Journal of Chromatography A, 1216 (2009) 8067–8071

Contents lists available at ScienceDirect

Journal of Chromatography A

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

Gel permeation chromatography clean-up for the determination of gestagens in kidney fat by liquid chromatography–tandem mass spectrometry and validation according to 2002/657/EC

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ARTICLE INFO

Article history:
Available online 22 April 2009

Keywords:
Gestagens
Fat
Validation
GPC
Liquid chromatography–tandem mass spectrometry (LC–MS/MS)

ABSTRACT

A specific and sensitive method based on liquid chromatography–tandem mass spectrometry using atmospheric pressure chemical ionization (LC–APCI–MS/MS) has been developed for the determination of gestagens in kidney fat (medroxyprogesterone acetate, megestrol acetate and melengestrol acetate). The procedure involved a clean-up procedure with gel permeation chromatography (GPC). The analytes were analyzed by reversed-phase LC–MS/MS, in positive multiple reaction monitoring (MRM) mode, acquiring two diagnostic product ions from the chosen precursor for the unambiguous confirmation of the gestagens. The method was validated at the validation level of 1.0 ng/g. The accuracy and precision of the method were satisfactory. The decision limits CCα ranged from 0.20 to 0.22 ng/g while the detection capabilities CCβ ranged from 0.33 to 0.38 ng/g. The method proved to be sensitive and reliable and thus renders an appropriate mean for residue analysis studies.

1. Introduction

The synthetic progestagens medroxyprogesterone acetate (MPA), megestrol acetate (MA) and melengestrol acetate (MGA) have been widely used in clinical gynaecology. Medroxyprogesterone acetate is a synthetic form of the naturally occurring female sex hormone, progesterone. Gestagens form a group of steroids comprising some of the oldest anabolic compounds, all related to the natural progestagenic steroid progesterone. The chemical structure of MPA closely resembles the chemical structure of progesterone as it is produced naturally in the human body. Progesterone also possesses anabolic activity and therefore can be used in animal production in order to improve muscle gain. Medroxyprogesterone is an analogue, a "look alike", of progesterone, not truly progesteronel, but rather a progestin. But, even a slight difference in the molecular configuration of a compound can produce a totally different response from its natural counterpart. For several years now, the use of gestagens and other anabolic steroids, in animal fattening has been prohibited in the European Union, because of their toxic effect on public health [1].

Usually, sample preparation methods are based upon extensive extraction of fat with organic solvents followed by procedures for defatting of the extracts obtained. After saponification (alkaline hydrolysis), samples are extracted with an organic solvent. The extract is cleaned with SPE and subsequent liquid/liquid extraction (LLE). The use of high performance liquid chromatography for further purification has also been reported [2]. Alternatively, novel approaches for the extraction using accelerated solvent extraction (ASE) or supercritical fluid extraction (SFE) are developed [3,4]. For the ASE method the detection capability was 0.5 ng/g and for the SFE method <2 ng/g. For the unambiguous identification and sensitive detection of the gestagens in such complex matrices hyphenated techniques are used, namely gas chromatography–mass spectrometry (following analyte derivatisation) [5–10] and liquid chromatography coupled to mass spectrometry (LC–MS) [2–4,11–14]. One of the analytical problems is associated with the fact that the gestagens are administered to the animal in the form of acetates, which, as a rule, do not hydrolyze upon circulation in the target animal. Therefore, analytical methods have to focus on the detection and identification of the acetylated gestagens. However, since the behaviour of most gestagens acetates in gas chromatography is very poor, an additional step, that of chemical hydrolysis, is used in most procedures applied before analyte derivatisation. The use of LC–MS/MS offers a rapid, simplified, specific and sensitive alternative to GC–MS methods involving simpler extraction procedures and removing the need for derivatisation reactions and alkaline hydrolysis.

The European Committee, suggests that both muscle tissue and kidney fat can be used for the monitoring of gestagens in foods of animal origin. Since in the literature muscle tissue has been

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doi:10.1016/j.chroma.2009.04.050

assayed more frequently compared to kidney, we deemed it useful to invest our efforts on the determination of gestagens in kidney fat. Furthermore, many of the current analytical procedures are multi-methods which analyze gestagens together with other prohibited anabolic steroids. The goal of the present study was to develop a highly sensitive and automated method. For the analysis a method coupling liquid chromatography to tandem mass spectrometry utilizing atmospheric pressure chemical ionization (APCI) in the positive ionization mode was developed. The sample preparation procedure involved a clean-up procedure using gel permeation chromatography (GPC). The developed methodology gave satisfactory recoveries and clean final extracts. As a whole the method proved to be simple, automated, and reliable and reached the required sensitivity. Hence it provides a suitable means for the determination and confirmation of the presence of gestagens in kidney fat and can be used effectively in residue control programs.

2. Experimental

2.1. Chemicals and reagents

Medroxyprogesterone acetate, megestrol acetate, melengestrol acetate and megestrol acetate-d3 were purchased from RIVM (Bilthoven, The Netherlands).

Methanol (HPLC grade) was obtained from Merck (Darmstadt, Germany), *n*-hexane, and dichloromethane were from Sigma (Sigma–Aldrich, Steinhem, Germany).

