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Control of levamisole residues in milk using a validated liquid chromatography-tandem mass spectrometry method

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Abstract

Concentrations of the anthelmintic agent levamisole were measured in a total of 85 raw cow milk samples collected during 2014 from dairy farms across Croatia. The liquid chromatography-tandem mass spectrometry (LC-MS/MS) method used for levamisole quantification was validated according to the criteria set by Commission Decision 2002/657/EC. The following validation parameters were determined: limit of decision (CCα) 0.55 μg kg^{-1}, detection of capability (CCβ) 0.59 μg kg^{-1}, limit of detection (LOD) 0.06 μg kg^{-1}, limit of quantification (LOQ) 0.22 μg kg^{-1}, precision and accuracy (expressed as recovery) 97.3-100 %, intra-laboratory reproducibility (RSD) 4.2-5.6 %. Levamisole levels for 45 of the total of 85 samples were below the LOD value (0.06 μg kg^{-1}). In the remaining 40 milk samples, levamisole was measured in the range from 0.061 to 0.142 μg kg^{-1} and the mean value was 0.092 μg kg^{-1}. Accordingly, all concentrations analysed were below the LOQ value (0.22 μg kg^{-1}) and limit of decision (CCα) of the method used.

Key words: levamisole, anthelmintic, milk, validation, LC-MS/MS

Introduction

During their lifetime, food-producing animals are nursed with different veterinary drugs for treatment, prevention or control of pests and infectious and non-infectious livestock diseases or to prevent losses during transportation (Stolker et al., 2007). For prophylaxis and treatment of endoparasite infections and ectoparasite infestations in domestic livestock, a wide range of anthelmintic drugs are administered, including benzimidazole compounds, imidazothiazoles (particularly levamisole), macrocyclic lactones and flukicidal compounds (Cooper et al., 2011). Anthelmintic treatments are commonly used to protect animals from parasites, such as intestinal worms, lungworms and liver fluke and to minimise production losses (Stolker and Brinkman, 2005).

Levamisole [*levo* isomer of 2,3,5,6-tetrahydro-6-phenylimidazo (2,1-*b*) thiazole] is primarily used as a broad-spectrum anthelmintic, an agent against endoparasites. Clinical and experimental trials have shown that levamisole might have an immunomodulative stimulation action on the immune and inflammatory functions of leukocytes and is applicable in the treatment of certain diseases (Brunner and Muscoplat, 1980; Mulcahy et al., 1986). Also, it was shown that the administration of levamisole in animals in the recommended immunostimulatory dose for three consecutive days stimulated an elevation of the total leukocyte count and percentage of neutrophil cells in healthy and unstressed boars (Bilandžić and Šimić, 2010).

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The most frequently used anthelmintic compounds are levamisole and therefore due to its extensive usage residues might be found in milk (Stolker and Brinkman, 2005). In general, improper or negligent use of veterinary drugs in domestic animals might cause the occurrence of residues in food products. Levamisole is classified in the group of anthelmintics (B2a) in "other veterinary drugs" (B2), which includes a variety of veterinary medicinal products classified according to their pharmacological action (EC, 1996). Therefore, monitoring of levamisole and other veterinary drug residues is required in food-producing animals and animal products (meat, milk, eggs and honey) under the National Surveillance Schemes of European Union Member States pursuant to Council Directive 96/23/EC (EC, 1996). Intensive use of anthelmintic agents, particularly in the absence of epidemiological evidence of therapy, provokes the development of resistance and represents an animal health issue. A limited number of anthelmintic agents are permitted for the treatment of lactating animals and have an established maximum residue limit (MRL) (EC, 2010). However, no MRL has been set for levamisole in milk, while levels were established for animal tissues (EC, 2010).

