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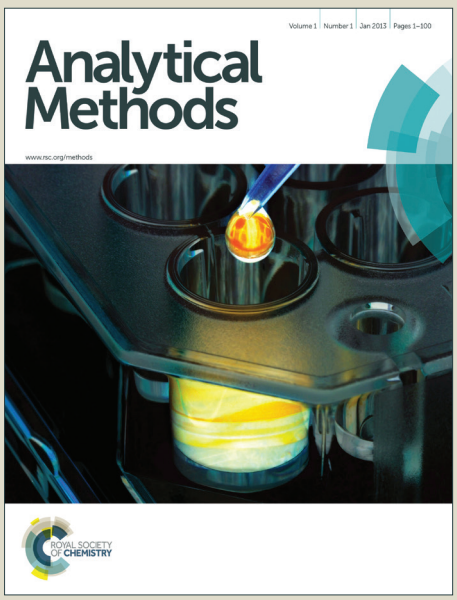

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A simple and sensitive high performance liquid chromatography assay with fluorescence detector for determination of canagliflozin in human plasma

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Abstract

Canagliflozin is the first sodium-glucose co-transporter-2 inhibitor approved for the treatment of type 2 diabetes mellitus. In this study, a simple and sensitive HPLC assay with florescence detector was developed for accurate quantification of canagliflozin in human plasma using telmisartan as internal standard (IS). Plasma samples were extracted by liquid liquid extraction method using diethyl ether as an extracting solvent. Chromatographic separation of canagliflozin and IS was performed on Nucleodur Isis C_{18} column with an isocratic mobile phase of 20mM potassium dihydrogen orthophosphate: acetonitrile (45:55, v/v) at a flow rate of 1 mL/min. Canagliflozin and IS were eluted at 2.8 and 5.8 min, respectively and detected at 280 and 325 nm for excitation and emission, respectively. The plasma calibration curve displayed excellent linearity over the concentration range of 16.13-6000 ng/mL. The assay was fully validated in terms of selectivity & specificity, linearity of calibration curve, accuracy & precision, recovery and stability in various storage conditions. To the best of our knowledge, this is the first validated HPLC-florescence detector assay for the quantification of canagliflozin in human plasma.

Key words: Canagliflozin; HPLC; Florescence; Human plasma

1. Introduction

Sodium-glucose co-transporter-2 (SGLT-2) inhibitor is a new class of oral antidiabetic drugs, which acts via insulin independent mechanism. [1] It produce effects by blocking the reabsorption of glucose from the proximal tubules of the kidney, leading to lowered renal threshold for glucose (RTG) and increased urinary glucose excretion.[2,3] Canagliflozin is the first SGLT-2 inhibitor which was approved in both USA and European countries for the treatment of type 2 diabetes patients.[4-6] It is highly selective to SGLT-2, and was found to be highly efficacious with good safety profile in clinical trials.[3] Being a novel mechanism of action, canagliflozin is not only suitable for monotherapy, but also in combination with other glucose-lowering agents, including insulin in patients who have not achieved glycemic targets with their initial therapies.[7]

The oral absorption of canagliflozin is rapid, with peak plasma concentrations achieved within 1-2 hours and steady-state levels within 4-5 days after once daily administration.[3,4,8,9] Absolute oral bioavailability of canagliflozin is 65%, with almost 99% bound to plasma protein, mainly albumin.[10] The reported terminal half-life ranged from 10.6-13.1 hours with a large volume of distribution, 119 L.[9,11] The *O*-glucuronidation is the main metabolic pathway for canagliflozin metabolism which produced two inactive metabolites via enzymes UGT1A9 and UGT2B4.[4,12]

A conducted literature search revealed only two reported assays for the analysis of canagliflozin. In the first reported technique, Devineni et al (2013) summarized the LC-MS/MS assay for the determination of canagliflozin in form of its ammonium adduct ion using electrospray ionization (ESI) in positive mode [12]. Recently we have also reported a UHPLC-MS/MS assay using ESI in negative mode for the determination of canagliflozin in rat plasma to

avoid adducts ion formation .[13] While the MS/MS could be advantageous in terms of sensitivity, selectivity and speed of analysis, but it would not be economical due to expensive instrumentation and the need for highly skilled technical expertise. Moreover, it is not usually available in most clinical, bioanalytical or educational research laboratories. So in a resource limited setting, a more convenient HPLC with UV or florescence detector (FLD) assays are usually preferred, provided lower limit of quantification (LLOQ) is sufficient for application. Additionally, HPLC-FLD assay allows more sensitivity compared to HPLC-UV assay.

