Original Article

METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS ESTIMATION OF METFORMIN HCl AND MYO-INOSITOL IN PHARMACEUTICAL DOSAGE FORM BY USING RP-HPLC

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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 pharmaceutical dosage form. RP-HPLC separation was achieved on an Enable C-18 250mm x 4.6mm, 5μm column. The mobile phase composed of Acetonitrile: Water (70:30 % v/v) [HPLC Grade] at flow rate 1ml/min with UV detection at 228 nm. The retention times of Metformin HCl and Myo-Inositol were found to be 2.752 min and 3.626 min respectively. Linearity was established for Metformin HCl and Myo-Inositol in the range of 25-150μg/ml and 60-360μg/ml respectively. System precision and method precision was found to be within the limits of the acceptance criteria. Relative standard deviation of Metformin HCl and Myo-Inositol for System precision was found to be 0.22 and 0.02 respectively and method precision was found to be 0.23 and 0.257 respectively. The percentage recoveries for Metformin HCl and Myo-Inositol were found to be in the range of 100.15-102.0% and 99.75-100.04% respectively. The limit of detection was 0.17μg/ml and 1.08μg/ml respectively. The limit of quantification was 0.52μg/ml and 0.37μg/ml respectively and the method was found to be specific. 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 and suitable for routine estimation of Metformin HCl and Myo-Inositol tablet formulation.

KEYWORDS: RP-HPLC, Metformin HCl, Myo-Inositol, formulation.

INTRODUCTION

Metformin HCl

Metformin HCl [figure1] 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. It is not used to treat type 1 diabetes.[1] It is also used for treatment of gestational diabetes, polycystic ovary syndrome (PCOS).[1] 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. 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. Literature survey revealed the HPLC methods for estimation of Metformin HCl in Bulk, human plasma and pharmaceutical dosage forms.[2–7] LC-MS-MS method was reported for the determination of Metformin in human plasma.[8] Literature survey reveals several Analytical and Bioanalytical methods for the analysis of Metformin. These methods reported with Metformin alone or in combination with other drug. These include HPLC[9-11] and spectrophotometric analysis of Metformin in tablets.[12-15]

Myo-inositol

Myo-inositol is a 6-carbon cyclic polyalcohol (Figure 2) 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.[16] 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.[17] The diagnostic criteria for PCOS include chronic oligomenorrhea or anovulation, hyperandrogenism, and polycystic ovarian morphology.[18] PCOS is associated with an increased risk of developing hypertension, dyslipidemia, type 2 diabetes, and heart disease.[19-21] Insulin resistance is another common feature of PCOS in both overweight and lean women,[22] and it is often treated with insulin sensitizers like metformin.[23,24] 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. 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.[25] 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. 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 other drug. These include, HPLC and spectrophotometric analysis of Myo-inositol in tablets.

MATERIALS AND METHODS

Chemicals and reagents

Metformin HCl and Myo-Inositol pure drugs were provided by Aurobindo Pharma Hyderabad, formulation tablets were purchased from local pharmacy. HPLC grade Water and Acetonitrile were purchased from Merck specialties Pvt. limited, Mumbai. 0.45μm nylon membrane filter papers were obtained from Pall Life Sciences, Mumbai. A combined dosage tablet METITAL was purchased from local market.

Instrumentation

Isocratic Shimadzu HPLC instrument on an Enable C-18 250mm x 4.6mm, 5μm column. The Instrument is equipped with binary pump and variable wavelength PDA detector. A 20μL Hamilton syringe was used for injecting the samples. Data was analysed by using LC Solutions software. Shimadzu UV-Visible spectrophotometer was used for spectral studies. Degassing of the mobile phase was done by using a Loba ultrasonic bath sonicator. A Shimadzu balance was used for weighing the materials. All samples were filtered through 0.45μm membrane filter. Mobile phase and sample/standard preparations were degassed using a sonicator.

Chromatographic conditions

Reverse phase high performance liquid chromatographic method was developed on a 5μm Enable C-18 250mm x 4.6mm, 5μm column using mobile phase containing Acetonitrile: Water (70:30 % v/v) at ambient temperature. The elution was carried out isocratically at flow rate of 1ml/min. The UV detector was set at 228nm.

Preparation of standard solution

Accurately Weighed and transferred 25mg of Metformin HCl and 60mg of Myo-Inositol working Standards into a 100ml clean dry volumetric flask, add 3/4th volume of HPLC graded water sonicated for 5 minutes and make up to the final volume with diluents to get concentrations like 0.25μg/ml of Metformin HCl and 0.6μg/ml of Myo-Inositol . Mix well and filter through 0.45μm filter.

Preparation of sample solution

Accurately transfer the contents of 20 tablets of Metformin HCl and Myo-Inositol, ground into a fine powder and calculate the average weight. Weigh and transfer the sample equivalent to 10mg of sample into a 100ml volumetric flask add about 50 mL of mobile phase as diluent, sonicate to dissolve it completely and filter through 0.45μm filter.

RESULTS AND DISCUSSION

Method Development

A Reverse phase HPLC method was developed considering the criteria the system suitability parameters i.e. resolution factor (Rf) between peaks, tailing factor (T), number of theoretical plates (N), run time and the cost effectiveness. The optimized method developed resulted in the elution of Metformin at 2.752min, Myo - Inositol at 3.626 min. Figures 3, 4 and 5 represent chromatograms of blank solution, mixture of sample and standard solutions respectively. The total run time is 8 minutes with all system suitability parameters meeting acceptable criteria for the mixture of standard solution.

System Suitability

System Suitability tests are an integral part of method development and are used to ensure adequate performance of the chromatographic system. Retention time (Rt), number of theoretical plates (N), peak resolution (Rs) and peak Tailing factor (T) were evaluated for six replicate injections of the mixture of standards at working concentration. The results were given in Table 2 within acceptable limits. In order to test the applicability of this developed method to a commercial formulation, 'METITAL' was chromatographed at a concentration equivalent to working standards concentration and it is shown in Figure 6. The sample peaks were identified by comparing the relative retention times with the standard drugs mixture (Figure 4). System suitability parameters were within the acceptance limits, ideal for the

chromatographed sample. Integration of separated peak area was done and each drug concentration was determined by using the peak area concentration relationship obtained in the standardization step.

