DETECTION OF CHLORAMPHENICOL RESIDUES IN MILK SAMPLES USING ELISA AND LC/MS-MS

Fatmira SHEHU

Department of Veterinary Public Health , Faculty of Veterinary Medicine, Agricultural University of Tirana, s_fatmira@yahoo.it

Enkela ZANI

Food Safety and Veterinary Institut, Tirana, Albania, enkela.zani@yahoo.com

Suzana KOLA

Food Safety and Veterinary Institut, Tirana, Albania, kolasuzana@hotmail.com

Ermira MARKU

Food Safety and Veterinary Institut, Tirana, Albania, elmiramarku@hotmail.com

Bizena BIJO

Department of Veterinary Public Health , Faculty of Veterinary Medicine, Agricultural University of Tirana , bizena.bijo@yahoo.com

Abstract

The use of Chloramphenicol in food producing animals is prohibited in many countries including the United States, Canada, the European Union, and Australia due to the high potential risk of severe effects such as aplastic anemia, allergic reactions, and the promotion of antibiotic resistance. The monitoring of food products, such as milk, meat and honey, for antibiotic residues is necessary to ascertain that these compounds are not misused and do not present a danger to human or animal health.

244 milk samples were collected in 12 regions of Albania during 2017. In this study, an analytical method for screening and confirmation of CAP residues in milk samples is described. The ELISA was carried out to screen milk samples, and LC/MS-MS was applied to confirm suspect samples. Through the use of Elisa, the presence of Choramphenicol residues was found in 4 milk samples. These samples subsequently subjected the test to the confirmatory method, which resulted in: 2 positive and 2 false positive samples. The method has been validated according to the criteria of the 2002/657/EC Decision

Key words: Chloramphenicol, ELISA, milk, LC-MS/MS

Introduction

Chloramphenicol is an effective antibiotic widely used in the past to treat several diseases in humans and animals (Rocha Siqueira et al., 2009).

Today, the use of the broad spectrum antibiotic Chloramphenicol (CAP) is illegal for the administration in food-producing animals in the EU and many other countries worldwide. It is still frequently employed in animal production because of its excellent antibacterial and pharmacokinetic properties and low price. The mechanism of action of Chloramphenicol is bacteriostatic, inhibiting the protein synthesis in bacterial ribosomes. Illegal use of this antibiotic can increase the risk of introducing harmful residues into the human food chain (Penney et al., 2005; Chen et al., 2011).

The widespread use of antibiotics in foodproducing animals can be a potential hazard for human health. Furthermore, the indiscriminate use of chloramphenicol can lead to bacterial resistance, allergic reactions, disruption of the balance of the gastrointestinal microbial flora, and hemotoxic effects, such as aplastic anemia, bone marrow depression and gray baby syndrome. Since it undergoes biotransformation to the inactive metabolite chloramphenicol glucuronide in the liver, individuals with subnormal liver function and infants are also at risk (Guidi LR et al 2015)

No safe residue level could be established for these side effects. In order to ensure consumer health this led to a complete ban of chloramphenicol for the treatment of animals used for food production and a zero-tolerance for chloramphenicol residues. Based upon scientific reports about chloramphenicol, an acceptable daily intake (ADI) has never been allocated and a maximum residue limit (MRL) has not been assigned. Chloramphenicol was banned for use in food-producing animals in the European Union and in many other countries including Brazil as a means to eliminate it from the food production chain and related goods (European Commission , 2010)

The European Union introduced the concept of the minimum required performance limit (MRPL) of 0.3 μg/kg, the highest concentration level at which the screening and confi rmatory method shall demonstrate satisfactory performances regarding the sensitivity, accuracy and precision (Commission Decision 2003/181)

