CROATICA CHEMICA ACTA
CCACAA, ISSN 0011-1643, e-ISSN 1334-417X
Croat. Chem. Acta **82** (3) (2009) 685–694.
CCA-3361

Original Scientific Paper

Optimized Method for Determination of Amoxicillin, Ampicillin, Sulfamethoxazole, and Sulfacetamide in Animal Feed by Micellar Electrokinetic Capillary Chromatography and Comparison with High-Performance Liquid Chromatography

Rade Injac,* Nina Kočevar, Borut Štrukelj

Faculty of Pharmacy, The Chair of Pharmaceutical Biology, University of Ljubljana, Askerceva 7, 1000 Ljubljana, Slovenia

RECEIVED APRIL 6, 2008; REVISED JUNE 30, 2008; ACCEPTED SEPTEMBER 16, 2008

Abstract. Antibiotics as additives in animal feedstuffs are forbidden in many countries in the world, but they are still abused. A micellar electrokinetic capillary chromatography method was performed at 25 °C and 30 kV (under pressure 15 mbar) using 25 mmol dm^{-3} phosphate buffer (pH = 8.0) containing 70 mmol dm^{-3} sodium dodecysulfate (SDS) and 10 % (volume fraction) methanol as the background electrolyte for separation and determination of amoxicillin, ampicillin, sulfamethoxazole, and sulfacetamide. UV detection was set at 210 nm. The method was validated and antibiotics were quantitatively determined as additives in spiked animal feedstuffs. Results were compared with a new HPLC method for the evaluation of four antibiotics in real samples. Both developed methods can be used for routine analysis of amoxicillin, ampicillin, sulfamethoxazole, and sulfacetamide as additives in animal feedstuffs.

Keywords: amoxicillin, ampicillin, sulfamethoxazole, sulfacetamide, micellar electrokinetic capillary chromatography, high-performance liquid chromatography

INTRODUCTION

Penicillins and sulfonamides are widely used antibiotics in today's human and veterinary medicine practice. Amoxicillin (AMO) and ampicillin (AMP) are β-lactam antibiotics that belong to the group of penicillins. They are semi-synthetic, broad-spectrum, acid stable, orally absorbed antibiotics that inhibit bacterial cell synthesis and are normally used for the treatment of common bacterial infections both in humans and animals.[1] They are normally the only penicillins added to feedstuffs at the maximum level of 500 mg kg^{-1}.[2]

Sulfamethoxazole (SMX) and sulfacetamide (SAC) are N-substituted derivatives of sulfanilamide and compete with p-aminobenzoic acid in enzymatic synthesis of dihydrofolic acid. This leads to a decreased synthesis of nucleic acids.[3] SMX and SAC have been widely used for both prevention and treatment of diseases and as feed additives to promote growth in animal feeding operations and concentrated animal feeding operations. Sulfonamides as additives are used at the level of 100 mg kg^{-1}.[4]

In 1999, the European Union has forbidden the use of these antibiotics as additives in animal feed,[5] but they are still in use in the non-EU countries.

Simultaneous extraction and analysis of tetracycline and sulfonamide by solid-phase extraction and liquid chromatography electrospray ionization tandem mass spectrometry (LC/EI/MS) in water was reported[6] as well as analysis of sulfonamide residues in honey by LC-fluorescence detection.[7] Salem *et al.*[8] used NMR spectroscopy to analyze SMX in pharmaceuticals and urine. High-performance liquid chromatography (HPLC) was used to determine SMX and SAC in bovine milk,[9] honey,[10] and ophthalmic preparations.[11] AMO and AMP were separated and determined by thin-layer chromatography (TLC) in pharmaceuticals,[12,13] reverse-phase liquid chromatography (RPLC) in human plasma,[14] LC with fluorescence detection in animal tissues,[15] LC/EI/MS/MS in honey and bovine milk,[16,17] solid-phase fluorescence immunoassay (SPFIA) in bovine and porcine kidneys,[18] LC/UV-DAD in water,[19] LC/MS/MS in bovine muscle, kidney and milk,[1] LC in feed,[20] micellar enhanced spectrophotometric

* Author to whom correspondence should be addressed. (E-mail: injacrade@gmail.com)

determination,[21] and by LC/EI/MS as a strategy for quality control.[22]

The commonly used CE modes in determination and quantification of antibiotics from different matrices are capillary zone electrophoresis (CZE) and micellar electrokinetic capillary chromatography (MEKC), which is efficient for separation of both ionic and neutral analytes. A screening method for analytical control of antibiotic residues in water by MEKC was developed for eight penicillins including AMO and AMP.[23] Determination of SAC was reported in pharmaceuticals and animal feedstuffs with another compound by MEKC[24–26] as well as SMX in pharmaceuticals and human serum by MEKC[27,28] and pharmaceuticals by CZE.[29] Yongxin *et al.*[30] used both CE methods for the analysis of AMP and its degradation products.

We studied influence of the applied pressure, surfactant's concentration, and organic modifier on separation of AMO, AMP, SAC, and SMX, and validated the method. The final aim of our study was to optimize the MEKC method, which was used in our previously published paper[31] and to compare it with a new HPLC method for the evaluation of four antibiotics as additives in animal feedstuffs. The model mixture is also interesting since β-lactams (penicillins and cephalosporins) have been reported to demonstrate antimicrobial synergy with a variety of sulfonamides (penicillins and other β-lactams are examples of bactericidal drugs; sulfonamides are bacterostatics),[32,33] and, as such, the prophylactics in feed could be covered with these representative drugs.

