

Development and Validation of Stability Indicating HPTLC Method for the Determination of Metformin Hydrochloride and Benfotiamine in Bulk and Combined Dosage Form

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ABSTRACT

Context: A Simple, selective, precise, and Stability indicating High Performance Thin Layer Chromatography (HPTLC) method of analysis of Metformin Hydrochloride (MET) and Benfotiamine (BENT) both as a bulk drug and in their combined formulation has been developed. **Method:** The basic aim of this method is to separate both the drugs by HPTLC and measure their spots at 249 nm. The separation was carried out on TLC aluminium sheets of silica gel 60F 254 using Benzene: Methanol: Triethylamine (8.5:1:0.5, v/v/v) as a mobile phase. Stability of MET and BENT was carried out by forced degradation study. **Result:** MET and BENT gave distinct and well defined peak at R_f 0.26 and 0.72, respectively. Calibration curves were linear in range of 500-3000 and 75-450 ng/spot for MET and BENT, respectively. Method was successively applied to tablet formulation. Stability study shows that the chromatograms of samples degraded with acid, base, hydrogen peroxide, light and dry heat showed well separated spots of pure MET and BENT as well as some additional peaks at different R_f values. **Conclusion:** The HPTLC method was also able to selectively quantitate Metformin hydrochloride and Benfotiamine in presence of their degradation products obtained in forced degradation study. Hence, the method can be used as stability indicating. The method was validated as per ICH guidelines and it is applied for the analysis of pharmaceutical dosage form containing these two drugs.

Key words: HPTLC, MET, BENT, Method validation, Forced Degradation, Stability Indicating.

INTRODUCTION

Metformin hydrochloride (MET) is chemically N, N-dimethylimidodicarbonimidic diamide hydrochloride (1,1-dimethylbiguanide hydrochloride) which suppresses the excessive hepatic glucose production helpful for improving glucose clearance from the body. It is official in Indian Pharmacopoeia,¹ British Pharmacopoeia,² European Pharmacopoeia,³ and United States Pharmacopoeia;⁴ A literature survey revealed that there are different methods are available which can estimate the Metformin Hydrochloride in their pharmaceutical dosage form e.g. Spectrophotometry,⁵⁻⁸ HPLC,⁹⁻¹³ LC-MS/MS,¹⁴ ion

pairing HPLC,¹⁵ Stability indicating HPTLC,^{16,17} Stability indicating P-HPLC,¹⁸⁻²⁴ LC-MS. Benfotiamine (S-benzoylthiamine O-mono-phosphate) is a member of allithiamines group which is a synthetic S-acyl derivative of thiamine (vitamin- B1). It is a lipid-soluble form of the Vitamin B-1, prescribed for treating painful nerve conditions by blocking AGEs (Advanced Glycation End products). Literature survey reveals that few HPLC methods have been reported for the estimation of benfotiamine individually and combination with other drugs²⁵⁻²⁷ The chemical

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structures of both drugs are shown in Figure 1 and Figure 2.

However no references have been found for stability indicating HPTLC method for simultaneous estimation of Metformin Hydrochloride and Benfotiamine. The International Conference on Harmonization (ICH) stability test guideline requires that analytical test procedures used for samples should be stability indicating and validated.²⁸⁻³⁰ The present work describes new method for simultaneous estimation of Metformin Hydrochloride and Benfotiamine.

MATERIALS AND METHODS

Materials

Active pharmaceutical ingredient (API) working standards of Metformin HCl and Benfotiamine were received as a gift sample from Alkem Pharmaceuticals Ltd., Mumbai, and Aquatic Remedies Pvt. Ltd., Hyderabad, respectively. All the reagents used were of analytical grade (S. D. Fine Chemicals, Mumbai, India) and used without further purification.

