Journal of Chromatography B, 878 (2010) 1480–1486

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Simultaneous determination of clopidogrel and its carboxylic acid metabolite by capillary electrophoresis

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ARTICLE INFO

Article history:
Received 29 December 2009
Accepted 23 March 2010
Available online 31 March 2010

Keywords:
Capillary electrophoresis
Clopidogrel
Carboxylic acid metabolite
Method validation

ABSTRACT

A capillary electrophoresis method was developed and validated for the first time for the analysis of clopidogrel and its carboxylic acid metabolite. Prior to method optimization, the pH dependence of effective mobility of both compounds was determined in order to define the initial pH of the running buffer. The optimized method demonstrated to be selective, and linear in the concentration range of 2–100 μM for both compounds. The method limits of detection and quantification were, respectively, 1.2 and 3.7 μM for clopidogrel and 1.1 and 3.2 μM for the carboxylic acid metabolite. Moreover, method validation demonstrated acceptable results for method repeatability (RSD < 7%), intermediate precision (RSD < 7%) and accuracy (85–96%) and is suitable for the quantitative analysis of clopidogrel and its metabolite in serum samples. The validated method was also applied to the determination of the kinetic parameters of the enzymatic hydrolysis of clopidogrel. An apparent K_m of 145 ± 30 μM and V_{max} of 0.4, 1.5 and 3.4 μM/min, respectively for the enzyme concentrations 1.0, 2.0 and 4.0 U/ml, were obtained.

1. Introduction

Clopidogrel (Fig. 1A), a thienopyridine derivative, is a potent antiplatelet and antithrombotic agent which inhibits the binding of adenosine diphosphate (ADP) to its platelet receptor and blocks subsequent platelet aggregation [1,2]. Clopidogrel is a prodrug, requiring oxidation by the hepatic cytochrome P450 (CYP450) and subsequent hydrolysis to generate the active metabolite, a thiol compound. However, after oral administration in humans, the main metabolite circulating in serum (85%) is the inactive carboxylic acid metabolite (Fig. 1B), formed by hydrolysis of the ester function by human carboxylesterasel [2,3].

A few analytical methods have been reported in the literature for the determination of clopidogrel and/or its carboxylic acid metabolite. These are based on high-performance liquid chromatography (HPLC) with UV absorbance [4–7] or mass spectrometry (MS) detection [8–15] and gas chromatography with mass spectrometry detection (GC-MS) [16]. Recently a capillary electrophoresis (CE) study has been published for the determination of clopidogrel and its impurities in pharmaceutical formulations [17] but no CE method has been described for clopidogrel analysis in biological samples.

CE has emerged as an alternative to the use of HPLC and as a powerful tool in the pharmaceutical industry, with a wide range of applications, such as drug formulation (active principle and drug related impurities quantification), determination of physicochemical properties (log *P*, solubility, etc.) or determination of pharmacokinetic parameters. Several methods can be used for pK_a determination but due to its simplicity and selectivity, CE has been frequently used for this purpose [18–24]. Besides the recent improvements of liquid chromatography techniques, like UPLC, CE still offers one major advantage, which is the ability to separate both charged and non-charged molecules. Other advantages include high separation efficiency, short analysis time, low sample and electrolyte (buffer) consumption, low waste generation and easy implementation of chiral separations.

The aim of the present study was to develop and validate a fast electrophoretic method for the quantification of clopidogrel and its carboxylic acid metabolite, which could be useful in metabolism and kinetic studies and also in the analysis of biological samples. Prior to the study, the pH dependence of effective mobility of both compounds was obtained (also by capillary electrophoresis) in order to determine the best working pH range.

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doi:10.1016/j.jchromb.2010.03.044

