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HPLC Clean-up for Urine Samples with Disturbing Background for Confirmation of 17ß-Methyl-5ß-androst-1-ene-3α,17α-diol (Epimetenediol)

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Introduction

In dope analysis, the confirmation of anabolic androgenic substances and their respective metabolites are a field of great interest. This substances are widely misused in numerous and variety of different sports. The main goal is to improve the detection limit and thus enhance the retrospection of a pharmacological manipulation. One of the main metabolites of Metandienon, the 17ß-methyl-5ß-androst-1-ene-3α,17α-diol (epimetenediol), is confirmed by gas chromatography-high-resolution mass spectrometry (GC-HRMS). If the substance appears in low concentrations, it is necessary to improve the sample cleaning-up procedure. To avoid elution of disturbing substances with the compound of interest a cleaning step by high performance liquid chromatography (HPLC) is added to the routine sample cleaning steps in screening procedure for conjugated anabolic androgenic substances (screening IVa). Utilizing this method, it is possible to enhance the detection limit compared to non HPLC treated samples. On the other hand it is possible, in addition with the confirmation by single ion monitoring (SIM) as bis-TMS m/z 448, 433, 358, 343 to obtain full scan mass spectra from urine samples with low concentrations of epimetenediol (e.g. 2 ng/ml). All suspicious samples from routine screening will be treated in a second analysis by the described procedure [1, 2].

Material and Method

Steroids

All steroids were obtained from different commercial sources or were synthesized in our laboratory. All other reagents and solvents were of analytical grade or if indicated of HPLC grade quality.

HPLC system

A liquid chromatograph series 1090 from Hewlett Packard (Böblingen, D) with automatic injection system and diode array detector was used for all samples. A fraction collector, (Gilson FC 205, Middleton, USA) with 3-way-valve was connected on-line to the HPLC system and specified to collect by defined time windows. Each time window was created individually considering the retention time and the peak width of the substance of interest. The fractions were collected in separate glass tubes. According to this procedure it was possible to run several samples automatically in sequence. A reversed phase column, LiChrospher® RP18, 5 µm, 125x4 mm I.D. (Merck, Darmstadt, D) was used for separation with solvent (A) water and solvent (B) acetonitrile (Scharlau, Barcelona, E) with a flow of 1 ml/min. All solvents were HPLC grade quality and were degassed and filtered through a 0.45 µm Teflon membrane under reduced pressure prior to use. A linear gradient starting with 30% B was increased linear to 100% B in 17.5 minutes, this corresponds to an increase of 4% B/min. The overall run time was 18.5 minutes followed by a post time of 4.2 minutes to equilibrate the column before the next injection started. The injection-volume was 50 µl, the diode array detector was set to a wavelength of 200 nm (reference wavelength 450 nm).

Sample preparation

The dry residue of the urine sample (8-16 ml), after separation of the free fraction, hydrolysis and n-pentane extraction, as described elsewhere [2, 3, 4], was resolved in 150 µl MeOH. The methanolic layer was transferred to an HPLC auto sampler vial for maximum volumes of 150 µl (Löwinger, Münnerstadt, D). The methanol was concentrated to approximately 55-60 µl. Before the sample collection by defined time windows started, the retention time of Epimetenediol had to be estimated under the described HPLC conditions. Fifty µl of an epimetenediol standard solution in methanol (5 µg/ml) was injected three times to estimate the mean retention time. A delay time for collection has to be considered. This delay time is required for liquid to move from the flow cell of the diode array detector to the dispense head of the collector. The delay time (minutes) is calculated by the formula:

$$\underset{min}{delay\ time} = (\underset{cm}{length\ of\ capillary} \times \underset{cm^2}{area}) \times \underset{ml/min}{flow\ rate}^{-1}$$

A protocol of the whole sequence for confirmation of a positive urine sample is described in table 1. In addition to the first fraction collected corresponding to the elution time of epimetenediol a second fraction is collected (17.5 min-18.4 min) to avoid any remaining contamination at the dispenser head of the sample collector regarding the next injected sample.

