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PROFILING SPECIFIC METABOLITES OF DEUTERIUM LABELED DEHYDROEPIANDROSTERONE (DHEA)

ABSTRACT

During recent years a number of products containing the steroid dehydroepiandrosterone (DHEA) have appeared on the food supplement market. The International Olympic Committee placed this steroid on the list of forbidden substances. However, establishment of oral DHEA administration in doping control is aggravated by extensive metabolic clearance, and an assumed non-specific metabolism. In this study, a preliminary investigation is performed to construct a DHEA-selective profile of non-oxygenated metabolites (androsterone, etiocholanolone, testosterone, epitestosterone, androst-5-ene-3β,17β-diol, 5α-androst-3α,17β-diol and 5β-androstan-3α-17β-diol) and oxygenated DHEA metabolites (7α-hydroxy-DHEA, 7β-hydroxy-DHEA, 16α-hydroxy-DHEA, 7-keto-DHEA, 16α-hydroxy-androsterone, 16α-hydroxy-etiocholanolone and 7-keto-androsterone).

Labeled DHEA (3β-hydroxy-[16,16-$^{2}H_{2}$]androst-5-en-17-one) was administered to 2 male volunteers (25.5 and 52.5 mg, respectively) and urine samples were collected. Sample analysis was performed on the total fraction of glucuronides, sulfates and non-conjugated steroids with gas chromatography-mass spectrometry. The most sensitive parameters during the first 10 hours after administration were androsterone, etiocholanolone, testosterone, 7β-hydroxy-DHEA and 7-keto-DHEA. Etiocholanolone and 5α-androst-3α,17β-diol showed the highest sensitivity during 10 to 30 hours after administration. The total conversion of DHEA to the studied metabolites was 39.7-42.8%. The conversion of DHEA to oxygenated and non-oxygenated metabolites was 0.8-1.32% and 38.4-42.0%, respectively. Although the conversion to oxygenated metabolites is limited, profiling these specific steroids may lead to additional information about the identity of the administered steroid.

INTRODUCTION

An increasing number of steroid containing products has appeared on the food supplement market. The first supplements sold contained dehydroepiandrosterone (DHEA) and androst-4-ene-3,17-dione (Δ4-AEDIONE), later followed by androst-5-ene-3β,17β-diol (Δ5-AEDIOL), androst-4-ene-3β,17β-diol (Δ4-AEDIOL), the nor-steroids 19-nor-androst-4-ene-3,17-dione and 19-nor-androst-4-ene-3β,17β-diol [1,2]. The proclaimed pharmacological effect by metabolism to steroids with potential anabolic activity made these products mostly popular amongst bodybuilders. During the years 1997 to 1999 the mentioned steroids have been placed on the IOC list of forbidden substances.

Analytical methods have been developed to establish the abuse of steroid supplements as DHEA by profiling of main metabolites as androsterone (AO), etiocholanolone (EO), testosterone (T) and androstanediols [1-6]. However, establishment of DHEA abuse in doping control is aggravated by extensive first-pass metabolism after oral administration, fast renal and metabolic clearance of DHEA and metabolites, and an assumed non-specific metabolism compared to other orally administered steroids as Δ4-AEDIONE [1,2].

The generally applied determination of glucuronides and/or sulfates of non-specific metabolites of DHEA as AO, EO, T and Δ4-AEDIONE [1-6], is generally insufficient to obtain quality information about the identity of the administered steroid. A study performed by Shackleton *et al.* [7] described a method based on gas chromatography-isotope ratio mass spectrometry to confirm administration of T, E, DHEA and dihydrotestosterone. However, the described method was also not specific for any of the studied steroids.

Several studies have been performed for the use of the testosterone/epitestosterone ratio (T/E ratio) as a marker of DHEA or Δ4-AEDIONE abuse [4,5,8]. In theory, the T/E ratio could be a non-specific but sensitive parameter since T is a known metabolite of DHEA, Δ4-AEDIONE, Δ5-AEDIONE, Δ4-AEDIOL and Δ5-AEDIOL and no E production has been proven after administration of testosterone. In reality, the T/E ratio appears to be an insensitive parameter of DHEA abuse [8].

The complication for doping analysis caused by the wide spread use of steroid supplements demands for profiling procedures of higher specificity. Determination of oxygenated metabolites of potentially used steroids could supply the necessary information for identification of abused steroids.

In this study, a preliminary investigation is performed to construct a conclusive profile of selected oxygenated and non-oxygenated DHEA metabolites, that are commercially available, in order to look for conclusive parameters for the detection of

DHEA abuse. Therefore, 3β-hydroxy-[16,16-$^2H_2$]androst-5-en-17-one (d_2-DHEA) was administered to 2 healthy male volunteers and urine samples were collected at set times before and after administration. Analysis of the collected urine samples was performed with gas chromatography-mass spectrometry (GC-MS).