The problem is more visible with milk due to its role in infant and overall human nutrition and its widespread consumption. Furthermore, chloramphenicol in milk can be transfered to dairy products, specially those rich in fat (Tian, H. 2011). Aslo, on a practical level for dairy products such as cheese made with starter cultures, antibiotic residues would reduce the intended microbial growth and therefore reduce the acid production. For all these reasons and more, the FDA has strict regulations on antibiotic residues in human food. Because of these regulations, antibiotic contaminated milk, including milk products and meat are considered to be adulterated. Although the evidence is considered limited, chloramphenicol has been categorized by the International Agency for Research on Cancer (IARC) as probably carcinogenic in humans, classified as group 2A (JECFA 2014, IARC 1990). Tolerance levels are stablished for some antibiotics, while others have a zero tolerance (as is the case for nitrofurans and chloramphenicol).Chloramphenicol for instance is an antibiotic of considerable current concern in the USA, Europian Union, and athers countries. Because of the adverse health effects humans, the FDA has banned the use of chloramphenicol in animal raised for food production and set zero tolerance in human food (21 CFR 522.390). Therefore, the analytical methods need to be as sensitive and selective as possible.

The control of chloramphenicol in foods can be performed by screening or confirmatory procedures. Screening methods only provide semi-quantitative analysis and can give rise to false positives, but they are used due to simplicity in sample preparation, sensitivity, speed and low cost. On the other hand, confirmatory methods, such as those employing liquid chromatography (LC) coupled to mass spectrometry (MS) are the approaches of choice for determination of antibiotics, because they allow definitive identification, quantitative determination at very high level of specificity and sensitivity

Material and Methods

ELISA Analysis

To measure the amount of CAP in milk, a commercial ELISA kit (Ridascreen, R1511; R-Biopharm AG, Darmstadt, Germany) was used. The kit had a specificity of 100% for CAP. The detection limit (LOD) of the Ridascreen chloramphenicol test was 24 ng/L and the recovery rates were 93% for all samples.

The milk samples was vortexed for 10 min and centrifuged at 3,000 × g for 10 min at room temperature (20–25°C). Following centrifugation, 4 mL of ethyl acetate supernatant (corresponding to 2 g of sample) was transferred into a new centrifuge tube and dried at 60°C under a weak stream of N2. The residue was redissolved in 1 mL of n-hexane and 0.5 mL of the CAP buffer was added to this solution and vortexed for almost 1 min. The solution was centrifuged at 3,000 × g for 10 min at room temperature (20–25°C) and 50 µL of the aqueous (upper) layer was used for analysis. The absorbance was measured at 450 nm using an ELISA plate reader (BioTek, tip/model Elx800). The concentrations of CAP were calculated according to the percentage of their mean absorbance divided by the absorbance of the maximum binding (B/BO%) using the standard curve. The values were multiplied by the dilution factor (0.25) as suggested by the kit manual.

Confirmation of CAP in milk by LC-MS/MS

According to the Commission Decision 2002/657/EC (European Commission, 2002), the confirmation of suspect positive samples must be carried out by mass spectrometry (MS) coupled to adequate chromatographic separation. This is the most reliable analytical method for the unambiguous confirmation of zero tolerance residue limit substances in products of animal origin.

Prior to LC-MS/MS analysis, sample preparation is needed to properly extract chloramphenicol from the food matrix. Concentration of the analyte and removal of interfering compounds may also be needed (Guidi LR et al 2015)

Extraction of milk - A 5 g portion of milk sample was diluted with 20 ml of acetonitryle, homogenized and centrifuged at 3 500g for 10 min at about 6°C. The top layer was taken and evaporated until dry using nitrogen, and dissolved in 6 ml of water. The whole solution was cleaned up by solid phase extraction (SPE) technique.

Clean up- SPE C-18 cartridges were preconditioned with 3 ml of methanol and 3 ml of water. After percolation of the whole solution, the column was washed with 6 ml of water, 3 ml of 20% methanol, and dried under negative pression for 5 min. CAP was eluted with 3 ml of 60% methanol. The eluate was diluted with 5 ml of water, the solution was mixed and passed through a new C18 SPE column, and the elution was made with 3 ml of methanol.

LC–MS/MS - The eluate was dried under gentle nitrogen stream at 45°C. The dry residue was dissolved in 200 µl of 5mM ammonium formate and shaken vigorously with a vortex to retrieve the whole residue, and then it was transferred to a vial and analyzed by LC–MS/MS.

