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Development and Validation of a Novel HPLC Method for the Determination of Ephedrine Hydrochloride in Nasal Ointment

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Abstract: A simple, precise, and cost-effective reverse phase ion pair chromatographic (RP-IP-HPLC) method was developed and validated for the determination of Ephedrine Hydrochloride in a nasal ointment. A simple and fast extraction protocol was developed for the effective recovery of the analyte, and for this purpose, Bromhexine Hydrochloride was used as the internal standard. The mobile phase consisted of MeOH, Sodium Lauryl Sulfate (SLS) 49.8 mM, triethylamine (ET_3N) in the ratio of 65:34.6:0.4%, respectively, with pH = 2.20. The detection of the compounds was carried out at 206 nm, and we used a PDA detector. A short run time was achieved with retention times of 6.3 min and 9.8 min for ephedrine hydrochloride and the internal standard, respectively. The proposed method was validated according to ICH guidelines. Linearity was confirmed in the range of 50–150 μg/mL. Recoveries results were within the range of 98–102% and precision < 2% for the analyte in spiked blank matrix. Robustness testing was conducted via a fractional factorial experimental design. The method was found to fulfill the required specifications for specificity and stability for both standard solutions and samples, as well and applied to the determination of ephedrine hydrochloride in nasal ointments produced by the Greek Military Pharmaceutical Laboratories.

Keywords: ephedrine; nasal ointment; extraction; ion pair chromatography; robustness testing; experimental design

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1. Introduction

Ephedrine is a sympathomimetic compound found in plants of *Ephedra genus*. Often referred to as a mixed acting sympathomimetic, ephedrine causes the release of norepinephrine from storage vesicles and at the same time stimulates alpha and beta adrenoceptors. In clinical practice, ephedrine is mainly used to prevent bronchospasm during surgical procedures, to treat acute hypotension, and as a nasal decongestant. On the other hand, the isomer of ephedrine called "pseudoephedrine" is used as nasal decongestant in oral or nasal formulation. The topical nasal ointment of ephedrine is widely produced and consumed by Greek military services as a decongestant [1–3].

The number of published protocols for the determination of Ephedrine Hydrochloride (EH) in nasal ointment is very limited, and also no relevant pharmacopoeia monograph is available. Most procedures proposed for the determination of EH alone or in mixtures, utilize reverse phase liquid chromatography (RPLC) with C8, C18, or cyano columns and mobile phases consisting of acetonitrile–buffer (phosphate, ammonium acetate) or methanol–buffer or acetonitrile–methanol–buffer [4,5]. However, none of these refer to the determination of EH in nasal ointment.

The development of an assay method for such formulations is challenging, due to the sample preparation protocol. Furthermore, using the aforementioned conventional aqueous/organic mobile phases constitutes another challenge, when hydrochloric salts of basic compounds, such as EH, are the active pharmaceutical ingredients (APIs) to be determined, due to the short retention time under such conditions. Hence, we managed to develop a simple, precise and cost effective reverse phase ion pair chromatographic (RP-IP-HPLC) method for the determination EH in a topical ointment dosage form by using a PDA detector. This new HPLC method was successfully validated per ICH guidelines and proved to be suitable for routine quality control use in Greek Military Pharmaceutical Laboratories in Athens, Greece. To the best of our knowledge, this is the first method that can assay EH in a nasal ointment matrix, providing an effective extraction protocol and a fast chromatographic analysis. Therefore, it differentiates substantially from other existing methods describing determination of EH in 'simpler' pharmaceutical formulations. In fact, the current method could be considered as a model one for the extraction and analysis of hydrochloric salts of basic compounds from nasal ointments and ointments in general. Furthermore, the validation part included robustness testing that was conducted via a fractional factorial experimental design. This way the factors that should be carefully controlled can be identified, by both graphical and statistical methods, as the ones having a significant effect for each response examined (area, retention time etc.)

2. Materials and Methods

2.1. Reagents and Solvents

Ephedrine hydrochloride nasal ointment (Lot 20093646—Exp. November 2023), blank matrix of ointment (Lot 1912364), home working standards (HWS) of EH, Bromhexine (Brx) hydrochloride (internal standard, IS), and sodium lauryl sulfate (SLS) were provided by Greek Military Pharmaceutical Laboratories. With regard to HPLC-grade solvents, MeOH and water were purchased from Sigma-Aldrich (St. Louis, MO, USA). Hydrochloride acid, trimethylamine (TEA), orthophosphoric acid, and diethyl ether were of analytical grade and obtained from Sigma-Aldrich, too. HPLC-grade water was obtained by a Merck Millipore Milli-Q device (Merck S.A. Hellas, Athens, Greece).

2.2. Instrumentation

Analyses were carried out using a VWR Hitachi HPLC Chromaster, equipped with photodiode-array detection unit, column oven, autosampler 100 μL, delivery solvent system and online degasser. The chromatographic separation was performed on a Pinnacle DB Cyano column 250 × 4.6 mm, 5 μm (Restek Corporation, Bellefonte, PA, USA) [6]. During validation process, a second HPLC system was employed. In particular, the Merck-Hitachi HPLC system was equipped with a 200 μL loop auto sampler, a pump, an external column oven and a variable wavelength (190–600 nm) PDA Detector Merck-Hitachi HPLC system. The chromatographic data were collected and recorded using Clarity Chromatography 8.1 software (DataApex®) for both HPLC systems.

2.3. Chromatographic Conditions

The reverse phase HPLC analysis was carried out in isocratic elution mode. The mobile phase was prepared by mixing MeOH, SLS and TEA solutions in varying proportions. Before mixing with organic solvent (MeOH), the final pH of aqueous solution (SLS & TEA) was adjusted to the desired value (pH 2.20) with orthophosphoric acid. The optimum mobile phase consisted of MeOH:SLS (49.9 mM):TEA in proportions 65:34.6:0.4% (v/v). This mobile phase was degassed by means of vacuum filtration and filtered through a 0.45 μm membrane, prior to use. The operating temperature of the column (Pinnacle II Cyano 5 μm, 250 mm × 4.6 mm) was set at 25 °C and the flow rate was maintained at 0.6 mL/min. The analyte and the internal standard were quantified at 206 nm. The injection volume was set at 20 μL, and we used a sample loop.

