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In Vitro Protein Binding of Cefonicid and Cefuroxime in Adult and Neonatal Sera

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The levels of in vitro protein binding of cefonicid and cefuroxime in human adult and neonatal sera were compared. Binding parameters for each drug were determined within the concentration range of 25 to 3,000 μg/ml. Cefonicid exhibited concentration-dependent protein binding in both types of sera, with more extensive binding in adult serum at all concentrations. Two classes of binding sites were found: a high-affinity, saturable site and a low-affinity, nonspecific site. Cefuroxime also showed two-class, concentration-dependent protein binding in adult serum, but binding in neonatal serum was to a single class and was independent of drug concentration. Parameters for class 1 binding sites for cefonicid indicated one binding site per albumin molecule in both adult and neonatal sera, with association constants of 7.0×10^4 and 1.3×10^4 M^{-1}, respectively. These parameters were also derived for cefuroxime in adult serum and were 0.15 and 7.1×10^4 M^{-1}, respectively. In neonatal serum, the combined value (number of binding sites per molecule × equilibrium association constant) was similar to combined values calculated for class 2 binding sites for cefuroxime in adult serum and for cefonicid in neonatal serum (287 versus 195 and 261 M^{-1}, respectively). Cephalosporins have been shown to compete with bilirubin for albumin binding sites. Lower levels of protein binding of cefuroxime in the therapeutic range may mean a lower potential for drug displacement of bilirubin in neonates, on the basis of these results. It may be prudent to select less highly protein-bound agents when treating neonatal infections.

Serum protein binding significantly affects the pharmacokinetic and pharmacodynamic behaviors of drugs. Many drugs bind in vivo to serum proteins, usually albumin, forming a reversible complex through hydrogen bonding (18). The proportion of bound drug depends on a number of factors, including drug and protein concentrations, the affinity of protein for the drug, and the number of protein binding sites (20). Generally, only unbound drug is pharmacologically active (21); therefore, changes in serum protein binding can have important effects on clinical outcome.

Protein binding can determine the distribution, tissue penetration, clearance, and other pharmacokinetic properties of drugs (6, 7, 10, 15). Interactions due to protein binding may become significant when two or more highly bound (>90%) agents with a common binding site are combined (10, 12). The displacement that occurs from competition for binding sites results in elevated free concentrations of one or both drugs (12), resulting in increased pharmacological effects or toxicities, increased tissue distribution, increased clearance, or a combination of these.

Displacement interactions may occur between drugs and protein-bound endogenous substances, such as bilirubin, resulting in increased free bilirubin concentrations (4, 11, 16). Such a result is of particular importance in neonates, in whom the albumin concentration and binding affinity of albumin for bilirubin are already lower than those in adults (14). A further reduction in bilirubin binding by competition for binding sites, resulting in increased levels of unbound bilirubin, will increase bilirubin penetration into the central nervous system and may lead to the development of kernicterus. In adults, the levels of plasma protein binding of cefonicid and cefuroxime have been reported to be 96 and 33%, respectively, at therapeutic concentrations (8, 9). However, the effect of various drug concentrations on serum protein binding and differences in the levels of protein binding of these antibiotics between adult and neonatal patients have not been well studied. Before we can fully investigate antibiotic displacement of bilirubin in neonates, we need to understand better the binding characteristics of these agents in adult and neonatal sera.

The objectives of this study were (i) to determine the effects of various drug concentrations on the protein binding of cefonicid and cefuroxime in pooled adult and neonatal sera, (ii) to characterize the protein binding parameters for the antibiotics, and (iii) to compare the protein binding parameters for cefonicid and cefuroxime in neonatal serum with those in adult serum.

