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Ergosteroids
VI. Metabolism of dehydroepiandrosterone by rat liver in vitro: a liquid chromatographic–mass spectrometric study

Ashok Marwah, Padma Marwah, Henry Lardy*

University of Wisconsin – Madison, Institute for Enzyme Research and Department of Biochemistry, 1710 University Avenue, Madison, WI 53705-4908, USA

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Abstract

Because relatively large amounts of dehydroepiandrosterone (DHEA) are required to demonstrate its diverse metabolic effects, it is postulated that this steroid may be converted to more active molecules. To search for the possible receptor-recognized hormones, DHEA was incubated with whole rat liver homogenate and metabolite appearances were studied by LC–MS as a function of time to predict the sequence of their formation. An array of metabolites has been resolved, identified and characterized by highly specific and accurate technique of LC–MS, and several of these steroids were analyzed quantitatively. Their identities were established by comparison with pure chemically synthesized compounds and by chemical degradation of isolated fractions. In the present study, we have reasonably established that DHEA was converted to 7α-OH-DHEA, 7-oxo-DHEA, and 7β-OH-DHEA in sequence. These metabolites were further reduced at position 7 and/or 17 to form their respective diols and triols, which were also sulfated at 3β-position. DHEA and its 7-oxygenated derivatives were also converted to their respective 3β-sulfate esters. Several of these steroids are being reported for the first time. 16α-Hydroxy-DHEA, androst-5-ene-3β,16α,17β-triol, androst-4-ene-3,17-dione, 11-hydroxy-androst-4-ene-3,17-dione, androst-5-ene-3,17-diol and testosterone were also identified and characterized. In all, 19 metabolites of DHEA are being reported in this extensive study. We have also detected the formation of 12 additional metabolites including several conjugates, which are the subject of current investigation.

Keywords: Ergosteroids; Dehydroepiandrosterone

1. Introduction

In addition to serving as an intermediate in the conversion of cholesterol to testosterone and estrogens, dehydroepiandrosterone (DHEA, I) is an effective treatment for obesity in mice [1], rats [2], and dogs [3], increases metabolic rate and thermogenesis in rats [4], induces the synthesis of thermogenic enzymes in the liver of rats [5], depresses blood glucose in diabetic animals [6], decreases blood cholesterol [7], influences the activity of the immune system [8,9] reduces the incidence of tumors in mice [10], and enhances memory function in old mice [11,12]. These metabolic effects are

*Corresponding author. Tel.: +1-608-262-3372; fax: +1-608-265-2904.

E-mail address: halardy@facstaff.wisc.edu (H. Lardy).

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manifest only when relatively large amounts of DHEA (I) are administered which indicates that this steroid is probably converted metabolically to more active metabolites that might qualify for the appellation ''steroid hormone''. While it is well known that DHEA (I) and its sulfate ester (DHEA-3β-sulfate, DHEAS, II) have direct effects on neuroreceptors [13], no receptor for it has been found in somatic cells [14].

The metabolic conversions of DHEA to steroid metabolites have been studied in many laboratories [15–30]. 7α-Hydroxylation of DHEA has been reported to occur in a wide variety of tissues [16–23]. The technique of GC–MS has been used by several workers [19–23]. Both 7α- and 7β-hydroxy derivatives (III and V) are formed and, although only 7α-hydroxylases have been reported [22] it was suggested that a direct 7β-hydroxylation might occur [24,25]. In vitro, biotransformation of DHEA by mouse liver homogenate yielded less polar DHEA metabolites like DHEA acetate [26]. Recently, Prough et al. studied the formation of 7α-OH-DHEA (3β,7α-dihydroxyandrost-5-en-17-one, III), 7-oxo-DHEA (IV, 3β-hydroxyandrost-5-ene-7,17-dione) and 16α-OH-DHEA (IX, 3β,16α-dihydroxyandrost-5-en-17-one) by rat and human liver microsomal fractions [28] using the technique of liquid chromatography–mass spectrometry.

In the present study, we decided to use highly specific and selective technique of LC–MS for quick identification and quantification of trace-level components in a complex biomatrix such as liver. The steroid molecules and their polar conjugates are good analytes for LC–MS instruments using soft ionization techniques. LC–MS provides molecular mass and structural information depending upon fragmentation patterns. It complements traditional HPLC detectors and permits direct analysis of intact polar non-volatile conjugates without derivatization and/or hydrolysis. HPLC and LC–MS analysis of DHEA has been reviewed [31].

We examined the transformation of DHEA in whole liver homogenate to predict the type of reactions that might occur in vivo where the product of one organelle may be the substrate for another. We have also observed metabolite appearances as a function of time to predict the sequence of their formation. We have reasonably established that 7-hydroxylation of DHEA leads to 7α-OH-DHEA (III), and the 7β-stereoisomer, i.e. 7β-OH-DHEA (3β,7β-dihydroxyandrost-5-en-17-one, V) is formed by the reduction of 7-oxo-DHEA (IV) which had been produced by the oxidation of 7α-OH-DHEA (III). Metabolites were resolved, identified, characterized and several were quantitated. Nineteen metabolites of DHEA are being reported in this study. 7β-OH-DHEA-3-S (XVIII, 3β-sulfooxy-7β-hydroxyandrost-5-en-17-one), 7α-triol-3-S (XX, 3β-sulfooxy-androst-5-en-7α,17β-diol), and 7β-triol-3-S (XXI, 3β-sulfooxy-androst-5-en-7α,17β-diol) are being reported for the first time, and several more will be reported in a subsequent publication. To the best of our knowledge, the metabolism of DHEA (I) has not been studied so extensively before.

2. Experimental

2.1. Chemicals

DHEA (I) and 16α-OH-DHEA (IX) were purchased from Steraloids, USA. DHEAS (II), 7α-OH-DHEA (III), 7-oxo-DHEA (IV), 7β-OH-DHEA (V), 7-oxo-diol (VI, 3β,17β-dihydroxyandrost-5-en-7-one), and 7-oxo-DHEAS (XVII, 3β-sulfooxyandrost-5-ene-7,17-dione) were synthesized in our laboratory by known procedures [32–36]. 7α-Triol (VII, androst-5-ene-3β,7α,17β-triol), 7β-triol (VIII, androst-5-ene-3β,7β,17β-triol), and 16α-triol (X, androst-5-ene-3β,16α,17β-triol) were synthesized by the reduction of the corresponding 17-oxo derivatives [37]. The structures of the synthesized compounds were confirmed by nuclear magnetic resonance (^{1}H and ^{13}C) and mass spectrometric data; their purity was checked by TLC and by LC–MS, and was found to be more than 99%. 7α-OH-DHEA-3-S (XVI, 3β-sulfooxy-7α-hydroxyandrost-5-en-17-one), 7β-OH-DHEA-3-S (XVIII), 7-Oxo-diol-3-S (XIX, 3β-sulfooxy-17β-hydroxyandrost-5-en-7-one), 7α-triol-3-S (XX) and 11-OH-Δ^4-dione (XV, 11β-hydroxyandrost-4-ene-3,17-dione) were synthesized in our laboratory and their identity and structure confirmed by their mode of synthesis [37] and by on line DAD-UV spectra and LC–MS. Δ^4-Diol (XIII, androst-4-ene-3β,17β-diol) was synthesized by reduction of testosterone (XII) and Δ^5-diol (XIV,

androst-5-ene-3β,17β-diol) by reduction of DHEA (I). [1,2-^{3}H]-7-Oxo-diol, [1,2-^{3}H]-7α-triol and [1,2-^{3}H]-7β-triol were purchased from ARC Inc. (St. Louis, MO). [4-^{14}C]-7-Oxo-DHEA-3-acetate was supplied by Amersham (UK). Δ^{4}-Dione (XI, androst-4-ene-3,17-dione), testosterone (XII), ATP, β-NADPH, NAD, NADH, malic acid, lactic acid and glucose-6-phosphate were purchased from Sigma. All solvents were of HPLC grade (Aldrich). Solid phase extraction (SPE) cartridges (Oasis-HLB, 3 c.c.) were purchased from Waters Associates. Liquid scintillation cocktail was purchased from Packard Instrument Co. (Merridan, CT).

2.2. Instrumentation

The chromatographic system consisted of an Agilent 1100 series LC–MS system, comprised of a capillary pump (G1376A) operated in normal mode, column oven (G1313A), autosampler (G1315A), diode array detector (G1315A), a mass detector (G1946A) and a Gilson fraction collector (FC-203B). Data were acquired and processed using LC/MSD Chemstation version A.08.03 software.

