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A new hydrophilic interaction liquid chromatographic (HILIC) procedure for the simultaneous determination of pseudoephedrine hydrochloride (PSH), diphenhydramine hydrochloride (DPH) and dextromethorphan hydrobromide (DXH) in cough-cold formulations

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Abstract

A new HILIC method has been developed for the simultaneous determination of pseudoephedrine hydrochloride (PSH), diphenhydramine hydrochloride (DPH) and dextromethorphan hydrobromide (DXH) in cough-cold syrup. Mobile phase consists of methanol:water (containing 6.0 g of ammonium acetate and 10 mL of triethylamine per liter, pH adjusted to 5.2 with orthophosphoric acid), 95:5 (v/v). Column containing porous silica particles (Supelcosil LC-Si, 25 cm × 4.6 mm, 5 μm) is used as stationary phase. Detection is carried out using a variable wavelength UV–vis detector at 254 nm for PSH and DPH, and at 280 nm for DXH. Solutions are injected into the chromatograph under isocratic condition at constant flow rate of 1.2 mL/min. Linearity range and percent recoveries for PSH, DPH and DXH were 150–600, 62.5–250, 75–300 μg/mL and 100.7%, 100.1% and 100.8%, respectively. Method is stability indicating and excipients like saccharin sodium, sodium citrate, flavour and sodium benzoate did not interfere in the analysis. Compounds elute in order of increasing ionization degree caused by cation-exchange mechanism in a run time of less than 15 min. Mobile phase pH is manipulated to regulate ionization and ion-exchange interaction and thereby retention of compounds.
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Keywords: Hydrophilic interaction liquid chromatography (HILIC); Cough-cold formulations; Pseudoephedrine hydrochloride; Diphenhydramine hydrochloride; Dextromethorphan hydrobromide; Stability indicating; Method validation

1. Introduction

Pseudoephedrine hydrochloride, (1*S*,2*S*)-2-(methylamino)-1-phenylpropan-1-ol hydrochloride [1], is a direct and indirect acting sympathomimetic agent. It is a stereoisomer of ephedrine and causes stimulation of central nervous system. Diphenhydramine, 2-(diphenylmethoxy)-*N*,*N*-dimethylethanamine hydrochloride [1], is an antihistamine used for symptomatic relief of hypersensitive reactions and for control of nausea, vomiting and vertigo of various causes. Dextromethorphan hydrobromide, *ent*-3-methoxy-17-methylmorphinan hydrobromide monohydrate [1], is an antitussive agent. It is a cough suppressant, which has a central action on cough center in medulla. Several formulations of these drugs, alone and in combination, are available in pharmaceutical market, which are used for the treatment of cough and cold.

Many analytical methods for quantitation of medications containing these three drugs are available, either alone, or combination of any two or in combination with various drugs. Derivative ultraviolet spectrophotometry [2–8], colorimetry [9], gas chromatography [10,11], nuclear magnetic resonance spectroscopy [12], atomic absorption spectrophotometry [13], atomic emission spectroscopy [14], near-IR transmittance analysis [15], capillary electrophoresis [16,17] or HPLC [18–25] have been used for quantitation of these components. Pharmacopoeial HPLC methods reported for each drug are inappropriate for their simultaneous determination because of interferences due to corresponding chromatographic peaks [26]. According to bibliographical revision performed, no liquid chromatographic method applied over those medications containing combination of these three drugs has been found. However, HPLC analytical method has been

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described for simultaneous determination of diphenhydramine, dextromethorphan, pseudoephedrine, phenylephrine, and phenylpropanolamine [27]. But, this method provides very poor chromatographic resolution between DPH, DXH and PSH peaks and may not be a stability indicating method. Resolution between peaks may further decrease with increased column life. HPLC method has been described for determination of benzoic acid, pseudoephedrine, chlorpheniramine, dextromethorphan, doxylamine and diphenhydramine and butylparaben by RP-HPLC using ion-pairing agent, acetontirile, methanol and tetrahydrofuran [28]. Ion-pairing agents are usually expensive and tetrahydrofuran is highly unstable reagent.

Hydrophilic interaction liquid chromatography (HILIC) has been proved to be useful technique for separation of polar and basic compounds because of its complementary selectivity as against reversed phase (RP) HPLC. Cough-cold formulations usually contain mixes of acidic and basic compounds and therefore difficult to separate in RP-HPLC using silica based columns (e.g. C18). Mobile phase condition optimized for analysis of a base has been found to be unsuitable for an acid or another base, and vice versa. Unmodified silica columns usually employ mobile phase with non-polar hydrocarbon solvent (heptane or hexane) mixed with relatively small percentage of more polar solvent. Non-polar compounds elute from the column first, while polar solutes show stronger interaction with silanol groups on silica surface. This polar selectivity of silica is very helpful in separating polar amine bases from weak acids (e.g. cough formulation matrix). In 1990, Alpert first described HILIC, also called reverse reversed-phase or aqueous normal phase chromatography [29]. In HILIC, stationary phase is polar material such as silica and mobile phase is highly organic (e.g. acetonitrile or methanol) with a small amount of aqueous polar solvent and counter ion (e.g. ammonium acetate) where compounds elute in order of increasing hydrophilicity. HILIC approach was applied to separate active compounds to determine its suitability for the analysis of basic amines present in cough-cold formulations.

Purpose of this study is to develop and validate a rapid, simple, specific, precise, and accurate liquid chromatographic method for simultaneous determination of PSH, DPH and DXH for use in stability studies and quality control applications associated with these drugs.

Present method describes a new method to analyse active components in cough-cold formulations by HILIC. Proposed isocratic HILIC method is stability indicating and provides improved analytical procedure with superior resolution and better chromatographic system suitability parameters (Fig. 1), used for determination of these medications. Various method parameters were evaluated for their effect on chromatography.

