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Analytical Method Development and Validation of Multicomponent Mucolytic Action in Pharmaceutical Formulation by Using HPLC Techniques

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Abstract:

In the present work, we have successfully developed analytical methods namely HPLC for bulk and formulation. The proposed RP-HPLC methods were simple, accurate, precise, rapid, specific and highly sensitive; developed and validated for the determination of GEM and AMB in bulk and in dosage form. The results were comparable with the corresponding labeled amounts. Hence, the proposed method can be successfully used for routine quality control analysis of pharmaceutical formulation and bulk drug in marketed preparations.

Gemifloxacin, aflouroquinoline derivative has antibacterial activity. Ambroxol dibromoaminobenzyl derivative, GEM and AMB are available in tablet dosage form (G-cin A,lupin) for mucolytic action. The present work dealt with simultaneous estimation of GEM and AMB from bulk and tablet formation by RPHLC. The developed HPLC method was validated in terms of accuracy, repeatability, and precision.

Key words: Gemifloxacin (GEM), Ambroxol (AMB), **HPLC** high performance liquid chromatography, Mucolytic action.

1. INTRODUCTION

The development of commercially available high performance liquid chromatography system has opened up this powerful

analytical method to many kinds of application fields. HPLC overcome many of these difficulties and provide excellent resolution with high speed and sensitivity for separating the types of compounds mentioned above. This technique can be useful not only in agricultural chemistry but also in inorganic, bioclinical and environmental chemistry.

Gemifloxacin (GEM) and Ambroxol (AMB) combination is recently been approved for Mucolytic action[1]. The present project utilizes the need and requirement for quality control of new combination of drugs in market and maintain the quality of marketed formulations.

A good method linear relationship was observed for GEM in concentration ranges of (10 – 60 μg/ml) .the correlation coefficients for GEM was found to be (0.9991).

The inter – day and intra – day precision results were good enough to indicate that the proposed method was precise and reproducible. The assay experiment showed that the content of GEM estimated in the tablet dosage form was free from the interferences of percipients.

Heinanen M. Barbas. *et al*, reported HPLC determination of ambroxol hydrochloride in human plasma and pharmacokinetics[1]Column used is 5 m Beckman – Ultrasphere ODS column (25cm 4.6mm i.d) with distilled water/phosphate buffer of pH 7 (73:27) as mobile phase at 1ml/min and detection at 246 nm.

Zarzuelo, A. *et al,* developed Reversed phase HPLC method for the determination of ambroxol in human plasma[2]Column used is 5 m Resolve C18 column (25cm 4.6mm i.d) operated at 250C with acetonitrile/distilled water/10mM – phosphate buffer of pH 7/THF (14:14:11:1) as mobile phase at 1.5 ml/min and detection at 242 nm.

Benli, P. *et al.*, reported ambroxol hydrochloride by HPLC [3]Reversed – phase LC was employed, using acetonitrile

/10mM – diammonium phosphate buffer (7:3) adjusted to pH 6 with phosphoric acid as a mobile phase and detected at 247 nm.

Martinez –Lozano. *et al*, reported Anodic voltammetric behavior of ambroxol at carbon paste electrode behavior of ambroxol at carbon paste electrode[4] Portions of a standard ambroxol hydrochloride (I) solution were mixed with 0.5M – sulfuric acid and mixed solution was transferred to an electrochemical cell combined with a carbon paste-working electrode. Anodic voltammetry was performed and the peak current was measured at 1.1V (Vs SCE).

Demircigil, B. T. *et al*, reported Flow – injection analysis of ambroxol in pharmaceutical tablet [5]Portions of an aqueous extract of tablet containing known amount of ambroxol (I), was injected into a fused – silica capillary (75 in i.d.) having been previously washed with distilled water, water, 0.1M – Phosphoric acid, and 0.1M NaOH. Water is used as a solvent and the absorbance of the resulting stream was monitored at 209 nm.

