Reprint from

RECENT ADVANCES IN DOPING ANALYSIS (12)

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(Editors)

Sport und Buch Strauß, Köln, 2004

S.A. APPOLONOVA, N.V. ARHIPENKO, V.A. SEMENOV:
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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) 383-387

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Use of ion trap liquid chromatography–mass spectrometry for detection and confirmation of stanozolol metabolites at trace levels in doping analysis

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Abstract

In present work a high-performance liquid chromatographic – electrospray ionization - ion trap mass spectrometry (LC-ESI/MS Ion Trap "SL") method was applied to the detection of 3'-hydroxystanozolol (major metabolite of stanozolol) on the low concentration level in human urine. A positive ion mode was used in the detection. The influence of the pH of mobile phase, condition of fragmentation, LC parameters on the intensively of signal were investigated. MS-MS parameters were optimized to achieve a high mass spectrometric sensitivity. Reference compound of commercially available 3'-OH-Stan and real samples were analyses by LC-ESI/MS ion trap and their comparison were carried out. The results demonstrate that LC-ESI/MS method is quite sensitive for confirmation of 3'-OH-Stan. The limit of detection is 0.2 – 0.5 ng ml^{-1} in human urine.

Introduction

3'-Hydroxystanozolol (3'-OH-Stan) is main metabolite of stanozolol in human urine. In 1998, the IOC stated that each accredited laboratory should confirm the presence of five anabolic agents (3'-OH-stanozolol, clenbuterol, norandrosterone, 17β-methyl-5β-androst-1-ene-3α,17α-diol and 17α-methyl-5β-androstane-3α,17β-diol) at a concentration level of at least 2 ng ml^{-1} in human urine. The standard of testing for these five anabolic agents are GC-MS-MS or GC-HRMS. However, stanozolol and its metabolites have poor gas chromatographic behavior. Therefore there has been an increasing interest in application of advance detection technique such as LC-MS-MS for screening and confirmation of 3'-OH-Stan in urine [1-3].

Experimental

The positive samples with 3'-OH-Stan and stock solutions (0.1 ng ml^{-1}, 0.5 ng ml^{-1}, 1 ng ml^{-1}, 2 ng ml^{-1}) were prepared by standard procedures for anabolic. After extraction procedure the residue was dissolved in 50 μl of methanol and 20 μl was injected on column.

All the experiments were carried out on an 1100 Series LC/MSD Trap "SL" system – Agilent Technologies (Palo Alto, CA, USA) equipped with an autosampler and an autoinjector. Chromatographic separations were performed using a Kromasil® C18 (2.1 × 100 mm, 80 Å, 5 μm) column connected to a guard column (cartridge 2.1 × 12.5 mm) filled with the same packing material.

The mobile phase was a mixture of **A**, 20 *mM* formic acid (pH = 2.5), and **B**, methanol, in a gradient elution mode. The starting mobile phase was 90 % **A** and 10 % **B**, and the linear gradient was run over 10 min to a proportion of 25 % **A** and 75 % **B** then the linear gradient was run over 20 min to a proportion of 10 % **A** and 90 % **B**. The flow rate was 0.25 ml min^{-1}.

The Agilent Technologies "SL" ion trap mass spectrometer (LC/MSD Ion Trap "SL") with an atmospheric pressure electrospray ionization (AP-ESI) was used for quantification in a positive ionization mode. Nitrogen gas was generated from a nitrogen tank (Jun-Air, Denmark) with output pressure of 80 p.s.i. and ion source (nebulizer) inlet pressure at 40 p.s.i. A drying gas was heated to 350 °C at a flow 9 l min^{-1}. The capillary voltage was – 4000 V. The skim trap drive and capillary exit were 46.4 and 104.0 V, respectively. The ion accumulation time was 300 ms with a scan range of 150 to 350 *m/z*.

Results and discussion

The first step of the work involved the comparison analysis of different chromatographic columns (*Zorbax C18* (USA), *Kromasil C18* (Russia), *Hypersil C18* (USA) and *Diasorb C18* (Russia)). The comparison analysis showed that only using column **Kromasil C18** gave very sensitive results by determination of 3'-OH-Stan in human urine. Also we carried out investigation of influence of pH mobile phase from the signal strange (Fig. 1). For electrospary ionization technique needs that substance which we want analyzing have any charge. Using program ACD/Labs 7.0 (Advanced Chemistry Development, Inc) we calculated that molecule of 3'-OH-Stan has plus (+1) on the nitrogen atom at pH 2-2.5. And the best results of analyzing of 3'-OH-Stan was at pH=2-2.5, which agree with calculated data.