Techniques such as high- performance liquid chromatography (HPLC) with various detectors (Takeba et al., 2000; Msagati and Nindi, 2001; El-Kholy et al., 2003), liquid chromatography-tandem mass spectrometry LC-MS/MS (De Ruyck et al., 2002; Jedziniak et al., 2009; Kinsella et al., 2009), ultra-performance liquid chromatography-tandem mass spectrometry UHPLC-MS/MS (Aguilera-Luiz et al., 2008; Garrido Frenich et al., 2010; Whelan et al., 2010a,b) or ultra-performance liquid chromatography combined with time-of-flight mass spectrometry (UPLC-ToF-MS) (Kaufmann et al., 2007; Stolker et al., 2008) are used today in the analysis and detection of anthelmintics. Some of the methods are also intended for the simultaneous determination of benzimidazoles and levamisole in milk (Jedziniak et al., 2009; Kinsella et al., 2009; Stolker et al., 2008).

This paper describes the validation of a new method for quantification of levamisole residues in milk using LC-MS/MS. The aim of the study was to perform surveillance of levamisole residues in milk samples collected from throughout Croatia over a one-year period.

Materials and methods

Sample collection

A total of 85 raw cow milk samples were collected from dairy farms across Croatia during 2014. The volume of collected raw milk samples was approximately 0.5 litres. 90 % of the taken samples were collected for analysis and frozen at -20 °C. In cases when samples arrived and analysis for levamisole was planned to be performed on the following day, the samples were not frozen but stored in a refrigerator at 2-8 °C.

Chemicals and standards

HPLC-grade solvents acetonitrile, formic acid (ACS reagent ≥96 %) and ethyl acetate were purchased by Sigma Aldrich Chemie GmbH (Germany). Sodium sulphate was purchased from Merck (Darmstadt, Germany). Dimethyl sulfoxide (DMSO) was obtained from J.T. Baker (USA). Sodium chloride and ethanol were obtained from Carlo Erba (Val de Reuil, France). Sodium hydroxide was obtained from Kemika (Zagreb, Hrvatska). Nitrogen 5.0 and 5.5 were purchased from SOL spa (Monza, Italy). Ultra-purified water was obtained using the Milli-Direct Q® 5UV (Millipore, Bedford, USA).

The analytical standard levamisole hydrochloride was obtained from Sigma Aldrich Chemie GmbH (Germany). A stock solution of 1 mg mL^{-1} was prepared in DMSO. It was stable for one year at -20 °C. Working solutions (10 μg mL^{-1}, 1 μg mL^{-1}, 0.1 μg mL^{-1}) were prepared by appropriate dilution of the stock solution with ethanol, they were stable for 1 year, 6 and 3 months at refrigerator (2-8 °C), respectively. For the first extraction approach, the working solution 0,1 μg mL^{-1} for spiking (LEV-spike) of milk samples of was prepared by diluting a working solution at 1 μg mL^{-1} with mobile phase A (0.1 % formic acid). The internal standard tetramisole-d5-hydrochloride was obtained from BVL (Berlin, Germany). Stock solution of 1 mg mL^{-1} was prepared in ethanol. Working solutions of 10 μg mL^{-1} and 1 μg mL^{-1} were prepared by appropriate dilution of the stock solution and 10 μg mL^{-1} solution, respectively, with ethanol. For the first extraction approach, the working solution of 1 μg mL^{-1} for spiking (TETR-d5-spike) of milk samples was prepared by diluting a working solution of 10 μg mL^{-1} with mobile phase A. Storage conditions were the same as for the levamisole stock, working and spike solution.

Sample extraction procedure

5 g of milk was weighed into a 50 mL polypropylene centrifuge tube and spiked with 25 μL of internal standard working solution of 1 μg mL^{-1}. Extraction of levamisole was performed by adding of 100 μL 10M NaOH and 15 mL ethyl acetate and vortex for 5 min. Then, 10 g of NaCl and 10 g of anhydrous Na_2SO_4 were added and left to rest without mixing. After centrifugation (3,500 x g, 10 min at room temperature) the upper organic layer (10 mL) was transferred into a 15 mL polypropylene centrifuge tube.

