Journal of Steroid Biochemistry, Vol. 9, pp. 359–363

0022-4731/78/0501-0359$02.00/0

A RADIOIMMUNOASSAY FOR SERUM MEDROXYPROGESTERONE ACETATE

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(*Received* 20 *June* 1977)

SUMMARY

A radioimmunoassay for measuring medroxyprogesterone acetate in serum or plasma is described using an antiserum produced against the steroid-3-carboxymethyloxime coupled to bovine serum albumin. The method has adequate accuracy, precision and sensitivity and the reliability criteria of the method are compared with those of other radioimmunoassays described for this steroid. Seven days after the intramuscular injection of 150 mg Depo-Provera, serum levels of medroxyprogesterone acetate ranged from 1750–9000 pg/ml and by 75 days these levels had decreased to 680–2600 pg/ml.

INTRODUCTION

Depo-Provera is a microcrystalline suspension of medroxyprogesterone acetate (MPA, 17α-hydroxy-6α-methylpregn-4-ene-3,20-dione-17α-acetate). When injected intramuscularly in a dose of 150 mg, Depo-Provera will provide a contraceptive effect for at least three months. Depo-Provera has been widely used and shown to be an effective and acceptable long-acting contraceptive [1]. Because of the use of this formulation in clinical trials organised by the World Health Organisation a sensitive radioimmunoassay for MPA was developed. The method which has been in use in this laboratory for the past two years is described in this paper. Other investigators [2–4] have also described radioimmunoassays for MPA.

METHODOLOGY

Preparation of antibodies to medroxyprogesterone acetate

MPA was converted to the 3-O-carboxymethyloxime (MPA-3-CMO) and the oxime was conjugated with bovine serum albumin (BSA) by the mixed anhydride method [5]. Spectrophotometric analysis showed that 22 residues of MPA-3-CMO were coupled per molecule of BSA. Antibodies against MPA-3-CMO-BSA were raised in New Zealand White rabbits as described previously [6]. Four rabbits were immunised. A high titre of MPA antibodies was detected 6 months after immunization. The rabbits were bled at this time and after clotting, the blood was centrifuged to obtain serum which was stored at −20°C. Only serum from the rabbit with the highest titre of antibodies to MPA was used.

Radioimmunoassay of medroxyprogesterone acetate

Serum (0.5–1 ml) was pipetted into extraction tubes (50 ml capacity) containing about 500 c.p.m. [^{3}H]-MPA (S.A. 40 mCi/mmol; New England Nuclear) in 0.1 M phosphate buffer pH 7.0 containing 0.1% gelatin (Sigma) and 10% methanol (PGM buffer). Samples were mixed and equilibrated at room temperature for 30 min. Extraction was carried out with 10 ml freshly distilled diethyl ether. The ether extracts were evaporated to dryness in a vacuum oven and the residues were dissolved in 1.7 ml PGM buffer. After 30 min, 0.5 ml of the solution was counted and used to correct for extraction losses. Two other 0.5 ml samples were pipetted into 16 × 75 mm glass tubes for assay. For the standard curve, 0.5 ml PGM buffer containing various concentrations of MPA (range 10–1000 pg/ml) were pipetted into assay tubes. Antiserum solution (0.1 ml of a 1:28000 dilution in plain phosphate buffer) and 0.1 ml PGM buffer containing 10000 d.p.m. [^{3}H]-MPA were pipetted into all the assay tubes and the contents were mixed thoroughly.

After incubation for 18 h at 4°C the tubes were transferred to an ice-bath and free and bound MPA were separated by addition of 0.5 ml 2% charcoal in 0.2% dextran T-70 solution. Each sample was mixed for 2 s on a vortex mixer and after 10 min centrifuged at 500 ***g*** for 10 min at 4°C. The bound fraction of MPA contained in the clear supernatant was decanted directly into counting vials containing 8 ml scintillant (1 l. toluene, 500 ml Triton X-100, 6 g PPO and 0.2 g POPOP) and the radioactivity was estimated. Duplicate samples of a pooled plasma not containing MPA as well as samples from this pool containing known amounts of MPA (varying from 100 pg/ml to 2000 pg/ml) were analyzed with each batch of assays.

RESULTS

Reliability criteria of the assay

Specificity. A few possible metabolites of MPA were tested for their interaction with the antiserum and only three (metabolites IV, VI and VII) showed a sig-

Table 1. Cross-reaction of possible metabolites of MPA with antiserum

Steroid	% Cross-reactivity (MPA = 100)
Metabolite I (11α,17α-dihydroxy-6α-methyl-4-pregnene-3,20-dione-17-acetate)	2
Metabolite II (6β,17,21-trihydroxy-6-methyl-4-pregnene-3,20-dione-17-acetate)	<0.01
Metabolite III (17,21-dihydroxy-6α-methyl-4-pregnene-3,20-dione-17-acetate)	1
Metabolite IV (17α-hydroxy-6α-methyl-5β-pregnane-3,20-dione-17-acetate)	62
Metabolite V (17α-hydroxy-6α-methyl-4-pregnene-3,20-dione)	<0.01
Metabolite VI (3β,17α-dihydroxy-6α-methyl-4-pregnene-20-one-17-acetate)	79
Metabolite VII (17α-hydroxy-4-pregnene-3,20-dione-17-acetate,-17-acetoxyprogesterone)	14

nificant cross-reaction (Table 1). A number of naturally occurring steroids (progesterone, 17α-hydroxyprogesterone, cortisol, oestradiol, testosterone and DHA) were also tested and their cross-reactivities were all less than 0.01%.

