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Orastan-A: Structural elucidation and detection in urine

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Introduction

The story of Orastan began with Orastan-E, one of market names for prostanozol. Later, Orastan-A was introduced initially containing the same compound. But then, Gaspari Nutrition changed the steroid on label to "5α-androstano[2,3-c]furazan-17β-tetrahydropyranol ether". This one, also advertised as "Furazadrol" (or "Winadrol", or "Furaguno"), is claimed to be furazabol without 17α-methyl group. Orastan-A from Gaspari Nutrition was tested for its composition by GC-MS EI, NMR and LC-high resolution MS. Excretion study was also performed.

Materials and Methods

Reagents: β-glucuronidase was obtained from Roche Diagnostics GmbH (Mannheim, Germany). N-methyl-N-(trimethylsilyl)trifluoroacetamide, ammonium iodide, and dithiothreitol were purchased from Sigma-Aldrich (USA). Dibasic sodium phosphate and monobasic potassium phosphate were supplied from Riedel-de-Haën (Germany). Diethyl ether, potassium carbonate and hydrocarbonate were from Chimmed, Russia. Methanol was purchased from Merck (Germany). All solutions were prepared using Milli-Q water.

Sample preparation: Urines were subjected to common screening procedure for anabolic steroids including enzymatic hydrolysis with E. Coli at pH 6.4, extraction with diethyl ether at pH 9.6, evaporation to dryness and either trimethylsilylation with MSTFA/NH_4I/dithiothreitol (1000/2/1.5) followed by GC-MS analysis on Agilent MSD 5973 or reconstitution in methanol followed by HPLC-MS analysis on Thermo Finnigan LTQ Orbitrap under the conditions of ESI MS or ESI MS/MS.

Results and Discussion

Identity Testing

Our preliminary examination of the identity of Orastan-A done by GC-MS revealed the presence of several compounds, and none of them could be structurally attributed to declared 5α-androstano[2,3-c]furazan-17β-tetrahydropyranyl ether. Based on GC-MS EI data (not shown), it was supposed that supplement contains something that resembles **danazol** in its structure, namely, 5α-androstane[3,2-d]isoxazol-17β-ol and its heterocyclic isomer, each as tetrahydropyranyl ether, acetate and native compound. We performed 1D and 2D NMR (data not shown) and found that 2 isomeric forms, most probably [3,2-d]isoxazol, Fig. 1, and [2,3-c]isoxazol isomers, Fig. 2, are present in the supplement in ratio 2:1.

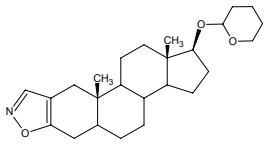

Fig. 1.

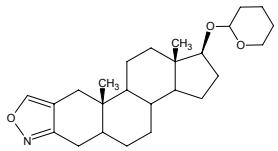

Fig. 2.

The gross formula (elemental composition) was also confirmed by high-resolution HPLC-MS (resolution 30000, mass accuracy 2 ppm in fullscan mode) performed with filtered methanolic extract of supplement contents. Six peaks were detected on the chromatogram as 3 isomeric pairs (see Table). The ESI MS/MS spectrum of peak 1 is presented on Fig. 3 and almost the same spectrum was obtained for peak 2. MS/MS spectra were recorded at lower resolution. Elemental composition was calculated from measured MW using software *Molecular Fragment Calculator v. 1.0*. Not more than 3 hits were proposed by software for each MW measured, and only one corresponded to steroidal backbone.

Peak #	MW (experimental)	Elemental composition	MW (theory)	Tentative Identity
1	315.2200	$C_{20}H_{29}NO_2$	315.2198	5α-androstane[3,2-d]isoxazol-17β-ol
2	315.2200	$C_{20}H_{29}NO_2$	315.2198	5α-androstane[2,3-c]isoxazol-17β-ol
3	357.2308	$C_{22}H_{31}NO_3$	357.2304	5α-androstane[3,2-d]isoxazol-17β-ol acetate
4	357.2308	$C_{22}H_{31}NO_3$	357.2304	5α-androstane[2,3-c]isoxazol-17β-ol acetate
5	399.2772	$C_{25}H_{37}NO_3$	399.2773	5α-androstane[3,2-d]isoxazol-17β-ol THP ether
6	399.2772	$C_{25}H_{37}NO_3$	399.2773	5α-androstane[2,3-c]isoxazol-17β-ol THP ether

