O.J. Pozo, K. Deventer, P. Van Eenoo, F.T. Delbeke

Endogenous interferences in the detection of anabolic steroids by liquid chromatography tandem mass spectrometry

DoCoLab, UGent, Department of clinical chemistry, microbiology and immunology, Technologiepark 30, B-9052 Zwijnaarde, Belgium

Introduction

The use of liquid chromatography tandem mass spectrometry (LC-MS/MS) is an alternative to the common GC-MS methods in the analysis of anabolic steroids. It has the general advantage that the required sensitivity is achieved without derivatization. However, the detection of anabolic steroids by LC-MS/MS presents also some disadvantages such as the lack of either acidic or basic moieties in the general steroid structure, which limits their ionization by atmospheric pressure [1].

The presence of endogenous steroids is an additional problem for the urinary detection of exogenous steroids by LC-MS/MS. Although in some applications [2] this effect was not noticed, these endogenous compounds can interfere with the LC-MS/MS detection of the analytes due to their similar structure, mainly if unspecific transitions are used. Deventer et al [3] observed difficulties in the LC-MS/MS determination of trenbolone due to the presence of almost co-eluting endogenous interferences.

On the other hand, the development of useful approaches in the identification of unknown anabolic steroids is increasing in the last years. Different alternatives have been described for that purpose. The use of precursor ion scanning is an attractive approach as it allows the determination of the mass of an analyte which produces a specific fragment [4]. The studies on the ionization [1] and fragmentation [5] of different anabolic steroids have also been described as useful tools to identify and characterize unknown or modified steroids.

The aim of this study is the evaluation of the interferences produced by urinary endogenous components in the screening for the anabolic steroids 17α-trenbolone (17TREN), 1-testosterone(1T), 5β-androst-1-ene-17β-ol-3-one (BOLDm) and oxandrolone (OXA). Different approaches based on mass spectrometry have been evaluated in order to circumvent these interferences. A methodology for the elucidation of anabolic-androgenic steroids and corticosteroids is also proposed and applied for these endogenous interferences.

Experimental

Instrumentation

A Finnigan TSQ Quantum Discovery Max LC-MS/MS triple quadrupole mass spectrometer (Thermo) was used with electrospray interface. The LC separation was performed using a Varian Omnispher C_{18} column (50 x 2 mm i.d., 3μm) (Varian, Sint-Katelijne-Waver, Belgium), at a flow rate of 300 μL/min. For the detection of 17TREN, an isocratic method with 0.1% aqueous formic acid:0.1% methanolic formic acid (60:40) was used as described elsewhere [3]. For 1T, BOLDm and OXA, 1mM ammonium acetate was used as modifier in both aqueous solvent and methanol. The percentage of organic solvent in the gradient program was changed linearly as follows: 0 min., 20%; 0.5 min., 20%; 2 min., 55%; 10.5 min., 55%; 13 min., 95%; 14 min., 95%; 15 min, 20%, 17 min 20%. Nitrogen was used as sheath gas, ion sweep gas and auxiliary gas, which were set at flows of 70, 2 and 5 units, respectively. Spray voltages of 4000 V and 3000 V were used in positive and negative ionization mode, respectively. The capillary temperature was set to 300 ºC and the source collision-induced dissociation (CID) to 2 units.

Sample preparation

5 mL of urine were hydrolysed after addition of 1 mL phosphate buffer (pH=7), 50 μL of β-glucuronidase solution and 50μL of internal standard (oxymetholone, 10μg/mL). After cooling to room temperature, the pH was adjusted to 9.2 by addition of 1g sodium bicarbonate: potassium carbonate (2:1). Liquid-liquid extraction was performed with 5mL diethyl ether. After centrifugation, the organic layer was evaporated under nitrogen at 40°C. The remaining residue was dissolved into 200μL of mobile phase. 20μL were injected into the system

SRM method development

Product ion spectra at different collision energies were obtained for each analyte by direct infusion of the standard at 1 μg/mL using the built-in syringe. The most sensitive product ion of each analyte was selected for the SRM screening method. The specificity of other abundant product ions was also checked.

