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# *In vivo* microdialysis sampling coupled to liquid chromatography for the study of acetaminophen metabolism

DENNIS O. SCOTT, LORI R. SORENSEN and CRAIG E. LUNTE*
*Department of Chemistry, University of Kansas, Lawrence, KS 66045 (U.S.A.)*

## SUMMARY

*In vivo* microdialysis sampling coupled to liquid chromatography is a powerful tool for the study of drug metabolism. This technique is illustrated by investigating the pharmacokinetics of acetaminophen in the blood and liver of an anesthatized rat. The pharmacokinetics of the sulfate and glucuronide metabolites as well as the parent acetaminophen can be determined with high precision using microdialysis sampling. Microdialysis samples can be collected at a high rate from several sites without fluid loss with a single animal. Because the animal serves as its own control better data can be obtained. Liquid chromatography provides determination of multiple analytes per sample for metabolic profiling. This technique will provide more accurate and precise pharmacokinetic data while requiring fewer animals.

## INTRODUCTION

The pharmacokinetics of a drug are typically determined by administering a known dose and withdrawing blood samples at timed intervals[1]. These samples are then analyzed for the drug of interest. To study tissue levels of a drug, many animals must be dosed with the same concentration of the drug and several animals sacrificed at each desired time point. Tissue samples from each animal are analyzed for the drug and the data from all of the animals at each time point averaged to generate the pharmacokinetic curve. Using microdialysis sampling coupled to liquid chromatographic detection allows the entire pharmacokinetic curve to be obtained from a single animal at several anatomical sites. This results in data of higher precision and accuracy while using fewer animals.

Microdialysis sampling is accomplished by implanting a small probe in the tissue of interest. The probe consists of a short length of dialysis tubing through which the sampling solution is slowly perfused. Molecules of molecular weight below the cutoff of the dialysis tubing will diffuse into the probe because of the concentration differences between the tissue and the perfusion medium. The perfusion medium is collected and analyzed by liquid chromatography to determine what compounds were present in the tissue during the dialysis experiment. Because the cutoff is typically such that proteins are excluded, microdialysis samples can be injected directly into the

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chromatographic system. This technique has found great utility for the *in vivo* study of neurotransmitters[2–5].

This report describes the application of *in vivo* microdialysis perfusion to the determination of the pharmacokinetics of acetaminophen (APAP) in the blood and liver of an anesthetized rat. 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. Well defined pharmacokinetic curves could be constructed using a single animal while experiments on multiple animals were highly reproducible. Simultaneous sampling at different sites in the animal was accomplished by using multiple dialysis probes. The relative rates of clearance from various organs could therefore be determined.

## EXPERIMENTAL

### *Materials*

APAP, β-glucuronidase (type B-1 from bovine liver) and sulfatase (type H-1 from *Helix pomatia*) were purchased from Sigma (St. Louis, MO, U.S.A.). HPLC-grade acetonitrile was obtained from Fisher (Fair Lawn, NJ, U.S.A.). All other chemicals were reagent grade or better and were used as received.

### *Microdialysis apparatus*

Microdialysis sampling was performed using a CMA/100 microinjection pump from Bioanalytic Systems (BAS)/Carnegie Medicin (West Lafayette, IN, U.S.A.) coupled to the dialysis probe which was implanted in the animal (Fig. 1). The perfusion medium was pumped through the probes at a flow-rate of 5 μl/min for all experiments. This perfusion rate was chosen to provide both reasonable recoveries and sampling times. As the perfusion rate is increased, the recovery decreases as does the time needed to collect sufficient sample for chromatographic analysis. The perfusion rate used for any given experiment depends upon several factors including the type of chromatographic system (*i.e.* conventional or microbore) and sensitivity of the detector. For intravenous sampling a BAS/Carnegie Medicin dialysis probe of the "cannula" type was used (Fig. 1A). These probes have a 4-mm dialysis membrane made of polycarbonate ether with a molecular weight cutoff of 20 000. For intravenous insertion of the microdialysis probe a probe guide from BAS/Carnegie Medicin was used. For tissue sampling the probe was constructed in-house from dialysis tubing purchased from Spectrum Medical Industries (Los Angeles, CA, U.S.A.). The tubing was regenerated cellulose with an I.D. of 150 μm, a wall thickness of 9 μm and a molecular weight cutoff of 9000 daltons. The ends of a *ca.* 5 mm length of dialysis tubing were sealed to pieces of 26-gauge stainless steel with "hot melt" glue. "Hot melt" glue (Ridlen/AAI Adhesives, Dallas, TX, U.S.A.) is a wax-based adhesive and was found to be easy to use and provide leakproof seals. This probe design is illustrated in Fig. 1B.

