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Bioanalytical Method Development Of Tirzepatide In Rat Plasma Using Lixisenatide As Internal Standard Using Liquid Chromatography Coupled With Tandem Mass Spectroscopy And Application To Pharmacokinetic Studies

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ABSTRACT

A rapid, sensitive and reproducible LC-MS/MS was developed and validated for the quantification of Tirzepatide in Rat plasma using Lixisenatide as internal standard. The reconstituted samples were chromatographed on a Phenyl, 150mm x 4.6mm, 3.5µm column using a Methanol: 0.1% Formic acid 50:50 v/v as the mobile phase at a flow rate of 1.0 mL/min. Good response was found in positive ion mode using MRM technique. The total chromatographic run time was set at 5 min. The calibration curve was found to be linear over the concentration range of 2.50–50.00 ng/mL for Tirzepatide with a coefficient of correlation of >0.99. No significant matrix effect was observed in all the six batches of rat plasma for the analyte at LQC and HQC concentrations. Accuracy, precision, recovery, matrix effect and stability results were found to be within the suitable limits. Simple and efficient method was developed and utilized in pharmacokinetic studies to see the investigated analyte in body fluids. The application denotes all the parameters of system suitability, specificity, linearity and accuracy are in good agreement with USFDA guidelines and applied effectively for the investigation of

pharmacokinetic studies in rat

Introduction

Tirzeptide, Synthetic peptide analog, is used for the treatment of type 2 diabetes. It is an Glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) are hormones involved in blood-sugar control has garnered significant attention due to its potential therapeutic applications, particularly in combating bacterial infections. This peptide demonstrates promising antimicrobial activity against a wide range of pathogens, making it a compelling candidate for further investigation and development in the field of infectious disease therapeutics [1]. In order to facilitate the clinical translation and pharmacological characterization of tirzeptide, robust analytical methods are essential for accurate quantification and characterization in biological matrices. Bioanalytical methods play a crucial role in elucidating the pharmacokinetic and pharmacodynamic properties of therapeutic agents, providing valuable insights into their efficacy and safety profiles [2]. In this study, we present a comprehensive bioanalytical method for the quantification of tirzeptide in biological samples, employing advanced analytical techniques and instrumentation. The method described herein offers high sensitivity, selectivity, and reliability, enabling precise determination of tirzeptide concentrations in various biological matrices such as plasma, serum, and tissue homogenates [3]. The analytical approach outlined in this work encompasses sample preparation, chromatographic separation, and detection, providing a holistic framework for tirzeptide analysis. Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) serves as the cornerstone of our analytical strategy, offering enhanced specificity and sensitivity for the quantification of tirzeptide [4]. Moreover, the method development includes rigorous validation procedures in accordance with regulatory guidelines, ensuring the robustness and reproducibility of the analytical results. Validation parameters such as linearity, accuracy, precision, and stability are meticulously evaluated to establish the suitability of the method for its intended application in pharmacokinetic studies, bioequivalence assessments, and preclinical and clinical investigations [5]. By leveraging state-of-the-art analytical methodologies and adhering to rigorous validation criteria, our bioanalytical method provides a reliable platform for advancing the understanding of tirzeptide pharmacokinetics and pharmacodynamics. Ultimately, this method contributes to the ongoing efforts aimed at harnessing the therapeutic potential of tirzeptide in the management of bacterial infections, addressing critical healthcare challenges worldwide.

MATERIALS AND METHODOLOGY

Instrumentation

Chromatography was performed with waters 2695 HPLC provided with high speed auto sampler, column oven, and degasser and SCIEX QTRAP 5500 mass spectrometer to provide a compact and with class AB SCIEX software, Phenyl, 150mm x 4.6mm, 3.5µm column was used for the present studies..

Reagents and chemicals

The reference sample was provided as Tirzepatide & Lixisenatide samples from Glenmark, Mumbai. HPLC grade Acetonitrile, HPLC grade Methanol and all other chemicals were obtained from Merck chemical division, Mumbai. HPLC grade water obtained from Milli-Q water purification system was used throughout the study.

Tirzepatide Standard Stock solution preparation

Weigh 5mg of Tirzepatide working standard and transferred into a 10ml volumetric flask diluted to

volume with diluent. Further diluted 0.2ml to 10ml with diluent. From this solution take 0.1 ml and transferred into 10 ml flask. This is the Tirzepatide stock solution.

Lixisenatide (IS) Standard Stock solution preparation

Weigh 10mg of Lixisenatide working standard and transferred into a 10ml volumetric flask diluted to volume with diluent. Further diluted 0.2ml to 10ml with diluent. From this solution take 0.1 ml and transferred into 10 ml flask. This is the Lixisenatide stock solution.

Preparation of Standard Solution (25 ng/ml of Tirzepatide)

Transferred 500µl of standard stock solution into 2ml centrifuged tube. To this add 200µl of plasma, 500µl of internal standard, 300µl of Methanol and 500µl of diluent. Centrifuge it to 20 min. Filter the supernatant liquid and transfer into HPLC vial.

Tirzepatide Sample Stock solution preparation

Take 1 mL of Tirzepatide sample and transferred into a 10ml volumetric flask diluted to volume with diluent. Further diluted 0.2ml to 10ml with diluent. From this solution take 0.1 ml and transferred into 10 ml flask. This is the Tirzepatide sample stock solution.

Extraction procedure

Label the Centrifuged and treated plasma samples accordingly to their time intervals. To about 200 µL of plasma add 500 µL of diluent and mix well. Further add 300 µL of Methanol to precipitate all the proteins and mix in vortex cyclo mixture. Centrifuge at 4000 RPM for 15 – 20 min. collect the supernatant solution in HPLC vial and inject into the chromatograph.

Buffer Preparation

1 mL of Formic acid is dissolved in 1 Lt of HPLC grade water. Filtered through 0.45µ filter paper.

Mobile phase preparation

Mix Methanol and 0.1% Formic acid in 50: 50 v/v and filtered through 0.45 µ filter paper.

Preparation of Linearity solution:

Prepare the linearity solutions of concentration ranging from 2.50 nanogram to 50 nanogram per ml of Tirzepatide prepared in a similar way as above. Centrifuge at 4000 RPM for 15 – 20 min. collect the supernatant solution in LC vial and inject into the chromatograph.

Methodology for Analysis:

Inject blank (Plasma solution), Linearity solutions and sample solutions into the chromatographic system and record the chromatograms. Measure the Peak area counts for due to Tirzepatide peak. Determine the concentration ofTirzepatide present in the plasma sample from the equation obtained from the Linearity Curve.

METHOD DEVELOPMENT AND OPTIMIZATION OF THE CHROMATOGRAPHIC CONDITIONS

For developing the method for the assay of Tirzepatide, a systematic study of the effect of various factors were undertaken by varying one parameter at a time and keeping all the other conditions constant. The following studies were conducted for this purpose. Phenyl column (150mm length 4.6 mm ID 3.5 µm) column was chosen as the stationary phase for this study. The mobile phase and the flow rate in order to get sharp peaks and base line separation of the components, the author has carried out a number of experiments by varying the commonly used solvents, their compositions and flow

rate. To effect ideal separation of the drug under isocratic conditions, mixtures of commonly used solvents like water, methanol and acetonitrile with or without buffers in different combinations were tested. A binary mixture of Mix Methanol and 0.1% Formic acid in 50: 50 v/v was proved to be the most suitable of all the combinations since the chromatographic peaks obtained were well defined and resolved and free from tailing. A mobile phase flow rate of 1.0 mL/min. was found to be suitable in the study range of 0.8 -1.5 mL/min. Detection was carried out by using mass detectors. The drug molecule was tuned on the mass spectrometer for the detection of the parent ion and the daughter ion (precursor ions) by injecting 10 µg/mL concentration. The tuning was carried out in both positive and negative modes of ionization, but better sensitivity with more reproducibility was found to be observed in the positive polarity mode. All the optimized potential parameters in the positive polarity mode have been given in the Table 1.

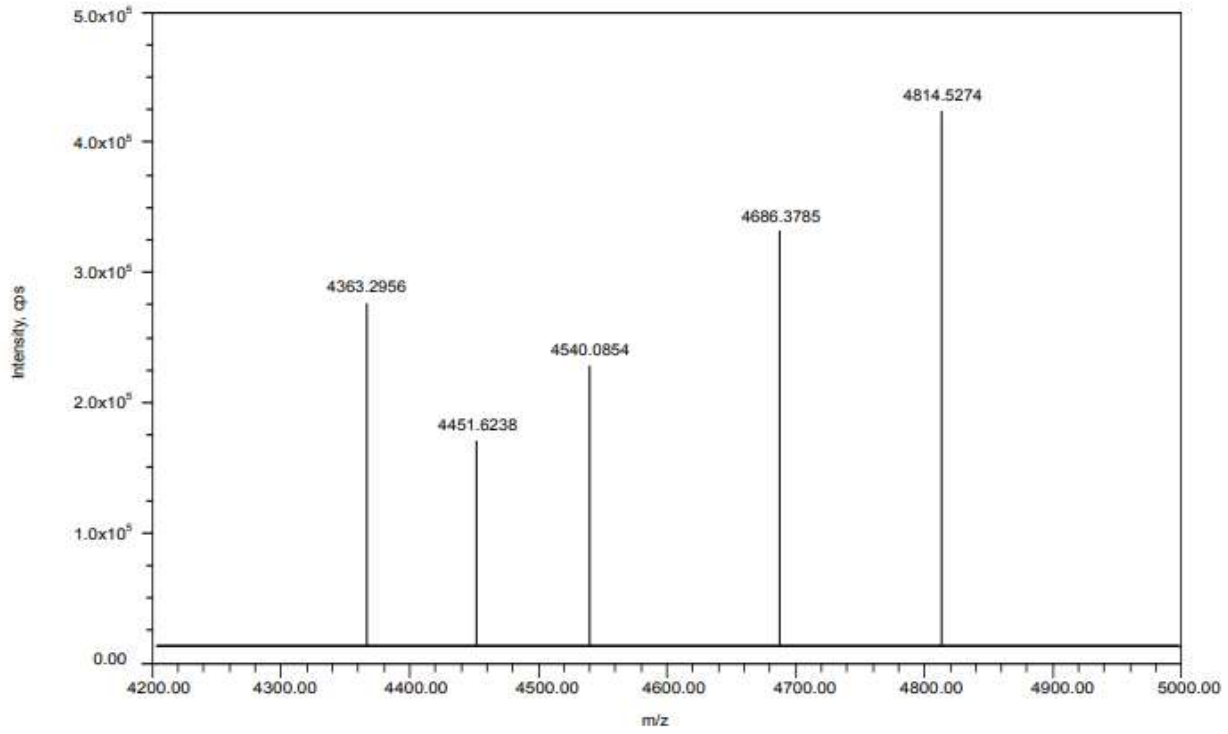

Figure 1: Multiple Reaction Monitoring-MRM of the Tirzepatide using Positive Polarity: Precursor Ion (m/z) 4814.5274 Daughter Ion with the Highest Intensity (m/z) 4686.3785

