Christian Horstkötter[1]
Eulalia Jiménez-Lozano[2]
Dolores Barrón[2]
José Barbosa[2]
Gottfried Blaschke[1]

[1]Institute of Pharmaceutical and Medicinal Chemistry, University of Münster, Münster, Germany
[2]Department of Analytical Chemistry, University of Barcelona, Barcelona, Spain

Determination of residues of enrofloxacin and its metabolite ciprofloxacin in chicken muscle by capillary electrophoresis using laser-induced fluorescence detection

A method for the residue analysis of the veterinary antimicrobial agent enrofloxacin and its active desethyl metabolite ciprofloxacin in chicken muscle tissue has been developed and validated. The detection of the analytes was performed by laser-induced fluorescence (LIF) detection using a HeCd laser (λ_{ex} = 325 nm) providing an enhancement in sensitivity and selectivity compared to conventional UV detection. The assay has been validated with satisfying results. The limits of quantification for enrofloxacin and ciprofloxacin were 5 μg/kg and 20 μg/kg, respectively, with a fivefold preconcentration yielded by a sample clean-up with a simple liquid-liquid extraction procedure. Calibration graphs were linear from 5 to 1000 μg/kg for enrofloxacin and from 20 to 1000 μg/kg for ciprofloxacin. The assay allows the detection of contaminated muscle samples at the required maximum residue limit of the European Union, which is 100 μg/kg for the sum of enrofloxacin and ciprofloxacin.

Keywords: Chicken muscle / Ciprofloxacin / Enrofloxacin / Laser-induced fluorescence detection
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1 Introduction

Antibacterial agents are frequently used in the treatment of food-producing animals with either curative or prophylactic aim. Intensive use of antibiotics in general, and quinolones in particular, in humans as well as in industrial farming has led to a significant increase in antimicrobial resistance, having therefore important consequences on public health [1–3]. To this effect monitoring is necessary to ensure that antibacterial agents are not present at levels that may pose risks to the public health. To ensure consumer safety, the European Union (EU) in council regulation 2377/90 and its later modifications [4, 5] has established maximum residue limits (MRL) for residues of veterinary drugs in animal tissues and derivative foodstuffs entering the human food chain. Among the antimicrobial agents, enrofloxacin is a fluoroquinolone developed exclusively for use in veterinary medicine, and it is extensively used in poultry, pigs and cattle in Europe. The active metabolite of enrofloxacin in several species is ciprofloxacin, therefore, the assay must also be capable to distinguish between these drugs. The MRL fixed for the sum of enrofloxacin and its main metabolite ciprofloxacin is 100 μg/kg (0.1 ppm) in chicken muscle [4].

Several methods have been reported for the determination of residues of enrofloxacin and ciprofloxacin in various biological matrices [6]. They are mainly based on HPLC determinations with ultraviolet [7–10], fluorescence [11–14] and mass spectrometry detection [15–18]. However the literature reports only a few methods using capillary electrophoresis (CE) to analyze quinolones in body fluids, pharmaceutical formulations and animal tissues [19–25]. CE has become a very useful tool for pharmaceutical analysis because of its high resolution, speed and the extremely small sample volume required [26]. However, the use of CE in the analysis of drugs is restricted due to the low concentration sensitivity of this technique, and further preconcentration and clean up of the samples are needed involving different treatments like liquid-liquid or solid-phase extractions [9, 13, 23]. However, the use of laser-induced fluorescence detection (LIF) in CE does not only improve the limit of quantification (LOQ) of analytes with native fluorescence but also allows the analysis in biological matrices with enhanced selectivity [21, 27].

In this work, we report the use of CE and LIF detection using a 325 nm HeCd laser in order to establish a methodology that allows the determination of enrofloxacin and ciprofloxacin in chicken muscle at concentration below of the permissible MRL, established products by the EU.

