Journal of Pharmaceutical Research and Innovation (JPRI)
Volume 5, Issue 2, July 2025

e-ISSN: 2583-0023

Analytical Method Development and Validation for the Simultaneous Estimation of Imeglimin, Metformin, Sitagliptin by RP-HPLC

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Abstract

Introduction: Analytical method development and validation are crucial for ensuring drug quality and efficacy. As combination therapy is common in type 2 diabetes, a reliable method is needed for the simultaneous estimation of Metformin, Sitagliptin, and Imeglimin in pharmaceutical formulations. This study aims to develop and validate a simple, precise, and robust RP-HPLC-UV method for the simultaneous estimation of Metformin, Sitagliptin and Imeglimin in single combined dosage forms.

Materials & Methods: Metformin, Sitagliptin, and Imeglimin were analyzed using a Shimadzu RP-HPLC system with a C18 column and UV detection at 260 nm. Stock and working solutions were prepared in aqueous media, and calibration curves were constructed over relevant concentration ranges. The optimized mobile phase (Methanol: Cyanomethane: 15 mM Ammonium Formate, 10:40:50 v/v, pH 6.5) under isocratic flow (0.9 mL/min) provided well-resolved peaks with retention times of 3.7, 4.7, and 8.3 min. The method was validated as per ICH guidelines for specificity, linearity, accuracy, precision, LOD, LOQ, robustness, and ruggedness.

Results: The developed RP-HPLC-UV method enabled efficient and simultaneous estimation of Sitagliptin, Metformin, and Imeglimin with well-resolved peaks at retention times. The method exhibited high specificity, with no interference from diluents or other components. The LOD and LOQ were 85/265 ng/mL for Sitagliptin, 25/80 ng/mL for Metformin, and 55/175 ng/mL for Imeglimin. Accuracy, assessed via recovery studies, ranged from 98.6–99.9%, and precision studies showed low %RSD, confirming repeatability and reproducibility. System suitability parameters, including theoretical plates, tailing factor, and resolution, were within acceptable limits, indicating the method's reliability for routine analysis of these antidiabetic drugs.

Conclusions: A validated RP-HPLC-UV method was developed for simultaneous estimation of Sitagliptin, Metformin, and Imeglimin. The method showed high specificity, accuracy, and precision, suitable for routine pharmaceutical analysis.

Keywords

Analytical method, Imeglimin, Metformin, RP-HPLC, Sitagliptin and Validation.

INTRODUCTION

Analytical method development and validation are fundamental aspects of pharmaceutical research and quality assurance, ensuring the identity, purity, potency, and performance of drug substances and formulations. Chromatographic techniques, particularly High-Performance Liquid Chromatography (HPLC), play a vital role in the qualitative and quantitative analysis of Active Pharmaceutical Ingredients (APIs) and their formulations due to their accuracy, sensitivity, and reproducibility [1] [2] [3]. Type 2 Diabetes Mellitus (T2DM) is a chronic metabolic disorder characterized by insulin resistance and impaired glucose regulation, often requiring combination therapy for effective management. Sitagliptin phosphate is an orally active Dipeptidyl Peptidase-4 (DPP-4) inhibitor presented as a white to off-white crystalline, non-hygroscopic powder. It exhibits good solubility in water and N,N-dimethylformamide, slight solubility in methanol, very slight solubility in ethanol, acetone, and acetonitrile, and is practically insoluble in isopropanol. Metformin hydrochloride, a biguanide-class oral hypoglycemic agent, is a white to off-white crystalline, non-hygroscopic powder that is freely soluble in water, slightly soluble in alcohol, and practically insoluble in acetone and methylene chloride. Imeglimin hydrochloride, a novel oral antidiabetic compound, occurs as a white to off-white solid and shows high solubility in water, ethanol, and DMSO, with limited solubility in other organic solvents. The combined use of these agents offers synergistic benefits in achieving better glycemic control in diabetic patients [4] [5] [6]. However, limited analytical methods are available for the simultaneous estimation of these three drugs in bulk and combined dosage forms. High-Performance Liquid Chromatography (HPLC) is a vital analytical technique in pharmaceutical research, with Reverse Phase HPLC (RP-HPLC) being the most widely used due to its high accuracy, reproducibility, and efficiency [7] [8]. RP-HPLC employs a non-polar stationary phase and a polar mobile phase, making it ideal for separating and quantifying a wide range of drug substances, impurities, and

