DETECTION AND QUANTIFICATION OF 7-HYDROXYDEHYDROEPIANDROSTERONE EPIMERS IN THREE BODY FLUIDS

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Received October 5, 2001
Accepted December 27, 2001

Dedicated to the memory of Dr Václav Černý.

3β,7α-Dihydroxyandrost-5-en-17-one (**1**) (7α-OH-DHEA) and its 7β-hydroxy epimer **2** (7β-OH-DHEA) – 7α- and 7β-hydroxydehydroepiandrosterone – were detected and quantified in three human body fluids: in blood serum, saliva and ejaculate. Specific radioimmunoassay and gas chromatography-mass spectrometry have been used. For the first time the data on changes of these dehydroepiandrosterone metabolites are reported for a representative group of healthy subjects of both sexes (172 females and 217 males) during the life span. The serum levels of both 7-hydroxydehydroepiandrosterone epimers in serum and also in semen were in the low nanomolar range, while concentrations by one order of magnitude lower were found in saliva, but still within the detection limit. The results will serve as a basis for comparative studies of 7-hydroxydehydroepiandrosterone levels under various pathophysiological conditions, with a particular respect to autoimmune disorders.
Keywords: Steroids; Hormones; Metabolites; Radioimmunoassay; GC-MS; Blood; Saliva; Semen; 7-Hydroxydehydroepiandrosterone.

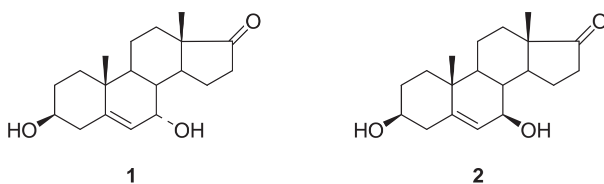
As early as in 1961 one of us (L. S.) published in this journal an original synthesis[+] of 7α-hydroxydehydroepiandrosterone **1** (3β,7α-dihydroxyandrost-5-en-17-one, 7α-OH-DHEA) and its 7β-epimer **2** (3β,7β-dihydroxyandrost-5-en-17-one, 7β-OH-DHEA). It was based on selective one-step acyloxylation of the allylic carbon with *tert*-butyl peroxybenzoate, under catalysis of transition metals[1].

Later on, enzymatic 7α-hydroxylation has been found in many mammalian tissues, liver being the main site of this reaction[2]. We have shown that 7α-hydroxylation took place even in various human fetal tissues at early

+ The work was done as a part of his PhD. Thesis, under tutorship of late Dr V. Černý.

doi:10.1135/cccc20020010

stages of fetal development[3]. The physiological significance of 7-hydroxylation of C_{19}- and C_{21}-steroid substrates has been unknown for decades. The finding of high 7-hydroxylating activity in mammary tumorous tissue led Skinner *et al.* in 1980 to investigation, whether 7α-OH-DHEA, present also in blood, could be used as a marker for prognosis of the course of the disease. Their results, however were not convincing[4]. For this purpose the authors developed the first radioimmunoassay for determination of 7α-OH-DHEA. The method was based on using a hapten prepared *via* 17-oxogroup and its specificity was poor, because the antisera did not distinguish the structure at C and D rings of the steroid molecule[5].



An interest for 7-hydroxylated metabolites rose again in the nineties, in connection with the discovery of immunomodulatory and immunoprotective effects of dehydroepiandrosterone[6] (DHEA), especially when Morfin and others brought evidence that not DHEA itself, but just its 7-hydroxylated metabolites could be the locally active steroids[7,8] (for review of the literature until 1997, see also ref.[9]). Further reports appeared on the enzymatic conversion of DHEA and its precursors to 7-hydroxylated metabolites[10], and the genes encoding for the enzymes responsible for 7-hydroxylation have been characterized[11].

