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DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF MYO-INOSITOL AND METFORMIN HYDROCHLORIDE IN BULK AND DOSAGE FORM

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Keywords:

Myo-Inositol, Metformin HCl, C18 column, Retention time, Validation, ICH guidelines

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ABSTRACT: A simple, sensitive, specific, accurate reversed phase high performance liquid chromatographic method was developed for the simultaneous estimation of Metformin HCl and Myo-Inositol in bulk and pharmaceutical dosage form. RP-HPLC separation was achieved on C-18 (250mm x 4.6mm, 5µm) column. The mobile phase composed of Water: Acetonitrile: Methanol (50:30:20 % v/v/v) [HPLC Grade] at flow rate 1ml/min with UV detection at 228 nm. The mobile phase consists of Ph 4. The retention times of Myo-Inositol and Metformin HCl were found to be 4.627 min and 4.002 minutes respectively. Assay of Myo-inositol and Metformin Hydrochloride was found to be 99.86% and 99.82% respectively. This method can be successfully employed for simultaneous quantitative analysis of Metformin HCl and Myo-Inositol in bulk drugs and formulations. The results indicate that there is no interference from excipients for the proposed method, thus making the method simpler, less time consuming. Chromatography parameters were validated as per ICH guidelines and the method can be applicable for routine quantitative analysis of drugs in combined dosage form.

INTRODUCTION: Myo-inositol is a 6-carbon cyclic polyalcohol **Fig. 1** that occurs as anhydrous, hydroscopic crystals. Myo-inositol has a sweet taste, is soluble in water, and is slightly soluble in alcohol. It is insoluble in ether and other organic solvents [1]. Inositols are pseudovitamin compounds that are falsely said to belong to the B-complex family. Inositol or its phosphates and associated lipids are found in many foods, in particular fruit, especially cantaloupe and oranges. PCOS is one of the most common endocrine disorders, affecting up to 20% of women of reproductive age [2].

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The diagnostic criteria for PCOS include chronic oligomenorrhea or anovulation, hyperandrogenism, and polycystic ovarian morphology [3]. PCOS is associated with an increased risk of developing hypertension, dyslipidemia, type 2 diabetes, and heart disease [4-6]. Insulin resistance is another common feature of PCOS in both overweight and lean women, and it is often treated with insulin sensitizers like metformin [7]. Over the last decade, myo-inositol, an isomerized and dephosphorylated precursor of glucose-6-phosphate, has been used more and more as a natural insulin sensitizer [8, 9].

High doses (usually in the 12-18g range) are required for any neurological effects while lower doses (2-4g) are sufficient for fertility and insulin sensitizing effects [10]. Literature survey revealed The HPLC methods for estimation of Myo-inositol in Bulk, human plasma and pharmaceutical dosage forms. LC-MS-MS method was reported for the

determination of Myo-inositol in human plasma [11]. Literature survey reveals several Analytical and Bioanalytical methods for the analysis of Myo-inositol. These methods reported with Myo-inositol alone or in combination with another drug. These include, HPLC and spectrophotometric analysis of Myo-inositol in tablets.

Metformin HCl **Fig. 2** is an oral antidiabetic drug in the biguanide class. It is most widely prescribed antidiabetic drug in the world used to treat type 2 diabetes. Metformin HCl helps to control the amount of glucose (sugar) in blood. It decreases the amount of glucose and also increases body's response to insulin, a natural substance that controls the amount of glucose in the blood [12]. It is not used to treat type 1 diabetes. It is also used for treatment of gestational diabetes, polycystic ovary syndrome (PCOS). It works by decreasing hyperglycemia primarily by suppressing glucose production by the liver (hepatic gluconeogenesis). It helps to reduce LDL cholesterol and triglyceride levels, and is not associated with weight gain. Metformin HCl comes as a liquid, as a tablet, and as an extended-release (long-acting) tablet taken orally [13]. It is used alone or with other medications. Very rare but serious side effect with Metformin HCl is lactic acidosis. Other than that common side effect are gastrointestinal irritations, including diarrhea, cramps, nausea, vomiting and increased flatulence [11, 12].

