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Human Albumin Binding of Tamoxifen in the Presence of a Perfluorochemical Erythrocyte Substitute

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Abstract—The binding of tamoxifen (an HSA site IV ligand) to human serum albumin (HSA) in the presence of a perfluorochemical (PFC) erythrocyte substitute has been examined. Standard centrifugation followed by supernatant ultrafiltration was used to study the binding of 0·1 and 0·5 $\mu g\ mL^{-1}$ tamoxifen at ambient conditions. Tamoxifen was extensively bound (>99%) to the PFC emulsion through an association with the emulsifiers of the droplets. Tamoxifen was also extensively bound (>99%) to HSA. The percent free tamoxifen increased upon HSA dilution. Tamoxifen was extensively bound by various mixtures of HSA and the PFC emulsion and the percent free drug was similar to those obtained with HSA alone. However, the position of drug binding (PFC emulsion vs HSA) varied significantly with changes in the ratio of PFC emulsion to HSA. This could be important in terms of the different distribution of HSA and PFC emulsion in the body. Studies with PFC emulsion components indicated that any displacement of HSA-bound tamoxifen by the PFC emulsion was due to the oleic acid and, to a much smaller degree, Pluronic F-68 components. Other HSA site IV ligands are expected to be similarly displaced.

The lack of donor blood and possible complications with blood transfusions have resulted in an increasing interest in developing suitable erythrocyte substitutes for a variety of medical applications (Ravis et al 1991). Perfluorochemicals (PFCs) are efficient solvents for gases and are among the most inert materials ever invented (Riess & LeBlanc 1988). The first generation of PFC emulsions includes 20% Fluosol-DA, developed by the Green Cross Corporation (Osaka, Japan) in 1977 (Lowe 1988). This was the first PFC emulsion investigated in man (Vercellotti & Hammerschmidt 1988). Recently, this emulsion was approved for use in preventing myocardial ischaemia during percutaneous transluminal coronary angioplasty (Anonymous 1990).

PFC emulsions may alter the pharmacokinetics of administered drugs (Ravis et al 1991); the plasma transport of drugs may be altered through dilution of plasma proteins, PFC emulsion binding of drug, or displacement of plasma protein bound drug by PFC emulsion components. The effect of a PFC emulsion on ligand binding by site I on human serum albumin (HSA) was examined using warfarin (Parsons et al 1985; Parsons 1987). Warfarin was weakly bound by the PFC emulsion, but the percent free warfarin increased when PFC emulsion was added to HSA solutions (Parsons et al 1985). The increase was due to HSA dilution and warfarin displacement by the oleic acid and, to a much smaller degree, Pluronic F-68 emulsion components (Parsons & Nadkarni 1987).

In similar studies with diazepam (Graben & Parsons 1988) and L-tryptophan (Sathe & Parsons 1990) (HSA site II-specific ligands) the percent free ligand increased in mixtures of HSA with PFC emulsion. In addition to the HSA dilution effect, the increases were due to displacement of HSA bound ligand by the oleic acid and, to a small extent, Pluronic F-68 components of the emulsion. In salicylate (HSA sites I and II) studies, displacement of HSA bound salicylate was also observed in addition to the HSA dilution effect (Parsons & Sathe 1991). This displacement was due only to the oleic acid component of the emulsion.

The present study has further evaluated PFC emulsion effects on drug transport using an HSA binding site-specific approach. Tamoxifen was used as a marker for binding site IV on HSA (Sjoholm 1986).

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Materials and Methods

The 0·1 M, pH 7·4±0·02 buffer containing Na_2HPO_4 and KH_2PO_4 (Fisher Scientific Co., Fair Lawn, NJ, USA) was prepared immediately before use with triple distilled water and passed through a 0·22 μm filter. Albumin solutions were prepared with essentially fatty acid-free (<0·005%) HSA (Sigma Chemical Co., St Louis, MO, USA) to which one mol of oleic acid (sodium salt, Sigma Chemical Co., St. Louis, MO, USA) per mol of HSA was added to simulate physiological conditions (Guyton 1981). All studies employed two concentrations of tamoxifen (Amersham, Arlington Heights, IL, USA), the average therapeutic concentration of 0·1 $\mu g\ mL^{-1}$ (McEvoy 1987) and 0·5 $\mu g\ mL^{-1}$. Tamoxifen was quantified using a trace amount of [*N*-methyl-^{3}H]tamoxifen (96·4% pure) with a specific activity of 220 $mCi\ mg^{-1}$ (Amersham, Arlington Heights, IL, USA) and liquid scintillation counting (LS-5000 TD, Beckman Instruments Inc., Fullerton, CA, USA).

