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Research Article

DEVELOPMENT AND VALIDATION OF AN LC-MS/MS METHOD FOR SIMULTANEOUS DETERMINATION OF METFORMIN AND SEMAGLUTIDE IN HUMAN PLASMA

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ABSTRACT

Objective: To develop a method capable of simultaneous quantification of metformin and semaglutide studied as a potential combination therapy for treating diabetes.

Methods: A refined protein precipitation extraction technique was used with warfarin as an internal standard for metformin and semaglutide. The two compounds were separated on a Kinetex Polar C18 (50 mm × 2.1 mm, 5 µ particle size) column, with a positive polarity electro spray ionization on a Liquid chromatography with Tandem Mass Spectrometry instrument. The estimation was done through a multiple reaction monitoring method and a gradient program utilizing acetonitrile and 0.1% formic acid in water as mobile phases to achieve a separation in 2.2 min.

Results: The established method performed linearly over a working range of 10.0–10,000 ng/mL for metformin (r^2>0.98) and 1.00–1000 ng/mL for semaglutide (r^2>0.98) in human plasma. The specificity, selectivity, precision, accuracy, recovery, matrix effects, and stability were within acceptable limits as necessitated by the guideline on bioanalytical method validation, as mentioned in the International Council for Harmonisation of Technical Requirements of Pharmaceuticals for Human Use M10.

Conclusion: This highly selective and sensitive method, in which 10.0 ng/mL was employed for metformin and 1.00 ng/mL was employed for semaglutide as the Lower Limit of Quantification (LLOQ), can be utilized for estimation in human plasma and will facilitate further application to bioequivalence and population pharmacokinetics studies. This method has the advantage of a lower LLOQ over other existing methods.

Keywords: Metformin, Semaglutide, Electro spray ionization, Method validation, Mass spectrometer.

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INTRODUCTION

Insulin resistance is the major manifestation of type 2 diabetes, classified as a long-term metabolic disorder [1]. Metformin is an oral hypoglycemic drug usually recommended as monotherapy [2]. However, there are instances where this alone would not be sufficient. Combination therapy with a complementary mechanism of action is the ideal way forward to achieve acceptable glucose levels [3,4]. There are a few reported Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) methods for determining metformin in plasma alone and combination with various gliflozins [5,6]. Semaglutide is used in type 2 diabetes and belongs to the incretin mimetics classification. It is a receptor agonist for glucagon-like peptide-1 (GLP-1). It facilitates insulin release from the pancreas to counter high blood glucose levels [7]. It is also being employed for weight management due to its anorexic properties and ability to reduce body fat. Semaglutide cannot be administered in diabetic ketoacidosis or type 1 diabetes. The exploratory studies conducted by Goldenberg *et al*. [8] and Li *et al*. [9] indicate that it enhances the growth of β cells in the pancreas. The United States Food and Drug Administration (USFDA) and the European Medicines Agency in 2017 and 2018 approved subcutaneous once-a-week for type 2 diabetes treatment [10-16]. Lee *et al*. have developed a related method for quantifying semaglutide in rat brains and plasma [17]. Kapitza *et al*. have studied the effect of semaglutide on combined oral contraceptive medications [18].

This combination of metformin and semaglutide is important due to their synergistic effect on reducing blood glucose levels. Although there are reported methods for bioanalytical analysis of metformin and semaglutide individually, and in combination with other compounds, there is no method reported so far for the simultaneous estimation of both metformin and semaglutide in human plasma employing LC-MS/MS.

This combined bioanalytical method was projected and validated in human plasma and can be directly employed in clinical studies to estimate metformin and semaglutide simultaneously. This method validation was carried out by the USFDA Guidance on Bioanalytical Method Validation of 2018 [19] and the International Council for Harmonisation (ICH) M10 guidelines applicable for the bioanalytical method validation and study sample analysis [20].

METHODS

Reagents and chemicals

Methanol and acetonitrile were procured from J.T. Baker (LC-MS grade). Formic acid (LC-MS grade), Sigma Aldrich, was used. Type 1 Milli-Q Water from the Merck Millipore water system was used. Human blood and plasma lots were procured from Delta Laboratories. Anticoagulant Dipotassium Ethylene Diamine Tetraacetic acid (K2EDTA) was procured from Merck. Semaglutide, metformin, and warfarin Analytical Standards were obtained as gift samples.

Instrumentation

All the weighings were carried out using a Mettler Toledo microbalance MX5. The solutions were degassed using an ultrasonicator for 5 min. Ultra performance liquid chromatography (LC) (Shimadzu Nexera) coupled with a Sciex 4500 LC-MS/MS with Analyst Software 1.7 was employed for chromatography, and a Vortex mixer and Sorvall centrifuge were used for extraction. This study employed a positive polarity multiple reaction monitoring (MRM) for this estimation.

