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# Method development and validation for the simultaneous estimation of ambroxol HCL and levofloxacin by using RP HPLC method

**B.Jyothsna[*1], V.Swapna[1]**

[1]*Chilkur balaji college pharmacy, Aziznagar (v),Moinabad(M)-5000075 Telangana, India*

***Corresponding Author: B.Jyothsna**
**Email: b.jyothsna369@gmail.com**

## ABSTRACT

A new, simple, precise, accurate and reproducible RP-HPLC method for Simultaneous estimation of bulk and pharmaceutical formulations. Separation of Ambroxol HCL and levofloxacin was successfully achieved by Inertsil C18, 250 X4.6, 5µm or equivalent in an isocratic mode utilizing 0.1% OPA: methanol (60:40) at a flow rate of 1mL/min and eluate was monitored at 238nm, with a retention time of 2.173 and 4.344 minutes for Ambroxol HCL and levofloxacin respectively. The method was validated and there response was found to be linear in the drug concentration range of 50µg/ml to150 µg/ml for Ambroxol HCL and 50µg/ml to150 µg/ml for Levofloxacin. The values of the correlation coefficient were found to0.999for Ambroxol HCL and 1for levofloxacin respectively. The LOD and LOQ for Ambroxol HCL were found to be 0.035and 0.116 respectively. The LOD and LOQ for Levofloxacin were found to be 0.1884 and 0.6281 respectively. This method was found to be good percentage recovery for Ambroxol HCL and Levofloxacin were found to be 99.1% and 99.7% respectively indicates that the proposed method is highly accurate. The specificity of the method shows good correlation between retention times of standard with the sample so, the method specifically determines the analyte in the sample without interference from excipients of tablet dosage forms. The method was extensively validated according to ICH guidelines for Linearity, Accuracy, Precision, Specificity and Robustness

**Keywords:** *Ambroxol and Levofloxacin, Validation, HPLC*

## INTRODUCTION

Analytical methods development and validation play important roles in the discovery, development, and manufacture of pharmaceuticals. The current good manufacturing practice (CGMP) and Food Drug Administration (FDA) Guidelines insist for adoption of sound methods of analysis with greater sensitivity and reproducibility. Development of a method of analysis is usually based on prior art (or) existing literature, using the same (or) quite similar instrumentation .It is rare today that an HPLC-based method is developed that does not in same way relate (or) compare to existing, literature based approaches. Today HPLC (High performance liquid chromatography) is the method of choice used by the pharmaceutical industry to assay the intact drug and degradation products. The

appropriate selection and chromatographic conditions ensure that the HPLC method will have the desired specificity. UV spectroscopy is also a simple analytical tool widely used for routine assay of drugs. Hence for the assay of the selected drugs HPLC and UV spectroscopy has been chosen for these proposed methods.

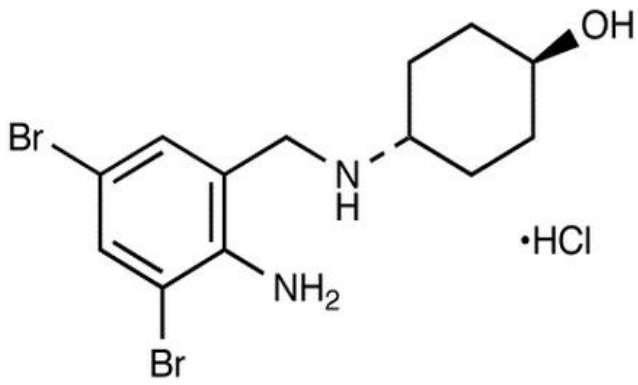
The developed chromatographic methods further validated as per ICH or USFDA guidelines for all the critical parameters. To access the precision and to evaluate the results of analysis the analyst must use statistical methods. These methods include confidence limit, regression analysis to establish calibration curves. In each analysis the critical response parameters must be optimized and recognized if possible

## DRUG PROFILE

### Ambroxol

- Expectorants

### Structure




**Structure of Ambroxol**

**IUPAC Name** : 4-{[(2-amino-3,5-dibromophenyl)methyl]amino}cyclohexan-1-ol hydrochloride
**Molecular formula** : $C_{13}H_{19}Br_2ClN_2O$
**Molecular Weight** : 414.56376
**Solubility** : Soluble in methanol, water, ethanol, and DMSO. Insoluble in ether.
**Pka** : 15.26

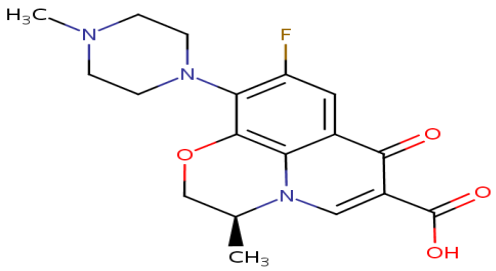
### Mechanism of action

Ambroxol is a mucolytic agent. Excessive Nitric oxide (NO) is associated with inflammatory and some other disturbances of airways function. NO enhances the activation of soluble guanylate cyclase and cGMP accumulation. Ambroxol has been shown to inhibit the NO-dependent activation of soluble guanylate cyclase. It is also possible that the inhibition of NO-dependent activation of soluble guanylate cyclase can suppress the excessive mucus secretion, therefore it lowers the phlegm viscosity and improves the mucociliary transport of bronchial secretions.

### Levofloxacin

- Quinolones

### Structure




### Stucture of Levofloxacin

**IUPACNAME**

(2S)-7-fluoro-2-methyl-6-(4-methylpiperazin-1-yl)-10-oxo-4-oxa-1-azatricyclo[7.3.1.0$^{5}$,$^{13}$]trideca-5(13),6,8,11-tetraene-11-carboxylic acid

**Molecular formula**

$C_{18}H_{20}FN_3O_4$

**Molecular Weight**

361.3

**Solubility**

Soluble in water

**PKA**

5.45

**Mechanism of action**

Levofloxacin inhibits bacterial type II topoisomerases, topoisomerase IV and DNA gyrase. Levofloxacin, like other fluoroquinolones, inhibits the A subunits of DNA gyrase, two subunits encoded by the gyrA gene. This results in strand breakage on a bacterial chromosome, supercoiling, and resealing; DNA replication and transcription is inhibited.

