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Quantitative determination of dehydroepiandrosterone fatty acyl esters in human female adipose tissue and serum using mass spectrometric methods

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ABSTRACT

Dehydroepiandrosterone-fatty acyl esters (DHEA-FAE) are naturally occurring water-insoluble metabolites of DHEA, which are transported in plasma exclusively by lipoproteins. To find out whether DHEA, like estradiol, might be stored in adipose tissue in FAE form, we set up a mass spectrometric method to quantify DHEA-FAE and free DHEA in human adipose tissue and serum. The method consists of chromatographic purification steps and final determination of hydrolyzed DHEA-FAE and free DHEA, which was carried out by gas chromatography–mass spectrometry (GC–MS) or liquid chromatography–tandem mass spectrometry (LC–MS/MS). Our results showed that no detectable amounts of DHEA-FAE could be found in adipose tissue although 32–178 pmol/g of free DHEA were determined by GC–MS and LC–MS/MS. The DHEA-FAE concentrations in serum quantified by GC–MS were 1.4 ± 0.7 pmol/ml in premenopausal women ($n = 7$), and 0.9 ± 0.4 pmol/ml in postmenopausal women ($n = 5$). Correspondingly, the free DHEA concentrations were 15.2 ± 6.3 pmol/ml and 6.8 ± 3.0 pmol/ml. In addition, the mean proportions of DHEA-FAE of total DHEA (DHEA-FAE + free DHEA) in serum were 8.6% and 11.2% in pre- and postmenopausal women, respectively. Serum DHEA-FAE concentration was below quantification limit for LC–MS/MS (signal-to-noise ratio, $S/N = 10$), while free DHEA concentrations varied between 5.8 and 23.2 pmol/ml. In conclusion, the proportion of DHEA-FAE of total DHEA in serum was approximately 9%. However, in contrast to our previous findings for estradiol fatty acid esters in adipose tissue which constituted about 80% of total estradiol (esterified + free), the proportion of DHEA-FAE of total DHEA was below 5%. Four to ten times higher concentrations of free DHEA were quantified in adipose tissue compared to those in serum.

1. Introduction

Dehydroepiandrosterone-fatty acyl esters (DHEA-FAE) belong to a unique family of naturally occurring steroid derivatives with hydrophobic properties due to the presence of long fatty acid carbon chains [1]. Endogenous DHEA-FAE have been determined in many animal and human tissues, such as blood [2,3], adrenal [4] and brain [5]. In plasma, DHEA-FAE are formed from DHEA in a reaction catalyzed by lecithin-cholesterol acyltransferase associated with high-density lipoprotein (HDL) and water-insoluble DHEA-FAE are exclusively transported in lipoproteins [6–8]. DHEA-FAE are hormonally inactive and capable of function only after hydrolysis to free DHEA. Lipoprotein-associated DHEA-FAE can be transferred via lipoprotein receptors into peripheral cells and serve as precursors of DHEA and its metabolites, such as androst-5-ene-3β,17β-diol [9], 5α-androstanedione and androstenedione [10]. Our group recently showed that the HDL-associated DHEA-FAE can enhance the vasodilatory effect of HDL ex vivo [11].

The concept of fat tissue as an endocrine organ in which the steroid uptake, synthesis and metabolism take place has been established [12,13]. Adipose tissue possesses all of the enzymes necessary for production of active androgens and estrogens from DHEA [14,15], the important prohormone which is secreted by

Abbreviations: DHEA, dehydroepiandrosterone; DHEA-FAE, dehydroepiandrosterone-fatty acyl esters; GC–MS, gas chromatography–mass spectrometry; LC–MS/MS, liquid chromatography–tandem mass spectrometry.

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adrenal glands. We and others have reported picomolar estradiol (E_2)-FAE concentrations in human blood [16] and other tissues, a large proportion residing in fat tissue [17,18]. Most steroids, including DHEA and E_2, are present in higher concentrations in fat tissue than in plasma [19], and after the menopause, the overwhelming majority of E_2 exists in the form of E_2-FAE in fat tissue [18]. This raises the possibility that fat tissue may store also other steroids in FAE form.