Considering the information mentioned above, herein, we aimed at developing a simple, reliable and economical HPLC-FLD assay, which enables easy determination of canagliflozin for routine analysis in all laboratories irrespective of whether they are equipped with sophisticated instruments or not. The developed assay was validated according to the USFDA and EMEA guideline.[14,15] The included validation parameters were selectivity, linearity and sensitivity, precision and accuracy, recovery, carryover effects and stability. The validation results were also compared with previously reported UHPLC-MS/MS assay and were successfully applied in incurred sample reanalysis.

2. Experimental

2.1 Chemicals and reagents. Canagliflozin (purity >99 %) was purchased from Beijing Mesochem Technology Co. Ltd. (Beijing, China); telmisartan (purity ≥ 98 %) used as an internal standard (IS) was obtained from Ranbaxy Research Laboratory, Gurgaon, India (Fig. 1). HPLC grade methanol and acetonitrile were obtained from Fisher Scientific UK Limited, Leicestershire UK, whereas potassium dihydrogen phosphate and ethyl acetate of analytical grade were obtained from Qualikems Fine Chem. Pvt. Ltd. Vadodara, India. Formic acid of analytical grade was obtained from Loba Chemie Pvt Ltd. Mumbai India. All aqueous solutions were obtained

from Milli-QR Gradient A10R (Millipore, Moscheim Cedex, France) having pore size 0.22 µm. Blank EDTA human plasma was obtained from King Khalid University Hospital, Riyadh, Saudi Arabia.

2.2 Stock solution preparation. Fresh standard stock solutions of canagliflozin (400 µg/mL) and IS (500 µg/mL) were prepared in methanol by dissolving accurately weighted amounts of their pure materials. Separate stock solution of canagliflozin was prepared for calibration curve and quality controls (QCs) samples from two separate weightings. All stock solutions were stored in refrigerator at 4°C temperature.

2.3 Calibration standards, QC and IS sample preparation. A series of working standards for calibration curve and QCs were prepared by further diluting of their respective stock solution with acetonitrile: water (50:50, v/v). The plasma calibration standards of canagliflozin at eight different concentration levels were prepared by spiking the 15 µL of their respective working standards in 150 µL of plasma to obtain final concentration levels ranged 16.13-6000 ng/mL. Similarly four QCs, at 17.50, 50.0, 500 and 5000 ng/mL were also prepared by spiking the 15 µL of their respective working standards in blank plasma and treated as lower limit of quantification for QC (LLOQ QC), low QC (LQC), mid QC (MQC) and high QC (HQC), respectively. Both plasma standards and QC samples were kept at −80°C until used during validation and/or samples analysis. The IS working solution of 20 µg /mL for routine use was freshly prepared by diluting the IS stock solution in acetonitrile-water (50:50, v/v) and stored in refrigerator.

2.4 Instrumentation and Chromatographic conditions. The analysis was performed on Waters Alliance HPLC 2695 separation module connected to Waters 2475 Multi Lambda florescence detector. The LC system consisted of a water binary pump, model 1525 (Milford,

MA, USA), with an autosampler model 717. Waters solvent delivery system was used to operate the isocratic flow. Data acquisition was carried out using Empower software. The chromatographic separation was performed using a Nucleodur C18 Isis column (150 mm × 4.5 mm, 5 µm) manufactured by Macherey-Nagel, Duren, Germany. An isocratic mobile phase consisting of 20 mM potassium dihydrogen orthophosphate buffer, pH 3.2 ± 0.05: acetonitrile (45:55, v/v) eluted at a flow rate of 1 mL/min. The buffer was adjusted to pH 3.2 ± 0.05 using 50% formic acid, filtered through a Millipore membrane filter (0.22 µm) from Nihon, Millipore (Yonezawa, Japan) and degassed in an ultrasonic bath for 5 min. The injection volume was 20 µL and effluent was monitored using florescence detection set at 280 excitation and 325 nm emission wavelength, having column oven temperature of 40°C ± 0.2°C and sample cooler temperature of 8°C ± 0.2°C.