The frozen PFC stem emulsion (20% Fluosol-DA) was used as received. Since the bicarbonate buffer of the annex solutions does not provide adequate buffering capacity (Tomera & Geyer 1982) it was replaced with 0·1 M phosphate buffer. The particle size of this emulsion is 0·1–0·2 μm (Yang et al 1984).

The PFC emulsion component studies were conducted with glycerin (Fisher, Fair Lawn, NJ, USA) alone, Pluronic F-68 (BASF-Wyandotte, Parsippany, NJ, USA) alone, egg yolk phospholipids (Hepar, Franklin, OH, USA) plus Pluronic F-68 to enhance buffer solubility, oleic acid plus

Pluronic F-68 (also for solubility reasons), and a combination of all components. Component solutions contained 100% of the emulsion content except for phospholipids, where 1% of the emulsion content was used due to poor buffer solubility.

Tamoxifen partitioning into the pure PFC liquid was studied by removing all other components of the emulsion by extraction (Parsons 1986). Due to the poor buffer solubility of tamoxifen, a 1 : 1 mixture of buffer and anhydrous ethanol was used to prepare these solutions. A mixture of 0·5 mL each of this tamoxifen solution and the PFC liquids was vortexed intermittently for 30 min and then centrifuged for 30 min at 1475 *g*. This experiment was repeated with sample agitation on a mechanical shaker for 4 h. Tamoxifen in each layer was quantified.

Binding studies were conducted in the presence of PFC emulsion alone, HSA alone, mixtures of PFC emulsion with HSA, and HSA with PFC emulsion components. Studies utilized standard centrifugation followed by supernatant ultrafiltration using disposable devices (Centrifree with YMT membrane, Amicon Corp., Danvers, MA, USA). The corrected counts min^{-1} of samples from the glass centrifuge tubes and plastic ultrafiltration reservoirs were compared with those from the stock tamoxifen solutions to determine if tamoxifen loss had occurred. A rinse ultrafiltrate was then obtained followed by three successive ultrafiltrates (at least 125 μL each) using a centrifugal force of 480 *g*. Samples were obtained from the rinse ultrafiltrate and each of the subsequent ultrafiltrates. The radioactivity for the rinse and subsequent ultrafiltrates were compared for any significant difference due to tamoxifen adsorption by the ultrafiltration membrane and the O-ring. All experiments were conducted in quadruplicate at ambient room temperature (22.9 ± 0.6°C) and comparisons of data were based on one way analysis of variance. In all cases, the 5% level of significance ($P < 0{\cdot}05$) was used.

Results

In all tamoxifen binding studies the loss of tamoxifen to glass centrifuge tubes and ultrafiltration reservoirs was negligible. The concentration of free tamoxifen was not significantly different in any of the three successive ultrafiltrates. Thus, obtaining a rinse ultrafiltrate compensated for any non-specific tamoxifen loss to the ultrafiltration membrane and the O-ring. This also demonstrated that any unsettled emulsion droplets following centrifugation or the increase in HSA concentration during ultrafiltration did not significantly alter the percent free drug. The mean concentration of tamoxifen in the three successive ultrafiltrates was used to calculate the percent free drug.

Tamoxifen was extensively (> 99%) bound by the PFC emulsion (Table 1). The very small percent free tamoxifen generally increased upon PFC emulsion dilution and was independent of drug concentration. Since tamoxifen was extensively bound by the PFC emulsion, the partitioning of tamoxifen from a hydroethanolic solution into pure PFC liquids was evaluated. Tamoxifen did not partition into the pure PFC liquids. Thus, PFC emulsion bound tamoxifen was associated with the emulsifiers present on the emulsion droplets.

Table 1. The effect of PFC emulsion concentration on the concentration of free tamoxifen.

% v/v of PFC emulsion	Tamoxifen ($\mu g\ mL^{-1}$)	Mean (s.d.) percent free tamoxifen
25	0·1	0·80 (0·03)
25	0·5	0·80 (0·05)
50	0·1	0·69 (0·02)
50	0·5	0·73 (0·02)
75	0·1	0·83 (0·07)
75	0·5	0·95 (0·08)
100	0·1	0·54 (0·06)
100	0·5	0·56 (0·08)

Table 2. The effect of human albumin (HSA) concentration on the concentration of free tamoxifen.

