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DEVELOPMENT AND VALIDATION OF HPTLC BIOANALYTICAL METHOD FOR SIMULTANEOUS ESTIMATION OF DRUGS PRESCRIBED IN THE COMORBID CONDITION OF DIABETES AND CARDIOVASCULAR DISEASE

Vineeta V. Khanvilkar[1]. Darshana D. Pardeshi[2]. Deepali R. Kadwadkar[3].

Bharati Vidyapeeth's College of Pharmacy, (Department of Pharmaceutical chemistry),

Navi Mumbai, (Maharashtra), India

Abstract

Diabetes and cardiovascular disease are rising global health concerns and in around 85% of cases, patients are having both these diseases as a comorbid condition. Such patients administer drugs belonging to both antidiabetic and antihyperlipidemic class. Managing diabetes mellitus along with cardiovascular disease is done by controlling blood glucose level and cholesterol level by adapting antidiabetic and antihyperlipidemic drug therapy respectively with their simultaneous administration. Selection of the most accurate drugs and their effective dose regime is very important to avoid further severe complications. Empagliflozin is prescribed as a first-line drug for diabetes treatment and Atorvastatin is the preferred one in cardiovascular disease. The dual effect of Atorvastatin on the blood glucose level creates a need for monitoring of its concentration in patients consuming simultaneously an antidiabetic drug like Empagliflozin. The proposed method is an attempt to develop an HPTLC method for simultaneous estimation of Atorvastatin and Empagliflozin in human plasma. Chromatographic separation of drugs was performed over TLC plates pre-coated with silica gel $60F_{254}$ using toluene: ethyl acetate: methanol: formic acid (12:5:3:0.5 v/v/v/v) as the mobile phase via a linear ascending technique. Detection and quantification were carried out at a wavelength 235 nm. The method was validated according to the European Medicines Agency ICH guideline M10 on Bioanalytical Method Validation and gave satisfactory results. The developed method can be successfully employed for simultaneous estimation of Empagliflozin and Atorvastatin from human plasma of patients with the comorbid condition of diabetes and cardiovascular disease.

Keywords: High performance thin layer chromatography, Comorbid condition, Bio-analytical method development, Empagliflozin and Atorvastatin

1. Introduction

Cardiovascular diseases (CVDs) are the most common cause of death around the world. In 2019, 17.9 million people died from CVDs, accounting for 32 percent of deaths worldwide amongst which 85 percent of these deaths were caused by a heart attack. Hyperlipidemia is one of the major risk factors for CVD. According to statistics from a study of 1,492 physicians conducted by the Centers for Disease and Prevention, hyperlipidemia is the second most prevalent chronic illness seen [1]. Hyperlipidemia and CVD are the common comorbid conditions observed in the patient having diabetes mellitus [2, 3]. In comorbidity, the patients are often treated with multiple medications. A study of prescriptions reflected that Empagliflozin and Atorvastatin are usually prescribed for patients with this comorbid condition of diabetes mellitus and CVD. As both of these drugs belong to completely distinct drug classes, a combined formulation is not currently available in the market.

Statins are the first-line treatment for CVD which acts by inhibiting HMG CO-A reductase, an enzyme found in the liver that plays important role in producing cholesterol [4]. Statin therapy has been associated with 19-55% reduction in CVD events in patients with diabetes mellitus [5]. Also, statins have an effective result on diabetes and are found to reduce the rate of progression of diabetic retinopathy, nephropathy, and neuropathy [6, 7]. Atorvastatin [(3R,5R)-7-[2-(4-fluorophenyl)-3-phenyl-4-(phenylcarbamoyl)-5-propan-2-ylpyrrol-1-yl]-3,5-dihydroxyheptanoic acid] (Figure 1A) is a statin derivative commonly prescribed to prevent cardiovascular disease in those at high risk and abnormal lipid level [8]. Empagliflozin [D-Glucitol,1,5-anhydro-1-C-[4-chloro-3-[[4-[[(3S)-tetrahydro-3furanyl]oxy]phenyl]methyl]phenyl]-, (1S)] (Figure 1B) is a sodium-glucose co-transporter-2

(SGLT-2) inhibitor, used to treat type II diabetes. SGLT-2 accounts for about 90% of glucose reabsorption into the blood [9]. Inhibiting SGLT-2 reduces blood glucose by blocking glucose reabsorption in the kidney and thereby excreting blood sugar via urine [9–11].

