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Application of microdialysis to study the in vitro metabolism of drugs in liver microsomes

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Abstract

Current methods for studying in vitro drug metabolism involve add-incubate-separate-measure approach. Separation of the desired analytes requires removal of protein which is typically accomplished by precipitation and centrifugation and extraction of the analytes into an organic phase. The analysis scheme then becomes more complex resulting in a decrease in precision and an increase in assay time. Microdialysis sampling circumvents these problems by allowing researchers to sample the reaction mixture periodically and obtain the complete metabolic profile. In the present study, microdialysis sampling was used to investigate Phase I metabolism of salicylic acid, diazepam and ibuprofen in rat liver microsomes. The major metabolites of these drugs were profiled by LC. Michaelis-Menten enzyme kinetic parameters, K_m and V_{max} were obtained for the formation of diazepam metabolites by both microdialysis and conventional microsomal incubations and were in good agreement with the values reported in the literature. This study shows that microdialysis has considerable promise as a sampling technique for in vitro drug metabolism studies. By making minor modifications to the instruments, microdialysis can be applied to other in vitro systems such as isolated hepatocytes to study the Phase II metabolism or tissue slices to study drug distribution.

Keywords: Drug metabolism; Microdialysis; Cytochrome *P*450; Liver microsomes; Diazepam; Michaelis-Menten kinetics

1. Introduction

Pre-clinical drug metabolism studies using animal and human in vitro systems such as isolated enzymes, liver microsomes, isolated hepatocytes, and organ slices provide valuable information to the pharmaceutical industry on drug discovery and drug development [1]. Data obtained from such systems are being used for the interpretation of structure-activity relationships and prediction of pharmacokinetic properties in humans [2].

Use of the in vitro systems is not limited to the pharmaceutical industry. In vitro methods for measuring chemical absorption, metabolism, and evaluating organ toxicity are rapidly emerging as powerful tools in assessing the chemical safety of environmental chemicals. The Environmental

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Protection Agency's new guidelines will require regulators to incorporate information from pharmacokinetics and pharmacodynamics studies of potentially toxic environmental chemicals into risk assessments [3].

The majority of therapeutic drugs are metabolized by a group of enzymes known as cytochrome *P*450 [4]. These monooxygenases are mainly present in the liver microsomes and so, hepatic subcellular microsomal fraction containing cytochrome *P*450 enzymes is the most frequently used in vitro model system for drug metabolism studies [5]. In vitro microsomal incubations are very useful in metabolic profiling and mechanistic studies. One of the most useful applications of in vitro systems is the identification and study of toxic metabolites [6]. Advances in molecular biology have made it possible to identify the specific isoforms of cytochrome *P*450 responsible for the metabolism of a particular drug. In vitro studies using liver microsomes and known substrates for these specific isoforms of cytochrome *P*450 can provide information on drug-drug interactions [7].

Current techniques for studying microsomal metabolism rely on discontinuous sampling at specified time intervals. Discontinuous techniques require multiple samples, each sample representing a single time point to obtain enzyme kinetics data. In conventional microsomal studies, the parent drug is incubated with the liver microsomes containing cytochrome *P*450 enzymes. The reaction is usually started by adding the enzyme cofactor NADPH or an NADPH generating system. After a fixed time interval the reaction is terminated by adding a quenching agent or an inhibitor to stop the enzymatic process.

Samples collected by traditional means require extensive, time-consuming sample clean up procedures prior to the injection into the assay system for metabolic profiling. The enzymes present in these samples will continue the metabolic or degradation processes until they are removed. The majority of drug molecules are hydrophobic and require organic extraction before analytical detection. Since the metabolites are more hydrophilic than the parent drug, a fraction of the metabolites is lost during this extraction step. Removal of protein, typically accomplished by precipitation and centrifugation followed by the organic extraction, results in a more complicated analysis scheme with an inherent decrease in precision and increase in assay time. Because of the limitations associated with these conventional add-incubate-separate-measure techniques, more and more efforts are taken to develop add-incubate-measure techniques to eliminate the separation step.

