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Report

Microdialysis Sampling for Determination of Plasma Protein Binding of Drugs

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The use of microdialysis sampling to study the binding of drugs to plasma proteins was evaluated. Microdialysis sampling is accomplished by placing a short length of dialysis fiber in the sample and perfusing the fiber with a vehicle. Small molecules in the sample, such as drugs, diffuse into the fiber and are transported to collection vials for analysis. Larger molecules, such as proteins and protein-bound drugs are excluded by the dialysis membrane. Microdialysis was found to give values for *in vitro* protein binding in plasma equivalent to those determined by ultrafiltration. Microdialysis offers advantages in terms of maintaining equilibria and experimental versatility. Microdialysis sampling also provides potential use for *in vivo* determinations of protein binding.

KEY WORDS: microdialysis sampling; drug–protein binding determination; free drug concentration determination.

INTRODUCTION

Binding of drugs to serum proteins is an important factor in determining their pharmacokinetic and pharmacologic behavior. Serum protein binding studies of drugs have typically been performed using either equilibrium dialysis or ultrafiltration (1–5). Equilibrium dialysis requires a long equilibration procedure and can often be complicated by complex equilibria in the system. Ultrafiltration does not require the long equilibration time of equilibrium dialysis but can be complicated by reequilibration as the protein and drug concentrations change during filtration. Ultrafiltration also requires a centrifugation step during which control of the sample temperature is difficult. In addition, neither technique is suitable for *in vivo* determination of free drug concentrations during pharmacokinetic studies.

The use of microdialysis perfusion is proposed as an alternative to these techniques to study drug–protein interactions. Microdialysis perfusion is performed by perfusing a short length of small-diameter dialysis tubing with a carrier solution. Low molecular weight compounds in the sample can diffuse through the dialysis membrane into the carrier solution where they are transported to a collection vessel. Higher molecular weight compounds, such as protein, are excluded by the dialysis membrane. The dialysate can then be analyzed by any suitable technique. Microdialysis perfusion is as rapid as ultrafiltration and with a stirred sample does not suffer from reequilibration during separation of free from bound drug. Microdialysis has been shown to be applicable to the determination of *in vivo* serum drug concentrations for pharmacokinetic studies (6) and should also be capable of determining the extent of drug binding *in vivo*.

The present study describes the use of microdialysis perfusion to determine the extent of drug binding to human serum proteins *in vitro*. To validate the method several drugs with varying extent of protein binding were studied. The microdialysis results were compared to ultrafiltration determinations carried out concurrently and to previously reported values in the literature.

MATERIALS AND METHODS

Materials

Acebutolol, acetaminophen, cephalothin, chloramphenicol, isoniazid, phenytoin, salicylic acid, theophylline, and warfarin were purchased from Sigma Chemical Co. (St. Louis, MO). Human plasma was obtained from the Kansas City Community Blood Center. All other chemicals were of reagent grade or better and used as received.

Microdialysis probes were prepared from dialysis fibers obtained from Spectrum Medical Industries, Inc. (Los Angeles, CA). The dialysis fibers were regenerated cellulose with an inner diameter of 150 μm, a wall thickness of 9 μm, and a nominal molecular weight cutoff of 9000. The ends of a 5-mm length of fiber were connected to pieces of 26-gauge stainless-steel cannula by "hot-melt" glue. The probe inlet was connected to a microinjection pump (Bioanalytical Systems, Inc./Carnegie Medicin, West Lafayette, IN) with 120-μm-i.d. FEP tubing. FEP tubing was also connected to the outlet of the microdialysis probe and led to the collection vial.

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Analytical Procedures

Chromatography

The concentrations of the drugs in both ultrafiltrates and microdialysates were determined by liquid chromatography with either UV or electrochemical detection. The chromatographic system consisted of a Shimadzu LC-6A pump with an SCL-6A controller, an SPD-6AV UV-Vis detector (Shimadzu, Columbia, MD), and an LC-4B Amperometric detector (Bioanalytical Systems, Inc., West Lafayette, IN). Separation was performed with a Hypersil ODS 5-μm (4.6-mm × 15-cm) column. The mobile phase was 0.05 *M* ammonium phosphate buffer, pH 2.5, with acetonitrile added as organic modifier as needed for the specific drug (Table I). A flow rate of 1 ml/min and a 20-μl sample loop were used for all experiments. The detection parameters were optimized for each drug and are listed in Table I.