METHOD VALIDATION
Validation of the analytical method is the process that establishes by laboratory studies in which the performance characteristics of the method meet the requirements for the intended analytical application. RP-HPLC method developed was validated according to International Conference on Harmonization (ICH) guidelines for validation of analytical procedures. The method was validated for the parameters like system suitability, linearity, accuracy, precision, robustness, limit of detection (LOD) and limit of quantification (LOQ) found. The standard solution with Accuracy 50%, Accuracy 100% and Accuracy 150% were injected into chromatographic system and calculated the amount found and amount added for Metformin HCl and Myo-Inositol and further calculated the individual recovery values.

Linearity
It is the ability of the method to elicit test result that is directly proportional to analytic concentration within a given range. It is generally reported as variance of slope or regression line. It is determined by series of three to six injections of five of more standards. Different levels of solution were prepared and injected to the chromatographic system and the peak area was measured. Plotted a graph of peak area versus concentration (on X-axis concentration and on Y-axis Peak area) and calculate the correlation coefficient. The calibration curve was represented in fig. 13 & 14. (Table 3).

System Precision
It is a measure of degree of repeatability of an analytical method under normal operation and it is normally expressed as % of relative standard deviation (% RSD). The standard solution was injected for six times and measured the area for all six injections in HPLC. The % RSD for the area of six replicate injections was found to be within the specified limits. (Table 4).

Method Precision

The method precision of test method was done by performing assay on six replicate determination of sample preparation at test concentration level (as per method of analysis) and the relative standard deviation of assay results was obtained. The % RSD for the area of six replicate injections was found to be within the specified limits. (Table 4).

Accuracy
The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and value found. The standard solution with Accuracy 50%, 100% and 150% were injected into chromatographic system and calculated the amount found and amount added for Metformin HCl and Myo-Inositol and further calculated the individual recovery values. (Table 5 & 6).

Robustness
In all the deliberately varied chromatographic conditions (e.g., flow rate, Mobile phase and Temperature), the chromatogram for system suitability showed satisfactory resolution (%RSD <2) with no significant changes in chromatographic parameters as shown in Table 7.

Limit of Detection (LOD)
The LOD is the lowest limit that can be detected. Based on the S.D. deviation of the response and the slope, the detection limit of Metformin HCl and Myo-Inositol was found to be 0.17 µg/ml and 1.08µg/ml. (Table 8).

Limit of Quantification (LOQ)
The LOQ is the lowest concentration that can be quantitatively measured. Based on the Standard deviation of the response and the slope, the quantification limit of Metformin HCl and Myo-Inositol was found to be 0.52 µg/ml and 3.27µg/ml. (Table 8).

Analysis of marketed products
The validated method was applied for the analysis of Metformin HCl and Myo-Inositol tablet of strength 250mg and 600mg. In this case average purity obtained is 98.98% and 99.88% respectively and no interference of impurity peak observed in Metformin HCl and Myo-Inositol peak. The results of analysis are given in Table 9.

Table 1: Summary of Validation parameter.

System suitability	Metformin HCl	Myo-Inositol
Theoretical plates	9554	8365
Linearity range (µg/mL)	25-250	60-360
Retention time	2.751	3.626
Co-relation co-efficient	0.999	0.999
System precision %RSD	0.22	0.02
Method precision %RSD	0.23	0.257
Accuracy	100.15-102	99.75-100.04
LOD (µg/mL)	0.17	1.08
LOQ (µg/mL)	0.52	3.27

Table 2: System Suitability Parameter Results.

Name	Retention Time	Area	USP Resolution	USP Tailing	USP Plate Count
Metformin	2.751	277617	-	1.33	9554
Myo-inositol	3.628	2567293	6.67	1.21	8365

Table 3: Linearity data of Metformin HCl and Myo-Inositol.

	Metformin HCl		Myo-Inositol	
Level (%)	Conc. (µg/ml)	Peak Area	Conc. (µg/ml)	Peak Area
25	25	271217	60	2511293
50	50	273100	120	2528013
75	75	274862	180	2544018
100	100	276646	240	2560271
125	125	278255	300	2576000
150	150	280012	360	2589912
Correlation Coefficient	0.999		0.999	
Slope (m)	163615.8		163245.8	
Intercept	3209.95		3324.95	

Table 4: System Precision of Metformin HCl and Myo-Inositol.

	System precision		Method precision	
Inj. Number	Peak Area of Metformin HCl	Peak Area of Myo-inositol	Peak Area of Metformin HCl	Peak Area of Myo-inositol
1	274985	2507140	274985	2511293
2	274036	2508008	274036	2516982
3	274958	2508177	274958	2513439
4	273268	2507341	273268	2520272
5	274154	2508121	274154	2501292
6	273846	2508288	274280	2511596
Mean	274208	2507846	274280	2512479
SD	607.37	481.72	641.29	6469.38
%RSD	0.22	0.02	0.23	0.257

Table 5: Accuracy Results for Metformin HCl.

% level	Amount added (µg/ml)	Amount recovered (µg/ml)	% recovery	Mean % Recovery
50	50	50.0	100.00	100.15
	50	50.12	100.24	
	50	50.1	100.20	
100	100	100.12	100.12	100.17
	100	100.22	100.22	
	100	100.17	100.17	
150	150	152.89	101.92	102.00
	150	153.12	102.08	
	150	153.0	102.0	

Table 6: Accuracy Results for Myo-Inositol.

% level	Amount added (µg/ml)	Amount recovered (µg/ml)	%recovery	Mean % recovery
50	120	119.61	99.68	99.76
	120	119.82	99.85	
	120	119.71	99.76	
100	180	179.84	99.91	100.04
	180	180.23	100.12	
	180	180.17	100.09	
150	240	239.24	99.68	99.75
	240	239.54	99.81	
	240	239.42	99.76	

Table 7: Robustness Results for Metformin HCl and Myo-Inositol.

S.N	Condition	Changed condition	Metformin HCl %RSD	Myo-Inositol %RSD
1	**Flow rate:** 1ml/min	0.9ml/min 1.1ml/min	0.0 1.7	0.1 0.7
2	**Mobile phase composition:** 70:30	68:32 72:28	1.2 0.4	1.1 0.5
3	**Temperature:** 30℃	28℃ 32℃	0.1 0.0	0.4 0.2

Table 8: Results for LOD and LOQ.