Microdialysis sampling is a powerful new technique which offers several advantages for in vitro drug metabolism studies as well as for in vivo pharmacokinetic investigations. It allows continuous, real-time monitoring of metabolic processes. The basic principle of microdialysis is the diffusion of the analyte or drug through a semi-permeable dialysis membrane. Samples obtained using microdialysis do not require removal of proteins prior to analysis because proteins are excluded from the sample by the dialysis membrane. The dialysate samples can be directly injected into the chromatographic system. Since there is no net fluid change, microdialysis provides a way to sample the reaction mixture periodically and obtain the complete metabolic profile without terminating the reaction after a fixed time.

Microdialysis is accomplished by means of a dialysis probe. The probe is placed in the animal tissue, or in the vessel containing the in vitro system, and continuously perfused with a solution isotonic to the body fluid or medium. Small molecular weight compounds are diffused and swept away in the perfusing solution. In order to determine the concentration of the analyte in the sample from the concentration determined in the dialysates it is necessary to know the relative recovery. The recovery is a function of the membrane material, membrane length, perfusion flow rate, analyte, and sample matrix. The relative recovery of the analyte is defined as the ratio of concentration of the analyte in the dialysate to the concentration of the analyte in the vial.

A substantial effort is currently being directed towards eliminating the separation step from the conventional add-incubate-separate-measure approach to in vitro metabolism studies. In this article, we demonstrate the utility of microdialysis as a combined sampling/separation/clean up step. Metabolic profiles were obtained for ibuprofen,

diazepam, and salicylic acid using the microdialysis technique. Michaelis-Menten enzyme kinetics were obtained for both ibuprofen and diazepam to demonstrate the applicability of microdialysis in obtaining quantitative data. Diazepam was chosen to compare the microdialysis data to the conventionally obtained data because of its short assay time and ease of chromatographic separation. This approach is not limited to the microsomal systems. Using isolated hepatocytes or tissue slices, microdialysis can be used to study the Phase II pathways where the conjugation of Phase I metabolites takes place.

2. Experimental

2.1. Materials

Diazepam and the metabolites: oxazepam, temazepam and *N*-desmethyldiazepam, salicylic acid and its metabolites: 2,3- and 2,5-dihydroxybenzoic acid, ibuprofen, NADPH, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, and NADP were all purchased from Sigma (St. Louis, MO). The ibuprofen metabolites, hydroxyibuprofen and carboxyibuprofen were generously donated by Pharmacia-Upjohn (Kalamazoo, MI). All other reagents were of analytical grade or better and used as received. All the solvents were of HPLC grade. Rat liver microsomes, obtained from In Vitro Technologies. (Baltimore, MD) were stored at -80°C until use.

2.2. Equipment

HPLC was performed using a BAS 200-B integrated chromatographic system (Bioanalytical Systems, West Lafayette, IN) containing UV-VIS and electrochemical detectors. Reversed-phase C_8 or C_{18} columns of 3.1×100 mm (BAS) were used for chromatographic separations. Samples were injected either on-line using a CMA 160 injection valve (Bioanalytical Systems) or off-line using a CMA 240 autoinjector combined with a CMA 210 microsampler. The sample loop size was 5 μl in both on-line and off-line methods. The chromatographic data acquisition and analysis were accomplished by BAS ChromGraph® software. The pharmacokinetic and metabolic profiles were obtained using BAS ChromGraph PKA® software which retrieves the peak concentration-time data from the BAS ChromGraph®. This software uses curve fitting functions to obtain pharmacokinetic parameters such as Area-Under-the-Curve (AUC) and clearance time.

2.3. Chromatography

Diazepam and its metabolites were separated isocratically on a C_{18} column using 55% methanol as mobile phase and detected at 254 nm. A mobile phase of 75 mM monochloroacetic acid (pH 2.3) was used on a C_{18} column to separate salicylic acid and its metabolites, 2,3- and 2,5-dihydroxybenzoic acid. Salicylic acid was detected by UV at 254 nm because it is present in excess in the incubation mixture. The metabolites were detected electrochemically on glassy carbon electrodes at 650 mV.

