THE BINDING OF PREDNISOLONE IN HUMAN SERUM AND TO RECRYSTALLISED HUMAN ALBUMIN *IN VITRO*

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- 1 Prednisolone binding in human serum and to recrystallised human serum albumin was measured by continuous ultrafiltration.
- 2 At serum concentrations of prednisolone up to $0.6 \mu\text{M}$, 95.0% was bound but at higher concentrations the binding became non-linear falling to 80.5% at $1.8 \mu\text{M}$. At even higher concentrations binding in serum became linear again and paralleled the binding to albumin which was linear throughout the entire range of prednisolone concentrations.
- 3 A binding model was proposed which included a saturable component attributable to binding to transcortin and a non-saturable binding to albumin.
- 4 Computer simulations using the experimentally determined binding parameters of the model indicated that binding in serum was critically dependent on transcortin concentration and almost independent of albumin concentration.

Introduction

Prednisolone is a widely prescribed synthetic corticosteroid. Unbound prednisolone in serum has been implicated as the active drug (Lewis *et al.*, 1971; Uribe *et al.*, 1976), although estimates of this fraction have shown disparate results. Reported values of percentage bound drug to serum and plasma proteins have varied widely, ranging from 50 to 90% (Lewis *et al.*, 1971; Powell & Axelsen, 1972; Uribe *et al.*, 1976; Schalm *et al.*, 1977; Centeno *et al.*, 1978). There is also disagreement as to whether prednisolone serum binding is linear or non-linear (Agabeyoglu *et al.*, 1979; Pickup, 1979; Wagner *et al.*, 1981) over the concentration range observed *in vivo*. The nature of the prednisolone-serum protein interaction has been extensively studied. Binding to albumin is believed to be of low affinity and high capacity in contrast to transcortin-prednisolone binding which is reported to be non-linear over the serum concentrations attained in treatment and of higher affinity and lower capacity (Lewis *et al.*, 1971; Wagner *et al.*, 1981).

This study has measured by continuous ultrafiltration the percentage binding of prednisolone to recrystallised human serum albumin and in human serum at concentrations observed during drug therapy. The nature of this binding in serum is compared with the binding to albumin and an appropriate binding model is proposed. Some predictions of how these observations may relate to patient treatment are discussed.

Methods

Subjects

Serum was obtained from ten volunteers (age 20-34 years) with no clinical or laboratory manifestation of illness. Two hours before withdrawal of blood for protein binding studies one volunteer (MHM) had taken 500 mg of paracetamol. No medication had been taken by the other subjects on the study day or during the previous 48 h.

Binding studies were also performed on recrystallised human albumin (Sigma, essentially globulin free albumin) at protein concentrations ranging from 20-45 g l⁻¹.

Cellulose acetate electrophoresis

[³H]-prednisolone (Amersham International, Sp.act 33 Ci nmol⁻¹, 10 μ l; 250,000 d/min) was mixed with 200 μ l of pooled normal human serum containing 0.4 μM or 14 μM of unlabelled prednisolone. Cellulose acetate electrophoresis on 20 μ l of each of these solutions was performed at pH 8.6 (barbital buffer, I = 0.05). The cellulose acetate strips were longitudinally bisected and one half stained with Ponceau S (0.2% Ponceau S in 3.0% trichloroacetic acid) and washed with 5.0% acetic acid. The stained protein bands were used to locate the separated protein components in the unstained half of the strip which were removed from the strip and the radioactivity measured by liquid scintillation counting.

Protein binding

This was measured by continuous ultrafiltration from Amicon Multimicro-Concentrator (MMC) chambers using Diaflo XM50 ultrafiltration membranes. A pH of 7.4 was maintained using 0.1 M phosphate buffered saline at 37°C. An equilibrated gas (N₂) pressure of 40 p.s.i. facilitated a flow rate of 2.5 ml h⁻¹. Sulphosalicylic acid (1.0 M) was used to check for protein leakage through the membrane. Non-specific binding to membranes was determined by defining a reflection coefficient (σ) which related the drug concentration in the ultrafiltrate (Df) to the concentration in the MMC chamber (Dt) when buffer instead of serum was being used, as shown in equation 1:

$$\sigma = 1 - (Df)/(Dt) \quad (1)$$

At the flow rate employed in this study $\sigma = 0.02$. Further, the serum in the MMC chamber was analysed at the end of each experiment and only those experiments in which this concentration was within 10% of the estimated value were accepted. The total drug concentration (Dt) in the MMC chamber and that bound to serum proteins (Db) were calculated according to equations 2 and 3 as follows:

$$Dt = 1/V \sum_{i=1}^n ((v_i Dr) - (v_i Df_i)) \quad (2)$$

$$Db = Dt - Df \quad (3)$$

where V = volume of serum used in the MMC chamber, which remained constant throughout the experiment (μ l).

