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Application of HPTLC for Simultaneous Estimation of Ambroxol hydrochloride and Roxithromycin in Pharmaceutical Dosage Forms

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Abstract

A simple, rapid and reliable HPTLC method was developed for the determination of Ambroxol Hydrochloride and Roxithromycin in tablets. Stress testing and stability studies were carried out for both the drugs as per the ICH guidelines. Determinations were performed on aluminium backed silica gel 60 F_{254} HPTLC plates, with conc. Ammonia, 1-propanol,ethylacetate, hexane 0.5+20+20+70(*v/v*), as mobile phase. The validated calibration range was 300ng - 1500ng $spot^{-1}$ (r = 0.998) and 750 ng – 3750 ng (r = 0.997) for Ambroxol Hydrochloride and Roxithromycin respectively. The spots were scanned at λ = 254nm. The suitability of this HPTLC method for quantitative determination of compounds was proved by various validation parameters in accordance with the requirements of ICH guidelines (Q2B). Results from the stress testing studies indicated in the present research paper will form an integral part the development of new pharmaceutical formulations of Ambroxol hydrochloride and Roxithromycin and their estimation in biological fluids.

Keywords:

HPTLC; Pharmaceutical dosage forms; Ambroxol Hydrochloride; Roxithromycin

1. Introduction

Ambroxol hydrochloride, or *trans*-4-(2-Amino-3,5-dibromobenzylamino) cyclohexanol hydrochloride is a metabolite of bromhexine. It is an expectorant and mucolytic agent that is officially recognized in the British Pharmacopoeia indicated in cough associated with bronchitis, mucous plugging and problems of expectoration. It is administered in a daily dose of 30 to 120 mg, orally in 2 to 3 divided doses. Similar doses have been given by inhalation, injection, or rectally [1-3].

Roxithromycin is (3*R*,4*S*,5*S*,6*R*,7*R*,9*R*,11*S*,12*R*,13*S*,14*R*)-4-[(2,6-Dideoxy-3-*C*-methyl-3-*O*-methyl-α-L-*ribo*-hexopyranosyl)oxy]-14-ethyl-7,12,13-trihydroxy-10-[(E)-[(2-methoxy-ethoxy)methoxy]imino]-3,5,7,9,11,13-hexamethyl-6-[[3,4,6-trideoxy-3-(dimethyl-amino)-β-D-*xylo*-hexopyranosyl]oxy]oxacyclotetradecan-2-oneerythromycin9-(*E*)-[O-[(2-methoxy-ethoxy)methyl]oxime]) (Fig. 1). It is a semi-synthetic macrolide antibiotic and is given orally in a dose of 150mg twice daily before meals, in the treatment of susceptible infections. The drug is officially recognized in the European as well as the British pharmacopoeia [4-9].

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Literature survey reveals several bioequivalence studies and HPLC and HPTLC methods for analysis of Ambroxol hydrochloride [10-13]. A number of methods involving infrared reflectance spectroscopy, liquid chromatography–mass spectrometry, plasma analysis, HPLC–ECD and spectrofluorimetric determination of Roxithromycin are also reported [14-19]. Liquid chromatographic methods for the detection of Ambroxol hydrochloride in combination with Roxithromycin are also been reported [20-22]**.**

The present paper describes a reliable, rapid and accurate HPTLC method for the determination of Ambroxol hydrochloride in conjunction with Roxithromycin, in tablets. The proposed HPTLC assays were validated in accordance with the requirements of ICH guidelines (Q2B). The drugs were subjected to stability testing and various stress conditions to evaluate the stability of the analytes and assess the degradation profile of analytes under selected experimental conditions.

2. Experimental

2.1 Instrumentation

HPTLC was performed with Camag (Muttenz, Switzerland) Linomat V applicator, Camag twin trough TLC chamber, Camag TLC Scanner, Camag Wincats software and a Hamilton (Reno, Nevada, USA) syringe (100 µ L).

2.2 Materials and Reagents

Ambroxol hydrochloride and Roxithromycin were obtained as a gift sample from Megha Healthcare Pvt. Ltd., Mumbai, India, to be used as working standards. Conc. Ammonia, 1-propanol, ethyl acetate and hexane were used as solvents to prepare the mobile phase. All the chemicals used were of analytical reagent grade (S. D. Fine Chem. Ltd., Mumbai) used without further purification. Rambro- 150 (containing 150 mg of Roxithromycin and 60mg of Ambroxol hydrochloride) tablets, marketed by Megha Healthcare Pvt. Ltd., Mumbai, were used for assays.