The fact that Atorvastatin can cause a reduction in the blood glucose level creates a need for monitoring of its concentration in patients consuming simultaneously an antidiabetic drug like Empagliflozin. As such comorbid conditions are highly dose-dependent, even a small change in concentration of drugs used in treatment may result in a severe complication. This necessitates monitoring the plasma concentration of both drugs used in the treatment. For quantitative measurement of drugs and their metabolites in biological matrices, bioanalytical methods are commonly used. These methods play a major role in the determination and interpretation of bioequivalence, pharmacokinetic and toxicological studies.

Literature survey revealed that analytical methods have been reported for Empagliflozin and Atorvastatin either individually or in combination with other drugs. Analytical methods including UV spectrophotometry [13], [14], HPLC[15]–[20], LC-MS[21]–[27], and HPTLC[27–32] have been reported for the determination of Empagliflozin in pharmaceutical products and biological fluids. Atorvastatin is reported to be determined either alone or in combination with other drugs in pharmaceutical preparation and biological matrices by analytical methods involving UV spectrophotometry[33–38], HPLC[39–46], LC-MS[47–51], and HPTLC[52–57]. Although analytical methods are available for the estimation of Empagliflozin and Atorvastatin in a biological matrix individually no method is reported yet for their simultaneous estimation in human plasma. Such a method if developed can help in the determination of the concentration of these two drugs simultaneously in human plasma.

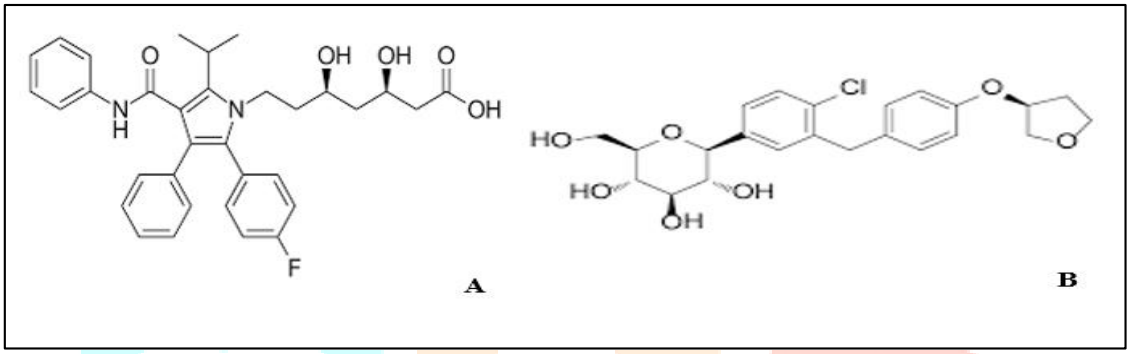

Figure 1: Chemical structure of A. Atorvastatin B. Empagliflozin

2. Experimental

2.1 Materials

Empagliflozin standard was procured as a gift sample from Dr. Reddy's laboratory Pvt. Ltd. (Hyderabad, India) with 99.8% purity for the drug, based on the company analysis certificate. Atorvastatin was obtained from S. D. Fine Chemicals Pvt. Ltd, (Mumbai, India). Drug-free human plasma was obtained from Kharghar Blood Bank, Kharghar (Navi Mumbai- India). All chemicals and reagents used in the study were of analytical grade and were purchased from S.D Fine chemicals, Pvt. Ltd. (Mumbai, India).

2.2 Instrumentation and software

A CAMAG HPTLC system (Muttenz, Switzerland) comprising of a CAMAG Linomat V semi-automatic sample applicator, CAMAG TLC Scanner IV, CAMAG TLC visualizer, flat bottom and twin-trough developing chamber (10×10 cm), UV cabinet with dual-wavelength UV lamp, Hamilton syringe (100 µL; Bonaduz, Switzerland), ultrasonic bath (Frontline FS-4, Mumbai, India) and CAMAG win CATS software was used in the study. Calculations were performed by the use of Microsoft Excel software for linear regression analysis for quantification of extracts and determination of validation parameters.

