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Original Article

DEVELOPMENT AND VALIDATION OF A SPECIFIC AND UNIQUE DUAL POLARITY ESI-LC-MS/MS METHOD FOR SIMULTANEOUS DETERMINATION OF SEMAGLUTIDE AND DAPAGLIFLOZIN IN HUMAN PLASMA

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ABSTRACT

Objective: To develop a method capable of simultaneous quantification and estimation of semaglutide and dapagliflozin which are being studied as a prospective combination therapy for treating Diabetes.

Methods: An intricate protein precipitation extraction technique was employed using verapamil and tolbutamide as internal standards for semaglutide and dapagliflozin, respectively. The two compounds were separated on a Kinetex C18 (50 mm x 2.1 mm, 5 µ Particle size) column, with a dual polarity ionization Electro Spray Ionization (ESI) on a Liquid chromatograph Tandem Mass Spectrometry (LC-MS/MS) instrument. The detection was carried out with a Multiple Reaction Monitoring (MRM) method, and a gradient program utilizing Acetonitrile and water as mobile phases to achieve a separation in 3 min.

Results: The method established was proved linear over a working range of 1.00 ng/ml to 1000 ng/ml and 2.00 ng/ml to 2000 ng/ml for semaglutide (r^2>0.98) and dapagliflozin (r^2>0.98) respectively in human plasma. The accuracy, recovery, and matrix effects were within acceptable limits. The stability was also established under various conditions as necessitated by the International Council for Harmonisation of Technical Requirements of Pharmaceuticals for Human Use (ICH) M10 guideline on Bioanalytical method validation.

Conclusion: This highly selective and sensitive method where 1.00 ng/ml for semaglutide and 2.00 ng/ml for dapagliflozin as the Lower Limit of Quantification (LLOQ) can be utilized for estimation in human plasma will facilitate the further application to pharmacokinetic and bioequivalence studies for combination of these two drugs in pharmaceutical dosage forms.

Keywords: Semaglutide, Dapagliflozin, Electro spray ionization, Method validation, Mass spectrometer

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INTRODUCTION

Semaglutide is an Incretin mimetics class of anti-diabetes medication used in type-2 diabetes. It is a Glucagon-like peptide-1 receptor agonist. It helps in the release of insulin from the pancreas in instances where there are high blood glucose levels [1]. It is also used as an obesity medication for weight management as it reduces hunger and body fat. Semaglutide injection is not an alternative to Insulin and cannot be used in Type 1 diabetes or Diabetic Ketoacidosis. It also appears to enhance the growth of pancreatic β cells which are mainly responsible for Insulin production and release [2, 3]. The subcutaneous once-a-week administration was approved by the United States Food and Drug Administration (USFDA) in 2017 and the European Medicines Agency (EMA) in 2018 for Type 2 diabetes treatment [4-10]. Lee *et al.* have developed a method for estimating semaglutide in rat plasma and brain [11]. Dapagliflozin is a Gliflozin class drug, an oral glucose-lowering drug used for the treatment of Type 2 diabetes. It is categorized as a Sodium/glucose co-transporter 2 (SGLT2) inhibitor [12-16]. Inhibition of SGLT2 transporter results in the increased excretion of both Sodium and Glucose in the urine thereby also exerting its diuretic effect which results in body weight and blood pressure reduction. There are some reported Liquid chromatograph Tandem Mass Spectrometry (LC-MS/MS) methods for determining dapagliflozin in rat plasma [17-18]. Though quite a few bioanalytical methods are reported for semaglutide and dapagliflozin individually, there is no bioanalytical method reported so far for simultaneous estimation of semaglutide and dapagliflozin in human plasma employing LC-MS/MS.

As there is an unmet need for estimating semaglutide and dapagliflozin in human plasma, this combined bioanalytical method is developed and validated for the first time in human plasma and can be directly applied in clinical studies. This method was validated as per The USFDA Guidance on Bioanalytical method validation of 2018 [19] and the International Council for Harmonisation of Technical Requirements of Pharmaceuticals for Human Use (ICH) M10 guidelines on Bioanalytical method validation and study sample analysis [20].

MATERIALS AND METHODS

Reagents and chemicals

The Methanol and Acetonitrile were procured from J. T. Baker. Type 1 Milli-Q Water procured internally from the water purification system Millipore. Human Blood and Plasma lots were procured from Delta Laboratories. Anticoagulant Dipotassium Ethylene Diamine Tetraacetic acid (K2EDTA) was procured from Merck, semaglutide, dapagliflozin, verapamil, and tolbutamide Analytical Standards were obtained as gift samples.

