<u>**Research Article**</u>

DEVELOPMENT AND VALIDATION OF RP-HPLC AND HPTLC METHOD OF ANALYSIS FOR SIMULTANEOUS ESTIMATION OF AMBROXOL HCL, DEXTROMETHORPHAN HBR AND GUAIFENESIN IN PHARMACEUTICAL COUGH COLD PREPARATION AND STATISTICAL COMPARISON OF DEVELOPED METHODS

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ABSTRACT

Pharmaceutical Cough Cold Preparations like syrup are widely used worldwide and mostly in countries like India. Aim and Objective of the study is to develop simple, precise, accurate, rapid and sensitive Method of Reverse Phase High Performance Liquid Chromatography (RP-HPLC) and High Performance Thin Layer Chromatography (HPTLC) for simultaneous estimation of Ambroxol HCl (AMB), Dextromethorphan HBr (DEX) and Guaifenesin (GUA) in Pharmaceutical Cough Cold Preparation and statistical comparison of both method was done.

For RP-HPLC method Hibar RP-C18 (250mm × 4.6mm i.d. with particle size of 5μm) analytical column was used. The mobile phase composition used for RP-HPLC method was Acetonitrile - Methanol - 10mM Phosphate Buffer – 0.3% Triethyl Amine (25:15:60 v/v) and pH was adjusted to 3 using Orthophosphoric Acid. Flowrate was kept 1 mL/min. Detection was done at 205 nm using PDA detector. Linearity range for RP-HPLC method was found to be 3-10.5 μg/ml, 2-7 μg/ml and 10-35 μg/ml for AMB, DEX and GUA respectively.

In HPTLC method Merck HPTLC plates precoated with 60F254 silica gel on aluminium plates were used as stationary phase. Mobile phase used in HPTLC method was Toluene – Methanol – Chloroform – Glacial Acetic Acid (6.5:1.5:1.5:0.5 v/v/v/v). Detection was done using Camag TLC scanner at 275 nm. Linearity range was found to be 1.5-3 μg/band, 1-2 μg/band and 5-10 μg/band for AMB, DEX and GUA respectively for HPTLC method.

Statistical comparison of RP-HPLC and HPTLC was done by application of t-Test to recovery data of both methods. Finally concluded that A novel RP-HPLC and HPTLC methods were developed for simultaneous estimation of AMB, DEX and GUA in syrup dosage form. Both the method gives the good resolution for all three drugs. The developed method was validated and found to be sensitive, accurate, specific and reliable for simultaneous estimation of AMB, DEX and GUA in syrup dosage form. The proposed method can be used effectively in routine analysis.

KEY WORDS: Ambroxol HCl, Dextromethorphan HBr, Guaifenesin, Reverse Phase – High Performance Liquid Chromatography, High Performance Thin Layer Chromatography.

INTRODUCTION

Cough is protective reflex of human body and removes the foreign materials from the respiratory tract. It is a very common effect of *angiotensin-converting enzyme inhibitors* [1]. Cough and cold is very common disease in the human. It is generally treated with combination dosage form. Combination of Ambroxol HCl (AMB), Dextromethorphan HBr (DEX) and Guaifenesin (GUA) has been recently developed and marketed by Percept Health Care under the brand name *Cotussin DM.*

4-[[(2-Amino-3,5-dibromophenyl) methyl]amino]cycophexanol Hydrochloride (AMB) is mucolytic agent. It depolymerises the mucous. 3-methoxy-17-methyl-9α, 13α, 14α-morphinan hydrobromide monohydrate (DEX) is nonopoid antitussive and acts by cough centre suppression. 3-(2-methoxyphenoxy)propane-1,2-diol (GUA) is bronchial secretion enhancer and it increases the mucociliary function of respiratory tract [2] and [3].
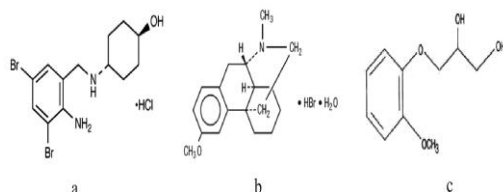

