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A sensitive LC-MS/MS method for tirzepatide in rat plasma with comprehensive pharmacokinetic evaluation

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Abstract

Tirzepatide, a first-in-class dual glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) receptor agonist, has been approved for the treatment of type 2 diabetes mellitus and obesity. Despite its remarkable commercial success and expanding global use, validated bioanalytical methods for tirzepatide quantification remain limited. This study aimed to develop and validate a sensitive liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for the quantification of tirzepatide in rat plasma and to characterize its pharmacokinetic profile. Plasma samples were prepared by protein precipitation with methanol. Semaglutide, a structurally related GLP-1 analog, was used as the internal standard. Chromatographic separation was achieved using gradient elution with mobile phases consisting of 0.1% formic acid in water and acetonitrile on a C18 column. The lower limit of quantification (LLOQ) of 0.5 ng/mL in rat plasma was achieved. The method was validated according to FDA guidelines. Intra- and inter-day accuracy ranged from 89.32% to 112.01%, and precision was within 9.51% across the concentration range of 0.5 to 1000 ng/mL. Recovery and matrix effects were acceptable, and tirzepatide was stable under various storage and handling conditions. The validated method was applied to pharmacokinetic studies following intravenous (IV, 0.5 mg/kg) and subcutaneous (SC, 1 and 2 mg/kg) administration in rats. Plasma concentration-time profiles showed multi-exponential decline with dose-proportional pharmacokinetics. The terminal elimination half-life following IV injection was 13.75 ± 1.80 h, consistent with the long-acting formulation design. Subcutaneous bioavailability was 61.54–74.92%, indicating efficient absorption from the injection site. This validated LC-MS/MS method provides a quantitative tool for tirzepatide analysis in preclinical pharmacokinetic studies and may support future investigations of this dual incretin agonist.

Keywords LC-MS/MS, Dual GIP/GLP-1 agonist, Tirzepatide, Peptide, Pharmacokinetics, Bioavailability

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Introduction

Tirzepatide is a first-in-class dual glucose-dependent insulintropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) receptor agonist for type 2 diabetes mellitus and obesity (Coskun et al. 2018, Nauck and D'Alessio 2022, Block et al. 2023, Sokary and Bawadi 2025). The development of tirzepatide represents a significant advance beyond selective GLP-1 receptor agonists such as liraglutide, dulaglutide, and semaglutide, which have been the cornerstone of incretin-based therapy over the past decade (Frías et al. 2021, Nicholls et al. 2024, Billings 2025, Aronne et al. 2025). While GLP-1 receptor activation enhances glucose-dependent insulin secretion, suppresses glucagon release, and delays gastric emptying, the additional activation of GIP receptors by tirzepatide contributes complementary metabolic benefits (Nauck and D'Alessio 2022, Cai et al. 2024, France et al. 2024, Samms et al. 2021). GIP is responsible for a significant portion of the incretin effect, including insulin secretion (Asmar et al. 2010, Nauck and Meier 2016), and reduces potential lipotoxicity by storing free fatty acids in adipose tissues via re-esterification (Cai et al. 2024, Asmar et al. 2010). GIP also stimulates glucagon secretion to prevent hypoglycemic conditions (Cai et al. 2024) and may act on brain receptors, potentially alleviating adverse effects such as nausea and vomiting (Nauck and Meier 2016). Importantly, the combination of GIP and GLP-1 agonism produces synergistic rather than merely additive effects, with evidence suggesting enhanced central appetite regulation and improved peripheral metabolism compared to selective GLP-1 agonism alone (Coskun et al. 2018, Cai et al. 2024, Cerchi et al. 2025).

The mechanistic advantages of tirzepatide translate into superior clinical outcomes. In head-to-head trials, tirzepatide demonstrated significantly greater weight loss and more pronounced reductions in glycated hemoglobin (HbA1c) levels compared to semaglutide, the most effective GLP-1 receptor agonist (Frías et al. 2021, Aronne et al. 2025). The SURPASS clinical trial program for type 2 diabetes mellitus and the SURMOUNT

program for obesity management have consistently shown dose-dependent improvements in both glycemic control and body weight reduction across diverse patient populations (Frías et al. 2021, Jastreboff et al. 2022). Notably, treatment with the highest dose of tirzepatide (15 mg) resulted in mean weight reductions exceeding 20% of baseline body weight in obesity trials, a magnitude of effect previously achievable only through bariatric surgery (Jastreboff et al. 2022, Garvey et al. 2023). Furthermore, tirzepatide demonstrated favorable effects on cardiometabolic risk factors, including improvements in blood pressure, lipid profiles, and markers of hepatic steatosis (Garvey et al. 2023). Based on this unrivaled efficacy and comprehensive metabolic benefits, tirzepatide has been approved for the treatment of both type 2 diabetes mellitus (Mounjaro®) and obesity (Zepbound®). Owing to its clinical effectiveness, Zepbound® became the most prescribed new-to-brand drug for obesity in the US only a few months after its approval in 2023.

Like the injectable formulation of semaglutide (Ozempic® and Wegovy®), tirzepatide (Mounjaro® and Zepbound®) is administered subcutaneously once a week. These extended dosing intervals are attributed to the optimized pharmacokinetic profiles of both therapeutic peptides. The half-life ($t_{1/2}$) of tirzepatide is 5.4 days in humans (Schneck and Urva 2024), which is comparable to that of semaglutide in humans ($t_{1/2} = 168$ h) (Jensen et al. 2017), enabling once-weekly dosing. Deliberate structural modification has been applied to overcome the short half-lives of native GIP and GLP-1 (Fig. 1A) (Coskun et al. 2018, Kurtzhals et al. 2023, Lau et al. 2015). Rapid degradation of the native hormones (within just a few minutes) is mainly the result of cleavage after the alanine residue (Ala) at the second residue from the N-terminus by the dipeptidyl peptidase-4 (DPP-4) present in plasma. Thus, Ala at position 2 is replaced with aminoisobutyric acid (Aib²) in semaglutide and tirzepatide to prevent recognition by DPP-4 and provide steric hindrance, thereby conferring resistance against the peptidase (Coskun et al. 2018, Lau et al. 2015). To further reduce clearance, the

