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Comparative method validation for closantel determination in cattle and sheep milk according to European Union Volume 8 and Veterinary International Conference on Harmonization guidelines

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ABSTRACT

A specific and sensitive LC–MS/MS method was developed for quantitative determination of closantel in bovine and ovine colostrum and tank milk. Sample preparation consisted of extracting milk samples with acetonitrile/acetone (80/20, v/v) followed by SPE clean-up with Oasis® mixed anion exchange columns. After elution with 5% formic acid in acetonitrile and evaporation to dryness, the residue was reconstituted in acetonitrile and water. HPLC separation was achieved on a Zorbax® Eclipse Plus C18 column and a gradient elution program with 1 mM ammonium acetate in water and acetonitrile. For closantel determination in bovine milk, the method was validated according to EU Volume 8 guidelines whereas for ovine milk both EU Volume 8 and VICH GL49 criteria were applied. The linear range of the method is between 10 and 2000 μg/kg, the limit of quantification 10 μg/kg and limit of detection is 0.63 and 0.32 μg/kg for sheep colostrum and tank milk and 1.27 and 1.24 μg/kg for cattle colostrum and tank milk, respectively. Both guidelines cover a similar set of parameters (linearity, accuracy, precision, limit of detection and limit of quantification), although the acceptance criteria might differ (accuracy and precision) or no specific acceptability ranges are specified in neither guidelines (LOD and LOQ). For some parameters, only one of the guidelines indicates acceptance criteria: EU Volume 8 for applicability, practicability and susceptibility and VICH GL 49 for linearity, specificity and analyte stability.

1. Introduction

Closantel (CLO, Fig. 1) is a broad-spectrum salicylanilide antiparasitic drug active against several adult and developmental stages of trematodes, nematodes and arthropods. It is mainly indicated to control fascioliasis (liver fluke) in sheep and cattle. To limit the potential public health hazard by intake of veterinary drugs through the food chain, maximum residue limits (MRLs) of various drugs in edible tissues were established by the European Commission (EC). For CLO, MRLs in muscle, fat, liver and kidney were already put forward in 2010 [1]. At that time, it was prohibited to treat dairy cattle and lactating sheep of which the milk was intended for human consumption with CLO. To meet the demands of several stakeholders, the EC established, in 2012, a provisional MRL for ovine and bovine milk of 45 μg/kg, which has been made final in December 2013 [2]. This implied the development of suitable analytical methods to determine residues of CLO in ovine and bovine milk, in order to study the residue depletion in milk and to set appropriate withdrawal times. European guidelines to validate methods used for such depletion studies are described in the Rules Governing medicinal products in the European Union Volume 8: Notice to applicants and guideline on the establishment of maximum residue limits (MRLs) for residues of veterinary medicinal products in foodstuffs of animal origin [3].

Few methods are currently available to determine CLO in milk, and consequently also to screen milk samples for possible violative residues of CLO. Some of the methods are based on HLPC with either ultraviolet [4] or fluorescence detection [5,6]. However, these detection techniques do not offer the same sensitivity and selectivity as mass spectrometry. Therefore, HPLC coupled to mass spectrometry (HPLC–MS) or tandem mass spectrometry (HPLC–MS/MS) can be considered as the preferred method for quantitative determination of drug residues [7]. To the best of our knowledge, only three studies describe the determination of CLO residues in milk using MS detection [7–9]. All these methods were developed prior to 2012, at a time when no MRL was established

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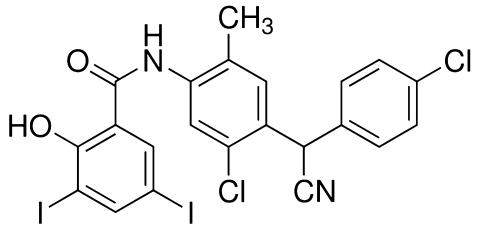

Fig. 1. Chemical structure of closantel.

for CLO in milk. Furthermore, with respect to method validation [8,9], validated their methods according to previous EU guidelines [10]. Others [7], validated their method using an in-house protocol covering linearity, limit of detection (LOD), limit of quantification (LOQ), precision and selectivity. The latter validation study did not specify, for example, any acceptability ranges for precision nor was accuracy determined. The most recent method validation guidelines issued by the International Cooperation on Harmonization on Technical Requirements for Registration of Veterinary Medicinal Products (VICH) date from 2012 [11]. VICH is a program, founded by the EU, Japan, USA, Australia, New Zealand and Canada, aimed at harmonizing technical requirements for veterinary product registration. More specifically, VICH GL49 stipulates the guidelines for the validation of analytical methods used in residue depletion studies. The guidance is developed to facilitate the mutual acceptance by national regulators of residue chemistry data for veterinary drugs used in food-producing animals.

The goal of present study was (a) to develop a sensitive LC–MS/MS method for the determination of CLO in bovine and ovine colostrum and tank milk, (b) to validate the method according to the most recent EU guidelines [3] alone (bovine) or in conjunction with the VICH GL49 guidelines [11] (ovine) and (c) to compare and discuss the validation parameters and acceptance criteria of both guidelines.

