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Determination of ciprofloxacin, enrofloxacin and flumequine in pig plasma samples by capillary isotachophoresis–capillary zone electrophoresis

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Abstract

Quinolones are a group of synthetic antibiotics that are widely used in veterinary medicine. Their residues may remain in tissues, milk, etc. intended for human consumption. The European Union fixes the maximum residue limits (MRLs) of veterinary medicinal products in foodstuffs of animal origin. Analytical methods are therefore needed to determine them in biological samples. In this study, we describe capillary isotachophoresis–capillary zone electrophoresis (ITP–CZE) to analyze three quinolones, enrofloxacin (ENR), ciprofloxacin (CPR) and flumequine (FLU), in pig plasma samples. We used solid-phase extraction with Oasis HLB cartridges as a sample pretreatment clean-up step. Capillary zone electrophoresis (CZE) requires low amounts of sample and is not as sensitive as one would wish. ITP–CZE is an easy way to increase the sample loadability and sensitivity. With this system sensitivity increases 40-fold. The detection limits for CPR, ENR and FLU were 70, 85 and 50 $\mu g\ l^{-1}$, respectively, which were lower than their MRLs in different kinds of samples. This method is simple and sensitive, and is therefore an alternative tool to the existing HPLC methods for analyzing the residuals of these quinolones in biological samples.

Keywords: Ciprofloxacin; Enrofloxacin; Flumequine

1. Introduction

Quinolones (Qs) are an important group of synthetic antibiotics with bactericidal action. They are derived from nalidixic acid, a naphthyridine derivative introduced for clinical applications in the livestock and farming industries usually to treat urinary, pulmonary and digestive infections [1]. Their bactericidal activity involves the selective inhibition of DNA gyrase [2].

Quinolones, however, are not limited to clinical applications. They are also widely used to treat and prevent veterinary diseases in animals intended for human consumption and commercially farmed fish such as salmon and catfish. Several agents have been specifically developed for veterinary medicine. For example, danofloxacin, enrofloxacin and sarafloxacin are used to treat respiratory and enteric bacterial infections in cattle, swine, chicken and turkey, and diseases in aqua-cultured fish [2,3]. The widespread administration of these drugs in veterinary medicine

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represents a potential risk because there may be residues of the drugs in edible tissues. Antibiotic residues in food are a cause for concern because resistant human pathogens are emerging and because there may be allergic hypersensitivity reactions in humans [4]. Restrictive measures and regulatory levels for quinolones are not widely established in veterinary infection despite calls for them by regulatory agencies. The European Union, on the recommendation of its Committee for Veterinary Medicinal Products, included a number of quinolones (enrofloxacin, ciprofloxacin, sarafloxacin, difloxacin, marbofloxacin, danofloxacin, oxolinic acid and flumequine) in Council Regulation (EC) No. 2377/90 [5], which establishes the maximum residue limits (MRLs) of veterinary medicinal products in foodstuffs of animal origin [3,6]. These limits demand analytical methods that can determine the presence of quinolone residues in biological samples and commercial products. The MRLs values are in the range 100–300 μg/kg for the sum of enrofloxacin and ciprofloxacin and in the range 200–1500 μg/kg for flumequine in pig tissue samples. For plasma samples MRLs are not fixed.

Current methods of analyzing Qs in biological samples are based on liquid chromatography (LC) [7,8]; few studies have determined quinolones by capillary electrophoresis (CE). CE is a good alternative to LC in drug analysis [9–15]. It combines high resolution and easy automation with modest sample requirements and low solvent consumption [16,17]. CE has a very good sensitivity based on mass detection. This is important when the size of the sample is very limited, as when analyzing a single cell. Thus a minute amount of sample is sufficient. However, CE is not sufficiently sensitive when based on concentration especially compared to high-performance liquid chromatography (HPLC). In most practical applications the latter feature is far more important, especially when routinely analyzing compounds present at low concentrations. To take full advantage of the separation power of CE for trace analysis of biological samples, we considered several methods of improving determination limits. One of these is to increase detection sensitivity. Several highly sensitive detection systems, such as a laser-induced fluorescence detection and electrochemical detection, have been reported [18,19].

