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Journal of Pharmacological and Toxicological Methods 49 (2004) 57–64
Journal of Pharmacological and Toxicological Methods
www.elsevier.com/locate/jpharmtox

Original article

An automated blood sampler for simultaneous sampling of systemic blood and brain microdialysates for drug absorption, distribution, metabolism, and elimination studies

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Received 17 June 2003; accepted 11 July 2003

Abstract

Introduction: A major problem in preclinical drug development where blood sampling from small animals is a routine practice is the time and labor involved in the serial sampling of small blood volumes from small animals such as rats for the duration of pharmacokinetic/pharmacodynamic (PK/PD) studies. The traditional method of manually drawing blood from the animal requires the animal to be anesthetized or restrained with some device, both of which cause stress to the animal. **Methods:** An automated blood sampler (ABS) was developed to simultaneously collect blood and brain microdialysate samples at preprogrammed time points from awake and freely moving animals. The samples are delivered to fraction collectors and stored at 4 °C until use. The lost blood volume during collection is replaced with sterile saline to prevent fluid loss from the animal. In addition, the system is capable of collecting urine and feces for metabolism studies and monitoring the animal activity for behavioral studies. In the present study, blood samples were collected for 24 h after dosing rats orally with a 5 mg/kg dose of olanzapine (OLAN). Brain dialysates were collected for the same duration from a microdialysis probe implanted in the striatum. **Results:** The pharmacokinetic parameters, obtained after an oral dose, are in good agreement with reported values in literature. The pharmacodynamic information obtained from brain dialysates data show that OLAN elevates the concentration of dopamine (DA) in the brain and remains in the brain even after it is cleared from the plasma. **Discussion:** The ABS described here is a very useful tool in drug development to accelerate the pace of preclinical in vivo studies and to simultaneously provide pharmacodynamic and physiological information.

Keywords: Automated blood sampler; Pharmacokinetics; Pharmacodynamics; Olanzapine; Microdialysis; Neurotransmitters

1. Introduction

Combinatorial chemistry and high throughput screening have contributed to the enormous number of drug candidates now entering preclinical mammalian studies. In vitro screening is largely used to weed out the likely failures and narrow the pool of drug candidates in early stages of drug discovery. Integrated in vivo studies are needed to understand the pharmacology and toxicity of the selected drug candidates for further development. Obtaining absorption, distribution, metabolism, and elimination (ADME) parameters from small animals is essential early on in preclinical studies to avoid future failures. Because the pharmacological effect of a drug is directly influenced by the amount of drug at the target site, when developing drugs for the brain and central nervous system (CNS), it is also important to study the distribution of the drug in the brain.

Significant advances have been made for in vitro screening, sample preparation, and analysis using robotic workstations in the 96-well format. On the other hand, automation of in vivo techniques remains far behind and is highly labor intensive.

In preclinical drug development, blood sampling from small animals is a routine practice. One snag in drug discovery pipeline is the time and labor involved in the serial sampling of small blood volumes from small animals such as rats for the duration of pharmacokinetic/pharmacodynamic (PK/PD) studies. The traditional method of manually drawing blood from the animal requires the animal to be anesthetized or restrained with some device, both of which cause stress to the animal. It is known that stress affects the pharmacokinetics by reducing the absorption and altering the metabolism (Jamali & Kunz-Dober, 1999). Stress delays gastric emptying and slow the absorption of

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1056-8719/$ – see front matter © 2003 Elsevier Inc. All rights reserved.
doi:10.1016/S1056-8719(03)00058-3

drugs through the gastrointestinal tract (Lee & Sarna, 1997). Another drawback of manual blood collection is the requirement of trained personnel and manual labor.

