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OPTIMIZATION AND VALIDATION OF CAPILLARY ELECTROPHORETIC METHOD FOR THE ANALYSIS OF AMPHENICOLS IN POULTRY TISSUES

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Abstract: A simple, rapid capillary electrophoretic (CE) method for simultaneous analysis of three amphenicols (chloramphenicol, thiamphenicol and florfenicol) in poultry tissues has been developed and validated. The separation condition were: buffer solution composed with 25 mM sodium tetraborate decahydrate and 10 mM sodium dodecylsulfate, an uncoated fused-silica capillary (57 cm × 75 μm i.d.), voltage 20 kV, and temperature 22°C. The method involved simple deproteinization by acetonitrile and SPE extraction procedure. The analytical method was validated according to the FDA bioanalytical method guidance. The method was linear ($r > 0.999$) at concentrations ranging from: 0.005 – 1 for chloramphenicol, 0.01 – 1 for thiamphenicol, and 0.025 – 5 μg/g for florfenicol. The precision values were less than 9.8 for intra- and 14.8% for inter-day variability, and accuracies ranged from 92.0 to 106.0% for analyzed amphenicols. The overall recoveries of all antibiotics from tissue samples were above 82.2%. Some new parameters were calculated as limit of decision (CCα) and detection capability (CCβ). The CE method is simple and reliable for simultaneous determination of residues of chloramphenicol, thiamphenicol and florfenicol in muscle with a total run time of less than 7 min.

Keywords: Capillary electrophoresis, amphenicol antibiotic residues, muscle, solid-phase extraction

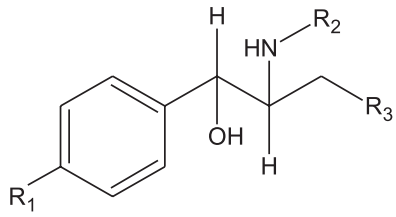
The widespread use of antimicrobial agents in animal husbandry may lead to the presence of the drugs and their metabolites in foodstuffs, which is a problem from the viewpoint of public health. These substances may be directly toxic or cause resistant human pathogens and possible allergic reaction in humans. To ensure the safety for consumers, the European Union (UE) has set a definitive maximum residual limit (MRL) for the drug residues and minimum required performance limit (MPRL) for banned in veterinary medicine substances, in food products of animal origin. Therefore, a sensitive and reliable methods are needed for the routine monitoring and detection of these antimicrobial residues in products of animal origin.

Chloramphenicol (CAP) and its two analogues: thiamphenicol (TAP) and florfenicol (FF) have a board-spectrum activity against both Gram-positive and Gram-negative bacteria as well as other groups of microorganisms. Amphenicols exert their action through protein inhibition and are effective in the treatment of several infectious diseases, therefore they are often used for therapeutic and prophylactic purposes in veterinary medicine as well as to enhance feed efficiency and promote animal growth (1, 2). TAP is an analogue of CAP, in which the *p*-nitro group at the benzene ring is replaced by a methylsulfonyl group, while FF is a fluorinated derivative of CAP and TAP, which has a fluorine atom instead of the hydroxyl group located at C-3. Their structural formula are presented in Figure 1. Because CAP has adverse reactions and side effects on humans, such as aplastic anemia, its clinical use was discontinued in many countries (3). TAP and FF appear to be viable substitutes for CAP in veterinary medicine, because the nitro group, which is responsible for hematological side effect, is absent.

There are only a few methods for simultaneous determination of amphenicols in biological matrix, mainly using liquid chromatographic techniques, coupled with ultraviolet absorbance (UV) detection (4) or mass spectrometry (MS) (5, 6). Measurements of these residues with gas chromatography (GC) were carried out also using electron capture detection (ECD) (7) and MS (8, 9). Unfortunately, the mentioned methods present sometimes a lack of selectivity, a need of efficient matrix cleanup procedures or a very selective detector. Moreover, some of them involve a time consuming derivatization step and expensive instrumentation. In recent years, methods using electrokinetic capillary chromatography (MEKC) for the determination of three amphenicol antibiotics in pharmaceutical prepara-

Table 1. MPRL and MRL values for amphenicol antibiotics for selected animal species in force in the EU.