e-ISSN: 2583-0023

degradation products. It is extensively applied in drug development, formulation analysis, and quality control to ensure the purity and performance of pharmaceutical products. The distinct physicochemical profiles of these three drugs provide a strong foundation for their simultaneous estimation using a validated RP-HPLC method [9]. This study aims to develop and validate a simple, precise, and robust RP-HPLC-UV method for the simultaneous estimation of Metformin, Sitagliptin and Imeglimin in combined dosage forms.

MATERIALS & METHODS

Solvent and Chemicals Used

Table 1. Solvents, Chemicals used

Cyanomethane	**S.D fine chemicals**
Methyl Alcohol	
Ammonium Formate	
Triethylamine	
Metformin	**Micro labs limited**
Sitagliptin	
Imeglimin	

Equipment and Instruments Used

Ultra Sonicator, Systronics - pH meter, Shimadzu 1700 UV-Visible spectrophotometer, Shimadzu single pan digital balance (BL 220 H), Shimadzu HPLC - LC-10 AD-QP system with Lab solution data station and Shimpack C18 column (150× 4.6,5)

Analytical Method Development

- Preliminary Studies
- Solubility Studies

The solubility of the Metformin, sitagliptin and Imeglimin was determined by using different low volatile solvents and it was found to be freely soluble in Aqueous, and soluble in Methyl alcohol.

Selection of Wavelength

The solutions of working standards Metformin, sitagliptin and Imeglimin in 10µg/ml concentration were prepared by dissolving in aqua and UV spectrum of all these drug standards were recorded and found out the Isosbestic point. (Metformin - 234nm, Sitagliptin – 242nm and Imeglimin – 262nm). It was found that the isosbestic point as 260nm.

Optimization of Chromatographic Conditions

The approach was chosen according to the nature and solubility of the drug substances. It was found that the drug substances were polar. Thus, methods of the reverse phase, ion pair, or ion- exchange can be used but we chose to create the system with reverse phase HPLC.

Eluent Preparation

The eluent was prepared by dissolving accurately weighed 0.180g of Ammonium Formate in 200ml Millipore Aqua (15mM strength and pH adjusted to 6.5 by using 0.1% Triethylamine), Methyl alcohol and Cyanomethane (60:10:40%v/v respectively). The eluent was sonicated and used for the separation in the HPLC system.

Preparation of Stock (1mg/ml) and Working Standard Solution (10µg/ml)

The stock solution of Metformin, sitagliptin and Imeglimin (1.0mg/ml) were prepared individually by accurately weighing 0.01g and dissolved in Aqua. The working standard solutions (10µg/ml) were prepared from the standard stock solution individually, labelled, and kept under at 2 to 8° C.

Preparation of Stock Calibration Curve Samples (CC)

The concentration range was selected based upon the Cmax value of the respective drugs of interest. The stock solution of 10µg/ml for each drug of Sitagliptin, Metformin and Imeglimin was prepared by using Aqus. The following calibration curve solution of Sitagliptin of concentration 1900, 1720, 1520, 1140, 950, 580, 380, 190, 95 ng/ml, Metformin concentration within the range of 660, 590, 530, 400, 330, 200, 130, 70, 35ng/ml, and Imeglimin concentration 1720, 1550, 1380, 860, 690, 520, 350, 170, 85 ng/ml were prepared from the standard stock solution. The containers were labelled and stored at 2.0 to 8° C until analysis.

Initial Chromatographic Conditions

This condition was used for sitagliptin, Metformin and Imeglimin separation initially. The peak observed was not good initially, and several tests are carried out to select the appropriate separation process.