EXPERIMENTAL

Chemicals

Reference steroids: Androst-5-en-3β-ol-17-one (dehydroepiandrosterone, DHEA), androst-4-en-17α-ol-3-one (epitestosterone, E), 5α–androstan-3α-ol-17-one (androsterone, AO), 5β–androstan-3α-ol-17-one (etiocholanolone, EO), androst-4-ene-3,17-dione (androstenedione, Δ4-AEDIONE), androst-5-ene-3β,17β-diol (androstenediol, Δ5-AEDIOL) and 17α-methyl-androst-4-en-11α,17β-diol-3-one (11α-hydroxy-methyltestosterone, 11α-OH-MeT) were obtained from Sigma, St. Louis, Missouri, USA. Androst-4-en-17β-ol-3-one (testosterone, T), androst-5-ene-3β,7α-diol-17-one (7α-hydroxy-dehydroepiandrosterone, 7α-OH-DHEA) and androst-5-ene-3β,7β-diol-17-one (7β-hydroxy-dehydroepiandrosterone, 7β–OH-DHEA) were obtained through courtesy of Organon, Oss, The Netherlands). Androst-5-en-3β-ol-7,17-dione (7-keto-dehydroepiandrosterone, 7-keto-DHEA), 5α-androstan-3α-ol-7,17-dione (7-keto-androsterone, 7-keto-AO), androst-5-ene-3β,16α-diol-17-one (16α-hydroxy-dehydroepiandrosterone, 16α–OH-DHEA), 5α–androstane-3α,16α-diol-17-one (16α-hydroxy-androsterone, 16α-OH-AO), 5β–androstane-3α,16α-diol-17-one (16α-hydroxy-etiocholanolone, 16α-OH-EO) were obtained from Steraloids, Newport, Rhode Island, USA. 3β-Hydroxy-[16,16-$^2H_2$]androst-5-en-17-one (isotopic purity 97.3%) was obtained from C/D/N Isotopes, Pointe-Claire, Quebec, Canada.

Chemicals: *N*-Methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA), ethanethiol (97%), phosphorous pentoxide, *tert*-butyldimethylsilyl chloride (TBDMSCl) and imidazole were obtained from Sigma-Aldrich Chemie Company, Steinheim, Germany. Ammonium iodide was obtained from Fluka Chemie, Buchs, Switzerland. Potassium hydroxide, sodium acetate trihydrate, glacial acetic acid (96%), diethyl ether, acetic acid and methanol were obtained from Merck, Darmstadt, Germany. Acetonitril was obtained from J.T. Baker B.V., Deventer, The Netherlands. All chemicals were of analytical grade, unless indicated otherwise.

Hydrolysis was performed with Helix pomatia (type HP-2, containing 110.000 IU/ml β-glucuronidase and 1000-5000 IU/ml arylsulfatase), obtained from Sigma-Aldrich, Steinheim, Germany.

Columns for solid-phase extraction were IST Isolute C_{18} columns (200 mg, non-endcapped) obtained from Sopachem, Lunteren, The Netherlands.

Sample collection

Two male volunteers (subject 1: 29 years, 88 kg; subject 2: 21 years, 75 kg), collected urine samples every 2 hours during 3 days. On the second day at 9 a.m. 3β-hydroxy-[16,16-$^2H_2$]androst-5-en-17-one (d_2-DHEA) was orally administered (volunteer 1, 25.5 mg; volunteer 2, 52.5 mg). For each collected urine sample the exact void time, volume and specific gravity (Urine Specific Gravity Refractometer, Atago, Japan) were recorded. Samples were stored at -20°C until time of analysis.

Sample preparation

The volume of urine that was sampled for the analysis was determined on the basis of specific gravity (d) of the urine, according to:

$$\text{Volume} = \frac{0.020}{d-1} * 5 \text{ ml} \qquad \textbf{(1)}$$

A maximum volume of 15 ml was applied. This was done to enable quantitative analysis of metabolites in low concentration in diluted urine samples. Solid-phase extraction was performed with C_{18} columns. Before applying urine samples, the column material was preconditioned by washing with 4 ml of methanol and equilibrating with 2 ml of water. Inorganic material was removed from the column by washing with 2 ml of water. The organic material was collected by elution with 4 ml of methanol. After evaporation to dryness, 2 ml of acetate buffer (0.1 M, pH 5.2) was added to the extract. Hydrolysis was performed by adding 100 µl of Helix pomatia and incubating for 1 hour at 55°C. Steroids were isolated from the buffer solution by extracting with 5 ml of diethyl ether twice. After extraction the phase separation was stimulated by centrifugation (4000 rpm, 5 min). The organic layer was removed and evaporated to dryness under a gentle stream of nitrogen at 40°C. The extract was dried overnight over P_2O_5/KOH under reduced pressure.

To retain the deuterium atoms in the steroid structures and to obtain suitable peakshape and mass spectrometric fragmentation, a non-enolizing derivatization procedure to *tert*-butyldimethylsilyl-derivatives was selected (Method A). A modified procedure of Dehennin *et al*. [4] was applied. Derivatization was performed by addition 100 µl of TBDMSCl/imidazole/acetonitrile (30:30:1000; m/w/v) to the extract and incubating at 60°C for 60 min. Afterwards the reaction was stopped by addition

of 10 drops of water and extraction was performed with 1.6 ml of heptane. After vortexing, the organic layer was removed and evaporated to dryness. To the dry extract 50 µl of heptane were added and transferred in autosampler vials after vortexing.

Due to co-elution a different silylation method based on enolization was selected for derivatization of T, E, 5α,3α-ADIOL and 5α,3β-ADIOL (Method B). Enolizing conditions could be applied in this case, because these are steroids with an androstan-17β-ol moiety and therefore the deuterium atoms are retained under enolizing conditions. Loss of deuterium atoms would have occurred, in case of androstan-17-one steroids. The same cleanup procedure was performed. Derivatization was performed by incubation of the extract in 100 µl of MSTFA/NH_4I/ethanethiol (1000:2:3; v/w/v).

Gas chromatography and mass spectrometry

Selected ion monitoring was performed with a Hewlett Packard gas chromatograph (Model 5890, Agilent Technologies, Waldbronn, Germany) coupled to a Hewlett Packard quadrupole mass spectrometer (Model 5972A). Ionization was performed in electron impact mode at 70 eV. Analysis was performed by selected ion monitoring of specific ions, representing the respective deuterated and non-deuterated metabolites (see Table 1). A check for possibly incomplete derivatization of Method B was performed by detection of ions representing mono-TMS derivatives of AO and EO at m/z 362.