2. Experimental

2.1. Instrumentation

Integrated high performance liquid chromatographic system LC-2010A from Shimadzu Corporation (Chromatographic and Spectrophotometric Division, Kyoto, Japan) consisted of four-liquid gradient system, high speed auto-sampler, column oven and UV–vis detector. Chromatograms were recorded and integrated on PC installed with Class-VP version 6.13 (Shimadzu, Kyoto, Japan) chromatographic software.

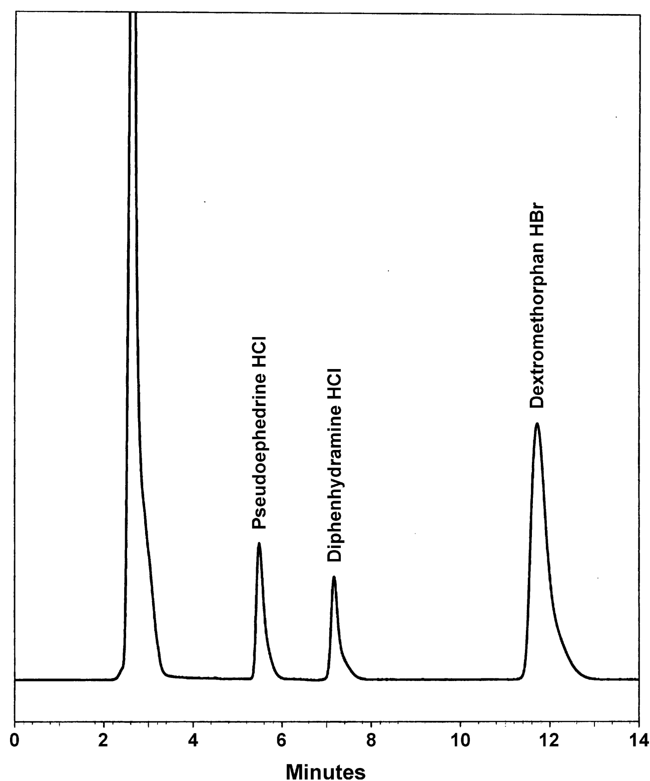

Fig. 1. Chromatogram of test solution showing separated peaks of PSH, DPH and DXH (initial wavelength 254 nm; changed at 9 min to 280 for DXH detection).

2.2. Reference substances, reagents and chemicals

Diphenhydramine hydrochloride, pseudoephedrine hydrochloride and dextromethorphan hydrobromide reference standards were obtained from United States Pharmacopoeial Convention, Rockville, MD, USA. Ammonium acetate and orthophosphoric acid (85%, w/w), of analytical reagent grade were supplied by E. Merck, Germany. Triethylamine of analytical reagent grade and methanol of HPLC grade were obtained from Riedel-de Haen, Germany. Water used was deionized and passed through Milli Q system, Millipore, USA.

2.3. Chromatographic condition

Mobile phase consisted of mixture of methanol and water (containing 6.0 g of ammonium acetate and 10 mL of triethylamine per liter, pH adjusted to 5.2 with orthophosphoric acid), in the ratio of 95:5 (v/v). Apparent pH of mobile phase was found to be about 7.2. Supelcosil™ LC-Si column, 25 cm × 4.6 mm, packed with 5 μm silica particles (Supelco, Switzerland; Part No. 58295) was used as stationary phase. A constant flow rate

of 1.2 mL/min was employed throughout the analysis. Variable UV–vis detector was set at 254 nm for PSH and DPH detection and programmed to change to 280 nm at about 9.0 min for detection of DXH. All analyses were performed at room temperature and volume of solution injected on to the column was 10 μL.

2.4. Diluent

Mixture of methanol and water was prepared in the ratio of 50:50 (v/v). The mixture was sonicated, cooled to room temperature and filtered through membrane filter of porosity 0.45 μm before use.

2.5. Samples

Test samples were commercially available syrup with following composition per 5 mL: DPH 12.5 mg, PSH 30.0 mg and DXH 15.0 mg and excipient quantity sufficient to produce 5 mL. Other active ingredients were menthol and sodium citrate with excipients like saccharin sodium and citric acid, flavour and preservative as sodium benzoate. Test samples used included accelerated stability samples with similar composition.

2.6. Solution preparation

2.6.1. Standard solution

Portions of 60 mg of PSH, 25 mg of DPH and 30 mg of DXH reference standards were transferred into a 200 mL volumetric flask. These substances were dissolved and diluted to volume with diluent to provide concentrations having 300 μg of PSH, 125 μg of DPH and 150 μg of DXH, per mL. Standard solution was mixed by hand, filtered through 0.45 μm membrane filter and 10 μL was injected.

2.6.2. Test solution

A 5.0 mL portion of syrup was transferred into a 100 mL volumetric flask with the help of pipette. Pipette was rinsed three times with diluent collecting rinsed diluent in same volumetric flask and volume was completed with diluent. Test solution was mixed by hand, cooled to room temperature, filtered through 0.45 μm membrane filter and 10 μL was injected.

2.7. Quantitation

Peak areas were recorded for all peaks and respective peak areas were taken into account to calculate amounts in milligram per 5 mL of syrup by the formula:

$$(R_t/R_s) \times 0.5 \times C$$

where R_t is the area of PSH/DPH/DXH in test solution, R_s the area of PSH/DPH/DXH in standard solution, C the weight, in mg, of respective PSH/DPH/DXH reference standards taken to prepare standard stock solution; 0.5 the factor obtained from dilution factors of standard and test solutions.

