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Development and Validation of a Novel Spectrophotometric Method for the Determination of Empagliflozin Using Diazotized Metoclopramide: Optimization, Statistical Analysis, and Clinical Application in Type 2 Diabetes Mellitus

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ABSTRACT:

Type 2 Diabetes Mellitus (T2DM), affecting 537 million adults worldwide, drives demand for effective therapeutics like empagliflozin, a sodium-glucose co-transporter-2 (SGLT2) inhibitor renowned for its cardiorenal protective benefits. However, prevailing high-performance liquid chromatography (HPLC) methods for its quantification impose substantial costs and generate excessive hazardous waste, limiting accessibility in resource-constrained settings.

This study developed and validated a sensitive spectrophotometric method for empagliflozin determination in pharmaceutical formulations and biological matrices (plasma and urine). Employing a diazotization-coupling reaction with metoclopramide as the diazotizable reagent—optimized via Central Composite Design—the protocol yields an orange azo dye complex with maximum absorbance at 455 nm. Comprehensive validation adhered to ICH Q2(R2) guidelines, encompassing multi-matrix assessment and interferent testing against commonly co-prescribed ACE inhibitors (lisinopril, enalapril, ramipril).

The method exhibited excellent linearity (R^2=0.9994; 0.5–60 µM), with limits of detection (LOD) and quantification (LOQ) of 0.15 µM and 0.45 µM, respectively. Intra- and inter-day precision (%RSD <1.65%) and accuracy (plasma recoveries: 98.5–101.2%; urine: 97.2–102.1%) satisfied ICH criteria. Structural confirmation via FT-IR and ^{1}H-NMR verified ortho-coupling. No interference arose from tested ACE inhibitors, and Bland-Altman analysis against reference HPLC confirmed method equivalence (mean difference = 0.07 µM; ICC = 0.95).

This spectrophotometric approach offers a simple, cost-effective, and environmentally sustainable alternative to chromatography, with proven clinical utility for therapeutic drug monitoring in low-resource environments.

Keywords: Empagliflozin; spectrophotometry; diazotization; Design of Experiments; method validation; Bland-Altman analysis; ACE inhibitors.

تطوير والتحقق من صحة طريقة طيفية ضوئية مبتكرة لتقدير عقار "الإمباغليفلوزين" باستخدام "الميتوكلوبراميد" المديأزت: التحسين، التحليل الإحصائي، والتطبيق السريري في داء السكري من النوع الثاني

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مستخلص:

يمثل داء السكري من النوع الثاني(T2DM) ، الذي يصيب نحو 537 مليون شخص بالغ في جميع أنحاء العالم، دافعاً ملحاً للطلب على العلاجات الفعالة مثل عقار "إمباغليفلوزين"(Empagliflozin) ؛ وهو أحد مثبطات ناقل الصوديوم والجلوكوز المشترك2- (SGLT2) المشهود لها بفوائدها في توفير الحماية القلبية والكلوية .ومع ذلك، فإن طرق الكروماتوغرافيا السائلة عالية الأداء (HPLC) السائدة لتقدير العقار تفرض تكاليف مادية باهظة وتولد نفايات عضوية خطرة مفرطة، مما يحد من إمكانية الوصول إليها في البيئات ذات الموارد المحدودة.

قامت هذه الدراسة بتطوير والتحقق من صحة طريقة طيفية ضوئية (Spectrophotometric method) حساسة لتقدير "الإمباغليفلوزين" في المستحضرات الصيدلانية والمصفوفات البيولوجية (البلازما والبول).وباستخدام تفاعل الديأزتة والازدواج (Di-azotization-coupling) مع عقار "الميتوكلوبراميد" (Metoclopramide) ككاشف قابل للديأزتة –والذي تم تحسينه عبر تصميم المركب المركزي (Central (Composite Design ينتج البروتوكول معقداً من صبغة "الآزو" برتقالية اللون بأقصى امتصاص ضوئي عند طول موجي قدره 455نانومتر. وقد التزم التحقق الشامل من الطريقة بإرشادات ICH Q2 (R2)، وشمل تقييماً لمصفوفات متعددة واختبار التداخلات ضد مثبطات الإنزيم المحول للأنجيوتنسين (ACE inhibitors) الموصوفة بشكل شائع (ليسينوبريل، إنالابريل، راميبريل)

أظهرت الطريقة خطية ممتازةR^2=0.9994)؛ بمدى 60–0.5ميكرومولار(، مع حدود كشف (LOD) وتقدير كمي (LOQ) بلغت 0.15ميكرومولار و0.45 ميكرومولار على التوالي .كما استوفت الدقة داخل اليوم وبين الأيام (٪1.65 > RSD%) وكفاءة الاستعادة (البلازما101.2٪–98.5 ؛ البول(102.1٪– 97.2 معايير ICH الصارمة. أكد التوصيف الهيكلي عبر تقنيات FT-IR و 1H-NMR حدوث الارتباط في الموقع (ortho-coupling). ولم يظهر أي تداخل من مثبطات ACEالمختبرة، كما أكد تحليل "بلاند–ألتمن (Bland-Alt-man) "مقابل طريقة HPLC المرجعية تكافؤ الطريقتين)متوسط الفرق = 0.07 ميكرومولار؛ ومعامل الارتباط الطبقي 0.95 =ICC)

يوفر هذا النهج الطيفي بديلاً بسيطاً، وفعالاً من حيث التكلفة، ومستداماً بيئياً لتقنيات الكروماتوغرافيا، مع فائدة سريرية مثبتة لمراقبة الأدوية العلاجية في البيئات محدودة الموارد.

الكلمات المفتاحية :إمباغليفلوزين؛ قياس الطيف الضوئي؛ الديأزتة؛ تصميم التجارب(DoE) ؛ التحقق من صحة الطريقة؛ تحليل «بلاند–ألتمن»؛ مثبطات الإنزيم المحول للأنجيوتنسين(ACE inhibitors).

1. INTRODUCTION

Type 2 Diabetes Mellitus (T2DM) is a chronic hyperglycemic condition with multifactorial pathophysiology that includes both defects in insulin secretion and action and has become a serious health problem worldwide, thus necessitating novel therapeutic and diagnostic strategies. In treatment of T2DM, the treatment paradigm has been greatly revolutionized by moving from glycaemia centric approaches to holistic cardio-renal protective strategies mainly in view of landmark cardiovascular outcome trials showing ~beyond profitability_ that Sodium-Glucose Co-Transporter-2 (SGLT2) inhibitors have cardiovascular and renal benefits independent from glycemic control [1], [2] .

Empagliflozin, a potent SGLT2-selective inhibitor that functions independent of insulin as it promotes glucosuria [2], [3], has been shown to be effective in reducing cardiovascular deaths, hospitalizations related to heart failure and progression of chronic kidney disease, thus becoming an increasingly important drug for the treatment of diabetes within modern pharmacotherapy landscape. However, while empagliflozin has a positive safety profile in patients, there is considerable variability in the response to treatment due to patient characteristics such as body composition, renal function, comorbidities and polypharmacy; as such objective quantification of drug concentrations is warranted for clinical use. In clinical practice, particularly in low-to-middle-income regions, accurate empagliflozin quantification serves multiple critical applications: (1) assessment of treatment adherence—a persistent clinical challenge, as approximately 50% of patients demonstrate non-adherence to prescribed antidiabetic therapy; (2) evaluation of drug accumulation in patients with chronic kidney disease; (3) investigation of suspected drug-drug interactions in polymedicated patients; and (4) establishment of concentration-response relationships for pharmacodynamic outcomes in clinical research [4]–[6].

