Simultaneous Determination of Carbinoxamine, Pholcodine, and Ephedrine in Antitussive Preparation by High-Performance Liquid Chromatography and Thin-Layer Chromatography–Densitometry

Azza Aziz Moustafa, Hesham Salem, Maha Hegazy, and **Omnia A. Mahmoud***

Key Words

Carbinoxamine
Pholcodine
Ephedrine
Reversed-phase high-performance liquid chromatography
Thin-layer chromatography–densitometry

Summary

Simple, accurate, precise, sensitive, and validated high-performance liquid chromatography (HPLC) and thin-layer chromatography (TLC)–densitometric methods were developed for the simultaneous determination of carbinoxamine (CAR), pholcodine (PHL), and ephedrine (EPH) in antitussive syrup. In method A, reversed-phase (RP)-HPLC analysis was performed on an Inertsil CN-3 column (250 mm × 4.6 mm, 5 μm), using a mobile phase consisting of acetonitrile–distilled water (pH 3.5) using orthophosphoric acid in the ratio 70:30 (*v*/*v*) and flow rate of 1.5 mL min^{-1}. Quantitation was achieved with ultraviolet (UV) detection at 220 nm. In method B, TLC analysis was carried out on an aluminum-backed sheet of silica gel 60 F_{254} layer using chloroform–propanol–ammonia (6:4:0.1, *v*/*v*) as the mobile. Quantification was carried out with UV detection at 245 nm. The validation of the proposed methods was applied according to the International Conference on Harmonization (ICH) guidelines. The suggested methods were successfully applied for the determination of the cited drugs in bulk powder and commercial dosage form.

1 Introduction

The pharmaceutical trend for cough treatment is to use oral liquid medication that contains two or three active ingredients acting synergistically to give the most appropriate clinical effect as antitussive, decongestant, and antihistaminic [1]. In oral formulations, carbinoxamine (CAR), 2-[(4-chlorophenyl)-pyridin-2-yl-methoxy]-*N*,*N*-dimethyl-ethanamine, is an antihistamine with anticholinergic and sedative properties used for the relief of allergic conditions such as rhinitis and is a common ingredient of compound preparations for symptomatic treatment of coughs and common cold [2]. Pholcodine (PHL), 7,8-didehydro-4,5α-epoxy-17-methyl-3-[2-(morpholin-4-yl)ethoxy] morphinan-6α-ol, is a centrally acting cough suppressant that has actions and uses similar to those of dextromethorphan. It helps to suppress unproductive coughs and also has a mild sedative effect but has little or no analgesic effects [2]. Ephedrine (EPH), (1R,2S)-2-(methylamino)-1-phenylpropan-1-ol, is a sympathomimetic amine commonly used as decongestant and bronchodilator. It works by reducing swelling, constricting blood vessels in the nasal passages, and widening the lung airways, allowing easier breathing [2]. The chemical structures of these drugs are shown in **Figure 1.** The combination of CAR, PHL, and EPH is indicated for the relief of nonproductive cough and upper respiratory symptoms associated with allergy and common cold.

Literature survey reveals that CAR, PHL, and EPH can be determined either alone or in combination with other drugs by several methods. Numerous analytical methods were developed for the assay of CAR as spectrophotometric methods [3–6], high-performance liquid chromatography (HPLC) [7–10], capillary elec-

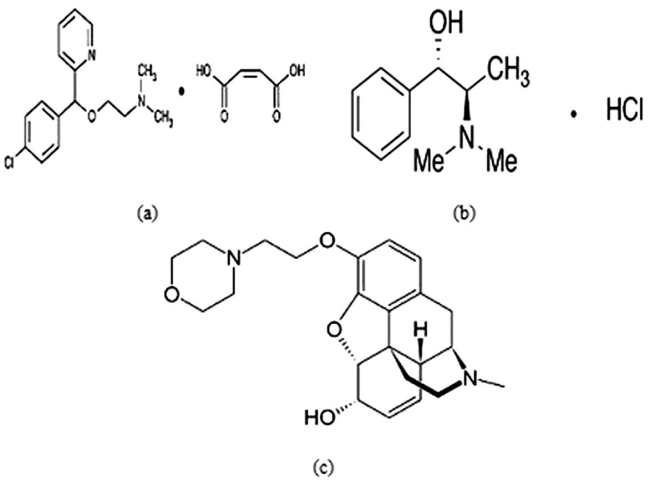

Figure 1

The chemical structures of (a) CAR, (b) EPH, and (c) PHL.

A.A. Moustafa and M. Hegazy, Analytical Chemistry Department, Faculty of Pharmacy, Cairo University, Kasr-El Aini Street, 11562 Cairo, Egypt; H. Salem, Analytical Chemistry Department, Faculty of Pharmacy, Deraya University, 14511 Cairo, Egypt; and O.A. Mahmoud, Analytical Chemistry Department, Faculty of Pharmacy, October University for Modern Sciences and Arts (MSA), 11787 6th October City, Egypt.
E-mail: dr.omniali@gmail.com

trophoresis [11], gas chromatography [12, 13], and liquid chromatographic method with mass spectrometric detection [14]; for PHL, HPLC [15–20], capillary electrophoresis [21], gas chromatography [22, 23], and liquid chromatographic method with mass spectrometric detection [24]; for EPH, spectrophotometric methods [25–27], HPLC [28–30], capillary electrophoresis [31–33], gas chromatography [34, 35], and liquid chromatographic method with mass spectrometric detection [35–37].

While the only method reported for the determination of ternary mixture was HPLC method [38], no TLC–densitometric methods have been reported for the analysis of this ternary mixture.

The aim of this work was to develop and validate RP-HPLC and TLC–densitometric methods for resolving and quantitation of the ternary mixture. The methods were simple, accurate, precise, and robust. The developed methods were compared to reference and reported methods, showing their advantages.

