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Simultaneous Determination of Nicotine and Cotinine Pharmacokinetics in the Blood and Brain using a Combination of Automated Blood Sampling and In Vivo Microdialysis in Sprague Dawley Rats

Pursuing central nervous system (CNS) active therapeutics is one of the most expensive and labor-intensive areas in drug development. An increase in the speed of CNS drug development could create a substantial savings for pharmaceutical companies. A common feature of all CNS drugs is that they must penetrate the blood brain barrier (BBB). The purpose of the current study was to determine the utility of in vivo microdialysis in combination with automated blood sampling as a screening tool when delineating BBB permeability to a CNS active compound such as nicotine, and its major metabolite, cotinine. The results of the current study suggest the combination of in vivo microdialysis with automated blood sampling can determine whether a drug readily crosses the blood brain barrier and is useful as a screening tool for CNS active drugs.

A study performed by the Tufts Center for the Study of Drug Development found that drugs whose primary therapeutic area is the central nervous system (CNS), compared to other areas such as analgesic/anesthetic, anti-infective, and cardiovascular, cost substantially more to develop. CNS drugs cost an average of $527 million and 114.6 months to develop, compared to an average of $375 million and 61.8 months to develop analgesic/anaesthetic drugs (1). Given the high cost and time involved in developing CNS drugs, advances in any phase of development could potentially save a pharmaceutical company millions of dollars.

Unlike drugs that target the periphery, CNS-active drugs must cross the BBB in order to reach their intracerebral locations. The BBB is a semi-permeable barrier made up of a tightly formed network of capillary endothelial cells that selectively restricts the movement of molecules between the brain and the periphery. The BBB has been called "the bottleneck in brain drug development" (2). Indeed, a common misconception is that most small molecules readily cross the BBB. In reality, only an estimated 2% of all small-molecule and nearly no large-molecule drugs cross the blood brain barrier. Thus, during drug development a quick screening tool giving large amounts of information from a relatively small labor cost could save a great deal of financial and employee resources.

The BASi Culex® Automated Pharmacology System (APS), in conjunction with the Empis microinfusion pump, is a highly effective system for simultaneous serial blood and brain dialysate collection in a single, conscious, unrestrained animal. Use of the Culex APS and Empis allows the user to create and compare time-concentration curves for both blood and brain dialysate, giving the experimenter a clear picture of a compound's BBB penetration.

The purpose of the current study was to illustrate the usefulness of intracranial microdialysis coupled with automatic blood sampling as a screening tool for BBB permeability in awake, freely-moving animals. Nicotine was chosen as the test compound due to its established ability to cross the BBB, a well documented pharmacokinetic curve, and a known major metabolite, cotinine.

Methods

Surgical Procedure

Four male Sprague Dawley rats (Harlan, Indianapolis, IN) (n = 2/experimental group) weighing between 300 and 350 g at the time of testing were used as subjects. Prior to surgery, the rats were divided into two groups: the nucleus accumbens, or the ventricle group. Animals were anesthetized and stereotaxically implanted with a guide cannula (MD-2250; Bioanalytical Systems, Inc, West Lafayette, IN) into either the left hemisphere shell of the nucleus accumbens or ventricle. The guide cannula was anchored to the skull by three stainless steel screws cemented together with Sun-Schein ultraviolet light sensitive dental acrylic. Prior to microdialysis experiments, a stylet was placed into the guide cannula to prevent blockage and reduce the risk of infection.

Three days after microdialysis guide cannula implantation, the left femoral and right jugular vein were ligated. Then, both veins were cannulated with a catheter with an internal 32 and 56 µL volume (jugular and femoral catheter, respectively) (CS 2011S and DX 2020S; Bioanalytical Systems Inc., West Lafayette, IN). After surgery, the animals were immediately placed into the Culex APS bowl cage, connected to the Culex APS, and allowed 24 hrs of post-surgery recovery. The APS maintained venous catheter patency throughout the experiment by flushing 20 µL of sodium heparinized saline (10 units/ml) every 12 min.

Drug Dosing

(-)-Nicotine hydrogen tartrate salt (N5260; Sigma-Aldrich) was dissolved in 0.9% saline and administered intravenously

(0.5 mg/kg) into the jugular vein at a flow rate of 50 µL/min for approximately 5-6 min via a Baby Bee Syringe Pump (MD-1001; Bioanalytical Systems, Inc., West Lafayette, IN) equipped with 1mL syringe. Drug doses were calculated with nicotine as a free base.