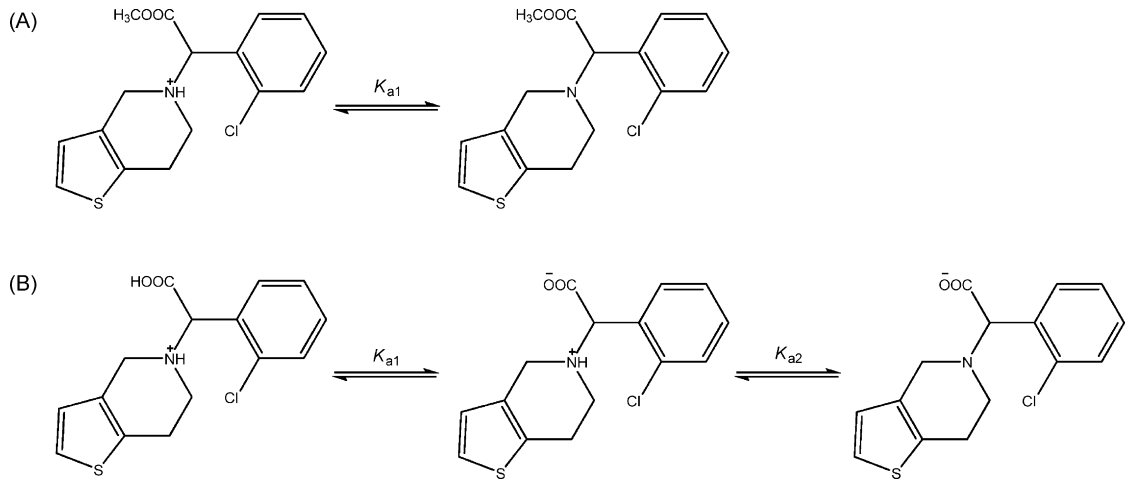

Fig. 1. Protonation/deprotonation equilibria of clopidogrel (A) and clopidogrel carboxylic acid metabolite (B) according to the estimation by the MarvinSketch software.

2. Materials and methods

2.1. Chemicals

Clopidogrel bisulfate was obtained from Hygro Chemical Pharmtek Pvt. Ltd. (Hyderabad, India). Clopidogrel carboxylic acid derivative was synthesised from clopidogrel by alkaline hydrolysis. Clopidogrel bisulfate (23.0 mg, 0.07 mmol, 1 eq.) was dissolved in MeOH (0.5 ml) to which LiOH (8.0 mg, 0.35 mmol, 5 eq.) and water (0.2 ml) was added. The mixture was stirred for 24 h at 40 °C. The reaction solvent was evaporated under reduced pressure and the residue was partitioned between aqueous 1 N HCl (2 ml) and diethyl ether (2 ml). The two layers were separated and the aqueous phase was further extracted with diethyl ether (2 ml). The combined organic layers were dried with $MgSO_4$ and filtered. The organic solvent was gently evaporated to dryness under a stream of nitrogen to yield a residue (18.6 mg; yield: 91.2%; purity: 98.6%). The residue was confirmed to be clopidogrel carboxylate by NMR assignments based on peak integration and multiplicity for 1D 1H spectra, and MS. δ_H (400 MHz, DMSO): 7.79 (1H, d, J = 7.6), 7.34 (1H, d, J = 7.6), 7.19–7.24 (3H, m), 6.71 (1H, d, J = 4.8), 4.33 (1H, s), 3.65–7.69 (2H, m) 10.8, 2.58–2.81 (4H, m). δ_C (100 MHz, DMSO): 25.35 ($\underline{C}H_2$), 48.10 ($CH_2\underline{C}H_2N$), 50.48 ($C\underline{C}H_2N$), 71.82 ($\underline{C}COOH$), 122.63 ($SCH\underline{C}HC$), 125.64 ($S\underline{C}H$), 126.38 ($ClC\underline{C}HCHCH\underline{C}H$), 127.72 ($ClCCHCH\underline{C}H$), 128.56 ($ClC\underline{C}HCH$), 130.79 ($ClCCH\underline{C}H$), 132.79 ($S\underline{C}CH_2$), 133.76 ($Cl\underline{C}CH$), 134.75 ($\underline{C}CHCOOH$), 138.72 ($CH\underline{C}CH_2$), 171.81 ($\underline{C}OOH$). m/z 308.8 (MH^+).

2.2. Instrumentation

Analytical separations were carried out in a Beckman P/ACE MDQ capillary electrophoresis system coupled with diode array detector (DAD) (Palo Alto, CA, USA).

NMR spectra used for characterization of products were recorded on a Bruker Avance 400 instrument, operating at 400 MHz for 1H spectra and at 100 MHz for 13C. The reference used for the ^{1}H NMR measurements was tetramethylsilane. Peak assignments are based on peak integration and multiplicity for 1D 1H spectra.

Mass spectrometry analysis was performed by direct infusion of methanolic solution in a LCQ ion trap mass spectrometer from Thermo Finnigan, equipped with an atmospheric pressure chemical ionization source (positive mode) and controlled by Excalibur version 1.3 software (Thermo Finnigan–Surveyor, San Jose, CA, USA).