	Metformin HCl	Myo-Inositol
LOD	0.17	1.08
LOQ	0.52	3.27

Table 9: Assay results of Metformin HCl and Myo-Inositol.

Drug in formulation	Label claim	Amount found	Assay%
Metformin HCl	250mg	247.45mg	98.98
Myo-Inositol	600mg	599.30mg	99.88

Table 11: List of abbreviations.

Sr. No.	Abbreviation	Full form
1	μg	Microgram
2	mg	Milligram
3	μ	Micron
4	μl	Micro liter
5	ml	Milliliter
6	μm	Micro meter
7	mm	Millimeter
8	nm	Nanometer
9	N	Normal
10	λmax	Wavelength of maximum absorbance
11	R.T.	Retention time
12	r2	Correlation coefficient
13	RSD	Relative standard deviation
14	SD	Standard deviation
15	CC	Calibration curve
16	IUPAC	International Union of Pure and Applied Chemistry
17	API	Active pharmaceutical ingredient
18	DP	Drug product
19	UV/VIS Spectrophotometer	Ultra violate/visible spectrophotometer
20	FTIR	Fourier transmission infra-red spectroscopy
21	RP-HPLC	Reverse phase-high performance liquid chromatography
22	HPTLC	High performance thin layer chromatography
23	GC-MS	Gas chromatography-Mass spectrometry
24	LC-MS	Liquid chromatography-Mass spectrometry
25	LC-MS-MS	Liquid chromatography-Tandem mass spectrometry
26	ICH	International Conference on Harmonization
27	FDA	Food and Drug Administration
28	US-FDA	United States Food and Drug Administration
29	DL	Detection limit
30	QL	Quantitation limit
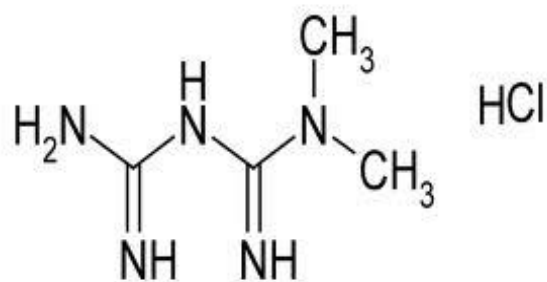
Figure 1: Structure of Metformin HCl.
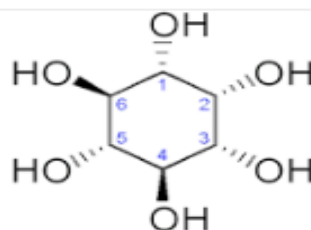
Figure 2: Structure of Myo-Inositol.
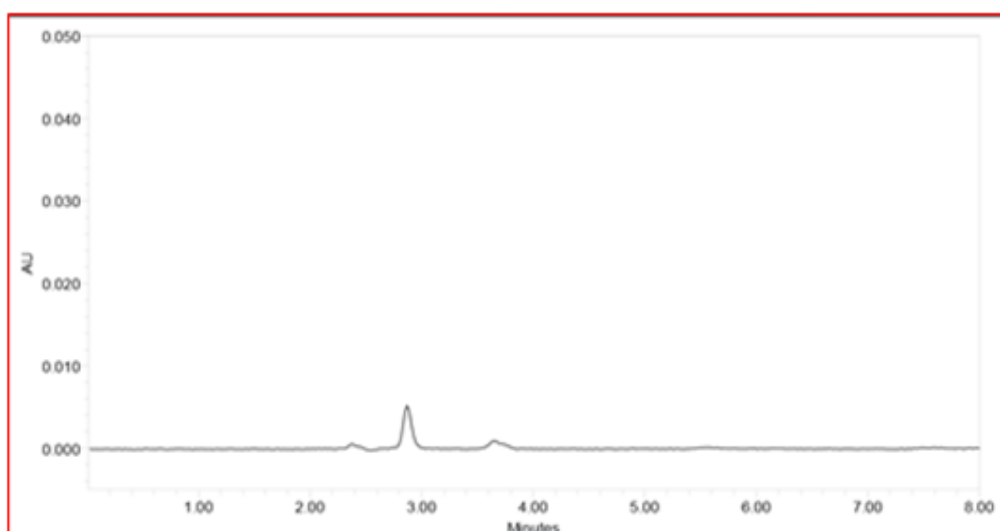
Figure 3: Chromatogram of Blank.
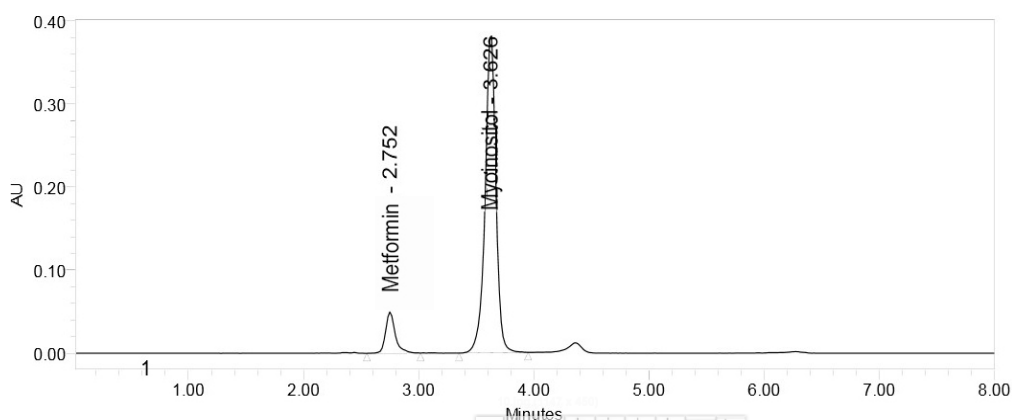
Figure 4: Standard Chromatogram of Metformin HCl & Myo-Inositol.
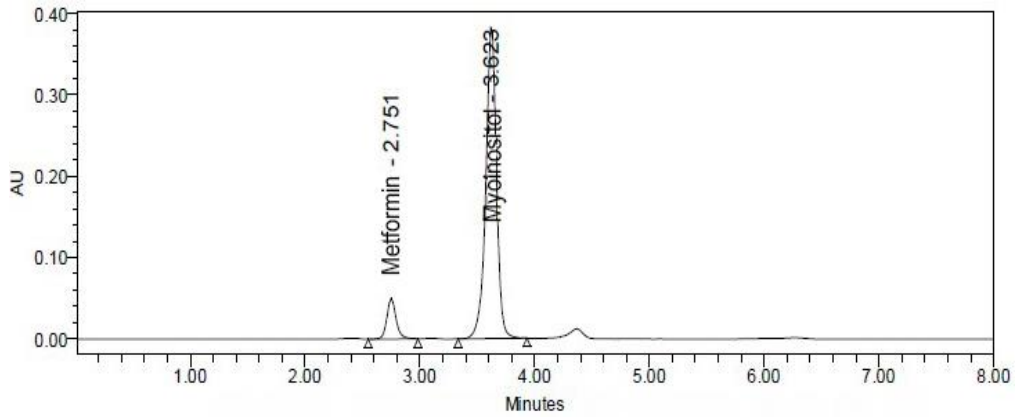
Figure 5: Sample Chromatogram of MetforminHCl & Myo-Inositol.
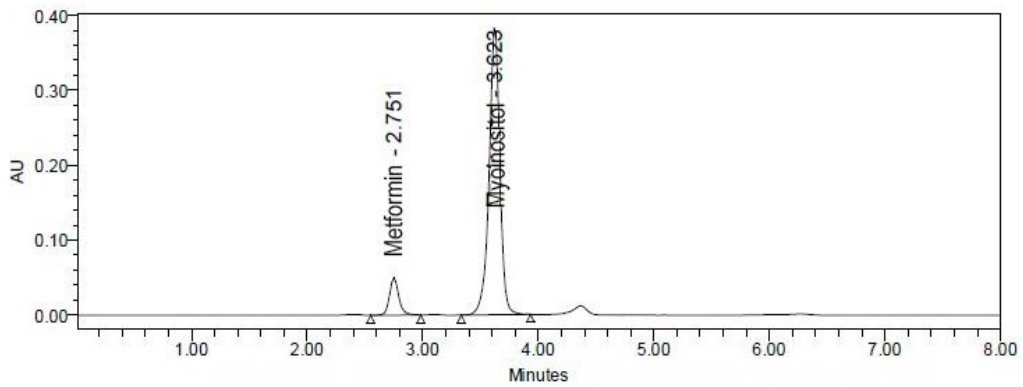
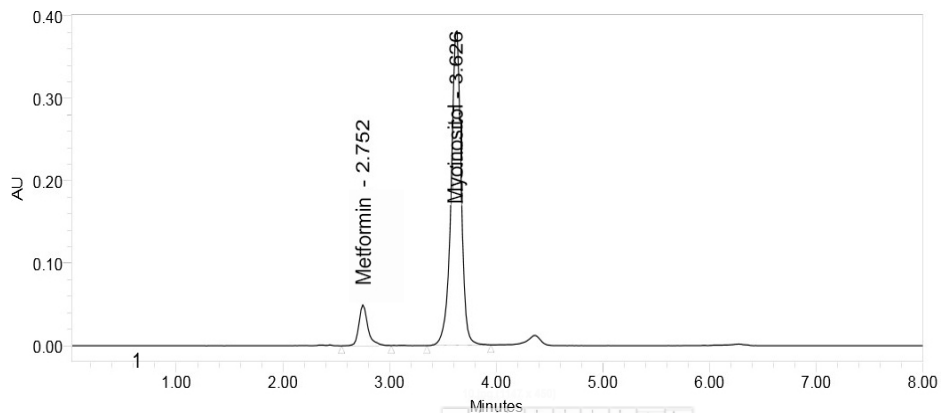**Figure 6: Chromatogram for System suitability**

