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Pharma

Advancing anti-obesity drug development with robust quantitation of GLP-1 analogs using human plasma

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Keywords

GLP-1, glucagon-like peptide-1, semaglutide, liraglutide, TSQ Certis MS, Vanquish UHPLC, Hypersil GOLD Peptide column, SOLAμ SPE, SAX, Chromeleon software, bioanalysis, quantitation, diabetes, obesity, SRM

Application benefits

- Sensitive and reproducible quantitation of semaglutide and liraglutide using human plasma by an LC-SRM method using the Thermo Scientific™ TSQ Certis™ Triple Quadrupole Mass Spectrometer coupled to the Thermo Scientific™ Vanquish™ Horizon UHPLC System
- Consistent recovery of semaglutide and liraglutide extracted from human plasma using Thermo Scientific™ SOLAμ™ SAX SPE Plates
- Minimal carryover for the bioanalysis of semaglutide and liraglutide using the Thermo Scientific™ Hypersil GOLD™ Peptide Column
- A compliance-ready Thermo Scientific™ Chromeleon™ Software solution for bioanalysis of GLP-1-like therapeutic peptides using human plasma

Goals

- Demonstrating the use of a liquid chromatography coupled to mass spectrometry (LC-MS) method for reliable, sensitive, and accurate quantitation of semaglutide and liraglutide using human plasma
- Developing a solid phase extraction (SPE) process for reproducible extraction of semaglutide and liraglutide using human plasma as a matrix
- Demonstrating minimal carryover, below 20% of the lower limit of quantitation (LLOQ), for the bioanalysis of semaglutide and liraglutide

Introduction

Recently, there has been considerable growth in glucagon-like peptide-1 (GLP-1) drug development, driven by their effectiveness for both type 2 diabetes and weight loss,[1] leading to significant demand for bioanalytical solutions to support their development and monitoring. Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) has become the preferred platform for quantifying GLP-1 analogs using biological matrices due to its high specificity, sensitivity, and dynamic range. However, developing a robust LC-MS/MS method for GLP-1 analogs requires careful orchestration of sample preparation, chromatographic separation, and mass spectrometric detection to overcome matrix complexity, peptide instability, and adsorption losses.

To address the above challenges, we have developed a complete bioanalytical solution for two prominent GLP-1 analogs, semaglutide and liraglutide, using human plasma. The solution includes the following:

- A robust extraction protocol using a mixed mode, strong anionic exchange (SAX) SPE plate for consistent recovery of semaglutide and liraglutide
- A high fidelity separation using a low-adsorption column tailored specifically for therapeutic peptides
- A highly sensitive triple quadrupole mass spectrometer for reliable and reproducible detection of both GLP-1 analogs extracted using human plasma.

Experimental

Sample preparation

Semaglutide and liraglutide were obtained from Adipogen Life Sciences™. The 1 mg/mL stock solutions and all calibration standards ranging from 0.5 to 1,000 ng/mL were prepared in pure methanol. All samples were extracted using an optimized protocol utilizing protein crash followed by SPE using SOLAμ SAX 96-well plates as shown in Figure 1. Liraglutide was used as an internal standard for the analysis of semaglutide, and vice versa. In both cases, the internal standard was kept at 10 ng/mL.

For recovery evaluation, two types of samples were prepared with 0.05, 0.1, and 0.2 ng/mL concentrations of both GLP-1 analogs. Experimental samples were prepared in the same way as detailed in the protocol. Theoretical samples were made by extracting 200 µL human plasma without adding the standards. After elution in low protein bind 96-well plates, the standards were added to the extracted sample to mimic 100% recovery. The % recovery was calculated by taking the measured peak area ratio of quantifier ions (e.g., *m/z* of 1064 for liraglutide, and *m/z* of 1238.4 for semaglutide) from experimental samples over the theoretical samples, and the ratio was multiplied by 100%.

