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PROFILING SPECIFIC METABOLITES OF DEUTERIUM LABELED ANDROST-4-ENE-3,17-DIONE

ABSTRACT

Androst-4-ene-3,17-dione (Δ4-AEDIONE) is a steroid that is easily obtainable as food supplement product. Because of assumed anabolic properties, the International Olympic Committee has placed Δ4-AEDIONE on the list of forbidden substances. So far, no techniques have become available to detect Δ4-AEDIONE abuse in a selective way. In this study, a preliminary investigation is performed to construct a Δ4-AEDIONE selective profile of non-oxygenated metabolites (dehydroepiandrosterone, androsterone, etiocholanolone, testosterone, epitestosterone, androst-5-ene-3β,17β-diol, 5α-androst-3α,17β-diol and 5β-androstan-3α-17β-diol) and oxygenated metabolites (6α/β-hydroxy-Δ4-AEDIONE, 4-hydroxy-Δ4-AEDIONE, 16α-hydroxy-Δ4-AEDIONE, 6-keto-Δ4-AEDIONE, 6α/β-hydroxy-testosterone, 16α-hydroxy-androsterone, 16α-hydroxy-etiocholanolone, 11β-hydroxy-androsterone and 11β-hydroxy-etiocholanolone).

Deuterium labeled Δ4-AEDIONE ([2,2,4,6,6,16,16-$^{7}H_2$]-androst-4-ene-3,17-dione) was administered to 2 healthy male volunteers (25.0 and 52.0 mg, respectively) and urine samples were collected. Sample analysis was performed of 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, 6α/β-hydroxy-Δ4-AEDIONE, 4-hydroxy-Δ4-AEDIONE and 6α/β-hydroxy-testosterone. Differences in excretion kinetics were observed between the studied metabolites. The total conversion of Δ4-AEDIONE to the studied metabolites was 39.3-42.1% after 48 hours. The conversion to oxygenated and non-oxygenated metabolites was 0.24-0.90% and 39.1-41.2%, respectively. Although the conversion to oxygenated metabolites was limited, the sensitivity and expected specificity for Δ4-AEDIONE detection of the corresponding metabolites make these potential parameters for possible use in doping analysis applications.

INTRODUCTION

The introduction of steroid containing food supplements on an easily accessible market had a great impact on doping analysis and the doping control system. Androst-4-ene-3,17-dione (Δ4-AEDIONE) was one of the first supplement steroids that was commercially available for athletes. The International Olympic Committee (IOC) placed Δ4-AEDIONE on the List of Forbidden Substances in 1997. The steroids that appeared on the food supplement market extended to androst-5-ene-3β,17β-diol, androst-4-ene-3β,17β-diol and nor-steroids 19-nor-androst-4-ene-3,17-dione and 19-nor-androst-4-ene-3β,17β-diol [1,2].

Non-specific metabolism, extensive first-pass metabolism and fast renal and metabolic clearance aggravate the analysis of orally administered supplement steroids. Analytical procedures for detecting Δ4-AEDIONE have mostly been limited to straightforward steroid profiling techniques, as originally developed for other endogenous steroids as testosterone (T), epitestosterone (E) [3,4] and 5α-dihydrotestosterone [5,6] in urine samples. A typical steroid profile that is analyzed in doping analysis includes, besides the mentioned mother compounds, metabolites as androsterone (AO), etiocholanolone (EO) and androstanediol epimers. This approach leads to mostly unspecific and therefore insufficiently conclusive information about the identity of the administered steroid.

In this study, a preliminary investigation is performed to construct a more steroid specific profile of selected oxygenated and/or non-oxygenated metabolites of Δ4-AEDIONE. Therefore, [2,2,4,6,6,16,16-$^{7}H_2$]-androst-4-ene-3,17-dione (d_7-Δ4-AEDIONE) 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), 5α-androstan-3α,11β-diol-17-one (11β-hydroxy-androsterone, 11β-OH-AO), 5β-androstan-3α,11β-diol-17-one (11β-hydroxy-

etiocholanolone, 11β-OH-EO) 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) was obtained through courtesy of Organon, Oss, The Netherlands. Androst-4-en-6α-ol-7,17-dione (6α-hydroxy-androstenedione, 6α-OH-Δ4-AEDIONE), androst-4-en-6β-ol-7,17-dione (6β-hydroxy-androstenedione, 6β-OH-Δ4-AEDIONE), androst-4-ene-6,7,17-trione (6-keto-androstenedione, 6-keto-Δ4-AEDIONE), androst-4-en-6β,17β-ol-7-one (6β-hydroxy-testosterone, 6β-OH-T), androst-4-en-4-ol-3,17-dione (4-hydroxy-androstenedione, 4-OH-Δ4-AEDIONE), androst-4-en-16α-ol-3,17-dione (16α-hydroxy-androstenedione, 16α-OH-Δ4-AEDIONE), 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. [2,2,4,6,6,16,16-$^{7}H_2$]-Androst-4-ene-3,17-dione (d_7-Δ4-AEDIONE, isotopic purity 98.4%) was obtained from C/D/N Isotopes, Pointe-Claire, Quebec, Canada.

Chemicals: *N*-Methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA), ethanethiol (97%) and phosphorous pentoxide 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. 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, St. Louis, Missouri, USA.

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. d_7-Δ4-AEDIONE was orally administered (subject 1, 25.0 mg; subject 2, 52.0 mg). For each collected urine sample the exact void time, volume and specific gravity (Urine Specific Gravity Refractometer, Atago, Japan) was recorded. Samples were stored at -20°C until time of analysis.

