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Application News

Liquid Chromatograph Mass Spectrometer LCMS-8060

Highly Sensitive Single LC-MS/MS Method for Cleaning Validation of Synthetic Peptides Using Shimadzu LCMS-8060

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User Benefits

- Six synthetic peptides quantified using a single LC-MS/MS method with LLOQ of 0.5 ng/mL
- Customized analytical method for peptides of different therapeutic categories
- Non-specific binding and low sensitivity issues addressed to make the method compatible for biological sample analysis

1. Introduction

As per supplementary guidelines on good manufacturing practices by WHO - "Cleaning validation is required as documented evidence to establish that cleaning procedures are removing residues to predetermined levels of acceptability, taking into consideration factors such as batch size, dosing, toxicology and equipment size". The objectives of good manufacturing practices (GMP) include the prevention of possible contamination and cross-contamination of pharmaceutical starting materials and products. Pharmaceutical products can be contaminated by a variety of substances such as contaminants associated with microbes, previous products (both active pharmaceutical ingredients (API) and excipient residues), residues of cleaning agents, airborne materials, such as dust and particulate matter, lubricants and ancillary material, such as disinfectants, and decomposition residues from:
A rapid, simple, sensitive, single method for,

- Product residue breakdown occasioned by, e.g. the use of strong acids and alkalis during the cleaning process; and
- Break down products of the detergents, acids and alkalis that may be used as part of the cleaning process.

Adequate cleaning procedures play an important role in preventing contamination and cross-contamination. Validation of cleaning methods provides documented evidence that an approved cleaning procedure will provide clean equipment, suitable for its intended use [(1)].
The analytical methods required for cleaning validation of active pharmaceutical ingredients require a different approach for development of different drug substances. In case of critical pharmaceutical products like peptides, these methods has to address the challenges posed by peptides viz poor ionisation, non-specific adsorption and carry-over. Shimadzu Application Development Centre (ADC) has developed a highly sensitive LC-MS/MS method for quantitation of six synthetic peptides i.e., abaloparatide, glucagon, cosyntropin, semaglutide, ganirelix acetate and liraglutide. The developed method is applicable to detect an LLOQ of 0.50 ng/mL for all 6 analytes and can be utilized for conducting the cleaning validation studies.

2. SALIENT FEATURES

- A rapid, simple, sensitive, single method for estimation of six synthetic peptides is developed to support the cleaning validation studies.
- 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 at 0.50 ng/mL
- Customized flow with gradient method satisfied the peak shape, retention time and background noise

3. Experimental

3.1 Sample preparation and analytical conditions

A primary stock solution of 1 mg/mL of each individual peptide was prepared by dissolving the 10 mg of working standard in a 10 mL of mixture containing DMSO: Methanol (10 : 90, v/v) in a clean and dry volumetric flask. Subsequent intermediate stock solution of 1 µg/mL was prepared in diluent containing Methanol : Water - (50 : 50, v/v).

Calibration curve standards were prepared in acetonitrile: water (50 : 50, v/v) containing 1.0% formic acid at nominal concentrations ranging from 0.50 - 250.00 ng/mL for abaloparatide & cosyntropin and 0.50-500.00 ng/mL for ganirelix, glucagon, liraglutide & semaglutide respectively.

An analytical investigation was performed using a Shimadzu LCMS-8060 mass spectrometer system (Fig.1). Chromatographic separation was carried out using a Shim-pack Velox™ column with a set column temperature of 60 °C. The mobile phases consisted of 1.0% formic acid in water (phase A) and 1.0% formic acid in acetonitrile (phase B). A gradient elution method was employed, starting at 0% B and reaching 100% B after 10 minutes, with a flow rate of 0.20 ml/min. Subsequently, the gradient was reset to 0% B within the following 0.50 minutes, and followed by a 1.00 minute equilibration step at 0% B.

The ionization of the molecule was carried out using electrospray ionization (ESI) in positive-ion mode, with an interface temperature and voltage set at 300 °C and 4 kV respectively. Mass spectrometry parameters were fine-tuned by introducing a solution of the analytes at a concentration of 1 ng/ml through the LC column into the ion source, and subsequently optimizing the MS parameters manually using LabSolutions™ software. The mass spectra for the synthetic peptides were recorded within the mass range of *m/z* 100 to 1200. The compounds were differentiated based on their characteristic product ions. The acquired data was processed using LabSolutions software, and calibration curves were generated by utilizing the analyte peak area and fitting them with a linear regression model.

3.2. Instrument parameters on LCMS-8060

Refer to Table 1 for analytical conditions and instrument parameters and Table 2 for MRM transition.