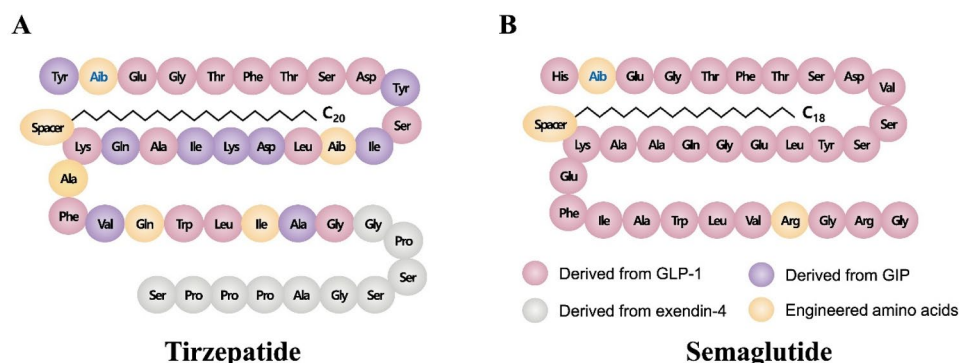


Fig. 1 Structures of **A** tirzepatide and **B** semaglutide

peptide backbone is engineered with a fatty acid chain. This lipidation promotes reversible binding to albumin, thereby reducing renal clearance, a strategy that has been proven effective for liraglutide and semaglutide (Fig. 1) (Kurtzhals et al. 2023). In tirzepatide, a C₂₀-fatty diacid chain was attached to the lysine residue at position 20 via a hydrophilic linker, which compensates for the hydrophobicity of the lipid moiety (Coskun et al. 2018).

According to a population pharmacokinetic analysis of pooled data from 19 studies, the PK profiles of healthy and patient groups were statistically indistinguishable (Schneck and Urva 2024). Briefly, tirzepatide is absorbed slowly, with an absorption rate constant of 0.0373 h⁻¹ and a high bioavailability of 80%. Plasma concentrations of tirzepatide subsequently reach their maximum after 8–72 h (T_{max}). The apparent volume of distribution and apparent clearance of tirzepatide were 10.3 L and 0.061 L/h, respectively. These values are close to those reported for semaglutide (12.5 L and 0.05 L/h) (Carlsson Petri et al. 2018). The limited volume of distribution suggests that, like semaglutide, tirzepatide predominantly resides in the intravascular space, reflecting its extensive binding to plasma albumin. The similar clearance values account for the comparable half-lives observed for both peptides. Tirzepatide is cleared predominantly by metabolism rather than excretion (Martin et al. 2024). The primary metabolic pathways include the proteolytic degradation of the peptide backbone and beta-oxidation of the fatty acid moiety. The resulting metabolites are then excreted as urine and feces. The systemic exposure of tirzepatide, as measured by the area under the curve (AUC), increased linearly with dose (Schneck and Urva 2024).

Despite the extensive clinical characterization and ever-increasing usage of tirzepatide, selective and sensitive bioanalytical methods for its quantification remain limited in the published literature. Several analytical approaches have been reported, including UV absorption-based detection (Vaishnavi and Vinayak 2025), radio-labeled substrate methods (Martin et al. 2024), and fluorimetry (Mansour et al. 2024), though these methods have limitations in selectivity or accessibility. More recently, LC–MS/MS methods have also been developed for tirzepatide quantification (Arumugam et al. 2025, Vijayamma and Nirmala 2024, Choi et al. 2025); however, their sensitivity may be insufficient for certain preclinical applications (LLOQ of 1–2.5 ng/mL).

Although the human pharmacokinetics of tirzepatide have been well characterized, preclinical studies remain essential for supporting emerging research areas. The development of novel formulations—such as oral, transdermal, or alternative delivery systems—requires sensitive and reliable quantification methods to support formulation optimization and bioavailability studies.

Investigation of tirzepatide pharmacokinetics across a broader range of dose regimens and in drug-drug interaction studies also demands analytical methods with sufficient sensitivity and selectivity. Furthermore, the growing interest in biosimilar development and generic formulations will necessitate the validation of analytical procedures for comparative pharmacokinetic and bioequivalence studies. Analytical methods with enhanced sensitivity are particularly important for pharmacokinetic characterization to capture the full concentration-time profile.

Therefore, this study aimed to develop and validate a simple and sensitive LC–MS/MS method for the quantification of tirzepatide in rat plasma and to demonstrate its utility through comprehensive pharmacokinetic characterization. This fully validated analytical procedure is expected to facilitate formulation development research and support comparative bioavailability assessments of tirzepatide. Furthermore, this study incorporates tirzepatide into a comparative pharmacokinetic dataset of GLP-1 receptor agonists generated under consistent in-house experimental conditions, enabling direct comparison of pharmacokinetic parameters across structurally related compounds.

Materials and methods

Materials

Tirzepatide (purity 100.2%) was purchased from Sinopep-Allsino Biopharmaceutical Co., Ltd. (Hangzhou, China). The internal standard (IS), semaglutide (purity 98%), was purchased from Zhejiang Peptides Biotech Co., Ltd. (Hangzhou, China). All solvents, including methanol, acetonitrile, and distilled water, were of high-performance liquid chromatography (HPLC) grade and purchased from J.T. Baker, Inc. (Phillipsburg, NJ, USA) unless otherwise noted. Formic acid was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

LC–MS/MS instrumentation

The LC–MS/MS analysis was performed using a 1290 Infinity II Bio LC system coupled to a 6495D Triple Quadrupole mass spectrometer (Agilent Technologies, Santa Clara, CA, USA). Chromatographic separation was achieved on an Acquity Premier Peptide CSH C18 column (130 Å, 1.7 µm, 2.1 × 150 mm; Waters, Milford, MA, USA), and the column oven temperature was maintained at 50 °C. The mobile phase consisted of 0.1% (v/v) formic acid in distilled water (A) and 0.1% (v/v) formic acid in acetonitrile (B). Gradient elution was performed at a flow rate of 0.3 mL/min as follows: initially 35% B, linearly increased to 55% B over 5 min, followed by a step increase to 90% B, which was held for 1 min (5–6 min). The mobile phase was then returned to 35% B over the next 3 min (6–9 min). For column washing, the flow rate

was increased to 0.4 mL/min for 1.5 min (9–10.5 min) while maintaining 35% B. Finally, the system was re-equilibrated for 1.5 min (10.5–12 min) by returning the flow rate to 0.3 mL/min.

The mass spectrometer was equipped with an electrospray ionization (ESI) source operating in positive ion mode. Tirzepatide and the IS were detected using multiple reaction monitoring (MRM) at transitions m/z 1204.3→396.2 and 1029.5→1238.8, respectively, with a dwell time of 200 ms per MRM channel. The collision energies were optimized to 25 eV for tirzepatide and 32 eV for the IS, while the fragmentor voltage was 166 V for both analytes. The source conditions were set to a gas temperature of 220 °C, a gas flow of 17 L/min, and a nebulizer pressure of 40 psi. Agilent MassHunter Quantitative Analysis 12.0 software (Agilent Technologies) was used to process mass spectrometric data.