Binding Experiments

Stock solutions of each drug were prepared in Ringer's solution at a concentration 100-fold that desired for the binding experiment. An aliquot of the stock solution was then pipetted into sufficient plasma to give the desired plasma concentration. The plasma concentrations of the various drugs used were chosen to be within their therapeutic range and are listed in Table II. The spiked plasma samples were thoroughly mixed by vortexing and allowed to equilibrate for 8 hr at 4°C. After equilibration the spiked plasma samples were divided into two fractions, one for analysis by microdialysis and the other for analysis by ultrafiltration.

Ultrafiltration

Ultrafiltration was performed with an MPS-1 micropartition system with a YMT-membrane filter (Amicon, Lexington, MA). The spiked plasma sample was brought to 37°C and 1-ml aliquots were then ultrafiltered by centrifugation at 1600*g* for 15 min. The ultrafiltrate was analyzed for free drug by liquid chromatography as described above. Nonspecific adsorption of each drug to the ultrafiltration membrane was determined by ultrafiltering a drug standard in Ringer's solution. The extent of nonspecific adsorption to the ultrafiltration membrane is listed in Table I for each drug. The free drug concentrations were corrected for this nonspecific adsorption.

Microdialysis

Microdialysis was performed using a microdialysis probe (Fig. 1) built in-house as described above. The probe was first calibrated at 37°C for recovery of the specific drug using standards in Ringer's solution. The standard used was of the same concentration as the plasma concentration to be studied. The recoveries of the drugs used in this study expressed as the concentration of the drug in the microdialysate divided by the concentration in the stock solution are listed in Table I. A 1-ml aliquot of the spiked plasma sample was then brought to 37°C in a shaker bath set at 50 rpm. Microdialysis was performed, with continuous shaking, using the calibrated probe at a perfusion rate of 5 μl/min. Samples were collected for 10-min intervals until at least five consecutive samples had consistent free drug concentrations. Free drug was determined by direct injection of the microdialysate into the chromatographic system using the conditions described previously. The free drug concentration in the plasma sample was then determined by dividing the concentration in the microdialysate by the recovery.

RESULTS AND DISCUSSION

The processes occurring during microdialysis are illustrated in Fig. 2. Because of the concentration gradient of free drug existing from the outside to the inside of the microdialysis fiber, free drug will diffuse into the probe. The molecular weight cutoff of the dialysis membrane is such that protein, and therefore protein-bound drug, cannot cross the membrane. Convection continually renews the solution

Table I. Analytical Conditions

Drug	Therapeutic range (μg/ml)	Mobile phase[a] (% ACN)	Detection conditions	Nonspecific adsorption[b] (%)	% Recovery[c]
Acebutolol	5–20	20	240 nm UV	3.5	10.6
Acetaminophen	10–20	10	250 nm UV	0	22.5
Cephalothin	10–20	25	250 nm UV	0	8.2
Chloramphenicol	3–13	30	280 nm UV	5.3	14.5
Isoniazid	3–5	0	+1.0 V EC	2.5	9.9
Phenytoin	>10	40	240 nm UV	0	15.6
Salicylic acid	150–300	10	250 nm UV	0	23.3
Theophylline	5–20	10	270 nm UV	2.6	19.1
Warfarin	2.2 ± 0.4	50	250 nm UV	0	21.3

[a] Mobile phase is 0.05 *M* ammonium phosphate buffer, pH 2.5, with the percentage (v/v) acetonitrile listed.
[b] Nonspecific adsorption to ultrafiltration membrane determined at the concentration of free drug found during the binding experiment. Reported as percentage adsorbed relative to the total concentration.
[c] Recovery of the microdialysis probe at the concentration of free drug found during the binding experiment. Reported as percentage concentration in the dialysate relative to the concentration in the sample.

Table II. Percentage of Drug Bound to Human Plasma Proteins as Determined by Microdialysis and Ultrafiltration

Drug	Concentration (μg/ml)	Percentage bound to protein			Ref. No.
		Ultrafiltration[a]	Microdialysis[a]	Literature	
Acebutolol	10	25.5 ± 4.1	26.8 ± 1.9	26 ± 3	8
Acetaminophen	5	18.1 ± 1.2	19.3 ± 2.8	15–20	9
	10	20.4 ± 3.2	19.7 ± 2.8		
	50	16.8 ± 3.4	19.5 ± 0.6		
Cephalothin	10	79.4 ± 1.5	82.8 ± 0.9	71 ± 3	10
Chloramphenicol	4	58.8 ± 5.8	55.1 ± 4.3	53 ± 5	11
Isoniazid	5	10.3 ± 7.0	13.0 ± 4.1	0	12
Phenytoin	20	85.6 ± 1.1	80.2 ± 3.9	89 ± 23	13
Salicylic acid	50	74.3 ± 0.5	68.4 ± 1.3	Variable	1
	100	65.0 ± 0.8	64.5 ± 0.7		
	150	48.7 ± 0.8	50.6 ± 1.9		
	300	42.5 ± 4.6	39.9 ± 1.3		
	500	37.7 ± 2.2	36.1 ± 2.7		
Theophylline	10	56.1 ± 2.9	58.3 ± 2.8	56 ± 4	14
Warfarin	2.5	98.3 ± 0.5	98.2 ± 0.5	98.1–99.5	15–18

