Natural Product Sciences
12(1) : 55-61 (2006)

HPLC Chromatographic Methods for Simultaneous Determination of Pholcodine and Ephedrine HCl with Other Active Ingredients in Antitussive-Antihistamine Oral Liquid Formulations

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Abstract- A description of simple, isocratic and precise reversed phase HPLC methods is given for simultaneous quantification of pholcodine and ephedrine hydrochloride together with either carbinoxamine maleate or terfenadine in antitussive-antihistaminic oral pharmaceutical formulations. Separations were carried out on X-Terra and symmetry shield C18 column (250 × 4.6 mm, 5 μm). The used isocratic elution systems were either 0.02 M KH_2PO_4-acetonitrile in the ratio of 75 : 25 and pH adjusted to 7.70 with orthophosphoric acid or sodium hydroxide, for syrup (method A), or 0.02 octanesulphonic acid sodium salt solution-acetonitrile-acetic acid in the ratio of 75 : 25 : 0.5 for suspension (method B). The elution of both mixtures was achieved with a flow rate of 1 ml/min. Detection was carried out by UV absorbance at wavelengths of 220 and 250 nm for syrup and suspension, respectively. The quantification of the components in synthetic mixtures and actual syrup and suspension were calculated using the internal standard technique with metoclopramide HCl and codeine phosphate as internal standards (IS), respectively. The methods, for both mixtures, were validated and met all the requirements for the quality control analysis recommended by FDA and ICH.

Keywords- pholcodine, ephedrine HCl, carbinoxamine maleate, terfenadine, HPLC

Introduction

The pharmaceutical trend for cough treatment is to use oral liquid medication that contain two or three active ingredients acting synergistically to give the most appropriate clinical effect as antitussive, decongestant and antihistaminic (Parfitt, 2002). In oral formulations, pholcodine (PC) (Fig. 1), an opiate alkaloid is used as a potent antitussive agent that centrally suppress cough reflex without any other significant analgesic or addictive actions on CNS (Parfitt, 2002). The antitussive activity of PC is similar to or somewhat greater than that of codeine but it has much safer profile. In contrast to codeine, PC is devoid of addiction liability in man since it is not metabolised into morphine (Findlay, 1988). Ephedrine HCl (EP) (Fig. 1), a common alkaloid of many Ephedra species, is widely used as an ingredient in antitussive formulations acting as a decongestant and also as a bronchodilator in the treatment of asthma (Parfitt, 2002). A comprehensive review for the physical and chemical properties of EP has been published (Ali, 1986).

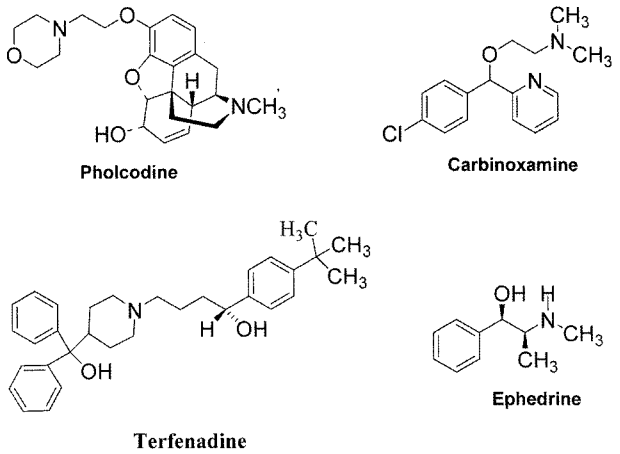

Fig. 1. Structure of pholcodine (PC); ephedrine (EP); carbinoxamine (CM) and terfenadine (TR).

Antihistamine active ingredients commonly incorporated into cough-cold preparations are carbinoxamine maleate (CM) and terfenadine (TR) (Fig. 1). TR has been considered as the first commercially available non-sedating antihistaminic agent devoid of any anti-cholinergic, -serotoninergic and -adrenergic effects (Badwan *et al* 1990; Parfitt, 2002).