Although nanomolar concentrations of DHEA-FAE circulate in human blood [2,3], it is not known whether DHEA-FAE, like E_2-FAE, are stored in the adipose tissue. However, some studies indicate uptake of DHEA-FAE by peripheral tissues. For example, studies involving administration of tritium-labeled HDL-associated DHEA-FAE showed an unexpectedly rapid clearance rate [20], possibly indicating effective uptake by peripheral tissues. Moreover, intravenous administration of insulin by hyperinsulinaemic-euglycaemic clamp increased the esterification of DHEA but decreased the concentrations of DHEA and DHEA-FAE in serum, also suggesting enhanced DHEA-FAE uptake by peripheral tissues [21].

In earlier studies, a radioimmunoassay method was used to measure the concentration of DHEA-FAE in plasma [2,3] and in adrenal tissue [4] after hydrolysis to free DHEA. While immunoassays suffer from cross-reactivity with other steroids [22], mass spectrometric methods, such as gas chromatography–mass spectrometry (GC–MS) and liquid chromatography–tandem mass spectrometry (LC–MS/MS), are considered as the gold standards for steroid hormone quantification due to their high accuracy and specificity [23].

Our aim was to clarify to what extent DHEA resides in female human adipose tissue, and what proportion of DHEA, if any, is in FAE form. Furthermore, we report a novel quantitative method using isotope dilution GC–MS in the selected ion monitoring mode (GC–MS-SIM) to determine DHEA-FAE and free DHEA in human serum and adipose tissue. In addition, we compared the GC–MS-SIM method with the LC–MS/MS method which is routinely used in the clinical laboratory of the hospital.

2. Materials and methods

2.1. Subjects

The study was approved by the Ethics Committee of Gynecology and Obstetrics, Pediatrics and Psychiatry of Helsinki University Central Hospital. All subjects volunteered and signed their informed consents. Samples of visceral adipose tissues ($n = 14$) and blood ($n = 20$) were obtained from patients undergoing gynecological surgery for nonmalignant conditions. Serum was separated by centrifugation within 1 h. The serum and adipose tissue samples were stored at −20 °C or −80 °C until analyzed.

2.2. Materials and chemicals

The radiolabeled steroid [1,2,6,7-[^{3}H]-(N)]DHEA (94.5 Ci/mmol), was obtained from PerkinElmer (Boston, MA, USA). DHEA-d_2 was from C/D/N Isotopes Inc. (Pointe-Claire, Quebec, Canada) and DHEA-d_5, ammonium iodide and N-methyl-N-trimethylsilyltrifluoroacetamide were from Sigma–Aldrich (St. Louis, MO, USA). DHEA was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Hexane, methanol, and diethyl ether were HPLC grade from Rathburn Chemicals Ltd. (Walkerburn, Scotland). Chloroform (Uvasol) and dithioerythritol were purchased from Merck (Darmstadt, Germany). Sephadex LH-20 was from Amersham Pharmacia Biotech AB (Uppsala, Sweden), and DEAE Sephadex A-25 from Amersham Biosciences (Uppsala, Sweden).

2.3. Instrumentation

The GC–MS system (Folkhälsan Research Center) was equipped with Fisons Instrument MD 1000 quadrupole mass spectrometer coupled to GC 8000 gas chromatograph (Fisons Instrumentation, Inc., Milan, Italy). A BP-1 column (length 15 m, I.D. 0.22 mm, film thickness 0.25 μm; SGE International Pty Ltd., Ringwood, Australia) with helium as carrier gas (flow 1 ml/min) was used for separation. Data were acquired and processed using XcaliburConfig MFC Application (version 1.0.0.1). LC–MS/MS (Laboratory of Women's clinic) was API 4000 triple quadrupole mass spectrometer (MSD Sciex, Concord, CA). Peripherals included an Agilent series 1200 HPLC system with a binary pump (Waldbronn, Germany) and a SunFire C18 column (2.1 × 100 mm; 3.5 μm; Waters, Milford, MA). Data were acquired and processed with the Analyst Software (MSD Sciex).