2.3 Preparation of stock solution and working standard solutions

10 mg of Empagliflozin and Atorvastatin was weighed accurately and transferred to a 10 mL volumetric flask. Methanol was used for dissolving drugs and volume was made up to the mark of 10 mL to obtain a stock solution of 1000 µg/mL of each drug sample. Working standard solutions were prepared fresh by appropriately diluting the stock solution (1000µg/mL) with methanol in a 10 mL volumetric flask to get the concentration of 50, 100, 150, 200, 250, 300, and 350 µg/mL of each Empagliflozin and Atorvastatin. All solutions were kept at 4-6°C and brought to room temperature before use.

2.4 Preparation of plasma samples and the quality control samples

An aliquot of 0.2 mL centrifuged drug free plasma was pipetted into a 2 mL of Eppendorf tube, to which 20 µl of each working standard solution of Empagliflozin and Atorvastatin was added to obtain calibration standards of 50, 100, 150, 200, 250, 300, and 350 ng/spot. The Quality Control (QC) samples are prepared in plasma having concentrations of 100 ng/ml (Low QC), 250 ng/ml (Mid QC), and 350 ng/ml (High QC). Protein precipitation and extraction were carried out by the addition of 1.2 mL of acetonitrile which was then vigorously vortexed in a cyclomixer for 2 min and after that, it was centrifuged at 4000 rpm for 10 min at 4°C. An aliquot of 1 mL of supernatant was collected and filtered through a 0.45 µm syringe filter and was used for HPTLC analysis using optimized chromatographic conditions.

2.5 Chromatographic condition

The samples were spotted in the form of narrow bands having a bandwidth of 8 mm with a 100µL microsyringe (Linomat syringe 659.0014) on pre-coated silica gel HPTLC plate 60 F254 (10 × 10 cm), 100 µm thickness, E. Merck, Darmstadt, Germany) employing a CAMAG Linomat V sample applicator. The mobile phase used was a mixture of Toluene, Ethyl

acetate, Methanol, and Formic acid in the ratio of (12:5:3:0.5, v/v/v/v). The time for chamber saturation was optimized to 20 min. The length of chromatographic development was 90 mm. The densitometric scanning was performed at 235 nm in absorbance mode. The slit dimensions were set at 0.50 mm × 0.45 mm, the scanning speed at 20 mm/s, and the data resolution at 100µm/step. The results were evaluated to achieve an optimum separation between spots and migration of spots to ensure separation reproducibility

2.6 Method validation procedure The developed method was validated as per the European Medicines Agency guideline on bioanalytical method validation for sensitivity, selectivity, precision, accuracy, linearity, recovery, carryover, and stability. The selectivity of the method was performed by analyzing six blank plasma samples. Each blank sample was tested for interference using an optimized extraction method. Linearity was performed over the range 50-350ng/spot for Empagliflozin and Atorvastatin. Co-efficient of regression, slope, and y-intercept were estimated by plotting a graph of drug concentration against peak area for each standard to determine linearity. Limit of detection (LOD) and limit of quantification (LOQ) were calculated from the slope and standard deviation obtained from the graph of linearity. Repeatability or intra-day precision was investigated by analyzing three replicate of QC samples at three different concentrations on the same day and inter-day precision was assessed by injecting the same three QC samples over three different days. Accuracy studies were studied at 80%, 100%, and 120% levels. The robustness was studied in triplicate at LQC (100 ng/spot) and HQC (350 ng/spot) for Empagliflozin and Atorvastatin by deliberately making small changes in chromatographic conditions. Recovery was studied by comparing the peak areas of extracted LQC, MQC, and HQC samples of Empagliflozin and Atorvastatin with the unextracted sample. A stability study was performed at two different stability QC concentrations (LQC, HQC) as per EMA Guidelines for bioanalytical method development.

3. Result and discussion

3.1 Optimization of chromatographic condition

Based on the literature, and the solubility of Empagliflozin and Atorvastatin in various solvents, combinations of solvents were prepared. These were then checked for their ability to quantitatively separate these two drugs. The mixture of Toluene: Ethyl acetate: Methanol: Formic acid in the ratio 12:5:3:0.5 v/v/v/v was found to give well separated and resolved peaks and was therefore selected as mobile phase. The isobestic wavelength 235 nm was selected as the detection wavelength for further analysis.

