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# Simultaneous multiresponse optimization applied to epinastine determination in human serum by using capillary electrophoresis

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**Abstract**

Experimental design and optimization techniques were implemented for the development of a rapid and simple capillary zone electrophoresis method (CZE) for the determination of epinastine hydrochloride in human serum. The effects of five factors were studied on the resolution between the peaks for the target analyte (epinastine hydrochloride) and lidocaine hydrochloride, used as internal standard, as well as on the analysis time. The factors were the concentration and pH of the buffer, the injection time, the injection voltage and the separation voltage. The separation was carried out by using an uncoated silica capillary with 50 μm i.d. and total length 64.5 cm (150 μm of path length) and UV detection (200 nm).

Multiple response simultaneous optimization by using the desirability function was used to find experimental conditions where the system generates desirable results. The optimum conditions were: sodium phosphate buffer solution, 16.0 mmol $L^{-1}$; pH 8.50; injection voltage, 20.0 kV; injection time, 30 s; separation voltage, 26.7 kV.

The method was confirmed to be linear in the range of 2.0–12 ng $mL^{-1}$. The injection repeatability of the method was evaluated by six injections at three concentration levels, while intra-assay precision was assessed by analysing a single concentration level, yielding a CV's of ca. 1% for standard and 2% for serum samples. Accuracy was evaluated by recovery assays and by comparing with an HPLC method, the results being acceptable according to regulatory agencies. The rudgeness was evaluated by means of an experimental Plackett–Burman design, in which the accuracy was assessed when small changes were set in the studied parameters. Clean-up of human serum samples was carried out by means of a liquid–liquid extraction procedure, which gave a high extraction yield for epinastine hydrochloride (93.00%).


*Keywords:* Multiresponse optimization; Capillary electrophoresis; Epinastine

## 1. Introduction

Whenever a new capillary electrophoretic method is being developed, optimization is usually applied to reduce the analysis time and efforts, without losing the resolution between the peaks originated by the analyte migration. Moreover, the need of simultaneously taking into account different aspects of the analysis calls for the use of multi-criteria optimization. In order to carry out this type of study, experimental design is a valuable tool, specifically response surface analysis [1]. In addition, when different objective functions have to be optimized, the so-called Derringer's desirability function is a valuable tool to be considered [2]. The latter function requires to define which results are acceptable for each individual response, and which results are not acceptable at all.

Epinastine hydrochloride (EPN) (9,13b-dihydro-1*H*-dibenz[*c*,*f*]imidazo[1,5-*a*]azepin-3-amine hydrochloride, CAS 80012-43-7) is a novel anti-allergic, non-sedative drug, that acts as histamine $H_1$ receptor antagonist [3]. The use of EPN is gaining importance owing to the fact that it does not penetrate the blood/brain barrier (based on its physicochemical properties, such as hydrophilicity and cationic charge at the physiological pH range). Therefore, it is not expected to induce side effects of the central nervous system [4]. On the other hand, EPN ophthalmic solutions are applied to prevent itching of the eyes caused by allergic conjunctivitis (a condition in

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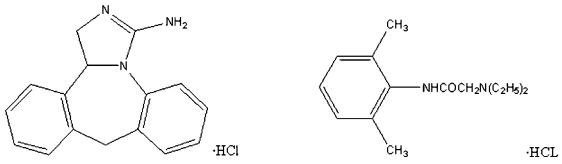


Epinastine hydrochloride Lidocaine hydrochloride

Fig. 1. Chemical structures of epinastine hydrochloride and lidocaine hydrochloride.

which the eyes become itchy, swollen, red, and teary when they are exposed to certain substances in the air) [5].

A recent pharmacokinetic study in pediatric patients, in which dosage was determined based on the body weight, showed that the average plasmatic concentration ($C$) is $25.6 \pm 6.9$ ng mL$^{-1}$, similar to those for adults after administration of 20 mg ($C = 26.9 \pm 9.1$ ng mL$^{-1}$) [4]. The pharmacokinetic properties of EPN make it a potential replacement for conventional non-sedating antihistamines, and provide it with great clinical relevance. In conclusion, precise pharmacokinetic properties should be investigated under several clinical states.

Remarkably, a limited number of publications deal with methods for the determination of EPN in human serum. They are exclusively based in high performance liquid chromatography (HPLC) with UV detection, most of them presenting poor sensitivity [6–8]. HPLC is an established technique with concentration sensitivity in the nanomolar range. However, capillary electrophoresis (CE), due to its high efficiency, offers a real and attractive alternative to HPLC, and appears as an appropriate technique for the analysis of biological samples, as demonstrated in several published papers in this area, in which CE has been shown to be a valuable alternative technique for their separation [9–12].

In this work, a CE method was developed, optimized and validated for the determination of EPN in human serum, reaching a sufficiently high sensitivity to follow the drug kinetics. The multiple response criteria were successfully used to optimize the separation of two analytes: EPN and lidocaine hydrochloride (LID), used as internal standard (Fig. 1). To the best of our knowledge, there seems to be no reports concerning methods for the determination of EPN in human serum by CE.

## 2. Experimental

### 2.1. Apparatus

All experiments were carried out on a capillary electrophoresis system (Agilent Technologies), equipped with a diode array detector. The instrument was operated under positive polarity (injection end of capillary). A PC Athlon 2.2 microcomputer was used for data handling. Electrophoretic separation was carried out with uncoated fused-silica capillary provided by Agilent Technologies with an inner diameter of 50 μm (150 μm of path length) and a total length of 64.5 cm (56 cm to detector). The pH of the buffers were adjusted by means of an Orion 9165 BN model 710a with Ag/ClAg, KCl electrode.

