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## **Screening for Synthetic Steroids in Human Urine by LC-ESI-MS/MS**

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### **Introduction**

The problem of unknown synthetic steroids in sports has become evident during the last few years. The anabolic steroid norbolethone (13-ethyl-17-hydroxy-18,19-dinor-17 $\alpha$ -pregn-4-en-3-one) was detected in a doping control urine sample in 2002 [1] although this compound has never been commercially available, and tetrahydrogestrinone (THG, 13-ethyl-17-hydroxy-18,19-dinor-17 $\alpha$ -pregn-4,9,11-trien-3-one) substantiated the fact that designer steroids are abused in the world of sport [2-5] in 2004. In February 2005, it was found that another steroid termed 'madol' (17 $\alpha$ -methyl-5 $\alpha$ -androst-2-ene-17 $\beta$ -ol, also known as desoxy-methyltestosterone (DMT)) [6, 7] may also have been used by athletes.

The primary methods of drug screening are based on the mass spectrometric identification of known compounds. Conventional assays will not detect unknown compounds differing by as little as 1 or 2 Daltons from previously identified compounds, especially for MS/MS experiments where defined precursor ions are selected for collision-induced dissociation (CID). In this study a complementary screening procedure is reported allowing the detection of new or unknown steroids with distinct nuclei in human urine. Complementary urinary "steroid profiles" are generated from precursor ion scan experiments using LC-ESI-MS/MS, a strategy also commonly employed also in metabolite identification studies. Product ions generated by common steroid nuclei including androst-4-en-3-one (e.g. testosterone), androst-1-en-3-one (e.g. 1-testosterone), estr-4-en-one (e.g. norbolethone), and androst-4,9,11-triene-3-one (e.g. trenbolone and gestrinone) were characterized [8] and used to develop a screening protocol described elsewhere [9].

## Experimental

*Steroids.*  $5\alpha$ -Androst-1-en-17 $\beta$ -ol-3-one (1-testosterone), estra-4,9,11-trien-17 $\beta$ -ol-3-one (trenbolone) and 17-hydroxy-18-homo-19-nor-17-pregna-4,9,11-trien-20-yn-3-one (gestrinone) were obtained from Thinker Chemical Co. Ltd. (Hangzhou, China).  $17\alpha$ -Methyl-androst-4-en-17 $\beta$ -ol-3-one (methyltestosterone) was purchased from Sigma-Aldrich (Steinheim, Germany).  $17\alpha$ -Ethyl-androst-4-en-17 $\beta$ -ol-3-one (ethyltestosterone), 13-ethyl-17-hydroxy-18,19-dinor-17 $\alpha$ -pregn-4-en-3-one (norbolethone), 17-hydroxy-18-homo-19-nor-17-pregna-4,9,11,20-tetraen-3-one (dihydrogestrinone), 18 $\alpha$ -homo-pregna-4,9,11-trien-17 $\beta$ -ol-3-one (tetrahydrogestrinone, THG), 20,20,21,21-<sup>2</sup>H<sub>4</sub>-18 $\alpha$ -homo-pregna-4,9,11-trien-17 $\beta$ -ol-3-one (d<sub>4</sub>-tetrahydrogestrinone, d<sub>4</sub>-THG),  $17\alpha$ -methyl-estra-4,9,11-trien-17 $\beta$ -ol-3-one (methyltrenbolone),  $17\alpha$ -ethyl-estra-4,9,11-trien-17 $\beta$ -ol-3-one (ethyltrenbolone) and  $17\alpha$ -propyl-estra-4,9,11-trien-17 $\beta$ -ol-3-one (propyltrenbolone) were synthesized in our laboratory.

*ESI-MS/MS.* All product ion scan experiments were performed using an Applied Biosystems Qtrap 4000 mass spectrometer (Darmstadt, Germany) equipped with an electrospray ionization interface (ESI) using nitrogen as collision gas. The instrument was operated in the positive mode at a spray voltage of 5500V, and declustering potentials as well as collision offset voltages were adjusted to generate product ion mass spectra containing precursor ions at relative abundances of approximately 10%. The scan rate was 1000 u/s and Q1 was operated at unit resolution.

