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7-Hydroxydehydroepiandrosterone epimers in human serum and saliva
Comparison of gas chromatography–mass spectrometry and radioimmunoassay

M. Hill[a,*], O. Lapčík[a], H. Havlíková[a], R. Morfin[b], R. Hampl[a]

[a]*Institute of Endocrinology, Národní třída 8, Prague 116 94, Czech Republic*
[b]*Conservatoire National des Arts et Metiers, 2 rue Conté, 75003 Paris, France*

Abstract

Recent reports demonstrated that 7-hydroxylated metabolites of dehydroepiandrosterone (DHEA) possess immunomodulatory and antiglucocorticoid properties. Increased 7α-OH-DHEA levels were found in patients with Alzheimer's disease. Hence, measurement of steroids in patients with autoimmune diseases or disturbances in the central nervous system could be of interest. A new sensitive GC–MS method for the determination of 7-hydroxydehydroepiandrosterone epimers was developed and compared with previously developed radioimmunoassays. Besides serum, these steroids were, for the first time, measured in saliva where their concentrations were about five times lower. 7α- and 7β-epimer levels correlated well in both body fluids and they were larger in male.

Keywords: Statistical analysis; Hydroxydehydroepiandrosterone; Steroids; Sterones

1. Introduction

Since the early 1960s it has been known that a significant portion of circulating dehydroepiandrosterone (DHEA) is metabolized to its 7α- and 7β-hydroxy derivatives. 7α-hydroxydehydroepiandrosterone was detected in human urine as early as 1959 by Fukushima [1]. Subsequently, 7α- and 7β-hydroxylation was demonstrated in many mammalian tissues, especially liver, due to the presence of ubiquitous 7-hydroxylases [2]. For more than two decades however, the biological significance of these metabolites was unknown. Due to the high activity of 7-hydroxylase in neoplastic mammary tissue, Skinner et al. suggested that 7α-OH-DHEA might serve as a marker for prognosis of mammary cancer [3]. Accordingly, they developed the first radioimmunoassay for 7α-OH-DHEA, using a hapten prepared through the carboxylic group at carbon 17. The specificity of the method was poor and the results were not convincing. A new impetus for further investigation of 7-OH-DHEA was the discovery of immunomodulatory and immunoprotective actions of dehydroepiandrosterone, many of which could be ascribed to its non-genomic antiglucocorticoid activity [4]. Various DHEA metabolites were tested and, surprisingly, it was found that in some instances 7-hydroxylated DHEA metabolites were much more potent agents than the parent steroid (DHEA itself) [5]. 7-Hydroxylating activity was found in many

*Corresponding author. Tel.: +420-2-24905-267; fax: +420-2-24905-325.

E-mail address: mhill@endo.cz (M. Hill).

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tissues including brain [5] and the enzyme species responsible for 7-hydroxylation of DHEA were studied more in detail [6]. In 1995–1997 the gene encoding for Cyp7b, responsible for 7α-hydroxylation, was described [7,8]. Recently, a series of papers appeared presenting further evidence for immunomodulatory action of 7-OH-DHEA epimers in man and mouse [9–11]. Our group has developed specific radioimmunoassay (RIA) for each epimer. We have produced steroid haptens coupled to bovine serum albumin via the C_{19} position for raising antibodies used in RIA [12,13]. The GC–MS technique used in this study was previously used for determination of 7-OH-DHEA in human sperm [14]. A different GC–MS method was used in determination of 7-OH-DHEA in murine plasma [15]. In this paper, both GC–MS and RIA techniques are compared for determination of 7-OH-DHEA epimer levels in human serum and also, for the first time, in saliva.

2. Experimental

2.1. Subjects and serum samples

Blood samples were obtained from five females (10–32 years), and 14 males (10–45 years). The subjects were recruited from screening of iodine deficiency in the Czech Republic. The saliva samples were obtained from personnel of the Institute of Endocrinology in Prague. They included 16 females (21–64 years), 11 males (23–59 years) and one sample pooled from both sexes. All subjects were free of major medical problems and/or medication known to affect steroid metabolism. All participants signed an informed consent.

2.2. Steroids and chemicals

The non-radioactive steroids and their conjugates were purchased from Steraloids (Wilton, NH, USA). The solvents for extraction and high-performance liquid chromatography (HPLC), and pyridine, were of analytical grade, from Merck (Darmstadt, Germany). The derivatization agent Sylon BFT was purchased from Supelco (Bellefonte, PA, USA).

2.3. Instruments

The GC–MS system was from Shimadzu (Kyoto, Japan). It consisted of a GC17A gas chromatograph equipped with automatic flow control; an AOC-20 autosampler and a QP5050A mass spectrometer, equipped with quadrupole electron impact detector with a fixed electron voltage of 70 eV.