For the matrix matched calibration the sample extracts were spiked'with 16.6, 33.2, 49.9 and 66.4 μL of levamisole solution of 0.1 μg mL^{-1} to obtain 0.5, 1, 1.5 and 2 ng mL $^{-1}$ and 25 μL of internal standard working solution of 1 μg mL^{-1} to obtain 5 ng mL^{-1} in the samples. Extract was evaporated to dryness under a stream of nitrogen at a temperature of 50±5 °C. The dry residue was dissolved in 50 μL of acetonitrile and 150 μL of 0.1 % formic acid, vortexed for 30 sand placed for 5 min in ultrasonic bath. After centrifugation at 3,500 x g for 10 min, clear supernatant was filtered through PVDF (Polyvinylidene Difluoride) filter (0.45 mm filter) and transferred to a vial in a final volume of 200 μL.

Instrumentation

For purposes of sample preparation, the following equipment was used: neoLab Vortexer RM-2M (Heidelberg, Njemačka), Waring Commercial Blender 7011HS (Torrington, CN, USA), IKA® Vortex model MS2 Minishaker (Staufen, Germany), Iskra ultrasonic bath (Šentjernej, Slovenia), centrifuge Rotanta 460R (Hettich Zentrifugen, Tuttlingen, Germany) and Nitrogen evaporation system N-EVAP® model 112 (Organomation Associates Inc., Berlin, MA, USA).

Levamisole quantification was performed using LC-MS/MS system comprising of HPLC 1260 and Triple Quad LC/MS 6410 mass spectrometer (Agilent, Palo Alto, CA, USA).

Chromatographic and MS parameters

Gradient elution using column Poroshell 120 EC C18, 50x3 mm, 2.7 μm particle size, column at 40 °C (Agilent, USA) was used for chromatographic separation. The mobile phase consisted of two components: A, 0.1 % formic acid; B, 0.1 % formic acid in acetonitrile and the elution was: 0 min, MFA-MFB (60:40, v/v); 2.0 min, MFA-MFB (30:70, v/v); 3.0 min, MFA-MFB (60:40, v/v). Elution was performed at a constant flow rate of 0.3 mL min^{-1} and injection volume was 5 μL. Chromatographic run was 3 min followed by 2 min of equilibration time.

Mass spectrometry analysis was performed operating in positive electrospray ionization mode (ESI+). The ion source was heated to 450 °C with a gas flow of 5 L min^{-1}, nebulizer pressure 15 psi and capillary voltage at 5000 V. Fragmentor voltage (F) and collision energy (CE) were optimized by injecting the standard at a concentration of 10 μg mL^{-1} directly to the MS/MS source. The data was acquired by the multiple reaction monitoring (MRM), by selecting the two most intense ion transitions from the precursor to product ions (Table 1).

Method validation

The method used for milk analysis was validated according to the criteria laid down by Commission Decision 2002/657/EC (EC, 2002). The parameters determined were: limit of decision (CCα), detection of capability (CCβ), specificity, linearity, precision and accuracy (expressed as recovery), limit of detection (LOD), limit of quantification (LOQ). Specificity was tested by analysing 20 representative blank samples. Response linearity was calculated from the five-point calibration curve (5, 25, 50, 100 and 150 μg L^{-1}) with internal standard at concentration of 200 μg L^{-1}. Results were calculated from the four-point matrix-matched calibration curve (0.5, 1.0, 1.5 and 2.0 μg kg^{-1}) where the lowest quantification level (C_0) was set at 0.5 μg kg^{-1}.

Table 1. Mass spectrometry transitions and optimised collision energy (CE) and fragmentor voltage (F)

Analyte	Ion precursor	Ion product	F (V)	CE (V)
LEV	205.1	177.9*	100	20
		122.9		28
TETR-d5	210.2	183.0	105	22

*the underlined transition was applied in the quantification

Method trueness and precision was determined by fortifying blank milk samples with levamisole at 0.5 (C_0), 1 and 1.5 μg kg^{-1}, in eight replicates for each level, and analysed on three different days by different analysts. It is expressed as the relative standard deviation (% RSD), being the ratio between the standard deviation (SD) and the mean concentration. CCα and CCβ were calculated by applying the calibration curve procedure. CCα was expressed as the sum of the average concentration of samples spiked at the C_0 level and 2.33 times the reproducibility standard deviation at C_0. CCβ was calculated as CCα value plus 1.64 times reproducibility standard deviation at CCα. The limit of detection (LOD) and limit of quantification (LOQ) were calculated by fortifying samples at the lowest quantification level, C_0 (0.05 μg kg^{-1}). LOD is defined as the standard deviation of the repeated samples at the lowest tested level multiplied by the *t*-value at the 98 % probability, where LOQ is 10 times the standard deviation of the lowest level (C_0).