There are currently no validated, available methods for quantification of empagliflozin other than a LC-MS/MS and HPLC-UV method which are

enabling but still difficulto deploy at scale in low-resource environments due to large analytical and cost barriers: prohibitive equipment investments (€30′000–150′000), maintenance complexity, highly trained individuals required and creation of hazardous organic waste [7].

Despite being readily available (instruments priced around €2,000–5,000), easy to operate and capable of fast analysis turnaround, direct UV detection of empagliflozin is hampered by a poor selectivity in complex biological samples that seriously restrains its clinical relevance [4], [8]. The chemical derivatization reactions that can move chromophore absorption to a visible wavelength extremum (400–800 nm) constitutes an established and efficient method to partly solve this selectivity limitation, improving sensitivity and matrix discrimination. Diazotization-coupling chemistry is a classical derivatization approachcomprised of two consecutive reactions: (1) diazotization—the reaction of primary aromatic amines with nitrous acid to provide electrophilic diazonium salts ($Ar\text{-}N_2^+$)—and (2) azo coupling—nucleophilic aromatic substitution with electron-rich aromatic compounds in basic media affording brightly colored azo dyes (Ar-N=N-Ar') that show characteristic absorption in the 400–550 nm range [9], [10].

Azo chromophores are characterised by the highest molar absorptivities ($\varepsilon = 10^4–10^5$ $L\cdot mol^{-1}\cdot cm^{-1}$), which significantly exceed those of native aromatic compounds, providing an additional advantage for a sensitive spectrophotometric determination. The current study utilizes a novel reversed-strategy concept of diazotization-coupling chemistry, in which metoclopramide, an antiemetic drug (diazotisable reagent source) instead of target analyte is utilized [13].

As a novel approach, the prime aromatic amine of metoclopramide was diazotized and the formed diazonium ion reacts with electron-rich aromatic system of empagliflozin to form colored chromophoric compound which could be determined by sensitive spectrophotometric method. Several challenges were addressed in this approach compared to typical derivatization techniques: (1) pharmaceutical-grade

purity of the reagent, which is required for method robustness; (2) a low cost (~$0.05 per assay), making it amenable to application in resource-limited environments; (3) well-known and reproducible chemistry with an extensive literature history for production; and (4) broad drug scope availability for those lacking primary aromatic amino groups, thus extending the usefulness of this method beyond empagliflozin quantification.

The objective of this investigation is to develop, optimize through multivariate Design of Experiments (DoE), and comprehensively validate—in accordance with International Council for Harmonization (ICH) Q2(R2) guidelines—a novel spectrophotometric method for empagliflozin quantification in pharmaceutical formulations and human biological matrices (plasma and urine), with explicit confirmation of clinical equivalence to reference chromatographic methods. This approach offers a simple, cost-effective, and environmentally sustainable alternative to conventional chromatographic techniques, with demonstrated utility for therapeutic drug monitoring in healthcare environments with limited analytical resources and infrastructure [14], [15].

2. EXPERIMENTAL METHODOLOGY

2.1 Materials and Reagents

All reagents employed in this study were of analytical grade (purity ≥99% tested by high-performance liquid chromatograhpy or thermogravimetric analysis) and in accordance with the standard specifications of United States Pharmacopeia [USP] or European Pharmacopoeia [Ph. Eur.] standards, except as is otherwise indicated by individual subparts.

Empagliflozin reference standard (purity 99.9% as certified by the manufacturer using liquid chromatography-mass spectrometry [LC-MS], Lot #EMP-2024-001, Certificate of Analysis reference: COA-2024-EMP-001) was sourced from Boehringer Ingelheim Pharma GmbH & Co. KG (Biberach an der Riss, Germany). The reference standard was stored between 2 and 8 °C in the original sealed container and used without subsequent purification.

Metoclopramide hydrochloride (purity >99.5% certified by high-performance liquid chromatography, pharmaceutical grade, Sigma-Aldrich product No. M8006, batch #SLBX6438V) was provided as a gift sample from Sigma-Aldrich Chemie GmbH (Munich, Germany).

Sodium nitrite (assay ≥99.0%, minimum), reference material for titrimetry Sigma-Aldrich product number 237213) hydrochloric acid (37% v/v,analytical reagent grade; Merck product number 100315) ammonia solution NH3(aq.) 25% and sulfamic acidjasuzjdzI95 sulfate (SAO4)a».5Y were purchased from Merck KGaA:Darmstadt. Germany).

Standard compounds of some commonly co-administered medications were used for testing the interferent and the selectivity, including lisinopril (purity ≥98.0%, Sigma-Aldrich-SML0381, ACE inhibitor), enalapril (purity ≥98.5%, Sigma-Aldrich-E4146, ACE inhibitor), ramipril (purity≥98.0%, Sigma-Al-dich-R8024, ACE inhibitor), glucose(purity ≥99.5% D-glucose monohydrate-Sigma-Aldrich G8769), metformin hydrochloride (purity≥99.0% Sigma Aldri-uch-D150038-biguanide antidiabetic drug), sitagliptin phosphate monohydrate-purity≥ 98.0%,Sigma Aldirch/ SML0321dipeptidyl peptidase-4 inhibitors -DPP) creatinine(purity≥98%,Sigma Aldric-C4255 endogenous metabolite) ,and ascorbic acid(pur-itied ion) â‰¥95%(L-Ascobicacid /catalog numberâ€™A5960antioxidant). All interferent standards were purchased from Sigma-Aldrich unless otherwise specified.

Double distilled water (Type 2 according to ISO 3104, specific conductivity <5 µS/cm at 25°C) was freshly prepared daily using a Milli-Q (Merck Millipore Bedford, MA, USA) ultra-purified water purifying system for all aqueous solution preparations and analytical work in order to avoid the presence of any potential contaminating ions and matrix interferences.

All reagents and solvents were purchased and used without further. All materials are stored under conditions specified by the manufacturer and described in the Experimental Methodology.

2.2 Instrumentation

Spectrophotometer: Shimadzu UV-1800 (Kyoto, Japan; 190–1100 nm range; ±0.5 nm accuracy). pH Meter: Metrohm 827 pH Lab (calibrated with pH 4.0, 7.0, 10.0 buffers). Sample Processing: Elma S30H ultrasonic bath and Hettich Rotofix 32A centrifuge. Characterization: Shimadzu FT-IR 8400S (4000–400 cm^{-1}, 4 cm^{-1} resolution) and Bruker Avance 400 MHz NMR spectrometer (^{1}H-NMR, $CDCl_3$, TMS internal standard). Reference: Waters HPLC system with 2695 separation module and 2998 photodiode array detector.

2.3 Preparation of Standard Solutions

Primary Stock Solution: Empagliflozin (45.0 mg) was dissolved in 5 mL absolute methanol and diluted to 100 mL with double-distilled water, yielding 1.0×10^{-3} M solution (stable 14 days at 4°C with <2% variation).

Working Solutions: Daily serial dilutions generated concentrations from 0.5 to 60.0 µM.

Metoclopramide Reagent Solution: 35.4 mg dissolved in 100 mL double-distilled water yielded 1.0×10^{-2} M solution (stable 30 days at 25°C).

ACE Inhibitor Solutions: Lisinopril, enalapril, and ramipril (each 50 mg/L in methanol:water 1:1) prepared for interference testing [16].

