In Vivo Microdialysis Sampling for Pharmacokinetic Investigations

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In vivo microdialysis sampling coupled to liquid chromatography was used to study acetaminophen disposition in anesthetized rats. The pharmacokinetics of acetaminophen and its sulfate and glucuronide metabolites were determined using both microdialysis sampling and collection of whole blood. For microdialysis, samples were continuously collected for over 5 hr without fluid loss using a single experimental animal. Microdialysis sampling directly assesses the free drug concentration in blood. The pharmacokinetic results obtained with microdialysis sampling were the same as those obtained from blood collection. The administration of heparin, necessary when collecting blood samples, was found to double the elimination half-life of acetaminophen. Microdialysis sampling is a powerful tool for pharmacokinetic studies, providing accurate and precise pharmacokinetic data.

KEY WORDS: microdialysis sampling; *in vivo* analysis; pharmacokinetics; free drug concentration; acetaminophen.

INTRODUCTION

This report describes the application of *in vivo* microdialysis perfusion to the determination of the pharmacokinetics of acetaminophen (APAP) in anesthetized rats. The dialysis samples were analyzed by liquid chromatography with UV absorbance detection. This allowed the detection of APAP as well as its major metabolites, the sulfate and glucuronide conjugates. Because no fluid is removed from the animal, continuous sampling without disturbing the pharmacokinetics is possible. The protein-free samples are amenable to direct injection into the chromatographic system greatly simplifying the analytical method. Finally, only the free drug concentration is sampled by microdialysis, resulting in more meaningful pharmacokinetic parameters. Well-defined pharmacokinetic curves could be constructed using a single experimental animal, while experiments on multiple animals were highly reproducible.

MATERIALS AND METHODS

Chemicals

Acetaminophen, β-glucuronidase (type B-1 from bovine liver), and sulfatase (type H-1 from *Helix pomatia*) were purchased from Sigma Chemical Company (St. Louis, MO). Acetaminophen-4-*O*-sulfate was prepared by the procedure of Feigenbaum and Neuberg (7). Acetaminophen-4-*O*-glucuronide was isolated from human urine. HPLC-grade acetonitrile was obtained from Fisher Scientific (Fair Lawn, NJ, USA). All other chemicals were reagent grade or better and were used as received.

Apparatus

Microdialysis sampling was performed using a CMA/100 microinjection pump from Bioanalytical Systems, Inc./ Carnegie Medicin (West Lafayette, IN) coupled to a microdialysis probe which was inserted into the jugular vein of the experimental animal. The perfusion medium was pumped through the probes at a flow rate of 5 μl/min for all experiments. "Cannula"-type dialysis probes with 4 mm of dialysis fiber from BAS/Carnegie Medicin were used. For intravenous insertion of the microdialysis probe a probe guide from BAS/Carnegie Medicin was employed.

The liquid chromatographic system consisted of an LC-6A pump, an SPD-6AV variable-wavelength UV-Vis absorbance detector (Shimadzu Scientific Instruments, Inc., Columbia, MD), and a CMA/200 autosampler (BAS/Carnegie Medicin) with an injection volume of 10 μl. Separation was achieved using two coupled Brownlee 5-μm ODS (2.1 mm × 10-cm) columns and a flow rate of 0.5 ml/min. For all experiments the UV detector was operated at 250 nm.

Microdialysis Probe Characterization

In order to determine the *in vivo* concentration of APAP giving rise to the concentration detected in the perfusion medium, it is necessary to know the recovery of the dialysis probe. The recoveries were determined from both spiked whole blood samples and Ringer's solution. The free drug concentration in the blood samples was determined by ultrafiltration. There was no difference in the recoveries from the two matrices. The average recoveries of the analytes for all probes used in these experiments were as follows: APAP, 11.8 ± 1.7%; APAP-sulfate, 12.0 ± 1.0%; and APAP-glucuronide, 4.56 ± 0.86%. The recovery of each dialysis probe was determined both before and after implantation. The recovery found after the implantation was used to calculate *in vivo* concentrations, although the two recoveries never differed by more than 5% relative (i.e., ±0.6% for APAP and APAP-sulfate and ±0.2% for APAP-glucuronide).

Protein Binding

The extent of binding of APAP to rat blood proteins was determined by both ultrafiltration and microdialysis. Ultrafiltration was performed with an MPS-1 micropartition system with a YMT-membrane filter (Amicon, Lexington, MA). Samples of both heparinized and unheparinized rat blood were spiked with a known concentration of APAP and equilibrated for 1 hr. Each sample was divided into two aliquots. One aliquot was analyzed by ultrafiltration and the other aliquot by microdialysis to determine the concentration of free APAP in the blood.