2.4. Preparation of Standard Solution

2.4.1. Stock Solution

The appropriate amount of both analytes was firstly dissolved in 20 mL of MeOH in a 50 mL volumetric flask, which was placed in ultrasonic bath for 8 min and then the volume was filled with 0.1 N HCL. Therefore, stock solutions of 500 μg/mL of EH and Brx were prepared in HCl 0.1 N:MeOH, 60:40 v/v, for each analyte. Stock solutions were stored in refrigerator at 5 °C.

2.4.2. Working Standard Solution

4 mL of EH stock solution and 2 mL of Brx stock solution were further diluted in 20 mL volumetric flask with the same mixture of solvents in order to obtain the following final concentrations: 100 μg/mL and 50.0 μg/mL for EH and Brx, respectively (100% concentration level).

2.5. Sample Preparation

The major problem found in the analysis of semi-solid dosage forms is interference from the lipophilic components. As a result, prior to chromatographic analysis, an extraction method is required to sufficiently separate the analyte from the excipients and avoid relevant problems. After several trials, sample preparation was conducted based on an irrelevant USP Pharmacopeia monograph that describes the extraction of Dibucaine Hydrochloride from ointment sample [7]. The new protocol replaced the time consuming and complicated extraction method that the laboratory used to apply for several years. The novel Liquid-Liquid Extraction (LLE) protocol included the following simple and fast steps (Figure 1): 2 g of ointment, equivalent to 20 mg of EH (10 mg/g), and 5 mL of Brx solution (2000 μg/mL), used as IS, were added in a separator. Then 50 mL of diethyl ether were added, mixed till the ointment was dissolved with the assistance of an ultrasonic bath for 5 min. After that, 50 mL, 40 mL and 30 mL portions of 0.1 N HCl were added consecutively to extract the analytes, as less HCl is needed for the efficient extraction of a basic drug in the 2nd and 3rd step. The aqueous phase extracts, containing EH and BRx, were combined in a 200 mL volumetric flask. The expected final concentrations were 100 μg/mL and 50 μg/mL for EH and Brx, respectively, for 100% theoretical extraction recovery. The solution was diluted with MeOH to volume and mixed. Finally, prior to HPLC injection, the solution was passed through a 0.45 μm PVDF filter.

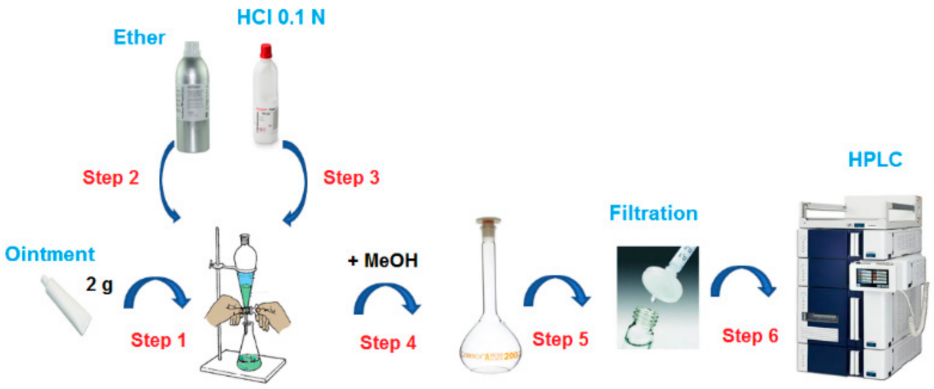


Figure 1. Graphical presentation of the extraction protocol for the nasal ointment.

2.6. Validation Process

The proposed method was validated according to the International Conference on Harmonization of Technical Requirements for Registration for Human Use (ICH) guidelines Q2 (R1) [8]. Experiments were performed in blank matrix of ointment, and the assay method was validated for system suitability, specificity, linearity, accuracy, precision, stability, and robustness.

2.6.1. System Suitability

System suitability testing was carried out to verify the system, method, and column performance. After five replicates injections of the same working standard solution, %RSD (system precision or instrument precision), retention factor, theoretical plates, tailing factor and resolution were calculated for both EH and Brx by "Clarity Chromatography 8.1 Software" [9].

2.6.2. Specificity

To assess the method specificity and exclude interference from other matrix components, four different chromatographs were evaluated and compared: the working standard solution of EH and Brx in nominal concentration (100%), an extracted blank free matrix of ointment, a spiked sample with EH and Brx and the diluent (HCl 0.1 N:MeOH 60:40). In particular, spiked samples were obtained after spiking specific amounts of solutions of EH and Brx during sample pretreatment in order to obtain concentrations corresponding to a 100% level. Both the spiked sample and the blank free matrix were analyzed after sample preparation procedure.

In addition, except from the evaluation of method specificity, the UV spectra for two peaks identification and peak purity was determined by a PDA detector. The spectrum was scanned from 200–400 nm and 206 nm was the wavelength selected for quantitation [10].

2.6.3. Linearity

The working standard solution-based calibration curve was evaluated at five concentration levels by diluting stock solution to give A, B, C, D, E, working standard solutions at level 50%, 75%, 100%, 125% and 150% of the target assay concentration (100 μg/mL). Brx (IS) was added in all solutions, in order to obtain 100% level concentration (50.0 μg/mL). These solutions were injected in triplicate and calibration curve was obtained by plotting peak area ratio (EH/IS) vs. EH concentration by Microsoft Excel software.