Instrumentation

The chemicals were weighed using a Mettler Toledo microbalance MX5. The solutions were degassed with an ultrasonicator. Ultra performance Liquid chromatography equipment (Shimadzu Nexera) coupled to a Sciex 5500 LC-MS/MS was used for Chromatography and Centrifuge Sorvall, and a Vortex mixer were used for extraction. This study employed a novel dual polarity Multiple Reaction Monitoring (MRM) method in mass spectrometry, which was not explored previously.

Preparation of stock and working solutions for analyte and internal standard

Stock solutions of semaglutide and dapagliflozin (2 mg/ml) were prepared by dissolving 10 mg in a 5 ml volumetric flask. These stocks were prepared separately in Methanol. The calibration standards were prepared from the first stock of both analytes and quality control samples were prepared from the respective second stocks. The Internal standards stocks of verapamil and tolbutamide

were prepared by dissolving 5 mg in a 5 ml volumetric flask with Methanol to prepare a 1 mg/ml stock. The intermediate stocks were followed by spiking solutions containing semaglutide and dapagliflozin (20 to 20000 and 40 to 40000 ng/ml respectively) prepared using a serial dilution approach using 50% methanol in water. 125 µl** of internal standard stock solution was added to a 50 ml measuring flask and made up to volume with 50:50 v/v Methanol: Water to obtain 2.5 µg/ml working solution.

Sample preparation

Protein Precipitation (PPT) extraction method was employed for sample preparation. To prepare calibration samples of semaglutide and dapagliflozin in the plasma, 95 µl of the blank human plasma was mixed with 5 µl of the standard working solution, 10 µl of the IS working solution and precipitated using 750 µl of methanol in a 1.5 ml microcentrifuge tube. After mixing on a vortex mixer for 10 min, the mixture was centrifuged at 4500 rpm for 15 min at 8 °C. After centrifugation approximately 500 µl was aliquoted into a 1 ml autosampler vial. An aliquot of 3 µl of the supernatant was injected into the LC-MS/MS.

Chromatographic condition

In gradient mode, a reversed-phase chromatographic separation was achieved on Kinetex C18 (50 mm x 2.1 mm, 5 µ Particle size) at 35 °C using mobile phase (A: Milli-Q water and B: Acetonitrile). The autosampler temperature was sustained at 8 °C. The Liquid Chromatograph (LC) binary gradient program was employed with a run time of 3.0 min. The flow rate employed was 0.5 ml/min for the run. All the mobile phases were filtered using a 0.22 µM membrane filter to prevent clogging of mobile phase lines.

Mass spectrometric conditions

An Atmospheric Pressure Ionization (API) 5500 with an Electro Spray Ionization (ESI) interface operated in MRM (alternate between negative and positive scans). The semaglutide is fragmented in positive mode and dapagliflozin in negative mode. The instrument was optimized for semaglutide, dapagliflozin, and internal standards verapamil and tolbutamide during tuning at a concentration of 100 ng/ml prepared in Acetonitrile and water solution (50:50) and infused at a flow rate of 15 µl**/min through a Hamilton pump infused into the mass spectrometer. The MRM transitions chosen were m/z 1029.2→1302.6 for semaglutide, 407.2→329.1 for dapagliflozin and 455.2→165.2 for verapamil (Internal Standard for semaglutide) and 269.1→106.0 for tolbutamide (Internal standard for dapagliflozin). The optimized mass spectrometric conditions for semaglutide and dapagliflozin for quantification were: ESI probe with a source temperature of 500 °C; ion spray voltage 5500; curtain gas, 20 psi, nebulizing gas (GS1) 45 psi, heater gas (GS2) 45 psi, Declustering Potential (100eV)and Collision energy (35eV) for semaglutide and verapamil. The dapagliflozin and tolbutamide were analysed with a Declustering Potential (-80eV), and Collision energy of (-17 and-10 eV). The entrance potential and cell exit potential were maintained at 10 eV for positive mode analytes and-10 eV for negative mode analytes. Ultra-high pure nitrogen gas was employed as collision gas. The optimized parameters were sufficient to attain linearity for the selected calibration curve range.

Data analysis

Data processing was carried out using Analyst 1.6.3 software. The calibration curves were constructed using the response factor (area ratio of analyte peak area and IS peak area) to the analyte concentration using linear regression model y = mx+c where y denotes the observed area ratio, m is for slope and c is for intercept respectively, with weighting factor $1/x^2$. The acceptance criteria were established to be>0.98 for the coefficient of determination (r^2) with a minimum of 6 non-zero calibration curve standards i. e, at least 75% of the standards should be acceptable (±20% bias for Lower Limit of Quantification (LLOQ) and ±15% bias for other standards) for a calibration curve.