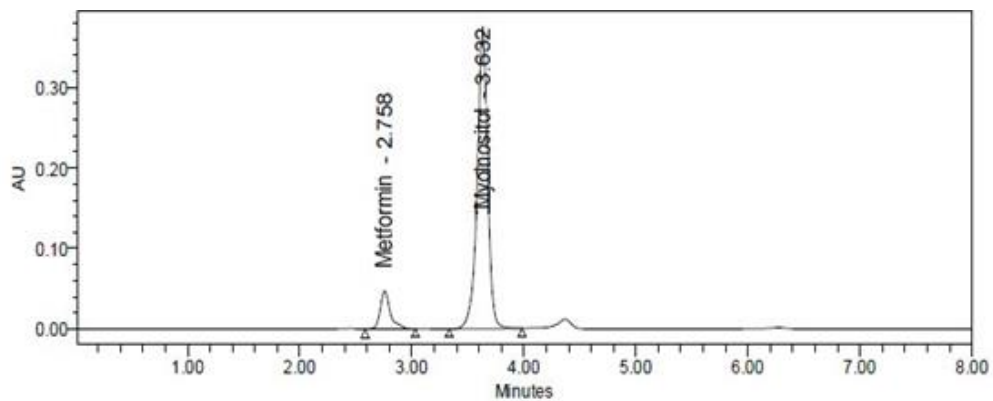**Figure 7: Typical Chromatogram for Linearity 25%**