2. Materials and methods

2.1. Chemicals, products and reagents

The analytical standard of CLO ($pK_a = 4.18$ [5]; purity 99.55%) was obtained from Janssen Pharmaceutica (Beerse, Belgium) and stored at room temperature. The internal standard (IS), $^{13}C_6$-CLO (purity > 99.4%), was obtained from Witega Laboratories (Berlin, Germany) and stored at 2–8 °C. Water, methanol and acetonitrile (ACN) were of LC–MS grade and obtained from Biosolve (Valkenswaard, The Netherlands), whereas acetone, ammonium acetate, ethyl acetate, formic acid and tetrahydrofuran were of analytical grade and obtained from Merck (Darmstadt, Germany).

2.2. Preparation of standard solutions

Stock solutions of CLO and the IS were prepared in 2% tetrahydrofuran in ACN (analyte concentration of 0.1 mg/mL). Working solutions of 10 μg/mL CLO and IS and 1.0 μg/mL CLO were prepared by appropriate dilution of the stock solution with ACN. Stock and working solutions were stored at 2–8 °C.

2.3. Sample preparation

Colostrum and tank milk from untreated sheep were provided by the Centre d'Economie Rurale (CER, Marloie, Belgium), whereas blank cattle colostrum and tank milk were provided by the Laboratory of Parasitology and Biocentre Agri-Vet (both from Faculty of Veterinary Medicine, Ghent University, Belgium). To five gram of milk, 12.5 μL of IS working solution and different concentration levels of CLO were added. Samples were vortex mixed and 15.0 mL of ACN/acetone (80/20, v/v) were added. The samples were vortex mixed for 15 s, shaken for 10 min and centrifuged (4750 rpm, 10 min, 4 °C). The supernatant was filtered through a Whatman® cellulose filter (Fisher Scientific, Aalst, Belgium) and was loaded onto an Oasis® MAX®-solid phase column (mixed anion exchange, 3 mL, 60 mg, Waters, Milford, USA), previously conditioned with 2 mL of ACN/acetone (80/20, v/v). The MAX®-column was rinsed subsequently with 2.5 mL of ethyl acetate and 3 mL of methanol. Next, the column was dried for 5 min under vacuum. CLO and the IS were eluted with 3 mL of 5% formic acid in ACN. The elute was evaporated to dryness at 40 ± 5 °C under a gentle nitrogen stream. The dry residue was reconstituted in 125 μL of ACN and vortex mixed. This reconstituted residue was diluted with 125 μL of water and vortex mixed again. The diluted solution was transferred into an autosampler vial and a 2 μL aliquot was injected onto the HPLC column.

2.4. Liquid chromatography

The chromatographic system consisted of an Alliance type 2695 HPLC separations module with column heater and cooling device (Waters). Chromatographic separation was achieved using a Zorbax® Eclipse Plus C18 column (100 mm × 3.0 mm i.d., d.p.: 3.5 μm), in combination with a guard column of the same type, from Agilent (Santa Clara, CA, USA). Mobile phase A was 1 mM ammonium acetate in water, while mobile phase B was ACN. A gradient elution program was performed: 0–1.0 min: 80% A/20% B, 1.0–1.5 min: linear to 40% A, 1.5–7.0 min: 40% A/60% B, 7.0–7.5 min: linear to 10% A, 7.5–9.5 min: 10% A/90% B, 9.5–10.0 min: linear to 80% A, 10–20 min: 80% A/20% B. The flow rate was set at 0.3 mL/min. This resulted in a retention time (RT) of 10 min. Nevertheless, an evaluation of the relative RT (RRT) is preferred over the absolute RT. The RRT is a parameter controlled in each batch of samples. The RRT should be within ±3% of the lowest and highest RT of the calibrator samples. If not, the batch will be rejected.

2.5. Mass spectrometry

The LC column effluent was pumped to a Quattro Ultima® triple quadrupole mass spectrometer (Micromass, Altrincham, UK), equipped with an electrospray ionization (ESI) ion source, operated in the negative mode. The instrument was tuned by direct infusion of a 1 μg/mL working solution of CLO and IS. The following parameters were retained for optimal CLO detection: capillary voltage: 3 kV, cone voltage: 25 V, source temperature: 120 °C, desolvation temperature: 250 °C, cone gas flow: 55 L/h, desolvation gas flow: 845 L/h.

Acquisition was performed in the selected reaction monitoring (SRM) mode. Following SRM transitions were monitored and used for identification and quantification of CLO: m/z 660.7 > 344.6 and 660.7 > 314.8 (collision energy: 35 eV), respectively. For the IS, the following transition was used for quantification: m/z 666.8 > 350.7 (collision energy: 40 eV). Quantification was performed with the MassLynx® software v4.0 (Micromass), using the above mentioned product ions.

2.6. Method validation

The method for analysis of CLO in cattle milk was validated prior to 2012, by a set of parameters which are in compliance with the requirements as defined in the Rules Governing medicinal products in the European Union, Volume 8 [3]. The method for analysis of CLO in sheep milk was validated after 2012, when both EU Volume 8 [3] and VICH GL49 [11] guidelines were published. More