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Plasma Protein Binding of Prednisolone in Normal Volunteers and Arthritic Patients

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Summary. The plasma binding of prednisolone was studied in twenty normal volunteers and twenty rheumatoid arthritis patients. An in vitro assessment of the binding following the addition of prednisolone, prednisone, and hydrocortisone to the plasmas obtained from the subjects showed significant differences in the percentage of prednisolone bound. However, the differences observed were regarded as clinically insignificant. The plasma protein binding was determined by an in vitro equilibrium dialysis of the individual plasma samples at 37° C. Prednisolone levels on both sides of the dialysis membrane were determined using radioactivity and HPLC analytical methodologies. The percentages of prednisolone bound calculated from the analytical results of either the radiochemical or HPLC method were not significantly different. The change in the percentage of prednisolone bound to plasma proteins was studied as a function of the total prednisolone plasma concentration in a normal volunteer and in a systemic lupus erythematosis patient. As a result of prednisolone binding to both transcortin and albumin, the binding of prednisolone changes as a function of prednisolone concentration. The binding data were fitted using nonlinear least squares regression, and the affinity constants for the binding of prednisolone to transcortin and albumin were estimated.

Key words: prednisolone, prednisone, rheumatoid arthritis; plasma protein binding, transcortin, albumin

Prednisolone is a potent corticosteroid that is extensively used for the treatment of rheumatoid arthritis and various other diseases. Prednisone, on the other hand, is considered a pro-drug for prednisolone [1, 2], and is employed for similar purposes as prednisolone.

It is now recognized for many drugs [3], that the unbound fraction in the plasma constitutes the biologically active form of the drug. Thus, the determination of the free or bound portions of various drugs in the plasma has important implications concerning the drug's therapeutic activity [1]. However, disease states may modify the protein binding of a particular drug [4, 5]. Hence, changes due to the disease state may be reflected in a drug's pharmacological effect.

Prednisolone has been shown to bind to both albumin and transcortin [4, 6–10]. To quantify the extent of prednisolone binding to these plasma proteins, and to determine the effect of disease state on the plasma protein binding, a kinetic study of the total plasma concentration and fraction free is necessary.

A recent report by Rocci and Jusko [6] has shown that prednisolone binding to plasma proteins can be described by a two term function of the following form:

$$C_b = \frac{n_t K_t P_t C_f}{1 + K_t C_f} + n_a K_a P_a C_f \qquad (1)$$

where C_b and C_f are the bound and free concentrations of prednisolone, n is the number of binding sites per protein molecule, K is the association (binding) constant, and P is the protein concentration. The subscripts t and a refer to transcortin and albumin respectively. Thus the binding of prednisolone to plasma proteins is nonlinear with respect to transcortin and linear with respect to albumin. The transcortin binding sites are high affinity and low capacity whereas the albumin sites are low affinity but high capacity [5].

Equation [1] can be written in the form:

$$C_b = \frac{P(1)C_f}{P(2) + C_f} + P(3)C_f \qquad (2)$$

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Table 1. Albumin levels and sex of normal volunteers and arthritic patients

Normal volunteers			Rheumatoid arthritis patients			
Subject No.	Sex	Plasma albumin g/dl	Subject No.	Sex	Plasma albumin g/dl	Drugs[a]
1	M	4.3	21	F	3.2	1
2	M	4.3	22	M	3.5	2, 3
3	M	4.4	23	F	3.6	2, 3, 4, 5, 6
4	F	4.2	24	M	3.7	2
5	F	4.2	25	M	4.3	7, 8
6	F	4.0	26	F	3.3	3, 9, 10
7	F	3.9	27	M	3.7	2, 11
8	M	4.2	28	F	4.2	12
9	M	4.1	29	F	3.8	2, 7
10	M	4.1	30	M	3.9	2, 13
11	M	3.9	31	F	2.9	2, 7
12	M	4.1	32	F	3.8	2, 6, 14
13	M	4.2	33	F	3.9	15
14	M	4.1	34	F	3.6	2
15	F	4.3	35	F	3.8	2
16	F	4.1	36	F	3.9	7
17	M	4.3	37	F	4.4	
18	M	4.5	38	F	4.1	2, 3, 7, 11
19	F	3.8	39	M	4.0	2
20	M	4.3	40	M	3.9	2, 11