## MATERIALS AND METHODS

### Equipment and Apparatus used

### Electronic balance

- HPLC Waters Separation Module LC-20AT Prominence Liquid Chromatography
- UV Detector
- Chromatographic data Software : EMPOWER
- SymmetryColumn C18 (250 X 4.6cm, Id- 5µ)
- Vacuum filter pump
- Mobile phase reservoir
- Ultra Sonicator , Membrane filter(0.45 and 0.2 microns)

### Reagents

- Acetonitrile HPLC grade
- Water (HPLC)
- Potassium Dihydrogen Phosphat

## METHOD VALIDATION

### System suitability

Tailing factor for the peaks due to Ambroxol and Levofloxacin in standard solution should not be more than 2.0.Theoretical plates for the Ambroxol and Levofloxacin peaks in standard solution should not be less than 2000.

### Specificity

Solution of standard, sample, blank and placebo were prepared as per test procedure and injected into the HPLC system.

### Acceptance criteria

Chromatogram of standard and sample should be identical with near Retention time.

### Blank interference

A study to establish the interference of blank was conducted. Diluent was injected into HPLC system as per the test procedure.

### Acceptance criteria

Chromatogram of blank should not show any peak at the retention time of analyte peak. There is no interference due to blank at the retention time of analyte. Hence the method is specific.

### Linearity

Prepare a series of standard solutions and inject into HPLC system. Plot the graph of standard

versus the actual concentration in µg/ml and determine the coefficient of correlation and basis for 100% response.

### Acceptance criteria

Linearity regression coefficient of average peak area response of replicate injections plotted against respective concentration should not be less than 0.999. The % y-intercept as obtained from the linearity data (without extrapolation through origin 0, 0) should be within ±2.0.

### Statistical evaluation

A graph between the concentration and the average area was plotted. Points for linearity were observed. Using the method of least squares, a line of best fit was taken and the correlation Coefficient, slope and, y-intercept were calculated.

## PRECISION

### Preparation of sample

- Transfer the 1044.5mg of sample into a 100ml of volume at flask and add 10ml of water and 10ml of Methanol and sonicate 20min and makeup with water. Transfer the above solution into 2ml into 100ml volume metric flask dilute to the volume with water.
- The method precision parameters were evaluated from sample chromatograms obtained, by calculating the % RSD of peek areas from 6 replicate injection.

### Acceptance criteria

The injection reproducibility requirements are met if the %RSD for peak areas is not more than 2.0 and for retention times is not more than 2.0.

### Recovery/accuracy

Recovery study can be performed in the concentration range of 80% to 120% of the target concentration of the test. Minimum 3 concentrations are recommended.

### Acceptance criteria

The average percentage recovery was between 98-102% and Relative standard deviation of these recovery concentrations was less than 2%.

### Limit of detection

The sensitivity of measurement of Ambroxol and Levofloxacin by use of proposed method was estimated in terms of the limit of detection (LOD). The LOD was calculated by the use of signal to noise ratio. In order to estimate the LOD value, the blank sample was injected six times and peak area of this blank was calculated as noice level. The LOD was calculated as three times the noise level.

$LOD = 3.3\ \sigma / S$

Where,

$\sigma$ = standard deviation of intercepts of calibration curves

S = mean of slopes of the calibration curves

The slope S may be estimated from the calibration curve of the analyte.

### Limit of quantitation

The sensitivity of measurement of Ambroxol and Levofloxacin by the use of proposed method was estimated in terms of limit of quantitation (LOQ). The LOQ was calculated by the use of signal to noise ratio. In order to estimate the LOQ value, the blank sample was injected six times and the peak area of this blank was calculated at noise level. The LOQ was calculated as ten times the noise value gave the LOQ.

$LOQ = 10\ \sigma / S$

Where,

$\sigma$ = standard deviation of intercepts of calibration curves

S = mean of slopes of the calibration curves

The slope S may be estimated from the calibration curve of the analyte.

## ROBUSTNESS

### Effect of variation in flow rate

Prepare the system suitability solution as per the test method and inject into the HPLC system with ±0.2 ml of the method flow. Evaluate the system suitability values as required by the test method for both flow rates. Actual flow rate was 1.0 ml/min and it was changed to 0.8ml/min and 1.2ml/min and inject into HPLC and system suitability was checked.

### Effect of variation in wavelength

Prepare the system suitability solution as per the test method and injected into the HPLC with

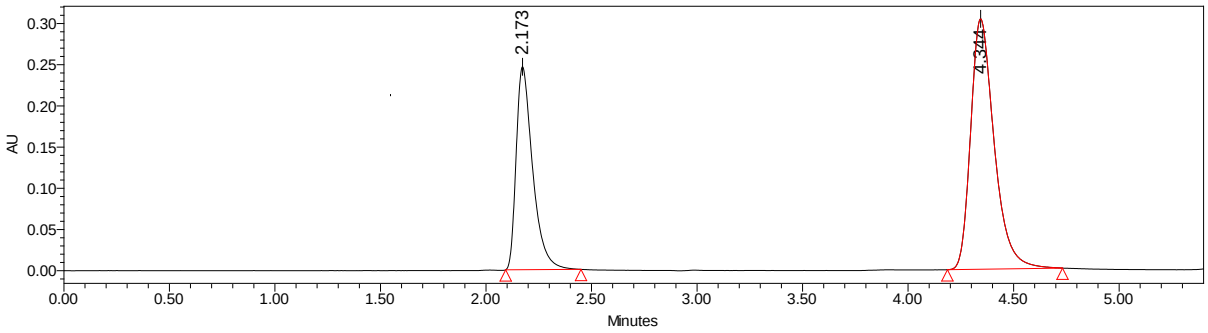
±2nm variation in wavelength. Evaluate the system suitability values as required by the test method for both wavelengths

## Optimized method

Mobile Phase : 0.1%OPA: Methanol (60:40)
Column : Inertsil C8, 250X4.6, 5um
Flow rate : 1.0ml /min
Temperature : 30$^0$C
Injection Volume : 10ul
Detector : PDA

## Procedure

Inject 10µL of standard, sample into chromatographic system and measure the areas for the Ambroxol and Levofloxacin peaks and calculate the % assay by using the formula




**Chromatogram for optimized method**

| Name | Retention Time | Area | USP Resolution | USP Tailing | USP Plate Count |
|---|---|---|---|---|---|
| Ambroxol | 2.173 | 1334998 | | 1.58 | 3841 |
| Levofloxacin | 4.344 | 2350999 | 12.42 | 1.36 | 7552 |

## Observation

RT was found to be good and the peak symmetry of both drugs were good. And the resolution theoretical plate count and tailing were within the limits and it is used for validation of the method.