Since, with exception of Skinner's results, no data were available on the levels of circulating 7-hydroxylated metabolites of DHEA in healthy subjects and under various pathological conditions, we have developed sensitive immunoassays for measurement of both 7-hydroxyisomers[12,13], taking advantage of haptens coupled to bovine serum albumin *via* the position 19 for raising antibodies[14]. In some instances determination of salivary hormones may be more advantageous over analyses of blood, thanks to its noninvasive sample collection[15,16]. Therefore, we used the above methods of analysis also for saliva samples, using gas chromatography-mass spectrometry (GC-MS) for confirming the identity of the analyzed steroids[17]. Finally, we have detected both 7-OH-DHEA isomers in human semen[18]. We hypothesized that they may serve as additional factors contributing to maintaining favorable immunological conditions for protecting male germ

cells from infection, and, at the same time, providing optimum conditions for fertilization.

Here, further data are given on circulation of 7-OH-DHEA in a large group of healthy subjects of both sexes through the life, along with the results of analysis of saliva and ejaculate for these steroids.

EXPERIMENTAL

Steroids and Reagents

The non-radioactive steroids were purchased from Steraloids (Wilton (NH), U.S.A.). The solvents for extraction and HPLC, as well as pyridine, were of analytical grade, from Merck (Darmstadt, Germany). The derivatization agent Sylon BFT (bis(trimethylsilyl)trifluoroacetamide, BTFSA and trimethychlorosilane (TMCS), 99 : 1 v/v) was purchased from Supelco (Bellefonte (PA), U.S.A.). The compounds used for synthesis of immunogens, 3β,7β-dihydroxy-17-oxoandrost-5-en-19-al 19-*O*-(carboxymethyl)oxime (7β-OH-DHEA-19-CMO) and its 7α-hydroxy epimer were prepared by Pouzar *et al.*[14]. The hapten was coupled to bovine serum albumin by the method of Yatsimirskaya *et al.*[19] with small modifications[12,13]. All other chemicals were purchased from Sigma (St. Louis (MI), U.S.A.), with the exception of Dextran T-70 obtained from Pharmacia (Uppsala, Sweden). [1,2,6,7-^{3}H]Testosterone, specific radioactivity 2.9 TBq/mmol and carrier-free Na[^{125}I] used for labeling of the radioligands were purchased from Radiochemical Center (Amersham, U.K.). The homologous tracers, methyl[^{125}I]iodotyrosinate (TME) conjugates with 7α- and 7β-OH-DHEA-19-CMO, were prepared as described previously[12,13].

Determination of 7-OH-DHEA Epimers in Blood Serum

7-OH-DHEA epimers were determined in sera obtained from 172 females aged 10–72 years and 217 males aged 10–91 years, without endocrine disorders, collected in screening the iodine deficiency in three regions of the Czech Republic during 1999–2000. In addition, sera from 35 healthy senior men (65–91 years) investigated in the frame of screening prostate disorders at the Clinics of Urology, First Medical School, Charles University, Prague, were analyzed. The subjects who took any medication during screening were excluded from the study.

Sera (50 μl) were extracted with diethyl ether (1 ml), followed by freezing out the water phase in solid carbon dioxide bath and evaporation of the solvent. The steroids were determined in dry residues by recently developed radioimmunoassays[12,13].

Determination of 7-OH-DHEA Epimers in Saliva

Extraction and solvent partition: Frozen saliva (2 ml) from healthy volunteers (5 females, 10–32 years and 14 males, 10–45 years) were thawn and centrifuged at 3 000–4 000 *g* to get rid of debris, and then extracted with diethyl ether (5 ml). The water phase was frozen out in solid carbon dioxide and the organic phase was evaporated. The dry residue was partitioned between 1 ml of 80% aqueous methanol and 1 ml of petroleum ether to remove the lipids. The petroleum ether phase was discarded, while the methanol–water phase containing steroids was evaporated in a speed-vac centrifuge. The dry residue was dissolved in etha-

nol (400 μl). An aliquot (200 μl) was transferred to the vials for derivatization and analysis by GC-MS, while the remaining portion was used for radioimmunoassay.

Derivatization: Pyridine (30 μl) and Sylon BFT (10 μl) were added to the dry residues from salivary extracts, mixed briefly and heated at 65 °C for 45 min. Acetonitrile (50 μl) and isooctane (200 μl) were then added and the mixture was shaken for 1 min. The isooctane phase was recovered and evaporated under nitrogen. The dry residue was rinsed with isooctane (50 μl) and the mixture was evaporated again. Finally, steroid derivatives were dissolved in isooctane (20 μl), and 2 μl portions were injected into GC-MS system.

