



“Analytical Method Development and Validation In Pharmaceutical Dosage Form Of Doxofylline And Ambroxol Hydrochloride In Bulk And Tablet Dosage Form By UV Spectroscopy & RP-HPLC Method.”

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1. INTRODUCTION

1.1 INTRODUCTION TO ANALYTICAL CHEMISTRY¹

Analytical Chemistry constitutes both theoretical and practical science and it is practical in a large number of laboratories in many different ways. The analytical procedure is the technique of performing the analysis. Analytical method validation is indeed necessary for herbal procedure, new process and reaction, new molecules, active ingredients, residues, impurity profiling and component of interest in different matrices. An analytical methodology comprises of the techniques, method, procedure and protocol. This methodology includes the required data for a given analytical problem, necessary sensitivity, requisite accuracy, mandatory range of analysis and requisite precision to the Analyst.

1.2 WHY VALIDATION ANALYTICAL PROCEDURE²

There are many reasons to validate analytical procedures. Among them are regulatory requirements, good science, and quality control requirement. The Code of Federal Regulations (CFR) 311.165c explicitly states that “accuracy, sensitivity, specificity, and reproducibility of test methods employed by the firm shall be established and documented”. Of course as Scientists we would want to apply good science to demonstrate that the analytical method used had demonstrated accuracy, sensitivity, specificity and reproducibility. Finally the management methods had demonstrated uses to release its product are properly validated for its intended use so the product will be safe for human use. Analytical methods need to be validated, verified or revalidated in the following instances.

- use in routine testing
- When transferred to another laboratory
- Whenever the conditions or method parameters for which the method has been validated change.

1.3 PROCESS OF ANALYTICAL METHOD VALIDATION

Process of the analytical method validation is listed below:

1. Preparation of the development on the method validation programme
2. To write the method validation protocol and get the approval
3. Implementation of the method validation protocol
4. Investigation of the method validation data
5. Reporting the analytical method validation
6. Finalizing the analytical method procedure

1.4 ICH GUIDELINES FOR ANALYTICAL METHOD VALIDATION^{3,4}

Method validation is the way to authenticate that the analytical procedure applied for a specific test is appropriate for its intended purpose. Methods need to be validated or revalidated. The International Conference of Harmonization (ICH) of technical requirements for the registration of pharmaceutical for human use has developed and provided a consensus text on validation of analytical procedures.

The parameters as defined by the ICH and by other organizations

- ✓ Specificity
- ✓ Accuracy

✓ Precision

- Repeatability
- Intermediate precision
- Reproducibility

✓ Accuracy

✓ Linearity

✓ Range

✓ Limit of detection

✓ Limit of quantitation

✓ Robustness

✓ Ruggedness

1. 4.1. SPECIFICITY

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to present. An investigation of specificity should be conducted during the validation of identification tests, the determination of impurities and assay.

1.4.2. 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 on an accepted reference value and the value found.

1.4.3. PRECISION

The precision of an analytical procedure expresses the closeness of the agreement between a series of measurements obtained from multiple sampling of same homogeneous sample under the prescribed conditions. Validation of tests for assay and for quantitative determination of impurities includes an investigation of precision.

Repeatability (Intra- assay precision)

Express the precision under small operating conditions over a short interval of time. It should be assessed using a minimum of nine determinations.

Intermediate Precision

The extent to which intermediate precision should be established depends on the circumstances under which the procedure is intended to be used. Typical validation to be studied includes days, Analysts, equipments, etc.

Reproducibility

Reproducibility measures the precision between laboratories. Reproducibility should be considered in case of the standardization of an analytical procedure, for insistence inclusion of procedure in Pharmacopoeias.

1.4.4. LINEARITY

Linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte sample.

1.4.5. RANGE

Range of an analytical procedure is the interval between the upper and lower concentration of analyte in the sample including these concentrations for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity.

1.4.6. LIMIT OF DETECTION

The detection limit is determined by the analysis of samples with known concentration of analyte and by establishing that minimum level at which the analyte can reliably detected.

- a. Based on visual evaluation
 - b. Based on signal-to-noise ratio
 - c. Based on the standard deviation of the response and the slope
- Based on the standard deviation of blank
 - Based on the calibration graph

1.4.7. LIMIT OF QUANTITATION

The quantitation limit is generally determined by the analysis of samples with the known concentrations of analyte and by establishing the minimum value at which the analyte can be quantified with acceptable accuracy and precision

- a. Based on visual evaluation
- b. Based on Signal-to- Noise ratio
- c. Based on the tandard deviation of the response and the slope

- Based on the standard deviation of blank
- Based on the calibration graphs

1.4.8. ROBUSTNESS

The evaluation of robustness should be considered during the development phase and depends on the type of procedure under study. It shows the reliability of an analysis with respect to deliberate variations in the method parameters.

1.4.9. RUGGEDNESS

The USP define ruggedness as the degree of reproducibility of test results obtained by the analysis of the same sample under a variety of normal test conditions such as different laboratories, different analysis, different lots of reagents etc. Ruggedness is a measure of reproducibility of test results under normal expected operational conditions from laboratory to laboratory and from Analyst to Analyst.

1.5. SYSTEM SUITABILITY TESTS⁵

System suitability tests are an integral part of Gas and Liquid Chromatography. They are used to verify that the resolution and reproducibility of the chromatographic system and are adequate for the analysis to be done. These tests are based on the concept that the equipment, electronics, analytical operations, samples to be analyzed and constitute an integral system that can be evaluated as such.

There are numerous guidelines which detail the expected limits for typical chromatographic methods. In the current FDA guideline on “Validation of Chromatographic Methods” the following acceptance limits are proposed as initial criteria.

1.5.1. Capacity Factor (K')

It is the measure of a sample peak in the chromatogram being specific for a given compound, a parameter which specifies the delay of a substance to be separated.

$K' = t-1/t_a$ Where,

t = retention time measured from time of injection to time of elution of peak maximum. t_a = retention time of non retarded component, air with thermal conductivity detection. Limit = generally the value of K' is > 2

1.5.2. Resolution (Rs)

The resolution R_s is a function of column efficiency N and is specified to ensure that closely eluting compounds are resolved from each other to establish the general resolving power of the system and to

ensure that internal standards are resolved from the drug.

$$R_s = t_2 - t_1 / 0.5(w_1 - w_2)$$

Where t_1 and t_2 = retention times of first and second adjacent bands.

Limit = R_s of >2 between the peak of interest and the closest potential interfering peak is desirable.

1.5.3. Tailing Factor (T)

The tailing factor T, a measure of peak symmetry, is unity for perfectly symmetrical peaks and its value increases as tailing becomes more pronounced.

In some cases, values less than unity may be observed. As peak asymmetry increases, integration, and hence precision becomes less reliable.

$$T = W_{0.05} / 2f$$

Where $W_{0.05}$ = width of peak at 5% height

f = Distance from the peak maximum to the leading edge of the peak, the distance being measured at a point 5% of the peak height from the baseline.

Limit: ≤ 2 is preferable.

1.5.4. Theoretical plates (N)

The number of theoretical plates, N is a measure of column efficiency. For Gaussian peaks, it is calculated by the equations.

$$N = 16(t / w)^2 \text{ or } N = 5.54(t / W_{1/2})^2$$

Where

t = retention time of substance.

w = width of the peak at its base, obtained by extrapolating the relatively straight sides of the peak to the baseline.

$W_{1/2}$ = width of the peak at half height, obtained directly by electronic integrators.

The value of 'N' depends upon the substance being chromatographed as well as the operating conditions such as mobile phase, temperature etc.

Limit = $N > 2000$ is desirable.

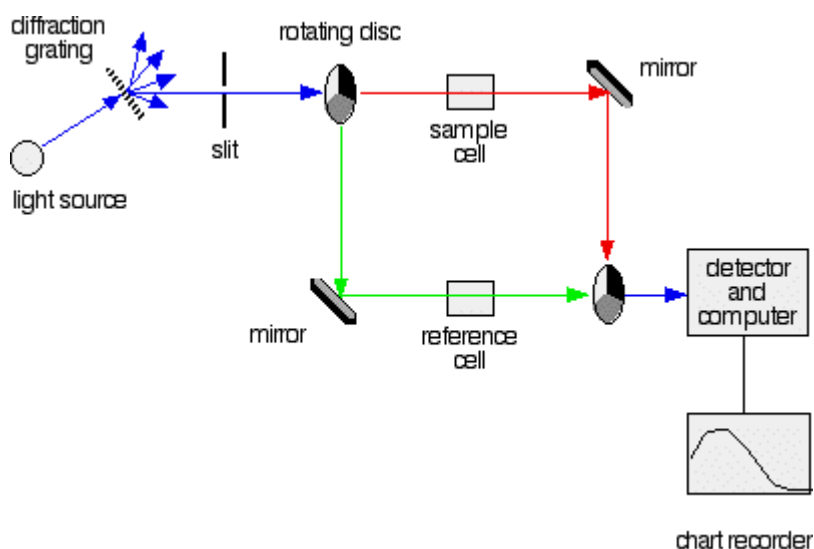
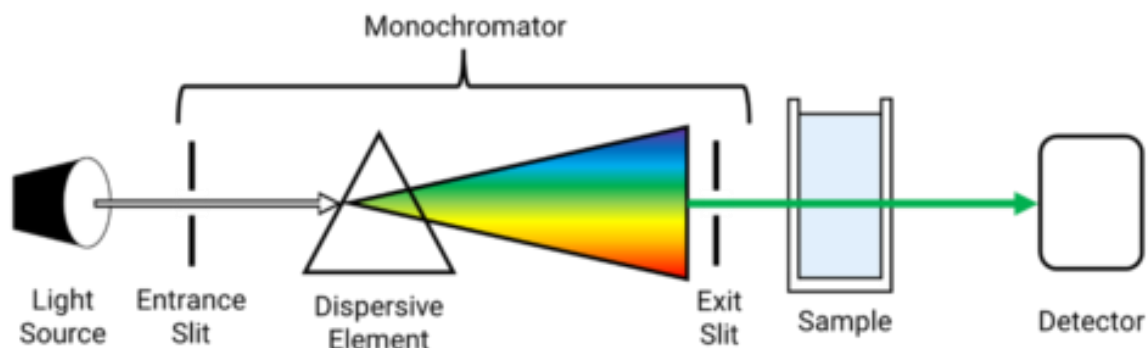
1.6 ULTRA VIOLET SPECTROSCOPY⁶

Ultraviolet spectroscopy deals with the measurement of energy absorbed when electrons are promoted to higher energy state. On passing electromagnetic radiation in the ultraviolet and visible regions through the compound with multiple bonds, a portion of the radiation is normally absorbed by the compound. The

amount of absorption depends on the wavelength of the radiation and the structure of the compound.

Absorption of the electromagnetic radiation in the visible and ultraviolet region of spectrum results in changes of electronic structure of ions and molecules.

Diagram of UV-Visible Spectrophotometer



Optical Diagram of a Double Beam UV-Visible Spectrophotometer

Quantitative Spectrophotometric Assay of Medicinal Substances

1. Use of a standard absorptivity value
2. Use of a calibration graph
3. Single-or double-point standardization

Methods of Multicomponent Analysis using UV-Visible Spectrophotometer⁷

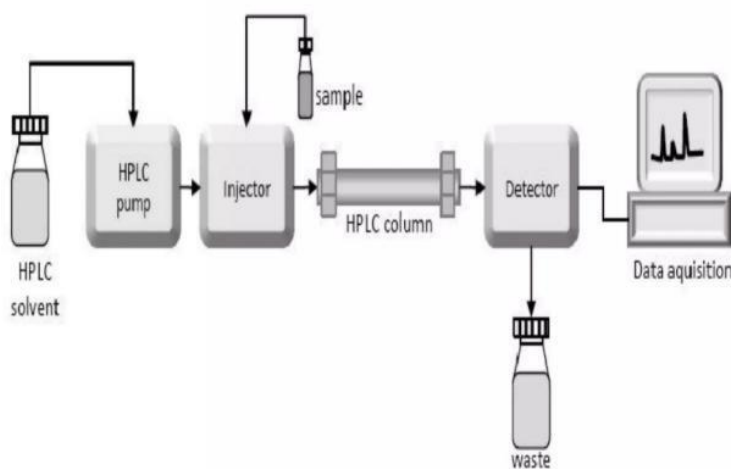
1. Simultaneous Equation Method
2. Absorbance Ratio or Q-analysis method.
3. Absorbance Correction Method

1.7 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY⁸

HPLC is a form of liquid chromatography to separate compounds that are dissolved in solution. HPLC instrument consists of four basic parts

- The column
- Detector
- Injection system and Mobile-phase pump system

A Schematic Diagram of HPLC Equipment



1.7.1 Principle of Separation in HPLC⁹ Normal phase chromatography

Mechanism: Retention by interaction of the stationary phase's polar surface with polar parts of the sample molecules.

Stationary phase: SiO₂, Al₂O₃, -NH₂, -CN, -Diol, -NO₂.

Mobile phase: Hectane, Hexane, Cyclohexane, CHCl₃, CH₂Cl₂, Dioxane, Methanol.

Application: Separation of non-ionic, non-polar to medium polar substances.

Reverse phase chromatography

Mechanism: Retention by interaction of the stationary phase's non-polar hydrocarbon chain with non-polar parts of the sample molecules.

Stationary Phase: n-octadecyl (RP-18), n-octyl (RP-8), ethyl (RP-2), phenyl, (CH₂)_n-CN, (CH₂)_n-diol.

Mobile phase: Methanol or Acetonitrile/Water or Buffer (sometimes with additives of THF or Dioxane)

(Rule of thumb: Increase of water content by 10% results in doubling the K' value.) **Application:**

Separation of non-ionic and ion forming non-polar to medium polar substances (carboxylic acids ->

hydrocarbons). If ion forming substances (as carboxylic acid) are to be separated, a pH control by buffers is necessary.

2. Review Of Literature

1. Lagana A *et al.*¹⁹ (2023), reported “**Solid Phase Extraction and High Performance Liquid Chromatographic Determination of Doxofylline in Plasma**”. The developed sensitive and selective High Performance Liquid Chromatographic Doxofylline assay used ultraviolet detection for plasma samples. The drug is isolated from biological samples with a reversed phase C₁₈ disposable extraction column. Plasma standard curves are linear for concentrations of Doxofylline from 0.03 to 10 mg/L.
2. Xie Zi-li²⁰ (2023), reported “**Determination of Doxofylline in Doxofylline Injection by GC Method**”. The capillary column used was DM-17 (30 mm X 0.32 mm X 0.25 μm). The carrier gas was nitrogen, and the detector was FID. The column temperature was 265°C and Papaverine Hydrochloride was selected as internal standard. The assay of Doxofylline was calculated by internal standard method.
3. Kamila MM *et al.*²² (2022), reported “**Development and Validation of Spectrophotometric Method for Estimation of Anti-Asthmatic Drug Doxofylline in Bulk and Pharmaceutical Formulation**”. The developed method utilized Double beam UV-Visible Spectrophotometer (UV-2450, Shimadzu, Japan) and has showed absorption maximum at 272 nm in water.
4. Kan Quan-cheng *et al.*²⁵ (2022), reported, “**HPLC Determination of Doxofylline and Pharmacokinetic Study in Serum of Patients with Chronic Obstructive Pulmonary Disease**”. The Doxofylline in serum after intravenous injection was determined by HPLC at 273 nm. The sample was separated on Waters C₁₈ column (150 mm X 3.9 mm, 4 μm) with mobile phase consisting of 0.1% triethylamine- 0.02 mol/L NaH₂PO₄ buffer (pH 6.8 ± 0.1) (15: 85) at a flow rate 1.0 ml/min. The pharmacokinetic parameters were analyzed by 3P97.
5. Narendra G. Patre *et al.*²⁶ (2021), reported “**A Validated, Stability-Indicating HPTLC Method for Analysis of Doxofylline**”. The developed method used aluminum plates coated with silica gel 60 F₂₅₄ as stationary phase and toluene-methanol (8:2) as mobile phase, followed by densitometric measurement at 254 nm. The R_F value of Doxofylline was 4.3. The drug was subjected to acidic, alkaline, oxidative, and photolytic stress to establish a validated stability-indicating HPTLC method.

6. Ashu Mittal *et al.*²⁷ (2021), reported “**Development and Validation of Rapid HPLC Method for Determination of Doxofylline in Bulk Drug and Pharmaceutical Dosage Forms**”. The chromatographic separation was achieved on HiQ Sil C₁₈ column using a mobile phase of acetonitrile: buffer (50: 50), pH 3, at a flow rate of 1 ml/min with detection of analyte at 272 nm. The separation was achieved within 3.1 ± 0.3 min for Doxofylline.
7. Liu Yifang *et al.*²⁹ (2020), reported “**Determination of Theophylline and Doxofylline in Human Plasma by HPLC**”. The developed method used C₁₈ column with caffeine as internal standard and the mobile phase of methanol-water (23:77). The detection wavelength was 273 nm. The plasma samples were injected directly after precipitation by methanol. The calibration curves of Theophylline and Doxofylline were linear in the concentration range of 2.5-40 µg/ml. The intra-and interday RSDs were less than 5.2%.
8. Venkatesan S *et al.*³⁰ (2020) reported “**A Simple HPLC Method for Quantitation of Doxofylline in Tablet Dosage Form**”. The developed Reverse-Phase High Performance Liquid chromatographic method used inertsil octyl decyl column in isocratic mode with mobile phase consisting of Methanol: Water (30:70) at a flow rate of 1.5 ml/min. The eluents were monitored at 274 nm.
10. Akhilesh G *et al.*³¹ (2019), reported “**Method Development and Acid Degradation Study of Doxofylline by RP-HPLC and LC-MS/MS**”. The developed and validated Reverse Phase High Performance Liquid Chromatography used acetonitrile: 0.05M formic acid in the ratio of 90:10, pH 3.0 as mobile phase and monitored at 274 nm. The acid degradation product as well as pathway was characterized by LC-MS/MS.
11. Atkuru Veera Venkata Naga Krishna Sunil Kumar *et al.*³² (2019), reported “**Development and Validation of Novel Analytical Methods for Estimation of Doxofylline in Bulk and Dosage Forms**”. Three methods were developed. The first method is based on charge-transfer complex formation of the drug with p-chloranilic acid and second method involves the formation of colored chloroform extractable ion-pair complex of the drug with bromophenol blue under acidic condition. The third method is based on ternary complex formation of the drug with molybdenum (V) thiocyanate binary complex. The colored products are quantitated spectrophotometrically at 540 nm, 390 nm and 690 nm for first, second and third method respectively.

