RADIOIMMUNOASSAY OF 16α-HYDROXY-DEHYDROEPIANDROSTERONE AND ITS SULFATE

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- ABSTRACT -

A simple and reliable radioimmunoassay for plasma 3β,16α-dihydroxy-5-androsten-17-one(16α-OH-DHEA) and its sulfate has been developed. The antiserum against 16α-OH-DHEA and its sulfate (16α-OH-DHEA-3-sulfate) was produced in rabbits immunized with 16α-OH-DHEA-3-succinate-bovine serum albumin. This antiserum reacted well with both 16α-OH-DHEA and its sulfate and only slightly cross reacted with DHEA and its sulfate.

The coefficient of variation (C.V.) of the intra assay was 10.26% for 16α-OH-DHEA and 12.32% for 16α-OH-DHEA-S. The C.V. of the inter assay were 14.34% for 16α-OH-DHEA and 15.64% for 16α-OH-DHEA-S.

The umbilical artery concentrations for 16α-OH-DHEA and 16α-OH-DHEA-S were 7.20 ± 6.71 ng/ml and 4490 ± 2140 ng/ml, and the umbilical vein concentrations were 14.20 ± 11.27 ng/ml and 2970 ± 1450 ng/ml respectively.

- INTRODUCTION -

In pregnancy a remarkable increase in estriol can be observed, preceded by an increase in the supply of its precursors, 3β,16α-dihydroxy-5-androsten-17-one and its 3-sulfate (hereafter abbreviated as 16α-OH-DHEA and 16α-OH-DHEA-S). In order to understand the regulatory mechanism of estriol production, it is necessary to know the quantitative be-

haviour of its precursors.

16α-OH-DHEA and its sulfate have been determined previously by various methods including calorimetry utilizing the Pettenkopfer reaction (1), fluorometry after converting them into estrogen by the placental aromatizing enzyme (2), and by gas chromatography (3,4,5,). However, these methods are inadequate for clinical use because of their poor sensitivity and accuracy as well as being complicated procedures.

The radioimmunoassay of 16α-OH-DHEA was first reported by Buster et al (6,7) and was far superior to previous chemical means, but they were unable to measure 16α-OH-DHEA-S, which is present in large amounts in the peripheral blood, because the antiserum used was prepared by using 16α-OH-DHEA-3, 16-dihemisuccinate as antigen, and showed a high affinity for 16α-OH-DHEA, but did not react with 16α-OH-DHEA-S.

The authors synthesized 16α-OH-DHEA-3-succinate-bovine serum albumin, which was postulated as capable of producing an antiserum structurally reactive with both 16α-OH-DHEA and its sulfate.

The antiserum produced by inoculating New Zealand white rabbits with the antigen reacted with both 16α-OH-DHEA and its sulfate as predicted. The results obtained are as described below.

- MATERIAL -

Non-radioactive steroids

3β-hydroxy-5-androsten-17-one (Dehydroepiandrosterone): Teikoku Hormone Mfg. Co., Ltd. 3β-hydroxy-5-androsten-17-one-3-sulfate (Dehydroepiandrosterone-3-sulfate): Kanebo Yakuhin. 3β,16α-dihydroxy-5-androsten-17-one (16α-OH-dehydroepiandrosterone): Sigma Chemicals.

Radioactive steroids

Dehydroepiandrosterone-7-^{3}H (s.a. 10 Ci/m mole) and dehydroepiandrosterone-7-^{3}H-sulfate ammonium salt (s.a. 10 Ci/m mole): Both are products of New England Nuclear.

Reagents

Phridine, succinic anhydride, tri-n-butylamine and isobutylchlorocarbonate: Wako Junyaku Kogyo. Dioxane: Wako Junyaku Kogyo, used after redistillation. Bovine serum albumin crystalline fraction V (BSA): Nutritional biochemical corporation. And complete Freund's adjuvant: Difco Laboratory.

Solutions

0.05 M borate assay buffer: 1/5 M H_3BO_4 and 1/5 M KCL were dissolved in 50 ml of distilled water, and 2.6 ml of 1/5 M NaOH and 107.39 ml of distilled water were added, and then the pH was adjusted to 7.8 at 20°C, after adding γ-globulin and BSA at concentrations of 0.05 (W/V) and 0.06 (W/V) respectively. 0.1 M phosphate buffer: 20 ml of 0.1 M KH_2PO_4 was mixed with 160 ml of 0.1 M Na_2HPO_4 and the pH of the resultant mixture was adjusted to 7.0 at 20°C. Dioxane scintillator; prepared by dissolving 250 mg of POPOP, 10 g of PPO and 100 g of naphthalene in 1000 ml of dioxane. 50% ammonium sulfate solution; prepared by dissolving ammonium sulfate in distilled water at a concentration of 50% (W/V).