3. Results and discussion

3.1. Chromatography

Different mobile phases comprising different combinations of phosphate buffer, ammonium acetate buffer, methanol and acetonitrile with different stationary phases (octadecylsilane, octylsilane and nitrile groups chemically bonded to porous silica particles) did not provide sufficient resolution between PSH, DPH and DXH peaks. Interferences from other matrix components like saccharin sodium, sodium benzoate, citric acid, flavour and sodium citrate present in formulation were observed, and therefore, separation of active components from matrix components was key issue during method development process. These interferences in RP-HPLC are attributed to ionization constant of active components (basic pK_a) and other matrix components (acidic pK_a) like saccharin sodium (pK_a 1.8), citric acid (pK_a 3.15, 4.77 and 6.40) and sodium benzoate (pK_a 4.2). However, basic pK_a of active ingredients and acidic pK_a of matrix components were proved to be very useful in present HILIC procedure employing slightly basic mobile phase because of their ionization characteristics.

3.2. HILIC separation

In HILIC, polar column particles (silica) get coated by a thin layer of H_2O from the mobile phase. Separation occurs based on a combination of analyte partitioning between aqueous polar layer and a less polar organic layer, ion-exchange interactions and reversed phase retention. Retention can be regulated by ionic strength of mobile phase (promoting ion-exchange interactions) or water content (decreasing partitioning). Selectivity can be regulated by addition of another protic organic solvent.

3.3. Separation mechanism of PSH, DPH and DXH

Separation mechanism is multi-modal on silica [30]. The unique selectivity usually is resulted from combination of hydrophilic interaction, ion-exchange and reversed-phase retention [30]. Ion exchange interaction was found to be main mechanism for achieving highest selectivity.

3.3.1. Hydrophilic interaction

HILIC mechanism involves partitioning between adsorbed polar component of mobile phase and remaining hydrophobic component of the mobile phase. In this, polar analyte partitions into and out of adsorbed water layer on negatively charged silica surface [29]. PSH is very soluble, DPH is freely soluble and DXH is sparingly soluble in water. In hydrophilic interaction, we expect an elution order of DXH, DPH and PSH (increasing RT) because of their solubilities. This effect was not observed since elution order was PSH, DPH and DXH (increasing RT), where DXH is the most hydrophobic. Reason for this behavior can be the fact that hydrophilic interaction holds good for highly polar and hydrophilic compounds, while PSH, DPH and DXH are considerably hydrophobic.

Increase in organic content (methanol) in mobile phase resulted in increased retention of all compounds in the same elution order. This indicates hydrophilic interaction of PSH, DPH and DXH but this effect was too low to overcome the effect due to ion exchange interaction, which provided retention in order of increasing ionization degree (more ionized compounds eluted first).

3.3.2. Ion exchange

3.3.2.1. Ionization of compounds. Manipulation of mobile phase pH is a technique that works well for ionizable compounds, because the retention characteristics of ionizable compounds are a function of pH of mobile phase [31,32]. Ionization of compounds play important role in optimizing liquid chromatographic methods, and hence an understanding of ionization process is necessary. Salts of weak bases (PSH, DPH and DXH), like other salts are essentially completely ionized in solution. PSH (salt of weak base pseudoephedrine and stronger acid HCl), DPH (salt of weak base diphenhydramine and stronger acid HCl) and DXH (salt of weak base dextromethorphan and stronger acid HBr) exist in aqueous solution in the form of conjugate acid of respective weak bases and counter ions Cl^-, Cl^- and Br^-. Their ionization in solution is highly dependent on pH. Percentage of ionization at a given pH can be calculated by the equation [33]:

$$\%\ \text{ionization} = 100/[1 + \text{antilog}(\text{pH} - \text{p}K_\text{w} + \text{p}K_\text{b})] \quad (1)$$

where pH is the value at which % ionization is calculated, $\text{p}K_\text{w}$ is ionic product of water (14.00 at 25 °C) and $\text{p}K_\text{b}$ is basicity or dissociation constant.

Conjugate acid–base pairs are linked by the expression:

$$\text{p}K_\text{w} = \text{p}K_\text{a} + \text{p}K_\text{b} \quad (2)$$

Hence, at 25 °C, Eq. (1) can be written as:

$$\%\ \text{ionization} = 100/[1 + \text{antilog}(\text{pH} - \text{p}K_\text{a})] \quad (3)$$

This equation is used to calculate degree of ionization of components, if $\text{p}K_\text{a}$ value is known. Value of antilog can be easily calculated by scientific calculator.

For example, % ionization for DXH at pH 7.5, can be calculated as $100/[1+\text{antilog}(7.5-8.3)] = 100/[1+\text{antilog}(-0.8)] = 100/[1+0.15849] = 86.30\%$.

Basic drugs PSH, DPH and DXH are virtually completely ionized at pH values up to 2 units below their $\text{p}K_\text{a}$, and virtually completely unionized at pH values greater than 2 units above their $\text{p}K_\text{a}$. Degree of ionization (% ionization) of PSH, DPH and DXH at different mobile phase pH are presented in Table 1.

3.3.2.2. Retention and elution order. Suppression of ionization by addition of buffer with pH at least 2 units apart of $\text{p}K_\text{a}$ of components shifts the equilibrium of ionization. Presence of counter ions present in mobile phase promotes ion-exchange interaction and causes lower retentions.

Compounds having their $\text{p}K_\text{a}$ values lower than mobile phase pH, will elute according to their ionization conditions. PSH, DPH and DXH are basic analytes and are positively charged in the ionization. These positively charged species undergo cation exchange with negatively charged silanol groups [34]. Therefore, more charged species will interact more and elute earlier. PSH is the highest ionized species (Table 1) and elution of PSH at first (after matrix peaks) indicates that this basic compound has got highest ion-exchange interaction with residual silanols on silica surface.

Fig. 2 shows effect of pH on retention behavior as a result of cation exchange mechanism caused by varying degree of ionization. Buffer containing 6.0 g ammonium acetate and 10 mL triethylamine per liter was prepared and pH of buffer was adjusted in the range of 4.0–5.5. Mobile phase containing methanol and buffer was mixed in the ratio 95:5 (v/v) and apparent pH was checked for each buffer pH (Table 1). Fig. 3 shows that retention (capacity factor) of PSH and DXH are influenced more than that of DPH.