Blood Sampling Procedure

As seen in **F1**, blood sampling was controlled by the Culex APS. The APS and associated automated blood sampling process (**F2**) has been described in detail elsewhere (3). Briefly, the APS is a robotic system that has the capability of collecting serial blood samples from an awake and freely-moving rodent (3). During our experiment, immediately following the intravenous infusion of nicotine, blood samples (250 µL/sample) were taken at varying time points between 0-360 min post-drug infusion time.

Microdialysis Procedure

The microdialysis probes (MD-2200; Bioanalytical Systems, Inc., West Lafayette, IN) were pin style with a probe volume of 2.1 µL. The probe membrane extended 2 mm beyond the tip of the guide cannula. The probes were inserted into the guide cannula immediately following surgery, while the animal was still anesthetized, and locked into place using a small elastic band. The perfusion medium was a Ringer's solution (147 mM Na^+, 2 mM Ca^{2+}, 4mM K^+, 155 mM Cl^-, pH 6.0). Ringer's solution was perfused at a rate of 2 µL/min, controlled by an Empis infusion pump (CX-300; Bioanalytical Systems, Inc., West Lafayette, IN.) Dialysate was collected into a Honeycomb refrigerated fraction collector (MD-1201; Bioanalytical Systems, Inc., West Lafayette, IN) that maintained the samples at 4° C. After two hours of perfusion to establish an equilibrium between the probes' internal and external environment, microdialysis samples were taken every 30 min for 6 hrs following the nicotine infusion.

Behavioral Activity

As seen in **F3**, the Raturn® is equipped with a sensor assembly consisting of a left (clockwise activity) and right (counterclockwise activity) sensor that monitors the animal's movement. The movement is recorded by the APS software as previously described (4-6). After 24 hrs of post-surgical recovery and habituation to the chamber, pre-drug administration baseline motor activity was recorded for a 6 hr period using the Raturn (4-6). Six hours of post-activity was also recorded following the nicotine infusion. Clockwise and counterclockwise activity was combined to form "total rotational behavior."

Sample Extraction

Blood samples consisted of 13 individual vials containing 250 µL of whole blood each. The samples were centrifuged at 2000 RPM for 10 min using a Beckman Coulter (Fullerton, CA) Allegra 6R centrifuge. 100 µL aliquots of plasma were transferred into a 96-well plate by an automated robotic system (Multiprobe II, PerkinElmer, Inc., Boston, MA). The samples were then frozen at -80°C until analysis.

Liquid-liquid extraction was accomplished as follows. The 100 µL samples were brought to room temperature and 100 µL of 5 M NaOH/1.1 M NaCl+IS was added to each sample and shaken gently for 30 sec. Then, 650 µL of 1:1 methyl t-butyl ether: CH_2Cl_2 was added to each sample and the plate was vortexed for 4 min. The samples were then centrifuged 10 min at 3250 RPM. 550 µL of the upper (organic) layer was siphoned off using the robotic system and transferred to a fresh 96-well plate. Next, 20 µL of 1 M HCl was added and the samples vortexed for 1 min, then centrifuged for 1 min at 3250 RPM. Samples were brought to dryness under a stream of N_2 at 40°C for 30 min. 100 µL of 50 mM formic acid was added to each sample. The samples were shaken for 1 min and, after a 2 min wait, shaken for an additional 1 min. Aliquots of 75 µL were then transferred to injection vials via the robotic system.

Nicotine and Cotinine Analysis

Nicotine and cotinine were assayed by liquid chromatography and mass spectrometry (LCMS) using isocratic conditions. The mobile phase consisted of pH 3.0, 50 mM formic acid and acetonitrile (60/40, v/v). An Ultra PFP column, 5 µm, 2.1 X 150 mm (Restek, Bellefonte, PA) was used at a flow rate of 0.4 mL/min at ambient temperature. Samples (25 µL) were injected using a Bioanalytical Systems, Inc Sample Sentinel autosampler with a 20 µL loop. The ion trap Finnigan LCQ Deca (ThermoSeparations, San Jose, CA) 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 250°C. Helium was used as the target gas for collision-induced dissociation. Positive ion LCMS chromatogram was obtained by monitoring the molecular ions of nicotine at m/z 163.3 and cotinine at m/z 177.4. The deuterated internal standard for nicotine was monitored at m/z 166.30, while deuterated cotinine was at m/z 180.4.

