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Optimization of a capillary zone electrophoresis method by using a central composite factorial design for the determination of codeine and paracetamol in pharmaceuticals☆

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Abstract

Capillary zone electrophoresis was optimized to quantitatively determine codeine and paracetamol via central composite factorial design. Critical parameters (concentration, buffer, pH, voltage) assessed effects on resolution, analysis time and efficiencies. Optimum separation conditions were achieved using phosphate buffer 20 mM (pH 6.8) and voltage (15 kV). The optimized procedure easily determined codeine and paracetamol with separation in less than 3 min. Calibration curves ($R > 0.999$) were prepared, with LODs of 13.5 and 340 ng mL^{-1} for codeine and paracetamol, respectively, and a good R.S.D.% (<3%). This method was applied to determine codeine and paracetamol in pharmaceutical formulations; recoveries coincided with stated contents.

Keywords: Central composite design; Capillary zone electrophoresis; Codeine; Paracetamol; Pharmaceuticals

1. Introduction

Codeine belongs to a group of medicines called opioids (Fig. 1) that mimic the effects of naturally occurring pain-reducing chemicals (endorphins) that are found in the brain and spinal cord. They act on the opioid receptors in the brain and block the transmission of pain signals [1]. Codeine has mild analgesic and sedative effects and is used for mild to moderate pain relief which is not relieved by non-opiate analgesics. On the other hand, codeine is also used either alone or in combination with other antitussive agents or expectorants in the symptomatic relief of non-productive coughs. Since the cough reflex may be a useful physiological mechanism which clears the respiratory passages of foreign material and excess secretions and may aid in preventing or reversing atelectasis, cough suppressants should not be used indiscriminately [2]. Paracetamol (acetaminophen or *N*-acetyl-*p*-aminophenol) is a synthetic non-opiate derivative of *p*-aminophenol (Fig. 1), and a major metabolite of phenacetin which is associated with analgesic nephropathy. It produces analgesia and antipyresis by a mechanism similar to that of salicylates. Paracetamol is a widely used drug to treat fever and pain. Although the drug is very safe at therapeutic doses, overdoses are known to cause severe liver damage, and adverse effects include rashes, blood dyscrasias, and pancreatitis [3]. The combination of paracetamol and codeine is used to reduce fever and to relieve mild to moderate pain caused by a variety of conditions (e.g. headache, toothache, neuralgia, migraine and period pains).

The simultaneous determination of paracetamol and codeine in pharmaceuticals using various techniques, such as HPLC [4–7], TLC [8] and diode-array spectroscopy, has been reported [9]. Capillary electrophoresis (CE) has been established as a powerful method for drug analysis and it has been used to determine codeine and paracetamol separately in pharmaceutical preparations [10–14] with UV detection, and biological fluids [15–21] using UV [15,17,18,20,21], electrochemical [19] and MS [16] detection, as well as in the detection of opium [22–25]. Capillary zone electrophoresis (CZE) could be inter-

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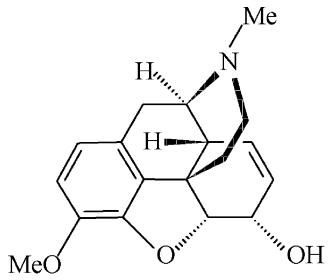
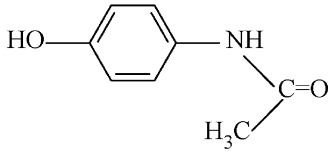


Codeine (pKa = 8.21)



Paracetamol (pKa = 9.71)

Fig. 1. Structure and pK_a values of codeine and paracetamol.

esting as a faster, economic, sensitive and selective analytical method [26]. Nevertheless, an optimization procedure for the simultaneous analysis of codeine and paracetamol by CZE is still lacking.

In this study, our objective was to develop, optimize and validate a specific, accurate, precise and reproducible quantitative method to determine not only codeine and paracetamol as a pharmaceutical substance but also their binary combination in pharmaceutical formulations. The optimization of the method was performed using an interpretative strategy, and more specifically, a central composite circumscribed (CCC) design that supplied data to obtain a fitted polynomial model for drawing a response surface in all the variable space. This proposed method is highly sensitive and specific, and can also be used for the routine analysis of pharmaceutical formulations consisting of codeine and paracetamol with short sample preparation and analysis times.

