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Simple Spectrophotometric Assay for Measuring Protein Binding of Penem Antibiotics to Human Serum

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The binding of antibiotics to plasma (serum) proteins through hydrogen bonding can significantly influence the biological characteristics of these drugs. A rapid spectrophotometric assay has been developed that measures the level of free (unbound) penem antibiotic in serum ultrafiltrates. Whole human serum was adjusted to a standard concentration of antibiotic and then filtered by centrifugation through a Centrifree (Amicon Corp., Lexington, Mass.) filter that retained >99.9% of serum protein. The degree of penem protein binding was determined spectrophotometrically by measuring the level of unbound drug in the ultrafiltrate at 322 nm. At this wavelength, no interfering absorption from residual protein was detected in the ultrafiltrate, and penem absorption was linear over a wide concentration range. The method gave protein-binding values comparable to those obtained by a high-pressure liquid chromatography assay but was more rapid, since it did not require solvent extraction and high-pressure liquid chromatography calibration procedures. The spectrophotometric assay has been used to assay over 100 penems to determine the structure-activity relationships that are involved with the high serum protein binding of these agents. As with penicillins and some cephalosporins, the nonpolar nature of the penem side chain at the C-2 position strongly influenced the degree of penem binding to serum proteins.

The binding of antibiotics to plasma (serum) proteins through hydrogen bonding significantly influences the biological characteristics of these drugs. Binding predominantly involves the albumin fraction of plasma and is essentially reversible. High binding (>90%) of antibiotics to albumin decreases their rate of elimination from the kidney via glomerular filtration and can play a major role in determining the distribution of drug throughout the body. High plasma binding can also influence the effectiveness of antibiotics in vivo, since only the unbound drug is believed to inhibit the growth of microorganisms. The binding of many antibiotics to both plasma and serum is similar; thus, serum is frequently used in protein-binding studies for convenience.

Several methods have been developed to measure the serum binding of antibiotics. The two principal methods are equilibrium dialysis (16) and ultrafiltration (8, 9, 12, 14, 15, 17). Ultrafiltration is the more rapid method and has been adapted for measuring the binding of beta-lactam antibiotics to serum (12, 15). Both procedures can be used with detection methods that measure the amount of unbound drug by microbiological, high-pressure liquid chromatography (HPLC), or spectrophotometric assays (12, 15). Although spectrophotometric assays can be very sensitive, they have suffered from high background absorbances of protein between 255 and 280 nm, coinciding with the maximum absorbance wavelength of many beta-lactams. Penem antibiotics are unique among beta-lactams in that their absorption maximum is around 322 nm (4). As part of our penem research effort, we aimed to develop a simple spectrophotometric assay for use in protein-binding determinations in which the amounts of free drug in the ultrafiltrates could be measured directly. The use of Centrifree (Amicon Corp., Lexington, Mass.) micropartition units that retained >99.9% of serum protein and an assay wavelength of 322 nm allowed the measurement of free penem antibiotic directly in the ultrafiltrate. As has been shown for penicillins and some cephalosporins, the hydrophobic nature of the C-2 substituent strongly influenced the binding of penems to human serum proteins.

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MATERIALS AND METHODS

Antimicrobial agents. Sch 34343 was obtained from the Schering Corp., Bloomfield, N.J. Sch 29482 and all other penems were synthesized as sodium salts at Pfizer Central Research.

Ultrafiltration of serum. The serum binding of each penem was determined in triplicate by adjusting 4.0 ml of pooled human serum (pH 7.4; Biological Specialty Corp., Lansdale, Pa.) to a final drug concentration of 6.73×10^{-5} M (approximately 20 μg/ml). Drug was initially dissolved in 0.05 M phosphate buffer, pH 7.0, and 0.08 ml of a concentrated stock solution was added to 3.92 ml of serum to produce the final adjusted 4.0-ml volume that was 98% serum. After being allowed to equilibrate for 5 min at 25°C, the entire sample was placed in Centrifree micropartition units, and the units were centrifuged at 3,500 rpm for 30 min at 25°C (Dupont Sorvall RC-5 centrifuge with SS-34 rotor). Drug-free ultrafiltrate was prepared similarly to serve as a blank in the spectrophotometric assay of penem in the ultrafiltrate.