2.6.4. Accuracy

The accuracy of the method was expressed as % recovery. For the evaluation of method accuracy, a freshly prepared blank matrix of ointment was spiked with various amounts of EH reference standard solution (500 μg/mL) and with the same amount of Brx reference standard solution (500 μg/mL), in order to obtain specific levels (75%, 100% and 125%) of the target concentration (100 μg/mL). Prior to HPLC analysis, the spiked samples for each level (in triplicate) were extracted according to Section 2.5 and the obtained areas were compared with the respective ones from working standard solution at 100% level concentration. The % Recovery was calculated according to the following Equation:

$$\%R = \frac{\text{AreaEH spiked}/\text{AreaBrx spiked}}{\text{AreaEH STD}/\text{AreaBrx STD}} \times \frac{\text{CEH STD}}{\text{CEH spiked}} \times 100 \quad (1)$$

where AreaEH (spiked or STD) and AreaBrx (spiked or STD) refer to the Areas of EH and IS obtained after analysis of spiked samples and working standard solutions, respectively, and CEH (spiked or STD) is the concentration of API in spiked sample or in working standard solution.

2.6.5. Precision

Precision was evaluated in terms of repeatability and intermediate precision. The repeatability was investigated by spiking blank matrix of ointment with EH solution at 100% concentration level and Brx solution and treated as previously described. The procedure was repeated five more times (6 replicate samples) in the same day and by the same analyst. As far as intermediate precision is concerned, the aforementioned procedure was repeated in three consecutive days which simulate different conditions, such as different analyst (day 3) and different HPLC system (day 2). Reproducibility was not estimated, as the current analytical method is not intended to be transferred to another laboratory.

2.6.6. Stability of Solutions

The stability of working standard solutions and spiked sample solutions was evaluated in triplicate for a period of 2 days (0 h, 5 h, 24 h and 48 h) and under different storage conditions (25 °C and 5 °C).

2.6.7. Robustness Testing

Robustness testing constitutes an integral part of method validation and is conducted to examine the effect of minor but substantial variations of method parameters on its chromatographic responses. In projected procedure the robustness of the method was investigated by a Fractional Factorial Design (FFD) with 2^{k-3} factorial runs, where k is the number of factors. These factors are varied in an interval which can be expected when transferring a method between different instruments or laboratories. A study was carried out at three levels for all variables (+1, 0, −1) to measure the robustness of HPLC procedure (Table 1).

Table 1. Levels of the factors selected for the determination of method robustness.

Experimental Factor	Interval		
	−1	**0 (Nominal Value)**	**+1**
A: Flow	0.5	0.6	0.7
B: pH	2.0	2.2	2.4
C: C_{salt}	48	49.9	51.8
D: T	23	25	27
E: % MeOH	63	65	67
F: λ_{max}	205	206	207
G: % Et_3N	0.35	0.40	0.45

Given the number of factors (k = 7), the experiments that provide a 2^{k-3} FFD design are 16, with 3 additional runs of nominal conditions, totally 19 experiments (Table 2). During experimental design, the responses considered were: Area of EH, Area of Brx, tailing factor (T_f) of EH, tailing factor (T_f) of Brx, resolution between the two peaks (Rs), theoretical plates (N) of EH, theoretical plates (N) of Brx, retention time (t_R) of E HCL, and retention time (t_R) of Brx HCL. The evaluation of method robustness was conducted after statistical and graphical analysis (Pareto charts, half normal and normal plots). Finally, the specific limits in which the parameters should be retained in order to provide a robust method were assessed. Design-Expert v. 10 trial version (Stat-Ease Inc., Minneapolis, MN, USA) was used for graphical analysis.

Table 2. Experiments of FFD proposed by Design Expert software.

Run	Experimental Factor						
	A (Flow)	**B (pH)**	**C (C_{salt})**	**D (T)**	**E (% MeOH)**	**F (λ_{max})**	**G (% Et_3N)**
1	0.7	2.0	51.8	27	63	205	0.45
2	0.6	2.2	49.9	25	65	206	0.4
3	0.7	2.0	48.0	27	67	207	0.35
4	0.6	2.2	49.9	25	65	206	0.4
5	0.5	2.4	48.0	23	67	207	0.35
6	0.5	2.4	48.0	27	67	205	0.45
7	0.7	2.4	51.8	23	67	205	0.35
8	0.7	2.4	51.8	27	67	207	0.45
9	0.5	2.0	48.0	27	63	207	0.45
10	0.7	2.0	51.8	23	63	207	0.35
11	0.5	2.0	51.8	27	67	205	0.35
12	0.5	2.0	48.0	23	63	205	0.35
13	0.5	2.4	51.8	27	63	207	0.35

Table 2. *Cont.*

Run	Experimental Factor						
	A (Flow)	B (pH)	C (C_{salt})	D (T)	E (% MeOH)	F (λ_{max})	G (% Et_3N)
14	0.7	2.4	48.0	27	63	205	0.35
15	0.5	2.0	51.8	23	67	207	0.45
16	0.6	2.2	49.9	25	65	206	0.4
17	0.5	2.4	51.8	23	63	205	0.45
18	0.7	2.4	48.0	23	63	207	0.45
19	0.7	2.0	48.0	23	67	205	0.45

3. Results and Discussion

3.1. Optimization of the Chromatographic Conditions

The aim of this study was to develop a simple and fast isocratic HPLC method for the assay of EH in nasal ointment. For this purpose, during the development of the method, four columns (Pinnacle II Cyano 5 µm, 250 mm × 4.6 mm; Pinnacle DB Biphenyl 3 µm, 150 mm × 4.6 mm; Kinetex Hilic 2.6 µm, 150 mm × 3 mm; Roc C_{18} USP L1 3 µm, 150 mm × 3 mm), two organic modifiers (acetonitrile and methanol) and various pH values ranging from 2.2–6.8, with and without ion pairing agent (SDS), were tested. RP-IP-HPLC is often used for the analysis of polar analytes (cationic or anionic species). The addition of an ion pair agent such as SDS in the mobile phase clearly affects their elution profile on reversed phase chromatography. Alternatively, micellar chromatography may be used instead. Here, the concentration of ion pair reagent in the mobile phase is so high that micelles are formed; i.e., the concentration of the ion pair reagent in the mobile phase is above the critical micelle concentration (CMC).