Prior to use, the dialysis tubing was activated by pretreatment with sodium hydroxide. The tubing was primed with a solution of isopropyl alcohol and 20% (v/v) glycerol for 5 min. The tubing was then hydrolyzed for 2 min with 0.1 *M* methanolic sodium hydroxide. A neutralizing solution containing 1% (v/v) acetic acid and 20% (v/v) glycerol in isopropanol was next pumped through the probe for 5 min. The probe was finally perfused with distilled water for several hours to remove residual glycerol.

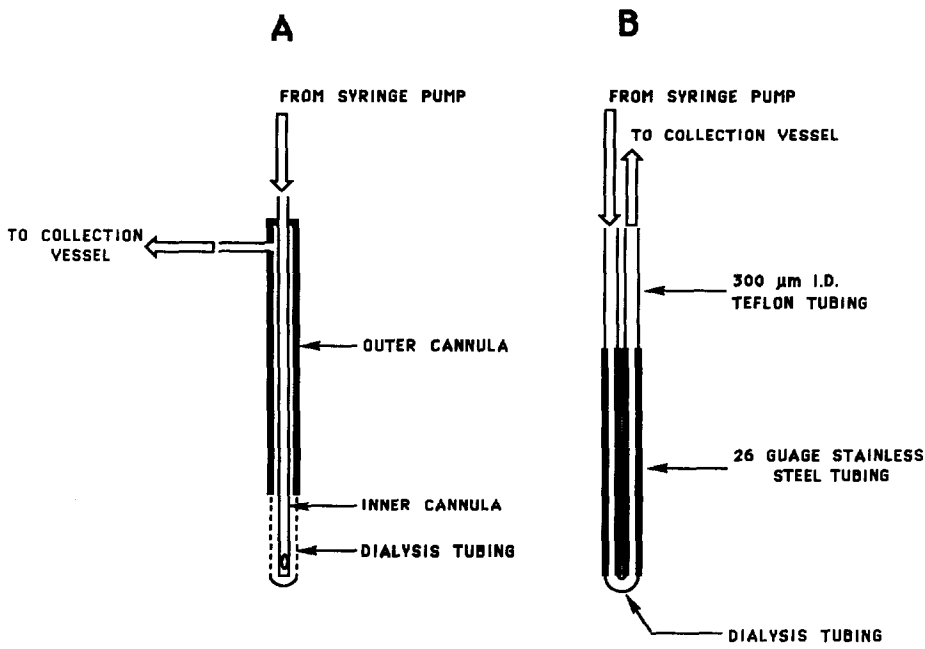


Fig. 1. Diagrams of (A) the "cannula"-type microdialysis probe and (B) the "loop"-type microdialysis probe. Dimensions are given in the text.

*Chromatographic system*

The liquid chromatographic system was a BAS LC-400 Chromatographic System equipped with a Shimadzu SPD-6AV variable-wavelength UV–VIS absorbance detector (Shimadzu, Columbia, MD, U.S.A). A Hypersyl ODS 5-μm (4.6 mm × 15 cm I.D.) column was used to achieve separation. The mobile phase was 0.05 *M* ammonium phosphate buffer, pH 2.7, with 10% (v/v) acetonitrile. A flow-rate of 1 ml/min and a 20-μl sample loop were used 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. This is typically expressed as the relative recovery, which is the ratio of the concentration in the perfusion medium to the concentration in the sample. Each probe must be calibrated individually because the recovery is different for each probe, even one of the same nominal length used at the same perfusion rate. Recovery was determined by spiking fresh rat whole blood with a known concentration of APAP. An aliquot of this spiked blood was centrifuged, filtered through a 22-μm filter and analyzed chromatographically. The rest of the spiked blood was used as a dialysis sample. The perfusion medium was a Ringer's saline solution as used for the *in vivo* experiments. Several dialysis samples were collected and analyzed chromatographically. APAP concentrations were selected to bracket the expected *in vivo* concentrations. 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 were never found to differ by more than 5%.

