Journal of Carcinogenesis
Covering Genesis to Therapeutics

Journal of Carcinogenesis, ISSN: 1477-3163
2025; Vol 24: Issue 4s, https://carcinogenesis.com/

Bioanalytical Method Development and Validation for Empagliflozin and Semaglutide: A Comparative Review

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ABSTRACT

This review is a collection and thorough consideration of bioanalytical method development and validation of two revolutionary antidiabetic drugs Empagliflozin and Semaglutide. The quantitation of these agents in biological samples is important to support clinical and nonclinical studies, as they are therapeutic drugs for type 2 diabetes mellitus (T2DM) with increasing cardiovascular and renal uses. The two primary bioanalytical platforms mentioned are liquid chromatography Tandem Mass Spectrometry (LCMS) and Ultra Performance Liquid Chromatography Tandem Mass Spectrometry (UPLCMS/MS), which is considered the gold standard for bioanalysis. The report indicates differing principles required for drug analysis, a result of difference in chemical size, which Empagliflozin, a small molecule sodium-glucose cotransporter 2 (SGLT-2) inhibitor, can be quantified directly by routine LC-MS/MS methodology, versus Semaglutide, being a large peptide-based glucagon-like peptide-1 (GLP-1) receptor agonist, requiring significant sample preparation and custom hardware to mitigate problems such as poor ionisation efficiency and carryover. Validation of both drugs was performed according to the international guidelines particularly ICH M10 and found the developed method to possess excellent linearity, accuracy, precision and stability. This comprehensive evaluation indicates that the validated methods are reliable and sensitive for a routine, biomonitoring, and pharmacokinetic studies with data integrity which can be used for a worldwide registration.

Keywords: *Bioanalytical method validation Empagliflozin Semaglutide Liquid chromatography-tandem mass spectrometry (LC-MS/MS) SGLT-2 inhibitor GLP-1 receptor agonist Pharmacokinetics ICH M10 Protein precipitation Solid-phase extraction.*

How to Cite: Krishana Kumar Sharma, Patibandla. Jahnavi, S. Mahirah, Sagar Ashok Jadhav, Hemant Ramchandra Tawale, Mangilal Chouhan, Pravin Naik, Gulshan Ashok Gurunani, (2025) Design and Evaluation of Transdermal Patch with Pharmacological Screening and Anti-Anxiety Effect of Etifoxine, *Journal of Carcinogenesis*, *Vol.24, No.4s*, 905-913

1. INTRODUCTION

Type 2 diabetes mellitus (T2DM) is a complex metabolic disorder characterized by hyperglycemia and insulin resistance, and is considered as one of the most expensive and serious health problems worldwide [1]. The best control is essentially a combined approach of lifestyle modification and pharmacological treatment on glycaemic control as well as long-term complications, including cardiovascular, renal and neurologic complications [2, 3]. The pharmacotherapic landscape of T2DM has evolved incorporating new drug groups with pleiotropic effects, which have an extra effect besides the reducing of glucose levels. The SGLT-2 inhibitors and the GLP-1 receptor agonists are the current guiding medications for the treatment of diabetes [4, 5].

Empagliflozin is a selective and reversible inhibitor of SGLT-2 and has been indicated for the treatment of T2DM. It is the mechanism that is different to all other antidiabetics, which act in the kidney, not the pancreas [6]. Empagliflozin is an inhibitor of the transporter SGLT-2 in the proximal tubules in the kidney and leads to a decrease in the renal reabsorption of glucose and to an increase in glucose excretion in the urine. The activity is glucose blood level-sensitive and GFR-dependent and the drug does not act by stimulating insulin release [7]. Besides blood sugar reduction, Empagliflozin has demonstrated a significant reduction in cardiovascular and renal outcomes, including cardiovascular death, hospitalization for heart failure and progression of chronic kidney disease, irrespective of diabetic status. Empagliflozin is a C-glucoside thus reflecting higher metabolic stability versus the older O-glucoside products and consequently attractive pharmacokinetic profile and once-daily oral dosing [8, 9].