EH, is a salt of a strong acid (HCl) and a weak organic base (Figure 2), with a pKa value of 9.6, which means that its retention time remains stable in the working pH of conventional alkyl bonded stationary phases (pH 2.0–7.0). During method development, the retention time of EH increased in the working pH range, following the addition of SDS in the mobile phase and this led to a better chromatographic efficiency compared to those achieved with conventional aqueous-organic mobile phases. In addition, in a micellar system, the adsorption of a surfactant such as SDS on the stationary phase, reduces the problematic interaction of free silanol groups with basic compounds. As a result, due to sterical impediment, the interaction between analytes and free silanols is lower, contributing to stable retention times and acceptable peak symmetry [11–13].

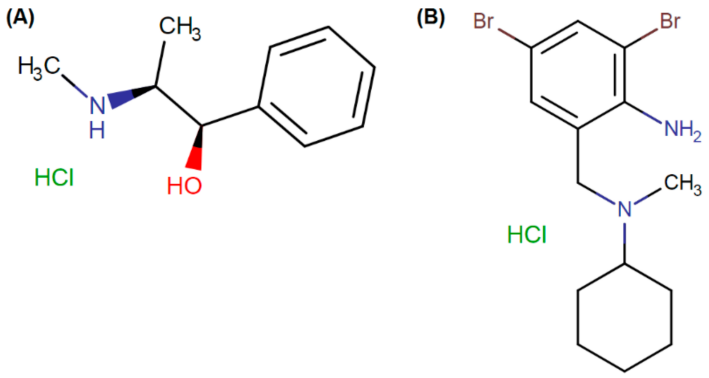


Figure 2. Chemical structures of (**A**) EH and (**B**) Brx.

With regards to preliminary experiments with the above-mentioned columns, the Biphenyl column (250 mm × 4.6 mm, 5 µm) [14] was abandoned, due to interferences from other matrix components present in ointment formulation that could not be resolved. The next trial involved a hydrophilic interaction liquid chromatography (HILIC) column (Kinetex Hilic 2.6 µm, 150 mm × 3 mm) with various mobile phases containing

acetonitrile—aqueous ammonium acetate buffer solutions [15,16]. The working pH was set at 6.22, flow rate at 1 mL/min and detection of the analyte was conducted in four different wavelengths via PDA detector (206, 215, 224 and 260 nm). The present method was rejected due to the extra need of equilibration time between injections and poor retention of EH, close to the solvent front. Next, trials were carried out by using a most conventional column with non polar stationary phase, the Roc C_{18} 3 μm, 150 mm × 3 mm, with and without ion pairing agent (SDS). The use of phosphate or ammonium acetate buffer solution led to poor retention of EH and therefore, the addition of SDS in the mobile phase was necessary in order to improve the chromatographic efficiency. Mobile phase, with aqueous phase (27 mM of SDS, pH = 2.15 adjusted with orthophosphoric acid): acetonitrile in portion 45:55 v/v, provided satisfactory results (t_R = 4.60 min) [17].

As mentioned previously, the addition of an appropriate internal standard (IS) during sample pretreatment is necessary, especially in the case of semi-solid formulations, such as nasal ointment. IS, a molecule with similar properties to the target analyte, is added in fixed concentration to the sample before analysis. Before selecting Brx as IS, experiments were performed with other substances, such as methyl paraben, which were abandoned due to poor recovery of these substances after sample preparation and their short retention time on C_{18} column. Brx had acceptable recovery results; however, its simultaneous analysis with EH on the C_{18} column was not as expected. To this purpose, a fourth column was utilized to finalize and optimize the HPLC conditions. In this case, a cyano stationary phase was selected (Pinnacle II Cyano 5 μm, 250 mm × 4.6 mm), often referred as moderately polar columns and recommended as an excellent choice for the analysis of hydrochloric salts of bases, like EH. The optimized mobile phase was constisted of methanol: water phase (containing 50 mM of SDS, 0.4 % v/v Et_3N and pH adjusted to 2.20 with orthophosphoric acid) in portion 65:35 v/v. Methanol substituted acetonitrile, since ACN is usually not recommended in the case of cyano-columns, as both dipole and π-π interactions (between polar aromatic solutes and the column) are strongly inhibited. The increase of SDS concentration from 27 mM to 50 mM is relevant to micellar chromatography, a subcategory of ion pair chromatography. In combination with cyano propyl column, satisfactory retention of the two analytes, good resolution, shorter run times, and satisfactory asymmetry factors were obtained (Figure 3). The presence of SDS in the mobile phase, along with low pH values are considered to have negative impact on a column life. However, for a 6-month period that development and validation of the method took place, the column had a constant behavior.

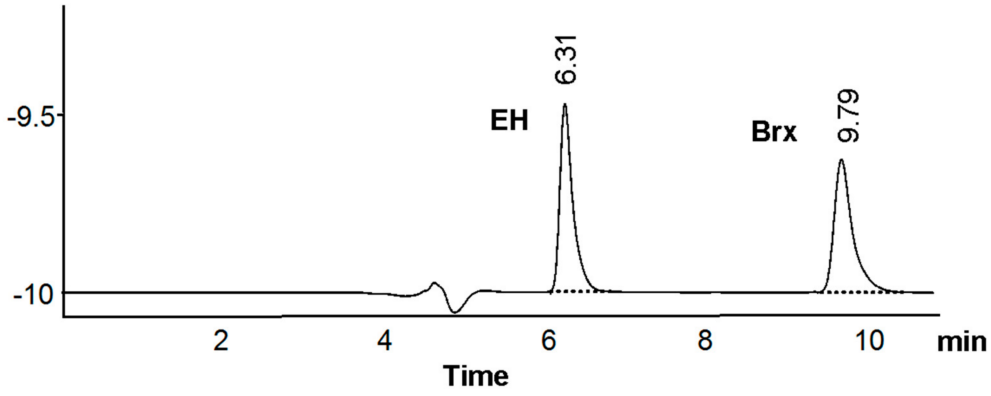


Figure 3. HPLC chromatogram of a working standard solution (0.1 mg/mL and 0.05 mg/mL for EH and Brx, respectively).