Elucidation of interferences with steroidal and corticosteroidal structure

An approach based on the use of different scan modes has been developed. As a first step, a precursor ion scan of the interfered product ion is used to establish the mass of the interference. Subsequently, full scan MS in both positive and negative modes is performed. According to a previous study [6] corticosteroids have a molecular weight higher than 350

and exhibit signal in both ionization modes. On the other hand, steroids are only ionised in positive ionization mode [1] and normally have a molecular weight lower than 350. Then, a possible molecular formula for the interference was obtained by applying several restrictions regarding their structure. Thus, for anabolic steroids, a minimum of 4 double bond equivalents (DBE) due to the 4 rings, 18 carbon atoms and 2 oxygen atoms was selected. In the case of corticosteroids, the DBE and the minimum number of carbon and oxygen atoms were restricted to 4, 21 and 4 respectively. Finally, the product ion spectrum of the interference is acquired and compared with reported endogenous compounds sharing the established molecular formula.

Results and discussion

SRM method

Figure 1 shows the structures and the product ion spectrum obtained for selected analytes. In the development of target screening methods for selected anabolic steroids, one SRM transition was selected for each analyte. MS parameters were optimized for the most abundant transition (Table 1).
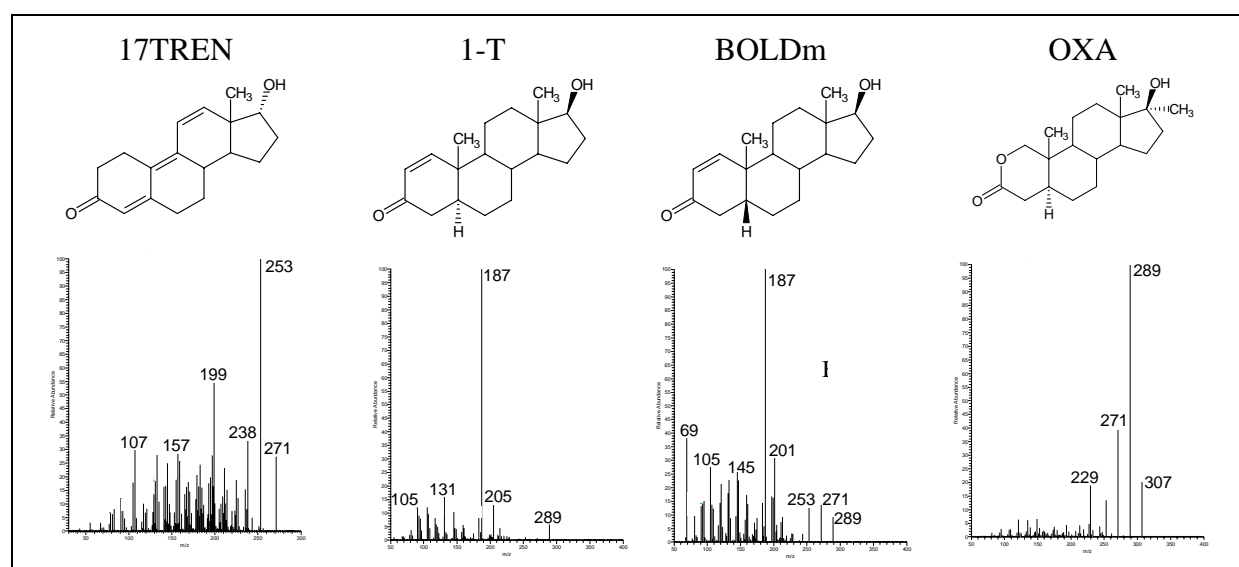

