Development and Validation of a 96-Well Equilibrium Dialysis Apparatus for Measuring Plasma Protein Binding

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ABSTRACT: A 96-well equilibrium dialysis block was designed and constructed that is compatible with most standard 96-well format laboratory supplies and instruments. The unique design of the dialysis apparatus allows one to dispense and aspirate from either or both the sample and dialysate sides from the top of the apparatus, which is not possible with systems currently on the market. This feature permits the investigator to analyze a large number of samples, time points, or replicates in the same experiment. The novel alignment of the dialysis membrane vertically in the well maximizes the surface-to-volume ratio, eliminates problems associated with trapped air pockets, and allows one to add or remove samples independently or all at once. Furthermore, the design of the apparatus allows both the sample and dialysate sides of the dialysis well to be accessible by robotic systems, so assays can be readily automated. Teflon construction is used to minimize nonspecific binding of test samples to the apparatus. The device is reusable, easily assembled, and can be shaken in controlled temperature environments to decrease the time required to reach equilibrium as well as facilitate dissolution of test compounds. Plasma protein binding values obtained for 10 diverse compounds using standard dialysis equipment and the 96-well dialysis block validates this method. © 2003 Wiley-Liss, Inc. and the American Pharmaceutical Association J Pharm Sci 92:967–974, 2003

Keywords: protein binding; equilibrium dialysis; high-throughput screening; volume shifts

INTRODUCTION

Only the fraction of drug that is unbound by blood and tissue components is available for pharmacological interaction.[1–6] Plasma proteins are major contributors to drug binding in blood. Therefore, to better understand the relationship between drug concentrations and pharmacological effects, one must determine the unbound fraction of the total drug concentration. The two methods generally used to determine plasma binding of compounds are ultrafiltration and equilibrium dialysis.[7,8] The advantages provided by the ultrafiltration method include a shorter experimental time. The primary disadvantage of the ultrafiltration method is nonspecific binding of the drug to the filtration apparatus and concentration of plasma proteins during centrifugation. Most equipment designed for ultrafiltration is constructed of polymers that are prone to nonspecific binding of drug-like compounds. Conversely, most laboratory equipment developed to conduct equilibrium dialysis is constructed of Teflon to minimize nonspecific binding. Equilibrium dialysis studies require longer incubation times to reach equilibrium and are associated with volume shifts from the dialysate to the plasma side due to differences in osmotic pressure. This shifting results in plasma protein dilution and has been shown to reduce the *in vitro* fractional binding of ligand to plasma proteins.[9–11] Using either method, one must be careful to control variables that could generate experimental artifacts. Assay temperature, type of dialysis membrane and membrane

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preparation, volume shifts, drug concentration, ligand stability, plasma source (fresh, frozen, individual donors), and buffer (pH, concentration, composition) have been shown to alter the *in vitro* fractional binding of a ligand to plasma components.[12–14]

Recent advances in high-throughput screening (HTS), combinatorial chemistry, and high-speed analoging have increased the number of compounds identified as potential leads in drug discovery projects.[15,16] Similar strategies are being used to develop high-throughput ADME (absorption, distribution, metabolism, and excretion) and toxicology assays in an effort to evaluate and select the most promising lead structures and reduce compound attrition.[17–20] The extent to which a compound is bound to plasma proteins is a critical component to predicting how a potential drug will interact with its intended target *in vivo* and with the clearance mechanisms of the organism.[3]

The Spectrum 5 or 20 cell equilibrium dialyzer is the current industry standard to which novel dialysis systems are compared. Several limitations of the current Spectrum system are addressed by the development of the 96-well dialysis block. The Spectrum dialyzer allows one to conduct up to only 20 dialysis events in a single experiment. The equipment is expensive, and the setup is time consuming. Samples are inserted and withdrawn from the dialysis cells with a syringe, making it less adaptable to laboratory automation. One is unable to access or remove independent samples easily during the experiment. Sample volumes are typically 0.250–1.36 mL.

EXPERIMENTAL PROCEDURES

Reagents

Ammonium acetate, amitriptyline-HCl, dexamethasone, diazepam, imipramine-HCl, ketamine-HCl, lidocaine-HCl, sulfamethoxazole, tolbutamide, warfarin, and monobasic sodium phosphate were purchased from Sigma Chemical Company (St. Louis, MO). Propranolol-HCl and dibasic sodium phosphate were obtained from Aldrich Chemical Company (Milwaukee, WI). Sodium chloride, HPLC-grade acetonitrile, and water were purchased from J.T. Baker (Phillipsburg, NJ). Fresh blood was collected from male and female donors into vacutainers containing sodium heparin and centrifuged to obtain plasma.