Correspondence: Prof. Dr. Gottfried Blaschke, Institute of Pharmaceutical and Medicinal Chemistry, University of Münster, Hittorfstraße 58–62, D-48149 Münster, Germany
E-mail: blaschg@uni-muenster.de
Fax: +49-251-83-32144

Abbreviations: CIP, ciprofloxacin; **DIF**, difloxacin; **ENR**, enrofloxacin; **MRL**, maximum residue limits

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2 Materials and methods

2.1 Chemicals and reagents

Enrofloxacin (1-cyclopropyl-7-(4-ethyl-1-piperazinyl)-6-fluoro-1,4-dihydro-4-oxo-3-quinolinecarboxylic acid; ENR) was obtained from Cenavisa S. A. (Tarragona, Spain), ciprofloxacin (1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinolinecarboxylic acid; CIP) from Ipsen-Pharma (Barcelona, Spain) and difloxacin (6-fluoro-1-(*p*-fluorophenyl)-1,4-dihydro-7-(4-methyl-1-piperazinyl)-4-oxo-3-quinolinecarboxylic acid; DIF) used as internal standard (IS) from Abbott S. A. (Madrid, Spain). Structures of enrofloxacin, ciprofloxacin and difloxacin are shown in Fig. 1. All chemicals used in the preparation of buffers and solutions were analytical reagent grade. Phosphoric acid (85%), triethylamine, glacial acetic acid, sodium hydroxide, dichloromethane and hexane were obtained from J. T. Baker (Deventer, Holland), potassium dihydrogen phosphate and sodium hydroxide solution (0.1 M) were supplied by E. Merck (Darmstadt, Germany). Water was purified by bidistillation.

2.2 Instrumentation

A Beckman P/ACE System 2100 equipped with a LIF detector (Beckman Instruments, Fullerton, CA, USA) was used with an untreated fused-silica capillary of 50 μm ID × 375 μm OD, 20 cm effective length and 27 cm total length (Polymicro Technologies, Phoenix, AZ, USA). Fluorescence excitation was provided by a HeCd laser (Omnichrome, Series 74, Laser 2000, Wessling, Germany) with 20 mW and a wavelength of 325 nm. The laser was connected to the LIF detector of the CE system by an optical fiber (Omnichrome POS FDS-A1/2, Laser 2000). Data collection and processing were performed using System Gold software 7.11 (Beckman Instruments). For pH adjustment, a digital pH-meter (pH522, WTW, Weilheim, Germany) with a pH electrode (SenTix 50, WTW) was used. Homogenization of the muscle tissue samples was approached by an Ultra-Turrax (Janke und Kunkel, Staufen, Germany). Centrifugation steps were performed either (4000 rpm) by a Labofuge 400R (Heraeus Instruments, Osterode, Germany) or (12 000 rpm) by a Jouan MR 1812 centrifuge (Jouan, St. Nazaire, France).

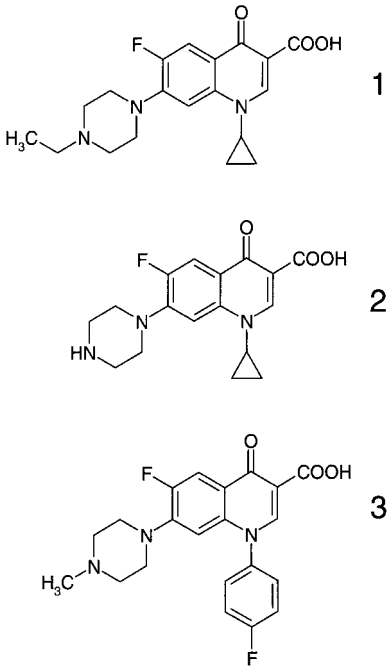

Figure 1. Chemical structures of (1) enrofloxacin, (2) ciprofloxacin and (3) difloxacin (IS).

2.3 Preparation of reagents and stock solutions

For preparation of the stock solutions of the analytes ENR and CIP, about 1 mg of each compound, exactly weighed, was dissolved in 20.0 mL acetic acid (50 mM) by ultrasonic treatment. The IS difloxacin was dissolved in 50 mM acetic acid at a concentration of about 50 μg/mL. The buffer used for the extraction of the chicken muscle samples was prepared from a 50 mM potassium dihydrogen phosphate solution adjusted to pH 7.0 by dropwise addition of 3 M sodium hydroxide solution. The electrophoresis buffer was a 100 mM phosphoric acid solution adjusted with triethylamine to pH 2.2.

