Research Paper

Stability-indicating HPLC Method for Simultaneous Determination of Terbutaline Sulphate, Bromhexine Hydrochloride and Guaifenesin

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The aim of the present study was the development and subsequent validation of a simple, precise and stability-indicating reversed phase HPLC method for the simultaneous determination of guaifenesin, terbutaline sulphate and bromhexine hydrochloride in the presence of their potential impurities in a single run. The photolytic as well as hydrolytic impurities were detected as 3,5-dihydroxybenzoic acid, 3,5-dihydroxybenzaldehyde, 1-(3,5-dihydroxyphenyl)-2-[(1,1-dimethylethyl) amino]-ethanone from terbutaline, 2-methoxyphenol and an unknown impurity identified as (2RS)-3-(2-hydroxyphenoxy)-propane-1,2-diol from guaifenesin. The chromatographic separation of all the three active components and their impurities was achieved on Wakosil II column, using phosphate buffer (pH 3.0) and acetonitrile as mobile phase which was delivered initially in the ratio of 80:20 (v/v) for 18 min, then changed to 60:40 (v/v) for next 12 min, and finally equilibrated back to 80:20 (v/v) for 10 min. Other HPLC parameters were: Flow rate at 1.0 ml/min, detection wavelengths 248 and 280 nm, injection volume 10 μl. The calibration graphs plotted with five concentrations of each component were linear with a regression coefficient R^2 >0.9999. The limit of detection and limit of quantitation were estimated for all the five impurities. The established method was then validated for linearity, precision, accuracy, and specificity and demonstrated to be applicable to the determination of the active ingredients in commercial and model cough syrup. No interference from the formulation excipients was observed. These results suggest that this LC method can be used for the determination of multiple active ingredients and their impurities in a cough and cold syrup.

Key words: Bromhexine hydrochloride, degradation products, guaifenesin, stability-indicating method, terbutaline sulfate, validation

Many pharmaceutical formulations against the common cold and cough syrup used to contain a combination of a bronchodialator terbutaline sulphate (TBN), mucolytic agent bromhexine hydrochloride (BHN) and an expectorant guaifenesin (GFN). They are chemically known as bis[(1RS)-(3,5-dihydroxyphenyl)-2-[(1,1-dimethylethyl)amino] ethanol]sulphate, N-(2-amino-3,5- dibromobenzyl)-N-methylcyclohexanamine hydrochloride, and (2RS)-3-(2-methoxyphenoxy)-propane-1,2-diol, respectively. TBN is a synthetic β_2−adrenoceptor agonist and enhances mucocillary transport to help expectoration. It is used as a bronchodialator in the treatment of bronchial asthma. GFN stimulate the bronchial glands lining of airway to produce a thin secretion that lubricates any thick mucous and making it easier to expel with coughing. It is known to increase the volume and reduce the viscosity of tenacious sputum[1]. BHN is a mucous modifying drug helps to improve the flow properties of bronchial mucous and eases expectoration. The excipients used are preservative, sweeteners, acidulates, artificial coloring and flavoring agent which are present in different proportions in the formulations. The simultaneous determination of the three components having different physicochemical properties is difficult and becomes complicated due to the matrix effect of inactive ingredients and with the presence of impurities.

To establish the stability characteristics, degradation studies (stress studies) were carried out on these three active ingredients under heat, water, acid, base, UV radiation and oxidative stress conditions, as recommended in the ICH guideline Q1A (R2)

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on stability testing of new drug substances and products[2].

A literature survey reveals some HPLC methods reported for the simultaneous determination of GFN[3-10] and BHN[11] along with some other active ingredients which exist as various combinations in cough-cold mixture and excipients in a multicomponent liquid dosage formulation as anticipated with the variation of mobile phase, column and detector. Some stability-indicating assay methods (SIAM) of GFN and TBN along with other active ingredients, excipients and impurities in liquid and solid dosage forms are reported[12-15]. Different HPLC methods for individual assay and related substances are available for TBN, GFN and BHN in official pharmacopoeia[16-18]. A few related impurities of TBN, BHN and GFN were mentioned in British Pharmacopoeia[17,18]. A few degradation products of TBN and a LC separation method were mentioned by Ahuja[19].

From the detailed literature survey it was found that no stability-indicating assay method reported yet for this combination of liquid dosage form, developed using the ICH approach of stress testing. A major demethylated photoproduct (2RS)-3-(2-hydroxyphenoxy)-propane-1,2-diol (IMP G), from GFN was not reported elsewhere. The structure of this impurity was confirmed by spectral (IR, ^{1}H NMR, MS) studies and derivatizing into its acetate. The developed analytical procedure was validated to justify the suitability for the detection and quantification of degradation products as emphasizes in the ICH guideline Q3B (R2) entitled "Impurities in new drug products"[20]. Furthermore the method has been tested for liquid formulation.

MATERIALS AND METHODS

Terbutaline sulphate was purchased from Neuland Laboratories Limited, India, bromhexine hydrochloride was purchased from Ven Petrochem Pvt. Ltd., India and guaifenesin was purchased from Granules India Limited, India. 1-(3,5-dihydroxyphenyl)-2-[(1,1-dimethylethyl)amino]-ethanone (IMP-T1) was purchased from Toronto Research Chemicals Inc. Canada; 3,5-dihydroxybenzoic acid (IMP-T2) and 3,5-dihydroxybenzaldehyde (IMP-T3) were obtained from Aldrich, Germany. (2RS)-3- (2-hydroxyphenoxy)-propane-1, 2-diol (IMP-G1) was synthesized in our laboratory; 2-methoxyphenol (IMP-G2) was obtained from Fluka, Germany. High purity water was prepared using Milli Q purification system from Millipore (Peenya, Bangalore, India).

