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Analytical Method Development and Validation for Sumultaneous Estimation of Metformine Hydrochloride and Pioglitazone by Using RP-HPLC

N. Geetha*, D. Sireesha, Dr. Vasudha bakshi

Department of Pharmaceutical Analysis and Quality assurance, Anurag group of institutions (Formerly Lalitha college of pharmacy), Hyderabad, Telangana, India

A B S T R A C T

A simple, Accurate, precise method was developed for the simultaneous estimation of the Metformin and Pioglitazone in Tablet dosage form. Chromatogram was run through C18, 4.6 mm, 5µm Make:x Terra Mobile phase containing Acetonitrile and Water in the ratio of 50:50 was pumped through column at a flow rate of 1ml/min. Optimized wavelength for Metformin and Pioglitazone was 242nm. %RSD of the Metformin and Pioglitazone were and found to be less than 2. Lnearity range of Metformin and Pioglitazone 25-125µg/ml. Correlation coefficient is 0.999**.**

Keywords: Metformin, Pioglitazone, RP-HPLC

A R T I C L E I N F O

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***Corresponding Author**
N. Geetha
Department of Pharmaceutical Analysis & Quality Assurance, Anurag Group of Institutions, Hyderabad, Telangana, India
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1. Introduction

Metformin is chemically named as 1-carbamimidamido-N,N-dimethylmethanimidamide[1]. Metformin decreases blood glucose levels by decreasing hepatic glucose production, decreasing intestinal absorption of glucose, and improving insulin sensitivity by increasing peripheral glucose uptake and utilization [2]. These effects are mediated by the initial activation by metformin of AMP-activated protein kinase (AMPK), a liver enzyme that plays

an important role in insulin signaling, whole body energy balance, and the metabolism of glucose and fats. Activation of AMPK is required for metformin's inhibitory effect on the production of glucose by liver cells[3]. Increased peripheral utilization of glucose may be due to improved insulin binding to insulin receptors. Metformin administration also increases AMPK activity in skeletal muscle [4].

Pioglitazone is chemically named as 5-({4-[2-(5-ethylpyridin-2-yl)ethoxy] phenyl} methyl)-1,3-thiazolidine-2,4-dione[5]. Pioglitazone acts as an agonist at peroxisome proliferator activated receptors (PPAR) in target tissues for insulin action such as adipose tissue, skeletal muscle, and liver[6]. Activation of PPAR-gamma receptors increases the transcription of insulin-responsive genes involved in the control of glucose production, transport, and utilization. In this way, pioglitazone both enhances tissue sensitivity to insulin and reduces hepatic gluconeogenesis. Thus, insulin resistance associated with type 2 diabetes mellitus is improved without an increase in insulin secretion by pancreatic β cells [7].

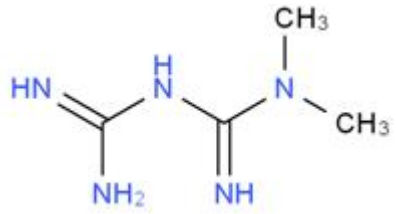

Figure 1: Structure of Metformin

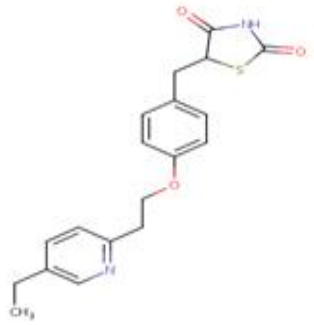

Figure 2: Structure of Pioglitazone

2. Materials and Methods

Preparation of standard solution:

10 mg of Metformin and 10mg of Pioglitazone were accurately weighed and transferred into a 10 ml clean dry volumetric flask, about 7 ml of diluent was added, sonicated to dissolve it completely and the volume was made up to the mark with the same solvent to give a concentration of 1000 µg/ml. (Stock solution) Further 0.75ml was pipetted out from the above stock solution into a 10ml volumetric flask and diluted up to the mark with diluent to give a concentration of 75 µg/ml and 75 µg/ml respectively[8].