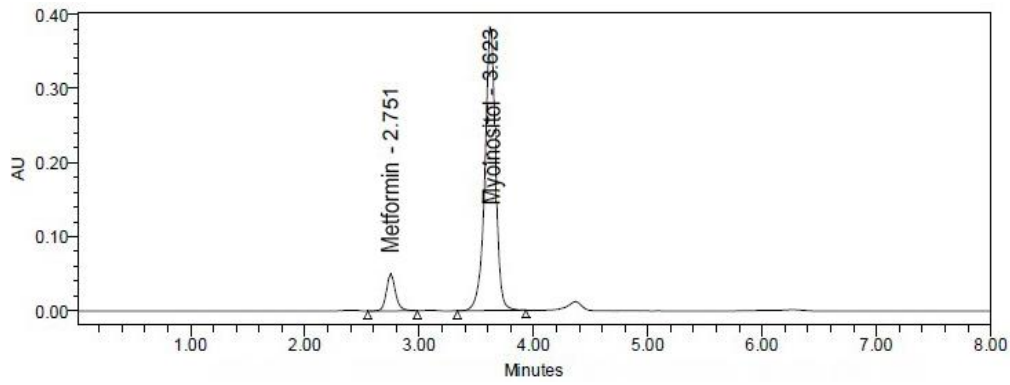**Figure 8: Typical Chromatogram for Linearity 50%.**
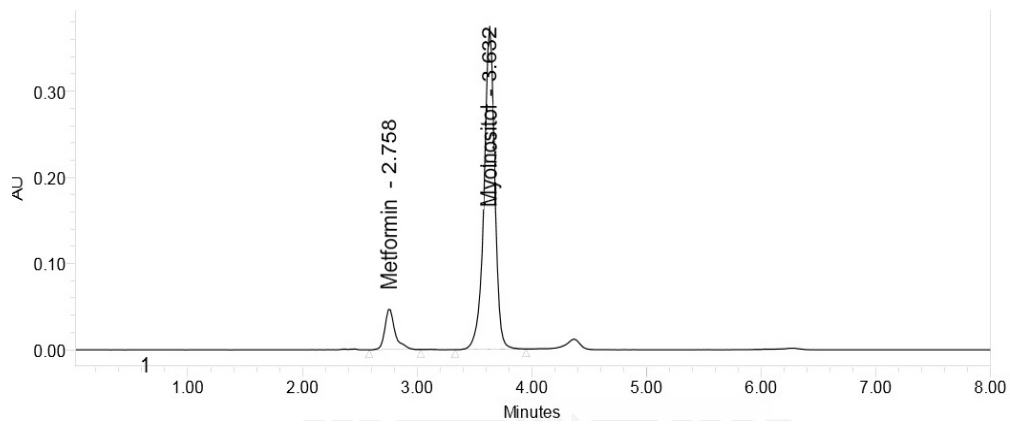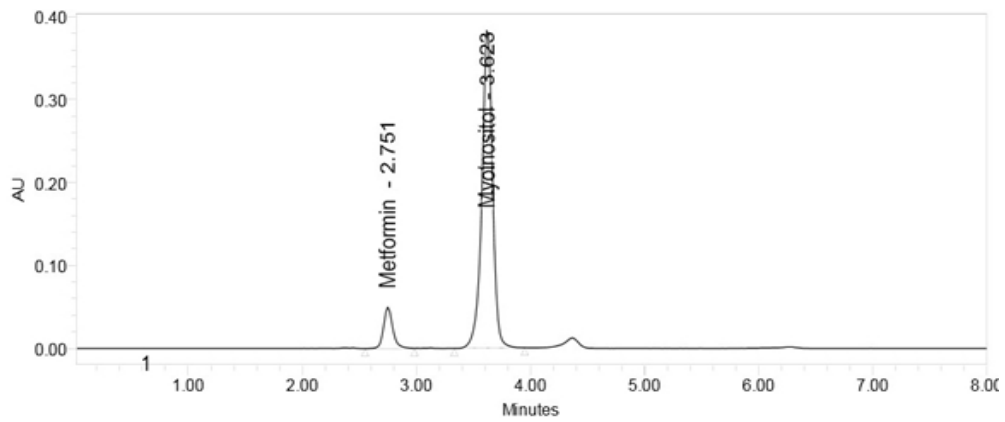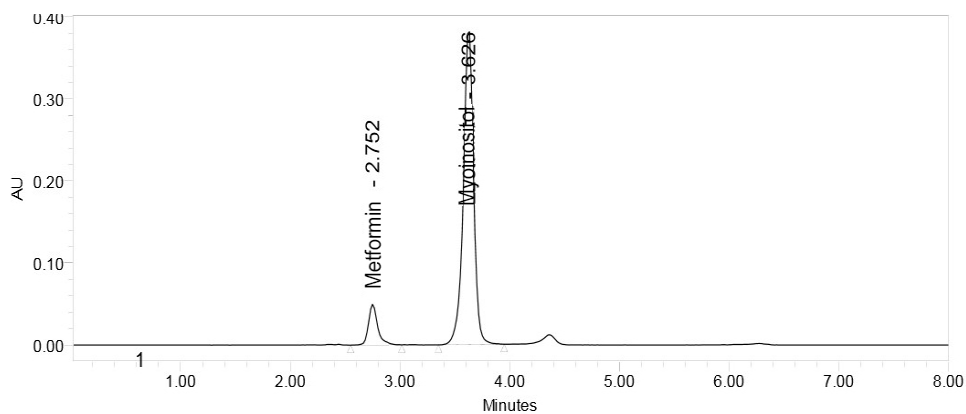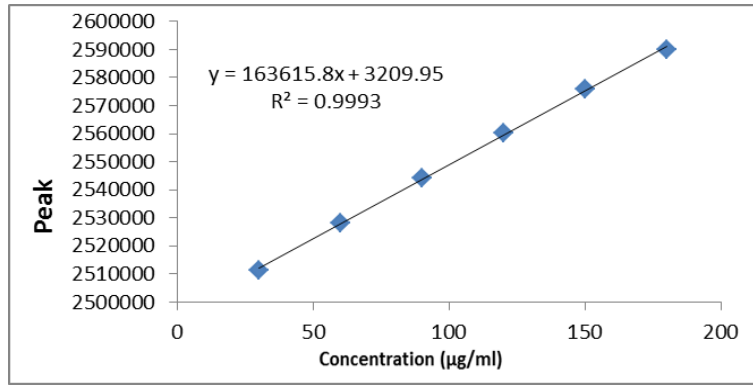
Figure 9: Typical Chromatogram for Linearity 75%.

Figure 10: Typical Chromatogram for Linearity 100%

Figure 11: Typical Chromatogram for Linearity 125%

Figure 12: Typical Chromatogram for Linearity 150%.

