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CEFTRIAXONE BINDING TO HUMAN SERUM ALBUMIN

INDIRECT DISPLACEMENT BY PROBENECID AND DIAZEPAM

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Abstract—*In vitro* protein binding studies were conducted to examine the interaction between ceftriaxone (CEF), probenecid (PROB) and diazepam (DIAZ). The presence of PROB and DIAZ at concentrations equal to molar albumin concentration caused a decrease in CEF affinity from $3.7 \times 10^4\ M^{-1}$ (control) to 1.1×10^4 (PROB) and 2.6×10^4 (DIAZ) M^{-1}, but not in binding capacity in pooled human plasma. PROB and DIAZ at five times the molar albumin concentration also caused a decrease in CEF affinity from $4.5 \times 10^4\ M^{-1}$ (control) to 0.45×10^4 (PROB) and 3.0×10^4 (DIAZ) M^{-1} in isolated human serum albumin. DIAZ and PROB displaced one another, confirming their common binding site (Site II, the benzodiazepine site) on serum albumin. By contrast, CEF was unable to displace either PROB or DIAZ from defatted albumin. In the presence of elevated free fatty acid concentrations (four times the albumin concentration), CEF decreased the binding of both drugs. CEF free fraction (fp) in isolated human serum albumin (CEF fp = 7.7%) was increased by drugs which bind to Site I: sulfisoxazole (CEF fp = 68.1%), warfarin (CEF fp = 56.0%) and furosemide (CEF fp = 55.0%). At ten times the molar concentration of albumin, CEF displaced both warfarin (warfarin fp from 0.99 to 2.20%) and phenytoin (phenytoin fp from 17.7 to 23.4%) from defatted albumin. CEF appeared to bind to Site I (the warfarin site) on human serum albumin, and was displaced by PROB and DIAZ via a mechanism which did not involve direct competition at a common binding site.

Ceftriaxone is a third generation cephalosporin possessing broad antimicrobial activity [1–4]. Ceftriaxone is unique among beta-lactam antibiotics in that it possesses a long biological half-life ($T_{1/2}$ = 8 hr). The long $T_{1/2}$ is a result of the limited renal tubular secretion and high degree of plasma protein binding of ceftriaxone [3, 4].

Recently [5], we reported on the interaction of ceftriaxone and probenecid. Probenecid effectively blocks both tubular secretion and biliary excretion of weak acids [6–8], and has been used to prolong the time course of beta-lactam antibiotics [6]. Somewhat unexpected results, however, were found with the ceftriaxone–probenecid study. Probenecid shortened the $T_{1/2}$ of ceftriaxone rather than prolonging it [5]. On closer examination, probenecid partially blocked both the renal (the portion due to active tubular secretion) and non-renal (biliary excretion) clearance of unbound ceftriaxone. However, the total clearance of ceftriaxone increased due to an increase in the plasma free fraction (fp) of ceftriaxone. Preliminary binding studies suggested that probenecid displaced ceftriaxone by a simple competitive mechanism. Previous studies have shown that probenecid displaces indomethacin [8] and methotrexate [9] from their protein binding sites.

The purpose of the present study was to characterize the protein binding interaction between ceftriaxone and probenecid. Since probenecid binds to the benzodiazepine site (Site II) on albumin [10], a characterization of a potential interaction between diazepam and ceftriaxone was also undertaken.

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MATERIALS AND METHODS

Chemicals. Radiolabeled [^{14}C]probenecid (30.1 mCi/mmol; 99% radiochemically pure) and diazepam (55.7 mCi/mmol; 99% radiochemically pure) were synthesized at Hoffmann–La Roche (Basel). Radiolabeled [^{14}C]warfarin (46 mCi/mmol; 97% radiochemically pure) was obtained from Amersham (Arlington Heights, IL) and [^{3}H]phenytoin (46 mCi/mmol; 98% radiochemically pure) was obtained from New England Nuclear (Boston, MA). Human serum fatty acid free albumin (FAF; Fraction V, fatty acid free) and Fraction V human serum albumin (HSA; Fraction V) were obtained from Miles Scientific (Naperville, IL). Diazepam (DIAZ), furosemide, warfarin, salicylic acid, phenytoin, probenecid (PROB), sulfisoxazole and ceftriaxone (CEF) were obtained from Hoffmann–La Roche. Oleic acid was obtained from the Sigma Chemical Co. (St. Louis, MO). Plasma was obtained from four healthy volunteers.