### 2.2. Software

A CE chemstation (Hewlett–Packard) was used for instrument control and data acquisition. Experimental design, data analysis and desirability function calculations were performed by using the software Stat-Ease Design-Expert trial Version 7.0.3.

### 2.3. Reagents

All the reagents were of analytical-reagent grade. They were preserved at 4 °C in the darkness during the experiments. Milli-Q quality water was used in all the CE experiments. Sodium phosphate, sodium hydroxide, sodium carbonate and lidocaine hydrochloride were obtained from Merck. All the buffers were filtered through a 0.45 μm nylon membrane (Sartorius-Germany) and degasified before use. The EPN standard was obtained from the commercial tablet Flurinol (Boehringer Ingelheim) by extraction and subsequent purification.

### 2.4. Electrophoretic conditions

The capillary, when new, was washed for 10 min with filtered 1 mol L$^{-1}$ sodium hydroxide solution, for 10 min with 0.1 mol L$^{-1}$ sodium hydroxide solution, for 10 min with Milli-Q water and for 10 min with electrolyte buffer solution.

At the beginning of the working day, the capillary was washed with sodium hydroxide 0.1 mol L$^{-1}$ solution, Milli-Q water and finally with running buffer solution during 10 min.

Between runs, the capillary was washed successively with 0.1 mol L$^{-1}$ sodium hydroxide solution, followed by Milli-Q water and then with running buffer solution for 2 min. At the end of the day, a last washing with 0.1 mol L$^{-1}$ of sodium hydroxide solution and with Milli-Q water was performed.

All the solutions were degassed in an ultrasonic bath and filtered though 0.45 μm membrane filter before use. The electrolyte buffer solution was prepared at the beginning of the day. Samples were introduced into the capillary via electrokinetic injection by applying 20.0 kV during 30 s. A constant voltage of 26.7 kV was used for all experiments. The wavelength used for recording the electropherograms was 200 nm. The capillary was thermostated at 25.0 °C.

### 2.5. Method validation

EPN and LID were dissolved in water reaching final concentrations of 0.20 and 0.57 mg L$^{-1}$, respectively and stored as stock solutions, in the darkness at 4 °C. The standard solutions were prepared every day by dilution in Milli-Q water.

#### 2.5.1. Calibration curves

The calibration curves were built by dilution of known amounts of analyte standard solutions in Milli-Q water. The concentration levels were: 2.0, 4.0, 6.0, 8.0, 10.0 and 12.0 ng mL$^{-1}$.The ratios between the peak areas for EPN and LID were plotted against the corresponding concentrations

(expressed in ng $mL^{-1}$), and the line was fitted by least-squares. The values of quantitation limit (LOQ) and detection limit (LOD) were calculated according to IUPAC recommendations [13].

#### 2.5.2. Precision assay

Three standards at three different concentration levels (level 1: 4.0 ng $mL^{-1}$, level 2: 6.0 ng $mL^{-1}$, and level 3: 8.0 ng $mL^{-1}$) were analysed six times each within the same day in order to obtain the repeatability (intra-day precision). In addition, standard level 2 was analysed during three different weeks to calculate the intermediate precision (inter-day precision) of the method. ANOVA test were performed to analyse the data.

#### 2.5.3. Accuracy

Accuracy was evaluated by means of recovery assays and comparing with a HPLC based method [6]. Known amounts of analyte standard solution were added to 200 μL of serum from healthy patients to reach the following concentrations: 20.0, 25.0 and 30.0 ng $mL^{-1}$. The mixtures were then analysed performing six replicates, and the averages obtained were used to compute the recovery of the method. The central level was also analysed by HPLC and an average comparison Student's *t*-test was performed.

### 2.6. Human serum sampling

The serum sample (200 μL) was mixed with 600 μL of 0.1 mol $L^{-1}$ $Na_2CO_3$ solution in a 10 mL round bottom polypropylene tube. The sample was briefly shaken by vortex, followed by addition of 5 mL of dichloromethane. The tube was capped, then shaken and afterward centrifuged at 4000 rpm during 5 min. The lower organic layer was transferred to a clean 2 mL tube and evaporated to dryness at 45 °C in a stream bath. Finally, 30 μL of internal standard 0.24 mg $L^{-1}$ stock solution were added, completing the reconstitution of the residue in 1.0 mL of running buffer [4].

## 3. Results and discussion

### 3.1. Injection mode

A common problem in CE is represented by the low sensitivity assessed when compared with HPLC. This problem stems from two sources: the low sample injection volume and the short optical path length for on-capillary detection [14]. Considering its applicability, an interesting way to concentrate samples is the on-line (or on-capillary) chemical approach. It consists in performing a sample stacking by using electrokinetic injection. This procedure provided us with larger sensitivity enhancements compared with hydrodynamic injection, and thus it was the injection mode selected for the present work.

### 3.2. Screening phase

The separation by CE depends on many factors, but the simultaneous study of all the potential factors is too complex, and would imply a prohibitely long experimental time. Consequently, an experimental Plackett–Burman design was built for the determination of the main factors affecting the peak resolution between EPN and LID, as well as the analysis time. The resolution can be defined according to Eq. (1):

$$R = 2 \times \left( \frac{tm_2 - tm_1}{w_1 + w_2} \right) \quad (1)$$

where $tm_1$ and $tm_2$ are the migration times, and $w_1$ and $w_2$ are the electrophoretic peak widths. When the resolution is higher than 1.5, the two species are considered to be resolved at the baseline [15].