Instrumentation

The 96-Well Equilibrium Dialysis Block was manufactured by Pfizer Inc. The Spectrum 20-Cell Equilibrium Dialyzer, Spectra/Por £2 membrane discs (MWCO: 12–14,000), and Spectra/Por £2 dialysis membrane (MWCO: 12–14,000) were purchased from Spectrum Laboratories Inc. (Laguna Hills, CA).

Assay Conditions

Prior to use, dialysis membranes were soaked in distilled water for 20 min and then in distilled water with 30% ethanol for 15 min, rinsed three times with distilled water, and finally soaked in isotonic sodium phosphate buffer until time of use. Fresh heparinized blood was collected from male and female donors, centrifuged to obtain plasma, and adjusted to pH 7.5 by adding 1 M phosphoric acid. The dialysate sides of the Spectrum apparatus and 96-well dialysis apparatus were loaded with 1.0 and 0.15 mL of phosphate buffer (0.05 M sodium phosphate in 0.07 M NaCl, pH 7.5), respectively. The same volume of undiluted or diluted (70 and 85%, respectively, by volume in buffer) plasma spiked with 10 μM test compound was pipetted into the sample side of each apparatus. After the 96-well dialysis unit was loaded with sample and buffer, an easily removable adhesive/piercible cover was placed over the top of the wells to prevent evaporation and pH change during the incubation. Both units were initially incubated at 37°C for 2, 4, 6, and 24 h, to determine the equilibration rate, and then for 8 h for all subsequent experiments. The 96-well and Spectrum units were incubated in a revolving incubator and water bath, respectively, at 37°C. Post-dialysis plasma and buffer volumes were recorded for all samples in the Spectrum apparatus and checked in the 96-well equilibrium dialysis device. Samples were all assayed similarly; that is, 90 μL of phosphate buffer was added to 10 μL of plasma, and then precipitated with two volumes of acetonitrile. After use, the Teflon cells/wells were washed with a nonionic detergent in water and rinsed with ethanol.

Chromatographic and Mass Spectroscopy (MS) Conditions

Gradient chromatographic conditions (4-min run time; 0–100% B) were employed using an Advantage Armor C18 (30 × 2.1 mm, 5 μm) analytical

column. The mobile phases used for analysis were (A) 90% 5 mM ammonium acetate, pH 4.5, with formic acid/10% acetonitrile and (B) 10% 5 mM ammonium acetate, pH 4.5, with formic acid/90% acetonitrile. The mobile phases were delivered at a 0.4-mL/min flow rate. A Perkin-Elmer Sciex API-3000 mass spectrometer in the positive-ion mode, with a Turbo Ion Spray source and multiple reaction monitoring, was used to quantitate peak area. Data collection and processing were performed with MacQuan PE-Sciex software.

Calculation of Fraction Bound (fb)

The fraction of drug bound was calculated using the Boudinot formula:

$$\mathrm{fb} = \frac{(D_{\mathrm{Te}} - D_{\mathrm{F}}) \cdot V_{\mathrm{pe}}/V_{\mathrm{pi}}}{\left[(D_{\mathrm{Te}} - D_{\mathrm{F}}) \cdot V_{\mathrm{pe}}/V_{\mathrm{pi}}\right] + D_{\mathrm{F}}} \times 100\% \quad (1)$$

where D_{Te} and D_{F} represent the total plasma concentration at equilibrium (sample side) and the free concentration (dialysate side), respectively, and V_{pi} and V_{pe} represent the initial and equilibrium plasma volumes, respectively.[11]

RESULTS

The 96-Well Dialysis Block

The 96-well dialysis block is constructed from Teflon and measures $14 \times 8.5 \times 2.5$ cm (Figure 1). The dialysis block contains 96 wells, in an 8×12 array, that conform to the spacing and dimensions of standard 96-well format laboratory supplies and instruments. Each dialysis cell has a 7-mm diameter and is 17.8 mm deep. The 8×12 array consists of nine segments that vertically bisect the wells. The apparatus is held in alignment by stainless steel pins that pass through the nine Teflon segments. Eight 2×12-cm sheets of dialysis membrane inserted between the segments during assembly of the block create 96 dialysis wells. The assembled dialysis block is then clamped together to prevent leaking. The resulting dialysis wells contain 0.56 cm^2 of membrane surface area when 0.15 mL of sample is added to each half-well. The surface area-to-half-cell volume ratio is 3.7 compared with 3.3 cm^2/mL in the Spectrum dialyzer (assuming 1.36 mL per half-cell).