Photo degradation was carried out in a photostability chamber (Thermolab, India) equipped with a light bank consisting of two UV (TUV 15W) and four fluorescent (TLD 15W) lamps. The HPLC system consisted of waters 1525 binary pump, waters 717 plus auto sampler, a waters 2487 dual λ absorbance detector (Waters, Milford, MA, USA). Acquisition of Chromatographic data was made with Empower version 2.0 software. The chromatographic separations were achieved on a Wakosil II C_{18} (150×4.6 mm, i.d., 5μ) column from Waters Corporation. IR experiments were performed with a FT-IR spectrometer (Perkin-Elmer Spectrum 2000, Wellesley, M.A. USA). NMR experiments were carried on a NMR spectrometer (BRUKER 300 MHz, Milton, Ontario, Canada). The proton chemical shifts were referenced to the TMS signal at 0 ppm. Electrospray mass spectrometry measurements were performed on a MS-TOF micromass spectrometer (Waters, USA).

Optimized chromatographic conditions:
HPLC studies were carried out on all the reaction solutions individually and on a mixture of the solutions in which decomposition was observed. The separations were achieved by gradient elution using phosphate buffer (25 mM disodium hydrogen phosphate dodecahydrate and 25 mM potassium dihydrogen orthophosphate, pH 3.0) and acetonitrile as the mobile phase. The phosphate buffer was prepared by dissolving 8.95 g of disodium hydrogen phosphate dodecahydrate and 3.40 g of potassium dihydrogen orthophosphate in 1000 ml water and final pH was adjusted by adding orthophosphoric acid and 2N sodium hydroxide. It was filtered through 0.45 μm nylon filter and degassed before use. The diluting solvent was prepared by mixing the same phosphate buffer (after raising pH to 3.5) with acetonitrile in the ratio of 80:20. The injection volume was 10 μl and mobile phase flow rate was 1.0 ml/min. The detection was carried out at 248 nm for BHN and 280 nm for TBN and GFN.

Preparation of stock and standard solutions:
For the preparation of stock solutions, 100 mg each of terbutaline sulphate, bromhexine hydrochloride and guaifenesin working standard (WS) was taken

in a 100 ml volumetric flask and it was made up to volume with the diluting solvent (Stock solution A). For the calibration of the assay method, 0.1, 0.5, 1.0, 2.0 and 5.0 ml aliquots of the stock solution A were diluted to 10 ml with the diluting solvent to give the final concentrations of 10, 50, 100, 200 and 500 μg/ ml.

Calibration solutions for the impurity method were prepared containing each of the related compounds at concentration ranging 0.5 μg/ml to 20.0 μg/ml (0.5, 1.0, 5.0, 10.0 and 20.0 μg/ml) and the active ingredients at the concentrations ranging from 10 μg/ ml to 500 μg/ml.

Preparation of sample solution:
Accurately measured volumes of the cough syrup equivalent to 10 mg each of BHN, TBN and GFN was taken in a 50 ml volumetric flask and made up the volume with diluting solvent. Final solution had a concentration of 200 μg/ml of each component.

Specificity and stress study:
Specificity is the ability of the method to measure the analyte response in the presence of its potential impurities. The specificity of the LC method developed for TBN, BHN and GFN was determined in the presence of their impurities, namely IMP-T1, IMP-T2, IMP-T3, IMP-G1 and IMP-G2. Stress studies were performed for TBN, BHN and GFN in bulk drug individually and in synthetic mixtures to assess the suitability of the method as stability-indicator. Forced degradation under stress conditions of UV light (254 nm), heat (70°), acid (0.1N HCl), base (0.1 NaOH) and oxidation (3.0% H_2O_2) was used to evaluate the ability of the proposed method to separate three active ingredients (AI) from the degradation products. All degradation experiments on TBN, BHN and GFN were performed at a drug concentration of 500 μg/ml.

Linearity:
Linearity test solutions for the assay method and for the related substance method were prepared at five concentration levels in the range of 10 μg/ml to 500 μg/ml and from 0.25% to 10.0% of target analyte concentration (TAC), 200 μg/ml (0.5, 1.0, 5.0, 10.0 and 20.0 μg/ml), respectively. The peak area versus concentration data were analyzed by least square linear regression.

Precision:
Assay method precision was evaluated by carrying out six independent assays of test sample of TBN, BHN and GFN at 100% level of the test concentration, 200 μg/ml. The precision of the related substance method was checked by injecting six individual preparations of TBN, BHN and GFN (200 μg/ml), spiked with 2.5% each of IMP-T1, IMP-T2, IMP-T3, IMP-G1 and IMP-G2. The % RSD of the area for each impurity was calculated. The intermediate precision of the method was also evaluated using different analyst and different instruments in the same laboratory.

Accuracy:
The accuracy of the assay method was evaluated in triplicate at three different concentration levels of every compound, 100, 200 and 400 μg/ml (50%, 100% and 200% of the analyte concentration). The accuracy study for impurities was carried out in triplicate at 0.25%, 0.5% and 1.0% of the TBN, BHN and GFN analyte concentrations, (200 μg/ml). The percentage recovery at each level was determined by comparison to the known amount added.

Limit of detection and limit of quantitation:
The limit of detection (LOD) and limit of quantitation (LOQ) were determined by the slope method by injecting a series of dilute solutions with known concentrations. A precision study was also carried at the LOQ level by injecting six individual preparations of IMP-T1, IMP-T2, IMP-T3, IMP-G1 and IMP-G2 and calculating the % RSD of the area.