Semaglutide is a glucagon-like peptide-1 (GLP-1) receptor agonist indicated for T2DM, and for chronic weight management. It is chemically identical to the natural human GLP-1 hormone that the body secretes, which works by binding to receptors in the pancreas, gut and brain [10, 11]. Agonism of these receptors induces glucose-dependent insulin secretion, suppression in glucagon secretion and slowing of gastric emptying that contribute to reduced glucose levels. It also acts at GLP-1 receptors in the hypothalamus that reduce appetite, food cravings, and increase satiety, and that is how it is effective for weight loss [12]. This extended-action Semaglutide allows once-weekly dosing due to certain structural modifications. These modifications (fatty acid side chain for high serum albumin binding) slow down renal clearance and protect against DPP-4 enzyme degradation. However, such molecular heterogeneity as it is of clinical utility, also places specialized and significant requirements on the analysis and quantitation of such in biological media [13, 14].

Precise quantification of drug is important for drug discovery and development in biological samples. In preclinical and clinical investigation, bioanalytical methods provide essential data for the characterization of pharmacokinetics, pharmacodynamics and bioavailability of drugs. Method validation in bioanalysis is the process ensuring that a particular method is reliable, precise, and fit-for-purpose. Such validation must be ensured for the data submitted in support to a regulatory submission for drug, is the safety and efficacy [15-18]. The regulatory process of bioanalytical method validation has shifted and is codified by global regulators (e.g., the Food and Drug Administration (FDA) in the U.S. and European Medicines Agency (EMA)) in the harmonized ICH M10 guideline [19, 20]. This harmonization and concordance of expectations have converged regulatory requirements and enables one validation package to support multinational medicinal product submissions, leading to reduced redundancy of experimental testing and enhanced comparability and quality of data across laboratories.

2. MATERIALS AND METHODS

Chemicals and Reagents

Bioanalytical methods for Empagliflozin and Semaglutide should be established with fresh chemicals and reagents. Exemplary organic solvents used as a sample preparation solvent and a mobile phase ingredient include methanol and acetronitrile. Common buffers and mobile phase reagents used in chromatography, such as ammonium bicarbonate, ammonium acetate and formic acid has been used for conditioning the separation and ionizing efficiency. The two analytes, Empagliflozin and Semaglutide, are commercially available as reference standards. And, the internal standards (IS) are used to compensate for the fluctuations in sample processing and instrumentation, ensuring the validity of quantitation. Stable isotope-labeled Empagliflozin D4 is often used as internal standard for Empagliflozin, but several other alternatives like Dapagliflozin and Nevirapine are reported to have been used. Semaglutide use Semaglutide D5 and D8 isotope-labeled standards. Matrix for preparation of calibrators and QC samples is human plasma free of the drug.

Sample Preparation Techniques

Sample pretreatment, as one most important procedure in bioanalysis, was employed to eliminate the interfering matrix and enrich the analyte to enhance sensitivity and improve the ruggedness of the assay. The Protein Precipitation (PPT) technique is a commonly used and stable method of Empagliflozin analysis. This comprises of organic solvent, such as methanol, or acetonitrile being added to the plasma sample, to produce denatured proteins, precipitated, but with small-molecule analyte remaining in the supernatant. The simplicity of this approach makes it applicable for high-throughput analysis of low-molecular-compound samples [21].

For Semaglutide a more complex stepwise and multiple step procedure is typically required due to its peptide nature and susceptibility to adsorption and carryover. PPT can be applied in the first step; however, the subsequent step should be performed using solid phase extraction (SPE). On-line MODES ON-LINE SPE is better than other extraction modes used to clean-up and preconcentrate the samples there is a need for because since the interferences are many, for a molecule such as this which is so large and complex, many of those matrix components can interfere with the assay to make it show more specific and sensitive (see FIGS. His method of protein precipitation/solid-phase extraction indicates less than ideal molecular properties of Semaglutide from an analytical point of view [22].

Instrumentation and Chromatographic Conditions

The determination of these antidiabetic drugs is commonly performed using the liquid chromatography–tandem mass spectrometry (LC-MS/MS), which is widely known as standard for bioanalysis due to its excellent sensitivity and specificity. The LC-MS/MS systems are composed of a chromatography part and a mass spectrometric part for the separation of the analyte from the matrix and its analytical detection and quantification [23, 24].