## Preparation of mobile phase

Transfer 500ml of HPLC water into 500ml of beaker add 0.1%OPA. Transfer the above solution 600ml of 0.1%OPA, 400ml of Methanol is used as mobile phase. They are mixed and sonicated for 20min.

## Preparation of the ambroxol and levofloxacin standard and sample solution

## Preparation of standard solution

Accurately weigh and transfer 75mg Ambroxol and 500mg Levofloxacin into 100ml of volumetric flask and add 10ml of Methanol and sonicate 10min (or) shake 5min and make with water. Transfers the above solution into 2ml into 100ml volumetric flask dilute to volume with water.

## Preparation of sample stock solution

Commercially available 20 tablets ware weighed and powdered the powdered equivalent to the 1044.5mg of Ambroxol and Levofloxacin of active ingredients were transfer into a 100ml of volumetric flask and add 10ml of Methanol and sonicate 20min (or) shake 10min and makeup with water.

Transfers above solution 2ml into 100ml of the volumetric flask dilute the volume with Methanol. And the solution was filtered through 0.45µm filter before injecting into HPLC system.

## Uv spectroscopy wavelength determination

The UV spectrums of Ambroxol and Levofloxacin under these mobile phase conditions were shown below and from these spectrums, Lambda Max 238 nm were observed.

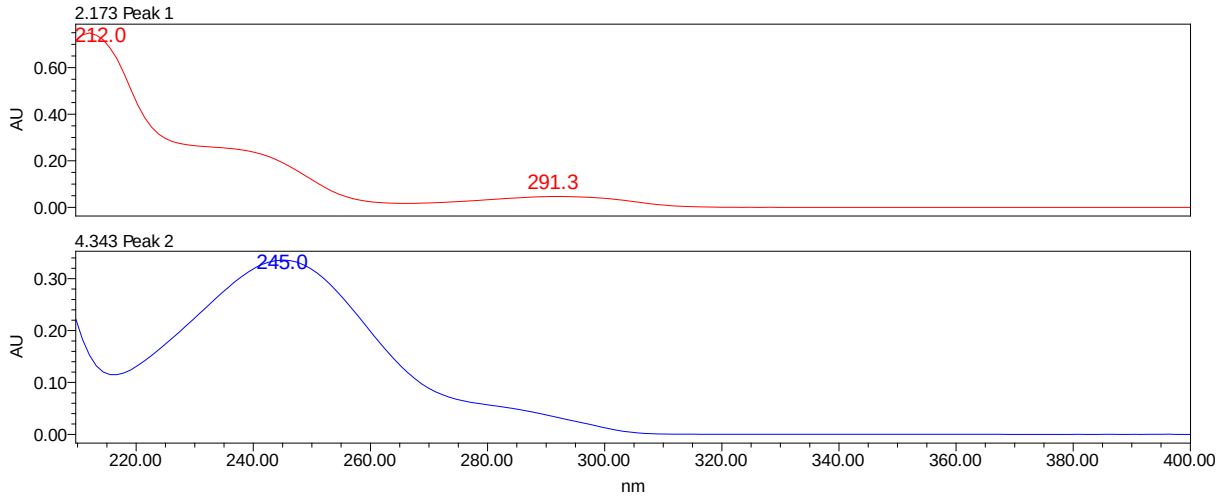
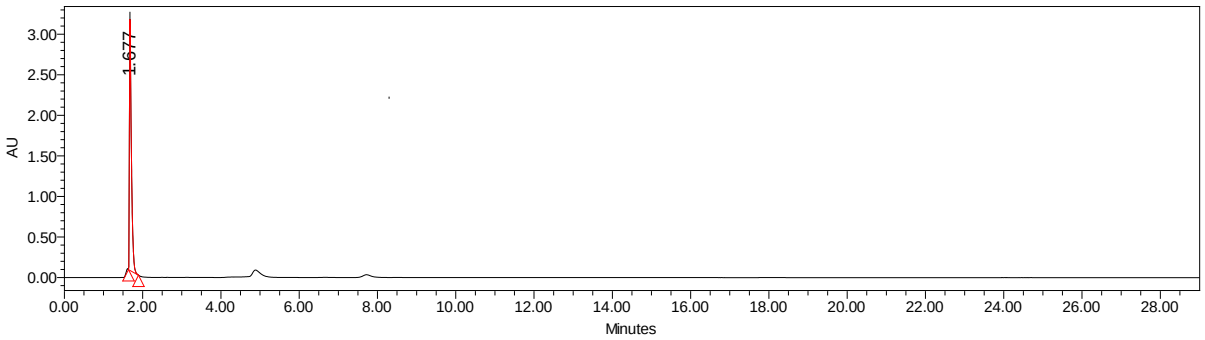



**Fig 5: Uv spectrum of standard Ambroxol and Levofloxacin Peak**

## Method development trails

### Trail 1

Mobile Phase: 0.1%OPA: Methanol (60:40)
Column: Phenomenex C18, 150X4.6, 5um
Flow rate: 1.0ml /min
Temperature: $30^0$C
Injection Volume: 10ul
Detector: PDA



