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Blood–Brain Barrier Penetration of 3-Aminopropyl-*n*-butylphosphinic Acid (CGP 36742) in Rat Brain by Microdialysis/Mass Spectrometry

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The detection and quantitation of the novel drug 3-aminopropyl-*n*-butylphosphinic acid (APBP), also known as CGP 36742, was performed *in vivo* using microdialysis and tandem mass spectrometry. This drug is a GABA-B antagonist with high specificity for GABA-B receptors. Animals received doses of 100, 200, 500 and 1000 mg kg^{-1} of the drug either intravenously or per os (p.o.). Microdialysis probes, placed by stereotaxis in either the frontal cortex or third ventricle of the rat, were used to collect dialyzate samples over several hours. Samples were then analyzed by micro-electrospray tandem mass spectrometry to achieve a molecular mass and structure specific analysis. For example, animals receiving a dose of 100 mg kg^{-1} p.o. showed a peak concentration of approximately 10 μM in the dialyzate. For comparison, tissue and plasma samples of the drug were measured under the same conditions using gas chromatography/mass spectrometry. This work demonstrates that the microdialysis technique in combination with the molecular specificity and high sensitivity of micro-electrospray tandem mass spectrometry can be used to study the time course of the appearance of unmodified drug in the brain of a single animal. © **1998 John Wiley & Sons, Ltd.**

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INTRODUCTION

Bioavailability and pharmacokinetics, conventionally determined by means of plasma or serum concentration measurements, are important decision criteria for the development of new drugs. Often, these two parameters only become available relatively late in the development process because their procurement is labor and cost intensive. The concentration of a drug and its time course in target tissue often are far more relevant than plasma or serum concentrations, but are hardly ever determined and used as decision criteria. This is particularly true for CNS-active compounds, where the ability to penetrate the blood–brain barrier is a prerequisite. Recently, microdialysis coupled with high-performance liquid chromatography (HPLC) has been used to determine concentrations of neuroactive compounds in the extracellular fluid of the brain as a measure of brain penetration.[1–4]

Microdialysis has gained wide recognition as a sampling technique for the measurement of many neurochemicals *in vivo*[5] and is based on the principle that it imitates the function of a capillary blood vessel. The microdialysis probe, a hollow microfiber with a semipermeable membrane, is implanted within the extracellular space of the tissue of interest in a living animal and perfused with an aqueous fluid. The perfusate exiting the probe reflects the composition of the extracellular fluid because of diffusion of compounds through the membrane, allowing direct sampling of the extracellular fluid in anesthetized or freely moving animals. Microdialysis can be accomplished in nearly every tissue or organ including blood and, furthermore, it is possible to sample repeatedly and continuously for hours or days from a single animal with no fluid loss and without killing the animal. The time course of the concentration of a drug in the brain can thus be followed for a single animal. Microdialysis also excludes from the sample large molecules such as proteins (proteases, peptidases) and glycoproteins, owing to the molecular mass cut-off limit of the membrane, thus providing relatively clean samples where compounds are not further enzymically degraded after dialysis. Among the major disadvantages of microdialysis sampling are the relatively low analyte concentrations obtained and the high salt content of the perfusate. Depending on the (spectral) characteristics and perfusate concentrations of

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the compound of interest, the sensitivity and specificity of detection methods used in HPLC can be inadequate.

Recently, micro-electrospray mass spectrometry (micro-ESMS), which utilizes low flow rates (nl min^{-1}) for flow-injection analysis and achieves high sensitivity, has been developed.[6] This technique is especially suited for the analysis of low-volume samples (0.5–10 μl) such as those usually obtained from microdialysis experiments.[7,8] The sensitivity of micro-ESMS has been shown to be in the amol μl^{-1} range for peptides and the fmol μl^{-1} range for proteins.[6,9] The use of tandem MS (MS/MS) methods further enhances both the specificity and the sensitivity of the analysis. In this technique, in the case of the triple-quadrupole instrument, the first mass analyzer (quadrupole) is set to transmit only a specific analyte ion into the instrument, providing the first level of specificity of the analysis. The selected ion is subsequently subjected to energetic collision with an inert gas in the second quadrupole region of the instrument. These collisions yield characteristic structurally informative fragment ions. One or more of the fragment ions is subsequently selected for transmission by the third quadrupole. This second selection adds structural specificity to the analysis and furthermore greatly reduces the contribution of chemical background noise to the signal, enhancing the limit of detection.