Table 2. Initial Chromatographic condition for simultaneous estimation of Sitagliptin, Metformin and Imeglimin by HPLC

Adsorbent	Shimpack C18 150× 4.6mm, 5
Eluent	Cyanomethane:15mM Ammonium Formate buffer (pH-5.6) (40:60 % v/v)
Mode	Isocratic
Temperature	Ambient
Flow rate (ml/min)	1
Detection wavelength	260 nm
Loop Volume	20µl
Retention time	Sitagliptin- 3.2mins Metformin -4.2mins Imeglimin -7.4 mins

The Effect of the Adsorbent

For the method development many brands of C18 columns were used but the better Resolution was achieved with the Shimpack (150 × 4.6 mm ,5) column.

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Effect of Solvent

First, the solvent was chosen as methyl alcohol for the separation of sample Sitagliptin, Metformin, and Imeglimin. Then Cyanomethane was selected as the separation and chromatogram were better and improved. The ratio of eluent was kept as 40:60%v/v for the initial separation of the sample mixture and found separation was not much efficient. So, the ratio of the eluent was changed to 40:10:50%v/v to improve the separation and the retention time was obtained as 3.7, 4.7, 8.3 for sample sitagliptin, Metformin, and Imeglimin respectively. System suitability parameters are calculated after performing the separation.

Effect of pH

The samples were chromatographed for 10min using Methyl alcohol, Cyanomethane and ammonium Formate (40:10:50%v/v) and tried with different pH range from 5.6 to 6.5 and flow rate of 0.9ml/min using Shimpack C18 (150mm× 4.6.,5) At Ph of 6.5, good Separation, Retention time, and peak shape were achieved efficiently.

Final Chromatographic Conditions

Therefore, the below method is applicable for the estimation of Sitagliptin, Metformin and Imeglimin by HPLC individually or in fixed combinations.

Table 3. Finalised Chromatographic condition for concurrent estimation of Sitagliptin, Metformin and Imeglimin by RP-HPLC-UV.

Adsorbent	Shimpack C18 column (150× 4.6, 5)
Column temperature	Ambient
Eluent	Methyl alcohol: Cyanomethane: 15mM Ammonium Formate buffer (pH-6.5) (10:40:50% v/v)
Mode	Isocratic
Flow rate (ml/min)	0.9
Detection wavelength	260nm
Loop volume	20µl
Retention time	Sitagliptin- 3.7mins Metformin-4.7mins Imeglimin -8.3 mins

Analytical Method Validation

The method developed applicable for the estimation of Sitagliptin, Metformin and Imeglimin was validated as per ICH guideline to prove the method is efficient and will be useful for the separation of these drugs and further study.

- Specificity/Sensitivity
- Robustness / Ruggedness.
- Detection Limit
- Quantification Limit
- Linearity
- Accuracy
- Precision

Specificity

According to the specification of ICH guideline procedure, need to perform identification tests, impurities determination, and testing, a specificity test should be carried out. First, the HPLC method was developed. Concerning the method, mobile phases and stationary phases were selected. The percentage of the organic solvent, ionic strength, pH, flow rate, etc. was modified in HPLC and further peaks if any. The second method is testing peak purity by a diode array detector. The PDA detector is used to compare the peak purity and impurity of the drugs. PDA spectrum, UV spectrum, absorbance ration curve, and the initial deprival spectra of Standard and sample peaks were recorded.

Ruggedness & Robustness

Ruggedness and robustness of the methods were studied by changing the experimental conditions.

Detection and Quantification Limit

The limit of detection (LOD) values for Sitagliptin, Metformin and Imeglimin were found to be and 85ng/ml, 25ng/ml and 55ng/ml respectively and their limit of quantification (LOQ) value were 265ng/ml, 80ng/ml and 175ng/ml respectively.

Linearity

A range of 95 to 1900ng/ml for Sitagliptin, 35 to 660ng/ml for Metformin, 85 to 1720ng/ml for Imeglimin and based on their Cmax were selected for demonstrating of linearity by dilution from the stock solution. Linearity is evaluated by instrumental method- linear relationship.

Accuracy

Triplets of Sitagliptin, Metformin and Imeglimin, have been replicated in the tests and the mean, standard difference, and coefficients of variation have been reported RSD.

Precision

Typical variations are days, analysts, equipment, etc. Analysis of standard and sampling solutions 6 times through assay procedure and chromatogram were obtained. Interday precision was assessed similarly over two weeks.