Instrumentation and Chromatographic conditions

The samples were applied in the form of bands of width 6mm with 100 µl sample syringe on precoated silica gel aluminium plate 60F-254 (20 cm x 10 cm) with 250µm thickness; (E. MERCK, Darmstadt, Germany) using a Camag Linomat V sample applicator. The plates were washed prior to chromatography with methanol and then activated at 110°C for 15 min. A constant application rate of 150 nl/sec was employed and space between two bands was 85 mm. The slit dimension was kept at 6 mm x 0.45 mm. The mobile phase consists of Benzene: Methanol: Triethylamine (8.5:1:0.5, v/v/v). Linear ascending development was carried out in 20 cm x 10 cm twin trough glass chamber (Camag, Muttenz, Switzerland). The optimized chamber saturation time for mobile phase was 20 min, at temperature (25°C ± 5%); the chromatogram was run and its length was found to be 8 cm and TLC plates were air dried. Densitometric scanning was performed on Camag TLC Scanner III controlled with CATS 1.4.2 software at 249nm. The source of radiation utilized was deuterium lamp. Evaluation was performed using peak area with linear regression.

Preparation of Standard solutions and calibration graphs

Combined standard stock solution containing 500 µg/ml of MET and 75 µg/ml of BENT was prepared in methanol. Calibration standards were spotted by using Camag Hamilton 100 µl syringe with the help of auto-

matic sample applicator Linomat V on TLC plates that gave concentration 500-3000 ng/spot of MET and 75-450 ng/spot of BEN, respectively. Each concentration was spotted six times on the TLC plates. The plates were developed using previously described mobile phase. The calibration curve was plotted as peak areas versus corresponding concentration.

Method Validation

Precision

Repeatability of the method was assessed by spotting 500 ng/spot for MET and 75 ng/spot for BENT of drug solution six times on a TLC, followed by development of plate. The 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 a week.

Limit of detection and limit of quantification

In order to determine detection and quantification limit, concentrations in the lower part of the linear range of the calibration curve were used. Stock solution of MET and BENT was prepared and different volume of stock solution in the range 500-3000 ng of MET and 75-450 ng for BENT were spotted triplicate. The amount of both the drugs by spot versus average response (peak area) was graphed and the equation for this was determined. The standard deviations (S.D.) and average of standard deviations was calculated (A.S.D.). Detection limit was calculated by $(3.3 \times \text{Standard deviation})/b$ and quantification limit was calculated by $(10 \times \text{Standard deviation})/b$, where "b" corresponds to the slope obtained in the linearity study of method.

Specificity

Specificity of the method was ascertained by analysing standard drug and sample. The mobile phase resolved both the drugs very efficiently, as shown in (Figure 2). The spot for MET and BENT was confirmed by comparing the RF and spectra of the spot with that of standard.

Accuracy

Recovery study was carried out by over spotting 80%, 100% and 120% of the standard drug solution of MET and BENT and the mixtures were reanalyzed by the proposed method. The experiment was conducted in triplicate. This was done to check the recovery of the drug at different levels in formulations.

Robustness

Robustness was studied in six replicate at the concentration level of 500 ng/spot for MET and 75 ng/spot for BENT. Robustness of the developed method was evaluated by the analysis of sample solution after making small changes in mobile phase composition and mobile phase saturation time. The low value of % RSD shows that the method is robust and that a slight change in mobile phase volume and mobile phase saturation time does not affect the results.

Analysis of MET and BENT in marketed formulation

To determine the content of MET and BENT simultaneously in conventional tablets (label claim 500mg MET and 75 mg BENT); twenty tablets were accurately weighed, average weight was determined and ground to a fine powder. A quantity of powder equivalent to 500 mg MET and 75 mg BENT was transferred into 100 ml volumetric flask containing 50 ml of methanol, sonicated for 15 min and diluted up to the mark with the same solvent. The resulting solution was filtered using 0.45µm filter (Millifilter, MA). 0.4 µL of the above solution was applied on TLC plates followed by development and scanning as described in section 2.2. The analysis was repeated for six times. MET and BENT gave sharp and well defined peaks at Rf 0.26 and 0.72, respectively, when scanned at 249 nm. The results are shown in Table 1 indicate that there was no interference from the excipients commonly present in the tablets.