Ibuprofen and its metabolites were assayed by LC using a gradient elution method on a C_{18} reversed-phase column with UV detection at 220 nm. Mobile phase A was prepared by adding 1 ml of concentrated phosphoric acid to 900 ml of water. After adjusting the pH to 2.8 and filtering this solution, 100 ml of acetonitrile was added with thorough mixing. Mobile phase B contained 1 ml of concentrated phosphoric acid in 600 ml of water. The pH was adjusted to 2.8 and 400 ml of acetonitrile was added. Mobile phases were degassed with helium. The column was initially equilibrated with 65/35 mixture of eluant A and eluant B. After sample injection (5 min) the composition was changed linearly to 100% B over a 2-min period and maintained at 100% B for another 6 min. At the end of the separation the composition was changed abruptly to 65/35 mixture of A and B and re-equilibrated for 10 min before the next sample injection.

Stock solutions of diazepam and ibuprofen (1 mg ml^{-1}) were prepared in methanol and later diluted to desired concentrations in 0.05 M phosphate buffer (pH 7.4). Salicylic acid and metabolite standards were prepared in the same phosphate buffer. All stock solutions were stored

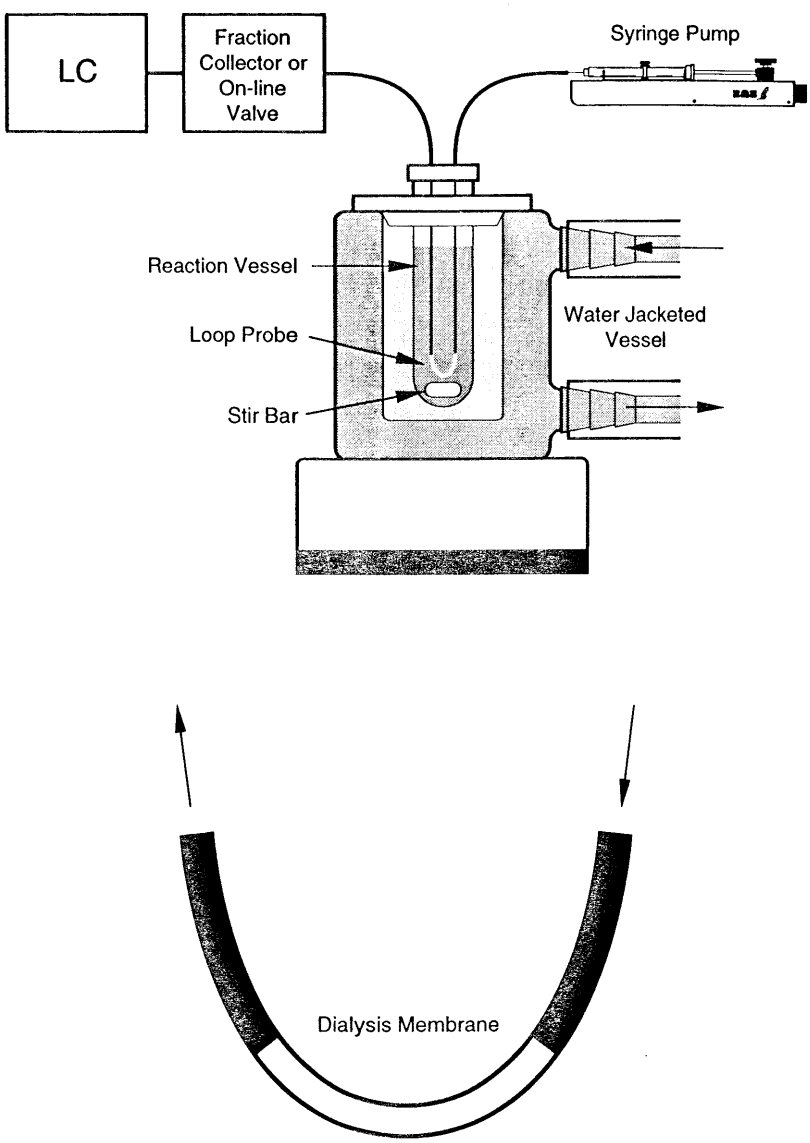

Fig. 1. Illustration of the microdialysis system for in vitro microsomal incubations.

in a freezer. Standards for in vitro recovery studies and calibrations were prepared daily from these stock solutions.

