MULTI-CLASS PROCEDURE FOR ANALYSIS OF ANTIBACTERIAL COMPOUNDS IN EGGS BY LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY

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Abstract

A liquid chromatographic method coupled with tandem mass spectrometry for determination of residues of β -lactams, macrolides, tetracyclines, quinolones, sulfonamides, and lincosamides in eggs has been described. Analytes were isolated from egg samples by solvent extraction method and extracts were cleaned by filtration on OASIS HLB cartridges. The whole procedure was validated according to European Commission Decision 2002/657/EC. The recovery ranged between 86% and 110%. The repeatability was below 16% and within-laboratory reproducibility was lower than 20%. The method was successfully applied in the official control of antibacterial compounds residue in Poland.

Key words: eggs, antibiotics, HPLC-MS/MS.

Antibiotics and some other antimicrobial compounds are widely administered to food-producing animals for the purpose of prevention or treatment of bacterial diseases. Despite the positive effects of veterinary medicaments, inadequate use of antibiotics poses a potential health risk for consumers. For this reason the European Commission has set maximum residue limits (MRLs) for antibiotics in animal tissues, milk, and eggs (5). For some antibiotics such as chlortetracycline, oxytetracycline, tetracycline, and tylosin, MRL of 200 $\mu\text{g/kg}$ in eggs have been set, whereas 150 $\mu\text{g/kg}$ has been established for erythromycin, 50 $\mu\text{g/kg}$ for lincomycin, and 500 $\mu\text{g/kg}$ for neomycin. However, for other classes of antibacterials such as β -lactams, fluoroquinolones, and sulfonamides, MRLs have not been set at all. Residues of antibiotics in eggs are controlled in National Residue Control Programme according to Council Directive 96/23 EC (6).

The screening for the presence of antibacterial compounds has been dominated by microbiological (3, 18), receptor-based (1), or immunological tests (12). They are easy to perform and inexpensive, but the main drawback of the common screening tests is their lack of specificity, thus positive results may not reveal, which antibiotic is present. Liquid chromatography coupled with UV and/or fluorometric (FL) detection has been applied for analysis of single antibiotic groups in eggs (8, 9, 16, 17). Over the past years, liquid chromatography - mass spectrometry (LC-MS) has been developed for the analysis of some groups of

antibacterial compounds in eggs (2, 7, 9-11, 13, 14, 17). Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) has become one of the most promising techniques for the analysis of antibiotics in eggs due to the determination of forbidden compounds that require limits detection as low as possible. Moreover, mass spectral analysis provides higher specificity than UV or FL detection. Furthermore, Commission Decision 2002/657/EC states that "methods based only on chromatographic analysis without the use of molecular spectrometric detection are not suitable for the use as confirmatory methods" (4).

The aim of this study was to develop a reliable and simple multi-class LC-MS/MS method for the analysis of antibiotics belonging to six different classes: β -lactams (penicillins and cephalosporins), macrolides, tetracyclines, quinolones, sulfonamides, and lincosamides. The procedure was validated according to European Commission Decision 2002/657/EC and was found suitable not only for identification of antibiotics but also for confirmation of positive results from immunochemical and microbiological screening systems used for routine control of antibiotics in eggs.

Material and Methods

Reagents. All organic solvents were HPLC grade and all chemicals were analytical grade. Acetonitrile, methanol, and sodium hydroxide were from J.T. Baker (the Netherlands). Heptafluorobutyric

acid (HFBA), trifluoroacetic acid (TFA), and pentafluoropropionic acid (PFPA) were from Sigma-Aldrich (USA). Oxalic acid and disodium versenate dihydrate (Na_2EDTA) were from POCH (Poland). Water was deionised ($>18 \text{ M}\Omega \text{ cm}^{-1}$) in-house by Millipore system (Millipore, France). The SPE copolymer cartridges OASIS HLB (3 mL/60 mg) were obtained from Waters (USA) and PVDF syringe filters (0.45 μm , 13 mm) were received from Restek (USA).

Analytical standard and standard solutions.

