

Simultaneous analysis of dapagliflozin and its three related impurities by stability-indicating UPLC method and *in vitro* toxicity evaluation

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A new stability- indicating analytical method by UPLC was developed for the simultaneous determination of dapagliflozin and three of its synthesis impurities. A Waters® Acquity UPLC H- Class model was used for method development and validation. The separation was achieved in a Zorbax phenyl column (50 x 3.0 mm, 1.8 μ m), using a mixture of acetonitrile: water (70:30, v/v) as mobile phase in isocratic mode. All the peaks were well detected by a photodiode array detector (PDA) at 230 nm. The method was properly validated according ICH guidelines with respect to linearity, specificity, precision, accuracy and robustness. The calibration curves of each analyte showed determination coefficients (r^2) > 0.99 and the method was linear at the concentrations range 30-70 μ g/mL for dapagliflozin and 1-10 μ g/mL for the impurities. Lastly, this UPLC method presented low limits of detection (LOD) and quantification (LOQ) for both dapagliflozin and impurities, being a technique with high sensitivity. The toxicity evaluation of dapagliflozin and its related impurities were evaluated using 3T3 cells. MTT reduction and neutral red uptake assays were performed in cytotoxicity tests. In addition, mitochondrial membrane potential ($\Delta\psi$ M), measurement of intracellular reactive oxygen and DNA damage (measured by comet assay) were evaluated. The impurity 3 showed significant damage in cytotoxicity tests at a concentration of 0.5 μ M, being even more expressive at higher concentrations. On the other hand, under the conditions tested, DNA damage was not detected and the compounds tested do not induce significant cell death.

Keywords Keywords: dapagliflozin, UPLC, analytical validation, drug impurities, toxicity assay.

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Introduction

Dapagliflozin (Forxiga®), chemically defined as (1s)-1, 5-anhydro-1-C-[4-chloro- 3-[(4-ethoxyphenyl) methyl] phenyl]-D-glucitol (Fig 1a), is a novel drug intended for the treatment of adult patients with type 2 diabetes mellitus. It belongs to a class of drugs called gliflozins, which reduces the blood glucose levels by inhibiting the sodium-glucose cotransporter 2 protein (SGLT2) in the kidneys, leading to a reduction in the glucose reabsorption and, hence, increasing urinary glucose excretion (1-4). Forxiga® is produced by AstraZeneca as coated tablets containing 5 or 10 mg of dapagliflozin and was approved in Europe and United States in 2012 and 2014, respectively.

To control the quality of a pharmaceutical product and ensure its safety is necessary to make use of appropriate analytical techniques according to each type of analysis. Methods that attend to their pretended uses are fundamental in the routine of the pharmaceutical industries.

One of the current trends in the quality control area is to investigate and monitor potential impurities in drug substances and products, once the presence of these compounds in the final product may affect both its efficacy and safety (5-7). These impurities may come

from different sources, such as residual solvents, synthesis intermediates or degradation products, for example (8). The organic impurities, as synthesis intermediates and degradation products, are the most commonly found in pharmaceutical products (9).

Literature data describes some chromatographic (HPLC) and spectroscopic (UV) methods for the quantification of dapagliflozin alone (10-12) or dapagliflozin with other anti diabetic agents in bulk and dosage forms (13-15). HPLC method has also been developed for the determination of dapagliflozin and its degradation products (11). However, there are no published analytical methods for the simultaneous determination of dapagliflozin in the presence of its related impurities.

Impurity profile methods must be, essentially, fast, simple and highly sensitive. Ultra performance liquid chromatography (UPLC) meets this purpose, since this technique has advantages such as: rapid analysis time, lower solvent consumption and high efficiency and detectability, 2 to 3 times higher when compared to HPLC, for example (16). In addition to identifying the presence of possible contaminants in raw materials and pharmaceutical products, it is also necessary to assess the toxicity of these impurities, in order to ensure patient safety throughout treatment. The cytotoxic effects of a chemical substance can be assessed by different interdependent techniques. Besides, the evaluation of the

genotoxic potential of such compounds also contributes to the evaluation of pharmacological safety, since impurities with genotoxic potential induce mutations, chromosome breaks and rearrangements that can lead to the development of cancer. In the process of synthesizing dapagliflozin, these related substances are usual intermediates that can remain in the final product as contaminants, leading to a decrease in its quality (17-19). In our study, we aimed to quantify and qualify the dapagliflozin and three organic related impurities, to ensure that the presence of these undesirable compounds is not above the limits recommended by regulatory agencies and to evaluate their toxic potential, considering the chronic use of these drugs. For that, it was established a stability-indicating method by ultra performance liquid chromatography to simultaneously determine dapagliflozin in the presence of its potential related impurities (fig 1b, 1c and 1d). Also, forced degradation studies were conducted to evaluate the specificity of the proposed method. The method development focused on the simplicity of the chromatography conditions to ensure its reproducibility in the laboratory routine. Drug and impurities toxicity were evaluated by cytotoxicity and genotoxicity assay, which indicate the potential of these compounds to cause cellular and DNA damage.

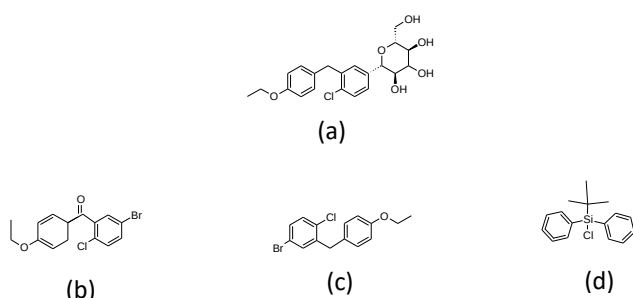


Figure.1 Chemical structures of Dapagliflozin (a), Impurity 1 (b), Impurity 2 (c) and Impurity 3 (d).