Sample preparation

The volume of urine 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. 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 equilibrated with 2 ml of demineralized water. Inorganic material was removed from the column by washing with 2 ml of water. The remaining compounds were 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 addition 100µl of Helix pomatia followed by incubation for 1 hour at 55°C. Steroids were isolated from the buffer solution by extraction with 5 ml of diethyl ether twice. After extraction the phase separation was optimized by centrifugation (4000 rpm, 5 min). The organic layer was removed and evaporated to dryness under a stream of nitrogen at 40°C. The extract was dried overnight over P_2O_5/KOH under reduced pressure. Derivatization was performed by incubation of the extract in 100 µl of MSTFA/NH_4I/ethanethiol (1000:2:3; v/w/v) at 80°C for 30 min.

Gas chromatography and mass spectrometry

Selected ion monitoring (SIM) 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 ionization mode at 70 eV. Analysis was performed by SIM analysis of specific ions representing the respective deuterated and non-deuterated metabolites (see Table 1). The number of deuterium atoms in Table 1 was based on maximum response after screening of all possible m/z values from $[M]^{\bullet+}$ to $[M+7]^{\bullet+}$. A check for possibly incomplete derivatization 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 at 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_7-Δ4-AEDIONE was checked by gas chromatography-mass spectrometry (GC-MS) in full scan mode prior to administration. No impurities could be detected in a concentrated sample.

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

Calibration samples were prepared by adding 100 µl of methanolic standard solutions to samples of 4 ml of demineralized water. 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: Δ4-AEDIONE (10-50 ng), AO (645-3227 ng), EO (629-3147 ng), T (80-400 ng), E (20-100 ng), DHEA (493-2467 ng), Δ5-AEDIOL (432-2160 ng), 16α-OH-Δ4-AEDIONE (25-125 ng), 6α-OH-Δ4-AEDIONE (10-50 ng), 6β-OH-Δ4-AEDIONE (10-50 ng), 6β-OH-T (12-60 ng), 16α-OH-AO (347-1733 ng), 16α-OH-EO (339-1016 ng), 6-keto-Δ4-AEDIONE (20-100 ng). The second calibration curve contained 6 data-points and covered the respective ranges: Δ4-AEDIONE (50-960 ng), AO (3227-61951 ng), EO (3147-60415 ng), T (400-7680 ng), E (100-1,920 ng), DHEA (2467-47360 ng), Δ5-AEDIOL (2160-41472 ng), 16α-OH-Δ4-AEDIONE (125-2400 ng), 6α-OH-Δ4-AEDIONE (50-960 ng), 6β-OH-Δ4-AEDION (50-960 ng), 6β-OH-T (60-1152 ng), 16α-OH-AO (1733-33280 ng), 16α-OH-EO (1016-32512 ng), 6-keto-Δ4-AEDIONE (100-1920 ng). 11α-OH-MeT (500 ng) was used as internal standard. Weighted regression analysis ($1/x^2$) was used for calculation of the calibration curve.

As described in Chapter 8, androst-4-ene-3-one steroids that are hydroxylated on C^6 lose the stereochemical integrity at this position after derivatization under enolizing conditions [7,8]. This resulted in the conversion of 6α-OH-Δ4-AEDIONE and 6β-OH-Δ4-AEDIONE to identical 3,5,16-triene-3,6,17-triol TMS products (6α/β-OH-AEDIONE). This was also the case for 6α-OH-T and 6β-OH-T (6α/β-OH-T). The applied range of quantification for 6α/β-OH-Δ4-AEDIONE was therefore 20-100 ng and 100-1920 ng, respectively for the used calibration curves.

Table 1: *Monitored m/z values of labeled and non-labeled derivatives of Δ4-AEDIONE and metabolites.*

Steroid derivative	m/z value non-labeled	m/z value labeled	number of deuterium atoms in derivative (n)
Δ4-AEDIONE	430.3	435.3	5
AO	434.3	440.3	6
EO	434.3	440.3	6
DHEA	432.3	437.3	5
Δ5-AEDIOL	434.3	440.3	6
T	432.3	438.3	6
E	432.3	438.3	6
5α,3α-ADIOL	241.2	246.2	5
5α,3β-ADIOL	241.2	246.2	5
6α/β-OH-Δ4-AEDIONE	518.4	522.4	4
4-OH-Δ4-AEDIONE	518.4	522.4	4
16α-OH-Δ4-AEDIONE	503.4	507.4	4
6-keto-Δ4-AEDIONE	516.4	520.4	4
16α-OH-AO	507.4	512.4	5
16α-OH-EO	507.4	512.4	5
6α/β-OH-T	520.4	525.4	5
11β-OH-AO	522.4	528.4	6
11β-OH-EO	522.4	528.4	6

Quantification of labeled steroids

The concentration of labeled steroids [d_n-M^{exo}] that were excreted could only be quantified in an indirect way, since synthetic labeled metabolites were not available. The values of [d_n-M^{exo}] were established by calculation of the concentration of unlabeled metabolites [d_0-M^{endo}], and the area ratio of the detected labeled metabolites (A(M+n)) vs. the respective non-labeled metabolites (A(M)). [d_0-M^{endo}] was established by calibration as described above. Calculation of [d_n-M^{exo}] was performed with equation (2) (see also Dehennin *et al.* [9]).

$$R = \frac{A(M+n)}{A(M)} = 0.795 * \frac{[d_n - M^{exo}]}{[d_0 - M^{endo}]} + R^0 \qquad \textbf{(2)}$$

Equation (2) was deduced by applying the mean area ratio A(M+2)/A(M), as determined in pre-administration samples (=R^0) and A(M+n)/A(M) in standard of Δ4-AEDIONE and d_7-Δ4-AEDIONE (=0.795*([d_n-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 4-6 deuterium atoms were present in the steroid structure of the metabolites.

