Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Pharmaceutical and Biomedical Analysis
32 (2003) 937–947
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Capillary electrophoresis in analysis of veterinary drugs

P. Kowalski, I. Olędzka, H. Lamparczyk *

Medical University of Gdańsk, Hallera 107, PL-80-416 Gdansk, Poland

Received 24 April 2002; received in revised form 25 February 2003; accepted 27 February 2003

Abstract

Antibiotics are extensively applied in veterinary medicine for the treatment of various bacterial infections. Because of their use in food producing animals, the risk of occurrence of unwanted residues in edible products exists. To ensure human food safety, The European Union has defined maximum residue limits (MRLs) for veterinary drug residues in food products. Analytical methods need to be developed to confirm the presence of antibiotics at the MRL level. A capillary electrophoresis (CE) method with UV detection is proposed for the quantitative determination of residues from poultry and porcine tissues. Eight of the most frequently used antibiotics and nifursol, which routinely used as poultry coccidiostat, were analysed. CE technique permitted analysing substances to be separated from muscle, liver, kidney and skin with fat after a simple extraction with acetonitrile or ethyl acetate under basic conditions. Proposed method is capable to identify drug residues in tissues at level below 20 μg/kg.

Keywords: Antibiotics; Residues in animal tissues; Capillary electrophoresis

1. Introduction

Antibiotics have for decades continued to play an important role in veterinary medicine as feed additives because of the broad spectrum and their economic advantages. In general, they are both used in livestock farm industries as active disease treatment agents for prophylactics and in cases of pulmonary, urinary and digestive infections as feed additives.

The use of antibiotics in veterinary began in the 1950s with the use of oxytetracycline and chlortetracycline as feed additives [1]. In the 1980s, it was estimated that at least 60% of all animals used for food were exposed to antibiotics at some point in their lives [2]. A significant and progressive increase in the use of antibiotics and quinolines in animal production was noted over the present decade. The use of these drugs to control and treat animal disease and to promote fast, more efficient growth of livestock is a common practice. An estimated 80% of livestock and poultry receive some animal drugs during their lifetime. With current intense animal husbandry practices, this figure may be higher. Improper use of animal drugs may cause residues in the edible tissues of slaughtered animals that could be hazardous to consumers and become a serious problem.

Despite their low toxicity, even residual amounts of antibiotics in food derived from

* Corresponding author. Tel./fax: +48-58-349-3130.

0731-7085/03/$ - see front matter
doi:10.1016/S0731-7085(03)00195-X

Table 1
MRLs for antibiotics in force in the EU

Substance	Species	MRLs (μg/kg)	Tissue
Amoxicillin	Poultry (chicken and hen) Porcine	50	All edible tissues
Doxycycline hydrochloride	Poultry Porcine	100 200 300 600	Muscle Eggs Liver, skin and fat Kidney
Streptomycin sulfate (sum dihydrostreptomycin and streptomycin)	Poultry	500 1000	Muscle, liver, skin and fat Kidney
Thiamphenicol	Poultry Porcine	40	All edible tissues
Florphenicol	Poultry (chicken and hen) Porcine	200 300 3000	Muscle, fat Kidney Liver
Nifursol	Turkey	No MRL has yet been established	
Enrofloxacin (sum of enrofloxacin and ciprofloxacin)	Poultry Porcine	30	All edible tissues
Norfloxacin	Porcine	No MRL has yet been established	

treated animals may be directly toxic or cause resistant human pathogens and possible allergic hypersensitivity reactions in humans [3].

Because of this concern, The European Union (EU) and the Joint FAO/WHO Expert Committee on Food Additives (JECFA) have established a maximum residue limit (MRL) for residues of these drugs in edible animal tissues to protect the consumer. Since the beginning of 2000 year, an MRL is required for every new substance that might be commercialised in the EU for veterinary use [4]. Therefore, development or improvement of analytical methods for monitoring their levels in farm animals and their primary products is of increasing interest.

Moreover, the procedure must be sensitive and accurate when it is designed for drug residue analysis. In this study, we developed an analytical procedure suitable for the determination of eight of antibiotics in tissues and one of coccidiostatic–nifursol (salfnriele). Nifursol [3,5-dinitro-*n*-(5-nitrofurfurylidene)-salicylohydrazide] is considered to be especially useful as a preventative treatment for a condition known as 'blackhead'.

The efficiency of the analytical procedure was assessed by the calculation of absolute recovery values. As a consequence of the widespread use of antibiotics and coccidiostatics, there are many methods published for their determination in animal tissues incorporating a variety of analytical techniques. Microbiological assays are most commonly used for the measurement of antibiotics in foods, but they cannot identify antibiotics and their precision appears to be variable. Furthermore, these tests often lack the specificity and sensitivity required for residue detection at MRLs, may be affected by non-specific inhibitors and often have a 24 h or more incubation time [5].

