Journal of Chromatography A, 1360 (2014) 229–239

Contents lists available at ScienceDirect

Journal of Chromatography A

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

A novel approach to the quantitative detection of anabolic steroids in bovine muscle tissue by means of a hybrid quadrupole time-of-flight-mass spectrometry instrument☆

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ARTICLE INFO

Article history:
Received 30 April 2014
Received in revised form 17 July 2014
Accepted 27 July 2014
Available online 4 August 2014

Keywords:
Anabolic steroids
Hybrid MS instrument
MicroLC
Chemometric analysis sample extraction
CD 2002/657/EC

ABSTRACT

In recent years, the analysis of veterinary drugs and growth-promoting agents has shifted from target-oriented procedures, mainly based on liquid chromatography coupled to triple-quadrupole mass spectrometry (LC-QqQ-MS), towards accurate mass full scan MS (such as Time-of-Flight (ToF) and Fourier Transform (FT)-MS). In this study, the performance of a hybrid analysis instrument (i.e. UHPLC-QuadrupoleTime-of-Flight-MS (QqToF-MS)), able to exploit both full scan HR and MS/MS capabilities within a single analytical platform, was evaluated for confirmatory analysis of anabolic steroids (gestagens, estrogens including stilbenes and androgens) in meat. The validation data was compared to previously obtained results (CD 2002/657/EC) for QqQ-MS and single stage Orbitrap-MS. Additionally, a fractional factorial design was used to shorten and optimize the sample extraction. Validation according to CD 2002/657/EC demonstrated that steroid analysis using QqToF has a higher competing value towards QqQ-MS in terms of selectivity/specificity, compared to single stage Orbitrap-MS. While providing excellent linearity, based on lack-of-fit calculations (*F*-test, $\alpha = 0.05$ for all steroids except 17β-ethinylestradiol: $\alpha = 0.01$), the sensitivity of QqToF-MS proved for 61.8% and 85.3% of the compounds more sensitive compared to QqQ-MS and Orbitrap-MS, respectively. Indeed, the CC_α values, obtained upon ToF-MS/MS detection, ranged from 0.02 to 1.74 μg kg^{-1} for the 34 anabolic steroids, while for QqQ-MS and Orbitrap-MS values ranged from 0.04 to 0.88 μg kg^{-1} and from 0.07 to 2.50 μg kg^{-1}, respectively. Using QqToF-MS and QqQ-MS, adequate precision was obtained as relative standard deviations for repeatability and within-laboratory reproducibility, were below 20%. In case of Orbitrap-MS, some compounds (i.e. some estrogens) displayed poor precision, which was possibly caused by some lack of sensitivity at lower concentrations and the absence of MRM-like experiments. Overall, it can be concluded that QqToF-MS offers good quantitative and confirmatory performance using the ToF-MS/MS mode whereas the full scan HR-ToF-MS allows screening for potential new designer drugs.

1. Introduction

Anabolic steroids may be used to boost the mass and quality of livestock carcasses in meat production for increasing profit margins [1,2]. Although countries such as the USA have authorized the use of certain steroid preparations, the use of these growth promoters within the EU is banned [3]. This ban, highlighted in two reports from the European Commission in 1999 and 2002, is based on the fact that the presence of hormones in meat products might potentially be harmful to human health through endocrine disrupting or carcinogenic effects. As a result, survey plans were developed by the individual member states to monitor the abuse of anabolic steroids. Within this context, the development of sensitive, specific and multi-residue analytical methods has been considered crucial for adequate control of possible illegal use of growth promoters in meat production. Additionally, these analytical methods must be in compliance with the criteria of CD 2002/657/EC [4].

☆ Presented at the 13th International Symposium on Hyphenated Techniques in Chromatography and Separation Technology, Bruges, Belgium, 29–31 January 2014.

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http://dx.doi.org/10.1016/j.chroma.2014.07.087
0021-9673/

Current national control laboratories have the tendency of relying on LC-triple quadrupole tandem MS (QqQ-MS/MS) based methods, this for their high sensitivity and selectivity when operated in the multiple reaction-monitoring (MRM) mode [5,6]. The introduction of UHPLC (ultrahigh performance liquid chromatography) and fast-switching QqQ-MS/MS instruments has significantly increased the number of compounds that can be analyzed in one run, and this at higher signal-to-noise (S/N) ratios [7]. Nowadays, a vast number of (UHP)LC-MS/MS applications have been described for the detection of anabolic steroids in different livestock derived matrices, i.e. urine, serum, bovine hair, kidney fat and muscle tissue [8–20]. A vast majority of these methods, however, only monitor a limited selection (four to maximum 23) of known anabolic steroids [8–18], not always comprising the three steroid classes [8,18].

The discovery of new designer drugs, intended to elude monitoring plans, cannot take place with a targeted LC-MS/MS approach. In this way, the use of full scan MS surveys offers a promising alternative to retrospectively screen acquired data, for non-"a priori" selected analytes. Within this context, a previous study already comprised the analysis of anabolic steroids on a bench top Fourier Transform Orbitrap mass spectrometer (Exactive™, mass resolution up to 100,000 full width at half maximum (FWHM)) [20]. In terms of selectivity/specificity, the UHPLC-Orbitrap-MS instrument obtained similar results as with the QqQ-MS, sensitivity proved somewhat inferior [20]. One potential way to overcome this shortfall is to exploit both full scan HR and MS/MS capabilities within a single analytical platform. For this particular purpose, hybrid analytical instruments with high or medium-high mass resolution, i.e. Q Exactive (up to 140,000 FWHM) and QqTime-of-Flight (≥25,000 FWHM), can be employed [21,22].