Sample preparation

A simple protein precipitation method was utilized to prepare plasma samples for LC–MS/MS analysis. Initial stock solutions of tirzepatide and the IS, semaglutide, were prepared by dissolving each compound in methanol to a final concentration of 1 mg/mL. The stock solution of tirzepatide was then serially diluted with methanol to yield a range of standard working solutions. The tirzepatide working solutions had concentrations of 0.5, 1, 5, 10, 50, 100, 500, and 1000 ng/mL. The working solution for the IS was prepared at a concentration of 1000 ng/mL by diluting the stock solution.

Calibration standards were prepared by combining 50 μ L of blank rat plasma with 50 μ L of a tirzepatide standard working solution, 50 μ L of the IS working solution, and 350 μ L of methanol in a 1.5 mL Protein LoBind tube (Eppendorf, Hamburg, Germany). For in vivo samples, 50 μ L of plasma collected from study rats was combined with 50 μ L of the IS working solution and 400 μ L of methanol in a 1.5 mL LoBind tube. All mixtures were vortexed for 10 min to ensure thorough mixing and protein precipitation, followed by centrifugation at 15,000 rpm for 10 min. Finally, a 10 μ L aliquot of the clear supernatant from each tube was injected into the LC–MS/MS system for analysis.

Quality control (QC) samples were prepared using the same procedure as tirzepatide standard working solutions, at final concentrations of 0.5, 8, 40, and 800 ng/mL as LLOQ, low, medium, and high QC, respectively.

Assay validation

The analytical method underwent a comprehensive validation process in accordance with the bioanalytical method validation guidance issued by the US Food and Drug Administration (FDA) (U.S. Food and Drug Administration, 2018). Specificity, sensitivity, linearity, accuracy,

precision, extraction recovery, matrix effect, stability, and dilution integrity were evaluated.

To confirm specificity, chromatograms from blank rat plasma were compared against those from plasma spiked with either tirzepatide or the IS. The linearity of the calibration curve was established over the concentration range of 0.5–1000 ng/mL. Accuracy and precision were evaluated under both intra-day ($n=5$ replicates analyzed on a single day) and inter-day ($n=15$; 3 replicates per day over five consecutive days) conditions using QC samples.

The extraction recovery was determined by comparing the analyte response from samples spiked before protein precipitation to those spiked after extraction. The matrix effect was investigated by comparing the signal of analytes in post-extraction plasma to the signal of analytes in neat solution.

Analyte stability was tested under four distinct conditions ($n=3$): short-term (4 h at room temperature), long-term (7 days at -20 °C), autosampler (24 h at 4 °C), and freeze-thaw (three cycles). The integrity of sample dilution was verified by analyzing plasma samples spiked at 1500 ng/mL, which were then diluted 4-, 5-, 10-, and 20-fold ($n=5$) with blank plasma.

Pharmacokinetic studies in rats

All animal experiments were performed in compliance with standard operating procedures under a protocol approved by the Institutional Animal Care and Use Committee (IACUC) of Sungkyunkwan University (SKKU-ICUC2025-06-29-1). Male Sprague–Dawley rats (7–8 weeks old; body weight 269 ± 26 g; Samtako Bio Korea, Osan, Korea) were fasted overnight prior to the study. For serial blood sampling, rats were anesthetized with isoflurane and polyethylene (PE) tubing was intubated into the jugular vein. The rats were allowed to recover from surgery for at least 24 h. Tirzepatide, formulated in normal saline, was administered to the rats at an injection volume of 1 mL/kg. One group ($n=4$) received a 0.5 mg/kg intravenous (IV) dose via the penile vein, while two other groups ($n=4$ and 6) received subcutaneous (SC) injections at doses of 1 and 2 mg/kg, respectively.

Following drug administration, venous blood samples (approximately 0.3 mL) were collected from the jugular vein at multiple time points: 0, 5, 15, 30, and 45 min, and 1, 2, 4, 6, 8, 12, 24, 36, 48, 72, 96, and 120 h post-dose. The collected blood was centrifuged at 15,000 rpm for 10 min to separate plasma, which was then stored at -20 °C until it was subjected to sample preparation and analysis. Any plasma samples found to have analyte concentrations above the upper limit of quantification (ULOQ) were diluted by a factor of 4, 5, 10, or 20, as appropriate, to bring the concentration within the quantifiable range.

Pharmacokinetic analysis

Pharmacokinetic parameters were calculated using non-compartmental analysis (NCA). The maximum plasma concentration ($C_{\max}$) and the time to reach $C_{\max}$ ($T_{\max}$) were obtained directly from the observed data. The area under the plasma concentration-time curve from time zero to the last measurable concentration (AUC_{all}) was calculated using the linear trapezoidal rule. The area under the curve extrapolated to infinity (AUC_{inf}) was estimated as $AUC_{\text{all}} + C_{\text{last}}/\lambda_z$, where C_{last} is the last measurable concentration and λ_z is the terminal elimination rate constant determined by log-linear regression of the terminal phase. The terminal half-life ($t_{1/2}$) was calculated as $0.693/\lambda_z$. For SC administration, the absolute bioavailability (F) was calculated as $(AUC_{\text{inf, SC}} \times \text{Dose}_{\text{IV}})/(AUC_{\text{inf, IV}} \times \text{Dose}_{\text{SC}})$.

Results and discussion

Establishing analytical methods with LC-MS/MS

Figure 2A shows the full mass scan spectrum of tirzepatide. Although tirzepatide is a relatively large molecule (average molar mass = 4813.5 Da), its large size also allows for the formation of multiply charged species that can be detected within the scan range of the instrument. In the full scan spectrum, multiple protonated species were observed, including $[M+3H]^{3+}$ at m/z 1605.2 and $[M+4H]^{4+}$ at m/z 1204.3. The $[M+4H]^{4+}$ ion at m/z 1204.3, corresponding to tetra-protonated tirzepatide (calculated m/z = 1204.14), exhibited the highest response and was selected as the precursor ion for MRM. In the product ion mass scan spectrum of the m/z = 1204.3 ion, several product ion candidates for MRM were observed (Fig. 2B). The MRM transition m/z 1204.3 $\rightarrow$ 396.2 was chosen for the quantification of tirzepatide, because it had the highest signal intensity and minimal interfering noise. The MRM transition for the IS (semaglutide) was chosen as m/z 1029.5 $\rightarrow$ 1238.8 according to the previous study (Lee et al. 2023).

Chromatographic conditions used for the analysis of tirzepatide were slightly modified from those of semaglutide used in our previous study (Lee et al. 2023). Briefly, the Acquity Premier Peptide CSH C18 column was used for optimal separation and higher response. The addition of 0.1% (v/v) formic acid to both mobile phases (distilled water and acetonitrile) was essential for achieving sharper peak shapes and improved intensity. Elution with a high organic solvent content (90%) for 1 min was introduced after the separation gradient to minimize analyte carryover. The mobile phase composition gradually returned to its initial condition, and the system was re-equilibrated for the next analysis. The representative MRM chromatograms of tirzepatide and the IS in a typical rat plasma sample are shown in Fig. 3. The retention times of tirzepatide and the IS were 4.9 min and 3.3 min, respectively.

