Vol. 14, No. 4, 2023
ISSN 2233-4203/ e-ISSN 2093-8950
ARTICLE www.msletters.org | Mass Spectrometry Letters

Determination of Liraglutide in Rat Plasma Using Selective Liquid Chromatography–Tandem Mass Spectrometry

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Received October 7, 2023; Revised November 7, 2023; Accepted November 9, 2023
First published on the web December 31, 2023; DOI: 10.5478/MSL.2023.14.4.141

Abstract : Liraglutide is a medication prescribed for the management of type 2 diabetes and chronic obesity. A simple, sensitive, and selective liquid chromatography–tandem mass spectrometry (LC–MS/MS) method was developed and validated for the quantitative analysis of liraglutide in rat plasma. After a simple protein precipitation step, liraglutide was chromatographically separated using the ACQUITY Premier Peptide BEH C18 Column with mobile phases comprising 50% acetonitrile and 50% methanol, and water with 0.3% FA. Positive ion electrospray ionization in multiple reaction monitoring mode was used to achieve detection. Good linearity was observed in the 5–600 ng/mL concentration range ($R^2 > 0.99$). Liraglutide had intra- and inter-day precision values of 2.13%–9.86% and 4.14%–8.36%, respectively. The accuracy ranged from −2.36% to 2.58%. The recovery and matrix effect were within acceptable limits. This selective LC-MS/MS method was used to study the pharmacokinetic properties of liraglutide after subcutaneous administration in rats.

Keywords : liraglutide, LC-MS/MS, rat, pharmacokinetics, carryover

Abbreviations : GLP-1, glucagon-like peptide-1; ELISA, enzyme-linked immunosorbent assay; LC-MS/MS, liquid chromatography–tandem mass spectrometry; SPE, solid-phase extraction; QC, quality control.

Introduction

Intestinal epithelial L-cells release a peptide hormone called glucagon-like peptide-1 (GLP-1) in response to hormonal, neurological, and trophic stimulation.[1,2] GLP-1 has a therapeutic potential because of its physiological role. However, its pharmacological value remains limited because it is rapidly degraded by the enzyme dipeptidyl peptidase-IV and neutral endopeptidase and it has a half-life of 2 min.[3-6] Many GLP-1 derivatives have been developed to improve the pharmacokinetic properties of natural GLP-1. Among them, liraglutide has been commercially successful. Liraglutide is a recombinant human GLP-1 derivative, a member of GLP-1 classes of drugs, and a GLP-1 receptor agonist[7-9] that shares 97% sequence similarity with human GLP-1.

Liraglutide was created by substituting Lys34 in the GLP-1 peptide with Arg34 and adding a fatty acid chain to Lys26.[5,6,10] Liraglutide can self-associate and form heptamers because of its fatty acid side chains, enabling it to be absorbed slowly via the subcutaneous route. Liraglutide is used to treat type 2 diabetes mellitus at low doses and improve obesity through weight loss at high doses.[11-13] In particular, the weight loss effect of liraglutide is because of its direct influence on the stomach, hypothalamus, and other central appetite centers; it can delay gastric emptying and decrease food intake via signals through the vagus nerve, resulting in a sensation of fullness.[14,15]

Various liraglutide formulations are being developed for widespread clinical use. Thus, pharmacokinetic studies and quantification of blood liraglutide concentrations are essential. Antibody-based immunoassays such as enzyme-linked immunosorbent assay (ELISA) have been used to measure blood liraglutide concentrations.[16] However, ELISA has limitations when used in pharmacokinetic studies of liraglutide. ELISA is a complex and laborious procedure that is prone to errors, has a low limit of detection, and requires

