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Immunoassay of 7-hydroxysteroids: 2. Radioimmunoassay of 7α-hydroxy-dehydroepiandrosterone

Oldřich Lapčík*, Richard Hampl, Martin Hill, Luboslav Stárka

Institute of Endocrinology, Národní tř. 8, 116 94, Praha 1, Czech Republic

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Abstract

High sensitivity radioimmunoassay of 3β,7α-dihydroxy-5-androsten-17-one (7α-OH-DHEA) has been developed and evaluated. The method is based on polyclonal rabbit antisera raised against 19-*O*-(carboxymethyl)oxime bovine serum albumin conjugate and bridge- and position homologous [^{125}I]iodotyrosine methyl ester as a tracer. Sensitivity of the assay amounted to 3.12 fmol (0.95 pg)/tube, precision as a mean intra- and interassay coefficient of variation was 7.1 and 10.6%, respectively, and the average recovery of the analyte added to steroid-free serum was 110%. Out of the steroids occurring in human serum which may interfere with the assay, the only important cross-reactants were dehydroepiandrosterone and 3β,7β-dihydroxy-5-androsten-17-one (7β-OH-DHEA) with cross-reactivities of 1.95 and 1.16%, respectively. The levels of free (unconjugated) 7α-OH-DHEA have been determined in 29 sera from healthy volunteers (23 females and 6 males), and from 48 patients (43 females and 5 males) in which dehydroepiandrosterone and its sulfate (DHEA/S) had been measured for various endocrinopathies. The levels in healthy subjects ranged from 0.21 to 6.57 (mean 2.33 ± 1.50) nM, while those of the patients from 0 to 5.99 (mean 1.46 ± 1.52) nM. The levels of 7α-OH-DHEA in patients significantly correlated with those of DHEA and its sulfate.

1. Introduction

Recently, we have published in this journal a radioimmunoassay of free (unconjugated) 3β,7β-dihydroxy-5-androsten-17-one (7β-OH-DHEA) [1]. 7-Hydroxylation of 3β-hydroxy-5-ene steroids of both C_{19}- and C_{21}-series is an ubiquitous reaction occurring in many mammalian tissues, while 7α- and 7β-hydroxylating enzymes are different *P*450 species, which also differ in their tissue localization (for the literature until 1997, see [2]).

The physiological meaning 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. [3], in 1980, to testing whether circulating 7α-OH-DHEA could be used as a marker for prognosis of the course of the disease, but no convincing results were obtained. Only recently, the discovery of immunoprotective and immunomodulatory properties of dehydroepiandrosterone and its sulfate (DHEA/S), of which some, but not all, may be explained by their non-genomic antiglucocorticoid activity, raised the question whether not only DHEA itself, but some of its metabolites, were responsible for these effects. Evidence has been brought that just 7-hydroxylated derivatives of DHEA and of other 3β-hydroxy-5-ene steroids may be the locally active agents, responsible for at least some of these activities [2,4–7].

Only a few data are available on the levels of circulating 7-hydroxylated metabolites of DHEA in healthy subjects and in patients. There is the paper of Skinner et al. [3] on 7α-OH-DHEA in women with breast cancer and the recent one of Attal-Khémis et al. [8] on 7α-OH-DHEA in patients with Alzheimer's disease. In

* Corresponding author. Tel.: +420-2-24905-289; fax: +420-2-24905-325.

E-mail address: olapcik@endo.cz (O. Lapčík).

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the latter case GC-MS techniques have been used for steroid determination.

The first, and to the best of our knowledge, the only radioimmunoassay of 7α-OH-DHEA has been reported as early as 1977 by Skinner et al. [9]. The method used polyclonal antiserum to the immunogen prepared through the carboxylic group at C_{17} and, therefore, it was not specific enough. Here a novel, specific radioimmunoassay for free 7α-OH-DHEA is presented.

