Stability-Indicating HPTLC Method for Determination of Milnacipran Hydrochloride in Pharmaceutical Formulations

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Key Words

Milnacipran hydrochloride
HPTLC
Stress studies
Validation
Pharmaceutical formulation
Method comparison

Summary

A simple, specific, precise and accurate stability-indicating assay method using high performance thin-layer chromatography (HPTLC) is described for estimation of Milnacipran hydrochloride (MIL) in bulk drug and in pharmaceutical formulations. The separations were achieved on prepared TLC plates precoated with silica gel 60 F_{254}. The mobile phase developed and optimized for bringing out the separation involves chloroform–methanol–ammonia in the ratio of 6.4:2.5:0.2 *v/v/v*. The densitometric scanning wavelength selected was 220 nm. The compact bands of MIL were obtained at R_F value of 0.45 ± 0.02. The method developed was able to separate peaks of all the degradation products formed in ICH-prescribed stress conditions with sufficient difference in their R_F values. The developed method was validated for linearity and range, specificity, precision, accuracy and robustness, and the results were found to be within acceptance criteria. The reliability of the method was evaluated when it was applied for the estimation of MIL in pharmaceutical capsule formulation, and assay results gave good recovery when statistically compared with the reversed-phase high-performance liquid chromatography (RP-HPLC) method.

1 Introduction

Serotonin-selective reuptake inhibitors (SSRIs) and serotonin noradrenalin reuptake inhibitors (SNRIs) have been used widely to treat mental disorders such as depression, anxiety disorders and psychosomatic pain [1]. Milnacipran hydrochloride (MIL) ((*Z*)-1-diethylaminocarbonyl-2-aminoethyl-1-phenyl-cyclopropane hydrochloride) is an antidepressant drug belonging to the class of serotonin noradrenalin reuptake inhibitors. In the treatment of major depression, MIL has achieved a similar efficacy to tricyclic antidepressants and a similar tolerability to selective serotonin reuptake inhibitors [2].

The extensive literature search reveals that determination of MIL in human serum and plasma is carried out using different chromatographic procedures [3–6]. Studies have also been reported for chiral determination, enantiomeric excess and chemical purity of MIL [7, 8]. A stability-indicating RP-HPLC method is developed and validated by authors for determination of MIL in pharmaceutical capsule dosage forms [9]. The parent drug stability test guidelines issued by the ICH and WHO [10, 11] suggest that stress studies should be carried out for better understanding of the stability behavior of the drug molecule. The stability-indicating high performance thin-layer chromatography (HPTLC) method for MIL is yet not reported; hence, the integral aim of the present investigation is development and validation of stability-indicating HPTLC method for the analysis of MIL in presence of degradation products formed in different ICH-prescribed stress conditions. The developed method is able to quantify MIL in pharmaceutical capsule formulations with good recovery values when compared with the reported RP-HPLC method.

2 Experimental

2.1 Chemicals and Materials

Analytically pure MIL was obtained as a gift sample from M/s Torrent Pharmaceutical Ltd. (Ahmedabad, India). Chloroform, methanol, ammonia, hydrochloric acid, sodium hydroxide and hydrogen peroxide (30% *w/v*) used for solvent preparation and stress studies were of analytical reagent grade (CDH Chemicals Delhi, India). Syringe filters (0.45 μm) used for the sample filtration were supplied by Millipore (Millipore Ltd. Bangalore, India). Capsule formulations A (Milnace 50, CC968002, 06/2011, Torrent Pharmaceuticals Ltd., India) and B (Milborn 50, GK90988, 05/2011 Sun Pharmaceuticals Ltd., India) containing labeled amount of 50 mg of MIL were procured from local market.