Spectrophotometric assay of penems. Penem beta-lactams have a characteristic absorption maximum at 322 nm. The concentration of penems in the ultrafiltrates was determined by measuring their A_{322}s in a spectrophotometer (Lambda 3b; The Perkin-Elmer Corp., Norwalk, Conn.) compared with drug-free ultrafiltrate as blank. A portion of the blank ultrafiltrate was adjusted to the standard concentration (6.73×10^{-5} M) with each test penem. The A_{322} of this standard was measured and recorded. The linearity of the absorption of penems in ultrafiltrate at this wavelength was established between 2.2×10^{-6} and 1.4×10^{-4} M (0.78 and 50 μg/ml, respectively). The percent binding of penem in the original pooled-serum preparation was determined in triplicate assays by using the following calculations: (i) percent penem unbound in ultrafiltrate = (absorption of penem in test

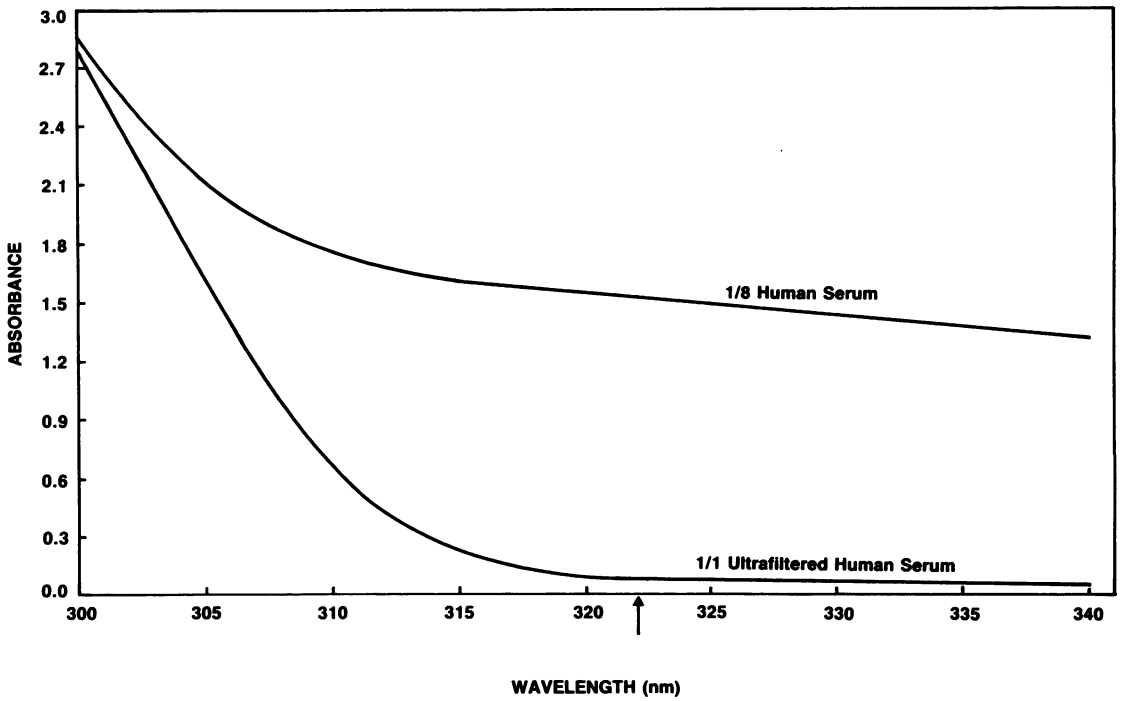


FIG. 1. Spectrophotometric scans of human serum diluted 1:8 in 0.1 M phosphate buffer and undiluted human serum ultrafiltrate versus phosphate buffer. Arrow indicates wavelength (322 nm) used to measure penem concentrations in the protein-binding assay.

ultrafiltrate)/(absorption of penem in standard ultrafiltrate) × 100 and (ii) percent penem bound to pooled serum = 100 − (percent penem unbound).

Protein determinations. The ability of the Centrifree micropartition units to remove protein from pooled human serum was measured by a standard Lowry procedure (11). The amount of protein in a sample of pooled human serum was compared with that remaining in the serum ultrafiltrate after centrifugation.

HPLC analysis of penem in ultrafiltrate. The concentration of penem in 98% human serum and serum ultrafiltrate was determined by an HPLC assay with a C18 column and a mobile phase of CH_3CN-0.05 M sodium acetate (20:80; pH 4.1) at a flow rate of 1.25 ml/min. The extraction of penem from serum was performed by a modification of the method of Kim et al. (10). The quantitation of penem was made from a standard curve of the compound in whole serum.