3.2. Development of Extraction Protocol for Ointment Sample

Initially, an extraction with three heating/cooling cycles was tested by using ethanol and adding IS before analysis [10]. The sample was heated and cooled in order to achieve adequate separation between the lipophilic excipients and API. After 3 cycles, the liquid layer which contained the analyte of interest was received and diluted with ethanol.

An aliquot of the liquid layer was filtered and entered the HPLC system. This extraction protocol was not adopted, due to interfering peaks in the relevant chromatograms.

Another extraction protocol including a heating/cooling/centrifugation approach was tested [18]. In this protocol, grams of ointment and glacial acetic acid in methanol were added in a centrifuge tube that was heated. After heating, the tube was allowed to cool, capped and centrifuged at 3000 rpm for 10 min. After centrifugation, an aliquot of liquid layer was received, filtered and placed in HPLC autosampler. This protocol was rejected due to inadequate recovery results for the analytes of interest.

In addition, an extraction protocol that was tested included the use of ultrasonic bath and mobile phase as solvent [9]. In this method, a particular amount of ointment was placed in a volumetric flask and diluted with mobile phase. The flask was entered into bath and was left for 15 min. The resulting solution was further diluted using the same solvent in order to achieve the desired concentration. After that, an aliquot of solvent was filtered and placed in HPLC system. The method was rejected because of poor recovery.

Finally, the last extraction protocol [5] that was tested included dilution of 1.0 g of pomade in 50.0 mL of methanol-water (30:20, v/v). The solution was mixed at 500 rpm for 4 h at 50 °C, and an aliquot was injected to HPLC. The extraction protocol was rejected due to the presence of many interfering peaks.

Finally, the protocol described in Section 2.5 was adopted, as it provided accurate and precise data with a relatively simple extraction protocol and a fast run time.

3.3. Method Validation

As described above, method validation was performed according to ICH guidelines in blank matrix of ointment, using internal standard to minimize errors during sample pretreatment.

System suitability test is an integral part of analytical methods. It was evaluated to verify that the chromatographic system is adequate for the assay of EH. A number of parameters were investigated and the results for EH and Brx are presented in Table 3, with the acceptance criteria by different sources [9,18,19].

Table 3. System suitability testing of optimum conditions.

Parameter	EH *	Brx *	Limits
Retention factor	0.33	1.05	-
Resolution	9.94	9.94	$R \geq 2$
Tailing factor	1.62	1.41	$0.8 < Tf < 2$
Theoretical plates	7149.7	9933	$N \geq 2000$
Injection precision (Area)-%	0.2	0.4	%RSD ≤ 2
Retention time (min)	6.30	9.76	-

* Results obtained as mean of five replicates in the same working standard.

Specificity expresses the capability of the method to distinguish the analytes of interest among the other components of ointment and being unaffected by the presence of them. Test was performed by comparing the chromatograms of blank matrix and spiked sample after extraction, working standard solution and diluent. In chromatograms of blank matrix and diluent, no significant peaks were observed at the respective retention times (Figure 4A) and resolution between API and IS was ≥2 (Figure 4B).

With regard to peak purity, Figure 5 provides data on peak purity of EH and Brx.

In addition, no significant variation of retention times and Areas (%RSD ≤ 2) was observed between spiked sample and working standard solution. These data demonstrated that that excipients did not interfere with the API and IS peaks, indicating specificity of the method.

The linearity of an analytical method can be explained as its capability to show results that are directly proportional to the concentration of the analyte in the sample.

As described in Section 2.6.3, five standard solutions were prepared and three injections of each concentration level were made to determine the linearity of the response of the system. Calibration curve for EH was plotted and the results of the regression analysis are presented in Table 4. The correlation coefficient r = 0.9996 met the requirements (>0.9990) and demonstrated a significant correlation between the concentration and response. Finally, the evaluation of the residual plot confirmed the absence of outliers, indicating the goodness of fit of linear model to data.

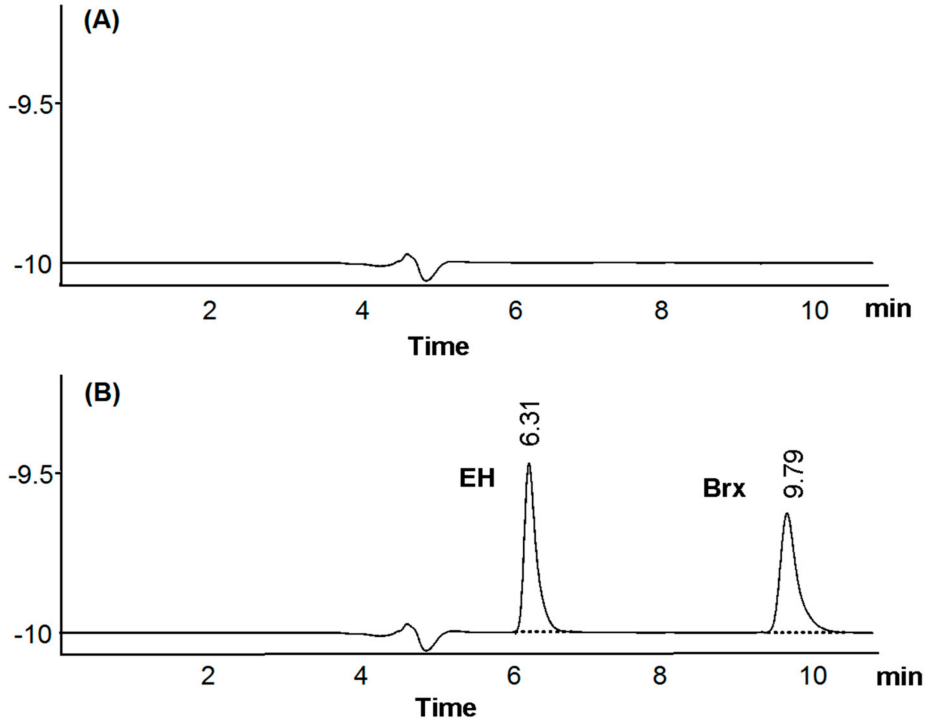


Figure 4. HPLC chromatogram of (**A**) blank matrix of ointment and (**B**) ointment sample.