2.4. Microdialysis

Microdialysis was accomplished using loop probes with a 1-cm long polyacrylonitrile (PAN) membrane, shown in Fig. 1, (BAS) and a CMA 100 syringe pump and CMA 140 fraction collector. The in vitro recoveries of the probe for a particular drug and its metabolites were determined prior to actual metabolism studies. Each probe was soaked in distilled deionized water for at least 15 min prior to flowing any solution. The probes were then flushed with distilled deionized

water for 1 h followed by Ringer's solution or buffer for another 30 min. The probes were immersed in a standard solution containing the drug and metabolites and perfused with 0.05 M potassium phosphate buffer at pH 7.4 for 2 h at a flow rate of 2 μl min^{-1}. The samples were collected every 30 min for a 2 h period. They were either assayed, as described above, right after collection or stored frozen until assay. The relative recovery for each analyte was obtained from the equation: $\% \ R = 100 \times (C_{dialysate}/C_{sample})$ where $C_{dialysate}$ is the concentration of the dialysate and the C_{sample} is the concentration of the analyte in the vial. The probes were thoroughly washed with water to remove any adsorbed analyte before they were used in metabolism studies.

2.5. Metabolism studies

Commercially obtained rat liver microsomes were used for incubations. These microsomal preparations usually contained about 680 pmol of cytochrome *P*450s mg^{-1} of protein. Loop microdialysis probes with 1 cm membrane length were used for sampling. Prior to use in the incubation mixture, the in vitro relative recovery of the probe for the parent drug and the metabolites were determined as described previously.

The desired drug (concentration range 10–500 μM) was incubated in a 1.5 ml polypropylene vial with 1 mg of microsomes in 0.05 M potassium phosphate buffer at pH 7.4 containing 10 mM magnesium chloride at 37°C with magnetic stirring. The microdialysis probe was placed in this solution and perfused with the same buffer as in the incubation mixture (Fig. 1). After collecting two 5-min blank dialysates, the reaction was initiated by adding a 100 μl of 10 mg ml^{-1} NADPH or an NADPH generating system consisting of 14 mM glucose-6-phosphate, 1 mM NADP and 4.3 U of glucose-6-phosphate dehydrogenase. The final volume of the sample was 1 ml. Dialysate samples were collected every 5 min and injected into the assay system or frozen until assay.

In order to investigate the enzyme kinetics, the same incubations were carried out at different substrate concentrations ranging from 10–500 μM. Enzyme kinetics parameters (K_m and V_{max}) were obtained by fitting the data into Lineweaver-Burke plots.

Conventional microsomal incubations were carried out with diazepam for comparison purposes. Different concentrations of diazepam were incubated with rat liver microsomes without a dialysis probe as described above. The reaction was stopped after 15 min by adding 40 μl of 2 M perchloric acid. After centrifugation, the supernatant was removed and 5 μl of the supernatant was injected directly into the LC system.

3. Results and discussion

Acetyl salicylic acid is widely used as an analgesic, antipyretic and a nonsteroidal anti-inflammatory drug. It is rapidly hydrolyzed to salicylic acid primarily in the liver. The major Phase I metabolites of salicylic acid are 2,5- and 2,3-dihydroxybenzoic acid (DHBA). Hydroxylation of salicylic acid by hydroxy radicals in biological systems has also been used as a sensitive measure of free hydroxyl radicals [8,9].

In vitro recovery of the probe for salicylic acid was found to be $57.4 \pm 1.3\%$. The recoveries for the metabolites were $54 \pm 2\%$ for 2,5-DHBA and $52.5 \pm 2.4\%$ for 2,3-DHBA. Fig. 2 shows a typical chromatogram of a dialysate sample. Conven-

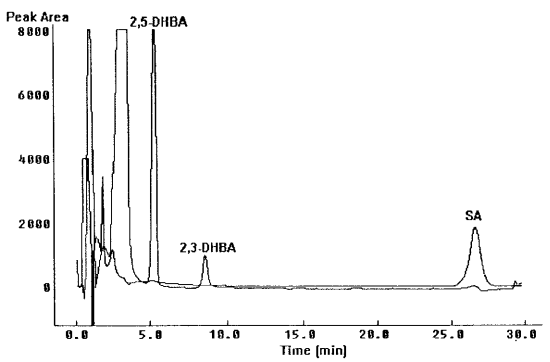

Fig. 2. A chromatogram of a dialysate sample from salicylic acid incubation. Rat liver microsomes (1 mg ml^{-1}) were incubated with 500 μM salicylic acid at 37°C. The metabolites, 2,5-DHBA and 2,3-DHBA were detected electrochemically at 650 mV on glassy carbon electrodes and salicylic acid was detected by UV at 254 nm.