Substances	Species	MPRL (µg/kg)	Tissue
CAP	Poultry and porcine	0.3	All edible tissues
		MRL (µg/kg)	
TAP	Poultry and porcine	50	All edible tissues
FF	Poultry	100	Muscle,
		200	Skin and fat,
		750	Kidney
		2500	Liver
	Porcine	300	Muscle,
		500	Kidney, skin and fat
		2000	Liver



	R_1	R_2	R_3
Chloramphenicol	$-NO_2$	$-COCHCl_2$	-OH
Thiamphenicol	H_3C-SO_2-	-H	-OH
Florfenicol	H_3C-SO_2-	$-COCHCl_2$	-F

Figure 1. Chemical structures of chloramphenicol, thiamphenicol and florfenicol.

tions (10) and bovine milk as a biological matrix (11) have been described. No CE methods have yet been applied to the amphenicol residue analysis in edible animal tissues.

In this paper, a CE method with UV detection was developed to separate and quantitate three antibiotics (chloramphenicol, thiamphenicol and florfenicol) in poultry muscle. The present method was fully validated and the detection limits obtained in this work are low enough to determine concentrations below the permissible MRL in animal products for analyzed antibiotics in force in the EU (Table 1).

EXPERIMENTAL

Chemicals and standards solutions

All reagents were analytical-reagent grade and were purchased from Merck (Darmstadt, Germany). Electrophoretic buffer solutions were prepared using sodium tetraborate decahydrate, sodium dodecylsulfate (SDS) and triple distilled water. Acetonitrile and methanol (analytical grade) were supplied by Merck (Darmstadt, Germany). FF, TAP and flumequine (internal standard) were obtained from Vetos-Farma (Bielawa, Poland), while CAP was purchased from Pliva (Kraków, Poland). A stock standard solutions (1mg/mL) of the compounds were prepared in sodium tetraborate decahydrate buffer (0.01 M) and stored in the dark at temp. below 4°C. Mixed working solutions at level of 1.0 µg/mL and 10.0 µg/mL were prepared fresh daily in deionized water.

Sample preparation

Samples (muscle from poultry) used for the method validation came from experimental farm (untreated animals), while the commercial samples were obtained from local supermarket (Gdańsk, Poland). The samples were stored in different containers and kept frozen until processing. Thawed muscle samples were homogenized using a Polytron TM (Germany), at high speed for 3 min and an accurately weighed 3 g amount. After fortification, equilibration and addition of the internal standard (flumequine), the samples were extracted with 6.0 mL of acetonitrile. The homogenate was centrifuged, decanted and evaporated to dryness in water bath at 40-45°C. After reconstitution with 3

