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Concentrations of veterinary drug residues in milk from individual farms in Croatia

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Summary

A total of 119 raw milk samples collected at individual small milk-producing facilities and collection tanks of milk routes from five counties of east and north continental Croatia were examined for chloramphenicol, sulfonamides, tetracyclines, gentamicin, streptomycin, dihydrostreptomycin, flumequine and enrofloxacin from January to March of 2011. Immunoassay methods used for drug determination were validated according to the guidelines laid down by European Commission Decision 2002/657/EC. Data indicated that the methods are appropriate for the detection of antibiotics measured. Measured mean values ($\mu g\ L^{-1}$) of antibiotics were: 0.005 for chloramphenicol, 3.67 for sulfonamides, 2.83 for tetracyclines, 1.10 for gentamicin, 2.64 for streptomycin, 7.67 for dihydrostreptomycin, 10.4 for flumequine and 4.11 for enrofloxacin. None of samples analyzed showed the presence of veterinary drug residues above the maximum residues levels (MRLs) established by European Union and Croatian legislation. The calculated estimated daily intakes (EDIs) for the average daily milk consumption of 300 mL for an adult in Croatia for examined antibiotics showed levels 20 to 1640 times lower than the values of acceptable daily intakes (ADIs) fixed by European Medicines Agency and World Health Organization. This suggested that toxicological risk associated with the consumption of analysed milk could not be considered a public health issue with regards to these veterinary drugs.

Key words: milk, veterinary drug residues, ELISA, estimated dietary intake

Introduction

Antimicrobial agents are widely used in dairy cattle management. Improper administration for disease therapy and as growth promoting agents can result in antibiotic residues in milk and dairy products and can contribute to the development of microbial drug resistance and the spread of resistant bacteria, including those with serious health consequences in animals (Stolker et al., 2007). Residues also present a potential risk to the consumer, particularly with the development of allergic reactions and interference of intestinal micro-flora (Dewdney et al., 1991). From the technological perspective, antimicrobial agent residues in milk can produce important losses in fermented products by inhibition of bacterial fermentation in the production processes of cheese or yoghurt (Molina et al., 2003).

Due to the harmful effects of veterinary medicinal residues, surveillance systems are enforced in the European Union pursuant to the requirements laid down in Council Directive 96/23/EC (EC, 1996) and Commission Decision 97/747/EC (EC, 1997). In line with these requirements, the Croatian legislation was fully aligned with the EU legislation concerning residues of veterinary medicinal prepa-

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rations in foodstuffs of animal origin in accordance with Council Regulation 2377/90/EC (EC, 1990) and subsequently with Council Regulation 37/2010/EU (EC, 2010), which lays down the Community procedure for the establishment of maximum residue limits (MRLs) of veterinary medicinal products in foodstuffs of animal origin.

Therefore, accurate detection of low levels of antimicrobial drug residues in milk is of great importance for the dairy industry and also for farmers, to ensure that contaminated milk from individual cows is not consigned to the bulk tank (Mitchell et al., 2003). Milk may also be contaminated with compounds of one of the antimicrobial drug classes: beta-lactams, sulphonamides (e.g., sulphadiazine), tetracyclines (e.g., oxytetracycline), macrolides (e.g., gentamicin), quinolones (flumequine, enrofloxacin) and aminoglycosides (e.g., neomycin).

Consequently, it is necessary to monitor a large number of milk samples for the presence of the most important antimicrobial drug residues, by using inexpensive, rapid and simple microbiological and immunological screening methods. In recent years, a series of methods has been developed to enable quick detection of antimicrobial agents in milk: microbial screening assays such as the tube test and (multi-) plate test systems (e.g. Delvotest®/DSM; Charm Cowside/Charm Sciences Inc.; Eclipse/Zeu-Inmunotech) and different immunological methods such as ELISA, RIA or biosensor methods (Ferguson et al., 2002; Pikkemaat et al., 2009). All methods used for that purpose have to detect antibiotics at or below their permissible limits or MRLs and also have to be validated in accordance with the Council Directive 2002/657/EC (EC, 2002).

As in other countries, milk is a widely consumed product in Croatia, with consumption around 170 litres per capita (Bosnić et al., 2003). In 2009, as compared to 2008, the collected cows' milk increased by 2.7 % and was 675 289 tons. However, the import of whole milk by dairymen increased in 2009 compared to 2008 by 8.5 % (CBS, 2010). More recent statistics show that in March 2011 compared to March 2010, the quantity of collected cows' milk has decreased by 6.3 %, while compared to the 2010 average, it has increased by 2.1 % (CBS, 2011). In the last decade there were a few studies about screening of antibiotic residues in milk collected for the market in Croatia (Kaša, 2004; Pintić et al., 2006).