MATERIALS AND METHODS

Materials. Pooled human neonatal serum was obtained from umbilical cord samples sent to the hospital laboratory for routine analysis at the time of birth. Adult serum was obtained from the blood bank. Serum chemistry profiles, including albumin and protein concentrations, were determined for a portion of the pooled sera. The serum albumin concentration in pooled adult serum was 48 g/liter (6.96×10^{-4} M), and that in pooled neonatal serum was 38 g/liter (5.51×10^{-4} M). Cefazolin (Ancef; SmithKline Beecham), cefonicid (Monocid; SmithKline Beecham), and cefuroxime (Zinacef; Glaxo, Inc.) were purchased from the hospital

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pharmacy. Sodium acetate, glacial acetic acid, 8-chlorotheophylline, disodium phosphate, triethylamine, phosphoric acid, acetonitrile, methanol, and high-pressure liquid chromatography (HPLC)-grade water were obtained from J. T. Baker (Phillipsburg, N.J.).

Protein binding. A stock solution of each drug was prepared daily. Buffer samples, which served as free-drug controls for the protein binding studies, were prepared with aliquots of the drug stock solutions added to Sorenson's phosphate buffer (pH 7.4). The antibiotic concentrations in the buffer samples ranged from 2.5 to 5,000 μg/ml. Serum samples were prepared with 2 ml of pooled serum, and aliquots of the drug stock solutions were added to obtain final antibiotic concentrations ranging from 25 to 3,000 μg/ml. High antibiotic concentrations were used to better characterize the binding of the drugs to serum proteins. Limitations in assay sensitivity prohibited the assessment of protein binding at lower drug concentrations. After incubation at room temperature for 30 min, free cefonicid and cefuroxime were separated from protein-bound drug by use of an ultrafiltration system with YMT filters (Amicon, Danvers, Mass.). One milliliter of spiked serum at each concentration was placed in the ultrafiltration system and centrifuged (Centra 4; International Equipment Co., Needham Heights, Mass.) at 3,000 rpm for 6 min, and the ultrafiltrate, containing free drug, was collected. All assays were done in duplicate.

Drug analysis. Concentrations of cefuroxime and cefonicid were assayed by previously published HPLC techniques (3, 5). For cefuroxime, a 0.1 M acetate buffer (pH 3.85)–acetonitrile (90%:10%) mobile phase was used with 8-chlorotheophylline as the internal standard. For cefonicid, the mobile phase consisted of a 0.05 M phosphate buffer (pH 7.2)–methanol–acetonitrile (86%:6%:8%) mixture, and cefazolin was used as the internal standard. Mobile phases were delivered at a rate of 1.5 ml/min. HPLC analyses were performed with a Waters 510 pump, a WISP 710B sample processor, and a Lambda-Max model 481 (Waters, Milford, Mass.) variable-wavelength UV-visible light absorbance detector set at 254 nm. The analytical column used was a reversed-phase (C_{18}) column (250 by 4.6 mm; particle size, 5 μm) (Econosphere; Alltech Associates, Deerfield, Ill.).

The standard curves were linear in the antibiotic concentration range of 0.5 to 5,000 μg/ml for cefonicid and 1 to 5,000 μg/ml for cefuroxime. The lower limits of quantitation for the assay were 0.5 μg/ml for cefonicid and 1 μg/ml for cefuroxime. The intra- and interday coefficients of variation for the assays were less than 10% for both drugs at all drug concentrations measured.

Data analysis. The Akaike's Information Criterion (23) and lack of systematic deviations around fitted curves were used to select model equations to fit the data. The protein binding of cefonicid in adult and neonatal sera and the protein binding of cefuroxime in adult serum were characterized by the relationship (17)

$$F_B = D_B/(D_B + D_F) \quad (1)$$

where

$$D_B = [(N_1K_1P_AD_F)/(1 + K_1D_F) + N_2K_2P_AD_F \quad (2)$$

and F_B is the fraction of drug bound; D_B, D_F, and P_A are the molar concentrations of bound drug, unbound drug, and albumin, respectively; N is the number of binding sites per molecule; and K is the equilibrium association constant. Subscripts 1 and 2 refer to the first and second classes of binding sites, respectively. The protein binding parameters N_1, K_1, and N_2K_2 were estimated by nonlinear least-squares regression (13) with equations 1 and 2.