2.4 Extraction procedure

Five grams of thawed and minced chicken muscle tissue were accurately weighed and placed into a 50 mL centrifuge tube. In order to prepare reference solutions, samples were spiked by adding appropriate volumes of stock solutions of ENR and CIP and 50 μL of IS solution to the samples. Then, water was added to obtain a final spiking volume of 1 mL. The samples were allowed to stand for 20 min in the dark before extraction. Afterwards 1.5 mL of extraction buffer pH 7.0 were added before homogenizing the mixture with an Ultra-Turrax for 3 min. Dichloromethane (20 mL) was added to the sample in order to extract the quinolones. After shaking for 10 min, the mixture was centrifuged at 3500 rpm (5 min). The sample was re-extracted with another portion of 10 mL of dichloromethane and again centrifuged. The organic phases were combined and transferred into a 50 mL heart-shaped flask, before 1 mL of 100 mM phosphoric acid was added. The dichloromethane was evaporated under vacuum at 30°C in a rotary evaporator and the residue

(phosphoric acid phase) was defatted by extraction with 10 mL of hexane. The mixture was transferred into a 15 mL centrifuge tube and, in order to compensate the losses of aqueous phase during the evaporation, double-distilled water was added until the lower phase was refilled up to 1 mL. After centrifugation for 10 min at 4000 rpm to achieve a complete phase separation, the lower aqueous phase was transferred into an Eppendorf cap and finally again centrifuged for 10 min at 12 000 rpm. An aliquot of this solution was injected into the CE system.

2.5 Capillary electrophoresis

Half an hour before starting a series, the laser was shut on and the capillary was rinsed with 0.1 M NaOH. Rinsing procedures were always performed at a pressure of 20 psi (= 1379 mbar). Prior to each sample solution, the capillary was flushed for 3 min with running buffer. Samples were introduced into the capillary at the anodic side by pressure injection with 0.5 psi (= 34.5 mbar) for 18 s. Separation was carried out by applying a voltage of 18 kV (667 V/cm). The resulting current was at about 80 μA. The temperature of the capillary cartridge was maintained at 20°C by a liquid cooling system. Detection was performed by LIF using a HeCd laser with an excitation wavelength of 325 nm. A 450 nm interference filter was used in order to select the most appropriate emission wavelength and to suppress the excitation light scattering of the laser beam. After each run, the capillary was rinsed with 0.1 M NaOH and double-distilled water for 1 min each before again re-equilibrating with running buffer. The running buffer was daily exchanged. At the end of a series the capillary was always rinsed with 0.1 M NaOH and double-distilled water for 5 min each, and was dried afterwards with nitrogen/air for 5 min.

2.6 Calibration and validation

Validation was performed according to the FDA guideline for bioanalytical assay validation [28]. For the calibration of the assay, spiked standard samples at eight different concentration levels covering the range from 5 μg/kg to 1 mg/kg for ENR (0.005, 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, and 1 mg/kg) and from 20 μg/kg to 1 mg/kg for CIP (0.02, 0.05, 0.1, 0.2, 0.3, 0.5, 0.8, and 1 mg/kg) were prepared and extracted according to Section 2.4. Each calibration sample was analyzed three times, and the mean corrected peak areas were evaluated. Calibration lines were obtained by plotting the concentration on the abscissa *versus* corrected peak area ratios (corrected peak area of the analyte related to the corrected peak area of the IS) on the ordinate and applying a $1/x^2$-weighted linear regression model on the data. To assess intraday accuracy and precision of the assay, five standard samples at three concentration levels each (0.005, 0.1 and 1 mg/kg for ENR and 0.02, 0.3 and 1 mg/kg for CIP) were spiked, extracted and analyzed. The procedure was repeated on three consecutive days to determine interday variability. Each day, separately weighed stock solutions of the analytes were prepared. Recovery experiments for ENR, CIP and DIF were performed by comparing the analytical results for extracted standard samples at the same concentration levels as above (0.005, 0.1 and 1 mg/kg for ENR and 0.02, 0.3 and 1 mg/kg for CIP) with unextracted standards prepared at the same concentrations in blank extract representing 100% recovery. For DIF however, recovery was naturally determined only at one concentration level (0.5 mg/kg). A total absence of matrix interference was confirmed through analysis of two different lots of blank extract.