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In Vivo Pharmacokinetic Experiments

Four- to five-month-old Sprague-Dawley rats weighing approximately 400 g were used. Rats were anesthetized with the inhalation anesthetic isoflurane. The choice of anesthetic is critical for metabolism experiments, as most anesthetics are extensively metabolized and will interfere with the metabolism of other compounds. Isoflurane is reported to be exhaled 95% unchanged, with less than 0.17% being metabolized, and does not effect renal or hepatic function (9,10). The rat's respiration was closely monitored during the entire experiment and maintained at a rate of approximately 50 min^{-1}. The animal's body temperature was maintained with a heating pad beneath its body and a heat lamp above.

To validate the microdialysis sampling technique, whole-blood samples were simultaneously collected. This was done by cannulation of the femoral vein. In order to prevent clotting of blood in the cannula, the rat was pretreated with heparin. A 100-µl sample was collected every 30 min. The whole-blood sample was treated with 100 µl of acetonitrile to precipitate proteins and centrifuged for 10 min. The supernatant was injected into the chromatographic system.

Pharmacokinetic experiments were performed by perfusing the implanted probe with a Ringer's solution consisting of 155 m*M* NaCl, 5.5 m*M* KCl, and 2.3 m*M* $CaCl_2$. A perfusion rate of 5 µl/min was used with samples collected for 10-min intervals. Dialysis samples were diluted with Ringer's solution as needed to keep the concentration in the range of calibration. Blanks were collected for at least 1 hr following insertion of the microdialysis probes. No chromatographic interferences were observed in the blanks. The animal was then dosed with APAP (10–100 mg/kg) in 0.5 ml of Ringer's solution i.p. at 37°C. Dialysis samples were collected until less than 1% of the maximum concentration of APAP remained in the blood. This was typically 6 to 7 hr after dosing. The *in vivo* concentrations of APAP and its metabolites were calculated by determining their concentrations in the dialysate from a standard curve and then accounting for the recovery of the microdialysis probe.

Microdialysis is a continuous sampling technique, therefore each sample represents the average concentration of analyte in the blood during the sampling interval. This is compared to taking discrete blood samples, which represent the concentration in the blood only at the time of sampling. Because of the continuous sampling, microdialysis is an integrating technique which is less prone to fluctuations than an instantaneous sampling technique. Pharmacokinetic parameter calculations are also simplified. For example, area-under-the-curve (AUC) calculations are performed by summing the product of the concentration (µg/ml), perfusion rate (ml/min), and sample interval (min) for all samples instead of interpolating between points with the trapezoidal rule.

RESULTS

Intravenous Microdialysis Sampling

Typical chromatograms of blood dialysate obtained by *in vivo* sampling are shown in Fig. 1. *In vivo* dialysis samples were either directly injected into the chromatograph or injected after appropriate dilution. As can be seen (Fig. 1), no interferences occur in the blanks obtained prior to dosing of the animal with APAP. The identities of the glucuronide and sulfate conjugates of APAP were confirmed both by coelution with authentic compounds and by enzymatic hydrolysis. Enzyme hydrolysis was carried out by previously described procedures (12). The disappearance of the metabolite and increase in APAP upon incubation with the appropriate enzyme were taken as confirmation of identity (Fig. 1). As β-glucuronidase contains sulfatase activity, both metabolites disappear in this incubation. The glucuronide and sulfate conjugates are the two major metabolites previously found for APAP (11,12).

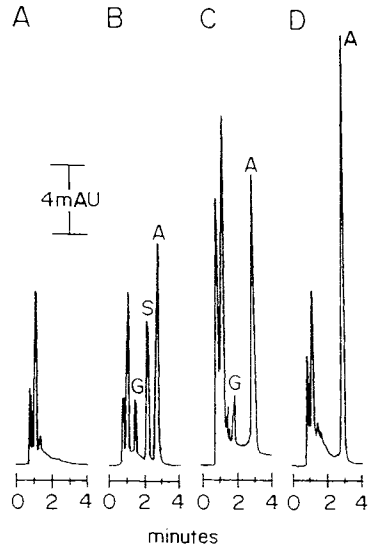

Fig. 1. Chromatograms of *in vivo* blood dialysate using UV detection. (A) Blank (prior to dosing with APAP); (B) 1 hr after a 100-mg/kg i.p. dose of APAP; (C) after incubation with sulfatase; (D) after incubation with β-glucuronidase. Peak identities; A, APAP; G, glucuronide conjugate; S, sulfate conjugate. Chromatographic conditions are given in the text.