12. Revathi R *et al.*³⁵ (2019), reported “**High Performance Liquid Chromatographic Method Development for Simultaneous Analysis of Doxofylline and Montelukast Sodium in a Combined Form**”. The chromatographic analysis was performed on inertsil C₈ column (4.6 mm X 250 mm, 5 µm) in isocratic mode with mobile phase consisting of Methanol-Sodium phosphate buffer (75:25), pH 6.5 at a flow rate of 1 ml/min. The eluents were detected at 230 nm.

13. Gadapa Nirupa *et al.*³⁶ (2018), reported “**Novel LC Method Development and Validation for Simultaneous Determination of Montelukast and Doxofylline in Bulk and Pharmaceutical dosage form**”. The chromatographic separation was carried out on C₁₈ column (150 mm X 4.6 mm, 5 µm) with the mobile phase comprised of methanol- phosphate buffer, pH 4.5 (90:10) at a flow rate of 1 ml/min and the eluents were detected at 280 nm.

14. Maulik Oza *et al.*³⁷ (2018), reported “**Development and Validation of Solvent Extraction Spectrophotometric Method for Simultaneous Estimation of Doxofylline and Terbutaline sulphate in their Combined Dosage Form**”. UV 2080 plus model, silicon photodiode detector controlled by UV Analyst software was utilized in this method. Solvent extraction method was performed at 277 nm .

15. Dincer Z *et al.*³⁹ (2017), reported “**Quantitative Determination of Ambroxol in Tablets by Derivative UV Spectrophotometric Method and HPLC**”. The Ambroxol was determined by First-order derivative UV-spectrophotometric method at 255 nm. The chromatographic method was performed on C₁₈ column with a mixture of aqueous phosphate (0.01 M), acetonitrile and glacial acetic acid in the ratio of 59:40:1.

16. Meiling Qi *et al.*⁴⁰ (2016), reported “**Simultaneous Determination of Roxithromycin and Ambroxol Hydrochloride in a New Tablet Formulation by Liquid Chromatography**”. The chromatographic method was carried out on a Diamonsil TM C₁₈ column. The mobile phase comprised of a mixture of acetonitrile, methanol and 0.5% ammonium acetate (39:11:50). Detection was carried out at 220nm.

17. Pai PNS *et al.*⁴¹ (2016), reported “**Determination of Ambroxol Hydrochloride using Dithiocarbamic acid Colorimetric method**”. A new simple colorimetric method was developed on the basis of a chemical reaction of amine group in Ambroxol Hydrochloride with carbon disulphide to form Dithiocarbamic acid, which on further reaction with cupric chloride forms a coloured copper chelate. The

yellowish-orange chromophore has absorption maxima at 448 nm.

18. Bhatia NM *et al.*⁴³ (2015), reported “**RP-HPLC and Spectrophotometric Estimation of Ambroxol Hydrochloride and Cetirizine Hydrochloride in Combined Dosage Form**”. The chromatographic methods were standardized using a HIQ SIL-C₁₈ column (250 X 4.6 mm i.d., 10 µm) with UV detection at 229 nm and mobile phase consisting of methanol-acetonitrile-water (40:40:20).

19. Lakshmana Prabhu S *et al.*⁴⁴ (2015), reported “**Simultaneous UV Spectrophotometric Estimation of Ambroxol Hydrochloride and Levocetirizine Dihydrochloride**”. The developed method was found to be simple, accurate and reproducible. A Shimadzu UV/Visible spectrophotometer, model 1601 was used in this method and the measurement of absorbance at 242 and 231 nm for Ambroxol Hydrochloride and Levocetirizine Dihydrochloride respectively.

20. Krishna Veni Nagappan *et al.*⁴⁵ (2014), reported “**A RP-HPLC Method for Simultaneous Estimation of Ambroxol Hydrochloride and Loratidine in Pharmaceutical Formulation**”. The developed Reverse Phase HPLC method is simple, selective, rapid, precise and economical. This method utilized Phenomenex Gemini C₁₈ (25 cm X 4.6 mm i.d., 5 µ) column with a mobile phase comprised of acetonitrile: 50mM Ammonium Acetate (50:50) at a flow rate of 1.0 ml/min, with detection at 255 nm.

21. Deshpande MM *et al.*⁴⁶ (2014) reported “**Application of HPLC and HPTLC for the Simultaneous Determination of Cefixime Trihydrate and Ambroxol Hydrochloride in Pharmaceutical Dosage Form**”. The methods employed are 1. High Performance Thin Layer Chromatography and 2. High Performance Liquid Chromatography. In HPTLC followed by densitometric measurements, the spots were made at 254 nm. The chromatographic separation was carried out on HPTLC aluminium sheets of silica gel 60 F254 and mobile phase containing acetonitrile: methanol: triethylamine in the ratio of 8.2:1:0.8. In HPLC method, the chromatographic separation was made on C₁₈ column using mobile phase as acetonitrile: methanol in the ratio of 50:50.

22. Dhiraj S. Nikam *et al.*⁴⁷ (2013) reported “**Stability Indicating RP-HPLC Method for Simultaneous Estimation of Ambroxol Hydrochloride and Roxithromycin in Bulk and Tablet Dosage Form**”. The chromatographic separation was made on Phenomenex Gemini C₁₈ column (250 cm X 4.6 mm, 5 µm) and mobile phase consisted of water: acetonitrile: orthophosphoric acid (50:50:0.1), at a flow rate of 1 ml/min

and monitored at 210 nm. Jain PSI⁴⁸ (2010), reported “**Stability-Indicating HPTLC determination of Ambroxol Hydrochloride in bulk drug and pharmaceutical dosage form**”. The Chromatography separation was carried out on HPTLC aluminium plates precoated with silica gel 60F-254 and the solvent system consisted of methanol-triethylamine (4:6). Densitometric analysis of Ambroxol Hydrochloride was carried out in the absorbance mode at 254 nm.

23. Prashanthi NL *et al.*⁴⁹ (2013), reported “**Estimation of Ambroxol Hydrochloride and Guiaphensin in Tablet Dosage Form by Simultaneous Equation Method**”. The absorbance for Ambroxol Hydrochloride and Guiaphensin were measured at 242 and 272 nm respectively. Beer’s law was obeyed at the concentration range of 5-50 µg/ml for Ambroxol and 10-80 µg/ml for Guiaphensin. The molar absorptivity for Ambroxol and Guiaphensin were 9742 ± 0.894 and 1015 ± 0.707 respectively.

25. Maithani M *et al.*⁵¹ (2012), reported “**Simultaneous estimation of Ambroxol Hydrochloride and Cetirizine Hydrochloride in Tablet Dosage Form by RP-HPLC Method**”. The developed Reverse Phase High Performance Liquid Chromatographic method is simple, specific and accurate. The chromatographic separation was made on Princeton C-8 (4.6 X 25 mm, 5 µm) column and mobile phase comprised of methanol and potassium dihydrogen phosphate buffer in the ratio of 80:20 adjusted to pH 3.5 ± 0.02 with orthophosphoric acid, at a flow rate of 1.0 ml/min and were measured at 276 nm.

26. Senthil Raja M *et al.*⁵² (2012), reported “**RP-HPLC Method Development and Validation for the Simultaneous Estimation of Azithromycin and Ambroxol Hydrochloride in Tablets**”. The chromatographic separation was carried out using mobile phase consisting of acetonitrile and mono basic potassium phosphate buffer of pH 8.5 in the ratio of 65:35. The column used was C₁₈ phenomenex Gemini 5m, 250cm X 4.6mm id with flow rate of 2 ml/min using PDA detection at 220 nm.

27. Ilangovan Ponnilarvarasan *et al.*⁵⁴ (2011), reported “**Simultaneous Estimation of Ambroxol Hydrochloride and Loratadine in Tablet Dosage Form by using UV Spectrophotometric Method**”. The spectrophotometric method developed is rapid, simple, accurate, sensitive and specific. The absorbance for Ambroxol and Loratadine was measured at 308 nm and 245 nm respectively.

27. Wankhede SB *et al.*⁵⁷ (2010), reported “**Simultaneous Spectrophotometric Estimation of Gemifloxacin Mesylate and Ambroxol Hydrochloride in Tablets**”. The developed two UV-spectrometric methods were simple, sensitive and rapid. In the simultaneous equation method, the absorbance of

Gemifloxacin and Ambroxol were measured at 272 and 249.5 nm respectively. In the first order derivative spectroscopy method, wavelengths selected for quantitation were 216 nm for Gemifloxacin and 279 nm for Ambroxol.

28. Patel PA *et al.*⁵⁸ (2010), reported “**Spectrophotometric Simultaneous Estimation of Salbutamol and Ambroxol in Bulk and Formulation**”. The two developed methods are 1. Simultaneous equation method and 2. Area under the curve method. In simultaneous equation method, the measurement of absorbance was made at 223 nm and 244 nm for Salbutamol sulphate and Ambroxol Hydrochloride respectively. In area under the curve method, the wavelength range was 232-217 nm for Salbutamol and 252-237 nm for Ambroxol.

29. Dhaneshwar SR *et al.*⁶¹ (2009), reported “**Validated HPTLC Method for Simultaneous Estimation of Amoxicillin Trihydrate and Ambroxol Hydrochloride in Pharmaceutical Dosage Form**”. Chromatographic separation was made on aluminium plates precoated with silica gel 60 F254 and solvent system consisted of N-butanol: 1.0 M Ammonium acetate: Methanol in the ratio of 7.5:2.0:1.5. Densitometric evaluation of the separated zone was performed at 222 nm.

30. Avinash V. Deosarkar *et al.*⁶² (2009), reported “**Simultaneous Quantification of Salbutamol Sulphate and Ambroxol Hydrochloride by RP-HPLC and HPTLC in Bulk Drug and Dosage Form**”. Two methods were developed 1.Reverse Phase High Performance Liquid Chromatography and 2. High Performance Thin Layer Chromatography. In the RP-HPLC method, Inertsil, ODS-3V C₁₈ (250 mm X 4.6 mm, 5µm) column in isocratic mode was used with mobile phase comprising of acetonitrile: 50 mM disodium hydrogen phosphate buffer (containing 0.1% triethylamine, pH 4.2) (28:72) at a flow rate of 1mL/min. In the HPTLC method, the chromatograms were developed using a mobile phase of methanol: ethyl acetate: toluene: ammonia (4:1.5:5.6:1.0) on precoated plate of silica gel 60 F₂₅₄ and quantified by densitometric absorbance mode at 231 nm.

31. Gopalakrishnan S *et al.*⁶⁴ (2009), reported “**Development of RP-HPLC Method for the Simultaneous Estimation of Ambroxol Hydrochloride, Cetirizine Hydrochloride and Antimicrobial Preservatives in Combined Dosage Form**”. The developed RP-HPLC method for simultaneous determination of Ambroxol Hydrochloride, Cetirizine hydrochloride, Methylparaben and Propylparaben in combined liquid pharmaceutical formulation was carried out on Hypersil BDS C₁₈ (200 mm X 4.6mm, 5µm) column using

acetonitrile: 0.05 M potassium dihydrogen orthophosphate, pH 3.5 (33:67) at a flow rate of 1 ml/min and effluent was detected at 230 nm.

32. Jigar Goswami *et al.*⁶⁵ (2008), reported “**RP- HPLC Method Development and Validation for Simultaneous Estimation of Ambroxol Hydrochloride and Cefpodoxime Proxetile in Pharmaceutical Dosage Form**”. The Chromatographic analysis was performed on Phenomenex Luna C₁₈ column with mobile phase containing Acetonitrile: 0.05 M Potassium Dihydrogen Ortho Phosphate Buffer (70:30), pH 6.7 at a flow rate of 1.0 ml/min and detected at 245 nm.

33. Nidhi Dubey *et al.*⁶⁷ (2008), reported “**Development of HPLC Method for Simultaneous Estimation of Ambroxol in Single Dose Form**”. The chromatographic separation was achieved on C₈ column (250 mm X 4.6 mm, 5 µm) in isocratic mode with mobile phase consisting of disodium hydrogen orthophosphate buffer: methanol, pH 4.5 at a flow rate of 1 ml/min. Scanning was performed at 220 nm.

3. AIM AND OBJECTIVES AIM:

To develop a simple, precise and accurate methods for the estimation of Doxofylline & Ambroxol Hydrochloride in bulk and in combined Pharmaceutical Dosage form and to validate the developed methods by UV Spectrophotometry, Reverse Phase High-Performance Liquid Chromatography or by both methods.

Objectives

Doxofylline & Ambroxol combination is used as an Antiasthmatic agent.

In the view of the literature cited for the quantification of above mentioned combination of drugs, it was found that the methods for the estimation of Doxofylline, Ambroxol Hydrochloride tablets individually and in combination with other drugs were available. No method available for the simultaneous estimation of the combined dosage forms with the solvents employed for the analytical studies.

To develop a simple, precise and accurate methods for the estimation of Doxofylline & Ambroxol Hydrochloride in bulk and in combined Pharmaceutical Dosage form and to validate the developed methods by UV Spectrophotometry, Reverse Phase High-Performance Liquid Chromatography or by both methods.

The combined dosage forms selected for the present study are Doxofylline & Ambroxol Hydrochloride tablets.

4. SCOPE AND PLAN OF WORK

The overall scope and plan of the research work is to develop the methods for new drug combinations enter into the market and to validate the newer analytical methods as per ICH guidelines. The parameters used to validate the developed method are: Accuracy, Precision, Linearity, Range, Repeatability, Reproducibility, Limit of Detection, Limit of Quantitation and Ruggedness. The system suitability test parameters like Capacity factor, Asymmetry factor, Tailing factor, Theoretical plates, HETP and Resolution should be calculated for RP-HPLC chromatograms and compared with standard values.

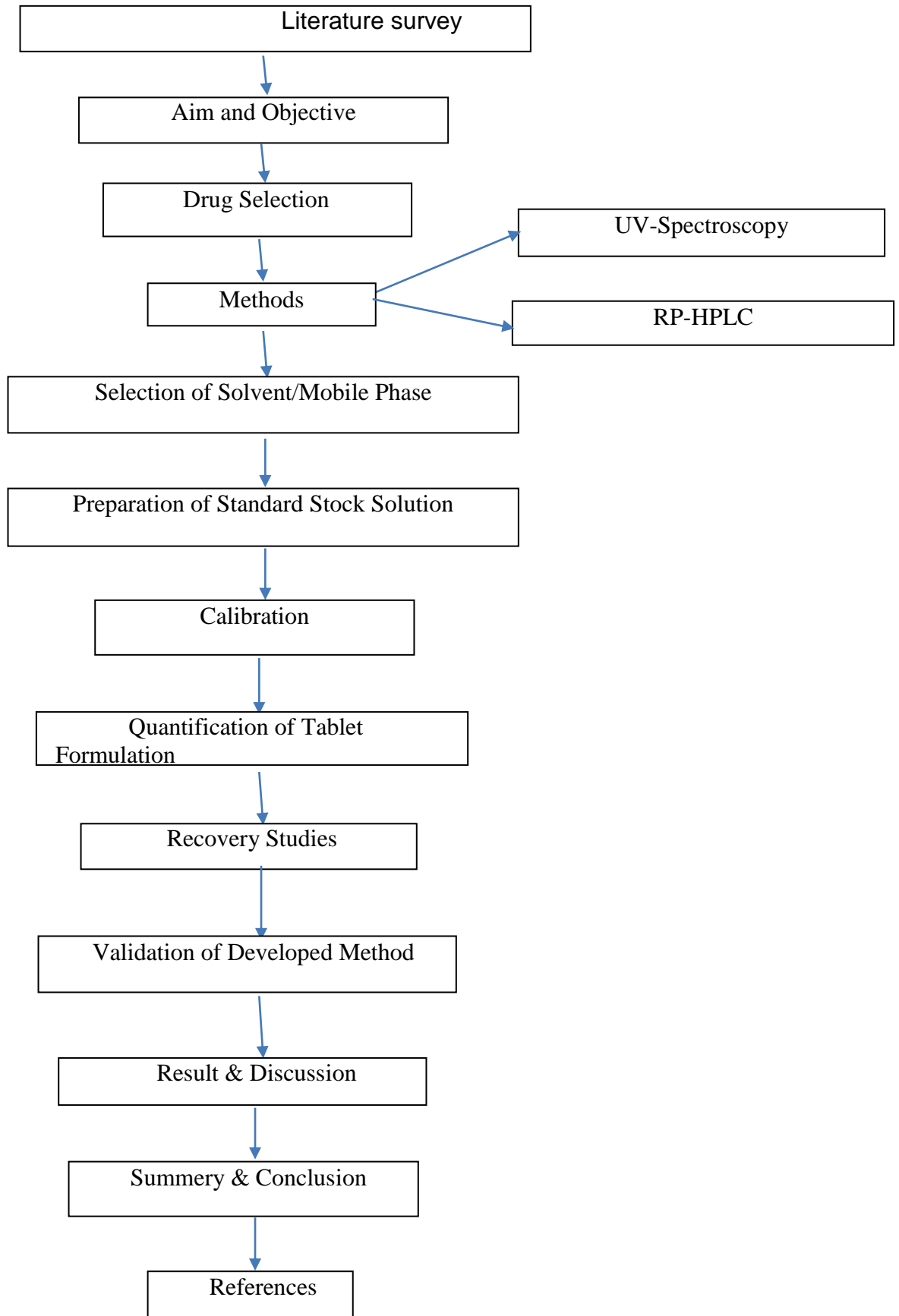
The plan of the present work is listed below:

For UV method:

- ✓ Find the solubility of drugs in various solvents
- ✓ To determine maximum absorbance and selection of wavelengths for detection.
- ✓ To determine the stability of drugs in the selected solvent at the specified wavelength
- ✓ Determining the standard absorbance for all selected wavelengths for each drug
- ✓ Development of simple, precise, accurate and sensitive methods
- ✓ Validation of developed methods as per ICH guidelines.

For RP-HPLC:

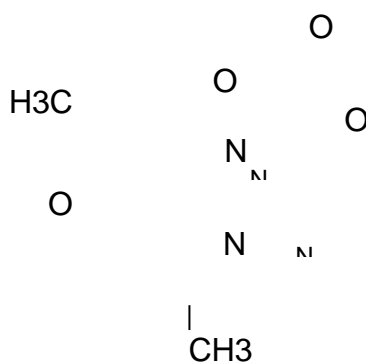
- ✓ Selection of suitable mobile phase and common wavelength for two drugs with proper resolution and short duration of time
- ✓ To determine the stability of the drugs in the mobile phase at the selected wavelength.
- ✓ Relating the area of chromatogram with respect to concentration for individual drugs
- ✓ Determination of percentage purity of physical mixture and in formulation
- ✓ Validation of the developed method



5 DRUG PROFILE

5.1.1. DOXOFYLLINE^{5,10,11,12} Chemical

Structure



Chemical name

7-(1,3-Dioxolan-2-yl methyl)-3,7-dihydro-1,3-dimethyl-1H-Purine-2,6-Dione.