2.4. Recovery standards

Our method determines the mixture of DHEA fatty acyl esters as DHEA-FAE in serum or fat tissue without distinguishing between different fatty acyl esters. Therefore, the recovery standards may be any of the different esters such as [^{3}H]-DHEA-linoleate, [^{3}H]-DHEA-oleate or [^{3}H]-DHEA-FAE (obtained by synthesis or incubation of [^{3}H]-DHEA with plasma as described previously [10,11]). Before each assay, the radiolabeled recovery standard was repurified by chromatography on Sephadex LH-20 on the same day. The recovery of the radiolabeled standards through the whole analytical process was 80 ± 7.7% ($n = 13$) for adipose tissue samples and 72 ± 18% ($n = 50$) for serum samples, respectively.

2.5. Quantification of DHEA-FAE and free DHEA in adipose tissue

2.5.1. Analytical procedures

The outline of the method is shown in Fig. 1.

2.5.2. Extraction and separation chromatography

The thawed adipose tissue samples were prepared as described in [18]: the visceral fat samples (~400 mg) were rinsed with 1 ml of sterile saline and homogenized (Ultra Turrax T8 Ika-Werke) in 1 ml distilled water in disposable glass tubes. Recovery standard ([^{3}H]-DHEA-linoleate or [^{3}H]-DHEA-oleate) in 5–10 μl hexane was added to each sample. The adipose tissue homogenates were extracted four times with 4.5 ml of diethyl ether by mixing for 3 min. After centrifugation at 1734 × *g* for 10 min, the water phases were frozen in a dry ice-ethanol bath, and the organic phases collected. The combined organic phases were evaporated under nitrogen flow and subjected to Sephadex LH-20 chromatography (Fig. 1).

The first chromatography on Sephadex LH-20 (0.5 × 6 cm in Pasteur pipettes) to separate FAE and free fractions was performed as follows: the samples were dissolved in 300 μl of hexane and applied on the columns. The sample tubes were rinsed twice with 200 μl hexane, which were also applied on the columns. The DHEA-FAE fraction was collected by further eluting with 4 ml of hexane, after which free DHEA was eluted with 5 ml of hexane: chloroform (1:1, v/v). Both fractions were evaporated to dryness under nitrogen and the free DHEA fractions were stored at −20 °C until analyzed by GC–MS and LC–MS/MS. The DHEA-FAE fractions were subjected to saponification.

2.5.3. Saponification and purification of DHEA-FAE

The DHEA-FAE fractions obtained from the first chromatography on Sephadex LH-20 were saponified in 1 ml of potassium hydroxide in methanol (1 mol/l) at 60 °C for 2 h. One millilitre of distilled water was added to the samples, which were then adjusted to pH 3 with hydrochloric acid (approximately 200 μl of 4 mol/l). Methanol