2.3. Chromatographic conditions

Chromatography of DHEA and its neutral metabolites (ether extract) was performed on a Zorbax-SB C_{18} analytical column (4.6×75 mm, 3.5 μm), protected with a Zorbax-SB C_{18} guard column and maintained at 20 °C. The flow-rate was set at 0.8 ml/min and the eluent was monitored at 205 and 240 nm with a reference wavelength of 360 nm. An acetonitrile–water linear gradient (20 to 45% acetonitrile in 25 min, 94% at 32 min and back to 20% at 34 min, followed by a 10 min post run time; mobile phase A) was used to analyze ether extracts. Sulfate conjugates were resolved on a Zorbax-SB C_{18} column (3.0×150 mm) at a flow-rate of 0.4 ml/min using a gradient comprised of acetonitrile (containing 3% acetic acid) and 3% aqueous acetic acid. The linear gradient started from 10% acetonitrile to reach 40% acetonitrile in 30 min, and 96% acetonitrile in 38 min; it was brought back to 10% at 40 min (mobile phase B).

The neutral compounds were analyzed using electro-spray ionization (ESI) in positive mode. Operating conditions optimized by flow injection analysis (FIA) of 7-oxo-DHEA (IV) and 7β-OH-DHEA (V) were: drying gas (N_2) 12 l/min; drying gas temperature 350 °C; nebulizer pressure 40 p.s.i.; capillary voltage 4500 V; and fragmentor voltage 80 V. The samples were run in scan mode. The sulfate conjugates were analyzed in negative ion mode. Operating conditions were (FIA): drying gas (N_2) 8.0 l/min; drying gas temperature 350 °C; nebulizer pressure 40 p.s.i.; fragmentor 80 V and capillary voltage 3000 V.

2.4. Tissue preparations

Male Sprague–Dawley rats (Harlan, Indianapolis, USA) weighing 200–300 g were housed in pairs in Plexiglas cages in a temperature-controlled room (25 °C) with a 12 h light cycle. Rats were acclimatized for 1 week prior to study. The rats were sacrificed by decapitation between 10 AM and noon and the whole liver was removed and placed on ice. It was rinsed twice with saline and immediately homogenized in sucrose solution (0.25 *M*) to yield liver homogenate (0.4 g/ml).

The liver samples were incubated at 37 °C. Each sample consisted of liver homogenate (1 ml) mixed with equal volume of incubation solution and DHEA (10 μg in 20 μl of methanol) or methanol (20 μl). The incubation solution consisted of magnesium sulfate (1.0 m*M*), glucose-6-phosphate (1.0 m*M*), potassium chloride (150 m*M*), ATP, β-NADPH and/or NADH (25 μ*M* each), malic acid, lactic acid and/or pyruvic acid (2.0 m*M* each). Methanol (10–20 μl) was added to compensate for the presence of methanol in liver samples incubated with DHEA. Metabolism of DHEA (I) was studied in whole liver homogenate in the presence of malic acid using β-NADPH and ATP; in the presence of lactic acid and NADH; and in the presence of malic and pyruvic acid, NADPH and NAD. Metabolism of DHEAS (II) was studied in whole liver homogenate in the presence of malic acid and β-NADPH.

2.5. Extraction procedure

After incubation of samples (2 ml) for the desired time period, the metabolic processes were inhibited

by adding an equal volume of acetonitrile. The aq. acetonitrile-homogenate was centrifuged, and the residue was extracted with acetonitrile (2 ml). The combined aqueous organic layer was evaporated to ~0.5 ml volume at 40 °C under a slow stream of nitrogen, diluted with methanol (3 ml) and centrifuged. The supernatant was evaporated under nitrogen and the residue was reconstituted into water (2 ml), centrifuged and applied to a solid-phase extraction cartridge containing hydrophilic–lipophilic reversed-phase sorbent (Oasis-HLB, 3 c.c.) preconditioned by washing with methanol (2 ml) and water (2 ml). The cartridge was then washed with water (2×2 ml) and eluted with methanol (2 ml). The methanol eluent was evaporated at 40 °C under a slow stream of nitrogen, water (1 ml) was added and the aqueous layer was extracted with ether (2×3 ml). The combined ether extract (Fraction A) was evaporated to dryness under nitrogen. The residue was dissolved in methanol–water (200 μl, 50:50 v/v) and 10 to 20 μl was injected on Zorbax-SB C_{18} column (4.6×75 mm). The aq. layer was reapplied to a preconditioned SPE cartridge (Oasis-HLB, 3 c.c.). It was washed with water–methanol (9:1, 2 ml) and eluted with methanol (2 ml). The methanol was evaporated to dryness at 37 °C under nitrogen. The residue (Fraction B) was dissolved in water (200 μl) and 10 to 20 μl was injected on Zorbax-SB C_{18} column (3.0×150 mm).

2.6. Calibration and quantitation

The standard stock solutions (1 mg/ml) were prepared by dissolving separately 10 to 25 mg of steroid samples in methanol. Working solutions were prepared by serial dilution in the concentration range of 1 to 1000 ng in 20 μl methanol. Liver samples prepared for incubation (2 ml) were inactivated by addition of an equal volume of acetonitrile, and were used to prepare standard samples using 1, 2, 10, 20, 100, 500, and 1000 ng concentrations (Table 1). External standard method was used for the quantitation of DHEA and its metabolites. The peak area or height of the compound of interest was correlated with the concentration of the steroid injected on column, using weighted linear and curve (quadratic or cubic) regression analysis of peak area or height and steroid concentration using SPSS 10.0.7 statistical software supplied by SPSS Inc., USA. A separate curve was plotted for each of the DHEA derivatives.

2.7. Recoveries

The recoveries of various DHEA derivatives were determined by comparing peak heights from inactivated liver extracts spiked with known amounts of various DHEA metabolites versus peak heights of the same concentrations obtained by spiking the

Table 1
Curve fitting ($x = ay + b.10^{-5}\ y^2 + c.10^{-10}y^3 + d$) for DHEA and its derivatives extracted from liver homogenate inactivated with acetonitrile[a]

Compd.[b]	m/z[c]	Conc. range (ng)	a	b	c	d	r^2 [d]
I[e]	311	20–1000	0.072[f]	–	–	0.97	0.9973
III	269	2–500	0.166	−0.654	–	1.41	0.9953
IV	303	2–500	0.447	0.412	2.201	1.04	0.9987
V	269	2–500	0.190	2.023	–	−2.77	0.9997
VI	327	2–500	0.033	2.107	−19.43	0.30	0.9999
VII	271	2–500	0.149	2.644	–	−5.45	0.9990
VIII	271	2–500	0.106	4.744	–	−2.2	0.9990
XVII	381	1–500	0.0136	−0.023	0.025	−0.89	0.9996

[a] Based on extracted ion chromatograms of mass spectra obtained in positive ion mode (I, III–VIII) and negative ion mode (XVII).
[b] Compounds: I, DHEA; III, 7α-OH-DHEA; IV, 7-oxo-DHEA; V, 7β-OH-DHEA; VI, 7-oxo-diol; VII, 7α-triol; VIII, 7β-triol; XVII, 7-oxo-DHEAS.
[c] Most abundant ion in the mass spectrum was used for quantitation.
[d] Correlation coefficient, adjusted.
[e] Height based.
[f] Weighted linear regression, $1/(\text{height})^2$.

extracted matrix. The recoveries of 7-oxo-diol (VI), 7α-triol (VII) and 7β-triol (VIII) were determined by spiking with tritium labeled steroid and by subjecting the various extracts to radioactive analysis.