2. Materials and methods

2.1. Instrumentation and equipment

Capillary electrophoresis runs were performed with a Beckman P/ACE System MDQ (Beckman Instruments, Inc., Fullerton, CA, USA) equipped with a DAD detector. The polyimide coating of the capillary was partially removed by burning at the point of detection, and the uncovered portion of the capillary was aligned on the detector block. A personal computer connected to the instrument through Beckman 32 Karat software was used for instrumental control and acquisition of chromatographic data.

Electrophoretic runs were carried out at 25 ± 0.2 °C. Samples were injected by hydrodynamic pressure. The fused silica capillaries (50 μm I.D., 375 μm O.D.) were obtained from Polymicro Technologies (Phoenix, AZ, USA) and the length of the capillaries used was 40.2 cm. The capillary was conditioned initially by flushing NaOH 0.1 M for 15 min followed by water for 15 min, and finally running buffer through it for 15 min, all at 20 psi. The capillary was rinsed for 2 min with water and 3 min with electrolyte solution between each injection to ensure a consistent electro-osmotic flow (EOF). pH measurements were performed with a Crison GLP 22 (Barcelona, Spain), equipped with a combined Ag/AgCl/glass electrode. The vortex shaker and sonification unit were obtained from Selecta (Abrera, Barcelona).

2.2. Reagents

Codeine and paracetamol were obtained from Sigma (St. Louis, MO, USA). Stock solutions containing 100 μg mL^{-1} of each compound were prepared in distilled water. The solutions were suitably diluted for analysis purposes. Buffer solutions were prepared with phosphoric acid, sodium dihydrogenphosphate, disodium hydrogenphosphate, and trisodium phosphate (Sigma–Aldrich). Distilled-deionized water (Barnstead, Sybron, Boston, MA, USA) was used to perform all the optimization and analysis experiments. Sodium hydroxide 0.1 M (99% purity, Merck, Darmstadt, Germany) was used for capillary conditioning.

2.3. Sample preparation

The pharmaceuticals were presented as powders (Algidol, Propalgina), capsules (Termalgin codeína), oral solutions (Bisolvon, Fludan codeína, Toseína, Apiretal), syrups (Lasa con codeína) and tablets (Cod-Efferalgan, Dolgesic codeína, Fludeten). For the analyses, 10 tablets were weighed, ground to a fine powder and homogenized. Several portions were taken and weighed, dissolved and diluted with water to an adequate concentration. The powders were treated similarly. An aliquot of solutions and syrups was diluted with water. All sample solutions were filtered into autosampler vials through 0.45 μm nylon membranes 12 mm in diameter.

2.4. Computer modeling

The SPSS program (SPSS Inc. Chicago, IL, USA) was used for the non-linear regression analysis of the data and to obtain the empirical mathematical model to represent the response surface. The surface plot was produced by Surfer software, a contouring and 3D surface mapping software program (RockWare Europe, Cureglia, Swiss).

3. Results and discussion

3.1. Screening parameters

Preliminary results revealed that the factors which affected the migration time and peak width responses the most were the pH of the running buffer and the voltage (*V*) applied. The ionic strength of the buffer was studied in the 5–40 mM range, where it was observed that a high ionic strength (from 30 mM) causes a warm-up of capillary that means in wide peaks and in a low electrophoretic mobility, thus leading to low migration times and low resolution. On the other hand, a low ionic strength causes an adsorption of codeine and paracetamol leading to a low recov-

ery and short peaks means. Thus, the best ionic strength was obtained at a concentration of 20 mM. The capillary length was also studied; when the capillary length increased, the analysis time also increased, thus a length was selected which was capable of determining the two substances with analysis times lower than 4 min. The presence of an organic modifier (methanol, acetonitril or propanol) only reduces the electro-osmotic flow, and therefore increases the migration time of paracetamol and codeine. The pH of the buffer electrolyte has a marked influence on the resolution since it is known that the ionization of the acidic silanol groups on the capillary surface is slight at a lower pH. Therefore, the EOF rate is not significant, while the EOF increases at high pH values.

