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Doping control screening procedure for trenbolone analogues in human urine by LC-(APCI)-MS/MS

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1. Introduction

In the recent years the misuse of synthetically modified steroids (designer steroids) has been reported in professional sport repeatedly [1]. The virtually unlimited variety of these compounds places a challenge for target analysis in doping control. One class of substances based on a 4,9,11-triene steroidal structure with a methyl residue at C-13 and different modifications at C-17 are trenbolone analogues, providing anabolic properties, and, thus are prohibited in sport according to regulations of the World-Anti-Doping-Agency (WADA) [2].
To improve screening for new target substances in doping control a qualitative assay based on LC-(APCI)-MS/MS was developed enabling the sensitive and selective detection of trenbolone (17β-hydroxy-estra-4,9,11-trien-3-one), epitrenbolone (17α-hydroxy-estra-4,9,11-trien-3-one) and the chemically modified analogues methyltrenbolone (17β-hydroxy-17-methylestra-4,9,11-trien-3-one), ethyltrenbolone (17β-hydroxy-17-ethylestra-4,9,11-trien-3-one), propyltrenbolone (17β-hydroxy-17-propylestra-4,9,11-trien-3-one), "17-ketotrenbolone" (17-oxo-estra-4,9,11-trien-3-one) and altrenogest (17β-hydroxy-17-(2-propen-1-yl)estra-4,9,11-trien-3-one) in human urine.

2. Methods

Reference compounds

While trenbolone, epitrenbolone and altrenogest were obtained from commercial sources, methyl-, ethyl-, propyltrenbolone and 17-ketotrenbolone were prepared in-house [3].

Sample Preparation

Briefly, after enzymatic hydrolysis (1h, 50ºC, pH=7.0) with β-glucuronidase from E. Coli, analytes were extracted with TBME at a pH of 9.6. The separated organic layer was evaporated, and the dry residue was reconstituted in 60 µl of a mixture of 5 mM ammonium acetate buffer containing 0.1 % glacial acid and methanol (1:1, v:v) [2].

LC-MS/MS

All analyses were performed on a Series 1100 liquid chromatograph (Agilent, Waldbronn, Germany) coupled to an API 2000™ triple quadrupole mass spectrometer (Applied Biosystems, Darmstadt, Germany). The LC was equipped with a Merck Purospher STAR RP-18e column (55 x 4 mm; particle size 3 µm). A reverse phase gradient was employed using A: ammonium acetate buffer (5 mmol/L, 0.1 % glacial acid; pH 3.5) and B: acetonitrile starting at 15 % B and changing to 100 % B within 7 min (post-time 1.25 min). The flow rate was set to 0.5 mL/min.

For mass spectrometric characterization of the analytes product ion spectra were recorded via direct infusion of pure reference compounds and positive electrospray ionisation (ESI) followed by collisionally activated dissociation (CAD) with nitrogen as collision gas (2.6e-3 Pa). For MRM experiments different parameters such as declustering potential and collision energy were optimized for maximum sensitivity of each ion transition of the analytes.

For screening purposes ionisation was accomplished by means of APCI in positive mode with an interface temperature of 450 ºC. Diagnostic ions of the analytes were detected in the multiple reaction monitoring mode of the instrument as described in Table 1.

3. Results

LC-MS/MS Analyses

For mass spectrometric characterization ESI product ion spectra of all analytes were acquired. Exemplarily, the product ion spectra of methyltrenbolone, ethyltrenbolone and 17-ketotrenbolone are depicted in Fig. 1-3. In accordance to earlier studies on trenbolone and propyltrenbolone characteristic fragment ions are observed at *m/z* 227 for all trenbolone analogues, confirming the common fragmentation pathway for 4,9,11-triene based steroids with a methyl residue at C-13 [3].