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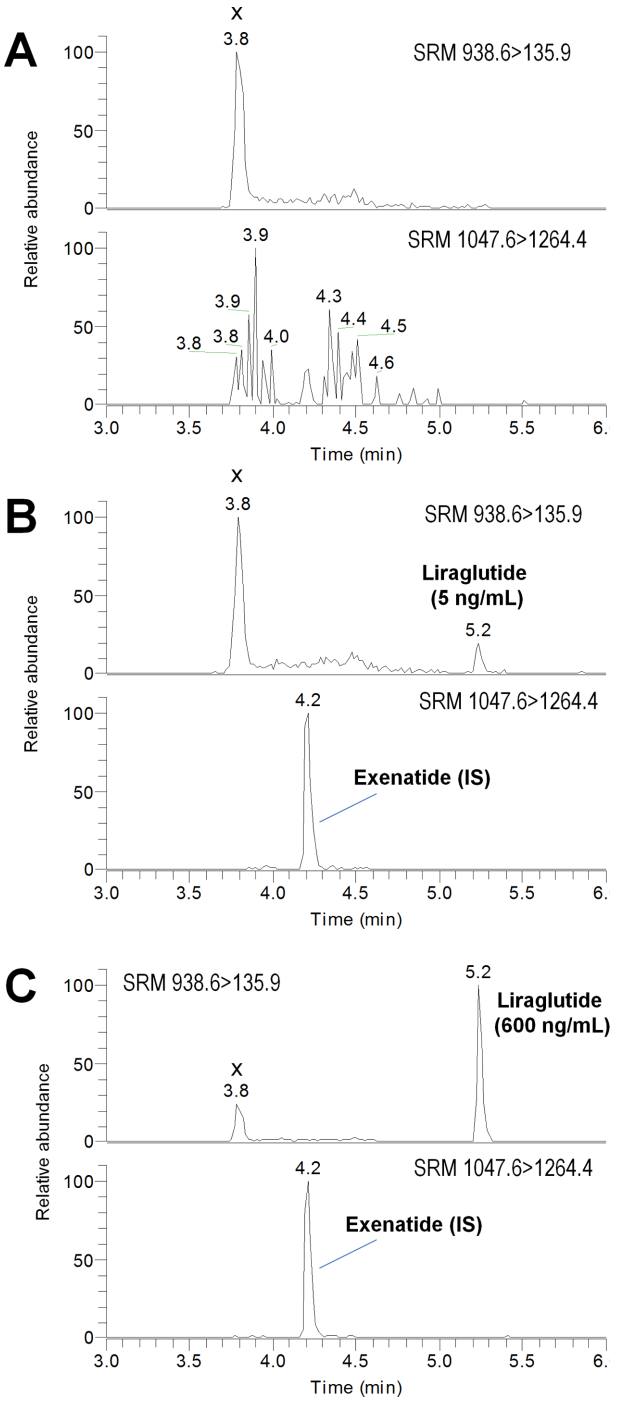

Figure 1. Representative SRM chromatogram of liraglutide (5.2 min) and exenatide (IS, 4.2 min). (A) Blank rat plasma, (B) blank plasma spiked with 5 ng/mL of liraglutide and IS, and (C) blank plasma spiked with 600 ng/mL of liraglutide and IS. x, interference peak.

practice to ensure consistency and a large amount of sample for the experiment.[17] Conversely, liquid chromatography–tandem mass spectrometry (LC-MS/MS) assays that can improve upon the shortcomings of ELISAs can be used for drug development and quantification of therapeutic peptides such as liraglutide.[18-22] At present, many LC-MS/MS-based methods for liraglutide quantification have been developed. However, they have disadvantages including the large amount of plasma required for quantification, complexity of pretreatment, and low sensitivity or short linearity range of the assay. In this study, a precise and selective LC-MS/MS method was developed and validated to measure liraglutide concentrations in rat plasma, that column carryover of liraglutide had been solved. This method was used to quantify the amount of liraglutide in a preclinical pharmacokinetic study, where rats were subcutaneously administered with liraglutide.