3.2 Optimization of the plasma extraction procedure

In bioanalytical method development, the critical step is the separation of spiked drugs from the complex matrix with high and reproducible recovery. Being the simplest, time-saving, and most reported extraction technique, the protein precipitation method was selected as an extraction technique for sample preparation. Different solvents were tried for the extraction of Empagliflozin and Atorvastatin from human plasma by protein precipitation. The process was carried out by spiking the known amount of drugs in plasma and comparing the % recovery of the extracted sample with the unextracted sample. Initially, methanol and tetrahydrofuran were tried which gave recovery about 40-50 %. Acetonitrile, however was found to give 70-80 % recovery with minimal plasma interference at R_f of the drugs and so was selected as the extracting solvent. The proposed method was able to separate the two drugs in human plasma with R_f values of 0.31± 0.02 and 0.51± 0.02 for Empagliflozin and Atorvastatin, respectively.

3.3 Method validation

The developed bioanalytical method was validated as per the validation protocol prepared using EMA guidelines on bioanalytical method validation. Performance characteristics of the bioanalytical HPTLC method were statistically validated for the selectivity, linearity, lower limit of quantification (LLOQ), LOD, accuracy, precision, robustness, carryover, and stability.

a) Linearity and Range

The standard stock solution was appropriately diluted and applied on HPTLC plate to obtain linearity of standard solutions containing Empagliflozin and Atorvastatin 50-350 ng/spot. Linearity was observed by plotting the graph of drug concentration (x) against peak area (y) for each standard. The standard deviation (SD), coefficient of determination (r^2), slope, and intercept of the calibration curves were estimated to determine the method's linearity. Linearity was found over the range 50-350 ng/spot for both Empagliflozin and Atorvastatin. Linearity data is given in table 1 and calibration curves are shown in Figure 1. LOD and LOQ were calculated based on the standard deviation of the response and slope. The estimated values for LOD and LOQ for Empagliflozin were found to be 20.20 ng/spot and 61.23 ng/spot respectively. LOD and LOQ for Atorvastatin are 9.31 ng/spot and 28.23 ng/spot respectively.

Table 1: Linearity parameters of Empagliflozin and Atorvastatin

Sr. No.	Concentration (ng/spot)	Empagliflozin		Atorvastatin	
		Mean Area	% RSD	Mean Area	%RSD
1	50	224	1.314	390	1.425
2	100	321	1.416	586	0.978
3	150	410	1.701	762	0.386
4	200	500	1.296	1013	0.716
5	250	560	1.257	1142	0.842
6	300	660	0.642	1290	0.395
7	350	800	0.270	1450	0.439
Correlation Coefficient (r^2)		0.9911		0.9928	

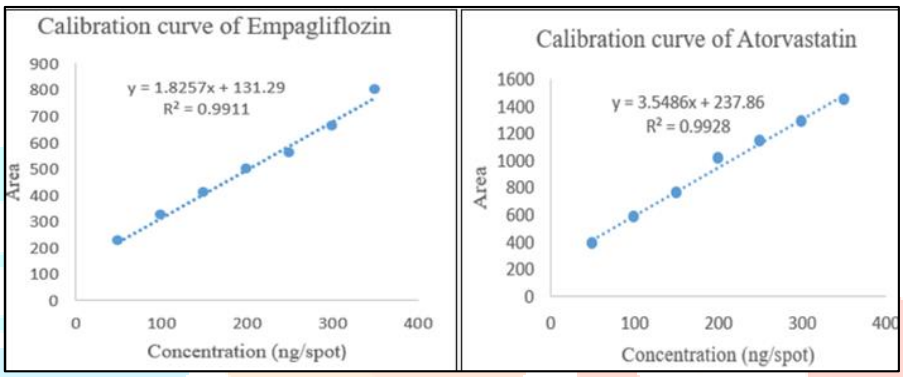

Figure 2: Calibration curve of Empagliflozin and Atorvastatin

b) Selectivity

The selectivity of the method was investigated by analyzing six blank plasma samples. Each blank sample was tested for interference with plasma spiked with Empagliflozin and Atorvastatin at the concentration of their LLOQ in human plasma. Figure 3 shows the typical chromatograms of blank plasma and plasma samples spiked with Empagliflozin and Atorvastatin. The method was found to be selective as there was no significant interference of biological matrix found at R_f value of the drugs.