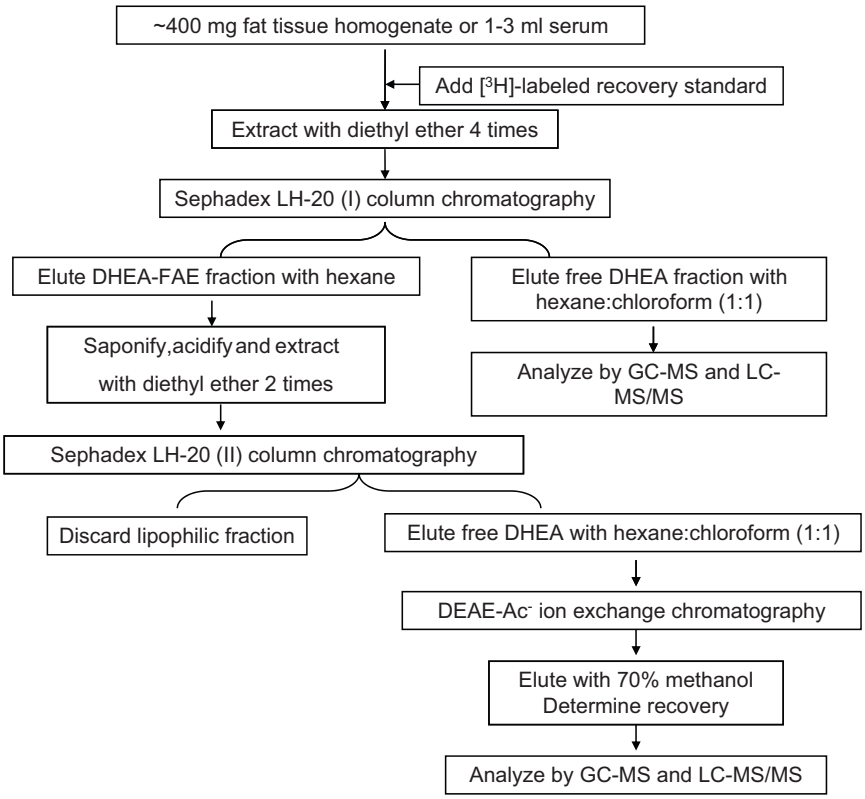

Fig. 1. The outline of the method.

was removed by evaporation, and the samples were extracted twice with 4.5 ml diethyl ether. After this, the extracts were evaporated under nitrogen, dissolved in hexane and subjected to a second chromatography on Sephadex LH-20 using a larger column (1 × 6 cm). The hydrophobic fraction was eluted with 6 ml of hexane followed by elution of the free DHEA with 9 ml of hexane: chloroform (1:1, v/v). After evaporating the organic solvents, the latter DHEA fraction was dissolved in 1 ml of 70% methanol and applied to a DEAE-Ac^- ion exchange column (0.5 × 3 cm) (Fig. 1), followed by elution with 4 ml of 70% methanol and evaporation to dryness. The dry residue was then dissolved in 1 ml of methanol and 50 μl was removed for determination of recovery. The rest was evaporated to dryness under nitrogen and stored at −20 °C until GC–MS and LC–MS/MS analysis.

2.5.4. GC–MS analysis

The DHEA calibration curve with concentrations in the range of 0.38–114 ng in 50 μl (corresponding to 22.8–6840 pg injected on-column) was prepared. After addition of internal standard (DHEA-d_5), all samples and calibrators were silylated to form trimethylsilyl (TMSi) derivatives as follows: the dry samples and calibrators were incubated with 50 μl of N-methyl-N-trimethylsilyltrifluoroacetamide: ammonium iodide: dithioerythritol (1000:2:4; v/w/w) for 30 min at 60 °C. A 3 μl volume of samples and calibrators was injected into the GC–MS using splitless injection mode. The calibration curve was linear with a mean correlation coefficient of 0.998 ± 0.002 (n = 5). The quantification limit of GC–MS was 17 pg of DHEA standard on-column based on a signal-to-noise ratio (S/N) of 5.

The GC temperature was programmed as follows: from 150 °C (for 1 min) increased at 50 °C/min to 180 °C, then at 1 °C/min to 200 °C, kept for 1 min, and finally at 20 °C/min to 290 °C and kept for 10 min. The injector, the ion source and the interface temperatures were 290, 250 and 270 °C, respectively. The detection was performed by GC–MS-SIM method utilizing 70 eV EI+ ionization. The ions monitored were m/z 432.6 and 417.6 for DHEA-diTMSi, and m/z 437.6 and 422.6 for DHEA-d_5-diTMSi (IS).

2.5.5. LC–MS/MS analysis

The DHEA calibration curve with concentrations in the range of 1–100 nmol/l (corresponding to 7.21–721 pg injected on-column) was prepared and it was linear with a mean correlation coefficient of 0.999 ± 0.0003 (n = 6). The limit of quantification of LC–MS/MS was 7.2 pg of DHEA standard on-column (S/N = 10).

Samples (prepared in Section 2.5.2) were dissolved in 1 ml of methanol, from which 250 μl were taken for LC–MS/MS analysis. Before analysis, 30 μl of deuterated (d_2) DHEA internal standard in 50% methanol (v/v) was added and the samples were mixed and extracted with 5 ml of diethyl ether: hexane (90:10; v/v). After mixing for 3 min and letting to stand for 15 min the upper layers were collected and evaporated to dryness under nitrogen. The residues were dissolved in 250 μl of 50% methanol. Twenty-five microlitres of samples and calibrators were analyzed on a LC–MS/MS system. The mobile phase was a linear gradient consisting of methanol (B) and 500 μmol/l ammonium acetate in water (A), at a flow rate of 300 μl/min. The column was eluted with 50% B and at 3–9 min a linear gradient was run to 100% B. Afterwards the column was re-equilibrated by washing with 50% B for 6 min. The column was directly connected to the electrospray ionization probe. DHEA was detected as ammonium adduct in the positive mode with the following transitions: m/z 306 to m/z 253 and IS, m/z 308 to m/z 255. The mass calibration and resolution adjustments (at 0.7 atomic mass units at full width and half height) on both the resolving quadrupoles were optimized using a polypropylene glycol solution with an infusion pump.