[a] Drugs that the arthritic patients were taking: 1 naproxen, 2 aspirin, 3 iron, 4 propranolol, 5 isosorbide dinitrate, 6 multiple vitamins, 7 ibuprofen, 8 amitriptyline, 9 fenoprofen, 10 folic acid, 11 prednisone, 12 triamcinolone, 13 cimetidine, 14 vitamin C, 15 choline salicylate

Table 2. Percentage of prednisolone bound[a] to plasma proteins

Normal volunteers				Arthritic patients			
	Percent bound				Percent bound		
Subject No.	1. run	2. run	Average	Subject No.	1. run	2. run	Average
1	65.9	74.6	70.3	21	73.3	76.2	74.7
2	69.5	78.3	73.9	21	70.3	70.2	70.2
3	68.1	76.2	72.2	23	75.6	79.7	77.7
4	68.4	75.5	72.0	24	74.9	70.4	72.6
5	68.2	78.4	73.3	25	75.0	74.8	74.9
6	69.8	71.5	70.7	26	71.8	73.9	72.9
7	68.1	69.3	68.7	27	72.7	74.2	73.5
8	71.4	73.5	72.5	28	77.5	78.0	77.7
9	70.5	72.4	71.5	29	73.1	73.5	73.3
10	68.6	69.7	69.2	30	72.1	76.4	74.3
11	66.9	71.3	69.1	31	70.9	74.4	72.6
12	73.7	75.4	74.6	32	72.5	77.7	75.1
13	71.1	74.1	72.6	33	76.1	76.5	76.3
14	73.1	75.7	74.4	34	71.4	74.4	72.9
15	72.7	76.6	74.7	35	77.6	78.7	78.1
16	74.3	78.0	76.1	36	74.9	78.4	76.7
17	71.6	72.9	72.2	37	76.5	78.0	77.2
18	74.4	77.1	75.7	36	73.9	73.1	73.5
19[b]	83.2	83.1	83.1	39	73.1	73.6	73.4
20	71.8	70.9	71.4	40	72.7	73.4	73.0
Average:			72.4[c]				74.5[c]
Variance:			4.78				4.59
C.V. (%):			3.02				2.87

[a] Determined by equilibrium dialysis and radiochemical assay of ^{3}H-prednisolone in plasma and buffer compartments
[b] Omitted from statistical analysis (see text)
[c] Difference between means is significant ($t = 13.0$, $p<0.001$)

where $P(1) = n_t P_t$ (3)
$P(2) = 1/K_t$ (4)
$P(3) = n_a K_a P_a$ (5)

The objective of the research reported in this paper was to study the plasma protein binding of prednisolone considering the effect of disease state on this process.

Materials and Methods

HPLC Assay and Radioactivity Assay

The steroid concentrations of prednisolone, prednisone, and hydrocortisone were determined in plasma and dialysis buffer by high performance liquid chromatography (HPLC) (Waters Associates, Inc., Model 204, Milford, Mass.). The samples were extracted with methylene chloride and the extracts were evaporated to dryness. The dried extracts were reconstituted with mobile phase before injection onto the HPLC column. The mobile phase contained hexane, methylene chloride, absolute ethanol, and acetic acid (688:250:60:2). A μ-Poracil column (Waters Associates, Inc., Milford, Mass.) 4 mm × 30 cm was used to separate the steroids. Triamcinolone acetonide was used as an internal standard. The full details of the HPLC method will be published elsewhere.