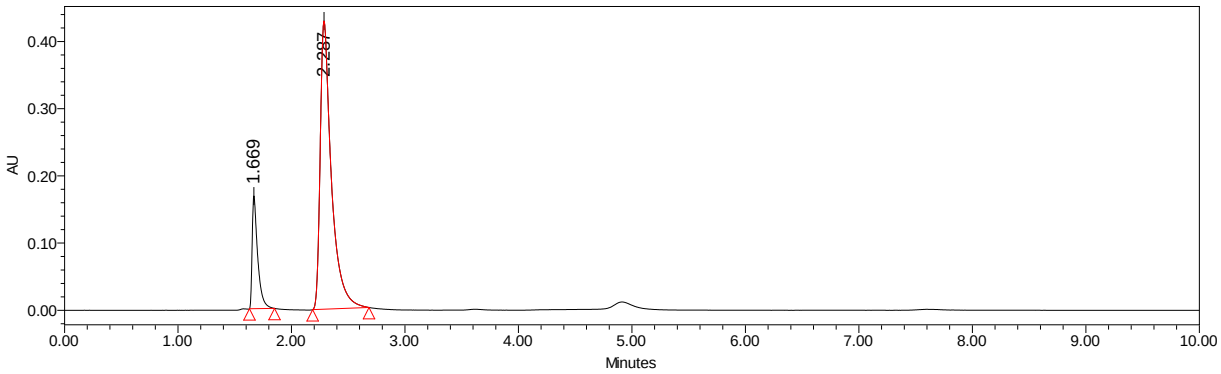

| Name | Retention Time | Area | USP Resolution | USP Tailing | USP Plate Count |
|---|---|---|---|---|---|
| Ambroxol | 1.677 | 10643613 | | 2.44 | 5831 |

### Typical chromatogram of trail 1

Observation: second peak was not detected & tailing was observed
Reason: may be concentration
Corrective Action: Change the concentration

### Trail 2

Mobile Phase: 0.1%OPA: Methanol (60:40)
Column: Phenomenex C18, 150X4.6, 5um
Flow rate: 1.0ml/min
Temperature: $30^0$C
Injection Volume: 10ul
Detector: PDA



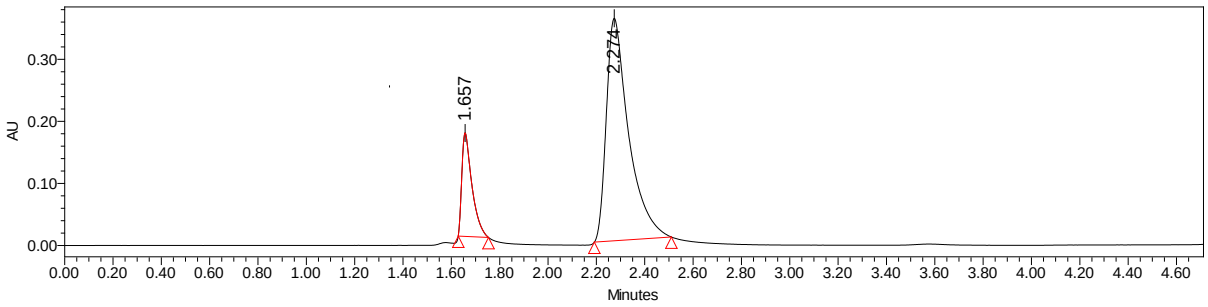

| Name | Retention Time | Area | USP Resolution | USP Tailing | USP Plate Count |
|---|---|---|---|---|---|
| Ambroxol | 1.669 | 566860 | | 2.11 | 6043 |
| Levofloxacin | 2.287 | 2934510 | 4.57 | 1.91 | 2949 |

## Typical chromatogram of trail 2

Observation: we got two peaks but tailing was failed
Reason: may be due to injection volume
Corrective Action: Change injection volume

## Trail 3

Mobile Phase: 0.1%OPA: Methanol (60:40)
Column: Phenomenex C18, 150X4.6, 5um
Flow rate: 1.0ml/min
Temperature: $30^0$C
Injection Volume: 8 ul
Detector: PDA



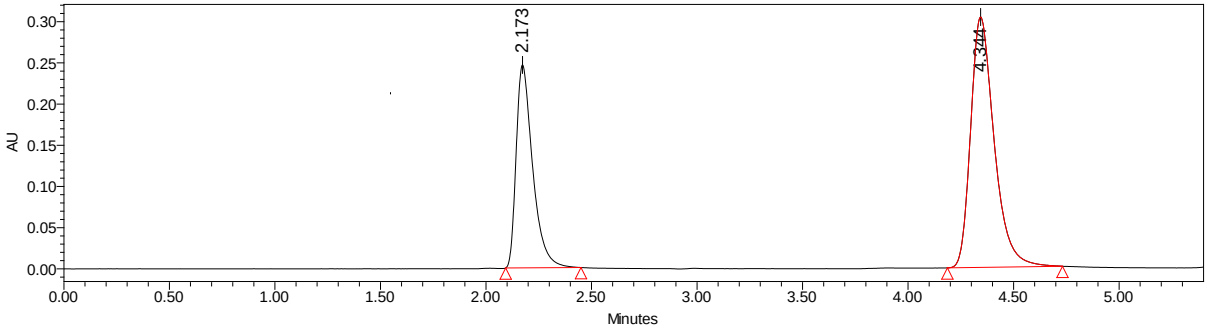

| Name | Retention Time | Area | USP Resolution | USP Tailing | USP Plate Count |
|---|---|---|---|---|---|
| Ambroxol | 1.657 | 482769 | | 1.99 | 7309 |
| Levofloxacin | 2.274 | 2299518 | 4.83 | 1.79 | 3070 |

## Typical chromatogram of trail 3

Observation: we got two peaks but tailing was low
Reason: may be due to column
Corrective Action: Change the column

## Trail 4

Mobile Phase: 0.1%OPA: Methanol (60:40)
Column: Inertsil C8, 250X4.6, 5um
Flow rate: 1.0ml /min
Temperature: $30^0$C
Injection Volume: 10ul
Detector: PDA




| Name | Retention Time | Area | USP Resolution | USP Tailing | USP Plate Count |
|---|---|---|---|---|---|
| Ambroxol | 2.173 | 1334998 | | 1.58 | 3841 |
| Levofloxacin | 4.344 | 2350999 | 12.42 | 1.36 | 7552 |

## Typical chromatogram of trail 9

## Observation

RT was found to be good and the peak symmetry of both drugs were good. And the resolution theoretical plate count and tailing were within the limits and it is used for validation of the method.

