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Bioanalytical method development and validation for simultaneous quantification of Empagliflozin and Linagliptin in human plasma by RP-HPLC

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Abstract
A simple, rapid and sensitive reversed-phase high-performance liquid chromatographic (RP-HPLC) method was developed for simultaneous determination of empagliflozin and linagliptin in human plasma using telmisartan as an internal standard. Chromatographic separation was achieved on a Discovery C18 (250 × 4.6 mm, 5 µm) column using an isocratic mobile phase consisting of 0.1% perchloric acid buffer (pH 4.5) and acetonitrile (68:32, v/v), at a flow rate of 1.0 mL/min. Detection was performed at 218 nm with a 10 µL injection volume and a total run time of 10 min. Plasma samples were processed by simple protein precipitation with acetonitrile. The retention times of telmisartan, empagliflozin and linagliptin were approximately 4.10, 4.76 and 6.46 min, respectively. Both analytes showed linearity over 0.01-10 µg/mL, with reported correlation coefficients of 0.999 for empagliflozin and 0.998 for linagliptin. The method showed acceptable selectivity, system suitability, precision, accuracy, ruggedness and stability under the conditions evaluated. Extraction recovery was 55.16-66.12% for empagliflozin and 54.39-67.72% for linagliptin. The method uses low plasma volume, a single-step extraction procedure and a short isocratic run, making it potentially useful for routine bioanalytical quantification and pharmacokinetic or bioequivalence studies.

Keywords: Empagliflozin, Linagliptin, Telmisartan, RP-HPLC, Human plasma, Bioanalytical method, Validation

Introduction

Empagliflozin is a sodium-glucose cotransporter-2 (SGLT2) inhibitor belonging to the oral hypoglycemic drug class. It is used in the management of type 2 diabetes mellitus and is characterized by the molecular formula C23H27ClO7 and molecular weight 450.91. Linagliptin is a dipeptidyl peptidase-4 (DPP-4) inhibitor used as an adjunct to diet and exercise for glycemic control in adults with type 2 diabetes mellitus. It has the molecular formula C25H28N8O2 and molecular weight 472.54. The fixed-dose combination of empagliflozin and linagliptin provides complementary pharmacological mechanisms for glycemic control.

The supplied literature survey identified analytical methods for these drugs individually and in combination using UPLC, HPLC, UV spectrophotometry and LC-MS/MS. The source material specifically notes that, at the time of the study, no method had been reported for simultaneous estimation of empagliflozin and linagliptin in human plasma. Accordingly, the present work was undertaken to develop a simple, rapid, reproducible and validated RP-HPLC method for their simultaneous determination in human plasma using telmisartan as an internal standard.

Empagliflozin is described as soluble in organic solvents such as ethanol and dimethylformamide and sparingly soluble in aqueous buffer, whereas linagliptin is described as soluble in methanol, sparingly soluble in ethanol and very slightly soluble in water.

Materials and Methods

1. Chemicals and reagents

Empagliflozin, linagliptin and telmisartan were procured from Selleck Chem LLC through Prolab Marketing, India. HPLC-grade acetonitrile, methanol and other chemicals were obtained from Merck Chemical Division, Mumbai. Milli-Q water was used throughout the study. Human plasma was obtained from Vimtalabs, Hyderabad, India and stored at 2-8 °C until use.

2. Instrumentation and chromatographic conditions

Chromatographic analysis was performed using a Waters 2695 HPLC system equipped with a quaternary pump, autosampler, column oven, degasser and Waters 2996 PDA detector, operated using Empower-2 software. A Discovery C18 column (250 × 4.6 mm, 5 µm) was used for separation. A double-beam Elico SL-159 UV-visible spectrophotometer was used for spectral studies. Additional equipment included a Remi cyclomixer, ultrasonic bath, centrifuge, 0.45 µm membrane filters, analytical balance and variable micropipettes.

Parameter	Optimized condition
Column	Discovery C18, 250 × 4.6 mm, 5 µm
Mobile phase	0.1% perchloric acid buffer (pH 4.5): acetonitrile (68:32, v/v)
Elution	Isocratic
Flow rate	1.0 mL/min
Injection volume	10 µL
Run time	10 min
Detection wavelength	218 nm
Column temperature	25 °C
Sample temperature	5 °C
Diluent	Water: acetonitrile (50:50, v/v)

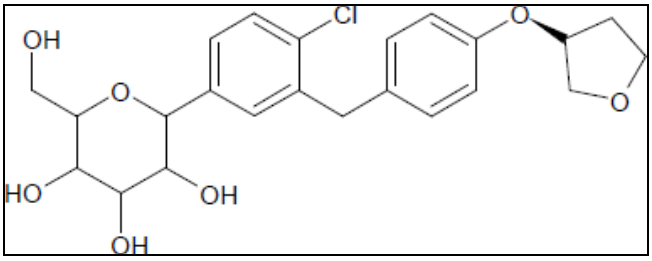

Fig 1: Chemical structure of empagliflozin.