Assay validation

Specificity, linearity, and sensitivity

No interfering signals were detected around the retention times of tirzepatide and the IS, indicative of high specificity of the analytical method (Fig. 3). The calibration curve of tirzepatide was linear over the concentration range of 0.5–1000 ng/mL in plasma samples. The typical linear equation was $y = 2.281415x + 0.00001270921$ ($R^2 > 0.9999$), with a weighting factor of $1/x$. The LLOQ of tirzepatide was 0.5 ng/mL, representing a 2-fold improvement in sensitivity over the most recently published LC-MS/MS method for tirzepatide in rat plasma (1 ng/mL) (Choi et al. 2025). This enhanced sensitivity enabled reliable quantification through the extended terminal elimination phase, as demonstrated by the plasma concentration-time profiles extending to 120 h post-dose in the present pharmacokinetic study. The improved sensitivity is particularly advantageous for low-dose preclinical studies, such as those required for novel formulation development and comparative bioavailability assessments, where

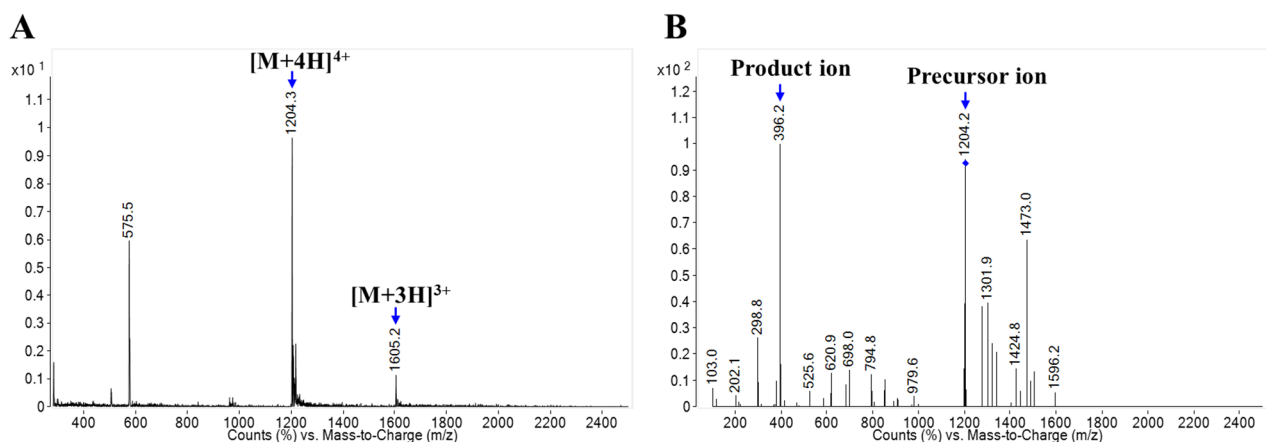


Fig. 2 Full mass scan **A** and product ion scan for the precursor ion m/z = 1204.3 **B** spectra of tirzepatide

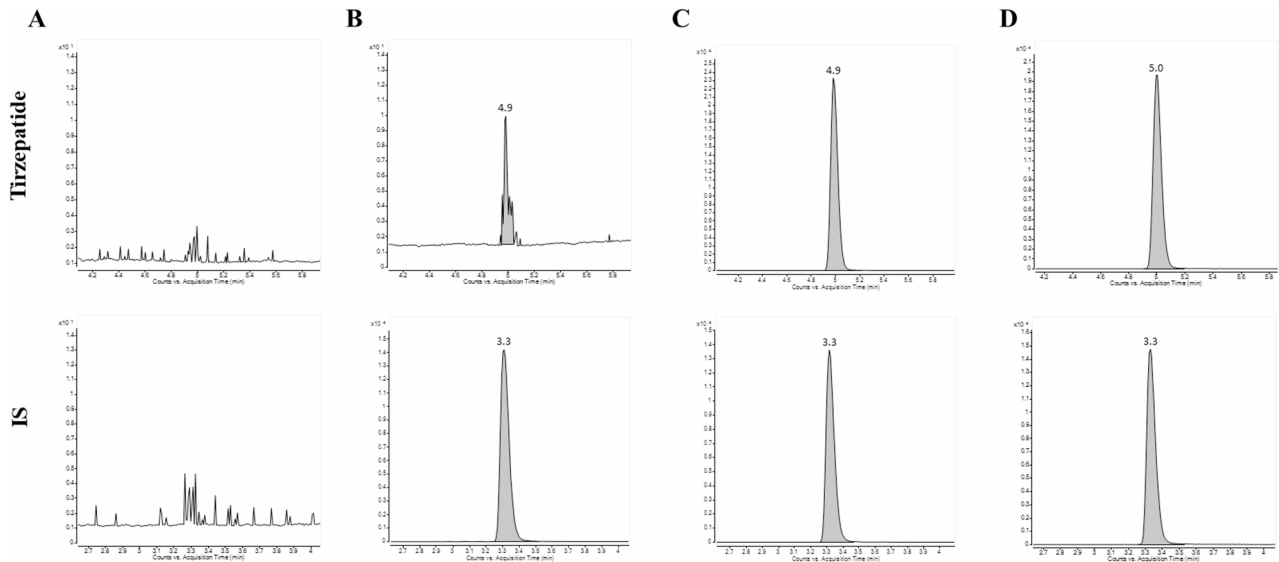


Fig. 3 Chromatograms of tirzepatide (upper panel) and the internal standard (IS, lower panel) in **A** blank rat plasma, **B** blank rat plasma spiked with tirzepatide at the LLOQ concentration (0.5 ng/mL) and IS, **C** blank rat plasma spiked at the ULOQ concentration (1000 ng/mL) and IS, and **D** a representative plasma sample from a pharmacokinetic study (IV 0.5 mg/kg, 1 h post-dose)

Table 1 Intra- and inter-day accuracy and precision values of the concentration measurement of tirzepatide in rat plasma samples

Concentration (ng/mL)	Intra-day (n = 5)		Inter-day (n = 15)	
	Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)
800	97.37	1.87	96.43	3.71
40	100.22	2.77	92.34	5.41
8	99.16	1.67	89.32	5.25
0.5	103.18	7.39	112.01	9.51

Table 2 Extraction recovery of tirzepatide in rat plasma with the protein precipitation method

Analyte	Concentration (ng/mL)	Extraction recovery (%)	Matrix effect (%)
Tirzepatide (n = 3)	800	99.23 ± 2.63	63.85 ± 0.72
	40	95.88 ± 0.62	74.02 ± 18.26
	8	91.74 ± 6.28	64.84 ± 9.39
IS (n = 9)	1000	101.56 ± 6.74	102.08 ± 5.76

plasma concentrations may fall below the detection limits of less sensitive methods (Choi et al. 2025, European Medicines Agency, 2022).

