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Development and validation of a multi-residue method for determination of 18 β-agonists in bovine urine by UPLC–MS/MS

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ABSTRACT

Ultra performance liquid chromatography (UPLC) hyphenated to tandem mass spectrometry (MS/MS) was used for the development of an analytical method capable of simultaneous identification and quantification of 18 β-agonist compounds namely, brombuterol, chlorbrombuterol, cimaterol, cimbuterol, clenbuterol, clencyclohexerol, clenisopenterol, clenpenterol, clenproperol, hydroxymetylclenbuterol, isoxsuprine, mabuterol, mapenterol (clenbuterol-like compounds), ractopamine, ritodrine, salbutamol, salmeterol (salbutamol-like compounds) and zilpaterol in bovine urine. In compliance with the Commission Decision 2002/657/EC (CD 2002/657/EC), the method was validated applying a matrix-comprehensive in-house validation approach based on a fractional factorial design. Six experimental factors varied on two levels were selected for this purpose. The relevant validation parameters such as decision limit CCα (range, 0.24–0.51 μg L^{-1}) and detection capability CCβ (range, 0.27–0.71 μg L^{-1}), within-laboratory reproducibility (<20%) as well as recovery (range, 92–109%) were in agreement with the performance criteria set in the CD 2002/657/EC. Overall, the proposed method enabled both screening and confirmatory detection of the β-agonist compounds in the framework of official monitoring plans.

1. Introduction

Veterinary drugs are therapeutically used to treat and prevent diseases in farm animals and in some cases, as growth-promoting agents to stimulate animal growth enhancement. As a consequence of veterinary drug administration to food-producing animals, the presence of their residues in animal food products is a critical public health concern.

In order to ascertain the high quality of food products of animal origin and to protect consumers by reducing their exposure to veterinary drug residues, several Directives and Regulations are stipulated by the European Union (EU). The EU introduced the procedure to establish maximum residue limits (MRLs) for veterinary drugs in the Council Regulation 1990/2377/EEC [1]. In the Annex of the subsequent Council Regulation 2010/37/EU, which repealed the Regulation 1990/2377/EC, veterinary drugs are distinguished in allowed and prohibited veterinary substances [2]. As reported by the EU, the residues of these prohibited veterinary drugs in food animal products constitute a risk for consumer health at any level of concentration and consequently, these substances do not require MRL values.

The prohibition on the use of veterinary drugs having a hormonal or thyrostatic action as well as β-agonist compounds improperly exploited as growth-promoting agents has been stipulated in the Council Directive 1996/22/EC, subsequently amended by Directive 2008/97/EC of the European Parliament and the Council [3]. As previously mentioned, these veterinary substances may be dangerous for consumers and in addition, they negatively affect the foodstuff quality, since the legal tolerance by the EU for their residues in animal food products is "zero".

Community legislation on veterinary drugs also involves guidelines for the official monitoring of their residues in animals and animal products in each State Member of the EU. The Council Directive 1996/23/EC classifies the veterinary drugs included in the official monitoring plans into two main groups namely, substances having anabolic effects and prohibited substances (Group A) as well as veterinary drugs and contaminants (Group B) [4]. Among prohibited substances included in the European Union monitoring plans, β-agonist compounds are one of the focuses of the scientific community. Their association with human poisoning cases from consumption of contaminated meat and liver animal products illicitly treated with clenbuterol are highlighted by other authors [5–9]. Clenbuterol and compounds belonging to the family of β-agonists are therapeutically administered to farm animals as bronchodilatants and tocolitic agents to accomplish relaxation of smooth muscle cells. Nevertheless, β-agonists are well-known as repartitioning agents illicitly employed in veterinary framework as growth promoters. On the one hand, these compounds are capable of reducing fat deposition stimulating lipolysis and decreasing at the same time, fatty acid synthesis. On the other hand, β-agonist

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administration to animals has been reported to increase muscle accretion modifying the rates of protein degradation and synthesis [10–12].

A high number of analytical methods are developed in order to quantify and confirm β-agonists in animals and animal products. Screening determinations of β-agonist residues are achieved using high sample throughput approaches such as enzyme and radio immunoassay, immunoaffinity chromatography, surface plasma resonance (SPR) and high performance thin layer chromatography (HPTLC) [13–19]. In addition, more sensitive and specific mass spectrometry techniques hyphenated to gas and liquid chromatography are performed for screening investigations especially by official laboratories involved in both screening as well as confirmatory analysis. In the framework of confirmatory purpose, that is, in the case of a positive response after a screening procedure, mass spectrometry is the only analytical technique taken into account by the European legislation [20]. Confirmatory methods for detection of β-agonists in animals and their products are carried out using gas chromatography–mass spectrometry (GC–MS) or tandem mass spectrometry (GC–MS/MS) and liquid chromatography–mass spectrometry (LC–MS) or tandem mass spectrometry (LC–MS/MS) [21–26]. MS/MS instrumentation coupled with a liquid chromatographic separation that does not require a derivatization procedure (i.e. gas chromatography) recently made LC–MS/MS the main instrumental method performed by official laboratories for confirmatory investigation of non volatile and polar compounds such as β-agonists [27–33].