Robustness:
To determine the robustness of the method developed, the experimental conditions were deliberately altered and the chromatographic parameters viz., capacity factor (K'), tailing factor (T), no. of theoretical plates (N) and percent recoveries were recorded. The flow rate of the mobile phase was 1.0 ml/min. To study the effect of flow rate on the resolution, 0.05 units flow was changed, from 0.95 to 1.05 ml/min. The effect of mobile phase was studied by varying acetonitrile from –10% to +10% and the pH of the mobile phase was studied by varying pH by –0.12 to +0.12, while other mobile phase components were held constant.

Solution stability and mobile phase stability:
The solution stability of TBN, BHN and GFN and their impurities in the related substance method was

investigated by leaving sample solutions in tightly capped volumetric flask at room temperature for 72 h. The contents of impurities were determined every 24 h up to the study period. The mobile phase stability was also investigated for 48 h by injecting the freshly prepared solutions every 6 h. Contents of impurities and active ingredients were checked in the test solutions.

RESULTS AND DISCUSSION

The main purpose of the chromatographic method was to separate IMP-T1, IMP-T2, IMP-T3, IMP-G1, IMP-G2 (fig. 1) and the degradation products generated during stress studies from the three active ingredient peaks. To achieve better separation of all the analytes in the chromatogram several trials were done with the variation of column, composition of mobile phase and diluting solvent, pH of the buffer, mode of elution either with isocratic or gradient. The results are summarized in Table 1. Octadecylsilyl silica gel column with different mobile phase is recommended for the determination of individual assay and related substance of TBN, BHN and GFN in different international pharmacopoeia[16,17]. Two different types of octadecylsilyl silica gel columns: Atlantis d C_{18} (150×4.6 mm, 3 μm) and Wakosil II C_{18} (150×4.6 mm, 3 μm), were compared on the basis of retention factor of very neighbouring component peaks to evaluate the column performance. A polar column Hypersil silica (250×4.6 mm, 3 μm) was also tried with. Results of this comparison showed that the Wakosil II column was the most suitable for the separation of three active components and their potential impurities in a single run and this column was used for all other experiments. By employing Wakosil II C_{18} column (150×4.6 mm, 3 μm), mobile phase with a phosphate buffer and acetonitrile (60:40, v/v) enabled to elute three active components with bad resolution between GFN and TBN (exp no. 8). Increasing the phosphate buffer proportion to 80% reasonable resolution between GFN and TBN was achieved with long retention of BHN (exp no. 9). To compromise these differences of elution in isocratic mode a need for gradient elution mode was felt. Due to the serious differences in polarity of the active ingredients, the pH of a chosen mobile phase and the diluting solvent had a significant influence on

TABLE 1: METHOD OPTIMIZATION DATA

Mobile phase component	pH	Column	Diluting solvent	Flow rate (ml/min)	Remarks
Water:buffer[a]:acetonitrile (25:50:25 v/v)	6.5[1]	Hypersil silica (250×4.6 mm; 5 μm)	Mobile phase	0.7	No separation between GFN and IMP-G2 and fast elution of both the components
Acetic acid (0.1%v/v):acetonitrile (40:60, v/v) (pH adjusted with TEA)	6.5[1]	Hypersil silica (250×4.6 mm; 5 μm)	Mobile phase	0.7	High tailing factor for BHN and bad resolution between GFN and TBN.
Acetic acid (0.1%v/v): acetonitrile (55:45) (pH adjusted with TEA)	6.5[1]	Atlantis dC_{18}, (150×4.6 mm; 4 μm)	Mobile phase	1.5	Bad resolution between the peaks TBN and BHN.
Buffer[b]: acetonitrile: methanol (20:20:60,v/v)	4.0[1] 4.2[2]	Atlantis dC_{18}, (150×4.6 mm; 4 μm)	Mobile phase	1.0	Bad resolution between GFN and TBN, no elution for BHN
Buffer[b]:acetonitrile (80:20,v/v)	4.0[1] 4.2[2]	Atlantis dC_{18}, (150×4.6 mm; 4 μm)	Mobile phase	1.0	Fast elution of GFN and TBN, bad resolution between the the peaks of GFN and IMP-G2, no elution for BHN
Acetic acid (1% v/v):acetonitrile (80:20,v/v)	3.4[1]	Atlantis dC_{18}, (150×4.6 mm; 4 μm)	Mobile phase	1.0	Bad resolution between TBN and IMP-T1, no elution for BHN
Phosphoric acid (0.05%v/v):acetonitrile (20:80,v/v)	7.0[1]	Atlantis dC_{18}, (150×4.6 mm; 4 μm)	Methanol	2.0	No elution for GFN and TBN
Buffer[b]:acetonitrile (60:40,v/v)	4.0[1] 4.2[2]	Wakosil II C_{18} (150×4.6 mm; 3 μm)	Mobile phase	1.0	Bad resolution between GFN and TBN peaks
Buffer[b]:acetonitrile (80:20,v/v)	4.0[1] 4.2[2]	Wakosil II C_{18} (150×4.6 mm; 3 μm)	Mobile phase	1.0	Long retention time for BHN peak
Buffer[b]:acetonitrile (gradient*)	4.0[1]	Wakosil II C_{18} (150×4.6 mm; 3 μm)	ACN:buffer[b] (20:80,v/v, pH 4.0)	1.0	Bad resolution among TBN, IMP-T2 and IMP-T1 peaks
Buffer[b]:acetonitrile (gradient*)	3.0[1]	Wakosil II C_{18} (150×4.6 mm; 3 μm)	ACN:buffer[b] (20:80,v/v, pH 3.5)	1.0	Acceptable retention time for all peaks. Resolution and peak characteristics were good.

