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Sitagliptin phosphate: isolation and identification of two degradation products formed under acid conditions and determination of *in vitro* cytotoxicity

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Sitagliptin phosphate (STG), used for the treatment of type 2 diabetes, is a drug that belongs to a new class called inhibitors of dipeptidyl peptidase IV (DPPIV). It is known to be the first agent of the class approved by the FDA. Currently, many studies describe the determination of STG in plasma and pharmaceutical formulations but there are few studies on the drug stability. Forced degradation studies were conducted to isolate and identify the main degradation products. It was possible to observe the formation of two degradation products (DP1 and DP2) in hydrochloric 2.5M acid at 60°C. The structural characterization by UPLC-UV/MS of the degradation products was important to understand the degradation mechanism of STG under acid conditions. Purified degradation products were tested *in vitro* for cytotoxicity with a mononuclear cell assay. No cytotoxicity was observed at the concentration range of 10.0–250.0 $\mu g.mL^{-1}$. The structural characterization of the degradation products was important to understand the degradation mechanism of STG under acid conditions. Today, there are some resolutions and consensus, national and international, which show the importance of knowing and identifying the degradation products and also to evaluate the cytotoxic potential of these molecules.

Keywords: Sitagliptin phosphate, Indicating-stability method, Degradation product, Cytotoxicity

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Introduction

The incretin hormones glucagon-like peptide 1 (GLP1) and glucose-dependent insulinotropic polypeptide (GIP) are important prandial stimulators of insulin secretion and regulators of blood glucose levels. The inhibition of Dipeptidyl Peptidase IV (DPPIV) by gliptins extends the endogenous activity of GLP1 and GIP, decreasing the blood glucose concentration in diabetic patients (1). Sitagliptin phosphate (Figure 1) was approved by FDA in 2006 as a DPP-IV inhibitor for the control of type 2 diabetes mellitus (2,3). It is the first gliptin to be commercially available and has clinically relevant advantages: a) once-a-day oral dosage, b) administration at any time of day, independently of glucose levels (2,4).

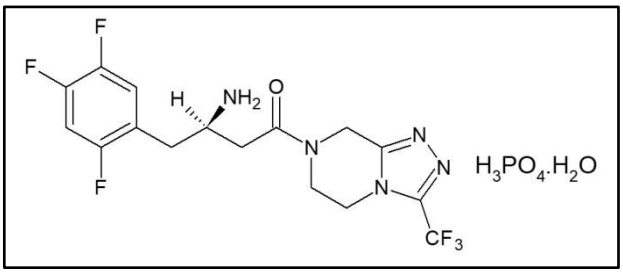

Figure 1. Chemical structure of sitagliptin phosphate.

An increasing number of publications can be found describing the development of analytical methods for determining sitagliptin phosphate in the plasma and urine of humans, rats, and dogs by LC-UV (5) and LC-MS (6-9), and pharmaceutical preparations by spectrophotometric methods (10-11).

Shokouhi et al. recently described a technique using net analyte signal and radial basis function neural network to simultaneously determine metformin and sitagliptin by UV-Vis spectrophotometry (12). El-Bagary et al. published stability-indicating methods for determining sitagliptin and its degradation products in alkaline conditions (13,14). In 2020, Li et al. developed a sensitive method for detecting quinoline-derivatized sitagliptin in small volumes of human plasma by MALDI-TOF mass spectrometry (15).

Gumieniczek et al. studied the chemical stability of sitagliptin in several mediums by LC-UV, LC-MS and FT-IR methods (16). The UV degradation kinetics in ultrapure water and wastewater effluent was described by Krakko et al. Quality by design tools led to the development of a solvent-modified micellar electrokinetic chromatography method for determining sitagliptin and its related compounds by Pasquini et al. (18).

Our group previously developed methods by LC-UV and capillary electrophoresis to characterize sitagliptin and its degradation products (19, 20). In this study, we evaluate the stability of sitagliptin phosphate under acidic conditions at 60 °C, purify the degradation products, and estimate the degradation kinetics. Additionally, the degradation products (DPs) were tested *in vitro* for cytotoxicity using mononuclear cells.

