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ANALYTICAL AND BIOANALYTICAL UHPLC-MS METHOD VALIDATION FOR DETERMINATION OF METFORMIN, A BIGUANIDE AND SITAGLIPTIN, A DPP-4 INHIBITOR

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ABSTRACT: A very sensitive, rapid and simple Ultra High Performance Liquid Chromatography-Mass Spectrometry (UHPLC-MS) method has been developed and validated for the simultaneous estimation of Metformin Hydrochloride and Sitagliptin Phosphate in standard and tablet formulation. The chromatographic separation of the drugs was achieved on a Hypersil Gold C18 analytical column (150x3mm, 5µ) with an isocratic elution of methanol and water with 0.2% formic acid (48:52 v/v) at a constant flow rate of 500µL/min on a UHPLC and the retention time of Metformin Hydrochloride and Sitagliptin Phosphate were 1.40±0.01 and 2.38±0.01 min respectively. Elutes were detected online on an Orbitrap mass spectrometer in ESI (+) mode. The method was validated according to ICH guidelines. The calibration plot was linear with correlation coefficient (r^2) of 0.9998 for Metformin Hydrochloride in the concentration range of 0.04-0.28ng/mL and 0.9992 for Sitagliptin Phosphate in the concentration range 0.04-0.28 ng/mL respectively. The LLOD of Metformin Hydrochloride and Sitagliptin Phosphate were 0.2 and 0.02pg/mL respectively. This method was validated with respect to linearity, accuracy, precision, specificity and robustness. The developed method was successfully applied and validated for the bioanalytical study in rat plasma according to US-FDA guidelines.

INTRODUCTION: Metformin Hydrochloride is chemically, 3-(diaminomethylidene)-1, 1-dimethylguaniine Hydrochloride **(Figure 1)**. It is a biguanide drug known as oral anti-diabetic drug. Inhibition of the mitochondrial respiratory chain (complex I), activation of AMP-activated protein kinase (AMPK), inhibition of glucagon-induced elevation of cyclic adenosine monophosphate (cAMP) and consequent activation of protein kinase A (PKA) and an effect on gut microbiota have been proposed as potential mechanisms of Metformin.

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It is the only currently available oral anti-diabetic/hypoglycemic agent that acts predominantly by inhibiting hepatic glucose release. As patients with type 2 diabetes often have excess hepatic glucose output, use of Metformin is effective in lowering glycosylated hemoglobin (HbA1c) [1, 2]. Metformin Hydrochloride was first extracted from an herb Galega officinalis and it was synthesized in 1922 in Dublin as a blood glucose lowering agent [3]. Metformin Hydrochloride is a white crystalline powder, soluble in water and methanol. The molecular weight of Metformin Hydrochloride is 165.63.

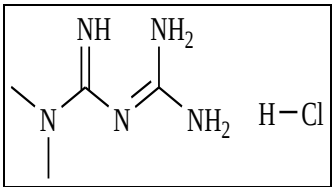

FIGURE 1: CHEMICAL STRUCTURE OF METFORMIN HYDROCHLORIDE

Sitagliptin Phosphate is chemically, (3R)-3-amino-1-[3-(trifluoromethyl)-5,6 dihydro[1,2,4] triazolo [4,3-a]pyrazin-7(8H)-yl]-4-(2,4,5-trifluorophenyl) butan-1-one Phosphate hydrate **(Figure 2)**. It is an oral hypoglycaemic drug that belongs to dipeptidyl-peptidase 4 (DPP-4) inhibitor classes. This enzyme breaks down the incretins GLP-1and GIP, gastrointestinal hormones released in response to a meal. By preventing GLP-1 and GIP inactivation, it is able to increase the secretion of insulin and suppress the release of glucagon by the alpha cells of the pancreas [4].