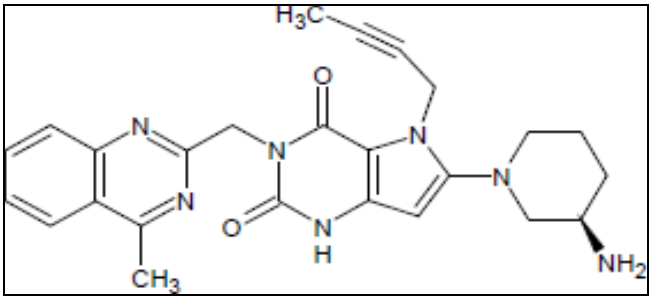

Fig 2: Chemical structure of linagliptin.

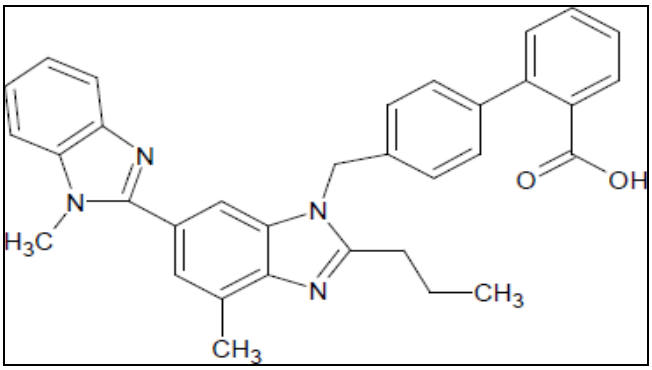

Fig 3: Chemical structure of telmisartan (internal standard)

3. Internal standard selection

Telmisartan was selected as the internal standard. The supplied study reports pKa values of 12.57 for empagliflozin, 1.9 and 8.6 for linagliptin, and 4.45 for telmisartan. Telmisartan was therefore employed to improve analytical accuracy during plasma analysis.

4. Preparation of buffer and mobile phase

A 0.1% perchloric acid solution was prepared by transferring 1 mL perchloric acid to a 1000 mL volumetric flask and diluting with Milli-Q water. The pH was adjusted to 4.5 using triethylamine and the solution was filtered through a 0.45 µm nylon membrane. The mobile phase was prepared by mixing the buffer and acetonitrile in a 68:32 (v/v) ratio. Water: acetonitrile (50:50, v/v) was used as the diluent.

5. Preparation of stock and working solutions

Individual stock solutions of empagliflozin and linagliptin were prepared by dissolving 10 mg of each pure drug in 10 mL of diluent to obtain nominal 1 mg/mL solutions. Working/spiking solutions were prepared by appropriate dilution. Calibration and quality-control plasma samples were prepared by spiking 10 µL of the appropriate analyte solution into 250 µL plasma together with 50 µL of internal-standard solution. Telmisartan stock solution was prepared similarly at 1 mg/mL and diluted to a 10 µg/mL working solution.

6. Plasma sample preparation

Protein precipitation was used for plasma extraction. Spiked plasma samples were mixed with 2 mL acetonitrile, cyclomixed for 15 s, vortexed for 2 min and centrifuged for 3 min at 3200 rpm. The resulting organic layer was collected and 10 µL was injected into the HPLC system. The final calibration concentrations were 0.01, 0.05, 0.10, 0.50, 1.0, 2.0, 5.0 and 10 µg/mL for both analytes.

7. Method development and optimization

The method was optimized by systematically evaluating detection wavelength, stationary phase, mobile-phase composition and flow rate. Individual UV spectra of the analytes indicated a common detection wavelength of 218 nm. Different octadecyl and octyl stationary phases were evaluated, with Discovery C18 providing the desired peak shape and separation. Several mobile-phase compositions were investigated; the supplied study identified the 68:32 (v/v) buffer: acetonitrile system at pH 4.5 as optimal. Flow rates between 0.5 and 2.0 mL/min were evaluated, and 1.0 mL/min was selected.

Under the optimized conditions, no interfering peaks were observed in blank or placebo samples at the analyte retention times. Reported retention times were approximately 4.100 min for telmisartan, 4.755 min for empagliflozin and 6.457 min for linagliptin.

