Journal of Chromatography B, 876 (2008) 225–235

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Validation of an extended method for the detection of the misuse of endogenous steroids in sports, including new hydroxylated metabolites

P. Van Renterghem[a,*], P. Van Eenoo[a], W. Van Thuyne[a], H. Geyer[b], W. Schänzer[b], F.T. Delbeke[a]

[a] DoCoLab – UGent, Department of Clinical Chemistry, Microbiology and Immunology, Technologiepark 30, 9052 Zwijnaarde, Belgium
[b] Deutsche Sporthochschule Köln, Institut für Biochemie, Carl-Diem-Weg 6, 50933 Cologne, Germany

ARTICLE INFO

Article history:
Received 3 June 2008
Accepted 29 October 2008
Available online 6 November 2008

Keywords:
Endogenous steroids
Steroid profiling
Doping

ABSTRACT

Endogenous steroids are amongst the most misused doping agents in sports. Their presence poses a major challenge for doping control laboratories. Current threshold levels do not allow for the detection of all endogenous steroid misuse due to great interindividual variations in urinary steroid concentrations. A method has been developed and validated to screen for traditionally monitored endogenous steroids in doping control as well as specific hydroxylated/oxygenated metabolites in order to enhance the detection capabilities for the misuse of endogenous steroids.

1. Introduction

Anabolic androgenic steroids (AAS) are misused by athletes to increase muscle mass and to enhance physical performance. This has led to a ban of AAS by international sports federations and the World Anti-Doping Agency (WADA) [1] which encouraged sports drug testing laboratories to develop screening methods for detecting the misuse of AAS. These steroids are derived from the naturally occurring steroid testosterone (T), which is considered as the most important androgenic steroid.

Notwithstanding increased popularity of synthetic steroids, naturally occurring steroids are still widely misused in sports. Intake of endogenous steroids alters one or more parameters of the urinary steroid profile [2] which currently includes T, epitestosterone (E), 5α-dihydrotestosterone (DHT), androsterone (Andro), etiocholanolone (Etio), dehydroepiandrosterone (DHEA), androstenedione (Adion), 5α-androstane-3α,17β-diol (5ααβ-Adiol), 5β-androstane-3α,17β-diol (5βαβ-Adiol), 5α-androstane-3β,17β-diol (5αββ-Adiol), 11β-OH-androsterone (11β-OH-Andro) and 11β-OH-etiocholanolone (11β-OH-Etio). Due to the natural presence of endogenous steroids and their metabolites in urine, threshold values must be set to distinguish normal steroid concentrations from elevated levels caused by endogenous steroid administration. Actually, increased ratios of certain metabolite concentrations, e.g. T/E, Andro/Etio, DHT/E) are indicative for the misuse of the endogenous steroids [2,3].

At present, the mere urinary presence of endogenous steroids does not constitute a doping offence. Only when threshold values are exceeded, doping control laboratories consider samples as suspicious. One of the main problems is that these threshold concentrations or ratios are based upon population statistics whereas natural urinary concentrations can show great interindividual [4] as well as ethnical variations [3,5–7]. Since doping control samples are anonymous, WADA accredited laboratories have no information on the athlete's identity nor her/his personal reference ranges for the monitored steroids. Therefore, the raise of individual steroid concentrations due to endogenous AAS misuse might remain unnoticed in doping control tests. This indicates that the currently used techniques to detect administration of endogenous steroids can be improved [8,9]. Currently, the screening for endogenous steroids is still based on the first steroid profiling method developed by Donike et al. [10,11]. These steroids monitored in the traditional steroid profile [3] are the major metabolites and are consequently present in urine in relatively high concentrations. More recent studies have revealed that after the administration of endogenous steroids other more specific metabolites occur in low urinary concentrations [12,13]. Administration of endogenous steroids may lead to a saturation of the major metabolism pathways of steroids and thereby emphasizing the role of minor metabolic pathways which yields detectable concentrations of other metabolites [14]. These new metabolites are mainly hydroxylated and oxygenated steroids at C4, C6, C7 and C16. Screening for and evaluation of these specific metabolites can be used to determine which endogenous

* Corresponding author. Tel.: +32 9 3313293; fax: +32 9 3313299.
E-mail address: Pieter.VanRenterghem@Ugent.be (P. Van Renterghem).
URL: http://www.docolab.ugent.be (P. Van Renterghem).

1570-0232/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2008.10.047

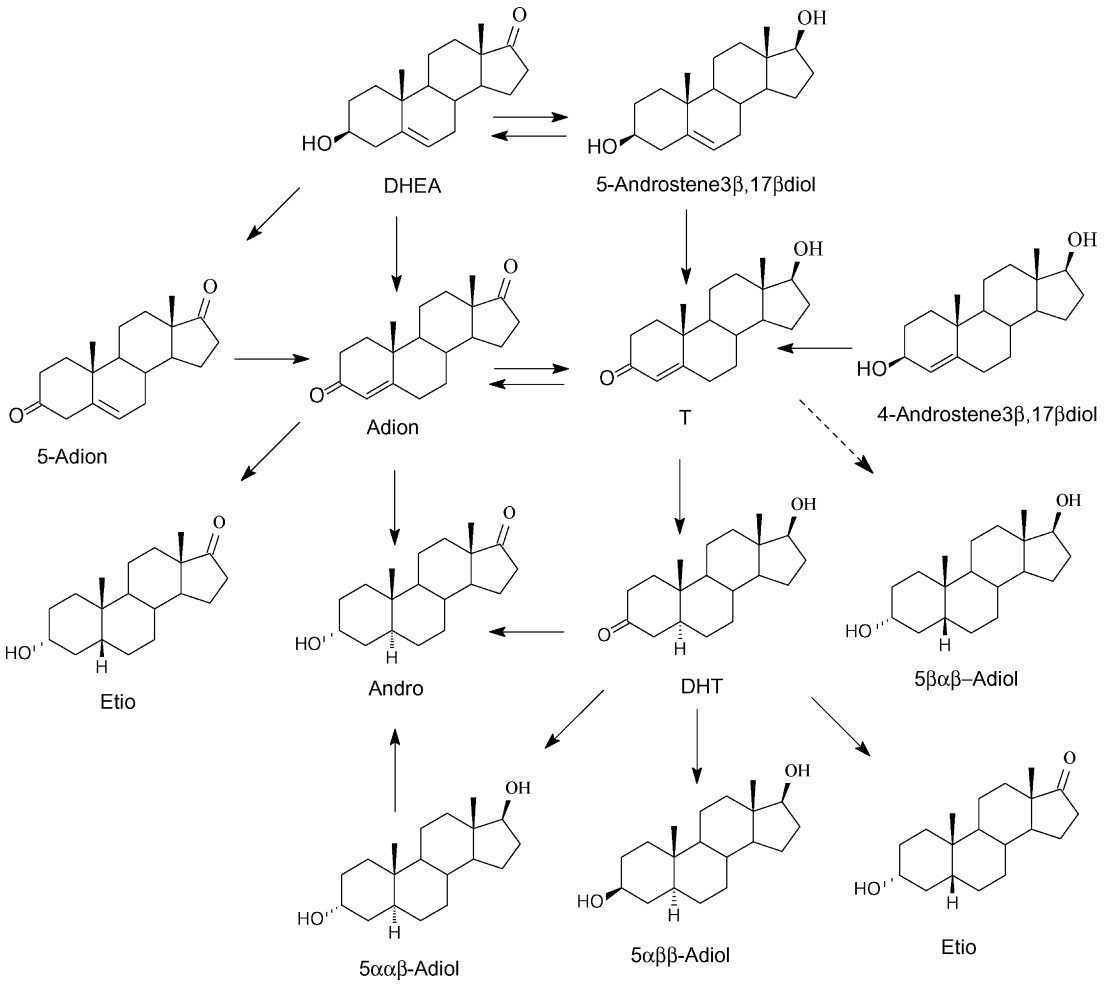

Fig. 1. Non-specific metabolism of testosterone, DHEA and androstenedione.

steroids were administered [13,15–17]. The official WADA technical document on endogenous steroids has already incorporated some of these specific urinary metabolites [2].

Fig. 1 shows the major metabolites of the steroids T, Adion and DHEA. Separately, these major pathways are extended in Fig. 2 with the specific metabolites of the minor pathways including the most relevant hydroxylated and oxygenated metabolites.

