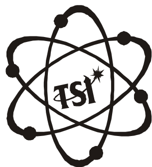

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Development and validation of RP-HPLC method for simultaneous estimation of gemifloxacin mesylate and ambroxol hydrochloride in pharmaceutical formulation

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ABSTRACT

A simple, precise, sensitive, and validated reverse phase high performance liquid chromatographic method has been developed and validated for simultaneous estimation Gemifloxacin mesylate (GFM) and Ambroxol hydrochloride (AMB) in tablets. Chromatographic seperation was performed on agilent ODS C_{18} (250 × 4.6 mm i.d., 5µm) column with a mobile phase comprising mixture of 25 mM potassium dihydrogen orthophosphate buffer (pH 3.5, adjusted with orthophosphoric acid) : acetonitrile (75:25 v/v) at a flow rate 1 ml/min, with UV-detection at 246 nm. Separation was completed in less than 10 min and retention time for GFM and AMB by proposed method was found to be 7.38 and 8.73 min, respectively. The validated calibration range for GFM and AMB was 64-320 µg/ml and 24-120 µg/ml, respectively. The LODs were 2.920 and 0.767 µg/ml and the LOQs were 8.85 and 2.32 µg/ml for GFM and AMB, respectively. The suitability of this HPLC method for quantitative determination of the compounds was proved by validation in accordance with the ICH guidelines. Statistical analysis proved that the method was accurate, precise, and reproducible. To establish stability indicating nature of the LC method, forced degradation of drug substances was performed under different stress conditions viz. acid and base hydrolysis, dry heat degradation and oxidation. Since the method is able to selectively quantitate these drugs in presence of their degradation products it can be used as a stability indicating method.

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KEYWORDS

RP-HPLC;
Gemifloxacin mesylate;
Ambroxol hydrochloride;
Validation.

INTRODUCTION

Gemifloxacin mesylate, chemically is 7-[(4*Z*)-3-(aminomethyl) - 4-methoxyimino-pyrrolidin-1-yl]-1-cyclopropyl-6-fluoro-4-oxo-1, 8-naphthyridine-3-carboxylic acid methane sulphonate[1]. It is an oral broad-spectrum quinolone antibacterial agent used in the treatment of acute bacterial exacerbation of chronic bronchitis and mild-to-moderate pneumonia[2,3]. Ambroxol hydrochloride, chemically is trans-4-[(2-amino-3,5dibromobenzyl) amino] cyclohexanol hydrochloride[4]. It is a mucolytic expectorant and used to reduce viscosity of mucous sercretions[5]. Literature survey revealed UV-Spectrophotometric, RP-HPLC and

HPTLC methods reported for estimation of GFM[6-10] and AMB[11-15] individually or in combination with other drugs in biological samples or in pharmaceutical formulations. However, there is no analytical method reported for the simultaneous determination of these drugs in a pharmaceutical formulation. Objective of the present work was to establish inherent stability of GFM and AMB through stress studies under variety of ICH recommended test conditions[16] and to develop a stability indicating assay[17]. This paper describes a simple, rapid and precise RP-HPLC method for simultaneous estimation of GFM and AMB in tablets. The proposed method was validated as per ICH guidelines[18,19].

EXPERIMENTAL

Reagents and chemicals

Standard gift samples of analytically pure Gemifloxacin mesylate and Ambroxol hydrochloride were provided by Hetero Drugs Ltd, Baddi, H.P., India and Inventia Healthcare Pvt. Ltd, Ambarnath, Thane, MS, India, respectively. Combined dose tablet formulation containing Gemifloxacin mesylate (320 mg) and Ambroxol hydrochloride (75 mg), manufactured by Hetero Drugs Ltd, was purchased from local market. Methanol and acetonitrile HPLC grade were obtained from Thermo Fischer Scientific India Ltd., Mumbai. Potassium dihydrogen phosphate and orthophosphoric acid of analytical reagent grade was used. Double distilled water was used for preparation of buffer solution.

Chromatographic system and conditions

A binary gradient HPLC (Agilent 1120 Compact LC) system with double reciprocating pump, manual injector, RP C_{18} column (Agilent TC-$C_{18,}$ 250 × 4.6 mm i.d., packed with 5µm particle size) and variable wavelength detector was used. The gradient pump includes an integrated dual-channel degasser with continuous vacuum using Teflon AF technology. The HPLC system was equipped with EZChrom Elite Compact software. For chromatographic separation, a mixture of 25mM phosphate buffer (pH 3.5 adjusted with orthophosphoric acid) and acetonitrile in the ratio of 75:25 v/v was used as mobile phase; it was filtered through 0.45 µm membrane filter before use and pumped from the respective solvent reservoirs to the column at a flow rate of 1 ml/min. The run time was set at 10 min and column was maintained at 27^{0}C with use of column oven. The volume of injection was 20µl. Prior to the injection of drug solution, the column was equilibrated with the mobile phase flowing through the system. The eluent was monitored at 246 nm and data acquired was stored and analyzed with the software.