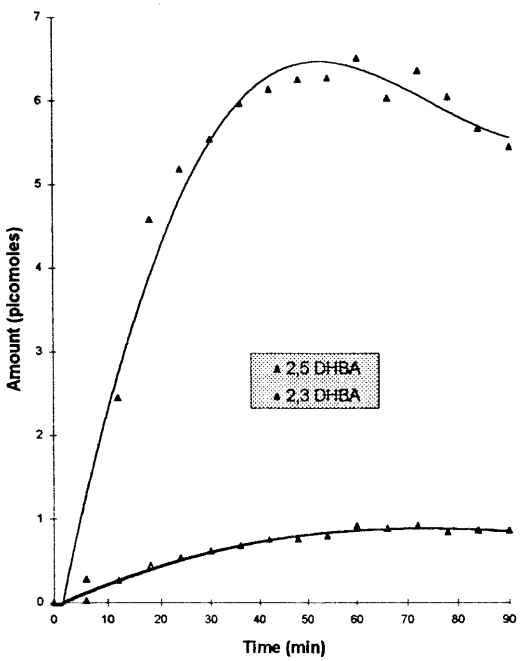

Fig. 3. Metabolic profiles of salicylic acid metabolites: 2,5-DHBA and 2,3-DHBA. Salicylic acid concentration is 500 μM.

tional microsomal studies carried out by Haliwell et al. [9] have shown only the presence of 2,5-DHBA in rat liver microsomes. In contrast, our studies have shown that both 2,5- and 2,3-DHBA form (Fig. 3). The electrochemical detection employed here was sensitive enough to indicate the minor presence of 2,3-DHBA.

Ibuprofen, 2-(4-isobutylphenyl)propionic acid, is another widely used anti-inflammatory drug that undergoes aliphatic hydroxylation and carboxylation by cytochrome *P*450s. The two major metabolites produced in this process are 2-[4(-2-hydroxy-2-methylpropyl)phenyl]propionic acid (hydroxy ibuprofen) and 2-[4(-2-carboxy-2-methylpropyl)phenyl]propionic acid (carboxy ibuprofen) [10]. These two metabolites and their respective conjugates account for approximately 60% of the metabolite products in urine with the ratio of carboxyibuprofen to hydroxyibuprofen being about 1.5 [10,11].

Fig. 4 shows a characteristic chromatogram of a dialysate sample from a microsomal incubation of ibuprofen. Profiles of the major metabolites from 100 μM ibuprofen are illustrated in Fig. 5. In vitro recovery of ibuprofen was 52.4 ± 1.7. The recoveries for the metabolites were 61.6 ± 2.8 for hydroxyibuprofen and 17.2 ± 2.1 for carboxy-ibuprofen. In the microsomal incubations mixture, the carboxy metabolite is found to be present about two-fold more than the hydroxy metabolite. Metabolic profiles were obtained for 10, 25, 50, 100, 200 and 500 μM of ibuprofen. The enzyme reaction was linear up to about 25 min in the whole concentration range (10–500 μM). Substrate concentration, [S], and velocity, V, data were fitted into Lineweaver-Burke equation as follows:

$$\frac{1}{V} = \frac{K_m}{V_{max}} \cdot \frac{1}{[S]} + \frac{1}{V_{max}}$$

where K_m is the Michaelis-Menten constant. The Lineweaver-Burke plots for the ibuprofen metabolites were linear as shown in Fig. 6. The K_m and V_{max} data for these metabolites are given in Table 1.

Diazepam, another commonly used drug was used in our study to demonstrate the utility of microdialysis in obtaining quantitative data. Diazepam has a low in vitro recovery because it is a

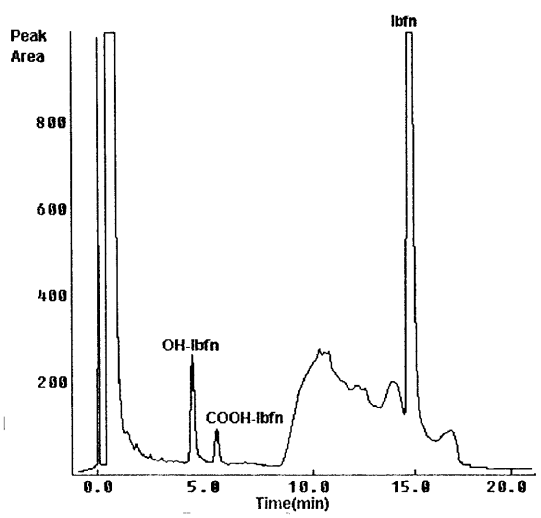

Fig. 4. Chromatogram of a dialysate from ibuprofen incubation. Rat liver microsomes (1 mg ml^{-1}) were incubated with 200 μM ibuprofen at 37°C.

