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DEVELOPMENT OF A RAPID CAPILLARY ZONE ELECTROPHORESIS METHOD TO QUANTIFY LEVOFLOXACIN AND MELOXICAM FROM TRANSDERMAL THERAPEUTIC SYSTEMS

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ABSTRACT. Capillary zone electrophoresis (CZE) could be a useful technique for the quantification of active substances from transdermal therapeutic systems (TTSs). TTSs are pharmaceutical forms in development that may release one or more active substances with some significant advantages as increased compliance to treatment, avoidance of first hepatic passage and low manufacturing costs. A simple, reliable, efficient, and low-cost CZE method was developed and validated for the simultaneous determination of levofloxacin (fluoroquinolone) and meloxicam (non-steroidal anti-inflammatory) from TTSs. The selected experimental parameters were 50 mM borax (pH 9.3) as background electrolyte, +25 kV applied voltage, 50 mbar/5 seconds hydrodynamic injection and 40°C temperature, using an uncoated fused-silica capillary with (51 cm total length/43 cm effective length, 50 µm i.d.). CZE experiments were performed in less than four minutes with a resolution of 7.79 at a wavelength of 335 nm. Validation of the method presented good linearity data, precision (RDS% < 1 for migration times and RDS% < 2 for peaks area) and sensitivity (LOD 3.43 and 16.05 $\mu g \cdot mL^{-1}$, LOQ 10.38 and 54.55 $\mu g \cdot mL^{-1}$ for levofloxacin and meloxicam, respectively). Recovery of the active substances ranged between 85.14% and 96.38%. Our developed CZE method proved its applicability for analysis of the two substances from TTSs.

Keywords: *levofloxacin, meloxicam, capillary zone electrophoresis, transdermal therapeutic systems*

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INTRODUCTION

Capillary electrophoresis (CE) is an analytical method increasingly used in the analysis of pharmaceutical products due to several advantages related to its simplicity, rapid method development and the low-costs of operation. Besides, this method may also be appropriate for the analysis of the complex samples, where analytes can be separated due to their different electrophoretic behaviour [1]. Capillary Zone Electrophoresis (CZE) is the simplest CE technique in which the analytes are separated using a simple buffer, without any additives, the separation taking place due to the differences between the own electrophoretic mobilities of the analytes. Our primary objective was the development, optimization and validation of a simple CZE method for the quantification of two active substances from new experimental transdermal therapeutic systems (TTSs).

TTSs are generally referred to as "patches". These are innovative pharmaceutical forms that may include one or more pharmaceutically active substances. Although there are many challenges in the TTS design (e.g. permeability and skin irritation) the advantages of using these devices are unquestionable as the administration of these pharmaceutical forms can improve the patient's adherence and compliance to treatment by reducing the frequency of dosing (a TTS per day up to a TTS per week) in the conditions where the first hepatic passage is avoided (thus requiring lower doses), which makes it possible to reduce both treatment and manufacturing costs [2,3]. The two selected active substances in this study are levofloxacin (LVF) and meloxicam (MLX) (Figure 1).

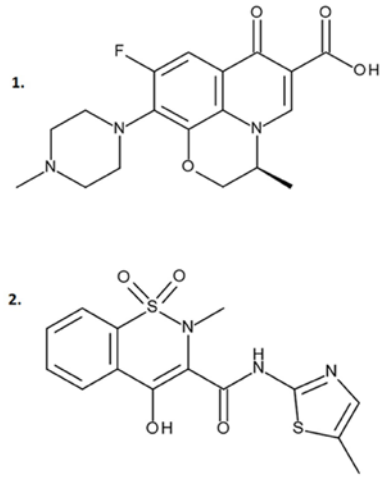

Figure 1. Chemical structures of LVF **(1)** and MEL **(2)**.

LVF ((*S)*-9-fluoro-2,3-dihydro-3-methyl-10-(4-methylpiperazin-1-yl)-7-oxo-7H-pyrido[1,2,3-de]-1,4-benzoxazine-6-carboxylic acid), is a third-generation fluoroquinolone, the levorotatory isomer of ofloxacin. The antibacterial spectrum of LVF includes Gram-positive bacteria, Gram-negative bacteria and atypical bacteria. LVF is indicated in the treatment of a variety of bacterial infections and is administered both internally and externally [4,5]. Several studies and patents for topical pharmaceutical products with LVF are already published in the literature [6-9].

