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How A Rodent Is Dosed and Sampled Is Equally Important to How Samples Are Analyzed: Automated Dosing, Sampling and LC/MSMS With Ion Traps and Triple Quads

The preclinical bioanalytical process with animal models begins with dosing and then sampling biological fluids and tissue. How an animal is housed, dosed and then sampled is equally important to how the samples are analyzed. Animal science and bioanalytical chemistry are very different professions, but integrating them is essential to the process of searching for new medicines to improve human health. The goal is to understand oral absorption kinetics, distribution, metabolism, excretion, blood-brain barrier penetration, drug-drug interactions, and the influences on biomarkers, hematology, electrophysiology, cardiology, blood pressure and behavior. In a typical pharmacokinetic and pharmacodynamic study, 8-12 samples are collected over a time span of 10-24 h. Urine, feces, bile, ultrafiltrates and microdialysates can augment the information available from whole blood. In the last decade, LC/MSMS augmented by automation of sample preparation has saved labor and improved precision for smaller volume/lower concentration samples. To improve both quality and throughput, while providing for reduced numbers of animals and enhanced animal comfort, we have implemented a robotic system (automated blood sampling system) that can accomplish most of the above goals. This review is a progress report on this evolving research program for automated blood and microdialysis sampling, which demonstrates that good preclinical bioanalytical chemistry requires proper sampling from low-stress laboratory animals.

In the 1950s and before, undergraduate analytical chemists were always taught the importance of obtaining a "representative sample" before proceeding in the laboratory to determine the amount of a metal in an ore. In those days, the concept of sampling was nearly always presented as a subject of inorganic analysis. By 1960, the analytical chemistry discipline began to separate from a long association with inorganic chemistry and students learned far more about the challenges of organic analysis and the great fun of gas chromatography, infrared and NMR. By 1980, liquid chromatography, electrophoresis, immunoassays and later on liquid chromatography / mass spectrometry had matured and bioanalytical chemistry replaced copper mines as the driver of analytical enthusiasms. Along the way, rigorous attention to sampling received less attention while the modern concepts of extractions from biological matrix evolved. We now have robots for nearly everything and 96-well plates are the new test tubes. With all of this technology in hand, we asked a question in 1999 that has occupied our attention ever since: What is the best way to get a representative sample from a rodent while preserving the data quality we need? We formed a hypothesis that the then common methods for dosing and sampling rodents were distorting the very data we needed. Our interests are specifically focused on pharmacokinetics (PK) and pharmacodynamics (PD).

In today's preclinical environment, LC/MSMS augmented by robotic sample preparation tools has played a dominant role in sample analyses (1). Better data in less time is a constant goal of the pharmaceutical industry. Experiments performed in parallel rather than in series are a key ingredient. It became very clear that sample preparation and sample analysis had advanced far beyond the process of sample acquisition. Sampling a stress-free animal provides more valuable and meaningful data. If individual animals are also automatically dosed, one can obtain reliable ADME, PK and PD information to interweave with *in vitro* data. Conclusions can be drawn more quickly than if this information was obtained from manually handled animals, in a separate department, by separate sets of researchers, often acting months apart. Following these principles the number of animals required to arrive at a conclusion can be dramatically decreased by refining the experimental protocols.

This is made possible by using advanced instrumentation and software to sample animals in a manner that substantially reduces pain and stress. In fact, both are virtually eliminated versus conventional techniques such as oral gavage and intraperitoneal dosing, tail vein sampling or suborbital bleeds *via* syringe. It was an earlier advance to confine cannulated animals in restrainers after periodically transferring them from a home cage for blood draws. Pain is eliminated by the use of catheters; however, stress from the animal-human interaction remains substantial and the process is very labor intensive.

With automation, it is straightforward to monitor behavior, collect urine and feces and carry out parallel *in vivo* ultrafiltration and microdialysis sampling to monitor endogenous markers such as glucose and neurotransmitters. It is possible to sample from two catheters simultaneously to assess first pass metabolism and even to collect bile as well (2).

Animal science and analytical science are mutually dependent. It is not helpful to use sophisticated techniques for bioanalytical chemistry on samples from stressed animals where the data may be of little value. Likewise, it is not helpful to improve the animal science without the good bioanalytical protocols necessary to obtain quality data. Better animal welfare results in better science, and better analytical

chemistry allows for reduced sample volumes. Experiments should be coordinated so that samples are processed quickly and the benefits of rapid decision making are realized.

Sampling

Historically, sampling or obtaining a "representative sample" has long been a topic for a course in analytical chemistry. But how should an animal be sampled? Where and when? And how many animals should be used?

The discovery phase of pharmaceutical research involves synthesizing libraries and screening them *in vitro* for effectiveness, metabolism and potential toxicity. Following those screens, the much-reduced number of compounds needs to be tested in mammalian species. The pharmaceutical industry and the FDA do not yet have full confidence in proceeding to clinical trials based on *in vitro* data or predictive software alone. Animal studies remain of primary importance to compound evaluations, but they are expensive and until recently have not produced much opportunity for automation.

