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Comparative evaluation between capillary electrophoresis and high-performance liquid chromatography for the analysis of florfenicol in plasma

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Abstract

A capillary electrophoresis (CE) and a reversed phase high-performance liquid chromatography (RP-HPLC) method with UV detection have been developed for florfenicol analysis in plasma samples. The suitabilities of both methods for quantitative determination of florfenicol were approved through validation specification, such as linearity, precision, selectivity, accuracy, limit of detection and quantification. The capillary electrophoresis (CE) and high-performance liquid chromatography (HPLC) assay were compared by analyzing a series of plasma samples containing florfenicol in different concentrations using the two methods. The extraction procedure is simple and no gradient elution or derivatization is required. Furthermore, the analysis time of the CE method is two times shorter than the respective parameter in HPLC and solvent consumptions is considerably lower. The calibration curve were linear to at least 0.05–10 μg/ml ($r = 0.9998$) and 0.1–10 μg/ml ($r = 0.9998$) for CE and HPLC, respectively. The separation efficiency are good for both methods. The detection limits for florfenicol were 0.015 μg/ml with CE and 0.03 μg/ml with HPLC and CE method gave lower value, even though UV detector was applied in the both cases. The both methods were selective, robust and reliable quantification of florfenicol and can be useful for clinical and biomedical investigations.

Keywords: CE; HPLC; UV detection; Florfenicol; Validation

1. Introduction

The more efficient therapeutic application of many drugs has given rise to a need for reliable, fast and efficient analytical procedures to measure their serum and plasma levels. At the beginning of 21st century, high-performance liquid chromatography (HPLC) with reversed phase and capillary electrophoresis (CE) are two of the most frequently used modern separation techniques. The determination of drugs in biological fluids in many clinical laboratories are generally performed by HPLC which is an established technique with highly automated instrumentation and with concentration sensitivity in the nanomolar range, it is ideal for bioanalytical assays. However, many pharmaceutical analysis laboratories have an increasing presence of CE equipment, so CE offers a real and attractive alternative to HPLC. The major strength of CE is that the basic separation principles are different from those of HPLC and other chromatographic techniques. Likewise, CE in many instances can have distinct advantages over HPLC in terms of simplicity, rapid method development, and reduced cost of the operation, because no packed column, pumps and mobile phase gradient are used. Many of the common problems associated with HPLC, such as high pressure, solvent leakage and the high cost of column may be eliminated. Concentration sensitivity is typically an order of magnitude less than in HPLC, but peak efficiencies are much higher. Higher peak efficiencies generally mean

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greater resolution in short run times by CE [1,2]. The range of CE applications in biomedical and pharmaceutical analysis is at least as extensive as that of HPLC. In this paper, we present the development, validation and comparison of a CE and an RP-HPLC method for determination of florfenicol in plasma.

Florfenicol (D-(threo)-1-(methylsulphonylphenyl)2-dichloroacetamide-3-fluoro-1-propanol) is a primarily bacteriostatic broad-spectrum antibiotic against many Gram-negative and Gram-positive bacteria and is used exclusively in veterinary medicine. Florfenicol is a fluorinated derivative of chloramphenicol and thiamphenicol, which has a fluorine atom instead of the hydroxyl group located at C-3. Therefore, it does not carry the risk of inducing human aplastic anemia. Florfenicol was approved in the European Union for the control of bacterial respiratory tract infections in cattle and pigs in 1995 and in 2000, respectively [3]. This drug is characterised by high bioavailability, good tissue penetration and rapid elimination, which are important for systemic treatment of animals in the food production industry.

The determination of florfenicol in biological fluids presents several analytical problems. For example, florfenicol like the other compounds adsorb UV light insufficiently to determine at nanogram level. A number of chromatographic methods have been described for quantitative determination of florfenicol in biological fluids. These include gas chromatography [4,5] and liquid chromatography–mass spectrometry [6–9]. More recently, micellar electrokinetic capillary chromatographic separation of florfenicol and florfenicol amine in pharmaceutical formulations has been reported with modified borate buffer containing sodium dodecyl sulphate [10]. However, the use of CE in the determination of florfenicol in biological matrix has not been reported.