Protein binding methods. Protein binding was determined by equilibrium dialysis methods established previously for CEF, phenytoin, DIAZ, and warfarin [3, 11, 12] and a method established herein for PROB. Teflon or plexiglass dialysis cells and presoaked dialysis membranes with a molecular weight cut-off of 10,000 daltons were used. The conditions of the dialysis procedure (e.g. equilibration time, concentration range) have been established previously. No significant volume shifts have been noted for this system and conditions in our laboratory.

Ceftriaxone Rosenthal studies. Human plasma and fatty acid free albumin (FAF: 40 g/L in phosphate

buffer) containing various CEF concentrations were used to generate Rosenthal plots [13]. To aliquots of these protein solutions, DIAZ or PROB was added to achieve concentrations that were multiples of the molar albumin concentration. Replicates (N = 2–4) of each plasma sample or albumin solution were dialyzed against an equal volume (1 mL) of isotonic phosphate buffer (0.13 M; pH 7.4). The cells were rotated at 37° for 4 hr. Aliquots from both sides of the membrane were removed and frozen until analyzed.

Displacement studies. Replicates (N = 2–4) of each plasma sample, albumin (HSA; 40 g/L in phosphate buffer) or fatty acid free albumin (FAF) containing the displacing agent (e.g. PROB, DIAZ, CEF) at 0, 1, 5 or 10 times the molar albumin concentration were used. Replicates (N = 2–4) of each plasma or albumin solution were dialyzed against phosphate buffer containing the drug to be displaced [CEF (80 μg/mL) or radiolabeled DIAZ (4.5 μg/mL), PROB (4.5 μg/mL), warfarin (2 μg/mL) or phenytoin (10 μg/mL)]. Post-dialysis samples of both buffer and protein solutions were removed for subsequent analysis.

Analysis. CEF concentrations were determined in buffer, plasma and protein solutions by an established HPLC method [14]. Post-dialysis concentrations of radiolabeled DIAZ, PROB and phenytoin were determined by liquid scintillation counting. Warfarin concentrations in buffer and protein solutions were also measured via liquid scintillation counting following a TLC isolation procedure [11].

Pre-dialysis albumin concentrations were determined by Bromcresol green [15] with total protein analysis by the Biuret method [16].

Statistics. CEF binding parameters were obtained from simple linear regression of Rosenthal plots [13], each plot contained ten data points. A test of parallel lines [17] was performed to identify statistically significant differences in the slope (i.e. affinity constant) of the Rosenthal plots. In addition, 95% confidence intervals around the estimates of the binding parameters were constructed [17].

One-way analysis of variance (ANOVA) was used in the simple displacement studies to establish a statistically significant influence of displacer concentration on the binding of DIAZ, PROB or CEF. Two-way ANOVA was used to assess the impact of free fatty acid and CEF concentration on the binding of PROB and DIAZ. If an F-ratio was found to be significant, a subsequent *t*-statistic was computed for pairs of treatment means to establish which treatments were different from one another. Throughout the data analysis, statistical significance was established at the $P < 0.05$ level.

RESULTS

CEF displacement from plasma proteins by PROB and DIAZ. The influence of PROB on the binding of CEF to plasma proteins is shown in Fig. 1. CEF appeared to interact with one class of binding sites. Statistical analysis revealed a significant decrease in the slopes of the Rosenthal plots with increasing PROB concentration (Table 1). The K_a of CEF decreased from 3.81×10^4 M^{-1} (control) to 1.08×10^4 M^{-1} [PROB at 0.9 M × ALB (0.9 × ALB)]. DIAZ also decreased the binding of CEF to plasma proteins (Table 1). DIAZ caused a significant decrease in the K_a values of CEF, from 3.65×10^4 M^{-1} (control) to 2.58×10^4 M^{-1} (PROB at 1.2 × ALB). Binding capacity parameters for CEF (nP, Table 1) were unaffected by either PROB or DIAZ.