Forced degradation of MET and BENT

Acid and Base induced degradation

From the standard solution of MET (5000 µg/ml), 1 ml of solution was mixed with 1ml of 0.1N HCl and 8 ml of methanol. The solution was refluxed at 80°C for 4 hrs. 3 µl volume of this solution was applied on TLC plate to get concentration 1500 ng/band. Similarly solution having final concentration 75 ng/band for BENT was prepared from the standard stock solution (750 µg/ ml) and 3 µl volume of resulting solution was applied on TLC plate. The chromatograms were run as described in section 2.2. and it is shown in Figure 4-7.

Hydrogen peroxide-induced degradation

From the standard solution of MET (5000 µg/ml), 1 ml of solution was mixed with 1ml of 3 % H₂O₂ and 8 ml of methanol. The solution was refluxed at 80°C for 4 hrs. 3 µl volume of this solution was applied on TLC plate to get concentration 1500 ng/band. Similarly solution having final concentration 75 ng/band for BENT was prepared from the standard stock solution (750 µg/ml)

and 3 µl volume of resulting solution was applied on TLC plate. The chromatograms were run as described in section 2.2 and it is shown in Figure 8-9.

Dry heat degradation

Dry heat studies were performed by keeping drug sample as individual in oven (80° C) for a period of 2 hour. Samples were withdrawn, dissolved in methanol and diluted appropriately to get concentration of 500 µg/ml for MET and 75 µg/ml for BENT. 3 µl volumes were applied on TLC plate to get concentration 1500 ng/band for MET and 450 ng/band for BENT. The resulting solutions were applied on TLC plate and analysed under optimized chromatographic conditions. The chromatograms were run as described in section 2.2 and it is shown in Figure 10-11.

Photolytic degradation

Photolytic studies were also carried out by exposure of drug individually to UV light up to 200 watt hours/square meter for period of 4 hrs. Sample was weighed, dissolved and diluted get respective concentrations. 3 µl of solutions were spotted. The chromatograms were run as described in section 2.2. and it is shown in Figure 12-13.

RESULTS AND DISCUSSION

Optimization of HPTLC method

Initially, trials were carried out using chloroform: methanol, toluene: methanol, methanol: ethyl Acetate, benzene: methanol: triethylamine in various proportions, to obtain the satisfactory resolution between the two drugs along with desired system suitability parameters. After several trials, benzene: methanol: triethylamine (8.5: 1: 0.5, v/v/v) selected as optimum mobile phase with chamber saturation time 20 min, which gave good resolution and sharp peaks for both the drugs. Other chromatographic conditions like run length, sample application volume, sample application positions, distance between tracks, detection wavelength, were optimized to give reproducible R_f values and symmetrical peak shape for the drug peak.

Validation of the method

Linearity

Linearity responses for MET and BENT were assessed in the concentration range 500-3000 ng/spot and 75-450 ng/spot, respectively. The linear equations for the calibration plots were $y = 1.279x + 3026$ and $y = 8.927x + 605.4$, with correlation coefficient (r) being 0.999

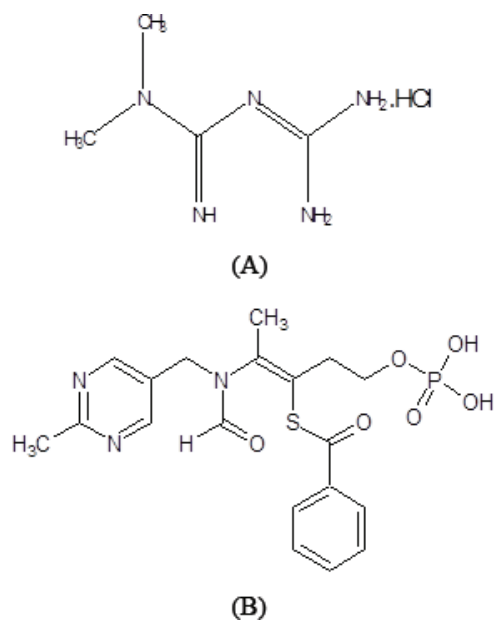


Figure 1: Chemical structures of MET (A) and BENT (B)

