Journal of Steroid Biochemistry, 1976, Vol. 7, pp. 795–799. Pergamon Press. Printed in Great Britain

A RADIOIMMUNOASSAY FOR BETAMETHAZONE PREPARATION OF A SPECIFIC ANTISERUM TO BETAMETHAZONE-3 (O-CARBOXYMETHYL) OXIME-BOVINE SERUM ALBUMIN AND EVALUATION OF METHOD

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(*Received* 22 *April* 1976)

SUMMARY

The preparation of a highly specific antiserum for betamethazone is described giving details of the preparation of the immunogen betamethazone-3- (O-carboxymethyl) oxime-BSA conjugate, immunisation schedule, antibody titres and the cross reactions of related physiological and synthetic corticosteroids. Details of a radioimmunoassay of betamethazone with and without extraction are given showing negligible cross-reaction with cortisol.

Concentrations in peripheral human venous plasma before, during, and after an intravenous infusion of betamethazone used to induce labour are also reported.

INTRODUCTION

The antiserum to betamethazone (9α-Fluoro-16β-methyl-1,4-pregnadiene-11β,17,21-triol,-3,20-dione), reported within was developed to enable measurement of levels of the steroid in patients receiving preparations of betamethazone.

Recent publications [1, 2] have stressed that specific antisera to corticosteroids can be produced by employing antigens where the steroid is coupled to the protein carrier through an existing functional group providing that the structurally unique features of the steroid are accessible to determine antibody specificity. Thus corticosteroid antigens coupled through the "3" position have generally produced antibodies of greater specificity than those utilising a 21-hemisuccinate linkage. For this reason the antigen betamethazone-3- (O-carboxymethyl) oxime-BSA was prepared.

MATERIALS AND METHODS

Betamethazone was a gift from Glaxo Research Limited and samples of pure betamethazone-17-valerate, dexamethazone, beclomethazone, and triamcinolone were donated by Dr. G. H. Phillips of Glaxo Research Limited, United Kingdom. [1,2,4-^{3}H]-Betamethazone, specific activity 40 Ci/mmol was obtained from the Radiochemical Centre, Amersham, United Kingdom. Other materials used for the preparation of the steroid–protein conjugate and radioimmunoassay technique were as previously reported [3].

Standards

Betamethasone 9-fluoro-16β-methyl-1,4-pregnadiene-11β,17,21-triol-3,20-dione; Dexamethasone 9-fluoro-16α-methyl-1,4-pregnadiene-11β,17,21-triol-3,20-dione; Beclomethasone 9-chloro-16β-methyl-1,4-pregnadiene-11β,17,21-triol-3,20-dione; Triamcinolone 9-fluoro-1,4-pregnadiene-11β,16α,17,21-tetrol-3,20-dione; Prednisolone 1,4-pregnadiene-11β,17,21-triol-3,20-dione; Prednisone 1,4-pregnadiene-17,21-diol-3,11,20-trione; Cortisol 4-pregnene-11β,17,21-triol-3,20-dione; Cortisone 4-pregnene-17,21-diol-3,11,20-trione; Corticosterone 4-pregnene-11β,21-diol-3,20-dione; Deoxycortisol 4-pregnene-17,21-diol-3,20-dione.

Preparation of betamethazone-3- (O-carboxymethyl) oxime

One gram of betamethazone and 5 μCi of [1,2,4-^{3}H]-betamethazone were dissolved in 20 ml of 10% aq. methanol. To this solution 2 ml of 2 M Sodium acetate and 720 mg of carboxymethoxylamine-hemihydro-chloride were added and the solution stirred at room temperature. Progress was followed by t.l.c. of the reaction products on silica gel (t.l.c. system 1 dichloromethane–acetone acid, 70:29:1 by vol.), which indicated about 80% after 10 h. The solution was then acidified with 10% HCl., extracted with ethyl acetate three times and the ethyl acetate washed three times with 50% $NaHCO_3$ to extract the betamethazone-3-(O-carboxymethyl)oxime. The extracted aq. phase was then acidified to pH 3.0 with dilute HCl and extracted three times with ethyl ace-

tate. The latter ethyl acetate extracts were dried ($NaSO_4$) and evaporated under reduced pressure and the residue re-crystallised from ethanol to give needles m.p. 231–232. The product moved as one spot in the following t.l.c. system: (i) Dichloromethane–acetate–acetic acid 70:29:1 (by vol.) $R_f = 0.6$ (betamethazone = 0.8) (ii) Ethyl × 3 acetate–benzene–acetic acid 80:16:4 (by vol.) $R_f = 0.6$ (betamethazone = 0.9) Spectral data: I.R. max. 5.78, 5.87, 6.03, 6.21, 6.33.