System Suitability Testing

System suitability parameters such as column efficiency, resolution factor of the optimized methods was found satisfactory.

RESULTS AND DISCUSSION

For the estimation of Metformin, sitagliptin and Imeglimin using the HPLC framework, this analytical method has been developed and validated. The drug standards were tested preliminarily and the solubility and wavelength of the was determined.

Journal of Pharmaceutical Research and Innovation (JPRI)
Volume 5, Issue 2, July 2025
e-ISSN: 2583-0023

Selection of Wavelength

The λmax was found to be as follows:

Table 4. Selection of isosbestic point

Drug name	Λmax
Metformin	234nm
Sitagliptin	242nm
Imeglimin	262nm

It was found that the isosbestic point was 260nm.

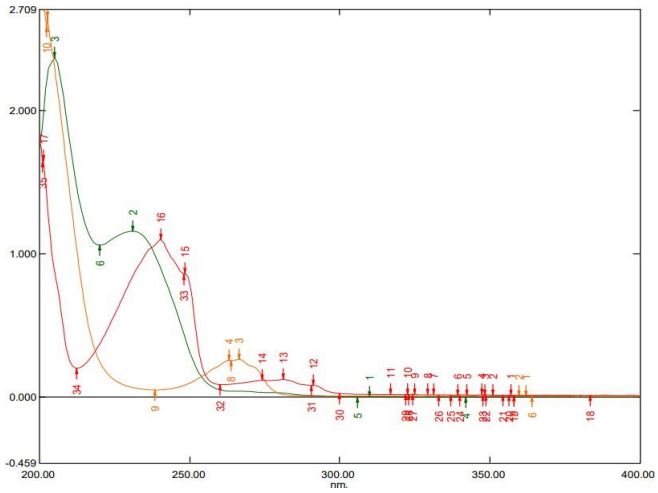

Figure 1. overlay spectrum for three drugs of sitagliptin, metformin and Imeglimin

Method Development

The optimized Chromatographic conditions were to achieve efficient separation and to enhance good resolution, run time, sensitivity, peak symmetry.

Final Chromatographic Conditions

Table 5. Final chromatographic conditions for Sitagliptin, Metformin, Imeglimin

Adsorbent	Shimpack C18 column (150× 4.6, 5)
Column temperature	Ambient
Eluent	Methyl alcohol: Cyanomethane: 15mM Ammonium Formate buffer (pH-6.5) (10:40:50 % v/v)
Mode	Isocratic
Flow rate (ml/min)	0.9
Detection wavelength	260nm
Loop volume	20µl
Retention time	Sitagliptin- 3.7mins Metformin-4.7mins Imeglimin -8.3 mins

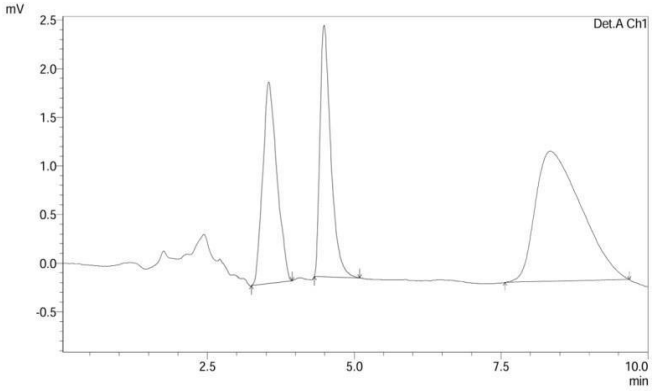

Figure 2. Typical chromatogram of simultaneous estimation of sitagliptin, Metformin, and Imeglimin

Validation of the Developed RP-HPLC-UV Method

The main concept of validation is to challenge the method and to evaluate the limits of variations were within the specified levels. The parameters for the validation were discussed earlier and here we discuss the results obtained.

Specificity / Selectivity

By analysing a solution containing Sitagliptin, Metformin and Imeglimin, the specificity/selectivity of the RP-HPLC-UV system established was evaluated. At the time of retention of Sitagliptin, metformin, Imeglimin and diluent solution there were no interferences.