Our goal was to be able to screen animal samples in a high-throughput format using 96-well plates, similar to investments in sample preparation for LC/MSMS and immunoassays. **F1** illustrates one format for a pharmacokinetics protocol utilizing eight rats with ten blood draws for each. Robotics for 96-well plates are available in virtually all laboratories pursuing drug development. Such plates can be added to, shaken or centrifuged and the samples transferred to other plates. Samples on 96-well plates can be processed automatically by liquid-liquid extraction or by solid phase extraction. Excellent 96-well autosamplers for LC/MSMS can then manage multiple-well plates for completely automatic processing overnight. Today, virtually all pharmaceutical research laboratories have implemented such sample preparation automation systems.

Conventional Sampling Methods

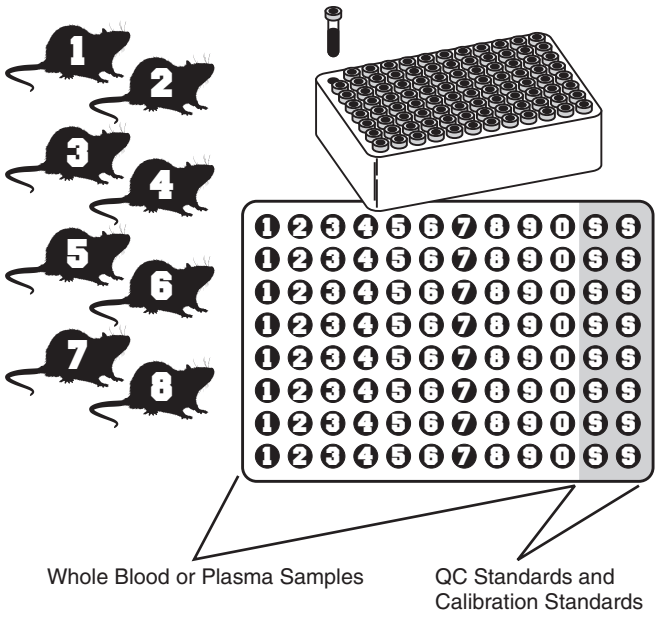
Traditional pharmacokinetics studies involve manual intermittent blood sampling and subsequent determination of blood or plasma drug concentrations (3-5). Because of difficulties associated with collecting blood over time from a single rodent, often multiple animals are used to generate a single PK curve. Manual blood drawings with a syringe is a primitive technique that brings stress and pain to animals and also wastes blood. Serial blood sampling from rodents can be a logistical challenge when conducted by hand over 12-48 hours. Intravenous catheters with heparin-saline locks are used for both anesthetized and awake, restrained rats. In other examples, an animal is sacrificed at each time point. **F2** shows the manual sampling of a restrained rat from its tail vein. Sampling or infusing rodents with indwelling catheters advanced slowly over several decades, beginning with anesthetized animals, moving to awake but restrained animals, and then finally to freely moving animals.

Automated Blood Sampling

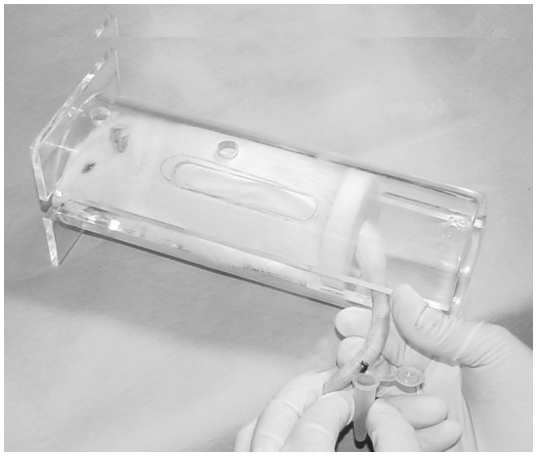
Over the last several years, we developed an automated blood sampler (ABS), a robotic system that collects serial blood samples from awake and freely-moving rats (6-10). This instrument also collects urine and feces, providing additional ADME information from the *same* subject. The automation adds another parameter by monitoring animal activity during the experiment, providing a look at possible behavioral anomalies associated with the drug much earlier in the screening process. Microdialysis samples can also be collected simultaneously (11).

The automated system generates data for the entire PK experiment using one animal. **F3** illustrates a four-animal system based on a cart about one meter square. **F4** shows a benchtop unit for a single animal. **F5** outlines schematically how the system takes each sample and prepares for subsequent samples. Each animal has its own independent blood sampling protocol, start/stop time and collection facilities. All functions are controlled by a single notebook computer. Animal damage to catheters is eliminated by affixing the catheter to a tether assembly mounted to a counter-balanced arm that keeps the catheter out of the animal's reach or view. The catheter tubing is protected from being twisted by animal movement through use of the Raturn® system, a movement-responsive cage that monitors the direction of movement and then rotates the cage, and animal within, in the opposite direction (2,12). The key innovation here is total elimination of need for a liquid swivel. Swivels are expensive, prone to leak, and inconvenient to sterilize. They also add unnecessary dead volume and prohibit use of more than one catheter.

