Potential Use of Microdialysis in Pharmacokinetics: A Protein Binding Study

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INTRODUCTION

The development of the microdialysis technique (1) has opened the possibility of multiple sampling of extracellular fluid directly in blood and other tissues. Since the use of microdialysis in pharmacokinetic/pharmacodynamic studies requires that true unbound concentrations can be estimated, it must be compared with established techniques for protein binding. Dubey and co-workers (2) presented data showing good agreement with equilibrium dialysis for diazepam protein binding. Herrera *et al.* (3) compared microdialysis to ultrafiltration and found similar binding for a number of drugs. The purpose of the present study was to compare the microdialysis with equilibrium dialysis methods by studying the protein binding of four model drugs with different physicochemical and binding characteristics, furosemide, phenytoin, racemic aminoglutethimide, and racemic disopyramide. Further, the time needed to reach a stable dialysate concentration was studied.

MATERIALS AND METHODS

Serum/Plasma

Pooled human serum from healthy subjects was extracted in Venoject tubes (Terumo, Leuven, Belgium). Owing to subsequent *in vivo* studies, plasma from Sprague Dawley rats (ALAB, Sollentuna, Sweden) was used in the aminoglutethimide study (Venoject tubes containing EDTA, 0.38 *M*). The serum/plasma was frozen at −20°C. Before use, pH was adjusted to 7.4 with 0.1 *N* HCl. In human serum, the concentration of albumin was measured with a bromocresolgreen method (4) and α_1-acid glycoprotein was measured with a standard immunological method. A corresponding method for rat α_1-acid glycoprotein was not available.

Chemicals

Table I shows the properties of the drugs. Furosemide and the sodium salt of phenytoin were commercially available solutions for injection. Racemic disopyramide phosphate and racemic aminoglutethimide were gifts from AB Hässle, Mölndal, Sweden, and Ciba-Geigy, Basel, Switzerland, respectively. All solvents and reagents were of analytical grade.

Experimental Procedure

Protein binding was performed in triplicates at 37°C at each of three concentrations, encompassing therapeutic levels. These concentrations were 5, 10, and 50 μg/ml furosemide, 7, 14, and 60 μg/ml phenytoin, 1, 5, and 20 μg/ml aminoglutethimide, and 2, 4, and 8 μg/ml disopyramide.

Microdialysis. Microdialysis probes, Microinjection Pump CMA/100, and Microfraction Collector CMA/140 were obtained from Carnegie Medicine, Stockholm, Sweden. The polycarbonate membrane of the probes had a molecular cut off below 20,000 daltons (1).

The microdialysis studies were performed in three steps. Steps 1 and 3 consisted of estimations of relative recovery (probe efficiency) from a Ringer solution and step 2 consisted of the estimation of the unbound concentration from serum/plasma. The time needed to reach a stable dialysate concentration was also established.

Relative recovery was estimated from 1.0 ml Ringer solution with a known concentration of drug at 37°C and under constant stirring. The probes were perfused with 2 μl/min of Ringer solution. Four fractions of 20 μl dialysate each were collected. Relative recovery was estimated according to Eq. (1).

$$\text{relative recovery} = \frac{\text{dialysate concentration}}{\text{surrounding concentration}} \quad (1)$$

In the protein binding studies four to eight samples were collected, depending on the time needed to reach a stable dialysate concentration for the different drugs. The local environment consisted of 5% CO_2 in technical air and pH was measured after the dialysis.

In the phenytoin microdialysis experiments, 2% propylene glycol in the Ringer solution was necessary to prevent crystallization of phenytoin at the probe surface.

Equilibrium Dialysis. Serum/plasma, 0.5 ml, was dialyzed against the same volume of buffer in an atmosphere of 5% CO_2 in air. Chambers of 1 ml were used. The dialysis membrane (Technicon chemicals) had a pore size between 20 and 60 Å. The dialysis was continued for 8 hr (furosemide), 5 hr (phenytoin), 4 hr (disopyramide), and 12 hr (aminoglutethimide). After dialysis, pH was measured. Samples were collected from both chambers.

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Table I. Properties of the Four Drugs

Drug	MW	pK_a	Fraction bound	Drug binding protein
Aminoglutethimide	232	4.2/11.9	25–30%	α_1-Acid glycoprotein
Furosemide	331	3.8	98–99%	Albumin
Phenytoin	252	8.3	90%	Albumin
Disopyramide	438	8.36	Concentration dependent in the therapeutic range	α_1-Acid glycoprotein Albumin

Chemical Assays

All drug concentrations were determined by high-performance liquid chromatography. The microdialysis and buffer samples were assayed without sample preparation.