Experimental

2.1. Materials

STG reference standard (99.5%) was supplied by Sequoia Research Products (Oxford, UK). Sitagliptin-coated tablets (Januvia® 50 mg) were purchased in the pharmaceutical market. All chemical reagents were of pharmaceutical or analytical grade. HPLC-grade acetonitrile and methanol were purchased from Tedia (Fairfield, USA). Reagent grade hydrochloric acid, triethylamine, and phosphoric acid were obtained from Merck (Darmstadt, Germany). Purified water was obtained by a Millipore® Direct-Q 3UV (Molsheim, France).

2.2. STG and DP Quantification

STG was determined by a previously validated chromatographic method using a Waters-Xbridge™ RP-8 column (250mm×4.6mm i.d., particle size 5 µm) operated isocratically at 25°C (19). The mobile phase consist of a solution of triethylamine (TEA) 0.3% and acetonitrile (ACN) (75:25 v/v), adjusted to pH 4.0 with phosphoric acid at a flow-rate of 1.0 ml/min, and UV detection at 207 nm. The injection volume was 20 µl. To isolate the DP, the triethylamine was withdrawn from the mobile phase and water was used with a pH adjusted to 4.0 employing formic acid and ACN in gradient (80:20 v/v).

2.3. UPLC-UV/MS Characterization

The UPLC-UV/MS system consisted of a Waters Acquity UPLC with an Acquity Photo Diode Array (DPA) detector and Micromass Q-Tof mass spectrometer, controlled with MassLynx 4.1 software (Waters, Milford, MA, USA). The optimal UPLC conditions for analysis of the sitagliptin and the degradation products formed in acid medium were: mobile phase composed of water pH 4.0 ajusted with formic acid and acetonitrile in gradient (80:20 v/v), flow rate of 0.3 mL.min^{-1}, temperature of 25 °C and injection volume was 0.5 µL. Chromatographic analyses were performed using an Agillent C_{18} column Zorbax Eclipse Plus (50 x 2.1mm; 1.8µm). The conditions used in the mass spectrometer were: capillary voltage of 2.8 kV, sample cone 40 V, extraction cone 2.5 V, source temperature 120°C and collision energy 4 V.

2.4. Stress condition and degradation kinetics

Stock solution at a 5.0 mg/ml concentration of STG was prepared with a mixture of methanol, water and HCl (final concentration HCl 2.5*M*). An aliquot of 1.0 mL of the degraded acid solution was periodically transferred to a volumetric flask (20 mL) and neutralized with 1.0 mL of sodium hydroxide (NaOH 2.5*M*). The final volume was completed with water and kept at 60 °C. The degradation kinetics was performed for 6 hours, and samples were analyzed at 0, 0.5, 1, 2, 3, 4, and 6 hours.

The kinetics order of degradation was determined by plotting the remaining drug concentration versus time (zero order kinetics), log of concentrations versus time (first-order kinetics) and the reciprocal of concentration (second-order kinetics) versus time. The regression coefficients (R^2) were obtained and the best-observed fit indicated the reaction order. Some kinetics parameters, such as the apparent order degradation rate constant (k), t_{50} and t_{90} were calculated. The degradation kinetics was observed for both the sample of coated tablets and the standard STG solution.

2.5. Isolation of degradation product

A gradient chromatographic method was developed to purify the degradation products and consisted of a mixture of water pH 4.0:acetonitrile (80:20 v/v) for 7 minutes, 100% of aqueous phase at 12 minutes, 100% of organic phase at 23 minutes. The system was equilibrated between 23 and 27 minutes returning to the initial condition (water pH 4.0:acetonitrile 80:20 v/v). This method allowed the collection of fractions to be further analyzed by UPLC-UV/MS. The degradation products isolated were called DP1 and DP2.

2.6. Cytotoxicity assay

Cellular injury was quantified by measuring lactate dehydrogenase (LDH) released into the medium. LDH is a cytosolic enzyme that is released into the cytoplasm upon membrane integrity loss. The activity was determined using a commercial kit (Doles Reagents, Goiânia, Brazil). LDH activity was evaluated using a colorimetric assay at 492 nm.