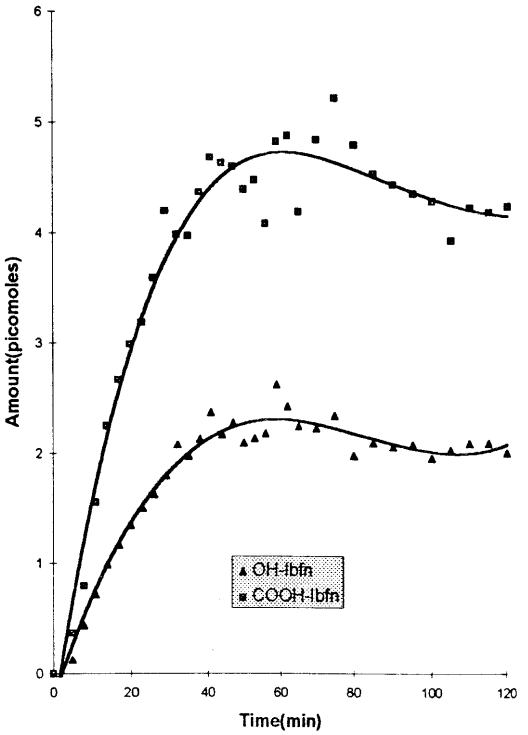

Fig. 5. Metabolic profiles of ibuprofen metabolites; OH-Ibfn, hydroxyibuprofen and COOH-ibfn, carboxyibuprofen. The ibuprofen concentration is 200 μM.

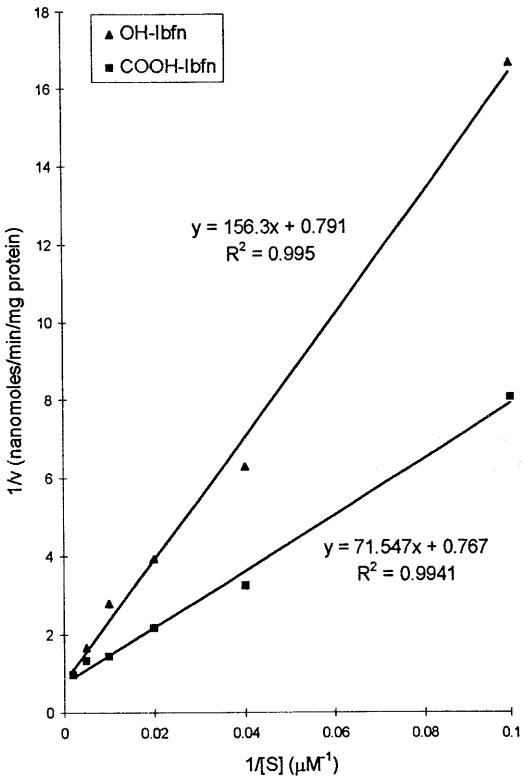

Fig. 6. Lineweaver-Burke plots for ibuprofen metabolism. Ibuprofen (concentration ranged from 10–500 μM) was incubated with rat liver microsomes (1.0 mg ml^{-1}) for 15 min at 37°C.

hydrophobic compound. It is worthwhile to investigate the applicability of microdialysis sampling for such a drug. On-line injection of the microdialysate samples were possible in this case because of the simple and short chromatographic separation of diazepam and its metabolites. These features made diazepam a good candidate for comparing microdialysis with the conventional incubation methods. In vitro recoveries of the probe for diazepam, temazepam, *N*-desmethyldiazepam and oxazepam were found to be 14.0 ± 1.8, 17.8 ± 1.9, 14.6 ± 1.3, and 16.2 ± 2.1, respectively.

