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Oxandrolone: its reaction with methanol during storage: metabolites and artifact formation due to the incorporation of an ethylmercapto group into persilylated oxandrolones

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Introduction

Oxandrolone (17β-hydroxy-17α-methyl-2-oxa-5α-androstan-3-one) is excreted mainly unchanged or metabolized to its 17-epimer [1,2]. The presence of the δ–hydroxy acid (formed by hydrolysis) was also detected, but no relation could be established between the presence of the steroid and the pH or the specific gravity of the corresponding urine samples [2]. Problems with extraction of oxandrolone and its metabolites from urine at pH values higher than 8.0 are already reported: the A ring (δ-lactone) of these compounds is stable in the pH range from 5.2 to 8.0, but it is degraded at higher pH resulting in a lower efficiency of the screening for oxandrolones [2]. The aim of this study was to verify the stability of oxandrolone in methanolic working solutions and the possibility of artifact formation due to the incorporation of an ethylmercapto group into persilylated oxandrolone.

Experimental

GC-MS analyses were performed using a Hewlett Packard (HP) gas chromatograph (6890 series) equipped with a 7673 HP auto sampler and coupled to a mass selective detector (Agilent 5973 Network). An in house deactivated [1] split/splitless glass single-taper liner from HP (79 mm x 7 mm O.D., cup 6 mm length x 1 mm hole) with a volume of 0.9 ml was used. Inside the liner, approx. 0.02 mg of deactivated glass wool were well compacted between 23 and 33 mm measured from its top. The injector temperature was 280°C. 3 μL were injected in the split mode (1:10) and pulsed with a pressure of 50 psi for 0.8 min. The column

was a HP-1 capillary column (100% methylsilicone, 0.2 mm I.D., film thickness 0.11 μm) with a length of 17 m. The oven temperature was programmed as follows: 0 min 140°C, +40°C/min, 0 min 180°C, +3°C/min, 0 min 240°C, +40°C/min, 3 min 300°C. The mass spectrometer operating conditions were: transfer line 280°C, ion source 220°C, quadrupole 150°C, SEV 200 V higher than in the manual tune; ionization energy 70 eV and scan range 50 to 800 Da.

Methyltestosterone was used as internal standard (I.S.), dissolved in methanol at 1000 ng/μL and diluted to 20 ng/μL. Oxandrolone and epioxandrolone were also prepared in methanol at 1000 ng/μL. These solutions were further diluted to yield standard solutions at concentration levels of 10 ng/μL and 0.1 ng/μL. Excretion urine samples of oxandrolone were prepared according to the method described by Geyer *et al.* [3]. The final residue was derivatized with 100 μL of a mixture of MSTFA/NH4I/ethanethiol (1000:2:6, v/w/v) and heating at 60°C [4].

Results and discussion

Working solution of oxandrolone: The GC-MS analysis showed limited stability for oxandrolone solutions in methanol even if stored at low temperature. A compound (artifact 1) eluting at 12.84 min shows ions at m/z 482, 467, 289 and 143 (Fig. 1). The structure of this compound can be explained by the reaction of oxandrolone with methanol (Fig. 2). After one year of storage the ratio of oxandrolone to this artifact is less than 1. The presence of the δ-hydroxy acid was also observed. This compound, eluting at 15.02 min, shows diagnostic ions at m/z 540, 525, 360 and 347 (Fig. 3). It was reported to be formed by chemical and/or enzymatic hydrolysis (*E. coli*) in urine [2]. The same behavior was observed with epioxandrolone.

Excretion urine of oxandrolone: Another study was performed with a urine positive for oxandrolone. As expected, the following metabolites were detected using screening procedure 4B: 16α-hydroxyoxandrolone, the δ-hydroxy acid and epioxandrolone. An excretion study urine sample from a male subject who ingested oxandrolone showed the presence of a compound with a molecular ion of 510 Da eluting at 17.2 min. The major ions in the spectrum are, in order of decreasing intensity, m/z 510, 143 and 481 (Fig. 4). The formation of this compound (artifact 2) can be explained by the silylation of oxandrolone to its bis-TMS derivative ($M^{+\cdot}$ = 450) followed by incorporation of an ethylmercapto group (SCH_3CH_2; $M^{+\cdot}$ = 510) if ethanethiol is used in the derivatizing mixture (Fig. 5).

Conclusion

Oxandrolone and epioxandrolone are unstable in methanolic solution, forming the δ-hydroxy acids and their methyl esters (artifact 1). Besides that the presence of another compound (artifact 2: most probably generated by incorporation of an ethylmercapto group) during the silylation reaction was observed. This work shows that artifacts are of great concern in doping analysis and the validation of standard solutions is mandatory.