Application of the microdialysis sampling technique combined with micro-ESMS/MS to the analysis of neuropeptides in the extracellular fluid of rat brain has been successful.[7,8] However, use of these combined methodologies for the study of other neuroactive compounds has not previously been demonstrated.

In the present study, microdialysis and micro-ESMS/MS were employed for the detection and quantitation of the novel drug 3-aminopropyl-*n*-butylphosphinic acid (APBP), also known as CGP 36742, $H_2N(CH_2)_3P(O)(OH)(CH_2)_3CH_3$, a GABA-B antagonist ($M_r$ 179.19) with high specificity for GABA-B receptors.[10,11]

The UV and fluorescence properties of this drug do not provide the analytical sensitivity required to measure low concentrations. However, mass spectrometry was successfully used to measure this drug quantitatively in rat brain after administration intravenously (i.v.) or per os (p.o.). The microdialyzates containing the drug were analyzed directly by micro-ESMS/MS without prior sample purification. For comparative purposes, tissue and plasma concentrations of APBP were determined in parallel with one of the drug administration schedules using gas chromatography/mass spectrometry (GC/MS) after appropriate derivatization.

This paper provides data showing that APBP crosses the blood–brain barrier and can be detected in rat brain by utilizing *in vivo* microdialysis and structure-specific micro-ESMS/MS.

EXPERIMENTAL

Microdialysis

Male Sprague–Dawley rats (200–325 g) were anesthetized with 45 mg kg^{-1} sodium pentobarbital (Nembutal; Abbott Laboratories, N. Chicago, IL, USA) given intraperitoneally (i.p.) and secured in a stereotaxic apparatus (Stoelting, Wood Dale, IL, USA). The rats were kept anesthetized by a maintenance i.p. dose of pentobarbital (15–20 mg kg^{-1}) every 90 min. Microdialysis probes (CMA/10, CMA Microdialysis, Sweden) with 4.0 mm dialysis membranes (M_r cut-off 20 000 Da) were implanted into the frontal cortex with the following coordinates after establishing the zero point over the bregma skull suture: anterior–posterior (AP) +3.8 mm, medial–lateral (ML) +3.25 mm and depth from dura [dorsal–ventral (DV)] −5.0 mm.[12] Distilled water was perfused through the probe at a rate of ~0.44 μl min^{-1}. This medium was chosen over artificial salts medium because it provides a solution more amenable to mass spectrometric analysis and, since only relative recoveries are calculated, it does not compromise evaluation of the data. The probe was allowed to equilibrate for 3 h prior to beginning the experiment. The dialysate collected during this time was used to test the biological background of the system and to dilute standards that were used to generate calibration graphs. The experiment was begun by i.v. injection via the tail vein of either 50 mg kg^{-1} (two rats), 100 mg kg^{-1} (three rats) or 200 mg kg^{-1} (two rats) of APBP. Two doses were given p.o., 500 mg kg^{-1} (2 rats) or 1000 mg kg^{-1} (two rats), of APBP dissolved in 0.9% saline. After injection, microdialyzate samples were collected every 30 min (13 μl each) for 4–8 h. In two rats given an i.v. injection of 100 mg kg^{-1} APBP, a second i.v. injection of 100 mg kg^{-} was given after 6 h and samples were collected for another 6 h. In addition, two rats were given 100 mg kg^{-1} APBP, where the microdialysis probe was implanted in the third ventricle with the following coordinates: AP −2.8, ML +0, DV −7.5 mm.[12] All samples were collected in siliconized 500 μl polypropylene microfuge tubes, which were placed in an ice-bath. Samples were immediately frozen in liquid nitrogen after collection and stored at −70 °C until analyzed. After the experiment was finished, the animal was killed, the brain removed and the probe placement was verified by microscopic examination of brain sections.