Due to the UV spectral pattern of florfenicol, the detection limit can be significantly improved at 200 nm region. Unfortunately, within a short UV wavelength range most of the commonly used organic solvents absorb UV light strongly. Hence, application of chromatographic methods which use organic solvents for liquid phases are strongly limited. Nevertheless, most of the HPLC determinations were performed on a reversed stationary phase and all utilize UV at 223 or 224 nm using liquid–solid extraction have already been published [11,12]. Many HPLC methods based on single extraction with ethyl acetate [13–17] and with acetone [18] have been studied for quantitative analysis of florfenicol.

On the contrary to HPLC mobile phases, the electrophoretic buffers usually have a minimal background in whole UV region. This allows the use of capillary electrophoresis techniques with UV detectors operating in 195–215 nm, where many organic analytes including florfenicol have a significantly higher adsorption [19].

Accordingly, the aim of our study was to develop a rapid, simple and robust quantitative procedure, validate, and compare CE and HPLC assays with UV detection for the determination of florfenicol in plasma.

2. Experimental

2.1. Reagents

Florfenicol used as the working standard was kindly provided by Vetos-Farma Factory (Bielawa, Poland). Ephedrine hydrochloride and lamotrygine (used as internal standard) were supplied by Farm-Impex (Gliwice, Poland) and Biovena Pharma (Warsaw, Poland), respectively. Reagents used for the preparation of samples and mobile phases were ethyl acetate, ortho-phosphoric acid 85%, sodium hydroxide, hydrochloric acid by POCh (Gliwice, Poland), and acetonitrile by Merck (Darmstadt, Germany). Sodium tetraborate decahydrate (pH 9.26; 25 mM) and sodium dihydrogenphosphate (pH 5.73; 25 mM) were purchased from POCh (Gliwice, Poland). The buffer solutions were prepared according to standard method, using triple distilled water. All chemicals were of analytical grade and used as received without further purification. The working solutions were also prepared in glass volumetric flasks by appropriate dilution just before use and they were stored in the dark under refrigeration to avoid possible decomposition.

2.2. Instrumental parameters and conditions

2.2.1. CE technique

All capillary electrophoresis experiments were carried out on an automated CE system (P/ACE 2100, Beckman, Fullerton, CA), with UV detection at 200 nm, and thermoregulated at 25.0 (±0.1) °C. Data were collected and analyzed on System Gold Chromatography Software. The fused-silica separations capillary had an internal diameter of 75 μm, a total length of 57 cm and a length from inlet to detector of 50 cm. A voltage of 25 kV was used for separation, with injection at the positive end (anode) and detection at the negative end (cathode). The samples were loaded with a 7 s argon pressure injection at 3.45 kPa. Each experiment was run duplicate, with acetonitrile as the neutral marker. The capillary was flushed between runs with 0.1 M hydrochloric acid (0.5 min), 0.1 M sodium hydroxide (1 min) and triple distilled water (1 min). Before injection the capillary was rinsed with the running buffer for 1 min. Likewise, before analysis and between each run, the voltage of capillary was pre-conditioned in order to give high precision. The migration times were 2.0 and 2.7 min for ephedrine hydrochloride (internal standard) and florfenicol, respectively.

2.2.2. HPLC technique

The high-performance liquid chromatographic system was purchased from Knauer (Berlin, Germany), and was equipped with a solvent pump Mini-Star K-500, an K-2500 UV detector. Separation was achieved on RP-18 LiChrospher column (5 μm particle size, 125 mm × 4 mm) from Merck (Darmstadt, Germany) which was placed in column thermostat jet stream with injection valve, delivered by Knauer (Berlin, Germany). The chromatographic data were collected

and processed with a computer system for data acquisition (EUROCHROM 2000). Other laboratory equipments used was temperature controlled centrifuge and instrument for mechanic shaking of samples.