v_i = the volume leaving the MMC chamber in the i th collection period (μ l); since the system is interconnected this exactly equals the volume entering the chamber during this period.

Dr = the prednisolone concentration in the reservoir (μ M).

Df_i = the prednisolone concentration in the ultrafiltrate of the i th collection (μ M). A series of three binding experiments were performed by this procedure using different reservoir concentrations:

Binding to serum Dr = 1.0 μ M

A reservoir concentration of 1.0 μ M permitted ten sequential estimates of percentage bound drug over a range of concentrations from 0.1 to 0.6 μ M. i.e. concentrations of drug found in serum during therapy.

Binding to serum or albumin Dr = 3.0 μ M

Protein binding measurements extending beyond the peak concentration observed in treatment were made in four serum samples using a reservoir concentration

of 3.0 μ M enabling total concentrations of 0.18 to 1.80 μ M to be studied.

Binding to serum or albumin Dr = 360 μ M

Sixteen sequential measurements of protein binding over the range 15 to 330 μ M of prednisolone were made. Precipitation of drug from solution occurred if higher reservoir concentrations were used.

Data analysis

Estimates of the apparent binding parameters for albumin-prednisolone binding and serum-prednisolone binding were made using a non-linear least squares regression of Db on Df (BMDP-81, 1981) using the models shown in Table 1. Provision was made for binding by non-specific partitioning only (Model 1) and for binding to one class of binding site plus non-specific partitioning (Model 2). The most appropriate model was selected by examining the asymptotic standard deviation observed after fitting the data to the different models and also comparing the residual sum of squares using the 'F' ratio test (Boxenbaum *et al.*, 1974).

Table 1 Models used in the analysis of protein binding data where: Db = concentration of prednisolone bound; Df = concentration of prednisolone free; Kp = 'non-specific' partitioning factor; P = concentration of protein; NP = hybrid capacity factor; K = affinity constant.

Model	Equation	Description
1	Db = Kp.P.Df.	Partitioning only
2	Db = NP.K.Df/(1+K.Df) + Kp.P.Df.	One class of binding site plus partitioning

Results

a) Cellulose acetate electrophoresis

After electrophoresis of serum 65% of the total radioactivity was recovered in the separated protein bands. The electrophoresis indicated that prednisolone was bound predominantly to albumin. At a concentration of 0.41 μ M, 90.6% of the bound drug was associated with albumin while at 138 μ M, 95.0% was bound to albumin. The remaining fraction was bound almost exclusively to α -globulins; β - and γ -globulins contributed minimally to the total binding. These results are shown in Table 2.

b) Binding by continuous ultrafiltration

The percentage binding of prednisolone to serum proteins was dependent on the concentration of the drug.

Table 2 The percentage of total radioactivity recovered in the protein bands separated after cellulose acetate electrophoresis.

Serum concentration (μM)	Albumin	Protein fraction		
		α	β	γ
0.41	90.60	9.28	0.07	0.05
138.00	95.00	4.78	0.12	0.10

Binding to serum $D_r = 1.0 \mu\text{M}$

The percentage bound over the concentration range obtained using this reservoir solution was essentially linear, varying by only $\pm 1.5\%$ (s.d.) with a mean figure at $0.6 \mu\text{M}$ of $95.0\% \pm 1.35$ (s.d.). However, the value of the percentage of prednisolone bound to the serum of MHM was much lower and was linear even up to a concentration of $600 \mu\text{M}$ with a mean value over this range of $69.2\% \pm 2.3$ (s.d.). These results are indicative of a displacement interaction by paracetamol and require further study.