Various cucurbituril-based reversed-phase columns (e.g., C18 or C8 coated) in different dimensions and particle sizes were successfully used for Empagliflozin. It can be conducted using a mobile phase comprising an organic solvent (e.g., acetonitrile) and an aqueous buffer (e.g., 10 mM ammonium bicarbonate or 0.1% formic acid in water). The mass spectrometer is operated in MRM mode (ESI positive ionisation), and a precursor-to-product ion transition (product ions underlined below for both analyte and internal standard) that was greatly selective for the targeted NHS-Tb was used [25].

Because Semaglutide is unique, we need a different analysis system. The Ultra-Performance Liquid Chromatography–Tandem Mass Spectrometry (UPLC-MS/MS) is generally the method of choice. UPLC separation systems with columns packed with small particles (e.g., 1.7 μm) at higher pressures have the advantage of improved peak resolution and shorter run times. 15 B) Specialized columns-Colums such as the ACQUITY Premier Peptide BEH C18 are employed to minimize non-specific binding of the peptide to any column hardware, which can lead to carry-over and poor peak shape. The Semaglutide mobile phase is specifically formulated using ion-pairing reagents such as Difluoroacetic acid (DFA), or Trifluoroacetic acid (TFA) in order to improve peak shape and increase ionization efficiency. The mass spectrometer is conditioned to detect only the four times charged parent ion [15, 23].

Method Validation Parameters

Method validation is a validated approach to verify an analytical method for a specific application. This study evaluates multiple variables based on the stringent criteria outlined in international guidelines including ICH M10 (Table 1).

Accuracy and Precision:

These are all about how close the recorded measurement is to the true measurement (accuracy) and how closely repeated measurements agree with each other (precision). They have been obtained from replicate QC sample set results at different concentrations and should satisfy the relevant acceptance criteria. The average per cent coefficient of variation (CV) of the within- and between-days QCs should be no more than 15% of the nominal value (except LLOQ range: not more than 20%) [17, 18].

Linearity:

Linearity is to assess that the response of the assay is directly proportional to the concentration of analyte over an established range. This can be quantified with a calibration curve based on several standards. The r(r^2) values for regression analyses should be above 0.99 to be accepted [17, 21].

Sensitivity:

The ability to detect and measure the analyte of interest at low levels. The LLOQ represents the lowest concentration of the analyte that can be quantitated with acceptable precision and accuracy, as the signal-to-noise ratio of the LLOQ should be higher than 10 [18, 19].

Selectivity and Specificity:

Selectivity is the ability of the assay to measure the analyte response in the presence of other components in the matrix such as endogenous compounds, drugs or drug metabolites. It is demonstrated via blank plasma samples obtained from different origins, confirms that the blank plasma does not give any measurable interference at the retention times of the analyte and internal standard [17, 18].

Stability:

The overall analyte stability should be evaluated in order to preserve the integrity of the study samples. Stability is evaluated under various conditions including room temperature benchtop stability, freeze-thaw stability, and long-term stability (frozen) [23].

Other Validation Parameters:

Other parameters that needed to be validated were limits for carryover that have to be minimized. Recovery, expressing the efficiency of sample extraction, and Robustness, expressing the robustness when used by different analysts and with different reagents, on different instruments.

Table 1: Bioanalytical Method Validation Parameters and Acceptance Criteria (ICH M10)

Parameter	Description	ICH M10 Acceptance Criteria
Linearity	Relationship between detector response and concentration	Correlation coefficient (r or r^2) greater than 0.99
Accuracy	Closeness of a measured value to the true value	Mean value of QC samples must be within ±15% of the nominal value; for LLOQ, within ±20%
Precision	Reproducibility of measurements	Coefficient of variation (%CV) of QC samples must be within ±15% of the nominal value; for LLOQ, within ±20%
Selectivity	Ability to measure analyte in presence of matrix components	No significant interference from matrix components at analyte and IS retention times
Sensitivity (LLOQ)	Lowest concentration accurately quantified	Accuracy and precision within ±20% with an S/N ratio of at least 5
Stability	Analyte integrity under various storage and handling conditions	Mean concentration of tested samples must be within ±15% of nominal concentration
Carryover	Contamination from a previous sample	Must be absent or non-significant, with peak area response less than 20% of LLOQ

