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Research Article

DEVELOPMENT AND VALIDATION OF HPTLC METHOD FOR SIMULTANEOUS DETERMINATION OF AMBROXOL HYDROCHLORIDE AND DOXOFYLLINE IN BULK AND IN PHARMACEUTICAL DOSAGE FORMS

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ABSTRACT

A simple, accurate and precise densitometric method for the simultaneous estimation of Ambroxol Hydrochloride and Doxofylline in Bulk and Pharmaceutical Dosage forms has been developed and validated. Separation of drugs was carried out using Ethyl acetate: Methanol: Glacial acetic acid (9: 1: 0.05 v/v/v) as mobile phase on precoated Silica Gel 60F254 plates. The densitometric evaluation of spots was carried out at 251 nm. The Rf value for Ambroxol Hydrochloride and Doxofylline were found to be 0.27 ± 0.02 and 0.68 ± 0.02, respectively. The method was validated with respect to linearity, accuracy, precision and robustness as per the International Conference on Harmonisation (ICH) guidelines. The drug response with respect to peak area was linear over the concentration range 120-420 ng/spot (n=6) and 1600-5600 ng/spot(n=6) for Ambroxol Hydrochloride and Doxofylline respectively. The limit of detection and limit of quatitation were found to be 8.95 ng/spot and 27.15 ng/spot for Ambroxol Hydrochloride and 78.91ng/spot and 239.14 ng/spot for Doxofylline. The percentage recovery of Ambroxol Hydrochloride and Doxofylline was found to be 100.062 and 99.692 respectively. The %R.S.D. values for intra-day precision study and inter-day study were <1.0%, confirming that the method was sufficiently precise. The method can be successfully employed for the simultaneous determination of Ambroxol hydrochloride and Doxofylline in pharmaceutical formulations.

Keywords: Ambroxol hydrochloride, Doxofylline, HPTLC, simultaneous determination, validation.

INTRODUCTION

Ambroxol hydrochloride (AMB) is chemically Trans-4- [(2-amino-3, 5-dibromobenzyl)amino] cyclohexanol HCL (Fig. 1) is a Mucolytic drug [1-7]. It is official in Indian Pharmacopoeia (IP), British Pharmacopoeia(BP) and European Pharmacopoeia(EP). It is estimated by potentiometric titration method as per IP, BP & EP. [8-10]. Literature review reveals that HPLC [12, 13, 14, 24, 25], UV [12] spectrophotometric and HPTLC [15] methods has been reported for estimation of AMB in pharmaceutical dosage forms. Doxofylline (DOX) is chemically **7-(1, 3-Dioxolan-2-yl-methyl)-3, 7-dihydro-1, 3-dimethyl-1H-purine-2, 6-dione** (Fig.2) used as Bronchodilator by inhibiting Phosphodiesterase-4 enzyme. [1-7] DOX and its tablet dosage form is only official in IP and estimated by Liquid Chromatographic method [8]. Literature review also reveals that HPLC [16, 18-21], UV [22] spectrophotometric and HPTLC [16, 17] methods has been reported for the estimation of DOX in pharmaceutical dosage forms. Literature survey does reveals only UV Spectrophotometric [23] methods have been developed and reported, But does not any HPTLC method for simultaneous determination of AMB and DOX in Pharmaceutical dosage form. The present developed method is simple, precise and accurate for simultaneous determination of both drugs in their Pharmaceutical Dosage forms as per International Conference on Harmonization (ICH) guidelines [11].

