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# The separation of unbound prednisolone in plasma by centrifugal ultrafiltration

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To study variable plasma protein binding of prednisolone in children with nephrotic syndrome we have devised a simple rapid method for measuring unbound prednisolone. The plasma was initially ultrafiltered at 37 °C in a 35 ° fixed angle head at 1500 g for 30 min then the filtrate was analysed by high pressure liquid chromatography. The effects of variable ultrafiltration conditions were studied. The method was used to compare the AUC unbound prednisolone in a nephrotic child (plasma albumin concentration 18 g $L^{-1}$) with a control (plasma albumin concentration 43 g $L^{-1}$); their respective AUC unbound prednisolone values were 4·02 mg h $L^{-1}$ and 1·07 mg h $L^{-1}$.

The unbound fraction of total plasma prednisolone is considered to be the pharmacologically active component of the drug (Pickup 1979) and it may also be related to its toxicity (Lewis et al 1971). In plasma, prednisolone binds avidly to corticosteroid binding globulins (a low capacity protein) but less avidly to albumin, quantitatively the most important binding protein for pharmacological concentrations of glucocorticoids (Ballard 1975).

Children with idiopathic nephrotic syndrome respond well to prednisolone treatment but some develop signs of steroid toxicity. Those children frequently are severely hypoalbuminaemic and this may enhance the toxic effects of treatment. Pharmacokinetic studies of prednisolone in hypoalbuminaemic patients require the measurement of both the total and unbound fraction. Equilibrium dialysis is widely used to measure unbound prednisolone but it is time-consuming and the results are difficult to interpret because of fluid shifts between compartments (Smith & Jubiz 1980).

Ultrafiltration separation is a more rapid method of preparation and has been used in other protein binding studies (Shah et al 1974; Jerkunica et al 1980). We have developed a simple method to measure unbound prednisolone using ultrafiltration separation and have assessed its potential use for clinical studies in children with nephrotic syndrome.

## METHODS

*Ultrafiltration procedure.* Heparinized plasma (1 mL), maintained at 37 °C after collection, was placed in a Centrifree ultrafiltration device (Amicon Corp. Massachussets) consisting of a YMT membrane sealed between a sample reservoir and filtrate cup. Ultrafiltration was performed within 4 h of venesection by centrifugation using a 35° fixed angle head. Albumin was not detected using a bromcresol green method in any (of 10) samples that appeared clear. During the study six filtrates were either cloudy or yellow these contained albumin as detected with Albustix (Ames Corporation) and were discarded.

*Chromatography.* A minimum volume of 200 μL of filtrate was extracted by an ether–dichloromethane mixture (60 : 40 v/v) evaporated to dryness under nitrogen and reconstituted in 100 μL of mobile phase.

The prednisolone concentration was measured by high performance liquid chromatography (HPLC) using a Waters Assoc. Model 6000A constant volume pump, a U6K universal loop injector and a Model 450 UV detector as described by Hartley & Brocklebank (1982). The mobile phase was prepared daily and consisted of dichloromethane–ethanol–water–(500 : 30 : 30) which was mixed for 1 h and separated in a separating funnel. Glacial acetic acid (1%) was added to the organic rich layer* and was passed at a flow rate of 2 mL $min^{-1}$ through a 30 cm × 3·9 mm i.d. 10 μm porous silica column (u Porasil, Waters Assoc.). Detection was achieved by ultraviolet absorption at 254 nm.

Dexamethasone was used as the internal standard, peak area prednisolone/dexamethasone ratios were calculated and compared with a standard curve using a Hewlett Packard 3390A integrator.

*Recovery experiments.* A solution of [2,4,6,7-$^3$H]prednisolone (Amersham Int.) with a final concentration 5·9 mg $mL^{-1}$ (specific activity 1 μCi μL) was prepared in Ringers lactate solution to give a final specific activity of 1 μCi $mL^{-1}$. One mL was placed in a Centrifree sample reservoir and incubated at 37 °C for 25 min to allow the prednisolone to bind to the membrane. After centrifugation, the membrane was removed, washed in 10 mL ether–dichloromethane (60 : 40 v/v) for 1 h. The washings were evaporated to dryness under nitrogen and reconstituted in 1 mL of Ringers lactate. This together with 1 mL of the filtrate was added to 5 mL aqueous scintillation fluid (Scintran Cocktail T, BDH Chemicals, Poole) and counted for 60 s with automatic quench correction on a β counter (Philips PW 4550LS Counter).