RESULTS

Determination of protein in ultrafiltrates. The amount of protein in a sample of pooled human serum was compared with that remaining in the serum ultrafiltrate after centrifugation. The effectiveness of the Centrifree units in removing serum proteins was verified by the >99.9% decrease in the protein content of the sample after ultrafiltration, which confirmed that the quantity of penem in the ultrafiltrate represented the unbound fraction.

Spectral scans of serum and ultrafiltrate. The spectral scans of human serum and serum ultrafiltrate versus 0.05 M phosphate buffer as reference are shown in Fig. 1. The high background absorbance of whole serum diluted 1:8 in phosphate buffer is evident from 300 to 340 nm. In comparison, undiluted serum ultrafiltrate shows minimal absorption beyond 320 nm, although considerable absorption of residual protein exists at wavelengths below 320 nm. A zero baseline was obtained with the ultrafiltrate measured against itself as reference, establishing the lack of background interference in the 322-nm region required for penem measurement.

Standard curves of penem in phosphate buffer or serum ultrafiltrate. Standard curves were prepared by measuring the absorbances of penem 1 solutions in 0.05 M phosphate buffer or in human serum ultrafiltrate at concentrations between 2.2×10^{-6} and 1.4×10^{-4} M (0.78 and 50 μg/ml, respectively) against buffer or ultrafiltrate, respectively. The curves are shown in Fig. 2. Each point represents the mean of triplicate determinations, with standard deviations of ≤0.03 absorbance units. The slopes for the buffer and ultrafiltrate curves were 7,784.2 and 7,657.1, respectively, as determined by regression analysis. Correlation coefficients were 0.999 and 1.000 for the buffer and ultrafiltrate curves, respectively. The curves are essentially identical, indicating that serum ultrafiltrate does not interfere with the penem A_{322}. Figure 2 also shows the linear absorbance of penem in serum ultrafiltrate across a physiologically relevant concentration range (0.78 to 50 μg/ml). Nonspecific binding of penem 1 to the Centrifree filter membrane was essentially zero at 6.73×10^{-5} M in phosphate buffer.

Protein binding of penems in whole serum. The percent protein binding of several penems was determined by ultrafiltration of whole serum containing 6.73×10^{-5} M penem (~20 μg/ml) and measurement of the A_{322} of free (unbound) compound in the resulting ultrafiltrate. Table 1 shows the mean of triplicate protein-binding determinations for each compound. Two Schering penems, Sch 34343 and Sch 29482, were used as controls. The protein-binding values of 63 and 93% for these compounds, respectively, agree well with values of 65 and 95% reported in the literature (3, 6). The protein-binding values obtained for the experimental penems ranged between 5 and 95%. The standard deviations obtained from the triplicate assays were ≤1.2%.