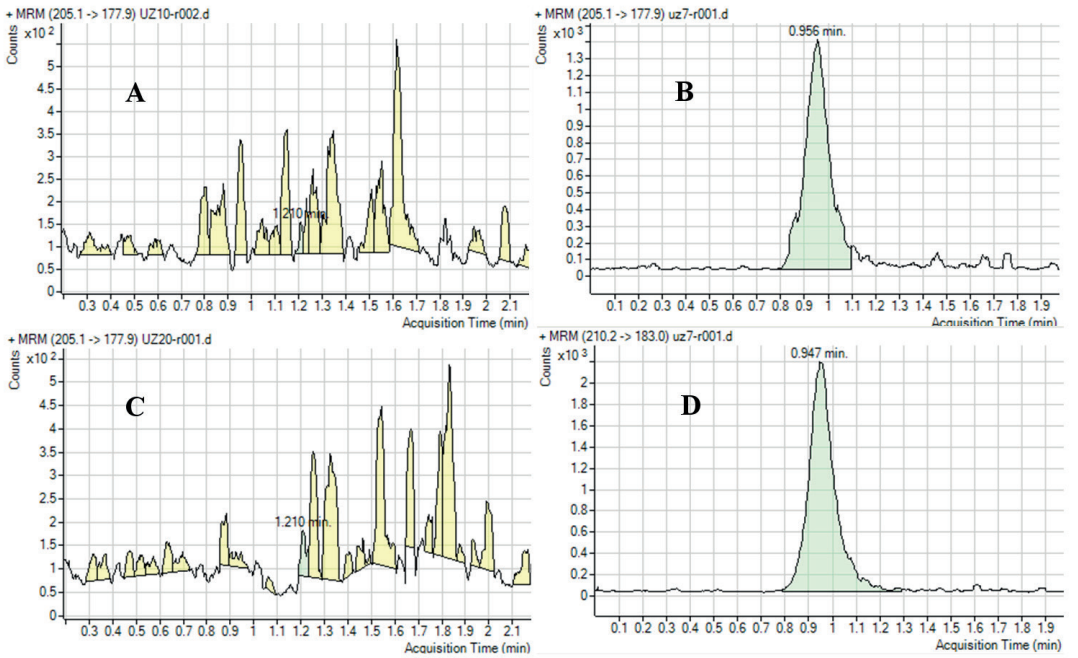
Statistical analysis

Statistical analysis was performed using the SPSS Statistics 17.0 software package. Concentrations were expressed as mean ± standard deviation.

Results and discussion

Residues of veterinary drugs in foods of animal origin can be harmful for human consumption and may cause allergic reactions in hypersensitive individuals or may cause problems indirectly through the induction of resistant bacterial strains (Stolker et al., 2008). Therefore, residue control is focused foremost on consumer protection and is based on risk analysis (EC, 2009). The laboratories responsible for the control of these substances are required to implement a sensitive and reliable method for the detection and evaluation of the levels of legal and illegal veterinary drugs. The performance parameters LOD, LOQ, CCβ, CCα, recovery, precision and intra-laboratory reproducibility of the LC-MS-MS method validated for quantification of levamisole milk are summarized in Table 2. The linearity of the standard calibration curve was evaluated by calculating the coefficient of the regression curve (R^2) which was ≥0.998. Recovery was calculated for three concentration levels (C_0, $2C_0$ and $3C_0$) and ranged between 97.3 (for spiking level at 3 C_0) and 100 % (for spiking level at C_0). Satisfactory values for precision and intra-laboratory reproducibility were achieved. Relative standard deviations of the intra-laboratory reproducibility were ≤5.6 %RSD. Calculated values

Figure 1. Chromatogram of levamisole in blank cow's milk (A), spiked milk at a level of 0.5 μg kg^{-1} (B), and actual sample (C) showing peak of quantifier 177.9, and chromatogram of tetramisole-D5 at level of 5 μg kg^{-1} (D)



for CCα and CCβ were (μg kg^{-1}): 0.55 and 0.59, respectively. The chromatogram of levamisole in blank milk sample, spiked sample at 0.5 μg kg^{-1} and actual sample, as well as chromatogram of the internal standard is shown in Figure 1. The obtained results indicated that the validated LC-MS/MS method was reliable for the quantification of levamisole in milk and meets the criteria laid down by the Commission Decision 2002/657/EC (EC, 2002).