At the beginning of the experiment, human mononuclear cells were separated from the peripheral blood of three healthy donors. Heparinized venous blood was diluted 1:1 with Hank's medium. Mononuclear cells were isolated by plasma separation on Ficoll-Paque gradient and washed twice in Hank's medium. The total cells were counted in a Neubauer chamber. The mononuclear cells were washed and resuspended in Hank's medium to a concentration of 10^7 viable cells in 1.0 mL. Pipetting the reagents into a 96-well plate was done in the following order: ferric alum, substrate and sample solution. Concurrently, a blank test was performed in triplicate with ferric alum and substrate (the sample was not added at this point). A positive control assay was also performed with the maximum amount of 2.5M HCl, 2.5M NaOH, and methanol to prepare the samples and the Triton X-100 (100% cell death). After the samples were added, the plates were incubated at 37 °C. The blank test, the control assay, and the sample tests were simultaneously prepared. Then stabilization solution was added every 10 seconds. The absorbance reading of the blank is subtracted from the readings obtained for each sample. In each well plate the final concentration analyzed was 10.0, 50.0, 150.0 and 250.0 µg.mL^{-1}, in triplicate. The STG sample and degradation product samples were prepared in methanolic solutions and diluted in Hank's medium.

A one-way analysis of variance (ANOVA) followed by Dunnet's test was used to compare the means of the tests. A difference was considered statistically significant when $p<0.05$. Data analyses were performed using Prism 5.0 (GraphPad Software, Inc., CA, USA).

Results and Discussion

Preliminary stability studies were conducted to evaluate the behavior of STG in the acid medium at conditions, room temperature and 60 °C. Initially, the STG solution was maintained under acid conditions (2.5 M HCl) for 6 hours at room temperature, but only 4% degradation was observed. The degradation process increased to about 20% when the solution was maintained in 2.5 M HCl at 60°C. The kinetic degradation study was performed on STG-coated tablets and reference standard solutions.
Figure 2 shows a typical chromatogram of STG samples degraded under acid conditions. STG retention time was close to 7 min and two major degradation products were observed at 3.4 and 5.7 minutes. STG peak area decreased by 20% after 6 hours under acid conditions at 60°C, while DP1 and DP2 increased to 9% and 7%, respectively. The chromatographic peak purity tool was applied to DP1 and DP2, indicating the absence of another coeluting substance at the same retention time at 207 nm.

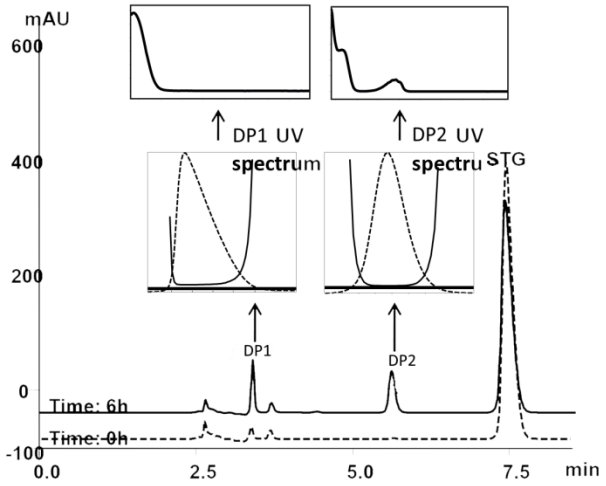

Figure 2. Chromatograms of STG reference standard solution during the degradation process in acid medium (HCl 2.5M) at 60°C (time 0-6 hours).

The degradation rate of STG was evaluated and approximately 20% of STG-coated tablets and 30% STG reference standard were degraded after 6 hours of exposure under acid conditions at 60 °C.
The degradation follows second-order kinetics under the experimental conditions applied. This reaction order is like the order published by Lange et al., for the photodegradation kinetics of sitagliptin quantified by capillary electrophoresis (UV-C light) (20).
The kinetic parameters such as rate constant k, t1/2, and t90 for STG coated tablets and STG reference standard were calculated using second model order and are summarized in Table 1.

Table 1. Degradation rate constant (k), half-life ($t_{1/2}$) and t_{90} for STG coated tablets and STG reference standard in solutions submitted to degradation in 2.5 M HCl at 60 °C.

Kinetic parameters			
Sitagliptin			
	k (l/mg.h)	**$t_{1/2}$ (h)**	**t_{90} (h)**
Coated tablets	$3.88.10^{-4}$	25.77	2.86
Reference standard	$6.89.10^{-4}$	14.52	1.61

Figure 3 shows STG samples and purified DPs analyzed by UPLC-UV/MS.

