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Article

# Gradient HPLC–DAD Determination of Two Pharmaceutical Mixtures Containing the Antihistaminic Drug Ebastine

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**This work describes the development, validation and application of a simple and reliable high-performance liquid chromatography–diode array detection (HPLC–DAD) procedure for the analysis of two pharmaceutical mixtures. The first mixture contains the antihistaminic drug ebastine (EBS) and the famous sympathomimetic drug pseudoephedrine hydrochloride (PSD), and the second mixture is composed of EBS and another sympathomimetic agent, phenylephrine hydrochloride (PHR). Effective chromatographic separation of EBS, PSD and PHR was achieved using a Zorbax SB-C8 (4.6 × 250 mm, 5 μm) column with gradient elution of the mobile phase composed of 0.05M phosphoric acid and acetonitrile. The gradient elution started with 20% (by volume) acetonitrile, ramped up linearly to 90% in 5 min, then kept constant until the end of the run. The mobile phase was pumped at a flow rate of 1 mL/min. The multiple wavelength detector was set at 254 (for EBS and PSD) and 274 nm (for PHR) and quantification of the analytes was based on measuring their peak areas. The retention times for PHR, PSD and EBS were approximately 2.5, 2.9 and 7.1 min, respectively. The reliability and analytical performance of the proposed HPLC procedure were statistically validated with respect to linearity, ranges, precision, accuracy, selectivity, robustness and detection and quantification limits. Calibration curves were linear in the ranges 5–100, 100–1,000 and 10–200 μg/mL for EBS, PSD and PHR, respectively, with correlation coefficients > 0.9996. The validated HPLC method was applied to the analysis of the two pharmaceutical mixtures in laboratory-made tablets in which the analytes were successfully quantified with good recovery values and no interfering peaks were encountered from the inactive ingredients. Finally, the proposed method made use of DAD as a tool for peak identity and purity confirmation.**

## Introduction

Ebastine (EBS) (Figure 1), chemically known as 1-[4-(1,1-dimethylethyl)phenyl]-4-[4-(diphenylmethoxy)piperidin-1-yl]butane-1-one (1), is a non-sedating antihistamine drug. It is given for the symptomatic relief of allergic conditions, including rhinitis, and in pruritic skin disorders (2). The British Pharmacopoeia (BP) describes a non-aqueous titration procedure with potentiometric detection for the assay of EBS (1). Alternatively, other methods have been described in the literature for the determination of EBS in its pharmaceutical dosage forms or in biological samples. Examples of these methods are partial least-squares spectrophotometric analysis (3), spectrofluorimetry (4), high-performance thin-layer chromatography (HPTLC) (5), high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection (6–8) and liquid chromatography–tandem mass spectrometry (LC–MS-MS) for the determination of EBS and its metabolites in human plasma (9, 10).

Pseudoephedrine hydrochloride (PSD) (Figure 1), chemically known as (1S,2S)-2-(methylamino)-1-phenylpropan-1-ol hydrochloride (1), is a direct-acting and indirect-acting sympathomimetic drug. It is given orally for the symptomatic relief of nasal congestion. It is commonly combined with other ingredients in preparations intended for the relief of cough and cold symptoms (2). The PSD monograph in the BP describes a potentiometric titration procedure for the assay of the bulk powder, and HPLC for PSD oral solution and tablets (1). On the other hand, HPLC is predominant in the United States Pharmacopeia (USP) monographs of PSD powder, as well as its single and multi-ingredient formulations (11). The quantification of PSD in various drug formulations and/or biological samples has been addressed in many reports. Analytical methodology in these reports involved the use of potentiometric membrane sensors (12, 13), chemometric spectrophotometry (14, 15), derivative spectrophotometry (15, 16), HPTLC (17), HPLC with UV detection (18, 19), gas chromatography (GC) with flame ionization detection (20), GC–MS (21), capillary electrophoresis with UV detection (22, 23), capillary electrophoresis with laser-induced fluorescence detection (24) and micro-emulsion electrokinetic chromatography (25).