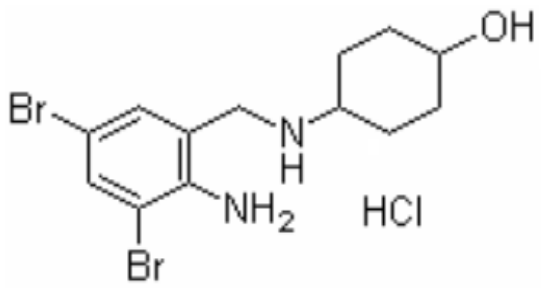

Fig. 1: Structure of Ambroxol hydrochloride (AMB)

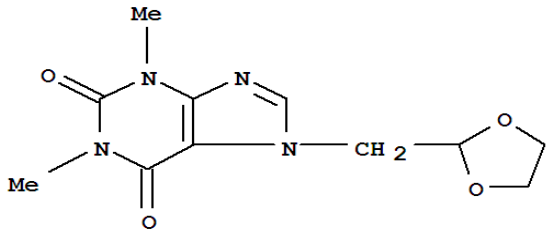

Fig. 2: Structure of Doxofylline (DOX)

MATERIALS AND METHODS

Chemicals and reagents

Pure drug samples of Ambroxol hydrochloride & Doxofylline were provided as a gift sample by Ami Life Science, Mumbai, Maharashtra, India. Commercial pharmaceutical tablets **Synasma-AX** (Ranbaxy Laboratories, India) was procured from local pharmacy. Methanol, Ethyl acetate and Glacial acetic acid of AR Grade and all other chemicals were obtained from Allied Chemical Corporation, Vadodara, Gujarat, India.

Instrumentation and chromatographic conditions

The HPTLC system (Camag, Muttenz, Switzerland) consisted of Linomat V autosprayer connected to a nitrogen cylinder, a twin trough chamber (10 × 10 cm), a derivatization chamber, and a plate heater. Pre-coated silica gel 60 F254 TLC plates (10 × 10 cm, layer thickness 0.2 mm (E. MerckKGaA, Darmstadt, Germany) was used as stationary phase. TLC plates were pre-washed with methanol and activated at 80^{0}C for 5 min prior to sample application. The standard and formulation samples of AMB and DOX in mixture were spotted on Pre-coated TLC plates in the form of narrow bands of lengths 6 mm. Samples were applied under continuous drying stream of nitrogen gas at constant application rate of 150 nL/s. The mobile phase consists of Ethyl acetate: Methanol: Glacial acetic acid (9:1:0.05 v/v/v). Linear ascending development was carried out in twin trough chamber (10 x 10 cm). The optimized chamber saturation time for mobile phase was 30 min, at ambient

temperature, the length of chromatogram run was 7 cm. Densitometric scanning was performed on CAMAG TLC scanner 3 in Absorbance/Reflectance mode, operated by winCATS 1.3.4 planar chromatography software. The spots were analyzed at a wavelength of 251 nm. The slit dimensions used in the analysis were length and width of 5 mm and 0.45 mm, respectively, with a scanning rate of 20 mm/s. The parameters were selected as recommended by the CAMAG TLC scanner 3 manual. Evaluation was performed using linear regression analysis of peak areas.

Preparation of standard stock solutions and calibration curves

Accurately weighed Ambroxol Hydrochloride (10 mg) was transferred to 10 ml volumetric flask, dissolved in and diluted with methanol up to the mark (1000 µg/ml). This solution was further diluted with methanol to obtain final concentration of AMB 100 µg/ml. For preparation of DOX stock solution, accurately weighed Doxofylline(10 mg) was transferred to 10 ml volumetric flask, dissolved in and diluted with methanol up to the mark (1000 µg/ml). For preparation of working standard solution, 3 ml of stock solution of AMB(100 µg/ml) and 4 ml of stock solution of DOX(1000µg/ml) were transferred to 10 ml volumetric flask and diluted with methanol upto the mark to obtain final concentration containing 30µg/ml of AMB and 400µg/ml of DOX. Calibration was done by applying mixture of standard solutions ranging from 4.0 – 14.0 µl by Hamilton syringe with the help of Linomat V autosprayer on TLC plate that gave concentration 120-420 ng/spot for AMB and 1600-5600 ng/spot for DOX, respectively. Each concentration was spotted six times on TLC plates. From the developed plates calibration curve was plotted as peak areas versus corresponding concentrations (Fig. 5 and 6).