R^0 was equal to zero for most metabolites, as [d_n-M^{endo}] was negligible. Due to insufficient specificity of the applied GC-MS method, $R^0>0$ in case of Δ4-AEDIONE, DHEA, E, 16α-OH-Δ4-AEDIONE and 6α/β-OH-Δ4-AEDIONE.

Validation

Quality control (QC) samples were prepared according the following procedure: A healthy male subject was administered two capsules of each 100 mg of Δ4-AEDIONE. 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 16α-OH-AO (QC_H) and 6α/β-OH-T (QC_L and QC_M) were <15%. Δ4-AEDIONE (all QC samples) was <20% and 6-keto-Δ4-AEDIONE (QC_M) was <40%. The respective inter-assay precisions were <20 %; except for AO and EO (QC_H), 4-OH-Δ4-AEDIONE and 6α/β-OH-Δ4-AEDIONE (QC_L) and 16α-OH-steroids that were <25%; Δ4-AEDIONE and 6-keto-Δ4-AEDIONE (QC_L and QC_M) were <35%. Based on these results the decision was made to exclude Δ4-AEDIONE and 6-keto-Δ4-AEDIONE from the quantification procedure. The relatively high variability of AO and EO in QC_H was caused by column overloading.

The recovery was >95% for all steroid parameters. All steroid derivatives were stable at room temperature for at least 70 hours. As also described in Chapter 4, the significance of side-reactions occurring in the Helix pomatia incubation mixture was tested. No products from 3β-hydroxylase-Δ5-4-isomerase activity were detected. Artificial hydroxylation reactions were not significant (<1%).

Statistical analysis

The calculated [d_n-M^{exo}]+[d_0-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. Assumed normality of excretion rates was tested with the Kolmogorov test ($p<0.05$).

RESULTS

In Figure 1 the recorded A(M+n)/A(M) ratio of Δ4-AEDIONE is shown as a function of time for both subjects. In blank urine samples only low concentrations of Δ4-AEDIONE were detected. Also, after administration minor quantities of d_7-Δ4-AEDIONE were recovered.

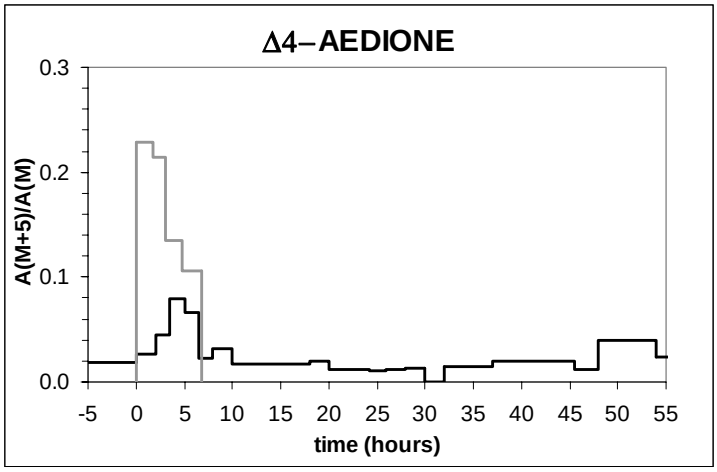


Figure 1: The area ratio A(M+n)/A(M) of labeled vs. non-labeled Δ4-AEDIONE as a function of time, in subject 1 (—) and subject 2 (—). d_7-Δ4-AEDIONE was administered at time-point 0 hours.

In Figures 2a and b the recorded A(M+n)/A(M) ratio is shown as a function of time for the non-oxygenated and oxygenated metabolites. In these results [d_0-6-keto-Δ4-AEDIONE] was below the limit of detection in both subjects, and was therefore excluded. Due to chromatographic co-elution of 5α,3α-ADIOL and 5β,3α-ADIOL could only be determined in samples of one subject.

Excretion of the labeled steroids returned to baseline within 20 hours, except for 5α,3α-ADIOL, 5β,3α-ADIOL, Δ5-AEDIOL and EO that were increased for more than 30 hours after time of administration. The maximum response of AO was around 10% higher than that of EO, but between 5-35 hours after time of administration the response of EO was higher. The parameters that showed the largest response in A(M+n)/A(M) were AO, EO, T, 6α/β-OH-Δ4-AEDIONE, 6α/β-OH-T and 4-OH-Δ4-AEDIONE.

In Figures 3a and b the total excretion rate of labeled and endogenous metabolites ([d_n-M^{exo}]+[d_0-M^{endo}]) is presented as a function of time for both subjects. The parameters Δ4-AEDIONE, 6-keto-Δ4-AEDIONE, 5α,3α-ADIOL, 5β,3α-ADIOL, 11β-OH-AO and 11β-OH-EO were not included in the quantification procedure. As the excretion of labeled Δ4-ADIONE metabolites is superposed on the biological variation of non-labeled steroid excretion, the presented response in Figures 3a and b is less pronounced as presented in Figures 2a and b. The excretion rate of all metabolites was back to baseline level at around 10 hours after administration. The metabolites that show the most significant effect were AO, EO, T, 6α/β-OH-Δ4-AEDIONE, 4-OH-Δ4-AEDIONE and 6α/β-OH-T.

