Journal of Pharmaceutical & Biomedical Analysis
Vol. 7, No. 12, pp. 1837–1846, 1989
Printed in Great Britain

0731–7085/89 $3.00 + 0.00
© 1990 Pergamon Press plc

Residue determination of two co-administered antibacterial agents — cephalexin and colistin — in calf tissues using high-performance liquid chromatography and microbiological methods*

PIERRE LEROY,† DOMINIQUE DECOLIN,† SERGE NICOLAS,‡ PHILIPPE ARCHIMBAULT‡ and ALAIN NICOLAS†§

†*Laboratoire de Chimie Analytique, URA CNRS 597, Faculté des Sciences Pharmaceutiques et Biologiques, B.P. 403, 54001 Nancy Cedex, France*
‡*Laboratoires VIRBAC S.A., B.P. 27, 06511 Carros Cedex, France*

Abstract: Residues of two antibacterial agents, cephalexin and colistin, co-administered by intramuscular injection to calves, were quantified in four different tissues (muscle, fat, liver and kidney) by column switching HPLC and by a microbiological method. For cephalexin assay, tissue samples with cephradin as internal standard were homogenized in a 5% trichloroacetic acid solution and filtrates were injected onto a concentration pre-column filled with LiChroprep RP-18 (25–40 μm). A clean-up step was incorporated by flowing a mobile phase (methanol–0.01 M phosphate buffer (pH 3.0); 15:85, v/v) through the enrichment column before elution on a LiChrospher RP-18e (5 μm) column with a methanol–phosphate buffer (30:70, v/v) at a flow rate of 1 ml min^{-1}. Spectrometric detection was at 260 nm. An additional "off-line" washing step of extracts with methylene chloride was operated to achieve higher selectivity in the case of liver and kidney samples. The limit for quantitative assay was 0.045 μg g^{-1} with relative standard deviations in the range 5–8% and recoveries within 70%.

For microbiological assay of colistin, samples were homogenized in 0.1 M hydrochloric acid–acetonitrile mixtures (3:1, v/v, for kidney and liver; 3:2, v/v, for fat and muscle). The supernatants were assayed by the cylinder plate method after evaporation to dryness under vacuum. *Bordetella bronchiseptica* ATCC 4617 was chosen as test organism. After a 3-h diffusion step at room temperature, the medium was incubated at 37°C for 18 h and then the diameter of the growth inhibition zones was measured. Sensitivity reached 0.10–0.15 μg g^{-1}. Results from the analysed samples over a 7–28 day period after drug administration show that no cephalexin was found at concentrations higher than the quantitation limit in the four test tissues and that colistin was found in muscle (injection site only) for 15 days and in kidney for 21 days.

Keywords: *Cephalexin; colistin; residues; calf tissues; reversed-phase HPLC; switching technique; microbiological assay; cylinder plate method;* Bordetella bronchiseptica.

*Presented at the "Third International Symposium on Drug Analysis", May 1989, Antwerp, Belgium.
§To whom correspondence should be addressed.

Introduction

Modern intensive animal breeding demands permanent suppression of different diseases caused by bacteria. Chemotherapeutic drugs are available for the prevention and control of these diseases. There is therefore the risk of unwanted residues in edible products from animals for food. Present regulations require the development of selective and sensitive analytical methods for the determination of undesirable veterinary drug residues contained in food stuffs. Withdrawal periods are fixed on the basis of elimination studies. Detection limits for the techniques used are of particular importance since they assume the character of tolerance levels for residues in meat at meat inspection.

At the present time, little information is available on cephalexin and colistin residues levels after intramuscular administration. This study deals with their determination in four different calf tissues (muscle, fat, liver and kidney) after treatment with a single veterinary formulation.

Literature surveys of β-lactam antibiotics [1–6] show that HPLC seems more efficient than other analytical techniques, especially for the detection of impurities, stability studies and therapeutic monitoring. Official or most accepted assays are now performed by HPLC with good correlation with microbiological assays [2]. However, conventional HPLC techniques are not in themselves suitable for trace analysis of cephalexin in tissues and should include either additional sample pretreatment steps or derivatization procedures. This work demonstrates that a pre-concentration step applied "on-line" with the use of a switching device gives the necessary selectivity and sensitivity without further development for analytical detection.