GC-MS analysis: The GC-MS system was from Shimadzu (Kyoto, Japan). It consisted of a GC17A gas chromatograph equipped with automatic flow control, AOC-20 autosampler and the mass spectrometer QP5050A, equipped with quadrupole electron impact detector with a fixed electron voltage of 70 eV. GC separation was carried out using a Zebron ZB-50 capillary column (15 m × 0.25 mm) with 0.15 μl film thickness, cat No. 7EG-G004-05, (Phenomenex, St. Torrance (CA), U.S.A.). The temperature of the injection port was 300 °C. The following protocol was used: Splitless high-pressure injection at 60 kPa for 1 min, 1 min delay at 120 °C and 30 kPa, then 40 °C/min and 10 kPa/min up to 210 °C and 53 kPa. The temperature gradient used was 1.8 °C/min and 0.5 kPa/min up to 221.9 °C and 56.3 kPa, and then 40 °C/min and 10 kPa/min up to 300 °C and 75 kPa. The duration of the analysis was 11.82 min. The response was recorded in the SIM mode using the fragments *m/z* 358 (M – 90) and *m/z* 359 (M – 91) for quantification, while for identification of the substances, the *m/z* 358, 359 and 343 were used. Standard mixtures of 7α- and 7β-OH-DHEA (in three concentrations: 1 000 pg/μl, 100 pg/μl and 10 pg/μl) were derivatized in the same way as the samples and used for calibration using an external standard method.

Radioimmunoassay: The diethyl ether extract of saliva was dissolved in 20 mM sodium phosphate buffer (200 μl), pH 7.1 and analyzed for 7α- and 7β-OH-DHEA by radioimmunoassay as described above for serum.

Determination of extraction efficiency: The efficiency of extraction steps for both 7-OH-DHEA epimers was determined using HPLC separation of the standards extracted in the same way as the samples, but in amounts sufficient for UV detection. The details of HPLC separation are described elsewhere[17]. The average final recovery after all separation steps was 78.2 and 77.9% for 7α-OH-DHEA and 7β-OH-DHEA, respectively.

Determination of 7-OH-DHEA in Semen

Semen samples were obtained by masturbation from 34 men (21–46 years) attending the Out-Patient Fertility Center of the Clinic of Obstetric and Gynecology, First Faculty of Medicine, Charles University, Prague, for problems with fertility. The samples after liquefaction were left frozen in solid carbon dioxide and stored frozen at –20 °C until analyzed. 7α- and 7β-OH-DHEA were determined by recently developed radioimmunoassays[12,13] in modification described for this material elsewhere[18], with only exception that HPLC pre-purification was omitted. In brief, the ejaculate (0.5 ml) was spiked with 20 000 dpm of ethanolic solution of pure [^{3}H]testosterone and extracted twice with diethyl ether (2.5 ml). The water phase was separated by freezing in solid carbon dioxide and the solvent was evaporated to dryness. The dry residue was dissolved in methanol (1.3 ml), water (1 ml) and light petroleum ether (0.3 ml) and stirred. After separation of the phases, the upper organic phase was sucked off carefully with a Pasteur pipette and the water-methanolic phase was evaporated in speed-vac centrifuge. The dry residue was re-dissolved in 0.5 ml of buffer for RIA (20 mM

sodium phosphate, pH 7.2, containing sodium azide and BSA, 0.1 g/100 ml each, in saline) and the solution was divided as follows: 4 × 50 μl were taken for RIA of each 7-OH-DHEA isomer in duplicates, and 100 μl were used for recovery by measuring [^{3}H]testosterone radioactivity by liquid scintillation counting.

Statistics

The data concerning dependence of serum levels of 7-OH-DHEA on age were evaluated using two-way ANOVA with sex and age group as the first and the second factor. Due to non-Gaussian data distribution and non-homogeneous variances, the data were treated by power transformation to minimum skewness prior to tests and the results were re-transformed to original scale. The circles with error bars in the Fig. 1 (see Results and Discussion) represent re-transformed group mean values with 95% confidence intervals. Number of subjects in each subgroup (1. females, 2. males) is given in parentheses. No overlapping of the confidence intervals between the subgroups or between the sexes within each age group means statistical significance of the differences on 95% level.