Chromatography

Sephadex LH-20: A product of Pharmacia Fine Chemicals, used after swelling overnight in benzene: methanol (95 : 5). Silica gel: Wako gel C 100, Wako Junyaku Kogyo. Celite: Wako Junyaku Kogyo.

Actinomyces

Streptomyces roseochromogenes; used a stock strain of I.F.O. 13080 stored at the Foundation of Institute of Zymology.

Liquid scintillation counter: Counting was carried out for 10 minutes with a Liquid Scintillation Counter Model ALOKA LSC-650.

- METHOD -

Preparation of antigen

Preparation of DHEA-3-succinate: One gram of succinic anhydride was dissolved in 50 ml of pyridine, to which one gram of dehydroepiandrosterone (DHEA) was added and refluxed for 72 hours. Pyridine was removed by a rotary evaporator and water was added to the residue and the solition was extracted with ethyl acetate. After washing with 1N HCL and water, the extract was dehydrated with sodium sulfate and evaporated to dryness. The residue was transferred to a Silica gel column chromatograph (3 × 30 cm). The non-reacting DHEA was removed with chloroform and the remainder was eluted with 0.5% methanol-chloroform and evaporated to dryness. The resultant crystals formed a single spot on TLC, which could be identified as DHEA-3-succinate by instrumental assay.

Preparation of 16α-OH-DHEA-3-succinate (Fig. 1): 100 ml of the medium (Czapek-Dox containing Yeast extract) was transferred into each of 40 shaker flasks (500 ml) and sterilized for 20 min. at 120°C. To the sterilized medium, *Streptomyces roseochromogenes*, which selectively hydroxylates the α position of carbon 16 steroids was introduced and incubated for 20 hours at 28°C under constant shaking. Then, DHEA-3-succinate dissolved in 3% (W/V) of dimethylformamide was added to the culture and incubated for a further 48 hours. At the completion of the incubation, the culture solution was centrifuged for 10 min, at 3000 rpm to remove vegetative cells. Thus obtained acellular culture solutions were adjusted to pH 6 with 1N HCl and extracted three times with equal volumes of ethyl acetate. The organic layer was dehydrated with sodium sulfate and evaporated to dryness. 800 mg of crude extract was yielded, and transferred to a column chromatograph (3 × 30 cm) with 30 g of silica gel and eluted with a mixed solvent consisting of benzene-ethyl acetate. The elutes containing 16α-OH-DHEA-3-succinate were combined and evaporated to dryness. The dried residue was transferred to a silica gel TLC and developed with benzene: methanol (95 : 5). 44 mg of 16α-OH-DHEA-3-succinate with a melting point of 211° ∿ 218°C was yielded.

Streptomyces roseochromogenes (I.F.O. 13080)

↓ preincubation for 20 hrs.
15 mg of DHEA-3-succinate/flask
shaking culture, 28°, 48 hrs.

supernat.

↓ pH 6.0 adjust with 1N HCl
shaking with ethyl acet. 3 vol.
centrifugation

ethyl acet.

↓ dehydration with Na_2SO_4
evap. to dryness

residue

↓ Silica gel column chromatography
(C_6H_6: EtOAc)

eluate

↓ TLC separation

16α-OH-DHEA-3-succinate

Fig. 1 Preparation of 16α-OH-DHEA-3-succinate.

Preparation of 16α-OH-DHEA-3-succinate-BSA: 16α-OH-DHEA-3-succinate was conjugated to BSA by the method of Erlanger et al (7). 18 mg of 16α-OH-DHEA-3-succinate were dissolved in 0.5 ml of dioxane, to which was added 9 µl of tri-n-butylamine and 5 µl of isobutylchlorocarbonate. The reaction was allowed to proceed for 20 min. at 10°C. In another procedure, 52.5 mg of BSA was dissolved in 1.37 ml of distilled water, 0.052 ml of 1N NaOH and 0.94 ml of dioxane. Subsequently, the former was gradually poured into the latter, and stirring and cooling were continued for a total of 4 hours. The mixture was dialyzed against running water overnight. The dialysate was then adjusted to pH 4.5 with 1N HCl which caused precipitation, and the reaction was continued overnight at 4°C. The precipitate was centrifuged for 20 min. at 1000 rpm at 0°C. The precipitate was suspended in 10 ml of distilled water and redissolved by adding a minimal quantity of 8% $NaHCO_3$ solution. This solution was dialyzed against distilled water in a cold room overnight, the dialysate was lyophilized yielding 49 mg of 16α-OH-DHEA-3-succinate-BSA. By the pettenkopfer reaction, it was confirmed that each molecule of BSA was bound to 15 molecules of 16α-OH-DHEA-3-succinate.