In the case of natural peptide antibiotics, the use of HPLC is not satisfactory because of polymorphism and detection problems. So, microbiological assays are still primarily involved for their quality control and measurement in biological matrices. For these, immunoassay techniques are more promising [7] but they need a long development time when antibodies are not commercially available. A classical microbiological assay was preferred in this study with few improvements for the extraction of colistin from tissues.

Experimental

Reagents, chemicals and standards

All chemicals and solvents used were of analytical reagent grade. Cephalexin monohydrate and colistin sulphate were obtained from VIRBAC S.A. (Carros, France). Biological potency of cephalexin and colistin were measured by a bacteriological assay (90.4 and 97.2%, respectively). Cephradin monohydrate was a generous gift of Smith Kline RIT S.A. (Rixensart, Belgium); the batch content of cephalexin (the main impurity) was measured and reached 3%.

Stock solutions of cephalosporins were prepared each day at a concentration of 0.1 mg ml^{-1} in 0.01 M phosphate buffer pH 3.0; they were diluted in the same buffer to obtain solutions for the determination of calibration curves in tissues and stored at +5°C pending analysis. Cephalexin concentrations of these standard solutions ranged from 0.45 to 18 $\mu g\ ml^{-1}$. They were added with a constant amount of cephradin used as internal standard (20 $\mu g\ ml^{-1}$ for kidney and liver samples, and 5 $\mu g\ ml^{-1}$ for muscle and fat). Aqueous stock solutions and dilutions of colistin were prepared daily at a concentration range of 2–20 $\mu g\ ml^{-1}$.

Treatment of animals

Twelve calves of Montbeliard or crossbred Montbeliard breed weighing between 60–80 kg, were selected for this study. They were isolated for 14 days prior to administration and were fed with a milk replacer. The oily suspensions of both antibiotics (containing 150 and 12 mg ml^{-1} of cephalexin as monohydrate, respectively, and colistin as sulphate) were administered by intramuscular injection in the neck. Animals received a 0.1 ml injection per kg every 12 h for 3 days in a single site localized alternately on each side. Three calves were killed at each time interval (i.e. 7, 14, 21 and 28 days) after the last injection. Tissue samples were frozen and stored at −20°C until they were analysed.

Sample preparation of calf tissues for cephalexin analysis

Minced tissue (10 g) was placed in a 100 ml flask and spiked with 1 ml standard solution of cephalexin and cephradin for calibration assays or internal standard solution alone for test samples. The flasks were left at room temperature for 30 min, 19 ml of a 5% trichloroacetic acid (TCA) solution previously chilled (+5°C) were added and the mixture was homogenized thoroughly using a Virtis model 45 fitted with U-shaped blades. The homogenate was centrifuged for 5 min at 1000***g***. The supernatant was filtered through a 0.2 μm Dynagard filter (Merck-Clevenot) and 2 ml of the filtrate was injected onto the enrichment column of the HPLC system.

For kidney and liver samples, an additional procedure was performed as follows: 10 ml of the supernatant was stirred with 20 ml methylene chloride–isopropanol mixture (95:5, v/v) for 2 min and centrifuged for 5 min at 1000***g***. The organic phase was discarded and the washing procedure was repeated after alkalination of the aqueous extract with about 0.25 ml concentrated ammonia solution. The initial pH value was then restored by adding concentrated hydrochloric acid.