Thin-layer chromatography screening methods using derivatization of antibiotics have also been

developed [6], these methods are rather selective and sensitive but are not reproducible enough. Gas chromatography methods are very sensitive and specific [7] but routine application of these methods for a large number of samples is not easy because of the many purification and derivatization steps required.

Likewise, many HPLC analysis methods for antibiotics in food have been reported in the literature over the past 20 years or more [8–16]. However, most of them require multi-step sample preparation and complicated procedures. Conventional multi-step residue extraction procedures achieve high purity at the cost of being time consuming and labour intensive and result in low residue recovery. Nevertheless, there is not much information available about techniques that could be useful at the same time for different animal species and for a wide range of tissues.

Capillary electrophoresis (CE) allows the selective extraction and concentration of veterinary residues from complex materials, often in a single step operation, with a comparatively high yield. CE has become a very useful tool for pharmaceutical analysis because of its high resolution, speed and the extremely small sample volume required. However, the use of CE in the bioanalysis of drugs is restricted due to the low concentration sensitivity of this technique. In order to determine analytes at a low concentration range some preconcentration procedures have proved useful. Nonetheless, the detection limits obtained in this work are low enough to determine concentrations below the permissible MRL in animal products. MRLs for drugs in force in the EU are shown in Table 1.

2. Experimental

2.1. Chemicals and stock solutions

Methanol, acetonitrile, ethyl acetate were of analytical-reagent grade (Merck, Darmstadt, Germany). Sodium tetraborate decahydrate (pH 9.26; 25 mM), sodium dihydrogenphosphate (pH 5.73; 25 mM) and boric acid (pH 6.94; 25 mM) buffer solutions was prepared according to standard method, using triple distilled water.

Theophylline, ethacridine lactate, ephedrine hydrochloride and procaine hydrochloride (used as internal standards) were purchased from Sigma (Deisenhofen, Germany). All drugs were obtained from Pharmaceutical Enterprise (Polfa, Poland) and Vetos-Farma Meat Processing Factory (Bielawa, Poland). The calibration of the pH meter was done with standard buffer solutions purchased from POCh (Gliwice, Poland).

Individual stock solutions (1 mg/ml) were prepared by dissolving the appropriate amount of each of substances in 0.5 ml of methanol and diluting up to 10 ml with triple deionized water.

The working solutions were also prepared in glass volumetric flasks by appropriate dilution just before use. They were stored in the dark under refrigeration to avoid possible decomposition.

2.2. Instrumental parameters

For preliminary UV investigations, a Philips UV/Vis spectrophotometer was used in the 190–300 nm band. All investigations were performed on a P/ACE System model 2100 (Beckman Instruments, Fullerton, CA) equipped with an autosampler, a fluid-cooled capillary cartridge, automatic injector and UV detector. Beckman System Gold software was used for system control, data collection and integration of the electrophoregrams.

The temperature ($25 \pm 0.1\ ^{\circ}C$) was controlled using a fluorocarbon-based cooling fluid. The applied voltage was 25 kV. All fused-silica capillaries used were obtained from Beckman and had an i.d. of 75 or 100 μm and o.d. 375 μm. Analytes were introduced into the capillary at anode via a 7 s, at 3.45 kPa argon pressure injection, whereas the detector was set on the cathode end of the capillary.

2.3. Tissue sample procedure

Samples (tissues from poultry and porcine) used for the method validation came from untreated animals that were slaughtered in order to collect samples from the different tissues. All the samples were obtained from VETOS-FARMA Meat Pro-

Table 2
Electrophoretic parameters of analysis and sample preparation for drugs analysis

Substance	Wavelength detection UV (nm)	Running buffer	Sample preparation
Amoxicillin	200	30 mM NaH_2PO_4 5 mM $Na_2B_4O_7$ 5 mM H_3BO_3	Protein precipitation by acetonitrile and liquid–liquid extraction with ethyl acetate
Doxycycline hydrochloride	280	20 mM $Na_2B_4O_7$	
Streptomycin sulfate	200	20 mM $Na_2B_4O_7$	
Thiamphenicol	200	25 mM NaH_2PO_4 25 mM $Na_2B_4O_7$	
Florphenicol	200	25 mm NaH_2PO_4 25 mM $Na_2B_4O_7$	
Nifursol	214	25 mM NaH_2PO_4 25 mM $Na_2B_4O_7$ 25 mM H_3BO_3	Protein precipitation by acetonitrile
Enrofloxacin	280	25 mm NaH_2PO_4 25 mM $Na_2B_4O_7$ 25 mM H_3BO_3	
Ciprofloxacin	280	25 mM NaH_2PO_4 25 mM $Na_2B_4O_7$ 25 mM H_3BO_3	
Norfloxacin	280	20 mM NaH_2PO_4 25 mM $Na_2B_4O_7$	

cessing Factory (Bielawa, Poland). Samples were stored in different containers and kept frozen until processing.