Figure 13: Linearity plot for Metformin HCl.
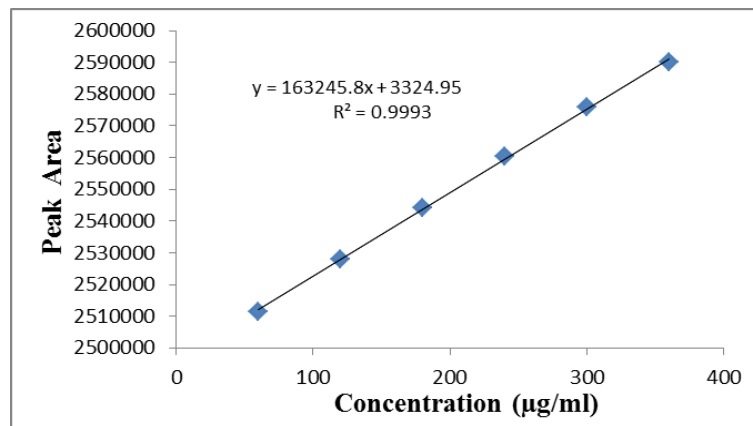
Figure 14: Linearity plot for Myo-Inositol.
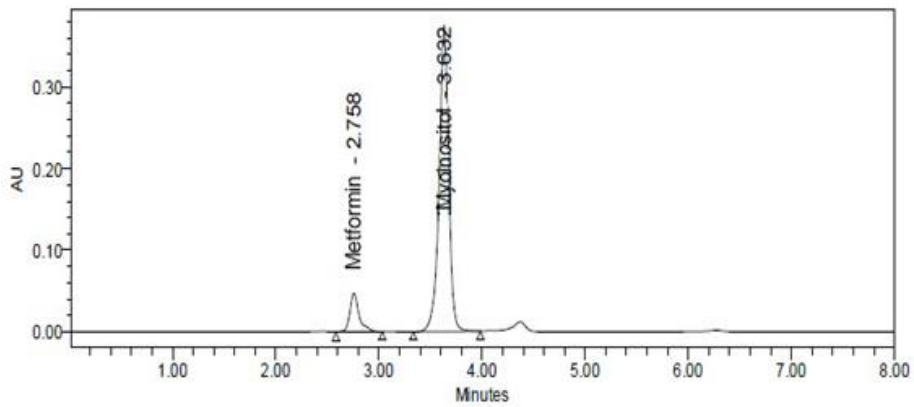
Figure 15: Typical Chromatogram for System precision.
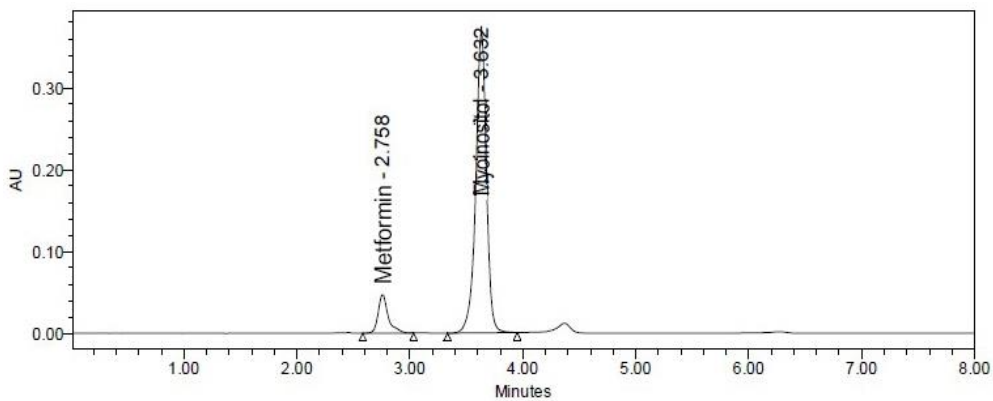
Figure 16: Typical Chromatogram for Method precision.
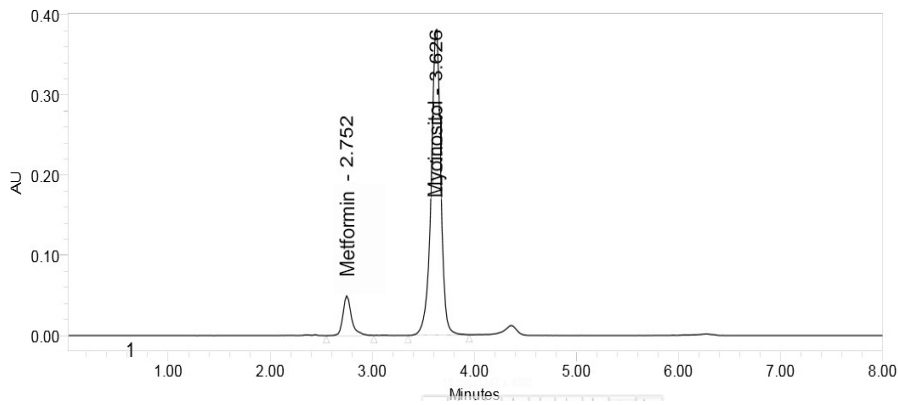
Figure 17: Typical Chromatogram for Accuracy 50%.
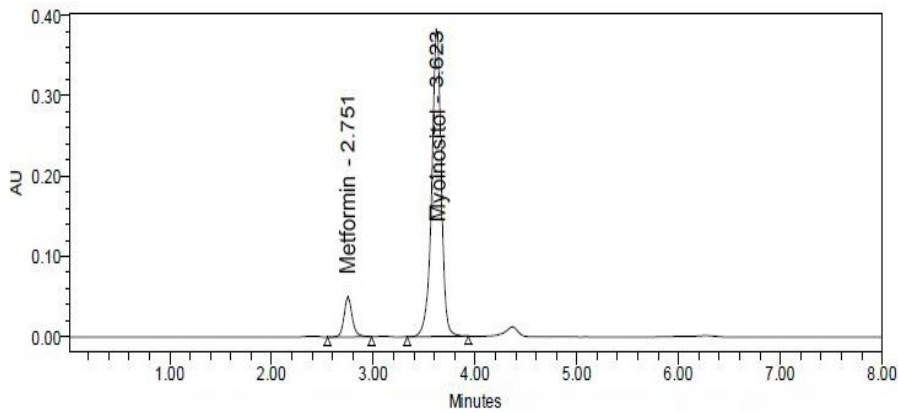
Figure 18: Typical Chromatogram for Accuracy 100%.
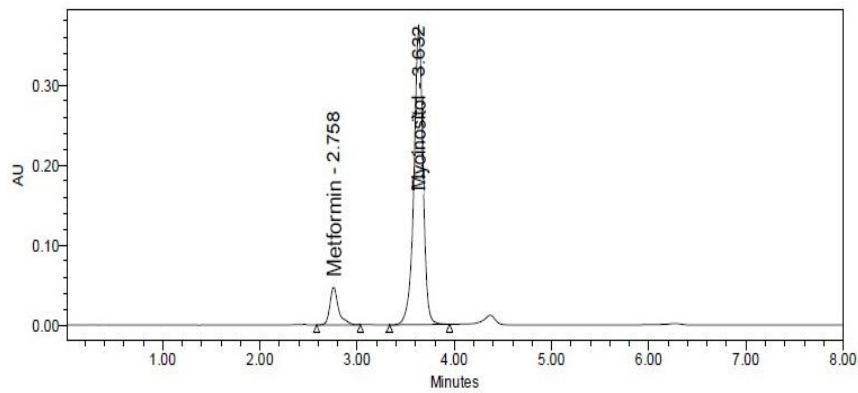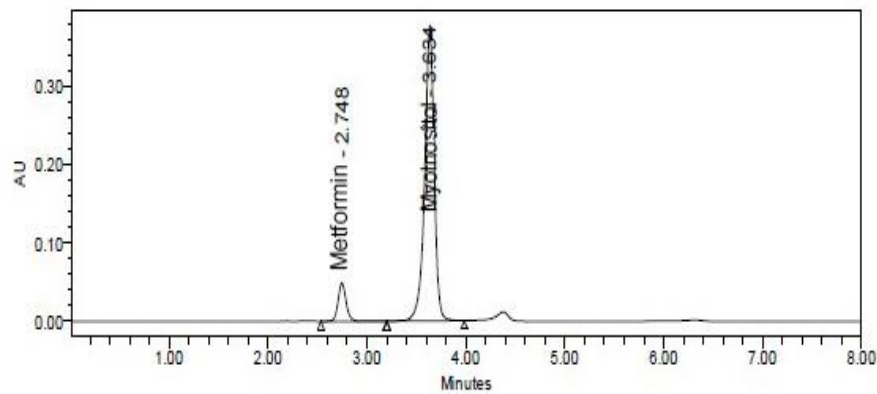