A LiChrospher-100 column C18, 5 μm, 125 mm × 4 mm, column from Merck was used for the separation of analytes at 25 °C temperature. As a mobile phase was used a binary mixture of acetonitrile–water (25:75 v/v) adjusted to pH 2.7 with 85% ortho-phosphoric acid. The flow rate was maintained at 1.5 ml/min, and the compound thus was eluted and recorded with a UV detector set at the wavelength of 224 nm. Under these conditions the retention times for florfenicol and the internal standard (lamotrygine) were 3.0 and 5.4 min, respectively. The total time of single determination including regeneration of column was 8 min.

2.3. Sample preparation

2.3.1. CE method

To the swine plasma samples (0.5 ml) was added the internal standard (10 μl of the ephedrine hydrochloride at concentration 1 mg/ml) and 3 ml acetonitrile for deproteinisation. Samples were agitated on the rotary mixer, centrifuged during 5 min at 8000 rpm/min, and the supernatant was evaporated to dryness at 50–55 °C under a gentle stream of air. Subsequently, the samples were extracted with ethyl acetate (3 ml) in the presence of 200 μl 0.1 M sodium hydroxide. The organic layers were collected and evaporated under the same conditions. The residue was reconstituted in 2 mM sodium tetraborate decahydrate (0.5 ml), centrifuged for 5 min at 8000 rpm/min and injected (7 ml) into the CE system.

2.3.2. HPLC method

Briefly, the swine frozen plasma was thawed at a temperature of 25 °C (room temperature) and 0.5 ml was transferred with the use of a pipette into a clean test tube, spiked with 20 μl of lamotrygine at a concentration of 100 μg/ml to obtain 4 μg/ml of internal standard in sample, and 0.2 ml 1 M sodium hydroxide and 3 ml of ethyl acetate were added. The resulting mixture was shaked during 10 min and centrifuged for 15 min at a 3500 rpm/min. The organic layer was evaporated until dried at 50–55 °C in a water bath and supernatant was redissolved in 200 μl of the mobile phase. After centrifugation at 10,000 rpm/min during 7 min, 20 μl was injected on the HPLC column.

Standard samples were prepared by spiking blank plasma with known amounts of florfenicol and used for construction of calibration curves.

2.3.3. Liquid–liquid extraction (LLE)

The proposed sample preparation procedures for CE and HPLC methods were optimized to offer the most reliable, effective, and rapid approach for routine analysis as well to permit elimination of time consuming purification steps, including expensive solid-phase extraction (SPE). Preparation of biological samples for florfenicol analysis was simply in both methods, based only on single liquid–liquid extraction.

2.4. Validation and analytical methods

Quantification procedures for both techniques were based on the internal standard method. Concentration of component of interest in biological material was determined by plotting peak-height ratios (florfenicol/I.S.) versus florfenicol concentrations (μg/ml). For the within-day and between-day precision and accuracy, pools of plasma was spiked with florfenicol standard to obtain final concentrations of 0.1, 0.5, 1.0, 2.0, 5.0 and 10.0 μg/ml and with a fixed concentration of internal standard for both techniques. The limit of quantification was 0.05 and 0.1 μg/ml for CE and HPLC, respectively. Assay validation results obtained from within-run and between-run experiments for florfenicol analysed by CE and HPLC are presented in Table 1. Specificity of the assay was determined on the basis of different plasma samples. The stability for both methods has been controlled using the plasma samples containing 0.05, 2.0 and 5.0 μg/ml of florfenicol and stored at a temperature of −20 °C during 2 months.

Both proposed methods for determination of florfenicol were validated with respect to specificity, linearity range, limit of detection and quantitation, precision and accuracy.