Another method is to increase the sample loadability of the system. Electrophoretic analyte-focusing techniques are an elegant way of increasing loadability in CZE. These techniques, such as the coupling of isotachophoresis (ITP) with CZE and field-amplified injection procedures [20,21], are based on applying local differences in electrical field strength during the injection or focusing step to enable the analyte ions to stack [21–25]. ITP uses a discontinuous electrolyte system made up of two different electrolytes: the leading electrolyte and the terminating electrolyte. The leading electrolyte contains ions with high mobility whereas the terminating electrolyte contains ions with low mobility. The sample ions therefore have an intermediate mobility, so in the capillary there are stacked isotachophoretic zones with sharp boundaries. During separation, the concentration of analytes in the sample changes until the sample ions reach equilibrium with respect to the concentration of the leading ions. When equilibrium is reached the leading and sample ions are moving at the same velocity. Moreover, in this zone, ion mobility is inversely proportional to the electric field [22,26]. This technique can be used with many compounds, ranging from small charged analytes to proteins. The main interest of the ITP–CZE system lies in analyzing samples that are not only dissolved in water but that have some conducting ions, as in biological samples [27]. The concentrating and separating power of ITP means that detection limits (LOD) could be lowered by at least two orders of magnitude [27].

Two techniques have been described for coupling ITP to CZE. The first uses on-column transient ITP in a single capillary, and the second couples a second CZE column to the ITP column [21,23–25,27–30]. The loadability in the second technique is lower than in the first but the instrumentation is much simpler using a single capillary. Several types of automated ITP–CZE have been developed for anionic and cationic separations in a single capillary using back-pressure programming [27,30]. A hydrodynamic pressure was used to remove the terminating electrolyte before the CZE started. The ITP conditions prevented excessive zone broadening.

Because of the complexity of biological samples they cannot generally be injected directly into the analytical instrument. A sample pretreatment must be carried out to obtain them without interferences and in a suitable medium. The bibliography contains

several pretreatment methods to analyze quinolones in biological samples; for example, sample deproteinization [11,12,31], liquid–liquid extraction (LLE) [7,10,22,26] and solid-phase extraction (SPE) [9,20,27,32–35]. Deproteinization, with a mixture of phosphoric acid and phosphate-buffered saline, achieved high recoveries (around 80% in tissue [31] and over 90% in plasma and urine [11,12]), but under these conditions the sample matrix is not suitable for ITP–CZE. LLE, however, obtained low recoveries for ciprofloxacin (CPR), mainly because such multiple extraction steps cause loss of molecules with low recoveries. Moreover, when the extraction step was carried out using organic solvents like ethyl acetate or methylenechloride, the recoveries were very low for Qs with piperazinyl moiety (<40%), like CPR and enrofloxacin (ENR) [35]. On the other hand, SPE was more selective and simpler to operate than LLE when pretreating complex matrices [9,20,27,32–35]. In a previous study we determined several quinolones in pig plasma samples using SPE with C_{18} cartridges as sample pretreatment and we have obtained high recoveries (90–100%) [9]. To our knowledge, apart from our previous study, there have been no other studies of SPE as a pretreatment for determining Qs in biological samples by CZE.

The main purpose of this paper was to determine conditions for isotachophoretic preconcentration in a single capillary using hydrodynamic backpressure programming to enhance the loadability of the sample and thus achieve lower detection limits for determining three quinolones, ciprofloxacin (CPR), enrofloxacin (ENR) and flumequine (FLU) in pig plasma samples. As far as we know, this system has not yet been used to analyze Qs. Before carrying out the analysis, we had to clean-up the sample by solid-phase extraction with Waters Oasis HLB cartridges.

2. Experimental

2.1. Apparatus

Electrophoretic experiments were performed using a Hewlett-Packard 3D CE (Waldbronn, Germany), with a DAD detector (Diode Array Detection). Data were collected with the software provided with the HP 3D Chemstation (Hewlett-Packard), which was operated under Windows NT (Microsoft). The capillary was fused-silica (64.5 cm×75 μm I.D.) supplied by Supelco (Bellefonte, PA, USA). A detection window was prepared by burning off the polyimide coating 56 cm from the capillary inlet.