EXPERIMENTAL

Instrumentation and Operating Conditions

An Agilent 3D-CE capillary electrophoresis system (Waldbronn, Germany) with a diode-array detector and HP ChemStation software was used to perform MEKC. Compounds were determined on a 56 cm (50 cm to the detector) × 50 μm i.d. fused silica capillary (with bubble cell, 150 μm) (Agilent, Waldbronn, Germany).

The capillary was conditioned prior to its first use by flushing with 0.1 mol dm^{-3} NaOH for 20 min and then with water for 10 min. In the optimized method, the capillary was conditioned at the beginning of each day with methanol under high pressure (930–950 mbar) for 3 min, water for 1.0 min, and then rinsed for 2 min with 0.1 mol dm^{-3} NaOH, and 3 min with the background electrolyte (BGE). This was followed by a hydrodynamic sample injection at 600 mbars (injection time was 30 s; pressure was 20 mbar). Determination was performed at 30 kV and 25 °C (under applied pressure of 15 mbar) in 10 min; under these conditions the current was 49–50 μA. UV detection was at 210 nm. Peak area was used for the quantification.

HPLC analyses were performed using a Knauer HPLC system: a Well Chrom K-2500 detector, a Well Chrom K-501 pump, and a Knauer degasser (Knauer Wissenschaftliche Gerätebau, Berlin, Germany), equipped with an EuroChrom® 2000 Basic Edition software, version 2.05. A Chromolith® Performance RP-1 endcapped column, 4.6 mm × 100 mm (Merck, Darmstadt, Germany) was used and detection was performed at 220 nm. The optimal operating conditions: injection volume 10 μL, flow rate 3 mL min^{-1}, mobile phase A distilled water with 0.1 % trifluoroacetic acid (TFA), mobile phase B 100 % acetonitrile with 0.1 % TFA, elution gradient 0–1 min 100 % A, 1–4 min 100–89 % A, 4–5 min 89 % A, 5–10 min 89–88 % A, 10–11 min 88–100 % A, 11–13 min 100 % A.

A Metrohm 691 pH meter by Herisau (Switzerland) was used for the pH measurement.

Reagents and Chemicals

All solvents and reagents were of analytical grade unless indicated otherwise. Solutions were prepared with deionized water (Milli-Q-quality). Acetonitrile, methanol, and TFA (HPLC grade) were obtained from Sigma (Deisenhofen, Germany). Amoxicillin (· $3H_2O$) was obtained from Krka (Novo mesto, Slovenia), ampicillin sodium and sulfamethoxazole were from Sigma (Deisenhofen, Germany), and sulfacetamide sodium was obtained from Vetprom (Belgrade, Serbia). The quality of the all standards was of BP requirements.

Buffer solutions were prepared by dissolving the appropriate amount of NaH_2PO_4 and/or $Na_2B_4O_7$ in deionized water and the pH was adjusted with NaOH or HCl. $NaH_2PO_4 \cdot H_2O$ and $Na_2B_4O_7 \cdot 10H_2O$ were from Kemika d.d. (Zagreb, Croatia). Sodium dodecylsulphate (SDS) was from Riedel-de Haën AG (Seelze, Germany). The BGE was 25 mmol dm^{-3} phosphate buffer, pH = 8.0, containing 70 mmol dm^{-3} SDS and 10 % (volume fraction) methanol.

Animal diet and feedstuff mixtures for cow K-19 (protein, crude fat, crude fiber, calcium, phosphorus, sodium, vitamins A, D_3, E, zinc, manganese, selenium, cobalt, copper, iron, and iodine), pigs from 25 to 60 kg BEK-1 (protein, crude fiber, vitamins A, D_3, E, B-complex, zinc, selenium, manganese, iodine, cobalt, copper, iron, and antioxidants Sanox), and chicken feed NSK-1 (protein, crude fiber, vitamins A, D_3, E, K_3, B-complex, zinc, selenium, manganese, iodine, cobalt, copper, iron, carophyll red, and antioxidants Sanox) were manufactured by JATA EMONA (Ljubljana, Slovenia).

Preparation of Standard Stock Solutions

Stock solutions of antibiotics were prepared by weighing 50 mg of drugs and dissolving in 50 mL of methanol. The stock solutions were diluted with the BGE for MEKC to obtain the concentration ranges required (1–300 mg L^{-1} for each drug).

The stock standard solutions were diluted with methanol to give 100 mg L^{-1} concentration of AMO, AMP, SAC, and SMX and were injected onto the HPLC column (2.5–15 μL). The peak area responses were obtained.

A method of the standard calibration was used. Linear standard curves for AMO, AMP, SAC, and SMX were obtained separately by plotting concentration versus area (for both MEKC and HPLC method).

The solutions were stored under refrigeration until use: They were stable for 7 days.

Sample Preparation and Extraction

Spiked animal feed was prepared by grinding 500 g of feedstuff and adding 50 mg of each antibiotic. Blank feed or spiked feed with added antibiotics were weighed (100 g) and extracted with methanol (5 × 20 mL), first by shaking, then in an ultrasonic bath for 15 min. The extracts were combined, filtered (nylon 0.2 μm), transferred to 100 mL volumetric flask, and filled up with methanol. Different known aliquots (10–750 μL) were placed in 1.5 mL calibrated vials and filled up to volume (by an automatic pipette) with the BGE (MEKC) or methanol (HPLC).

RESULTS AND DISCUSSION

Preliminary Studies

The MEKC method has been employed for the analysis of neutral and ionic drugs in a number of publications. The commonly used real samples were pharmaceuticals. There are numerous recently published papers with penicillins and sulfonamides in different matrices, but not in animal feedstuffs.[34]

To optimize the determination, a preliminary study was carried out using a solution containing 100 mg L^{-1} of each compound.