Furosemide. Furosemide was determined with fluorescence detection (5) with some modifications: the mobile phase consisted of acetonitrile/0.02 *M* phosphate buffer, pH 2.5 (38/62). A column (200 × 4.5-mm i.d.) with ODS-Hypersil (C18) was used. At 0.05 μg/ml the coefficient of variation (CV) was 5% for dialysate and buffer. The serum samples were deproteinized with equal volumes of acetonitrile. All furosemide samples were kept out of light in order to minimize hydrolysis (6).

Phenytoin. Phenytoin was determined with UV detection (7). A column (150 × 4.5-mm i.d.) with ODS-Hypersil (C18) was used. For dialysate and buffer, the mobile phase consisted of acetonitrile/methanol/phosphate buffer, pH 3.7 (12/31/57). At 0.5 μg/ml the CV was 2.8%. For serum, the mobile phase consisted of triethylamine/methanol/phosphate buffer, pH 6 (0.2/54/46). The serum samples were deproteinized with equal volumes of acetonitrile.

Aminoglutethimide. Aminoglutethimide was determined with UV detection (8). Separation was performed on a Nova-Pak (C18) column with acetonitrile/0.005 *M* 1-heptanesulfonic acid in water (35/65). At 0.3 μg/ml the CV was 2.1%. Plasma (50 μl) was deproteinized with acetonitrile (75 μl). At 0.5 μg/ml the CV was 5.7%.

Disopyramide. Disopyramide was determined with UV detection (9) with some modifications: a column (150 × 4.5-mm i.d.) with ODS-Hypersil (C18) was used. The mobile phase consisted of triethylamine/distilled water/acetonitrile (0.25/78/22). Serum (250 μl) was deproteinized with trichloroacetic acid (25 μl). At 0.5 μg/ml the CV was 7.6%.

Calculations

The unbound fraction of drug (f_u) was calculated according to Eqs. (2) and (3).

Microdialysis

$$f_u = \frac{\text{(dialysate concentration/relative recovery)}}{\text{serum or plasma concentration}} \quad (2)$$

Relative recovery is the mean value of determinations before and after each experiment.

Equilibrium Dialysis

$$f_u = \frac{\text{buffer concentration}}{\text{serum or plasma concentration}} \quad (3)$$

Statistics

The estimates of unbound fraction obtained with microdialysis were compared to those obtained with equilibrium dialysis by analysis of variance.

RESULTS

A stable dialysate concentration was reached within the second 10-min collection period for furosemide, aminoglutethimide, and disopyramide. The time needed to reach a stable dialysate concentration was longer for phenytoin (Figs. 1 and 2).

The estimates of unbound fractions obtained with microdialysis and equilibrium dialysis are shown in Table II. Equal unbound fractions were found for aminoglutethimide and phenytoin with the two methods, while microdialysis gave significantly higher unbound fractions for furosemide and disopyramide. Concentration-dependent binding was observed for disopyramide. The concentrations of albumin and α_1-acid glycoprotein after pH adjustment were within normal range; albumin, 46–56 g/L; and α_1-acid glycoprotein, 0.56 g/L.

In the microdialysis studies from serum with furosemide and phenytoin, the initial dialysate concentrations were higher than at steady state (Fig. 2). This was not observed in any of the experiments with Ringer solution.

DISCUSSION

A good agreement in unbound fraction was obtained between microdialysis and equilibrium dialysis with racemic

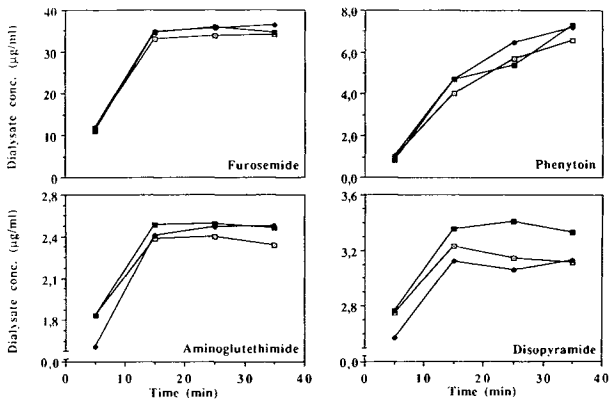

Fig. 1. Individual dialysate concentrations from Ringer solution with three probes for each drug. Furosemide, 50 μg/ml; phenytoin, 10 μg/ml; aminoglutethimide, 5 μg/ml; disopyramide, 4 μg/ml.