2.3 Preparation of combined standard solutions of Ambroxol Hydrochloride and Roxithromycin

A combined standard stock solution containing 1 mg mL^{-1} of Ambroxol hydrochloride and 2.5 mg mL^{-1} of Roxithromycin was prepared in methanol. Calibration solutions were prepared by diluting the stock solution, so that application of 10 µL volumes gave a series of spots, covering the calibration range 300 – 1500 ng for Ambroxol hydrochloride and 750-3750 ng for Roxithromycin.

2.4 Sample Preparation

Twenty Rambro-150 tablets were weighed and powdered in a glass mortar and an amount of powder equivalent to 60 mg of Ambroxol hydrochloride and 150 mg of Roxithromycin was transferred to a 100 ml calibrated volumetric flask, extracted with methanol for 10 minutes by shaking mechanically, diluted to volume with the same solvent and filtered with Whatman Filter paper No.40 (manufactured by Whatman International Ltd. Maidstone, England). A sample solution (10 µL, containing 600 ng of Ambroxol hydrochloride and 1500 ng of Roxithromycin) was spotted for assay of Ambroxol hydrochloride and Roxithromycin.

2.5 Chromatographic Conditions

Chromatography was performed on 10 cm x 20 cm aluminium backed silica gel 60 F_{254} HPTLC plates (E. Merck, Darmstadt, Germany) stored in a dessicator and prewashed with methanol. The spotting was done by means of a Hamilton micro syringe (Switzerland),

mounted on a Linomat V applicator (Camag, Muttenz Switzerland) by keeping a distance of 10mm from the lower edge of the plate. Ascending development of the plate, migration distance 70 mm, was performed at 25 ± 2^0C , with conc.Ammonia,1-propanol,ethylacetate,hexane 0.5+20+20+70(*v/v*), as the mobile phase, in a Camag chamber previously saturated for 10 minutes which required 15mL of the solvent mixture. The total solvent mixture volume required for development was about 10mL. Samples were applied as 6 mm wide band at a spraying rate of 15 s μL^{-1}; and the distance between the bands was 13.0 mm. The average development time was 20 minutes. After development the plate was dried at 50^0 C in an oven for 5 minutes. Densitometric scanning was then performed with a Camag TLC Scanner equipped with Wincats Software Version 1.3.0 at λ = 254 nm using deuterium light source, the slit dimensions were 6.00 x 0.45 mm.

2.6 Validation of the HPTLC method

The assays of the present method were carried out and validated as per the various parameters proposed by the ICH guidelines (Q2B).

2.6.1 Linearity

Separate Standard solutions equivalent to 300, 600, 900, 1200 and 1500 ng $spot^{-1}$ of Ambroxol hydrochloride acid and 750, 1500, 2250, 3000 and 3750 ng $spot^{-1}$ of Roxithromycin were spotted on the prewashed HPTLC plates. A minimum of ten determinations of each concentration per spot were performed. The plates were developed, dried and scanned as described above. The calibration curve was constructed by plotting peak area against the corresponding concentration of both the drugs (ng $spot^{-1}$) individually. The linearity of response for Ambroxol hydrochloride was assessed in the concentration range 300 to 1500 ng $spot^{-1}$, in terms of slope, intercept and correlation coefficient values (by linear regression).

2.6.2 Precision

Intermediate Precision studies were performed by establishing the effects of random events on the precision of the analytical procedure. Interday precision (%C.V.) was assessed by carrying out replicate analysis of sample solutions and reference standard solutions with concentrations covering the entire calibration range. The analysis was carried out by solutions of concentration 600ng, 900ng and 1200ng for Ambroxol hydrochloride and 1500ng, 2250ng and 3000ng of Roxithromycin on three days over a period of one week.

2.6.3 Accuracy

The accuracy of the method was determined by the use of standard additions at three different levels, i.e. multiple level recovery studies. Sample stock solution of tablet formulation of 600 ng mL^{-1} of Ambroxol hydrochloride and 1500 ng mL^{-1} of Roxithromycin was prepared. To the above prepared solutions, 80%, 100% and 120% of the standard drug solutions were spiked and the percentage recoveries were estimated.