Molecular formula

C₁₁H₁₄N₄O₄

Molecular weight

266.26

Category Anti-asthmatic Description

White crystalline powder

Solubility

Soluble in water, acetone, ethyl acetate, benzene, chloroform, dioxane, hot methanol and hot ethanol;

Practically insoluble in ethyl ether or petroleum ether.

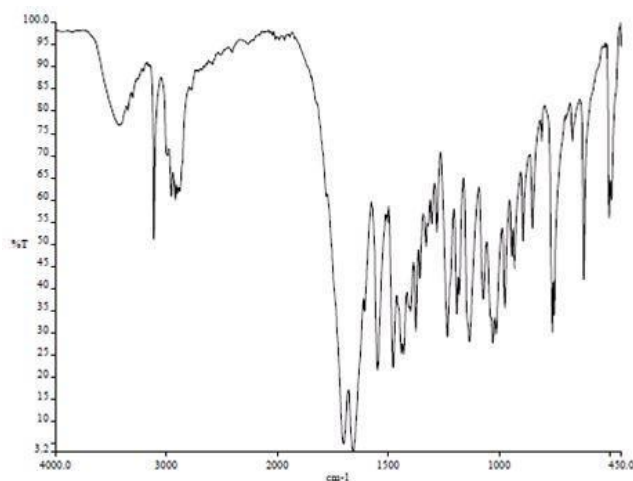
Identification

1. Melting point

Standard value	Observed average value*
144 °C – 145.5°C	145°C

*Average of six observations

2. Infra red spectrum



Storage

Store in a cool, dark and dry place

Indication

Doxofylline is primarily indicated for Bronchial asthma, Bronchospasm and Chronic asthmatic bronchitis.

Mode of action:

Doxofylline is methyl xanthine derivatives and plays the direct role in relaxation of bronchial smooth muscle and thus acts as bronchodilator.

Doxofylline is the inhibitor of Phosphodiesterase and thus increases the intracellular level of cyclic-3',5'-adenosine monophosphate (cAMP) which produce bronchodilation and thus achieving suppression asthma role.

Pharmacokinetics

Plasma protein binding is 48%. Renal excretion accounts for less than 4% and plasma half life is 7.42 hours.

Adverse Reaction

Nausea, vomiting, epigastric pain, cephalgia, irritability, insomnia, tachycardia, extrasystole, tachypnea, hyperglycemia, albuminuria.

Contraindication

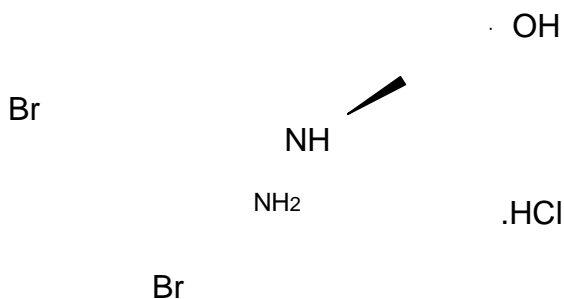
Doxofylline is contraindicated in conditions like Acute Myocardial infarction, Hypersensitivity to xanthine derivatives.

Route of administration

1. It is given by mouth in doses upto 1200 mg daily
2. It may also be given by slow intravenous injection

Special Precaution:

Liver disease, Congestive Heart Failure, Chronic Obstructive Lung Disease, Concomitant Infections.

5.1.2 AMBROXOL HYDROCHLORIDE^{5,10,11,12} Chemical Structure**Chemical Name**

1 ({[2-Amino-3, 5 dibromo phenyl]-methyl} amino) cyclohexanol monohydrochloride

Molecular formula

$C_{13}H_{18}Br_2N_2O.HCl$

Molecular weight

414.6

Category

Mucolytic agent; Expectorant

Description

A white or yellowish crystalline powder

Solubility

Sparingly soluble in water; Soluble in methanol and practically insoluble in methylene chloride

pH

A 1% solution in water has a pH of 4.5 to 6.0

Standard

Ambroxol Hydrochloride contains not less than 99.0% and not more than 101.0% of $C_{13}H_{18}Br_2N_2O$, calculated on the dried basis

LOD

NMT 0.5%, determined on 1.0 gm by drying in an oven at 105°C

Assay

Dissolve 0.3 gm in 70 ml of ethanol. Titrate with 0.1 M NaOH, determining the end point potentiometrically. Carry out blank. 1 ml of 0.1 M NaOH is equivalent to 0.04146 gm of Ambroxol Hydrochloride.

Melting point

Standard value	Observed average value*
232 °C -234°C	233°C

❖ Average of six observations

Storage

1. Protect from light. Following reconstitution, aliquot and freeze at -20°C. This product is stable for 2 years as supplied
2. Stock solutions are stable for 4 months at -20°C

Indication:

It is primarily indicated in conditions like Bronchitis, Chronic bronchitis, Cystic fibrosis

Mode of action

The substance is a mucoactive drug with several properties including secretolytic and secretomotoric actions that restore the physiological clearance mechanisms of the respiratory tract which play an important role in the body's natural defense mechanisms. It stimulates synthesis and release of surfactant by type II pneumocytes. Surfactants act as an anti-glue factor by reducing the adhesion of mucus to the bronchial wall, in improving its transport and in providing protection against infection and irritating agents.

Adverse drug reaction

The symptomatic adverse reactions produced by Ambroxol HCl are more or less tolerable and if they become severe, they can be tolerated symptomatically, these include Hypersensitivity reactions and Contact allergy.

Overdosage

No symptoms of over dosage have been reported in man due to date. If they occur, symptomatic treatment should be provided.

Drug Interactions

1. Administration of Ambroxol together with antibiotics (Amoxicillin, Cefuroxime, Erythromycin, Doxycycline) lead to higher antibiotic concentration in the lung tissue.
2. No clinically relevant unfavourable interaction with other medications has been reported.

Contraindication

Ambroxol should not be used in patients known to be hypersensitive to Ambroxol or other components of the formulation.

5. MATERIALS AND METHODS

5.1 INSTRUMENTS SPECIFICATIONS

1. Shimadzu AUX- 200 digital balance
2. Shimadzu 1700 double beam UV-visible spectrophotometer with a pair of 10 mm matched quartz cells
3. Shimadzu HPLC system (LC-10ATVP)
4. Elico SL-210 double beam UV-visible spectrophotometer with a pair of matched quartz cells
5. Remi centrifuge apparatus
6. Sonicator model 2120 MH
7. Cyberlab micropipette
8. Elico LI 120 pH meter
9. Melting point apparatus - Guna enterprises Chennai

5.1.1 SPECIFICATIONS (TERMS) OF INSTRUMENTS

a) **Shimadzu AUX-200 digital balance:** (Shimadzu instruction manual)

SPECIFICATIONS	
Weighing capacity	200 gm
Minimum display	0.1 mg
Standard deviation	≤ 0.1 mg
Operation temperature range	5 to 40°C

b) Shimadzu UV-Visible spectrophotometer: (Shimadzu instruction manual) Model: Shimadzu, UV-1700, pharماسpec; Cuvetts: 1 cm matched quartz cells

Specifications	
Light source	20 W halogen lamp, Deuterium lamp. Light source position automatic adjustment mechanism.
Monochromator	Aberration-correcting concave holographic grating
Detector	Silicon Photodiode
Stray Light	0.04% or less (220 nm: NaI 10 g/l) 0.04% or less (340 nm: NaNO ₂ 50 g/l)
Measurement wavelength range	190~1100 nm
Spectral Band Width	1 nm or less (190 to 900 nm)
Wavelength Accuracy	± 0.5 nm automatic wavelength calibration mechanism
Recording range	Absorbance : -3.99~3.99 Abs Transmittance : -399~399%
Photometric Accuracy	± 0.004 Abs (at 1.0 Abs), ±0.002 Abs (at 0.5 Abs)
Operating Temperature/Humidity	Temperature range : 15 to 35°C Humidity range : 35 to 80% (15 to below 30° C) 35 to 70% (30 to 35° C)

c) Shimadzu High Performance Liquid Chromatography:

(Shimadzu instruction manual)

Detector Specifications	
Light source	Deuterium Arc lamp
Measurement wavelength range	190 to 700 nm
Spectral Band Width	5 nm
Wavelength Accuracy	± 1 nm

Cell path length	10 nm
Cell volume	20 μ l
Operating temperature range	4 to 35° C (39 to 104° F)
Recording range	0.0001 to 4.000 AUFS
Operating temperature/Humidity	4 to 35° C / 75 %
Pump Specifications	
Pump type	Double reciprocating plunger pump
Pumping method	Constant flow delivery and constant pressure delivery
Suction filter	45 μ m
Line filter	5 μ m mesh
Operating temperature	4 to 35° C (39 to 104° F)

5.2 REAGENTS AND CHEMICALS USED IN THE STUDY:

All the chemicals used were of analytical reagent grade and HPLC grade procured from Qualigens India Pvt. Ltd., Mumbai. The chemicals used for the study were

- Distilled water
- Acetonitrile (HPLC Grade)
- Methanol (Spectral and HPLC Grade)
- Water (Spectral and HPLC Grade)
- Orthophosphoric acid (Analytical Grade)

5.3 MATERIALS AND METHODS FOR DOXOFYLLINE AND AMBROXOL HYDROCHLORIDE COMBINATION DOSAGE FORM:

Drugs

Pharmaceutically pure sample of Doxofylline (DOX) and Ambroxol Hydrochloride (AMB) were generously gifted by Shine Pharmaceuticals Pvt. Ltd., Chennai and Apex Pharmaceuticals Pvt. Ltd., Allathur. Combination product (SYNASMA-AX, Ranbaxy Laboratories Ltd.) containing 400 mg Doxofylline and 30 mg Ambroxol Hydrochloride was procured from a local Pharmacy.

Methods Employed

The methods employed for the simultaneous estimation of Doxofylline and Ambroxol Hydrochloride in combination are

1. UV Spectrophotometric method
 - a. Simultaneous equation method
 - b. Absorbance correction method and
 - c. Absorbance Ratio Method
2. Reverse Phase High-Performance Liquid Chromatography method

5.3.1. UV SPECTROPHOTOMETRIC METHODS:

Selection of solvent

The solubility of drugs were determined in a variety of solvents as per Indian Pharmacopoeial standards. Solubility was carried out in non polar to polar solvents. The common solvent was found to be distilled water for the analysis of DOX and AMB for the proposed method.

Preparation of standard stock solution

Accurately weighed drug samples of both DOX and AMB (50 mg each) were transferred to a suitable standard volumetric flask separately, dissolved and diluted to mark with distilled water. Both the drug solutions were diluted so as to get 10 µg/ml. The solutions were scanned in the UV region of 200-400 nm in 1cm cell against distilled water as blank and the overlain spectra was recorded.

Selection of wavelengths for estimation and stability studies

From the overlain spectra, by the observation of spectral characteristics of DOX and AMB, the drugs were simultaneously estimated by Simultaneous equation method, Absorbance correction method and Absorbance Ratio method. The wavelengths selected for Simultaneous equation method were 274 nm and 244.5 nm for DOX and AMB respectively.

For Absorbance Correction Method, it was observed that DOX has zero absorbance at 308 nm, where as AMB has substantial absorbance. Thus AMB was estimated directly at 308 nm without interference of DOX. For estimation of DOX, the absorbance of AMB was measured at 274 nm using standard solution of 10 µg/ml. The contribution of AMB was deducted from the total absorbance of sample mixture at 274 nm. The calculated absorbance was called as corrected absorbance for DOX. To estimate the amount of DOX, the absorbance of AMB were corrected for interference at 274 nm by using absorptivity values.

For Absorbance Ratio Method, the wavelengths selected were 244.5 nm (λ_{\max} of AMB) and 233.5 nm (wavelength of equal absorptivity of two components i.e. iso-absorptive point).

Preparation of calibration graph

From the above stock solution, aliquots were drawn and suitably diluted so as to get the final concentration range of 7-35 μ g/ml of DOX and 1-5 μ g/ml of AMB. Absorbances of these solutions were recorded in the respective wavelengths.

Quantification of tablet formulation

Twenty tablets were weighed and the average weight was found. The tablets were triturated to get a fine powder. An accurately weighed quantity of powder equivalent to 70 mg of DOX was transferred into a 100 ml volumetric flask, sufficient distilled water was added and the solution was sonicated for 15 minutes and diluted to the mark with distilled water. It was filtered through Whatmann filter paper No. 41 and the filtrate was suitably diluted to get final concentration of 14 μ g/ml of DOX and 1 μ g/ml of AMB with distilled water. The absorbance of sample solution was measured at all selected wavelengths. The content of DOX and AMB in sample solution of tablet was calculated. This procedure was repeated six times.

Recovery studies

The recovery experiment was done by adding known concentrations of DOX and AMB raw materials to the 50% preanalyzed formulation. Standard DOX and AMB in the range of 80%, 100% and 120% to the 50% preanalyzed formulation into a series of 10 ml volumetric flasks and diluted with distilled water and made up to the mark with the same. The contents were sonicated for 15 minutes. After sonication, the solutions were filtered through Whatmann filter paper No. 41. The absorbances of the resulting solutions were measured at their selected wavelengths for determination of DOX and AMB. The amount of each drug recovered from the formulation was calculated for all the drugs by Simultaneous equation method, Absorbance correction method and Absorbance ratio method. The procedure was repeated for three times for each percentage recovery.

Validation of developed method

The methods were validated with respects to linearity, LOD (Limit of Detection), LOQ (Limit of Quantitation), precision, accuracy and ruggedness.

Linearity

Linearity was checked by diluting standard stock solution at five different concentrations. DOX was linear with the concentration range of 7-35 µg/ml and AMB showed linearity in the range of 1-5 µg/ml and the calibration curves [mean value of six determinations] were plotted between concentration and absorbance of drugs. Optical parameters were calculated.

Accuracy (Recovery studies)

To check the accuracy of the developed method and to study the interference of formulation excipients, analytical recovery experiments were carried out by using standard addition method in three different concentrations. From the total amount of drug found, the percentage recovery and %RSD were calculated.

Precision:

The precision of the method was confirmed by repeatability and intermediate precision. The repeatability was performed by the analysis of formulation and it was repeated for six times with the same concentration. The amount of each drug present in the tablet formulation was calculated. The %RSD was also calculated. The intermediate precision of the method was confirmed by intraday and interday analysis i.e. the analysis of formulation was repeated three times in the same day and on three successive days. The amount of drugs and %RSD were determined.

Ruggedness:

The ruggedness test of analytical assay method is defined as degree of reproducibility of test results obtained by the analysis of the same samples under a variety of normal test conditions such as different laboratories, different Analysts, different lots of reagents etc. Ruggedness is a measure of reproducibility of test results under normal expected operational conditions from laboratory to laboratory and from Analyst to Analyst. In present study, determination of the DOX and AMB were carried out by using different instruments and different Analysts.

5.3.2 HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD:

Chromatographic method depends upon the nature of the sample, molecular weight and solubility. The drugs selected for the present study was polar compound; hence it can be separated either by normal phase or reverse phase chromatography. Reverse phase chromatographic technique was selected for initial separations with the knowledge of properties of compounds. C₁₈ column was chosen as stationary phase and

various mixtures of phosphate buffer (pH 3.0), acetonitrile and methanol were selected as mobile phase.

Selection of mobile phase and λ_{\max}

Different mixtures of mobile phase with different ratios were selected and their chromatograms were recorded. From this, the mobile phase selected for the study was 10 mM Phosphate buffer, Acetonitrile and Methanol in the ratio of 70: 20: 10 and the pH is adjusted to 3.0 with orthophosphoric acid, since these two drugs were eluted with sharp peak and with better resolution. Hence, this mobile phase was used to optimize the chromatographic conditions. The detection wavelength was measured by scanning the 10 μ g/ml solution of Doxofylline and Ambroxol HCl in the mobile phase in UV- Spectrophotometry, and overlaid spectra was recorded. The detection wavelength selected was 224 nm (isoabsorptive point of two drugs).

Optimized Chromatographic Conditions

The following parameters were used for RP-HPLC analysis of DOX and AMB Mode of operation – Isocratic Stationary phase - C₁₈ column (150 mm X 4.6 mm I.d., 5m)

Preparation of the Standard stock solution Standard Doxofylline stock solution

Accurately weighed 35 mg of DOX was transferred into a 10 ml standard volumetric flask separately and dissolved with minimum quantity of HPLC water and the volume was made up to the mark with HPLC water. From the above solution, 1 ml was transferred into a 50 ml volumetric flask and diluted with HPLC water to get the concentration of 70 μ g/ml of DOX.

Standard Ambroxol Hydrochloride solution

Accurately weighed 25 mg of AMB was transferred into a 10 ml standard volumetric flask separately and dissolved with minimum quantity of HPLC water and the volume was made upto the mark with HPLC water. From the above solution, 1 ml was transferred into a 10 ml volumetric flask and diluted with HPLC water to get the concentration of 250 μ g/ml. From this solution, 1 ml was transferred into a 50 ml volumetric flask and diluted with HPLC water to get the final concentration of 5 μ g/ml of AMB.

Linearity and Calibration

Aliquots (1-5 ml) of mixed working standard solutions of DOX and AMB were transferred into a series of 10 ml volumetric flasks, and the volume was made up to the mark with distilled water. An aliquot (20 μ l) of each solution was injected under the operating chromatographic condition as described above and the responses were recorded. Calibration curves were constructed for each drug by plotting peak area versus concentration, and the regression equations were calculated. Each response was average of three determinations.

Quantification of tablet formulation

Twenty tablets containing DOX 400 mg and AMB 30 mg were accurately weighed. Weighed content of drug equivalent to 35 mg of DOX was transferred into a 10 ml volumetric flask and dissolved with HPLC water and sonicated for 15 minutes. The above solution was filtered through Whatmann filter paper No. 41 and the clear solution was collected. HPLC water is added to make up to the required volume to get the concentration of 3.5 mg/ml. 1 ml was pipetted into a 50 ml volumetric flask and made up to the mark with HPLC water to get the concentration of DOX (70 μ g/ml) and AMB (5 μ g/ml). Accurately measured 2 ml of the sample solution was transferred into a 10 ml volumetric flask, and diluted up to the mark with HPLC water to get the final working concentration of DOX (14 μ g/ml) and AMB (1 μ g/ml). The peak area measurements were done by injecting sample six times and the amount of DOX and AMB were calculated from their respective calibration curve.