Bioanalytical method validation

Calibration and quality control samples

Calibration curves were made by spiking 5 µl** of spiking solution to 95 µl** of blank human plasma. The final concentrations in the plasma samples were 1.00, 3.00, 10.6, 106, 303, 485, 775, 1000 and 2.00, 6.00, 21.2, 212, 605, 970, 1550, 2000ng/ml. The Quality control (QC) samples were set at concentrations of 1.01, 2.82, 469, 750, and 2.02, 5.65, 940, and 1500 ng/ml (Lower Limit of quantification QC, Low QC, Mid QC, High QC) for semaglutide and dapagliflozin respectively.

Preparation of plasma calibration standards and quality control samples

5 µl** of the cocktail working solutions prepared above are spiked in 95 µl** of blank human plasma. This was the standard solution.

Calibration curve

The calibration curve was prepared using 8 standards excluding standard zero encompassing the range (1.00–1000 ng/ml) and (2.00–2000 ng/ml) for semaglutide and dapagliflozin respectively. The linearity was assessed by a weighted ($1/x^2$) least squares regression analysis.

Linearity was assessed by plotting calibration curves in human plasma in each quantitative run.

Table 1: Acceptance criteria for calibration standards

Level	Accuracy
LLOQ	±20%
All standards except LLOQ	±15%

Accuracy and precision

To evaluate the Accuracy and Precision of this method, Quality Control samples at four concentrations (Lower Limit of Quantification Quality control (LLOQQC), Low Quality Control (LQC), Mid Quality Control (MQC), and High Quality Control (HQC) were analyzed in six replicates for each analyte on three different days from extracted plasma. The acceptance criteria are as follows

Table 2: Acceptance criteria for quality control samples

QC level	Accuracy	Precision
LLOQQC	±20%	≤20%
LQC, MQC and HQC	±15%	≤15%

Specificity and selectivity

The specificity of the method was gauged by studying six different plasma lots to investigate the likely interferences at the retention time for both analytes and internal standards. The six blank lots were processed without analytes or internal standards. The acceptance criteria were that at least five out of the tested six blanks must have<20% area interference to that of the LLOQ level response at the corresponding retention time. Selectivity of the assay was established by analyzing six individual blank plasma lots with individual analyte spiking and checking for interference at other analyte and Internal Standard retention time.

Recovery

Recoveries were evaluated at three QC levels LQC, MQC and HQC (2.82, 469, and 750 ng/ml for semaglutide and 5.65, 940, and 1500 ng/ml for dapagliflozin) by comparing the peak area in spiked

pooled human plasma samples with those of analyte spiked in neat solutions.

Matrix effect

The matrix effect assessment was optimally suggested by Matuszewski *et al.* [21]. However, the latest approach suggested by ICH M10 guidelines was adopted wherein the back-calculated concentrations were obtained from 6 different interference-free lots and the mean concentration across the 6 lots was calculated. In this methodology, the matrix effects were assessed to check the impact of co-eluting matrix components which might trigger potential ion suppression or enhancement.

The matrix effect was evaluated at the LQC and HQC levels. Each of the selected blanks including one hemolyzed plasma lot were spiked with LQC and HQC and were quantified under a calibration curve. The acceptable criteria for the matrix factor should be a mean accuracy of 85% to 115%.

Stability experiments

The stability of semaglutide and dapagliflozin in human plasma was assessed by analyzing six replicates of QC samples at low and high levels in four different exposure conditions as follows; (1) The spiked samples at room temperature (ambient temperature for>18 h; (2) The processed samples at autosampler temperature i. e. 8 ℃ for 24 h, (3) four freeze and thaw cycles (24 h per cycle), and (4) For 14 d in the deep freezer at-80 °C. Samples were considered stable if the relative error was within 85–115% of the nominal concentration.

