Reverse Phase Chromatographic Method of Analysis for Assay and Content Uniformity Estimation of Drug Sitagliptin, Metformin and Empagliflozin from Available Marketed Formulation

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ABSTRACT

A new method of analysis with reverse phase chemistry was designed and developed. Validation for method of analysis was performed for its intended use to calculate assay and content uniformity of drug substance sitagliptin, metformin and empagliflozin in the drug products. The method has a run time of 10 min on X-bridge C18 column having 250 mm length, 4.6 mm internal diameter and Particle Size of 5 μm, by the use of 0.1% Trifluoroacetic acid Buffer 40%: Methanol 40%: Acetonitrile 20% ratio as constituent composition in the proposed mobile phase and chromatography run at wavelength of 224 nm. The retention time of Metformin, Empagliflozin and Sitagliptin, were 3.383, 5.571 and 6.429 min respectively. International Conference on Harmonization guideline was referred for validation. The method showed adequate sensitivity for precision, linearity and accuracy parameter (between the range 25-75 µg/mL, 250-750 µg/mL and 2.5-7.5 µg/mL for sitagliptin, metformin and empagliflozin respectively). The percentage recoveries obtained for sitagliptin, metformin and empagliflozin are in the range of 98.0–102.0%. As results are within the acceptance[1], hence the new developed and proposed method is suitable for quantification of one, two or three component drugs, separately or in combination.

Keywords: Sitagliptin, Metformin, Empagliflozin, RP-HPLC, Content Uniformity.

INTRODUCTION

Sitagliptin, metformin drugs are used to treat type 2 diabetes health issues, along with diet, exercise, and for overweight patients either alone or in combination with other types of oral hypoglycemic agents and empagliflozin drug is used in patients having type 2 diabetes along with diet and exercise. The drugs are taken either single or in combination with each other and are taken orally[2-3].

The chemical structures of sitagliptin, metformin and empagliflozin are as follows:
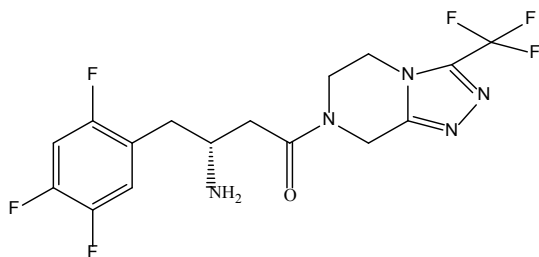
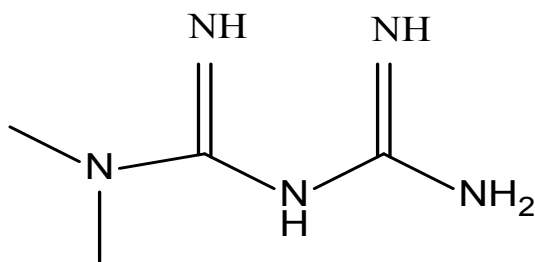**Fig. 1. Representing the Structure of Sitagliptin**

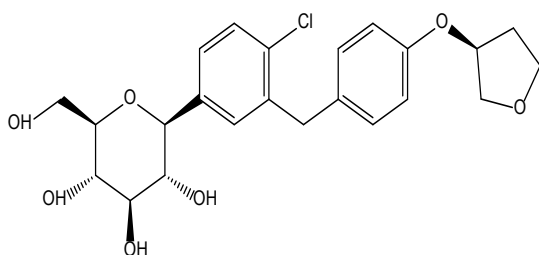**Fig. 2. Representing the Structure of Metformin**

Fig. 3. Representing the Structure of Empagliflozin

Initially, trials were taken to optimize an analytical method for metformin and empagliflozin. A method of analysis with reversed-phase chemistry using high-performance liquid chromatographic instrument was designed to develop and calculate contents of metformin and empagliflozin by using INNO C18 column (4.6 mm internal diameter with 150 mm length and particle size of 5 µm).The constituents in mobile phase mixture are pH 3.0 phosphate buffer 30% and methanol 70% ratio at a constant flow in the instrument i.e.1.0 mL/min and injection volume of 10 µL in the waters manufactured HPLC, with auto sampler and type of separation module 2695,with PDA detector 2996. Results for the developed method showed that there are no interfering peaks from blank solution at the specified retention time of metformin and empagliflozin. Retention time for metformin was observed at about 1.8 minute with tailing factor of not more than 2.0 & plate counts of more than 2000 and for empagliflozin

was observed at about 2.7 min with tailing factor not more than 2.0 & plate counts of more than 2000. A specific and simple RP-HPLC method was developed. As, metformin & empagliflozin are available in marketed formulation in single as well as in different combinations as empagliflozin tablets, metformin tablets, empagliflozin & metformin tablets, sitagliptin & metformin tablets. Hence, the research further continued by including one more drug substance sitagliptin. The aim was to achieve novelty in research by developing a new method for assay & content uniformity test for multicomponent analysis of drugs and for two tests (Assay and content uniformity).