3. Results and discussion

3.1. Sample preparation

DHEA (I) is known to undergo metabolism by several pathways leading to the formation of androgens, estrogens et al. as well as their polar conjugates. A less understood pathway is 7-hydroxylation. The 7-oxygenated Δ^5-steroids are particularly prone to dehydration, the driving force being formation of resonance-stabilized dienones, dienes and trienes. So our first concern was to extract all the metabolites from the incubation mixture without causing any side reactions. This was accomplished by precipitating proteins from the incubation mixture by addition of acetonitrile. This avoided use of acids. Crude extracts were subjected to solid-phase extraction to remove inorganic salts and water-soluble protein matter. In order to facilitate the chromatography, the complex mixture of products was partitioned between ether and water. The ether extract (Fraction A) contained the DHEA as well as its unconjugated, moderately polar metabolites. During initial exploratory studies using [4-^{14}C]-7-oxo-DHEA, it was observed that a single extraction of aqueous layer with ether resulted in ~90% recovery of 7-oxo-DHEA (IV). Therefore the aqueous layer was extracted twice with ether to maximize the recovery, which was found to be 95–97%. The recovery of polar metabolites of DHEA (I) such as androstenetriols was found to be of the order of 80–85% after two ether extractions (Fig. 1). The recovery of these polar metabolites of DHEA could be improved by using a more polar solvent such as ethyl acetate or a mixture thereof for the extraction but this also caused extraction of unwanted substances of unknown structures from the liver blanks, which were found to interfere with subsequent analysis by mass detector. Hence we decided to use diethyl ether for the extraction process.

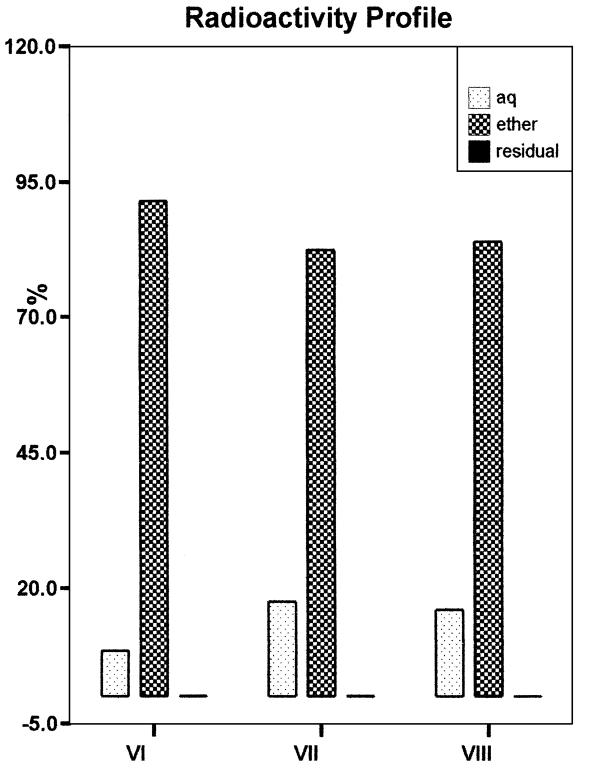

Fig. 1. Extraction recoveries for 7-oxo-diol (VI), 7α-triol (VII), and 7β-triol (VIII) using [1,2-^{3}H] labeled steroids.

3.2. Chromatography and LC–MS

3.2.1. Analysis of neutral molecules

A methanol–water linear gradient was used initially to analyze ether extracts on Zorbax Eclipse column. This solvent system resolved many different steroids but failed to separate 7α-triol (VII) from its 7β isomer (VIII). Subsequently we were able to resolve these stereoisomers on a Zorbax-SB C_{18} column using acetonitrile–water gradient as mentioned under the experimental conditions (mobile phase A). A representative chromatogram showing resolution of DHEA and its derivatives, i.e. 7β-triol (VIII, 5.66 min), 7α-triol (VII, 6.64 min), 7-oxo-diol (VI, 7.88 min), 7β-OH-DHEA (V) and 16α-triol (X) 8.94 min), 7α-OH-DHEA (III, 10.70 min,) 7-oxo-DHEA (IV, 12.21 min), 16α-OH-DHEA (IX, 13.65 min), 11β-OH-Δ^4-dione (XV, 16.25 min), Δ^4-dione (XI, 26.60 min), testosterone (XII 24.30 min) and DHEA (I, 25.09 min) is shown in Fig. 2. All of the

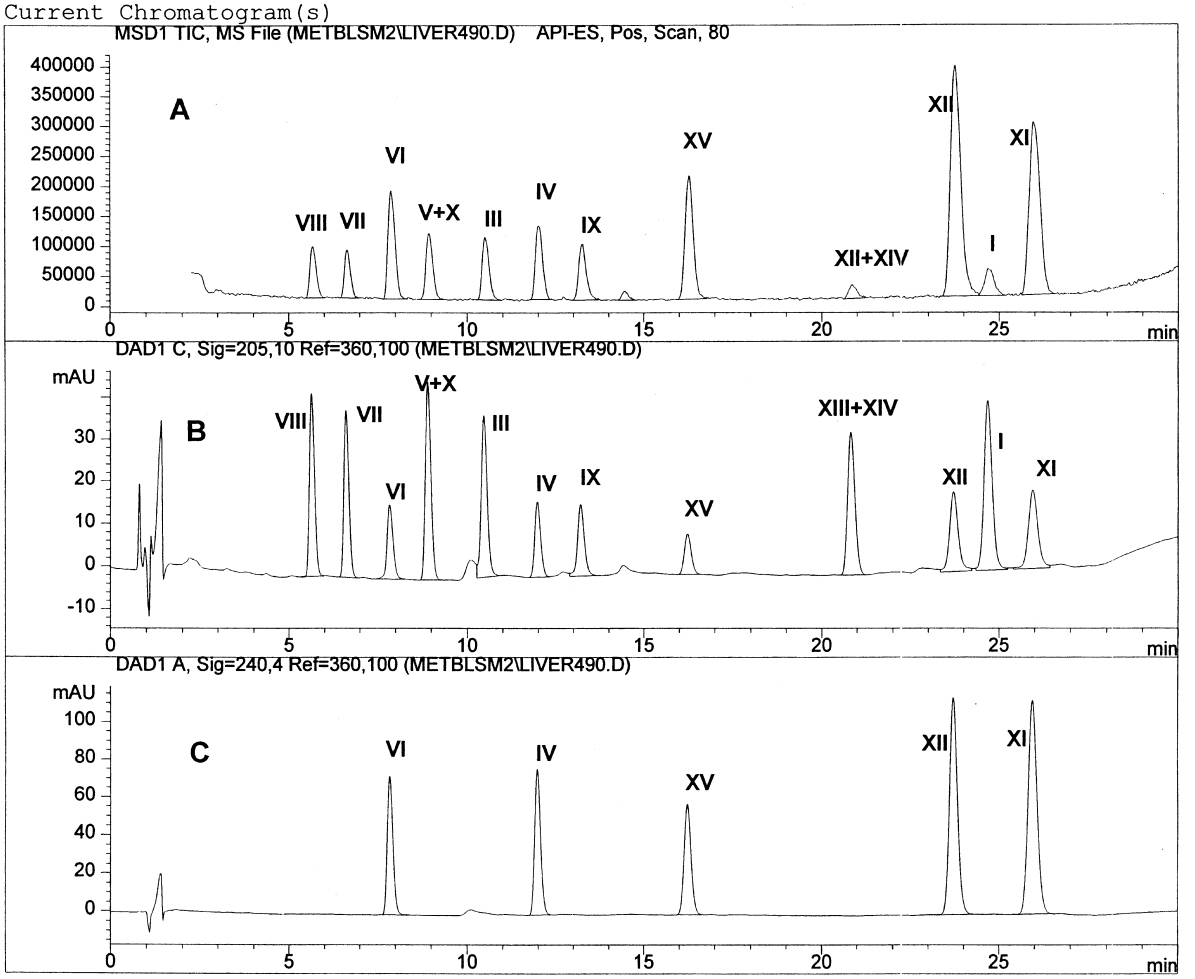

Fig. 2. LC–MS profile of a synthetic mixture of DHEA (I) and 12 of its metabolites. A: Mass detector (ESI-TIC); B: at 205 nm; and C: at 240 nm. LC–MS profile: Zorbax-SB C_{18} column eluted with acetonitrile–water gradient (20 to 45% in 25 min and 94% at 32 min); positive ion mode at 4500 V. Peak identification: I, DHEA; III, 7α-OH-DHEA; IV, 7-oxo-DHEA; V, 7β-OH-DHEA; VI, 7-oxo-diol; VII 7α-triol; VIII, 7β-triol; IX, 16α-OH-DHEA; X, 16α-triol; XI, Δ^4-dione; XII, testosterone; XIII, Δ^4-diol; XIV, Δ^5-diol; XV, 11-OH-Δ^4-dione.