Ultrapure water was produced with a Pure Lab system (Sation 9000, Spain).

Stock standard solutions (1 mg/ml) were prepared in methanol and stored at −20 °C in the dark. Working solutions were prepared by appropriate dilution of the stock standard solutions with methanol and were stored at 4 °C in the dark for a maximum period of six months.

2.2. Samples

Kidney fat samples collected from untreated bovine animals (both male and female) at slaughterhouses were used as blank and, after fortification with the gestagens, as quality control samples. Kidney fat samples from bovine animals were collected as part of the national program for residue control in Greece, were assayed for the presence of the gestagens. Incurred kidney samples from pork were provided by RIVM through a proficiency test (see Section 3.5). The samples were received in frozen condition and were kept frozen (−20 °C) until analysis.

2.3. Instrumentation

LC–MS/MS analysis was performed on a ThermoElectron TSQ Quantum AM mass spectrometer equipped with a Finnigan Surveyor MS pump Plus, a Finnigan Surveyor Autosampler plus and a Dell computer system with Xcalibur data acquisition software (ThermoElectron, San Jose, CA, USA).

The clean-up was performed on a GPC system equipped with a marathon III pump (RigasLabs, Thessaloniki, Greece), an automatic sampler of large volume Midas (SparkHolland, Emmen, The Netherlands), a Glass column Omni fit (1000 mm), a UV scanning detector Fasma 506 (RigasLabs), two Rheodyne valves (Cotati, CA), a fraction collector CHF 122SB ADVANTEC MFS, INC. (Toyo Seisakusho Kaisha, Japan) and a Clarity Data chromatography software (DataApex, Prague, Czech Republic).

2.4. GPC clean-up

For GPC glass chromatographic columns with the following characteristics were used: 1000 mm × 25 mm ID, and filling material Bio Beads (S-X3) 200–400 mesh, 70 g (Bio-Rad Laboratories, Richmond, CA). The flow was applied in upward direction (flow rate was kept at 5 ml/min to reach a maximum pressure of ca. 150 psi) with dichloromethane used as the mobile phase. Injection volume was 5 ml throughout the study. The wavelength of the UV scanning detector (used to monitor the GPC column effluent in order to collect the fractions) was 254 nm. The samples run time was 45 min and the time frame for the collection of the gestagens was from 35 to 44 min (9 min).

2.5. LC–MS/MS analyses

A reversed-phase Hypersil ODS column (150 mm × 4.6 mm i.d., 5 μm; ThermoElectron) was used for the analyses. The mobile phase was composed of deionised water as solvent A and methanol as solvent B. The gradient program used was as follows: 40% methanol as solvent B at the start (t = 0 min), increased linear to 70% (t = 12 min, held for 10 min), increased to 85% (t = 22.10 min, held for 1 min) and equilibrated for 3.5 min at the initial conditions. The flow rate was kept at 0.7 ml/min. Injection volume was 15 μl throughout the study. The Ionization of each compound was tested in APCI positive multiple reaction monitoring (MRM) mode. The source conditions were optimized to obtain four identification points (two product ions) for each compound, according to the criteria of the Commission Decision 2002/657/EC. A capillary temperature at 335 °C was employed. The nitrogen sheath and auxiliary gas flow rates were set at 40 and 0 arbitrary units, respectively. Vaporizer temperature was set at 450 °C, the discharge current at 5 μA. The peak width for quadrupoles Q1 and Q3 was set at 0.70 amu. The collision energy (CE) and tube lens were optimized for each compound (see Section 3.1 and Table 1).

2.6. Sample preparation

Two grams of fat were weighed in a 10 ml volumetric flask A-class and diluted with 1 ml *n*-hexane. Then the volumetric flask was filled up to 10 ml with dichloromethane. The sample was transferred in a 10 ml vial of the Midas autosampler for GPC. The sample was injected and the fraction of the gestagens (eluted between 35 and 44 min) was collected according to the program of the GPC system. The fraction was evaporated to dryness in a water bath at 40 °C under a stream of nitrogen. The residue was dissolved in 0.6 ml methanol, transferred to an injection vial, evaporated under a stream of nitrogen at 55 °C to dryness, redissolved in 0.1 ml of methanol and analyzed on the LC–MS/MS.

The developed procedure for the extraction–purification of the gestagens in fat samples is shown in Fig. 1.

3. Results and discussion

3.1. Liquid chromatography–mass spectrometry conditions

Acquisition parameters of the mass spectrometer were optimized in ion spray mode by direct continuous pump infusion (at a flow rate of 10 μl/min) of standard working solutions of the analytes at a concentration of 10 ng/μl in the mass spectrometer. Data acquisition was performed preliminary on the standard compounds in full scan, to choose an abundant precursor $[M+H]^+$. In preliminary studies of the ionization mode we have observed higher detection signals with the application of APCI compared to electrospray ionization. Hence APCI was applied for the rest of the study. MS–MS product ion scans were then recorded in full scan. Finally, all the analyses, were carried out by multiple reaction monitoring mode monitoring of the product ions of the steroids in order to obtain higher detection specificity and sensitivity. Table 1 lists the precursor ions and the product ions of each compound with their optimum