Influence of pH

Determination was carried out at different pH values (5−10). The influence of pH was examined over the range of 5.0−10.0 by using phosphate buffer and/or borate buffer in different ratios as electrolytes in deionised water and adjusting with HCl or NaOH to the required pH. The results show that determination was best with the phosphate buffer at pH 8.0 with BGE containing SDS.

Influence of an Organic Modifier

Peaks of antibiotics in preliminary studies were overlapped and showed shoulders. Also the symmetry of the peaks was not good. The addition of different organic modifiers can be essential for the purity of peak. Methanol, ethanol, and acetonitrile were tested in concentrations from 0 to 15 %. The presence of 10 % (volume fraction) methanol in the BGE resulted in better symmetry of peaks and shoulders disappeared.

Influence of Phosphate and SDS Concentration

The phosphate buffer molarity varied from 5 to 40 mmol dm^{-3} using the experimental conditions mentioned above. With a 25 mmol dm^{-3} concentration, suitable peak shape was achieved. Influence of SDS concentration in the BGE on migration time (n = 6) is given in Figure 1a. The results demonstrate that SDS concentration influences the mobility of AMO, AMP, SLA, and SLX (10 − 120 mmol dm^{-3}). A concentration of 70 mmol dm^{-3} was selected for the further experiments (Figure 1b) to give the best shape and symmetry of the peak and resolution (AMO/AMP 7.0, AMP/SMX 2.8., and SMX/SAC 2.4).

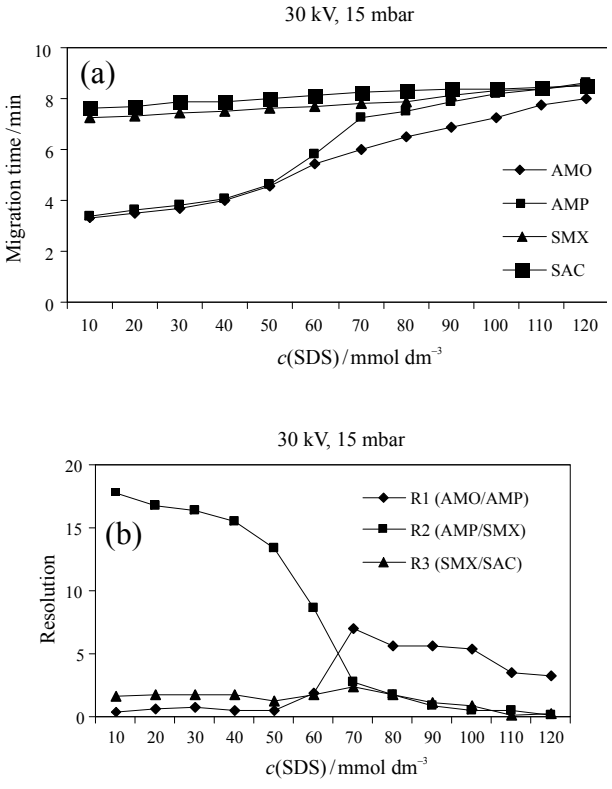

Figure 1. Effect of SDS concentration on: migration time (a) and resolution (b). The BGE was 25 mmol dm^{-3} phosphate buffer, pH = 8.0, containing 70 mmol dm^{-3} SDS and 10 % (vol. fraction) methanol, the temperature and voltage were 25 °C and 30 kV, respectively, with the applied pressure 15 mbar.