# RESULTS AND DISCUSSIONS

## System suitability

**System suitability data of Ambroxol and Levofloxacin**

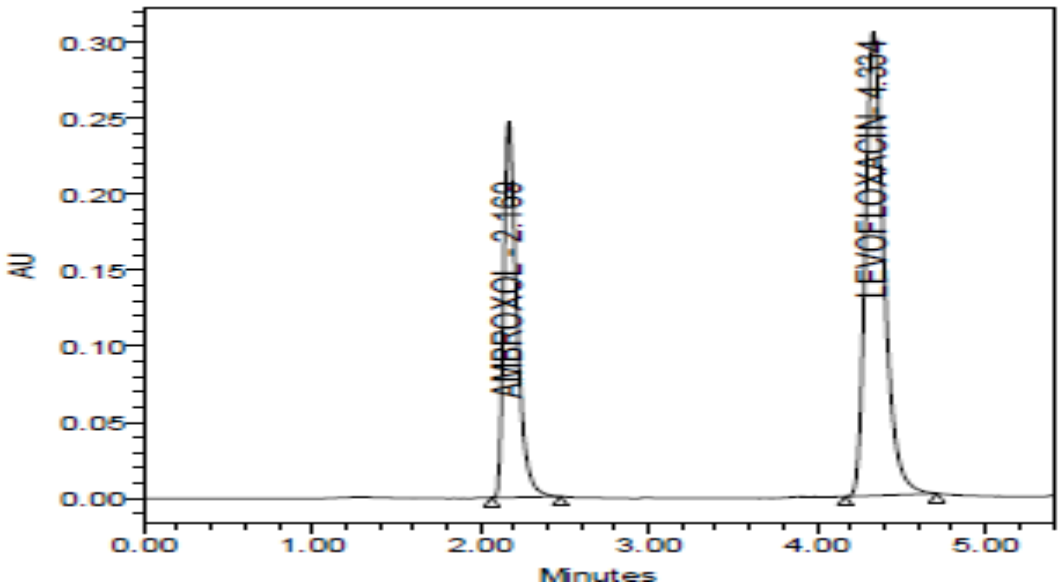
| parameter | Ambroxol | Levofloxacin | Acceptance criteria |
|---|---|---|---|
| Retention time | 2.171 | 4.342 | +-10 |
| Theoretical plates | 3815 | 7547 | >2500 |
| Tailing factor | 1.59 | 1.35 | <2.00 |
| % RSD | 0.2 | 0.5 | <2.00 |

**Standard Results of Ambroxol**

| S.no | Sampl name | RT | Area | USP plate count | USP tailing |
|---|---|---|---|---|---|
| 1. | Injection1 | 2.169 | 1341010 | 3848 | 1.57 |
| 2. | Injection 2 | 2.170 | 1342391 | 3858 | 1.58 |
| 3. | Injection 3 | 2.176 | 1342732 | 3804 | 1.56 |
| 4. | Injection 4 | 2.175 | 1337571 | 3840 | 1.56 |
| 5. | Injection 5 | 2.175 | 1341222 | 3855 | 1.57 |

**Standard Results of Levofloxacin**

| S.no | Sample name | RT | Area | USP plate count | USP tailing |
|---|---|---|---|---|---|
| 1. | Injection 1 | 4.334 | 2341300 | 7701 | 1.36 |
| 2. | Injection 2 | 4.347 | 2361615 | 7565 | 1.37 |
| 3. | Injection 3 | 4.353 | 2350663 | 7658 | 1.37 |
| 4. | Injection 4 | 4.355 | 2337632 | 7751 | 1.36 |
| 5. | Injection 5 | 4.342 | 2336473 | 7760 | 1.38 |




## RESULT

Results of system suitability study are summarized in the above table. Six consecutive injections of the standard solution showed uniform retention time, theoretical plate count, tailing factor and resolution for both the drugs which indicate a good system for analysis.

### Specificity

**Specificity data for Ambroxol and Levofloxacin**

| S no | Sample name | Ambroxol area | Rt | Levofloxacin Area | Rt |
|---|---|---|---|---|---|
| 1 | Standard | 1341010 | 2.169 | 2341300 | 4.334 |
| 2 | Sample | 1351169 | 2.171 | 2349438 | 4.342 |
| 3 | Blank | - | - | - | - |
| 4 | Placebo | - | - | - | - |

### Accuracy

**Accuracy data for Ambroxol**

| S.NO | Accuracy level | Injecton | Sample area | RT |
|---|---|---|---|---|
| 1 | 50% | 1 | 670989 | 2.172 |
| | | 2 | 670987 | 2.178 |
| | | 3 | 670544 | 2.174 |
| 2 | 100% | 1 | 1348025 | 2.175 |
| | | 2 | 1343803 | 2.174 |
| | | 3 | 1344430 | 2.179 |
| 3 | 150% | 1 | 2016609 | 2.175 |
| | | 2 | 2013780 | 2.177 |
| | | 3 | 2016871 | 2.175 |