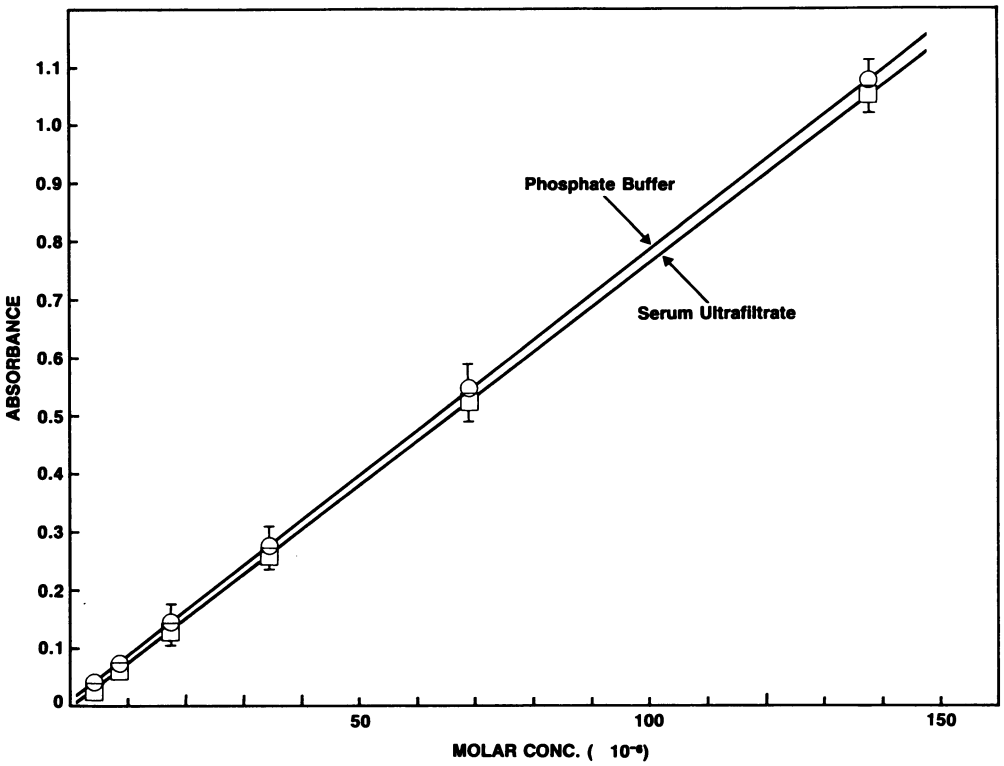


FIG. 2. Standard curves for penem 1 prepared in human serum ultrafiltrate or phosphate buffer. One standard deviation for any point was ≤ 0.03 A_{322} unit.

Protein-binding determinations for three penem compounds were compared by using the spectrophotometric and HPLC methods (Table 1). Penem 1, which showed a low binding of 5 (±1.2)% by the spectrophotometric method, gave a value of 0% by the HPLC method. An intermediate-binding compound, penem 5, and a high-binding compound, Sch 29482, gave comparable values by both methods. With the compounds tested, the accuracy and reproducibility of the spectrophotometric method compared well with those of the reference HPLC method.

Penem structure-activity relationships associated with high protein binding. It has previously been shown that the degree of binding of penicillins to serum proteins increases with the hydrophobic character of the side chain (13). To determine whether this relationship is true for penems, a number of penem analogs were tested for binding by the spectrophotometric assay (Table 2). The results supported the hypothesis that the presence of a hydrophobic side chain at C-2 of the penem nucleus promotes high binding to human serum. The penems tested fell into two groups on the basis of the extent of their binding to human serum protein. Penems in group 1 were not highly bound and carried polar groups at C-2 that were either polar aliphatic or charged ring structures (Table 2). The second group of penems comprised those with nonpolar side chains that conferred high serum protein binding, as determined by the assay. The penems in the second group demonstrated that the degree of protein binding is not highly influenced by the size of the side chain at C-2 or the chemical nature of its terminal moiety. The simplest side chain used was the ethyl moiety of Sch 29482, which imparted a degree of protein binding that was similar to that observed for the other nonpolar penems in this group. Hydrophobic substitution on the aliphatic side chain at C-2 permits a similar degree of protein binding, as demonstrated by the *m*-chloroanilide moiety of penem 9, the aminothiazole amide moiety of penem 15, and the ethyl ester moiety of penem 11. Results with compounds taken from a sulfone series indicate that the oxidation state of sulfur in the side chain has little influence on the degree of binding (compare values for penems 12 through 14 and penems 8 and 9). Thus, a group of penems that are widely diverse structurally demonstrated similar degrees of protein binding.

TABLE 1. Human serum protein binding of penems by spectrophotometric method

Compound	% Protein binding by[a]:	
	Spectrophotometric assay	HPLC assay
Penem 1	5.0 (1.2)	0
Sch 34343	62.9 (1.0)	ND[b]
Penem 5	52.7 (0.6)	45.9 (8.7)
Penem 8	93.9 (0.5)	ND
Penem 9	93.5 (0.5)	ND
Penem 11	95.2 (0.2)	ND
Sch 29482	93.1 (0.2)	94.8 (1.7)

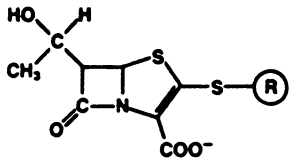
[a] Values are means (standard deviations) of triplicate assays.
[b] ND, Not determined.

