between 21 and 33 years. After an overnight fast each received aminophylline B.P. orally in a dose of 3–5 mg kg⁻¹ of body weight. At 3, 4, 5 and 6 h after this administration 5 ml blood samples were taken into plain glass tubes and simultaneous saliva samples were obtained. Salivary flow was stimulated by chewing parafilm and salivary flow rate was measured by the weight difference in the preweighed collection tubes. After centrifugation serum and saliva samples were stored frozen at –20°C until analysis by EMIT.

The mean saliva/serum theophylline ratio was 0.46 ± 0.12 (s.d.) and although there were marked inter-individual differences the mean varied little with time. The degree of correlation between serum and saliva theophylline concentrations altered markedly with time, the correlation coefficient, *r*, increasing from 0.33 (*P* > 0.1) at 3 h to 0.81 (*P* < 0.001) at 6 h. The method of Deming (1943) and Smith *et al.* (1980) was used to calculate the slope and intercept to the regression line, serum on saliva, at each time point. It was found that the slope decreased while the intercept increased with time (Table 1). The individual scattergrams are shown in Figure 1. The saliva/serum theophylline ratio was unrelated to the salivary flow rate.

The original aim of this study was to provide a simple method of calculating theophylline half-life in patients on oral therapy and thereby predict a dose of theophylline which would maintain an individual

serum level within the therapeutic range, but because of the low correlation between serum and saliva theophylline levels at earlier time points this would prove impractical. It is probable that variations between subjects in the rates of absorption and distribution of theophylline contribute to this early lack of correlation. However Koup *et al.* (1979) and Slattery *et al.* (1980) have demonstrated that only one plasma concentration of theophylline is required accurately to predict a patient's maintenance dose. It might therefore be possible to use one saliva theophylline concentration measured 6 h after a single oral dose in the same fashion, since our results suggest that at that time serum theophylline concentration can be predicted from salivary concentration with some accuracy. It is intended to investigate this possibility further.

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ESTIMATION OF TRANSCORTIN CONCENTRATION BY MEASUREMENTS OF PLASMA PROTEIN-BINDING OF PREDNISOLONE AND BY ELECTROIMMUNODIFFUSION

Since the binding of prednisolone to plasma proteins occurs at transcortin and albumin, prednisolone exhibits nonlinear kinetics in the therapeutic dose

range (Jusko *et al.*, 1980; Frey *et al.*, 1980, 1981). Therefore, the concentrations of albumin and transcortin are mandatory for the interpretation of pred-

nisolone kinetics in clinical studies. The concentrations of transcortin are usually obtained indirectly on the basis of *in vitro* binding studies; for these studies the equilibrium dialysis method is used and the binding of prednisolone to plasma proteins is determined (Agabeyoglu *et al.*, 1979; Frey *et al.*, 1981; Wagner *et al.*, 1981). The determination of the transcortin concentration by *in vitro* binding studies is time consuming, because about ten individual measurements of bound and of free concentrations of prednisolone have to be performed for the calculation of a single concentration of transcortin in plasma (Frey *et al.*, 1981; Wagner *et al.*, 1981). Therefore, a simple method to screen for abnormal capacities of transcortin to bind prednisolone should be available. For that purpose we propose to assess the concentration of transcortin by electroimmunodiffusion according to Laurell (1966). The purpose of the present study was to establish, whether or not values of transcortin concentration calculated on the basis of *in vitro* binding of prednisolone (i) are identical with those obtained by the electroimmunodiffusion method (ii).

(i) The transcortin concentrations were assessed in ten stable renal transplant patients (plasma creatinine concentration < 160 $\mu\text{mol/l}$) and in twelve patients treated chronically with prednisone for an autoimmune disease. Patients were studied following their usual oral or intravenous doses on two consecutive days. All doses were administered in the morning with the patients having fasted overnight. On each day, eight to fourteen blood samples were collected in heparinised tubes over an 8 to 12 h period. The tubes were placed in an ice bath and centrifuged within 30 min of sampling to separate out plasma. All plasma samples were analysed for prednisolone by a high performance liquid chromatographic procedure we have developed (Frey *et al.*, 1979). Equilibrium dialysis was performed on all plasma samples to quantify the unbound prednisolone concentration as described previously (Frey *et al.*, 1980). Dialysis of plasma was performed against isotonic Krebs Ringer buffer (pH 7.4) spiked with tracer quantities (3 ng/ml; 46 Ci/mmol; 98% radiochemical purity, Amersham) of prednisolone-6,7- ^3H (N) at 37°C for 14 h. The *in vitro* fraction of unbound prednisolone was calculated by dividing the concentration in the buffer half-cell by the concentration in the plasma half-cell. Since prednisolone protein binding is nonlinear (Figure 1), it is necessary to correct the binding parameters for the changes in concentration which result due to diffusion from plasma to buffer during equilibrium dialysis. This was done following the method of Behm *et al.* (1979) (equation 14).