[1]pH of Buffer; [2]pH of Mobile phase; [a]Buffer of pH 6 prepared by dissolving 1.36 g KH_2PO_4 in 500 ml water and by adjusting pH by NaOH or H_3PO_4. [b]Buffer of pH 4 prepared by dissolving 8.95 g $Na_2HPO_4 12H_2O$ and 3.40 g KH_2PO_4 in 1000 ml water and by adjusting pH by NaOH or H_3PO_4;* Gradient: 0-18 min; Acetonitrile 20%; 19-30 min, Acetonitrile 20-40%; 31-40 min, Acetonitrile 40-20%.

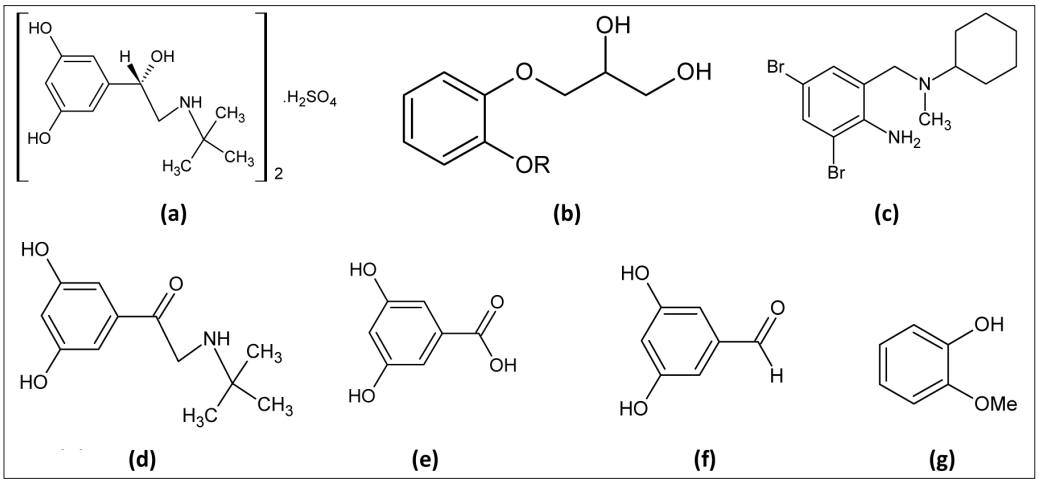

Fig. 1: Chemical structures of (a) – (g)
(a) terbutaline sulphate (TBN) (b) guaifenesin (R=CH_3, GFN), 3-(2-hydroxyphenoxy) propane-1,2-diol (R=H, IMP-G1) (c) bromhexine hydrochloride (BHN) (d) 1-(3,5-dihydroxyphenyl)-2-[(1,1-dimethylethyl) amino]-ethanone (IMP-T1) (e) 3,5-dihydroxybenzoic acid (IMP-T2) (f) 3,5-dihydroxybenzaldehyde (IMP-T3) (g) 2-methoxyphenol (IMP-G2).