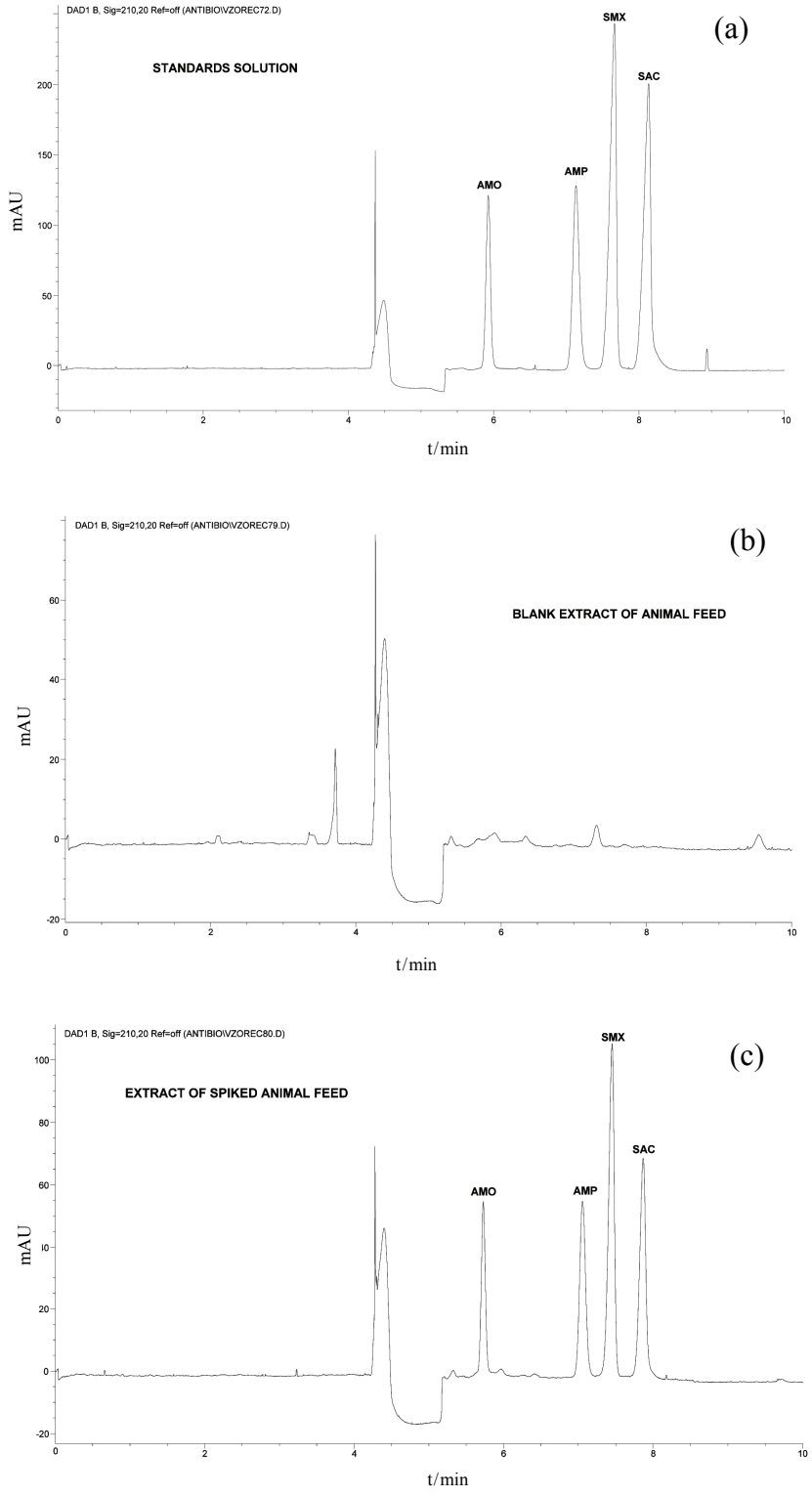

Figure 2. Electropherogram obtained for (a) 0.25 mg mL^{-1} of AMO, AMP, SMX, and SAC as a standard solution, (b) blank animal feedstuff (mixtures for cow K-19), and (c) 0.1 mg mL^{-1} of AMO, AMP, SMX, and SAC from spiked animal feedstuff; under the optimized conditions, at 210 nm. The BGE was 25 mmol dm^{-3} phosphate buffer, pH = 8.0, containing 70 mmol dm^{-3} SDS and 10 % (volume fraction) methanol, the temperature and voltage were 25 °C and 30 kV, respectively, with the applied pressure 15 mbar.

Influence of Running Voltage, Applied Pressure, and Temperature

Running voltage effects in the range of 5−30 kV were tested using 25 mmol dm^{-3} of phosphate buffer containing 70 mmol dm^{-3} sodium dodecylsulphate and 10 % (volume fraction) methanol, pH = 8, without running pressure, at 25 °C. The best results were at 30 kV and an acceptable level of baseline noise was achieved by performing experiments at 25 °C.

The applied pressure was tested in the range of 0−30 mbar using the above experimental conditions. Migration times decreased with increasing the applied pressure. 15 mbar of pressure can be selected as optimum. It gives the best symmetric peak and acceptable level of baseline noise.

The electropherograms obtained in the determination under selected conditions are presented in Figure 2.

HPLC Method Optimization

To improve peak resolution, operating conditions were optimized varying the elution gradient and the column parameters (column type, injection volume, and flow rate). Most chromatographic methods based on UV detection for the analysis of penicillins, especially for feed samples, require a derivatization reaction using toxic reagents such as mercury (Hg).[19] Due to the complex reaction and toxicity, some authors have developed separation methods without derivatization using C18 columns and gradient elution profiles with acetonitrile/water mixtures containing TFA.[19,35] In this study, we developed a short separation method without derivatization that enables efficient separation of the four selected antibiotics. Two chromatographic columns were tested: a C18 Kromasil 100 column (4.6 mm×250 mm, 5 μm; BIA Separations, Ljubljana, Slovenia) and a Chromolith® column. The first belongs to the group of the most widely used conventional particle-packed C18 chromatographic columns, whereas the second is a monolithic silica column. The main advantages of monoliths are their superior performance for fast separations with low pressure at high flow rates and, technically, they are more easily prepared in comparison with their polymeric counterparts and provide larger surface areas for small molecule separations. In our study, the monolithic column enabled more than two times faster analysis with comparable separation efficiency; therefore, this column was selected for the further studies. Applications of higher injection volumes (outside the linearity range) resulted in significant peak broadening and, moreover, significantly lower separation efficiency of SMX and AMP. We also examined the influence of different flow rates (1.5, 3, and 5 mL min^{-1}). Lower flow rates markedly prolonged the analysis time. Higher flow rates additionally shortened the analysis time, but resulted in poor separation of SMX and AMP. The separation under optimal conditions resulted in chromatograms shown in Figure 3.

Validation of the methods

The characteristics and the procedures used for validation were those described in USP 24,[36] the International Conference of Harmonization (ICH) Guidelines,[37,38] and other literature.[31,39,40]

Selectivity

Selectivity of the method was investigated by observing interfering peaks from matrix present in animal feed. Three different matrices were tested for the interferences. There was no interference in MEKC or HPLC results with matrix ingredients in any of the tested sample, which indicates that the method is selective (Figures 2 and 3).

Linearity

Linearity of the assay has to be determined by analysis of a series of standards with at least five different concentrations.[38,39] Linearity for the MEKC method was checked in the range from 0.5 to 300 mg dm^{-3} for each investigated compound (0.5, 1.0, 3.0, 5.0, 10.0, 25.0, 50.0, 75.0, 100.0, 125.0, 150.0, 200.0, 250.0, and 300.0 mg dm^{-3}). The linearity of calibration curves (peak area vs. concentration) for antibiotics is shown in Table 1.