Here, we describe the use of an automated blood sampling (ABS) system, shown in Fig. 1, for serial blood sampling from awake and freely moving rats for pharma-

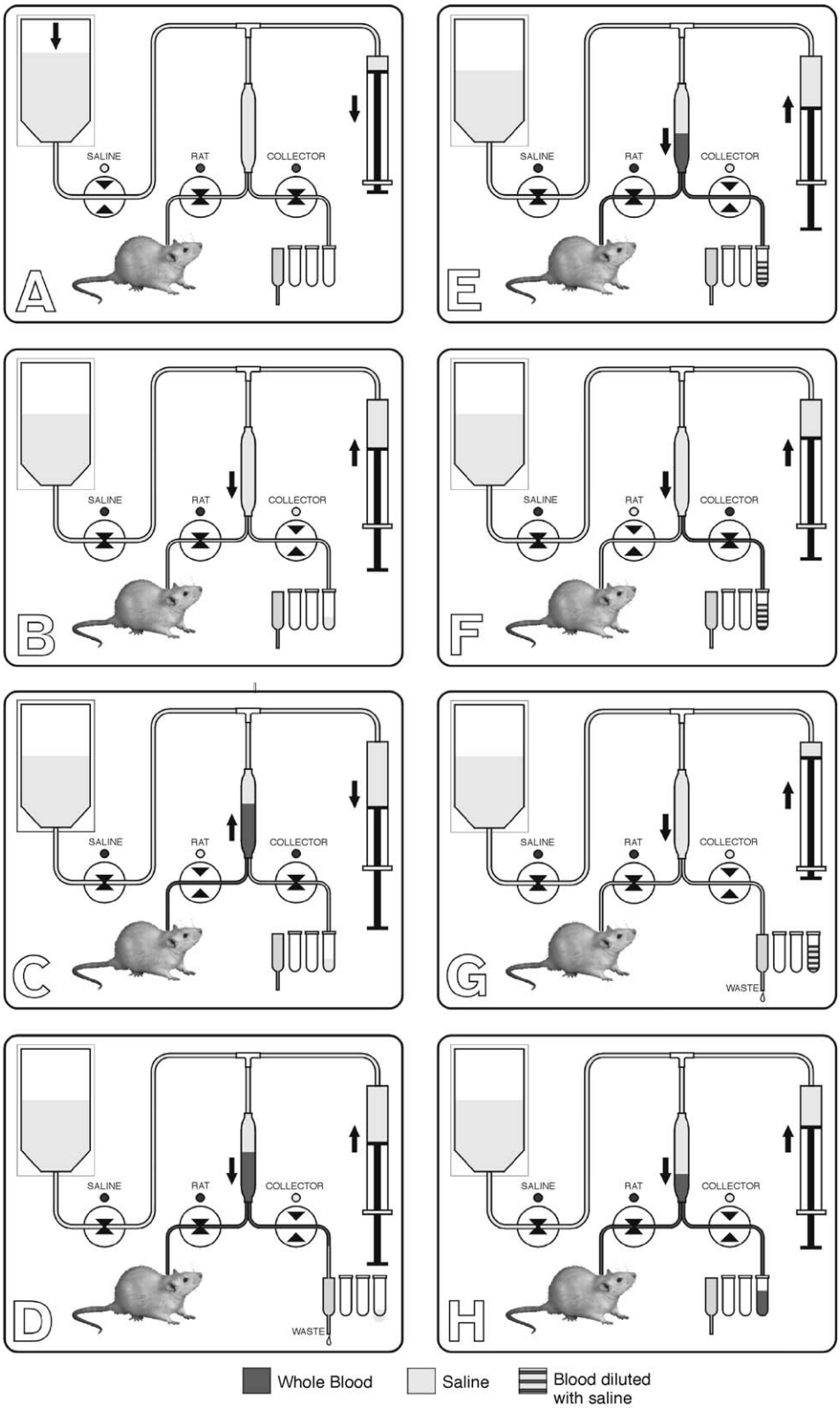

Fig. 1. Schematic illustration of the blood sampling process in the ABS. Heparinized saline is used as a motive force to transfer blood through the system.

cokinetic studies. We have used the same system to simultaneously sample brain microdialysates from the same animal. The ABS system is capable of collecting blood, urine, and feces while monitoring the animal activity during the experiment. The system collects blood in sealed, refrigerated vials, which are directly transferable to a 96-well plate. Urine is collected in chilled scintillation vials. Feces are collected separately on a stainless steel screen. The automated sampler withdraws blood from a freely moving animal according to a programmed schedule, stores the samples in an integrated refrigerated fraction collector, and replaces the withdrawn volumes with physiological saline.