[a] $n = 4$.

around the probe, keeping the concentrations of all components outside the probe at the bulk solution concentration. Because little drug is actually removed from the sample, the overall drug concentration remains essentially constant during the experiment. Also, because microdialysis does not change the fluid volume, the protein concentration remains constant. Therefore, the drug binding equilibrium is not disturbed by this technique.

Problems of nonspecific adsorption are less severe with microdialysis relative to ultrafiltration because of the smaller surface area of the membrane involved. The surface area of the microdialysis membranes used in these experiments was approximately 0.016 cm^2, while that of the ultrafiltration membranes was 1.5 cm^2. If the membranes are similar in the extent of nonspecific adsorption sites, then the ultrafiltration membrane will bind two orders of magnitude more drug than the microdialysis membrane. In addition, collecting dialysis samples until a constant value is reached eliminates any error due to nonspecific adsorption. Any adsorption sites that may exist are quickly saturated, while the solution around the probe is renewed by convection. Because relatively little material is removed from the sample, the overall equilibrium is not disturbed.

The data for the binding studies are listed in Table II. Both microdialysis and ultrafiltration gave degrees of binding consistent with previously reported values. The precision of the microdialysis data is similar to the precision achieved by ultrafiltration. When the microdialysis data and the ultrafiltration data are compared (Fig. 3), it is clear that the results are equivalent. From Fig. 3, there appears no bias in the microdialysis results relative to determination of drug binding by ultrafiltration.

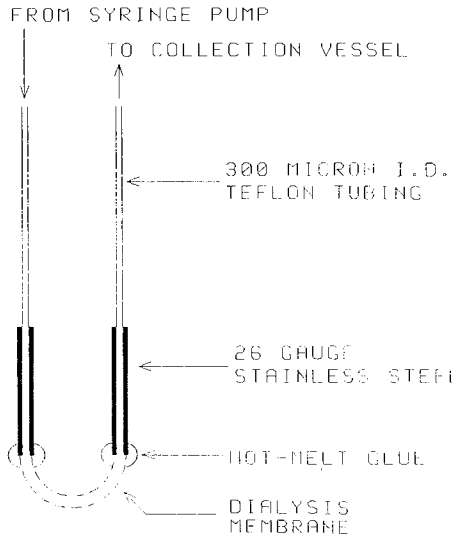

Fig. 1. Diagram of the microdialysis probe.

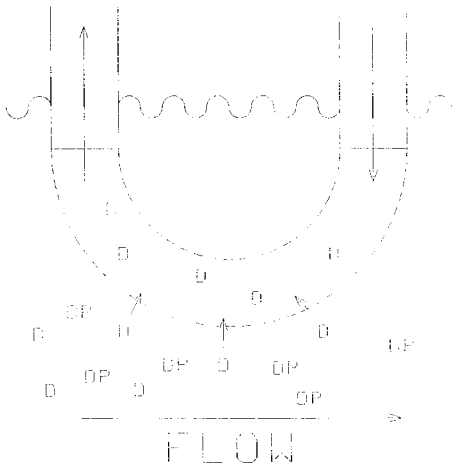

Fig. 2. Processes occurring during microdialysis sampling. Arrows indicate direction of perfusion through the probe. D, free drug; DP, drug–protein complex.

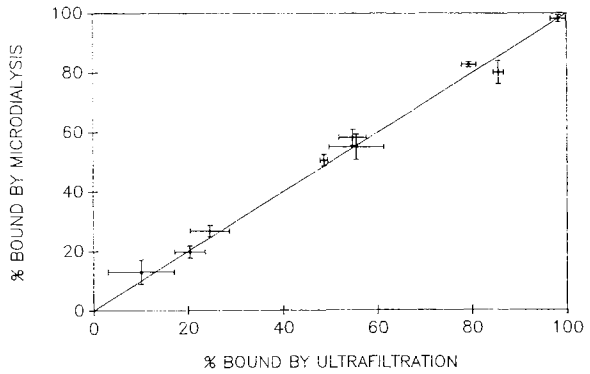

Fig. 3. Comparison of determination of drug binding to plasma protein by microdialysis and ultrafiltration.