Recovery Studies

The accuracy of the method was determined by calculating the recoveries of DOX and AMB by the standard addition method. Known amounts of standard solutions of DOX and AMB were added at 80%, 100% and 120% level to prequantified sample solution of DOX (14 μ g/ml) and AMB (1 μ g/ml). The amounts of DOX and AMB were estimated by applying obtained values to the respective regression equations.

Limit of Detection and Limit of Quantitation

Preparation of calibration curve for the serial dilution of standard was repeated for six times. The limit of detection and limit of quantitation were calculated by using the average value of slope and standard deviation of response (Intercept).

System Suitability Studies

The system suitability studies were carried out as specified in I.P. and U.S.P. The parameters like Column efficiency, Tailing factor, Asymmetric factor and Theoretical plate number were calculated.

7. RESULTS AND DISCUSSION

In order to quench the thirst for the analysis of the new drug combinations, Doxofylline & Ambroxol Hydrochloride was taken for our studies. Simultaneous estimation of multiple drug formulations have advantage that the methods were less time consuming and the usage of solvent is minimized. To ensure the percentage purity in combined dosage forms of the drugs, the UV-spectroscopy, RP-HPLC or both were developed. These methods were found to be simple, economic and applicable for routine analysis.

DOXOFYLLINE AND AMBROXOL HYDROCHLORIDE COMBINATION DOSAGE FORM:

The methods employed for the analysis of Doxofylline and Ambroxol Hydrochloride were

1. UV-Spectroscopic Methods
 - a. Simultaneous equation method
 - b. Absorbance correction method and
 - c. Absorbance Ratio Method
2. Reverse-Phase High Performance Liquid Chromatography

7.1 UV-SPECTROSCOPIC METHODS:

The solubility of DOX and AMB were determined in a variety of solvents as per ScHefter and Higuchi method¹⁵⁹. 10 mg of samples were taken in test tube and checked their solubility with variety of solvents as per IP and the profiles are shown in Table-1.

Table-1
SOLUBILITY PROFILE OF DOXOFYLLINE AND AMBROXOL HYDROCHLORIDE IN POLAR AND NON POLAR SOLVENTS

S.NO	SOLVENTS	DOXOFYLLINE	AMBROXOL HYDROCHLORIDE
1	Distilled Water	Soluble	Sparingly Soluble
2	0.1M Sodium hydroxide	Sparingly Soluble	Insoluble
3	0.1 M Hydrochloric acid	Soluble	Slightly Soluble
4	Methanol	Soluble	Freely soluble
5	Acetone	Soluble	Insoluble
6	Acetonitrile	Freely Soluble	Slightly soluble
7	Ethanol	Sparingly Soluble	Slightly soluble
8	Chloroform	Soluble	Insoluble

9	Dimethyl formamide	Freely Soluble	Very Slightly Soluble
10	Isopropyl alcohol	Insoluble	Insoluble
11	Benzene	-	Insoluble
12	n-butanol	-	Very Slightly Soluble
13	Dichloroethane	Very Freely Soluble	Very Slightly Soluble
14	Diethyl ether	Slightly Soluble	Insoluble
15	Ethyl acetate	Sparingly Soluble	Insoluble
16	Cyclohexane	Sparingly Soluble	Insoluble
17	Pyridine	-	Soluble
18	Petroleum ether	Insoluble	-
19	Toluene	Sparingly Soluble	-
20	n-hexane	Insoluble	-
21	Carbon tetrachloride	Sparingly Soluble	-

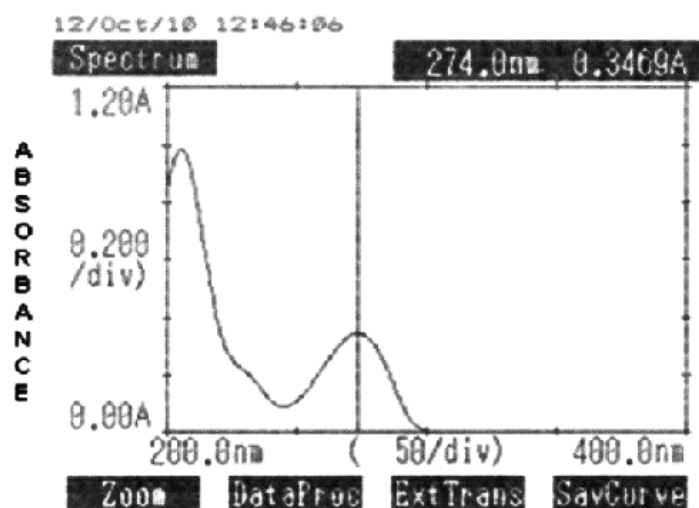
The numeral polar and non-polar solvents were attempted to dissolve the drugs. From the solubility profile, the distilled water was chosen as a common solvent for the estimation of DOX and AMB in bulk and in formulation.

Based upon its easy availability, cost factor and stability condition, distilled water was selected as solvent.

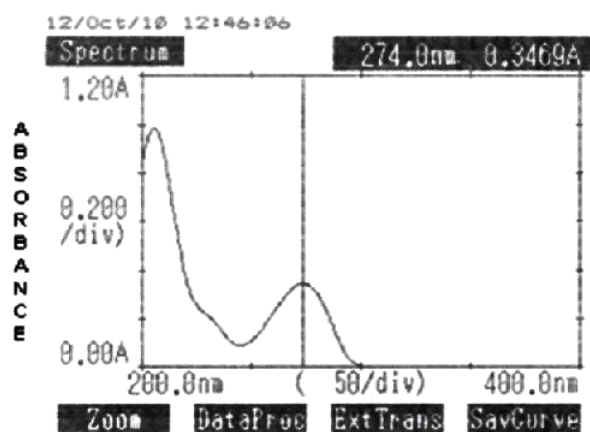
Three accurate, simple and rapid UV methods, namely Simultaneous equation method, Absorbance correction method and Absorbance ratio method were selected.

The drugs were dissolved in distilled water to produce 10 µg/ml. Scanned in the UV- region of 200-400 nm by using distilled water as blank, it shows constant wavelength at 274 nm for DOX and 244.5 nm for AMB, and overlain spectra was made. This is shown in Figures-1,2&3.

FIGURE-1



UV SPECTRUM OF DOXOFYLLINE IN DISTILLED WATER CONCENTRATION: $10 \mu\text{g ml}^{-1}$



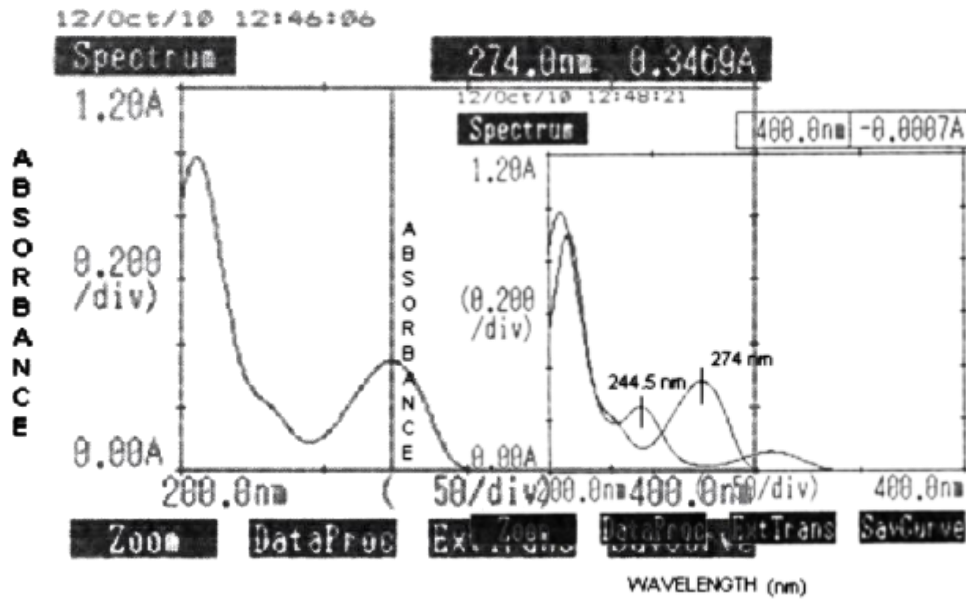
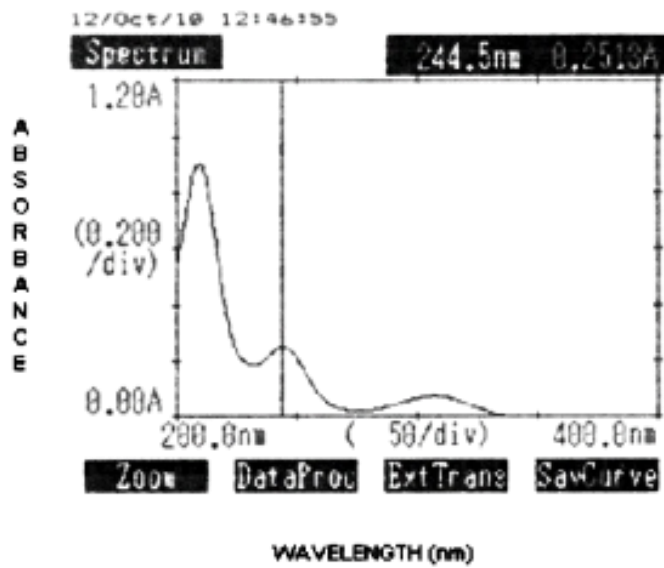


FIGURE-2



UV SPECTRUM OF
HYDROCHLORIDE
IN DISTILLED
CONCENTRATION:

AMBROXOL

WATER
10 \square g ml⁻¹

FIGURE-3

OVERLAID SPECTRUM OF DOXOFYLLINE AND AMBROXOL HYDROCHORIDE IN DISTILLED WATER

The stability study of DOX and AMB were performed by observing the absorbance of both at the concentration of 10 µg/ml at their wavelengths, at various time intervals 0 min, 10 min, 20 min, 30 min, 40 min, 50 min, 1 hr, 1 hr 15 min, 1 hr 30 min, 1 hr 45 min, 2 hr, 2 hr 30 min, 3 hr, 3 hr 30 min, 4 hr and 24 hr.

The stability study of DOX and AMB are tabulated in Table-2

Table-2
STABILITY STUDY OF DOXOFYLLINE AND AMBROXOL HYDROCHLORIDE FOR UV SPECTROSCOPIC METHODS

Solvent: Distilled Water

Concentration of Ambroxol Hydrochloride and Doxofylline: 10 µg/ml

S.No	Time	Absorbance of Doxofylline (274 nm)	Absorbance of Ambroxol Hydrochloride (244.5 nm)
1	0 min	0.348	0.252
2	10 min	0.347	0.250
3	20 min	0.350	0.254
4	30 min	0.351	0.256
5	40 min	0.354	0.253
6	50 min	0.353	0.255
7	60 min	0.349	0.252
8	1 hour 15 min	0.350	0.252
9	1 hour 30 min	0.348	0.253
10	1 hour 45 min	0.348	0.251
11	2 hours	0.350	0.249
12	2 hours 30 min	0.351	0.247
13	3 hours	0.351	0.253
14	3 hours 30 min	0.349	0.254
15	4 hours	0.352	0.253
16	24 hours	0.349	0.247

From the data shown, it was observed that DOX and AMB were stable in distilled water at their wavelengths.

7.1.1 Simultaneous equation method:

The individual and overlaid spectra of DOX and AMB were recorded as shown in Figure 1, 2 and 3. From the spectrums, 274 nm was λ_{\max} of DOX and 244.5 nm was λ_{\max} of AMB and these two wavelengths were used for the simultaneous estimation of DOX.

Different aliquots of DOX in distilled water were prepared in the concentration range of 7-35 [g ml⁻¹. The

absorbances of these solutions were measured at 244.5 nm and 274 nm. The calibration curves were plotted

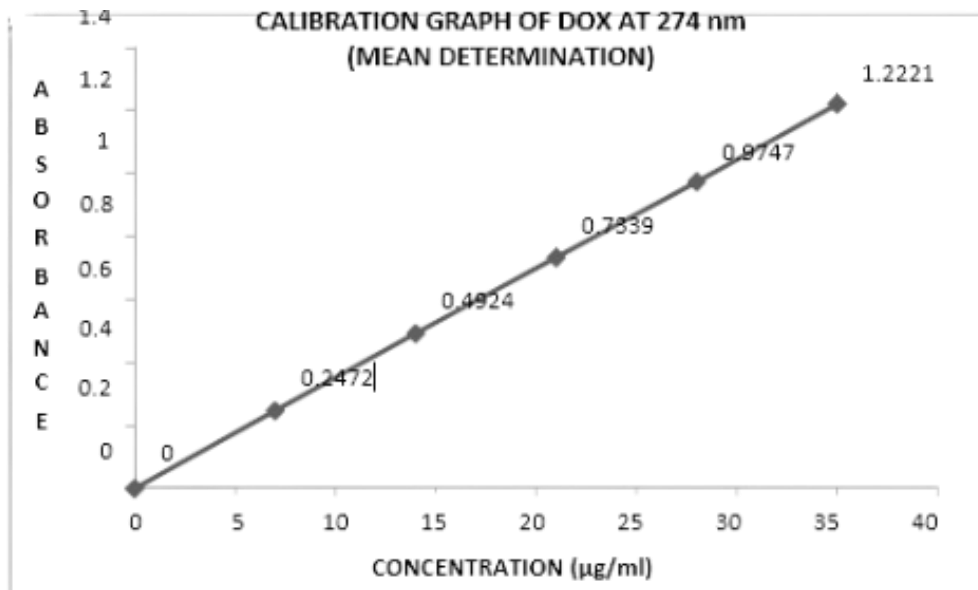
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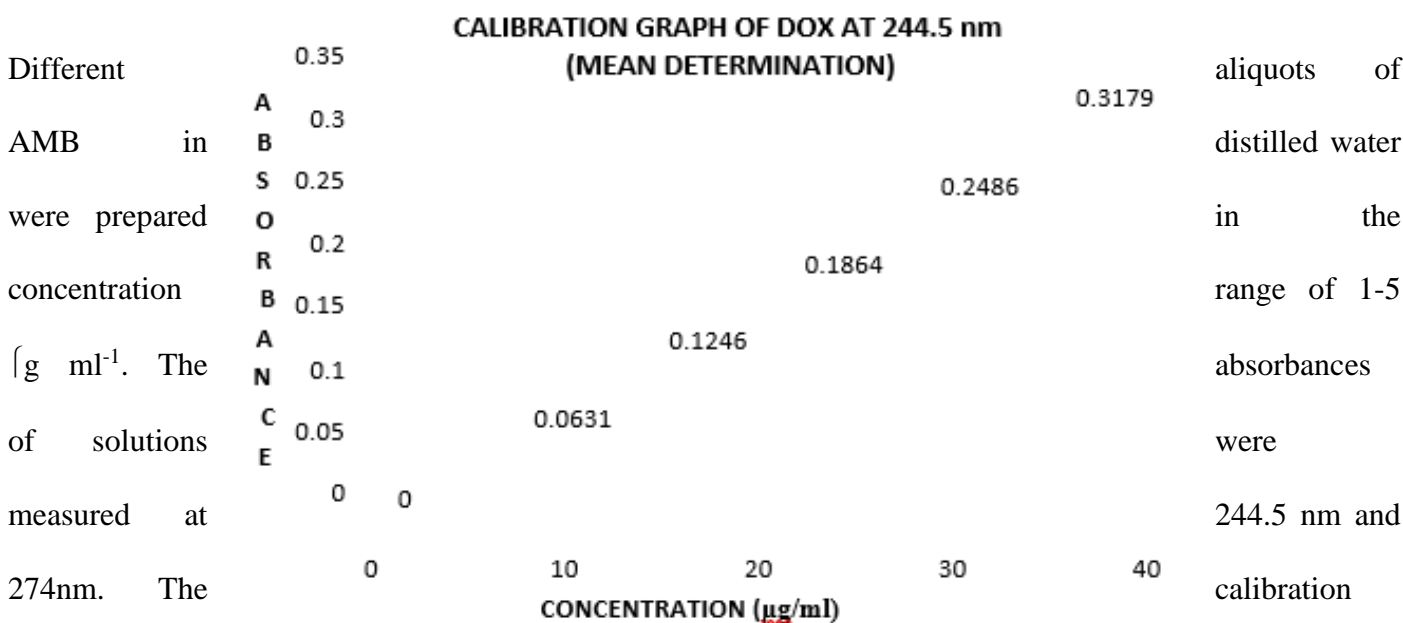
graphs

six

determinations) were plotted and are shown in Figures-4&5.

FIGUR-4
CALIBRATION CURVE OF DOXOFYLLINE IN DISTILLED WATER AT 244.5 nm
CONCENTRATION (µg/ml)

FIGURE-5
CALIBRATION CURVE OF DOXOFYLLINE IN DISTILLED WATER AT 274 nm



curves were plotted using concentration against absorbance. The calibration graphs (mean value of six determinations) at 244.5 nm and 274 nm are shown in Figure- 6&7.