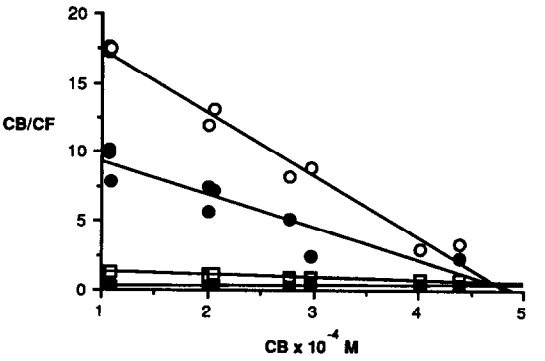

Fig. 1. Representative Rosenthal plots for the protein binding of CEF in human plasma in the absence and presence of increasing concentrations of PROB. Displacer concentrations are expressed as molar equivalents of albumin: (○) control, (●) PROB 1 × ALB, (□) PROB 5 × ALB, and (■) PROB 10 × ALB.

CEF displacement from albumin by PROB and DIAZ. Graphically the results of the influence of PROB and DIAZ on the binding of CEF to fatty acid free albumin (FAF) were similar to the plasma data. Analysis of the CEF–FAF interaction revealed a significant decrease in the slope of the Rosenthal plot in the presence of either PROB or DIAZ (Table 1). The K_a values of CEF decreased from 4.52×10^4 M^{-1} (control) to 0.45×10^4 M^{-1} (PROB at 5 × ALB). Although not as large as for PROB, DIAZ caused a significant decrease in the apparent K_a for CEF from 4.53×10^4 M^{-1} (control) to 1.87×10^4 M^{-1} (DIAZ at 10 × ALB).

PROB displacement from albumin by DIAZ and CEF. DIAZ decreased the binding of PROB to human FAF (Fig. 2). The mean fp of PROB increased from 2.4% (control) to 12.3% (DIAZ at 10 × ALB). Statistical analysis revealed that the differences for all of the DIAZ treatments were significant. CEF caused a small, but statistically significant increase in PROB fp in albumin (Fig. 2). The mean fp of PROB increased from 2.4% (control) to 2.9% (CEF at 10 × ALB) in albumin. All of the differences between the CEF treatments and the controls were statistically significant.

DIAZ displacement from albumin by PROB and CEF. PROB decreased the binding of DIAZ to human serum albumin (Fig. 3). The mean fp of DIAZ increased from 0.16% (control) to 6.2% (PROB at 10 × ALB) in albumin. The differences for all of the PROB treatments were statistically significant. CEF (5 and 10 × ALB) caused a small, but statistically significant increase in DIAZ fp in albumin (Fig. 3). The mean fp of DIAZ increased

Table 1. Ceftriaxone binding parameters in plasma or albumin solution in the presence and absence of various concentrations of probenecid or diazepam

Protein solution	Displacer	Displacer concn (M × ALB)	nP (M × 10^4)	K_a ($M^{-1} \times 10^{-4}$)
Plasma	Probenecid	0	4.45 [4.11–4.79]	3.81 [3.53–4.09]
		0.2	4.44 [4.18–4.72]	2.07* [1.94–2.19]
		0.4	4.16 [3.79–4.53]	1.63* [1.48–1.78]
		0.9	3.81 [2.66–4.96]	1.08* [0.76–1.40]
Plasma	Diazepam	0	5.34 [5.01–5.71]	3.65 [3.37–3.87]
		0.1	5.72 [5.51–5.93]	3.16* [3.03–3.29]
		1.2	5.46 [5.24–5.68]	2.58* [2.47–2.69]
Albumin	Probenecid	0	4.85 [4.28–5.42]	4.52 [3.95–5.09]
		1	4.37 [3.93–4.81]	2.93* [2.58–3.29]
		5	3.26 [2.78–3.74]	0.45* [0.37–0.53]
		10	Non-significant regression line	
Albumin	Diazepam	0	4.82 [4.55–5.09]	4.51 [4.26–4.80]
		1	4.57 [4.23–4.81]	4.90* [4.62–5.18]
		5	4.03 [3.73–4.33]	2.99* [2.74–3.24]
		10	4.12 [3.41–4.83]	1.87* [1.51–2.23]

