A RADIOIMMUNOASSAY METHOD FOR PREDNISOLONE: COMPARISON WITH THE COMPETITIVE PROTEIN BINDING METHOD

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1 Cross reactivity of prednisolone with an antiserum raised against dexamethasone-21-hemisuccinate conjugated with bovine serum albumin was utilized to develop a radioimmunoassay for prednisolone in unextracted plasma.

2 The results are in good agreement with those obtained with a standard competitive protein binding method employing prior extraction and separation of the synthetic from endogenous steroids.

Introduction

Prednisolone (11 β , 17 α , 21-trihydroxypregna-1, 4-diene-3, 20-dione), a synthetic analogue of cortisol has been used in corticosteroid therapy for many years. However, because of the limited knowledge of prednisolone pharmacokinetics, the dosage regimes and doses used vary widely and are chosen rather arbitrarily. With the recent development of sensitive competitive protein binding (CPB) and radioimmunoassay (RIA) methods for prednisolone (Sandberg, Bacallao & Cleveland, 1970; Turner, Carroll, Runkus, Charles & Chatloraj, 1973; Colburn & Buller, 1973; English, Chakraborty & Marks, 1974) it has been possible to assess the bioavailability of different formulations of prednisolone and prednisone (Leclarcq & Copinschi, 1974; DiSanto & DeSante, 1975; English, Chakraborty, Marks, Trigger & Thompson, 1975). Some observations with important clinical implications have also been made by monitoring plasma prednisolone levels in liver disease patients especially those who develop corticosteroid side-effects (Powell & Axelson, 1972; Kozower, Veatch & Kaplan, 1974). It is likely that the clinical response to prednisolone therapy in various diseases considered in the light of the plasma prednisolone concentrations will assist in the choice of the prednisolone preparation to be used, the dose, route and the frequency of its administration.

The prednisolone radioimmunoassay developed by Colburn & Buller (1973) used an antiserum raised against prednisolone-21-hemisuccinate conjugated to bovine serum albumin (BSA) and showed roughly 10% cross-reactivity with endogenous cortisol. The present paper describes a

method for measuring prednisolone using an antiserum raised against dexamethasone-21-hemisuccinate-BSA conjugate in sheep and which reacts poorly with endogenous steroids. The results of the RIA procedure were compared with those obtained by using a standard CPB assay (English *et al.*, 1974) on plasma samples from patients receiving prednisolone therapy.

Methods

Radio tracers, namely [1,2-³H] cortisol (45 Ci/mmol), and [6,7-³H] prednisolone (47 Ci/mmol) were obtained from the Radiochemical Centre, Amersham and stored in ethanol, at 4°C, at a concentration of 10 μ Ci/ml. Cortisol was obtained from the Sigma Chemical Co. Ltd, London and prednisolone from Steraloids Ltd. 6 β Hydroxycortisol, 3 α , 5 α tetrahydrocortisol and 3 α , 5 β tetrahydrocortisol were generous gifts from the Medical Research Council Steroid Reference Centre, Westfield College, London. 20-Dihydro-prednisolone and 20-dihydrocortisol were made by reducing prednisolone and cortisol with sodium borohydride and were purified by thin layer chromatography (t.l.c.) on Eastman-Kodak Chromagram sheets (13181) in the solvent system:- methylene dichloride: methanol: water 150:10:1 (v/v).

The antiserum raised against dexamethasone-21-hemisuccinate-BSA conjugate in the sheep (Dumasia, Chapman, Moss & O'Connor, 1973) was a gift from Mr M. Moss, Racecourse Security Services Research Laboratories, Newmarket.

Aqueous solutions containing 1% Norit A charcoal or 0.1% Dextran T70 were stored at 4°C. Immediately before use equal volumes of the two solutions were mixed and were constantly stirred before and during use. All counting was carried out in a Packard liquid scintillation counter model no. 2425 and the external standard ratios method was used for quench correction. In this system tritium was counted with an efficiency of 20-25%. The scintillation mixture (6 ml) consisted of two volumes toluene and one volume lissapol containing 0.6% butyl PBD [2-(4'-tert-butyl-phenyl)-5-(4''-biphenyl)-1,3,4-oxadiazole].