2.2 Chromatographic Conditions

Chromatography was performed on 10 × 10 cm aluminum HPTLC plates precoated with 250 μm layers of silica gel (E. Merck, Darmstadt, Germany; supplied by Merck India, Mumbai,

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India). Before chromatography, the plates were prewashed with methanol and activated at 100°C for 25 min. Samples were applied as 5-mm wide bands, under a continuous flow of nitrogen, by means of a CAMAG (Muttenz, Switzerland) Linomat V sample applicator fitted with 100 µL applicator syringe (Hamilton, Bonadauz, Switzerland). A constant application rate of 0.1 µL s^{-1} was used and the distance between the adjacent bands was 10 mm. The plates were then conditioned for 20 min in a presaturated twin-trough glass chamber (10 × 10 cm^2) with the mobile phase of chloroform–methanol–ammonia (6.4:2.5:0.2, *v/v/v*), in one trough and plates in the other trough. The plates were then placed in the mobile phase and ascending development was performed to a distance of 80 mm from the point of application at ambient temperature, and the development time was 12 min. Subsequent to the development, the plates were dried in a current of air with the help of an air dryer and spots were visualized in CAMAG UV cabinet with dual wavelength UV lamp (254 and 366 nm); densitometric scanning was performed at 220 nm with CAMAG TLC scanner III operated in reflectance–absorbance mode and controlled by WinCats software. The slit dimensions were 4 × 0.20 mm at 10 mm s^{-1} scanning speed. The concentrations of compound and degradation behavior were studied from the intensity of diffusely reflected light. Evaluation was based on linear regression of peak areas.

2.3 Preparation of Standard Solution

A stock solution of MIL was prepared by dissolving accurately weighed 100 mg of MIL with 100-mL methanol. Aliquots of this solution were suitability diluted with methanol to get working standard solutions of MIL in the concentration of 0.1 mg mL^{-1}. Standard solutions of MIL were prepared to get concentration range of 500–6000 ng per spot by dilution of working standard solution with methanol.

2.4 Stress Studies

The stress studies were carried out as per ICH guidelines, by forcibly degrading MIL under different stress conditions such as hydrolytic, oxidative, thermal and photolytic degradation.

Hydrolytic degradation was carried out by preparing MIL solutions (2 mg mL^{-1}) in 1 N HCl, 0.1 N NaOH and distilled water and refluxing at 100°C for 4 h. For oxidative studies, MIL solution (2 mg mL^{-1}) was prepared in 3% hydrogen peroxide and was refluxed at 100°C for 4 h. Thermal degradation was carried out by exposing the MIL in Petri dish in hot air oven at 120°C for two days; similarly, for photo degradation the aqueous solution of MIL (2 mg mL^{-1}) was exposed to direct sunlight for 6 h. All the stressed samples were suitably diluted with methanol (acidic and alkaline hydrolytic stressed samples were appropriately neutralized) to get concentration of 0.1 mg mL^{-1}. For the evaluation of degradation behavior of MIL, the stress samples were applied (20 µL, 2000 ng per spot) on the activated chromatoplates and were analyzed as described in Section 2.2.

2.5 Method Validation

The developed method was validated for linearity and range, specificity, precision, accuracy and robustness per ICH guidelines [12].

2.5.1 Linearity and Range

Each concentration in the range of 500–6000 ng per spot was spotted three times on individual plates and response was measured after scanning. For evaluation of linearity, peak area and concentrations were subjected to least square regression analysis to calculate calibration equation and correlation coefficient.

2.5.2 Specificity (Interference from Excipients and Degradation Products)

The specificity of the method was ascertained by analyzing MIL in presence of excipients of MIL capsule formulation and their degradation products. The bands of MIL in the sample were confirmed by comparing R_F values and respective spectra of the sample with those of the standard. The peak purity of MIL was assured by comparing the spectra at three different levels, that is, peak start (s), peak apex (m) and peak end (e) positions.

2.5.3 Precision

To check the precision of the method, MIL bulk drug was weighed and transferred into six different volumetric flasks and dissolved using methanol to get concentration of 0.1 mg mL^{-1} and 20 µL of each prepared sample was applied on chromatographic plate in duplicate. The plate was developed and analyzed as per the procedure given in Section 2.2. The precision of the method was evaluated by calculating the percent relative standard deviation (%RSD) of mean peak areas obtained from each spot of sample. For intermediate precision of the method, the same procedure was performed at different time intervals on the same day, on different days and by different analysts.

2.5.4 Accuracy

The accuracy of the method was determined by recovery studies using standard addition method to laboratory-made synthetic mixture. Known amounts of MIL were added at three different levels (approximately 80, 100 and 120% of label claim) to constant weight of synthetic mixture of placebo, respectively. All the prepared samples were dissolved in 50 mL methanol and further analysed as described in Section 2.2.