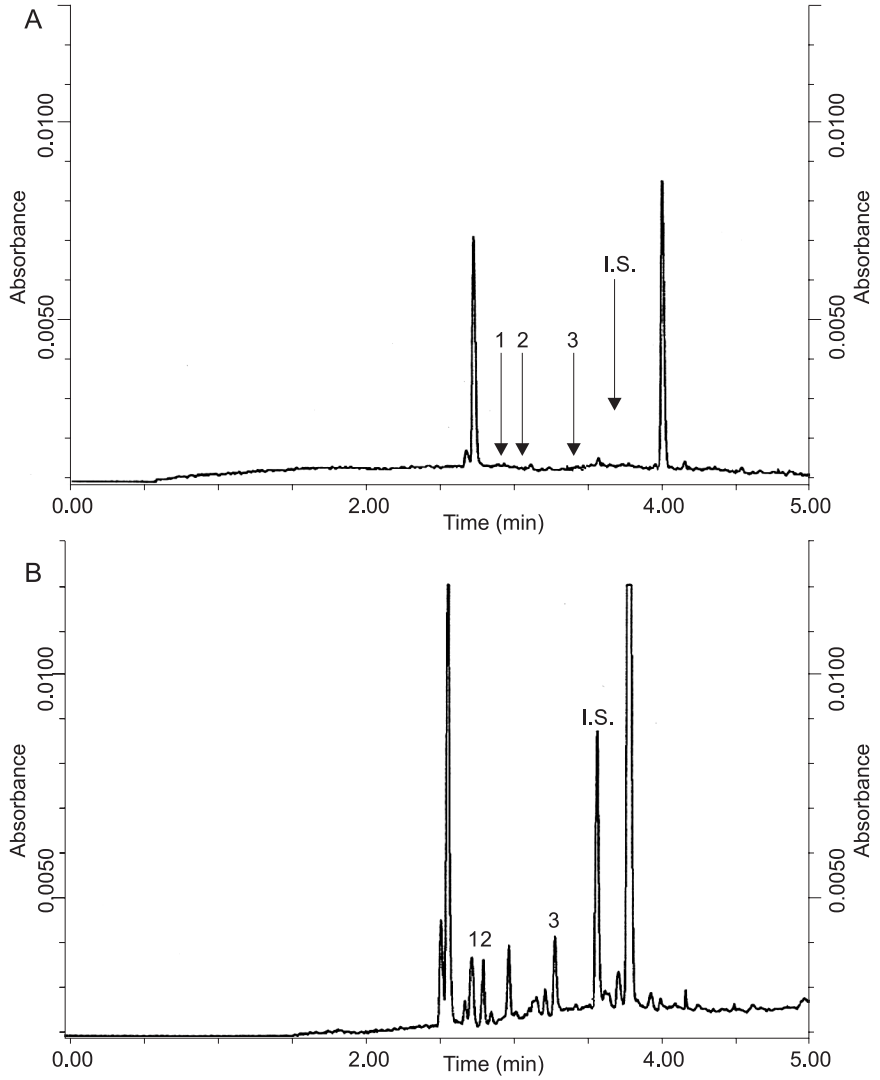


Figure 2. (**A**) Electropherogram of drug-free animal tissue. (**B**) Electropherogram of blank animal tissue spiked with florfenicol (**1**), thiamphenicol (**2**), chloramphenicol (**3**) at the concentration of 150 ng/g and flumequine as internal standard (1000 ng/g) (**I.S.**).

mL of water, these extracts were applied to a C-18 solid-phase extraction (SPE) column and washed with 2 mL of deionized water, and eluted with 300 µL of methanol. The eluate was evaporated to dryness in water bath at 40 – 45°C. Finally, the residue was reconstituted with 200 µL of 2 mM sodium tetraborate decahydrate and centrifuged at 8000 *g*. The supernatant was stored at -20°C until the time of analysis.

Instrumentation

The experiments were performed on Beckman P/ACE 2100 (Fullerton, CA, USA) system, equipped with an autosampler, a fluid-cooled capillary cartridge and UV detector. Separations were carried out using fused-silica capillary of 75 µm internal diameter and 57 cm total length (51 cm effective length). Quantitative analysis was conducted at 20 kV, in order to keep the total current below 50 µA. All experiments were performed at 22.0 ± 0.1°C. Injections were performed hydrodynamically into the capillary at anode via a 2 s pneumatic injection at 3.45 kPa argon pressure. The best separation of analytes was obtained using a buffer solution, composed of 25 mM SDS and 10 mM sodium tetraborate decahydrate.

Validation of method

Calibration was performed by adding known amounts of CAP, TAP and FF to blank edible ani-

Table 2. Summary of precision and validation data for CAP, TAP and FF obtained with CE calibrations.