Analysis of AMB and DOX in marketed Tablet Formulation

To determine the content of AMB and DOX simultaneously in conventional tablets (Synasma-AX label claim 30 mg AMB and 400 mg DOX); twenty tablets were accurately weighed, average weight determined and ground to fine powder. A quantity of powder equivalent to 30 mg (AMB) and 400 mg (DOX) was transferred into 100 ml volumetric flask containing 50 ml methanol, sonicated for 30 min and diluted to mark with same solvent to obtain 0.3 mg/ml of AMB and 4 mg/ml of DOX. The resulting solution was filtered using 0.45 µm filter (Millifilter, MA). From the above solution 1ml was transferred into 10 ml volumetric flask and diluted to mark with same solvent. So, Resultant solution was found to contain 30µg/ml (30 ng/µl)Ambroxol Hydrochloride and 400µg/ml(400 ng/µl)Doxofylline. 6µl of this solution applied on TLC plate followed by development and scanning at 251 nm. The analysis was repeated for three times.

Method Validation

Linearity

For the linearity study the 4-14 µl from the working standard solution containing 30 ng/spot of AMB and 400 ng/spot of DOX was injected. So, linearity responses for AMB and DOX were assessed in the concentration range 120-420 ng/spot and 1600-5600ng/spot, of working standard solutions, respectively.

Precision

Precision of the method was determined in the terms of intra-day and inter-day variation (%RSD). Intra-day precision (%RSD) was assessed by analyzing standard drug solutions within the calibration range, three times on the same day. Inter-day precision (%RSD) was assessed by analyzing drug solutions within the calibration range on three different days over a period of 7 days.

Accuracy

To the pre-analyzed sample a known amount of standard solution of pure drug (AMB and DOX) was spiked at three different levels(80%, 100% and 120%). These solutions were subjected to re-analysis by the proposed method.

Sensitivity

The sensitivity of measurement of AMB and DOX by the use of proposed method was estimated in terms of Limit of Detection (LOD) and Limit of Quantitation (LOQ). The LOD and LOQ were calculated by equation. Based on the standard deviation of the response and the slope, LOD and LOQ were estimated using the formulae:

$LOD = 3.3\ \sigma/S$

Where, σ = the standard deviation of the response

S = the slope of the calibration curve

$LOQ = 10\ \sigma/S$

Where, σ = the standard deviation of the response

S = the slope of the calibration curve

LOD and LOQ were determined from the standard deviations of the responses for six replicate determinations.

Specificity

Specificity of the method was ascertained by analyzing standard drug and sample. The mobile phase resolved both the drugs very efficiently as shown in Fig. 7. The spot for AMB and DOX was confirmed by comparing the R_f and spectra of the spot with that of standard. The wavelength 251 nm for detecting peak purity of AMB and DOX was assessed by comparing the spectra at three different levels, i.e., peak start (S), peak apex (M) and peak end (E) positions of the spot.

Repeatability

Repeatability of sample application was assessed by spotting 10µL (300 ng/spot of AMB and 4000 ng/spot of DOX) of drug solution six times on a TLC, followed by development of plate and recording the peak area for six spots.

RESULTS AND DISCUSSION

Method development

The TLC procedure was optimized for simultaneous determination of AMB and DOX. The mobile phase Ethyl acetate: Methanol: Glacial acetic acid (9:1:0.05v/v/v) resulted in good resolution and sharp and symmetrical peaks of R_f 0.27 ± 0.02 for AMB and 0.68 ± 0.02 for DOX. It was observed that pre-washing of TLC plates with methanol (followed by drying and activation) and pre-saturation of TLC chamber with mobile phase for 30 min (optimum chamber saturation time) ensured good reproducibility and peak shape of both the drugs. (Fig. 3)

Validation

Linearity

Linear regression data for the calibration plots revealed good linear relationships between area and concentration over the ranges 120-420 ng/spot for AMB and 1600-5600 ng/spot for DOX. The linear equations for the calibration plots were $y = 3.3888x + 37.462$ and $y = 2.7044x + 1625.4$ with Regression(r^2) being 0.9972 and 0.9954 for AMB and DOX, respectively (Fig. 4, 5, 6) (Table 1, 2 and 3).

