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Simultaneous Determination of Dapagliflozin and Bisoprolol by LC–MS/MS: Method Development, Validation, and Forced Degradation Studies

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ABSTRACT

The Central Drugs Standard Control Organization (CDSCO) has approved the use of fixed dosages of bisoprolol fumarate and dapagliflozin propanediol monohydrate to treat Type 2 Diabetes Mellitus in patients with underlying hypertension and cardiovascular risk. The current work developed and validated a selective, sensitive, and stability-indicating LC–MS/MS technique for the simultaneous measurement of bisoprolol and dapagliflozin in tablet dosage forms and bulk pharmaceutical substances. Using a mobile phase of 5 mM ammonium formate with 0.1% formic acid in water (Mobile Phase A) and 5 mM ammonium formate with 0.1% formic acid in methanol (Mobile Phase B), chromatographic separation was accomplished on a Phenomenex Kinetex 2.6 μm C18 (100 Å, 150 $\times$ 4.6 mm) column. A SCIEX Triple Quadrupole 4500 mass spectrometer in positive ion mode was used for detection, using Multiple Reaction Monitoring (MRM). According to ICH recommendations, forced degradation studies were carried out using 0.1 N hydrochloric acid (acidic), 0.1 N sodium hydroxide (basic), and 3% hydrogen peroxide (oxidative), as well as thermal and photolytic stress conditions. With a correlation value $R^2 > 0.999$, the technique efficiently separated analytes and showed acceptable stability. For bisoprolol and dapagliflozin, linearity was seen over concentration ranges of 250–2000 ng/mL and 500–4000 ng/mL, respectively. In accordance with ICH Q2(R2) requirements, the approach demonstrated appropriate accuracy, precision, and robustness, demonstrating its suitability for regular analysis.

Keywords: LC-MS/MS, forced degradation studies, dapagliflozin, bisoprolol

1. Introduction

Dapagliflozin has the chemical formula $C_{21}H_{25}ClO_6$ and a molecular weight of 408.9 g/mol. Its IUPAC name is (2S,3R,4R,5S,6R)-2-[4-chloro-3-[(4-ethoxyphenyl)methyl]phenyl]-6-(hydroxymethyl)oxane-3,4,5-triol (PubChem 2024). Dapagliflozin propanediol monohydrate acts as an inhibitor of the type 2 sodium–glucose cotransporter (SGLT2). It was the first SGLT2 inhibitor to receive approval for the treatment of type 2 diabetes mellitus. It improves glycaemic control in adults by reducing glucose reabsorption in the proximal tubule of the nephron, thereby promoting glycosuria when used in combination with diet and exercise. It can be administered either as monotherapy or in combination with other oral antidiabetic agents or insulin. On January 8, 2014, U.S. Food and Drug Administration (FDA) approved dapagliflozin. Subsequently, in April 2021, its indications were expanded to include reduction in renal function decline, kidney failure, cardiovascular mortality, and hospitalisation for heart failure in adults with chronic kidney disease (Kashyap et al. 2024,Gu et al. 2025)

The chemical name of bisoprolol fumarate is 1-(propan-2-ylamino)-3-[4-(2-propan-2-yloxyethoxymethyl)phenoxy]propan-2-ol. It has the molecular formula $C_{18}H_{31}NO_4$ and a molecular weight of 325.4 g/mol. It is the fumarate salt of a phenoxy-2-propanol derivative, a synthetic β_1 -adrenergic receptor antagonist with cardioprotective and antihypertensive effects. Bisoprolol exhibits high selectivity toward cardiac β_1 -adrenergic receptors and lacks intrinsic sympathomimetic activity. By competitively blocking β_1 receptors in myocardial tissue, it reduces heart rate and myocardial contractility, thereby decreasing cardiac output and systemic blood pressure. In addition to its direct cardiac effects, bisoprolol suppresses renin release from juxtaglomerular cells in the kidneys, leading to attenuation of the renin–angiotensin system and further contributing to blood pressure reduction(PubChem 2024).

The combination therapy of dapagliflozin and bisoprolol is particularly beneficial in patients with type 2 diabetes mellitus who also present with hypertension or increased cardiovascular risk. This dual approach simultaneously targets hyperglycaemia and cardiovascular stress through complementary mechanisms of action, offering an integrated therapeutic strategy for high-risk patients (Chaudhari et al. 2023,Prajapati et al. 2025)

Extensive literature studies have reported the quantitative determination of dapagliflozin and bisoprolol using various analytical techniques such as RP-HPLC [Murugesan and Mathrusri Annapurna 2022,Barot et al. 2025,Hassan et al. 2020,Darji et al. 2025,Gibril and Rudwan 2023], HPTLC [Patel et al. 2023,Shukla et al. 2024,Suma et al. 2019,Shubhangee et al.

2025, Abdelrahman et al. 2020, Gunjal et al. 2024, Patel et al. 2025], RP-UPLC-PDA [Elzayat et al. 2024], and LC–MS/MS [Patel et al. 2023, Surendran et al. 2019, Logoyda et al. 2020], both individually and in combination with other drugs. The chemical structures of dapagliflozin and bisoprolol are shown in Figure 1A and Figure 1B [Valiya et al. 2025, Ermer 2025]

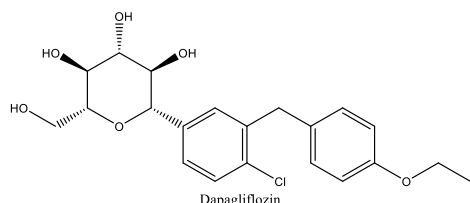


Figure 1A. Chemical Structure of Dapagliflozin

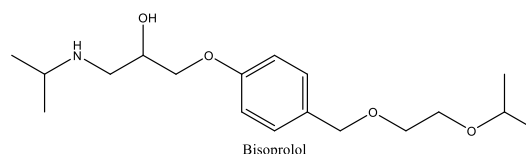


Figure1B. Chemical Structure of Bisoprolol

Table 1: Comparison of previously reported analytical methods for dapagliflozin and bisoprolol

S.No	Method	Analytes	Matrix	Linearity range	LOD / LOQ	Major limitation
1	RP-HPLC	Dapagliflozin + Bisoprolol	Synthetic mixture	DAPA: 5–25 µg/mL; BISO: 5–25 µg/mL	DAPA: 0.155 / 0.465 µg/mL; BISO: 0.224 / 0.680 µg/mL	UV detection; no molecular-mass or fragmentation information for degradation products (Patel et al. 2026)
2	HPTLC	Dapagliflozin + Bisoprolol	Combined oral formulation	DAPA: 200–1200 ng/band; BISO: 100–600 ng/band	DAPA: 25.87 / 78.38 ng/band; BISO: 17.68 / 53.60 ng/band	Densitometric detection does not provide <i>m/z</i> or MS/MS fragmentation information (Ninama and Koradia 2025)
3	RP-HPLC	Dapagliflozin + Bisoprolol	Combined synthetic mixture	DAPA: 5–15 µg/mL; BISO: 5–15 µg/mL	DAPA: 3.02/3.23 µg/mL; BISO: 0.99/1.06 µg/mL	PDA/UV detection; method is based on a synthetic mixture rather than mass-spectral characterization (Joshi and Patel 2025)
4	RP-HPLC	Dapagliflozin + Bisoprolol	synthetic mixture	DAPA: 5–15 µg/mL; BISO: 2.5–7.5 µg/mL	DAPA: 2.40 / 3.15 µg/mL; BISO: 1.30 / 2.55 µg/mL	Relatively high detection/quantification limits and no MS-based structural information (Vaghasiya et al. 2025)
5	RP-HPLC	Dapagliflozin + Bisoprolol	Pharmaceutical dosage form	DAPA: 5–100 ng/mL; BISO: 5–100 ng/mL	DAPA: 0.9 / 3.5 ng/mL; BISO: 1.0 / 3.5 ng/mL	Conventional UV/HPLC approach; no precursor-to-product ion information (Raut et al. 2026)

