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Effect of a perfluorochemical emulsion on prednisolone binding by human albumin

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Perfluorochemical (PFC) liquids are immiscible with most other liquids but are good solvents for oxygen and carbon dioxide [1, 2]. This has led to the experimental use of emulsified PFC in blood loss and in the treatment of decompression sickness, sickle cell anemia, and myocardial and brain ischemia [3]. One PFC emulsion (Fluosol DA, 20%) is undergoing clinical trials in several countries and has been used in over 800 patients in Japan* and 240 patients in the U.S.A.†

In highly exchanged transfused rats, PFC emulsions alter the pharmacokinetics of phenytoin [4], aspirin and salicylic acid [5], antipyrine,‡ morphine [6] and penicillin, but not diazepam [7]. Little data exist on the direct interaction of PFC emulsions with drugs normally transported by plasma proteins. Whether PFC emulsions will assume this transport function or alter the normal binding of drugs by plasma proteins is unknown. Any emulsion effect may be dependent on the ionization state of the drug. Therefore, a basic study of drug binding by PFC emulsion and by human albumin in the presence of PFC emulsion was conducted. Prednisolone was chosen as a model nonionic drug.

Prednisolone is bound with high affinity but low capacity by transcortin, with low affinity but high capacity by albumin [8], and to a limited extent by α_1-acid glycoprotein [9]. Binding to albumin is linear and is especially important at prednisolone levels greater than 400 ng/ml [10]. Prednisolone is 51.3% bound by 4% fatty acid free human albumin at 37° [8].

Methods

Fraction V human albumin (HSA) and prednisolone (Sigma Chemical Co., St. Louis, MO, U.S.A.) as well as [^{3}H]prednisolone (Amersham, Arlington Heights, IL, U.S.A.) were utilized. The PFC emulsion (Fluosol DA, 20%, Green Cross, Osaka, Japan) was a gift from Alpha Therapeutic Corp., Los Angeles, CA, U.S.A. As used, it contained 14.0% perfluorodecalin, 6.0% perfluorotripropylamine, 2.7% Pluronic F-68, 0.4% yolk phospholipids, 0.032% potassium oleate and 0.8% glycerol, where all percentages are w/v. The average particle size is 0.1 to 0.2 μm [11]. All other chemicals were of reagent grade.

Experiments were conducted at ambient room temperature (23.4 ± 0.6°). Ultrafiltration was performed with disposable ultrafiltration tubes (Centrifree, Amicon Corp., Danvers, MA, U.S.A.). Perfluorochemicals were quantitated by gas chromatography (model 9500, Carle Instruments Inc., Fullerton, CA, U.S.A.) and prednisolone was quantitated by liquid scintillation counting (LS-150, Beckman Instruments, Inc., Fullerton, CA, U.S.A.) with quenching examined using an external standard.

All solutions were prepared immediately prior to use in a 0.1 M, pH 7.4, phosphate buffer composed of Na_2HPO_4, KH_2PO_4 and triple distilled water. Since the frozen stem emulsion of Fluosol DA, 20% should not be subjected to repetitive thawing and freezing [12], it was thawed once, divided into 10-ml portions, and refrozen in glass vials. Only the volume needed for a given experiment was subjected to rethawing. The stem emulsion is supplied with annex solutions of electrolytes, buffer and hydroxyethylstarch. These were replaced by the phosphate buffer to decrease the number of emulsion components which could be responsible for any observed changes in drug binding by HSA. The phosphate buffer was also necessary since the bicarbonate buffer of the annex solutions does not provide adequate pH control [13].