2.5. Sample preparation. A simple liquid liquid extraction (LLE) method was employed to extract both analyte and IS from plasma. All samples (stored at −80 °C) were thawed before sample preparation. An aliquot of 150 µL of plasma sample, 15 µL of IS solution (20 µg/mL of telmisartan) was added into a clean glass tube and vortex-mixed for 30 sec followed by addition of 2 mL of diethyl ether into each tube. The vortex-mixing was repeated for further 2 min followed by centrifugation at 4000 *g* for 5 min at 4 °C. After centrifugation, the upper organic layer was transferred into a clean tube and evaporated to dryness under the gentle stream of nitrogen at 35 °C. The residue was reconstituted with 200 µL of mobile phase, out of which 20 µL was injected into the HPLC for analysis.

2.6 Method validation. Assay validation was performed in human plasma in accordance with international guidelines for bioanalytical method validation recommended by USFDA and

EMEA.[14,15] The validation parameters included selectivity and specificity, linearity and sensitivity, precision and accuracy, recovery, carry-over effects and stability.

2.6.1. Selectivity and specificity. Assay selectivity was evaluated in six blank plasma samples which were obtained from six different lots to test for any possible endogenous interference at the retention time of canagliflozin and IS. These blank plasma samples were processed by the proposed extraction method and the response of co-eluting interferences was evaluated by comparing their chromatograms with plasma spiked with analyte at LLOQ (16.13 ng/ mL) level and IS at 2 µg/ mL level.

2.6.2. Assay linearity and sensitivity. The assay linearity was determined by preparing calibration curves in human plasma in triplicate, ranging from 16.13 to 6000 ng/mL concentration of the analyte. Calibration curves were obtained by plotting the peak area ratios of analyte to IS (y-axis) versus the nominal concentration of analyte (x-axis) using weighted linear regression. The determination coefficient (r^2) for each calibration curve was required to be ≥ 0.99 and the linearity was evaluated by duplicate processing of calibration curve and the back calculated concentrations at each point have to be within ±15% deviation from the nominal value. The LLOQ was defined as the lowest concentration of analyte in plasma calibration curve that could be detected with an acceptable precision (≤20%) and accuracy ± 20 %.

2.6.3. Precision and accuracy. Precision and accuracy was determined in human plasma at four different QC concentrations (LLOQ QC, LQC, MQC and HQC). The intraday precision and accuracy were determined by analyzing 6 replicates of each QCs on the same day, while the inter-day precision and accuracy were determined in by analyzing 18 replicates over three consecutive days. The deviation in mean value of precision was limited to < 20 % for the LLOQ

QC and 15 % for the other QC samples and accuracy was limited to be within ± 20 % for the lowest QC samples and ± 15 % for the other QC samples.

2.6.4. Recovery. The extraction recovery of canagliflozin was determined at three QC levels (LQC, MQC and HQC) by comparing peak area response ratio of plasma spiked with analyte prior to extraction with those spiked with analyte after the extraction Same procedure was followed to assess percentage extraction recovery for IS at single concentration of 2 µg/ mL.

2.6.5. Carry-over effects. For a robust and long term performance of the assay, it is important to eliminate any expected carry-over effect. The carry-over effects in respect to autosampler contamination was assessed in triplicate by injecting double blank processed plasma samples after an upper limit of quantification (ULOQ) processed sample. It was calculated by comparing the percentage peak area of double blank plasma to the area of the LLOQ sample. The same procedure was followed for IS also. The peak area response for blank plasma extract was limited to ≤ 20% of the peak area of the LLOQ and ≤ 5% of the IS to rule out any carry-over effect.

2.6.6. Stability. The stability of canagliflozin was evaluated in human plasma by analyzing six replicates of QC samples (LQC and HQC) under various storage conditions. All stability parameters were evaluated against freshly prepared plasma calibration curves. Bench-top stability of canagliflozin was determined by processing and analyzing QC samples after keeping them for 8 h at room temperature. Freeze-thaw stability was determined after storing the spiked QC samples at −80 °C and thawing at 25 °C for three cycles. In injector stability was determined by analyzing the reconstituted QC samples in mobile phase after keeping it for 2 days in the auto-sampler at 8 °C. Long-term stability was determined by analyzing the spiked QC plasma samples which were stored at −80 °C for 90 days. The stock solutions and working solutions of

canagliflozin and IS were also evaluated for their stability at room temperature for 12 h and at refrigerator temperature (4 °C) for 15 days. The peak area ratio of stock solutions and working solutions were compared to peak area ratios after injection of freshly prepared solution .The stability samples were considered stable if the deviation from the mean calculated concentration of quality control samples were found to be within the limits of accuracy (±15%) and precision (≤15%).