Approximately 3–6 g of thawed and minced ground tissues were weighed accurately into a 50-ml tube. To the samples was added the internal standard and 5 ml acetonitrile. The tube was tightly capped and manually shaken until the tissue was dislodged from the bottom of the tube and mechanically homogenized using a tissue homogenizer (Polytron 3000, Kinematica, Germany) for 20 s at medium speed. The sample was placed on a platform shaker at high speed for 10 min, macerated and centrifuged for 8 min at $10\,000 \times g$; then the supernatant was decanted to a clean tube and evaporated to dryness under a gentle stream of air at 45–50 °C.

The samples included enrofloxacin, ciprofloxacin, norfloxacin and nifursol were deproteinzed by acetonitrile, without liquid–liquid extraction, but the samples contained amoxicillin, doxycycline hydrochloride, streptomycin sulfate, thiam- and florphenicol were deproteinized by acetonitrile and subsequently extracted with ethyl acetate in the presence of 1 M NaOH. The organic layer was separated, transferred to clean test tube and evaporated to dryness in a water bath 45–50 °C under a stream of air. The dry residue was reconstituted in 2 mM sodium tetraborate decahydrate (0.5 ml), centrifuged for 5 min at $8000 \times g$ and was injected into the CE system or stored at −20 °C until the time of analysis.

The modification concerned of liquid–liquid extraction of some of the drugs was introduced to reduce background effects from interfering components in the samples. Additionally, obtained extracts were more clear.

Electrophoretic parameters of analysis and sample preparation are shown in Table 2. The same tissue sample procedure was used for muscle, liver, kidney and skin with fat of the two different studied species (porcine and poultry). The tissues

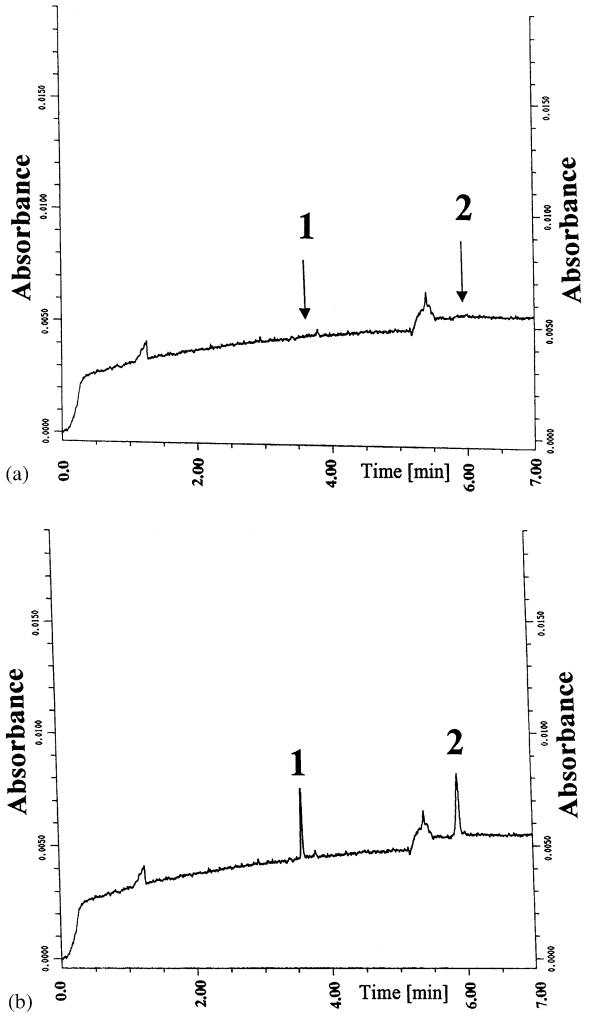

Fig. 1. Some examples of electrophoretic resolution obtained in the analysis, respectively, of a blank sample (a) and a poultry skin with fat sample spiked with 3 μg/g of ephedrine hydrochloride (I.S.) (1) and 2 μg/g of amoxicillin (2) (b).