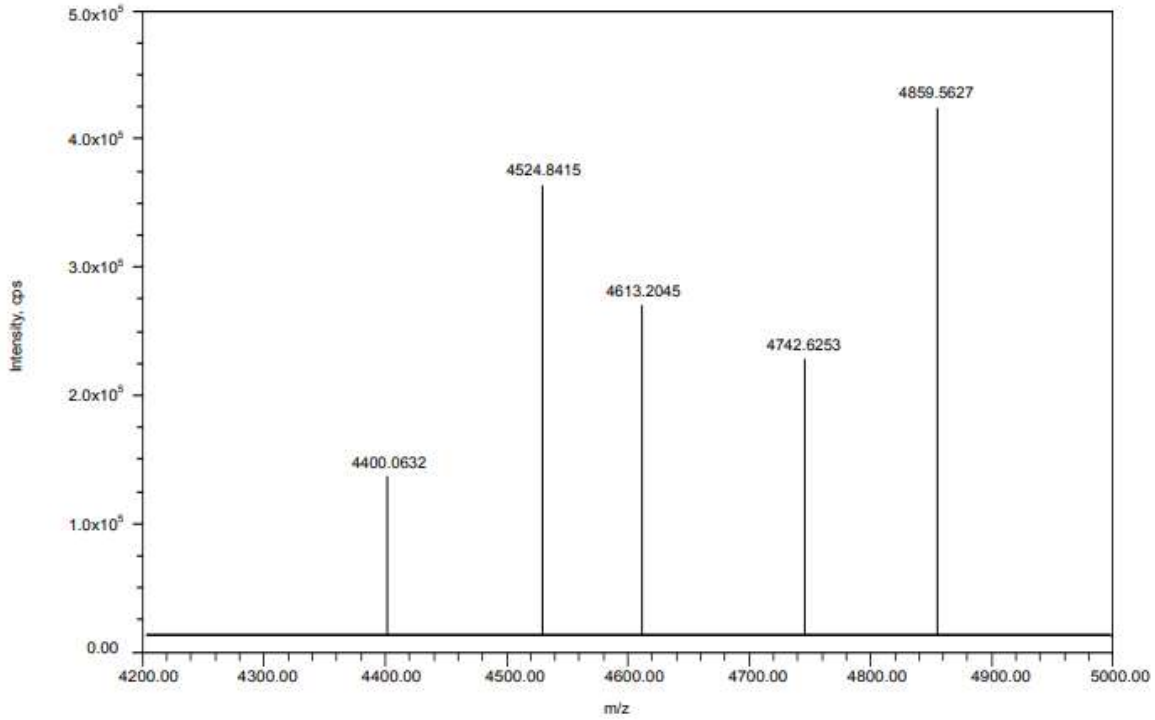

Figure 2: Multiple Reaction Monitoring-MRM of the Lixisenatide using Positive Polarity Precursor Ion (m/z) -4859.5627 Daughter Ion with the Highest Intensity (m/z) -4524.8415

Extraction Process of Plasma Samples and Their Drying:

Extracted Sample Preparation:

Step-1

Retrieve required number of plasma samples from the deep freezer, thaw them at room temperature and vortex the tubes.

Step-2

Arrange the pre-labelled empty tubes as per the batch sequence and aliquot 200.000 µL of plasma then add 300.000 µL of MeOH vortex for 15min

Step-3

Add 500.000 µL of STD Stock then add 500.000 µL of IS STD Stock vortex for 15min and add 500.000 µL of Diluent

Step-5

Vibromax the samples for 5 min at 2500 rpm.

Step-6

Centrifuge the samples at 4000 rpm, 10^0C for 5 min.

Step-7

Collect approximately 1.000 ml of supernatant.

UN Extracted Sample Preparation:

Step-1

Take 500.000 µL of STD Stock solution into pre-labelled tubes.

Step-2

Add 500.000 µL of ISTD working solution and vortex to mix.

Step-3

Add 1000.000 µL of mobile phase and vortex to mix.

Step-4

Transfer appropriate volume into pre-labelled auto-sampler vials and injects 10.000 µL into LC-MS/MS

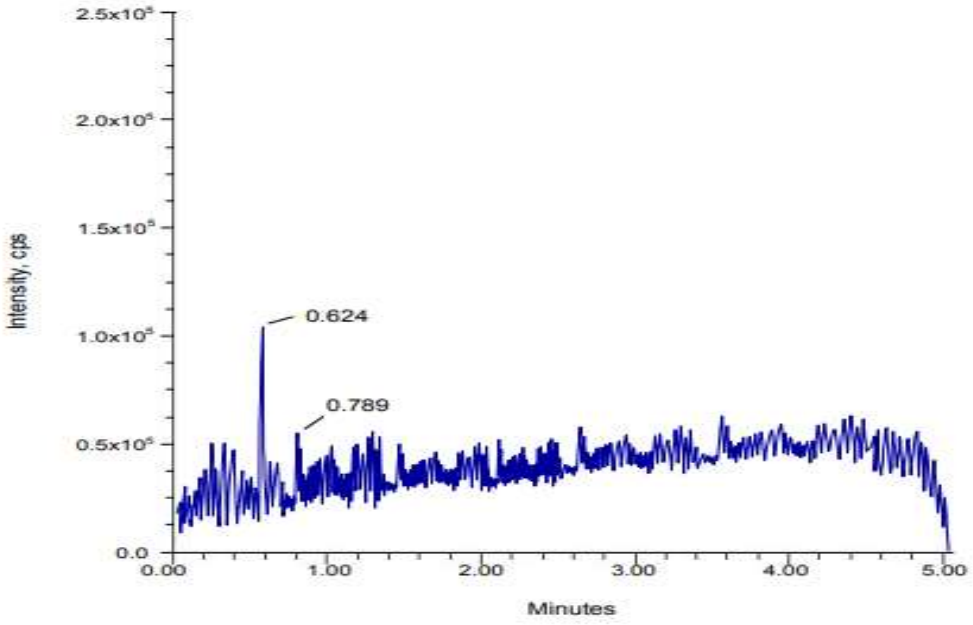

Figure 3 : Chromatogram of Blank plasma

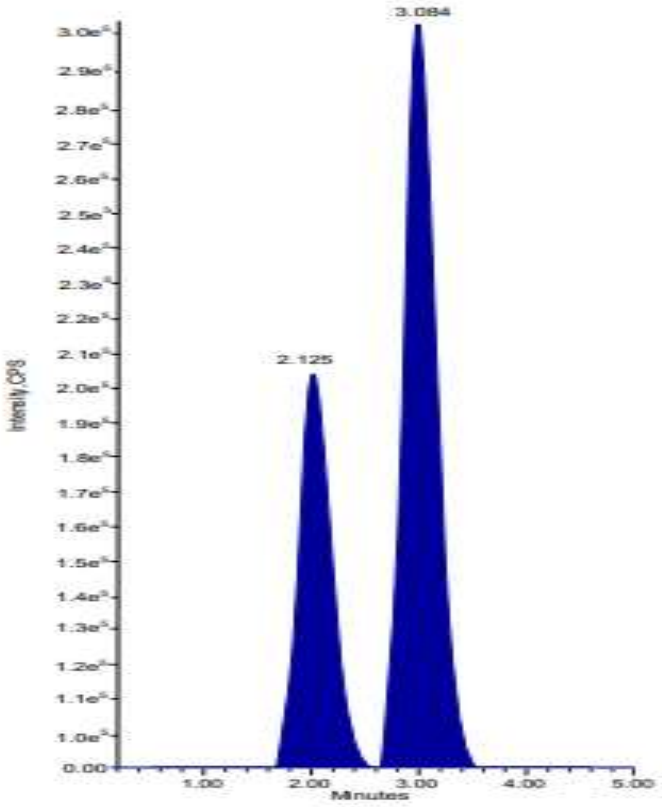

Figure 4: Chromatograms of Tirzepatide and Internal standard

Research ethics

This investigation was conducted in strict line with the Committee for Control and Supervision of Experiments on Animals (CPCSEA) recommendations and regulations. The CPCSEA is a statutory agency created by the Indian government that regulates animal research. This has allowed the pharmacokinetic performance methods at the Animal House Facility of Flair labs (City: Surat, State: Gujarat, Country: India. The protocol of animal study was approved by institute of animal ethics committee (Reg.No:1250/PO/RcBi/S/23/CPCSEA). The animals were housed in identical laboratory circumstances, with a temperature of 20-26ºC and a humidity of 50-60%. All animals were fasted overnight before to the experiment and given water adlibitum. Pharmacokinetic evaluation was performed for Tirzepatide Injection.

RESULTS AND DISCUSSIONS:

System suitability:

System suitability experiment was performed by injecting 6 consecutive injections using aqueous standard mixture equivalent to MQC concentration of the calibration curve. The % CV of peak area ratio (analyte area/ISTD area) and %CV of retention times for analyte and ISTD were within acceptance criteria. The results are summarized in the following table 3.