Table 1
Composition of running buffers used for the determination of the pK_a by CE (I = 0.05 M).

pH range	Constituent	Stock solutions
2.5–3.0	Phosphate	0.5 M H_3PO_4, 1.0 M NaH_2PO_4
4.0–5.0	Acetate	1.0 M CH_3COONa, 1.0 M CH_3COOH
6.0–7.5	Phosphate	1.0 M NaH_2PO_4, 0.5 M Na_2HPO_4
8.0–9.0	Tris	0.2 M Tris, 0.2 M Tris.HCl
9.2–10.5	Ammonium	0.1 M NH_3, 0.1 M NH_4Cl
11.0–11.5	Phosphate	0.1 M K_3PO_4, 0.5 M K_2HPO_4

2.3. Determination of the pH dependence of effective mobility

The apparent mobilities of clopidogrel and its carboxylic acid were determined using running buffers with an ionic strength of 0.05 and pH in the range of 2.5–11.5, prepared as described in Table 1. Sodium dihydrogenphosphate (99%, Merck, Darmstadt, Germany), sodium hydrogenphosphate (99%, Riedel-de Haën, Seelze, Germany), *o*-phosphoric acid (85%, Panreac, Castellar del Vallès, Spain), potassium hydrogenphosphate (99%, Sigma–Aldrich, Steinheim, Germany), potassium dihyrogenophosphate (≥98%, Sigma–Aldrich, Steinheim, Germany), sodium acetate (99%, Riedel-de Haën, Seelze, Germany), acetic acid (99.79%, Fisher Scientific Ltd., Loughborough, UK), ammonia (25%, Panreac, Castellar del Vallès, Spain), ammonium chloride (>99.5% %, Merck, Darmstadt, Germany), Tris (Sigma–Aldrich, Steinheim, Germany) and Tris HCl (99%, Sigma–Aldrich, Steinheim, Germany) were used to prepare the buffer solutions.

Analyte solutions at 10 μM were prepared in water by dilution from stock solutions (1000 μM in dimethylsulfoxide (DMSO, 99.5%, Lab-Scan, Dublin, Ireland)). DMSO was used as neutral marker (1%, v/v). A 75 μm, 50 cm (40 cm to detector) fused silica capillary was used and maintained at 25°C.

The pH dependence of the mobilities was evaluated and the ionization constants were determined based on the relationship between the electrophoretic mobility and the degree of dissociation of species over a range of electrolyte pHs. The effective mobility, M_e ($cm^2\ s^{-1}\ V^{-1}$) of an ionic species at a particular pH, defined as the difference between the apparent mobility (M_{app}) and the mobility due to the electroosmotic flow (M_{EOF}), can be calculated using the following equation:

$$M_e = M_{app} - M_{EOF} = \frac{L_c L_d}{V}\left(\frac{1}{t_{app}} - \frac{1}{t_{EOF}}\right) \quad (1)$$

where L_c is the length of the capillary to the detector (cm), L_d is the total capillary length (cm), V is the applied voltage (V) and t_{app} and

t_{EOF} are the migration times of the analyte and the neutral marker compound (DMSO), respectively. A plot of effective mobility of an ionic species against the pH of the running buffer, affords a sigmoidal curve whose inflection point corresponds to the analyte pK_a.

Prior the assay of each compound, the capillary was rinsed with 0.1 mM NaOH, followed by water and the more basic running buffer. Mobilities were then determined in this buffer. Before proceeding to the determination of mobility with the buffer immediately following in the pH scale, the capillary was rinsed with that buffer for 5 min. Injection was by pressure (1.5 psi) for 6 s and a potential of 20 kV was applied throughout the run.

Each sample was tested three consecutive times at each pH and the mobilities were calculated based on the migration time of the sample and of the t_{EOF}, using Eq. (1). The average of the mobilities of each sample was fitted by non-linear regression to a sigmoidal curve using GraphPad Prism 5.0 (GraphPad software, Inc.).

2.4. Method development and validation

The method for the separation and quantification of the two test compounds (clopidogrel and its carboxylic acid) was developed and optimized by studying different variables, such as buffer pH, modifier concentration (SDS and HP-β-CD), applied voltage and capillary temperature. For that purpose a design of experiment (DoE) and analysis of variance were performed with the support of Minitab® Statistical Software.

A multilevel factorial design with two factors (voltage and capillary temperature) was developed using respectively 5, 7.5, 10, 15 and 20 kV for voltage and 15, 20 and 25 °C for capillary temperature. Experiments with different combinations of these variables were conducted in triplicate and their effect on peak area, migration time, peak width and resolution was studied. Stock solutions of clopidogrel bisulfate and its carboxylic acid derivative were prepared in methanol (MeOH, HPLC grade, Lab-Scan, Dublin, Ireland) at 2 mM, and kept in the dark at 4 °C. Working standards for calibration were prepared by dilution from the stock solutions in running buffer in the range 2–100 μM.

After optimization, a 25 mM phosphate buffer pH 7.0 was chosen as background electrolyte (BGE). In order to improve separation and peak shape, 150 mM of sodium dodecyl sulfate (SDS, >98.0%, Fluka, Buchs, Switzerland) and 50 mM of hydroxypropyl-β-cyclodextrin (HP-β-CD, average M_w ~1460, Steinheim, Germany) were added. The applied voltage was 10 kV and the capillary was operated at 20 °C.