The analysed factors were: concentration and pH of the buffer, injection voltage, injection time and separation voltage. These factors were evaluated at two levels each (see Table 1). The evaluation consisted in analyzing a stock standard solution in all the cited conditions. In each case, the peak resolution between EPN and LID as well the as analysis time were evaluated.

Table 1
Plackett–Burman design built for factor selection

| Experiment number | Buffer concentration (mmol $L^{-1}$) | pH | Vi[a] (kV) | Ti[b] (s) | Vs[c] (kV) | Resolution | Analysis time (min) |
|---|---|---|---|---|---|---|---|
| 1 | 40 | 6.95 | 15 | 25 | 20 | 1.06 | 4.64 |
| 2 | 40 | 6.95 | 25 | 35 | 20 | 0.00 | 4.41 |
| 3 | 40 | 6.95 | 25 | 25 | 30 | 0.90 | 3.14 |
| 4 | 20 | 6.95 | 25 | 35 | 20 | 0.00 | 3.89 |
| 5 | 20 | 6.95 | 15 | 35 | 30 | 0.87 | 2.80 |
| 6 | 20 | 6.95 | 15 | 25 | 20 | 1.66 | 4.84 |
| 7 | 40 | 9.16 | 25 | 25 | 30 | 6.94 | 3.48 |
| 8 | 40 | 9.16 | 15 | 35 | 20 | 8.48 | 6.32 |
| 9 | 40 | 9.16 | 15 | 35 | 30 | 9.09 | 3.58 |
| 10 | 20 | 9.16 | 15 | 25 | 30 | 11.20 | 3.31 |
| 11 | 20 | 9.16 | 25 | 25 | 20 | 5.14 | 4.31 |
| 12 | 20 | 9.16 | 25 | 35 | 20 | 2.73 | 4.00 |

[a] Vi, injection voltage.
[b] Ti, injection time.
[c] Vs, separation voltage.

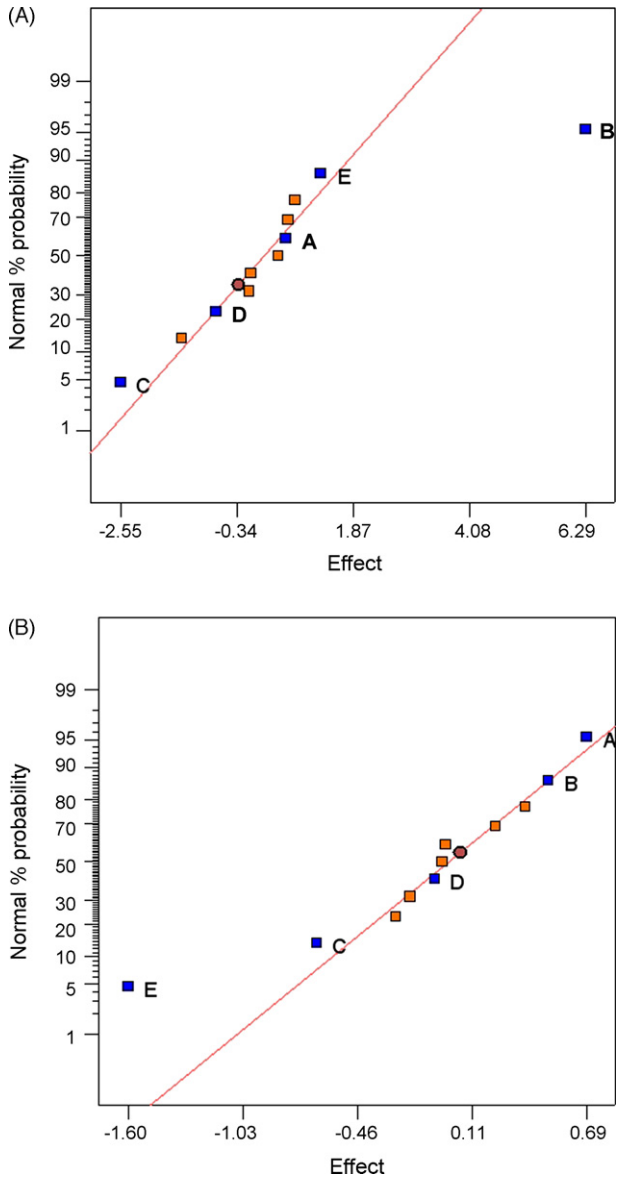


Fig. 2. Normal plots when analysing effects for both responses: (A) resolution and (B) analysis time.

An ANOVA test was applied to the experimental data, using the effects of dummy variables to obtain an estimate of standard errors in the coefficients. As a conclusion of this analysis, concentration and pH of the buffer, separation voltage and injection voltage were shown to be significant ($p < 0.05$) and should be considered in the further optimization analysis. On the other hand, in order to get a deeper insight, normal probability plots for both analysed responses were built, which allowed us to reach a similar conclusion. These plots can be appreciated in Fig. 2(A and B).