Sample preparation of calf tissues for colistin analysis

Minced tissue (15 g) was spiked with 0.75 ml of colistin standard solution for calibration and left at room temperature for 30 min. Tissue samples were homogenized with the Virtis homogenizer for two periods of 5 min, first in 22.5 ml of 0.1 M hydrochloric acid and then in an additional volume of acetonitrile (7.5 ml for muscle and fat samples, 15 ml for liver and kidney samples). After centrifugation at 6500***g*** at +5°C for 10 min, the supernatant was filtered through a G-4 glass-filter. To the filtrate was added 50 μl of antifoaming agent (Rhodorsyl 70426R, Rhône Poulenc) followed by evaporation to dryness under vacuum at a temperature of 50°C with a rotatory evaporator. The dried residue was dissolved in 3 ml of 0.2 M phosphate buffer pH 8.0 and the resulting suspension centrifuged at 1000***g*** for 5 min. The supernatant was used for the microbiological assay.

Chromatographic equipment and operating conditions

The HPLC system used for cephalexin assay consisted of two solvent delivery pumps (pump A: model 300, Scientific Systems Inc., PA, USA; pump B: model Spectroflow 400 Kratos-ABI, Ramsey, NJ, USA), two six-way valves (valve 1: model 7125, Rheodyne, Cotati, CA, USA; valve 2: model 7010, Rheodyne), a UV variable wavelength spectrometric detector (model LC 233, Merck-Clevenot, Nogent-sur-Marne, France) and an integrator (model D-2000, Hitachi, Merck-Clevenot).

The guard- (Hibar RT 4 × 4 mm, Merck, Darmstadt, FRG) and analytical- (Hibar RT 250 × 4 mm) columns were prepacked with LiChrospher 100 CH-18 (5 μm) and always

used in-line. The enrichment column (25 × 4 mm) was dry-packed with LiChroprep RP 18 (25–40 μm) and fitted on valve 1 instead of the injection loop.

Mobile phases were methanol–0.01 M phosphate buffer, pH 3.0, mixtures (mobile phase A, 15:85, v/v; mobile phase B, 30:70, v/v), filtered through a 0.2-μm microfilter (model Sartorlon, Sartorius, France), degassed and used at a flow rate of 1 ml min^{-1}. Spectrometric detection was operated at 260 nm. Connections between the different parts of the HPLC system and successive operating steps occurring during the assay are more comprehensively described in Fig. 1. To avoid an excess of substances injected, the enrichment and guard columns were changed and the whole HPLC system was flushed with 60 ml of methanol–water (30:70, v/v) then with 30 ml of pure methanol after every 30 injections.

Microbiological assay

The stock suspension of the test organism *Bordetella bronchiseptica* ATCC 4617 was diluted 1:5 in Tryptic soy broth so that its transmittance at 550 nm in a 1-cm cell averaged 75%. Of the inoculum, 0.6 ml was mixed with 120 ml of culture medium No. 10 (ref. 0463-17-1) from Difco. 18 ml of the harvested suspension was distributed into Petri dishes (120 × 120 mm). Stainless steel cylinders (8 mm, i.d., × 10 mm, height) were used; 200 μl of the solution resulting from tissue extraction was poured into each cylinder. After 3-h diffusion at room temperature and incubation for 18 h at 37°C, inhibition zones were measured with a precision of 0.5 mm.

Results and Discussion

Cephalexin residues analysis

HPLC techniques used for the analysis of cephalexin and other cephalosporins in biological fluids usually imply therapeutic monitoring at concentration levels higher than 0.5 μg ml^{-1} in urine or plasma. Few reports are available concerning tissue determination in meat-producing animals.

The usual analytical procedures include a simple sample preparation with protein precipitation using TCA [8–10], methanol [4, 9, 11, 12] or acetonitrile [13], chromatographic separation in reversed-phase mode with the possible addition of an ion-pairing reagent in the mobile phase and UV detection. A detailed description of all the chromatographic conditions for HPLC of cephalosporins has been already given [8, 9, 14]. Trace analysis of cephalexin in tissues using conventional methods [11, 12, 15] results in lack of sensitivity due to spectrometric detection and selectivity being compromised by endogenous compounds.