Careful selection and identification of suitable metabolites for the detection of endogenous steroids are crucial to find sensitive key markers for endogenous steroid misuse. Therefore, there is compelling need for a screening method for all those steroids.

2. Materials and methods

2.1. Materials

2.1.1. Chemicals

$NaH_2PO_4{\cdot}H_2O$, $Na_2HPO_4{\cdot}2H_2O$, Na_2SO_4, NH_4I and K_2CO_3 were from Merck (Darmstadt, Germany). Diethyl ether, LC–MS grade methanol and $NaHCO_3$ were from Fisher scientific (Leicestershire, UK) and β-glucuronidase (*E. coli*) from Roche Diagnostics (Mannheim, Germany). *N*-Methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) was obtained from Karl Bucher Chemische Fabrik GmbH (Waldstetten, Germany) and ethanethiol was from Acros Organics (Geel, Belgium). XAD-2 was from Serva (Heidelberg, Germany).

2.1.2. Reference standards

5α-Androstane-3α,17β-diol (5ααβ-Adiol), 5α-androstane-3β,17β-diol (5αββ-Adiol), 4-androstene-3,17-dione (Androstenedione, Adion), 11β-OH-androsterone (11β-OH-Andro), 11β-OH-etiocholanolone (11β-OH-Etio), 5β-androstane-3α,17β-diol (5βαβ-Adiol), androsterone (Andro), etiocholanolone (Etio), testosterone (T), epitestosterone (E), 4-OH-4-androstene-3,17-dione (4-OH-Adion) and 3α,5cyclo-5α-androstan-6β-ol-17-one (5cyclo) were obtained from Sigma (St. Louis, MO, USA). Dehydroepiandrosterone (DHEA) was from Serva (Heidelberg, Germany). 7-Keto-DHEA, 4-androstene-3,6,17-trione(6-oxo-Adion), 7α-OH-testosterone (7α-OH-T), 7β-OH-DHEA, 7α-OH-DHEA, 6α-OH-androstenedione (6α-OH-Adion), 6α-OH-testosterone (6α-OH-T), 16α-OH-etiocholanolone (16α-OH-Etio), 16α-OH-DHEA and 16α-OH-androstenedione (16α-OH-Adion) were purchased from Steraloids (Newport, USA). 5α-Dihydrotestosterone (DHT) was obtained from Piette International Laboratories (Drogenbos, Belgium). 17α-Methyltestosterone (17α-Me-T) was from Organon (Oss, The Netherlands). Androsterone glucuronide, etiocholanolone glucuronide, testosterone glucuronide, 16,16,17,d3 testosterone, epitestosterone glucuronide, dihydrotestosterone glucuronide, 5α-androstane-3β,17β-diol glucuronide, 16α-OH-androsterone (16α-OH-Andro), 6β-OH-androsterone (6β-OH-Andro), 4β-OH-DHEA, 4-OH-testosterone (4-OH-T), 6β-OH-etiocholanolone (6β-OH-Etio) and testosterone-d3 were purchased from the National Measurement Institute (Pymble, Australia). All steroid

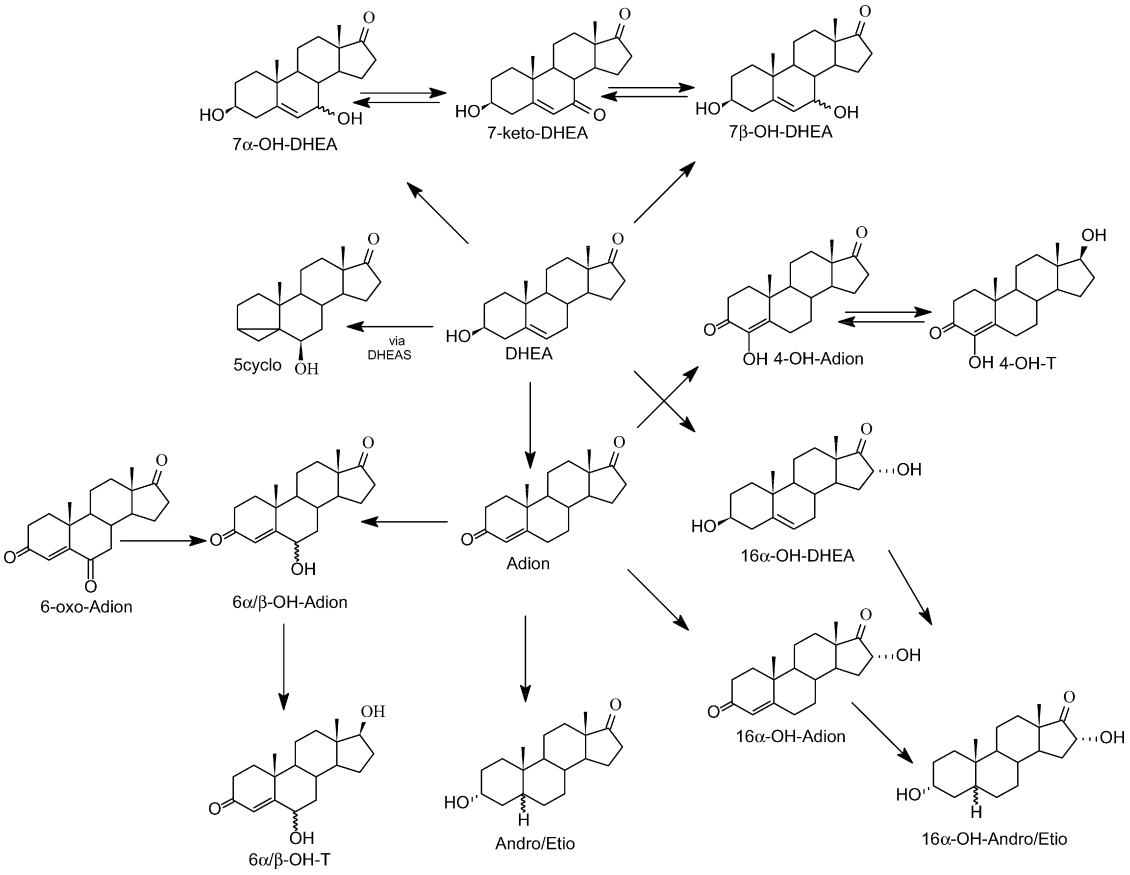

Fig. 2. Specific metabolism of DHEA, androstenedione and their precursors.

standards contained less than 1% impurities. All standard solutions were made in methanol and stored at 4 °C.

2.2. Extraction from urine

The extraction procedure is based upon the methods developed by Donike et al. [20,21]. To 5 ml urine 50 μl internal standard (17α-Me-T, 2 μg/ml), 1 ml of phosphate buffer (0.1 M $Na_2HPO_4{\cdot}2H_2O/NaH_2PO_4{\cdot}H_2O$ solution, pH 7) and 50 μl β-glucuronidase were added.

Hydrolysis was performed for 2.5 h at 56 °C. After cooling, 200 mg of $NaHCO_3/K_2CO_3$ (2/1, w/w) buffer and 5 ml of freshly distilled diethyl ether were added and a liquid–liquid extraction was performed by rolling for 20 min. The tubes were centrifuged at 1200 × *g* or 2700 rpm for 5 min and the organic layer was separated, dried over anhydrous Na_2SO_4 and evaporated under oxygen free nitrogen (OFN). The dry residue was derivatised with 100 μl MSTFA/NH_4I/ethanethiol (1150/3/6, v/w/v) for 1 h at 80 °C.

2.3. GC–MS conditions

The GC–MS analysis was performed on an Agilent 6890 GC system coupled to a 5975B Vl MSD mass spectrometer from Agilent Technologies (Palo Alto, USA). The instrument was equipped with a 17 m J&W Ultra1 column (internal diameter 0.2 mm, film thickness 0.11 μm) (Palo Alto, USA). The GC temperature program was: 120 °C–70 °C/min → 177 °C–4 °C/min → 231 °C–30 °C/min → 300 °C (2 min). The temperatures of other instrument parts were 270 °C for the injector, 250 °C for the transfer line, 230 °C for the ion source and 150 °C for the quadrupole. 0.5 μl was injected splitless. Helium was used as carrier gas which was under constant pressure of 84.9 kPa. The instrument was operated in full scan mode for qualitative purposes between the *m*/*z* 50 and 650. For steroid quantification Selective Ion Monitoring (SIM) was used with a dwelltime of 20 ms for all monitored ions (Table 1). Additionally, *m*/*z* 272 was monitored to screen for possible presence of the mono-trimethylsilyl derivatives of Andro and Etio which is an indication of incomplete derivatisation by the MSTFA/NH_4I/ethanethiol mixture.

