Reprint from

RECENT ADVANCES IN DOPING ANALYSIS (12)

W. Schänzer
H. Geyer
A. Gotzmann
U. Mareck
(Editors)

Sport und Buch Strauß, Köln, 2004

M. MACHNIK, M. THEVIS, C. VON KUK, S. GUDDAT, W. SCHÄNZER:
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In: W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck (eds.) Recent advances in doping analysis (12). Sport und Buch Strauß, Köln (2004) 149-157

M. Machnik, M. Thevis, C. v. Kuk, S. Guddat, W. Schänzer

Gestrinone Analysis in Equine Urine by LC-MS/MS

Institute of Biochemistry, German Sport University Cologne, Germany

Abstract

Initiated by the THG doping scandal in human sports a method for the detection of 3-keto-4,9,11-triene steroids in horse urine has been developed. The method comprises a sample preparation established for anabolic steroids in horse urine and LC-MS/MS detection.
A pilot excretion study with gestrinone was conducted to verify the method capability for the analysis of post administration urine samples. Therefore a single dose of 12.5 mg of gestrinone (Nemestran® , Hoechst-Roussel, Switzerland) was administered to two geldings by oral ingestion. Urine was collected in regular intervals over a period of 10 days.
Peak urine concentrations were measured at 8 hours after administration and ranged between 215 and 595 ng/ml. Baseline values were achieved within 48 hours after administration.
Further investigations on the metabolism of gestrinone revealed a not yet confirmed hydroxylated metabolite of gestrinone, which is excreted as the glucuronide. A comparison of the elimination of parent gestrinone and the metabolite showed that the metabolite can be detected much longer than the parent compound.

Introduction

Since the latest doping affairs triggered by designer steroids such as tetrahydrogestrinone (THG), efforts have been undertaken to identify and detect these illegal drugs [1-3].
Gestrinone has been regarded as a precursor in the synthesis of THG [1-3], both bearing a 3-keto-4,9,11-triene steroid nucleus.

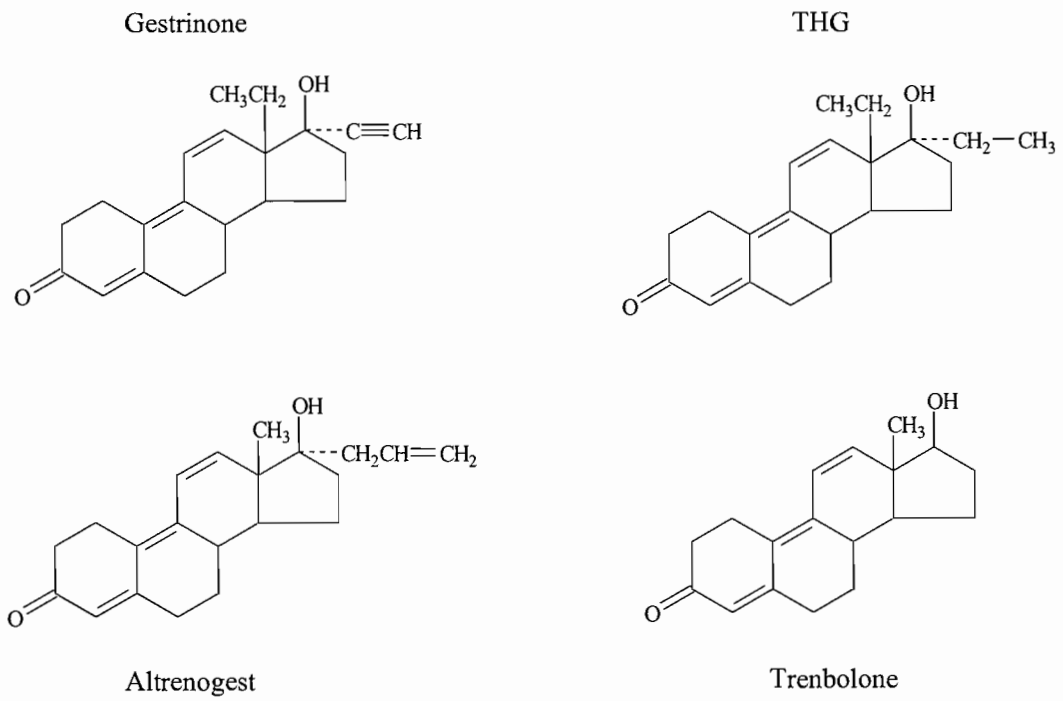

Figure 1: Chemical structures of gestrinone and some derivatives with 3-keto-4,9,11-trien steroid nucleus.