Figure 1. Structure and product ion spectra of selected anabolic steroids

Table 1. MS/MS parameters resulting in the most abundant transition

Analyte	Precursor (m/z)	Precursor (ion)	TLV (V)	Product (m/z)	Col. Energy (eV)
17TREN	271	[M+H]$^+$	150	253	22
1T	289	[M+H]$^+$	120	187	22
BOLDm	289	[M+H]$^+$	120	187	22
OXA	324	[M+NH$_4$]$^+$	100	289	20

In the case of 17TREN, the most abundant product ion was obtained after the unspecific loss of water from the protonated ion. The similar structures of 1T and BOLDm (they only differ on the position of the 5-hydrogen) makes their product ion spectra of the $[M+H]^+$ having the same ions, only differing in relative intensity. In both cases the most abundant product ion (m/z 187) was selected for the SRM method. This product ion resulted from the fragmentation of the carbon skeleton and can be considered as more specific. Finally, for OXA the most abundant transition occurred when $[M+NH_4]^+$ was used as precursor ion and by selecting $[M+NH_4-NH_3-H_2O]^+$ as product ion (m/z 289).
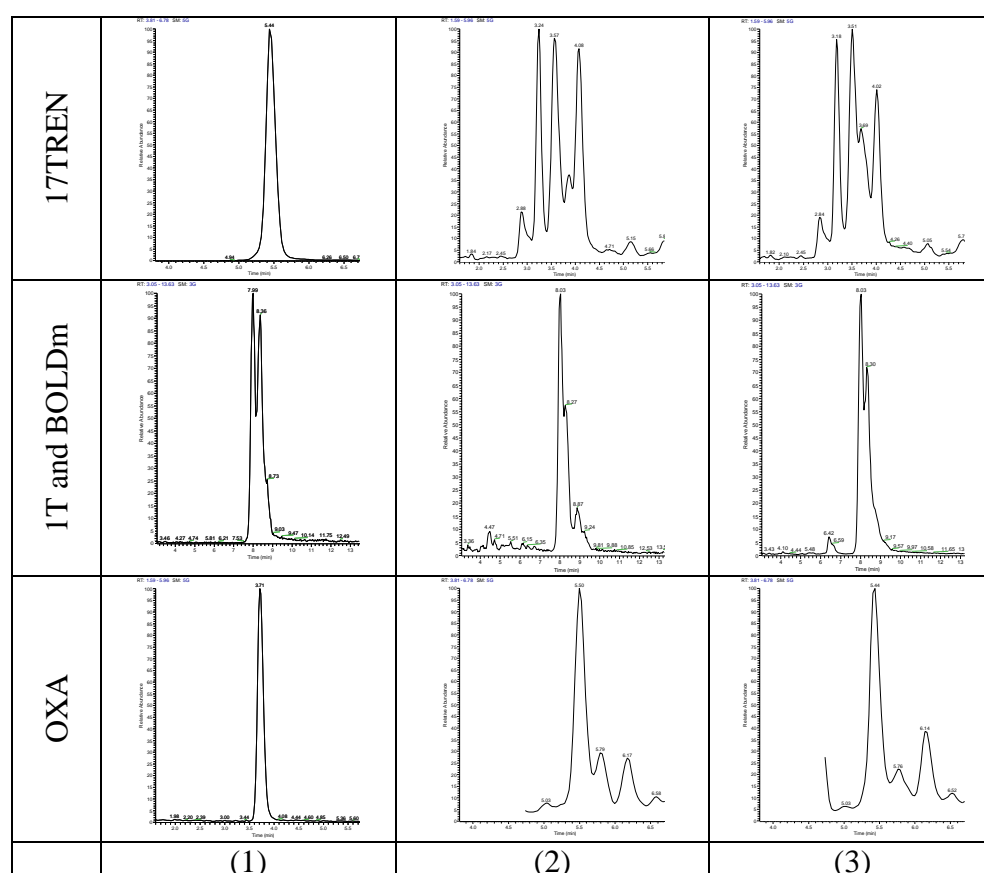

Figure 2. LC-MS/MS chromatograms for selected steroids: (1) Standard, (2) negative urine and (3) urine spiked at the MRPL (10 ng/ml).