Instrumentation

Electrospray ionization was performed on a Finnigan TSQ70 triple-quadrupole mass spectrometer, upgraded with TSQ700 software and a 20 kV dynode (Finnigan MAT, San José, CA, USA). The mass spectrometer was equipped with a micro-ES source, modified from a commercial electrospray source (Vestec Products, PerSeptive Biosystems, Boston, MA, USA) as described previously.[6] The micro-ES source utilizes flow-injection techniques at flow rates of 50–1000 nl min^{-1} and provides sensitivity in the amol μl^{-1} range for peptides. Briefly, microspray needles were constructed from a fused-silica capillary of i.d. 50 μm and o.d. 220 μm. One end of this needle was the spray orifice and the other end was attached to a stainless-steel zero dead volume (with a 0.02 in through-hole) where the high voltage connection was made. Standard electrospray operating conditions were as follows: needle voltage, 3.2 kV;

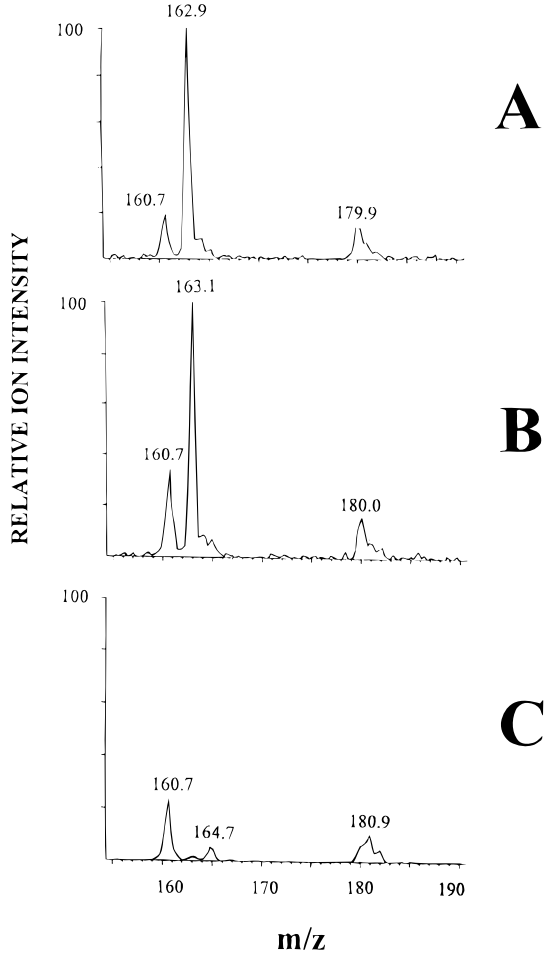

Figure 1. (A) Tandem mass spectrum of 2 μl of a 6 pmol μl^{-1} solution of APBP in 50% acetonitrile, 0.5% formic acid. Data were acquired in the positive MS/MS product ion mode from the $[M + H]^+$ ion of APBP, with *m/z* 180.1. The most prominent fragment ion at *m/z* 163 was monitored in all subsequent analyses. (B) tandem mass spectrum of a microdialysis sample from the frontal cortex of the rat brain after an i.v. infusion of APBP (50 mg kg^{-1}) and (C) a tandem mass spectrum of a microdialysis sample from rat frontal cortex prior to an i.v. infusion of APBP.

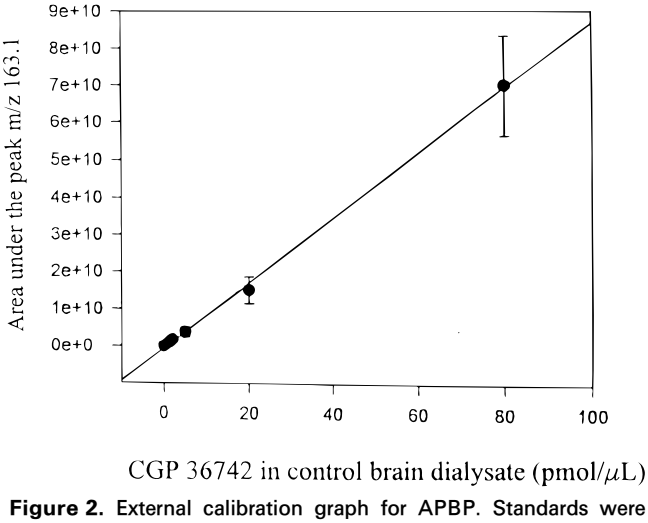

Figure 2. External calibration graph for APBP. Standards were dissolved in microdialyzate samples obtained prior to drug administration to the rat.

nozzle voltage, 205 V; repeller voltage, 9–10 V; and block temperature, 230–240 °C. The drying gas was ultra-pure, dry N_2 at ~1 atm.