In order to monitor veterinary drug contamination in raw milk samples from dairy farms and individual production facilities, residual concentrations of chloramphenicol, sulfonamides, tetracyclines, gentamicin, streptomycin, dihydrostreptomycin, flumequine and enrofloxacin were examined. Furthermore, an estimation of the dietary intake of veterinary drugs residues derived from milk consumption was performed.

Materials and methods

Sampling

A total of 119 raw milk samples were collected from January to March 2011 at individual small milk-producing facilities and from the collection tanks of milk routes in five counties of east and north continental Croatia: Vukovar-Srijem, Požega-Slavonia, Osijek-Baranya, Varaždin and Krapina. Samples were stored at 4-8 °C until analysed. For further investigations, milk samples were stored at -20 °C for 3 weeks.

Reagents and standard solutions

The Chloramphenicol kit (type E.G.1) was provided by the Laboratory of Hormonology (Marloie, Belgija). The Multi-Sulfonamides (sulfisoxazole, sulfachloropyridazine, sulfamethazine, sulfamerazine, sulfadiazine) EIA kit (type 5101SULMp), Streptomycin and dihydrostreptomycin EIA kit (type 5111STREP1p), Gentamicin EIA kit (type 5111GEN), Flumequine EIA kit (5101FLUM1p) and Enrofloxacin EIA kit (5101 ERFX1p) were provided by Euro-Diagnostica B.V. (Arnhem, The Netherlands). The RIDASCREEN® Tetracyclin kit (type R3501) was provided by R-Biopharm AG (Darmstadt, Germany).

Sulfisoxazole, sulfachloropyridazine, tetracycline and quinolones (flumequine, enrofloxacin) were obtained from Fluka (St. Louis, USA), while neomycin trisulfate salt hydrate, dihydrostreptomycin sesquisulfate, gentamicin solution, tylosin, chloramphenicol, chlortetracycline hydrochloride, sulfamethazine, sulfamerazine, sulfadiazine, Tween

80, succinic acid and triethylamine were purchased from Sigma (St. Louis, USA).

Methanol, ethanol, potassium dihydrogen phosphate and ethyl acetate were obtained from Carlo Erba (Milan, Italy). Potassium chloride was purchased from Merck (Darmstadt, Germany). Trichlorometane, sodium hydroxide, disodium hydrogen phosphate dihydrate, hydrochloric acid, sodium chloride, isooctane and Tween 80 were obtained from Kemika (Zagreb, Croatia). Ethylene diamine tetraacetic acid disodium salt hydrate was from Riedel-De-Haen (Seelze, Germany).

Ultra high purity water was processed through a purification system NIRO VV UV UF 20 (Nirosta d.o.o. Water Tehnologies, Osijek, Croatia).

Phosphate buffers PBS were prepared by dissolving 8.94 g sodium chloride, 0.77 g disodium hydrogen phosphate and 0.18 g potassium dihydrogen phosphate in 1000 mL of ultra pure water and adjusted to the proper pH using 1M hydrochloric acid or 1 M sodium hydroxide. The SDB buffer was prepared by dissolving 30 g sodium chloride, 0.2 g potassium chloride, 1.15 g disodium hydrogen phosphate, 0.2 g potassium dihydrogen phosphate and 0.5 mL of Tween 80 in 1000 mL of ultra pure water and adjusting to the pH 7.4.

Standard solutions and spiking of samples

Standard stock solutions were prepared on a weekly basis by dissolving the analyte in phosphate buffer PBS pH 8 (for neomycin), methanol (for chloramphenicol and sulfonamides), 1 mM ethylene diamine tetraacetic acid disodium salt hydrate (for streptomycin, dihydrostreptomycin and gentamicin), 0.03 N sodium hydroxide (for flumequine and enrofloxacine) or in 70:30, v/v ethanol and phosphate buffer PBS pH 7.4 mixture (for tetracyclines).

Intermediate working solutions were prepared prior to each analysis by diluting stock solutions in the same solvent. Working solutions were used for spiking blank milk samples at different levels. Following fortification, samples were allowed to equilibrate for 15 minutes before extraction.

Instruments

The following instruments were used in sample preparation: IKA® Ultra-Turrax® model T25 and IKA® Vortex model Genius 3 (IKA® -WERKE GMBH & CO.KG, Germany), centrifuge model Mistral model 200R (SANYO, Gallenkamp PLC, Leicester, UK), water bath GFL model 1083 (Gesellscaft fur Labortechnik mbH, Burgwedel, Germany), nitrogen evaporation system N-EVAP model 111 (Orgamonation Associates Inc., Berlin, USA), pH meter inoLAB WTW (Willheilm, Germany).

