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Application News

Liquid Chromatograph Mass Spectrometer LCMS-8060

Selective and Sensitive Method for Estimation of Liraglutide in Human Plasma using Shimadzu LCMS-8060

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User Benefits

- Simple, novel and most sensitive method with LLOQ - 0.5 ng/mL
- Low plasma volumes in sample extraction extends the life of mass spectrometer
- Quick sample extraction method increased sample productivity

1. Introduction

Liraglutide is a glucagon-like peptide-1 receptor agonist (GLP-1 receptor agonist) also known as incretin mimetics. It works by increasing insulin release from the pancreas and decreases excessive glucagon release [(1)]. Liraglutide is a medication used for treatment of type 2 diabetes or obesity. The prolonged action of liraglutide is achieved by attaching a fatty acid molecule at one position of the GLP-1-(7-37) molecule, enabling it to both self-associate and bind to albumin with-in the subcutaneous tissue and blood stream. The active GLP-1 is then released from albumin at a slow, consistent rate. Albumin binding also results in slower degradation and reduced renal elimination compared to GLP-1-(7-37).

Following subcutaneous administration, a mean C max of 35 ng/mL was achieved after 8-12 hours of dosing with an absolute bioavailability of 55 %. It indicates that the method required for pharmacokinetic evaluations need to achieve a sensitivity limit of 0.50 ng/mL.

Such method should address many problems posed by peptides viz., poor ionization, non-specific adsorption, carry-over and low extraction recovery.

We have therefore developed a method with high chromatographic resolution, good sensitivity with lowest limit of quantification (LLOQ) of 0.50 ng/mL for liraglutide in human plasma using LCMS-8060. Method was developed keeping some key criteria in focus namely simpler extraction procedure, highly optimized chromatography and enhanced sensitivity. These factors enable selective and high-throughput analysis of liraglutide for the pharmacokinetic investigation.

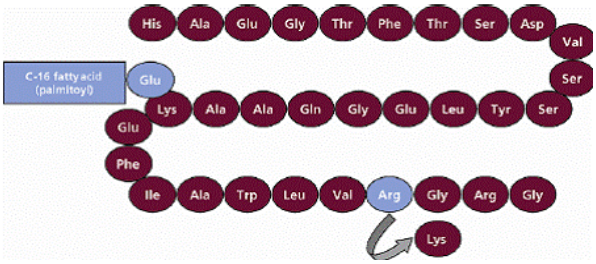

Fig. 1 Structure of Liraglutide [(2)]

2. Salient Features

- A rapid, sensitive, and high throughput method for quantification of liraglutide in human plasma
- Ready to use validated method easy to transfer to customers laboratories
- Single step SPE method increased sample throughput
- Heated ESI along with New UF-Qarray ion guide technology contributes by increasing ion production and enhancing transmission respectively. This ensures sensitive and selective quantification of liraglutide at 0.50 ng/mL.
- Less plasma volume (200 µL) avoided unnecessary wastage of plasma samples and at the same time increase the instrument life.

3. Method Validation

Liraglutide LCMS method was validated as per US major guidelines. All method validation parameters evaluated met the acceptance criteria. Summary of liraglutide method validation results are given in Table 1.

Table 1 Method Validation Summary

Calibration curve range		0.50 ng/mL to 202.70 ng/mL
Intraday precision and accuracy (For LLOQ-QC)	Accuracy (% Nominal)	102.63
	Precision (% CV)	14.52
Intraday precision and accuracy (For LQC, MQC and HQC)	Accuracy (% Nominal)	96.10 to 101.01
	Precision (% CV)	2.85 to 12.79
Global precision and accuracy (For LLOQ-QC)	Accuracy (% Nominal)	109.98
	Precision (% CV)	18.52
Global precision and accuracy (For LQC, MQC and HQC)	Accuracy (% Nominal)	102.40 to 106.78
	Precision (% CV)	7.45 to 13.09
Global % recovery	Recovery (%)	50.92
	Precision (% CV)	13.06

Note: LLOQ QC- Lower Limit of Quantification Quality Control, LQC- Lower Quality Control, MQC_ Middle Quality Control and HQC- Higher Quality Control

4. Experimental

4.1. Sample preparation and analytical conditions

- Samples were processed using positive pressure solid phase extraction. 200 µL of analyte spiked in plasma samples were pre-treated with 600 µL buffer and vortexed to mix. Samples were loaded on pre-conditioned SPE cartridges, washed with organo-aqueous solution and eluted in a solution of methanol-acetonitrile. This eluted solution was injected directly on a Shimadzu LCMS-8060 system.