### 3.3. Response surface design

Systematic optimization procedures are carried out by selecting an objective function, finding the most important factors and investigating the relationship between responses and factors by the so-called response surface methods (RSM). Once the conditions that ensure the analyte separation were established, an optimization procedure was applied in order to find out the exact values of the most important factors for a correct separation and a rapid analysis.

A central composite design was used, consisting of 30 experiments: combinations of the (selected) independent variables in the following ranges: buffer concentration 10–50 mmol $L^{-1}$, pH 6.70–9.70, injection voltage 15–25 kV, separation voltage 17.5–27.5 kV (see Table 3). These ranges were selected based on prior knowledge about the system under study. On the other hand, the injection time was set at 30 s. The detection wavelength was set at 200 nm and the temperature was fixed at 25 °C. All experiments were performed in random order to minimize the effects of uncontrolled factors that may introduce a bias on the measurements.

The peak resolutions ($R$) and analysis times (At) for all the 30 experiments were fitted to polynomial models, once outliers were removed by analysing the Cook's distance, i.e. those experimental data that exert disproportionate influence on the model [1] (see Table 2). The model coefficients were calculated by backward multiple regression [1], and validated by the analysis of variance (ANOVA). As can be seen in Tables 3 and 4, modified cubic models are those which better explain the behaviour of resolution and analysis time under the studied factors, although irrelevant main terms were maintained in order to fit hierarchical models. These tables also show the statistical parameters corresponding to the fitting for resolution and analysis time respectively. As can be observed, most model terms are significant ($p < 0.05$) and the lack of fit is not significant ($p > 0.05$) in both cases. These models were selected because they exhibit low standard deviation [0.20 for resolution (CV% = 8.0) and 0.10 for analysis time (CV% = 2.6)] and high adjusted $R$-squared (0.991 for resolution and 0.974 for analysis time) values, indicating a good relationship between the experimental data and the fitted models.

When a simple response is being analysed, the model analysis indicates areas in the design region where the process is likely to give desirable results, which is a relatively easy task. However, the *desirability* is a function of more than one response. The method proposes a desirability function which includes the researcher's priorities and desires on building the optimization procedure. One- or two-sided functions are used, depending on whether each of the $m$ responses has to be maximized or minimized, or has an allotted target value. The procedure involves creating a function for each individual response $di$ and finally obtaining a global function $D$ that should be maximized choosing the best conditions of the designed variables.

The function $D$ varies from 0 (value totally undesirable) to 1 (all responses are in a desirable range simultaneously), and it is defined by the Eq. (2):

$$D = \{d_1 \times d_2 \times d_3 \times \cdots \times d_m\}^{1/m} \quad (2)$$

where $d_1, \ldots, d_m$ correspond to the individual desirability function for each response being optimized.

Table 2
Central composite design used for the optimization of peak resolution and analysis time

| Experiment | Factors | | | | Responses[c] | |
|---|---|---|---|---|---|---|
| | Buffer concentration (mmol $L^{-1}$) | pH | Vi[a] (kV) | Vs[b] (kV) | Resolution | Analysis time (min) |
| 1 | 50 | 8.25 | 20 | 22.5 | 3.78 | 4.56 |
| 2 | 10 | 8.25 | 20 | 22.5 | 1.58 | 3.07 |
| 3 | 30 | 6.72 | 20 | 22.5 | 0.76 | 3.85 |
| 4 | 30 | 9.72 | 20 | 22.5 | 5.59 | 4.39 |
| 5 | 40 | 7.50 | 15 | 20.0 | 1.13 | 4.30 |
| 6 | 40 | 7.50 | 25 | 25.0 | 0.00 | 3.62 |
| 7 | 40 | 7.50 | 25 | 20.0 | 0.00 | 4.41 |
| 8 | 40 | 7.50 | 15 | 25.0 | 0.83 | 3.23 |
| 9 | 20 | 7.50 | 15 | 25.0 | 0.99 | 3.13 |
| 10 | 20 | 7.50 | 25 | 25.0 | 0.00 | 3.34 |
| 11 | 20 | 7.50 | 15 | 20.0 | 0.96 | 2.96 |
| 12 | 20 | 7.50 | 25 | 20.0 | 0.00 | 2.88 |
| 13 | 40 | 9.02 | 25 | 25.0 | 3.69 | 4.14 |
| 14 | 40 | 9.02 | 15 | 25.0 | 6.00 | 4.27 |
| 15 | 40 | 9.02 | 15 | 20.0 | 6.71 | 5.11 |
| 16 | 40 | 9.02 | 25 | 20.0 | 5.39 | 4.75 |
| 17 | 20 | 9.02 | 25 | 25.0 | 1.86 | 3.24 |
| 18 | 20 | 9.02 | 15 | 20.0 | 6.36 | 4.43 |
| 19 | 20 | 9.02 | 15 | 25.0 | 4.94 | 3.49 |
| 20 | 20 | 9.02 | 25 | 20.0 | 1.93 | 4.53 |
| 21 | 30 | 8.25 | 20 | 22.5 | 1.69 | 3.37 |
| 22 | 30 | 8.25 | 20 | 22.5 | 2.02 | 3.65 |
| 23 | 30 | 8.25 | 20 | 22.5 | 1.73 | 3.41 |
| 24 | 30 | 8.25 | 20 | 22.5 | 2.07 | 3.63 |
| 25 | 30 | 8.25 | 20 | 22.5 | 2.12 | 3.56 |
| 26 | 30 | 8.25 | 20 | 22.5 | 2.18 | 3.68 |
| 27 | 30 | 8.25 | 20 | 27.5 | 2.60 | 3.19 |
| 28 | 30 | 8.25 | 20 | 17.5 | 3.17 | 4.99 |
| 29 | 30 | 8.25 | 10 | 22.5 | 5.15 | 4.08 |
| 30 | 30 | 8.25 | 30 | 22.5 | 0.97 | 3.94 |

[a] Vi, injection voltage.
[b] Vs, separation voltage.
[c] Outliers: #13, 16 and 17, 18 and 20.