% v/v of 4% HSA solution	Tamoxifen ($\mu g\ mL^{-1}$)	Mean (s.d.) percent free tamoxifen
25	0·1	0·79 (0·04)
25	0·5	0·72 (0·05)
50	0·1	0·56 (0·03)
50	0·5	0·57 (0·02)
75	0·1	0·58 (0·03)
75	0·5	0·54 (0·04)
100	0·1	0·49 (0·03)
100	0·5	0·48 (0·04)

The results for tamoxifen binding by HSA (Table 2) indicated that tamoxifen was very highly (> 99%) bound by HSA. The very small percent free tamoxifen generally increased upon HSA dilution and was independent of drug concentration. Comparison of the HSA data (Table 2) with the PFC data (Table 1) showed that the drug may have a slightly greater overall binding by HSA.

Tamoxifen was also extensively bound by mixtures of HSA and PFC emulsion (Table 3). The percent free drug was independent of tamoxifen concentration and did not greatly differ from those with HSA alone (Table 2). After standard centrifugation of the HSA and PFC mixtures, samples were obtained of the supernatant before ultrafiltration to obtain an approximate value of the percent of total tamoxifen associated with HSA and with the settled emulsion droplets. These results (Table 3) also demonstrated that tamoxifen had a higher overall affinity for HSA than for the PFC emulsion droplets. This was especially evident from the results at the 50:50 ratio of PFC emulsion and HSA. While the percent free drug in these mixtures did not vary greatly from those with HSA alone, the position of tamoxifen binding (PFC emulsion vs HSA) varied significantly with a change in the ratio of PFC emulsion to HSA.

With decreasing concentrations of HSA in the mixtures of HSA and PFC emulsion, the emulsion binding of tamoxifen increased (Table 3). Whether this was due to the overall affinity of the PFC emulsion for tamoxifen or displacement of HSA bound tamoxifen by a PFC emulsion component could not be determined from this data. For this reason, the effect of each emulsion component on the binding of tamoxifen by HSA was examined (Table 4). Compared with the 1% HSA control, glycerin had no effect on tamoxifen

Table 3. Tamoxifen binding by mixtures of 4% human serum albumin (HSA) solutions with PFC emulsion (PFCE).

% of PFCE	% of 4% HSA	Tamoxifen (μg mL^{-1})	% (s.d.) free tamoxifen	% bound tamoxifen	
				PFCE	HSA
25	75	0·1	0·56 (0·02)	9·63	89·81
25	75	0·5	0·57 (0·02)	8·78	90·65
50	50	0·1	0·61 (0·02)	23·58	75·82
50	50	0·5	0·60 (0·03)	22·70	76·70
75	25	0·1	0·57 (0·04)	84·17	15·26
75	25	0·5	0·64 (0·06)	84·10	15·26

Table 4. The effect of PFC emulsion components on tamoxifen binding by human albumin (HSA).

Component[a]	Mean (s.d.) percent free tamoxifen	
	0·1 μg mL^{-1} tamoxifen	0·5 μg mL^{-1} tamoxifen
1·0% HSA control	0·79 (0·04)	0·72 (0·05)
Glycerol	0·83 (0·03)	0·85 (0·03)[b]
Pluronic F-68	1·13 (0·20)[b]	1·02 (0·05)[b]
Pluronic F-68 + phospholipids	0·95 (0·04)	0·98 (0·06)
Pluronic F-68 + oleic acid	2·19 (0·26)[b,c]	2·11 (0·18)[b,c]
All components combined	1·55 (0·07)[b,c,d]	1·61 (0·15)[b,c,d]

[a] All results are with 1·0% HSA and component concentrations of 100% (1% for phospholipids) of the emulsion content. [b] Significantly different from 1·0% HSA control ($P<0·05$). [c] Significantly different from Pluronic F-68 alone ($P<0·05$). [d] Significantly different from Pluronic F-68 plus oleic acid ($P<0·05$).

binding at 0·1 μg mL^{-1} tamoxifen, but had a significant and unanticipated displacement effect at 0·5 μg mL^{-1}. This displacement could be due to an interaction of glycerin with HSA or a change in solvent dielectric constant. However, this apparent displacement may have been coincidental since such a small change (0·13) in percent free drug is difficult to measure accurately.