	CAP	TAP	FF
Linearity range (μg/g)	0.005 – 1	0.01 – 1	0.025 – 5
Sample linearity: slope	2.430 (± 0.0028)	2.049 (± 0.0051)	1.695 (± 0.0021)
intercept	0.0023 (± 0.0012)	-0.0025 (± 0.0022)	0.007 (±0.0042)
Correlation coefficient (r)	0.999	0.999	0.999
Standard error	0.003	0.004	0.010
n	8	7	8
LOD (ng/g)	1.5	3.2	7.4
LOQ (ng/g)	4.9	10.7	24.8
CCα, (ng/g)	5.1	2.6	15.8
CCβ, (ng/g)	7.1	6.2	22.7

Table 3. Assay validation results obtained from within-run and between-day experiments for CAP, TAP and FF.

Nominal concentration (ng/g)	Within-run			Between-run			Overall recovery (%)	Overall RSD (%)
	Concentration found (n = 6) (ng/g)	Precision RSD (%)	Accuracy (%)	Concentration found (n = 6) (ng/g)	Precision RSD (%)	Accuracy (%)		
CAP								
10	9.2 ± 0.9	9.8	92.0	10.6 ± 1.6	14.8	106.0		
50	50.4 ± 3.3	6.6	100.8	49.2 ± 3.5	7.2	98.4	85.4 ± 5.5	6.4
250	251.2 ± 7.6	3.0	100.7	250.0 ± 11.2	4.5	100.0		
TAP								
10	9.3 ± 0.9	9.7	93.0	10.1 ± 1.5	14.5	101.0		
50	46.8 ± 3.6	7.7	93.5	49.8 ± 4.5	9.1	99.6	82.2 ± 4.4	5.3
250	243.9 ± 11.3	4.6	97.6	250.0 ± 13.6	5.5	100.0		
FF								
50	47.4 ± 4.3	9.1	94.8	48.0 ± 6.1	12.8	96.0		
250	255.7 ± 16.9	6.6	102.3	253.9 ± 11.8	4.6	101.6	86.5 ± 4.9	5.7
1000	1010.6 ± 25.6	2.5	101.1	1010.0 ± 35.1	3.5	101.0		

mal tissue to yield concentrations over the range 0.005 – 1, 0.01 – 1, 0.025 – 5 μg/g for CAP, TAP and FF, respectively, and flumequine (internal standard) at the concentration of 1 μg/mL. These standards were then extracted according to the above procedure. Selectivity of the method was determined by comparing the electropherograms corresponding to the samples of blank muscle tissues and revealed no peak interfering with analyzed antibiotics and internal standard (n = 6). Intra- and inter-day precision and accuracy of the assay have been evaluated by the analysis of quality control (QC) samples at each concentration level (0.01, 0.05; 0.25 for CAP and TAP; 0.05; 0.25; 1 for FF, respectively) on the same day (within-day) and on ten different days (between-day variability). The precision was determined and expressed as relative standard deviation (RDS%). Accuracy of the method was defined as an agreement of obtained assay result with the nominal concentration value. The recovery was calculated by comparing assay of concentrations obtained from standard solutions and from animal muscle samples (n = 6). The limit of detection (LOD) and quantification (LOQ) of antibiotics were determined based on the standard deviation (SD) of the detector response (σ) and the slope (S). LOD = 3σ / S, LOQ = 10σ / S. The value of σ was calculated based on the experimental calibration curves being the SD of y – intercepts of the regression line. The decision limit (CCα) means the limit at and above which it can be concluded with an error probability of α that a sample is non-compliant. Detection capability (CCβ) means the smallest content of the substance that may be detected, identified and/or quantified in a sample with an error probability of β. For MRL substances the α error and the β error are 5%. Decision limit (CCα) and detection limit (CCβ) were evaluated according to ISO 11483-2 (12). Additionally, test of freeze-thaw stability of

all antibiotics in poultry tissues has been performed using quality control samples at the concentrations of analyzed compounds measuring three replicates at each level during 2 months.