Preparation of antiserum

2.5 mg of 16α-OH-DHEA-3-succinate-BSA was dissolved in 1.25 ml of physiological saline and the resultant solution was emulsified with an equal volume of complete Freund's adjuvant. This solution was divided into four equal portions; one portion injected into interphalangeal areas of each of the hind paws of New Zealand white rabbits weighing an average of 3 kg, the remaining two portions were injected subcutaneously into the backs divided into more than 10 sites. Injections of the back were made fortnightly for the first 2 months and then once a month. Blood was sampled 10 days after each injection and centrifuged. The antiserum was stored at -80°C.

Preparation of radioactive and non-radioctive 16α-OH-DHEA and their sulfate

Preparation of 16α-OH-DHEA-^{3}H (Fig. 2): Preincubation of *Streptomyces roseochromegenes* was carried out in the above described medium for Actinomyces for 48 hours as is shown in Fig. 2. When culturing of organisms was completed, incubation products from 3 Sakaguchi flasks were pooled and centrifuged for 10 min. at 3000 rpm to obtain free cells. These free cells were suspended in 5 ml of phosphate buffer containing 1% (W/V) of glucose (Suspension of resting cells). Two hundred and fifty µCi (7.2 µg) of DHEA-^{3}H was transferred to conical glass tubes and evaporated to dryness, to which 2 ml of the suspension of resting cells (*Streptomyces roseochromogenes*) prepared previously was added. The mixture was incubated continuously for one hour at 28 °C under air bubbling with a capillary pipette. Then, 4 ml of acetone was added to the medium and the solution was extracted twice with 2 volumes of ether. The organic layer was evaporated under N_2 gas. The residue was dissolved in methanol and subjected to Silica gel TLC. Area corresponding to 16α-OH-DHEA-^{3}H was removed and 16α-OH-DHEA-^{3}H was extracted with methanol: chloroform (1:2). The extract was purified through Sephadex LH-20 micro-column chromatography (1 × 10 cm) and 62.5 µCi of 16α-OH-DHEA-^{3}H was obtained (34.9% of yield).