Sample analysis

Microdialysis samples were kept frozen (−70 °C) until analysis. Samples were thawed on ice and diluted 1:4 to a final concentration of 50% acetonitrile, 0.5% formic acid. Dilution was necessary in order to reduce the concentration of the biological salts that were present in the microdialyzate sample. The concentrations of the drug in standards or diluted microdialyzates were determined by injection of 2 μl of sample into the stream of continuously infused carrier solvent [acetonitrile–distilled water–88% formic acid (100:100:1, v/v)] flowing at ~0.8 μl min^{-1}. Data were acquired in the positive MS/MS product ion mode from the $[M + H]^+$ ion of APBP at *m/z* 180.1. The most prominent fragment ion at *m/z* 163 produced by collision with argon at 1.8–2.2 mTorr (1 Torr = 133.3 Pa) and a collision offset potential of −10 eV was monitored by scanning a 150–190 u window. This fragment resulted from the neutral loss of NH_3 from the parent $[M + H]^+$ ion. A typical tandem mass spectrum of the pure drug is shown in Fig. 1(A) and is compared with that from an *in vivo* microdialysate in Fig. 1(B). A background spectrum (i.e. biological microdialysate prior to drug administration) is shown in Fig. 1(C).

External calibration graphs were generated by diluting aqueous standard solutions with the appropriate volumes of baseline control microdialysate and diluents and processing the standard solutions in the same manner as the microdialyzates. Quantitation was effected by manual integration of the reconstructed ion chromatogram profile for the *m/z* 163 fragment ion of the drug.

The relative *in vitro* recoveries for APBP by microdialysis were determined by placing microdialysis probes into microvials containing a 500 μl aliquot of a 10 pmol μl^{-1} solution (22–23 °C) of drug. The probes were perfused with distilled water at ~0.44 μl min^{-1} and equilibrated for 30 min. Samples were then taken after 30 and 60 min and analyzed using the micro-ESMS/MS system. The relative recovery ($recovery_{in\ vitro} = C_{out}/C_i$) was calculated by comparing the areas under the peaks in the reconstructed ion chromatograms, where C_{out} is the concentration in the dialysate and C_i the concentration in the medium.

Determination of tissue and plasma concentrations

APBP was determined by GC/MS in tissue extracts or plasma of male Tif:RAlf(SPF) rats (Tierfarm Sisseln, Switzerland) weighing 160–200 g as reported previously.[13] Briefly, tissues were homogenized in appropriate volumes of 0.1 M HCl containing the internal standard, 3-aminopropyl-*n*-propylphosphinic acid, frozen at −80 °C for 24 h, thawed and centrifuged. Plasma was acidified with 2 M HCl containing the internal standard. Tissue extracts and plasma were derivatized sequentially with pentafluorobenzoyl chloride and