Phenylephrine hydrochloride (PHR) (Figure 1), chemically known as (1R)-1-(3-hydroxyphenyl)-2-(methylamino)ethanol hydrochloride (1), is a sympathomimetic with primarily direct effects on alpha-adrenergic receptors. Similar to PSD, PHR is most commonly used for the symptomatic relief of nasal congestion, and it is frequently included in preparations intended for the relief of cough and cold symptoms (2). PHR is an official drug in most international pharmacopoeias. The BP suggests a potentiometric titration with 0.1M ethanolic sodium hydroxide for the assay of the bulk form, while HPLC and spectrophotometric $A_{max}$ methods have been proposed for PHR eye drops and injections, respectively (1). The USP recommends a bromometric titration method for the assay of PHR, whereas several HPLC methods have been applied for PHR dosage forms (11). Moreover, PHR was assayed by a wide variety of analytical techniques, including the use of an electrochemical sensor (26), anodic voltammetry on a modified glassy carbon electrode (27), spectrophotometry (28, 29), derivative spectrophotometry (28), chemometric spectrophotometry (30, 31), flow-injection spectrophotometry (32, 33), flow injection analysis with chemiluminescence detection (34), capillary electrophoresis (35, 36),

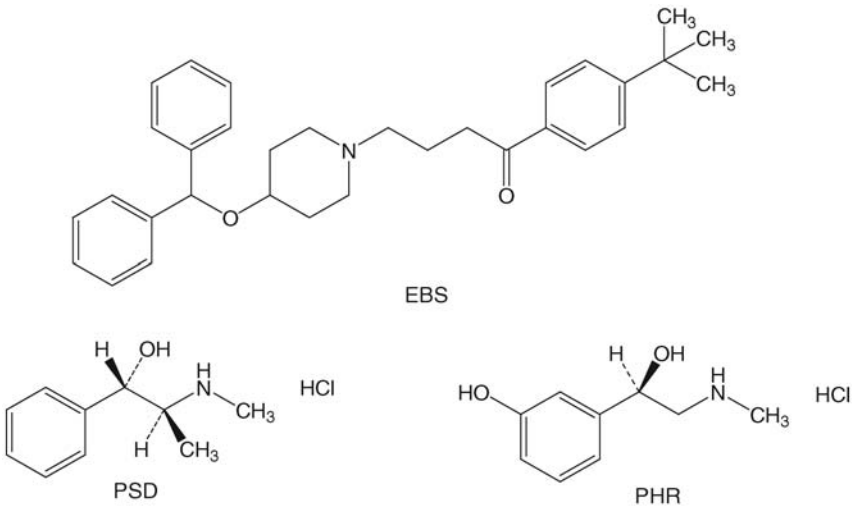


**Figure 1.** Chemical structures of EBS, PSD and PHR.

micellar electrokinetic capillary chromatography (37) and HPTLC (38). In addition, liquid chromatography using various detection modes has been widely applied. Examples of these reports are HPLC with UV detection (39, 40), HPLC with fluorescence detection (41), HPLC with electrochemical detection (42) and LC–MS-MS (43).

EBS is co-formulated with sympathomimetic compounds such as PSD or PHR for the symptomatic relief of common cold (2, 44, 45). To the best of our knowledge, no reports have been published yet dealing with the assay of the two pharmaceutical mixtures (EBS–PSD and EBS–PHR). The fact that up till now the analysis of these drug combinations has not been tackled yet encouraged us to develop a simple and reliable HPLC–diode array detection (DAD) procedure for quality control purposes.

## Experimental

### *Instrumentation*

The HPLC–DAD system consisted of an Agilent 1200 series (Agilent Technologies; Santa Clara, CA) (quaternary pump, vacuum degasser and diode array and multiple wavelength detector G1315 C/D and G1365 C/D) connected to a computer loaded with Agilent ChemStation Software. A Rheodyne manual injector with 20-μL loop was used. The column used was a Zorbax SB-C8 (4.6 × 250 mm, 5 μm particle size) (Agilent).

