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Determination of sitagliptin in the presence of its organic impurities using Box-Behnken experimental design for robustness assessment

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A simple HPLC method was developed and validated for the quantitative determination of sitagliptin and its organic impurities from the synthesis process. The method was carried out in an XBridge™ Phenyl column (250 mm X 4.6 mm i.d., 5 μm) with a mobile phase consisting of an acetonitrile/formic acid (0.05% aqueous solution) mixture (40:60, v/v), with isocratic elution. Flow rate was 1.0 mL/min and detector wavelength was 207 nm. The validation process, in accordance with international guidelines, shows that the method was linear (R^2 = 0.9997-0.9999) and ANOVA showed a non-significant linearity deviation (p>0.05). Precision RSD was <4% (n=6) and method accuracy ranged between 97.52-102.85%. Limits of detection (1.4 and 0.5 μg mL^{-1}) and quantification (2.8 and 2.1 μg mL^{-1}) were determined for impurities 1 and 2, respectively. Critical factors were selected to examine method robustness with a three-level Box Behnken experimental design and no significant factors were detected. The HPLC method for impurity determination in sitagliptin was precise, accurate and robust. The separation of the compounds presented an adequate resolution even in the presence of the main degradation product proving to be effective for routine analyses in the pharmaceutical industry.

1. Introduction

Control and evaluation of impurities in the pharmaceutical industry are essential to ensure the quality, safety and efficacy of drug development. The assessment of an impurity profile through identification and quantification has received significant attention from regulatory authorities and several pharmacopoeias have incorporated limits with regard to permissible levels of impurities present in the formulations (Roy 2002; Agentine et al. 2007; Nagpal et al. 2011; Raman et al. 2011; Holm and Elder 2016). Constant surveillance of drug impurities is necessary as they may have many implications on population health, including teratogenic and mutagenic potential, and carcinogenic effects (Ahuja 2007; Bercu et al. 2009; Balasubrahmany et al. 2012).

Therefore it is necessary to establish methods that can contribute to the improvement of quality control, safety and efficacy of drugs to identify and quantify impurities which can influence the therapy and health of patients undergoing chronic treatments.

Among the diseases which require continuous treatment, diabetes mellitus type 2 has a complex pathophysiology and its pharmacotherapy requires a drug combination to maintain basal glucose levels in the blood (Duez et al. 2012; Neumiller and Setter 2012; Irons and Minze 2014). The gliptin class is one of the drug therapies used for diabetes mellitus type 2 and there are few studies on evaluation and quantification of its impurities. Sitagliptin (STG) is part of this group and acts by inhibiting the DPP-4 enzyme, which enhances the action of incretins, allowing the maintenance of glucose levels (Duez et al. 2012; Neumiller and Setter 2012).

Due to the small number of studies regarding the identification and quantification of impurities related to STG, combined with the fact that it represents substantial consumption in current therapy, the development and research of analytical methods is a relevant contribution to ensure reliability and safety in the chronic use of these drugs for an effective treatment of type 2 diabetes mellitus.

Researchers described a product of STG alkaline degradation which is also part of its synthetic pathway and an active metabolite of the drug: 3-(trifluoromethyl)-5,6,7,8-tetrahydro [1,2,4]triazolo [4,3a]pyrazine. The structure was elucidated by different spectroscopic techniques and it corresponds to impurity 1 described in this paper. The determination of STG, metformin and the STG alkaline degradation used high performance liquid chromatography (HPLC) with the detection of 220 nm and run time program of 15 min (El-Bagary et al. 2011). Another study developed a stability-indicating HPLC method of STG tablets using detection at 207 nm as in the case of this search. The main degradation product was identified under UV-C light, cytotoxicity test by LDH of this product using mononuclear cells to show a toxic effect at concentrations above 50 μg mL^{-1} (Lange et al. 2012). Peraman et al. (2013) simultaneously evaluated STG and metformin stability in pharmaceutical dosage forms using HPLC and the run time analysis was 38 minutes. This method detected fifteen degradation products; four of these structures have been identified where one of them corresponds to impurity 1 of this research.

Recently, a research developed a methodology by HPLC for evaluation of process impurities and degradants of STG, but the analysis time was 65 minutes in a gradient model (Vuyyuru et al. 2017).

No study is available for separation and quantification of STG and its main impurities of synthesis in the presence of major degradation product by UV-C light so far. Until now, no research was performed with impurity 2 of this study.

Impurity profiling by analytical methods in new drug substances is a key piece of the current guidelines and the most important issues in recent pharmaceutical analysis. This is similar to forced degradation studies which supply information on possible degradants and intrinsic stability of a drug substance (Jain and Basniwal 2013). In this case, the impurities were selected according to the synthesis route.