RESULTS AND DISCUSSION

Method optimization

The aim was to develop a robust bioanalytical method best suited for the simultaneous estimation of semaglutide and dapagliflozin in a single bioanalytical run on an LC-MS/MS platform. During the development of the extraction method due to the difference in physiochemical parameters between semaglutide and dapagliflozin, we adopted a protein precipitation method to maximize the recovery of both analytes. The existing methods for semaglutide in human plasma and related pharmacokinetic studies employed an LLOQ of 3 ng/ml [16-24]. During tuning of semaglutide in positive mode, the most prominent precursor ion 1029.2 was selected as a multiply charged [M+4] considering the large molecular weight of 4114 daltons. The product ion of 1302.6 was the chosen fragment which yielded the best chromatographic response. The main difficulty was overcoming dapagliflozin ionization, forming an adduct in formic acid. Therefore formic acid and its salts like Ammonium formate were not used in the mobile phase. Dapagliflozin was tuned in negative mode with a precursor ion of 407.2. The product of 329.1 along with the precursor of 407.2 yielded the best response. As semaglutide was ionisable in positive mode and dapagliflozin in negative mode we adopted a dual polarity ESI LC-MS/MS method for their simultaneous estimation. The MRM parameters for MS/MS determination were optimized to maximize the response for both analytes. The currently available method can quantify plasma concentrations of semaglutide in human plasma from 3 to 250 ng/ml by Kapitza *et al.* [22]. The validated method in this article has a wider linear dynamic range from 1 to 1000 ng/ml helpful even in cases of dose reduction due to other comorbidities. The currently available method for dapagliflozin utilizes an Acetate adduct for the parent ion which was 467.1 as reported by Shah PA *et al.* [23]. However, we have chosen a mobile phase to yield a parent ion 407.2 devoid of adduct and an enhanced selectivity at the chosen LLOQ of 2 ng/ml. Van der Aart-van der Beek AB *et al.* [24] reported an upper limit of quantification as 500 ng/ml for dapagliflozin utilizing a 200 µl** processing volume. However, we have achieved an upper limit of 2000 ng/ml with a 100 µl** sample processing volume.