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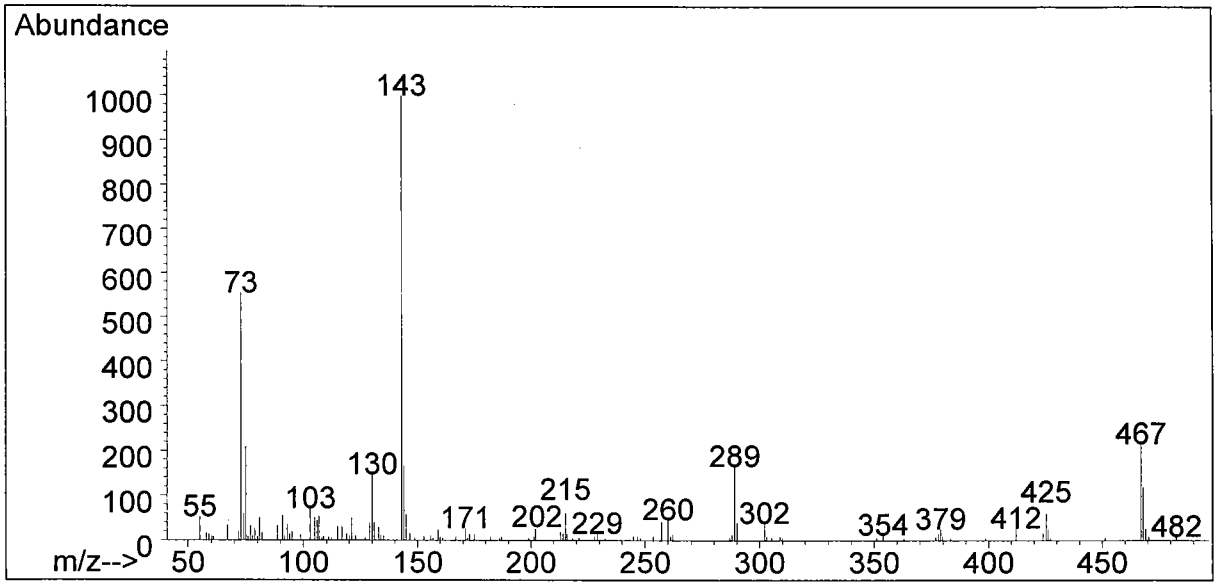

Figure 1. EI mass spectrum of the TMS derivative of the artifact 1 formed by methanolysis.