Improvement in sensitivity can be obtained by using either electrochemical detection or a derivatization reaction. However, common β-lactam antibiotics do not exhibit electrooxidative properties on a glassy carbon electrode over a potential range of 0–1.2 V, except for cephalosporins with an aminothiazole substituent in the side chain [13]. Post-column bromination [16] or photolytic degradation [17] were proposed to produce electroactive species but the poor selectivity of these derivatization reactions were a limiting factor for the analysis of biological material containing antibiotics.

The widely used ortho-phthalaldehyde (OPA) reaction for primary aliphatic amines has been applied to various β-lactam antibiotics in a post-column derivatization mode but there are difficulties in reaching optimum fluorescence [18, 19].

Another fluorogenic reagent for amino groups, flourescamine, may be more suitable

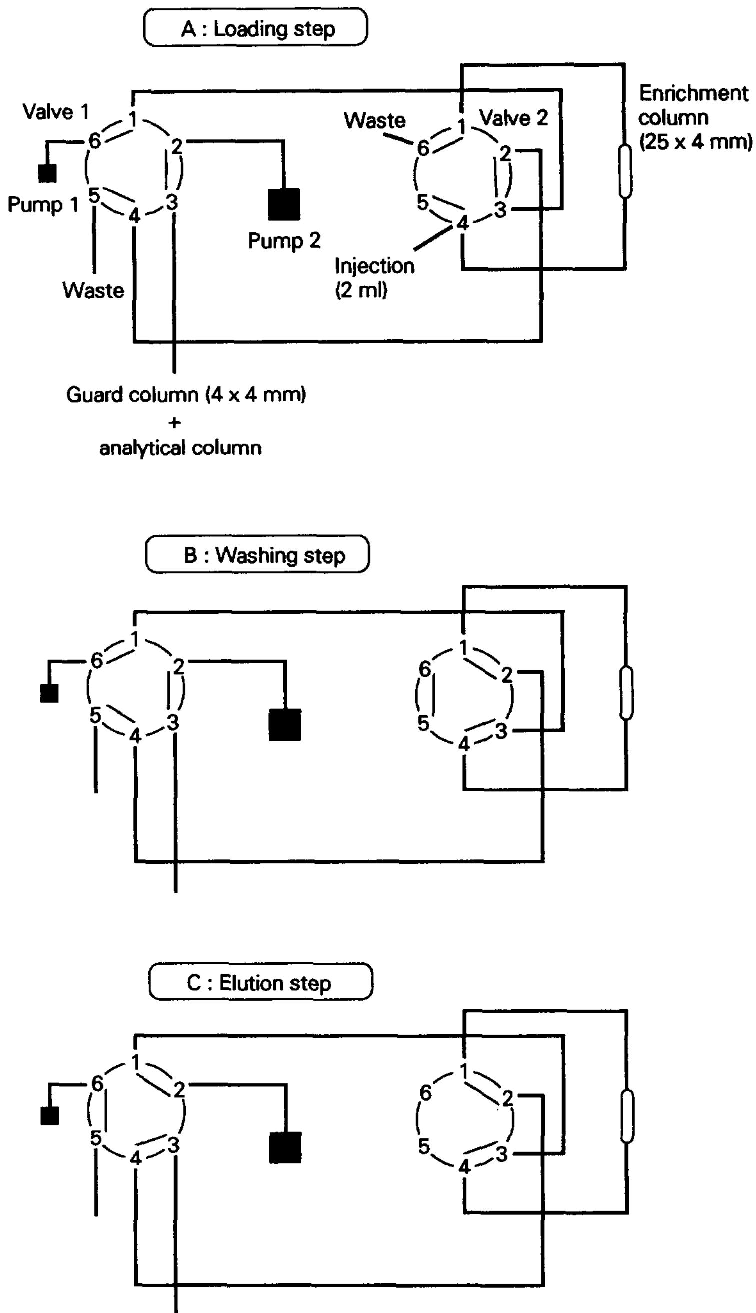

Figure 1
Scheme of the column-switching HPLC system and successive operations performed during the cephalexin assay.

for cephalosporin derivatization [10, 16, 20] since the reaction occurs at lower pH values (i.e. 7–8) than for OPA.