Accuracy and precision

Table 1 presents the intra- and inter-day accuracy and precision for the quantification of tirzepatide in rat plasma. Intra- and inter-day accuracies for concentrations excluding the LLOQ ranged from 89.32% to 100.22%, while the accuracies at the LLOQ were 103.18% (intra-day) and 112.01% (inter-day). The precision of the measurement was less than 5.41% for the QC samples

and 9.51% for the LLOQ samples. The accuracy and precision values satisfy the recommended criteria of the FDA method validation guidance (U.S. Food and Drug Administration, 2018).

Extraction recovery and matrix effects

Recovery of tirzepatide after extraction by simple protein precipitation was evaluated with the low, medium, and high QC samples (Table 2). The average extraction recovery was greater than 91.74% in all QC samples with the simple protein precipitation method. A high degree of extraction recovery is directly related to the efficient transfer of tirzepatide from the biological matrix to the analyte, helping to ensure reproducible results. The average matrix effect levels calculated for the low, medium, and high QC samples were 63.85%, 74.02%, and 64.84%, respectively. The observed matrix effects indicate moderate ion suppression for tirzepatide, which is not uncommon for large lipidated peptides analyzed under ESI conditions. The IS (semaglutide) showed minimal matrix effect (~102%) under the same conditions. Since calibration standards were prepared in the same rat plasma matrix as study samples, the consistent matrix effect was effectively accounted for during quantification, as confirmed by the accuracy and precision values meeting FDA acceptance criteria across all QC concentration levels.

Stability

The stability of tirzepatide was evaluated under various conditions, and the results are presented in Table 3. Neither short-term storage at room temperature nor

Table 3 Stability of tirzepatide in rat plasma under different storage or handling conditions

Concentration (ng/mL) (n = 3)	Short-term (%) (4 h at room temperature)	Long-term (%) (7 days at -20 °C)	Autosampler (%) (24 h in the autosampler)	Freeze-thaw (%) (3 freeze-thaw cycles)
800	95.13 ± 8.15	100.90 ± 4.26	93.31 ± 2.11	97.69 ± 3.01
40	103.56 ± 4.70	98.56 ± 3.32	100.40 ± 4.00	102.40 ± 5.81
8	101.17 ± 1.77	101.17 ± 3.19	95.31 ± 3.15	96.68 ± 9.45

Table 4 Dilution Integrity of tirzepatide in rat plasma at different dilution factors

Initial concentration (ng/mL)	Dilution factor (n = 5)	Accuracy (%)	Precision (%)
1500	4	91.13	1.54
	5	92.84	2.47
	10	94.13	4.00
	20	91.89	3.98

long-term storage at -20 °C had a significant impact on sample stability. No signs of degradation were found when the samples were left standing inside the autosampler for 24 h. Tirzepatide remained intact during three freeze-thaw cycles. These results demonstrate that tirzepatide is stable under various storage and handling conditions, ensuring the reliability of this method for routine preclinical studies.

Dilution integrity

Blank plasma spiked with 1,500 ng/mL tirzepatide was diluted by factors of 4, 5, 10, and 20, and analyzed to confirm the integrity of the diluted samples. These dilution factors were selected to encompass the range of concentrations expected to exceed the ULOQ in early post-dose pharmacokinetic samples. The average accuracies and precisions of the measured concentrations ranged from 91.13 to 94.13% and 1.54–4.00%, respectively (Table 4). These results confirm the analytical integrity of diluted samples with high original concentrations.

Pharmacokinetics of tirzepatide in rats

Plasma concentration-time profiles of tirzepatide following IV and SC injections are depicted in Fig. 4. Non-compartmental analysis was conducted to obtain relevant pharmacokinetic parameters as summarized in Table 5. The plasma concentration of tirzepatide after IV

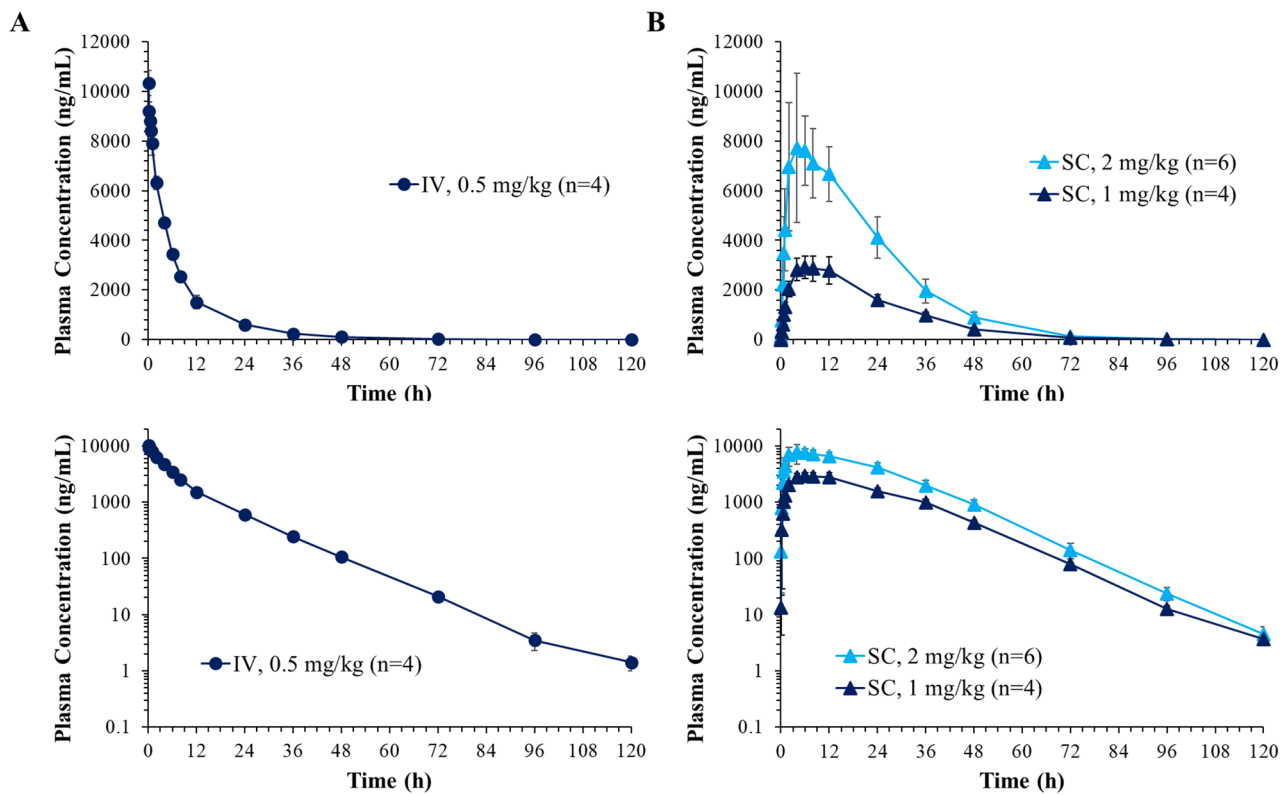


Fig. 4 Plasma concentration vs. time profiles of tirzepatide after **A** intravenous injection (IV, 0.5 mg/kg) and **B** subcutaneous injection (SC, 1 and 2 mg/kg) in rats (mean ± SD, n = 4–6). The upper panels show a linear scale, and the lower panels show a semi-logarithmic scale