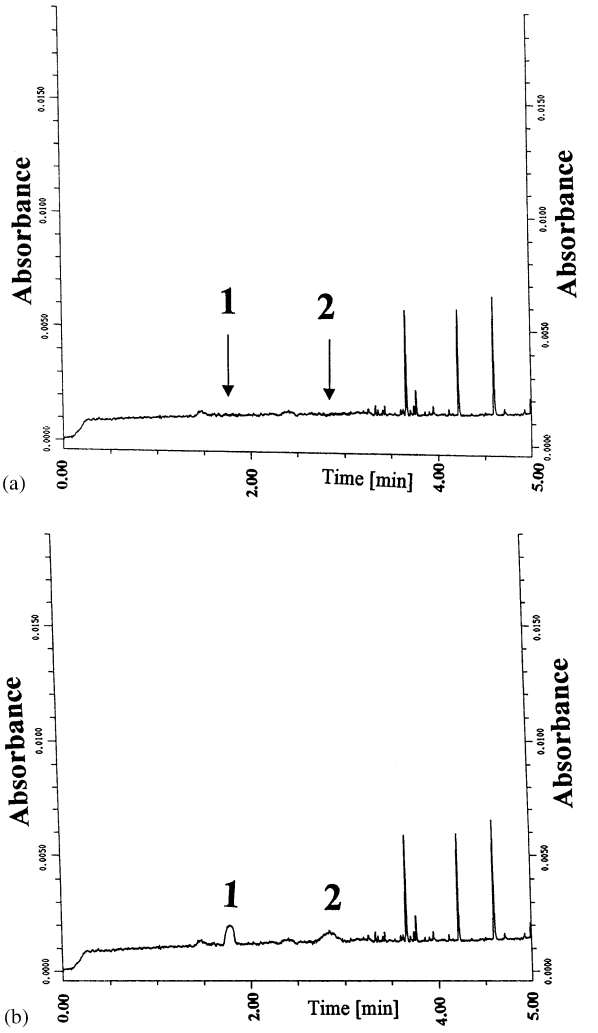

Fig. 2. Some examples of electrophoretic resolution obtained in the analysis, respectively, of a blank sample (a) and a porcine muscle sample spiked with 20 μg/g of ethacridine lactate (1.5.) (1) and 1 μg/g of doxycycline hydrochloride (2) (b).

samples obtained from animals under treatment with all of drugs were treated in the same way.

2.4. Preparation of the capillary

The capillary was conditioned every day with an initial wash cycle consisting of 1 M NaOH for 15 min, deionized water for next 10 min and running electrolyte for 5 min. In order to equilibrate the capillary and minimise hysteresis effects, the capillary was flushed between each run with 1 M NaOH for 2 min, deionized water for 1.5 min, followed by the running buffer for 1 min. The separation buffer was refreshed after ten analyses. Daily after finishing the experiments, the capillary was washed with 1 M NaOH for 5 min and deionized water for 5 min and purged with air for 2 min.

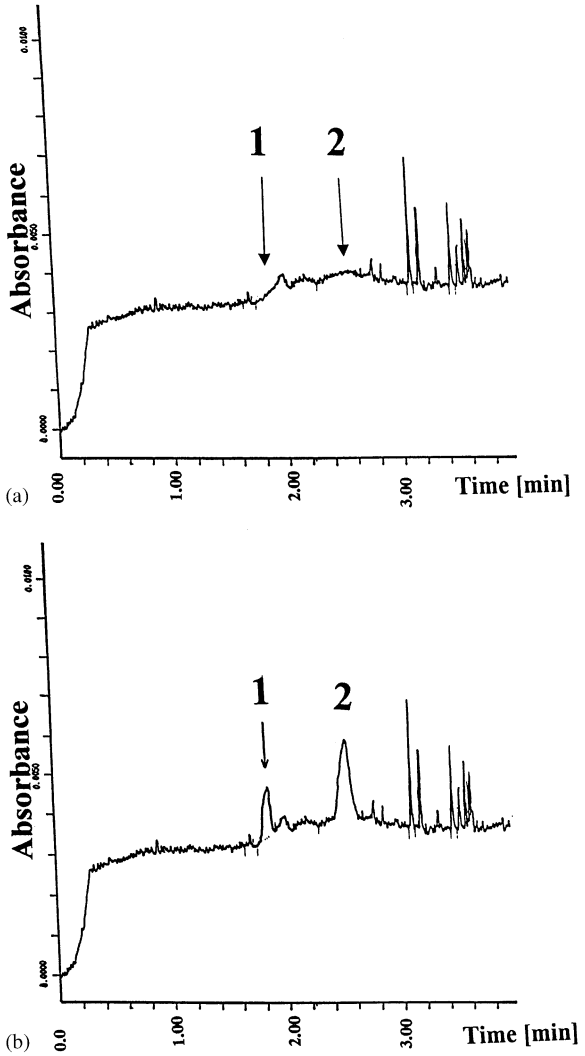

Fig. 3. Some examples of electrophoretic resolution obtained in the analysis, respectively, of a blank sample (a) and a poultry muscle sample spiked with 500 ng/g of streptomycin sulfate (1) and 1 μg/g of amoxicillin (I.S.) (2) (b).