As can be seen in Fig. 2, at lower mobile phase pH (6.1), decreased retention and resolution are observed because of very small differences in degree of ionization of PSH (99.96% ionization), DPH (99.90% ionization) and DXH (99.37% ionization). Mobile phase pH lower than this will force ionization equilibrium of compounds approaching 100%, and hence decreasing retention and overlapping peaks can be seen.

Similarly, at higher mobile phase pH (7.5), because of least ionization of DXH, it showed delayed retention as compared to lower mobile phase pH. Same behavior was seen for PSH and DPH but shift in their retention was comparatively lesser than that of DXH (Fig. 3). Further increase in mobile phase pH will lead to prolonged retention and broader peak shapes.

Table 1
Ionization of compounds at different mobile phase pH

Buffer pH	Mobile phase pH[a]	PSH ($\text{p}K_\text{a}$ 9.5)		DPH ($\text{p}K_\text{a}$ 9.1)		DXH ($\text{p}K_\text{a}$ 8.3)	
		pH–$\text{p}K_\text{a}$	% Ionization	pH–$\text{p}K_\text{a}$	% Ionization	pH–$\text{p}K_\text{a}$	% Ionization
5.5	7.5	−2.0	99.01	−1.6	97.55	−0.8	86.32
5.3	7.3	−2.2	99.37	−1.8	98.44	−1.0	90.91
5.2	7.2	−2.3	99.50	−1.9	98.76	−1.1	92.64
5.0	7.0	−2.5	99.68	−2.1	99.21	−1.3	95.23
4.6	6.7	−2.8	99.84	−2.4	99.60	−1.6	97.55
4.2	6.3	−3.2	99.94	−2.8	99.84	−2.0	99.01
4.0	6.1	−3.4	99.96	−3.0	99.90	−2.2	99.37

[a] Apparent pH of mobile phase consisting of methanol and buffer (6.0 g ammonium acetate and 10 mL triethylamine per liter, at specified pH) in the ratio 95:5 (v/v).

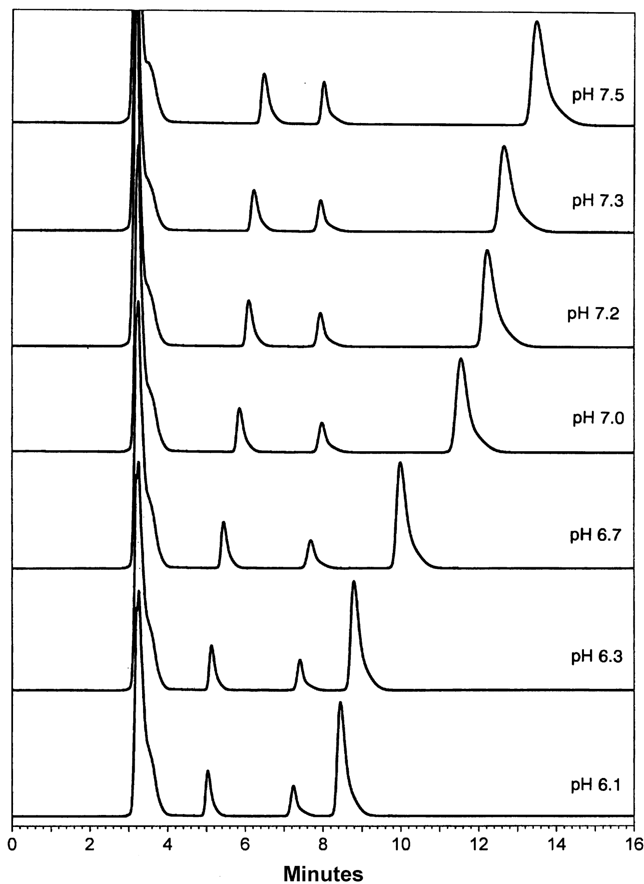

Fig. 2. Overlaid chromatograms of test solution at different mobile phase pH showing their retention behavior.

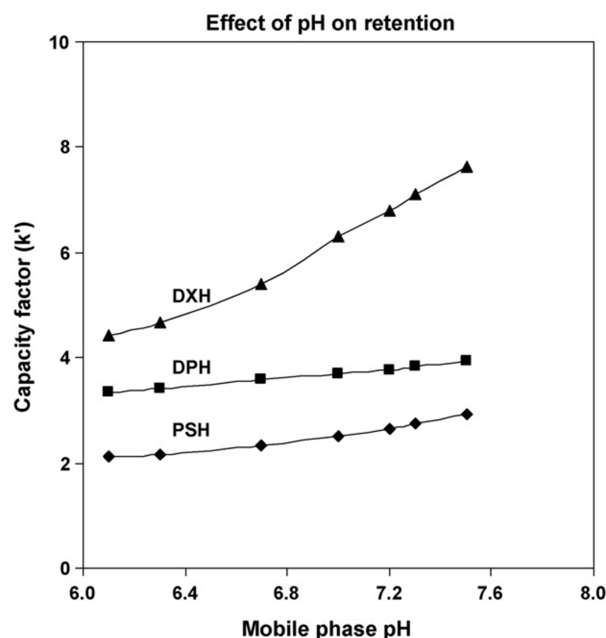

Fig. 3. Plot of pH vs. capacity factor.

Also, stability of silica columns are limited to pH higher than 7.5, where silica will be dissolved creating voids in the column, causing changes in retention and loss of resolution.

