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Analytical HPTLC-Densitometry Method for the Development and Validation of Citicoline Sodium and Methylcobalamin in Combined Dosage Form.

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ABSTRACT

A simple, precise, rapid, selective, and economic high-performance thin layer chromatography (HPTLC) method has been established for simultaneous analysis of Citicoline Sodium and Methylcobalamin. HPTLC method was developed using on precoated silica gel F254 G60 plates as stationary phase, using methanol: acetonitrile: water: triethylamine (8.5:1.5:1:0.5 v/v/v) as mobile phase. The plates were scanned at approximately 254 nm for both Citicoline sodium and Methylcobalamin respectively. In HPTLC method both the drugs were resolved using proposed mobile phase and Rf value was found to be 0.39 for Citicoline sodium and Rf 0.61 for Methylcobalamin. The method was found to linear in the range 1000-6000 ng/band for citicoline sodium and methylcobalamin respectively. This HPTLC procedure is economic, sensitive, and less time consuming than other chromatographic procedures. It is important tool for analysis of combined dosage form. Proposed method can be successfully applied for the quantitative determination of Citicoline Sodium and Methylcobalamin in Bulk drug and Pharmaceutical dosage form.

Keywords: Citicoline (CITI), Methylcobalamin(MCA), HPTLC, Method Development, Validation.

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INTRODUCTION

Citicoline is an intermediate in the generation of phosphatidyl choline from choline. It ischemically 5'-O [hydroxyl ({hydroxyl [2(trimethylammonio) ethoxy] phosphoryl}oxy) phosphoryl] cytidine(figure 1). Citicoline is a white or off white amorphous, hygroscopic powder having molecular weight 488.3g/mol1. It helps to improve focus and mental energy and may possibly be useful in the treatment of attention deficit disorder [1]. Methylcobalamin (MCA) is a coenzyme of Vitamin B12 which is biologically active[2](figure. 2). The importance of the combination of citicoline and methylcobalmine known to health care system to boost memory, production of brain energy, sustain cognitive function and motor skill and enhance focus and decision making thought processes. The major indication of the combination is diabetic neuropathy, diabetic gastropathy, memory loss, brain injury, anorexia, nerve rejuvenation, nerve regeneration and alertness[3].

Several method were cited in literature for determination of CITI and MCA individually or in combination with another drug by UV-Vis spectroscopy, HPLC, LC-MS and HPTLC but for combination only UV Spectrophotometric method was reported. Hence there is need to develop a simple, economic, precise and accurate high performance thin layer chromatographic method for Citicoline Sodium in the presence of methylcobalamin in tablet dosage form.

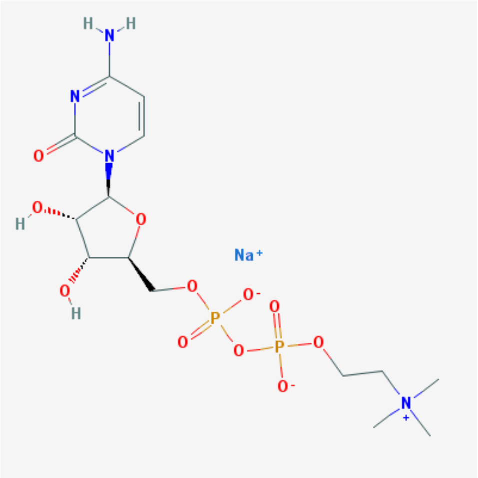

Figure 1: Structure of citicoline sodium

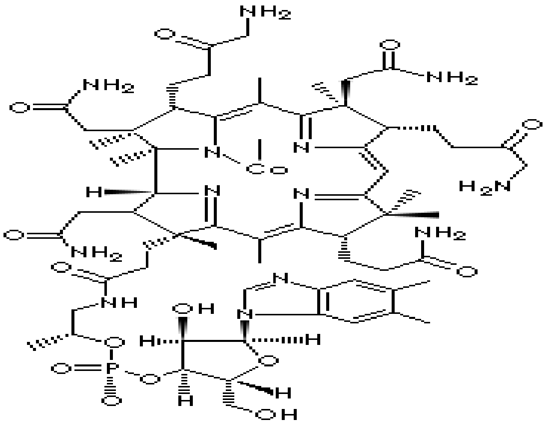

Figure 2: Structure of Methylcobalamin

MATERIALS AND METHODS

Instrument:

High performance Thin Layer liquid chromatography (CamagLinomat 5) with Semiautomatic application, band application (2 - 500µl) and Camag twin trough glass chamber (10 cm x 10 cm) were used for all measurements. Electronic balance (Shimadzu AX-200, Japan was used for weighing the samples**.** Double distilled water and Whatmann filter paper (no.41) were used throughout the experimental work.