The radioactivity of the tritium labeled prednisolone was determined in plasma and dialysis buffer using a scintillation counter (Packard Tri-Carb Liquid Scintillation Spectrophotometer, Model 3320, Downers Grove, Ill.). Appropriate samples of the plasma or buffer were transferred into 10 ml of a scintillation cocktail fluid (Unogel, Schwarz/Mann, Orangeburg, N.Y.) and counted three times for a period of 10 min. All counts were corrected for background and quenching and the three results were averaged for each sample. The average dpm, $\overline{\text{dpm}}$, were used for the percent bound calculations.

Volunteers and Patients

Ten ml blood samples were drawn from 20 normal, healthy volunteers and 20 rheumatoid arthritis patients. The blood samples were immediately centrifuged at 2000 rpm (Sorvall, Inc., Model RC-3 Refrigerated Centrifuge, Newtown, Conn.) and the plasma samples obtained were immediately frozen at

Table 3. Comparison of the plasma protein binding of prednisolone as determined by radioactivity or HPLC analysis of the same dialysis samples

Sample No.	Percent bound	
	Radiochemical assay average[a]	HPLC assay
N-1[b]	70.3	64.5
N-2	73.9	75.8
N-3	72.2	69.1
N-4	72.0	70.6
N-5	73.3	70.9
A-1[c]	74.7	77.2
A-2	70.2	74.8
A-3	77.7	80.3
A-4	72.6	77.8
A-5	74.9	80.6
Average:	73.2[d]	74.2[d]
Variance:	5.12[e]	27.4[e]
C.V. (%):	3.09	7.06

[a] Average of two determinations
[b] N refers to normal volunteers
[c] A refers to arthritic patients
[d] The means are not significantly different ($t = 0.543$, $p>0.025$, $df = 13$) (see ref. 18)
[e] Variances are not homogeneous. ($F = 5.36$, $p<0.05$)

Table 4. Change in the percentage of prednisolone bound to plasma proteins as the total concentration of prednisolone changes

Normal R. B.[a]		Patient W. W.[b]	
Prednisolone concentration[c] μM	Percent bound[d]	Prednisolone concentration[c] μM	Percent bound[d]
0[e]	90.9		
0.0821	89.6	0.0152	84.6
0.235	88.0	0.560	74.3
0.344	83.2	0.444	76.3
0.577	76.2	0.411	77.1
0.718	67.4	0.222	82.0
0.899	66.0	0.171	83.1
1.19	63.0	0.108	84.6

[a] Prednisolone, prednisone, and hydrocortisone (ratio 4:1:1) were added to the plasma in vitro
[b] Patient received a 20 mg dose of prednisone. Plasma samples were obtained at 0.6, 1, 2, 4, 6, and 8 hours after the dose. Prednisolone concentrations were determined by HPLC
[c] The total equilibrium concentrations (bound and free) of prednisolone
[d] Determined by equilibrium dialysis with radiolabeled prednisolone
[e] Only radioactive prednisolone added to the sample

−20° C until further use. The sex and the plasma albumin levels of the subjects as well as the drugs the arthritic patients were taking are listed in Table 1. The mean age of the normal group was 32 with a range of 24 to 49. The mean age of the arthritic group was 51 with a range of 21 to 72.

Three of the arthritic patients were receiving prednisone at the time of the study. For these patients, the steroid levels in the plasma were first determined by HPLC. The results for the prednisolone levels were 0.0508, 0.219, and 0.299 μM. Nevertheless, during the protein binding equilibrium dialysis study, the same amounts of the three steroids were added to these samples as in all other samples.

For the concentration dependent protein binding study, plasma samples from a male, normal, healthy volunteer, R. B., and a male, systemic lupus erythmatosis (SLE) patient, W. W., were used.

Protein Binding Determination

A freshly prepared pH 7.4 phosphate (0.0175 M) buffer (contained: 0.696 g dibasic potassium phosphate, 0.138 g monobasic sodium phosphate, 2.25 g sodium chloride, pH adjusted to 7.4, q. s. distilled water 500 ml) was used for all dialysis experiments and preparations. Cellophane dialysis tubing (Union Carbide, Chicago, Ill.) with a flat diameter of 9.5 mm was cut into 15 cm lengths and soaked in phosphate buffer for a minimum of 24 h at 4° C. Immediately before being used, the strips of dialysis tubing were removed from the soaking buffer and the excess buffer was wiped away as completely as possible.

