Journal of Chromatography A, 1216 (2009) 8059–8066

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Confirmatory method for the determination of nandrolone and trenbolone in urine samples using immunoaffinity cleanup and liquid chromatography–tandem mass spectrometry

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ARTICLE INFO

Article history:
Available online 3 May 2009

Keywords:
Nandrolone
Trenbolone
Urine
Immunoaffinity chromatography
Mass spectrometry

ABSTRACT

A confirmatory method for the simultaneous determination of nandrolone (α and β) and trenbolone (α and β) in urine samples by liquid chromatography electrospray mass spectrometry (LC–MS-MS) was developed. After an enzymatic deconjugation, the urine was subjected to a one-step cleanup on a commercially available immunoaffinity chromatography cartridge. The analytes were detected by liquid chromatography–positive ion electrospray tandem mass spectrometry using deuterium labelled internal standards. The analytical procedure was applicable to bovine and swine urine samples. The procedure was validated as a quantitative confirmatory method according to the Commission Decision 2002/657/EC criteria. The results obtained showed that the method was suitable for statutory residues testing regarding the following performance characteristics: instrumental linearity, specificity, precision (repeatability and intra-laboratory reproducibility), recovery, decision limit (CCα), detection capability (CCβ) and ruggedness. The decision limits (CCα) obtained, were between 0.54 and 0.60 $\mu g\,L^{-1}$; the recovery was above 64% for all the analytes. Repeatability was between 1.6% and 5.7% and within-laboratory reproducibility between 1.6% and 6.0% for all the steroids.

1. Introduction

Nandrolone (17-α-19-nortestosterone and 17-β-19-nortestosterone) and trenbolone (17-α-trenbolone and 17-β-trenbolone) are two steroidal synthetic androgens (Fig. 1) with important anabolic activities in animal productions, where they have been widely used to increase mass gain and improve feed conversion. Until the 90's nandrolone (NT) has been one of the most used anabolic steroids in food-producing animals, and has been frequently found in illegal injectable preparations containing β-NT fatty acid esters. Its metabolic pathway in urine includes the formation of the β-NT and α-NT compounds [1–3]. Also trenbolone (TB) has been administered to animals as illegal growth promoter by subcutaneous implantation in the form of β-TB acetate, which is rapidly hydrolysed to the active compound β-TB. Subsequently β-TB undergoes an oxidation followed by a reduction leading to the formation of α-TB in urine [4,5]. Both NT and TB are eventually excreted in urine as α- and β- glucuronide or sulphate conjugates. Since 1986, in view of the intrinsic properties of hormones and related scientific findings, the use of NT, TB and other natural and synthetic hormones as growth promoters in food-producing animals has been banned within the European Union (EU). This prevents consumers from being exposed to unforeseeable risks from the intake of hormone residues and their metabolites [6]. EU member states are required to monitor animals, fit for human consumption, for possible abuse [7]. Furthermore, EU confirmatory methods for the determination of anabolic residues in animal tissues and biological fluids must be validated according to the criteria set by Commission Decision 2002/657/EC [8]. Analytical methods, suitable for the analysis of NT, TB or both in urine samples using gas chromatography–tandem mass spectrometry (GC–MS) are published [1,3,9–16]. However GC–MS is a time-consuming technique when applied to hormone determination because it always requires derivatization step. Due to its structure, TB gives problems using the common derivatizing agents [17], and the instability of derivatized TB in the reaction mixture also causes the formation of other species with time [18]. A robust, easy and sensitive alternative to GC–MS is liquid chromatographic–tandem mass spectrometry (LC–MS-MS), where the derivatization step can be avoided, although there could be problems about ion-suppression phenomena. LC–MS-MS is therefore the best choice for the simultaneous analysis of TB and NT, in order to reach detection limits fit for purpose and increase specificity. LC–MS-MS methods determining TB, NT or both using atmospheric pressure chemical ionisation (APCI) [5,19] and electrospray ionisation (ESI) [20–24], most of them in MRM mode [5,19–22,24], are described. Most of them are applicable to the

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doi:10.1016/j.chroma.2009.04.075

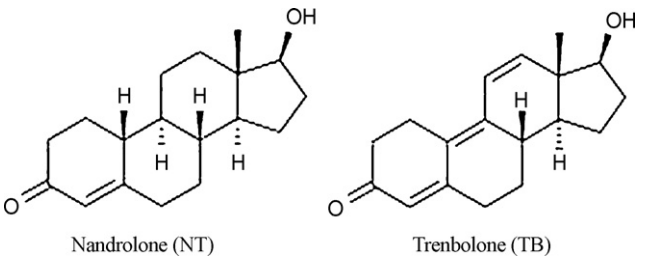

Fig. 1. Nandrolone and trenbolone chemical structures.