Experimental section

Chemicals

Dapagliflozin (98%), Impurity 1 (98.4%): (5-Bromo-2-chlorophenyl) (4-ethoxyphenyl) methanone and Impurity 2 (98%): 4-bromo-1-chloro-2-(4-ethoxy benzyl) benzene were purchased from Ontario Chemical (Canada), while Impurity 3 (98%): tert-butyl diphenyl chlorosilane was purchased from Carbosynth (United Kingdom). Forxiga® (dapagliflozin 10 mg tablets, AstraZeneca, were obtained from local market. Acetonitrile and purified water, both of analytical grades were used for sample preparation and mobile phase constitution.

Apparatus description

A Waters® Acquity UPLC H-Class model, equipped with a quaternary pump, an auto sampler, compartment with thermostat and diode array detector was used for method

development and validation. Empower™ chromatography software was used for data acquisition and results analysis. The chromatographic separation was achieved in isocratic mode.

Stock solution preparation

Standard solution

Standard solutions of dapagliflozin and its impurities were individually prepared in acetonitrile, in a final concentration of 100 µg.mL⁻¹ each. Further dilution of the stock solutions were made with the diluent (acetonitrile: water 50:50, v/v).

Sample solution

Twenty Forxiga® tablets were accurately weighed and finely powdered. A quantity equivalent to 10 mg of dapagliflozin was transferred to a 10 mL volumetric flask containing acetonitrile, which was kept in ultrasonic bath for 30 minutes, in order to extract the dapagliflozin from the dosage form. The volume was completed with the same solvent and the solution was filtered using paper. This stock solution was diluted to 500 µg.mL⁻¹ and filtered with 0.22 µm membrane before injection.

Placebo solution

All the excipients contained on the dosage form (microcrystalline cellulose, anhydrous lactose, crospovidone, silica dioxide, magnesium stearate, polyvinyl alcohol, titanium dioxide, macrogol and talc), were weighed in their usual concentration according the Handbook of Pharmaceutical Excipients and prepared by the same way of sample solution.

Method validation

The analytical method was validated according to the Official Guides, which establish the evaluation of the following parameters: specificity, linearity, limit of detection (LOD) and limit of quantification (LOQ), precision, accuracy and robustness (20-22).

Specificity

The specificity of the method was evaluated by the injection of the placebo solution at a theoretical concentration of 100 µg.mL⁻¹ and the chromatogram was compared with the standard solutions. Besides, forced degradation of the sample solution was carried out under the following conditions: for acid, alkali and oxidative degradations, aliquots of 5 mL of the sample solution were transferred to 20 mL volumetric flasks with 5 mL of HCl 1M, NaOH 1M or H₂O₂ 3%, and the final volume completed with the mobile phase. Photolytic studies were done by exposing aliquots of the sample solution to mirror chambers (100 x 18 x 17 cm) equipped with UVA (352 nm) and UVC (254 nm) lamps. For thermal

degradation, the sample was kept under 60 °C in the oven. Every one hour, for five hours, an aliquot of each solution, in all stress conditions, was diluted with mobile phase and injected in the chromatographic system in a concentration of 100 µg/mL. All the solutions were filtered using 0.22 µm membrane filters before UPLC analysis.

Linearity

The linearity of the method was checked by the injection of the analytes in a range corresponding to their individual calibration curves, in three different days. The impurities calibration curves were prepared at seven points (1.0 to 10.0 µg.mL⁻¹), while the dapagliflozin curve was made with five points (30.0 to 70.0 µg.mL⁻¹). All the points were injected in triplicate and the media of the values were used in the statistic analysis by linear regression.

LOD and LOQ

Limits of detection and quantification of dapagliflozin and its impurities were obtained from linearity data, based on the standard deviation of the response (σ) and the slope (S), and was calculated using the formulas: $LOD = 3.3 \sigma / S$ and $LOQ = 10 \sigma / S$.

Precision

Precision of the method was assured considering intraday (repeatability) and inter-day (intermediate precision) analysis. For this assay, six individual sample solutions were prepared in a final concentration of 50 µg.mL⁻¹, which corresponds to the test concentration. Thereafter, each of these solutions was spiked with 5 µg.mL⁻¹ of the impurities standard solutions. All the individual spiked solutions were injected in triplicate, and the same procedure was performed on three successive days. RSD values were evaluated.

Accuracy

Known amounts of the impurities and dapagliflozin standards were spiked in the sample solution that was kept in a fixed concentration of 50 µg.mL⁻¹. The impurities and dapagliflozin standard concentrations were modified at three levels, corresponding to 1, 5 and 10 µg.mL⁻¹ and 30, 50 and 70 µg.mL⁻¹, respectively. Each solution was prepared in triplicate and the values are expressed as percentage of the mean experimental concentration by the theoretical concentration.

Robustness

The verification of robustness was carried out during the development of the method, in order to evaluate if characteristics chromatography parameters as resolution, theoretical plates and symmetry of peaks, do not be compromised in front of minimal variations in analytical

conditions. The main modifications were with respect to the mobile phase proportion, flow rate and injection volume.

Cell culture

Cell viability assays were performed using mouse fibroblast type 3T3 cell line. The cells were cultured in DMEM culture medium (Dulbecco's Modified Eagle Medium) supplemented with 10% fetal bovine serum. For the control of microbiological contamination in cell culture, 100 U.mL⁻¹ of penicillin and 100 µg.mL⁻¹ of streptomycin were added. The 75 cm² culture flasks were placed in a CO₂ oven (atmosphere: CO₂ 5% and O₂ 95%). Upon reaching 70 to 80% confluence, the cells were subcultured to allow their continuous multiplication.

Cytotoxicity assay

Cytotoxicity was assessed using MTT reduction and Neutral Red (NR) uptake assays. Cells were seeded in 96-well plates containing 10,000 cells per well. The samples of dapagliflozin and its impurities were prepared in DMSO, and tested at concentrations of 0.1; 0.25; 0.5; 1.0; 10.0 and 50.0 µM. As negative controls, culture medium and DMSO were used, and triton X-100 as a positive control.