The ABS is equipped with a sensor assembly, which consists of a left sensor to sense the clockwise movements and a right sensor to sense the counterclockwise movements of the animal in the cage. The animal activity software records the direction and the duration of the sensor movements. All the features and the operation of the system are described in detail elsewhere (Bohs, Cregor, Gunaratna, & Kissinger, 2000; He, Kramp, Ramos, & Bakhtiar, 2001; Peters et al., 2000).

Microdialysis is a well-utilized technique in neurosciences to sample the extracellular space in brain to obtain information on neurochemicals. This technique is becoming a valuable tool to study the drug distribution in brain and blood–brain barrier penetration by drug molecules (Deguchi & Morimoto, 2001; De Lange & Danhof, 2002). These studies are essential for development of drugs that would be used for the treatment of neurological and psychological diseases. A well-studied antipsychotic drug, olanzapine (OLAN), was selected to demonstrate the utility of this approach for both pharmacokinetic and microdialysis sampling.

OLAN is well absorbed and reaches peak concentrations in $\sim$ 6 h following an oral dose in humans. It is eliminated extensively by first-pass metabolism, with $\sim$ 40% of the dose metabolized before reaching the systemic circulation. The major circulating metabolites are 10-*N*-glucuronide and 4′-*N*-desmethylolanzapine (NDMO) (Kassahun et al., 1997; Mattiuz et al., 1997).

2. Methods

2.1. Materials

OLAN, its major metabolite NDMO, and the internal standard LY17022 were all generous donations from Eli Lilly and Company (Indianapolis, IN, USA). Dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), 5-hydroxyindoleacetic acid (5-HIAA), and serotonin (5-HT) were all purchased from Sigma (St. Louis, MO, USA). Distilled, deionized water was generated from NANO Pure ultrapure water system (Barnstead/Thermolyne, Dubuque, IA, USA).

2.2. Apparatus

Liquid chromatography/mass spectrometry (LCMS) studies were carried out using a Finnigan LCQ Deca ion-trap mass spectrometer with electrospray ionization (ESI) (ThermoFinnigan, San Jose, CA, USA) equipped with a BAS (West Lafayette, IN) PM-80 gradient pump and a BAS Sample Sentinel autosampler with a 20-μl injection loop (Bioanalytical Systems, West Lafayette, IN, USA). For neurotransmitter assay, a LC/electrochemistry (LCEC) system equipped with a BAS PM-92e pump and a BAS Epsilon dual-channel electrochemical detector with ChromaGraph software was used. Serial blood sampling from rats was accomplished by the BAS Culex automated blood sampler.

2.3. Brain microdialysis

The Internal Animal Care and Use Committee (IACUC) approved all the experimental procedures using animals. Guidelines in the Public Health Service Policy on Humane Care and Use of Laboratory Animals (NIH Publication, revised 1986) were followed.

Adult Sprague–Dawley rats were anesthetized with ketamine/xylazine (90 mg/kg + 10 mg/kg) by intraperitoneal injection. A brain probe guide cannula (MD-2250, BAS) was stereotaxically implanted in the cortex or in the striatum. After at least 3-day postsurgery recovery period, a BAS brain microdialysis probe (MD-2204, BAS) was inserted into the guide cannula 24 h before sampling was to begin. The probe was perfused using a BAS syringe pump with Ringer's solution (147-mM Na^+, 2.0-mM Ca^{2+}, 4-mm K^+, 155-mM Cl^-, pH 6.0) at a 1 μl/min flow rate. Two 30-min blank dialysates were collected before dosing the animal. After dosing, dialysates were collected at 30-min intervals for the first 7 h and then at 1-h intervals for the duration of blood sampling. Dialysates were collected into 300-μl vials with an equal volume of 0.2-M acetic acid to prevent degradation of neurotransmitters. Dialysates were split in two fractions. One fraction was assayed for OLAN and the other was assayed for neurotransmitters to obtain pharmacodynamic data.