Materials:

Multicomponent tablet Storax plus (500mg CITI and 0.75mg MCA) manufactured by Intas Pharmaceutical Ltd. All chemicals and reagents used were of AR grade.

Reagents:

All the chemicals used were of AR grade.

METHODS

Preparation of Mobile Phase:

Mobile phase was prepared by mixing methanol: acetonitrile: water: Triethylamine (8.5: 1.5: 1: 0.5, v/v/v/v) in a mobile phase chamber. Mobile phase tank was saturated with mobile phase for 20 minute.

Preparation of Standard Stock Solutions:

CITI(10 mg) and MCA(10 mg) were weighed and transferred to 10 ml volumetric flasks and dissolved in few ml of methanol. Volumes were made up to the mark with methanol to yield a solution containing 1000 µg/ml of CITI and 1000 µg/ml MCA, respectively.

Selection of Analytical wavelength

The sensitivity of HPLC method that uses UV detection depends upon proper selection of detection wavelength. An ideal wavelength is the one that gives good response for the drugs that are to be detected. Overlay UV spectra of both the drugs showed that CITI and MCA absorbed appreciably at 254 nm, so detection was carried out at 254 nm (Figure. 3).

Optimization of Mobile Phase

To develop the HPTLC method for estimation of CITI and MCA in combined dosage form, selection of the mobile phase was carried out on the basis of polarity. A mobile phase that would give a dense and compact band with an appropriate Rf value for CITI and MCA was desired. Various mobile phases such as methanol, hexane, methanol–ethyl acetate, methanol- toluene, methanol- acetone, Methanol- acetonitrile and methanol- acetonitrile and water were evaluated in different proportions but mobile phase consisting of methanol: acetonitrile: water: triethylamine (8.5: 1.5: 1: 0.5, v/v/v/v) gave good separation of CITI and MCA from its matrix. So, methanol: acetonitrile: water: triethylamine (8.5: 1.5: 1: 0.5, v/v/v/v) mobile phase with a chamber saturation time of 20 min at ambient condition and solvent migration distance of 70 mm was selected as an optimum condition.

Chromatographic conditions:

A Pre-coated silica gel G_{60} – F_{254}aluminum TLC sheet (100×100 mm, thickness layer 0.2 mm) pre washed with methanol was used as stationary phase. The linear ascending development was carried out in a CAMAG twin-trough glass chamber (10×10 cm) equilibrated with the mobile phase methanol: acetonitrile: water: Triethylamine (8.5: 1.5: 1: 0.5, v/v/v/v) for 20 min at room temperature. The plate was allowed to developed up to 70 mm. Quantitative evaluation of the plate was performed in absorbance mode at 254 nm. The slit dimensions were 6 mm length and 0.45 mm width, with a scanning rate of 20mm/s with a computerized CAMAG TLC scanner -3 integrated with winCATS4 software.

Densitometric analysis

Densitometric scanning was performed in the absorbance mode under control by winCATS4 planar chromatography software. The source of radiation was the deuterium lamp, and bands were scanned at 254 nm. The slit dimensions were 6 mm length and 0.45 mm width, with a scanning rate of 20 mm/s. Concentrations of the compound chromatograph were determined from the intensity of diffusely reflected light and evaluated as peak areas against concentrations using a linear regression equation.

CALIBRATION CURVE FOR CITI AND MCA

Working standard solution were filled in the syringe and 1, 2, 3, 4, 5, 6 µl (1000 to 6000 ng/band of CITI and MCA each) loaded on a TLC plate, using Linomat V applicator under nitrogen stream.