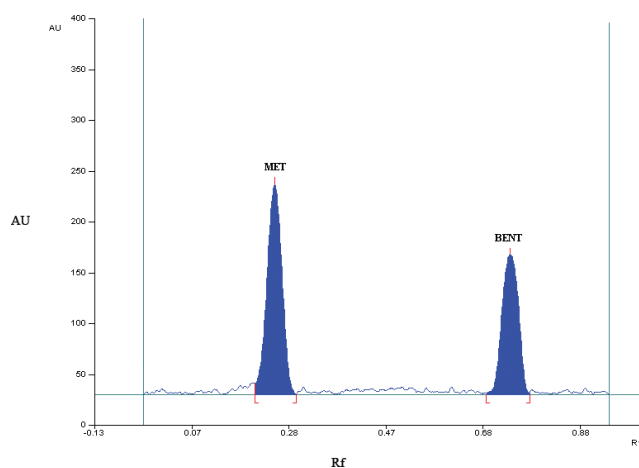


Figure 2: Densitogram of standard MET and BENT

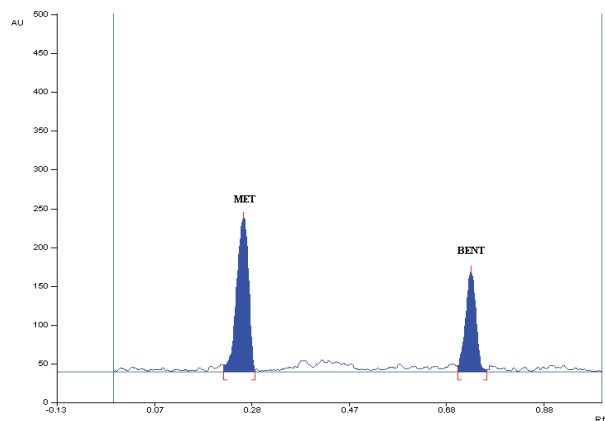


Figure 3: Representative densitogram of mixed standard solution MET(1500 ng/band, $R_f = 0.26 \pm 0.04$) and BENT (225 ng/band, $R_f = 0.72 \pm 0.03$)

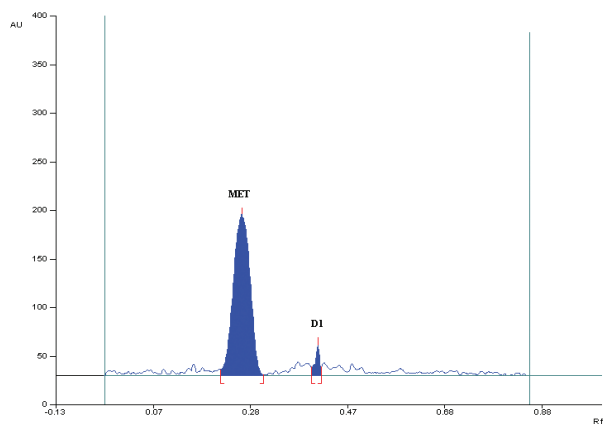


Figure 4: Representative densitogram of MET after acid degradation D1 (Rf = 0.44)