After the dialysis cells are filled, an easily removable adhesive/piercible cover is placed over the top of the wells to prevent evaporation during

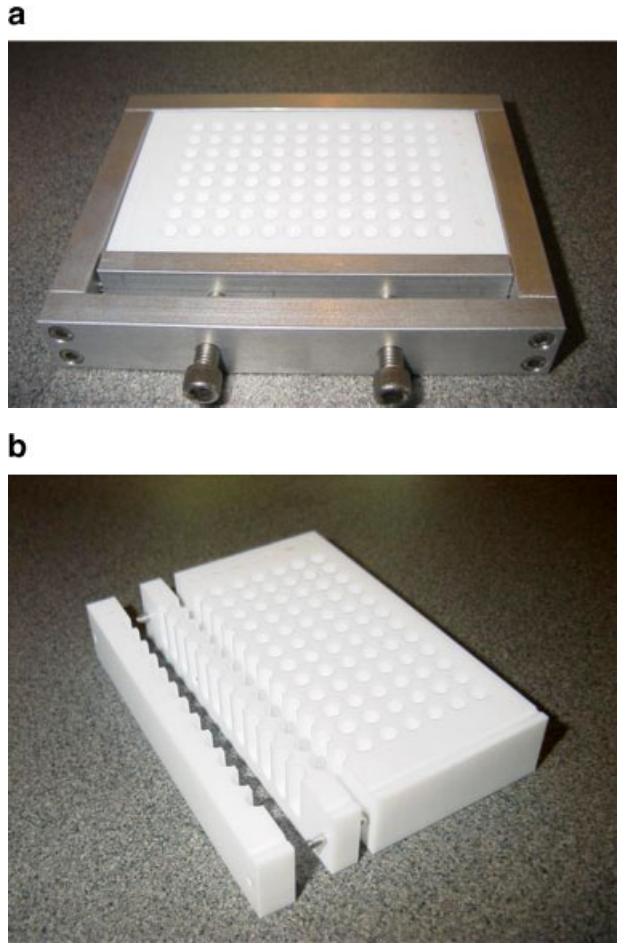

Figure 1. Photograph of the 96-well equilibrium dialysis block: (a) completely in clamp and (b) dialysis block with partially separated bars.

incubation. The temperature of the samples can be controlled using a variety of mechanisms, such as an incubator, a water bath, or a cold room. The apparatus can be shaken to increase the rate of dialysis. Following dialysis, the samples are collected from the sample and dialysate sides with standard single- or multiwell pipetting equipment. After use, the dialysis apparatus is disassembled (discarding the used dialysis membranes), washed with a nonionic detergent, rinsed with ethanol, and is then ready for reuse. Several units can assembled and loaded in <30 min, thereby permitting one to analyze thousands of dialysis events per day, provided appropriate analytical support is available.

The vertical alignment of the dialysis membrane within the well is a key feature that sets this invention apart from other dialysis devices. Vertical placement of the dialysis membrane provides several advantages over the existing cross-

sectional arrangements.[16] The vertical arrangement of the membrane allows one to dispense or remove from both sides of the dialysis well at any time during the experiment without disassembling the apparatus or terminating the assay. Unlike systems that insert the dialysis membrane horizontally across the well, our design maintains a constant surface area-to-volume ratio for any chosen experimental volume within the apparatus range. The volume-to-surface area ratio determines the length of time required to reach equilibrium. Furthermore, the vertical alignment of the membrane minimizes potential problems with trapped air bubbles. Air bubbles trapped against the dialysis membrane can significantly increase the time required to reach equilibrium by reducing the effective surface area. A final advantage is compatibility with current single-, multi-, and 96-well laboratory equipment and ease of adapting to robotic systems.

