Stability-Indicating HPTLC Method for Simultaneous Estimation of Amoxicillin Trihydrate and Ambroxol Hydrochloride in Bulk and Pharmaceutical Dosage Form

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Abstract

A rapid, precise, selective and sensitive HPTLC method for simultaneous determination of Amoxicillin trihydrate (AMOX) and Ambroxol hydrochloride (AMBRO) in pharmaceutical dosage form was developed and validated in the present work. The chromatographic separation was performed on precoated silica gel 60 F_{254} plates with Ethyl acetate: methanol: toluene: water: glacial acetic acid (6.0: 3.0: 2.0: 1.0: 0.5 v/v) as the mobile phase with UV detection at 237nm. Retention factor for AMOX and AMBRO were found to be 0.32 ± 0.04 and 0.70 ± 0.05 respectively. Linearity was observed in the concentration range of 2000-12000ng/band for AMOX and 500-2500 ng/b and for AMBRO and the coefficient of regression for both the drugs was 0.9986 and 0.995 respectively. The method was validated for precision, robustness, and recovery and the values obtained were within the ICH limits. The LOD and LOQ values for AMOX were 105 and 220 ng per band respectively and for AMBRO 50 and 120 ng per band respectively. Drugs were subjected to oxidation, acid hydrolysis, base hydrolysis and sun light to apply stress condition for degradation studies as per ICH guidelines. The degradation products were well resolved from the pure drug with significantly different Rf values. Since the method could effectively separate the drug from its degradation products, it could be used as a stability-indicating method for analysis of individual drugs and the combined dosage form.
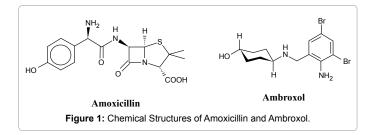
Introduction

Amoxicillin trihydrate (AMOX) is chemically a (2S, 5R,6R) [[(2R)-2 - amino-2(4- hydoxyphenyl) acetyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo [3.2.0]heptane-2-carboxyic acid, and it belongs to class of broad spectrum antibiotics. AMOX is official in IP, BP, Eur Pharmacopoeia and USP [1-4]. Literature survey reveals that AMOX has been determined by spectrophotometry [5-7], HPLC [8-11], HPLC with fluorimetric detection [12], HPLC with photo diode array detection [13], voltametry [14].

Ambroxol hydrochloride (AMBRO) [trans-4-(2-amino-3,5-dibromobenzylamino) cyclohexanol Hydrochloride] [15] is semi-synthetic derivative of vasicine obtained from Indian shrub *Adhatoda vasica*. It is a metabolic product of bromhexine. It is used as broncho secretolytic and expectorant drug. It stimulates the transportation of the viscous secretions in the respiratory organs and reduces the stand stillness of the secretions [16]. It is official in IP [1]. Few methods have been reported in the literature for the determination of AMBRO individually or in combination with other drugs [17-19].

Combination of AMOX and AMBRO is used for Respiratory system and as anti-allergic. The chemical structures of AMOX and AMBRO are shown in Figure 1.

Study has been reported on simultaneous determination of AMOX and AMBRO in human plasma by LC-MS/MS and HPLC method [19,20]. Literature survey reveals that there is no TLC densitometry method reported for the determination of these analytes in combination; therefore the aim of the present study was to develop rapid, accurate and reproducible TLC densitometry method for simultaneous estimation of AMOX and AMBRO from its formulation [21]. The attempts were made to establish the stability indicating, forced degradation methods [22-27]. Based on their results, we thought worthwhile to ascertain a new analytical method. To establish stability indicating nature of the TLC method, forced degradation of drug substances was performed under stress conditions (acid and base hydrolysis, oxidation). The proposed methods were validated as per the ICH guidelines [28,29].

Materials and Methods

Chemicals

Gift samples of AMOX and AMBRO were provided by Elder Pharmaceuticals Pvt. Ltd., Mumbai. Solvents were obtained from Qualigens laboratory, Mumbai. All chemicals and reagents used were of AR grade. All solutions were prepared daily.