2 Experimental

2.1 Apparatus and Software

2.1.1 HPLC System

Agilent 1100 series chromatographic system equipped with quaternary pump, microvacuum degasser, thermostatted column compartment, and variable wavelength ultraviolet–visible (UV–vis) detector was used. Sample injections were made through an Agilent 1100 series autosampler. Data collection and processing were performed using Agilent ChemStation software, version A.10.01. An Inertsil CN-3 column (250 mm × 4.6 mm, 5 µm) was from Agilent Technologies (Polo Alto, CA, USA). A Jenway 3505 pH-meter (Jenway, UK), equipped with combined glass electrode, was used for pH adjustment.

2.1.2 TLC–Densitometry System

CAMAG TLC Scanner 3 S/N 130319 operated with winCATS software, Linomat 5 autosampler (CAMAG, Muttenz, Switzerland), CAMAG microsyringe (100 µL), and TLC aluminum sheets (20 × 20 cm) precoated with silica gel 60 F_{254} (Merck, Darmstadt, Germany) were used.

Calculations were performed using the Excel program.

2.2 Materials

2.2.1 Samples

2.2.1.1 Pure Sample

CAR, PHL, and EPH were kindly supplied by Amoun Pharmaceutical Co., El-Obour city, Cairo, Egypt. Their purities were found to be 101.01 ± 0.83 for CAR by reported method [38], 99.68 ± 1.04 and 100.37 ± 0.15 for PHL and EPH, respectively, by official methods [39].

2.2.1.2 Pharmaceutical Dosage Form

Cyrinol® syrup (batch No. 135275) was manufactured by Amoun Pharmaceutical Co. (El-Obour city, Cairo, Egypt) and purchased from a local market. Each 5 mL syrup contains 2 mg CAR, 4 mg PHL, and 7 mg EPH.

2.2.2 Chemicals

Propanol, 33% ammonia solution, and orthophosphoric acid (85%) used were from Adwic-El Nasr Pharmaceutical Chemicals Co. (Egypt); chloroform was from Lobachemie (India); and acetonitrile (HPLC grade) was from LabScan Limited (Dublin, Ireland). The water used was of distilled grade.

2.3 Standard Solutions

2.3.1 For HPLC

Stock standard solutions of CAR, PHL, and EPH were prepared using distilled water of concentration 1 mg mL^{-1}. Working standard solutions were freshly prepared by dilution from the stock standard solutions with the mobile phase to obtain a concentration of 250 µg mL^{-1} for CAR and 400 µg mL^{-1} for EPH and PHL.

2.3.2 For TLC

Stock standard solutions of concentration 2 mg mL^{-1} for CAR and PHL and 10 mg mL^{-1} for EPH were prepared using distilled water as the solvent. Working standard solutions were freshly prepared by dilution from the stock standard solutions with the mobile phase to obtain a concentration of 1 mg mL^{-1} for CAR and PHL and 5 mg mL^{-1} for EPH.

2.4 Procedure

2.4.1 Chromatographic Conditions

2.4.1.1 RP-HPLC Method

RP-HPLC was carried out at ambient temperature on an Inertsil CN-3 column (250 mm × 4.6 mm, 5 µm). The mobile phase consisted of acetonitrile–distilled water (pH 3.5) using orthophosphoric acid in the ratio 70:30 (*v*/*v*). The mobile phases were filtered using 0.45 µm Millipore membrane filter (Billerica, MA) and delivered at a rate of 1.5 mL min^{-1}. The injection volumes were 20 µL, and the detections were done at 220 nm.

2.4.1.2 TLC–Densitometric Method

TLC aluminum sheets 20 × 20 cm precoated with 0.25 mm silica gel 60 F_{254} were used. The samples were applied to the TLC plate as bands (band width: 6 mm; bands were spaced 1 cm apart from each other and 1 cm from the bottom edge of the plate). The applied volume per band was 10 µL using a 100-µL syringe. The developing system was chloroform–propanol–ammonia (6:4:0.1, *v*/*v*). Linear ascending development was done in a chromatographic tank previously saturated with the developing system at room temperature for 1 h, to a distance of approximately 8 cm from the lower edge. The developed plates were air-dried and scanned at 254 nm.

Detection was done using CAMAG TLC Scanner 3 operated in the absorbance mode, with deuterium lamp as the source of radiation; the slit dimension was kept at 3 mm × 0.45 mm, and 20 mm s^{-1} scanning speed was employed.

2.4.2 System Suitability

2.4.2.1 RP-HPLC Method

Twenty microliters of the working standard solutions were injected and chromatographic conditions were applied. The system suitability parameters including retention time (t_R), tailing

Table 1

Statistical analysis of parameters required for system suitability of HPLC and TLC–densitometric methods.

Parameter	RP-HPLC method			TLC–densitometric method			Reference value [40]
	PHL	EPH	CAR	EPH	PHL	CAR	
t_R (RP-HPLC) R_F (TLC)	2.623	4.291	7.343	0.12 ± 0.01	0.3 ± 0.01	0.42 ± 0.01	$t_R > 1$ (HPLC)
N (column efficiency)	8467	12,457	5650				$N > 2000$ Increases with efficiency of the separation
HETP (height equivalent to theoretical plates)	0.029 cm^{-1}	0.020 cm^{-1}	0.044 cm^{-1}				The smaller the value, the higher the column efficiency
Selectivity factor (α)	1.79	1.81		3.14	1.69		$\alpha > 1$
T (tailing factor)	0.95	0.95	1	0.72	0.71	0.94	$T < 2$ $T = 1$ for symmetric peak
R_s (resolution)	3.34	6.78		3.61	2.19		Rs > 2

factor (T), theoretical plate count (N), height equivalent to theoretical plate (HETP), and resolution were calculated according to the United States Pharmacopeia (USP) guidelines [40] listed in **Table 1.**

2.4.2.2 TLC–Densitometric Method

Parameters including retention factor (R_F), selectivity factor (α), tailing factor (T), and resolution (Rs) were calculated for both mixtures according to USP guidelines [40], listed in Table 1.