Results and Discussion

Profile of Nicotine and Cotinine in the Plasma and Dialysate

As seen in F4-7 (A, B), nicotine and cotinine were readily detectable in both the plasma and dialysate. This finding suggests that intracranial microdialysis provided by an Empis and fraction collector, coupled with automatic blood sampling provided by the Culex APS, is useful as a screening tool for BBB permeability in awake, freely-moving animals. In relation to the current study, a greater amount of nicotine than cotinine was detected in the dialysate during the 6-hr time frame, while in the plasma, a greater amount of cotinine was detected compared to nicotine. As seen in **T1**, this finding was confirmed as nicotine's area under the curve (AUC 0-∞; µg-min/ml) for all animals, compared to cotinine, was consistently higher in the dialysate, and vice versa in the plasma. **T1** lists values for nicotine to cotinine ratios (ratio = nicotine AUC/cotinine AUC), as a value of 1 indicates equal nicotine to cotinine AUC values. A value greater than 1 indicates a larger nicotine AUC compared to cotinine. **T1** indicates that cotinine AUC was 6-17 times that of nicotine in the plasma. In dialysate, nicotine AUC was 1-5 times that of cotinine. Our data is consistent with previous literature that used *in vivo* microdialysis of both the blood and brain, showing that nicotine more readily crosses the BBB compared to its metabolite cotinine (7, 8).

Also important to note is the apparent lack of difference in nicotine and cotinine recovery between animals implanted in

the NAC compared to the VEN. It appears that both brain areas are suitable for microdialysis studies. Thus, the density of the brain area does not appear to significantly reduce recovery of nicotine and cotinine. However, this finding may not generalize to all compounds at all concentrations in the brain.

Activity Monitoring

As seen in **F4-7 (C, D)**, locomotor activity does not appear to be directly associated with nicotine concentrations in either the plasma or brain. Only two rats appeared to have increases in motor activity post-drug administration, Rats #117 and #121. In these two, motor activity increased in a delayed manner with an increase in activity up to 90-120 min post-drug administration, and then declining over time. While nicotine levels immediately peak, activity shows a delayed reaction. Interestingly, cotinine levels appear to be more closely associated with activity than nicotine. However, it is currently unknown if cotinine increases locomotor activity alone. Thus, caution is suggested when evaluating locomotor activity in relation to plasma or dialysate drug concentrations.

Conclusions

The current study demonstrates that the combination of automated blood sampling in conjunction with intracranial microdialysis provides a powerful system for primary drug and metabolite BBB penetration screening in awake and freely-moving animals. Our data is consistent with prior published papers showing after intravenous nicotine administration, nicotine more readily crosses the BBB compared to cotinine and that in the blood, cotinine levels are higher than nicotine levels (7, 8). Thus, the Culex APS in combination with the Empis microinfusion pump provides equivalent data to that classically seen in the scientific literature.

Our study also demonstrated that the targeted brain region does not significantly alter the detected amount of drug in the dialysate. No apparent concentration differences in nicotine or cotinine were detected between dialysates taken from the NAC compared to the VEN, suggesting that tissue density plays a limited role in drug recovery with microdialysis. However, our data is limited to a specific compound and a single dose of nicotine (0.5 mg/kg). It is possible that our dose has saturated the brain to its maximum capacity and that lower doses could show recovery variations depending on dose and brain region.