Table 3: System suitability Results of Tirzepatide

Sample Name MQC (50ng/ml)	Analyte Area (cps)	Analyte RT (min)	ISTD Area (50ng/ml)	ISTD RT (min)	Area Ratio
MQC-1	$2.025x10^5$	2.123	$3.016x10^5$	3.088	0.671
MQC-2	$2.022x10^5$	2.127	$3.028x10^5$	3.082	0.677
MQC-3	$2.034x10^5$	2.124	$3.015x10^5$	3.080	0.674
MQC-4	$2.018x10^5$	2.121	$3.026x10^5$	3.085	0.666
MQC-5	2.02610^5	2.124	$3.029x10^5$	3.082	0.668
MQC-6	$2.033x10^5$	2.127	$3.029 x10^5$	3.089	0.671
Mean	$2.026x10^5$	2.124	$3.023x10^5$	3.084	0.671
SD	0.006	0.002	0.006	0.003	0.003
%CV	**0.29**	**0.09**	**0.19**	**0.09**	**0.44**

Results: The %CV forTirzepatide and ISTD area ratio was found to be 0.44. Hence it passed the system suitability.

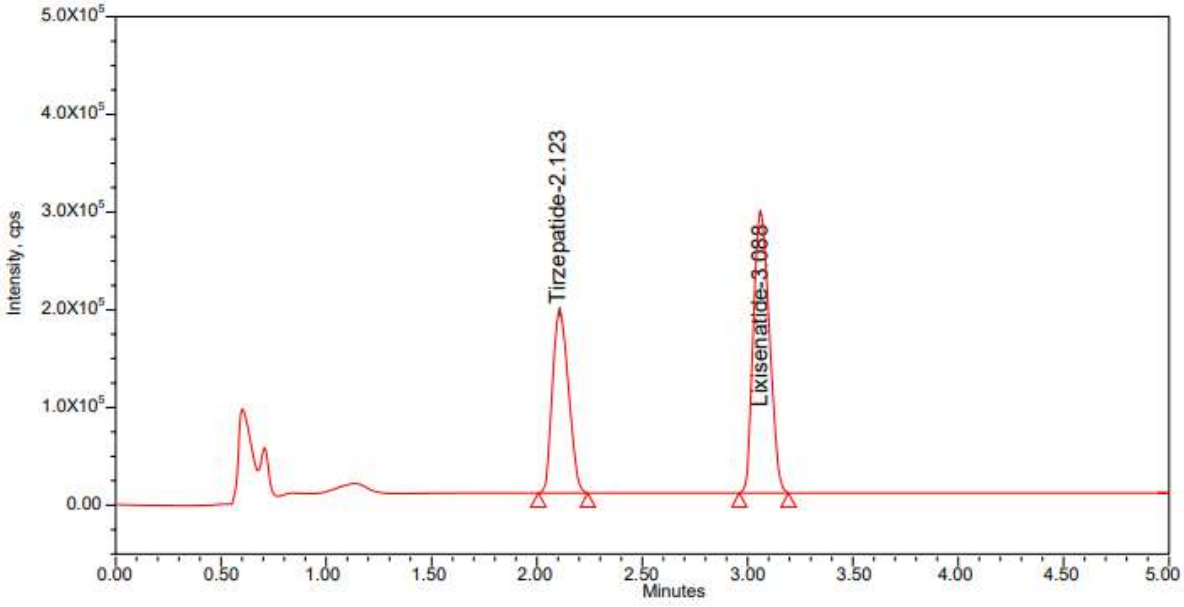

Figure 5: Chromatogram of System suitability

Carryover Effect

No significant carry over observed during this experiment. The results are summarized in the Table 4 The carryover area response in subsequent injections is found to be <20%. Hence the method passed the carryover effect.

Table 4 Auto sampler carryover of Tirzepatide

Sample ID	Peak Area		% Recovery	
	Drug	ISTD	Drug	ISTD
Un Extracted Samples				
Std Blk	0	0	N/A	N/A
HQC			0.00	0.00
Std Blk	0	0		
LLOQ			N/A	N/A
Extracted Samples				
Std Blk	0	0	N/A	N/A
HQC			0.00	0.00
Std Blk	0	0		
LLOQ			N/A	N/A

Specificity and screening of biological matrix:

No interfering peaks were found in six different random blank Rat plasma samples at the retention times of either Tirzepatide or ISTD.

Table: 5 Specificity and screening of biological matrix of Tirzepatide

S. No.	**Sample ID**	**Intensity(cps)**		**% Interference**		**Pass/ Fail**
		Drug	**ISTD**	**Drug**	**ISTD**	
1.	Std Blk 1	0	0	0	0	Pass
2.	LLOQ 1 (2.5ng/ml)	$0.204x10^5$	$3.022x10^5$	0	0	Pass
3.	Std Blk 2	0	0	0	0	Pass
4.	LLOQ 2 (2.5ng/ml)	$0.209x10^5$	$3.036x10^5$	0	0	Pass
5.	Std Blk 3	0	0	0	0	Pass
6.	LLOQ 3 (2.5ng/ml)	$0.205x10^5$	$3.017x10^5$	0	0	Pass
7.	Std Blk 4	0	0	0	0	Pass
8.	LLOQ 4 (2.5ng/ml)	$0.201x10^5$	$3.047x10^5$	0	0	Pass

9.	Std Blk 5	0	0	0	0	Pass
10.	LLOQ 5 (2.5ng/ml)	$0.203x10^5$	$3.034x10^5$	0	0	Pass
11.	Std Blk 6	0	0	0	0	Pass
12.	LLOQ 6 (2.5ng/ml)	$0.201x10^5$	$3.079x10^5$	0	0	Pass

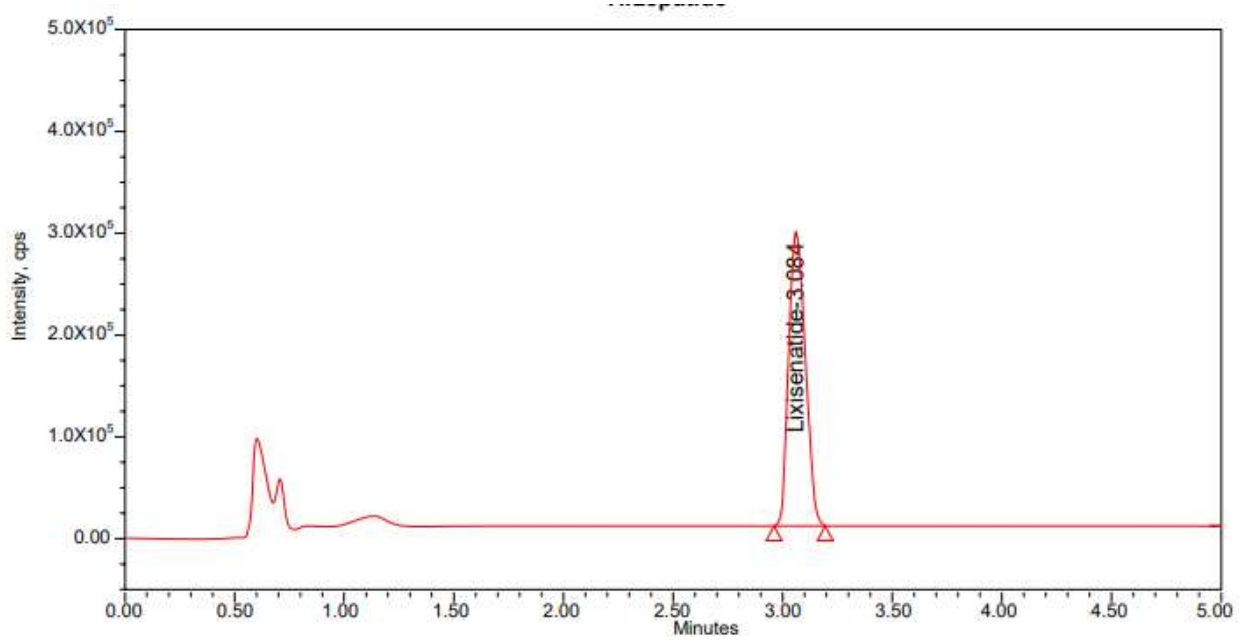

Figure 6: Specificity Chromatogram of Internal Standard

Sensitivity

The Sensitivity of the method was evaluated by analyzing 6 LLOQ samples (2.5 ng/mL). The % CV and mean accuracy for analyte at LLOQ level were found to be 0.98 and 94.79 respectively. The results are summarized in the Table.6

Table 6 Sensitivity Results of Tirzepatide

Replicate Number	**LLOQ**
	Nominal Concentration(ng/ml)
	2.5
	Analyte peak area
1	$0.204x10^5$
2	$0.209x10^5$
3	$0.205x10^5$
4	$0.201x10^5$

5	$0.203x10^5$
6	$0.201x10^5$
n	6
Mean	$0.203x10^5$
SD	0.002
% CV	0.98
% Mean Accuracy	94.79%

Acceptance Criteria:

At least 67 % (4 out of 6) of samples should be within 80.00-120.00 %.% Mean accuracy should be within 80.00-120.00 %. % CV accuracy should be ≤ 20.00 %.