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Numerous methods have been published for the quantification of the above mentioned four compounds either individually or in combination with other drugs. In compound PC oral solutions, PC with pseudoephedrine HCl and chlorpheniramine were determined by HPLC and TLC after extensive extraction (Yu and You, 2002). In cough mixtures, PC was determined simultaneously with guaiaphenesin, ephedrine and other antitussive agents by ion-pairing technique at 210 nm without previous extraction (Cao *et al.*, 1999; Lau *et al.* 1989; Carnevale, 1983). For EP determination, a non-aqueous and acidimetric titrations are described in the USP 27 (The United States Pharmacopoeia, 2004) and BP (British Pharmacopoeia, 2004), respectively. Numerous HPLC procedures employing ion-pairs or buffer systems had been reported for the assay of EP in combination with other antitussive or antihistamine in cough cold pharmaceutical dosage forms (Gasco-Lopez *et al.*, 1997; Gil-Augsti *et al.*, 2001a; Hood and Cheung, 2003; Lau and Cheung, 1990; Lau and Mok, 1995). The official analysis of CM as a bulk or in pharmaceutical dosage forms is described only in the USP (The United States Pharmacopoeia, 2004). Quantification of CM in the presence of other antihistamines in anti-cold preparation was carried out using HPLC with micellar mobile phase of sodium dodecyl sulphate-pentanol mixture (Gil-Augsti *et al.*, 2001b), using semi-polar cyano column (Gil-Augsti *et al.*, 2001c) or with ion-pairing gradient elution (Masuda *et al.*, 1997). Also, derivative spectrophotometric methods have been used for simultaneous determination of CM with phenylpropanol amine HCl (Le-Hazif *et al.*, 1996), phenylephrine HCl, or diphenhydramine (Shoukrallah, 1991). In the presence of pseudoephedrine HCl, CM was simultaneously quantified by HPLC (Mansour, 1998). In tablet forms, TR and pseudoephedrine HCl were simultaneously determined using reversed phase HPLC with (Argekar *et al.*, 1998) or without (Raman *et al.*, 2001) ion-pairing.

Two forms of compound liquid oral preparations containing PC and EP with either CM as syrup form, or TR as suspension form are commercially available. Although the above listed analytical procedures have been validated and applied in routine analysis of those drugs in combination with other medicinal agents, none of them addressed their simultaneous one step quantification. The present work describes the development of two reversed phase HPLC methods (method A and B) using simple isocratic mobile phases with simple HPLC equipment. Also, the validation work for the developed methods is described as per guidelines recommended by USP-27 monograph (USP, 2004) and ICH (ICH, 2003).

Experimental

Reagents and materials – HPLC grade acetonitrile (AcCN) was purchased from Ried-de Haen (Seelze, Germany). Analytical grade potassium dihydrogen phosphate and octanesulphonic acid sodium salt were purchased from Merck (Darmstadt, Germany). The used deionised water was obtained from in-house Millipore Milli-Q 50 ultra pure water system (Millipore, Bedford, MA, USA). Primary reference standards for the standardization of authentic powder materials to be used as working reference standards were purchased from USP or BP offices. Raw materials to prepare the secondary reference standards were provided as gifts from the pharmaceutical companies. PC, CM and EP were obtained from Apic (Amoun) Pharmaceutical Company (El-Obour City, Cairo, Egypt). TR was a gift from GlaxoWellcome-Egypt (El-Salam City, Cairo, Egypt). The obtained raw materials were all of either BP or USP quality and accompanied with their certificates of analysis. The materials were used after standardization, against the primary standards, without further purification.

Commercial pharmaceutical preparation – Cyrinol (Apic (Amoun) Pharmaceutical Company, El-Obour City, Cairo, Egypt) and Davenol (Wyeth Pharmaceutical, CA, USA) Syrups labelled to contain 4 mg PC, 7 mg EP and 2 mg CM. Marynol Suspension (Glaxo Wellcome-El Salam City, Cairo, Egypt) labelled to contain 4 mg PC, 7 mg EP and 30 mg TR. All the products were purchased from local pharmacy stores.