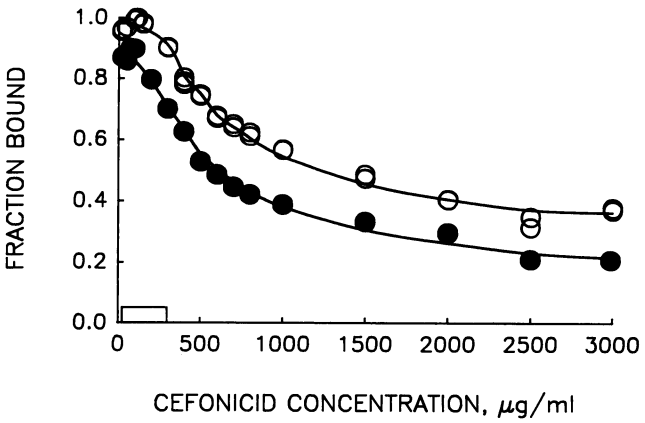

FIG. 1. Cefonicid serum protein binding in adult (○) and neonatal (●) sera. The lines depict the NONLIN least-squares regression fitting of these data. The open box represents the therapeutic range (25 to 300 mg/liter) of cefonicid.

The protein binding of cefuroxime in neonatal serum, however, was independent of drug concentration and was characterized by the relationship

$$F_B = D_B/(D_B + D_F) \quad (3)$$

where

$$D_B = NKP_AD_F \quad (4)$$

and the protein binding parameter NK was estimated by nonlinear least-squares regression (13) with equations 3 and 4.

RESULTS

The fraction of cefonicid bound to serum proteins as a function of total drug concentration in adult and neonatal sera is illustrated in Fig. 1. The protein binding of cefonicid was dependent on drug concentration in both adult and neonatal sera, although the extent of binding in adult serum was greater than that in neonatal serum at all concentrations studied. The extent of serum protein binding decreased with increasing concentrations of antibiotic. The binding of cefonicid to both adult and neonatal serum proteins was best described by two classes of binding sites (equations 1 and 2). The protein binding parameters generated by NONLIN least-squares regression analysis are presented in Table 1. In both matrices, cefonicid serum protein binding was characterized by a high-affinity, saturable binding site and a low-affinity, nonspecific binding site. On the basis of the assumption that cefonicid associates with serum albumin, the number of high-affinity cefonicid binding sites per molecule was approximately 1 for both adult and neonatal sera. However, the equilibrium association constants differed significantly, with the neonatal binding sites having approximately 19% of the affinity for cefonicid of the adult binding sites. The combined value for the second class of cefonicid binding site (N_2K_2) differed approximately twofold between adult and neonatal sera. In addition, the albumin concentration in neonatal serum was approximately 20% lower than that in adult serum.

TABLE 1. Protein binding parameters for cefonicid and cefuroxime in adult and neonatal sera

Drug and serum	Albumin concn[a] (10^{-4} M)	N_1[b]	K_1[b], M^{-1}	N_2K_2[b], M^{-1}	% Binding
Cefonicid					
Adult	6.96	0.93 (0.04)	7.0×10^4 (2.1×10^4)	537 (34)	31.5–99.9
Neonatal	5.51	1.0 (0.08)	1.3×10^4 (0.2×10^4)	261 (52)	20.9–90.0
Cefuroxime					
Adult	6.96	0.15 (0.023)	7.1×10^4 (4.4×10^4)	195 (36)	8.8–88.0
Neonatal	5.51			287 (17)	10.9–21.9

[a] The albumin concentrations were measured chemically.
[b] NONLIN least-squares parameter estimate (SE).