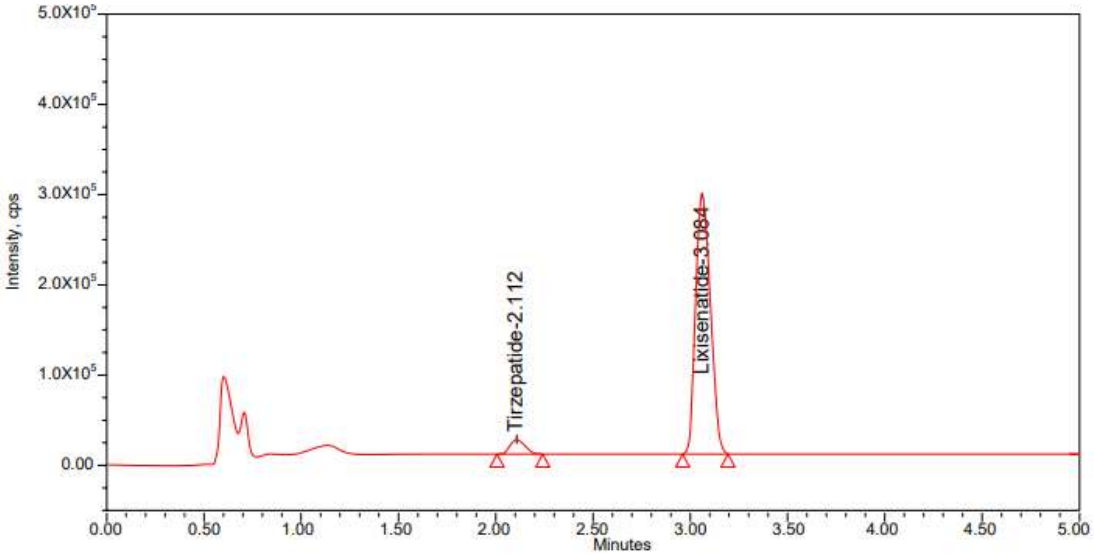

Figure 7: Sensitivity Chromatogram of LLOQ

Matrix effect:

The matrix of plasma constituents over the ionization of analyte was determined by comparing the response of post-extracted plasma standard MQC samples (25 ng/ml of Tirzepatide) with the response of analyte from neat samples at equivalent concentrations. The matrix effect intended method was assessed by using chromatographically screened Rat plasma.

The % CV of back calculated concentrations for the HQC and LQC samples of all the investigated lots were found to be 0.23 and 1.57 respectively. The % mean accuracy of back calculated concentrations for the HQC and LQC samples of all the investigated lots were found to be 99.88 and 99.37 respectively. The results are summarized in the Table :7

Table 7: Matrix **effect Results of Tirzepatide (**HQC-37.5ng/ml, LQC-12.5ng/ml)

S.No.	Plasma Lot No.	HQC	LQC
		Nominal Concentration(ng/ml)	
		37.5	12.5
		Analyte peak area	
1.	Lot 1	$3.031x10^5$	$1.012x10^5$
		$3.024x10^5$	$1.011x10^5$
		$3.019x10^5$	$1.015x10^5$
2.	Lot 2	$3.014x10^5$	$1.017x10^5$
		$3.026x10^5$	$1.005x10^5$
		$3.011x10^5$	$1.006x10^5$
3.	Lot 3	$3.015x10^5$	$1.015x10^5$
		$3.033x10^5$	$1.017x10^5$
		$3.024x10^5$	$1.008x10^5$
4.	Lot 4	$3.012x10^5$	$1.015x10^5$
		$3.035x10^5$	$1.025x10^5$
		$3.034x10^5$	$1.016x10^5$
5.	Lot 5	$3.027x10^5$	$1.015x10^5$
		$3.025x10^5$	$1.028x10^5$
		$3.014x10^5$	$1.012x10^5$
6.	Lot 6	$3.031x10^5$	$1.008x10^5$
		$3.020x10^5$	$1.078x10^5$
		$3.028x10^5$	$1.010x10^5$
n		18	18
Mean		$3.023x10^5$	$1.017x10^5$
SD		0.007	0.016
%CV		0.23	1.57
% Mean Accuracy		99.88%	99.37%
No. of QC Failed		0	0

Acceptance Criteria

The % mean accuracy of back calculated concentration of LQC and HQC samples prepared from different biological matrix lots should be within 85.00-115.00 %.

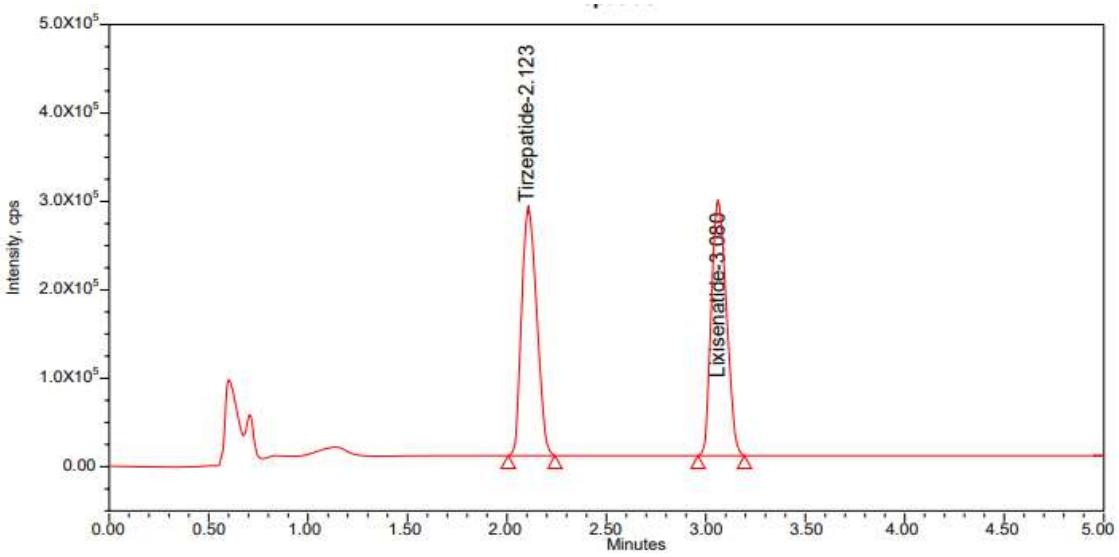

Figure 8: Matrix Effect Chromatogram of HQC

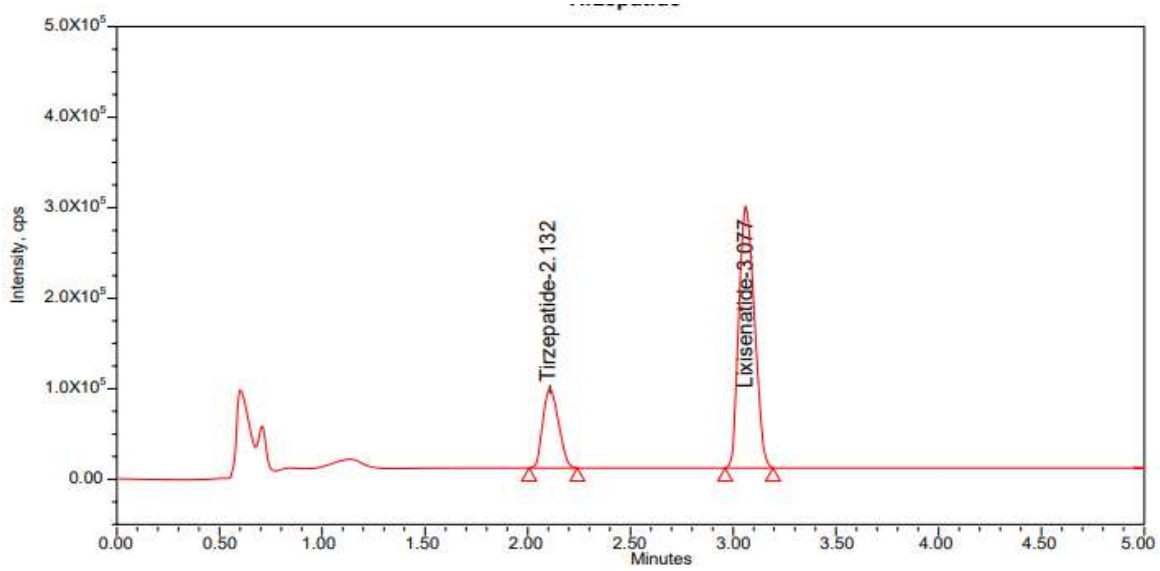

Figure 9: Matrix Effect Chromatogram of LQC

Linearity:

The standard curves were linear over the concentration range of 2.50-50 ng/mL of Tirzepatide Maralixibat. The mean correlation coefficient was 0.999. Samples were quantified using the ratio of peak area of analyte to that of IS. Peak area ratios were plotted against plasma concentrations. And results were tabulated in Table :8 and Figure 10:

Table :8 Linearity Results of Tirzepatide)

Final Conc. (ng/ml)	Tirzepatide Peak Response	IS Response	Peak Area Ratio
0	0	0	0
2.50	0.215	3.047	0.071
6.25	0.517	3.052	0.169
12.50	1.022	3.036	0.337
18.75	1.564	3.022	0.518
25.00	2.049	3.045	0.673
31.25	2.561	3.031	0.845
37.50	3.027	3.042	0.995
50.00	4.082	3.033	1.346
Slope	0.0268		
Intercept	0.00334		
R^2 Value	0.99975		