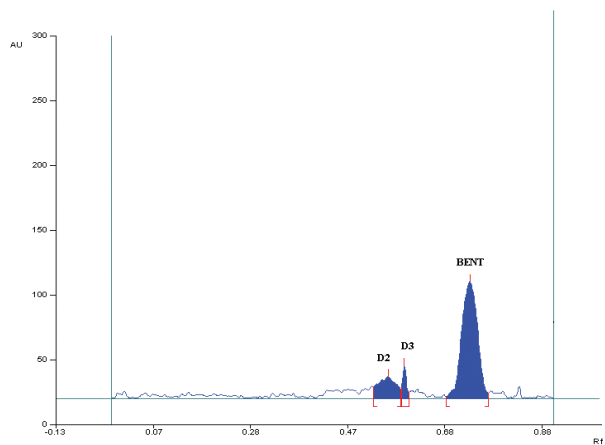


Figure 5: Representative densitogram of BENT after acid degradation D2 (Rf = 0.54) and D3 (Rf = 0.59)

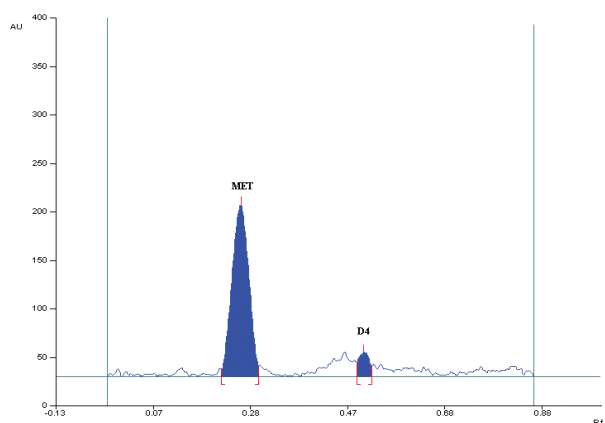


Figure 6: Representative densitogram of MET after alkaline hydrolysis D4 (Rf = 0.51)

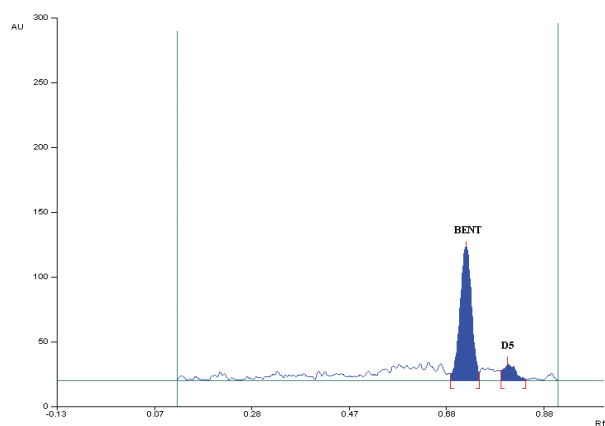


Figure 7: Representative densitogram of BENT after alkaline hydrolysis D5 (Rf = 0.82)

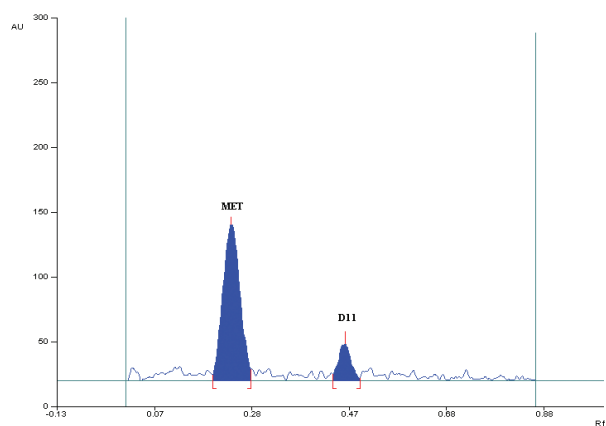


Figure 10: Representative densitogram of MET after dry heat degradation D11 (Rf = 0.47)

