Multi residue screening of intact testosterone esters and boldenone undecylenate in bovine hair using liquid chromatography electrospray tandem mass spectrometry

Michel W.F. Nielen a,∗, Johan J.P. Lasaroms a, Patrick P.J. Mulder a, Johan Van Hende b, J.(Hans)A. van Rhijn, Maria J. Groot a

a RIKILT Institute of Food Safety, P.O. Box 230, 6700 AE Wageningen, The Netherlands
b Ghent University, Faculty of Veterinary Medicine, Salisburylaan 133, B-9820 Merelbeke, Belgium

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Abstract

The abuse of esters of natural androgenic steroids in cattle fattening and sports is hard to control via routine urine testing. The esters are rapidly hydrolysed in vivo into substances which are also endogenously present in urine. In veterinary control strange findings of 17α/β-testosterone and 17α/β-testosterone in urine are often ignored because of the lack of statistically sound reference data of naturally occurring levels. An interesting alternative for inconclusive urine analyses in veterinary control can be provided by the analysis of the administered steroids themselves, i.e. the analysis of intact steroid esters in hair. Unfortunately, the analysis of intact steroid esters is complicated not only by the vulnerability of the esters which precludes alkaline hydrolysis of the hair, but also by the wide polarity range of short and long-chain esters yielding very poor recoveries for either the one or the other. In this study, a multi-steroid esters LC/MS/MS screening method is presented for trace analysis of the synthetic intact esters of 17α/β-testosterone and the undecylenate ester of 17β-boldenone in bovine hair. The method, requiring only 200 mg of pulverised hair, features a mild digestion procedure using tris(2-carboxyethyl)phosphine hydrochloride (TCEP) and the use of four deuterium-labelled steroid esters as internal standards covering the wide polarity range of the analytes. In spiked hair samples for most of the analytes the limit of detection and the accuracy using isotope dilution were 2–5 ng/g and 97–105%, respectively. The applicability was demonstrated using hair samples from a controlled experiment in which six bovines were injected intramuscularly with two different doses of two commercial mixtures of testosterone esters, and with two different doses of boldenone undecylenate. Depending on the dose all administered testosterone- and boldenone esters were found to be incorporated in bovine hair following a single intramuscular injection, except testosterone propionate which dose might have been too low.

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Keywords: Liquid chromatography; Mass spectrometry; Hair; Testosterone ester; Anabolic steroid; Boldenone

