J. steroid Biochem. Vol. 23, No. 1, pp. 103–106, 1985
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0022-4731/85 $3.00 + 0.00
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A NEW RADIOIMMUNOASSAY FOR SERUM 16α-HYDROXYANDROST-4-ENE-3,17-DIONE WITH SPECIFIC ANTISERUM

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(*Received* 31 *August* 1984)

Summary—A radioimmunoassay system for serum 16α-hydroxyandrost-4-ene-3,17-dione was developed with the use of rabbit antiserum against 16α-hydroxyandrost-4-ene-3,17-dione-3-(*O*-carboxymethyl)oxime which was conjugated with bovine serum albumin. The antiserum was highly specific for 16α-hydroxyandrost-4-ene-3,17-dion, with cross reactions to other steroids being less than 0.8% except for androst-4-ene-3,17-dione(3.4% cross reaction). Use of LH-20 column chromatography, however, clearly separated these two steroids. Pregnancy sera were measured with this assay system after an addition of labelled internal standard, extraction and separation by column chromatography. The lower limit of detection for 16α-hydroxyandrost-4-ene-3,17-dione was 2 pg/tube. The mean recovery rate of the added standard was 98.3 ± 8.8% (mean ± SE). Intra- and inter-assay coefficients of variation were 8.6% ($n = 6$) and 12.1% ($n = 7$), respectively.

INTRODUCTION

High concentrations 3β,16α-dihydroxyandrost-5-ene-17-one(16-α-hydroxy-DHA) and its sulfated form 16α-hydroxy-DHA sulfate are circulating in the umbilical vessels at the time of birth [1, 2]. The steroid 16α-hydroxy-DHA is converted to estriol during incubation experiments with full-term placental preparation and with midterm placentas perfused *in situ* [1, 3]. In these studies, an intermediary metabolite, 16α-hydroxyandrost-4-ene-3,17-dion (16α-hydroxyandrostenedione) was detected. The clinical significance of estriol and 16α-hydroxy-DHA and their sulfated forms, which are produced in large amounts during human pregnancy, is not absolutely clear at the moment. Due to the lack of a routine assay method for 16α-hydroxyandrostenedione, its role in human pregnancy has remained obscure. This paper describes a new method for quantitating 16α-hydroxyandrostenedione from plasma extracts. LH-20 column chromatography was used for steroid separation prior to quantitation by specific radioimmunoassay (RIA).

EXPERIMENTAL

Tritiated 16α-hydroxyandrostenedione was prepared as follows. [1,2,6,7-^{3}H]DHA (Amersham International Ltd; sp.act. 60–90 Ci/mmol) was incubated with *Streptomyces roseochromogenes* resulting in the production of [1,2,6,7-^{3}H]16α-hydroxy-DHA. This product was first purified by thin layer chromatography (TLC; ethyl acetate–*n*-hexane–acetic acid, used as the solvent system, 15:4:1, by vol). The 16α-hydroxy-DHA fraction was further incubated with the 10,000–105,000 *g* precipitate of porcine corpora lutea. Incubation was carried out at 37°C in air for 60 min. Tritiated 16α-hydroxyandrostenedione thus formed was extracted with ethylether. The extract was first separated on TLC (cyclohexane–ethylacetate used as the solvent system, 1:1) and then the 16α-hydroxyandrostenedione spot was eluted and the extract was applied to the LH-20 column prepared in *n*-hexane–benzene–methanol (8:1:1, by vol). The 16α-hydroxyandrostenedione fraction thus obtained was used for the internal standard to correct losses during extraction and separation, and for RIA.

Standard non-radioactive steroids were purchased from Steraloids Inc. and their purity was checked by TLC prior to use. Steroids stock solutions were prepared in redistilled ethanol and stored at −20°C. Organic solvents used were of reagent grade. Tap water was passed through an ion exchange resin and distilled 3 times in all-glass stills.

