Continuous Blood Withdrawal as a Rapid Screening Method for Determining Clearance and Oral Bioavailability in Rats

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Purpose. To develop a methodology for continuous blood withdrawal in rats suitable for drug discovery screening purposes and perform limited validation studies with a series of test compounds.

Methods. A reliable methodology for continuous blood withdrawal in rats was developed. The method is dependent on continuous heparin infusion during withdrawal and the minimization of constrictive, thrombogenic sites. Plasma drug concentrations from either intermittent sampling or continuous withdrawal experiments were determined with HPLC analysis.

Results. The continuous withdrawal method was successfully adapted to rats such that blood samples could be reliably collected over a 6-hr experiment. The clearance and oral bioavailability values for theophylline, atenolol, propranolol, warfarin, BMS-182874 and BMS-A were determined from continuous withdrawal or intermittent sampling experiments. The results from the two methods were comparable, with each compound reliably placed in the same low, medium or high category based on clearance or oral bioavailability characteristics.

Conclusions. The continuous withdrawal method proved to be a viable alternative to the classic intermittent sampling technique. The method should prove useful in drug discovery screening, where the evaluation of large numbers of compounds for systemic clearance or oral bioavailability is often necessary.

KEY WORDS: drug discovery; continuous withdrawal; rapid screening methods.

INTRODUCTION

Drug metabolism studies are now widely recognized as an integral part of the discovery phase of pharmaceutical research and there is an ever increasing need to gain pharmacokinetic/metabolism information on promising compounds. Combinatorial chemistry has dramatically increased the output of synthetic programs and new methodologies must be adopted to be able to provide pharmacokinetic/metabolism information in a timely fashion.

Traditional pharmacokinetic studies involve intermittent blood sampling (also referred to as serial sampling) and subsequent determination of blood or plasma drug concentrations. To get an adequate representation of the concentration vs. time curve, and thus an accurate AUC determination, 6–12 samples per animal must be taken. When this number is multiplied by several animals per compound and by many compounds, the number of samples to be collected, processed and analyzed quickly adds up. Even with rapid analytical procedures, such as LC-MS and LC-MS/MS, the time of analysis can become a major time constraint for pharmacokinetic experimentation. In order to decrease the number of samples for analysis, and thus total study time, the continuous blood withdrawal approach was investigated.

The continuous withdrawal approach was first applied by Kowarski and coworkers as a means of accurately measuring an "integrated concentration" in humans without having to draw intermittent samples (1). The method was shown to provide accurate determinations of AUC values in humans and dogs (2–4) but there are no published reports of use of the method in rats, presumably due to technical issues related to animal size. Indeed, many obstacles were encountered before a reliable procedure was found that allowed for prolonged blood withdrawal at a suitable flow rate. The prime advantage of the method with respect to drug discovery research is the dramatic decrease in blood/plasma sample number, from 6–12 samples per animal to a single sample. The objectives of the current study were to: 1) develop a reliable methodology for continuous blood withdrawal in rats and 2) perform limited validation studies with a series of test compounds that have a wide range of oral bioavailability, protein binding, and Tmax (Table I).

EXPERIMENTAL

Materials

D,L-Propranolol, atenolol, theophylline, and warfarin were obtained from Sigma Chemical Co. (St. Louis, MO) and were at least 95% pure. Sodium heparin (1000 USP units/ml) was purchased from Fujisawa, USA, Inc. (Deerfield, IL). BMS-182874 and BMS-A were synthesized at Bristol-Myers Squibb Pharmaceutical Research Institute (Princeton, NJ) and were at least 97% pure.

Animals

Male Harlan-Sprague Dawley rats (320–340 g) were obtained from Hilltop Lab Animals, Inc. (Scottdale, PA). For the continuous withdrawal experiments, the rats were instrumented with a jugular vein cannula (0.030″ I.D. × 0.065″ O.D. silicone tubing, Baxter SP, McGaw Park, IL) and femoral artery cannula (0.020″ I.D. × 0.037″ O.D. silicone tubing). The cannulae were made long enough (*ca.* 60 cm) to reach directly to a syringe pump. For the intermittent sampling experiments, the rats were instrumented with a jugular vein cannula (0. 020″ I.D. × 0.037″ O.D. silicone tubing) and femoral artery cannula (0.020″ I.D. × 0.037″ O.D. silicone tubing). All cannulae were shipped charged with polyvinylpyrrolidone (PVP), 0.9% saline, and 1000 U/ml sodium heparin (1 g PVP: 1 ml saline: 0.5 ml heparin) and tucked into a subcutaneous pocket. After surgery, the rats were allowed to recover for one day and then shipped in an environmentally-controlled vehicle, with access to food and liquid. Within several days of their arrival the cannulae were exteriorized and rats transferred to metabolism cages (Nalgene, Milwaukee, WI). The PVP was withdrawn and cannulae were flushed with sodium heparin solution (25 U/ml in saline).