1. Introduction

Anabolic steroids are banned substances in the European Union but might still be illegally applied as growth promoters in cattle fattening [1,2]. In the search for suitable sample matrices bovine hair has shown to be an attractive sample matrix for prolonged detectability of residues of anabolic steroids and beta-agonists. Such substances can be incorporated into hair from blood via the hair follicle, incorporated from sweat via the hair shaft, absorbed from sweat on the outside of the hair shaft, and/or absorbed from exogenic sources, such as (environmental) contamination or intentional illegal pour-on treatment [3–5]. Beta-agonists, such as clenbuterol disappear in urine within a few days following administration but might be detected in hair for more than 2 months [6]. Basic anabolic steroids, such as stanozolol are incorporated in hair as well [7,8]. Gleixner and Meyer [9] determined the natural steroid hormones estradiol and testosterone in hair of cattle by high-performance liquid chromatography (HPLC) and specific enzyme immuno assays (EIA). In hair from calves estradiol and testosterone levels were, depending on sex, in the range of 5–9 ng/g and 3–7 ng/g, respectively. In the hair of bulls, an influence of pigmentation was seen for testosterone in...
black hair, concentrations were approximately four times higher. Since then, the potential of hair analysis for the determination of neutral steroids, such as androgens, estrogens and corticosteroids has been demonstrated in several papers [10–16]. Natural steroids are usually administered as synthetic steroid esters but these are rapidly hydrolyzed in vivo into natural steroids: following oral intake of testosterone undecanoate the unchanged ester could be found in plasma from athletes for 6 h only [17]. In urine it is hard to differentiate between the (metabolites of) endogenous natural steroids always present and the (metabolites of) natural steroids from the hydrolysed esters. In doping control this problem is usually addressed by establishing the 17β-testosterone/17α-testosterone urinary ratio (so-called T/E ratio) [18] and/or the application of $^{13}$C/$^{12}$C isotope ratio mass spectrometry [19]. In veterinary control strange findings of 17β-testosterone and 17α-testosterone in urine are often ignored because of the lack of statistically valid reference data of naturally occurring background levels. An interesting alternative for inconclusive urine analyses in veterinary control can be provided by the analysis of the administered synthetic steroids themselves, i.e. the analysis of intact esters of natural steroids in hair. The first problem to be solved is the digestion and extraction of the hair: usually hair samples are digested under alkaline or acidic conditions, which is adequate for beta-agonists and steroids but less suitable for the vulnerable steroid esters. Several authors applied direct solvent extraction with methanol or diethyl ether in order to prevent hydrolysis of the esters but only the adsorbed substances will be accessible and the recovery for incorporated steroid esters might be questioned [3,20,21]. Gleixner and Meyer digested hair samples using dithiothreitol to break up the longitudinal chains of the keratin protein of the hair by reducing the disulfide bonds and applied that method to the determination of the natural steroids estradiol and testoster-
one [9]. Recently, Hooijerink et al. developed an LC/MS/MS method for the determination of intact estradiol benzoate in hair [22]. Following a mild digestion using the reducing agent tris(2-carboxyethyl)phosphine hydrochloride (TCEP) and subsequent solid phase extraction (SPE) samples were analysed by liquid chromatography (LC) with positive ion electrospray ionization (ESI) tandem mass spectrometry (MS/MS) in the MRM mode using 16,16,17-d3-estradiol-3-benzoate as internal standard. The development of a similar method for intact esters of testosterone is even more demanding due to the wide range of fatty acid esters available and their very different polarities. In this work we coped with the polarity-range problem by developing dedicated SPE and LC procedures and the synthesis and application of a range of deuterium-labelled testosterone esters as internal standards. Moreover, we included the synthetic 17β-boldenone undecylcyl ester which detection is of particular interest because of the scientific debate about the origin of its main urinary metabolite 17α-boldenone [23]. The EU legislation for veterinary control has set different requirements concerning the performance of analysis methods and the interpretation of results [24]. Methods are classified for screening or confirmation, and can be qualitative or quantitative. For banned substances having no tolerance level usually qualitative (multi)screening methods are applied. These methods range from immunoassays to multi-residue GC/MS and LC/MS/MS instrumental screening methods. The MS-based multi-residue screening methods typically measure only one ion or MRM transition per analyte. When a sample is screened suspect, confirmation of identity by mass spectrometry is required: the analyte in the suspect sample should comply with criteria for a minimum number of identification points (data acquisition should include at least four ions or two MRM transitions), ion ratios and relative retention time should be within defined limits [24]. Detection limit, recovery, accuracy and within-laboratory reproducibility of this multi-residue screening method were determined using spiked bovine hair samples and finally the feasibility of application to real-life samples was investigated using incurred hair samples from a controlled animal treatment experiment.