2.7. Incurred samples reanalysis Since we are in academic institution and don't have a hospital or clinical pharmacology unit, and also due to ethical issues pertaining to use of drugs in human subjects, the study in patients or normal volunteers could not be taken up. However performance of this newly developed assay in real plasma sample was confirmed by reanalysis of some spared rat plasma samples of previous pharmacokinetic study of canagliflozin in rats.[13] Before analysis, a partial validation of this assay in term of precision and accuracy was performed in rat plasma. Approximately 30 % of rat plasma samples stored in a freezer at −80 ◦C for 200 days were randomly selected, thawed and processed for re-analysis.

3. Results and discussion

3.1. Florescence sensitivity test and internal standard selection. As compared to UV, FLD usually offer high selectivity and more sensitivity. Moreover the signal intensities are very low compared to UV absorption and hence can measure ideally with a very low background noise level. In this study, canagliflozin was identified as florescence sensitive as it produced maximum absorbance at 280 nm and the corresponding emission at 325 nm. For the selection of an appropriate internal standard, some florescence sensitive compounds e.g. motelukast, gemifloxacin, valsartan and telmisartan were screened, but most of them showed low response or

being interfered by endogenous matrix. Telmisartan was chosen as it was co-eluted with canagliflozin at appropriate retention time with minimum interferences of plasma matrix and high resolution under the proposed chromatographic conditions.

3.2. Optimization of the chromatographic conditions. This HPLC-FLD assay aimed to develop a reverse phase sensitive chromatographic system capable of eluting both analyte and IS from plasma samples. Chromatographic separation by using different composition of mobile phase at different ratios, pH of the buffer and different analytical columns were tried to achieve good resolution by optimizing the chromatographic parameters like retention factor, selectivity (separation factor) and efficiency. The retention factor was optimized by adjusting the strength of organic modifiers in mobile phase and separation factor by optimizing the ratio of organic modifiers in mobile phase, pH of mobile phase and temperature of column. Considering the all above parameters, an isocratic mobile phase containing buffers e.g. disodium monohydrogen phosphate, potassium dihydrogen orthophosphate, ammonium acetate) at different pH with organic modifiers e.g. methanol, acetonitrile were tried. Finally, the mobile phase consisting of acetonitrile and 20 mM potassium dihydrogen orthophosphate buffer adjusted to pH 3.2 ± 0.05 using 50% formic acid showed best separation of both analyte and IS and was selected for analysis. Although the retention factor was comparatively lower with the optimized chromatographic condition, but high selectivity and efficiency resulted to separation of analyte and IS with high resolution. Flow rate and ratio of buffer to organic phase were also optimized to achieve maximum possible short runtime with enhanced analytical signal response. A number of reversed-phase C_{18} columns, such as Nucleosil, Bondapak, Nucleodur Isis and Novapak were tested and compared to achieve high efficiency of chromatographic separation. Finally

Nucleodur Isis (150 mm × 4.5 mm, 5 µm) was chosen as it produced better peak shape with high efficiency and sensitivity.

3.3. Sample preparation optimization. Sample extraction optimization is the most critical step to improve sensitivity and recovery of assay for accurate determination of analytes in biological fluids. With the time-saving advantage and its simplicity, the protein precipitation method was tried initially using acetonitrile and methanol as protein precipitating agent. Unfortunately, the result was not satisfactory because of its high noise and interferences by endogenous plasma at the retention time of analyte and IS. Consequently, the LLE method was tried using ethyl acetate, diethyl ether, n-hexane and dichloromethane. Ethyl acetate and diethyl ether produced better results with minimum interferences at the retention time of analyte and IS. Finally diethyl ether was selected as it produced maximum recovery compared to ethyl acetate. The percentage extraction recoveries of canagliflozin and IS using diethyl ether was ≥87.7% and 77.1%, respectively (section 3.4.4), which was greater than ethyl acetate as extracting agent.

3.4. Method validation

3.4.1 Selectivity and specificity. There was no evidence of endogenous interfering peak nearby the retention times of canagliflozin and IS in all tested lots of blank plasma samples under the proposed chromatographic condition. Representative chromatograms of blank plasma and plasma spiked with IS only are shown in fig. 2 A and 2B, respectively.