Materials and Methods

Chemicals, reagents, and materials

Liraglutide and exenatide were purchased from PolyPeptide Laboratories Inc. (Torrance, CA, USA) and Toronto Research Chemicals (Toronto, Ontario, Canada), respectively. Formic acid (FA), ammonia solution, and other chemical reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). The Oasis WAX µElution plate was purchased from Waters (Milford, MA, USA). MS grade water, methanol (MeOH), and acetonitrile (ACN) were bought from Thermo Fisher Scientific (Waltham, MA, USA).

Preparation of calibration curve standards and quality control (QC) samples

Working solutions with eight different concentrations were prepared by diluting liraglutide stock solution with 50% ACN. Primary working standard solutions with concentrations of 100 ng/mL, 1 µg/mL, and 10 µg/mL were also prepared by diluting liraglutide stock solution with 50% ACN. Eight concentrations of secondary working standard solutions were prepared by diluting the primary working standard solution. Exenatide was diluted with 50% ACN to a final concentration of 5 µg/mL. Plasma was spiked with the secondary working standard solution to construct calibration curves at final concentrations of 5, 10, 25, 50, 100, 250, 400, and 600 ng/mL. Plasma was spiked with the working standards to prepare QC samples with final concentrations of 5, 25, 100, and 400 ng/mL. These samples were used to evaluate inter- and intraday precision and accuracy. The stock solution was stored at −20°C, and each working solution was freshly prepared and used.

Sample preparation

The supernatant was prepared after centrifugation (4°C, 1,600 g) with 200 µL of plasma mixed with 5 µg/mL of exenatide (internal standard, IS) and 200 µL of ACN and 200 µL of water for precipitation prior to clean-up by solid-phase extraction (SPE). The Oasis Method Development 96-well Elution Plate was used to allow the best-perform-

ing type of Weak Anion eXchange (WAX) to be selected. This basic operating method was conducted in accordance with the manufacturer's instructions. In summary, SPE plates were conditioned with 500 μL of MeOH, and 500 μL of water was introduced to establish equilibrium and condition the SPE sorbent. The previously prepared sample was loaded onto the SPE and subjected to sequential washing steps using 500 μL of water and 50% MeOH. Then, the target analyte was eluted using a 200 μL solution containing 60% ACN and 40% MeOH with 6% ammonia, followed by an additional elution step involving 100 μL of a solution containing 6% FA in water. Finally, the two eluted solutions were mixed, and 20 μL of pooled solution was analyzed directly by LC-MS/MS.

Method validation

The analysis procedure was verified in accordance with the Food and Drug Administration (FDA)'s 2018 guidelines for "Bioanalytical Method Validation"[23] and the Ministry of Food and Drug Safety (MFDS)'s 2013 "Guideline on Bioanalytical Method Validation".[24] The developed LC-MS/MS method's selectivity and specificity were defined as (1) no interference of endogenous plasma components with liraglutide and exenatide as IS retention times and (2) no cross interference between liraglutide and IS. To construct the calibration curves for rat plasma samples, the nominal concentration of the calibration standard was plotted against the peak area ratio of plasma samples containing liraglutide–IS for three days at seven concentrations ranging from 5 ng/mL to 600 ng/mL. The calibration curves were fitted with the linear regression model ($y = ax + b$) weighted by 1/x using Xcalibur 4.1 (Waltham, MA, USA).

For intraday assay, the accuracy and precision were evaluated using six replicates on the same day. Meanwhile, for interday assay, the accuracy and precision were evaluated for three consecutive days using four different concentrations (5, 25, 100, and 400 ng/mL). Liraglutide's stability in rat plasma samples was evaluated under various temperature and time conditions in triplicate at lower QC (LQC, 25 ng/mL) and higher QC (HQC, 400 ng/mL): (1) stability after three freeze–thaw cycles at −80°C, (2) long-term stability for 15 days at −80°C, and (3) short-term stability at room temperature for 6 h.