Chromatographic equipments – For Method A (PC, EP and CM mixture): A Waters Module I plus (Waters, Milford, MA, USA) system, equipped with auto injector, was used. The data were acquired and processed using a Millennium 2010 Chromatography Manager Software version 2.1 (Waters, Milford, MA, USA).

For Method B (PC, EP and TR mixture): An Agilant 1110 HPLC (Palo Alto, CA, USA) system was used. Injection was performed manually with a Rheodyne Model 7125 injector (Rheodyne, Cotati, CA, USA) with a fixed 20 μl sample loop. Integration was accomplished with HP 3396 automatic integrator. The integrator conditions were set as follows: Chart speed 0.5 cm/min; attenuation 4; peak width 0.04 and area rejection 2000. All the chromatographic runs were carried out at ambient temperature of 20 ± 2 °C. The pH measurements were carried out using Thermo Orion (Beverly, MA, USA) pH meter model 550A which was calibrated with two points 4 and 7.

Chromatographic conditions – The chromatographic

columns (250 × 4.6 μm i.d.) were packed with X-Terra C18 (method A) or symmetry shield C18 (method B) (both from Waters, Milford, MA, USA) with 5 μm particle size. In method A, the mobile phase consisted of 0.02 M potassium dihydrogen phosphate-acetonitrile (AcCN) in ratio of 75:25 and pH was adjusted to 7.70 with orthophosphoric acid. The flow rate was set at 1 ml/min with a typical back pressure of 1200 psi. Exactly 20 μl of the standard and sample solutions were injected to the column and UV detection of analytes was applied at 220 nm. In method B, the mobile phase consisted of 0.02 M solution of octanesulphonic acid sodium salt-acetonitrile (AcCN)-acetic acid in ratio of 75:25:0.5. The flow rate was 1 ml/min with a typical back pressure of 1650 psi; 20 μl of the standard and sample preparations were injected and UV detection was applied at 250 nm. Prior to injecting solutions, both columns were equilibrated for at least 30 min with the mobile phase flowing through the system.

System suitability – The chromatographic systems were in agreement with the following parameters, calculated from six injections of a freshly prepared solution test mixture: minimum of theoretical plates in the chromatographic columns > 2000 plates/m, calculated on the basis of the CM or TR peaks for each system; the relative standard deviation (RSD) of analytes peak areas of 1.0%; tailing factor for analytes peaks < 1.5; resolution between the adjacent peaks > 2.

Preparation of standards – For mixture A, stock solutions of EP and CM were prepared by dissolving in water at concentration of 0.16 and 0.6 mg/ml, respectively; PC stock solution was prepared by dissolving the material in methanol at concentration of 0.6 mg/ml. Stock standard solutions were pipetted into five 100 ml separating funnels followed by the addition of 5 ml of metoclopramide HCl (IS) solution in a concentration of 1 mg/ml. To the solutions in the separating funnels, 10 ml of water was added followed by 2N sodium hydroxide to render the solution alkaline. The alkaline solutions, in the separating funnels, were extracted by shaking with three 15 ml portions of chloroform. The combined chloroform extracts were passed through small beds of anhydrous calcium sulphate to 100 ml beakers. Under steady stream of nitrogen, the chloroform extracts were evaporated to dryness. The residues left in the five beakers were dissolved in the mobile phase and transferred quantitatively to 20 ml volumetric flasks. Each flask was completed to volume with the mobile phase and mixed well. For HPLC injection, portions of the samples were filtered through nylon bulk membrane filters (pore size 0.45 μm).

For mixture B. stock solution for EP and PC were prepared as mentioned for mixture A, but TR standard was dissolved in methanol to give a concentration of 100 mg/ml. A 5 ml portion of aqueous solution of codeine phosphate (10 mg/ml) was used as an internal standard (IS). The rest of the preparation procedures were followed exactly as mentioned under preparation of mixture A.