3.4.2. Linearity and lower limit of quantification. Excellent linear relationship was observed between peak area ratios of canagliflozin and IS versus plasma concentrations over a range of 16.13-6000 ng/mL. The best linear fit with lowest total bias was achieved using $1/X^2$ weighing factor and was used to back calculation of plasma calibration curve concentrations. The good linearity of plasma calibration curves was evident from the mean ±SD values of

determination coefficient (r^2) 0.994±0.002; slope 0.0012 ±0.00033 and intercept 0.1054± 0.058. The back calculated concentrations at all point on the plasma calibration curves were within the ±15% of the nominal concentrations (Table 1). Usually, LLOQ (sensitivity) is one of the limitations of HPLC assay for its application in pharmacokinetic study. This assay offered LLOQ 16.13 ng/mL in human plasma. The reported C_{max} of canagliflozin after oral administration of its lowest recommended therapeutic dose, i.e. 50 mg, is 426 ng/mL in diabetic patient [12], i.e. 26 times higher than the LLOQ of this assay. That means this assay is adequate sensitive for the quantitation of canagliflozin in resource limited laboratory for routine pharmacokinetic study. Representative chromatograms of plasma spiked with LLOQ and MQC levels are shown in fig. 3A and 3B, respectively. Both canagliflozin and IS were eluted at 2.8 and 5.8 min, respectively with a total run time of 8 min only. The linearity in plasma was achieved up to 6000 ng/mL (ULOQ) concentration, which was 14 times greater than the C_{max} of lowest therapeutic dose.

3.4.3. Precision and accuracy. The comparative results of intra- and inter-day precision and accuracy for canagliflozin at four QC levels (LLOQ QC, LQC, MQC and HQC) by HPLC-FLD in human plasma and by using UHPLC-MS/MS assay in rat plasma[13] are presented in table 2. In the present HPLC-FLD assay, the intra-day and inter-day precision values (expressed as % CV) were ≤ 12.8% and ≤ 11.2%, respectively. Similarly, the intra-day and inter-day accuracy ranged from 93.3-108.5% and 94.9-110.4%, respectively. The results showed that the assay met the desired level of acceptance criteria similar to previously reported UHPLC-MS/MS assay and hence was considered accurate and precise for application in determination of canagliflozin in human plasma.

3.4.4. Recovery. The % mean extraction recovery of canagliflozin using the proposed extraction method at LQC, MQC and HQC levels were found to be 92.6%, 84.8% and 87.7% with % CV of 3.9%, 7.2% and 4.7%, respectively . Similarly for IS it was 77.1% with % CV of 4.8%. This result indicates that the recovery of canagliflozin by LLE method using diethyl ether as extracting solvents was in the acceptable range, consistent and concentration independent. So the extraction process of plasma sample was sufficient to isolate the canagliflozin and IS from plasma.

3.4.5. Carry-over effects. No significant peaks were observed in the chromatograms of blank plasma samples, injected just after highest concentration level of calibration curve. Therefore, the carryover effects were considered to be negligible for this assay. However, a flush solvent of 80:20 v/v acetonitrile:water is recommended for the injection needle washing, after every 10-15 unknown samples.

3.4.6. Stability. The comparative stability results of canagliflozin under different storage conditions (freeze–thaw, autosampler, in injector, short-term and long-term) by HPLC-FLD assay in human plasma and UHPLC-MS/MS assay in rat plasma[13] are summarized in table 3. The results demonstrated that canagliflozin spiked plasma was stable during three cycles of freeze-thaw, 48 h in autosampler after post preparation, at least 8 h at room temperature, and up to 90 days at around −80 °C in both assay. The stock solutions and working standard of canagliflozin and IS were also found to be stable for 15 days at refrigerator temperature (below 10 °C). The deviation of mean test responses (% CV) was within ±15% at LQC and HQC concentration level. Thus, there is no indication of canagliflozin instability in human plasma samples which can affect the assay performance.

3.5. Incurred sample reanalysis. The % difference in the concentrations of canagliflozin achieved at different time points in rats (n=6) using this HPLC assay and our previously reported UPLC-MS/MS assay was presented in table 4. The results indicate that approx. 77% of derived concentrations data for canagliflozin using both assay were a % difference of <20%. These results imply the suitability of this assay for quantitative deamination of canagliflozin in real plasma samples.

3.6. Assay performance of HPLC-FLD and UHPLC-MS/MS

Detection in MS is based on the determination of the mass of a molecule by measuring the m/z of its protonated or deprotonated ion which produced depending upon its ionization in positive or negative mode, respectively. Although both positive and negative ions can be analyzed by MS, the majority of instruments are used to investigate positive ions because in most ion sources they are produced in larger number than negative ions. Our UHPLC-MS/MS assay was based on ESI negative mode, because in positive mode canagliflozin produced sodium and ammonium adducts ion. So the chromatographic condition was simple acetonitrile and water (80:20) to avoid adducts ions formation. However, in this HPLC-FLD assay where ionization is not an issue chromatographic condition was optimized by using different buffers with organic modifiers as mobile phase. Similarly in sample preparation procedure, previously used protein precipitation using acetonitrile produced high noise and interferences by endogenous plasma at the retention time of analyte and IS under proposed chromatographic condition. Hence, sample preparation in HPLC-FLD assay using liquid liquid extraction was preferred for the extraction of analyte and IS from plasma samples. In spite of above differences in assay performance, we have successfully developed this HPLC-FLD assay having almost similar range of plasma calibration curve (16.13-6000 ng/mL).