3.2. Experimental design

A primary interest in the development of a new method for the separation and quantitation of analytes of a pharmaceutical interest is the amount of time and the number of trials required to implement the method, which are obviously cost related. To minimize the experiments and to shorten the method development time, a modeling strategy might well be effective. Thus, an interpretative strategy was chosen to investigate the separation of codeine and paracetamol.

Two parameters (pH and voltage) affecting the peak resolution were considered and a minimum of three levels are necessary to apply the response surface method. The experimental strategy chosen for the optimization procedure was a central composite circumscribed design. This strategy consists of a combination of a factorial design and an additional design (star design) in which the center of both designs coincide. The star points are at α distance from the center and establish new extremes for the low and high settings for both factors. This design has circular symmetry and requires five levels for each factor. To maintain rotatability, the α value depends on the number of experimental runs in the factorial portion of the central composite design.

3.3. Response surface method

The CCC design requires nine runs. The parameter settings in the design are provided in Table 1, while the design is reproduced in Table 2. The design runs were carried out in a randomized sequence. The migration time and widths of peaks were measured. Replications of factor combinations were necessary to estimate the experimental error. Thus, the center point was run five times.

Table 1
Parameters for CE method for determination of codeine and paracetamol

Capillary	40.2 cm (30 cm effective length) × 75 μm (I.D.)
Detection	UV at 214 nm
Temperature	25 °C
Electrolyte	20 mM phosphate buffer at pH 6.7
Preconditioning	10 min with 100 mM NaOH followed by water for 10 min and finally with running buffer for 30 min. All at 20 psi
Rinse time	After each run the capillary was rinsed with water for 2 min and running buffer for 3 min at 20 psi
Injection	Hydrodynamic injection at 1 psi for 10 s
Applied voltage	15 kV
Runtime	5 min

Table 2
Factor settings in the design

CE factor	$-\alpha$	−1	0	+1	$+\alpha$
pH	3.5	4.5	6.8	9.2	10.2
voltage (kV)	7.9	10	15	20	22.1

The peak resolution was calculated using Eq. (1):

$$\mathrm{Rs} = \frac{2(t_2 - t_1)}{(w_2 + w_1)} \tag{1}$$

where w_1 and w_2 are the width at the peak base of two consecutive peaks, measured as time units. The numerator in Eq. (1) describes the separation process in terms of differential migration and the denominator describes the dispersive processes acting against it. The minimum resolution value was considered as 1 (one peak starts immediately after the second peak has finished), and the peaks would be overlapped between 0 and 1. A good resolution in the present work has been taken as being 1.5 to ensure full resolution with some distance between the two peaks.

A response surface method was used to quantify and interpret the relationships between responses and factor effects. The general empirical model is a second order polynomial, where the response y is related to the variables (factors) x as follows:

$$y = b_0 + \sum_{i=1}^{k} b_i x_i + \sum_{1 \le i \le j}^{k} b_{ij} x_i x_j + \sum_{i=1}^{k} b_{ii} x_i^2 \tag{2}$$

where k is the number of variables (factors), b_0 the intercept parameter, and b_i, b_{ij}, b_{ii} are the regression parameters for linear, interaction and quadratic factor effects, respectively. The SPSS program carried out the non-linear regression analysis of the data, and the model obtained was:

$$\mathrm{Rs} = 19.55 - 5.75\,\mathrm{pH} + 0.58\,\mathrm{V} + 0.42\,\mathrm{pH}^2 - 0.01\,\mathrm{V}^2 - 0.04\,\mathrm{pHV} \tag{3}$$

An R^2 factor of 0.7862 was obtained to fit this equation, where R^2 is the coefficient of multiple determination and its square root (R) is the coefficient of multiple correlation, which vary between −1 and +1, and therefore indicate how closely the factor explains the data. This factor demonstrates that the fitting to the quadratic model is not correct, and therefore a cubic model is required to evaluate the relation among factors.

$$\mathrm{Rs} = -30.91 + 0.01\,\mathrm{pH} + 8.16\,\mathrm{V} + 0.62\,\mathrm{pH}^2 - 0.29\,\mathrm{V}^2 - 1.03\,\mathrm{pHV} - 0.02\,\mathrm{pH}^2\mathrm{V} + 0.04\,\mathrm{pHV}^2 \tag{4}$$