6	HPTLC	Dapagliflozin	Bulk drug and marketed tablet	100–1000 ng/band	DAPA: 16.7/50.5 ng/band	Single-analyte method and densitometric detection; no bisoprolol determination or MS characterization (Karmankar and Tajne 2019)
7	HPTLC	Dapagliflozin + Vildagliptin	Fixed-dose combination	DAPA: 0.6–1.4 µg/band	DAPA: 0.02 / 0.07 µg/band	Densitometric method; different combination and no MS-based degradation-product characterization (Krishnamoorthy et al. 2025)
8	HPTLC	Dapagliflozin + Metoprolol	Pharmaceutical formulation	1000–6000ng/band	DAPA- 21.97/66.60ng/band	Different β-blocker combination; HPTLC cannot provide molecular-mass/fragmentation information (Saiyed et al. 2024)
9	LC-MS/MS	Bisoprolol	Human plasma	1–100 ng/mL	LLOQ: 0.99 ng/mL	Highly sensitive bioanalytical method, but limited to bisoprolol and biological matrix (Peste et al. 2009)
10	UHPLC-MS/MS	Bisoprolol	Human plasma	0.0200–40.0 ng/mL	LLOQ: 20.0 pg/mL	Excellent sensitivity but designed for pharmacokinetic analysis rather than simultaneous pharmaceutical dosage-form analysis with dapagliflozin (Jiang et al. 2025)

Table 1 summarizes the analytical methods reported in the literature for the determination of dapagliflozin and bisoprolol. Most of the simultaneous methods reported are based on RP-HPLC or HPTLC with UV/PDA or densitometric detection and are primarily applied for routine quantitative estimation in pharmaceutical formulations. The reported LC–MS/MS methods are mainly oriented to the individual drug determination in biological matrices, not for the simultaneous analysis of dapagliflozin and bisoprolol in pharmaceutical dosage forms. Furthermore, to the best of our knowledge, a simultaneous LC-MS/MS method for the determination of dapagliflozin and bisoprolol in pharmaceutical dosage forms incorporation forced degradation studies has not been reported.

The current study aims to develop and validate a sensitive and selective LC-MS/MS method for the simultaneous detection of dapagliflozin and bisoprolol in pharmaceutical dosage forms, in accordance with the ICH Q2(R2) guidelines. The new method was applied to forced degradation tests under specific stress conditions to assess the stability of both drug substances. These forced degradation studies were conducted under acidic, alkaline, oxidative, thermal, and photolytic conditions, with the resulting samples analysed using the developed LC-MS/MS technique. These studies provide information on the chemical stability of dapagliflozin and bisoprolol under different stress conditions and demonstrate the applicability of the developed analytical method for stability assessment (ICH 2023, Gangurde et al. 2026, Vyas et al. 2023, Jain et al. 2025, Sulthana et al. 2026a, Vadnere et al. 2025, Foudah et al. 2025).

2. Chemicals and Reagents

2.1 Chemicals and Reagents

Bisoprolol fumarate (98.8%) and dapagliflozin (98%) were obtained from PADM Laboratories, Bangalore India. Empagliflozin (99.5%) and metoprolol (99.1%) were used as internal standards and purchased from the same source as the sample. DapaBiso 10mg/5mg (Zydus) combined tablet of dapagliflozin (10 mg) and bisoprolol (5 mg) was procured from the local market. Methanol, acetonitrile were purchased from J.T. Baker (India) while hydrochloric acid, sodium hydroxide and hydrogen peroxide were purchased from Rankem India (India).

2.2 Instrumentation

Analytical method development and validation was performed on Sciex Triple Quad 4500 LCMS/MS system. Accurate weighing of material used was done with a Radway AS 220 R2 Plus analytical balance. The separation was done on a Phenomenex Kinetex C18 column 150 mm × 4.6 mm, 2.6 µm particle size, 100 Å pore size.

2.3 Chromatographic Conditions

The simultaneous determination of dapagliflozin and bisoprolol has been conducted in a Kinetex C18 column (150 × 4.6 mm, 2.6 µm, 100 Å) from Phenomenex. The aqueous mobile phase (Mobile Phase A) was 0.1% formic acid in 5 mM ammonium formate and the organic mobile phase (Mobile Phase B) was 0.1% formic acid in methanol. Methanol was used as a diluent. The quantification was performed in positive-ion multiple reaction monitoring (MRM) mode in Sciex Triple Quad 4500 LC-MS/MS system.

2.4 Preparation of Mobile Phase A(5 mM Ammonium Formate Containing 0.1% Formic Acid in Milli-Q Water)

Accurately weighed 0.3153 g of ammonium formate was placed in a 1 L volumetric flask for the preparation of Mobile Phase A. Now the salt was dissolved by adding milli-Q water. Then 1.0 mL formic acid was added to get 0.1% (v/v) final concentration. The solution was finally diluted to the 1 L mark using Milli-Q water.

2.5 Preparation of Mobile Phase B (Ammonia-formic acid containing 5 mM Ammonium Formate (AF) in Methanol (MeOH))

The ammonium formate was accurately weighed and transferred to a 1 litre volumetric flask to make up the Mobile Phase B. To dissolve the salt, methanol was added and then 1.0 mL of formic acid was added to get the stock solution to 0.1% (v/v) concentration. This volume was then brought to 1 L with methanol.

2.5 Preparation of Dapagliflozin and Bisoprolol Standard Stock Solution-I 1000 µg/mL

100mg of dapagliflozin and bisoprolol were transferred into different 100 mL volumetric flasks respectively. The drugs were dissolved in methanol; the following solutions were diluted to volume with methanol to give 1000 µg/mL of the stock solution for each drug.

2.6 Preparation of Dapagliflozin and Bisoprolol Standard Stock Solution- 2 (100µg/mL)

To achieve a concentration of 100µg/mL, an aliquot of 10 mL from standard stock solution I was put into a 100 mL volumetric flask and diluted to volume with methanol.

2.7 Preparation of Dapagliflozin and Bisoprolol Working Stock Solution

To obtain a working solution containing 10µg/mL of dapagliflozin, 10mL of standard stock solution II was placed into a 100mL volumetric flask and diluted to volume with methanol.

To obtain a working solution containing 5µg/mL of bisoprolol, 5 mL of standard stock solution II was placed into a 100 mL volumetric flask and diluted to volume with methanol.

2.8 Preparation of Test Solution

The dapagliflozin and bisoprolol were accurately weighed 20 tablets with the help of average weight calculation. Five (5mg) tablets were transferred into a 500 mL volumetric flask and the powder was dissolved. Methanol was used to supplement the sample, and it was sonicated prior to making up to the mark with methanol. The primary stock solution that was obtained had 0.1 mg/mL of dapagliflozin and 0.05 mg/mL of bisoprolol. To prepare the initial working solution, 100µL of

the stock solution was placed in a 1 mL volumetric flask and diluted with methanol. This was then diluted 10 fold with 1 mL of methanol to create the final working stock solution. The final concentrations of dapagliflozin and bisoprolol were 1 µg/mL and 0.5 µg/mL, respectively.

2.10 Method Validation

2.10.1 System Suitability

Six blank preparations, as well as standard preparations of dapagliflozin and bisoprolol, were injected into the LC-MS system to evaluate system suitability. The %CV values attained were used to test the suitability of the analytical system.

2.10.2 Solution Stability

The 10 µg/mL dapagliflozin and 5 µg/mL bisoprolol working stocks were tested for stability at 0, 24 and 48 hrs. The combined standard stock solution (both analytes) was also kept for 48 hours for stability testing.