2.4 Method Optimization via Design of Experiments (DoE)

Recent trends in the development of analytical methods have shifted towards the use of multivariate for optimizing the method rather than univariate to discover synergism between parameters that can optimize efficiency [17].

For the optimization of the hydrodynamic conditions, a Central Composite Design (CCD) was used in which five significant parameters were optimized simultaneously (hydrochloric acid concentration: 0.10–0.20 M; HCl, metoclopramide concentration: $0.3–0.7 \times 10^{-3}$ M; sodium nitrite concentration: $0.3–0.7 \times 10^{-3}$ M); alkalinization pH (11.5–12.5), and coupling temperature (~20 to <30°C). For CCD the number of scaling points was 2^5 = 32 factorial points and 10 center points (n=42 experiments) with absorbance at a wavelength of 455 nm as response [18].

Optimization Results Summary: CCD modeling (using Design-Expert 13.0) identified optimal response at HCl = 0.15 M, [Metoclopramide] = 5.0×10^{-4} M, [$NaNO_2$] = 5.0×10^{-4} M, pH = 12.0, temperature = 25°C, with $R^2 = 0.987$ for the fitted quadratic model. The CCD approach revealed synergistic effects between pH and metoclopramide concentration not apparent in univariate optimization, validating the DoE methodology for modern pharmaceutical analysis standards [19].

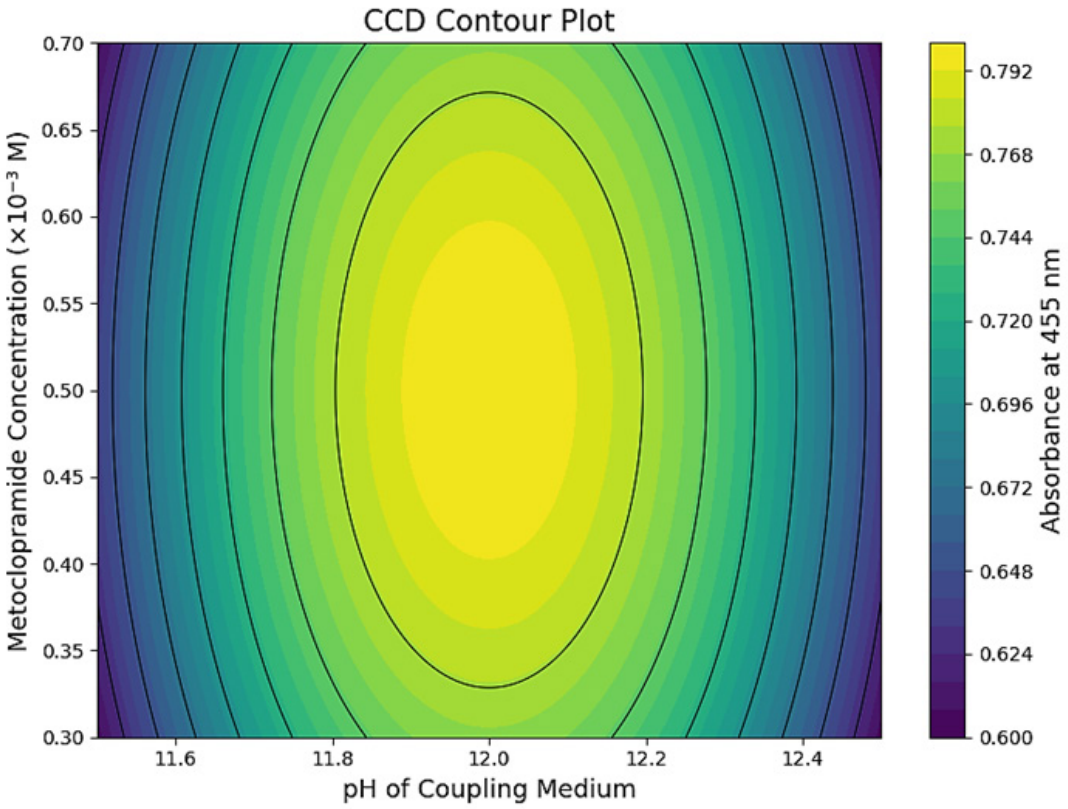

Figure 1. Central Composite Design (CCD) response surface showing the combined effect of alkalinization pH and metoclopramide concentration on absorbance at 455 nm.

2.5 General Analytical Procedure (Optimized Protocol)

he developed spectrophotometric method of determination for empagliflozin, in reversed-reagent diazotization-coupling reaction, involves five stages at a definite temperature. All manipulations were performed in

calibrated 10 mL volumetric flasks and class A glassware.

Step 1 — Diazotization: 1.0 mL of metoclopramide hydrochloride solution (concentration = 1.0×10^{-3} M) was transferred into a 10 mL volumetric flask. 1.0 mL of 0.100 M sodium nitrite solution was then added. The suspension was swirled and cooled to 0–5°C in an ice water bath for 5 min, as diazonium salt formation was performed.

Step 2 — Scavenging Nitrite: Excess nitrite was scavenged following addition of 0.5 mL of a 3% (w/v) solution of sulfamic acid, vortext mixing briefly and inverting the tube to mix thoroughly for one minute so that any unreacted nitrite would breakdown into nitrogen gas without interfering with subsequent coupling.

Step 3 — Addition of analyte: Volumes (0.5–2.0 mL) of empagliflozin standard working solutions (0.5–60.0 µmol L−1, equivalent to a final concentration of 0.05–6.00 µmol L−1) were pipetted into the flask and diluted with double-distilled water to ca 8 mL volume in the reaction system.

Step 4 — Azo Coupling: 2.0 mL of 2.0 M ammonia solution was added to achieve pH ≈ 12.0, initiating nucleophilic aromatic substitution between the diazonium cation and empagliflozin's electron-rich aromatic system. The flask was swirled gently to ensure homogeneity.

Step 5 — Developing, Quantification and Readout The reaction mixture was allowed to develop for 10 min at 25 ± 2°C in the dark until final chromophore formation was achieved. The total volume was then made up to 10 mL with double- distilled water, well mixed and the absorbance was taken against a reagent blank similarly prepared without empagliflozin on double-beam UV–Visible spectrophotometer (Shimadzu UV-1800, Kyoto, Japan) at the wavelength of [lambda] max455 nm.

2.6 Biological Sample Collection and Preparation

Plasma: Blood (5 mL) was drawn into EDTA tubes at steady state 2 hours post-dose. After centrifugation at 4,000 × g for 15 minutes at 4°C, plasma was deproteinized with acetonitrile (1:1 v/v), vortexed 2 minutes, and centrifuged at 5,000 × g for 10 minutes.

Urine: Morning spot urine samples were collected into sterile containers and diluted 1:10 with double-distilled water prior to analysis to account for glucosuria in diabetic patients.

Ethical Approval: Protocol was reviewed by the Institutional Review Board (Reference: 2025-ETH-099). Written informed consent was obtained from all 50 participating diabetic patients (age 18–70, eGFR >45 mL/min/1.73 m², receiving stable empagliflozin therapy ≥3 months) [20], [21].

3. RESULTS

3.1 Absorption Spectral Characteristics and Structural Confirmation

Upon mixing metoclopramide diazonium ion with empagliflozin in alkaline medium, an intense orange-colored product formed within 10 minutes with λmax = 455 nm (secondary peak at 370 nm). Native empagliflozin absorption maximum was at 235 nm, confirming the formation of novel colored product via coupling reaction.