2.4.3 Construction of Calibration Curves

2.4.3.1 RP-HPLC Method

Separate aliquots were transferred from the working standard solution of each drug to prepare solutions of different concentrations. The corresponding chromatographic conditions were applied for these solutions, and the chromatograms were recorded. The calibration curve of each drug was constructed by plotting the relative peak area (the peak area found to that of a standard of the same drug) against the corresponding concentration, from which the regression equations were calculated. The calibration curves for CAR, PHL, and EPH were constructed using the standards 50, 80, and 120 μg mL^{-1} of each drug, respectively. The calibration curves were constructed in the range of 5–100 μg mL^{-1} for CAR, 12–200 μg mL^{-1} for PHL, and 20–200 μg mL^{-1} for EPH, using the average of three experiments.

2.4.3.2 TLC–Densitometric Method

Separate aliquots were accurately transferred from the working standard solutions of each drug to prepare solutions of different concentrations. The corresponding chromatographic conditions were applied, and the chromatograms were recorded. The calibration curves were constructed by plotting the relative peak area against the corresponding concentration, from which the regression equations were calculated. The calibration curves were constructed using the standards (3 μg mL^{-1} for CAR, 5 μg mL^{-1} for PHL, and 35 μg mL^{-1} for EPH). Calibration curves were constructed in the range of 0.5–9 μg $band^{-1}$ for CAR, 1–10 μg $band^{-1}$ for PHL, and 5–45 μg $band^{-1}$ for EPH, using the average of three experiments.

2.4.4 Assay of Laboratory-Prepared Mixtures

Different aliquots of the drugs were accurately transferred from their working standard solutions and mixed to prepare solutions of different ratios. The chromatographic conditions of both methods were adopted for each laboratory-prepared mixture, and the concentrations of each drug were calculated from the corresponding regression equation. Each concentration was conducted from the average of three experiments.

2.4.5 Application to Pharmaceutical Dosage Form

2.4.5.1 Cyrinol® Syrup

Five milliliters of Cyrinol® syrup were accurately transferred to a 100-mL separating funnel and diluted by 20 mL distilled water. To the solutions in the separating funnels, 5 mL 2 N sodium hydroxide to render the solution alkaline was added. The alkaline solutions, in the separating funnels, were extracted by shaking with three 15 mL portions of chloroform. Under vacuum using Rota-Vap, the chloroform extracts were evaporated to dryness [38]. The residue left was dissolved in 10 mL volumetric flask; the volume was completed with distilled water to get 200 μg mL^{-1} for CAR, 400 μg mL^{-1} for PHL, and 700 μg mL^{-1} for EPH.

For HPLC method, an appropriate dilution was made with the mobile phase to prepare the working standard solution to obtain a solution of 80 μg mL^{-1} for CAR, 160 μg mL^{-1} for PHL, and 280 μg mL^{-1} for EPH. For TLC–densitometric method, an appropriate dilution was made with distilled water to prepare the working standard solution to obtain a solution of 160 μg mL^{-1} for CAR, 320 μg mL^{-1} for PHL, and 560 μg mL^{-1} for EPH.

The prepared working standard solutions of dosage form were filtered through 0.45 μm Millipore syringe membrane filter. The corresponding chromatographic conditions were applied for each working standard solution. Six replicates of each experiment were done. The concentration of each drug was calculated from its corresponding regression equation. The standard addition technique was applied by adding different known concentrations of pure standard drugs to the pharmaceutical formulation before proceeding in the previously mentioned methods.

3 Results and Discussion

The UV absorption spectra of CAR, PHL, and EPH display a considerable overlap **(Figure 2).** RP-HPLC and TLC–densitometric methods were successfully applied for the determination of ternary mixture. These methods could effectively separate CAR, PHL, and EPH in their laboratory mixtures and have been successively applied to the pharmaceutical formulation without the interference from the excipients.

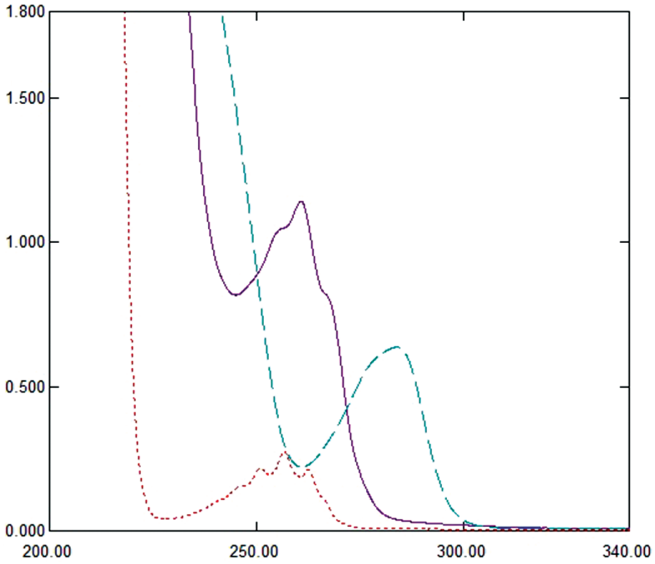

Figure 2

Zero order UV spectra of 80 µg mL^{-1} of CAR (·····), 160 µg mL^{-1} of PHL (—), and 280 µg mL^{-1} EPH (- - -).

3.1 RP-HPLC Method

The development of an HPLC method for the simultaneous determination of CAR, PHL, and EPH in antitussive syrup is a great challenge because of the great differences in the polarity of the components. Also, the selectivity needed for separating each compound from the other and from the excipients, such as dyes, preservatives, and sweeteners (parabens, sodium saccharine, and others) is considered a great challenge.