Preparation of stock and working solutions for Analyte and Internal Standard

Stock solutions of metformin and semaglutide (2 mg/mL) were prepared by weighing 20 mg in a 10 mL volumetric flask. These stocks were prepared individually in methanol. The calibration standards and the quality control samples were prepared from distinctly separate stocks to rule out weighing differences that could wreck the accurate estimation. The internal standards stock of warfarin was prepared by dissolving 5 mg in a 5 mL volumetric flask with methanol to prepare a 1 mg/mL stock. The spiking solutions containing both analytes were prepared using a serial dilution process from an intermediate cocktail stock containing both analytes. A 50% methanol in water solution was used for the serial dilution steps. 250 µL stock solution of internal standard was added to a 100 mL measuring flask and made up to volume with a 50% acetonitrile in water solution to achieve a 2.5 µg/mL working solution.

Sample preparation

The protein precipitation (PPT) extraction method was used for sample preparation. PPT was chosen over liquid–liquid extraction and solid phase extraction due to its cost-effectiveness and ideal sample cleanup. To prepare calibration samples of metformin and semaglutide in the plasma, 95 µL of blank human plasma was mixed with 5 µL of the standard working solution, and 10 µL of the IS working solution was added with a handy step and precipitated using 1000 µL of acetonitrile in a 2 mL microcentrifuge tube. Mixing was carried out using a vortex mixer for 15 min, and the mixture was centrifuged at 3500 rpm for 10 min at 8°C. After centrifugation, a 600 µL aliquot of supernatant was transferred into a 1 mL autosampler vial. An injection volume of 2 µL was optimized considering the response and to avoid peak tailing.

Chromatographic condition

In gradient mode, a reversed-phase chromatographic separation was achieved on a Kinetex Polar C18 (50 mm × 2.1 mm, 5 µ particle size) column at 40°C using mobile phase (A: 0.1% formic acid in water and B: acetonitrile). The autosampler temperature was maintained at 8°C. The LC binary gradient program was employed with a run time of 2.2 min, customized for this combination of analytes. The flow rate employed was 0.65 mL/min for the run. All the mobile phases were filtered using a 0.22 µM membrane filter to prevent fine particles from clogging the mobile phase lines. The gradient program was designed to overcome signal suppression and thereby matrix effect.

Mass spectrometric conditions

An atmospheric pressure ionization 5500 with an electro spray ionization (ESI) interface operated in MRM mode. The metformin and semaglutide were detected and fragmented in positive mode. The instrument was optimized for semaglutide, metformin, and internal standard warfarin during tuning at a concentration of 150 ng/mL prepared in acetonitrile and water solution (50:50) and infused at a flow rate of 10 µL/min through a Hamilton infusion pump. The MRM transitions chosen were m/z 130.0→60.1 for metformin, 1029.1→1302.6 for semaglutide, and 309.3→163.2 for warfarin used as an Internal Standard. The structure of metformin, semaglutide and tuning information are available in Figs. 1-3 respectively. Warfarin was selected as the internal standard due to its easy ionization in positive mode. The mass spectrometric conditions were augmented for quantification of metformin and semaglutide using an: ESI probe with a source temperature of 400°C; ion spray voltage of 5000; curtain gas, 40 psi, nebulizing gas (GS1) 35 psi, heater gas (GS2) 45 psi, declustering potential (80 eV) and a collision energy (17 and 35 eV). Warfarin was analyzed with a declustering potential (80 eV) and a collision energy (10 eV). The entrance potential and cell exit potential were maintained at 10 eV. Ultra-high-purity inert nitrogen gas was the collision gas employed. The optimized parameters resulted in acceptable linearity in the identified range.

Data analysis

Data processing and regression analysis were carried out using Analyst software version 1.6.3. The calibration curves were constructed using the response factor (area ratio of analyte peak area and IS peak area) to the analyte concentration using a linear regression model, y = mx + c, where y denotes the observed area ratio, m is for slope, and c is for intercept, respectively, with a 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 a 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 into 95 µL of blank human plasma. The final concentrations in the plasma samples were 10.0, 30.0, 102, 1020, 3300, 4850, 7750, and 10,000 for metformin and 1.00, 3.00, 10.2, 102, 310, 509, 790, and 1000 ng/mL for semaglutide. The quality control (QC) samples were set at concentrations of 10.1, 30.2, 5050, 7850, and 1.01, 3.01, 495, and 780 ng/mL (LLOQ QC, Low QC, Mid QC, and High QC) for metformin and semaglutide, respectively.