Fig. 1 Chemical structures of (a) Ambroxol HCl, (b) Dextromethorphan HBr and (c) Guaifenesin

Literature review reveals that analytical HPLC and HPTLC methods have been reported for simultaneous estimation of AMB and GUA in combination dosage forms [4], [5], [6], [7], [8], [9] and [10]. Analytical HPLC methods have been reported for simultaneous estimation of DEX and GUA [11], [12], [13] and [14]. There are also analytical methods has been reported for estimation of AMB alone or with combination of other drugs [15], [16], [17], [18], [19], [20], [21] and [22]. For estimation of DEX by HPLC few methods are reported [23], [24], [25], [26] and [27] likewise for GUA in combination with other drugs HPLC methods has been reported [28], [29], [30] and [31].

Literature review also revealed that no any method has been developed for simultaneous estimation of AMB, DEX and GUA in single combination dosage form. In this study simple, rapid and sensitive RP-HPLC and HPTLC has been developed. Validation has been done by the ICH guideline Q2R1 [32].

After development statistical tests has been applied to both methods for comparison. Because of two methods t-Test was applied to compare the methods [33].

MATERIALS AND METHODS

Materials

AMB, DEX and GUA were obtained from the store of Department of Pharmaceutical Sciences, Saurashtra University, Rajkot, Gujarat, India and used without further purification. All other chemicals and reagents were used of HPLC grade and purchased from the company Merck Chemicals, India. Cotussin DM syrup was obtained from the Percept Health Care, Gujarat, India as a gift sample.

Instrumentation and Chromatographic Conditions

RP-HPLC Method

HPLC system used was consisting of two pump, manual injector, detector and column. HPLC system used was of Shimadzu LC solution-20AD, Kyoto, Japan and software used was LC solution. Sample injector used having 20μl fixed volume capacity. Detector

used in HPLC system was SPD-M20A-PDA detector and column used was Hibar RP-C18 Column (250mm×4.6mm i.d. with particle size of 5µm). Various mobile phases have been tested in order to obtain the best suitable mobile phase for the RP-HPLC method. The composition of optimized mobile phase was Acetonitrile - Methanol - 10mM Phosphate Buffer – 0.3% Triethyl Amine (25:15:60 v/v) [pH was adjusted to 3 using Orthophosphoric Acid]. The flow rate was kept 1mL/min and detection was done at 205nm.

HPTLC Method

HPTLC system was consisting of Applicator, TLC scanner and TLC Visualizer. HPTLC system used was of Camag, Muttenz, Switzerland and software used was Wincat. The samples were applied using applicator Linomat 5 using Hamilton syringe having capacity of 100µl. Samples were applied on aluminium plates precoated with silica gel 60F254(20×10 cm and 10×10 cm) which were purchased from Merck, India. Scanning was done using TLC scanner of Camag, Muttenz, Switzerland at wavelength 275nm. TLC chamber used are also of Camag, Muttenz, Switzerland and saturation time was kept 15min. TLC plates were prewashed with methanol and activated at 110ºC for 5 min before use. Different mobile phase has been used to obtained the best mobile phase and best suitable result was obtained in the mobile phase Toluene – Methanol – Chloroform – Glacial Acetic Acid (6.5:1.5:1.5:0.5 v/v/v/v).

Extraction Process

In this method dosage form used is in syrup form. Hence, extraction of drugs from the syrup is required before the estimation. Each 5ml of Cotussin DM syrup contains 15mg of AMB, 10mg of DEX and 50mg of GUA. Drugs were extracted by liquid-liquid extraction method. For extraction 5ml of syrup was taken in separating funnel and syrup was diluted with 10ml of water. Then 20ml of chloroform was added to the separating funnel and extracted. Then chloroform layer was separated from the aqueous layer. Again successive extraction of aqueous layer was done two times using 20ml of chloroform. Then 0.5ml of ammonia was added to the aqueous layer. Again aqueous layer was extracted three times using 20mll of Chloroform. Chloroform layer was separated and all the chloroform layers are combined in beaker. Then chloroform was evaporated till the dryness. After evaporation the remaining residues are dissolved in 7-8ml of methanol. Then volume was made upto 10ml with methanol. The final solution contains 1500 µg/ml of AMB, 1000 µg/ml of DEX and 5000 µg/ml of GUA.