2.4. Calibration curves

The calibration curves were performed in steroid stripped urine which shows best resemblance for the matrix of natural urine [22]. Removal of the naturally present androgenic steroids was necessary to measure the spiked reference mixtures quantitatively. Steroid stripped urine was prepared by pouring negative urine on a preconditioned XAD-2 column. Aliquots of 5 ml were spiked at five levels per compound. The selection of the calibration ranges was based upon older studies containing similar steroids [14,16]. An overview of the concentration ranges for each compound is given in Table 2. Calibration curves were obtained by plotting the peak area ratios of the analytes over the internal standard (IS) versus the spiked concentration.

Each concentration was analysed in triplicate.

2.5. Hydrolysis efficiency and extraction recovery

Hydrolysis efficiency was evaluated by spiking six steroid stripped urine samples with the glucuronide conjugates of Andro, Etio, T, E, DHT and 5αββ-Adiol at the concentrations of the third

Table 1
Retention times, relative retention times and monitored ions (*m*/*z*).

Compound	RT (min)	RRT	Target ion (*m*/*z*)	Qualifier ions	
5Cyclo	8.45	0.574	432	417	327
Andro	11.22	0.759	419	434	329
Etio	11.45	0.775	419	434	329
5ααβ-Adiol	11.54	0.781	241	256	
5βαβ-Adiol	11.61	0.786	256	241	
DHEA	12.26	0.829	432	417	
5αββ-Adiol	12.67	0.857	241	421	
E	12.68	0.858	432	417	
7α-OH-DHEA	12.79	0.869	415	430	325
DHT	12.88	0.871	434	419	
6β-OH-Andro	12.95	0.880	522	507	327
Adion	13.09	0.886	430	415	
6β-OH-Etio	13.1	0.891	522	507	327
7α-OH-T	13.26	0.901	430	415	
T	13.37	0.905	432	417	
11β-OH-Andro	13.75	0.930	522	507	
11β-OH-Etio	13.97	0.945	522	507	
4β-OH-DHEA	14.54	0.988	520	430	415
7β-OH-DHEA	14.61	0.993	415	520	430
16α-OH-Etio	14.69	0.999	507	522	
17α-Me-T	14.71	1	301	446	
16α-OH-Andro	14.88	1.012	507	522	417
6-Oxo-Adion	15.18	1.032	516	501	411
7-Keto-DHEA	15.19	1.033	518	503	413
6α-OH-Adion	15.38	1.046	518	503	
6α-OH-T	15.52	1.055	520	505	430
4-OH-Adion	15.62	1.062	518	503	
16α-OH-DHEA	15.71	1.068	505	520	415
4-OH-T	15.77	1.072	520	505	
16α-OH-Adion	16.03	1.090	503	518	206

calibrator. This analysis was performed in parallel with six samples spiked with the free steroid fractions.

Hydrolysis efficiency was also checked by comparing six identically spiked urines with both free and glucuronidated steroid fractions subjected to 2.5 h and 17 h hydrolysis.

Table 2
Range, equation and correlation coefficient (*r*) of calibration curves of all screened compounds.

Compound	Calibration range (ng/ml)	Equation	Correlation coefficient, *r*
5Cyclo	5–500	$y = 0.1784x - 0.0880$	0.9938
Andro	125–5000	$y = 0.0490x - 1.1787$	0.9990
Etio	125–5000	$y = 0.0415x - 0.5094$	0.9993
5ααβ-Adiol	5–500	$y = 0.0225x + 0.001$	0.9996
5βαβ-Adiol	5–500	$y = 0.0231x - 0.0161$	0.9975
DHEA	5–200	$y = 0.0311x - 0.0394$	0.9998
5αββ-Adiol	5–200	$y = 0.0135x - 0.012$	0.9994
E	5–200	$y = 0.0891x - 0.1379$	0.9995
7α-OH-DHEA	5–100	$y = 0.0161x - 0.0278$	0.9995
DHT	5–200	$y = 0.0554x + 0.0366$	0.9969
6β-OH-Andro	5–100	$y = 0.0240x - 0.0354$	0.9989
Adion	5–200	$y = 0.0850x - 0.1554$	0.9996
6β-OH-Etio	5–100	$y = 0.0202x - 0.0089$	0.9990
7α-OH-T	5–100	$y = 0.0111x + 0.0035$	0.9992
T	5–200	$y = 0.0916x - 0.1158$	0.9995
11β-OH-Andro	100–4000	$y = 0.0243x - 1.1493$	0.9991
11β-OH-Etio	100–4000	$y = 0.0225x - 1.1156$	0.9994
4β-OH-DHEA			
7β-OH-DHEA			
16α-OH-Etio	5–500	$y = 0.1398x - 0.2743$	0.9992
16α-OH-Andro	5–500	$y = 0.1261x - 0.2755$	0.9993
6-Oxo-Adion	5–100	$y = 0.0355x - 0.0865$	0.9992
7-Keto-DHEA	5–100	$y = 0.0230x - 0.0679$	0.9980
6α-OH-Adion	5–100	$y = 0.0693x - 0.0595$	0.9991
6α-OH-T	5–100	$y = 0.0703x - 0.1098$	0.9993
4-OH-Adion	5–100	$y = 0.0913x - 0.1166$	0.9984
16α-OH-DHEA	5–100	$y = 0.0930x - 0.1858$	0.9992
4-OH-T	5–100	$y = 0.1968x - 0.1715$	0.9991
16α-OH-Adion	5–100	$y = 0.0811x - 0.1522$	0.9993

For extraction recovery experiments, six urine samples were spiked at the level of the third calibrator and extracted as described above. Additionally six blank urine samples were extracted and afterwards the transferred organic layer was spiked at the same level, simulating a 100% recovery. To both sets of samples d3-testosterone were added as an external standard (20 ng/ml) after extraction. Both sets of extracts were then analysed with the described GC–MS method. The extraction recovery was calculated by comparison of the relative area ratios (compounds to the external standard) obtained for the samples spiked before and after extraction.

2.6. Method validation

Validation was carried out according to the Eurachem guidelines [23]. For each calibration curve linearity ($n = 3$) was checked at five levels by least squares fit. The bias, repeatability (within-day) and reproducibility (between-day and different analysts) were determined at three levels; the lowest, midrange and highest point of the calibration curve, respectively. Bias ($n = 18$) was defined as the percentual difference between the average of the measured concentrations and the theoretical value. Repeatability ($n = 6$) and reproducibility ($n = 18$) were calculated as the relative standard deviation RSD and expressed as percentages. The limit of quantitation (LOQ) was considered as the lowest point of the calibration curve where precision and bias were within the tolerated interval, the limit of detection (LOD) was set arbitrarily at half LOQ.

Long-term evaluation of method performance was done by statistical analysis of a large set of spiked quality control samples accompanying routine samples and determination of the mean observed value and standard deviation over a period of 8 months.

Selectivity was tested by the analysis of steroid stripped urine spiked with structurally related compounds (27 exogenous anabolic steroids, 11 corticosteroids) and other routinely monitored doping agents (7 β-agonists, 54 stimulants, 28 diuretics, 16 narcotics, 21 beta blockers).

Matrix interferences were checked by analysing steroid stripped urine.

3. Results and discussion

3.1. Hydrolysis efficiency and extraction

The use of β-glucuronidase from *E. coli* for hydrolysis enables the quantification of the free and glucuronidated fraction of the steroids. Although the sulphated fraction can be deconjugated using a juice from Helix Pomatia (HP), this mixture causes several unwanted side effects [24,25]. Because of these side effects doping control laboratories avoid the application of HP in their screening procedures. Because the WADA technical document [2] on endogenous steroids refers to the glucuronide conjugates concerning the steroids of interest, this method was only tested with the bacterial β-glucuronidase. Comparing the results of the glucuronidated steroids with the free fraction duplicates and with the 17 h hydrolysis protocol shows that the 2.5 h hydrolysis was complete. The differences in measured concentrations were never significantly different from 100% ($\alpha = 0.05$) which proves excellent hydrolysis efficiency.