Preparation of plasma calibration standards and QC samples

The standards were prepared by spiking 5 µL of the cocktail working solutions to 95 µL of interference-free blank human plasma.

Calibration curve

A cocktail calibration curve was prepared using eight non-zero standards encompassing the range (10.0–1000 ng/mL) for metformin and (1.00–1000 ng/mL) for semaglutide. The linearity assessment was conducted through a weighted ($1/x^2$) least squares regression. The linearity was assessed by plotting calibration curves (area ratio of analyte/IS versus concentration) in human plasma for all the qualification runs. The acceptance for the accuracy of the standards was set as ±20% for standard-01 (the lowest standard) and ±15% for all other remaining standards. At least six out of eight non-zero standards, excluding the lowest and highest standards, were used in all the acceptable validation runs.

Precision and accuracy

To evaluate the precision and accuracy of the optimized method, QC samples at four concentrations were analyzed in six replicates for both analytes on three different days from extracted plasma. The acceptance for the accuracy of the standards was set as ±20% at the LLOQ QC (LLOQQC) and ±15% for all other remaining QCs. The acceptable precision was ≤20% at LLOQQC and ≤15% at all remaining QC levels. 67% of overall QCs and 50% at each QC level were necessary for the run to be acceptable. All the QC samples were included without exception to calculate the global mean and accuracy.

Specificity and selectivity

The assessment for specificity was conducted using six different plasma lots to examine the interferences for analytes and the internal standard at their retention time. The identified lots for this study were processed as per the expected procedure without analytes or an internal standard. The acceptance criteria were set as at least five out of the tested six blank lots must have <20% and <5% of interference to the LLOQ area response and internal standard response at their respective retention times. Selectivity was established by employing the identified six blank plasma lots screened for specificity. Each of these lots was spiked once separately with metformin, semaglutide, and warfarin to ensure there were no cross contributions across the tested analytes.

Extraction recovery

Recoveries were evaluated at the low, mid, and high QC levels by comparing the peak area in spiked pooled human plasma samples (extracted samples) with those of the analyte spiked in neat solutions. The recovery at multiple levels is performed to ascertain that there

were no recovery differences across the validated range that could influence the results obtained.

Matrix effect

The matrix effect was carried out by the latest quantitative assessment approach (ICH M10) using six independent interference-free lots, and the accuracy of the six lots was estimated to determine the absence of suppressive or enhancement effect in the quantitation process.

The evaluation of the matrix effect was at the low quality control (LQC) and high quality control (HQC) levels. The selected blank lots and the hemolyzed plasma lot spiked with LQC and HQC, and the back-calculated concentrations were calculated to ensure no matrix effect in the chosen methodology. The acceptable criteria for the matrix factor should be a mean accuracy of 85–115%.

Stability experiments

The stability of metformin and semaglutide in human plasma was assessed by analyzing six replicates of QC samples at LQC and HQC in four diverse exposure conditions as follows; (1) the bench top stability where the spiked samples were kept at room temperature (ambient temperature for >10 h; (2) the autosampler stability at autosampler temperature, i.e., 8°C for 24 h, (3) the freeze thaw stability using five freeze and thaw cycles (24 h for first cycle and 12 h for subsequent cycles), and (4) long term stability for 15 days in the deep freezer at −80°C. Samples were considered stable if the obtained accuracy was within 85–115% of the nominal concentration.

RESULTS AND DISCUSSION

Method optimization

The aim was to develop a reproducible and robust bioanalytical method for simultaneously estimating metformin and semaglutide using a distinct methodology on the LC-MS/MS system. During the development of the extraction method, a PPT approach was found to be best suited to get the most out of the recovery of metformin and semaglutide. The main effort was to optimize metformin ionization, with a relatively smaller Q1 fragment. Metformin was tuned in positive mode with a precursor ion of 130.0. The product of 60.1 with the precursor of 130.0 yielded the best response. The currently available methods for metformin were using an LLOQ of 50 ng/mL. We have optimized an LLOQ of 10 ng/mL in the current method. Likewise, for semaglutide in human plasma and related pharmacokinetic studies, an LLOQ of 3 ng/mL was employed. For the tuning of semaglutide (molecular weight: 4114 daltons), the [M + 1] positive charge was beyond the mass range for suitable detection in the selected instrument. Therefore, a multiply charged prominent [M + 4] precursor ion 1029.1 was selected during tuning in positive mode. The product ion of 1302.6 was the chosen fragment, resulting in the best chromatographic response. The currently available method can quantify plasma concentrations of semaglutide in human plasma from 3 to 250 ng/mL, by Kapitza *et al*. does not have an adequate LLOQ. The method validated in this article ensures a wider linear dynamic range from 1 to 1000 ng/mL, which is handy in single ascending dose and multiple ascending dose studies. Moreover, the sample processing volume had been optimized to 100 µL from the existing 200 µL, thereby ensuring lower sample volume consumption during analysis.