Linearity for the HPLC method was checked in the range from 0.1 to 100 mg dm^{-3} for each investigated compound (0.1, 0.2, 0.5, 1.0, 2.5, 5.0, 10.0, 15.0, 20.0, 25.0, 50.0, 75.0, and 100.0 mg dm^{-3}).

Limits of Detection (LODs) and Limits of Quantification (LOQs)

LODs and LOQs, respectively, were estimated in accordance with the base line noise method. The base line noise was evaluated by recording detector response in a time period that was ten times longer as the peak width. LOD was calculated as the sample concentration that causes a peak three times higher as the base line noise level and LOQ was calculated as the concentration that is ten times higher as the base line noise level.[41] LODs and LOQs are shown in Table 1 for each compound, for both methods.

Accuracy

Accuracy of the method was determined by analyzing a solution of known concentration (working standard solution) and comparing the measured and the known values. The mean recoveries were 99.78±0.2 %, 99.80±0.3 %, 99.99±0.2 %, and 99.84±0.2 % ($n = 5$) for AMO, AMP, SMX, and SAC, respectively, which proves good accuracy of the method (Table 2). The MEKC results for accuracy were compared with that for

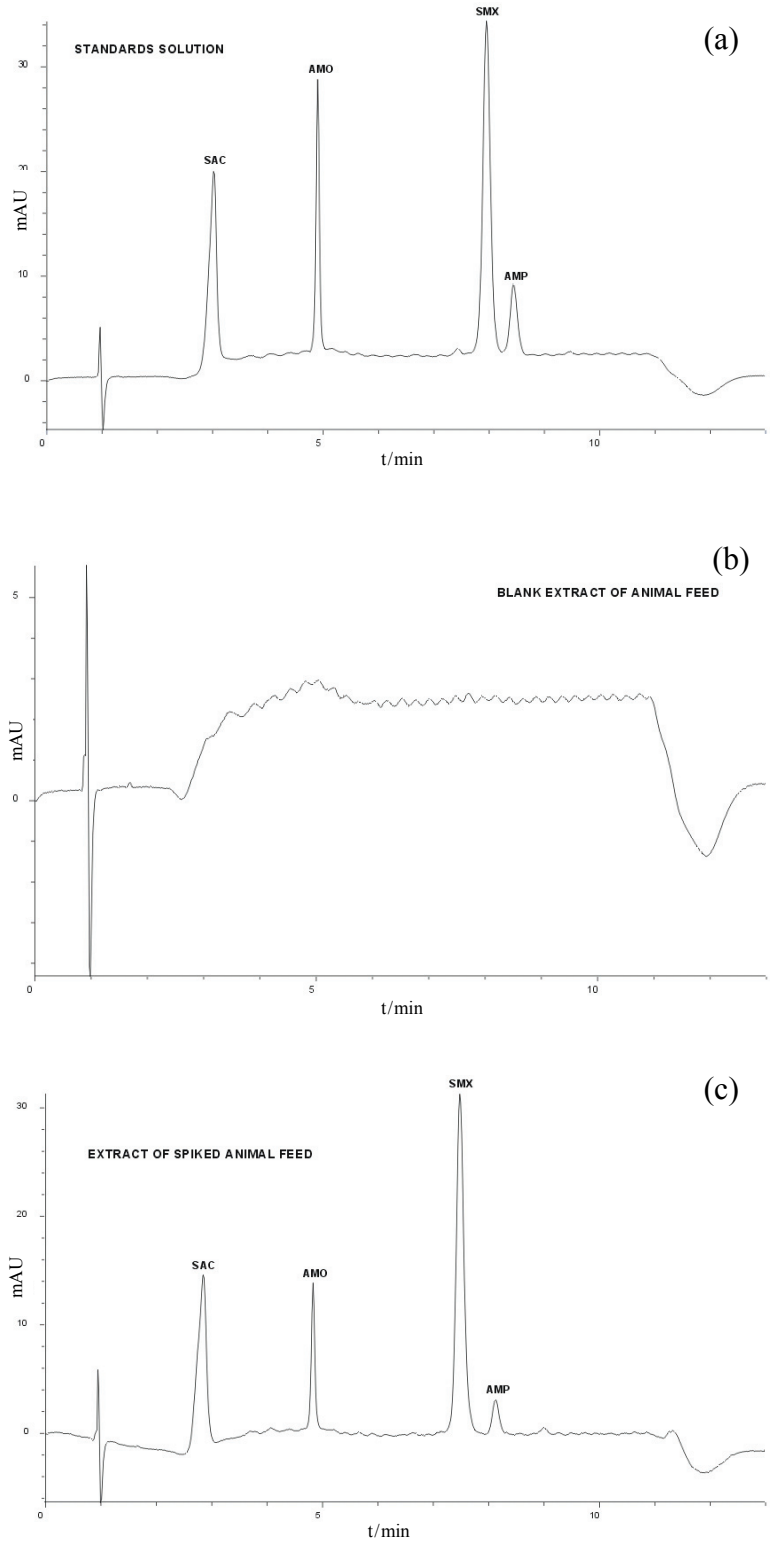

Figure 3. Chromatograms obtained for (a) 0.1 mg mL^{-1} of AMO, AMP, SMX, and SAC as a standard solution, (b) blank animal feedstuff (mixture of all three feedstuffs), and (c) 0.1 mg mL^{-1} of AMO, AMP, SMX, and SAC from spiked animal feedstuff; under the optimized conditions, at 220 nm. The optimal operating conditions: injection volume 10 μL, flow rate 3 mL min^{-1}, mobile phase A distilled water with 0.1% TFA, mobile phase B 100% acetonitrile with 0.1% TFA, elution gradient 0–1 min 100% A, 1–4 min 100–89% A, 4–5 min 89% A, 5–10 min 89–88% A, 10–11 min 88–100% A, 11–13 min 100% A.