Ruggedness & Robustness

By changing the Chromatographic conditions like flow rate (0.8 & 1.0 ml), and eluent ratio (40:20:40), chromatograms were observed for changes and recorded. All the parameters pass the criteria within determined limits and thus it proves that the method developed is robust.

Detection & Quantification Limit

The LOD and LOQ were determined at 3 and 10 times the baseline noise, respectively.

Table 6. LOD and LOQ For Sitagliptin, Metformin and Imeglimin

Drug substance	Detection	Quantification
Sitagliptin	85ng/ml	265ng/ml
Metformin	25ng/ml	80ng/ml
Imeglimin	55ng/ml	175ng/ml

Linearity

The data of linearity over the concentration range of 95 to 1900ng/ml for Sitagliptin, 35 to 660ng/ml for metformin, 85to 1720ng/ml for Imeglimin based on their Cmax and the calibration curve was plotted against concentration versus response factor given below and correlation coefficient (r2) of be 0.9939, 0.9918, 0.9950 respectively, demonstrating that the developed RP-HPLC-UV method has adequate linearity to the concentrations of Sitagliptin, Metformin and Imeglimin.

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Volume 5, Issue 2, July 2025
e-ISSN: 2583-0023

Table 7. Linearity for Sitagliptin

Con(ng/ml)	Peak Area
0	0
95	745
190	1632
380	2863
580	4094
950	5979
1140	7220
1520	9092
1720	10473
1900	11814

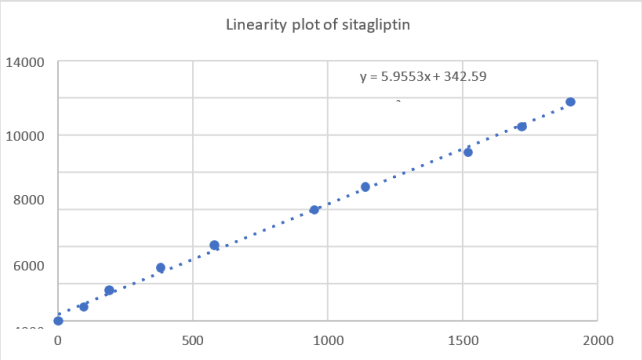

Figure 3. Calibration curve for sitagliptin

Table 8. Linearity for Metformin

Con(ng/ml)	Peak Area
0	0
35	533
70	1663
130	2926
200	4079
330	5316
400	6679
530	8175
590	9354
660	10632

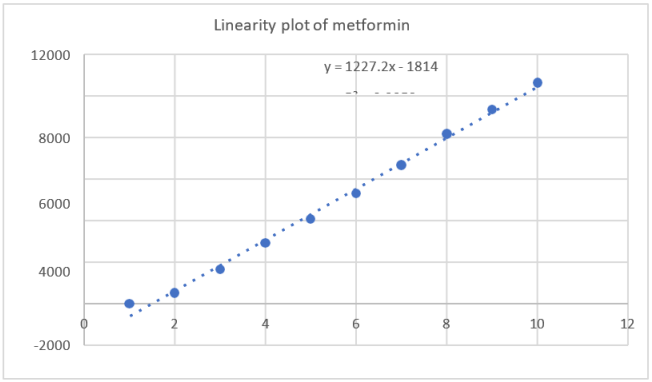

Figure 4. Calibration curve for metformin

Table 9. Linearity for Imeglimin

Con (ng/ml)	Peak Area
0	0
85	4232
170	12082
350	18160
520	27045
690	34202
860	41090
1380	59958
1550	68130
1720	75816

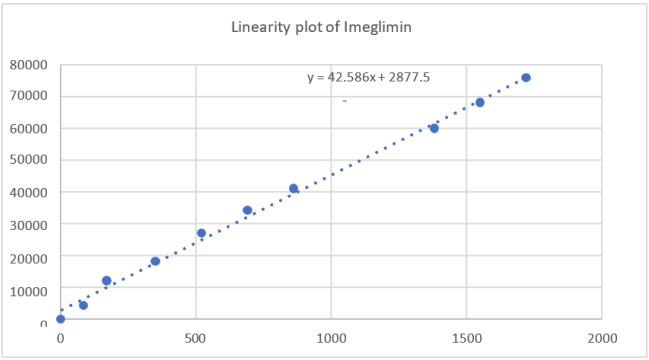

Figure 5. Calibration curve for Imeglimin

Accuracy

Standard addition and recovery experiments (6 times) were conducted to determine the accuracy respectively and coefficient of variation was calculated and results were found to be accurate.