Therefore, mobile phase pH must be optimized in order to obtain appropriate retention in considerable run time. This was achieved at mobile phase pH 7.2 (buffer pH 5.2) that provided optimum selectivity with fair resolution and considerable retention. The compounds at this pH show adequate retention and resolution with acceptable peak shapes, because of their ionization degree (PSH 99.50%, DPH 98.76 and DXH 92.64%) suitable for optimum cation exchange interactions.

3.3.3. Reversed-phase retention

It occurs by interaction of analytes with siloxane bridges on the silica surface. However, this interaction is rather weak as compared to retention on C18 bonded phases. Reversed phase retention was not observed in proposed method, as increased organic content in mobile phase showed increased retention of PSH, DPH and DXH.

3.4. Mobile phase optimization

3.4.1. Ammonium acetate buffer

Methanol and buffer containing varying amounts of only ammonium acetate (3, 4, 6, 8, 10 g/L) [pH of each adjusted to 5.2 with acetic acid] in the ratio 95:5 (v/v) at a flow rate of 1.2 mL/min was used to check retention. Mobile phase with 3 g/L ammonium acetate buffer provided greatest retention and that containing 10 g/L provided least retention of compounds. Increased ammonium acetate promotes more cation exchange interactions of positively charged basic compounds with negatively charged silanols. Decreased resolution, capacity factor and peak tailing were observed with increasing buffer strength in the mobile phase.

Increase in ammonium acetate buffer pH 5.2 concentration (for example, from 5% to 10%) in the mobile phase causes much lesser retention of compounds with decreasing resolution and capacity factor, as compared to that exhibited by change in ionic strength (different ammonium acetate amounts). This is due to lower hydrophilic interaction of analytes with increase in net water content in mobile phase.

3.4.2. Triethylamine phosphate buffer

Similar trends were observed with the use of only triethylamine (TEA) buffer. Methanol and buffer solutions containing 1%, 2%, 3% and 4% TEA, pH of each adjusted to 5.2 with orthophosphoric acid in the ratio 95:5 (v/v). Least retention and resolution were observed in case of 4% TEA. Increase in the ratio of buffer in mobile phase (90:10, methanol and buffer) showed decreased retention and resolution. This retention behavior seen above is indicative of hydrophilic interaction liquid chromatography (HILIC) because of opposite chromatographic effect than that found in reversed phase (RP) HPLC.

It is important to add buffer in the mobile phase to control the ionization of both the analytes and the stationary phase, since the ionic state of both effects the acid–base equilibrium between analyte and stationary phase.

Basic compounds interact with residual silanols on the silica surface and thus exhibit excessive peak tailing. Use of triethylamine, an amine modifier helps in reducing peak tailing by the suppression of silanols. Various combinations of ammonium acetate and triethylamine buffers (pH adjusted to 5.2 with orthophosphoric acid) were used to establish optimum quantities of these buffers and determined to be 6 g of ammonium acetate with 10 mL of TEA per liter water.

3.4.3. Choice of organic solvent

Under HILIC conditions, acetonitrile is weaker solvent that provides higher retention than methanol. Solvent strength from weakest to strongest: tetrahydrofuran < acetone < acetontrile < isopropanol < ethanol < methanol < water, where water is the strongest eluting solvent.

Use of acetonitrile in the mobile phase (as organic component) is deemed to be unsuitable because of its limitation to provide good solubility of compounds present in formulation. Mobile phase containing acetonitrile (as organic modifier), methanol and buffer (2:3:90, v/v/v) provided slightly more retention for PSH, DPH and DXH, but with inferior resolution between PSH and DPH.

The separation of active compounds PSH, DPH and DXH was found to be unique. Under HILIC conditions, mobile phase comprising methanol and water containing 6 g ammonium acetate and 10 mL triethylamine per liter pH adjusted to 5.2 (95:5, v/v), provided retention order (increasing) DXH, DPH and PSH, because of predominant cation exchange mechanism. In HILIC, atleast 5% polar solvent (containing buffer) ensures silica particle is always hydrated. Phosphate buffers were not used due to their low solubility in high organic mobile phases and may cause precipitation. Further, lack of volatility renders them incompatible with electrospray ionization mass spectrometry (ESI-MS).

3.5. Sample diluent, injection volume and peak shape

Like RP-HPLC, diluent has great effect on peak shape and sensitivity of peaks. Solutions containing PSH, DPH and DXH in different diluents were injected. No change in peak shape was observed in diluents having methanol and water, 50:50 (v/v) and 70:30 (v/v). Injection volumes of 5, 10, 20 and 30 μL provided no significant change in peak shape, other than slight increase in peak broadening with increasing injection volume, as expected (Fig. 4).

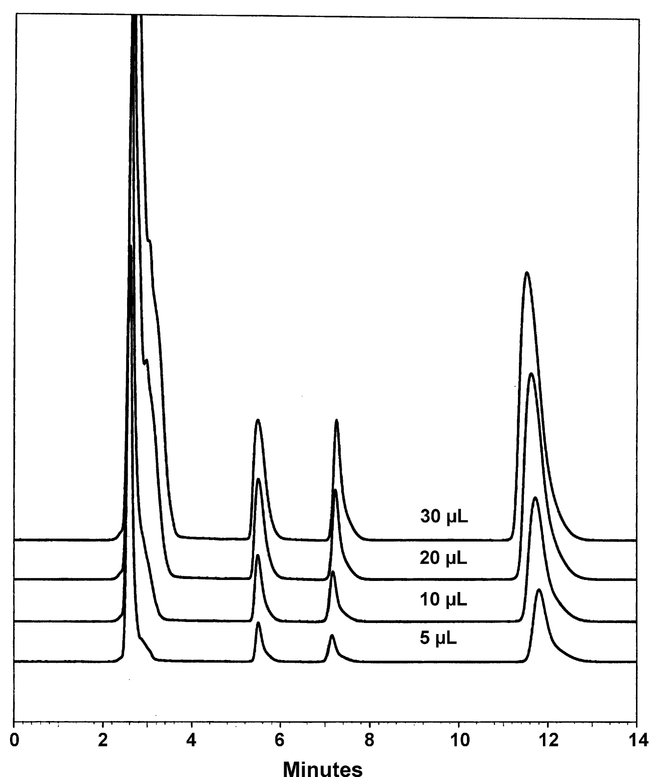

Fig. 4. Overlaid chromatograms of test solution following injection volume of 5, 10, 20 and 30 μL.