The efficiency of extraction was assessed with a HPLC system from Gilson (Villiers le Bel, France), which consisted of a master pump 305 with manometric module 805, a slave pump 306, a dynamic mixer 811C, an autoinjector 234 and a fraction collector FC 203B. The UV detector LCD 2082 and column oven LCO 100 were from Ecom (Czech Republic). The reversed-phase column (ET 250/4 Nucleosil 100-5 C_{18}) was from Macherey-Nägel (Germany). A CSW APEX system DataApex (Czech Republic) was used for collecting and manipulating chromatographic data. A vacuum centrifuge from HETO (Melsungen, Germany) was used for evaporation of solvents.

2.4. Methods

2.4.1. Sample preparation

Serum (500 μl) was extracted with 1.5 ml diethyl ether and the organic phase was removed and evaporated. The dry residue was partitioned between 1 ml of 80% methanol with water and 1 ml of light petroleum (b.p. 60–80°C) to eliminate the majority of lipids and sterols. The light petroleum phase was discarded, while the methanol–water phase containing steroids for analysis was evaporated in a vacuum centrifuge. The dry residue was derivatized as described below.

Saliva was kept frozen at −20°C until analysis. Frozen samples were thawed, centrifuged at 3000–4000 *g* for 10 min, and the clear supernatant (2 ml) was extracted with 5 ml of diethyl ether. The water phase was frozen in a mixture of solid carbon dioxide and ethanol and the combined organic extracts were decanted into glass tubes and evaporated to dryness. The dry residue was dissolved in ethanol (400 μl). An aliquot (200 μl) of the solution was transferred to the vials to be used for derivatization and analysis by GC–MS. The remaining portion was used for radioimmunoassay.

2.4.2. Derivatization

Pyridine (30 μl) and Sylon BFT [99% bis-(trimethylsilyl)trifluoroacetamide (BTSFA)+1% trimethylchlorosilane (TMCS)] (10 μl) were added to the dry residues from serum or salivary extracts, mixed briefly and heated at 65°C for 45 min. Acetonitrile (50 μl) and isooctane (200 μl) were then added and shaken for 1 min. The isooctane phase was recovered and evaporated under nitrogen. The sides of the tubes containing the dry residue were rinsed with isooctane (50 μl) and the mixture was evaporated again. Finally, steroid derivatives were dissolved in 20 μl isooctane, and 2 μl portions were injected into the GC–MS system.

2.4.3. GC–MS analysis

Standard mixtures of 7α- and 7β-OH-DHEA (in three concentrations: 1000 pg/μl, 100 pg/μl and 10 pg/μl) were derivatized in the same way as the samples and used for calibration by an external standard method.

GC separation was carried out with a middle polar (50% phenylpolysiloxane in the stationary phase) capillary column Zebron ZB-50 (15 m×0.25 mm) with 0.15 μm film thickness, catalogue no. 7EG-G004-05, (Phenomenex, St Torrance, CA, USA). The temperature of the injection port was 300°C. The following protocol was used:

Temperature gradient–plateau at 120°C (1 min), linear gradient 40°C/min from 120°C to 210°C (2.25 min), linear gradient 1.8°C/min from 210°C to 221.9°C (6.61 min) and linear gradient 40°C/min from 221.9°C to 300°C (1.95 min).

Pressure gradient–high-pressure (''pulsed splitless'') injection at 60 kPa (1 min), linear gradient 10 kPa/min from 30 to 53 kPa (2.3 min), linear gradient 0.5 kPa/min from 53 to 56.3 kPa (6.60 min) and linear gradient 10 kPa/min from 56.3 to 75.0 kPa (1.87 min). The duration of the analysis was 11.8 min.

The response was recorded in the selected ion monitoring (SIM) mode monitoring the fragments *m/z* 358 (M-90) and *m/z* 359 (M-89) for calculation and verification of the results, respectively. For a clear identification of the substances, the *m/z* 358, 359 and 343 were used. In addition, three different GC gradients were used to check the correct identification of the substances. The detector voltage was at 1.4 kV and the sampling rate was 0.25 s. The temperature of the interface and the ion source was 310°C and 240°C, respectively.

2.4.4. Radioimmunoassays

The radioimmunoassays previously described [12,13] were used for determination of 7α- and 7β-OH-DHEA in serum. The diethylether extract of saliva was dissolved in 200 μl of 20 m*M* sodium phosphate buffer (pH 7) and analyzed for 7α- and 7β-OH-DHEA with the same radioimmunoassays.