The combination of myo-inositol and metformin HCl is often used to manage Polycystic Ovary Syndrome (PCOS). Here's a brief overview of their pharmacology: Myo-inositol is a vitamin-like substance that plays a crucial role in cellular signaling and insulin sensitivity. Myo-inositol helps improve insulin sensitivity, which can lead to better ovulation and menstrual cycle regulation in women with PCOS. Metformin is an antidiabetic medication belonging to the biguanide class [13]. Metformin works by reducing glucose production in the liver, improving insulin sensitivity, and enhancing peripheral glucose uptake. Combining myo-inositol with metformin can enhance the body's response to insulin and improve hormonal balance. This combination is particularly effective in regulating menstrual cycles and improving ovulation in women with PCOS. Studies have shown that this combination can be more effective than either agent alone in improving metabolic and hormonal parameters.

As combined dosage forms become increasingly vital in treatment, the necessity for accurate and reliable analytical methods to quantify these active pharmaceutical ingredients (APIs) is paramount. This project centers on the development and validation of RP-HPLC methods for the simultaneous estimation Myo-inositol and Metformin hydrochloride in combined dosage form. Reverse-phase high-performance liquid chromatography (RP-HPLC) is chosen for its established efficacy in separating and quantifying pharmaceutical compounds, offering high resolution, sensitivity, and accuracy. Literature review suggests few HPLC determinations were performed [14]. These methods reported with Metformin alone or in combination with another drug. These include HPLC and spectrophotometric analysis of Metformin in tablets [15, 16, 17].

Therefore, this was an attempt to develop simple, robust, reproducible RP-HPLC method for the determination of efficacy and safety of myo-inositol and metformin hydrochloride combination. This method was fully validated according to International Conference on Harmonization (ICH) and ready for the application in routine analysis without interference of an excipients.

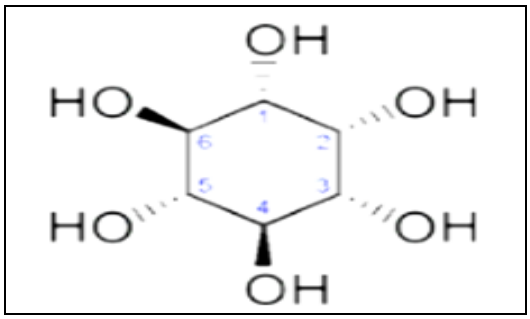

FIG. 1: STRUCTURE OF MYO-INOSITOL

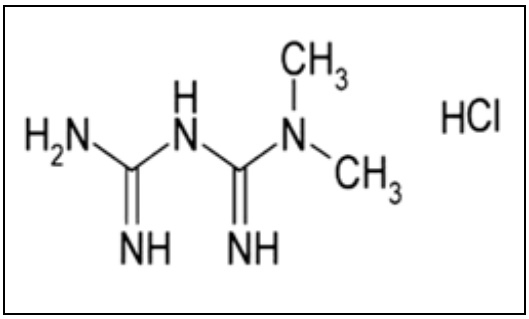

FIG. 2: STRUCTURE OF METFORMIN HCL

MATERIALS AND METHODS:

Instruments: A Systronics LC 138 chromatographic system is used for the quantitative analysis. It consists of a prominence solvent delivery module, a manual injector with a 20µL fixed loop, Pressure pump, UV-visible detector, operated by computer software Clarify 2.0. A separation was performed on Hibar® (Merck Germany) C18 column with dimensions 250×4.6mm, particle size 5µm at an ambient temperature. A Fast Clean ultrasonicate Sonicator was used for the degassing purpose. Weighing balance Sansui Vibra DJ-150S-S was used for the weighing of samples and reagents, pH meter (Equiptronics EQ 621) was used for the checking and maintaining pH of the mobile phase, and filter papers of Sartorius Stedim grade 292 was used for the filtration of mobile phase and other chemical reagents.

Chemicals and Reagents: Myo-inositol (20 gm) and metformin HCL (20 gm) pure drugs were obtained as a gift sample fromEris lifesciences limited Amingaon, north Guwahati. A combined formulation Metital (600mg/500mg) of two drugs purchased from Permanand pharmacy Jalgaon. Analytical grade methanol purchased from Merck chemicals Pvt. Ltd. Mumbai.