2.6.4 Specificity

Analytically pure drug samples of Ambroxol Hydrochloride and Roxithromycin were obtained from Megha Healthcare Pvt.Ltd. Mumbai, and they were found to be free from degradation products and impurities**.** The mobile phase designed for the method resolved both the drugs very efficiently (Fig. 2). The R_f value of Roxithromycin and Ambroxol hydrochloride was found to be 0.5 and 0.2 respectively. The wavelength 254 nm was selected for detection as it showed better detection sensitivities for both the drugs. The spot for Roxithromycin and Ambroxol Hydrochloride from tablet formulation was confirmed by

comparing their R_f and absorbance/reflectance spectrum with those of standard Roxithromycin and Ambroxol hydrochloride.

2.6.5 System Suitability

According to the USP 23 method (621), system suitability test are an integral part of a chromatographic analysis and should be used to verify that the resolution and reproducibility of the chromatographic system are adequate for the analysis. To ascertain the effectiveness of the method developed in this study system, suitability tests were performed on freshly prepared standard stock solutions of Ambroxol hydrochloride and Roxithromycin.

2.6.6 Robustness

Robustness is a measure of the capacity of a method to remain unaffected by small but deliberate variations in the method conditions, and is an indication of the reliability of the method. The change in the development distance was used as a parameter to check the robustness of the method.

2.7 Stability-indicating Assays

The assessment of the stability of drugs was also along with the various parameters used to validate the proposed method. The standard drug solutions were found to be stable at room temperature in the solvent (methanol) used for preparing the solutions. The stability of the drugs in solution was assessed by performing assay of drugs in solution exposed to room temperature conditions up to a period of 48 hours. The analytes in the solvent were assessed by investigating three aliquots of each drug solution at high and low concentrations.

2.7.1 Analysis for the degradation product(s) and related substances (Stress testing):

2.7.1.1 Forced degradation of Drugs

Sample stock solution of 600 ng mL^{-1} of Ambroxol hydrochloride and 1500 ng mL^{-1} of Roxithromycin were prepared and used for forced degradation studies.

2.7.1.2 Preparation of acid-induced degradation products

To above mentioned solutions of analytes, 20 ml of 1M methanolic HCl each was added and the solutions were kept for 72hrs at room temperature in the dark in order to exclude the possible degradative effect of light. The solutions were then used to analyze the possible effect of acid-induced degradation in the drugs.

2.7.1.3 Preparation of base-induced degradation product

To sample stock solutions of analytes, 10 mL of 0.1 N NaOH was added. The resultant solutions were kept for 72hrs at room temperature in the dark in order to exclude the possible degradative effect of light and were used for analysis of any possible degradation products that may be formed due to exposure to a base.

2.7.1.4 Preparation of hydrogen peroxide-induced degradation product

To sample stock solutions of analytes, 20 ml of hydrogen peroxide (30% $^{v}/_{v}$), was added and the mixtures were refluxed for 4 hrs at 65^0 C. After an interval of 1hr, 1 ml samples were withdrawn and used for chromatographic analysis.

2.7.1.5 Photochemical degradation product

The photochemical stability of the drugs was studied by exposing the prepared stock solutions to UV light (Philips, 50–60 Hz, 40 W, 50 cm distance) for 8 hrs. The resultant solutions were used for chromatographic analysis.

2.7.2. Detection of the related impurities

The related impurities were determined by spotting a higher concentration of the drugs so as to detect and quantify the related impurities in drugs if present. About 20-mg of each analyte were dissolved in 10 mL of methanol and 20 µL of these solutions were spotted on the TLC plate, developed and scanned.