Sitagliptin Phosphate is used for the improvement of glycemic control in patients with type II diabetes mellitus as monotherapy or combination therapy with Metformin Hydrochloride. Recently the combination of two drugs proved to be effective in controlling the metabolic syndrome and resulted in significant weight loss, reversal of insulin resistance, islet and adipocyte hypertrophy and achieved hepatic steatasis. Sitagliptin Phosphate is a white crystalline powder soluble in water and methanol. The molecular weight of Sitagliptin Phosphate monohydrate is 523.33.

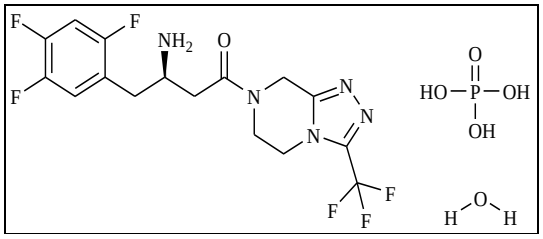

FIGURE 2: CHEMICAL STRUCTURE OF SITAGLIPTIN PHOSPHATE

The literature survey revealed that there are many methods reported for Metformin Hydrochloride and Sitagliptin Phosphate in single and combined dosage form. Simultaneous UV spectrophotometric method for estimation of Sitagliptin Phosphate and Metformin Hydrochloride in bulk and tablet dosage form [5-7], Spectrofluorimetric method for determination of Sitagliptin Phosphate in formulation and spiked human urine [8], validated HPTLC (High performance thin layer chromatography) method for the simultaneous determination of Metformin Hydrochloride and Sitagliptin Phosphate in marketed formulation [9, 10] and simultaneous estimation of Sitagliptin Phosphate monohydrate and Metformin Hydrochloride in bulk and pharmaceutical formulation by RP-HPLC (Reverse phase high performance liquid chromatography) [11-14] were reported.

Bioanalytical methods were also reported for HPLC-UV determination of Metformin in human plasma for application in pharmacokinetics and bioequivalence studies [15], a molecularly imprinted polymer was developed for selective extraction followed by liquid chromatographic determination of Sitagliptin in rat plasma and urine [16, 17], Sitagliptin Phosphate [18], in combination of both the drugs [19] and in combination with some other drugs [20] were reported.

An UPLC (Ultra performance liquid chromatography) [21] and capillary electrophoresis [22] methods were also reported for the estimation of drugs in combination. A simultaneous quantitation of Metformin Hydrochloride and Sitagliptin Phosphate in mouse and human dried blood spots using laser diode thermal desorption tandem mass spectrometry [23] was also reported.

To date, according to literature survey no UHPLC-MS method was reported for simultaneous determination of Metformin and Sitagliptin in tablet formulation form or in rat plasma. The present work describes a simple, sensitive, selective, accurate, precise and robust UHPLC-MS method for simultaneous estimation of Metformin Hydrochloride and Sitagliptin Phosphate in tablet formulation and in rat plasma. The novelty of the method is its very low level of detection of drugs and low volume of plasma sample used.

MATERIALS AND METHODS:

Materials and Reagents:

Metformin standard was purchased from Sigma Aldrich. Sitagliptin standard was procured as a gift sample from VerGo Pharma research laboratories Pvt. Ltd Goa. LC-MS grade solvents Methanol and Water were purchased from J.T. Baker® Chemicals, Formic acid was purchased from Fluka analytical. Janumet tablets, containing Metformin Hydrochloride (500mg) and Sitagliptin Phosphate (50mg) manufactured by MSD Pharmaceuticals pvt.ltd was purchased from local pharmacist.

Instrumental and Chromatographic conditions:
The analysis of Metformin Hydrochloride and Sitagliptin Phosphate was carried out on Q-Exactive Orbitrap UHPLC-MS system. Method development and validation was carried out on UHPLC system, consisting of a LC-pump (Accela), degasser and autosampler. UHPLC system was coupled to a quadrupole-orbitrap mass spectrometer by electrospray ionization (ESI) source. Data acquisition and processing were performed using Thermo Xcalibur Qual browser (Version 2.2).