2.4. Pharmacokinetic studies

The jugular vein of the same animal implanted with the brain guide cannula was cannulated and a catheter was implanted for blood collection. During the 24-h postsurgery recovery period in the Culex ABS, the catheter patency was maintained automatically by frequently flushing the catheter with sterile saline. After collecting two blank blood samples, the animal was dosed orally with 5 mg/kg of OLAN dissolved in 0.1-M HCl and then adjusted to pH 6 with 0.1-N NaOH. Blood samples (100 μl) were collected at 5 min, 15 min, 30 min, 45 min, 60 min, 90 min, 2 h, 4 h, 6 h, 8 h, 12 h, and 24 h after dosing. The samples were diluted twofold with an equal volume of saline during the collec-

tion. Animal rotational behavior was monitored during the blood collection and for another 3–4 h after the 24-h sample. Noncompartmental pharmacokinetic parameters were calculated using PK Solutions 2.0 (Summit Research Services, Montrose, CO, USA).

2.5. Sample extraction

Blood samples were centrifuged and plasma was separated. After adding 5 μl of a 1-μg/ml solution of the internal standard to a 100-μl plasma sample (50 ng/ml in final volume), 400 μl of 2% NH_4OH in ethyl acetate was added to precipitate protein. The sample was then vortexed for 2 min and centrifuged for 10 min. The supernatant (400 μl) was dried under N_2 and reconstituted in 100 μl of 0.1-M ammonium acetate/ACN (50:50 v/v). Dialysate samples were assayed without further sample treatment.

2.6. OLAN and metabolite assay

OLAN and NDMO were assayed by LCMS using isocratic conditions. The mobile phase consisted of 40% 0.1-M ammonium acetate/50% ACN and 10% methanol. A YMC basic column (150 × 4.6 mm, 5 μm) was used for the isocratic separation at a flow rate of 0.8 ml/min at ambient temperature. Samples (10 μl) were injected manually. The MS was operated in positive ESI mode. Nitrogen was used as both sheath and auxiliary gas at a pressure of 80 and 20 arbitrary units, respectively. The spray voltage was set at 5.0 kV and the capillary temperature was at 300 °C. Helium was used as the target gas for collision-induced dissociation. Positive ion LC/MS/MS chromatogram was obtained by monitoring the daughter ions of NDMO (MH^+ 299.0, 38%) at m/z 230.2, OLAN (MH^+ 313.2, 38%) at m/z 256.4, and internal standard (MH^+ 327.2, 36%) at m/z 270.3.

Stock solutions of OLAN and internal standard were prepared by dissolving 1.0 mg of each compound in methanol. Standard NDMO solutions (10 μg/ml) were used as received from Lilly Laboratories. Working solutions were made by appropriately diluting stock solutions in ACN/NH_4AC (50:50 v/v) mixture. Plasma standards for the calibration curves were prepared by adding appropriate aliquots of diluted OLAN and NDMO standards to rat plasma to give appropriate concentrations (1, 5, 10, 25, 50, and 100 ng/ml). These standard samples were extracted after adding the internal standard (equal to 50 ng/ml) by the same method as described previously for pharmacokinetic samples. The working solutions and calibration standards were prepared fresh daily.

2.7. Neurotransmitter assay

Neurotransmitters in the dialysates, mainly DA, DOPAC, HVA, 5-HIAA, and 5-HT, were detected by LCEC on glassy carbon electrodes at two potentials, at 750 and 650 mV with a Ag/AgCl reference electrode. Dialysate samples (5.3 μl) were directly injected to the LC system using an autosampler. The compounds were separated isocratically at room temperature on a microbore ODS column (150 × 1.0 mm, 3 μm) (BAS Unijet) using a mobile phase containing 25-mM NaH_2PO4, 20-mM citric acid, 60-mM NaCl, 10-mM diethylamine hydrochloride, 0.5-mM octyl sodium sulfate, and 0.03-mM Na_2EDTA (pH 3.2) mixed with acetonitrile and dimethyl acetoamide in the ratio of 91.9:5:3.1 (v/v) at a flow rate of 100 μl. Neurotransmitter stock solutions, DA, DOPAC, HVA, 5-HIAA, and 5-HT (1 mg/ml each) were prepared in 0.2-M acetic acid. Calibration standards were prepared by appropriately diluting the stock solutions in 0.2-M acetic acid to give DA/DOPAC/HVA/5-HIAA/HT concentrations ranging from 0.5/1.0/0.5/1.0/1.0 to 10.0/20.0/10.0/20.0/20.0 ng/ml.