Under these MS conditions, satisfactory sensitivity was obtained for all analytes when reference standards were analysed (Figure 2.1). However, no significant differences were obtained when a blank urine sample was compared with the same urine spiked at the minimum required performance limit (MRPL). Abundant peaks present in the urine matrix were observed irrespective of the blank urine indicating the endogenous character of these interferences. Therefore, the developed methods could not be applied for the reliable detection of these anabolic steroids in urine.

Different alternatives can be used in order to circumvent the presence of these interferences such as the improvement in chromatography. In our case, the selection of optimum MS/MS ions was used for the circumvention of endogenous interferences.

17a-trenbolone

Use of a more specific transition

Substantial interferences were found at the retention time of 17TREN, possibly due to the low specificity of the selected transition i.e. loss of water. Other ions (such as 165 or 141) were obtained after increasing the collision energy. However, despite their higher specificity, their use did not reduce the presence of interfering peaks, showing the structural similarity between 17TREN and the urinary interferences (Figure 3).
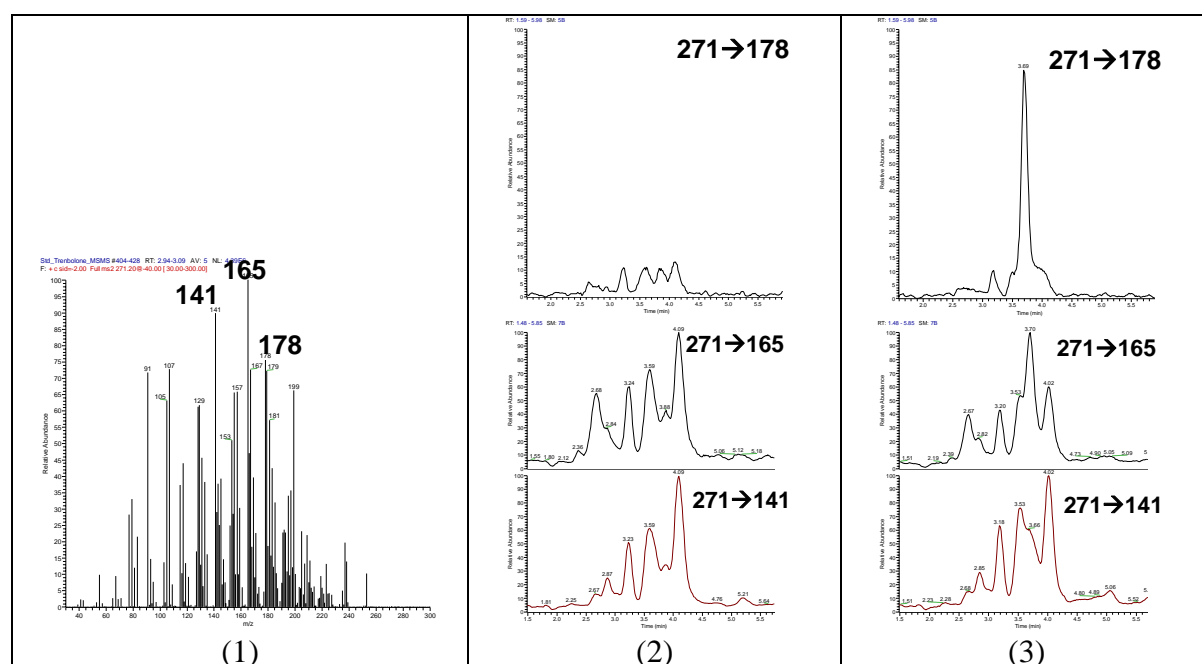