3.6. Equilibration time, column storage and its stability

Used Supelcosil™ LC-Si column for normal phase chromatography is shipped saturated with hexane:ethylacetate (98:2). While using this column in HILIC mode for the first time, column must be flushed with intermediate solvent isopropyl alcohol (IPA) for atleast 30 min in order to avoid miscibility problem. More equilibration time is expected at first use, however, 45–60 min is sufficient for equilibration during routine use. After use, column should be flushed with methanol and water (95:5, v/v) and stored in same solvent. It is appropriate to use above solvent mixture as injector rinsing solvent for needle wash. Since, operating pH range of these columns is 2.0–7.5, mobile phase pH 7.2 is safer for analysis and does not provide a risk to column life. Expected column life of this silica column is similar to other silica based columns and depends on proper usage and care.

3.7. Method validation

Test method for simultaneous determination of PSH, DPH and DXH was validated to include requirements of International Conference on Harmonization (ICH) guidelines [35]. Parameters like specificity, linearity, accuracy, precision, range, robustness and system suitability were examined and found to be acceptable.

3.7.1. Specificity

No interferences were observed due to presence of components like saccharin sodium, sodium benzoate, citric acid, menthol, ammonium chloride, flavour and sodium citrate.

3.7.1.1. Forced degradation study. Degradation of PSH, DPH and DXH in mixed standard, individual standard and test solutions prior to final dilution was stimulated by subjecting the solutions with stress condition such as 0.1 N HCl, 0.1 N NaOH, 3% H_2O_2 and UV-light and heat. Solutions were heated occasionally in order to accelerate degradation. Degraded solutions were treated according to standard and test solution after final dilution with diluent.

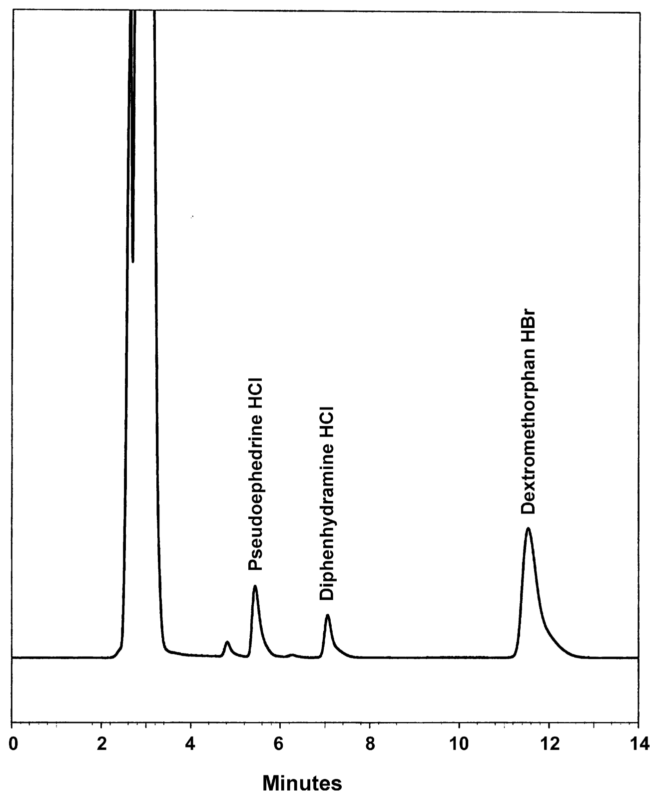

Fig. 5. Chromatogram of test solution subjected to forced degradation by oxidation (H_2O_2) showing degradation peaks.

Degraded mixed standard, individual standard and degraded test solutions were examined under same chromatographic conditions of analysis but using photo-diode array detector. Purity of analyte peaks was calculated and found to be close to 100%. Fig. 5 represents chromatogram of test solution subjected to oxidation, which showed maximum degradation. Extent of degradation of components in standard solutions was comparatively more than that in syrup test solutions under similar degradation conditions. This difference in the extent of degradation may be attributed to resistance of pharmaceutical formulation to degradation, where excipients used may slow down the degradation process.

3.7.2. Linearity

Peak areas versus concentrations in μg/mL were plotted for PSH, DPH and DXH at the concentration range between 50% and 200% of target levels. PSH, DPH and DXH showed linearity in the range of 150–600 μg/mL, 62.5–250 μg/mL, 75–300 μg/mL. Values of slope were found to be 852.7, 1619.1 and 6912.0 and values of intercept were −452.9, −127.0 and −4146.9 for PSH, DPH and DXH, respectively. The correlation coefficient for these compounds ($R^2 > 0.999$) suggests that method provides good linear dynamic range.

3.7.3. Accuracy

Accuracy and precision of the proposed HILIC determination were evaluated from assay result of components [35]. Accuracy was done by performing assay of components calculated from peak area responses of different samples by analyte recovery and standard addition methods.

3.7.3.1. By analyte recovery. Into blank syrup matrix, three components were spiked from a standard stock solution with 50%, 75%, 100%, 125% and 150% of the target level in the syrup. Each of the spiked solution was injected in triplicate. Mean recovery and R.S.D. were 100.7% and 0.99% for PSH, 100.1% and 0.75% for DPH and 100.8% and 0.61% for DXH (Table 2).