3. Results

3.1. Pharmacokinetics

A marked difference in the animal's behavior was observed after the oral administration of OLAN to the rat. The animal became catatonic a short time after the drug administration and remained inactive for several hours. During this time, the animal showed no interest in food or water and there was no urine output. The animal regained its normal behavior about 8 h after dosing. The sensor movements recorded by the system shown in Fig. 2 are in agreement with the visual observations. All four data sets show an increase in activity shortly after drug administration followed by a inactive period. More information on the animal behavior can be obtained by examining the number of sensor activations and their duration times (Gunaratna, Cregor, & Kissinger, 2000).

The ABS is designed to collect whole blood. If desired, anticlotting agents can be deposited in the sampling vial prior to placing whole blood to avoid coagulation. In these studies, the blood samples were collected with an equal volume of heparinized saline. Plasma concentration–time profiles obtained after administrating the animals with a single 5-mg/kg oral dose are shown in Fig. 3. The noncompartmental mean pharmacokinetic parameters listed in Table 1 are comparable with values reported in literature (Richelson & Souder, 2000).

3.2. Brain microdialysis

A microdialysis probe is a sampling device that contains a specific length of a semipermeable dialysis membrane. An implanted probe samples the extracellular fluid around the implanted tissue site. Small unbound drug or neurotransmitter molecules diffuse through the dialysis membrane into the perfusing medium, which is isotonic to the extracellular fluid. The percentage recovery of the analyte is dependent