Post-column alkaline degradation of penicillins and β-lactam antibiotics with mercuric ions [21] has been reported to afford derivatives with UV spectral properties between 300–350 nm, and for this reason a better selectivity from endogenous compounds is claimed.

A pre-column derivatization has also been proposed using imidazole and metallic ions but was only applied to standards [19]. Recently, benzylpenicillin residues were measured in milk [22] and in other biological fluids [23] by this method.

Since no great improvement of detection sensitivity in biological fluids was reported with the derivatization procedures mentioned above, in this work attention has been focused on the development of sample preparation and clean-up steps. UV spectrometry has been retained for detection. The amphoteric properties of cephalexin preclude a liquid–liquid extraction with organic solvents as the usual concentration step carried out during this operation in drug analysis. Thus the protein precipitation has to be retained as initial pretreatment of tissue samples. For this purpose, methanol and acetonitrile were tested as solvents for tissue homogenization but no recovery of cephalexin was observed in dried residues resulting from the evaporation of the organic layers. TCA was therefore preferred because of its ability to cleave protein–drug bonds without significant sample dilution. Moreover, the fully aqueous solution obtained after tissue homogenization is suitable for a concentration step on a column filled with an hydrophobic stationary phase such as LiChroprep RP-18. This column operation was efficient for increasing the sensitivity of spectrometric detection when standards were tested, but a large frontal peak coelutes with cephalosporins when tissue samples are used. The enrichment column had to be washed to eliminate interfering compounds before the elution took place. The whole procedure was conducted "on-line" in the HPLC system and did not increase handling and analysis time. The elaborated switching device operates in a "fore-flush" mode. The resulting peak-broadening was reasonable with the decrease in the number of plates for cephalexin and cephradin being <10% when the enrichment column was used instead of a 100-μl loop. No elution of cephalosporins from the enrichment column occurs during the injection of acidic extracts and washing step, which were optimized: elution period, 1.5 min, and the proportion of methanol in the mobile phase A, 15% less than in the mobile phase B used for the elution onto the analytical column.

An additional clean-up step of extracts from kidney and liver samples was performed before injection onto the HPLC system in order to remove endogenous compounds eluting close to the analysed cephalosporins and found in higher amounts than in other tissues. Successive washings of acidified and alkalinized extracts with an apolar organic solvent mixture were found to be successful for the selective elimination of interfering substances.

The HPLC analysis was performed on a 5-μm octadecylsilane endcapped silica column (LiChrospher 100 CH-18e). Throughout the experiment, three columns of different batches were used and gave no significant variations in chromatographic profiles of tissue extracts. Typical chromatograms from the four tissues are shown in Figs 2 and 3. Parameters for validation of the cephalexin assay are given in Table 1.

Colistin residues analysis

An extraction procedure of colistin from tissues has been developed in this study. It