Figure 3. Product ion spectrum of 17TREN at 50 eV of collision energy (1) and LC-MS/MS chromatograms for 17TREN using different transitions: negative urine (2) and urine spiked at 10 ng/ml (3)

This product ion spectrum also showed an ion at m/z 178. Due to the absence of any nitrogen in the 17TREN structure, ions with even m/z can be considered as exceptional as they involve a homolytic fragmentation, which is not common in CID mechanisms where an even electron ion is normally selected as precursor ion. Hence, although less abundant (ion at m/z 178 has abundance of around 15% relative to the most intense ion at m/z 253), this product ion can be considered as more specific increasing the specificity of the method (Figure 3).

Elucidation of interferences

In order to know if these interferences were caused by any steroid and/or corticosteroid, a precursor ion scan of m/z 253 was performed showing 4 different interferences (Figure 4). Two of them presented m/z 367 (a and b) and the others 365 (c) and 339 (d) respectively. None of these interferences thus shared the molecular weight with 17TREN. The high m/z values for the $[M+H]^+$ ions of these compounds and their retention time could be indicative for a corticosteroid structure. This was confirmed by an acquisition in negative ionization mode where an important $[M+HCOO]^-$ which is typical for corticosteroids [6] was found. This adduct ion was obtained for interferences (a), (b) and (c) but not for (d). Therefore, interferences (a), (b) and (c) were considered as corticosteroids.
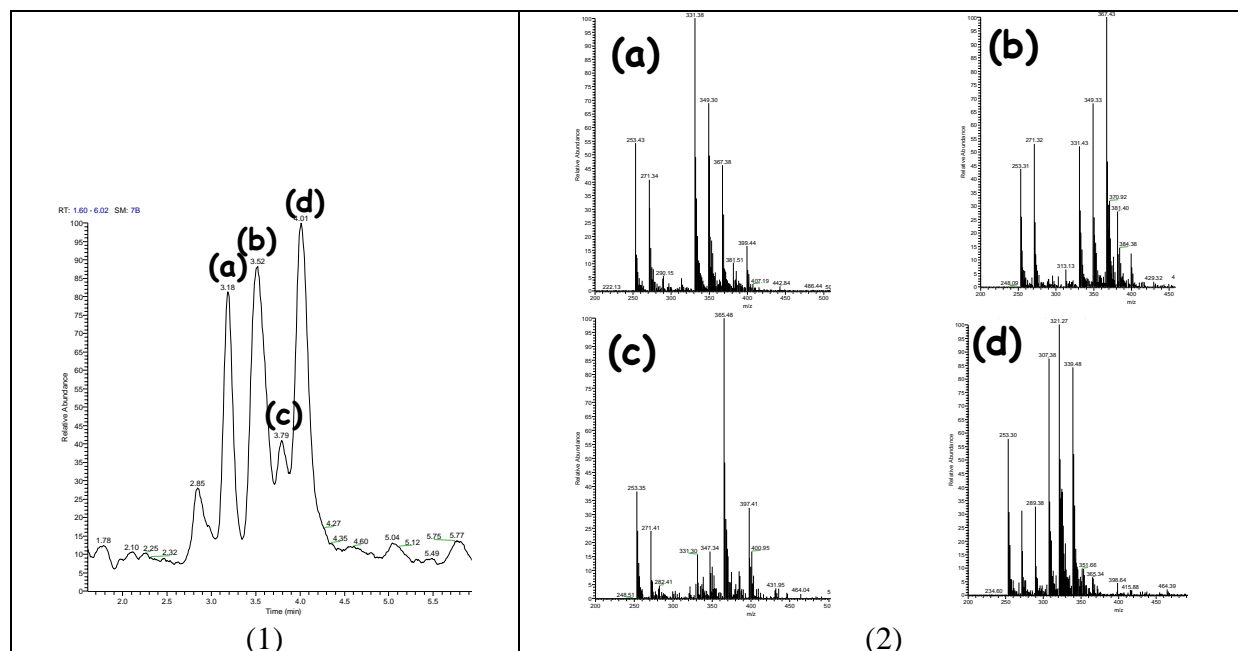

Figure 4. (1) Precursor ion scan chromatogram of m/z 253 showing the 4 interferences, (2) Spectra obtained for each interference.