Statistical design of experiments (DoE) was applied to evaluate the analytical method robustness by multifactorial approach. It allows a smaller number of experiments and also provides a better understanding of the effect of controllable variables on critical

parameters. The assessment of method robustness is essential to verify the statistical significance of critical factors and their interactions on the experimental region and method parameters. In order to accomplish a well fitted DoE, the number of factors must be considered (Ferreira et al. 2017). Although robustness assessments of analytical methods are commonly conducted using inexpensive techniques such as Plackett-Burman, the reduced number of controllable factors to be modeled allowed the application of the Box-Behnken design with a small number of experiments (Cavazzuti 2013).
In accordance with international demand and the need for pharmaceutical industries to qualify their products, this paper aims to use the HPLC technique to determine STG and its main synthesis impurities (Fig. 1) in order to obtain the best analytical methodology. Additionally, the use of the Box-Behnken design allows evaluating the modifications made in the parameters and the interaction that occurs between them.

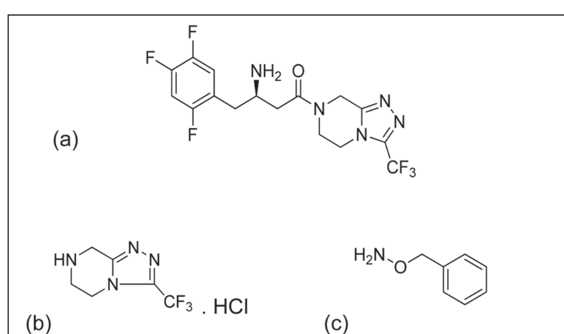

Fig. 1: Chemical structures of sitagliptin (a), impurity 1 (b) and impurity 2 (c).

2. Investigations, results and discussion

The pharmacological and toxicological profile is responsible for the safety of a drug and its adverse effects may be caused by impurities present in the drug. Accordingly, it is important to monitor and control impurities to ensure quality and safety of a drug by the analytical activities that represent a key component of modern pharmaceutical analysis (Jain and Basniwall 2013). Regulatory agencies are increasingly attentive to this issue and have sought different strategies in order to ensure the quality and safety of pharmaceutical preparations (ICH Q3AR2 2006; ICH Q3BR2 2006).
Among the analytical techniques, HPLC is extensively used in order to get high resolution, reproducible responses, easy instrument operation and data analysis with adaptability to major procedures (Gumustas et al. 2013).
The main objective of the chromatographic method used here was to achieve a separation of STG from its two impurities with adequate detection at trace levels. The ability to separate and quantify it in the presence of major degradation product improves the method and allows the control of the pharmaceutical formulation.
A reported stability-indicating HPLC method for the determination of STG revealed that fast drug degradation was observed in UV-C light and the basic, oxidative, thermal and photolytic conditions also detected degradation products but not as fast as under UV-C light (Lange et al. 2012). This study used a C8 column, mobile phase of water with 0.3% of triethylamine pH 4 and acetonitrile (75:25, v/v) at 207 nm (Lange et al. 2013).
Our research started with this condition but all compounds remained very close to each other and changing the pH ratio of the mobile phase did not provide better results. Using a C18 column under the same conditions tested before, impurities did not interact with the column, probably because of polarity. After, changing the mobile phase to water with 0.1% of formic acid pH 3.5 and acetonitrile, all compounds were at the same retention time.
The literature shows that in reversed phase HPLC, a stationary phase with less polarity and a mobile phase with greater polarity are strongly applied to separate solutes with different polarities. In this case, drug and impurities are polar and it is necessary to change the stationary phase in order to provide separation with an adequate resolution between all the substances.
Testing the phenyl column, water with 0.1% formic acid and acetonitrile with changes in proportions of mobile phase, the best condition found was 60% aqueous phase with separation of drug and its impurities in short analysis time and acceptable system suitability (resolution, theoretical plates, symmetry and retention factor). Mobile phase was applied to dilute samples.
The method was validated according to ICH guidelines (ICH Q2R1 2005) and specificity was evaluated by comparing the chromatograms of the STG and its impurities with a simulated excipient mixture (Fig. 2). It was found that the excipients in the formulation did not interfere in the analysis of STG and impurities and there was no additional peak eluting at the same retention time. Additionally, impurities did not interfere with the detection of STG. A chromatographic peak purity tool demonstrated that they were pure in all cases. Since STG and its two impurities peaks could not be attributed to any other peak, the method was confirmed suitable for analysis.

