Distribution of Tacrine Across the Blood–Brain Barrier in Awake, Freely Moving Rats Using *in Vivo* Microdialysis Sampling

Martin Telting-Diaz[1] and Craig E. Lunte[1,2]

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Microdialysis was used to sample simultaneously the distribution of THA (9-amino-1,2,3,4-tetrahydroacridine; Tacrine), a potential anti-Alzheimer agent, both in blood and across the blood–brain barrier of anesthetized and awake, freely moving rats. Microdialysis probes were implanted in the jugular vein and dorsal hippocampus and dialysis samples were simultaneously collected from both sites. Dialysis samples were analyzed using a microbore column chromatographic assay with a detection limit of 0.3 ng/ml. Pharmacokinetic parameters were calculated after a 1 mg/kg intravenous dose of THA. Plasma pharmacokinetics followed a biexponential mode, with $t_{1/2}$(dis.) = 8.4 ± 2.7 min and $t_{1/2}$(elim.) = 76.7 ± 24.2 min for awake, freely moving rats. THA rapidly penetrated the blood–brain barrier, with maximum concentrations attained within 60 min post-dose. In the brain of awake, freely moving rats $t_{1/2}$(abs.) was 26.0 ± 5.2 min and $t_{1/2}$(elim.) was 99.1 ± 17.7 min. THA levels in hippocampus extracellular fluid were 10 times lower than those in plasma. For anesthetized rats, the $t_{1/2}$(elim.) in blood was 154.8 ± 46.8 min, while in the hippocampus $t_{1/2}$(elim.) was 159.5 ± 31.7 min. The binding of THA in rat plasma was 56.2 ± 5.0%, while the fraction bound to rat whole blood was 73.3 ± 4.1% as determined by microdialysis and ultrafiltration.

KEY WORDS: Tacrine; microdialysis; blood–brain barrier.

INTRODUCTION

THA (9-amino-1,2,3,4-tetrahydroacridine; Tacrine) is a cholinergic drug capable of centrally inhibiting choline acetylesterase. Clinically, THA has been used for the treatment of a variety of indications (1–4). In recent years, much attention has centered on THA due to studies describing improvement in memory and functional performance of patients with Alzheimer's disease (5,6).

Alzheimer's disease is a highly disabling neuropsychiatric disorder characterized by an irreversible deterioration of memory and intellectual behavior. While the etiology of Alzheimer's disease remains unknown, evidence has been presented that the hippocampus (an essential brain structure for memory and learning) is one of the principal areas affected by Alzheimer's disease (7). A specific loss of cholinergic neurons and deficits of choline acetyltransferase have been suggested to play a major role in the primary cognitive symptoms of the disease (8). Decreased central cholinergic activity has received major attention from investigators in search of a biochemical approach that supports a pharmacotherapy for the disease.

To establish rational dose regimens and drug concentrations, accurate pharmacokinetics profiles are necessary not only in the vascular system but also in the extracellular space of target pharmacological sites. Classical techniques employed to sample drug distribution in discrete brain areas are often time-consuming, are mostly invasive, and perturb tissue structures. Furthermore, the anesthesia commonly associated with these procedures may interfere with the true hemodynamics and metabolism implicated in that organism (9,10).

In vivo microdialysis sampling has been commonly used to monitor neurochemicals in the extracellular fluid (ECF) of discrete brain regions (11,12). More recently, the technique has been applied to pharmacokinetic and metabolism studies (13–17), as well as for sampling other living tissues (18,19).

This report describes the application of *in vivo* microdialysis to determine the pharmacokinetics of THA in blood and its transport across the blood–brain barrier into the dorsal hippocampus of anesthetized and awake, freely moving rats. A recent report has described the use of microdialysis to sample discrete brain regions in combination with classical blood sampling to study the distribution of zidovudine in the rabbit (20).

MATERIALS AND METHODS

Chemicals

THA (9-amino-1,2,3,4-tetrahydroacridine) was purchased from Sigma Chemical Company (St. Louis, MO). Chloral Hydrate C-IV and HPLC-grade methanol were obtained from Fisher Scientific (Fair Lawn, NJ). All other chemicals were reagent grade and were used as received.