The metabolism of diazepam is complex and known to be species, age and sex dependent [12]. The two major Phase I metabolites common to all species are temazepam and *N*-desmethyldiazepam. It has been reported that diazepam's other potential metabolite, 4-hydroxydiazepam is not detectable under normal incubation conditions [13]. Oxazepam, the secondary metabolite, from temazepam and *N*-desmethyldiazepam is considered to be a minor product. A typical chromatogram of a dialysate sample (Fig. 7) shows the minor presence of oxazepam.

Metabolic profiles of temazepam and *N*-desmethyldiazepam are shown in Fig. 8. The ki-

Table 1
Michaelis-menten kinetics parameters for ibuprofen metabolites

Metabolite	K_m (μM)	V_{max} (nanomoles/mg-protein per min)
Hydroxyibuprofen	199.5 ± 2.6	1.24 ± 0.14
Carboxyibuprofen	95.1 ± 4.9	1.13 ± 0.37

All values are mean ± S.E.M. for $n = 3$.

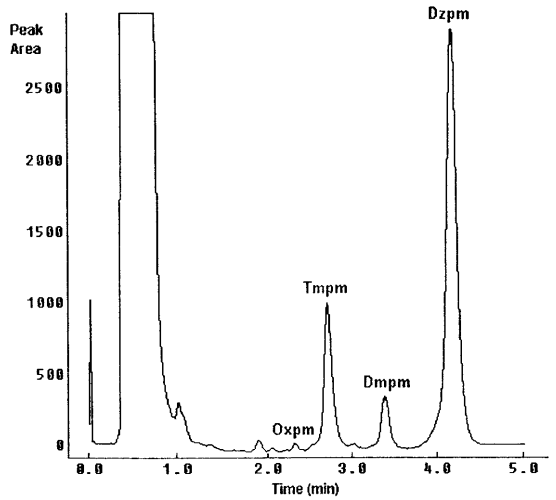

Fig. 7. Chromatographic separation of diazepam and its metabolites in a dialysate sample. In this incubation 29.5 μM diazepam was incubated with 1 mg of rat liver microsomes at 37°C; Tmpm, temazepam; Dmpm, *N*-desmethyldiazepam, Oxpm, oxazepam, Dzpm, diazepam.

netic studies of the formation of temazepam and *N*-desmethyldiazepam were performed using both microdialysis and conventional sampling techniques. Fig. 9 shows the representative Lineweaver-Burke plots for the two metabolites. It has been shown that V_{max} for the formation of temazepam is about 5-fold higher than the V_{max} for *N*-desmethyldiazepam [13]. Good linearity for each metabolite has been observed up to about 20 min in the concentration range studied (25–400 mM). The K_m and V_{max} values obtained by both techniques are compared in Table 2. These values are in agreement with the reported K_m and V_{max} values for diazepam metabolite formation [14,15].

On-line microdialysis sampling with LC has been used by Zhou et al. to study the enzyme kinetics of 2′, 3′, 5′-triacetyl-6-azauridine with porcine liver esterase and *N*-acetylphenylalanyl-3,5-diiodotyrosine with pepsin [16]. Enzyme kinetics data from both manual and microdialysis methods for initial rate of pepsin hydrolysis were fitted to the same function to obtain K_m and V_{max} parameters. These data were reproducible and in good agreement.

4. Conclusion

We have demonstrated that microdialysis can be used successfully to obtain enzyme parameters for Phase I xenobiotic metabolism. Metabolic profiles were obtained for hydrophobic drugs with low recovery (diazepam) as well as for drugs with high recovery (ibuprofen and salicylic acid). Although it has been reported that the 2,3-dihydroxybenzoic acid, a metabolite of salicylic acid is not detectable in microsomal fractions, the electrochemical detection employed with microdialysis sampling was able to detect this metabolite. Similarly, the metabolic profiles of diazepam show the presence of the secondary metabolite, oxazepam.

The Michaelis-Menten kinetics data were obtained for diazepam metabolism by both microdialysis and manual sampling for quantitative comparison. These data are in good agreement with the data reported in the literature.

