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Rapid multi-method for the determination of growth promoters in bovine milk by liquid chromatography–tandem mass spectrometry

George Kaklamanos [a], Georgios Theodoridis [b,*]

[a] Veterinary Laboratory of Serres, Terma Omonoias, 62110 Serres, Greece
[b] Department of Chemistry, Aristotle University, Thessaloniki 54124, Greece

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ABSTRACT

A rapid multi-method was developed for the determination of 21 growth promoters from different classes, including gestagens, corticosteroids, RALs, stilbenes, steroids in bovine milk by liquid chromatography–tandem mass spectrometry (LC–MS/MS). All compounds were eluted from the analytical column in less than 8.5 min and were subsequently analyzed with atmospheric pressure chemical ionization (APCI) using both positive and negative mode. Sample preparation included extraction of the compounds with acetonitrile and purification with solid-phase extraction (SPE). The method was validated according to Commission Decision 2002/657/EC, at a validation level of 1 ng/ml. The specificity, accuracy, precision, decision limit (CCα) and the detection capability (CCβ) were satisfactory evaluated. The recoveries ranged from 80.7% to 118.8% and reproducibility represented as coefficient of variance (CV) was from 1.8% to 13.0%. The CCα and CCβ values were in the ranges 0.06–0.10 ng/ml and 0.11–0.17 ng/ml, respectively. The developed method was applied in real samples proving its rapidness and sensitivity for the determination of the 21 growth promoters.

1. Introduction

Growth hormones are administrated to animals, in order to make them gain weight faster, thus producing meat products for consumers at a faster rate. Growth promoters help reduce the waiting time and the amount of feed eaten by an animal before slaughtering. In dairy cows, hormones can also be used in order to increase milk production. Growth hormones typically act by triggering cells to produce other chemicals, the so-called growth factors. These growth factors actually cause the increase in growth rate and milk production. Thus, hormones can increase the productivity and profitability of the meat and dairy industries. However growth promoters have been banned from use in livestock production because of the risk to humans and wildlife associated with many diseases such as carcinogenesis in humans and their impact as endocrine distributing chemicals [3–7]. As a result the use of growth promoters in animal production is prohibited in the European Union by Directive 96/22/EC [1] and the control of compliance is regulated by Directive 96/23/EC [2] by applying the National Residue Control Plans between individual member states.

At farm level, misuse of steroid hormones in living animals is being monitored by analyses of the animal's urine and serum. As it concerns imported and commercial products, matrices such as muscle tissue, fat and milk are all considered as specimens for analysis. With regard to milk, growth promoters can pass from the bloodstream and can be finally excreted in milk by the mammary gland which synthesizes milk proteins and lactose during late pregnancy and lactation [8]. As milk is a primary source of food and dairy products and a major constituent of human diet, it can be a key source for the entry of growth promoters at retail and farming level. Despite its widespread use and high importance in human diet milk as a matrix has never been a priority specimen and only a limited number of publications have been reported in the literature [9–19]. Furthermore milk is a complex matrix as it contains several lipids (triacylglycerols, phospholipids), carbohydrates (lactose, glucose, galactose, and other oligosaccharides), proteins (casein micelles), salts, minerals and vitamins. For these reasons, there is a need for the development of multi-methods for the determination of growth promoters in milk.

High performance liquid chromatography coupled to mass spectrometry (LC–MS and LC–MS/MS) is the main technique applied for residue analysis in milk as it overcomes the need for derivatization reactions (needed in GC–MS) while at the same time if offers high selectivity, specificity and sensitivity. LC–MS/MS is mostly used in electrospray ionization (ESI) mode; atmospheric pressure chemical ionization (APCI) has been used only once for the determination of five corticosteroids reaching calculated limit of detection (LOD) from 0.02 to 0.07 μg/kg [18].

* Corresponding author. Tel.: +30 2310 997718; fax: +30 2310 997719.
E-mail address: gtheodor@chem.auth.gr (G. Theodoridis).

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LC–MS/MS offers the possibility for the development of multi-methods which could reach up to 50 anabolic hormones [15]. The reported LC methods include the identification of eight phytoestrogens [9] following extraction with acetone, hydrolysis for 4 h at 52 °C and further purification with C18 and SiOH solid-phase extraction (SPE) cartridges reaching an identification limit under 1 ng/ml. Another LC–MS/MS method achieved the determination of 11 natural and synthetic steroids with LODs ranging from 1 to 50 ng/ml. Samples were extracted with methanol, incubated overnight and concentrated using Oasis HLB, silica and amino-propyl SPE cartridges [10]. Automated on-line SPE-LC–MS on C_{30} cartridges was employed after extraction and cleanup with primary secondary amine for the determination of 5 estrogens and bisphenol A, reaching LODs in the range from 0.05 to 0.30 ng/ml [13]. Bisphenol A and 16 growth promoters were also the subject of another LC–MS/MS study following purification on SPE using C18 cartridges. Validation level was 1 ng/ml, CCα ranged from 0.14 to 0.28 μg/kg and CCβ from 0.24 to 0.48 μg/kg [16].

LC–MS/MS analysis typically follows extraction on SPE, which often employs more than one type of cartridges. SPE cartridges of different chemistries have been utilized for the purification of milk: Oasis HLB [10,19], C18 [9,16,17], SiOH [9,10], C_{30} [13] graphitized carbon-black [15] and amino cartridges [10,15,19]. Anion exchange mechanisms have also been exploited for the purification of hormones using Oasis MAX SPE followed by UPLC–MS/MS for the analysis of 6 resorcylic acid lactones (LOD in the range 0.01–0.05 μg/l) [14]. A mixed-mode polymeric strong ion exchange and reverse phase SPE cartridge (MCX) was also used for the purification of corticosteroids extracted from milk; the samples were hydrolysed for 16 h, extracted with acetonitrile, introduced to MCX-SPE and analyzed by APCI-LC–MS/MS.