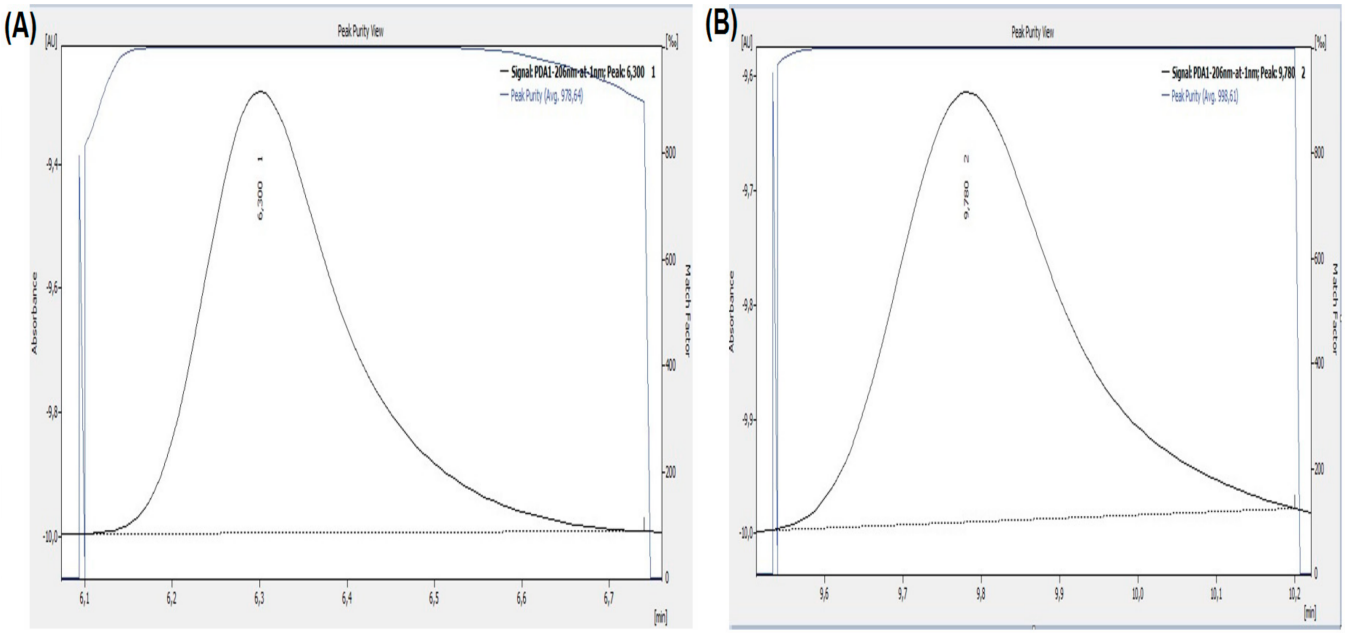


Figure 5. Peak purity data of (**A**) EH peak at 6.30 min (Average = 978.64) and (**B**) Brx peak at 9.78 min (Average = 998.61).

Table 4. Validation results of HPLC method for quantitation of EH in ointment.

Validation Parameter	Results	Criteria
Linearity (*n* = 3) (y:Area EH/Area Brx, x:EH concentration)	y = 14.093x + 0.008 r = 0.9996	r > 0.9990
Specificity, Peak Purity	EH: 978.69 Brx: 998.61	≅ 1000
Accuracy (*n* = 3)		
	75.0 µg/mL (75%) Recovery (%): 101.97	X = 100 ± 2%
	100 µg/mL (100%) Recovery (%): 101.81	X = 100 ± 2%
	125 µg/mL (125%) Recovery (%): 101.06	X = 100 ± 2%
	Average Recovery: 101.62	X = 100 ± 2%
	$RSD_{3\ levels}$ (%): 0.44	%X < 2
Precision		
Repeatability (*n* = 6)	100 µg/mL (100%) RSDr (%): 1.35	%X < 2
Intermediate Precision	100 µg/mL (100%) RSDip (%): 1.2	%X < 3
(*n* = 18, different analyst & day)	HorRat: 0.43 *	X < 2
Spiked sample stability		
25 °C (after 48 h)	Recovery (%): 99.69	X = 100 ± 2%
5 °C (after 48 h)	Recovery (%): 99.50	X = 100 ± 2%

* HorRat: Horwitz ratio.

The accuracy of an analytical method is the closeness of the test results to the true value. Recoveries (%R) of extracted spiked samples (made by adding known amounts of EH and IS in blank matrix) were calculated and the results are presented in Table 5. Recoveries values met the requirements (98% ≤ %R ≤ 102% for each level and $\%RSD_{total} \leq 2$) and demonstrated that the method was accurate within the range.

Table 5. Results obtained after conduction of experiments.

Run	Response								
	Area EH	**Area Brx**	**T_f EH**	**T_f Brx**	**R_s**	**N_{EH}**	**N_{Brx}**	**t_R EH (min)**	**t_R Brx (min)**
1	6749.4	4272.62	1.20	1.45	9.76	4365	8439	5.45	8.90
2	7702.26	6095.67	1.60	1.58	9.71	7165	9339	6.23	9.58
3	6840.6	5244.46	1.53	1.54	8.12	7665	8263	5.17	7.46
4	7606.06	6096.18	1.60	1.56	9.95	7195	8901	6.24	9.62
5	9412.09	7051.03	1.59	1.50	9.51	8029	10,369	7.30	10.96
6	9244.43	6382.82	1.61	1.58	8.87	7812	10,644	7.26	10.52
7	6568.75	4629.22	1.52	1.51	7.48	6470	6969	5.24	7.56
8	6663.99	5185.93	1.52	1.50	7.83	6404	7413	5.21	7.56
9	9367	7162.84	1.56	1.55	12.39	8412	10,604	7.79	12.83
10	6660.39	5042.12	1.39	1.40	10.65	5324	7682	5.58	9.43
11	9053.91	6437.70	1.52	1.54	8.31	6937	8405	7.31	10.64
12	9194.87	6025	1.52	1.46	12.94	8571	10,389	7.86	13.28
13	9222.29	6902.07	1.43	1.45	11.04	6438	9130	7.72	12.72
14	9181.06	4574.15	1.42	1.57	10.95	6690	9451	5.56	9.087
15	9187.26	7328.10	1.39	1.57	8.84	7116	8633	7.40	11.05
16	7613.12	6070.78	1.57	1.56	9.82	7180	9430	6.24	9.62
17	9009.74	6293.74	1.38	1.49	11.14	5521	8609	7.78	13.14
18	6764.02	5272.55	1.52	1.53	11.45	7215	8650	5.53	9.22
19	8291.28	4580.49	1.48	1.56	8.46	7053	8254	5.23	7.72

