the ECF concentration of amantadine is considerably lower than its *in vitro* potency at the NMDA receptor and other mechanisms of action seem likely. After comparing different methods of estimating the free brain ECF concentration (ZNF, MT and brain homogenates), it can be concluded that the microdialysis ZNF method is the most appropriate.

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