RESULTS AND DISCUSSION

The dependence of serum levels of 7α-OH-DHEA (A), its 7β-isomer (B) and their ratio (C) on age in 172 females from 10 to 72 years and 252 males (10–91 years), is shown in Fig. 1. The data represent individual age groups divided by five-years intervals (above 20 years). The circles with error bars represent retransformed group mean values with 95% confidence intervals calculated by the least significance difference multiple comparison. Overlapping of the confidence intervals denotes statistical insignificance between groups and *vice versa*.

Physiological levels of 7α-OH-DHEA and 7β-OH-DHEA in saliva from 19 healthy subjects of both sexes as measured by GC-MS and RIA are shown in Table I. The concentrations of 7α-OH-DHEA and 7β-OH-DHEA in ejaculate from 34 men, either healthy semen donors or patients with problems of sexual function or infertility are shown in Table II. Individual values are shown in Fig. 2.

Both epimers of 7-OH-DHEA have been detected and quantified not only in blood serum, but also in two other body fluids, in saliva and semen. Using recently developed specific immunoassays[12,13], the circulating levels of these steroids were reported throughout the life span in both sexes in a representative groups of healthy subjects for the first time. This will enable us to compare the results with those of sex- and age-matched patients sufferring from various endocrine disorders. With respect to supposed immunomodulatory and immunoprotective properties of 7-OH-DHEA, of particular importance will be endocrinopathies with autoimmune etiology.

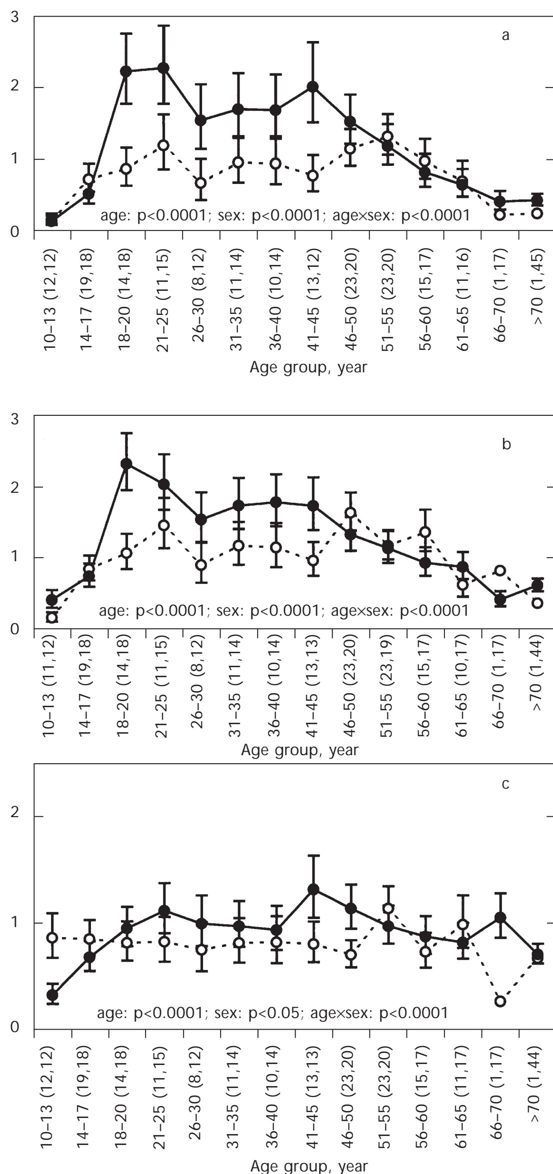


FIG. 1

Dependence of serum levels of 7α-OH-DHEA (a), 7β-OH-DHEA (b) (in mmol/l) and their ratio (c) on age. The data are given for five-year age groups (above 2 years); ● males, ❍ females.