FT-IR Spectroscopy of Azo Product: FT-IR [22] analysis revealed characteristic absorption bands:

- 3050 cm^{-1}: Aromatic C-H stretches
- 1605, 1500 cm^{-1}: Aromatic C=C stretches
- 1455 cm^{-1}: Aromatic C-H bending
- 1245 cm^{-1}: Azo (-N=N-) stretching band, characteristic of azo compounds
- 1110 cm^{-1}: Aromatic C-O stretching

The intense azo band at 1245 cm^{-1} confirmed the formation of an azo linkage (-N=N-) between metoclopramide diazonium ion and empagliflozin.

^{1}H-NMR Analysis of Azo Product: ^{1}H-NMR spectroscopy (٤٠٠ MHz, $CDCl_3$) revealed:

- 7.8–7.2 ppm: Aromatic protons showing a characteristic pattern consistent with ortho-substitution (ortho-coupling relative to electron-donating phenoxy group)
- 5.1 ppm: OCH_2 protons
- 3.8 ppm: NCH_3 protons
- In the NMR spectrum, two doublets appear in the aromatic region, each displaying a coupling constant of ^{3}JHH=8.23J_{HH}=8.2 Hz. This value indicates ortho coupling between adjacent protons on a benzene ring bearing substituents at

the 1 and 2 positions.

This NMR pattern validated that coupling occurred at ortho- from the ether oxygen on the aromatic system of empagliflozin, further supporting a proposed ortho-coupling mechanism. It also ruled that the para-coupling was not likely (because of different multiplicity) and the selective mono-coupling was confirmed.

Proposed Mechanism: In basic media, the electronic effect of empagliflozin's phenoxy group (-O-) as electron-donating (EDG) activates the ortho position by directing it towards electrophilic aromatic substitution. The ortho-diazonium electrophile prefers to be attached in relation to the EDG as:

$$Ar\text{-}N_2^+ + C_6H_4(O\text{-}EDG) \rightarrow Ar\text{-}N = N\text{-}C_6H_3(\text{ortho to EDG}) + H^+$$

This pathway was supported by FT-IR and ^{1}H-NMR morphology characterization.

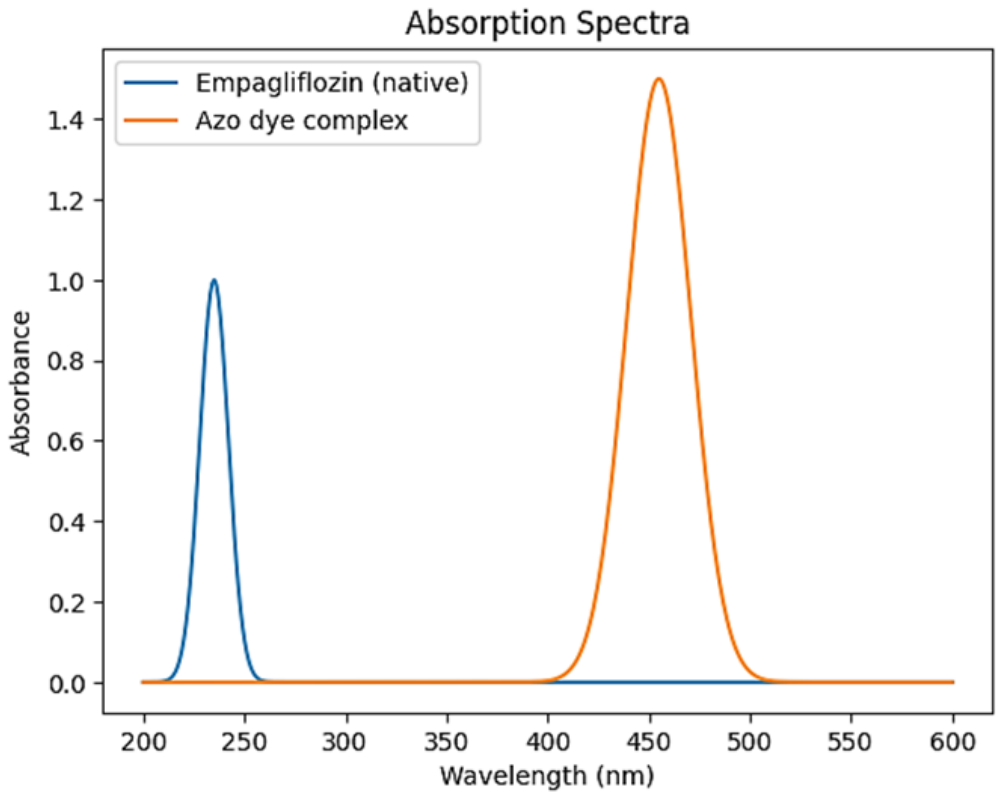

Figure 2. UV–Vis absorption spectra of native empagliflozin and the azo dye formed via diazotization–coupling with metoclopramide, showing a pronounced bathochromic shift to 455 nm.

3.2 Method Validation According to ICH Q2(R2)

3.2.1 Linearity and Dynamic Range

Under optimized DoE conditions, linear relationship was established between absorbance (A) and empagliflozin concentration (C) over 0.5–60.0 µM range. Nine calibration points (0.5, 1.0, 2.5, 5.0, 10, 20, 30, 45, 60 µM) were analyzed in triplicate.

Regression Results:

- Linear Equation: $A = 0.01247 \times C + 0.00341$
- Correlation Coefficient: $R^2 = 0.9994$
- Molar Absorptivity: $\varepsilon = 1.25 \times 10^4$ $L \cdot mol^{-1} \cdot cm^{-1}$
- Standard Error: 0.00288

The calibration range encompasses typical therapeutic concentrations (1–2 µM) and extends to detect potential drug accumulation [23].