Therefore, two responses, peak resolution and analysis time were simultaneously optimized by using the desirability function. Table 5 shows the criteria which were followed for the optimization of the individual responses. They were selected based on prior knowledge about the system under study. As can be seen, the resolution was adjusted to a fixed value (2.0), i.e. resolution values under 1.5 corresponded to a desirability of 0, while values between 1.5 and 2.0 corresponded to desirabilities ranging from 0 to 1. This resolution corresponded to excellent separation between peaks. On the other hand, the analysis time was minimized.

Following the conditions and restrictions previously discussed, the optimization procedure was carried out and the response surfaces obtained for the global desirability function are presented in Fig. 3(A–F). These plots were obtained for a given pair of factors, while maintaining the other two fixed at their optimal values. As can be seen in Fig. 3A, D and E, when the pH is lower than 7.86, the desirability is 0. This fact is caused by a resolution lower than 1.5, although the migration time is reasonably good. As the pH increases, the desirability becomes better, reaching the optimum at pH 8.23. Similar observations can be made considering the buffer concentration (Fig. 3A–C), where the desirability is higher for smaller concentration values, a fact that corresponds to minimum times, and resolutions near 2.0. On the other hand, when analysing Fig. 3B, D and F, the desirability is a maximum when Vi is near to 20 kV, coinciding with a resolution of $R = 2.0$. Finally, observing Fig. 3C, E and F, an increase of the desirability can be seen for higher values of Vs, owing to its influence on the analysis time (Vs does not influence the resolution).

The experimental conditions corresponding to one maximum in the desirability function ($D = 0.98$) are: 16.1 mmol $L^{-1}$ of buffer concentration, pH 8.23, separation voltage of 24.5 kV, injection voltage of 20 kV and 30 s of injection time. The individual response values corresponding to the latter value of $D$ are: resolution = 1.86 and analysis time = 2.98 min. The suggested values during the optimization procedure were experimentally corroborated, and the corresponding electropherogram is shown in Fig. 4.

Considering that the separation order corresponds to an alkaline pH, one can analyze if the order matches the mass/charge ratio. The p$K_a$'s and molecular weights are as

Table 3
ANOVA for the response surface reduced cubic model fitted for resolution

| Source[a] | Sum of squares | d.f. | Mean square | $F$ value | Prob > $F$[b] | Coefficient estimate | Standard error |
|---|---|---|---|---|---|---|---|
| $A$ | 0.01 | 1 | 0.01 | 0.17 | 0.6962 | −0.08 | 0.20 |
| $B$ | 1.13 | 1 | 1.13 | 29.67 | 0.0016 | 1.67 | 0.31 |
| $C$ | 2.05 | 1 | 2.05 | 53.9 | 0.0003 | −1.01 | 0.14 |
| $D$ | 0.01 | 1 | 0.01 | 0.29 | 0.6116 | 0.17 | 0.31 |
| $AB$ | 1.26 | 1 | 1.26 | 33.15 | 0.0012 | 2.63 | 0.46 |
| $AC$ | $1.25 \times 10^{-5}$ | 1 | $1.25 \times 10^{-5}$ | $3.28 \times 10^{-4}$ | 0.9861 | $-2.5 \times 10^{-3}$ | 0.14 |
| $AD$ | 0.01 | 1 | 0.01 | 0.36 | 0.5720 | −0.17 | 0.28 |
| $BC$ | 0.20 | 1 | 0.2 | 5.16 | 0.0635 | 0.93 | 0.41 |
| $BD$ | 0.12 | 1 | 0.12 | 3.03 | 0.1322 | 0.93 | 0.53 |
| $CD$ | 0.01 | 1 | 0.01 | 0.24 | 0.6422 | 0.07 | 0.14 |
| $A^2$ | 0.82 | 1 | 0.82 | 21.48 | 0.0036 | −2.89 | 0.62 |
| $B^2$ | 2.36 | 1 | 2.36 | 61.96 | 0.0002 | 1.26 | 0.16 |
| $C^2$ | 1.79 | 1 | 1.79 | 46.9 | 0.0005 | 0.27 | 0.04 |
| $D^2$ | 1.26 | 1 | 1.26 | 33.07 | 0.0012 | 0.92 | 0.16 |
| $ABC$ | 0.57 | 1 | 0.57 | 14.94 | 0.0083 | 1.50 | 0.39 |
| $ABD$ | 0.13 | 1 | 0.13 | 3.35 | 0.1170 | 0.86 | 0.47 |
| $BCD$ | 0.46 | 1 | 0.46 | 11.97 | 0.0135 | 1.85 | 0.53 |
| $A^2B$ | 4.18 | 1 | 4.18 | 109.77 | <0.0001 | 9.93 | 0.95 |
| $A^2C$ | 0.83 | 1 | 0.83 | 21.73 | 0.0035 | 2.23 | 0.48 |
| $A^2D$ | 0.20 | 1 | 0.20 | 5.14 | 0.0639 | 2.73 | 1.20 |
| Residual | 0.23 | 6 | 0.04 | | | | |
| Lack of fit | 0.01 | 1 | 0.01 | 0.32 | 0.5980 | | |
| Pure error | 0.22 | 5 | 0.04 | | | | |
| Total | 112.08 | 26 | | | | | |

[a] $A$, buffer concentration (mmol $L^{-1}$); $B$, pH; $C$, injection voltage (kV); $D$, separation voltage (kV).
[b] Considered significant when $p < 0.05$.

follows: EPN, 11.2 and 249.31 and LID, 7.9 and 234.34, respectively. As can be seen, EPN is positively charged, while LID is negatively charged. This fact explains the separation order observed in Fig. 3, i.e. EPN first and LID second.