Using Eq. (4), R^2 is 0.9655. Fig. 2A shows a wide zone of good resolution within lower pH values and in all the whole voltage range. For example, if we take into account that results overlap in a resolution with a value below 1, it is observed that the resolution is 1.43 at pH 9.2, V 20 kV (Table 3). Therefore, it

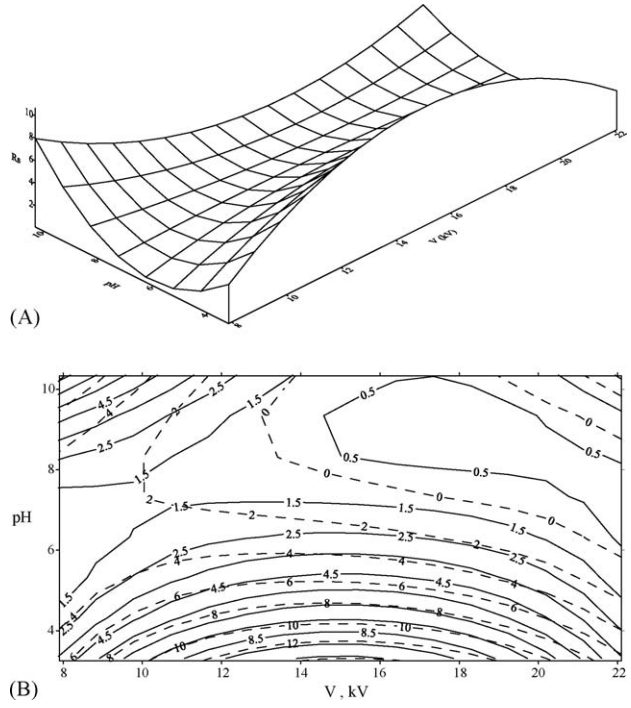


Fig. 2. (A) Response surface of the resolution of codeine and paracetamol; (B) contour map of resolution (–) and migration time (- -) of paracetamol vs. pH and applied voltage.

is possible to obtain several conditions with a good resolution. On the other hand, the second objective of the separation is to achieve a minimum analysis time. Thus, it is also important to study the relation between the migration time and the effects of pH and V. The mathematical model which takes the last compound into account, allows for this relation, and was:

$$\mathrm{Tm} = -45.60 + 10.82\,\mathrm{pH} + 4.78\,\mathrm{V} - 0.35\,\mathrm{pH}^2 + 0.38\,\mathrm{V}^2 - 1.69\,\mathrm{pHV} + 0.05\,\mathrm{pH}^2\mathrm{V} - 0.01\,\mathrm{pHV}^2 \quad (5)$$

In Fig. 2B, the contour map of resolution and analysis time are shown as being overlapped. The isolines mark the points with the same resolution values (solid line) and the equal migration time (broken line). The line numbered as 0 migration time indicates a value lower than 1 min for the substance migration, although zero also appears as the interpolation result of the mathematical model (Eq. (5)). Under such conditions, a peak is observed in less than 1 min. The optimum conditions were pH 6.8 and voltage 15 kV, which allow a good resolution in a short analysis time (below 3 min).

Table 3
Central composite circumscribed design and responses (resolution and migration time) obtained

Run	pH	*V*	Rs	tm
1	−	−	2.00	4.4
2	+	−	2.96	3.0
3	−	+	2.17	3.5
4	+	+	1.43	1.6
5	0	−α	0.93	3.8
6	−α	0	10.63	15.7
7	0	+α	1.28	1.5
8	+α	0	2.52	2.5
9	0	0	1.54	2.1

3.4. Selected conditions

After the modeling procedure, the optimum conditions were selected according to the good resolution and short analysis time criterion. Thus, if Fig. 2B is taken into account in the search for a resolution above 1.5 units and the shortest analysis time, as mentioned above, the best conditions used to separate and determine codeine and paracetamol were: a total capillary length of 40.2 cm with a 50 μm I.D. and a length to the detector of 30.2 cm, a temperature of 25 °C, injection at 1 psi for 10 s, a wavelength at 214 nm, running buffer 20 mM phosphate at pH 6.8, and an applied voltage of 15 kV. Under these conditions, the migration time of both compounds is 1.9 and 2.5 min for codeine and paracetamol, respectively.