By application of the structural restrictions for corticosteroids, only two molecular formulae could be assigned to a molecular weight of 366 for interferences (a) and (b): $C_{21}H_{34}O_5$ and $C_{22}H_{38}O_4$. Because endogenous corticosteroids normally contain 21 C and 5 O atoms, $C_{21}H_{34}O_5$ was selected as possible molecular formula for interferences (a) and (b). In the case of interference (c), also two molecular formulae accorded with m/z 364: $C_{21}H_{32}O_5$ and $C_{22}H_{36}O_4$. Identically to the previous case, $C_{21}H_{32}O_5$ was selected as possible molecular formula for interference (c). The product ion spectrum of the $[M+H]^+$ ion of the three interferences ((a), (b) and (c)) showed different ions corresponding to several losses of water (up to four losses of water were observed) and α-hydroxyacetaldehyde. In negative mode, the

product ion spectrum of the [M+HCOO]$^-$ was dominated by losses of HCOOH and H$_2$CO typical for corticosteroids [6].

Two corticosteroids were found to be endogenously present in urine with C$_{21}$H$_{34}$O$_5$ as molecular formula: tetrahydrocortisol and allotetrahydrocortisol while tetrahydrocortisone was the only endogenous corticosteroid with C$_{21}$H$_{32}$O$_5$ as molecular formula. The theoretical elucidation was finally confirmed by the injection of tetrahydrocortisol, allotetrahydrocortisol and tetrahydrocortisone standards [7].

As no signal was observed for interference (d) in negative ionization mode, it was not considered as a corticosteroid. The product ion spectrum of the [M+H]$^+$ of this interference showed the loss of 4 molecules of water and one of methanol. These losses are not typical for steroids and therefore interference (d) can not be considered as anabolic steroid.

Improving the method reliability

The partial elucidation of the interferences confirmed their differences in molecular weight with 17TREN. Therefore, the interferences are produced after an in-source fragmentation of the endogenous corticosteroids. In order to avoid this in-source fragmentation, the acquisition was performed at lower tube lens voltage (TLV). The use of a TLV of 50 V minimised the in-source fragmentation increasing the sensitivity of the method. Under these conditions 17TREN was clearly detected at the MRPL using the more specific transition (Figure 5).
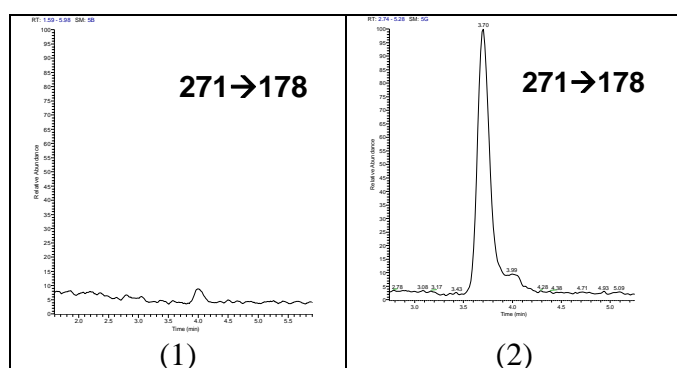

Figure 5. LC-MS/MS for the detection of (1) negative urine (2) Spiked urine at 10ng/ml.