The protein binding of cefuroxime as a function of total drug concentration in adult and neonatal sera is shown in Fig. 2. Concentration-dependent binding was found with cefuroxime in adult serum (range, 8.8 to 88%), whereas in neonatal serum, cefuroxime exhibited constant fractional protein binding over the drug concentration range studied. The fraction of cefuroxime bound in neonatal serum averaged 15.6 ± 3.8% (mean ± standard deviation). In adult serum, the binding of cefuroxime was best described by two classes of binding sites (equations 1 and 2); however, the binding of cefuroxime in neonatal serum was best described by a single, low-affinity binding site (equations 3 and 4). The serum protein binding parameters for cefuroxime in adult and neonatal sera are shown in Table 1. In adult serum, the number of high-affinity binding sites per molecule of albumin was 0.15. The cefuroxime high-affinity equilibrium association constant was similar to that observed for cefonicid (7.1×10^4 and 7.0×10^4 M^{-1}, respectively). The second class of cefuroxime binding site in adult serum had a combined value (N_2K_2) somewhat lower than that observed for cefonicid (195 and 537 M^{-1}, respectively). In neonatal serum, a single, nonsaturable binding site provided the best fit for the cefuroxime protein binding data; hence, only a combined constant (N_2K_2; Table 1) could be calculated. The N_2K_2 values for the low-affinity binding sites were similar in both sera.

DISCUSSION

The purpose of this investigation was to compare the protein binding of two cephalosporins, cefonicid and cefuroxime, in adult and neonatal sera. Cefonicid has been reported to be highly bound to serum proteins (8), while cefuroxime has been reported to be moderately bound (9). However, few investigations have examined the serum protein binding of drugs in the neonatal population. The protein binding of the antibiotics was studied over a wide range of drug concentrations, including those exceeding therapeutic levels, to accurately characterize and determine protein binding parameters. The results of this study demonstrate notable differences between the binding of these two cephalosporins in adult and neonatal sera.

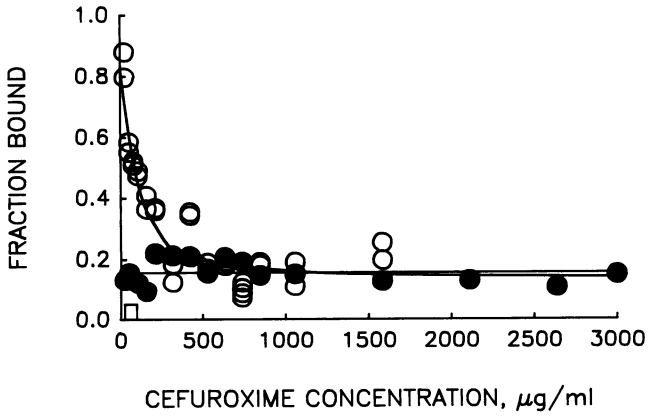

FIG. 2. Cefuroxime serum protein binding in adult (○) and neonatal (●) sera. The lines depict the NONLIN least-squares regression fitting of these data. The open box represents the therapeutic range (25 to 100 mg/liter) of cefuroxime.

The binding of cefonicid to both adult and neonatal serum proteins was highly dependent on drug concentration, with a decreased fraction of cefonicid being bound as drug concentration increased. Over the cefonicid concentration range of 25 to 3,000 μg/ml, the percentage of drug bound to serum proteins decreased from 99.9 to 31.5% and from 90.0 to 20.9% in adult and neonatal sera, respectively. Within the therapeutic range, binding was independent of drug concentration at lower concentrations (25 to 150 μg/ml) but became nonlinear at higher concentrations (150 to 300 μg/ml, Fig. 1). Cefonicid protein binding in adult serum within the therapeutic range was similar to that reported previously (90 to 98%) (1, 8). Cefonicid was found to bind to a high-affinity, saturable binding site as well as to a low-affinity site that was not saturated over the range of drug concentrations studied. Previous investigations suggested that cephalosporins associate with a single high-affinity binding site on serum albumin (19). The results of the present study examining the binding of cefonicid support these earlier findings. However, a second class of low-affinity binding site was also detected. This second class of binding site was observed in the present study because of the wide range of drug concentrations used.