2.4.5. Determination of extraction efficiency

The efficiency of both extraction steps for each steroid was determined with HPLC separation of the standards extracted in the same way as the samples, but in amounts sufficient for UV detection. The separation of epimers was carried out with a column (ET 250/4 Nucleosil 100-5 C_{18} from Macherey–Nägel) as follows: (1) Standard solution (25 μl) containing 2.5 μg of each steroid was either directly injected into the HPLC system. (2) Dry residue of the standard solution (75 μl) with 7.5 μg of each steroid was partitioned between 1 ml of water and 2.5 ml of diethylether and the ether phase was dried, dissolved in 75 μl of methanol and 25 μl of the solution was injected into HPLC system. (3) Dry residue of the standard solution (75 μl) with 7.5 μg of each steroid was partitioned between 800 μl of methanol, 200 μl of water and 1 ml of light petroleum and the methanol–water phase was processed as previously. A binary gradient was used at a constant flow-rate of 1 ml/min. Mobile phase A was 15% acetonitrile in water containing 100 mg/l ammonium hydrogen carbonate. Mobile phase B was methanol. The dotted line in Fig. 1 illustrates the shape of the binary gradient. The column temperature was kept at 40°C. The chromatographic conditions and separation of 7-OH-DHEA epimers are shown in Fig. 1.

2.5. Statistical evaluation of the data

The GC–MS analysis and radioimmunoassay were compared with two-parameter linear regression. The results from GC–MS were chosen as the independent variable. If the 95% confidence interval of the intercept included zero, the hypothesis of intercept

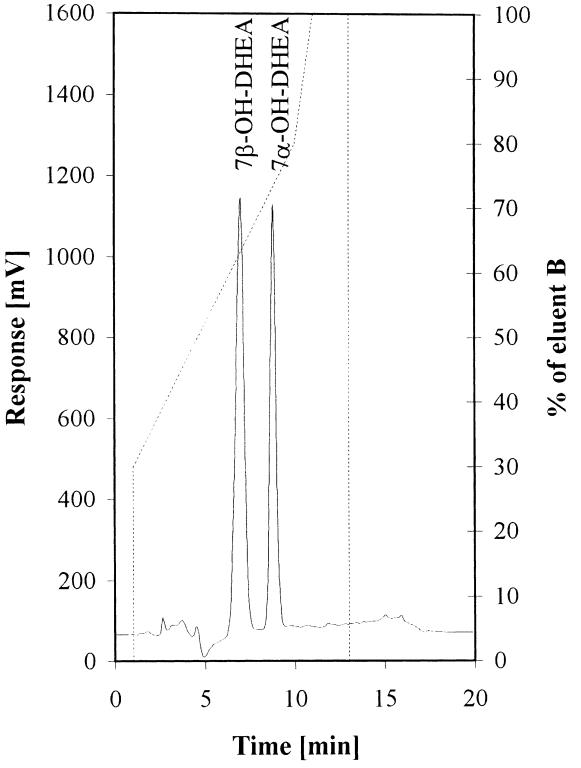

Fig. 1. HPLC separation of 7-hydroxydehydroepiandrosterone epimers on column ET 250/4 Nucleosil 100-5 C_{18} from Macherey–Nägel. Standard solution (25 μl) containing 2.5 μg of each steroid was injected. Binary gradient was used at a constant flow-rate of 1 ml/min. Mobile phase A was 15% acetonitrile in water containing 100 mg/l ammonium hydrogen carbonate. Mobile phase B was methanol. The dotted line illustrates the shape of the binary gradient. The column temperature was kept at 40°C.

equal to zero was accepted. Correspondingly, if the 95% confidence interval of the slope of the regression line included 1, the hypothesis of a slope equal to 1 was accepted. If both conditions were met, the compatibility of the methods was affirmed. The influential points and outliers were detected by use of standard regression diagnostic plots [16].

In addition, the differences in steroid concentrations measured by GS–MS analysis and by radioimmunoassay in individual samples were evaluated with Wilcoxon's paired non-parametric test (with respect to non-Gaussian distribution of the data). The sex differences were evaluated with the Mann–Whitney robust test.

The relations between steroids were evaluated using Pearson's correlations. To avoid non-constant variance, non-Gaussian distribution of the data and to straighten the simple monotonic curvilinear relationships between variables, power transformation to minimum skewness in each of both dimensions was applied [16]. The principal axis and 95% confidence ellipsoids were computed in Excel 97 using the method described elsewhere [17]. The results obtained were retransformed to original scale.

3. Results

3.1. Extraction efficiency

The extraction efficiency with diethylether was 97.8±3.8% and 97.3%±4.3%, $n=6$ for 7α-OH-DHEA and 7β-OH-DHEA, respectively. No detectable concentration of the steroids was found in the petroleum ether fraction and the efficiency was thus considered as 100% for both steroids. The final recovery after all separation steps was 78.2% and 77.9% for 7α-OH-DHEA and 7β-OH-DHEA, respectively.