1-testosterone and 5β-androst-1-ene-17β-ol-3-one

Use of a more specific transition

Contrary to 17TREN, no homolytical fragmentations were found for 1T and BOLDm and only product ion at m/z 205 was found to be slightly more specific. In the full scan spectrum, an abundant [M+H+MeOH]$^+$ was found (Figure 6). The formation of these adducts is not very common for anabolic steroids although it is very abundant in 1-ene-3-keto anabolic steroids

and in some unconjugated 3-keto anabolic steroids [1]. In this case, both analytes presented a double bond in the position 1 and therefore the use of [M+H+MeOH]$^+$ as precursor ion was possible. As can be seen in Figure 6, in the detection of 1T and BOLDm in human urine, the selection of [M+H+MeOH]$^+$ as precursor ion was found to be necessary and no significant interferences were observed in the blank urine samples and both analytes could be detected in the spiked urine. The specificity of the method was even further increased by combining both the use of [M+H+MeOH]$^+$ as precursor and selecting the most specific product ion (m/z 205).
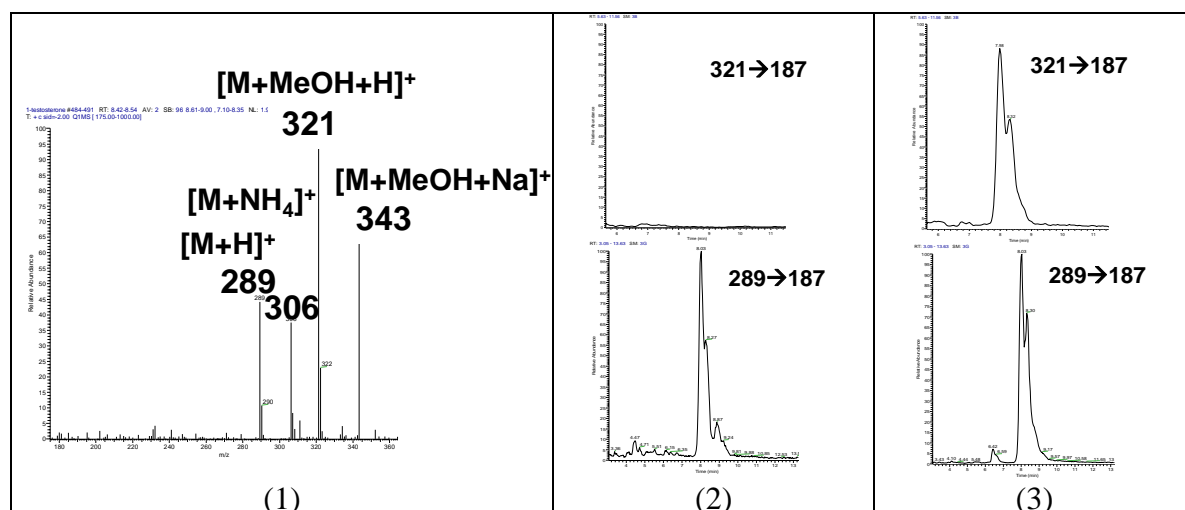

Figure 6. (1) Full scan spectrum for 1-testosterone. (2) negative urine using different precursor ions (3) spiked urine using different precursor ions.

Elucidation of interferences

One of the two interferences showed for these analytes was elucidated as epitestosterone while the other remained unknown. The molecular formula for this last interference can be $C_{18}H_{24}O_3$ or $C_{19}H_{28}O_2$. The search for endogenous steroids with these molecular formulae was unproductive. More details about these elucidations can be found elsewhere [7].