The binding of prednisolone to plasma proteins occurs at two major sites, transcortin and albumin (Rocci & Jusko, 1978). This may be expressed by the

Figure 1 Plot of bound *v* unbound plasma prednisolone concentrations in a renal transplant patient. At lower concentrations a larger fraction of prednisolone is bound than at higher concentrations. This is best explained by the presence of two proteins binding prednisolone, i.e. one with high affinity and low capacity (transcortin) and one with low affinity and high capacity (albumin). The capacity of transcortin to bind prednisolone was derived by a computer fit of the data obtained following intravenous (Δ) and oral ($\blacktriangle$) dosing to the binding relation (Equation 2).

following equation assuming that prednisolone only binds to transcortin and albumin and that there is only one binding site per molecule of protein:

$$B_{P_0} = \frac{CAP_T \times F_{P_0}}{KD_T + F_{P_0}} + \frac{CAP_A \times F_{P_0}}{KD_A + F_{P_0}} \quad (1)$$

where B_{P_0} is the concentration of prednisolone bound to both sites; CAP_T , KD_T and CAP_A , KD_A are the binding capacities and the dissociation constants for transcortin and albumin respectively, and F_{P_0} is the unbound or free concentration of prednisolone. When $KD_A \gg F_{P_0}$, then Equation 1 reduces to:

$$B_{P_0} = \frac{CAP_T \times F_{P_0}}{KD_T + F_{P_0}} + \frac{CAP_A}{KD_A} \times F_{P_0} \quad (2)$$

The binding parameters for prednisolone, CAP_T , KD_T and CAP_A/KD_A , were estimated using Equation 2 by non-linear least squares regression with unit weighting (MULTIFUN) (Holford, 1979). The coefficients of determination (r^2) for the fits of data to Equation 2 ranged from 0.911 to 0.997.

(ii) The transcortin concentrations were assessed by electroimmunodiffusion in the plasma samples obtained prior to glucocorticoid dosage. The mean values of the two measurements are reported. The electroimmunodiffusion was performed according to Laurell (1966). Details of our technique are given elsewhere (Frey *et al.*, 1977). Briefly 5 μl plasma, 1:4 and 1:8 diluted with isotonic saline, are applied in an

Agarose gel (1% Agarose in H₂O/75 mM barbitone buffer pH 8.6, 1:2 v/v) of uniform thickness containing a monospecific antihuman transcortin rabbit antibody (gift from Dr van Baelen, Leuven). The electrophoresis is run with 20 volts/cm for 5 h at 10°C (electrophoresis apparatus LKB 2117 Multiphor with direct voltage stabilizer PS 10, Werner Meier, Luzern). After electrophoresis the gel is pressed (10 g/cm² for 10 min), washed with saline and water, dried at 50°C overnight, stained with coomassie blue (0.5%) and destained with ethanol: H₂O: acetic acid glacial 45/45/10 v/v. The peak height of the rockets measured varies linearly with the amount of transcortin applied within 1 to 11 mg/l. Dilutions of standard serum are used for calibration. Twelve samples can be run in duplicate on each gel of 10 × 10 cm size and two gels can be run simultaneously on the electrophoresis apparatus. The method exhibits an intraday and an interday variability of < 5% (CV) and < 10%, respectively.