4. Conclusion

In this study, a simple sensitive and reliable HPLC-FLD assay was developed and validated for the quantification of canagliflozin in human plasma. To the best of our knowledge this is the first validated HPLC-FLD assay for the determination of canagliflozin in plasma samples. Compared to previously reported MS/MS assays, this is more simple and economical, which make it suitable for use in pharmacokinetic study in all laboratories irrespective of whether they are equipped with sophisticated instruments or not.

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References

1. C.H. Jung, J.E. Jang and J.Y. Park, *Diabet. Metab. J.,* 2014, **38**, 261-73.
2. C.J. Bailey, *Trends Pharmacol. Sci.,* 2011, **32**, 63-71.
3. M. Rosiak, S. Grzeszczak, D.A. Kosior and M. Postuła, *Ther. Clin. Risk Manag.,* 2014, **10**, 683-9.
4. S. Elkinson, and L.J. Scott, Drugs, 2013, **73**, 979-88.
5. *U.S. FDA approves canagliflozin (TA-7284) for the treatment of adult patients with type 2 diabetes*, 2013, Janssen Pharmaceuticals, Inc: USA.
6. *INVOKANA™ (canagliflozin) Approved In The European Union For Treatment Of Adults With Type 2 Diabetes*, 2013, Janssen-Cilag International NV: Belgium.
7. R. Lajara, Expert Opin. Pharmacother., 2014, 1-21.
8. A.J. Scheen, *Clin Pharmacokinet*, 2014, 53, 295-304.
9. *InvokanaTM (canagliflozin) tablets, US prescribing information* 2013.
10. Y.Toderika and N. Ferguson, *Cardiol. Rev*., 2014, **22**, 97-104.
11. S. Sarnoski-Brocavich and O. Hilas, *P T,* 2013. **38**, 656-666.
12. D. Devineni, C.R. Curtin, D. Polidori, M.J. Gutierrez, J. Murphy, S. Rusch and P.L. Rothenberg, *J. Clin. Pharmacol*, 2013, **53**, 601-10.
13. Iqbal M, E.Ezzeldin., K.A. Al-Rashood , Y.A. Asiri, and N. L. Rezk, *Talanta,* 2015, **132**, 29-36.
14. *Guidance for Industry on Bioanalytical Method Validation*, Center for Drug Evaluation and Research (CDER), Editor 2001, US FDA: Rockville, MD.
15. *Guideline on bioanalytical method validation*, CHMP, Editor 2011, EMA.

Figure legends

Figure 1. Chemical structure of canagliflozin and telmisartan (IS).

Figure 2. Chromatographic behaviour of blank extracted plasma (A) and blank plus IS plasma (B).

Figure 3. Representative HPLC chromatograms of canagliflozin and IS in extracted plasma spiked at LLOQ level (A) and MQC level (B).

Table 1. Intra-day reproducibility of standard plasma calibration curve of canagliflozin obtained by HPLC-FLD assay

Nominal Concentration (ng/mL)	Mean*± SD (ng/mL)	CV %	Accuracy (%)
16.13	18.60±2.01	10.8	115.3
46.08	49.67±3.21	6.5	107.8
115.2	119.7±1.53	9.4	103.9
288.0	260.3±13.6	7.0	90.4
720.0	714.3±38.11	8.2	99.2
1800	1893±125.1	6.6	105.2
3600	3555±261.6	7.4	98.8
6000	5763 ± 89.1	1.5	96.1

*Average of three replicates

Table 2. Comparative intra-day and inter-day precision and accuracy values for canagliflozin by HPLC-FLD assay in human plasma and UHPLC-MS/MS assay in rat plasma