The obtained levels of CCα and CCβ were below 1 μg kg^{-1} and lower than those previously reported in a study using sample preparation and liquid-liquid extraction that was similar to this study in certain steps and same the technique LC-MS/MS for levamisole determination in milk (μg kg^{-1}): 1.2 and 1.4 (De Rujck et al., 2002). However, the recovery rates obtained in the present study and the cited study were similar: 89.6-98.1 % and 89.6-102 %, respectively. In other studies focused on the determination levamisole and other anthelmintic compounds using the QuEChERS procedure for sample preparation and detection by UPLC-MS/MS, a recovery of 75.3-92.8 % was obtained (Aguilera-Luiz et al., 2008). That study also showed intermediate precision expressed as RSD of 9.8 %, which was similar to value obtained in the present study.

Some authors studied the use of liquid-liquid extraction sample preparation with ethyl acetate or liquid extraction with an additional solid-phase sample extraction step procedure followed by quantification by LC-MS/MS and UHPLC-MS/MS, whereby method recoveries were determined in the ranges: 84-89 % (Jedziniak et al., 2009) and 94-104 % (Whelan et al., 2010b). Furthermore, CCα and CCβ values determined using UHPLC-MS/MS analysis were (μg kg^{-1}): 0.83 and 1.4 (Whelan et al., 2010b). In a recent study using solvent extraction with acetonitrile, citric acid and EDTA, and quantification with UHPLC-MS/MS, the recovery rates were 94.8-118.5 % (Garrido Frenich et al., 2010).

In the present study, low values were obtained for LOD and LOQ. Garrido Frenich et al. (2010) obtained a similar LOQ value of 0.1 μg kg^{-1} using UHPLC-MS/MS. However, in another study using UPLC-MS/MS, the performance parameters LOD and LOQ were 1 μg kg^{-1} and 3 μg kg^{-1} (Aguilera-Luiz et al., 2008).

In the present study, 85 samples of raw milk were collected in Croatia during 2014 and analysed. For 45 samples, levamisole concentrations were below the LOD value (0.06 μg kg^{-1}). For the remaining 40 milk samples, levamisole levels were determined in the range from 0.061 to 0.142 μg kg^{-1} and all concentrations were below the LOQ value (0.22 μg kg^{-1}) (Table 3).

It was previously concluded that levamisole is rapidly excreted from milk after intramuscular dose administration (7 mg levamisole hydrochloride per kg body weight) and the maximum concentration was measured 1 h after the treatment (Österdahl et al., 1986). Furthermore, the half life of levamisole in milk was between 5 h and 29 h after treatment and levels were below the limit of determination (0.04 μg mL^{-1}). In a recent study of the persistence of levamisole residues in bovine milk after the

Table 2. Validation parameters of the LC-MS/MS method for quantification of levamisole in milk

Spiking level	Precision (n=8) 1st day		Precision (n=8) 2nd day		Precision (n=8) 3rd day		Intra laboratory reproducibility (n=24)		Trueness (n=24)
(μg kg^{-1})	Mean±SD (μg kg^{-1})	RSD (%)	Mean±SD (μg kg^{-1})	RSD (%)	Mean±SD (μg kg^{-1})	RSD (%)	Mean±SD (μg kg^{-1})	RSD (%)	Recovery (%)
C_0=0.5	0.50±0.03	5.9	0.49±0.01	2.6	0.52±0.01	2.3	0.50±0.02	4.2	100.0
$2C_0$=1.0	0.99±0.03	2.9	1.01±0.01	5.9	0.94±0.01	1.3	0.99±0.05	5.0	99.0
$3C_0$=1.5	1.50±0.07	4.5	1.48±0.02	2.5	1.36±0.02	3.7	1.46±0.08	5.6	97.3