The extraction of the active substances, using chloroform from alkaline solution, was found to be essential to overcome the coelution of some excipients with the eluting analytes. Recoveries of the analytes from the extracted samples were determined by comparing the peak areas of solutions obtained from directly

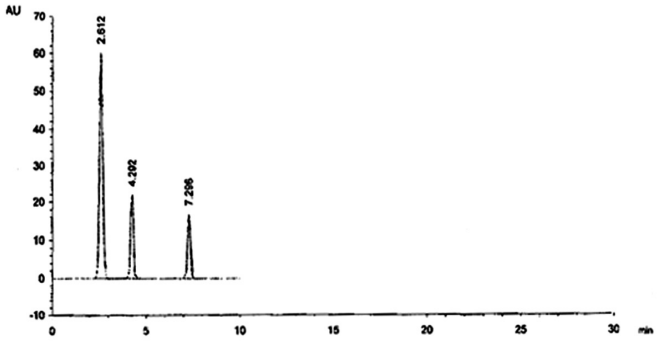

Figure 3

RP-HPLC chromatogram of 120 µg mL^{-1} PHL (t_R = 2.612), 90 µg mL^{-1} EPH (t_R = 4.292), and 50 µg mL^{-1} CAR (t_R = 7.296), using Inertsil CN-3 column and mobile phase of acetonitrile–distilled water (pH 3.5) in ratio of 70:30, *v*/*v*, and flow rate of 1.5 mL min^{-1} at 220 nm.

Table 2

Assay parameters and validation sheet obtained by applying the RP-HPLC and TLC–densitometric methods to the ternary mixture.

Parameters	RP-HPLC method			TLC–densitometric method		
	CAR	PHL	EPH	CAR	PHL	EPH
Calibration range[a)]	5–100	12–200	20–200	0.5–9	1–10	5–45
Slope	0.0203	0.0138	0.0083	0.3005	0.2067	0.0244
Intercept	−0.0081	−0.0958	0.0026	0.1157	−0.0228	0.1504
Standard error of slope	1.18×10^{-4}	1.02×10^{-4}	4.58×10^{-5}	0.00167	0.0017	0.000124
Standard error of intercept	0.0068	0.0124	0.0056	0.0088	0.0113	0.0038
SD of residuals	0.00986	0.01636	0.00715	0.01260	0.01327	0.00427
Mean[b)]	100.03	100.28	99.54	100.34	99.81	100.04
RSD	0.798	1.061	0.735	1.086	1.209	0.865
LOD[a)]	1.603	3.912	2.843	0.138	0.212	0.578
LOQ[a)]	4.857	11.855	8.614	0.419	0.642	1.750
Accuracy[c)] (mean ± SD)	100.12 ± 0.874	100.02 ± 0.921	100.38 ±1.332	100.57 ± 1.052	100.73 ± 0.947	100.57 ± 1.165
Correlation coefficient (*r*)	0.9999	0.9999	0.9999	0.9999	0.9999	0.9999
Precision						
Intra-day precision[c)]	99.89 ± 0.87	100.46 ± 0.92	99.67 ± 0.87	100.75 ± 1.22	101.91 ± 1.03	100.12 ± 1.59
Inter-day precision[c)]	99.93 ± 1.07	100.35 ± 0.72	100.47 ± 0.91	101.82 ± 1.58	101.43 ± 0.97	101.73 ± 1.31
RMSEP	0.1652	0.4153	0.3471	0.0140	0.0214	0.0609
Robustness[e)]	1.32	0.83	0.73	1.33	1.12	1.11

[a)]RP-HPLC method: in µg mL^{-1}; TLC–densitometric method: in µg band^{-1}
[b)]Average of three experiments
[c)]Relative standard deviations (RSD) of three samples

Table 3

Determination of CAR, PHL, and EPH in laboratory-prepared mixture by RP-HPLC and TLC–densitometric method.

Mixture No.	RP-HPLC method						TLC–densitometric method					
	Concentration (μg mL^{-1})			Recovery[a] %			Concentration (μg mL^{-1})			Recovery[a] %		
	CAR	PHL	EPH	CAR	PHL	EPH	CAR	PHL	EPH	CAR	PHL	EPH
1[b]	20	40	70	98.27	101.40	100.82	2	4	7	101.84	100.40	101.54
2	20	20	28	99.42	99.24	101.20	3	5	35	99.10	101.05	100.46
3	15	32	40	99.64	100.46	100.78	0.5	1	10	101.96	99.30	101.77
4	60	80	100	101.80	99.79	99.83	6	3	25	99.14	99.70	100.19
5	50	120	90	100.86	101.63	99.25	2	1.5	30	98.58	101.52	99.07
Mean ± SD				100.00 ± 1.365	100.50 ± 1.023	100.38 ± 0.805				100.13 ± 1.634	100.39 ± 0.917	100.60 ± 1.094

[a]Average of three determinations
[b]Ratio of CAR, PHL, and EPH in Cyrinol® syrup

prepared samples. The extraction recoveries of all the used analytes were quantitative. It was found that the extraction process was effective without any loss.

To optimize the RP-HPLC methods, it was necessary to test the effect of different variables. Different stationary phases, detection wavelengths, and flow rates were also tested. A good resolution and linearity were obtained using an Inertsil CN-3 column and acetonitrile–distilled water (pH 3.5) using orthophosphoric acid in the ratio 70: 30 (*v*/*v*) as the mobile phase. The flow rate was kept at 1.5 mL min^{-1}, and detection was done at 220 nm **(Figure 3).**

The method was successfully applied for the simultaneous determination of CAR, PHL, and EPH in less than 8 min, permitting the quantification of the studied drugs in their combined dosage forms without prior separation.