2.5.5 Robustness [13, 14]

In order to establish the robustness of the method, small deliberated changes were made in the experimental conditions and chromatographic parameters like change in plate activation time, chamber saturation time (±20% change from set time), volume of mobile phase (±10% change from set volume) and development distance (±10% change from set distance). In the above changed conditions, 20 µL of 0.1 mg mL^{-1} MIL stock solution was analyzed as described in Section 2.2 and results of robustness studies were expressed in term of %RSD of peak areas in each changed condition and were compared with similar results obtained in unchanged experimental conditions.

2.5.6 Limits of Detection and Limit of Quantitation

Limits of detection and limit of quantitation were determined on the basis of the mathematical terms mentioned in ICH guidelines for method validation from triplicate results of linearity. Limit of detection was determined using equation $3.3\sigma/s$ and limit of quantification was determined using equation $10\sigma/s$,

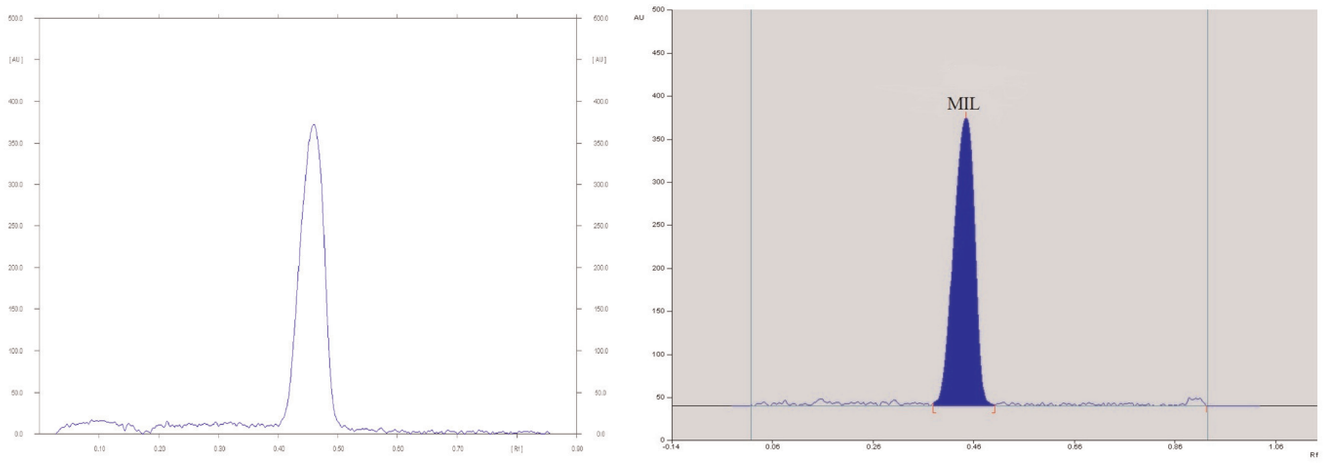

Figure 1

HPTLC chromatogram of MIL (2000 ng per spot) with R_F 0.45.

where s is the slope of calibration curve and σ is standard deviation of responses.

2.5.7 Solution Stability

The solutions at analytical concentration (0.1 mg mL^{-1}) were prepared and stored at room temperature protected from light for 24 h and analyzed at interval of 0, 6, 12 and 24 h for the presence of any band other than that of MIL and the results were simultaneously compared with the freshly prepared MIL standard solution of the same concentration in the form of change in %RSD of the response obtained.

2.5.8 Application of Validated Method to Pharmaceutical Formulation

For confirming the applicability of developed and validated method, 20 capsules of each brand were weighed and net content of each capsule was calculated. Capsule powder equivalent to 50 mg MIL was accurately weighed and transferred to a 50-mL volumetric flask with addition of about 40 mL of methanol. The mixture was sonicated for 30 min with shaking, and volume was made up to the mark with methanol. The above solution was centrifuged at 2500 rpm in a research centrifuge for 15 min and filtered through 0.45-µm syringe filter. The resulting solution was further diluted to get working concentration of 0.1 mg mL^{-1} for MIL and 20 µL was analyzed as described in chromatographic conditions. The analysis was repeated in triplicate and amount of MIL recovered for each formulation was found out by regression equation. To check the validity of the developed method, marketed formulations were also analyzed by reported method [9] and results were compared using paired sample t test [15, 16].