Precision of a method presents the degree of agreement among individual test results when the procedure is applied repeatedly to multiple samples. Initially, repeatability data met the requirements (RSDr ≤ 2, r: repeatability) after assay of six different extracted spiked samples. Regarding Intermediate Precision (data from 3 days), requirements were fulfilled (grand%RSD ≤ 2) and results obtained by one way Anova test were very

close to grand%RSD of 18 determinations. Last, Horwitz ratio (calculated by formula $\%RSD_{HorRat} = 2^{*}C^{-0,15}$) presented an acceptable value for the method precision. All precision results are summarized in Table 4.

With regards to stability of the solutions, for reference standard solution at 100% level concentration, results of %R and %RSD met the requirements ($\%RSD \leq 2$, $98 \leq \%R \leq 102$) only in the case of storage at 5 °C for 5 h, indicating the storage condition in order to avoid errors of results. On the other hand, spiked samples remain stable for 48 h at 5 °C and 25 °C, respectively. Stability results are shown in Table 4.

3.4. Robustness Testing Results

The 19 experiments described in Table 2 were conducted and the obtained responses are presented in Table 5.

Then the calculation of the factor effects on the considered responses is performed either by a graphical or/and statistical interpretations [20–23]. Initially, in the statistical approach, the effect E_x of a factor X on a response Y was calculated via Equation (2):

$$E_x = \frac{\sum Y(+1)}{n} - \frac{\sum Y(-1)}{n} \quad (2)$$

where $\Sigma Y(+1)$ and $\Sigma Y(-1)$ represent the sum of the responses when factor X is set at (+1) and (−1) level, respectively and n is equal to 2^{k-3}, where k is the number of the examined parameters. The normalized effect of factor X, $E_x(\%)$, was also calculated:

$$E_x(\%) = \frac{Ex}{\overline{Y}} \times 100\% \quad (3)$$

where $\overline{Y}$ is the average nominal result. The results are presented in Table 6.

Table 6. The normalized effect $E_x(\%)$ on the responses.

Factors	$E_x(\%)$								
	Area EH	**Area Brx**	**T_f EH**	**T_f Brx**	**Rs**	**N_E**	**N_{Brx}**	**t_R EH**	**t_R Brx**
A	−26.1%	−30.4%	−3.3%	−0.5%	−10.6%	−13.3%	−15.8%	−35.0%	−36.7%
B	1.18%	0.41%	3.16%	0.40%	−1.52%	−1.50%	0.77%	−0.38%	−0.72%
C	−8.5%	−0.4%	−6.9%	−3.0%	−9.7%	−22.4%	−15.4%	−0.03%	−0.1%
D	2.02%	−0.12%	0.03%	1.18%	−4.09%	−1.00%	3.79%	−0.90%	−3.44%
E	−1.45%	2.7%	5.8%	3.2%	−29.1%	8.6%	−5.4%	−6.3%	−19.7%
F	−5.2%	12.3%	2.1%	−0.8%	2.4%	5.5%	−0.6%	0.0%	0.5%
G	−1.40%	1.18%	−1.98%	1.99%	−0.34%	−3.88%	0.80%	−0.18%	−0.25%

Then the critical effect that will lead to the determination of the significant factors was calculated through the replicate experiments of the nominal values. The *t*-test statistic is utilized, as shown below:

$$t = \frac{|Ex|}{(SE)e} \leftrightarrow t_{critical} \quad (4)$$

where $(SE)e$ is the standard error of an effect and $t_{crtical}$ is a tabulated value that depends on the number of degrees of freedom (d.f.) and determined at a significance level a = 0.05. The t-value based on the effect of the factor E_x (experimental value) and on the standard error (SEe), is then compared to $t_{crtical}$ (theoretical value). The effects with *t*-value greater than or equal to $t_{crtical}$ are characterized significant. Equation (4) was rewritten in such a way that $E_{critical}$ (critical effect) was used instead of a t-value. All effects in absolute value that were larger than or equal to this $E_{critical}$ ($|Ex| > Ecritical$) were characterized as ''significant'':

$$|Ex| \leftrightarrow E_{critical} = t_{critical}\,(SE)_e \quad (5)$$

$(SE)_e$ was estimated as follows:

$$(SE)_e = \sqrt{\frac{4s^2}{N}} \quad (6)$$

with N being the number of experiments performed at each factor level. For 2 degrees of freedom (d.f. = n − 1, where n = 3 is the number of replicates at nominal level) and significant level a = 0.05 then $t_{crtical}$ = 4.303. As a result, the Equation (5) was rewritten as follows:

$$|Ex| \leftrightarrow E_{critical} = 4.303 \times (SE)_e$$

or normalized to $\overline{Y}$

$$|\%Ex| \leftrightarrow \%E_{critical} = \frac{E_{critical}}{\overline{Y}} \times 100\% \quad (7)$$

According to equations above the calculated values are presented in Table 7.

Table 7. The calculation of $E_{critical}$ and $\%E_{critical}$ values for each response.