The fraction corresponding to epimetenediol (12.3-12.8 min) was collected for each sample in separate glass tube, evaporated to dryness under water vacuum, dried in a vacuum desiccator over KOH/P_2O_5 and prepared for GC-HRMS analysis [2].

Table 1: Sequence protocol to confirm a suspicious urine sample for epimetenediol

Inject.No.	sample	Inject.vol. [µl]	R.T. [min]	fraction collected [min]	fraction collected [min]
1	epimetenediol (5 ppm)	50	12.215	-	-
2	epimetenediol (5 ppm)	50	12.210	-	-
3	epimetenediol (5 ppm)	50	12.213	-	-
4	Methanol	50	-	-	-
5	epimetenediol (0.25 ppm)	50	-	12.3-12.8	17.5-18.4
6	Methanol	50	-	12.3-12.8	17.5-18.4
7	Blank urine	50	-	12.3-12.8	17.5-18.4
8	MeOH	50	-	12.3-12.8	17.5-18.4
9	suspicious sample	50	-	12.3-12.8	17.5-18.4

Results and Discussion

All urine samples, prepared routinely for procedure IVa (conjugated anabolic androgenic steroids and their respective metabolites) will be handled as follows:

Scr IVa (conjugated fraction)
↓
analysis by GC-MS quadrupole
↓
re-analyses by GC-HRMS
if suspicious for epimetenediol:
↓
new sample clean up
↓

8-16 ml urine separation of the free fraction hydrolysis n-pentane extraction dry residue is prepared for HPLC

Table 2: HPLC retention times (R.T.) of different endogenous and anabolic steroids, their respective metabolites and other substances in screening procedure IVa.
Column: LiChrospher® RP18, 5 μm, 125x4 mm I.D., flow: 1 ml/min,
solvent A: water, solvent B: acetonitrile; gradient: 30%B linear to 100%B in 17.5 min

R.T.	Endogenous and anabolic steroids and other substances in screening IVa	
1.603	Fluoxymesterone-M1	9α-Fluoro-17α-methyl-androst-4-ene-3α,6ß, 11ß,17ß-tetrol
1.846	Pemoline	
3.550	Metandienone-M2	6ß-Hydroxymetandienone
3.969	Salbutamol	
4.140	Tetrahydrocortisol	
5.175	Dehydrochloro-methyltestosterone-M1	6ß-Hydroxy-dehydrochloromethyltestosterone
5.396	11-Keto-isoandrosterone	
5.560	Morphine	
5.803	11ß-Hydroxyetiocholanolone	
5.831	Stanozolol-M1	3'-Hydroxystanozolol
6.034	11ß-Hydroxyandrosterone	
6.274	11-Keto-etiocholanolone	
6.304	Stanozolol-M2	4ß-Hydroxystanozolol
6.393	11-Keto-androsterone	
6.562	Boldenone	
7.749	Stanozolol-M3	16ß-Hydroxystanozolol
7.896	Testosterone	
8.611	Dehydroepiandrosterone	
8.630	Boldenone-M1	5ß-Androst-1-en-17ß-ol-3-one
8.646	Epitestosterone	
8.717	5ß-Pregnane-3α,17α,20α-triol	
8.783	5ß-Androstane-3α,17ß-diol	
8.790	Methyltestosterone	
8.903	Metenolone	
8.923	Metandienone-M3	17-Epimetandienone
9.039	Oxymesterone	
9.076	Methyltestosterone-M2	17α-Methyl-5ß-androstane-3α,17ß-diol
9.076	Nandrolone-M2	5ß-Estran-3α-ol-17-one
9.220	4-Chlorodehydro-methyltestosterone	
9.347	5α-Androstane-3α,17ß-diol	
9.510	5α-Dihydrotestosterone	
9.693	Etiocholanolone	
9.886	Nandrolone-M1	5α-Estran-3α-ol-17-one
10.143	Methyltestosterone-M1	17α-Methyl-5α-androstane-3α,17ß-diol
10.183	Metenolone-M1	1-Methylene-5α-androstan-3α-ol-17-one
10.277	Norethandrolone-M1	17α-Ethyl-5ß-estrane-3α,17ß-diol
10.347	Clostebol-M1	4-Chloro-androst-4-en-3α-ol-17-one
10.422	Androsterone	
11.602	5ß-Pregnane-3α,20α-diol	
11.679	Stanozolol	
11.878	Mesterolone-M1	1α-Methyl-5α-androstan-3α-ol-17-one
12.201	Metandienone-M1	17ß-Methyl-5ß-androst-1-ene-3α,17α-diol
12.390	Drostanolone -M1	2α-Methyl-5α-androstan-3α-ol-17-one