2.4.1. Specificity

As a first step, the specificity of both analytical methods was confirmed on the basis of different blank and extracts samples ($n = 6$) from plasma. Typical electropherograms and chromatograms of blank plasma extract (Figs. 1A and 2A) and plasma obtained from extract of plasma spiked with suitable florfenicol and internal standard concentration are shown in Figs. 1B and 2B. No interference was observed in the region of interest where the analytes were eluted, as is shown in the blank sample electropherogram and chromatogram, which indicates that the reported methods are selective. The identification of florfenicol was performed by characterizing the sample peak in term of the migration or retention times and

Table 1
Summary of precision and validation data for florfenicol, obtained with HPLC and CE calibrations

	HPLC	CE
Linearity range [μg/ml]	0.1–10	0.05–10
Sample linearity		
Slope	0.18 (±0.001)	0.205 (±0.002)
Intercept	0.005 (±0.005)	0.012 (±0.007)
Correlation coefficient	0.9998	0.9998
Standard error	0.01	0.01
n	6	7
LOD [μg/ml]	0.03	0.015
LOQ [μg/ml]	0.1	0.05
Separation time [min]	8	4
Sample pretreatment	Liquid–liquid	Liquid–liquid

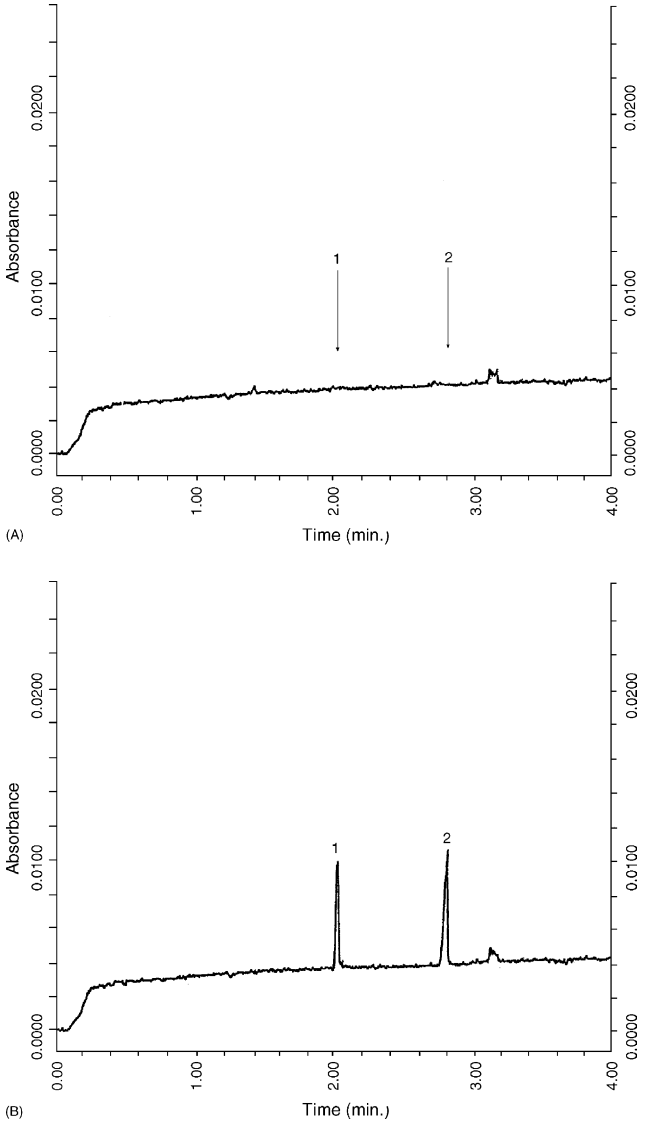

Fig. 1. Typical electropherograms of blank swine plasma extract (A) and plasma spiked with ephedrine hydrochloride(1) (internal standard) (10 μg/ml) and florfenicol (2) (5 μg/ml) and (B).

UV spectrum. The separation efficiency is higher with the CE method, which leads to shorter analysis times.