2.2. Chemicals and reagents

Ciprofloxacin (CPR) and enrofloxacin (ENR) were donated by Cenavisa (Reus, Spain), and lomefloxacin and flumequine were purchased from Sigma (St Louis, USA). Standard stock solutions of 1000 mg l^{-1} were prepared in NaOH 0.1 N and stored under refrigeration. Standard working solutions were prepared weekly or daily, depending on their concentration, by diluting the standard stock solution with water which had been purified by a Milli-Q system (Millipore, Bedford, MA, USA).

Disodium hydrogen phosphate (Probus, Barcelona, Spain), sodium chloride (Sigma, MO, USA) and β-alanine (Sigma) were used to prepare the electrophoretic solutions. Sodium hydroxide (Prolabo, Bois, France) and phosphoric acid (Probus) were used to adjust the pH of the electrolytes. Methanol (MeOH) (SDS, Peypin, France) was used during solid-phase extraction.

2.3. Capillary isotachophoresis–capillary zone electrophoresis conditions

Before use, the capillary was rinsed with 1 *M* NaOH (Probus) (1000 mbar pressurized flow) for 15 min, and then with H_2O Milli-Q for 15 min. Finally it was flushed with running buffer for 10 min. Before each analysis, the capillary was rinsed with the running buffer for 3 min.

During isotachophoresis, we used two different electrolytes: the leading one was 10 m*M* sodium monohydrogen phosphate and 5 m*M* NaCl (pH 9.0, adjusted with diluted phosphoric acid) and the terminating one was 10 m*M* β-alanine (pH 9.0, adjusted with 0.1 N NaOH). Sample injection was performed hydrodynamically at a pressure of 50 mbar for 110 s. In the focusing step, 10 kV were applied as negative voltage in conjunction with 50 mbar of positive pressure for 1 min. In the fourth step of the isotachophoretic procedure, we applied a negative voltage of 10 kV for 1 min in the analysis

of standard solutions and we applied a negative voltage of 10 kV for 3 min in the analysis of pig plasma samples. Electrophoretic separation was developed using the leading electrolyte as the background electrolyte (BGE). The detector was set at 260 nm. The capillary temperature was 25 °C and the separation voltage was 25 kV.

2.4. Capillary zone electrophoresis conditions

As before, 10 m*M* sodium monohydrogen phosphate and 5 m*M* NaCl (pH 9.0, adjusted with diluted phosphoric acid) were used as BGE. Sample injection was carried out hydrodynamically at a pressure of 50 mbar for 6 s. Running buffer was then introduced to the capillary at 50 mbar for 6 s to diminish the dispersion of the sample during the analysis. The detector was set at 260 nm. The capillary temperature was 25 °C and the separation voltage was 25 kV.

2.5. Sample pretreatment

Solid-phase extraction (SPE) was used for pretreatment with Waters Oasis HLB cartridges (60 mg). The cartridge was activated with 5 ml of MeOH followed by 5 ml H_2O Milli-Q at a flow-rate of 1–2 ml/min using a water aspirator as a vacuum source connected to the cartridge pack; 1 ml of sample was passed through the cartridge. The cartridge was washed with 1 ml H_2O Milli-Q and the compounds were eluted from the cartridge with a 1-ml aliquot of MeOH.

Pig plasma samples were used in all experiments.

3. Results and discussion

3.1. ITP–CZE system

Capillary electrophoresis (CE) is characterized by the low amount of sample that needs to be injected (between 10 and 100 nl). Higher injections lengthen the injection volume and consequently broaden the band. Therefore, the injection length of the sample can be reduced by stacking procedures, which narrow the sample zone before separation. This makes the CE method more sensitive. In this paper we have used an on-column stacking system prior to capillary electrophoresis separation. The stacking system we used was ITP, which is based on a discontinuous electrolyte system made up of two different electrolytes: the leading electrolyte and the terminating electrolyte. The difference between the electrolytes are their mobilities.

The ITP system involves the following steps:

1. The inlet and outlet vials contain leading electrolyte (L). The capillary is filled with L.
2. The inlet vial is replaced with the sample vial. The sample is injected hydrodynamically.
3. The sample vial is replaced with the terminating electrolyte vial (T). The focusing step is then started: negative voltage and positive hydrodynamic backpressure are applied. The term ‘‘positive backpressure’’ means that the backpressure induces a flow in the direction of the capillary outlet. ‘‘Negative backpressure’’, on the other hand, induces a flow in the opposite direction. The negative voltage resulted in an electroosmotic flow in the direction of the capillary inlet, while the positive backpressure opposed this movement to produce the stack of sample analytes. Moreover, because of the characteristics of the ITP zones, the peak did not broaden.
4. The T vial is replaced with the L vial, and then a negative voltage is applied. As a result, the concentrated analyte ions approach the capillary inlet and T is removed before the CZE separation.
5. CZE separation is begun.