Detection in Urine

Healthy volunteer participated in this excretion study. Only 4 urines (blank, 1 hr 40 min, 5 hrs 50 min and 8 hrs 10 min) were available. After administration of 2 capsules of Orastan-A (supposedly 100 mg), no changes were visible on a TIC chromatogram in GC-MS anabolic screening, and only a small peak with *m/z* 545 appeared in the window of 3'-hydroxystanozolol TMS derivative. This peak was then interpreted as a silylation artefact of

the metabolite tentatively identified as 5α-androstane[3,2-d]isoxazol-16ξ-ol-17-one (Fig. 4). Upon silylation the metabolite produces O,O'-bis-TMS derivative (MW 473 Da), but also N,O,O'-tris-TMS artefact (MW 545 Da). The same behaviour was observed for parent steroid(s). The ratio O,O'-bis-TMS derivative to N,O,O'-tris-TMS artefact is about 3 to 1. However, the bis-TMS derivative elutes in the corticosteroid area, and background severely complicates its detection in GC. EI mass spectra of TMS derivatives are not informative and contain mostly molecular and $[M\text{-}CH_3]^+$ ions in the ratio of *ca.* 1:3.

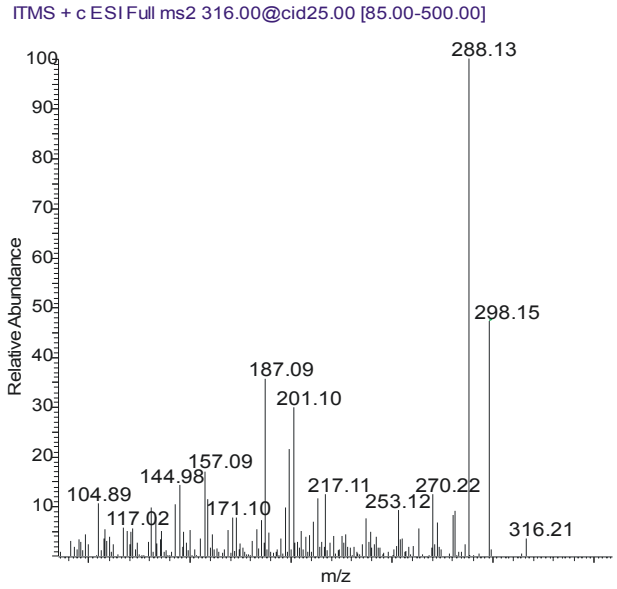

Fig. 3. ESI MS/MS spectrum of major component of Orastan-A supplement

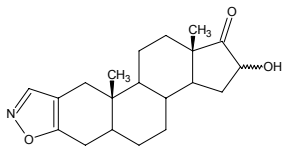

Fig. 4. Tentative structure of main metabolite, MW 329

Once tentative structure of the metabolite was suggested, we performed HPLC-HRMS with excretion urines. Not surprisingly, HPLC was found to be much more suitable for detection of these metabolites than GC. Based on HPLC-HRMS data, 6 peaks were detected that correspond to molecular weight of the proposed metabolite (see Fig. 5). Parent compound as two isomers was also detected in urine (data not shown). Obviously, each parent compound gives 3 metabolites, supposedly 6β-ol- (4ξ-ol-?), 16α-ol- and 16β-ol-17-ones.

ESI MS and ESI MS/MS spectra were recorded for each peak. The ESI MS/MS spectra for peaks 1, 3, and 5 are shown in Fig. 6A to 6C. The spectra were recorded under the same MS/MS conditions and indicate different stability of metabolites corresponding to peaks 1, 3

and 5, and therefore, different structure.