Preparation of betamethazone-3- (O-carboxymethyl) oxime-bovine serum albumin

The betamethazone-3-CMO was coupled to BSA using the mixed anhydride technique [4]. From determination of the radioactivity in the conjugate it was calculated that 26.5 mol of steroid were coupled per mol of protein.

Immunisation schedule

Four New Zealand White rabbits were used for the immunisation. To each an initial dose of 2 mg was given distributed among several sites along the back of the animal. Thereafter booster injections were similarly made at four week intervals using 1 mg of the antigen. All injections were made up in 1 ml of saline emulsified with 1 ml of Freund's Complete Adjuvant.

Blood was collected from the animals periodically, beginning ten days after the second booster injections (approximately ten weeks from the start).

Radioimmunoassay

Standard curves were set up by "drying down" the appropriate aliquots, in triplicate, of an acetone solution of betamethazone (1 pg/μl in assay tubes to give standards of 0, 10, 20, 50, 100, 200, and 250 pg) to standards and samples 100 μl of diluted antisera (working dilution 1/10,000) was added and mixed. 100 μl of labelled betamethazone (containing 32,000 d.p.m.) was then added to the tubes which were then mixed and left overnight. Separation of bound and free steroid was effected by addition of 1 ml of Dextran-coated charcoal in assay buffer. Supernatants containing the bound fraction were decanted into 10 ml of toluene-triton scintillant.

Cross reactions were determined by constructing "standard curves" for related steroids and calculated accordingly:

$$\frac{\text{Betamethazone displacing 50\% labelled betamethazone}}{\text{Steroid displacing 50\% labelled betamethazone}} \times 100$$

Specificity was further shown by determining the blank value for other extractable steroids from 10 samples of pregnancy plasma. 1 ml sample of late pregnancy plasma was extracted with 2 × 10 ml of ether. Extracts were dried down and made up in 1 ml acetone from which 100 μl aliquots were taken in duplicate and assayed.

The accuracy was assessed by adding 1, 2, 5 and 10 ng of betamethazone to 1 ml aliquot of (1) deionised-distilled water, (2) pooled pregnancy plasma (3) deionised-distilled water containing 100 ng/ml of cortisol using methods A and B.

In method "A" 10 μl of the sample was taken in duplicate and assayed.

In method "B" 100 μl of these samples were extracted once with 5 ml dichloromethane, dried down, and made up to 1 ml with acetone from which 100 μl was taken in duplicate and dried down in assay tubes.

Cortisol was measured using a specific antisera raised against Cortisol-3- (O-carboxymethyl) oxime-BSA [5].

Clinical study

One patient at term received an intravenous infusion of 2 mg betamethazone phosphate/h for 8 h by a slow infusion pump (Scientifica & Cook) to induce labour. Heparinised cubital vein blood samples were taken before, during and after the infusion at varying intervals for determination of cortisol [5] and betamethazone using the methods described previously. The onset of early labour was diagnosed on clinical criteria—62 h after the infusion had stopped, and 2½ h before the last sample was taken.

RESULTS

Antibody Titres

Of the four animals immunised only one developed antibodies of high titre after ten weeks giving a working dilution, binding 50% of labelled betamethazone, at 1/4000. After fifteen weeks the antibody titre rose to 1/12,000 in this animal. Of the other three animals two showed some binding: 50% at 1/200 and 1/100 after ten weeks but failed to increase after fifteen weeks.

Standard curve

Figure 1 shows a typical standard curve for betamethazone. The sensitivity of the standard curve was below 10 pg.

Specificity

Figure 2 shows the per cent cross-reactions of eleven structurally similar steroids. Other steroids tested were 17-hydroxyprogesterone, progesterone and testosterone all of which showed less than 0.001% cross-reaction. The largest cross-reactions were to dexamethazone (5.5%) and beclomethasone (3.5%) where in the former the structural difference is in the orientation of the 16-methyl group and in the latter is in the size of the halogen substituent at C-9. The cross-reaction of triamcinolone where a hydroxyl group replaces the 16-methyl was two orders of magnitude less. The cross-reactivities of corticosteroids present in plasma were all below 0.005%.