3.2. Analytical criteria

3.2.1. GC–MS analysis

The mass spectra of the trimethylsilyl (TMS) derivatives of both 7-hydroxydehydroepiandrosterone epimers are shown in Fig. 2. The relatively low patterns of both epimers with a dominant fragment at m/z 358 (M-90) resulted in a relatively high sensitivity of electron impact detection and enabled us to analyze the substances in relatively small samples of body fluids. The response of the GC–MS system was linear from 10 to 10 000 pg of injected sample for both epimers. The correlation coefficients of the log(response) vs. log(mass of injected steroid) were 0.9985 and 0.9998 for 7α-OH-DHEA and 7β-OH-DHEA, respectively. The reproducibility in the mixture of the epimers dissolved in phosphate buffer (pH 7) was tested using 10 samples containing 200 pg of the each substance. The test was performed without tuning of the detector. The RSDs were 2.02% and 2.23% for 7α-OH-DHEA and 7β-OH-DHEA, respectively. The second test of reproducibil-

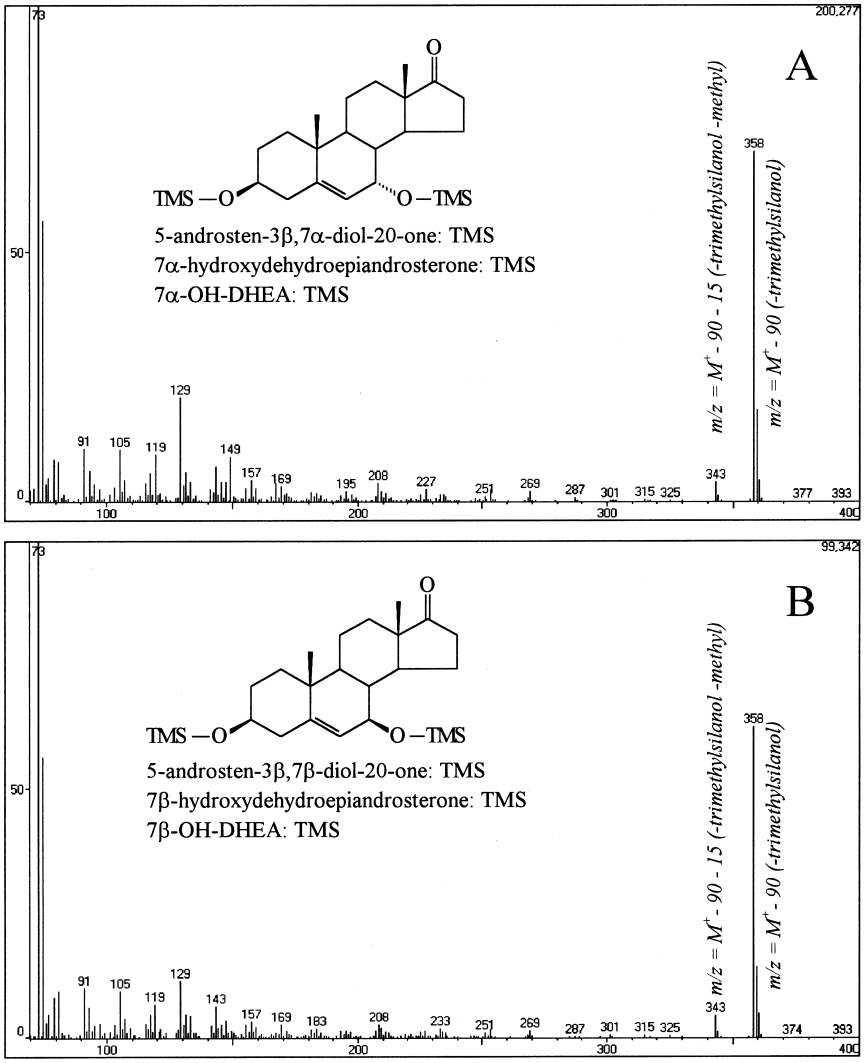

Fig. 2. Mass spectra of the TMS derivatives of 7-hydroxydehydroepiandrosterone epimers. A quadrupole electron-impact detector from Shimadzu (QP 5050A) with a fixed electron voltage of 70 eV was used for the measurements.

ity was performed on five samples of saliva (1 ml) with tuning of the detector prior each analysis. The RSDs were 2.35% and 4.03% for 7α-OH-DHEA and 7β-OH-DHEA, respectively. The response corresponding to a ''signal-to-noise ratio'' (S/N) 2:1 (as was evaluated from the chromatogram of the standards using the software CLASS 5000, a component of the GC–MS system) corresponded to 0.039 and 0.025 pg for 7α-OH-DHEA and 7β-OH-DHEA, respectively.