2.10.3 Linearity

Seven dapagliflozin concentration levels were used for the linearity range (500 to 4000 ng/mL), and seven bisoprolol concentration levels were used for the linearity range (250 to 2000 ng/mL). The solutions were standard solutions at the particular concentrations, and the coefficient of determination (R^2) were obtained by using the regression equation $Y = bX + C$.

2.10.4 Accuracy

Recovery experiments at a concentration of 80%, 100% and 120% were used to test the accuracy of the method. Pre-analysed samples received dose spiked amounts of respectively dapagliflozin and bisoprolol. A total of six replicate injections ($n = 6$) were analysed for each concentration level, and the percentage recovery was determined.

2.10.5 Precision

Intra and inter-day precision was assessed with concentrations of dapagliflozin of 500, 1500 and 2000 and bisoprolol of 250, 750 and 1000. The samples in each concentration were analysed six times ($n = 6$). Precision was given as the %CV of the measured peak areas.

2.10.6 Limit of Detection and Limit of Quantification

Limit of detection (LOD) and limit of quantification (LOQ) determinations were determined using a signal-to-noise ratio. The equations used for calculation of the LOD and LOQ were $3.3 \times (\sigma/s)$

and $10 \times (\sigma/s)$, respectively. The signal to noise ratios were 3:1 and 10:1 for LOD and LOQ, respectively.

2.10.7 Robustness

Analytical procedure robustness was tested by introducing deliberately some variation with the selected chromatographic and instrumental parameters: flow rate, column and source temperature. The impact of these intentional changes was captured and evaluated.

2.10.8 Forced Degradation Study

Forced degradation studies were conducted to elucidate the stability of dapagliflozin and bisoprolol when exposed to acid, base, oxidative, heat and light in both bulk drugs and drug products. Working solutions of 10 µg/mL dapagliflozin and 5 µg/mL bisoprolol were used for solution based stress studies. For acid, alkaline and oxidative degradation experiments, 2 mL of each working solution was subjected to the respective stress treatment. Thermal and photolytic degradation was conducted in the solid state with 10 mg of each active ingredient and powdered tablet formulation, which is equivalent.

2.10.8.1 Acid Hydrolysis

Two mL aliquots of dapagliflozin (10 µg/mL) and bisoprolol (5 µg/mL) working solutions were placed separately in 10 mL volumetric flasks for acid hydrolysis. 0.1N hydrochloric acid (2 mL) was added to each preparation and heated in the water bath at 60°C for 2 hours. Once the stress treatment was completed the solutions were cooled to room temperature and neutralised by the addition of 2 mL of 0.1 N sodium hydroxide. Methanol was then used to adjust the volume to the mark.

Tablet formulation samples were subjected to the same stress conditions to study the degradation behaviour of the analytes in the presence of excipients. A reagent blank (no analytes) is treated in the same manner as the samples and analyzed to ensure that no interfering components are detected.

2.10.8.2 Alkali Hydrolysis

The dapagliflozin (10 µg/mL) and bisoprolol (5 µg/mL) working solutions were each placed in a separate 10 mL volumetric flask for the procedure of acid degradation reported herein. Two millilitres of 0.1 N hydrochloric acid were added to each of these solutions and incubated

at 60°C for 2 hours in a water bath. The samples were neutralised after exposure with 2 mL of 0.1 N sodium hydroxide. Then some methanol was added to the volume up to the mark.

The tablet formulation preparations were also treated to evaluate the effect of excipients on the degradation behavior. The reagent blank (no analyte) was treated in the same way as the sample and analysed to ensure that there was no interference from the reagents or treatment methods.

2.10.8.3 Oxidative Hydrolysis

To perform oxidative stress tests, the dapagliflozin (10 µg/mL) and bisoprolol (5 µg/mL) working solutions were added separately to the 10 mL volumetric flasks. The 2 mL of 3% hydrogen peroxide was added to each solution and boiled in a water bath at 60°C for 2 hours. The samples were then allowed to cool to room temperature and then to the volume with the diluent.

Tablet formulation samples were treated in the same way as above for oxidative treatment to understand the effect of excipients on the oxidative stability of the analytes. A reagent blank (no analyte in it) was treated and analysed under the same conditions to ensure that there were no interfering peaks present.

2.10.8.4 Thermal Degradation

Thermal stress testing was performed for 10 mg of dapagliflozin, 10 mg of bisoprolol and equivalent amount of powdered tablet formulation by dry heat at 65°C in a hot-air oven for 30 minutes. Following the exposure time, stressed samples were individually placed in 10 mL volumetric flasks and reconstituted with the diluent. This was followed by sample preparation for LC-MS/MS processing using the previously mentioned sample preparation method. The blank, which was processed under the same conditions and in parallel, was also analysed to ensure the absence of interference.

2.10.8.5 Photodegradation

Ten milligram samples of dapagliflozin, bisoprolol as well as an equal amount of powdered tablet formulation were exposed to UV light for 30 minutes, followed by photolytic stress testing. Stressed materials were transferred individually to 10 mL volumetric flasks after exposure material and then brought back to the diluent. The obtained solutions were then prepared prior to LC-MS/MS analysis according to the sample preparation protocol.

A blank (no analytes) preparation was subjected to the same UV treatment as the sample and treated simultaneously. The developed analytical method was checked through this preparation to confirm there are no interferences and to confirm the specificity of the developed analytical method. [Madhavaram and Jays 2026a, Madhavaram and Jays 2026b, Sulthana et al. 2026b]

3. Results and Discussion

3.1 Method Development and Chromatographic Optimization

The chromatographic parameters were adjusted to avoid carryover, increase the MS response to bisoprolol and dapagliflozin, and offer acceptable peak symmetry. A Phenomenex Kinetex C18 column (150 × 4.6 mm, 2.6 µm) was used. The core-shell C18 stationary phase provides excellent chromatographic efficiency, is ideal for peak characteristics, and works well with analytes of various polarity. To separate the drug compounds and their breakdown products while maintaining a reasonably quick analytical run-time, a gradient elution technique was utilised.

Initially, a 10 µL injection volume of Mobile Phase A (Milli-Q water with 0.1% (v/v) formic acid) and Mobile Phase B (methanol) were used to conduct a chromatographic separation. Formic acid was chosen to enhance peak shape and enable protonation of the analytes in positive electrospray ionisation (ESI) mode. However, due of the strong analyte response, metoprolol showed peak splitting under these circumstances, and carryover was seen in successive blank injections. As a result, these circumstances were deemed inappropriate for additional examination.

In the second trial, 5 mM ammonium formate and 0.1% (v/v) formic acid were added to the aqueous and organic phases of the mobile phase to change its composition. To enhance chromatographic performance while preserving effective ionisation in positive ESI mode, ammonium formate, a volatile buffer compatible with LC-MS/MS, was used. Peak symmetry, signal intensity, and repeatability were all improved by the changed mobile phase.. A small carry-over was, however, noted for an injection volume of 10 µL. The chromatograms from Trials 1 and 2 are presented in Figure 2A.

In order to further reduce carry over and increase the robustness of the analytical procedure, the injection volume was decreased to 2 µL and the optimized mobile-phase composition remained unchanged. Also, the gradient program was optimized, decreasing the overall

analysis time from 12 to 10 min while maintaining the chromatographic resolution. Under the optimized conditions sharp and symmetrical peaks with good reproducibility were obtained along with satisfactory separation of the baseline separation of the analytes and also their degradation products. These conditions were subsequently used for method validation and forced degradation studies. A summary of the optimization trials is presented in Table [2A](#). The final optimised method chromatogram as shown in [Figure 2B](#).