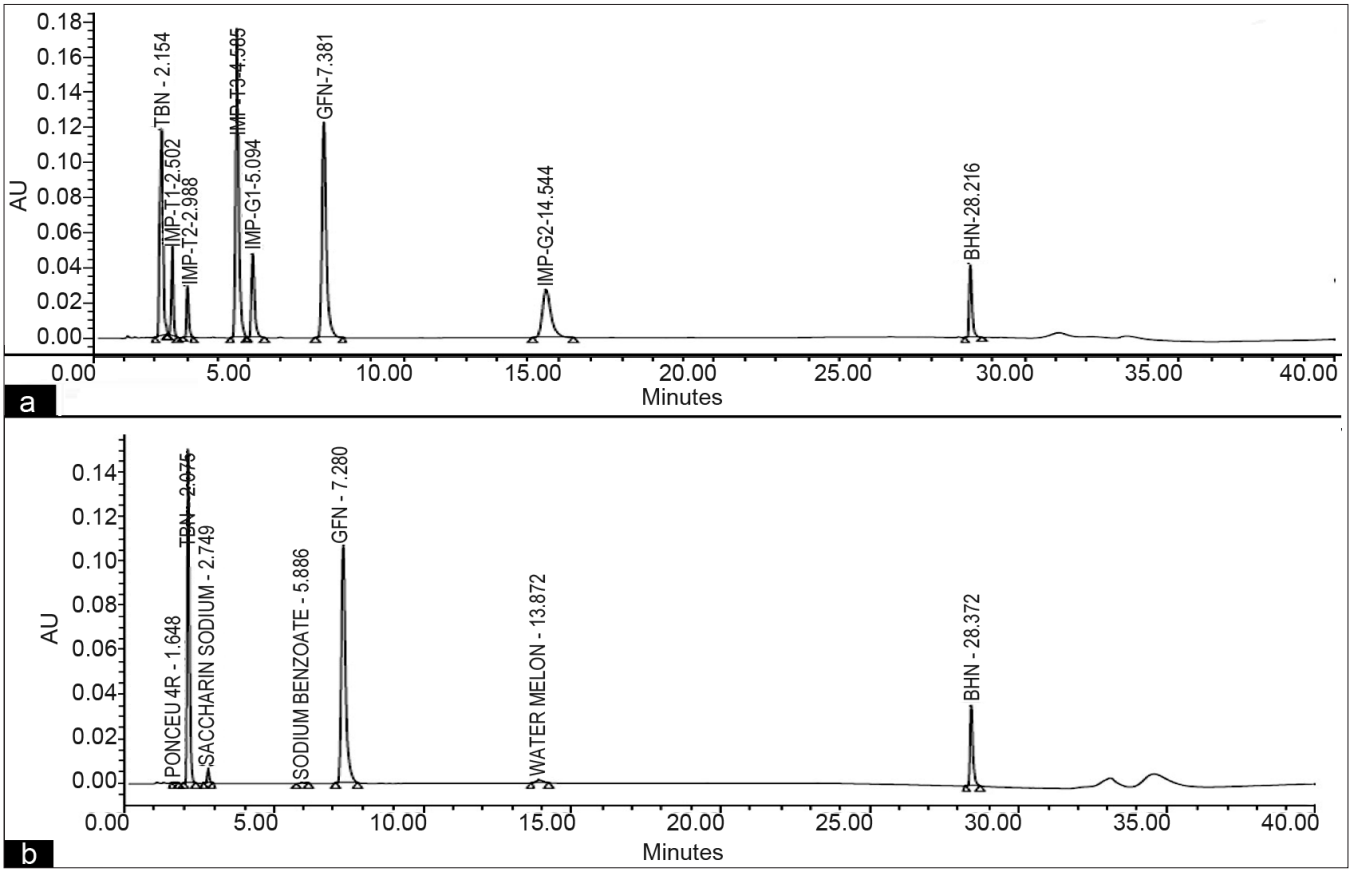

Fig. 2: HPLC chromatograms of mixture of pure samples with impurities and excipients
(a) synthetic solution of TBN, GFN, BHN and impurities IMP-T1, IMP-T2, IMP-T3, IMP-G1, IMP-G2 (b) a model cough syrup formulation with sweetener, coloring agent, preservative, flavoring agent in glycerin base.

separation (exp. no 10 and 11). The optimal gradient selected was the following: First a linear gradient to solvent A, (phosphate buffer, pH 3.0) – Solvent B, (acetonitrile) (80:20, v/v) mixture during 18 min, then a second linear gradient to solvent A – solvent B (60:40, v/v) during 12 min. Finally, the initial conditions were established in 10 min.

HPLC chromatograms in optimized chromatographic condition for mixtures of three active ingredients with

all the five impurities (fig. 2a) and in a model cough syrup formulation (fig. 2b) showed that all the peaks were well separated from the immediate neighboring peak.

To assess the suitability of the method as a stability indicator, stress testing was performed. In most of the different stressed conditions of bulk drugs, moderate to high degradation was found. In GFN and TBN, some impurities among the probable five mentioned impurities were detected, and no impurity peak was detected in BHN even after complete degradation. In neutral and acidic photolytic conditions major formation of IMP-T3 was found in contrast to the IMP-T2 in hydrolytic conditions. The same trend of photolytic degradation of each ingredient was observed by mixing three components together in the model cough syrup. The detailed data are as summarized in Table 2. HPLC chromatograms for all the degradation studies are given in fig. 3.

System suitability and system precision were performed daily by evaluating number of theoretical plates (N), USP resolution between neighboring peaks (Rs) and USP tailing factor (T), which were found well within the specified USP limit (Rs>2, N>4000 and T<1.5). The calibration curves were constructed covering both high level (10-500 µg/ml) and low level (0.5-20.0 µg/ml) concentration for the purpose of active ingredient (AI) and impurity analysis in cough syrup and plotting concentration (µg/ml) against peak area. For each component, a series of five concentration points were prepared and each solution was injected six times. A summary of the data showing the slopes, y-intercept values and 95% Confidence Interval (C.I.) for the slope and y- intercept values for assay and impurities is given in Table 3. The correlation coefficient for the assay of active components and analysis of impurities were all greater than 0.999. In addition, the analysis of residuals for the assay and the impurity showed that the values are randomly scattered around zero, which shows a good fit with the linear model.

The intraday precision showed overall percent recoveries in the range of 98.68 to 99.62% with RSD ranging from 1.51 to 2.08% for the three active ingredients and overall percent recoveries 99.03 to 100.36 with RSD ranging from 0.38 to 1.40% for the impurities. The interday precision showed overall percent recoveries in the range of 99.99 to 102.14% with RSD ranging from 0.90 to 2.30% for the three active ingredients and overall percent recoveries 99.28 to 101.98 with RSD ranging from 0.10 to 2.30% for the impurities. The intermediate precision for analysis of active ingredients and impurities showed percent recoveries in the range of 97.75 to 101.50 with %RSD less than 2.0. By applying analysis of variance (ANOVA) test to the recovery results, the statistical results with 95%

TABLE 2: SUMMARY OF FORCED DEGRADATION RESULTS

Stress condition	Time (h) Temp (°)	% Degradation*			Degradation product[a]			
		GFN	BHN	TBN	GFN		TBN	
					Relative retention	%	Relative retention	%
Base hydrolysis (0.1 N aq, NaOH)	20 80	8.79	98.13	84.62	_	_	1.1(IMP-T1) 1.97	0.015 0.01
Acid hydrolysis	20 80	10.90	10.90	77.75	_	_	1.47(IMP-T2)	1.67
Neutral hydrolysis (H_2O)	20 80	9.50	1.2	76.6	_	_	1.47(IMP-T2)	12.56
Photo degradation in H_2O	72 40	30 (41.68)	55.09 (79.4)	22.86 (23.91)	_	_	1.09 (IMP-T1) 2.13 (IMP-T3)	1.03 8.3
Photo degradation in 0.1 N aq. HCl	72 40	64 (43.68)	87.35 (64.96)	21.69 (39.47)	0.69 (IMP-G1) 0.75 1.94 (IMP-G2)	10.0 5.0 15.0	1.09 (IMP-T1) 2.13 (IMP-T3)	0.9 8.27
Photo degradation in 0.1 N aq. NaOH	72 40	55.85 (53.37)	100.0 (98.25)	80.00 (81.51)	0.69 (IMP-G1) 0.75 1.94 (IMP-G2)	0.5 1.6 36.0	1.17(IMP-T1) 2.18(IMP-T3)	10.79 1.41
Oxidation in H_2O_2	20 80	25.68	7.0	10.5	_	_	1.1(IMP-T1)	10.71