Table 1 Analytical conditions and instrument parameters

Parameter	HPLC
Column	Shim-pack Velox™ C18 column 100 x 2.1 mm, 2.7 µm, (P/N: 227-32009-03)
Mobile Phase	A: 1.0 % formic acid in water. B: 1.0 % formic acid in Acetonitrile
Flow Rate	0.20 mL/min
Oven Temp	60 ℃
Injection volume	40 µL
Parameter	**MS**
Interface	ESI
Interface temp and Voltage	300 ℃ and 4 kV
MS Mode	MRM, Positive
Heat Block Temp	400 ℃
DL Temp	250 ℃
Nebulizing Gas	3 L/min
Drying Gas	10 L/min
Heating Gas	10 L/min

Table 2 MRM transition and parameters of synthetic peptides on LCMS

Compound	MRM (*m/z*)	CE (V)
Abaloparatide	661.00-309.15	-37.0
Cosyntropin	587.20-223.10	-23.0
Ganirelix	524.20-170.05	-37.0
Glucagon	871.15-225.10	-37.0
Liraglutide	938.8-1128.60	-35.0
Semaglutide	1029.15-136.20	-41.0

Fig. 1 Nexera™ X2 with LCMS-8060 system

4. Result and Discussion

4.1. Optimization of ESI-MS/MS parameters

Regarding the mass spectrometry optimization, six synthetic peptides were subject to tuning using the LCMS-8060 system in positive ion mode. The fragmentation pattern and mass dependent parameters for each of the six synthetic peptides is presented in Table 2. The protonated precursor ion (Q1) of the synthetic peptides exhibited multiple charged states with corresponding *m/z* values of 661.00 (abaloparatide), 587.20 (cosyntropin), 524.20 (ganirelix), 871.15 (glucagon), 938.8 (liraglutide), and 1029.15 (semaglutide). Amongst the full scan of protonated precursors, the most dominant charge state Q1 masses were +6 (abaloparatide), +5 (cosyntropin), +4 (ganirelix), +3 (glucagon), +4 (liraglutide), and +4 (semaglutide). The product ion scan displayed predominant fragment ions at respective *m/z* values of 309.15 (abaloparatide), 223.10 (cosyntropin), 170.05 (ganirelix), 225.10 (glucagon), 1128.60 (liraglutide), and 136.20 (semaglutide). Detailed information on the multiple reaction monitoring (MRM) transitions utilized for the analysis can be found in Table 2.

4.2 Optimization of LC parameters

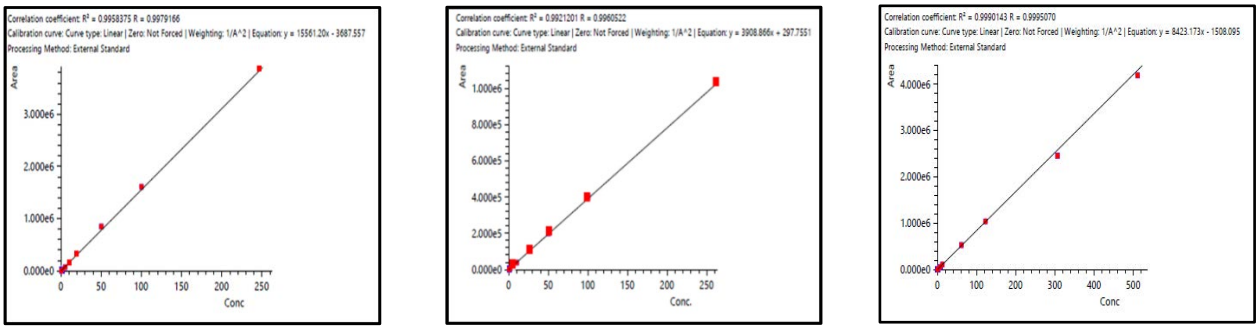
To optimize the chromatographic conditions, including the selection of column type, mobile phase composition, and nature, several trials were conducted to enhance retention and signal of the six synthetic peptides. The best chromatographic separation for simultaneous quantification of the six synthetic peptides in a single run was successfully achieved using customized gradient flow conditions on a Shim-pack Velox C18 column. To obtain well-defined peaks, the injection volume was optimized at 40 µL and the column oven temperature was set at 60 ℃.

- **Linearity**

  Linearity experiments was evaluated by analysing calibration curves of abaloparatide, cosyntropin, ganirelix, glucagon, liraglutide and semaglutide. The standard curves were calculated by a weighted ($1/x^2$) least squares linear regression method through the measurement of the peak-area. The correlation coefficient (r^2) for all the six peptides was found more than 0.98 as shown in Figure 2. Calibration curve was found linear in the range of 0.50 - 250.00 ng/mL for abaloparatide and cosyntropin and 0.50 - 500.00 ng/mL for ganirelix, glucagon, liraglutide and semaglutide, respectively.

