Control of chloramphenicol in samples of meat, meat products and fish

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Scientific paper

Summary

In the goal of controlling the illegal use and the control of chloramphenicol remains, during the year 2010 there were collected samples of muscle tissue of poultry (n=33), bovine (n=109) and pigs (n=46), meat products (n=21) and fish (n=7), from all areas of the Republic of Croatia. Concentrations of chloramphenicol were determined by the screening ELISA method and confirmatory method of liquid chromatography- tandem mass spectrometry (LC-MS/MS). Validation of methods proved their quantification capability below minimum required performance limit of method performance of 0.3 µg/ kg. The determined concentrations of chloramphenicol in bovine, pig and poultry meat, meat products and fish were in the range of minimum value of 0.177 to the highest value of 202.9 ng/ kg. The lowest mean concentration of 3.54 ng/ kg was determined in meat products and the highest of 15.8 ng / kg in bovine meat. There were no determined concentrations over 300 ng/ kg in any sample of the controlled meat and fish. Considering the determined low concentrations of chloramphenicol, it can be concluded that the meat of controlled animals is safe to consume. That means that the prohibition on use of that antibiotic is respected in the Republic of Croatia. In accordance with the determined cases of increased concentrations in meat, fish and shrimps originating from Asia, a constant control of imported shipments from the countries of that part of the world is necessary.

Key words: *chloramphenicol, meat, fish, ELISA, LC-MS/MS*

Introduction

Chloramphenicol as a veterinary preparation is used in treatments of different infections of animals which are used for the production of food for human nutrition. It is used because of its bacteriostatic activity against most Gram positive and Gram negative bacteria, ad well as because of prevention of growth and reproduction of rickettsias, chlamydiae and mycoplasmas (Dowling, 2006). Mechanism of action of chloramphenicol is the inhibition of protein synthesis in bacterial cells and in bone marrow cells of mammals (Kucers et al., 1997). After per oral, i.e. parenteral application, chloramphenicol is mostly biotransformed through glucoronidation in different organs including liver, kidney and muscle into metabolites which are transported in the whole body and excreted by kidneys (Anadon et al., 1994; Akhtar et al., 1996).

From the toxic point of view, chloramphenicol is a cytotoxic, genotoxic and hematotoxic compound which causes bone marrow depression and, in the worst case, aplastic anemia in people (Dowling, 2006). In most difficult cases, irreversible aplastic anemia can lead to leukemia which has a high mortality rate determined (> 50%). Chloramphenicol contributes to the development of resistant strains of bacteria too, which also contributes to serious health problems (Ferguson et al., 2005).

Because of the potential toxic effect on people and the possibility of finding metabolites in edible tissues, the application of chloramphenicol with animals whose products are intended for human consumption is banned in the European Union (EC, 1994). The European Union has also established the so- called "zero tolerance", so chloramphenicol was included in the group of substances for which a maximum allowed quantity in foodstuffs of animal origin can not be determined due to toxicological reasons (EC, 2009). In

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accordance with that, the Republic of Croatia banned the use of chloramphenicol with animals used for human nutrition by the Regulation on prohibition of certain veterinary drugs with food producing animals (Anon., 2002). Despite prohibition, chloramphenicol remains are found systematically in all kinds of products of animal origin, which causes a great concern in the EU (RASFF, 2002- 2011).

In the goal of determining chloramphenicol remains in the food of animal origin, specific and sensitive methods are used. The EU has a proscribed minimum required performance limit (MRPL) for chloramphenicol of 0.3 µg/ kg and it represents a minimum concentration of chloramphenicol in a sample which must be detected by the applied method (EC, 2003). The methods that are most often used to determine chloramphenicol are immunoenzymatic test (ELISA) and gas chromatography (Scortichini et al., 2005; Cerkvenik- Flajs, 2006). In cases of samples with increased concentrations for determining and quantification of chloramphenicol, confirmatory methods of gas and liquid chromatography- tandem mass spectrometry are used (GC-MS/MS, LC-MS/MS) (Gantverg et al., 2003; Impens et al., 2003; Mottier et al., 2003; Bogusz et al., 2004; Peng et al., 2006; Rønning et al., 2006).