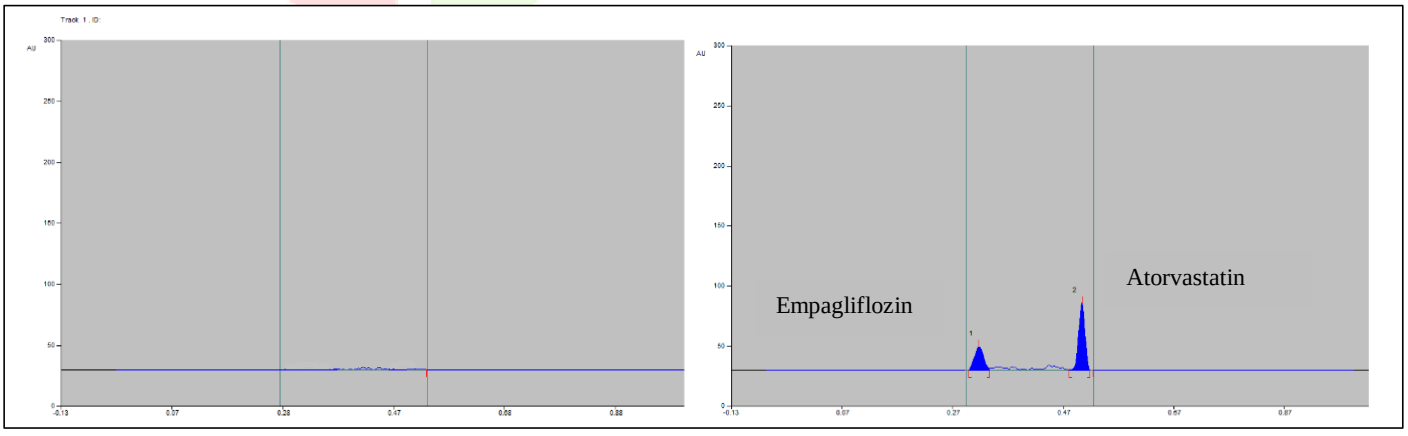

Figure 3: Densitogram of blank plasma and spiked plasma with Empagliflozin and Atorvastatin at LLOQ (50 ng/spot) depicting the selectivity of the proposed method

c) **Accuracy**

The accuracy of the method was studied by performing recovery studies of QC samples in triplicate, by spiking the known amount of drug solution at different concentration levels of 80%, 100%, 120% in QC samples. Accuracy was expressed as the percentage of the analytes recovered by the assay. The results are summarized in Table 2, indicating that the method was accurate.

Table 2: Results of Accuracy studies of Empagliflozin and Atorvastatin

	Empagliflozin			Atorvastatin		
% level	**80**	**100**	**120**	**80**	**100**	**120**
Initial amount (ng/spot)	100	100	100	100	100	100
Spiked amount (ng/spot)	80	100	120	80	100	120
Total amount (ng/spot)	180	200	220	180	200	220
Average area	452	514	549	865	941	1033
Concentration found (ng/spot)	175	209	228	177	198	224
Recovery (%)	97.113	104.5	103.63	98.33	99	101.81

d) Precision

Repeatability or intra-day precision was investigated by analyzing three replicate QC samples of each drug at three different concentrations. Inter-day precision was assessed by analyzing the same three QC samples over three days. The relative standard deviation (%RSD) of the obtained assay values at three different concentration levels was calculated. The resultant densitogram of quality control samples are given in Figure 4, 5, and 6. The statistical data for the precision study are summarized in Table 3. The %RSD values for the intra-day and inter-day precision confirmed that the method is precise.