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For the animals to be used in the continuous withdrawal experiments, both cannulae were connected to infusion pumps (Harvard Apparatus, South Natick, MA) at an infusion rate of 0.5 ml/hr overnight (25 U/ml sodium heparin in saline). The following morning, the jugular cannulae were hooked up to an infusion pump set to withdraw at 1.0 ml/hr, and the rats were infused through the femoral artery cannulae with 25 U/ml sodium heparin at the same rate. The withdrawal syringes were adapted such that the blood flowed directly into a 10 ml syringe barrel (LuerLok®, Becton Dickinson and Co., Franklin Lakes, NJ), because attachment of the tuhing to the syringe barrel *via* a metal needle was thrombogenic. This was accomplished by the following procedure: 1) threading the silicone tubing into the syringe barrel through the hub, 2) inserting a small piece of PE 190 tubing (*ca.* 1 cm, I.D. 1.19 mm, O.D. 1.70 mm, Becton Dickinson and Co.) into the silicone tubing, 3) feeding the silicone tubing back until the expanded tubing becomes loosely lodged in the hub, 4) adding *ca.* 0.1 mL of sodium heparin (1000 U/ml) and then inserting the syringe plunger and 5) pulling the silicone tubing until it forms an airtight seal.

The rats were dosed either orally or intraarterially (ia, bolus injection). For the continuous withdrawal experiments, blood was withdrawn from the jugular vein for 6 hr, at which time the animals were sacrificed. The intermittent blood samples (0.5 mL) were followed by a heparin/saline flush (0.5 ml, 25U/ml). The total blood withdrawn over the study period was 6.0 ml in the continuous withdrawal experiment and 4.5 ml in the intermittent sampling experiment.

Compounds were administered either singly or as mixtures. The dose levels were as follows: theophylline, 4 mg/kg ia, 10 mg/kg oral; atenolol, 3 mg/kg ia, 3 mg/kg oral; propranolol, 3 mg/kg ia, 3 mg/kg oral; warfarin, 1 mg/kg ia, 1 mg/kg oral; BMS-182874, 35 mg/kg ia, 100 mg/kg oral; BMS-A, 24 mg/kg ia, 24 mg/kg oral. When the mixture of theophylline, atenolol and propranolol was administered the dose level for each compound was the same as when they were dosed singly.

Blood samples were centrifuged for 20 min at 3,000 rpm, and plasma samples collected. Plasma proteins were precipitated by the following procedure: one volume of plasma was mixed with 1 volume of ethanol, spun, a second volume of ethanol added, and to that total supernatant was added 3 volumes of acetonitrile (ACN) and the final supernatant taken to dryness. For atenolol, the same basic extraction procedure was used except blood was used instead of plasma (extraction efficiencies were higher from blood than plasma) and the addition of ethanol was done in one step instead of two.

Table I. Oral Bioavailability, Protein Binding, and Tmax for Experimental Compounds

Compound	Oral bioavail. (%)	% Protein Binding	T_{max} (hr)
Theophylline	>100 (5)	23 (6)	1.5 (7)
Atenolol	45 (8)	negligible (9)	3 (9)
Propranolol	10 (10)	90 (9)	0.2 (9)
Warfarin	high	99 (11)	3.6 (12)
BMS-182874	100	99	0.3
BMS-A	8	ND[a]	0.5

[a] Not determined.

Analysis of Drugs

For analysis of propranolol, an HPLC system with fluorescence detection was used. The HPLC conditions were as follows: A C_{18} column (ODS-AQ, 4.6 × 150 mm, S-3 μ, YMC, Inc., Wilmington, NC) was used with a gradient system at a flow rate of 0.8 ml/min. The mobile phase consisted of solvent A (95:5; 50 mM ammonium acetate, pH 5.0: ACN) and solvent B (5:95; 50 mM ammonium acetate, pH 5.0: ACN), programmed in a linear gradient from 0% A to 20% A over 20 min. The eluate was monitored by fluorescence detection (Waters 474 scanning fluorescence detector, Waters, Inc., Milford, MA, excitation wavelength = 290 nm, emission wavelength = 360 nm).