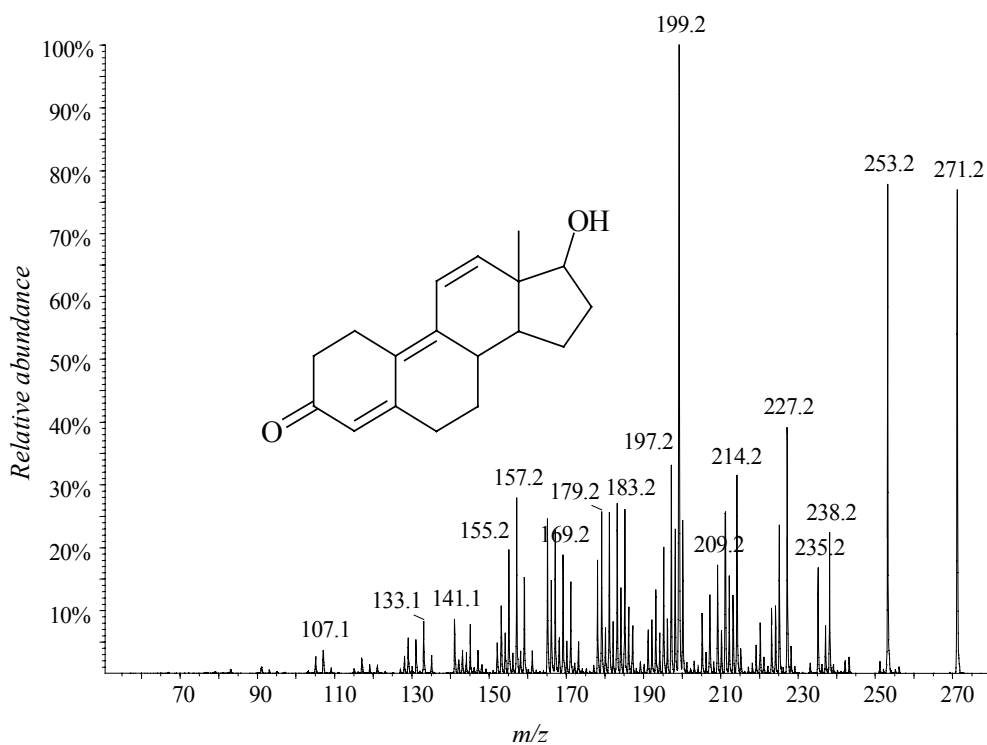
*LC-MS/MS.* All analyses were performed on an Agilent 1100 Series HPLC (Waldbronn, Germany) coupled to an Applied Biosystems Qtrap 4000 mass spectrometer (Darmstadt, Germany). The LC was equipped with a Macherey-Nagel Nucleodur C-18 Pyramid column (length 70 mm, inner diameter 4 mm, particle size 5  $\mu$ m), and the solvents A: 5mM ammonium acetate containing 0.1% acetic acid (pH 3.5) and B: acetonitrile were used with a gradient from 10% B to 100% B within 10 minutes. The flow rate was 800  $\mu$ L/min and the effluent was introduced into the mass spectrometer by means of ESI in the positive mode using a spray voltage of 5500 V. The ion source was operated at 350°C. Precursor ion scan experiments were conducted by selecting the product ions at  $m/z$  109, 187, 199, 227, and 241 at collision offset voltages of 30V, and unit resolution was used in both mass selection quadrupoles Q1 and Q3.