Oxandrolone

Use of a more specific transition

More than 10 different transitions were evaluated using both [M+H]$^+$ and [M+NH$_4$]$^+$ as precursor ion. For all of them some interference at a retention time close to OXA was noticed. As in the case of 1T and BOLDm, a significant [M+H+MeOH]$^+$ ion was observed during the ES ionization of OXA (Figure 7). The abundance of this ion represents only 40% of the base peak ([M+NH$_4$]$^+$). Despite its lower abundance, the use of this ion as precursor ion increased the sensitivity of the detection because the interferences were very much reduced due to the low affinity of most steroids to form this type of adduct. Under these conditions, negligible interference peaks were obtained at the OXA retention time in blank urine samples (Figure 7).
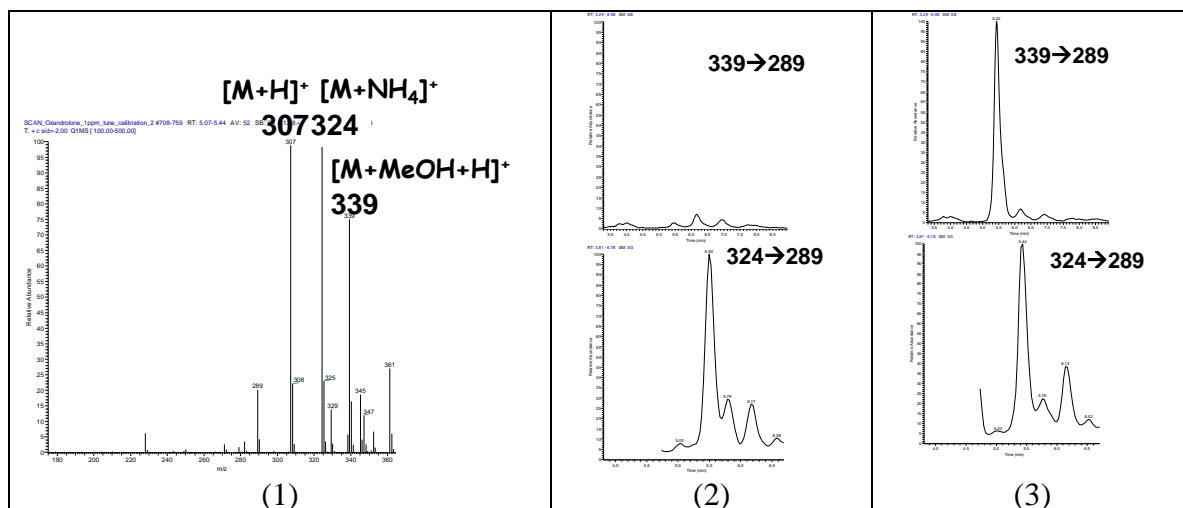
Figure 7. (1) Full scan spectrum for OXA. (2) negative urine using different precursor ions (3) spiked urine using different precursor ions.

Elucidation of interferences

Two of the interferences detected for OXA were elucidated after applying the approach described. These interferences shared the same m/z than OXA and most of the product ions. The presence of 11-hydroxy-androsterone and 16-hydroxy-androsterone was confirmed by the injection of standards as described elsewhere [7].

Acknowledgements

Conclusions

Although LC-MS/MS is a powerful tool for the urinary detection of anabolic steroids, it can be hampered by the presence of endogenous components. Hence, the straightforward use of the most abundant transition does not always result in a more sensitive determination due to these interferences. The selection of more specific transitions such as the use of unusual homolytical fragmentations can help to circumvent these endogenous interferences. The selection of uncommon precursor ions such as [M+H+MeOH]$^+$ is also useful in order to increase the specificity of the method in a matrix as complex as urine. Optimal conditions for selected analytes are shown in Table 2.

The developed approach for the identification of steroids and corticosteroids has shown its applicability as it allowed for the identification of 6 up to 9 of the detected interferences. Therefore, it can be useful in the identification of the molecular formula of unknown steroids

in other applications such as metabolism studies or in the identification of new designer steroids.

Table 2. MS/MS parameters optimized to circumvent endogenous interferences

Analyte	Precursor (m/z)	Precursor (ion)	TLV (V)	Product (m/z)	Col. Energy (eV)
17TREN	271	$[M+H]^+$	50	178	52
1T	321	$[M+H+MeOH]^+$	100	205	20
BOLDm	321	$[M+H+MeOH]^+$	100	205	20
OXA	339	$[M+H+MeOH]^+$	80	289	20

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