Journal of the American Association for Laboratory Animal Science
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Vol 47, No 6
November 2008
Pages 41–45

Validation of the Use of Nonnaive Surgically Catheterized Rats for Pharmacokinetics Studies

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Although large animals, such as dogs and nonhuman primates, often are used for more than 1 pharmacokinetics study, common practice is to use only naive rodents for pharmacokinetics studies. We undertook a series of studies to validate whether surgically cannulated nonnaive rats could be used again after a 7-d washout. When vascular catheters are cared for appropriately, we find that they remain patent for more than 2 wk, with negligible drug carryover. Hematocrit decreased approximately 11% after pharmacokinetics studies but rebounded to prestudy levels after a 7-d washout. We empirically tested whether drugs known to alter drug disposition (1-aminobenzotriazole and quinidine) had residual effects on drug disposition after a 7-d washout and found that they did not. This finding suggests that after a 7-d washout, nonnaive rats likely would produce pharmacokinetics data similar to those of naive rats. We also tested reference compounds in naive and nonnaive rats and found no difference in pharmacokinetics parameters. Using surgically cannulated rats for a second study was feasible because of the relatively noninvasive nature of pharmacokinetics sampling (unrestrained rats attached to automated blood samplers). In addition, reusing surgically altered animals yields considerable cost savings. Our studies indicate that pharmacokinetics parameters did not differ significantly between naive and nonnaive rats. Cost–benefit analysis, monetary considerations, and validation studies support using rats for a second study after a 7-d washout period.

Abbreviations: ABT, 1-aminobenzotriazole; AUC, area under the time–concentration curve; CYP, cytochrome P450; NCE, new chemical entity

Pharmacokinetics studies are an important part of the drug discovery process. In a typical pharmacokinetics study, a compound is administered to an animal, and then plasma samples are taken at intervals ranging from minutes to days. These samples are assayed for levels of the compound. The objective is to determine the rate of appearance or disappearance of the drug in the plasma. The shape of the curve of drug concentrations versus time allows determination of important pharmacokinetics parameters such as the half-life, volume of distribution, clearance, maximal concentration, and exposure (area under the time–concentration curve [AUC]). Relatively low drug doses are used, because the objective is to determine the body's effect on the drug, not the pharmacologic effects of the drug on the animal.

Typically rats are used for screening the pharmacokinetics properties of new chemical entities (NCEs), because rats are small enough not to require much compound but large enough to yield multiple blood samples. Commonly, only naive animals are used. In contrast, when pharmacokinetics studies are performed in large animals such as dogs and nonhuman primates, nonnaive animals are used after an appropriate washout period, during which the test compound is eliminated from the body. These larger species are considered too valuable to euthanize after a single, relatively noninvasive and nonharmful study. We undertook to determine whether using nonnaive rats for screening pharmacokinetics studies was possible.

Multiple factors including blood volume, trainability, and ready availability of clinical tests (to ensure that animals are clinically and metabolically normal) make the repeated use of large animals fairly easy. The amount of blood withdrawn (2 to 3 ml divided over 8 to 10 samples) is negligible for a 10-kg dog, whereas a similar amount removed from a rat can represent a clinically significant proportion of the total blood volume. Handling stress can have effects such as altered gastric emptying time,[9] increased heart rate, and altered metabolism, all of which can affect the pharmacokinetics of a drug. Variation in degree of reaction to handling stress can act as an uncontrolled variable in a study. Large animals such as dogs can be acclimated to blood withdrawal and trained to present a paw for venipuncture. For rats, such training is impractical, but the use of automated blood samplers to withdraw blood through surgically implanted catheters offers a way to obtain samples from unstressed animals.[8] Finally, serum chemistry and complete blood counts can routinely be obtained from large animals to ensure that animals used in additional studies do not exhibit signs such as elevated liver enzymes or anemia that might indicate abnormal metabolism. For rats, the blood volume needed for such testing is prohibitive.

