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RECENT ADVANCES IN DOPING ANALYSIS (12)

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Characteristics and Abundance of various Conjugated Metabolites Commending the Analysis of the Sulphate Fraction

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1. Introduction

Screening and confirmation methods of positive cases in human doping analysis are often targeted to the free and/or glucuronide metabolites, whereas phenomena like epimerisation in 17-methylsteroids (fig. 1) and formation of long-term-metabolites are known to depend on sulphate conjugation. Analysing a variety of positive human urines, further sulphated metabolites are found on which a confirmation possibly can rely on. Some abundances are surpassing the concentrations of the glucuronides, making detection more sensitive.

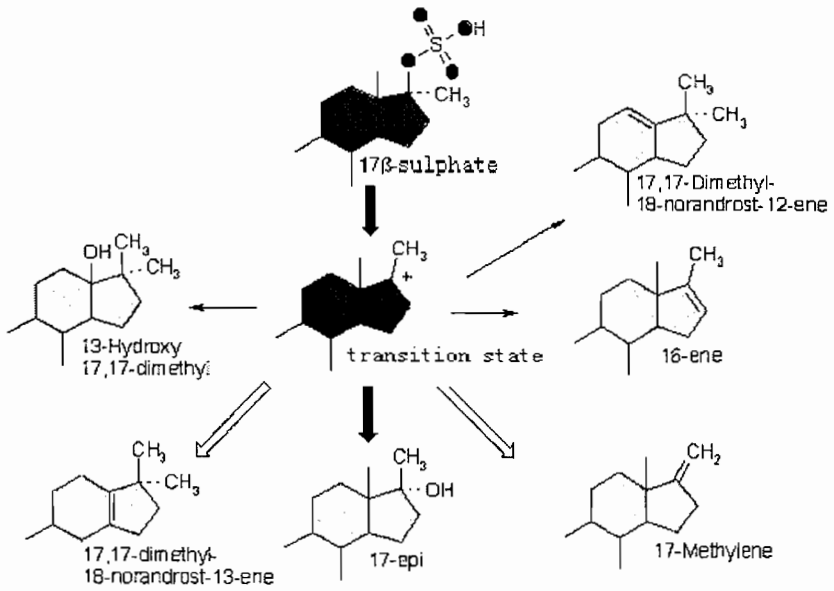

Fig. 1 : possible products of 17-methylsteroids after formation of a carbenium ion from 17-sulphate in urine [1, 2]

Abbreviations: ISTD: internal standard, PC: parent compound, MSTFA: N-Methyl-N-Trimethylsilyltrifluoracetamide, M: metabolite, MG: molecular weight, THC: Tetrahydrocannabinol, THG: Tetrahydrogestrinone, TMS: Trimethylsilyl

2. Sample preparation

Flow scheme:

2 ml urine, add 0,75 ml phosphate buffer 0.8 M (→ pH 7) + internal standard (each 1 µg):
[2,2,4,4-$^2H_4$]- Etiocholanolone, [2,2,3,4,4-$^2H_5$]-Androsterone,-17ß-D-glucuronide,
[2,2,4,4-$^2H_4$]-Androsterone,-17ß-D-Sulphate

↓

Enzymatic hydrolysis with 25 µl ß-glucuronidase E.coli 50 °C, 60 min
+ 250 µl K_2CO_3/ $KHCO_3$ (20 %); extract with 5 ml tert.-Butylmethylether (pH 9)

↓

5 ml organic layer: evaporate/derivatize with 100 µl MSTFA-NH_4I-TMS-S-ethanol
(15 min, 60 °C) transfer in vials ► GC/MS of **free and glucuronated metabolites**

↓

3 ml aqueous layer: remove ether residue + 1 ml sodium acetate-buffer (1 M, pH 4,9→ pH 7),
centrifuge, decant on C18-column (conditioned); 2 wash steps (H_2O; n-heptane)
elution (1 ml Methanol) + [2,2,4,4-$^2H_4$]-Etiocholanolone