RP-HPLC method has the advantage, over the reported method [38], of using an Inertsil CN-3 column; no buffers have been used which will save the column and increase its life time; further advantages: higher sensitivity with lower concentration range, LOD and LOQ, reduced peak tailing and sharp peaks, higher column efficiency (*N*), complete separation within 7 min compared to reported method (14 min) – this can reduce the volume of organic solvents used which is more economic in industrial scale and routine work. Thermo Scientific Inertsil CN-3 column (250 mm × 4.6 mm, 5 μm) is an excellent reversed-phase material for a wide range of applications and is one of the most popular packing materials available, being very robust with long column lifetimes with excellent reproducibility. On the other hand, ruggedness was studied in reported method [38].

System suitability parameters were calculated and are listed in Table 1. The assay parameters and validation sheet are listed in **Table 2.** The method was successfully applied to determine the selected drugs in the laboratory-prepared mixtures without any interference from each other or from excipients, after chloroform extraction; the results are shown in **Table 3.**

3.2 TLC–Densitometric Method

This method offers a simple way to quantify directly on TLC plate by measuring the optical density of the separated bands. The amounts of compounds are determined by comparison to a standard curve from reference materials chromatographed simultaneously under the same condition. Different solvent systems were tried for the separation of ternary mixture. Satisfactory results were obtained by using a mobile phase composed of chloroform–propanol–ammonia (6:4:0.1, *v*/*v*) which gave good resolution and sharp symmetrical peaks.

In order to minimize band diffusion, the optimum band width chosen was 6 mm. Different scanning wavelengths were tried; on using 254 nm, the separated peaks were more sharp and symmetrical with minimum noise. The R_F values were 0.12, 0.3, and 0.42 for CAR, PHL, and EPH, respectively.

A typical chromatogram of the three components is shown in **Figures 4a and b,** in which separation allows the determination of ternary drugs without any interference from each other or from excipients, after chloroform extraction.

TLC–densitometric method has the advantages, over the reported method [38] and the proposed RP-HPLC method, of being simpler (simple developing systems with no pH adjustments); several samples can be run simultaneously using a small quantity of the mobile phase, thus lowering analysis time, cost per analysis, and it has higher sensitivity.

3.3 Application to Pharmaceutical Dosage Form

The suggested RP-HPLC and TLC–densitometric methods were valid and applicable for the analysis of CAR, PHL, and EPH in Cyrinol® syrup. The validity of the proposed methods was further assessed by applying the standard addition technique, which showed accurate results. The results for the ternary mixture were compared with those of the official British Pharmacopoeia (BP) and reported method as shown in **Table 4.** The results confirm the suitability of the proposed methods for the routine determination of these components in their combined formulations.

3.4 Validation of the Methods

Method validation was performed according to the International Conference on Harmonization (ICH) guidelines [41] for all the proposed methods as follows:

3.4.1 Range and Linearity

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample [41].

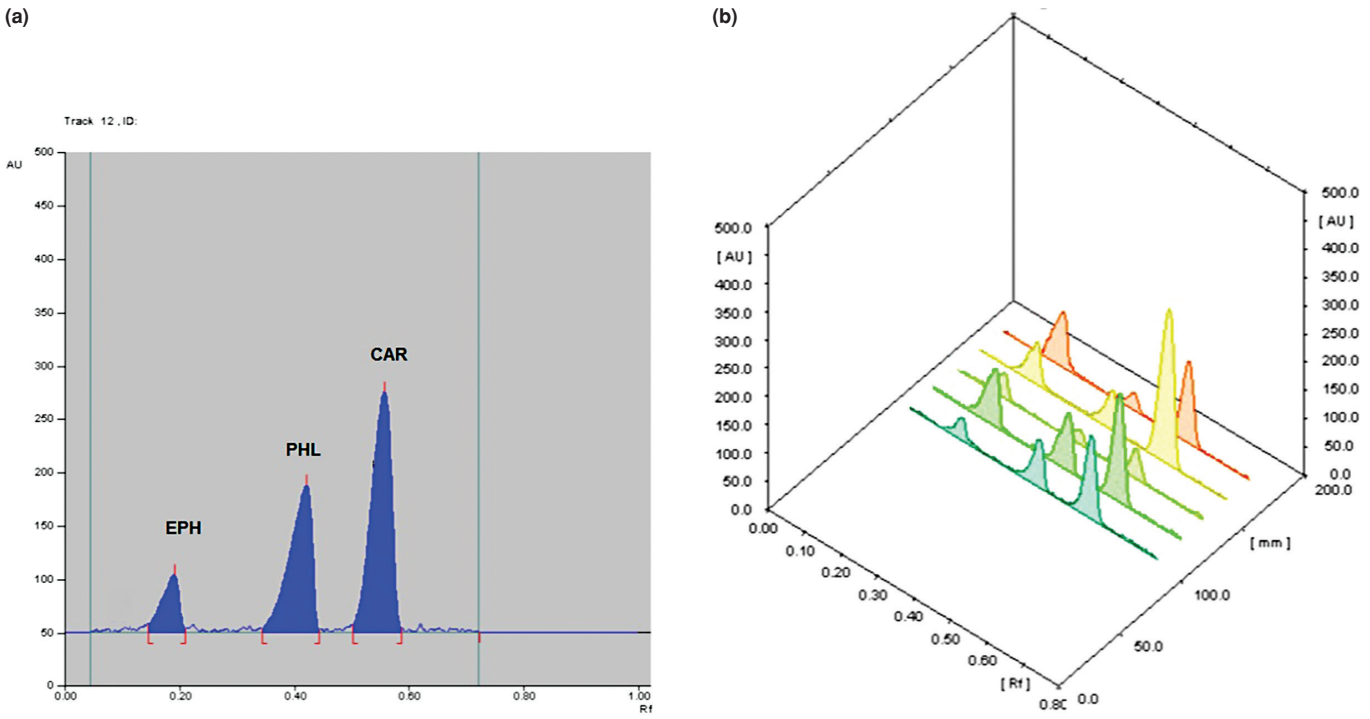

Figure 4

a) TLC chromatogram of 14 µg band^{-1}of EPH, 8 µg band^{-1} of PHL, and 4 µg band^{-1} of CAR, using chloroform–propanol–ammonia (6:4:0.1, *v*/*v*) as the developing system. b) TLC chromatograms of different laboratory-prepared mixtures of CAR, PHL, and EPH using chloroform–propanol–ammonia (6:4:0.1, *v*/*v*) as the developing system.

