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Research Article

VALIDATED RP-HPLC METHOD FOR SIMULTANEOUS DETERMINATION OF CANAGLIFLOZIN, DAPAGLIFLOZIN, EMPAGLIFLOZIN AND METFORMIN

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ABSTRACT

The first reversed phase high performance liquid chromatographic method for simultaneous determination of Empagliflozin, Canagliflozin, Dapagliflozin and Metformin has been developed and validated to be a simple, sensitive, rapid, specific, precise, and accurate method. Chromatographic separation was achieved on C18 column (250×4.6 mm-5µm p.s) Inertsil® ODS through isocratic elution using acetonitrile and 0.05 M potassium dihydrogen phosphate buffer PH 4 in a ratio [65:35, v/v] as a mobile phase at flow rate of 1ml/min. UV detection was operated at 212 nm and injection volum was 10 µl. Linearity range for Canagliflozin, Dapagliflozin, Empagliflozin and Metformin was 7.5-225, 5-150, 6.5-187.5 and 10-1000 µg/ml, respectively. The proposed method showed good linearity, accuracy, precision and was successfully applied for determination of the four drugs in laboratory prepared mixtures and in the seven pharmaceutical dosage forms and so it is suitable for quality control of them.

Keywords: Empagliflozin [EMPA], Canagliflozin [CANA], Dapagliflozin [DAPA] and HPLC.

INTRODUCTION

Generally, glycemic control in patients with diabetes mellitus type-2[T2DM] is poor, with only approximately 53 % of patients achieving glycemic goals with their current treatment regimen[1]. However, even in patients with good glycemic control, the progressive nature of T2DM will lead to that most patients will need to multiple anti-diabetic medications to manage their disease[2].Till now, most type 2 diabetic patients therapeutic goals are not achieved[3], and so they still need for efficient new therapeutic strategies for treating type 2 diabetes, including combination therapy.

Recently, sodium-glucose co-transporter-2 [SGLT-2] inhibitors have been developed as novel class of therapeutic agents for the treatment of type 2 diabetes[4,5]. Sodium glucose co-transporter 2 [SGLT2] inhibitors are introducing an alternative mechanism for control of hyperglycemia in T2DM by reducing glucose reabsorption in the kidney and thereby increasing urinary glucose excretion [UGE][6,7]. Besides their proven efficacy in lowering plasma glucose levels additional benfits are associated with their mechanism of action such as a low risk of hypoglycemia, weight loss and reductions in blood pressure [8,9,10]. Canagliflozin [CANA] Figure 1], Dapagliflozin [DAPA] [Figure 2] and Empagliflozin [EMPA] [figure 3], are SGLT2 inhibitors approved by FDA and the Committee for Medicinal Products for Human Use [CHMP] of the European Medicines Agency alone or in combination with metformin [MET] [Figure 4].There are many methods have been developed for the analysis of these compounds alone, in combination with metformin or in combination with other drugs in different pharmaceutical dosage forms and biological fluids. A literature review showed many methods have been described for determination of EMPA alone including high performance liquid chromatography HPLC[11,12,13], LC-MS/MS[14] and also in combination with MET including

HPLC[15,16,17,18], spectrophotometry[19,20,21] and by LC-MS/MS[22,23]. Few methods were reported for determination of EMP in combination with Linagliptin[24,25,26] and in other chemical mixtures with Metformin and Linagliptin by UPLC[27]. Many methods have been described for the determination of CANA alone in pharmaceutical preparations or biological fluids including HPLC[28,29,30,31,32] spectrophotometry[33,34,35] HPTLC[36] , LC-MS/MS[37] and also in combination with MET[38,39,40]. Literature survey reveals that a number of methods were reported for determination of DAPA alone[41-47] in combination with MET[48-53] and in other combination as with Saxagliptin[54]. There is no any analytical method has been reported for the simultaneous determination of MET, EMPA, DAPA and CANA. This proposed method will be useful in their determination in the same time in bulk and in different combination of their pharmaceutical dosage forms in quality control laboratories, saving time and solvents with simple mobile phase.