Instrumentation and chromatographic conditions

Thin layer chromatography was performed on 10 cm×10 cm

Figure 1: Chemical Structures of Amoxicillin and Ambroxol.

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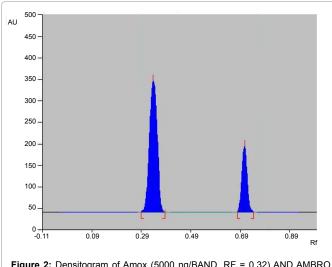
aluminum-backed TLC plates coated silica gel 60F$_{254}$ (E. Merck, Darmstadt, Germany; supplied by Merck India, Mumbai, India). The plates were prewashed by methanol and activated at 105-110°C for 15 min before use for chromatography. The samples in methanol were applied as bands 6 mm wide, 10 mm from the bottom and 20 mm from the sides of the plate, under a continuous flow of nitrogen, by means of a CAMAG Linomat-5 sample applicator fitted with a 100-µL syringe. A constant application rate of 150 nL s^{-1} was used. The plate was then placed in pre-saturated twin-trough chamber (CAMAG; 10×10 cm^2) containing the mobile phase, Ethyl acetate: methanol: toluene: water: glacial acetic acid (6.0: 3.0: 2.0: 1.0: 0.5 v/v) and ascending development was performed to a distance of 80 mm from the point of application at ambient temperature. After development, plates were air dried, observed under UV chamber and densitometric scanning was performed at 237 nm with a CAMAG TLC scanner III operated in the reflectance–absorbance mode and controlled by WinCATS software version 4. The slit dimensions were 5 mm×0.45 mm and the scanning speed was 20 mm s^{-1}. Evaluation was by linear regression of peak area against the amount of sample per band.
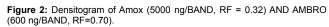
Preparation of standard solutions

Standard stock solution of AMOX and AMBRO were prepared by dissolving quantity of 5.0 mg of AMOX and 5.0 mg of AMBRO separately in methanol in separate 10.0 ml volumetric flask and final volume of both solutions were made up to mark with methanol to get a stock solution of 500 µg/ml.
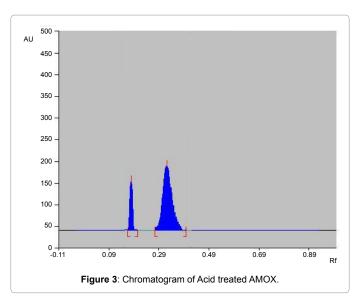
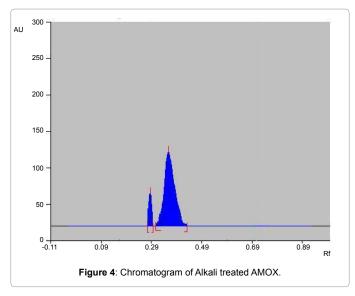## Preparation of the sample solutions

Twenty tablets were weighed and finely powdered; an accurately weighed powder equivalent to one tablet containing 3 mg of AMBRO and 25 mg of AMOX was taken and dissolved in the mobile phase and sonicated for 20 min. and then volume was made up to the mark with mobile phase. The resulting solution was mixed and filtered through Whatmann filter paper No. 41. From the filtrate sample solution were applied to the TLC plate to give spot concentration 600 ng/band of AMBRO and the 5000 ng / band of AMOX. The plate was developed in the previously described chromatographic conditions. The peak areas of the spots were measured at 237 nm and concentrations in the samples were determined using multilevel calibration.

Figure 2: Densitogram of Amox (5000 ng/BAND, RF = 0.32) AND AMBRO (600 ng/BAND, RF=0.70).

Figure 3: Chromatogram of Acid treated AMOX.

Figure 4: Chromatogram of Alkali treated AMOX.

Method validation

The method was validated in compliance with ICH guidelines [23].

Procedure for forced degradation study

For forced degradation studies, samples of AMOX and AMBRO were prepared separately under separate degraded conditions to maintain the % degradation between 5-20% as per ICH guidelines [22,23].