A good correlation was found between RIA and GC–MS measurements in serum of 7α-OH-DHEA ($r=0.856$, $P<0.0001$, $n=19$), and 7β-OH-DHEA ($r=0.967$, $P<0.0001$, $n=19$). The correlation in saliva was good for 7α-OH-DHEA ($r=0.785$, $P<0.001$, $n=15$) and weaker for the 7β-epimer ($r=0.739$, $P<0.01$, $n=14$). The chromatograms of three 7α-OH-DHEA and 7β-OH-DHEA samples of human serum and saliva are shown in Fig. 3. Retention times from 30 measurements were 7.644±0.017 and 9.276±0.025 min (mean±SD) for 7β- and 7α-OH-DHEA, respectively. The compatibility of the GC–

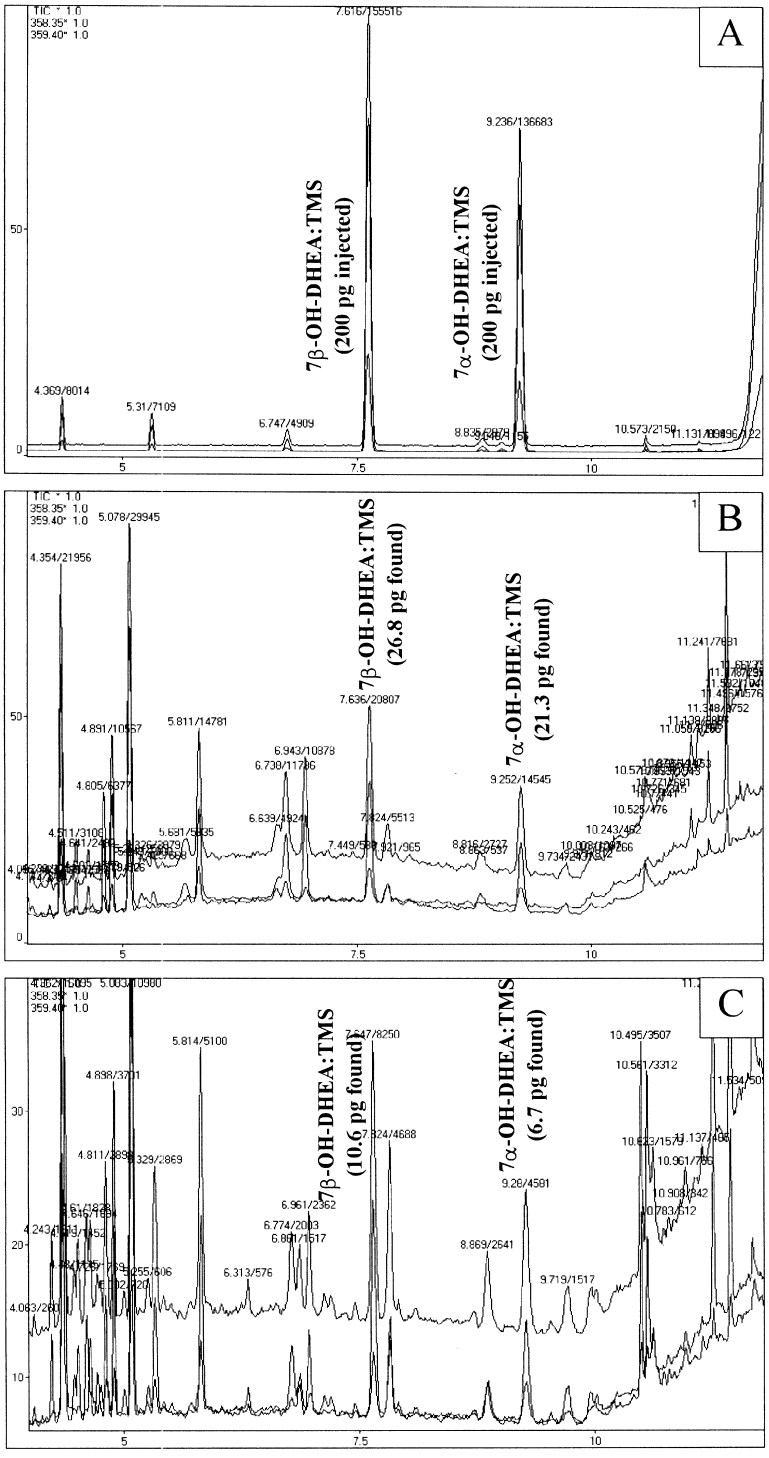

Fig. 3. Gas chromatograms of 2 μl of standard solution containing 7α- and 7β-OH-DHEA:TMS at 100 pg/μl (A), 2 μl of samples obtained from 50 μl of serum (B) and to 100 μl of saliva (C) after extraction and derivatization. Time scales in min.