A scheme of this system is shown in Fig. 1.

During the ITP step, the ions with more mobility than the leading ions and those with less mobility than the terminating ions are removed from the capillary. This considerably cleans the sample [27]. It is therefore important to choose a suitable electrolyte system in which stable isotachophoretic zones are formed and the analytes are completely separated. Several papers have used phosphate electrolyte as background electrolyte (BGE) for analyzing quinolones and obtained a good separation [13–15]. Phosphate is a fast ion, faster than quinolone compounds, and is therefore considered a suitable leading ion. We then had to choose a terminating

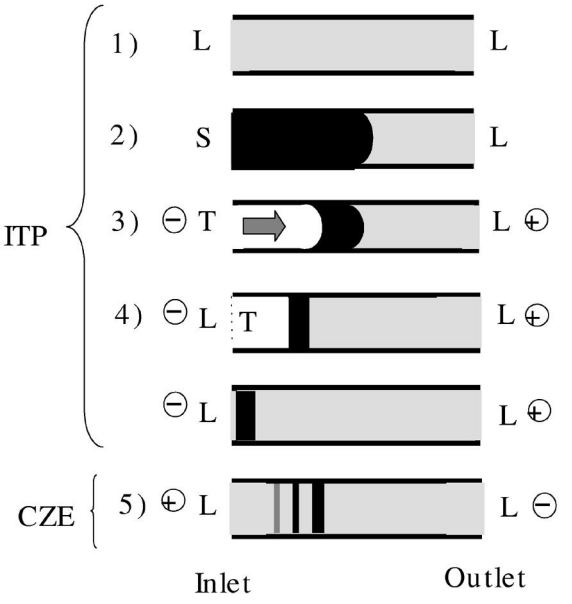

Fig. 1. Scheme of ITP–CZE system. The voltage is indicated by the negative and the positive symbols, which mark the cathode and anode, respectively. The arrow marks the sense of the pressure applied. Nomenclature: S, sample; L, leading electrolyte vial and T, terminating electrolyte.

electrolyte (T). β-Alanine is often used and is recommended as the terminating electrolyte in anionic ITP [23,27]. It also has less mobility than quinolone compounds. For this reason, we chose it as terminating electrolyte.

For capillary electrophoretic separation we can use a terminating electrolyte (T), a leading electrolyte (L) or an electrolyte that is different from T and L as background electrolyte. For several reasons, we chose the L as the background electrolyte in CZE. Firstly, it is better for the sample clean-up because the sample ions with less mobility than the terminating ions—including neutral species and counter ions—are removed in the inlet vial during the focusing step. Secondly, as the conductivity of L is always higher than that of analyte ions, band broadening will be less when L is used as the BGE in the CZE step than when T is used. Thirdly, with L it is easy to monitor the current for precisely timing when to switch from ITP to CZE because, when it is switched from ITP to CZE, the current will be high. If we had used T as BGE, the current would have been considerably lower. Also, it would have fluctuated due to ripples in the power supply and made it difficult to time the moment precisely [30].

ITP is a stacking system that is used before CZE analysis to improve the sensitivity of capillary electrophoresis. In the bibliography [21,23–25,27,30], there are descriptions of improvements in determination limits as a result of using ITP as the stacking system. However, the loadability is limited by the capillary volume. To establish the optimum conditions we must study the following parameters: injection volume, voltage and pressure applied during the third step (focusing step) and the duration of this step.

During the optimization of the ITP, it is important to take into account when to stop the negative voltage applied in the fourth step and start CZE. If we start CZE too early, some terminating ions remain inside the capillary, which makes the electric field inhomogeneous and affects the efficiency and migration time during CZE. Therefore, if CZE starts so late, all or part of the sample ions are removed from the capillary. We could control this step by monitoring the electric current [30]. The voltage is stopped when the electric current generated is 90% of the electric current generated when the capillary is full of L. At this moment, we consider the sample matrix is almost completely out of the column.