2.6. Quantification of DHEA-FAE and free DHEA in serum

The quantitative method for DHEA-FAE and free DHEA in serum was performed similarly to that for adipose tissue (Fig. 1) with some modifications. In short, after adding the tritiated recovery standard ([^{3}H]-DHEA-FAE or [^{3}H]-DHEA-oleate), the samples were extracted four times with 2.5 ml of diethyl ether. The water phases were frozen in dry ice-ethanol bath, after which the organic phases were collected and evaporated to dryness under nitrogen. The chromatographic separation of DHEA-FAE from free DHEA, as well as saponification of the DHEA-FAE fraction were carried out as described for adipose tissue samples. The hydrolyzed DHEA-FAE fraction was further purified by the second hydrophobic chromatography (similar to the first separation chromatography, LH-20 column size, 0.5 × 6 cm). The DEAE-Ac$^-$ ion exchange chromatography utilized in the adipose tissue method was omitted in the analysis of serum. The concentration of DHEA was determined by GC–MS or alternatively by LC–MS/MS, as described above.

2.7. Statistical analyses

Data are expressed as the mean ± standard deviation (SD) values unless otherwise stated. The non-parametric tests (Mann–Whitney test and Spearman's rank correlation) were performed using SPSS Statistics 17.0 software. Statistical significance was assumed if a null hypothesis could be rejected at $P = 0.05$.

3. Results

3.1. Quantitative determination of DHEA-FAE and free DHEA in adipose tissue

3.1.1. Determination by GC–MS

We monitored two ion pairs for DHEA-diTMSi/DHEA-d$_5$-diTMSi, m/z 432/437 and m/z 417/422. Since the results calculated from both ion pairs were similar, we used the molecular ions (m/z 432 and m/z 437) to present the final results. We first tested the fat tissue method by adding 9.3 ng of DHEA-FAE to two samples of 327 mg of pooled adipose tissue, from which quantifiable peaks with recoveries of 82.5% and 92.5% were obtained, respectively. In fat tissue (initial sample sizes 200 mg and 340 mg), no DHEA-FAE could be definitely quantified. The smallest amount of DHEA that could be detected in these sample sizes is 373 pg when considering limit of quantification of GC–MS, dilution factor, and recovery. Typical GC–MS-SIM chromatograms of the DHEA-FAE fraction in fat tissue are shown in Fig. 2. In order to increase the amount of DHEA for analysis, a greater initial weight of fat tissue sample (1200 mg) was used. However, the 1200 mg fat sample caused distortion of internal standard (DHEA-d$_5$) making quantification impossible.

GC–MS analysis of 7 individual samples and 2 adipose tissue pools indicated that the concentration of DHEA-FAE was below the limit of quantification (3.8 pmol/g or 1.1 ng/g, using sample size of 340 mg), while free DHEA was measurable (Table 1). The free DHEA concentrations varied between 38.0 and 85.4 pmol/g in premenopausal women ($n = 5$), and were 86.4 and 158 pmol/g in postmenopausal women ($n = 2$). The free DHEA concentration was 178 pmol/g in an adipose tissue pool from 4 premenopausal women and 108 pmol/g in another adipose tissue pool from 3 postmenopausal women.

3.1.2. Determination by LC–MS/MS

No DHEA-FAE could be detected in the adipose tissue, by using LC–MS/MS ($S/N = 10$), as shown in a typical chromatogram in Fig. 3. While DHEA-FAE concentration in adipose tissue was below the sensitivity of the assay (3.0 pmol/g or 0.9 ng/g, using sample size of 420 mg), the free DHEA concentrations varied between 32.2 and 101 pmol/g in premenopausal women ($n = 5$), and were 99.7 and 162 pmol/g in postmenopausal women ($n = 2$) (Table 1). The relation between the values obtained by GC–MS and LC–MS/MS in adipose tissue samples ($n = 7$) was evaluated by Spearman's rank correlation showing a strong correlation (Spearman's rho = 0.964, $P < 0.001$) (Fig. 4).