Review of literature study done for development of a new method[4-10]. From the literature study[11-19] as tabulated in Table 1, it is observed that there are few methods available only for assay test determination of single or two drug components and single method for assay and content uniformity test is not available. To the best of our knowledge, it was observed that a single method to estimate three drug components for two tests i.e., assay and content uniformity (content of single dosage unit) test to estimate and calculate the drug substance sitagliptin, metformin and empagliflozin in the marketed drug product is not available. Hence, the research work was initiated for development and to validate a new, simple, accurate and economical method by RP-HPLC equipped with PDA detector. Sample preparation, diluent, and mobile phase in the proposed method is easy to prepare and economical and this method of analysis can be used for routine determination and calculation of drug components as well as to estimate stability batches analysis in quality control and research laboratory for assay and content uniformity calculation of drug substance sitagliptin, metformin and empagliflozin in available marketed drug product.

Method development trials

Trials were performed to optimize a new HPLC method for multicomponent analysis and are summarized in below Table 2. Chromatograms are shown in Figure 4 & 5.

Table 1: Summary of method development trials

| Trial No.	Method details	Observation & Conclusion
1	Column: Phenomenex C18, 250*4.6mm, 5umMobile phase: Phosphate Buffer 30%: Methanol 60%: Acetonitrile 10% ratioFlow of system: 1.0 mL/min Diluent: Water: Methanol (50:50) v/v	Resolution between sitagliptin and empagliflozin is 0.90. As resolution is not achieved above 2.0, solvent acetonitrile to be removed in mobile phase to achieve separation.
2	Column: Phenomenex C18, 250*4.6mm, 5umMobile phase: Phosphate Buffer 30%: Methanol 70% ratio Flow of system: 1.0 mL/min Diluent: Water: Methanol (50:50) v/v	Resolution between sitagliptin and empagliflozin is 1.49. As resolution is improved however not achieved above 2.0, column brand to be changed to check effect on separation.
3	Column: X Bridge C18,250*4.6mm, 5umMobile phase: Phosphate Buffer 30%: Methanol 70% ratio Flow of system: 1.0 mL/min Diluent: Water: Methanol (50:50) v/v	Resolution between sitagliptin and empagliflozin is 1.46. As resolution is not improved by changing column, mobile phase buffer to be changed and composition to be increased to 40% in mobile phase to achieve resolution.
4	Column: X Bridge C18,250*4.6mm, 5um Mobile phase: 0.1% Trifluoro acetic acid 40%: Methanol 40%: Acetonitrile 10% ratio Flow of system: 1.0 mL/min Diluent: Water: Methanol (50:50) v/v	Resolution between sitagliptin and empagliflozin is 4.04. Resolution is achieved however Sitagliptin retention time is 7.9 minutes & to achieve early elution, acetonitrile % to be increased to 20%.
5	Column: X Bridge C18, 250*4.6mm, 5um Mobile phase: 0.1% Trifluoro acetic acid 40%: Methanol 40% : Acetonitrile 20% ratio Flow of system: 1.0 mL/min Diluent: Water: Methanol (50:50) v/v	Resolution between sitagliptin and empagliflozin is 3.57. Resolution is achieved. All peaks are well separated.
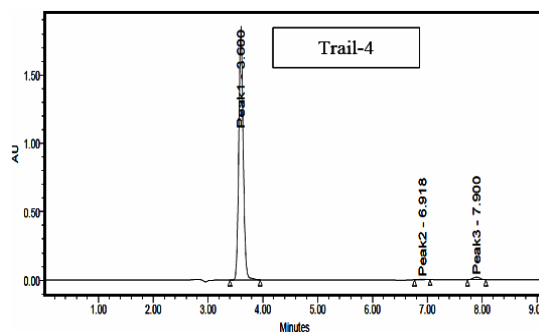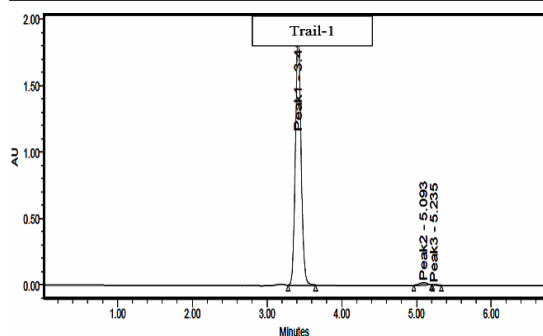
Fig. 4. representing chromatogram of development trial no. 1 to 4
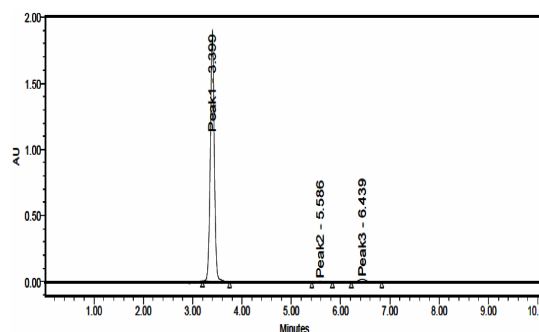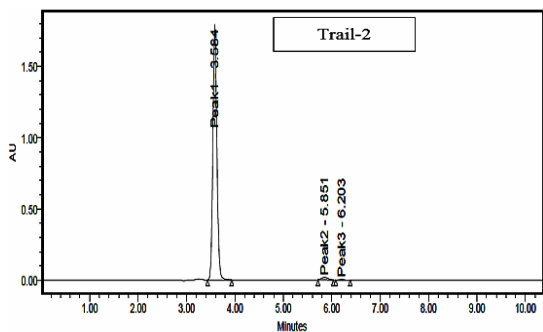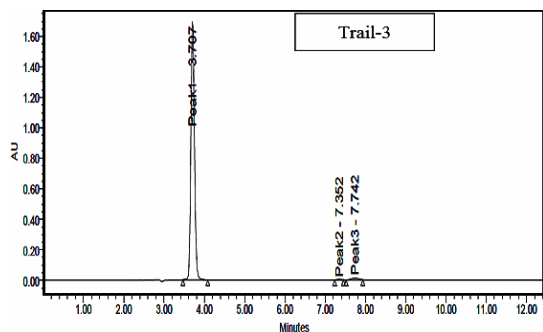