A fused silica capillary with 31 cm length and 75 μm internal diameter was used. In order to have shorter analysis times, samples were introduced at the anode in the detector side (10 cm to the detector), by hydrodynamic injection (1.5 psi, 4.0 s). Detection was carried out at 240 nm.

Each new capillary was treated with 0.1 M sodium hydroxide and water for 30 min each. At the beginning of the day, the capillary was rinsed with 0.1 M sodium hydroxide, water and the background electrolyte for 10 min each. Prior to the injections, the capillary was rinsed with 0.1 M sodium hydroxide, water and the background electrolyte for 1 min each.

The method was validated for linearity, limits of detection and quantification, precision, accuracy and robustness under the optimized analytical conditions.

Calibration curves were constructed using the peak areas obtained at different concentrations of clopidogrel and its carboxylic acid metabolite in the range of 2–100 μM. Analyses were conducted in triplicate. The slope and intercept were determined by least-squares linear regression and the results were evaluated based on the determination coefficient of the linear regression (r^2) and residual analysis.

The limits of detection (LOD) and quantification (LOQ) were calculated with the lower concentration standards (2 μM) using the signal-to-noise ratio approach.

Instrumental repeatability was determined by calculating the relative standard deviation (RSD%) of the peak area of six analyses of three different standards (2, 25 and 100 μM), performed on the same day and under the same experimental conditions. Precision of the method was measured as system repeatability and intermediate precision.

For determination of method precision and accuracy, three replicates of human serum samples were spiked with different amounts of the test compounds and vortex-mixed for 30 s to obtain final concentrations of 5, 25 and 50 μM. 100 μl of diethyl ether (99.5%, Sigma–Aldrich, Steinheim, Germany) were added to 100 μl aliquots of each sample and the test tubes were vortex-mixed for 30 s and centrifuged at 2400×*g* for 10 min. The organic layer was transferred to a clean test tube and evaporated under a gentle stream of nitrogen. This procedure was repeated twice. The dried residue was redissolved in 100 μl of running buffer. Method repeatability was determined by analysing three replicates of each spiked sample in the same day. Intermediate precision was studied by analysing spiked serum samples on three different days. Robustness of the method was evaluated from the results obtained in the experimental designs described before.

2.5. In vitro metabolism study

Clopidogrel was diluted in phosphate buffer pH 7.0, with a final concentration of 5% DMSO. The mixture was incubated at 37 °C for 5 min in the sample compartment of the capillary electrophoresis system and after that time the reaction was started by adding porcine liver carboxylesterase (Sigma–Aldrich, St. Louis, MO, USA). The reaction was done in triplicate and followed for approximately 60 min by multiple injections along time.

The Michaelis–Menten constant (K_m) and the maximum velocity (V_{max}) of hydrolysis of clopidogrel by porcine liver carboxylesterase were determined. Five substrate concentrations (10, 25, 50, 75 and 100 μM) and three enzyme concentrations (1.0, 2.0 and 4.0 U/ml) were tested. Controls were prepared by incubation of reaction mixtures without enzyme and/or substrate in the same conditions.

3. Results and discussion

3.1. Determination of the pH dependence of effective mobility

Despite being a well known compound, there was no published data relative to the ionization constant (pK_a) of clopidogrel except for one reference in the product description of Plavix® [25]. No studies of this kind were found for clopidogrel carboxylic acid either.

Since this information would be very useful in order to define the pH for the running buffer of the analytical method, the pH dependence of effective mobility of both compounds were determined. The ionization curves were obtained by non-linear regression fitting of a sigmoidal curve to the electrophoretic mobilities of the test compounds as a function of buffer pH, in the range 2.5–11.5 and are shown in Fig. 2.

As indicated by the schematic diagram showing the protonation/deprotonation equilibria of both compounds (Fig. 1) one positively charged species together with a neutral species is expected for clopidogrel, while for its carboxylic acid derivative two charged species and a zwitterionic are predicted. However, for this compound, only one inflexion point was observed in the ionization profile. It was not possible to determine experimentally the second inflexion point, because it would be outside of the exper-

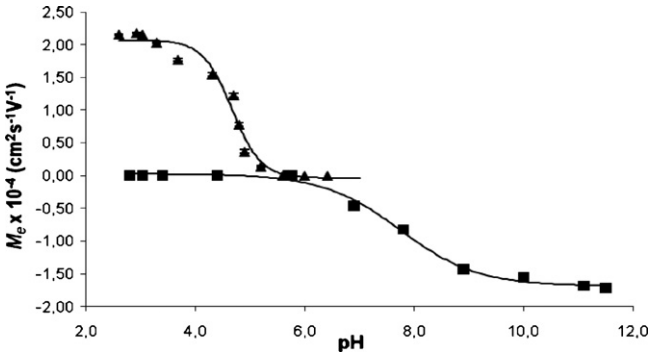

Fig. 2. pH dependence of the mobility of clopidogrel (▲) and clopidogrel carboxylic acid metabolite (■) determined by capillary electrophoresis at 25 °C (n = 3) using different buffer solutions. Other analytical conditions described in materials and methods.

imental pH range of this methodology (2.0–12.0). The ionization constants determined are shown in Table 2.