*Enzymatic hydrolysis*

For cleavage of conjugates by β-glucuronidase, 100 μl of enzyme solution, 1250 U/ml in 0.1 *M* sodium phosphate buffer, pH 4, was added to 100 μl of dialysate. Hydrolysis was carried out for 8 h at 40°C. Cleavage of sulfate conjugates was achieved by adding 100 μl of enzyme solution, 200 U/ml sulfatase in 0.1 *M* sodium acetate, pH 5, to 100 μl of dialysate. The sample was incubated for 5 min at 34°C. The two hydrolysis samples and an untreated dialysate were analyzed consecutively by liquid chromatography.

*In vivo experiments*

Four- to five-month old Spargue-Dawley rats weighing *ca* 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% unchangd with less then 0.17% being metabolized and does not effect renal or hepatic function[6,7]. The rat's respiration was closely monitored during the entire experiment and maintained at a rate of *ca.* 50 $min^{-1}$. The animal's body temperature was mantained with a heating pad beneath its body and a heat lamp above.

For intravenous sampling a cannula-type probe was inserted into the jugular vein. A small incision was made in the side of the neck. The pectoral muscle was gently lifted and an intravenous probe guide inserted through the pectoral muscle into the jugular vein. The muscle closed around the probe guide and held it securely in place. The dialysis probe was inserted into the vein through the probe guide. The incision was then packed with damp gauze. There is no bleeding from around the probe if the insertion is done properly.

For implantation into the liver a "loop"-type probe was used. A small incision was made in the peritoneal cavity to expose the liver. A small incision was made into the liver and the dialysis probe carefully inserted. The skin was closed securely around the probe and the incision was packed with damp gauze. The probe cannot be held rigidly by an external clamp or it will shift in the liver during the animals breathing. If done properly, this procedure should not cause much bleeding.

Metabolism experiments were performed by perfusing the implanted probes 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 being 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 h following insertion of the microdialysis probes. No interferences were observed in blanks from either blood or liver dialysates. The animal was then dosed intraperitoneally (i.p.) with APAP in Ringer's solution (100 mg/kg) at 37°C. Dialysis samples were collected until less than 1% of the maximum concentration of APAP remained in the blood or liver. This was typically 6–7 h after dosing. The *in vivo* APAP concentration was calculated by determining the APAP concentration in the dialysate

from a standard curve and then accounting for the recovery of the microdialysis probe. As no standards were available for either the sulfate or glucuronide conjugates, peak heights in mAU were used for these compounds. Pharmacokinetic parameters such as half-life of elimination could still be determined even without actual concentrations.

## RESULTS

### *Intravenous microdialysis sampling*

Typical chromatograms of blood dialysate obtained by *in vivo* sampling are shown in Fig. 2. *In vivo* dialysis samples were diluted ten-fold with Ringer's solution and directly injected into the chromatograph. As can be seen no interferences occur in the blank obtained prior to dosing of the animal with APAP (Fig. 2A). An advantage of microdialysis sampling is that the actual animal serves as its own control. Fig. 2B is a chromatogram of the dialysate after administration of APAP. The retention times of APAP, the sulfate and the glucuronide are 4.2, 3.2 and 2.6 min, respectively. The identities of the glucuronide and sulfate conjugates of APAP were confirmed by enzymatic hydrolysis. The chromatogram of a dialysate sample hydrolyzed with sulfatase shows that the peak tentatively identified as the sulfate conjugate is no longer present and also shows an increase in APAP (Fig. 2C). Following hydrolysis with β-glucuronidase both the peaks identified as the glucuronide and the sulfate conjugate