F1. Samples for a PK screen using eight animals and one 96-well plate.

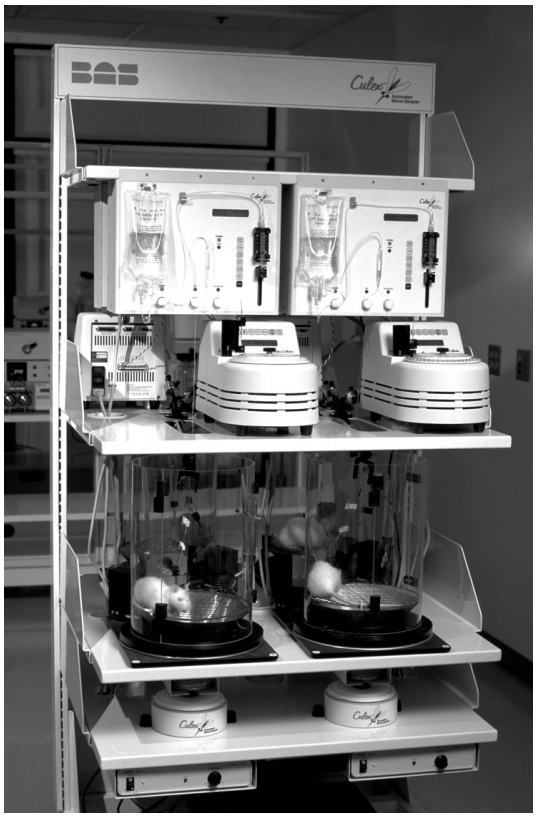


F2. Manual tail vein sampling for rat blood.

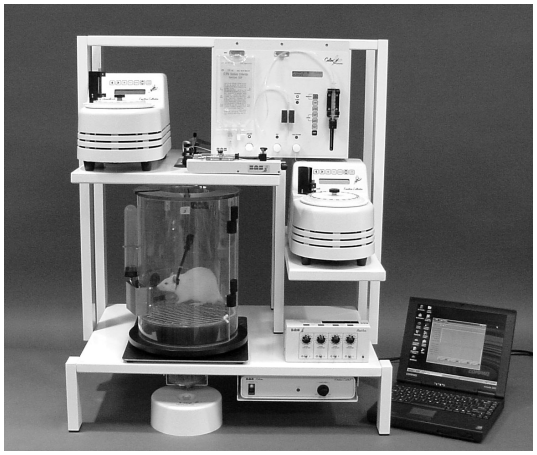


Positional errors in catheter placement may occur during repeated handling of the animal, which may cause a catheter tip to shift within the blood vessel. This is unavoidable during manual blood sampling as a rat is removed from its home cage, placed into a restraining device, and then returned to the home cage. Once an animal is installed in the ABS, the only time it is touched again is during a drug administration procedure that necessitates handling (e.g. gavage). Dosing methods such as IV infusions or use of gastric or duodenal catheters require no contact with the animal. Infusions, both bolus and continuous, can be programmed using an optional accessory designed in our laboratory. All blood sampling events are conducted hands-off, with the animal free to move at will.

F3. A robot station for sampling blood, urine, and feces while monitoring behavior in four rats. Samples are collected in refrigerated 300 µL vials directly transferable to 96-well plates as in **F1**.



F4. A benchtop system for simultaneous, automated sampling of blood, microdialysates, urine and feces.



Membrane Sampling Probes for Pharmacodynamics

One technology that attracted our attention more than 20 years ago is the use of implanted dialysis fibers as passive artificial blood vessels. Today, this takes the form of either microdialysis or ultrafiltration probes. This approach has been widely employed in recent years and is especially useful for small hydrophilic compounds, including glucose, amino acids, neurotransmitters and some drug substances (13-14). Membrane sampling is beneficial in that it does not collect protein (including protein-bound drugs) or cellular matter; but these are also limitations. In addition, the concentration recovery of analytes from the extracellular space surrounding a probe is very dependent on physical factors including membrane dimensions, perfusate flow rate, molecular weight and charge. Hydrophobic compounds are problematic in sticking to components of the probe and connecting tubing. Nevertheless, membrane sampling is quite reproducible under given circumstances and is especially useful for following changes in endogenous compounds in response to drugs. Glucose and neurotransmitters are favorite examples.

F6 shows a probe designed for continuous sampling of brain regions in mice and rats. **F7** illustrates a schematic for a linear probe used in peripheral tissue. Related designs are available for sampling the vasculature, bile and even bone marrow. The three essential components of such experiments are a syringe pump, a probe and a microfraction collector. Collection rates are on the order of 1 µL/min and, thus, it is rarely practical to collect samples more often than every 5-10 minutes. Fortunately, the same refrigerated microfraction collectors used for dialysates can also be used for blood. Coupling to LC/MSMS has been reported by several groups (15-16).