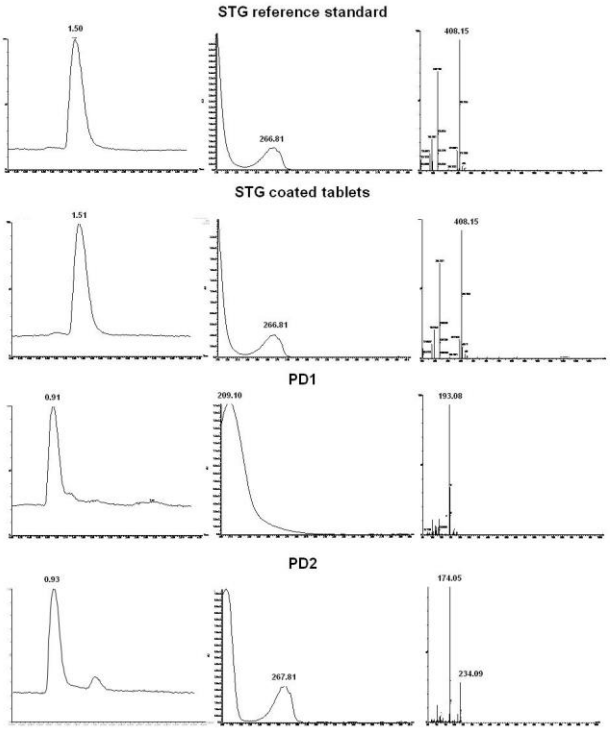

Figure 3. Analysis by UPLC-UV/MS of samples: STG reference standard, STG coated tablets, DP1 isolated and DP2 isolated.

Mass analysis was performed to identify the structure of the degradation products. The mass spectrometer conditions were optimized with injections of STG reference standard and subsequently applied to degradation product solutions DP1 and DP2.
The mass spectrum of STG showed the molecular ion (m/z 408.15), which agrees with the molecular weight $[M+H]^+$ of sitagliptin base. The mass spectrum of DP1 and DP2 showed the molecular ion at m/z 193.08 and m/z 234.09, respectively.
The UV spectra of the DP1 and DP2, recorded during the run, show a change in the maximum absorption indicating the loss of some chromophoric groups, mainly in the DP1.
The chemical structures of DP1 and DP2 were proposed according to high resolution molecular ion peaks and its fragmentation profiles (Figure 4).

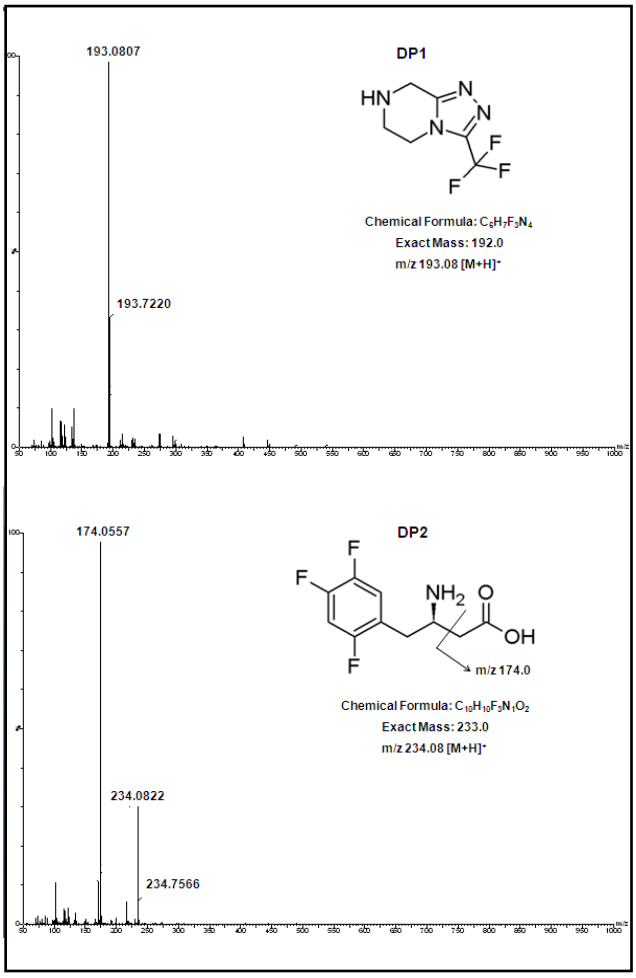

Figure 4. Mass spectrum and identification of produts degradation. The figure (A) refers to DP1 and (B) refers to DP2.