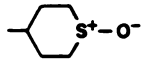
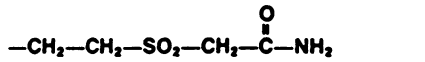
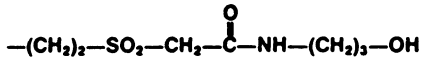
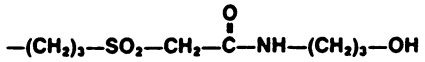
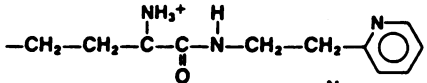
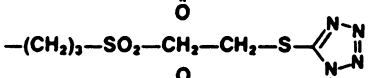
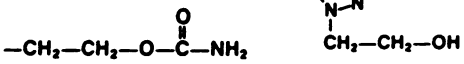
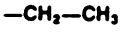
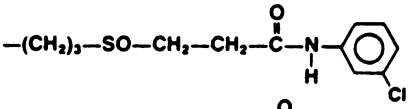
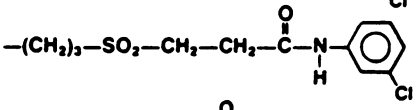
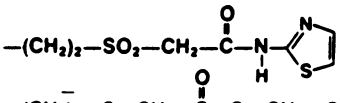
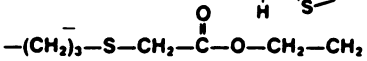
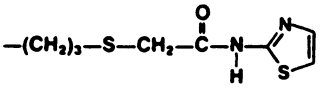
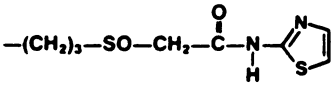
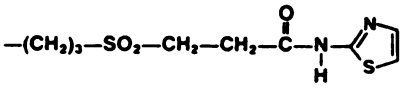
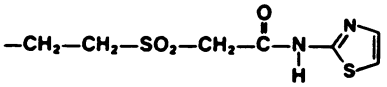
DISCUSSION

The spectrophotometric assay developed to study the binding of penem antibiotics to human serum protein is both simple and rapid. Good agreement was observed between the assays of serum ultrafiltrates for unbound penem by the

TABLE 2. Penem structures and percent bound to human serum protein

Penem Nucleus:



Penem	R Group	% Bound[a]
	Group I	
1		5
2		7
3		19
4		32
5		53
6		54
7		56
SCH-34343		63
	Group II	
SCH-29482		93
8		94
9		94
10		95
11		95
12		90
13		94
14		92
15		95

[a] Values rounded to the nearest whole percent.

spectrophotometric method and by HPLC. The results for two control penems, Sch 34343 and Sch 29482, were in good agreement with those reported in the literature (3, 6). Because of its simplicity, the spectrophotometric assay has an advantage over HPLC and microbiological methods for determining the serum protein binding of penems. Assay conditions are standard for all compounds, and there is no need for solvent extraction procedures that are necessary with HPLC techniques. We have used the spectrophotometric assay to analyze over 100 penem antibiotics having a broad range of protein-binding values to determine the structure-activity relationships involved with the high serum protein binding of these agents.

Earlier studies have described the physicochemical characteristics of penicillins and cephalosporins that influence their binding to serum proteins. Bird and Marshall (2) showed that the binding of a series of penicillin analogs correlates with their partition coefficients and, thus, the hydrophobic nature of the molecules. The degree of protein binding increases as the side group confers a more hydrophobic nature on the molecule. This is consistent with the hypothesis that the side groups are responsible for binding at a hydrophobic interface in the albumin fraction of serum protein through hydrophobic and ionic interactions (2, 13). Subsequent studies have characterized the serum binding of cephalosporins and penems and have shown that this binding is independent of the process of beta-lactam-ring hydrolysis mediated by serum albumin (4, 12). Our protein-binding results with penems support the importance of the hydrophobicity conferred on the molecule by the side chain at C-2. The size or composition of the side chain does not seem to affect the degree of protein binding of penems, except to decrease the polarity of the molecule. This is demonstrated by the structural diversity of the penems in group 2 of Table 2.

The pharmacological significance of high serum protein binding of beta-lactams is very difficult to predict (5, 18). While highly protein-bound compounds often produce high peak levels in serum, an effect on elimination half-life is evident only if renal elimination is primarily by glomerular filtration (1, 18). Ceftriaxone, for instance, is highly bound to human plasma protein and possesses an extended elimination half-life in humans of 8 h (7). The screening of beta-lactams for their binding to serum proteins is only a part of the effort to discover compounds with extended elimination half-lives. We have used the ultrafiltration and spectrophotometric assays described in this paper to measure the protein binding of a large number of penems in both human and animal sera. The results have been used to supplement animal pharmacokinetic studies aimed at finding penem antimicrobial agents with improved characteristics for clinical application. The spectrophotometric assay of unbound penem in serum ultrafiltrates is a simple and accurate method by which to obtain this data.

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