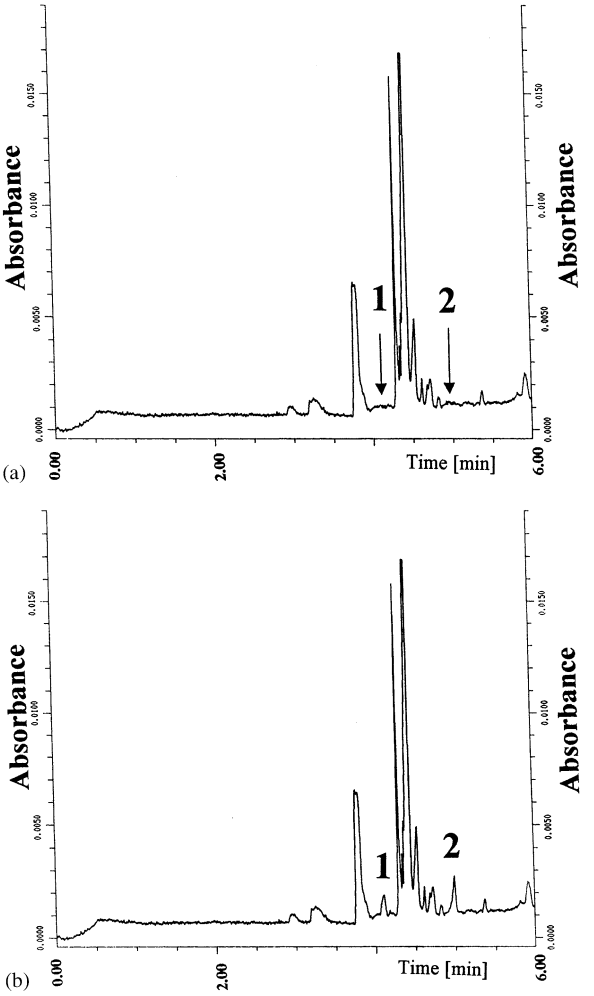

Fig. 4. Some examples of electrophoretic resolution obtained in the analysis, respectively, of a blank sample (a) and a poultry muscle sample spiked with 1 μg/g of thiamphenicol (1) and 10 μg/g of theophylline (I.S.) (2) (b).

3. Results and discussion

3.1. Electrophoretic analysis

The deprotenization with acetonitrile gave the best results and allowed, after a centrifugation step and evaporation, a direct injection into the CE system. The electrophoregram runtime was maximum 10–11 min, a time shorter than other studies described, where drugs migrated later. This fact permitted use to analyse a relatively high number of samples in a short period of time. Figs. 1–8 show the electrophoregrams from muscle, liver, kidney and skin with fat blank samples and samples spiked with antibiotic or nifursol and internal standard. Electrophoregrams showed a good resolution and no interference with other peaks, indicating a high specificity and sensitivity of this method.

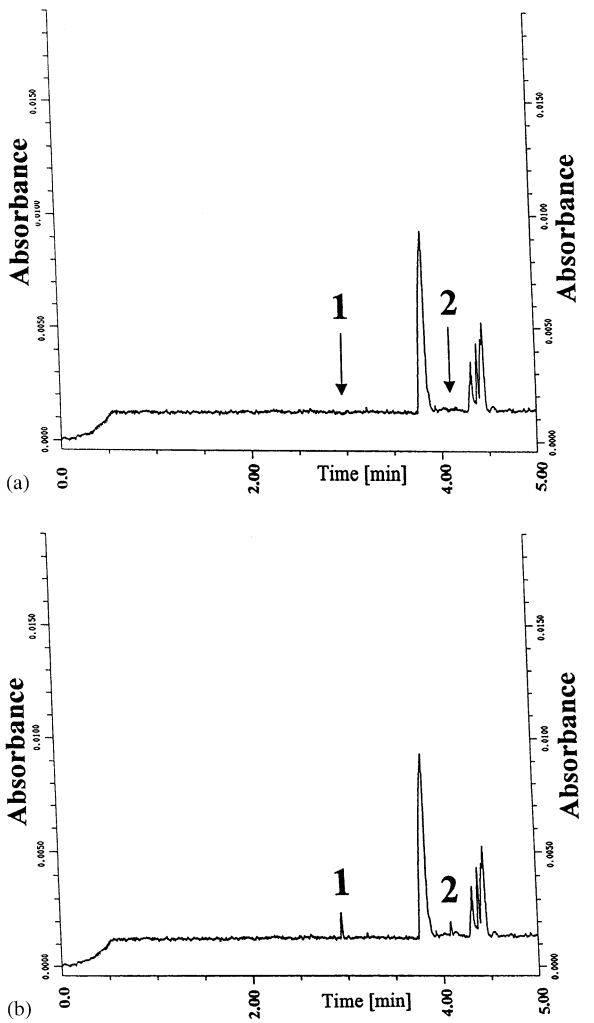

Fig. 5. Some examples of electrophoretic resolution obtained in the analysis, respectively, of a blank sample (a) and a poultry liver sample spiked with 10 μg/g of ephedrine hydrochloride (I.S.) (1) and 500 ng/g of florphenicol (2) (b).