	Intra-day (6 replicates)			Inter-day (18 replicates)		
Nominal Conc. (ng/mL)	Measured conc. (ng/mL ± SD)	Precision (CV, %)	Accuracy (%)	Measured conc. (ng/mL ± SD)	Precision (CV, %)	Accuracy (%)
HPLC-FLD assay						
17.50	18.99 ± 2.43	12.8	108.5	19.33±2.16	11.2	110.4
50	51.33±4.72	9.2	102.7	50.83±3.47	6.8	101.7
500	480.5±29.2	6.1	96.1	478.9±28.1	5.9	95.8
5000	4665 ± 267	5.7	93.3	4744±327	6.9	94.9
UHPLC-MS/MS assay						
4.31	4.69±0.44	9.3	108.9	4.60±0.39	8.5	106.8
12.3	11.9±0.53	4.5	96.9	12.1±0.44	3.6	98.3
320	338.3±26.8	7.9	105.7	330.2±18.5	5.6	103.2
5333	4873±327.4	6.7	91.4	4983.7±321.4	6.4	93.5

Table 3. Comparative stability data for canagliflozin by HPLC-FLD assay in human plasma and UHPLC-MS/MS assay in rat plasma

Stability parameters	Nominal concentration (ng/mL)	Mean* ±SD (ng/mL)	Precision (CV %)	Accuracy (%)
Bench top stability				
HPLC-FLD assay (8 h)	50	50.74± 4.13	8.1	101.5
	5000	4861.3 ± 523.5	10.8	97.2
UHPLC-MS/MS assay (6h)	12.3	11.9±0.53	4.5	96.8
	5333	5288±121	3.5	103.8
Freeze thaw stability (3 cycle)				
HPLC-FLD assay	50	49.25± 4.38	8.9	98.5
	5000	4918.5± 333.2	6.8	98.4
UHPLC-MS/MS assay	12.3	11.9±0.76	6.4	96.5
	5333	5535±193	8.3	89.3
In Injector stability (48 h)				
HPLC-FLD assay	50	56.09± 1.36	2.4	112.2
	5000	5130.3± 305.2	5.9	102.6
UHPLC-MS/MS assay	12.3	13.3±1.10	8.2	108.4
	5333	5730±154	2.7	107.5
Long-term stability at −80°C				
HPLC-FLD assay (90 days)	50	50.63± 2.64	5.2	101.3
	5000	4722.8± 295.7	6.3	94.5
UHPLC-MS/MS assay (30 days)	12.3	11.15±0.56	5.0	90.6
	5333	4989±231	4.6	93.6

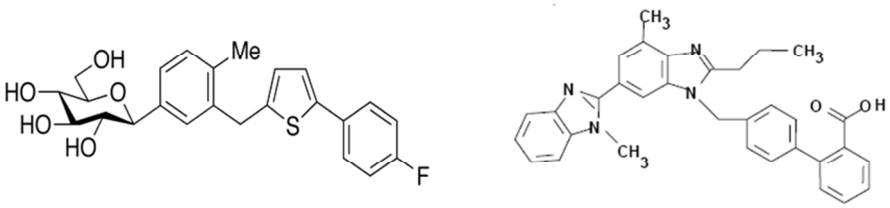
*Average of 6 replicates

Table 4. Comparison of concentrations of canagliflozin at different time points in rats (n=6) using HPLC-FLD assay and UPLC-MS/MS assay.

Rat no./ time Point (h)	Canagliflozin (ng/mL)		
	UPLC-MS/MS Assay	HPLC Assay	Percentage difference*
R1/ 2.5	4452.6	4239.1	4.9
R1/ 8	428.4	579.8	- 30.0
R1/ 24	341.4	378.6	-10.3
R2/ 1	305.4	379.4	-21.6
R2/ 3	400.0	488.0	-19.8
R2/ 6	1590.9	1356.6	15.9
R3/ 2	977.7	946.5	3.3
R3/ 6	226.3	244.0	-7.5
R3/ 24	378.2	424.9	-11.6
R4/ 0.5	1199.1	1000.5	18.1
R4/ 1.0	1224.5	887.0	32.0
R4/ 6	555.8	643.1	-14.6
R5/ 1.5	631.7	742.7	-16.2
R5/ 3	798.4	749.4	6.3
R5/ 8	1715.3	1464.5	15.8
R6/ 1	1000.3	951.4	5.0
R6/ 3	1921.0	1490.6	25.2
R6/ 4	1273.6	1524.6	-17.9