Preparation of standard solution

Accurately weighed quantity of GFM (80 mg) and AMB (18.8 mg) was dissolved and diluted with mobile phase to obtain final concentration of 160 µg/ml and 37.6 µg/ml of GFM and AMB, respectively.

Assay sample preparation

Twenty tablets were weighed and average weight was calculated. The tablets were then crushed to obtain fine powder. Tablet powder equivalent to about 80.0 mg GFM and 18.8 mg of AMB was transferred to 50.0 ml volumetric flask, added 30 ml of mobile phase and ultrasonicated for 15 min, volume was then made up to the mark with mobile phase. The resulting solution was mixed and filtered through Whatmann filter paper No 42 and filtrate was appropriately diluted to obtain final concentration of about 160 µg/ml of GFM and 37.6 µg/ml of AMB. The diluted solutions were filtered through 0.20 µm membrane filter to get clear solutions.

METHOD VALIDATION

The proposed HPLC method was validated in accordance with ICH guidelines with respect to linearity, accuracy, precision, limit of detection (LOD), limit of quantification (LOQ), specificity and robustness of method.

Linearity

The standard stock solution of both the drugs were individually diluted in order to prepare five standard solutions, in the concentration range 64-320 µg/ml and 24-120 µg/ml for GFM and AMB, respectively. A 20-µl volume was then injected and chromatographed under above mentioned chromatographic system. A linear regression of the GFM/AMB peak area values *versus* the concentration in µg/ml was performed. Linearity was checked with correlation coefficient *r*2 and with

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homoscedasticity of calibration curve.

Accuracy

The accuracy of an analytical procedure expresses the closeness of agreement between the value, which is accepted either as a conventional true value or an accepted reference value and the value found. The accuracy of the assay method was evaluated by standard addition method at three concentration levels (80 %, 100 % and 120 % of final assay concentration). A known amount of drug substance was added to a preanalysed tablet powder and the sample solutions were prepared in mobile phase. The solutions were filtered through 0.2 µm membrane filter and analysed by proposed chromatographic method. The sample solutions at each concentration level were analyzed in triplicate, and the percent recovery and respective standard deviations were calculated.

Precision, limit of detection and limit of quantification

Intra-day precision (repeatblility) was determined by replicate analysis (n = 3) of the assay preparation on the same day; intermidiate precision was checked by replicate analysis of the solutions on three consecutive days. Assay precision was expressed as relative standard deviation. The LOD was determined from the calibration curve, using the following equation:

$$\mathbf{LOD = 3.3\frac{Sb}{a}}$$

where *S*b is the *y*-intercept standard deviation and *a* is the line slope.

The limit of quantification (LOQ) was calculated using

$$\mathbf{LOQ = 10\frac{Sb}{a}}$$

The test solutions at LOD and LOQ concentrations were injected six times in the chromatograph, and the % RSD of the peak area of replicate injections was calculated.

Specificity

The specificity of the method was assessed by comparing the chromatograms obtained from drug standards and from placebo solution prepared from the excipients most commonly used in pharmaceutical formulations, including microcrystalline cellulose, lactose monohydrate, starch, aerosil, carboxymethylcellulose, titanium dioxide, magnesium stearate and sodium starch glycolate.

Robustness

To demonstrate the robustness of the method, deliberate small changes were made in the optimized method parameters, such as: pH of phosphate buffer, flow rate and composition of mobile phase. The pH of phosphate buffer was changed by ± 0.2 pH units, flow rate was changed by ± 0.1 ml and the composition of mobile phase was veried by ± 2 v/v, of the used optimized conditions. The assay preparation was chromatographed under varied conditions and the effect on the retention time and peak assymetry was studied.

Forced degradation studies

The ICH Q1A (R2) guideline18 requires stress testing to be carried out, in order to elucidate the inherent stabilitycharacteristics of the active substance. Evaluation of susceptibility to oxidation, hydrolytic (acid and base), dry heat and photolytic stability are required. An ideal stability-indicating method is one that quantifies the standard drug alone, and also resolves its degradation products. Intentional degradation was tried by placing the sample drug powder in 3 separate 50.0 ml volumetric flask along with 3.0 ml of regent (0.1 N HCl, 0.1 N NaOH, and 3% hydrogen peroxide solution) for acidic, alkaline and oxidative degradation study, respectively. The flasks were kept in hot air oven at 60^0C for 24 h. For dry heat degradation, tablet powder sample was transferred to 50.0 ml volumetric flask and kept in oven at 60^0C for 24 h. For photolytic degradation, tablet powder sample in 50.0 ml volumetric flask was exposed to sun light for two days. Samples were withdrawn after specified time interval, cooled and analyzed by proposed HPLC method after suitable dilution.