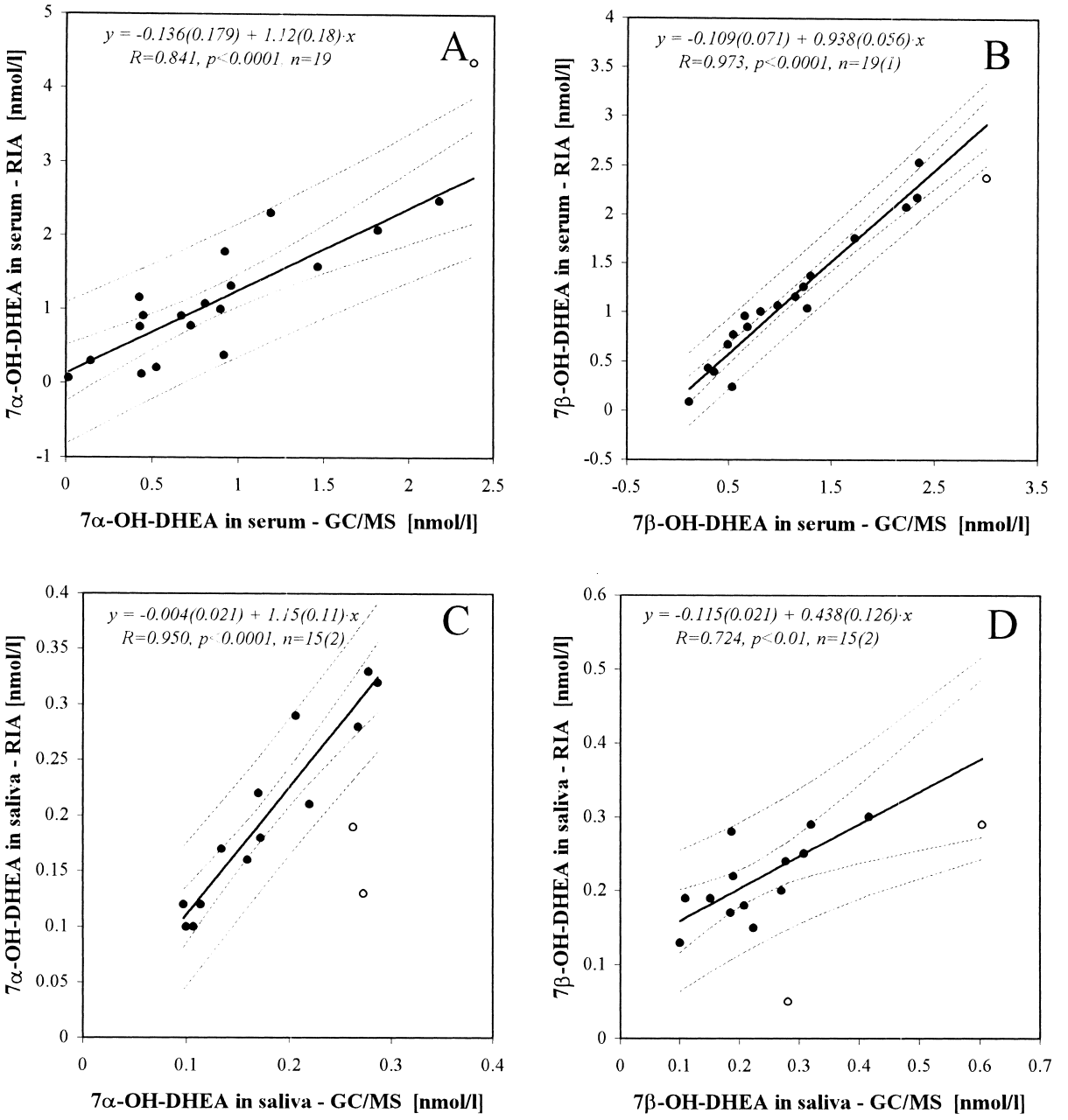

Fig. 4. Linear regression analysis between levels of 7α- (A and C), 7β-OH-DHEA (B and D) measured in serum (A and B) and in saliva (C and D) by radioimmunoassay (dependent variable) and GC–MS (independent variable). The full circles designate the experimental points included for calculation of regression line while the empty ones depict the outliers. The bold line is the regression line, the dotted lines close to regression line symbolize lower and upper 95% confidence interval of the fit and the farther dotted lines enclose lower and upper 95% confidence interval of predicted values. The values in parentheses in equations represent SDs of the regression line parameters. *r* is the correlation coefficient of regression, *P* is the probability level of the regression, *n* is the number of experimental points and the values in parentheses following depict the number of outliers.

MS and RIA was evaluated using two-parameter linear regression (Fig. 4). For 7α-OH-DHEA, the methods were compatible both in serum and in saliva (Fig. 4A and C) as documented also by the zero intercept and the slope equal to 1. The situation was different for 7β-OH-DHEA in serum and saliva, where the excellent correlation found in serum with both methods made them fully compatible (Fig. 4B) while in saliva, the correlation was weaker with intercept significantly higher than zero and only about 0.5 for the slope (Fig. 4D). In addition, the mean concentration of salivary 7β-OH-DHEA measured by GC–MS was almost 50% higher than those measured by RIA (Table 1). Three different gradients were used for the separation, but with the same result, indicating the presence of hardly separable artifact.