2. EXPERIMENTAL PART

2.1 Apparatus:-
Model: Jasco HPLC LC-2000 plus series
Specification: Isocratic mode
Pump: Jasco PU-2080Plus reciprocating intelligent pump
UV Detector: Jasco UV-2075 Plus detector
Column: C18 Column

2.2 Reagents and Chemicals:-
- Methanol HPLC grade
- Formic acid AR grade
- Doubled distilled water

2.3 Selection of Mobile phase: The pure drug of Gemiifloxacin was injected into the HPLC system and run in different solvent systems. Different mobile phases like methanol and water, acetonitrile and water, , methanol and 0.01M potassium dihydrogen orthophosphate buffer (KH2PO4) were tried in order to find the best conditions for the separation of Gemifloxacin so it was found that Methanol and 7% formic acid gives satisfactory results as compared to other mobile phases.. It was found that finally, mobile phase was determined to be methanol: 7% formic acid (80: 20v/v).

2.4 Preparation of Mobile phase: 7% Formic acid was prepared by dissolving accurately about 7 ml of formic acid in 100ml of glass double distilled water. Mobile phase was prepared by mixing(400ml of Methanol and 100ml of Formic acid) and its PH is adjusted to 2.1 This mobile phase was ultrasonicated for 20 min. and it was filtered through 0.45 µm Nylon 6.6(N66) 47 mm membrane filter paper.

2.5 Preparation of standard stock solution: Accurately about 10mg of Gemifloxacin was weighed and transferred to two separate 100ml volumetric flask. The drugs were dissolved in 50 ml of mobile phase with shaking and then volume was made up to the mark with mobile phase to get 100µg/ml of standard stock solution of drug. This stock solution was filtered through 0.2µ Nylon 6.6 (N66) membrane filter.

2.6 Selection of analytical wavelength: By appropriate dilution of standard stock solution with mobile phase. Various concentrations of GEM was prepared Solution is scanned using double beam UV visible spectrophotometer in the spectrum mode between the range of 400nm to 200nm and the wavelength selected was 260 nm.

2.7 Chromatographic condition: The optimum composition of the mobile phase containing Methanol and 7% formic acid (80:20v/v) and its PH adjusted to 2.1 was selected because it was found ideally resolve the peaks of GEM .The flow rate was set to 1.0ml/min and UV detection was carried out at 260 nm. The mobile phase and samples were degassed by ultrasonic vibration for 20 min. and filtered through 0.45µm Nylon 6.6 (N66) 47 mm filter paper. All determination was performed at constant room temperature.
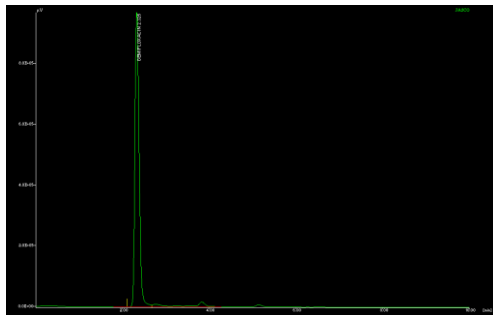

Figure 2.1   A Typical Chromatogram of Gemifloxacin

3. METHOD VALIDATION

3.1. Linearity: For GEM appropriate aliquots was pipetted out from each standard stock solution into a series of 10 ml volumetric flask. The volume was made up to the mark with mobile phase to get set solution for GEM having concentration range 10-60 mcg/ml. Triplicate dilution of concentration of drug were prepared . From these triplicate solutions, 20 µl injection of each concentration of drug were injected into HPLC system two times separately and chromatograph under condition as described above. Evaluation of both drugs was performed with UV detector at 260 nm. Working calibration curves for GEM was plotted with peak areas vs the respective concentration of GEM as shown in Figure below peak of GEM are shown in below. The Beer Lambert's law was obeyed in the concentration

range of 10-60 mcg/ml for GEM. The linearity of calibration graph and adherence of the system to Beer's law was validated by high value of correlation coefficient and also standard deviation (S.D) for intercept value was less than 2%.
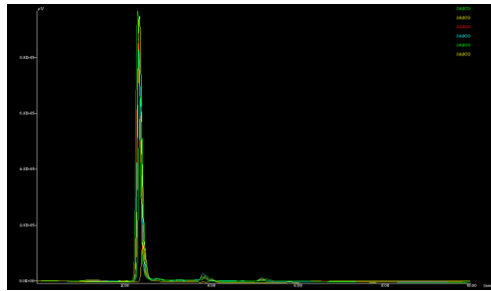