2.7 Application

The assay was applied to a sample of enrofloxacin-contaminated pig muscle tissue provided by Staatliches Veterinäruntersuchungsamt in Münster, Germany. In this case a real chicken muscle sample was not available, and the fact that analytical procedures in veterinary control centers are applied in general for all animal muscle tissues was considered. To an aliquot of 5 g of the minced sample, 50 μL of IS solution and 950 μL water were added. Afterwards, the sample was extracted and analyzed as described in Sections 2.4. and 2.5.

3 Results and discussion

3.1 Extraction procedure

The extraction procedure should be as simple as possible, as due to the high sensitivity of the LIF detection technique, no high preconcentration factors were necessary for the determination. The method described in this paper based on a previously published liquid-liquid extraction procedure, that has been modified in some details [12]. Recovery values for the tertiary amines ENR and DIF were quite good, with 68% and 64%, respectively; for CIP as secondary amine, however, it was low with 22%, thus, the extraction may be further improved. Anyway, the recovery was constant for both analytes over the entire working range as well as for the IS DIF. Performing the extraction it was of great importance to add the acidic aqueous phase to the dichloromethane extract before the evaporation, otherwise the secondary amino group of CIP seemed to react with matrix compounds during the evaporation. This led to irreproducible and

nonlinear correlating results in the calibration of CIP. The tertiary amines ENR and DIF did not show this behavior. By the addition of phosphoric acid to the dichloromethane phase, CIP was protonated and mostly transferred into the aqueous phase, which obviously protected the secondary amine structure from chemical degradation.

3.2 Capillary electrophoresis

Electrophoretic conditions have been optimized in particular with regard to the requirements of the LIF detection. Since fluorescence intensities of the quinolones studied were low at alkaline conditions, an acidic electrophoresis buffer at pH 2.2 was chosen. The exact adjustment of the buffer pH however is of less importance, as it has been shown, that the electrophoretic mobilities of the quinolones hardly change within the pH range between 2.0 and 4.0 [23, 29, 30].

In order to minimize the run time of the assay and to increase the sample throughput, the capillary length (20/27 cm) was chosen as short as possible and the applied voltage was set at 18 kV. At a higher voltage and a resulting current of more than 100 μA current break-downs very often occurred in the system.

ENR, its metabolite CIP and the IS DIF were readily separated within 7 min with migration times of 5.7, 5.2 and 6.2 min, respectively (Fig. 2A). Due to the extraction procedure, a cleanup and a 5-fold preconcentration (5 g chicken muscle tissue led to 1 mL of the final extract solution) was realized. With HeCd-LIF detection, LOQs of 5 μg/kg and 20 μg/kg for ENR and CIP, respectively, were obtained (Fig. 2B). The differences in the LOQ observed for the two analytes are mainly related to the low recovery of CIP in this assay. Comparing to a previously published CE-UV assay for ENR and CIP in chicken muscle tissue with a 50-fold preconcentration [23], the LOQ was lowered for ENR (5-fold) as well as for CIP (2.5-fold). To exactly compare both assays with regard to their sensitivity, the relevant data is given in Table 1.

3.3 Validation data

Linearity of the method was assessed by a calibration in the concentration range between 0.005 and 1 mg/kg for ENR and 0.02 and 1 mg/kg for CIP. Calibration functions calculated by $1/x^2$-weighted linear regression were $y = 2.7461\,x + 0.0030$ for ENR ($r = 0.9986$) and $y = 0.4739\,x - 0.0028$ for CIP ($r = 0.9979$). The corresponding residual plots for ENR and CIP are presented in Fig. 3. The results of intra- and interday precision and accuracy experiments are summarized in Tables 2 and 3. According to [28] for