Preparation of samples – For mixture A, a 5 ml portion of commercial syrup was quantitatively pipetted and transferred to a 100 ml separating funnel. A 5 ml portion of metoclopramide HCl solution (IS) was added and the volume brought to the constant volume of 25 ml with water. The content of the separating funnel was alkalinised with 5 ml of 2N sodium hydroxide and subjected to extraction, filtration, evaporation and reconstitution processes as it was mentioned, above, in the preparation of the standards.

For mixture B, due to the high viscosity of the commercial suspension, its solution was prepared on a weight basis. Specific gravity was used to get the exact volume weighed. The same procedures mentioned in the preparation of sample for mixture A were followed but codeine phosphate as IS.

Chromatographic procedures – With the above chromatographic conditions, the standard and sample solutions were injected and the chromatograms were recorded (Fig.

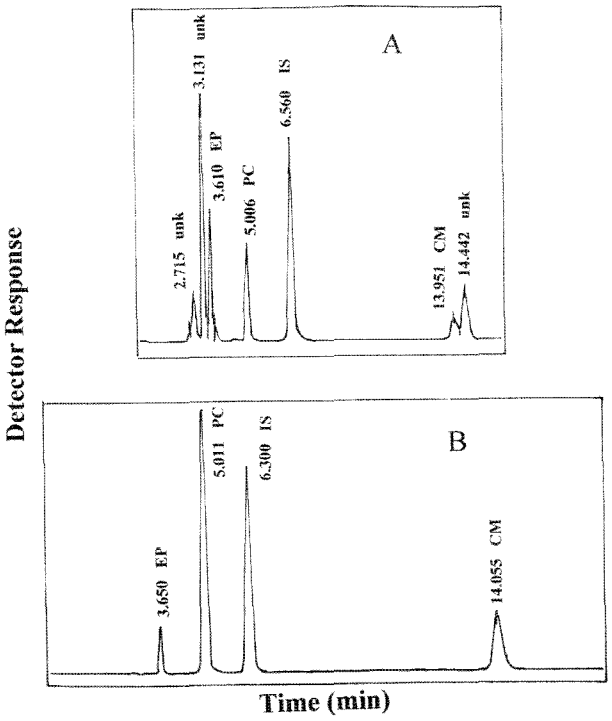

Fig. 2. A typical chromatogram of the antitussive-antihistamine syrup (A) before and (B) after chloroform extraction, [EP (3.650 min); PC (5.011 min); IS (6.300 min) and CM (14.055)].

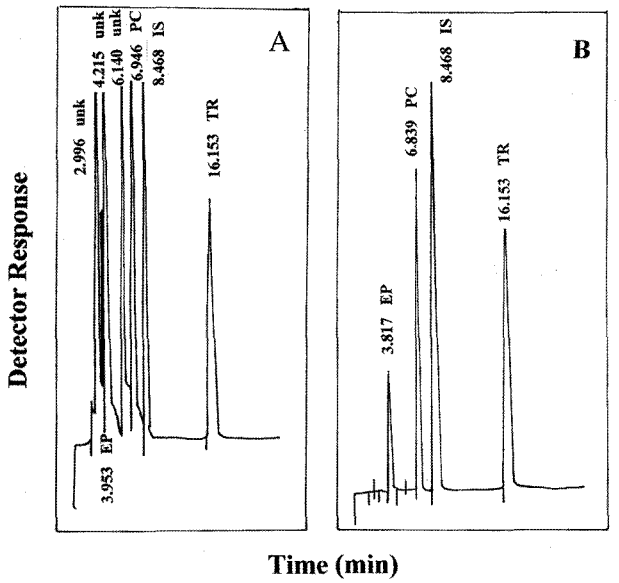

Fig. 3. A typical chromatogram of the antitussive-antihistamine suspension (A) before and (B) after chloroform extraction, [EP (3.817 min); PC (6.839 min); IS (8.468 min) and TR (16.153 min)].