Experimental

MATERIALS AND REAGENTS

All reference standards of Canagliflozin [CANA], Dapagliflozin [DAPA], Empagliflozin [EMPA] and Metformin [MET)] were obtained as a gift samples from Western pharmaceutical industries, Egypt. Acetonitril [Fisher, UK] was of HPLC grade. Potassium dihydrogen phosphate [Nice chemicals LTD, India], orthophosphoric acid [Honey Well, USA] and triethylamine [Acros organics] used were of analytical grade. Water was prepared by double-glass distillation and filteration was through 0.45µm cellulose nitrate membrane filters [Chemlab, Spain].

Invokamet® containing 50 mg of CANA and 500 mg of MET, Invokana® containing 100 mg of CANA [manufactured by Janssen-Canada], Xigduo® containing 10 mg of DAPA and 500 mg of MET, Forxiga® containing 10 mg of DAPA [manufactured by Astrazeneca pharmaceutical company-USA], Synjardy® containing 12.5 mg of EMPA and 500 mg of MET, Jardiance® containing 25 mg of EMPA [manufactured by Boehringer Ingelheim pharmaceutical company-Germany] and Glucophage® containing 1000 mg of MET per tablet [manufactured by Merck Serono -Egypt] were purchased.

Instrumentation and chromatographic conditions

Shimadzu [Japan] HPLC system was used in analysis. It was equipped with a model [2LC-10AD vp] pumps, autosampler [sil-10ADvp], column oven [CTO-10A (C) vp] and UV detector [UV SPD-10A (V) vp]. The chromatographic analysis was performed on a reversed phase C18 column [250×4.6 mm-5µm p.s] Inertsil® ODS. A UV-visible spectrophotometer [UV-1650PC Shimadzu, Japan] was used for selecting the prefered wave length for detection on HPLC which selected to be λ 212 nm. Isocratic elution was done using a mobile phase consisting of acetonitrile and 0.05 M potassium dihydrogen phosphate buffer PH 4 in a ratio [65:35, v/v]. The mobile phase was degassed for 30 min by sonication using an ultrasonic bath, Ultra Sonik [Ney-28].

The buffer was prepared by dissolving 6.8 gm of potassium dihydrogen phosphate in 1000 ml of distilled water. PH of the buffer was adjusted using Jenway 3510 PH meter [UK] to be 4 using orthophosphoric acid. The same buffer was used in preparation of a solvent which was consisted of acetonitrile and 0.05 M phosphate buffer PH4 in a ratio 50:50, v/v. Vibra balance [Japan] was used for weighting the studied drugs. Injection volum was 10 µl with flow rate of 1 ml/min. All determinations of the chromatographic separation were performed at ambient temperature.

Procedures

Preparation of standard solutions

Standard stock solutions of CANA [1.5 mg/ml], DAPA [1 mg/ml], EMPA [1.25 mg/ml] and MET [1 mg/ml] were prepared by dissolving 150 mg of CANA, 125 mg of EMPA and 100 mg of DAPA and MET in the solvent using 100 ml volumetric flasks separately. Any required concentration could be prepared by serial dilution of standard stock solutions.

Preparation of working solutions

Working solution was prepared by transferring of 5 ml of each stock solution to 50 ml volumetric flask and volum was completed with the solvent to have standard mixture solution containing 150, 100, 125 and 100 µg/ml of CANA, DAPA, EMPA and MET respectively.

Preparation of calibration curve

Solutions for calibration graphs were prepared by serial dilution of the standard stock solutions with the solvent to cover the concentration ranges of 7.5-225, 5-150, 6.25-187.5 and 10-1000 µg/ml for CANA, DAPA, EMPA and MET respectively. Triplicate of 10 µl injections were made for each concentration and chromatographed according to the chromatographic conditions that were mentioned previously. The peaks area of each standard solution were plotted against their corresponding concentrations, A linear relationship was obtained.

Preparation and assay of synthetic mixtures

Accurately measured aliquots from standard stock solutions of CANA, DAPA, EMPA and MET were transferred to a series of 10 ml volumetric flask to prepare eight synthetic mixtures and the volum completed with solvent. Eight concentrations were chosen from each studied drug to be fall within the calibration linearity range mentioned previously.