The calculated AO/EO and T/E ratio based on [d_n-M^{exo}]+[d_0-M^{endo}] values are shown in Figure 4. In subject 1 the AO/EO showed an increase during the first 10 hours with a maximum of 40%, followed by a decrease (40% maximum) until 25-30 hours after time of administration. In subject 2 the AO/EO ratio was only decreased (70% maximum decrease) during 25-30 hours after time of administration. For subject 1 and 2, the T/E ratio was increased from 0.9 to 2.4 (170%) and 2.5 to 14.2 (470%), respectively.

In Tables 2 and 3 an outline is given of the calculated conversion of Δ4-AEDIONE to the respective metabolites after 24 and 48 hours, as calculated by the total excreted amount for each metabolite vs. the applied dose. For the major part, Δ4-AEDIONE was converted to AO and EO. Only 1-2 % was converted to other studied metabolites. Conversion to oxygenated metabolites was 0.2-0.9%. The total recovery of Δ4-AEDIONE that was converted after 48 hours was 39-42%. Of the total conversion, 90-95% occurred within the first 24 hours after administration.

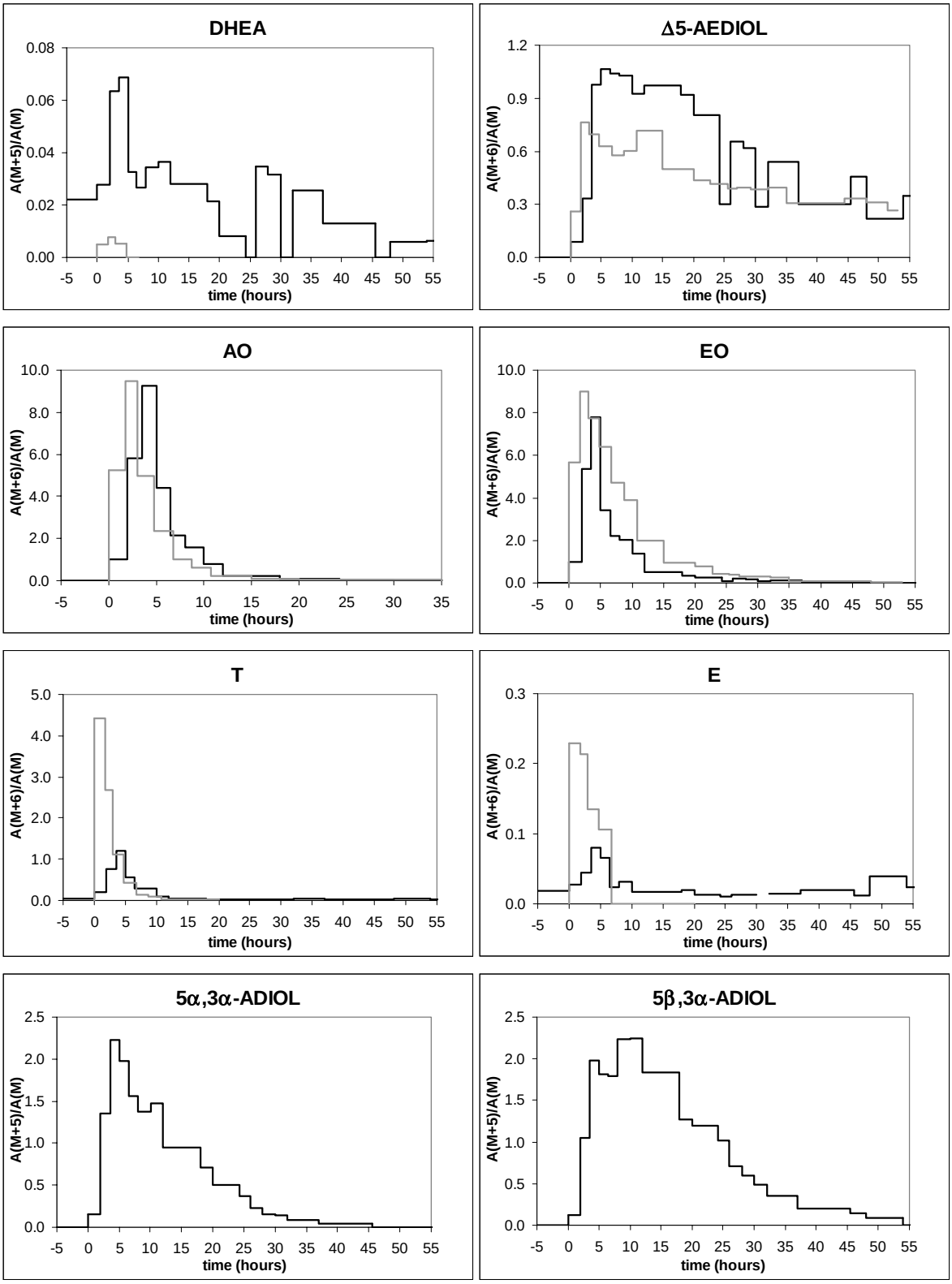


Figure 2a: The area ratio A(M+n)/A(M) of labeled vs. non-labeled, non-oxygenated metabolites of Δ4-AEDIONE as a function of time, in subject 1 (▬) and subject 2 (▬). d_7-Δ4-AEDIONE was administered at time-point 0 hours.