Chromatographic separation was carried out on a Hypersil Gold C18 analytical column (150x3mm, 5µ) with a isocratic elution of the mobile phase system consisting of methanol (A) and water with 0.2% formic acid (B), (48:52, v/v) at a constant flow rate of 500µL/min, at ambient temperature. The autosampler was set to inject 4µL of sample with a chromatographic run time of 10min. Mass detection was carried out in positive electrospray ionization (ESI) mode. The tuning parameters for the ESI-MS were set as follows: capillary temperature 320ºC, spray voltage 3.60 kV, heater temperature 350ºC, sheath gas flow rate 45, Aux gas flow rate 10 and Sweep gas flow rate is 2.

Preparation of mobile phase:
Different mobile phase trials were taken with combination of methanol: water and acetonitrile: water to optimize mobile phase. Mobile phase A was 100% Methanol and Mobile phase B was prepared by adding 0.2% Formic acid in water then degassed.

Preparation of standards:
The stock solutions of Metformin Hydrochloride and Sitagliptin Phosphate were prepared in methanol at concentrations of 1mg/mL and 0.1 mg/mL respectively. 1µL of solution was pipetted out from the above stock to make up the volume up to 1mL with methanol.

Preparation of Sample solution:
Twenty tablets of Janumet™ containing 500 mg of Metformin Hydrochloride and 50 mg of Sitagliptin Phosphate were accurately weighed and crushed in to a fine powder. An amount of powder equivalent to 10mg of Metformin Hydrochloride and 1mg of Sitagliptin Phosphate was transferred in to a 10 mL volumetric flask and 5mL of methanol was added. It was spinixed to dissolve and then further diluted with methanol up to 10mL mark. This was used as stock solution. 1µL of solution was pipetted out from the above stock to make up the volume to 1mL with methanol.

Preparation of plasma sample & extraction:
The collected rat plasma sample was stored at -80ºC and allowed to thaw gradually to room temperature before processing. An aliquot of 10 µL of each stock solution of the drugs and 20µl plasma were taken into eppendrof tubes. Metformin Hydrochloride and Sitagliptin Phosphate were extracted by protein precipitation technique using acetonitrile as a precipitating solvent then vertexed for 30 sec and centrifuged at 10,000 rpm for 10 min. The supernatant containing drugs was collected and analysed on UHPLC-MS.

Analytical method validation:
Method validation was performed as per ICH guidelines for simultaneous estimation of Metformin Hydrochloride and Sitagliptin Phosphate in combined dosage. The following validation was addressed: system suitability, specificity, linearity, accuracy, recovery and precision, limit of detection and limit of quantification.

System suitability:
The system suitability was assessed by analyzing five replicate injections for the drugs at concentration of 1.6ng/mL of Metformin Hydrochloride and 0.16ng/mL of Sitagliptin Phosphate. The acceptance criteria should not be more than 2.0% RSD for the peak areas of the both the drugs.

Specificity:
The diluents chromatograms and sample chromatograms were compared for the interference of any extra peak at the same retention time of the sample. If there is no interference of any extra peak then this indicates that diluents used in sample preparation do not interfere in the estimation of Metformin Hydrochloride and Sitagliptin Phosphate.

Calibration curve and Linearity:
The calibration curves were plotted between peak area and concentration, regression analysis was performed by calculating slope, intercept and correlation coefficient (R^2). The drugs were evaluated by making three replicate measurements in the concentration range of 0.4 to 2.8ng/mL for Metformin Hydrochloride and 0.04 to 0.28ng/mL for Sitagliptin Phosphate.

Accuracy:
The accuracy of the method was evaluated at three concentration levels i.e. 75%, 100% and 125% for standard and sample in triplicate. The calculated values of percentage recovery should be between 98.00% -102%.

Precision:
The precision of the method was carried out by repeatability of injection. Intra-day (repeatability) and inter-day (intermediate) precision were studied by calculating the percentage relative standard deviation (%RSD) for six determinations of peak areas of Metformin Hydrochloride (1.6 ng/mL) and Sitagliptin Phosphate (0.16 ng/mL).