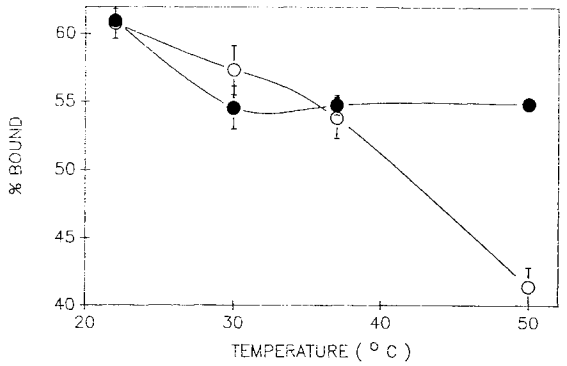

Fig. 5. Temperature dependence of the binding of chloramphenicol to plasma protein. Symbols as in Fig. 4.

These results also show that microdialysis is valid over a wide concentration range and wide range of degrees of drug binding. From drugs which did not bind (isoniazid) through drugs exhibiting very high binding (warfarin), microdialysis was usable to determine the free drug concentration. Total concentrations ranging from 2.5 to 500 μg/ml, which gave free concentrations from 50 to 300 μg/ml, were amenable to determination by microdialysis. The concentrations used in this study were chosen to be in the therapeutic range for each drug and do not reflect a limitation of the microdialysis technique. There is no reason this method cannot be used for drugs whose therapeutic concentrations are outside of this range if an appropriate analytical method is available. Figure 4 shows the results of a concentration study of the binding of salicylate to human plasma proteins. These results are consistent with previous reports (1) and further illustrate the utility of microdialysis to drug binding studies.

Because the determination of the free drug concentration does not require a centrifugation step when using microdialysis, it is possible to control the sample temperature much more precisely. The dependence of protein binding of chloramphenicol as a function of temperature is shown in Fig. 5. The determination was done by both microdialysis and ultrafiltration. Using microdialysis the sample could be continuously incubated in a constant-temperature bath throughout the experiment for precise temperature control.

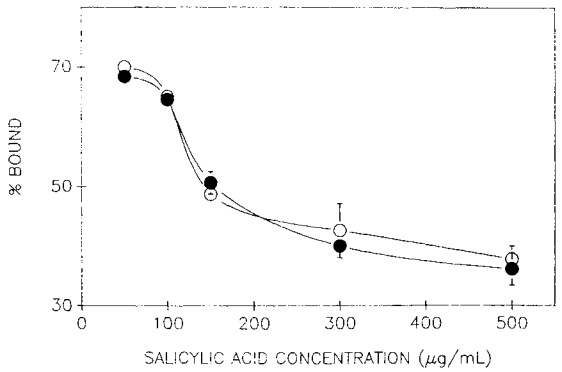

Fig. 4. Dependence of binding of salicylate to plasma protein on the concentration of salicylate. (○——○) Microdialysis; (●——●) ultrafiltration.

Using ultrafiltration, the sample temperature could not be controlled during the centrifugation step. The initial sample temperature and the temperature immediately after centrifugation are listed in Table III. The final temperature is roughly the same for each of the ultrafiltration experiments regardless of the initial sample temperature. This is further illustrated in Fig. 5, where binding appears independent of temperature by ultrafiltration but clearly does depend on temperature using microdialysis. The ability to control precisely the temperature of the binding experiment provides a method for determination of the thermodynamics of the binding equilibrium.

CONCLUSION

This data demonstrates that microdialysis is a valid method of determining the binding characteristics of drugs to proteins. Microdialysis offers the advantage of not changing the volume of the sample and not greatly changing the concentration of the components in the sample. Therefore the binding equilibrium is not disturbed by changes in the sample during the course of the experiment. Because there is no need for a centrifugation step, the sample temperature can be maintained at any desired value during the entire separation procedure using microdialysis. However, because only a small amount of the free drug is sampled, each probe must be characterized for its recovery of drug to determine accurately the free drug concentration in the sample. This requires more chromatographic analyses than are necessary for ultrafiltration and, therefore, a somewhat longer analysis time. Because microdialysis probes are readily implanted intravenously (7), binding of drugs to proteins could be studied *in vivo* under physiological conditions following dosing.

Table III. Temperature Change on Centrifugation

Initial temperature[a]	Final temperature[a]	Temperature change[a]
22	24	+2
31	27	−4
38	29	−9
51	32	−19

[a] Degrees centrigrade.

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