Time Required to Achieve Equilibrium in the 96-Well Dialysis Apparatus

The time course to equilibrium was examined for 10 drugs. Each compound was evaluated at 10 μM in phosphate buffer and dialyzed against phosphate buffer. Samples were removed for analysis following 2, 4, 6, and 24 h of dialysis. Equilibrium percentage was determined by dividing drug concentration on the dialysate side by drug concentration on the sample side multiplied by 100. The average percent of duplicate determinations is presented in Figure 2. All compounds reached equilibrium within 6 h. The time to reach equilibrium was similar for each drug and equivalent to that previously determined for the Spectrum dialyzer. All subsequent validation studies were conducted for 8 h to ensure equilibrium was achieved.

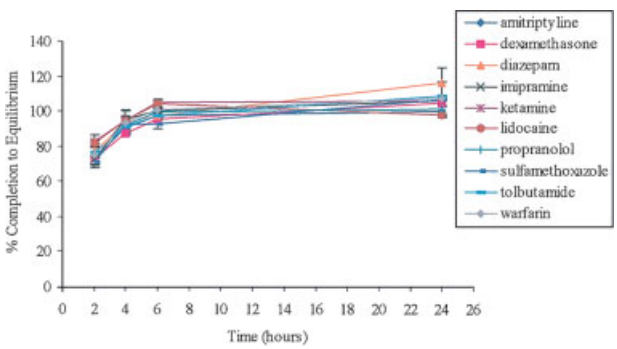

Figure 2. Time course to reach equilibrium in the 96-well dialysis equilibrium block.

Validation of the 96-Well Dialysis Apparatus

A direct comparison between the 96-well dialysis block and the Spectrum dialyzer was conducted by determining the plasma fraction bound values for 10 diverse compounds. Making a single stock of each compound at 10 μM in fresh plasma minimized experimental variation. A compound was dialyzed for 8 h in both the 96-well dialysis block and the Spectrum dialyzer. Following dialysis, samples were collected, sample volumes determined, and compound concentration quantitated by liquid chromatography/mass spectrometry (LC/MS/MS). Fraction bound values were calculated using the Boudinot formula[11] to help minimize experimental distortions caused by volume shifts. The formula for calculating fraction bound values and effects of volume shifts are discussed in more detail later.

Fraction bound values obtained using the 96-well dialysis block and the Spectrum apparatus were very similar to each other and consistent with literature values[21–30] (Table 1). There were minor shifts in fraction bound values between successive experiments that were attributed to intersubject variations in plasma protein concentrations.[31–34] However, the same few drugs (propranolol, ketamine, and imipramine) consistently had a higher percentage of drug bound in the 96-well block relative to the Spectrum dialyzer. Subsequent time course experiments demonstrated that the small number of consistently higher fraction bound values observed for these drugs when using the 96-well dialysis block were not due to a failure to reach equilibrium (data not shown).

Effect of Volume Shifts

One of the main differences observed between the two dialysis apparatuses is the extent of the volume shift from the dialysate side to the sample side during dialysis caused by osmotic pressure. Following 8 h of dialysis, plasma in the sample side of the Spectrum dialyzer was diluted to 87% of its original concentration compared with 95% in the 96-well dialysis block. Dilution of plasma has been shown to reduce the fractional binding of compounds to plasma components.[35,36] In the past, dextran has been added to the dialysate side to equalize the osmotic pressure and minimize the volume shift.[35] The need to demonstrate that the test compound does not bind to dextran makes this approach inconvenient for higher throughput approaches.

Table 1. Comparison of Plasma Binding Values in the 96-Well Dialysis Block and the Spectrum Dialyzer

	Literature Value	96-Well Dialysis Block		Spectrum Dialyzer		Difference between Dialysis Units
Drug	%Bound	%Bound	SD	%Bound	SD	%Bound
Amitriptyline	95	95.4	0.7	93.0	0.8	2.4
Dexamethasone	68	77.8	3.3	77.1	0.2	0.8
Diazepam	99	98.6	0.6	98.7	0.0	−0.1
Imipramine	90	90.0	0.7	87.2	0.5	2.8
Ketamine	47	54.4	2.0	49.8	2.0	4.5
Lidocaine	70	83.8	1.0	81.7	0.6	2.1
Propranolol	87	87.1	0.4	84.0	0.5	3.1
Sulfamethoxazole	62	75.6	1.6	74.1	2.6	1.4
Tolbutamide	99	98.5	0.2	98.4	0.0	0.0
Warfarin	99	99.4	0.0	99.4	0.0	0.0