Preparation of sample solution:

10 Tablets of contents were weighed and triturated in glass mortar. The quantity of powder equivalent to 10 mg of active ingredient present in Metformin and Pioglitazone was transferred into a 10 ml clean dry volumetric flask, 7 ml of diluent was added to it and was shaken by mechanical stirrer and sonicated for about 30 minutes by shaking at intervals of five minutes each and was diluted up to the mark with diluent to give a concentration of 1000 µg/ml and allowed to stand until the residue settles before taking an aliquot for further dilution (stock solution)[9]. 0.75 ml of upper clear solution was transferred to a 10 ml volumetric flask and diluted with diluent up to the mark to give the respective concentrations as par with standard solution. The solution was filtered through 0.45 µm filter before injecting into HPLC system [10].

Preparation of Placebo:

The amount of powdered inactive ingredient supposed to be present in 10 tablets were accurately weighed and transferred in to 10 ml volumetric flask, 7 ml of diluent was added and shaken by mechanical stirrer and sonicated for about 30 minutes by shaking at intervals of five minutes and was diluted up to the mark with diluent and allowed to stand until the residue settles before taking an aliquot for dilution [11]. 0.1 ml of upper clear solution was transferred to a 10 ml volumetric flask and diluted with diluent up to the mark and the solution was filtered through 0.45 µm filter before injecting into HPLC system [12].

Method Validation

The objective of validation of an analytical procedure is to demonstrate that it is suitable for its intended purpose. According to ICH guidelines, typical analytical performance characteristics that should be considered in the validation of the type of methods are:

Linearity:

Preparation of sample stock solution:

About 50 mg of Metformin and 1.5 mg of Piogltazone samples was weighed in to 10 ml volumetric flask, it was dissolved with diluent and the volume was made up to the mark with same diluent (5000µg/ml of Metformin and 150µg/ml of Piogltazone)[13].

Preparation of Level – I (25µg/ml of Metformin & 1.5µg/ml of Piogltazone)

0.25 ml of stock solution had taken in 10ml of volumetric flask diluted up to the mark with diluent.

Preparation of Level–II (50µg/ml of Metformin &3µg/ml of Piogltazone)

0.5 ml of stock solution had taken in 10ml of volumetric flask diluted up to the mark with diluent.

Preparation of Level–III (75 µg/ml of Metformin &4.5 µg/ml of Piogltazone)

0.75 ml of stock solution had taken in 10ml of volumetric flask diluted up to the mark with diluent.

Preparation of Level–IV (100 µg/ml of Metformin &6 µg/ml of Piogltazone)

1.0 ml of stock solution had taken in 10ml of volumetric flask diluted up to the mark with diluent.

Preparation of Level–V (125 µg/ml of Metformin &7.5 µg/ml of Piogltazone).

1.25 ml of stock solution had taken in 10ml of volumetric flask diluted up to the mark with diluent. 10µl of each level were injected & placed into the HPLC and UV systems and recorded the peak response and absorbances.

Procedure:

Each level solution was injected into the chromatographic system and the peak area was measured[13]. A graph of peak area versus concentration (on X-axis concentration and on Y-axis Peak area) was plotted and the correlation coefficient was calculated.

The linearity of the method was demonstrated over the concentration range of 25-125µg / ml. Aliquots of five levels were prepared from sample solution and labeled as solution 1, 2, 3, 4 and 5 respectively. The solutions were injected in to HPLC system as per test procedure [14].

Each level solution was placed into the UV system and the Absorbance was noted. A graph of absorbance versus concentration (on X-axis concentration and on Y-axis absorbance) was plotted and the correlation coefficient was calculated [15].

Acceptance criteria

1. Correlation Coefficient should be not less than 0.9990.
2. % RSD of peak areas for Solution 1, 2, 3, 4 and 5 should be not more than 2.0 %.

Precision

Preparation of stock solution:10 mg of Metformin and 10mg of Piogltazone were accurately weighed and transferred into a 10 ml clean dry volumetric flask, about 7 ml of diluent was added and sonicated to dissolve it completely. The volume was made up to the mark with the same solvent to give the concentration of 1000 µg/ml. (Stock solution) .Further 0.75 was pipette out from the above stock solutions into a 10ml volumetric flask and diluted up to the mark with diluent to give the concentration of 75 µg/ml and 75 µg/ml respectively[16].

Procedure:

The standard solutions were injected & placed into the HPLC and UV systems and recorded the peak response and absorbance for five times and the areas for all five injections were measured in HPLC[17]. The %RSD for the area of five replicate injections was found to be within the specified limits [18].

