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Optimization and validation of a micellar electrokinetic chromatographic method for the analysis of florfenicol

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Abstract

We have optimized a micellar electrokinetic capillary chromatographic method for the separation of florfenicol and florfenicol amine, its degradation product. The separation was carried out using a 50 mM sodium borate buffer (pH 9.0) containing 25 mM of sodium dodecyl sulphate. The method selectivity was proven by the simultaneous separation of florfenicol and two structural antibiotics, chloramphenicol and thiamphenicol. The same system can also be applied for the quantitative determination of these antibiotics. The method was then validated regarding linearity, precision and accuracy.

Keywords: Florfenicol; Florfenicol amine; Chloramphenicol; Thiamphenicol; MEKC

1. Introduction

Florfenicol is a synthetic, broad-spectrum antibiotic that has been specially developed for veterinary use. It is a fluorinated analogue of thiamphenicol, a chloramphenicol analogue (Fig. 1), and its structural modification confers advantages in activity, particularly against bacteria resistant to thiamphenicol and chloramphenicol. Florfenicol is more active than chloramphenicol against *Haemophilus somnus*, a major pathogen in bovine meningitis [1,2].

Numerous chromatographic methods have been developed for the determination of this antibiotic and its residues in biological fluids and tissues, but our attention has been directed to the pharmaceutical formulations. The literature shows that high performance liquid chromatography (HPLC) is the most common method for determination of florfenicol [3–13] in (biological) matrices, but as yet, no determination methods in pharmaceuticals have been published.

Capillary electrophoresis (CE) offers an alternative technique. Although analysis by means of CE has been achieved for residues of several veterinary drugs from poultry and porcine tissues [14], the literature shows no selective single method able to determine and quantify florfenicol without derivatization and specific sample pre-treatment in pharmaceuticals.

The aim of the present study was to develop a selective CE method that was capable of determining florfenicol and florfenicol amine. To prove the method selectivity, the same system was also applied for the qualitative determination of two structural antibiotics, chloramphenicol and thiamphenicol. The system is also appropriate for quantitative determination of these antibiotics in different pharmaceutical formulations without specific sample pretreatment. The method was then validated regarding linearity, precision and accuracy.

2. Experimental

2.1. Instrumentation and electrophoresis procedure

Experiments were performed on a Waters Quanta 4000 (Millipore, Milford, USA). A fused-silica capillary was used, 60 cm in total length (52.5 cm to the detector) and 75 μm

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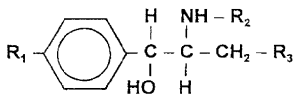

	R_1	R_2	R_3
Chloramphenicol	NO_2 -	-$COCHCl_2$	-OH
Florfenicol	H_3C-SO_2-	-$COCHCl_2$	-F
Florfenicol amine	H_3C-SO_2-	-$COCHCl_2$	-F
Thiamphenicol	H_3C-SO_2-	-H	-OH

Fig. 1. Chemical structure of chloramphenicol, florfenicol, florfenicol amine, and thiamphenicol.

internal diameter (i.d.). Hydrostatic injections were performed by lifting the sample vial approximately 10 cm above the height of the buffer vial for 10 s. For detection, the absorbance was measured by means of an online fixed-wavelength UV detector with a zinc discharge lamp and a 214-nm filter. The experiments were performed at 15 kV at room temperature (20 ± 2 °C). Data were collected on a Hewlett-Packard Integrator (HP 3396 – series II, Avondale, USA). The pH measurements were performed on a calibrated Metrohm 744 pH Meter (Herisau, Switzerland).

2.2. Chemicals and reagents

Chloramphenicol was obtained from Laboratoria Flandria (Ghent, Belgium), florfenicol and florfenicol amine from Schering-Plough Animal Health (Brussels, Belgium), and thiamphenicol and sodium dodecyl sulphate (SDS) from Sigma (St. Louis, MO, USA). Sodium dihydrogen phosphate monohydrate (p.a.) and disodium hydrogen phosphate dihydrate (p.a.) were obtained from Merck (Darmstadt, Germany), sodium tetraborate decahydrate from Janssen Chimica (Beerse, Belgium), and boric acid from UCB (Brussels, Belgium).