2. Materials and methods

2.1. Steroids and reagents

7α-OH-DHEA, 7β-OH-DHEA and 7-oxo-DHEA were prepared by the original procedure of Stárka [10]. All other steroids and reagents were purchased from Sigma (St Louis, MI, USA) with the exception of Dextran T-70 obtained from Pharmacia (Uppsala, Sweden). Carrier-free Na[^{125}I] was from the Institute of Radioisotopes, the Hungarian Academy of Science (Budapest, Hungary).

2.2. Synthesis of the immunogen

The derivative, 3β,7α-dihydroxy-17-oxo-5-androsten-19-al 19-*O*-(carboxymethyl)oxime (7α-OH-DHEA-19-CMO) was prepared by the method of Pouzar et al. [11]. The hapten was coupled to bovine serum albumin (BSA, Bioveta-Ivanovice, Czech Republic) according to the method of Yatsimirskaya et al. [12] in a later modification [1]. The hapten-BSA molar ratio was assessed as 15 by titration of free amino groups by trinitrobenzenesulfonic acid [13].

2.3. Immunization

Female rabbits were immunized by a standard procedure [14] using 100 μg doses of the immunogen emulsified in a mixture of complete Freund's adjuvant-saline 1:1 (100 μl) in three week-intervals. The sera were collected from the ear vein and lyophilized.

2.4. Preparation of the tracer

Tyrosine methyl ester (TME) conjugate with 7α-OH-DHEA-19-CMO was prepared by a modified *N*-hydroxysuccinimide method, as described in detail in [15]. [^{125}I]Iodinated tracer was prepared by a convenient radioiodination using chloramine-T in aquous dioxane [14]. The tracer was separated from the conjugate by chromatography on TLC plates (DC-Alufolien Art 5593, Merck, Darmstadt, Germany) in system dichloromethane-2-propanol, 92:8 (by vol.). The distribution of radioactivity on the plate was measured by scanning with an automatic TLC linear analyzer Tracemaster 40 with a counting tube LB2821 (Berthold, Wildbad, Germany). The average yield of iodination was 35%.

2.5. High performance liquid chromatography (HPLC)

The standards or sample extracts, direct as well as after solvolysis, were chromatographed on an HPLC using the reverse C_{18} column ET 250/4 Nucleosil 100-5 C_{18} (Macheray-Nagel, Germany), exactly as described previously for 7β-OH-DHEA (see also the legend to Fig. 1) [1]. The same procedure has been used for determination of the losses during extraction and chromatography, using [^{3}H]7α-OH-DHEA, prepared biosynthetically, as described previously [1].

2.6. Separation of free and sulfate fractions of 7α-OH-DHEA

Free (unconjugated) 7α-OH-DHEA and its esters with fatty acids were extracted from the serum with five volumes of isooctane-ethyl acetate mixture. The water phase, containing mono- and disulfates, was solvolysed using ethyl acetate saturated with sulfuric acid as described previously [1].

2.7. Radioimmunoassay

The radioimmunoassay (RIA) system consisted of the antiserum (No. 37-2) at a dilution of 1:150,000, the tracer (ca 15,000 cpm/tube) and standards or serum extracts in a total volume 0.3 ml of 20 mM sodium phosphate buffer, pH 7.1 containing sodium azide and BSA (1 g/l each) in saline. For determination of the free (unconjugated) 7α-OH-DHEA diethyl ether extracts from serum (50 μl) were prepared prior to RIA and evaporated to dryness under nitrogen. The standard curve prepared by a serial dilution of 7α-OH-DHEA in a steroid-free serum (3.1–200 pg in 50 μl, corresponding to 0.20 to 13.1 nmol/l) was processed in the same way as the samples. After incubation at 37°C and for 30 min and a further 1 h at 2–4°C, the free/bound fraction was separated by adsorption on dextran-coated charcoal (0.025 g Dextran T-70 and 0.25 g Norit A in 100 ml of the RIA buffer). Radioactivity in supernatants was measured on a 12-Channel Gamma Counter (Type LB-2104, Berthold).