One reason that naive animals are favored over nonnaive animals is that exposure to drugs can alter subsequent drug disposition. Many drug interactions are well documented and have clinical importance. For example, phenobarbital is well known to increase levels of the enzyme that metabolizes it, so that progressively higher and higher doses of the drug must be administered to achieve the same effect[13]. The antifungal drug ketoconazole is a potent inhibitor of cytochrome P450 (CYP) 3A4 enzyme, and caution must be used when patients are given this drug, because it can reduce the metabolism of concurrent medications.[14] Grapefruit juice can inhibit intestinal CYP3A4 and P-glycoprotein transporters, significantly affecting intestinal absorption of drugs.[18] Tamoxifen has enhanced bioavailability when coadministered with quercetin, which

Received: 31 Mar 2008. Revision requested: 12 May 2008. Accepted: 16 Jul 2008.
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promotes the drug's intestinal absorption and reduces its first-pass metabolism.[16]

Quinidine (which inhibits P-glycoprotein)[1,7] and 1-aminobenzotriazole [ABT] (which inhibits CYPs)[2,12] are used experimentally to investigate drug disposition because they selectively inhibit specific types of drug transport and metabolism. However, the effects of these drugs fade over time and eventually disappear ('wash out'). The effects of grapefruit juice last less than 3 d in humans.[4] The capability of ketoconazole to inhibit the elimination of budesonide is reduced by 50% by a 12-h separation of their administration times.[15] For large animal studies, washout periods typically are 2 to 4 wk. The challenge in surgically cannulated rats is to find a washout period that is long enough to ensure that pharmacokinetics parameters are not affected by the first drug, yet short enough that the catheter is still likely to be patent.

Materials and Methods

Animals. Male Sprague–Dawley rats (specific pathogen-free; CD, Charles River Laboratories, Wilmington, MA) with vendor-placed jugular vein or femoral artery catheters (or both) were obtained. Rats were approximately 225 g at time of arrival. Rats were singly housed in individually ventilated plastic shoebox-style microisolation caging, on maple wood-chip bedding (Harlan Teklad, Indianapolis, IN), and with ad libitum chow (2918, Harlan Teklad) and autoclaved tap water. Environmental conditions in the housing room: 21.1 to 21.7 °C, 30% to 50% relative humidity, and 12:12-h light:dark cycle. All experiments were approved by the institutional animal care and use committee.

Pharmacokinetics study. Rats were dosed intravenously by means of jugular vein catheters or orally by gavage. Blood samples were obtained through surgically implanted femoral arterial catheters. Samples (230 µl) were drawn either manually by connecting a syringe to the catheter by means of a 22-gauge stub adaptor or automatically by connecting the catheter to a commercially available automated blood sampler (Accusampler, Di-Lab, Littleton, MA, or ABS, Instech-Solomon Laboratories, Plymouth Meeting, PA). After blood withdrawal, the catheters were flushed with an equal volume of heparinized saline (10 U/ml). For a typical study, 230 µl of whole blood was withdrawn immediately prior to dosing and at 5 (intravenously administered drugs only), 15, and 30 min and 1, 2, 4, 6, 8, 12, and 24 h after drug administration. After the completion of a study, catheters were locked by injection of 40 µl of heparin lock solution (100 U/ml; Hep-Lock, Baxter Healthcare, Deerfield, IL) and left untouched until a subsequent study 1 wk later. Antipyrine (Acros Organics USA, Morris Plains, NJ) was dosed intravenously at 2 mg/kg as a solution of 2 mg/ml. Fexofenadine (Sigma, St Louis, MO) was dosed orally at 20 mg/kg as a solution of 40 mg/ml. NCEs were dosed at 0.5 to 2 mg/kg in a variety of vehicles suitable for intravenous or oral dosing. The NCEs used represented a wide variety of chemical structural classes from drug discovery programs for more than 5 molecular targets in the fields of oncology and Alzheimer disease.