Fig. 5. representing chromatogram of development trial no. 5

MATERIALS AND METHODS

Chemicals and Reagents

Sitagliptin, metformin, empagliflozin standard and active pharmaceutical ingredients were received upon request from Fortune Pharma Lab., located at Hyderabad., India. The commercially

available marketed drug product with the brand name Jardinance 10 mg (Empagliflozin 10 mg) and Istamet tablet 50 mg/500 mg of strength (Sitagliptin-50 mg drug substance and Metformin-500 mg drug substance) purchased from the nearby local medical store. All solvents, chemicals and required reagents indented for this research work were of highly pure chromatographic i.e., pure grade water, methanol, acetonitrile, and trifluoroacetic acid was procured, manufactured by Merck.

Instrumentation

A Waters manufactured HPLC system type of model 2695 having photodiode array detector, inbuilt autosampler injector with Empower-2 qualified software was used. Weighing instrument, sonicator bath, oven for drying purpose was used for the experiments.

Chromatography of the method of analysis

Resolution and separations for three drug components was achieved on the X-Bridge C18 column, 250 mm length with 4.6 mm internal diameter and 5μm of Particle Size. The mobile phase mixture utilized to achieve resolution and separation to calculate content determination was 0.1% Trifluoroacetic acid buffer 40%: methanol 40%: acetonitrile 20% at a flow of system 1.0 mL/minute and auto injection volume is 10μL. The inbuilt column oven of the system was maintained at ambient temperature, and the drugs were subjected for detection at fixed wavelength of 224 nm.

Mobile phase of method of analysis

Liquid mobile phase was prepared by inclusion and mixing 400 mL of 0.1% trifluoroacetic acid buffer, 400 mL methanol and 200 mL of acetonitrile (40:40:20% ratio). The constituent mobile phase was subjected for sonication up to 15 min and filtered with filtration assembly by the use of 0.45μm membrane filter.

Diluent in the method of analysis

A composition of mixture in the volume 400 mL 0.1% trifluoroacetic acid buffer, 400 mL methanol and 200 mL of acetonitrile (40:40:20 % ratio) as a diluent in the method of analysis.

Standard stock solution of method of analysis

Precisely as well as carefully weighed, and transferred 50 mg of sitagliptin standard, 500 mg metformin standard and 5 mg of empagliflozin standard into a 50 mL previously dried and clean volumetric certified flask. Added a small quantity of diluent and subjected to sonication to dissolve the components in it completely and made volume of the flask up to the mark by diluent (1000 ppm of sitagliptin, 10000 ppm of metformin and 100 ppm of empagliflozin).

Standard solution of method of analysis

Further pipetted 2.5 mL of the above Sitagliptin, Metformin and Empagliflozin stock containing solution into 50 mL previously dried and clean volumetric certified flask and filled up to the line of flask with diluent. Mixed very well (50 ppm of sitagliptin, 500 ppm of metformin and 5 ppm of empagliflozin).

Sample solution for assay method of analysis

Step1: Accurately weighed 10 tablets of Jardinance 10 mg (Empagliflozin 10 mg) formulation and crushed in mortar and pestle. Then transferred powder of tablet which is crushed, equivalent to the drug substance10 mg of empagliflozin into 100 mL previously dried and clean volumetric certified flask. Added the diluent mixture to about 70 mL, and subjected to sonication for 30 min to extract the drug totally in the solution and made volume of the flask up to the line marked, with the diluent mixture. This solution was subjected to filtration through 0.45-micron PVDF type of syringe filter (100 ppm of empagliflozin, sample stock-1).

Step2: Precisely as well as carefully weighed 10 tablets of Istamet 50mg/500 mg (Sitagliptin-50 mg drug substance and Metformin-500 mg drug substance) formulation and crushed in mortar and pestle. Then transferred powder of tablet which is crushed, equivalent to the drug substance 50 mg of sitagliptin and drug substance 500 mg metformin into 50 mL previously dried and clean volumetric certified flask. Added the diluent mixture to about 35 mL, and subjected to sonication for 30 min to extract both the drug completely in the solution and made volume of the flask up to the line marked, with the diluent mixture. This solution was subjected to filtration through 0.45-micron PVDF type of syringe filter (1000 ppm of sitagliptin & 10000 ppm of metformin, sample stock -2).

Step3: Further pipetted 2.5 mL each of sample, stock-1 & stock-2 and then transferred into

50 mL clean and previously dried volumetric certified flask and made volume of the flask up to the line marked, with the diluent mixture and mixed well (50 ppm of sitagliptin, 500 ppm of metformin and 5 ppm of empagliflozin).