In contrast to THG, gestrinone is a legally marketed substance and is used to treat endometriosis in humans [4]. It belongs to the group of gestagens, which have anti-progestagenic and anti-estrogenic properties. Common preparations are Dimetriose® and Nemestran® (Hoechst-Roussel, Switzerland) available as oral capsules. For humans the therapeutic dose of gestrinone is 2.5 mg twice weekly. THG and gestrinone are considered to have anabolic effects [5] and are on the list of prohibited substances of the World Anti Doping Agency (WADA). They are also forbidden in equine sports according to the rules of national and international horse federations. One objective of the present study was to provide information about the excretion and metabolism of gestrinone in the horse. Therefore two geldings were orally given gestrinone and urine was collected at regular intervals. A detection method was employed comprising enzymatic hydrolysis, acidic solvolysis, liquid-liquid and solid phase extractions to yield extracts for the injection into the liquid chromatograph-tandem mass spectrometer (LC-MS/MS).

Experimental

Study design

A single dose of 12.5 mg gestrinone (5 tablets of Nemestran®) were orally given to two geldings (552 kg, 7 a; 538 kg, 4a). The dose was calculated from the metabolic bodyweight on the basis of the human therapeutic dose of 2.5 mg. Urine collecting was timed at 0, 2, 4, 8, 24, 36, 48, 97, 145, 193 and 241 hours after the administration. Samples were stored at – 20°C until analysis.

Sample preparation and LC-MS/MS analysis

For the analysis the glucuronides, the sulphates and the unconjugated steroids of gestrinone were separated. To 2 ml of urine were added 200 ng of the internal standard altrenogest and the unconjugated steroids were isolated by tert. buthylmethylether (TBME) extraction. After repeated addition of the internal standard glucuronides of gestrinone were enzymatically hydrolysed with β-glucuronidase and again extracted with TBME under moderate basic conditions (pH 9.6). The rest of the urine sample containing the sulphates was processed through a Chromabond® C_{18} cartridge (Macherey-Nagel, Düren, Germany) followed by solvolysis in ethylacetate with
0.08 % H_2SO_4. The final etheral layer was evaporated and the residue dissolved in 100 µl of methanol, of which 10 µl were injected onto the LC-MS/MS instrument (HP Series 1100 Liquid Chromatograph, Agilent Technologies, Waldbronn, Germany; API 2000 triple quadrupole mass spectrometer, Applied Biosystems, Darmstadt, Germany). Ionisation was achieved in the positive mode using nitrogen as collision gas. Ions were registered by multiple reaction monitoring. Liquid chromatography was performed on a 4x55 mm Purospher®Star 3 µm column (Merck, Darmstadt, Germany) with a flow of 0.3 ml/min using the following solvents: A = 5 mM ammonium acetate in H_2O, 0.1 % acetic acid; B = acetonitrile. Gradient: 10 % B → 90 % B, 8 min; 90 % B → 100 % B, 9 min; held for 2 min, reequilibration with 10 % B, held for 3.5 min, run time 14.5 min.

Results

The described method produced characteristic daughter ion scans for gestrinone (fig. 2), altrenogest and other 3-keto-4,9,11-trien steroids. For the quantification of gestrinone ion transitions m/z 309/241 and for the internal standard altrenogest m/z 311/227 were registered.