Method Development: Various pre-trials of the mobile phases have been carried out before selecting the proper mobile phase. Finally, acetonitrile, water and methanol with composition of 30:50:20(v/v/v) respectively, has been selected as a mobile phase and pH value for the same is 4.4 Flow rates between 0.5 ml/min to 1.5 ml/min has been studied, but optimal signal can't be obtained on those flow rates. So that, flow rate of 1.0 ml/min was selected, and it gave optimal signal with low signal to noise ratio and reasonable separation using C18 column. Total analysis time is 6 minutes. The maximum absorption of MIL and MET was detected at 254 nm. At this wavelength both Myo-inositol and Metformin Hydrochloride were showed complete resolution.

Selection of Stationary Phase: On the basis of reversed phase HPLC mode and number of carbon present in the molecule (analyte) RP-Purospher C_{18} column of following configuration was selected for further study. RP-Peanosphere C_{18}, 250·4.6mm, particle size 5µm.

Selection of Mobile Phase: Metformin Hydrochloride is soluble in methanol and Acetonitrile on ultrasonication, freely soluble in water, Myo-inositol is sparingly soluble in methanol, slightly soluble in 50% acetonitrile and water. These drugs on ultrasonication are soluble in methanol, Acetonitrile, and water mixture. Hence, mixture of Acetonitrile: water: methanol was taken in ratio 30:50:20 (v/v/v) and was used for initial separation.

Preparation of Mobile Phase: Acetonitrile, water and methanol were taken in the ratio of 30:50:20 v/v/v. Mobile phase was ultrasonicated for 15 minutes for degassing. pH adjusted between 4.3 - 4.5 using 10% o-phosphoric acid.

Preparation of Standard Solution and Stock Solution of MIL and MET:

Myo-inositol Standard Stock Solution [MIL]: An accurately weighed quantity of MIL (60 mg) was taken in 10 mL volumetric flask and was dissolved in methanol (6 mL). Then the volume was made up to 10ml using methanol to get MIL standard stock solution. Stock solution was filtered through a 0.45 µm membrane filter paper. The working standard solution of MIL was prepared from suitable aliquots of stock solution.

Metformin Hydrochloride Standard Stock Solution [MET]: An accurately weighed quantity of MET (50 mg) was taken in 10 mL volumetric flask and was dissolved in methanol (6 mL) with ultrasonication. Then the volume made up to 10 ml using methanol to get MET standard stock solution. Stock solution was filtered through a 0.45 µm membrane filter paper. The working standard solution of MET was prepared from suitable aliquots of stock solution.

Maintained Chromatographic Conditions:

Mobile Phase: Acetonitrile, Water and Methanol (30:50:20).

HPLC Column: Octadecylsilyl C18 Column (5µm, 4.6mm* 250mm).

Detection Wavelength: 254 nm.

Flow Rate: 1ml/min

Column Temperature: Ambient

Run Time: 6 minutes

Injection Volume: 20 µL

Diluent: Methanol

Selection of Detector and Detection Wavelength [18]: UV-Visible detector was selected, as it is reliable and easy to set at the correct wavelength. An overlay spectrum of Myo-Inositol (60mg/ml) and Metformin Hydrochloride (50mg/ml) in methanol was recorded. From the standard stock solution, further dilutions were made using methanol and scanned over the range of 200-400 nm. The individual spectrum was obtained and they were overlain. It was observed that the both drugs showed considerable absorbance at 254 nm. From the overlay spectrum 254nm nm was selected as a wavelength of measurement because at 218 to 231 the absorbance of met was to be intensely prominent as compare to Myo-inositol which will alter the detection of compound nearby therefore, isosbestic point at s254nm was selected as detection wavelength.