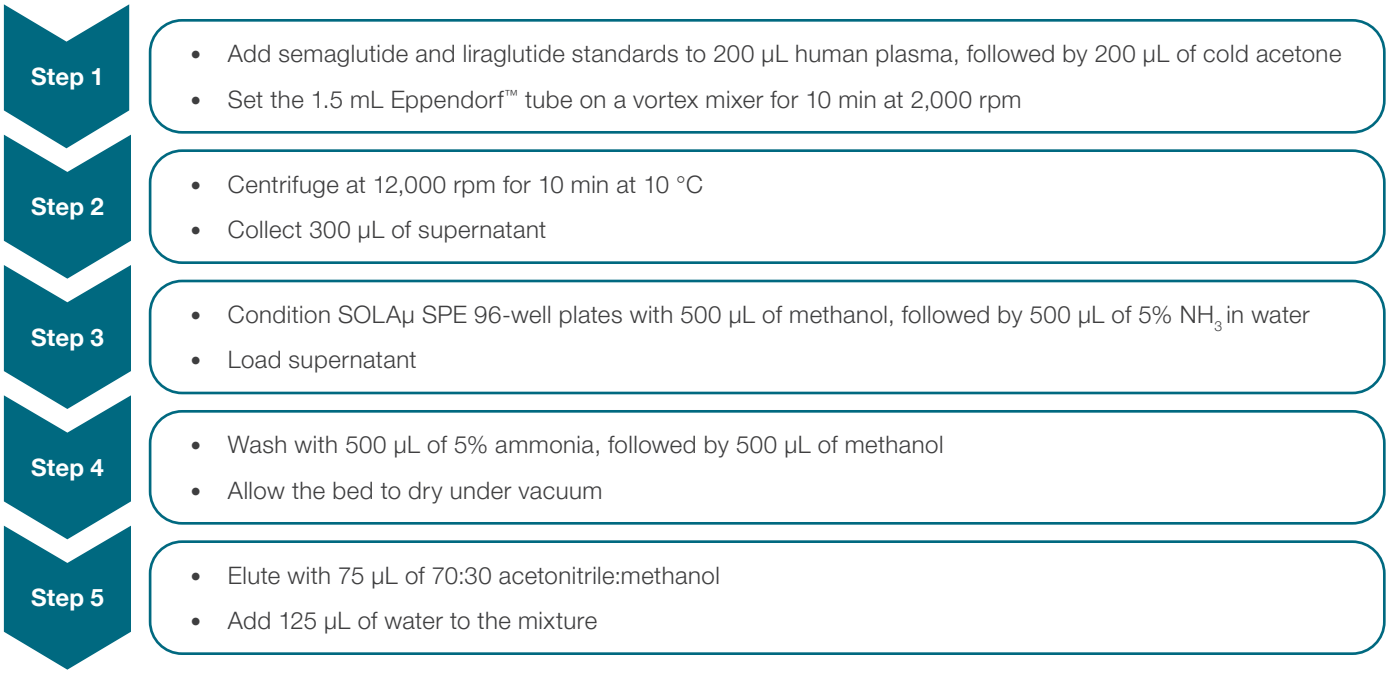


Figure 1. SPE protocol for the extraction of GLP-1 analogs in 200 µL of human plasma. SOLAμ SAX 2 mg/1 mL 96-well fixed plates (Cat. No. 60209-003) were used for all extractions.

Chromatography

Table 1 details the Vanquish UHPLC system conditions used for the separation of GLP-1 analogs.

Table 1. LC and autosampler conditions for separation of GLP-1 analogs.

Vanquish UHPLC system settings		
HPLC column	Hypersil GOLD Peptide, 2.1 x 50 mm, 1.9 µm (Cat. No. 26002-052130)	
Flow rate	0.25 mL/min	
Solvent A	0.4 % formic acid in water (v/v)	
Solvent B	0.1 % difluoro acetic acid in acetonitrile (v/v)	
Gradient	Time (min)	%B
	0	35
	5	60
	5.5	95
	7.5	95
	8	35
	10	35
Injection volume	20 µL	
Needle wash	After draw, 30 µL/s for 10 s with 30% methanol	
Column temperature	60 °C	
Divert to source	2.5–6.0 minutes	

Mass spectrometry

Table 2 provides the MS ion source and scan parameter settings applied, and Table 3 specifies the SRM transitions for the analysis of GLP-1 analogs on the TSQ Certis MS.

Table 2. MS ion source and scan parameter settings for the analysis of GLP-1 analogs.

