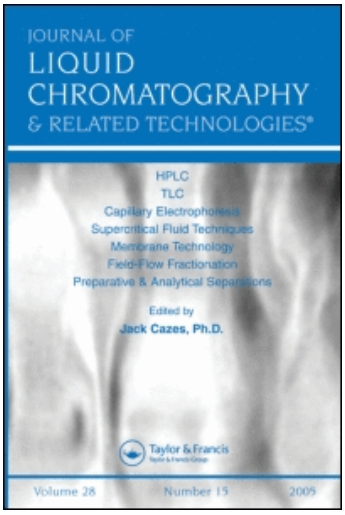

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Optimization of an RP-HPLC Method for Drug Control Analysis

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ABSTRACT

Optimization of important conditions for the reversed-phase high-performance liquid chromatographic method was done for the separation of the active ingredients in Marcaine® adrenaline injections (bupivacaine hydrochloride 2.5 mg and adrenaline 5.0 μg). Simultaneous influence of several conditions, such as the mobile phase composition, pH of the mobile phase, and temperature, on important chromatographic criteria for the separation, was investigated. The separation factor values defined the optimal conditions, which were confirmed by analysing the appropriate mathematical models. The 3-D graphs, constructed with sixty-four

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experimental points, were investigated, and the results showed that the optimal separation was achieved with the mobile phase of methanol–water (65 : 35 v/v), by adjusting pH to 3.5 and the temperature range from 20°C to 30°C. The optimized method was validated and the obtained results were statistically evaluated.

Key Words: Optimization; Reversed-phase high-performance liquid chromatography; Bupivacaine; Adrenaline; Drug analysis.

INTRODUCTION

Optimization of important conditions for the reversed-phase high-performance liquid chromatographic method was done for the determination of bupivacaine hydrochloride and adrenaline (2.5 mg : 5.0 μg) in a pharmaceutical dosage form. It defined the optimal conditions, such as the mobile phase composition, pH of the mobile phase, and temperature. Simultaneous influence of several conditions on important chromatographic criteria for the separation was investigated. The separation factor values defined the optimal conditions, which were confirmed by analysing the appropriate mathematical models.

It was found in the literature, that the combination of adrenaline and bupivacaine in injections, had been determined using the HPLC method[1] on L1 ODS column with water : methanol : sodium-monobasic-phosphate as a mobile phase and amperometric detection. Adrenaline, in different combinations, had been determined using spectrophotometric[2–10] or electrochemical methods,[11] and some separation techniques, such as GC,[12] HPLC[13–16] (in biological material, or metabolits, or after derivation), and CE.[17–21] Bupivacaine had been determined using spectrophotometric,[22] GC,[23–27] and HPLC[28–30] methods (in biological material, in the mixtures with some morphines derivates and in epidural solutions with lidocainehydrochloride).

These novel investigations attempt to optimize the conditions for the determinations. By applying the optimized RP-HPLC technique, it is possible to identify and simultaneously determine both bupivacaine hydrochloride and adrenaline in the Marcaine® adrenaline injections. Therefore, the aim of this paper was to optimize the RP-HPLC method, using the appropriate mathematical models.

EXPERIMENTAL

Reagents

All the chemicals and reagents were of an analytical reagent grade and water was redistilled and filtered through a membrane filter. Methanol–HPLC

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gradient grade (*Fluka*, Ulm, Germany), triethanolamine p. a. (*Fluka*, Ulm, Germany), and 85% phosphoric acid (*Carlo Erba*, Italy) were used to prepare mobile phases.

Chromatographic Conditions

The chromatographic system, Hewlett Packard 1100, consisted of a HP 1100 pump, thermostatted column compartment G 1316A, UV-VIS Detector G 1314A, and HP ChemStation integrator. Separations were performed on a Beckman Ultrasphere ODS 4.6 mm × 15 cm, 5 μm particle column. The samples were introduced through a Rheodyne injector valve with a 20 μL sample loop. The mobile phase was a mixture of methanol and water in different ratios. The pH was adjusted with phosphoric acid. The flow rate was 1 mL/min and UV detection was performed at 270 nm. Propyphenazone was used as an internal standard.

The optimization of the RP-HPLC method was performed with varying compositions of the mobile phase of methanol–water from (50 : 50 v/v) to (85 : 15 v/v), within the pH range from 2.0 to 5.5 and temperature range from 20°C to 55°C. The influence of triethanolamine, which was added to the water phase, was examined. The pH of the water phase was adjusted with triethanolamine from 7.8 to 9.2. The mixture of methanol-water (65 : 35 v/v) was prepared and the pH of such mobile phase was adjusted to 3.5 with phosphoric acid.