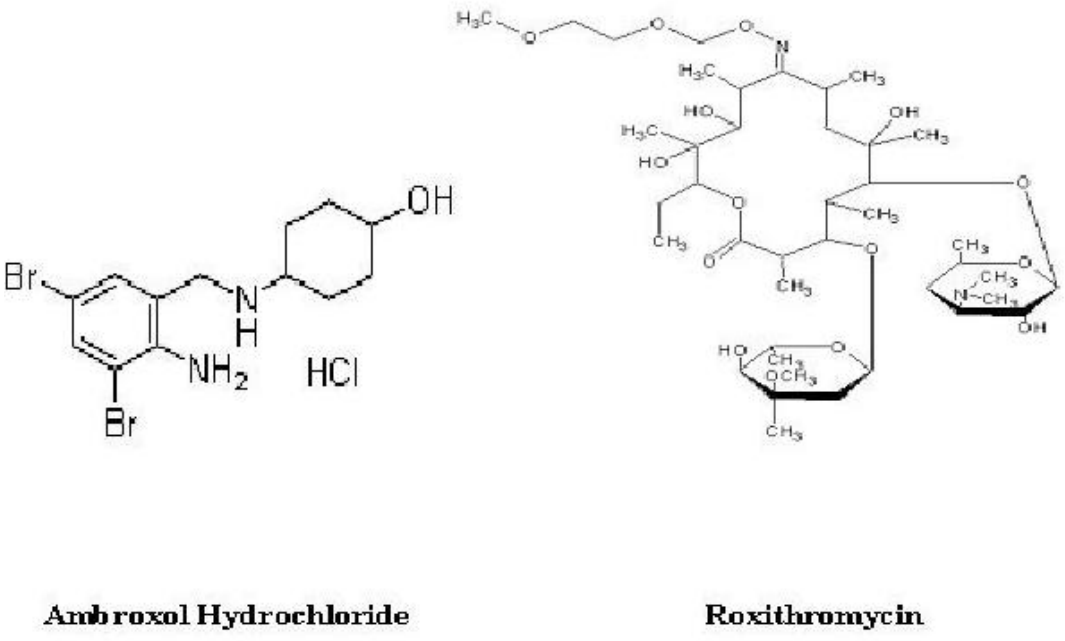
2.8 Repeatability

Repeatability of the method was assessed by performing 9 determinations of concentrations covering the calibration range. Individual sample solutions and reference standards were analysed 3 times per concentration and the results were used for determination of precision. Three concentrations of 600 ng, 900 ng and 1200 ng for Ambroxol hydrochloride and 1500 ng, 2000 ng and 2500 ng of Roxithromycin were applied on HPTLC plates and ten replicates of each were determined.

3. Results and Discussion

3.1 Linearity

The linearity of response for Ambroxol hydrochloride was assessed in the concentration range 300 to 1500 ng spot^{-1} and the calibration plots showed the correlation coefficient r = 0.997; slope = 3.414 ± 0.2011 (n = 10) over the concentration range studied. The linearity of response for Roxithromycin was assessed in the concentration range 750 to 3750 ng spot^{-1}, and the calibration plots showed the correlation coefficient r = 0.998 ; slope = 3.211 ± 0.08363 (n = 6) over the concentration range studied.



Figç 1: Structures of ambroxol hydrochloride and roxithromycin

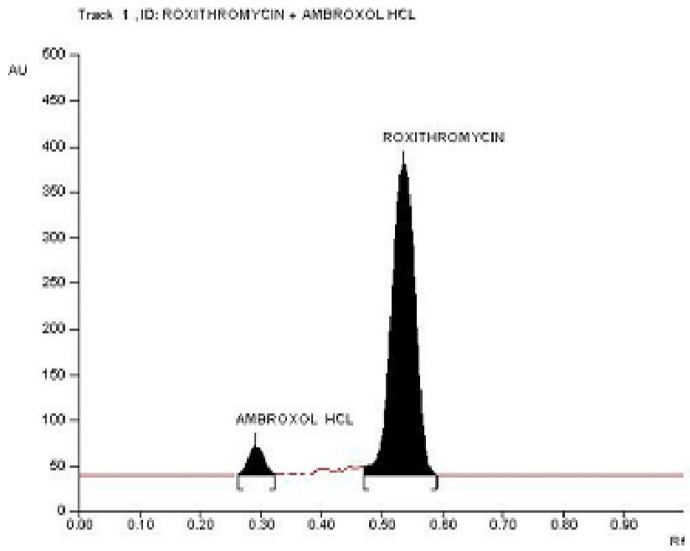


Fig. 2: HPTLC chromatogram for ambroxol hydrochloride and roxithromycin

3.2 Precision

The results of inter-day precision studies for Ambroxol hydrochloride and Roxithromycin for the different concentrations studied (Table 1). To ascertain the precision of the procedure experiments were carried out by two different analysts (Table 2).