Pharmaceutical formulation preparation and assay

Ten tablets of each pharmaceutical preparation were separately grounded in a morter. Accurately weighted amount of the finely homogenous powdered Invokamet®, Xigduo®, Synjardy®, Invokana®, Forxiga®, Jardiance® and Glucophage® tablets equivalent to [50 mg of CANA and 500 mg of MET], [10 mg of DAPA and 500 mg of MET], [12.5 mg of EMPA and 500 mg of MET], [100 mg of CANA], [10 mg of DAPA], [12.5 mg of EMPA] and 1000 mg of MET respectively were transferred in to a series of 100 ml volumetric flasks. The solvent was added till two thirds of each flask volum and were sonicated for 15 minute to dissolve the powdered tablets then volum was completed with solvent. The solutions were filtered through 0.45 µm nylon syringe filter. Serial dilution was done to the required concentrations for each tablet which were in the linearity range and then injected according to the previously descriped conditions.The recovery of each injected drug were calculated by its corresponding regression equation.

RESULTS AND DISCUSSION

The proposed method is the first for simultaneous determination of all SGLT-2 inhibitors alone or with MET which were approved by FDA in dosage forms .It can be applied for chromatographic analysis of seven dosage forms including Invokamet®, Xigduo®, Synjardy®, Invokana®, Forxiga®, Jardiance® and Glucophage® in the quality control laboratory. This method was saving time coupled with the use of the most abundant instrument in the quality control laboratories [HPLC].

Method development and optimization

The wave length was selected by scanning dilution from stock solution of each studied drug by UV-VIS spectrophotometer. It's notable that intensive overllaping of the absorption spectra of the studied drugs was between 210 to 225 nm as presented in figure 5. By repeatation of the chromatographic separation on different wave lengths in this range it is found 212 nm to be more favourable.

The PKa of EMPA, DAPA and CANA is 12.57 and so they were insoluble in polar solvent. The predicted solubility of EMPA, DAPA and CANA in water is 0.111, 0.173, 0.0045 mg/ml respectively[55-57]. A mixture of acetonitrile and phosphate buffer PH 4 gave a good solvent in the ratio of 50:50 respectively. The mobile phase was optimized to be composed from the same mixture with different ratio which was 65:35 of acetonitrile and phosphate buffer PH 4 respectively. When the organic solvent percent was increased the separation time would be decreased [less than 1.5] but the resolution was decreased leading to bad separation. When organic solvent was decreased the resolution was better but the separation took longer time. Adjusting the flow rate to 1ml/min which gave good resolution and also permit chromatographic separation of the four studied drugs in short time only 5 min.

Method validation

Linearity and range

According to the international conference on harmonization [ICH] guidelines[58], seven different concentrations of each drug were choosen and prepared by the developed method. Triplicate injections for each concentration injected where a linear releationship between the area under the curve [AUC] and the seven concentrations injected from each studied drug was obtained. The regression equation was obtained for each drug and validated by the high value of the regression coefficient as showen in Table 1.

System suitability

Six replicates of CANA, DAPA, EMPA and MET at concentrations of 150, 100, 125 and 100 µg/ml were injected respectively. Several chromatographic parameters were calculated from experimental data such as relative standard deviation [RSD], capacity factors [K′], tailing factor [Tf], selectivity factor [α], resolution factor [RS], number of the theoretical plates [N], height equivalent to a theoretical plate [HETP] and retention time of six injections. The column efficiency is a measure of peak sharpness and important for detection of trace compound and so assessed by these parameter as shown in Table 2.

All of the obtained values of these parameters meet the recommended values by BP guidelines[59] which verify that the chromatographic system performance and equipments were adequate to be used in the analysis.

Detection and quantitation limits [sensitivity]

The limit of detection [LOD] is the lowest concentration of the analyt which can be detected but not quantitated where the signal to noise ratio at the analytical wave length is 3.3.The limit of quantitation [LOQ] is the lowest measurable concentration where the signal to noise ratio is 10. According to Miller and Miller[60], LOD = [3.3 * response signal standard deviation]/calibration slope and LOQ = [10* response signal standard deviation]/calibration slope.