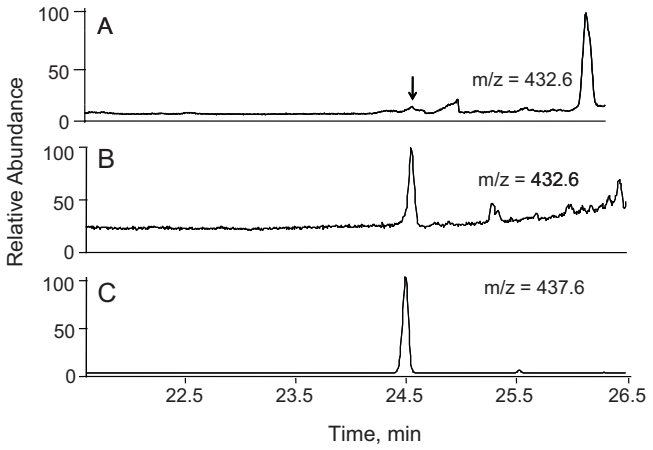

Fig. 2. GC–MS-SIM chromatograms of DHEA-FAE fraction in adipose tissue and in serum. Panels: ion chromatogram following the fragment 432.6 of DHEA-diTMSi (A, in adipose tissue; B, in serum), and 437.6 of DHEA-d$_5$-diTMSi, internal standard (C, in both adipose tissue and serum).

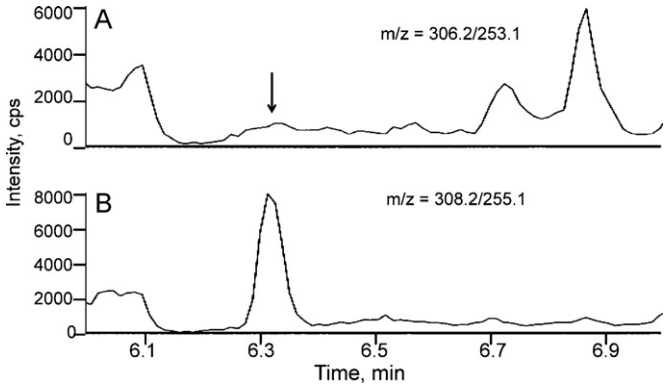

Fig. 3. LC–MS/MS chromatograms of DHEA-FAE fraction in adipose tissue. Panels: ion chromatogram following the fragmentation 306.2/253.1 of DHEA (A) and 308.2/255.1 of DHEA-d$_2$, internal standard (B).

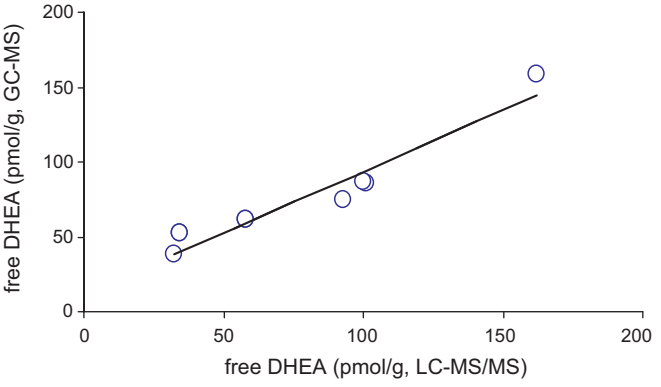

Fig. 4. The relation between adipose tissue DHEA concentrations measured by LC–MS/MS and GC–MS ($n = 7$). A significant correlation was found between the free DHEA values obtained by LC–MS/MS and GC–MS (Spearman's rho = 0.964, $P < 0.001$).

Table 1
The visceral adipose tissue free DHEA and DHEA-FAE concentrations in pre- and postmenopausal women.