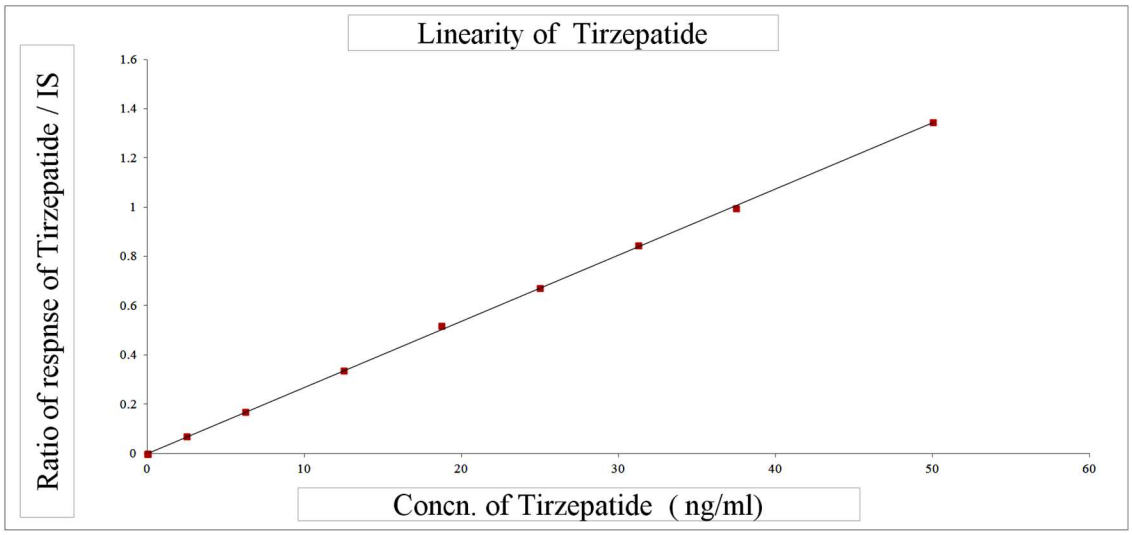

Figure 10: Calibration plot for concentration v/s Area ratio of Tirzepatide

Precision and accuracy

The intra-assay precision and accuracy were estimated by analyzing six replicates containing Tirzepatide

at four different QC levels. The inter-assay precision was determined by analyzing the four levels QC samples on four different runs. The criteria for acceptability of the data include, accuracy within 85–115% from the actual values and a precision of within ±15% relative standard deviation (RSD) except for LLQC, where it should be within 80–120% for accuracy and <20% of RSD. And results were tabulated in Table: 9

Table: 9 Precision and accuracy Results of Tirzepatide

Acquisition Batch ID	Date	HQC	MQC	LQC	LLQC
		Nominal Concentration (ng/ml)			
		37.5	25.0	12.5	2.5
		Analyte peak area			
110	05.05.2023	$3.014x10^5$	$2.025x10^5$	$1.015x10^5$	$0.204x10^5$
		$3.026x10^5$	$2.022x10^5$	$1.012x10^5$	$0.209x10^5$
		$3.011x10^5$	$2.034x10^5$	$1.016x10^5$	$0.205x10^5$
		$3.015x10^5$	$2.018x10^5$	$1.015x10^5$	$0.201x10^5$
		$3.033x10^5$	$2.026x10^5$	$1.012x10^5$	$0.203x10^5$
		$3.024x10^5$	$2.033x10^5$	$1.012x10^5$	$0.201x10^5$
n		6	6	6	6
Mean		3.020	2.026	1.018	0.2038
SD		0.008	0.006	0.001	0.002
% CV		0.26	0.29	0.09	0.98
% Mean Accuracy		99.78	99.88	99.18	94.79

Recovery of analyte

The recovery of drug and IS was evaluated at three concentration levels namely low, medium and high quality control. Recovery was calculated by comparing its response in replicate samples with that of neat standard solution responses. Analyte recovery from a sample matrix (extraction efficiency) is a comparison of analytical response from an amount of analyte added to that determined from sample matrix. Because of basic properties of Tirzepatide, extraction was carried out using mobile phase solvent.And results were tabulated in Table: 10,11,12 &13

Table: 10 Recovery of analyte Results of Tirzepatide (HQC)

Replicate Number	HQC (37.5 ng/ml)		
	Extracted Response	Un Extracted Response	Matrix Factor
1.	$3.031x10^5$	$3.049x10^5$	0.9940
2.	$3.024x10^5$	$3.034x10^5$	0.9967
3.	$3.019x10^5$	$3.031x10^5$	0.9960
4.	$3.014x10^5$	$3.028x10^5$	0.9953
5.	$3.026x10^5$	$3.036x10^5$	0.9967
6.	$3.011x10^5$	$3.024x10^5$	0.9957
N	6	6	6
Mean	$3.020x10^5$	$3.033x10^5$	0.9957
SD	0.007	0.008	0.001
%CV	0.23	0.26	0.10
% Mean Recovery	99.79%	100.21%	-

Table: 11 Recovery of analyte Results of Tirzepatide (MQC)

Replicate Number	MQC (25 ng/ml)		
	Extracted Response	Un Extracted Response	Matrix Factor
1.	$2.025\ x10^5$	$2.036\ x10^5$	0.9945
2.	$2.022\ x10^5$	$2.031\ x10^5$	0.9955
3.	$2.034\ x10^5$	$2.042\ x10^5$	0.9960
4.	$2.018\ x10^5$	$2.026\ x10^5$	0.9960
5.	$2.026\ x10^5$	$2.033\ x10^5$	0.9965
6.	$2.033\ x10^5$	$2.045\ x10^5$	0.9941
n	6	6	6
Mean	$2.026\ x10^5$	$2.035\ x10^5$	0.9954
SD	0.006	0.007	0.0009
%CV	0.29	0.34	
%Mean Recovery	98.88	98.99	-

Table: 12 Recovery of analyte Results of Tirzepatide (LQC)

Replicate Number	LQC (12.5 ng/ml)		
	Extracted Response	Un Extracted Response	Matrix Factor
1.	1.012 x10^5	1.018 x10^5	0.994
2.	1.011 x10^5	1.016 x10^5	0.995
3.	1.015 x10^5	1.024 x10^5	0.991
4.	1.017 x10^5	1.029 x10^5	0.988
5.	1.005 x10^5	1.015 x10^5	0.990
6.	1.006 x10^5	1.017 x10^5	0.989
n	6	6	6
Mean	1.011 x10^5	1.019 x10^5	0.991
SD	0.004	0.005	0.002
%CV	0.39	0.49	0.20
%Mean Recovery	98.92	99.78	-

Internal standard

Table: 13 **Internal standard Results of** Lixisenatide **(50 ng/ml)**

Rep No.	IS (50ng/ml)		
	Extracted Response	Un Extracted Response	Matrix Factor
1.	3.030 x10^5	3.031x10^5	0.999
2.	3.021 x10^5	3.022x10^5	0.999
3.	3.024x10^5	3.028x10^5	0.998
4.	3.027x10^5	3.035x10^5	0.997
5.	3.025x10^5	3.018x10^5	0.996
6.	3.023x10^5	3.016x10^5	0.992
n	6	6	6
Mean	3.025	3.025	0.996
SD	0.003	0.007	0.002
%CV	0.09	0.23	0.20
%Mean Recovery	99.73	99.73	-

Ruggedness on precision accuracy

It passed the Ruggedness on precision accuracy and the results were tabulated in **Table:14**

Table:14 Ruggedness on precision accuracy of Results of Tirzepatide

P & ID	**HQC (37.5 ng/ml)**	**MQC MQC (25ng/ml)**	**LQC (25ng/ml)**
Different column	Analyte Peak area		
	3.021x10^5	2.017x10^5	1.020x10^5
	3.025x10^5	2.021x10^5	1.024x10^5
	3.029x10^5	2.027x10^5	1.029x10^5
	3.032x10^5	2.020x10^5	1.023x10^5
	3.027x10^5	2.024x10^5	1.027x10^5
	3.021x10^5	2.027x10^5	1.020x10^5
N	6	6	6
Mean	3.025 x10^5	2.022 x10^5	1.023 x10^5
SD	0.004	0.004	0.003
% CV	0.13	0.19	0.29
% Mean Accuracy	99.99	98.70	100.17

Ruggedness on reinjection reproducibility:

The %CV for Tirzepatide were found to be 0.09%-0.19%. Hence it passed the Ruggedness on reinjection reproducibility. and the results were tabulated in Table:14

Table: 15 Ruggedness on reinjection reproducibility Results of Tirzepatide.

P & ID	HQC (37.5 ng/ml)	MQC MQC (25ng/ml)	LQC (25ng/ml)
Different column	Analyte Peak area		
	$3.018x10^5$	$2.015x10^5$	$1.022x10^5$
	$3.015x10^5$	$2.020x10^5$	$1.020x10^5$
	$3.019x10^5$	$2.024x10^5$	$1.024x10^5$
	$3.012x10^5$	$2.018x10^5$	$1.026x10^5$
	$3.017x10^5$	$2.022x10^5$	$1.028x10^5$
	$3.011x10^5$	$2.023x10^5$	$1.025x10^5$
N	6	6	6
Mean	3.015	2.020	1.024
SD	0.003	0.003	0.002
% CV	0.09	0.14	0.19
% Mean Accuracy	99.61	98.59	100.22

Stability experiments

By comparing the act of stock solution stability under the stability sample with the sample from the fresh stock sample preparation. Sample Stability studies in plasma were performed at the LQC and HQC concentration levels using six replicates at each level. Analyte was considered stable if the change is smaller amount than 15 % as per US FDA guidelines. The perfectness of spiked rat plasma stored at room temperature was evaluated for twenty-four hrs. The stability of spiked rabbit plasma stored at RT in auto sampler was evaluated for 24 h. The auto sampler stability (LQC, MQC and HQC) was evaluated by comparing the extract plasma samples that were injected immediately with the samples that were re-injected after storing with wet extract stability at room temperature after 12 h and 18 h at 2-8 °C. The reinjection reproducibility was evaluated by comparing the extracted plasma samples that were injected immediately with the samples that were re-injected after storing in the dry extract stability at room temperature after 12 h and 18h at-20 °±3 °C. The freeze-thaw stability was conducted by comparing the steadiness samples that had been frozen at-31 °C and thawed 3 times with freshly spiked internal control samples. The short-term stability was conducted 7 days 7 °C. For long-term stability evaluation, the concentrations obtained after 24 h were compared with the initial concentration.