APAP rapidly distributes in the blood, reaching a maximum concentration in approximately 60 min. Its concentration then slowly decreases over several hours. The pharmacokinetics of this process can be described by an open single-compartment model with first-order absorption according to Eq. (1).

$$C = A_1 e^{-\alpha t} - A_2 e^{-k_a t} \quad (1)$$

The half-lives of absorption and elimination were calculated from the slope of the semilog presentation of the concentration-versus-time curve by curve stripping using a biexponential fit. The half-life of absorption was found to be 31.3 ± 3.4 min and the half-life of elimination was found to be 36.0 ± 2.0 min ($n = 4$). The two metabolites appear after the APAP and mirror its rise in concentration. The pharmacokinetics of both metabolites exhibit plateau regions followed by elimination. The pharmacokinetic parameters calculated for APAP and its metabolites are listed in Table I.

Comparison to Whole-Blood Samples

Microdialysis sampling was validated by simultaneously

Table I. Pharmacokinetic Parameters for APAP Administered i.p. to Anesthetized Rats

Dose (mg/kg)[a]	$t_{1/2}$(abs.)[b] (min)	$t_{1/2}$(elim.)[b] (min)	AUC_{APAP} (mg/L · min)	AUC_{gluc} (mg/L · min)	AUC_{sulf} (mg/L · min)
No heparin					
100	31.3 ± 3.4	36.0 ± 2.0	3790 ± 550	1890 ± 560	2490 ± 220
50	20.8 ± 5.3	28.3 ± 3.1	1280 ± 400	830 ± 98	1090 ± 100
10	14.2 ± 4.2	19.5 ± 2.3	168 ± 9	103 ± 10	330 ± 48
With heparin					
100	17.8 ± 4.4	57.5 ± 11.4	3210 ± 850	3320 ± 940	3430 ± 700

[a] $n = 3$.
[b] APAP.

collecting blood samples through a catheter in the femoral vein. Pharmacokinetic curves obtained by the two methods were identical (Fig. 2). However, the half-life of elimination determined when both microdialysis and collection of blood were used was almost twice as long as when using microdialysis alone. For these simultaneous experiments the half-life of elimination of APAP determined from collected blood samples was 58.5 ± 7.9 min ($n = 3$) and that determined from microdialysis samples was 57.5 ± 11.4 min ($n = 3$). The major difference in the experimental protocol was the need to heparinize the animal to prevent blood clotting in the catheter. To determine if the heparin was responsible for the longer half-life of APAP, microdialysis alone was performed on a heparinized animal. The longer half-life was again found, suggesting that heparin greatly affects the observed half-life for APAP. The metabolite profiles were also considerably different when the rat was heparinized. While the peak concentrations of APAP was unchanged, the peak concentration of APAP-sulfate doubled and that of APAP-glucuronide nearly tripled in the presence of heparin (Table II).

While the half-life of elimination was the same for collection of blood samples and microdialysis sampling, the blood concentration determined at any given time point was different. The concentration determined by microdialysis was always lower than that determined in the collected blood sample by 14.8 ± 2.9%. This difference reflects the extent of binding of APAP to blood proteins. The concentration determined by microdialysis is the free APAP concentration, while that determined in the collected blood sample is the total concentration.

This assumption was verified by determining the extent of binding of APAP to blood proteins *in vitro* by ultrafiltration and microdialysis at concentrations bracketing those determined *in vivo* during the pharmacokinetic experiments. The extent of binding was found to be 16.4 ± 1.8% ($n = 5$) by ultrafiltration and 18.7 ± 1.3% ($n = 5$) by microdialysis whether the sample was treated with heparin or not and independent of APAP concentration. This corresponds well with the *in vivo* data.

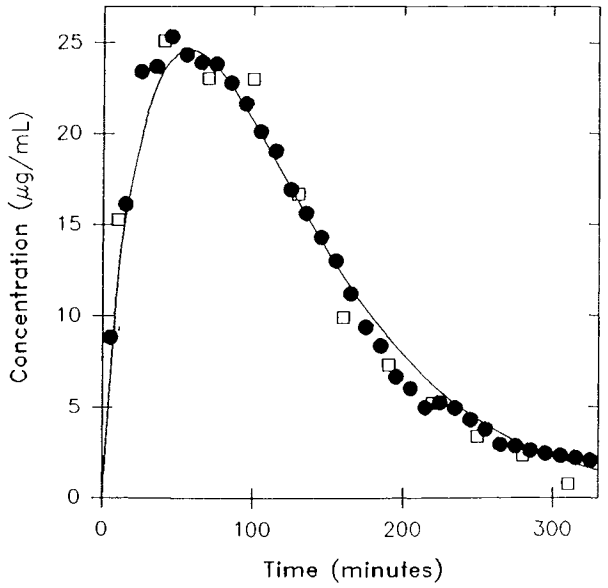

Fig. 2. Pharmacokinetics of APAP determined using microdialysis and blood sampling. (●——●) Microdialysis; (□——□) whole-blood sample.