Table 3: Results of Precision studies of Empagliflozin and Atorvastatin

	Intra-day precision of Empagliflozin				Inter-day precision of Empagliflozin			
Concentration levels	**LLOQ**	**LQC**	**MQC**	**HQC**	**LLOQ**	**LQC**	**MQC**	**HQC**
Concentration (ng/spot)	50	100	250	350	50	100	250	350
Average peak area	233	321.33	555.67	864	219.6	327	547	869
Standard deviation	4.54	5.557	8.219	7.4833	3.399	3.858	6.683	4.320
%RSD	1.95	1.729	1.479	0.866	1.547	1.180	1.22	0.497
	Intra-day precision of Atorvastatin				**Inter-day precision of Atorvastatin**			
Concentration levels	**LLOQ**	**LQC**	**MQC**	**HQC**	**LLOQ**	**LQC**	**MQC**	**HQC**
Concentration (ng/spot)	50	100	250	350	50	100	250	350
Average peak area	387.66	576	1133.67	1455	373.3	563.34	1101.34	1404.667
Standard deviation	7.717	8.6023	9.533	6.531	4.921	8.055	15.965	9.1772
%RSD	1.9907	1.493	0.840	0.448	1.318	1.429	1.4496	0.653

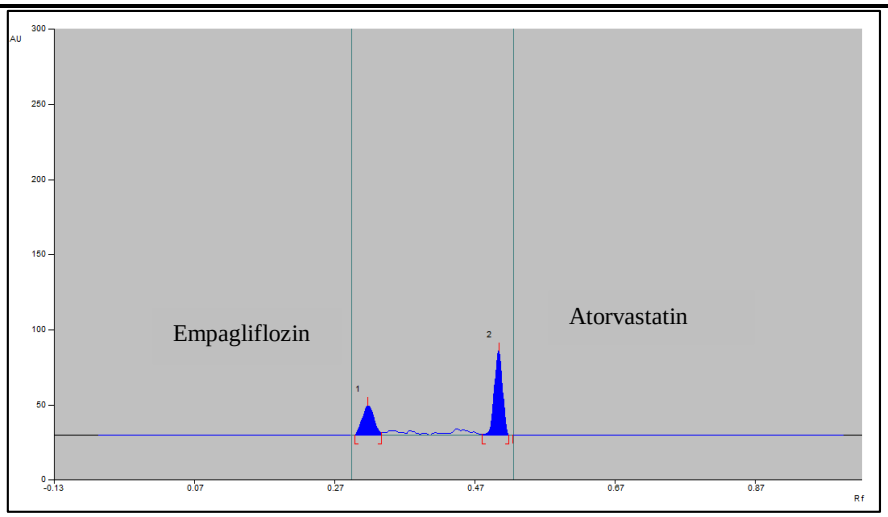

Figure 4: Densitogram of LQC sample (100 ng/spot) spiked in human plasma

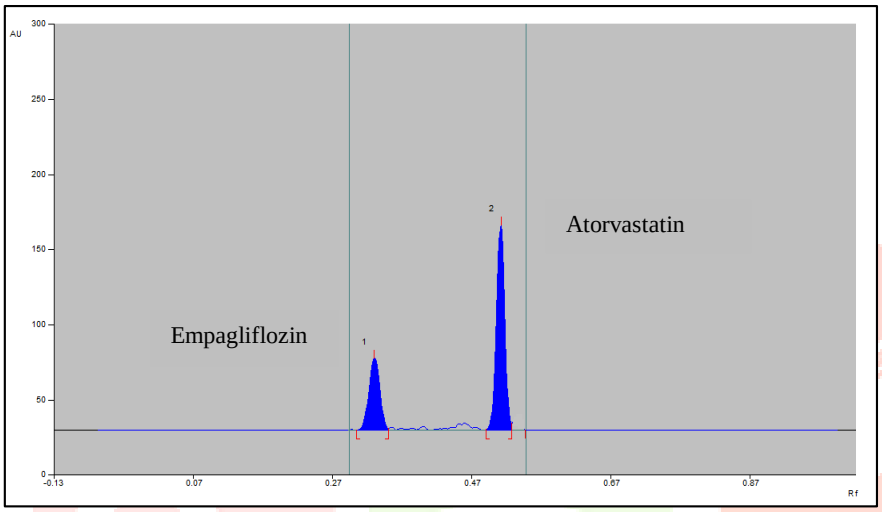

Figure 5: Densitogram of MQC sample (250 ng/spot) spiked in human plasma

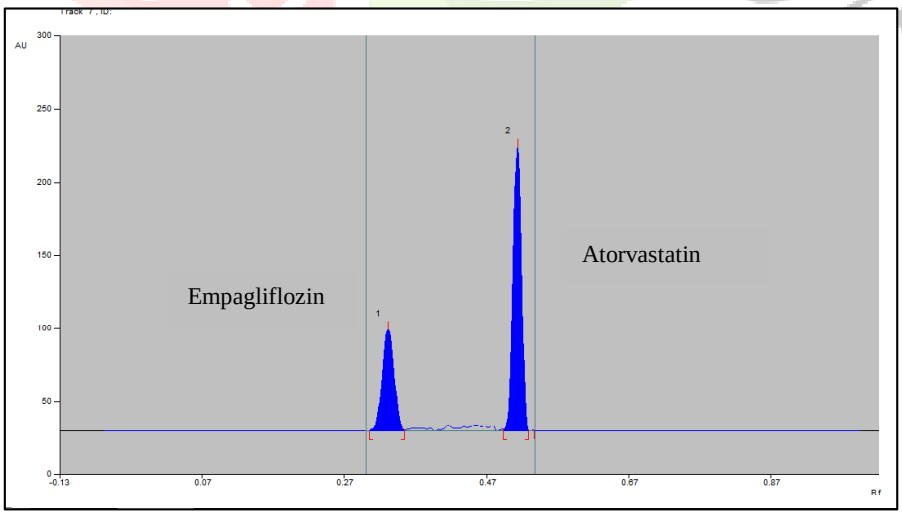

Figure 6: Densitogram of HQC sample (350 ng/spot) spiked in human plasma

e) **Robustness**

The robustness was studied in triplicate at the concentration of 100 (LQC) and 350 (HQC) ng/spot for Empagliflozin and Atorvastatin by deliberately making small changes in chromatographic conditions such as changes in mobile phase composition (± 0.2 ml), chamber saturation time (±5 min) and detection wavelength (±2 nm). Effects of these changes on peak area were evaluated by calculating the %RSD. Results of robustness are represented in Table 5, which indicates that the method is robust.

Table 5: Result of robustness of Empagliflozin and Atorvastatin

Method parameters	**Level of variation**	**Modified parameters**	**% RSD of area**			