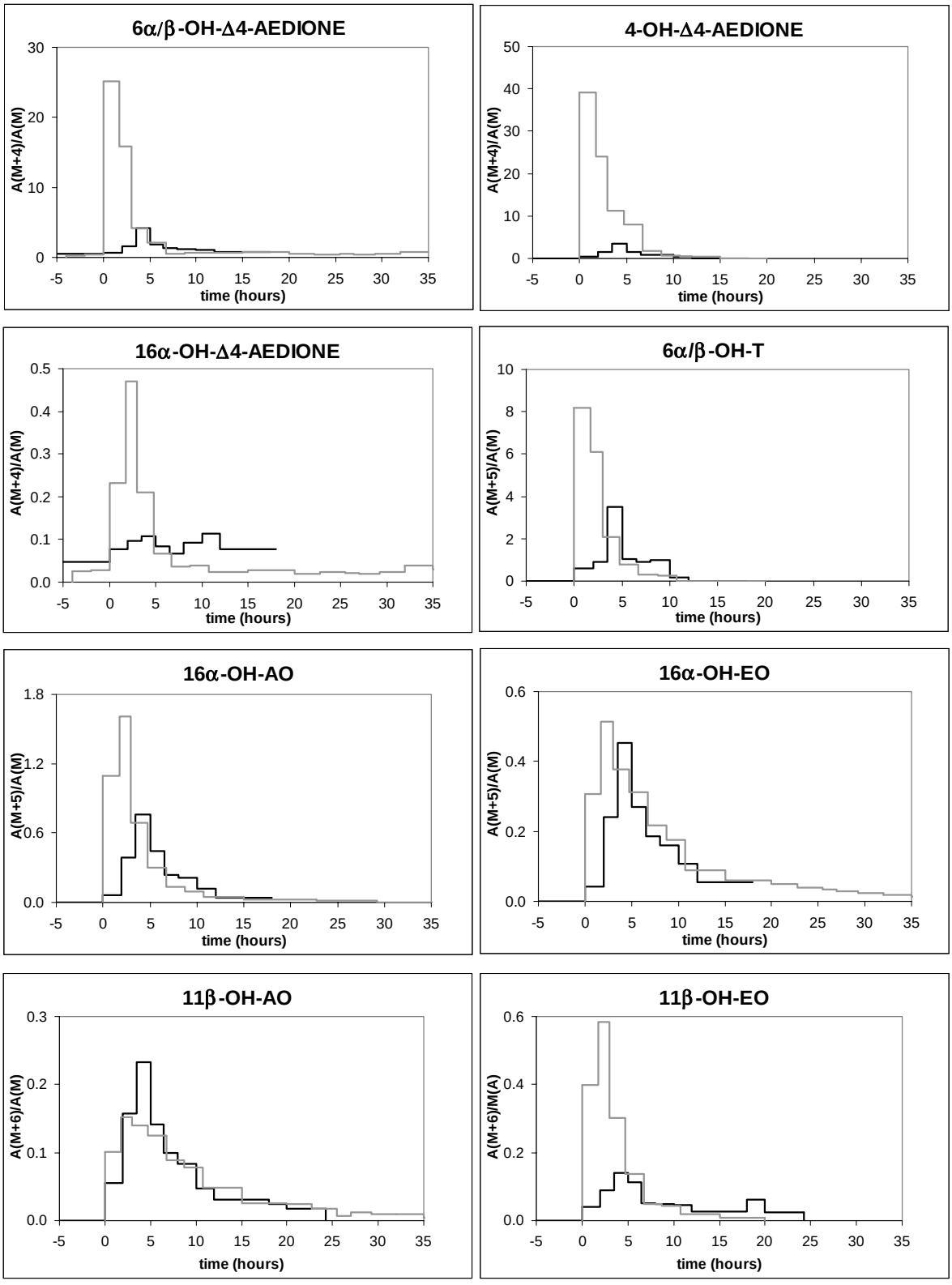


Figure 2b: The area ratio A(M+n)/A(M) of labeled vs. non-labeled, oxygenated metabolites of Δ4-AEDIONE as a function of time, in subject 1 (——) and subject 2 (——).