In this paper, concentrations of chloramphenicol were determined by immunoenzymatic method, and samples with higher concentrations were checked by liquid chromatography- tandem mass spectrometry. Chloramphenicol remains were determined in bovine meat, pork and poultry and meat products and fish in 2010.

Materials and Methods

Sampling

During the year 2010 there were collected samples of poultry, bovine and pig muscle tissues, and meat products (pâté, hamburger, sausages, frankfurters) and fish from all areas of the Republic of Croatia. Samples were refrigerated until delivered to a laboratory where they were chopped to smaller pieces and deep frozen at -18 °C until the analysis.

Table 1. **Recovery and accuracy results of ELISA and LC-MS/MS methods for chloramphenicol determination in meat**

Method	Spiked concentrations (µg/kg)	Measured concentrations (µg/kg)	Recovery (%)	Standard deviation	CV (%)
ELISA	0.15	0.16	108.4	0.021	10.6
	0.3	0.28	92.4	0.024	9.8
	0.45	0.35	78.8	0.035	9.5
	Mean		93.2	0.027	9.97
LC-MS/MS	0.15	0.155	103.8	0.022	10.5
	0.3	0.287	95.9	0.024	7.6
	0.45	0.458	101.8	0.031	6.3
	Mean		100.5	0.025	8.13

Table 2. **Chloramphenicol concentrations (ng/kg) in bovine, pig and poultry meat, meat products and fish controlled in 2010**

		Chloramphenicol concentrations (ng/kg)			
Meat	No. of samples	Minimum	Maximum	Mean	Standard error
Bovine	109	0.108	202.9	15.8*	2.65
Pig	46	0.263	92.7	11.3*	2.41
Poultry	33	1.01	139.2	15.5*	15.5
Products	21	0.177	17.9	3.54	0.965
Fish	7	5,59	18.9	10.9**	1.96

* $p < 0,05$

** $p < 0,01$

Chemicals and devices

For the analysis of chloramphenicol by the ELISA method there was used a test by the producer Laboratory of Hormonology (Marloie, Belgium) equipped with: microtiter plate (96 wells), standard solution of chloramphenicol of 0, 0.05, 0.1, 0.2, 0.5, 1 and 2 ng/ mL of chloramphenicol, concentrated conjugate (peroxidase- linked chloramphenicol), lyophilized antibodies anti-chloramphenicol, substrate / chromogen solution (peroxide / TMB), dilution buffer, pH 7.4, stop solution (6 N H2SO4), concentrated wash buffer. Ultra pure water (Milli-Q Millipore, 18.2 $MWcm^{-1}$) was used in the experiment.

Chloramphenicol standard was purchased from the producer Sigma (St. Louis, USA). Dichloromethane, ammonium acetate and petroleum ether, then methanol and diethyl ether (HPLC grade) and columns for purification of samples C_{18} SPE (500 mg per 3 mL) were purchased from J. T. Baker (Deventer, Netherlands). Acetone (HPLC grade) and monosodium phosphate (p.a.) were purchased from the producer Merck (Darmstadt, Germany), and methanol, diethyl ether and ethyl acetate (for the purification of samples for ELISA method), then sodium sulfate (anhydrous) from Carlo Erba (Milan, Italy). Isooctane, sodium chloride and disodium hydrogen phosphate dihydrate were purchased from Kemika (Zagreb, Croatia). Nitrogen 5.0

and 5.5 was purchased from SOL spa (Monza, Italy).

There was used ammonium acetate (CH3COONH4) prepared by a dissolution of 0.38 g of NH_4Ac in 1l of ultra pure water for the mobile phase A for the LC system. Methanol was used for the mobile phase B.

Column Zorbax XDB C18, 4.6 x 75 mm 3.5 µm (Agilent Technology, USA) was used for chromatographic separation. The following devices were used in the preparation of samples: a mixer IKA® Ultra-Turrax® (IKA® -WERKE GMBH & CO.KG, Germany), centrifuge Rotanta 460R (Hettich zentrifugen, Tuttlingen, Germany), N-EVAP model 111 Nitrogen Evaporator (Orgamonation Associates Inc., Berlin, USA).