Amoxicillin (AMOX), ampicillin (AMPI), penicillin G (PEN G), penicillin V (PEN V), oxacillin (OXA), cloxacillin (CLOX), nafcillin (NAF), dicloxacillin (DICLOX), cephapirin (CFPI), ceftiofur (CFT), cefoperazone (CFPE), cephalexin (CFLE), cefquinome (CFQ), cefazolin (CFZ), cefalonium (CFLO), sulfaguanidine (SGU), sulfadiazine (SDZ), sulfathiazole (STZ), sulfamerazine (SME), sulfamethazine (SMT), sulfamethoxazole (SMA), sulfamethoxypyridazine (SMP), sulfamonomethoxine (SMM), sulfadoxine (SDX), sulfaquinoxaline (SQX), sulfadimethoxine (SDMX), tylosin (TYL), erythromycin (ERY), spiramycin (SPI), tilmicosin (TIL), josamycin (JOS), danofloxacin (DAN), difloxacin (DIF), enrofloxacin (ENR), ciprofloxacin (CIP), flumequine (FLU), sarafloxacin (SAR), marbofloxacin (MAR), norfloxacin (NOR), oxolinic acid (OXO), nalidixic acid (NAL), chlortetracycline (CTC), tetracycline (TC), doxycycline (DC), oxytetracycline (OTC), and sulfaphenazole (internal standard) were from Sigma-Aldrich (USA).

Stock standard solutions (1 mg mL⁻¹) were prepared by weighing 10.0 ± 0.1 mg of each standard substances, followed by quantitative transfer to a 10 ml volumetric flask and filling to volume with methanol or water. The standards of macrolides, tetracyclines, quinolones, sulfonamides, and lincomycin were dissolved in methanol, whereas β -lactams were dissolved in ultra pure water and stored at -20°C . Working standard solutions and mixed standard solutions were prepared by diluting suitable aliquot of stock standard in ultra pure water and stored at approximately 4°C .

Sample preparation and extraction. Samples were prepared from fresh eggs. Egg shell was discarded and combined yolk and albumen were homogenised at room temperature and frozen at -20°C until the time of analysis.

An aliquot (1 g) of previously homogenised whole egg was placed in a 50 ml polypropylene centrifuge tube and 50 μL of 2 $\mu\text{g mL}^{-1}$ solution of sulfaphenazole (internal standard) was added. At that time, the control samples were spiked with mixed standard solution. One ml of 0.02 M of oxalic acid (pH 4), 0.5 ml of 0.1 M Na_2EDTA and 8 ml of acetonitrile were added to the centrifuge tubes and the samples were homogenised with Vortex mixer. The mixtures were centrifuged for 10 min at $4,200 \times g$ and supernatants were cleaned by passing through an OASIS HLB cartridges. The cleaned extracts were evaporated to dryness in nitrogen evaporator at 45°C . The dry residues were reconstituted in 1 ml of 0.025% HFBA in water and filtered through 0.45 μm PVDF filters.

LC-MS/MS. An Agilent Series 1200 HPLC system (Agilent Technologies, Germany) was connected to a AB Sciex API 4000 quadrupole mass spectrometer (AB Sciex, Canada). The mass spectrometer was operated in electrospray positive ionisation mode (ESI+) and two multiple reaction monitoring (MRM) transitions were monitored in order to give four identification points in compliance with Commission Decision 2002/657/EC. The mass spectrometer settings were optimised both with direct infusion of working standard solution from a syringe pump and with LC injection. The following mass spectrometer parameters were used: resolution Q1 and Q3 = unit; nebuliser gas = 40 psi; auxiliary gas = 50 psi, curtain gas = 20 psi; collision gas = 3 psi; ion spray voltage = 5,500; temperature = 500°C . The fragmentation reactions used for monitoring were selected on the basis of their significance in products ion spectra. The Analyst 1.5.1 software controlled the LC-MS/MS system and processed the data.

The chromatographic separation was performed on a Luna octadecyl (C18) column (150 x 2.0 mm, 3 μm) (Phenomenex®, USA) coupled with octadecyl guard column (2 x 4 mm) (Phenomenex®, USA). The mobile phase consisted of solvent A: 0.025% of HFBA in water (v/v) and solvent B: acetonitrile. The elution was performed in a gradient mode; the mobile phase starting conditions were 95% of eluent A for 2 min and then decreased to 10% within 9 min. This composition was stable for 3 min and then increased to 95% of eluent A. With the following equilibration time of 10 min, the resulting total run time was 24 min. The column was operated at 35°C with a flow rate of 0.25 mL min⁻¹, and the injection volume was 30 μL .

Evaluation of the procedure. The whole procedure was validated according to recommendations of the European Commission Decision 2002/657/EC. The validation study was performed in terms of selectivity, specificity, accuracy, precision (repeatability and within-laboratory reproducibility), as well as calculation of decision limit ($\text{CC}\alpha$) and detection capability ($\text{CC}\beta$). Five-points matrix-matched calibration curves spiked at the levels 0, 0.5, 1.0, 1.5, and 2.0 times of the validation level (VL) were obtained by plotting the response of respective analyte/internal standard peak area ratio *versus* the analyte/internal standard concentration. Validation level defined in this paper as the MRL for drugs with MRL or specific level of interest for drugs without MRL equalled 40 $\mu\text{g/kg}$.