Precision

Three independent concentrations for each drug were used to assess the repeatability and intermediate precision [reproducibility]. Triplicate were injected from each concentration in the same day to evaluate the repeatability [intra-day precision] and through three successive days to evaluate intermediate precision [inter-day precision].The relative standard deviation [RSD%] was calculated and found to be between [0.02-0.35] for intra-day and between [0.04-1.24] for inter-day precision in the three concentrations show excellent precision which represented in Table 3. Also RSD of retention time was found to be less than 1% during inter-day and intra-day precision.

Accuracy [recovery]

Accuracy of the method was validated by calculating the percent recovery of eight laboratory prepared synthetic mixtures of CANA, DAPA, EMPA and MET. Also it was validated by standard addition technique for Synjardy® 12.5/500 mg, Forxiga® 10mg and Invokana® 100mg.In standard addition method, standard is added directly to the aliquots of the analysed sample where sample matrix contributes to the analytical signal. In the proposed method, it involves the addition of five concentration levels of each analyte standard solutions to the preanalyzed pharmaceutical tablet samples using Synjardy, Forxiga and Invokana containing 500, 12.5, 10 and 100 µg/ml of MET, EMPA, DAPA and CANA respectively.

The results obtained from the laboratory prepared mixtures represented in Table 4 and the standard addition method represented in Table 5 explain the good validated accuracy of the proposed method which confirmed that all excepients in the pharmaceutical formulation did not contribute to the analytical signal obtained in the analysis.

Robustness

Robustness test is a measure of the capacity of the chromatographic method to remain unaffected by small deliberated variations in procedural parameters. Resolution factor was the most important parameter to be studied between the chromatographed drugs in the comparison between before and after changes. In this study, small deliberated changes to the chromatographic conditions were performed to investigate the robustness of the proposed method which were change in column, flow rate, buffer PH and organic composition of the mobile phase. The effect of the organic composition variation of the mobile phase was studied by varying the acetonitrile percent ± 1% [v/v]. PH of the mobile phase was varied through ± 0.2.The flow rate of the mobile phase was changed from 1ml/min between 1.05 and 0.95 ml/min. finally, column was changed to use C18 column [250×4.6 mm-5µm p.s] Inertsil® ODS-3V. Analysis were performed in triplicate injections and one factor only changed at each time while keeping the other factors constant. The results obtained in Table 6 indicate a good robustness as there was not any significant difference between all the results.

Specificity

The term selectivity expresses how much the results obtained by the proposed method for the studied drugs are influenced by the presence of foreign substances including other components of a combined formulation or excepients. The selectivity and specificity of this proposed method could be explained through the standard addition method where satisfactory recoveries and standard deviation were obtained in the presence of different pharmaceutical dosage forms which represented in Table 5. It can be investigated by eight synthetic laboratory prepared mixtures of the studied drugs indicating high selectivity for simultaneous determination of MET, EMPA, DAPA and CANA. Also it can be explained more by placebo injection which prepared by dissolving the tablet matrix components in solvent which did not produce any response and the chromatograms of the studied drugs in different tablet formulation explain that the excepients did not interfere with the studied drugs at the selected wavelength of the detection in that proposed method.

Ruggedness

It deals with how the method affected by systematic changes of each parameters of the method while keeping others constant including changing the analyst, equipments and the day of

analysis [inter-day and intra-day analysis]. The ruggedness of the proposed method can be investigated by comparison of the inter-day and the intra-day assay results for simultaneous determination of MET, EMPA, DAPA and CANA where the RSD did not exceed 1.24 as shown in Table 3.

Analytical solutions stability

The stability of each stock solution was determined by analyzing each after 12 h at room temperature for short term stability and after 1 month of storage under refrigeration at [2- 8°c] for long term stability assessment, and the results were compared with those of freshly prepared standard solutions. The recoveries percentage of the assay of these solutions were calculated and found to be within the acceptable limit [90-110%] indicating good stability.

Application of the proposed HPLC method to pharmaceutical formulations

The proposed method was validated and successfully applied to determine EMPA in Jardiance and Synjardy, DAPA in Forxiga and Xigduo, CANA in Invokana and Invokamet and MET in Synjardy, Xigduo, invokamet and Glucophage.