Absorbance values for ELISA method were measured at micro- plate reader Tecan model Sunrise (Tecan Austria GmbH, Austria). The system for liquid chromatography- mass spectrometry (LC-MS/MS) consists of HPLC 1200 and mass spectrometer Triple Quad LC/MS 6410 (Agilent Technology, USA).

Sample preparation for ELISA method

Meat is homogenized, 3 g are weighed and mixed with 3 mL of distilled water, and then 6 mL of ethyl acetate are added. It is mixed intensively for 10 minutes and then centrifuged for 10 minutes at 3000 x g. 4mL of the upper layer of ethyl acetate are pipetted and evaporated to dryness in a gentle stream of nitrogen at 50 ˚C. The rest is dissolved in 1 mL of mixture of isooctane / dichloromethane (2:3, v / v) and added 0.5 mL of dilution buffer (from the kit). It is mixed intensively for 1 minute and centrifuged for 10 minutes at 3000 x g. If emulsion appears in the upper layer, the test tube is put for a short time- for two minutes in a water bath at 80 ˚C and centrifuged again. 50 µL of watery upper layer are used for the test.

Sample preparation for LC-MS/MS method

Extraction of 10 g of sample is made through the mixture of acetone and dichloromethane in 50:50 (v / v) ratio. Supernatant is separated after the removal of water and after evaporation in a stream of nitrogen, the rest is dissolved by a phosphate buffer with pH value adjusted to 7.8. Then follows purification with SPE C18 columns.

Columns are activated by methanol and water. After sample application there follows a water wash and air drying of column fillers. Interferences are removed by petroleum ether which is followed by drying and elution of chloramphenicol by diethyl ether. It is necessary then to evaporate and reconstruct analyte by a mobile phase.

ELISA test

All chemicals are tempered at room temperature for 45 minutes before the analysis. Concentration of wash buffer is prepared before the usage (1 mL of concentrated buffer + 9 mL of distilled water). A concentrate of enzyme conjugate (CAP-HRPO) is diluted 100 x in a dilution buffer (e.g. 10 µL of conjugate + 990 µL of dilution buffer). Also, lyophilized antibody is reconstructed by adding 6 mL of dilution buffer, and it is stirred well.

The procedure of the test is conducted by the following order of adding reagent to wells: 50 µL of standard solution of 0 to 2 ng/ mL are added, and then 50 µL of sample solution in a duplicate. Then follow adding 100 µL of a prepared solution of enzyme- conjugate to each well and 100 µL of the prepared antibody solution to each well. The plate is closed well and shaken strongly for 1 minute and then incubated in the dark at 4 °C during the period of two hours. The solution from the plate is then removed and rinsed three times with a wash buffer (three times per 300 µL). After each pouring out of wash solution to a new clean blotter, wells are emptied completely by tapping a plate against it. 150 µL of the ready peroxide/ TMB solution is added to each well and stirred well. It is incubated for 30 minutes at room temperature (20- 25 ˚C) in the dark, 50 µL of a stop solution is added to each well and shaken well. Absorbance values are read at 450 nm within 30 minutes at a micro- plate reader (Tecan Austria GmbH, Salzburg, Austria).

Liquid chromatography-tandem mass spectrometry, LC-MS/MS

Column chromatography is separated through isocratic flow of mobile phase which consists of 30% 5 mM of ammonium acetate and 70% of methanol at a flow rate 0.5 mL / min. A volume of sample of 15 µL is applied at the column (temperature 40 °C). The duration of the analysis is 5 minutes with extra 2 minutes for the stabilization of the column.

Ionization of molecules for mass spectrometer is conducted by the source for ionization, Electrospray Ionization. Gas temperature in the source is 350 °C and gas flow is 5 L/ min. The pressure in the nebulizer is 35 psi and capillary is under voltage of 4000 V. The scanning mode is multiple reaction monitoring (MRM) at negative ionization where two molecular masses are monitored (chloramphenicol m/z=321 and internal standard of chloramphenicol m/z=326), out of which per two fragments of each original compound are used for quantification.