*Sample preparation.* Three mL of urine were buffered to pH 7.0 with 1 mL of a 0.8 M phosphate buffer ( $\text{Na}_2\text{HPO}_4$ :  $\text{NaH}_2\text{PO}_4$ , 1:2, w:w) and 25  $\mu\text{L}$  of  $\beta$ -glucuronidase from *E. coli* and 300 ng of  $\text{d}_4$ -THG (internal standard, ISTD) were added. The sample was heated at 50°C for 1 hour, and, after cooling to ambient temperature, 0.75 mL of an aqueous solution containing potassium carbonate and potassium bicarbonate (20%, 1:1, w:w) plus 6 mL of *tert.*-butyl methyl ether were added. The mixture was shaken mechanically for 5 minutes, and subsequently centrifuged at 600 g for 10 minutes. The organic layer was transferred to a fresh glass tube, evaporated to dryness at 50°C, and the dry residue was dissolved in 60  $\mu\text{L}$  of a mixture of methanol and 5mM ammonium acetate containing 0.1% acetic acid (1:1, v:v). A total of 10  $\mu\text{L}$  was injected into the LC-MS/MS system.

## Results and discussion

The common dissociation routes of steroids with androst-4-en-3-one, androst-1-en-3-one, estr-4-en-one, and androst-4,9,11-triene-3-one were investigated using structurally related as well as isotopically labeled compounds [8]. For instance, trenbolone fragmentation pathways were elucidated using synthesized analogues methyltrenbolone, ethyltrenbolone and propyltrenbolone as well as gestrinone and THG. The product ion mass spectra of trenbolone and its 17-alkylated analogues are shown in Figure 1 demonstrating the influence of side chain extension on the precursor ion and the general fragmentation pattern. Abundant product ions such as  $m/z$  227 and 199 are found in all spectra depicted in Figure 1. Based on studies on the fragmentation behavior of selected steroids, common product ions for gestrinone, trenbolone, 1-testosterone, testosterone, and nandrolone as well as related compounds were identified as listed in Table 1. In addition, the fragment ion at  $m/z$  199 was observed with various steroidal structures such as androsterone and etiocholanolone and included in the list of product ions relevant for steroid profile measurements. The selected product ions were incorporated in a precursor ion scan procedure consisting of six parallel experiments allowing the simultaneous measurement of analytes generating considerable fragment ions upon CID at a fixed collision offset voltage of 30V. An example of a mixture of reference compounds analyzed under the conditions described above is shown in Figure 2.

a)



b)

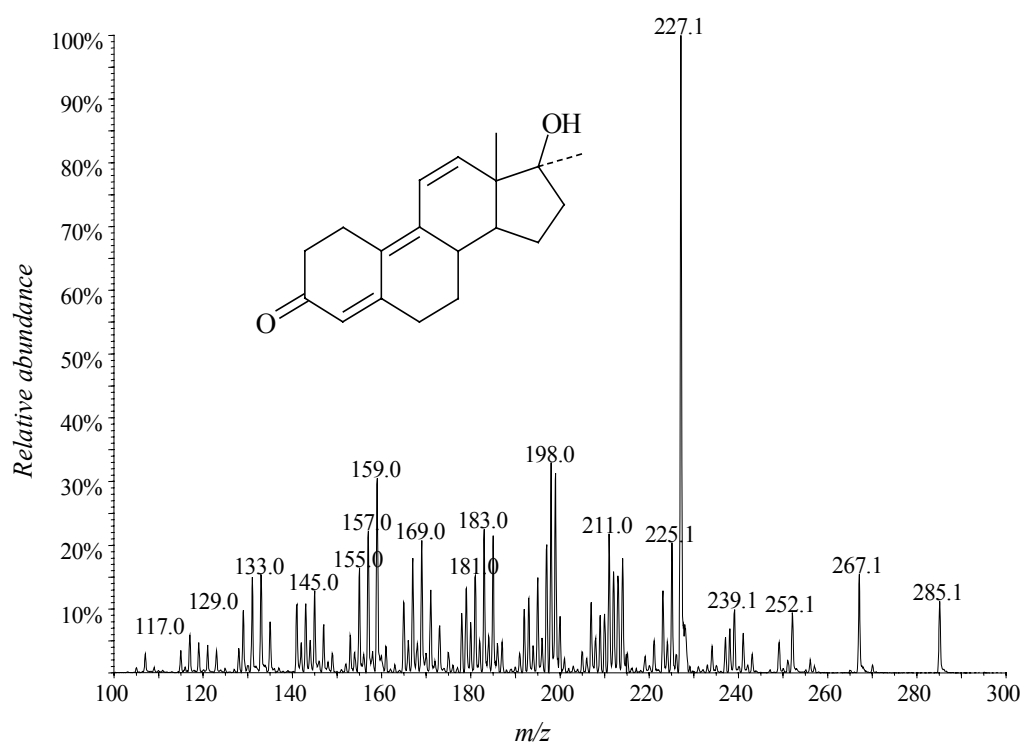


Figure 1: ESI product ion spectra of a) trenbolone, b) methyltrenbolone, c) ethyltrenbolone, and d) propyltrenbolone.