3.3. Levels of 7-hydroxydehydroepiandrosterone epimers in serum and saliva

The physiological levels of 7α-OH-DHEA and 7β-OH-DHEA in serum and in saliva of subjects of both sexes measured with GC–MS and RIA, are presented in Table 1. The levels of both steroids in saliva were about 5 times lower than in blood.

The sex differences in serum levels of the both epimers were studied in detail in the forthcoming paper and we evaluated the differences only in saliva. Significantly higher levels of 7α-OH-DHEA ($P<0.05$, Mann–Whitney test) were found in men (0.231 ± 0.027 nmol/l, mean±SEM) than in women (0.169 ± 0.015 nmol/l), and significantly higher levels of 7β-OH-DHEA ($P<0.05$) were found in men (0.382 ± 0.053 nmol/l) than in women (0.238 ± 0.025 nmol/l).

3.4. Relationship between the levels of 7-hydroxydehydroepiandrosterone epimers in serum and saliva

Correlations between the 7-hydroxydehydroepiandrosterone epimers were examined both in serum ($r=0.905$, $n=19$, $P<0.0001$) and in saliva ($r=0.712$, $n=27$, $P<0.0001$). The distribution of data in both dimensions was non-Gaussian (Fig. 5). Accordingly, the data were transformed to minimum skewness to approximate Gaussian data distribution. The principal axes and 95% confidence ellipsoid constructed were re-transformed to original scale. Correlations between 7α-OH-DHEA and 7β-OH-DHEA levels were found stronger in serum than in saliva.

4. Discussion

Because of pronounced sex and age differences [18] and because of their immunomodulatory and antiglucocorticoid properties [4,9–11], determination of 7-hydroxysteroid concentrations in humans is of particular interest. Increased levels of 7α-OH-DHEA were found in patients with Alzheimer's disease

Table 1
Levels of 7α-OH-DHEA and 7β-OH-DHEA measured by GC–MS and RIA in serum and saliva of subjects of both sexes

	Serum				Saliva			
	7α-OH-DHEA (nmol/l)		7β-OH-DHEA (nmol/l)		7α-OH-DHEA (nmol/l)		7β-OH-DHEA (nmol/l)	
	GC–MS	RIA	GC–MS	RIA	GC–MS	RIA	GC–MS	RIA
Number of samples	19	19	19	19	28	15	28	15
Mean	0.913	1.242	1.163	1.171	0.197	0.195	0.304	0.209
SD	0.646	1.043	0.817	0.722	0.078	0.079	0.153	0.069
SEM	0.148	0.239	0.187	0.166	0.015	0.020	0.029	0.018
Median	0.809	1.000	0.983	1.040	0.181	0.180	0.280	0.200
Quartiles	0.438; 1.19	0.38; 1.78	0.536; 1.73	0.670; 1.76	0.144; 0264	0.120; 0.280	0.189; 0.386	0.170; 0.280
Absolute range	0.016–2.38	0.070–4.36	0.116–3.00	0.090–2.54	0.083–0.427	0.100–0.330	0.101–0.725	0.050–0.300
Differences	NS		NS		NS[a]		NS[a]	

The data were measured in both sexes. The differences were evaluated with use of Wilcoxon's paired nonparametric test. NS, not significant.

[a] Only the differences between samples that were measured by both methods were considered in Wilcoxon's paired test ($n=15$).