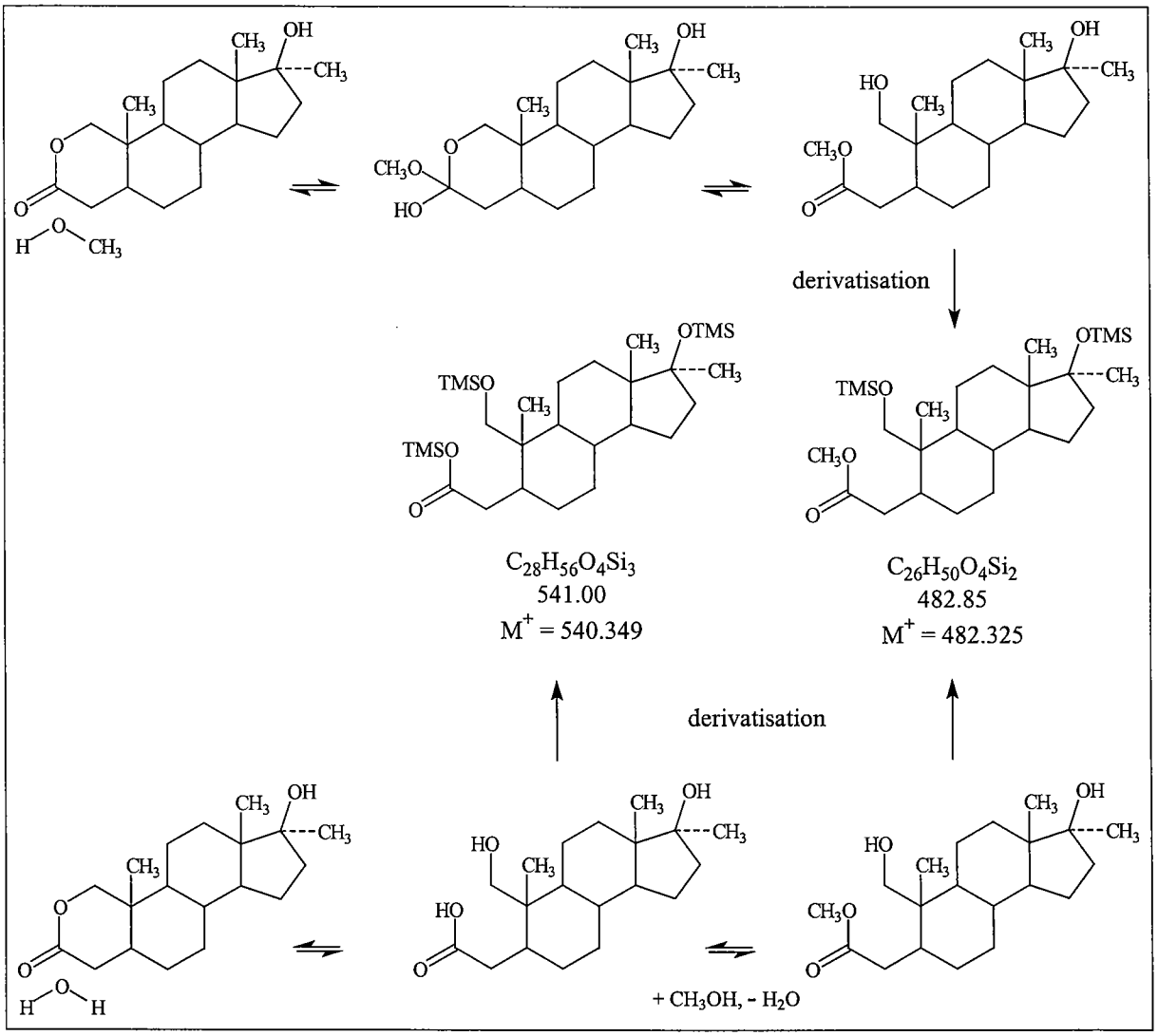

Figure 2. Reaction schemes for the formation of artifact 1 and the δ-hydroxy acid.

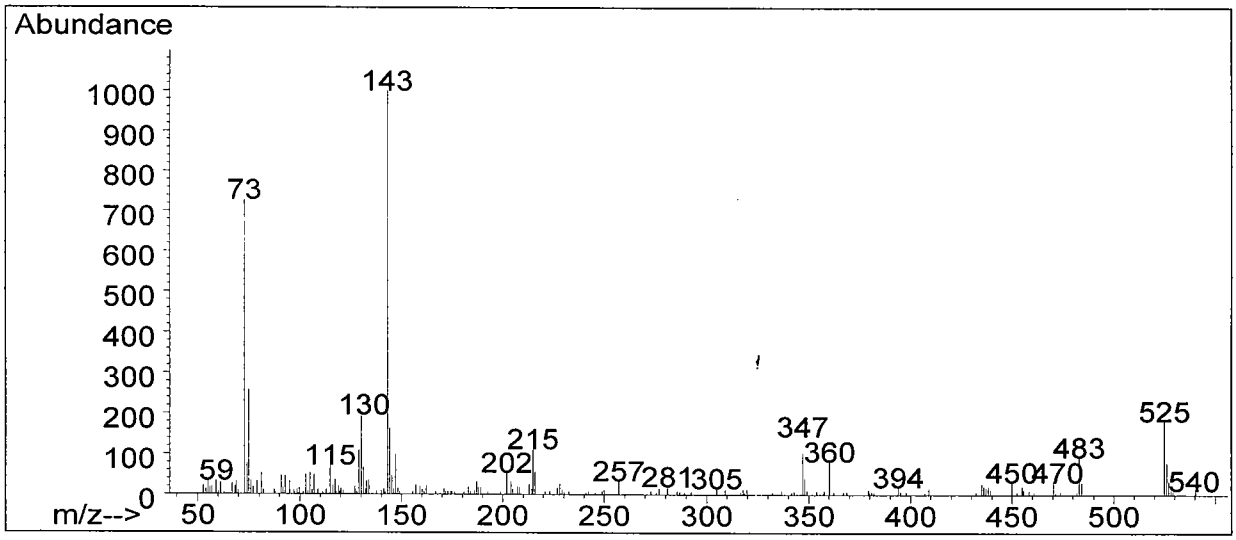

Figure 3. EI mass spectrum of the TMS derivative of the δ-hydroxy acid.