Acceptance criteria:

% Relative standard deviation of peak areas and R_t should not be more than 2.0

Intermediate Precision/Ruggedness

To evaluate the intermediate precision (also known as Ruggedness) of the method Precision was performed on different day by using different make column of same Dimensi ons [19].

Preparation of stock solution:

10 mg of Metformin and 10mg of Piogltazone accurately weighed and transferred into a 10 ml clean dry volumetric flask, about 7 ml of diluent was added ,sonicated to dissolve it completely and the volume was made up to the mark with the same solvent to give the concentration of 1000 µg/ml. (Stock solution) Further pipette out 0.75 ml from the above stock solution into a 10ml volumetric flask and diluted up to the mark with diluent to give the concentration of 75µg/ml and 75 µg/ml respectively[20].

Procedure: The standard solution was injected for five times and the area was measured for all five injections in HPLC. The %RSD for the area and R_t of five replicate injections was found to be within the specified limits [21].

Acceptance criteria

1. % Relative standard deviation of peak areas and R_t should not be more than 2.0

% **Accuracy**

Assay was performed in triplicate for various concentrations of Metformin and Piogltazone equivalent to 50, 100, and 150 % of the standard amount was injected into the HPLC system as per the test procedure [22].

Preparation of Standard stock solution:

10 mg of Metformin and 10mg of Piogltazone accurately weighed and transferred into a 10 ml clean dry volumetric flask, about 7 ml of diluent was added, sonicated to dissolve it completely and volume was made up to the mark with the same solvent to give the concentration of 1000 µg/ml. (Stock solution)

Preparation Sample solutions:

Preparation of 50% solution (37.5 µg/ml of Metformin and 37.5 µg/ml of Piogltazone):

From the above stock solutions take 0.375ml into 10 ml dry volumetric flask, make up to the mark with diluent**.**

Preparation of 100% solution (75 µg/ml of Metformin and 75 µg/ml of Piogltazone):

From the above stock solutions take 0.3 ml and 1.8 ml into 10 ml dry volumetric flask, make up to the mark with diluent

Preparation of 150% solution (102.5 µg/ml of Metformin and 102.5 µg/ml of Piogltazone):

From the above stock solutions take 1.025 ml into 10 ml dry volumetric flask, make up to the mark with diluent. These solutions were filtered through 0.45µ membrane and then each concentration; three replicate injections were made under the optimized conditions

Procedure:

The standard solution, Accuracy -50%, Accuracy -100% and Accuracy -150% solutions were injected. The amount found and amount added for Metformin and Piogltazone individual recovery and mean recovery values were calculated and the results were found to be within the limit.

Acceptance criteria

The mean % recovery of the Metformin and Piogltazone each spike level should be not less than 98.0 % and not more than 102.0 %[23].

Robustness

The robustness of the proposed method was determined by analysis of aliquots from homogenous lots by differing physical parameters like flow rate and mobile phase composition, temperature variations which may differ but the responses were still within the specified limits of the assay[24].

a) Effect of variation of flow rate*:*

A study was conducted to determine the effect of variation in flow rate. The flow rate was varied at 1.0 ml/min to 1.2 ml/min. Standard solution 75 ppm Metformin and 75 ppm Piogltazone were prepared and analysed using the varied flow rates along with method flow rate [25]. The results are summarized on evaluation of the above results, it can be concluded that the variation in flow rate affected the method significantly [26]. Hence it indicates that the method is robust even by change in the flow rate ±10%.

The method is robust only in less flow condition. The effect of variation of flow rate was evaluated.

Acceptance criteria

a. The tailing factor for Metformin and Piogltazone should be not more than 2.0 for Variation in flow.
b. The % RSD of Asymmetry and retention time for Metformin and Piogltazone should be not more than 2.0 % for variation in flow.

b) Effect of variation of mobile phase composition:

A study was conducted to determine the effect of variation in mobile phase ratio by changing the ratio of mobile phase [27]. The Organic composition in the Mobile phase was varied from 40 % to 60 %.Standard solution 75 µg/ml of Metformin and 75 µg/ml Piogltazone were prepared and analysed using the varied mobile phase composition along with the actual mobile phase composition in the method [28,29].