↓

Chemical cleavage: + 5 ml ethylacetate/H_2SO_4 (250 ml/200mg), 60 °C, 30 min
+ 0.5 ml KOH 1M, evaporate ethylacetate, + 0,75 ml phosphate buffer 0.8 M
+ 250 µl K_2CO_3/ $KHCO_3$ (20 %)→ pH 9; extract with 5 ml tert.-Butylmethylether

↓

Decant organic phase, evaporate, derivatize with 100 µl MSTFA-NH_4I-TMS-S-ethanol (15
min 60 °C), transfer in vials of sulphated metabolites ►GC/MS of **sulphated metabolites**

A sample preparation method with additional chemical cleavage of sulphate conjugates has been basically introduced [3,4] as an optimized clean-up for anabolic steroid conjugates. It is suited well for the presented compounds. Alkaline extraction is performed twice to exclude contamination of the sulphates with not extracted glucuronide metabolites. Internal standards are added to the glucuronide and sulphate fraction prior to cleavage for control of cleavage, retention times and calibration.

3. Results of metabolites in the sulphate fraction

3.1. Parameter of analysis:

GC-System: HP 5890; MS: HP 5971; Aquisition mode: SIM; TMS-derivatives;

Oven program: 180 °C → 3°C/min →230 °C, 230 °C → 30°C/min →310 °C

3.2. Remarks:

- Results have been confirmed with up to 10 positive cases
- Results represent only sulphated or glucuronidated metabolites, no results concerning mixed conjugates are investigated
- Concentrations of metabolites are only estimated via unconjugated standards
- Recovery of some metabolites is poor (salbutamol, THC [5])
- Calculation of excretion rates is not possible as no excretion studies were conducted, results are case studies from positive urines
- Interindividual variability of phase-II-metabolism (degree of sulphatation or glucuronidation) is to be considered and has been obvious in analyzed samples, results show trends or means

3.3. Major metabolites

In the samples analysed, concentrations of sulphate-metabolites of cocaine, spironolactone and morphine outrange the corresponding glucuronide-metabolites:

metabolite found	ratio sulphate: glucuronide =
Benzoylecgonine (fig. 2)	2,4 : 1
Morphine (fig. 3)	1 : 1 – 1 : 6
Canrenone (fig. 4)	5 : 1

Table 1: major metabolites found in the sulphate fraction of positive human urines

More data is needed for the metabolites of morphine. Procedures differing from chemical cleavage with methanol + ethylacetate + H_2SO_4 had more or less drawbacks. Hydrolysis with enzyme (H.pomatia) is incomplete, hydrolysis in HCl at 80 °C is unsatisfactory due to degradation of unconjugated metabolite, also extraction is incomplete.

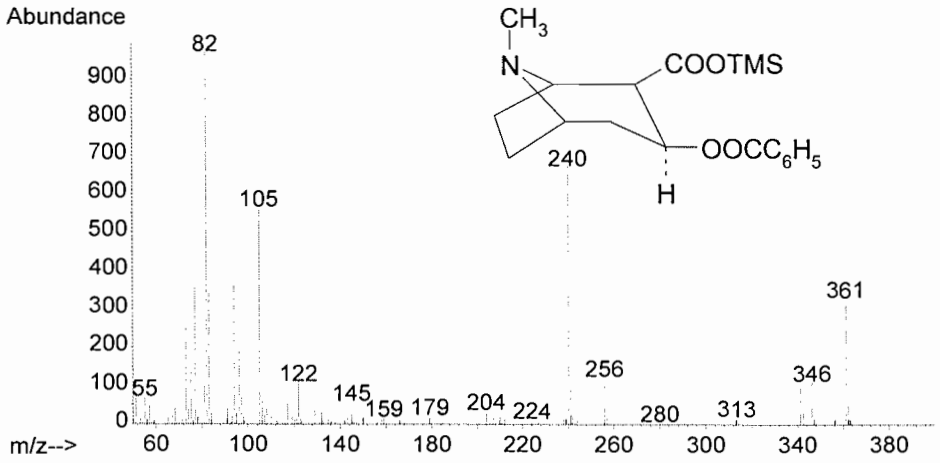

Fig. 2: mass spectrum and structure of benzoylecgonine-O-TMS, MG = 361, detected metabolite of cocaine in the sulphate fraction