Pharmacokinetic study

Male Sprague–Dawley rats (weighing 240–260 g) were obtained from Koatech (Laboratory Animal Supplying Facility, Pyeongtaek, Gyeonggi, Republic of Korea). They were received at 7 weeks of age and used after a seven-day acclimatization period. Accordingly, 8-week-old rats were used in this study. All animals were fed with a normal rodent pellet diet and had unlimited access to water. The animal quarters were strictly maintained at 23 ± 3°C under a relative humidity of 55 ± 15%. A 12-h light/dark cycle was used with an intensity ranging from 150 lux to 300 lux. Three rats were subcutaneously administered with a single dose of 360 μg/kg of liraglutide. Subsequently, 500 μL of blood was collected from the external jugular vein of each rat at 0, 0.5, 1, 2, 4, 6, 8, 10, 12, 24, 30, 48, and 72 h. All samples were centrifuged for 5 min at 15,000 × g and 4°C to immediately separate plasma. The plasma was then transferred to tubes and stored at −80°C until analysis.

LC-MS/MS conditions

Ultrahigh-performance LC (UHPLC)-MS/MS analyses were performed using a triple-stage quadrupole (TSQ) Vantage™ mass spectrometer (Thermo Fisher Scientific) equipped with a heated electrospray ionization (HESI) unit and coupled to Vanquish UHPLC (Thermo Fisher Scientific). Gradient elution conditions were maintained in the UHPLC system. Mobile phase A comprised 0.3% FA in water, whereas mobile phase B comprised a 1:1 mixed solution of ACN and MeOH. The mobile phase gradient conditions in terms of % solvent B were as follows: 0–0.2 min, 20%; 0.2–1 min, 20%–50%; 1–3 min, 50%–80%; 3–4 min, 80%–90%; 4–5 min, 90%; 5–6 min, 90%–20%; and 6–8 min, 20%. The ACQUITY Premier Peptide BEH C18 Column (2.1 × 150 mm, 1.7 mm; particle size, 300 Å; Waters) was used to separate peptides.

The mobile phase solutions were filtered using a 0.22 mm membrane and degassed via ultrasound. The flow rate was 0.25 mL/min. The column and autosampler temperatures were maintained at 80°C and 10°C, respectively. The mass spectrometer was operated in selected reaction monitoring (SRM) mode with ± 0.5 Da mass selection window. The fragmentation conditions for the two analytes exhibited collision energies (CEs) of 41% and 23%, with a Q1 peak width of 0.7 and cycle time of 1 s. The electrospray voltage was 4,000 V, the sheath gas pressure was 35 Arb units, the auxiliary gas pressure was 10 Arb units, the ion source vaporizer temperature was 300°C, and the capillary temperature was 350°C. Several multicharged precursors were observed for liraglutide and exenatide.

Pharmacokinetic parameters

The noncompartmental model in WinNonlin software version 2.1 (Scientific Consulting, Inc., Apex, NC, USA) was used to determine the pharmacokinetic parameters of liraglutide in plasma.

Results and Discussion

Liraglutide determination using LC-MS/MS

The Vanquish UHPLC coupled to a TSQ vantage system equipped with a HESI source detected liraglutide as a multiply charged precursor ion (+4 at *m/z* 938.4). The precursor ion intensity was higher in positive mode than in negative mode. The SRM mode for quantification increased the selectivity and sensitivity of the target ions while lowering matrix interference. The intensities of product ions were

evaluated by increasing the precursor ion CE of the compound of interests to determine the target ions. Since *m/z* 135.8 had the highest intensity when CE was 41 units, it was selected as the target ion in SRM mode (Figure S1A). The SRM condition of exenatide used as IS was also determined to be m/z 1047.6 > 1264.4 in the same way (Figure S1B).