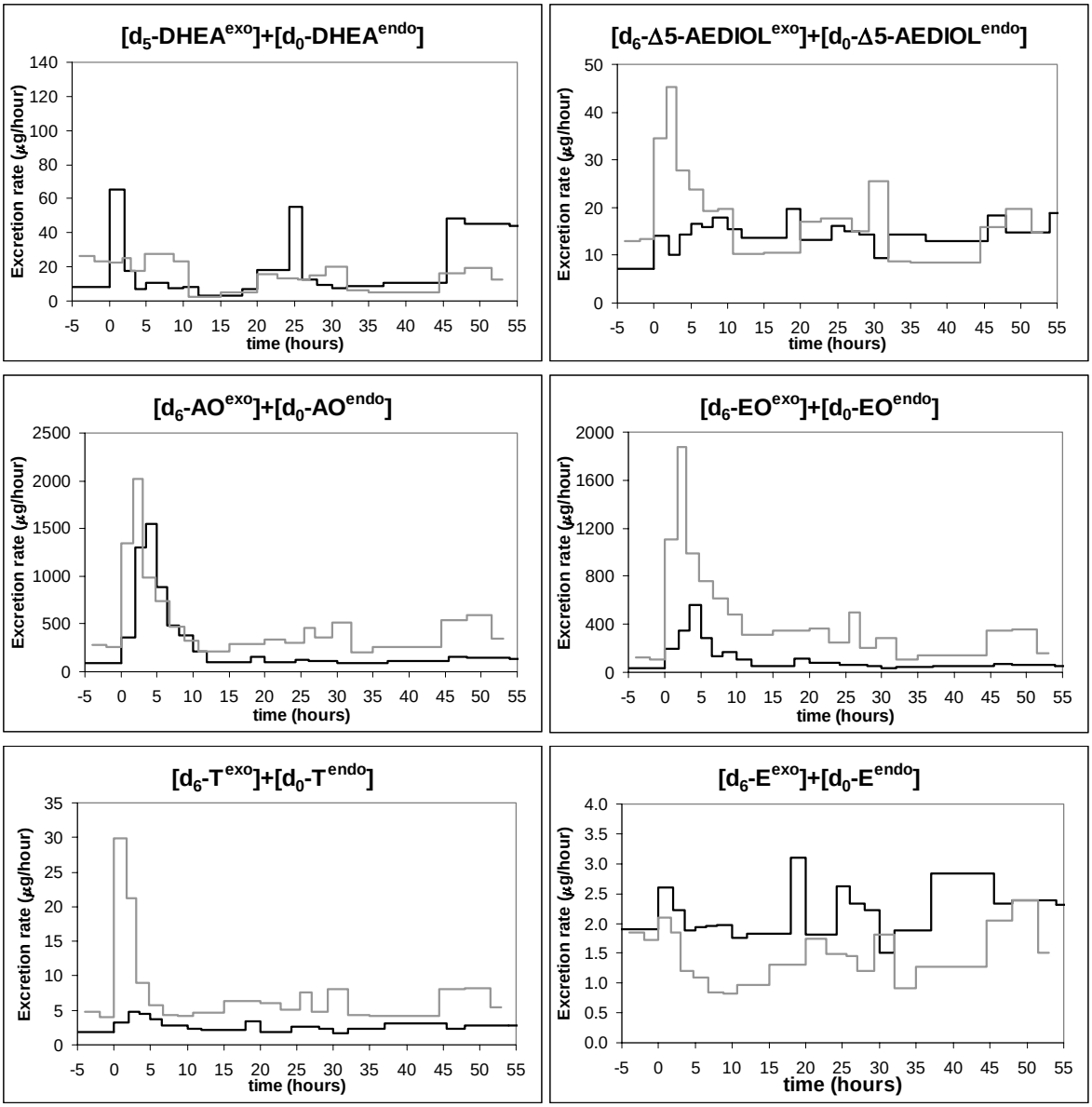
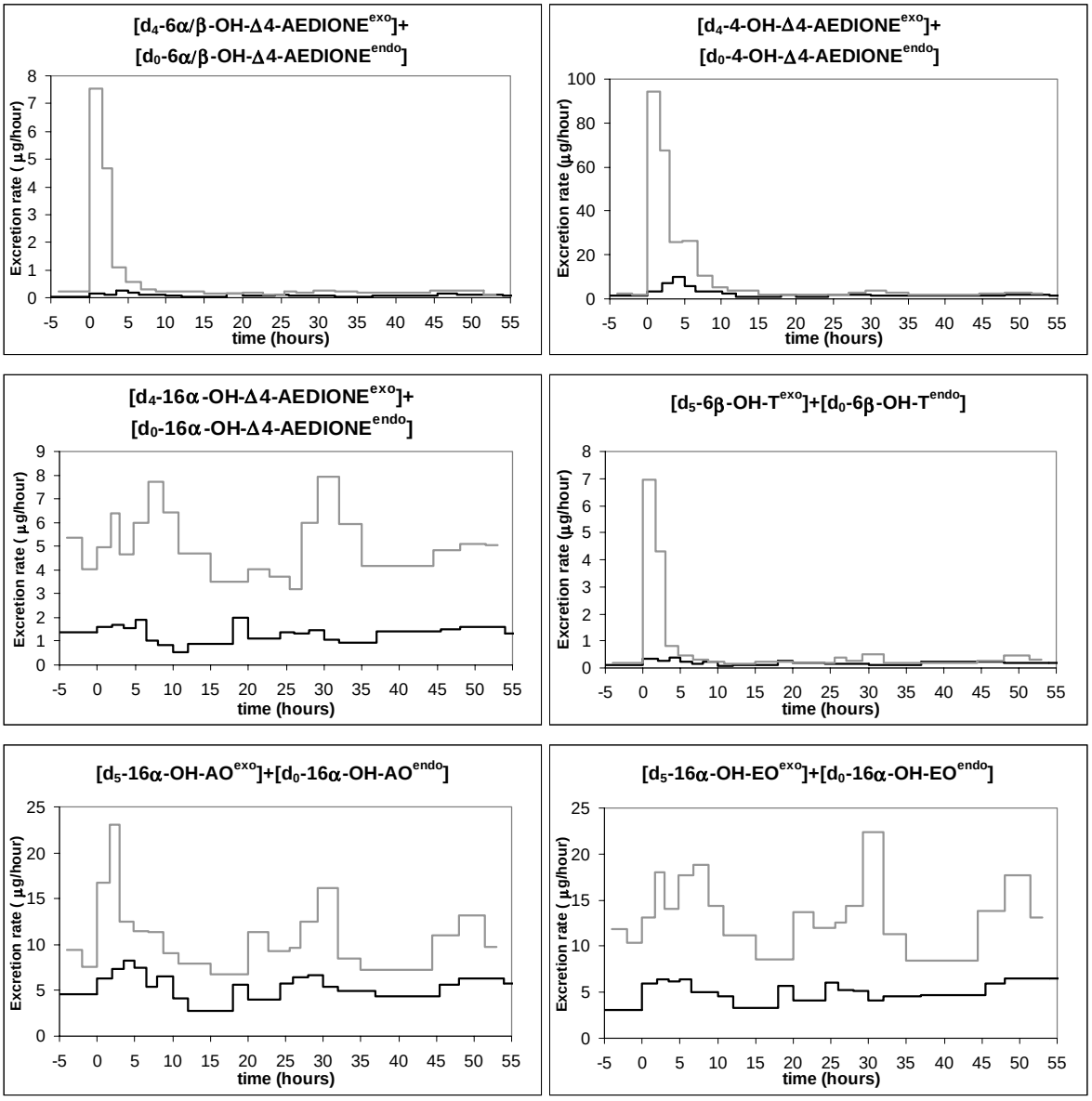


Figure 3a: Excretion rates of the combined labeled [d_n-M^{exo}] and non-labeled [d_0-M^{endo}], non-oxygenated metabolites of Δ4-AEDIONE as a function of time, in subject 1 (——) and subject 2 (——). d_7-Δ4-AEDIONE was administered at time-point 0 hours.