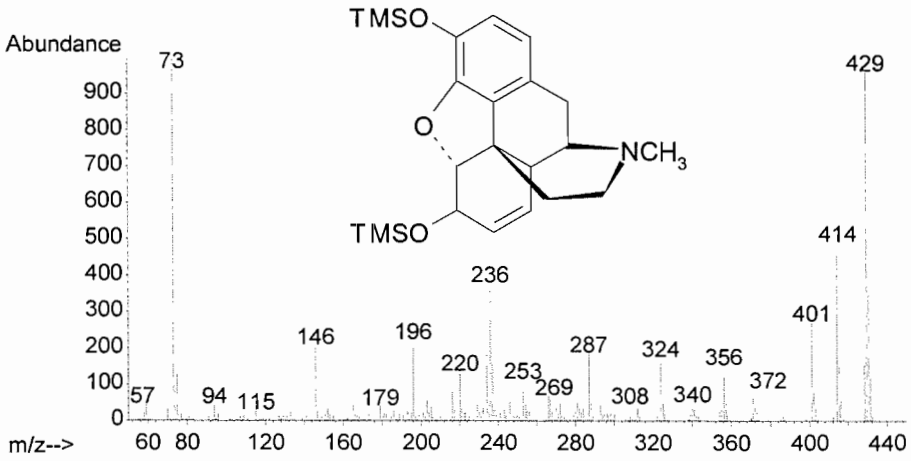

Fig. 3: mass spectrum and structure of morphine-O-TMS, MG = 429, detected metabolite of morphine in the sulphate fraction

3.4. Minor metabolites

The term "minor" metabolites relates to the amount of metabolite found, especially compared to that in the glucuronide fraction. Most of the "minor" metabolites in the sulphate fraction are neglectable, but the interindividual variability of phase-II-metabolism has to be remembered. A clostebol-long-term-metabolite (O-TMS: M+ 470, fig. 5, table 2) is found only as sulphate. Sulphated parent compounds and those metabolites known from the glucuronide fraction were investigated allowing easy integration of metabolites into existing screening procedures, but there might be other metabolites beyond that worth while analyzing as their sulphates.

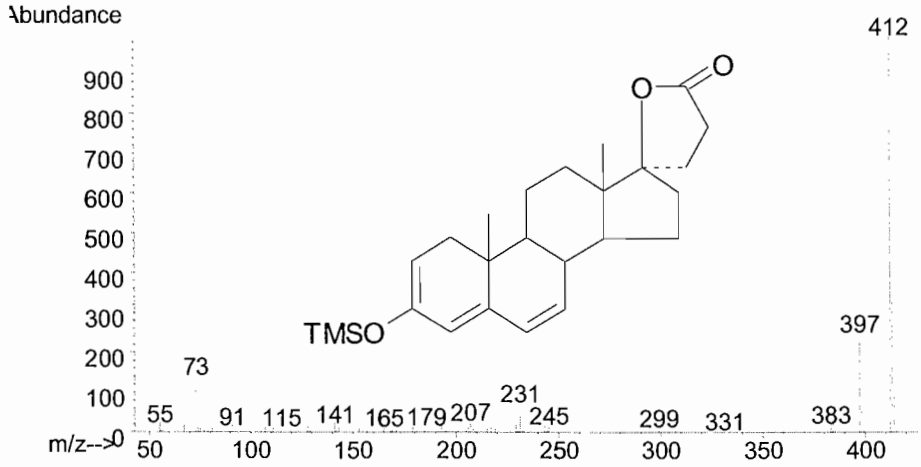

Fig. 4: mass spectrum and structure of canrenone- O-TMS, MG = 412, detected metabolite of Spironolactone in the sulphate fraction