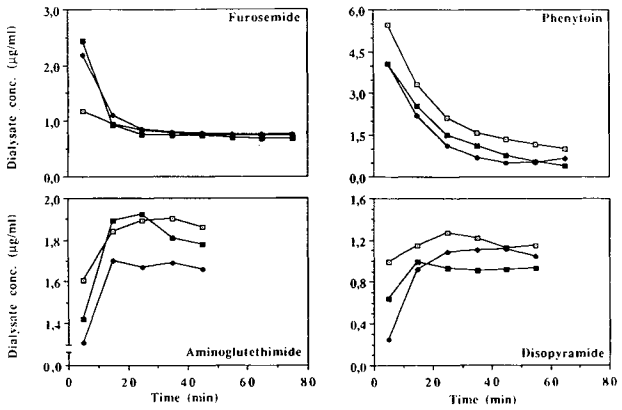

Fig. 2. Individual dialysate concentrations from serum/plasma with three probes for each drug. Drug concentrations as in Fig. 1.

aminoglutethimide (0.73 and 0.72), substances showing low degrees of binding, primarily to α_1-acid glycoprotein. The concentration-dependent binding of racemic disopyramide, also bound to α_1-acid glycoprotein, was verified with both methods; however, microdialysis showed significantly higher unbound fractions. Lima (10) reported values for unbound fractions of disopyramide that were considerably higher after equilibrium dialysis (0.55 to 0.86 at total concentrations of 2 to 8 μg/ml) than those obtained with the methods in the present study. Possible differences in α_1-acid glycoprotein concentrations might be one cause for the results different to our study.

The unbound fraction of furosemide was, on average, 30% higher with microdialysis compared to equilibrium dialysis (0.019 vs 0.014). Reported unbound fractions of furosemide are 0.02 to 0.04, as determined with equilibrium dialysis (11). Whether the most reliable estimate of "true" unbound fraction is obtained with microdialysis or equilibrium dialysis can not be stated.

The unbound fractions of phenytoin were equal between microdialysis and equilibrium dialysis (0.11 and 0.10) and agree well with values reported in the literature (12,13). The unbound fractions of phenytoin in the study by Herrera *et al.* (3) were higher (microdialysis, 0.20; equilibrium dialysis, 0.14) than in the present study. A regenerated cellulose membrane was used in their microdialysis studies, while the membrane used in the present study was made of polycarbonate. Herrera and co-workers (3) did not report any crystallization on the dialysis membrane.

There has yet been little validation of the microdialysis technique for the determination of true unbound drug concentrations *in vivo*. The theoretical aspects of correct determination of tissue interstitial concentrations have been addressed by several investigators (14–17). Benveniste and coworkers (17) concluded that correction factors other than the *in vitro* recovery, such as extracellular volume fraction, tortuosity factor (determined by the extent of prolongation of the diffusion pathway), and diffusion coefficient, should be used when estimating the true unbound concentration in tissues. The initially higher dialysate concentrations of furosemide and phenytoin obtained from serum compared to Ringer solution (Fig. 2) might be explained by some of these factors. In particular, electrostatic forces may influence the diffusion over the membrane.

Based on our results and those by other authors (2,3), microdialysis is a valuable method for estimation of unbound concentrations of drugs in plasma. The technique, however, is not suitable for all compounds, due to their physicochemical properties and analytical limitations. The use of *in vivo* microdialysis, working at steady-state conditions with low perfusion rates, could be particularly suitable for determining unbound tissue concentrations.

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Table II. Unbound Fractions (Mean ± SD) of the Four Drugs Obtained with Microdialysis and Equilibrium Dialysis at 37°C

Drug	Microdialysis	Equilibrium dialysis	Relative recovery[a] (membrane length)
Aminoglutethimide[b]	0.73 ± 0.07[c]	0.72 ± 0.03[d]	47–58% (10 mm)
Furosemide[b]	0.019 ± 0.002[c]	0.014 ± 0.002***,[c]	70–83% (16 mm)
Phenytoin[b]	0.11 ± 0.04[c]	0.10 ± 0.01[c]	53–65% (16 mm)
Disopyramide			
2 μg/ml	0.32 ± 0.01[e]	0.23 ± 0.01**,[e]	63–87% (16 mm)
4 μg/ml	0.35 ± 0.04[e]	0.34 ± 0.02[e]	
8 μg/ml	0.60 ± 0.04[e]	0.48 ± 0.01***,[e]	

[a] Range of relative recovery for individual probes.
[b] Mean value for the three different concentrations.
[c] $n = 9$.
[d] $n = 8$.
[e] $n = 3$.
** $P < 0.01$.
*** $P < 0.001$.

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