In order to completely eliminate the cross-reaction of DHEA (see Results, Table 1), an alternative assay system has been checked in which an excess (1 ng/tube) of this major cross-reactant was added to the assay system in order to saturate unspecific populations of antibodies [16].

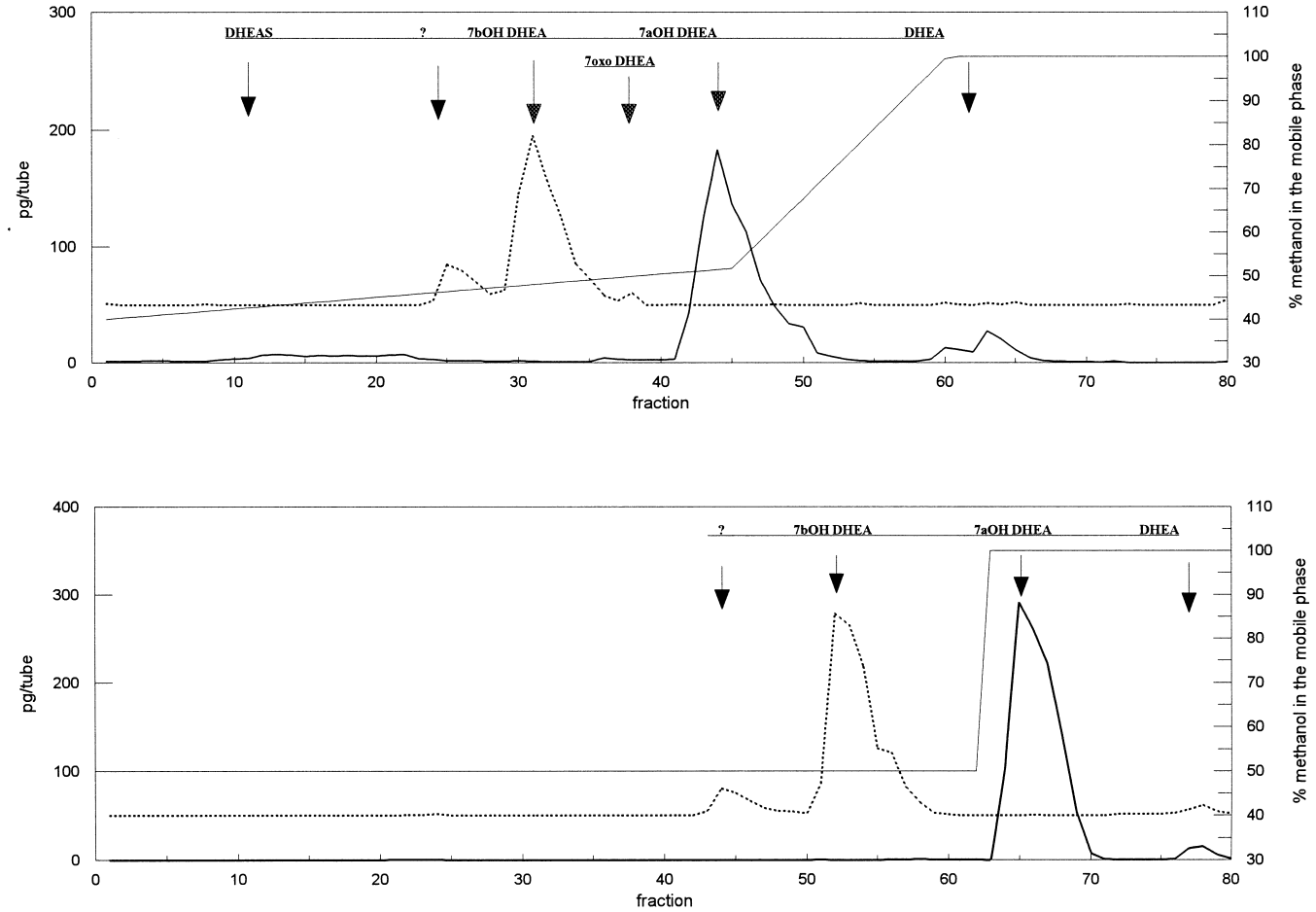

Fig. 1. Immunoreactivities of 7α-OH-DHEA and its 7β-hydroxy isomer following HPLC of the extract from a serum pool using two gradient systems (upper and lower panel). Gradient 1: for the first 14 min the elution was carried with 50% methanol, followed by pure methanol for a further 4 min (until the 18th minute) after which it was returned again to 50% methanol, and elution went on for an additional 6 min (until the 24th minute). Gradient 2: solvent A, 40% methanol, solvent B, pure methanol. From 100% A at time 0 the concentration of methanol was linearly increased up to 20% B (resulting in 52% methanol) until the 15th minute, followed by a further increase up to 100% B (pure methanol) at the 20th minute. Thick full lines show the immunoreactivity of 7α-OH-DHEA, the dotted lines of the 7β-OH-DHEA, respectively, thin full lines show the percents of methanol in the mobile phase and arrows indicate the mobility of authentic standards.

Table 1
Cross-reactivity of chemically related steroid present in human circulation with two rabbit antisera to 3β,7α-dihydroxy-17-oxo-5-androsten-19-al 19-*O*-(carboxymethyl)oxime:BSA (7α-OH-DHEA-19-CMO:BSA)

Steroid competitor	Cross-reaction (%)	
	Antiserum No. 37-2	38-2
3β,7α-Dihydroxy-5-androsten-17-one (7α-OH-DHEA)	100.00	100.00
3β-Hydroxy-5-androsten-17-one (DHEA)	1.95	4.05
3α,7β-Dihydroxy-5-androsten-17-one (7β-OH-DHEA)	1.16	1.41
3β-Hydroxy-5-androsten-17-one-3-sulfate (DHEAS)	0.95	1.16
5-Androstene-3β,7α,17β-triol	0.49	0.07
4-Androstene-3,17-dione (androstenedione)	0.38	0.68