Administration of	Metabolite detected (as sulphate)	Ratio sulphate : glucuronide	TMS-derivatives [m/z]
Cannabinoids	Δ9-Carboxy-THC	1 : 18	488, 473, 371
Metenolone	1-Methylen-5α-androstane-3α-ol-17-one-	1 : 2,2	446, 431, 341
	PC-	1 : 7 (PC)	208, 195 (PC)
Clostebol	4-Chloro-androst-4-ene-3α-ol-17-one-(M1)	1 : 30 (M1)	466, 468, 451 (M1)
	4-Chloro-5α-androstane-3α-ol-17-one-(M3)	1 : 28 (M3)	453, 468, 455 (M3)
	4-Chloro-3ß-hydroxy-5α-androstane-17-one-	Only sulphate (fig 5)	470, 468
Tamoxifen	PC-	1 : 10	58, 72, 489
Oxymetholone	17α-Methyl-5α-androstane-3α,17β-diol-	1 : 6	435, 345, 270

Table 2: Characteristics of metabolites in human positive urines (3 – 10 cases)

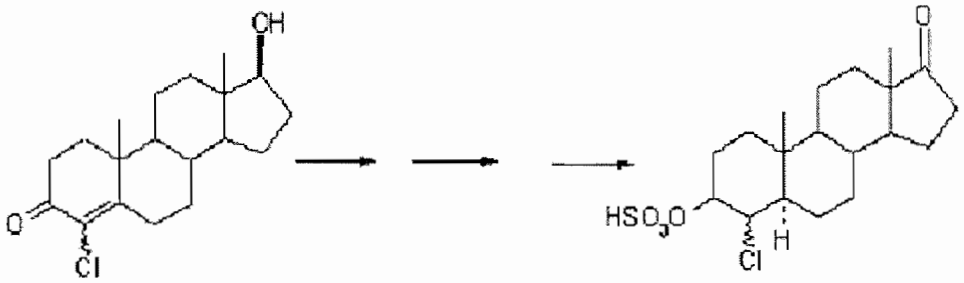

Fig. 5: 4-Chloro-3β-hydroxy-5α-androstane-17-one, a long-term tetrahydro-metabolite of clostebol, according to [6]

3.5. Metabolites in horse urine

In the horse phase-II-metabolism in the form of sulphatation is very prominent, a lot of metabolites of endogenous compounds, such as steroids are excreted as sulphates; pharmaceuticals are often mainly metabolised to their parent compound sulphates (e.g. metabolites of MT and metandriol which are found as PC-sulphate as well as their 17β-epimers). Chemical cleavage of sulphates is therefore suited for detection of drug abuse in the horse. The results of a positive case with allyltrenbolone (Altrenogest) are presented. Allyltrenbolone (fig. 6) shares the 4,9,11-trien-3-one-structure with steroids of interest like trenbolone, gestrinone and the latest designer drug THG. It is used for the synchronisation of estrus in mares mainly for breeding and is believed to posess anabolic properties.

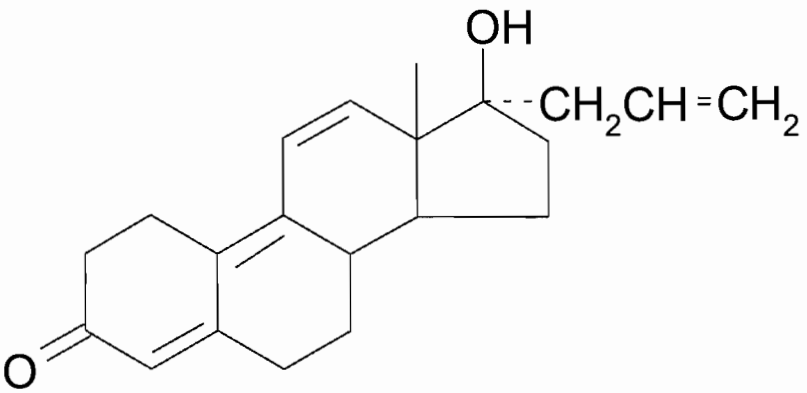

Fig. 6: 17α-Hydroxy-17-(2-propenyl)estra-4,9,11-trien-3-one (allyltrenbolone), MG: 310