Gas chromatography was performed with a HP-1 fused silica column (length 18 m, inner diameter 0.2 mm, film thickness 0.11 µm). Via electronic pressure control the column flow (helium) was constant 1 ml/min. Sample injection of 1 µl was performed in split mode (ratio 1/10). A Hewlett Packard autosampler (Model 7673, Agilent Technologies, Waldbronn, Germany) was used for auto-injection. The injector temperature was set to 250°C. The oven temperature program used was: initial temperature 180°C, 2°/min to 225°C, 30°/min up to 310°C, held for 5 min. The interface temperature was set to 280°C.

The quality of d_2-DHEA was checked by GC-MS in full scan mode prior to administration. No impurities could be detected in a concentrated sample.

Table 1: *Monitored m/z values of labeled and non-labeled steroids. Method A (TBDMS-derivatization) was used for the analysis of labeled androstan-17-one steroids. Method B (TMS-enolTMS derivatization) was used for all non-labeled steroids and labeled androstan-17β-ol steroids.*

Steroid derivative	m/z value non-labeled Method A	m/z value labeled Method A	m/z value non-labeled Method B	m/z value labeled Method B	number of deuterium atoms in derivative (n)
AO	347.3	349.3	434.3	-	2
EO	347.3	349.3	434.3	-	2
DHEA	345.3	347.3	432.3	-	2
Δ5-AEDIOL	461.3	463.3	434.3	-	2
T	-	-	432.3	434.3	2
E	-	-	432.3	434.3	2
5α,3α-ADIOL	-	-	241.2	243.2	2
5α,3β-ADIOL	-	-	241.2	243.2	2
7α-OH-DHEA	399.3	401.3	430.3	-	2
7β-OH-DHEA	400.3	402.3	430.3	-	2
7-keto-AO	361.3	363.3	430.3	-	2
7-keto-DHEA	359.3	361.3	517.4	-	2
16α-OH-DHEA	343.3	344.3	505.4	-	1
16α-OH-AO	345.3	346.3	507.4	-	1
16α-OH-EO	345.3	346.3	507.4	-	1

Quantification of non-labeled endogenous metabolites ([d_0-M^{endo}])

Calibration samples were prepared by adding 100 µl of methanolic standard solutions to 4 ml demineralized water samples. These samples were included in the cleanup procedure with the urine samples. Quantification of non-labeled endogenous steroids ([d_0-M^{endo}]) was performed using two calibration curves for each steroid.

One curve containing five data-points, covered respective ranges: AO (467-2333 ng), EO (469-2347 ng), T (20-100 ng), E (10-50 ng), DHEA (459-2293 ng), Δ5-AEDIOL (509-2547 ng), 16α-OH-DHEA (248-1240 ng), 7α-OH-DHEA (25-125 ng), 7β-OH-DHEA (75-375 ng), 16α-OH-AO (307-1533 ng), 16α-OH-EO (195-973 ng), 7-keto-AO (15-75 ng), 7-keto-DHEA (40-200 ng). The second calibration curve contained 6 data-points and covered the respective ranges: AO (2333-56000 ng), EO (2347-56320 ng), T (100-2400 ng), E (50-1200 ng), DHEA (2293-55040 ng), Δ5-AEDIOL (2547-61120 ng), 16α-OH-DHEA (1240-29760 ng), 7α-OH-DHEA (125-3000 ng), 7β-OH-DHEA (375-9008 ng), 16α-OH-AO (1533-36800 ng), 16α-OH-EO (973-23360 ng), 7-keto-AO (75-1808 ng), 7-keto-DHEA (200-4800 ng). 11α-OH-MeT (500

ng) was used as internal standard. Weighted regression analysis ($1/x^2$) was used for calculation of the response curves.

Quantification of labeled steroids

The labeled steroids [d_2-M^{exo}] were quantified in an indirect way, since synthetic labeled metabolites were not available and construction of calibration curves for the labeled steroids was impossible except for DHEA. The concentration of the labeled metabolites was established by quantification of the concentration of the non-labeled metabolites [d_0-M^{endo}] and recording the area ratio of the detected labeled metabolite (A(M+2)) vs. the non-labeled metabolite (A(M)). The concentration of labeled DHEA was calculated with equation (2).

$$\frac{A(M+2)}{A(M)} = 0.27 * \frac{[d_2 - M^{exo}]}{[d_0 - M^{endo}]} + 0.072 \qquad \textbf{(2)}$$

Equation (2) was deduced by applying the mean area ratio A(M+2)/A(M) determined in pre-administration samples (=0.072 for DHEA) and A(M+2)/A(M) in a standard of DHEA and d_2-DHEA (=0.57*([d_2-M^{exo}]/[d_0-M^{endo}]). The calculation was performed with the assumption that no proton-deuterium exchange occurred, so no non-labeled metabolites of exogenous origin were detected ([d_0-M^{exo}]=0). This was considered valid, as 2 deuterium atoms were present in the steroid structure of the metabolites, with the exception of the 16α-hydroxy-steroids. Metabolites 16α-OH-DHEA, 16α-OH-AO and 16α-OH-EO were therefore excluded from the quantification procedure. Equation (2) was corresponding to the results of Dehennin *et al.* [4]. Comparable equations were used for other metabolites, assuming that the isotope effect was constant for all metabolites. The mean area ratio A(M+2)/A(M) determined in pre-administration samples was specifically calculated for each metabolite.

Validation

Quality control (QC) samples have been prepared according the following procedure: A healthy male subject was administered two capsules of each 100 mg of DHEA. Three pooled urine samples were prepared: QC_L (collected during 16 hours before time of administration), QC_H (collected during 0-8 hours after time of administration) and QC_M (collected during 8-16 hours after time of administration). The QC samples were divided over sample tubes and stored with the other urine samples of the experiment at –20°C until time of analysis.