Prednisolone binding was determined by standard centrifugation followed by supernatant fraction ultrafiltration. Ultrafiltrate was obtained through centrifugation. PFC emulsion and 4% HSA solutions were diluted either with each other or with phosphate buffer. Samples (3 ml) of each solution, containing either 0.2 or 1.0 μg/ml prednisolone and a trace amount of [^{3}H]prednisolone, were placed in a glass tube and centrifuged at 1475 *g* to settle emulsion droplets. This is similar to the centrifugation of blood in the study of drug binding by erythrocytes. Directly subjecting the PFC emulsion to ultrafiltration results in partial blockage of the filter and prolonged filtration times. Centrifugation times varied from 10 to 20 min for the smallest to largest PFC emulsion concentrations examined. Two 25-μl samples of supernatant fraction were obtained for prednisolone quantitation. Approximately 1 ml of supernatant fraction was added to an ultrafiltration tube and filtered at 480 *g* in an ultracentrifuge to collect approximately 125 μl of rinse ultrafiltrate. Use of an ultracentrifuge was necessary for adequate temperature control. The rinse ultrafiltrate was discarded and the solution remaining in the ultrafiltration tube was removed and replaced with approximately 1 ml of fresh supernatant. This procedure compensated for an initial loss of prednisolone to the ultrafiltration membrane. Ultrafiltration was repeated to collect three successive ultrafiltrates of approximately 100 μl each. Two 25-μl samples of each ultrafiltrate were obtained for free prednisolone quantitation, and the collection cup was cleaned before collecting the next ultrafiltrate sample. Binding studies were conducted in quadruplicate.

To study prednisolone partitioning into the PFC liquids, all other constituents of the stem emulsion were removed by extraction. Samples (4 ml) of emulsion were centrifuged at 1475 *g* for 30 min, and the aqueous supernatant fraction was discarded. Methanol (8 ml) was added to break the emulsion and dissolve emulsifiers. After centrifugation at 181 *g* for 4 min, the methanol portion was discarded. This process was repeated with two 4-ml portions of methanol. The PFC interface was gently rinsed four times with 4-ml portions of chloroform. The PFC layer was then washed three times with 4-ml portions of anhydrous ethanol and four times with 4-ml portions of triple distilled water. The PFC mixture was examined by gas chromatography [14] and stored at −15°.

Prednisolone partitioning into the purified PFC was examined by mixing 0.5 ml each of PFC and phosphate buffer for 30 min on a vortex mixer. The buffer contained either 0.2 or 1.0 μg/ml prednisolone and a trace amount of [^{3}H]prednisolone. Samples were centrifuged at 1475 *g* for 30 min, and five 25-μl samples of both the buffer and PFC layers were obtained for prednisolone quantitation. This experiment was repeated with sample agitation on a shaker bath for 5 hr. Both experiments were conducted in quadruplicate for each prednisolone concentration.

Results and discussion

There was no detectable loss to the glass centrifuge tubes of either free prednisolone or prednisolone bound by HSA

* Anonymous, *F-D-C Reports*, June 13, T & G-3 (1983).

† Personal communication, Nov. 9, 1984, cited with permission of Laurie L. West, Alpha Therapeutic Corp.

‡ S. G. White, W. A. Wargin and R. P. Shrewsbury, *A.Ph.A. Acad. Pharm. Sci.* (Abstr.) **14**(2), 232 (1984).

and/or PFC emulsion. A previous study in this laboratory showed no detectable loss of either HSA or PFC emulsion to the ultrafiltration tubes. Prednisolone alone in buffer was also not significantly bound by the ultrafiltration tubes. However, the prednisolone concentration in the first ultrafiltrate was slightly low (approximately 95%) due to membrane binding and/or dilution with the glycerin used to preserve the membrane. Subsequent filtrates contained the original concentration of prednisolone. These results were confirmed in each binding study since there was no detectable difference in free prednisolone concentration in the three successive ultrafiltrates. This also demonstrates that the increase in HSA concentration during ultrafiltration had no detectable effect on the free prednisolone concentration. Thus, the mean prednisolone concentration in the three successive ultrafiltrates was used to determine the percent prednisolone free for each sample.

Prednisolone binding by 4% HSA solutions diluted with buffer served primarily as controls. Prednisolone concentration was the same before and after centrifugation of these samples since the centrifugal force employed does not cause albumin sedimentation. Results are presented in Table 1. Prednisolone was more strongly bound than previously reported [8], probably due to the lower temperature and/or type of HSA employed in the present study. The percent prednisolone free increased as HSA concentration decreased.

The PFC emulsion did bind prednisolone. After centrifugation the prednisolone concentration was lower in the supernatant fraction than in the original sample demonstrating prednisolone association with settled emulsion droplets. The free prednisolone concentration in the ultrafiltrates was consistently slightly less than the supernatant prednisolone concentration. This is due to prednisolone binding by small emulsion droplets not settled by centrifugation but retained by the ultrafiltration membrane. Results are presented in Table 1. The percent prednisolone free decreased as the percent v/v of PFC emulsion increased. The percent free appears to be independent of total prednisolone concentration at any given concentration of PFC emulsion. While prednisolone binding by the PFC emulsion was relatively weak, 75% v/v PFC emulsion bound approximately as much prednisolone as a 25% v/v solution of 4% HSA.