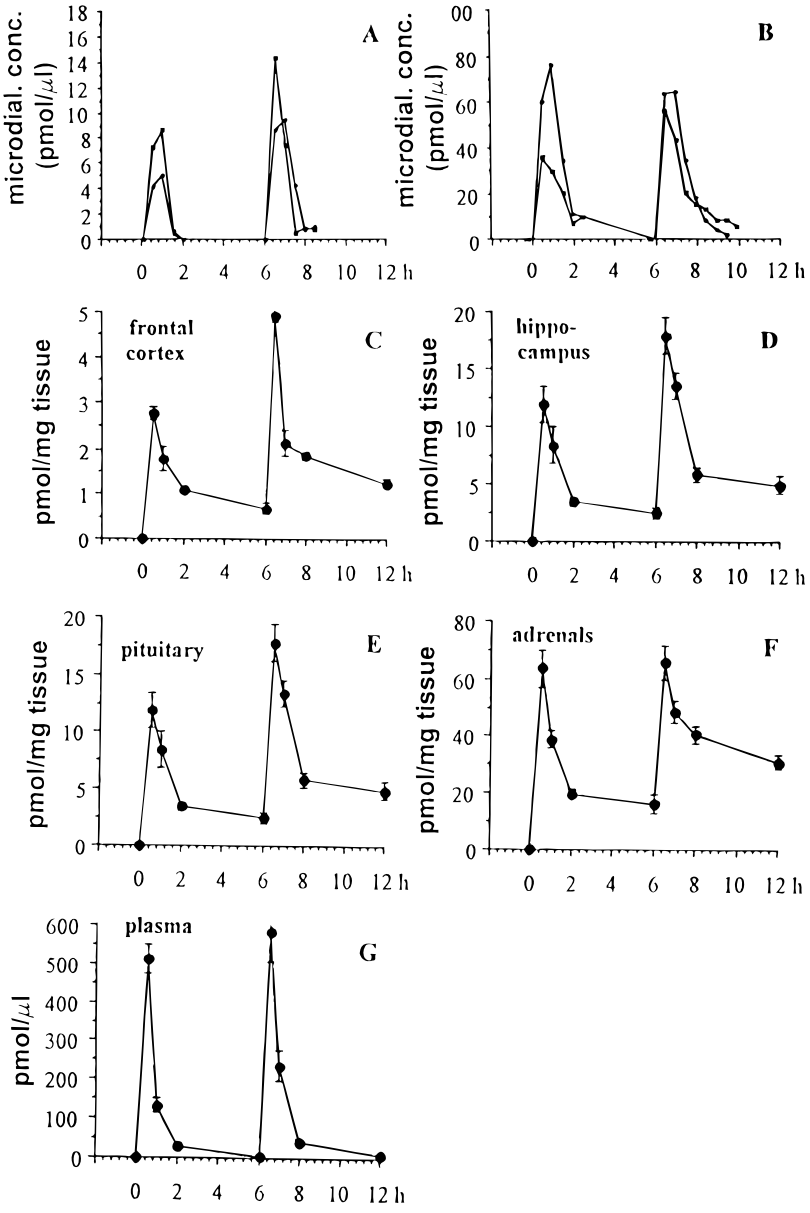

Figure 3. Kinetic analysis of APBP in microdialyzates of rat frontal cortex (A) ($n = 2$) and third ventricle (B) ($n = 2$), and tissue concentrations in rat frontal cortex (C) ($n = 5$), hippocampus (D) ($n = 5$), pituitary (E) ($n = 5$), adrenals (F) ($n = 5$) or plasma concentrations (G) ($n = 5$) of CGP 36742 after injection of 100 mg kg^{-1} i.v. at 0 and 6 h. For comparison with the microdialysis data, tissue concentrations are given in pmol mg^{-1} tissue and plasma concentrations in pmol μl^{-1}.

trimethyl orthoformate to yield methyl 3-(pentafluorobenzoyl) aminopropyl-*n*-butylphosphinate and the corresponding derivative of the internal standard. Quantitation was performed at *m/z* 367 (derivative of APBP) and *m/z* 353 (derivative of internal standard) on a Finnigan TSQ700 GC/MS instrument equipped with a Fisons Model 8560 gas chromatograph having a 12 m × 0.22 mm i.d. fused-silica capillary column (SGE, 12 QC2/BPX5), using helium as the carrier gas.

RESULTS

Sample analysis and calibration graphs

External calibration graphs were used for the quantitation of APBP in dialyzates. All standard solutions of the drug were made up in dialyzates that were collected from the animal prior to drug administration and were analyzed in the same way as authentic samples. Figure 2 represents a mean of the external calibration graphs obtained by micro-ESMS/MS, showing the linearity produced by direct injection of various concentrations of APBP in diluted microdialyzates. The calibration graphs yielded an average linear coefficient r^2 of 0.9930 ± 0.0076 ($n = 13$, mean $\pm$ SD) and a detection limit of 500 fmol μl^{-1} dialysate.