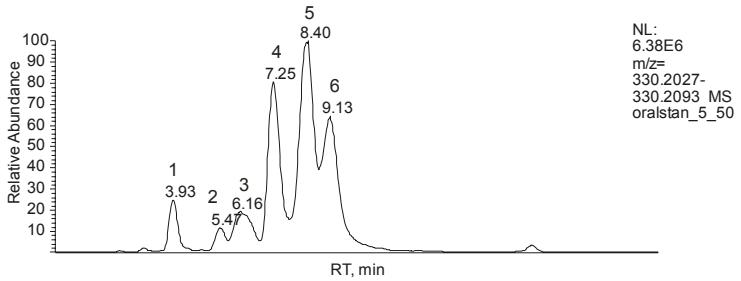

Fig. 5. Mass chromatogram plotted against the accurate mass of tentative metabolite

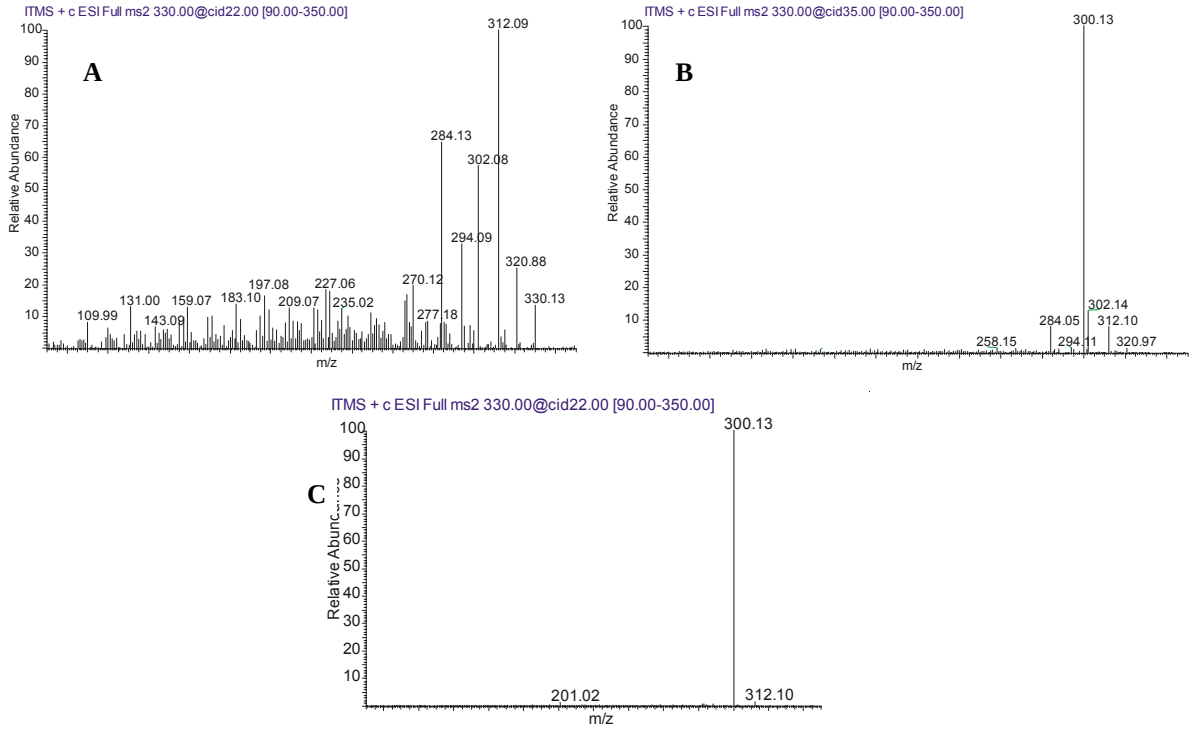

Fig. 6. ESI MS/MS spectrum for peak 1 (A) (protonated molecular ion is at *m/z* 330.21), 3 (B) and 5 (C) (protonated molecular ion at *m/z* 330.21 not detected)

Conclusion

The supplement Orastan-A from Gaspari Nutrition was demonstrated to contain a compound structurally related to **danazol**, but not **furazabol** as it was stated on the label. As the supplement contains (principally) **two** different isomeric compounds and each is subject to metabolize, it is impossible to unambiguously propose the structure of the main metabolite. However, we assume the metabolite detected in urine after administration of this supplement to be 5α-androstane[3,2-d]isoxazol-16ξ-ol-17-one. HPLC-MS data indicate the existence of other hydroxylated metabolites, but their structure is under investigation now.