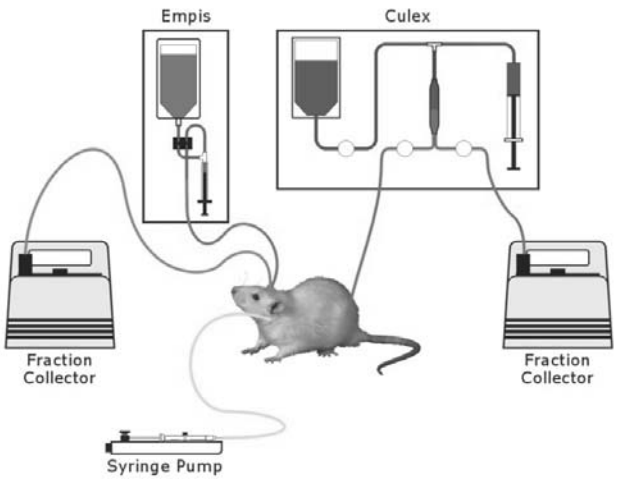
In addition, the Raturn provides locomotor activity that may indicate a CNS action, albeit our data suggests that locomotor activity may be delayed at times. However, the combination of the Culex APS, microdialysis, and the Raturn does provide an experimenter with the capability to correlate blood and brain drug concentrations with behavioral effects produced by the drug.

The combination of automated blood sampling with microdialysis provides several advantages compared to other techniques used to detect BBB penetration of CNS drugs. Some of the advantages of the system stem from the complete automation of drug dosing, blood sampling, and microdialysis. The ability to do automated serial blood and brain dialysate sampling allows for cost reductions, particularly when studying large numbers of animals. For example, to create a ten-time-point pharmacokinetic curve for three compounds using a single blood sample per time point per rat would mean that each animal would be dosed per compound (X3). Thus, the experiment would need a sample size of 30 rats. However, using the automated system, a single rat could be dosed and all blood samples taken from that animal per compound. A 30-rat study would decrease to a 3-rat study, a 10-fold factor. Hence, the automated system has an advantage over traditional means in that costs can be minimized by reduction in animal usage, the maximization of information obtained per animal (i.e., entire microdialysis and blood PK curves from a single rat), a decrease in variability due to the samples coming from a single source, a potential decrease in the number of surgeries, and an expanded amount of "run" time because the machine can be operated 24 hrs every day with little or no supervision. An additional advantage of the Culex APS in combination with microdialysis is that animals are awake and freely moving, whereas some *in vivo* techniques anesthetize the animals, a factor that could potentially alter PK, and the dialysates can be automatically injected on-line into the detection system so that degradable analytes can be assayed with little loss. Our experimental equipment combination gives a more complete picture of systemic and central drug time course allowing for a direct comparison between the two.

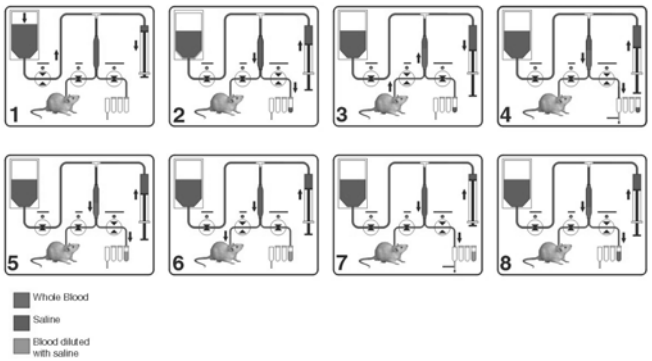
Use of the automated Culex APS with microdialysis also has potential pitfalls. The most evident problem is that the system would require relatively complex surgeries (i.e, implantation of multiple catheters and a microdialysis probe) and programming and coordinating multiple pieces of equipment (i.e, the Culex, Raturn, Empis, Bee Pumps, and fraction collectors). These problems may be minimized through training of staff and careful quality control systems.

The current study demonstrates the power of the combination of blood sampling and microdialysis using an automated system in the study of compound BBB penetration. The potential cost and time savings due to automation of the system makes any disadvantages seem marginal. Thus, the Culex APS in conjunction with microdialysis is a useful tool in the arsenal of a drug development program focused on the development of CNS active compounds.

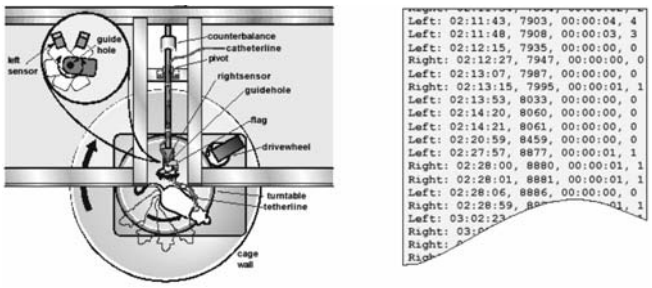
F1. Experimental setup diagram showing the combination of automated microdialysis and blood sampling and intravenous drug dosing in an awake and freely-moving animal. Drug dosing was provided by a BASi Bee pump directly into the jugular vein. An Empis connected to the microdialyis probe, feeding to a fraction collector, provided the dialysate samples for the study, and the Culex APS provided automated blood sampling.