3β-Hydroxy-5-androstene-7,17-dione (7-oxo-DHEA)	0.16	12.90
17β-Hydroxy-5α-androstan-3-one (dihydrotestosterone)	0.04	0.04
3β-Hydroxy-5-pregnen-20-one (pregnenolone)	0.04	0.03
3β-Hydroxy-5-pregnene-7,20-dione (7-oxo-pregnenolone)	0.02	0.03
5-Androstene-3β,17β-diol (androstenediol)	0.01	0.02
17β-Hydroxy-4-androsten-3-one (testosterone)	0.01	0.02

2.8. Radioimmunoassay of other steroids

7β-OH-DHEA was measured by the recently published RIA [1]. DHEA and its sulfate in serum samples were measured by RIA kits of Immunotech (France).

2.9. Serum samples

Sera were obtained from 29 volunteers (23 females and 6 males from the personnel of the Institute of Endocrinology, Prague), aged from 19–60 years and from 48 randomly selected outdoor patients of the Institute (43 females and 5 males) in which DHEA and its sulfate were determined previously for various endocrinopathies. Most of them (36) were patients with various thyroid disorders. Sera were stored frozen at −20°C until analyzed. In addition, 7α-OH-DHEA (free and sulfate fraction) have also been measured in sera from ten 12-week old rats (the control, untreated group) in the frame of a research project on the effect of DHEA on the cognitive function.

2.10. Statistics

Linear regression, Pearson's correlation and Student's *t*-test after the Fischer transformation was employed for evaluation of the relations between measured steroid levels and for determination of the significance of the correlation, respectively.

3. Results

3.1. Specificity of the antisera

Cross-reactivities of 11 selected potential cross-reactants present in human serum with two antisera are shown in Table 1. By the addition of an excess of DHEA (1 ng/tube) to the assay system, the cross-reaction of this major competitor could be eliminated, on the expense of assay sensitivity which was reduced by almost five times.