Precision

The precision of the method was expressed as relative standard deviation (RSD %). The %R.S.D. values for intra-day precision study and inter-day study listed in (Table 4 and 5) were <1.0%, confirming that the method was sufficiently precise.

Accuracy

When the method was used for accuracy and subsequent analysis of both the drugs from the pharmaceutical dosage form, and spiked with 80, 100, and 120% of additional pure drug, the recovery was found to be99.86- 100.34% for AMB and 99.31- 100.31% for DOX (Table 6 and 7).

Sensitivity

The LOD and LOQ were calculated by equation. The LOD and LOQ values were 8.95 and 27.15 ng/spot for AMB and and 78.91 and 239.14 ng/spot for DOX.

Repeatability

The % RSD for peak area values of AMB and DOX were found to be 0.8548and 0.9987 respectively, as given in Table 8.

Specificity

The peak purity of AMB and DOX were assessed by comparing their respective spectra at peak start, apex and peak end positions of the spot i.e., r (S, M) = 0.9994 and r (M, E) =0.9990 for AMB and r (S, M) = 0.9986 and r (M, E) = 0.9996 for DOX. Good match was obtained between standard and sample spectra of AMB and DOX respectively. (Fig. 7)

Analysis of AMB and DOX in marketed formulation

When the Synasma-AX tablets were analyzed, AMB and DOX gave sharp and well defined peaks at R_f 0.27±0.02 and 0.68±0.02, respectively, when scanned at 251 nm. The results in Table 9 indicate that there was no interference from the excipients commonly present in the tablet formulation. The % purity was 99.16% for AMB and 99.56% for DOX.