Apparatus

Dialysis System. Microdialysis probes used for blood sampling were constructed of fused silica tubing (75-μm i.d., 147-μm o.d.) with a 4-mm length of regenerated cellulose dialysis fiber (232-μm i.d., 250-μm o.d., 5000 MW cutoff). The construction of these probes has been described elsewhere (17). Brain dialysis experiments were conducted with CMA/12 microdialysis probes (polycarbonate membrane: 210-μm i.d., 500-μm o.d., 20,000 MW cutoff, 2-mm length; Bioanalytical Systems, Inc./CMA, West Lafayette, IN) supplied with an intracerebral guide cannula and a dummy probe. The perfusion medium was delivered at a flow rate of 1.00 μl min^{-1} for all experiments using a CMA/100 microinjection pump from BAS/CMA. Dialysate samples were collected over 10-min intervals by a CMA/200 refrigerated fraction collector into 250-μl polypropylene microcentrifuge tubes.

Chromatographic System. The analysis of samples was performed by HPLC. The system consisted of a Shimadzu LC-6A liquid chromatographic pump with an SCL-6A system controller (Shimadzu Scientific Instruments, Inc., Columbia, MD) and a Rheodyne 9125 injection valve with a 5-μl sample loop. A Chrompack Fluorescence detector (Raritan,

[1] Department of Chemistry, University of Kansas, Lawrence, Kansas 66045.
[2] To whom correspondence should be addressed.

NJ) operated at 330-nm excitation and 365-nm emission wavelengths, with a slit width of the Em monochromator set at 30 nm, was used to detect THA. Separation of the drug was achieved with an Alumina-CN microbore column (100 mm × 1-mm i.d) packed with 8-μm-particle size material from Bioanalytical Systems, Inc. THA was eluted with a mobile phase of 0.05 *M* ammonium phosphate buffer, pH 6.9, and 25% acetonitrile (v/v). A Datajet Integrator (Spectra-Physics, San Jose, CA) connected to a WINner/386 workstation was used to obtain the chromatographic data.

Surgical Procedure

Sprague–Dawley rats weighing 350–450 g were anesthetized with chloral hydrate, 400 mg/kg, via ip injection. Under anesthetic effect, surgical regions were shaved and an incision was made in the midline neck region, exposing the right jugular vein. A microdialysis probe was inserted ca. 2.5 cm into the vein toward the heart as described previously (17). The animal was then securely positioned in a stereotaxic instrument (David Kopf Instruments) with the incisor bar set at −3.3 mm from the interaural line. A midline incision of approximately 2 cm was made parallel to the sagittal suture. The position of the bregma was used as a reference point to locate the hippocampus. A hole 2 mm in diameter was made through the skull using a Trephine drill, and an intracerebral guide cannula was lowered into the right dorsal hippocampus using a micromanipulator attached to the stereotaxic equipment. The coordinates were −3.3 mm anterior, −20 mm lateral, and −4.0 mm ventral, according to the rat stereotaxic atlas of Paxinos and Watson (21). The position of the dialysis probes in the hippocampus was visually determined after each experiment. The brain was removed from the animal and sectioned to determine the precise placement of the probe.

For awake animal experiments, two other holes were drilled on the skull to place stainless-steel anchor screws (size: 1-mm diameter, 2-mm length). The guide cannula and anchor screws were affixed to the skull with dental cement (Dentsply Ltd., Surrey, UK). After implantation was completed, the incision was covered with the tissue and closed with a drop of glue so that only the guide cannula was exposed. A dummy probe was inserted through the cannula and a recovery time of 24 hr was permitted to elapse before experiments began. During this time the rat was allowed ad libitum access to food and water. After the recovery period, the animal was attached to an awake animal system (CMA/BAS) and the dummy probe was replaced by the working microdialysis probe. The awake animal system allows the animal full range of motion during dialysis sampling. The animal was allowed at least 2 hr after attachment to the awake animal system before a pharmacokinetic experiment was begun. In experiments performed on anesthetized rats, the intracerebral guide cannula was removed prior to insertion of the dialysis probe. The dialysis probe was inserted directly into the brain and held in position by the micromanipulator.