3.2. HPLC of the extracts from the serum

Diethyl ether extract from a serum pool was chromatographed by HPLC using two different solvent gradients, and in the individual fractions the immunoreactivities of both 7α- and 7β-OH-DHEA were determined. The distribution of immunoreactivities with depicted positions of authentic standards is shown in Fig. 1. Using the first solvent gradient, certain amounts of 7α-OH-DHEA immunoreactive material were recorded in the polar fractions corresponding to steroid sulfates and in the DHEA fraction. Almost no 7α-OH-DHEA immunoreactivity has been found in the fraction containing the opposite isomer and *vice versa*.

3.3. Reliability of the assay

The average recovery of the analyte during extraction amounted to 78.1%. The correction of the results to the losses could be avoided by preparation of the standard curve in steroid free serum processed in the same way as samples. When the HPLC step was included, the recovery after extraction and HPLC amounted on average to 74% and the results were corrected accordingly. The sensitivity of the assay (without addition of an excess of DHEA), expressed as the minimal amount of 7α-OH-DHEA distinguishable from the zero sample with 95% probability, was 3.12 fmol (0.95 pg/tube). The mean intra-assay coefficient of variation, determined from eight parallel analyses of three serum pools of the low, medium and high analyte content, was 7.1%; the mean interassay coefficient from three analyses of the same pools was 10.6%. The mean recovery of 25–400 pg (0.082–1.31 pmol)/tube added to the steroid-free serum was 110% (95% confidence interval 98.2–121%).

3.4. Distribution of serum 7α-OH-DHEA between free and sulfate fractions

7α-OH-DHEA concentrations have been determined by RIA in the free and solvolysed sulfate fraction from nine human serum pools and in 10 sera from untreated male rats. The HPLC purification step was included in this case for a complete separation of the analyte from potential cross-reactants expected especially in the sulfate fraction. The mean values are summarized in Table 2. No correlation was found between free and sulfate levels either in humans or in rats.

3.5. Free 7α-OH-DHEA in humans

Free (unconjugated) 7α-OH-DHEA and its 7β-isomer were determined in serum of 29 healthy subjects and from 48 out-patients with various endocrinopathies in which DHEA and its sulfate had been measured previously. The results are summarized in Table 3. The values of 7α-OH-DHEA in patients were compared with those of its 7β-isomer, DHEA and DHEAS. A highly significant correlation of 7α-OH-DHEA has been found with all these steroids ($p < 0.001$ in all instances), in the succession DHEA > 7β-OH-DHEA > DHEAS. The respective straight lines and their parameters are shown in Fig. 2.

Table 2
Serum levels of 7α-OH-DHEA in the free (isooctane-ethyl acetate) and sulfate fraction from human and rat sera following solvent extraction, solvolysis and HPLC[a]

Parameter	Humans ($n = 9$)			Rats ($n = 10$)		
	Free (nM)	Sulfate (nM)	Sulfate/Free	Free (nM)	Sulfate (nM)	Sulfate/Free
Median	1.33	5.37	4.04	1.79	1.00	0.56
Mean ± S.E.M.	1.34 ± 0.07	5.25 ± 1.38	3.91 ± 19.2	1.79 ± 0.13	1.00 ± 0.1	0.55 ± 0.74
Range	1.07–1.66	0.33–10.8	0.31–8.25	1.21–2.46	0.78–1.44	0.33–0.78

[a] The values were corrected for losses during processing.

4. Discussion

7-Hydroxylation of DHEA and of other 3β-hydroxy-5-ene steroids takes place in many mammalian tissues, and is increased considerably in sites of injury, where local immune response occurs [2]. These findings, as well as the results of experiments demonstrating the direct effect of 7-hydroxylated steroids on immune response, support the hypothesis that just these steroids are responsible for at least some immunoprotective and antiglucocorticoid effects ascribed to DHEA [2,4–7].