The described HPLC cleaning procedure is an appropriate method to improve the detection limit and to obtain full scan spectra in samples with low concentration of epimetenediol for the final confirmation of this compound by GC-HRMS (Figure 2). This method is not restricted to a single substance only. Additional compounds have been investigated under the above described conditions and their respective HPLC retention times have been estimated (Table 2). With the applied sample collector it is possible to collect up to 10 defined aliquots from the sample injected during one HPLC run by the so called 'time window' mode.

To estimate the retention time of epimetenediol it is necessary to use a standard solution of higher concentration (5 µg/ml). Because of the poor UV-absorbance of this compound the detection limit at wavelength 200 nm is at least 150 ng. The amount injected to estimate the retention time is 250 ng, which is roughly 15.5 times higher than the expected amount in the suspicious urine sample. Therefore it is imperative that the sample collector with 3-way-valve does not get in contact with the standard solution for estimating the retention time to avoid any contamination and possibility of carryover. The capillary has to be disconnected behind the DAD outlet and the solvent is collected in a separate waste container.

A high repeatability of the retention times is required to collect reproducible amounts of epimetenediol in each sample. An optical control is not possible during HPLC analysis because of the poor UV-absorption of epimetenediol and the very low concentration present in urine (Figure 1). The retention times of an epimetenediol standard solution (n=6) were calculated as mean: 12.212 min, standard deviation: ±0.003 min (=0.02%). For the future, an internal standard should be adapted to this cleaning-up procedure. To control the reproducibility of the HPLC system, sufficient attempts with trenbolone as internal standard have been made.

Another aspect of interest is the glass ware. All used glass tubes for HPLC sample collection have to be cleaned very carefully. It was performed in our laboratory according to the following procedure:

1) adding 1 ml of 6 N HCl and heat it at 110°C for one hour
2) cleaning by destilled water and afterwards by destilled methanol
3) drying
4) adding 250 µl MSTFA/NH4Cl /acetonitril (10:0.1:90; v:w:v) and heat it at 100°C for 1 hour
5) cleaning by destilled acetone
6) drying

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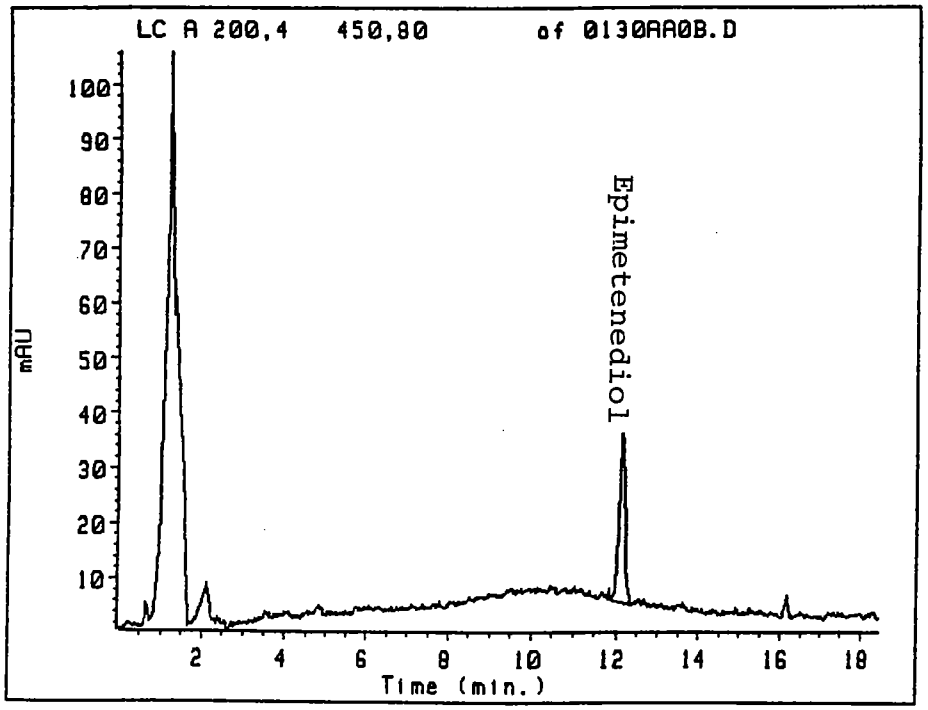
Appendix