Prednisolone assay Competitive protein binding method This method is based on the high affinity of prednisolone for plasma corticosteroid binding globulin (CBG). The details of the procedure have been described elsewhere (English *et al.*, 1974).

Radioimmunoassay

The plasma samples were treated at 70°C for 30 min prior to radioimmunoassay so as to eliminate interference by endogenous CBG with the binding of prednisolone to the antiserum (Foster & Dunn, 1974).

Tubes containing test plasma (0.1 ml) or pooled plasma (0.1 ml) containing known amounts (0-50 ng) of prednisolone and made up to 0.7 ml with 0.1 M phosphate buffer, pH 7.4, were heated at 70°C for 30 minutes. They were then cooled by placing them in an ice bath for 5 minutes. All further steps were carried out at 4°C. To each tube 0.1 ml of the diluted antiserum (1:300 with phosphate buffer) was added, mixed and incubated for 2 hours. [³H]-prednisolone (10,000 d/min (32 pg)) was then added to the mixture and incubated for a further 1 hour. For the separation of the free from antibody bound steroid, Dextran coated charcoal suspension (0.1 ml) was pipetted into each tube, mixed, allowed to stand for 10 min and then spun at 2,000 rev/min for 10 minutes.

The radioactivity in aliquots (0.5 ml) of the

supernatant was measured. The standard curve was prepared by plotting protein bound radioactivity against the mass of prednisolone present. The cross reaction of a compound was determined by comparing the amount required to reduce radio-label binding by 50% with the amount of prednisolone required to produce the same effect.

Results

Figure 1 shows a standard curve for the prednisolone radioimmunoassay. The smallest amount of prednisolone which can be assayed with confidence was 0.5 ng giving a usable range for the assay of 5-400 ng/ml in the plasma.

The within-batch precision of the assay was examined by replicate analyses of three pools of plasma to which prednisolone had been added to give concentrations of 10, 100 and 500 ng/ml. The results in Table 1 show the coefficient of variation was within 3-5%.

The cross-reactivity of the antiserum with various metabolites of prednisolone and some endogenous steroids is shown in Table 2. The cross reaction with cortisol and corticosterone was 6.4% and 3.8% respectively. In contrast, the metabolites of cortisol and prednisolone showed very little cross reaction.

The prednisolone concentration in thirty four plasma samples from patients receiving prednisolone therapy were assayed by both the CPB and RIA methods. The results are shown in Figure 2. Regression analysis gives a slope of 0.86 and a correlation coefficient of 0.95. The line of best fit does not pass through the origin but gives a blank for the CPB assay of 3.3 ng/ml.

Discussion

The data show that binding of prednisolone to dexamethasone antiserum is sufficiently avid for this to be used as the basis for a prednisolone

Table 1 The precision of the radioimmunoassay for prednisolone

Plasma pool	Mass of prednisolone added (ng/ml)	Mass of prednisolone assayed (ng/ml)	Coefficient of variation (%)
1	10	10.08 ± 0.10 (10)	3.13
2	100	102.70 ± 1.70 (10)	5.17
3	500	500.00 ± 4.80 (10)	3.05

The figures in parentheses indicate the number of determinations

Figure 1 Standard curve for the radioimmunoassay of prednisolone. Each point represents the mean of five determinations $\pm$ s.e. mean.

radioimmunoassay and that this is perfectly adequate for the measurement of prednisolone plasma levels in routine clinical investigations. Nevertheless, the technique is somewhat less sensitive than that described by Colburn & Buller (1973) which uses an antiserum raised against a prednisolone-21-hemisuccinate-BSA conjugate. The dexamethasone antiserum used did not cross

Figure 2 Comparison of plasma prednisolone values (in patients who had received oral doses of the drug) obtained by competitive protein binding (CPB) and radioimmunoassay (RIA) methods. $CPB = 0.86 \text{ RIA} + 3.33$.

react significantly with any of the several cortisol metabolites tested but its cross reactivity with cortisol itself was only slightly smaller than that observed by Colburn & Buller with a prednisolone antiserum. An interesting and potentially important difference between the present radioimmunoassay and that described by Colburn & Buller (1973), however, is that 20β -hydroxyprednisolone, a major metabolite of the drug, does not cross react with the antidexamethasone antiserum, and hence is not measured by the present method but interferes markedly with the technique based on the antiprednisolone conjugate. Whether these observations on the cross reaction characteristics are true for all antisera raised against dexamethasone-21-hemisuccinate-BSA conjugate in the sheep need confirmation.