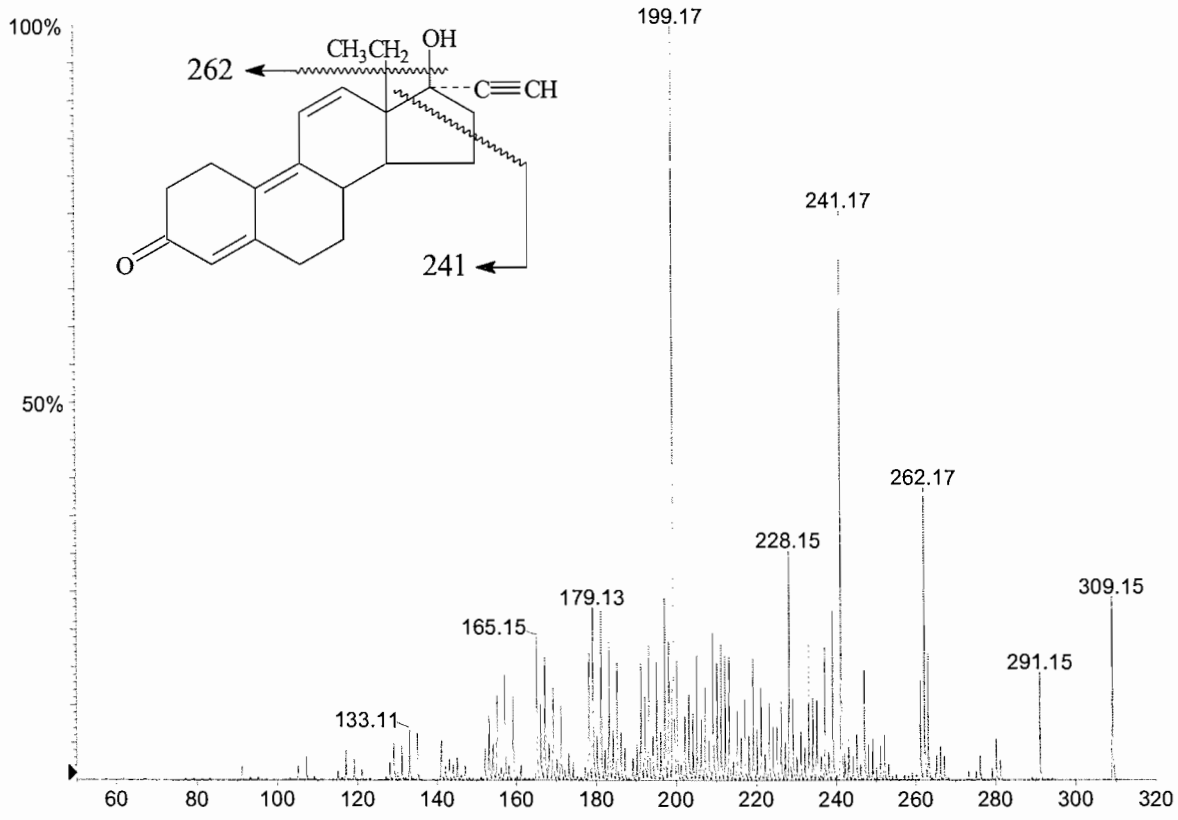

Figure 2: ESI-product ion spectrum and chemical structure of gestrinone, $(M+H)^+ = 309$.

The estimated urine levels of gestrinone of the two geldings are shown in figure 3. Gestrinone has not been detected in the free fraction. The majority of gestrinone was excreted as the glucuronide. Peak concentrations were measured 8 hours after administration with c_{max} values of 215 and 595 ng /ml. At 36 and 48 hours, respectively, concentrations were at or below the limit of detection, which was in the range of 2 – 4 ng/ml.