### 3.4. Method performance

#### 3.4.1. Linearity and related figures of merit

In order to verify the method linearity within a concentration range of 2.0–12.0 ng $mL^{-1}$ of EPN, three replicates were

Table 4
ANOVA for the response surface reduced cubic model fitted for analysis time

| Source[a] | Sum of squares | d.f. | Mean Square | $F$ value | Prob > $F$[b] | Coefficient estimate | Standard error |
|---|---|---|---|---|---|---|---|
| $A$ | 0.04 | 1 | 0.04 | 3.64 | 0.0828 | 0.19 | 0.10 |
| $B$ | 0.40 | 1 | 0.40 | 40.73 | <0.0001 | −0.88 | 0.14 |
| $C$ | 0.10 | 1 | 0.10 | 10.18 | 0.0086 | 0.14 | 0.04 |
| $D$ | 0.05 | 1 | 0.05 | 4.67 | 0.0536 | 0.23 | 0.11 |
| $AB$ | 0.18 | 1 | 0.18 | 18.31 | 0.0013 | 1.88 | 0.44 |
| $AD$ | 0.78 | 1 | 0.78 | 78.14 | <0.0001 | −1.25 | 0.14 |
| $BC$ | 0.17 | 1 | 0.17 | 16.78 | 0.0018 | −0.22 | 0.05 |
| $BD$ | 0.58 | 1 | 0.58 | 58.61 | <0.0001 | −1.20 | 0.16 |
| $CD$ | 0.05 | 1 | 0.05 | 4.89 | 0.0491 | 0.12 | 0.05 |
| $A^2$ | 0.34 | 1 | 0.34 | 33.82 | 0.0001 | −1.24 | 0.21 |
| $B^2$ | 0.53 | 1 | 0.53 | 53.04 | <0.0001 | 0.57 | 0.08 |
| $C^2$ | 0.33 | 1 | 0.33 | 33.70 | 0.0001 | 0.11 | 0.02 |
| $D^2$ | 0.46 | 1 | 0.46 | 46.53 | <0.0001 | 0.54 | 0.08 |
| $ABD$ | 0.80 | 1 | 0.8 | 80.36 | <0.0001 | 2.81 | 0.31 |
| $A^2B$ | 0.51 | 1 | 0.51 | 51.28 | <0.0001 | 3.00 | 0.42 |
| $AB^2$ | 0.09 | 1 | 0.09 | 8.87 | 0.0126 | −1.23 | 0.41 |
| Residual | 0.11 | 11 | 0.01 | | | | |
| Lack of fit | 0.02 | 6 | 3.95E−3 | 0.23 | 0.9484 | | |
| Pure error | 0.09 | 5 | 0.02 | | | | |
| Total | 10.36 | 27 | | | | | |

[a] $A$, buffer concentration (mmol $L^{-1}$); $B$, pH; $C$, injection voltage (kV); $D$, separation voltage (kV).
[b] Considered significant when $p < 0.05$.