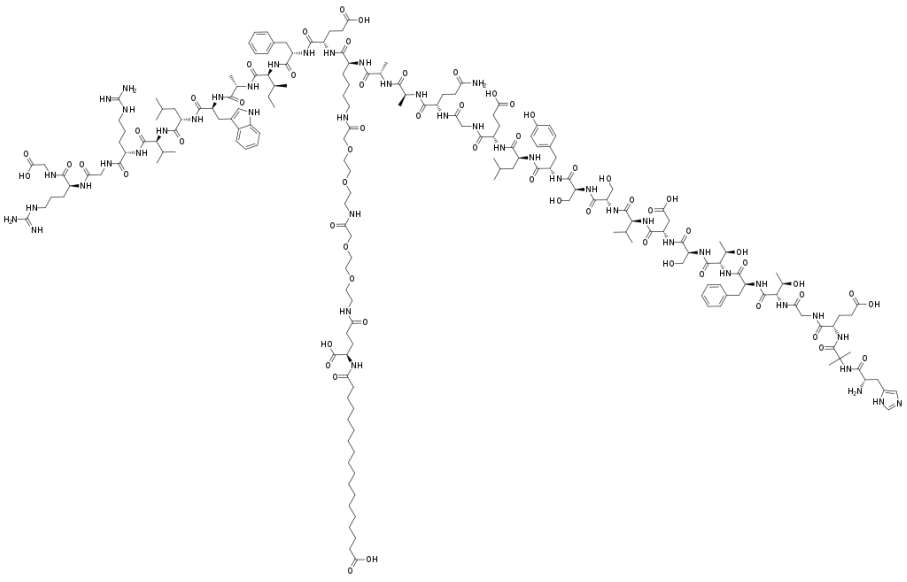

Fig. 1: Structure of semaglutide

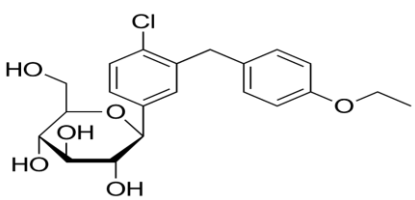

Fig. 2: Structure of dapagliflozin

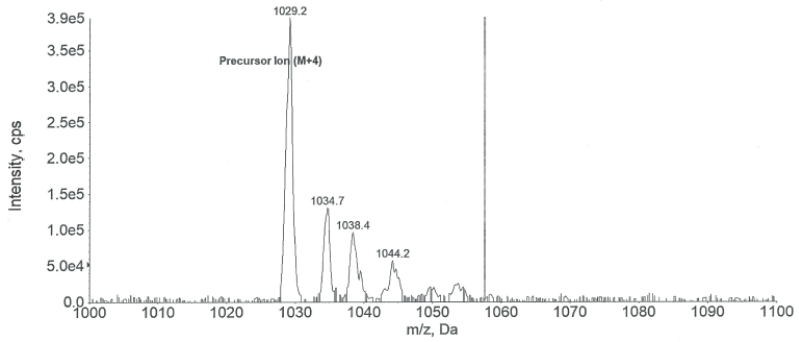

Fig. 3: Full scan mass spectrum of semaglutide

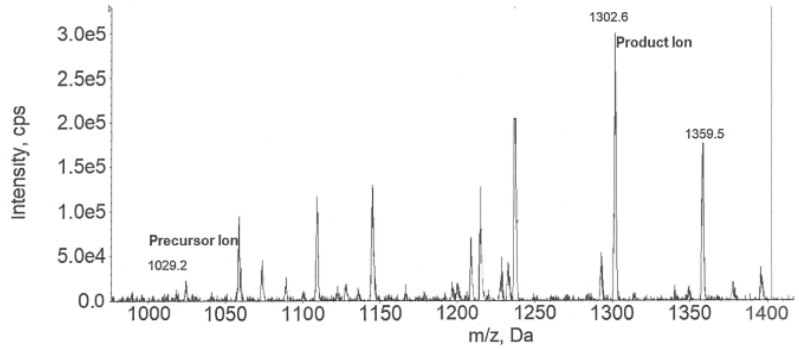

Fig. 4: Product ion mass spectrum of semaglutide

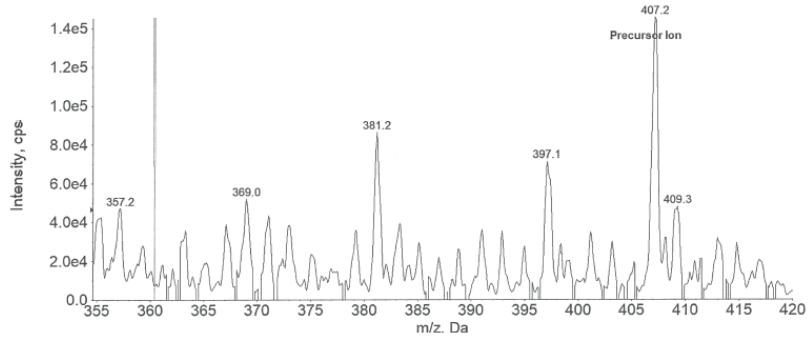

Fig. 5: Full scan mass spectrum of dapagliflozin

Table 3: Precision and accuracy data of calibration curve standards for semaglutide and dapagliflozin in human plasma

Matrix	Analyte	Nominal concentration (ng/ml)	Back calculated conc. (ng/ml)	n	CV (%)	RE (%)
Human Plasma	Semaglutide	1.00	0.97	3	13.52	97.0
		3.00	2.88	3	3.76	96.0
		10.6	9.57	3	3.19	90.3
		106	97.3	3	3.89	91.8
		303	292	3	3.14	96.4
		485	476	3	2.44	98.1
		775	788	3	2.04	101.7
		1000	1030	3	1.60	103.2
	Dapagliflozin	2.00	1.98	3	13.88	99.2
		6.00	5.55	3	9.14	92.5
		21.2	20.8	3	3.47	98.3
		212	206	3	6.04	97.0
		605	628	3	7.13	103.9
		970	943	3	4.28	97.3
		1550	1460	3	3.57	94.1
		2000	1930	3	3.84	96.6

n=3 replicates at each concentration, Data presented in mean, %CV, and %RE

Calibration curves

The calibration standards were analyzed at specified standard concentrations for semaglutide and dapagliflozin from 1.00 to 1000 ng/ml and 2.00 to 2000 ng/ml respectively. The calibration curve for both analytes displayed a good linear relationship across the concentration range. The eight-point calibration curves were plotted for both analytes with linear fit $1/x^2$ weighting regression factor. The mean correlation coefficient for both analytes in human plasma was 0.998. The calibration curve results for both analytes in human plasma are summarized in table 3.