Bench Top Stability

For benchtop stability experiment, stability of Tirzepatide in the rat plasma after 8 h exposure on benchtop was determined at three concentrations (LQC, MQC, and HQC) in six replicates. The results

are shown in Table :16

Table: 16 Bench Top Stability of Tirzepatide

Replicate No.	HQC	LQC	MQC
	Nominal Concentration(ng/ml)		
	37.50	12.50	25.0
	Analyte peak area		
1	$3.023x10^5$	$1.021x10^5$	$2.020x10^5$
2	$3.025x10^5$	$1.019x10^5$	$2.024x10^5$
3	$3.028x10^5$	$1.037x10^5$	$2.027x10^5$
4	$3.030x10^5$	$1.013x10^5$	$2.030x10^5$
5	$3.033x10^5$	$1.020x10^5$	$2.034x10^5$
6	$3.037x10^5$	$1.039x10^5$	$2.037x10^5$
n	6	6	6
Mean	$3.029 x10^5$	$1.024 x10^5$	$2.028 x10^5$
SD	0.005	0.010	0.005
%CV	0.16	0.97	0.24
% Mean Accuracy	100.02%	100.27%	99.00%

Auto Sampler Stability:

Auto sampler stability Samples of Tirzepatide in plasma were assessed by analyzing LQC, MQC, and HQC samples are injected every 1 h up to 24 h for the stability study. All samples compared with the fresh prepare samples of 0 Hr of different QC in six replicates. Samples were considered to be stable if assay values meet the compliance with the acceptable limits of accuracy (i.e., ±15% SD) and precision (i.e., ±15% RSD. The results are shown in Table ;17

Table 17: Auto Sampler Stability of Tirzepatide

Replicate No.	HQC	MQC	LQC
	Nominal Concentration (ng/ml)		
	37.50	25.0	12.50
	Analyte peak area		
1	$3.015x10^5$	$2.015x10^5$	$1.018x10^5$
2	$3.017x10^5$	$2.017x10^5$	$1.026x10^5$

3	$3.020x10^5$	$2.019x10^5$	$1.025x10^5$
4	$3.025x10^5$	$2.012x10^5$	$1.013x10^5$
5	$3.022x10^5$	$2.015x10^5$	$1.021x10^5$
6	$3.022x10^5$	$2.017x10^5$	$1.028x10^5$
7	$3.025x10^5$	$2.019x10^5$	$1.027x10^5$
8	$3.027x10^5$	$2.021x10^5$	$1.025x10^5$
9	$3.022x10^5$	$2.023x10^5$	$1.013x10^5$
10	$3.021x10^5$	$2.015x10^5$	$1.020x10^5$
11	$3.023x10^5$	$2.023x10^5$	$1.022x10^5$
12	$3.025x10^5$	$2.022x10^5$	$1.017x10^5$
13	$3.025x10^5$	$2.022x10^5$	$1.015x10^5$
14	$3.023x10^5$	$2.025x10^5$	$1.024x10^5$
15	$3.017x10^5$	$2.017x10^5$	$1.011x10^5$
16	$3.022x10^5$	$2.029x10^5$	$1.019x10^5$
17	$3.021x10^5$	$2.021x10^5$	$1.018x10^5$
18	$3.023x10^5$	$2.013x10^5$	$1.016x10^5$
19	$3.025x10^5$	$2.015x10^5$	$1.014x10^5$
20	$3.027x10^5$	$2.019x10^5$	$1.011x10^5$
21	$3.022x10^5$	$2.022x10^5$	$1.019x10^5$
22	$3.021x10^5$	$2.015x10^5$	$1.022x10^5$
23	$3.013x10^5$	$2.017x10^5$	$1.019x10^5$
24	$3.018x10^5$	$2.019x10^5$	$1.017x10^5$
N	24	24	24
Mean	$3.059x10^5$	$2.018x10^5$	$1.019x10^5$
SD	0.18	0.004	0.004
%CV	5.8	0.19	0.39
% Mean Accuracy	99.28 %	100.28%	98.25%

Freeze thaw stability:

Freeze-thaw stability Six replicates of each (LQC, MQC, and HQC) that were stored at −20°C were thawed completely thawing at room temperature and refrozen immediately to −20°C. This process was repeated twice and the samples were extracted for injection into LCMS. The results are shown in Table:18

Table 18: Freeze Thaw Stability of Tirzepatide

Replicate No.	HQC	LQC	MQC
	Nominal Concentration(ng/ml)		
	37.50	12.50	25.0
	Analyte peak area		
1	$3.018x10^5$	$1.025x10^5$	$2.021x10^5$
2	$3.035x10^5$	$1.014x10^5$	$2.031x10^5$
3	$3.029x10^5$	$1.012x10^5$	$2.023x10^5$
4	$3.037x10^5$	$1.029x10^5$	$2.029x10^5$
5	$3.028x10^5$	$1.026x10^5$	$2.035x10^5$
6	$3.027x10^5$	$1.021x10^5$	$2.028x10^5$
N	6	6	6
Mean	$3.029x10^5$	$1.021x10^5$	$2.027x10^5$
SD	0.006	0.006	0.005
%CV	0.19	0.58	0.24
% Mean Accuracy	100.06	99.15	98.96

Wet Extract stability

Prepare LQC, MQC and HQC solutions (each one six preparations) and inject into LCMS system after 12 hrs and 18 hrs. Results are presented in Table : 20 &21

Table 20: Wet Extract Stability of Tirzepatide at 12 Hrs

Replicate No.	HQC	LQC	MQC
	Nominal Concentration(ng/ml)		
	37.50	12.50	25.0
	Analyte peak area		
1	$3.011x10^5$	$1.028x10^5$	$2.020x10^5$
2	$3.014x10^5$	$1.038x10^5$	$2.032x10^5$
3	$3.015x10^5$	$1.024x10^5$	$2.022x10^5$
4	$3.025x10^5$	$1.025x10^5$	$2.038x10^5$
5	$3.029x10^5$	$1.022x10^5$	$2.035x10^5$
6	$3.021x10^5$	$1.020x10^5$	$2.031x10^5$
N	6	6	6
Mean	$3.019x10^5$	$1.026x10^5$	$2.029x10^5$
SD	0.006	0.006	0.007
%CV	0.19	0.58	0.34
% Mean Accuracy	99.73	100.40	99.05

Table 21: Wet Extract Stability of Tirzepatide Maralixibat at 18 Hrs

Replicate No.	HQC	LQC	MQC
	Nominal Concentration(ng/ml)		
	37.50	12.50	25.0
	Analyte peak area		
1	$3.015x10^5$	$1.015x10^5$	$2.019x10^5$
2	$3.029x10^5$	$1.025x10^5$	$2.022x10^5$
3	$3.011x10^5$	$1.024x10^5$	$2.026x10^5$
4	$3.019x10^5$	$1.032x10^5$	$2.032x10^5$
5	$3.026x10^5$	$1.028x10^5$	$2.034x10^5$
6	$3.011x10^5$	$1.022x10^5$	$2.029x10^5$
n	6	6	6
Mean	$3.018x10^5$	$1.024x10^5$	$2.027x10^5$
SD	0.007	0.005	0.005
%CV	0.23	0.48	0.24
% Mean Accuracy	99.60	100.22	98.92

Dry extract stability:

Prepare LQC, MQC and HQC solutions (each one six preparations) and inject into LCMS system after 12 hrs and 18 hrs. The %CV and mean accuracy for Tirzepatide was passed the Dry Extract stability.and results were tabulated in Table 22 &23

Table 22: Dry Extract Stability of Tirzepatide at 12 Hrs

Replicate No.	HQC	LQC	MQC
	Nominal Concentration(ng/ml)		
	37.50	12.50	25.0
	Analyte peak area		
1	$3.018x10^5$	$1.010x10^5$	$2.018x10^5$
2	$3.022x10^5$	$1.008x10^5$	$2.023x10^5$
3	$3.026x10^5$	$1.015x10^5$	$2.015x10^5$
4	$3.019x10^5$	$1.022x10^5$	$2.028x10^5$
5	$3.025x10^5$	$1.024x10^5$	$2.022x10^5$
6	$3.030x10^5$	$1.028x10^5$	$2.021x10^5$
n	6	6	6
Mean	$3.023x10^5$	$1.017x10^5$	$2.021x10^5$
SD	0.004	0.007	0.004
%CV	0.13	0.68	0.19

% Mean Accuracy	99.87	99.58	98.63

Table 23: Dry Extract Stability of Tirzepatide at 18 Hrs

Replicate No.	HQC	LQC	MQC
	Nominal Concentration(ng/ml)		
	37.50	12.50	25.0
	Analyte peak area		
1	$3.011x10^5$	$1.016x10^5$	$2.010x10^5$
2	$3.016x10^5$	$1.018x10^5$	$2.012x10^5$
3	$3.008x10^5$	$1.020x10^5$	$2.014x10^5$
4	$3.013x10^5$	$1.017x10^5$	$2.016x10^5$
5	$3.022x10^5$	$1.016x10^5$	$2.011x10^5$
6	$3.016x10^5$	$1.021x10^5$	$2.013x10^5$
n	6	6	6
Mean	$3.014x10^5$	$1.018x10^5$	$2.012x10^5$
SD	0.004	0.002	0.002
%CV	0.13	0.19	0.09
% Mean Accuracy	99.63	99.60	98.29

Short term Stability

Prepare LQC, MQC and HQC solutions (each one six preparations) and inject into LCMS system after 7 days stored at 5±3°C. The %CV and mean accuracy for Tirzepatide was passed the short term stability. Results are presented in Table : 24

Table 24: Short term Stability of Tirzepatide

Replicate No.	HQC	LQC	MQC
	Nominal Concentration(ng/ml)		
	37.50	12.50	25.0
	Analyte peak area		
1	$3.033x10^5$	$1.028x10^5$	$2.050x10^5$
2	$3.023x10^5$	$1.035x10^5$	$2.042x10^5$
3	$3.038x10^5$	$1.032x10^5$	$2.058x10^5$
4	$3.040x10^5$	$1.042x10^5$	$2.039x10^5$
5	$3.041x10^5$	$1.033x10^5$	$2.043x10^5$
6	$3.032x10^5$	$1.030x10^5$	$2.041x10^5$
N	6	6	6
Mean	$3.034x10^5$	$1.033x10^5$	$2.045x10^5$

SD	0.006	0.004	0.007
%CV	0.19	0.38	0.34
% Mean Accuracy	100.21	101.10	98.82

Long term Stability

Long-term stability was also performed at day 1, day 7, day 14, day 21, and day 28. The percentage mean accuracy was within limits (85–115%). These values indicating that Tirzepatide is stable for 28 days and results shown in Table :25, 26,27, 28 &29