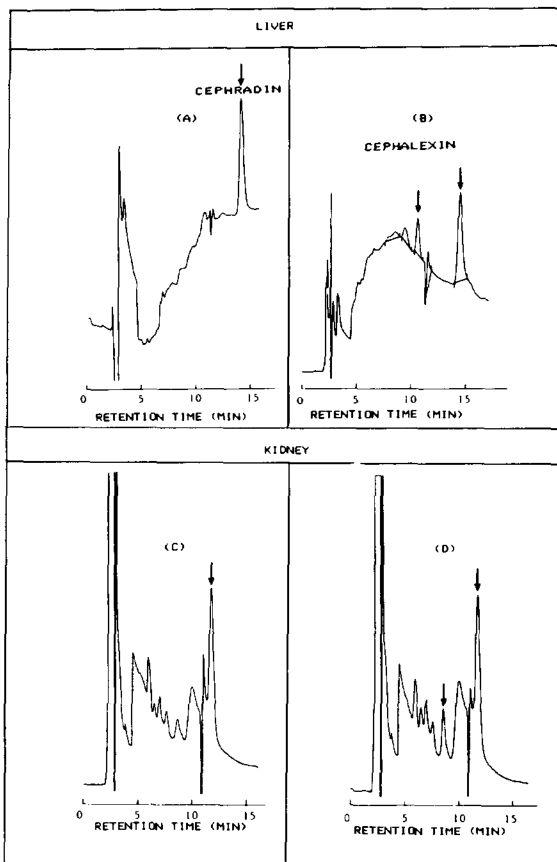

Figure 2
Chromatograms from samples of calf liver (A, B) and kidney (C, D). A, C: Blanks spiked with cephradin as internal standard. B, D: Sample spiked with cephalexin (0.255 $\mu g\ g^{-1}$) and cephradin. Chromatographic conditions are detailed in Fig. 1 and in the Experimental section.

allows the release of the antibiotic from tissues by homogenation in acidic medium with the addition of acetonitrile. The evaporation of the extract allows concentration of the sample in a 1:5 ratio. This together with the initial "prediffusion step" increases the sensitivity of the microbiological assay.

Prediffusion allows antibiotic diffusion to proceed at a temperature low enough to prevent growth of the test culture [25]. Linearity and accuracy results for this assay are reported in Table 2.

Residues quantitation

The developed techniques have quantitation limits of 0.045 and 0.10 $\mu g\ g^{-1}$ respectively for cephalixin and colistin. As the drugs were co-administered, the residues of the two antibiotics ought to be determined in the same samples of tissues. Usually, accepted requirements need the analysis of samples over a period related to the rate of elimination of the drugs. In the case of multi-drug administration, the one with the slower elimination rate fixes the withdrawal period. Few data are available concerning the disposition and metabolism of antibiotics in animal tissues. If hydrophobicity and

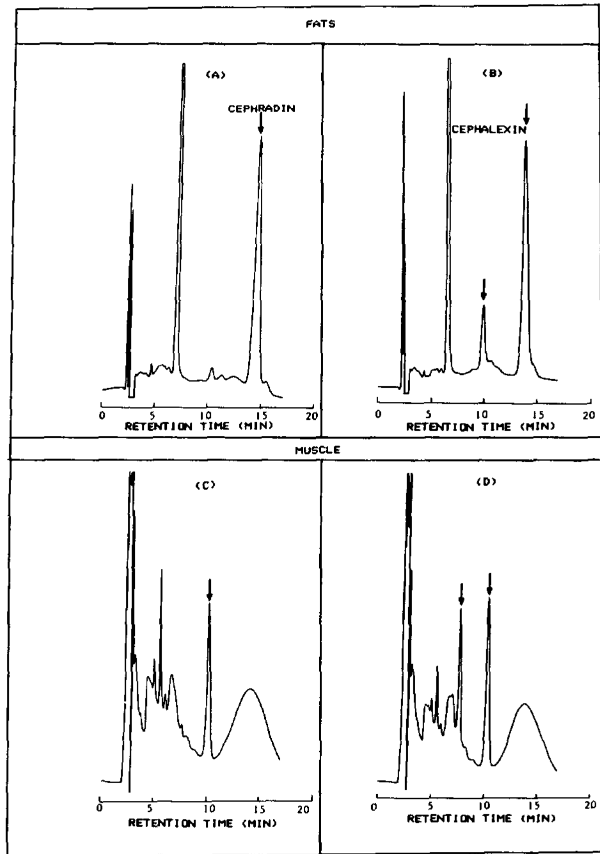

Figure 3
Chromatograms from samples of calf fats (A, B) and muscle (C, D). A, C: Blanks spiked with cephradin as internal standard. B, D: Sample spiked with cephalexin (0.225 μg g^{-1}) and cephradin. Chromatographic conditions are detailed in Fig. 1 and in the Experimental section.