Figure 19: Typical Chromatogram for Accuracy 150%.

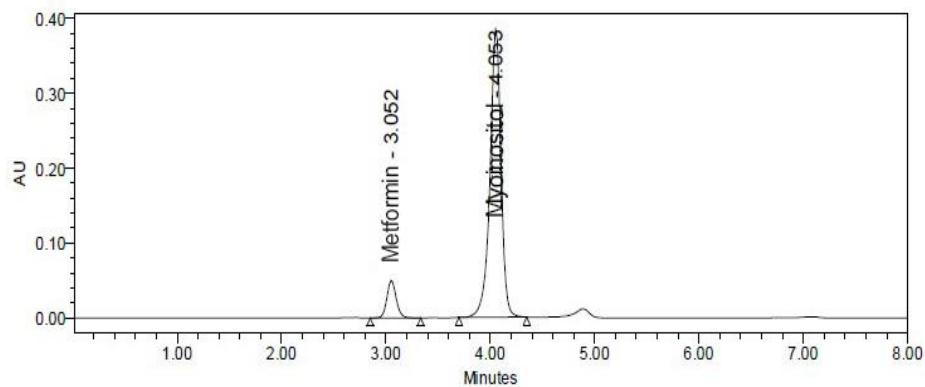**Figure 20: Typical Chromatogram for Flow rate of 0.9ml/min.**

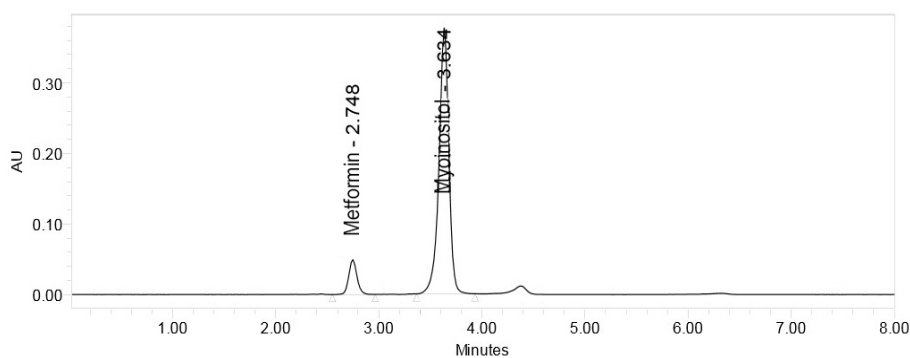**Figure 21: Typical Chromatogram for Flow rate of 1.1ml/min.**

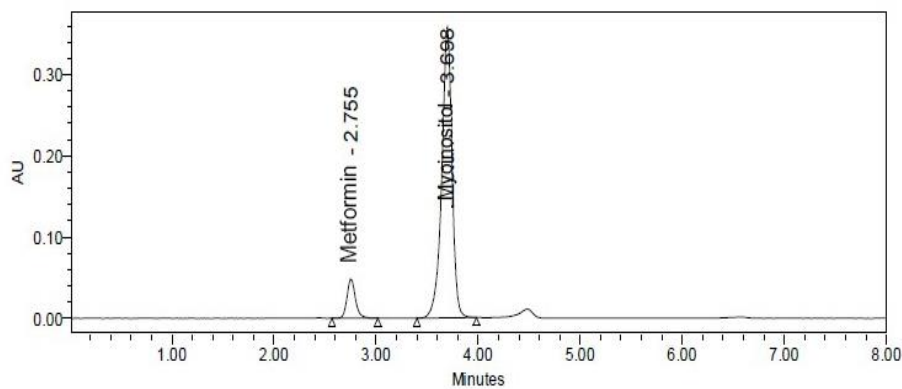**Figure 22: Typical Chromatogram for Effect of Mobile phase (68:32).**