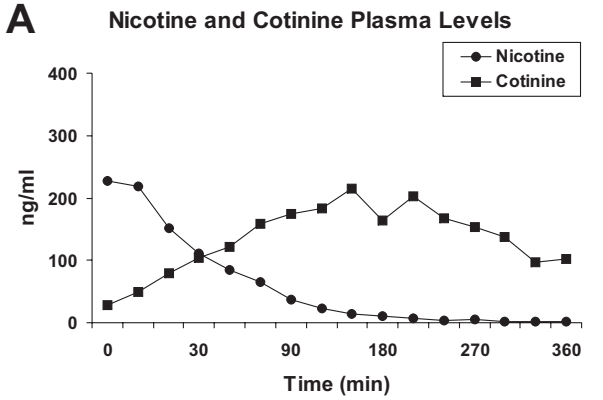
F2. The Culex APS uses heparinized saline as the moving force for blood transfer. This process has described in detail (6). The eight setups shown below make it possible to obtain serial blood samples.

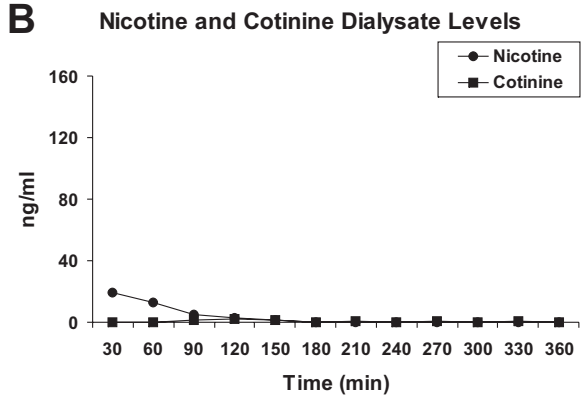


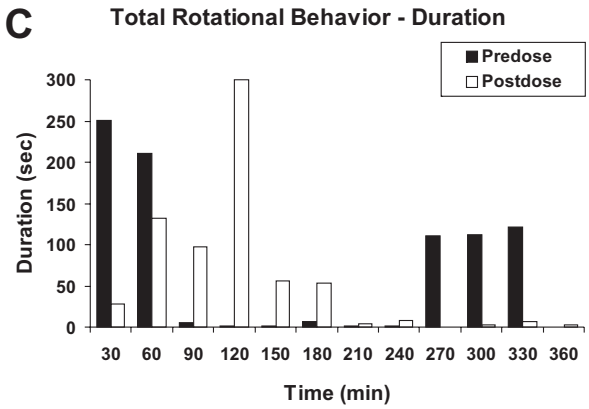
F3. The BASi Raturn animal activity monitoring system. As the animal rotates either clockwise or counterclockwise, the movement triggers a sensor located in the balance arm that then "turns" the animal into the opposite direction, preventing line tangling.

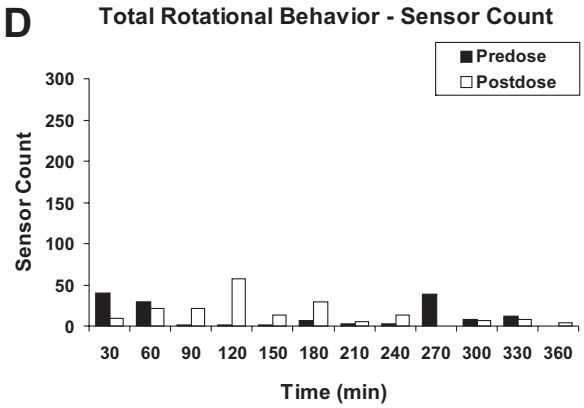


F4. Rat 117 was implanted in the NAC. Nicotine and cotinine plasma (A) and dialysate (B) levels are presented. In addition, total rotational behavior, duration (C) and sensor count (D) is shown.