The extraction procedure consists out of liquid–liquid extraction with diethylether which is more efficient than less polar solvents (e.g. *n*-pentane) and is also frequently used in other methods, followed by evaporation under OFN, because the use of OFN avoids unwanted oxidation reactions. The mean recoveries with their relative standard deviations are listed in Table 3 . As could be expected, the more polar hydroxylated and oxygenated metabolites show

Table 3
Bias, repeatability, reproducibility and tolerance limits of the method at lowest, middle.

Compound	Concentration (ng/ml)	Bias (%)	Repeatability (%)	Reproducibility (%)	RSDmax (%)	Extraction recovery (%)
Andro	125	−0.52	4.88	6.99	21.9	81.58 ± 8.05
	1250	4.94	2.48	5.02	15.5	
	5000	4.46	6.12	7.57	12.6	
Etio	125	10.60	3.44	6.88	21.9	82.14 ± 8.35
	1250	4.46	2.85	5.28	15.5	
	5000	1.12	2.54	3.43	12.6	
T	5	2.78	1.39	1.73	35.5	82.22 ± 4.15
	50	−1.54	2.20	2.55	25.1	
	200	1.43	1.64	2.36	20.4	
E	5	11.55	1.49	2.31	35.5	81.24 ± 4.71
	50	0.02	2.83	3.50	25.1	
	200	1.56	1.39	2.82	20.4	
5ααβ-Adiol	5	0.41	5.79	6.96	35.5	82.76 ± 8.63
	50	4.11	2.09	4.68	25.1	
	200	1.05	5.09	3.54	20.4	
	500	−4.42	6.24	4.40	17.8	
5βαβ-Adiol	5	13.02	4.88	7.76	35.5	80.18 ± 8.15
	50	2.77	2.92	5.13	25.1	
	200	2.44	2.73	5.07	20.4	
	500	−4.48	5.95	4.04	17.8	
5αββ-Adiol	5	10.64	5.18	5.17	35.5	79.27 ± 8.09
	50	0.64	2.40	3.53	25.1	
	200	−0.44	1.99	2.74	20.4	
DHEA	5	10.67	1.48	2.27	35.5	78.24 ± 9.64
	50	−0.03	2.65	3.37	25.1	
	200	1.87	2.65	2.82	20.4	
DHT	5	−18.28	2.45	2.96	35.5	117.33 ± 14.83
	50	−5.91	2.75	3.41	25.1	
	200	2.56	3.28	5.32	20.4	
Adion	5	16.63	2.51	2.51	35.5	81.88 ± 5.24
	50	−0.60	2.36	3.31	25.1	
	200	−3.49	1.79	2.35	20.4	
11β-OH-Andro	100	5.26	10.88	8.15	22.6	78.94 ± 9.04
	1000	2.99	3.04	3.32	16.0	
	4000	2.81	2.94	3.28	13.0	
11βOH-Etio	100	11.53	11.85	9.01	22.6	79.38 ± 10.71
	1000	4.02	3.07	3.54	16.0	
	4000	2.94	3.02	2.99	13.0	
7α-OH-DHEA	5	16.30	5.98	5.71	35.5	68.47 ± 8.33
	50	2.81	8.13	7.23	28.8	
	200	0.38	8.83	8.56	22.6	
6β-OH-Andro	5	8.51	5.17	4.18	35.5	69.65 ± 7.97
	20	0.25	3.21	2.26	28.8	
	100	1.55	3.17	3.55	22.6	
6β-OH-Etio	5	−2.93	3.47	4.15	35.5	60.45 ± 7.03
	20	1.93	4.71	3.57	28.8	
	100	4.48	4.06	5.65	22.6	
7α-OH-T	5	−10.08	7.63	7.30	35.5	54.82 ± 14.11
	20	7.02	8.22	9.83	28.8	
	100	8.79	3.28	13.99	22.6	
16α-OH-Etio	5	13.86	2.83	7.36	35.5	77.54 ± 1.47
	20	−0.96	1.96	4.43	28.8	
	100	−1.50	6.09	5.17	22.6	
	500	−8.05	6.51	6.57	17.8	
16α-OH-Andro	5	15.64	2.53	6.75	35.5	78.65 ± 0.23
	20	−1.82	2.08	4.54	28.8	
	100	−2.45	5.56	4.99	22.6	
	500	−8.05	6.51	6.57	17.8	
6-Oxo-Adion	5	5.26	4.09	5.78	35.5	72.23 ± 2.84
	20	−9.58	3.67	5.03	28.8	
	100	−0.03	2.40	2.79	22.6	

Table 3 (*Continued*)

Compound	Concentration (ng/ml)	Bias (%)	Repeatability (%)	Reproducibility (%)	RSDmax (%)	Extraction recovery (%)
7-Keto-DHEA	5	14.53	8.22	10.57	35.5	62.44 ± 8.25
	20	−16.34	7.90	11.95	28.8	
	100	−10.31	4.03	8.84	22.6	
6α-OH-Adion	5	2.41	9.21	8.31	35.5	55.57 ± 8.65
	20	2.00	5.05	5.99	28.8	
	100	6.42	6.14	5.70	22.6	
6α-OH-T	5	11.08	7.24	6.63	35.5	55.94 ± 7.51
	20	3.77	5.53	7.81	28.8	
	100	10.65	9.61	6.88	22.6	
4-OH-Adion	5	1.91	1.83	6.10	35.5	75.98 ± 10.73
	20	−3.42	3.13	5.64	28.8	
	100	−0.73	3.71	3.53	22.6	
16α-OH-DHEA	5	11.21	3.01	11.28	35.5	75.25 ± 1.74
	20	−1.14	3.64	9.58	28.8	
	100	−2.02	8.01	7.90	22.6	
4-OH-T	5	−5.71	4.38	6.06	35.5	76.97 ± 8.88
	20	−2.87	2.38	5.06	28.8	
	100	−2.25	2.41	2.86	22.6	
16α-OH-Adion	5	8.85	5.68	11.39	35.5	67.04 ± 3.93
	20	−0.99	2.52	11.23	28.8	
	100	1.71	6.74	8.73	22.6	
5Cyclo	5	−14.71	3.94	11.31	35.5	96.74 ± 17.07
	100	−7.95	9.11	13.59	22.6	
	500	−4.01	7.74	9.59	17.8	

lower extraction recoveries in the ether phase in comparison to the steroids containing only two oxygens.

3.2. Chromatography

The total runtime of the analytical method is 19 min. The retention times (RT) and mass spectra of all compounds were determined by full scan analysis. Based upon these findings the *m*/*z* values were selected for SIM analysis. All retention times and the selected ions for SIM are shown in Table 1. Quantification was performed using the ratio of the areas of the target ion and internal standard whereas the qualifier ions were applied to confirm the identity of the substance. From Fig. 3 it is clear that the compounds of interest elute between the 8th and the 16th minute. The compounds that coelute