Acceptance criteria

a. Tailing Factor of Metformin and Piogltazone drugs should not be more than 2.0 for Variation in composition of mobile phase.
b. The % RSD of tailing factor and retention times of Metformin and Piogltazone drugs should be not more than 2.0 for Variation in composition of mobile phase.

3. Results and Discussion

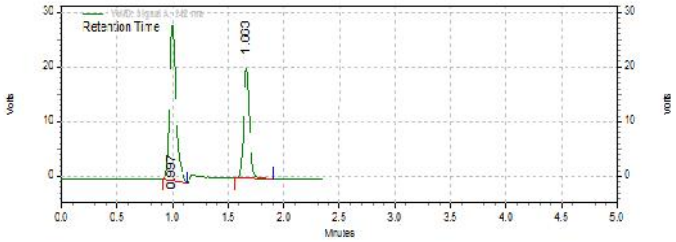

Figure 3: Chromatogram for Linearity level 1

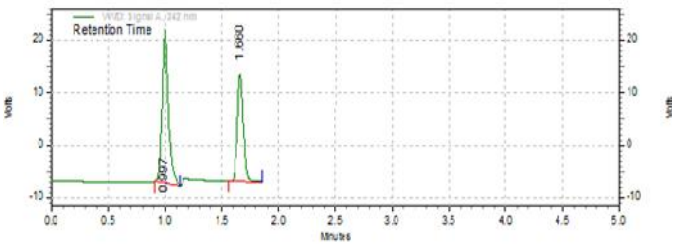

Figure 4: Chromatogram for Linearity level 2

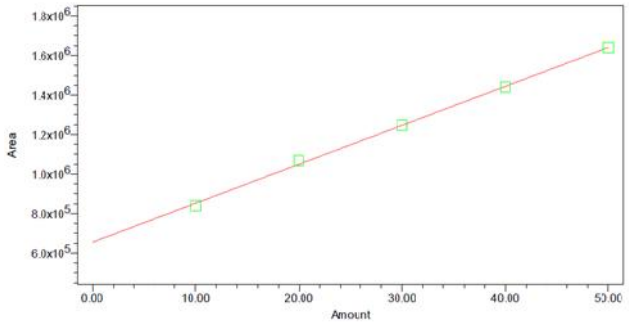

Figure 5: Calibration curve of Metformin

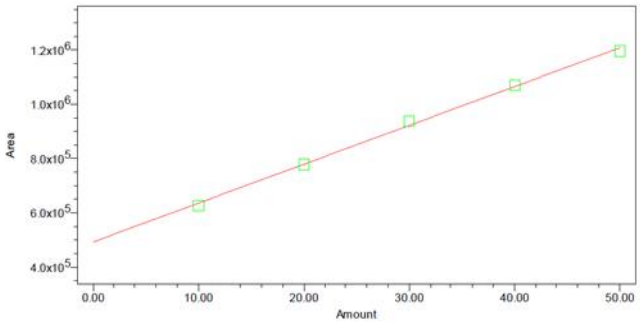

Figure 6: Calibration curve of Pioglitazone

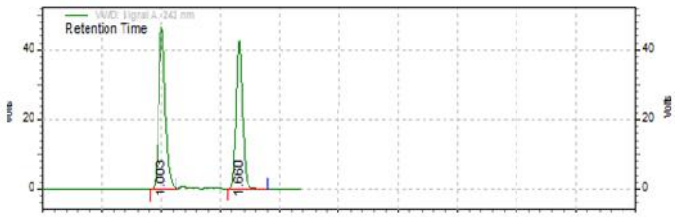

Figure 7: Sample Chromatograms for Repeatability

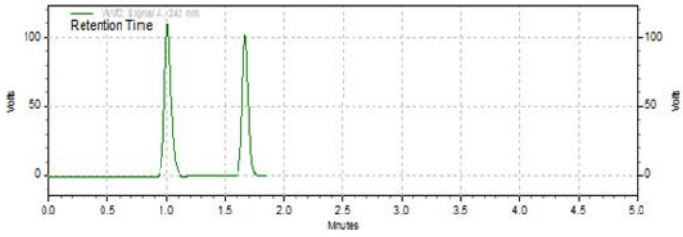

Figure 8: Chromatograms for Intermediate precision

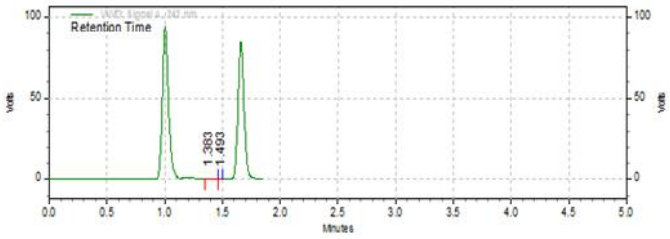

Figure 9: Chromatograms for Intermediate precision

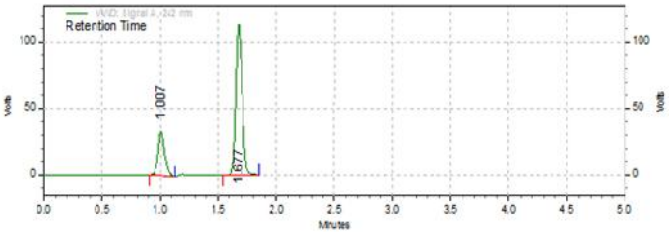

Figure 10: Standard Chromatogram for Accuracy

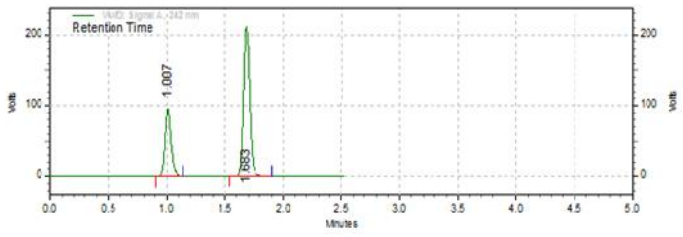

Figure 11: Chromatogram for Acc 50%

Table 1: List of Standard and Sample details

S.No	Name	Batch No.	Manufaturer/ supplier
1.	Metformin working standard	-	KP labs pvt. Ltd
2.	Pioglitazone working standard	-	KP labs pvt. Ltd

Table 2: List of Equipment/Instrument details

S.No	Instrument name	Model
1.	HPLC system	Waters, software: Empower, 2695 separation module. 2996 PDA detector.