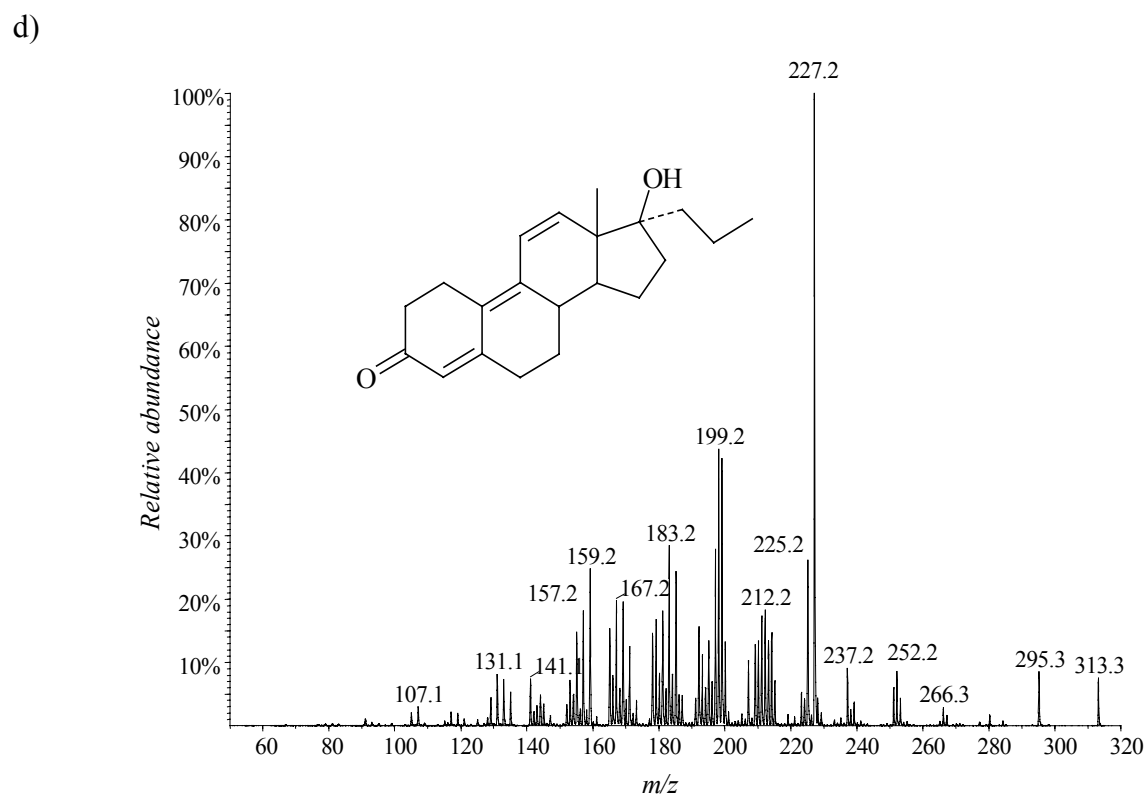
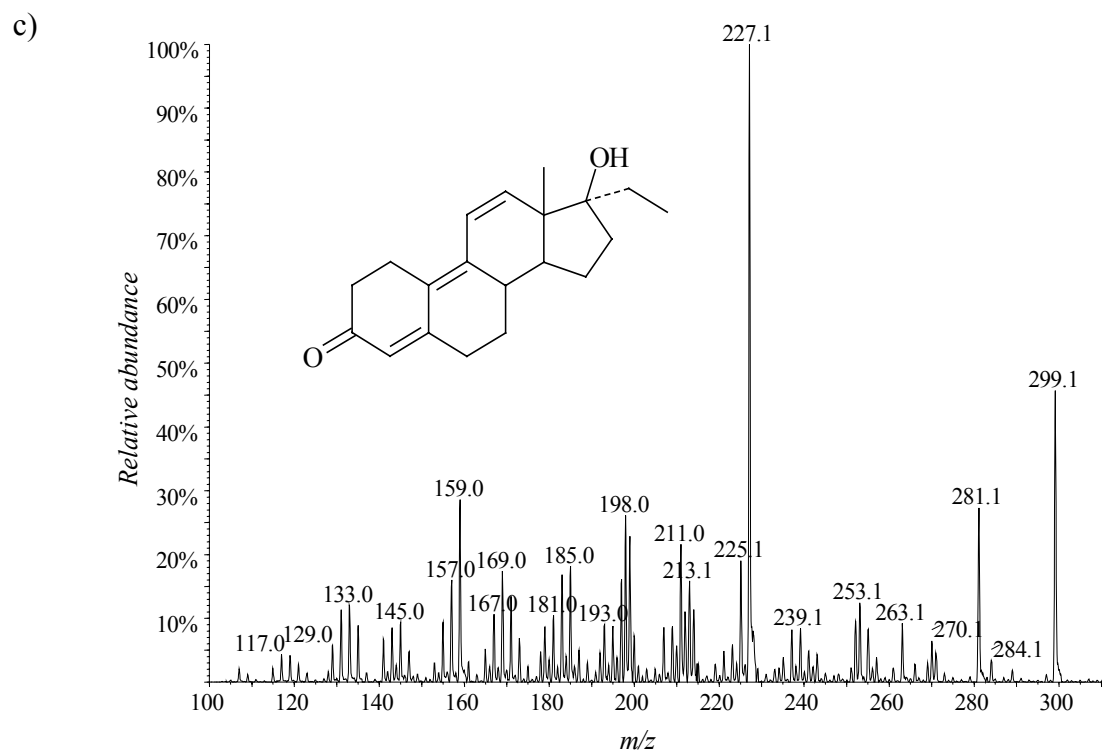