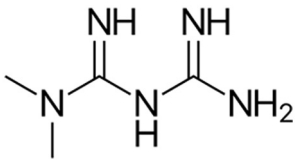

Fig. 1: Structure of metformin

Calibration curves

The calibration standards were set at standard concentrations of 10.0–10,000 for metformin and 1.00–1000 ng/mL for semaglutide. The calibration curve (8 non-zero standards) for both analytes displayed a good linear fit with a weighting factor of $1/x^2$ across the selected range over the 1/x. The eight-point calibration curves were plotted for both analytes with linear fit weighting regression factor. The mean correlation coefficient for the selected analytes in human plasma was 0.990. The calibration curve results for both analytes in human plasma are summarized in Table 1. The calibration curve for Metformin and Semaglutide are depicted in Figs. 4 and 5 respectively.

Specificity and selectivity

Specificity and selectivity for this plasma method were studied in six different lots, along with a hyperlipidemic and a hemolyzed lot to determine the extent of endogenous interferences. Each blank lot was processed in two replicates (one without any spiking and one 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 internal standard at the working solution level. As depicted in Figures 6 and 9, no interference was detected at the retention time of metformin and semaglutide in the tested lots. As few clinical samples could be hemolyzed or hyperlipidemic, the absence of interference in these lots ensures accurate analyte estimation in those samples also.

Accuracy and precision

The inter-day and intra-day accuracy and precision results for metformin in plasma are available in Table 2, and semaglutide is

Table 1: Precision and accuracy data of calibration curve standards for metformin and semaglutide in human plasma

Matrix	Analyte	Nominal concentration (ng/mL)	Back calculated Conc. (ng/mL)	n	CV (%)	Accuracy (%)
Human plasma	Metformin	10.0	10.4	3	13.24	104.0
		30.0	30.3	3	8.37	101.0
		102	98.0	3	3.69	96.1
		1020	1030	3	6.03	101.0
		3300	3140	3	7.18	95.2
		4850	4880	3	2.58	100.7
		7750	7380	3	1.69	95.2
		10000	9860	3	1.17	98.6
	Semaglutide	1	1.09	3	12.03	109.0
		3	3.00	3	3.61	100.0
		10.2	9.30	3	3.29	91.2
		102	100	3	3.79	98.0
		310	284	3	3.23	91.6
		509	468	3	2.48	91.9
		790	801	3	2.01	101.4
		1000	1020	3	1.62	101.6