These parameters were optimized with standard solutions of CPR (1), ENR (2) and FLU (3) dissolved in Milli-Q water. Fig. 2 shows their struc-

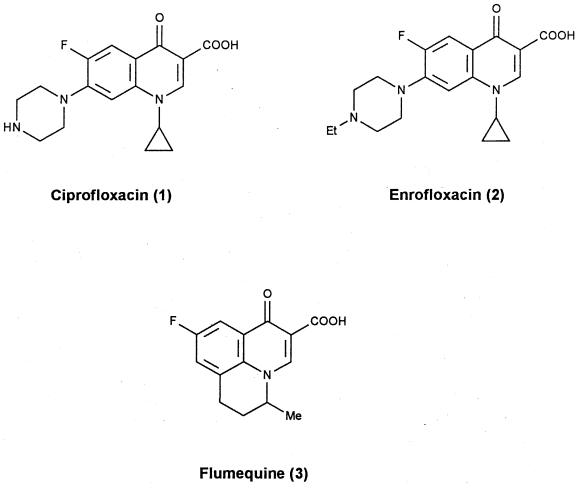

Fig. 2. Structures of the three quinolones.

tures. For developing their analysis, phosphate electrolyte at pH 9 was used as BGE. At this pH, a complete separation of several quinolones was obtained [13]. We therefore chose sodium monohydrogen phosphate as the leading electrolyte.

The first parameter to optimize was the injection volume. We fixed the maximum plug of sample that could be injected and then determined the best conditions of focusing. The volume injection was increased from 10 to 30%, while the parameters of the third step were fixed at 10 kV (negative voltage) and 50 mbar (positive backpressure) for 1 min. The sample amount that can be focused isotachophoretically depends on the compounds and the sample matrix. In the best conditions, the maximum sample volume that can be injected into ITP–CZE systems [23,27,30] is 55% of the total capillary volume. However, in this study injections above 30% obtained broadening peaks and in this case, the optimum injection volume was 30%.

After fixing the injection volume at 30%, and fixing the third step at 10 kV (negative voltage) and 50 mbar (positive backpressure), we studied the duration of the third step. The time necessary to accomplish the preconcentration was evaluated by increasing the focusing time until the peaks ceased to get higher in the CZE step. We studied several focusing times: 1, 5, 10 and 20 min. Lower focusing times (1 and 5 min) obtained well-defined peaks. For the highest focusing times (10 and 20 min) the sample is not more stacked, moreover both resolution and efficiency became worse. Fig. 3 shows the resolutions at the different focusing times. As the resolutions and the responses were similar for 1 and 5 min, and the stacking was not improved by increasing the focusing time from 1 to 5 min, we chose 1 min as the optimum. However, under these conditions quinolones were not completely separated.

As we have already explained, during the fourth step the analyte ions are stacked approaching the capillary inlet and the terminating ions are removed from the capillary. The inlet vial and the outlet vial contain leading electrolyte. This step takes 1 min at the optimum experimental conditions.

After optimizing the parameters that affect the stacking step, we had to finish optimizing the electrophoretic separation. It was important remember that all modifiers we added to the BGE could affect the stacking step.

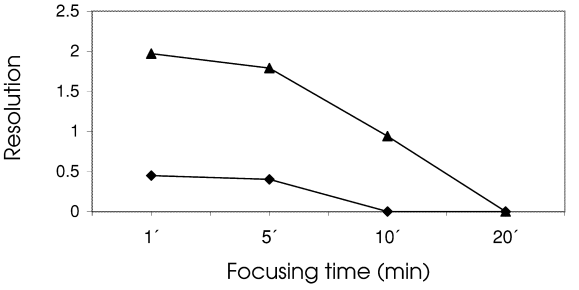

Fig. 3. Representative focusing time versus resolution from standard solution with 1 mg l^{-1} of CPR, ENR and FLU. The buffer system was: 10 m*M* sodium monohydrogen phosphate (pH 9.0, adjusted with diluted phosphoric acid) as leading electrolyte and 10 m*M* β-alanine (pH 9.0, adjusted with 0.1 N NaOH) as terminating electrolyte. Background electrolyte (BGE) was the same as the leading one. Injection: 50 mbar for 6 s. Capillary temperature: 25 °C. Separation voltage: 25 kV. Symbols: ♦, ciprofloxacin/enrofloxacin; ▲, enrofloxacin/flumequine.