Sample solution for content uniformity (the content of a single strength unit)

Step1: Accurately weighed one tablet of Jardinance 10 mg (Empagliflozin 10 mg) formulation and transferred tablet into 100 mL previously dried and clean volumetric certified flask. Added the diluent mixture to about 70 mL, and subjected to sonication for 30 min to extract the drug totally in the solution and made volume of the flask up to the line marked, with the diluent mixture. This solution was subjected to filtration through 0.45-micron PVDF type of syringe filter (100 ppm of empagliflozin, Sample stock-1).

Step2: Accurately weighed one tablet of Istamet 50 mg/500 mg (Sitagliptin-50 mg drug substance and Metformin-500 mg drug substance) formulation and transferred into a 50 mL previously dried and clean volumetric certified flask. Added the diluent mixture to about 35 mL, and subjected to sonication for 30 min to extract both the drug totally in the solution and made volume of the flask up to the line marked, with the diluent mixture. This solution was subjected to filtration through 0.45-micron PVDF type of syringe filter (1000 ppm of sitagliptin & 10000 ppm of metformin, sample stock-2).

Step3: Further pipetted 2.5 mL each of sample, stock-1 & stock-2 and then transferred into 50 mL clean and previously dried volumetric certified flask and made volume of the flask up to the line marked, with the diluent mixture and mixed well (50 ppm of sitagliptin, 500 ppm of metformin and 5 ppm of empagliflozin).

Validation of chromatography method

The optimized and developed chromato-graphy method was subjected to perform the validity of the method as per the guidelines available from ICH. The validation of the chromatography method was performed for system suitability, specificity of method, accuracy of method, precision of method, Intermediate precision of method, linearity of method, and robustness and detailed procedure is mentioned below with results.

System Suitability of method of analysis

The system suitability of method of analysis was confirmed by preparing and injecting standard solution of sitagliptin (50 µg/mL), metformin (500 µg/mL), and empagliflozin (5 µg/mL), and the solutions were subjected for injection into system six times and the parameters of chromatography like analyte retention time, analyte peak tailing, resolution between two components and USP criteria of plate count were noted.

Specificity of method of analysis

As mentioned in ICH stated guidelines, the term "Specificity" is the ability of the method of analysis to specifically separate or resolve the particular component or components of targeted drug substance analyte in the composition of other components or matrix.

Linearity of method of analysis

The stock solution containing standard of sitagliptin, metformin and empagliflozin, was made by using diluent mixture. From the stock solution containing standard, various levels of standard concentration solutions were made in the concentration range of 25-75 µg/mL, 250-750 µg/mL and 2.5-7.5 µg/mL for sitagliptin, metformin and empagliflozin respectively and injected into chromatography system. The calibration plot of linearity (peak area of analyte vs. concentration of drug substance) were created in calculation sheet by using analysis results (from number of readings and sets are 3) at specified concentrations of drug substance. The linearity of the method was subjected for evaluation with the least square calculation method by using calculation sheet.

Accuracy of method of analysis

The accuracy for proposed method of analysis was determined with addition of standard at three different predefined concentrations of drug substance in the method at 50%, 100% as well as 150%, and calculated for reporting purpose, the theoretical i.e., true value and found i.e., value obtained for comparison.

Precision of method of analysis

The precision for proposed method of analysis was confirmed from the peak area of drug substance, obtained by injecting six replicate samples prepared for sitagliptin (50 µg/mL),

metformin (500 µg/mL), and empagliflozin (5 µg/mL). The intermediate precision of method of analysis was also carried out. The results obtained were calculated in calculation sheet for relative standard deviation i.e., %RSD of method.

Robustness method of analysis

The Robustness of method of analysis was checked and confirmed by changing small however deliberately changing in method of analysis parameters like flow of system (± 0.1 mL/min), organic phase ratio in composition of mobile phase (± 10% of total volume). System suitability of method of analysis was checked.

Degradation check in the method of analysis

Specificity for method of analysis was confirmed by studying degradation in acid, base, peroxide, thermal, and UV stress conditions. The samples were exposed to the mentioned conditions and the peak obtained from the chromatography was checked for purity and demonstrated that the method is capable to resolve and separate all quantifiable degradation products in same chromatographic test method.

Acidic condition study

Further pipetted 2.5 mL each of sample stock-1 & sample stock-2 (empagliflozin, sitagliptin & metformin sample stock) and then transferred into a 50 mL clean and previously dried volumetric certified flask. Added 3.0 mL, 0.1N HCl and kept at room temperature for 24 hours. After 24 h refluxed for about 15 min at 60ºC and allowed to cool at room temperature. This solution was subjected to neutralize with 3.0 mL, 0.1N NaOH and diluted up to the marked line with diluent mixture. As a final step, this solution was subjected to filtration through 0.45-micron PVDF type of syringe filter (50 ppm of sitagliptin, 500 ppm of metformin and 5 ppm of empagliflozin).

Alkaline condition study

Further pipetted 2.5 mL each of sample stock-1 & sample stock-2 (empagliflozin, sitagliptin & metformin sample stock) and then transferred into a 50 mL clean and previously dried volumetric certified flask. Added 3.0 mL, 0.1N NaOH and kept at room temperature for 24 hours. After 24 h refluxed for about 15 min at 60ºC and allowed to cool at room temperature. This solution was subjected to

neutralize with 3.0 mL, 0.1N HCl and diluted up to the marked line with diluent mixture. As a final step, this solution was subjected to filtration through 0.45-micron PVDF type of syringe filter (50 ppm of sitagliptin, 500 ppm of metformin and 5 ppm of empagliflozin).