Table 10. Accuracy Result for sitagliptin

Concentration (ng/ml)	Mean (ng/ml) ±S. D	Recovery %	RSD %
LQC (400)	397.25±0.75	99.31	0.18
MQC (1150)	1148.65±0.85	99.88	0.073
HQC (2000)	1998.21±0.79	99.91	0.042

Table 11. Accuracy Result for metformin

Concentration (ng/ml)	Mean (ng/ml) ±S. D	Recovery %	RSD %
LQC (200)	197.18±0.88	98.59	0.44
MQC (400)	396.28±0.98	99.07	0.24
HQC (700)	692.52±0.74	98.93	0.10

Table 12. Accuracy Result for Imeglitpin

Concentration (ng/ml)	Mean (ng/ml) ±S. D	Recovery %	RSD %
LQC (500)	495.32±1.08	99.064	0.21
MQC (850)	847.46±1.43	99.70	0.16
HQC (1800)	1798.67±1.16	99.92	0.064

e-ISSN: 2583-0023

Precision

The precision for this method was checked by injecting six individual preparations by measuring the repeatability (intra-day precision) as well as the intermediate precision (inter- day precision) of concentration and peak areas for Sitagliptin, Metformin and Imeglimin by replicate injections (n=6) of 3 different concentration levels (400,1150 and 2000ng/ml) for Sitagliptin, (200, 400, and 700ng/ml) for Metformin, (560, 850, and 1800ng/ml) for Imeglimin, and were carried out. Precision was reported as a percentage coefficient of variation.

Table 13. Intra-day and Inter-day Precision- Sitagliptin

Concentration (ng/ml)	Mean ±S. D	RSD %
LQC (400)	3388.3±85.2	0.67
MQC (1150)	8997±71.2	0.78
HQC (2000)	12663±24	0.70
Concentration (ng/ml)	Mean ±S. D	RSD %
LQC (400)	3615±30.74	0.71
MQC (1150)	8944.6±71.93	0.80
HQC (2000)	12862.67±91.43	0.85

Table 14. Intra-day and Inter-day Precision- Metformin

Concentration (ng/ml)	Mean ±S. D	RSD %
LQC (200)	4147.3±338	0.81
MQC (400)	6572±52.9	0.80
HQC (700)	10311±93.2	0.90
Concentration (ng/ml)	Mean ±S. D	RSD %
LQC (200)	4279±37	0.86
MQC (400)	6409±48.5	0.75
HQC (700)	10645.3±77.3	0.72

Table 15. Intra-day and Inter-day Precision- Imeglimin

Concentration(ng/ml)	Mean ±S. D	RSD %
LQC (500)	27081±184.9	0.68
MQC (850)	46143±190.2	0.41
HQC (1800)	75976±445.6	0.58
Concentration(ng/ml)	Mean ±S. D	RSD %
LQC (500)	27392±175.2	0.63
MQC (850)	45852±156.7	0.34
HQC (1800)	76088±335.8	0.44

Table 16. System Suitability parameters

Validation Parameter/ System suitability	Sitagliptin	Metformin	Imeglimin
Number of theoretical plates	4862/meter	8750/meter	6168/meter
Tailing factor	1.37	1.7	1.4
Resolution	-	2.5	4.9

CONCLUSION

The developed RP-HPLC-UV method provides a reliable, simple, and efficient analytical approach for the simultaneous estimation of Sitagliptin, Metformin, and Imeglimin. The method was validated as per ICH guidelines, demonstrating excellent accuracy, precision, sensitivity, and linearity. Its robustness and suitability make it ideal for routine quality control, as well as for bioavailability, bioequivalence, and pharmacokinetic evaluations of these drugs in individual and combined dosage forms.

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