To evaluate possible interferences encountered in the method, the specificity of method was verified by analysing 20 different blank samples. Precision (repeatability and within-laboratory reproducibility) was determined by the repeated analysis ($n=6$) of egg samples spiked with analytes at concentrations corresponding to 0.5, 1.0, and 1.5 times of the validation level, from run-to run during 1 d and 3 d, respectively. Precision was evaluated by calculating the relative standard deviation (RSD) of the results obtained for each level of the target compound. The recoveries were evaluated in the same experiment as repeatability by comparing the measured concentrations to the fortified

concentrations of the samples. The $CC\alpha$ and $CC\beta$ were determined by the matrix calibration curve procedure.

Results

The developed procedure was designed to obtain qualitative and quantitative surveillance information about the antibacterial compounds belonging to different chemical groups and to analyse simultaneously the same analytical protocol. Identification was carried out by retention times, identification points of each analyte as required by the EU validation criteria, and relative ion ratio of selected MRM transitions. The optimal conditions obtained for analysis of antibacterial compounds by mass spectrometry including precursor ion (Q1), product ion (Q3), dwell time, declustering potential (DP), entrance potential (EP), collision energy (CE), cell exit potential (CXP), and retention time (RT) are listed in Table 1.

Gradient elution utilising an octadecyl chromatographic column was used for separation of multi-class drugs. At the first step of the study, trifluoroacetic acid (TFA), pentafluoropropionic acid (PFPA) and heptafluorobutyric acid (HFBA) solutions were studied to evaluate the most appropriate one. The chromatograms obtained showed that HFBA provided more symmetric peaks than the other ion-pairing reagents and the best peak shapes were obtained at 0.025% HFBA. The optimal separation of β -lactams, sulfonamides, macrolides, tetracyclines, fluoroquinolones, and lincomycin was obtained with the column Luna C18 (150 x 2.0 mm, 3 μ m) coupled with mobile phase consisted of acetonitrile and 0.025% HFBA in water.

The validation parameters were estimated on the basis of in-house validation concept in accordance with Commission Decision 2002/657/EC. Matrix calibration curves were used for quantification in order to reach a high accuracy. The basic value for the calculation of the parameters is the level of interest, *i.e.* the VL. The specificity of the method was checked by analysing blank egg samples of different origins and the specificity was 100% for all analytes, as no peak was detected in these samples at the retention time corresponding to each analyte. The recoveries were in the range between 86% and 110%. Precision (repeatability and within-laboratory reproducibility) of the procedure, as well as decision limit ($CC\alpha$) and detection capability ($CC\beta$) estimated for spiked egg samples are listed in Table 2. Fig. 1 demonstrates typical chromatograms from the analysis of antibacterial compounds belonging to β -lactams, sulfonamides, macrolides, tetracyclines, fluoroquinolones, and lincomycin (MRM 1 is presented). The stability of individual stock standard solutions of analytes stored at -20°C was maintained for at least 6 months, with the exception of penicillins, which were stable for 3 months. Working standard solutions and mixed standard solutions stored approximately at 4°C were stable for at least 1 month. The stability of the analytes in eggs matrix stored at -20°C was at least 4 weeks.

Discussion

The proper analysis of the residues of the veterinary drugs in eggs is very difficult, because of a very complicated composition of matrices of eggs. The albumen (egg white) is a polar medium with glycoproteins, while the ovum (egg yolk) is non polar because the presence of lipoproteins. Other analytical difficulties are associated with the different physico-chemical properties of the antibacterial compounds included in this study.

The significant binding between the lipoprotein and drugs contributes to poor isolation of analytes from matrices of eggs. To obtain optimal conditions for isolation of multi-class antibacterial compounds from eggs, the sample must be buffered before extraction procedure. Additionally, it must be mentioned that some classes of antibacterial compounds (tetracyclines, fluoroquinolones) can form complex with metal ions. Therefore, suitable reagents have to be included to the analytical procedure.

The extraction procedure, described in "Material and Methods", was adopted after extensive investigation of methods for the extraction of analytes of the interest from eggs. It was based on the researches by Garrido Frenich *et al.* (7) for the extraction of several classes of veterinary drugs in eggs. In our preliminary comparative studies, it was found that the use of acetonitrile extraction after treatment of sample with oxalic buffer and Na_2EDTA solution was sufficient for simultaneous isolation of penicillins, cephalosporins, macrolides, fluoroquinolones, sulfonamides, tetracyclines, and lincomycin from eggs' matrix. Only aminoglycosides, because of their polarity, were not isolated from eggs' matrix with satisfactory results, so it was decided not to include this group of antibacterial compound to the developed procedure and prepare the separate analytical protocol.