FIGURE-6
CALIBRATION CURVE OF AMBROXOL HYDROCHLORIDE IN DISTILLED WATER AT 244.5 n

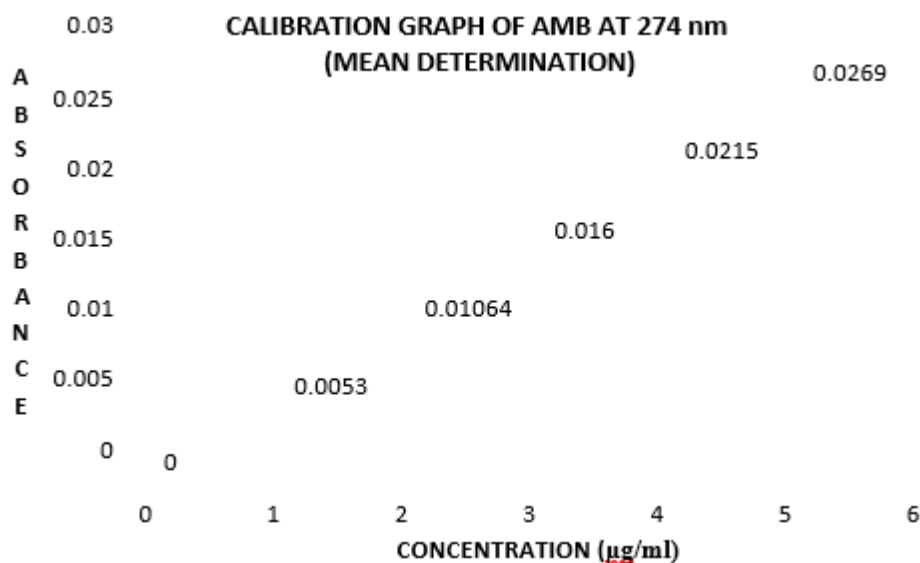
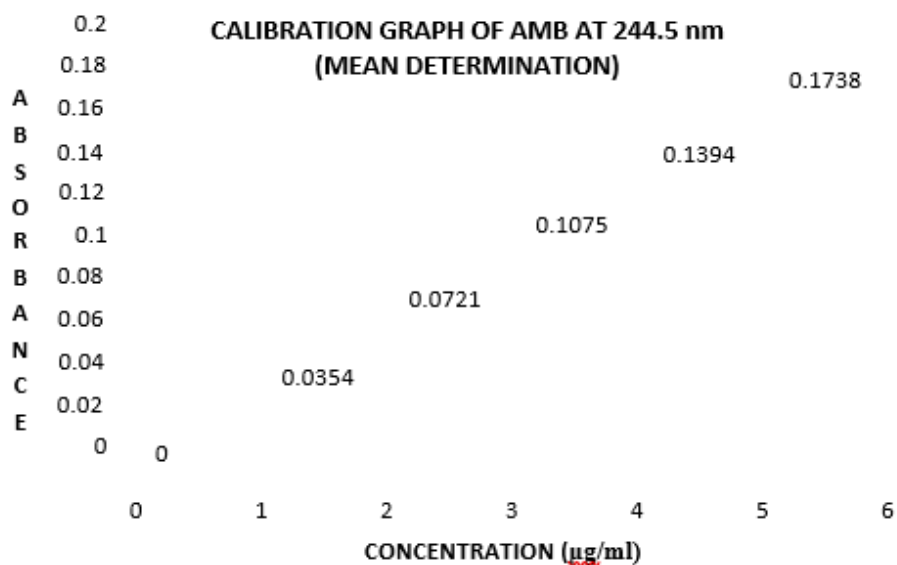


FIGURE-7

CALIBRATION CURVE OF AMBROXOL HYDROCHLORIDE IN DISTIL LED



The optical parameters

like Sandell’s sensitivity, Molar absorptivity, Correlation coefficient, Slope, Intercept, LOD, LOQ and Standard error were calculated. The correlation coefficient for the two drugs was found to be about 0.999. This indicates that all the drugs obey Beer’s law in the selected concentration range. Hence the curves were found to be linear. The optical characteristics of the two drugs at their selective wavelengths are shown in Table-3 for DOX and Table-4 for AMB.

Table-3
OPTICAL CHARACTERISTICS OF DOXOFYLLINE
(SIMULTANEOUS EQUATION METHOD)

PARAMETERS	AT 274 nm*	AT 244.5 nm*
Beer's law limit ($\mu\text{g mL}^{-1}$)	7-35 ($\mu\text{g mL}^{-1}$)	7-35 ($\mu\text{g mL}^{-1}$)
Molar absorptivity	9330.73755	2374.305627
Sandells sensitivity ($\mu\text{g}/\text{cm}^2/0.001 \text{ A.U}$)	0.02872	0.11104
Correlation coefficient (r)	0.999970	0.999390
Regression equation ($y = mx + c$)	$y = (0.03483)x + (0.00209)$	$y = (0.00901)x + (-0.00093)$
Slope (m)	0.03483	0.00901
Intercept (c)	0.00209	-0.00093
LOD ($\mu\text{g mL}^{-1}$)	0.216615	0.829938
LOQ ($\mu\text{g mL}^{-1}$)	0.656410	2.51496
Standard error	0.0004651	0.0014219

*Mean of six observations

Table-4
OPTICAL CHARACTERISTICS OF AMBROXOL HYDROCHLORIDE
(SIMULTANEOUS EQUATION METHOD)

PARAMETERS	AT 244.5 nm*	AT 274 nm*
Beer's law limit ($\mu\text{g mL}^{-1}$)	1-5 ($\mu\text{g mL}^{-1}$)	1-5 ($\mu\text{g mL}^{-1}$)
Molar absorptivity	14467.40119	1725.394095
Sandell's sensitivity ($\mu\text{g}/\text{cm}^2/0.001 \text{ A.U}$)	0.029669	0.302388817
Correlation coefficient (r)	0.999413	0.991096
Regression equation ($y = mx + c$)	$y = (0.03478)x + 0.001124$	$y = (0.00409)x + 0.00062$

Slope (m)	0.03478	0.00409
Intercept (c)	0.001124	0.00062
LOD ($\mu\text{g mL}^{-1}$)	0.129641	1.246725
LOQ ($\mu\text{g mL}^{-1}$)	0.392853	3.777957
Standard error	0.0002335	0.000230

The tablet containing DOX 400 mg and AMB 30 mg was selected for analysis. The nominal concentration of DOX from linearity i.e. $14 \text{ [g mL}^{-1}]$ was prepared and this contains $1 \text{ [g mL}^{-1}]$ concentration of AMB. The absorbance of the solution was measured at their respective wavelengths. The percentage label claim present in tablet formulation is given in Table-5 for DOX and AMB respectively.

Table-5
QUANTIFICATION OF FORMULATION
[SYNASMA-AX] (SIMULTANEOUS EQUATION METHOD)

Drug	Sample No.	Labeled amount (mg/tab)	Amount found (mg/tab)*	Percentage Obtained*	Average (%)	S.D	% R.S.D.	S.E.
DOX	1	400	399.81	99.95	99.97	0.32750	0.0818	0.13370
	2	400	400.04	100.01				
	3	400	399.56	99.89				
	4	400	400.21	100.05				
	5	400	399.50	99.87				
	6	400	400.27	100.06				
AMB	1	30	29.45	98.16	98.64	0.13841	0.46779	0.05651
	2	30	29.52	98.42				
	3	30	29.71	99.03				
	4	30	29.52	98.42				
	5	30	29.81	99.37				
	6	30	29.53	98.42				

The amount present in the tablet formulation was in good concord with the label claim and the % RSD values were found to be 0.0818 and 0.46779 for DOX and AMB respectively.

The low % RSD values indicate that the method has good precision. Further the precision of the method was confirmed by Intraday and Interday analysis. Analysis of the formulation was carried out for three times in the same day and one time in three consecutive days. The % RSD value of intraday and interday analysis were found to be 0.0638 and 0.0726 for DOX & 0.097 and 0.16348 for AMB. The results of the analysis are shown in Table-6. The results showed that the precision of the method was confirmed.

Table-6
INTRADAY AND INTERDAY ANALYSIS OF FORMULATION [SYNASMA-AX]
(SIMULTANEOUS EQUATION METHOD)

Drug	Sample No.	Labeled amount (mg/tab)	Percentage obtained*		S.D		% R.S.D.	
			Intraday	Interday	Intraday	Interday	Intraday	Interday
DOX	1	400	99.94	99.95	0.25520	0.290	0.0638	0.0726
	2	400	99.91	99.98				
	3	400	99.84	100.02				
	4	400	99.87	99.86				
	5	400	99.87	99.97				
	6	400	99.85	99.875				
Mean			99.88	99.94				
AMB	1	30	98.71	98.84	0.02888	0.29017	0.097	0.16348
	2	30	98.77	98.84				
	3	30	98.68	98.84				
	4	30	98.88	99.13				
	5	30	98.83	99.10				
	6	30	98.67	98.88				
Mean			98.75	98.94				

*Mean of three observations

The developed method was validated for Ruggedness. It refers to the specific of one lab to multiple days which may include multiple Analysts, multiple instruments and different sources of reagents and so on. In the present work it was confirmed by different Analysts and different instruments. The % RSD value by Analyst 1 and Analyst 2 were found to be 0.09651 and 0.13017 for DOX & 0.23489 and 0.26049 for AMB respectively. The

%RSD value by Instrument I and Instrument II were found to be 0.09929 and 0.0820 for DOX & 0.26661 and 0.46701 for AMB respectively. The low %RSD values indicate that the developed method was more rugged. The results are shown in Table- 7

Table- 7
RUGGEDNESS STUDY OF FORMULATION [SYNASMA-AX]
(SIMULTANEOUS EQUATION METHOD)

Drug	Condition	Average* % Obtained	S.D	% R.S.D	S.E.
DOX	Analyst 1	99.88	0.38557	0.09651	0.15741
	Analyst 2	99.89	0.52015	0.13017	0.21235
	Instrument 1	99.66	0.39635	0.09929	0.161811
	Instrument 2	99.97	0.32734	0.08200	0.13366

AMB	Analyst 1	98.91	0.06969	0.23489	0.02845
	Analyst 2	98.68	0.07711	0.26049	0.03148
	Instrument 1	98.68	0.07893	0.26661	0.03222
	Instrument 2	98.64	0.13851	0.46701	0.05655

*Mean of six observations

The accuracy of the method was performed by recovery studies. To the preanalyzed formulation, a known quantity of DOX and AMB raw material solutions were added at different levels. The absorbance of the solutions was measured and the percentage recovery was calculated.

The percentage recovery was found to be in the range of 99.98- 100.087 % for DOX and 98.417-99.86% for AMB. The recovery data is shown in Table-8.

Table-8.
RECOVERY STUDY DATA OF 50% PRE-ANALYSED FORMULATION [SYNASMAAX]
(SIMULTANEOUS EQUATION METHOD)

Drug	Percentage	Amount present* ($\mu\text{g ml}^{-1}$)	Amount added* ($\mu\text{g ml}^{-1}$)	Amount estimated* ($\mu\text{g ml}^{-1}$)	Amount recovered* ($\mu\text{g ml}^{-1}$)	% Recovery*	S.D.	% RSD	S.E.
DOX	80	13.9979	11.2	25.2058	11.2079	100.087	0.01126	0.10046	0.00650
	100	13.9979	14	27.9951	13.9971	99.98	0.01214	0.08673	0.07009
	120	13.9979	16.8	30.8028	16.8049	100.023	0.00081	0.00482	0.00047
AMB	80	1.013	0.8	1.80036	0.7874	98.417	0.00120	0.15240	0.00069
	100	1.013	1	2.0116	0.9998	99.86	0.00365	0.36507	0.21073
	120	1.013	1.2	2.2065	1.1935	99.4567	0.0021	0.18430	0.00121

*Mean of three observations

It indicates that there is no interference due to excipients present in the formulation. It can be easily and conveniently adopted for routine quality control analysis.

This method is accurate, simple, rapid, precise, reliable, sensitive, reproducible and economic, and is validated as per ICH guidelines.

7.1.1 Absorbance correction method

The individual and overlaid spectra of DOX and AMB were recorded and shown in Figure-1, 2 & 8. From the overlaid spectra, 308 nm was selected for the estimation of AMB without any interference from DOX, and 274 nm was selected for the estimation of DOX after the absorbance corrected for interference by

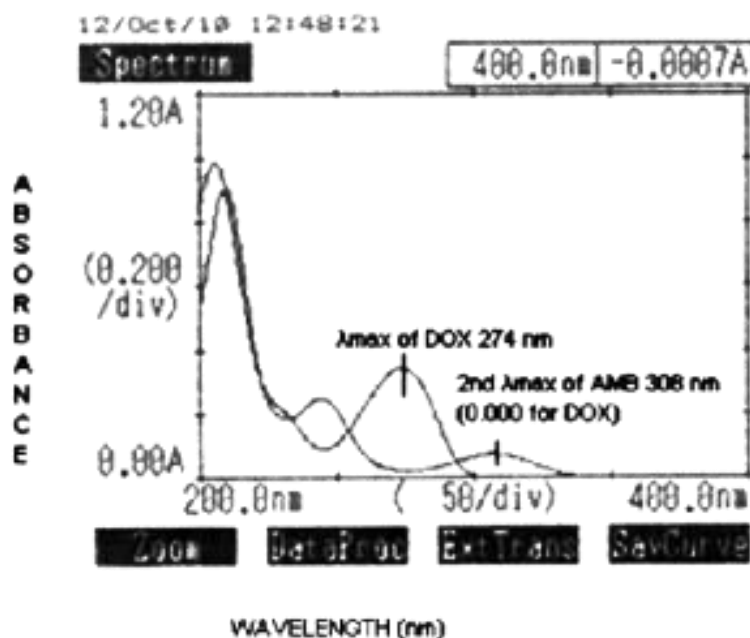
AMB. The

at 308 nm was

its λ_{\max} .

absorbance of DOX

zero and 274 nm was



**SPECTRUM OF
DOXOFYLLINE
AMBROXOL
HYDROCHORIDE
(ABSORBANCE
METHOD)**

**FIGURE-8
OVERLAID
AND
CORRECTION**

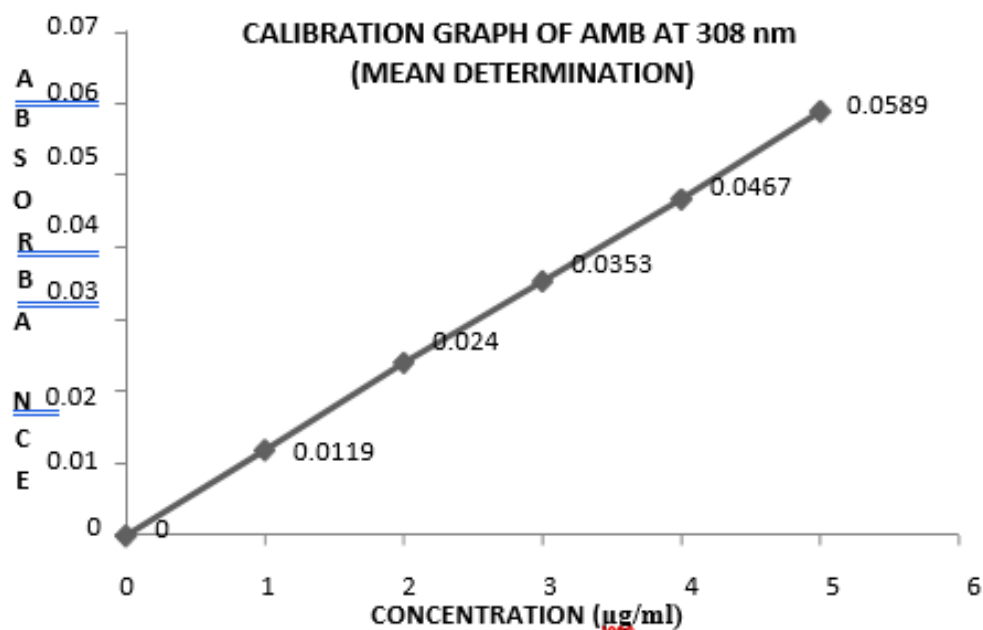
Different aliquots of DOX and AMB were diluted to the concentration range separately in distilled water.

The absorbance of each solution was measured at 274 nm and 308 nm. The calibration curve was plotted

using absorbance against concentration. The calibration graphs at 274 nm and 308 nm for AMB is shown in

Figures 5 & 9 and calibration graphs at 274 nm for DOX is shown in Figure-7.

FIGURE-9
CALIBRATION CURVE OF AMBROXOL HYDROCHLORIDE IN DISTILLED WATER AT 308 nm



The preparation of calibration curve was repeated six times for each drug at their selective wavelengths. The optical parameters like Sandell’s sensitivity, molar absorptivity, correlation coefficient, slope, intercept, LOD, LOQ and Standard error were calculated. The correlation coefficient for both the drugs were found to be about 0.999. This indicates that both the drugs obey Beer’s law in the selected concentration range. Hence the concentrations were found to be linear. The optical characteristics of DOX and AMB at selected wavelengths are shown in Table-9 and Table-10 respectively.

Table-9
OPTICAL CHARACTERISTICS OF DOXOFYLLINE
(ABSORBANCE CORRECTION METHOD)

PARAMETERS	AT 274 nm*
------------	------------

Beer's law limit ($\mu\text{g mL}^{-1}$)	7.35 ($\mu\text{g mL}^{-1}$)
Molar absorptivity	9330.73755
Sandell's sensitivity ($\mu\text{g/cm}^2/0.001 \text{ A.U}$)	0.02872
Correlation coefficient (r^2)	0.999970
Regression equation ($y = mx + c$)	$y = (0.03483)x + (0.00209)$
Slope (m)	0.03483
Intercept (c)	0.00209
LOD ($\mu\text{g mL}^{-1}$)	0.216615
LOQ ($\mu\text{g mL}^{-1}$)	0.656410
Standard error	0.0004651

*Mean of six observations

Table-10
OPTICAL CHARACTERISTICS OF AMBROXOL HYDROCHLORIDE
(ABSORBANCE CORRECTION METHOD)

PARAMETERS	AT 274 nm*	AT 308 nm*
Beer's law limit ($\mu\text{g mL}^{-1}$)	1.5 ($\mu\text{g mL}^{-1}$)	1.5 ($\mu\text{g mL}^{-1}$)
Molar absorptivity	1725.394095	4868.786
Sandell's sensitivity ($\mu\text{g/cm}^2/0.001 \text{ A.U}$)	0.302388817	0.08848
Correlation coefficient (r)	0.991096	0.99933
Regression equation ($y = mx + c$)	$y = (0.00409)x + 0.00062$	$y = 0.011726x + 0.000166$
Slope (m)	0.00409	0.011726
Intercept (c)	0.00062	0.000166
LOD ($\mu\text{g mL}^{-1}$)	1.246725	0.053809
LOQ ($\mu\text{g mL}^{-1}$)	3.777957	0.163058
Standard error	0.000230	9.37974 E-05

*Mean of six observations

The tablet containing DOX 400 mg and AMB 30 mg was selected for analysis. The nominal concentration of DOX from linearity i.e. $14 \mu\text{g mL}^{-1}$ ($1 \mu\text{g mL}^{-1}$ of AMB) was prepared and the absorbance of the solutions were measured at their selected

wavelengths. The percentage label claim of the tablet formulation was found to be 100.32

± 0.50529 for DOX and 99.60 ± 0.65582 for AMB. The amount present in the tablet formulation was in good concord with the label claim and the %RSD values were found to be 0.50529 and 0.65582 for DOX and AMB respectively. The low % RSD values indicate that the method has good precision. The result of formulation estimations is shown in Table-11.