Results and Discussion

The present study was undertaken for the screening of CAP residues in milk samples by using an ELISA method and for confirmation of the results by an LC-MS/MS method. A commercial ELISA kit was used for presumptive CAP detection and quantification. From 244 milk samples, through the use of ELISA, the presence of chloramphenicol residues was found in 4 (1,6 %) milk samples. These samples subsequently subjected the test to the confirmatory method, which resulted in: 2 positive and 2 false positive samples. The methods used for antibiotic determination in milk by competitive immunoassay proved to be rapid and reliable

Based on the findings of the study, the contamination level in milk samples were not alarming as only two samples out of total 244 (0.8%) were found to exceed the MRPL which on further dilution after pooling will not be having any adverse effect on human health. It suggests that the present status of

chloramphenichol contamination is within specified limits but need continuous monitoring to take timely remedial actions to prevent its detrimental effects on public health.

Conclusions

With food legislation constantly under review to combat the very real risk of food contamination, it is becoming more and more difficult for food testing laboratories to meet the specifications set down by the authorities. The high risk of infants suffering due to exposure to CAP has pushed identification of CAP in milk to the forefront (Food Safety magazine, 2003). There are several noted harmful effects of CAP on infants, whether that be through medication or the ingestion of contaminated food stuffs.

Antibiotic residues in edible animal products are of great concern to regulatory agencies and consumers, so reliable screening methods for rapid, selective and sensitive detection of these residues are necessary to ensure food safety. Therefore, monitoring and educational programs are needed to warrant safety of consumers and international trade (Gentili A., et al 2005)

References

Chen X. B., Wu Y. L., Yang T. (2011) Simultaneous determination of clenbuterol, chloramphenicol and diethylstilbestrol in bovine milk by isotope dilution ultra performance liquid chromatography-tandem mass spectrometry. J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.879:799–803

U.S. Food and Drug Administration : Chloramphenicol. 21 CFR 522.390 - CFR Title 21 –Chapter 1, Subchapter E Part 522 › Section 522.390 www.law.cornell.edu/cfr/21/522.390

Commission Decision 2002/657/EC of 12 August (2002) implementing Council directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. Off. J. Eur. Commun. L221:8–36.

Commission Decision 2003/181/EC of 13 March 2003 amending Decision 2002/657/ EC as regards the setting of minimum required performance limits (MRPLs) implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results OJ L 221, 17.8.(2002), p. 8–36.

European Commission, Council Regulation Nº 37/2010 of 22 dec 2009: on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin., *Off. J. Eur. Comm.* 1–72 (2010).

Food Safety magazine. (2013), The Danger of Chloramphenicol in Milk., June 7.

Gentili A., Perret S., and Marchese S., Fast screening methods to detect antibiotic residues in food samples *Trends Anal. Chem*., 24(7), 704–733, (2005).

Guidi LR, Silva LHM, Fernandes C, Engeseth NJ, Gloria MBA, (2015); LC-MS/MS determination of chloramphenicol in food, Scientia Chromatographica 7(4):287-295 *Instituto Internacional de Cromatografia* ISSN 1984-4433

IARC. Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans. Geneva: World Health Organization, International Agency for Research on Cancer, 1972-present. Available at: http://monographs.iarc.fr/ENG/Classification/index.php, 50, 1–171 (1990).

JECFA, *Who Food Additives Series*, 53, 1–40, (2014).

Rocha Siqueira S. R., Donato J. L. de Nucci G., Reyes F. G., 2009. A high-throughput method for determining chloramphenicol residues in poultry, egg, shrimp, fish, swine and bovine using LC-ESI-MS/MS. *J. Sep. Sci.* 32: 4012-4019

Penney L., Smith A., Coates B., Wijewickreme A., (2005). Determination of chloramphenicol residues in milk, eggs and tissues by liquid chromatography/mass spectrometry. *J. AOAC Int.* 88 :645-6534

Rocha Siqueira, S. R., J. L. Donato, G. de Nucci, and F. G. Reyes. (2009). A high-throughput method for determining chloramphenicol residues in poultry, egg, shrimp, fish, swine and bovine using LC-ESI-MS/MS. J. Sep. Sci. 32:4012–4019.

Tian, H. (2011). Determination of chloramphenicol, enrofloxacin and 29 pesticides residues in bovine milk by liquid chromatography-tandem mass spectrometry. *Chemosphere*, *83*, 349–355.