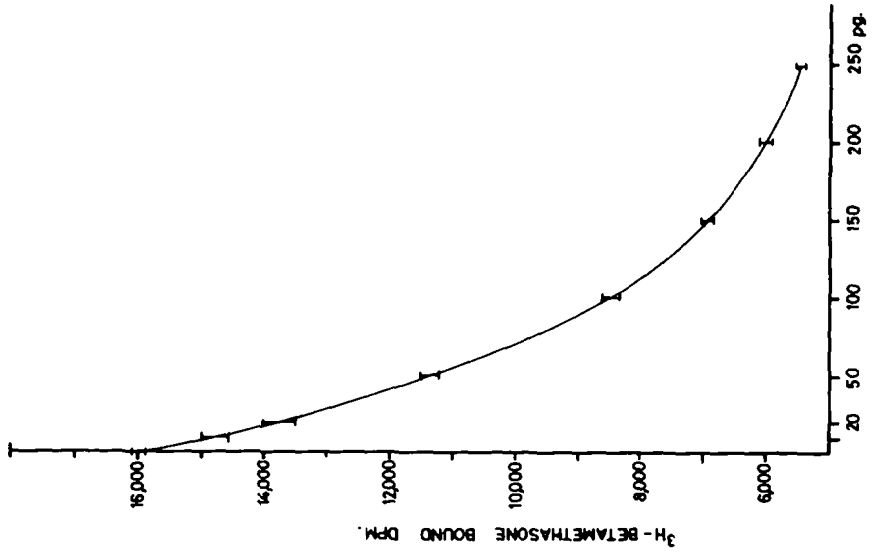

Fig. 1. Standard curve for betamethazone using antiserum to betamethazone-3 (O-carboxymethyl) oxime-BSA showing mean + SE for 5 determinations.

STEROID	STRUCTURE	CROSS-REACTION
Betamethasone		100
Dexamethasone		5·5
Beclomethasone		3·5
Triamcinolone		0·05
Betamethasone-17-valerate		0·001
Prednisolone		0·01
Prednisone		< 0·001
Cortisol		0·005
Cortisone		< 0·001
Deoxycortisol		< 0·001
Corticosterone		< 0·001

Fig. 2. Cross reactions of antiserum to betamethazone-3-CMO-BSA (betamethazone-21-disodium phosphate = 0.05%).

Table 1. Replicate analyses of unlabelled betamethazone added to: (1) deionised-distilled water (2) pooled pregnancy plasma (3) deionised-distilled water containing 100 ng/ml of cortisol using methods A and B (see text)

	Amount added ng/ml	Betamethazone measured ng/ml	S.E.	Coefficient of Variation
Method A (No extraction)				
(1) Water	1.0	1.1	0.06	5.0
	2.0	2.13	0.06	2.8
	5.0	5.08	0.18	3.5
	10.0	9.93	0.31	3.1
(2) Plasma	1.0	1.18	0.09	7.3
	2.0	1.90	0.07	3.7
	5.0	5.15	0.06	1.3
	10.0	9.75	0.10	1.1
(3) Water + Cortisol (100 ng)	1.0	1.0	0.07	
	2.0	1.93	0.05	2.5
	5.0	4.88	0.11	2.3
	10.0	9.78	0.10	1.1
Method B (With extraction)				
(1) Water	1.0	0.9	0.04	4.6
	2.0	1.8	0.04	2.3
	5.0	4.8	0.18	3.8
	10.0	9.6	0.15	1.5
(2) Plasma	1.0	1.1	0.04	3.7
	2.0	2.0	0.04	2.0
	5.0	4.9	0.13	2.7
	10.0	9.3	0.14	1.5
(3) Water + Cortisol (100 ng)	1.0	0.9	0.04	4.5
	2.0	2.0	0.03	1.3
	5.0	4.8	0.06	1.3
	10.0	9.7	0.19	2.0

The blank value obtained from analyses of ten late pregnancy plasmas gave a mean and S.D. of 13.3 ± 2.38 pg.

Betamethazone disodium phosphate has a cross-reaction of 0.05%.

Accuracy and precision

Table 1 shows accuracy of replicate analyses of betamethazone with added water, plasma, and water containing 100 ng/ml of cortisol.