CCα (μg kg^{-1})	CCβ (μg kg^{-1})	LOD (μg kg^{-1})	LOQ (μg kg^{-1})
0.55	0.59	0.06	0.22

Table 3. Concentrations of levamisole in milk samples above LOD values

No samples	Levamisole (μg kg^{-1})
1	0.0611
2	0.0649
3	0.0651
4	0.0651
5	0.0652
6	0.0652
7	0.0661
8	0.0672
9	0.0691
10	0.0693
11	0.0698
12	0.0707
13	0.0758
14	0.0786
15	0.0816
16	0.0819
17	0.0837
18	0.0841
19	0.0881
20	0.0886
21	0.0890
22	0.0925
23	0.0945
24	0.1030
25	0.1038
26	0.1060
27	0.1078
28	0.1093
29	0.1110
30	0.1115
31	0.1124
32	0.1149
33	0.1159
34	0.1166
35	0.1168
36	0.1184
37	0.1197
38	0.1272
39	0.1304
40	0.1417
Mean ± SD (μg kg^{-1})	0.092±0.022

treatment, with a combination product, levamisole residues were rapidly excreted and after several days were below 0.04 μg mL^{-1} (Whelan et al., 2010b). The highest levamisole levels (600 μg kg^{-1}) were determined at the first and the third milking.

The European Food Safety Authority (EFSA) published a summarized report for National Surveillance Schemes of European Union Member States as annual reports (EFSA, 2014, 2015). The summary report for 2012 presented a number of non-compliant samples for anthelmintics (B2a) for animal species and animal products (% non-compliant to the total number of analyzed samples) for: bovine 1 (0.02 %), pigs 3 (0.04 %), sheep and goats 11 (0.36 %), horses 1 (0.40 %), aquaculture 2 (0.29 %), milk 5 (0.09 %), rabbits 1 (0.64 %) and farmed game 1 (0.39 %) (EFSA, 2014). Levamisole was not found in milk, though other anthelmintics such as clorsulon, fenbendazole, ivermectin, ketotriclabendazole, triclabendazole, triclabendazolsulfon and triclabenzolsulfoxide were detected in Denmark, Belgium and France.

In conclusion, the present study of quantifying levamisole in milk was performed by using a validated sensitive method of LC-MS/MS. According to the obtained results, the levamisole concentrations in milk were below the LOQ and CCα of the method used.

Kontrola ostataka levamisola u mlijeku primjenom validirane metode tekućinske kromatografije-tandemske-spektrometrije masa

Sažetak

Koncentracije anthelmintika levamisola određene su u ukupno 85 uzoraka sirovog kravljeg mlijeka prikupljenih tijekom 2014. na mliječnim farmama iz cijele Hrvatske. Za kvantifikaciju levamisola korištena je metoda tekućinske kromatografije-tandemske-spektrometrije masa (LC-MS/MS) koja je validirana u skladu s kriterijima utvrđenim Odlukom Komisije 2002/657/EZ. Utvrđeni su sljedeći parametri validacije: granična koncentracija određivanja (CCα) 0,55 ug kg^{-1}, sposobnost dokazivanja (CCβ) 0,59 ug kg^{-1}, granica određivanja (LOD) 0,06 ug

kg^{-1}, granica kvantifikacije (LOQ) 0,22 ug kg^{-1}, preciznost i točnost (izraženi kao iskorištenje) 97,3-100,0 %, unutarlaboratorijska obnovljivost (RSD) 4,2-5,6 %. Koncentracije levamisola za 45 od ukupno 85 uzoraka bile su ispod vrijednosti LOD (0,06 ug kg^{-1}). U preostalih 40 uzoraka mlijeka, levamisol je izmjeren u rasponu od 0,061 do 0,142 ug kg^{-1}, a srednja vrijednost je bila 0,092 ug kg^{-1}. Zaključno, sve koncentracije analizirane su ispod LOQ vrijednosti (0,22 ug kg^{-1}) i CCα, granične koncentracije metode.

Ključne riječi: levamisol, antihelmintik, mlijeko, validacija, LC-MS/MS

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