2. Experimental

2.1. Chemicals

Water was purified using a Milli-Q system (Millipore, Bedford, MA, USA). Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) and subsequent solid phase extraction (SPE) samples were analysed by liquid chromatography (LC) with positive ion electrospray ionization (ESI) tandem mass spectrometry (MS/MS) in the MRM mode using 16,16,17-d3-estradiol-3-benzoate as internal standard. The development of a similar method for intact esters of testosterone is even more demanding due to the wide range of fatty acid esters available and their very different polarities. In this work we coped with the polarity-range problem by developing dedicated SPE and LC procedures and the synthesis and application of a range of deuterium-labelled testosterone esters as internal standards. Moreover, we included the synthetic 17β-boldenone undecylcyl ester which detection is of particular interest because of the scientific debate about the origin of its main urinary metabolite 17α-boldenone [23]. The EU legislation for veterinary control has set different requirements concerning the performance of analysis methods and the interpretation of results [24]. Methods are classified for screening or confirmation, and can be qualitative or quantitative. For banned substances having no tolerance level usually qualitative (multi)screening methods are applied. These methods range from immunoassays to multi-residue GC/MS and LC/MS/MS instrumental screening methods. The MS-based multi-residue screening methods typically measure only one ion or MRM transition per analyte. When a sample is screened suspect, confirmation of identity by mass spectrometry is required: the analyte in the suspect sample should comply with criteria for a minimum number of identification points (data acquisition should include at least four ions or two MRM transitions), ion ratios and relative retention time should be within defined limits [24]. Detection limit, recovery, accuracy and within-laboratory reproducibility of this multi-residue screening method were determined using spiked bovine hair samples and finally the feasibility of application to real-life samples was investigated using incurred hair samples from a controlled animal treatment experiment.

2.2. Synthesis of internal standards

The deuterated internal standards 16,16,17-d3-testosterone propionate, 16,16,17-d3-testosterone phenylpropionate, 16,16,17-d3-testosterone cyclopentylpropionate and 16,16,17-d3-testosterone decanoate were prepared using the following procedure, adapted from Francisco et al. [25]: 16,16,17-d3-testosterone (11.66 mg, 40 μmol) was dissolved in toluene (2 ml) and a solution of DMAP (10 mg/ml, 1 ml) in toluene was added. While stirring at room temperature the appropriate acyl chloride was added: propionyl chloride (11.0 μl, 120 μmol), phenylpropionyl chloride (11.9 μl, 80 μmol), cyclopentylpropionyl chloride (12.2 μl, 80 μmol) or decanoyl chloride (16.6 μl, 80 μmol). Upon addition of the acyl chloride a white precipitate started to form. The reaction mixtures were warmed to 60 °C using a water bath and the course of the reactions were monitored by analyzing aliquots by HPLC–UV (240 nm) at regular intervals. After 3–4 h ester formation was almost complete (>98%) and the reactions were stopped. To each reaction mixture hexane (20 ml) was added and the organic
layer was washed twice with water (20 ml) and three times with 1% ammonium (20 ml). The organic layer was dried on sodium sulphate and the solvent was removed under reduced pressure.

The d3-testosterone esters were obtained as a white crystal mass in a yield of 93–96% 9. HPLC-UV indicated a minimum chemical purity for each of the esters of 95%. LCMS and NMR analysis confirmed the formation of the testosterone esters and indicated 98% deuterium incorporation at positions 16, 16 and 2 of the steroid skeleton.

2.3. Samples

Hair samples were obtained from a national veterinary control program and from a controlled animal treatment experiment: three female and three male bovines of approximately 200–300 kg were injected intramuscularly with the following commercially available products: boldenone undecylenate (1 mg/kg and 2 mg/kg BW) and two mixtures containing testosterone propionate (0.1–0.2 mg/kg and 0.2–0.4 mg/kg BW), testosterone phenylpropionate (0.2–0.5 mg/kg and 0.5–0.8 mg/kg BW), testosterone isocaproate (0.2–0.5 mg/kg and 0.5–0.8 mg/kg BW), and testosterone decanoate (0.4–0.8 mg/kg and 0.8–1.3 mg/kg BW). In addition, samples were analysed from nortestosterone dodecanoate esters and the boldenone undecylenate were calculated using d3-testosterone phenylpropionate; the enanthate and cypionate esters using d3-testosterone cypionate; and the decanoate and undecanoate esters and the boldenone undecylenate were calculated using d3-testosterone decanoate. Unknown incurred samples were analysed with and without a 5 ng/g spike of the steroid (negative control) and hair spiked with 2 ng/g, 5 ng/g, 10 ng/g and 20 ng/g of the analytes (positive controls) were analysed in the same analysis series. Recoveries were calculated by comparing the peak areas in the reconstructed chromatograms of the 5 ng/g spiked hair samples and the corresponding response from direct injection of a 1 ppb standard solution, unless otherwise indicated. Accuracies were calculated using a 0–20 ng/g calibration curve from matrix-matched standards and the relative response versus the deuterium-labelled internal standards.