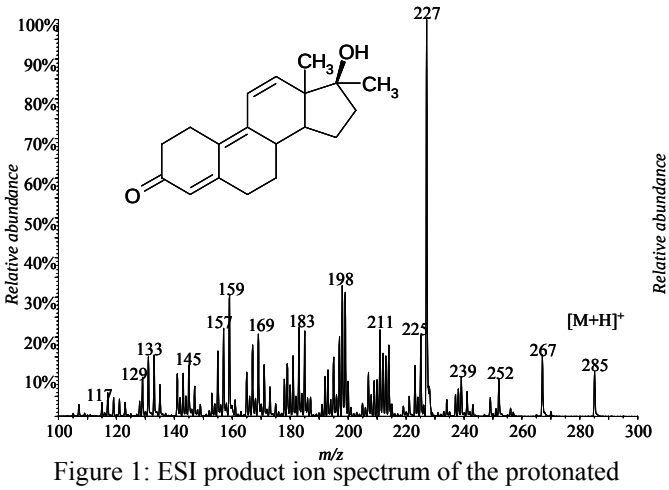

Figure 1: ESI product ion spectrum of the protonated molecule of methyltrenbolone (MW 284, CE=40eV)

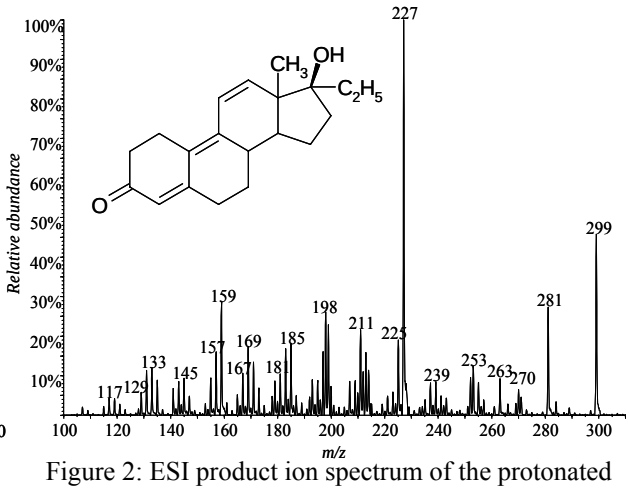

Figure 2: ESI product ion spectrum of the protonated molecule of ethyltrenbolone (MW 298, CE=40eV)

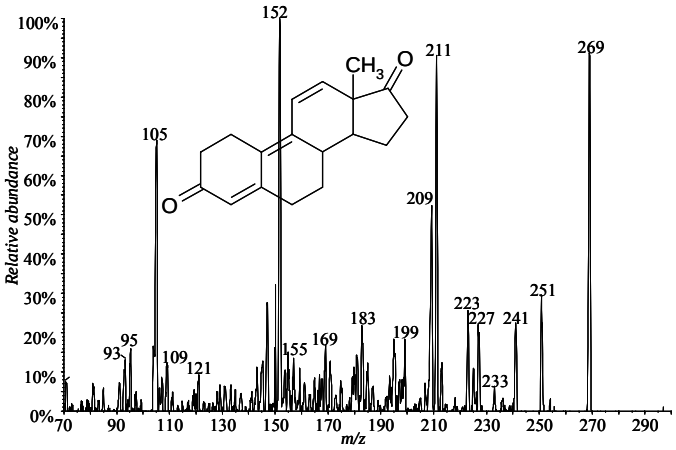

Figure 3: ESI product ion spectrum of the protonated molecule of 17-ketotrenbolone (MW 268, CE=30eV)

Validation

The qualitative assay was completely validated regarding the parameters specificity, assay limit of detection (LLOD), intra- and inter-assay precision and recovery (Tab. 1). The analysis of blank urine samples (n=10) demonstrated the specificity of the method as presented in Fig. 4, and all analytes were sufficiently detected at their minimum required performance limit (MRPL) of 10 ng/mL (Fig. 4).

Tab. 1: MS parameters and validation results of trenbolone and its analogues

	MS parameters		**validation results**			
	precursor/product ion (*m/z*)	collision energy (eV)	LLOD (S/N>3, ng/mL)	intra-assay precision CVs (%, n=6+6+6)	inter-assay precision CVs (%, n=18+18+18)	recovery (%)
trenbolone	271/199	39	10	13-17	14-16	105
epitrenbolone	271/199	39	5	9-14	11-12	95
methyltrenbolone	285/227	33	10	5-14	10-14	91
17-ketotrenbolone	269/211	21	10	7-20	18-20	86
ethyltrenbolone	299/227	30	2	4-5	6-12	72
propyltrenbolone	313/227	30	2	2-7	5-11	81
altrenogest	311/227	30	3	3-7	7-13	92