4.2 Instrument parameters on LCMS-8060

Refer to the Table 2 for analytical conditions and instrumental parameters. MRM transitions are described in the Table 3.

4.2 Instrument parameters on LCMS-8060

Table 2 Analytical conditions and instrument parameters

Parameter	HPLC
Column	Shim-pack™ Velox Biphenyl 2.7 µm, 2.1 × 100 mm column (P/N: 227-32015-03)
Mobile Phase	A: 0.1% formic acid in water B: 0.1% formic acid in Acetonitrile
Flow Rate	0.25 mL/min
Oven Temp	40 ˚C
Injection volume	25 µL
Parameter	**MS**
Interface	ESI
Interface Voltage and temp	5 kV and 400 ˚C
MS Mode	MRM, Positive
Heat Block Temp	400 ˚C
DL Temp	300 ˚C
CID Gas	270 kPa
Nebulizing Gas	3 L/min
Drying Gas	10 L/min
Heating Gas	10 L/min

Table 3 MRM transition and parameters of Liraglutide on LCMS

Compound	MRM (m/z)	CE (V)
Liraglutide	938.5-1128.4	-20.0

Fig. 2 Nexera™ X2 with LCMS-8060 system

5. Result and Discussion

5.1. Method Development

Liraglutide was dissolved in solvent consisting of 50/50 Acetonitrile/water. However poor linearity was observed in the range of 0.5 to 200 ng/mL when analyzing the working solution and we suspect that this was due to strong peptide adsorption. After enhancing the solvent strength, we got good linearity of the aqueous working solutions.

In this study, Shimadzu LCMS-8060 was selected to quantify liraglutide in human plasma. The m/z 938.5 was selected as a precursor with 4+ charge state. Many ion fragments with similar intensities were produced under a certain collision energy. Initially several possible ion fragments (m/z 1064, m/z 1129, m/z 1185 and m/z 523 were selected for investigation. .After careful MRM optimization and pre-analysis of biological samples, the transition m/z 938.5 → 1128.4 which showed high intensity and minimum interference was finally selected to quantify liraglutide in human plasma. Refer Table 3 for optimized MRM.

Optimization of chromatographic conditions was simple and focused on evaluating the composition of the mobile phase, buffer and type of analytical column.

Based on previous research experience, we found acetonitrile was more suitable than methanol as the organic phase and the presence of formic acid was beneficial for the sensitivity of liraglutide. Final mobile phase comprised of 0.1% formic acid in acetonitrile and 0.1% formic acid in water. Several HPLC columns were evaluated for better peak shape. However, Shimadzu's Shim-pack Velox Biphenyl column (100 x 2.1 mm, 2.7um pore diameter) provided minimum matrix interference and a good chromatographic resolution and sharp peak shape. The analysis time was set to 10.0 mins. Refer Fig. 3 for liraglutide extracted blank and extracted LLOQ chromatograms. Signal to noise of liraglutide at LLOQ level was found S/N > 20.

In terms of sample preparation, SPE was the only means tested. In our experience with quantitative bioanalysis of peptides in particular, the use of SPE represents the opportunity for the most selective extraction technique, the most tunable and most amenable to truly rugged methodology.

In any case, considering the emphatically polar nature of Liraglutide, it was easily predictable that any format of liquid–liquid extraction would afford negligible recovery. Protein precipitation with organic solvent may afford decent recovery, but this technique is highly unselective, only eliminating the largest proteins from the sample and leaving a myriad of potential interferences that will experience an equal concentration effect to what the analyte and internal standard experience during sample preparation

In a method where it is known that an extensive concentration of test article in a sample extract will be required in order to attain sufficient sensitivity for a given purpose, protein precipitation becomes all the less feasible in the context of matrix effect. Besides, our established LC limitation of phosphatidylcholines non-elution within a single gradient cycle meant that it would be required for the sample extraction to provide this selectivity. Selective over-retention of these interferences is readily achievable on SPE sorbents that involve reversed-phase retention and where optimization is performed fully and adequately.