Raw extracts obtained from eggs' matrix were very dirty, so the clean-up step with solid-phase extraction cartridge was included to the analytical protocol. The sample clean-up is not complicated and makes the method fast and simple. This enabled the analysis of a large number of samples in a one working day. Sensitivity achieved by this simple process was sufficient to determine the analytes at the concentration levels of interest. Five points matrix-matched standards calibration curve ensured correct quantification of samples, if possible ion suppression effects were corrected.

Based on previous results obtained in our laboratory (15), the Luna C18 column was chosen in the present study. One liquid chromatography gradient schedule was developed to separate of β -lactams, macrolides, fluoroquinolones, sulfonamides, tetracyclines, and lincomycin. Chromatographic separation is particularly important when isobaric compounds such as sulfamethoxypyridazine and sulfamonomethoxine, sulfadoxine and sulfadimethoxine, flumequine and oxolinic acid, tetracycline and doxycycline are present. In our method, these analytes were successfully separated, as shown in Fig. 1.

Fig. 1. Chromatograms for MRM 1 of egg sample spiked with each of the 46 target drugs at 0.5 VL concentration.

Table 1
Mass spectrometry parameters for quantitative and qualitative analysis of antibacterial compounds

Analyte	RT (min)	Precursor (m/z)	Products 1/2 (m/z)	DP (V)	EP (V)	CE 1/2 (V)	CXP 1/2 (V)	Dwell (ms)
Amoxicillin	10.2	366	208/349	45	10	18/14	4/8	5
Ampicillin	10.6	350	106/160	58	5	27/19	5/15	5
Penicillin G	12.4	335	160/176	60	5	17/19	15/17	5
Penicillin V	12.8	351	160/114	54	5	17/48	15/10	5
Oxacillin	13.1	402	160/243	50	10	18/18	15/15	5
Cloxacillin	13.4	436	160/277	50	10	20/20	15/15	5
Nafcillin	13.5	415	199/171	50	10	20/50	15/15	5
Dicloxacillin	13.8	470	160/311	50	10	20/20	15/15	5
Cephapirin	10.3	424	152/124	50	10	35/70	15/15	5
Cefoperazone	11.2	646	530/143	60	10	17/50	15/15	5
Cephalexin	10.6	348	158/106	50	10	10/23	15/15	5
Cefquinome	10.3	529	134/125	50	10	25/75	15/15	5
Cefazolin	10.7	455	323/156	50	10	15/23	15/15	5
Cefalonium	10.4	459	337/152	46	5	16/28	8/15	5
Ceftiofur	11.6	524	241/125	50	10	25/70	15/15	5
Sulfaguanidine	3.1	215	156/108	20	10	20/30	15/15	5
Sulfadiazine	9.6	251	156/108	53	10	22/30	15/15	5
Sulfathiazole	10.1	256	156/108	53	10	20/34	15/15	5
Sulfamerazine	10.3	265	156/108	45	10	25/37	15/10	5
Sulfamethazine	10.8	279	156/108	50	10	25/36	15/15	5
Sulfamethoxazole	11.7	254	156/108	50	10	23/35	15/10	5
Sulfamethoxypyridazine	10.9	281	156/108	60	10	25/35	15/15	5
Sulfamonomethoxine	11.2	281	156/108	50	10	23/37	15/15	5
Sulfadoxine	11.6	311	156/108	60	10	25/40	15/15	5
Sulfadimethoxine	12.2	311	156/108	50	10	23/37	15/15	5
Sulfaquinolaxine	12.1	301	156/108	50	10	23/40	15/15	5
Tylosin	11.6	916	174/772	110	10	52/42	10/20	5
Erythromycin	11.5	734	158/576	75	10	42/27	15/15	5
Spiramycin	10.9	843	174/540	120	10	52/44	16/14	5
Tilmicosin	11.2	869	174/696	135	10	61/56	17/18	5
Josamycin	12.2	828	174/229	80	10	46/44	16/14	5
Danofloxacin	10.7	358	340/255	60	10	33/50	15/15	5
Difloxacin	11.0	400	382/356	50	10	30/28	9/8	5
Enrofloxacin	10.8	360	342/286	72	10	30/50	15/15	5
Ciprofloxacin	10.7	332	314/231	61	10	30/47	15/15	5
Flumequine	12.9	262	244/202	44	10	25/45	15/15	5
Sarafloxacin	11.0	386	368/348	50	10	31/46	8/8	5
Marbofloxacin	10.6	363	345/320	70	10	30/22	15/15	5
Norfloxacin	10.6	320	302/231	60	10	33/50	15/15	5
Oxolinic acid	11.9	262	244/216	53	10	25/40	15/15	5
Nalidixic acid	12.7	233	215/187	42	10	30/35	15/15	5
Chlortetracycline	11.1	479	444/462	56	10	31/25	14/16	5
Tetracycline	10.8	445	410/427	36	10	27/19	12/18	5
Doxycycline	11.2	445	428/154	55	10	25/42	15/15	5
Oxytetracycline	10.7	461	426/443	41	10	27/19	14/20	5
Lincomycin	10.4	407	126/359	74	10	36/28	7/8	5
Sulfaphenazole (IS)	12.2	315	156	50	10	30	15	5