It has been demonstrated that implantation of a microdialysis probe does not permanently disrupt the blood–brain barrier (22). Approximately 30 min after implantation the membrane has sealed around the dialysis probe and the barrier is intact. In these experiments, the integrity of the blood–brain barrier was determined after the experiment by iv infusion of Cleave's acid. That this compound did not appear in subsequent brain dialysis samples was taken as evidence of the barrier's integrity.

Pharmacokinetic Experiments

Probes inserted in the jugular vein were perfused with freshly prepared Ringer's solution (155 m*M* NaCl, 5.5 m*M* KCl, and 2.3 m*M* $MgCl_2$) at a perfusion rate of 1.00 μl min^{-1} for all experiments. For sampling of the hippocampus an artificial cerebrospinal fluid (CSF) solution mimicking physiological concentrations of major electrolyte components was prepared, consisting of 140 m*M* Na^+, 3.0 m*M* K^+, 1.2 m*M* Ca^{2+}, 1.0 m*M* Mg^{2+}, 130 m*M* Cl^-, 20 m*M* HCO_3^-, and 0.25 m*M* HPO_4^{2-} at pH 7.6 (12). The perfusion rate through the intracerebral probes was also 1.00 μl min^{-1}.

All microdialysis probes were calibrated *in vitro* prior to *in vivo* use as described previously (17,18). Recovery was determined in either Ringer's solution or artificial CSF solution, depending upon the implantation site, maintained in a thermostated bath at 37°C. The recovery of THA ranged from 33 to 39% for the intravenous probes and from 21 to 25% for the intracerebral probes.

For all experiments, samples were continuously collected over 10-min intervals. Blank dialysis samples were collected for at least 1 hr prior to dosing with THA. THA standard solution prepared in 0.9% NaCl at 37°C was administered bolus to rats via the iv cannula in a volume no larger than 0.5 ml. Dialysis samples were continuously collected from both the intravenous probe and the hippocampus probe. Whole-blood samples of 100 μl were collected at 30-min intervals for the first hour and every hour there after.

Microdialysis samples were injected directly into the chromatographic system. Whole-blood samples were centrifuged for 10 min. THA was extracted from 90 μl of the resulting plasma following a modification of the method of Forsyth *et al.* (23). The plasma sample was treated with 50 μl of 1.5 *M* Tris · HCl buffer, pH 10. This was extracted with two 500-μl volumes of chloroform using vortex mixing for 45 sec. After centrifugation, the chloroform layers were removed, combined, and evaporated to dryness at 40°C under

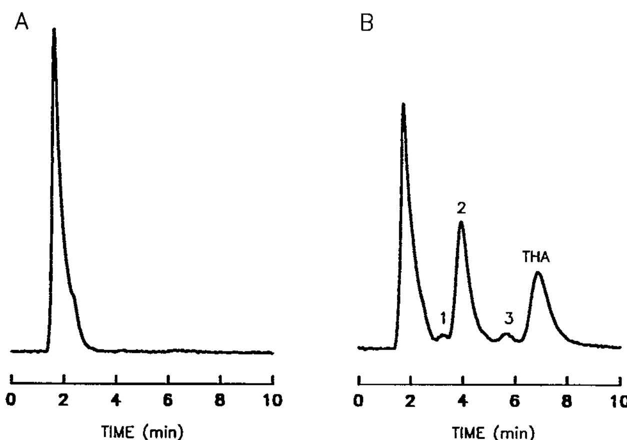

Fig. 1. Typical chromatograms of blood dialysate: (A) blank prior to dosing; (B) 1 hr after a 1 mg/kg iv dose of THA.

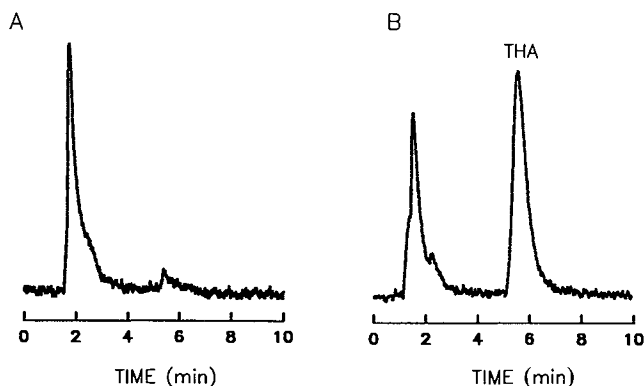

Fig. 2. Typical chromatograms of hippocampus dialysate: (A) blank prior to dosing; (B) 1 hr after a 1 mg/kg iv dose of THA.

a gentle stream of argon. The residue was reconstituted in 100 μl of saline solution, and 5 μl was injected into the chromatographic system. Recovery of THA from whole blood was found to be 91.2 ± 9.7% using this procedure.