The accuracy of testosterone–acetate and –propionate were calculated using the internal standard d3-testosterone propionate; the benzoate, phenylpropionate and isocaproate esters using d3-testosterone phenylpropionate; the enanthat and cypionate esters using d3-testosterone cypionate; and the decanoate and undecanoate esters and the boldenone undecylenate were calculated using d3-testosterone decanoate. Unknown incurred samples were analysed with and without a 5 ng/g spike of the steroid esters.

2.4. Sample preparation

The hair samples were cut into 0.5 cm pieces using a pair of scissors. Then 500 mg was pulverized in a Sartorius (Goettingen, Germany) model Mikro-dismembrator S ball mill as follows: the teflon vessel containing a grinding ball and the cut hair was cooled for 2 min in a Dewar vessel with liquid nitrogen followed by shaking in the mill at 3000 rpm for 2 min. Two hundred milligrams of the pulverized hair thus obtained was weighed by shaking in the mill at 3000 rpm for 2 min.

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4.1. Solid phase extraction

Solid phase extraction and LC/MS/MS of testosterone esters is not straightforward due to the wide polarity range of the different testosterone esters involved. Long alkyl chain esters, such as testosterone undecanoate do not elute from most C18 reversed phase LC columns, even at 100% modifier in the mobile phase. Shackleton et al. [26] concluded for example that LC of these esters was only possible following conversion to more polar Girard hydrazones. We described an LC/MS/MS identification method for the analysis of high levels of anabolic steroids in illegal cocktails in which we successfully applied a
Table 1  
Positive ion electrospray MS/MS conditions for the MRM acquisition of intact testosterone esters, their deuterated internal standards and boldenone undecylenate

<table>
<thead>
<tr>
<th>Component</th>
<th>Ion transition for screening</th>
<th>Second ion transition for confirmation</th>
<th>Collision energy (eV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testosterone acetate (T-C₂)</td>
<td>331 &gt; 109</td>
<td>331 &gt; 97</td>
<td>20</td>
</tr>
<tr>
<td>Testosterone propionate (T-C₃)</td>
<td>345 &gt; 109</td>
<td>345 &gt; 97</td>
<td>20</td>
</tr>
<tr>
<td>d₃-Testosterone propionate</td>
<td>348 &gt; 109</td>
<td>–</td>
<td>20</td>
</tr>
<tr>
<td>Testosterone benzoate (T-Bz)</td>
<td>393 &gt; 97</td>
<td>393 &gt; 109</td>
<td>20</td>
</tr>
<tr>
<td>Testosterone phenylpropionate (T-PhiC₃)</td>
<td>421 &gt; 97</td>
<td>421 &gt; 109</td>
<td>20</td>
</tr>
<tr>
<td>d₃-Testosterone phenylpropionate</td>
<td>424 &gt; 97</td>
<td>–</td>
<td>20</td>
</tr>
<tr>
<td>Testosterone isocaproate (T-iCpr)</td>
<td>387 &gt; 97</td>
<td>387 &gt; 109</td>
<td>20</td>
</tr>
<tr>
<td>Testosterone enanthate (T-C7)</td>
<td>401 &gt; 97</td>
<td>401 &gt; 109</td>
<td>20</td>
</tr>
<tr>
<td>Testosterone cypionate (T-Cyp)</td>
<td>413 &gt; 97</td>
<td>413 &gt; 109</td>
<td>20</td>
</tr>
<tr>
<td>d₃-Testosterone cypionate</td>
<td>416 &gt; 97</td>
<td>–</td>
<td>20</td>
</tr>
<tr>
<td>Boldenone undecylenate (17βBolundec)</td>
<td>453 &gt; 135</td>
<td>453 &gt; 121</td>
<td>20</td>
</tr>
<tr>
<td>Testosterone decanoate (T-C₁₀)</td>
<td>443 &gt; 97</td>
<td>443 &gt; 109</td>
<td>20</td>
</tr>
<tr>
<td>d₃-Testosterone decanoate</td>
<td>446 &gt; 97</td>
<td>–</td>
<td>20</td>
</tr>
<tr>
<td>Testosterone undecanoate (T-C₁₁)</td>
<td>457 &gt; 97</td>
<td>457 &gt; 109</td>
<td>20</td>
</tr>
</tbody>
</table>