Plate was developed using methanol: acetonitrile: water: Triethylamine (8.5: 1.5: 1: 0.5, v/v/v/v) at room temperature and dried in air. Developed plates were subjected to densitometric measurements at wavelength 254 nm using TLC Scanner 3.

METHOD VALIDATION[6,7]

The method was validated for accuracy, precision, linearity, detection limit, quantitation limit and robustness.

Linearity

Linearity of the method was evaluated by constructing calibration curves at six concentration levels over a range of 1000–6000 ng/band for CITI and MCA respectively. The calibration curves (n = 6) were developed by plotting peak area versus concentration.

Precision

Precision was evaluated in terms of intraday and interday precisions. Intraday precision was determined by analyzing sample solutions of CITI(1000, 3000, 6000 ng/band) and MCA(1000, 3000, 6000 ng/band) at three levels covering low, medium, and high concentrations of the calibration curve three times on the same day (n = 3). Interday precision was determined by analyzing sample solutions of CITI(1000, 3000, 6000 ng/band) and MCA(1000, 3000, 6000 ng/band) at three levels covering low, medium, and high concentrations over a period of 3 days (n = 3). Mean peak area was calculated and RSD was determined. Repeatability of measurement of peak area was determined by analyzing CITI and MCA sample (2000 ng/band) six times without changing the position of plate. Reproducibility study was carried out by measurement of peak area.

Accuracy

The accuracy of the method was determined by calculating recoveries of CITI and MCA by method of standard additions. Known amount of CITI (0, 1600, 2000 and 2400 and MCA (0, 1500, 1875, and 2250) were added to a pre quantified sample and the amount of CITI and MCA were estimated by measuring the peak area and by fitting these values to the straight-line equation of calibration curve.

LOD and LOQ

The limit of detection (LOD) is defined as the lowest concentration of an analyte that can reliably be differentiated from background levels. Limit of quantification (LOQ) of an individual analytical procedure is the lowest amount of analyte that can be quantitatively determined with suitable precision and accuracy. LOD and LOQ were calculated using following equation as per ICH guidelines.

$LOD = 3.3 \times \sigma / S$; $LOQ = 10 \times \sigma / S$; Where σ is the standard deviation of y-intercepts of regression lines and S is the slope of the calibration curve.

Robustness

Changes in the chamber saturation time and solvent migration distance were introduced and the effects on the results were examined. Robustness of the method was determined in triplicate at a concentration level of 2000 ng/band of CITI and MCA respectively. The mean and % RSD of peak areas were calculated.

Solution stability

The solutions at analytical concentration of CITI 2000 µg/ml and MCA 2000 µg/ml were prepared and stored at room temperature for 24 h and analyzed at interval of 0, 4, 8 and 24 hr. The results were simultaneously compared with the freshly prepared CITI and MCA standard solution of the same concentration.

System suitability

System suitability test was an integral part of the method development to verify that the system is adequate for the analysis of CITI and MCA to be performed. Retention Factor (R*f*) and peak purity were determined.

ANALYSIS OF MARKETED FORMULATION:

Twenty tablets were weighed accurately and finely powdered. Tablet powder equivalent to 500 mg CITI containing 750 µg of MCA was taken in 100 ml volumetric flask. Methanol (50 ml) was added to the above flask and the flask was sonicated for 15 minutes. The solution was filtered using Whatman filter paper No.41 and volume was made up to the mark with the mobile phase to obtain a concentration of 75 µg/ml of MCA. A 40 µl of above solution was applied to the plate to obtain final concentration of 3000 ng/band of MCA. 1 ml from the stock were taken in 10 ml volumetric flask and diluted with methanol to obtain final concentration of 500 µg/ml of CITI. A 6 µl of above solution was applied to the plate to obtain final concentration of 3000 ng/band of CITI. The plate was developed using mobile phase and it was subjected to densitometric measurement. The possibility of interference from other components of the tablet formulation in the analysis was studied. From the developed chromatogram spot area and Rf values were determined.

STORAX PLUS® manufactured by Intas Pharmaceutical Ltd. Containing 0.75 mg of Methylcobalamin and 500 mg Citicoline Sodium. Overages of Methylcobalamin is added.