Robustness:
The robustness of a method is the ability of the method to remain unaffected by making slight deliberate changes in chromatographic conditions. The robustness of the method was studied by making slight changes in ratio of the mobile phase, temperature and flow rate. The %RSD was calculated by taking average of three replicate injections.

Bioanalytical Method Validation:
The method was validated in accordance with US-FDA bioanalytical method validation guidelines with respect to specificity, recovery, precision, accuracy and stability.

Specificity:
The specificity was assessed by comparing chromatograms of three blank plasma samples and plasma sample spiked with Metformin Hydrochloride and Sitagliptin Phosphate.

Recovery: The recoveries were determined by comparing the peak area of standard (un-extracted sample) with the peak area of extracted drugs from plasma sample at three different concentration levels (low, medium, and high).

Precision and accuracy:
The intra-day and inter-day precision (%CV) and accuracy were determined by analysis of five replicates at three concentration levels (low, medium, and high). The intra-day and inter-day precision should not exceed 15% and the accuracy was required to be within ±15%.

Stability:
The stability was carried out by analyzing three replicates of stability samples at three concentration levels (low, medium, and high). Freeze thaw stability samples stored at $-80^{o}C$ for 24h were thawed at room temperature. After completely thawed, the samples should be refrozen for 24 h under the same conditions. The short-term temperature stability samples were kept at room temperature for 8 h. The long-term temperature stability samples were kept at refrigerator for 7 days. The acceptable precision and accuracy should be within ±15%.

RESULTS AND DISCUSSION:

Method Development:
A UHPLC method was developed for Metformin Hydrochloride and Sitagliptin Phosphate in bulk and tablet dosage form. The chromatographic conditions were optimized in order to provide a good performance of the assay. The mobile phase for drug was selected based on its polarity. Different trails were taken and finally the optimized mobile phase was methanol and water (with 0.2% formic acid) in the ratio of 48:52 v/v with a flow rate of 500µL/min. The retention time of Metformin Hydrochloride is 1.40±0.01min and Sitagliptin Phosphate is 2.38±0.01min respectively. The **Figure 3** and **Figure 4** represent the chromatograms and mass spectra of Metformin Hydrochloride and Sitagliptin Phosphate respectively.

Analytical Method Validation:
System suitability:
The system suitability was performed by analyzing five replicate injections of Metformin Hydrochloride (1.6ng/mL) and Sitagliptin

Phosphate (0.16ng/mL). The %RSD of Metformin Hydrochloride and Sitagliptin Phosphate was found to be 0.523 and 1.391respectively.