**Accuracy (%recovery) results of Ambroxol**

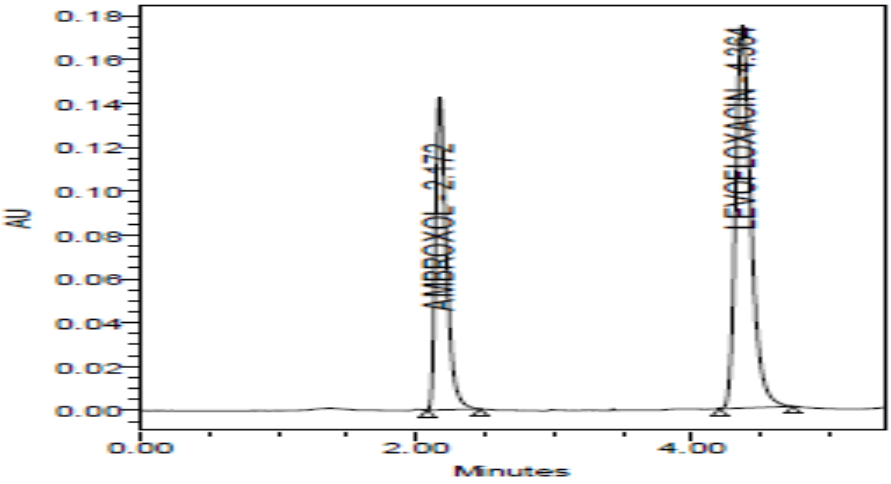
| S.NO | Accuracy level | Sample name | Sample weight | µg/ml added | µg/ml found | % Recovery | % Mean |
|---|---|---|---|---|---|---|---|
| 1 | 50% | 1 | 522.25 | 7.425 | 7.44 | 100 | 100 |
| | | 2 | 522.25 | 7.425 | 7.44 | 100 | |
| | | 3 | 522.25 | 7.425 | 7.43 | 100 | |
| 2 | 100% | 1 | 1044.50 | 14.850 | 14.94 | 101 | 100 |
| | | 2 | 1044.50 | 14.850 | 14.90 | 100 | |
| | | 3 | 1044.50 | 14.850 | 14.90 | 100 | |
| 3 | 150% | 1 | 1566.75 | 22.275 | 22.35 | 100 | 100 |
| | | 2 | 1566.75 | 22.275 | 22.32 | 100 | |
| | | 3 | 1566.75 | 22.275 | 22.36 | 100 | |

**Accuracy data for Levofloxacin**

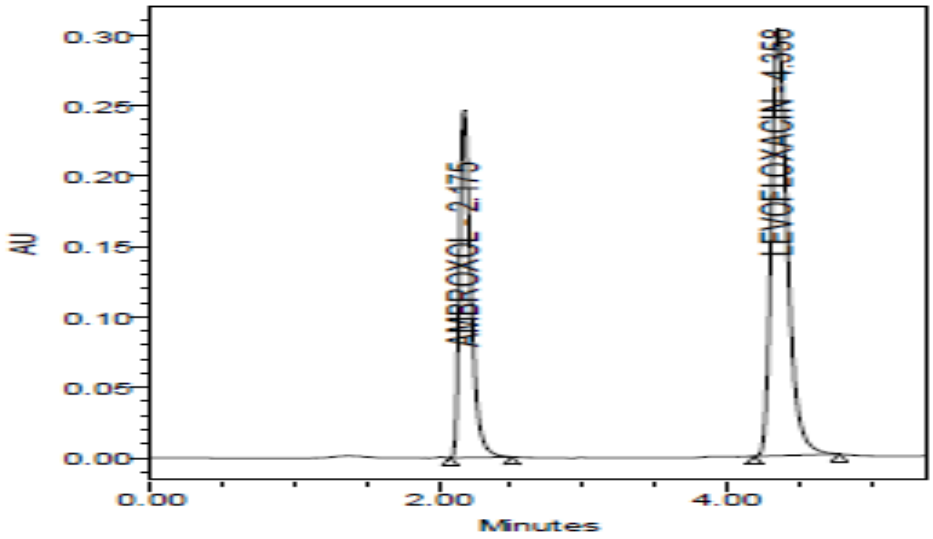
| S.NO | Accuracy level | Injecton | Sample area | RT |
|---|---|---|---|---|
| 1 | 50% | 1 | 1172024 | 4.364 |
| | | 2 | 1178580 | 4.361 |
| | | 3 | 1175503 | 4.362 |
| 2 | 100% | 1 | 2344198 | 4.358 |
| | | 2 | 2347708 | 4.363 |
| | | 3 | 2343797 | 4.362 |
| 3 | 150% | 1 | 3520171 | 4.360 |
| | | 2 | 3518383 | 4.364 |
| | | 3 | 3530859 | 4.361 |

**Accuracy (%recovery) results of Levofloxacin**

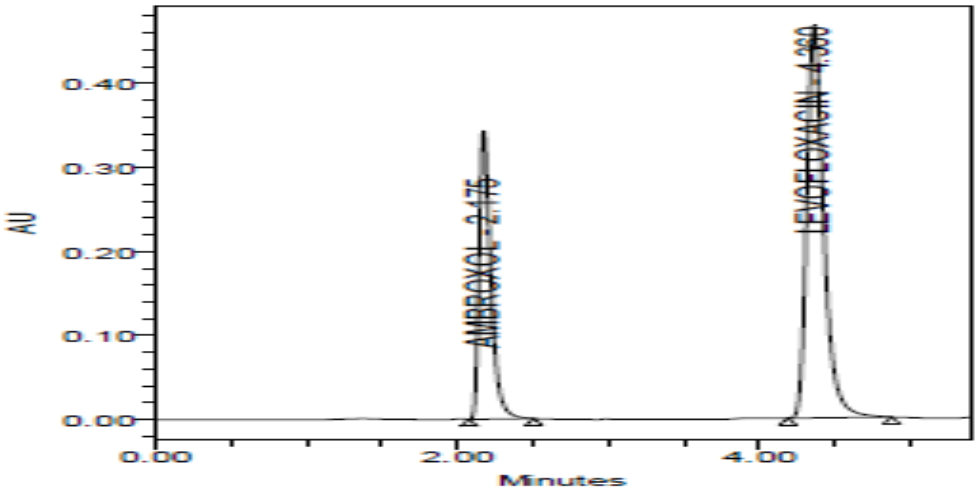
| S.NO | Accuracy level | Sample name | Sample weight | µg/ml added | µg/ml found | % Recovery | % Mean |
|---|---|---|---|---|---|---|---|
| 1 | 50% | 1 | 522.25 | 50.000 | 49.82 | 100 | 100 |
| | | 2 | 522.25 | 50.000 | 50.10 | 100 | |
| | | 3 | 522.25 | 50.000 | 49.97 | 100 | |
| 2 | 100% | 1 | 1044.50 | 100.000 | 99.64 | 100 | 100 |
| | | 2 | 1044.50 | 100.000 | 99.79 | 100 | |
| | | 3 | 1044.50 | 100.000 | 99.63 | 100 | |
| 3 | 150% | 1 | 1566.75 | 150.000 | 149.63 | 100 | 100 |
| | | 2 | 1566.75 | 150.000 | 149.55 | 100 | |
| | | 3 | 1566.75 | 150.000 | 150.08 | 100 | |