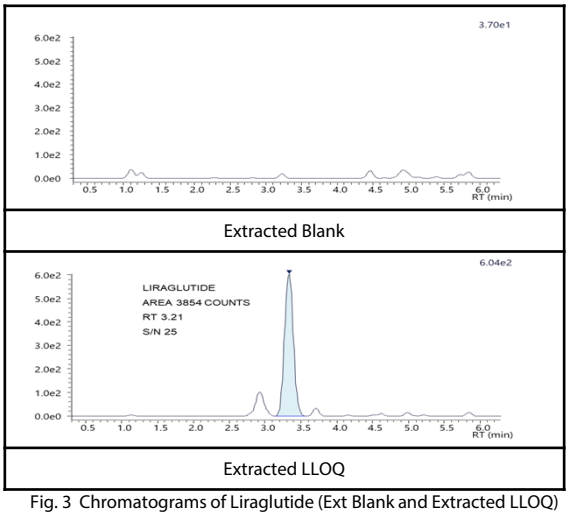

Fig. 3 Chromatograms of Liraglutide (Ext Blank and Extracted LLOQ)

5.2. Method Validation

- **Selectivity**

  Selectivity of the method was evaluated by extracting and analyzing 6 different lots of blank human plasma. Blank matrices from six different lots showed no significant interference at the retention time and MRM transition of liraglutide. Results are presented in Table 4.

Table 4 Selectivity

Plasma lot no.	Liraglutide		
	Area in blank matrix	LLOQ area	% Interference
V2533	335	1,699	19.72
V2528	169	3,597	4.70
P8072	54	2,880	1.88
V2534	510	2,842	17.95
V2537	87	2,049	4.25
P8200	189	3,961	4.77

- **Linearity**

  A linear equation was judged to produce the best fit for the concentration vs area response relationship. The regression type was 1/ Concentration^2 and peak area ration for an 8-points calibration curve was found linear from 0.5 to 202.70 ng/mL for liraglutide. The goodness of fit (r^2) was consistently greater than 0.99 during course of validation. Refer to Fig. 4 for a calibration curve.

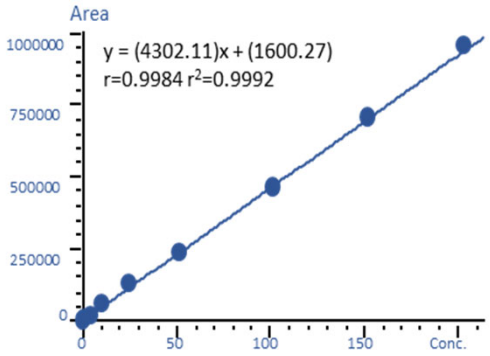

Fig. 4 Calibration curve

- **Intra-day accuracy and precision**

  Intraday accuracy and precision of liraglutide was determined for lowest limit of quantification (LLOQ QC), low (LQC), medium (MQC) and high (HQC) concentration of quality control samples in the biological matrix based on the expected range. Accuracy for intra-day was within 85-115% of the nominal value for all quality control samples except for LLOQ QC which was within 80-120%. For precision, the %CV was ≤ 15% for all quality control samples, except LLOQ QC which was ≤ 20%. Quantitative data is summarized in Table 5

Table 5 Intra-day accuracy and precision

Intra-day (n=6)			
Nominal Conc (ng/mL)	Observed Conc (ng/mL)	Accuracy (%)	Precision (%CV)
LLOQ QC (0.50 ng/mL)	0.51	102.63	14.52
LQC (1.52 ng/mL)	1.54	101.01	12.79
MQC (25.39 ng/mL)	24.40	96.10	2.85
HQC (152.03 ng/mL)	149.40	98.27	4.92

- **Global precision and accuracy**

  Inter-day accuracy and precision of liraglutide was evaluated on 3 PA batches. Accuracy for inter-day was within 85-115% of the nominal value for all quality control samples except for LLOQ QC which was within 80-120%. For precision, the %CV was ≤ 15% for all quality control samples, except LLOQ QC which was ≤ 20%. The results are presented in Table 6 for 18 QCs at each level, analyzed over 3 batches.