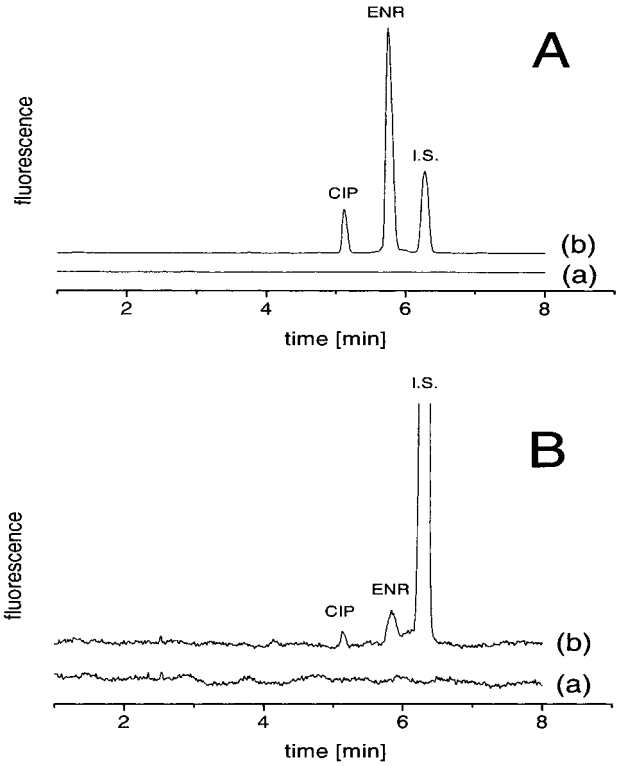

Figure 2. Electropherograms of (A) blank chicken muscle (a) and a spiked chicken muscle sample (b) containing 1000 μg/kg of ENR and CIP and (B) blank chicken muscle (a) and a spiked chicken muscle sample at the LOQ (b) with 5 μg/kg of ENR and 20 μg/kg of CIP (electrophoretic conditions: 20/27 cm capillary (ID = 50 μm); applied voltage, 18 kV; temperature, 20°C; running buffer, 100 mM phosphate/ triethylamine, pH 2.2; LIF detection, λ_{ex} = 325 nm, λ_{em} = 450 nm).

Table 1. Comparison of the sensitivity of the CE-LIF assay presented in this paper with a previously published CE-UV assay [23]

Parameter	CE-LIF assay		CE-UV assay [23] [a]	
	ENR	CIP	ENR	CIP
LOQ (μg/kg)	5 μg/kg	20 μg/kg	25 μg/kg	50 μg/kg
Extraction procedure	Liquid-liquid extraction		Liquid-liquid extraction and SPE	
Preconcentration factor	5		50	
Recovery	68%	22%	74%	54%
Total injected volume	35.2 nL		12.8 nL	
Optical path length (ID of the capillary)	50 μm		75 μm	

a) Electrophoretic conditions of the cited CE-UV assay: 40/47 cm capillary (ID = 75 μm); applied voltage, 20 kV; temperature, 25°C; running buffer, diethylmalonic acid buffer, pH 8.22; UV detection, λ = 275 nm

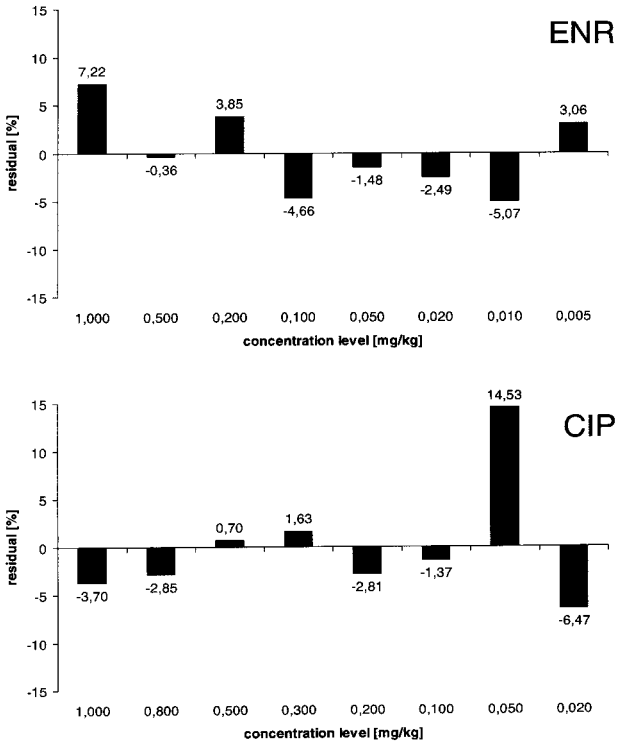

Figure 3. Residual plots of the calibration lines of ENR and CIP.