The estimation of linearity of the proposed methods was achieved by construction of the calibration curves. The peak area ratio (drug/standard) against the concentration in µg mL^{-1} for CAR, PHL, and EPH was obtained. In this study, six concentrations were chosen for each drug. Each concentration was repeated three times, in order to provide information on the variation in peak area values among samples of the same concentration.

The calibration range was established through consideration of the necessary practical range, according to ternary mixture concentration present in the pharmaceutical product, to give accurate, precise, and linear results. For RP-HPLC method, calibration curves were constructed in the range of 5–100 µg mL^{-1} for CAR, 12–200 µg mL^{-1} for PHL, and 20–200 µg mL^{-1} for EPH. For TLC–densitometric method, calibration curves were constructed in the range of 0.5–9 µg mL^{-1} for CAR, 1–10 µg mL^{-1} for PHL, and 5–45 µg mL^{-1} for EPH. The corresponding assay parameters and validation sheet for the proposed methods are listed in Table 2.

3.4.2 Limits of Detection and Quantification

According to the International Conference on Harmonization (ICH) [41] recommendations, the limit of detection (LOD) was determined by establishing the minimum level at which the analyte can be reliably detected. It was found to be 1.603 µg mL^{-1}, 3.912 µg mL^{-1}, and 2.843 µg mL^{-1} for CAR, PHL, and EPH, respectively, for HPLC method; while for TLC–densitometric method, it was found to be 0.138 µg band^{-1}, 0.212 µg band^{-1}, and 0.578 µg band^{-1} for CAR, PHL, and EPH, respectively. The limit of quantitation (LOQ) was determined by establishing the lowest concentration that can be measured according to ICH recommendations below which the calibration graph is non-linear. It was found to be 4.857 µg mL^{-1}, 11.855 µg mL^{-1}, and 8.614 µg mL^{-1} for CAR, PHL, and EPH, respectively, for HPLC method; while for TLC–densitometric method, it was found to be 0.419 µg band^{-1}, 0.642 µg band^{-1}, and 1.750 µg band^{-1}, for CAR, PHL, and EPH, respectively, as shown in Table 2, where:

$$\mathrm{LOD} = 3.3S_a/b,\ \mathrm{LOQ} = 10S_a/b$$

S_a is the standard deviation of the intercept of the calibration curve, and b is the slope of the calibration curve.

3.4.3 Accuracy

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found [41]. To study the accuracy of the proposed methods, procedures under linearity were repeated three times for the determination of five blind concentrations of pure CAR, PHL, and EPH. The accuracy expressed as percentage recoveries/RSD is shown in Table 2. The interference of excipients in the pharmaceutical formulations was studied by applying standard addition method to the pharmaceutical formulation. Good accuracy proved that the excipients in pharmaceutical formulations did not interfere in the analysis of these compounds, as shown in Table 4.

3.4.4 Precision

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision may be considered at three levels: repeatability, intermediate precision, and reproducibility [41].

Table 4

Application of standard addition technique to the analysis of CAR, PHL, and EPH in Cyrinol® syrup by RP-HPLC and TLC–densitometric methods compared to reported methods.

Pharmaceutical formulation	Drug	RP-HPLC method				TLC–densitometric method				Reference methods[b]
		Claimed amount taken (μg mL^{-1})	Added (μg mL^{-1})	Found (μg mL^{-1})	Recovery[a] %	Claimed amount taken (μg mL^{-1})	Added (μg mL^{-1})	Found (μg mL^{-1})	Recovery[a] %	
Cyrinol® syrup (batch No. 135275). Each 5 mL syrup contains 2 mg CAR, 4 mg PHL, and 7 mg EPH	CAR	20	10	10.09	100.85	1.6	1.4	1.43	101.97	101.39 ± 0.823
			20	19.81	99.04		1.6	1.61	100.73	
			30	30.34	101.14		1.8	1.79	99.28	
			Mean ± SD		100.34 ± 1.138		Mean ± SD		100.66 ± 1.349	
			Student's *t*-test 0.454 (2.26)[c]		*F* test 1.405 (6.26)[c]		Student's *t*-test 0.897 (2.26)[c]		*F* test 1.967 (6.26)[c]	
	PHL	40	30	29.78	99.28	3.2	2.2	2.20	100.12	100.76 ± 1.172
			40	40.47	101.17		3.2	3.17	99.11	
			50	50.87	101.74		4.2	4.25	101.08	
			Mean ± SD		100.73 ± 1.290		Mean ± SD		100.10 ± 0.983	
			Student's *t*-test 1.205 (2.26)[c]		*F* test 1.375 (6.26)[c]		Student's *t*-test 0.287 (2.26)[c]		*F* test 1.243 (6.26)[c]	
	EPH	70	60	59.37	98.94	5.6	5.2	5.21	100.16	99.69 ± 0.634
			70	69.82	99.75		5.6	5.67	101.17	
			80	80.50	100.62		6	6.12	102.05	
			Mean ± SD		99.77 ± 0.839		Mean ± SD		101.13 ± 0.946	
			Student's *t*-test 1.198 (2.26)[c]		*F* test 1.530 (6.26)[c]		Student's *t*-test 1.402 (2.26)[c]		*F* test 1.207 (6.26)[c]	