Table 6 Global precision and accuracy

Inter-day (n=18)			
Nominal Conc (ng/mL)	Observed Conc (ng/mL)	Accuracy (%)	Precision (%CV)
LLOQ QC (0.50 ng/mL)	0.55	109.98	18.52
LQC (1.52 ng/mL)	1.62	106.78	13.09
MQC (25.39 ng/mL)	26.00	102.40	7.45
HQC (152.03 ng/mL)	158.18	104.04	12.87

- **Recovery**

  Recovery experiments was evaluated by comparing peak area response of extracted QC samples with post extracted QC samples at three concentrations (LQC, MQC and HQC). Post extracted QC samples represent 100% recovery. The global recovery of liraglutide was 50.92%. Global recovery results are presented in Table 7. Recovery for liraglutide was found precise, consistent and reproducible at all levels.

Table 7 Recovery

QC level	Recovery
LQC (n=6)	43.24
MQC (n=6)	54.54
HQC (n=6)	54.97
Mean	**50.92**
SD	**6.65**
% CV	**13.06**

- **Matrix effect**

  Matrix factor was evaluated by comparing peak area ratio in presence of matrix ions with mean peak area ratio In absence of matrix ions.

  Un-extracted blank quality control samples were prepared from six different human blank plasma batches and processed, followed by reconstitution with aqueous LQC and HQC samples. Single injection was given from each lot. Peak area ratio in presence of matrix ions was obtained from these un-extracted LQC and HQC samples.

  The mean peak area ratio in absence of matrix ions was calculated from the results obtained from total recovery.

  Precision of LQC and HQC samples was 3.69% and 3.02% respectively, which was within the acceptance criteria of ≤15% .Representative data of matrix factor is shown in Table 8. The results confirm the suitability of the method for quantitative estimation of liraglutide in human plasma.

Table 8 Matrix effect

Liraglutide	Aqueous sample	Post extracted sample	Matrix factor
LQC	6,217	5,983	0.96
	5,883	5,777	0.98
	5,746	5,372	0.93
	6,024	5,723	0.95
	6,343	6,232	0.98
	5,718	5,936	1.04
Mean			**0.97**
SD			**0.04**
%CV			**3.69**

Liraglutide	Aqueous sample	Post extracted sample	Matrix factor
HQC	5,43,668	5,22,379	0.96
	5,81,895	5,76,332	0.99
	6,36,429	6,06,953	0.95
	6,19,884	5,61,864	0.91
	6,03,813	5,89,440	0.98
	5,81,429	5,50,638	0.95
Mean			**0.96**
SD			**0.03**
%CV			**3.02**

- **Carry-over effect**

  Carryover was evaluated by injecting extracted samples in the sequence of extracted blank, extracted highest calibrator, extracted blank and extracted lowest calibrator. No carryover was observed at the retention time and MRM transition of liraglutide in the extracted blank sample following the highest standard calibrator.

6. Conclusion

A Bioanalytical LCMS method has been successfully developed and validated for quantification of liraglutide in human plasma as per US major guidelines. This method is applicable for the determination of liraglutide in human plasma over the range of 0.50 to 202.70 ng/mL with a validated lower limit of quantification of 0.50ng/mL. LCMS-8060, along with special sample preparation and optimized chromatography provides a very selective and sensitive method for bioanalysis of liraglutide study samples in human plasma. Ultra-high speed and high-separation analysis was achieved on Nexera™ X2 UHPLC by using a simple mobile phase at a minimal gradient flow rate of 0.250 mL/min. By providing these ready to use solutions, we partner with your labs to achieve desired results in your scientific endeavors.

7. References

1. https://en.wikipedia.org/wiki/Liraglutide (accessed Jan 02, 2020)
2. https://www.rxlist.com/victoza-drug.htm (accessed Jan 02, 2020)

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