Binding to serum or albumin $D_r = 3.0 \mu\text{M}$

In the four subjects the percentage bound to serum proteins and recrystallised albumin was measured at total concentrations up to $1.8 \mu\text{M}$. In these experiments the binding was non-linear and decreased from 96.4 ± 0.9 (s.d.) at $0.2 \mu\text{M}$ to $80.5\% \pm 1.5$ (s.d.) at $1.8 \mu\text{M}$. These results are shown in Figure 1. In addition estimates of the apparent binding parameters were made by plotting the concentration of bound drug (Db) against the concentration of free drug (Df) as shown in Figure 2. These results were best described by Model 2 (Table 1). Selection of Model 2 was supported by a significantly smaller residual sum of squares ($P < 0.01$) and smaller asymptotic standard deviations of the parameters when compared with Model 1. These parameter

Figure 1 The percentage of prednisolone bound to serum at concentrations up to $1.8 \mu\text{M}$.

Figure 2 The concentration of prednisolone bound (Db) plotted against the concentration of free prednisolone (Df) (Δ , $\square$, $\circ$, $\bullet$, binding to serum; $\blacktriangle$ binding to albumin).

values are shown in Table 3. In contrast when the binding of prednisolone to recrystallised human serum albumin was examined at these concentrations no saturable component was detected, the data being best described by Model 1. These results are also shown in Figure 2.

Binding to serum or albumin $D_r = 360 \mu\text{M}$

When the binding to serum was examined at higher drug concentrations the data were again best described by Model 2. These experiments were designed to examine the apparent linear binding observed at higher concentrations. They did not permit the estimates of NP and K which were obtained at the lower reservoir concentrations. However, the mean KpP value was estimated to be $2.62 \mu\text{M} \pm 0.47$ (s.d.). When the binding to recrystallised human serum albumin was examined at these concentrations again no saturable component

Table 3 Parameter values for the binding of prednisolone to serum derived from Model 2, see text for details.

Subject	NP (μM)	K (μM^{-1})	KpP (μM)	Albumin (g l^{-1})
JWR	0.64	69.99	2.36	49.0
WHS	0.72	38.95	2.12	47.0
GMH	0.58	55.20	3.13	43.0
CSC	0.67	47.86	2.13	40.0
Mean	0.65	53.00	2.44	44.8
$\pm$ s.d.	0.06	13.13	0.47	4.0

was detected, the data being best described by Model 1. The mean KpP value in these experiments was $2.36 \mu\text{M} \pm 0.09$ (s.d.). It should be noted that irrespective of the starting reservoir concentration there was no statistically significant difference in any of the values obtained for KpP whether from serum or albumin. Further attempts were made to saturate the albumin binding, by reducing the albumin concentration in the MMC chamber, but this could not be achieved. In these experiments the KpP value was constant and again did not differ statistically from the previously obtained KpP values. These results are shown in Table 4.

Table 4 KpP values for the binding of prednisolone to albumin at different albumin concentrations.

Albumin concentration (g l^{-1})	KpP (μM)
20	2.44
25	2.36
30	2.38
35	2.44
40	2.40
45	2.19

Discussion

It is known that prednisolone binds to serum albumin and transcortin (Lewis *et al.*, 1971; Westphal, 1969; Angeli *et al.*, 1978). In the present study the results from the cellulose acetate electrophoresis showed that prednisolone was bound predominantly to albumin with 5 to 10% of the total binding to α -globulins. Since transcortin separates with α -globulins on cellulose acetate strips it is likely that binding to this fraction reflects binding to transcortin. It is unlikely that β - and γ -globulins bind significant quantities of prednisolone.

The binding of prednisolone in human serum can be expressed by binding to two components. A saturable 'non-linear' binding which was particularly important at lower concentrations such as those associated with therapy and a non-saturable 'linear' component which dominated the binding at higher concentrations up to the limit of solubility of the drug. Given that prednisolone binds to transcortin and also because of the electrophoretic results referred to above, the saturable binding of the drug at lower concentrations was attributed to transcortin-prednisolone binding. This saturable binding of prednisolone supports the recent work of Pickup *et al.* (1979), Agabeyoglu *et al.* (1979) and Wagner *et al.* (1981). However, since transcortin also binds other steroids such as cortisol, which is subject to diurnal variation, it is possible that prednisolone was studied in the presence of competing amounts of cortisol (Angeli *et al.*, 1978).