Validation of ELISA and LC-MS/MS method

Validation of methods was per-

formed according to the criteria of the EU regulations 2002/657/CE for screening and confirmatory methods (EC, 2002). Specificity of methods was determined by the analysis of 20 negative samples of muscle tissue of different animal species. For screening ELISA method there was determined the detection of capability CCβ by enriching 20 samples to a level below MRPL (0.15 µg/kg) and it was calculated as the sum of the calculated value of limit of decision CCα and standard deviation of the samples enriched at 0.15 µg/kg multiplied by 1.64. Critical concentration CCα for the ELISA method was determined as a sum of mean value of the concentration of blank samples and standard deviation of the responses of blank samples multiplied by 2.

Critical concentration CCα of LC-MS/MS method was determined by enriching samples in three series at 0.15 µg/ kg and it was calculated as the sum of values of the lowest concentration of the validation procedure (C_0) and standard deviation at C_0 (Sr/C_0) multiplied by 2.33. For calculation of detection of capability CCβ there were used the results at all three validation levels (n = 54), and the sum of CCα values and standard deviation at CCα multiplied by 1.64.

Recovery and accuracy of the methods were determined by enriching blank samples CAP in 3 series at 3 concentration levels (0.15, 0.3 and 0.45 µg/kg) per 6 repetitions.

Statistical analysis

Statistical analysis was conducted by the program Statistica© 6.1 (StatSoft©, Inc., Tulsa, USA). Chloramphenicol concentrations determined in muscle tissue were expressed as minimum and maximum concentration determined, mean value ± standard error. *T*-test for determining the difference between mean values of chloramphenicol concentrations between kinds of meat was used. Statistically significant differences were expressed at a significance level $p < 0.05$.

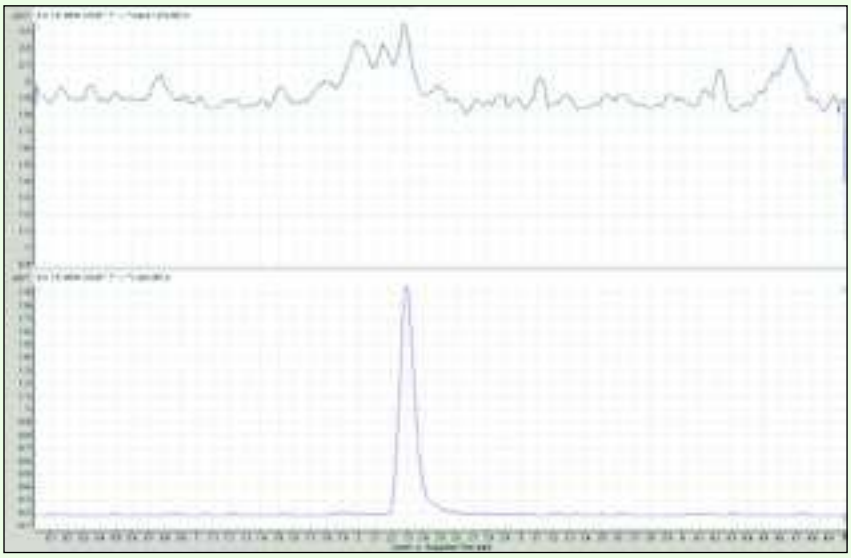

Figure 1. **LC-MS/MS chromatogram of blank sample and standard solution of chloramphenicol at 1 µg/mL**

Results and Discussion

Chloramphenicol concentrations in this paper were determined by the ELISA method, and the samples with higher values were checked by the LC- MS/MS method. Results of recovery, i.e. the accuracy of methods were presented in Table 1. There was determined a total recovery for the ELISA method of 93.2%, and 100.5% for the LC- MS/MS method. Dispersion of methods' results is not significant, which is indicated by coefficients of variation (CV %) of both methods which are less than 20%. Limit of decision CCα for confirmatory LC- MS/MS method is 170 ng/kg and its of detection of capability CCβ is 190 ng/kg, i.e. detection capability CCβ for the ELISA method is 280 ng/ kg.