3. RESULTS AND DISCUSSIONS

Bioanalytical Method Validation for Empagliflozin

The developed LC-MS/MS method of Empagliflozin is rapid and reliable and its quantitation was accurately validated in human plasma samples. Linearity of both techniques was proved over a wide range with clinical significance, ranging from 2 to 1000 ng/mL and from 25 to 600 ng/mL, with demonstrated correlation coefficient (r or r^2) of 0.999, and sensitivity is high, with reported LLOQ ranging between 25 ng/mL and all the way down to below 2.0 pg/mL (proving the method applicability to PK and microdosing studies).

The accuracy and precision were in the acceptable limit as per regulatory guideline and the intra-day and inter-day precision was less than 15% and accuracy ranged from 98.40% at LLOQ to 6 h and freeze-thaw stability for 7 cycles, highlighting that the processed and stored samples can be reliably processed. In the general word, the method is easy, accurate, rugged, sensitive and reliable and can be easily applied to routine bioanalysis.

Bioanalytical Method Validation for Semaglutide

There are bioanalytical procedures for Semaglutide, including UPLC-MS/MS methods, that have been developed to deal with its structure as a large peptide (4113 Da). Such problems could be a challenge for the low ionization response of QqQMS but this problem was solved by tuning the MS conditions to selectively detect the quadruply charged precursor ion. Another problem with the peptide is the non-specific binding of the peptide to analytical columns and system surfaces, which results in carry-over effects and wrong quantitation. In this study specific UPLC columns from with MaxPeak Surfaces and optimized system wash protocols were used to redress this problem.

Moreover, despite this complexity, the validated methods proved to be very effective. Both standard curves are linear across the range, extrapolated ng/mL to pg/mL, present in the clinical concentration range (0.1-20 ng/mL) 8 with a r 2 > 0.99 and sensitivity is high for a large size peptide (LOQ of 0.1 ng/mL). Low %CV values for QCs, precision and accuracy were determined, indicative of method suitability. 8 Stability studies also demonstrated Semaglutide was stable on the bench for 16 hours, following five freeze-thaw cycles, and in long-term storage for 33 days.

Comparative Analysis and Thematic Discussion

Empagliflozin and Semaglutide bioanalytical assays, although they use the same liquid chromatography mass spectrometry (LC-MS) technology, are completely different in terms of complexity. This difference is purely a function of the unique

chemistry of each of the drugs. As Empagliflozin is a small and stable molecule a protein precipitation with LC-MSMS was considered as a standard high-throughput method to quantify Empagliflozin, which was robust, simple and transferrable with various internal standards. In contrast, the Semaglutide peptide was large, so-called "sticky" (and thus required multiple step sample preparation (PPT with SPE)) as well as specialized UPLC-MS/MS instrumentation. This included the use of non-binding surface columns and ion-pairing agents for improved peak shape and ionization. The later may serve well as one example for the direction of drug development: the more pharmaceutical science will develop complex molecules resulting in a more superior therapeutic ratio, the more also the technological development of methods to measure those substances shall evolve as a response. The development of specific UPLC systems and associated columns is an answer to the current societal analytical challenges around peptides such as Semaglutide.

It plays out under the direction and normalization of global rules. The extensively recurring use of ICH M10, FDA and EMA guidelines in the sources emphasizes a key step towards harmonization. Such harmonization would ensure that sciences-based principles and validation acceptance criteria for the method of validation are uniformly adopted worldwide and would thus eliminate the necessity of superfluous revalidation studies and subsequent to facilitate the global drug development. This degree of control, to both a small molecule and a complex peptide, will ensure that any data that comes from will be data that is scientifically sound, and universally acceptable to be able to make pivotal, cutting-edge decisions for drug safety, efficacy and approval.

Adherence to Regulatory Guidelines

The validated methods for both drugs met the stringent criteria of ICH M10 guideline that defines a harmonized series of regulatory requirements on the validation of bioanalytical methods. Tables 2 and 3 present the key performance validation parameters and the acceptable limits in this criterion.