Oxidative degradation

Further pipetted 2.5 mL each of sample stock-1 & sample stock-2 (empagliflozin, sitagliptin & metformin sample stock) and then transferred into a 50 mL clean and previously dried volumetric certified flask. Added 3 mL 1% H_2O_2 solution and kept at room temperature for 24 hours. After 24 h diluted up to the mark with diluent. As a final step, this solution was subjected to filtration through 0.45-micron PVDF type of syringe filter (50 ppm of sitagliptin, 500 ppm of metformin and 5 ppm of empagliflozin).

Thermal degradation

Sitagliptin, metformin and empagliflozin samples were placed in uniform way in petri plate and subjected for heating at 110ºC (in oven) for 24 hours.

Photolytic degradation

The photostability nature of the drug substance in the drug product was studied by exposure of the sample to direct sunlight light up to 12 hours.

Further pipetted 2.5 mL each of sample, stock-1 & stock-2 and then transferred into 50 mL clean and previously dried volumetric certified flask and made volume of the flask up to the line marked, with the diluent mixture and mixed well (50 ppm of sitagliptin, 500 ppm of metformin and 5 ppm of empagliflozin).

Results and discussion for method validation parameters

Validation of method of analysis is completed to show for its intended use and to establish quality characteristics of the developed method of analysis, meet the predefined acceptance of the targeted analytical test method. Validation of method of analysis was completed referring ICH

published Q2 guideline. In this section, results of all parameters are tabulated and observations are noted.

Suitability of method of analysis

　　Suitability of method of analysis was achieved by evaluation of the parameters like tailing of analyte, theoretical plates, resolution between peaks, and %RSD of injections from multiple injections. The values obtained, shows acceptable results and are tabulated in Tables 2, 3, 4 and 5. Chromatogram of standard solution is shown in Figure 6.
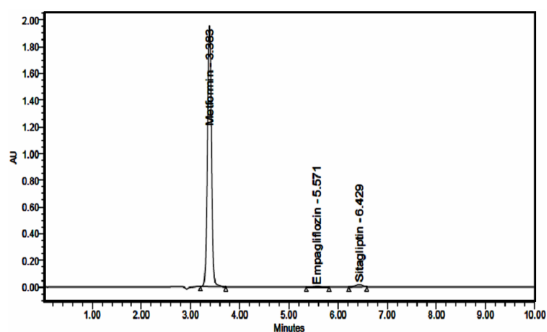

Fig. 6. Representing standard solution

Table 2: Results of system suitability from standard solution injection

No.	Analyte name	Concen-tration (ppm)	Analyte Rt	Analyte Area	Analyte plate count	Analyte Tailing	Resolution between analytes
1	Sitagliptin	50	6.415	152877	10112	0.96	3.64
2	Metformin	500	3.383	10893692	8328	1.02	NA
3	Empagliflozin	5	5.564	32258	11141	1.06	12.11

Table 3: Results of system suitability from standard solution injections for sitagliptin

No.	Analyte Rt	Analyte Area	Analyte name: Sitagliptin Analyte plate count	Analyte Tailing	Resolution between analytes
1	6.415	152877	10112	0.96	3.64
2	6.417	151496	10166	0.95	3.63
3	6.421	159298	10172	0.95	3.65
4	6.429	155765	10393	0.94	3.62
5	6.430	155944	10530	0.93	3.62
6	6.444	156039	10847	0.90	3.71
Mean		155236			
Std. Dev.		2737.9			
%RSD		1.7			

Table 4: Results of system suitability from standard solution injections for drug substance Metformin

No.	Analyte Rt	Analyte Area	Analyte name: Metformin Analyte plate count	Analyte Tailing
1	3.383	10893692	8328	1.02
2	3.383	10919204	8273	1.02
3	3.383	10879621	8224	1.01
4	3.385	10886278	8212	1.02
5	3.385	10883289	8344	1.02
6	3.386	10916881	8586	1.04
Mean		10896494		
Std. Dev.		65378965.3		
%RSD		0.2		

Table 5: Results of system suitability from standard solution injections for drug substance empagliflozin

No.	Analyte Rt	Analyte Area	Analyte name: Empagliflozin Analyte plate count	Analyte Tailing	Resolution between analytes
1	5.564	32258	11141	1.06	12.11
2	5.565	32931	11308	1.04	12.22
3	5.568	32801	11291	1.06	12.18
4	5.571	32919	10870	1.03	11.91
5	5.578	33990	11213	1.04	12.07
6	5.579	32507	11203	1.03	12.04
Mean	32901				
Std. Dev.	594.7				
%RSD	1.8				