2.4.2. Linearity

The linearity study verifies that the plasma sample solutions are in a concentration range where analyte response is linearly proportional to concentration. The calibration curves were established as dependence of peak-height ratio (florfenicol/IS) versus florfenicol concentration (μg/ml). The CE method was shown to be linear over the concentration range 0.05–10 μg/ml, while the HPLC method was linear from 0.1 to 10 μg/ml. The mean linear regression equations of standard curves for CE and HPLC were $H/H_{IS} = 0.205\ (\pm 0.002)C + 0.012\ (\pm 0.007)$ and $H/H_{IS} = 0.18\ (\pm 0.001)C - 0.005\ (\pm 0.005)$, respectively, where H/H_{IS} was peak-height of florfenicol/peak-height of internal standard

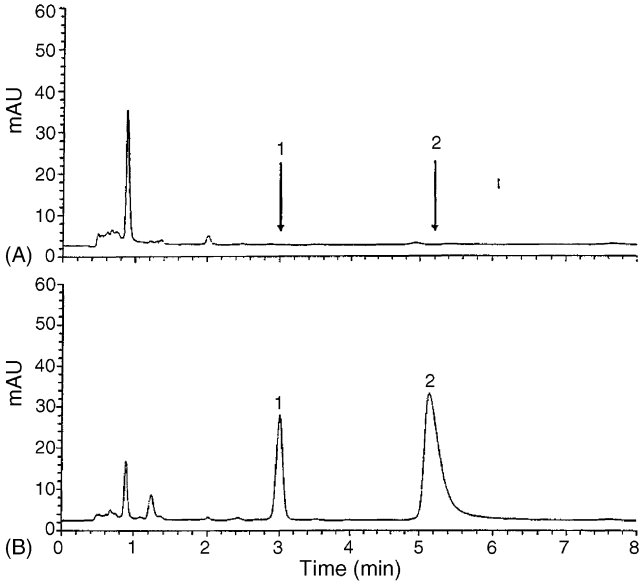

Fig. 2. Typical chromatograms of blank swine plasma extract (A) and plasma spiked with florfenicol (1) (5 μg/ml) and lamotrygine, internal standard (2) (4 μg/ml) (B).

and C was the concentration of florfenicol. In brackets were given standard errors at regression coefficients. These curves were constructed from six different concentrations. Low intercept number together with relatively large standard error, particularly for HPLC, additionally confirms the specificity of this method. Main validation parameters of the methods for linearity are shown also in Table 1.

2.4.3. Limit of detection and quantitation

The limit of detection (LOD) was defined as the sample concentration that allowed for an unambiguous detection of the corresponding peak signal in six consecutive experiments. The LODs were determined as the smallest concentration from which it is possible to deduce the presence of the substance with reasonable statistical certainty and were about 0.015 μg/ml for CE and 0.03 μg/ml for HPLC. The limit of quantitation (LOQ), defined as the lowest concentration level at which the assay was validated (precisely and accurately with R.S.D. less than 10%), were found to be 0.05 and 0.1 μg/ml for CE and HPLC, respectively. The LOD and LOQ were calculated for the calibration graphs of florfenicol as three and then times of the baseline noise level for LOD and LOQ, respectively.

2.4.4. Precision and accuracy

The precision of both methods was determined by calculating the relative standard deviation (R.S.D.) for the repeated measurements. Inter-assay precision data were obtained repeating the same experiment, on the same day and in the same laboratory, by the total analysis of six replicate samples, each of which were independently prepared according to the method procedure. The precision of CE assay, for inter-assay variability, ranged from 9.8% for 0.1 μg/ml to 3.6% for

Table 2
Assay validation results obtained from between-run and within-run experiments for florfenicol analysed by CE and HPLC

Nominal concentration [μg/ml]	Within –run			Between-run		
	Mean concentration [μg/ml]	CV [%]	Accuracy [%]	Mean concentration [μg/ml]	CV [%]	Accuracy [%]
CE						
0.1	0.09	9.8	90.0	0.09	9.8	90.0
0.5	0.51	8.2	102	0.48	9.2	96.0
1.0	1.08	7.2	108	1.09	7.8	108.0
2.0	1.90	6.5	95	1.95	6.3	97.5
5.0	5.12	5.6	102.4	5.10	4.8	102.0
10.0	10.00	3.6	100	10.06	4.5	100.6
HPLC						
0.1	0.11	10.0	110	0.12	10.0	120
0.5	0.53	9.2	106	0.52	9.4	104
1.0	1.04	7.8	104	1.03	8.1	103
2.0	1.97	6.6	98.5	1.99	8.9	99.5
5.0	4.92	5.1	98.4	4.97	7.0	99.4
10.0	10.03	3.1	100.3	10.18	5.1	101.8