Sample analysis. Hematocrit was determined by collecting blood in heparinized glass capillary tubes, spinning in a hematocrit centrifuge, and reading the result against a hematocrit chart. Drug concentrations in plasma samples (anticoagulant, lithium heparin) were analyzed by liquid chromatography–mass spectroscopy (API 5000, Applied Biosystems, Foster City, CA) using appropriate parameters for each compound. Half-life, volume of distribution, clearance, maximal concentration, and AUC were calculated by using Watson LIMS software (version 7.2, Thermo Electron Corporation, Philadelphia, PA).

Results

Hematocrit was analyzed before and after pharmacokinetics studies to determine whether rats were hematologically normal or anemic after a 7-d washout period. Hematocrit (mean ± SEM) prior to study was 36.2% ± 2.4% (n = 10). At the 24-h time point, after withdrawal of ten 230-µl blood samples, hematocrit in the same rats was 32.0% ± 2.8%, representing a decrease of 11%. After 7 d, hematocrit rebounded to 40.5% ± 3.7%.

To determine whether residual NCE was present in catheters after a 7-d washout, we sampled blood from dosing (jugular vein) and blood sampling (femoral artery) catheters after catheters had been heparin-locked for 7 d. The animals analyzed were from 11 different pharmacokinetics studies, representing 11 NCEs from several different chemical structural classes. Animals had been dosed with 0.5 to 2.0 mg/kg compound. None of the catheters had compound levels above the detection limit of 2 to 10 nM.

To determine whether prior exposure to drugs known to alter drug disposition (1-aminobenzotriazole and quinidine) would alter drug metabolism and disposition after a 7-d washout, we administered these inhibitors and then determined pharmacokinetics parameters for the reference compounds antipyrine and fexofenadine) immediately before and after a 7-d washout (Figure 1). ABT (Sigma, St Louis, MO) was dosed orally at 50 mg/kg, followed 2 h and 7 d later by intravenous dosing with antipyrine (drug whose clearance is dependent on metabolism by multiple CYPs).[3,6,11] In a different set of rats, quinidine (Sigma) was given orally at 50 mg/kg, followed 30 min and 7 d later by oral dosing with fexofenadine (a drug dependent on P-glycoprotein transporter for absorption and biliary efflux).[4, 5,17] Exposure (AUC_{norm}) of the reference compounds was compared among naive rats, ABT- or quinidine-treated rats, and ABT- or quinidine- treated rats after a 7-d washout. As expected, exposure was increased profoundly (Figure 1) in the pharmacokinetics studies immediately after dosing with the inhibitors. Antipyrine AUC for ABT-treated rats was 27.93 ± 36 µM·h·kg/mg compared with 12.5 ± 3.1 µM·h·kg/mg in naive rats ($P = 0.0006$). Fexofenadine AUC for quinidine-treated rats was 0.043 ± 0.0002 µM·h·kg/mg compared with 0.014 ± 0.005 µM·h·kg/mg in naive rats ($P = 0.00002$). However, after a 7-d washout, exposure for antipyrine (9.21 ± 9.41 µM·h·kg/mg) and fexofenadine (0.009 ± 0.000001 µM·h·kg/mg) in inhibitor-treated animals was not significantly greater ($P > 0.08$) than AUC_{norm} in naive rats (Figure 2).

To determine whether prior exposure to NCEs would result in subsequent alterations in drug metabolism and disposition, we determined pharmacokinetics parameters of the reference compounds antipyrine and fexofenadine in naive rats and nonnaive rats 7 to 10 d after exposure to NCEs during standard screening pharmacokinetics studies. The parameters measured for antipyrine and fexofenadine did not differ significantly between naive and nonnaive rats (Table 1, Figure 2).