Parameters were estimated from linear regression of Rosenthal plots (values in brackets represent the 95% confidence interval of parameter estimates).
* Significant difference in slope compared to control value.

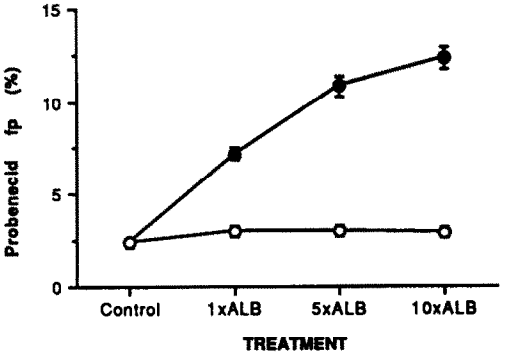

Fig. 2. Influence of DIAZ or CEF on the binding of PROB to an isolated, fatty acid free human serum albumin solution. Symbols represent mean (± one SD, N = 3) PROB free fraction values (fp). Displacer [DIAZ (●) or CEF (○)] concentrations are expressed as molar equivalents of albumin.

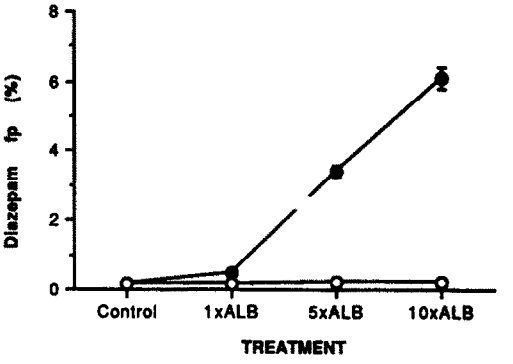

Fig. 3. Influence of PROB or CEF on the binding of DIAZ to an isolated, fatty acid free human serum albumin solution. Symbols represent mean (± one SD, N = 3) DIAZ free fraction values (fp). Displacer [PROB (●) or CEF (○)] concentrations are expressed as molar equivalents of albumin.

from 0.18% (control) to 0.21% (CEF at 10 × ALB) in albumin.

CEF displacement from albumin by various drugs. CEF was displaced by sulfisoxazole, warfarin and furosemide; salicylic acid and phenytoin also displaced CEF, but to a more limited extent (Fig. 4). The mean fp of CEF increased from a control value of 7.7% to a high of 68.1% (sulfisoxazole at 5 × ALB) and a modest increase to a value of 9.8% (phenytoin at 5 × ALB). Phenytoin solubility was limiting (actual phenytoin concentration was closer to 3 × ALB) and may have been responsible for the

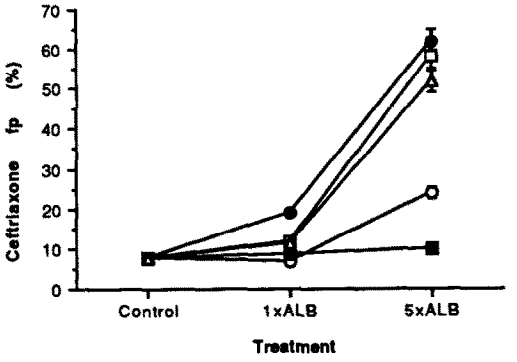

Fig. 4. Influence of a variety of drugs to displace CEF from an isolated albumin solution which was fatty acid free. Symbols represent mean (± one SD, N = 3) CEF free fractions values (fp). Displacer concentrations are expressed as molar equivalents of albumin: (○) salicylic acid, (●) sulfisoxazole, (□) warfarin, (■) phenytoin, and (△) furosemide.