The hereby obtained pK_a for clopidogrel was in agreement with the previously reported value [25].

3.2. Method development and validation

3.2.1. Optimization of the electrophoresis conditions

According to the determined ionization constants, at pH above 6.0 clopidogrel is in the neutral form and therefore should migrate with the EOF, while at pH under 6.0 the same happens with the carboxylic acid metabolite. Since it is not possible to find a pH where both compounds are ionized and in order to improve separation and reduce interference by neutral compounds, MEKC (micellar electrokinetic chromatography) had to be used. The use of surfactants is one of the most useful modes of CE to improve the separation of small molecules. One of the most used surfactants is SDS (sodium dodecyl sulfate), an anionic surfactant. At neutral to alkaline pH, EOF moves in direction of the cathode. However, if SDS is included in the buffer solution, the electrophoretic migration of the anionic micelles proceeds in the direction of the anode and the overall micellar migration is slowed compared with the bulk flow of solvent. Because different molecules have different affinities for micelles, requirements for an improvement in the separation are available.

The effect of the variation of the concentration of SDS on the separation of the two compounds was studied. The migration times of the analytes at each analytical condition were confirmed by individual injection. Because, at pH 7.0, clopidogrel is neutral, it has greater affinity for the micelle and has a slower migration compared to clopidogrel carboxylate which is slightly negatively charged at the same pH.

The use of cyclodextrins is also known to have impact in peak resolution, peak shape, migration time and, in some cases, even in migration order due to the formation of inclusion complexes with the solutes. In this case, better peak shape and resolution were achieved at 150 mM of SDS by adding 50 mM of HP-β-CD (Fig. 3). Although shortening the length of the capillary to 10 cm allowed a faster analysis, the separation was not efficient even at the highest SDS concentration. However, the separation and shape of the peaks was improved by the addition of HP-ß-CD. A multilevel factorial design with two factors (voltage and capillary temperature) was developed. Experiments with different combinations of these variables were conducted in triplicate and their effect on peak area, migration time, peak width and peak resolution was studied. Results are shown in Table 3 and surface plots for the peak area response are shown in Fig. 4. Relative to robustness, both voltage and capillary temperature affected significantly ($p < 0.05$) the peak area and the migration time of both compounds, as expected, bringing them to lower times. Migration time repeatability was improved but a significant ($p < 0.005$) decrease in peak resolution was noticed with the increase in temperature. Increased voltage had a detrimental effect on the sensitivity of the method (lower peak areas) and produced extremely high currents (180–200 μA) limiting the capillary lifetime.

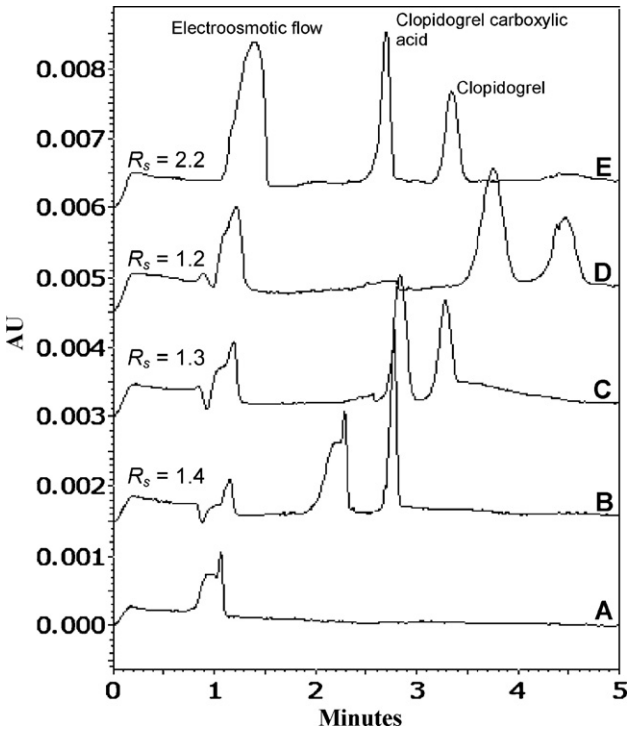

Fig. 3. Effect of the variation of concentration of surfactant in the running buffer, on the migration time of the two test compounds: (A) 25 mM phosphate buffer (pH 7.0); (B) 25 mM phosphate buffer (pH 7.0) with 50 mM of SDS; (C) 25 mM phosphate buffer (pH 7.0) with 100 mM of SDS; (D) 25 mM phosphate buffer (pH 7.0) with 150 mM of SDS; E–25 mM phosphate buffer (pH 7.0) with 150 mM of SDS and 50 mM of HP-β-CD. Analytes were at 50 μM. Other analytical conditions described in materials and methods.