Table-11

**QUANTIFICATION OF FORMULATION [SYNASMA-AX]
(ABSORBANCE CORRECTION METHOD)**

Drug	Sample No.	Labeled amount (mg/tab)	Amount found (mg/tab)*	Percentage Obtained*	Average (%)	S.D	% R.S.D.	S.E.
DOX	1	400	403.06	100.76	100.32	2.0275	0.50529	0.82775
	2	400	402.08	100.52				
	3	400	398.96	99.74				
	4	400	401.17	100.29				
	5	400	403.55	100.88				
	6	400	398.79	99.70				
AMB	1	30	30.04	100.14	99.60	0.19595	0.65582	0.08
	2	30	29.80	99.33				
	3	30	30.04	100.14				
	4	30	29.56	98.53				
	5	30	29.80	99.33				
	6	30	30.04	100.13				

*Mean of six observations

Further the precision of the method was confirmed by intraday and interday studies. The %RSD values of intraday and interday analysis were found to be 0.16602 and 0.10613 for DOX & 0.58398 and 0.62845 for AMB. The results of analysis are shown in Table-12. The results showed that the precision of the method was high.

**Table-12
INTRADAY AND INTERDAY ANALYSIS OF FORMULATION
[SYNASMA-AX] (ABSORBANCE CORRECTION METHOD)**

Drug	Sample No.	Labeled amount (mg/tab)	Percentage obtained*		S.D		% R.S.D.	
			Intraday	Interday	Intraday	Interday	Intraday	Interday
DOX	1	400	100.05	100.16	0.66545	0.42507	0.16602	0.10613
	2	400	100.20	100.12				
	3	400	100.18	100.17				
	4	400	100.18	100.14				
	5	400	100.36	99.99				
	6	400	100.28	100.09				
Mean			100.21	100.11				
AMB	1	30	98.78	99.71	0.17418	0.18831	0.58398	0.62845
	2	30	99.60	99.71				
	3	30	99.93	99.71				
	4	30	99.87	99.30				
	5	30	99.59	99.31				
	6	30	99.33	99.30				
Mean			99.51	99.51				

*Mean of three observations

The developed method was validated for ruggedness. In the present work it was confirmed by different Analysts and different instruments. The % RSD value by Analyst 1 and 2 were found to be 0.13601 and 0.15235 for DOX & 0.72059 and 0.72059 for AMB. The % RSD value by Instrument 1 and 2 were found to be 0.09097 and 0.5051 for DOX & 0.65957 and 0.6550 for AMB. The low %RSD values indicate that the developed method was more rugged. The results are shown in Table-13.

Table-13
RUGGEDNESS STUDY OF FORMULATION
[SYNASMA-AX] (ABSORBANCE CORRECTION METHOD)

Drug	Condition	Average* % Obtained	S.D	% R.S.D	S.E.
DOX	Analyst 1	99.95	0.54375	0.13601	0.22199
	Analyst 2	99.99	0.60929	0.15235	0.24874
	Instrument 1	99.89	0.36348	0.09097	0.14839
	Instrument 2	100.32	0.50671	0.5051	0.20686
AMB	Analyst 1	99.30	0.21466	0.72059	0.08764
	Analyst 2	99.30	0.21466	0.72059	0.08764
	Instrument 1	99.03	0.19596	0.65957	0.07999
	Instrument 2	99.60	0.19515	0.6550	0.07966

*Mean of six observations

The accuracy of the method was confirmed by recovery studies. To the pre-analyzed formulation, a known quantity of mixture of DOX and AMB raw material solutions were added at different levels. The absorbance of the solutions was measured at selected wavelengths and the percentage recovery was calculated. The percentage recovery was found to be in the range of 99.64-100 % for DOX and 99.73-100.55 % for AMB. The %RSD values were found to be less than 2 and this indicates that the method is accurate. The result of the recovery studies is shown in Table-14.

Table-14
RECOVERY STUDY DATA OF 50% PRE-ANALYSED FORMULATION
[SYNASMA-AX] (ABSORBANCE CORRECTION METHOD)

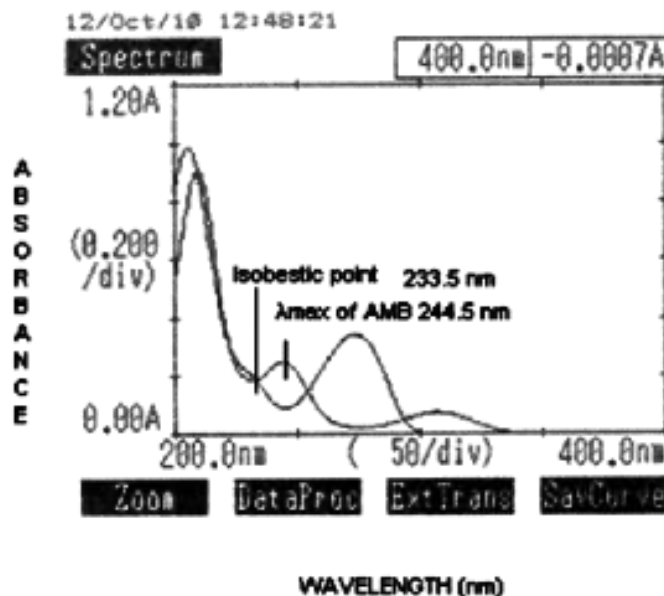
Drug	Percentage	Amount present* ($\mu\text{g ml}^{-1}$)	Amount added* ($\mu\text{g ml}^{-1}$)	Amount estimated* ($\mu\text{g ml}^{-1}$)	Amount recovered* ($\mu\text{g ml}^{-1}$)	%Recovery*	S.D.	%R.S.D.	S.E.
DOX	80	14.0471	11.2	25.24457	11.19747	99.97	0.00438	0.03907	0.00253
	100	14.0471	14	27.99667	13.94957	99.64	0.00443	0.03175	0.00256
	120	14.0471	16.8	30.84827	16.80117	100.00	0.00875	0.05208	0.00505
AMB	80	1.0460	0.8	1.8504	0.80443	100.55	0.00495	0.61534	0.00286
	100	1.0460	1.0	2.04373	1.00057	100.06	0.00491	0.49072	0.00283

	120	1.0460	1.2	2.24277	1.19677	99.73	0.00491	0.41027	0.00284
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*Mean of three

7.1.1 Absorbance

The individual and DOX and AMB were Figures-1, 2 & 10. spectra, the were 244.5 nm (λ_{max} (iso-absorptive point).



observations

ratio method

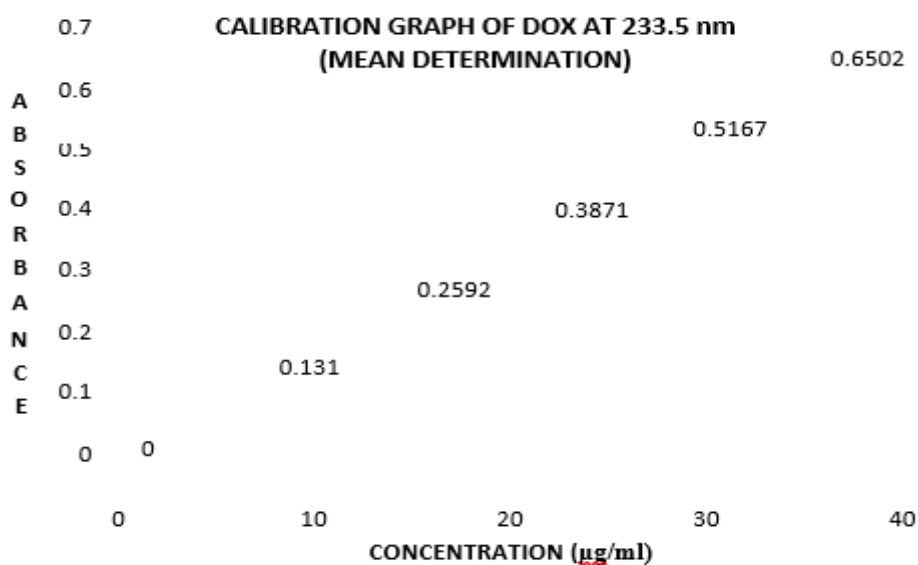
overlaid spectra of recorded and shown in From the overlaid wavelengths selected of AMB) and 233.5 nm The linearity of DOX

and AMB was constructed in the range of 7-35 $\mu\text{g/ml}$ and 1-5 $\mu\text{g/ml}$ and their calibration curves are shown in Figures 11 & 12.

**FIGURE-10
OVERLAID SPECTRUM OF DOXOFYLLINE AND AMBROXOL HYDROCHORIDE
(ABSORBANCE RATIO METHO**

**FIGURE-11
CALIBRATION CURVE OF DOXOFYLLINE IN DISTILLED WATER AT 233.5 nm**

FIGURE-12
CALIBRATION CURVE OF AMBROXOL HYDROCHLORIDE IN DISTILLED WATER AT 233.5



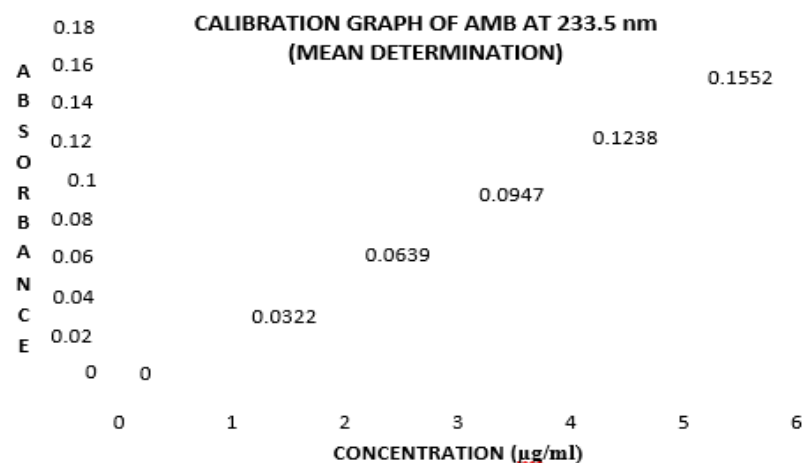
The optical characteristics such as Beer's law limit (7-35 and 1-5 µg/ml), molar extinction coefficient, Sandell's sensitivity,

characteristics Beer's law limit 1-5 µg/ml), extinction Sandell's correlation co-efficient,

slope and intercept were calculated and are shown

intercept were calculated in Tables 15 & 16.

Tables-15
OPTICAL
OF
RATIO



CHARACTERISTICS
DOXOFYLLINE
(ABSORBANCE
METHOD)

PARAMETERS	AT 233.5 nm*	AT 244.5 nm*
Beer's law limit ($\mu\text{g ml}^{-1}$)	7-35 ($\mu\text{g mL}^{-1}$)	7-35 ($\mu\text{g mL}^{-1}$)
Molar absorptivity	4930.64917	2374.305627
Sandell's sensitivity ($\mu\text{g/cm}^2/0.001 \text{ A.U}$)	0.054045077	0.111044398
Correlation coefficient (r^2)	0.999916585	0.999390328
Regression equation ($y = mx + c$)	$y = (0.018514)x + (3.88889 \text{ E-}05)$	$y = (0.00901034)x + (-0.000930952)$
Slope (m)	0.018514	0.00901034
Intercept (c)	3.88889 E-05	-0.000930952
LOD ($\mu\text{g mL}^{-1}$)	0.466573211	0.829938025
LOQ ($\mu\text{g mL}^{-1}$)	1.413858215	2.514963713
Standard error	0.000632051	0.001421926

*Mean of six observation

Table-16
OPTICAL CHARACTERISTICS OF AMBROXOL HYDROCHLORIDE
(ABSORBANCE RATIO METHOD)

PARAMETERS	AT 233.5 nm*	AT 244.5 nm*
Beer's law limit ($\mu\text{g mL}^{-1}$)	1-5 ($\mu\text{g mL}^{-1}$)	1-5 ($\mu\text{g mL}^{-1}$)
Molar absorptivity	12853.45552	14467.40119
Sandell's sensitivity ($\mu\text{g/cm}^2/0.001 \text{ A.U}$)	0.033610515	0.029669537
Correlation coefficient (r^2)	0.999299449	0.999413463
Regression equation ($y = mx + c$)	$y = (0.030895714)x + 0.001063492$	$y = (0.034782381)x + 0.001124603$
Slope (m)	0.030895714	0.034782381
Intercept (c)	0.001063492	0.001124603
LOD ($\mu\text{g mL}^{-1}$)	0.218077361	0.129641597
LOQ ($\mu\text{g mL}^{-1}$)	0.660840488	0.392853325
Standard error	0.000264883	0.000233506

*Mean of six observations

The amount present in the formulation was determined by calculating the average of six replicate analysis and its percentage purity was found to be in the range of 99.52-99.67% for DOX and 98.002-99.90% for AMB. The amount present in the tablet formulation was in good concord with the label claim and the %RSD values were found to be 0.05979 and 0.52331 for DOX and AMB respectively. The low %RSD values indicate that the method has good precision. The result of analysis is shown in Table-17.

Table-17
QUANTIFICATION OF FORMULATION [SYNASMA-AX]
(ABSORBANCE RATIO METHOD)

Drug	Sample No.	Labeled amount (mg/tab)	Amount found (mg/tab)*	Percentage Obtained*	Average (%)	S.D	% R.S.D.	S.E.
DOX	1	400	398.74	99.67	99.57	0.23814	0.05979	0.09722
	2	400	398.08	99.52				
	3	400	398.36	99.59				
	4	400	398.14	99.54				
	5	400	398.20	99.55				
	6	400	398.25	99.56				
AMB	1	30	29.71	99.03	98.88	0.15579	0.52331	0.0636
	2	30	29.47	98.23				
	3	30	29.40	98.00				
	4	30	29.62	98.73				
	5	30	29.67	99.90				
	6	30	29.82	99.40				

Precision of the method was studied by making repeated analysis of the same sample and it was carried out three times in a day and for three days. The %RSD values of intraday and interday analysis were found to be 0.13374 and 0.12245 for DOX & 0.70482 and 0.66480 for AMB. The results of the analysis are shown in Table-18. The results showed that the precision of the method was high.

Table-18
INTRADAY AND INTERDAY ANALYSIS OF FORMULATION [SYNASMA-AX]
(ABSORBANCE RATIO METHOD)

Drug	Sample No.	Labeled amount (mg/tab)	Percentage obtained*		S.D		% R.S.D.	
			Intraday	Interday	Intraday	Interday	Intraday	Interday

DOX	1	400	99.62	99.69	0.53349	0.48811	0.13374	0.12245
	2	400	99.70	99.52				
	3	400	99.62	99.57				
	4	400	99.90	99.74				
	5	400	99.69	99.54				
	6	400	99.90	99.65				
Mean			99.74	99.62				
AMB	1	30	98.93	99.43	0.20973	0.19799	0.70482	0.66480
	2	30	99.28	99.03				
	3	30	99.91	99.17				
	4	30	99.15	98.70				
	5	30	98.70	99.65				
	6	30	99.16	99.55				
Mean			99.19	99.26				

*Mean of three observations

The developed method was validated for ruggedness. In the present work it was confirmed by different Analysts. The % RSD values for Analyst 1 and Analyst 2 were The low % RSD values indicate that the developed method was more rugged. The results are shown in Table-19.

Table-19
RUGGEDNESS STUDY OF FORMULATION [SYNASMA-AX]
(ABSORBANCE RATIO METHOD)

Drug	Condition	Average* % Obtained	S.D	% R.S.D	S.E.
DOX	Analyst 1	99.61	0.43500	0.10918	0.17759
	Analyst 2	99.67	0.42239	0.10595	0.17244
	Instrument 1	99.61	0.43349	0.10879	0.17697
	Instrument 2	99.65	0.44678	0.11213	0.18239
AMB	Analyst 1	99.72	0.20096	0.67175	0.08204
	Analyst 2	99.41	0.22669	0.76009	0.09254
	Instrument 1	99.43	0.23091	0.77409	0.09427
	Instrument 2	99.51	0.24123	0.81303	0.09848

The accuracy of the method was confirmed by recovery studies. The percentage recovery was found to be in the range of 100.30-100.50% for DOX and 99.00-99.83% for AMB. The %RSD values were found to be less than 2 and thus indicate that the method is accurate. The result of recovery study was shown in Table-

20

Table-20
RECOVERY STUDY DATA OF 50% PRE-ANALYSED FORMULATION [SYNASMA-AX]
(ABSORBANCE RATIO METHOD)

Drug	Percentage	Amount present* ($\mu\text{g ml}^{-1}$)	Amount added* ($\mu\text{g ml}^{-1}$)	Amount estimated* ($\mu\text{g ml}^{-1}$)	Amount recovered* ($\mu\text{g ml}^{-1}$)	% Recovery*	S.D.	% R.S.D.	S.E.
DOX	80	13.9429	11.2	25.19887	11.25597	100.50	0.013979	0.12419	0.00807
	100	13.9429	14	27.98503	14.04213	100.30	0.004102	0.02921	0.002368
	120	13.9429	16.8	30.82167	16.87877	100.47	0.017989	0.106578	0.10385
AMB	80	1.0367	0.8	1.82873	0.79203	99.00	0.00665	0.83999	0.00384
	100	1.0367	1.0	2.034267	0.99757	99.76	0.00648	0.64998	0.00374
	120	1.0367	1.2	2.234633	1.19793	99.83	0.01019	0.85072	0.00588

*Mean of three observations

7.1 REVERSE-PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY FOR DOXOFYLLINE AND AMBROXOL HYDROCHLORIDE

An involvement was made in this project to devise a simple, accurate, less expensive and sensitive RP-HPLC method of the estimation of DOX and AMB in solid dosage form. Since the drugs are polar, Reverse Phase High Performance Liquid Chromatography was selected.

Selection of mobile phase

The standard solutions containing DOX and AMB were injected into HPLC system and run in different solvent systems. By studying the literature survey, different mobile phases in different proportions and different pH were tried in order to find the best conditions for the separation.

Each mobile phase was sonicated for 10 minutes and filtered through 0.45 μ membrane filter. The mobile phase was allowed to equilibrate until steady baseline was obtained. The standard solutions containing DOX and AMB were run and combinations of solvents were tried to get a good separation and stable peak. From the various mobile phase tried, mobile phase containing 10 mM Phosphate buffer: Acetonitrile: Methanol in the ratio of 70: 20: 10 (pH adjusted to 3.0 with orthophosphoric acid) was selected, since it gave sharp peak

with symmetry and reproducible retention time for DOX and AMB.