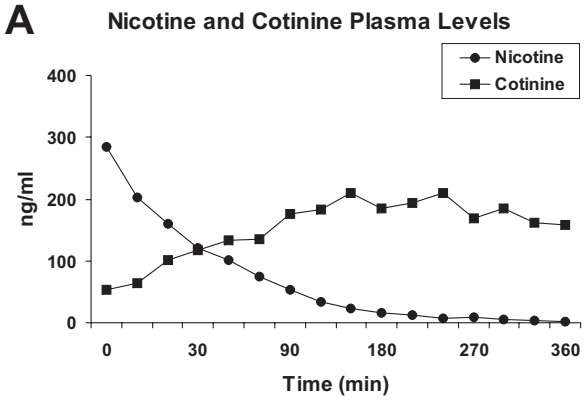


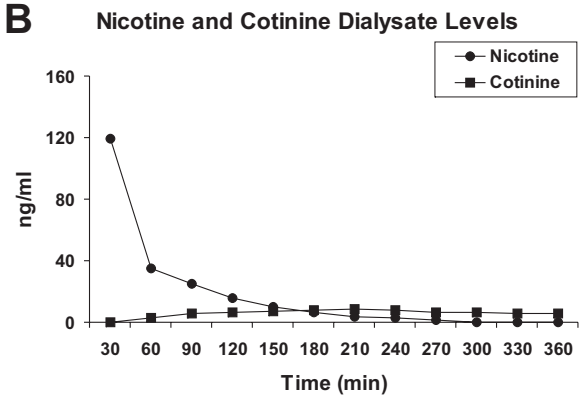


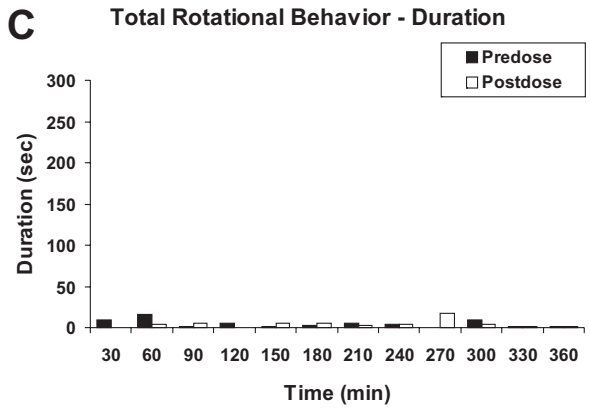


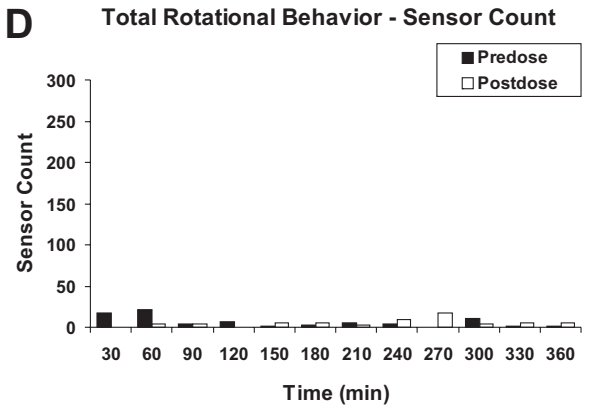


F5. Rat 119 was implanted in the NAC. Nicotine and cotinine plasma (A) and dialysate (B) levels are presented. In addition, total rotational behavior, duration (C) and sensor count (D) is shown.