Table 25: Long term Day-1 Stability of Tirzepatide

Replicate No.	HQC	LQC	MQC
	Nominal Concentration(ng/ml)		
	37.50	12.50	25.0
	Analyte peak area		
1	$3.013x10^5$	$1.008x10^5$	$2.018x10^5$
2	$3.015x10^5$	$1.012x10^5$	$2.016x10^5$
3	$3.031x10^5$	$1.012x10^5$	$2.015x10^5$
4	$3.022x10^5$	$1.028x10^5$	$2.014x10^5$
5	$3.028x10^5$	$1.017x10^5$	$2.015x10^5$
6	$3.017x10^5$	$1.015x10^5$	$2.012x10^5$
N	6	6	6
Mean	$3.021x10^5$	$1.015x10^5$	$2.015x10^5$
SD	0.007	0.006	0.002
%CV	0.23	0.59	0.09
% Mean Accuracy	99.79	99.34	98.33

Table 26: Long term Day-7 Stability of Tirzepatide

Replicate No.	HQC	LQC	MQC
	Nominal Concentration(ng/ml)		
	37.50	12.50	25.0
	Analyte peak area		
1	$3.052x10^5$	$1.008x10^5$	$2.038x10^5$
2	$3.058x10^5$	$1.008x10^5$	$2.032x10^5$
3	$3.042x10^5$	$1.001x10^5$	$2.030x10^5$
4	$3.046x10^5$	$1.019x10^5$	$2.045x10^5$
5	$3.039x10^5$	$1.016x10^5$	$2.042x10^5$

6	$3.036x10^5$	$1.018x10^5$	$2.044x10^5$
N	6	6	6
Mean	$3.045x10^5$	$1.011x10^5$	$2.038x10^5$
SD	0.008	0.007	0.006
%CV	0.26	0.69	0.29
% Mean Accuracy	100.60	98.98	99.15

Table 27: Long term Day-14 Stability of Tirzepatide

Replicate No.	HQC	LQC	MQC
	Nominal Concentration(ng/ml)		
	37.50	12.50	25.0
	Analyte peak area		
1	$3.058x10^5$	$1.008x10^5$	$2.028x10^5$
2	$3.059x10^5$	$1.008x10^5$	$2.022x10^5$
3	$3.047x10^5$	$1.001x10^5$	$2.020x10^5$
4	$3.056x10^5$	$1.009x10^5$	$2.025x10^5$
5	$3.049x10^5$	$1.006x10^5$	$2.022x10^5$
6	$3.046x10^5$	$1.011x10^5$	$2.024x10^5$
n	6	6	6
Mean	$3.052x10^5$	$1.007x10^5$	$2.022x10^5$
SD	0.005	0.003	0.001
%CV	0.16	0.29	0.049
% Mean Accuracy	100.78	98.54	98.75

Table 28: Long term Day-21 Stability of Tirzepatide

Replicate No.	HQC	LQC	MQC
	Nominal Concentration(ng/ml)		
	37.50	12.50	25.0
	Analyte peak area		
1	$3.069x10^5$	$1.045x10^5$	$2.055x10^5$
2	$3.065x10^5$	$1.042x10^5$	$2.032x10^5$
3	$3.045x10^5$	$1.032x10^5$	$2.033x10^5$
4	$3.053x10^5$	$1.059x10^5$	$2.049x10^5$
5	$3.044x10^5$	$1.048x10^5$	$2.025x10^5$
6	$3.072x10^5$	$1.033x10^5$	$2.048x10^5$
n	6	6	6

Mean	$3.058x10^5$	$1.043x10^5$	$2.040x10^5$
SD	0.01	0.01	0.01
%CV	0.32	0.95	0.49
% Mean Accuracy	101.01	102.06	99.57

Table 29: Long term Day-28 Stability of Tirzepatide

Replicate No.	HQC	LQC	MQC
	Nominal Concentration(ng/ml)		
	37.50	12.50	25.0
	Analyte peak area		
1	$3.079x10^5$	$1.048x10^5$	$2.001x10^5$
2	$3.061x10^5$	$1.045x10^5$	$2.012x10^5$
3	$3.055x10^5$	$1.042x10^5$	$2.013x10^5$
4	$3.059x10^5$	$1.055x10^5$	$2.019x10^5$
5	$3.047x10^5$	$1.043x10^5$	$2.005x10^5$
6	$3.077x10^5$	$1.038x10^5$	$2.008x10^5$
n	6	6	6
Mean	$3.063x10^5$	$1.045x10^5$	$2.009x10^5$
SD	0.01	0.005	0.006
%CV	0.32	0.47	0.29
% Mean Accuracy	101.18	102.26	97.99

Pharmacokinetic Studies:

The liquid-liquid extraction method was used to isolate Tirzepatide in rat plasma. For this, 200µl of plasma sample (respective concentration) were added into labelled polypropylene tubes and vortexed briefly after that 500µl of standard stock and 500 µl of Internal standard stock was added and vortexed for approximately 10min followed by centrifuged at 4000rpm at 20°C. Supernatant from each sample was transferred to labelled via tube and evaporated at 40°C until dryness. These samples were reconstituted with 800µl of Methanol vortexed briefly and then transferred the sample into auto sampler vials for injection.

Tirzepatide sample was injected into rat body collected samples at different time intervals like 0.5, 1, 2, 3, 4, 5 and 6 days in six different rats. After that samples are prepared as per test method injected into chromatographic system record their values. And results wew tabulated in Table 30&31

Table 30: Pharmacokinetic studies of Tirzepatide

Time Intervals (Days)	Tirzepatide (ng/ml)
0.5	12.793
1	22.491
2	18.596
3	13.050
4	6.353
5	2.207
6	0.000

Table 31: Pharmacokinetic parameters of Tirzepatide

Pharmacokinetic parameters	Tirzepatide
AUC_{0-t} (ng h/ml)	95
C_{max} (ng/ml)	22.5
$AUC_{0-\infty}$ (ng h/ml)	95
$T_{1/2}$ (h)	5 days
T_{max} (h)	1.0 day

$AUC_{0-\infty}$: Area under the curve extrapolated to infinity
AUC_{0-t}: Area under the curve up to the last sampling time
C_{max}: The maximum plasma concentration
T_{max}: The time to reach peak concentration
$T_{1/2}$: Time the drug concentration

A single dose of Tirzepatide injection was administered rats, samples were collected at 0.5, 1, 2, 3, 4, 5 and 6 days' post-dose. An aliquot of 5 ml blood was collected at each time point in K2 EDTA vacutainer tubes. Additionally, a predose sample was collected to check the possible interferences from the plasma. The collected samples were centrifuged to obtain the plasma and stored at −70} 10 °C. Plasma samples were spiked with the IS and processed along with QC samples at four concentrations. Pharmacokinetic parameters of Tirzepatide was calculated using WinNonlin (Version 5.2) software package. Stability of the study samples were established by incurred sample reanalysis (ISR). For ISR two samples from each subject were selected near *C*max and the elimination phase in the pharmacokinetic profile. The samples were considered stable; the percent difference should not be more than} 20%.

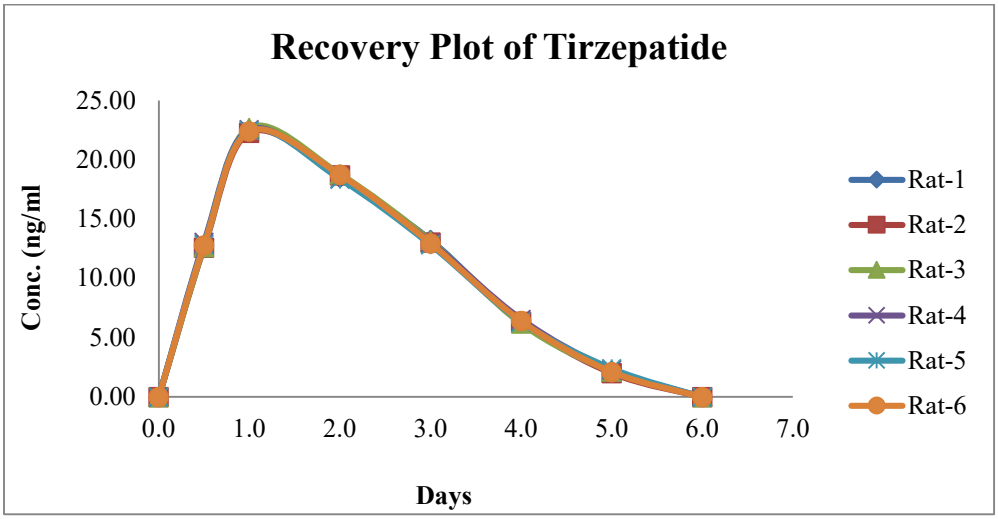

Figure 11: Recovery plot for Tirzepatide in Rat plasma

CONCLUSION

For the primary time higher sensitive HPLC-ESI-LCMS/MS method was developed and validated for the determination of Tirzepatide in Rat plasma. Here the described method is rugged, fast, reproducible bioanalytical method. This method was validated according to USFDA guidelines. Simple and efficient method was developed and may be utilized in pharmacokinetic studies and to see the investigated analyte in body fluids.