For analysis of atenolol, a similar HPLC gradient system to the one described above was used with the following modifications: the linear gradient was programmed from 100% A to 20% A over 20 min and the fluorescence detection was with an excitation wavelength of 230 nm and an emission wavelength of 300 nm.

For analysis of theophylline, the same HPLC column and solvents were used, but with UV detection. The linear gradient was programmed from 100% A to 85% A over 20 min and was monitored at 280 nm.

For analysis of warfarin, BMS-182874, and BMS-A the linear gradient was programmed from 90% A to 20% A and UV detection was used. Warfarin was monitored at 310 nm. The BMS compounds were monitored at 270 nm.

Data Analysis

AUC values for the continuous withdrawal method were calculated from the following equation:

$$\text{AUC} = \text{integrated concentration} \times \text{withdrawal period.}$$

The integrated concentration is the single plasma level determined after the 6 hr continuous withdrawal. AUC values for the intermittent sampling method were calculated with standard trapezoidal rule techniques. Clearance values were calculated as follows:

$$\text{Cl} = \text{Dose/AUC.}$$

Compounds were placed into low, medium or high clearance based on liver plasma flow considerations. High clearance, >15 ml/min/kg, was defined as greater than 50% of liver flow. Medium and low clearance were then assigned ranges of 5–15 ml/min/kg or <5 ml/min/kg, respectively. Low, medium and high oral bioavailability categories were assigned as 0–33%, 33–67%, or 67–100%.

RESULTS

Blood Withdrawal Procedure

In order to not deplete the animal's blood supply too drastically (<30% of total blood volume), the maximum allowable withdrawal rate that could be used over a 6-hr period is *ca.* 1 ml/hr, so an experimental design that allowed for this was necessary. Early experiments in rats failed at this withdrawal rate due to coagulation in the catheters that could not be prevented by simple heparin treatment of the catheters (13).

The same experiment was successful when rats received a continuous infusion of heparin/saline solution (25 U/ml) during the 6-hr blood withdrawal period. The infusion also helped to replace fluids. Although this infusion protocol will lead to some dilution of blood and decrease in hematocrit, the changes should be nearly equivalent to those seen after intermittent sampling with heparin/saline flushes after each blood sample. The most consistent results were obtained when blood withdrawal was *via* the jugular vein and the cannula I.D. was as large as possible (I.D. > 0.030"). Also, it was important that the blood never flowed through any points of constriction (*e.g.* a metal needle) between the animal and the syringe barrel.

Compound Stability

Because the blood samples were collected over a 6-hr period, there was a concern that there may be compound degradation in the collection syringe. To investigate this, the compounds were incubated in whole blood for 6 hr and the results are shown in Table II. At concentrations similar to those observed *in vivo*, there was no significant breakdown of any of the compounds. However, there was a large time-dependent loss of atenolol if the compound was sitting in blood for long periods of time before plasma was prepared. This loss, presumed to be slow uptake of the drug by the cellular components of blood, reached 50% after several hours, the time frame in which the experiments were heing run. The compound could be completely recovered if the assay was run after direct solvent extraction of whole blood, so the atenolol assay was modified accordingly.

Determination of Pharmacokinetic Parameters

The 0–6 hr ia and oral AUC values were determined by intermittent sampling (integration by trapezoidal rule) or by continuous withdrawal. Compounds were chosen for investigation that had a wide range of oral bioavailability, plasma protein binding and Tmax values (Table I). The plasma vs. time profiles after intermittent sampling and the integrated concentration found after continuous withdrawal for theophylline and atenolol are shown in Figure 1. The results for AUC values, systemic clearance and oral bioavailability for all the compounds are shown in Table III. There is generally a reasonably good agreement between the two methods. In all cases, the values determined by the two methods placed the clearance or oral bioavailability value of each compound in the same category of low, medium or high (categories defined in Methods section).

Table II. Stability of Compounds in Blood at Room Temperature

Compound	Concentration in blood (μM)	% of drug recovered after 6 hr[a]
Theophylline	50	98
Atenolol	2	110[b]
Propranolol	0.7	88
Warfarin	50	94
BMS-182874	30	110
BMS-A	10	105

[a] Average of duplicate determinations.
[b] Solvent extraction done directly from whole blood, see Experimental section.