The optical density at 450 nm was measured by microplate reader Tecan model Sunrise Absorbance Reader (Tecan Austria GmbH, Salzburg, Austria).

Sample preparation for immunoassay analyses

Sample pre-treatment procedures were carried out according to immunoassay kit (EIA) manufacturer instructions. For chloramphenicol determination, samples were simply defatted by centrifugation for 15 min (3000 rpm at 4 °C) and the supernatant was diluted 4 times in dilution buffer (obtained by the manufacturer).

For sulfonamide determination, 1 mL of milk samples was taken and 5 mL ethyl acetate was added and stirred for 15 minutes. Samples were centrifuged (5 min at 2000 rpm) and 1 mL was evaporated to dryness under a mild stream of nitrogen at 50 °C. Residues were dissolved in 1 mL PBS buffer, samples were defatted with 1 mL of iso-octane/trichloromethane (2:3, v/v) and vortexed. After centrifugation (5 min, 2000 rpm), 100 μL of the upper layer was taken and 300 μL of PBS buffer pH 7.4 added.

Milk samples for tetracycline determination were defatted by centrifugation for 10 min (3000 rpm at 4 °C). Defatted samples were diluted 10 times with dilution buffer (obtained by the manufacturer).

For gentamicin, streptomycin and dihydrostreptomycin determination, samples were defatted by centrifugation for 10 min (2000 rpm at 4 °C). Defatted samples were diluted 10 times with SDB buffer and pH was adjusted to 7.4±0.4. For enrofloxacin and flumequine determination, 0.5 mL of milk sample was added into a test tube, 4.5 mL of 8 % methanol in sample dilution buffer was added, mixed for 30 minutes and centrifuged for 10 min at 2000 rpm. For flumequine determination, the aqueous part below the fat layer was additionally diluted 10 times in sample dilution buffer (obtained by the manufac-

turer). In the case of enrofloxacin, the aqueous part was directly pipetted into the EIA wells. For each EIA test, 50 μL portions of prepared samples were used. Immunoassays were performed according to the manufacturer instructions. The absorbance for all assays was measured at 450 nm.

Validation study of EIA method

Performance characteristics of EIA methods were determined as prescribed for qualitative screening methods in Commission Decision 2002/657/EC. Also, the limit of detection (LOD) and limit of quantification (LOQ) were obtained by adding 3 and 10 times the standard deviation of 20 blank samples to the mean blank value. Recovery was assessed by performing tests where fortified milk samples at 0.5, 1 and 2 times the MRL level were analyzed (six replicates, respectively). The decision limit CCα was evaluated by analyzing 20 blank milk samples fortified with the analyte at MRL. The concentration at the fortified level plus 1.64 times the corresponding standard deviation equals the decision limit CCα (α = 5 %). The value of the decision limit plus 1.64 times the corresponding standard deviation equals the detection capability CCβ (β = 5 %).

Calculation of the estimated daily intake

The estimated daily intake (EDI) was calculated by the equation given by Juan et al. (2010):

EDI = [(Mean of mg antibiotic per kg of food) per (Daily Intake of food)] divided by [adult body weight (60 kg)]

Average daily milk consumption for an adult of 300 mL was used according to literature data for Croatia (Antonić et al., 2007).

Results and discussion

The objective of this study was to determine the residual levels of chloramphenicol as a prohibited substance and other veterinary drugs throughout three months of raw milk samples monitoring in Croatia, to reveal if the maximum residue limits have been exceeded in terms of consumer health protection.

All veterinary drugs analyses were performed using an in-house enzyme immunoassay validated to the criteria of Commission Decision 2002/657/EC. Validation data corresponding to EIA method performance (limit of detection, LOD; detection capability, CCb; recovery) are shown in Table 1. All EIA methods used obtained CCβ values less than the fixed MRL values and recoveries higher than 70 % in accordance with regulations set by Commission Decision 2002/657/EC. Data indicated that the methods are appropriate for the detection of antibiotics measured.