Since microdialysis requires no sample preparation, instability of the metabolites during sample

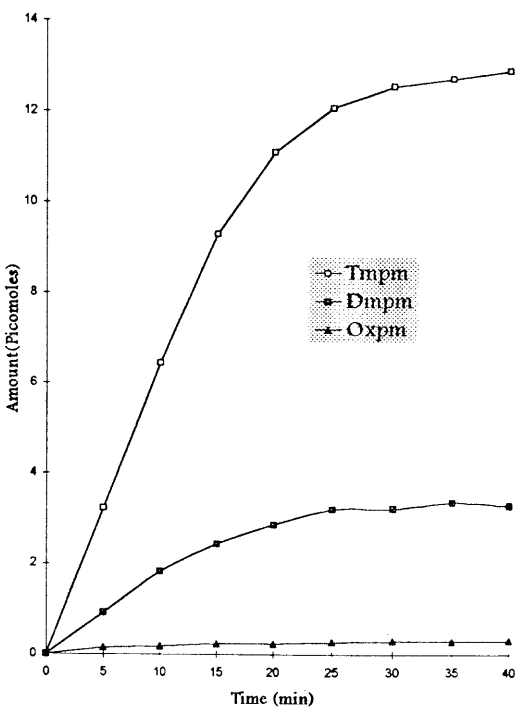

Fig. 8. Metabolic profiles of diazepam metabolites. Diazepam concentration was 145 μM.

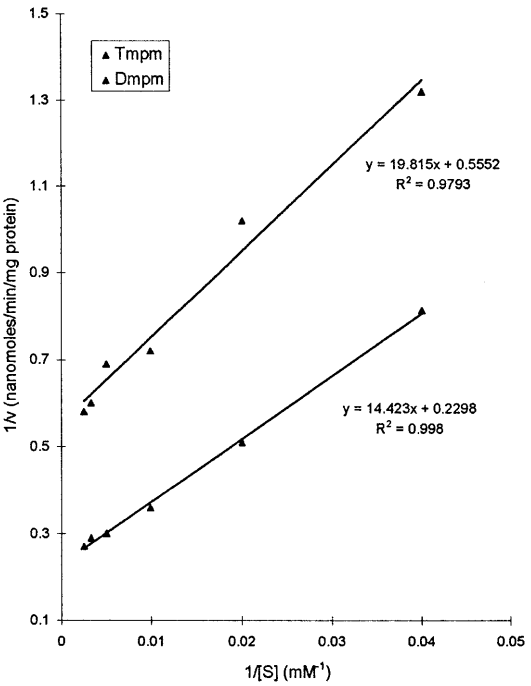

Fig. 9. Lineweaver-Burke enzyme kinetics plots for diazepam metabolism. Diazepam (concentration ranged from 25–400 μM) was incubated with rat liver microsomes (1.0 mg ml^{-1}) for 15 min at 37°C.

preparation is not a problem. The whole metabolic profile can be obtained from one sample incubation. The microdialysis system described above can be adapted to any other in vitro system, such as isolated cells or liver slices, with minor modifications of the instruments. It has been established that the 2-arylpropionic drugs, ibuprofen and ketopropfen, undergo metabolic chiral inversion in which the inactive (−)-*R* isomer converts to active (+)-*S* isomer in hepatic microsomes [17]. In the future we plan to use microdialysis to investigate the enzyme kinetics of this process.

Since the temporal resolution of the biological processes depends on the chromatographic separation time, measures to reduce the assay time are essential. Microbore chromatography coupled to mass spectrometric detection can be used to shorten the assay time and improve the temporal resolution. In these microsomal incubations, the total volume of the incubation mixture and the amount of reagents can be reduced by employing small coaxial type microdialysis probes with a 4 mm length and 5–10 μm outer diameter. By employing on-line injection of the microdialysate samples, a completely automated system can be developed to study the drug metabolism process in vitro or in vivo in near real-time.

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Table 2
Comparison of Michaelis-Menten kinetics parameters for diazepam metabolites from microdialysis and conventional methods

Metabolite	K_m (μM)		V_{max} (nmol/mg^{-1}(protein) min^{-1})	
	Microdialysis	Conventional	Microdialysis	Conventional
Temazepam	57.6 ± 5.2	62.6 ± 4.1	4.36 ± 0.93	5.35 ± 0.66
N-desmethyldiazepam	35.7 ± 3.4	40.8 ± 2.7	1.83 ± 0.37	4.78 ± 0.19

All values are mean ± S.E.M. for $n = 3$.

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