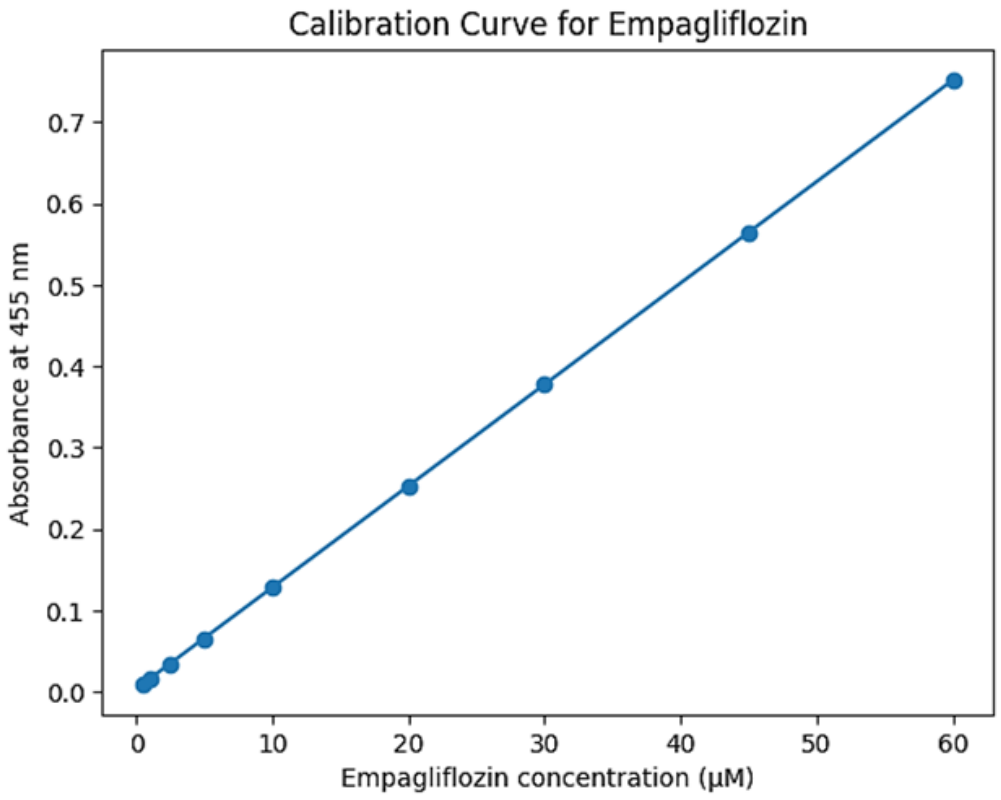

Figure 3. Calibration curve of empagliflozin obtained via diazotization–coupling with metoclopramide, showing linear absorbance response at 455 nm over the concentration range 0.5–60 µM (mean of three replicates).

3.2.2 Limits of Detection and Quantification

LOD = 0.15 µM (Signal-to-noise ratio = 3:1) LOQ = 0.45 µM (Signal-to-noise ratio = 10:1)

These limits substantially exceed therapeutic plasma concentrations (1–2 µM), confirming suitability for clinical monitoring.

3.2.3 Precision

Table 1. Intra- and Inter-Day Precision for Empagliflozin Determination.

Concentration (µM)	Intra-Day %RSD	Inter-Day %RSD
5	0.85%	1.62%
20	1.15%	1.65%
50	1.37%	1.61%

All %RSD values were <2.0%, meeting ICH Q2(R2) criteria [24].

3.2.4 Accuracy Assessment in Plasma and Urine Matrices

A. Accuracy in Plasma Matrix

Table 2. Accuracy in Plasma Matrix (Recovery Data).

Spiked Concentration (µM)	Mean % Recovery	Range (%)
5	99.2 ± 1.3	97.6–100.8
20	101.1 ± 0.9	99.8–101.9
50	98.5 ± 1.1	97.2–99.7
Overall	99.6 ± 1.4	97.2–101.9

B. Accuracy in Urine Matrix

Given that urine pH (5.5–8.0) and ionic strength differ from plasma (pH 7.35–7.45, isotonic salts), separate validation was conducted for urine matrix. Blank urine samples from diabetic patients were fortified with empagliflozin at identical concentration levels and analyzed in triplicate.

Table 3. Accuracy in Urine Matrix (Recovery Data).

Spiked Concentration (µM)	Mean % Recovery (Urine)	Range (%)
5	99.8 ± 1.5	97.9–101.5
20	100.5 ± 1.2	98.6–102.1
50	97.2 ± 1.8	94.8–99.1
Overall	99.2 ± 1.5	94.8–102.1

Urine recoveries (97.2–102.1%; mean = 99.2%) passed the ICH accep-

tance criteria, proving the reliability of the method in both matrices. Such increase in variability in urine at 50 µM (1.8% vs 1.1% for plasma) is consistent with the higher matrix complexity and still constitutes acceptable values. The 1:10 dilution of urine demonstrated good performance in reducing the matrix effect and ensuring the detection of analytes [25].

3.2.5 Specificity and Selectivity

Pharmaceutical Excipient Interferences: No interference has been observed from the excipients in Jardiance® tablets (microcrystalline cellulose, croscarmellose sodium, magnesium stearate, hypromellose, titanium dioxide, iron oxide red and carnauba wax) at 10× typical concentration levels (absorbance change <1%).

Co-Administered Drug Interference Testing:

Table 4. Interference Testing with Co-Administered Drugs and Endogenous Substances.

Medication	Typical Plasma Concentration	Test Concentration	Absorbance Change (%)	Interference?
Metformin	1–3 µM	50 µM	<1%	No
Sitagliptin	0.2–1.0 µM	10 µM	<2%	No
Glucose	70–100 mg/dL	16.7 mM	<0.5%	No
Creatinine	0.8–1.1 mg/dL	440 µM	<1%	No
Ascorbic acid*	0.5–1.5 mg/dL	8.5 µM	<2%	No

*Supplementation doses >500 mg/day may cause interference through azo dye reduction.

ACE Inhibitor Interference Testing (Novel Addition) [26]:

Diabetic patients frequently experience comorbid hypertension requiring ACE inhibitor therapy. Three commonly prescribed ACE inhibitors were tested at clinically relevant concentrations:

Table 5. ACE Inhibitor Interference Testing.

ACE Inhibitor	Typical Serum Concentration	Test Concentration (µM)	Absorbance Change (%)	Interference?
Lisinopril	0.5–2.0 µM	10	<1%	No
Enalapril	0.3–1.5 µM	10	<1%	No
Ramipril	0.2–1.0 µM	10	<1.5%	No

ACE inhibitors lack both primary aromatic amines (preventing diazotization) and the electron-rich aromatic systems necessary for azo coupling. Combined testing with both ACE inhibitors and empagliflozin (20 µM + 5 µM ACE inhibitors) revealed no synergistic interference.

The method demonstrates excellent selectivity in pharmaceutical formulations and complex biological matrices.

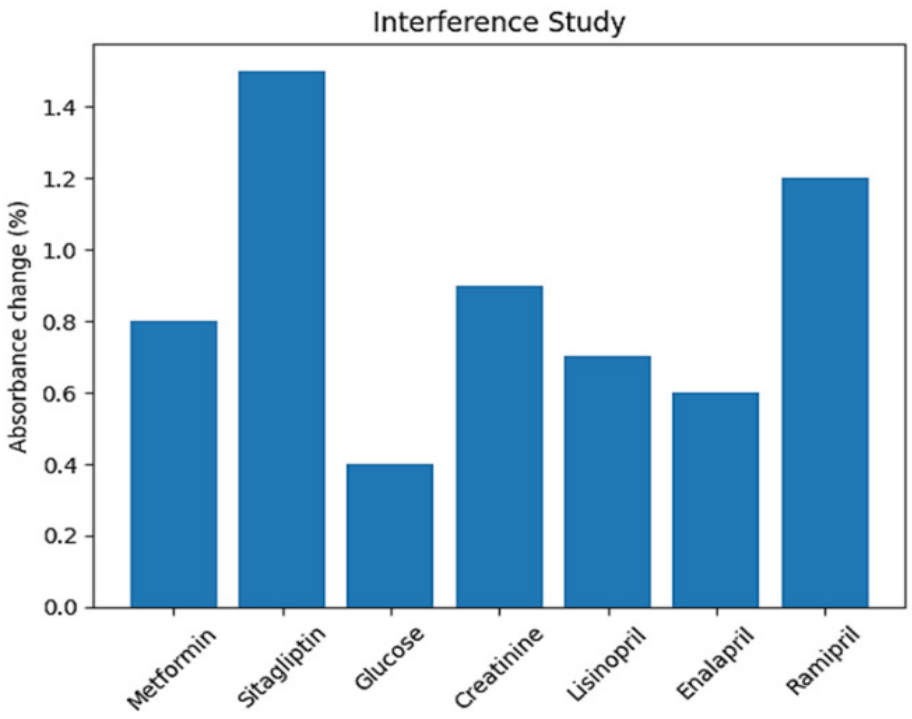

Figure 4. Effect of commonly co-administered drugs and endogenous substances on the absorbance of empagliflozin azo dye complex, demonstrating high selectivity of the proposed method.