n=3 replicates at each concentration. Data presented in Mean, %CV, and %Accuracy

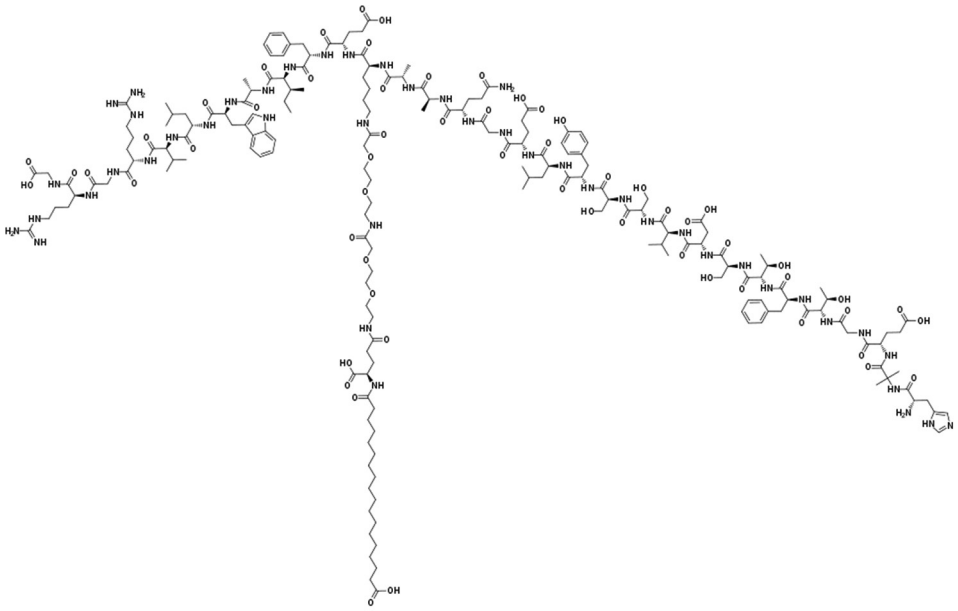

Fig. 2: Structure of semaglutide

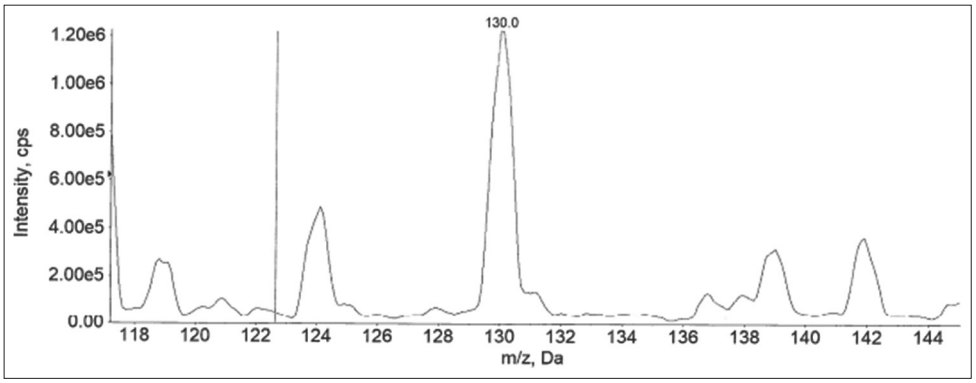

Fig. 3: Full scan mass spectrum of metformin

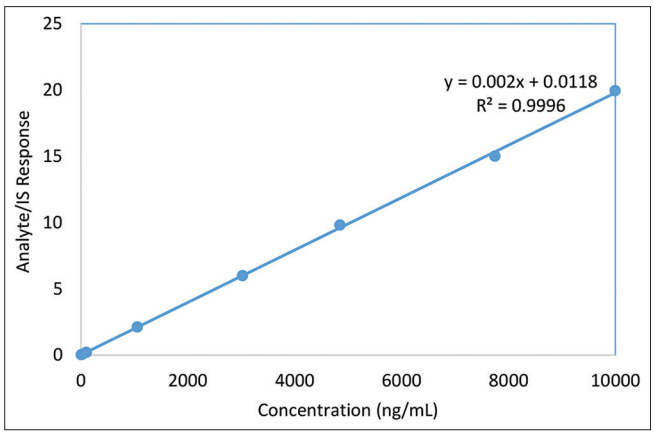

Fig. 4: Linearity plot of metformin

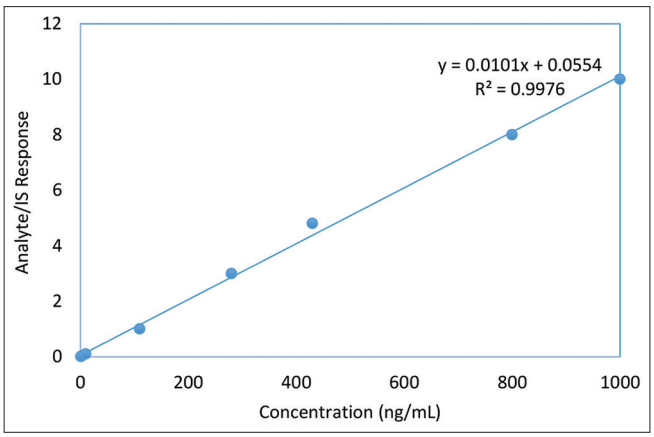

Fig. 5: Linearity plot of semaglutide

presented in Table 3, respectively. The precision and accuracy were assessed at the four QC levels using six replicates at each level on a single day for intra-day results and across three batches on 2 separate days for inter-day precision and accuracy. The chromatograms for extracted lower limit and upper limit of quantification for metformin and semaglutide are presented in Figs. 7,8,10 and 11 respectively.

For metformin at each QC level, the intra-day accuracy was between 93.3% and 95.0% and precision was between 3.55% and 7.10%. In the case of inter-day, the accuracy was between 91.4% and 97.3% and the precision was between 2.59% and 11.31%.