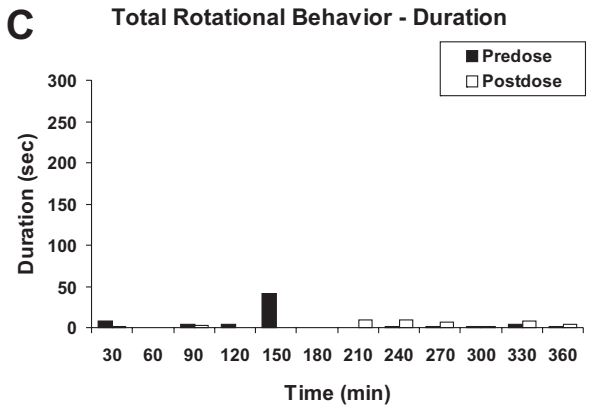


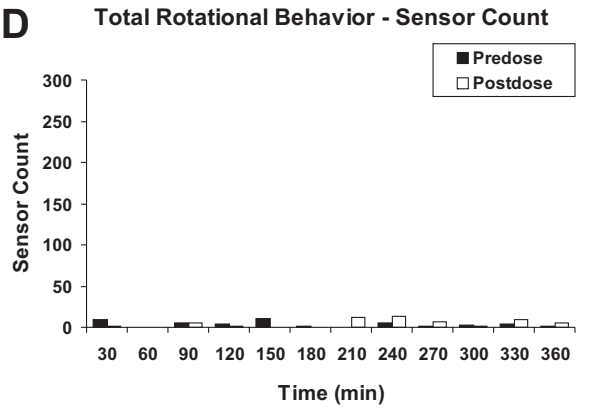




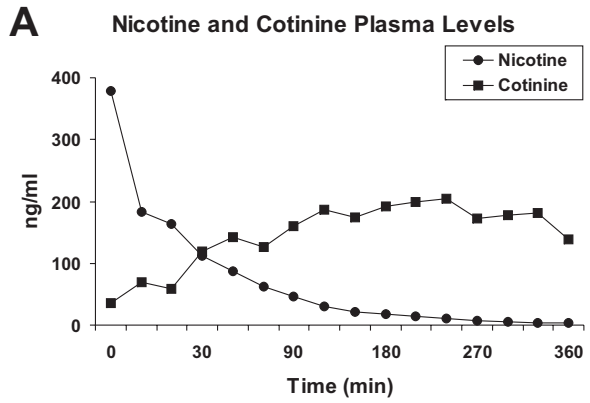


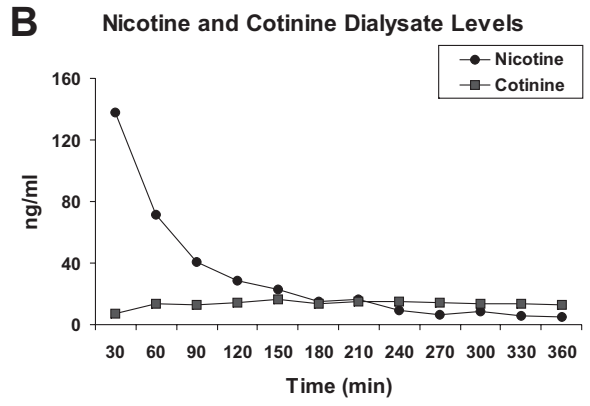
F6. Rat 118 was implanted in the VEN. Nicotine and cotinine plasma (A) and dialysate (B) levels are presented. In addition, total rotational behavior, duration (C) and sensor count (D) is shown.