3.7.3.2. By standard addition. Known amounts of three components were spiked at different levels into syrup sample matrix that already contained some quantity of analytes. A 5.0 mL portion of syrup was transferred into six 100 mL volumetric flasks marked A, B, C, D, E and F. Flask A was completed to volume with diluent without any addition and treated according to test solution preparation. Amounts of PSH, DPH and DXH were determined by analysis to know initial concentration of these components (regarded as 100%). Flasks B through F were spiked with varying known amounts of PSH, DPH and DXH from a stock solution at levels of 50%, 75%, 100%, 125%, and 150% of the target concentration to contain overall levels of 150%, 175%, 200%, 225% and 250%. Volume was completed with diluent and treated according to test solution preparation. Each of spiked samples was analyzed and measured amounts reported were compared with total theoretical amount present. Mean recovery was 100.9% for PSH, 101.2% for DPH, and 101.1% for DXH.

3.7.4. Precision

Instrumental precision was determined by analyzing test sample by six replicate determinations. Relative standard deviations from these determinations were 0.56% for PSH, 0.66% for DPH, and 0.59% for DXH.

Method precision or intra-assay precision was performed by preparing six different standard solutions involving different weighings and dilutions. Each solution was injected in triplicate under same conditions and mean value of peak area response for each solution was taken. Corrections in area were made for each weight taken to prepare six standard solutions and relative standard deviation of peak area response were calculated from the six solutions. Relative standard deviations were 0.53% for PSH, 0.80% for DPH and 0.74% for DXH.

Intermediate precision was performed by analyzing samples by two different analysts using different instruments. Standard solution and ten different samples at 100% target level were prepared by each analyst. Relative standard deviations obtained from 20 assay results by three analysts were 0.66% for PSH, 0.64% for DPH and 0.91% for DXH.

3.7.5. Range

Range of a method is defined as lower and higher concentrations for which the method has adequate accuracy, precision and linearity.

To demonstrate the range of the proposed method, six samples each of lower concentration (50% of target level) and higher

Table 2
Accuracy data (analyte recovery)

Number	Theoretical amount (mg/5 mL)	Theoretical (% of target level)	Determined amount (mg/5 mL)	Recovered (%)	Bias (%)
Pseudoephedrine hydrochloride					
1	15.00	50.0	15.24	101.6	+1.6
2	22.50	75.0	22.68	100.8	+0.8
3	30.00	100.0	30.33	101.1	+1.1
4	37.50	125.0	37.88	101.0	+1.0
5	45.00	150.0	44.56	99.0	−1.0
	Overall mean ($n = 5$)			100.7	
	Overall %R.S.D.			0.99	
Diphenhydramine hydrochloride					
1	6.25	50.0	6.21	99.4	−0.6
2	9.38	75.0	9.46	100.9	+0.9
3	12.50	100.0	12.40	99.2	−0.8
4	15.63	125.0	15.72	100.6	+0.6
5	18.75	150.0	18.82	100.4	+0.4
	Overall mean ($n = 5$)			100.1	
	Overall %R.S.D.			0.75	
Dextromethorphan hydrobromide					
1	7.50	50.0	7.56	100.8	+0.8
2	11.25	75.0	11.32	100.6	+0.6
3	15.00	100.0	15.27	101.8	+1.8
4	18.75	125.0	18.92	100.9	+0.9
5	22.50	150.0	22.53	100.1	+0.1
	Overall mean ($n = 5$)			100.8	
	Overall %R.S.D.			0.61	

concentration (150% of target level) similar to accuracy samples by spiking drug substance into blank matrix (placebo) were prepared. Each sample was analyzed in duplicate. Recoveries and R.S.D. were found to be 99.8–100.9% and less than 1.20%, respectively (Table 3).

3.7.6. Robustness

Robustness of proposed method was performed by keeping chromatographic conditions constant with following changes:

(i) Mobile phase composition changed from methanol:buffer, ‘95:5’ to ‘93:7’ (v/v).
(ii) Increasing flow rate of mobile phase from 1.2 to 1.5 mL/min.
(iii) Using another column (Waters, Silica, 3.9 mm × 300 mm, 5 μm).

Standard solution was injected six times in replicate for each change. System suitability parameters like resolution, peak asymmetry, theoretical plates, capacity factor and relative standard deviation were recorded for each peak and found to be within acceptable limits.

Six test samples at target concentration level were prepared and analyzed in duplicate for each change. Recoveries and relative standard deviations were calculated for each component during each change and found to be 98.8–101.6% and <1.2%, respectively.

Table 3
Data of range

Compound name	Lower range (50% level)		Higher range (150% level)	
	Mean recovery (%)	%R.S.D.	Mean recovery (%)	%R.S.D.
PSH	101.4	0.74	99.8	1.14
DPH	99.9	1.12	100.6	0.93
DXH	100.5	0.81	100.9	1.18

3.7.7. Limit of quantitation (LOQ) and limit of detection (LOD)

LOQ and LOD for the determination of PSH, DPH and DXH in the proposed HILIC method were established by signal-to-noise ratio (S′/N ratio) obtained from serial dilution of test solution and injection of blank solution. Ratio of signal size to that of noise is termed S′/N ratio and was calculated by equation $2H/h$, where H is the height of peak corresponding to component concerned, in the chromatogram obtained with the prescribed reference solution, h, is the range of the background noise in a chromatogram obtained after injection of a blank.

LOQ is defined as lowest concentration of analyte that can be determined with acceptable precision and accuracy under stated experimental conditions. LOD is a parameter that provides lowest concentration of analyte in a sample that can be detected, but not quantitated, under stated experimental conditions.