compounds were monitored in UV at 205 nm, but only those (IV, VI, XI, XII and XV) having α,β-enone system were seen at 240 nm. Their retention times, molecular masses, λ_{max} as seen in diode array UV and mass fragmentation pattern are given in Table 2. It may be noted that 7β-hydroxy isomers eluted before 7α-hydroxy isomers. In the chromatogram based on mass detector in total-ion current (TIC) mode DHEA (I), Δ^4-diol (XIII) and Δ^5-diol (XIV) had poor sensitivity but were detected in extracted ion mode. This mode was extensively used to identify and quantify various steroid derivatives as discussed subsequently. The molecular ion $(M+H)^+$ was the most abundant ion for 7-oxo-DHEA (IV, m/z 303), testosterone (XII, m/z 289), and Δ^4-dione (XI, m/z 287). Δ^4- and Δ^5-diols yielded most abundant ion at m/z 273 by each loosing a water molecule $(M+H-H_2O)^+$, while the most abundant ion for 7α-OH-DHEA (III) and 7β-OH-DHEA (V) was obtained at m/z 269 by loss of two water molecules from the parent ion $(M+H-2\times H_2O)^+$. 7α-Triol (VII) and 7β-triol (VIII) behaved similarly by yielding most abundant ion at m/z 271 $(M+H-2\times H_2O)^+$. It may be noted that the sodium ion adducts

Table 2
Comparison of LC–MS analysis of standard DHEA and its unconjugated derivatives and metabolites of DHEA produced by incubation with whole liver homogenate[a]

Compound[b]	t_R (min)	Characteristic ions in mass spectra (m/z)					UV λ_{max} (nm)
		$(M+Na)^+$	$(M+H)^+$	$(M-H_2O)^+$	$(M-2\times H_2O)^+$	$(M-3\times H_2O)^+$	
I[c]	25.16	311	289	271	253	–	–
III	10.75	327	–	287	269	251	–
IV	12.26	325	303	285	–	–	242
V	9.11	327	305	287	269	251	–
VI	7.91	327	305	287	269	–	242
VII	6.69	329	–	289	271	253	–
VIII	5.63	329	–	289	271	253	–
IX	13.47	327	305	287	269	251	–
X	8.94	329	307	289	271	253	–
XI	26.55	309	287	–	–	–	244
XII	24.19	311	289	–	–	–	246
XIII + XIV	21.15	–	–	273	255	–	–
XV	16.30	325	303	285	267	–	244

[a] Ether extracts of metabolic reaction mixtures were subjected to LC–MS on a Zorabax-SB C_{18} column using diode array detector (200 to 400 nm). Mass spectra were obtained in the range m/z = 200–400.

[b] Compounds: I, DHEA; III, 7α-OH-DHEA; IV, 7-oxo-DHEA; V, 7β-OH-DHEA; VI, 7-oxo-diol; VII, 7α-triol; VIII, 7β-triol; IX, 16α-OH-DHEA; X, 16α-triol; XI, Δ^4-dione; XII, testosterone; XIII, Δ^4-diol; XIV, Δ^5-diol and XV, 11β-OH-Δ^4-dione.

[c] Data obtained from incubated samples. Standard samples exhibited same characteristic ions in mass spectra and same λ_{max} in DAD-UV. Retention times of the compounds were in good agreement ($<\pm 2\%$) with those of the standard compounds.

were the most abundant ions for DHEA (I, m/z 311), 7-oxo-diol (VI, m/z 327) and 16α-OH-DHEA (IX, m/z 327), a phenomenon usually observed in electrospray ionization mass spectrometry [38]. 7β-OH-DHEA (V) and 16α-triol (X) did not resolve in this system. However it was possible to differentiate them on the basis of extracted ion chromatograms of their most abundant ions (at m/z 269 $(M+H-2\times H_2O)^+$ and 329 $(M+Na)^+$, respectively). Δ^4-Diol (XIII) and Δ^5-Diol (XIV) did not resolve in the present study nor was it possible to differentiate them based on mass spectra since both had identical fragmentation patterns and retention times. Subsequently we were able to resolve them on an ODS-AQ column (2.0×150 mm, 3 μ, YMC, Inc.) using methanol–water gradient. This allowed us to prove the absence of Δ^4-Diol (XIII) in the incubation mixture.

Fig. 3 shows a representative extracted ion chromatogram (at m/z 269, 271, 303, 327, 329, 309, 311, and 273) of ether extract of liver homogenate incubated with DHEA (I) in the presence of lactic acid and NADH for 60 min at 37 °C. 7α-Triol (VII) and 7β-Triol (VIII) were detected at m/z 271 $(M+H-2\times H_2O)^+$; 7α-OH-DHEA (III) and 7β-OH-DHEA (V) at m/z 327 $(M+Na)^+$ and 269 $(M+H-2\times H_2O)^+$. Their structure was further confirmed by isolating the fractions containing the respective compounds, and by subjecting them to dehydration under acidic conditions. The LC–MS profile of these products was found to be similar to that obtained by similar treatment of pure substances, which were either obtained from commercial sources or synthesized in our laboratory as discussed earlier in Section 2.1. 7-Oxo-DHEA (IV) and 7-oxo-diol (VI) were identified at m/z 303 $(M+H)^+$ and 327 $(M+Na)^+$, respectively, and their structures were further confirmed by converting the isolated fractions to androsta-3,5-diene-7,17-dione and 17β-hydroxyandrosta-3,5-dien-7-one under acidic conditions. 16α-OH-DHEA (IX, m/z 327 $(M+Na)^+$) and 16α-triol (X, m/z 329 $(M+Na)^+$ and 271 $(M+H-2\times H_2O)^+$) were identified on the basis of retention times and mass fragmentation pattern. It may be mentioned that 7-hydroxy metabolites of DHEA yielded the most abundant ion by loss of two water molecules, the driving force being formation of a conjugated triene system in the molecule. By contrast 16-hydroxylated DHEA derivatives yielded $(M+Na)^+$ as most abundant ion. Testosterone (XII, m/z 311) and Δ^4-dione