Wavelength selection

The UV spectra of individual drugs were recorded in the wavelength range from 200-400 nm and compared. The choice to use a common wavelength set at 224 nm was considered satisfactory, permitting the detection of drugs with adequate sensitivity.

System suitability

The system suitability studies were carried out to determine Tailing factor, Asymmetrical factor, Theoretical plates and Capacity factor. The results are given in Table-21. The values obtained demonstrated the suitability of the system for the analysis of investigated drug combination and the system suitability parameters may fall within $\pm 3\%$ standard deviation range during routine performance of the method.

Table-21
SYSTEM SUITABILITY PARAMETERS FOR THE OPTIMIZED CHROMATOGRAM OF DOXOFYLLINE AND AMBROXOL HYDROCHLORIDE FOR RP-HPLC METHOD

PARAMETERS	DOXOFYLLINE	AMBROXOL HYDROCHLORIDE
Tailing factor	1.55	1.44
Asymmetrical factor	1.67	1.62
Theoretical plates	3951	4519
Capacity factor	1.87	3.48

Stability

The stability of the drugs in the proposed mobile phase was checked by monitoring the absorbance of DOX and AMB at the selected wavelength over a period of 5 hours at room temperature. The result is reported in Table-22. The result shows that the absorbance of both the drugs remained almost unchanged and no significant degradation within the indicated period. Thus revealed that both the solutions were stable for at least 5 hours, which was sufficient to complete the whole analytical process

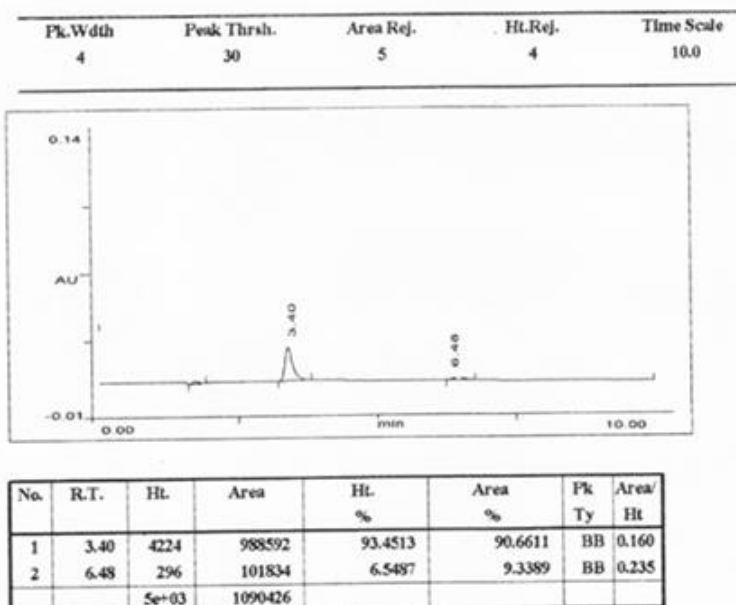
Table-22
STABILITY STUDY OF DOXOFYLLINE AND AMBROXOL HYDROCHLORIDE FOR HPLC

METHOD**DRUG: Doxofylline + Ambroxol Hydrochloride****Mobile phase: Phosphate buffer: Acetonitrile: Methanol Ratio: 70: 20: 10**

S.No	Time	Doxofylline at 274 nm	Ambroxol Hydrochloride at 244.5 nm
1	0 min	0.354	0.263
2	10 min	0.353	0.264
3	20 min	0.350	0.263
4	30 min	0.355	0.261
5	40 min	0.353	0.262
6	50 min	0.352	0.263
7	1 hour	0.353	0.264
8	1 hour 15 min	0.352	0.260
9	1 hour 30 min	0.353	0.261
10	1 hour 45 min	0.351	0.262
11	2 hours	0.354	0.264
12	2 hours 30 min	0.352	0.265
13	3 hours	0.350	0.264
14	3 hours 30 min	0.351	0.267
15	4 hours	0.355	0.271
16	5 hours	0.354	0.273

Linearity

The linearity of the method was determined at five concentration levels ranging from 7- 35 µg/ml for DOX and 0.5-2.5 µg/ml for AMB. The linearity chromatogram is recorded in Figures 13-17.

FIGURE-13**LINEARITY CHROMATOGRAM OF DOXOXYLLINE AND AMBROXOL HYDROCHLORIDE (7, 0.5 µg ml⁻¹) – FIRST SET [1/3]****FIGURE-14**

LINEARITY CHROMATOGRAM OF DOXOXYLLINE AND AMBROXOL HYDROCHLORIDE

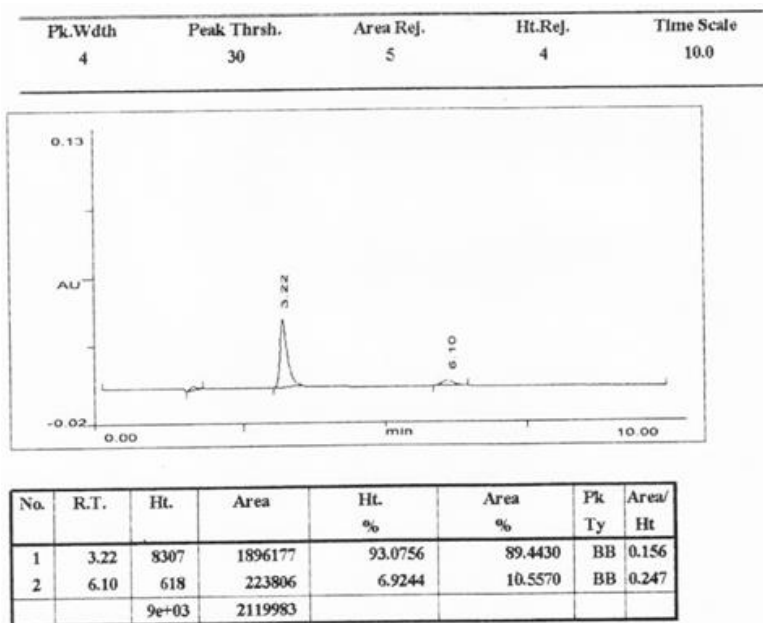


FIGURE-15

LINEARITY CHROMATOGRAM OF DOXOXYLLINE AND AMBROXOL HYDROCHLORIDE (21, 1.5 µg ml⁻¹) - FIRST SET [1/3]

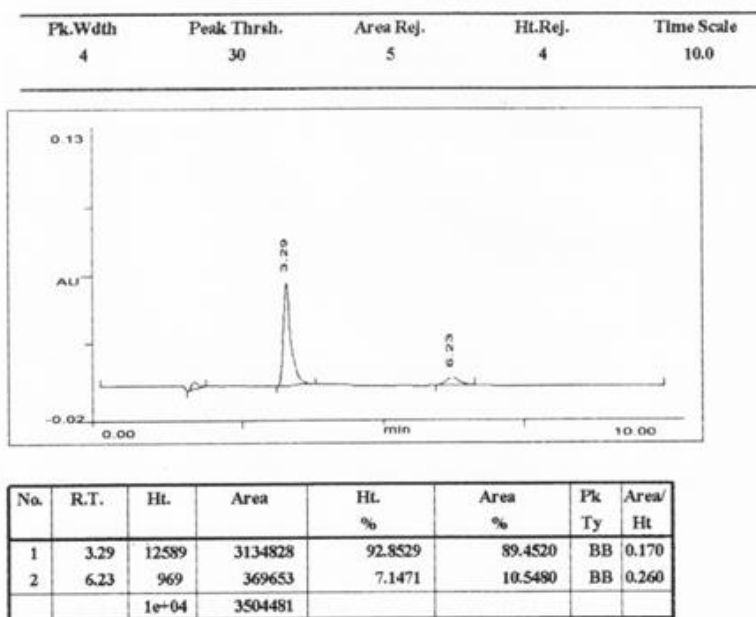


FIGURE-16
LINEARITY CHROMATOGRAM OF DOXOXYLLINE AND AMBROXOL HYDROCHLORIDE
(28, 2 µg ml⁻¹) - FIRST SET [1/3]

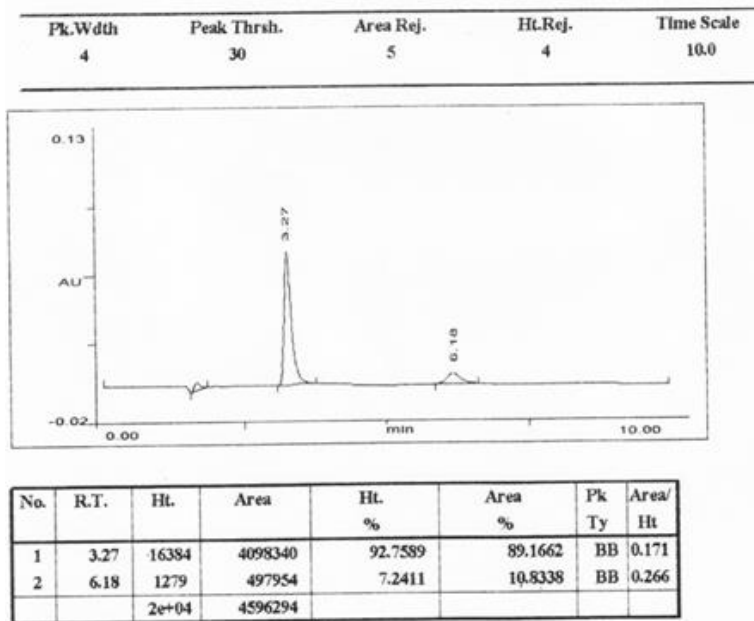


FIGURE-17
LINEARITY CHROMATOGRAM OF DOXOXYLLINE AND AMBROXOL HYDROCHLORIDE
(35, 2.5 µg ml⁻¹) - FIRST SET [1/3]

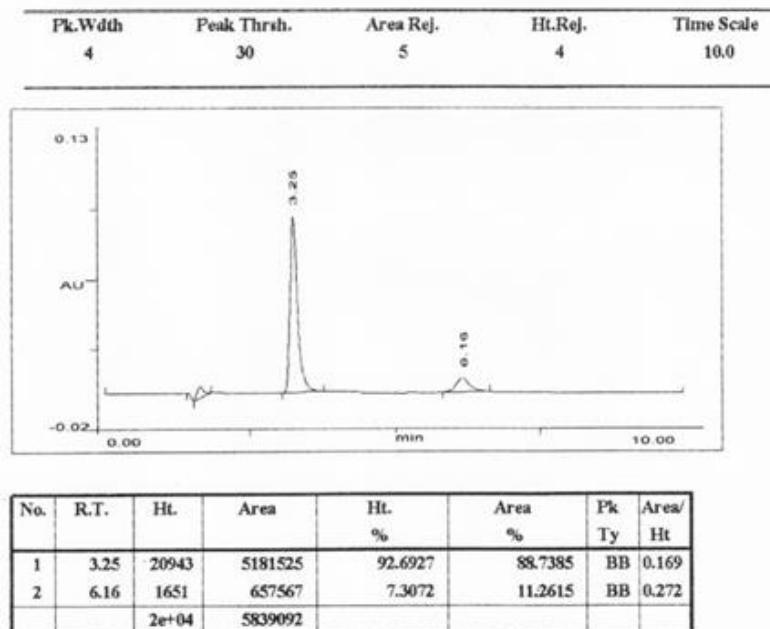


FIGURE-18
CALIBRATION CURVE OF DOXOPHYLLINE BY RP-HP

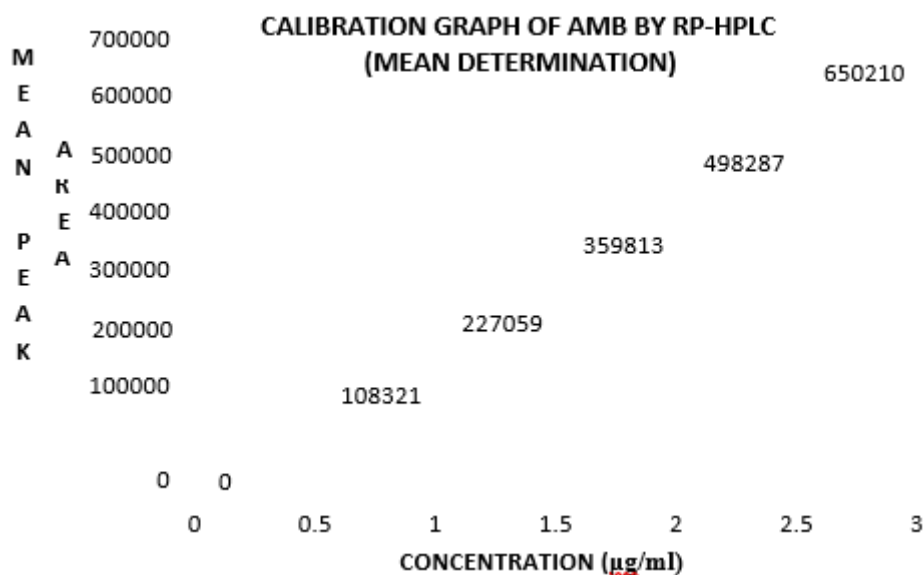
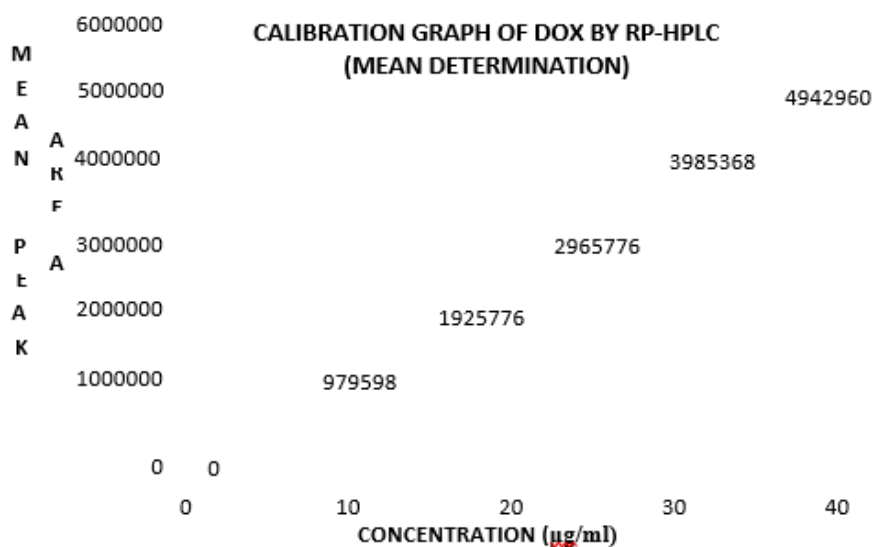


FIGURE-19
CALIBRATION CURVE OF AMBROXOL HYDROCHLORIDE BY RP- HPLC



The calibration curves were plotted between the mean peak areas vs. respective concentrations and are shown in Figures-18&19 for DOX and AMB respectively. The corresponding linear regression equation was $y = 270225.0286 x + (- 3.70062 E-09)$ with square of correlation coefficient r^2 of 0.999765561 for AMB and $y = 169886.3316 x + (-17753.96429)$ with square of correlation coefficient r^2 of 0.999707379 for DOX respectively. The results showed that an excellent correlation exists between the peak area and concentration of the drugs within the concentration range indicated above and is represented in Table-23

Table-23

**OPTICAL CHARACTERISTICS OF DOXOFYLLINE AND AMBROXOL HYDROCHLORIDE
IN RP-HPLC METHOD**

PARAMETERS	DOXOFYLLINE*	AMBROXOL HYDROCHLORIDE*
λ_{max} (nm)	274 nm	244.5 nm
Beers law limit ($\mu\text{g mL}^{-1}$)	7 -35	0.5 – 2.5
Correlation coefficient (r^2)	0.999707379	0.999765561
Régression equation ($y = mx + c$)	$y = 169886.3316 x + (-17753.96429)$	$y = 270225.0286 x + (-3.70062 \text{ E-}09)$
Slope (m)	169886.3316	270225.0286
Intercept (c)	-17753.96429	-3.70062 E-09
Standard Error	68094.73019	6120.552086

Quantification

The tablet dosage form containing DOX 400 mg and AMB 30 mg was selected for the analysis. The ostensible concentration $14 \mu\text{g mL}^{-1}$ of DOX in the mobile phase was prepared, which contains $1 \mu\text{g mL}^{-1}$ of AMB. $20 \mu\text{l}$ of each solution was injected and chromatograms were recorded and shown in Figures 20-25. The assay procedure was repeated for six times. The percentage purity was found to be 100.82% and 100.12 for DOX and AMB (Table-24). The result of analysis showed that the amount of drugs were in good agreement with the label claim of the formulation.