Table 1: Results from Interday precision studies at three different concentrations.

Drug	Concentration (ng/spot)	Reference Standards % RSD (n=3)	Sample solutions %RSD (n=3)
Ambroxol hydrochloride	600	0.008	0.027
	900	0.094	0.025
	1200	0.093	0.013
Roxithromycin	1500	0.053	0.031
	2250	0.045	0.172
	3000	0.026	0.041

Table 2: The results of Precision studies by two different analysts.

Tablet sample solutions	(% C.V.)
Analyst I	100.8 (0.018)
Analyst II	100.9 (0.017)

3.3 Accuracy

Sample stock solution of tablet formulation of 600 ng mL^{-1} of Ambroxol hydrochloride and 1500 ng mL^{-1} of Roxithromycin was prepared. To this sample stock solutions 80%, 100% and 120% of the standard drug solutions were spiked and the percentage recoveries were found to be within the limits (Table 3).

3.4 Specificity

The peak purity of Roxithromycin was tested by correlating the spectra of Roxithromycin at the peak start (S), peak apex (A) and at the peak end (E) positions. Correlation between these spectra indicated purity of Roxithromycin peak {correlation r (S, A) = 0.999, r (A, E) =

0.998 (Fig. 3). The peak purity of Ambroxol hydrochloride was tested by correlating the spectra of Ambroxol hydrochloride at the peak start (S), peak apex (A) and at the peak end (E) positions. Correlation between these spectra's indicated purity of Ambroxol Hydrochloride peak {correlation r (S, A) = 0.993, r (A, E) = 0.994 (Fig. 4). Thus it can be concluded that the excipients did not interfere with the peaks of standard drug solutions. Typical absorption spectra of Ambroxol hydrochloride and Roxithromycin are shown in (Fig. 5).

Table 3: Results from recovery studies

Component	Label claim (mg/ tablet)	Initial amount (ng)	Amount added (%)	Amount recovered (ng)	% Recovered ±SD	%RSD
Ambroxol hydrochloride	60	600	0	595.62*	99.27	0.46 (0.447)
		600	80	1076.0*	99.63	0.12 (0.130)
		600	100	1187.89*	98.99	0.71 (0.714)
		600	120	1309.83*	99.23	0.54 (0.541)
Roxithromycin	150	1500	0	1497.0*	99.80	0.13 (0.142)
		1500	80	2691.36*	99.68	0.24 (0.25)
		1500	100	2992.80*	99.76	0.43 (0.452)
		1500	120	3290.76*	99.72	0.27 (0.205)

*: each reading is the mean of three replicate determinations.