Investigation of the three-D graphs, constructed with sixty-four experimental points, showed that the optimal separation was achieved with the mobile phase methanol–water (triethanolamine was added to the water phase up to pH 8.8) (65 : 35 v/v), adjusting pH of the mobile phase to 3.5, and in the temperature range of 20–30°C.

Standard Solutions for Optimization

Concentrations of standard solutions for optimization of the method were 250 μg/mL for bupivacaine hydrochloride and 50 μg/mL for adrenaline. All the solutions were prepared in the mobile phase.

Standard Solutions

Concentrations of standard solutions for calibration curves were from 125 to 575 μg/mL for bupivacaine hydrochloride and from 5 μg/mL to 100 μg/mL for adrenaline. All the solutions were prepared in the mobile

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phase. Propyphenazone (15 μg/mL) was added as an internal standard. All substances were USP reference standards.

Laboratory Mixtures

Laboratory mixtures of bupivacaine hydrochloride and adrenaline, in three concentration ratios (200 : 15 μg/mL, 250 : 50 μg/mL, and 450 : 90 μg/mL), were prepared in a mobile phase. For the chromatographic separation, propyphenazone was added as an internal standard (15 μg/mL).

Sample Solutions

The RP-HPLC optimized method was developed for determination of bupivacaine hydrochloride and adrenaline in Marcaine® adrenaline injections, *Astra*, Södertälje, Sweden. One milliliter of the injection solution contained 2.5 mg of bupivacaine hydrochloride and 5.0 μg of adrenaline (as epinephrine tartarate).

Three different concentrations of the sample solutions of Marcaine® adrenaline injections were prepared: 0.8, 1.2, and 1.5 mL, transferred to 10 mL volumetric flasks, and mixed with 0.3 mL of an internal standard solution ($c = 0.5$ mg/mL) and 1 mL of standard solution of adrenaline ($c = 0.5$ mg/mL). The resulting solutions were injected into the column.

RESULTS AND DISCUSSION

For the separation and determination of bupivacaine hydrochloride and adrenaline in Marcaine® adrenaline injections, the RP-HPLC method was applied. The concentration ratio of bupivacaine hydrochloride and adrenaline in Marcaine® adrenaline injections was 500 : 1, which is a potential problem of the separation and simultaneous determination of these two substances. Simultaneous determination was performed by applying the method of standard addition for the determination of adrenaline and the method of internal standard for the determination of bupivacaine hydrochloride. Retention time for adrenaline was 1.53 min and for bupivacaine hydrochloride 2.64 min (Fig. 1). The capacity factor for adrenaline was 2.74 and for bupivacaine hydrochloride 5.29, while the resolution factor was 6.62. The best resolution was obtained using propyphenazone as an internal standard.

Optimization of the chromatographic conditions for the RP-HPLC separation of bupivacaine hydrochloride and adrenaline defined optimal conditions: the composition of the mobile phase, pH of the mobile phase, and temperature.

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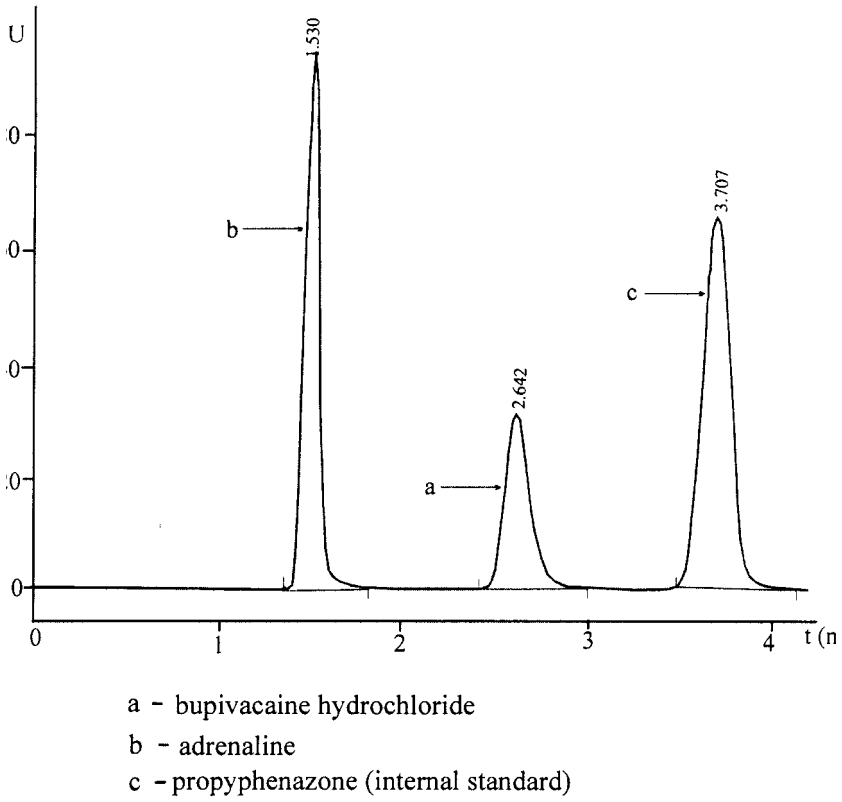

Figure 1. Representative chromatogram of Marcaine® adrenaline injections.