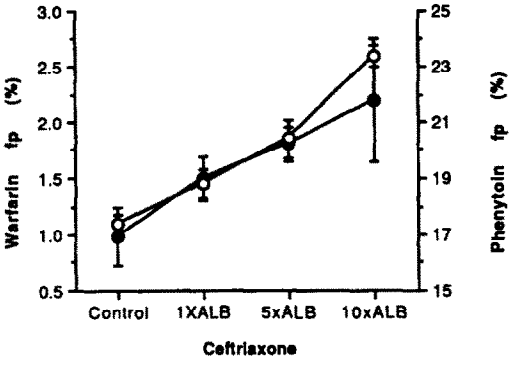

Fig. 5. Influence of CEF on the binding of warfarin (●) and phenytoin (○) to an isolated albumin solution which was fatty acid free. Symbols represent mean (± one SD, N = 3) drug free fraction values (fp). CEF concentrations are expressed as molar equivalents of albumin.

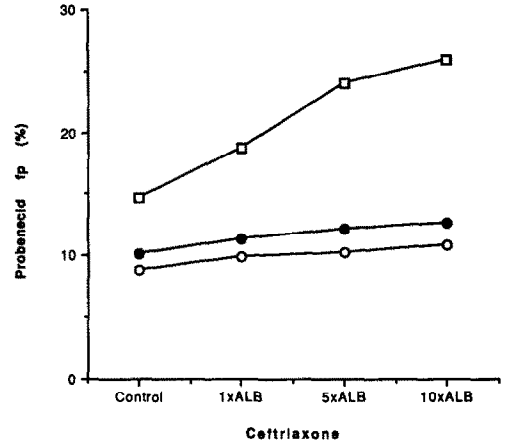

Fig. 6. Influence of CEF on the binding of PROB to an isolated albumin solution which was fatty acid free (○, FAF) or to the same FAF solution to which oleic acid had been added at one (●, FAF + ffa1) or four (□, FAF + ffa4) times the molar equivalents of albumin. Symbols represent mean PROB free fraction values (N = 3). CEF concentrations are expressed as molar equivalents of albumin.

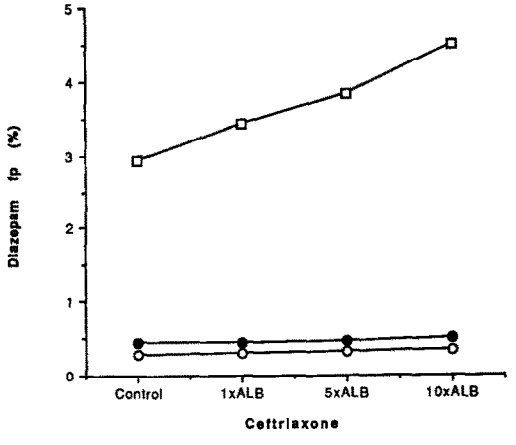

Fig. 7. Influence of CEF on the binding of DIAZ to an isolated albumin solution which was fatty acid free (○, FAF) or to the same FAF solution to which oleic acid had been added at one (●, FAF + ffa1) or four (□, FAF + ffa4) times the molar equivalents of albumin. Symbols represent mean DIAZ free fraction values (N = 3). CEF concentrations are expressed as molar equivalents of albumin.

diminished effectiveness of phenytoin in displacing CEF (Fig. 4).

Warfarin and phenytoin displacement from albumin by CEF. Warfarin was displaced by CEF from albumin; the mean warfarin fp increased from 0.99 to 2.20% in the presence of a CEF concentration 10 × ALB (Fig. 5). Phenytoin was also displaced by CEF (Fig. 5) with the mean fp increasing from 17.7% (control) to 23.4% (CEF at 10 × ALB concentration).