Recoveries and precision. Values obtained for the recovery of MPA added to plasma and assayed ranged from 72% to 120%. Mean values for recoveries at various levels of steroid added to plasma are shown in Table 2.

The intra-assay variation was also satisfactory. Replicate analyses of samples of pooled plasma to which MPA had been added in various concentrations gave the following values (mean ± % S.D.; no. of estimations in parentheses): 100 pg/ml, 94 ± 13.6 [5]; 200 pg/ml, 170 ± 6.8 [4]; 500 pg/ml, 428 ± 3.7 [6]; 2000 pg/ml, 1856 ± 4.4 [3]; 4000 pg/ml, 3156 ± 3.3 [6]; 8000 pg/ml, 6267 ± 2.1 [8]. Thus the intra-assay coefficient of variation for concentrations of MPA greater than 200 pg/ml was less than 5%. The inter-assay variation was derived from duplicate analyses carried out on different days of plasma samples containing various concentrations of MPA (from 100 to 3200 pg/ml). For duplicate analyses of 32 different samples of plasma the coefficient of variation was 6%.

Sensitivity. Under the conditions of the assay about 40% of [^{3}H]-MPA was bound by the antiserum when no unlabelled MPA was added to the assay tube. The binding was significantly reduced ($P < 0.05$) in the presence of 10 pg unlabelled MPA per assay tube so that the minimum detection limit of the assay was regarded as 10 pg/tube. In practical terms the sensitivity of the method was 50 pg/ml. Standard curves plotted as logit B/B_0 against log dose were linear from 10 to 1000 pg MPA.

Comparison of different extraction methods

The radioimmunoassay for MPA described by Hiroi *et al.*[4] utilised extraction of plasma by a benzene–*iso*-octane (2:1, by vol.) mixture. They suggested that use of this solvent might eliminate water-soluble metabolites of MPA which might cross-react with the MPA antiserum and that this was the possible reason for the lower values they observed compared to values reported previously [2] in similar subjects using a double antibody radioimmunoassay of MPA in unextracted serum samples. Hiroi *et al.* however did not publish a comparison of their extraction technique with the direct assay. Table 3 shows a comparison of results obtained using the extraction procedure described in this paper and that described by Hiroi *et al.* There was no difference between the two extraction techniques in the extraction of MPA or of the three metabolites cross-reacting with our antiserum. This observation was further supported by similar levels of MPA found using the two extraction techniques for plasma from women treated with MPA.

Kaiser *et al.*[7] reported that plasma levels of MPA measured by a double antibody radioimmunoassay in unextracted serum were five- to ten-fold greater than values obtained by a gas–liquid chromatographic assay. They suggested, therefore, the presence in serum of metabolites of MPA which cross-react

Table 2. Recovery of medroxyprogesterone acetate (MPA) for plasma

MPA added (pg/ml)	No. of determinations	MPA recovered (mean ± S.D.; pg/ml)	Coeff. of variation (%)
100	9	97.1 ± 17.9	18.5
200	10	185 ± 30.8	16.6
500	10	423 ± 29.2	6.9
2000	10	1801 ± 198	11.7
4000	9	3373 ± 394	11.7
8000	10	6590 ± 713	10.8

Table 3. Comparison of MPA values obtained by radioimmunoassay of plasma samples extracted with diethyl ether or benzene–*iso*-octane

Sample	'MPA' (pg/ml)	
	diethyl ether extraction	benzene–*iso*-octane extraction
Pooled plasma containing known amounts of MPA or metabolites:		
Pooled plasma	<50	<50
Pooled plasma + 200 pg/ml MPA	190	216
Pooled plasma + 2000 pg/ml MPA	2024	2091
Pooled plasma + 2000 pg/ml metabolite IV	827	930
Pooled plasma + 2000 pg/ml metabolite VI	1159	1054
Pooled plasma + 4000 pg/ml metabolite VII	815	704
Plasma from DMPA treated subjects:		
GE 18.4.	1038	1200
GE 25.7.	503	654
LB 19.3.	3752	4444
LB 12.6.	872	1008
VB 28.2.	1866	1839
VB 23.5.	486	512
LL 28.4.	3763	3054
LL 24.6.	446	592

with the antiserum. Table 4 shows a comparison of MPA levels in plasma samples from subjects treated with DMPA assayed by the method described here involving diethyl ether extraction and a radioimmunoassay in which MPA levels were estimated directly in plasma samples after methanol precipitation, similar to the technique described for the estimation of serum norethisterone oenanthate [8]. The results indicate that MPA values obtained by direct assay of unextracted plasma are two to three times higher than those measured after diethyl ether extraction. These results confirm the presence in plasma of metabolites of MPA which are not extracted by diethyl ether and which are responsible for the elevated levels of MPA found when a direct assay method is used on unextracted plasma samples. However the nature of the metabolites is unknown. When the possible metabolites of MPA available to us were added to plasma and assayed either after extraction with diethyl ether or benzene, *iso*-octane or assayed directly after methanol precipitation, there were no significant differences in MPA values obtained by the three different methods (Table 5).