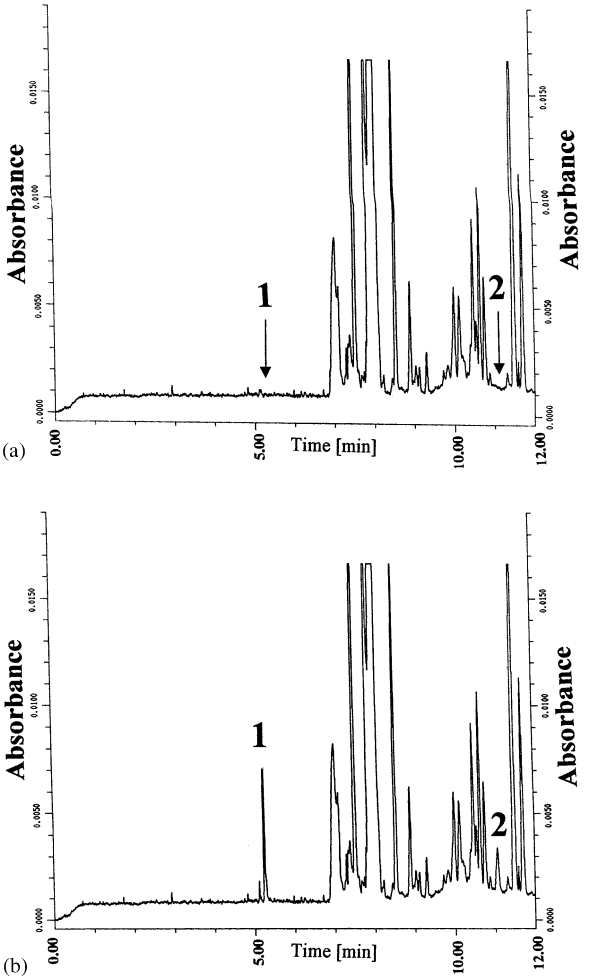

Fig. 6. Some examples of electrophoretic resolution obtained in the analysis, respectively, of a blank sample (a) and a turkey kidney sample spiked with 20 μg/g of ephedrine hydrochloride (I.S.) (1) and 1 μg/g of nifursol (2) (b).

In order to select the best conditions and internal standard, the electrophoretic behaviour of each of the substance were also studied. In order to predict the optimal buffer solution for the separation of drugs, it was necessary to select concentration and composition of buffer at which the differences between the mobilities of the studied substances are greatest and, hence at which these substances migrate with the best separation. Some of parameters of the electrophoretic analysis (analysis and migration times of antibiotics and their internal standards) are collected in Table 3.

Some of the drugs have similar migration times, and therefore it was not possible to validate all compounds simultaneously. In that case, validation experiments were performed separately for each compound considered.

3.2. Calibration procedure

Different internal standards were chosen for the analysed drugs. These were as follows: theophyl-

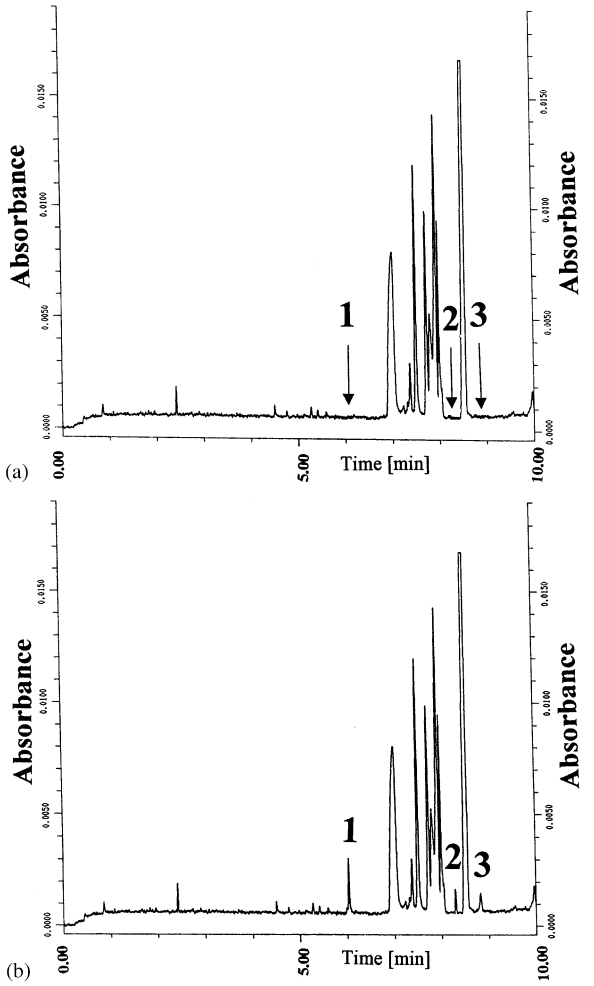

Fig. 7. Some examples of electrophoretic resolution obtained in the analysis, respectively, of a blank sample (a) and a porcine liver sample spiked with 4 μg/g of procaine hydrochloride (I.S.) (1) and 500 ng/g of ciprofloxacin (2) and 500 ng/g of enrofloxacin (3) (b).