RESULTS AND DISCUSSION

The chromatographic conditions produced a well-defined, compact band of CITI and MCA with Rf 0.39 ± 0.011 and 0.62 ± 0.007 respectively (Figure 4-7).

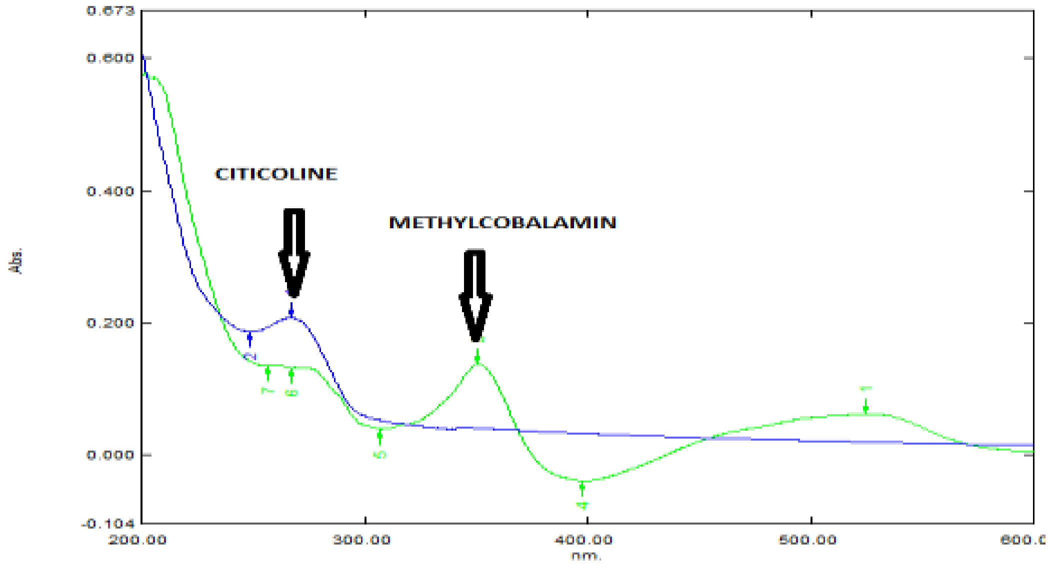

Figure 3: Overlain Spectra of 10ppm Solutions of Citicoline Sodium and Methylcobalamin

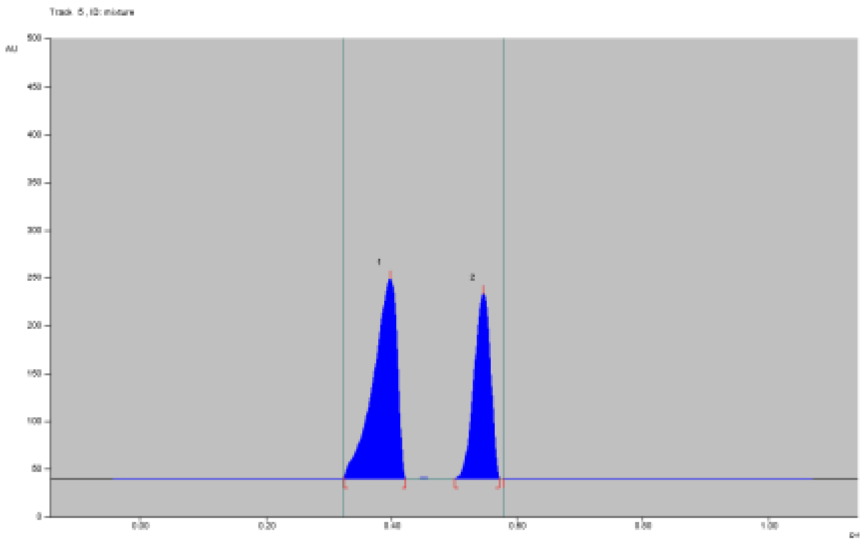

Figure 4: Densitogram of mixed standard solution of CITI and MCA(1000 and 1000 ng/band) using mobile methanol: acetonitrile: water: triethylamine (8.5:1.5:1:0.5, v/v/v/v)