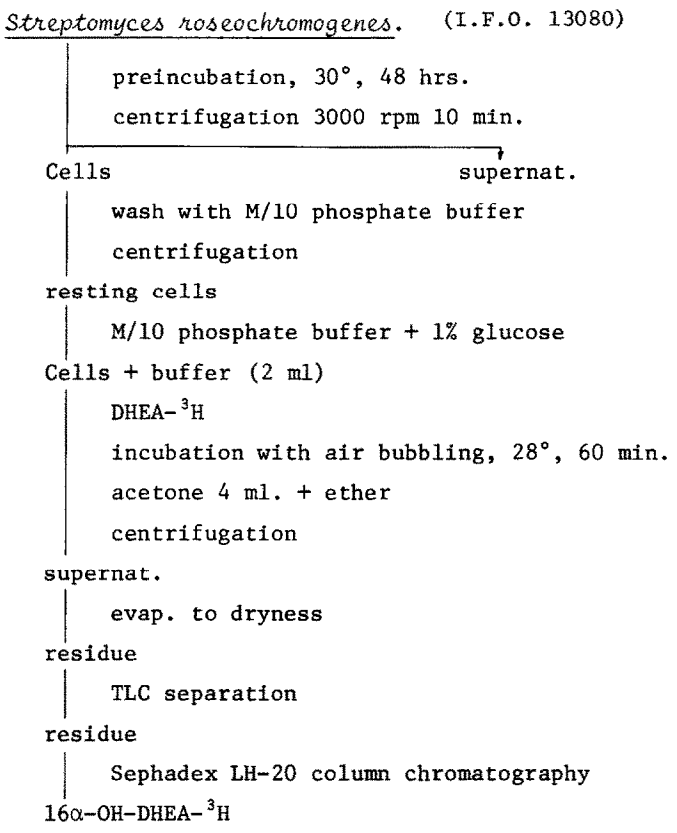

Fig. 2 Preparation of 16α-OH-DHEA-^{3}H

Preparation of 16α-OH-DHEA-sulfate (Fig. 3): The procedures are outlined as shown in Fig. 3. Each ml of the solution prepared by dissolving 44 mg of dehydroepiandrosterone sulfate (DHEA-S) in 5 ml of N. N-dimethylformamid was introduced into the medium on which preincubation was performed, and incubated under shaking for 24 hours at 28°C. Incubation solutions were centrifuged at 3000 rpm for 5 min, adjusted to pH 1.0 with 1N HCl, and extracted three times with ethyl acetate. The organic layer was dehydrated with sodium sulfate and evaporated to dryness in vacuo. The oily residue was separated on celite partition column chromatography (2.3 × 50 cm) after Younglai et al (9). and fractions corresponding to 16α-OH-DHEA-S were pooled and evaporated to dryness. The residue was dissolved in benzene and recrystallized with hexane yielding 4.5 mg of 16α-OH-DHEA-S. These crystals showed a positive blue tetrazolium reaction, had a melting point of 226° ∿ 234°C, reacted with the antiserum to 16α-OH-DHEA, and were identified as 16α-OH-DHEA-S by Infrared Spectrophotometry.

Preparation of 16α-OH-DHEA-^{3}H-sulfate: Two hundred and fifty μCi (9.6 μg) of DHEA-^{3}H-sulfate ammonium salt was transferred to conical glass tube and evaporated to dryness under N_2 gas, to which 2 ml of the

resting cell suspension as shown in Fig. 2 was added, and incubated under air bubbling for 60 min, at 28°C. The incubation solution was adjusted to pH 1.0 with 1N HCl as shown in Fig. 3 and extracted twice with 2 volumes of ethyl acetate. The organic layer was evaporated to dryness. The crude residue was purified on celite partition column chromatography, and 46.5 μCi of 16α-OH-DHEA-^{3}H-sulfate ammonium salt was obtained (18.6% of yield).

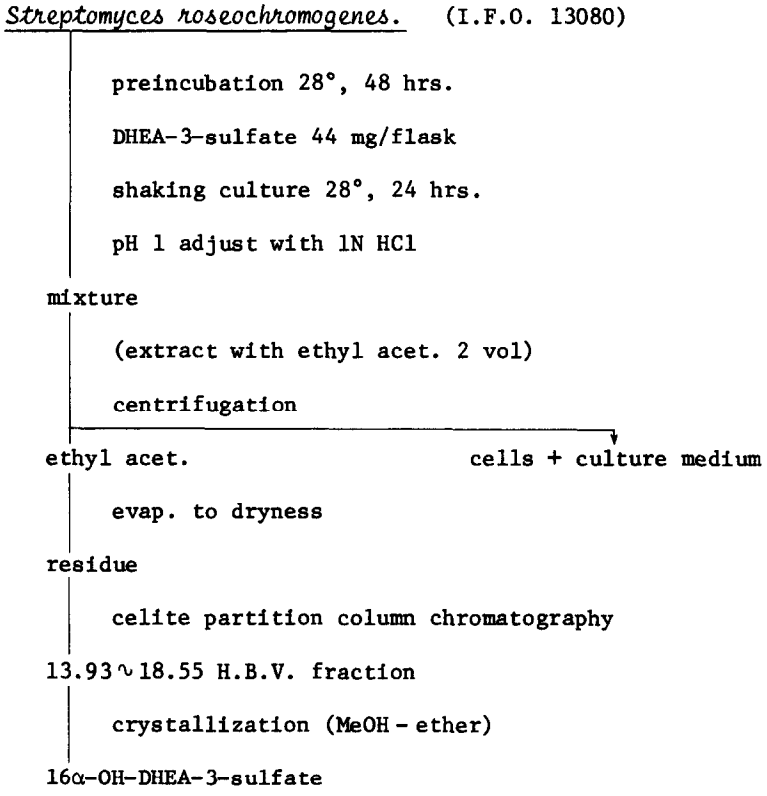

Fig. 3 Preparation of 16α-OH-DHEA-3-sulfate.