			EMP		**ATS**	
			LQC	**HQC**	**LQC**	**HQC**
Mobile phase composition Toluene: Ethyl acetate: Methanol: Formic acid (12:5:3:0.5 v/v/v/v)	+0.2 ml	Toluene: Ethyl acetate: Methanol: Formic acid (12.2: 5: 3: 0.5 v/v/v/v)	1.74	1.328	1.115	1.4985
	-0.2 ml	Toluene: Ethyl acetate: Methanol: Formic acid (11.8: 5: 3: 0.5 v/v/v/v)	1.903	1.561	1.445	0.945
Saturation time (20 min)	+2 min	22 minutes	1.9159	1.5375	1.815	1.1024
	-2 min	18 minutes	1.755	1.125	1.403	1.482
Detection wavelength 235nm	+2 nm	240 nm	1.429	1.007	2.2108	0.870
	-2 nm	230 nm	1.60	1.34	1.66	1.827

f) **Recovery studies**

Recovery was calculated by comparing the peak areas of freshly prepared and extracted LQC, MQC, and HQC samples of Empagliflozin and Atorvastatin with the unextracted sample. The recoveries of drugs were determined at the three concentrations LQC, MQC, and HQC, and results are shown in Table 4.

Table 4: Result of Recovery studies of Empagliflozin and Atorvastatin

	Empagliflozin			**Atorvastatin**		
QC sample	**LQC**	**MQC**	**HQC**	**LQC**	**MQC**	**HQC**
Mean peak area of standard solution	465	738	1103	730	1586	1920
Mean peak area of extracted solution	320	545	832	530	1135	1485
Mean % recovery	68.81	73.84	75.43	72.60	71.56	77.34

g) **Stability studies**

Benchtop stability, freeze thaw stability and long term stability studies were undertaken for Empagliflozin and Atorvastatin at the LQC and HQC concentration. In bench top stability samples were kept at room temperature for 6 hours. Freeze thaw stability was done by analyzing sample after three freeze thaw cycle. For long term stability, sample stored in freezer were evaluated. Results obtained were compared with those of the freshly prepared samples and are tabulated in Table 5. The result confirmed the stability of plasma samples under laboratory conditions.