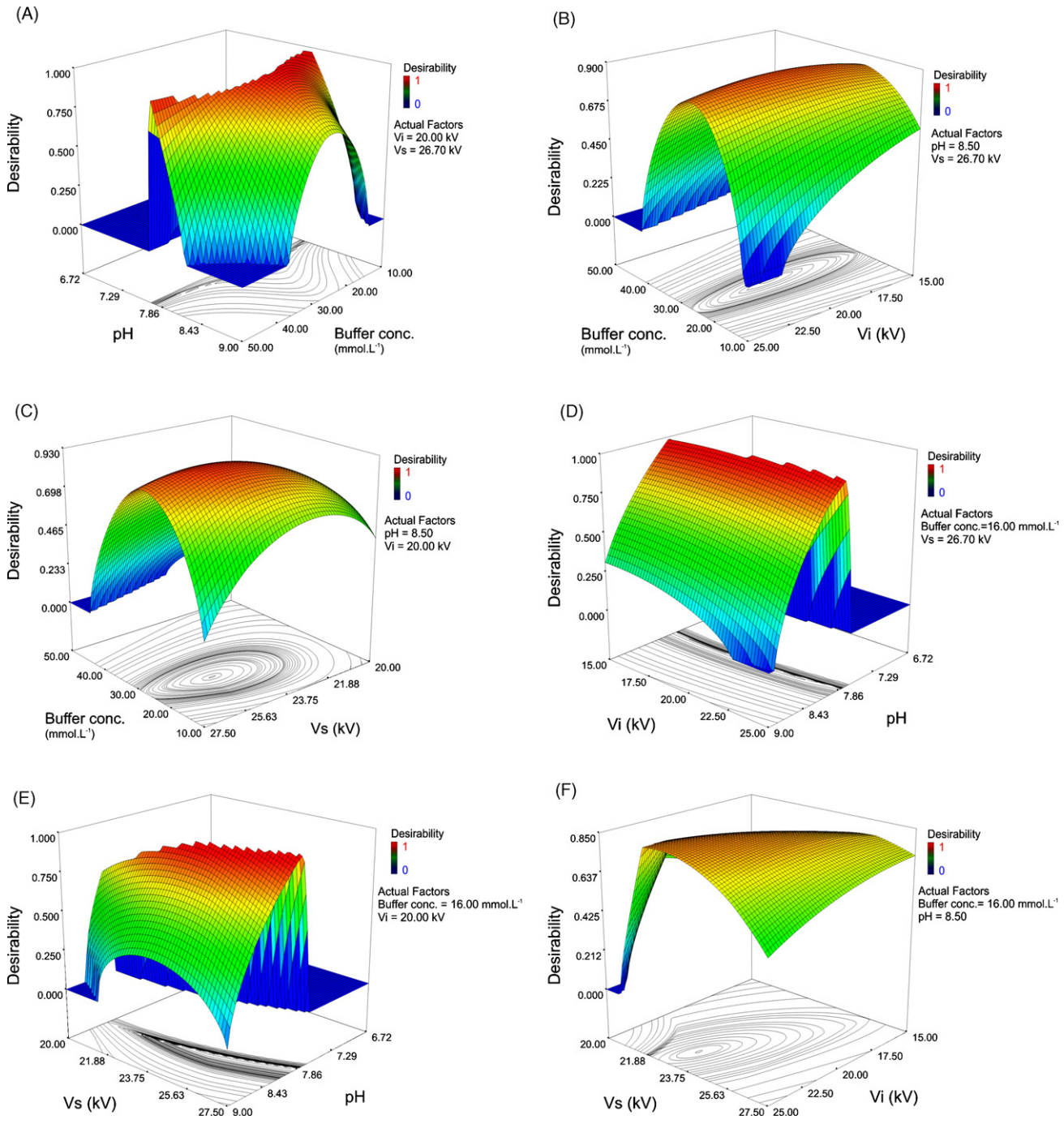


Fig. 3. Response surface plots corresponding to the desirability function when optimising the following pair of factors, while maintaining constant the remaining ones at their optimum values: (A) pH–buffer concentration, (B) buffer concentration–Vi, (C) buffer concentration–Vs, (D) Vi–pH, (E) pH–Vs, and (F) Vi–Vs.

prepared at six concentration levels and subjected to the analytical procedure. A least-squares fitting was performed with the obtained data (ratios of areas) and the results are the following: (a) the coefficient of determination (*R*-squared) was greater than 0.999, (b) the performed ANOVA of lack of fit test allows one to conclude that linearity exists within the range studied and (c) the computed LOQ and LOD were 0.3 ng $mL^{-1}$ and 0.1 ng $mL^{-1}$, respectively. Interestingly, these latter figures of merit are considerably lower than those obtained with the HPLC technique [6].

### 3.4.2. Precision

The intra-assay precision (repeatability) was determined by analysis of six replicate samples at three levels of concentration (levels 1, 2 and 3), under the same conditions, by the same analyst, and on the same day. The coefficient of variation (CV%) values obtained when computing the concentrations are shown in Table 5. As can be seen, these values are near 1%, indicating an excellent precision for the present method.

On the other hand, the intermediate precision was determined by total analysis of six replicates samples at level 2, under the

Table 5
Criteria for the optimization of the individual responses

| Response | Goal | Lower limit | Upper limit |
|---|---|---|---|
| pH | Is in range | 6.7 | 9.7 |
| Buffer concentration (mmol $L^{-1}$) | Is in range | 10 | 50 |
| Vi (kV)[a] | Is in range | 15 | 25 |
| Vs (kV)[b] | Is in range | 17.5 | 27.5 |
| Resolution | Is target (2.00) | 1.50 | 6.71 |
| Analysis time (min) | Minimize | 2.88 | 5.11 |

[a] Vi, injection voltage.
[b] Vs, separation voltage.

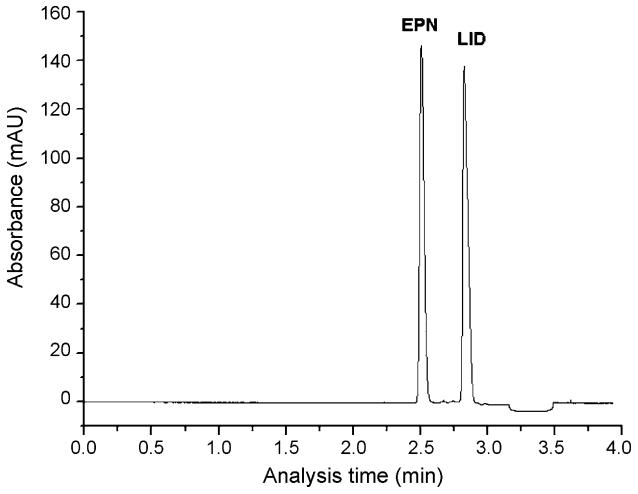


Fig. 4. Electropherogram corresponding to the extract from human serum containing 25.0 ng $mL^{-1}$ of EPN. The final concentrations were: EPN (2.4 min), 4.5 ng $mL^{-1}$ and LID (2.8 min) 7.2 ng $mL^{-1}$.

same conditions, by the same analyst, and on three different weeks (weeks 1, 2 and 3). An ANOVA test was applied to the experimental data, and the results can be also seen in Table 6. No significant differences exist between the obtained averages in 3 weeks.