Specificity

Any interfering peaks, from blank solution at retention times of sitagliptin, metformin and empagliflozin retention time are not observed in this method. Hence, this method was proved to be specific.
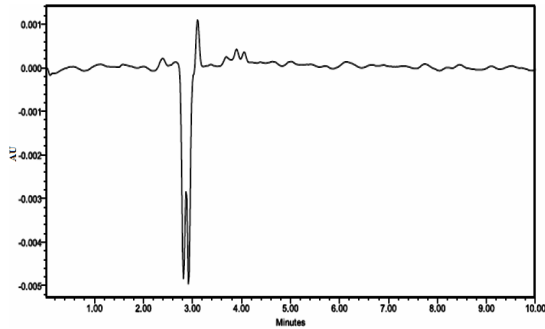
Fig. 7. representing blank (diluent)
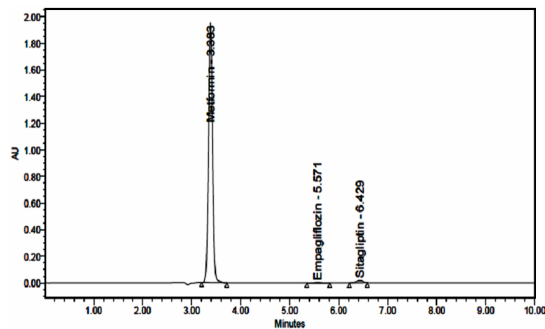
Fig. 8. Representing standard
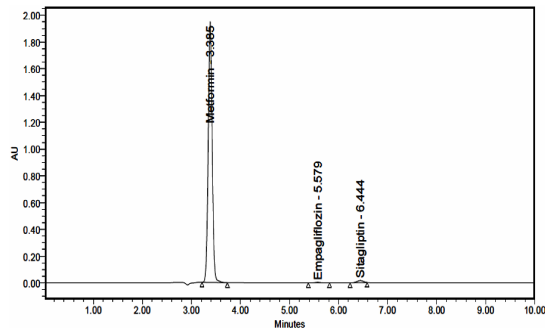
Fig. 9. representing sample

Retention times of metformin, empagliflozin, and sitagliptin, were 3.383, 5.571 and 6.429 min for standard solution and 3.385, 5.579 and 6.444 minutes for sample solution respectively.

Linearity of method of analysis

The linearity of method of analysis was subjected to evaluate at different range of concentration (50% to 150%) against standard level of sitagliptin, metformin and empagliflozin.

The calibration plot of linearity (peak area of analyte vs. concentration of drug substance in

μg/mL) was created in calculation sheet by analysis results obtained (from number of reading and sets are 3) at specified concentration of drug substance. R^2 value for sitagliptin, metformin and empagliflozin was calculated and observed 0.9992, 0.9994 and 0.9997 respectively. The summary of the results, is tabulated in Tables 6, 7 & 8 and shown in Figure. 10, 11, 12 & 13.

Fig. 10. Calibration Graph for Sitagliptin

Fig. 11. Calibration Graph for Metformin

Fig. 12. Calibration Graph for Empagliflozin

Fig. 13. Overlay of Linearity levels

Table 6: Linearity results for sitagliptin

S.No.	Linearity Level	Standard stock solution taken (µL)	Diluted volume up to mark (mL)	Concentration (ppm)	Area
1	50 %	1250	50	25.0	87438
2	75 %	1875	50	37.5	134113
3	100 %	2500	50	50.0	181266
4	125 %	3125	50	62.5	230245
5	150 %	3750	50	75.0	273289
			Correlation Coefficient		0.999

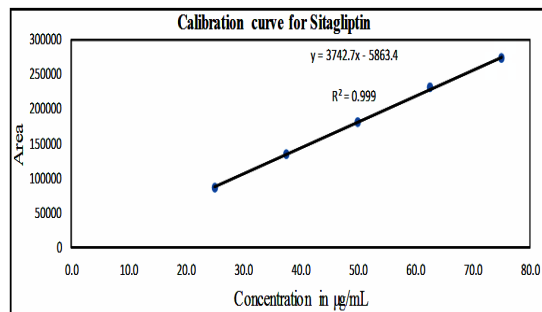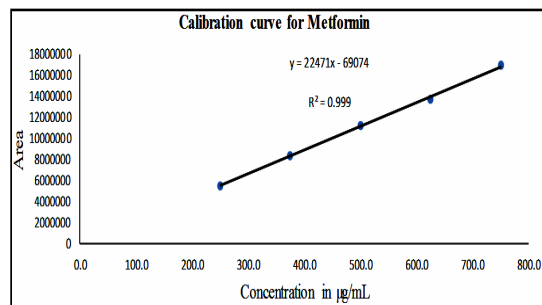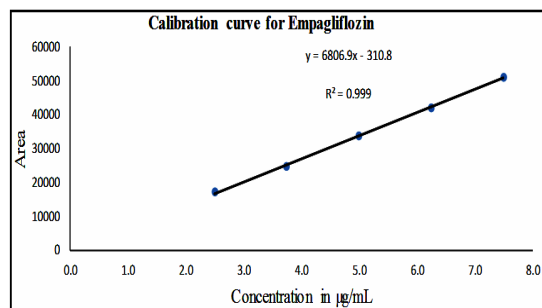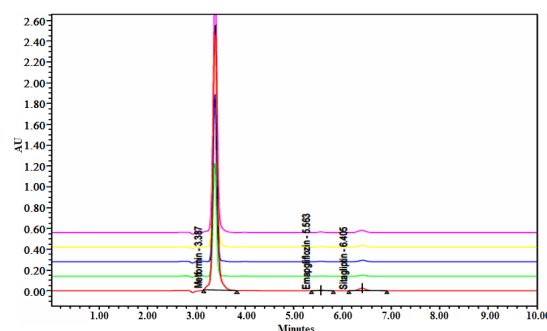**Table 7: Linearity results for metformin**