3 Results and Discussion

3.1 Method Development and Optimization of Chromatographic Conditions

Selection of best solvent system is the critical step in HPTLC method development. From the different solvent systems tried, the mobile phase consisting of chloroform and methanol in ratio of 6.4:2.5 *v/v* gave good separation between MIL and its degradation products with optimum R_F value for MIL; however, tailing of MIL peak was observed, which was avoided by addition of 0.2 mL ammonia in mobile phase. The optimized mobile phase was chloroform–methanol–ammonia, 6.4:2.5:0.2 *v/v/v*, which gave a symmetric peak of MIL with R_F of 0.45 **(Figure 1).** Well-defined bands were obtained when the chamber was saturated with mobile phase for 20 min at ambient temperature. Reproducible responses were obtained at optimized slit dimensions of 4 × 0.2 mm. For quantitative purpose, the densitometric scanning was carried at wavelength 220 nm where MIL and its degradation products exhibit sufficient UV absorption, and estimation of MIL was achieved without hampering sensitivity.

3.2 Stress Degradation Behavior

The stress degradation study shows that MIL is susceptible to hydrolytic and oxidative stress conditions. The representative chromatograms for the hydrolyzed and oxidative degraded samples of MIL are shown in **Figure 2** (a–d). In the hydrolytic degradation, the order of degradation behavior is alkaline $\wp$ acidic $\wp$ neutral. Two, three and one additional peaks were obtained with acid, alkaline and neutral hydrolysis, respectively. MIL being amide undergoes hydrolysis to acid and amine. On oxidative degradation of MIL, approximately 30% of reduction in the MIL peak area was observed (Figure 2d). MIL was found to be stable in thermal degradation and in photolytic degradation, as no marked reduction in the peak area was observed and no additional peak was seen in chromatograms.

One of the degradation products of MIL at R_F 0.80 ± 0.01 (represented as D5), obtained in all hydrolytic and oxidative degradation conditions, corresponds to the only degradation product peak obtained with neutral hydrolysis; it indicates that MIL is hydrolyzed in presence of water during acidic, alkaline and oxidative hydrolysis, which was also confirmed by scanning and comparing the spectra of each degradation spot **(Figure 3).** The degradation behavior of MIL in hydrolytic and oxidative stressed conditions is compiled in **Table 1**.

Similarly, the degradation product (represented as D1) formed in acidic and alkaline hydrolysis at R_F 0.04 ± 0.01 and 0.05 ± 0.01, respectively, may be the same as both the spots displayed similar spectra **(Figure 4).**