- ASSAY PROCEDURES -

Volume of plasma

Blood was obtained by using heparinized syringes and centrifuged for 5 min. at 3000 rpm. The volume of plasma used varied according to the concentration in the samples. Usually 0.5 to 2 ml of plasma were required for the measurements of 16α-OH-DHEA and 0.01 to 0.5 ml for those of 16α-OH-DHEA-S. Each determination was performed in duplicate.

Extraction and purification

16α-OH-DHEA: Plasma was transferred to test tubes containing 1000 dpm of 16α-OH-DHEA-^{3}H, to which 3 volumes of ether were added. The solutions were centrifuged and frozen in a bath of dry-ice and acetone. The ether phase was decanted, washed with distilled water and evaporated to dryness under N_2 gas. The residue was transferred to a micro-column (1 × 10 cm) containing 2 ml of Sephadex LH-20 and eluted in a solvent system of benzene: methanol (95 : 5). The first half of the eluate was used for recovery and the second half was used for RIA.

16α-OH-DHEA-S: 0.5 ml of plasma was transferred to glass test tubes containing 1000 dpm of 16α-OH-DHEA-^{3}H-S (when the volume of plasma was less than 0.5 ml, distilled water was added to make the total volume of 0.5 ml), and the unconjugated 16α-OH-DHEA in plasma was removed with 3 volumes of ether. Then, 150 mg of ammonium sulfate was added to the residue and the solution was extracted twice with 3 volumes of ethyl acetate. One half of the extract was used for recovery and the other half for RIA.

Radioimmunoassay

The above described samples were transferred to conical glass tubes containing 1×10^4 dpm of 16α-OH-DHEA-^{3}H or 16α-OH-DHEA-^{3}H-S. For obtaining standard curves, test tubes containing standard 16α-OH-DHEA (0-2 ng) or 16α-OH-DHEA-S (0-5 ng) were prepared simultaneously and evaporated to dryness under N_2 gas. Then, 0.25 ml of 1:2000 dilution of antiserum was added to each of the standard and sample tubes, and the tubes were gently aggitated on a vortex. After standing for 30 min, at room temperature, 0.25 ml of 50% ammonium sulfate was added to each tube, and the tubes were gently aggitated on a vortex and let stand for 10 min. The tubes were then centrifuged for 10 min. 0.2 ml of the supernatant (free fraction) was transferred into counting vials, and the radioactivity was measured following the addition of 12 ml of dioxane scintillator.

Direct method for determining 16α-OH-DHEA-S

0.5 ml of plasma (in case the volume of plasma was less than 0.5 ml, distilled water was added to make the total volume of 0.5 ml) was transferred to conical glass tubes containing 1×10^4 dpm of 16α-OH-DHEA-^{3}H-S. 0.25 ml of 1:2000 dilution of antiserum was added to the tubes, and the tubes were aggitated gently on a vortex and let stand for 30 min. at room temperature. Then, 0.25 ml of 50% ammonium sulfate was added, and the tubes were gently aggitated on a vortex and let stand for a further 10 min. The tubes were centrifuged for 10 min. 0.2 ml of the supernatant (free fraction) was transferred to counting vials, to which 12 ml of dioxane scintillator was added. This was followed by measurement of radioactivity.

Calculation

Concentrations of 16α-OH-DHEA or 16α-OH-DHEA-S per ml of plasma can be calculated by the following method. The rate of binding of 16α-OH-DHEA-^{3}H is derived by the following equation from the measured value of radioactivity (D):

% bound = $1/A\ (A-D) \times 0.5/0.2 \times 100$, where A represents dpm added. The per cent recovery can be obtained from the following formula. Recovery = $2R'/R \times 100$, where R is dpm added and R' is the value measured with samples for correction of recovery.

Plasma content of 16α-OH-DHEA and 16α-OH-DHEA-S can be calculated by the following formula based on the value (M) obtainable from the calculated binding rate and the standard curve plotted at the time of measurement, as well as the value (m) obtained with the water blank:

$$\text{Concentration per ml} = (M-m) \times \frac{100}{\text{recovery (\%)}} \times 2 \times \frac{1}{\text{plasma (ml)}}$$

- RESULT -

Titer of antiserum and specificity of the antibody

Blood was sampled starting one month after the initial injection. The reactivity or potency of the antiserum is as depicted in Fig. 4, which reveals that at one month 0.25 ml of a 1:500 dilution of the antiserum had a binding rate of 76% for 1×10^4 dpm of 16α-OH-DHEA-^{3}H followed by a gradual increase in reactivity, and that the binding rate became 63% with 1:1000 dilution at two months, and 70% at three months, and at four months it became 85% with 1:2000 dilution. On the other hand, the potency of the same antiserum for 16α-OH-DHEA-S was also shown to increase from the second month when the binding rate was 28% and a binding rate of 43% could be observed with a 1:2000 dilution at four months.