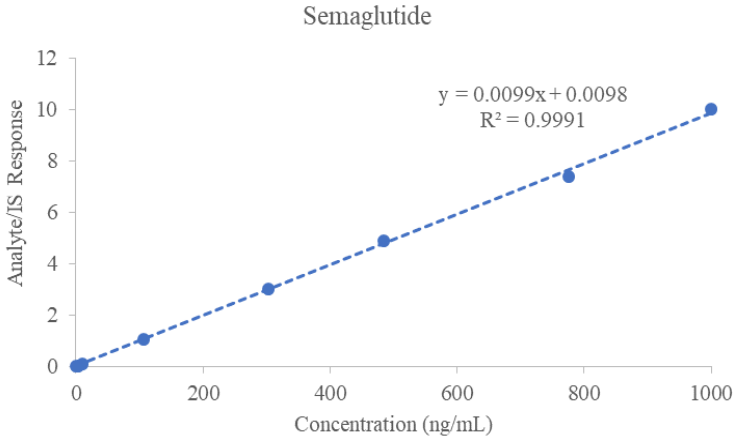

Fig. 6: Linearity plot of semaglutide

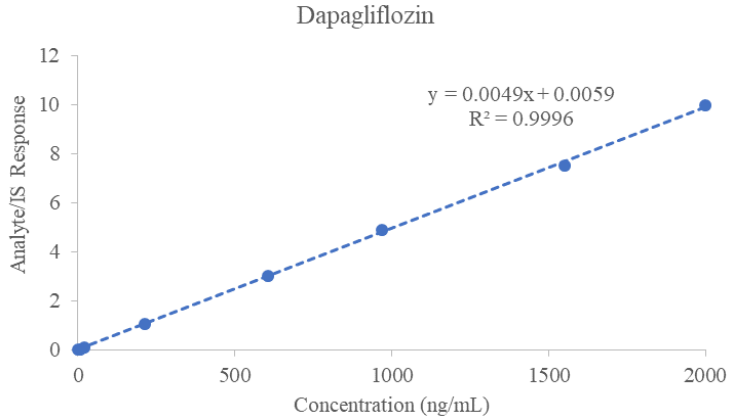

Fig. 7: Linearity plot of dapagliflozin

Specificity and selectivity

Specificity and selectivity employing this method in plasma were studied in six different lots along with a hyperlipidaemic and a hemolyzed lot. This is done to determine the degree of endogenous interferences that might contribute to the chromatographic area count of the analyte or internal standard. Each blank lot was processed as 2 replicates (1 devoid of any spiking and 1 spiked with the spiking solution of LLOQ and Internal Standard). The interference in the blank sample corresponding to each lot was compared against the average peak response at the LLOQ level and respective internal standard. As shown in fig. 8 and 11, no interference was detected at the retention time of semaglutide and dapagliflozin and its internal standards, verapamil and tolbutamide in any of the blank samples.