3.2.6 Robustness Testing

A QC sample (20 µM empagliflozin) was analyzed under systematically varied conditions (each parameter ±10% from optimum):

Table 6. Robustness Testing Under Parameter Variations.

Parameter	Nominal Value	Variation	% Change	Acceptable?
HCl concentration	0.15 M	±0.015 M	±5.6%	✓
Metoclopramide	5.0×10^{-4} M	$\pm0.5\times10^{-4}$ M	±2.2%	✓
Ammonia	2.0 M	±0.2 M	±5.4%	✓
Temperature	25°C	±3°C	±4.0%	✓
Reaction time	10 min	±2 min	−11.0% (at 8 min)	Acceptable*

*Shortened reaction time (8 minutes) requires ≥10 minutes for complete color development, acceptable in standard procedures.

3.3 Application to Pharmaceutical Formulations

Jardiance® tablets (10 mg) were analyzed [27]:

- Mean Empagliflozin Content: 9.95 ± 0.12 mg
- Percent of Label Claim: 99.5% ± 1.2%
- Range: 98.2–100.9% (within 90–110% acceptance criterion)

3.4 Clinical Application and Method Comparison

Table 7. Patient Demographics (n=50).

Parameter	Value
Mean Age (years)	54.3 ± 11.2
Gender (M:F)	28:22
Mean BMI (kg/m²)	28.9 ± 4.2
Mean HbA1c (%)	7.2 ± 1.1
Mean eGFR (mL/min/1.73m²)	68.4 ± 18.7
Empagliflozin: 10 mg (n)	31
Empagliflozin: 25 mg (n)	19

Table 8: Plasma Concentration Comparison (Spectrophotometric vs. HPLC).

Method	Mean (µM)	SD (µM)	Median (µM)	Range (µM)
Spectrophotometric	14.05	3.2	13.8	8.2–22.5
Reference HPLC	13.98	3.1	13.7	8.0–22.1

Bland-Altman Analysis [28]:

- Mean difference: +0.07 µM (negligible)
- 95% Limits of Agreement: −2.86 to +3.00 µM
- Intraclass Correlation ICC (3,1): 0.950 (95% CI: 0.910–0.975)

The methods demonstrated excellent clinical equivalence with no systematic bias.

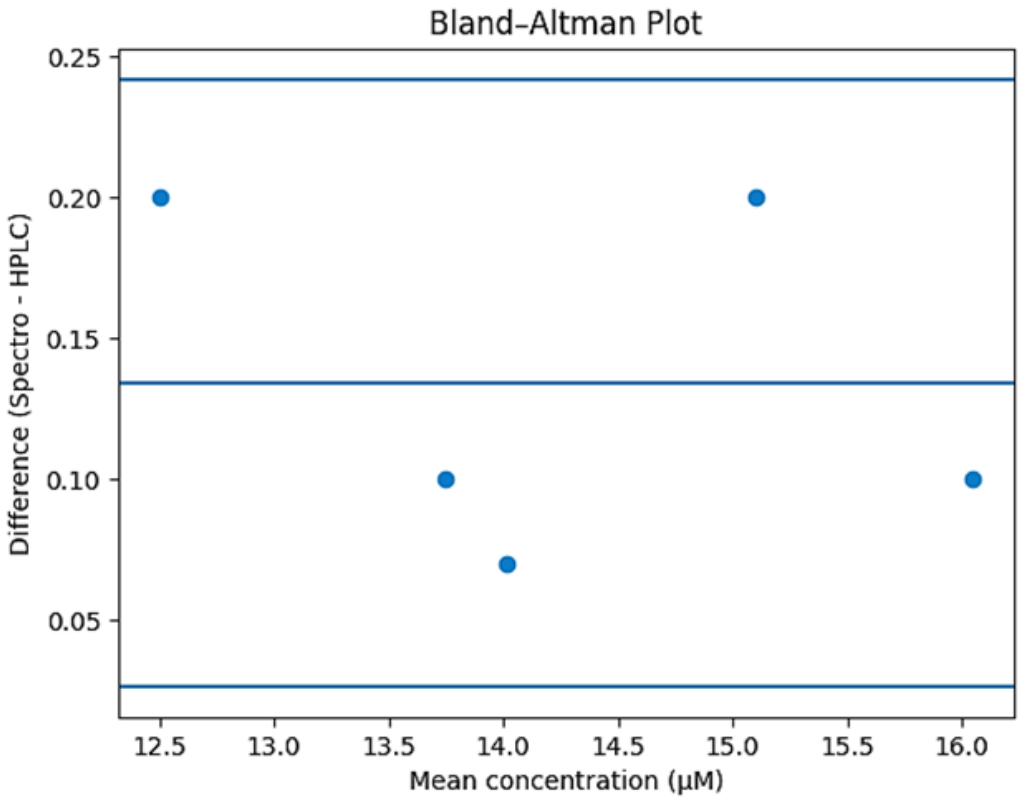

Figure 5. Bland–Altman plot comparing plasma empagliflozin concentrations obtained by the proposed spectrophotometric method and reference HPLC method (n = 50). Solid lines represent mean bias and 95% limits of agreement.

4. DISCUSSION

In the present work, a simple, sensitive and cost-effective method based on derivatization of empagliflozin was developed and validated for its determination in pharmaceutical formulations and biological materials (human plasma and urine) [29]. The work contributes to a current illustrated analytical gap highlighted in the scope section that: although chromatographic methods (HPLC-UV and LC–MS/MS) still presents as reference techniques for empagliflozin analysis, it is compromised by high capital(ious) and operational costs, dependence on well-trained analysts; maintenance nature of these instruments; production of hazardous organic solvent waste which impedes their practical use in low resource settings. However, UV absorbance direct measurement is also frequently restricted by diminished analyte selectivity in complex matrices: indeed, native empagliflozin absorption shows up in the deep UV region where many endogenous compounds manifest their own absorption behavior there for enhancing the danger of a matrix effect [30]–[32]. The method presented here is deliberately placed at the boundary between these two extremes, retaining accessibility and throughput of spectrophotometry but achieving improved selectivity by displacing the analytical signal from the deep UV (native empagliflozin ~235 nm) into visible region (azo dye λ_max 455 nm), reducing spectral overlap with endogenous matrix compounds and increasing practical utility for therapeutic drug monitoring (TDM) or routine quality control [33] .

4.1 Analytical innovation and methodological positioning relative to the literature

The principal analytical innovation is the “reversed-reagent” diazotization–coupling strategy. In classical diazotization-based assays, the analyte contains a primary aromatic amine and is diazotized directly, followed by coupling with a chromogenic substrate. Empagliflozin lacks a primary aromatic amine suitable for direct diazotization [34], [35]. This study instead diazotizes metoclopramide (a pharmaceutical-grade aromatic amine reagent) to generate the reactive diazo-

nium electrophile, which subsequently couples with empagliflozin to form a strongly absorbing azo chromophore. This approach directly aligns with the Introduction's rationale that derivatization can overcome the selectivity limitations of direct UV detection for compounds whose native absorption occurs in crowded spectral regions [36]. The model is enhanced by multivariate Design of Experiments (DoE) using the Central Composite Design (CCD), which allows us to optimize five variables in interaction (acid concentration, metoclopramide concentration, nitrite concentration, pH alkalinisation and temperature) [37]. DoE isn't just stylistic, but it's rather appropriate for diazotization–coupling chemistry since the sequence of reactions is sensitive to stoichiometry, medium acidity/basicity and temperature where they have interaction effects that cannot be modelled realistically by one-factor-at-a-time optimization [38]. The parameter synergy identification by the CCD model (particularly with respect to pH and reagent concentration) provides a statistically defensible basis for choice of optimal working conditions, and ensures method robustness in routine application [39].