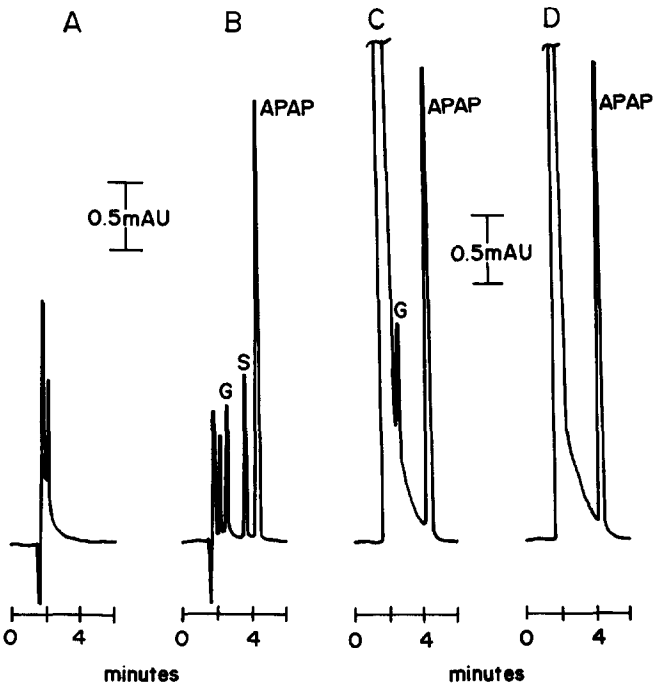


Fig. 2. Chromatograms of *in vivo* blood dialysate. (A) Blank (prior to dosing with APAP), (B) 1 h after a 100-mg/kg i.p. dose of APAP, (C) treated with sulfatase, (D) treated with β-glucuronidase. Peaks: APAP = acetaminophen; G = glucuronide conjugate; S = sulfate conjugate. Chromatographic conditions are given in the text.

are no longer present again with an increase in APAP (Fig. 2D). Commercial β-glucuronidase contains some sulfatase activity[8] so this result is not surprising. These results provide strong evidence for the identity of the two APAP metabolites detected even though no standards are available. The glucuronide and sulfate conjugates are the two major metabolites previously found for APAP[9,10].

A typical pharmacokinetic curve for elimination of APAP from blood following a 100-mg/kg i.p. dose is shown in Fig. 3. APAP rapidly distributes in the blood, reaching a maximum concentration in *ca.* 60 min. The concentration then slowly decreases over several hours. The pharmacokinetics of this process can be described by an open single-compartment model which exhibits first order kinetics. From the slope of the semi-log presentation of the elimination phase, the half-life of elimination was found to be 34.1 ± 1.0 min ($n=4$). The two metabolites appear 20 min after the APAP and mirror its rise in concentration. The pharmacokinetics of both metabolites exhibit plateau regions followed by elimination (Fig. 3). The elimination phase can again be modeled by an open single-compartment model. The half-life of elimination was determined to be 37.8 ± 5.6 min ($n=3$) for the glucuronide and 51.8 ± 14.5 minutes ($n=3$) for the sulfate conjugate.

*In vivo microdialysis in the liver*

Typical chromatograms of liver dialysate obtained by *in vivo* sampling are shown in Fig. 4. As with the blood dialysate, the liver dialysate could be directly injected into the liquid chromatograph. The chromatogram of the blank sample prior to dosing with APAP shows no interferences. The chromatogram of a sample obtained 1 h after a 100-mg/kg i.p. dose of APAP contains APAP as well as its sulfate and glucuronide conjugates. Identities of the conjugates were again confirmed by enzymatic hydrolysis.

The pharmacokinetics of elimination of APAP from both the liver and blood following a 100-mg/kg i.p. dose of APAP are shown in Fig. 5. These data were obtained simultaneously by implanting two probes, one in the liver the other intravenous, in the same animal. Uptake of APAP into the liver is clearly slower than in the blood with the peak concentration not reached until almost 2 h after administration. However the half-life of elimination was found be the same as from the blood, 35.1 min.

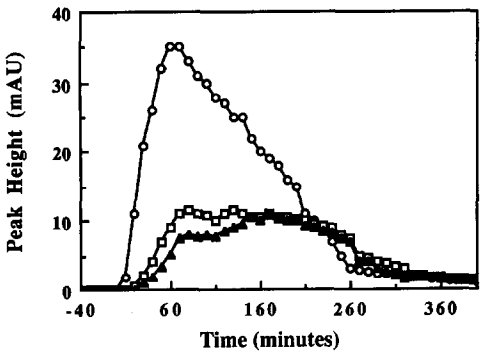


Fig. 3. Relative time course of blood concentration of APAP and its metabolites. Symbols: ○ = APAP; □ = sulfate conjugate; ▲ = glucuronide conjugate.

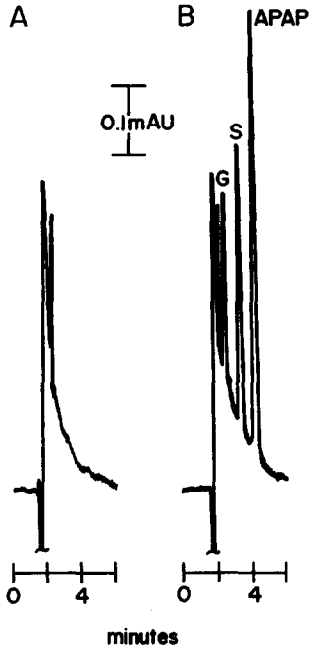


Fig. 4. Chromatograms of *in vivo* liver dialysate. (A) Blank (prior to dosing with APAP, (B) 1 h after a 100-mg/kg i.p. dose of APAP. Peaks as in Fig. 2.