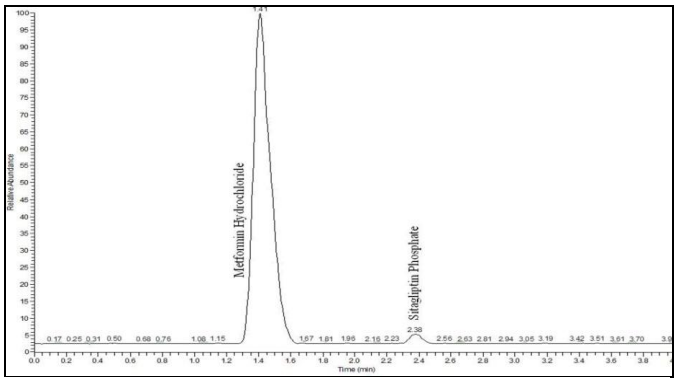

FIGURE 3: CHROMATOGRAM OF STANDARD METFORMIN HYDROCHLORIDE AND SITAGLIPTIN PHOSPHATE

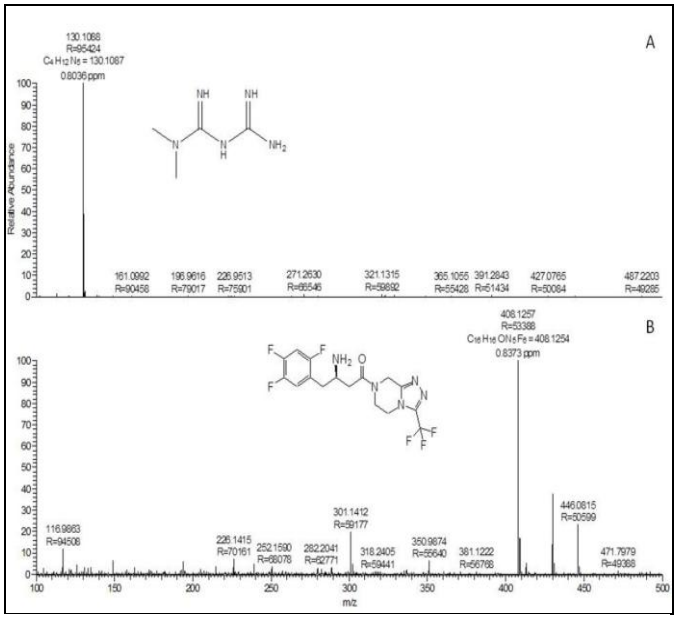

FIGURE 4: MASS SPECTRUM OF METFORMIN HYDROCHLORIDE [A] AND SITAGLIPTIN PHOSPHATE [B]

Specificity: The developed UHPLC-MS method used for the analysis and identification of Metformin Hydrochloride and Sitagliptin Phosphate was shown to be specific. The retention time for Metformin Hydrochloride and Sitagliptin Phosphate was 1.40±0.01 and 2.38±0.01 min respectively. No interfering peaks were observed within the same retention time of the analytes.

Linearity:

The calibration curves plotted against the peak area and concentration over a working standard range of seven samples, 0.4 to 2.8ng/mL for Metformin Hydrochloride and 0.04 to 0.28ng/mL for Sitagliptin Phosphate (n=3) has shown linear response, with a correlation coefficient (R^2) of 0.9998 and 0.9992 respectively **(Table 1)**.

TABLE 1: LINEARITY OF METFORMIN HYDROCHLORIDE AND SITAGLIPTIN PHOSPHATE

Parameters	Metformin Hydrochloride	Sitagliptin Phosphate
Linearity range (ng/spot) n=3	0.4-2.8ng/mL	0.04-0.28ng/mL
Regression equation	Y=200000000x +20000000	Y=100000000x +651684
Correlation coefficient (r)	0.9998	0.9992

Accuracy:

The accuracy studies of Metformin Hydrochloride and Sitagliptin Phosphate were determined at 75%, 100% and 125% concentration levels in triplicates. The percentage recovery of Metformin Hydrochloride and Sitagliptin Phosphate was found to be in the range of 100.10% to 100.91% and 98.93% to 99.75% respectively **(Table 2)**.

TABLE 2: PERCENT RECOVERY OF METFORMIN HYDROCHLORIDE AND SITAGLIPTIN PHOSPHATE

%Level	Metformin Hydrochloride			Sitagliptin Phosphate		
	%Recovery	%Mean Recovery	%RSD	%Recovery	%Mean Recovery	%RSD
75	100.66			98.93		
75	100.20	100.32	0.29	99.75	99.30	0.41
75	100.10			99.24		
100	100.54			99.32		
100	100.62	100.69	0.19	99.54	99.28	0.27
100	100.91			99.00		
125	100.40			98.47		
125	100.14	100.30	0.14	99.34	98.98	0.46
125	100.38			99.15		

Precision:

The precision of the analytical method was studied by determining the concentration of each drug in the tablet in six replicates. The results of the precision study indicate that the method is reliable and the %RSD for the precision study was 0.181% and 0.181% (inter-day precision), 0.197% and 0.451% (intra-day precision) for Metformin Hydrochloride and Sitagliptin Phosphate respectively **(Table 3)**.