MLX (4-hydroxy-2-methyl-N-(5-methylthiazol-2-yl)-2H-1,2-benzothiazine-3-carboxamide 1,1-dioxide) is an effective non-steroidal anti-inflammatory drug (NSAID) which is used in the treatment of rheumatoid arthritis, osteoarthritis, and ankylosing spondylitis. In the last decade, several studies have been published regarding preparation and testing TTSs containing MLX with encouraging results in skin permeability [10-13].

Co-administration of a fluoroquinolone with an NSAID in a TTS may be beneficial in both local and systemic treatment. However, few previous studies have followed this possible association. At the administration of MLX, a significant decrease of plasma antioxidant activity occurs and also when LVF is co-administered with MLX in rabbits; the body weight of the rabbits was not influenced [14]. Other similar studies present the effects on oxidative balance in blood and the immunolocalisation of ABCG-2 transporter protein in rabbit retina [15,16]. Another research shows that co-administration of MLX with ofloxacin in goats does not require any adjustment in dosage regimens, but in another study addressed on cross-bred calves, it is recommended the change of LVF dosage when is co-administered with MLX [17,18].

The aim of our study was the development of a new CZE method applicable for the simultaneous quantification of two active substances (LVF and MLX) from three different experimental TTS formula.

RESULTS AND DISCUSSION

Several previously published CE methods analysed both LVF and MLX. The used background electrolyte (BGE) solutions are summarized in Table 1.

Table 1. The BGE composition used in different CE methods for the analysis of LVF and MLX *(Ref. – References).*

LVF		**MLX**	
BGE composition	***Ref.***	***BGE composition***	***Ref.***
25 mM borax (pH 9.2)	[19]	100 mM borax buffer (pH 8.5)	[22]
60 mM hydroxylpropyl-beta-cyclodextrin in 50 mM phosphate buffer (pH 2.30)	[20]	10 mM Tris buffer with 60 mM sodium octane-sulfonate and 20% acetonitrile (pH 11)	[23]
20 mM phosphate buffered (pH 8.0)	[21]	18 mM sodium phosphate buffer (pH 5.90)	[24]

The pK_a values for LVF and MLX are very similar (as HA - protonated form a weak acid). Also, the results of previous research regarding the separation of a large number of antibacterial quinolones have been taken into consideration, research in which the selected BGE was 25 mM borax [25]. By correlating the information presented in Table 1 with the physical properties of the two active substances comprised in Table 2 it was considered that an appropriate BGE for our separation method might be a solution containing 25 mM borax at pH 9.3.

Table 2. Physical properties of LVF and MLX *(MW – molecular weight, HA – a protonated form a weak acid, BH^+ -a protonated form of a weak base, DMF – dimethylformamide).*

Compound	**MW ($g \cdot mol^{-1}$)**	**Solubility**	**pK_a**	**Log*P***	**Ref.**
LVF	361.37	freely soluble in glacial acetic acid, chloroform, sparingly soluble in water	(HA) 5.59 (BH^+) 7.94	1.268	[26,27]
MLX	351.4	insoluble in water, soluble in DMF, very slightly in methanol; $1.736\ M \cdot 10^{-3}$ (in 0.2 M phosphate buffer pH 7.4 at 37 °C)	(HA) 4.5 (BH^+) 3.05	2.71	[28-31]

LVF shows maximum absorption in UV at 300 nm and an additional maximum absorption of 327 nm [32,33]. MLX shows maximum absorption in UV light at 360 nm [34,35]. Thereby, the appropriate wavelength for the determination of both substances was set at 335 nm. To optimise the CZE method, the influence of BGE concentration, pH, applied voltage, injection pressure, injection time, and system temperature on the separation were analysed systematically using a “one factor at a time” optimization approach. The maximum current flow was set to 150 µA, to avoid instability of the electrophoretic system [36].