*Degradation in the mixture of the components is shown in bracket; [a]The recovery of unknown degradation products was calculated using respective active ingredient (GFN and TBN) as a reference standard

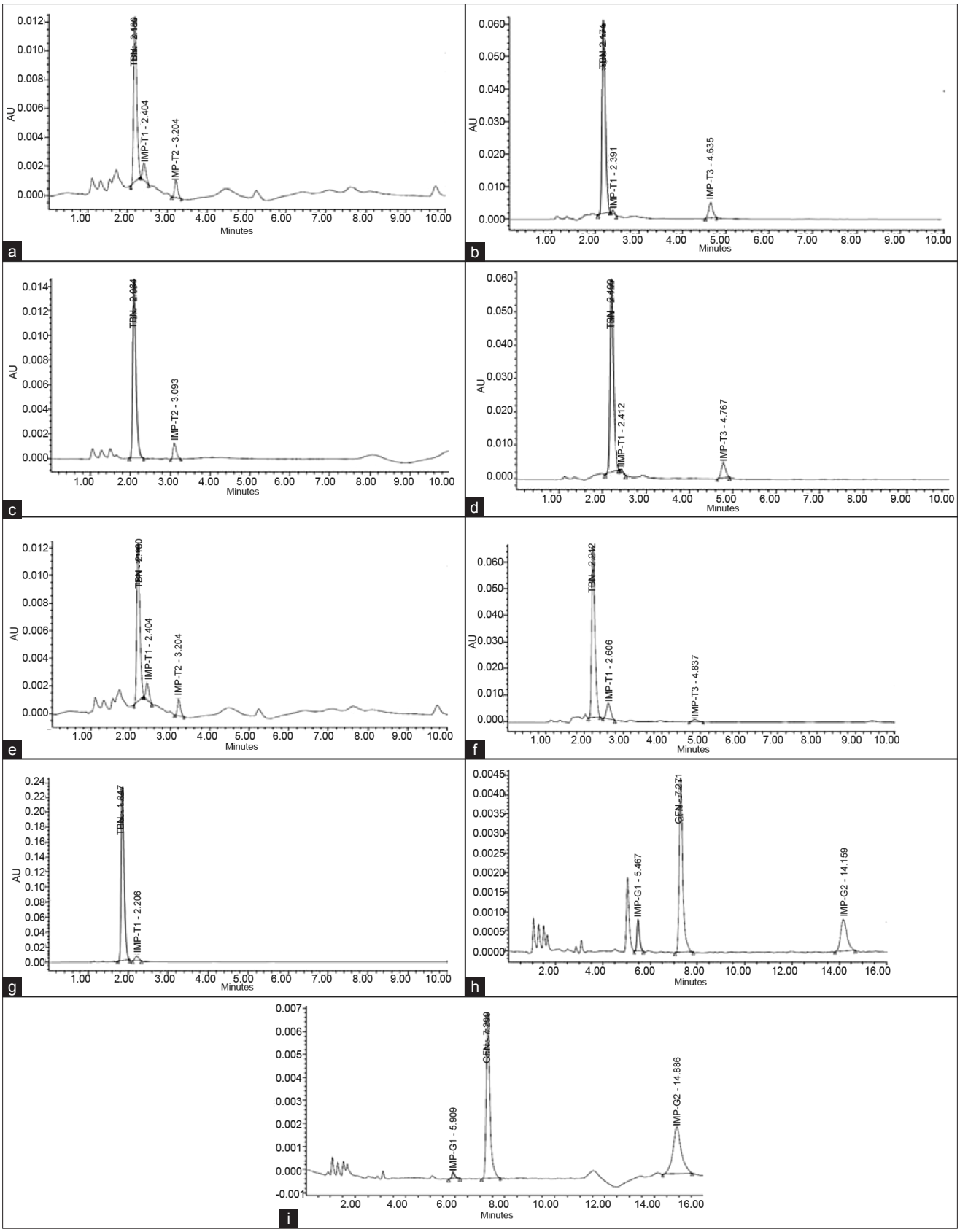

Fig. 3: HPLC chromatograms for stressed samples

(a) TBN in water hydrolytic condition (b) TBN in water photolytic condition (c) TBN in acidic hydrolytic condition (d) TBN in acidic photolytic condition (e) TBN in basic hydrolytic condition (f) TBN in basic photolytic condition (g) TBN in oxidative condition (h) GFN in acidic photolytic condition (i) GFN in basic photolytic condition.

confidence limit indicate that there was no significant differences between inter and intra-day analysis results in respect of tabulated F value. The results are summarized in Table 4.

The accuracy for the applied HPLC method was confirmed by applying it to the synthetic mixtures of TBN, BHN, and GFN and their impurities in different concentration levels of 100, 200, 400 μg/ml. Mean recovery of nine (3×3) replicates of GFN ranged from 100.10 to 100.83% with RSD range 0.30 to 0.80%, overall recovery 100.50% and a 95% confidence interval (CI) of ±0.21. Mean recovery of nine replicates of TBN ranged from 98.81 to 100.63% with RSD range 0.30 to 1.40%, overall recovery 99.66% and a 95% confidence interval (CI) of ±0.71. Mean recovery of nine replicates of BHN ranged from 98.80 to 101.15% with RSD range 0.80 to 1.56%, overall recovery 100.08% and a 95% confidence interval (CI) of ±0.91. The accuracy of the method for the impurity analysis was determined by fortifying placebo and three active drug substances with known amount of the five impurities at three different concentration levels ranging from 0.5 to 2.0 μg/ml. The mean recovery of impurities of three replicates remained within 93.00% to 100.5% and % RSD less than 5.0. This indicates that the assay value obtained accurately represented the true drug content in the formulation over the method range 10-500 μg/ml. No difference in precision between the upper and lower limits of the method ranges was found using an ANOVA at the 95% confidence level as evidenced by the F ratio for each component. The results are summarized in Table 5.