Table 1. Statistical parameters of the calibration curve for each compound (linear regression), with LODs and LOQs

		AMO	AMP	SMX	SAC
MEKC	Intercept	19.9 ± 1.2	18.2 ± 1.5	48.4 ± 2.4	35.6 ± 1.6
	Slope	2144.1 ± 2.1	3354.4 ± 4.5	6208.3 ± 11.3	5670.8 ± 9.8
	R	0.9995	0.9992	0.9997	0.9998
	Linear range (mg L^{-1})	4–250	3–265	2–280	2–285
	LOD (mg L^{-1})	0.9	0.6	0.3	0.4
	LOQ (mg L^{-1})	3.1	2.1	1.1	1.2
HPLC	Intercept	–0.241 ± 0.022	–0.101 ± 0.011	–0.705 ± 0.017	–0.042 ± 0.008
	Slope	0.019 ± 0.003	0.009 ± 0.002	0.057 ± 0.006	0.036 ± 0.005
	R	0.9996	0.9991	0.9997	0.9991
	Linear range (mg L^{-1})	5–75	10–100	2–100	2–100
	LOD (mg L^{-1})	1.8	3.0	0.5	0.8
	LOQ (mg L^{-1})	5.9	10.0	1.7	2.6

the HPLC method (Table 2) and it was found that MEKC had not better accuracy for investigated concentrations as compared to the HPLC calculating by ANOVA one-way test followed with LSD post-hoc statistical analysis ($p > 0.05$).

Precision

Precision can be measured as repeatability, reproducibility, and intermediate precision. In this work, only repeatability and intermediate precision were studied.

Repeatability

Repeatability test was performed to determine intra-day variation in peak areas, retention and migration times. Standard solutions with concentrations of 20, 40, and 60 mg L^{-1} were analyzed ($n = 6$). RSD values for migration times (between 0.25 and 0.98 %), retention times (between 0.29 and 0.46 %), and for peak areas (between 0.42 and 1.86 % for MEKC, and 0.59 and 1.66 % for HPLC) indicate that the repeatability of both methods is acceptable.

Intermediate Precision

Intermediate precision was evaluated over three days (inter-day repeatability) with working solutions of concentrations 10−50 mg L^{-1}. These solutions were injected on each of the three days under the same conditions and the results were used for the repeatability study. The solutions were stored at room temperature (25±2 ºC during the experimental period) in sunlight, which caused a decrease of recovery values for maximum of 2.3 % for all drugs in methanol. When stored refrigerated in the dark, the recovery decreased for maximum of 0.7 % in three days, for all drugs. RSD values (MEKC: 0.15−0.31 % for AMO, 0.12−0.32 % for AMP, 0.09−0.21 % for SMX, and 0.19−0.39 % for SAC; HPLC: 0.49−0.81 % for AMO, 0.30−0.62 % for AMP, 0.35−0.71 % for SMX, and 0.36−0.69 % for SAC) indicate acceptable peak area, migration, and retention time intermediate precision for both MEKC and HPLC method.

Robustness

The optimum MEKC conditions set for this method have been slightly modified in order to evaluate the robustness.[42] The effects of different concentrations of SDS (70±1 mmol dm^{-3}) and organic modifier (10±0.5 % methanol) in the BGE as well as the effects of buffer pH (8.0±0.06), capillary temperature (25±5 °C), running pressure (15±1 mbar), running voltage (30±1 kV), and detection wavelength (±3 nm) were determined. The design applied was the fractional factorial design.[43] No significant variations in accuracy, specificity, and precision (RSDs were ≤ 2.0 % in all cases) were found in the tested ranges, which indicated that the MEKC method conditions are robust.

The effects of different concentrations of organic modifier (±0.5 %; acetonitrile) in the mobile phase as well as the effects of column temperature (±5 °C), flow rate (±0.1 mL min^{-1}), injection volume (±0.1 μL), and detection wavelength (±3 nm) were determined. No significant variations in specificity, accuracy, and precision were found in the tested ranges, which indicates good robustness of the HPLC method (RSDs were lower than 1.5 % for migration time and peak area).

Stability of Antibiotics

Stability of AMO, AMP, SMX, and SAC in methanol was checked during seven days at room temperature and refrigerated in dry and dark place. Recoveries of each compound were ≥ 99.6 %. This indicates good stability.

Table 2. Determination of accuracy in samples of known concentration

	$\gamma_{theor.}$[(a)]	$\gamma_{exp.}$[(b)]				Recovery / %			
		AMO	AMP	SMX	SAC	AMO	AMP	SMX	SAC
MEKC	20	19.9	19.9	20.0	20.0	99.50	99.50	100.00	100.00
	40	39.9	40.0	40.0	39.9	99.75	100.00	100.00	99.75
	60	59.8	59.9	60.2	59.8	99.67	99.83	100.33	99.67
	80	79.9	80.2	79.8	80.0	99.88	100.25	99.75	100.00
	100	100.1	99.4	99.9	99.8	100.11	99.42	99.89	99.76
HPLC	15	14.6	14.7	14.8	14.7	97.33	98.00	98.67	98.00
	30	29.9	29.8	30.1	30.0	99.67	99.33	100.33	100.00
	45	45.1	45.0	45.1	44.9	100.22	100.00	100.22	99.78
	60	59.9	59.7	60.0	60.0	99.83	99.50	100.00	100.00
	75	74.6	74.7	75.2	74.9	99.47	99.60	100.27	99.87