Figure 2 shows the influence of BGE concentration, pH of the BGE, voltage, and temperature on the migration times in the optimisation process.

The pH of the BGE was adjusted by addition of NaOH 1M and boric acid 1M. Thus, the best separation conditions were selected to provide adequate migration time and good peak symmetry, resolution, and selectivity (Figure 3).

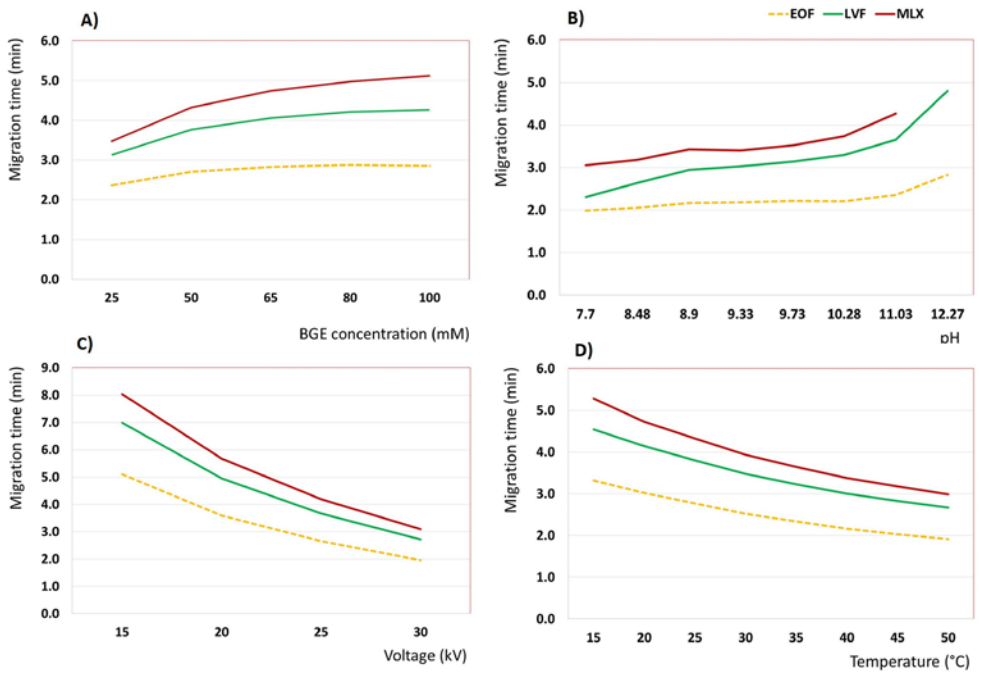

Figure 2. The influence of **A)** BGE concentration, **B)** pH of the BGE, **C)** voltage and **D)** temperature on the migration times in the optimisation process of the CZE method.

The best separation using the optimised analytical conditions of LVF and MLX by CZE is represented in Figure 4 and the parameters of the CZE experiment in Table 3.

Method validation. The method was verified regarding validation parameters according to ICH Q2(R1) Guideline [37]. The specificity of the method was proved using the UV spectra and the migration times of the analytes Thereby, the LVF and MLX could be identified simultaneously from a mixture without any interference. The migration order was established as being: LVF followed by MLX. As in the preliminary experiments, the EOF migration time values were constantly similar; we considered that it was not necessary to use an internal standard. Linearity and detectability (limit of detection – LOD, limit of quantification – LOQ) of the method were also determined. Thus, an excellent linear signal-concentration relationship for the two compounds was demonstrated (Table 4).