2 and 3). The retention time of EP, PC, CM and metoclopramide (IS), method A, were found to be 3.650, 5.011, 14.055 and 6.300 min, respectively. In method B, the retention times were 3.817, 6.839, 16.153 and 8.468 min for EP, PC, TR and Codeine phosphate (IS), respectively. The response factors of the standard solution (peak area ratio of the standard and IS) and the sample solution were calculated and the concentrations of the drugs were calculated using the following formula:

Conc. of drug = response factor of sample/response factor of standard

× conc. of the standard

Results and Discussion

For mixture A, the mobile phase was chosen to be phosphate buffer and AcCN. At pH 4.80, the resolution between EP and PC was not adequate for quantification and working in alkaline pH, ca 8.1, showed a good separation between the early eluting analytes (EP, PC and metoclopramide, IS), but CM retention time was delayed to 34.705 min and it is not practical or appropriate for routine work. However, at pH 7.70, all the three analytes with the IS showed a good resolution between each other and reasonable retention time for fast and reliable analysis. Decreasing the concentration of AcCN (organic phase) led to a significant increase in the retention time of CM (24 min) and co-elution of the two peaks of PC and IS. The final composition of the mobile phase as 0.02M

Table 1. Performance of the HPLC separation involving the three analytes of each mixture[a]

	N	ASF	R_S	R_t (± SD)	%RSD (n = 6)
mixture A:					
EP	3280	1.02	----	3.650(0.15)	1.10
PC	3478	1.10	2.47	5.011(0.05)	0.26
IS	5497	1.05	2.01	6.300(0.20)	0.40
CM	5404	0.95	9.70	14.055(0.12)	0.82
mixture B:					
EP	2400	1.01	----	3.817(0.05)	1.05
PC	2993	1.03	3.63	6.839(0.18)	0.95
IS	3187	1.00	2.05	8.468(0.32)	1.04
TR	3450	1.03	6.50	16.153(0.24)	0.99

[a]N is the number of the theoretical plates of the columns; ASF is the asymmetry factor of the peaks; R_S is the resolution between each two consecutive peaks; R_t is the retention time with their standard deviation; %RSD is the relative standard deviation.

KH_2PO_4-AcCN (75 : 25 (v/v), pH 7.70) was a compromise between reasonable retention times, sharp peaks and good resolution.

The same principles were applied in choosing the composition of the mobile phase for the mixture of method B. The addition of octanesulphonic acid, as an ion- pairing agent, to the mobile phase was essential to considerably reduce the retention time of TR, and to improve the tailing of the peak. The other analytes (EP, PC and codeine phosphate, IS) were almost not affected. The final mobile phase composition of 5 mM octanesulphonic acid sodium salt-acetonitrile-acetic acid (75:25:0.5) and C18 symmetry bonded stationary phase provided a steady base line and the specificity required for the simultaneous quantification of EP, PC and TR in presence of codeine phosphate as IS.

To compensate for the disparity in the UV absorbance between the different components in both mixtures wavelengths of 220 and 250 nm were chosen for mixture A and B, respectively. The retention time for all the analytes in both mixtures, mentioned under the chromatographic procedures, did not vary to any considerable degree during- and in-between analysis (%RSD less than 2%). Resolution of the analytes peaks from each other was recorded in Table 1. All the resolution values (Rs) met the acceptance criteria for resolution of greater than or equal to two.

The development of an HPLC method for the simultaneous determination of PC, EP, CM or TR in antitussive-antihistamine syrups or suspensions is a great challenge because of the great differences in polarity of the components. Also, the selectivity needed for separating each compound from the other two and from the excipients, such as dyes, preservatives and sweeteners (parabens, sodium

saccharine, sodium benzoate, and others) which also absorbs strongly at the selected detection wavelengths (Fig. 2 and 3). The extraction of the active substances, using chloroform from alkaline solution, was found to be essential to overcome the co-elution of some excipients with the early eluting analyte (EP). Recoveries of the analytes from the extracted samples were determined by comparing the peak areas of solutions obtained from direct prepared samples. The extraction recoveries of all the used analytes were almost quantitative. For more assurance that the extraction process was effective without any loss, the internal standard technique was used for the quantification.