### *Materials*

EBS was supplied by Mulji Mehta Pharma (Mumbai, India). PSD and PHR were donated by Pharco Pharmaceuticals Co. (Alexandria, Egypt). HPLC-grade acetonitrile (Scharlau Chemie S.A.; Sentmenat, Spain), HPLC-grade methanol (Sigma–Aldrich Chemie GmbH; Buchs, Switzerland), analytical grade orthophosphoric acid and high-purity distilled water were used. Inactive ingredients used in the preparation of tablets (maize starch, microcrystalline cellulose "Avicel," magnesium stearate, hydroxypropylmethylcellulose (HPMC) and colloidal silica "Aerosil") were obtained from Pharco Pharmaceuticals Co.

### *General procedure*

A gradient mobile phase system consisting of 0.05M phosphoric acid (A) and acetonitrile (B) was used. The separation was achieved with a linear gradient program as follows: 20% v/v B at zero time; from 0 to 5 min, ramp up to 90% v/v B; from 5 to 10 min, hold 90% v/v B. After 10 min, the gradient program was returned to the initial conditions and the analytical column was reconditioned for 3 min. The flow rate was 1.0 mL/min. The injection volume was 20 μL. The eluant was monitored by the diode array detector from 190 to 400 nm, and chromatograms were extracted at 254 and 274 nm. All determinations were performed at 25°C.

EBS stock solution (1,000 μg/mL), PSD stock solution (2,000 μg/mL) and PHR stock solution (1,000 μg/mL) were prepared in acetonitrile–methanol (90:10, v/v). The working solutions were prepared by dilution of the stock solutions with HPLC-grade acetonitrile to reach the concentration ranges 5–100 μg/mL, 100–1,000 μg/mL and 10–200 μg/mL for EBS, PSD and PHR, respectively. Triplicate injections were made for each concentration and chromatographed under the previously described LC conditions. The peak areas were plotted against the corresponding concentrations to construct the calibration graphs.

### *Assay of the pharmaceutical dosage form*

For the EBS–PSD mixture, a total of 10 tablets (laboratory-made tablets containing 10 mg EBS and 120 mg PSD per tablet, in addition to maize starch, microcrystalline cellulose, magnesium stearate, HPMC and colloidal silica as tablet fillers) were weighed and finely powdered. To an accurately weighed quantity of the powder equivalent to the average weight per tablet, 5 mL methanol followed by 25 mL acetonitrile were added, stirred for 20 min then filtered into a 50-mL volumetric flask. The residue was washed with two 5-mL portions of acetonitrile and washings were added to the filtrate and diluted to volume with acetonitrile. Aliquots of the tablet solution were diluted with acetonitrile to obtain final concentrations within the

previously mentioned concentration ranges, and then chromatographed using the conditions mentioned earlier.

For the EBS–PHR mixture, a total of 10 tablets (laboratory-made tablets containing 10 mg EBS and 10 mg PHR per tablet, in addition to the same tablet additives) were weighed and finely powdered, and then an accurately weighed quantity of the powder equivalent to the average weight per tablet was treated as explained in the previous paragraph.

## Results and Discussion

### *Optimization of chromatographic conditions*

A gradient liquid chromatographic method coupled with diode array detection was developed to provide a suitable procedure for the routine quality control analysis of mixtures of EBS–PSD and EBS–PHR. The most important aspect in LC method development is the achievement of sufficient resolution with acceptable peak symmetry in a reasonable analysis time. To achieve this goal, several experiments were carried out to optimize both the stationary and mobile phases. For optimization of the stationary phase, several reversed-phase columns [Zorbax SB-C8 (4.6 × 250 mm), Zorbax SB-C18 (4.6 × 250 mm), Zorbax Eclipse XDB-C18 (4.6 × 150 mm) and Waters Symmetry C18 (3.9 × 150 mm)] were tested. The Zorbax SB-C8 column was found to be optimum because it provided the best resolution between the two structurally related drugs, PSD and PHR; hence, it became the column of choice for this study. Several mobile phases were evaluated using various proportions of different aqueous phases and organic modifiers. The best mobile phase combination was 0.05M phosphoric acid solution and acetonitrile. Methanol was tried as an organic modifier, and it resulted in a broad and delayed EBS peak. EBS has a tribenzenoid structure, which is expected to show a strong affinity to the reversed-phase columns, therefore, acetonitrile was found suitable as organic modifier to overcome the strong affinity of the drug to the C8 surface. Further trials revealed that acetonitrile in a high ratio (> 80% by volume) was an important factor that led to the elution of EBS in a reasonable retention time with acceptable peak asymmetry. On the other hand, using a mobile phase with a high proportion of acetonitrile in an isocratic mode resulted in a complication for the two shortly-eluting PHR and PSD peaks. Insufficient retention and obvious co-elution between the two peaks were observed. To overcome these problems and to ensure complete resolution between PSD and PHR, gradient elution starting with low percentage of acetonitrile was applied. Several gradient programs were tried and the best compromise between adequate resolution, reasonable retention times and acceptable peak shapes was achieved using a gradient system starting with 20% (by volume) acetonitrile ramped up linearly to 90% in 5 min, then kept at this percentage thereafter. The flow rate was kept constant at 1.0 mL/min throughout the run.