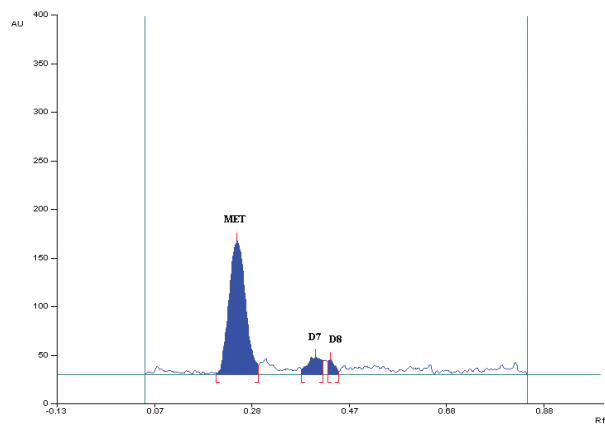


Figure 8: Representative densitogram of MET after oxidative degradation D7(Rf = 0.40) and D8 (Rf = 0.45)

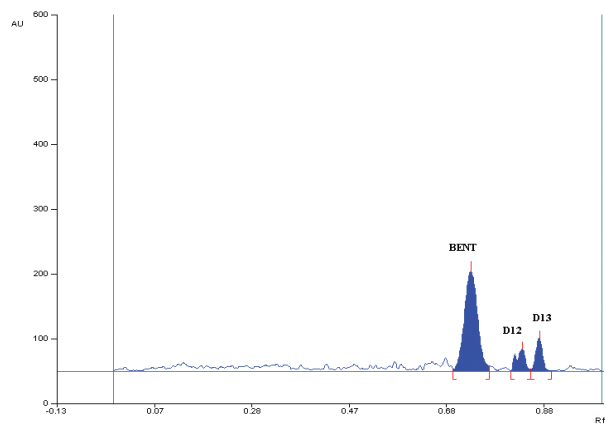


Figure 11: Representative densitogram of BENT after dry heat degradation D12 (Rf = 0.83) and D13 (Rf = 0.88)

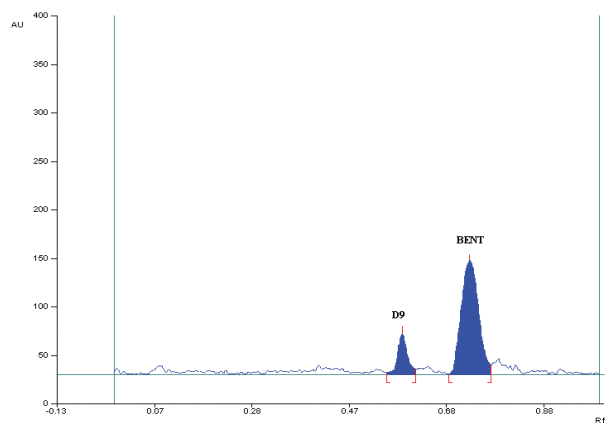


Figure 9: Representative densitogram of BENT after oxidative degradation D9 (Rf = 0.58)

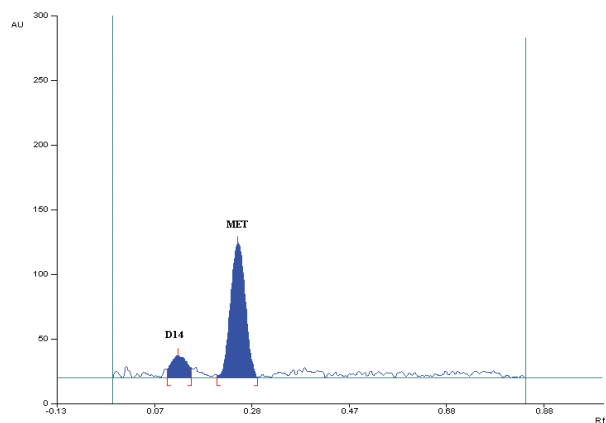


Figure 12: Representative densitogram of MET after exposing to light D14 (Rf = 0.14)