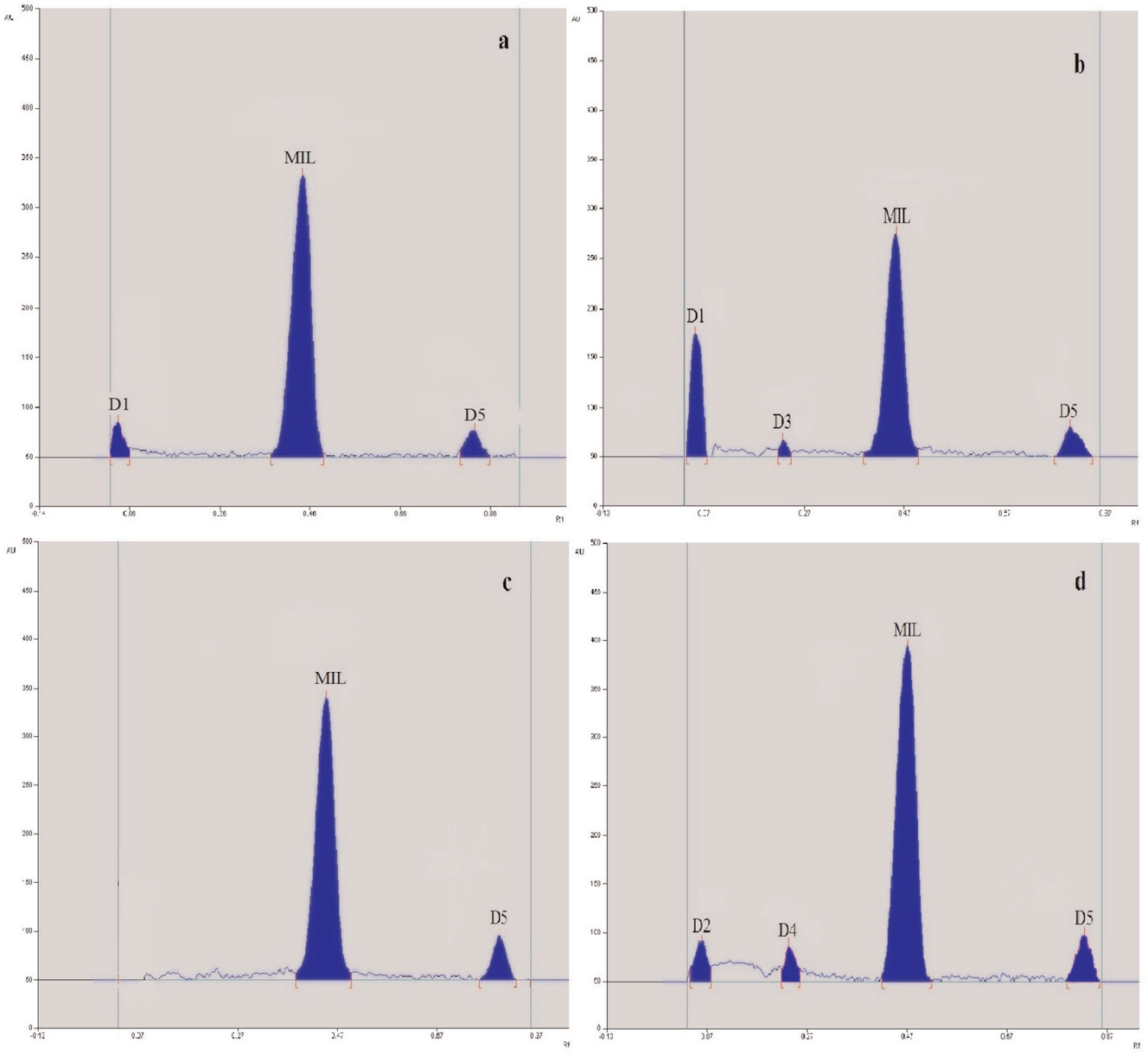

Figure 2

HPTLC chromatogram of MIL: (a) acid degraded; (b) alkaline degraded; (c) neutral degraded; (d) oxidative degraded. D1, D2, D3, D4 and D5 are the degradation products of MIL.

3.3 Method Validation

3.3.1 Linearity and Range

Linearity was observed over the concentration range 500–6000 ng per spot confirming adherence of the system to Beer's law. The regression analysis equation was $y = 2.753x + 1322.283$ and correlation coefficient (r) was 0.9966 **(Figure 5).**

3.3.2 Specificity

Specificity of the method for MIL was proved from the spectral scan **(Figure 6),** and peak purity correlation (r) results **(Table 2)** for MIL in bulk and in two capsule formulations indicate that there is no merging or co-elution of interfering peaks with MIL, so there is no interference from any excipients present in capsule formulation of MIL. Similarly, stability-indicating power of the method was also proved by forcibly degrading MIL in different stress conditions and subsequently analyzing MIL in presence of degradation products. The results indicate that the method is able to quantify MIL in presence of its degradation products.

3.3.3 Precision

For determination of precision of MIL by the proposed method, same homogeneous samples of MIL (real samples) were prepared repeatedly and analyzed. Intermediate precision was evaluated at different times on same day, on different days and even by different analysts. Low values of RSD (less than 2%) obtained in the precision studies **(Table 3)** indicate that the method is precise and reproducible.

3.3.4 Accuracy

Accuracy of the proposed method was studied by preparing synthetic mixtures of capsule excipients having a known amount of

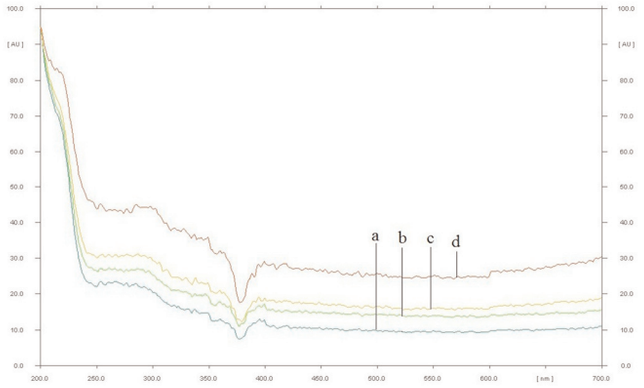

Figure 3

Overlaid band spectra of D5 in acid-degraded (a, with peak purity 0.99945), alkaline-degraded (b, with peak purity 0.99965), neutral-degraded (c, with peak purity 0.99915) and oxidative-degraded (d, with peak purity 0.99908) MIL samples, respectively.