MTT reduction assay

To determine cell viability by the MTT assay, 100 µL aliquots of the cell culture suspension containing the culture medium were incubated for 24 hours at 37°C. Subsequently, the supernatant containing the culture medium was removed, and each treatment was added, separately (drug and impurities), in different concentrations in the wells of the culture plates, which were kept incubated again at 37 °C for 24 or 96 hours.

After this period, the samples were removed from the wells and washed with sterile phosphate buffered saline (PBS). Then, the 0.5 µg.mL⁻¹ MTT solution was added to the wells containing the post-treatment cells and the plates incubated for 24 hours. Afterwards, the MTT solution was removed and the DMSO added to solubilize the formazan crystals. The plates were shaken for 5 minutes, protected with aluminum foil and put to rest for another 5 minutes.

The absorbances were read in a plate reader at 550 nm. To reduce inter-experimental variability, data were normalized and scaled between 0% (negative control) and 100% effect (positive control). The experiments were carried out in triplicate and each concentration was tested with three replications.

Neutral red uptake assay

The assay was performed as previously described in 2.6.1 item. After 24 or 96 hours of incubation of the cells with drug or impurities, the medium was removed, washed with phosphate buffer and replaced with a 50 µM neutral

red solution (Sigma-Aldrich, St. Louis, USA), which was kept for a period of 3 hours at 37 °C. Two washes were made with sterile PBS solution, followed by a wash with 1% CaCl_2 in 0.5% formaldehyde. Then, the cells were lysed with ethanol/ glacial acetic acid 50:1 (v/v), with agitation for 10 minutes followed by 5 minutes of rest. The absorbance was measured at 540 nm and the percentage of cell death in relation to the control cells was used as a measure of cytotoxicity.

Measurement of intracellular reactive oxygen

The intracellular reactive oxygen (ROS) species production was monitored by means of the DCFH-DA assay. For this determination, the cells were seeded at a density of 10,000 cells per well in 96 well plates and allowed to grow for 24 h. On the day of the experiment, the cells were pre-incubated with 10 μM of DCFH-DA for 30 min at 37 °C in the dark. The cells were rinsed with PBS and incubated with the drugs and their impurities at 0.10; 0.25; 0.50; 1.0; 10.0 e 50.0 μM for 24 or 96 h. Tert-butyl hydroperoxide (500 μM) was used as a positive control. Fluorescence was recorded in a fluorescence microplate reader (SpectraMax M2e, SoftMax® Pro 5, Molecular Devices, Sunnyvale, CA, USA) set at 485 nm excitation and 530 nm emission. The data obtained were calculated as the percentage of control conditions for each experiment from at least three independent experiments with each concentration tested in three replicates within each experiment.

Assessment of mitochondrial membrane potential ($\Delta\psi\text{m}$)

The mitochondrial integrity was evaluated by measuring tetramethyl rhodamine ethyl ester perchlorate (TMRE) inclusion. To perform this assay, cells were seeded at a density of 10,000 cells per well. After 24 h of incubation at 37 °C, the medium was gently aspirated and the cells were incubated with the drugs and their impurities at 0.10; 0.25; 0.50; 1.0; 10.0 e 50.0 μM for 24 or 96 h. Carbonyl cyanide m-chlorophenyl hydrazine (CCPC) 100 μM was used as positive control. Then, the medium was substituted by a new medium containing 2 μM of TMRE for 30 min at 37 °C in the dark. Afterwards, the medium was gently aspirated and replaced by phosphate buffer. Fluorescence was measured in a fluorescence microplate reader set to 544 nm excitation and 590 nm emission. Three replicates were made in four independent experiments.

Comet assay

The cells were seeded for 24 hours at a density of 60.000 cells per well. After this time, the medium was aspirated and the cells were incubated with the analytes at 0.1; 1.0 and 10.0 μM at 37 °C. After 24 h of incubation time, the cells were harvested by trypsinization (0.05% trypsin/EDTA). The cell suspensions were centrifuged (400×g, 5 min, 4 °C), the obtained cell pellets were resuspended in low-melting point agarose (0.75%, 150 μL) (Sigma-

Aldrich (St. Louis, USA) and 60 μL aliquots were distributed on two slides coated with 1% normal-melting agarose. The samples were incubated in lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris-HCl, distilled water, 10% DMSO, and 1% Triton X-100) at 4 °C for 24 h in the dark. The slides were then incubated with alkaline electrophoresis running buffer (300 mM NaOH and 1 mM EDTA, pH 13) for 20 min at 4 °C before electrophoresis, which was carried out for 20 min at 25 V and 300 mA. After that, the slides were neutralized with 0.4 M Tris-HCl for 15 min in the dark. The DNA was fixed by immersing the slides in 70% ethanol for 15 min and in absolute ethanol for a further 15 min and left to dry overnight. For the microscopy analysis, the dried slides were stained with gel red (20 μg $\cdot$ L^{-1}) and DNA migration was observed in at least 100 cells at 400x magnification using a fluorescence microscope (Olympus, Japan) equipped with a 510–550 nm excitation filter of connected to a camera. The images were evaluated by Open Comet™ software. The percentage of DNA in the comet tail (% DNA in tail) was the parameter evaluated to describe comet formation. Concurrently with the comet assay, an extra and identical replicate comet slide was prepared, lysed, and immediately fixed and stained without electrophoresis for evaluation of the cytotoxicity using the low molecular weight (LMW) DNA diffusion assay.