For semaglutide at each QC level, the intra-day accuracy was between 92.1% and 100.8%. Moreover, precision was between 2.85% and

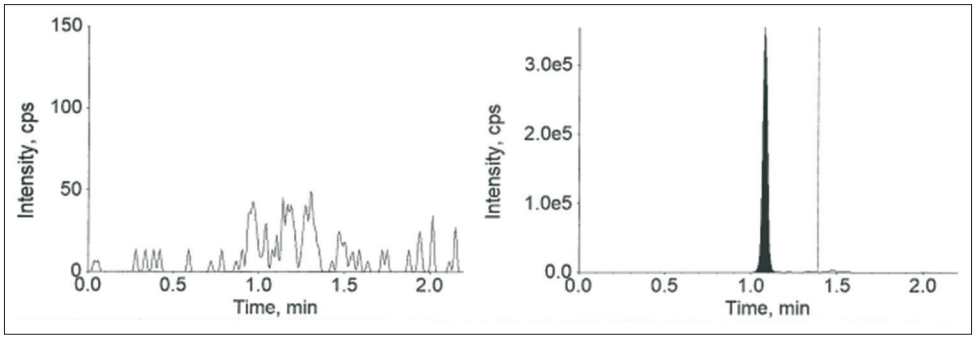

Fig. 6: Extracted standard zero for metformin

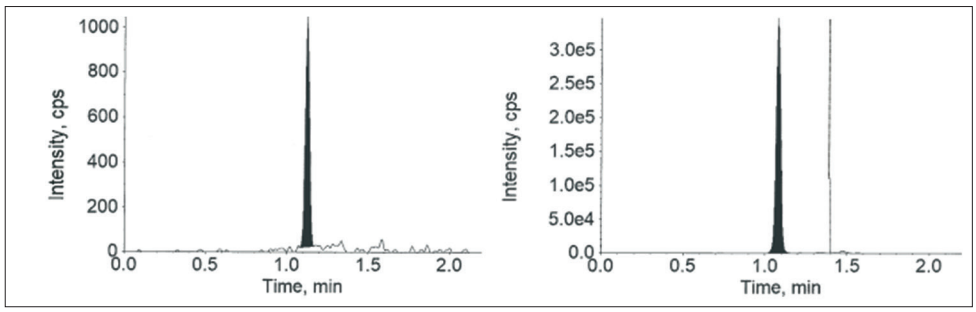

Fig. 7: Extracted lower limit of quantification of metformin

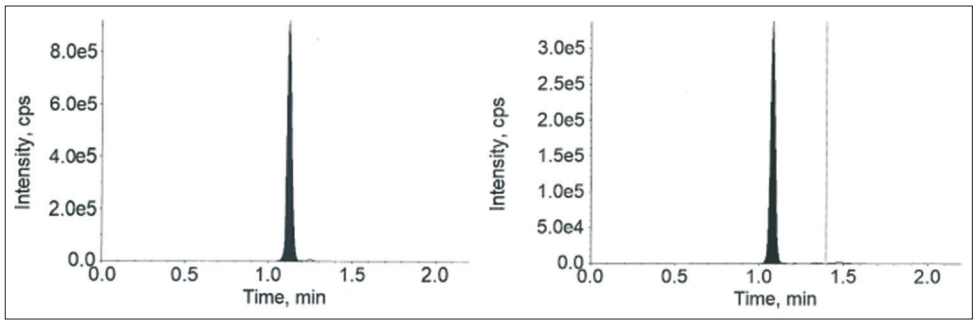

Fig. 8: Extracted upper limit of quantification of metformin

Table 2: Precision and accuracy for the estimation of metformin

Experiment	QC level	Nominal Conc. (ng/mL)	Back calculated Conc. (ng/mL)	Precision (%)	Accuracy (%)
Intra day	LLOQC	10.1	9.60±0.68	7.10	95.0
	LQC	30.2	28.2±1.86	6.61	93.3
	MQC	5050	4790±170	3.55	94.9
	HQC	7850	7390±358	4.84	94.1
Inter day	LLOQC	10.1	9.83±1.11	11.31	97.3
	LQC	30.2	27.6±1.13	4.08	91.4
	MQC	5050	4730±190	4.02	93.6
	HQC	7850	7560±195	2.59	96.3

n=6 replicates in each concentration. Data presented in (mean±SD). LQC: Low quality control, HQC: High quality control, MQC: Medium quality control

Table 3: Intra-day precision and accuracy for the estimation of semaglutide

Experiment	QC level	Nominal Conc. (ng/mL)	Back calculated Conc. (ng/mL)	Precision (%)	Accuracy (%)
Intra day	LLOQC	1.01	1.02±0.10	9.81	100.8
	LQC	3.01	2.77±0.10	3.78	92.1
	MQC	495	474±18.7	3.95	95.7
	HQC	780	759±21.6	2.85	97.3
Inter day	LLOQC	1.01	1.01±0.02	2.24	99.7
	LQC	3.01	2.74±0.15	5.64	90.9
	MQC	495	473±20.2	4.29	95.5
	HQC	780	751±14.8	1.97	96.3

n=6 replicates in each concentration. Data presented in (mean±SD). LQC: Low quality control, HQC: High quality control, MQC: Medium quality control

9.81%. In the case of inter-day, the accuracy was between 90.9% and 99.7% and the precision was between 1.97% and 5.64%.