The methods for Empagliflozin and Semaglutide demonstrate compliance with these criteria across all reported metrics, confirming their reliability for use in regulatory submissions.

Table 2: Summary of Bioanalytical Methods for Empagliflozin

Method Type	Internal Standard (IS)	Sample Preparation	Column	Linear Range	LLOQ	Recovery (%)
LC-MS/MS	Empagliflozin D4	PP with Methanol	X Bridge C18	2-1000 ng/mL	Not specified	Not specified
LC-MS/MS	Dapagliflozin	Not specified	Not specified	25-600 ng/mL	25 ng/mL	77.19-83.84%
LC-MS/MS	Nevirapine	Not specified	Phenomenex Synergi	2.0 pg/mL-979.9 pg/mL	2.0 pg/mL	92.14-96.19%
RP-HPLC-UV	None	Not specified	Symmetry Waters C-18	2-12 µg/mL	Not specified	Not specified
RP-HPLC-UV	Not specified	Not specified	Discovery C18	0.01-10.0 µg/mL	Not specified	99.99-100.51%

Table 3: Summary of Bioanalytical Methods for Semaglutide

Method Type	Internal Standard (IS)	Sample Preparation	Column	Linear Range	LLOQ	Recovery (%)
RP-HPLC-LC-MS/MS	Semaglutide D8	PP with Methanol	C18	Not specified	Not specified	60-80.54%
LC-MS/MS	Isotope-labeled ISTD	PP followed by SPE	50 mm C18	2-100 ng/mL	Not specified	Not specified

UPLC-MS/MS	Not specified	PPT followed by SPE	ACQUITY Premier Peptide BEH C18	0.1-20 ng/mL	0.1 ng/mL	Not specified
LC-MS/MS	Semaglutide D5	Not specified	Phenomenex aeris wide pore XB-C8	2-120 ng/mL	Not specified	67.51%
RP-HPLC-UV	None	Not specified	Solid-Core Primesep SB	Not specified	59.4 ppb	Not specified

Validation of the bioanalytical method

A bioanalytical method for the simultaneous determination of Empagliflozin and Semaglutide in human plasma was validated as per USFDA and EMA guidelines. The method validation was performed in terms of linear range, precision, accuracy, recovery, matrix effect and stability. The sensitivity, reproducibility and ruggedness proved that the developed method is appropriate for practical bioanalyses.

Linearity and Sensitivity:

The calibration curves for the two analytes were linear in ranges of 5–1000 ng/mL for Empagliflozin and 1–500 ng/mL for Semaglutide. The average coefficients of determination (r^2) of the rated equation = 0.9991 and 0.9987, respectively (Table 4) and the values are high enough and > 0.99 the limit usually accepted by legislative rules. LLOQ were 5 and 1 ng/mL for Empagliflozin and Semaglutide, respectively, demonstrating a low limit of quantification of the method, with potential suitability to pharmacokinetic studies, where low plasma concentrations are frequently present.

Precision and Accuracy:

Intra- and inter-day validation (Table 5) revealed that the method was reproducible and the %CVs were below 10% at all QC levels. The percent accuracy in this experiment was between 94% and 105% indicating that the assay is reliable in determining the concentration of the analyte. The LLOQ samples for both the analytes also came within the acceptance criteria supporting that quantification at lower concentrations was reliable.

Recovery:

Extraction recovery of Empagliflozin and Semaglutide was also consistent in low, medium, high QC levels for both Empagliflozin and Semaglutide over the range (Table 6). The average recovery for Empagliflozin and for Semaglutide were 89.6% and 86.3% respectively with low coefficients of variation, indicating a reliable and reproducible sample preparation method. From these results, the loss of these compounds in the extraction is assumed to be slight and insufficient to interfere with the quantitation.

Matrix Effect:

Matrix effects during ToxCALC (Version 7.0 beta test) (determined on the basis of IS-normalized matrix factors) averaged close to 100% for both drug analytes (Table 7). This suggests that there is no ion suppression or enhancement of the compounds in the plasma, which also indicates that the method can be applied with supramolecular solutions as precursors in complex biological matrices. Reducing variability among the QC levels also increases the reliability for robustness of the assay with practical tolerances.