Bioanalytical Method Validation

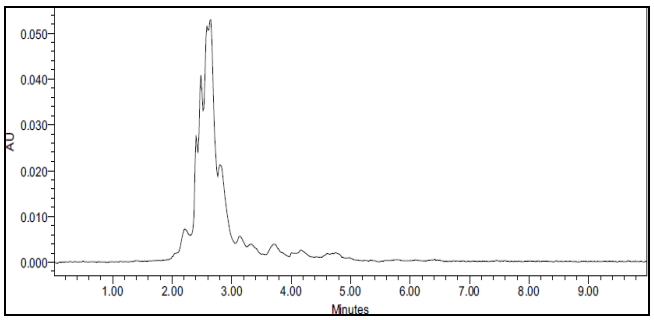

Fig 4: Representative chromatogram of blank in human plasma

The method was evaluated for specificity/selectivity, linearity, matrix effect, system suitability, carryover, precision, accuracy, recovery, reinjection reproducibility, ruggedness and stability, following the validation approach described in the supplied study.

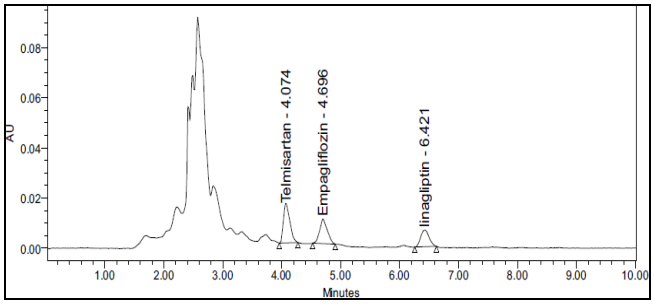

Fig 5: Representative chromatogram of empagliflozin, linagliptin and telmisartan in human plasma.

1. Specificity and selectivity

Blank plasma from six sources was examined for endogenous interference. LLOQ samples were injected six times and spiked LLOQ samples were evaluated alongside clean plasma. The study reported no endogenous interference at the retention times of empagliflozin, linagliptin or the internal standard. The LLOQ was 0.01 µg/mL for both analytes.

Replicate	Empagliflozin LLOQ (µg/mL)	Linagliptin LLOQ (µg/mL)
1	0.009	0.009
2	0.011	0.011
3	0.009	0.010
4	0.010	0.011
5	0.010	0.009
6	0.011	0.010
Mean	0.0100	0.0100
SD	0.00092	0.00089
%CV	9.19	8.94
% mean accuracy	99.83	100.00

2. Matrix effect

Matrix effect was assessed using LQC and HQC samples prepared in six different plasma lots. The study reported no significant matrix effect for either analyte or the internal standard.

Analyte	Level	Mean (µg/mL)	SD	%CV	% mean accuracy
Empagliflozin	LQC 0.10	0.1010	0.00874	8.66	100.95
Empagliflozin	HQC 5.00	5.0276	0.33414	6.65	100.55
Linagliptin	LQC 0.10	0.100	0.00546	5.46	100.00
Linagliptin	HQC 5.00	5.0423	0.23096	4.58	100.85

3. System suitability and carryover

Six homogeneous MQC injections were used for system-suitability assessment. Retention-time %RSD values were 0.26%, 0.35% and 0.47% for telmisartan, empagliflozin and linagliptin, respectively. Peak-area %RSD values were 0.40%, 0.30% and 0.51%. Resolutions of 3.32 and 7.08 were reported for empagliflozin and linagliptin, respectively. Theoretical plate counts were 10086.83 for telmisartan, 7073.67 for empagliflozin and 10483.67 for linagliptin, with tailing factors of 1.19, 1.12 and 1.21, respectively. No autosampler carryover was observed.

Parameter	IS	Empagliflozin	Linagliptin	Acceptance criterion
Retention time %RSD	0.26	0.35	0.47	≤2%
Peak area %RSD	0.40	0.30	0.51	≤5%
Resolution	—	3.32	7.08	>2
Theoretical plates	10086.83	7073.67	10483.67	Higher indicates efficiency
Tailing factor	1.19	1.12	1.21	≤2
HETP (cm/plate)	0.0025	0.0035	0.0024	Lower indicates higher efficacy
Capacity factor (k′)	1.39	1.76	2.77	1-10

4. Linearity

An eight-point calibration curve covering 0.01-10 µg/mL was constructed using the ratio of analyte peak area to internal-standard peak area.

The supplied study reports correlation coefficients of 0.999 for empagliflozin and 0.998 for linagliptin. The plotted calibration Figs show R^2 values of 0.9990 and 0.9987, respectively.