Preparation of Standard Stock Solution

10mg of DEX, 15mg of AMB and 50mg GUA was accurately weighed then transferred to 100ml volumetric flask. 70-80ml of Methanol was added and sonicated to dissolve the drugs. Volume was made up to the mark with Methanol. The final was solution contained 100µg/ml of DEX, 150µg/ml of AMB and 500µg/ml of GUA.

Preparation of Standard Test Solution

5ml of syrup was taken and extracted the drug using the extraction process described in **2.2.3**. After extraction the extract contained 1000µg/ml of DEX, 1500µg/ml of AMB and 5000µg/ml of GUA. 1ml above solution was pipette out and was transferred it to 10ml volumetric flask and volume was adjusted upto the mark using Methanol. The final solution contained 100µg/ml of DEX, 150µg/ml of AMB and 500µg/ml of GUA.

Method Validation

RP-HPLC and HPTLC methods were validated as per the guideline of International Conference on Harmonization Q2R1. Linearity, Precision, Accuracy, Specificity, Robustness, LOD and LOQ were taken in consideration for the validation of both the methods.

Linearity

Six points calibration curve were generated for RP-HPLC and HPTLC methods. Working standards for both the methods were prepared from the standard stock solution and calibration curves were generated. In RP-HPLC method linearity range was found to be 3-10.5 µg/ml, 2-7 µg/ml and 10-35 µg/ml for AMB, DEX and GUA

respectively. The calibration range for HPTLC method was found to be 1.5-3 µg/band, 1-2 µg/band and 5-10 µg/band for AMB, DEX and GUA respectively.

Precision

Precision was done by taking chromatogram of six replicates of same concentration made from the working standard for both the methods. Then %RSD was calculated from the area of peak. Likewise Intraday and Interday precisions were taken and %RSD was calculated.

Accuracy

Accuracy was done by the standard addition method. Accuracy is % of analyte which is recovered from the added known amount of drug. For both RP-HPLC and HPTLC methods 50%, 100% and 150% of the analyte was added as standard addition and then chromatogram was taken and % Recovery was calculated.

Specificity

For specificity chromatogram of standards are compared with the chromatogram of the drug after adding the excipient. In this RP-HPLC and HPTLC method Menthol was added as an excipient as it was one of the component of syrup and then chromatogram was compared with the standard chromatogram.

Robustness

For robustness various chromatographic conditions are changed and then the chromatogram of standard is compared with the altered condition chromatogram. For RP-HPLC method two altered wavelength 203nm and 207nm and two altered pH 2.8 and 3.2 were taken and then chromatograms were taken and compared with the standard. And for HPTLC two altered wavelength 273nm and 277nm and two altered saturation time 13min and 17min were taken.
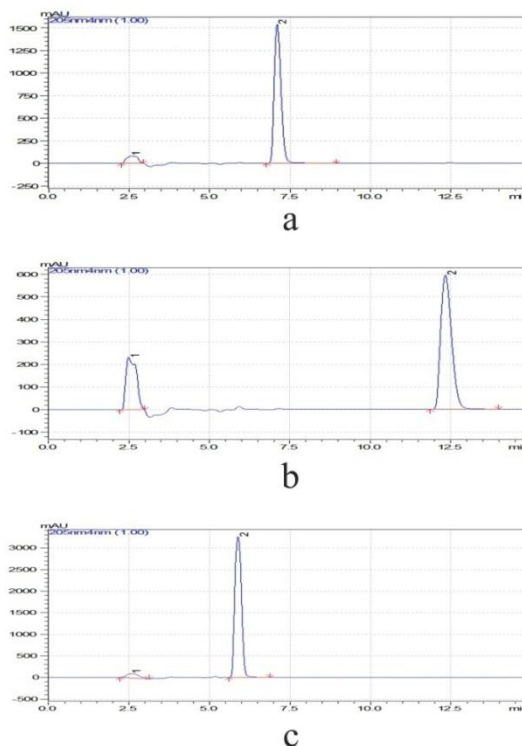