Figuur 3b: Excretion rates of the combined labeled [d_n-M^{exo}] and non-labeled [d_0-M^{endo}], non-oxygenated metabolites of Δ4-AEDIONE as a function of time, in subject 1 (▬) and subject 2 (▬).

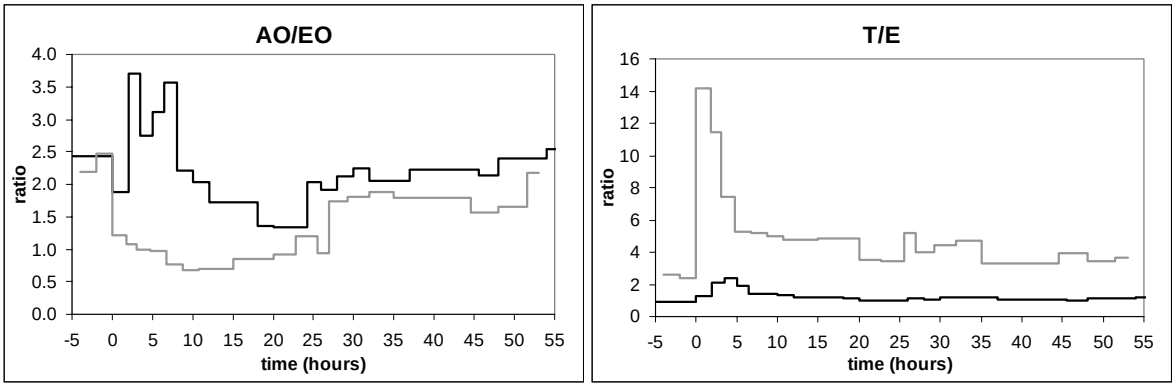


Figure 3: Ratio's of metabolite concentrations calculated as $[d_n\text{-}M^{exo}]+[d_0\text{-}M^{endo}]$, as a function of time, in subject 1 (▬) and subject 2 (▬). d_7-Δ4-AEDIONE was administered at time-point 0 hours.

Table 2: *Conversion (%) of Δ4-AEDIONE to the respective metabolites after 24 hours.*

Metabolite (M)	Subject 1 25.0 mg) after 24 hours	Subject 1 25.0 mg) after 48 hours	Subject 2 (52.0 mg) after 24 hours	Subject 2 (52.0 mg) after 48 hours
DHEA	0.02	0.02	<0.01	<0.01
Δ5-AEDIOL	0.70	1.2	0.35	0.57
AO	26.8	27.0	16.1	16.5
EO	10.3	10.8	21.6	24.0
T	0.05	0.05	0.16	0.16
E	<0.01	<0.01	<0.01	<0.01
6α/β-OH-Δ4-AEDIONE	<0.01	<0.01	0.04	0.04
6α/β-OH-T	<0.01	<0.01	0.03	0.03
4-OH-Δ4-AEDIONE	0.12	0.12	0.59	0.59
16α-OH-Δ4-AEDIONE	<0.01	<0.01	0.01	0.01
16α-OH-AO	0.07	0.07	0.10	0.11
16α-OH-EO	0.05	0.05	0.10	0.11

Table 3: *Conversion (%) of Δ4-AEDIONE to different groups of metabolites.*

Metabolites	Subject 1 (25.0 mg) after 24 hours	Subject 1 (25.0 mg) after 48 hours	Subject 2 (52.0 mg) after 24 hours	Subject 2 (52.0 mg) after 24 hours
AO and EO	37.1	37.8	37.7	40.5
non-oxygenated	37.9	39.1	38.2	41.2
oxygenated	0.24	0.24	0.87	0.89
Total	38.1	39.3	39.1	42.1

DISCUSSION

Excretion kinetics

Our results show different patterns for the excretion of produced metabolites (Figures 2a and b). DHEA, E, 16α-OH-Δ4-AEDIONE, 11β-OH-AO and 11β-OH-EO showed no, or a limited response to Δ4-AEDIONE administration (compare class 1 in Chapter 4). A rapid and short increase of excretion was observed for AO, T, 6α/β-OH-Δ4-AEDIONE, 4-OH-Δ4-AEDIONE, 6α/β-OH-T and 16α-OH-AO (class 2). Within 10 hours after administration, the respective metabolites were cleared in high speed, leading to relatively fast return to baseline values. The other studied metabolites Δ5-AEDIOL, EO, 5α,3α-ADIOL, 5β,3α-ADIOL and 16α-OH-EO showed a rapid and prolonged increase of excretion (class 3). Within 10 hours after administration the respective metabolites were cleared in relatively low speed, leading to a slow return to baseline level. This was illustrated most clearly in case of labeled Δ5-AEDIOL, which could be detected up to 100 hours post-administration (data not shown).