Enzymatical hydrolysis with either ß-glucuronidase from E. coli or arylsulfatase/glucuronidase from H. pomatia resulted only in marginal amounts of cleaved metabolite. In addition silylation is inappropriate since enolization of 4,9,11-trien-3-one-structures leads to by-products and decomposition [7]. As the main urinary metabolite is proposed to be the glutathion-conjugated allyltrenbolone, an effective analytical method based on chemical cleavage and LC/MS/MS-detection with chemical ionisation was developed.

3.5.1. Analysis parameters

Model LC: Agilent 1100 LC; MS : PerkinElmer API 2000

flow parameter: 0,5 ml/min; column: Purospher Licrochart C_{18}

Gradient: 90% NH4-Acetate + 10% CH3CN ⇒ 10% NH4-Acetate + 90% CH3CN in 8 min

Declustering potential (V): analyte: 26; ISTD (Fluoxymesterone): 16

3.5.2. Result of equine urine of a horse positive with allyltrenbolone

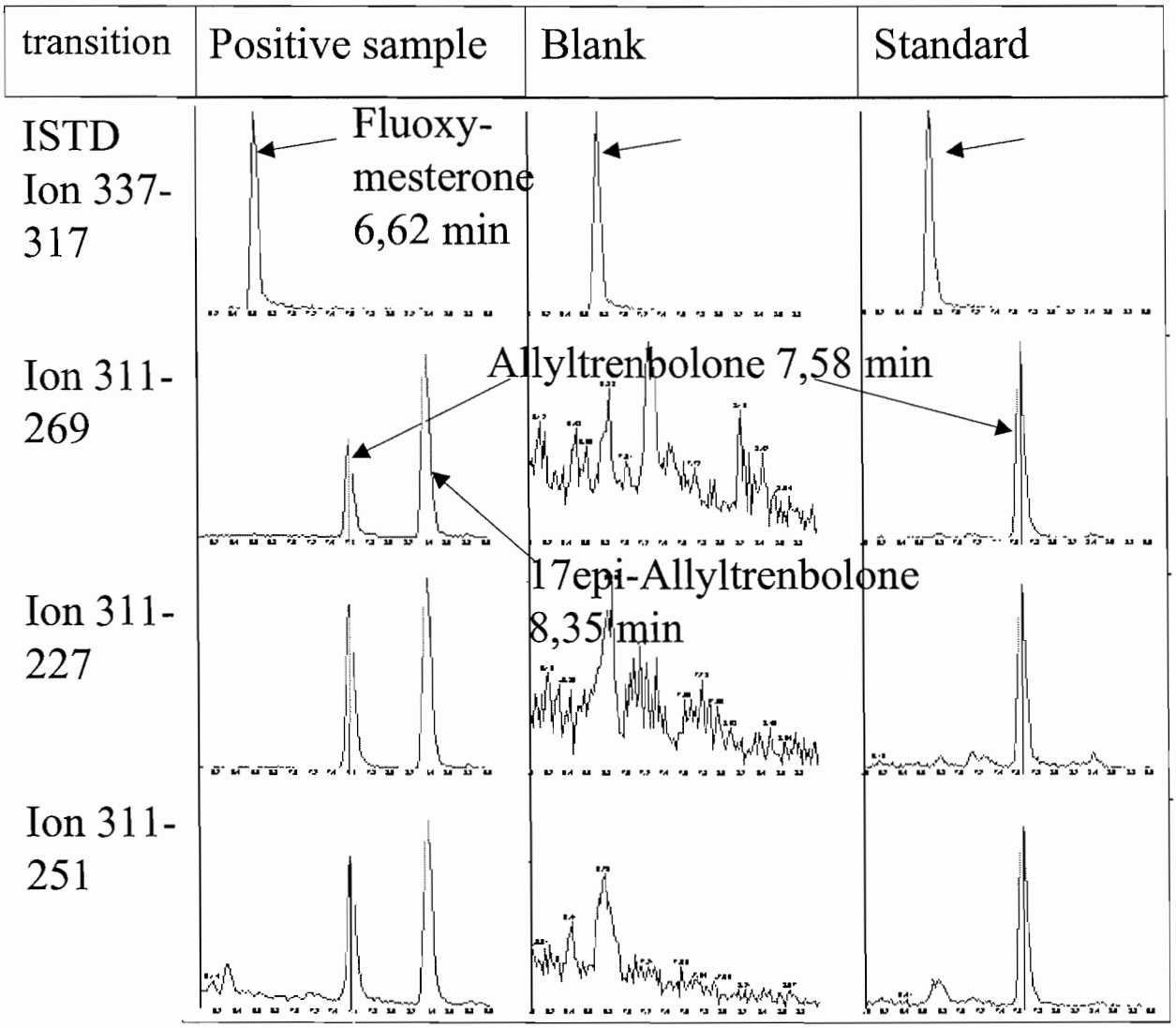

Fig. 7: LC/MS/MS-result of equine urine of a horse positive with allyltrenbolone, a blank and a standard urine after chemical cleavage

Ion transition m/z (311 = M+ H^+)	Collision energy (V)	Electron potential (V)	Relative intensity 17α-allyltrenbolone RT 7,58 min	Relative intensity 17β-allyltrenbolone RT 8,35 min
ISTD (337-317) Fluoxymesterone	30	-7		
311-227	33	-12	100	100
311-269	19	-12	47	82
311-251	251	-12	43	49

Table 3: relative intensity of fragment ions and LC/MS/MS-parameter for ion transitions in the detectionof deconjugated allyltrenbolone

With LC/MS/MS it was possible to confirm allyltrenbolone parent compound as well as the 17ß-epimer of allyltrenbolone in the conjugated fraction (fig. 7), cleavable with methanol, ethylacetate and H_2SO_4. The relative intensities of fragment ions from precursor ion 311 in positive mode are identical with a standard allyltrenbolone (table 3), the maximal relative standard deviation being 4.2%. The resulting metabolites could be confirmed in a controlled excretion study, but the structure of the conjugated metabolites has still to be confirmed.