A total of 119 milk samples were subjected in parallel to immunological screening methods. Antibiotic concentrations analyzed by EIA methods in raw milk samples and values for MRLs are summarized in Table 2. All concentrations found were lower than the established MRLs set by Council Regulation 37/2010/EU (EC, 2010). In previous study, residues of beta-lactam and sulphonamide antibiotics

Table 1. Method performance data for EIA methods (limit of detection, LOD; limit of quantification LOQ; detection capability, CCb; recovery) of the antibiotics studied in milk

Analyte	LOD	LOQ	CCβ	Recovery
	(μg kg^{-1})	(μg kg^{-1})	(μg kg^{-1})	(%)
Chloramphenicol	<0.01	<0.01	0,23	74,5
Sulfonamides	1,8	3,1	69,5	93,2
Tetracycline	9,4	23,5	12	94,3
Streptomycin	4,5	7,8	24,2	119,7
Dihydrostreptomycin	6,3	14,2	29,1	92,8
Gentamicin	2,2	4,4	38,6	120,5
Flumequine	10,2	26	42,3	95,4
Enrofloxacin	5,4	12,1	13,7	79,3

Table 2. Veterinary drug residues (range and mean) in milk samples collected in three months period in 2011 and maximum residues levels (MRL) regulated by Croatian legislation (Ordinance, 2011)

Analyte	n	Range	Mean	MRL
		(μg L^{-1})	(μg L^{-1})	(μg kg^{-1})
Chloramphenicol[a]	119	0.0004-0.05	0,005	0,3
Sulfonamides	119	0.211-45.8	3,67	100
Tetracycline	119	0.025-49.5	2,83	100
Streptomycin	119	0.50-25.6	2,64	200
Dihydrostreptomycin	119	0.026-187.6	7,67	200
Gentamicin	119	0.005-4.05	1,1	100
Flumequine	119	1.72-49.8	10,4	50
Enrofloxacin	119	0.56-22.3	4,11	100

[a]Chloramphenicol is not authorized for use in food producing animals in the European Union and in Croatia (in MRL column is indicated MRPL value)

by screening diffusion test (Delvotest, DSM Food Specialties, The Netherlands) were detected in total 1.17 % of milk samples collected during seven months in 2004 and 2005 in Croatia (Pintić et al., 2006).

Chloramphenicol is a broad spectrum antibiotic active against both Gram-positive and Gram-negative bacteria and an effective therapeutic agent for the treatment of mastitis in cattle. Due to the potential risk to human health, the use of chloramphenicol is prohibited in food-producing animals destined for human consumption in the European Community (Council Regulation 37/2010/EU) and Croatia (Ordinance, 2002). The European Union introduced the concept of the minimum required performance limit (MRPL) of 0.3 μg kg^{-1} as the minimum content of chloramphenicol in a food of animal origin to be detected and confirmed (EC, 2003; Ordinance, 2005).

In the present study, the measured chloramphenicol mean concentration of 5 ng L^{-1} was substantially lower than the MRPL level. However, in the past decade, there have been reports of veterinary drug residues in other European countries: <0.2 μg kg^{-1} in 2000 and 0.5 μg kg^{-1} in 2001 in Slovenia, 0.72 μg kg^{-1} in 2006 in Poland, 0.3 μg kg^{-1} in 2002 in Lithuania, 0.14 μg kg^{-1} in 2004 in Estonia (Dolajš et al., 2007). During the past decade, the presence of chloramphenicol residues found in 39 samples ranging from 0.3 to 1.27 mg kg^{-1} has been reported in milk and dairy products mainly from eastern European countries such as Estonia, Latvia and Lithuania (RASFF, 2011). The highest measured concentration of 1.27 mg kg^{-1} chloramphenicol was detected in Germany in yoghurt imported from Lithuania.

Sulfonamides play an important role as effective chemotherapeutics of bacterial and protozoan diseases and as growth promoters in veterinary medicine. The Committee for Veterinary Medicinal Products considers that the sum of all substances belonging to the sulfonamide group in bovine milk should not exceed 100 μg kg^{-1} (EMEA, 1995a). In the present study, the concentration of determined sulfonamides was 27 times lower than the MRL.

Gentamicin is the most commonly used aminoglycoside for the treatment of a variety of bacterial infectious diseases in cattle in Europe (EMEA, 2001). Concentrations obtained in this study presented the lowest concentration measured among all veterinary drugs monitored. On the other hand, the highest measured level of any veterinary drug determined was for dihydrostreptomycin (187.6 μg kg^{-1}). Streptomycin and dihydrostreptomycin are active against many Gram-negative bacteria and used to treat bacterial diseases in cattle, sheep, pigs and poultry, parenterally or via drinking water (EMEA, 2002b).