As has also been described in Chapter 4, kinetic differences can be explained by the extensive first and second phase metabolism of Δ4-AEDIONE. Our results show that Δ4-AEDIONE is almost completely metabolized, to mostly non-oxygenated metabolites. As in agreement with Uralets *et al.* [1], negligible amounts of labeled Δ4-AEDIONE itself were recovered.

A low renal clearance was shown previously for sulfate conjugates of DHEA, T, pregnenolone, estrone and cholesterol as compared to glucuronides, because in blood those steroids are mainly bound to albumin [10-13]. In contrast to DHEA metabolism, insufficient data are available about the second phase metabolism of orally administered Δ4-AEDIONE. As no separate analysis of the different conjugates was performed in this experiment, no further evidence was obtained.

As also reported by Uralets *et al*. [1] AO and EO are main metabolites of Δ4-AEDIONE. In the present experiment, data show a more prolonged excretion of EO compared to AO, leading to an AO/EO ratio that was significantly decreased until 30 hours after administration. This can be accounted to a relatively high production of the EO sulfate as compared to AO sulfate. Another explanation could be found in enterohepatic circulation of EO-glucuronide, resulting in lower clearance efficiency [14].

Sensitivity and selectivity

When sensitivity of a parameter is defined as the maximum response of either A(M+n)/A(M) or ([d_n-M^{exo}] + [d_0-M^{endo}]), then AO, EO, T, 6α/β-OH-Δ4-AEDIONE, 4-

OH-Δ4-AEDIONE and 6α/β-OH-T can be regarded as most sensitive (see Figures 1 and 2). The T/E ratio can also be considered as a sensitive parameter in this experiment.

The detection of 6α-OH-Δ4-AEDIONE glucuronide after Δ4-AEDIONE administration was previously reported in a case study [15]. However, regarding the applied derivatization procedure the identification is unclear as 6α-OH-Δ4-AEDIONE and 6β-OH-Δ4-AEDIONE form an identical derivative (see Chapter 8). Also no excretion data were reported. In our results the combined 6α/β-OH-Δ4-AEDIONE showed a relatively high sensitivity. This was also the case for 6α/β-OH-T. As explained in Chapter 3, the sensitivity of these steroids can most likely be assigned to the 6β-OH-metabolites.

4-OH-Δ4-AEDIONE was also detected as a sensitive metabolite, as illustrated by its higher relative response in subject 2 than for AO and EO. The endogenous origin of this steroid has not been described previously. It has, however, frequently been applied as an effective aromatase inhibitor in cancer therapy. In a clinical trial report, no endogenous 4-OH-Δ4-AEDIONE was detected in plasma before administration of this steroid [16]. However, hydroxylation at C^4 is a relevant metabolic route for estrogens, leading to catecholestrogens [17,18]. As described in Chapter 3, insufficient evidence is available to exclude the endogenous origin of 4-OH-Δ4-AEDIONE or the conversion to this steroid after oral administration of Δ4-AEDIONE.

No production of Δ5-steroids was expected, as the conversion of Δ5- to Δ4-steroids is usually considered as an irreversible step in steroid biosynthesis. However, limited amounts of labeled Δ5-AEDIOL and negligible amounts of labeled DHEA were formed. This could suggest a relatively slow conversion of Δ4-AEDIONE to DHEA, possibly followed by immediate other metabolic steps as 17-hydrogenation to Δ5-AEDIOL or hydroxylation (as have been studied in Chapter 4).

In a previously reported case study [19], 11β-OH-AO and 11β-OH-EO were mentioned as suspected metabolites of Δ4-AEDIONE. The involved steroid 11β-hydroxylation is an important step in cortisol biosynthesis. In this case, the relative response of those steroids was very limited, which can be explained by the localization of 11β-hydroxylase activity in adrenal tissue [20].

Specificity for Δ4-AEDIONE was in the first place expected for oxygenated derivatives of this steroid, as 6α/β-OH-Δ4-AEDIONE, 4-OH-Δ4-AEDIONE, 16α-OH-Δ4-AEDIONE and 6-keto-Δ4-AEDIONE. All those parameters except 6-keto-Δ4-AEDIONE extensively responded to Δ4-AEDIONE administration. Prior to the experiment also 19-hydroxy-androst-4-ene-3,17-dione was evaluated in a pilot study (data not shown). This potential metabolite [21-23] could not be identified in blank

and post-administration samples, and was therefore excluded from this experiment. The reduced metabolites 16α-OH-AO and 16α-OH-EO were expected to be either reduced metabolites of 16α-OH-Δ4-AEDIONE or 16α-hydroxy metabolites of AO and EO, respectively. Whether 6α/β-OH-T are reduced metabolites of 6α/β-OH-Δ4-AEDIONE or are 6α/β-OH-metabolites of testosterone, is also not clear.

Care should be taken with the extrapolation of these data to a situation of non-labeled Δ4-AEDIONE administration. Significant physiological isotope effects have been reported in case of deuterium labeled substance administration [24]. Due to these physiological isotope effects the rate of metabolic deuterium removal is lower than of equivalent hydrogen removal, leading to a possible limitation of 4-, 6α-, 6β and 16α-hydroxylation in this particular case.

SUMMARY

Several oxygenated steroids were introduced that could provide sensitive and specific information about administration of Δ4-AEDIONE, although the conversion to those steroids was limited (<1%). The highest sensitivity for detection of Δ4-AEDIONE was obtained for AO, EO, T, 6α/β-OH-Δ4-AEDIONE, 4-OH-Δ4-AEDIONE and 6α/β-OH-T.

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