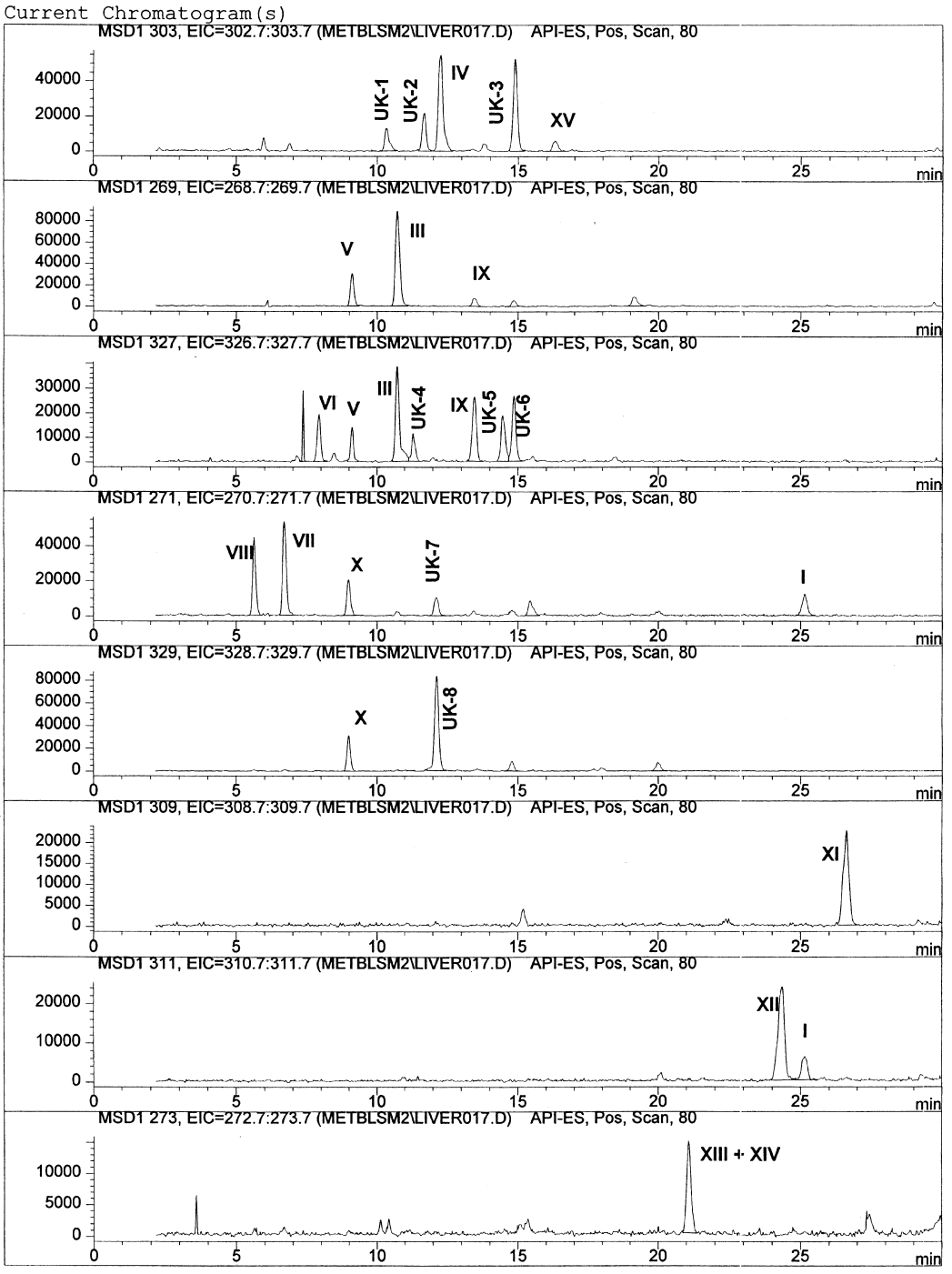

Fig. 3. Representative chromatograms (extracted ions at m/z 303, 269, 327, 271, 329, 309, 311 and 273) from LC–MS analysis of ether extract of DHEA incubation with liver homogenate in the presence of lactate and NADH. LC–MS profile: Zorbax-SB C_{18} column eluted with acetonitrile–water gradient (20 to 45% in 25 min and 94% at 32 min); positive ion mode at 4500 V. Peak identification: I, DHEA; III, 7α-OH-DHEA; IV, 7-oxo-DHEA; V, 7β-OH-DHEA; VI, 7-oxo-diol; VII 7α-triol; VIII, 7β-triol; IX, 16α-OH-DHEA; X, 16α-triol; XI, Δ^4-dione; XII, testosterone; XIII, Δ^4-diol; XIV, Δ^5-diol; XV, 11-OH-Δ^4-dione. UK-1 to UK-8: unknown metabolites of DHEA.

(XI, m/z 309) were identified on the basis of mass fragmentation pattern and UV spectrum, as was a small peak at t_R 16.3 min (m/z 303) as 11-OH-Δ^4-dione (XV). Unreacted DHEA (I, t_R 25.1 min) could be seen at m/z 271 $(M+H-H_2O)^+$ as well as at m/z 311 $(M+Na)^+$.

3.2.2. Analysis of sulfate conjugates

The aqueous extract (Fraction B) contained polar conjugates such as sulfates, which gave heavily tailing peaks under normal chromatographic conditions; therefore acetic acid (3.0% v/v) was added to acetonitrile and to water to suppress peak tailing and to achieve better separation and resolution of these polar compounds. Addition of stronger acids like trifluoroacetic acid, and even formic acid was found to cause peak suppression in mass detection. Addition of acetic acid was found to be ideal since it was able to suppress peak tailing caused by the presence of highly polar sulfate moiety without unduly suppressing mass signal. Since sulfate conjugates are excellent substrates for proton abstraction, the aqueous extracts were analyzed in negative ion mode for these conjugates.

The extracted ion analysis of electrospray negative ion mass scans of aq. extracts of liver incubated with DHEAS (II) in the presence of malic acid showed the presence of several sulfate conjugates as shown in Fig. 4. The following sulfate derivatives were identified by comparison (t_R and mass spectra) with authentic samples: DHEAS (II, 33.32 min, $(M-H)^-$ at m/z 367), 7-oxo-DHEAS (XVII, 23.12 min, $(M-H)^-$ at m/z 381); 7β-OH-DHEA-3-S (XVIII, 19.06 min, $(M-H)^-$ at m/z 383); 7α-OH-DHEA-3-S (XVI, 23.81 min, $(M-H)^-$ at m/z 383); 7-oxo-diol-3-S (XIX, 18.12 min, $(M-H)^-$ at m/z 383); 7α-triol-3-S (XX, 18.92 min, $(M-H)^-$ at m/z 385); and 7β-triol-3-S (XXI, 13.52 min, $(M-H)^-$ at m/z 385). 7-Oxo-diol-3-S (XIX) was not detected in the incubation of DHEA in the presence of lactic acid and NADH nor in the incubation of DHEA in the presence of malic acid and NADPH, while only 7β-OH-DHEA-3-S (XVIII) and 7β-triol-3-S (XXI) were detected in the incubation of DHEA in the presence of malic and pyruvic acids and NADPH.

In the present study involving incubation of DHEA with whole liver homogenate, we have identified 19 metabolites of DHEA including several sulfate conjugates. 7-oxo-diol-3-S (XIX), 7α-triol-3-S (XX) and 7β-triol-3-S (XXI), to the best of our knowledge, have not been reported earlier in the studies concerning metabolism of DHEA. However Starka et al. did report the formation of 7α-triol-3-S (XX) from DHEAS (II) by rat liver microsomal fractions [29], and an androstenetriol sulfate of unknown structure and configuration has been reported [30] in the urine of rats injected with ^{14}C-labeled DHEAS (II). Various metabolic pathways are shown in Fig. 5.

3.2.3. Unknown metabolites

An examination of the extracted ion chromatogram as shown in Figs. 3 and 4, as well as other data obtained during the study revealed the presence of several other major and minor metabolites of DHEA in ether and aqueous extracts. They have been designated UK-1 to UK-8 in Fig. 3. They are most probably oxygenated (UK-1 to UK-3, m/z 303), and/or hydroxylated (UK-4 to UK-6 m/z 327, UK-7 m/z 271 and UK-8 m/z 329) derivatives of DHEA. However the presence of 5α-reduced derivatives of DHEA cannot be ruled out. Attempts to identify them by matching with several synthetic derivatives have not been successful until now, and further work is under way to establish their identity. But based on the present work we found that the following DHEA derivatives were not present in the incubation mixture: 3β-hydroxyandrost-4-en-17-one (27.46 min, m/z 271 and 311), the Δ^4-triols (androst-4-ene-3α,11β,17β-triol, 10.31 min, and androst-4-ene-3β,11β,17β-triol, 12.32 min, m/z 329 [37]), and the Δ^5-triols (androst-5-ene-3β,11β,17β-triol, 10.28 min, and androst-5-ene-3β,11α,17β-triol 11.04 min, m/z 329 [37]). Several new steroidal sulfate conjugates were also found to be present in the aq. extracts of liver homogenate incubated with DHEA and DHEAS. Further work on the structural elucidation of these compounds will be reported in a subsequent communication.

3.3. Quantification

It may be noted that ESI-MS has a relatively narrow linear dynamic range. Above a certain point a critical ionization concentration may be exceeded