The cross reactivity of various steroids to this antiserum was checked with the method as described by Abraham (10), the results are summarized in Table 1.

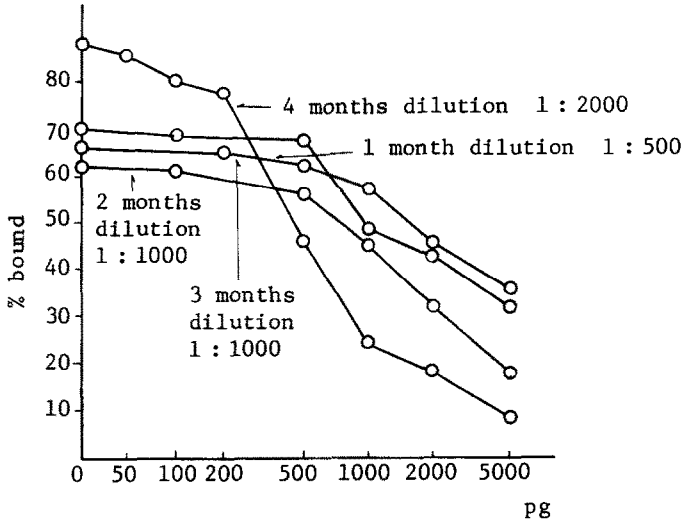

Fig. 4 Variation in the 16α-OH-DHEA Standard curve of diluted antiserum by immunizing period.

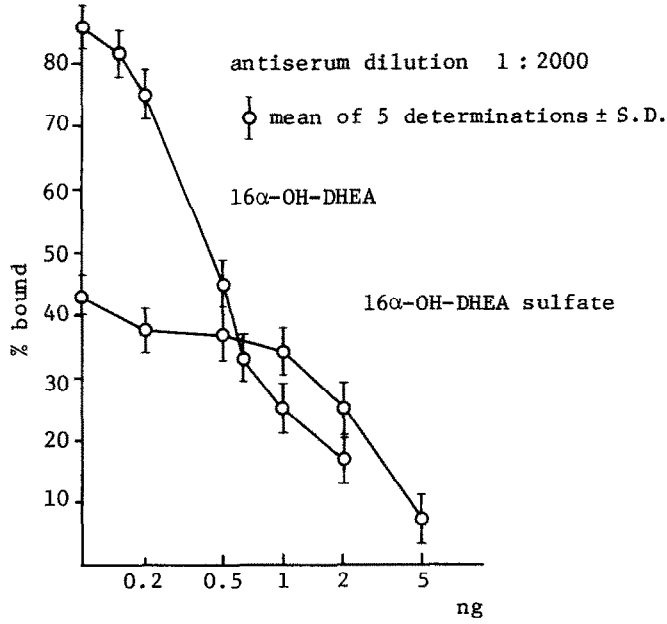

Fig. 5 Standard curve of 16α-OH-DHEA and 16α-OH-DHEA-S

Table 1 % cross reaction of various steroids against anti 16α-OH-DHEA-3-succinate-BSA serum.

Steroids	by 16α-OH-DHEA-^{3}H	by 16α-OH-DHEA-^{3}H-S
16α-OH-DHEA	100	
16α-OH-DHEA-S	16.59	100
DHEA sulfate	0.93	4.8
DHEA	1.17	5.55
3β, 17β-dihydroxy-5-androsten-16-one	0.64	0.32
17β-hydroxy-5-androstan-3-one	0.50	0.78
4-androsten-3, 17-dione	0.16	1.19
16α-hydroxy-progestrone	0.15	0.15
Androsterone	0.11	0.09
Corticosterone	0.05	0.05
3β, 16α-dihydroxy-5-pregnen-20-one	0.01	<0.01
Cortisol	0.02	<0.01
Androsterone sulfate	<0.01	<0.01
3α-hydroxy-5β-androstan-17-one-3-glucuronide	<0.01	<0.01
Cortisone	<0.01	<0.01

Sensitivity of the standard curve

The bound radioactivity was plotted against the logarithm of the dose of 16α-OH-DHEA and 16α-OH-DHEA-S. In Fig. 5 is presented graphically the standard curve derived from five fold determinations. At the 95% confidence 200 pg of 16α-OH-DHEA and 1 ng of 16α-OH-DHEA-S were significantly different from 0.