Protein Binding

Both microdialysis and ultrafiltration were used to determine the extent of THA binding to blood proteins. The procedure for microdialysis determination of protein binding has been described previously (24). Determinations performed by ultrafiltration were made with an MPS-1 micropartition system with a YM-T membrane filter (Amicon, Lexington, MA). Binding studies were carried out in both rat whole blood and rat plasma samples. The samples were spiked at 20, 50, and 100 ng/ml, equilibrated at 37°C for 1 hr, and analyzed in triplicate by microdialysis and ultrafiltration.

Pharmacokinetic Calculations

Sample concentrations were determined from the chromatographic peak area by fitting to a standard curve. The standard curve was prepared from standards of THA in Ringer's solution over the range 0.5 to 200 ng/ml. The *in vivo* concentration of microdialysis samples were then corrected for the dialysis probe recovery.

Noncompartmental analysis was used to determine pharmacokinetic parameters. The pharmacokinetic parameters for blood samples were calculated as described previously (17). To include absorption as well as distribution and elimination, brain dialysate data were fitted to a triexponential equation of the form

$$C(t) = Ce^{-\gamma t} - Ae^{-\alpha t} - Be^{-\beta t}$$

where γ is the absorption rate constant and α and β are the distribution rate constant and the elimination rate constant, respectively. All results are expressed as the mean ± standard deviation.

RESULTS AND DISCUSSION

Protein Binding

THA was found to be 56.2 ± 5.0% bound to rat plasma and 73.3 ± 4.1% bound to rat whole blood. The binding was concentration independent over the range studied, 20–100 ng/ml. The difference observed between plasma and whole blood indicates that, at least *in vitro,* more than 10% of THA distributes into blood cells. Several authors (25,26) have demonstrated binding of THA to membrane-bound choline acetylesterase of human and bovine erythrocytes *in vitro*. In light of the difference between whole blood and plasma, one must stress the need to use whole-blood recovery values when assessing the performance of dialysis probes.

Pharmacokinetics in Awake, Freely Moving Rats

Intravenous THA (1 mg/kg) was given to rats which had dialysis probes inserted in the jugular vein and dorsal hippocampus. Blank blood and hippocampal dialysate samples were collected for at least 1 hr prior to dosing and the chromatograms obtained during this time showed no interferent peaks (Figs. 1A and 2A). After THA administration, samples were collected for at least 4 hr. Levels of the drug could still be detected in both blood and brain after this time at the dose given. Peaks 1, 2, and 3 in Fig. 1B are presumably THA metabolites, since they are not present in blank samples. These compounds appeared in blood dialysates soon after drug administration and roughly followed the concentration–time curve of THA. Hydroxylated metabolic products of THA, which have been implicated in the adverse effects reported with this drug (27), have been observed in human and animal studies using HPLC with fluorescence detection (28). The identity of these metabolites has not been confirmed at this point.

The calculated pharmacokinetic parameters, mean values and standard deviations ($n = 3$), for awake, freely mov-

Table I. Pharmacokinetic Parameters for THA in the Blood and Hippocampus Extracellular Fluid of Awake and Anesthetized Rats[a]

	Awake, freely moving		Anesthetized	
	Blood	Hippocampus	Blood	Hippocampus
$t_{1/2}$(dis.) (min)	8.4 ± 2.7	9.8 ± 6.9	13.8 ± 9.3	10.7 ± 3.8
$t_{1/2}$(elim.) (min)	76.7 ± 24.2	99.1 ± 17.7	154.8 ± 46.8	159.5 ± 31.7
$t_{1/2}$(abs.) (min)		26.0 ± 5.2		49.8 ± 11.3
MRT (hr)	0.9 ± 0.4		1.2 ± 0.6	
AUC (μg ml/mL)	31.6 ± 22.6	24.0 ± 6.2	60.7 ± 17.0	29.6 ± 15.1
V_β (L)	1.5 ± 0.9		0.9 ± 0.5	
CL (ml/min)	12.4 ± 5.2		6.8 ± 2.1	