Results and Discussion

UPLC method development

During the development of a chromatography method for the simultaneous analysis of two or more compounds, it is necessary to attend, at least, two main criteria: adequate resolution between the analytes, as well as reduced total run time – especially if the proposed method intends to be applied in the quality control routine. However, when it comes to a technique for the quantification of the drug and its related impurities, another challenge is to achieve a method with high sensitivity, able to detect the presence of these impurities at trace levels. Based on this, the initial point in the method development was to test the chromatography conditions in which all the analytes are detected without coelution and with a satisfactory retention time. The first step was to define the mobile phase composition and proportions. Since the solvent used to dissolve the dapagliflozin and the impurities standards was the acetonitrile, it was chosen to test it as a component of the mobile phase. In addition, one of the goals in optimizing the chromatography method was to keep its simplicity, avoiding the use of buffers or organic modifiers, while it is possible. Therefore, after many tests, a mixture of acetonitrile and water (70:30, v/v) was chosen as the mobile phase composition. Likewise, tests with different stationary phases were conducted starting from the most usual, as C18, C8 columns, and also a more polar column as the cyano. However, any of these had satisfactory results, especially with respect to the extensive retention time of the analytes.

After several trials, the column that presented the best performance, both in retention time and resolution between the peaks, was a Zorbax phenyl column (50 x 3.0 mm, 1.8 μm). Modifications in the flow rate and injection volume were also verified in order to achieve better results in the most important chromatographic parameters for the analysis of drugs and related substances. The flow rate was fixed in 0.1 mL.min⁻¹, injection volume of 2 μL , and the column temperature kept constant at 25 °C. The absorbance detection wavelength was 230 nm, in which all the analytes of interest showed satisfactory absorption, in a fast analysis time of 8 minutes (Figure 2). To verify the application of the method in coated tables of dapagliflozin, a sample solution, prepared as describe at 2.3.2 item, was injected in the UPLC in a concentration of 500 $\mu\text{g.mL}^{-1}$. The batch tested did not show de presence of any of its potential impurities.

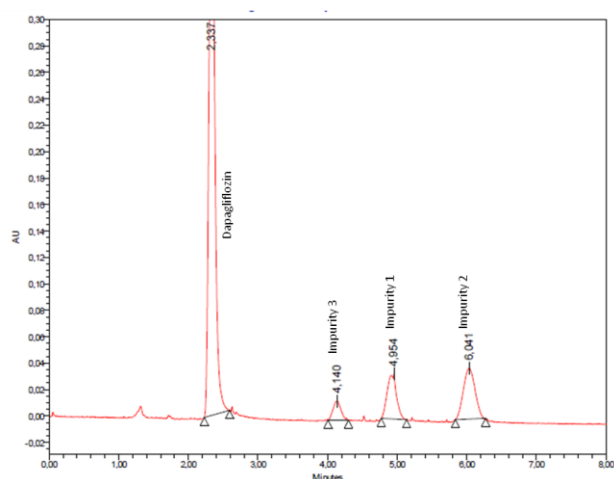


Figure 2. Representative chromatogram of dapagliflozin and its synthesis impurities at 50.0 and 5.0 $\mu\text{g.mL}^{-1}$, respectively. Chromatographic conditions: Zorbax phenyl column (50 x 3.8 mm, 1.8 μm) at 25 °C, with a mobile phase consisting of acetonitrile: water (70:30 v/v), at a flow rate of 0.1 mL.min⁻¹ and injection volume of 2 μL . The detection was made at a wavelength of 230 nm by using PDA detector.

Analytical validation

Specificity

The specificity of the developed method was proved by the absence of any significant interference from placebo on dapagliflozin determination. Besides, stability studies were carried in the dapagliflozin dosage form to confirm the degradation products eventually formed do not compromise the impurities analysis. Figure 3 shows representative chromatograms of dapagliflozin under acid, alkaline, oxidative and UVC degradations. As can be seen, any degradation product had the same retention time of the related impurities. After 5 hours at UVA light and 60°C exposing, was not observed any degradation, demonstrating the drug is stable under this condition.

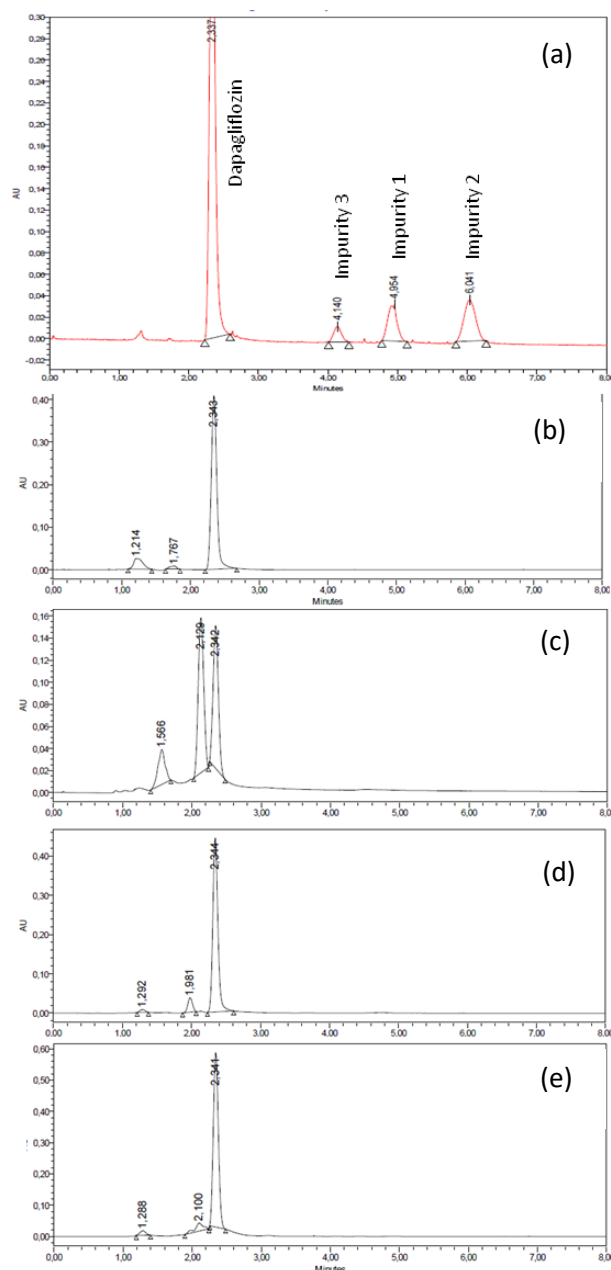


Figure 3. Representative chromatograms of dapagliflozin under stress conditions: dapagliflozin non-degraded sample and synthesis impurities (a); acidic degradation (1M HCl, 5h) (b); alkaline degradation (1M NaOH, 5h) (c); oxidative degradation (3% H₂O₂, 5h) (d) and UVC degradation, 5h (e).