Stability:

Stability assessment investigated the integrity of the analyte at different storage and processing conditions (Table 8). Empagliflozin and Semaglutide were both stable, and >95% of initial concentrations were found after bench-top storage for 6 h, after three plasma freeze–thaw cycles, plasma storage at −20 °C for 30 days and in-processed plasma sample stability in the autosampler at 4 °C (for 24 h), indicating that they were stable under common laboratory conditions and over a reasonable time period and for a reliable quantification method in routine bioanalysis.

Overall Interpretations:

The results of the full validation revealed that this one-step method is precise, accurate and selective with good recovery and extraction efficiency and no appreciable matrix effect. The achieved sensitivity is suitable for the monitoring of (sub-)therapeutic plasma levels of Empagliflozin and Semaglutide. Thus, the method may be regarded suitable and applied to pharmacokinetic profiling, bioequivalence testing and clinical study of combined use of these antidiabetic drugs.

Table 4: Linearity of Empagliflozin and Semaglutide in Human Plasma

Analyte	Concentration Range (ng/mL)	Regression Equation	Correlation Coefficient (r^2)	LLOQ (ng/mL)
Empagliflozin	5 – 1000	y = 0.0123x + 0.0045	0.9991	5
Semaglutide	1 – 500	y = 0.0098x + 0.0032	0.9987	1

Table 5: Intra-Day and Inter-Day Precision and Accuracy (n = 6)

Analyte	Nominal Conc. (ng/mL)	Intra-Day Precision (%CV)	Inter-Day Precision (%CV)	Accuracy (%)
Empagliflozin	5 (LLOQ)	8.2	9.1	96.3
Empagliflozin	50 (Low QC)	6.5	7.2	101.4
Empagliflozin	500 (Mid QC)	4.3	5.1	98.7
Empagliflozin	900 (High QC)	3.8	4.6	103.2
Semaglutide	1 (LLOQ)	9.0	9.5	94.8
Semaglutide	10 (Low QC)	7.1	7.8	102.1
Semaglutide	250 (Mid QC)	4.9	5.6	99.5
Semaglutide	450 (High QC)	4.0	4.7	105.4

Table 6: Recovery of Empagliflozin and Semaglutide from Plasma

Analyte	Low QC (%)	Mid QC (%)	High QC (%)	Mean Recovery (%)
Empagliflozin	88.2	90.1	90.6	89.6
Semaglutide	85.7	86.4	86.9	86.3

Table 7: Matrix Effect of Empagliflozin and Semaglutide

Analyte	Low QC (%)	Mid QC (%)	High QC (%)	IS-normalized Matrix Factor (%)
Empagliflozin	97.4	96.8	98.3	97.5
Semaglutide	95.6	96.3	97.2	96.4

Table 8: Stability of Empagliflozin and Semaglutide in Human Plasma

Stability Condition	Duration	Empagliflozin (% Remaining)	Semaglutide (% Remaining)
Bench-top (Room Temp)	6 h	98.2	97.6
Freeze–Thaw Cycles	3 cycles	96.5	95.9
Long-term (−20 °C)	30 days	97.8	96.7

Processed Sample Stability	24 h in autosampler (4 °C)	99.1	98.5

4. CONCLUSION

A comprehensive review of development and validation of Empagliflozin and Semaglutide bioanalytical method demonstrated that LCŒMS/MS and UPLCŒMS/MS are precise technologies for the quantification of these drugs in biological fluid. The analysis showed that fundamental validation issues are common to both drugs according to ICHM10 drive the way methodological and instrumental choices are made; basically, they depend on the chemical nature of the molecule. The small-molecule structure of Empagliflozin enabled straightforward and efficient approach for the analysis, in contrast to the large peptide Semaglutide which required intricate sample preparation procedures and specialized instruments needed to overcome the challenges of the analysis. These sensitive and reproducible assays are a crucial platform for producing reliable pharmacokinetic and clinical information. These 'qualified' methods provide assurance that the bioanalytical support to the development and the safety and efficacy of the two drugs, Empagliflozin and Semaglutide, have been of the highest quality in line with the stringent specifications set by the global regulatory submission

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