Troubleshooting to remove column carryover

To quantify liraglutide in the LC-MS/MS system, 300 Å (ACQUITY Premier Peptide BEH C18 Column) was selected because large pore size columns show high selectivity for peptide analysis. However, the peptides did not completely pass through the pores of the stationary phase and were adsorbed on the surface and simultaneously desorbed from their critical concentration in the organic solvent comprising 100% ACN with 0.01% FA. Based on previous studies 25, 26, the content of FA was increased to 0.3% to remove column carryover. Furthermore, the mobile phase composition was adjusted and finalized as 50% ACN and 50% MeOH to analyze 500 ng/mL of liraglutide.27 Finally, a symmetric liraglutide peak appeared, and the analyte's column carryover was eliminated (Figure S2). The final analytical method was proposed by optimizing the column length, run time, change in the composition ratio of the mobile phases over time, and column temperature (Table S1).

Method validation

Specificity and selectivity

Figure 1 shows that no endogenous interference was observed in liraglutide and exenatide (IS) retention times, and the optimized LC-MS/MS method conditions provided complete separation of liraglutide and IS. The method's selectivity was demonstrated by the absence of interference with the retention times of analyte and IS in blank plasma. Figure 1 shows the chromatograms for (A) blank rat plasma, blank plasma spiked with 5 ng/mL at the lower limit of quantification (LLOQ) (B), and blank plasma spiked with 600 ng/mL at the upper limit of quantification (C) of liraglutide or IS.

Calibration curve and lower limits of quantification

Linear calibration curves were established between 5 ng/mL and 600 ng/mL for liraglutide (Fig. S3). The calibration curve included a double blank (blank plasma without liraglutide or IS), zero blank (blank plasma with IS only), and eight calibration standards with concentrations of 5 (LLOQ), 10, 25, 50, 100, 250, 400, and 600 ng/mL for liraglutide (blank plasma with IS and liraglutide). A weighted linear regression model (1/x) for liraglutide was used to determine the relationship between the analyte:IS peak area ratio and the nominal analyte concentration in the calibration standard. According to FDA guidelines, the back-calculated concentrations of calibration standards must be within ±15%, whereas the LLOQ must be within ±20% of the nominal value. At least 75% and six standard samples for calibration curves must meet the above criteria. In this study, the back-calculated concentrations for each calibration standard were within the range recommended by the FDA (i.e., 2.13%–9.86% of the nominal concentration). The coefficient of determination (R^2) for each three-day calibration curve was ≥0.99. The LLOQ was 5 ng/mL, whereas the signal-to-noise ratio was >10.

Accuracy and precision

Table 1 shows the results of liraglutide intra- and interday assay accuracy and precision. The intraday accuracy and precision of the liraglutide analysis method ranged from 97.64% to 101.07% and from 2.13% to 9.86%, respectively. Meanwhile, the interday precision and accuracy ranged from 4.14% to 8.36% and from 99.60% to 102.58%, respectively. All accuracy (%) and precision (%) results satisfied the range recommended by the MFDS and FDA guidelines for bioanalytical method validation.

Stability

All stability studies in plasma were performed in triplicate using 25 ng/mL as LQC and 400 ng/mL as HQC, whereas stability studies in stock solution were performed in six replicates using 25, 100, and 400 ng/mL as LQC, MQC, and HQC respectively: (1) stability of the stock solution (30 days at −20°C), (2) freeze–thaw stability (three freeze–thaw cycles), (3) short-term stability (6 h at room temperature) of samples in rat plasma, and (4) long-term

Table 1. Intra- and inter-day accuracy and precision of the LC-MS/MS method for liraglutide in plasma.

Nominal Concentration (ng/mL)	Intra-day (*n = 6*)			Inter-day (*n = 6, 3* days)		
	Calculated Concentration* (Mean ± SD, ng/mL)	Precision (RSD, %)	Accuracy (%)	Calculated Concentration* (Mean ± SD, ng/mL)	Precision (RSD, %)	Accuracy (%)
5	5.05 ± 0.50	9.86	101.07	4.98 ± 1.21	8.36	99.60
25	24.93 ± 1.53	6.13	99.71	24.98 ± 5.82	4.14	99.91
100	97.64 ± 3.23	3.31	97.64	100.11 ± 23.46	4.90	100.11
400	403.17 ± 8.59	2.13	100.79	410.32 ± 96.74	5.59	102.58

*Values represent the means ± SEs of six determinations. RSD, relative standard deviation; SD, standard deviation.