The course of 7-OH-DHEA levels during life was different for each sex. While in males the levels of both epimers declined continuously, two distinct maxima were found round the age 22 and 53 in females. In our previous study on age dependence of DHEA and its sulfate (DHEA/S), DHEA in females exhibited two maxima around the age 20 and 45 years, respectively, while the levels of DHEAS declined continuously after reaching the peak around 25 years[20]. As demonstrated here, in the case of 7-OH-DHEA epimers the second maximum appeared almost ten years later, as late as in the postmenopausal period. Also the ratio of the epimers reflecting the 7α- and 7β-hydroxylating activities (see Fig. 1c) differed for males and females. In boys and also in men from roughly the sixth decenium, 7β-OH-DHEA was the major isomer, while in adult men until approximately 55 years of

TABLE I
Concentrations of 7α-OH-DHEA and its 7β-isomer (nmol/l) in saliva from 19 healthy volunteers of both sexes

Parameter	7α-OH-DHEA		7β-DHEA	
	GC-MS	RIA	GC-MS	RIA
Mean	0.197	0.195	0.304	0.209
S.D.	0.078	0.079	0.153	0.069
S.E.M.	0.015	0.020	0.029	0.018
Median	0.181	0.180	0.280	0.200
Range	0.083–0.427	0.100–0.427	0.101–0.725	0.050–0.300

TABLE II
Concentrations of 7α-OH-DHEA and its 7β-isomer in randomly collected ejaculates from 34 men (in nmol/l)

Parameter	7α-OH-DHEA	7β-OH-DHEA
Mean	3.72	3.50
S.D.	1.67	2.69
Median	3.21	2.54
Range	1.29–7.88	1.08–15.7

age the ratio of the epimers was constant, close to 1, similarly to females. This is in agreement with the fact that 7α- and 7β-hydroxylases are two different enzyme species, differing not only in genes encoding for them, but also by their tissue localization[8,10,11]. More detailed evaluation of the changes of 7-OH-DHEA in the life is in preparation.

Concentrations of both 7-OH-DHEA isomers in saliva were by about one order of magnitude lower than in blood, similarly to other steroids found in this material so far[15,16]. The sensitivity of the radioimmunoassays used here was sufficient to detect even these low levels, which may be advantageous in situations, when non-invasive sample collection is preferred.

Of particular importance may be the finding of 7-OH-DHEA epimers in the ejaculate: The concentrations of both epimers were close to- or even higher than those found in blood, indicating a certain role of these immunomodulatory DHEA metabolites for maintaining the internal milieu in semen. The results are preliminary and further studies, focused on various disorders of male reproductive function, with a particular respect to autoimmune ones, are in preparation. Many questions remain: What is the source of these metabolites in ejaculate? Does semen itself contain steroid-metabolizing enzymes, in particular those responsible for the DHEA metabolism? Do concentrations of these steroids correlate with other immunomodulatory factors, such as interleukins and other signal molecules present in semen? We hope to shed the light on at least some of these questions soon.

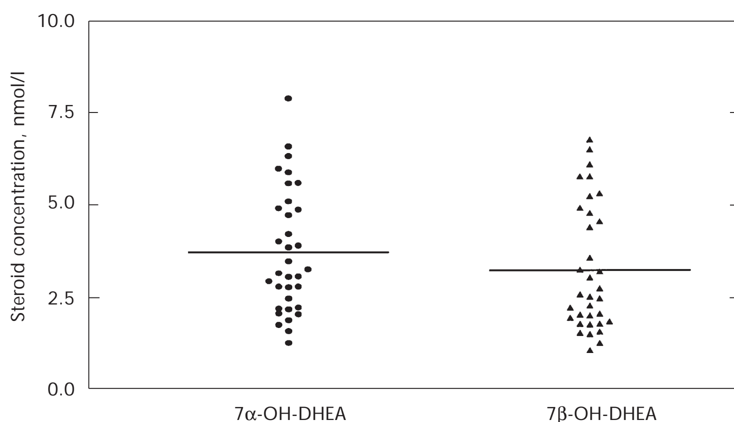


FIG. 2
Individual concentrations of 7α-OH-DHEA and 7β-OH-DHEA in semen from 34 men. Horizontal bar shows the median

This work was supported by the Research Project (Grant No. 000000023761) of the Ministry of Health of the Czech Republic.

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