Antiserum was raised in rabbits against 16α-hydroxyandrostenedione-3-(*O*-carboxymethyl)oxime conjugated with bovine serum albumin (BSA). This antigen was synthesized in our laboratory by the reaction of 16α-hydroxyandrostenedione (Sigma Chemical Co.) with *O*-(carboxymethyl)hydroxylamine followed by the procedures to form steroid–protein conjugates described elsewhere [7]. The saline solution of the antigen was mixed with the equal volume of complete Freund's adjuvant, and the emulsified solution was injected intracutaneously to rabbits. Following 5 successive intracutaneous injections at

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multiple sites on the back (1 month intervals), a satisfactory titer of the antiserum was obtained. The anti-serum was subjected to precipitation procedures to obtain the gamma globulin fraction. Ten milliliters of the serum were mixed with the equal volume of 50% ammonium sulfate, the precipitate was dissolved in water and added with the same volume of 33% ammonium sulfate. The precipitate formed was dissolved in 50 ml of distilled water and stored in a refrigerator at 4°C. This stock solution of antiserum was diluted 1:2,000 with the assay solution; the final dilution factor of the antiserum being 1:10,000 per assay tube. Phosphate buffered saline solution containing 0.06% gamma globulin and 0.05% BSA was used as the assay solution.

RIA procedures were essentially the same as those published for other steroids [4]. One milliliter of plasma, containing 1,000 dpm of [^{3}H]16α-hydroxyandrostenedione as a tracer, was extracted twice with 5 ml of water-saturated peroxide free ethylether. The combined extract was then washed once with water (10:1, v/v), evaporated under a stream of nitrogen, and the residue transferred to the LH-20 mini-column prepared in a 2 ml Tuberculin syringe. The solvent system now used was *n*-hexane–benzene–methanol (8:1:1, by vol). The column was eluted with the solvent and the 16α-hydroxyandrostenedione fraction was collected into an assay tube (9 × 50 mm).

The residue was dissolved in 1 ml of ethanol, half of which was taken for calculating percent recovery, 10,000 dpm of [^{3}H]16α-hydroxyandrostenedione was added per tube, and the organic solvent was dried *in vacuo*. Each assay tube contained tritiated ligand plus either standard or plasma extract. The antibody solution (250 μl) was then added and mixed well.

The tubes were then incubated at 25°C for 30 min, after which 250 μl of 50% ammonium sulfate was added, mixed well, and incubation was continued for 10 min. Upon completion of incubation, the tubes were centrifuged at 1,000 *g* for 15 min at 4°C. An aliquot of the supernatant (250 μl) was transferred to a counting vial and 10 ml of scintillator (PPO 4 g, POPOP 0.05 g and toluene up to 1,000 ml) were added and the tubes were shaken vigorously for 1 min. Counts were performed in an Aloca Liquid Scintillation Spectrometer (Model SC-900).

RIA data were processed using a four parameter logistic curve fitting routine with use of an IBM call system [5].

Accuracy, precision and specificity of the RIA method were checked by recovery experiments, multiple determinations of the quality control samples, and by measuring each of various steroids in this assay system.

RESULTS

Titer of the antiserum

The final dilution factor of the antiserum per assay tube was 1:10,000.

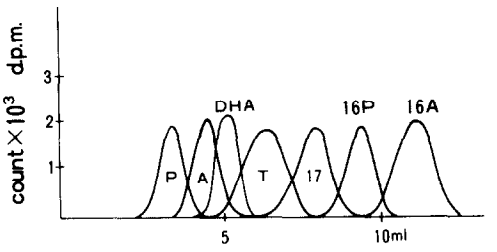

Fig. 1. Elution of 16α-hydroxyandrostenedione and other steroids by Sephadex LH-20 Column. The solvent system used was *n*-hexane–benzene–methanol (8:1:1, by vol.) P = progesterone; A = androstenedione; DHA = dehydroepiandrosterone; T = testosterone; 17 = 17α-hydroxyprogesterone; 16P = 16α-hydroxyprogesterone; 16A = 16α-hydroxyandrostenedione. (Estrogens were eluted far behind the 16A peak).