Discussion

A practical reason for using only naive rodents in pharmacokinetics studies was that formerly the volume of blood needed for analysis required terminal sampling. Because of improvements in analytic sensitivity, a pharmacokinetics study involving 10 time points can be conducted with the use of less than 3 ml blood from a single rat. Improvements in materials

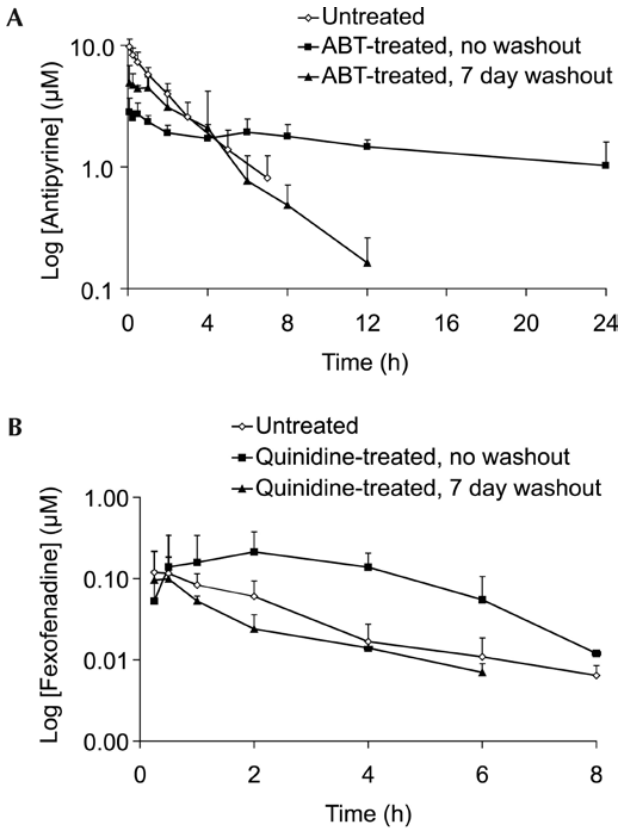

Figure 1. Plasma concentration–time profiles of antipyrine and fexofenadine in naive, inhibitor-treated, and nonnaive rats. (A) Plasma concentration of antipyrine after intravenous administration of 2 mg/kg antipyrine in naive rats (◊), rats treated with 50 mg/kg ABT followed by intravenous antipyrine 2 h later (■), and ABT-treated rats given a second dose of antipyrine after a washout period of 7 d (▲). Rats pretreated with ABT show prolonged high levels of antipyrine in the plasma because of ABT-induced inhibition of CYP-mediated metabolism. After a 7-d washout, the curve of the nonnaive rats resembles that of naive rats, with no significant difference in AUC between these groups. (B) Plasma concentration of fexofenadine after oral administration of 20 mg/kg fexofenadine in naive rats (◊), rats treated with 50 mg/kg quinidine followed by oral fexofenadine 30 min later (■), and quinidine-treated rats given a second dose of fexofenadine after a 7-d washout period (▲). Compared with naive animals, quinidine-treated rats showed higher fexofenadine concentrations in the plasma at the 2-, 4-, and 6-h time points because of quinidine's inhibitory effect on intestinal transport by means of the P-glycoprotein efflux transporter. After a 7-d washout, the curve of the nonnaive rats resembles that of naive rats, with no significant difference in AUC between these groups.