[a]Average of three experiments
[b]Reference methods: for CAR, HPLC method is reported [38], while for PHL and EPH, BP methods are to be found in the literature [39]
[c]Figures between parentheses represent the corresponding tabulated values of *t* and *F* at $P = 0.05$

The precision of the proposed methods, expressed as RSD, was determined by the analysis of three different concentrations of pure drugs within the linearity range. Intra-day precision (repeatability) was performed through replicate analysis of three concentrations of the studied drugs on three successive occasions (15, 50, and 80 μg mL^{-1} for CAR; 40, 80, and 160 μg mL^{-1} for PHL and EPH) for RP-HPLC method and (1, 5, and 7 μg band^{-1} for CAR and PHL while 15, 25, and 40 μg band^{-1} for EPH) for TLC–densitometric method. The results obtained showed small values of relative standard deviation indicating good precision within a day. Inter-day precision (intermediate precision) was carried out through replicate analysis of the same three concentrations of the studied drugs on three successive days. The relative standard deviations were found to be small indicating reasonable repeatability and intermediate precision of the proposed method. Comparing the results of the proposed methods for each mixture, it was found that RP-HPLC methods were more precise than TLC–densitometric methods (lower RSD). The results are given in Table 2. The root mean square error of prediction (RMSEP) was calculated for both chromatographic methods (Table 2). RMSEP was used as a diagnostic tool for examining the prediction errors; the small values indicate good accuracy and precision.

3.4.5 Specificity

The specificity of a method is the extent to which it can be used for analysis of a particular analyte in a mixture or matrix without interference from other components. The specificity of the proposed methods was tested by the analysis of five laboratory-prepared mixtures containing different concentrations of CAR, PHL, and EPH within the linearity range. The laboratory-prepared mixtures were analyzed according to the previous procedures described under each of the proposed methods. The specificity was demonstrated by the chromatograms recorded for mixtures of CAR, PHL, and EPH, indicating that the methods enabled highly specific analysis of the drug mixture. Well-resolved peaks for CAR, PHL, and EPH were observed (Figures 3 and 4a). Satisfactory results were obtained (Table 3), indicating the high selectivity of the proposed methods for the simultaneous determination of CAR, PHL, and EPH.

3.4.6 Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage [41].

Table 5

Statistical comparison between the results obtained by the proposed methods and reference methods for the determination of CAR, PHL, and EPH in pure powder form.

Parameters	RP-HPLC method			TLC–densitometric method			Reference methods[b]		
	CAR	PHL	EPH	CAR	PHL	EPH	CAR	PHL	EPH
Mean	100.03	100.28	99.54	100.34	99.81	100.04	100.08	99.95	100.38
RSD	0.798	1.061	0.735	1.086	1.209	0.865	0.858	0.982	0.928
Variance	0.64	2.77	0.54	1.19	1.46	0.75	0.74	0.96	0.87
No. of experiments	6	6	6	6	6	6	5	5	5
Student's *t*-test	0.094 (2.26)[a]	0.155 (2.26)[a]	1.679 (2.26)[a]	0.426 (2.26)[a]	0.186 (2.26)[a]	0.621 (2.26)[a]			
F test	1.155 (5.19)[a]	1.376 (6.26)[a]	1.612 (5.19)[a]	1.613 (6.26)[a]	1.209 (6.26)[a]	1.158 (5.19)[a]			

[a]Figures between parentheses represent the corresponding tabulated values of t and F at $P = 0.05$
[b]Reference methods: for CAR, HPLC method is reported [38], while for PHL and EPH, BP methods are to be found in the literature [39]

The robustness of the proposed methods was investigated by the analysis of samples under a variety of experimental conditions. For RP-HPLC method, small changes in the pH (3.5 ± 0.1) and small changes in proportions of acetonitrile by up to ±2% were introduced to the mobile phases. A slight change in the retention time and peak parameters was observed; however, the peak areas were conserved. For TLC–densitometric method, small changes in proportions of chloroform by up to ±2% were introduced to the developing systems. R_F values and peak symmetry were slightly changed; however, the peak areas were conserved. The effect of robustness was more observed in the TLC method (higher RSD) as listed in Table 2, which proved that RP-HPLC method was more robust upon changing the experimental conditions.

3.5 Statistical Analysis

The results obtained by the proposed methods for the determination of pure samples of CAR, PHL, and EPH were statistically compared to those obtained by the reference methods [38, 39] . The values of the calculated t and F were less than the corresponding tabulated ones, which revealed that there was no significant difference with respect to accuracy and precision between the proposed methods and the official ones as shown in **Table 5.**

4 Conclusion

By applying the RP-HPLC and TLC–densitometric methods for the analysis of CAR, PHL, and EPH, it was found that the proposed methods were simple, sensitive, accurate, precise, repeatable, specific, and robust. The methods could be applied for the analysis of the studied drugs in their synthetic mixtures and their combined dosage forms without interference from common excipients, and the results were in good agreement with comparison methods [38, 39]. The proposed RP-HPLC method had the advantage of being more precise and robust than the proposed TLC–densitometric method. It was capable of accurate determination of ternary mixture quantitatively in a wider concentration range (5–100 μg mL^{-1} for CAR, 12–200 μg mL^{-1} for PHL, and 20–200 μg mL^{-1} for EPH) compared to the TLC–densitometric method. The proposed RP-HPLC had also the advantage of separating the ternary mixture, using a simple and rapid isocratic mobile phase, without the need of precolumn derivatization or gradient elution method. The TLC–densitometric method had the advantages, over the reported and proposed RP-HPLC method, of being simpler, sensitive, and economic, as it saves cost and time, as up to 20 samples could be applied to a single plate and analyzed per one development. The developed methods are quite sensitive for the quantitative detection of CAR, PHL, and EPH in pharmaceutical preparation and can thus be used for routine analysis and in quality control.