Linearity

The linearity of the method was evaluated by linear regression analysis of each analyte separately and the results were all satisfactory (Table 1). The calibration curves of dapagliflozin, impurity 1, impurity 2 and impurity 3 showed determination coefficients (r^2) ≥ 0.99 and the residual analysis indicated absence of atypical samples.

LOD and LOQ

The limits of detection and quantification of the drug and its impurities were obtained through the linearity curves, by the standard deviation of the response (σ) and the slope (S). The results founded for dapagliflozin were $1.7 \mu\text{g.mL}^{-1}$ (LOD) and $5.7 \mu\text{g.mL}^{-1}$ (LOQ), impurity 1 were $0.08 \mu\text{g.mL}^{-1}$ (LOD) and $0.25 \mu\text{g.mL}^{-1}$ (LOQ), impurity 2 were $0.09 \mu\text{g.mL}^{-1}$ (LOD) and $0.3 \mu\text{g.mL}^{-1}$ (LOQ) and for impurity 3 were $0.26 \mu\text{g.mL}^{-1}$ (LOD) and $0.8 \mu\text{g.mL}^{-1}$ (LOQ). These results were considered very satisfactory according to the ICH Guideline Q3A (R2), which establishes the notification, identification and qualification limits for impurities in drug substances. It is important that the LOQ is below the notification limit of the impurity in order to guarantee the sensibility of the method to the intended use.

Precision

Table 2 presents the data of precision studies, obtained from the intra-day (repeatability) and inter-day (intermediate precision) results. The RSD values were calculated and all are $\leq 3\%$, which is considered adequate for analytical methods for quantitative determination of impurities.

Accuracy

The accuracy of the method was assessed by spiking known amounts of the dapagliflozin and impurities standards in the drug samples in three different levels and injected in triplicate into chromatographic system. The results are reported in Table 3 as the percent recovery of each analyte in the sample. All the recovery values were considered satisfactory, with acceptable relative standard deviations $\leq 3\%$.

Table 1. Statistical data for the linearity parameters of dapagliflozin, impurity 1, impurity 2 and impurity 3 obtained from validation study of a quantitative UPLC-DAD.

	Dapagliflozin	Impurity 1	Impurity 2	Impurity 3
Concentration range ($\mu\text{g.mL}^{-1}$)	30.0 – 70.0	1.0 – 10.0	1.0 – 10.0	1.0 – 10.0
Regression equation	$25518x + 8024.9$	$65899x - 2623.2$	$72182x - 5601.4$	$24672x - 1684$
Determination coefficient (r^2)	0.9996	0.9999	0.9999	0.9992
F	8437,0133	65669,2385	41526,7498	6102,085424
Significance F	2,84448E-06	1,71723E-11	5,39973E-11	6,51397E-09
Lower 95%	-32514,7963	-6682,66793	-11193,2083	-6670,1864
Upper 95%	48565,39639	1436,460012	-9,62336033	3301,7573

Table 2. Intraday and inter-day precision results of dapagliflozin and impurities 1, 2 and 3 obtained from validation study of a quantitative UPLC-DAD.

Days	1	2	3		1	2	3
Dapagliflozin				Impurity 1			
Repeatability (%)	95.05	95.62	94.19	Repeatability (%)	95.9	96.06	97.49
RSD %	1.32	0.49	2.67	RSD %	2.28	1.05	2.18
Intermediate Precision (%)	94.94			Intermediate Precision (%)	96.48		
RSD %	1.75			RSD %	1.96		
Impurity 2				Impurity 3			
Repeatability (%)	92.67	92.53	94.43	Repeatability (%)	95.46	98.24	100.37
RSD %	2.63	1.60	0.47	RSD %	2.02	1.70	0.36
Intermediate Precision (%)	93.21			Intermediate Precision (%)	98.02		
RSD %	1.93			RSD %	2.54		

Table 3. Recovery data at three levels (R) for dapagliflozin and its impurities obtained from validation study of a quantitative UPLC-DAD.

	Theoretical concentration ($\mu\text{g.mL}^{-1}$)	Experimental concentration ($\mu\text{g.mL}^{-1}$)	% Recovery	Statistical data
Dapagliflozin				
R1 (30 $\mu\text{g.mL}^{-1}$)	80.0	82.85	103.3	Mean = 102.3
R2 (50 $\mu\text{g.mL}^{-1}$)	100.0	103.27	103.0	SD = 0.0088
R3 (70 $\mu\text{g.mL}^{-1}$)	120.0	121.85	101.5	RSD% = 0.854
Impurity 1				
R1 (1 $\mu\text{g.mL}^{-1}$)	1.0	1.02	102.8	Mean = 101.7
R2 (5 $\mu\text{g.mL}^{-1}$)	5.0	4.98	99.3	SD = 0.0211
R3 (10 $\mu\text{g.mL}^{-1}$)	10.0	10.36	103.0	RSD% = 2.077
Impurity 2				
R1 (1 $\mu\text{g.mL}^{-1}$)	1.0	1.00	100.4	Mean = 102.2
R2 (5 $\mu\text{g.mL}^{-1}$)	5.0	5.2	103.8	SD = 0.0202
R3 (10 $\mu\text{g.mL}^{-1}$)	10.0	10.26	102.6	RSD% = 1.973
Impurity 3				
R1 (1 $\mu\text{g.mL}^{-1}$)	1.0	1.03	103.9	Mean = 103.5
R2 (5 $\mu\text{g.mL}^{-1}$)	5.0	5.04	100.7	SD = 0.0298
R3 (10 $\mu\text{g.mL}^{-1}$)	10.0	10.66	105.9	RSD% = 2.875