Influence of fatty acids on the binding of PROB and DIAZ. Two commercially available albumin preparations exhibited different behavior in their ability to bind PROB and DIAZ. FAF bound both PROB and DIAZ to a greater extent than the non-defatted albumin preparation (HSA) (data not shown). Moreover, CEF exhibited a greater degree of PROB and DIAZ displacement from HSA than from the FAF system.

The addition of oleic acid to FAF at concentrations that were one and four times the molar albumin concentration (+ffa1 and +ffa4 respectively) resulted in an increase in the mean control fp values for PROB and DIAZ (Figs. 6 and 7 respectively). Moreover, the greater the fatty acid content the more pronounced was the effect of CEF. In the higher oleic acid solution (+ffa4), the mean fp of PROB increased from 14.8% (control) to 25.8% (CEF at 10 × ALB) (Fig. 6), and the mean fp of DIAZ increased from 2.9% (control) to 4.5% (CEF at 10 × ALB) (Fig. 7). Two-way ANOVA revealed a significant influence of both free fatty acid and CEF concentration on mean PROB and DIAZ fp values, as well as a significant interaction between the two factors.

DISCUSSION

Although the binding of drugs to albumin has been examined for over 50 years, confusion remains with

regard to the actual number of independent binding sites and the potential for interaction between these sites. Reports have suggested that there exist at least three and perhaps as many as six or more independent sites with the major drug binding sites being the warfarin, diazepam and digitoxin sites [10, 18–22]. The warfarin site (Site I) is reported to be relatively broad and may actually include one (azapropazone) or more (azapropazone and bilirubin) subsets of binding sites [18, 19]. In contrast, the diazepam site (Site II) appears to be a single, narrowly defined site [10, 18–28]. Some investigators believe that the diazepam site is the primary free fatty acid binding site [28], whereas others maintain that it is not [18, 19].

The presence of these binding sites have been established by the displacement of one drug by another. Such observations have been viewed as evidence for the occupation of a common binding site. However, this hypothesis assumes that the displacement occurs via a simple competitive mechanism involving a single class of binding sites. While appropriate for some cases, other mechanisms (i.e. multiple binding sites and conformational albumin changes) cannot be ruled out in these experiments.

Albumin has been called a "breathing" molecule [23]. The tertiary and quarternary structures of albumin are not rigid and can be influenced by a number of factors (e.g. pH, Cl^- and albumin concentration) [23–25]. One such effect, known as the N → B transition, is decidedly influenced by pH [23–25]. The B conformation causes drugs to bind more avidly to both the warfarin (Site I) and diazepam (Site II) binding sites than the N form of albumin, with the warfarin site being more sensitive to this transition [25]. A number of researchers [25–29] have shown that the presence of medium chain-length free fatty acids (C8-C18) produced a "stabilizing" conformational change of albumin such that at low free fatty acid/albumin ratios (<2) the binding of Site I drugs was enhanced, while the binding of Site II drugs decreased [26, 27]. Higher free fatty acid concentrations (free fatty acid/albumin ratio > 2) caused diminished binding at both sites [25–27]. Wanwimolruk and Birkett [25] maintain that the conformational changes induced by free fatty acids and the N → B transition are different, but have similar and additive effects at Site I. The presence of other drugs may also alter the binding capability of albumin [30–35].

In our original *in vivo* interaction study [5], the displacement of ceftriaxone from plasma proteins by probenecid was unexpected since probenecid was thought to bind to the diazepam binding site (Site II) on albumin [10, 18, 19], while many of the beta-lactam antibiotics appear to bind to the warfarin site (Site I) [10, 18]. The interaction of ceftriaxone with bilirubin [36] would also suggest that ceftriaxone binds to the warfarin site on HSA. However, the present Rosenthal analyses of ceftriaxone binding in the presence of probenecid or diazepam was consistent with a hypothesis of competitive inhibition at Site II on albumin. Both probenecid and diazepam caused a decrease in the apparent affinity of ceftriaxone whereas binding capacity remained largely unaffected.