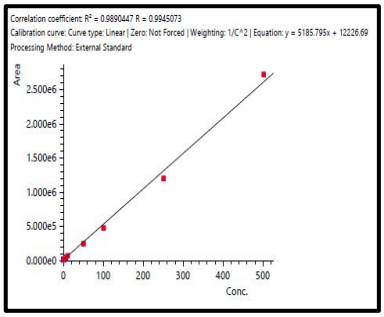
Table 3 Calibration curve data

Analyte	Calibration curve range (ng/mL)	Weighing factor	Correlation coefficient (r^2)	Accuracy (%)	SNR of LLOQ (0.5 ng/mL)
Abaloparatide	0.50-250.00	$1/x^2$	0.9958	98 to 101	25
Cosyntropin	0.50-250.00	$1/x^2$	0.9921	99 to 105	22
Ganirelix	0.50-500.00	$1/x^2$	0.9990	95 to 99	15
Glucagon	0.50-500.00	$1/x^2$	0.9890	96 to 103	18
Liraglutide	0.50-500.00	$1/x^2$	0.9979	98 to 106	35
Semaglutide	0.50-500.00	$1/x^2$	0.9989	97 to 102	19

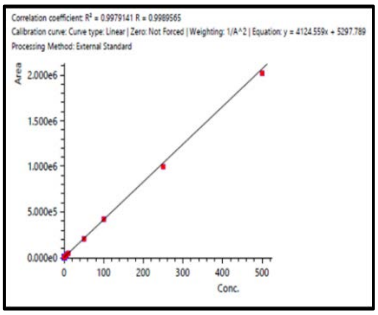


(a) Abaloparatide (b) Cosyntropin (c) Ganirellix

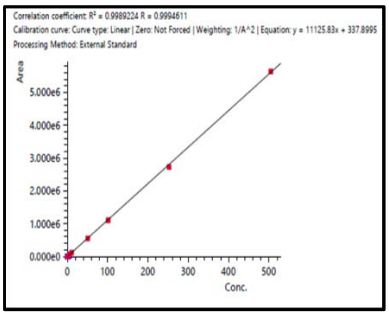
Fig. 2 Representative Calibration Curve



(d) Glucagon



(e) Liraglutide



(f) Semaglutide

Fig. 2 Representative Calibration Curve (continued….)

5. Conclusion

A highly selective and sensitive single LCMS method has been developed with the LLOQ of 0.50 ng/mL, which is more than an order of magnitude (refer to Fig.3). The method uses simple and customized gradient program. The developed method is sensitive enough to support the cleaning validation of regulatory studies. Shimadzu LCMS-8060, along with optimized chromatography provides a very selective and sensitive method for simultaneous quantification of abaloparatide, cosyntropin, ganirelix, glucagon, liraglutide and semaglutide. Ultra-high speed and high-separation analysis was achieved on Nexera X2 UHPLC by using a simple mobile phase at a gradient flow rate of 0.200 mL/min. By providing these ready to use solutions, we partner with your labs to achieve desired results in your scientific endeavors.

6. References

1. https://www.who.int/medicines/areas/quality_safety/quality_assurance/SupplementaryGMPValidationTRS937Annex4.pdf?ua=1

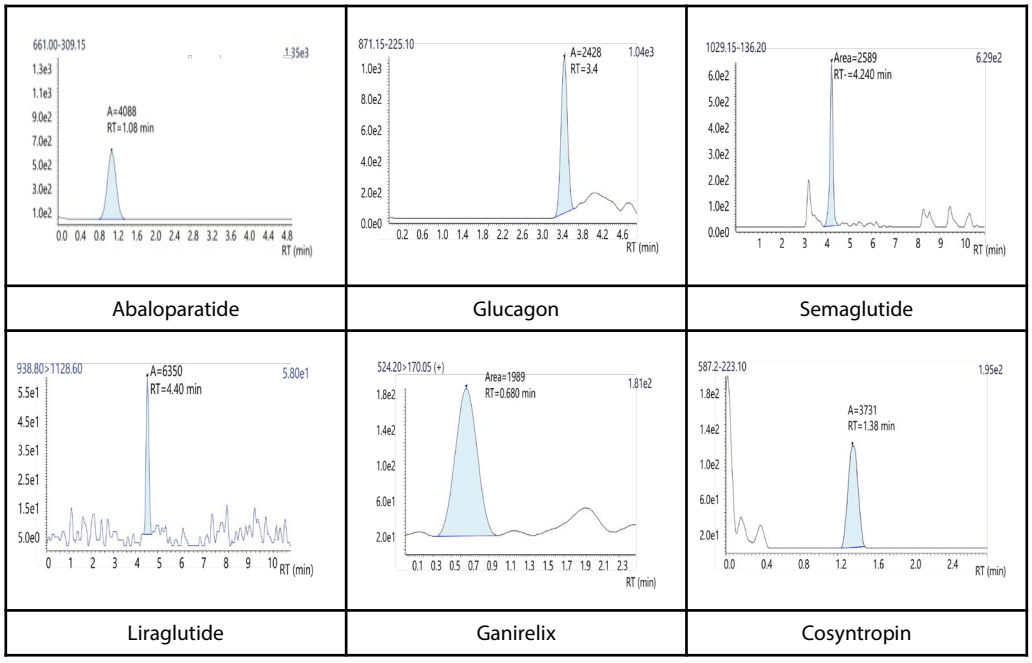


Fig. 3 Chromatograms

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