RESULTS AND DISCUSSION

Optimization of chromatographic conditions

As most of the compounds of interest are predominantly polar and of low molecular mass, a reverse phase HPLC column Agilent TC C_{18} (250 mm X 4.6 mm i.d) was selected for analysis. Several mobile phases with

different solvents in varying proportions were tried in order to resolve the two drug peaks with acceptable peak asymmetry, number of theoretical plates and resolution. Mobile phase containing acetonitrile and 25 mM phosphate buffer was found to resolve both drugs, but sharp tailing was observed in GFM peak. The retention behavior and peak shape of both the drugs was then studied with respect to pH of buffer solution. Finally, a mobile phase containing 25mM potassium dihydrogen phosphate buffer (pH 3.5, adjusted with orthophosphoric acid) and acetonitrile in the ratio of 75:25 (v/v) with a flow rate of 1.0 ml/min was selected for analysis as it gave good peak shape for both GFM and AMB with a retention time of 7.37 and 8.73 minutes, respectively. System suitability tests were carried out to determine resolution, column efficiency, peak asymmetry and tailing factor. Results of system suitability parameters are summarized in TABLE 1.

TABLE 1 : System suitability parameters for GFM and AMB

System suitability parameter	GFM	AMB
Retention time (min) (mean ± S.D., n = 5)	7.38	8.73
Tailing factor (peak assymetry)	1.31	1.15
USP Plate count	13130	15256
Resolution	4.96	

Linearity

Calibration graphs were constructed in the ranges of 64-320 μg/ml for gemifloxacin hydrochloride and 24-120 μg/ml for for ambroxol hydrochloride. Peak areas were found to have good linear relationship with the concentration. For both GFM and AMB calibration curves the r^2 was found 0.999. The correlation coefficients, y-intercepts and slopes of the regression lines of the two compounds were calculated and presented in TABLE 2.

TABLE 2 : Calibration data of GFM and AMB

Analyte	Concentration (μg/ml)	Peak area[a]	% RDS	Type	Slope	Intercept	r^2
GFM	64	1950.8820	0.13	linear			0.999
	128	4675.0701	0.04				
	192	6872.1275	0.96				
	256	9235.8113	0.31				
	320	11600.5837	0.22				
AMB	24	978.2456	1.02	linear			0.999
	48	2239.0541	0.69				
	72	3272.1821	0.75				
	96	4403.1462	0.27				
	120	5470.4438	0.08				

[a]Mean of 3 runs, the area values were divided by 10000

Given considerable extent of the calibration range, the homoscedasticity of the analytical method was evaluated with Cochran's test. In order to achieve homoscedasticity, the Cochran *C* of 4 standards with 3 replicates of each standard should be less than the critical values of 0.768[20]. Since the largest and smallest values of variance usually appear at the extremities of the calibration curve in the heteroscedastic case, the two lowest concentrations and the two highest concentration standards were included in the tests. The results are shown in TABLE 3.

The two calibration curves pass the homoscedasticity test since the Cochran'C values were less than the critical value.

TABLE 3 : Cochran's test results

Concentration (μg/ml)	S.D.	Square of S.D.	Sum of square of S.D.[a]	C[b]
Calibration data of GFM				
64	2.56	6.55	1459.36	0.564
128	1.64	2.69		
256	28.69	823.12		
320	25.04	627		
Calibration data of AMB				
24	9.94	98.8	492.18	0.478
48	15.35	235.62		
96	11.83	139.95		
120	4.22	17.81		

[a]standard deviation of 3 replicates, C[b]: Cochran's C

Accuracy and precision

The percentage recovery for GFM and AMB ranged from 99.14 % to 100.49 % and 99.16 % to 101.32 %, respectively. Thereby suggesting that there was no interference from the excipients commonly present in the tablets. The low % RSD values indicated the suitability of this method for routine analysis. The results of recovery studies are summarized in TABLE 4.

The devoloped method was found to be precise as the percent relative standard deviations for repeatability and intermediate precision were found to be less than 2 as recommended by ICH guidelines (TABLE 4).