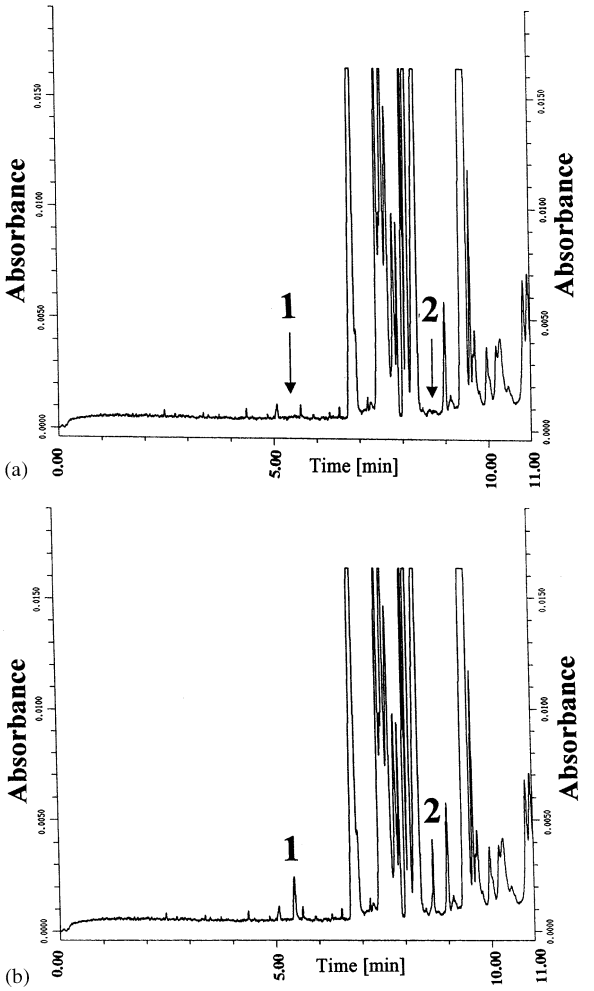

Fig. 8. Some examples of electrophoretic resolution obtained in the analysis, respectively, of a blank sample (a) and a porcine kidney sample spiked with 4 μg/g of procaine hydrochloride (I.S.) (1) and 1 μg/g of norfloxacin (2) (b).

line (I.S.) for amoxicillin and thiamphenicol, ethacridine lactate (I.S.) for doxycycline hydrochloride, amoxicillin (I.S.) for streptomycin sulfate, ephedrine hydrochloride (I.S.) for florphenicol and nifursol, finally procaine hydrochloride (I.S.) for enrofloxacin, ciprofloxacin and norfoxacin. Using the standard solutions of drugs and internal standards, samples of blank control tissues (5.0 g) were spiked with both compounds at appropriated concentrations. Numerical data of calibration characteristics were shown in Table 4.

All the samples were prepared and analysed using the same procedures, which were described, in Section 2. Calibration curve based on the peak-height ratios of analysed substance to internal standard was constructed using six or seven different concentrations of substance analysed six times for each concentration. The data were subjected to linear regression analysis in order to achieve the appropriate calibration factors.

Regression equations together with calibration ranges, quantification and detection limits as well

Table 3
Some of parameters of the electrophoretic analysis

Substance	Analysis time (min)	i.d. of capillary (μm)	Length of capillary (cm)
Amoxicillin	7.0	75	67
Doxycycline hydrochloride	5.0	100	47
Streptomycin sulfate	4.0	100	47
Thiamphenicol	6.0	75	57
Florphenicol	5.0	75	57
Nifursol	12.0	75	77
Enrofloxacin	10.0	75	77
Ciprofloxacin	10.0	75	77
Norfloxacin	11.0	75	77

as mean recoveries for the investigated substances were listed in Table 4.

3.3. Detection and quantification limits

The limit of detection (LOD) was determinated as the sample concentration that produces a peak with a height three times the level of the baseline noise and the limit of quantification (LOQ) was calculated as the sample concentration that produces a peak with a 10 times the signal-to-noise ratio. Numerical data of the LOD and LOQ for the analysed antibiotics are collected in Table 4. The detection limits obtained in this work are low enough to determine concentrations below the permissible MRL in animal products. The LOD/LOQ values characterizing the described procedure should be sufficient for food screening or for future residue depletion studies involving drugs.

3.4. Recovery

Recoveries for all compounds were determined from comparison of the data obtained from extracted samples to the data obtained by direct analysis of each compound at the respective concentrations without extraction. Recovery studies were performed at six different concentrations of each of substance. The mean recovery values calculated for muscle, liver, kidney and skin with fat samples are reported in Table 4.

3.5. Stability studies

The effect of freezing and thawing cycles was studied using six parallel samples at two different concentrations. The evaluation of the data was the same as for the long-term stability test. The results indicate that there was no significant or relevant change in concentration of each of drug during two cycles of freezing and thawing.