The formula currently utilized to calculate fractional binding values compensates for the decrease in fractional binding caused by the volume shift. The concentration of drug bound is increased by a proportion related to the extent of the volume shift. Derivation of the formula (Figure 3) shows that for all compounds, the concentration of drug bound is corrected by the factor "V_{pe}/V_{pi}," where "V_{pe}" represents the volume of sample at equilibrium and "V_{pi}" represents the initial volume of sample before the start of dialysis. The "V_{pe}/V_{pi}" correction factor is intended to compensate for the decrease in fraction bound caused by the dilution of plasma during dialysis.[11]

The mathematical formula (Figure 3) assumes that the fractional plasma binding of all compounds is equally affected by the dilution of plasma components. However, theoretical simulations have shown that osmotic fluid shifts produce the largest changes in fractional binding for compounds that are bound by low capacity proteins with low affinity constants.[11] Human plasma contains >60 different proteins with a wide range of concentrations and binding affinities/capacities for test compounds. There is also a relative abundance of intersubject genetic variation and fluctuation in these plasma proteins. In general, the importance of this intersubject variability is minimized because two plasma components (albumin and α-acid glycoprotein) comprise 60% of the total protein and account for the majority of drug binding.[19]

A study was conducted to determine the effect of plasma dilution on the fractional binding of the 10 compounds in this study. The fractional binding of these compounds was determined at 100, 85, and 70% of the original plasma protein concentration by diluting the plasma with buffer prior to adding drug. The test compounds were then dialyzed against buffer for 8 h ($n = 3$) in the 96-well dialysis block. The percent fraction bound values were determined for each sample (Table 2). The results

Table 2. Plasma Dilution Effects on the Fractional Binding of Test Drugs to Plasma Components

	100% Plasma		85% Plasma		70% Plasma		Change in Percent Fraction Bound in Diluted Plasma	
Drug	%Bound	SD	%Bound	SD	%Bound	SD	100–85%	100–70%
Amitriptyline	93.0	0.9	92.1	0.4	91.2	0.7	0.9	1.8
Dexamethasone	72.4	1.2	71.8	1.5	72.1	4.7	0.6	0.3
Diazepam	98.4	0.0	98.4	0.1	98.2	0.3	0.0	0.2
Imipramine	89.9	0.5	87.1	1.0	83.9	0.5	2.8	6.0
Ketamine	53.1	1.7	50.1	1.7	46.9	7.4	3.0	6.2
Lidocaine	69.3	1.8	67.9	1.6	60.9	2.7	1.4	8.4
Propranolol	83.7	1.7	79.7	1.8	76.2	2.3	4.0	7.5
Sulfamethoxazole	70.9	5.8	68.8	2.1	66.6	3.5	2.1	4.3
Tolbutamide	96.7	1.0	97.3	0.2	97.0	0.3	−0.6	0.3
Warfarin	99.3	0.3	99.4	0.1	99.3	0.0	−0.1	0.0

$$\text{A. Fraction Bound} = \frac{\text{[drug bound] (corrected for volume shift)}}{\text{[total drug]}}$$

$$\text{B. Fraction Bound} = \frac{\text{[drug bound]} * \frac{\text{volume of plasma (at equilibrium)}}{\text{volume of plasma (initial)}}}{\text{[drug bound]} * \frac{\text{volume of plasma (at equilibrium)}}{\text{volume of plasma (initial)}} + \text{[drug free]}}$$

$$\text{C. [drug bound]} = \text{[total drug]} - \text{[free drug]}$$
$$= \text{[drug on plasma side at equilibrium]} - \text{[drug on buffer side]}$$

$$\text{D. Fraction Bound} = \frac{(DTe - DF) * \frac{Vpe}{Vpi}}{(DTe - DF) * \frac{Vpe}{Vpi} + DF}$$

Figure 3. Boudinot formula for calculating fraction bound.

indicate that the fractional binding of some of the compounds is more sensitive to plasma dilution than for others (Table 3). For example, essentially no decrease was observed in the fractional binding of tolbutamide, warfarin, and diazepam, even when plasma was diluted to 70% of its original concentration. However, the fractional binding of propranolol, ketamine, and imipramine decreased 4.0, 3.0, and 2.8% respectively, when plasma was diluted to 85% of its original concentration. The fractional binding of these drugs is further reduced when the plasma is diluted to 70% of its original concentration.