To improve electrophoretic separation we studied several parameters: adding an organic solvent, adding a salt (NaCl) to the BGE, and changing the analysis voltage.

When we add an organic solvent to the separation buffer several of its characteristics are modified, i.e. selectivity, viscosity and zeta-potential. It is difficult to predict how adding organic solvents affects electrophoretic mobility, but in general, adding 2-propanol (IPA) or methanol (MeOH) seems to raise electrophoretic mobility and accentuate differences in mobility [16,26]. So we added up to 10% (v/v) of MeOH and IPA; not only was separation not improved but there were broadening peaks.

Adding a salt increased the efficiency of the peaks. We added several concentrations of NaCl to the BGE: 2.5, 5 and 10 m*M*. Results were best when 5 m*M* NaCl was added. The addition of a salt could affect the stacking step. This is because the salt contains chloride which is a fast ion. However, its addition did not make the separation worse and by adding 5 m*M* NaCl to the leading electrolyte (BGE) and applying 15 kV during the CZE step, all peaks were clear and had high resolution, as we can see in Fig. 4.

After optimizing the separation we determined the calibration parameters. Linearity was between 100

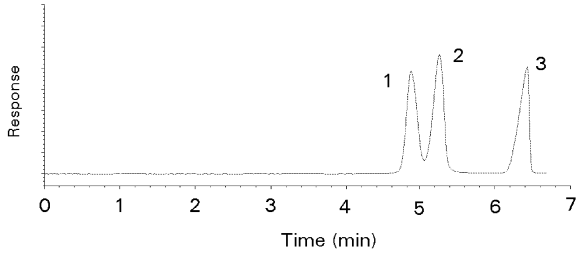

Fig. 4. Electropherogram of 1 mg l^{-1} of CPR, ENR and FLU from standard solution. The separation voltage during CZE analysis was 15 kV. The buffer system was: 10 m*M* sodium monohydrogen phosphate (pH 9.0, adjusted with diluted phosphoric acid), 5 m*M* NaCl. For other conditions, see Fig. 3.

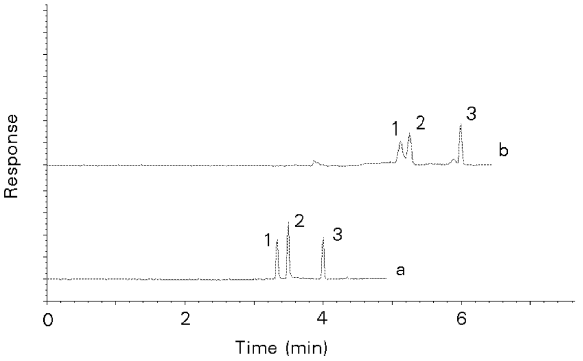

Fig. 5. Electropherogram of 8 mg l^{-1} of CPR, ENR and FLU from standard solution analyzed by CZE system (a) and a standard solution of 0.2 mg l^{-1} analyzed by ITP–CZE system (b). For other conditions, see Fig. 4.

and 1500 μg l^{-1} for ciprofloxacin and enrofloxacin, while for flumequine it was between 100 and 1000 μg l^{-1}. We injected these solutions five times, starting with the least concentrated one and ending with the most. The area values obtained were successively analyzed using ULC (Univariate Linear Calibration) software [36] to evaluate the correlation coefficient (*r*), the relative standard deviation (RSD) within solutions and the limit of detection (LOD). Linearity was good, and the correlation coefficient was >0.999. The detection limit (LOD) was calculated according to the method of Widefordner and Long [37] using the ULC program with *K* equal to 3. LOD was 50 μg l^{-1} for ciprofloxacin and flumequine, and 10 μg l^{-1} for enrofloxacin. The RSD of the areas were between 0.6 and 2.9% and the RSD of the migration time were between 1.1 and 4.5% for 10 repeated injections of standard solutions of 1000 μg l^{-1}. A fresh buffer was used after each sequence of three injections. These results are shown in Table 1.