The aim of this study was to develop an analytical method capable of simultaneous identification and quantification of 18 β-agonists namely, brombuterol, chlorbrombuterol, cimaterol, cimbuterol, clenbuterol, clencyclohexerol, clenisopenterol, clenpenterol, clenproperol, hydroxymetylclenbuterol, isoxsuprine, mabuterol, mapenterol (clenbuterol-like compounds), ractopamine, ritodrine, salbutamol, salmeterol (salbutamol-like compounds) and zilpaterol in bovine urine by means of ultra performance liquid chromatography (UPLC) hyphenated to tandem mass spectrometry. The proposed procedure based on an advanced chromatographic technology with high peak resolution and rapid separation properties could be successfully applied for both screening and confirmatory detection of β-agonists in the framework of official monitoring plans [34–36]. In order to estimate the performance level of this method, an in-house validation approach was undertaken in compliance with the "validation according to alternative models" section of the CD 2002/657/EC [20]. A factorial design was performed to evaluate the possible influence of selected experimental factors (e.g. operator, SPE cartridge size, NH_3 percentage in elution, sample pH value during application on cartridge, final volume in sample extraction, sample storage duration before measurement) on analytical results of each β-agonist compound.

2. Experimental

2.1. Reagents and materials

Hydrochloride salts of bromclorbuterol, brombuterol, clencyclohexerol, clenisopenterol, clenpenterol, hydroximethylclenbuterol, mabuterol and mapenterol as well as cimaterol, cimbuterol, and clenproperol were purchased from Witega (Berlin, Germany). Clenbuterol, isoxsuprine, ractopamine, ritodrine and salbutamol as hydrochloride salts were purchased from Sigma Aldrich (Germany) and zilpaterol was obtained from TRC (Toronto, Canada). Internal standards namely brombuterol-d9, clenbuterol-d9, clenpenterol-d5, mabuterol-d9 and mapenterol-d11 as hydrochloride salts and cimaterol-d7, cimbuterol-d9 and clenproperol-d7 were purchased from Witega (Berlin, Germany). Finally, hydrochloride salts of isoxsuprine-d5, ractopamine-d5 were obtained from Rikilt (Wageningen, The Nederlands) as well as hydrochloride salt of salbutamol-d9 was purchased from Sigma Aldrich (Germany). The stock standard solutions (from 1 g L^{-1} to 10 mg L^{-1}) were prepared in methanol and stored at −20 °C. The working mixed standard solutions (at 100 μg L^{-1} and 10 μg L^{-1}) of β-agonist compound were prepared by dilution of the stock standard solutions and subsequently kept at 4 °C. As regards internal standards, mixed standard solutions including all deuterated compounds were prepared as well. Deionized water employed in all solutions was obtained by a Milli-Q system (Millipore, USA). Methanol, acetonitrile, 2-propanol of high performance liquid chromatography (HPLC) grade, and β-glucoronidase/arylsulfatase (from *Helix pomatia*, Merck, Germany) were obtained from Merck (Germany). Glacial acetic acid, formic acid, sodium acetate anhydrous, sodium hydroxide and dichloromethane were purchased from Carlo Erba (Italy). As far as SPE cartridges are concerned, Bond Elute Certify 300 mg (Agilent, USA) were applied for the cleanup procedure.

2.2. Analytical procedure

Samples of bovine urine were collected from a local slaughterhouse, frozen at −20 °C and kept at this temperature until analysis. Pooled urine obtained from twenty calves previously analyzed for each β-agonist under investigation was used as blank urine to quantify the validation samples.

As regards the sample preparation procedure, 2.5 ml of urine was placed in 15 ml centrifuge tubes and subsequently spiked with 5 μg L^{-1} of each internal standard and β-agonists in relation to the level of fortification required. After an addition of 30 μL of β-glucoronidase/arysulfatase as well as 1 ml of acetate buffer (pH 4.8; 2 M), urine samples were placed in a thermoregulated bath (GFL, Germany) at 37 °C for 12 h. Afterwards, the urine samples were adjusted to a pH between 7.5 and 8 using a solution of NaOH (10 N and 1 N) for the subsequent SPE purification process. Samples were applied on SPE cartridges previously activated with 2 ml of methanol and 2 ml of water and placed on a vacuum manifold device (Supelco, Germany). Subsequently, SPE cartridges were washed with 1 ml of water and a following addition of 2 ml of acetate buffer (pH 4.0; 0.1 M) was employed. SPE cartridges were washed with 2 ml of methanol and finally β-agonists were eluted with 4 mL of dichloromethane and 2-propanol in a ratio of 80:20, v/v with 3% of NH_3. After the elution process, the obtained solutions were evaporated at 40 °C to dryness under a gentle stream of nitrogen by means of a Thermo Scientific (USA) evaporator. Finally, the samples were dissolved in 400 μL of a water/methanol mixture (60:40, v/v) for UPLC–MS/MS analyses.

2.3. LC–MS/MS system

A Waters UPLC system was used to perform a reverse phase chromatography separation of β-agonists. An Acquity ethylene bridged hybrid (BEH) C18 column (100 mm × 2.1 mm and 1.7 μm particle size) was used as stationary phase and a mobile phase consisting in deionized water with 0.1% of formic acid and acetonitrile with 0.1% of formic acid were employed. An injection volume of 5 μL, a flow rate of 0.35 ml/min and a linear gradient from 2% to 95% of acetonitrile in 5.5 min were applied for chromatographic separation. The LC system was coupled with a Waters Xevo TQ MS mass spectrometer equipped with an ESI source operating in positive ionization mode (ESI+). The MassLynx 4.11 software (Waters, USA) was used in order to control the UPLC–MS/MS system. Capillary voltage, source temperature, desolvatation gas flow rate and its temperature were set at 1 kV, 150 °C, 900 L/h and 600 °C, respectively. Cone voltage (CV) in the range of 16–28 eV as well as collision energy (CE) in the range of 12–40 eV were optimized for each protonated molecular ions [M + H]+ and different product ions, respectively. In Table 1 are summarized [M + H]+, product ions, and β-agonist-specific MS/MS parameters.

For the quantification purpose, six concentration levels were prepared by spiking blank urine samples at 0, 0.2, 0.5, 1, 2, 5 μg L^{-1} with a mixture of 18 β-agonists and 11 internal standards (5 μg L^{-1}).