The multiple wavelength detector offers the advantage of measuring each analyte at its maximum wavelength, thus improving sensitivity. In addition, diode array detection enhances the power of HPLC and is an elegant option for assessing method specificity by comparison of recorded spectra during peak elution. Quantification was achieved based on peak area measurement. EBS shows an absorption spectrum with a maximum at 254 nm. PSD is well known as a weak UV-absorbing compound; however, it shows an adequate absorption at 254 nm, therefore, 254 nm was found to be suitable for the quantification of both compounds. PHR was measured at 274 nm, which corresponds to a well-defined absorption peak.

Figure 2 shows a typical chromatogram for the separation of the three analytes. PHR, PSD and EBS eluted at retention times $2.46 \pm 0.012$, $2.91 \pm 0.013$ and $7.14 \pm 0.023$ min, respectively. Resolution ($R_s$) is a measure of the degree of separation between adjacent peaks. A value of 1.5 for resolution implies a complete separation of any two consecutive peaks (1). Resolution was calculated between the two adjacent peaks of PHR and PSD and found to be 2.37, which revealed an adequate baseline separation. Column performance (apparent efficiency) can be expressed by the number of theoretical plates ($N$), which equals approximately 5,580, 3,510 and 17,970 for PHR, PSD and EBS, respectively.

### *Analytical performance of the proposed method*

#### *Linearity and concentration ranges*

The linearity of the proposed HPLC procedure was evaluated by analyzing a series of different concentrations for each compound. The linear regression equations were generated by least-squares treatment of the calibration data. Under the optimized conditions described previously, the measured peak areas were found to be proportional to concentrations of the analytes. Table I presents the performance data and statistical parameters, including linear regression equations, concentration ranges, correlation coefficients, standard deviations of the intercept ($S_a$), slope ($S_b$) and standard deviations of residuals ($S_{y/x}$). Regression analysis shows good linearity, as indicated by the correlation coefficient values (>0.9996). In addition, deviation around the slope can be further evaluated by calculation of the percentage relative standard deviation (RSD%) of the slope ($S_b$%), which were found to be less than 1.3 %. The analysis of variance test for the regression lines reveals that, for equal degrees of freedom, an increase in the variance ratio (F values) means an increase in the mean of squares due to regression and a decrease in the mean of squares due to residuals. The greater the mean of squares due to regression, the steeper the regression line. The smaller the mean of squares due to residuals, the less the scatter of experimental points around the regression line. Consequently, regression lines with high F values (low significance F) are much better than those with lower ones. Good regression lines show high values for both r and F statistical parameters (46).

#### *Detection and quantification limits*

According to the pharmacopoeial recommendations (1, 11), the limit of detection (LOD) is defined as the concentration that has a signal-to-noise ratio of 3:1, while for limit of quantification (LOQ), the ratio under consideration is 10:1. The LOD and LOQ values for the studied analytes were calculated and presented in Table I.