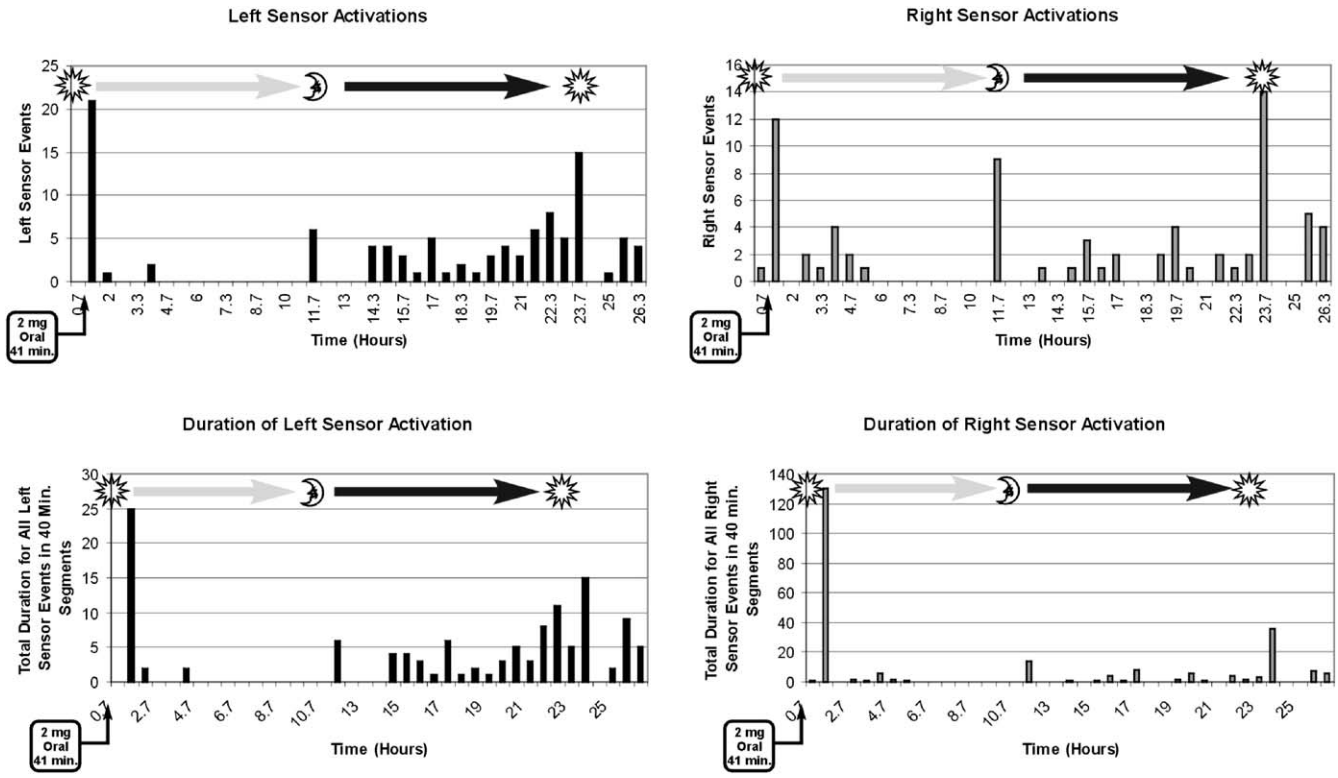

Fig. 2. Animal behavior was monitored after oral administration of 2-mg OLAN for a period of >27 h. Each bar represents sensor data within a 40-min segment, starting with the initiation of behavioral monitoring 41 min before dosing. The sun/moon symbols and the light/dark arrows indicate the time of day when these activity measurements were made.

on membrane length, perfusing flow rate, and nature of the analyte. In these experiments, the probes implanted in the rat striatum contained a 4-mm length of membrane. In vitro percentage recovery for both OLAN and NDMO through the brain probe was found to be $<10\%$.

Microdialysis samples collected from the same animal during the pharmacokinetic study were assayed for both OLAN and the metabolite NDMO and for major neurotransmitters present in the brain. OLAN and NDMO profiles in the brain (Fig. 4) show that OLAN peaks in the brain about 2 h after an oral dose. OLAN levels remain elevated in the brain long after the plasma levels started to decline.

It is known that antipsychotic drugs such as OLAN bind to dopaminergic receptors and increase the turnover of DA in the brain, which increases the amounts of HVA and DOPAC, primary metabolites of DA (Aravagiri, Teper, & Marde, 1999). A typical chromatogram of a brain dialysate is shown in Fig. 5. In this study, the neurotransmitter DA was barely detectable in the dialysates, whereas DOPAC (Fig. 6) and HVA (Fig. 7) were present in large amounts, indicating the increased release of DA in rat brain due to OLAN. The levels of DOPAC and HVA increased after OLAN administration and started to decline after about 10 h, long after the OLAN is cleared from plasma. In contrast, the levels of 5-HIAA, a metabolite produced by the deamination of 5-HT by monoamine oxidase, remained un-

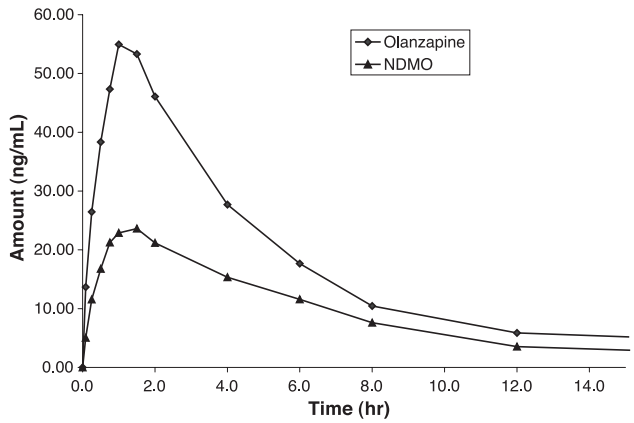

Fig. 3. Mean plasma OLAN concentration–time profiles ($n=6$) of OLAN and NDMO after administering an oral dose of 5 mg/kg to Sprague–Dawley male rats.

Table 1
Noncompartmental pharmacokinetic parameters ($n=6$) for OLAN

C_{max}	169 ± 31 ng/ml
t_{max}	0.8 ± 0.2 h
AUC_{∞}	316 ± 27 ng h/ml
V_d	14.35 ± 2.1 l