**FIGURE-20
CHROMATOGRAM FOR ANALYSIS OF FOMULATION [SYNASMA-AX]
REPEATABILITY -1**

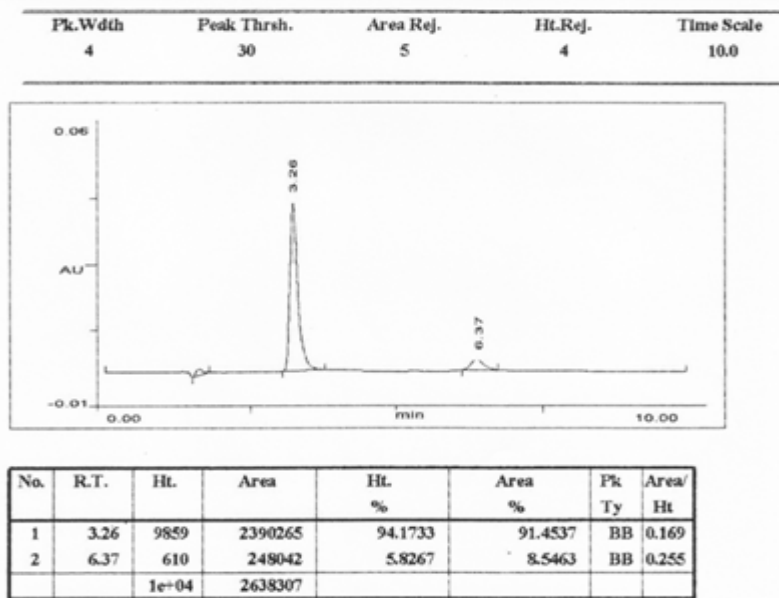


FIGURE-21
CHROMATOGRAM FOR ANALYSIS OF FORMULATION [SYNASMA-AX] REPEATABILITY-2

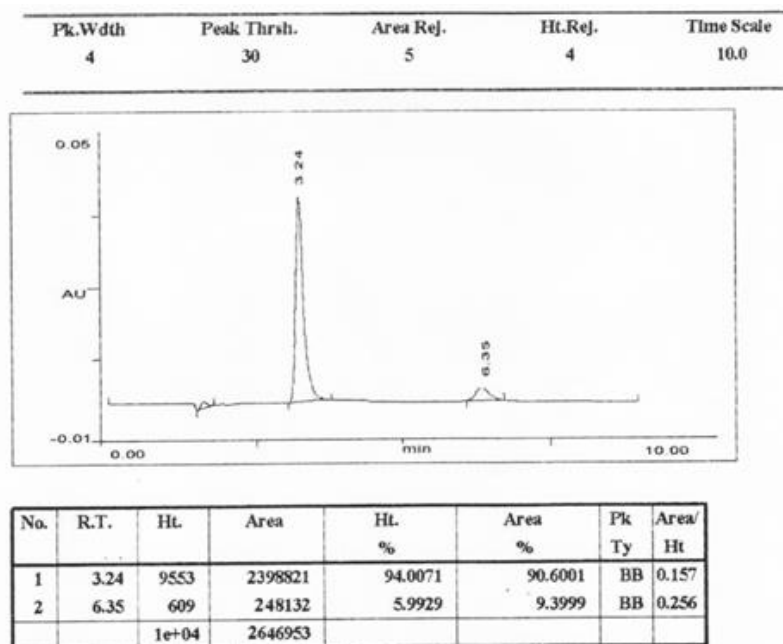


FIGURE-22
CHROMATOGRAM FOR ANALYSIS OF FORMULATION [SYNASMA-AX] REPEATABILITY-3

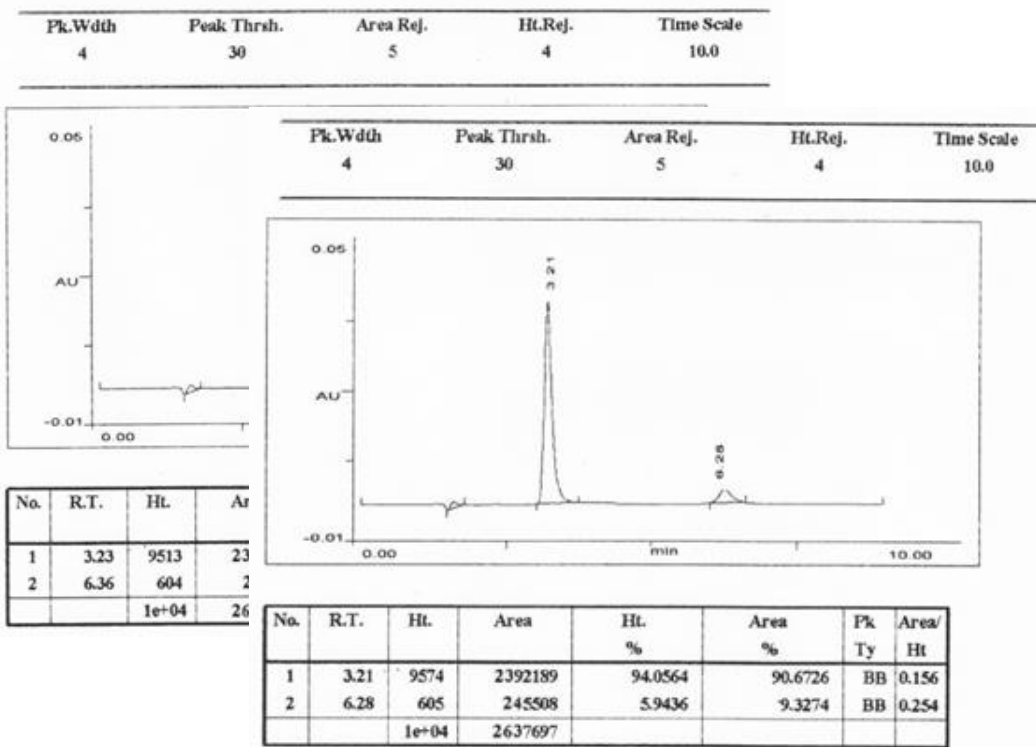


FIGURE-23
CHROMATOGRAM FOR ANALYSIS OF FORMULATION [SYNASMA-AX] REPEATABILITY-
4

FIGURE-24
CHROMATOGRAM FOR ANALYSIS OF FORMULATION [SYNASMA-AX]

REPEATABILITY-5

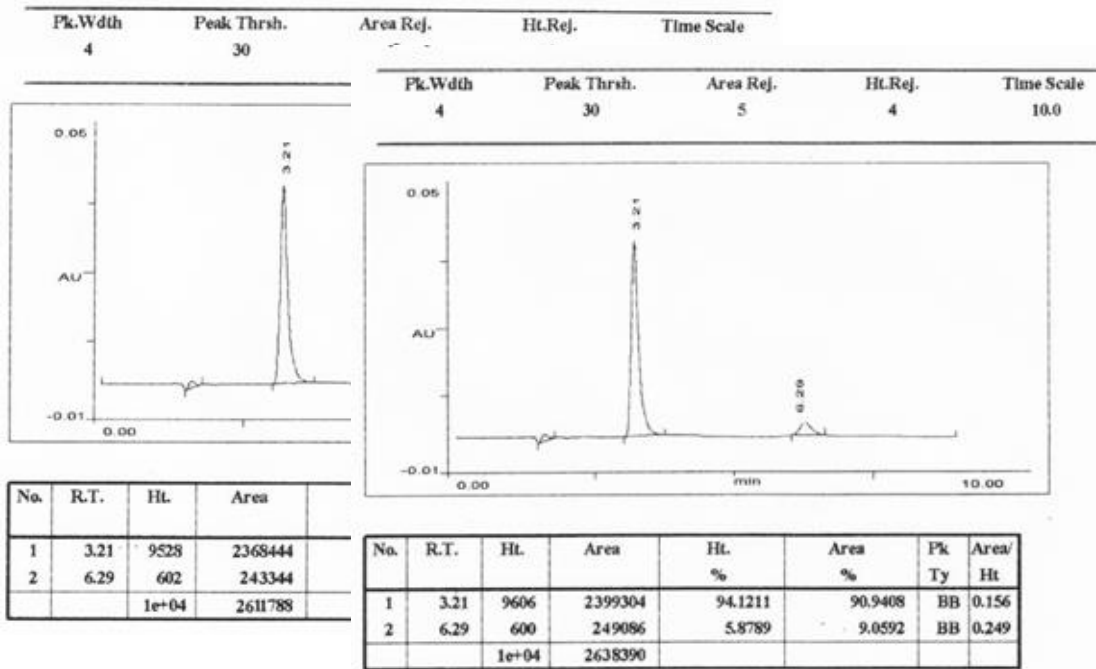


FIGURE-25
 CHROMATOGRAM FOR ANALYSIS OF FORMULATION [SYNASMA-AX] REPEATABILITY-6

Table-24
 QUANTIFICATION OF FORMULATION

**[SYNASMA-AX]
BY RP-HPLC**

Drug	Sample No.	Labeled amount (mg/tab)	Amount found (mg/tab)	Percentage Obtained*	Average (%)	S.D.	% R.S.D.	S.E.
DOX	1	400	404.2	101.05	100.82	1.92215	0.47582	0.78472
	2		405.6	101.40				
	3		403.3	100.82				
	4		400.5	100.12				
	5		404.5	101.12				
	6		405.7	101.42				
AMB	1	30	30.14	100.46	100.12	0.51887	1.71792	0.21183
	2		30.15	100.50				
	3		30.16	100.53				
	4		29.64	98.80				
	5		29.88	99.60				
	6		30.25	100.83				

Method validation

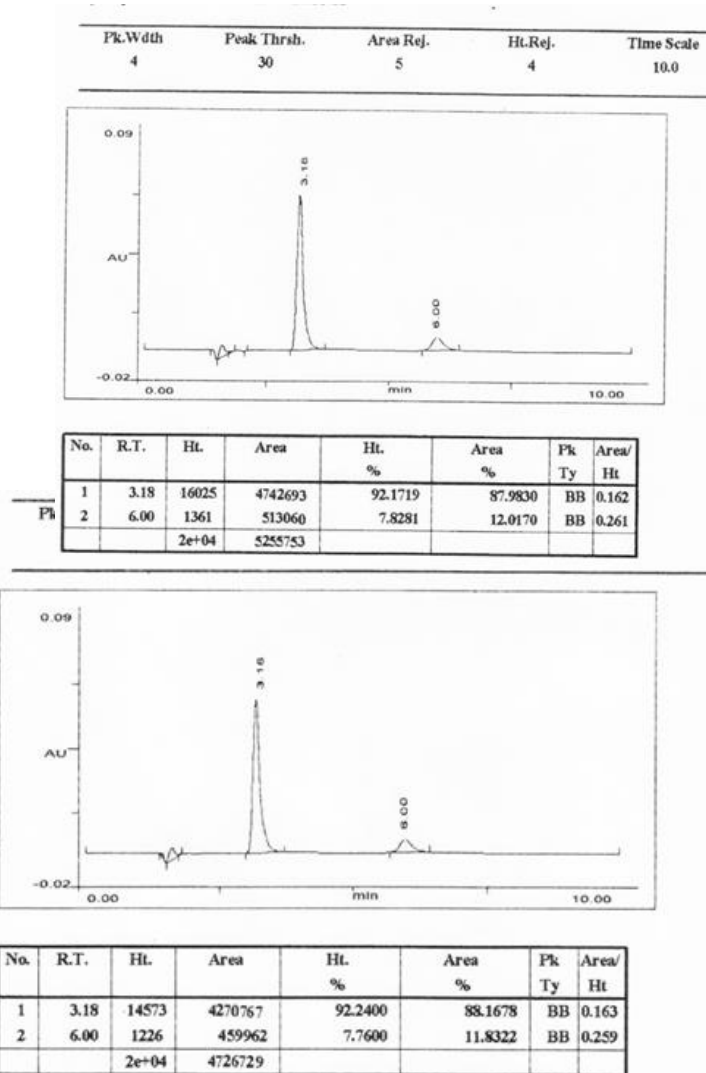
The proposed HPLC method was validated as per the guidelines of the International Conference on the Harmonization of Technical Requirements for the Registration of Pharmaceuticals for Human use (ICH).

The accuracy of the method was performed by recovery experiments. The recovery studies were carried out by standard addition method at three different levels 80%, 100% and 120% by injecting the solutions. The chromatograms are recorded as shown in the Figures 26-28. The percentage recovery was found to in the range between 98.36-99.76% for AMB and 98.54-99.43% for DOX. The low percentage of RSD values for recovery experiment indicates that the method is accurate. The values are given in the Table-25. The high percentage recovery revealed that there was no interference due to the excipients used in the formulation. Therefore the developed method was found to be accurate.

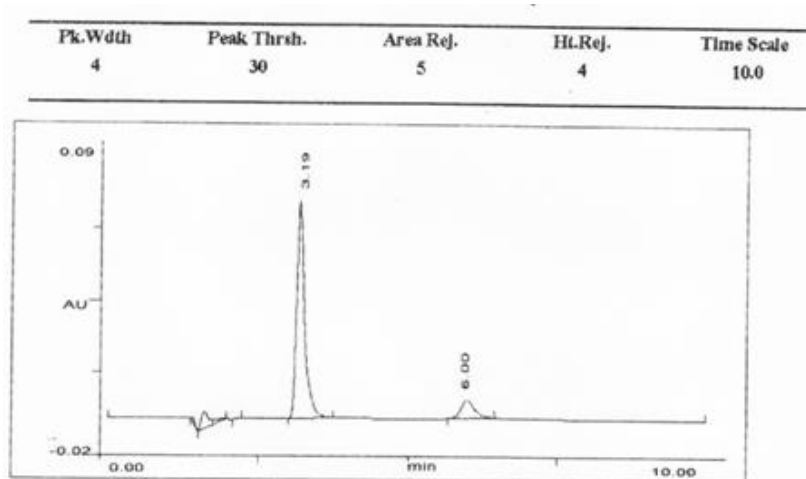
**FIGURE-26
CHROMATOGRAM FOR 80% RECOVERY OF FORMULATION [SYNASMA-AX]**

**FIGURE-27
CHROMATOGRAM
RECOVERY OF
[SYNASMA-AX**

**FOR 100%
FORMULATION**



**FIGURE-28
CHROMATOGRAM FOR 120% RECOVERY OF FORMULATION
[SYNASMA-AX]**



No.	R.T.	Ht.	Area	Ht. %	Area %	Pk Ty	Area/Ht
1	3.19	18237	5226995	92.1852	88.1255	BB	0.167
2	6.00	1546	570561	7.8148	11.8745	BB	0.265
		2e+04	5797556				

Table-25

RECOVERY

OF 50% PREANALYSED FORMULATION [SYNASMA-AX] BY RP-HPLC

STUDY DATA

Drug	Percentage	Amount present* (µg ml ⁻¹)	Amount added* (µg ml ⁻¹)	Amount estimated* (µg ml ⁻¹)	Amount recovered* (µg ml ⁻¹)	% Recovery*	S.D.	% R.S.D.	S.E.
DOX	80	14.1671	11.2	24.8706	11.0368	98.54	0.03429	0.31075	0.01980
	100	14.1671	14	28.0212	13.8541	98.96	0.00035	0.00249	0.00019
	120	14.1671	16.8	30.8721	16.70503	99.43	5.7735E-05	0.00035	0.00003
AMB	80	1.0533	0.8	1.8401	0.7869	98.36	0.00173	0.22010	0.00099
	100	1.0533	1.0	2.04017	0.98687	98.36	0.00214	0.21644	0.00123
	120	1.0533	1.2	2.25049	1.1972	99.76	0	0	0

The precision of the method was demonstrated by interday and intraday variation studies. In the intraday study, six repeated injections of standard and sample solutions were made and % RSD was calculated. In the Interday variation studies, six repeated injections of standard and sample solutions were made for three consecutive days and the % RSD was calculated. The results are presented in Table-26. From the data obtained, the developed

TABLE-26
INTRADAY AND INTERDAY ANALYSIS OF FORMULATION [SYNASMA-AX]

BY RP-HPLC METHOD

Drug	Sample No.	Labeled amount (mg/tab)	Percentage obtained*		S.D		% R.S.D.	
			Intraday	Interday	Intraday	Interday	Intraday	Interday
DOX	1	400	100.70	100.50	0.50817	0.46576	1.69804	1.57263
	2	400	100.79	97.64				
	3	400	97.80	98.03				
Mean			99.76	98.72				
AMB	1	30	99.12	99.69	2.77081	1.53285	0.69469	0.62578
	2	30	99.55	98.97				
	3	30	100.47	99.56				
Mean			99.71	99.41				

All the above parameters with the ease of operations ensure that the projected methods could be applied for the routine analysis of DOX and AMB in pure form and in tablet dosage form.

SUMMARY AND CONCLUSION**DOXOFYLLINE AND AMBROXOL HYDROCHLORIDE**

Doxofylline is chemically 7-(1, 3-Dioxolan-2-yl methyl)-3, 7-dihydro-1, 3-dimethyl-1H- Purine-2, 6-Dione. It is a novel bronchodilator. Ambroxol Hydrochloride is chemically 1 ([2-Amino-3, 5 dibromo phenyl]-methyl) amino) cyclohexanol monohydrochloride which is a semi synthetic derivative of vasicine from the Indian shrub "Adhatoda Vasica". It is a mucolytic agent and expectorant. Ambroxol Hydrochloride is an N-desmethyl metabolite of bromohexine.

Ambroxol Hydrochloride and Doxofylline in combination are used as an antiasthmatic agent. The simple, rapid, precise and reproducible analytical methods for the simultaneous estimation of Ambroxol Hydrochloride and Doxofylline in formulation were developed.

The tablet dosage form (SYNASMA-AX) containing 400 mg of Doxofylline and 30 mg of Ambroxol Hydrochloride has been selected for the study.

The methods adopted for studies were

7.0 UV SPECTROSCOPIC METHOD:

UV spectrophotometric method for the estimation of Doxofylline and Ambroxol Hydrochloride in combined tablet dosage form by

1. Simultaneous Equation Method
2. Absorbance Correction Method

From the solubility data, distilled water is used as a common solvent. Doxofylline and Ambroxol Hydrochloride were prepared separately ($10 \text{ [g ml}^{-1}\text{]}$) and scanned in the UV region of 200-400 nm. From the overlaid spectra, by the observation of spectral characteristics of Doxofylline and Ambroxol Hydrochloride, they were selected for Simultaneous equation method, Absorbance correction method and Absorbance ratio method. The wavelengths selected for simultaneous equation method were 274 nm and 244.5 nm, 274 nm and 308 nm for the absorbance correction method and 233.5 nm and 244.5 nm for absorbance ratio method.

1. Simultaneous Equation Method

The percentage label claim present in tablet formulation was found to be 99.97% and 98.64% for Doxofylline and Ambroxol Hydrochloride respectively. The percentage recovery was found to be in the range of 99.98-100.09% and 98.42-99.86% for Doxofylline and Ambroxol Hydrochloride respectively.

2. Absorbance Correction Method

The percentage label claim present in tablet formulation was found to be 100.32% and 99.60% for Doxofylline and Ambroxol Hydrochloride respectively. The percentage recovery was found to be in the range of 99.64-100.00% and 99.73-100.55% for Doxofylline and Ambroxol Hydrochloride respectively.

3. Absorbance Ratio Method

The percentage label claim present in tablet formulation was found to be 99.57% and 98.88% for Doxofylline and Ambroxol Hydrochloride respectively. The percentage recovery was found to be in the range of 100.30-100.50% and 99.00-99.83% for Doxofylline and Ambroxol Hydrochloride respectively.

7.2 REVERSE PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY METHOD:

RP-HPLC method has been developed for the estimation of both drugs in bulk and in formulation. The proposed method gives reliable assay results with short analysis time, using mobile phase Phosphate buffer, pH 3.0: Acetonitrile: Methanol in the ratio of 70: 20: 10. The percentage label claim present in tablet formulation was found to be 100.82% and 100.12% for Doxofylline and Ambroxol Hydrochloride respectively. The percentage recovery was found to be in the range of 98.54-99.43% and 98.36-99.76% for Doxofylline and Ambroxol Hydrochloride respectively. The contents of drug present in the formulation were found to be satisfactory and system suitability parameters are in desired limit.

All the above methods do not suffer from any interference due to common excipients. It indicates that the methods were accurate. Therefore the proposed methods could be successfully applied to estimate commercial pharmaceutical products containing Doxofylline and Ambroxol Hydrochloride.

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