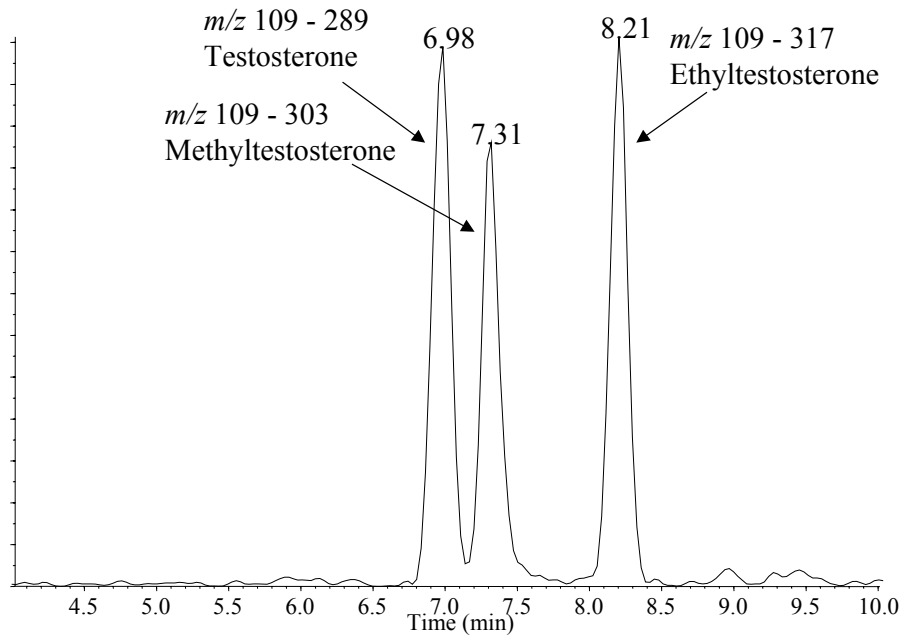