Reliance on simple binding displacement studies or graphical analysis to identify the mechanism of interaction (i.e. competitive or non-competitive) has been reported to be, at best, suggestive [31]. Like the present ceftriaxone interactions, Zini *et al.* [30] observed that indomethacin diminishes the affinity of warfarin for albumin while minimally affecting the capacity constant. This observation together with the lack of indomethacin displacement by warfarin was taken as evidence for a conformational change in the structure of albumin. Other drugs believed to bind to the diazepam site of albumin [10, 18, 19] also can displace warfarin [33–35].

A similar mechanism appears likely for the ceftriaxone–probenecid/diazepam interactions since ceftriaxone was ineffective at displacing either agent from albumin. Moreover, ceftriaxone displaced drugs known to bind solely (warfarin, sulfisoxazole and furosemide) or in part (salicylic acid and phenytoin) to the warfarin site [10, 18–20]. The displacement of warfarin and phenytoin by ceftriaxone confirms the hypothesis that ceftriaxone binds to the warfarin site rather than the benzodiazepine site on albumin. Ceftriaxone displacement by probenecid and diazepam, therefore, is consistent with a conformational change in albumin (and altered drug binding at Site I) brought about by the occupation of binding Site II by these two drugs.

Interestingly, the mechanism of the ceftriaxone-probenecid displacement was not as evident when these same studies were performed in whole plasma (data not shown) or in albumin solutions in the presence of elevated free fatty acids. As stated earlier, free fatty acids exert multiple effects on drug binding to albumin. In the present study, the binding of probenecid and diazepam decreased as the concentration of free fatty acids increased, supporting the hypothesis that free fatty acids compete directly at the benzodiazepine site [28]. A second effect of free fatty acids in the present study observed at the higher molar ratios was a greater decrease in the binding of probenecid and diazepam in the presence of increasing concentrations of ceftriaxone. This observation suggests that ceftriaxone diminished the binding of probenecid and diazepam indirectly. Ceftriaxone may have displaced free fatty acids from Site I, resulting in even more fatty acids available to compete with probenecid and diazepam for binding at Site II. To our knowledge, such a cascade mechanism has not been proposed for a specific drug–drug interaction, although this type of interaction would be consistent with the more general concern of binding variability of albumin samples from different sources [23]. This type of interaction undoubtedly adds to the complexity of interpreting drug displacement studies.

The clinical implications of the present studies are limited. Given the long $T_{1/2}$ of ceftriaxone, concomitant administration of probenecid is not therapeutically relevant. Moreover, the displacement of ceftriaxone by diazepam is also unlikely to occur in a clinical setting since diazepam would have to be present at concentrations (>100 μg/mL) which greatly exceed those concentrations (<1 μg/mL) encountered following pharmacological doses of diazepam. By contrast, ceftriaxone at higher therapeutic

doses (>1 g/day) may act as a competitive displacer of other drugs, or endogenous ligands binding at the warfarin site [36]. However, it should be noted that the impact of drug displacement from plasma proteins is usually limited to their pharmacokinetic profile and is rarely of clinical relevance [37, 38].

In conclusion, the original intent of these binding studies was to characterize more fully the observed *in vivo* interaction between ceftriaxone and probenecid. Evidence presented herein suggested that ceftriaxone binds to the warfarin site on albumin and that its displacement by probenecid (and diazepam) was through a conformational change in albumin which subsequently caused a change in the affinity of the warfarin (ceftriaxone) binding site. In the process of characterizing this interaction, several fundamental principles were re-examined. First, the reliance on Scatchard or Rosenthal analysis, or simple displacement studies without reverse displacement studies to confirm the mechanism can be misleading. Second, the dynamic nature of the albumin molecule (i.e. conformational changes due to free fatty acids, other molecules bound to various sites) may make the interpretation of the drug interactions very difficult and may contribute to the variability observed from laboratory to laboratory. Third, a cascade mechanism of displacement (ceftriaxone → free fatty acid → probenecid) may also add to the complexity of studying drug–drug binding interactions.

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