Fig. 2: HPLC Chromatograms of elution of (a) Ambroxol HCl, (b) Dextromethorphan HBr (c) Guaifenesin

LOD and LOQ

LOD (Detection Limit) and LOQ (Quantitation Limit) for both methods are obtained by calculation method [32]. LOD and LOQ are calculated by the following equations.

LOD = 3.3σ/S; LOQ = 10σ/S

Where σ = standard deviation of the response

S = slope of the calibration curve

RESULT AND DISCUSSION

RP-HPLC Method

RP-HPLC method was developed for simultaneous estimation of AMB, DEX and GUA. It can be used for estimation of drug in market formulation in routine analysis. All the chromatographic conditions were optimized to get the best separation of drugs. Optimized mobile phase was found to be Acetonitrile - Methanol - 10mM Phosphate Buffer – 0.3% Triethyl Amine (25:15:60 v/v) and pH was adjusted to 3 using Orthophosphoric Acid and flow rate was kept 1mL/min. **Fig. 2** shows the chromatograms of elution of AMB, DEX and GUA. The retention time was found to be 5.87 min, 7 min and 12.33 min respectively for GUA, AMB and DEX respectively. **Fig. 3** shows the overlay spectra of linearity of AMB, DEX and GUA.

Table 1 Shows the system suitability parameters for RP-HPLC method. By six point calibration curve the linearity of AMB was found to be 3-10.5 μg/ml and the linearity equation was found to be y=3.3617x + 3992.5.

The correlation coefficient was found to be 0.9978 for AMB. The linearity of DEX was found to be 2-7 μg/ml with correlation coefficient 0.9982 and linearity equation was found to be y=3.0595x + 1399.9. The linearity of GUA was found to be 10-35 μg/ml with correlation coefficient 0.9984 and linearity equation was found to be y=1.1559x + 25960. Hence, the method was found to be linear for the above concentration range for all three drugs. The method was found to be simple, sensitive, accurate and robust.
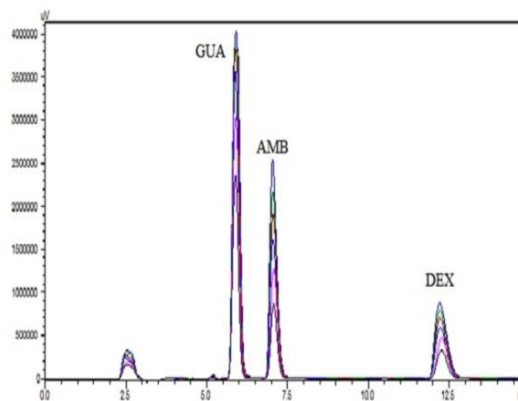

Fig. 3: HPLC overlay spectra of Ambroxol HCl, Dextromethorphan HBr and Guaifenesin

Table 1: System suitability parameters for HPLC

System suitability parameters	AMB	DEX	GUA
Retention Time (minutes)	7.0	12.33	5.87
Theoretical Plates	5048.384	5982.814	3904.307
Tailing Factor	1.3	1.3	1.214
Resolution	2.44	9.63	5.95

Table 2 shows the data of precision and **Table 3** shows the accuracy data for RP-HPLC method. % recovery was found to be 99.77-101.44%, 99.53-100.0 and 99.30-100.19% for AMB, DEX and GUA respectively.