5 μm C8 column with an acetonitrile gradient for the separation of, amongst others, testosterone phenylpropionate, testosterone cypionate and testosterone decanoate [27]. In the present study we used again a C8 column but from a different brand, cf. Section 2.5. In the clean up via solid phase extraction a 100 mg C18 cartridge was used in order to have sufficient retention, even for the most polar ester, testosterone acetate. Different combinations of methanol/water mixtures for sample application and elution solvents (acetonitrile, methanol, ethyl acetate, and mixtures thereof) were investigated for optimum recovery for the highest number of testosterone esters. Finally, sample application from methanol/water (40:60) and elution with 2 ml acetonitrile and 2 ml ethyl acetate yielded the best compromise and was chosen in all further experiments. Increasing the elution

Fig. 1. Common fragmentation behaviour of intact testosterone- and boldenone esters in positive ion electrospray ionisation tandem mass spectrometry.
volume towards 5 ml acetonitrile and 5 ml ethyl acetate might improve the recoveries of the least polar esters even more, but at the cost of a longer evaporation time.

The screening of testosterone esters with positive ion electrospray ionisation MS/MS is facilitated by a common fragmentation behaviour (Fig. 1). Apart from characteristic ions related to ester cleavage testosterone-specific low mass ions are obtained at \( m/z \) 97 and 109 and a minor ion at \( m/z \) 123 which originate from ring A and B cleavages. \( m/z \) 97 includes all A-ring carbons except C5 but including C19, and \( m/z \) 109 probably includes carbon atoms C1–7. Recently, it was demonstrated that low abundant D-ring fragments are also formed in 3-keto-4-ene steroids having a substitution at C17 [26–28].

The common fragmentation behaviour of different testosterone esters was applied in this work for multiresidue analysis in hair. An MRM acquisition method was set up based on the transition of the respective \([M + H]^+\) ions towards either \( m/z \) 97 or 109, depending on the optimum sensitivity obtained in infusion experiments (Table 1). A precursor ion scanning approach would be preferred of course but, unfortunately, that option is not sensitive nor selective enough for trace analysis in a complex matrix as digested hair. Boldenone and esters thereof fragment towards the B-ring fission product ions \( m/z \) 121 and 135 [23]. \( m/z \) 135 provided the best sensitivity in the present screening method. A typical reconstructed LC/MS/MS chromatogram of a hair sample spiked with 5 ng/g boldenone undecylenate is given in Fig. 2, together with the blank signal. The detection limit of boldenone undecylenate in this hair sample can be estimated to be in the order of 2 ng/g. For most of the analytes the detection limit in real samples is typically 2–5 ng/g depending on the sensitivity of the MS/MS system and the complexity of specific hair samples, except for testosterone enanthate which suffers occasionally from signal interference. Note that the final performance data in terms of detection capability (CC\(_{\text{b}}\)) and decision limit (CC\(_{\text{l}}\)) might be even better since we intend to install a more sophisticated MS/MS system in 2006. From LC/MS/MS data of hair samples spiked at a level of 5 ng/g the recovery, accuracy and within-lab reproducibility were determined, the results are summarised in Table 2. The mean recoveries (\( n = 5 \)) are in the range of 20–64% which is considered quite acceptable, given the wide polarity range and the level of spiking. Sources for incomplete recovery might be
the centrifugation of the hair digest, the solid phase extraction and the redissolution step. The most polar steroid ester, testosterone acetate, and the most nonpolar steroid ester, testosterone undecanoate, showed relatively low recoveries indicating that the method developed did not selectively discriminate against either polar- or nonpolar steroid esters, thereby offering a good compromise for a multi-analyte screening method. The mixture of deuterium-labelled intact steroid esters used as internal standards was very well capable to correct for the incomplete recoveries and the ionisation suppression of co-eluting analogues: the accuracies were 97–103% for most of the esters at the 5 ng/g level, except for testosterone phenylpropionate, -isocaproate and cypionate (87%, 82% and 110%, respectively) which were relatively close to the LOD in this hair matrix. At higher signal-to-noise levels (10 ng/g spiked hair) the accuracy data of the latters improved to 100%, 97% and 105%. The within-lab reproducibility was in the range of 10–16% R.S.D. (n = 5) at the 5 ng/g level, except for testosterone cypionate (35%). Again, this value improved (to 15% R.S.D.) at the 10 ng/g level.