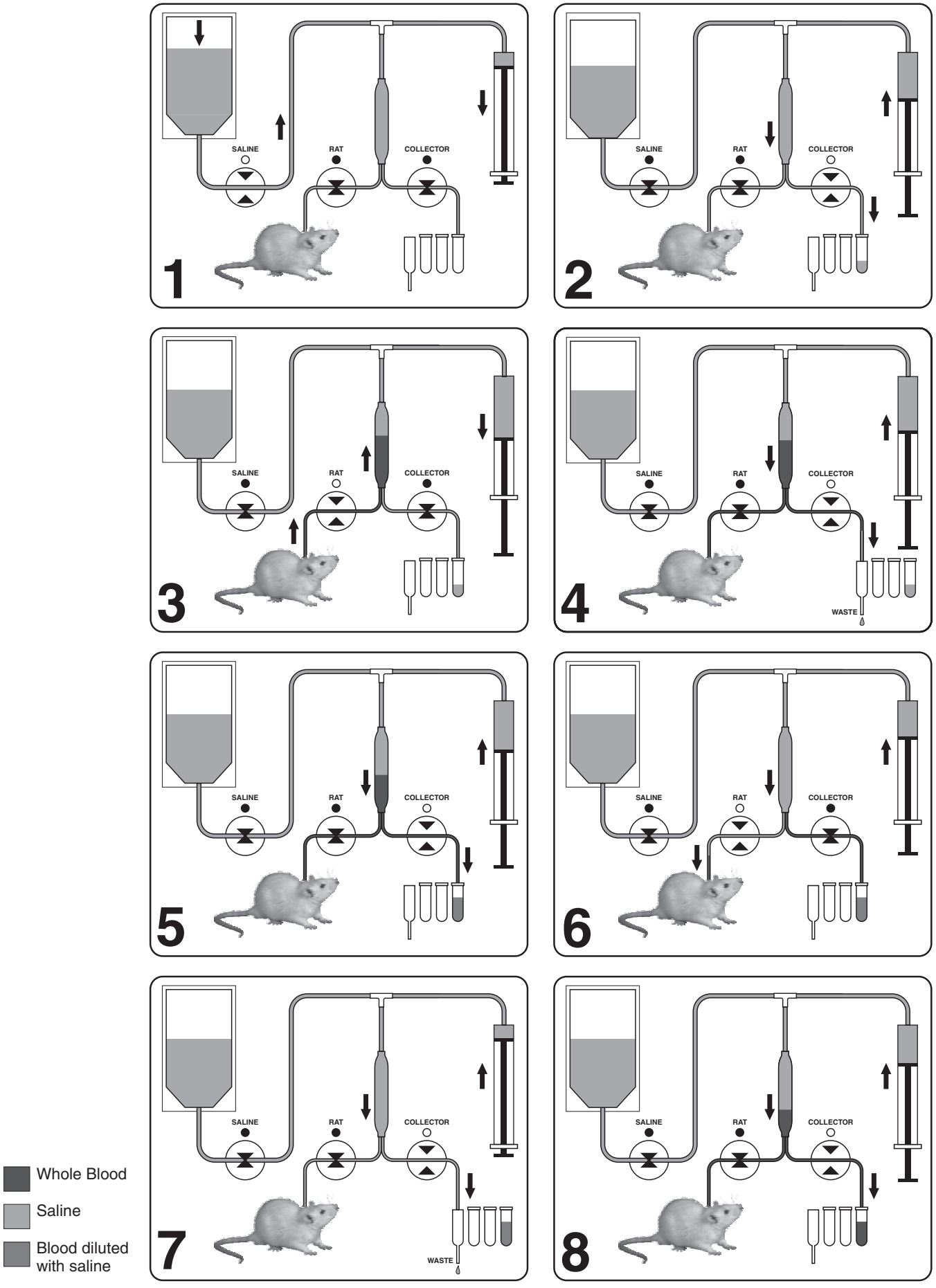
Automated Dosing

Properly dosing animals is a very important step for pharmacokinetic and pharmacodynamic studies. Manual dosing partly defeats the benefit of automatic sampling, especially for the early time points. Different types of automated dosing can be performed on the automated blood sampling system. As with whole blood and dialysis sampling, by eliminating animal-human interaction, the data quality improves.