	Area EH	Area Brx	T_f EH	T_f Brx	Rs	N_{EH}	N_{Brx}	t_R EH	t_R Brx
$\overline{Y}$	7640.48	6087.54	1.59	1.57	9.83	7180.00	9223.33	6.24	9.61
SD	53.62	14.52	0.014	0.013	0.12	15.00	282.83	0.0040	0.025
S^2	2875.04	210.82	0.00021	0.00018	0.015	225.00	79,994.33	0.000016	0.00064
(SE)e	26.81	7.26	0.0072	0.0066	0.060	7.50	141.42	0.0020	0.013
$E_{critical}$	115.36	31.24	0.031	0.029	0.26	32.27	608.51	0.0087	0.055
$\%E_{critical}$	1.51%	0.51%	1.95%	1.82%	2.64%	0.45%	6.60%	0.14%	0.57%

On the other hand, a graphical interpretation of the estimated effects may be used to present, easily, their significance on the responses [21]. The graphical interpretation consists of Pareto charts, normal probability and half normal probability plots obtained from Design Expert software. The normal probability plot presents the expected values of effects from a normal distribution, and the half normal plot provides the absolute values of estimated effects, derived from a normal distribution. In these plots, the nonsignificant effects are found on a straight line through zero, while the significant effects deviate from it. In addition, a normal plot can provide information about the kind of effects of factors on responses. With regard to Pareto charts, factors that exceed the Bonferroni limit have a significant effect on the response, factors above t-value and bellow Bonferroni limit are potentially significant and the ones that do not exceed t-value are non-significant.

The next step was to present all factors that significantly influence the responses after statistical and graphical analysis and compare the results between two approaches. The results are presented in Table 8.

Table 8. Summarization of significant (√) and non-significant (-) effect of the factors A-G on each response, after (a) statistical and (b) graphical interpretation of the estimated effects.

	Area EH		Area Brx		T_f EH		T_f Brx		Rs		N_E		N_{Brx}		t_R EH		t_R Brx	
	a	b	a	b	a	b	a	b	a	b	a	b	A	b	a	b	a	b
A	√	√	√	√	√	-	-	-	√	√	√	√	√	√	√	√	√	√
B	-	-	-	-	√	-	-	-	-	-	√	-	-	-	√	-	√	-
C	√	-	-	-	√	√	√	√	√	√	√	√	√	√	-	-	-	-
D	√	-	-	-	-	-	-	-	√	√	√	-	-	-	√	-	√	√
E	-	-	√	√	√	√	√	√	√	√	√	√	-	√	√	√	√	√
F	√	-	√	√	√	-	-	-	-	-	√	-	-	-	-	-	-	-
G	-	-	√	-	√	-	√	-	-	-	√	-	-	-	√	-	-	-

As presented in Table 8, differences and similarities appear between the two approaches. More factors appeared to have a significant effect on the responses according to the statistical approach. This is meaningful, as mathematical equations perform a more accurate and strict study of factors' influence than graphical approach.

The last step was to set restrictions on the levels of the factors that were recognized as statistically significant [24]. When factor X has a significant effect, the initial interval is reduced and the non-significant limits are estimated as follows:

$$\left[X_{(0)} - \frac{\left| X_{(+1)} - X_{(-1)} \right| \times E_{critical}}{2 \times |E_X|} \, , \, X_{(0)} + \frac{\left| X_{(+1)} - X_{(-1)} \right| \times E_{critical}}{2 \times |E_X|} \right] \quad (8)$$

where $X_{(0)}$, $X_{(+1)}$ and $X_{(-1)}$ are the values of factors at levels 0, +1 and −1, respectively. For example, the effect of factors A, C, D and F on response Area EH were found as statistically significant. The non-significant limits are shown in Table 9. Similarly, limits were estimated for all factors with significant influence on responses.

Table 9. Non-significant limits for factors A, C, D and F that influence the response Area EH.

Significant Factors	Non-Significance Limits
A	[0.59, 0.61]
C	[49.56, 50.24]
D	[23.50, 26.50]
F	[205.71, 206.29]

To sum up, as observed after performing the robustness testing, it is clear that factors A (flow rate) and D (column temperature) had a significant influence on the majority of responses, except of the tailing factor of IS peak. In some cases, the limits that factors A and D should be restricted are very short in order to succeed a robust method. However, in a calibrated HPLC system it is normal to expect that such parameters as flow rate and column temperature are maintained stable during analysis without affecting the robustness of the method. The same holds true for factor E (%MeOH) that influences a lot of responses, but since its mixture with the aqueous part of the mobile phase is performed by the HPLC system, the robustness of the method can be ensured in this case, too. Finally, factors such as B (pH), C (Csalt) and G (%ET_3N), which are involved in the preparation of the mobile phase, affect many responses, as well. This means that the analyst must pay special attention during the preparation of the mobile phase (salt weighing, pH adjustment and addition of triethylamine), in order to reduce the random errors and ensure the method robustness.

4. Conclusions

A simple, precise, robust and cost–effective reverse phase ion pair chromatographic method was developed for the determination of Ephedrine HCL in a topical ointment dosage form, in association with simple and reliable sample preparation. The new HPLC method was successfully validated according to ICH guidelines and proved to be suitable for its purpose (fitness for purpose). The validation part included robustness testing that was conducted via a fractional factorial experimental design. This way the factors that should be carefully controlled can be properly identified. The analytical procedure was successfully applied for the routine analysis of ointments formulation containing ephedrine HCL in Greek Military Pharmaceutical Laboratories and offers advantages in terms of fast chromatographic analysis.

To the best of our knowledge, this is the first validated HPLC method in references that can assay Ephedrine HCL in nasal ointment in a simple and fast way. Moreover, the addition of ion pair reagent (SDS) in mobile phase proved to be notably useful for the analysis of polar analytes. The addition of internal standard during sample preparation, provided a reproducible and robust method, avoiding errors during the process. Finally,

due to the short time of sample pretreatment and assay, this method can be very useful for the extraction and analysis of hydrochloric salts of basic compounds in nasal ointments.

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