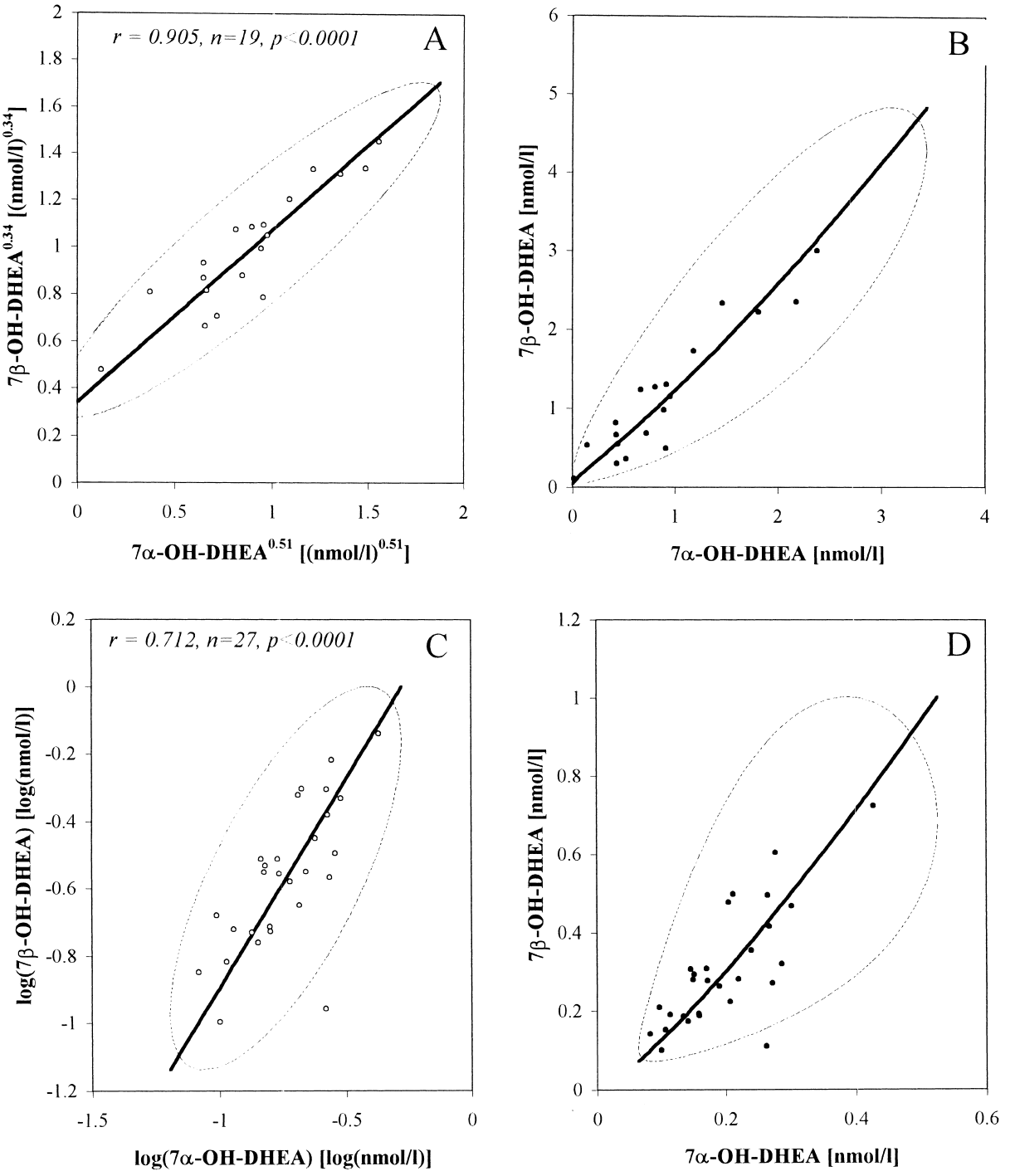

Fig. 5. Correlations between 7α- and 7β-OH-DHEA in serum (A and B) and in saliva (C and D). Full bold lines represent principal axes, while the thin dotted lines enclose 95% confidence ellipsoids. A and C illustrate the correlation after transformation of the variables in each dimension to minimum skewness while the B and D represent the correlation after retransformation of the results to original scale.

[19]. Hence, steroid measurements in patients with autoimmune diseases or disturbances of the central nervous system could be of interest. Therefore, a new sensitive GC–MS method for the determination of 7-hydroxydehydroepiandrosterone epimers was developed, verified and compared with previously described radioimmunoassays [12,13]. For the first time, these steroids were also measured in saliva.

When compared with serum, about five times lower levels of both steroids were found in saliva. Strong correlations between the 7α- and 7β-epimers were established both in serum and in saliva. The close correlations between the epimers indicate that the expectant diagnostic meaning of both epimers is similar and there is a good predictability in the blood or saliva concentrations of the one isomer from the second one. They should correlate both due to their formation from the common precursor (DHEA) and due to possible inter-conversion via 7-oxo-DHEA involving reversible oxido-reduction. Salivary concentrations of both epimers were higher in men than in women, and resembled circulating levels of their DHEA precursor [20]. We suggest the use of saliva for measurement of 7-hydroxysteroid levels, well reflecting those found in serum and because of the non-invasive collection of samples.

Trimethylsilyl ether derivatives of both epimers of 7-hydroxydehydroepiandrosterone were readily made without loss of substrate. In contrast, to produce ester derivatives involving acidic conditions destroy the steroids via dehydration and fission of steroid ring B. Surprisingly high sensitivity of the GC–MS measurement could be ascribed to the low fragmentation of the steroid TMS derivatives, with a dominant fragment at m/z 358. The m/z 359 is the isotope fragment associated with m/z 358. We have used it due to lack of the other fragment (except m/z 358) fulfilling a sufficiently high relative intensity and, at the same time, a relatively low noise caused by the matrix effect. The ratio of the fragments gives information about the number of carbons and monitoring of the additional fragment lower the risk of false signals.

Despite the similarity in chemical structure of both epimers, we have found a remarkable difference in their polarity as apparent from the marked difference in retention times applied to a column with medium polarity, thus enabling a good separation of both epimers. However, certain difficulties occurred in separation of the 7β-isomer from artifacts in saliva that probably caused falsely higher results by GC–MS than by RIA.

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