Table 2: Precision Data for HPLC

		AMB (6μg/ml)	DEX (4μg/ml)	GUA (20μg/ml)
Intraday Precision	SD (n=6)	222.38	241.14	346.16
	%RSD	0.95	1.67	0.82
	SD (n=6)	76.87	103.11	122.81
	%RSD	0.27	0.71	0.25
	SD (n=6)	430.9	136.3	136.3
	%RSD	1.5	0.96	0.96
Interday Precision	SD (n=6)	331.69	189.92	532.61
	%RSD	1.4	1.4	1.2
	SD (n=6)	263.8	139.2	312.85
	%RSD	0.93	0.95	0.63
	SD (n=6)	300.85	240.12	724.4
	%RSD	1.1	1.7	1.5

Table 3: Accuracy Data for HPLC

	Standard addition	Amount of Drug (μg/ml)	Amount of API added (μg/ml)	Avg. Amount of Drug recovered (μg)	Avg. % Recovery
AMB	50%	4.5	2.25	2.247	99.86
	100%	4.5	4.5	4.56	101.44
	150%	4.5	6.75	6.73	99.77
DEX	50%	3	1.5	1.49	99.57
	100%	3	3	3	100.00
	150%	3	4.5	4.48	99.53
GUA	50%	15	7.5	7.45	99.30
	100%	15	15	14.97	99.78
	150%	15	22.5	22.54	100.19

Calculated LOD and LOQ shows that the method is sufficiently sensitive and LOD and LOQ data are shown in **Table 4**. All the data of result shows that the developed method was sufficiently accurate, precise and sensitive for the routine use.

Table 4: Validation parameters for HPLC and HPTLC

Validation Parameters	AMB	DEX	GUA
HPLC			
Range (µg/ml)	3 - 10.5	2 - 7	10 - 35
Regression equation	y=3.3617x + 3992.5	y=3.0595x + 1399.9	y=1.1559x + 25960
Correlation coefficient (r^2)	0.9978	0.9982	0.9984
Limit of Detection(µg/ml)	0.147	0.068	1.36
Limit of Quantitation (µg/ml)	0.445	0.205	4.12
HPTLC			
Range (µg/band)	1.5 - 3	1 - 2	5 - 10
Regression equation	y=2112x + 1050.7	y=2441.4 + 785.79	y=981.11x + 5139.1
Correlation coefficient (r^2)	0.9977	0.9962	0.9961
Limit of Detection(µg/band)	0.056	0.056	0.612
Limit of Quantitation (µg/band)	0.171	0.171	1.853

Table 5: Precision Data for HPTLC

		AMB (2.1µg/band)	DEX (1.4µg/band)	GUA (7µg/band)
Intraday Precision	SD (n=6)	29.42	57.25	143.06
	%RSD	1.05	1.87	1.13
	SD (n=6)	20.05	43.45	132.22
	%RSD	0.76	1.43	1.10
	SD (n=6)	36.63	41.68	144.33
	%RSD	1.41	1.62	1.12
Interday Precision	SD (n=6)	47.6	35.9	70.66
	%RSD	1.32	1.03	0.59
	SD (n=6)	52.31	43.5	95.92
	%RSD	1.55	1.45	0.79
	SD (n=6)	36.13	22.0	176.2
	%RSD	1.16	0.78	1.55

Table 6: Accuracy Data for HPLC

	Standard addition	Amount of Drug (µg/band)	Amount of API added (µg/band)	Avg. Amount of Drug recovered (µg)	Avg. % Recovery
AMB	50%	1.5	0.75	0.745	99.31
	100%	1.5	1.5	1.49	99.61
	150%	1.5	2.25	2.228	99.02
DEX	50%	1	0.5	0.493	98.69
	100%	1	1	0.997	99.7
	150%	1	1.5	1.527	101.8
GUA	50%	5	2.5	2.49	99.73
	100%	5	5	4.935	98.70
	150%	5	7.5	7.46	99.43

LOD and LOQ are obtained by calculation method and shown in **Table 4.** The developed HPTLC method was found to be accurate, precise, reliable and sensitive for estimation of drug in routine analysis.