Renal transplant patients and patients with autoimmune diseases have similar plasma concentrations of transcortin (Table 1). The mean transcortin concentration in plasma calculated on the basis of binding studies of prednisolone to transcortin (ED) is not different from the mean concentration of transcortin measured immunochemically (EID) (Table 1, *t*-test for paired observations; Zar, 1974). Except for one transplant patient (number 6, Table 1) and for two patients with an autoimmune disease (numbers 5 and 6, Table 1) the differences between the results obtained by the two methods may be explained by the variability of the methods used. Why the two

methods provide different results in the three patients mentioned is not known. For all patients a linear relationship was found between the transcortin values obtained by the two methods: ED (μmol/l) = 0.859·EID (μmol/l) + 0.125 (*r* = 0.58; *P* < 0.01), where ED and EID means results obtained by equilibrium dialysis and electroimmunodiffusion, respectively. Calculating a linear relationship between ED and EID assumes that the magnitude of the variable ED is determined by the magnitude of the variable EID (Zar, 1974). Obviously the binding of prednisolone to transcortin (ED) is only possible if the protein transcortin is present (EID). Thus ED values may be considered at least to some extent as dependent upon EID values. However other variables may determine the binding of prednisolone to plasma proteins and influence the transcortin values derived from binding studies (ED). Therefore it may be more meaningful to determine the intraclass correlation coefficient (*R*₁), a procedure which does not require designation of one variable as dependent and one variable as independent (Zar, 1974). *R*₁ for all patients investigated is 0.441. Omission of case 7 (transplant) and cases 5 and 6 (autoimmune disease) yields an *R*₁ value of 0.72. These *R*₁ values are well below 1 despite the fact, that the mean transcortin concentrations obtained for both populations are not different when assessed by EID or when assessed by ED (Table 1: mean). Note that a *R*₁ value of 1 indicates ideal concordance and a *R*₁ value of 0 indicates no concordance between the two methods.

Our results of transcortin measurement fall within the range observed in 17 patients with various

Table 1 Transcortin concentrations (μmol) in renal transplant patients and patients with autoimmune diseases. Transcortin was assessed by electroimmunodiffusion (EID) and by equilibrium dialysis (ED).

Patients Method	Renal transplant		Autoimmune disease	
	ED	EID	ED	EID
Patient number			Patient number	
1	0.64	0.54	1	0.56
2	1.10	0.98	2	0.70
3	0.45	0.56	3	0.81
4	0.67	0.46	4	0.61
5	0.29	0.52	5	1.21
6	0.42	0.83	6	1.04
7	0.40	0.43	7	0.40
8	1.18	1.01	8	0.53
9	0.70	0.39	9	0.79
10	0.61	0.60	10	0.45
			11	0.57
			12	0.62
Mean	0.65	0.62		0.69
± s.d.	0.29	0.23		0.24
CV (%)	45	37		34
				18

diseases by Wagner *et al.* (1981) (range: 0.06–2.31 μmol ; mean 0.51 μmol); these authors measured the binding capacity of transcortin for prednisolone by an equilibrium dialysis method similar to ours. The intersubject variability observed in the 17 patients studied by Wagner *et al.* (1981) (coefficient of variation > 100%) is larger than the variability observed in our patients (Table 1). The clinical importance of these variabilities is unknown, because until now the relation between transcortin and prednisolone concentration was investigated in a limited number of patients only (Wagner *et al.*, 1981; Frey *et al.*, 1981). This may be due to the time consuming equilibrium dialysis procedure and to the calculations mandatory for determining the transcortin concentration by the conventional dialysis method: the free fraction of prednisolone changes as a function of the total plasma concentration (Figure 1). Due to diffusion of prednisolone from the plasma half-cell to the buffer half-cell the free fraction of prednisolone at the end of equilibrium dialysis is lower than the true free fraction *in vivo*. Therefore one must calculate the free drug concentration corresponding to the *in vivo* total plasma concentration by use of the estimated parameters, relating total with free prednisolone concentrations in each patient. For the estimation of these parameters (affinity and capacity constants of albumin and transcortin for prednisolone) more than about 10 individual measurements are required (Figure 1). Definitely a simpler screening procedure to estimate the transcortin capacity for prednisolone binding should be available.