Both 7-hydroxylated metabolites of DHEA are present in circulation [9] and, therefore, specific methods for their determination in body fluids are needed. The described method, together with the previously reported one on 7β-OH-DHEA, fulfil this task. Though specificity of the antiserum raised against the 19-*O*-CMO derivative is high, in sera of high DHEA content the cross-reaction of DHEA present in the blood is in one order-of-magnitude higher concentration and may result in overestimation of the results. In our recent paper on the immunoassay of 17α-hydroxy-pregnenolone, using an antiserum with a high cross-reaction with 17α-hydroxyprogesterone, we have demonstrated by a careful mathematical analysis that the effect of the major cross-reactant could be eliminated by its addition in an excess to the assay system, resulting in saturation of less specific populations of antibodies, on the only little expense of assay sensitivity [16]. This approach could be used here, as well, by the addition of an excess of DHEA (1 ng/tube). The sensitivity of the method was then reduced about five times, but it still remained sensitive enough with respect to expected analyte concentrations.

7α-OH-DHEA has been measured in healthy subjects and in patients with as much as possible, a wide range of expected values. In addition, it was measured in the control group of rats in the frame of another study. The values of 7α-OH-DHEA were close to those reported by Skinner et al. [9] in healthy women and very close to those of the 7β-isomer, with which they correlated well. Even more tight correlation, however, has been found with unconjugated DHEA and slightly less with its sulfate. On the other hand, there was found to be no correlation between the free 7α-OH-DHEA and its levels in the sulfate fraction after solvolysis in the plasma pools. This may be explained by the fact that circulating 7α-OH-DHEAS is produced by peripheral conjugation (mainly in the liver) of 7α-OH-DHEA with sulfuric acid, resulting in mono- and disulfates and not by 7α-hydroxylation of DHEAS, while circulating DHEAS is secerned by adrenals in the sulfate form and then hydrolysed in the periphery [17].

The concentrations of free 7α-OH-DHEA in humans are only ca four times lower than in the sulfate fraction, in contrast to its 7β-isomer, approximately 94%

Table 3
Serum levels of free (unconjugated) 7α-OH-DHEA, 7β-OH-DHEA, DHEA and DHEAS in healthy volunteers and in selected patients, in which DHEA and its sulfate were measured for various endocrinopathies

	7α-OH-DHEA (nM)	7β-OH-DHEA (nM)	DHEA (nM)	DHEAS (μM)
Healthy subjects (29)				
Median	2.33	2.04	25.10	7.52
Mean ± S.E.M.	2.33 ± 1.5	2.26 ± 1.15	26.5 ± 25.5	7.69 ± 4.62
Range	0.21–6.57	0.43–4.21	3.2–70	1.2–13.1
Patients (48)				
Median	1.05	1.17	14.80	3.45
Mean ± S.E.M.	1.46 ± 1.52	1.58 ± 1.60	19.3 ± 16.0	3.94 ± 2.82
Range	0–5.99	0–8.06	1.1–72.7	0.24–9.6

of which is present in the sulfated form. In rats the serum concentrations of free and sulfated 7α-OH-DHEA were very close to each other, in agreement with the recent findings of Attal-Khémis et al. [18] in mice.

Having now the methods for determination of both 7-OH-DHEA isomers, the studies on their levels in healthy subjects during the lifespan, as well as in patients with various endocrinopathies, should follow. With regard to hypothesized immunomodulatory effects of these steroids, the investigation of patients with autoimmune endocrine disorders will be the first at stake.