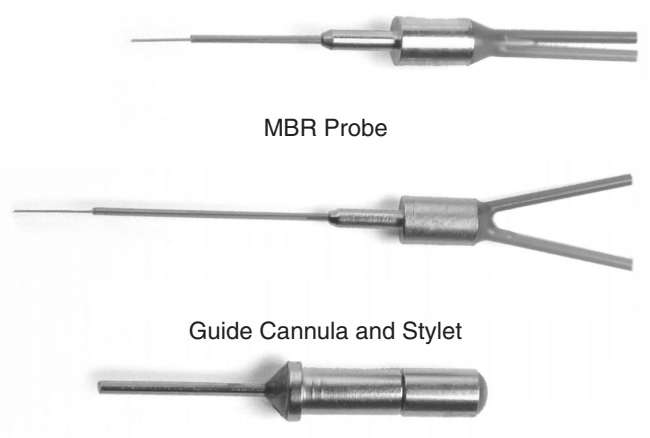
Intravenous

To do this right, we had to develop a new type of infusion pump that allowed for programming concentration of a single drug over time (e.g., apply a "loading dose"), or to apply two drugs to explore an *in vivo* interaction. One of our goals was to be able to initiate a protocol at any time over a rat's diurnal cycle. Continuous infusion also provides a convenient route to getting clearance data (CL), which results from the infusion rate divided by the steady-state plasma concentration (Ro/Css). **F8** shows the Empis® hardware which includes three stepping motor-driven syringes and reservoirs for physiological saline and up to two dosing solutions. Empis operations are driven by convenient software *(www.empis.net),* allowing for a dose to be initiated at any time. Bolus doses, continuous infusion or combinations of these are permitted. **F9** illustrates a schematic for automated dosing.

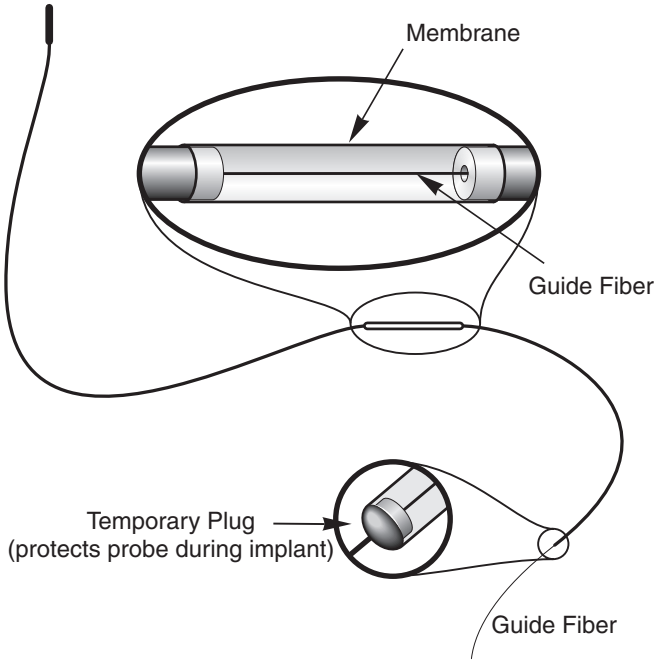
F5. Schematic illustration of the automated blood sampling process. Heparinized saline is used as a motive force to transfer blood through the system.



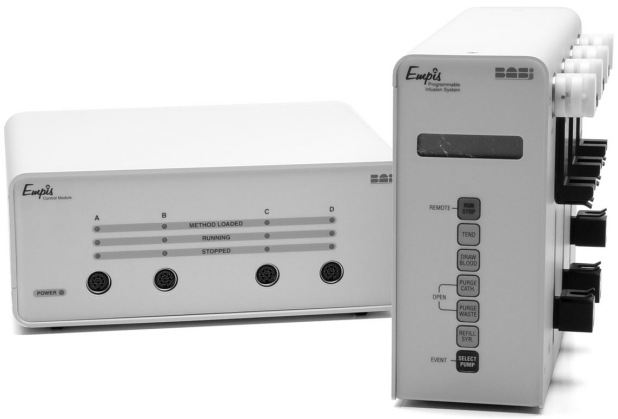
F6. Microdialysis brain probe (MBR) and accessories for sampling brain regions in mice and rats. A guide cannula is first implanted under anesthesia. Later (24 h) the stylet is removed and the probe is inserted into the guide. The membrane at the probe tip is 0.22 mm in outside diameter.



F7. A linear microdialysis probe for sampling soft peripheral tissue. The membrane length in such probes normally ranges from 5-10 mm. The diameter is 0.32 mm.



F8. Hardware for an automated dosing system (Empis).



Gastric and Duodenal

A PO (by mouth) dose requires a gavage. Anyone who has seen a gavage procedure will recognize that this is extremely stressful for the animal. It makes a lot of sense to ask how this stress can distort the data being sought. We've not yet figured out how to get a rodent to reliably take a tablet on command, and thus PO dosing requires handling the animal. On the other hand, gastric dosing *via* catheter is possible, and we do that as an alternative. Dosing directly into the duodenum, by bypassing much of the digestive tract, is also feasible and can provide useful new information when contrasted with both PO and IV dosing. Detailed protocols are available from the authors.

Combinatorial Pharmacology

There are several very compelling new technologies now available that include: (i) whole-animal imaging; (ii) protein biomarker monitoring by multichannel immunoassays; (iii) flow cytometry of blood components; (iv) metabonomic component monitoring using *in vivo* microdialysis and *in vivo* ultrafiltration; (v) automated blood sampling for awake, freely-moving animals for pharmacokinetics and biomarkers; and (vi) parallel monitoring of physiological (blood pressure, body temperature, electroencephalogram [EEG], and electrocardiogram [ECG] and psychological [e.g., turning and rearing behavior]) parameters. While not all of these data sources can be enabled simultaneously, many of them can be accomplished automatically, raising the quality of parallel information available from animal models.