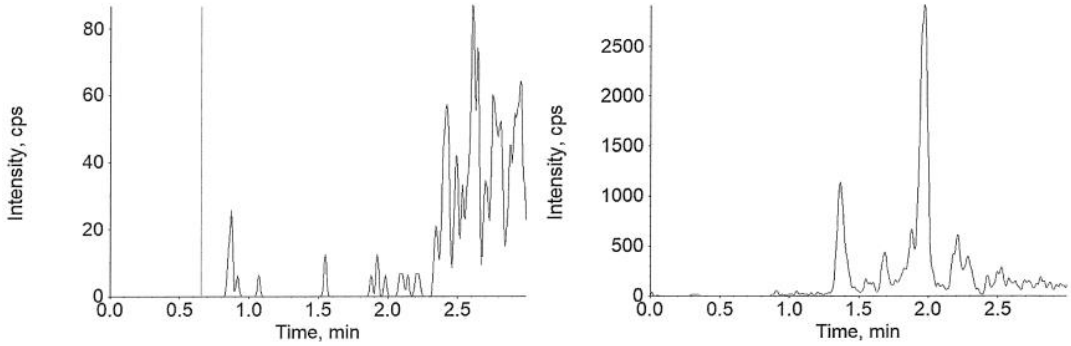

Fig. 8: Extracted blank of semaglutide

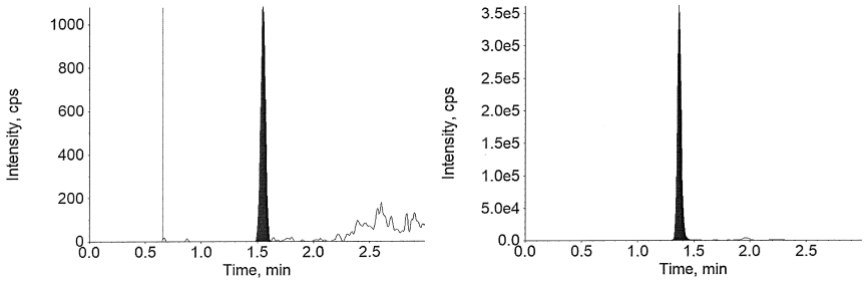

Fig. 9: Extracted LLOQ of semaglutide

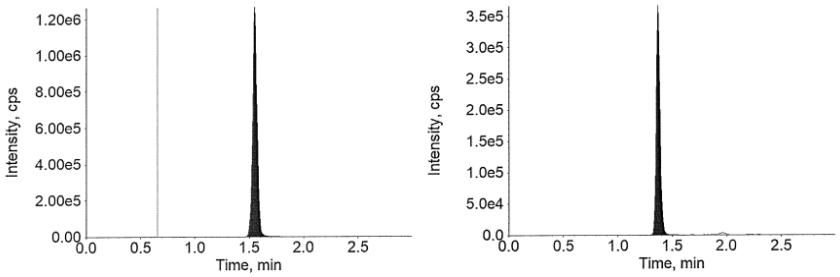

Fig. 10: Extracted ULOQ of semaglutide

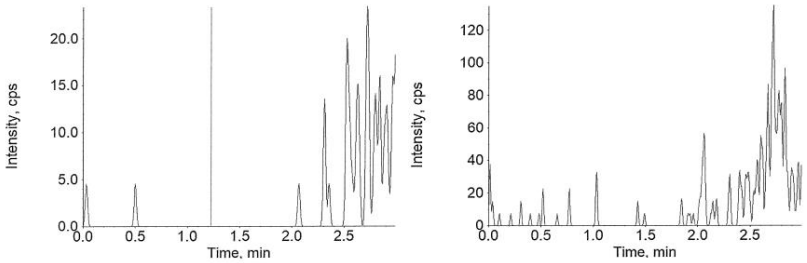

Fig. 11: Extracted blank of dapagliflozin

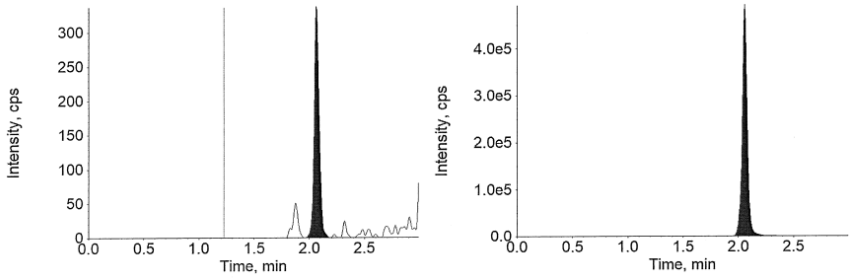

Fig. 12: Extracted LLOQ of dapagliflozin

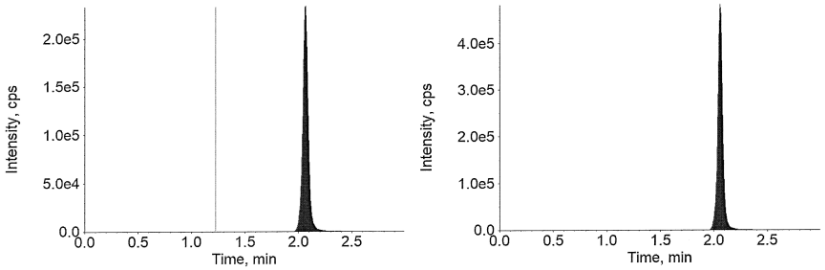

Fig. 13: Extracted ULOQ of dapagliflozin

Accuracy and precision

The intra-day and the inter-day accuracy and precision outcomes for semaglutide in plasma are accessible in tables 4 and 5 and dapagliflozin is presented in tables 6 and 7 respectively. The accuracy and precision were evaluated at the selected four levels of QCs by using six replicates at each QC level. Quality Control levels employed were (1.01, 2.82, 469, and 750ng/ml) for semaglutide and (2.02, 5.65, 940, and 1500ng/ml) for dapagliflozin.

For semaglutide at each QC level, the intra-day precision was between 3.19% and 11.28 %, and accuracy was between 95.2% and 103.0%. In the case of inter-day, the precision was between 3.31% and 7.68%and accuracy was between 97.6 % and 100.6% for human plasma.

For dapagliflozin at each QC level, the intra-day precision was between 3.55% and 7.10% and accuracy was between 95.0% and 102.0%. In the case of inter-day, the precision was between 3.83% and 10.47 % and the accuracy was between 98.4 % and 101.0% for human plasma.

Extraction recovery and matrix effect

As shown in table 8 and table 9, the extraction recovery for semaglutide was 75.4±10.71% to 73.4±4.39 and 75.5±5.29 in human plasma at LQC, MQC, and HQC respectively. Similarly, the extraction recovery for dapagliflozin in human plasma was 80.1±4.12 to 82.9±5.02 and 81.8±3.88 in human plasma at LQC, MQC, and HQC respectively. The mean Matrix effect calculated from the six independent lots for human plasma at Low QC and High QC levels were within the acceptance range of 85% to 115% as shown in tables 10 and 11.

Table 4: Intra-day precision and accuracy for the estimation of semaglutide

QC level	Nominal conc. (ng/ml)	Back calculated conc. (ng/ml)	Precision (%)	Accuracy (%)
LLOQC	1.01	1.00±0.11	11.28	98.5
LQC	2.82	2.69± 0.21	7.99	95.2
MQC	469	460±43.12	9.37	98.2
HQC	750	772±24.64	3.19	103.0

n=6 replicates in each concentration, Data presented in (mean±SD)

Table 5: Inter-day precision and accuracy for the estimation of semaglutide

QC level	Nominal conc. (ng/ml)	Back calculated conc. (ng/ml)	Precision (%)	Accuracy (%)
LLOQC	1.01	1.00±0.06	6.32	98.9
LQC	2.82	2.75±0.21	7.68	97.6
MQC	469	469±28.80	6.15	99.9
HQC	750	755±24.94	3.31	100.6

n=6 replicates in each concentration, Data presented in (mean±SD)