The intra-assay precision was below 10% for all parameters, except for 7α-OH-DHEA (QC_L) that was <15%. The respective inter-assay precisions were all <20% except for AO and EO (QC_H), 7-keto-AO (QC_L) and the 16α-OH-steroids that were <25%. A relatively high variability of AO and EO in QC_H was caused by column overloading. Due to efficiency reasons no re-analysis of AO and EO was performed after dilution.

The recovery was >95% for all steroid parameters except 16α-OH-DHEA (>90%). All steroid derivatives were stable at room temperature at least 70 hours, except 7-keto-DHEA that was stable at least 24 hours.

Previously, significant 3β-hydroxylase-Δ5-4-isomerase activity in Helix pomatia was reported, resulting in an artificial 20% T production from Δ5-AEDIOL [9]. Use of Helix pomatia for the analysis of a total fraction of non-conjugated steroids and their glucuronides and sulfates, has been validated in this experiment by checking side product formation, after incubation of reference steroids, for every used batch (incubation for 2 hours). No products from 3β-hydroxylase-Δ5-4-isomerase activity were detected. Artificial hydroxylation reactions were not significant (<1%).

Statistical analysis

The calculated $[d_2\text{-}M^{exo}]+[d_0\text{-}M^{endo}]$ values of post-administration samples compared to the mean and standard deviation values of pre-administration samples were tested for significance with Student's one tailed t-test, with $p<0.05$ considered as significant. Testing of differences in A(M+2)/A(M) values were also tested with Student's one tailed *t*-test, with $p<0.01$ considered as significant. Assumed normality of excretion rates was tested with the Kolmogorov test ($p<0.05$).

RESULTS

The relative effect of labeled DHEA administration to two male subjects was established by recording the area ratio A(M+n)/A(M) of the ions at m/z values described in Table 1. In Figures 1a and b the area ratio A(M+n)/A(M) is graphically presented as a function of time, before and after oral administration of labeled DHEA, respectively for non-oxygenated and oxygenated metabolites. Clear differences were observed in kinetic behavior. The response of most metabolites returned to baseline before 30 hours after time of administration. Exceptions were DHEA, Δ5-AEDIOL, EO, 5α,3α-ADIOL and 5β,3α-ADIOL that were still significantly elevated at 50 hours

after time of administration ($p<0.01$). Oxygenated metabolites all approached baseline level before 30 hours after time of administration.

Compounds that showed a large increase in A(M+n)/A(M) were DHEA, AO, EO, T, 5α,3α-ADIOL, 5β,3α-ADIOL, 7β-OH-DHEA, 7-keto-DHEA and 7-keto-AO. Metabolites showing a moderate to no response were Δ5-AEDIOL, E, 7α-OH-DHEA, 16α-OH-DHEA, 16α-OH-AO and 16α-OH-EO. Concentration of labeled 7-keto-AO was below limit of detection during the pre-administration period in subject 2 and during the complete collection period in subject 1. Labeled 7α-OH-DHEA was below limit of detection during the whole collection period in subject 2 and could only be established in some urine samples of subject 1.

The total excretion rate of labeled DHEA metabolites and the excretion rate of endogenous metabolites ($[d_2\text{-}M^{exo}]+[d_0\text{-}M^{endo}]$) is graphically presented as a function of time in Figures 2a and b. The metabolites 5α,3α-ADIOL, 5β,3α-ADIOL, 16α-OH-DHEA, 16α-OH-AO and 16α-OH-EO were not quantified. The effect of labeled DHEA administration is less pronounced than as presented in Figures 1a and b, as it is superposed on the bias caused by random biological variation and circadian rhythm in steroid excretion. Circadian rhythm is most clearly visible in case of DHEA and Δ5-AEDIOL. In Figure 3, the AO/EO and T/E ratios of the calculated $[d_2\text{-}M^{exo}]+[d_0\text{-}M^{endo}]$ values are presented as a function of time.

From the data of $[d_2\text{-}M^{exo}]$ the conversion of d_2-DHEA to the respective metabolites after 24 hours was calculated (see Tables 2 and 3). The conversion to oxygenated metabolites is relatively small compared to non-oxygenated metabolites. The conversion to the latter is, however, mostly contributed by the production of AO and EO.