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*Examination of optimum ultrafiltration conditions.* Heparinized blood was collected from healthy volunteers 2 h after taking 60 mg oral prednisolone. To avoid changes in binding characteristics the blood was maintained throughout at 37 °C. To examine the optimum ultrafiltration force a sample of plasma was divided into 1 mL aliquots and placed into Centrifree units. Four groups of 8 units were centrifuged at 500, 1000, 1500 and 2000*g*, respectively, for 30 min at 37 °C in a 35° fixed angle rotor. Prednisolone concentrations in the filtrates were measured by HPLC.

The effects of the duration of ultrafiltration were then investigated. One mL aliquots of a sample of plasma were pipetted into Centrifree units and divided into 3 groups of 10. The groups were centrifuged at 1500*g* for 15, 30 and 45 min, respectively, and the prednisolone concentration of the filtrates measured. The influence of storage on the binding of prednisolone was examined by comparing the prednisolone concentration of the filtrates of plasma maintained at 37 °C and analysed within 4 h of collection with identical samples stored at −20 °C for 28 days before ultrafiltration.

The effect of variable pH of the plasma was studied by comparison of 9 aliquots of plasma analysed after gassing with carbon dioxide and placed in the Centrifree units with a 100% $CO_2$ atmosphere, and 10 aliquots of the same plasma analysed without gassing.

Possible effects of temperature on the binding of prednisolone were also studied. Aliquots of blood samples were separated and ultrafiltered at 37, 22 and 4 °C.

*Clinical studies of prednisolone pharmacokinetics.* The disappearance from plasma of total and unbound prednisolone was studied in a 10 year old boy with relapsing steroid responsive nephrotic syndrome. Blood samples were taken through an indwelling intravenous catheter at 10, 20, 30, 45, 60, 90, 180, 240 and 360 min after an intravenous bolus infusion of 60 mg $m^{-2}$ prednisolone (Codelsol, Merck, Sharpe and Dohme). A similar study was done in a normal adult volunteer after a 60 mg $m^{-2}$ intravenous dose of prednisolone. The disappearance from plasma of both total and unbound prednisolone was analysed using the C-STRIP computer program (Sedman & Wagner 1976). From the areas under the curves (AUC) the degree of the protein binding was calculated

$$\% \text{ AUC}_{\text{unbound}} = \frac{\text{AUC}_{\text{unbound}}}{\text{AUC}_{\text{total}}} \times 100$$

Statistical analysis was by Student's *t*-test.

## Results

*Ultrafiltration recovery of [$^3$H]prednisolone.* The non-specific binding of prednisolone to the filter was small averaging 2·1% (n = 5) and recovery in the filtrate was 94 ± 6% (n = 5).

Filtrations at 500 and 2000*g* were unsatisfactory due to the small volume of filtrate and protein leakage but there was no significant difference between the prednisolone concentrations of the filtrates after centrifugation at 1000 or 1500*g*.

Protein binding decreased in samples separated at 4 °C (ng $mL^{-1}$: 59 ± 5·8 s.d., n = 10, $P < 0{\cdot}001$) but there was no significant difference from plasma samples separated at 37 °C or room temperature (20 °C) before ultrafiltration (ng $mL^{-1}$: 44 ± 3·1, 44 ± 1·6 resp., n = 10).

There was also a significant increase in protein binding in 10 whole plasma samples stored at −20 °C for 28 days. The mean prednisolone concentration of the filtrate maintained at 37 °C and examined within 4 h of collection was 146 ± 7·9 ng $mL^{-1}$ (n = 10). In contrast, the average prednisolone concentration of the filtrates of duplicate plasma samples ultrafiltered after storage for 28 days at −20 °C was 131 ± 11·2 ng $mL^{-1}$ ($P < 0{\cdot}001$). Storage of filtrates for 1 month at −20 °C had no adverse effects.