Behavior Monitoring

Turning behavior has been evaluated in rodents for over 30 years. Rearing is also a good measure. The balance beam in the Raturn allows for both. Software developed in our lab tracks both events and their duration. There are specific rotational models for central nervous system (CNS) pharmacology that predispose an animal to one direction or the other. These models are readily accommodated. Simply tracking general activity over a diurnal cycle and comparing with respect to control animals and dose gives an early indication of CNS activity (blood-brain barrier [BBB] penetration) that might be either pharmacological or toxicological. Monitoring activity simultaneously with serial blood sampling has not been possible until recently. This is now easily done and costs virtually nothing once an animal is instrumented for collecting other data *(www.culex.net)*.

Temperature

Tracking temperature in an awake, freely-moving rodent is easily done using miniature thermocouples or thermistors. Thermocouple probes have a relatively simple construction and can be made as small as a grain of sand. Thermocouples, which produce a voltage output proportional to temperature, require more signal-processing electronics than thermistors and can be more prone to disturbances from external electrical noise. Thermistor probes, which provide a resistance change proportional to temperature, may be made as small as a grain of rice. The probe construction is more complex, but the signal-processing electronics are simpler. To monitor temperature in an awake, freely-moving rodent successfully, it is essential that the temperature probe and connecting leads be

small and yet rugged enough to withstand flexure within the body and at the point of externalization. The probe tip-lead junction must also be protected against body fluids, which could affect the output signal.

Blood Pressure

Tracking blood pressure data has also been achieved in awake animals using commercially available, medical-grade blood pressure transducers. These low-cost, disposable blood pressure transducers are located externally to the animal and monitor blood pressure through a catheter. This has advantages over telemetry devices, since the surgery necessary to install a catheter is much less invasive than the procedure normally required to install a telemetry device. Blood pressure can be monitored continuously, as power supply and signal generation is external to the animal and is not limited by battery life. Because the catheter can be tended continuously, preventing catheter tip occlusion, blood pressure can be monitored indefinitely.

EEG and ECG

Electrophysiology can also now be instrumented in awake rats as a PD tool ongoing simultaneously with sampling fluids for corresponding chemical data. As with liquid channels, multiple electrical channels can also be exteriorized from the Raturn animal containment system without need for rotating commutators. This presents a number of possibilities for correlating chemistry with electrical activity in brain or heart. In one example, ECG signals were monitored for QTc interval prolongation in response to a drug interaction (17).

LC/MSMS Compatibility

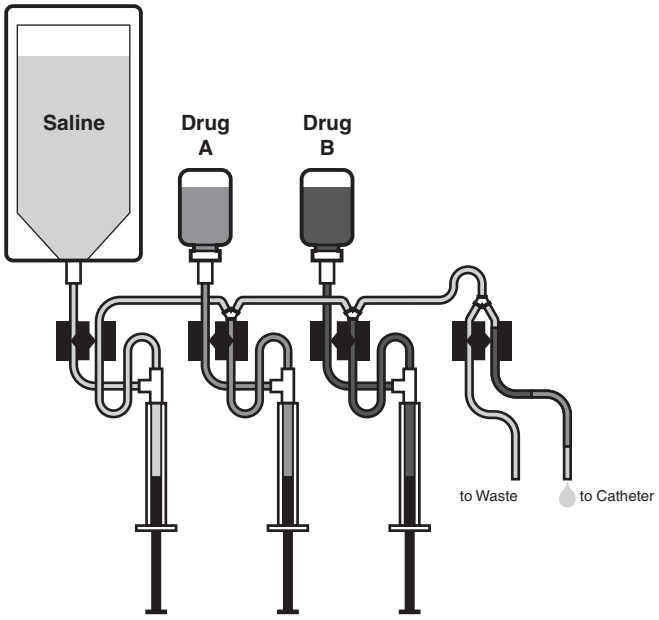
Ion Traps and Triple Quads

Our early development work all relied on the Thermo LCQ instrument (**F10**), which has been a real workhorse for us. We especially like the MS^n capability that ion traps provide. Contrary to some opinions, our years of experience with ion traps indicate they are excellent for preclinical PK quantitation. More recently we have also grown fond of the Thermo TSQ Quantum Ultra platform (**F11**) which affords greatly improved detection limits, excellent m/z resolution and highly selective reaction monitoring (h-SRM) which reduces interference from matrix and can improve S/N. This was achieved due to the HyperQuad Quadruple technology used on the Quantum platform. The precursor ion from the analyte is selected at a higher resolution, typically at 0.1 – 0.3 FWHM, without significant loss of transmission to the collision cell. **F12** shows the quantitative analysis of steroid in rat plasma using SRM and h-SRM. It is clearly indicated that H-SRM can eliminate chemical noise, lower detection limits, and reduce the likelihood of generating false positives (18). Thermo Electron Corporation includes a more thorough explanation and other examples on their web site.