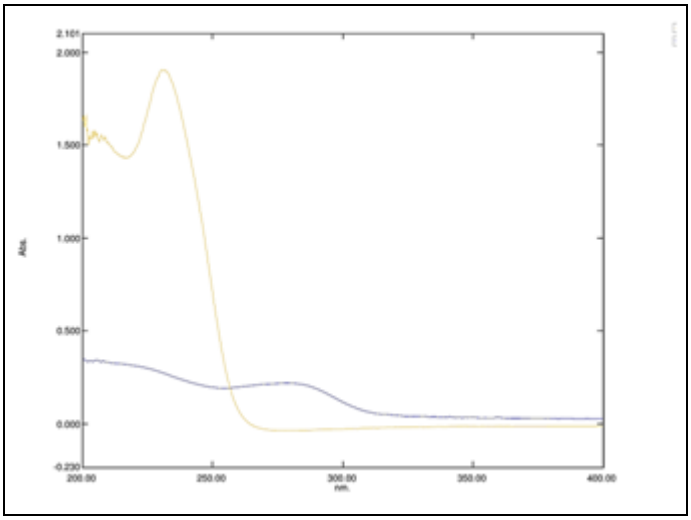

FIG. 3: OVERLAY SPECTRA OF MYO-INOSITOL AND METFORMIN HCL

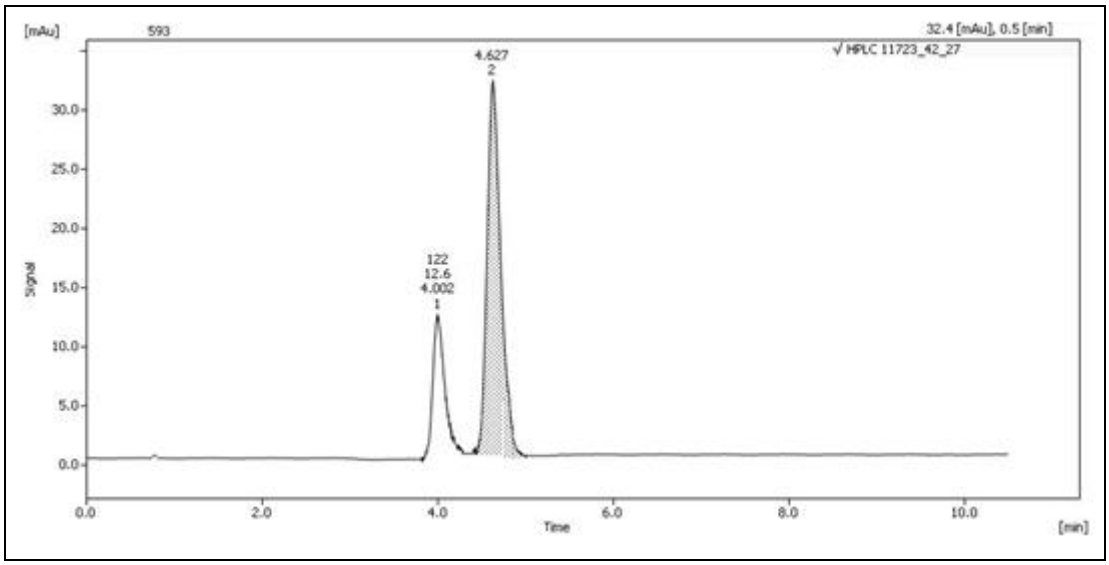

FIG. 4: TYPICAL LIQUID CHROMATOGRAM OBTAINED FOR A 20 ML INJECTION OF TABLET OF MYO-INOSITOL AND METFORMIN HYDROCHLORIDE

Calibration Curve (Linearity):

Linearity Study for Myo-inositol: An accurately measured aliquot portion of working standard solution of Myo-inositol was transferred to five separate 10 mL volumetric flasks. The volume was made up to the mark using 85 % v/v methanol to

obtain concentrations of Myo-inositol (15µg/ml, 30µg/ml, 45µg/ml, 60µg/ml, 75µg/ml). Absorbance of these solutions was measured at 254 nm. Chromatograms of each solution were recorded for 15 min. The results are shown in the