The limit of detection (LOD) of IMP-T1, IMP-T2, IMP-T3, IMP-G1 and IMP-G2 were found to be 0.156, 0.033, 0.135, 0.061 and 0.025 μg/ml at a signal to noise ratio 3:1. The limits of quantitation (LOQ) of IMP-T1, IMP-T2, IMP-T3, IMP-G1 and IMP-G2 were found to be 0.483, 0.099, 0.411, 0.182 and 0.077 μg/ml. The precision of the impurities at LOQ level was below 5.0% RSD.

In the robustness study with the small but deliberate variations in method parameters e.g., pH of buffer in mobile phase (pH 3.12, 3.00, 2.88), mobile phase ratio (buffer: ACN, v/v 82:18, 80:20, 78:22) and flow rate (0.95, 1.00, 1.05 ml/min) there were no dramatic changes in the chromatographic parameters (K', T and N) for active ingredients found. Percent recoveries of three active ingredients remained within the range 98.69-101.81 with %RSD ranged between 0.1 to 2.0.

The RSD of the assay of all the three active ingredients namely TBN, BHN and GFN during solution stability and mobile phase stability study was within 1.40 % assuring that sample solutions and mobile phase used during assays can be preserved for 72 h and 48 h, respectively.

TABLE 3: LINEARITY AND REGRESSION DATA

Parameter	Compound							
	GFN	TBN	BHN	IMP-T1	IMP-T2	IMP-T3	IMP-G1	IMP-G2
Range (μg/ml)	10-500	10-500	10 -500	0.5-20.0	0.5-20.0	0.5-20.0	0.5-20.0	0.5-20.0
Slope	0.520	0.409	0.142	0.1624	2.733	2.39	6.25	8.90
95% CI for mean slope	±0.007	±0.01	±0.002	±0.0013	±0.002	±0.01	±0.06	±0.01
Intercept	4.913	2.470	1.142	0.05	-0.61	1.52	-1.04	-3.76
95% CI for mean intercept	±1.48	±0.99	±0.40	±0.02	±0.04	±0.43	±0.18	±0.27

Number of points on each regression line is five, TBN - bronchodialator, terbutaline sulphate; BHN - mucolytic agent, bromhexine hydrochloride; GFN - expectorant, guaifenesin

TABLE 4: REPEATABILITY AND INTERMEDIATE PRECISION DATA

Substance	Added conc. (μg/ml)	Repeatability		Intermediate precision			
		Intra-day measured concentration		Inter-day measured concentration		Different analyst (day, column) measured concentration	
		Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
TBN	200	98.68	1.51	100.78	0.9	101.5	1.3
BHN	200	98.91	1.79	99.99	1.3	101.68	1.5
GFN	200	99.62	2.08	102.14	2.3	101.18	1.3

Repeatability and intermediate precision data of HPLC assay of TBN, BHN and GFN (n=6), TBN - bronchodialator, terbutaline sulphate; BHN - mucolytic agent, bromhexine hydrochloride; GFN - expectorant, guaifenesin

TABLE 5: RECOVERY DATA

Substances	Added (μg/ml)	Found (μg/ml)	Recovery (%)	RSD (%)
TBN	100.00	98.81	98.81	0.40
BHN	100.00	101.15	101.15	0.80
GFN	100.00	100.58	100.58	0.30
IMP T-1	0.50	0.515	103.00	2.30
IMP T-2	0.50	0.499	99.80	2.40
IMP T-3	0.50	0.492	98.40	0.40
IMP G-1	0.50	0.513	102.60	0.60
IMP G-2	0.50	0.500	100.00	1.30
TBN	200.00	201.26	100.63	1.40
BHN	200.00	197.76	98.80	1.56
GFN	200.00	201.67	100.83	0.40
IMP T-1	1.00	0.93	93.00	4.60
IMP T-2	1.00	1.00	100.00	0.70
IMP T-3	1.00	1.00	100.00	0.50
IMP G-1	1.00	0.99	99.74	0.20
IMP G-2	1.00	1.00	100.00	0.50
TBN	400.00	398.22	99.55	0.3
BHN	400.00	401.16	100.29	1.52
GFN	400.00	400.4	100.10	0.80
IMP T-1	2.00	1.99	99.55	4.20
IMP T-2	2.00	2.01	100.50	0.80
IMP T-3	2.00	1.98	99.15	0.90
IMP G-1	2.00	2.00	100.00	1.40
IMP G-2	2.00	2.00	100.00	0.70

Results of recovery analysis of TBN, GFN, BHN and its impurities at different concentration level (n=3), TBN - bronchodialator, terbutaline sulphate; BHN - mucolytic agent, bromhexine hydrochloride; GFN - expectorant, guaifenesin

The applicability of the validated method was also tested by analyzing samples of a model cough syrup and a commercial cough syrup containing the three active ingredients and excipients namely sodium benzoate, saccharin sodium, ponceau 4R supra and water melon flavor in glycerin base. The chromatogram of the model cough syrup analysis is shown in fig. 2b. The recovery (99.94% to 100.01%) and %RSD (0.025 to 1.56) of the assay method were well within the limit and indicated that the proposed method can be used for quantitation of TBN, BHN and GFN and impurities in a single run, in routine quality control analysis of cough-cold syrup.