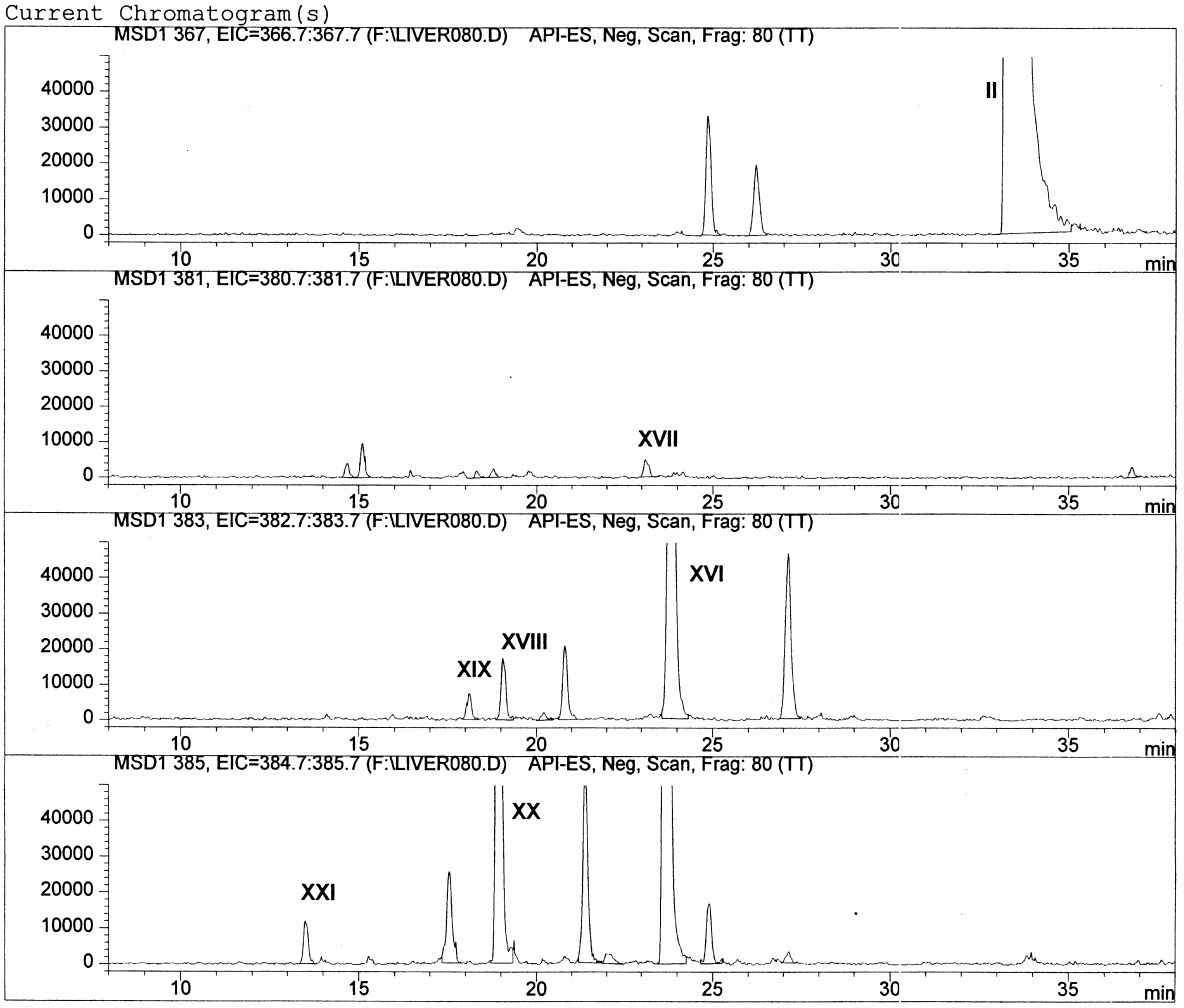

Fig. 4. Representative chromatograms (extracted ions at m/z 367, 381, 383, and 385) from LC–MS analysis of aqueous extract of DHEA sulfate incubation with liver homogenate in the presence of malate and NADPH. LC–MS profile: Zorbax-SB C_{18} column eluted with acetonitrile–3% aqueous acetic acid gradient (10 to 40% in 30 min); negative ion mode at 3000 V. Peak identification: XVI, 7α-OH-DHEA-3-S; XVII, 7-oxo-DHEAS; XVIII, 7β-OH-DHEA-3-S; XIX, 7-Oxo-diol-3-S; XX, 7α-triol-3-S; and XXI, 7β-triol-3-S.

resulting in non-linear ESI response. It has been our experience [38] as well as reported in the literature [39] that even at low (sub ng concentrations) marked deviation from a straight line may be observed, thereby necessitating a second or higher order fitting. This is in marked contrast to the broad linear range of a UV detector. Consequently we subjected the data to linear as well as curve fitting regimes and appropriate calibration curves were selected. The regression analysis parameters (concentration range, curve fitting parameters and correlation coefficient, r^2 adjusted) for DHEA (I) and several of its derivatives are given in Table 1.

DHEA (I) and the following seven metabolites were quantitatively estimated in the ether extract at various time points under three different incubation

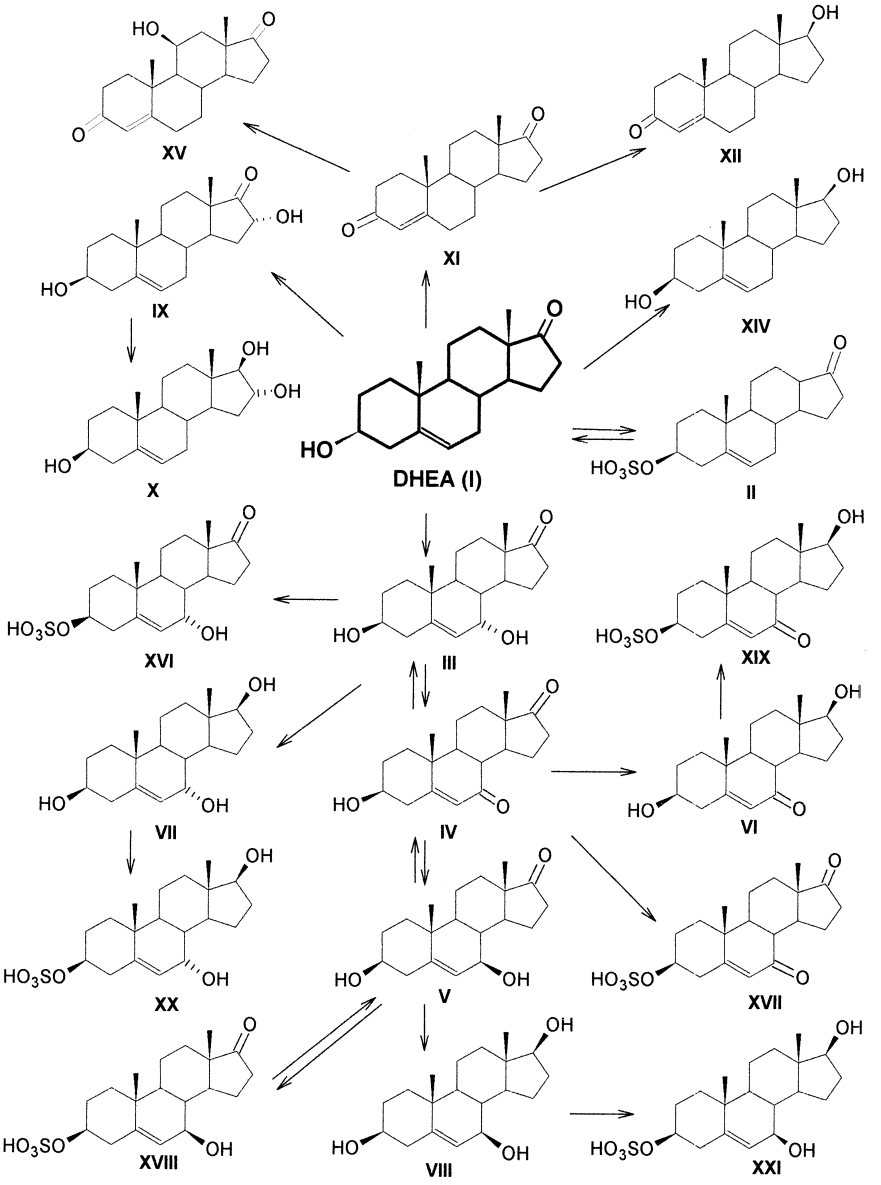

Fig. 5. Metabolism of dehydroepianderosterone by rat liver in vitro.

conditions: 7β-triol (VIII), 7α-triol (VII), 7-oxo-diol (VI), 7β-OH-DHEA (V), 7α-OH-DHEA (III), 7-oxo-DHEA (IV) and 7-oxo-DHEAS (VII). The quantities of the other sulfate conjugates reported herein viz. 7α-OH-DHEA-3-S (XVI), 7β-OH-DHEA-3-S (XVIII), 7-oxo-diol-3-S (XIX), 7α-triol-3-S (XX) and 7β-triol-3-S (XXI) are approximate only since they were calculated based on the calibration curve created for 7-oxo-DHEAS (Table 3 and 4).