2.	Semi micro balance	Sartorius ME235P
3.	P^H Meter	Lab India ph. Meter
4.	Sonicator	Ultrasonic cleaner power sonic 420
5.	UV/VIS spectrophotometer	LABINDIA UV
6.	Constant temperature water bath	Thermolab GMP

Table 3: List of Chemicals and Reagents

S.No	Name	Manufacturer	Grade
1.	Potassium dihydrogen ortho phosphate	Merck	GR
2.	Sodium perchlorate	Merck	GR
3.	Perchloric acid	Merck	GR
4.	Ortho phosphoric acid	Merck	GR
5.	Methanol	Merck	HPLC
6.	Acetontrile	Merck	HPLC
7.	Water	Milli-pore	Milli-Q
8.	0.45 µm Nylon filter	Axivia	S0761009
9.	0.45µm PVDF filter	Rankem	D004A07

Table 4: Linearity results for Metformin

S.No	Linearity Level	Concentration	Area
1	I	25 ppm	2013603
2	II	50 ppm	2343280
3	III	75 ppm	4773302
4	IV	100 ppm	6244426
5	V	125 ppm	755227
Correlation Coefficient			0.999

Table 5: Linearity results for Pioglitazone

S.No	Linearity Level	Concentration	Area
1	I	25 ppm	3243280
2	II	50 ppm	4773302
3	III	75ppm	6244426
4	IV	100ppm	7552271
5	V	125 ppm	8916832
Correlation Coefficient			0.999

Table 6: Calibration parameters for Metformin and Pioglitazone

Parameter	Results for Metformin	Results for Pioglitazone
Slope	19718	14311
Intercept	65498	49120
Correlation co-efficient	0 .999	

Table 7: Linearity results for Metformin:

S.No	Linearity Level	Concentration	Abs
1	I	25 ppm	0.166
2	II	50 ppm	0.30
3	III	75 ppm	0.468
4	IV	100 ppm	0.610
5	V	125 ppm	0.767
Correlation Coefficient			0.999