Lewis *et al.* (1971) have demonstrated that patients with low concentrations of albumin exhibit more often prednisolone related side-effects, when compared with patients with higher concentrations of albumin. The high incidence of side-effects in the

former patients was hypothetically and in part attributed to the high free fraction of prednisolone as a consequence of the low albumin concentration. However, following commonly used oral doses of prednisolone ($\sim 0.3 \text{ mg/kg}$), the most important determinant of the unbound fraction of prednisolone is not the albumin concentration but the capacity of transcortin to bind prednisolone (Figure 1, equation 1). Thus, one may anticipate that the transcortin capacity alone or in combination with the albumin concentration may be of greater predictive value for prednisolone related side-effects and/or efficacy, than the albumin concentration alone. Because we have demonstrated a close relationship between results obtained by the two methods for transcortin measurements, we propose to use the simpler immunochemical one as a screening method.

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PLASMA PROTEIN BINDING AND HALF-LIFE OF SULPHISOXAZOLE (SULPHAFURAZOLE)

Tse (1980) reported a method for rapid estimation of half-life changes as a result of altered plasma protein binding for drugs whose distribution is confined to extracellular fluids. Sulphisoxazole (SSZ), an antibacterial agent is reported to be one such drug and its apparent volume of distribution (V_d) is shown to be 25–30% of the body weight (Marshall, 1948). A study (Shastri, 1980) carried out on the kinetics of this drug in malnourished adults with low serum albumin levels suggests that theoretical calculations for half-life of a drug like SSZ may not be valid for actual experimental situations. A summary of the data already reported (Shastri, 1980) is presented in Table 1, along with theoretical calculations as proposed by Tse (1980). It may be mentioned that the undernourished subjects in the plasma protein binding experiment, had not participated in the kinetic study earlier, but they were comparable in all respects, including anthropometry and serum albumin concentrations, to the undernourished subjects in the kinetic study.

It is seen that in spite of a reduction in the plasma protein binding of SSZ in the undernourished subjects there is no change either in the actual V_d or the half-life of this drug in these subjects as against changes in theoretical V_d and half-life. The theoretical half-life for SSZ is reduced by more than two-thirds in the undernourished subjects and is significantly different as compared to the wellnourished (Table 1).

The method suggested by Tse (1980) presupposes a change in V_d consequent on a change in plasma protein binding of a drug. It is in fact seen that (Table 1) the theoretical V_d for SSZ expressed per unit of body weight is about 20% higher in the undernourished as compared to that in the wellnourished but when expressed in absolute volumes is actually lower by 23% in the undernourished. This, however, may not occur in an actual situation (Shastri, 1980) for it is seen that V_d expressed in absolute volumes or per unit body weight is similar in the two groups (Table 1) thereby suggesting that some of the assumptions made in the equation may not be valid. One such assumption may be that SSZ is distributed only in the extracellular fluid. In fact, a figure of 0.35 l/kg for the V_d of SSZ (Table 1) supports the possibility that the distribution of this drug may not be confined to extracellular fluid alone. If, indeed, there is some amount of tissue binding for this drug it is possible that in malnutrition it may be reduced, as was earlier suggested for tetracycline (Shastri & Krishnaswamy, 1976), and this could be one of the reasons why the actual V_d has not showed the expected increase in the undernourished subjects. Further, the model used by Tse (1980) may be simplistic, for in malnutrition and other situations where plasma protein binding of SSZ may be reduced, there could be qualitative and quantitative changes in body composition which probably would invalidate the assumptions in the calculation of

Table 1 Plasma protein binding, volume of distribution (V_d) and half-life ($T_{1/2}$) of sulphisoxazole

Groups	Body weight (kg)	% Plasma protein binding	V_d (l)	V_d (l/kg)	Theoretical V_d (l)	Theoretical V_d (l/kg)	$T_{1/2}$ (h)	Theoretical $T_{1/2}$ (h)
Wellnourished	58.6 ±5.52 (6)	87.67 ±2.109 (6)	17.95 ±4.59 (6)	0.35 ±0.092 (6)	6.78 ±0.549 (6)	0.12 ±0.003 (6)	7.69 ± 1.987 (6)	—
Undernourished	39.6 ±6.26** (5)	64.00** ±6.471 (5)	13.05 ±4.67 (8)	0.38 ±0.126 (8)	5.52* ±0.740 (5)	0.14** ±0.005 (5)	6.91 ±0.857 (8)	2.14† ±0.138 (5)

Values are means ± s.d.

Figures in parentheses indicate number of observations.

* $P < 0.02$, ** $P < 0.001$

† $P < 0.001$ compared with actual $T_{1/2}$ in undernourished subjects.