#### *Precision and accuracy*

The within-day (intra-day) precision and accuracy for the proposed method were studied at three concentration levels for

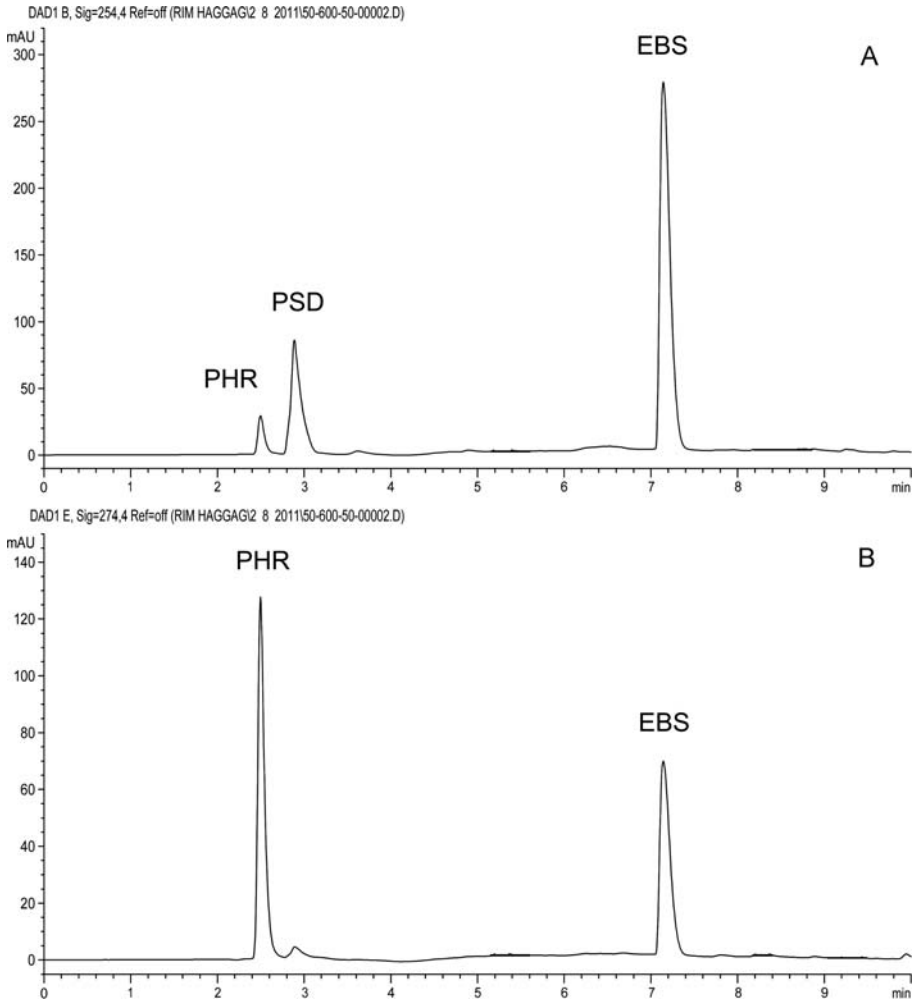


**Figure 2.** HPLC chromatograms of 20 μL injection of 50 μg/mL PHR, 600 μg/mL PSD and 50 μg/mL EBS mixture at 254 nm (A) and 274 nm (B).

**Table I**
Regression and Statistical Parameters for the Determination of the Three Analytes using the Proposed HPLC–DAD Method

| Parameter | EBS | PSD | PHR |
|---|---|---|---|
| Wavelength (nm) | 254 | 254 | 274 |
| Concentration range (μg/mL) | 5–100 | 100–1,000 | 10–200 |
| Intercept (a) | −8.46 | −2.27 | −5.49 |
| $S_a$ | 24.26 | 9.47 | 10.70 |
| Slope (b) | 49.79 | 1.313 | 13.96 |
| $S_b$ | 0.43 | 0.016 | 0.18 |
| RSD% of the slope ($S_b$%) | 0.86 | 1.22 | 1.29 |
| Correlation coefficient (r) | 0.99981 | 0.99972 | 0.99968 |
| $S_{y/x}$ | 38.47 | 12.17 | 13.74 |
| F | 13,320 | 7,077 | 6,280 |
| Significance F | $9.26 \times 10^{-10}$ | $1.20 \times 10^{-7}$ | $1.52 \times 10^{-7}$ |
| LOD (μg/mL) | 0.29 | 6.05 | 0.76 |
| LOQ (μg/mL) | 0.97 | 20.15 | 2.53 |

each compound using three replicate determinations for each concentration within one day. Similarly, the between-day (inter-day) precision and accuracy were tested by analyzing the same three concentrations for each compound using three replicate determinations repeated on three days. Recoveries were calculated using the corresponding regression equations, which were satisfactory. The RSD% and percentage relative error ($E_r$%) did not exceed 1.5%, proving the high repeatability and accuracy of the developed method for the estimation of the analytes in their bulk form (Table II).