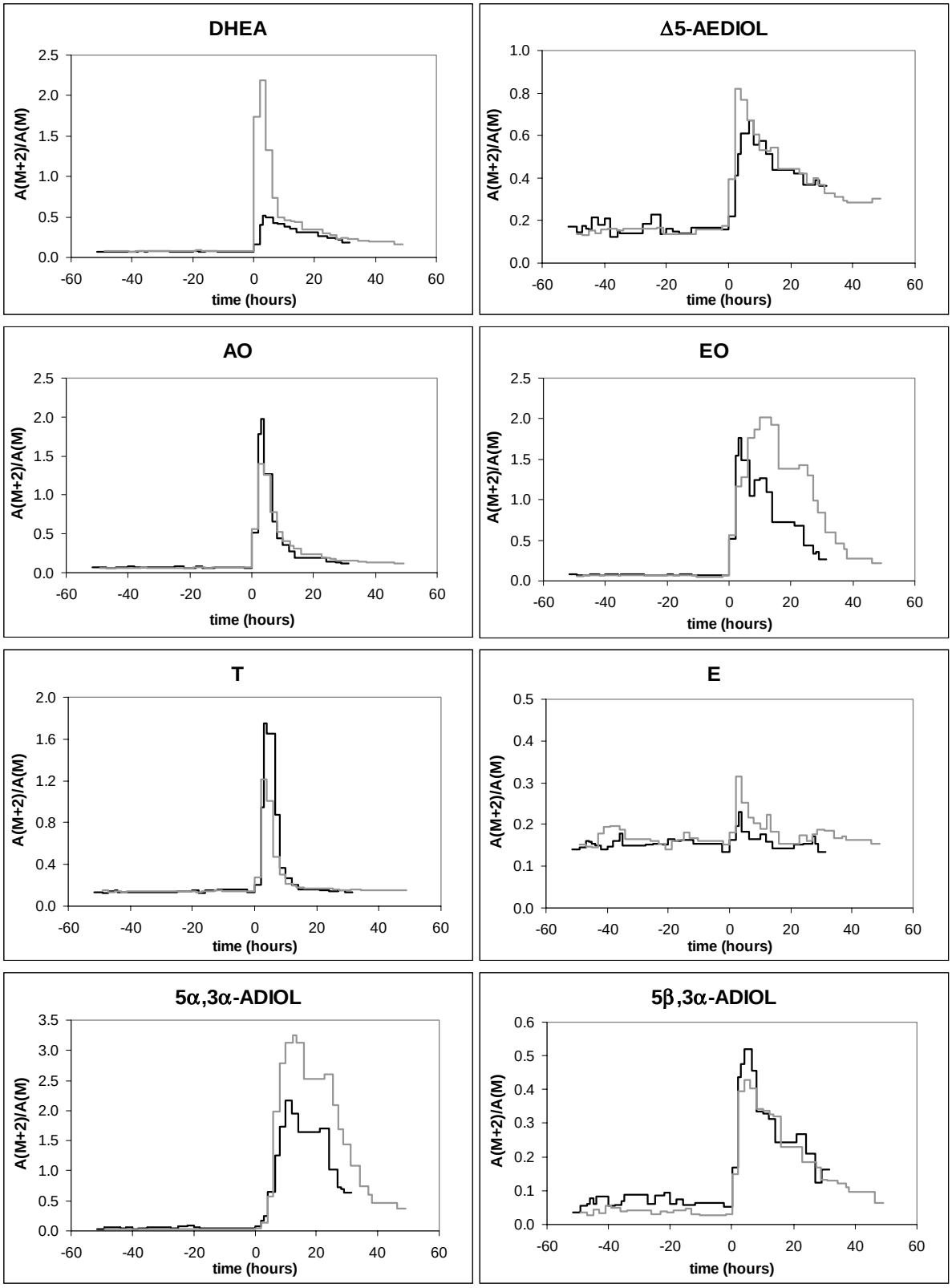


Figure 1a: The area ratio A(M+n)/A(M) of labeled vs. non-labeled DHEA or non-oxygenated metabolites of DHEA as a function of time, in subject 1 (▬) and subject 2 (▬). d_2-DHEA was administered at time-point 0 hours.