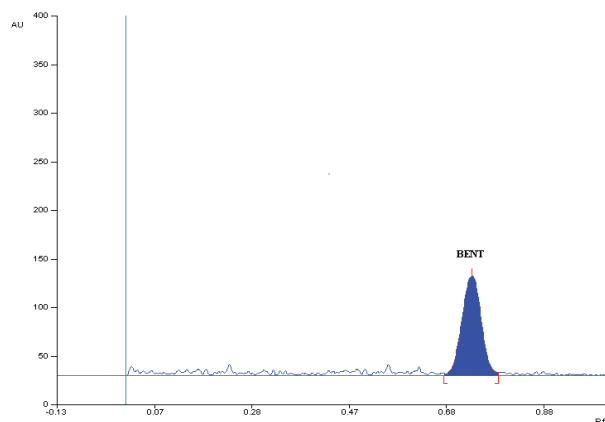


Figure 13: Representative densitogram of BENT after exposing to light

and 0.999 for MET and BENT, respectively. Range was established with five replicate readings of each concentration.

Precision

Precision of the method was determined in the terms of intraday and interday variations (%RSD). Intraday precision (%RSD) was assessed by analysing standard drug solutions within the calibration range, three times on the same day, %RSD was found to be 0.56-0.64 for MET and 0.69-1.07 for BENT.

Interday precision (%RSD) was assessed by analysing standard drug solutions within the calibration range on three different days over a period of week. %RSD

was found to be 0.70-0.84 for MET and 1.14-1.43 for BENT. This indicates that adequate preciseness of the method.

Limit of detection and limit of quantitation

Detection limit and quantification limit was calculated by the method as described in Section 2.4.2. The LOQ and LOD for MET were 426 mg and 141 mg/spot. For BENT, LOQ and LOD were found to be 59 mg and 20 mg, respectively. This indicates that adequate sensitivity of the method.

Accuracy

To the preanalysed sample a known amount of standard solution of pure drug (MET and BENT) was over spotted at three different levels. These solutions were subjected to reanalyze by the proposed method and results of the same are shown in Table 2.

Robustness

The standard deviation of peak areas was calculated for each parameter and %RSD was found 1.18-1.28. The low %RSD indicates robustness of the method. The summary of Robustness parameters were listed in Table 3.

Repeatability

Repeatability of sample application was assessed by spotting (300 ng/spot) of drug solution seven times on a TLC, followed by development of plate and recording

Table 1: Assay of tablet formulation.

Component	Label claim (mg)	%Label claim*	%RSD
MET	500	99.98	1.06
BENT	75	99.41	1.58

* mean of six estimations

%RSD- % Relative Standard Deviation

Table 2: Results of recovery studies.

Component	Label claim (mg/tablet)	(%) Amount of standard drug added	% Drug recovered *	%RSD
MET	500	80	99.82	0.6342
	500	100	100.08	0.6263
	500	120	100.26	0.8535
BENT	75	80	100.26	1.3614
	75	100	99.88	0.6372
	75	120	100.31	0.6231

* mean of three estimations at each level.

%RSD- % Relative Standard Deviation

Table 3: Robustness of MET and BENT

Sr. No.	Parameters	Variation	% R.S.D*	
			MET	BENT
1.	Mobile phase composition	± 1% Methanol	1.18	1.28
2.	Change in wavelength	± 1 nm	0.75	0.53

*Average of three determinations

Table 4: Summary of validation parameter.

Parameter	MET	BENT
Linearity range (ng/spot ⁻¹)	500-3000	75-450
Correlation coefficient	0.999	0.999
Limit of Detection (ng/spot ⁻¹)	140.74	19.60
Limit of quantitation (ng/spot ⁻¹)	426.48	59.39
% Recovery (n=9)	100.05	100.15
Precision (%R.S.D.)		
Interday (n=3)	0.70-0.84	1.14-1.43
Intraday (n=3)	0.56-0.64	0.69-1.07
Robustness	Robust	Robust
Specificity	Specific	Specific