Table 8: Linearity results for Pioglitazone

S.No	Linearity Level	Concentration	Abs
1	I	25 ppm	0.150
2	II	50 ppm	0.310
3	III	75 ppm	0.176
4	IV	100 ppm	0.627
5	V	125 ppm	0.810
Correlation Coefficient			0.999

Table 9A: Sample Chromatogram values for Repeatability of Metformin

Injection No	Peak Area	R_t
1	6249423	1.007
2	6249423	1.006
3	6249423	1.007
4	6249423	1.004
5	6249423	0.995
6	6225772	1.005
Avg	6198494	
SD	26810.4	
% RSD	0.43	

Table 9B: Sample Chromatogram values for Repeatability of Pioglitazone

Injection No	Peak Area	R_t
1	5147832	1.77
2	5147832	1.82
3	5147832	1.95
4	5147832	1.75
5	5147832	1.79
6	5147832	1.81
Avg	5169153	
SD	25816.7	
% RSD	0.49	

Acceptance Criteria: The % RSD for the area and Rt of five standard injections results should not be more than 2%

Table 10A: Sample Chromatogram values for intermediate Precision of Metformin

Injection No	Peak Area	R_t
1	6243294	1.077
2	6243294	1.065
3	6243294	1.074
Mean	6209408	
SD	35056.4	
%RSD	0.56	

Table 10B: Sample Chromatogram values for intermediate Precision of Pioglitazone

Injection No	Peak Area	R_t
1	5144782	1.775
2	5144782	1.810
3	5144782	1.810
Mean	5151041	
SD	20905.4	
% RSD	0.40	

Acceptance Criteria: The % RSD for the areas and Rt's of three standard injections results should not be more than 2%

Table 12: Chromatogram Values for Accuracy of Metformin

Sample No.	Spike Level	Amount (µg/ml) added	Amount (µg/ml) found	% Recovery	Mean % Recovery	Stdev	% Rsd
1	50 %	0.75	0.74	99.8%	99.0%	0.79	0.80
		0.75	0.74	99.18%			
		0.75	0.73	98.2%			
2	100 %	1	0.99	98.8%	99.13%	0.56	0.57
		1	1.00	99.1%			
		1	0.98	99.5%			
3	150 %	1.25	1.24	99.2%	99.69%	0.26	0.26
		1.25	1.24	99.0%			
		1.25	1.24	99.79%			

Acceptance Criteria: The % Recovery for each level should be between 98.0 to 102.0%.

Table 13: Chromatogram Values for Accuracy of Pioglitazone

Sample No.	Spike Level	Amount (µg/ml) added	Amount (µg/ml) found	% Recovery	Mean % Recovery	Stdev	% Rsd
1	50 %	0.75	0.74	99.9%	99.4%	0.27	0.27
		0.75	0.74	99.6%			
		0.75	0.74	99.9%			
2	100 %	1	0.99	99.8%	99.65%	0.13	0.13
		1	0.99	99.1%			
		1	0.99	99.5%			
3	150 %	1.25	1.23	98.2%	98.89%	0.24	0.24
		1.25	1.23	99.0%			
		1.25	1.23	98.79%			

Acceptance Criteria: The % Recovery for each level should be between 98.0 to 102.0%

4. Conclusion

High performance liquid chromatography and spectroscopy are at present one of the most sophisticated tool of the analysis. The estimation of Metformin and Pioglitazone was done by RP-HPLC and UV Methods. The mobile phase was optimized with consists of Acetonitrile: Water mixed in the ratio of 50:50 % v/ v. A $C1_8$ column C18 (4.6 x 150mm, 5µm, Make: XTerra) or equivalent chemically bonded to porous silica particles was used as stationary phase.The solutions were chromatographed at a constant flow rate of 1.0 ml/min. the linearity range of Metformin and Pioglitazone were found to be from 25-125 µg/ml. of Metformin and Pioglitazone. Linear regression coefficient was not more than 0.999.The maximum absorbance are found to be at 242 nm.The values of % RSD are less than 2% indicating accuracy and precision of the method. The percentage recovery varies from 97-102% of Metformin and Pioglitazone. LOD and LOQ were found to be within limit. The results obtained on the validation parameters met ICH and USP requirements. It inferred the method found to be simple, accurate, precise and linear. The method was found to be having suitable application in routine laboratory analysis with high degree of accuracy and precision.