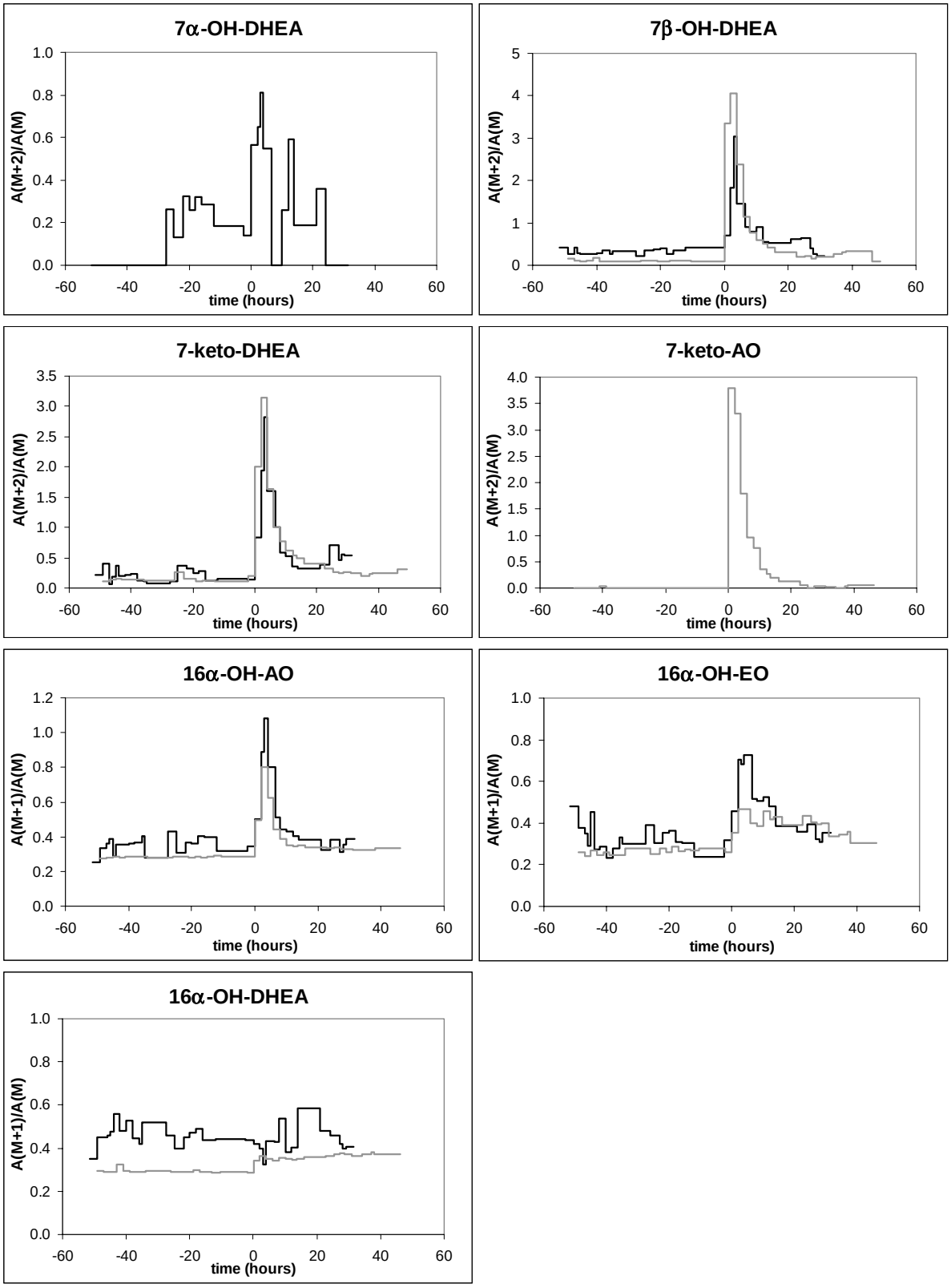


Figure 1b: The area ratio A(M+n)/A(M) of labeled vs. non-labeled DHEA or oxygenated metabolites of DHEA as a function of time, in subject 1 (▬) and subject 2 (▬).

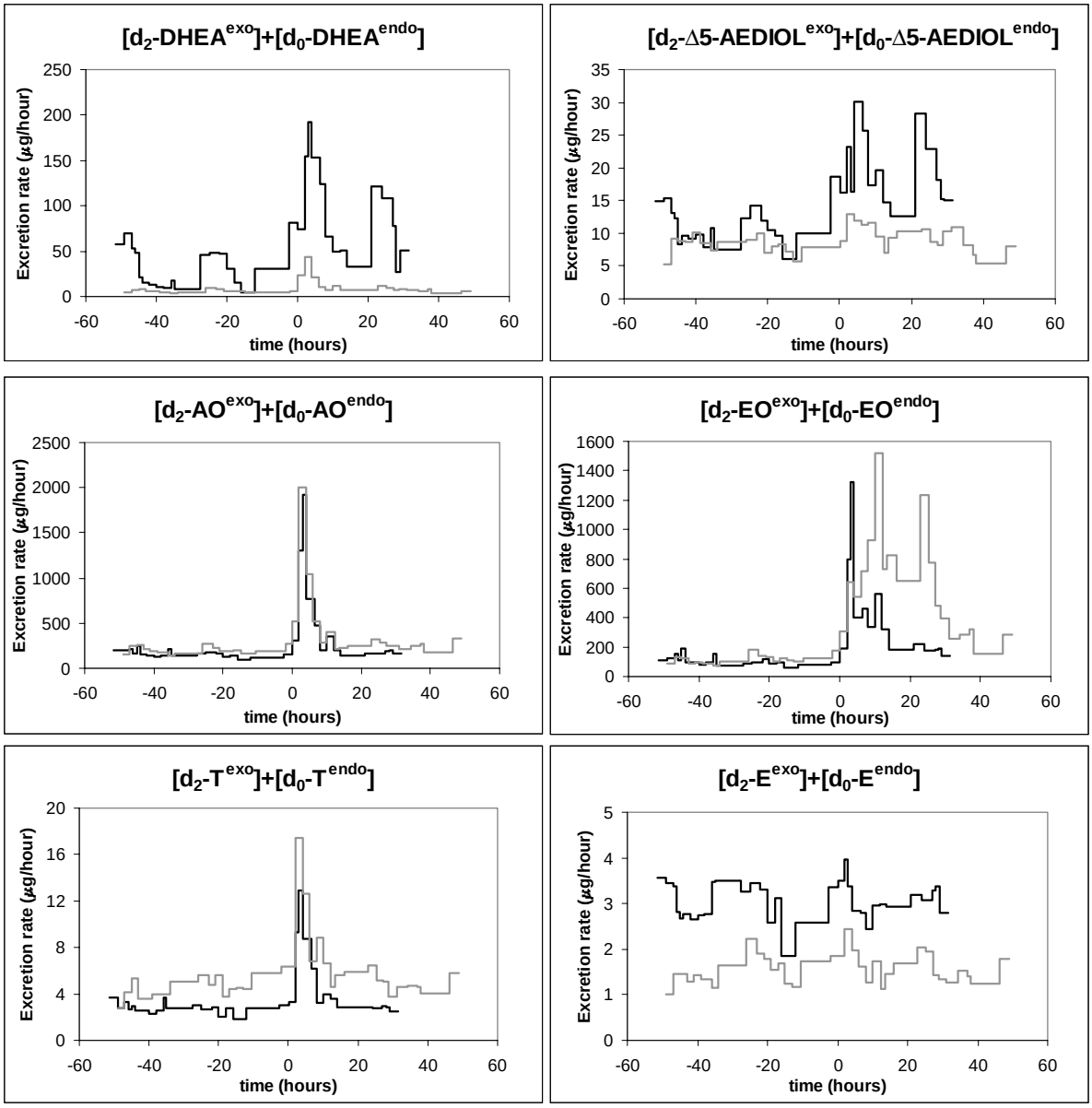
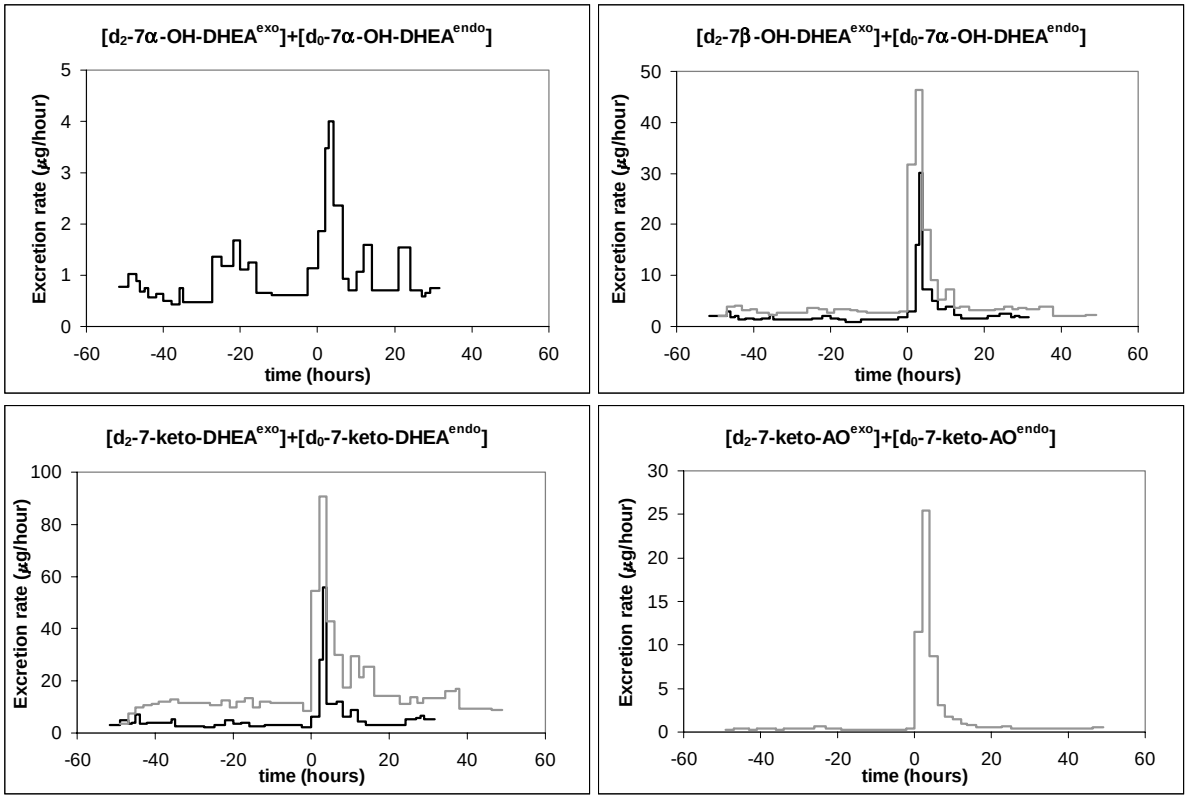