Table 2. Pharmacokinetic parameters of liraglutide in rats after subcutaneous administration (SD, standard deviation).

Parameters	Individual			Average (± SD)
	#1	#2	#3	
C_{max} (ng/mL)	878.0	523.6	1194.3	865.3 ± 335.5
T_{max} (min)	0.3	0.3	0.3	0.3 ± 0.0
$T_{1/2}$ (min)	0.3	0.3	0.3	0.3 ± 0.0
AUC_{last} (ng·min/mL)	615.0	438.1	619.7	557.6 ± 103.5

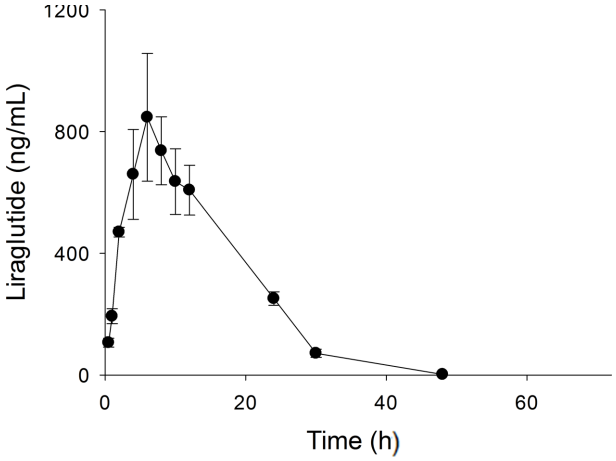

Figure 2. Concentration–time curve of liraglutide in rat plasma. Plasma was obtained over time after rats were subcutaneously injected with 360 μg/kg of liraglutide.

stability in plasma stored in a freezer (15 days at −80°C). Table S2 shows that liraglutide was stable in plasma for up to 6 h at room temperature (short term) and exhibited freeze–thaw stability (104.67% and 92.50%) after three freeze–thaw cycles. Moreover, liraglutide exhibited long-term stability (97.84% and 103.60%) in plasma at −80°C for 15 days. No significant degradation was observed in any condition examined, and all plasma samples were stored at −80°C and thawed over ice.

Pharmacokinetic study of liraglutide in rat plasma using LC-MS/MS

The plasma liraglutide concentration was measured quantitatively using the validated LC-MS/MS method. The mean plasma concentration–time curve for liraglutide and the associated pharmacokinetic parameters were obtained after a single subcutaneous administration of liraglutide (350 μg/mL). Figure 2 shows the pharmacokinetic profiles, and Table 2 lists the pharmacokinetic parameters. After intraperitoneal administration, AUClast and Cmax were 557.6 ± 103.5 ng·min/mL and 865.3 ± 335.5 ng/mL, respectively.

The method described in this study can be used to monitor the therapeutic effects of liraglutide and the accuracy, precision, and sensitivity required for determining the pharmacokinetic parameters.

Conclusion

A rapid, specific, sensitive, and reproducible method based on LC-MS/MS coupled with SPE and protein precipitation preparation was developed to determine the pharmacokinetics of liraglutide in rat plasma. The final analysis method was established by selecting through column comparison to measure the accurate quantification of liraglutide in plasma. Based on the results, liraglutide has a linear range of 5–600 ng/mL and an LLOQ of 5 ng/mL. This analytical method successfully determined the pharmacokinetic profile of liraglutide in rat plasma.

Conflict of interest

The authors declare to no competing financial interest.

Acknowledgement

This research was supported by the Dongkook Pharmaceutical Research Fund.

Supplementary information

Electronic Supplementary Information (ESI) is available [Table S1-S2 and Figure S1-S3]. See DOI: 10.5478/MSL.2023.14.4.178

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