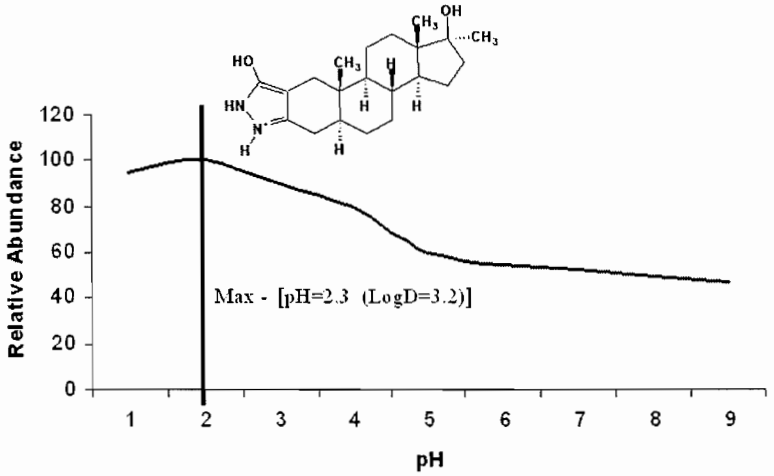

Fig. 1. Influence of pH mobile phase from the signal strange.

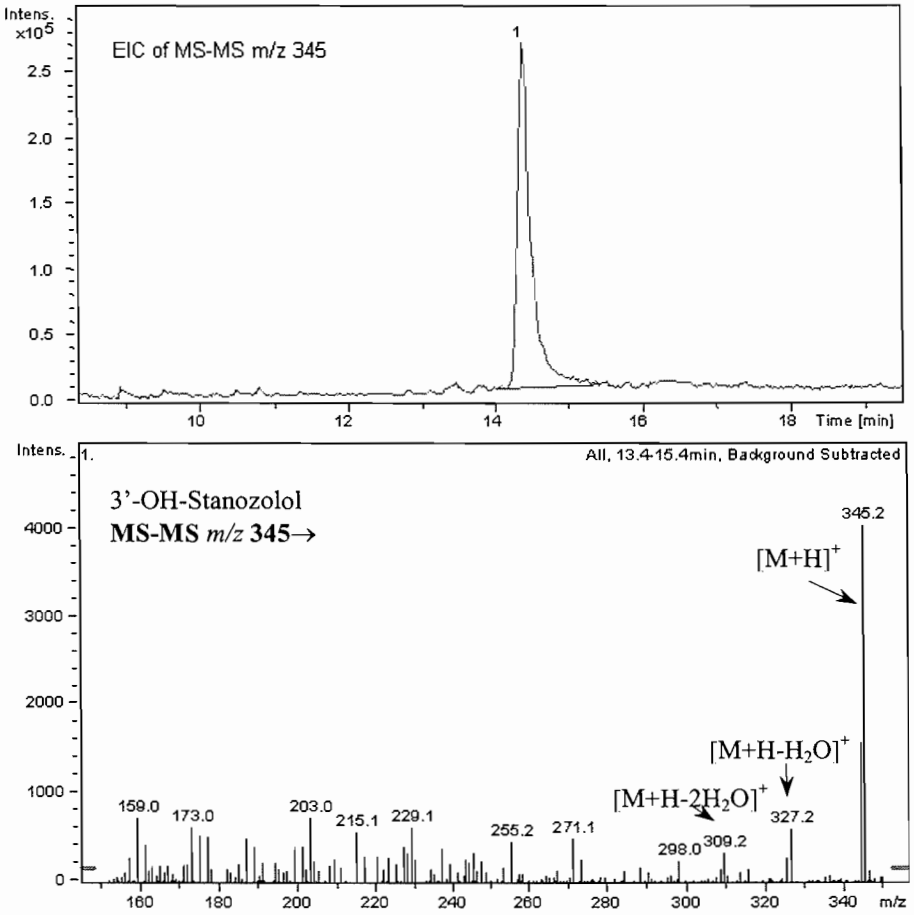

Fig. 2. Extracted-ion MS-MS chromatograms of *m/z* 345 of standard solution of 3'-hydroxy-stanozolol and its mass spectrum.

The second step in the work involved the characterization of the mass spectrum properties of the pure of 3'-OH-Stan. For that the 3'-OH-Stan solution (1 ml, 10 ng ml^{-1}) was carried out direct inlet injection. The MS-MS spectrum by *m/z* 345 without optimization of

fragmentation parameters showed a lot of diagnostic ions that were present in a specific pattern of clusters.