3.6. Withdrawal times

Results of drugs determinations in poultry and porcine tissues showed, that the residues of antibiotics and coccidiostatic (nifursol) relatively rapidly disappeared from animal organisms, most quickly from muscles, slowly from liver and kidney. The Food and Drug Administration (FDA) establishes withdrawal times, which are times after drug treatment when edible tissue of animals are not to be used for food and during which animals are not to be slaughtered. Determination of veterinary drugs residues in poultry and porcine tissues showed, that the withdrawal times are different for various substances (from 3 days for doxycycline hydrochloride to 18 days for thiam- and florphenicol).

4. Conclusions

A CE method with UV detection has been developed for quantitative determination of eight antibiotics and one coccidiostatic (nifursol) resi-

Table 4
Quantitative parameters (calibration characteristics) obtained for the analysis of antibiotics by CE in animal tissues

Substance	Calibration range (μg/ml)	Slope of the calibration (a)	Intercept (b)	Regression coefficient (r)	Detection limit (μg/kg)	Quantification limit (μg/kg)	Mean recoveries (%)
Amoxicillin	0.05–2	0.345 ($\pm$0.005)	0.0027 ($\pm$0.004)	0.9992	15	50	66
Doxycycline hydrochloride	0.05–2	0.476 ($\pm$0.009)	0.003 ($\pm$0.001)	0.9992	15	50	75
Streptomycin sulfate	0.016–4.8	0.21 ($\pm$0.004)	0.022 ($\pm$0.009)	0.9991	35	120	84
Thiamphenicol	0.025–6	0.042 ($\pm$0.0005)	0.0014 ($\pm$0.001)	0.9995	8	25	74
Florphenicol	0.05–6	0.107 ($\pm$0.002)	0.01 ($\pm$0.006)	0.9989	15	50	73
Nifursol	0.02–2	0.00032 ($\pm 3 \times 10^{-6}$)	0.009 ($\pm$0.003)	0.9997	5	20	72
Enrofloxacin	0.02–2	0.0015 ($\pm 9 \times 10^{-6}$)	0.025 ($\pm$0.007)	0.9998	5	20	79
Ciprofloxacin	0.02–2	0.0017 ($\pm 1 \times 10^{-5}$)	0.04 ($\pm$0.01)	0.9998	5	20	82
Norfloxacin	0.01–1	0.0015 ($\pm 5 \times 10^{-5}$)	0.004 ($\pm$0.006)	0.9998	3	10	77

dues in poultry and porcine tissues. The method is sensitive enough to perform determinations below the MRL values established by the EU. Therefore, it can be used for withdrawal time calculations. The analytical procedure developed in this work is fast, specific, accurate, precise, reproducible and sensitive, although simple UV detection was used. The proposed simplified clean-up procedure, including deprotenization by acetonitrile and liquid–liquid extraction with ethyl acetate of drug substances at concentrations below 20 μg/kg in a variety of food types. Cost of analysis is decreased because less solvent is needed and fewer laboratory technicians need to undergo training. Finally, data is generated more quickly because of the ease of the process and its potential to be automated.

References

[1] R.H. Gustafson, in: W.A. Moast (Ed.), Agricultural Uses of Antibiotics (ACS Symposium Series), American Chemical Society, Washington, DC, 1986 (chapter l).
[2] R.W. Fedeniuk, P.J. Shand, J. Chromatogr. A 812 (1998) 3–15.
[3] D. Barron, E. Jimenez-Lozano, J. Cano, J. Barbosa, J. Chromatogr. B 759 (2001) 73–79.
[4] J.A. Hernandez-Arteseros, et al., J. Chromatogr. A 945 (2002) 1–24.
[5] C.C. Walker, H.M. Lott, S.A. Barker, J. Chromatogr. 642 (1993) 225–242.
[6] J.P. Abjean, J. Planar Chromatogr. 6 (1993) 147–154.
[7] S.J. Stout, W.A. Steller, A.J. Manuel, M.O. Poeppel, A.R. da Cunha, J. Assoc. Off. Anal. Chem. 67 (1984) 142–149.
[8] A. Posyniak, I. Zmudzki, J. Niedzielska, J. Chromatogr. A 914 (2001) 59–66.
[9] G. Suhren, K. Knappstein, Analyst 123 (1998) 2797–2801.
[10] M.D. Rose, J. Bygrave, G.W.F. Stubbings, Analyst 123 (1998) 2789–2796.
[11] R.E. Hornish, J.R. Wiest, J. Chromatogr. A 812 (1998) 123–133.
[12] J.C. Yorke, P. Froc, J. Chromatogr. A 882 (2000) 63–77.
[13] C. Marasehiello, F. Cusido, M. Abellan, J. Vilageliu, J. Chromatogr. B 754 (2001) 311–318.
[14] H. Oka, Y. Ito, H. Matsumoto, J. Chromatogr. A 882 (2000) 109–133.
[15] D. Hurtaud, R. Delepine, P. Sanders, Analyst 119 (1994) 2731–2738.
[16] E. Dreassi, P. Corti, F. Bezzini, S. Furlanetto, Analyst 125 (2000) 1077–1081.