* Percentage difference=[(UPLC-MS/MS–HPLC assay)/ Average]×10

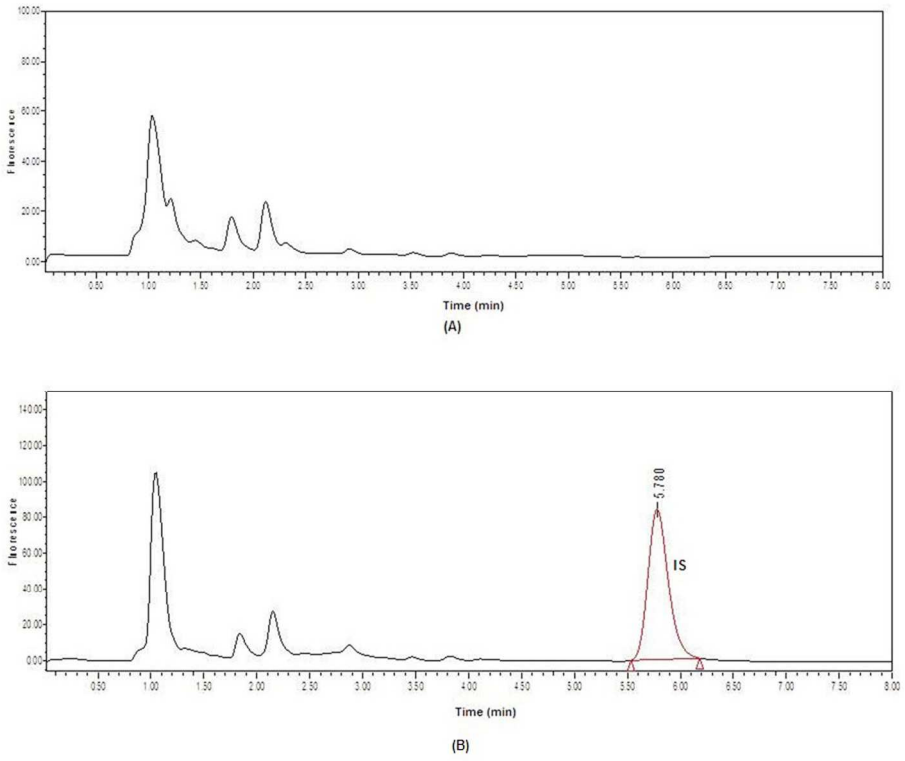
Figure 1



Canagliflozin **Telmisartan**

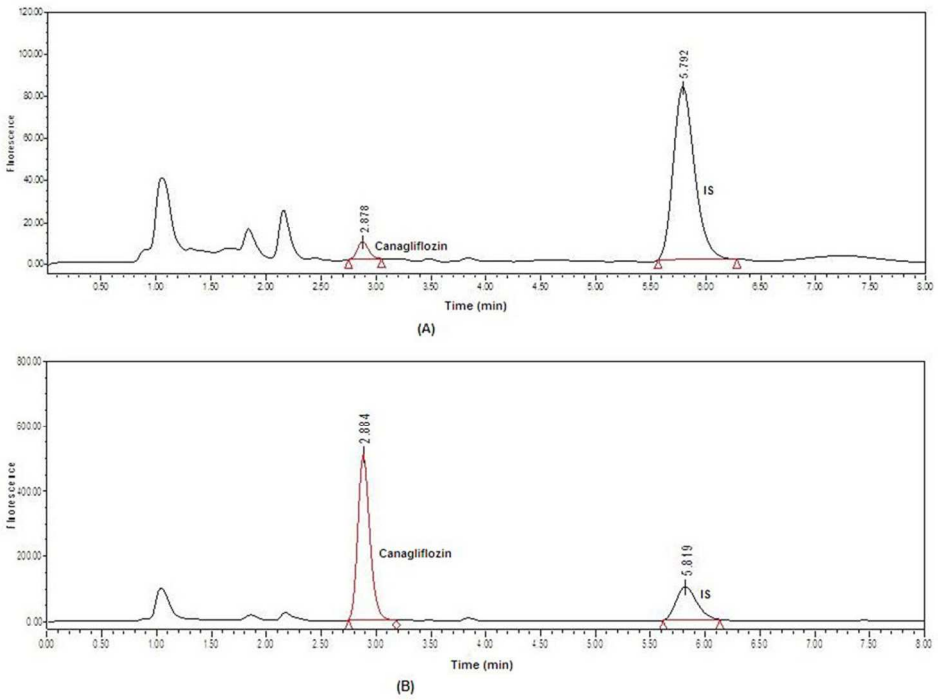
254x190mm (96 x 96 DPI)

Figure 2



232x208mm (300 x 300 DPI)

Figure 3



203x162mm (300 x 300 DPI)