5. References

[1] Campbell DB, Lavielle R, Nathan C. The mode of action and clinical pharmacology of gliclazide: a review. Diab Res Clin Prac.**1991**; 14:S21-S36.

[2] Hassa Saad S.M., Mahmoud Wagiha H., Elmosallamy Mohamed A.F, Othman Abdel Hammeed M. Determination of metformin in pharmaceutical preparations using potentiometry, spectrofluorimetry & UV–vis spectrophotometry. Anal. Chimica Acta. **1999**, 378(1-3): 299-311.

[3] AbuRuz, S.,Millership J,McElnay J. The development and validation of liquid chromatography method for the simultaneous determination of metformin and glipizide, gliclazide, glibenclamide or glimperide in plasma. J Chromatogr. B, **2005**, 817(2): 277-286.

[4] Herman G, Bergman A, Liu F, Stevens C, Wang A, Zeng W, Chen L, Snyder K, Hilliard D, Tanen M, Tanaka W, Meehan A, Lasseter K, Dilzer S, Blum R, Wagner J. Pharmacokinetics and pharmacodynamic effects of the oral DPP-4 inhibitor sitagliptin in middle-aged obese subjects.

J.Clin.Pharmacol. **2006**, 46 (8): 876–886

[5] Florentin T, Monica A .Specificity of an analytical hplc assay method of metformin hydrochloride. Revue Roumaine de Chimie.**2007**, 52(6):603–609.

[6] Georgita C, Albu F, David V, Medvedovici A.Simultaneous assay of metformin and glibenclamide in human plasma based on extraction-less sample preparation procedure and LC /(APCI)MS. J.Chromatogr. B. **2007**, 854(1-2): 211-218.

[7] Wei Zeng, Donald Musson G, Alison Fisher L, Li Chen, Michael Schwartz S, Eric Woolf J, Amy Qiu Wang. Determination of sitagliptin in human urine and hemodialysate using turbulent flow online extraction and tandem mass spectrometry. J. Pharm. Biomed.Anal. **2008**, 46(3): 534-542.

[8] Ramakrishna Nirogi, Vishwottam Kandikere, Koteshwara Mudigonda, Prashanth Komarneni, Raghupathi Al eti, Rajeshkumar Boggavarapu. Sensitive liquid chromatography tandem mass spectrometry method for the quantification of sitagliptin, a DPP-4 inhibitor, in human plasma using liquid–liquid extraction. Biomed. Chromatogr. **2008**, 22(2): 214–222.

[9] Jain D, Jain S,Jain D ,Maulik A. Simultaneous Estimation of Metformin Hydrochloride, Pioglitazone Hydrochloride, and Glimepiride by RP-HPLC in Tablet Formulation . Journal of Chromatogr. Sci. **2008**, 46: 501-504.

[10] Patil S.S., Bonde C. .Development and Validation of analytical method for Simultaneous Estimation of Glibenclamide and Metformin HCl in Bulk and Tablets using UV visible spectroscopy, Int. J. ChemTec. Res. **2009**, 1(4): 905-909.

[11] Lakshmi KS, Rajesh T, Sharma S, Lakshmi S. Development and Validation of Liquid Chromatographic and UV Derivative Spectrophotometric Methods for the Determination of Metformin, Pioglitazone and Glimepiride in Pharmaceutical Formulations. Der Pharma Chemica. **2009**, 1 (1): 238-246.

[12] Robert Moses, Fixed combination of repaglinide and metformin in the management of type 2 diabetes, Diabetes, Metabolic syndrome and obesity. Targets and Therapy Dove press, open access to scientific and medical research. **2009**, 2: 101-9.

[13] Al-Rimawi F, Development and validation of an analytical method for metformin hydrochloride and its related compound (1-cyanoguanidine) in tablet formulations by HPLC-UV.Talanta. Epub **2009**, 79(5): 1368-71.

[14] Bala Sekaran C, Prameela Rani A. Development and Validation of spectrophotometric method for the determination of DPP-4 Inhibitor, Sitagliptin, in its pharmaceutical preparations. Int. J. Pharm. Pharm. Sci. **2010**, 2(4): 138-142.