Specificity of the method

A typical elution pattern for the seven tritiated steroids recovered from the LH-20 column is shown in Fig. 1. Androstenedione, which showed the highest cross reaction (3.4%) with the antiserum, was clearly separated from the 16α-hydroxyandrostenedione. Table 1 shows cross reactivities of some related steroids with the antiserum. No steroid tested showed more than 3.4% cross reaction.

Characteristics of the calibration curve

The slopes of the straight lines produced by logit-log transformation were located between 0.5 and 0.6, showing that uniformity was satisfactory. The 50% intercept was located between 28 and 79 pg. The minimal detectable doses ranged from 0.6 to 2.0 pg. In the absence of steroids, the initial bound to total ratio (B/T) ranged from 70 to 80%. Each of 6 standard curves analysed by 4 parameter sigmoidal curve fit with variable weighting method demonstrated that this assay system provides a relatively stable radioimmunoassay method.

Table 1. Cross reactions of various steroids with the antiserum against 16α-hydroxyandrostenedione-3-CMO-BSA

Compounds	Per cent cross reaction
16α-Hydroxyandrostenedione	100.00
Androst-4-ene-3,17-dione	3.40
Testosterone	0.57
16α-Hydroxyprogesterone	0.80
17α-Hydroxyprogesterone	0.50
Progesterone	0.47
16α-Hydroxyestrone	0.12
16α-Hydroxytestosterone	0.16
Androsterone	0.11
16α-Hydroxypregnenolone	<0.01
17α-Hydroxypregnenolone	<0.01
Dehydroepiandrosterone	<0.01
16α-Hydroxydehydroepiandrosterone	0.04
16-Keto-androst-5-ene-3β,17β-diol	<0.01
Estrone	<0.01
Estradiol	<0.01
Estriol	<0.01
Cortisol	<0.01

Figure 2 illustrates one standard curve with the 90% confidence limits.

Accuracy and reproducibility of the method

Accuracy of the method was assessed by recoveries of known quantities (2 and 5 ng) of 16α-hydroxyandrostenedione added to 1 ml of plasma; $95.7 \pm 7.4\%$ ($n = 10$) and $102.0 \pm 9.1\%$ ($n = 10$) of the added steroid was recovered, respectively. The coefficient of variance for intra- and inter-assays was computed to be 8.6% ($n = 6$) and 12.1% ($n = 7$) when 2 ng of 16α-hydroxyandrostenedione were added to one ml of plasma.

Blood concentrations of 16α-hydroxyandrostenedione

Peripheral plasma concentrations of 16α-hydroxyandrostenedione for pregnant women at various gestational stages are shown in Table 2. Up to 11 weeks the mean value was low, from 24 weeks it rose up and at term the average titer of the steroid increased further.

Immediately following normal vaginal delivery, the mean concentration of the steroid in umbilical arterial blood was slightly lower than that in umbilical venous blood. The concentration in the amniotic fluid, collected during amniotomy at term delivery, was still lower than that in the umbilical arterial blood, but it was much higher than that in the maternal peripheral blood (Table 3).

DISCUSSION

The radioimmunoassay method described here provides a specific assay system for 16α-hydroxyandrostenedione in biological fluids. The accuracy and precision of the method have been shown to be satisfactory.

Although the antigen used to generate the antibody was coupled with BSA at position C-3, the resulting antiserum discriminated a 5-ene-analogue very nicely (16α-hydroxy-DHA).

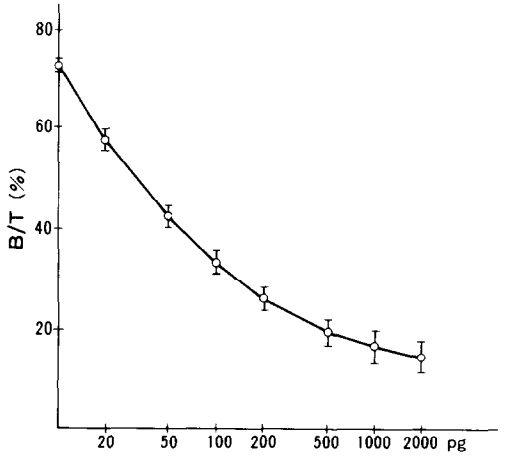

Fig. 2. RIA dose–response curve obtained using anti-16α-Hydroxyandrostenedione-3-CMO-BSA serum and 16α-Hydroxyandrostenedione-[1,2,6,7-^{3}H] ♀ represents 90% confidential limits.