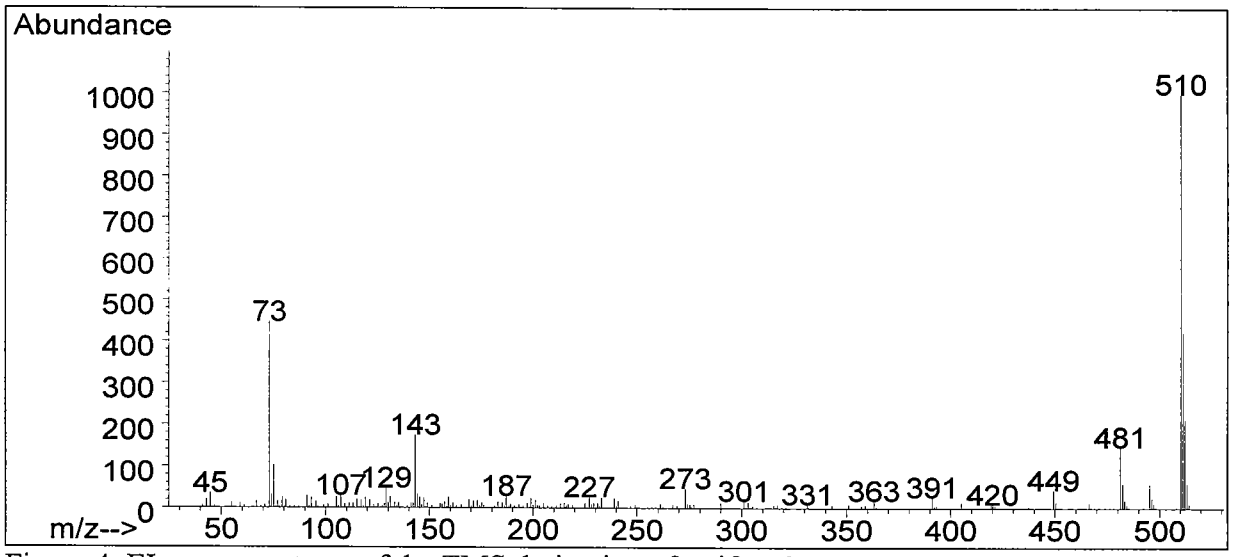

Figure 4. EI mass spectrum of the TMS derivative of artifact 2.

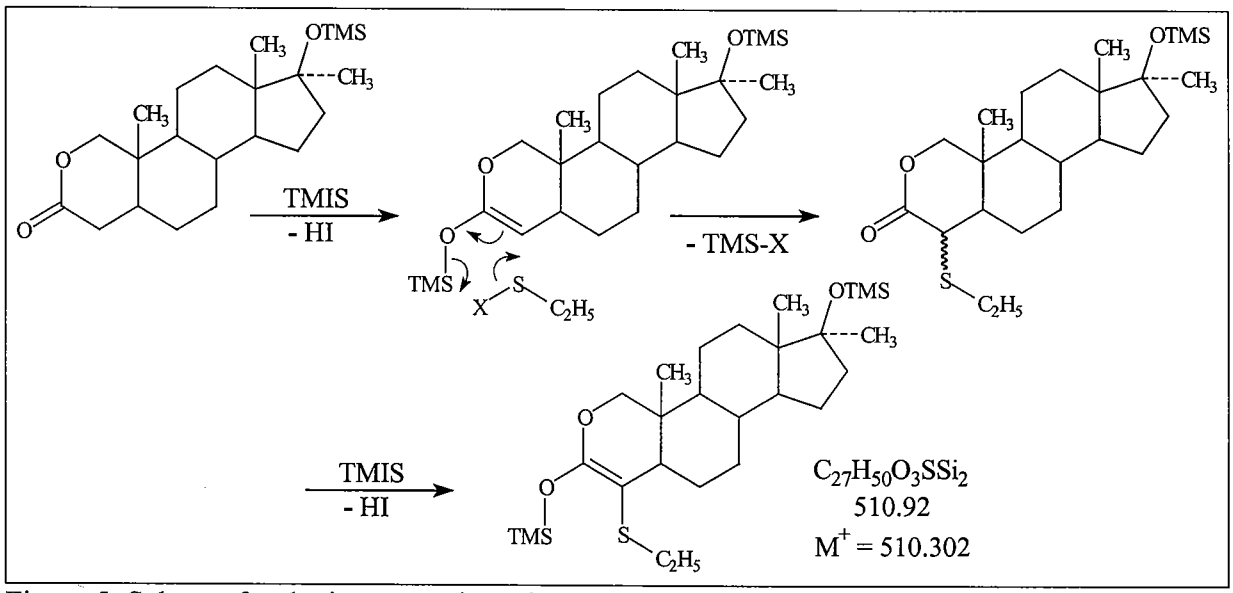

Figure 5. Scheme for the incorporation of an ethylmercapto group into oxandrolone, bis-TMS