3.5. Method validation

3.5.1. Linearity

Calibration curves were constructed using the standard stock solution (100 μg mL^{-1}), which was taken and diluted to a suitable ratio with running buffer to obtain six increasing concentrations (in triplicate for each concentration) in the 0.1 to 20 μg mL^{-1} range. Peak areas were measured and linear regression calculation gave the slopes, intercepts and regression coefficients shown in Table 4. Calibration parameters were adequate for codeine and paracetamol determination.

3.5.2. Limits of detection (LOD) and quantification (LOQ)

The limit of detection of a method is the lowest analyte concentration that produces a response detectable above the noise level of the system, which is typically taken as being three times the noise level. The LODs were 13.5 ng mL^{-1} for codeine and 340 ng mL^{-1} for paracetamol, determined by successive dilutions until a detectable one is obtained. The LODs were well below those required for codeine and paracetamol analysis in the pharmaceutical preparations (Table 4). The limit of quantification (LOQ) corresponds to a signal equal to 10 times the standard deviation of the background noise. The LOQs were 35 ng mL^{-1} for codeine and 1050 ng mL^{-1} for paracetamol (Table 4).

3.5.3. Precision

Intra-day precision (average of the peak area of 10 determinations performed on the same day), and the intermediate precision

Table 4
Calibration parameters and limits of detection (3 s criterion) in ng mL^{-1} for codeine and paracetamol

Compound	Slope	Intercept	*r*	LOD
Codeine	1152 ± 45	45 ± 14	0.9998	10
Paracetamol	1319 ± 52	66 ± 10	0.9995	340

Table 5
Repeatability and intermediate precision (R.S.D.%, $n = 10$) at three different concentrations ($\mu g\ mL^{-1}$): $c_1 = 1$, $c_2 = 5$ and $c_3 = 10$

Compound	Repeatability			Intermediate precision		
	c_1	c_2	c_3	c_1	c_2	c_3
Codeine	2.41	1.28	1.76	1.77	1.00	0.92
Paracetamol	2.24	3.57	2.35	1.45	2.26	1.80

(average of intra-day values taken for 10 days over a 2-month period) were determined at three different drug concentrations (1, 5 and 10 $\mu g\ mL^{-1}$). The relative standard deviations (R.S.D.) were always below 3.6% (Table 5).

3.6. Analysis of pharmaceuticals

Once the conditions for separation and quantitation were established, the CE method was applied on different pharmaceutical formulations (capsules, tablets, powders, oral solutions and syrups) for codeine and paracetamol determination, both together in the same pharmaceutical (in Algidol, Cod-Efferalgan efervescente, Dolgesic codeína, Fludeten comprimidos efervescentes, and Termalgin codeína) and individually (for codeine in Bisolvon, Fludan codeína, Lasa con codeína, and Toseína; and for paracetamol in Apiretal gotas and Propalgina). The pharmaceutical extract, obtained as indicated in Section 2.3, was injected into the CE system by triplicate. The results are shown in Table 6. In those pharmaceuticals with both substances, paracetamol concentration is much higher in relation to codeine, thus two dilutions were analyzed in order to quantify the two analytes.

Fig. 3 shows the electropherograms obtained in determining codeine and paracetamol in the pharmaceuticals Dolgesic codeína, Fludeten, Termalgin codeína, Fludan codeína, Lasa con codeína, and Apiretal, using the optimum conditions described herein.

The other compounds contained in the pharmaceutical preparations, such as ascorbic acid, sucrose, aspartame, saccharine, diphenhydramine hydrochloride, bromhexine, sorbitol, glycerine, sodium benzoate, pseudoephedrine hydrochloride, chlorpheniramine maleate, benzoic acid, azorubine, polyethylenglycol, glycerol, phenylephrine hydrochloride, dextromethorphan hydrobromide, and sodium cyclamate, did not interfere with the codeine and paracetamol determination. Fig. 3 shows the other compounds present in the formulations, such as saccharine, sodium benzoate, chlorpheniramine, and pseudoephedrine, which do not overlap with codeine and paracetamol. The results of the analysis indicate that the optimized electrophoretic method is suitable for the assay of drugs in pharmaceuticals given the satisfactory recoveries obtained.