After tying off one end of the dialysis tubing, a one ml sample of plasma from an individual patient was placed inside the tubing from the open end. Then 100 μl of a solution containing three unlabeled steroids was added to the plasma sample in the tubing. As a result of this addition, the plasma concentration inside the dialysis tubing for the three steroids were prednisolone, 1.09 μM, prednisone, 0.277 μM, and hydrocortisone, 0.281 μM.

Finally, 10 μl of the tritium labeled prednisolone solution (approximately 1000 dpm/10 μl) (New England Nuclear, Boston, Mass.) was added to the plasma sample in the dialysis tubing, the end of the tubing was tied off, and the tubing was inverted several times to achieve mixing. After being folded into a V shape, the dialysis tubing was placed into a 1 cm × 10 cm test tube containing 3 ml of the phosphate buffer. The top of the tube was sealed with parafilm to prevent loss by evaporation and the outside of the test tube was covered with aluminum foil to minimize light exposure.

The prepared dialysis test tubes were oscillated in a 37° C water bath (Lab-Line Instruments, Inc., Model 3581, Melrose Park, Ill.) for 16 h. After removal from the water bath, the dialysis tubing was removed from the buffer and the outside of the dialysis tubing was gently dried with chemwipes.

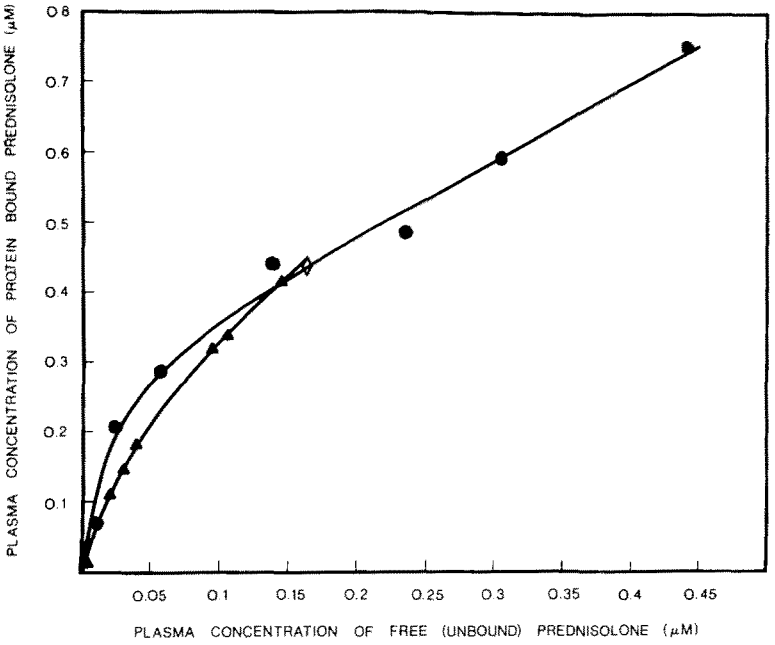

Fig. 1. Plot of Bound versus Free Concentrations of Prednisolone in Plasma. Key: ● normal male volunteer R. B.: ▲ SLE patient W. W.: ◇ Point is for the 19 normals. (These plasmas initially contained 1.09 μM prednisolone. The total equilibrium concentration was 0.597 μM. The average percent bound by the 19 normals was 72.4%). The points plotted are the observed free and bound equilibrium concentrations. The lines are the estimated values based on Eq. 1 and the least square estimates of the parameters in Table 5

Table 5. Least squares estimates of parameters based on the fitting of prednisolone plasma protein binding data