Figure 1 (cont.): ESI product ion spectra of a) trenbolone, b) methyltrenbolone, c) ethyltrenbolone, and d) propyltrenbolone.

a)



b)

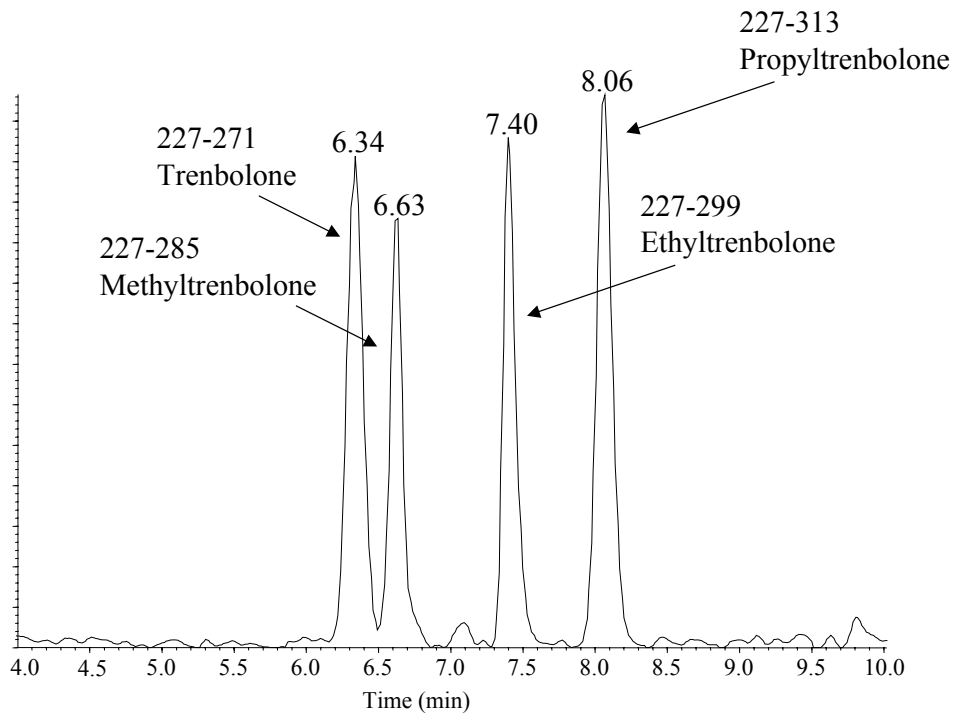


Figure 2: Base peak chromatograms of precursor ion scan experiments on a)  $m/z$  109 and b)  $m/z$  227 using reference compounds in acetonitrile at 2  $\mu\text{g/mL}$ .

Blank urine samples were analyzed according to the protocol described above and evaluated employing base peak chromatograms for improved visualization of analytes. Peaks for synthetic steroids were obtained in urine samples at concentrations of 50 ng/mL, and by selective extraction of detected precursor ions the sensitivity and selectivity of the employed

mass spectrometric strategy becomes evident (Figure 3, inset). In case of “unknown” signals that are usually not observed in urine samples, subsequent product ion scan experiments are conducted to provide information that may indicate a structure related or not related to steroids. If a suspicious compound is detected, enormous effort is required to verify the analyte’s structure and unambiguously determine its composition as performed in previous designer steroid cases [5, 6]. In Figure 3, a blank urine sample (a) is compared to a urine specimen fortified with 50 ng/mL of gestrinone, dihydrogestrinone, ethyltestosterone and propyltrenbolone (b) giving rise to signals in respective precursor ion scan experiments in addition to commonly observed peaks resulting from endogenous compounds.

## **Conclusion**

The threat of designer steroids in sports requires new, complementary strategies to cope with the possibility of chemically modified drugs. Precursor ion scan options of modern triple-quadrupole mass spectrometers provide a tool that allows the detection of compounds generating fragment ions commonly generated from specific steroid nuclei with sufficient proton affinities, and thus indicating the presence of new, unknown drugs. Once a suspicious signal is observed, follow-up studies including various mass spectrometric analyses including derivatization, different ionization techniques, etc. as well as synthesis and characterization of the suspected compound are necessary to prove the structure of the potentially prohibited substance.