Plasma concentrations

Levels of betamethazone and cortisol found in a patient receiving betamethazone-disodium phosphate (Betnesol) 16 mg, given intravenously over 8 h for induction of labour are shown in Fig. 3. Betamethazone concentrations increased from undetectable values to reach their highest levels at the conclusion of the infusion, rapidly declining thereafter. Twenty-four h after completion of the infusion betamethazone was again unrecordable.

Cortisol values were depressed during the infusion but returned to values found in late pregnancy at the time of sampling thirty-eight h after the end of the infusion.

DISCUSSION

Methods published on the radioimmunoassay of dexamethazone have shown marked interference from cortisol from unsuppressed plasma samples, Meikle *et al.* [6] used an antiserum to dexamethazone-3-(O-carboxymethyl) oxime BSA which showed a cross-reaction of 0.4% with cortisol. This relied on paper chromatography to purify dexamethazone prior to assay. Lee and Oshawa[7] employed an antiserum to dexamethazone-21-hemisuccinyl-BSA with a cortisol cross-reaction of 3.5% (based on relative displacement of label at the 1 ng level). A purification step was also employed: endogenous corticosteroids bound to corticosteroid-binding globulin were separated from dexamethazone by absorption of the latter with Florisil. The antiserum to betamethazone-3-(O-carboxymethyl) oxime-BSA described in this paper showed a high degree of specificity. Generally the specificity was two orders of magnitude better than that achieved by Meikle *et al.* [6] for dexamethazone, and sufficient to obviate the need for purification steps in the presence of relatively high levels of cortisol and other endogenous corticosteroids. Furthermore, since the presence of a fluorine atom in the B ring of the structure of betamethazone results in negligible binding to corticosteroid binding globulin [8] betamethazone could be measured with accuracy using a direct non-extraction method.

The results obtained on human plasma samples indicate the method is accurate in detecting betamethazone concentrations after therapy and suggests the infusion of 16 mg betamethazone over eight h has a

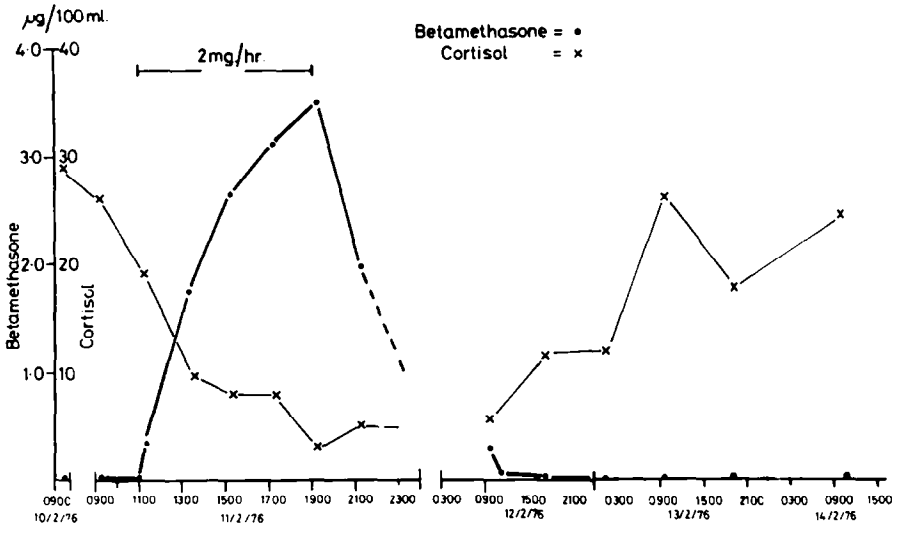

Fig. 3. Levels of betamethazone and cortisol following intravenous infusion of 2 mg of betamethazone disodium phosphate hourly from 11.00 h to 19.00 h for induction of labour.

transient adrenal suppressive effect lasting up to thirty-eight h. The evalution of plasma sex steroid levels in this subject on the same samples showed no effect of betamethazone on circulating progesterone values but a significant decline in oestradiol occurring concomittantly with the betamethazone therapy, and a subsequent rise above pre-influsion values. This finding, together with a marked reduction in urinary oestriol excretion in this patient, indicates a marked inhibitory effect on the supply of oestrogen precursors for placental metabolic conversion.

Acknowledgements– We wish to thank Dr. Heulwen Morgan of Queen Charlotte's Maternity Hospital for assistance with supplying the plasma samples from the patient described and Glaxo Laboratories Limited for their gift of steroids.

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