Extraction recovery

As shown in Tables 4 and 5, the extraction recovery for metformin ranged from 63.0% to 65.1% across the different QC levels. The % CV for metformin ranged from 8.20% to 10.9%. Similarly, the extraction recovery for semaglutide in human plasma ranged from 73.1% to 77.5% across the different QC levels. The %CV for semaglutide ranged from 1.93% to 9.91%. Although the recovery of metformin is slightly lower than that of semaglutide, it is uniform across the tested levels.

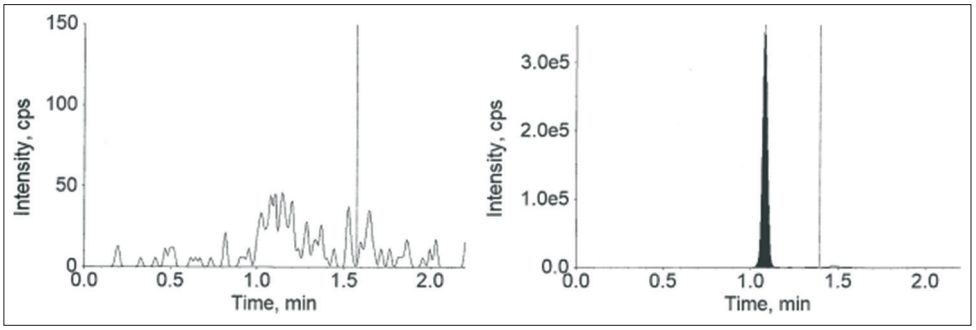

Fig. 9: Extracted standard zero for semaglutide

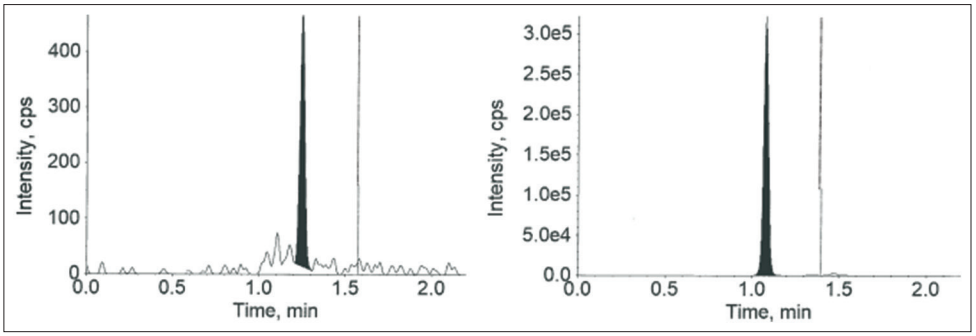

Fig. 10: Extracted lower limit of quantification of semaglutide

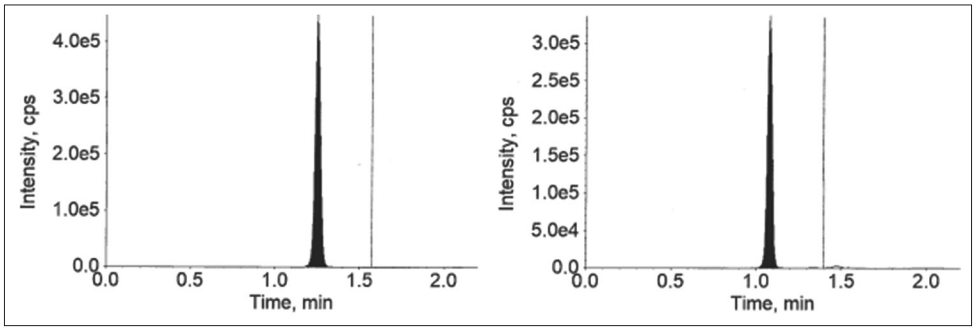

Fig. 11: Extracted upper limit of quantification of semaglutide

Table 4: Extraction recovery of metformin in human plasma

Level	Mean recovery (%)	CV (%)
LQC	65.1	8.30
MQC	63.0	10.9
HQC	64.1	8.20

n=6 replicates in each concentration. Data presented in Mean and CV%.
LQC: Low quality control, HQC: High quality control, MQC: Medium quality control