A second multilevel factorial design was developed in order to study the influence of pH in combination with temperature. The 25 mM phosphate buffer pH was set at 6.0, 7.0 and 7.5. It was determined that, in the range studied, the buffer pH has little effect on peak area and width ($p > 0.05$) but affects significantly the migration time of both compounds ($p < 0.05$).

Optimum separation conditions were set in order to achieve a compromise between lower migration times, higher sensitivity and better resolution. With a voltage of 10 kV and a capillary temperature of 20 °C, short analysis time is achieved without compromising method sensitivity and peak resolution.

3.2.2. Method validation

Results obtained for method validation are presented in Table 4.

Linearity was studied under the optimized analysis conditions in the range of 2–100 μM. The calibration curves obtained for clopi-

Table 2
Experimental ionization constants determined by capillary electrophoresis.

	Clopidogrel		Clopidogrel carboxylic acid
	Experimental*	Literature	Experimental*
pK_{a1}	4.62 ± 0.28	4.55 [25]	n.d.
pK_{a2}	–	–	7.70 ± 0.11

* Sigmoidal adjustment of CE mobilities against pH (GraphPad Prism 5).

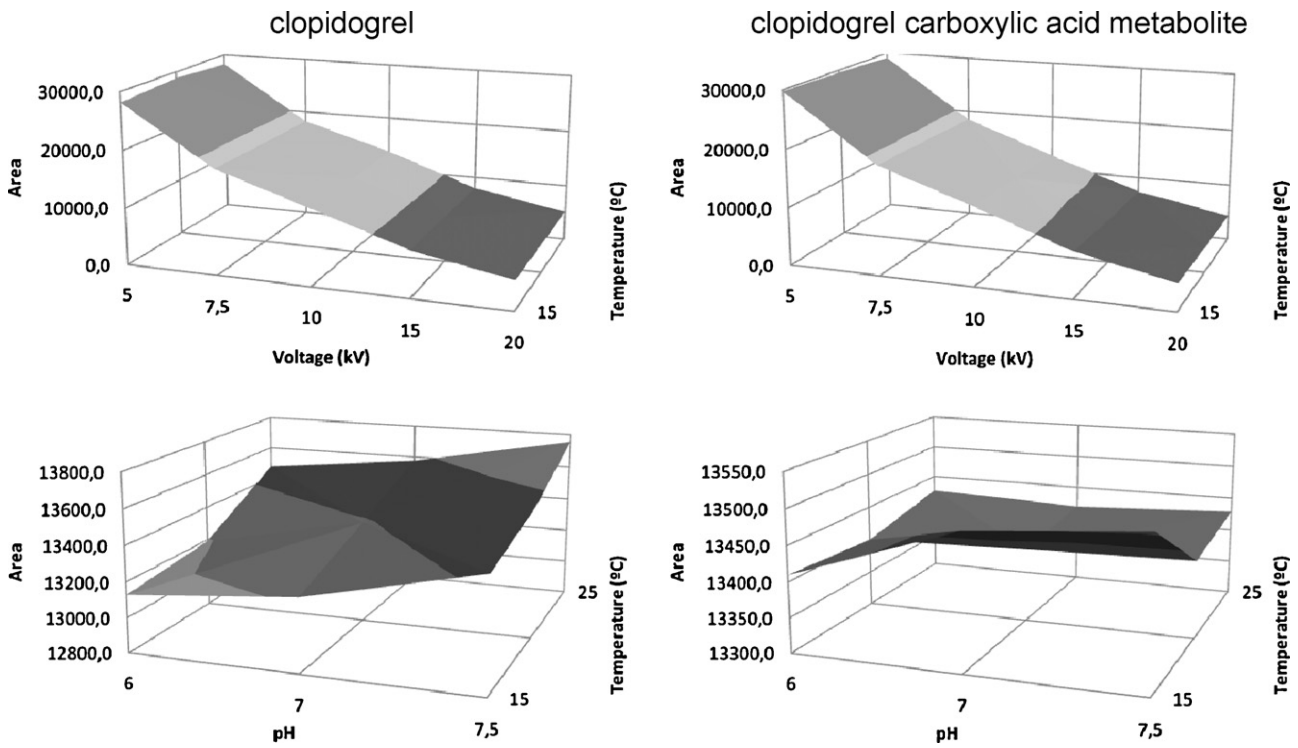

Fig. 4. Surface plots of the response of the peak area of clopidogrel and its carboxylic acid metabolite as a function of the parameters buffer pH, voltage and capillary temperature.

dogrel and its carboxylic acid derivative were linear in this range and $r^2 < 0.95$. All residuals were under 10%.