As the pK_a of codeine and paracetamol are 8.21 and 9.71, respectively at pH 6.8 (the pH selected for the buffer), these two substances are not protonated. For this reason, both substances can co-migrate with the electro-osmotic flow. There is a risk that small water-soluble neutral impurities will co-migrate with them. If this happens, an overestimation of codeine and paracetamol concentrations is expected. Results in Table 6 show that this did not happen with the proposed method. Thus, the method is useful to determine both substances in pharmaceuticals preparations.

Table 6
Determination of codeine and paracetamol in pharmaceutical preparation

Commercial name and manufacturer	Composition	Recovery ± C.V. %
Algidol (Almirall Prodesfarma)	Per packet: codeine phosphate 10 mg, paracetamol 650 mg, ascorbic acid 500 mg, sucrose, orange coloring (E-110), excipient c.s.	100.1 ± 2.1, 98.4 ± 1.6
Cod-Efferalgan efervescente (Upsamedica)	Per tablet: codeine phosphate 30 mg, paracetamol 500 mg, aspartame 30 mg and other excipient c.s.	104.4 ± 2.2, 99.5 ± 0.9
Dolgesic codeine (Ferrer grupo)	Per tablet: codeine phosphate 0.015 g, paracetamol 0.5 g, excipients c.s.	99.4 ± 1.4, 97.7 ± 1.5
Fludeten comprimidos efervescentes (Alter)	Per tablet: codeine phosphate 30 mg, paracetamol 500 mg, sodium saccharine 10 mg, and effervescent excipient c.s.	98.7 ± 1.1, 98.5 ± 2.5
Termalgin codeine (Novartis)	Per capsule: codeine phosphate 14.05 mg, paracetamol 300 mg, excipients c.s.	103.1 ± 2.6, 97.6 ± 1.4
Bisolvon (Fher)	Per 5 mL solution: ephedrine hydrochloride 7.5 mg, diphenhydramine hydrochloride 7.5 mg, bromhexine hydrochloride 2.5 mg, codeine hydrochloride 10 mg, ethanol and excipients c.s.	100.5 ± 0.5
Fludan codeine (Ipsen Pharma)	Per mL solution: codeine phosphate 2 mg, sorbitol, glycerine (E-422), sodium benzoate (E-211), carmoisine coloring (E-122), excipients c.s.	101.8 ± 2.2
Lasa con codeine (Lasa)	Per 5 mL syrup: pseudoephedrine hydrochloride 30 mg, chlorpheniramine maleate 2 mg, codeine phosphate 10 mg and excipients	96.8 ± 1.8
Toseína (Italfarmaco)	Per mL solution: codeine phosphate 10 mg, sorbitol, sodium benzoate (E-211), aspartame (E-921), azorubine (E-122), excipients c.s.	99.8 ± 0.8
Apiretal goats (ERN)	Per mL: paracetamol 100 mg, polyethylenglycol, glycerol, benzoic acid (E-210), azorubine (E-122), other excipients	99.8 ± 1.2
Propalgina (Roche)	Per packet of 5 g: paracetamol 500 mg, chlorpheniramine maleate 2 mg, phenylephrine hydrochloride 7.5 mg, dextromethorphan hydrobromide 10 mg, ascorbic acid (vitamin C) 200 mg, sodium cyclamate 180 mg, sodium saccharine 20 mg, sucrose 3.67 g, excipients c.s.	98.5 ± 2.4