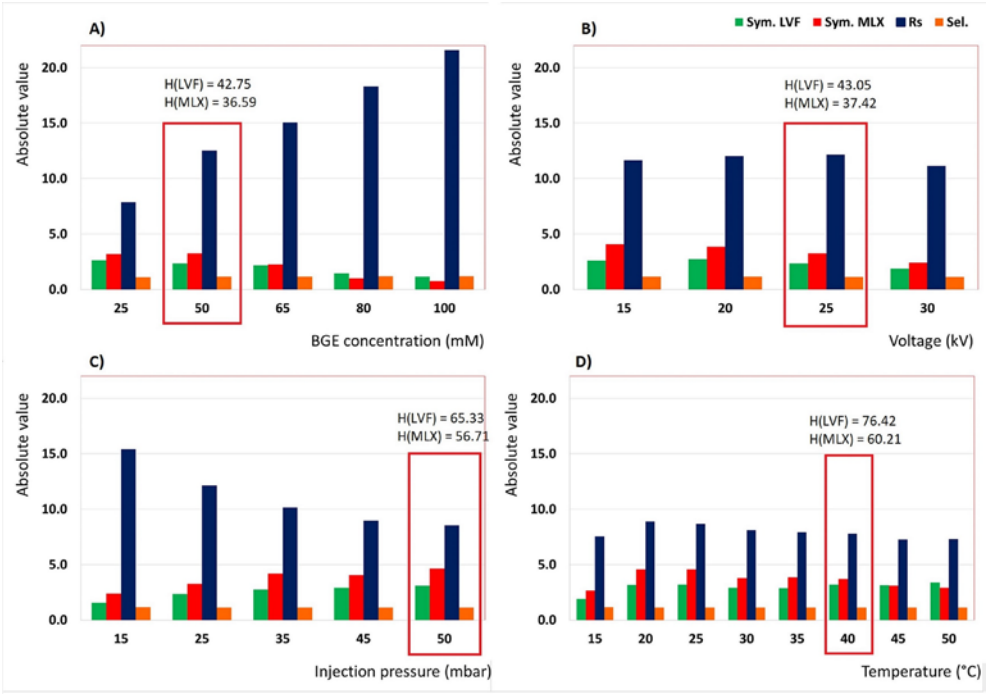

Figure 3. Variation of symmetry, resolution and selectivity of analytes signals in terms of **A)** BGE concentration, **B)** voltage, **C)** injection pressure and **D)** temperature *(Sym. – symmetry, Rs – resolution, Sel. – selectivity, H – height of peak)*.

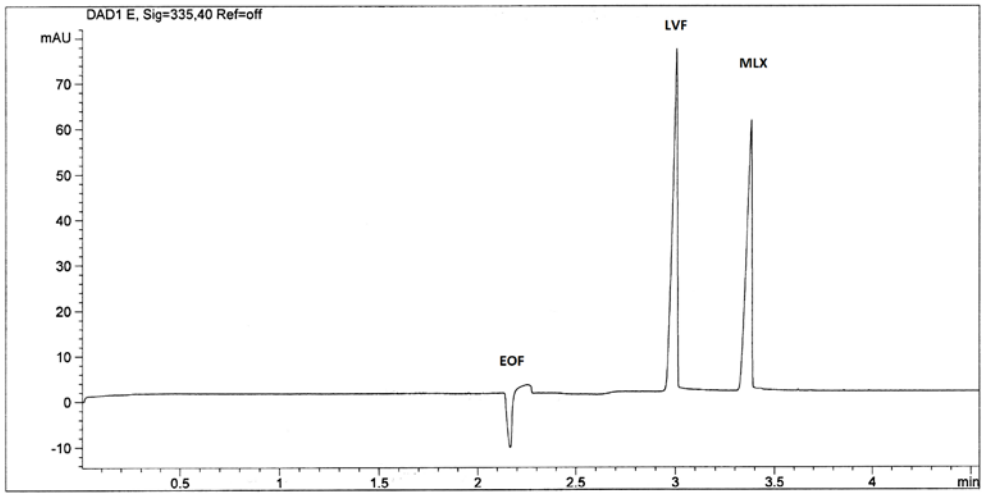

Figure 4. The electropherogram of LVF and MLX separation (BGE 50 mM borax, +25 kV applied voltage, 50 mbar/5 seconds hydrodynamic injection, 40°C temperature).