The fraction of cefonicid bound to neonatal serum proteins was smaller than that bound to adult serum proteins at all drug concentrations studied. This difference was relatively constant, with the fraction bound in neonatal serum being approximately 0.1 lower than that in adult serum. We attribute the lower level of fractional binding of cefonicid in neonatal serum to the 20% lower serum albumin concentration and the 80% lower equilibrium association constant in neonatal serum. The lower association constant in neonatal serum may be due to conformational differences between neonatal and adult albumin. Alternatively, the lower association constant may result from the presence of endogenous compounds, such as 2-hydroxybenzoylglycine, that competitively inhibit cefonicid binding to this class of binding site in neonatal serum and that are not present or are present in lower concentrations in adult serum (22). Thus, both the lower binding capacity (N_1P_A) and the lower binding affinity

(K_1) in neonatal serum result in the lower level of fractional binding of the antibiotic in this serum than in adult serum.

Cefuroxime protein binding in adult serum was concentration dependent throughout the concentration range tested, with the percentage bound varying from 88.0% at 25 μg/ml to 8.8% at 700 μg/ml. Unlike cefonicid, cefuroxime exhibited concentration-dependent binding in adult serum over the entire therapeutic range. The extent of fractional binding found within the therapeutic range (88 to 40%) in the present study was somewhat greater than the previously reported values of 50 to 33% (2, 9). This result may be due to the assessment of cefuroxime protein binding at lower concentrations in the present study. Like cefonicid, cefuroxime was found to bind to a high-affinity ($K_1 = 7.1 \times 10^4$ M^{-1}), saturable binding site as well as to a low-affinity ($N_2K_2 = 195$ M^{-1}) binding site that was not saturated over the range of drug concentrations studied. Interestingly, however, the number of binding sites per albumin molecule was only 0.15. The number of binding sites on the albumin molecule for other cephalosporins (19), including cefonicid, has been shown to be 1. These results suggest that the protein responsible for the high-affinity binding of cefuroxime may not be albumin but rather another serum protein. The binding capacity for this class of binding site was 1.0×10^{-4} M. Another possible explanation for N_1 being less than 1 is that serum contains compounds that noncompetitively inhibit cefuroxime protein binding.

There was a marked contrast between adult and neonatal sera in the fraction of cefuroxime bound to serum proteins (Fig. 2). Cefuroxime binding in neonatal serum was concentration independent, averaging 15.6% over the entire concentration range studied. The high-affinity binding site demonstrated in adult serum was not present in neonatal serum. The relatively low level of constant fractional binding of cefuroxime in neonatal serum indicates a binding site with a large capacity but a low affinity. The values for the nonspecific binding parameter, N_2K_2, were similar for neonatal and adult sera; thus, the levels of fractional binding at high drug concentrations, at which the high-affinity binding site is saturated in adult serum, were similar for both types of sera. The percentage of cefuroxime bound to neonatal serum within the therapeutic range was lower than that for adult serum because of the lack of the high-affinity binding site in neonatal serum. This result further suggests that albumin is not the serum protein responsible for the high-affinity binding of cefuroxime in adult serum. The absence of a high-affinity binding site in neonatal serum suggests that the protein responsible for this binding is not developed in neonates or that neonatal serum contains an endogenous compound (22) that competitively inhibits the association of cefuroxime with protein.

The differences in protein binding of these cephalosporins between adult and neonatal sera are important for several reasons. One is the potential for drug interactions. When two or more molecules compete for the same binding site and the concentration of one or more of them approaches the saturation point for that particular binding site, the potential for displacement of one substance by another exists. For drugs such as the cephalosporins, the large therapeutic concentration range decreases the likelihood of any such interaction becoming clinically important. However, with substances such as bilirubin, such an interaction can be potentially devastating in neonates. In the selection of drugs for use in neonates, one must consider protein binding and whether such binding is likely to interfere with the protein binding of bilirubin. In neonatal serum, cefonicid exhibited saturation of binding sites in the therapeutic range. Cefonicid has been shown to compete with bilirubin for albumin binding sites in vitro (16). The risk of a clinically significant interaction occurring between cefonicid and bilirubin in neonates appears high. In contrast, cefuroxime showed no saturation of protein binding sites at the concentrations studied and would not therefore be expected to displace bilirubin significantly. The latter finding has been confirmed by others (16). When a decision regarding the appropriate antibiotic choice in neonatal infections must be made, it may be prudent to choose an agent that is less extensively bound to albumin.

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