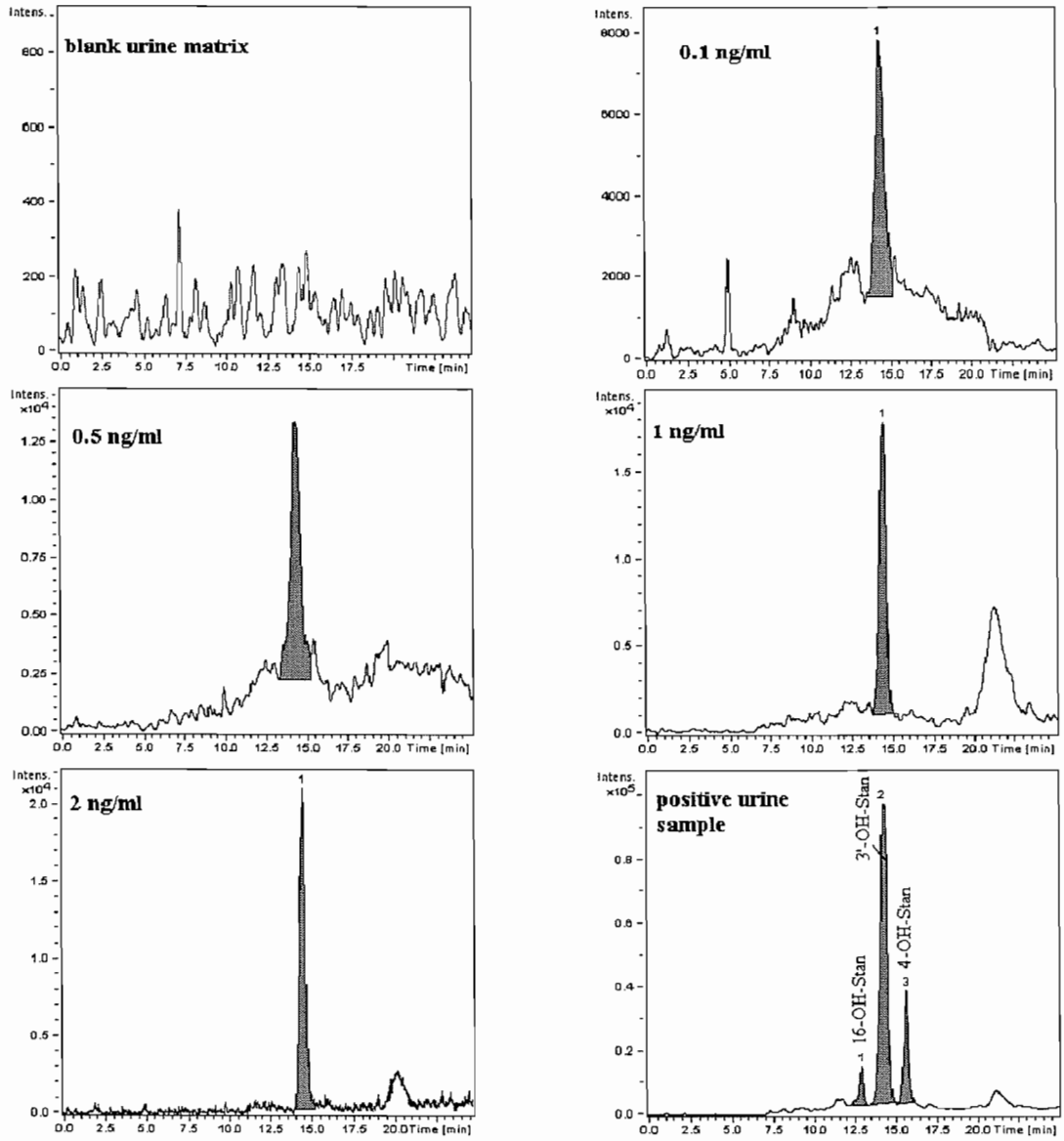

Fig. 3. LC-ESI(+)/MS^2 (*m/z* 345->) chromatograms of 3'-hydroxystanozolol (EIC of MS-MS *m/z* 345) at different concentration spiked in urine and real urine sample.

Such MS-MS spectrum is not informative and when *m/z* 345 completely subjects to a fragmentation we strongly lose in sensitivity. When the optimization of the mass spectrometer parameters was completed, MS and MS-MS measurements were made. Conditions of a fragmentation (CutOff and Ampl.) have been picked up so as protonated ion ($[M+H]^+$, m/z 345) remained in MS-MS spectrum of 3'-OH-Stan. And at the same time peaks interference's from endogenous material (with m/z 345) collapsed completely under these conditions of the fragmentation (Fig. 2, 3). In the LC-ESI/MS/MS experiments, the detection

was based on selected ion monitoring (SIM); and isolation and further fragmentation of the $[M+H]^+$ ion, respectively.

Conclusions

A sensitive and reproducible LC-ESI (+)/MS^2 method for the determination of 3'-OH-Stan in human urine has been developed. The limit of detection is 0.2-0.5 ng ml^{-1} in human urine.

References

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