Figure 1: HPLC chromatogram at wavelength 200 nm (HPLC conditions as described)
A: Epimetenediol standard solution (500 ng)
B: Urine sample, 8 ml (sample preparation as described)
Fractions collected: 12.3-12.8 min and 17.5-18.4 min.

Figure 2: EI mass spectra of 17ß-Methyl-5ß-androst-1-ene-3α,17α-diol bis-TMS.
Urine (2 ml) liquid-liquid extraction (B) and urine (8 ml) extraction followed by HPLC fractionation (C). Synthesized reference standard (M+ 448) is shown in (A).
Injection in B (split mode 1:20) and C (splitless)

A


LC A 200,4 450,80 of 0130AA0B.D
mAU
100
90
80
70
60
50
40
30
20
10
2
4
6
8
10
12
14
16
18
Time (min.)
Epimetenediol

B

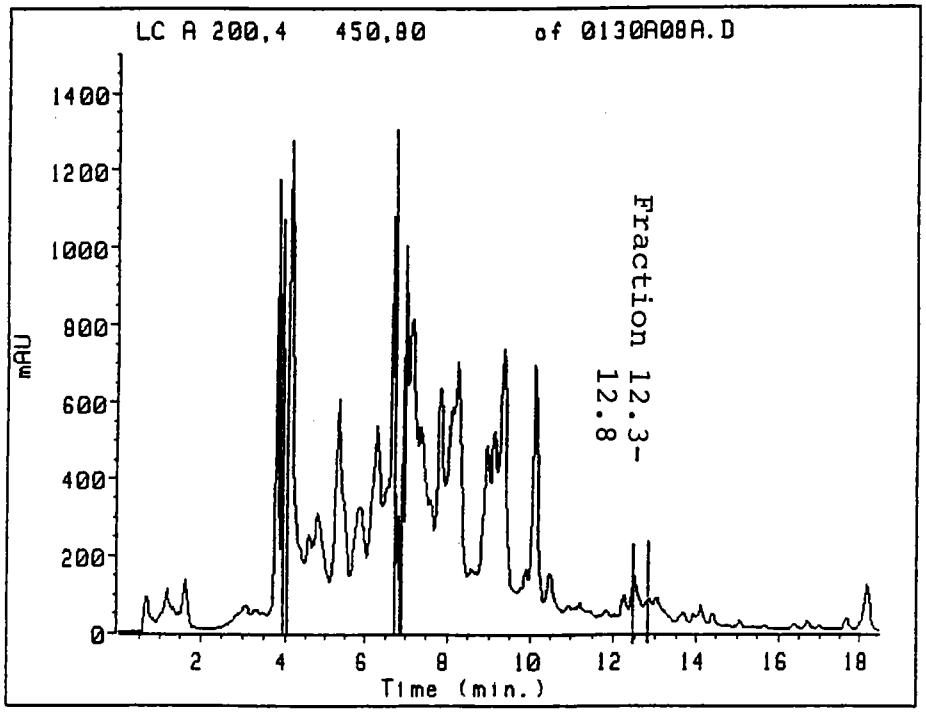
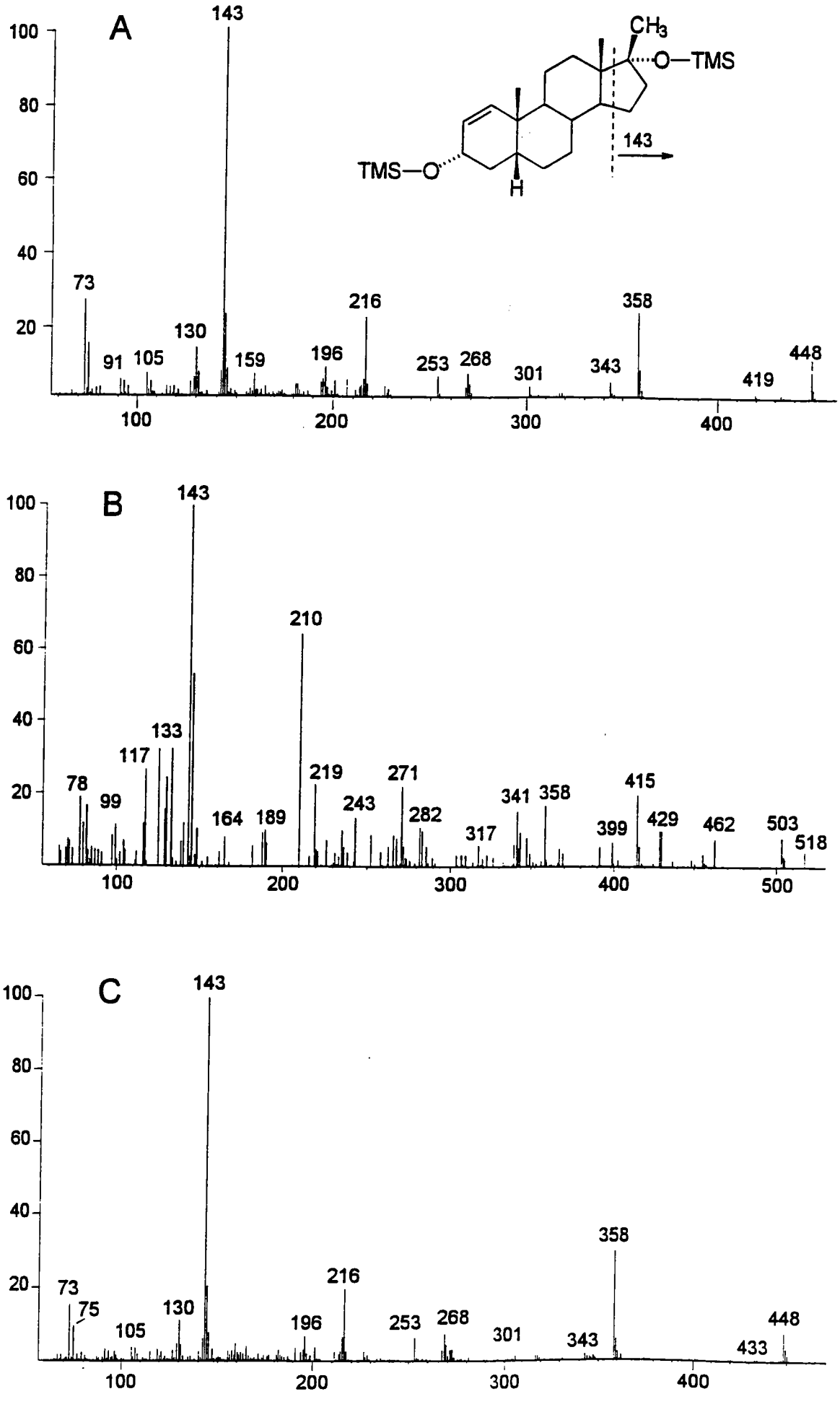

LC A 200,4 450,80 of 0130A08A.D
mAU
1400
1200
1000
800
600
400
200
0
2
4
6
8
10
12
14
16
18
Time (min.)
Fraction 12.3-
12.8

Figure 1


A
143
CH3
O—TMS
TMS—O
H
143
100
80
60
40
20
73
91
105
130
159
196
216
253
268
301
343
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419
448
100
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B
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429
462
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518
100
200
300
400
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C
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433
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100
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Figure 2