The mean pH of 9 samples gassed with $CO_2$ was 6·31 ± 0·02 and the mean pH of the ungassed samples was 7·55 ± 0·01. The unbound prednisolone concentrations were 139 ± 10·6 and 135 ± 10·1 ng $mL^{-1}$, respectively, showing that pH within the ranges studied had no significant effect on protein binding of prednisolone.

From these studies the final procedure was established for ultrafiltration using 1 mL of plasma maintained at 37 °C after collection then centrifuged in a 35° fixed angle head at 1500*g* for 30 min at 37 °C. The filtrate was stored at −20 °C for later analysis.

*Clinical studies.* The plasma concentration-time curves for both total and unbound prednisolone are shown for a nephrotic boy and a healthy volunteer (Fig. 1). In both individuals the total and unbound curves are characterized by an initial rapid distribution phase followed by a slower elimination phase. Analysis of these data with the C-STRIP program suggested biexponential curves in all four instances. From this, the area under the concentration time curves (AUC) for both total and unbound prednisolone together with the terminal half-life for total prednisolone ($t_{\frac{1}{2}}$) were calculated; the results are shown in Table 1. The AUC of the unbound prednisolone was remarkably higher in the hypoalbuminaemic nephrotic than the healthy volunteer. This was more apparent when the AUC of the unbound fraction

Table 1. Pharmacokinetic data from a hypoalbuminaemic-nephrotic boy compared with normoalbuminaemic control.

| | Pred. dose mg $m^{-2}$ | Plasma albumin (g$L^{-1}$) | AUC mg h $L^{-1}$ Total | AUC mg h $L^{-1}$ Unbound | % AUC unbound | $t_{\frac{1}{2}}$ h total |
|---|---|---|---|---|---|---|
| Nephrotic | 60 | 18 | 6·18 | 4·02 | 65 | 2·42 |
| Volunteer | 60 | 43 | 4·37 | 1·07 | 24 | 3·83 |

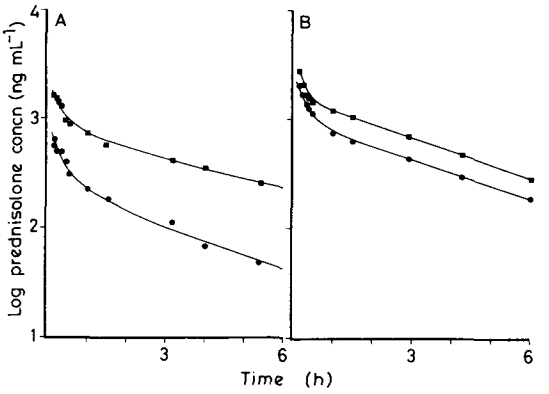


FIG. 1. Plasma concentration-time curves for a healthy volunteer nephrotic boy (A) and a (B) ● unbound, ■ total.

was expressed as a percentage of the total AUC (% AUC unbound). The $t_{\frac{1}{2}}$ of total prednisolone was also shorter in the hypoalbuminaemic nephrotic, probably due to the higher proportion of unbound drug facilitating clearance.

*Discussion*

There is a clinical association between hypoalbuminaemia and an increased incidence of glucocorticoid toxicity (Lewis et al 1971) which might be a consequence of altered pharmacokinetics due to reduced protein binding.

The method using a Centrifree micropartition system (Amicon) is suitable for the analysis of small plasma samples and has been verified for several other systems (Whitlam & Brown 1981). Ultrafiltration was shown to be a simple, rapid (taking approx. 1 h to perform), reproducible method suitable for the analysis of 1 mL samples of plasma. There was minimal binding of prednisolone to the membrane. Protein binding was unchanged in plasma maintained at room temperature providing it was ultrafiltered within 4 h of sampling. The pH of the sample did not alter the degree of protein binding.

The method produced reliable and meaningful results. As expected there was a remarkable increase in the proportion of prednisolone in the unbound form and a shorter half-life of total prednisolone in the hypoalbuminaemic nephrotic patient. The main objection to ultrafiltration is its expense, each Centrifree unit costs approximately £2, though a reusable system is available which approximately halves this cost which must be related to its advantages over equilibrium dialysis.

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