Correlation Between the Decrease in Fractional Binding Caused by Dilution of Plasma and Differences Between Fractional Bound Values Determined Using the 96-Well Block and Spectrum Dialyzer

A review of data comparing the fraction bound values obtained using the Spectrum dialyzer and the 96-well dialysis block suggests a correlation between the fraction bound differences obtained using the two systems and the sensitivity of a drug to dilution of plasma proteins. Drugs like tolbutamide, warfarin, and diazepam, whose fractional binding is less sensitive to plasma dilution, also have very similar fractional binding values in both dialysis methods (Table 3). In contrast, drugs whose fractional binding demonstrates a greater sensitivity to plasma dilution (imipramine, propranolol, and ketamine) have a significantly higher apparent fractional binding when determined with the 96-well block relative compared with results obtained with the Spectrum dialyzer. This result suggests that the different values obtained using the two dialysis methods can be accounted for by the greater dilution of plasma in the Spectrum dialyzer. The decrease in fractional binding observed when plasma is diluted to 85% of it original concentration is roughly equivalent to the difference in fractional binding determined using the two systems. Keeping in mind that we are comparing values close to the range of inherent experimental variability, the correlation we observe appears significant (Table 3 and Figure 4).

CONCLUSIONS

The design of the 96-well dialysis apparatus successfully overcomes several limitations of currently available equilibrium dialysis equipment. The 96-well dialysis block is easy to assemble, use, and clean, and is automation friendly. The vertical design has advantages over current 96-well formats on the market both in terms of surface-to-volume ratio and access to samples. Plasma fraction bound values obtained using the 96-well dialysis apparatus are comparable to literature

Table 3. Apparatus Specific Differences Versus Plasma Dilution Effect

Drug	Difference between Percent Fraction Bound in Spectrum Versus the 96-Well Block in 100% Plasma (Data from Table 1)	Change in Percent Fraction Bound in Diluted Plasma in 96-Well Dialysis Block (Data from Table 2)	
		100–85%	100–70%
Diazepam	0.0	0.0	0.2
Tolbutamide	0.0	0.6	0.3
Warfarin	0.0	0.1	0.0
Dexamethasone	0.8	0.6	0.3
Sulfamethoxazole	1.4	2.1	4.3
Lidocaine	2.1	1.4	8.4
Amitriptyline	2.4	0.9	1.8
Imipramine	2.8	2.8	6.0
Propranolol	3.1	4.0	7.5
Ketamine	4.5	3.0	6.2

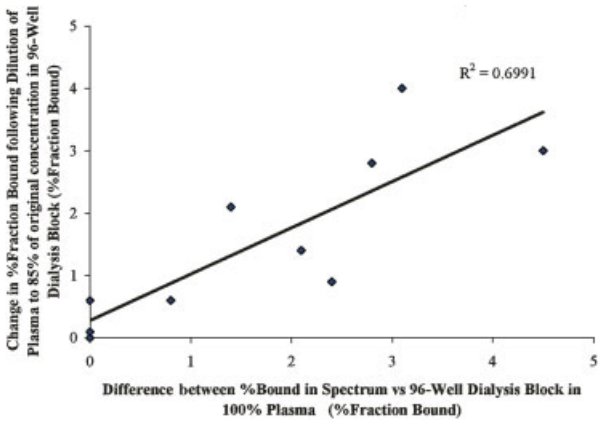

Figure 4. Apparatus-specific differences versus plasma dilution effect.

values. The minor, yet consistent differences observed between the 96-well dialysis block and the standard Spectrum dialyzer appear to be due to increased plasma dilution during dialysis in the Spectrum dialyzer. The 96-well dialysis block can be used for any equilibrium dialysis application currently carried out with the Spectrum dialyzer. The higher throughput format provides an opportunity to conduct a wide range of analyses that have been frequently omitted in the past because they were too time consuming. These studies include plasma protein binding at multiple concentrations, comparing plasma from multiple species, drug binding to specific components in plasma, partitioning of compounds into components of whole blood, microsome protein binding, relative binding affinities of compounds to different tissues or macromolecular subfractions, and receptor binding studies. In addition, facile access to the sample and dialysate sides of the dialysis well without the need to terminate the experiment greatly enhances the ability to conduct time-course experiments.

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