The appearance of the APAP conjugates in the liver directly corresponds to the uptake of APAP (Fig. 6). The concentrations of both the glucuronide and the sulfate conjugate take longer to peak in the liver than in the blood, mirroring the behavior of APAP itself. As in the blood, a plateau region followed by elimination is observed. The half-life of elimination for both conjugates was the same as the elimination from blood, 38.4 min for the glucuronide and 52.6 min for the sulfate conjugate.

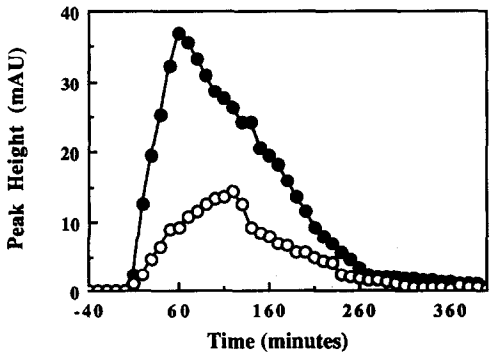


Fig. 5. Relative time course blood and liver APAP following a 100-mg/kg i.p. dose. Symbols: ● = blood concentration; ○ = liver concentration.

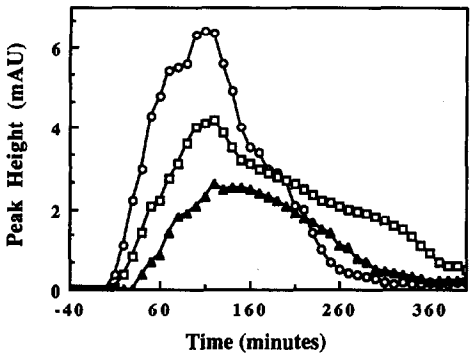


Fig. 6. Relative time course of liver concentration of APAP and its metabolites. Symbols as in Fig. 3.

## CONCLUSIONS

*In vivo* microdialysis sampling coupled to liquid chromatography is a powerful tool for the study of the pharmacokinetics of drugs. The technique is not limited to APAP but has general applicability. A strength of the technique is that the detection method is not directly coupled to the sampling method. Indeed, it is possible to use multiple detection methods on a single sample to optimize detection of several compounds for metabolic profiling. 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 or longer times are easily achieved. 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. Two probes were used for these experiments, one intravenous and one in the liver, but probes can be implanted at other sites such as the kidney, brain, or in the intestine with little difficulty. The major limitation on the number of probes that can be used and the temporal resolution that can be achieved is the ability to analyze the large number of samples generated. 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.

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## REFERENCES

1 M. Rowland and T. N. Tozer, *Clinical Pharmacokinetics: Concepts and Applications*, Lea & Febiger, Philadelphia, PA, 1989.
2 U. Ungerstedt, in C. A. Marsden (Editor), *Measurement of Neurotransmitter Release In Vivo*, Wiley-Interscience, Chichester, 1984, p. 81.
3 U. Ungerstedt, C. Forster, M. Herrera-Marschitz, I. Hoffman, U. Jungnelius, U. Tossman and T. Zetterstrom, *Neurosci. Lett. (Suppl.)*, 10 (1982) 493.

4 M. Sandberg and S. Lindstrom, *J. Neurosci. Methods*, 9 (1983) 65.
5 U. Ungerstedt and A. Hallstrom, *Life Sci.*, 41 (1987) 861.
6 *USP Drug Information*, United States Pharmacopeial Convention Inc., Rockville, MD, 9th, ed., 1989, p. 230.
7 *Drug Evaluation*, 6th ed., American Medical Association, Philadelphia, PA, 1988, p. 296.
8 G. F. Weirich, *Arch. Insect. Biochem. Physiol.*, 3 (1986) 109.
9 J. A. Hinson, L. R. Pohl, T. J. Monks and J. R. Gillette, *Life Sci.*, 29 (1981) 107.
10 M. Hamilton and P. T. Kissnger, *Anal. Biochem.*, 125 (1982) 143.