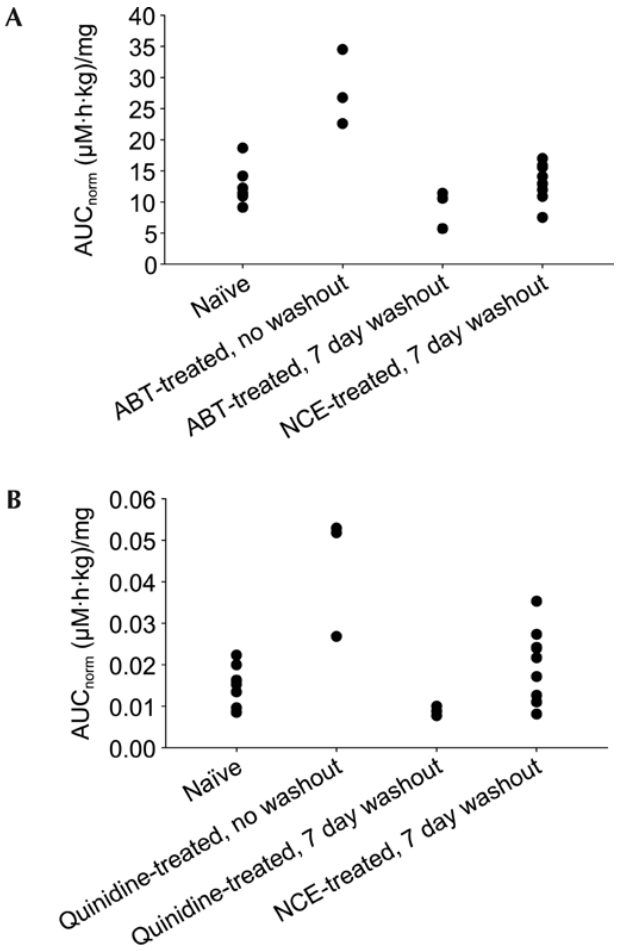

Figure 2. Scatter plots of the AUC of antipyrine and fexofenadine. To determine whether prior exposure to NCEs alters subsequent drug metabolism and disposition, we determined pharmacokinetic parameters of the reference compounds antipyrine and fexofenadine in naive and nonnaive rats 7 to 10 d after exposure to NCEs during standard screening pharmacokinetic studies. (A) AUC values for antipyrine dosed intravenously in naive rats, rats given antipyrine 2 h after pretreatment with 50 mg/kg ABT, given a second dose of antipyrine 7 d after pretreatment with ABT, and given antipyrine 7 to 10 d after a pharmacokinetics screening study of an NCE. The AUC of antipyrine was increased in ABT-treated rats compared with naive rats. However, the AUC did not differ significantly between naive rats and 1-aminobenzotriazole- or NCE-dosed rats after a 7-d washout.(B) AUC values for fexofenadine dosed orally in naive rats, rats dosed 30 min after pretreatment with 50 mg/kg quinidine, given a second dose of fexofenadine 7 d after pretreatment with quinidine, and given fexofenadine 7 to 10 d after a pharmacokinetics screening study of an NCE. The AUC of fexofenadine was increased in quinidine-treated rats compared with naive rats but did not differ significantly between naive rats and quinidine- or NCE-dosed rats after a 7-d washout.

and implantation techniques allow catheters in rats to stay patent routinely for durations sufficient to conduct more than a single pharmacokinetics study. Therefore the use of rats for multiple pharmacokinetics studies has now become feasible. We did not investigate the feasibility of maintaining rats for more than 2 studies, because the animals grow too large and thus require too much compound. However, this concern might not be valid with female rats or with strains or stocks that are smaller than the male Sprague–Dawley rats we typically use for our pharmacokinetics studies.

The concern that prior exposure to a compound might affect subsequent drug disposition will always be present. Exposure to a strong CYP inducer or inhibitor or an inhibitor of P-glycoprotein transporters might alter subsequent metabolism or drug distribution even after the inducer's or inhibitor's complete elimination from the body. We tested empirically whether 2 known inhibitors—1 of drug metabolism enzymes and another of drug transporters—affected drug disposition after a 7-d washout and found that they did not. We also saw no evidence that altered drug disposition occurred in the course of normal pharmacokinetics screening studies with investigational compounds. Still, a particular NCE might be an even more potent or irreversible inhibitor than ABT or quinidine, but we consider that this possibility is not sufficiently likely to preclude using the rats a second time. In the unlikely event that such a potent

Table 1. Pharmacokinetics parameters in naive rats and those previously dosed with various compounds