Table 3. Electrophoretic parameters of LVF and MLX separation using the optimized analytical conditions (EOF migration time 2.17, concentration 0.5 mg · mL-1) *(MT – migration time, A – area, H – height, Sym. – symmetry, P – plates, Res. – resolution, Sel. – selectivity).*

Compounds	**MT** (min)	**A** (mAU*s)	**H** (mAU)	**Sym.**	**P**	**Res.**	**Sel.**
LVF	3.00	128622	76.30	3.19	67497	-	-
MLX	3.38	112586	57.99	3.71	73023	7.79	1.13

LOD and LOQ were calculated based on the standard deviation of the response (Sy.x) and the slope (S). Excellent correlation coefficients and low detection limits for LVF and MLX were obtained. The LOD and LOQ for LVF are comparable with those previously published in the literature [19], while for MLX are slightly higher than other in published materials [23]. The concentration range of the method is between 6.25 μg·mL^{-1} – 1000 μg·mL^{-1}. Also, the robustness of the CZE method is supported by the variation of internal (system temperature, applied voltage, injection parameters) and external (BGE composition, concentration, pH) parameters in the optimisation part of the research.

Table 4. Linearity and detection limits of the separation CZE method.

Linearity parameters and detection limits	LVF	MLX
Regression equation	y = 267.44x + 0.1345	y = 238.48x - 0.8388
Correlation coefficient	0.9990	0.9983
Y intercept	0.1345	-0.8388
Slope of the regression line (S)	267.44	238.48
Standard deviation of residuals from the line (Sy.x)	0.2777	1.3010
LOD (μg·mL^{-1}), calculated LOD = $\frac{3.3\ Sy.x}{S}$	3.43	16.05
LOQ (μg·mL^{-1}), calculated LOQ = $\frac{10\ Sy.x}{S}$	10.38	54.55

Table 5. The obtained RDS% values for intra-day and inter-day precision *(Conc. – concentration, RSD – residual standard deviation).*

Compound	Conc. (μg·mL^{-1})	Intra-day precision (RSD%) n = 6			Inter-day precision (RSD%) n= 18, days 3		
		MT_{EOF}	MT	A	MT_{EOF}	MT	A
LVF	**500**	0.228	0.167	1.456	0.849	0.824	1.396
	300	0.765	0.807	1.366	0.738	0.629	1.969
	100	0.218	0.273	1.514	0.378	0.398	1.740
MLX	**500**	0.228	0.219	1.401	0.849	0.623	1.353
	300	0.765	0.699	1.977	0.738	0.562	1.982
	100	0.218	0.299	0.423	0.378	0.465	1.963

The precision of the method was evaluated regarding repeatability and intermediate precision (Table 5). The obtained RDS% values are comparable with previously published data and support a good precision (intra-day and inter-day) of the method [19,23,24].

Application of the CZE method for the quantification of LVF and MLX from TTSs. Three experimental TTSs containing LVF and MLX (Table 6) were evaluated using the optimized CZE method. Dissolving the TTSs in the appropriate solvent represented a significant challenge taking into consideration both active substances and excipients. Ethylcellulose (EC) is insoluble in water but is freely soluble in chloroform. If EC contains

not less than 46.5% of ethoxy groups, this is freely soluble in ethanol (96°) and methanol. Hydroxypropyl methylcellulose (HPMC) is soluble in cold water, practically insoluble in chloroform, ethanol (96°) but soluble in a mixture of ethanol and dichloromethane, methanol and dichloromethane, water and ethanol (96°) [38]. The TTS samples mentioned in Table 6 were introduced in 25 mL volumetric flasks and dissolved in a mixture of organic solvents, with or without distilled water. Based on known solubility's of LVF, MLX, and polymers, several combinations of solvents were tested to obtain an appropriate solution for injection in the CE system.

Table 6. Composition of three experimental TTSs *(HPMC E5 - hydroxypropylmethylcellulose type E5, HPMC 15000 - hydroxypropylmethylcellulose type 15000, EC 10 - ethylcellulose type 10)*.