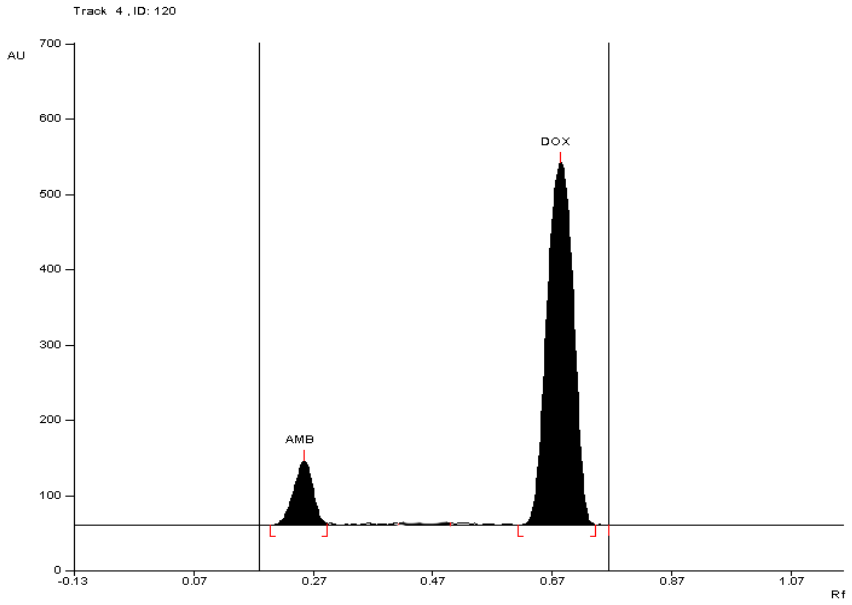

Fig. 3: HPTLC Chromatogram of Standard AMB and DOX in mixture

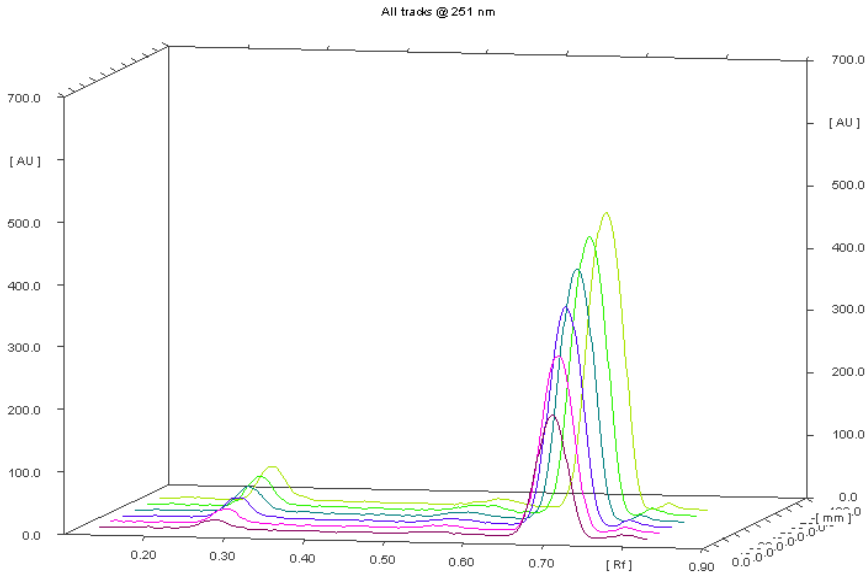

Fig. 4: 3D Representation of Densitogram for Calibration curve of AMB and DOX

Table 1: Result of Calibration readings for AMB

Concentration (ng/spot)	R_f	Area Mean (n=6) ± SD	%RSD
120	0.26	424.450.± 5.745	1.3536
180	0.27	643.666± 5.968	0.9272
240	0.27	879.610± 8.364	0.9509
300	0.27	1075.150± 9.190	0.8548
360	0.28	1242.333± 12.602	1.015
420	0.28	1449.455± 10.501	0.7248

Table 2: Result of Calibration readings for DOX

Concentration (ng/spot)	R_f	Area Mean (n=6) ± SD	%RSD
1600	0.66	5596.21±44.61	0.7972
2400	0.67	8315.32±84.51	1.0164
3200	0.67	10567.66±76.67	0.7255
4000	0.67	12650.53±131.78	1.0417
4800	0.68	14310.71±115.21	0.8051
5600	0.68	16726.95±116.91	0.699