The commercially available drugs Nuflor® (Florfenicol, Schering-Plough), Cebenicol® (Chloramphenicol, Chauvin/Lundbeck), and Urfamycine® (Thiamphenicol, Zambon) were used for quantitative determinations.

All solutions were prepared using distilled water obtained from deionized water.

2.3. Running buffers

During the development of the method, sodium borate buffers of different pH and molarity were used. The sodium borate buffers were prepared by mixing a sodium tetraborate decahydrate solution and a boric acid solution. These buffers were used as solvent for the preparation of stock, standard, and sample solutions.

Running buffer solutions were prepared at different SDS concentrations.

2.4. Internal standard solutions

To compensate for differences in injection volume during the quantitative determination, an internal standard was used. As chloramphenicol, florfenicol, and thiamphenicol are baseline separated, they can all be used as internal standards with regard to each other. Selection had to be made based on the substance to be examined. Although each antibiotic can be combined, the nearest migrating compound was chosen as the internal standard. Internal standards were used at 0.1 mg/ml in sample solutions (Table 1).

2.5. Reference solutions

Reference solutions were prepared at the concentration of 0.1 mg/ml in solvent.

2.6. Sample preparation for the quantitative determination

Quantitative sample solutions were prepared at the concentration of 0.1 mg/ml in solvent (Table 1).

All samples and buffers were filtered by passing them through 0.45 μm membrane filters (Millipore, Bedford, USA).

3. Results and discussion

3.1. Optimization of the method

Capillary zone electrophoresis (CZE) is the simplest mode of CE, and the most widely used. Therefore, initially, CZE was considered as a separation method. Because florfenicol contains no functional groups which are ionized between pH 2 and pH 12, this antibiotic is uncharged in this pH-range. Consequently, the separation and determination of florfenicol by CZE would be non-selective.

The introduction of micellar electrokinetic capillary chromatography (MEKC) has overcome the difficulty of separating neutral analytes using CZE, and has increased the selectivity in the separation of charged molecules. Compounds having the same charges and similar structures often migrate

Table 1
Sample preparation for the quantitative determination

	Sample solution (per 100 ml)	Internal standard solution (mg/ml)	Diluted sample solution (mg active substance/ml)
Chloramphenicol [Cebenicol®] 20 mg/5 ml solution	±2.5 ml	Thiamphenicol: 0.10	±0.10
Florfenicol [Nuflor®] 300 mg/ml solution	±190 mg	Thiamphenicol: 0.10	±0.10
Thiamphenicol [Urfamycine®] 250 mg capsules	±115 mg	Chloramphenicol: 0.10	±0.10

at almost the same velocity in CE, whereas differences in their distribution constants in the micellar phase lead to baseline separations [15]. Many examples demonstrating an improved resolution using MEKC when compared to CE have been published [16]. Therefore, MEKC was investigated as a separation method.

The factors that could most affect the response migration time were the pH value, the molarity of the running buffer, and the concentration of the micelle-forming agent. The pH of the separation buffer plays an important role. It affects the observable migration velocity of the solutes by changing the effective electrophoretic mobility of the solutes by affecting the degree of dissociation (or protonation), and by changing the velocity of the electro–osmotic flow (EOF) by affecting the zeta potential at the capillary walls.

At ordinary temperatures, florfenicol possesses stability over a wide pH range. A borate buffer was selected as running buffer to improve the selectivity of the determination of florfenicol. Chloramphenicol and thiamphenicol, the two structural analogues of florfenicol, have vicinal hydroxyl groups and can form a complex with the borate ions so that they become charged [17]. Florfenicol, having no such group, cannot react with the borate ions and remain uncharged. Moreover, chloramphenicol is susceptible to general acid/base catalysis because of buffer ingredients. Therefore, careful selection of buffers for chloramphenicol solutions is indicated [18]. Because both phosphate and citrate buffers catalyze hydrolysis of this antibiotic, a borate buffer was recommended. The best selectivity and peak shapes were obtained at pH 9.0. Different concentrations of the running buffer (10–50 mM) were tested to optimize the separation. When the concentration of the electrolyte increased, the selectivity of the separation and the migration times remained the same. Because of the higher buffering capacity, a 50 mM buffer was selected.