Microdialysis recovery

Estimates of the *in vitro* relative recoveries for APBP were performed to check the permeability of the dialysis probe membranes to the analyte and to provide a normalization factor for comparison of results obtained

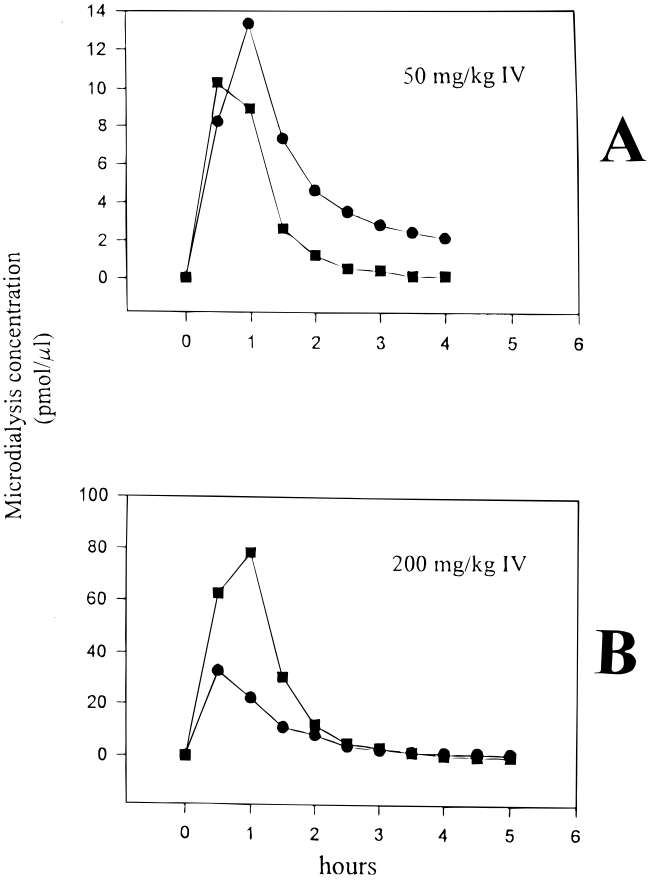

Figure 4. (A) Kinetic analysis of APBP in rat frontal cortex ($n = 2$) after i.v. administration of 50 mg kg^{-1} or (B) 200 mg kg^{-1} ($n = 2$). The points marked by squares and circles represent duplicate experiments.

with different probes. A constant perfusion was performed at a flow rate of ~0.44 μl min^{-1}. An exact amount of drug was dissolved in artificial CSF and the microdialysis probe was lowered into the solution (10 pmol μl^{-1}) at room temperature. After the probe had been allowed to equilibrate for 30 min, the perfusate was analyzed to determine the concentration of drug in the outflow. From these measurements, the relative recovery of drug was determined to be 45 ± 5% ($n = 12$, mean ± SD). However, the generally used procedure of correcting *in vivo* dialysis data using *in vitro* calibration may not be accurate because it does not take into account the differing diffusion and transport characteristics of the drug and individual variability of microdialysis probes.[14] Therefore, amounts of APBP reported were not corrected for the average *in vitro* recovery.

Drug accumulation in the frontal cortex and third ventricle

Figure 3(A) shows the results of two 12 h experiments with 100 mg kg^{-1} APBP administered i.v. at 0 and 6 h. The appearance of the intact drug in the rat brain frontal cortex after i.v. injection was seen in the first 30 min dialyzate collected and peaked in the 1 h dialysate. The compound was also removed and/or metabolized from the brain, such that the drug was at the limit of detection in dialyzates collected 3 h post-injection. The presence of drug was again seen 30 min after the second 100 mg kg^{-1} i.v. injection administered 6 h after the first injection. Again, levels decreased over the next 3 h to the limit of detectable amounts. Figure 3(B) shows the appearance of APBP in the third ventricle of the rat brain after two i.v. injections of 100 mg kg^{-1} with an interval of 6 h. The time course for the accumulation of the drug in the ventricle was very similar to that seen in the frontal cortex. The amount of drug in the third ventricle increased and began to decrease at the same time points as in the frontal cortex, but was approximately five-fold higher. After the first injection, the drug was detectable for an additional 30 min and was detectable for an additional 1.5 h after the second injection as compared with the frontal cortex. Figure 4(A) and (B) show the results for the 50 and 200 mg kg^{-1} i.v. administration of APBP, respectively, where the highest concentrations of the drug were seen 30 min or 1 h post-injection. Peak levels were not different between 50 and 100 mg kg^{-1} i.v., but were markedly (3–7-fold) higher after 200 mg kg^{-1} i.v. The p.o. administration of APBP showed that the highest concentrations were seen 1.5 or 2 h after the administration (Fig. 5). Interestingly, the peak levels after 1000 mg kg^{-1} p.o. were 10-fold or more higher than those after 500 mg kg^{-1}.