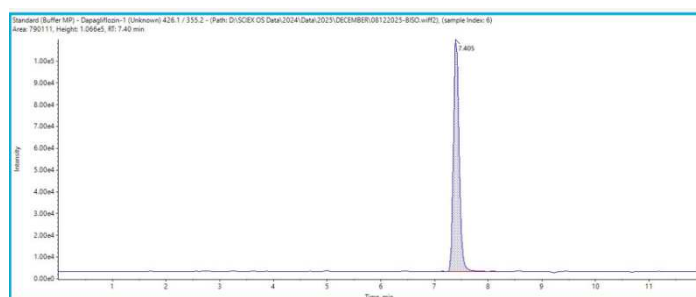


Figure a: Chromatogram of Dapagliflozin under Trial 1

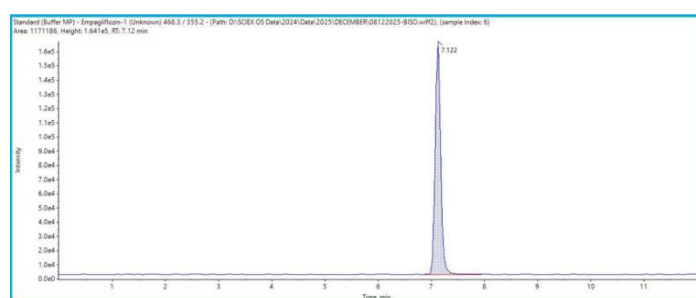


Figure b: Chromatogram of Empagliflozin (IS) under Trial 1

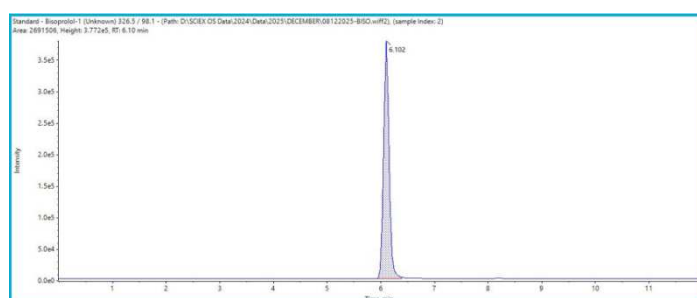


Figure c: Chromatogram of Bisoprolol under Trial 1

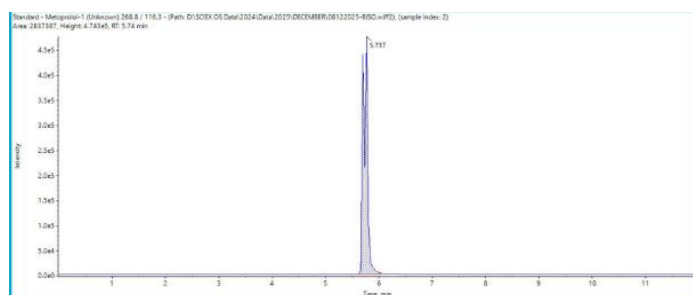


Figure d: Chromatogram of Metoprolol (IS) under Trial 1

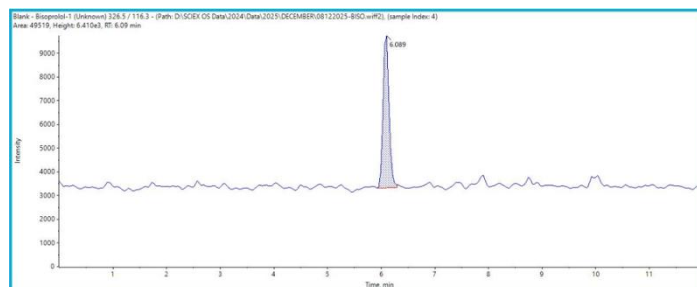


Figure e: Chromatogram of blank injection under Trial 1 showing carryover effect

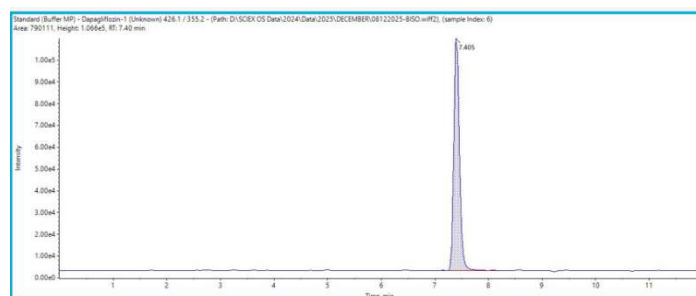


Figure f: Chromatogram of Dapagliflozin under Trial 2

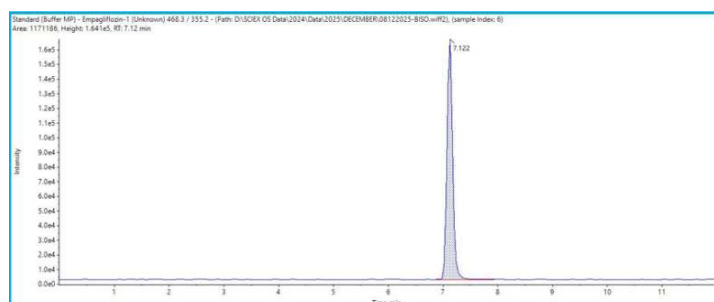


Figure g: Chromatogram of Empagliflozin (IS) under Trial 2

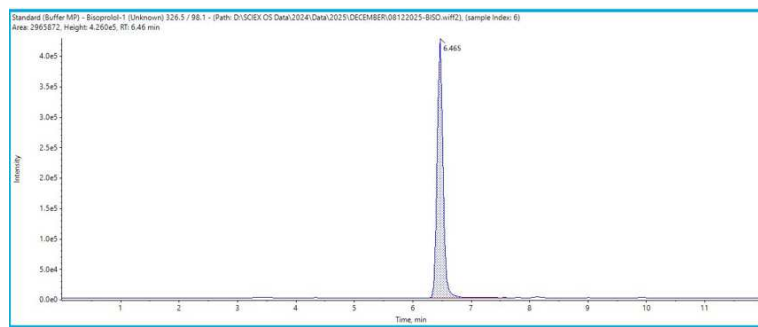


Figure h: Chromatogram of Bisoprolol under Trial 2

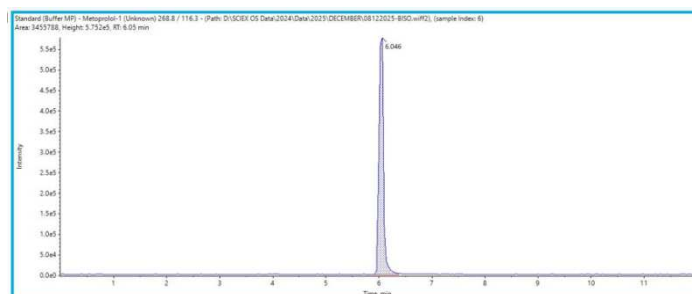


Figure i: Chromatogram of Metoprolol (IS) under Trial 2

Figure 2A: LC-MS/MS chromatograms obtained under Trial 1 and Trial 2

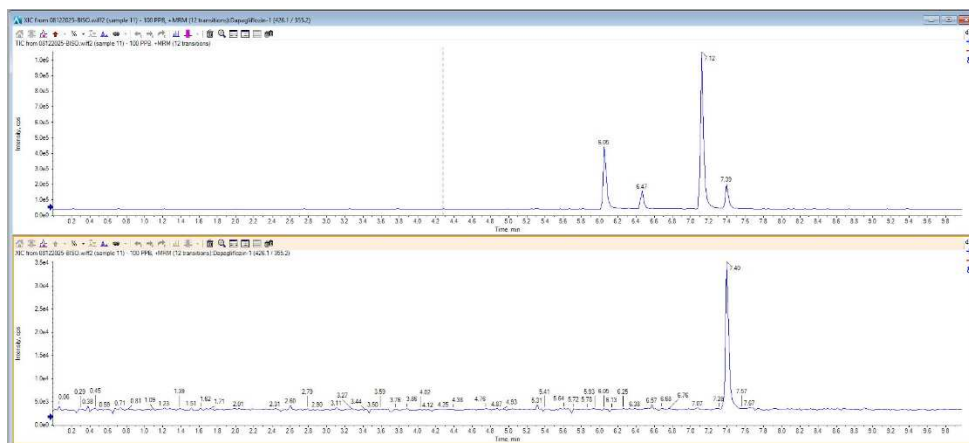


Figure a: Optimised chromatogram of Dapagliflozin

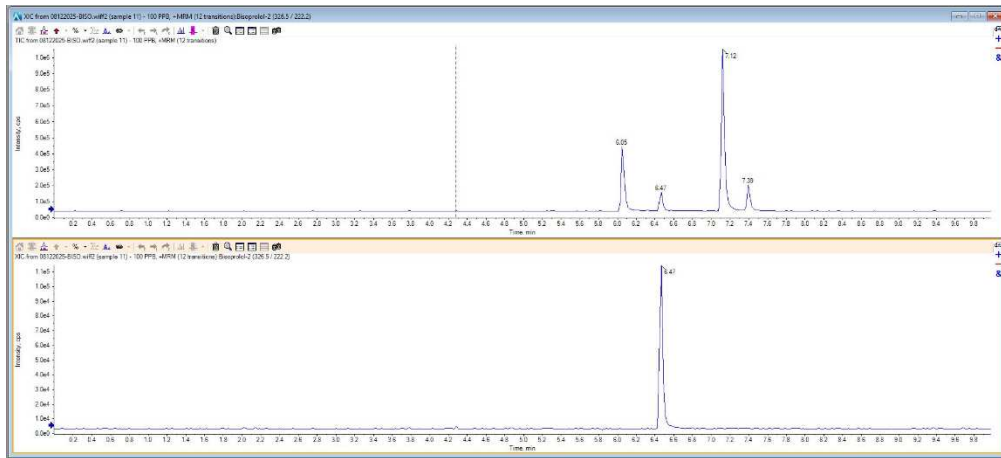


Figure b: Optimised chromatogram of Bisoprolol

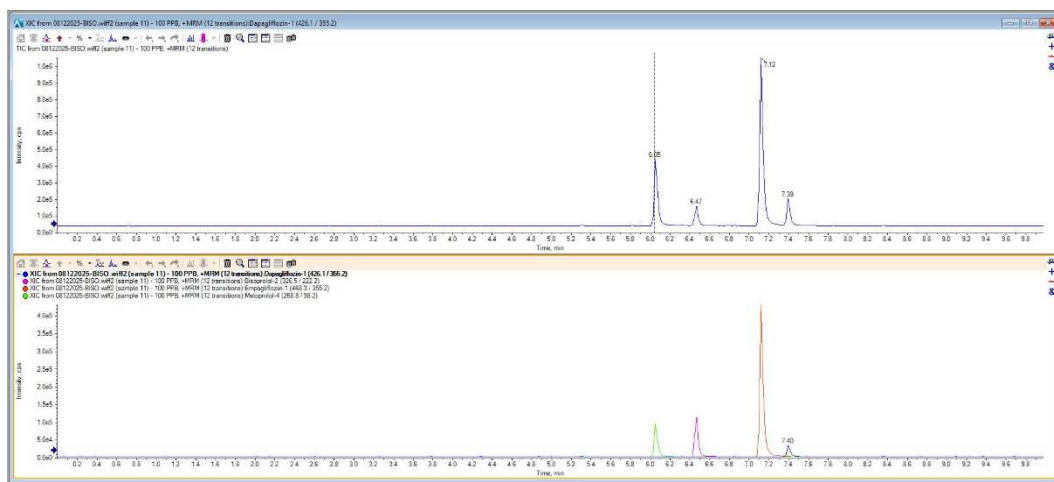


Figure c: Optimised chromatogram of Dapagliflozin and Bisoprolol

Figure 2B: Optimised chromatograms of Dapagliflozin and Bisoprolol along with internal standards

Table 2A: Summary of LC-MS Optimisation Trials

Trial	Mobile Phase A	Mobile Phase B	Injection Volume and Run Time	Observation
1	0.1% (v/v) formic acid in Milli Q Water	Methanol	10µL and 12 Minutes	Peak Splitting and Carryover effect
2	5 mM of ammonium formate with 0.1% (v/v) formic acid in water	Five mM of ammonium formate and 0.1% (v/v) formic acid in methanol	10µL and 12 Minutes	Higher Response
3	Ammonium formate in water with 0.1% (v/v) formic acid	Ammonium formate 5 mM in methanol with 0.1% (v/v) formic acid	2µL and 10 Minutes	Good Peak shape

3.1 Chromatography and mass spectrometry conditions

During method development, chromatographic conditions have been optimised to achieve good separation of dapagliflozin and bisoprolol. This resulted in the gradient elution shown in Table 2B with a run time of 10 minutes

Table 2B. Gradient Elution

Time (minutes)	A%	B%	Flow (mL/min)
0.00	90	10	0.5
1.00	90	10	0.5
4.00	20	80	0.5
5.00	5	95	0.5
7.00	5	95	0.5
8.00	5	95	0.5
8.50	20	80	0.5
9.00	90	10	0.5
10.00	90	10	0.5