Values of chloramphenicol concentrations in kinds of meat, meat products and fish are presented in Table 2. The determined concentrations of chloramphenicol in all kinds of meat and products were in the range from 0.111 to the highest value of 202.9 ng/kg determined in bovine meat. There were no determined concentrations above the set-point values for MRPL of 300 ng/kg. The lowest mean concentration of 3.54 ng/ kg was determined in meat products and the highest of 15.8 ng /kg in bovine meat. Statistical analysis determined chloramphenicol concentrations determined in meat products to be significantly lower than the values determined in bovine, pig and poultry meat ($p< 0.05$), and fish ($p< 0.01$).

The determined highest concentrations of 202.9 and 139.2 ng/ kg were analyzed by the confirmatory method LC-MS/MS. The determined concentrations were below values of critical concentration CCα of 190 ng/kg. Chromatograms of the negative sample are shown in Figure 1.

Despite the prohibition of using, chloramphenicol remains are constantly found in all kinds of products of animal origin, which is indicated by the data of the RASFF (Rapid Alert System for Food and Feed) in the European Union. In the last decade there has been recorded a significant number of reports on using chloramphenicol in the products of animal origin (RASFF 2002- 2011). The highest number of samples with

increased concentrations of chloramphenicol in meat (12 cases) was recorded during the year 2003, then in poultry meat (4 reports) and fish (10 cases) in 2002 and 2004. Packages which contained chloramphenicol remains mostly originated from China and Southeast Asia (WHO, 2004). Using the RASFF system, during the last decade there were reported increased chloramphenicol concentrations in: 33 samples of meat of different animals, 12 samples of poultry meat and 19 in fish and fish products.

The highest chloramphenicol concentrations determined by RASFF system were determined in Spain in 2002 and 2007 in samples of eel (3.5 µg/kg) and dry- cured pork (> 8 µg/kg) originating from China. Also, chloramphenicol concentration of 10 µg/ kg was determined in fresh chicken originating from Belgium in 2006. For the sake of comparison, an expressedly high chloramphenicol concentration of 297 µg/ kg was determined in Spain in 2002 in shrimps from China.

During the first three months of the year of 2011 there were reported the following cases of increased chloramphenicol concentrations in the EU: red mullet (0.858 µg/ kg) from Vietnam, salted pork (0.45 and 0.31 µg/ kg) from China, frozen eel (2.26 and 1.01 µg/ kg) from India, and three packages of frozen shrimps (0.17, 0.23 and 0.24) from India. In recent researches, increased chloramphenicol concentrations were determined in poultry tissue in northeastern Iran and there were determined concentrations of 7 µg/ kg in muscle, 55 µg/ kg in kidney, and the highest concentration of 155.2 µg/ kg in liver (Tajik et al., 2010).

The results of this study indicate that bovine, pig and poultry meat, and meat products and fish from Croatia are safe to consume considering the determined very low chloramphenicol concentrations. Also, in accordance with a large number of determined cases in the EU in meat and fish, and shrimps from Asia, we think that there is a need for constant control of this antibiotic in imported products from this area.

Conclusion

Validated and sensitive methods, immunoenzymatic ELISA and confirmatory method of liquid chromatography- tandem mass spectrometry LC- MS/MS were used in this paper for determining remains of chloramphenicol in meat, meat products and fish. It has been proven by validation that both methods are satisfactory, i.e. that they have a quantification capability below the setpoint MRPL value of 300 ng/ kg.

Determined chloramphenicol concentrations in bovine, pig and poultry meat, meat products and fish were in the range from 0.177 to the highest value of 202.9 ng/ kg, i.e. there were no determined concentrations over 300 ng/ kg. Considering the determined low chloramphenicol concentrations, it can be concluded that meat of the controlled animals is safe to consume, i.e. the conducted control indicates that there is no illegal use of that antibiotic in the Republic of Croatia.

In accordance with the determined cases of increased concentrations in the EU in packages of meat, fish and shrimps from Asia, we consider that a constant control of imported packages from the countries of that part of the world is necessary.

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