TABLE 4 : Accuracy and precision results

	Accuracy					Precision			
						repeatability		Intermediate Precision	
Analyte	Level of recovery	Amount of drug added[a]	Amount of drug recovered[a]	% recovery[a]	S.D.[b]	% Label claim[a]	% RSD	% Label claim[a]	% RSD
GFM	80 %	48.13	47.89	99.50	±0.57	100.20	0.272	99.76	0.261
	100 %	80.23	80.20	99.96	±0.46				
	120 %	112.17	111.93	99.79	±0.54				
AMB	80 %	11.30	11.31	100.09	±1.07	99.75	0.363	100.06	0.340
	100 %	18.87	18.75	99.36	±0.69				
	120 %	26.30	26.16	99.47	±0.35				

[a]Mean of 3 replicates, [b]Standard deviation of 3 replicates

Limit of detection and limit of quantification

The LOD of GFM and AMB were 2.92 µg/ml and 0.77 µg/ml, respectively. The LOQ of GFM and AMB were 8.85 µg/ml and 2.32µg/ml, respectively.

Specificity

No excipient peaks co-eluted with the analytes, indicating the method is selective and specific in relation to the excipients used in this study.

Robustness

In all the deliberate varied chromatographic conditions (pH of buffer, flow rate, composition of mobile phase variation) the retention time and peak asymmetry of both the drug peaks was not significantly affected indicated by the low standard deviation values (below 2) for each parameter. Hence, the developed LC method was robust for the determination of GFM and AMB in combined dose tablet formulation.

Results of analysis of tablet formulation

The LC method developed in this study was used for determination of the GFM and AMB content in combined dose tablet formulation. Total chromatographic analysis time per sample was 10 min with GFM and AMB eluting at retention times of 7.38 and 8.73, respectively (Figure 1). Assay (% of active drug content present in the tablets with respect to its label claims) results from six replicate analyses of G-CIN A tablets were 100.01 % (± 0.18) and 99.91 % (± 0.31) for GFM and AMB, respectively. There was good agreement between assay results and the label claim of the product. This indicates that distribution of the drug in tablets is uniform without significant variation.

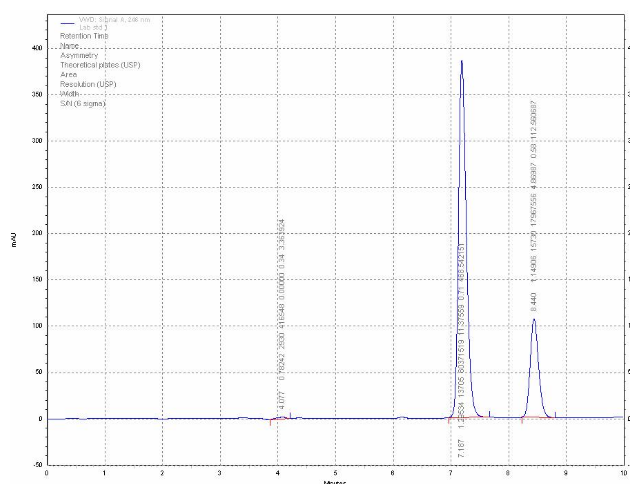

Figure 1 : Typical HPLC chromatogram of GFM and AMB

Forced degradation studies

GFM and AMB were found to be stable under conditions such as photolytic stress, oxidative and thermal stress conditions. Both the drugs were found to degrade under acidic and basic hydrolysis conditions

(Figure 2 and 3, respectively). The rate of degradation in acid was slower as compared to alkaline hydrolysis. The degradation reaction was more intense and quicker in alkaline condition. Both the drugs were found stable under oxidative, thermal and photo degradation stress conditions. Results of forced degradation studies are shown in TABLE 5. The proposed liquid chromatographic system was able to resolve the peak of degradation products thus confirms the stability indicating of the developed LC method.

Figure 2 : HPLC chromatograms of GFM and AMB exposed to acidic hydrolysis (0.1 M HCl).

Figure 3 : HPLC chromatograms of GFM and AMB exposed to basic hydrolysis (0.1 M NaOH).

TABLE 5 : Results of forced degradation studies

Stress condition	Period of study	% Assay of active substance	
		GFM	AMB
Acid hydrolysis (0.1 M HCl at 60^0C)	24 h	51.64	98.99
Base hydrolysis (0.1 M HCl at 60^0C)	24 h	31.10	42.27
Oxidation (3% H2O2 at 60^0C)	24 h	99.82	99.47
Thermal degradation at 60^0C	24 h	99.86	99.68
Photodegradation (exposure to sunlight)	2 days	99.98	99.59

CONCLUSION

Based on the results it can be concluded that the RP-HPLC method is simple, sensitive, accurate, precise, specific and reproducible. The method is also able to selectively quantitate gemifloxacin mesylate and ambroxol hydrochloride in presence of their degradation products expressing the stability indicating property of the method. Statistical analysis proves that the method is suitable for the analysis of gemifloxacin mesylate and ambroxol hydrochloride in pharmaceutical formulation without any interference from the excipients. Hence, the developed RP-HPLC method can be used for routine quality control of pharmaceutical formulations contiaining gemifloxacin mesylate and ambroxol hydrochloride alone or in combination.

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