TABLE 3: PRECISION STUDY OF METFORMIN HYDROCHLORIDE AND SITAGLIPTIN PHOSPHATE

Precision(n=6)	Metformin Hydrochloride		Sitagliptin Phosphate	
	Intra-day	Inter-day	Intra-day	Inter-day
Injection-1	100.52	100.35	99.32	99.10
Injection-2	100.17	100.39	99.35	99.02
Injection-3	100.32	100.63	99.03	99.28
Injection-4	100.62	100.32	99.54	99.00
Injection-5	100.19	100.38	99.50	99.12
Injection-6	100.28	100.01	99.36	99.68
Average	100.35	100.34	99.35	99.03
Standard Deviation	0.181	0.198	0.180	0.446
%RSD	0.181	0.197	0.181	0.451

Robustness:

The result of the robustness study indicates that the method is robust and is unaffected by small variations in the chromatographic conditions. The %RSD was found less than 2%.

Lower limit of detection (LLOD) and Lower limit of quantification (LLOQ):

Limit of detection of Metformin Hydrochloride and Sitagliptin Phosphate was found to be 0.2 and 0.02pg/mL. Limit of Quantitation of Metformin Hydrochloride and Sitagliptin Phosphate was found to be 400pg/mL and 40pg/mL.

BIOANALYTICAL METHOD VALIDATION:

The specificity, recovery, precision, accuracy and stability of the bioanalytical method were validated according to US-FDA guidelines. The chromatograms and mass spectra of Metformin Hydrochloride and Sitagliptin Phosphate are represented by **Figure 5** and **Figure 6** respectively.

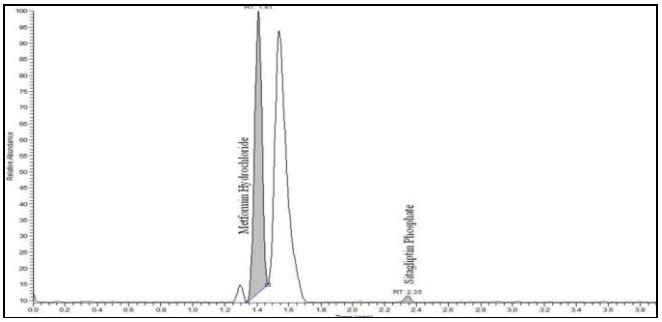

FIGURE 5: CHROMATOGRAM OF SPIKED PLASMA SAMPLE (METFORMIN HYDROCHLORIDE AND SITAGLIPTIN PHOSPHATE)

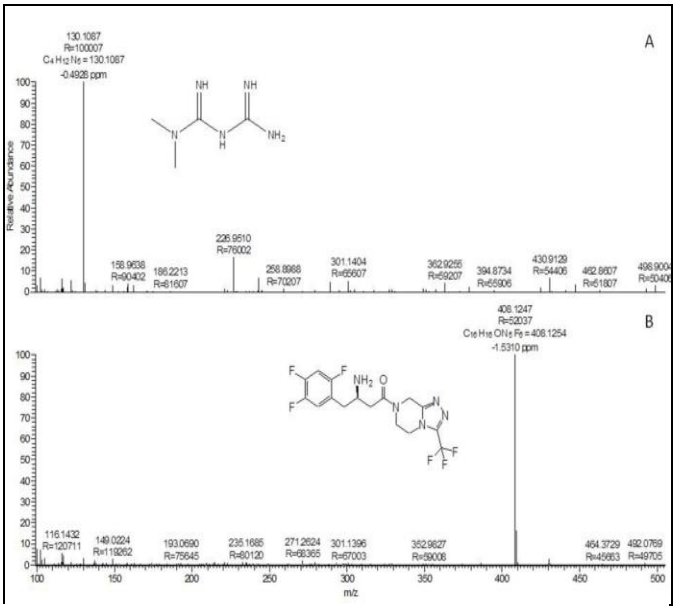

FIGURE 6: MASS SPECTRA OF SPIKED PLASMA SAMPLE, METFORMIN HYDROCHLORIDE (A) AND SITAGLIPTIN PHOSPHATE (B)

Specificity:

The Specificity of three plasma samples was checked for endogenous components which might interfere with Metformin Hydrochloride and Sitagliptin Phosphate peak detection. No interferences were observed in retention time range of Metformin Hydrochloride and Sitagliptin Phosphate in rat plasma samples.