Concentration (µg/mL)	Empagliflozin mean ± SD	%CV	% accuracy	Linagliptin mean ± SD	%CV	% accuracy
0.01	0.010 ± 0.0010	10.00	100.00	0.010 ± 0.0010	10.00	100.00
0.05	0.0513 ± 0.0032	6.26	102.67	0.050 ± 0.0021	4.11	101.33
0.10	0.0999 ± 0.0105	10.64	99.00	0.101 ± 0.0074	7.32	100.67
0.50	0.4973 ± 0.0156	3.14	99.47	0.501 ± 0.0300	6.00	100.20
1.00	1.0183 ± 0.0754	7.40	101.83	1.008 ± 0.0818	8.11	100.77
2.00	1.9777 ± 0.1188	6.01	98.88	1.999 ± 0.0912	4.56	99.97
5.00	5.0683 ± 0.0663	1.31	101.37	5.028 ± 0.0841	1.67	100.56
10.00	9.9707 ± 0.2820	2.83	99.71	10.195 ± 0.6913	6.78	101.95

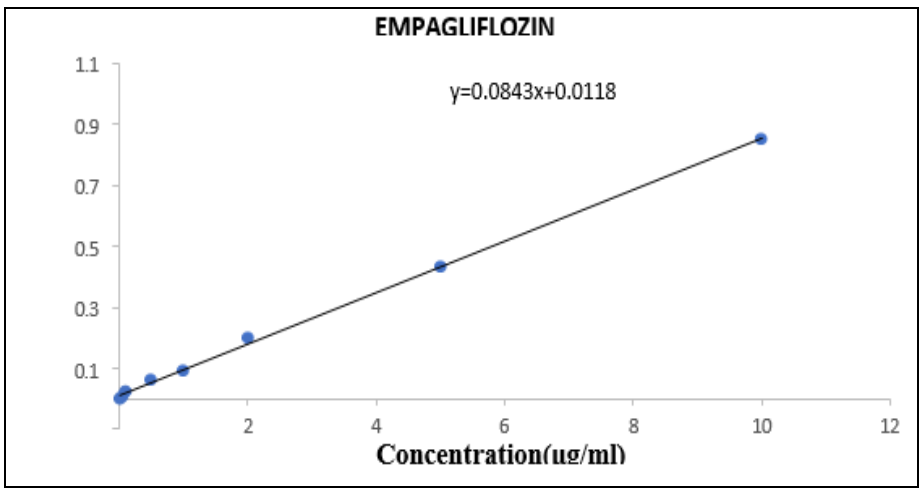

Fig 6: Calibration relationship for empagliflozin over 0.01-10 µg/mL.

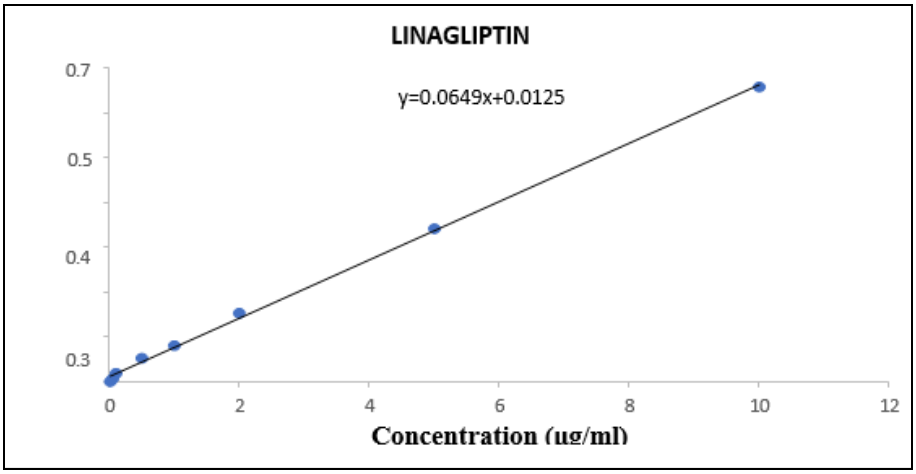

Fig 7: Calibration relationship for linagliptin over 0.01-10 µg/mL.

5. Precision and accuracy

Intra-day and inter-day precision and accuracy were assessed at LLOQ, LQC, MQC and HQC levels. The supplied study reports precision, ranges of 3.65-10.14% CV for empagliflozin and 4.07-9.16% CV for linagliptin. Accuracy ranged from 98.17-101.82% for empagliflozin and 98.00-101.22% for linagliptin.