Table 1

Results of stress degradation study of MIL.

Stress degradation condition	Approx. observed degradation of MIL (%)	R_F values of major degradation products
Acid hydrolysis	16	0.04, 0.81
Alkaline hydrolysis	35	0.05, 0.23, 0.80
Neutral hydrolysis	10	0.81
Oxidative degradation	30	0.06, 0.24, 0.80

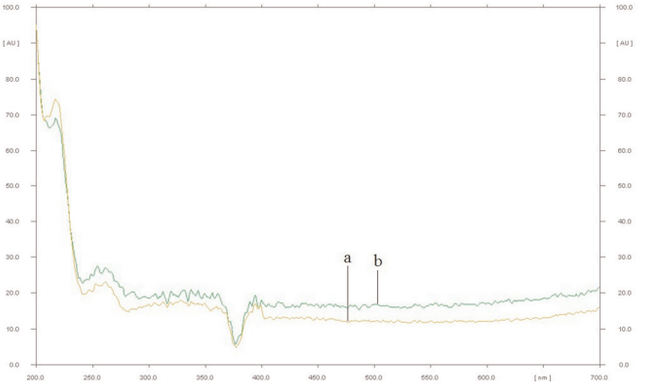

Figure 4

Overlaid band spectra of D1 in acid-degraded (a, with peak purity 0.99933) and alkaline-degraded (b, with peak purity 0.99945) MIL samples, respectively.

MIL corresponding to approximately 80–120% of the label claim. Mean recovery **(Table 4)** for MIL was between 99.2% and 99.8% indicating that the developed method was accurate for the determination of MIL in pharmaceutical formulations.

3.3.5 Robustness

Acceptable %RSD values obtained after making small deliberate changes in the developed HPTLC method indicate that the method is robust for the intended purpose **(Table 5).**

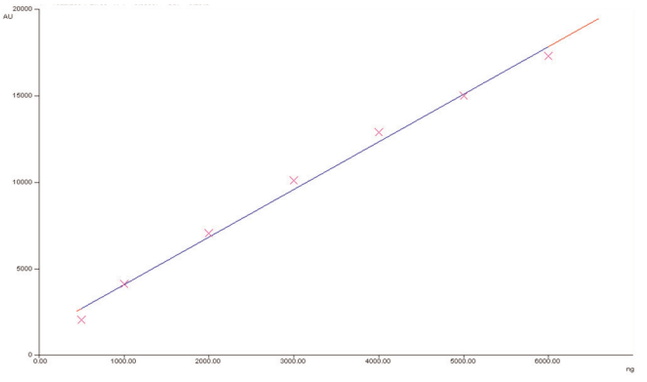

Figure 5

Calibration plot of MIL by the developed HPTLC method.

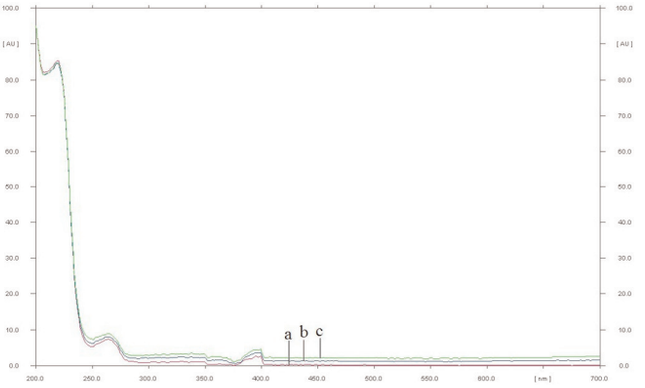

Figure 6

Spectra comparison of MIL. (a) Bulk, (b) capsule formulation A and (c) capsule formulation B.

Table 2

Peak purity correlation results of MIL in bulk and in two formulations at peak start, middle and end.