*Selectivity*

Method selectivity was examined by preparing several laboratory-prepared mixtures of EBS–PSD and EBS–PHR at various concentrations within the linearity ranges mentioned in Table I. The laboratory-prepared mixtures were analyzed according to the previously described procedure. The analysis results were satisfactory, including RSD% and the $E_r$% values shown in Table III, thus validating the selectivity, precision and accuracy of the developed method and demonstrating its capability to resolve and quantify the analytes in different ratios. Also, selectivity of the proposed HPLC–DAD method can be assessed by the system suitability parameters like retention time ($t_R$) and $R_s$, which were found to be stable during the course of the study.

*Robustness*

Robustness was examined by evaluating the influence of small variations in different conditions such as concentration of phosphoric acid solution ($\pm$ 0.005M), ratio of acetonitrile in

**Table II**
Precision and Accuracy for the Determination of EBS, PSD and PHR in Bulk Form using the Proposed HPLC–DAD Method

| Analyte | Nominal value (μg/mL) | Within-day | | | Between-day | | |
|---|---|---|---|---|---|---|---|
| | | Found ± SD* (μg/mL) | RSD (%) | $E_r$ (%) | Found ± SD (μg/mL) | RSD (%) | $E_r$ (%) |
| EBS | 20 | 19.86 ± 0.13 | 0.66 | −0.70 | 19.79 ± 0.21 | 1.06 | −1.05 |
| | 40 | 40.06 ± 0.30 | 0.75 | 0.15 | 40.36 ± 0.51 | 1.26 | 0.90 |
| | 80 | 79.17 ± 0.51 | 0.64 | −1.04 | 79.86 ± 0.89 | 1.12 | −0.17 |
| PSD | 200 | 198.06 ± 2.02 | 1.02 | −0.97 | 197.32 ± 3.20 | 1.62 | −1.34 |
| | 400 | 404.96 ± 4.28 | 1.06 | 1.24 | 404.60 ± 5.16 | 1.28 | 1.15 |
| | 800 | 788.48 ± 7.04 | 0.89 | −1.44 | 793.92 ± 11.96 | 1.51 | −0.76 |
| PHR | 20 | 19.75 ± 0.18 | 0.91 | −1.25 | 19.81 ± 0.29 | 1.46 | −0.95 |
| | 40 | 39.94 ± 0.41 | 1.03 | −0.15 | 39.77 ± 0.55 | 1.38 | −0.57 |
| | 100 | 99.38 ± 0.77 | 0.78 | −0.62 | 99.13 ± 1.23 | 1.24 | −0.87 |

*Mean ± standard deviation for three determinations.

**Table III**
Determination of EBS–PSD and EBS–PHR Laboratory-Prepared Mixtures using the Proposed HPLC-DAD Method

| Nominal value (μg/mL) | | Found ± SD* (μg/mL) | | RSD (%) | | $E_r$ (%) | |
|---|---|---|---|---|---|---|---|
| EBS | PSD | EBS | PSD | EBS | PSD | EBS | PSD |
| 40 | 600 | 39.73 ± 0.37 | 601.74 ± 8.22 | 0.93 | 1.37 | −0.67 | 0.29 |
| 40 | 480 | 39.91 ± 0.45 | 483.96 ± 4.70 | 1.13 | 0.97 | −0.22 | 0.83 |
| 40 | 400 | 39.32 ± 0.35 | 403.76 ± 3.08 | 0.89 | 0.76 | −1.70 | 0.94 |
| 40 | 200 | 39.96 ± 0.51 | 199.12 ± 2.34 | 1.28 | 1.19 | −0.10 | −0.44 |
| 100 | 100 | 100.87 ± 1.21 | 101.83 ± 1.77 | 1.20 | 1.74 | 0.87 | 1.83 |
| EBS | PHR | EBS | PHR | EBS | PHR | EBS | PHR |
| 20 | 80 | 20.34 ± 0.24 | 80.38 ± 0.59 | 1.18 | 0.73 | 1.70 | 0.48 |
| 40 | 80 | 39.80 ± 0.35 | 78.56 ± 1.06 | 0.88 | 1.35 | −0.50 | −1.80 |
| 80 | 80 | 81.05 ± 0.98 | 79.87 ± 0.74 | 1.21 | 0.93 | 1.31 | −0.16 |
| 80 | 40 | 81.26 ± 0.54 | 40.55 ± 0.42 | 0.67 | 1.04 | 1.58 | 1.38 |
| 80 | 20 | 80.75 ± 0.74 | 19.93 ± 0.28 | 0.92 | 1.41 | 0.94 | −0.35 |