The selectivity/specificity of the method was checked by the analysis of hair samples from bovines not being suspect for steroid abuse. The selectivity of the LC/MS/MS analysis operated in the MRM mode was fit for purpose: no or minor interferences appeared at the retention times of interest, except for testosterone enanthate. Note that the ruggedness and stability of the method can be monitored in each individual sample analysis by the signal intensity of the deuterated internal standards.

### 3.3. Applicability

Spiked samples do not represent the physiological situation in which steroid esters might not be only absorbed but also incorporated into the growing hair. Definitely the use of certified reference hair samples containing known incorporated

<table>
<thead>
<tr>
<th>Component</th>
<th>LOD (ng/g)</th>
<th>Recovery at 5 ng/g mean (%)</th>
<th>Accuracy a at 5 ng/g mean (%)</th>
<th>Within-lab reproducibility at 5 ng/g, n = 5 (% R.S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-acetate</td>
<td>2–5</td>
<td>20</td>
<td>99</td>
<td>16</td>
</tr>
<tr>
<td>T-propionate</td>
<td>2–5</td>
<td>36</td>
<td>103</td>
<td>13</td>
</tr>
<tr>
<td>T-benzoate</td>
<td>2–3</td>
<td>34</td>
<td>97</td>
<td>13</td>
</tr>
<tr>
<td>T-phenylpropionate</td>
<td>2–3</td>
<td>47</td>
<td>87</td>
<td>12</td>
</tr>
<tr>
<td>T-isocaproate</td>
<td>2–5</td>
<td>21</td>
<td>82</td>
<td>11</td>
</tr>
<tr>
<td>T-cypionate</td>
<td>2–5</td>
<td>46</td>
<td>110</td>
<td>35</td>
</tr>
<tr>
<td>Bol-undecylenate</td>
<td>2–5</td>
<td>64</td>
<td>102</td>
<td>10</td>
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<tr>
<td>T-decanoate</td>
<td>2–5</td>
<td>34</td>
<td>99</td>
<td>10</td>
</tr>
<tr>
<td>T-undecanoate</td>
<td>2–5</td>
<td>23</td>
<td>102</td>
<td>14</td>
</tr>
</tbody>
</table>

n.d., not determined.

a Using isotopically labelled internal standards.

b Due to occasional signal interference in real samples, see text.

c Recovery data at 20 ng/g spiked hair.