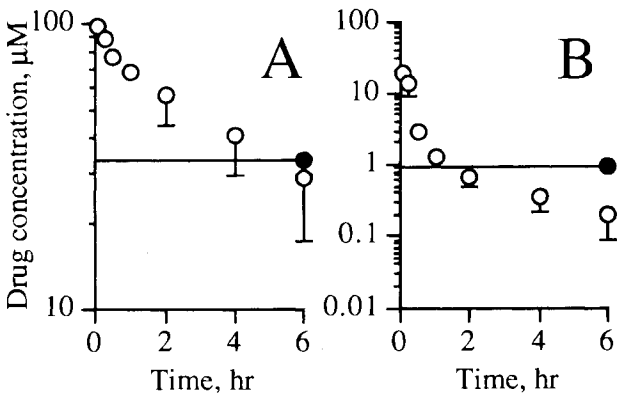

Fig. 1. Drug concentration vs. time plots for theophylline (A) and atenolol (B) with the intermittent sampling method (○) and the integrated concentration determined by continuous withdrawal (●).

The method was also tested in conjunction with multiple compound administration, and these results are shown in Table IV. The excellent agreement between the single and multiple compound administration show that there are no inherent problems with combining this method and continuous withdrawal.

DISCUSSION

Although the method of continuous withdrawal has been previously applied to dogs and humans, there are no reports of the application of the method in smaller animals. For drug screening as part of drug discovery efforts, large animal experiments may not feasible because of compound supply issues and the numbers of compounds that need to be evaluated. Rats are most often the animal model used for the initial screening of *in vivo* pharmacokinetics, and there has been a great deal of recent interest in high throughput *in vivo* assays using multiple compound administration (14–15). A screening method using continuous withdrawal in rats would also provide significant enhancements in productivity and could provide an accurate, rapid initial determination of clearance and oral bioavailabilty.

The previous dog and human studies employed withdrawal rates of 1–5 ml/hr and were done for up to 24 hr. These studies showed that this method provided accurate AUC values (2–4). Indeed, it can be argued that the continuous withdrawal method provides superior accuracy relative to intermittent sampling because there is no numerical approximation involved in the integration of discreet plasma concentration-time points for calculation of AUC values. Also, after oral dosing the Cmax is always sampled with continuous withdrawal (as long as it is within the experimental time frame), whereas it can easily be missed when intermittent sampling is used. Because this method will ultimately be applied to compounds with unknown absorption characteristics, a protocol for blood collection that allowed coverage of a large portion of the AUC after oral administration for a wide variety of compounds was necessary. A withdrawal time of 6 hr was judged to be adequate based on past experience (a survey of 50 BMS-compounds revealed that 94% had a Tmax of <3 hr in rats, R. White, personal communication).

For screening purposes, rigorous pharmacokinetic characterization of compounds is not necessary, but information that groups compounds into low, medium or high clearance and oral

Table III. Comparison of AUC, Clearance and Oral Bioavailability Values After IA or Oral Administration with Either Intermittent Sampling or Continuous Withdrawal

	IA AUC ± SD (μM × hr)	Clearance (ml/min/kg) (category)[a]	Oral AUC ± SD (μM × hr)	Oral bioavailability (category)[a]
Theophylline				
intermittent	315 ± 46	1.2 (L)	325 ± 24	103 (H)
continuous	202 ± 37	1.6 (L)	237 ± 26	117 (H)
Atenolol				
intermittent	10.5 ± 0.3	18 (H)	4.2 ± 1.3	40 (M)
continuous	5.4 ± 1.4	35 (H)	1.8 ± 0.1	33 (M)
Propranolol				
intermittent[b]	4.0 ± 0.3	43 (H)	0.44 ± 0.06	11 (L)
continuous	4.4 ± 1.0	39 (H)	0.5 ± 0.3	11 (L)
Warfarin				
intermittent	140 ± 38	0.39 (L)	138 ± 41	99 (H)
continuous	96 ± 26	0.56 (L)	93 ± 16	97 (H)
BMS-182874				
intermittent	150 ± 5	11 (M)	159 ± 9	100 (H)
continuous	182 ± 6	9.2 (M)	179 ± 14	98 (H)
BMS-A				
intermittent	6.8 ± 1.1	130 (H)	0.54 ± 0.06	8 (L)
continuous	7.2 ± 0.6	120 (H)	1.5 ± 0.2	21 (L)

[a] L = low, M = medium, H = high, categories defined in Methods section.
[b] Data from (6).

bioavailability is sufficient. If the clearance or oral bioavailability of the compound is categorized as either low, medium or high, in all cases the two methods place the compound in the same category.