The Infrared spectrum of IMP-G1 revealed a sharp band at 3650 cm^{-1} in very dilute solution of carbon tetrachloride due to the presence of "free" phenolic hydroxyl group. The other characteristic bands in KBr are as follows (cm^{-1}): 3800-3200 (O-H stretching mode associated with hydroxyl groups), 1920-1600 (ortho- substituted aromatic overtones), 1350-1263 (C=C stretching of aromatic group), 1266-1092 (C-O stretching vibration of phenolic and aliphatic hydroxyl group).

The assignments of signals of the NMR spectra of IMP-G1 and its acetate derivative (prepared by usual procedure of acetylation) are given as follows:

^{1}H NMR of IMP-G1 (300 MHz, $CDCl_3$): δ 6.79-6.94 (4H, m, Ar-H), 4.89 (1H, brs, Ar-OH), 4.10 (1H, d, J=6 Hz, hydroxyl proton of -CHOH-), 3.95-4.05 (3H, m, methylene and methine protons of Ar-OCH_2-CHOH-), 3.70 (2H, d, J=4.5 Hz, methylene protons of –CH(OH)OCH_2-), 3.32 (1H, s, hydroxyl proton of -CH_2OH).

^{1}H NMR of acetate of IMP-G1 (300 MHz, $CDCl_3$): δ 6.84-7.12 (4H, m, Ar-H), 5.31 (1H, m, methine protons of Ar-OCH_2-CHOH-), 4.28 (1H, dd, J_1=12 Hz and J_2=3.9 Hz, H_a proton of Ar-OCH_2-), 4.14 (1H, dd, J_1=12 Hz and J_2=5.7 Hz, H_b proton of –ArOCH_2-), 4.03 (2H, d, methylene protons of -CH_2OH), 2.2, 2.0, 1.99 (3 × 3H, s, methyl protons of three -$OCOCH_3$).

The presence of three hydroxyl groups in the IMP-G1 was confirmed by the appearance of three distinct acetate singlets at 2.2, 2.0 and 1.99 ppm in its acetate derivative. The absence of any signal for o-methoxy

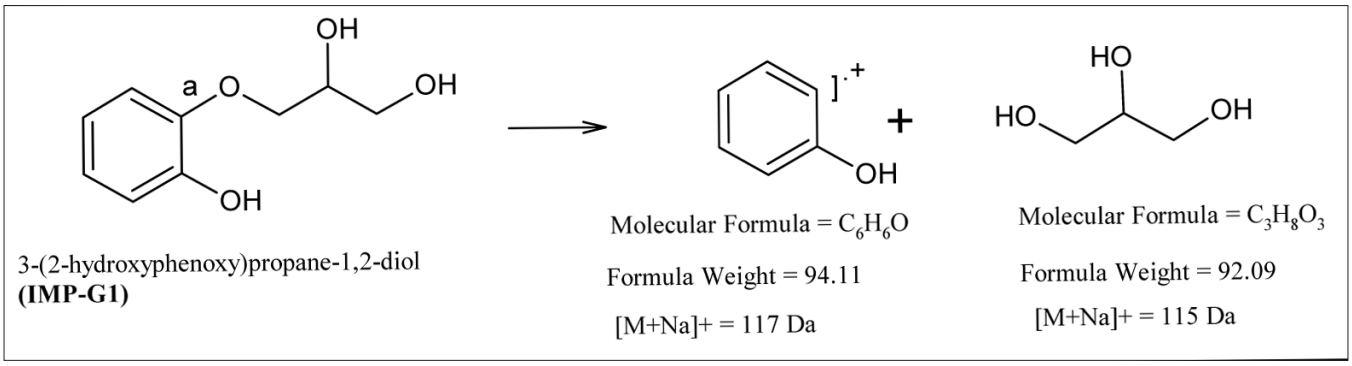

Fig. 4: Mass fragmentation pattern of IMP-G.

group further supports the demethylation leading to the formation of IMP-G1. The two geminal protons H_aH_b of the aromatic $–OCH_2$ group are diastereotopic and so anisochronous, and showed two different chemical shift values. The equatorial H_a proton exhibited geminal coupling with axial H_b proton and also with the vicinal proton of –CHOH at 4.28 ppm (J_1=12 Hz, J_2=3.9 Hz) as AB quartet. The axial proton H_b coupled similarly with equatorial counterpart H_a and also with vicinal pseudoaxial proton of -CHOH at 4.14 ppm as AB quartet. The pseudoaxial proton of –CHOH group appeared as a multiplet at 5.31 ppm. The other two geminal protons of the $–CH_2OH$ group appeared as doublet at 4.03 ppm.

The positive ion Electrospray Ionization (ESI) mass spectrum of IMP-G1 exhibited a sodiated molecular ion, $[M+Na]^+$ at m/z 207 confirming the molecular weight 184 Da. The decrease of 14 mass units from GFN molecular weight 198, suggested the demethylation of GFN. To obtain additional structural information, ESI- MS/MS analyses were performed. The MS/MS spectrum of IMP-G1 contained the sodiated product ions, m/z 115, 117 (100%), 145 (70%), 133 (20%), 99.9 (15%), 174 (20%) (rounded value given). Cleavage of bond (a) resulted in product ions at m/z 115 $[M+Na]^+$, representing the aliphatic portion and m/z 117 $[M+Na]^+$ representing the demythylated aromatic portion of the molecule (fig. 4). Therefore the MS/MS data confirmed the demethylated structure for IMP-G1. Based on the combination of the IR, NMR and MS data, IMP G-1 was identified as (2RS)-3-(2-hydroxyphenoxy)-propane-1,2-diol.

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