In Figure 4, ion m/z 193.08 refers to a fragmentation already described in the literature (5, 8, 12). Based on the comparison of UV spectrum and mass spectrum, it can be suggested that DP1 is the same degradation product isolated in basic medium by El-Bagary and collaborators (13). The compound is known as 3-(trifluoromethyl)-5,6,7,8-tetrahydro[1,2,4]triazolo[4,3-a]pyrazine.

The DP2, with m/z 234.08, is formed when the amidic group in STG cleavages in acid conditions (Figure 5). The new acid formed is called (3R)-3-amino-4-(2,4,5-trifluorophenyl)butanoic acid.

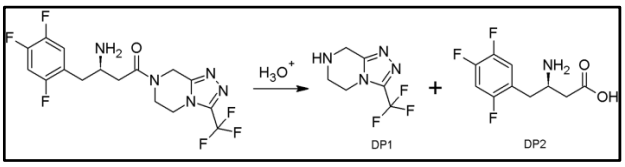

Figure 5. Chemical reaction of sitagliptin in acid medium.

Cytotoxicity assays are among the most used in vitro methods to predict the potential toxicity of a substance in a cell culture (21). The cytotoxic effects of STG were determined by the release of the cytosolic lactate dehydrogenase. The initial concentrations of degradation solution containing DP1 and DP2 were: 10.0 (A10), 50.0 (A11), 100.0 (A12) and 250 µg/mL (A13) and the concentration of intact STG was 250 µg/mL (A14). All concentrations given are the final ones. The LDH activity in the supernatant was measured and values of 100% indicate total cellular viability (control data). Triton X-100 at 1% was used as positive control. In order to detect the cytotoxic potential of DPs in mononuclear cells, the evaluation was performed on DPs against the intact drug. In this experimental protocol, non-significant differences ($p>0.05$) were observed for DPs (A10, A11, A12 and A13) and for the drug (A14) at all concentrations studied, indicating that the presence of DPs did not reduce the cell viability. The results are summarized in Figure 6.

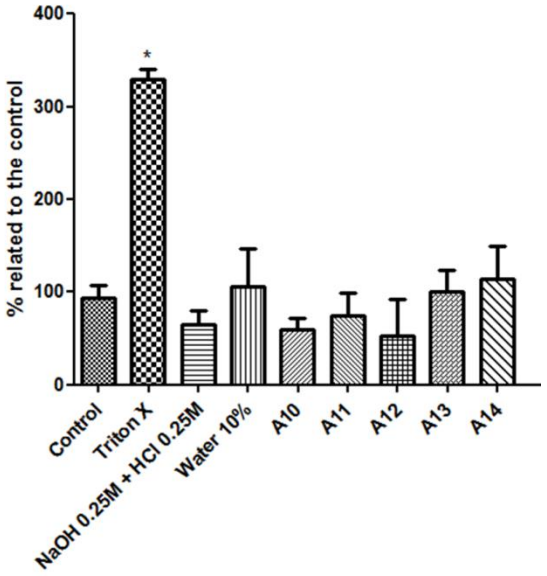

Figure 6. Cytotoxicity study of STG and DPs at concentrations of 10.0, 50.0, 100.0 and 250.0 µg.mL-1. Values of 100% indicates total cellular viability. Triton X-100 1% represents cell injury (positive control). A10, A11, A12 and A13: samples of the degradation products after degradation in HCl 2.5M for 6h); A14: sample of STG reference standard. *Significant differences between means ($p<0.05$).

Conclusions

The STG degradation follows a second-order kinetics for both STG coated tablets and STG reference standard. The two major DPs observed under acid conditions at 60°C were collected and identified by UPLC-UV/MS, and STG hydrolysis was proposed. In vitro cytotoxicity assay showed non-significant differences for DPs and the drug at all concentrations studied, indicating that the DPs were not cytotoxic.

Acknowledgments

In memory of Professor Elfrides Schapoval, who dedicated her life for pharmaceutical sciences.

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