Drug concentrations in tissue and plasma

The concentrations of APBP in frontal cortex, hippocampus, pituitary, adrenals, and plasma 30 min and 1, 2 and 6 h after two consecutive i.v. injections of 100 mg kg^{-1}, 6 h apart, were determined in groups of five rats per time in parallel to the microdialysis experiment described above. The results are depicted in Fig. 3(C)–(G) in pmol of drug per mg of tissue to facilitate comparison with the microdialysis data. In all tissues and in plasma, the time course of the concentrations of APBP closely resembled that seen in microdialyzates. There were large differences in the absolute amounts, however, in hippocampus and cortex, the tissue concentrations expressed in pmol mg^{-1} were about half those in microdialyzates expressed in pmol μl^{-1}. In the pituitary, adrenals, and plasma, they were about 3-, 25-, and 200-fold higher than in the brain areas, respectively. It is of interest that, as in the microdialysis experiments, the peak concentrations after the second injection tended to be higher than those after the first injection.

DISCUSSION

We have studied the brain bioavailability of the GABA-B antagonist APBP in the cerebral cortex and the third ventricle of rats using *in vivo* microdialysis and micro-ESMS/MS. Small concentration changes of analyte superimposed on a high background could be measured without any sample clean-up other than dilution with a solvent appropriate for ESMS. The mass-specific detection of analytes provided direct molecular analysis, eliminating ambiguities inherent in other

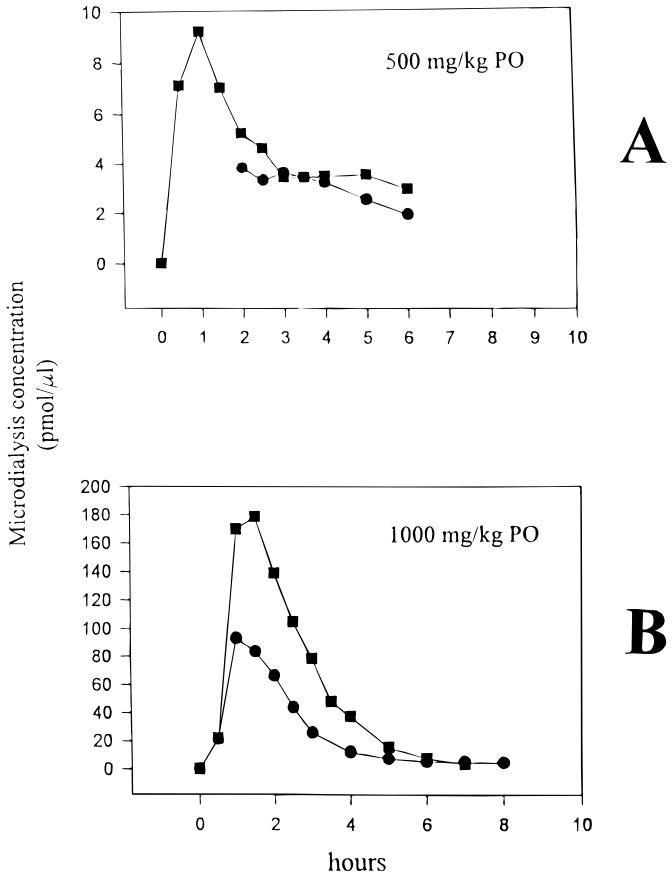

Figure 5. (A) Kinetic analysis of APBP in rat frontal cortex ($n = 2$) after p.o. administration of 500 mg kg^{-1} or (B) 1000 mg kg^{-1} ($n = 2$). The points marked by squares and circles represent duplicate experiments.