Validation of the method – Validation of HPLC methods means evaluating the performance parameters of the method, which include the system suitability, accuracy, precision, specificity or selectivity, linearity, limit of quantification and limit of detection. System suitability testing is an integral part of any HPLC procedure. The tests are based on the concept that equipments, electronics, column packaging, and detectors constitute a single system that is amenable to an overall test of system function. Reliable chromatographic performance, for example, column efficiency (N) and peak tailing (ASF) are mentioned in Table 1. For completing the system suitability testing, pump and detector performance were checked by injecting standard solution 6 replicates.

Results of linearity test give assurance that the methods are valid for their intended use throughout the specified range. The range of the methods was done by analysing mixed standard solutions containing 160 - 480 μg/ml of PC, 280 - 840 μg/ml of EP, and 80 - 240 μg/ml of CM or 1200 - 3600 μg/ml of TR (50 to 150% of targeted level of the assay concentration) containing 12 μg/ml metoclopramide HCl or 50 μg/ml of codeine phosphate, as internal standards for mixture A and B, respectively. The prepared standard mixtures were injected in triplicate and the response factors were calculated. Results were inputted into a Microsoft excel spread sheet program so calibration curves could be plotted. The intercepts, slopes and the square of the correlation coefficients for the curves are shown in Table 2. All six r^2 values are greater than 0.999, indicating their acceptability. Mixtures containing a known amount of the analytes (in both mixtures) were used for the determination of the recovery of the compounds. The quantifications were performed using the slope and intercept data of the regression analysis for each analyte (Table 4). The limit of detection (LOD) and limit of quantification (LOQ) of both methods were determined based on calculation using the standard deviation of the slopes of both calibration curves (Table 2). Precision (method repeatability) was investigated using one batch (from each dosage forms) and performing six-replicate assays (these six separate sample solutions were prepared as per procedure), each was injected twice and the response factors obtained were used to calculate the mean and %RSD values. Freshly prepared standard solutions (mixture A or B) were injected ten times, and the mean and %RSD values were calculated in order to evaluate injection (system) repeatability. The repeatability (within-run precision) was evaluated within a day, whereas the reproducibility (between-run precision) was evaluated on two separate days. The results obtained are shown in Table 3. In all instances the accepted criteria of % RSD of less than 2% was met.

Accuracy of the method was studied by recovery investigation. Placebo of syrup solution containing some syrup excipients (Gennaro, 1990) apart from all the active ingredients was used. Mixtures containing a known amount of the analytes (in both mixtures) were used for the determination of the recovery of the compounds (Table 4). Known amount of each of the active ingredients (in both mixtures) were spiked into separate 25 ml aliquots of placebo to give pseudo sample solution of approximately 80, 100 and 120% of the stated labelled strength values. These samples were then analysed according to each

Table 2. Linearity study results[a]

analyte	range (μg/ml)	intercept	slope $(\mu g/ml)^{-1}$	r^2	LOQ (μg/ml)	LOD (μg/ml)
mixture A:						
PC	160-480	3.1×10^{-2}	0.004	0.9998	18.60	6.13
EP	280-840	-3.6×10^{-5}	0.001	0.9997	3.95	1.30
CM	80-240	-9.0×10^{-3}	0.003	0.9999	6.00	1.98
mixture B:						
PC	160-480	-7.0×10^{-4}	0.003	0.9999	1.20	0.39
EP	280-840	2.0×10^{-3}	0.012	0.9998	1.20	0.66
TR	1200-3600	-8.0×10^{-3}	0.007	0.9999	26.00	8.40

[a]LOQ is the limit of quantification; LOD is the limit of detection; r^2 is the squared correlation coefficient.