Three replicates determination were made for each tablet. Percentage of drugs recovery and relative standard deviation were calculated. Satisfactory results were obtained for each compound in good agreement with regard to the ICH guidlines of 90-110% of the corresponding label claims. The results obtained represented in Table 7 were compared statistically by Student's t-test, and variance ratio F-test at 95% confidence level with the reported methods [12,31,42]. The results in Table 6 showed that the t and F values were smaller than the tabulated values indicating that there was no significant difference between the proposed and reported methods, indicating that the proposed method is as accurate and precise as the reported methods.

CONCLUSION

The proposed HPLC method provide simple, accurate and reproducible analysis for simultaneous determination of MET, EMPA, DAPA and CANA in seven pharmaceutical dosage forms and in combined laboratory prepared mixtures without any interference from the excipients. The method was completely validated and owing to its sensitivity, simplicity, and short analysis time makes it suitable for routine analysis tests in quality control laboratories of different anti-diabetic pharmaceutical formulations with reliable single method.

Table 1: Characteristic Parameters for the regression equations of the proposed LC method for the simultaneous determination of MET, EMPA, DAPA and CANA

Parameter	Metformin	Empagliflozin	Dapagliflozin	Canagliflozin
Linearity range(µg/ml)	10-1000	6.5-187.5	5-150	7.5-225
LOD (µg /ml)	3.2	1.4	0.7	2.1
LOQ (µg/ml)	9.6	2.3	2.2	6.5
Regression equation*				
n	7	7	7	7
Slope (b)	30266	30247	30572	29591
SD of slope	56.79	115.7	74.05	142.46
RSD of the slope (%)	0.188	0.383	0.242	0.481
Confidence limit of slope at 95% confidence limit	(30425.33-30107.67)	(30329.09-30163.91)	(30680.5-30464.5)	(29699-29483)
Intercept (a)	15905	15399	8633.8	18831
Standard deviation of intercept	29177.868	12926.7952	6618.4	19100.71
Confidence limit of intercept at 95% confidence limit	(33784.37--2339.37)	(21198.88-9598.12)	(16276.28-990.72)	(23944-13717)
Correlation coefficient (r)	1	1	1	1

*Y = a + bC , where C is the concentration of the standard substance in µg/ml and Y is the area under curve

Table 2: Chromatographic characteristic required for system suitability testing using the proposed LC method

Parameter (min)	Metformin	Empagliflozin	Dapagliflozin	Canagliflozin	Recommended values
Retention time	1.898	3.004	3.560	4.414	
Tailing factor (T_F)	1.398	1.185	1.136	1.072	0.8 ≤TF≤1.5
Capacity factor (K′)	1.893	3.579	4.427	5.729	0.5 <K′<1.5
Theoretical plates (N)	2741.234	4283.577	5253.636	6065.003	The more plate the better
HETP (cm/plate)	54.725	35.018	28.552	24.732	The smaller value the better
Relative standard deviation (RSD* %)	0.067	0.105	0.126	0.019	≤1
Resolution (R_S)	0.000	6.731	2.928	4.036	>1.5
Selectivity factor (**α**)	0.000	1.891	1.237	1.294	Always>1

*Relative standard deviation for six replicates injections of MET, EMPA, DAPA and CANA

Table 3: Evaluation of intra-day and inter-day precision for the determination of MET, EMPA, DAPA and CANA using the proposed LC method

Analyte	Theoretical concentration added (µg/ml)	Intra-day concentration*			Inter-day concentration †		
		Mean of found amount (µg/ml)	Recovery (%) ± SD	RSD (%)	Mean of found amount (µg/ml)	Recovery (%) ± SD	RSD (%)
MET	40	39.712	99.28 ± 0.031	0.08	40.161	100.403 ± 0.053	0.132
	400	403.558	100.89 ± 0.25	0.06	404.891	101.223 ± 5.923	1.463
	850	843.38	99.22 ± 0.88	0.10	841.351	99.705 ± 2.244	0.267
EMPA	25	25.04	100.16 ± 0.05	0.20	24.914	99.656 ± 0.170	0.683
	100	98.06	98.06 ± 0.11	0.11	101.120	101.120 ± 0.481	0.475
	175	173.08	98.90 ± 0.17	0.10	173.225	98.986 ± 0.072	0.041
DAPA	10	10.12	101.21 ± 0.0023	0.02	10.069	100.694 ± 0.029	0.284
	75	73.96	98.61± 0.26	0.35	73.806	98.408 ± 0.124	0.168
	150	151.25	100.83 ± 0.48	0.31	150.131	100.087 ± 0.556	0.371
CANA	30	29.94	99.80 ± 0.13	0.35	30.127	100.424 ± 0.061	0.205
	105	104.40	99.43 ± 0.11	0.10	104.871	99.877± 0.150	0.143
	210	209.87	99.94 ± 0.27	0.13	211.099	100.523 ± 0.732	0.347

*Three replicates of each concentration on the same day.
†Three replicates of each concentration on three consecutive days.