### Precursor ion scans

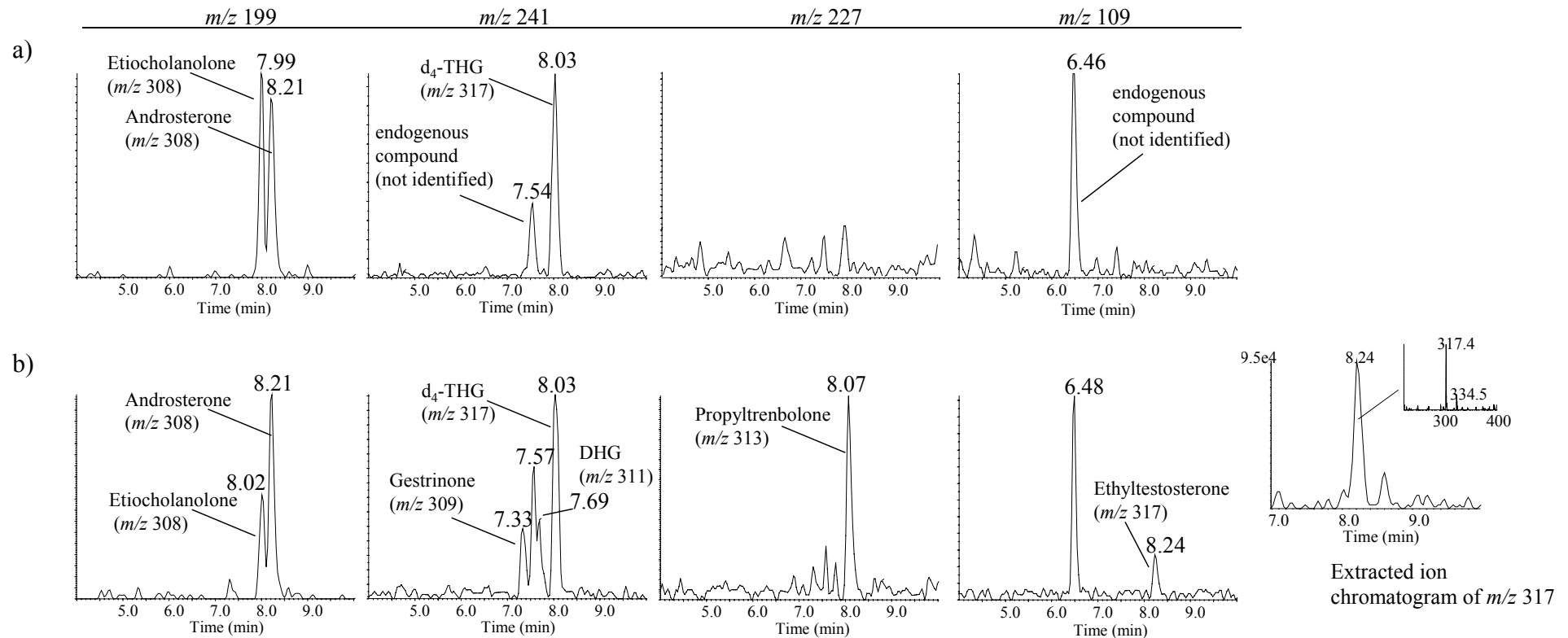


Figure 3: a) blank urine specimen fortified with  $d_4$ -THG as ISTD and analyzed for precursors of  $m/z$  199, 241, 227 and 109. Androsterone and etiocholanolone are detected in addition to two endogenously generated compounds; b) blank urine sample fortified with 50 ng/mL of gestrinone, dihydrogestrinone, ethyltestosterone and propyltrenbolone. Distinct signals are observed allowing an extraction of detected precursor ions and subsequent product ion experiments to obtain information whether the analyte's structure may be related to a steroid or not.



## References

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