In mixtures of 4% HSA solution and PFC emulsion, the supernatant prednisolone concentration was always less than the original concentration. This difference increased as the percent v/v of 4% HSA solution decreased (e.g. supernatant concentration was 95 and 75% of the original for 75 and 25% v/v HSA solution respectively). This demonstrates significant prednisolone binding by settled emulsion droplets even in the presence of HSA. The percent prednisolone free in each mixture is presented in Table 1.

Comparison of the results presented in Table 1 also demonstrates significant prednisolone binding by PFC emulsion in the presence of HSA. The percent prednisolone free was statistically significantly lower ($P < 0.0005$) when HSA solutions were diluted with PFC emulsion rather than buffer, based on analysis of variance. The contribution of the PFC emulsion to overall prednisolone binding increased as the PFC emulsion concentration increased and HSA concentration decreased. Thus, the increase in free prednisolone was less than expected for simple dilution of HSA. However, PFC emulsion bound prednisolone less avidly than HSA so the net result of HSA dilution with PFC emulsion was still an increase in free prednisolone.

Changes in percent prednisolone free alone do not determine if a PFC emulsion component(s) is directly affecting prednisolone binding by HSA. Displacement of HSA bound prednisolone could be hidden by prednisolone binding by the PFC emulsion. To investigate this possibility, the equation $C_b = nkAP$, previously found applicable to prednisolone binding by HSA, was utilized [15]. C_b and A are the molar concentrations of bound and free prednisolone, respectively; k is the affinity constant; n is the number of binding sites; and P is the molar HSA concentration. Application to prednisolone binding by HSA alone yielded $nk = 4.55 \times 10^3 \pm 1.11 \times 10^2\ M^{-1}$. No trend of nk with HSA concentration was evident. The value of nk at 37° is approximately $2 \times 10^3\ M^{-1}$ [16]. Linear ligand binding by surfactant micelles is described by $C_b = CA$ [17]. C is analogous to nkP, but the molar concentration of the PFC-surfactant emulsion droplets cannot be defined. Application of this equation to binding of prednisolone by PFC emulsion alone yielded values of C of 0.281, 0.429 and 0.576 for 25, 50 and 75% v/v PFC emulsion respectively. Combining the equations for prednisolone binding by HSA alone and for binding by PFC emulsion alone (i.e. $C_b = nkAP + CA$) allows prediction of the percent prednisolone free in the mixtures of HSA and PFC emulsion. Results are presented in Table 1 along with the ratios of predicted to observed values. The observed and predicted values are similar, implying that no significant change in prednisolone binding parameters (nk and C) occurred upon mixing of PFC emulsion and HSA.

Prednisolone bound by the PFC emulsion could be partitioning into the liquid PFC core of the emulsion droplets and/or interacting with the emulsifiers. To determine if only one of these mechanisms is occurring, prednisolone partitioning into the PFC was examined. PFC from Fluosol DA, 20% rather than those obtained elsewhere was used since PFC purity has been a problem [18]. After extraction of emulsifiers, the yield of PFC was approximately 70% of the labeled amount. Gas chromatograms of the PFC liquids were similar before and after the extraction. In studies of prednisolone partitioning between PFC and buffer, pred-

Table 1. Prednisolone binding by solutions of human albumin, PFC emulsion and their mixtures

Prednisolone concentration (μg/ml)	% v/v when present		Percent prednisolone free			
	PFC emulsion	4% Albumin solution	PFC emulsion alone	4% Albumin solution alone	Mixtures of albumin solution and PFC emulsion	
					Observed	Predicted*
2	25	75	77.8 ± 1.4	33.6 ± 0.4	31.1 ± 0.4	30.7 (0.987)†
10	25	75	78.3 ± 1.0	34.0 ± 0.4	30.8 ± 0.6	30.7 (0.997)
2	50	50	70.1 ± 1.6	42.6 ± 1.0	33.8 ± 0.9	36.4 (1.08)
10	50	50	69.9 ± 1.0	42.2 ± 1.0	34.3 ± 0.7	36.4 (1.06)
2	75	25	62.5 ± 1.5	60.7 ± 1.3	47.3 ± 2.0	44.7 (0.945)
10	75	25	64.4 ± 0.8	60.9 ± 1.4	47.8 ± 1.0	44.7 (0.935)