Table 5 Pharmacokinetic parameters of tirzepatide following intravenous (IV, 0.5 mg/kg) and subcutaneous (SC, 1 and 2 mg/kg) injection in rats ($n=4-6$, mean $\pm$ SD)

Parameter	IV	SC	
	0.5 mg/kg ($n=4$)	1 mg/kg ($n=4$)	2 mg/kg ($n=6$)
$t_{1/2}$ (h)	13.75 $\pm$ 1.80	11.68 $\pm$ 0.49	9.79 $\pm$ 0.53
C_0 or C_{max} (ng/mL)	10955.87 $\pm$ 801.23	3069.33 $\pm$ 554.17	8360.86 $\pm$ 2342.66
T_{max} (h)	–	9.00 (6.00, 12.00)	5.00 (4.00, 8.00)
AUC_{all} (ng·h/mL)	71160.36 $\pm$ 1950.20	87556.35 $\pm$ 9715.43	213274.13 $\pm$ 39355.17
AUC_{inf} (ng·h/mL)	71188.90 $\pm$ 1958.02	87618.47 $\pm$ 9717.32	213339.07 $\pm$ 39373.80
CL (mL/h/kg)	7.03 $\pm$ 0.19	–	–
V_{ss} (mL/kg)	72.37 $\pm$ 2.82	–	–
Bio-availability (%)	–	61.54	74.92

$t_{1/2}$ terminal half-life, C_0 initial plasma concentration, C_{max} peak plasma concentration, T_{max} time to C_{max} , AUC_{all} area under the plasma concentration vs. time curve from time zero to the time of the last observation, AUC_{inf} AUC from time zero to infinity, CL clearance, V_{ss} volume of distribution
*Values were presented as the median (minimum, maximum)

injection followed a multi-exponential decline, as indicated by more than one linear phase in the semi-log plot of plasma concentration versus time. The half-life ($t_{1/2}$) of the terminal phase was determined as 13.75 $\pm$ 1.80 h. The average clearance (CL) and steady-state volume of distribution (V_{ss}) of tirzepatide were estimated as 7.03 mL/h/kg and 72.37 mL/kg, respectively. After SC injection, the plasma concentration of tirzepatide gradually increased, reached its maximum concentration in 4–12 h, and then decreased with an average $t_{1/2}$ of 9.79–11.68 h. The systemic exposure of tirzepatide, indicated by C_{max} , AUC_{all} , and AUC_{inf} following SC injection at 1 and 2 mg/kg, was approximately dose-proportional. The estimated absolute bioavailability of tirzepatide after SC injection was 61.54–74.92% in rats. It should be noted that dose-proportionality was assessed using only two dose levels (1 and 2 mg/kg), and the observed linearity in systemic exposure should be interpreted within this dose range. Dose-proportionality across a broader dose range would warrant further investigation in future preclinical studies.

The systemic clearance of tirzepatide, determined following IV administration in rats (6.80–7.27 mL/h/kg), is substantially greater than that observed in humans (0.47

mL/h/kg) (Schneck and Urva 2024). In accordance with this, the observed terminal half-life of approximately 12–16 h in rats is substantially shorter than the 5-day half-life in humans (Schneck and Urva 2024). Given the species-specific differences in metabolic rates, the variance in pharmacokinetic parameters between species is inherently natural, and faster elimination is expected in small animals such as rats (Kim et al. 2025, Boxenbaum 1982). Also noteworthy is the distinct binding capability of human and rat serum albumin, which might affect the renal clearance of therapeutic peptides (Kosa et al. 1997, Pistolozzi et al. 2014, Aubry et al. 1995).

Building upon our previously published analytical methods for semaglutide (Lee et al. 2023), liraglutide (Oh et al. 2023), and lixisenatide (Oh et al. 2023), the present study incorporates tirzepatide into a comparative pharmacokinetic dataset generated under consistent in-house experimental conditions using the same animal model and pharmacokinetic analysis approach (Table 6). This enables direct cross-analog comparison of pharmacokinetic parameters within a controlled experimental framework. Tirzepatide exhibited the lowest intravenous clearance among the tested therapeutic peptides. Consistent with the low clearance, tirzepatide had the longest intravenous half-life, being about 1.5 times longer than semaglutide. Since lixisenatide lacks a fatty acid side chain, the significantly longer half-life observed for tirzepatide, as well as for liraglutide and semaglutide, demonstrates that the albumin-binding strategy remains effective in rats. Considering that therapeutic peptides are mainly eliminated through non-specific proteolysis, a process that is universally conserved across species (Huh et al. 2011, Mordenti et al. 1991), it is reasonable that the same strategy to extend the half-lives of these peptides can be applied across various species.

The V_{ss} of tirzepatide, liraglutide, and semaglutide in rats were all within a comparable range, while lixisenatide (290 mL/kg) had a significantly larger V_{ss} . The distinction again can be explained by the confinement of the fatty acid-modified peptides to albumin, whereas lixisenatide, lacking such a moiety, distributes more widely. The V_{ss} of tirzepatide in rats is consistent with the reported value of 92.1 mL/kg in humans (sum of volume of distribution in central and peripheral compartments), a trend also observed for liraglutide and semaglutide (86 and 93.9 mL/kg in humans, respectively) (Watson et al. 2010, Overgaard et al. 2019).