Table 5: Result of Stability studies of Empagliflozin and Atorvastatin

	Empagliflozin						Atorvastatin					
	Short term stability		Freeze thaw stability		Long term stability		Short term stability		Freeze thaw stability		Long term stability	
	LQC	HQC	LQC	HQC	LQC	HQC	LQC	HQC	LQC	HQC	LQC	HQC
Mean	355.6	851.2	369.1	863.7	401.2	899.3	572.5	1536.3	575.9	1534.4	669.09	1468.1
SD	5.31	14.70	12.22	21.35	8.99	22.80	12.76	30.68	8.60	8.60	17.59	37.36
%RSD	1.49	1.72	3.31	2.47	2.24	2.53	2.22	1.99	1.49	1.49	2.62	2.54

h) Carryover

Carry-over was performed by injecting blank samples after a high concentration sample (ULOQ). As depicted in Figure 6 no extraneous peaks were observed in blank plasma at the R_f values of the drugs. The calculated value of carryover for Empagliflozin and Atorvastatin was 0%.

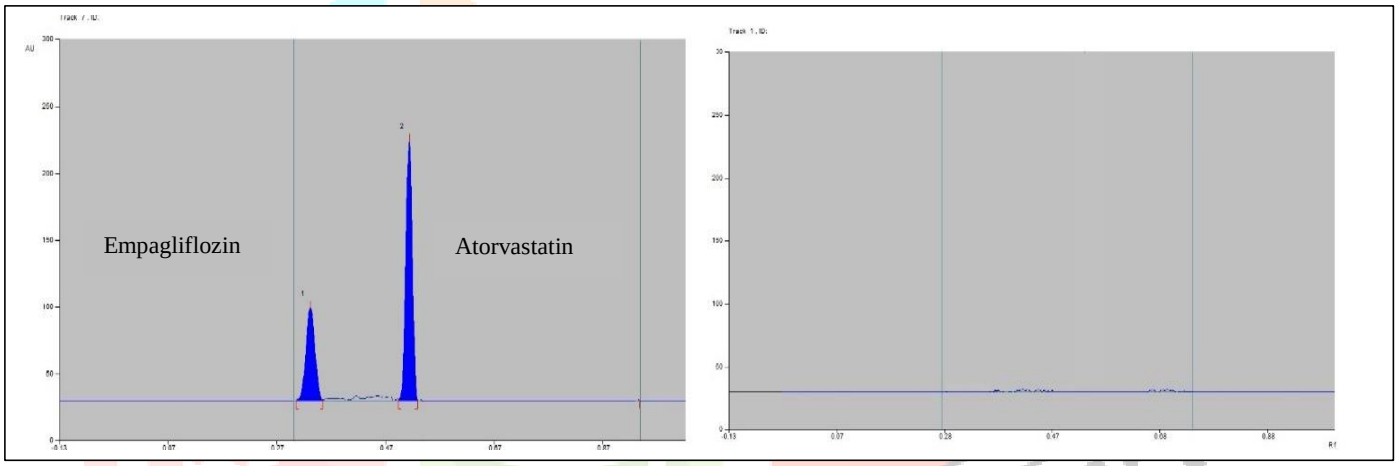

Figure 6: Densitogram of ULOQ sample and blank plasma sample applied after ULOQ depicting the carry over study

4. Conclusion The analytical method was developed for simultaneous estimation of Empagliflozin and Atorvastatin in human plasma and was validated as per EMA guidelines on bioanalytical method validation. The method was found to be specific, linear, accurate, precise, and reproducible which can be used for routine bio-analysis of Atorvastatin and Empagliflozin.

5. Declaration

Funding and/or Conflicts of interests/Competing interests
The authors did not receive support from any organization for submitted work. All authors certify that they have no affiliations with or involvement in any organization for submitted work. All authors certify that they
have no affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript.

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