In contrast to serum, the binding of prednisolone to recrystallised human serum albumin was linear and thus non-saturable at all concentrations studied. The mechanism of this binding is unclear but may be analogous to a partitioning phenomenon described by the 'non-specific' partitioning factor (Kp). Furthermore the term 'binding' should only be used in those circumstances where saturation of the binding sites can be shown. Defining a partitioning factor also avoids the assumptions implicit in determining the albumin-prednisolone apparent association constants (Wagner *et al.*, 1981; Agabeyoglu *et al.*, 1979; Lewis *et al.*, 1971) where it is assumed that the number of binding sites is unity.

The partitioning factor Kp obtained with recrystallised human serum albumin was not significantly different from that obtained in serum. It is probable therefore, that the linear binding observed in serum is due to an interaction with albumin and the saturable binding to that of transcortin. Thus the saturable term in Model 2 is assigned to the binding of prednisolone to transcortin while the linear term to that of albumin. For convenience the equation is repeated assuming these specific binding proteins:

$$\text{Db} = (\text{N.Pt.K.Df}/(1+\text{K.Df})) + \text{Kp.Pa.Df} \quad (2)$$

If it assumed that the average concentration of transcortin (Pt) is $0.8 \mu\text{M}$ then the number of binding sites on transcortin (N) is unity, since the mean N.Pt value was 0.65 (see Table 3). The affinity constant for binding to transcortin (K) has been determined and a value for Kp is obtained by dividing the mean capacity factor Kp.Pa determined experimentally (Table 3) by the mean albumin concentration (Pa) (Table 3). The effect on the concentration of bound prednisolone (Db) of independently changing the variables of free prednisolone (Df), transcortin (Pt) and albumin (Pa) concentrations can then be simulated. Such simulation showed that the degree of binding was extremely sensitive to the concentration

Figure 3 Simulated binding of prednisolone derived from Model 2. Effect of different prednisolone concentrations.

of prednisolone with the percentage bound drug falling from 96.6% to 82.2% bound over a range in concentration (Df) of 0.2 to 1.6 μM , (Figure 3). Likewise, when transcortin concentration was altered from 15 to 45 mg l^{-1} the value of percentage bound changed from 82.6% to 95.4% (Figure 4). In contrast little effect was observed when the albumin concentration was changed from 20 g l^{-1} to 50 g l^{-1} , the corresponding percentage bound changing from 91.1% to 93.2% (Figure 4).

Figure 4 Simulated binding of prednisolone derived from Model 2. Effect of different albumin (○) and transcortin (□) concentrations.

Reduced albumin levels have been correlated with an increased incidence of prednisolone adverse drug effects (Lewis *et al.*, 1971; Uribe *et al.*, 1976) and

indeed a reduction of prednisolone dosage has been recommended based on serum albumin concentrations (Centeno *et al.*, 1978). However, from the insight gained into the binding from the simulations, it is evident that gross changes in albumin concentrations should be tolerated without altering greatly the percentage bound drug. While at first this may seem surprising as albumin is the serum protein with highest binding capacity for prednisolone, on closer examination of the non-saturable nature of albumin–prednisolone interaction it is clear that a reduction in albumin concentration *per se* should not reduce the value for percentage bound. It is also evident on examination that since binding to transcortin is saturable a reduction in this protein concentration should reduce the percentage bound, particularly if it has only one binding site. In addition it is important to re-emphasise the dangers of making conclusions based on 'cause and effect' correlations: a correlation between albumin concentration and percentage bound does not prove that a reduction in albumin levels is responsible for a lowered percentage bound.

It is clear that many factors are interacting to reflect in the final value for percentage bound prednisolone. These factors include serum concentrations of albumin, transcortin and cortisol as well as prednisolone. It is evident that a meaningful interpretation of prednisolone–protein interaction is difficult without consideration of these factors. This must be particularly so when comparison of results in patients with disease states are to be made, since the concentrations of albumin, transcortin and cortisol may all be altered in many conditions, for example rheumatoid arthritis, nephrotic syndrome and Hodgkin's disease.

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