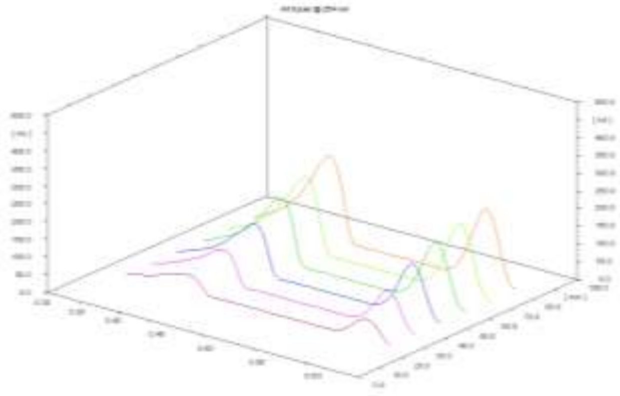

Figure 5: Three dimensional overlay of HPTLC densitograms of calibration bands of CITI and MCA

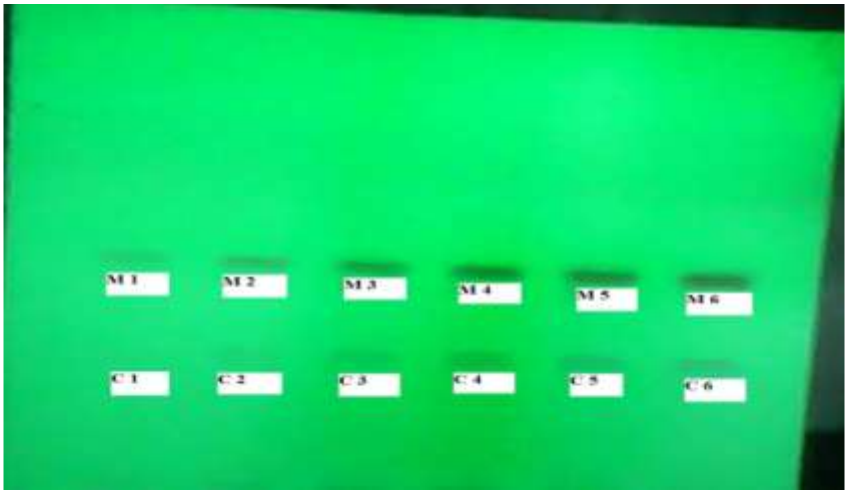

Figure 6: Photograph of developed TLC plate of CITI and MCA

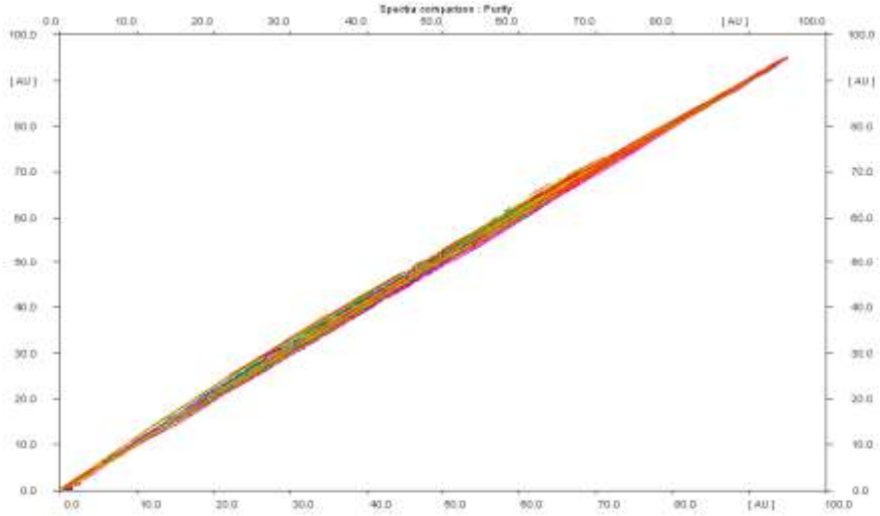

Figure 7: Peak Purity Correlation of CITI and MCA

Peak area versus concentration of the drug was plotted and regression equations were computed. Spectra of both drugs were recorded in the range of 200-400 nm and purity of chromatographic peak was checked by scanning individual peak at 3 different positions (peak start, peak apex and peak end) (figure. 8 & 9).