Comparison of the results obtained by the competitive protein binding method and the radioimmunoassay method showed good agreement, although over the whole range of concentrations, the latter technique always gave slightly lower values. This discrepancy can be due either to the presence of cortisol in the pooled plasma used for preparing the standard curve or to some metabolite of prednisolone which cross-reacts in the CPB method. It is scarcely necessary to draw attention to the fact that the prednisolone assay described cannot be used to measure prednisolone in patients who are already, for some reason, receiving therapy with another dexamethasone cross reactive synthetic steroid. It does, however,

Table 2 Cross-reactivity of various corticoids with the antiserum

Compound	Cross-reactivity (%)
Prednisolone	100
20β -hydroxyprednisolone (20 -dihydroprednisolone)	< 1.0
Cortisol	6.4
11-deoxycortisol	2.7
6β -OH cortisol	< 1.0
20 -dihydrocortisol	< 1.0
5α -tetrahydrocortisol	< 1.0
5β -tetrahydrocortisol	< 1.0
Corticosterone	3.8
Prednisone	< 1.0

permit measurement of prednisolone in the presence of prednisone since the latter, for all practical purposes does not cross react with the antiserum.

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References

- COLBURN, W.A. & BULLER, R.H. (1973). Radioimmunoassay for prednisolone. *Steroids*, **21**, 833-846.
- DISANTO, A.R. & DESANTE, K.A. (1975). Bioavailability and pharmacokinetics of prednisone in humans. *J. pharm. Sci.*, **64**, 109-112.
- DUMASIA, M.C., CHAPMAN, D.I., MOSS, M.S. & O'CONNOR, C. (1973). Production and properties of antisera to dexamethasone - protein conjugates. *Biochem. J.*, **133**, 401-406.
- ENGLISH, J., CHAKRABORTY, J. & MARKS, V. (1974). A competitive protein binding method for plasma prednisolone assay. *Ann. clin. Biochem.*, **11**, 11-14.
- ENGLISH, J., CHAKRABORTY, J., MARKS, V., TRIGGER, D.J. & THOMSON, A.G. (1975). Prednisolone levels in the plasma and urine: A study of two preparations in man. *Br. J. clin. Pharmacol.*, **2**, 327-332.
- FOSTER, L.B. & DUNN, R.T. (1974). Single anti-body technique for radioimmunoassay of cortisol in unextracted serum or plasma. *Clin. Chem.*, **20**, 365-368.
- KOZOWER, M., VEATCH, L. & KAPLAN, M.M. (1974). Decreased clearance of prednisolone, a factor in the development of corticosteroid side effects. *J. clin. Endocr. Metab.*, **38**, 407-412.
- LECLERCQ, R. & COPINSCHI, G. (1974). Patterns of plasma levels of prednisolone after oral administration in man. *J. Pharmac. Biopharm.*, **2**, 175-187.
- POWELL, L.W. & AXELSEN, E. (1972). Corticosteroids in liver disease: studies on the biological conversion of prednisone to prednisolone and plasma protein binding. *Gut*, **13**, 690-696.
- SANDBERG, D.H., BACALLAO, C.Z. & CLEVELAND, W.W. (1970). Measurement of plasma prednisolone by a competitive protein binding assay. *Biochem. Med.*, **4**, 383-390.
- TURNER, M.K., CARROLL, C.J., PUNKUS, J.L., CHARLES, D. & CHATTORAJ, S.C. (1973). Simultaneous competitive protein binding assay for cortisol, cortisone, and prednisolone in plasma and its clinical application. *Clin. Chem.*, **19**, 731-736.

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