**Typical chromatogram for Accuracy 50 %**




**Typical chromatogram for Accuracy 100 %**




**Typical chromatogram for Accuracy 150 %**

## RESULT

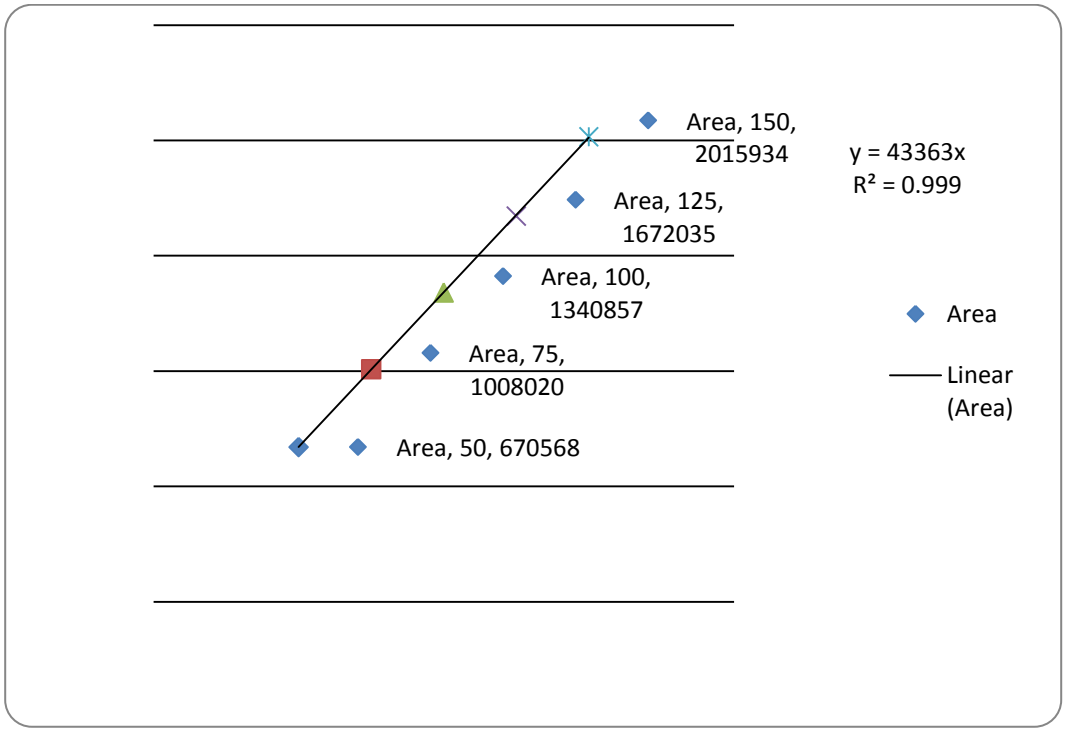
Results of accuracy study are presented in the above table. The measured value was obtained by recovery test. Spiked amount of both the drug were compared against the recovery amount.

% Recovery was 100.00% for Ambroxol and 100.00% for Levofloxacin. All the results indicate that the method is highly accurate.

## LINEARITY

**Linearity data for Ambroxol**

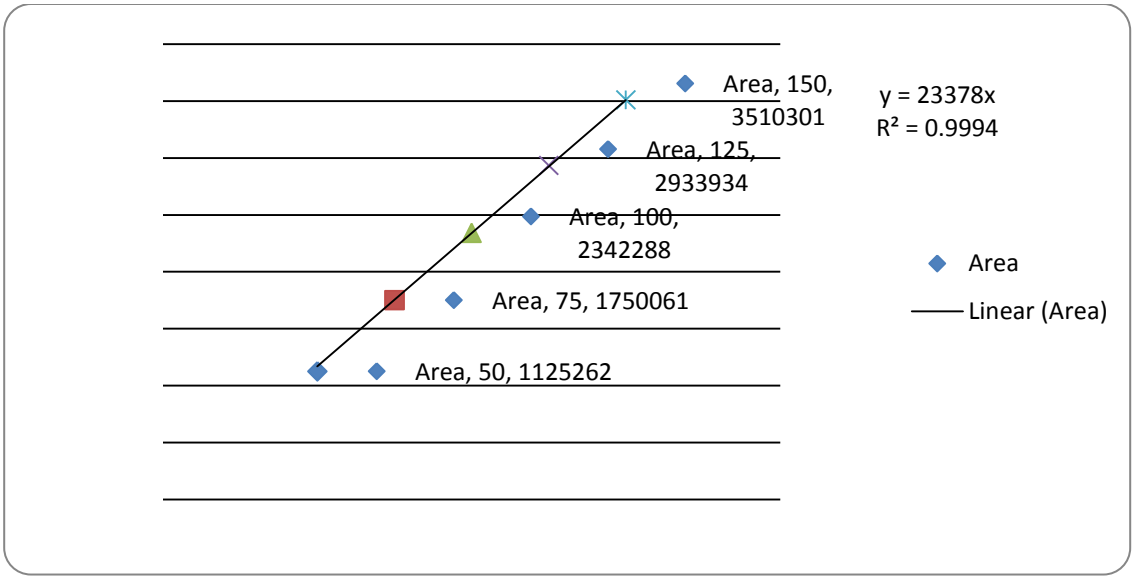
| S.no | Conc(µg/ml) | RT | Area |
|---|---|---|---|
| 1. | 50 | 2.171 | 670568 |
| 2. | 75 | 2.172 | 1008020 |
| 3. | 100 | 2.176 | 1340857 |
| 4. | 125 | 2.178 | 1672035 |
| 5. | 150 | 2.176 | 2015934 |
| Correlation coefficient ($r^2$) | | | 0.999 |




**Linearity plot of Ambroxol**

**Linearity data for Levofloxacin**

| S.no | Conc(µg/ml) | RT | Area |
|---|---|---|---|
| 1. | 50 | 4.360 | 1125262 |
| 2. | 75 | 4.359 | 1750061 |
| 3. | 100 | 4.363 | 2342288 |
| 4. | 125 | 4.373 | 2933934 |
| 5. | 150 | 4.363 | 3510301 |
| Correlation coefficient ($r^2$) | | | 0.999 |