Plasmas utilized	Parameters[a]			Measures of fit		Calculated parameters	
	P(1)	P(2)	P(3)	r^{2}[b]	corr.[c]	K_a (M^{-1})[d]	K_f (M^{-1})
Normal	0.293 (0.0314)[e]	0.0180 (0.00445)	1.05 (0.091)	0.998	0.995	1.8×10^3	5.6×10^7
Patient	0.235 (0.00585)	0.0414 (0.00086)	1.62 (0.0324)	1.000	1.000	2.7×10^3	2.4×10^7

[a] Least square estimates of the parameters of Eq. (2) when C_b and C_f were in μM units
[b] Coefficient of determination
[c] Correlation coefficient for the linear regression of estimated bound concentration versus observed bound concentration of prednisolone
[d] Assuming $n_a = 1$ for albumin, an albumin molecular weight of 66.500 (ref. 15), and albumin concentrations for normal (3.9 g/dl) and patient (4.0 g/dl)
[e] Standard deviation of the estimated parameters

Two 0.5 ml samples were obtained from the contents of the inside of the dialysis tubing and two 1.0 ml samples were obtained from the dialysis buffer (outside). After analysis by radiochemical assay (or HPLC) of each of the samples, the percentage of prednisolone bound to the plasma proteins was calculated using the following equation:

$$\text{percent bound} = \frac{(\overline{\text{dpm}})_i \cdot 2 - (\overline{\text{dpm}})_o}{(\overline{\text{dpm}})_i \cdot 2} \cdot 100 \qquad (6)$$

where the subscripts i and o refer to inside and outside samples, respectively. For HPLC analysis, the amount of prednisolone in the sample was used for the above calculation instead of the dpm. The HPLC analysis was only conducted on the samples from the first 5 subjects of both groups. Once equivalence of the two methods of assay was established only the radiochemical assay was used thereafter.

Protein Binding Versus Concentration

The endogenous hydrocortisone of the normal volunteer, R. B., was suppressed by the administration of 1 mg of dexamethasone 9 h prior to blood collection. The blood sample was drawn and the plasma was separated by centrifugation. Since the dexamethasone plasma concentration 9 h after administration would be exceedingly small, we considered the dexamethasone interference with prednisolone binding to be minimal. Different but known volumes of a steroid solution which contained prednisolone, prednisone, and hydrocortisone in the ratio of 4 : 1 : 1 were added to 1.0 ml aliquots of the normal's plasma.

The SLE patient, W. W., who was receiving pred-

nisone regularly, was given a 20 mg dose of prednisone at 8 a. m. Blood samples from the patient were taken at 0.6, 1, 2, 4, 6, and 8 h after the dose. The total prednisolone concentration of the plasma was determined for each of the timed samples by HPLC analysis.

The plasma samples from the patient and normal volunteer were used to evaluate the concentration dependent plasma protein binding of prednisolone. The procedure described above for protein binding was used for this evaluation except that no additional unlabelled steroids were added to the samples.

Results

Comparison of Prednisolone Protein Binding in Normals and Arthritics

The percentage of prednisolone bound to the plasma protein for each of the 20 normals and arthritics is given in Table 2 as determined by equilibrium dialysis and radiochemical analysis of the plasma and buffer compartments for ^{3}H-prednisolone.

The data for subject 19 were omitted from the parametric statistical analysis because the results from this subject differ by more than 4 standard deviations from the mean of the other normals and by more than 3 standard deviations from the next highest average observation of the normal group.

The variances of the two groups, normals and arthritics, were compared using an F test and were found not significantly different ($F = 1.04$ $p>0.25$). A Student t-test for differences between the means was significant. The statistical tests are summarized in Table 2. Additionally, the data for all 20 normal volunteers versus the 20 arthritic patients were compared using a nonparametric Mann-Whitney test and the two groups were shown to differ significantly ($u = 106$, $p<0.01$) in the percentages of prednisolone bound. However, the differences observed are regarded as clinically insignificant.