Source settings	
Positive ion (V)	4,100
Sheath gas (Arb)	35
Aux gas (Arb)	10
Sweep gas (Arb)	0
Ion transfer tube temperature (°C)	200
Vaporizer temperature (°C)	225
Scan settings	
Scan mode	SRM
Q1 resolution (FWHM)	1.2
Q3 resolution (FWHM)	1.2
Collision gas pressure (mTorr)	1.5
Source fragmentation (V)	0
Cycle time (s)	0.6

Table 3. SRM transitions for the analysis of GLP-1 analogs. *m/z* of 1064 represents the quantifier ion (Quan) for liraglutide, and *m/z* of 1238.4 represents the quantifier ion for semaglutide. The others are confirming ions (Conf1 and Conf2) for the analysis of liraglutide and semaglutide.

Compound	Start time (min)	End time (min)	Precursor (*m/z*)	Product (*m/z*)	Collision energy (V)	RF lens (V)
Liraglutide	3	6	938.8	1064.0 (Quan)	30	150
Liraglutide	3	6	938.8	1128.8 (Conf1)	30	150
Liraglutide	3	6	938.8	1185.4 (Conf2)	30	150
Semaglutide	3	6	1029.4	1238.4 (Quan)	35	150
Semaglutide	3	6	1029.4	1302.8 (Conf1)	35	150
Semaglutide	3	6	1029.4	1359.4 (Conf2)	35	150

Software

Chromeleon software, version 7.4 was used for all instrument control, data acquisition, processing, and reporting.

Results and discussion

Consistent recovery of semaglutide and liraglutide using human plasma as a matrix with the SOLAµ SPE 96-well plate

Semaglutide and liraglutide were extracted using human plasma as a matrix with a SOLAµ SAX 2 mg 96-well plate. The entire sample preparation process takes less than 1 hour and is automated when combined with a positive pressure dispenser for high-throughput sample cleanup. By reducing the elution solvent volume to 75 µL and eliminating the drying step after elution, we have achieved an average of 62% extraction recovery with high reproducibility for both GLP-1 analogs. This recovery was consistent for all tested concentrations ranging from 0.05 to 0.2 ng/mL as shown in Table 4.

Table 4. % extraction recovery evaluation for both GLP-1 analogs extracted using human plasma as a matrix with a SOLAμ SAX 2 mg 96-well plate. Three replicates with three concentrations (0.05, 0.1, and 0.2 ng/mL) were examined.

Concentration (ng/mL)	% recovery semaglutide	% recovery liraglutide
0.05	56	58
	50	63
	53	61
0.1	67	63
	64	68
	62	65
0.2	63	65
	65	70
	60	66

Highly sensitivity quantitation of semaglutide and liraglutide using human plasma as a matrix with the LC-SRM method

All samples were evaluated using a selective reaction monitoring (SRM) scan mode on the TSQ Certis MS. Despite multiple high intensity fragments (>50% abundance) obtained from higher-energy collisional dissociation of the precursors (e.g., *m/z* of 938.8 for liraglutide and *m/z* of 1029.4 for semaglutide), the most intense fragment ion (e.g., *m/z* of 1064 for liraglutide and *m/z* of 1238.4 for semaglutide) was selected for the quantitation while the others were selected for confirmation. Table 3 shows the list of quantitation and confirming ions for both GLP-1 analogs. Figure 2 demonstrates selective quantitation of 0.05 and 0.1 ng/mL semaglutide and liraglutide extracted using human plasma as a matrix against blank plasma matrix. Using the LC-SRM method on the TSQ Certis MS, a lower level of quantitation (LLOQ) of 0.05 ng/mL was achieved for both GLP-1 analogs. Figure 3 shows example calibration plots for the bioanalysis of semaglutide and liraglutide, demonstrating at least 3.5 orders of linear dynamic range (LDR) with excellent precision (i.e., ≤15% RSD) and accuracy (i.e., ≤20% difference) for concentrations ranging from 0.05 to 100 ng/mL. The ability to accurately and consistently quantify these low levels of GLP-1 analogs will allow pharmaceutical companies developing anti-obesity therapeutics to precisely characterize drug levels, metabolites, and impurities for optimal drug development.