Analyte	QC level	Nominal (µg/mL)	Mean calculated ± SD (µg/mL)	Mean recovery (%)
Empagliflozin	LLOQ	0.010	0.0099 ± 0.00086	99.39
Empagliflozin	LQC	0.10	0.0995 ± 0.00827	99.47
Empagliflozin	MQC	1.00	1.0048 ± 0.07351	100.48
Empagliflozin	HQC	5.00	5.0161 ± 0.20318	100.32
Linagliptin	LLOQ	0.010	0.0099 ± 0.00081	99.17
Linagliptin	LQC	0.10	0.0999 ± 0.00497	99.91
Linagliptin	MQC	1.00	0.9935 ± 0.06463	99.35
Linagliptin	HQC	5.00	4.9833 ± 0.22717	99.67

6. Extraction recovery

Extraction recovery was determined by comparing extracted QC samples with neat standards at equivalent concentrations. The reported mean recoveries were 65.03%, 66.12% and 55.16% for empagliflozin at LQC, MQC and HQC, respectively, and 67.72%, 62.04% and 54.39% for linagliptin. Thus, recovery was moderate in absolute terms but was reproducible across the tested concentration levels.

Analyte	QC level	Nominal (µg/mL)	Mean extracted response ± SD	Recovery (%)
Empagliflozin	LQC	0.10	16577 ± 108.81	65.03
Empagliflozin	MQC	1.00	60503 ± 730.2	66.12
Empagliflozin	HQC	5.00	285856 ± 985.58	55.16
Linagliptin	LQC	0.10	12553 ± 242.01	67.72
Linagliptin	MQC	1.00	53705 ± 197.76	62.04
Linagliptin	HQC	5.00	226272 ± 623.47	54.39

7. Reinjection reproducibility

LLOQ, LQC, MQC and HQC samples were retained at autosampler temperature for approximately 24 h and reinjected. For empagliflozin, mean accuracy was 100.00-101.77%; for linagliptin it was 98.33-102.58%. The corresponding CV values were 4.37-8.94% and 5.12-9.70%, respectively.

8. Ruggedness

Ruggedness was evaluated using different columns and different analysts. The reported mean recoveries for empagliflozin using different columns were 98.78-101.13% across QC levels and for linagliptin 99.43-100.00%. Across analysts, empagliflozin showed 98.33-101.10% and linagliptin 98.67-100.00% mean recovery.

9. Stability

Stability was evaluated at room temperature for 24 h, at −28 °C for 37 days, at −80 °C and after three freeze-thaw cycles. The reported results indicated that both analytes remained stable under the tested storage conditions. For freeze-thaw stability, mean accuracy ranged from 96.77-99.50% for empagliflozin and 96.29-99.22% for linagliptin.

Condition	Empagliflozin LQC accuracy (%)	Empagliflozin HQC accuracy (%)	Linagliptin LQC accuracy (%)	Linagliptin HQC accuracy (%)
Day zero	101.00	99.03	98.83	100.42
−28 °C	99.50	100.23	101.00	101.77
−80 °C	99.50	100.52	102.00	100.81
Freeze-thaw, 3 cycles	96.77	99.50	96.29	99.22

Discussion

The developed method provided chromatographic separation of empagliflozin and linagliptin in human plasma within a 10-min isocratic run. The selected C18 stationary phase, acidic aqueous phase and acetonitrile composition produced well-defined peaks with reported resolutions above 2. The low retention-time and peak-area variability observed during system suitability supports consistent chromatographic performance.

The method demonstrated a broad calibration range of 0.01-10 µg/mL for both analytes, with high reported linearity. Selectivity experiments indicated that endogenous plasma constituents did not interfere with the analytes or internal standard. Precision and accuracy results were close to nominal concentrations across the tested QC levels. The method also showed acceptable ruggedness and stability under the conditions evaluated.

A notable feature is the simple single-step protein-precipitation procedure, which requires only 250 µL plasma and uses acetonitrile as the precipitating solvent. Although the reported extraction recovery is moderate, it was reasonably consistent across QC levels and the internal-standard approach helps compensate for extraction and injection variability. For a final journal submission, the recovery and matrix-effect calculations should be independently checked against the original laboratory worksheets and chromatographic peak-area records.

Conclusion

A simple RP-HPLC method was developed for simultaneous determination of empagliflozin and linagliptin in human plasma using telmisartan as an internal standard.

The method employs a Discovery C18 column, isocratic buffer-acetonitrile elution, UV/PDA detection at 218 nm and a 10-min run time.

Validation results from the supplied study demonstrate specificity, linearity over 0.01-10 µg/mL, suitable precision and accuracy, acceptable ruggedness and stability, and reproducible extraction.

The method therefore provides a practical analytical procedure for plasma quantification and may be useful in pharmacokinetic and bioequivalence investigations.

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