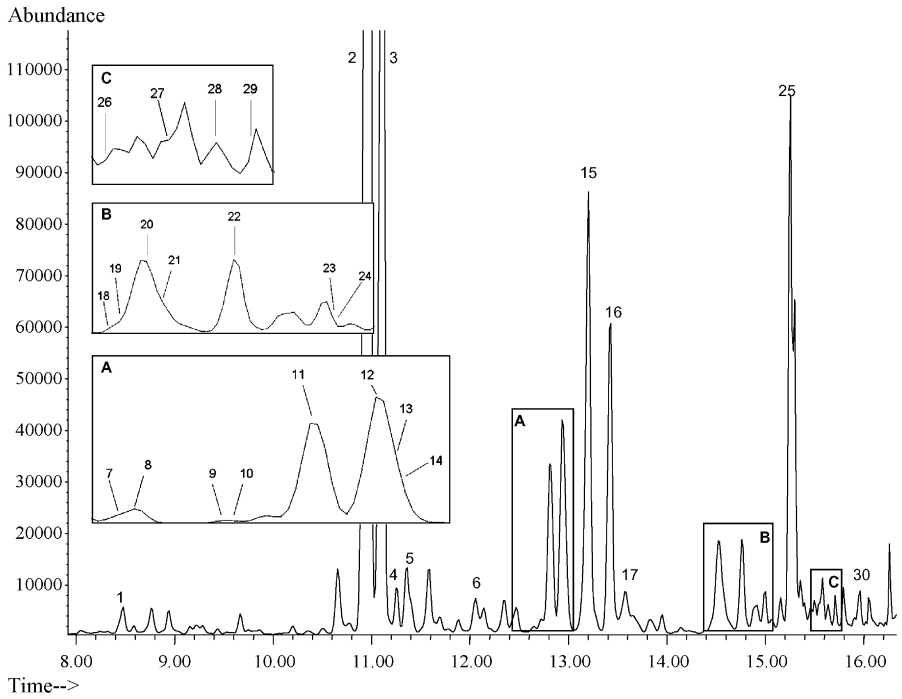

Fig. 3. Chromatogram of an excretion urine after intake of 6-oxo-androstenedione. (1) 5cyclo; (2) androsterone; (3) etiocholanolone; (4) 5α-androstane-3α,17β-diol; (5) 5β-androstane-3α,17β-diol; (6) DHEA; (7) 5α-androstane-3α,17β-diol; (8) epitestosterone; (9) 7α-OH-DHEA; (10) DHT; (11) 6β-OH-androsterone; (12) androstenedione; (13) 6β-OH-etiocholanolone; (14) 7α-OH-testosterone; (15) testosterone; (16) 11β-OH-androsterone; (17) 11β-OH-etiocholanolone; (18) 4β-OH-DHEA; (19) 7β-OH-DHEA; (20) 16α-OH-etiocholanolone; (21) 17α-methyl-testosterone; (22) 16α-OH-androsterone; (23) 6-oxo-androstenedione; (24) 7-keto-DHEA; (25) 6α-OH-androstenedione; (26) 6α-OH-testosterone; (27) 4-OH-androstenedione; (28) 16α-OH-DHEA; (29) 4-OH-testosterone; (30) 16α-OH-androstenedione.

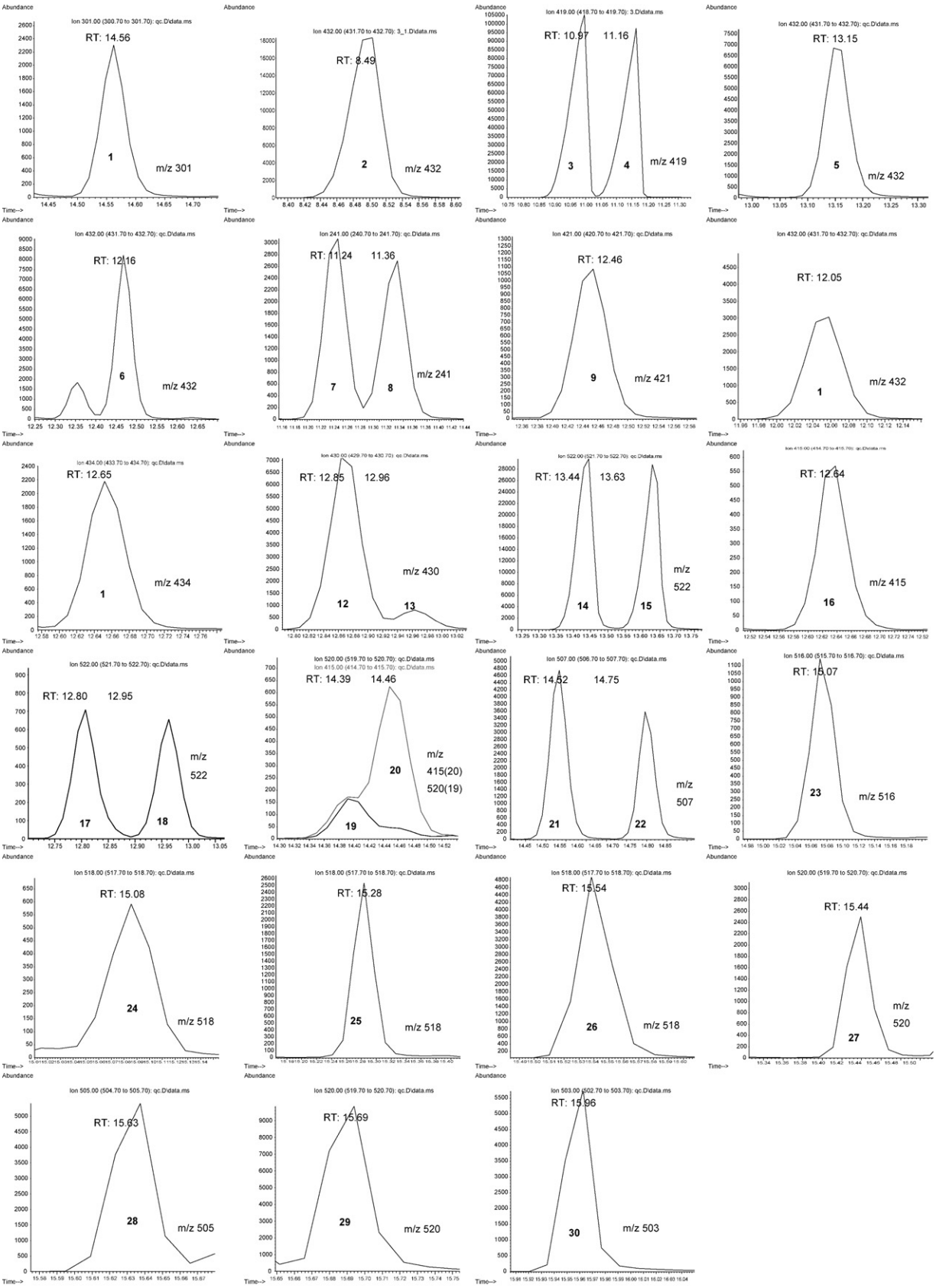

Fig. 4. The ion traces of all selected compounds in a calibration sample at the third level. (1) 17α-Methyl-testosterone; (2) 5cyclo; (3) androsterone; (4) etiocholanolone; (5) testosterone; (6) epitestosterone; (7) 5α-androstane-3α,17β-diol; (8) 5β-androstane-3α,17β-diol; (9) 5α-androstane-3α,17β-diol; (10) DHEA; (11) DHT; (12) androstenedione; (13) 7α-OH-testosterone; (14) 11β-OH-androsterone; (15) 11β-OH-etiocholanolone; (16) 7α-OH-DHEA; (17) 6β-OH-androsterone; (18) 6β-OH-etiocholanolone; (19) 4β-OH-DHEA; (20) 7β-OH-DHEA; (21) 16α-OH-etiocholanolone; (22) 16α-OH-androsterone; (23) 6-oxo-androstenedione; (24) 7-keto-DHEA; (25) 6α/β-OH-androstenedione; (26) 4-OH-androstenedione; (27) 6α/β-OH-testosterone; (28) 16α-OH-DHEA; (29) 4-OH-testosterone; (30) 16α-OH-androstenedione.

(Fig. 3) can be separated using their typical m/z-ratios, as shown in Fig. 4.

The ion traces of the individual steroids in Fig. 4 also show that all compounds with similar ions, excepting 4β-OH-DHEA and 7β-OH-DHEA, eluted separately indicating good selectivity of the method. The coelution of 4β-OH-DHEA and 7β-OH-DHEA, which share identical target ions makes it impossible to quantify both steroids. Consequently both steroids were excluded from the validation process. Nevertheless these steroids are included in the method for qualitative purposes.

3.3. Method validation

Each calibration curve was based upon five concentration levels. Different compounds required different calibration ranges [14] (Table 2). Equations and correlation coefficients are given in Table 2. All steroids yielded calibration curves with excellent correlation coefficients ($r \geq 0.99$) indicating good linearity for the validated compounds within their given range. For all points of the calibration curve bias and repeatability based upon three replicates were satisfactory.