REFERENCE:

1. Chavda VP, Ajabiya J, Teli D, Bojarska J, Apostolopoulos V. Tirzepatide, a new era of dual-targeted treatment for diabetes and obesity: a mini-review. Molecules. 2022 Jul 5;27(13):4315.
2. Nauck MA, D'Alessio DA. Tirzepatide, a dual GIP/GLP-1 receptor co-agonist for the treatment of type 2 diabetes with unmatched effectiveness regrading glycaemic control and body weight reduction. Cardiovascular diabetology. 2022 Dec;21(1):1-6.
3. Jastreboff AM, Aronne LJ, Ahmad NN, Wharton S, Connery L, Alves B, Kiyosue A, Zhang S, Liu B, Bunck MC, Stefanski A. Tirzepatide Once Weekly for the Treatment of Obesity. New England Journal of Medicine. 2022 Jun 4. PubChem. Bethesda (MD): National Library of Medicine (US), National Center for Biotechnology Information; 2004-. bChem Compound Summary for CID 156588324, Tirzepatide; [cited 2022 Dec. 4]
4. Erni F. Use of high-performance liquid chromatography in the pharmaceutical industry. Journal of Chromatography A. 1990 May 16; 507:141-9.
5. Nikolin B, Imamović B, Medanhodzić-Vuk S, Sober M. High performance liquid chromatography in pharmaceutical analyses. Bosn J Basic Med Sci. 2004 May;4(2):5-9
6. Alam, S.; Hasan, M.K.; Neaz, S.; Hussain, N.; Hossain, M.F.; Rahman, T. Diabetes mellitus: Insights from epidemiology, biochemistry, risk factors, diagnosis, complications and comprehensive management. Diabetology 2021, 2, 36–50. [Google Scholar] [CrossRef]
7. Sun, H.; Saeedi, P.; Karuranga, S.; Karuranga, S.; Pinkepank, M.; Ogurtsova, K.; Duncan, B.B.; Stein, S.; Basit, A.; Chan, J.C.N.; et al. IDF Diabetes atlas: Global, regional and country-

level diabetes prevalence estimates for 2021 and projections for 2045. Diabetes Res. Clin. Pract. 2022, 183, 109119–109132. [Google Scholar] [CrossRef]
8. Lim, S.; Bae, J.H.; Kwon, H.S.; Nauck, M.A. COVID-19 and diabetes mellitus: From pathophysiology to clinical management. Nat. Rev. Endocrinol. 2021, 17, 11–30. [Google Scholar] [CrossRef]
9. Sebire, S.J.; Toumpakari, Z.; Turner, K.M.; Cooper, A.R.; Page, A.S.; Malpass, A.; Andrews, A.R. "I've made this my lifestyle now": A prospective qualitative study of motivation for lifestyle change among people with newly diagnosed type two diabetes mellitus. BMC Public Health 2018, 18, 204. [Google Scholar] [CrossRef] [Green Version]
10. Ganesan, K.; Rana, M.B.M.; Sultan, S. Oral Hypoglycemic Medications; Updated 8 May 2022; StatPearls Publishing: Treasure Island, FL, USA, 2022. [Google Scholar]
11. Costello, R.A.; Shivkumar, N.S. Sulfonylureas; Updated 23 February 2022; StatPearls Publishing: Treasure Island, FL, USA, 2022. [Google Scholar]
12. Zeynaloo, E.; Stone, L.D.; Dikici, E.; Ricordi, C.; Deo, S.K.; Bachas, L.G.; Daunert, S.; Lanzoni, G. Delivery of therapeutic agents and cells to pancreatic islets: Towards a new era in the treatment of diabetes. Mol. Aspects Med. 2022, 83, 101063–101084. [Google Scholar] [CrossRef]
13. Marín-Peñalver, J.J.; Martín-Timón, I.; Sevillano-Collantes, C.; Del Cañizo-Gómez, F.J. Update on the treatment of type 2 diabetes mellitus. World J. Diabetes 2016, 7, 354–395. [Google Scholar] [CrossRef] [PubMed]
14. Al-Tabakha, M.M. Future prospect of insulin inhalation for diabetic patients: The case of Afrezza versus Exubera. J. Control Release 2015, 215, 25–38. [Google Scholar] [CrossRef] [PubMed]
15. Modi, P. Diabetes beyond insulin: Review of new drugs for treatment of diabetes mellitus. Curr. Drug Discov. Technol. 2007, 4, 39–47. [Google Scholar] [CrossRef] [PubMed]
16. Tupas, G.D.; Otero, M.C.B.; Ebhohimen, I.E.; Egbuna, C.; Aslam, M. Chapter 8—Antidiabetic Lead Compounds and Targets for Drug Development. In Phytochemicals as Lead Compounds for New Drug Discovery; Egbuna, C., Kumar, S., Ifemeje, J.C., Ezzat, S.M., Kaliyaperumal, S., Eds.; Elsevier: Amsterdam, The Netherlands, 2020; pp. 127–141. [Google Scholar]
17. Frias, J.P.; Nauck, M.A.; Van, J.; Kutner, M.E.; Cui, X.; Benson, C.; Urva, S.; Gimeno, R.E.; Milicevic, Z.; Robins, D.; et al. Efficacy and safety of LY3298176, a novel dual GIP and GLP-1 receptor agonist, in patients with type 2 diabetes: A randomised, placebo-controlled and active comparator-controlled phase 2 trial. Lancet 2018, 392, 2180–2193. [Google Scholar] [CrossRef]
18. Frias, J.P.; Nauck, M.A.; Van, J.; Benson, C.; Bray, R.; Cui, X.; Milicevic, Z.; Urva, S.; Haupt, A.; Robins, D.A. Efficacy and tolerability of tirzepatide, a dual glucose-dependent insulinotropic peptide and glucagon-like peptide-1 receptor agonist in patients with type 2 diabetes: A 12-week, randomized, double-blind, placebo-controlled study to evaluate different dose-escalation regimens. Diabetes Obes. Metab. 2020, 22, 938–946. [Google Scholar]
19. Apostolopoulos, V.; Bojarska, J.; Chai, T.-T.; Elnagdy, S.; Kaczmarek, K.; Matsoukas, J.; New, R.; Parang, K.; Lopez, O.P.; Parhiz, H.; et al. A Global review on short peptides: Frontiers and perspectives. Molecules 2021, 26, 430. [Google Scholar] [CrossRef]

20. Nauck, M.A.; Meier, J.J. The incretin effect in healthy individuals and those with type 2 diabetes: Physiology, pathophysiology, and response to therapeutic interventions. Lancet Diabetes Endocrinol. 2016, 4, 525–536. [Google Scholar] [CrossRef]
21. Müller, T.D.; Finan, B.; Bloom, S.R.; D'Alessio, D.; Drucker, D.J.; Flatt, P.R.; Fritsche, A.; Gribble, F.; Grill, H.J.; Habaner, J.F.; et al. Glucagon-like peptide 1 (GLP-1). Mol. Metab. 2019, 30, 72–130. [Google Scholar] [CrossRef]
22. Gupta, S.; Sen, U. More than just an enzyme: Dipeptidyl peptidase-4 (DPP-4) and its association with diabetic kidney remodelling. Pharmacol. Res. 2019, 147, 104391–104403. [Google Scholar] [CrossRef]
23. Frías, J.P. Tirzepatide: A glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) dual agonist in development for the treatment of type 2 diabetes. Expert Rev. Endocrinol. Metab. 2020, 15, 379–394. [Google Scholar] [CrossRef]
24. Andreadis, P.; Karagiannis, T.; Malandris, K.; Avgerinos, I.; Liakos, A.; Manolopoulos, A.; Beklari, E.; Matthews, D.R.; Tsapas, A. Semaglutide for type 2 diabetes mellitus: A systematic review and meta-analysis. Diabetes Obes. Metab. 2018, 20, 2255–2263. [Google Scholar] [CrossRef]
25. Bokvist, B.K.; Coskun, T.; Cummins, R.C.; Alsina-Fernandez, J. GIP and GLP-1 Co-Agonist Compounds. U.S. Patent 947478, 25 October 2016. [Google Scholar]
26. Jastreboff, A.M.; Aronne, L.J.; Ahmad, N.N.; Wharton, S.; Connery, L.; Alves, B.; Kiyosue, A.; Zhang, S.; Liu, B.; Bunck, M.C.; et al. Tirzepatide once weekly for the treatment of obesity. N. Engl. J. Med. 2022, 11, 127. [Google Scholar] [CrossRef]
27. Wang, L. Designing a dual GLP-I R/GIPR agonist from tirzepatide: Comparing residues between tirzepatide, GLP-I, and GIP. Drug Des. Dev. Ther. 2022, 16, 1547–1560. [Google Scholar] [CrossRef] [PubMed]
28. Cosku, T.; Sloop, K.W.; Loghin, C.; Alsina-Fernandez, J.; Urva, S.; Bokvist, K.B.; Cui, X.; Briere, D.A.; Cabrera, O.; Roell, W.C.; et al. LY3298176, a novel dual GIP and GLP-1 receptor agonist for the treatment of type 2 diabetes mellitus: From discovery to clinical proof of concept. Mol. Metab. 2018, 18, 3–14. [Google Scholar] [CrossRef]
29. Østergaard, S.; Paulsson, J.F.; Kofoed, J.; Zosel, F.; Olsen, J.; Jeppesen, C.B.; Spetzler, J.; Ynddal, L.; Schleiss, L.G.; Christoffersen, B.Ø.; et al. The effect of fatty diacid acylation of human PYY3-36 on Y2 receptor potency and half-life in minipigs. Sci. Rep. 2021, 11, 21179–21194. [Google Scholar] [CrossRef] [PubMed]
30. Sun, B.; Willard, F.S.; Feng, D.; Alsina-Fernandez, J.; Chen, Q.; Vieth, M.; Ho, J.D.; Showalter, A.D.; Stutsman, C.; Ding, L.; et al. Structural determinants of dual incretin receptor agonism by tirzepatide. Proc. Natl. Acad. Sci. USA 2022, 119, 211650611–211650622. [Google Scholar] [CrossRef] [PubMed]
31. Chow, E.; Chan, J.C.N. The emerging role of incretins and twincretins. Nat. Rev. Endocrinol. 2021, 18, 73–74. [Google Scholar] [CrossRef]
32. Fisman, E.Z.; Tenenbaum, A. The dual glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) receptor agonist tirzepatide: A novel cardiometabolic therapeutic prospect. Cardiovasc. Diabetol. 2022, 20, 225–230. [Google Scholar] [CrossRef]