Figure 2 shows a 3-D graph constructed with sixty-four experimental points, which represents the functional dependence of the selectivity factor of the composition of the mobile phase and pH of the mobile phase. A continuous decrease of the profile under defined pH values could be observed.

Figure 3. shows a 3-D graph, which represents the functional dependence of the selectivity factor of temperature and composition of the mobile phase. The obtained surface shows a continuous change with temperature and composition of the mobile phase.

Figure 4. shows a 3-D graph of the functional dependence of the selectivity factor of the pH and temperature. The optimal separation conditions were represented with so-called "reefs", and the most inappropriate, with a minimum. In the temperature range of 20–30°C, the optimal pH of the water

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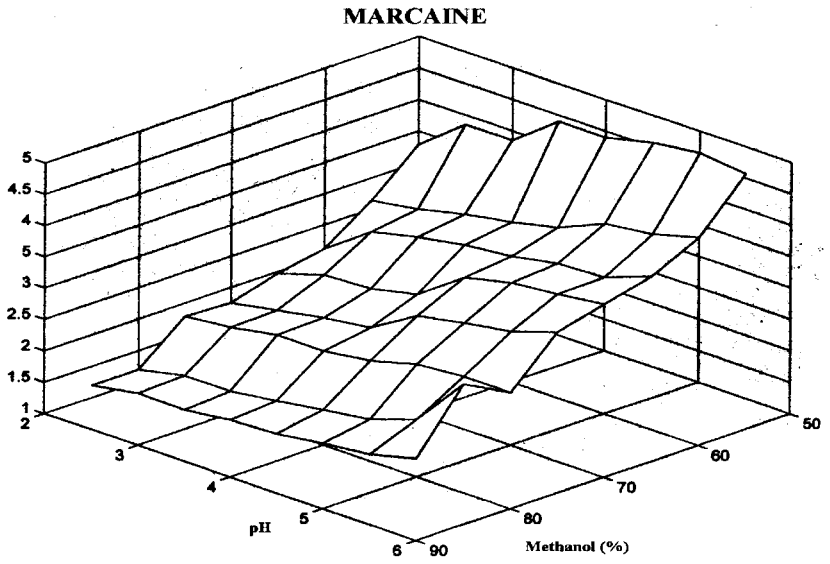

Figure 2. Three-D graph: $\alpha = f$ (%Methanol, pH).

was 8.8, while this range is wider for the pH 8.2. Low values of the selectivity factor were achieved for pH values of the water from 8.5 to 9.5, especially for the temperatures higher than 40°C, represented with an expressive minimum of the surface.

The results of the optimization showed that the optimal separation with defined peaks could be achieved using a mobile phase of methanol–water (65 : 35 v/v), pH of the mobile phase adjusted to 3.5 with phosphoric acid, and the flow rate of 1 mL/min. An addition of triethanolamine to the water up to pH 8.8 had a significant influence on the peak symmetry.

The proposed RP-HPLC method, after optimization, was validated. Robustness, selectivity, linearity, precision, limit of detection (LOD), and limit of quantitation (LOQ) were investigated.

The robustness of the method describes the effect of minor changes in the analytical parameters, such as pH value, eluent composition, temperature, flow rate, etc. It can be concluded that the proposed RP-HPLC method is robust, because slight variations in some experimental parameters had little or no effect on the results. Monotonous decreasing of the profile of three-D graphs, when temperature and composition of the mobile phase were changed, as well as when pH of the different mobile phases was changed, defined robustness of the method (Figs. 2 and 3).

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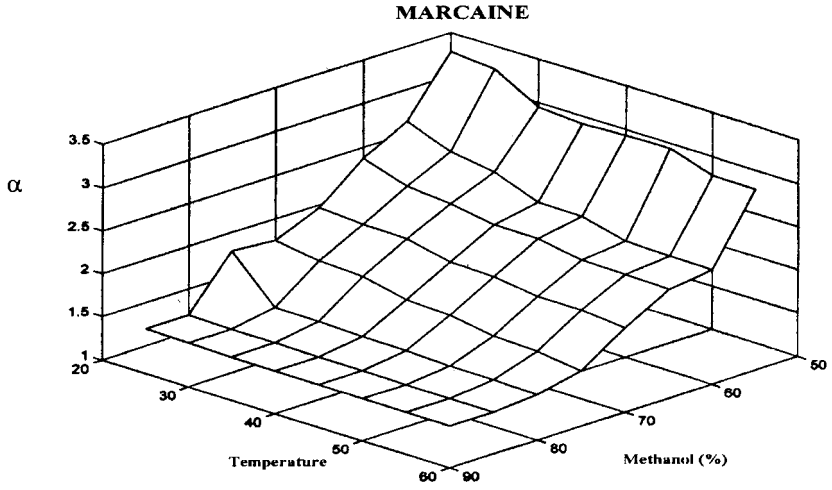

Figure 3. Three-D graph: $\alpha = f$ (%Methanol, t°C).