Figure 2a: Excretion rates of the combined labeled [d_2-M^{exo}] and non-labeled [d_0-M^{endo}] DHEA and non-oxygenated metabolites of DHEA as a function of time, in subject 1 (▬) and subject 2 (▬). d_2-DHEA was administered at time-point 0 hours.



Figuur 2b: Excretion rates of the combined labeled [d_2-M^{exo}] and non-labeled [d_0-M^{endo}] oxygenated metabolites of DHEA as a function of time, in subject 1 (▬) and subject 2 (▬).

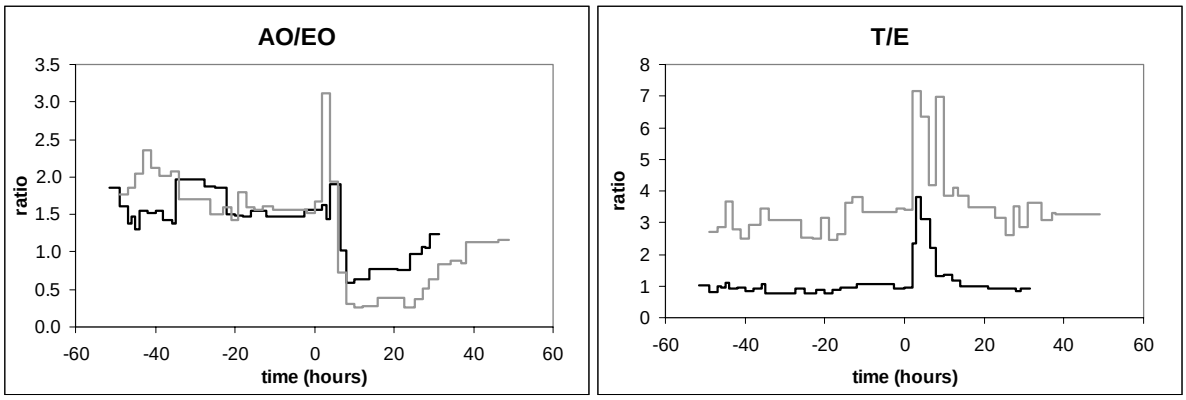


Figure 3: Ratios of metabolite concentrations calculated as [d_2-M^{exo}]+[d_0-M^{endo}], as a function of time, in subject 1 (▬) and subject 2 (▬). d_2-DHEA was administered at time-point 0 hours.

Table 2: *Conversion (%) of DHEA to the respective metabolites after 24 hours. N.D. = not determined.*

Metabolite (M)	Subject 1 (25.5 mg)	Subject 2 (52.5 mg)
DHEA	2.6	0.36
Δ5-AEDIOL	0.48	0.15
AO	19.9	12.0
EO	21.5	26.1
T	0.16	0.10
E	< 0.01	< 0.01
7α-OH-DHEA	0.03	N.D.
7β-OH-DHEA	0.34	0.45
7-keto-DHEA	0.46	0.72
7-keto-AO	N.D.	0.15

Table 3: *Conversion (%) of DHEA to different groups of metabolites after 24 hours.*

Group of metabolites	Subject 1 (25.5 mg)	Subject 2 (52.5 mg)
AO and EO	41.4	38.1
non-oxygenated	42.0	38.4
oxygenated	0.83	1.3
Total DHEA conversion	42.8	39.7
Total recovery DHEA + metabolites	45.4	40.0

DISCUSSION

Excretion kinetics

In this study the kinetics of DHEA and several of its metabolites were investigated. The excretion behavior of the sum of conjugated and non-conjugated DHEA and several of its metabolites as described in Figures 1a and b differ fundamentally. Roughly, the observed patterns can be divided in three classes:

1. *No change in excretion.* No or little production of the metabolite takes place after administration of d_2-DHEA, as accounts for E, 7α-OH-DHEA and 16α-OH-DHEA.
2. *Rapid and short increase of excretion*. Within 10 hours after administration of d_2-DHEA the respective compound is cleared in high speed, leading to a relatively fast return to baseline values, as accounts for AO, T, 7β-OH-DHEA, 7-keto-DHEA, 7-keto-AO, 16α-OH-AO and 16α-OH-EO.

