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Food safety evaluation: Detection and confirmation of chloramphenicol in milk by high performance liquid chromatography-tandem mass spectrometry

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Abstract

A simple and rapid procedure for extraction of chloramphenicol (CAP) in milk and analysis by high-performance liquid chromatography coupled with quadrupole mass spectrometry in tandem was developed. The method consisted of one step of liquid–liquid extraction using ethyl acetate and acidified water (10 mmol L^{-1} formic acid) and HPLC-MS/MS detection. CAP-D5 was used as internal standard. The method was validated according to Commission Decision 2002/657/EC. The calibration curves were linear, with typical r^2 values higher than 0.98. Absolute recovery of CAP from milk proved to be more than 95%, however CAP-D5 absolute recovery was 75%. The method was accurate and reproducible, being successfully applied to the monitoring of CAP in milk samples obtained from the Brazilian market. Decision limit (CCα) was 0.05 ng mL^{-1} and detection capability (CCβ) was 0.09 ng mL^{-1}.

Keywords: Chloramphenicol; Antibiotic; Food control; High performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS)

1. Introduction

Chloramphenicol (CAP, Fig. 1) is a broad-spectrum antibiotic, which is capable of causing fatal blood diseases in humans. It is reserved to the treatment of serious infections, when no other alternative is available. However, the use of CAP in animals can be very appealing, since it is well tolerated by them and it is largely distributed among tissues and fluids. Other properties of CAP can also be very interesting to producers. In fishery, for example, this drug is preferable because of its long half-life in solution; other antibiotics, like tetracyclines, are less stable, requiring larger doses and more frequent applications [1].

In Brazil, only recently the production, importation and commercialization of CAP for food-producing animals were banned [2]. Therefore, other drugs must be used for the treatment of infections of those animals. In the specific case of milking cattle, some diseases can cause a decrease in feed consumption and milk production, being mastitis the most worrisome [3].

The use of CAP in food-producing animals is prohibited, because it is not possible to establish a safe intake level for its residues or its metabolites' residues in food. The Joint FAO/WHO Expert Committee on Food Additives, considering the existence of fatal dose-independent effects of this substance, was not able to determine an acceptable daily intake (ADI) for CAP, neither a maximum residue limit (MRL). Therefore, in compliance with JECFA's recommendation, not only Brazil but also the USA and the European Union established a zero tolerance for CAP residues in food.

The possibility of disseminating resistant bacteria is also very important when discussing the impact of antibiotic residues in food. In general, these drugs cause a favorable selective pressure for the propagation of resistant bacteria. In veterinary medicine, the use of such agents can also cause this effect [4]. Consequently, resistant phenotypes have already been found in food products commercially available [5–7]. Nonetheless, it is difficult to determine unequivocally the source of these bacteria, since food handlers can present them in their flora [8].

To analyze veterinary drugs residues in food, at least two methods need to be used: one for screening and another for

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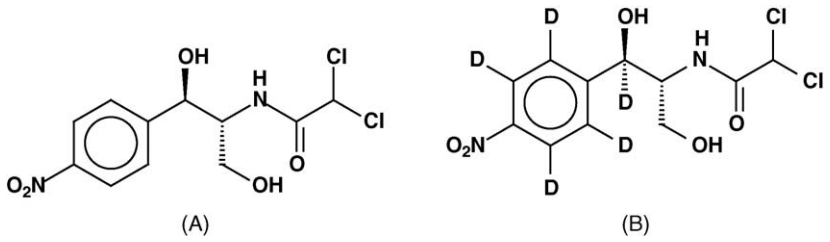

Fig. 1. Structures of (A) CAP and (B) CAP-D5.

confirmation. Screening methods must be optimized to avoid false-negative results, allow high sample throughput and present low cost. On the other hand, confirmation methods must be optimized to avoid false-positive results (that is, it must have superior specificity), provide structure elucidation and quantify the analyte [9].

It is possible to find in the literature several review articles on chromatographic methods to analyze antibiotics in different food matrixes [10–14]. There are also many specific articles on the analysis of CAP in milk [15–19,26,27]. However, official analyses require the use of methods which comply with qualitative and quantitative criteria established by local sanitary agencies. In Brazil, it is common to adopt the European regulation for this type of analysis.