The relatively high subcutaneous bioavailability (approximately 62–75%) suggests efficient absorption from the subcutaneous depot. Tirzepatide shows delayed subcutaneous T_{max} in rats compared to lixisenatide and liraglutide, which may reflect a relatively slower absorption process of the peptide. The mean absorption time (MAT) of tirzepatide in rats, calculated by subtracting the

Table 6 Comparison of analytical methods and selected pharmacokinetic parameters of various GLP-1 receptor agonists in rats (mean ± SD)

	Parameter	Lixisenatide (Oh et al. 2023)	Liraglutide (Oh et al. 2023)	Semaglutide (Lee et al., 2023)	Tirzepatide
Analytical method	Instrumentation	Agilent 1260 HPLC/ Agilent 6490 MS	Agilent 1260 HPLC/ Agilent 6490 MS	Agilent 1290 UPLC/ SCIEX QTRAP 6500 + MS	Agilent 1290 UPLC/ Agilent 6495D MS
	Matrix	plasma	plasma, brain	plasma, brain	plasma
	LLOQ (ng/mL)	10	1 (plasma), 2 (brain)	0.5 (plasma, brain)	0.5
	m/z transitions	810.8 → 129.2	938.5 → 1063.8	1029.3 → 1302.9	1204.3 → 396.2
IV	Dose (mg/kg)	1 (n = 5)	0.05 (n = 5)	0.02 (n = 7)	0.5 (n = 4)
	t _{1/2} (h)	0.37 ± 0.06	3.67 ± 0.40	9.26 ± 1.06	13.75 ± 1.80
	CL (mL/h/kg)	1358.78 ± 207.27	14.96 ± 3.82	12.41 ± 2.37	7.03 ± 0.19
	V _{ss} (mL/kg)	291.44 ± 78.29	73.12 ± 20.68	102.96 ± 10.38	72.37 ± 2.82
SC	Dose (mg/kg)	5 (n = 5)	1 (n = 5)	0.1, 0.2 (n = 4) [†]	1, 2 (n = 4–6) [†]
	t _{1/2} (h)	0.44 ± 0.08	3.55 ± 0.51	7.22–8.99	9.79–11.68
	T _{max} (h)*	0.25 (0.25, 0.50)	3.00 (2.00, 6.00)	6.00 (3.00, 12.00)	5.00 (4.00, 8.00)
	Bioavailability (%)	2.17	13.16	76.65–82.85	61.54–74.92

*Values were presented as the median (minimum, maximum); †, For compounds tested at multiple SC doses, ranges represent values across dose levels

average intravenous mean residence time (MRT_{IV}) from the average subcutaneous mean residence time (MRT_{SC}, estimated for 2 mg/kg dose group), was 9.01 h. Indeed, this MAT of tirzepatide is longer than that of lixisenatide, liraglutide, and semaglutide. Despite the protracted absorption profile (MAT = 9.01 h), tirzepatide maintained high subcutaneous bioavailability (62–75%), indicating efficient and sustained absorption from the depot site. This favorable absorption characteristic, combined with the extended elimination half-life, supports the feasibility of long-acting formulation development.

Conclusions

A simple and sensitive LC–MS/MS method has been developed and fully validated for the quantification of tirzepatide in rat plasma in accordance with the FDA bioanalytical method validation guidance. The method employs straightforward protein precipitation for sample preparation and uses semaglutide as the internal standard, achieving adequate sensitivity, specificity, accuracy, and precision for preclinical pharmacokinetic studies.

Application of this validated method successfully characterized the pharmacokinetics of tirzepatide in rats following intravenous and subcutaneous administration, demonstrating multi-exponential disposition, dose-proportional kinetics, and favorable bioavailability. These findings provide valuable preclinical data that support the development of novel tirzepatide formulations and alternative delivery systems. The analytical method established in this study can be readily adapted for various preclinical investigations and may serve as a foundation for clinical pharmacokinetic studies or therapeutic drug monitoring with appropriate modifications.

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Author contributions

S. Song: Conceptualization, Methodology, Investigation, Writing-original draft; MH: Conceptualization, Methodology, Investigation; SK: Investigation; YK: Investigation; SH: Investigation; EP: Investigation; BSS: Conceptualization, Methodology, Writing-original draft, Writing-Review & Editing; S. Shin: Conceptualization, Methodology, Writing-original draft, Writing-Review & Editing.

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Data availability

All data generated or analysed during this study are included in this published article.

Declarations

Competing interests

S. Shin was an associate editor of the Journal of Analytical Science and Technology while conducting this study. Editor status has no bearing on editorial considerations. Other authors declare that they have no competing interests.

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References

Aronne LJ, Horn DB, le Roux CW, Ho W, Falcon BL, Gomez Valderas E, et al. Tirzepatide as compared with semaglutide for the treatment of obesity. *N Engl J Med*. 2025;393(1):26–36.

Arumugam A, Lakshmi G, Srinivas G, Rao N. A single dose, bioavailability study Mounjaro (tirzepatide) solution for injection in a pre-filled pen 2.5 mg/0.5 ml in normal healthy adult human subjects under fasting condition. *Eur J Appl Sci*. 2025;13(03):270–6.

Asmar M, Simonsen L, Madsbad S, Stallknecht B, Holst JJ, Bülow J. Glucose-dependent insulinotropic polypeptide may enhance fatty acid re-esterification in subcutaneous abdominal adipose tissue in lean humans. *Diabetes*. 2010;59(9):2160–3.

Aubry A-F, Markoglou N, McGann A. Comparison of drug binding interactions on human, rat and rabbit serum albumin using high-performance displacement