Table 2. Intra- and interday accuracy and precision data of the determination of ENR

Nominal concentrations of enrofloxacin (μg/kg)			
	5	100	1000
Concentration found (arithmetic mean value, μg/kg)			
Day 1 (*n* = 5)	5.54	107.8	1006
Day 2 (*n* = 5)	5.14	102.7	1027
Day 3 (*n* = 5)	4.81	107.6	1061
Interday (*n* = 15)	5.16	106.0	1031
Accuracy (arithmetic mean value, %)			
Day 1 (*n* = 5)	110.8	107.8	100.6
Day 2 (*n* = 5)	102.7	102.7	102.7
Day 3 (*n* = 5)	96.1	107.6	106.1
Interday (*n* = 15)	103.2	106.0	103.1
Precision (relative standard deviation, %)			
Day 1 (*n* = 5)	16.86	5.31	1.22
Day 2 (*n* = 5)	16.48	3.41	2.73
Day 3 (*n* = 5)	9.41	2.89	1.54
Interday (*n* = 15)	14.69	4.37	2.90

bioanalytical assays, precision should be less than 15% (less than 20% at the LOQ) and accuracy should be within 85% and 115% (within 80% and 120% at the LOQ, respectively). In our experiments, precision was usually lower than 5.5% at the higher concentration levels and did not exceed 17% at the LOQ. Accuracy values always ranged between 96.1% and 113.3% indicating that the assay fulfilled the requirements. Results of the recovery experiments have just been presented and discussed in Section 3.1.

Table 3. Intra- and interday accuracy and precision data of the determination of CIP

Nominal concentrations of ciprofloxacin (μg/kg)			
	20	300	1000
Concentration found (arithmetic mean value, μg/kg)			
Day 1 (*n* = 5)	22.46	327.5	1049
Day 2 (*n* = 5)	21.38	311.9	971
Day 3 (*n* = 5)	21.39	328.6	1121
Interday (*n* = 15)	21.74	322.7	1047
Accuracy (arithmetic mean value, %)			
Day 1 (*n* = 5)	112.3	109.2	104.9
Day 2 (*n* = 5)	106.9	104.0	97.1
Day 3 (*n* = 5)	106.9	109.5	112.1
Interday (*n* = 15)	108.7	107.6	104.7
Precision (relative standard deviation, %)			
Day 1 (*n* = 5)	11.25	4.70	2.91
Day 2 (*n* = 5)	12.41	4.51	2.02
Day 3 (*n* = 5)	15.16	3.22	1.57
Interday (*n* = 15)	12.46	4.61	6.43

3.4 Application

As it was not possible to receive a muscle tissue sample from a chicken that was contaminated with enrofloxacin, a pig muscle sample was analyzed instead in the same way. The electropherogram is presented in Fig. 4. The sample was contaminated with 985 μg/kg ENR and 94 μg/kg CIP, which is more than 10-fold higher than the maximum residue limit of 100 μg/kg for the sum of ENR and CIP, that is allowed by the EU [5].

4 Concluding remarks

A sensitive CE-LIF method for the analysis of ENR and its main metabolite CIP in chicken muscle has been established. Owing to its higher sensitivity and selectivity the LIF detection in CE is a reliable substitute to UV detection for the analysis of quinolones as veterinary drug residues. The detection and quantification limits for the methodology proposed are low enough to determine residues of these drugs in animal tissues below the permissible MRL established by the European Commission.

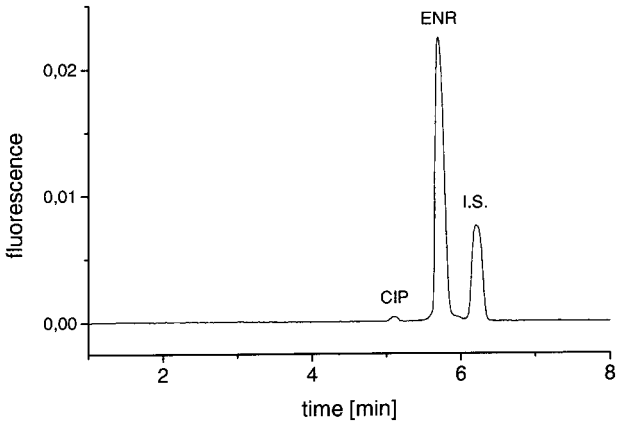

Figure 4. Electropherogram of a pig muscle tissue sample containing 985 μg/kg ENR and 94 μg/kg CIP; for electrophoretic conditions, see Fig. 2.

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