S.No.	Linearity Level	Standard stock solution taken (µL)	Diluted volume up to mark (mL)	Concentration (ppm)	Area
1	50 %	1250	50	250	5533712
2	75 %	1875	50	375	8409615
3	100 %	2500	50	500	11221992
4	125 %	3125	50	625	13768045
5	150 %	3750	50	750	16898907
			Correlation Coefficient		0.999

Table 8: Linearity results for empagliflozin

S.No.	Linearity Level	Standard stock solution taken (µL)	Diluted volume up to mark (mL)	Concentration (ppm)	Area
1	50 %	1250	50	2.5	17070
2	75 %	1875	50	3.8	24837
3	100 %	2500	50	5.0	33671
4	125 %	3125	50	6.3	42017
5	150 %	3750	50	7.5	51023
			Correlation Coefficient		0.999

Accuracy of method of analysis

To check the accuracy of method of analysis, analysis was carried at three multiple levels, 50%, 100%, 150%. Percentage accuracy in terms of recovery was calculated, and the result values obtained are noted in Table as below 9, 10 and 11.

Table 9: Representing accuracy values, Sitagliptin

Concentration in % (Specification Level)	Analyte Area	Drug substance Added (ppm)	Drug substance Amount obtained (ppm)	Recovery in %	Recovery (Avg.)
50 % Set 1	78762	25.00	25.37	101.47	101.14
50 % Set 2	78112	25.00	25.16	100.64	
50 % Set 3	78639	25.00	25.33	101.32	
100 % Set 1	159298	50.00	51.31	102.62	101.14
100 % Set 2	155765	50.00	50.17	100.34	
100 % Set 3	155944	50.00	50.23	100.46	
150 % Set 1	233730	75.00	75.28	100.38	100.58
150 % Set 2	233289	75.00	75.14	100.19	
150 % Set 3	235628	75.00	75.89	101.19	

Table 10: Representing accuracy results, Metformin

Concentration in % (Specification Level)	Analyte Area	Drug substance Added (ppm)	Drug substance Amount obtained (ppm)	Recovery in %	Recovery (Avg.)
50 % Set 1	5401809	250.00	247.87	99.15	99.49
50 % Set 2	5426824	250.00	249.02	99.61	
50 % Set 3	5432191	250.00	249.26	99.71	
100 % Set 1	10919204	500.00	501.04	100.21	100.08
100 % Set 2	10879621	500.00	499.23	99.85	
100 % Set 3	10916881	500.00	500.94	100.19	
150 % Set 1	16098907	750.00	738.72	98.50	98.39
150 % Set 2	16055545	750.00	736.73	98.23	
150 % Set 3	16088046	750.00	738.22	98.43	

Table 11: Representing accuracy results, Empagliflozin

Concentration in % (Specification Level)	Analyte Area	Drug substance Added (ppm)	Drug substance Amount obtained (ppm)	Recovery in %	Recovery (Avg.)
50 % Set 1	16665	2.50	2.53	101.30	100.94
50 % Set 2	16411	2.50	2.49	99.76	
50 % Set 3	16738	2.50	2.54	101.75	
100 % Set 1	32258	5.00	4.90	98.05	101.48
100 % Set 2	33919	5.00	5.15	103.09	
100 % Set 3	33990	5.00	5.17	103.31	
150 % Set 1	49023	7.50	7.45	99.33	99.62
150 % Set 2	49090	7.50	7.46	99.47	
150 % Set 3	49386	7.50	7.51	100.07	

Method precision & intermediate precision

Parameter was performed to check performance of method. Six samples at a concentration of 50 µg/mL of sitagliptin, 500 µg/mL of metformin and 5 µg/mL of empagliflozin are injected. The values tabulated in Tables 12, 13,14,15,16, &17. %RSD results observed below are noted.

Table 12: Representing repeatability values (Method precision), Sitagliptin

No.	Analyte Rt	Analyte Area	Analyte plate count	Analyte Tailing	Resolution between analytes
1	6.391	176519	9699	1.03	3.54
2	6.400	176202	9704	1.01	3.56
3	6.407	178059	9742	1.03	3.53
4	6.407	178530	9678	1.03	3.55
5	6.412	176142	9743	1.02	3.57
6	6.420	178382	9589	1.03	3.56
Avg		177306			
Std. Dev		1132.79			
% RSD		0.6			

Table 13: Representing repeatability values (Method precision), Metformin

No.	Analyte Rt	Analyte Area	Analyte plate count	Analyte Tailing
1	3.381	10884334	8594	1.02
2	3.382	10990504	8500	1.02
3	3.382	11000201	8461	1.02
4	3.382	10989075	8515	1.03
5	3.382	10926237	8566	1.02
6	3.383	10952941	8519	1.03
Mean		10957215		
Std. Dev		45300.24		
% RSD		0.4		

Table 14: Representing repeatability values (Method precision), Empagliflozin

No.	Analyte Rt	Analyte Area	Analyte plate count	Analyte Tailing	Resolution between analytes
1	5.552	32186	11492	1.06	12.26
2	5.555	32232	11698	1.05	12.16
3	5.556	32493	11648	1.01	12.11
4	5.559	32894	11549	1.04	12.07
5	5.563	33063	11461	1.04	12.25
6	5.568	33164	11186	1.07	12.21
Avg		32672			
Std. Dev		425.69			
% RSD		1.3			

Table 15: Representing repeatability values (Intermediate precision), Sitagliptin