Components (m/m %)	TTS 1	TTS 2	TTS 3
LVF	0.5	0.5	0.5
MLX	0.5	0.5	0.5
HPMC E5	3	-	-
HPMC 15000	-	2	1
EC 10	-	-	1
Ethanol (96% v/v)	30	30	30
Propylene glycol	10	10	10
Polysorbate 20	1	1	
Distilled water	up to 100	up to 100	up to 100

The solvent systems used were as follows: for TTS 1 - 2 ml DMF, 9 ml ethanol, and distilled water up to 100 ml; for TTS 2 - 2 ml DMF, 2 ml chloroform, and ethanol : dichloromethane (1:1) up to 100 ml; and for TTS 3 - 2 ml DMF, 5 ml chloroform, 5 ml methanol, and ethanol : dichloromethane (1:1) up to 100 ml. After 20 minutes stirring on the ultrasonic vibration, the mixtures were filtrated through 90 mm Whatman® qualitative filter paper and diluted adequately with methanol (theoretical concentration of the active substances in samples is 0.1 mg mL^{-1}). Although HPMC is soluble in a mixture of water and ethanol (96% v/v) the samples of TTS 2 and TTS 3 did not dissolve. The bulky precipitates were filtered, and the obtained solutions were additionally diluted with methanol. The collected quantitative results were unsatisfactory probably due to the retention of active substances in the precipitate. The obtained recovery results for LVF were satisfactory for all three experimental TTSs; however, MLX recovery values were lower (Table 7). According to some authors, recovery testing tends to be less critical, as long as the values of detection limits, precision and accuracy are acceptable [39].

Table 7. The composition of three experimental TTSs.

TTSs	Active substance	Theoretical concentration ($mg \cdot mL^{-1}$)	Found concentration ($mg \cdot mL^{-1}$)	Recovery (%) ± SD
1	LVF	0.1	0.096	96.38 ± 4.37
	MLX	0.1	0.091	91.23 ± 7.76
2	LVF	0.1	0.091	91.31 ± 5.05
	MLX	0.1	0.088	90.03 ± 9.45
3	LVF	0.1	0.090	90.10 ± 5.23
	MLX	0.1	0.085	85.14 ± 4.63

CONCLUSIONS

The developed CZE method proved to be appropriate to quantify the LVF and MLX simultaneously from TTSs. A BGE containing 50 mM borax (pH 9.3) was used and the following optimised experimental conditions were applied: +25 kV applied voltage, 50 mbar/5 seconds hydrodynamic injection, 40°C temperature, detection at a wavelength of 335 nm. The method validation parameters have been verified and also the recovery of the active substances. The main advantages of this new method consist in its simplicity, rapidity (under four minutes), and low volumes consumption of solvents and analytes with consequently low costs of operation. Thus, we consider that our newly developed CZE method may be an alternative to classical HPLC in quantitative determinations of TTSs.

EXPERIMENTAL SECTION

Apparatus and chemicals. The CZE experiments were performed on an Agilent 1600 CE (with diode-array detector). The software used to process the data was Chemstation 7.01 (Agilent). An uncoated fused-silica capillary (Agilent) with 51 cm total length (43 cm effective length) and 50 µm internal diameter was used in the experiments. The pH of the samples was measured with a Hanna Instruments HI2215 pH-meter.

Active substances and polymers were supplied as follows: LVF and EC 10 (EC 10 mPa·s) from Sigma Aldrich Co. (Germany), MLX from Techno Drugs & Intermediates Ltd. (India), HPMC 15000 (Metolose 90SH - 15000 mPa·s) from Shin-Etsu Chemical Co., Ltd. (Tokyo, Japan), and HPMC E5 (Methocel E5 - 5 mPa·s) from Dow Chemical Co. (Midland, USA). All chemicals and solvents were of analytical reagent grade, and ultrapure water was obtained using a Millipore Direct-QS water distiller.

The stock solutions were prepared daily using methanol for LVF and a mixture of methanol – DMF in ratio 9:1 for MLX, at a concentration of 1 mg mL^{-1}. The solutions were kept at +4°C in the refrigerator and diluted with methanol to obtain the appropriate concentrations.

Capillary preconditioning and conditioning. To ensure optimal run-to-run analytical conditions, reproducible migration times and a constant EOF, the capillary was conditioned by flushing with 1M NaOH (20 min), ultra-pure water (5 min), and BGE (20 min) at the beginning of each day. Between experiments, the capillary was preconditioned by flushing with BGE (2 min).

Preparation of TTSs. The samples of TTSs were obtained in the form of the thin matrices (polymeric films) by casting and solvent evaporation technique, according to a previously published method [40].

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