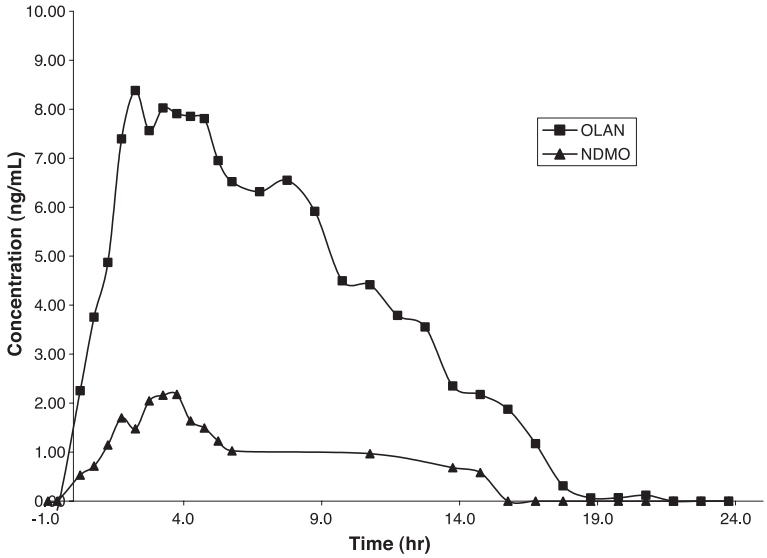

Fig. 4. OLAN and NDMO distribution in brain following an oral dose of 5 mg/kg to Sprague–Dawley male rats ($n = 6$).

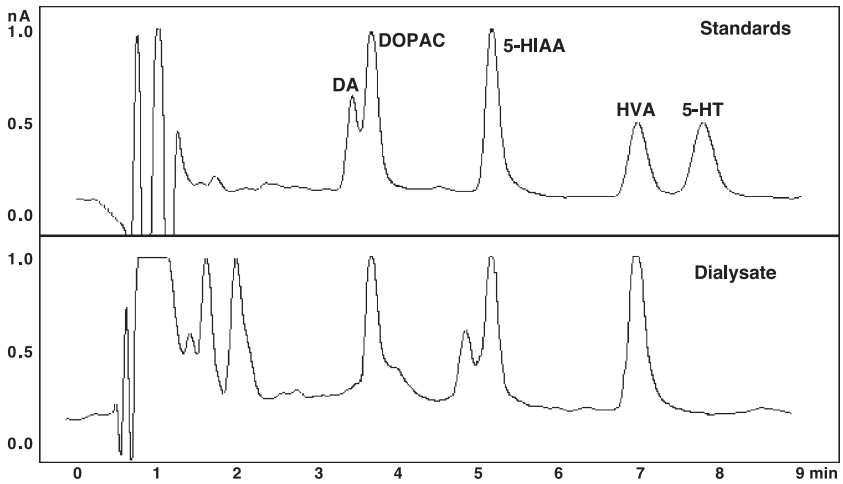

Fig. 5. A chromatogram of a mixture of neurotransmitter standards and a brain dialysate sample at 4-h time point after administering an oral dose of 5-mg/kg OLAN.

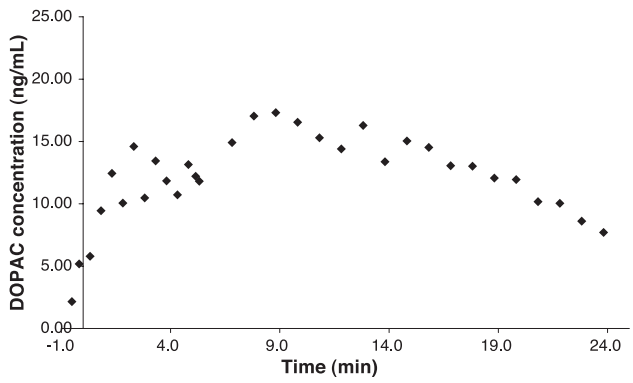

Fig. 6. DOPAC concentration in rat brain dialysates after administration of a 5-mg/kg oral dose of OLAN ($n = 6$). DOPAC levels remain elevated for >10 h, indicating the increased activity of dopaminergic neurons by OLAN.

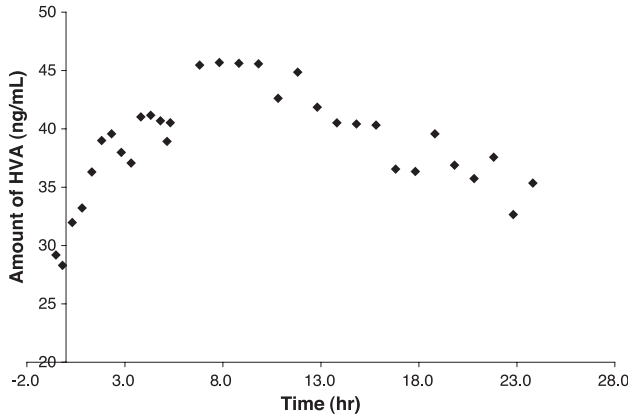

Fig. 7. Levels of HVA, a metabolite of DA, in rat brain dialysates after administration of a 5-mg/kg oral dose of OLAN ($n = 6$).