Figure 3.1 Linearity

Table 3.1 Observation table for calibration curve of GEM

Conc. mcg/ml	Peak area
10	120956
20	207321
30	340723
40	452187
50	538765
60	659876
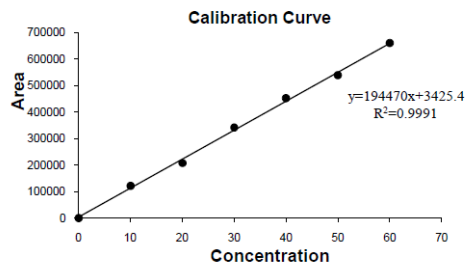
Figure 3.2 Calibration curve of GEM

Table 3.2 Validation and System suitability parameter

Parameters	GEM
Linearity range(mcg/ml)	10-60 mcg/ml
Slope	194470
Intercept	3425.4
Regression coefficient	0.9991

Retention time	2.36
Tailing factor	0.75
Limit of detection(mcg/ml)	0.05
Limit of Quantitation(mcg/ml)	0.17
Theoretical plate no.	18000

3.2. Limit of Detection (LOD) and Limit of Quantitation (LOQ):

LOD is the lowest amount of an analyte in a sample that can be detected but not necessarily quantitated an exact value. LOQ is the lowest amount of an analyte in a sample that can be quantitatively determined with suitable precision and accuracy.

$$3.3 \text{ x D, LOD} = \mathbf{S}, 10 \text{ x D, } \text{ LOQ} = \mathbf{S}$$

Where, **D** = standard deviation of the y-intercepts of regression line.
$\quad$ **S** = slope of the calibration curve.

3.3. Specificity: The specificity of the HPLC method was determined by complete separation of GEM as shown in Figure with parameters like retention time (tR), Resolution (RS) and tailing factor (Tf). Here tailing factor for peak of GEM was less than (2%) and Resolution was satisfactory. The peak obtained for GEM was sharp and have clear baseline separation.

3.4. Precision: Precision of the method was determined with the tablet sample. Twenty tablets of Gemifloxacin were weighed; their average weight was determined and finally crushed to fine powder. The tablet powder equivalent to of 320mg of GEM was weighed and transferred to 100 ml volumetric flask and Dissolved in mobile phase and the content was kept in ultrasonicator for 30 min. Finally the volume was made up to the mark with mobile phase. The solution was filtered through 0.2µ m Nylon6,6 (N66) 47mm membrane filter paper. This tablet solution was further diluted with mobile phase to obtain mixed sample solutions in Beer Lamberts'

range for each drug containing 32 mcg/ml of GEM. System repeatability was determined by six replicate application and three times measurement of sample solution at the analytical concentration. The repeatability of sample application and measurement of peak area for each drug were calculated by repeating the assay three times of six replicate dilutions of the same concentration in the same day for intraday precision. Interday precision was obtained by the assay of six sample sets on different days. The results are shown below:

Table 3.3 Intra-day precision

Drug	% mean*	S.D	% RSD	S.E
GEM	100.3	1.6075	1.6026	0.09

*n=3

Table 3.4 Inter-day precision

Drug	% mean*	S.D	% RSD	S.E
GEM	99.67	0.7916	0.7942	0.2209

3.5 Accuracy (Recovery studies): The solutions for recovery studies at 80%, 100% and 120% level were prepared in the same manner as given under simultaneous equation method. But in the method mobile phase was used as solvent. These solutions were filtered through 0.2µ m Nylon 6,6 (N66) 47 mm membrane filter paper. The solution was analyzed by RP-HPLC method as described above. The results for recovery studies and statistical evaluation are shown below:

Table 3.5 Recovery studies

Level of % Recovery	Amount present(mg/tab)	Amount of standard added(mg/tab)	Total amount recovered(mg)	% Recovery*
	GEM	GEM	GEM	GEM
80	320	256	575.59	99.93
100	320	320	638.84	99.82
120	320	384	703.92	99.99

Table 3.6 Statistical validation:

Component	Mean	Standard Deviation	Coefficient of Variation	Standard Error
GEM	99.98%	0.011	0.0112	0.1

3.6 Robustness of method: The evaluation of robustness should be considered during the development phase and depends upon the type of procedure under study. It should show the reliability of an analysis with respect to deliberate variations in the method parameters. The parameters included pH mobile phase, flow rate, percentage of Methanol in the mobile phase. The solution containing 32 mcg/ml of GEM was injected into sample injector of HPLC three times under different parameters like variation in pH, flow rate, percentage of Methanol in the mobile phase. The results are shown in table below:

Table 3.7 Robustness results Chromatographic changes

1.   Flow rate changes:

Flow rate(ml/min)	level	RT	Tailing factor
		GEM	GEM
0.5	-0.5	2.91	1.56
1	0	2.36	0.75
1.5	+0.5	2.11	1.67

Mean ± S.D (n=3)   GEM   2.46 ± 0.4092

2. pH changes:

pH	level	RT	Tailing factor
		GEM	GEM
1.9	-0.2	2.38	2.1
2.1	0	2.36	0.75
2.3	+0.2	2.39	2.00

Mean ± S.D (n=3)   GEM   2.37 ± 0.0152

3. (%) of methanol changes:

% of methanol	level	RT	Tailing factor
		GEM	GEM
70	-10	3.90	3.56
80	0	2.36	0.75
90	+10	1.61	4.89

4. ESTIMATION OF GEM FROM PHARMACEUTICAL DOSAGE FORM (ASSAY OF TABLET)

The tablet solution was prepared in the same manner as described in precision method. This tablet solution was further diluted with mobile phase to obtained sample solutions in Beer Lamberts' range for drug containing 32 mcg/ml of GEM. A 20 µl volume of sample solution was injected into sample injector of HPLC six times under chromatographic conditions as described above. Area of each peak was measured at 260 nm. The amount of each drug present in the sample (n=5) as determined from peak area of GEM present in the pure mixture.

Table 4.1 Analysis of tablet formulation

Sr. No.	Label Claim mg/tab	Amount Found mg/tab	% of Label Claim
	GEM	GEM	GEM
1	320	320.05	100.01
2	320	319.99	99.99
3	320	318.97	99.67
4	320	320	100
5	320	319.77	99.93

Table 4.2 Statistical validation

Component	Mean	Standard Deviation	Coefficient of Variation	Standard Error
GEM	99.92	0.1431	0.1432	0.06404

5. RESULTS AND DISCUSSION

HPLC method was developed, validated and used for quantitative determination of Gemifloxacin mesylate (GEM) formits tablet dosage form. Chromatographic separation was performed on Grace C$_{18}$ colum (250 mm × 4.6 mm, 5 µm), with a mobile phase comprising of mixture of methanol : 7% formic acid (80:20v/v)PH adjusted to 2.1 at a flow rate of 1 ml/min,

with detection at 260 m with R_t 2.36 min. Separation was completed in less than 10 min. As per (1CH) guidelines the method was validated for lineary, accuracy, precision, limit of quantitation, limit of detection, and robustness.

Linearity of GEM was found to be in the range of 10-60 µg/ml. the correlation coefficient was 0.9991. The results of tablet analysis (n=5) were found to be 99.92% with ± 0.1431% standard deviation for GEM .Percent recovery of GEM was found to be 99.98%. The assay experiment shows that the method is free of excipients, hence the method is specific.

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