4. Conclusion and summary

The processing of urine samples for the analysis of sulphate metabolites with the means of chemical cleavage in methanol, ethylacetate and H_2SO_4 is easy but more time consuming than enzymatic hydrolysis alone. To ensure complete extraction of glucuronide metabolites, these are extracted twice with *tert*-butylmethylether.

The phase II sulphate conjugates have already been described as their equivalent glucuronide conjugates or the parent compound is excreted as sulphate. Hence the investigated metabolites can be integrated into existing screening procedures with little expense, enabling more sensitivity or a higher degree of certainty in positive or suspicious cases. Sulphate metabolites of canrenone and cocaine are excreted in large amounts in human urine, strongly suggesting inclusion of sulphate analysis for more sensitive detection. In suspicious cases the sulphate metabolites of other drugs might give additional information.

There might also be supplementary sulphate metabolites which have not yet been observed. Long-term excretion of sulphate metabolites could be a helpful tool to detect abuse of banned substances, as is evidenced in the case of an known clostebol-long-term-metabolite.

Interindividual variability of phase-II-metabolism (degree of sulphation or glucuronidation) is high and has to be considered regarding ratios between sulphated and glucuronidated metabolites. Moreover this ratio can change with increasing time as has been seen after the application of norsteroids.

Allyltrenbolone and 17-epi-allyltrenbolone are excreted in urine as conjugates after application of allyltrenbolone in the horse. Both metabolites can be cleaved via chemical cleavage and detected with LC/MS/MS.

5. Literature

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