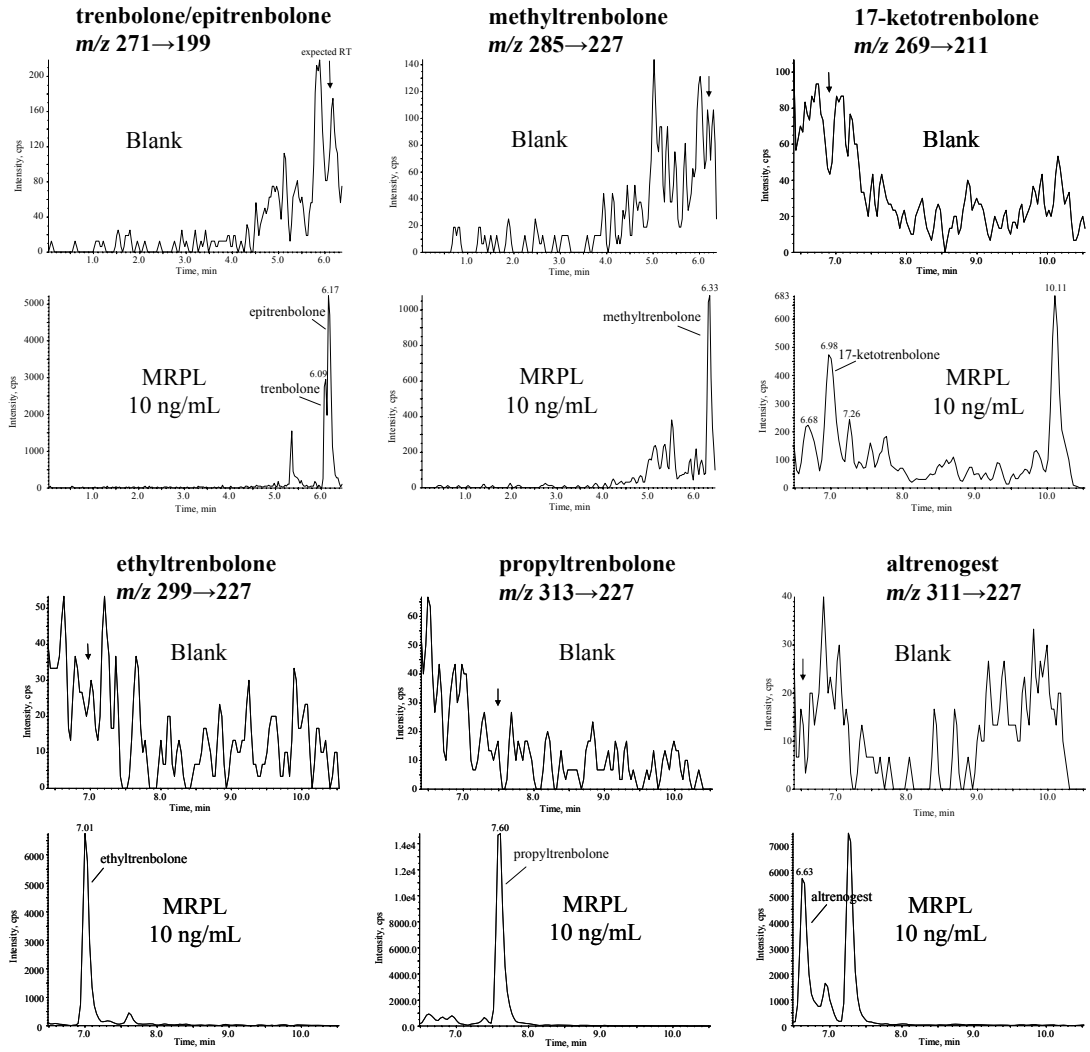

Figure 4: Chromatograms of blank urine specimens and spiked urine samples (conc. 10 ng/mL, MRPL) for trenbolone, epitrenbolone (*m/z* 271/199), methyltrenbolone (*m/z* 285/227), 17-ketotrenbolone (*m/z* 269/227), ethyltrenbolone (*m/z* 299/227), propyltrenbolone (*m/z* 313/227) and altrenogest (*m/z* 311/227) .

4. Discussion

The proceeding efforts of modifying anabolic steroids, being undetectable for target analyses in doping control require capable approaches to reveal a misuse in sports. A LC-(APCI)-MS/MS method is presented for the detection of partially new trenbolone analogues in human urine, providing good sensitivity and reliability. So far the analyses of 2000 routine doping control samples has demonstrated no adverse analytical findings.

References

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