Table 4: Determinations of MET, EMPA, DAPA and CANA in laboratory prepared mixtures using the proposed LC method

Mixture	Concentration (µg/ml)				Area				Amount found				Recovery (%) *			
	MET	EMPA	DAPA	CANA	MET	EMPA	DAPA	CANA	MET	EMPA	DAPA	CANA	MET	EMPA	DAPA	CANA
1	20	90	5	50	62277	2713952	162914.33	149414	20.05	89.22	5.05	49.86	100.26	99.13	100.93	99.71
2	50	100	110	180	1560765.5	3006172	3380532	5427306.5	51.04	98.88	110.29	182.77	102.09	98.88	100.27	101.54
3	80	50	50	75	2429631	1507678	1549405.5	2223060.57	79.75	49.34	50.40	74.49	99.69	98.67	100.80	99.32
4	150	75	140	90	4582205	2220601.5	4318958	2662177.5	150.87	72.91	140.99	89.33	100.59	97.21	100.71	99.25
5	250	150	20	210	7663274	4564578	628259.5	6165766	252.67	150.40	20.27	207.73	101.07	100.27	101.34	98.92
6	500	175	95	150	15272119	5331318	2877813.5	4408102	504.07	175.75	93.85	148.33	100.81	100.43	98.79	98.89
7	700	12.5	10	15	21300970	391345.3	315316.67	454824	703.27	12.43	10.03	14.73	100.47	99.43	100.32	98.23
8	850	25	75	30	25483511	768412.5	2300640.5	899011	841.46	24.90	74.97	29.75	99	99.58	99.96	99.15
Mean ± SD†													100.5 ± 0.86	99.2 ± 0.95	100.4 ± 0.73	99.4 ± 0.91
RSD† (%)													0.86	0.96	0.72	0.92

*Mean of triplicate injections
† Mean, SD and RSD calculated as the percentage recovery from the added amount.

Table 5: Application of standard addition technique on pharmaceutical formulation in the assay of MET, EMPA, DAPA and CANA by the proposed method

Analyte	Initial tablet sample (µg/ml)	Authentic amount added (µg/ml)	Claimed total amount (µg/ml)	Total amount found (µg/ml) ± SD	Recovery (%)	RSD (%)	Relative error (%)
MET	500	0	500	497.99 ±4.028	99.599	0.809	0.401
		150	650	641.253 ± 0.4612	98.654	0.072	1.346
		200	700	707.257 ± 0.252	101.037	0.036	-1.037
		250	750	752.525 ± 0.658	100.337	0.087	-0.337
		300	800	798.993 ± 1.085	99.874	0.136	0.126
		350	850	843.093 ± 2.875	99.187	0.341	0.813
EMPA	12.5	0	12.5	12.581 ± 0.102	100.650	0.816	-0.650
		12.5	25	25 ± 0.062	99.998	0.247	0.004
		37.5	50	49.735 ± 0.061	99.470	0.122	0.53
		62.5	75	74.385 ±0.066	99.180	0.089	0.82
		87.5	100	100.141 ± 0.114	100.141	0.113	-0.14
		137.5	150	151.877 ± 0.192	101.251	0.127	-1.25
DAPA	5	0	5	5.023 ± 0.016	100.4623	0.312	-0.462
		5	10	10.05 ± 0.027	100.5012	0.027	-0.501
		25	30	30.063 ± 0.016	100.210	0.055	-0.210
		45	50	50.427 ± 0.024	100.8545	0.048	-0.854
		95	100	99.589 ± 0.140	99.58924	0.140	0.411
		145	150	149.153 ± 1.041	99.43515	0.698	0.565
CANA	100	0	100	101.557 ± 0.396	101.5566	0.390	1.557
		20	120	119.967 ± 0.322	99.972	0.268	0.028
		50	150	149.672 ± 0.076	99.781	0.051	0.219
		80	180	179.957 ± 0.087	99.976	0.048	0.024
		100	200	199.045 ± 0.222	99.523	0.111	0.478
		125	225	223.005 ± 0.326	99.113	0.146	0.886