Sample	Correlation of center and slope spectra of MIL	
	r (s, m)	*r* (m, e)
MIL	0.99959	0.99987
MIL capsule formulation A	0.99978	0.99956
MIL capsule formulation B	0.99932	0.99951

3.3.6 Solution Stability

No significant change was observed in peak area of MIL when analyzed up to 24 h at different time intervals (RSD 1.03%), which indicates the solution stability within the period of evaluation.

3.4 Method Application

The proposed, developed and validated stability-indicating HPTLC method was successfully applied for determination of MIL in two marketed formulations of MIL. There was no interference of excipients commonly found in capsules as described

Table 3

Summary of validation parameters of developed HPTLC method.

Validation parameter		Observation for MIL
Specificity		Specific
Linear range (ng per spot)		500–6000
Precision (%RSD of response)	Method precision (n = 6)	1.23
	Intra-day (n = 6)	1.87
	Inter-day (n = 6)	1.56
	Different analyst (n = 6)	1.92
Limit of detection (ng per spot)		108.6
Limit of quantification (ng per spot)		329.0

Table 4

Results from accuracy study.

Level (%Label claim)	Amount of drug added (mg)	Amount of drug found (mg)	Mean recovery (%)[a)]	%RSD
80	39.3	38.9	99.4	1.7
	40.8	40.0		
	39.7	40.2		
100	50.8	49.9	99.2	1.0
	49.2	49.3		
	50.1	49.7		
120	61.1	61.3	99.8	0.6
	59.7	59.5		
	59.8	59.4		

[a)]Mean of three replicates

Table 5

Results from the robustness study of method.

Method parameter/Condition		%RSD of peak area of MIL (n = 6)
Plate activation time[a)]	20 min	1.88
	30 min	0.93
Chamber saturation time[a)]	16 min	1.74
	24 min	0.86
Volume of mobile phase[b)]	8.2 mL	1.22
	10.0 mL	1.79
Development distance from spot application[c)]	7.2 cm	0.98
	8.8 cm	1.56

[a)]±20% change in set time

[b)]±10% change in set volume

[c)]±10% change in set distance

Table 6

Assay results of MIL capsule dosage form using the proposed method.

Formulation	Amount of drug taken (mg)	Amount of drug obtained (mg)[a)]	%Label claim[a)]
A	50.0	49.1 ± 0.8	98.5 ± 0.60
B	50.0	50.2 ± 0.65	100.8 ± 0.70

A, Milnace (Torrent Pharmaceuticals Ltd. Ahmedabad, India); B, Milborn (Sun Pharmaceutical Ltd. Baroda, India)

[a)]Mean value ± standard deviation of three determinations

Table 7

Statistical comparison between two analytical methods for assay values.

Statistical Parameter	MIL Pharmaceutical Capsule Formulation			
	A		B	
	HPTLC	HPLC	HPTLC	HPLC
Mean (n = 3)	98.5	99.3	100.8	101.0
Variance	0.6	0.6	0.7	0.7
Pearson Correlation	0.998		0.532	
T stat ($t$cal)	1.633		0.161	
t Critical one tail	2.919		2.919	
t Critical two tail ($t$crit)	4.302		4.302	
$t$cal < $t$crit	Yes		Yes	
Null Hypothesis	Pass		Pass	

in specificity study. No degradation product peaks were obtained when marketed formulations were analyzed by this method. The assay results obtained were satisfactory, accurate and precise as indicated by the good recovery and acceptable standard deviation (SD) values **(Table 6).** The good performance of the method indicates that it can be used for the determination of MIL in drug substances and pharmaceutical preparations.

3.5 Statistical Method Comparison

The assay results of the developed HPTLC method were statistically compared with the results obtained in reported RP-HPLC method [9] by paired t-test at 5% level of significance. Results indicate that there is no significant difference between the assay results obtained by the two methods **(Table 7).**

4 Conclusion

This developed and validated stability-indicating HPTLC method is specific, precise and accurate and able to separate the drug from all of its degradation products. The method was successfully applied for determination of MIL in its pharmaceutical capsule formulations, which suggests good reliability of the method as no significant difference in assay results was obtained when the developed method was compared with the reported RP-HPLC method. The developed HPTLC method can be con-

veniently used for routine quality control analysis of MIL in industries for batch release.

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