Extraction and separation from plasma

In order to establish the extracting procedure for 16α-OH-DHEA and 16α-OH-DHEA-S from plasma, extraction was carried out under conditions described in Table 2, adding each of the labeled steroids to distilled water. 98% of 16α-OH-DHEA was extracted with ether, while 99% of 16α-OH-DHEA-S was extracted with ethyl acetate under an acidified condition after addition of 30% (W/V) of ammonium sulfate. Therefore, it was concluded that both compounds may be adequately extracted under these conditions.

Table 2 Extraction rate of 16α-OH-DHEA and 16α-OH-DHEA-S

Aq phase			Solvent phase	recovery %	
pH	salt (%)			16α-OH-DHEA	16α-OH-DHEA-S
7			ether	97	1.5
1	$(NH_4)_2SO_4$	10	"	98	15
1	"	30	"	98	70
7	"	0	EtOAc		66
1	NaCl	10	"		88
1	"	20	"		94
1	"	0	"		74
1	$(NH_4)_2SO_4$	10	"		95
1	"	30	"		99

As shown in Table 1, DHEA shows low cross reactivity to this antiserum, but DHEA is more abundant in human plasma. Thus, it may be necessary to separate DHEA. Sephadex LH-20 microcolumn chromatography was utilized with the solvent system of benzene: methanol (95 : 5). The elution pattern is illustrated in Fig. 6.

Comparison of the two procedures for measuring 16α-OH-DHEA-S (the first is to extract with ethyl acetate after removal of unconjugated 16α-OH-DHEA using ether, and the second is to make a direct measurement after removal of unconjugated 16α-OH-DHEA with ether) revealed that well correlated values are obtained with either, so the latter direct measuring method was employed as our routine.

Accuracy

Table 3 represents the results obtained by five separate measurements of 1 ml of distilled water containing standard 16α-OH-DHEA or 16α-OH-DHEA-S. The coefficient of variation for 16α-OH-DHEA was less than 12.9% for measurements with addition of more than 1 ng, while it became higher for those with less than 500 pg. In case of 16α-OH-DHEA-S, the coefficient of variation was found to be less than 14.65% when more than 2 ng was added. The figures obtained by subtracting the value of the

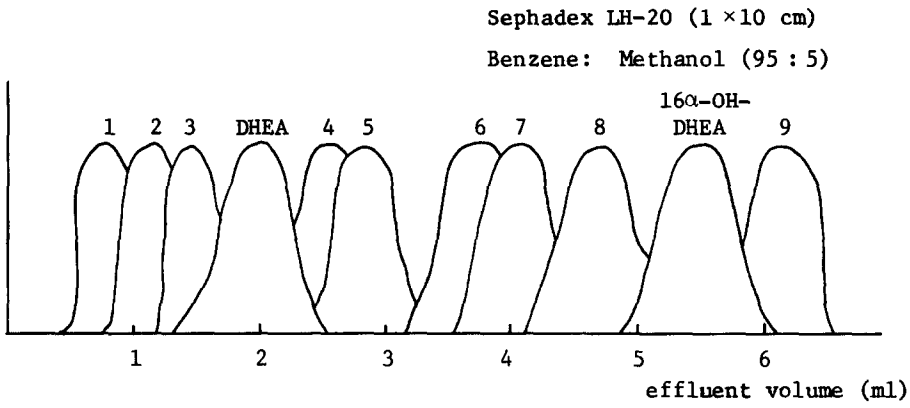

Fig. 6 Elution pattern of steroids by microcolumn chromatography.

1 - progesterone. 2 - androstendione and deoxycorticosterone.
3 - 3β, 16α-dihydroxy-5-pregnen-20-one (pregnenolone).
4 - 17α-OH-progesterone. 5 - testosterone. 6 - estrone
7 - corticosterone. 8 - cortisone and aldosterone.
9 - 16α-OH-progesterone.

water blank from the measured value correspond to 88.5 to 119% for the free form and 93 to 116% for the sulfate, indicating adequate accuracy of method.