Table 5: Forced degradation of MET and BENT

Sample exposure condition	Number of degradation products (Rf values)		Drug remained		Recovery (%)	
	MET	BENT	MET (1500 ng/spot)	BENT (750 ng/spot)	MET	BENT
0.1N HCl, 4 h, Reflux at 80°C	1(0.44)	2(0.54,0.59)	1387.95	574.87	88.53	76.64
0.1 N NaOH, Reflux 4h at 80°C	1(0.51)	0.82	1297.80	652.87	86.52	87.05
3% H ₂ O ₂ Reflux at 80° C	2 (0.40,0.45)	0.58	1173.45	552.82	78.23	73.71
Photo	1 (0.14)	-	1289.80	648.37	85.95	86.45
Heat 80 °C for 2 h	1 (0.47)	2 (0.83,0.88)	1289.25	540.82	85.32	72.06

the peak area for seven spots. The %RSD for peak area values of MET and BENT was found to be 1.21 and 0.57, respectively. The summary of validation parameters were listed in Table 4.

Stability-indicating Property

The chromatogram of samples degraded with acid, base, hydrogen peroxide and light showed well separated spots of pure MET and BENT as well as some additional peaks at different Rf values. The number of degradation product with their Rf values, content of MET and BENT remained, and percentage recovery were represented in Table 5.

CONCLUSION

The proposed developed HPTLC method is validated as per ICH guidelines. The low value of % RSD indicates high degree of precision of method. The results of the recovery studies performed shows high degree of accuracy for developed method. So it can be concluded that proposed HPTLC method is accurate, precise, specific and can be employed successfully for estimation of MET and BENT in pharmaceutical dosage forms.

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CONFLICT OF INTEREST

No conflict of interest are declared.

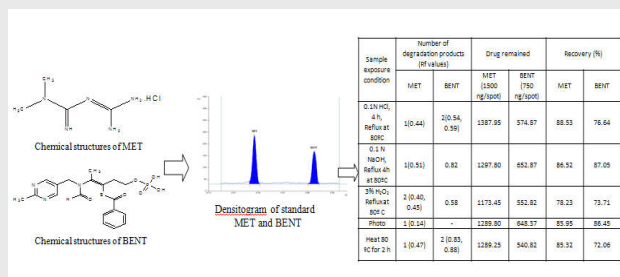
ABBREVIATION USED

MET: Metformine Hydrochloride; **BENT:** Benfotiamine; **Rf:** Retention Factor; **ICH:** International conference on Harmonization; **LOD:** Limit of Detection; **LOQ:** Limit of Quantitation; **S.D.:** standard Deviation; **%R.S.D.:** % Relative Standard Deviation.

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Pictorial Abstract



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SUMMARY

- Extensive literature survey revealed that no stability indicating HPTLC method has been reported so far for the simultaneous estimation of the MET and BENT in combined tablet dosage form. So the work was aimed at development and validation of a simple, accurate and precise stability indicating HPTLC method for simultaneous quantification of these compounds as bulk drugs and in combined tablet dosage form. Different mobile phases containing various ratios of Chloroform: methanol, Toluene: methanol, Methanol: Ethyl Acetate, Benzene: methanol: Triethylamine, Toluene: Methanol: Ethyl acetate were tried to achieve the separation of two drugs. Finally the mobile phase comprising Benzene: methanol: Triethylamine (8.5: 1: 0.5, v/v/v) was selected as optimal for obtaining well defined and resolved peaks for both the drugs. Results were found to be linear in the concentration range of 500-3000 ng/band for MET and 75-450 ng/band for BENT with high correlation coefficient. The recovery study results ranged from 99.82 to 100.26 for MET and 99.88 to 100.31 for BENT %. The method was found to be accurate and precise, as indicated by recovery studies as recoveries were close to 100 % and % R.S.D. not more than 2. Both the drugs were found to be susceptible to the stress conditions used when exposed to stress degradation as per ICH Guidelines.

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