Table 3
Amount (ng/1000 ng of DHEA or DHEAS added) of various sulfate conjugates of DHEA derivatives after incubation of DHEA and DHEAS with liver in the presence of malic acid and NADPH[a]

S. No	Concentration (ng) at time (min)								
		0	1	2	5	10	30	60	120	240
A.	With DHEA									
1.	XVI[b]	0	0	<0.5[c]	0.5	1.7	12.9	10.6	6.8	4.2
2.	XVIII	0	0	0	<0.5	<0.5	1.2	1.2	1.2	0.9
3.	XX	0	0	0	0.7	0.6	3.4	2.7	4.8	2.3
4.	XXI	0	0	0	0	<0.5	0.6	1.0	0.6	<0.5
B.	With DHEAS									
1.	XVI	0	1.0	1.2	2.5	8.3	27.8	22.5	13.0	6.3
2.	XVII	0	3.0	2.1	2.2	1.3	<0.5	<0.5	0	0
3.	XVIII	0	0.6	0.6	0.6	0.7	1.4	1.4	1.2	1.0
4.	XIX	0	<0.5	<0.5	0.7	1.0	0.7	0	0	0
5.	XX	0	0	0	0.7	1.8	27.0	41.4	21.4	10.1
6.	XXI	0	0	0	<0.5	<0.5	1.0	1.2	<0.5	<0.5

[a] Based on extracted ion chromatograms of mass spectra of aqueous extract obtained in negative ion mode.
[b] Compounds: XVI, 7α-OH-DHEA-3-S; XVII, 7-oxo-DHEAS; XVIII, 7β-OH-DHEA-3-S; XIX, 7-oxo-diol-3-S; XX, 7α-triol-3-S; XXI, 7β-triol-3-S.
[c] Detected but could not be quantified because of low concentration, i.e. <0.5 ng.

3.4. Biological studies

In the presence of malate, the native malic enzyme, and NADPH the P450 hydroxylases of rat liver are maintained active because of the presence of NADPH-P450-reductase. Under these conditions DHEA (I) is converted rapidly to 7α-OH-DHEA (III, Fig. 6a). 7α-OH-DHEA (III) increased in amount during the first 10 min of incubation and then declined as it was converted to other metabolites. No 7β-OH-DHEA (V) and only traces of 7-oxo-DHEA (IV) were formed in the first 2 min. 7β-OH-DHEA (V) appeared only after 7-oxo-DHEA (IV) had been formed; the latter reached a maximum concentration at 30 min (Fig. 6b) and then declined, whereas 7β-OH-DHEA (V) continued to increase. 7α-Triol (VII) was detected very early and continued to increase during the 2-h incubation. The 7β-Triol (VIII) first appeared at 30 min and continued to increase. Therefore it can be concluded that direct hydroxylation of DHEA (I) leads to the formation of 7α-OH-DHEA (III), which is oxidized to 7-oxo-DHEA (IV). The latter is then reduced to 7β-hydroxy-DHEA (V). It may be noted that the ability of these steroids to induce the hepatic thermogenic enzymes, mitochondrial glycerophosphate dehydrogenase and cytosolic malic enzyme, increases in that order. These findings are consistent with the original suggestion of Hampl and Starka [27] that 7β-OH-DHEA (V) is formed by enzymatic reduction of 7-oxo-DHEA. Metabolism of DHEAS (II) in the

Table 4
Amount (ng/1000 ng of DHEA added) of various sulfate metabolites of DHEA after incubation of DHEA with liver in the presence of lactic acid and malic and pyruvic acid[a]

	Compd.	Concentration (ng) at time (min)				
		0	5	30	60	120
A.	In the presence of lactate					
1.	XVI[b]	0	<0.5[c]	4.3	1.7	1.1
2.	XVIII	0	0	0.7	0.5	0.3
3.	XX	0	0	1.7	0.7	0.4
4.	XXI	0	0	<0.5	<0.5	0
B.	In the presence of malate and pyruvate					
1.	XVIII	0	0	0.8	<0.5	<0.5
2.	XXI	0	0	<0.5	<0.5	<0.5

[a] Based on extracted ion chromatograms of mass spectra of aqueous extract obtained in negative ion mode.
[b] XVI, 7α-OH-DHEA-3-S; XVIII, 7β-OH-DHEA-3-S; XX, 7α-triol-3-S; XXI, 7β-triol-3-S.
[c] Detected but could not be quantified because of low concentration, i.e. <0.5 ng.

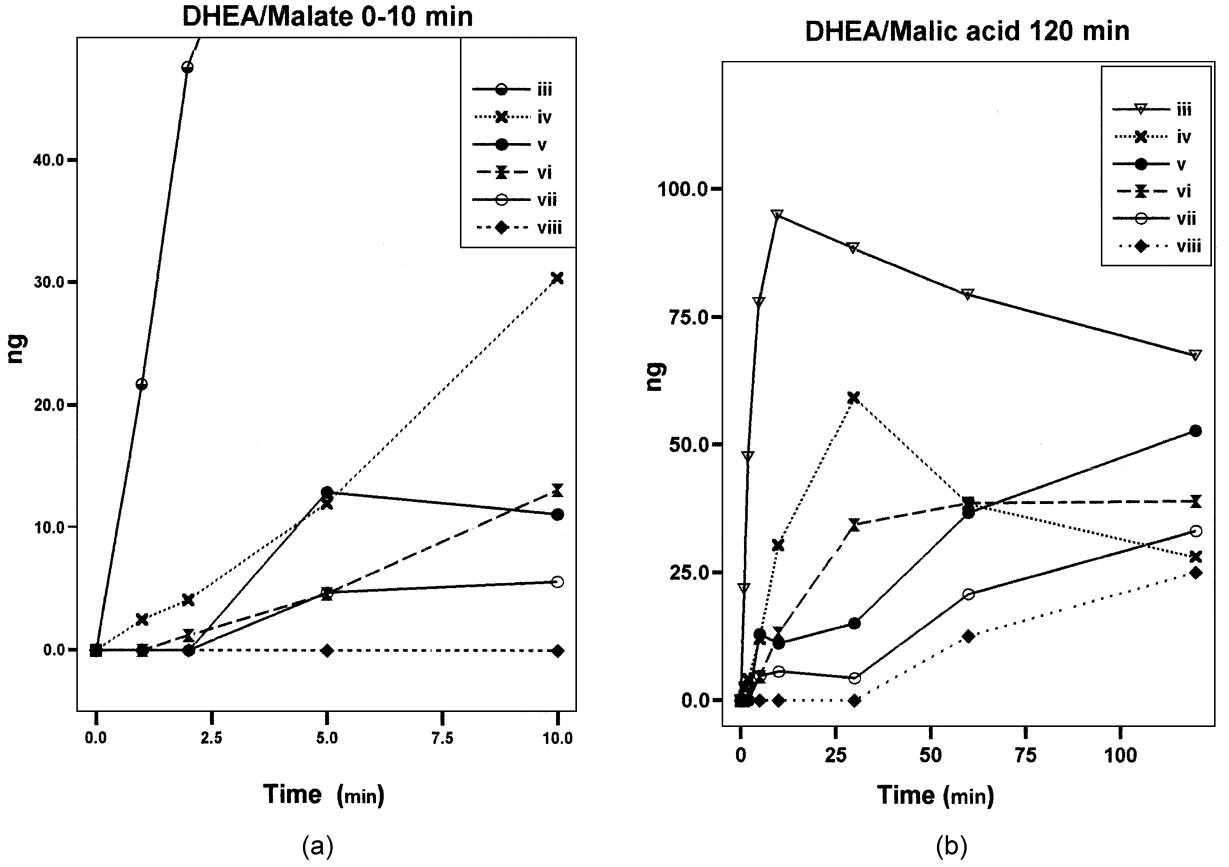

Fig. 6. (a) Formation of 7-oxygenated derivatives of DHEA in the presence of malic acid and NADPH. The *Y*-axis represents amount of metabolites in ng/μg of added DHEA, and the *X*-axis shows incubation time (0–10 min). Peak identification: III, 7α-OH-DHEA; IV, 7-oxo-DHEA; V, 7β-OH-DHEA; VI, 7-oxo-diol; VII, 7α-triol; VIII, 7β-triol. The study clearly brings out the following metabolic sequence: DHEA→7α-OH-DHEA→7-oxo-DHEA→7β-OH-DHEA. (b) Formation of 7-oxygenated derivatives of DHEA in the presence of malic acid and NADPH. The *Y*-axis represents amount of metabolites in ng/μg of added DHEA, and the *X*-axis shows incubation time (0–120 min). Peak identification: III, 7α-OH-DHEA; IV, 7-oxo-DHEA; V, 7β-OH-DHEA; VI, 7-oxo-diol; VII, 7α-triol; VIII, 7β-triol.

presence of malic acid resulted in the formation of the same product mixture but much lower concentrations of the metabolites were observed (Fig. 7). For example 7α-triol and 7β-triol were detected after 2 h of incubation. Sulfate conjugates were, however, relatively more abundant in this case (Table 3).