Table 6: Robustness testes for the proposed LC method

Parameter	Analytes*		
	EMPA	DAPA	CANA
Resolution (Rs)			
Normal	6.731	2.928	4.036
Change in column (ODS-3V)	6.728	2.9165	4.0185
PH 4.1	6.496	2.753	3.716
PH 3.9	6.674	3.047	4.356
Mobile phase organic strength +1% ACN	6.914	3.123	4.443
Mobile phase organic strength -1% ACN	6.854	3.100	4.429
Flow rates (ml/min) + 0.05	6.676	3.050	4.365
Flow rates (ml/min) - 0.05	6.766	3.063	4.401

*At concentration of 100, 125, 100 and 150 µg/ml for MET, EMPA, DAPA and CANA , respectively

Table 7: Statistical comparison between the results of the proposed HPLC method and the reported for the determination of MET, EMPA, DAPA and CANA in pharmaceutical formulations

Analyte	Proposed method		Reported methods		t-test†	F-test‡
	Recovery (%) ± SD*	RSD* (%)	Recovery (%) ± SD*	RSD* (%)		
MET	100.49 ± 0.68	0.67	100.25 ± 0.43	0.43	1.68	2.485
EMPA	99.933 ± 0.237	0.237	99.88 ± 0.290	0.290	0.23	1.494
DAPA	99.86 ± 0.647	0.648	99.83 ± 0.153	0.153	0.070	17.949
CANA	100.247 ± 1.507	1.503	99.98 ± 0.50	0.50	0.280	9.090

*Average of three determinations of three different concentrations.
†Tabulated t-value at 95% confidence limit = 4.303 and degree of freedom = 2.
‡Tabulated F-value at 95% confidence limit = 19 and degree of freedom = 2.

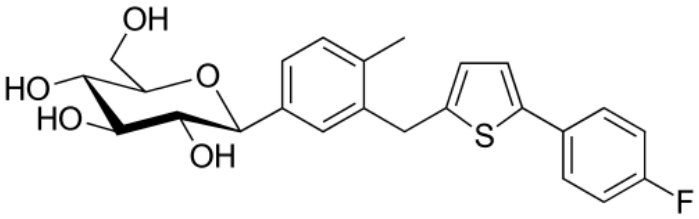

Fig. 1: Chemical structure of Canagliflozin [CANA]

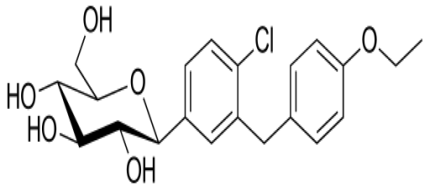

Fig. 2: Chemical structure of Dapagliflozin [DAPA]

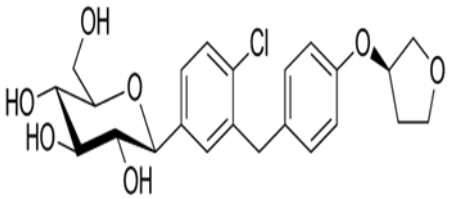

Fig. 3: Chemical structure of Empagliflozin [EMPA]

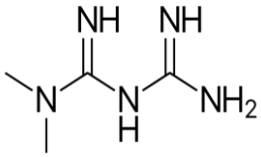

Fig. 4: Chemical structure of Metformin [MET]