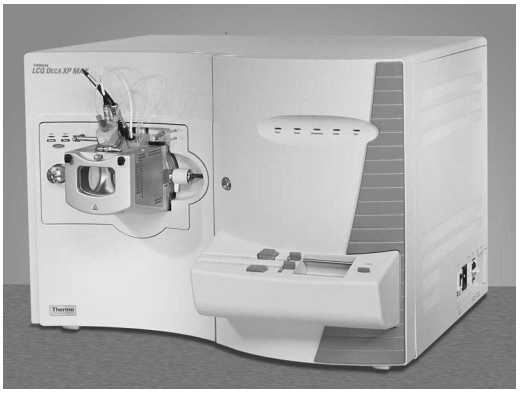
Representative PK and Drug-Dug Interaction Studies

Due to their small size and low blood volume, mice challenge surgical and analytical chemistry skills. Some things remain impossible at this size; for example, bioanalytical approaches for many analytes do not have the lower limit of quantitation (LLOQ) to achieve serial determinations in a single mouse. LC/MSMS methods enable

F9. Empis configured for bolus dose of two drugs. Full motion simulations of this and other Empis options are shown at *www.empis.net.*

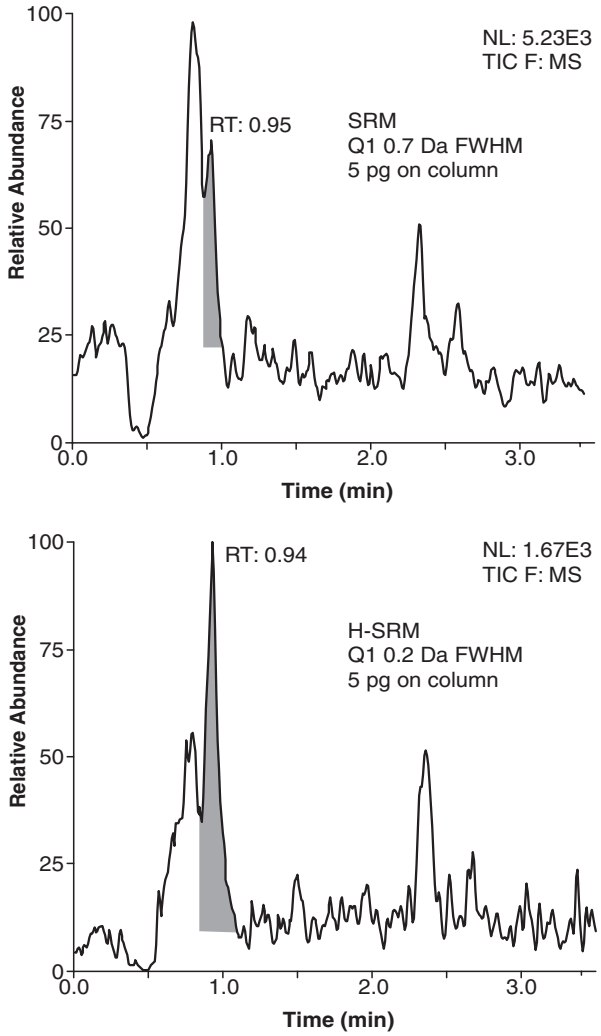


F10. The LC/MSMS LCQ Deca ion trap instrument.



F11. The LC/MSMS TSQ Quantum Ultra equipped with ESI, APCI and APPI interfaces.

F12. Quantitative analysis of steroid in rat plasma using SRM and h-SRM.