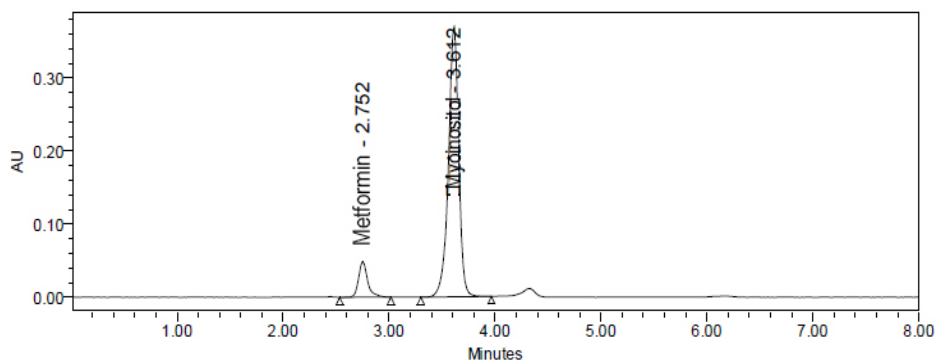**Figure 23: Typical Chromatogram for Effect of Mobile phase (72:28).**

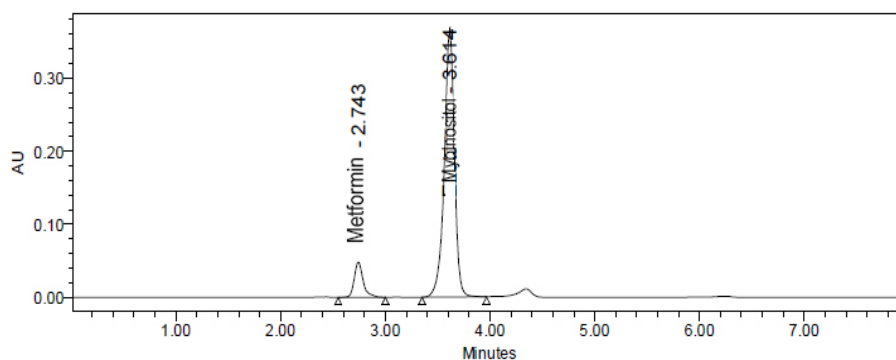**Figure 24: Typical Chromatogram for Temperature at 28° C.**

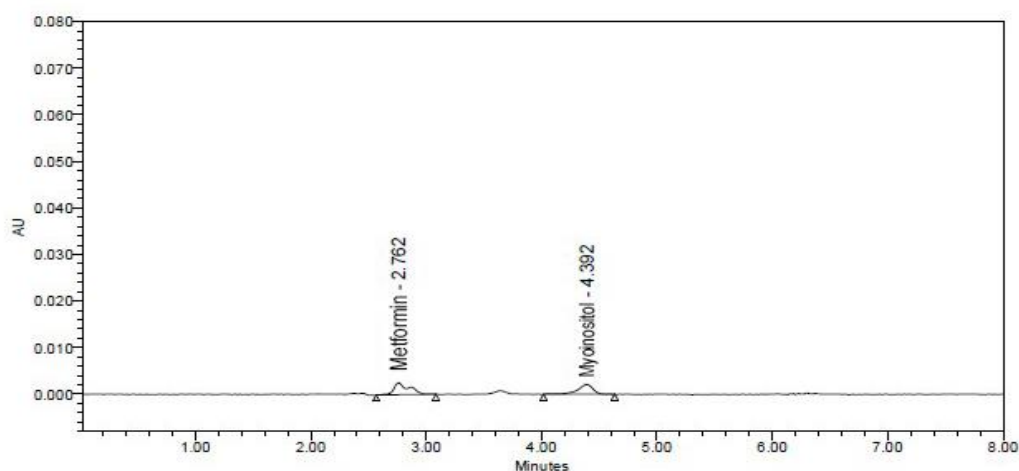**Figure 25: Typical Chromatogram for Temperature at 32°C.**

Figure 26: Typical Chromatogram for LOD of Metformin HCl and Myo-Inositol.
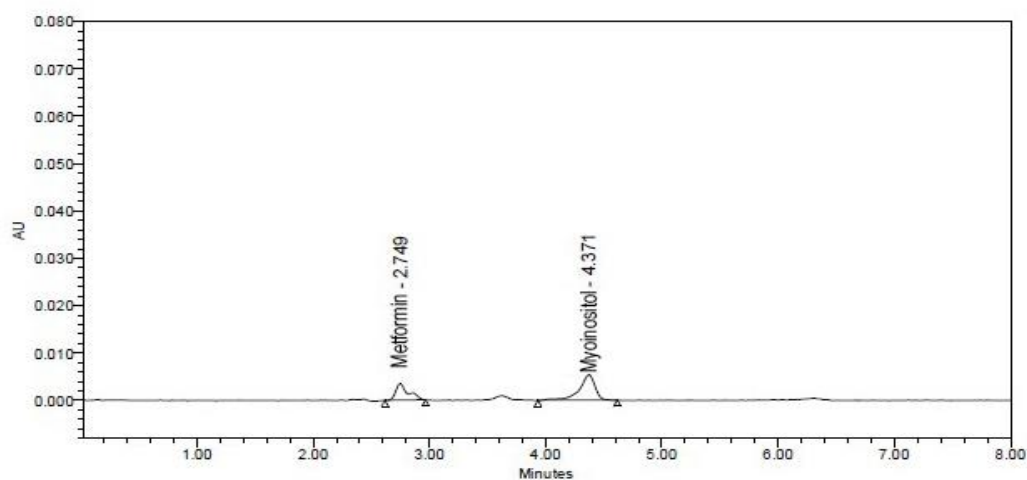

Figure 27: Typical Chromatogram for LOQ of Metformin HCl and Myo-Inositol.

CONCLUSION

A reverse phase HPLC isocratic method developed and has been validated as per ICH guidelines in terms of linearity, precision, accuracy, robustness, limit of detection and limit of quantification for the simultaneous quantitative estimation of Metformin HCl and Myo-Inositol. The correlation coefficients were greater than 0.999 for both the drugs. The System precision and

Method precision results were good enough to say that the method developed is precise and reproducible. Accuracy studies revealed that mean recoveries after spiking experiments of Metformin HCl and Myo-Inositol were between 100.15-102% and 99.75-100.04% respectively, an indicative of accurate method. Accordingly it can be concluded that the developed reverse phase HPLC method is accurate, precise, linear, robust and sensitive. Therefore the method can be used

for the routine analysis of Metformin HCl and Myo-Inositol in tablets. Based on this evidence the method can be stated as highly economical and it is recommended for routine use in quality control laboratories.

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