### 3.4.3. Accuracy

Known amounts of EPN standard solution were added to 200 μL of serum from healthy patients in order to reach concentrations of 20.0, 25.0 and 30.0 ng $mL^{-1}$ (i.e. 80, 100 and 120% of the average therapeutic serum concentration reported in the literature [4]). These mixtures were then analysed by performing six replicates. Table 6 shows the average recovery values, which are indicative of the high accuracy obtained in the three concentration levels studied considering the requirements for bioanalytical assays [16].

On the other hand, a new serum sample containing 25 ng $mL^{-1}$ was analysed by HPLC method performing six replicates ($\bar{x} = 22.18$ ng $mL^{-1}$, $s = 0.41$ ng $mL^{-1}$) and the studied CE method also performing six replicates ($\bar{x} = 22.41$ ng $mL^{-1}$, $s = 0.47$ ng $mL^{-1}$). Averages obtained by the two methods (for CE values see Table 7) were compared through a Student's *t*-test that allowed us to conclude that no statistical differences exist between the results achieved by both methodologies ($p > 0.19$).

Table 6
Results obtained in both the intra-assay and intermediate precision studies

| | Ra average[a] | CV (%) |
|---|---|---|
| Level 1 | 0.70 (1) | 1.12 |
| Level 2 | 1.05 (1) | 1.24 |
| Level 3 | 1.40 (2) | 1.11 |
| Level 2—week 1 | 1.044 (7) | 0.71 |
| Level 2—week 2 | 1.040 (10) | 1.12 |
| Level 2—week 3 | 1.041 (8) | 0.77 |
| Intermediate precision | 1.043 (9) | 0.84 |
| Mean comparison (ANOVA) | $p < 0.01$ | |

[a] Values between parenthesis correspond to the standard deviation. Ra is the relation between the EPN and LID electrophoretic areas.

Table 7
Results obtained in accuracy studies

| Concentration levels (%) | Amount added to serum blank (ng $mL^{-1}$) | Recovery of EPN[a] (ng $mL^{-1}$) | Recovery (%) |
|---|---|---|---|
| 80 | 20.0 | 19.4 (0.5) | 97.03 |
| 100 | 25.0 | 23.0 (0.5) | 91.91 |
| 120 | 30.0 | 27.0 (0.5) | 90.08 |

[a] Values between parenthesis are standard deviations.

### 3.4.4. Selectivity

Techniques such as electrophoresis tend to rely on selectivity in the separation process, often called separation selectivity [17]. In any case, with the aim of verifying that EPN and LID peaks correspond to the pure compounds, a purity test was carried out, which is based on the correlation between the spectra of the components recorded within the peaks. The correlation should be superior to 0.99 to conclude that a single compound is present. For the two peaks being analysed, the purity factor was greater than the established threshold limit.

### 3.4.5. Robustness

In order to evaluate the robustness of the developed method, an experimental Plackett–Burman design was built setting small changes in the studied parameters (see Table 8) and evaluating

Table 8
Plackett–Burman design used for robustness analysis

| Experiment | Concentration of buffer (mmol $L^{-1}$) | pH | Vi (kV) | Vs (kV) | Recovery of EPN (%) |
|---|---|---|---|---|---|
| 1 | 13 | 8.4 | 16 | 26 | 89.49 |
| 2 | 13 | 8.4 | 14 | 24 | 90.13 |
| 3 | 13 | 8.4 | 14 | 26 | 89.40 |
| 4 | 13 | 8.6 | 14 | 26 | 87.58 |
| 5 | 13 | 8.6 | 16 | 24 | 86.41 |
| 6 | 13 | 8.6 | 16 | 26 | 87.93 |
| 7 | 17 | 8.4 | 16 | 26 | 88.00 |
| 8 | 17 | 8.4 | 16 | 24 | 88.01 |
| 9 | 17 | 8.4 | 14 | 24 | 86.76 |
| 10 | 17 | 8.6 | 14 | 24 | 86.84 |
| 11 | 17 | 8.6 | 16 | 24 | 86.35 |
| 12 | 17 | 8.6 | 14 | 26 | 84.51 |

the effect that these changes produce on the accuracy of the method [18].

The evaluation consisted in analysing a stock standard solution and a real sample in all the cited conditions. In each case, the recovery of EPN was evaluated. An ANOVA test was applied to the experimental data, employing the effects of dummy variables to obtain estimates of standard errors, with the following result: concentration and pH of the buffer were found to be significant ($p < 0.05$). Consequently, the buffer preparation is an important issue to be considered when quantifying epinastine hydrochloride in human serum.

## 4. Conclusions

Epinastine hydrochloride can be quantitated in human serum by using capillary electrophoresis. Peak resolution and analysis time were simultaneously optimized by resorting to the useful tool of multiple response optimization. The use of experimental design and response surface methodology enhanced by the application of the desirability function allows for a rapid solution of analytical tasks such as the one studied in the present work. Good results with respect to linearity, precision, accuracy, selectivity and robustness were obtained in the concentration range studied for epinastine hydrochloride, and these results present better performance, especially for sensitivity, than those achieved when the reference high performance liquid chromatography technique is applied to human serum samples.

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