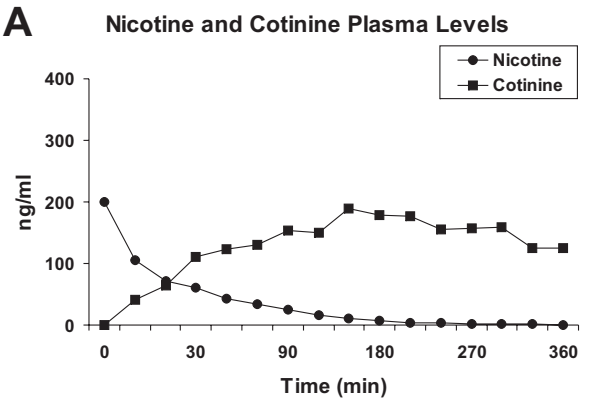


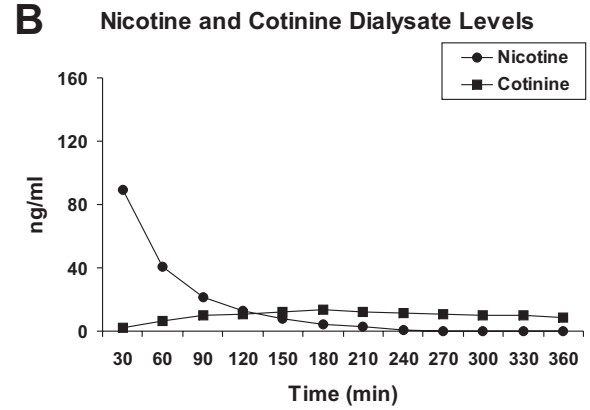


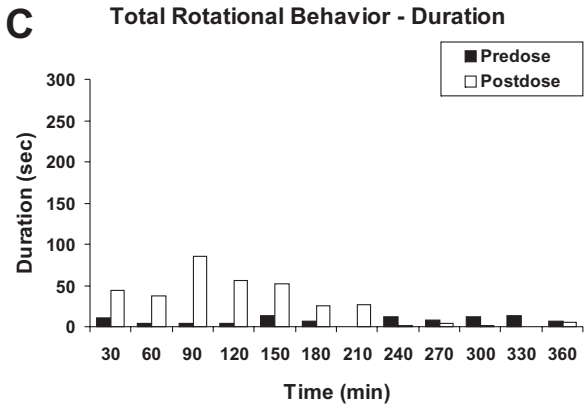
F7. Rat 121 was implanted in the VEN. Nicotine and cotinine plasma (A) and dialysate (B) levels are presented. In addition, total rotational behavior, duration (C) and sensor count (D) is shown.











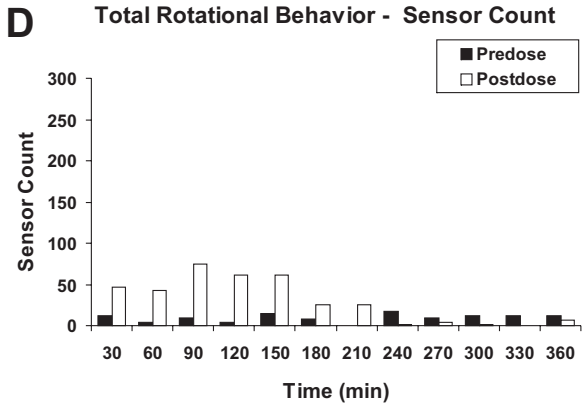


Table1. Area under the curve (AUC 0-∞; µg-min/ml) values and AUC ratio for plasma and dialysate in Sprague Dawley rats.

	Plasma			Dialysate		
	Nicotine	Cotinine	Nic/Cot Ratio	Nicotine	Cotinine	Nic/Cot Ratio
Rat #117	12340	76496	0.16	1762	818	2.15
Rat #119	14099	126565	0.11	23788	4348	5.47
Rat #118	13700	117082	0.12	22491	16468	1.37
Rat #121	6483	114173	0.16	9011	7701	1.17

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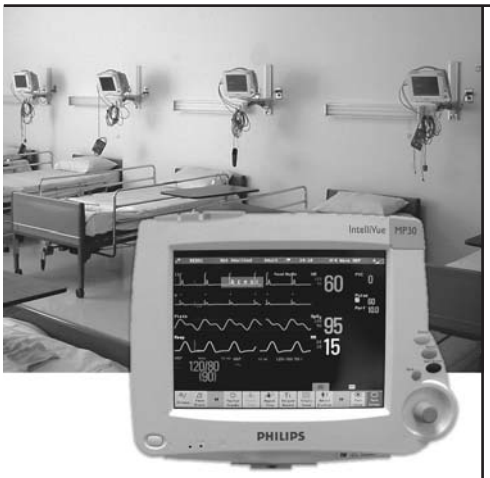
BASi Announces Opening of Intensive Monitoring and First-in-Man Facility



At the DIA Meeting in Philadelphia in June, BASi announced the opening of a new, state-of-the-art intensive monitoring and first-in-man facility at its Baltimore, Maryland Clinical Research Unit. The new facility increases capacity for advanced Phase I and Phase IIa studies including first-in-man, escalating dose, bioavailability, drug interaction and safety and tolerance. The 10-bed facility allows intensive monitoring of study participants including cardiac monitoring, blood pressure and O2 with central monitoring and observation using the Philips comprehensive cardiac monitoring system. Additional capabilities include infusion pumps and bedside oxygen. The new unit adds to the existing capacity at Baltimore which includes two independent clinical units with 96 beds and mixed-gender capabilities.

BASi Director of Clinical Operations, Patrick Ayd, commented,

"This new capability will allow us to expand the services we offer to the drug development process and provide enhanced capacity to meet the demands of pharmaceutical and biotechnology companies seeking approval for new proprietary drugs."

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