Dose Dependence of Elimination Kinetics

The pharmacokinetics of APAP were studied as a function of the dose administered (Fig. 3). As can be seen, the half-lives of both absorption and elimination decreased as the dose decreased. Microdialysis sampling was capable of following the blood concentration of APAP and its metabolites over a wide range of doses, which resulted in varying pharmacokinetic parameters. For all the doses studied the derived parameters fit the experimental data well.

DISCUSSION

Microdialysis offers several advantages for pharmacokinetic studies. The temporal resolution is much higher than for other methods. While 10-min intervals were used for these experiments, shorter times are easily achieved if needed. Since no blood is drawn, a large number of samples can be collected from a single animal without loss of fluid volume. Simultaneous sampling can be achieved using multiple dialysis probes. This provides the ability to monitor pharmacokinetics at multiple sites in a single animal. Because complete pharmacokinetic curves can be obtained for several organs using a single experimental animal, overall fewer animals will be necessary to obtain data on a given drug. Microdialysis samples only the free fraction of the drug in blood. In addition, microdialysis coupled to collection of whole-blood samples provides a technique to determine the

Table II. Peak Concentration and Time for APAP and Metabolites Following i.p. Injection in Anesthetized Rats

	APAP		APAP-glucuronide		APAP-sulfate	
Dose[a] (mg/kg)	C_p (mg/L)	T_p (min)	C_p (mg/L)	T_p (min)	C_p (mg/L)	T_p (min)
No heparin						
100	27.9 ± 1.7	55.0 ± 5.7	9.7 ± 2.0	76.0 ± 5.7	13.1 ± 3.5	55.0 ± 7.3
50	14.1 ± 2.9	43.3 ± 5.7	5.8 ± 0.2	46.6 ± 5.7	9.5 ± 0.2	45.0 ± 2.3
10	3.0 ± 0.3	21.6 ± 2.8	1.7 ± 0.3	45.0 ± 7.0	4.1 ± 1.7	42.0 ± 4.2
With heparin						
100	33.1 ± 2.7	28.3 ± 2.8	23.6 ± 2.3	56.6 ± 5.7	24.1 ± 2.4	53.3 ± 5.7

[a] $n = 3$.

degree of drug binding *in vivo* during a pharmacokinetic experiment. The major limitation of microdialysis sampling is the small sample volumes obtained. The sample volume, perfusion rate, recovery, and sampling interval are all interrelated. Slow perfusion rates provide higher recoveries but smaller samples per unit time. Therefore the detection method must be sufficient to determine directly the lowest drug concentration necessary.

The use of anesthetized animals in these experiments is not a requirement for microdialysis sampling. Further, the use of heparin, necessitated by blood collection through a femoral vein catheter, was found to affect the observed pharmacokinetic parameters.

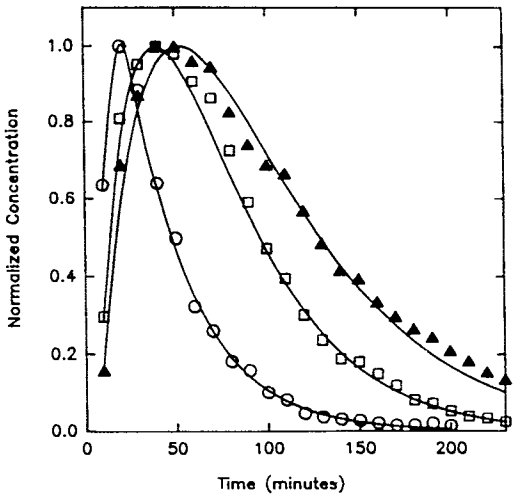

Fig. 3. Time course of blood [APAP] for various doses. The lines represent the fits to the data of the derived parameters using a single-compartment extravascular dosing model. (▲——▲) 100 mg/kg; (□——□) 50 mg/kg; (○——○) 10 mg/kg i.p.

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