Table 5: Extraction recovery of semaglutide in human plasma

Level	Mean recovery (%)	CV (%)
LQC	77.5	3.33
MQC	73.1	1.93
HQC	75.5	9.91

n=6 replicates in each concentration. Data presented in Mean and CV%.
LQC: Low quality control, HQC: High quality control, MQC: Medium quality control

Matrix effect

The mean accuracy from the six independent lots to measure the Matrix effect was 93.3% and 95.6%, with a CV of 5.77% and 4.85% at LQC and HQC, respectively, for metformin, and are captured in Table 6. The mean accuracy from the six independent lots to measure the Matrix effect was 95.1% and 95.7%, with a CV of 2.48% and 2.93% at LQC and HQC, respectively, for semaglutide, and are captured in Table 7. The results indicate that the matrix effect is alleviated and there would be no suppression or enhancement of signal, thereby ensuring correct estimation.

Table 6: Matrix effect of metformin in human plasma

Matrix lot	% Accuracy at LQC	% Accuracy at HQC
1	93.5	95.5
2	89.4	89.4
3	96.0	93.8
4	89.6	101.8
5	88.7	100.1
6	102.6	92.8
Mean	93.3	95.6
CV%	5.77	4.85

n=6 replicates in each concentration. Data presented in Mean and CV%.
LQC: Low quality control, HQC: High quality control

Stability

Table 7: Matrix effect of semaglutide in human plasma

Matrix lot	% Accuracy at LQC	% Accuracy at HQC
1	96.3	96.4
2	94.7	97.6
3	98.0	95.5
4	96.3	97.4
5	91.4	90.3
6	93.7	97.3
Mean	95.1	95.7
%CV	2.48	2.93

n=6 replicates in each concentration. Data presented in Mean and CV%.
LQC: Low quality control, HQC: High quality control

Table 8: Stability of metformin in human plasma

Experimental condition	Sample Conc. (ng/mL)	Measured Conc. (ng/mL)	CV (%)	Stability (%)
		Human plasma		
Bench top stability for 10 h at room temperature 24°C	30.2	28.2±1.63	5.77	93.3
	7850	7560±195	2.59	96.3
Autosampler stability for 24 h at 8°C	30.2	27.6±1.13	4.08	91.4
	5050	4720±208	4.41	93.5
	7850	7500±364	4.85	95.6
Five freeze-thaw cycles at −80°C	30.2	28.0±1.50	5.36	92.7
	7850	7480±304	4.08	95.3
Long-term stability for 15 days at −80°C	30.2	27.9±1.55	5.57	92.3
	7850	7520±274	3.65	95.8

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

Table 9: Stability of semaglutide in human plasma

Experimental condition	Sample Conc. (ng/mL)	Measured Conc. (ng/mL)	CV (%)	Stability (%)
		Human plasma		
Bench top stability for 10 h at room temperature 24°C	3.01	2.79±0.12	4.35	92.7
	780	752±19.2	2.56	96.4
Autosampler stability for 24 h at 8°C	3.01	2.74±0.15	5.64	90.9
	495	473±18.7	3.96	95.6
	780	746±21.9	2.93	95.7
Five freeze-thaw cycles at−80°C	3.01	2.86±0.10	3.78	95.0
	780	751±14.8	1.97	96.3
Long-term stability for 15 days at−80°C	3.01	2.82±0.10	3.46	93.6
	780	752±21.7	2.88	96.5

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

The stability of metformin and semaglutide was evaluated in human plasma and the results are presented in Tables 8 and 9, representing that any degradation if evident is within the acceptable stability of 15%, at room temperature, in the autosampler, after five freeze and thaw cycles and on long storage for 15 days at −80°C in freezers. The stability results indicate the applicability of the method within the set criteria as outlined by the ICH guidelines. The statistical analysis was conducted with n=6 for all stability measurements.

CONCLUSION

A strong method was established in the present study, which was systematically validated to quantify two antidiabetic drugs, metformin and semaglutide, simultaneously in human plasma using a cost-effective and viable LC-MS/MS approach. This method will support its application to pharmacokinetic studies and a blend of these drugs in pharmaceutical dosage forms and prospective new pharmaceutical formulations. This reproducible optimized extraction technique and the resultant chromatography resulted in ideal outcomes. The procedure captured is easy to adopt and was designed to ensure easy adaptability for therapeutic drug monitoring studies. This method could be used as a reference and could be used as a basis for other combinations of GLP-1 receptor agonists and metformin.

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AUTHORS CONTRIBUTIONS

Jagapathi Raju Vatsavayi- Conceptualization, Experimentation, Writing–Original draft, and Nalanda Baby Revu- Data Curation, Review, Editing.

CONFLICTS OF INTEREST

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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