Comparison of Radioactivity Versus HPLC Methods

The equilibrium dialysis samples from the first 5 normals and arthritic subjects were measured both by radioactivity counting of the ^{3}H-prednisolone and by HPLC quantification of the total amount of prednisolone in the samples. These measurements were used to calculate the percentage of prednisolone bound to the plasma proteins. The results from these two methods are presented in Table 3. The same dialysis test tube was sampled by both methods.

The variances of the two sets of data are not homogeneous by an F test. A paired Student t-test, corrected for unequal variances [18], was used to test the difference between the two methods and the difference between the mean percentages bound was found to be not significant. The statistical tests are summarized in Table 3.

Change in the Percentage of Prednisolone Bound to Protein Versus the Concentration of Prednisolone

The percentage of prednisolone bound to the plasma proteins changes as a function of the total concentration of prednisolone in the plasma due to the nonlinear protein binding of prednisolone as described by Eq. (1). Table 4 shows the change in the percent bound for the normal volunteer and the SLE patient where the plasma protein binding of prednisolone was studied at different total concentrations of prednisolone.

A protein binding plot of the concentration bound versus concentration free of prednisolone is shown for these subjects in Figure 1. The concentrations plotted on Figure 1 are the bound and free equilibrium concentrations. Also shown in Figure 1 is a single point representing the average of the concentration bound versus concentration free for the 19 normal subjects.

After obtaining the initial estimates of the parameters of Eq. (2) by manual stripping techniques, the data from the normal and SLE patient were fit to Eq. (3) using a digital computer and the program NONLIN (11). During this fitting, the concentrations were weighted as their recriprocals. The least square estimates of these parameters, the measures of fit, and some calculated parameters for the protein binding of prednisolone are shown in Table 5.

Discussion

The in vitro study of the 20 normals and 20 rheumatoid arthritis patients showed a significant difference in the percentage of prednisolone bound to the plasma proteins. However, at the level of binding observed, the differences between the two groups are not clinically significant as the fraction free would not be dramatically changed due to the additional mean binding of the arthritic group.

The results of this study must be viewed with caution. It must be emphasized that three steroids, prednisolone, prednisone, and hydrocortisone, were added to these plasmas. These steroids are all known to bind to albumin and transcortin [7]. Thus, the binding of each individual steroid will influence the binding of the others by competition for the same binding sites. Furthermore, the exact concentration ratio of the three steroids employed in these studies may not equate to clinically observed steroid concentrations. However, previous studies done in this laboratory [12] have shown the peak plasma concen-

tration of prednisolone to be approximately three times the prednisone concentrations. A similar ratio was used for the present study.

The difference between the albumin levels of the two groups should also be noted. The normal group had a significantly higher albumin level than the arthritic group ($t = 4.30$, $p<0.001$). However, besides the disease state, the mean age of the two groups was dissimilar. Plasma albumin levels are known to decrease with age [13] and in inflammatory disease [5, 14]. In addition, however, the structure of the protein may be different due to the disease state [16, 17]. In spite of the lower albumin levels in the arthritic group, the observed trend of the plasma protein binding of prednisolone was toward a greater percentage bound in the arthritic group. This trend is opposite to that which is expected based upon the change in albumin concentration alone. Thus, there may be a duplicity of effects resulting from the lower albumin levels occurring in conjunction with an altered albumin molecule which causes different affinities of the protein for prednisolone.

The results obtained by radiochemical or HPLC analytical methods in the determination of protein binding of prednisolone were not significantly different as shown in Table 3. The variance was greater for the HPLC method. However, this result was expected as the prednisolone concentrations in the dialysis buffer were near the limits of detection for the HPLC analysis. The agreement of the two analytical methods indicates that the radiochemical method is adequate. Because of the greater variance associated with the HPLC analysis method, the radiochemical method of determination is preferred.

The fitting of the normal volunteer's and SLE patient's concentration bound versus concentration free data shows the nonlinearity of the plasma protein binding of prednisolone. The affinity constants derived for albumin and transcortin are in agreement with other investigators [6]. These data confirm the high affinity and low capacity of prednisolone binding to transcortin and the low affinity but high capacity of albumin binding.

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