Robustness

During the development of the method, some variations in the analytical conditions were made in order to assure the reliability of the analysis against minimal changes in chromatography system. The mobile phase composition, flow rate and volume of injection were deliberately modified and the analytical run was evaluated with respect to the chromatography parameters which could compromise the analysis results, as resolution between the peaks, symmetry, theoretical plates and areas values. After this study, it was possible to assure the method resists to these variations without compromising the separation, detection and quantification of the analytes.

Toxicity evaluation

MTT reduction assay

The MTT assay (3 - [4,5-dimethyl-thiazol - 2-yl] - 2,5 - diphenyl-tetrazolium bromide) is based on the reduction of the MTT salt by the mitochondrial dehydrogenase enzyme to form formazan blue as a product. The test measures cell respiration, which is proportional to the amount of formazan produced and the number of viable cells in culture (23, 24). The results are represented by the

percentage of viable cells in relation to the control versus the six concentrations of each treatment.

The exposure of 3T3 cells to dapagliflozin did not show reduction in cell viability at any concentrations and incubation time evaluated, which suggests that the drug has no cytotoxic effect under these conditions. On the other hand, the results of its synthetic impurities indicate some toxicity for the cells. Impurities 1 and 3 showed a small reduction in cell viability only at the highest concentrations tested (1.0, 10.0 and 50.0 μM / 96h). Impurity 3 showed very significant results of damage to cells since the concentration of 0.5 μM in the acute exposure (24h) and even more in the chronic incubation time (96h), this damage being proportional to its concentration (Figure 4).

Neutral red uptake assay

The neutral red test is based on the ability of viable cells to incorporate the supravital neutral red dye. In dead or damaged cells, the dye cannot be retained (25).

The results found for both the drug and impurities were similar that of the MTT assay (Figure 5). Once again, dapagliflozin showed no reduction in cell viability, while its impurities demonstrated toxicity to cells at different magnitudes. In this assay, impurity 1 reduced cell viability significantly at concentrations of 10 and 50 μM ,

while impurity 3 showed the highest toxicity from the 0.25 to 50.0 μM .

Measurement of intracellular reactive species

The excess of reactive species may lead to adverse effects, such as membrane peroxidation, loss of ions, protein cleavage, and DNA strand breakage (26). The effect of the drug and its impurities on the generation of reactive oxygen and nitrogen species was evaluated by the DCFH-DA assay. This compound crosses cell membranes and it is enzymatically hydrolyzed by intracellular esterases in non-fluorescent dichlorodihydrofluorescein (DCFH) (27). The cells

exposure to the DCFH-DA induced an increase in the production of reactive oxygen and nitrogen species both in dapagliflozin and impurities samples. In the case of dapagliflozin, the formation of reactive species was observed only at 96 hours of incubation, in the concentrations of 1.0 and 10.0 μM ($p < 0.01$ and $p < 0.001$, respectively) (Figure 6). In the impurities samples, the results suggest that these compounds have a greater tendency to form reactive species, as shown in figure 6. At 96 hours of exposure, all impurities cause significant oxidative stress to 3T3 cells, suggesting that oxidative stress plays a role in its cytotoxicity.

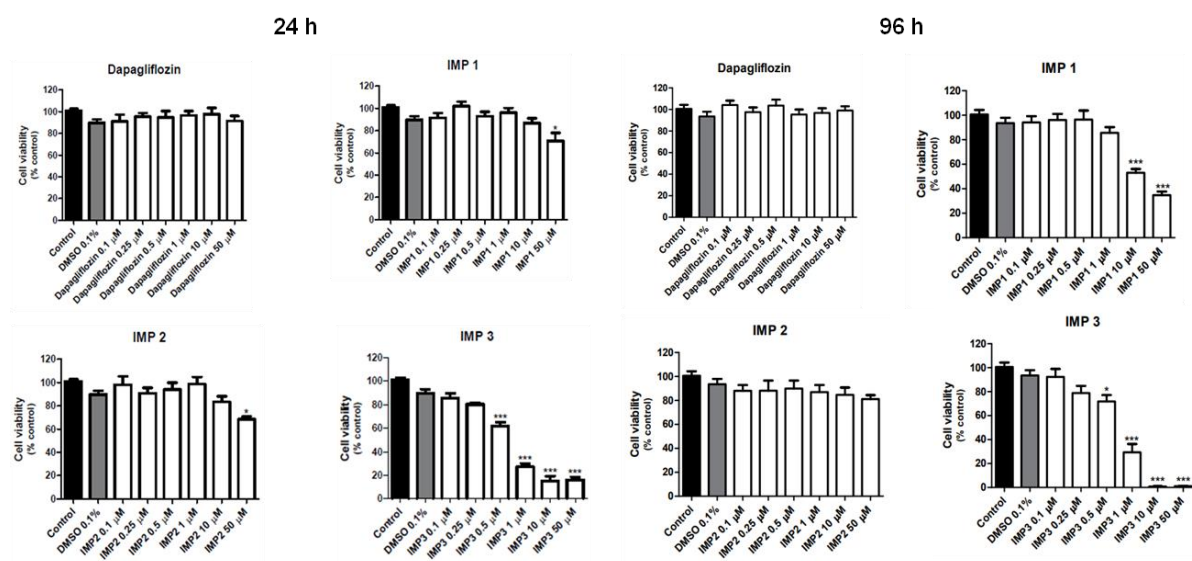


Figure 4. Cell viability evaluated by the MTT reduction assays in 3T3 cells after 24 and 96h incubation with dapagliflozin, impurity 1 (IMP 1), impurity 2 (IMP 2) and impurity 3 (IMP 3). Results are expressed as mean $\pm$ standard error of the mean. Statistical analysis performed through ANOVA/Bonferroni (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ versus control).