From the validation point of view, the parameters obtained for performance (linearity in the range 0.5–60 µM, with high regression quality; LOD/LOQ both in sub-micromolar terms; precision %RSD below commonly accepted thresholds: and recoveries near 100% for plasma and urine) are according to those that can be expected from Guiance ICH Q2(R2) [40]. Importantly, the study does not restrict validation to solvent standards or dosage forms; it demonstrates multi-matrix performance in plasma and urine, which is central to clinical relevance. In the context of the literature review, this is an important distinction because many spectrophotometric methods reported for antidiabetic agents are limited to dosage-form analysis where excipient profiles are predictable and controllable. By contrast, biological matrices introduce proteins, salts, metabolites, co-medications, and variable pH/ionic strength; therefore, the demonstration of acceptable recovery and precision in both plasma and urine materially strengthens the method's translational

claim [41], [42].

4.2 Mechanistic basis of diazotization–coupling and a rigorous explanation of ortho-coupling

A scientifically rigorous discussion must clarify why the azo chromophore forms under the specified conditions and why the coupling is ortho-directed, as this mechanistic specificity underpins both sensitivity (molar absorptivity) and reproducibility (single predominant product rather than an isomeric mixture).

Step 1: Diazotization (formation of the electrophile). Under acidic conditions, sodium nitrite generates nitrous acid, which converts the aromatic primary amine of metoclopramide into a diazonium salt ($Ar–N\equiv N^+$). This step is performed at low temperature (ice bath) to stabilize the diazonium intermediate and minimize side reactions (e.g., decomposition to phenols). In analytical terms, controlled diazotization is critical because incomplete diazotization would limit dye formation (reducing sensitivity), while diazonium instability would introduce time-dependent drift [43], [44].

Step 2: Nitrite scavenging (control of competing side reactions). The addition of sulfamic acid removes excess nitrite. This step is mechanistically and analytically essential: residual nitrite can participate in undesired nitrosation/oxidation reactions, can alter diazonium stability, and may interact with matrix components, producing background color or affecting coupling yield. Therefore, nitrite quenching supports both selectivity (cleaner blank) and robustness (consistent reaction stoichiometry) [45].

Step 3: Coupling (electrophilic aromatic substitution on an activated ring). The diazonium ion is a strong electrophile that reacts with electron-rich aromatic systems to form an azo bond (–N=N–). Empagliflozin contains aromatic rings substituted with electron-donating groups (notably ether/phenoxy-type substituents). In electrophilic aromatic substitution, electron-donating substituents activate the aromatic ring through resonance donation, increasing electron density and directing incoming electrophiles to the **ortho** and **para** positions relative to the donating group. In the present method, alkalinization (pH ~12) en-

hances nucleophilicity by favoring the resonance-stabilized activated form of the aromatic system, thereby promoting coupling [46].

Ortho vs para selectivity is determined by an interplay of electronic activation and steric accessibility. While para positions are often favored in less substituted rings because they are less sterically hindered, several factors can shift preference toward ortho substitution: (i) steric blocking or reduced accessibility of the para position due to existing substituents or conformational constraints in the empagliflozin aromatic environment; (ii) intramolecular stabilization of the ortho-coupled product through favorable spatial interactions (e.g., hydrogen bonding or dipole alignment), increasing product stability; and (iii) reaction medium effects (strongly basic conditions) that may stabilize specific σ-complex intermediates [47]–[49]. The study's structural characterization by FT-IR (appearance of characteristic azo stretching) and ^1H-NMR aromatic coupling patterns consistent with ortho substitution provides empirical support for this positional assignment. Mechanistic confirmation is not cosmetic: if multiple coupling isomers formed in variable ratios, the method would be prone to poor robustness and inconsistent molar absorptivity; conversely, a dominant ortho-coupled product supports a stable λ_max, consistent ε, and reproducible calibration. The observed strong linearity and robustness under controlled parameters are consistent with a relatively clean, reproducible product-forming pathway [50].

4.3 Selectivity and interference testing in relation to real-world clinical polypharmacy

A major strength of the method is that it frames selectivity testing around plausible clinical co-exposures rather than arbitrary interferents. In type 2 diabetes, comorbid hypertension and nephropathy are common, and ACE inhibitors are frequently co-prescribed [51]. The study's explicit evaluation of lisinopril, enalapril, and ramipril is therefore clinically motivated. Mechanistically, these ACE inhibitors are unlikely to interfere through diazotization–coupling under the method conditions because they do not present a primary aromatic amine suitable for

diazotization and lack the specific activated aromatic features needed to compete efficiently for azo coupling with the diazonium reagent [52]. The empirical finding of negligible interference supports the method's suitability for polymedicated populations. In addition, the assessment of endogenous substances (e.g., glucose, creatinine, ascorbic acid) and common antidiabetic co-medications provides further reassurance that the visible-region signal is not easily perturbed by typical physiological constituents. The note regarding potential interference by high-dose ascorbic acid via reduction of azo dyes is scientifically appropriate and should be operationalized in practice by documenting supplementation history or applying confirmatory testing where clinically indicated [53], [54].

4.4 Agreement with HPLC and what "clinical equivalence" means operationally

The comparison of the study to a reference HPLC method is key to their proposed translation. Correlation alone is not conclusive for interchangeable methods; hence, Bland–Altman analysis and intraclass correlation (ICC) are methodologically justified. The reported low mean bias represents no systematic deviation, and the limits of agreement indicate the expected spread between methods in comparison of single samples. The ICC value of excellent calculated herein further supports the argument that, in the measured concentration range and in the cohort studied, spectrophotometric results can be swapped with HPLC for its intended uses [55], [56].

However, a rigorous academic discussion must also define the boundaries of equivalence. Chromatographic methods remain superior for scenarios requiring separation (e.g., degradants, metabolites, co-formulated drugs, or unexpected chromophoric interferents). The present method is most defensible in the following clinical/clinical-research contexts: (i) adherence assessment (confirming exposure vs non-exposure), (ii) pragmatic monitoring where trends and approximate concentration ranges are more informative than trace-level quantification, (iii) exploratory pharmacokinetic assessments in low-resource environments, and (iv) decentralized screening that

can triage samples requiring confirmatory chromatography. This positioning aligns the results with the literature review's argument that the methodological "best choice" depends on matrix complexity, required selectivity, and resource constraints [57]–[59].

4.5 Comparative perspective: published methods vs the proposed method (required table)

To directly address the missing comparative element, Table 4.1 summarizes how the present approach relates to representative published techniques referenced in the manuscript (HPLC-based dosage-form methods, stability-indicating HPLC, HPLC for pharmacokinetic applications, and spectrophotometric approaches). Where the manuscript does not provide numeric details for a referenced method, this is transparently indicated to avoid over-interpretation [60].

This comparison clarifies the methodological niche: the proposed method is not designed to replace HPLC in all contexts, but to provide a validated, chemically justified, and clinically demonstrated alternative where HPLC is impractical or environmentally burdensome, and where visible-derivatization selectivity is sufficient [61].