Table 2. 16α-Hydroxyandrostenedione levels in peripheral blood of pregnant women

Duration of pregnancy	Mean ± SE (ng/ml)		N
5–11 weeks	1.53	0.36	10
12–23 weeks	1.71	0.51	20
24–36 weeks	2.85	0.59	39
Full-term	5.06	1.30	19

Likewise, 16α-hydroxyestrone, which has the same D-ring structure as 16α-hydroxyandrostenedione, cross reacts with the antiserum only minimally. Studies using column chromatography further established the specificity of this method. Indeed, progesterone, androstenedione, testosterone, 17α-hydroxyprogesterone and 16α-hydroxyprogesterone, which all exhibit more than 0.5% cross reactivity with 16α-hydroxyandrostenedione, were clearly separated after elution on an LH-20 column.

Plasma concentrations of 16α-hydroxyandrostenedione in peripheral venous blood from pregnant women tended to increase as gestation proceeded. These data suggest production of 16α-hydroxyandrostenedione by the feto–placental unit. In fact, the present results indicate that the levels of 16α-hydroxyandrostenedione in the amniotic fluid and the umbilical cord plasma are much higher than levels found in maternal peripheral blood at the corresponding gestational age. An elevated level of 16α-hydroxyandrostenedione is found in the umbilical venous blood as compared to the arterial sample. This indicates an extensive conversion. The precursor steroid 16α-hydroxy-DHA is mainly produced on the fetal side. The precursor is carried in the umbilical artery [2] to the placenta and is converted there to 16α-hydroxyandrostenedione by 5-ene,3β-hydroxysteroid dehydrogenase and 4,5-ene-isomerase.

The main source of 16α-hydroxyandrostenedione for the amniotic fluid is not quite clear. It is possible that a portion of this steroid, circulating between the fetus and the placenta, is eliminated in the fetal urine into the amniotic fluid. This steroid may also pass through the membranes and thus appear in the amniotic fluid. The present results show that the concentration of 16α-hydroxyandrostenedione in amniotic fluid is higher than that of maternal peripheral plasma at term, however, concentrations in umbilical cord plasma exceed the steroid level found in amniotic fluid.

Many components, including some steroids, increase markedly during the course of pregnancy.

Table 3. 16α-Hydroxyandrostenedione levels in cord blood and amniotic fluid at term

Source	Mean ± SE (ng/ml)		N
Umbilical artery	11.16	1.82	14
Umbilical vein	13.00	2.43	18
Amniotic fluid	7.92	1.68	11

Among steroids, considerable amounts of dehydroepiandrosterone, progesterone and oestrogens circulate in peripheral blood of pregnant women. If and when the antiserum cross reacts with any of those steroids, an over estimation of the 16α-hydroxyandrostenedione levels may result. Our present data indicate that the specificity of the antiserum is sufficiently high and that the column chromatography separates all the steroids which show slight cross reactivity. However, non specific reactions must always be kept in mind when relatively small quantities of a new substance is being measured.

The clinical significance of 16α-hydroxyandrostenedione for pregnancy lies mainly in the fact that it is an obligatory intermediate in the formation of estriol from 16α-hydroxy-DHA[6]. Now that a method for routine determination of 16α-hydroxyandrostenedione in biological fluids has been developed, more detailed examination of the physiology of 16α-hydroxyandrostenedione is possible.

Acknowledgements—The expert technical assistance of Hideko Okano is gratefully acknowledged. This work was supported in part by research grants from the Japan Ministry of Education, Science and Culture, and from the Morinaga Milk Industry Co., Ltd.

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