LOQ was identified as concentration that produced S′/N ratio of greater than 10, while analyte concentration that produced S′/N ratio greater than 3 was accepted as LOD. Concentration and calculated S′/N ratio are provided in Table 4. Fig. 6 represents overlaid chromatograms obtained from diluted test

Table 4
Concentrations (μg/mL) at LOQ and LOD levels

Name of compound	Limit of quantitation (LOQ)		Limit of detection (LOD)	
	Concentration (μg/mL)	S′/N ratio	Concentration (μg/mL)	S′/N ratio
PSH	1.500	16.5	0.7500	8.5
DPH	0.625	10.1	0.3125	3.3
DXH	0.750	32.7	0.3750	15.7

solutions at LOQ and LOD levels. Since described method employ simultaneous determination of components, LOQ and LOD levels were fixed relative to DPH peak whose height and S′/N ratio was found to be the least.

3.7.8. System suitability

System suitability tests were performed to chromatograms obtained from standard and test Solutions to check parameters such as column efficiency, peak asymmetry, capacity factor and resolution between PSH, DPH and DXH peaks. Results obtained from injection of standard solution as representative chromatogram are summarized in Table 5.

3.7.9. Solution stability

Standard and test solution stability was performed and found that the solutions were stable for at least 24 h. Six test solutions were prepared from the same stock test solution. Assay of freshly prepared test solution and that after 24 h storage at ambient room temperature in dark, were performed. Results of these samples indicate no significant change in assay values (Table 6).

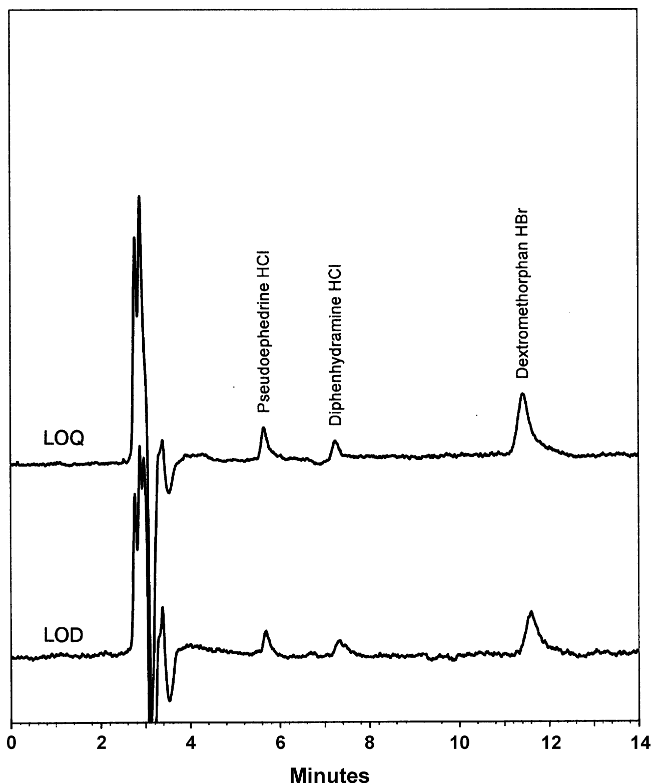

Fig. 6. Chromatograms at LOQ and LOD levels (overlaid).

Table 5
System suitability parameters

Component	Area R.S.D.[a] (%)	Tailing factor	Theoretical plates	Capacity factor	Resolution
PSH	0.25	1.93	4512	2.66	–
DPH	0.33	1.94	8427	3.78	5.260
DXH	0.18	1.96	4994	6.81	9.330

[a] Six replicate injections.

3.8. Comparison with pharmacopoeial methods and advantages

USP 26 has described several monographs containing PSH, DPH and DXH, alone and in combination with other drugs. For example, monograph "acetaminophen, dextromethorphan hydrobromide, doxylamine succinate, and pseudoephedrine hydrochloride oral solution" specifies tailing factor of not more than 2.5 and column efficiencies of not less than 500 theoretical plates. Similarly, monograph "diphenhydramine and pseudoephedrine capsules" mentions resolution of NLT 3.0 between PSH and DPH and tailing factor of NMT 2.0.

BP'2003 has described assay of PSH by HPLC under monograph "pseudoephedrine tablets", assay of DPH and DXH by titration under monographs "diphenhydramine oral solution" and "dextromethorphan hydrobromide". These methods are unsuitable for simultaneous determination of PSH, DPH and DXH in oral liquids.

System suitability parameters like resolution and tailing factor are found to be superior in the proposed HILIC method (Table 5), besides an ability to analyze these components simultaneously. Also, like other HILIC methods, this method can be used at higher flow rates because of low column back pressure exhibited by bare silica packings.

3.8.1. Advantage in ESI-MS

High organic mobile phase is ideal for efficient desolvation and compound ionization in ESI-MS. Proposed HILIC method for separation of active compounds PSH, DPH and DXH can provide enhanced ESI-MS sensitivity because of highly volatile mobile phase (methanol 95%) which may allow lower detection limit.

Table 6
Solution stability data

Number	PSH (%)		DPH (%)		DXH (%)	
	1	2	1	2	1	2
1	101.2	101.3	100.5	100.9	100.3	100.8
2	100.6	100.3	100.5	99.8	101.5	100.9
3	99.8	100.6	101.2	100.6	101.1	99.4
4	99.9	100.9	101.1	100.5	100.3	101.1
5	100.8	99.5	100.8	99.8	99.6	100.7
6	100.0	99.2	99.4	100.1	101.7	100.8

1, assay of fresh sample; 2, assay after 24 h at room temperature in dark.

4. Conclusion

Proposed new HILIC method is rapid, specific, accurate and precise for the simultaneous determination of PSH, DPH and DXH in cough-cold syrup. Hence, this method can be used for routine analysis and quality control of liquid pharmaceutical preparations containing PSH, DPH and DXH. The method can also be applied for determination of these drugs in tablets, capsules or other pharmaceutical preparations, alone and in combination. It can offer enhanced ESI-MS sensitivity because of high organic content in mobile phase.

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