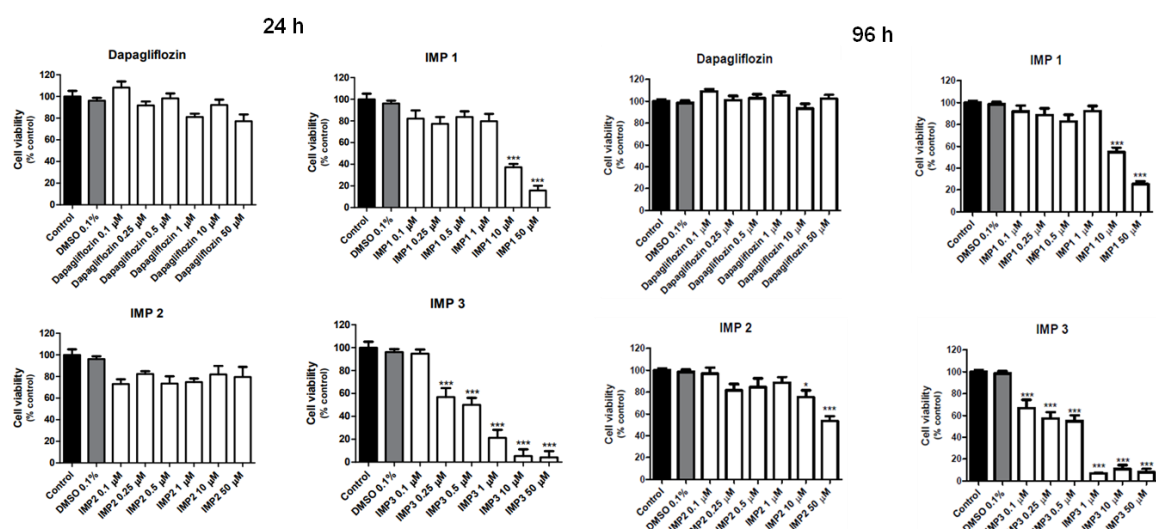


Figure 5. Cell viability by the neutral red uptake assay in 3T3 cells after 24 and 96h incubation with dapagliflozin, impurity 1 (IMP 1), impurity 2 (IMP 2) and impurity 3 (IMP 3). Results are expressed as mean $\pm$ standard error of the mean. Statistical analysis performed through ANOVA/Bonferroni (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ versus control).

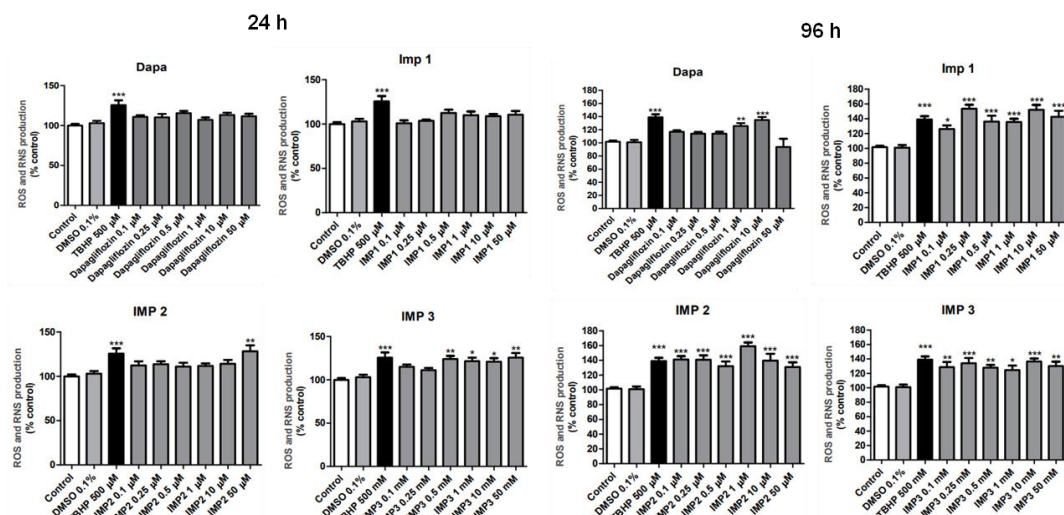


Figure 6. Results of intracellular reactive oxygen (ROS) and nitrogen (RNS) species in 3T3 cells incubated with dapagliflozin (Dapa), impurity 1 (IMP 1), impurity 2 (IMP 2) and impurity 3 (IMP 3) after 24 and 96h incubation-time at 36°C. Results are expressed as percentage (%) of control conditions. Statistical comparisons were made using one-way ANOVA/Bonferroni post-hoc test (*p < 0.05; **p < 0.01; ***p < 0.001 versus control).

Assessment of mitochondrial membrane potential ($\Delta\psi_m$)

The $\Delta\psi_m$ is the most important parameter controlling the accumulation of Ca^{2+} and ATP synthesis and its measurement is directly related to the normal cell function. Because of its crucial role in the maintenance of the physiological function of the respiratory chain generating ATP, changes in $\Delta\psi_m$ compromise oxidative phosphorylation by reducing cell energy and inducing cell death (28). Both depolarization or hyperpolarization of the mitochondrial membrane can be indicative of cell damage at different levels. As shown in figure 7, impurity 3 has a more pronounced effect on mitochondria compared to other analytes. As can be seen, the impurities caused an increase in $\Delta\psi_m$, which is indicative for mitochondrial hyperpolarization. This can stimulate the reverse proton flux through the respiratory chain and subsequent production of ROS and cell death.