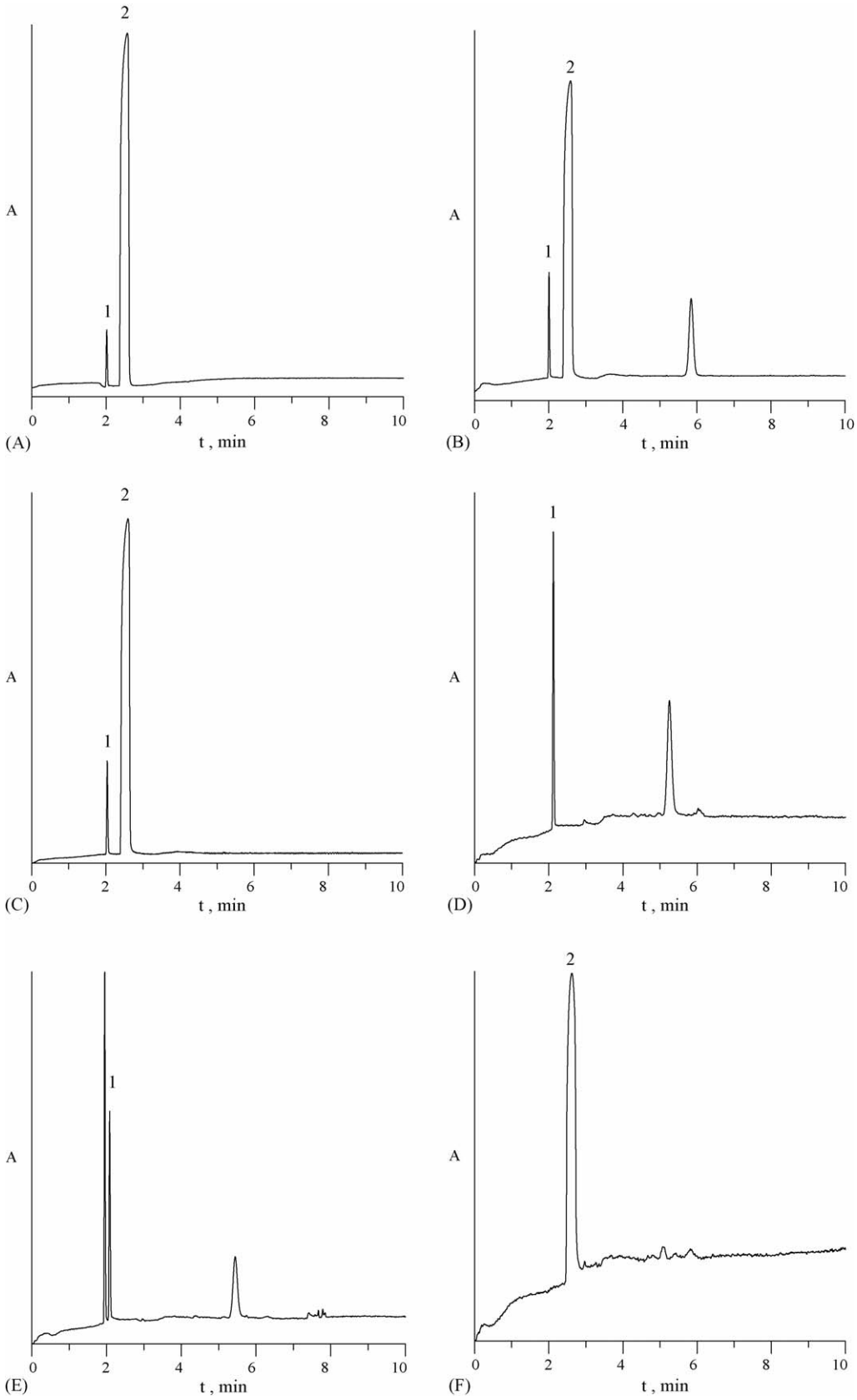


Fig. 3. Electropherogram of pharmaceutical preparation: (A) Dolgesic codeína; (B) Fludeten; (C) Termalgin codeína; (D) Fludan codeína; (E) Lasa con codeína; (F) Apiretal; (1) codeine and (2) paracetamol.

4. Conclusion

In this work a capillary zone electrophoresis method has been developed using an experimental design known as central composite design. This strategy allowed us to obtain a large response surface using only nine runs and to know the electrophoretic peaks behavior of codeine and paracetamol in all the variable space. With the response surface, the optimum electrophoretic conditions allowing us to obtain a good resolution between peaks with the minimum analysis time were easy to determine. The optimum conditions were buffer running phosphate 20 mM at pH 6.8 and voltage 15 kV. Under these conditions, the procedure was straightforward, sensitive, simple and fast for codeine and paracetamol determination in pharmaceutical samples. Thus, the method might well be suitable for quality control analyses of codeine and paracetamol in pharmaceuticals.

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