Table 7: t-Test comparison for HPLC and HPTLC methods

Parameter	Value		
	AMB	DEX	GUA
Name of test	Two tailed t-Test	Two tailed t-Test	Two tailed t-Test
No. of pairs	3	3	3
P value	0.1214	0.7360	0.4139
P value summary	Ns	Ns	Ns
Significance difference (p<0.05)	No	No	No
t value	2.602	0.3871	1.023
df (Degree of freedom)	2	2	2
Mean of differences	1.039	-0.3733	0.4700
95% confidence interval	-0.6792 to 2.758	-4.524 to 3.777	-1.507 to 2.447
R square	0.7720	0.06969	0.3436
Correlation coefficient (r)	0.8920	-0.2626	-0.3264
P value (one tailed)	0.1493	0.4154	0.3942

HPTLC Method

A rapid, sensitive and accurate HPTLC method was developed and validated for the simultaneous estimation of AMB, DEX and GUA in syrup dosage form. Mobile phase was optimized for effective separation of drugs by HPTLC. Optimized mobile phase used was Toluene – Methanol – Chloroform – Glacial Acetic Acid (6.5:1.5:1.5:0.5 v/v/v/v). **Fig. 4** shows the chromatogram of individual drugs, standard stock solution and test solution. By using six points calibration curve method linearity range of AMB was found to be 1.5-3 µg/band. Linearity equation was found to be y=2112x + 1050.7 and correlation coefficient was found to be 0.9977. Linearity equation for DEX was found to y=2441.4x + 785.79 and correlation coefficient was found to be 0.9962 and for GUA linearity equation was found to be y=981.11x + 5139.1 with correlation coefficient 0.9961.
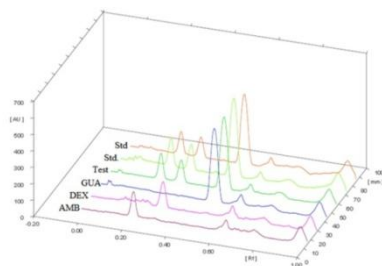

Fig. 4: HPTLC chromatogram of individual drugs, standard stock solution and test solution
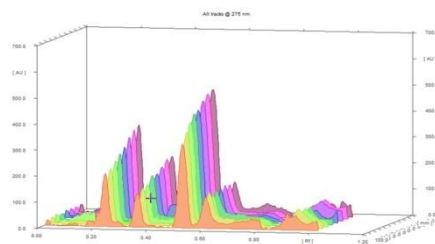

Fig. 5: HPTLC overlay spectra of Ambroxol HCl, Dextromethorphan HBr and Guaifenesin
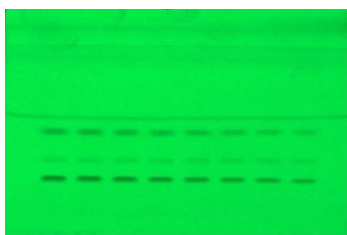

Fig. 6: Photograph of chromatographic plate of linearity under the UV light

Fig. 5: Shows the overlay spectra of linearity and Fig. 6 shows the photograph of chromatographic plate of linearity.

Precision data is shown in **Table 5** and accuracy data are shown in **Table 6.** From the accuracy %Recovery was found to be 99.02-99.61%, 98.69-101.8% and 98.7-99.73% for AMB, DEX and GUA respectively.

Statistical comparison

t-Test was applied to the accuracy data of the RP-HPLC and HPTLC of all three drugs and comparison was done. For comparison GraphPad Prism software (Prism 5 for Windows, Version 5.03) was used. The data of t-Test is shown in **Table 7.** P value was found to be 0.1214, 0.736 and 0.4139 for AMB, DEX and GUA respectively. t-Test shows that there is no significant difference between both the methods and both the methods can be used for estimation of drug in routine analysis as both the method gives the %Recovery nearly to 100%.

CONCLUSION

A novel RP-HPLC and HPTLC methods were developed for simultaneous estimation of AMB, DEX and GUA in syrup dosage form. Both the method gives the good resolution for all three drugs. The developed method was validated and found to be sensitive, accurate, specific and reliable for simultaneous estimation of AMB, DEX and GUA in syrup dosage form. The proposed method can be used effectively in routine analysis.

ACKNOWLEDGMENT

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