Tetracyclines are globally used as broad spectrum antibiotics in veterinary medicine against a wide range of Gram-positive and Gram-negative aerobic and anaerobic bacteria (JECFA, 1998). For example, monitoring of locally produced and im-

Table 3. Estimation of daily intakes (EDIs) of veterinary drug residues through milk consumption based on the mean concentrations found in three months period in 2011

Analyte	EDI[a]	ADI[b]
	(μg/kg BW/day)	(μg/kg BW/day)
Chloramphenicol[c]	0.28	NE
Sulfonamides	0.204	NE
Tetracycline	0.157	3
Gentamicin	0.061	100
Streptomycin	0.147	50
Dihydrostreptomycin	0.426	50
Flumequine	0.578	30
Enrofloxacin	0.228	372
Total value	1,801	605

NE: Not yet have been established
[a]EDI was calculated by the equation presented by Juan et al., 2010
[b]Acceptable daily intake (EMEA 1995a, 1995b, 2001, 2002a; WHO, 2002, 2004, 2006)
[c]EDI for chloramphenicol was expressed as ng/kg BW/day

ported milk and dairy products collected in different seasonal periods from farms and retail outlets in Kuwait indicated that 29.1 % of the analyzed fresh milk samples were above the MRL for tested residues, with tetracycline as the dominant residue (Alomirah et al., 2007). In this study, the highest tetracycline level detected was 49.5 μg kg^{-1}. However, the mean tetracycline concentration (2.83 μg kg^{-1}) was more that 35 times lower than the MRL level.

In the present study, the quinolones flumequine and enrofloxacin showed levels below the MRLs. However, the highest mean level in milk samples was determined for flumequine. These two veterinary drugs are synthetic antibiotics widely used to treat livestock diseases, such as gastrointestinal and respiratory tract infections (EMEA, 2002a).

For the purpose of evaluation of dietary exposure with veterinary antibiotic residues through the intake of the raw milk controlled in the present study, the daily intakes (EDIs) for consumers were estimated. Table 3 shows the EDIs of veterinary drug residues based on the concentrations found in this study, calculated with the presumption of average daily milk consumption for an adult of 300 mL (Antonić et al., 2007). Residue values of all drugs measured ranged from 0.061 to 0.578 mg/kg BW/day and were 20 to 1640 times lower than the set values of acceptable daily intakes (ADIs) (EMEA, 1995b, 2001, 2002a; WHO, 2002, 2004, 2006). However, ADIs values have not been established for chloramphenicol and sulfonamides (EMEA, 1995a; WHO, 2004). The total EDI value obtained was 1.801 mg/kg BW/day, substantially lower than the total acceptable daily intake. Also, the highest specified calculation for EDI was calculated for flumequine in general. Accordingly, toxicological risk associated with the consumption of analysed milk could not be considered as a public health issue with regards to these veterinary drugs.

Conclusions

The methods used for antibiotic determination in milk were validated according to Commission Decision 2002/657/CE and proved to be rapid and simple, and permitted good accuracy and repeatability, with recoveries higher than 70 %. In the three-month period of monitoring milk samples, the veterinary drugs residues measured were far below the maximum residue limits (MRLs) set by the legislation. The estimated daily intakes (EDIs) calculated showed that the contribution of milk to dietary intake of the investigated antibiotics were 20 to 1640 times lower than the ADIs proposed by EMEA and WHO. This indicates that raw milk in Croatia contains very low levels of veterinary drugs and is therefore safe for human consumption.

Koncentracije ostataka veterinarskih lijekova u mlijeku s individualnih farmi u Hrvatskoj

Sažetak

Tijekom 3 mjeseca analizirano je ukupno 119 uzoraka mlijeka na veterinarske lijekove: kloramfenikol, sulfonamide, tetraciklin, gentamicin, streptomicin, dihidrostreptomicin, flumekin i enrofloksacin. Imunoenzimske metode korištene za određivanje veterinarskih lijekova validirane su prema odredbama propisanim Odlukom Europske komisije 2002/657/EC. Rezultati validacije metoda pokazuju da su primijenjene metode prikladne za tu namjenu. Ni u jednom uzorku mlijeka nije utvrđena koncentracija ostataka veterinarskih lijekova iznad najviših dopuštenih količina (NDK) utvrđenih Europskom legislativom. Izračunate procjene unosa određivanih lijekova mlijekom su za 20 do 1640 puta niže od zadanih prihvatljivih dnevnih količina unosa. Prema dobivenim rezultatima može se zaključiti da određivani veterinarski lijekovi ne predstavljaju toksikološki rizik za potrošače s obzirom na rezultate analize mlijeka na ostatke antibiotika s malih farmi.

Ključne riječi: mlijeko, ostaci veterinarskih lijekova, ELISA, procjena unosa lijekova

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