Good sensitivity was achieved by using positive ionisation with the following settings for the mass spectrometric parameters: spray voltage, 5500V; Source temperature, 500°C; ion source gas 1 65 psi; Ion source gas 2, 65 psi. As shown in Figure 2B, the two compounds are well separated with the developed method. Empagliflozin and Metoprolol were used as internal standards for dapagliflozin and bisoprolol, respectively. They were selected based on their structural similarity to the corresponding analytes, comparable chromatographic retention behaviour, and suitable mass-spectrometric responses under the optimised LC-MS/MS

conditions. The internal standards were chromatographically separated from the corresponding analyte peaks and did not show significant interference. The internal standards were used to compensate for variations in sample preparation and instrumental response. The MRM transition settings for dapagliflozin, bisoprolol, and their respective internal standards, empagliflozin and metoprolol are summarized in Table 3

Table 3 : MRM transition setting for Dapagliflozin, bisoprolol and internal standards

ID	Q1(m/z)	Q3 (m/z)	Collision energy (v)
Dapagliflozin -1	426.1	355.2	13
Bisoprolol -1	326.5	222.2	12
Empagliflozin -1	468.3	355.2	12
Metoprolol -1	268.8	98.2	13

3.2 Method Validation

3.2.1 System suitability

To ensure the adequate efficiency of the LC-MS/MS system, system appropriateness was assessed prior to analysis. Six injections of standard stock solutions of bisoprolol and dapagliflozin were performed under ideal mass spectrometric and chromatographic conditions (n = 6). System suitability results are mentioned in Table 4

Table 4: System suitability

Analyte Name	Peak Area
Dapagliflozin	201504
	204156
	200789
	204758
	205281
	199544
Average	202672
Standard Deviation	2368.7
%RSD	1.17

Analyte Name	RT
Dapagliflozin	7.41
	7.39
	7.39
	7.40
	7.39
	7.40
Average	7.40
Standard Deviation	0.00816
%RSD	0.110

Analyte Name	Peak Area
Bisoprolol	320480
	323119
	322385
	318977
	329785
	321193
Average	322657
Standard Deviation	3782.1
%RSD	1.17

Analyte Name	RT
Bisoprolol	6.48
	6.47
	6.46
	6.48
	6.47
	6.48
Average	6.47
Standard Deviation	0.00816
%RSD	0.126

3.2.2 Specificity

Dapagliflozin and bisoprolol's specificity chromatograms show clear peaks with no extra peaks during their retention periods, suggesting that neither drug was affected by excipients or interference.