Table 6: Intra-day precision and accuracy for the estimation of dapagliflozin

QC Level	Nominal conc. (ng/ml)	Back calculated conc. (ng/ml)	Precision (%)	Accuracy (%)
LLOQC	2.02	1.92±0.14	7.10	95.0
LQC	5.65	5.64± 0.37	6.61	99.7
MQC	940	959±34.08	3.55	102.0
HQC	1500	1480±71.53	4.84	98.5

n=6 replicates in each concentration, Data presented in (mean±SD)

Table 7: Intra-day precision and accuracy for the estimation of dapagliflozin

QC level	Nominal conc. (ng/ml)	Back calculated conc. (ng/ml)	Precision (%)	Accuracy (%)
LLOQC	2.02	2.00±0.21	10.47	98.8
LQC	5.65	5.56±0.25	4.53	98.4
MQC	940	950±36.38	3.83	101.0
HQC	1500	1490±60.99	4.09	99.3

n=6 replicates in each concentration, Data presented in (mean±SD)

Table 8: Extraction recovery of semaglutide in human plasma

Concentration (ng/ml)	Mean recovery (%)	% CV
2.82	75.4	10.71
469	73.4	4.39
750	75.5	5.29

n=6 replicates in each concentration, Data presented in mean and %CV

Table 9: Extraction recovery of dapagliflozin in human plasma

Concentration (ng/ml)	Mean recovery (%)	%CV
5.65	80.1	4.12
940	82.9	5.02
1500	81.8	3.88

n=6 replicates in each concentration, Data presented in mean and % CV

Table 10: Matrix effect of semaglutide in human plasma

Concentration (ng/ml)	Mean matrix effect (%)	%CV
2.82	100.5	9.21
750	98.7	3.45

n=6 replicates in each concentration, Data presented in mean and % CV

Table 11: Matrix effect of dapagliflozin in human plasma

Concentration (ng/ml)	Mean matrix effect (%)	% CV
5.65	97.9	2.34
1500	100.0	4.85

n=6 replicates in each concentration, Data presented in mean and %CV

Stability

The stability of semaglutide and dapagliflozin was evaluated in human plasma and the results are shown in tables 12 and 13, representing that no significant degradation happened at ambient temperature, or for the processed samples in autosampler, after four freeze and thaw cycles or on long storage at-80 °C in freezers.

Table 12: Stability of semaglutide in human plasma

Experimental condition	Sample conc. (ng/ml)	Measured conc. (ng/ml)	CV (%)	Stability (%)
		Human plasma		
18 h at room temperature 24 °C	2.82	2.69±0.21	7.99	95.2
	750	772±24.6	3.19	103.0
24 h in the autosampler 8 °C	2.82	2.72±0.25	9.06	96.5
	469	459±43.5	9.50	97.8
	750	768±22.3	2.90	102.4
24 h at-80 °C then exposed to 4	2.82	2.74±0.15	5.64	97.0
freeze and thaw cycles	750	750±14.8	1.97	100.1
14 d at-80 °C	2.82	2.78 ±0.20	7.08	98.6
	750	740±22.8	3.07	98.8

Measured concentration provided in mean±SD, All stability measurements performed with n=6 replicates

Table 13: Stability of dapagliflozin in human plasma

Experimental condition	Sample conc. (ng/ml)	Measured conc. (ng/ml)	CV (%)	Stability (%)
		Human plasma		
18 h at room temperature 24 °C	5.65	5.64±0.37	6.61	99.7
	1500	1480±71.5	4.83	98.7
24 h in the autosampler 8 °C	5.65	4.92±0.37	7.49	87.0
	940	846±45.3	5.36	90.0
	1500	1550±47.0	3.03	103.3
24 h at-80 °C then exposed to 4 freeze	5.65	5.52±0.23	4.08	97.7
and thaw cycles	1500	1510±39.1	2.59	100.7
14 d at-80 °C	5.65	5.54±0.35	6.25	98.0
	1500	1480±74.4	5.03	98.7

Measured concentration provided in mean±SD, All stability measurements performed with n=6 replicates

CONCLUSION

In the current study, we have developed and thoroughly validated a robust, intricate, selective, and cost-effective LC-MS/MS method to quantify two antidiabetic drugs semaglutide and dapagliflozin simultaneously in human plasma. This method will facilitate the further application of pharmacokinetic and bioequivalence studies for these two drugs in pharmaceutical dosage forms or their new pharmaceutical formulation. This simple extraction technique and the optimized chromatographic conditions gave the best outcomes. This detailed procedure is user-friendly and will allow fast analysis procedures for further application to clinical studies.

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AUTHORS CONTRIBUTIONS

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CONFLICT OF INTERESTS

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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