Table 3 represents the validation results for the bias ($n = 18$), repeatability ($n = 6$) and reproducibility ($n = 18$) at the lowest, middle and highest point of the calibration curve. The bias should lay in between −20% and 20% for the lowest point. In case of higher calibrators the interval was limited to −15% to 15% [26]. The maximum allowed values for the repeatability and reproducibility were determined by the Horwitz-equation, $\text{RSDmax} = 2_{1-0.5 \log C}$ (C = concentration (ng/ml) $\times 10^{-9}$) [27]. Repeatability and reproducibility are acceptable if the calculated RSD does not exceed (2/3)RSDmax and RSDmax, respectively. For both bias and precision, the obtained results were well below the allowable tolerance limits.

The robustness and long-term accuracy and reproducibility were checked by analysing 214 spiked steroid free urines at the level of the third calibrator (QC level) for each compound. For the 5cyclo only 105 measurements were recorded. The results obtained from these quality control samples (analyzed under routine conditions over a period of 8 months) are shown in Table 4. It is clear that both the accuracy and reproducibility are excellent and well below the criteria as set for method validation. These results indicate that the method shows sufficient robustness for quantitative analysis under routine conditions.

Except for 7β-OH-DHEA and 4β-OH-DHEA the selectivity was satisfactory. No interferences from other routinely monitored doping agents and structurally related compounds could be detected indicating good selectivity. In addition, analysis of steroid stripped negative urine did not reveal any matrix interferences proving good specificity of the method.

Table 4
Results of the long-term evaluation (8 months) of quality control samples.

Compound	QC level	Number of measurements	Observed mean	SD%
5Cyclo	50	105	52.65	14.04
Andro	1250	214	1245.44	12.40
Etio	1250	214	1259.61	12.34
T	50	214	49.49	10.92
E	50	214	50.17	11.88
5ααβ-Adiol	50	214	49.04	13.94
5βαβ-Adiol	50	214	48.93	11.93
5αββ-Adiol	50	214	49.88	11.33
DHEA	50	214	48.78	11.61
DHT	50	214	46.92	11.95
Adion	50	214	47.83	12.42
11β-OH-Andro	1000	214	974.91	11.38
11β-OH-Etio	1000	214	1016.35	12.89
7α-OH-DHEA	20	214	20.68	9.94
6β-OH-Andro	20	214	20.81	10.10
6β-OH-Etio	20	214	20.53	10.54
7α-OH-T	20	214	19.71	11.66
16α-OH-Etio	20	214	19.99	10.87
16α-OH-Andro	20	214	18.97	9.36
6-Oxo-Adion	20	214	19.21	14.55
7-Keto-DHEA	20	214	20.66	12.47
6α-OH-Adion	20	214	19.64	14.67
6α-OH-T	20	214	18.48	9.37
4-OH-Adion	20	214	18.68	10.03
16α-OH-DHEA	20	214	19.29	11.42
4-OH-T	20	214	18.21	8.89
16α-OH-Adion	20	214	18.95	11.15

3.4. Monitored steroids and their importance

The developed method is capable of monitoring the steroids which are traditionally included in screening methods in doping control laboratories. These steroids are either the parent compounds (e.g. T, DHEA, Adion, DHT) or their major metabolites

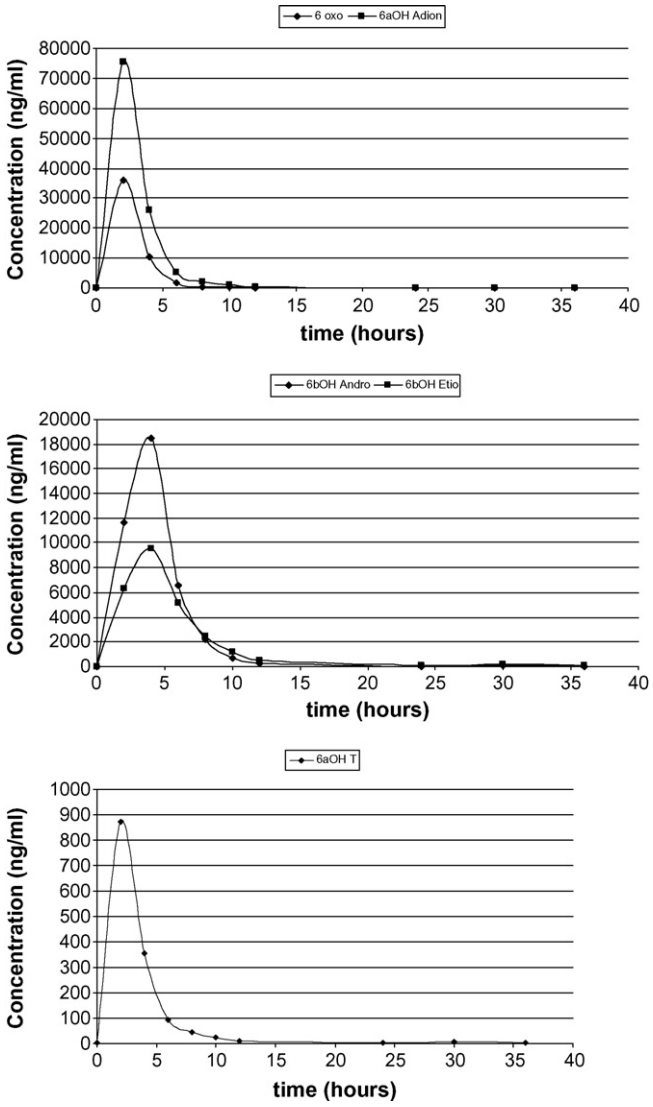

Fig. 5. Excreted concentrations of 6-oxo-androstenedione, 6α-OH-androstenedione, 6α-OH-testosterone, 6β-OH-androsterone, 6β-OH-etiocholanolone after administration of a single dose of 100 mg 6-oxo-androstenedione to a male volunteer.

(Andro, Etio and multiple stereoisomers of androstanediol) [28–31]. Andro and Etio have been used as sensitive but non-specific parameters for the detection of T, DHEA and Adion administration [13,14,32,33] and are excreted in relative high amounts after oral application of these steroids (see Fig. 3). Besides Andro and Etio, the raise in T/E ratio is also considered as indicative for endogenous steroid misuse. Administration of T, DHEA and Adion influence the urinary T concentration in contrast to the urinary concentration of E which remains approximately constant [10,30]. As previously described in literature [14,32,34,35], the conversion from 5-ene steroids (DHEA, 5-androstenediol) to 4-ene steroids (Adion, T, DHT) occurs unilaterally providing the possibility to distinguish between DHEA and Adion intake. Minor steroid metabolites reveal additional differences between DHEA and Adion metabolism. Based upon in vivo studies minor metabolites of DHEA and Adion, which can be identified as specific metabolites, were included in the method and presented in Fig. 1. Other methods have been developed combining a large number of steroids and corticosteroids [18,19]. However, this work combines for the first time the endogenous steroids which are traditionally monitored in doping control and a number of their hydroxylated/oxygenated metabolites in a comprehensive screening method which screens for 30 endogenous steroids. Using the present method differentiation between administered steroids might be possible.

Indeed, the most prominent specific metabolites of Adion are hydroxylated at C4, C6 and C16, although hydroxylation at C4 occurs only in very small amounts to form 4-OH-Adion [36]. The described method is able to detect 4OH-Adion, 6ζ-OH-Adion as well as 16α-OH-Adion. The hydroxylated metabolites at C4 and C6 are further reduced to 4-OH-T or 6α/β-OHT respectively, which are also monitored.

As shown in Fig. 4, no isomeric differentiation could be achieved between 6α/β-OH-Adion and 6α/β-OH-T in the current method. The loss of the stereo specificity is caused by the formation of the same 3,5-dienol product after derivatisation with MSTFA/NH_4I/ethanethiol [37,38]. In vivo studies revealed the presence of 6α-OH-Adion and 6α-OH-T [14,37,39] in contrast to in vitro experiments performed by Lévesque et al. [40] where 6β-hydroxylation prevails. These studies justify the derivatisation with MSTFA/NH_4I/ethanethiol mixture assuming that only the 6α isomers are monitored. 16α-OH-Adion breaks down to the respective Andro and Etio isomers. All these metabolites of Adion, 6α-OH-Adion, 6α-OH-T, 4-OH-Adion, 4-OH-T, 16α-OH-Adion, 16α-OH-Andro and 16α-OH-Etio are included in this method and the metabolic pathways are presented in Fig. 2. Hence, in contrast to previously published screening methods for anabolic steroids, the metabolites monitored in this method can indicate Adion misuse.