3.2.3 Solution Stability

Solution stability was performed for both drugs at 0h, 24h, and 48h and the solution was found to be stable for 48h. Solution stability results are shown in Table 5

Table 5: Dapagliflozin Solution Stability

Dapagliflozin	Storage Conditions	Peak Area Day -1	Peak Area Day -2	Mean	Std Deviation	%CV
	Dark (-20 deg C)-1	402992	393975	400526.5	472.1116276	0.117872757
	Dark (-20 deg C)-2	402510	398535			
	Dark (-20 deg C)-3	395076	410071			
	Mean	400192.7	400860.3			
	Storage Conditions	Peak Area Day -1	Peak Area Day -3	Mean	Std Deviation	%CV
	Dark (-20 deg C)-1	612798	585394	594400.0	2983.519	0.5019
	Dark (-20 deg C)-2	582206	592938			
	Dark (-20 deg C)-3	594525	598539			
	Mean	596509.7	592290.3			
Bisoprolol	Storage Conditions	Peak Area Day -1	Peak Area Day -2	Mean	Std Deviation	%CV
	Dark (-20 deg C)-1	687259	695695	694348.2	1199.488803	0.172750338
	Dark (-20 deg C)-2	695567	684981			
	Dark (-20 deg C)-3	697674	704913			
	Mean	693500.0	695196.3			
	Storage Conditions	Peak Area Day -1	Peak Area Day -3	Mean	Std Deviation	%CV
	Dark (-20 deg C)-1	781990	776144	773067.8	1741.604002	0.225284759
	Dark (-20 deg C)-2	765611	779350			
	Dark (-20 deg C)-3	767908	767404			
	Mean	771836.3	774299.3			

3.2.4 Linearity

Dapagliflozin exhibited linearity over the concentration range of 500-4000ng/mL(0.50-4.00µg/mL), with the regression equation $y=476.99x+1255.4$ and a correlation coefficient (r^2) of 0.999. Bisoprolol exhibited linearity over the concentration range of 250-2000ng/mL(0.25-2.00µg/mL), with the regression equation $y=1187x-49489$ and a correlation coefficient (r^2) of 0.999. The calibration curves for both dapagliflozin and bisoprolol are shown in Figure 3A and Figure 3B

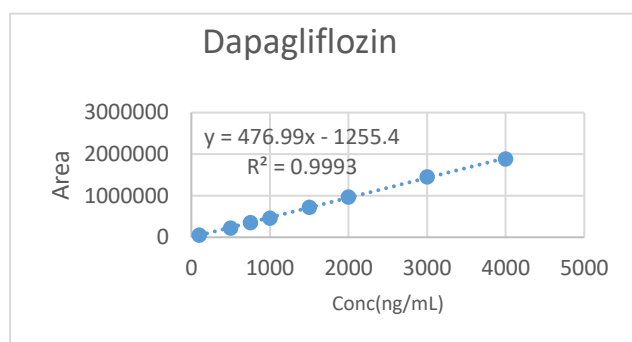


Figure 3A: Dapagliflozin Linearity Graph

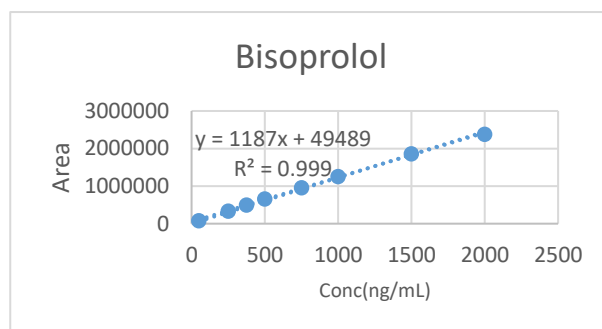


Figure 3B: Bisoprolol Linearity Graph

3.2.5 Precision

The percentage CV values for intraday and interday precision were found to be less than 2%, indicating that the proposed method is precise and reliable for the simultaneous estimation of dapagliflozin and bisoprolol. Tables 6A and Table 6B summarises the precision data.

Table 6A: Intraday precision results of Dapagliflozin and Bisoprolol

Drug(Intraday)	Spike Level (%)	Formulation Amount (ng/mL)	Standard Spiked (ng/mL)	Mean Conc (n=6)	SD	(CV %)
Dapagliflozin	50	1000	500	1492.4	8.139	0.55
Dapagliflozin	100	1000	1000	1996.0	15.222	0.76
Dapagliflozin	200	1000	2000	2991.7	38.824	1.30
Bisoprolol	50	500	250	753.9	5.769	0.77
Bisoprolol	100	500	500	999.0	12.593	1.26
Bisoprolol	200	500	1000	1495.4	16.141	1.08

Table 6B: Interday precision results of Dapagliflozin and Bisoprolol

Drug(Interday)	Spike Level (%)	Formulation Amount (ng/mL)	Standard Spiked (ng/mL)	Mean Conc (n=6)	SD	(CV %)
Dapagliflozin	50	1000	500	1517.6	12.084	0.80
Dapagliflozin	100	1000	1000	2013.0	19.686	0.98
Dapagliflozin	200	1000	2000	3000.6	33.331	1.11
Bisoprolol	50	500	250	758.5	3.197	0.42
Bisoprolol	100	500	500	999.9	11.445	1.14
Bisoprolol	200	500	1000	1485.9	16.788	1.13

3.2.6 Accuracy

The results demonstrated that the proposed method is accurate for the simultaneous quantification of dapagliflozin and bisoprolol, with percentage recoveries for both APIs within the acceptable range 98-102%. Table 7 summarises accuracy data.

Table 7: Accuracy study results

Drug	Spike Level (%)	Formulation Amount (ng/mL)	Standard Spiked (ng/mL)	Mean Conc	SD	(CV %)	Amount of drug(ng/mL)(Formulation+ standard)	Recovery %
Dapagliflozin	80	1000	800	1806.2	11.277	0.62	1800	100.3
Dapagliflozin	100	1000	1000	2016.1	17.714	0.88	2000	100.8
Dapagliflozin	120	1000	1200	2212.5	20.255	0.92	2200	100.6
Bisoprolol	80	500	400	897.1	11.331	1.26	900	99.7
Bisoprolol	100	500	500	1003.6	10.639	1.06	1000	100.4
Bisoprolol	120	500	600	1096.2	7.490	0.68	1100	99.7

3.2.7 LOD and LOQ

The limit of detection (LOD) was determined as 100ng/mL for dapagliflozin and 50ng/mL for bisoprolol. The practical limit of quantification (LOQ) was established at the lowest calibration concentrations that were reliably quantified, corresponding to 500ng/mL for dapagliflozin and 250ng/mL for bisoprolol.