The limits of detection and quantification were calculated using the signal-to-noise ratio approach. The values obtained were respectively, 1.2 and 3.7 μM for clopidogrel and 1.1 and 3.2 μM for the carboxylic acid metabolite. Since the determined instrumental limit of quantification was higher than the lowest concentrations in the calibration curves, the studies with spiked samples were conducted at higher concentrations.

RSD for instrumental repeatability ($n = 6$) were under 5% and therefore the use of an internal standard was considered not necessary.

Table 3
Results obtained for optimization of the electrophoretic method for separation and quantification of clopidogrel and its carboxylic acid metabolite.

T_{cap} (°C)	Voltage (kV)	Area		Width (min)		t_{mig} (min)		Resolution
		CLP	CLPcarb	CLP	CLPcarb	CLP	CLPcarb	
15	5	29759.7	28158.3	0.520	0.793	7.993	6.379	2.5
15	7.5	18761.7	18309.0	0.397	0.517	5.359	4.279	2.4
15	10	13492.3	13218.3	0.283	0.337	3.834	3.059	2.5
15	15	7861.7	8046.3	0.157	0.273	2.308	1.825	2.3
15	20	4632.7	5077.7	0.103	0.183	1.470	1.168	2.1
20	5	29585.0	28872.0	0.447	0.790	7.271	5.756	2.5
20	7.5	18605.7	18179.0	0.353	0.497	4.901	3.860	2.5
20	10	13405.0	13388.3	0.273	0.340	3.544	2.784	2.5
20	15	7317.3	8065.0	0.153	0.233	2.129	1.672	2.4
20	20	4427.3	5076.7	0.093	0.173	1.332	1.050	2.1
25	5	29127.0	28232.0	0.397	0.690	6.149	4.942	2.2
25	7.5	18341.3	18167.7	0.337	0.487	4.015	3.219	1.9
25	10	13418.2	13575.0	0.273	0.340	2.961	2.364	2.0
25	15	7317.0	7986.0	0.147	0.213	1.796	1.428	1.9
25	20	4341.3	5074.3	0.093	0.157	1.214	0.985	1.8
T_{cap} (°C)	Buffer pH	Area		Width (min)		t_{mig} (min)		Resolution
		CLP	CLPcarb	CLP	CLPcarb	CLP	CLPcarb	
15	7.5	13510.7	13436.5	0.280	0.338	3.929	3.111	2.6
15	7.0	13492.3	13218.3	0.283	0.337	3.834	3.059	2.5
15	6.0	13409.8	13128.7	0.282	0.332	3.750	2.993	2.5
20	7.5	13412.5	13550.5	0.278	0.343	3.650	2.824	2.7
20	7.0	13405.0	13388.3	0.273	0.340	3.544	2.784	2.5
20	6.0	13400.0	13191.7	0.273	0.345	3.461	2.740	2.3
25	7.5	13428.8	13757.7	0.277	0.342	3.057	2.409	1.9
25	7.0	13418.2	13575.0	0.273	0.340	2.961	2.364	2.0
25	6.0	13426.8	13474.7	0.273	0.342	2.904	2.307	2.0

Table 4
Statistical data obtained for the validation of the electrophoretic method.

	Clopidogrel	Clopidogrel carboxylic acid
Linearity		
Range (μM)	2–100	2–100
Linear regression equation	$y = 314x + 808$	$y = 314x + 67$
r^2	0.998	0.999
Residuals	< 10%	< 10%
	RSD %	RSD %
Instrumental repeatability ($n = 6$)		
2 μM	4.9%	3.1%
25 μM	2.6%	3.9%
100 μM	1.4%	1.2%
Method repeatability ($n = 3$)		
5 μM	5.5	6.7
25 μM	5.0	2.6
50 μM	2.0	5.6
Intermediate precision ($n = 3$)		
5 μM	5.2	6.8
25 μM	1.4	2.2
50 μM	0.2	4.2
Accuracy ($n = 3$)	E_r %	E_r %
5 μM	85.3 ± 5.6	83.1 ± 7.4
25 μM	92.9 ± 5.2	95.3 ± 2.5
50 μM	90.8 ± 1.9	86.6 ± 4.9
Limit of detection (μM)	1.2	1.1
Limit of quantification (μM)	3.7	3.2

Precision of the method was measured as method repeatability and intermediate precision, by analysing respectively three replicates of each spiked sample on the same day, and on three different days. For method repeatability RSD values were all under 5.5% for clopidogrel and 6.7% for clopidogrel carboxylate. Inter day coefficients of variation were under 5.2% for clopidogrel and 6.8% for clopidogrelcarboxylate.