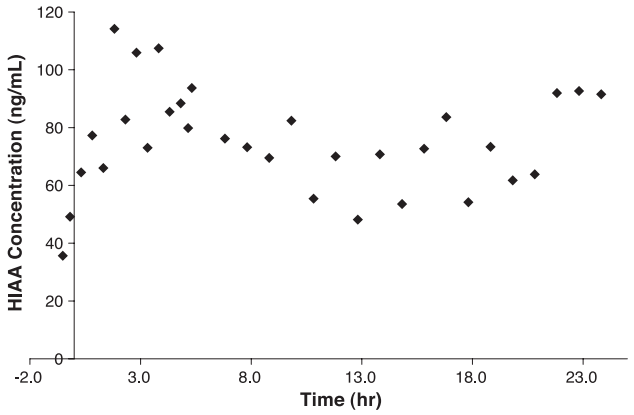

Fig. 8. Levels of 5-HIAA, a metabolite of 5-HT, in rat brain dialysates after administration of a 5-mg/kg oral dose of OLAN ($n = 6$).

changed in brain dialysates (Fig. 8). This is in agreement with the observation Scheepers, Gispen-de Wied, Westenberg, and Kahn (2001) made that OLAN significantly increased HVA concentrations and the HVA/5-HIAA ratio but did not alter 5-HIAA concentrations. The data obtained from this microdialysis study show the prolonged effect of OLAN in the brain even after the drug is cleared from plasma.

4. Discussion

OLAN is a selective monoaminergic antagonist with high-affinity binding to serotonergic 5-$HT_{2A/2C}$, dopaminergic DA_{1-4}, muscarinic M_{1-5}, histamine H_1, and α_1-adrenergic receptors (Bymaster, Hemrick-Luecke, Perry, & Fuller, 1996; Nyberg, Farde, & Halldin, 1997; Seeman, 2002). It has been reported that this drug's antipsychotic activity is mediated through a combination of DA and 5-HT_2 antagonism (Kapur et al., 1998; Wadenberg, Soliman, VanderSpek, & Kapur, 2001). OLAN's antagonism of histamine H_1 receptors may explain the somnolence observed in the animals with this drug (Meltzer, 1999).

After the oral dose, OLAN absorbs quickly as evident by the short t_{max} and readily distributes into the tissues. The high volume of distribution (V_d) is an indication of the high tissue distribution of OLAN.

Aravagiri et al. (1999) have studied the distribution of OLAN in various brain regions. After repeated oral administration of OLAN for 15 days, the animals were sacrificed and brain tissue samples were collected for OLAN analysis. They reported that OLAN levels in brain regions varied widely. After a 6-mg/kg/day oral dose of OLAN, the authors reported that the OLAN concentrations in caudate and cerebellum were twofold to threefold lower than the whole brain concentrations. In our study, we have implanted the probe in the striatum region. By implanting probes in different regions of the brain, the distribution of OLAN can be studied in awake, freely moving animals. The data obtained by microdialysis sampling provide near real-time information.

In conclusion, we have successfully demonstrated the utility of a new ABS for simultaneous PK/PD and blood–brain barrier studies of drugs. Because the animals are freely moving and not stressed by human handling or by anesthesia, the PK/PD studies conducted are less subjected to experimental artifact and would be more reliable and accurate. Furthermore, near real-time information on other physiological processes can be obtained from the same animal simultaneously without additional manpower. The system can be integrated with other studies involving animals such as behavior monitoring and electrophysiology. Because the ABS can sample four animals at a time, two systems can be used to collect blood samples from eight animals onto the rows of a 96-well plate to facilitate high throughput analysis. The ABS developed here bridges the gap between the in vitro screening phase and the analysis of in vivo samples to accelerate decision-making before costly chronic toxicology and clinical trials.

Acknowledgements

The authors thank Dr. Quin Zhou of Bioanalytical Systems for her technical assistance in neurotransmitter analysis and Meloney C. Cregor for her assistance in brain probe implantation.

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