3.2.8 Robustness

Small but intentional changes to flow rate, temperature, and source temperature were made in order to evaluate the resilience of the approach. These modifications' effects were recorded, and the outcomes were analysed. We changed the mobile phase's flow rate from 0.5 mL/min to 0.4 mL/min and 0.6 mL/min. The temperature in the column was changed from 45°C to 40°C and 50°C. A temperature adjustment was made from 500°C to 450°C and 400°C. The fact that the percentage RSD was less than 2 in each of these cases shows how robust the

strategy is and how little intentional changes have no effect on it. Robustness results are compiled in a Table⁸

Table 8: Robustness results

Analyte Name	Parameter Change	Average(n=3)	STDEV	CV (%)
Dapagliflozin	STANDARD AQ-1	1008.83	5.71	0.57
	STANDARD AQ-1 (0.4ML PER MIN)	997.06	11.86	1.19
	STANDARD AQ-1 (0.6ML PER MIN)	994.51	4.61	0.46
	STANDARD AQ-1 (Column oven 40 degree)	1001.83	12.93	1.29
	STANDARD AQ-1 (Column oven 50 degree)	997.75	8.24	0.83
	STANDARD AQ-1 (Source Temp 400 degree)	987.65	10.16	1.03
	STANDARD AQ-1 (Source Temp 450 degree)	988.47	6.36	0.64

Analyte Name	Parameter Change	Average	STDEV	CV (%)
Bisoprolol	STANDARD AQ-1	498.08	4.05	0.81
	STANDARD AQ-1 (0.4ML PER MIN)	505.42	7.80	1.54
	STANDARD AQ-1 (0.6ML PER MIN)	500.90	5.69	1.14
	STANDARD AQ-1 (Column oven 40 degree)	505.93	3.19	0.63
	STANDARD AQ-1 (Column oven 50 degree)	500.36	6.30	1.26
	STANDARD AQ-1 (Source Temp 400 degree)	497.91	3.71	0.75
	STANDARD AQ-1 (Source Temp 450 degree)	504.81	8.58	1.70

3.2.9 Result of Forced Degradation Study

Stability behaviour of dapagliflozin and bisoprolol under acid, alkali, oxidative, thermal and photolytic stress conditions were evaluated by forced degradation studies. Standard drug solutions and samples of tablet formulation were exposed to 0.1 N HCl, 0.1 N NaOH, 3% H₂O₂, thermal stress at 65 °C, and photolytic stress for one photo cycle. Stress studies were carried out for 2 h for acidic, alkaline, oxidative and thermal stress. Photolytic stress was applied for 24 h. Then, the stressed samples were analysed by the developed LC-MS/MS method.

The percentage of drug recovered for standard dapagliflozin samples was ranged from 98.09% to 99.86% under the stress conditions tested. Similarly, the drug recoveries of bisoprolol ranged from 98.08% to 99.24%. The recovery of dapagliflozin in tablet formulation was between 98.26% and 99.55%, and the recovery of bisoprolol was between 99.33% and 99.94%. The highest recovery was obtained under oxidation conditions for standard dapagliflozin (99.86%) and for bisoprolol in tablet formulation (99.94%).

The chromatographic evaluation of the stressed samples did not reveal any additional peaks due to degradation products under the selected stress conditions. No significant changes in the chromatographic profiles of dapagliflozin or bisoprolol compared to the respective unstressed

samples were observed. Signals corresponding to degradation were considered to be those that eluted away from the solvent front and not the parent ions/adducts.

In general, high percentage of drug recovery and no detectable extra degradation peaks indicate that dapagliflozin and bisoprolol are relatively stable under the chosen stress conditions. The results indicate that the developed LC–MS/MS method can be applied for monitoring chromatographic behaviour of both drugs during forced degradation studies. Table 9 summarises the forced degradation results of dapagliflozin and bisoprolol standards and in tablet formulation. The LC-MS/MS chromatograms of dapagliflozin and bisoprolol under different forced degradation conditions are shown in Figure 4A and Figure 4B.

Table 9: Forced degradation results of dapagliflozin and bisoprolol standards and in tablet formulation

Table 9A: Forced degradation results of dapagliflozin standard

For Standard of Dapagliflozin					
Conditions	Reagent	Temperature	Time	Drug amount recovered (%)	RT of Degradant (min)
Acid	0.1M HCl	65°C	2h	98.67	-
Alkali	0.1M NaOH	65°C	2h	98.09	-
Oxidative degradation	3%H ₂ O ₂	65°C	2h	99.86	-
Thermal Degradation	65°C		2h	98.09	-
Photo degradation	1 photo cycle		24h	99.23	-

Table 9B: Forced degradation results of Bisoprolol standard

For Standard of Bisoprolol					
Conditions	Reagent	Temperature	Time	Drug amount recovered (%)	RT of Degradant (min)
Acid	0.1M HCl	65°C	2h	98.61	-
Alkali	0.1M NaOH	65°C	2h	98.08	
Oxidative degradation	3%H ₂ O ₂	65°C	2h	99.24	-
Thermal Degradation	65°C		2h	98.24	-
Photo degradation	1 photo cycle		24h	99.21	-

Table 9C: Forced degradation results of dapagliflozin in tablet formulation

For Standard of Tablet formulation – Dapagliflozin					
Conditions	Reagent	Temperature	Time	Drug amount recovered (%)	RT of Degradant (min)
Acid	0.1M HCl	65°C	2h	98.64	-
Alkali	0.1M NaOH	65°C	2h	98.37	-
Oxidative degradation	3%H ₂ O ₂	65°C	2h	98.28	-
Thermal Degradation	65°C		2h	99.55	-

Photo degradation	1 photo cycle	24h	98.26	-
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Table 9D: Forced degradation results of bisoprolol in tablet formulation

For Standard of Tablet formulation – Bisoprolol					
Conditions	Reagent	Temperature	Time	Drug amount recovered (%)	RT of Degradant (min)
Acid	0.1M HCl	65°C	2h	99.33	-
Alkali	0.1M NaOH	65°C	2h	99.70	-
Oxidative degradation	3%H ₂ O ₂	65°C	2h	99.94	-
Thermal Degradation	65°C		2h	99.75	-
Photo degradation	1 photo cycle		24h	99.80	-