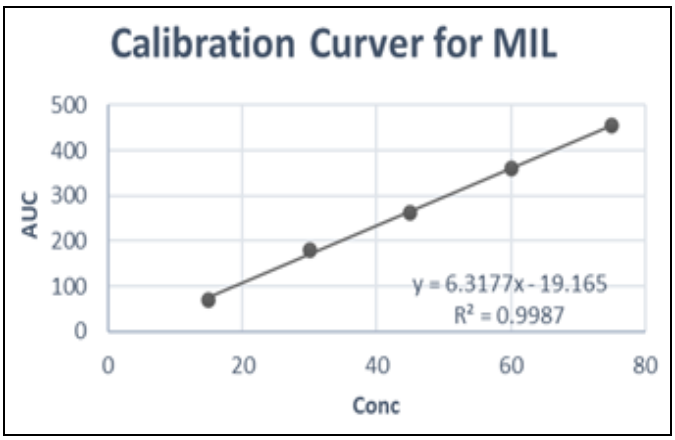

FIG. 5: CALIBRATION CURVE OF MYO-INOSITOL AT 254 NM

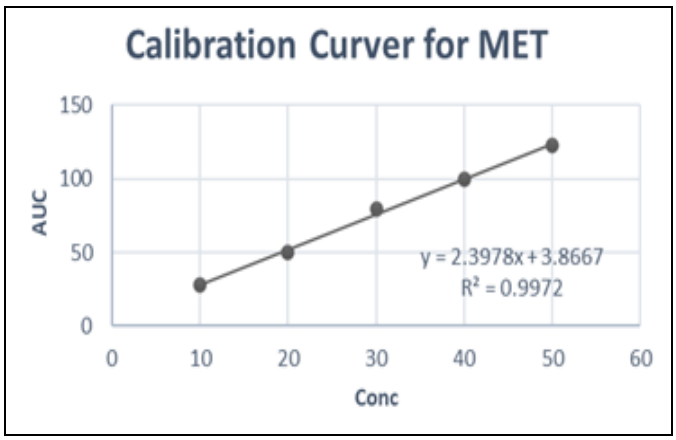

FIG. 6: CALIBRATION CURVE OF METFORMIN HCL AT 231NM

Linearity study for Metformin HCl: Accurately measured aliquot portions of working standard solution of Metformin Hydrochloride were transferred to five separate 10 mL volumetric flasks.

The volume was made up to the mark using 85% v/v methanol to obtain concentrations (10µg/ml, 20µg/ml, 30µg/ml, 40µg/ml, 50µg/ml) Absorbance of these solutions was measured at 231 nm. Chromatograms of each solution were recorded for 15 min. The results are shown in the **Fig. 4** Calibration curve of Metformin HCl at 231nm.

Standard Solutions: Stock standard solutions of Myo-Inositol (Mil) 1mg/mL and Metformin Hydrochloride (MET) 1mg/mL were prepared by dissolving 10 mg MIL and 10 mg MET in methanol. Working standard solutions of MIL 60 µg/mL and MET 50 µg/mL were prepared by diluting suitable aliquots of corresponding stock solutions with mobile phase **Table 1.**

TABLE 1: ASSAY RESULTS FOR THE COMBINED DOSAGE FORM USING THE PROPOSED HPLC METHOD (API)

Name of Drug	Percentage Purity	% RSD
Myo-Inositol	99.86 ± 0.257	±0.461
Metformin Hydrochloride	99.82 ± 0.357	±0.352

Sample Solution: The tablet quantity equivalent to MIL (60 mg) and MET (50 mg) was measured accurately, then it was transferred to a 100 mL volumetric flask containing methanol (50mL). Then the content was ultrasonicated for 20 min. and volume was made up to the mark using methanol. The above solution was filtered through Whatmann filter paper No.1. This solution was again filtered through 0.45µm Millipore membrane filter. From this solution (1mL) was diluted to 10 mL using mobile phase to get MYO (60 µg/mL) and MET (50µg/ml) solution. The content was ultrasonicated for 20 min **Table 2.**

TABLE 2: ASSAY RESULTS FOR THE COMBINED DOSAGE FORM USING THE PROPOSED HPLC METHOD (TABLET)

Name of Drug	Percentage Purity	% RSD
Myo-Inositol	99.86 ± 0.257	±0.461
Metformin Hydrochloride	99.82 ± 0.357	±0.352

Validation of Chromatographic Method: The analytical method was optimized and must be validated before practical use, the ICH Guidelines for analytical method validation Q2 (R_1) [18], SST were performed and parameters were categorized below.