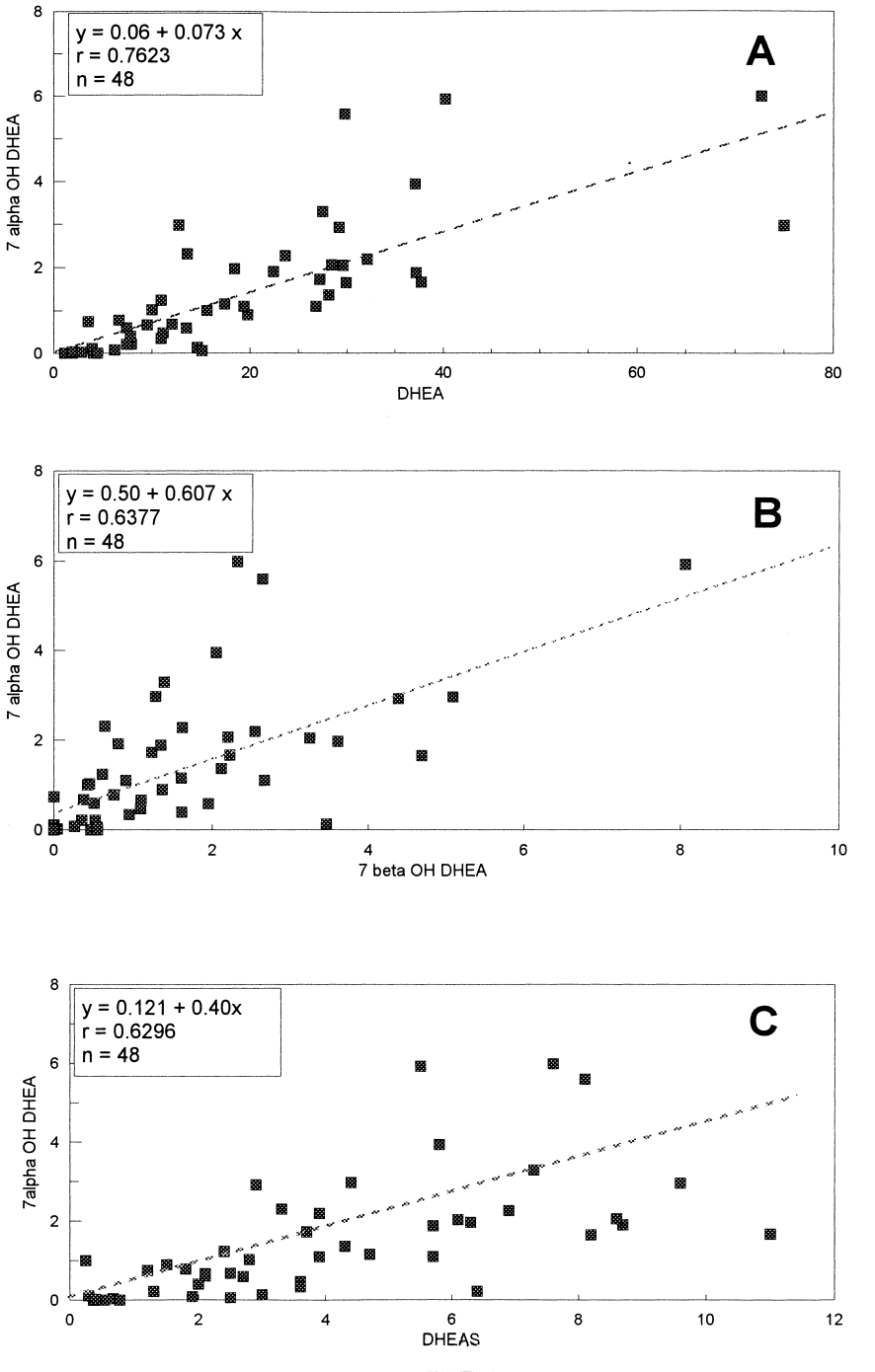

Fig. 2. The Pearson's correlation of 7α-OH-DHEA levels with those of DHEA (A), 7β-OH-DHEA (B) and DHEAS (C), respectively, in 48 sera from randomly selected patients with various endocrinopathies in which determination of DHEA/S had been indicated.

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