*Mean ± standard deviation for five determinations.

**Table IV**
Application of the Proposed HPLC–DAD Method to the Analysis of EBS–PSD and EBS–PHR Laboratory-Prepared Tablets

| | External standard | | Standard addition | |
|---|---|---|---|---|
| | EBS | PSD | EBS | PSD |
| %Recovery ± SD* | 100.70 ± 0.78 | 99.48 ± 1.29 | 100.21 ± 1.17 | 99.01 ± 1.15 |
| RSD% | 0.78 | 1.30 | 1.17 | 1.16 |
| | EBS | PHR | EBS | PHR |
| %Recovery ± SD | 100.88 ± 1.05 | 98.82 ± 1.14 | 99.52 ± 0.86 | 99.32 ± 1.07 |
| RSD% | 1.04 | 1.15 | 0.86 | 1.08 |

*Mean ± standard deviation for five determinations.

the gradient program (± 2% v/v), source of acetonitrile (Scharlau Chemie S.A., Spain; SDS, France; or Labscan, Poland), working wavelengths (± 3 nm) and flow rate (± 0.1 mL/min). These variations did not have any significant effect on the measured responses or the chromatographic resolution. RSD% for the measured peak areas using these variations did not exceed 3%.

### *Stability of solutions*

The stability of standard working solutions and sample solutions in the diluting solvent (HPLC-grade acetonitrile) was examined and no chromatographic changes were observed within 24 h at room temperature. Also, the stock solutions prepared in acetonitrile–methanol (90:10) were stable for at least two weeks when stored refrigerated at 4°C. Retention times and peak areas of the drugs remained unchanged and no significant degradation was observed during these periods.

### *Analysis of pharmaceutical dosage form*

Due to the unavailability of the commercial tablets in the Egyptian market, laboratory-made tablets were prepared and analyzed by the proposed HPLC–DAD method. The active ingredients were extracted with the same solvent used for the preparation of the standard stock solutions (acetonitrile–methanol 90:10), then dilution was made with HPLC-grade acetonitrile to reach concentration levels within the specified ranges. The active ingredients eluted at their specific retention times. No interfering peaks were observed from any of the inactive ingredients of the assayed tablets. The diode-array detection enables peak purity verification where no signs of co-elution from any of the inactive components were detected. Recoveries were calculated using both external standard and standard addition methods. The assay results revealed satisfactory accuracy and precision, as indicated from percent recovery, SD and RSD% values (Table IV). It is evident from these results that the proposed method is applicable to the assay of both fixed dose combinations with satisfactory levels of selectivity, accuracy and precision.

## Conclusions

In this study, a validated simple and reliable HPLC–DAD procedure was described for the assay of two new drug combinations indicated for the treatment of common cold and allergic rhinitis symptoms. To our present knowledge, no attempts have yet been made to assay these new drug mixtures by any analytical methodology. The analytes (ebastine, pseudoephedrine and phenylephrine) were successfully resolved and quantified using a RP-C8 column in a relatively short run time (less than 8 min); consequently, the described method can be considered cost and time-effective. The developed method made use of the diode-array detector as a tool for peak purity confirmation and multiple wavelength detection; however, the method can be adapted to conventional HPLC with UV detection, which is more popular in quality control laboratories. Reliability was guaranteed by testing various validation parameters of the method and successful application to laboratory-made tablets. Finally, the developed method was found accurate and precise; hence, it can be recommended for the routine quality control of the studied drugs, either in bulk form or in their combined tablet formulations.

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