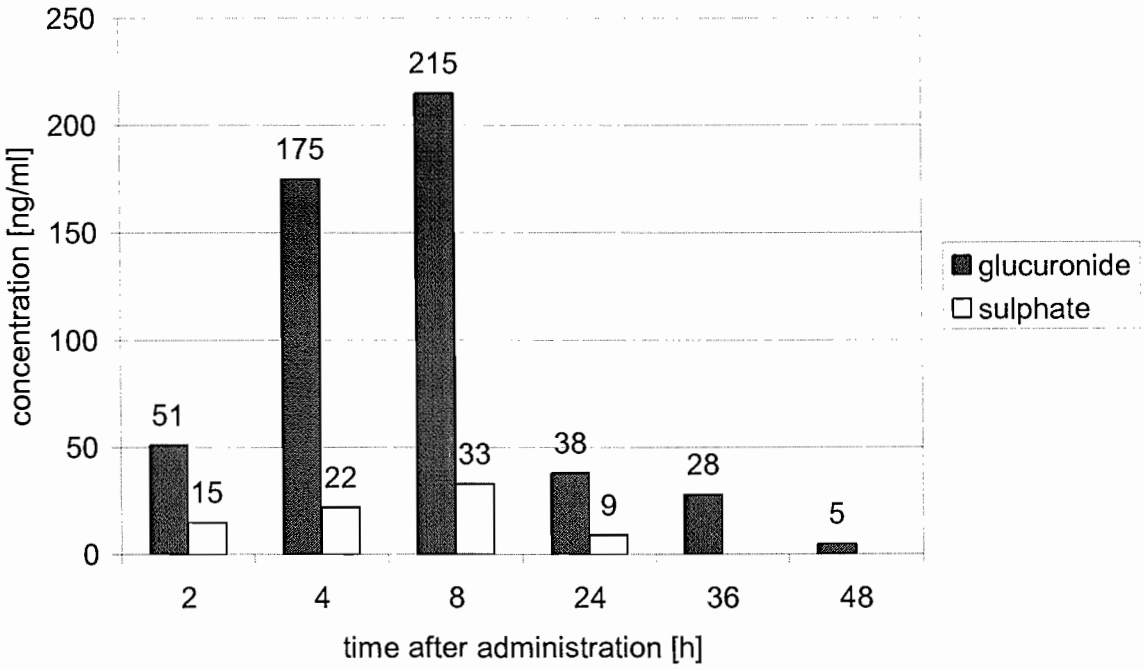

Figure 3A: Urine concentrations of gestrinone conjugates after single oral administration of 12.5 mg to horse 1.

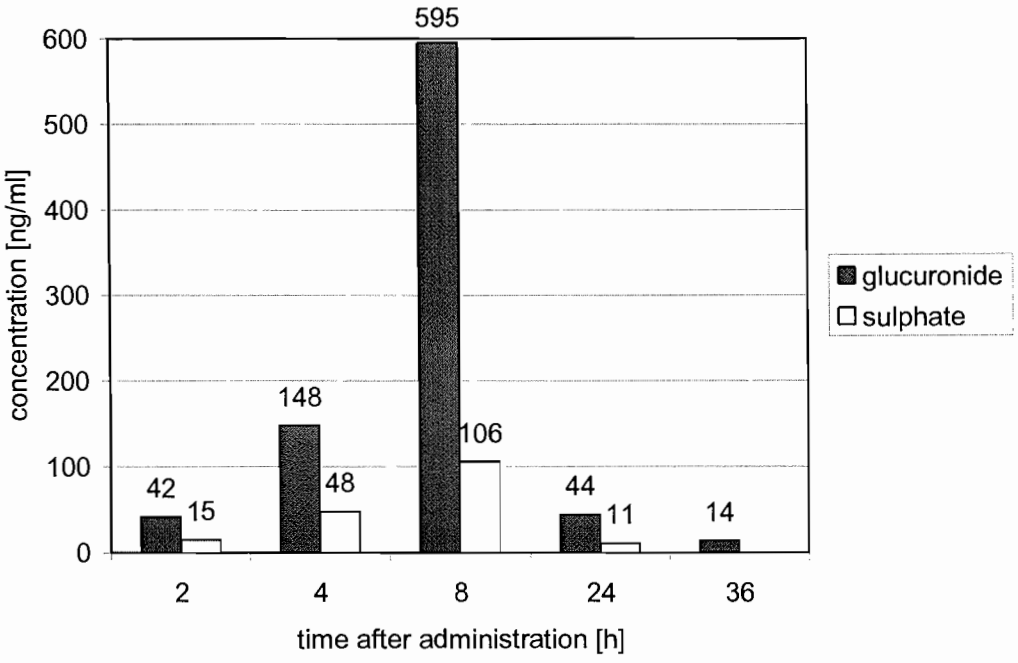

Figure 3B: Urine concentrations of gestrinone conjugates after single oral administration of 12.5 mg to horse 2.