Pluronic F-68 alone resulted in a small, but significant displacement effect at both tamoxifen concentrations (Table 4). No significant change in the percent free tamoxifen resulted from the addition of egg yolk phospholipids plus Pluronic F-68 compared with the addition of Pluronic F-68 alone. Pluronic F-68 combined with oleic acid caused significant displacement of HSA-bound tamoxifen (Table 4) compared with the effect of Pluronic F-68 alone; the percent free tamoxifen was twice that observed with Pluronic F-68 alone. The use of the four components combined resulted in a slightly smaller displacement effect than the use of Pluronic F-68 plus oleic acid.

Discussion

PFC emulsions are drugs with potentially wide ranging diagnostic and therapeutic uses beyond that of respiratory transport. The intravascular half-life of 20% Fluosol-DA is dose-dependent and highly variable (Lowe 1988). Thus, the determination of PFC emulsion effects on the disposition of co-administered therapeutic agents is potentially of great importance in designing appropriate dosage regimens for drugs in the presence of these agents. In the present study tamoxifen was selected to determine the effect of a PFC erythrocyte substitute on the transport of a drug specifically bound by site IV on HSA. Studies were conducted at ambient room temperature since drug binding to specific sites on HSA has normally been studied at this temperature (Sjoholm et al 1979; Sudlow et al 1975, 1976). While caution is required in extrapolating these results to those expected at body temperature, such studies have correlated well with studies conducted in-vivo (Kober et al 1978; Yoshikawa et al 1984).

Tamoxifen was very extensively bound (>99%) by the PFC emulsion through an association with the emulsifiers of the droplets. Thus, this or similar emulsions could potentially be used as drug delivery or removal systems for tamoxifen and similarly highly bound drugs. For reasons stated earlier, hydroxyethylstarch and other components of the annex solutions were not included in these studies. While the binding characteristics of tamoxifen to hydroxyethylstarch are unknown, any interaction in-vitro and in-vivo would be expected to be very minor relative to the extensive binding of tamoxifen by the PFC stem emulsion. The binding of various drugs to hydroxyethylstarch and other plasma substitutes has been shown to be negligible and not of clinical relevance in most cases (Borchardt et al 1987).

Tamoxifen was also extensively bound (>99%) by HSA alone. These results agree well with the reported binding of tamoxifen of greater than 98% (Lien et al 1989). HSA has approximately one binding site for tamoxifen with an estimated association constant of $1·3 \times 10^5$ M^{-1} (Lien et al 1989). The overall binding of tamoxifen was greater with HSA than with the PFC emulsion.

Tamoxifen binding by various mixtures of HSA and the PFC emulsion was also extensive (>99%). The percent free drug in the mixtures did not greatly differ from each other or from those obtained with corresponding concentrations of HSA alone. However, binding tamoxifen (PFC emulsion vs HSA) varied significantly with changes in the ratio of PFC emulsion to HSA. This could be important in terms of the different distributions of HSA and PFC emulsion in the body (Ravis et al 1991). Similar results on the varying distribution of bound drug were also obtained for diazepam (Graben & Parsons 1988) and propranolol (Fan & Parsons 1989; Parsons & Shih 1989) which are also highly bound by this PFC emulsion.

Finally, any direct or indirect displacement of HSA-bound tamoxifen in the presence of the PFC emulsion was largely attributed to the oleic acid component of the emulsion.

Pluronic F-68 also displaced HSA-bound tamoxifen, but only to a small extent. Other drugs bound by HSA binding site IV would also be expected to be displaced. Similar results were obtained for warfarin (a site I drug) (Parsons & Nadkarni 1987), diazepam (Graben & Parsons 1988) and tryptophan (Sathe & Parsons 1990) (site II drugs), and salicylic acid (a site I and II drug) (Parsons & Sathe 1991). Free fatty acids are known to have an inhibitory effect on the binding capacity of HSA (Tillement et al 1984). The decreased displacement of HSA-bound tamoxifen upon the addition of phospholipids (and glycerin) to Pluronic F-68 and oleic acid may be due to an increased attraction of oleic acid for the component micelles and away from HSA. Overall, it was apparent that oleic acid was the PFC emulsion component almost entirely responsible for any displacement of bound tamoxifen from site IV on HSA.

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