RT – retention time, DP – declustering potential, FP – focusing potential, CE – collision energy, CXP – cell exit potential.

Table 2
Validation parameters for antibiotics in spiked egg samples at 1 VL

Analyte	MRL/VL ($\mu\text{g/kg}$)	Repeatability (%)	Within-laboratory reproducibility (%)	CC α ($\mu\text{g/kg}$)	CC β ($\mu\text{g/kg}$)	Recovery (%)
Amoxicillin	40	15.9	18.5	30	43	97
Ampicillin	40	9.9	14.2	30	42	103
Penicillin G	40	7.7	11.9	28	34	94
Penicillin V	40	8.2	15.5	30	41	87
Oxacillin	40	12.7	14.8	30	39	90
Cloxacillin	40	15.3	17.3	30	41	88
Nafcillin	40	13.8	18.3	29	38	86
Dicloxacillin	40	12.6	13.0	28	37	101
Cephapirin	40	13.9	19.4	29	42	102
Cefoperazone	40	11.9	14.6	30	41	102
Cephalexin	40	7.5	10.3	27	34	108
Cefquinome	40	11.2	14.8	27	36	105
Cefazolin	40	14.6	16.5	28	38	103
Cefalonium	40	10.9	17.2	27	38	102
Ceftiofur	40	7.8	11.1	28	36	106
Sulfaguanidine	40	10.0	10.3	27	35	102
Sulfadiazine	40	12.4	15.9	29	39	102
Sulfathiazole	40	10.8	12.5	25	33	105
Sulfamerazine	40	13.6	15.1	28	39	105
Sulfamethazine	40	11.2	12.4	27	35	102
Sulfamethoxazole	40	10.6	12.3	25	33	103
Sulfamethoxypyridazine	40	9.8	11.4	29	39	105
Sulfamonomethoxine	40	9.7	13.7	28	37	103
Sulfadoxine	40	8.5	13.8	26	34	110
Sulfaquinoxaline	40	9.3	10.0	26	32	100
Sulfadimethoxine	40	11.0	12.3	26	33	101
Tylosin	200	9.2	11.5	231	275	100
Erythromycin	150	10.3	11.8	178	223	107
Spiramycin	40	11.0	11.7	26	31	99
Tilmicosin	40	13.3	14.8	25	33	103
Josamycin	40	9.1	15.8	28	36	98
Danofloxacin	40	11.6	14.1	27	36	103
Difloxacin	40	12.3	16.9	28	38	103
Enrofloxacin	40	11.3	14.5	27	36	103
Ciprofloxacin	40	13.3	13.5	28	37	104
Flumequine	40	9.3	14.5	26	34	98
Sarafloxacin	40	14.4	14.6	27	35	103
Marbofloxacin	40	12.7	16.8	27	36	103
Norfloxacin	40	14.0	15.2	27	37	104
Oxolinic acid	40	10.1	12.9	26	34	107
Nalidixic acid	40	7.0	12.0	25	31	101
Chlortetracycline	200	9.8	12.8	232	285	102
Tetracycline	200	8.1	12.8	235	281	101
Doxycycline	40	11.6	17.6	28	39	103
Oxytetracycline	200	9.5	9.6	238	277	103
Lincomycin	50	11.3	15.4	58	75	105

The procedure was successfully validated according to Commission Decision 2002/657/EC. The method is implemented in the National Residue Control Programme in Poland for confirmation of positive results from immunochemical and microbiological screening tests and for routine control of antibiotics in eggs (about 50 samples per year is analysed by this procedure). Among the samples analysed in 2011, three samples contained doxycycline or enrofloxacin. This was confirmed using the criteria described in "Material and Methods". Since doxycycline and enrofloxacin are forbidden to use in laying hens, the obtained results indicate that these samples might have been derived from farms using inappropriate drugs.

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