Table 1
Calibration curves and reproducibility results obtained for the measurement of cephalexin in four different calf tissues by the HPLC assay

Calf tissue	Calibration curve equation*	Regression coefficient	Recovery (%)	RSD %†
Liver	$y = 1.13x - 0.05$	0.997	60	7.9
Kidney	$y = 0.475x - 0.038$	0.999	65	7.3
Fat	$y = 2.535x - 0.038$	0.999	85	5.6
Muscle	$y = 3.68x - 0.23$	0.998	70	6.7

*Obtained for six different concentration values ranging from 0.045 to 1.8 μg g^{-1} of cephalexin.
†Calculated from five replicate assays performed on calf tissue sample blank spiked with 0.09 μg g^{-1} of cephalexin.

human plasmatic data [24] are considered, cephalexin must be eliminated first, but the oily vehicle of the medicinal preparation and the large injected volume (about 50 ml) could delay the diffusion step. Moreover, accumulation in specific organs and/or covalent binding to tissues might occur. Samples of tissue were collected over a 7–28 day

Table 2
Calibration curves and reproducibility results obtained for the measurement of colistin in four different calf tissues by the microbiological assay

Calf tissue	Calibration curve equation*	Regression coefficient	RSD %†
Liver	$y = 4.842 \log x + 17.050$	0.9642	12.5
Kidney	$y = 8.316 \log x + 18.664$	0.9894	10.4
Fat	$y = 6.176 \log x + 24.635$	0.9963	16.5
Muscle	$y = 4.842 \log x + 19.866$	0.9642	16.5

*Obtained for six different concentration values ranging from 0.10 to 1 $\mu g\ g^{-1}$ of colistin.
†Calculated for 10 replicate assays performed on calf tissue sample blank spiked with 0.15 $\mu g\ g^{-1}$ of colistin.

Table 3
Residual concentrations ($\mu g\ g^{-1}$) of colistin found in the four tested calf tissues at different intervals after the last i.m. administration

Interval time (days)	Animal number	Liver	Kidney	Fat	Muscle	
					Thigh	Injection site
	1	<0.1	33.9	0	0	0.7
7	2	<0.1	13.3	0	0	0.3
	3	<0.1	2.8	0	0	0.2
	4	0	0.9	<0.1	0	0.2
14	5	0	1.4	0	0	0.2
	6	<0.1	4.2	<0.1	0	0.3
	7	0	0.6	0	0	0
21	8	0	1.3	0	0	0
	9	<0.1	0.5	0	0	0
	10	<0.1	0	0	0	0
28	11	<0.1	0	0	0	0

period, earlier sampling was not necessary since colistin was taken to be the limiting drug for elimination purposes.

No cephalexin was found in the four tissues at concentrations higher than the quantitation limit. In spite of the oily excipient, this confirms its elimination from the site of injection and consequently from the other tissues. On the contrary, colistin recoveries in various tissue samples tested (Table 3) show a slower elimination of this antibacterial agent in kidney and in muscle (injection site).

The late elimination of colistin requires a withdrawal period of at least 4 weeks before meat can be consumed after intramuscular injection of cephalexin–colistin according to the dosage applied.

Conclusions

Determination of veterinary drug residues in tissues, which is necessary to assure the safety of consumers, is performed by still more sophisticated analytical systems to obtain the required selectivity, sensitivity and a high frequency of analysed samples. Moreover, the search for a versatile method which can be easily extended to the entire group of the drug considered here may look interesting with regard to the long time needed to achieve an analytical development with all cited criteria. In the present study, a quantitation limit

of 45 ppb of cephalexin, which stated a 10-fold improvement versus usual techniques, was obtained with a simple apparatus including only a switching device between enrichment and analytical columns, coupled with a conventional UV spectrometric detection. For higher sensitivity or better selectivity, improvement of the described HPLC system should be applied to the detection phase and numerical treatment of chromatographic data. Different papers published in the course of this study confirm our choice of these analysis procedures of β-lactam antibiotics residues: it consists of pre-column derivatization of benzylpenicillin and digital subtraction between assay and blank chromatograms for the measurement of very low concentration levels [22, 23, 26, 27].

A microbiological assay was selected for colistin since a HPLC procedure cannot be performed for this natural multicomponent antibiotic. Nevertheless, good sensitivity, and no cross interference with the co-administered antibiotic were obtained.