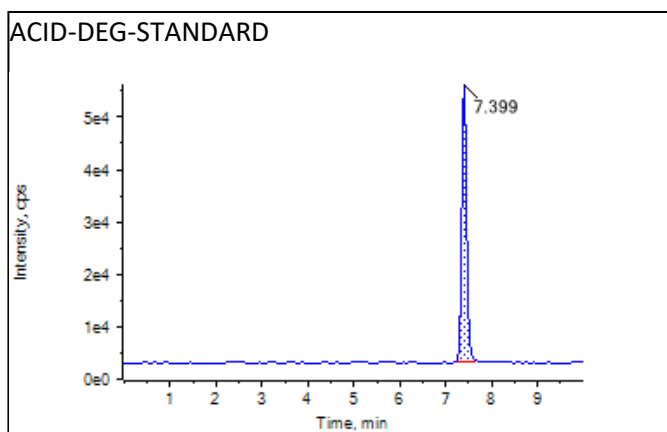


Figure a: Acidic

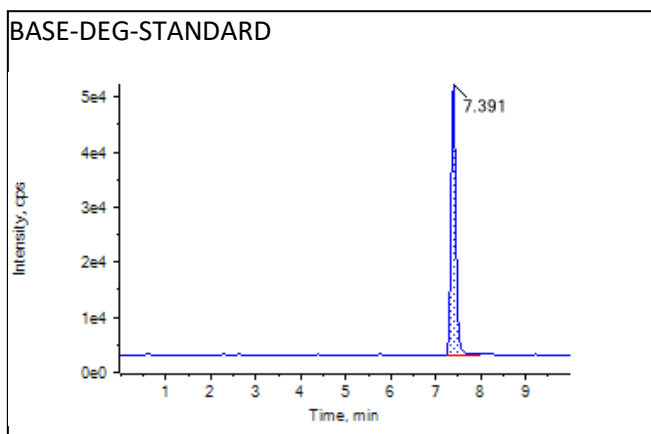


Figure b: Alkaline

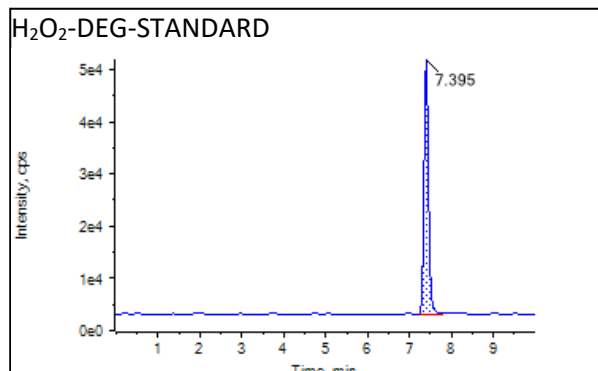


Figure c: Oxidative

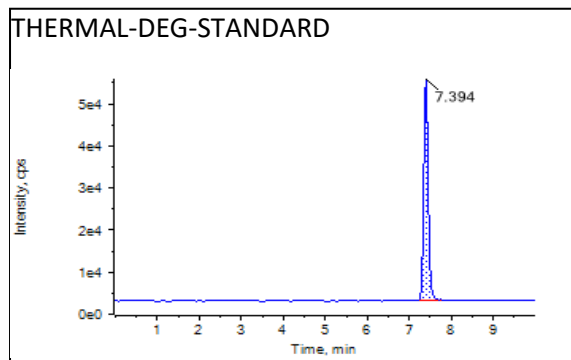


Figure d: Thermal

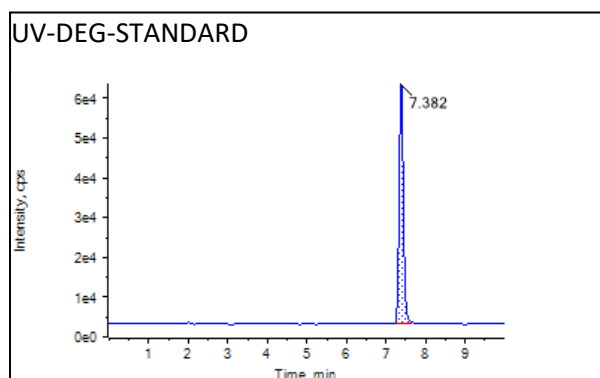


Figure e: Photolytic

Figure 4A: LC-MS/MS chromatograms of dapagliflozin under different forced degradation conditions

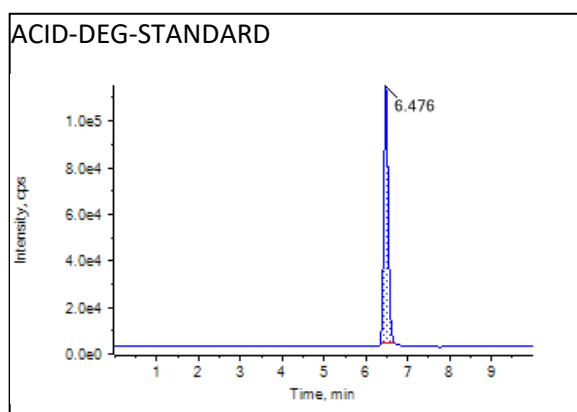


Figure a: Acidic

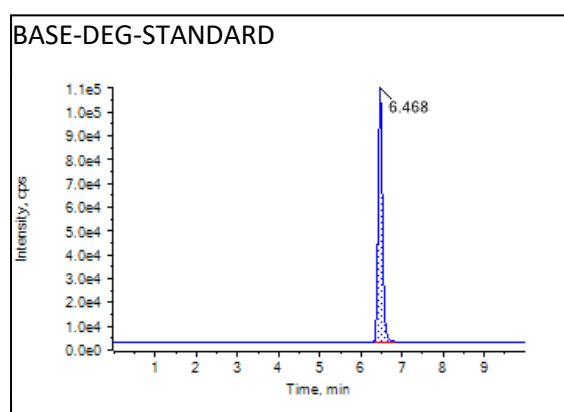


Figure b: Alkaline

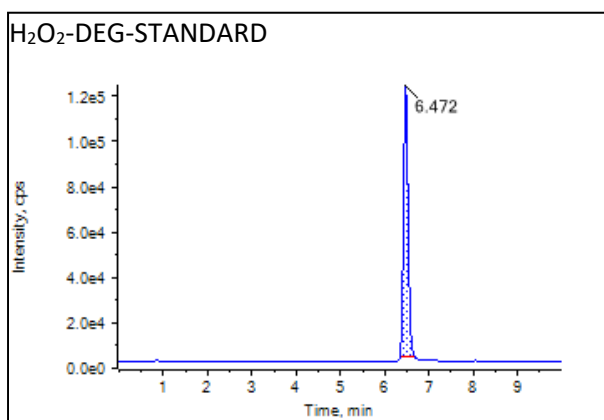


Figure c: Oxidative

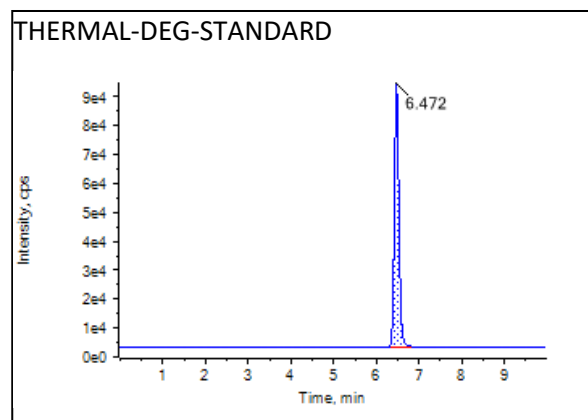


Figure d: Thermal

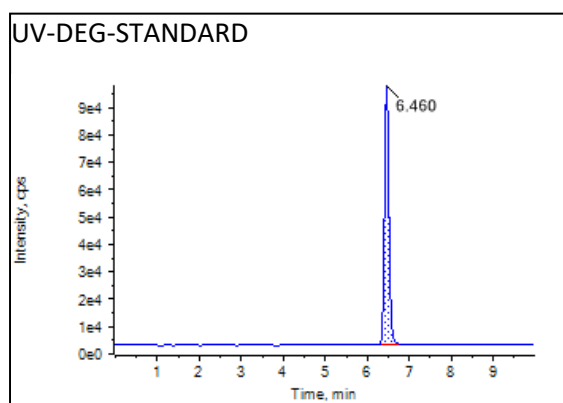


Figure e: Photolytic

Figure 4B: LC-MS/MS chromatograms of bisoprolol under different forced degradation conditions

4. Conclusion:

A sensitive, robust and stability-indicating LC–MS/MS method was developed for the simultaneous quantification of dapagliflozin and bisoprolol in API and tablet formulations. The chromatographic and mass spectrometric parameters were optimised to obtain an efficient separation with sharp and symmetrical peaks. The developed method showed good linearity, accuracy and precision and met the acceptance criteria given in the ICH Q2(R2) guidelines.

To evaluate the intrinsic stability behaviour of both analytes, forced degradation studies were carried out under acidic, alkaline, oxidative, thermal and photolytic stress conditions. No significant degradation of dapagliflozin or bisoprolol was detected in the applied stress conditions, which shows the stability of the compounds in the studied conditions. The robustness testing by deliberate variations of the analytical parameters showed negligible effect on the performance of the method, which further confirms the reliability and adequacy of the developed method for routine pharmaceutical quality control and stability studies.

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AUTHOR CONTRIBUTIONS

Sushma Madhavaram: Conceptualisation, data curation, formal analysis, investigation, writing original draft, resources. **Judy Jays:** editing, supervision, validation, formal analysis, conceptualisation.

CONFLICT OF INTEREST

The authors have no competing interests

ETHICS COMMITTEE APPROVAL

The authors declare that the ethics committee approval is not required for this study.

Funding Declaration

No Funding was received for this work

Clinical Trial Number : Not Applicable

Abbreviations

DAPA	Dapagliflozin
Biso	Bisoprolol
API	Active Pharmaceutical Ingredient
ICH	International Conference on Harmonization
LC-MS/MS	Liquid chromatography – Tandem mass Spectrometry
ACN	Acetonitrile
LOD	Limit of Detection
LOQ	Limit of Quantification
DEG	Degradation

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