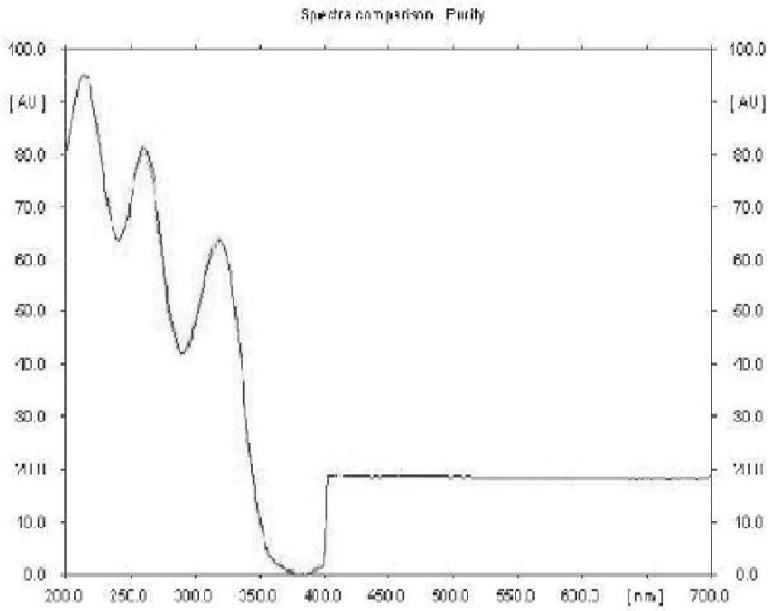


Fig. 3: Peak purity spectra of Roxithromycin

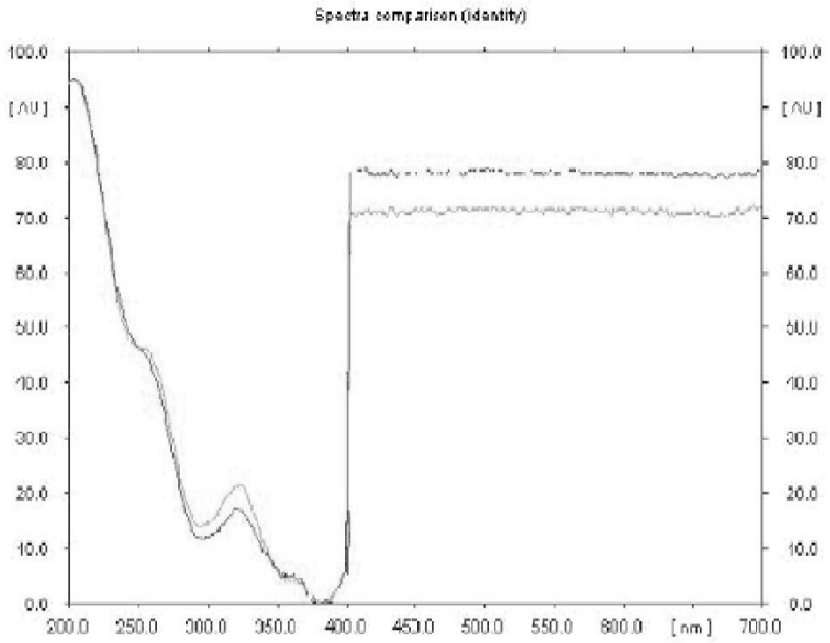


Fig. 4: Peak purity spectra of Ambroxol hydrochloride

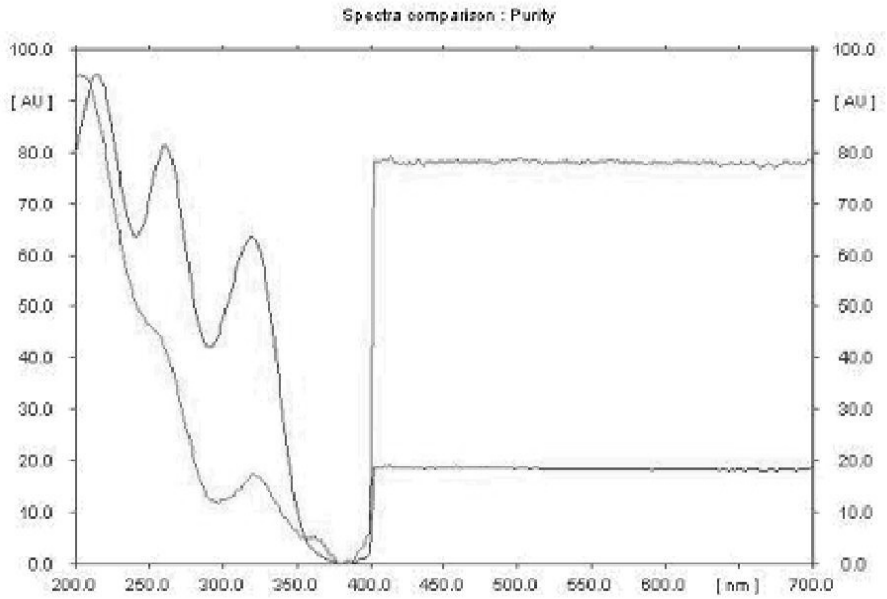


Fig. 5: Absorption spectra of Roxithromycin and ambroxol hydrochloride

3.5 Robustness

The results from robustness studies after changing the development distance for the plates (Table 4). The amount of drug being eluted remained unaffected, even after the change in migration distance. This suggests the robustness of the method after being subjected to varying chromatographic condition.