This study shows that CZE can be used to trace quinolone antibiotics. As far as we know, this is the first time that ITP–CZE has been used to decrease the limits of detection of quinolones by CE. Fig. 5 compares the electropherogram from analyzing 8 mg l^{-1} of standard solution by conventional CZE (Fig. 5a) with the electropherogram from analyzing 0.2 mg l^{-1} of standard solution by ITP–CZE system (Fig. 5b). The signals for each compound are similar. However, in the ITP–CZE system the analysis time increased for all compounds.

With this system, the sensitivity of the CE method increased 40-fold. Coupling ITP with CZE in a single capillary is therefore suitable for analyzing enrofloxacin, ciprofloxacin and flumequine at low levels.

We have therefore improved detection limits so that they are similar to those of liquid chromatography methods with UV detection [32,35,38].

3.2. Analysis of plasma samples

CE has a number of advantages over liquid chromatography and other techniques. For example, less sample preparation is needed, analysis is less expensive and there is less interference in the assay.

Table 1
Calibration data and precision studied with standard solutions

Compound	Linearity (μg l^{-1})	Correlation coefficient (r^2)	LOD (μg l^{-1})	LOQ (μg l^{-1})	%RSD[a] (area)	%RSD[a] (time)
CPR	100–1500	0.9996	50	100	0.6	0.2
ENR	100–1500	1.0000	10	20	2.8	0.3
FLU	100–1000	0.9973	50	100	2.9	0.5

[a] Calculated for 10 consecutive runs at 1000 μg l^{-1}.

CE methods sometimes analyze drugs or antibiotics in serum and plasma samples by directly injecting them [39]. Analysis time and the cost of consumables are therefore smaller. However, as plasma or serum samples contain large amounts of proteins, this could generate interfering peaks. They also contain many constituents at higher concentrations, so during ITP long zones are formed and, when CZE separation begins the analytes start from very different positions [39]. Consequently, the sample must be pretreated.

As a sample pretreatment we studied solid-phase extraction to remove the sample matrix. This type of pretreatment prepares samples for a wide range of analytes in complex matrices because it is more selective and simpler to operate, and because it uses less than other types of pretreatments like liquid–liquid extraction or deproteinization [16,17,26].

In a previous study [9] we determined several quinolones in pig plasma samples using SPE as the pretreatment and CE to carry out analysis. We used C_{18} cartridges for the SPE, and obtained high recoveries. As far as we know, no other study of quinolones has used SPE as the pretreatment and CE as the analysis. In this paper, we used Waters Oasis HLB cartridges to carry out the SPE pretreatment. This is because they contain a macroporous copolymer with lipophilic retention characteristics that provide the reversed-phase properties that are needed to retain the analyte. They also have hydrophilic retention characteristics that avoid the wettability problems of the C_{18} packings. As far as we know, this kind of cartridge has not been used to determine quinolones, but recoveries are high when they are used to determine antibiotics in pig plasma samples [38,40].

The parameters optimized for SPE pretreatment were the clean-up and elution steps. One milliliter of plasma sample was passed through the cartridge, and several volumes of water were tested (between 0.5 and 2 ml) to obtain a clean sample with good recoveries for all quinolones. One milliliter of Milli-Q water was enough for the clean-up step. To optimize the elution step we used MeOH. We also studied several volumes of MeOH for the elution of Qs from the Waters Oasis HLB cartridge. One milliliter of eluent was enough to carry out this step and recoveries were high (around 100%) for all compounds.

For accurate quantification we used an internal standard. This makes accurate injections unnecessary because a reference standard is included in each sample analysed. We chose an internal standard with a migration time that enables quinolones to be eluted in a suitable time and does not allow compounds to interfere in the electropherogram. We therefore analyzed a test sample containing known amounts of each component plus a predetermined amount of the internal standard (I.S.). The precision of the analysis depends on accurately measuring the peak areas. The internal standard should have similar physicochemical properties to the analyte. For this reason we investigated three quinolones: marbofloxacin, danofloxacin and lomefloxacin. The best results were for lomefloxacin, which was efficiently extracted from plasma samples, and had a migration time that correctly separated the three compounds in this study.

During the analysis of pig plasma samples by the ITP–CZE system described, the duration of the fourth step increased. This is because the complexity of the sample makes more difficult the approach of analyte ions to the capillary inlet. This step lasts for 3 min.