[(a)] Theoretical concentration expressed in mg L^{-1}

[(b)] Experimental concentration expressed in mg L^{-1}

Table 3. Application results

	Tested sample	$\gamma_{exp.}$[(a)]				Recovery / %			
		AMO	AMP	SMX	SAC	AMO	AMP	SMX	SAC
MEKC	K-19	10	10	10	10	99.9 ± 0.2	99.7± 0.2	100.1 ± 0.3	99.7 ± 0.5
	BEK-1	10	10	10	10	100.4 ± 0.3	99.8 ± 0.2	98.9 ± 0.1	99.3 ± 0.4
	NSK-1	10	10	10	10	99.5 ± 0.6	99.4 ± 0.5	99.1 ± 0.3	99.8 ± 0.2
HPLC	K-19	10	10	10	10	79.9 ± 0.8	69.1 ± 0.7	102.2 ± 0.2	99.0 ± 0.2
	BEK-1	10	10	10	10	80.3 ± 0.9	69.3 ± 0.9	99.3 ± 0.1	97.5 ± 0.9
	NSK-1	10	10	10	10	79.5 ± 0.5	69.4 ± 0.8	99.4 ± 0.5	97.8 ± 0.7

[(a)] Experimental concentration expressed in mg L^{-1}

Application

The presented methods were tested by determining AMO, AMP, SMX, and SAC in animal feedstuffs. When analyzing spiked commercial products, the amounts and recoveries obtained were determined by comparing the results with a standard solution containing the same concentration as expected in the spiked commercial products. Results for the MEKC method presented in Table 3 show strong agreement between the claimed and the found values. On the other hand, results for the HPLC method didn't show agreement between the claimed and the found values for AMO and AMP. According to LOQs for each drug, minimal amounts for quantification with the proposed MEKC method are 3.1, 2.1, 1.1, and 1.2 mg $kg^{-1}$of feed, and with the HPLC method 5.9, 10.0, 1.7, and 2.6 mg $kg^{-1}$of feed for AMO, AMP, SMX, and SAC, respectively.

Advantages and Disadvantages of the MEKC Over the HPLC Method

The advantage of the proposed MEKC method over the HPLC method for the analysis of four antibiotics in animal feedstuffs is its lower running costs and higher environmental friendliness. In the developed and proposed methods, 40 analyses with the MEKC require 2 mL of buffer containing 10 % (volume fraction) of methanol, while 40 analyses with HPLC require approximately 1000 mL of the mobile phase with acetonitrile and TFA. A disadvantage of a MEKC method is usually lower sensitivity in contrast to a HPLC method, but in our case of the analysis of AMO, AMP, SMX, and SAC in animal feedstuffs, sensitivity was even better for the proposed MEKC method. The HPLC method showed slightly better robustness as compared to the MEKC method.

CONCLUSION

The re-optimized MEKC method is a useful technique for a rapid separation (9 min) of amoxicillin, ampicillin, sulfamethoxazole, and sulfacetamide with SDS as a surfactant (70 mmol dm^{-3}) and pH = 8 (phosphate buffer). A pressure of 15 mbar was applied, which gives the best resolution, shape, and symmetric peaks in all cases. This system was applied successfully for their identification and quantitative determination in animal feedstuffs with different matrices, spiked with amoxicillin, ampicillin, sulfamethoxazole, and sulfacetamide. The MEKC results were compared with the new HPLC method. The advantage of the proposed MEKC method over the HPLC method for the analysis of four antibiotics in different animal feedstuffs is its lower running costs, better sensitivity (in our case), and environmental acceptability. Both developed methods can be used for routine analysis of drugs (antibiotics) abused as additives in animal feedstuffs.