To study the acid degradation of AMOX, 1ml of 0.02N HCl was added to 5mg of Amox and made upto mark with methanol. It was then stirred continuously (with magnetic stirrer) for 15 minutes. For alkali and hydrogen peroxide degradation of AMOX, 1 ml of 0.1N NaOH and 1 ml of 3%v/v of H$_2$O$_2$ were added to separate volumetric flasks containing 5 mg of AMOX and made up to mark with methanol. These solutions were allowed to stand for 6 hours at room temperature. For dry heat degradation of AMOX, the sample was exposed to sunlight for 2 hrs.

For the degradation studies of Ambroxol, 1 ml each of 0.1N HCl,

0.1N NaOH and 3% v/v hydrogen peroxide were added to 5 mg of the AMBRO and made up to mark with methanol. These solutions were refluxed for two hours at 80°C. For dry heat degradation of AMBRO; sample was exposed to sunlight for 12 hours.

Results and Discussion

Optimization of procedures

Different proportions of ethyl acetate, methanol, toluene and glacial acetic acid were tried while selecting the mobile phase. The addition of 1ml water to above combination improved the peak shape. Ultimately Ethyl acetate: methanol: toluene: water: glacial acetic acid (6.0: 3.0: 2.0: 1.0: 0.5 v/v) was finalized as mobile phase. The spots developed were dense, compact and typical peak of AMOX and AMBRO obtained was shown in Figure 2. The peak was symmetrical in nature and no tailing was observed when plates were scanned at 237 nm.

Linearity

Calibration curves were constructed in the concentration range of 2000-12000 ng/band for AMOX and 500-2500 ng/band for AMBRO. The beer's law is obeyed over this concentration range, and the coefficient of regression for both the drugs was 0.998 and 0.995 respectively.

Analysis of formulation

The spots at R_f 0.32 and 0.70 for AMOX and AMBRO were

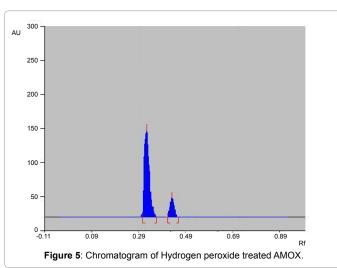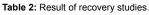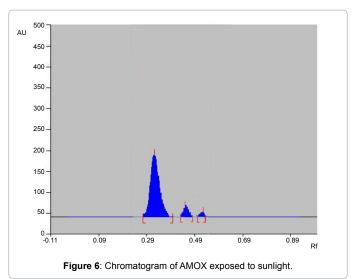| Label Claim (mg/tab)		Amount Found(mg/tab)*		%Amount Found*± S.D	
AMOX	AMBRO	AMOX	AMBRO	AMOX	AMBRO
25	3	25.12	2.99	100.48 ± 0.122	99.66 ± 0.512

*Mean of six estimations
S.D = Standard deviation

Table 1: Result of marketed formulation analysis.

Level of Recovery	Drug	% Recovery*	(±)Standard deviation*	%RSD*
80	AMOX	100.33	0.6923	0.6904
	AMBRO	99.66	0.2412	0.2398
100	AMOX	101.31	0.7311	0.7289
	AMBRO	101.26	0.4554	0.4362
120	AMOX	100.35	0.8129	0.8102
	AMBRO	98.82	0.3952	0.3911

Table 2: Result of recovery studies.

Figure 5: Chromatogram of Hydrogen peroxide treated AMOX.

Figure 6: Chromatogram of AMOX exposed to sunlight.

Figure 7: Chromatogram of Acid treated AMBRO.

Figure 8: Chromatogram of Alkali treated AMBRO.

observed respectively in the densitogram of the drug samples extracted from tablets. There was no interference from the excipients commonly present in the tablets. The AMOX and AMBRO content was found to be close to 100% and the results are summarized in Table 1. The low %RSD value indicated the suitability of this method for routine analysis.

Precision

Precision was evaluated by carrying out six independent sample preparation of a single lot of formulation.

Percentage relative standard deviation (%RSD) was found to be less than 2% for inter day and intraday variety of the both methods.