References

[1] A. Aszalos, *Trends. Analyt. Chem.* **1**, 387–393 (1982).
[2] A. H. Thomas, *J. Pharm. Biomed. Anal.* **5**, 319–324 (1987).
[3] R. D. Toothaker, D. C. Scottwright and L. A. Pachla, *Antimicrob. Agents Chemother.* **31**, 1157–1163 (1987).
[4] S. A. Signs, T. M. File and J. S. Tan, *Antimicrob. Agents Chemother.* **26**, 652–655 (1984).
[5] J. J. Kirschbaum and A. Aszalos, in *Modern Analysis in Antibiotics* (A. Aszalos, Ed.), pp. 239–322. Marcel Dekker, New York (1986).
[6] F. Kedzierewicz, C. Finance, A. Nicolas, P. Dixneuf and M. Hoffman, *J. Pharm. Sci.* **78**, 73–77 (1989).
[7] T. Kitagawa, W. Ohtani, Y. Maeno, K. Fujiwara and Y. Kimura, *J. Assoc. Off. Analyt. Chem.* **68**, 661–664 (1985).
[8] M. C. Rouan, F. Abadie, A. Leclerc and F. Juge, *J. Chromatogr.* **275**, 133–144 (1983).
[9] J. B. Lecaillon, M. C. Rouan, C. Souppart, N. Febvre and F. Juge, *J. Chromatogr.* **288**, 257–267 (1982).
[10] E. Crombez, G. Van Der Weken, W. Van Den Bossche and P. De Moerloose, *J. Chromatogr.* **177**, 323–332 (1979).
[11] K. Jsutsumi, H. Kubo and T. Kinoshita, *Analyt. Lett.* **14**, 1735–1743 (1981).
[12] M. C. Nahata, *J. Chromatogr.* **225**, 532–538 (1981).
[13] H. Fabre, M. D. Blanchin and W. Th. Kok, *Analyst* **133**, 651–655 (1988).
[14] I. Wouters, S. Hendrickx, E. Roets, J. Hoogmartens and H. Vanderhaeghe, *J. Chromatogr.* **291**, 59–80 (1984).
[15] T. Nakagawa, J. Haginaka, K. Yamaska and T. Uno, *J. Chromatogr.* **147**, 509–512 (1978).
[16] M. D. Blanchin, W. Th. Kok and H. Fabre, *Chromatographia* **24**, 625–627 (1987).
[17] C. M. Selavka, I. S. Krull and K. Bratin, *J. Pharm. Biomed. Anal.* **4**, 83–89 (1986).
[18] M. E. Rogers, M. W. Adlard, G. Saunders and G. Holt, *J. Chromatogr.* **257**, 91–100 (1983).
[19] M. E. Rogers, M. W. Adlard, G. Saunders and G. Holt, *J. Chromatogr.* **297**, 385–391 (1984).
[20] T. L. Lee, L. D'Arconte and M. A. Brooks, *J. Pharm. Sci.* **68**, 454–458 (1979).
[21] J. Haginaka and J. Wakai, *J. Pharm. Pharmac.* **39**, 5–8 (1987).
[22] B. Wiese and K. Martin, *J. Pharm. Biomed. Anal.* **7**, 95–106 (1989).
[23] B. Wiese and K. Martin, *J. Pharm. Biomed. Anal.* **7**, 107–118 (1989).
[24] L. P. Marrelli, in *Analytical Profiles of Drugs* (E. Florey, Ed.), pp. 42–43. Academic Press, New York (1975).
[25] T. B. Platt, in *Modern Analysis in Antibiotics* (A. Aszalos, Ed.), pp. 341–386. Marcel Dekker, New York (1986).
[26] B. Wiese and K. Martin, *J. Pharm. Biomed. Anal.* **7**, 67–68 (1989).
[27] B. Wiese, *J. Pharm. Biomed. Anal.* **7**, 79–93 (1989).

[*Received for review 21 April 1989; revised manuscript received 28 August 1989*]