REFERENCES

1. M. Becker, E. Zittlau, and M. Petz, *Anal. Chim. Acta* **520** (2004) 19–32.
2. L'Informatore Farmaceutico di Vet. e Zoot, 11th ed., OEMF s.p.a.
3. S. Bogialli, R. Curini, A. D. Corcla, M. Nazzari, and R. Samperi, *Anal. Chem.* **75** (2003) 1798–1804.
4. W. G. Huber, *Adv. Vet. Sci. Comp. Med.* **15** (1971) 101–132.
5. W. Molterer, *Off. J. Eur. Communities: Legis.* **41** (1998) 4–8.
6. S. Yang, J. Cha, and K. Carlson, *J. Chromatogr., A* **1097** (2005) 40–53.
7. G. F. Pang, Y. Z. Cao, C. L. Fan, J. J. Zhang, X. M. Li, and Z. Y. Li, *Anal. Bioanal. Chem.* **376** (2003) 534–541.
8. A. A. Salem, H. A. Mossa, and B. N. Barsoum, *J. Pharm. Biomed. Anal.* **41** (2006) 654–661.
9. A. V. Pereira and Q. B. Cass, *J. Chromatogr., B* **826** (2005) 139–146.
10. K. E. Maudens, G. F. Zhang, and W. E. Lambert, *J. Chromatogr., A* **1047** (2004) 85–92.
11. R. M. Rao, Y. M. Rao, and A. H. Shah, *J. Pharm. Biomed. Anal.* **20** (1999) 717–722.
12. S. A. Nabi, M. A. Khan, S. N. Khowaja, and Alimuddin, *Acta Chromatogr.* **16** (2006) 164–172.
13. G. Indrayanto and T. K. Sia, *J. Liq. Chromatogr. Relat. Technol.* **24** (2001) 1501–1510.
14. M. L. Storms and J. T. Stewart, *J. Liq. Chromatogr. Relat. Technol.* **25** (2002) 2433–2443.
15. W. Luo and C. Y. W. Ang, *J. AOAC Int.* **83** (2000) 20–25.
16. J. Wang, *J. AOAC Int.* **87** (2004) 45–55.
17. S. Riediker, A. Rytz, and R. H. Stadler, *J. Chromatogr., A* **1054** (2004) 359–363.
18. L. Okerman, K. De Wasch, J. Van Hoof, and W. Smedts, *J. AOAC Int.* **86** (2004) 236–240.
19. E. Beneto-Pena, A. I. Partal-Rodera, M. E. Leon-Gonzalez, and M. C. Moreno-Bondi, *Anal. Chim. Acta* **556** (2006) 415–422.
20. V. Gamba and G. Dusi, *Anal. Chim. Acta* **483** (2003) 69–72.
21. A. Fernandez-Gonzalez, R. Badia, and M. E. Diaz-Garcia, *J. Pharm. Biomed. Anal.* **29** (2002) 669–679.
22. M. Ashraf-Khorassani, L. T. Taylor, L. M. Koeth, and J. A. Roush, *Chromatographia* **62** (2005) 459–463.
23. L. Nozal, L. Arce, A. Rios, and M. Valcarcel, *Anal. Chim. Acta* **523** (2004) 21–28.
24. J. M. L. Gallego and J. P. Arroyo, *J. Chromatogr., B* **784** (2003) 39–47.
25. M. I. Bailon Perez, L. Cuadros Rodriguez, and C. Cruces-Blanco, *J. Pharm. Biomed. Anal.* **43** (2007) 746–752.
26. R. Injac, A. Mlinarić, V. Đorđević-Milić, K. Karljiković-Rajić, and B. Štrukelj, *Food Addit. Contam.* **25** (2008) 424–431.
27. R. Injac, J. Kac, K. Karljiković-Rajić, and B. Štrukelj, *J. Food Drug Anal.* **16** (2008) 18–25.
28. J. J. B. Nevado, G. C. Penalvo, and F. J. G. Bernardo, *J. Sep. Sci.* **28** (2005) 543–548.
29. J. J. B. Nevado, G. C. Penalvo, and F. J. G. Bernardo, *J. Chromatogr., A* **918** (2001) 205–210.
30. Z. Yongxin, C. Hoogmartens, A. Van Schepdael, E. Roets, and J. Hoogmartens, *J. Liq. Chromatogr. Relat. Technol.* **22** (1999) 1403–1415.
31. R. Injac, N. Kocevar, and S. Kreft, *Anal. Chim. Acta* **594** (2007) 119–127.
32. N. A. Simmons, *J. Antimicrob. Chemother.* **1** (1975) 257–261.
33. S. C. Sweetman, *Martindale: The complete drug reference*, 34th ed. London: Pharmaceutical Press, 2005.
34. L. Suntornsuk, *J. Chromatogr. Sci.* **45** (2007) 559–577.
35. www.sigmaaldrich.com/TheReporter (Reporter 18.1, 2000).
36. *United States Pharmacopoeia 24 - National Formulary 19*. United States Pharmacopoeial Convention, Rockville, MD 2000, pp. 185–186.
37. *International Conference on Harmonization, guideline Q2A: Text on validation of analytical procedures.* Federal Register 1995, 60, 11260.
38. *International Conference on Harmonization, guideline Q2B: Validation of analytical procedures: methodology*. Federal Register 1997, 62, 27463.
39. G. A. Shabir, *J. Chromatogr., A* **987** (2003) 57–66.
40. R. Brown, M. Caphart, P. Faustino, R. Frankewich, J. Gibbs, E. Leutzinger, G. Lunn, L. Ng, R. Ragagopalan, Y. Chiu. and E. Sheinin, *LC-GC* **19** (2001) 74–79.
41. I. Toro, J. F. Dulsat, J. L. Fabregas, and J. Claramunt, *J. Chromatogr., A* **1043** (2004) 303–315.
42. R. Injac, M. Bošković, N. Kočevar, and T. Vovk, *Anal. Chim. Acta* **620** (2008) 150–161.
43. Y. V. Heyden, A. Nijhuis, J. Smeyers-Verbeke, B. G. M. Vandeginste, and D. L. Massart, *J. Pharm. Biomed. Anal.* **24** (2001) 723–753.

SAŽETAK

Optimizirana metoda određivanja amoksiciklina, ampicilina, sulfametoksazola i sulfacetamida u hrani za životinje micelarnom elektrokinetičkom kromatografijom i usporedba s tekućinskom kromatografijom visoke djelotvornosti

Rade Injac, Nina Kočevar, Borut Štrukelj

Faculty of Pharmacy, The Chair of Pharmaceutical Biology, University of Ljubljana, Askerceva 7, 1000 Ljubljana, Slovenia

U mnogim zemljama svijeta zabranjeno je korištenje antibiotika kao aditiva u hrani za životinje, ali je zloupotreba i dalje veoma prisutna. Razvijena je nova metoda micelarne elektrokinetičke kromatografije pri 25 °C i naponu od 30 kV (radni tlak: 15 mbar) uz upotrebu fosfatnog pufera (pH = 8,0) koji sadrži 70 mmol L^{-1} natrijevog dodecilsulfata i 10 % metanola (φ_{MeOH} = 10 %) kao radnog elektrolita za razdvajanje i određivanje amoksiciklina, ampicilina, sulfametoksazola i sulfacetamida. Spojevi su detektirani pri 210 nm. Metoda je validirana i primijenjena za kvantitativno određivanje antibiotika dodanih u hranu za životinje. Rezultati su uspoređeni s novorazvijenom metodom tekućinske kromatografije visoke djelotvornosti za ispitivanja četiri antibiotika u realnim uzorcima. Obje metode mogu se koristiti za rutinsku analizu amoksiciklina, ampicilina, sulfametoksazola i sulfacetamida kao aditiva u hrani za životinje.