Sodium dodecyl sulphate is one of the most popular surfactants. Since SDS is widely used in the MEKC technique, the usefulness of this additive was also evaluated. The influence of SDS on the separation was studied by adding it to a 50 mM sodium borate buffer (pH 9.0). An apparent increase of selectivity was observed: if only the buffer was used, co-migration of florfenicol, florfenicol amine and formamide (marker) on one hand, and chloramphenicol and thiamphenicol on the other was observed. As the concentration of SDS increased, the mutual distance within and between the two groups of eluting compounds increased so the two groups began to merge (Fig. 2). Because of nearly equal mutual distances, the highest resolution was obtained with 25 mM SDS (Fig. 3).

It was found that the best conditions for the determination of florfenicol and florfenicol amine are provided by a 50 mM sodium borate buffer (pH 9.0) containing 25 mM SDS.

3.2. Quantitative determination in pharmaceutical formulations

The same 50 mM sodium borate buffer (pH 9.0) containing 25 mM SDS solution may be applied for the quantitative determination of chloramphenicol, florfenicol, and thiamfenicol in capsules and solutions. Using different placebo mixtures it was demonstrated that the following excipients do not adversely affect the results: lactose, talc, magnesium stearate, dextran, methyl parahydroxybenzoate, *N*-methyl-2-pyrrolidone, propylene glycol, macrogol 300, and hypromellose.

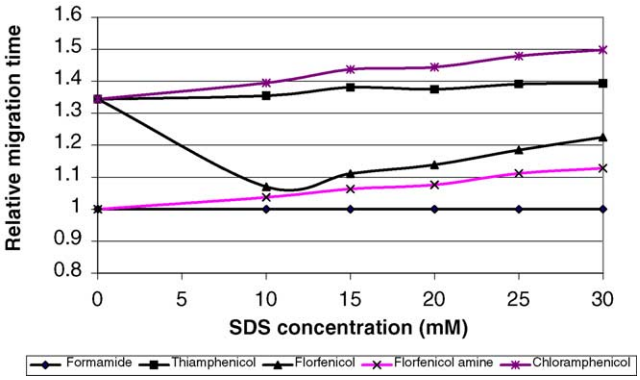

Fig. 2. Influence of SDS concentration. Experiments were carried out using a fused-silica capillary 60 cm (52.5 cm to the detector) × 75 μm i.d., and 50 mM sodium borate buffer (pH 9.0) containing different concentrations of SDS as running buffer; applied voltage, 15 kV; detection at 214 nm.

3.3. Validation of the method

3.3.1. Linearity

The detector responses were found to be linear for the three antibiotics in the concentration range of 0.025 to 0.125 mg/ml. Regression analysis data for the calibration curves were calculated using the peak areas (Table 2).

3.3.2. Precision

The error of the equipment, the accuracy of electrophoretic separation, and the relative standard deviations of estimations were determined by performing 10 consecutive injections of the same sample (0.10 mg/ml). The relative standard deviation of chloramphenicol, florfenicol and thiamphenicol were, respectively, 0.69, 1.08, and 0.70% (calculated with the areas).

The precision (repeatability) was determined by the analysis of 10 replicate samples under the same operating conditions, carried out by the same analyst, and on the same day. The mean value of the concentration and the relative standard deviation are summarized in Table 3.

The assay of the antibiotics was also performed with HPLC [2] so we could compare the results (Table 3).

Table 2
Linearity

	Correlation coefficient (r^2)	Regression equations
Chloramphenicol	0.9996	$y = 0.1343\,x - 0.0001$
Florfenicol	0.9999	$y = 0.1188\,x - 0.0002$
Thiamphenicol	0.9997	$y = 0.1056\,x + 0.0075$