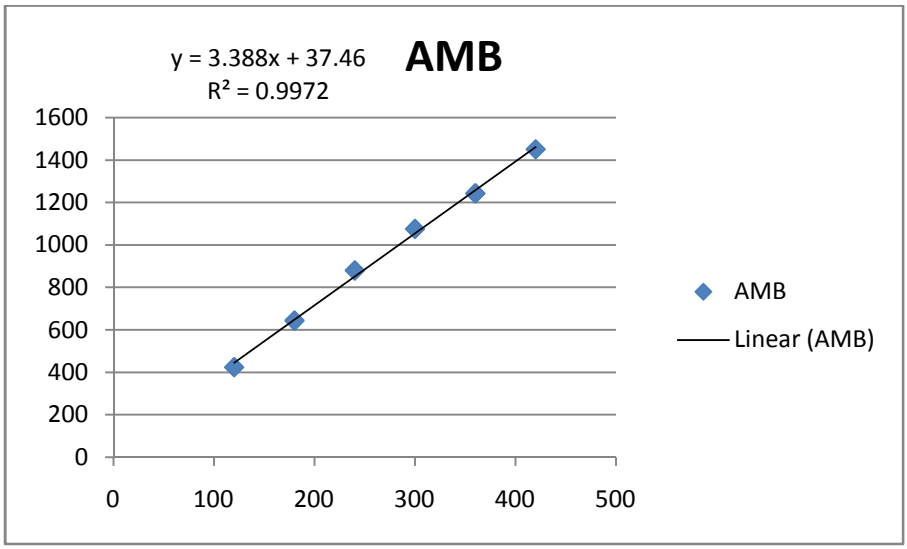

Fig. 5: Calibration curve of AMB in Methanol at 251 nm

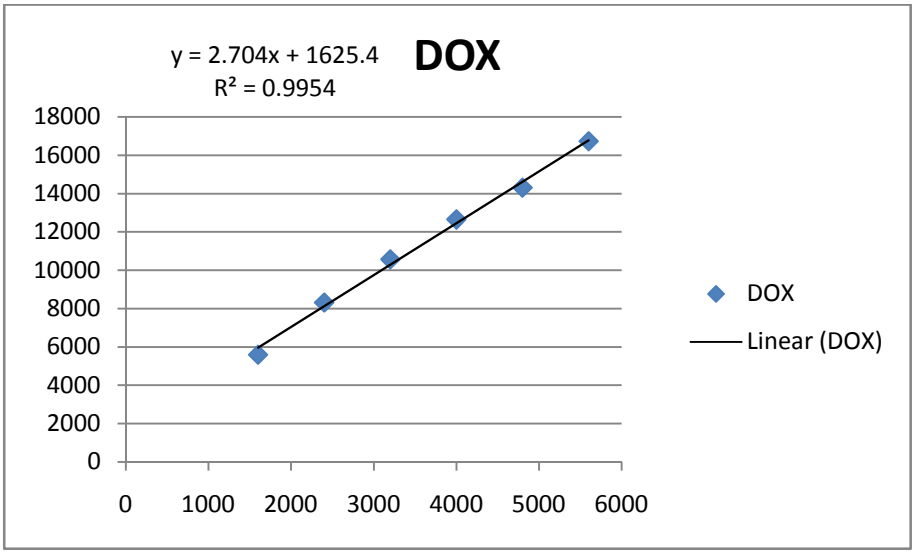

Fig. 6: Calibration curve of DOX in Methanol at 251 nm

Table 3: Statistical Data of AMB and DOX

Parameters	Results	
	AMB	DOX
Linear Range(ng/spot)	120-420	1600-5600
Slope	3.3886	2.7044
Intercept	37.4616	1625.41
Std. Deviation of Slope	0.04560	0.02439
Std. Deviation of Intercept	9.20	64.67
Limit of Detection(ng/spot)	8.95	78.91
Limit of Quantitication(ng/spot)	27.15	239.14
Regression Equation	y = 3.3888x + 37.462	y = 2.7044x + 1625.4
Co-Relation Co-Efficient (r)	0.9985	0.9976
Co-Efficient of Determination (r^2)	0.9972	0.9954

Table 4: Intra-Day and Inter-Day study of AMB

Concentration (ng/spot)	Intra-Day Area Mean (n=3) ± SD	%RSD	Inter-Day Area Mean (n=3) ± SD	%RSD
180	648.66± 5.75	0.8839	644± 5.53	0.8593
240	873.23± 7.28	0.8346	874.9± 8.16	0.9339
300	1070.93± 10.83	0.9980	1075.66± 6.57	0.6114

Table 5: Intra-Day and Inter-Day study of DOX

Concentration (ng/spot)	Intra-Day Area Mean (n=3) ± SD	%RSD	Inter-Day Area Mean (n=3) ± SD	%RSD
2400	8039.52± 53.08	0.6381	8297.40± 84.47	0.9981
3200	10570± 55.62	0.5263	10568.2± 78.121	0.7382
4000	12693.07± 73.26	0.5772	12676.27± 86.99	0.6833

Table 6: Determination of Accuracy for AMB

Concentration of Sample Taken (ng/spot)	Concentration of Pure API spiked (ng/spot)	Total Concentration (ng/spot)	Mean Total Concentration Found (n=3) (ng/spot)	%Recovery Mean (n=3)	%RSD
180	144	324	323.94	99.98	0.6721
	180	360	361.25	100.3472	0.5721
	216	396	395.45	99.86	1.05
Average				100.06	