10 μg/ml, while in the case of HPLC, ranged from 10.0 % for 0.1 μg/ml to 3.1% for 10.0 μg/ml. The intermediate precision data were obtained by repeating the intra-assay experiment on a different day with newly prepared samples, buffer solution for CE, and mobile phase for HPLC, respectively. Good repeatability and intermediate precision (no more than 10%) were shown in independent assays performed by two analyst in different days for both methods (Table 2).

The accuracy of the method was determined by assessing the agreement between the measured and known concentration of analysed samples.

2.4.5. Freeze–thaw cycles and recoveries

In both techniques, the effect of freezing and thawing cycles was studied using plasma samples at three different concentrations (0.5, 2.0, 5.0 μg/ml of florfenicol) measuring three replicates at each level during 2 months. The results of freeze–thaw stability (reported in Table 3) indicated that florfenicol is stable in plasma for three freeze–thaw cycles when frozen at −20 °C and thawed to room temperature. All the samples were stable at the stored conditions.

Table 3
Results for processed plasma sample freeze–thaw cycles during 2 months ($n = 3$)

Plasma concentration [μg/ml]				
Spiked	Initial	After I freeze–thaw cycle	After II freeze–thaw cycle	After III freeze–thaw cycle
CE method				
0.5	0.50	0.49	0.48	0.47
2	2.06	1.94	1.84	1.76
5	5.03	4.95	4.86	4.73
HPLC method				
0.5	0.51	0.50	0.49	0.48
2	1.99	1.98	2.01	1.99
5	5.03	5.02	5.00	4.97

2.4.6. Recovery

Recovery of florfenicol from plasma samples was studied at three (of the low, middle and high) concentration levels with six replicates comparing the detector response obtained from the analyte added to and extracted from the plasma to detector response obtained for plasma samples without extraction procedure, for both methods. Good recoveries were observed for florfenicol under investigation at all spiking levels and average recoveries were complied with the requirement over 90%. The recoveries obtained in CE method varied from 91 to 98.8% (R.S.D. 2.6%), whereas in HPLC were similar from 92.5 to 98.8 % (R.S.D. 2.1%).

3. Results and discussion

The CE and HPLC methods have been developed for identification and quantitative determination of florfenicol in plasma. Both methods exhibited satisfying validation results concerning sensitivity, linear range, detection limit, reproducibility, accuracy and precision. The results were encouraging, even there was a slight difference between the levels of analyte determined by CE and HPLC. In Table 2, the parameters of the HPLC and CE method are compared.

3.1. Wavelength

Typical biomedical or pharmacokinetics studies of drugs require analytical method with detection limit at nanogram level. Unfortunately, this compound does not have a strong ultraviolet chromophore. Sensitive quantification of florfenicol can be performed using UV detectors that are operating at wavelengths region between 190 and 210 nm. The main problem is that almost all of the HPLC mobile phases contain organic liquids, which absorb short wavelength UV light. Therefore, liquid phases composed of pure water are preferred. Fortunately, such as separation systems based on water instead organic-water mixtures are commonly used in

electrophoresis. By comparing the sensitivity of both techniques for florfenicol determination, it was observed that CE is more sensitive than HPLC. It is result of the instrumental conditions, which are necessary for quantitative analysis of this compound with maximum sensitivity. For CE optimum wavelength was established for 200 nm and for HPLC for 224 nm. Mostly, when operating chromatographic and electrophoretic techniques at the same wavelength, it may be necessary to use a few times more concentrated samples for CE to obtain an equivalent LOQ. Notwithstanding, as can be observed LOQ, the lower value in the range, was lower for CE than for HPLC in spite of being CE a technique considered less sensible due to the narrow inner diameter of the capillary and to the injection of very small volumes. It was related to the higher precision in the height of the corresponding peak in CE at lower levels of concentration than in HPLC and it could be due to the higher peak efficiency in CE that provides better signal to noise ratio at 200 nm than at 224 nm [20]. Furthermore, CE method allows determination of florfenicol at about 200 nm, because of water is solvent, and no interference peaks of background can be observed. It is impossible using HPLC method at about 200 nm with organic component, where signal of detector could be generated from tested substance and organic solvent. Nevertheless, both techniques provide a linear range wide enough for florfenicol determination.