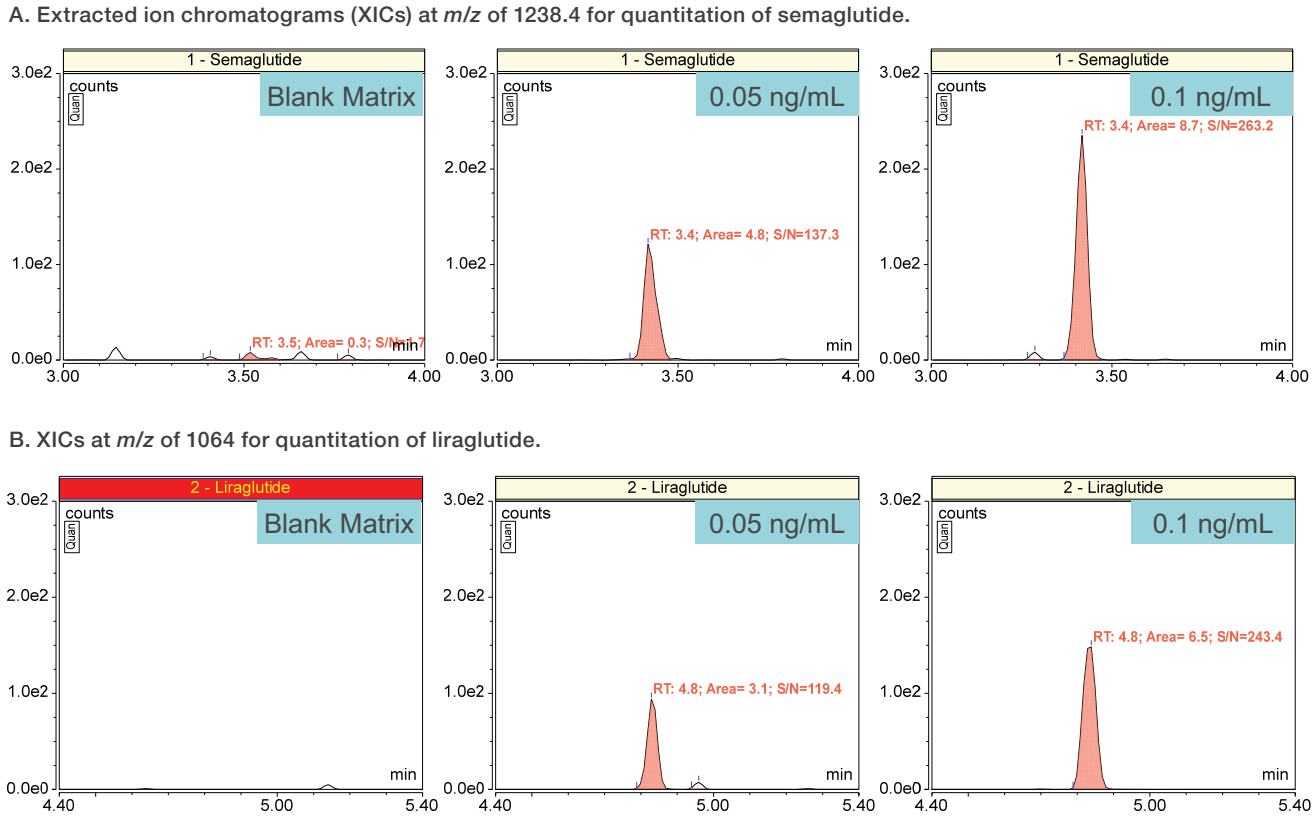
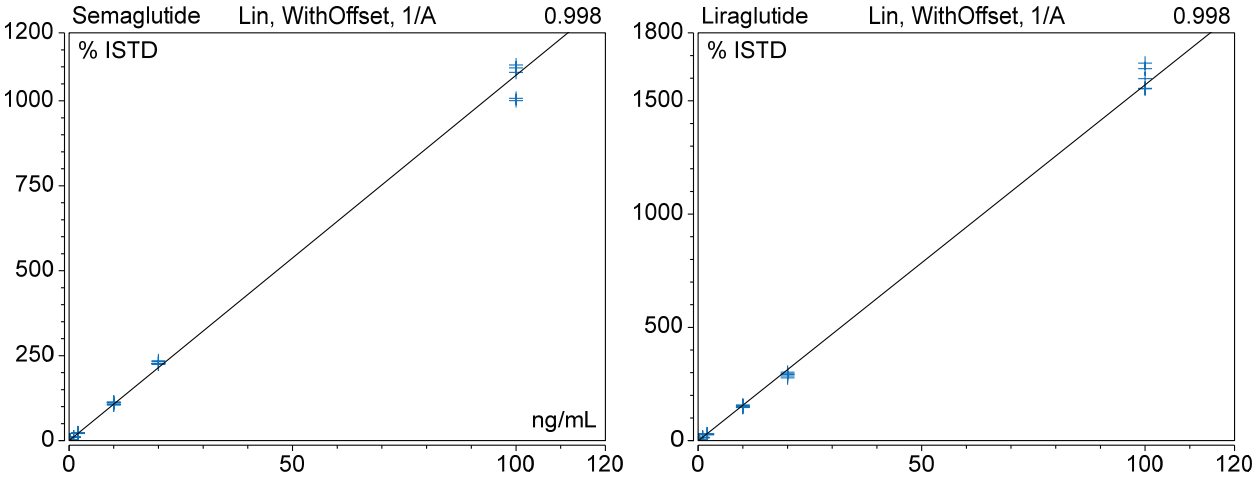