Accuracy (% Recovery Studies): Accuracy refers to the closeness of a measured value to a standard value. Accuracy studies were carried out by adding a known number of pure drugs of MIL and MET to the pre-analyzed sample solution. The percentage recovery studies were carried out by spiking 80%, 100% and 120% of respective drug, each level was injected 3 time. The recovery studies showed that the results were within acceptable limits, above 99% and below 101%. The results are given in **Table 3.**

TABLE 3: STANDARD ADDITION TECHNIQUES FOR DETERMINATION OF MYO-INOSITOL AND METFORMIN HYDROCHLORIDE (N = 6)

Drug	Weight of Drug in each Tablet powder taken (mg)	Recovery (%) mean
MIL	600	99.77
MET	500	99.73

Precision (Repeatability): The precision of the method has been evaluated by injecting the six replicate sample preparations. The percentage assay for both MIL and MET were calculated and tabulated which showed that results were within acceptable limit i.e. % RSD below 2.0 indicating that the method is reproducible. The results are shown in **Table 4.**

TABLE 4: RESULTS OF PRECISION STUDY REPEATABILITY STUDY OF MIL AND MET

Replicate	MIL	MET
Mean Peak Area	362.474	122.433
S. D	0.5405	0.5489
% RSD	0.4833	0.4589

*Mean of six Observations

Drug	% of drug found	S. D	% RSD
MIL	99.88	0.5551	0.4578
MET	99.58	0.5447	0.4577

*Mean of three observations

Drug	% of drug found	S. D	% RSD
MIL	99.97	0.4577	0.4577
MET	99.56	0.4154	0.4155

*Mean of three observations

Intermediate Precision (Reproducibility): The intraday and interday precisions of the proposed method were determined by estimating the corresponding responses 5 times on the same day and on 5 different days for present method.

The results are reported in terms of relative standard deviation (RSD).

Robustness of the Method: To ensure the insensitivity of the developed HPLC method to minor changes in the experimental conditions, it is important to demonstrate its robustness. None of the alterations caused a significant change in resolution between MIL and MET, retention time and Theoretical plates. The results are shown in **Table 5.**

TABLE 5: RESULTS OF ROBUSTNESS STUDY

Factor	Level	Retention Time		Theoretical Plates	
Flow Rate (mL/min)		MIL	MET	MIL	MET
Mean ± S. D		4.569 ± 0.221	4.041 ± 0.126	4586.07 ± 1.694	4271.25 ± 1.138
% of Methanol in the Mobile Phase (v/v)					
Mean ± S.D		4.627 ± 0.163	4.040±0.071	4586.694 ± 1.771	4271.30 ± 0.890
pH of Mobile Phase					
Mean ± S.D		4.614 ± 0.062	3.992 ± 0.281	4587.309 ± 1.264	4271.98 ± 1.387

Ruggedness of the Method: Ruggedness study was carried out using only one parameter i.e. different analyst. The result showed that the % RSD were below 2%.

The results are shown in **Table 6**. This study was signified the ruggedness of the method under varying conditions of its performance.

TABLE 6: RUGGEDNESS STUDIES

Drug	Label Claim (mg)	Amount Found (%) ± S.D. (n = 3)	
		Analyst I	Analyst II
MIL	600	599.88 ± 0.5747	599.97 ± 0. 4964
MET	500	499.92 ± 0.4754	499.99 ± 0.4182

Limit of Detection (LOD) and Limit of Quantification (LOQ): The limit of detection (LOD) and limit of quantitation (LOQ) for the procedure were performed. LOD was expressed by establishing the minimum level at which the analyte can be reliably detected. LOQ was considered as the lowest concentration of analytes in standard that can be reproducibly measured with acceptable accuracy and precision.

Limit of detection (LOD) and limit of quantitation (LOQ) were separately determined at a signal to noise ratio (S: N) of 3 and 10 and based on the calibration curves. The standard deviation of the y – intercept and slope of the regression line were used.