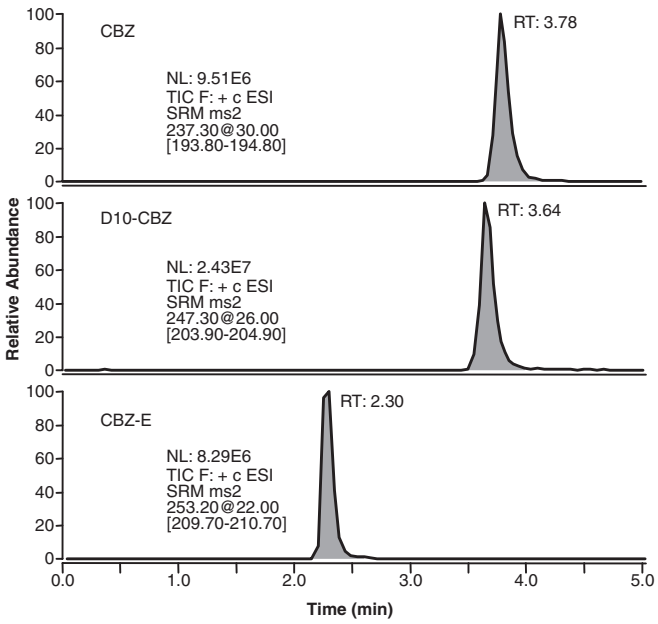


study of smaller volumes and lower concentrations. **F13** shows a representative chromatogram of carbamazepine (CBZ) and its epoxide metabolite (CBZ-E) from a blood sample (10 µL) after oral administration of CBZ at a dose of 20 mg/kg to a mouse. **F14** shows complete PK curves for CBZ and metabolite in an awake and freely-moving mouse sampled without human intervention. **F15** shows how to achieve a carbamazepine steady-state concentration in rat blood using programmed Empis dosing with a loading dose followed by continuous infusion. **F16** shows a typical drug interaction study where drug A was automatically dosed alone (lower PK curve) and then in the presence of drug B (top PK curve). The dramatic enhancement of bioavailability of drug A when B is present reflects a P450 interaction. The drugs investigated here are proprietary. Other PK examples are available on *www.culex.net*.

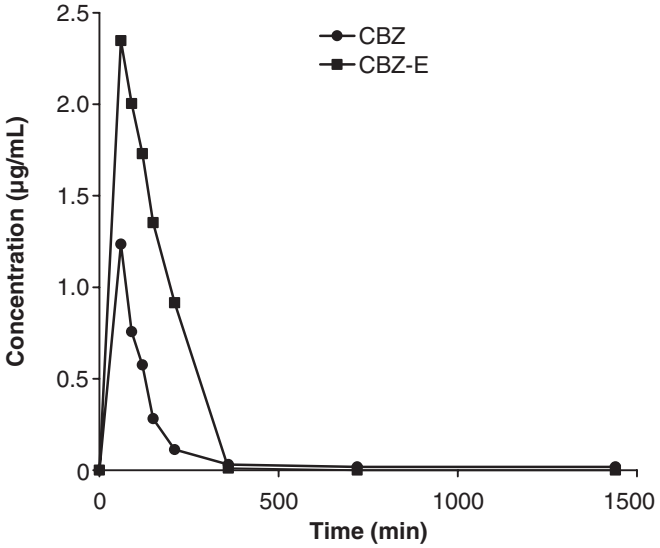
Conclusion

How a rodent is dosed and sampled can be very important to obtain consistent data. Automated *in vivo* dosing and sampling with membrane probes and indwelling catheters provide several advantages for both the research animal and the scientist. The sampling process is totally painless to the animal. The corresponding reduction in stress improves both data relevance and quality, enabling a reduction in the total number of animals required. Quality assurance is improved because sampling times and volumes are precisely controlled and logged by software. Automated sampling of freely-moving rodents wouldn't be practical were it not for very recent advances in bioanalytical chemistry that permit processing small-volume samples with precision and accuracy. Automated dosing and pharmacodynamic measurements only make sense because automated sampling is possible. This evolutionary process once again supports the

F13. Representative SRM chromatogram of CBE, metabolite CBZ-E and internal standard D10-CBE from a blood sample (10 µL) after oral administration of CBZ at a dose of 20 mg/kg to a mouse.



F14. Pharmacokinetics of CBZ and its epoxide metabolite from a 30-g mouse following an oral dose of 20 mg/kg. Blood samples (10 µL) were automatically collected and refrigerated over 24 h.



notion that better data comes from better instruments, and better data then suggests where more new instruments are needed. At the end of the day, patients will be better served. That is our focus.

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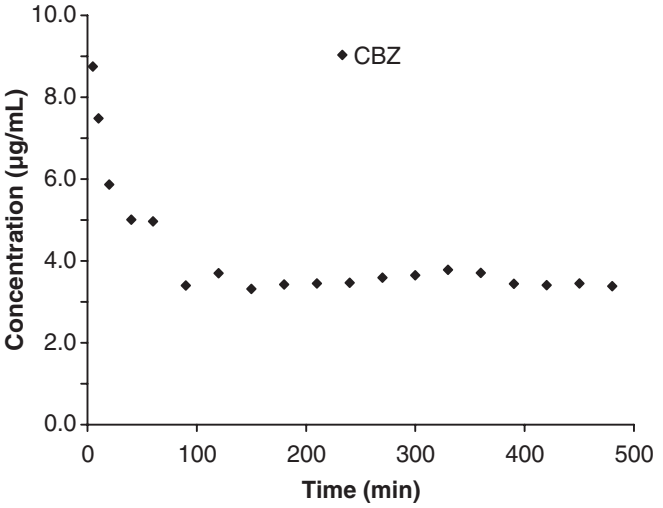
F15. Achieving a carbamazepine steady-state concentration in rat blood using programmed Empis dosing with a loading dose of 1.4 mL of 2 mg/mL of CBZ at 0.5 mL/min for 2.8 minutes followed by a steady flow of the same solution at 0.6 mL/hour over 8 hours.



F16. A typical drug interaction study where drug A was automatically dosed alone (bottom PK curve) and then in the presence of drug B (top PK curve).