[a] $n = 3$.

ing and anesthetized rats are summarized in Table I. THA given intravenously to awake, freely moving rats was characterized by a short initial distribution phase with a half-life of 8.4 ± 2.7 min and an elimination phase with a half-life of 76.7 ± 24.2 min. The time courses for blood concentrations of THA in the awake, freely moving rat are shown in Fig. 3. The AUC was 31.6 ± 22.6 μg min/ml, the volume of distribution was 1.5 ± 0.9 L, and the plasma clearance was 12.4 ± 5.2 ml/min. All of these parameters indicate a wide tissue distribution and rapid elimination in accordance with earlier reports from human studies (29).

As expected, THA readily crossed the blood–brain barrier, with maximum concentrations reached within 30–60 min, with a half-life of absorption of 26.0 ± 5.2 min and a peak concentration of 33.1 ± 6.4 ng/ml. Following absorption, the concentration of THA in hippocampus extracellular fluid followed a biexponential decrease parallel to that in plasma (Fig. 4), with a distribution half-life of 9.8 ± 6.9 min, an elimination half-life of 99.1 ± 17.7 min, and an AUC of 24.0 ± 6.2 μg min/ml. Baldwin *et al.* (30) have recently reported that following intraperitoneal injection of 5 mg/kg to rats, peak concentrations of THA in striatum ECF were about half those in plasma. In the present study, THA rapidly penetrated the hippocampus extracellular space, and while the brain–blood ratio was not as high as cited above, ECF concentrations could be monitored for nearly 4 hr.

Pharmacokinetics in Anesthetized Rats

In chloral hydrate-anesthetized rats, THA levels were similar to those found in the awake animal, although the half-life of elimination in blood increased to 154.8 ± 46.8 min and the AUC also had a twofold increase. A similar increase in the elimination half-life between awake and chloral hydrate-anesthetized rats has been observed for theophylline using microdialysis sampling (17). Comparison of blood dialysate samples to simultaneously collected whole-blood samples showed excellent agreement when binding of THA in blood was taken into account (Fig. 5). These results demonstrate that microdialysis sampling of the blood can be compared to classical serial blood removal methods recognizing that dialysis provides the free concentration, while whole blood provides the total concentration. In addition, it is again shown that anesthesia is extremely perturbing to the observed pharmacokinetics.

By comparison to the blood dialysates shown in Fig. 1, blood extracts were not nearly as interferent free (Fig. 6), even though the dialysates were directly injected into the chromatographic systems without any extraction or cleanup. It must be remembered that microdialysis directly samples

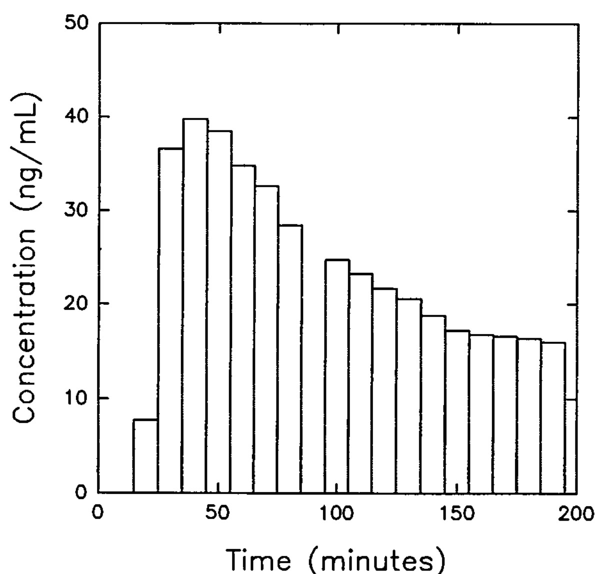

Fig. 4. Concentration-versus-time profile for THA in the hippocampus extracellular fluid of an awake, freely moving rat following a 1 mg/kg iv dose.