	Nonnaive rats	Naïve rats	p^a
Antipyrine			
AUC_{norm} (μM·h·kg/mg)	13.17 ± 2.89	12.51 ± 3.10	0.67
Volume of distribution (L/kg)	1.75 ± 0.36	1.39 ± 0.46	0.10
Clearance (mL/min· kg)	7.09 ± 2.00	7.38 ± 1.56	0.76
Half-life (h)	2.76 ± 0.55	2.30 ± 0.82	0.20
Fexofenadine			
AUC norm (μM·h·kg/mg)	0.020 ± 0.009	0.014 ± 0.005	0.10
Maximal concentration (μM)	0.22 ± 0.20	0.14 ± 0.08	0.31
Half-life (h)	2.0 ± 0.6	2.5 ± 1.4	0.33

Pharmacokinetics parameters for fexofenadine (20 mg/kg orally) were determined in 9 naive and 9 nonnaive rats 7 d after being given 1 of 7 different NCEs. Pharmacokinetics parameters for antipyrine (2 mg/kg intravenously) were determined in 7 naive and 9 nonnaive rats 7 d after being given 1 of 4 different NCEs.
[a]Calculated by using 2-sided *t* tests assuming equal variance.

inhibitor was synthesized, data from in vitro assays performed before or in parallel with pharmacokinetics studies likely would reveal this unique characteristic.

In large animals, extending the washout period to 2, 3, or 4 wk is common. We elected to study a 7-d washout period for 3 practical reasons. First, the catheters in rats do not reliably stay patent past 2 wk. Second, NCEs used for pharmacokinetics screening studies are generally available only in small amounts, making large rats undesirable for study. Young male SD rats grow very rapidly and likely would become too large after a washout period of longer than 7 d. In our studies, rats are used twice within 12 d of arrival, at a final weight of no more than 315 g. Finally, the 7-d period likely is sufficiently long that the compound from the first pharmacokinetics study is not detected just before the start of the next study so as to ensure minimal interference in the analysis of the second compound. We calculate that at a starting dose of 2 mg/kg and a molecular weight of 500, only drugs with half-lives longer than 24 h would still be detectable in the plasma (that is, present at 2-10 nM or greater) after 7 d of washout, assuming that the drug is evenly distributed throughout the animal; we chose a molecular weight of 500 for the calculation, because it is the optimal molecular weight predicted to have drug-like properties.[10] To mitigate against the possibility of residual drug interfering with detection of compounds in subsequent studies, repeated use of rats might be limited to compounds from different projects or compounds that are structurally distinct from each other. The present study assesses use in small-molecule drug programs only, for drugs given orally or parenterally at low (0.5 to 2 mg/kg) doses. Repeated use of rats given biologicals, highly toxic compounds, or compounds in extended-release formulation is not addressed by this study but probably is not advisable. However, for a lab working with both drug-like small molecules and other types of compounds, our results suggest that first performing a small-molecule screening pharmacokinetics study and later using the rats to evaluate a biological, toxin, or extended-release formulation is feasible.

Our current data suggest that scientifically valid results can be obtained from rats used in a second study. After a 7-d rest period, rats have normal hematocrit, lack detectable drug in plasma, and show no evidence that even high doses of known strong inhibitors of drug disposition affect subsequent studies. When they are appropriately managed, surgically implanted catheters routinely stay patent in rats for more than 2 wk. However using an animal for multiple procedures can raise ethical concerns. With regard to the risks and benefits of using 1 rat with 1 surgery and 2 studies, versus 2 rats with 1 surgery and 1 study each, surgical catheterization engenders associated risks and stresses to animals, whereas during the study itself rats are sampled unrestrained in their home cage and experience only brief manual restraint for dosing. For a typical throughput of 50 rats per week, allowing for a second use of 70% of the rats would require only 32 new surgically implanted rats each week. This scenario generates an annual savings of 900 rats, at a savings of approximately US$100,000 if commercially prepared rats are purchased. We conclude that the humane and financial cost–benefit analysis favors use of rats for 2 pharmacokinetics screening studies.

Acknowledgments

The authors would like to thank Andreas Harsch and Gloria Kwei (Department of Drug Metabolism and Pharmacokinetics, Merck Research Laboratories, Boston, MA) for valuable discussions.

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