3.2. Internal standard

Furthermore, in order to improve the quantitative performance of both methods and to ensure acceptable precision, an internal standard (IS) should be used which has migration position near to the peak of analyte. The ephedrine hydrochloride has been selected for CE analysis as the IS to compensate for injection errors and fluctuations of migration times, whereas for HPLC was chosen lamotrygine.

3.3. Buffer and mobile phase composition

Likewise, one important parameter for CE separation is the buffer system, particularly the pH applied. The optimisation of electrophoretic separation and migration time of analyte was conducted using number of borate and phosphate buffers in the pH range 6–10. The best results were obtained using a buffer solution composed with sodium tetraborate decahydrate solution (25 mM, pH 9.3) and sodium dihydrogenphosphate (25 mM, pH 5.7). These separation conditions eliminate many of the possible interferences, including the majority of the endogenous substances and allow for detection at 200 nm.

Optimisation of the chromatographic conditions was carried out with respect to mobile phase composition. Acetonitrile was added, because a poor peak symmetry was observed when using methanol. Adding 85% orthophosphoric acid to the mobile phase modified the retention time of endogenous plasma peaks to get clear peaks of interest. The best separation of florfenicol from plasma peaks was obtained when mobile phase was composed of acetonitrile-water (25:75 v/v) adjusted to pH 2.7 with 85% phosphoric acid. Due to that retention time for florfenicol and lamotrygine were too long (4.57 and 8.15, respectively) influence of mobile phase was changed from 1 ml/min to 1.5 ml/min.

3.4. Biomedical application of both methods

It can be stated that both methods are specific, precise and accurate enough for bioavailability and pharmacokinetic investigation of florfenicol. Likewise, the proposed methods can be applicable for quantitative determination of florfenicol residue in farming animal tissues. These methods are sensitive enough to perform determinations below the maximum residue limit (MRL) values for this drug and allow establishing the withdrawal period necessary to avoid any risk for human health after consuming foodstuffs proceeding from animals treated with this drug.

4. Conclusions

As a general conclusion the CE method for the determination of florfenicol in plasma is a interesting alternative tool to the HPLC method. Both methods allow direct and sensitive quantification of florfenicol in biological samples and have been shown to have good sensitivity, linearity (correlations > 0.999) with relative standard deviation (R.S.D.) values no more than 10%. Validation parameters for this particular situation were adequate for both methods, although linearity has resulted slightly better and precision slightly poorer for HPLC. Likewise, the separation efficiency is higher with CE methods, which leads to shorter separation time. The described procedure in the case of CE as well as HPLC is rapid and has been successfully applied to bioavailability investigations of florfenicol.

Overall, the applications of both methods in the analysis of dugs are broadly similar and cover areas, such as therapeutic drug monitoring, and pharmacokinetic studies. As is well known, both methods have some advantages and some drawbacks. However, HPLC is limited by sometimes poor separation efficiencies, expensive chromatographic columns, and the consumption of relatively high amounts of chemicals. Whereas, one of the major important weak points of CE is that sometimes its poor precision and sensitivity, because of the low sample injection volume and the short optical path-length, when on-line absorption detectors are used. Nevertheless, CE can offer benefits in term of quicker method development, significantly reduced analysis time and operating costs. Furthermore, separation efficiency in CE greater in case that sample volumes are very small. The cost of a capillary and electrolyte required for a specific number of analyses are evidently lower than in HPLC. Conversely, HPLC can offer performance advantages, such as improved injection precision and detection sensitivity.

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