Table 7: Determination of Accuracy for DOX

Concentration of Sample Taken (ng/spot)	Concentration of Pure API spiked (ng/spot)	Total Concentration (ng/spot)	Mean Total Concentration Found (n=3) (ng/spot)	%Recovery Mean (n=3)	%RSD
2400	1920	4320	4290.5	99.3172	0.384
	2400	4800	4815.2	100.31	0.245
	2880	5280	5250.8	99.44	0.634
Average				99.692	

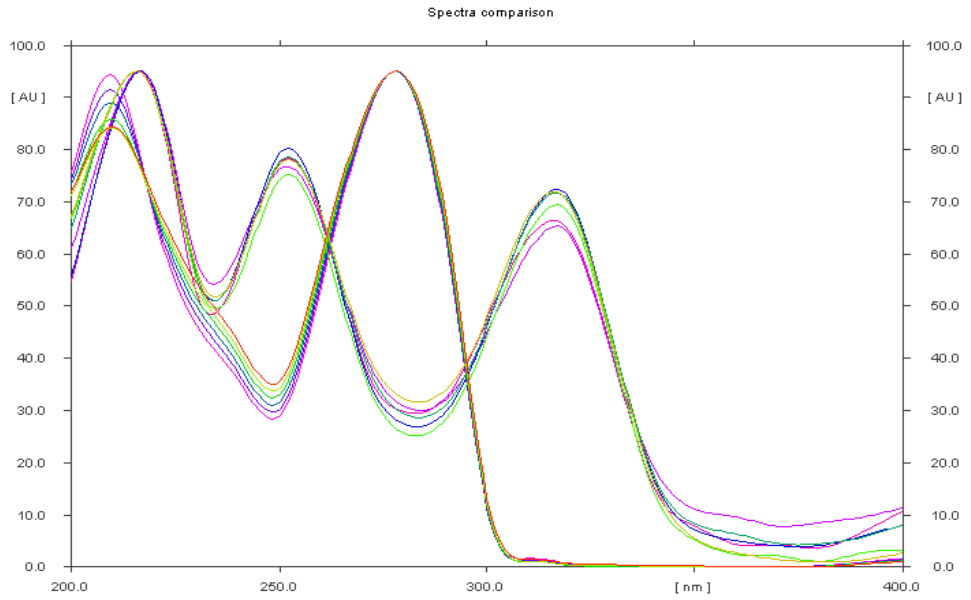

Fig. 7: UV Absorption (Reflectance Mode) of the corresponding spots for AMB and DOX

Table 8: Repeatability study of AMB and DOX

Concentration	AMB (300ng/spot)	DOX4000 (ng/spot)
Area	1065.2	12580.8.8
	1071.3	12500.3
	1075.4	12525.1
	1090.4	12780.8
	1068.2	12715.9
	1080.4	12800.3
Mean	1075.15	12650.53
± SD	9.1903	131.7866
%RSD	0.8548	0.9987

Table 9: Assay Result of Marketed Formulation

Parameters	Synasma-AX TAB	
	AMB	DOX
Actual Concentration (ng/spot)	30	400
Concentration Obtained (ng/spot)	29.70	398.25
%Purity	99.16	99.56
%RSD	0.669	0.518
Limit [3, 7]	Not less than 98.5%	98.5% -101.0%

Summary of Validation

Table 10: Validation Parameters

Parameters		
	AMB	DOX
Recovery (%)	100.062	99.692
Repeatability (%RSD)	0.8548	1.0417
Precision (CV)		
Intra-day (n=3)	0.0091	0.005805
Inter-day (n=3)	0.008013	0.008145
Specificity	Specific	Specific
Selectivity	Selective	Selective

CONCLUSION

The developed HPTLC method is simple, precise, accurate and reproducible and can be used for simultaneous determination of AMB and DOX in pharmaceutical dosage forms. The method was validated as per International Conference on Harmonization (ICH) guidelines.

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