Fig. 6a shows the electropherogram of a blank plasma sample and Fig. 6b shows the electropherogram of spiked plasma sample (1500 $\mu g\ l^{-1}$ of CPR, ENR and FLU) after the SPE procedure has been carried out. In Fig. 6b we can see that the analysis time increased slightly. This was because the com-

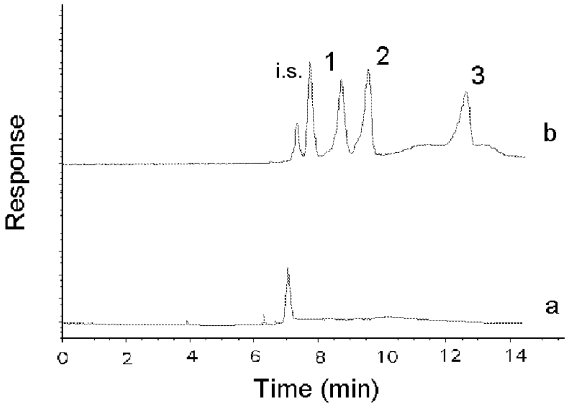

Fig. 6. Electropherogram of a blank plasma sample (a) and a plasma sample spiked with 1.5 mg l^{-1} of CPR, ENR and FLU (b). For other conditions, see Fig. 4.

Table 2
Calibration data and precision studied in several spiked plasma samples

Compound	Linearity ($\mu g\ l^{-1}$)	Correlation coefficient (r^2)	LOD ($\mu g\ l^{-1}$)	LOQ ($\mu g\ l^{-1}$)	%RSD[a] (area)	%RSD[a] (time)
CPR	100–1500	0.9988	70	140	4.0	1.2
ENR	100–1500	0.9968	85	170	4.0	1.3
FLU	100–1000	0.9958	50	100	5.0	1.8

[a] Calculated for five consecutive runs at 1000 $\mu g\ l^{-1}$.

plexity of the sample matrix increased in the fourth step, when the sample was concentrated near the capillary inlet. As we can see, there were no interferences at the analysis time of quinolones which are studied. We can therefore conclude that the pretreatment studied can be used to analyze these compounds in these kinds of samples.

Next we determined the calibration parameters in pig plasma samples, spiking blank pig plasma with different standard concentrations. Linearity was between 100 and 1500 $\mu g\ l^{-1}$ for ciprofloxacin and enrofloxacin, and for flumequine it was between 100 and 1000 $\mu g\ l^{-1}$. The LOD was 70 $\mu g\ l^{-1}$ for enrofloxacin, 85 $\mu g\ l^{-1}$ for ciprofloxacin and 85 $\mu g\ l^{-1}$ for flumequine (Table 2).

For pig plasma samples spiked with different concentrations of each quinolone, the recoveries were above 90% for all compounds. The RSD values were between 5 and 7% for pig plasma samples spiked with 200 $\mu g\ l^{-1}$, and between 4 and 5% for pig plasma samples spiked with 1000 $\mu g\ l^{-1}$ (Table 3).

Based on the good results of LODs obtained for plasma samples, the developed method could be applied for tissue samples after a sample pretreatment, which is now under study in our laboratory.

Table 3
Recoveries (%R) and repeatibility (%RSD) for CPR, ENR and FLU from pig plasma samples at several spiked level preconcentrations

Compound	Spiked level preconcentration			
	200 ($\mu g\ l^{-1}$)		1000 ($\mu g\ l^{-1}$)	
	%R	%RSD[a]	%R	%RSD[a]
CPR	128	6	90	4
ENR	126	5	101	4
FLU	98	7	92	5

[a] Calculated for five consecutive runs at each concentration spiked with CPR, ENR and FLU standards, respectively.

4. Conclusions

This study shows that an ITP–CZE system in a single capillary can be used to determine enrofloxacin, ciprofloxacin and flumequine in pig plasma samples. With this system, sensitivity is 40 times higher than with CZE. We used Oasis HLB cartridges to carry out SPE as a clean-up step. Recoveries were around 100%. As far as we know, this kind of cartridge has never been used to determine quinolones.

This system is simple and sensitive. It is also characterized by its repeatability as the low relative standard deviations (RSD) obtained throughout the procedure demonstrate.

We therefore conclude that this is a good alternative method to HPLC for analyzing quinolones in biological samples at low levels.

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