Table 4. Comparison of MPA values obtained by diethyl ether extraction or direct assay of serum samples from women receiving Depo-Provera

Sample	'MPA' (pg/ml)	
	ether extraction	direct assay
GE 25.4.	1032	2100
GE 16.5.	539	920
GE 7.11.	258	700
GE 12.12.	189	440
LL 21.4.	7185	10000
LL 13.5.	1741	3500
LL 18.6.	560	970
LL 5.8.	<200	<200
LB 13.3.	4360	9000
LB 14.5.	1313	3600
VB 27.3.	944	2400
VB 19.6.	316	1250

Application of the method

Ten healthy women attending the Anaemia and Malnutrition Research Centre, Chiangmai, Thailand were given an intramuscular injection of 150 mg Depo-Provera. Seven days after injection serum MPA levels ranged from 1750–9000 pg/ml (mean ± S.D., 3560 ± 2458 pg/ml). By 75 days after injection these levels had decreased and ranged from 680–2600 pg/ml (mean ± S.D., 1245 ± 617 pg/ml).

DISCUSSION

The radioimmunoassay for MPA described in this paper has satisfactory accuracy, precision and sensitivity. The sensitivity of our method (50 pg/ml) is similar to that (200 pg/ml) of Hiroi *et al.*[4] and could be improved by use of an iodinated (^{125}I) ligand [9]. The direct radioimmunoassays for MPA in blood [2, 3] appear much less sensitive (about 500 pg/ml).

The question of specificity cannot be satisfactorily answered. Both our method and that of Hiroi *et al.*[4] give similar values when applied to blood samples (Table 3) but the direct assays give much higher values (Table 4). Levels of MPA determined by direct radioimmunoassay on plasma samples from dogs were 5–10 times greater than values obtained by a gas–liquid chromatographic analysis [7]. Whether these higher values are due to the presence of MPA in a form which is estimated in the direct assay but not extractable by organic solvents or is due to the

Table 5. Comparison of values obtained in the MPA radioimmunoassay after diethyl ether extraction, benzene–*iso*-octane extraction or direct assay after methanol precipitation of samples of plasma containing MPA or its metabolites

Sample	Steroid (pg/ml)		
	ether extraction	benzene–*iso*-octane extraction	direct assay after methanol precipitation
Pooled plasma	< 50	< 50	< 50
Pooled plasma + MPA (200 pg/ml)	224	210	180
Pooled plasma + MPA (2000 pg/ml)	2100	2320	1900
Pooled plasma + MPA metabolite I (20,000 pg/ml)	1280	1000	1200
Pooled plasma + MPA metabolite II (400,000 pg/ml)	176	128	100
Pooled plasma + MPA metabolite III (40,000 pg/ml)	240	400	100
Pooled plasma + MPA metabolite IV (5,000 pg/ml)	2200	2560	2400
Pooled plasma + MPA metabolite V (400,000 pg/ml)	264	–	270
Pooled plasma + MPA metabolite VI (4,000 pg/ml)	2720	2960	2200
Pooled plasma + MPA metabolite VII (4,000 pg/ml)	1400	1560	1520

presence of MPA metabolites is unknown. Our preliminary studies have shown that the amount of MPA extractable from serum can be increased by incubating the sample with a β-glucuronidase preparation prior to extraction.

Despite its widespread use little is known about the metabolism of MPA in humans particularly when the drug is administered intramuscularly. Most of the studies reported were concerned with the drug administered either orally [10, 11] or intravenously [12, 13]. Two metabolites have been identified; 6α-methyl-6β,17α,21-trihydroxypregn-4-ene-3,20-dione-21-acetate is excreted in urine as a conjugate, almost certainly as a glucosiduronate [14, 15] and Ishihara *et al.*[16] observed the loss of the axial 17α-acetate group from MPA after its administration to baboons. Neither of these two metabolites showed any significant cross-reaction with our antiserum.

Jeppsson and Johansson[17] studied two women injected intramuscularly with 150 mg Dep-Provera. The levels of MPA they detected in serum at 7 days (about 3000 and 4000 pg/ml) and at 75 days (about 600 and 1100 pg/ml) after injection fell within the range of values found by us. The three women studied by Ortiz *et al.*[9] had lower values 7 days after injection (about 1500, 1200 and 2500 pg/ml) but similar values at 75 days after injection (about 1000, 1000 and 700 pg/ml).

Acknowledgements—This work was supported by a grant from the World Health Organization. We are also grateful to Dr. K. T. Kirton, Upjohn Co. Kalamazoo, U.S.A. for the gift of MPA metabolites and Dr. Kosin Amatyakul for supplying samples of serum from women receiving DMPA.

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