Recovery studies

To check the degree of accuracy of the method, recovery studies were performed in triplicate by the standard addition method at 80%, 100% and 120%. Known amounts of standard AMOX and AMBRO were added to the pre-analyzed samples and were subjected to the proposed method. Results of recovery studies for the both methods are shown in Table 2.

Robustness

The robustness of the TLC densitometry method were determined by variations in methanol composition (± 2%), chamber saturation period (± 10%), development distance (± 10%), the time from application to develop (0, 10, 20, 30 min), time from development to scanning (0, 10, 20, 30 min). One factor at a time was changed at a

concentration level of 600 ng/band and 5000 ng/band for AMBRO and AMOX respectively, to study the effect on the R_f and peak area of the drugs. The method was found to be unaffected by small changes with % RSD for all the parameters less than 2% indicating that the method is robust.
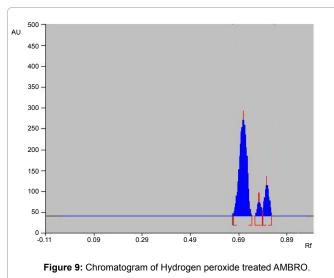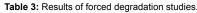
Stability-indicating property [22]

The stability study of AMOX and AMBRO under different stress conditions was carried out. AMOX underwent degradation faster than AMBRO owing to its susceptible beta lactum ring. AMBRO on the other hand was more stable and required harsher conditions to undergo degradation. The main peaks of both the pure drugs were well resolved from their degraded products. This shows the advantage of our mobile phase to effectively separate the peak of the pure drug from its degradation product. The amount of drug recovered after degradation studies and the Rf of degradation products are given in Table 3. The peaks obtained after degradation under various conditions have been shown in the figures 3-10.
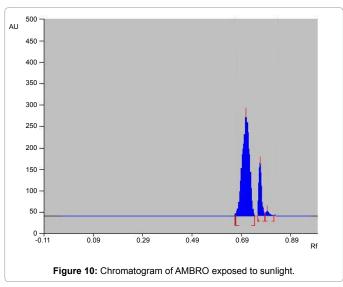
Conclusion

The proposed TLC densitometry method was validated as per ICH guidelines. The standard deviation, %RSD and standard error calculated for the method are low, indicating a high degree of precision of the method. The results of the recovery studies performed show a high degree of accuracy of the proposed method. The results of the stress studies indicated the specificity of the method. The method gives well-resolved peaks of AMOX and AMBRO even after exposure to different stress conditions when analyzed individually. The method

Stress conditions	AMOX % Assay of active substance	Rf values of degraded	Stress conditions	AMBRO % Assay of active substance	Rf values of degraded products
Acid hydrolysis (0.02N HCl 15 mnts stirring)	86.31	0.17	**Acid hydrolysis** (0.1N HCl, 2 hrs reflux at 80°C)	93.52	0.74
Base hydrolysis (0.1N NaOH 6 hrs at RT)	91.01	0.28	**Base hydrolysis** (0.1N NaOH, 2 hrs reflux at 80°C)	89.33	0.72,0.83
Oxidation (3% H_2O_2, 6 hrs at RT)	80.25	0.42	**Oxidation** (3% H_2O_2, 2 hrs reflux at 80°C)	83.21	0.77,0.81
Exposed to Sunlight (2 hrs)	84.21	0.46,0.52	**Exposed to Sunlight** (12 hrs)	81.46	0.79,0.81

Table 3: Results of forced degradation studies.

Figure 9: Chromatogram of Hydrogen peroxide treated AMBRO.

Figure 10: Chromatogram of AMBRO exposed to sunlight.

can be used to determine the purity of the drugs available from various sources by detecting the related impurities.

Statistical tests indicate that the proposed HPTLC and HPLC methods reduce the duration of analysis and appear to be equally suitable for routine determination of AMOX and AMBRO when compared with the reported methods. As the method is stability indicating one it may be extended to study the degradation kinetics of AMOX and AMBRO in combination. Hence, it can be concluded that the developed TLC-densitometry method is accurate, precise, and selective and can be employed successfully in the estimation of AMOX and AMBRO in bulk and in pharmaceutical formulation.

Acknowledgement

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