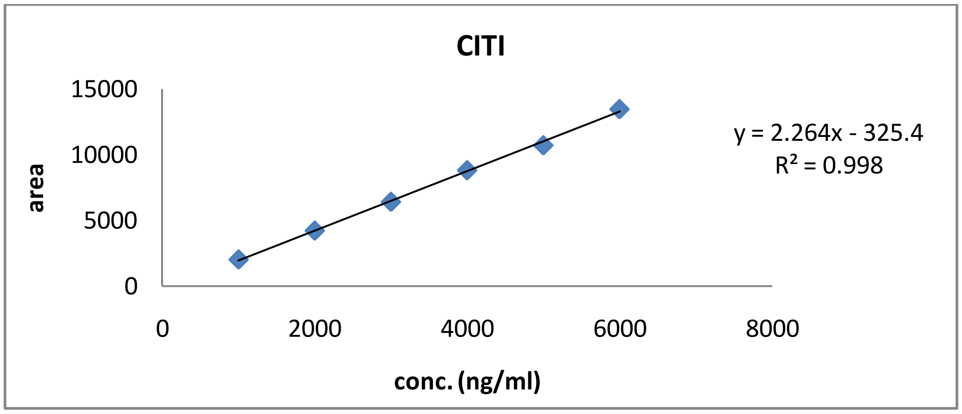

Figure 8: Calibration curve for CITI.

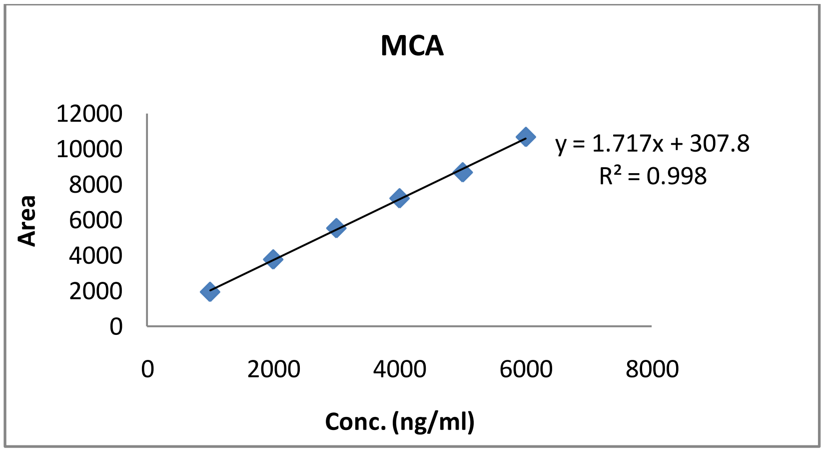

Figure 9: Calibration Curve for MCA

METHOD DEVELOPMENT

The proposed method was found to be simple, accurate and rapid for the routine determination of Citicoline sodium and Methylcobalamin in tablet formulation. This method can be successfully used for simultaneous estimation of Citicoline sodium and Methylcobalamin in combined dosage.

Linearity

Linearity was observed in the concentration range of 1000-6000 ng/band for Citicoline sodium and 1000-6000 ng/band for Methylcobalamin as shown in table 2. The proposed method was validated as per ICH guideline.(Table 1).

Table 1: Summary of Validation Parameters of HPTLC

Validation parameters	CITI	MCA
Linear range (ng per band)	1000-6000	1000-6000
Intraday (*n* = 3)	1.02 – 2.16	1.03 – 2.38
Interday (*n* = 3)	1.23 – 2.30	1.18 – 2.62
Repeatability of peak area (% RSD, *n* = 6)	1.81	1.80
Accuracy (%)	100.95 – 102.19%	111.97 – 112.81%
LOD (ng per band)	109.82	70.56
LOQ(ng per band)	366.09	235.20
Robustness	Robust	Robust
Specificity	Specific	Specific