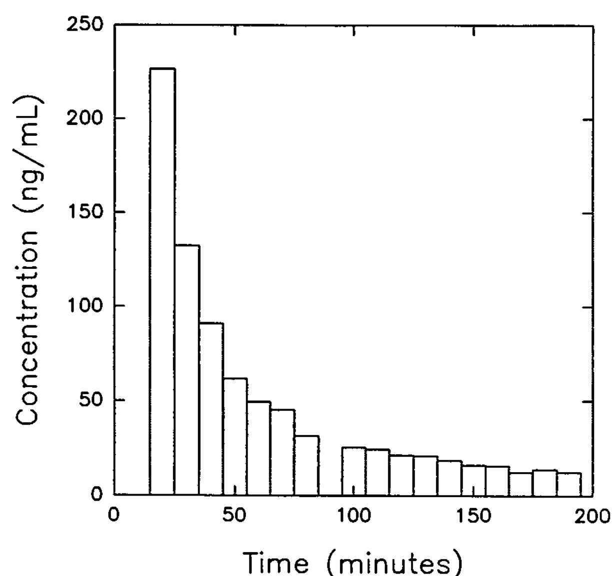

Fig. 3. Concentration-versus-time profile for THA in the blood following a 1 mg/kg iv dose.

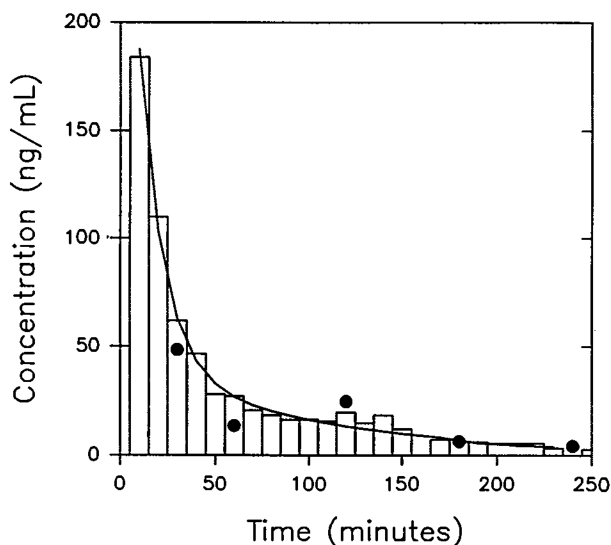

Fig. 5. Concentration-versus-time profile for THA in the blood of an anesthetized rat following a 1 mg/kg iv dose. The columns represent the microdialysis samples and the circles represent the whole-blood samples. The concentration of THA in whole blood samples is corrected for protein binding.

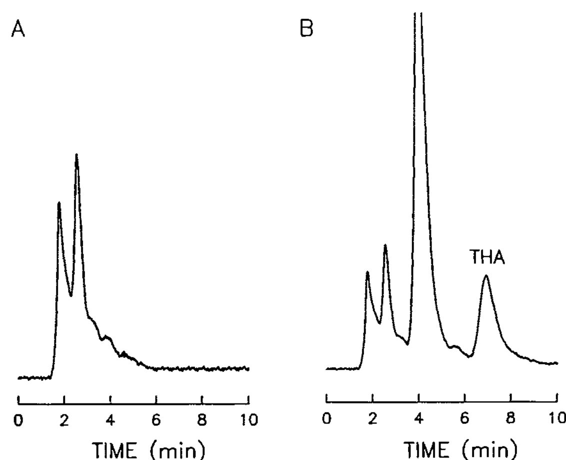

Fig. 6. Typical chromatograms of whole-blood extract: (A) blank prior to dosing; (B) 1 hr after a 1 mg/kg iv dose of THA.

only the unbound fraction of the drug in the blood, instead of the total concentration. As it is the unbound fraction that can partition into the tissues, this should be a more relevant determination. This will be particularly true in the case of drugs whose binding is concentration dependent.

As with the pharmacokinetics in blood, anesthesia profoundly perturbed the pharmacokinetics of THA in the brain. The half-life of absorption increased to 49.8 ± 11.3 min and the half-life of elimination increased to 159.5 ± 31.7 min, essentially mimicking the increase in blood elimination half-life.

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