Because DHEA can be partially considered as a precursor of Adion, its main metabolism yields the same 4-ene metabolites as Adion. The main specific conversions in DHEA metabolism are hydroxylation at C7 and C16, directly forming 7α-OH-DHEA, 7β-OH-DHEA, 16α-OH-DHEA and 16β-OH-DHEA [13,41,42]. Except for 7β-OH-DHEA all metabolites can be quantified using the developed method. Robinzon et al. [43] found that 7α-OH-DHEA reversibly converts to 7-keto-DHEA which is also marketed as a food supplement. In vitro studies showed that both C7 hydroxylated isomers are interconvertible via 7-keto-DHEA [44]. The 16α-OH derivate

Table 5
Quantitative results of an excretion study until 36 h post-administration of 100 mg 6-oxo-androstenedione to a male volunteer.

Time (h)	Concentration (ng/ml)									
	5Cyclo	Andro	Etio	T	E	5ααβ-Adiol	5βαβ-Adiol	5αββ-Adiol	DHEA	DHT
0	7.1	2481.05	2788.30	24.20	21.30	40.45	116.35	6.10	35.70	–
2	5.8	4737.50	655.73	72.65	31.65	75.00	437.65	13.45	73.15	–
4	6	4843.95	5079.70	77.00	24.50	68.25	380.45	15.25	58.75	–
6	5.6	3718.80	4026.92	48.10	25.75	56.00	333.85	12.50	47.85	–
8	6.25	3416.41	3981.28	49.55	28.75	76.80	333.15	8.30	45.70	–
10	10.4	4101.45	4791.05	49.60	28.10	92.40	398.10	11.35	67.45	5.35
12	7.5	965.96	1357.47	30.00	13.85	71.45	338.80	6.60	49.15	–
24	–	2362.34	2893.56	27.45	19.20	208.85	195.90	6.05	34.70	–
30	19	6016.88	7897.77	62.40	50.10	106.55	531.55	21.70	77.70	6.55
36	9.15	2176.99	2798.26	23.50	14.35	46.80	258.20	9.00	29.25	–

	Adion	11β-OH-Andro	11β-OH-Etio	7α-OH-DHEA	6β-OH-Andro	6β-OH-Etio	7α-OH-T	4β-OH-DHEA	7β-OH-DHEA	16α-OH-Etio
0	–	1191.90	100.25	–	–	31.20	–	–	–	94.00
2	–	1916.80	1074.60	33.30	11606.20	6290.65	–	–	–	261.50
4	–	1781.50	983.00	27.50	18514.75	9563.50	–	–	–	194.75
6	–	1862.75	607.10	14.50	6613.00	5125.35	–	–	–	177.85
8	–	1141.15	1286.75	9.05	2153.80	2420.55	–	–	–	156.50
10	10.30	1609.95	1350.95	11.45	637.75	1183.75	–	–	–	202.70
12	–	922.00	1067.10	7.70	226.10	540.45	–	–	–	162.50
24	5.05	1359.45	242.60	–	24.60	80.80	6.10	–	–	95.65
30	9.25	2552.25	462.40	10.25	57.25	166.70	14.70	–	–	253.25
36	–	703.55	241.40	–	22.80	78.45	–	–	–	110.65

	16α-OH-Andro	6-Oxo-Adion	7-Keto-DHEA	6α-OH-Adion	6α-OH-T	4-OH-Adion	16α-OH-DHEA	4-OH-T	16α-OH-Adion
0	99.00	–	–	–	–	–	20.40	–	5.70
2	415.05	36131.50	–	75340.75	872.15	–	60.20	–	–
4	288.45	10406.50	–	25985.70	353.45	–	39.70	–	13.75
6	219.35	1614.10	–	5348.75	94.45	–	25.10	–	12.25
8	197.90	407.20	–	1949.25	45.95	5.70	23.90	–	11.65
10	262.65	176.35	–	905.95	23.75	–	33.00	–	11.65
12	187.80	53.10	–	291.85	10.70	7.00	23.10	–	6.90
24	124.90	–	–	11.20	–	–	14.05	–	5.65
30	285.70	–	–	12.40	8.55	6.10	35.25	–	13.10
36	104.60	–	–	–	–	–	12.00	–	–

of DHEA is reduced to 16α-OH-Andro and 16α-OH-Etio which are also metabolites of Andro, Etio and 16α-OH-Adion [14]. Cawley et al. [12] mentioned the natural presence of 5cyclo in urine as metabolite from DHEA sulphate. The combined detection of these metabolites is possible in the described method and therefore will allow to differentiate between Adion and DHEA administration.

The oxidized metabolites also allow for the detection of other steroids which are available as prohormones in nutritional supplements. Together with their metabolites these steroids are also included in this method. Indeed, 7-keto-DHEA is distributed via internet for its antiageing effects and fat-reducing properties. The main metabolites of 7-keto-DHEA are the 7 hydroxy isomers [41,42,45,46] which are included in this method (Fig. 4). Another keto-steroid sold via internet as a supplement is 6-oxo-Adion. As expected 6-oxo-Adion itself and its reduced 6α-OH metabolites have been identified as main markers for misuse of this aromatase inhibiting steroid. Several urines of an administration study with 6-oxo-Adion, performed according a strict WADA research protocol and the Ethical Committee, University Hospital (Ghent, Belgium) (EC/2005-81/sdp), were analysed using the described method. The total ion chromatogram in Fig. 3 clearly shows the peak of 6α-OH-androstenedione, the main metabolite of 6-oxo-androstenedione. Additional to the metabolites presented by Van Thuyne et al. [37] and Deventer et al. [39], 6β-OH-androsterone and 6β-OH-etiocholanolone were also identified in this study as markers for misuse of 6-oxo-androstenedione. These metabolites also remained detectable up to 30 h post-administration. The maximum concentration of 6β-OH-andro and 6β-OH-Etio was reached after 4 h, which was 2 h later than for 6-oxo-adion, 6α-OH-adion and 6α-OH-T (see Fig. 5). In Table 5, the measured concentrations for each of the monitored steroids over the first 36 h post-administration and a blank urine collected prior to administration is shown. These results do not reveal marked variations in concentration of the other monitored steroids. The identification of two unreported metabolites after intake of the 6-oxo supplement indicates the possible use of the developed method to enhance detection and identify new markers for AAS-misuse.

Another aromatase inhibitor is formestane (4-OH-Adion), which is commonly used as a therapeutic agent in the treatment of breast cancer, is primarily excreted as 4-OH-Adion itself or as 4-OH-testosterone. 4-OH-testosterone is also available as a food supplement but is marketed as a potent steroid with enhanced anabolic effects. Both steroids have a similar metabolism are interconvertible [36] and as shown in Table 3 are quantitatively detectable with this method.

4. Conclusion

A quantitative GC–MS method has been developed for the detection of endogenous anabolic steroids and several hydroxylated and oxygenated metabolites. The method has been validated according to Eurachem guidelines. The method is the first comprehensive quantitative method which combines steroids from the traditional steroid profile and specific hydroxylated metabolites of endogenous steroids. The developed method is also the first method to encompass all endogenous steroids mentioned on the WADA technical document for monitoring endogenous steroids.

Acknowledgements

This project has been carried out with the support of WADA. The Flemish Ministry of Culture, Youth, Sports, Media and Brussels is gratefully acknowledged for its financial support. The technical assistance of W. Van Gansbeke is well appreciated.