RESULTS AND DISCUSSION

The method has been validated for linearity, precision, accuracy, selectivity, recovery and stability. The linearity of the method was evaluated by plotting calibration curves of peak height ratios of CAP, TAP and FF, respectively, to internal standard (flumequine) against the known concentrations of analyzed substances and calculated by weighted (1/x) linear regression analysis. Calibration curves were linear over a concentration range of 0.005 – 1; 0.001 – 1, and 0.025 – 5 µg/g for CAP, TAP and FF, respectively. The high values of correlation coefficients ($r > 0.999$) indicate an excellent linearity of the method in the considered concentration range (Table 2). The detection and quantitation limits determined were 1.5 and 4.9 ng/g for CAP; 3.2 and 10.7 ng/g for TAP, 7.4 and 24.7 ng/g for FF, respectively. After the analysis, the $CC\alpha$ values were established as 5.1, 2.6, and 15.8 ng/g, whereas $CC\beta$ values were found to be 7.1, 6.2, and 22.7 ng/g for CAP, TAP and FF, respectively. The intra- and inter-day precision and accuracy of the assay are presented in Table 3. The low RSD values below 9.8, 9.7 and 9.1% (within-day) and 14.8, 14.5 12.8% (between day) for CAP, TAP and FF, respectively, confirmed the good precision of the method. The intra- and inter-day accuracies were better than 92.0 and 96.0% for the analyzed antibiotics, respectively. The typical electropherograms of extract of blank animal tissue samples after extraction and extract of animal tissue samples spiked with CAP, TAP, FF at the concentration of 150 ng/g and internal standard (flumequine – 1000 ng/g) are presented in Figure 2 A and B. No interference was observed in the region of interest where the analytes were eluted which indicates that the reported method is selective. The migration times for FF, TAP, CAP and flumequine (I.S) were 2.7, 2.8, 3.3 and 4.1 min, respectively. The absolute mean recoveries for all amphenicols and internal standard in muscle samples were above 82.2%. The test of freeze-thaw stability of the analyzed antibiotics confirmed no significant degradation of compounds in animal tissue samples. All the samples were stable under the storage conditions.

As an application, 24 poultry tissue (muscle, kidney, liver and skin) samples purchased from different local markets were investigated using the proposed method. No sample contained detectable concentration of the analyzed drugs.

The results obtained in this work confirm that the CE method, when properly optimized and validated, is adequate to be used for identification and quantification of amphenicols in meat samples. Since the animal tissue samples are very complex mixtures with a variety of substances, the analytes have to be separated from matrix components before their electrophoretic separation. In this work a simple procedure for the isolation and extraction of amphenicol antibiotics from poultry muscle with different organic solvents followed by extract cleanup was investigated. The recoveries of the analyzed drugs were satisfactory using acetonitrile for deproteinization and solid phase extraction (SPE) technique with C 18 column to complete cleanup procedure of samples. Moreover, the time required for processing the sample was greatly shortened in comparison with tested liquid-liquid extraction.

Separation and determination of FF by conventional capillary zone electrophoresis (CZE) is difficult because it contains no functional groups which could be ionized in pH range between 2 and 12 (10). Therefore, to increase the selectivity of electrophoretic separation as well to improve the resolution, the micellar electrokinetic chromatography (MEKC) with SDS as surfactant, was chosen as optimal separation mode of CE. Because citrate and phosphate buffer solution can catalyze of amphenicol hydrolysis, a borate buffer was employed as electrolyte solution.

In spite of the fact that drug residue analysis of foodstuffs is currently performed predominantly using LC methods, many pharmaceutical analysis laboratories have an increasing CE instrumentation. Application of LC are limited by UV absorption properties of amphenicols, which require a low wavelength detection (200 nm), at which most of the organic solvents in mobile phase absorb UV light very well. Because water solutions are employed for separation in CE, it is possible to use low wavelength UV detection. In comparison with LC methods, the CE technique can offer benefits in term of significantly reduced total analysis time, lesser consumption of solvents and sample.

CONCLUSION

In this work a specific, precise and reliable method for the rapid determination of three amphenicol antibiotics in poultry tissues was developed and validated. The CE technique with UV detection is sensitive enough to TAP and FF identi-

fication below MRL values established by the EU. Likewise, the proposed method does not require time consuming, complex extraction and derivatization procedures, and may be suitable for routine screening investigation of foodstuffs.

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