3. *Rapid and prolonged increase of excretion*. Within 10 hours after administration of d_2-DHEA the excretion of the respective compound increases quickly and returns slowly to baseline values, as accounts for DHEA, EO, AEDIOL, 5α,3α-ADIOL and 5β,3α-ADIOL.

The differences between the kinetic patterns can be explained by the extensive first and second phase metabolic steps of DHEA. Our results show that orally administered DHEA is almost completely converted into metabolites. Through extensive first-pass metabolism, oral administration of DHEA will predominantly lead to production of DHEA sulfate (DHEA-S) [10]. In blood DHEA-S is mainly bound to albumin, in contrast to DHEA [11]. This results in much lower clearance rates for DHEA-S (13 L/day) compared to DHEA (2,000 L/day) and justifies our observation of prolonged excretion of small amounts of DHEA [12]. Conversion of DHEA to first phase metabolites followed by rapid sulfation and/or conversion of DHEA-S to other sulfated metabolites and their subsequent binding to albumin, could explain the prolonged excretion of certain metabolites [13-17]. Those metabolites that will be conjugated with glucuronic acid, are not bound to serum proteins and thus will be excreted rapidly. That could justify the rapid and short excretion of other metabolites.

Our data show a more prolonged excretion of EO than of AO, which indicates that a relation exists between EO and DHEA. It could suggest a relatively higher production of the sulfate of EO compared to that of AO. This presumption is however not supported by earlier results of Rosenfeld *et al.* [18] and Dehennin *et al.* [4] who performed separate analyses of glucuronides and sulfates after DHEA administration. Other possibilities are a rapid and stereoisomer specific conversion of relatively long circulating DHEA and an enterohepatic circulation of EO-glucuronide, resulting in lower clearance efficiency [19].

Sensitivity and specificity

When a parameter is selected as marker for substance abuse, the demands of sensitivity and selectivity should be fulfilled. In this respect, an evaluation of classical parameters for the detection of the abuse of endogenous steroids such as the ratios between AO and EO and between T and E, respectively, is of interest. Both ratios are known to have a limited specificity to establish administration of T, DHEA or Δ4-AEDIONE [1-5,8]. However, in terms of sensitivity they could be of use. In this investigation, the AO/EO ratio initially showed a small increase followed by a significant decrease below baseline level for at least 30-50 hours after DHEA administration (Figure 3). The T/E ratio was increased significantly during 10 hours and was therefore only of use for a shorter period than the AO/EO ratio. These characteristics have also been reported by Kazlauskas *et al*. [3].

The specificity and sensitivity of metabolites as parameter can be interpreted from Figures 1 and 2. The difference between the post-administration A(M+2)/A(M) ratio and the mean pre-administration ratio in Figure 1, is a measure of relative response towards DHEA administration.

Responding parameters, besides DHEA itself, in terms of sensitivity were the metabolites AO, EO, T, 5α3α-ADIOL, 5β,3α-ADIOL, Δ5-AEDIOL, 7β-OH-DHEA, 7-keto-DHEA and 7-keto-AO. Because of the high biological variation due to a high amplitude circadian rhythm, the response of DHEA and Δ5-AEDIOL resulted in only a limited sensitivity (Figure 2). 7-Keto-AO showed a large response in one subject, but could not be detected before and after administration in the other subject. The most sensitive parameters were therefore, AO, EO, T and the 7-oxygenated metabolites 7β-OH-DHEA and 7-keto-DHEA. No conclusions could be drawn about 5α,3α-ADIOL and 5β,3α-ADIOL as those were not quantified in this study.

Interpretation of the 16α-OH metabolites was not possible in these experiments as one deuterium was metabolically removed from the steroid structure due to the hydroxylation. A significant isotope effect due to deuterium-proton exchange can not be disregarded ($[d_2\text{-}M^{endo}]\neq 0$) for these metabolites. Equation (2) is therefore not applicable for 16α-OH-metabolites and have been excluded from further interpretation. Furthermore, significant physiological isotope effects have been reported in case of deuterium labeled substance administration [20]. Due to these physiological isotope effects the rate of metabolic deuterium removal is expected to be lower than the rate of equivalent proton removal, leading to a limitation of 16α-hydroxylation in this particular case.

In terms of specificity it could be argued that the non-oxygenated metabolites are not of interest, because they originate from various steroids. The oxygenated metabolites 7-keto-AO, 16α-OH-AO and 16β-OH-EO seem to be specific as they are reduced metabolites of 7-keto-DHEA and 16α-OH-DHEA. However, they could also be oxygenated metabolites of AO and EO. This is partly illustrated by 16α-hydroxylation of AO by human liver microsomes, as performed by Einarsson *et al.* [21]. The metabolites 7-keto-DHEA, 7α-OH-DHEA, 7β-OH-DHEA and 16α-OH-DHEA can theoretically be expected to be the most specific metabolites as they are directly converted from DHEA [21-25]. Moreover, as the double bond is retained at the Δ5-position in the steroid structure during metabolic conversion, a high sensitivity for DHEA and a low sensitivity towards Δ4-steroids as Δ4-AEDIONE and T can be expected. Based on this investigation at least the 7-oxygenated metabolites appear promising enough to study aspects of specificity in more detail.

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