Accuracy was evaluated as the extraction recovery of the three spiked serum samples (5, 25 and 50 μM), comparing the areas of the samples subjected to extraction with the areas of unprocessed reference solutions. Each sample was prepared in triplicate. Extraction recoveries (E_r) ranged from 85.3% to 92.9% for clopidogrel and between 83.1% and 95.3% for clopidogrel metabolite. No interferences or matrix effects were detected and since high recoveries were obtained, the use of an internal standard was not adopted.

To determine the robustness of the method, the results obtained from the experimental designs described before were used. Changes in the voltage and in the capillary temperature affected significantly the migration time and peak area ($p < 0.05$), but did not produce significant changes in resolution. Contrarily, different dilutions of the analytes affected the peak resolution, as the resolution factors ranged from 1.8 to 2.6, due to larger peak widths obtained with more concentrated solutions. Such an effect may be the result of a long injection plug (23.4 mm). Although solubilization in water was considered, in order to elicit a stacking effect, this was not possible due to the low solubility of clopidogrel.

3.3. In vitro metabolism study

The enzymatic hydrolysis of clopidogrel and formation of the carboxylic acid metabolite was tested using the validated method (Fig. 5) and the kinetic parameters for this reaction were determined.

The product concentrations were determined and the hydrolysis initial rates were calculated from the linear portion of the product concentration *versus* time curve. The kinetic parameters were obtained from the Lineweaver–Burk plot, which yielded an apparent K_m of 145 ± 30 μM. For the V_{max} 0.4, 1.5 and 3.4 μM/min, were obtained respectively for 1.0, 2.0 and 4.0 U/ml enzyme concentrations.

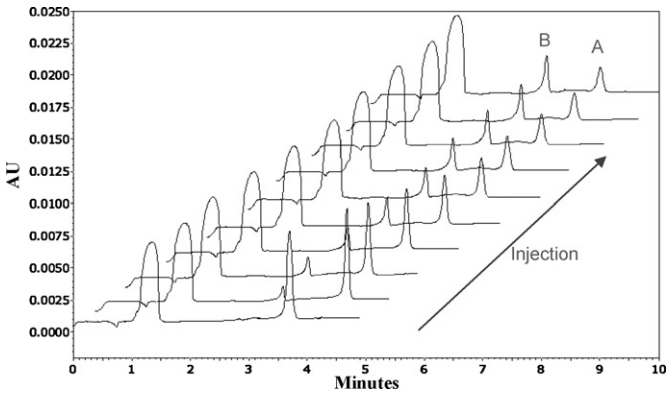

Fig. 5. Electropherograms obtained at different injection times following the hydrolysis reaction of clopidogrel (A) with porcine carboxylesterase and the formation of clopidogrel carboxylate (B). Other Analytical conditions described in materials and methods.

4. Conclusions

A capillary electrophoresis method was developed for the first time for the separation and quantification of clopidogrel and its carboxylic acid metabolite. Ionization profiles were also determined for the first time by CE for both compounds and demonstrated to be useful during method development. Optimization studies were performed by varying surfactant and cyclodextrin concentration, voltage, buffer pH and capillary temperature.

The optimized method was validated with respect to accuracy, precision, linearity, limits of detection and quantification and robustness. The developed method is quite simple, rapid and sensitive and can be applied for separation and determination of both compounds in serum samples in the range 4–100 μM. Although the limits of detection and quantification could be too high for detection of minute quantities of clopidogrel and its metabolite in biological samples, the sensitivity of the method can be improved by extracting the analytes from higher volumes of serum or by making lower dilutions when redissolving after the extraction. The use of staking by dissolution of the extracts in water was not possible due to the low solubility of clopidogrel. However, sensitivity can be improved by injecting the samples at the long end of the capillary (data not shown). In this case, longer migration times and higher peak areas are obtained and consequently, lower limits of detection and quantification can be achieved. However, longer running times would be unsuitable for metabolism studies since fewer injections would be possible in the same time interval. Taking into account the analysis time and capillary rinses, total time between injections was 8 min for the validated method which was sufficient to follow the kinetics of the hydrolysis of clopidogrel within the concentrations of enzyme and substrate used.

Acknowledgment

The authors wish to thank Fundação para a Ciência e Tecnologia for H. Serra doctoral fellowship (SFRH/BDE/15561/2005). We are also thankful to Dr. Rita Noronha for the NMR analysis, Dr. Ana Coelho for allowing us to use the MS facility and Ms. Júlia Oliveira for helpful discussions relative to DoE experiments.

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