References

[1] *K. Parfitt,* Martindale: The Complete Drug Reference, 33 edn., The Pharmaceutical Press, London, 2002.

[2] *S.C. Sweetman,* Martindale: The Complete Drug Reference, The Pharmaceutical Press, London, 2009, 1236–1237.

[3] *L. Monferrer-Pons, J. Esteve-Romero, G. Ramis-Ramos, M. Garcia-Alvarez-Coque,* Anal. Lett. **29** (1996) 1399–1413.

[4] *I. Shoukrallah,* Acta Pharm. Jugosl. **41** (1991) 107–115.

[5] *I. Shoukrallah,* Anal. Lett. **24** (1991) 2043–2058.

[6] *A. Ramadan H. Mandil,* Anal. Biochem. **353** (2006) 133–137.

[7] *J. Araujo, J. Boyer, M. Probecker,* STP Pharma Sci. **4** (1988) 598–602.

[8] *C.D.A. Bastos, M.A. de Oliveira,* Química Nova **32** (2009) 1951–1955.

[9] *I.M. Palabiyik, F. Onur,* J. Anal. Chem. **67** (2012) 56–63.

[10] *A.M. Mansour,* J. AOAC Int. **81** (1997) 958–962.

[11] *Y.F. Tang, H.L. Wu, S.M. Wu, S.H. Chen, H.S. Kou,* J. Microcolumn Sep. **12** (2000) 366–370.

[12] *D. Hoffman, M. Leveque, T. Thomson,* J. Pharm. Sci. **72** (1983) 1342–1344.

[13] *A. Ramadan, S. Antakli, I. Mahmoud,* Res. J. Aleppo Uni. **56** (2007) 97.

[14] *B.G. Bhavani, B. Gurupadayya, S. Sharfuddin,* J. App. Chem. **6** (2014) 46–51.

[15] *Z.R. Chen, D.M. Siebert, A.A. Somogyi, F. Bochner,* J. Chromatogr. B **424** (1988) 170–176.

[16] *A.T. Andresen, M. Krogh, K.E. Rasmussen,* J. Chromatogr. B **582** (1992) 123–130.

[17] *M, Johansen, F. Tønnesen, K.E. Rasmussen,* J. Chromatogr. B **573** (1992) 283–288.

[18] *A. Low, R. Taylor,* J. Chromatogr. B **663** (1995) 225–233.

[19] *A. Petkovska, H. Babunovska, M. Stefova,* Maced. J. Chem. Chem. Eng. **30** (2011) 139–150.

[20] *W.-G. Zhu, M. Li,* Chem. Bioeng. **7** (2011) 025.

[21] *J. Sheng, Q. Ping, J. Lei, H. Ju, C. Song, D. Zhang,* Anal. Lett. **45** (2012) 652–664.

[22] *M. Johansen, K.E. Rasmussen, A.S. Christophersen,* J. Chromatogr. B **532** (1990) 277–284.

[23] *C. Meadway, S. George, R. Braithwaite,* Forensic Sci. Int. **127** (2002) 131–135.

[24] *O.M. Denk, G.G. Skellern, D.G. Watson,* J. Pharm. Pharmacol. **54** (2002) 87–98.

[25] *M. El-Shahat, M. Badei, A. Daifullah,* J. Chem. Technol. Biot. **54** (1992) 175–181.

[26] *Z. Şentürk, N. Erk, S.A. Özkan, C. Akay, Ş. Cevheroğlu,* J. Pharm. Biomed. **29** (2002) 291–298.

[27] *P.C. Falcó, A.S. Cabeza, C.M. Legua,* Anal. Lett. **27** (1994) 531–547.

[28] *N. Okamura, H. Miki, T. Harada, S. Yamashita, Y. Masaoka, Y. Nakamoto, M. Tsuguma, H. Yoshitomi, A. Yagi,* J. Pharm. Biomed. **20** (1999) 363–372.

[29] *R.A. Niemann, M.L. Gay,* J. Agric. Food Chem. **51** (2003) 5630–5638.

[30] *Y.-M. Liu, S.-J. Sheu,* J. Chromatogr. A **600** (1992) 370–372.

[31] *C.L. Flurer, L.A. Lin, R. Duane Satzger, K.A. Wolnik,* J. Chromatogr. B **669** (1995) 133–139.

[32] *L. Mateus-Avois, P. Mangin, M. Saugy,* J. Chromatogr. B **791** (2003) 203–216.

[33] *K.W. Phinney, T. Ihara, L.C. Sander,* J. Chromatogr. A **1077** (2005) 90–97.

[34] *J. Betz, M. Gay, M. Mossoba, S. Adams, B. Portz,* J. AOAC Int. **80** (1996) 303–315.

[35] *E. Marchei, M. Pellegrini, R. Pacifici, P. Zuccaro, S. Pichini,* J. Pharm. Biomed. **41** (2006) 1633–1641.

[36] *R.L. Sheppard, X. Tong, J. Cai, J.D. Henion,* Anal. Chem. **67** (1995) 2054–2058.

[37] *M.L. Gay, R.A. Niemann, S.M. Musser,* J. Agric. Food Chem. **54** (2006) 285–291.

[38] *R.M. Abdallah,* Nat. Prod. Sci. **12** (2006) 55–61.

[39] British Pharmacopoeia, The Stationery Office on behalf of the Medicines and Healthcare Products Regulatory Agency (MHRA), © Crown Copyright, Vol. I & II, London, 2012.

[40] United States Pharmacopoeia Commission, United States Pharmacopeia – National Formulary, United States Pharmacopeial Inc., Rockville, MD, 2004.

[41] ICH Q2A, Note for Guidance on Validation of Analytical Methods: Definition and Terminology, International Conference on Harmonization, IFPMA, Geneva, 1994.

Ms received: December 16, 2014
Accepted: May 8, 2015