**Lineariity plot of Levofloxacin**

## RESULT

A linear relationship between peak areas versus concentrations was observed for Ambroxol and Levofloxacin in the range of 50% to 150% of nominal concentration. Correlation coefficient was 0.999 for both Ambroxol and Levofloxacin which prove that the method is linear in the range of 50% to 150

### Precision

**Precision data for Ambroxol**

| S.no | RT | Area | %Assay |
|---|---|---|---|
| injection1 | 2.174 | 1345018 | 99 |
| injection2 | 2.176 | 1341662 | 99 |
| injection3 | 2.177 | 1347766 | 100 |
| injection4 | 2.175 | 1344750 | 99 |
| injection5 | 2.177 | 1347036 | 100 |
| injection6 | 2.176 | 1342685 | 99 |
| Mean | | | 99 |
| Std. Dev. | | | 0.18 |
| % RSD | | | 0.18 |

**Precision data for Levofloxacin**

| S.no | RT | Area | %Assay |
|---|---|---|---|
| injection1 | 4.360 | 2344232 | 100 |
| injection 2 | 4.359 | 2348002 | 100 |
| injection 3 | 4.362 | 2342121 | 100 |
| injection 4 | 4.358 | 2341546 | 100 |
| injection 5 | 4.358 | 2347734 | 100 |
| injection 6 | 4.365 | 2347075 | 100 |
| Mean | | | 100 |
| Std. Dev. | | | 0.12 |
| %RSD | | | 0.12 |

## RESULT

Results of variability were summarized in the above table. % RSD of peak areas was calculated for various run. Percentage relative standard deviation (%RSD) was found to be less than 2% which proves that method is precise

## Limit of detction

Minimum concentration of standard component in which the peak of the standard gets merged with noise called the LOD

LOD = 3.3* σ/S

Where;

σ = standard deviation

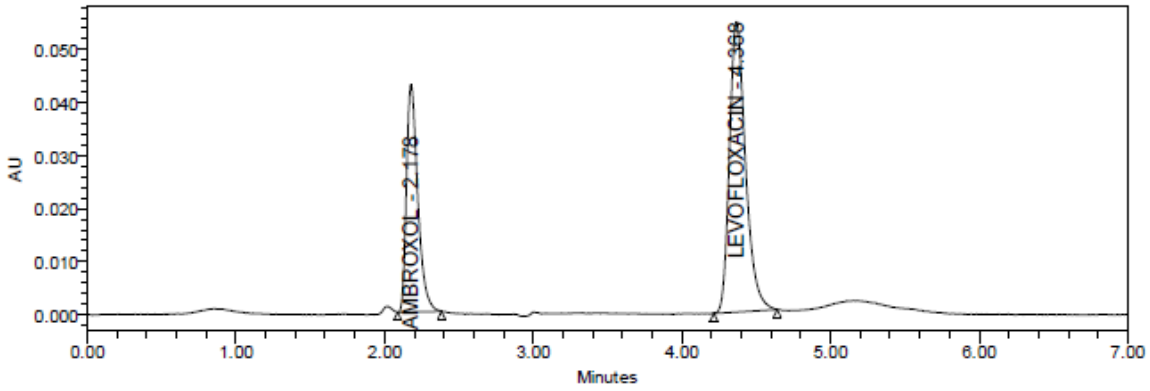
S = slope

LOD for Ambroxol = 0.035

LOD for Levofloxacin =0.1884

**LOD data for Ambroxol and Levofloxacin**

| S.no | Sample name | RT | Area |
|---|---|---|---|
| 1 | Ambroxol | 2.178 | 226664 |
| 2 | Levofloxacin | 4.368 | 410697 |




**Chromatrogram for LOD**

## Limit of quantification

Minimum concentration of standard component in which the peak of the standard gets detected and quantification

LOQ = 10*σ/S

Where;

σ = standard deviation

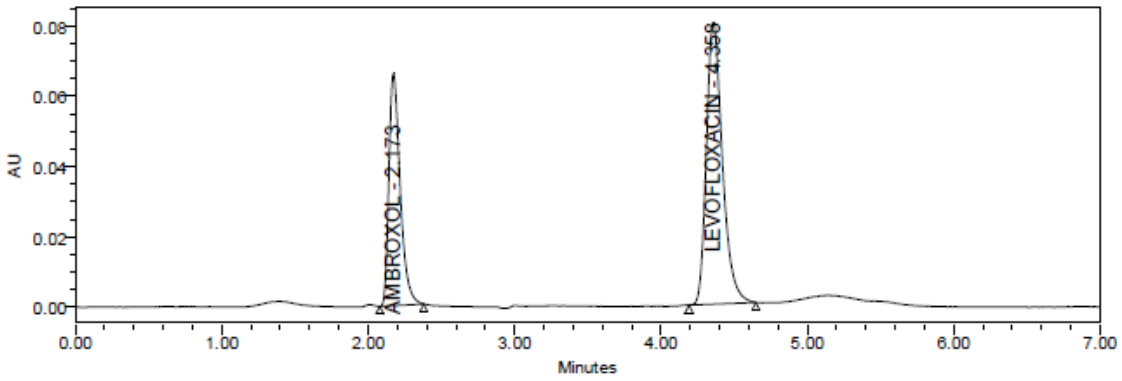
S = slope

LOQ for Ambroxol =0.116

LOQ for Levofloxacin =0.6281

**LOQ data for Ambroxol and Levofloxacin**

| S.no | Sample name | RT | Area |
|---|---|---|---|
| 1 | Ambroxol | 2.173 | 351847 |
| 2 | Levofloxacin | 4.358 | 606845 |




**Chromatogram for LOQ**

## CONCLUSION

The study is focused to develop and validate HPLC methods for estimation of Ambroxol and Levofloxacin in tablet dosage form.

For routine analytical purpose it is desirable to establish methods capable of analyzing huge number of samples in a short time period with good robustness, accuracy and precision without any prior separation steps. HPLC method generates large amount of quality data, which serve as highly powerful and convenient analytical tool.

The method shows good reproducibility and good recovery. From the specificity studies, it was found that the developed methods were specific for Ambroxol and Levofloxacin.

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