detection methods. The microdialysis samples in the present study were analyzed without prior sample purification. The dialyzates were thawed on ice and diluted 1:4. Dilution was necessary in order to reduce the concentration of the biological salts that were in the dialysate sample and to add solvent components necessary for stable ES ionization. Desalting with nanoflow capillary liquid chromatography was not done because the compound was not retained on the reversed-phase packings. All analyses were performed by MS/MS for two reasons: in the MS mode, a background ion of m/z 182 significantly interfered with measurement of the drug ion (m/z 180.2), and MS/MS provided much greater specificity for the intact parent compound and lowered the background significantly.

The time courses for the appearance and disappearance of APBP after i.v. administration as determined by micro-ESMS/MS were very similar in frontal cortex and in the ventricle and matched those determined in parallel in brain tissue, pituitary, adrenals and plasma, the half-lives being ~30 min. Concentrations of the drug in cerebral microdialyzates and in brain tissue were low compared with the administered dose, indicating poor brain penetration. This is underscored by the observations that the concentrations in the pituitary (which is at least partially outside the blood–brain barrier), adrenal glands, and plasma were about 3-, 25-, and 200-fold higher, respectively, than those in brain. Considering the incomplete recovery in microdialyzates, one may estimate that tissue concentrations were ~3–4-fold lower than those in the extracellular fluid, which may reflect a limited cell penetrability of the compound, probably a consequence of its hydrophilicity.

The peak concentrations of APBP in frontal cortical microdialyzates of rats treated with 100 mg kg^{-1} i.v. or 500 mg kg^{-1} p.o. were ~10 μM, not corrected for recovery. Based on the observation that the *in vitro* recovery of the probes was about 50%, we can therefore assume that the true extracellular peak concentrations of the compound were ~20 μM. At 200 mg kg^{-1} i.v. or 1000 mg kg^{-1} p.o., markedly higher amounts in microdialyzates were reached (30–80 and 90–180 μM, respectively), which again would have to be about doubled to indicate extracellular levels. The similarity between the tissue concentrations of the drug in frontal cortex and hippocampus makes significant regional differences in the brain unlikely.

In the microdialysis experiments, peak amounts of the drug in the ventricle were significantly higher (>5-fold) than in the frontal cortex. The increased amounts of APBP in the third ventricle as compared with the frontal cortex may be due to a combination of several factors. The ventricle is a more dynamic region in that the microdialysis probe is surrounded by a pool of CSF and not cells as in the frontal cortex. This absence of tissue tortuosity would greatly enhance the recovery of the drug across the microdialysis membrane.[14] The drug levels in the third ventricle may also be higher owing to a greater transfer of the drug to the CSF. There are seven small regions that line the ventricle that

lack a tight blood–brain barrier. These areas, the circumventricular organs, are passively permeable to hydrophilic solutes such as APBP.[15–17]

Compared with micro-ESMS/MS, determination of tissue concentrations has the advantage that it is possible to determine drug amounts in many tissues of brain regions if that is required. On the other hand, with micro-ESMS/MS in general it takes less time to develop the analytical methodology for an individual compound (once the technique is established) and several-fold fewer animals are required to obtain equivalent information. In the present case, the information obtained with 12 animals permitted the conclusion that the compound, after i.v. and p.o. administration, did not reach adequate concentrations in the brain to explain the observed cognitive effects on the basis of GABA-B receptor inhibition. Used early in drug development, this technique provides the possibility to determine rapidly whether a given compound reaches adequate tissue levels and thus fulfills an essential criterion for pursuing it further. For a more explicit assessment of blood–brain transfer of a drug, simultaneous microdialysis in brain and blood[1] or brain microdialysis combined with serial blood sampling[18] is indicated.

In conclusion, *in vivo* microdialysis in combination with micro-ESMS/MS provides molecular specific monitoring of systemically administered pharmacological agents in target tissues. This work demonstrated that microdialysis/micro-ESMS/MS could be applied effectively for a complete time course study of the appearance of the unmodified drug in rat brain in a single animal.

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