Table 3. Precision results

conc.determined (μg/ml)	within run		between run	
	mean	%RSD (n = 6)	mean	%RSD (n = 12)
mixture A:				
CP	321.2	0.20	322.5	0.57
EP	562.4	0.45	560.5	0.53
CM	161.5	0.33	162.3	0.64
mixture B:				
CP	319.8	0.25	322.0	0.68
EP	558.9	0.55	559.2	0.82
TR	2405.8	0.75	2408.1	0.66

procedure and percentage recoveries were calculated, results are given in Table 4. For all the three analytes in both mixtures at different concentration levels, the recovery values were found to meet the acceptance criteria of 100 ± 2%.

The specificity or selectivity refers to the extent to which a method can determine particular analyte in a mixture or matrix without the interferences from other components. In both methods, it was tested by running solutions containing the placebo of syrups in almost same quantities and conditions that in the samples to show that there are no peaks at retention times corresponding to any of the analytes. It is clear from the chromatograms (Fig. 2 and 3) that there are no interfering peaks from the excipients, after chloroform extraction.

Ruggedness of the methods was studied by using different sources of solvents, different HPLC systems and evaluation of the stability of standards and samples over a 7 day period. The studies showed that the chromatographic patterns did not significantly change when different solvent sources were used in conjunction with different HPLC systems (interchange of both systems between the two mixtures). Stability studies of standard and sample solutions found them to be stable for at least 7 days when stored at room temperature at 20 ± 2 °C.

Table 5. Results from quantification analysis of commercial dosage forms (n = 5)

	label (mg/ml)	found % ± RSD
cyrinol syrup:		
PC	4.00	101.33 ± 0.24
EP	7.00	99.68 ± 0.09
CM	2.00	99.24 ± 0.15
davenol syrup[a]:		
PC	4.00	85.15 ± 0.35
EP	7.00	78.25 ± 0.40
CM	2.00	88.35 ± 0.18
marynol suspension:		
PC	4.00	99.25 ± 0.35
EP	7.00	98.16 ± 0.18
TR	30.00	100.26 ± 0.36

[a]The tested syrup was manufactured by John Wyeth and Brother Ltd (Havard-England); Lot no. (E) 7780 Mfd Mar 1987 and Exp Mar 1990.

The proposed HPLC procedures were applied to the quantification of the above mentioned compounds in commercial syrups and suspension. Table 5 summarized the analytical results from those commercial dosage forms. The analysis of the supplied syrup or suspension formulations manufactured by local pharmaceutical companies showed consistent percentage level strength values for all the three analytes, in each mixture, in the 90 - 110% range as per US Pharmacopoeial requirements.

Table 4. Accuracy (recovery) study results[a]

% of target concentration*	% recovery		
	mixture A: PC	EP	CM
80	100.96 (0.25)	101.92 (0.62)	101.95 (0.63)
100	100.82 (0.34)	101.93 (0.28)	99.94 (0.21)
120	99.87 (0.44)	101.26 (0.55)	100.58 (0.79)
	mixture B: PC	EP	TR
80	100.48 (0.36)	101.01 (0.26)	99.89 (0.56)
100	99.75 (0.64)	100.95 (0.36)	100.15 (0.35)
120	100.11 (0.24)	101.15 (0.23)	100.05 (0.36)

[a]100% of target concentration is equivalent to 320 μg/ml PC, 560 μg/ml EP, 200 μg/ml CM, and 2400 μg/ml TR. The numbers in brackets represent % RSD values for three replicates.

The aim of the study was to develop a method for the simultaneous quantification of pholcodine, ephedrine hydrochloride, with carbinoxamine maleate or terfenadine in oral liquid dosage forms. The developed methods allowed the quantification of the three components in each mixture using the same dilution and injection volume. At the same time, the results of the validation showed that both methods were unaffected by the assay time or using different equipments. The methods are proved to be precise, convenient and accurate.

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(Accepted February 28, 2006)