Sample	*n*	DHEA-FAE[a]		Free DHEA[a]	
		GC–MS	LC–MS/MS	GC–MS	LC–MS/MS
Fat tissue, premenopausal women	5	N.D.[b]	N.D.[b]	30.8–85.4	32.2–101
Fat tissue, postmenopausal women	2	N.D.[b]	N.D.[b]	86.4–158	99.7–162
Pooled fat tissue, from 4 pre	1	N.D.[b]	–	178	–
Pooled fat tissue, from 3 post	1	N.D.[b]	N.D.[b]	108	–

[a] Results are expressed as pmol/g.
[b] N.D., not detectable, defined as below the limit of quantification, based on signal-to-noise ratio <5:1 in GC–MS and <10:1 in LC–MS/MS.

Table 2
Serum free DHEA and DHEA-FAE concentrations in pre- and postmenopausal women.

Subject	Method	*n*	DHEA-FAE[a]	Free DHEA[a]	Ratio of DHEA-FAE to free DHEA[b]
Premenopausal women	GC–MS	7	1.4 ± 0.7	15.2 ± 6.3[d]	9.5
	LC–MS/MS	12	N.D.[c]	12.6 ± 5.8	–
Postmenopausal women	GC–MS	5	0.9 ± 0.4	6.8 ± 3.0[d]	12.8
	LC–MS/MS	6	N.D.[c]	10.7 ± 5.4	–

[a] Results are expressed as pmol/ml DHEA mean ± SD.
[b] Calculated as (DHEA-FAE/free DHEA) × 100.
[c] N.D., see Table 1.
[d] $P = 0.01$ (Mann–Whitney test).

3.2. Quantitative determination of DHEA-FAE and free DHEA in serum

3.2.1. Determination by GC–MS

The GC–MS-SIM chromatograms of DHEA-FAE fraction in postmenopausal serum (DHEA-FAE concentrations 1.3 pmol/ml) are shown in Fig. 2. In our preliminary study we found the DHEA-FAE concentrations in both pre- and postmenopausal women to be relatively low. When starting from 1 ml of serum, the concentrations were near or below the limit of quantification of GC–MS-SIM analysis. By increasing the sample volume up to 3 ml serum, we were able to reach quantifiable levels.

The mean concentrations of DHEA-FAE and free DHEA in serum in 7 pre- and 5 postmenopausal women are shown in Table 2. In premenopausal women, the concentrations of DHEA-FAE varied from 0.7 to 2.8 pmol/ml (mean 1.4 pmol/ml) and those of free DHEA between 8.5 and 27 pmol/ml (mean 15.2 pmol/ml). The mean proportion of DHEA-FAE of total DHEA (DHEA-FAE + free DHEA) was 8.6%. After the menopause, the free DHEA concentrations (3.8–11.5 pmol/ml, mean 6.8 pmol/ml) were significantly lower ($P = 0.010$). The DHEA-FAE concentrations (0.5–1.4 pmol/ml, mean 0.9 pmol/ml) were slightly lower, and the proportion of DHEA-FAE of total DHEA slightly higher (11.2%), but these differences did not reach statistical significance.

3.2.2. Determination by LC–MS/MS

The serum DHEA-FAE and free DHEA concentrations in 12 pre- and 6 postmenopausal women are shown in Table 2. Serum DHEA-FAE in either pre- or postmenopausal women was not detectable by LC–MS/MS, and could not be influenced by changing the initial sample amount, mainly due to interference of serum matrix. The serum free DHEA concentrations were slightly, but not significantly lower after menopause (10.7 ± 5.4 pmol/ml vs.12.6 ± 5.8 pmol/ml). The values obtained by GC–MS and LC–MS/MS correlated significantly with each other (Spearman's rho = 0.839, $P = 0.002$).

4. Discussion

To our best knowledge, there has been no attempt to measure DHEA-FAE in human adipose tissue. In contrast to our earlier finding showing that fatty acylated E_2 constituted the overwhelming majority of E_2 in female adipose tissue [18], DHEA-FAE was not detectable in fat tissue in pre- or postmenopausal women, despite the presence of 4–10 times higher concentrations of free DHEA as compared to that in serum. In addition, 0.5–2.8 pmol/ml of DHEA-FAE concentrations in serum in pre-and postmenopausal women were quantified by GC–MS, and the mean proportion of DHEA-FAE of total DHEA was 9.7%.