determination only of NT [1–3,9–14,19,22], or TB [4,5,13,20,23,24]. Only a few of them can be applied to the simultaneous determination of NT and TB [15,16,21,25], and to the analysis of both bovine and swine urine samples [4,12,13,16,21,24]. Almost all of the published methods can, actually, analyse only one type of urine [1–3,5,14,15,19,20,25], or urine of other animal species [9–11,26]. In order to achieve the required specificity, many methods include time-consuming urine sample preparation procedures with enzymatic hydrolysis and SPE cleanup steps. The SPE columns used are C_{18} [5,9–16,19], the combination of C_{18} and NH_2 [1,3,16,20,21] and Oasis [23,24]. None of the methods reviewed reports the use of the immunoaffinity chromatography cartridge (IAC) in the cleanup step of the samples for LC–MS-MS confirmatory analysis. A few describe this procedure for screening HPLC analysis [25], for GC–MS quantification [1,12] and for an HPLC screening step followed by GC–MS quantification [2]. A minority of the GC–MS or LC–MS-MS methods reviewed [5,20,21,25] is validated according to the criteria set by the draft SANCO/1805/2000 [27] and only one is fully validated according to the criteria set by the Commission Decision 2002/657/EC [24]. The present paper describes a sensitive method for the simultaneous identification and quantification of NT (α and β) and TB (α and β) in urine samples. This method involves, after an enzymatic deconjugation step, a very simple and quick IAC cleanup of the samples. It is easily applicable to both bovine and swine urine. Liquid chromatography–positive ion electrospray mass spectrometry using deuterium labelled standards was applied for the confirmation of all four steroids. Furthermore, the procedure was validated as a quantitative confirmatory method according to Commission Decision 2002/657/EC [8]: instrumental linearity, specificity, precision (repeatability and intra-laboratory reproducibility), recovery, decision limit (CCα), detection capability (CCβ) and ruggedness were evaluated for all the analytes.

2. Experimental

2.1. Chemicals and materials

All reagents and solvents were of analytical or HPLC grade quality and were supplied by Sigma (Sigma–Aldrich, Milan, Italy). Ultrapure water was obtained by Milli-Q system Millipore (Bedford, MA, USA). Trenbolone/19-Nortestosterone immunoaffinity chromatography columns (1 mL) was purchased from Randox (Crumlin, United Kingdom). The concentrated IA column wash buffer and column storage buffer were provided in the IAC commercial package. The working diluted wash buffer was prepared using one part of concentrate buffer and 19 parts of ultrapure water and the working diluted storage buffer using one part of concentrate buffer and four parts of ultrapure water. α-TB, α-NT and β-NT-d3 (Internal Standard – ISTD) were purchased by NARL (Pymble, NSW 2073, Australia). β-TB, β-TB-d2 (ISTD) were bought from RIVM (Bilthoven, The Netherlands) and β-NT from Riedel-de-Haën (Seelze, Germany). Steroids stock solutions at the concentration of 1 mg mL^{-1} in methanol were prepared and stored at −18 °C. Suitable working standard solutions were obtained by appropriate dilution of the corresponding stock solution and stored at 4 °C. β-glucuronidase-arylsulphatase from Helix Pomatia was provided by Sigma (St. Louis, MO, USA).

2.2. Liquid chromatography–mass spectrometry (LC–MS-MS)

LC analysis was carried out by a Thermo Finnigan HPLC system (Thermo Fisher Scientific, San Jose, CA, USA) consisting of a Surveyor MS quaternary pump equipped with a degasser, a Surveyor AS autosampler equipped with a column oven and a Rheodyne valve with a 25 μL sample loop. Chromatographic separation was obtained using a Xterra MS C18 Waters (Milford, MA, USA) reversed-phase HPLC column (150 mm × 2.1 mm i.d. 3.5 μm) with a Xterra MS C18 Waters guard column (10 mm × 2.1 mm i.d. 3.5 μm). The LC eluents were: [*A*] 0.1% (v/v) aqueous formic acid solution and [*B*] acetonitrile. The injection volume was 20 μL and the separation of the different compounds was obtained using a 0.2 mL min^{-1} flow and a gradient program reported in Table 1 with an overall run time of 25 min. Column was kept in a column oven at 40 °C. The eluent from the column was directly transferred into the ion spray interface with no post-column split. The mass spectrometer was a TSQ Quantum Ultra (Thermo Fisher Scientific, San Jose, CA, USA) triple quadrupole equipped with an electrospray interface (ESI) set in the positive electrospray ionisation (ESI+) mode. Acquisition parameters were optimized in the ion spray mode by direct continuous pump infusion of standard working solutions of the analytes (10 ng μL^{-1} in methanol) at a flow rate of 10 μL min^{-1} into the ion source of the mass spectrometer. The setting of the optimized parameters was as follows: capillary voltage set at 3.0 kV and ion transfer capillary temperature set at 300 °C. Sheath, auxiliary and sweep gases (nitrogen) were fixed at 30, 20 and 1 (arbitrary unit), respectively. The collision gas was argon at 1.0 mTorr and peak resolution of 0.7 Da FWHM was used on Q1 and Q3. The scan time for each monitored transition was 0.1 s and the scan width was 1 Da. Data acquisitions were performed on the pure standard compounds in full scan mode (mass range 50–500 Da) using the first quadrupole to choose the precursor ion. In MS–MS experiments *m/z* product ion scans were recorded between 50 and 500 Da and three diagnostic product ions were chosen for each analytes to obtain high specificity and sensitivity. The multiple reaction monitoring (MRM) conditions for the analytes are given in Table 2. Analytes and internal standard MS–MS data points were recorded and elaborated using XcaliburTM version 1.4 SR1 software from Thermo.

2.3. Sample preparation

Five milliliters of urine sample were spiked at 2 ng mL^{-1} with 100 μL ISTDs solution (0.1 ng μL^{-1}). 1 M aqueous hydrochloric acid solution was added to adjust the pH value to 5.0. 50 μL β-glucuronidase-arylsulphatase was added and the enzymatic deconjugation was carried out overnight at 37 °C. The sample was cooled to room temperature and 5 mL diluted column wash buffer was added. 1 mol/L aqueous sodium hydroxide solution was added

Table 1
Gradient timetable.

Time (min)	[*A*]	[*B*]	Curve
0	75	25	
1	75	25	
15	30	70	Linear
18	30	70	
19	75	25	Linear
25	75	25	