[15] Zeng W, Xu Y, Constanzer M, Woolf EJ. Determination of sitagliptinin human plasma using protein precipitation and tandem mass spectrometry. J.Chromatogr. B. **2010**, 878(21): 1817-1823.

[16] Freddy H. Havaldar, Dharmendra L.Vairal. Simultaneous estimation of metformin hydrochloride, rosiglitazone and pioglitazone hydrochloride in the tablets dosage form. Int. J.Appl .Bio. Pharm.Tec. **2010**, 1(3): 1000-1005.

[17] Alexandar S, Diwedi R, Chandrasekar M. A RP-HPLC method for simultaneous estimation of metformin and pioglitazone in pharmaceutical formulation. Res. J.Pharm.BioChemica. Sci. **2010**, 1(4): 858-866.

[18] Pawar S, Meshram G, Jadhav R,Bansal Y.Simultaneous determination of Glimepiride and Metformin hydrochloride impurities in sustained release pharmaceutical drug product by HPLC .Der Pharma Chemica. **2010**, 2(4): 157-168.

[19] Havele S, Dhaneshwar S. Development and validation of a HPLC method for the determination of metformin hydrochloride, gliclazide and piogliglitazone hydrochloride in multicomponent formulation. Webmed central pharmaceutical sciences. **2010**, 1(10): 1078

[20] Havele S, Dhaneshwar S. Estimation of Metformin in Bulk Drug and in Formulation by HPTLC.J Nanomedic Nanotechnolo, **2010**, 1: 102.

[21] Parag Pathade, Imran Md, Vinod Bairagi,Yogesh Ahire. Development and Validation of Stability Indicating UV Spectrophotometric Method for the Estimation of Sitagliptin Phosphate in Bulk and Tablet Dosage Form. J. Pharm. Res. **2011**, 4(3): 871-873.

[22] Ghazala Khan, Dinesh Sahu Agrawal Y P, NeetuSabarwal, AvnishJain,Gupta A K. Simultaneous Estimation of Metformin and Sitagliptin In Tablet Dosage Form. Asian J. Biochem. Pharma. Res. **2011**, 1(2): 352-358.

[23] Ramzia El-Bagary I, Ehab Elkady F, BassamAyoub M. Spectroflourometric and Spectrophotometric Methods for the Determination of Sitagliptin in Binary Mixture with Metformin and Ternary Mixture with Metformin and Sitagliptin Alkaline Degradation Product. Int. J. Biomed. Sci. **2011**, 7(1): 62-69.

[24] Ravi PratapPulla, Sastry B S, Rajendra Prasad Y, AppalaRaju N. Simultaneous Estimation of Metformin HCl and Sitagliptin Phosphate in Tablet Dosage Forms by RP-HPLC. Res. J. Pharm. Tech. **2011**, 4(4): 646-649.

[25] Shyamala M, Mohideen S, Satyanarayana T, Narasimha Raju Ch, Suresh Kumar P, SwethaK. Validated RP-HPLC for simultaneous estimation of Sitagliptin phosphate and Metform in hydrochloride in tablet dosage form. American J. Pharm Tech. Res. **2011**, 1(2): 93-101.

[26] Tripathi K.D. Essential of Medical Pharmacology, 5th Edn, Jaypee Brothers Medical publisher New Delhi. pp: 515-516.

[27] Dubal Anil, Khatwal Rizwanbasha, Kosaraju Jayasankar, Meda Venkat, Samanta Malay.

Bioanalytical method development and validation of sitagliptin phosphate by RP-HPLC and its application to pharmacokinetic study. Int. J. Pharm Pharm Sci. **2012**, 4(2): 691-694.

[28] Govindasamy Jeyabalan Simultaneous estimation of saxagliptin hydrochloride and Metformin hydrochloride in active pharmaceutical ingrident by RP-HPLC. **2012**, vol.4.

[29] T.Raja and A.Lakshmana Rao. Validated RP-HPLC Method for Simultaneous Estimation of Metformin Hydrochloride and Sitagliptin Phosphate in Bulk Drug and Pharmaceutical Formulation. Int. Journal of Pharmaceutical, Chemical and Biological Sciences. **2012**, 2(4): 696-702.