Precision and Accuracy:

The Precision and accuracy were validated at three concentration levels. The intra-day and inter-day

variation, as well as the accuracy, were within the acceptable range, confirming that the current method is reproducible and accurate **(Table 4)**.

TABLE 4: ACCURACY, PRECISION STUDY OF METFORMIN HYDROCHLORIDE (A) AND SITAGLIPTIN PHOSPHATE (B) IN RAT PLASMA (N=5)

Drug	Spiked plasma Conc.(ng/mL)	Intra-day			Inter-day		
		Conc. M±SD	%CV	Accuracy (%RE)	Conc. M±SD	%CV	Accuracy (%RE)
	1.2	1.1943±0.05	6.13	-0.8	1.1394±0.05	5.88	8.3
A	1.6	1.5760±0.04	3.46	-1.0	1.6182±0.06	3.85	0.6
	2.0	1.9750±0.05	1.53	-1.5	1.9851±0.03	1.83	-10
B	0.12	0.1110±0.005	5.30	-7.5	0.1170±0.006	9.55	-2.5
	0.16	0.1534±0.006	4.10	-4.3	0.1594±0.005	5.16	-0.6
	0.20	0.2031±0.007	1.71	1.5	0.1974±0.003	2.88	-1.5

Recovery:

The extraction recoveries of Metformin Hydrochloride and Sitagliptin Phosphate were determined by comparing the peak area of standard (un-extracted sample) with the peak area of extracted plasma sample at three different concentration (low, medium, and high) levels and results are shown in **Table 5**.

TABLE 5: RECOVERY STUDY OF METFORMIN HYDROCHLORIDE AND SITAGLIPTIN PHOSPHATE IN RAT PLASMA (n=3)

Recovery	Metformin Hydrochloride			Sitagliptin Phosphate		
	1.2ng/mL	1.6ng/mL	2.0ng/mL	0.12ng/mL	0.16ng/ml	0.2ng/mL
Mean± SD	100.89±5.68	98.39±4.18	99.84±1.72	97.77±6.54	95.35±5.53	99.24±2.15
%RSD	5.6	4.2	1.7	6.69	5.8	2.1

Stability:

The stability of Metformin Hydrochloride and Sitagliptin Phosphate under the various storage conditions was investigated at three different concentration (low, medium, and high) levels. The evaluated results indicated that Metformin Hydrochloride and Sitagliptin Phosphate in the rat plasma sample were stable under short term, long term and freeze thaw experimental conditions **(Table 6)**.

TABLE 6: STABILITY STUDIES OF METFORMIN HYDROCHLORIDE AND SITAGLIPTIN PHOSPHATE UNDER DIFFERENT CONDITIONS (N=3)

Stability	Metformin Hydrochloride			Sitagliptin Phosphate		
	1.2ng/mL	1.6ng/mL	2.0ng/mL	0.12ng/mL	0.16ng/mL	0.2ng/mL
Short-term	1.223	1.649	2.026	0.124	0.155	0.198
Mean con	2.09	0.78	0.83	6.20	7.93	2.40
CV (%)						
Freeze-thaw	1.141	1.566	1.975	0.111	0.156	0.205
Mean con	0.47	0.93	1.23	3.63	5.39	4.09
CV (%)						
Long-term	1.118	1.545	1.945	0.117	0.162	0.198
Mean con	0.853	0.77	2.03	6.95	2.480	1.94
CV (%)						

CONCLUSION: A simple, fast and reliable UHPLC-MS analytical method was developed and validated for the simultaneous estimation of Metformin Hydrochloride and Sitagliptin Phosphate in bulk and tablet dosage form according to ICH guidelines. The developed method was successfully applied and validated for the bioanalytical study in rat plasma according to US-FDA guidelines. The developed method can be useful for bioavailability/bioequivalence studies and routine therapeutic drug monitoring with desired precision and accuracy. This method may be useful for detection of very low concentration of low molecular weight metabolites in biological fluids.

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