Our and others earlier findings suggested that fatty acylated E_2, in line with fatty acylated cholesterol, could represent storage forms in peripheral tissues [17,18]. Conversely, DHEA, the precursor of androgens and estrogens, resides mostly in the free form in adipose tissue. In humans and other primates de novo synthesis of steroids from cholesterol in adipose tissue is unlikely [12], but the adrenal glands provide a continuous supply of DHEA and, to a much greater extent, of DHEA-sulfate (DHEA-S) which can be metabolized to a variety of androgenic and estrogenic steroids by the enzyme machinery present in adipose tissue [24]. The quantitatively most important steroid hormone precursor DHEA-S is selectively taken up by adipose tissue cells and desulfated making further conversion to other steroids possible. As detectable amounts of fatty acyl esterified DHEA were not observed in adipose tissue, it appears that DHEA might not be esterified as effectively as E_2 in adipose tissue. Alternatively, after local formation or uptaken from circulation, DHEA-FAE are likely to be rapidly hydrolyzed, presumably by hormone-sensitive lipase (HSL) [25]. Our preliminary studies in human adipose tissue *in vitro* indicate that DHEA-FAE are hydrolyzed effectively by HSL (Wang et al., unpubl.). In contrast to E_2 which is almost an end-product of steroid metabolism, DHEA served as a substrate for steroid hormone synthesis. Therefore, it is natural that DHEA exists in the unconjugated form in adipose tissue, ready to be converted to active hormone metabolites when needed.

We showed that the free DHEA concentrations in serum are comparable with the values previously obtained by GC–MS analysis [26]. However, the serum DHEA-FAE values were much lower compared to the early immunoassay studies [2,3,27]. It is generally accepted that immunoassay methods, compared to mass spectrometric methods, tend to give higher values especially when low-level samples are analyzed, possibly due to cross-reactivity or interference by sample matrix and other factors [22,28]. While the earlier studies showed that the proportion of DHEA-FAE of total DHEA increased with aging [2,3,27], we found that the proportion of DHEA-FAE of total DHEA did not significantly differ between pre- and postmenopausal women. This might be attributed to

the relatively small number of samples analyzed in our study, high interindividual variation in postmenopausal serum DHEA levels [29] as well as methodological differences. One unexpected result was that DHEA-FAE in serum could not be quantified by the LC–MS/MS analysis. While LC–MS/MS is very specific and accurate and thus, a superior tool for most substances, in this study GC–MS proved to be more sensitive for the discovery and quantification of novel steroid metabolites at levels near to their detection limit [30]. This is partly because using a long capillary column and a slow temperature program GC separates substances with very closely related structures with a better resolution than LC [23].

Apart from serum and adipose tissue, some investigators using radioimmunoassay have reported the concentrations of DHEA-FAE in the adrenals of several animals and man [4], as well as in rat brain [5]. The reason for the scarcity of studies quantifying fatty acylated DHEA or other steroids in adipose tissue may lie in the difficulty of separating these hydrophobic substances from other lipophilic compounds abundant in adipose tissue. The presence of such contaminants may disturb the final quantitative assay. In our quantitative method, extensive purification of the DHEA-FAE fraction using three column chromatographic steps (Fig. 1) was necessary to abolish interference with contaminating substances. Unlike in the serum method, it was not possible to increase fat sample size despite intensive pre-purification prior to mass spectrometric analysis. Another limitation of the fat tissue method is that it is time-consuming, complicated and therefore limits the number to relatively few samples.

In conclusion, in contrast to E_2 whose fatty acylated form constituted 80% of total E_2 in adipose tissue in postmenopausal women, the DHEA-FAE concentration was less than 5% of total DHEA. Four to ten times more of free DHEA is stored in adipose tissue compared to serum. Free DHEA originating mainly from circulating DHEA-S after its desulfation, and from circulating free DHEA or, to a small extent, DHEA-FAE after its hydrolysis, is constantly available for metabolic conversions to hormonally active androgenic and estrogenic steroids in adipose tissue. In theory, this provides a machinery which can produce estrogens after the cessation of ovarian steroid production.

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