The aim of the present research was to develop a rapid and reliable LC–MS/MS multi-method for the detection and quantitative determination of growth promoters in milk; the method's applicability should be evaluated by testing real milk samples. Furthermore the method should cover the needs of our Laboratory which is the Greek National Reference Laboratory for the detection of steroids in samples of animal origin. Hence the method should be compliant with the current regulation and should be validated according to the criteria of the Commission Decision 2002/657/EC [20] for the unambiguous confirmation of the presence of the analytes under investigation. In the present paper a total of 21 growth promoters were investigated using ionization in APCI in both positive and negative mode. For the selection of the analytes under investigation care was taken to include growth promoters which could be considered as a minimum package for National Residue Control Plans based on groups A1 (stilbenes), A3 (steroids), A4 (resorcylic acid lactones) and B2f (corticosteroids) [21]. Analytes from these groups are commonly detected by EU Member States in the framework of National Residue Control Plans in other specimens (blood and urine) [22].

2. Experimental

2.1. Materials and reagents

Methanol and water were of LC–MS grade and acetonitrile, acetone of HPLC grade obtained from Merck (Darmstadt, Germany). Oasis HLB (60 mg, 3 ml) SPE cartridges were obtained from Waters (Milford, MA, USA).

Methyltestosterone (MTS), α/β-trenbolone (α/β-TB), triamcinolone acetonide (TRI), dexamethasone (DEX), flumethasone (FLU), diethylstilbestrol (DES), dienestrol (DNS), hexestrol (HEX) were purchased from Cerilliant (Promochem, Wesel, Germany). Chlormadinone acetate (CMA), melengestrol acetate (MGA), megestrol acetate (MA), medroxyprogesterone acetate (MPA), α/β-zearalanol (ZER/TAL) were purchased from NARL (Pymble, NSW, Australia). β-estradiol (E2), ethynylestradiol (EE2), α/β-boldenone (α/β-BO), α/β-nortestosterone (α/β-NO), were purchased from Sigma (Sigma–Aldrich, Steinhem, Germany). As internal standards Deuterated analogs of the analytes under investigation were selected: methyltestosterone-d3 (MTS-d3), testosterone-d3 (TS-d3), 17β-estradiol-d3 (β-E2-d3), diethylstilbestrol-d6 (DES-d6), triamcinolone acetonide-d6 (TRI-d6), megestrol acetate-d3 (MA-d3), α/β-zearalanol-d4 (ZER/TAL-d4) were purchased from RIVM (Bilthoven, The Netherlands).

Standard stock solutions were prepared in methanol at a concentration of 1000 μg/ml and for the deuterated standards at 100 μg/ml. Intermediate standard solutions were prepared by 10-fold dilution in methanol at a concentration of 10 μg/ml. A mixed standard solution containing the 21 compounds was prepared in methanol at a concentration of 1 μg/ml and 0.1 μg/ml. A mixed standard solution containing the 6 deuterated internal standards was prepared in methanol at a concentration of 1 μg/ml. The mixed standard solutions were prepared for spiking the samples. All standard solutions were stored at −20 °C.

The calibrators were prepared by adding 2.5, 5, 12.5, 25, 37.5, 50, of a 0.1 and 7.5, 10, 12.5, 15, 25 μl of a 1 ng/μl mixed standard solution in order to prepare the calibration levels needed from 0.1 to 10 ng/ml. The internal standards were set to a concentration of 4 ng/ml by adding 10 μl of a 1 ng/μl mixed solution to each calibrator. The same amount of the internal standards was added to each sample prior extraction and further clean-up. Quality control (QC) samples were prepared by spiking blank samples prior extraction with 12.5 and 50 μl from a mix solution of 0.1 ng/μl and 25 μl from a mix solution of 1 ng/μl to produce low, medium and high concentration levels (0.5 ng/ml, 2 ng/ml and 10 ng/ml). The QC samples were used to monitor for losses of analytes during the sample purification procedure. Each analytical run included solvent blanks, matrix blanks, and calibration standards at (0.5 ng/ml, 2 ng/ml and 10 ng/ml) after the batch of the calibration curve and at the end after running the samples in order to effectively control all the procedure.

2.2. Chromatographic conditions

A Surveyor MS pump with a Finnigan Surveyor automatic injector was used (Thermo Electron, San Jose, CA, USA). The analytes were separated on a Prevail C18 analytical column (150 mm × 4.6 mm, 3 μm – Alltech, Deerfield, IL) applying a gradient elution with the mobile phase consisted of water as solvent A and methanol as solvent B. Gradient elution started with 60% solvent A (v/v), decreased to 20% over 6 min, which was kept over a period of 2.5 min and then changed to 60% solvent A (v/v) from 8.5 to 12 min for re-equilibration of the analytical column. The total analysis time was 12 min with the injection volume was 15 μl. The flow rate was 700 μl/min and the temperature of the column was set at 20 °C. Data acquisition and integration were carried out by Xcalibur software version 1.4.

2.3. Detection conditions

A TSQ Quantum AM triple-quadruple mass spectrometer (Thermo Electron, San Jose, CA, USA) was used in APCI. Data acquisition was performed in separate injections, in positive and negative ionization mode. Nitrogen was used as sheath and auxiliary gas, at flow rates of 50 and 5 arbitrary units, respectively. Capillary and vaporizer temperatures were set at 300 and 450 °C, respectively. The discharge current was optimized at 7 μA. Argon was used as