Figure 2. Selective quantitation of (A) semaglutide and (B) liraglutide using human plasma. Example extracted ion chromatograms (XICs) at *m/z* of 1238.4 and *m/z* of 1064 are shown for three extracted plasma samples: blank plasma, 0.05 ng/mL, and 0.1 ng/mL.

A. Example calibration curves for semaglutide and liraglutide showing 3.5 orders of LDR.



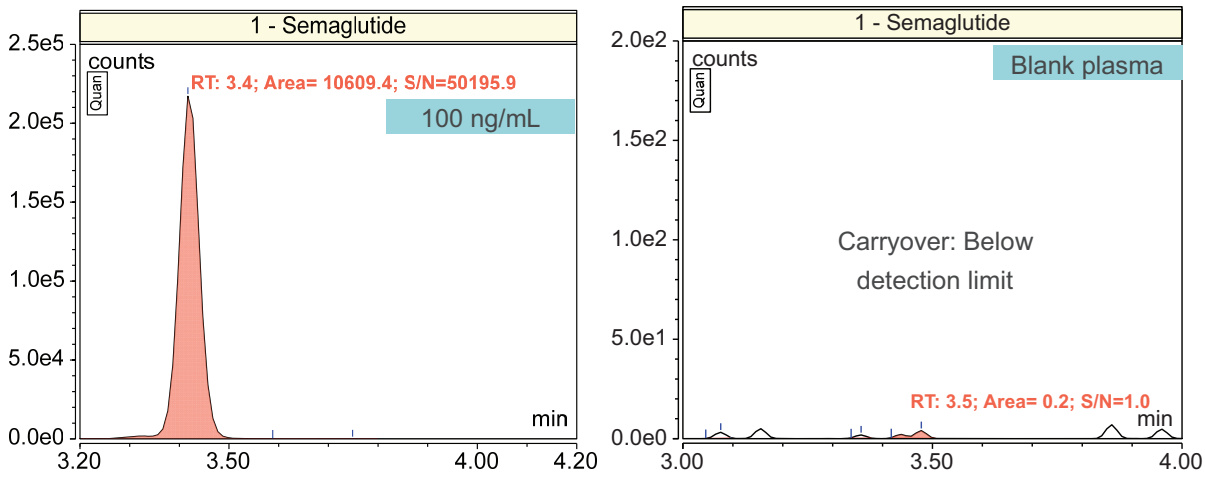
B. Example precision and accuracy measurements for concentrations ranging from 0.05 to 100 ng/mL of semaglutide and liraglutide using human plasma.

Concentration (ng/mL)	Semaglutide		Liraglutide	
	Accuracy (% Difference)	Precision (%Area RSD)	Accuracy (% Difference)	Precision (%Area RSD)
0.05	-0.1	13.6	14.5	5.3
0.1	-8.4	12.4	13.7	6.3
0.2	4.3	10.4	-3.4	5.4
1	-5.2	5.5	-8.2	5.3
2	2.4	3.9	-8.1	6.0
10	2.0	3.5	-3.1	2.6
20	6.5	1.9	-7.5	3.3
100	-1.5	4.8	2.0	3.2

Figure 3. Example calibration plots and precision and accuracy measurements for the analysis of semaglutide and liraglutide using human plasma. (A) Calibration curves were constructed by plotting the peak area ratio of quantitation ions (i.e., *m/z* of 1238.4 over *m/z* of 1064 and vice versa) against concentrations ranging from 0.05 to 100 ng/mL. (B) Average precision and accuracy measurements were obtained from at least four replicate injections for concentrations ranging from 0.05 to 100 ng/mL.