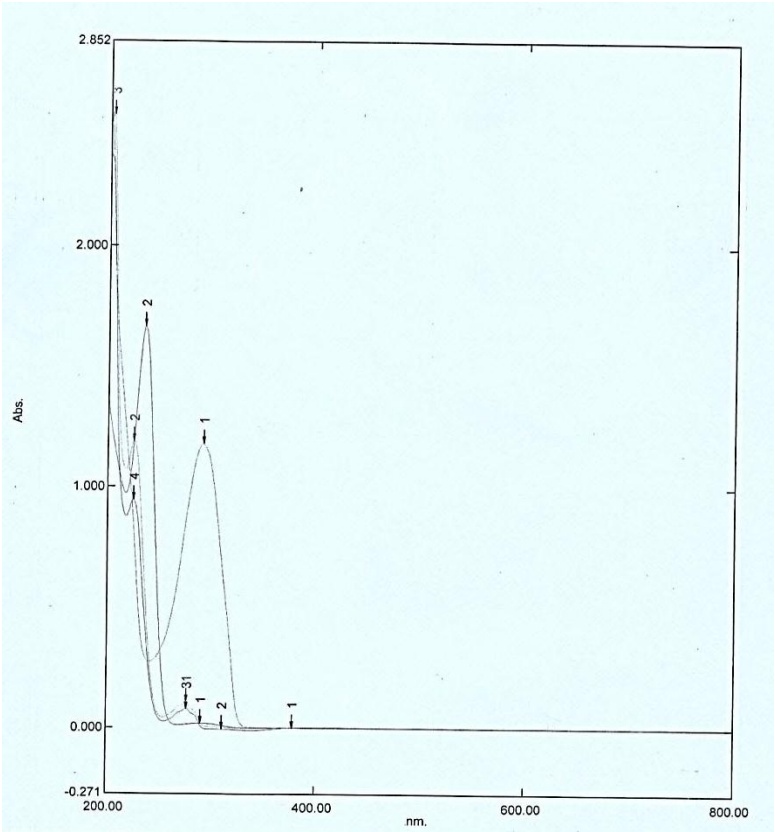

Fig. 5: UV absorption spectra of [1] CANA 30 µg/ml, [2] EMPA 25 µg/ml, [3] MET 20 µg/ml and [4] DAPA 10 µg/ml in solvent

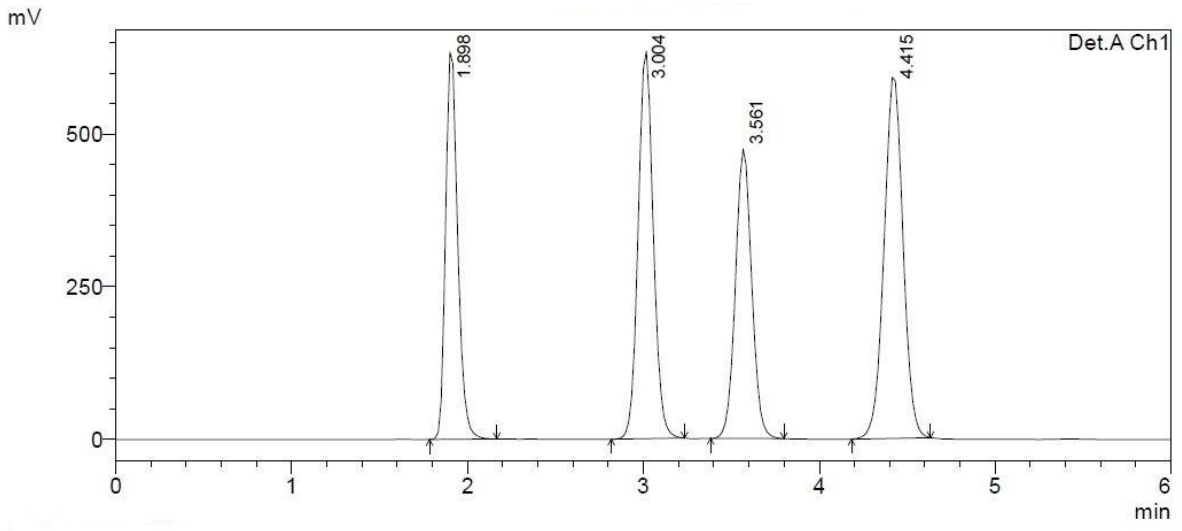

Fig. 6: HPLC chromatogram of laboratory prepared standard mixture of Metformin, Empagliflozin, Dapagliflozin and Canagliflozin at retention time 1.898, 3.004, 3.561 and 4.415 respectively

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