References

[1] WADA, The 2007 Prohibited List International Standard, 2007, URL: http://www.wada-ama.org/rtecontent/document/2007_List_En.pdf.
[2] WADA, Reporting and evaluation guidance for testosterone, epitestosterone, T/E ratio and other endogenous steroids, Technical Document TD2004EAAS, Version 1.0, 2004.
[3] M. Donike, M. Ueki, Y. Kuroda, H. Geyer, E. Nolteernsting, S. Rauth, W. Schänzer, U. Schindler, E. Völker, M. Fujisaki, J. Sports Med. Phys. Fitness 35 (1995) 235.
[4] H. Geyer, U. Mareck-Engelke, W. Schänzer, M. Donike, in: W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck-Engelke (Eds.), Proceedings of the Manfred Donike Workshop [15th] Cologne Workshop on Dope Analysis, Sport & Buch Strauß, Cologne, 1997, p. 107.
[5] M. van Houten, L. Gooren, Asian J. Androl. 2 (2000) 13.
[6] A. Kicman, D. Gower, Ann. Clin. Biochem. 40 (2003) 321.
[7] A. Heald, F. Ivison, S. Anderson, K. Cruickshank, I. Laing, J. Gibson, Clin. Endocrinol. 58 (2003) 262.
[8] N. Robinson, V. Castella, C. Saudan, P.E. Sottas, C. Schweizer, N. Dimo-Simonin, P. Mangin, M. Saugy, Forensic Sci. Int. 163 (2006) 148.
[9] C. Saudan, N. Baume, N. Robinson, L. Avois, P. Mangin, M. Saugy, Br. J. Sports Med. 40 (2006) 21.
[10] M. Donike, K.-R. Bärwald, K. Klostermann, W. Schänzer, J. Zimmermann, in: H. Heck, W. Hollmann, H. Liesen, R. Rost (Eds.), Sport: Leistung und Gesundheit, Deutscher Ärzte Verlag, Köln, 1983, p. 293.
[11] M. Donike, B. Adamietz, G. Opfermann, W. Schänzer, J. Zimmermann, F. Mandel, in: I.-W. Franz, H. Mellerowicz, W. Noack (Eds.), Training und Sport zur Prävention und Rehabilitation in der technisierten Umwelt, Springer-Verlag, Berlin/Heidelberg/New York/Tokyo, 1985, p. 503.
[12] A. Cawley, E. Hine, G. Trout, A. George, R. Kazlauskas, Forensic Sci. Int. 143 (2004) 103.
[13] J.-F. Lévesque, C. Ayotte, in: W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck-Engelke (Eds.), Recent Advances in Doping Analysis, Proceedings of the 17th Manfred Donike Workshop on Dope Analysis, vol. 7, Sport & Buch Strauß, Cologne, 1999, p. 169.
[14] D. van de Kerkhof, Steroid profiling in doping analysis, Ph.D. Thesis, Utrecht University, 2001.
[15] P. Van Eenoo, F. Delbeke, J. Steroid Biochem. Mol. Biol. 101 (2006) 161.
[16] C. Ayotte, D. Goudreault, A. Charlebois, J. Chromatogr. B 687 (1996) 3.
[17] J.-F. Lévesque, C. Ayotte, in: W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck-Engelke (Eds.), Proceedings of the Manfred Donike Workshop 17th Cologne Workshop on Dope Analysis, Sport & Buch Strauß, Cologne, 1999, p. 213.
[18] M. Thevis, H. Geyer, U. Mareck, U. Flenker, W. Schanzer, Ther. Drug Monit. 29 (2007) 236.
[19] C. Shackleton, J. Steroid Biochem. Mol. Biol. 45 (1993) 127.
[20] M. Donike, H. Geyer, A. Gotzmann, M. Kraft, F. Mandel, E. Nolteernsting, G. Opfermann, G. Sigmund, W. Schänzer, J. Zimmermann, in: P. Bellotti, G. Benzi, A. Ljungqvist (Eds.), Official Proceedings of the International Athletic Foundation World Symposium on Doping in Sport, International Athletic Foundation, Monte Carlo, Florenz, 1987, p. 80.
[21] H. Geyer, W. Schänzer, U. Mareck-Engelke, E. Nolteernsting, G. Opfermann, in: W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck-Engelke (Eds.), Proceedings of the Manfred Donike Workshop 16th Cologne Workshop on Dope Analysis, Sport & Buch Strauß, Cologne, 1998, p. 99.
[22] H. Geyer, U. Mareck-Engelke, E. Nolteernsting, G. Opfermann, M. Donike, in: M. Donike, H. Geyer, A. Gotzmann, U. Mareck-Engelke (Eds.), Proceedings of the Manfred Donike Workshop 12th Cologne Workshop on Dope Analysis, Sport & Buch Strauß, Cologne, 1995, p. 199.
[23] Eurachem, The Fitness for Purpose of Analytical Methods, A Laboratory Guide to Method Validation and Related Topics, vol. 1, 1998.
[24] G. Messeri, G. Cugnetto, G. Moneti, M. Serio, J. Steroid Biochem. 20 (1984) 793.
[25] E. Vanluchene, W. Eechaute, D. Vanderkerckhove, J. Steroid Biochem. 16 (1982) 701.
[26] F. Bressolle, M. Bromet-Petit, M. Audran, J. Chromatogr. B 686 (1996) 3.
[27] W. Verwaal, M. van Bavel, A. Boot, J. Bravenboer, F. de Goei, C. Maas, A. Van der Putten, De ware(n)-chemicus, Valideren van (fysisch-) chemische en (fysisch-) mechanische methoden op het niveau van de wettelijke eis, vol. 26, 1996.
[28] A. Maître, C. Saudan, P. Mangin, M. Saugy, J. Anal. Toxicol. 28 (2004) 426.
[29] S. Rendic, in: M. Donike, H. Geyer, A. Gotzmann, U. Mareck-Engelke, S. Rauth (Eds.), Proceeding 10th Cologne Workshop on Dope Analysis, Sport & Buch Strauß, Cologne, 1992, p. 27.
[30] O. Namba, Y. Miyachi, T. Kawahara, M. Irie, Y. Kuroda, Horm. Sports 55 (1989) 275.
[31] A. Kicman, R. Brooks, S. Collyer, D. Cowan, M. Nanjee, G. Southan, M. Wheeler, Br. J. Sports Med. 24 (1990) 321.
[32] V. Uralets, P. Gillette, J. Anal. Toxicol. 23 (1999) 357.
[33] V. Uralets, P. Gillette, J. Anal. Toxicol. 24 (2000) 188.
[34] P. Van Eenoo, F. Delbeke, N. Desmet, P. De Backer, in: W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck-Engelke (Eds.), Recent Advances in Doping Analysis, Proceedings of the 16th Manfred Donike Workshop on Dope Analysis, vol. 6, Sport & Buch Strauß, Cologne, 1998, p. 171.
[35] M. Garle, E. Palonek, in: W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck-Engelke (Eds.), Recent Advances in Doping Analysis, Proceedings of the 16th Manfred Donike Workshop on Dope Analysis, vol. 6, Sport & Buch Strauß, Cologne, 1998, p. 181.

[36] M. Kohler, M.K. Parr, G. Opfermann, M. Thevis, N. Schlörer, F.-J. Marner, W. Schänzer, Steroids 72 (2007) 278.
[37] W. Van Thuyne, P. Van Eenoo, P. Mikulcíková, K. Deventer, F. Delbeke, Biomed. Chromatogr. 19 (2005) 689.
[38] G. Opfermann, W. Schänzer, in: W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck-Engelke (Eds.), Proceedings of the Manfred Donike Workshop 14th Cologne Workshop on Dope Analysis, Sport & Buch Strauβ, Cologne, 1996, p. 247.
[39] K. Deventer, P. Van Eenoo, P. Mikulcíková, W. Van Thuyne, F. Delbeke, J. Chromatogr. B 828 (2005) 21.
[40] J.-F. Levesque, M. Gaudreault, R. Houle, N. Chauret, J. Chromatogr. B 780 (2002) 145.
[41] P. Van Eenoo, F. Delbeke, N. Desmet, P. De Backer, in: W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck-Engelke (Eds.), Recent Advances in Doping Analysis, Proceedings of the 19th Manfred Donike Workshop on Dope Analysis, vol. 9, Sport & Buch Strauβ, Cologne, 2001, p. 91.
[42] F. Delbeke, P. Van Eenoo, W. Van Thuyne, N. Desmet, J. Steroid Biochem. Mol. Biol. 83 (2003) 245.
[43] B. Robinzon, K. Michael, S. Ripp, S. Winters, R. Prough, Arch. Biochem. Biophys. 412 (2003) 251.
[44] S. Chalbot, R. Morfin, Drug Metab. Dispos. 33 (2005) 563.
[45] R. Hampl, M. Hill, L. Stárka, J. Steroid Biochem. Mol. Biol. 78 (2001) 367.
[46] L. Stárka, R. Hampl, M. Hanu], M. Matou]ková, M. Hill, Clin. Chem. Lab. Med. 43 (2005) 1218.