Further investigations on the metabolism revealed a metabolite of gestrinone with a molecular weight of 324. The product ion scan of the protonated parent ion m/z 325 generates a product ion at m/z 307, which is consistent with a loss of water. The secondary release of water leading to fragment m/z 289 presumbly originates from a second hydroxy group (fig. 4). Therefore we postulate a hydroxylated metabolite of gestrinone (I).

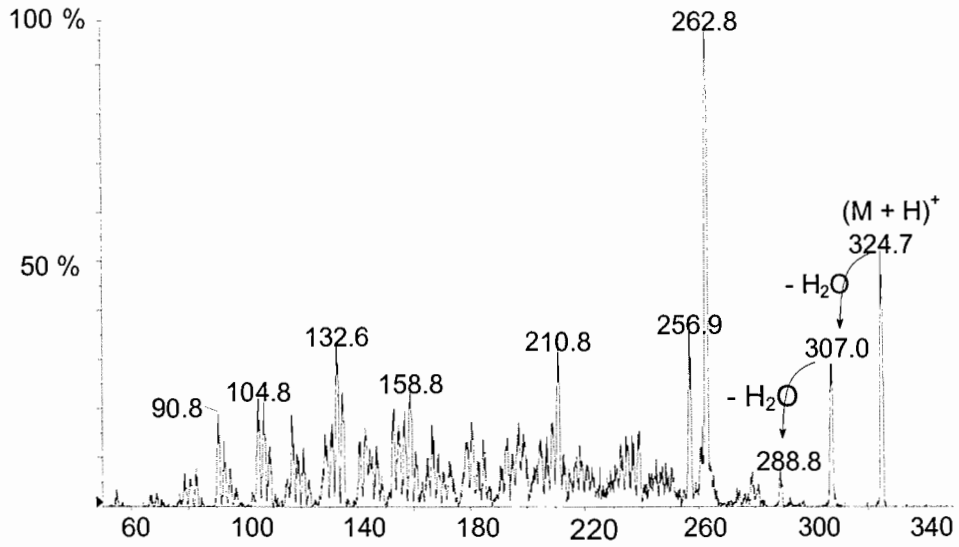

Figure 4: ESI-product ion spectrum of gestrinone metabolite I, $(M+H)^+ = 324.7$.

The presence of a second hydroxy-group could be confirmed by methoxim formation of the 3-keto-group and subsequent trimethylsilylation (TMS) of the hydroxy-groups. The EI mass spectrum of the 3-methoxim-O,O'-bis-TMS derivative is shown in figure 5. The molecular weight of 497 and two TMS-OH losses (- 90 u) confirm a structure with a keto- and two additional hydroxy-groups.