There was concern about the effect that continuous infusion of heparin would have on drug pharmacokinetics, and this is why compounds with a range of plasma protein binding levels were included. At least for these test compounds, there appears to be no noticeable affect on the clearance of highly bound drugs. Also of interest was the effect a prolonged Tmax might have on the determination of oral bioavailability in a 6-hr experiment. Both atenolol and warfarin have Tmax values around 3 hr and both gave very comparable results by the two methods.

There were some differences noted between the results from the two methods, the largest discrepancy was with atenolol, where the ia and oral AUC values after the continuous withdrawal experiment were *ca.* 50% less. This difference could be due to biological variation or to a real difference inherent in the two techniques. Several possible differences were investigated, including: 1) a poor recovery of drug after the continuous withdrawal procedure or 2) overestimation of the AUC found in the intermittent sampling protocol. Although there were problems encountered with the extraction efficiency of atenolol from plasma, the whole blood extraction procedure did not show a time-dependent loss so this is not likely to explain the discrepancy. The AUC found after intermittent sampling could be an overestimate because the plasma vs. time profile is quite curvilinear and trapezoidal integration may lead to extra area. However, when the AUC was calculated from a fitted biexponential curve the value was only 14% less than by trapezoidal integration. Also, this problem would only be expected to affect the ia AUC and the results show that the oral is also lower. Thus, the lower than expected AUC values for atenolol are currently unclear but do not appear to be related to any inherent problems in the experimental technique.

The disadvantages of the continuous withdrawal method are: 1) the loss of information regarding the elimination half-

Table IV. Comparison of AUC Values Found with the Continuous Withdrawal Method After IA or Oral Administration of Multiple Compounds

Compound	Single or multiple compound administered	IA AUC ± SD (μM × hr)	Oral AUC ± SD (μM × hr)	Oral bioavailability (%)
Theophylline	single	202 ± 26	237 ± 37	117
	multiple	258 ± 88	209 ± 108	81
Atenolol	single	5.4 ± 1.4	1.8 ± 0.1	33
	multiple	6.5 ± 0.6	2.8 ± 0.9	43
Propranolol	single	4.4 ± 1.0	0.5 ± 0.3	11
	multiple	4.0 ± 0.5	0.8 ± 0.2	20

life and volume of distribution of a compound, 2) the loss of data regarding absorption after oral administration (Cmax, Tmax) and 3) the present method requires the compound to be stable in blood for the duration of the experiment. The first two concerns may not be very important in the early screening mode where information regarding elimination half-life, volume of distribution, and Tmax is often not critical. Crude information on the elimination half-life and Tmax could be provided by the analysis of a single discreet plasma sample at the end of the continuous withdrawal period. The last concern is usually not a problem, but when compounds with moieties susceptible to plasma enzyme (*e.g.* esterases/amidases) degradation are being tested it could be alleviated by cooling the syringe pump or using a peristaltic pump with a cooled collection container (alternatively, a protease inhibitor could be added).

The major advantage is the large decrease in time spent during sample collection, processing and analysis. Once the continuous withdrawal experiment is set up, it runs unattended and alleviates the time consuming process of intermittent sample collection. Modern LC-MS and LC-MS/MS analysis generally require minimal sample processing and the analysis times are rapid, but with large numbers of samples even these rapid methods can become rate limiting. In these experiments, there were eight blood samples taken in the serial sampling experiment. So, for a single compound with N = 3 and two dose routes there were analyses done on 48 blood samples compared to analyses of 6 blood samples for the continuous withdrawal experiment. When continuous withdrawal and multiple compound administration are used simultaneously, then there are even larger decreases in sample analysis. Another advantage of the method is that it raises the limit of detection necessary to accurately determine a plasma AUC. The integrated concentration is relatively high compared to the concentrations encountered in the elimination phase of intermittent sampling studies (Figure 1), especially for compounds that show multicompartmental kinetics.

In summary, the continuous withdrawal method in rats reliably placed all compounds into the same category of low, medium or high clearance or oral bioavailability as the classic intermittent sampling method. The method should prove useful when a rapid screen of large numbers of compounds for systemic clearance characteristics or oral bioavailability is necessary.

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