- chromatography. *Comp Biochem Physiol Part C: Pharmacol Toxicol Endocrinol.* 1995;112(3):257–66.
- Billings LK. Comparison of dose escalation versus switching to tirzepatide among people with type 2 diabetes inadequately controlled on lower doses of dulaglutide. *Ann Intern Med.* 2025;178(5):609–19.
- Boxenbaum H. Interspecies scaling, allometry, physiological time, and the ground plan of pharmacokinetics. *J Pharmacokinet Biopharm.* 1982;10(2):201–27.
- Cai W, Zhang R, Yao Y, Wu Q, Zhang J. Tirzepatide as a novel effective and safe strategy for treating obesity: A systematic review and meta-analysis of randomized controlled trials. *Front Public Health.* 2024;12:1277113.
- Carlsson Petri KC, Ingwersen SH, Flint A, Zacho J, Overgaard RV. Semaglutide s.c. once-weekly in type 2 diabetes: A population pharmacokinetic analysis. *Diabetes Ther.* 2018;9(4):1533–47.
- Cerchi E, Santo P, de Oliveira MC, Janovsky C, Halpern B. Effects of tirzepatide on weight management in patients with and without diabetes: A systematic review and meta-analysis. *Int J Obes (Lond).* 2025.
- Choi HI, Jeong HC, Jeong JW, Lee J, Kim DH, Ko KC, et al. Development and validation of an LC-MS/MS method for tirzepatide, a dual GIP/GLP-1 receptor agonist, in rat plasma for application to a pharmacokinetic study. *J Chromatogr B Analyt Technol Biomed Life Sci.* 2025;1268:124836.
- Coskun T, Sloop KW, Loghin C, Alsina-Fernandez J, Urva S, Bokvist KB, 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.
- De Block C, Bailey C, Wysham C, Hemmingway A, Allen SE, Peleshok J. Tirzepatide for the treatment of adults with type 2 diabetes: An endocrine perspective. *Diabetes Obes Metab.* 2023;25(1):3–17.
- European Medicines Agency. Public assessment report, EMA/791310/2022. 2022.
- France NL, Syed YY. Tirzepatide. A review in type 2 diabetes. *Drugs.* 2024;84(2):227–38.
- Frias JP, Davies MJ, Rosenstock J, Manghi FCP, Landó LF, Bergman BK, et al. Tirzepatide versus semaglutide once weekly in patients with type 2 diabetes. *N Engl J Med.* 2021;385(6):503–15.
- Garvey WT, Frias JP, Jastreboff AM, le Roux CW, Sattar N, Aizenberg D, et al. Tirzepatide once weekly for the treatment of obesity in people with type 2 diabetes (SURMOUNT-2): A double-blind, randomised, multicentre, placebo-controlled, phase 3 trial. *Lancet.* 2023;402(10402):613–26.
- Huh Y, Smith DE, Feng MR. Interspecies scaling and prediction of human clearance: Comparison of small- and macro-molecule drugs. *Xenobiotica.* 2011;41(11):972–87.
- Jastreboff AM, Aronne LJ, Ahmad NN, Wharton S, Connery L, Alves B, et al. Tirzepatide once weekly for the treatment of obesity. *N Engl J Med.* 2022;387(3):205–16.
- Jensen L, Helleberg H, Roffel A, van Lier JJ, Bjornsdottir I, Pedersen PJ, et al. Absorption, metabolism and excretion of the GLP-1 analogue semaglutide in humans and nonclinical species. *Eur J Pharm Sci.* 2017;104:31–41.
- Kim J, Kim K, Lee TS, Cho H, Yoo S-D, Kim TH, et al. Prediction of the pharmacokinetics of the GLP-1 receptor agonist semaglutide in humans via a population pharmacokinetic model integrating interspecies scaling. *J Pharm Invest.* 2025;55(6):827–35.
- Kosa T, Maruyama T, Otogiri M. Species differences of serum albumins: I. Drug binding sites. *Pharm Res.* 1997;14(11):1607–12.
- Kurtzhals P, Ostergaard S, Nishimura E, Kjeldsen T. Derivatization with fatty acids in peptide and protein drug discovery. *Nat Rev Drug Discov.* 2023;22(1):59–80.
- Lau J, Bloch P, Schaffer L, Pettersson I, Spetzler J, Kofoed J, et al. Discovery of the once-weekly glucagon-like peptide-1 (GLP-1) analogue semaglutide. *J Med Chem.* 2015;58(18):7370–80.
- Lee TS, Park EJ, Choi M, Oh HS, An Y, Kim T, et al. Novel LC-MS/MS analysis of the GLP-1 analog semaglutide with its application to pharmacokinetics and brain distribution studies in rats. *J Chromatogr B Analyt Technol Biomed Life Sci.* 2023;1221:123688.
- Mansour NM, El-Masry AA, El-Sherbiny DT, Moustafa MA. White analytical insight for sensitive fluorescent determination of semaglutide and tirzepatide in pharmaceuticals and biological matrices. *Spectrochim Acta Part A Mol Biomol Spectrosc.* 2024;313:124159.
- Martin JA, Czeskis B, Urva S, Cassidy KC. Absorption, distribution, metabolism, and excretion of tirzepatide in humans, rats, and monkeys. *Eur J Pharm Sci.* 2024;202:106895.
- Mordenti J, Chen SA, Moore JA, Ferraioli BL, Green JD. Interspecies scaling of clearance and volume of distribution data for five therapeutic proteins. *Pharm Res.* 1991;8(11):1351–9.
- Nauck MA, D'Alessio DA. Tirzepatide, a dual GIP/GLP-1 receptor co-agonist for the treatment of type 2 diabetes with unmatched effectiveness regarding glycaemic control and body weight reduction. *Cardiovasc Diabetol.* 2022;21(1):169.
- Nauck MA, Meier JJ. The incretin effect in healthy individuals and those with type 2 diabetes: Physiology, pathophysiology, and response to therapeutic interventions. *Lancet Diabetes Endocrinol.* 2016;4(6):525–36.
- Nicholls SJ, Bhatt DL, Buse JB, Prato SD, Kahn SE, Lincoff AM, et al. Comparison of tirzepatide and dulaglutide on major adverse cardiovascular events in participants with type 2 diabetes and atherosclerotic cardiovascular disease: SURPASS-CVOT design and baseline characteristics. *Am Heart J.* 2024;267:1–11.
- Oh HS, Choi M, Lee TS, An Y, Park EJ, Kim TH et al. Pharmacokinetics and brain distribution of the therapeutic peptide liraglutide by a novel LC-MS/MS analysis. *J Anal Sci Technol.* 2023;14(1).
- Oh HS, Park EJ, Lee TS, An Y, Kim TH, Shin S et al. Pharmacokinetics of lixisenatide, a GLP-1 receptor agonist, determined by a novel liquid chromatography–tandem mass spectrometry analysis in rats. *Separations.* 2023;10(5).
- Overgaard RV, Delff PH, Petri KCC, Anderson TW, Flint A, Ingwersen SH. Population pharmacokinetics of semaglutide for type 2 diabetes. *Diabetes Ther.* 2019;10(2):649–62.
- Pistolozzi M, Fortugno C, Franchini C, Corbo F, Muraglia M, Roy M, et al. Species-dependent binding of tocinamide analogues to albumin: Affinity chromatography and circular dichroism study. *J Chromatogr B.* 2014;968:69–78.
- Samms RJ, Christie ME, Collins KAL, Pirro V, Droz BA, Holland AK et al. GIPR agonism mediates weight-independent insulin sensitization by tirzepatide in obese mice. *J Clin Invest.* 2021;131(12).
- Schneck K, Urva S. Population pharmacokinetics of the GIP/GLP receptor agonist tirzepatide. *CPT Pharmacometrics Syst Pharmacol.* 2024;13(3):494–503.
- Sokary S, Bawadi H. The promise of tirzepatide: A narrative review of metabolic benefits. *Prim Care Diabetes.* 2025;19(3):229–37.
- U.S. Food and Drug Administration. Bioanalytical method validation: Guidance for industry. 2018.
- Vaishnavi NB, Vinayak MG. Advancements in RP-HPLC methodology for tirzepatide estimation in human plasma: Development, validation, and applications. *Asian J Pharm Res Dev.* 2025;13(3):180–4.
- Vijayamma G, Nirmala S. 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. *Front Health Inf.* 2024;13(4).
- Watson E, Jonker DM, Jacobsen LV, Ingwersen SH. Population pharmacokinetics of liraglutide, a once-daily human glucagon-like peptide-1 analog, in healthy volunteers and subjects with type 2 diabetes, and comparison to twice-daily exenatide. *J Clin Pharmacol.* 2010;50(8):886–94.

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