In the European Communities, performance criteria for analytical methods are laid down by Commission Decision 2002/657/EC [20]. In the case of prohibited substances (Group IV, by Council Regulation 90/2377/CEE [21], which includes CAP), another requirement is the compliance of the minimum required performance limit (MRPL). Nowadays, the MRPL for the analysis of CAP in milk is 0.3 $\mu g\,kg^{-1}$ [22].

The main goal of this study was to establish and disseminate a rapid and innovative method for the detection of CAP in milk, based on liquid–liquid extraction and quadrupole HPLC-MS/MS detection. The method was validated according to Commission Decision 2002/657/EC and applied in the analysis of milk samples collected by a Brazilian health surveillance program.

2. Experimental

The following reagents were used: acetonitrile, methanol, ethyl acetate and formic acid. All reagents were HPLC-grade, being acquired from Tedia (Fairfield, USA). Water was purified by reverse osmosis (Milli-Q, Millipore).

2.1. Standard preparation

Standards were obtained from the following suppliers: chloramphenicol from United States Pharmacopoeia (USP, Rockville, MD, USA), chloramphenicol-D5 (CAP-D5) from Cambridge Isotope Laboratories (CIL, Andover, MA, USA). CAP-D5 was used as internal standard (IS).

Stock solutions of CAP were prepared in methanol/water (50:50, v:v) at a concentration of 1 $mg\,mL^{-1}$. These solutions were further diluted to yield appropriate working solutions for the preparation of the calibration standards. Working solutions of the IS were prepared in methanol/water (50:50, v:v) at 48 $ng\,mL^{-1}$. All standard solutions were sealed and kept at −20 °C, protected from light, for no longer than 3 months.

2.2. Instrumental conditions

The HPLC-MS/MS system consisted of Varian 1200L MS/MS detector with an ESI source, Varian ProStar 430 AutoSampler and Varian ProStar 210 Solvent Delivery Modules (Varian, Walnut Creek, CA, USA). The detector was tuned by infusing a polypropileneglycol solution at a rate of 20 $mL\,min^{-1}$, using a syringe pump, and optimized with a standard solution of CAP (0.1 $mg\,mL^{-1}$ in methanol/water 50:50, v:v, containing 10 $mmol\,L^{-1}$ of formic acid), using the same procedure.

Chromatographic separation was achieved using a Varian Pursuit column (100 μm × 20 μm × 5 μm) in combination with a Varian MetaGuard pre-column. The mobile phase consisted of 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B). Gradient elution was performed (0–3 min, 5% B; 3.5–6 min, 70% B; 6.5–15 min, 5% B) at a flow rate of 0.3 $mL\,min^{-1}$. The temperature of the autosampler and the column was 23 ± 2 °C.

The mass spectrometer was used in the positive ion MS/MS mode (ESI). The following instrument conditions were used for CAP and CAP-D5: needle, 3600 V; shield, 600 V; capillary, 76 V; nebulizing gas, 50 psi; drying gas, 30 psi, 300 °C; collision gas, argon, 1.80 mTorr; multiplier, 2000 V; scan time, 1 s; SIM width, 0.7 amu.

The instrument was operated in multiple reaction-monitoring (MRM) mode, using the following transitions *m/z* 323 → 275 (quantification ion) and *m/z* 323 → 165 (confirmation ion) for CAP and *m/z* 328 → 280 (quantification ion) for CAP-D5; with collision energies of −13.5, −23.0 and −11.0 V, respectively. Relative abundance of the two MRM transitions monitored for CAP was superior to 50% (Fig. 2).

2.3. Sample preparation

Two milliliters of milk samples were spiked with 50 μL of IS working solution (48 $ng\,mL^{-1}$) and vortexed for 10 s. After 10 min of equilibration, 0.8 mL of water acidified with 10 $mmol\,L^{-1}$ of formic acid and 4 mL of ethyl acetate were added. The samples were extracted for 10 min on a rotary mixer (Fanem, model 255B, São Paulo, SP), at 400 rpm. After that, they were centrifuged for 5 min at 3200 rpm. The supernatant