Table 3
Quantitative determination of the antibiotics by MEKC and HPLC

Substance to be examined	Theoretical amount	Amount found by MEKC (n = 10)	Amount found by HPLC (n = 10)
Chloramphenicol [Cebenicol®]	20 mg/5 ml	20.26 ± 0.10 mg or 101.3 ± 0.5%	20.27 ± 0.04 mg or 101.4 ± 0.2%
Florfenicol [Nuflor®]	300 mg/ml	296.6 ± 3.3 mg or 98.9 ± 1.1%	297.0 ± 3.3 mg or 99.0 ± 1.1%
Thiamphenicol [Urfamycine®]	250 mg	247.3 ± 2.3 mg or 98.9 ± 0.9%	246.8 ± 0.2 mg or 98.7 ± 0.1%

3.3.3. Accuracy

The accuracy of the method was determined by investigating the recovery of each component at three levels, ranging from 80 to 120% of the theoretical concentration, from placebo mixtures spiked with the active substance (Table 4).

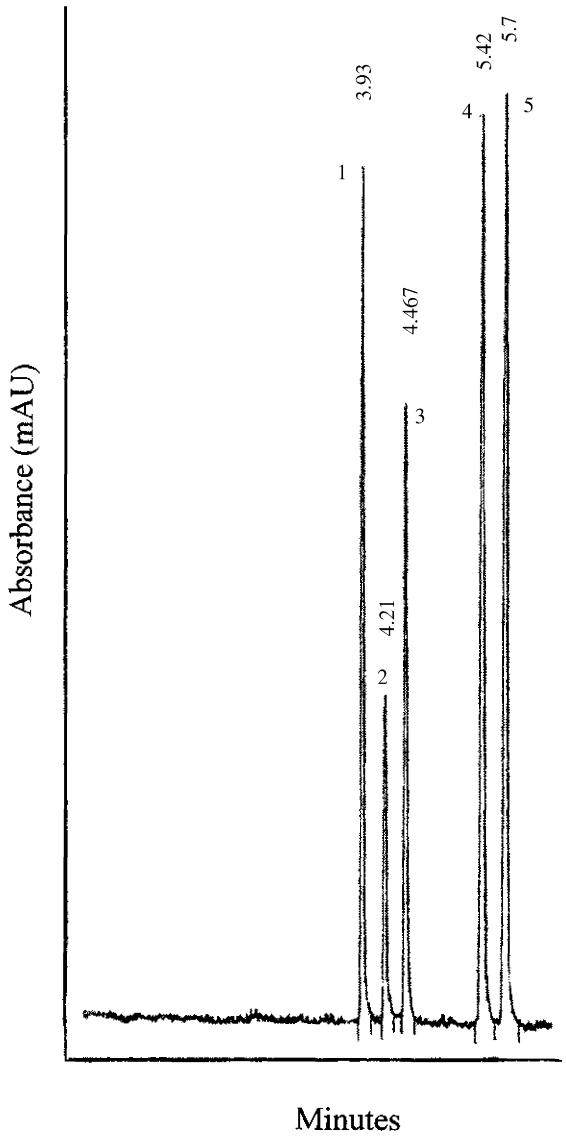

Fig. 3. Electropherogram of a mixture of formamide (1), florfenicol amine (2), florfenicol (3), thiamfenicol (4) and chloramphenicol (5) using a fused-silica capillary 60 cm in total length (52.5 cm to the detector) × 75 μm i.d. and 50 mM sodium borate buffer (pH 9.0) containing 25 mM SDS as running buffer; applied voltage, 15 kV; detection at 214 nm.

Table 4
Accuracy

	Recovery placebo + 80% (n = 3)	Recovery placebo + 100% (n = 3)	Recovery placebo + 120% (n = 3)
Chloramphenicol	102.0% ± 0.7%	99.9% ± 0.7%	100.5% ± 0.0%
Florfenicol	101.8% ± 0.6%	100.8% ± 0.5%	98.8% ± 0.9%
Thiamphenicol	101.9% ± 0.5%	99.8% ± 0.9%	98.4% ± 0.5%

4. Conclusions

Our results demonstrate that the micellar electrokinetic capillary chromatographic separation of florfenicol and florfenicol amine can be achieved using a 50 mM sodium borate buffer solution (pH = 9.0) containing 25 mM SDS. This system can also be applied successfully to the identification and the quantitative determination of chloramphenicol and thiamfenicol in pharmaceutical formulations.

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