In a system that provided NADH the same products were formed on a similar time scale as shown in Fig. 8. Again the first product was 7α-OH-DHEA and 7-oxo-DHEA peaked at 30 min, but 7β-OH-DHEA and 7α- and 7β-triols were produced continually and in greater amounts. Fig. 9 depicts formation of 7-oxygenated derivatives of DHEA in the presence of NADPH and with pyruvate present to temporarily minimize the conversion of NAD^+ to NADH. Product formation was much subdued compared with other two systems but the distribution was similar. In all three incubations involving DHEA, limited formation of sulfate conjugates was observed (Tables 3 and 4). It was observed that in all three cases the conversion of DHEA to 7α-OH-DHEA (III), 7-oxo-DHEA (IV) and 7-oxo-diol (VI) reached peak concentration at about 30 min after which a steady decline set in, whereas concentration of 7β-OH-DHEA, 7α-triol (VII) and 7β-triol (VIII) in-

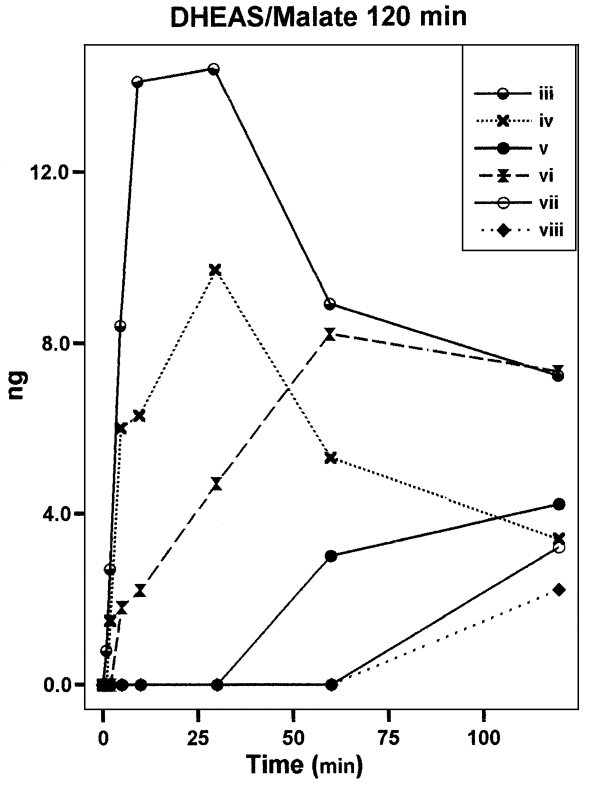

Fig. 7. Formation of 7-oxygenated derivatives of DHEAS in the presence of malic acid and NADPH. The *Y*-axis represents amount of metabolites in ng/μg of added DHEA, and the *X*-axis shows incubation time (0–120 min). Peak identification: III, 7α-OH-DHEA; IV, 7-oxo-DHEA; V, 7β-OH-DHEA; VI, 7-oxo-diol; VII, 7α-triol; VIII, 7β-triol. The concentration of metabolites is much lower than those obtained by incubation of DHEA under similar conditions (Fig. 6b) but the same trend is maintained.

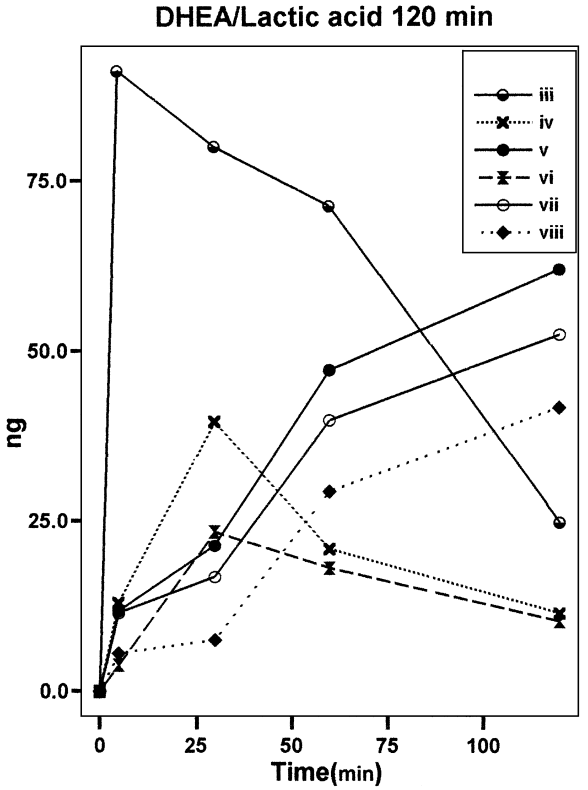

Fig. 8. Formation of 7-oxygenated derivatives of DHEA in the presence of lactic acid and NADH. The *Y*-axis represents amount of metabolites in ng/μg of added DHEA, and the *X*-axis shows incubation time (0–120 min). Peak identification: III, 7α-OH-DHEA; IV, 7-oxo-DHEA; V, 7β-OH-DHEA; VI, 7-oxo-diol; VII, 7α-triol; VIII, 7β-triol. 7β-OH-DHEA, 7α-triol and 7β-triol are formed at the expense of 7-oxo-DHEA, 7α-OH-DHEA and 7-oxo-diol.

creased during 2 h. This behavior is more pronounced in case of incubations using lactate and pyruvate (Figs. 8 and 9).

4. Conclusions

In the present study, metabolism of DHEA has been studied extensively. Metabolites were resolved, identified, and characterized using highly specific and selective technique of LC–MS, and several of these steroids were analyzed quantitatively. Nineteen metabolites of DHEA have been reported, several (XVIII, XX and XXI) of them for the first time. We have also studied metabolite appearances as a function of time for the first time. We have reasonably proved that 7-hydroxylation of DHEA leads to 7α-OH-DHEA (III), and the 7β-isomer, i.e. 7β-OH-DHEA (V) is formed by the reduction of 7-oxo-DHEA (IV) which had been produced by the oxidation of 7α-OH-DHEA (III). To the best of our knowledge, the metabolism of DHEA (I) has not been studied so extensively before.

Acknowledgements

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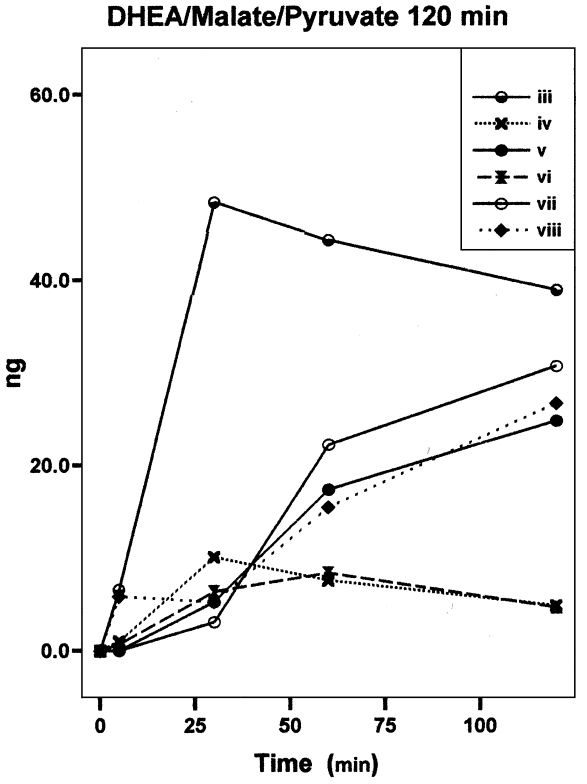

Fig. 9. Formation of 7-oxygenated derivatives of DHEA in the presence of malate, pyruvate and NADPH. The *Y*-axis represents concentration of metabolites in ng/μg of added DHEA, and the *X*-axis shows incubation time (0–120 min). Peak identification: III, 7α-OH-DHEA; IV, 7-oxo-DHEA; V, 7β-OH-DHEA; VI, 7-oxo-diol; VII, 7α-triol; VIII, 7β-triol. 7β-OH-DHEA, 7α-triol and 7β-triol are formed at the expense of 7-oxo-DHEA, 7α-OH-DHEA and 7-oxo-diol but at relatively lower concentrations (cf. Figs. 7 and 8).

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