* Based on binding by PFC emulsion alone and albumin alone.
† Ratio of predicted to observed.

nisolone was not detectable in PFC after 0.5 or 5 hr of agitation. Thus, PFC emulsion bound prednisolone is associated only with the emulsifiers of the emulsion droplets.

In summary, prednisolone was bound by the PFC emulsion through an interaction with emulsifiers. While this binding was relatively weak, prednisolone was significantly bound by the PFC emulsion even in the presence of HSA. This binding partially offset the increase in free prednisolone that occurred upon HSA dilution. Changes in prednisolone binding by HSA and the PFC emulsion upon mixing of their solutions appeared to be due to dilution alone.

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Department of Pharmacal Sciences DANIEL L. PARSONS
School of Pharmacy
Auburn University,
AL 36849, U.S.A.

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Complement-dependent stimulation of prostacyclin biosynthesis: inhibition by rosmarinic acid

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The complement system forms a defence mechanism concerned with recognition and elimination of microbes [1]. It consists of two cascades of sequentially activated serum proteins which converge at complement factor C3, from which activation may proceed through C5 to the lytic pathway. Activated complement components (C3a, C3b, C5a) mediate inflammatory processes, e.g. by causing chemotaxis, neutrophil activation, histamine release, increased vascular permeability, and by coating of the microbial cell wall with ligands for receptors on phagocytes [1]. In addition, we have shown that C5a and its metabolite C5a des Arg are potent stimuli of prostacyclin biosynthesis in rabbit mesothelial and endothelial cells [2, 3], and suggested that this could represent a mechanism for increasing local blood flow, thus promoting the development of an acute inflammatory response [2, 3].

Pharmacological modulation of the complement system could be of potential interest for the treatment or control of pathophysiological situations linked with complement activation [4]. Recently, it has been discovered that rosmarinic acid (2-[[3-(3,4-dihydroxyphenyl-1-oxo-2-propinyl]-oxy]-3-(3,4-dihydroxyphenyl)-propionic acid, Nattermann), a product extracted and purified from *Melissa officinalis*, inhibits the C3 convertase (C4b2a) of the classical pathway [5]. It is one of the few [4] compounds with *in vivo* activity, since rosmarinic acid (20 mg/kg i.v.) suppressed endotoxin-induced complement activation in a rabbit model of circulatory shock [6]. This was associated with less severe hypotension and thrombocytopenia and diminished generation of prostacyclin and thromboxane A_2 [6]. However, this experiment did not exclude the possibility that rosmarinic acid inhibited cyclo-oxygenase, prostacyclin or thromboxane synthase activities directly.

The purpose of the present experiments was to test whether rosmarinic acid suppressed activation of human serum complement by cobra venom factor (CVF), and whether this would be reflected in diminished stimulation of prostacyclin biosynthesis. CVF selectively activates the alternate pathway (consisting of factors B, D, C3b) by combining with Bb to form a stable C3 convertase (CVF, Bb) which can activate both C3 and C5 [1], in contrast to endotoxin which may activate both pathways as well as other defence systems. Rabbit peritoneal tissue, a rich source of prostacyclin [7] was used for incubation with the activated sera.

Serum from seven volunteers was pooled and made 1 M with ε-aminocaproic acid (fresh serum). An aliquot of this was heated at 56° for 15 min in order to inactivate complement factors C2 and B (heat-inactivated serum). Fresh and heat-inactivated serum were incubated with cobra venom factor (CVF, Sigma, 10 units/ml) for 120 min at 37° in the presence or absence of 1 mM rosmarinic acid. This concentration is based on the estimated initial plasma concentration achieved following i.v. administration of the non-toxic dose of 40 mg/kg, as extrapolated from pharmacokinetic studies in rats. Rosmarinic acid was used in purified crystalline form and dissolved in 0.9% NaCl by addition of 0.1 N NaOH to pH 7. Thereafter, the comp-