d Additional accuracy data from 10 ng/g spiked hair.
Fig. 4. Reconstructed LC/MS/MS chromatograms of bovine hair samples from a controlled animal treatment experiment. Samples were taken following administration of (a) boldenone undecylenate, sampled on day 8 after the first administration, two different mixtures of testosterone-propionate, -phenylpropionate, -isocaproate and -decanoate, sampled on (b) day 6 after the third administration and (c) day 14 after the first administration, and (d) nortestosterone dodecanoate (NorT-C₁₂), sampled on day 8 after the second administration, respectively. MRM acquisition and abbreviations according to Table 1. Arrows indicate the position of the negative results for testosterone propionate. Other conditions, see Section 2.
boldenone undecylenate. For testosterone esters the best choice was demonstrated by the analysis of incurred hair samples from a controlled bovine treatment experiment. Following a single injection of 1–2 mg/kg BW the long-chain steroid esters might be detected up to 14 days. Testosterone propionate was not detected and either does not seem to be incorporated in bovine hair, and/or was administered in a too low dose. The scope of the method can be easily extended when other steroid esters show up in illicit preparations, as demonstrated by the analysis of nortestosterone dodecanoate.

Sample extracts which are screened suspect might be re-injected into the LC/MS/MS system for confirmatory analysis by adding an appropriate second MRM transition to the data acquisition settings. More recent triple quadrupole mass spectrometers do allow more MRM transitions to be acquired simultaneously and the sensitivity loss, due to the inherently shorter dwell times, is compensated by increased overall sensitivity of the instruments. As a result the multi-ester LC/MS/MS screening method presented here might be simply converted into a confirmatory method, covering at least two MS/MS transitions per analyte and without requiring a second injection of the sample extract. The analysis of the synthetic intact steroid esters in bovine hair offers the multi-ester LC/MS/MS screening method the possibility to detect the origin of boldenone residues in bovines.

4. Conclusions

A multi-steroid esters LC/MS/MS screening method has been developed for a wide range of intact androgenic steroid esters. The applicability has been successfully demonstrated by the analysis of incurred hair samples from a controlled bovine treatment experiment. Testosterone propionate was not detected and either does not seem to be incorporated in bovine hair. Therefore, it can be concluded that the analysis procedure does not discriminate against testosterone propionate. Obviously, testosterone propionate is not incorporated in hair as the intact ester, and/or its dose (0.1–0.4 mg/kg BW) was simply too low. The absence of testosterone propionate esters is in agreement with a previous study in which a mixture of testosterone enanthate and testosterone propionate was administered to human volunteers: only testosterone enanthate could be detected in plasma and the propionate ester was obviously absent.

The testosterone decanoate ester, having the highest repeat dose in the mixtures (0.8 and 1.3 mg/kg BW), was detected for 14 days following a single dose (Fig. 4c). The other, lower dose, testosterone esters hardly showed up at day 14, compare for example Fig. 4b and c.

The method development did not include nortestosterone dodecanoate; however, evidently MS/MS transitions can be added in order to allow the detection of new steroid esters as demonstrated in Fig. 4d. The intact nortestosterone dodecanoate ester could be detected at least 8 days following a second administration of 2 mg/kg BW (first administration 22 days ago). The steroid esters results after a single dose administration obtained in this work compare favourably with the data from Segura et al. [30] in which no testosterone esters, nor nandrolone (nortestosterone) decanoate could be detected by GC/MS/MS. Probably either the digestion procedure and/or the overall sensitivity of that method was not appropriate for steroid esters in hair.

Although being developed as a multi-steroid LC/MS/MS screening method, confirmation of identity can be achieved by re-analysing the sample extract and acquiring at least two MS/MS ion transitions [24]. From a sensitivity point of view the second product ion at m/z 121 would be the best option for boldenone undecylenate. For testosterone esters the best choice is compound dependent: either m/z 109 or 97 might be selected as second product ion (Table 1). As an example the hair sample obtained on day 8 and incurred with boldenone undecylenate was subjected to confirmatory LC/MS/MS analysis with acquisition of the two MRM transitions m/z 453 > 135 and m/z 453 > 121. The ratio of the signals in the two reconstructed MS/MS ion transition chromatograms as well as the relative retention time were both within the limits set by spiked reference hair samples and fulfilled the confirmation of identity criteria [24].

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References