Linear relationships of the peak area over the mentioned concentration ranges for bupivacaine hydrochloride and adrenaline were obtained. Important parameters of calibration curves: slope (a), intercept (b), correlation coefficient (r), standard deviation of the slope (S_a), and standard deviation of the intercept (S_b) are presented in Table 1. LOD and LOQ were experimentally determined, and they are also presented in Table 1.

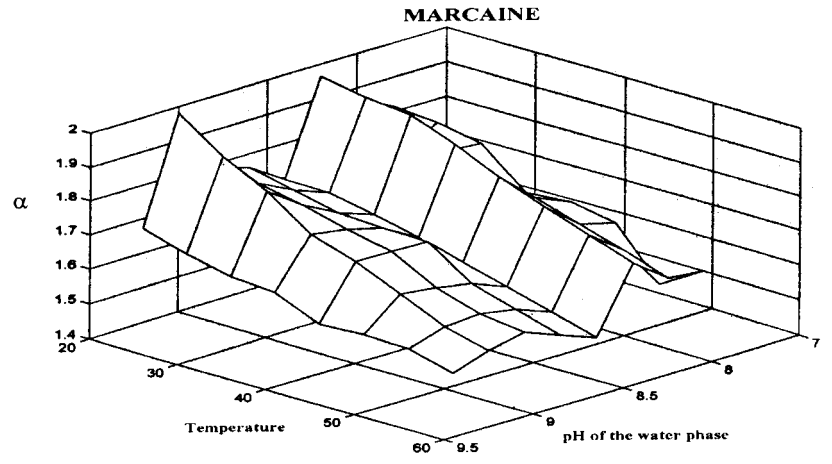

Figure 4. Three-D graph: $\alpha = f$ (pH_{H_2O}, t°C).

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Table 1. The important parameters for the calibration curves.

Compound	$y = ax + b$	r	S_a	S_b	LOD[a]	LOQ[a]
Bupivacaine hydrochloride	1.135×-2.18	0.9999	0.007	2.56	1.62 μg/mL	9.75 μg/mL
Adrenaline	$10.02 \times +5.24$	1.0000	0.03	1.47	3 ng/mL	0.60 μg/mL

Note: r, correlation coefficient; S_a, standard deviation of the slope; S_b, standard deviation of the intercept; LOD, limit of detection; LOQ, limit of quantification.
[a]Experimentally determined values.

Table 2. Determination of active ingredients in Marcaine® adrenaline injections.

Compound	Taken (mg/mL)	Found (mg/mL)	R (%)	CV (%)
Bupivacaine hydrochloride	200	199.9 ± 1.1[a]	99.97	0.57
	300	300.8 ± 0.8	100.27	0.28
	375	372.6 ± 0.6	99.37	0.16
Adrenaline	0.40	0.399 ± 0.005	99.99	1.25
	0.60	0.604 ± 0.004	100.65	0.66
	0.75	0.757 ± 0.004	100.99	0.53

[a]S_d ($n = 10$).

The concentration of bupivacaine hydrochloride was calculated using the method of internal standard, but the concentration of adrenaline was determined using the method of additional standard.

Shown in Table 2 are the results of the RP-HPLC determination of bupivacaine hydrochloride and adrenaline in Marcaine® adrenaline injections. Important statistical values, such as standard deviation and coefficient of variation, as well as good recoveries, are given in Table 2. Standard deviation and coefficient of variation have low values, which are required for a valid method.

The results show that the described optimized RP-HPLC method is reproducible and can be used for the determination of bupivacaine hydrochloride and adrenaline. The proposed method is rapid, accurate, sensitive, and the results are reproducible. The authors propose this method for the simultaneous determination of the mentioned compounds in their pharmaceutical formulations.

CONCLUSION

Optimization of the RP-HPLC method was performed by changing the composition of the mobile phase, pH of the water, pH of the mobile phase, and temperature. After choosing the optimal chromatographic conditions, the method was validated. It showed good selectivity, specificity, linearity, precision, and robustness related to pH, temperature, and composition of the mobile phase changes. Therefore, the authors propose this method for the separation, identification, and simultaneous determination of bupivacaine hydrochloride and adrenaline in pharmaceutical dosage forms.

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