No.		Analyte Rt	Analyte Area	Analyte plate count	Analyte Tailing	Resolution between analytes
1		6.415	152877	10112	0.96	3.64
2		6.417	151496	10166	0.95	3.63
3		6.421	159298	10172	0.95	3.65
4		6.429	155765	10393	0.94	3.62
5		6.430	155944	10530	0.93	3.62
6		6.444	156039	10847	0.90	3.71
	Avg		155236			
	Std. Dev		2737.9			
	% RSD		1.8			

Table 16: Representing repeatability values (Intermediate precision), Metformin

No.		Analyte Rt	Analyte Area	Analyte plate count	Analyte Tailing
1		3.383	10893692	8328	1.02
2		3.383	10919204	8273	1.02
3		3.383	10879621	8224	1.01
4		3.385	10886278	8212	1.02
5		3.385	10883289	8344	1.02
6		3.386	10916881	8586	1.04
	Mean		10896494.2		
	Std. Dev		17335.87		
	% RSD		0.2		

Table 17: Representing repeatability values (Intermediate precision), Empagliflozin

No.		Analyte Rt	Analyte Area	Analyte plate count	Analyte Tailing	Resolution between analytes
1		5.564	33258	11141	1.06	12.11
2		5.565	33931	11308	1.04	12.22
3		5.568	33801	11291	1.06	12.18
4		5.571	33919	10870	1.03	11.91
5		5.578	33990	11213	1.04	12.07
6		5.579	33507	11203	1.03	12.04
	Avg		33734			
	Std. Dev		290.23			
	% RSD		0.9			

Robustness of method of analysis

The robustness is studied to check, flow of system (± 0.1 mL/min) and solvent mixture composition (± 10%), and its effect on the area, tailing, and plate count. The results are tabulated in Tables 18, 19 & 20.

Table 18: Representing robustness, Sitagliptin

Parameter evaluation	Analyte Area	Analyte Rt	Analyte plate count	Analyte Tailing	Resolution between analytes
Less Flow in the system, 0.9 mL/min	206052	7.103	10168	1.01	3.63
Actual Flow in the system, 1.0 mL/min	152877	6.415	10112	0.96	3.64
More Flow in the system, 1.1 mL/min	169796	5.833	9183	1.02	3.49
Less organic phase	205002	7.003	10158	1.01	3.61
More organic phase	168790	5.840	9145	1.02	3.42

Table 19: Representing robustness, Metformin

Parameter evaluation	Analyte Area	Analyte Rt	Analyte plate count	Analyte Tailing
Actual Flow rate of 0.9 mL/min	12619965	3.746	9160	1.03
Less Flow rate of 1.0 mL/min	10893692	3.383	8238	1.02
More Flow rate of 1.1 mL/min	10173220	3.078	8548	1.02
Less organic phase	12629865	3.741	9142	1.01
More organic phase	10172520	3.072	8531	1.02

Table 20: Representing robustness, Empagliflozin

Parameter evaluation	Analyte Area	Analyte Rt	Analyte plate count	Analyte Tailing	Resolution between analytes
Less Flow rate of 0.9 mL/min	39137	6.164	11774	1.02	12.38
Actual Flow rate of 1.0 mL/min	32258	5.564	11141	1.06	12.11
More Flow rate of 1.1 mL/min	30696	5.056	11089	0.98	11.96
Less organic phase	39047	6.154	11572	1.02	12.23
More organic phase	30196	5.013	11007	0.99	12.01

All system suitability parameters are within the pre-defined acceptance criteria and there is no much shift in retention time for deliberately changed parameters as compared to the parameters in as such method. From the results obtained, it is proved method is robust.

Degradation observations

As no interference in blank and at degradant peaks, results showed purity of sitagliptin, metformin and empagliflozin drug substances, and hence method of analysis is specific and stability-indicating. The values are summarized in below Table 21.

Table 21: Representing values of forced degradation studies-sitagliptin, metformin & empagliflozin

Sr.No.	Stress parameter	Stress Condition	Degradation by Area %	Peak Purity
1	Standard	NA	NA	Passes
2	Acidic	0.1 N, HCl 24 hours	0.19	Passes
3	Basic	0.1 N, NaOH 24 hours	1.60	Passes
4	Oxidative	1 %, H2O2 24 hours	2.26	Passes
5	Thermal	110°C, 24 hours	2.40	Passes
6	Photolytic	Sunlight, 12 hours	2.69	Passes

The three drug components, when exposed to above mentioned stress degradation conditions showed less degradation even for harsh degradation conditions and proved stability indicating nature to perform stability studies in the proposed method.

Advantages

Advantages of the new proposed, developed and validated method are, Other reported methods determine only assay, however our new method determines assay and content uniformity (content of single dosage unit). As assay and content of a single unit can be determined in a single analysis, correlation of result between content of sample matrix from the whole as well as a single unit can be determined. This data is useful in the evaluation of manufactured batches.

CONCLUSION

A new method of analysis with reverse phase chemistry was designed and developed to calculate assay and content uniformity of sitagliptin, metformin and empagliflozin pharmaceuticals. The method of analysis was validated for specified test parameters as mentioned in ICH published guidelines. From validation data and results obtained, it can be concluded that the target of developing a common chromatographic method for assay and content uniformity test has been achieved. This specific, economical, and efficient method can be used to estimate sitagliptin, metformin and empagliflozin as individual components as well as in combination in pharmaceuticals.

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