Precision

Ten measurements were carried out on a given sample obtained from pregnant women, and it was revealed that measurements of 16α-OH-DHEA had a coefficient of variation of 10.26% for simultaneous measurements and 14.34% for alternate day measurements, while measurements of 16α-OH-DHEA-S had a coefficient of variation of 12.32% for simultaneous measurements and 15.64% for alternate day measurements.

Measured value

Levels of 16α-OH-DHEA and 16α-OH-DHEA-S were determined in maternal peripheral and umbilical blood at 38 to 40 weeks of pregnancy. The results are given in Table 4.

Table 3 Accuracy on the radioimmunoassay of 16α-OH-DHEA and 16α-OH-DHEA-S (water 1 ml)

added steroid	(ng)	mean ± SD (ng)	C.V. (%)	mean-water blank (ng)	recovery (%)
16α-OH-DHEA	0	0.25 ± 0.61	41.8	0	
	0.5	0.68 ± 0.17	27.3	0.432	86.4
	1.0	1.27 ± 0.16	12.6	1.015	101.5
	2.0	2.64 ± 0.34	12.9	2.385	119.2
	5.0	4.68 ± 0.25	5.3	4.425	88.5
	10.0	10.90 ± 0.76	7.0	10.655	106.5
16α-OH-DHEA-S	0	0.11 ± 0.05	47.8	0	
	1.0	0.89 ± 0.26	29.0	0.778	77.8
	2.0	2.32 ± 0.34	14.6	2.203	110.1
	5.0	5.92 ± 0.27	4.5	5.803	116.1
	10.0	9.74 ± 0.85	8.79	9.62	96.2
	20.0	20.90 ± 1.72	8.23	20.85	104.2

Table 4 16α-OH-DHEA and 16α-OH-DHEA-S level in maternal and umbilical plasma (ng/ml)

	16α-OH-DHEA	16α-OH-DHEA-S
maternal periferal vein of pregnancy 38∿40 W	3.36 ± 1.91	870 ± 220
umbilical artery	7.20 ± 6.71	4490 ± 2140
umbilical vein	14.20 ± 11.27	2970 ± 1450

- DISCUSSION -

16α-OH-DHEA and its sulfate are steroids of significance as precursors of estriol which increases in pregnancy. However, because of the complexity of determination, little clinical application was possible. In 1972 Buster *et al* (6, 7) first attempted the RIA of 16α-OH-DHEA. The antibody used reacted to 16α-OH-DHEA, but not to 16α-OH-DHEA-S. Thus, RIA has not been used successfully to measure 16α-OH-DHEA-S which is much more abundant in blood than is 16α-OH-DHEA.

Considering the relationship between an antigen and its antiserum, the authors synthesized an antigen for obtaining an antiserum which

reacts to both 16α-OH-DHEA and its sulfate, by coupling BSA to hydroxy group of carbon 3 of 16α-OH-DHEA without affecting the 16-OH group.

There are various processes for preparing 16α-OH-DHEA-3-succinate, such as directly succinating 16α-OH-DHEA or preparing DHEA-3-succinate from DHEA followed by 16α-hydroxylation. By the first method, most of the 16α-OH-DHEA is converted into 16α-OH-DHEA-3-16-dihemisuccinate, and it is almost impossible to isolate 16α-OH-DHEA-3-succinate. Thus, the second method was selected to prepare 16α-OH-DHEA-3-succinate.

As shown in Table 1, this antibody preserves the properties of the 16α-OH group and shows low cross reaction to either DHEA or DHEA-S, which suggests that the RIA with this antiserum may be little influenced by DHEA or DHEA-S which are so abundant in blood.

Organic solvents with high polarity such as butyl alcohol are commonly used for the extraction of 16α-OH-DHEA-S (11), but our studies of the salting out methods show that the good extraction of up to 99% was obtained with 30% (W/V) of ammonium sulfate and ethyl acetate. Further, as this antiserum shows a low cross reaction to DHEA-S, an attempt was made to use it for the determination of 16α-OH-DHEA-S by the so-called 'direct method', in which RIA for 16α-OH-DHEA-S is performed directly after extraction of 16α-OH-DHEA with ether. The results obtained were adequately consistent with those by previous method of extraction. This suggests that one step may be omitted during the assay procedures.

Utilizing our method, measurements were made for 16α-OH-DHEA and 16α-OH-DHEA-S in maternal peripheral and umbilical blood during the last trimester of pregnancy. Our results are similar to those reported previously (2, 3, 4, 5, 6, 7, 10).

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