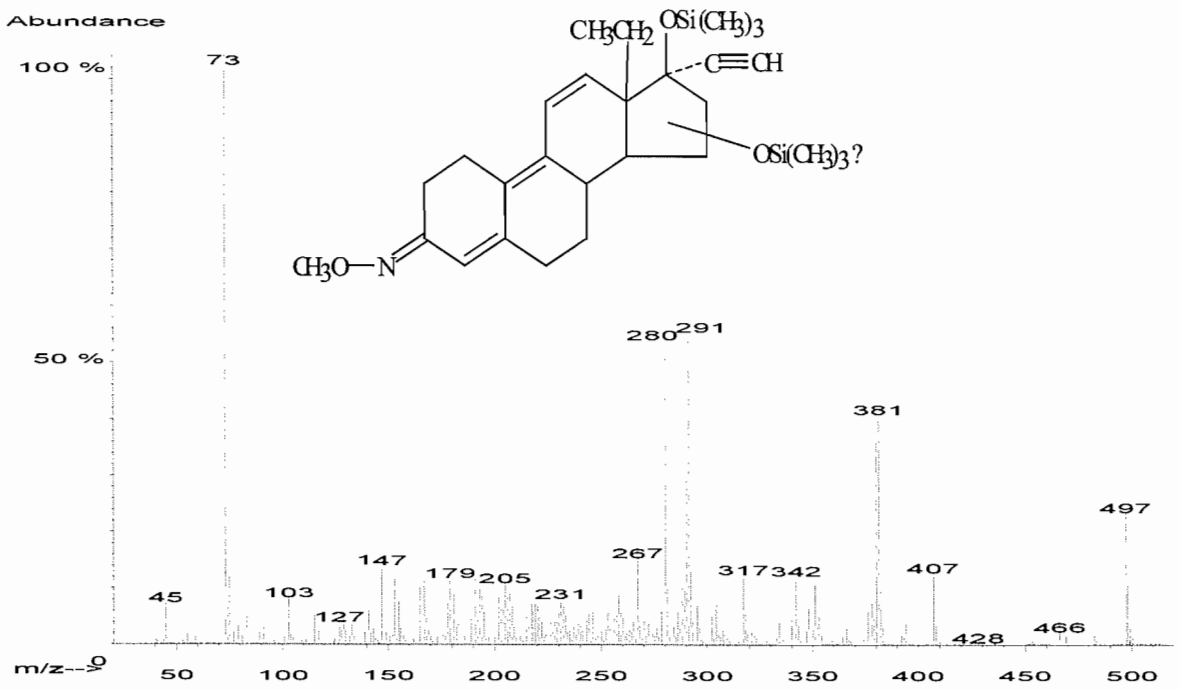

Figure 5: Electron ionisation (EI) full scan mass spectrum of the 3-methoxim-O,O'-bis-TMS derivative of metabolite I, $M^+ = 497$.

However, the position of the second hydroxy-group remains unknown. A 16-hydroxylation, which has been reported for many other steroids in equine metabolism [6,7] appears less probable because the collision induced dissociation of the metabolite does not generate a fragment at m/z 241 (fig. 4) which would be present in case of a 16-hydroxylation. This has been already published by Kim et al. [8], who identified a 16-hydroxylated metabolite exhibiting a fragment m/z 241 in human post administration urines following gestrinone intake. Metabolite I was monitored over the entire elimination. It turned out that compound I was excreted as the glucuronide and could be detected in p. a. urines longer than parent gestrinone (Tab. 1).

Time after	Horse 1		Horse 2	
administration /h	parent gestrinone	gestrinone metabolite	parent gestrinone	gestrinone metabolite
2	yes	yes	yes	yes
4	yes	yes	yes	yes
8	yes	yes	yes	yes
24	yes	yes	yes	yes
36	yes	yes	yes	yes
48	yes	yes	no	yes
97	no	yes	no	yes
145	no	yes	no	yes
193	no	yes	no	yes
241	no	yes	no	yes

Table 1: Detectability of parent gestrinone and metabolite I in horse urine after single oral application of 12.5 mg gestrinone.

Conclusions

The described method allows the detection of 3-keto-4,9,11-triene steroids in horse urine with a detection limit of approximately 2 – 4 ng/ml for gestrinone. In an excretion study with two geldings it could be shown that gestrinone is mainly excreted as glucuronide, no gestrinone was excreted unconjugated. Gestrinone could be detected in horse urine over a period of 36 and 48 hours, respectively, after oral administration of 12.5 mg.

The method also enables the discovery of a hydroxylated metabolite, which can be detected up to 10 days after the administration. The configuration of this glucuronidated metabolite is still unknown.

Acknowledgements

We thank the German Federation Equestre Nationale (FN), Warendorf, for organizing and performing the excretion study.

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