A new feature with chromatography is the development of MicroLC systems. These miniaturized systems are working with microLC columns, characterized by a smaller inner diameter (0.5 mm ID), leading to a reduction of all volume-based system characteristics (i.e. void volume of 1–3 μL). In this way, higher absolute peak intensities may be offered, which can lead to a higher sensitivity compared to conventional (UHP)LC systems [23]. Moreover, this system is characterized by a flow rate between 5 and 200 $\mu L\,min^{-1}$ vs. the UHPLC flow rate between 50 and 2000 $\mu L\,min^{-1}$, which offers ecological and economical benefits by drastically reducing the volume of mobile phase consumption.

In this study, the aim was to increase the sensitivity and selectivity of anabolic steroids analysis in bovine meat by switching to a hybrid instrument that enables the combination of full scan HRMS analysis and MS/MS experiments. To this extent, the high-throughput detection, confirmation and quantification of 34 anabolic steroids in bovine muscle tissue, covering the classes of gestagens, estrogens (including stilbenes) and androgens, was evaluated for a QqToF instrument preceded by UHPLC separation. Moreover, to shorten the sample extraction procedure, a fractional factorial design was performed for optimization purposes. Additionally, a miniaturized MicroLC system was investigated regarding its capacity to circumvent potential sensitivity issues by increasing peak intensities. Finally, the newly developed method was validated and its performance was evaluated towards well-established analytical methods, using QqQ-MS/MS and single-stage Orbitrap-MS [20].

2. Materials and methods

2.1. Reagents and chemicals

Standards of 17β-nortestosterone, fluoxymesterone, progesterone, estrone, estriol, 17α-estradiol, 17β-estradiol, 17α-hydroxyprogesterone, 17β-ethinylestradiol, diethylstilbestrol, dienestrol, α-zearalanol, β-zearalanol, 17α-testosterone, 17β-testosterone, hexoestrol, norgestrel, acetoxyprogesterone, medroxyprogesterone acetate, methyltestosterone and the internal standard androstadieendione were purchased from Sigma–Aldrich (St. Louis, MO, USA). Methylboldenone (or methandienone), norethandrolone, α-nortestosterone, methandriol, β-boldenone, 4-androstenedione and caproxyprogesterone were provided by Steraloids Inc. (Newport, RI, USA). α-boldenone and the internal standards 17β-estradiol-d_3, medroxyprogesteron acetate-d_3, methyltestosterone-d_3, 17β-testosterone-d_2 and hexoestrol-d_4 were obtained from RIKILT (Wageningen, The Netherlands). β-trenbolone, flugestone acetate, trenbolone acetate, megestrol acetate, chlormadinone acetate and melengestrol acetate were kindly provided by the Scientific Institute of Health, Food Chain Safety and Environment (WIV-ISP, Brussels, Belgium). Solvents were of analytical grade when used for extraction and purification purposes, and of LC-MS Optima grade for UHPLC and MicroLC-MS applications. They were obtained from VWR International (Merck, Darmstadt, Germany) and Fisher Scientific (Loughborough, UK), respectively. Ultra pure water was obtained by usage of a purified-water system (VWR International, Merck, Darmstadt, Germany).

Primary stock solutions were prepared in methanol at a concentration of 1000 $ng\,\mu L^{-1}$. Working solutions were prepared by 100 and 1000 times dilution in a mixture of methanol and water, for which the ratio depended on the type of LC. When necessary, sonication was used to ensure complete dissolution of compounds. Primary stock and working solutions were stored in dark glass bottles at −20 °C.

2.2. Instrumentation

Two Eskigent Ekspert™ LC systems were compared, i.e. an UHPLC UltraLC 100-XL with degasser and a MicroLC 200 (AB Sciex, California, USA), both consisting of an Eskigent pumping system and autosampler. Chromatographic separation of the anabolic steroids was achieved by reversed phase chromatography and gradient elution [19]. The type of columns, mobile phases, gradients, flow rates and column oven temperatures are summarized in Table 1.

The analysis was performed on a TripleToF® 4600 mass analyzer (AB Sciex, USA), operating separately in positive and negative ion mode. The 4600 mass analyzer may reach a resolution in full scan up to 30,000 FWHM at *m/z* 956, the ToF-MS/MS can reach a resolution up to 25,000 FWHM at an *m/z* of 195. With respect to the ionization efficiency of the anabolic steroids, the DuoSpray™ and the TurboV™ ionization source were compared. The DuoSpray™ is equipped with an electrospray (ESI) as well as an atmospheric pressure chemical ionization (APCI) inlet, whereas the TurboV™ contains only a single inlet, equipped with an APCI probe. The working conditions for both ionization sources are shown in Table 1. For the full scan ToF-MS analysis, a scan range of *m/z* 100–600 with an accumulation time of 100 ms was selected, whereas with the ToF-MS/MS experiment a scan range of *m/z* 80–600 and an accumulation time of 50 ms (positive mode) and 80 ms (negative mode) were used. After optimization of the detection method, a scheduled MRM^{HR} (multiple reaction-monitoring$^{high\ resolution}$) workflow was used in combination with a ToF-MS survey scan for the positive mode, while the negative mode relied on a dedicated MRM^{HR} algorithm in combination with a ToF-MS survey. Instrument control was carried out by Analyst® TF 1.6 software (AB Sciex, California, USA). For quantitative data interpretation MultiQuant™ software (AB Sciex, California, USA) was required. The room was air-conditioned and kept at a constant temperature of 22 °C to avoid any drifts in mass calibration, since both the power supply output and the length of the flight tube are a function of temperature [24].