GLP-1 analogs are known to have non-specific binding issues, causing high system carryover. To evaluate the carryover, blank plasma matrix was injected following the 100 ng/mL sample, and the peak area of quantitation ion obtained from the blank plasma injection was compared to the LLOQ (i.e., 0.05 ng/mL). As shown in Figure 4, carryover was below 10% of the LLOQ for both GLP-1 analogs using the LC-SRM method and needle wash condition as layout in Table 1. The ability to ensure minimal carryover for these "sticky" peptides without performing additional wash cycles is critical for high-throughput analysis and meeting the regulatory requirements for bioanalytical method development.

A. XICs at *m/z* of 1238.4 in 100 ng/mL sample, followed by a blank plasma injection shown as an inset for carryover evaluation, and the peak area results are compared with the LLOQ sample.



B. XICs at *m/z* of 1064 in 100 ng/mL sample, followed by a blank plasma injection shown as an inset for carryover evaluation, and the peak area results are compared with the LLOQ sample.

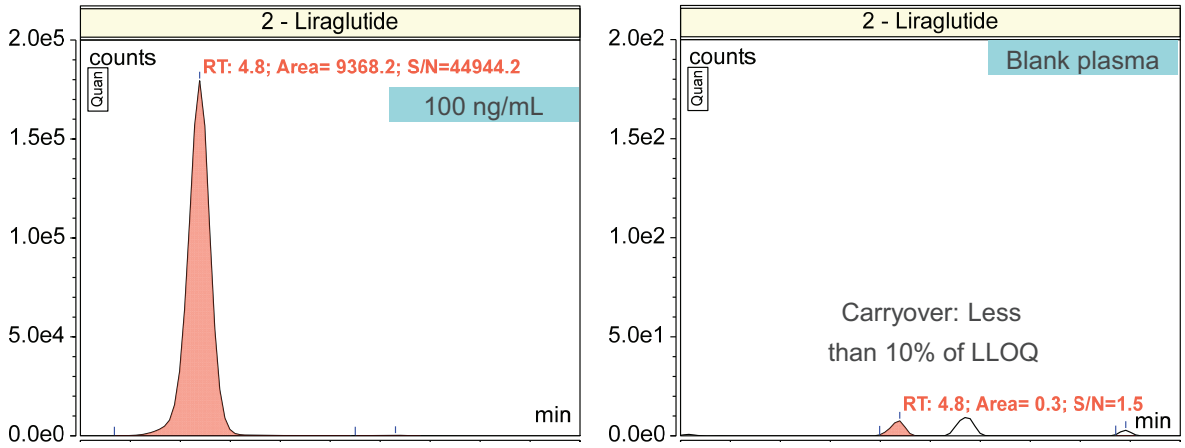


Figure 4. Carryover analysis for (A) semaglutide and (B) liraglutide extracted using human plasma. For both GLP-1 analogs, carryover was less than 10% LLOQ using the LC-SRM method.

Conclusions

Here, we present a comprehensive LC-MS solution, developed with the Vanquish UHPLC system coupled to the TSQ Certis MS with Chromeleon software, for highly sensitive quantitation of GLP-1 analogs in using human plasma as a matrix. The solution enables:

- Sensitive quantitation of semaglutide and liraglutide using human plasma with a LLOQ of 0.05 ng/mL
- Excellent precision and accuracy across three orders of LDR for the analysis of GLP-1 analogs
- Consistent, up to 70% recovery, of semaglutide and liraglutide using the SOLAμ SAX SPE plates
- Minimal carryover below 15% of the LLOQ for semaglutide and liraglutide using the Hypersil GOLD Peptide column

Reference

1. Drucker, D.J.; Habener, J.F.; Holst, J.J. Discovery, characterization, and clinical development of the glucagon-like peptides. *Journal of Clinical Investigation* **2017**, 127(12), 4217-4227. https://doi.org/10.1172/JCI97233

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