Table 2: Accuracy study of CITI and MCA by the proposed HPTLC method

Amount of sample taken(ng/ml)		Amount of standard drug added (ng/ml)		Amount of drug recovered (ng/ml)		% Recovery ±SD (n = 3)		%RSD	
CITI	MCA	CITI	MCA	CITI	MCA	CITI	MCA	CITI	MCA
2000	1875	0.0	0.0	2043.86	2115.27	102.19 ±1.52	112.81 ± 1.60	1.49	1.42
2000	1875	1600	1500	3632.37	3612.91	101.61 ±1.24	112.72 ± 1.38	1.22	1.23
2000	1875	2000	1875	4018.93	3973.61	100.94 ± 1.21	111.97 ±1.49	1.19	1.33
2000	1875	2400	2250	4425.25	4360.96	101.26 ± 0.95	112.64 ± 1.19	0.94	1.06

Precision

Precision was calculated as interday and intraday variations (% RSD found to be less than 3 for both drugs as shown in table 1.

Accuracy

The accuracy of method was determined at 80, 100 and 120 % level. The percent recovery ranges from 100.94%to 102.19% for CITI and 111.97% to 112.81% for MCA as shown in Table 2.

LOD & LOQ

The LOD was found to be 109.82 ng/band and 70.56 ng/band for CITI and MCA respectively and LOQ was found to be 366.09 ng/band and 235.20 ng/band for CITI and MCA respectively.(Table 1).

Robustness

The low values of RSD (Table 4) obtained after introducing small, deliberate changes in parameters of the developed HPTLC method confirmed its robustness.(Table 3).

Table 3: Robustness studies of HPTLC method

Method parameter/Condition	Deliberate changes	Peak area Mean± SD (n = 3) CITI	MCA	%RSD CITI	MCA
Chamber saturation time	Actual (20 min)	4205.67 ± 88.14	3786.58 ± 77.63	2.09	2.05
	18 min	4187.003 ±81.67	3862.14 ±52.62	1.95	1.36
	22 min	4201.747 ±79.74	3829.403 ±39.77	1.89	1.03
Development distance from spot application	Actual (7 cm)	4205.67 ± 88.14	3786.58± 77.63	2.09	2.05
	7.7 cm	4136.77 ±72.02	3873.59 ±54.10	1.74	1.39
	6.3 cm	4231.11 ± 82.61	3806.04 ±49.13	1.95	1.29
Mobile Phase Ratio	(Actual) Methanol: Acetonitrile: Water: Triethylamine (8.5:1.5:1:0.5)	4205.67 ± 88.14	3786.58 ± 77.63	2.09	2.05
	Mehanol: Acetonitrile: Water: Triethylamine (8:2:0.5:0.5)	4226.68 ±60.22	3800.44 ±51.86	1.42	2.01
	Mehanol: Acetonitrile :Water:Triethylamine(8:1.5:0.5:1)	4230.9 ± 76.40	3805.13 ± 63.56	1.22	1.67

Solution Stability

Stability of sample solutions were studied at ambient condition for 24 hr. The obtained results are shown in (Table 4). The prepared solution was found to be stable for 24 hr.

Table 4: Solution stability study

Time (Hrs.)	Area CITI 2000 ng/band	MCA 2000 ng/band	% of drug found CITI	MCA
0	2039.16	2001.59	100	100
3	2006.96	1976.59	99.51	98.82
6	1955.96	1954.71	98.06	97.73
24	1948.47	1938.89	96.93	96.94

System Suitability

System Suitability parameters were shown in table 5.The obtained result showed that the instrument is suitable for estimation of CITI and MCA in combination.

Table 5: System Suitability Studies of HPTLC Method

Sample	Peak purity	Rf Value
CITI	0.9997	0.39
CITI formulation	0.9996	0.42
MCA	0.9997	0.62
MCA formulation	0.9989	0.63

Accuracy

Marketed brand of tablet (Storax plus) was analyzed and amount of drug determined by proposed method was found to be 101.63±0.71% for CITI & 111.80±0.68% for MCA as shown in Table 6.

Table 6: Analysis of marketed formulation

Formulation	Labeled Amount (mg)		Amount found (mg)		% of drug found ±SD (n=3)	
	CITI	MCA	CITI	MCA	CITI	MCA
STORAX PLUS	3000	3000	3049.08	3354	101.636 ±0.71	111.8 ± 0.68

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