Comet assay

The more useful approach for assessing DNA damage is the single-cell gel (SCG) or Comet assay. It is a simple method to measure the DNA strand breaks in eukaryotic cells and is widely used to assess the safety of drugs (29). The electrophoretic comet is the normal comet assay, which indicates single or double breaks in the DNA strand; the comet without electrophoresis is also called low molecular weight DNA, and is related to the degradation of DNA resulting from cell death and not necessarily with genotoxic effects (30). Although some impurities have some toxicity, none have been shown to cause DNA damage (Figure 8).

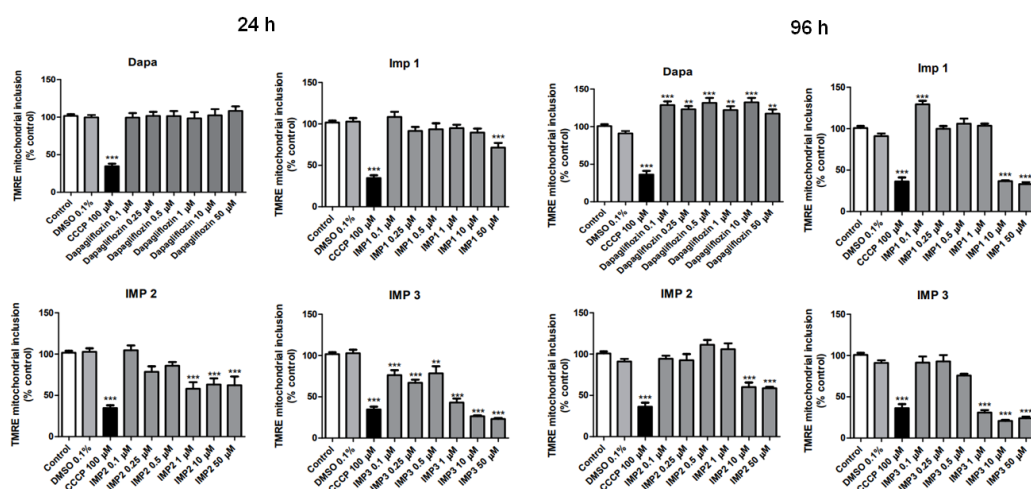


Figure 7. Evaluation of mitochondrial membrane potential ($\Delta\psi_m$) in 3T3 cells incubated with dapagliflozin (Dapa), impurity 1 (IMP 1), impurity 2 (IMP 2) and impurity 3 (IMP 3) after 24 and 96h incubation-time at 36°C. Results are expressed as mean $\pm$ standard error of the mean. Statistical analysis performed through ANOVA/Bonferroni.

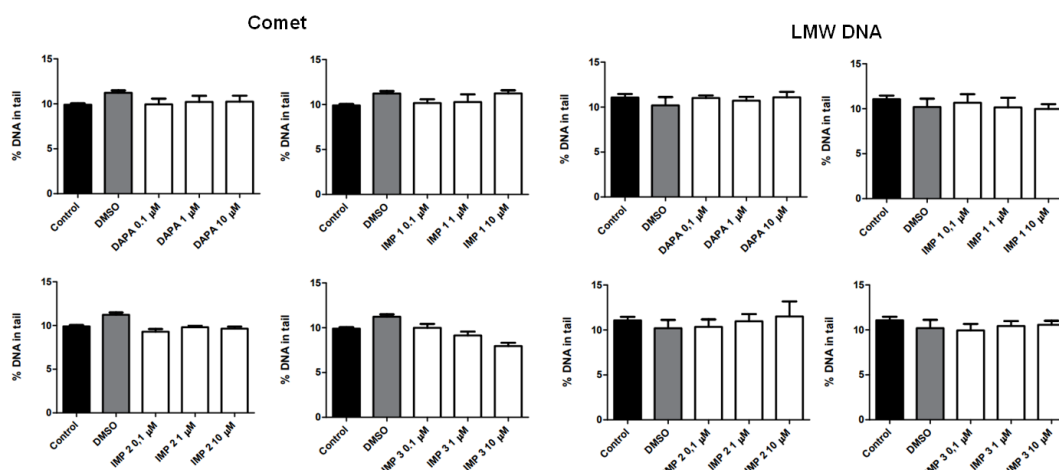


Figure 8. DNA damage measured by comet assay and LMW DNA diffusion assay in 3T3 cells incubates with dapagliflozin (Dapa), impurity 1 (IMP 1), impurity 2 (IMP 2) and impurity 3 (IMP 3) after 24 h incubation-time at 36°C. Results expressed as mean $\pm$ standard error of the mean. Statistical analysis performed through ANOVA/Bonferroni.

Conclusions

The present method met all the requirements of the analytical validation protocol and can be used for the simultaneous analysis of dapagliflozin and its related impurities. This UPLC method demonstrated to be an adequate technique to the routine quality control analysis, once it is a simple, fast and sensitive method to quantify, with reliability, the presence of impurities in dapagliflozin tablets.

The qualification of the drug and its related impurities, performed by different toxicity assay, is important and mandatory, mainly in chronic use due to their daily accumulation. The present results showed that dapagliflozin did not cause any type of cellular or DNA damage in any of the tested concentrations. In contrast, its contaminants, especially Impurity 3, showed a drastic reduction in cell viability, suggesting a high cytotoxic potential for this substance.

Since the dapagliflozin is used in the treatment of a chronic disease, these results contribute significantly to quality control throughout the entire production process. It is extremely important that manufacturers carefully monitor the synthesis processes of the active pharmaceutical ingredient, as well as the production stages of the final product, in order to ensure its safety and therapeutic effect.

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Conflict of interest

The authors declare no conflicts of interest.

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