3.6 Stability studies

To test the stability of both the drugs on the HPTLC plates, each analyte was tested against freshly prepared solutions and the following results were obtained. No decomposition of the drugs was observed during chromatogram development. No decrease in the concentration of drugs on the plate was observed within 3 hours. Decrease in concentration of Roxithromycin and Ambroxol Hydrochloride was observed 3 hours after development. Therefore, chromatograms were scanned within 2 hours after development. The results of stability studies of the analyte in the solution are indicated in (Table 5).

Table 4: Results from Robustness studies

Development distance (cm)	Ambroxol Hydrochloride Assay (%) (600 mg)	Roxithromycin Assay (%) (1500 mg)
7.0	99.32	98.38
7.5	98.97	98.59
8.0	99.66	98.89

Table 5: Stability studies of drugs

Drug	% Drug Loss ±SD		
	after 3 Hours	after 24 Hours	after 48 Hours
Ambroxol hydrochloride	No Loss	2.38 ± 0.7	3.57± 0.47
Roxithromycin	No Loss	3.89 ± 0.4	5.82 ± 0.43

3.7 Analysis for the degradation product(s) and related substances:

The amount of drug degraded due varying forced degradation factors and the amount of drug loss is indicated in **(Table 6 and 7).** The degradation products formed in the studies were found in very minor detectable concentrations in Densitometric scanning.

Table 6: Analysis of Forced Degradation Studies of Ambroxol hydrochloride

Condition of exposure for Ambroxol hydrochloride	Number of degraded products with (R_f values)	Drug Recovered (%)	Drug Degraded (%)
Acid degradation	1 (0.71)	92.00	8.0
Base degradation	1 (0.79)	89.50	10.5
H_2O_2 degradation	1 (0.82)	87.90	12.10
UV degradation	2 (0.89, 0.90)	86.91	10.09, 3.0

Table 7: Analysis of Forced Degradation Studies of Roxithromycin

Condition of exposure for Roxithromycin	Number of degraded products with (R_f values)	Drug Recovered (%)	Drug Degraded (%)
Acid degradation	1 (0.75)	91.19	8.81
Base degradation	1 (0.81)	90.50	9.5
H_2O_2 degradation	1 (0.72)	88.90	11.10
UV degradation	2 (0.80, 0.95)	80.91	10.09, 9.0

3.8 Repeatability

Applications of three concentrations of 600 ng, 900 ng and 1200 ng concentration of Ambroxol hydrochloride and 1500 ng, 2000 ng and 2500 ng of Roxithromycin were done on HPTLC plates and ten replicates of each were determined (Table 8).

Stress testing has been accepted as an important part of the drug development process. Efforts by the ICH with context to impurities and stability have brought an increased regulatory examination of impurities that require identification and toxicological qualification at particular levels.

Table 8: Results of Repeatability Studies

Drug	Concentration (ng/spot)	Reference Standards % RSD (n=3)	Sample solutions %RSD (n=3)
Ambroxol hydrochloride	600	0.005	0.032
	900	0.098	0.035
	1200	0.092	0.013
Roxithromycin	1500	0.037	0.032
	2000	0.049	0.133
	2500	0.027	0.041

The chief mechanisms of chemical decomposition of active pharmaceutical ingredients include hydrolysis/dehydration, oxidation, isomerization/epimerization, rearrangements, decarboxylation, dimerization/polymerization, and photolysis and transformation products involving reaction with excipients/salt forms. Stressing the parent analyte under particular conditions can generate degradation products. The generated degradation products in the stressed samples can be termed as ‘‘potential’’ degradation products that may or may not be possibly formed under relevant storage conditions.

The stability assays play a vital role in the drug development process to facilitate: stability indicating method development, drug dosage form design, selection of storage conditions and packaging, better understanding of the potential liabilities of the drug molecule chemistry, and facilitates the development of stability indicating analytical methodology.

The results of the validation assays indicate that the proposed method can serve as a better chromatographic method as compared to the routine analysis methods. The method can be effectively used for determination of purity of the drugs and their related impurities. The method may also be extended to study the degradation products, and can thus be employed to indicate the stability of the drugs.

The developed method proves as a suitable and economic alternative chromatographic method for stability-indicating analysis and bulk drug and formulation analysis of Ambroxol hydrochloride and Roxithromycin. Results from the studies indicated in the present research paper will form an integral part the development of new pharmaceutical formulations of Ambroxol hydrochloride and Roxithromycin and its estimation in biological fluids.

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