The LOD and LOQ were calculated using the formulas,

$$LOD = 3.3 \times D / S$$

$$LOQ = 10 \times D / S$$

Where, S = Slope of regression line, D = Standard deviation of y- intercept on the regression line

The results are shown in **Table 7.**

TABLE 7: RESULTS OF LOD AND LOQ BY RP-HPLC METHOD

Parameter	Myo-inositol	Metformin hydrochloride
LOD (µg/mL)	0.1416	0.5898
LOQ (µg/mL)	0.4291	1.9662

System Suitability Parameters: In the system suitability test, the binary solution of 60 µg/ml of MIL and 50 µg/ml of MET (n=6) was prepared and injected. Then the system suitability parameters like retention time, theoretical plates, tailing factor and resolution were calculated from the chromatogram. Results are shown in **Table 8.**

TABLE 8: SYSTEM SUITABILITY TEST PARAMETERS

System Suitability Parameters	Proposed Method	
	MIL	MET
Retention Time (t_R)	4.627	4.022
Capacity Factor (k)	3.627	3.002
Theoretical Plate Number (N)	4588.660	4272.220
Asymmetry factor	1.787	1.925
Resolution Factor (R)	2.418	

Specificity: Specificity of an analytical method is its ability to measure accurately, and specifically, the concentration of analyte without interference from other API, diluents, mobile phase, Specificity was checked by determining MIL and MET in laboratory prepared binary mixture and in binary mixture containing different degradation products.

RESULTS AND DISCUSSION: The absorption spectra of MIL and MET greatly overlap; so conventional determination of these compounds in mixture is not possible. To optimize the LC parameters, several mobile phase compositions were tried. A satisfactory separation and good peak symmetry for MIL and MET were obtained with a mobile phase consisting of Acetonitrile: Methanol: Water (30:50:20 v/v/v), pH 4.0 adjusted using 10% o-phosphoric acid. Quantification of the drugs was performed at 254 nm. Resolution of the components with clear baseline separation was obtained.

Validation of the Chromatographic Method:

Linearity: Linear correlation was obtained between peak areas and concentrations of MIL and MET in range of 15µgm/ml to 75µgm/ml, for both drug compounds. The linearity of calibration curves was found to be acceptable over the concentration ranges of 10 µgm/ml to 50 µgm/ml for Myo-inositol and Metformin Hydrochloride,

with a R^2 values 0.9987 and 0.9972. The results show that good correlation existed between the peak area and concentration of the analysts.

Accuracy: The recovery experiments were performed by the standard addition method. The recoveries obtained were 99.78% and 99.75% for MIL and MET, respectively. The high values indicate that the method was accurate.

Precision: Precision study was carried out using parameter like method repeatability study which showed that results were within acceptable limit of both the drugs MIL and MET respectively 0.4833 and 0.4589 i.e. % RSD below 2.0 indicating that the method is reproducible.

Intermediate Precision: The intraday RSD values for MIL and MET were 0.4578 and 0.4577 respectively. The interday RSD values for MIL and MET were 0.4577 and 0.4155 respectively. The % RSD (< 2%) values indicate that the method was sufficiently precise.

Robustness: The method was found to be robust with no significant changes on test result upon change of analytical conditions like different flow rate, 85% methanol in mobile phase and pH of mobile phase with the standard deviation was found to be below 1 and % RSD is less than 2 for all results. It was found that under small deliberate changes of chromatographic factors, there was no considerable change in under study parameters.

Ruggedness Study: Ruggedness study of MIL and MET are carried out and results are found to be S.D. is not less than 0.4184 accurate by statistical manner and obeys ICH guidelines.

LOD and LOQ: LOD values for MIL and MET were found to be 0.1416µg/mL and 0.5898µg /mL, respectively. LOQ values for MIL and MET were found to be 0.0.4291µg/mL and 1.9662µg/mL, respectively. These data showed that the method was sensitive enough for the determination of MIL and MET.

System Suitability Test: A binary solution of 60µg/mL of MIL and 50µg/mL MET (n=5) was prepared and same was injected, then the system suitability parameters were calculated from the chromatogram. The parameters, retention times, resolution factor, tailing factor and theoretical plates were evaluated. The results obtained from system suitability tests are in agreement with the official requirements.

CONCLUSION: The proposed LC method presented in this paper has advantages of simplicity, accuracy, precision and convenience for separation and quantitation of MIL and MET in combination and can be used for the assay of their respective dosage form. Moreover, the proposed HPLC method is a stability indicating assay method that can determine MIL and MET in presence of their degradation products. Thus, the proposed HPLC method can be used for the quality control of MIL and MET in typical laboratories.

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CONFLICTS OF INTEREST: Nil

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