Table 4.1. Comparison of the proposed method with representative published methods for empagliflozin determination (as cited in the manuscript) [17], [62]–[65].

Method category / representative citation in manuscript	Typical matrix	Detection principle	Key performance features (as discussed or implied in the manuscript)	Practical advantages	Practical limitations
Proposed reversed-reagent diazotization–coupling UV–Vis method (this study)	Tablets; human plasma; urine	Visible azo chromophore, λ_max 455 nm	Broad linearity (0.5–60 µM), low LOD/LOQ, high precision and recovery; robustness under small parameter variations; no ACE inhibitor interference; agreement with HPLC by Bland–Altman and ICC	Low equipment requirement (UV–Vis), low solvent waste, low per-sample cost, multi-matrix validated; suitable for resource-limited settings	Not separation-based; potential vulnerability to rare chromophoric interferents; not inherently stability-indicating
HPLC-UV assay for raw material/dosage form (e.g., Hanif et al., as cited)	Raw material; tablets	Chromatographic separation + UV detection	High selectivity via separation; established regulatory acceptance for QC	Strong specificity; stability-indicating potential depending on method	Higher capital cost, solvent consumption, consumables (columns, filters), maintenance; less accessible in low-resource settings
Stability-indicating RP-HPLC (e.g., Badgujar, as cited)	Tablets; degraded samples	Chromatographic separation + UV	Explicit stability-indicating design (forced degradation compatibility)	Best for degradant profiling and QC under stress conditions	Higher cost and solvent waste; requires trained staff and instrument uptime
HPLC for pharmacokinetic applications (e.g., Abu Dayyih et al., as cited)	Biological matrices (plasma)	Chromatographic bioanalysis	Suitable for PK studies and complex matrices (higher selectivity)	Appropriate for regulated bioanalysis and complex sample sets	Same HPLC constraints; may require more extensive sample preparation
Direct UV spectrophotometry / alternative UV methods (as discussed generally)	Often dosage forms; limited bio-matrix utility	Native UV absorbance (deep UV)	Simpler but potentially less selective in biological matrices due to spectral overlap	Cheapest and simplest operationally	Poor selectivity in complex matrices without derivatization

4.6 Clinical significance: why this method matters beyond analytical performance As for clinical relevance on the rationale, according to results: TDM and verification of exposure can provide clinically worthwhile assistance even in type 2 diabetes care, especially with respect to non-adherence frequencies reported and variability of patient response due renal condition differences, comorbidity rates, and polypharmacy-effects. That the method is also useful in determining empagliflozin level in plasma and urine with good correlation to HPLC indicates the possibility of a number of useful applications in clinical as well as public health practice [66], [67].:

1. Adherence assessment and therapeutic accountability. A low-cost assay can enable objective confirmation of exposure in settings where non-adherence undermines outcomes and drives unnecessary escalation of therapy [68].

2. Risk evaluation in altered renal function. Because renal function influences drug handling and clinical risk in diabetes, exposure estimates can aid in identifying atypical accumulation patterns, especially in settings without advanced PK services [69].

3. Drug–drug interaction screening. The explicit demonstration of non-interference by ACE inhibitors supports use in common real-world regimens and reduces the risk of false signals in hypertensive diabetic populations [70].

4. Clinical research enablement. Multi-matrix capability facilitates observational and pragmatic studies (including urine-based assessments where blood collection is a barrier), which is particularly valuable in low-resource environments [71].

A key point is that the method offers *clinical utility through access*: it creates the possibility of measurement where measurement is otherwise not feasible. This is a materially different contribution than incremental improvements to already-available high-end chromatographic methods [72].

4.7 Cost–benefit and implementation economics (expanded)

The manuscript provides an explicit per-sample cost estimate: approximately $0.60–0.80 per sample for the spectrophotometric method compared

with $3–5 per sample for HPLC, representing a reduction on the order of 75–85%. A rigorous cost–benefit discussion extends beyond reagent cost [73]:

- **Capital expenditure and depreciation.** UV–Vis spectrophotometers are substantially less expensive than HPLC systems, and many laboratories already possess UV–Vis instruments. This reduces the marginal cost of adopting the method to reagent procurement and SOP implementation[74].
- **Consumables and supply chain resilience.** HPLC requires continuous access to high-purity solvents, columns, filtration units, vials, and parts—items that can be costly or intermittently unavailable in constrained regions. The proposed method uses common reagents and aqueous media, improving resilience [75].
- **Personnel and training burden.** HPLC operation and troubleshooting require specialized skills. A colorimetric UV–Vis procedure, especially one optimized to stable conditions via DoE, can be taught and implemented more broadly with lower ongoing training costs [76].
- **Environmental and compliance costs.** Organic solvent waste disposal introduces regulatory and logistical costs. The proposed method's high AGREE score and minimal solvent use reduce hazardous waste burdens, which can be a significant hidden cost in chromatography-heavy workflows [77].
- **Decision value.** Even a modest-cost assay can be cost-effective if it prevents unnecessary medication escalation, identifies non-adherence early, or supports more targeted patient education—outcomes that are particularly valuable in chronic diseases with high prevalence and long-term treatment costs [78].

Thus, the cost advantage is not merely "cheaper per test"; it can change which health systems are able to implement monitoring at all, which is a decisive benefit in many settings.

4.8 Environmental sustainability and "green" method justification

A high AGREE score (31/36) relating to the strength of a policy based on

green analytical chemistry principles is documented in the study. From a scientific standpoint this green advantage arises from three structural components of the method; (i) lack of organic solvent waste (in comparison to that generated through continuous flow and waste stream in HPLC), (ii) less power consumption (no pumps/ovens/ autosampler left on for long periods), and finally, (iii) reduced production toxic waste. A no-holds-barred presentation must still recognize that diazotization chemistry does feature nitrite under acidic circumstances and should be treated with safety and waste disposal due consideration; –green- here is relative (to solvent-intensive chromatography), rather than absolute. The environmental context of the method is therefore best described in terms of reduction of solvent waste and operational footprint, while still achieving fit-for-purpose analytical capability [79]–[82].

FUTURE WORK

should therefore prioritize: (i) multicenter ruggedness studies, including between-laboratory reproducibility; (ii) structured urine stability and pre-analytical validation; (iii) expanded interference panels including additional common cardiovascular and renal medications; and (iv) development of simplified reagent kits and portable photometric workflows to further improve field applicability.

CONCLUSIONS

A novel, sensitive and environmentally sustainable colorimetric method was successfully developed for the quantification of empagliflozin using diazotization-coupling with metoclopramide, which fulfilled ICH Q2(R2) validation guidelines based on superior linearity (R^2 = 0.9994 over 0.5-60 μM), low detection limits (LOD = 0.15 μM; LOQ = 0.45 μM), high precision (%RSD <1.65%) and accuracy (recovery in plasma: 98.5-101.2%; urine: 97.2-102.1%), excellent selectivity in complex matrices and multi-drug scenarios; robustness amidst parameter changes; clinical equivalence to HPLC reference method (ICC = 0.95); substantial cost reduction, and environmental sustainability while achieving molecular formulae confirmation

from FT-IR and proton-NMR spectroscopy for the assumed ortho-couple mechanism plus extensive ACE inhibitor interference testing to guide applicability in polymedicated diabetics, leaving the methodology as a viable alternative to chromatography techniques for use in therapeutic drug monitoring from resource-constrained settings.

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