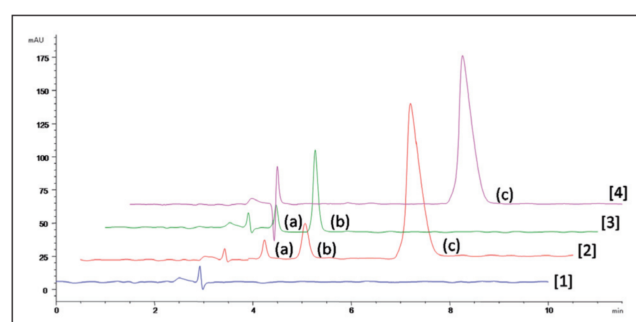

Fig. 2: Chromatogram of simulated excipient mixture [1]; standard preparation of impurity 1 - 5.0 μg/mL (a), impurity 2 - 5.0 μg/mL (b) and sitagliptin - 60.0 μg/mL (c) with simulated excipient mixture [2]; standard preparation of impurity 1 - 10.0 μg/mL (a) and impurity 2 - 10.0 μg/mL (b) [3]; standard preparation of sitagliptin - 60.0 μg/mL (c) [4].

The STG main photodegradation product (Lange et al. 2012) was tested and it also did not interfere in the analysis of STG and its impurities (Fig. 3) and it elutes at distinct retention times demonstrating the selectivity of the method which is also useful for quality control in pharmaceutical industry.

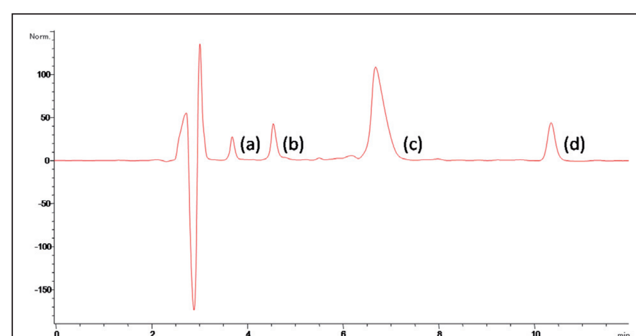

Fig. 3: Chromatogram of standard preparation of impurity 1 - 15.0 μg/mL (a), impurity 2 - 15.0 μg/mL (b) and sitagliptin - 60.0 μg/mL (c) in the presence of a main degradation product (d).

The high resolution attained allows preparing solutions of the STG drug at an elevated concentration. This increases the possibility of analysis by the pharmaceutical industry since impurities are only present in trace levels.
The first method for simultaneous determination of STG and metformin by HPLC in the presence of alkaline degradation product of STG led to the elucidation of the structure by different spectroscopic techniques presenting the same chemical structure than that of impurity 1 from this study. It can be regarded as an impurity of synthesis or as a degradation product.

Similarly, Peraman et al. (2013) developed a stability-indicating liquid chromatographic method for simultaneous analysis of STG and metformin with detection of fifteen degradation products. The structures of four were elucidated where two appear during degradation of STG (Peraman et al. 2013). One of these chemical structures is like impurity 1 and the other presented a structure similar to impurity 2 from this study.

Analytical methods developed for impurity analysis must have the highest sensitivity possible for the detection and quantification of trace levels of these contaminants in drug samples. The LOD, LOQ, and RSD of LOQ values determined for two impurities of STG are reported in Table 1. In this case, the values obtained were very low among the values established for international guidelines (ICH Q3AR2 2006).

Table 1: Limits of detection, quantification and relative standard deviation of impurities 1 and 2. Linearity of sitagliptin and its impurities.

Limit of detection		Limit of quantification		RSD* of LOQ	
Impurity 1	Impurity 2	Impurity 1	Impurity 2	Impurity 1	Impurity 2
1.4 μg mL^{-1}	0.5 μg mL^{-1}	2.8 μg mL^{-1}	2.1 μg mL^{-1}	3.38	3.64
Linearity					
	Slope	Intercept	R^2		
Sitagliptin	36.953	-0.331	0.9999		
Impurity 1	14.056	4.801	0.9997		
Impurity 2	54.907	-13.331	0.9999		

*Relative standard deviation of the areas at LOQ.

The analytical method proposed is sensitive enough to measure small amounts of impurities in order to ensure the quality and safety of pharmaceutical formulations together with the high resolution found in the chromatogram. Additionally, RSD obtained for impurities 1 and 2 for limit of quantification is much smaller than 10%. This qualifies the method since these are drug impurities that allow quantification with reproducibility.

According to official guideline specifications, the limits of detection and quantification acquired are below the reporting threshold considering the amount of drug substance administered per day (ICH Q3BR2).

The linearity standard plot for the assay method was obtained over the concentration ranges tested, i.e, 5.0- 65.0 μg mL^{-1} for STG, 3.0- 30.0 μg mL^{-1} for impurity 1 and 2.5- 30.0 μg mL^{-1} for impurity 2. All determination coefficient obtained were greater than 0.999. The results are within the acceptance criteria (ICH Q2R1 2005) and demonstrate that there is a correlation between peak area and concentration of analytes confirmed linearity (Table 1) (ICH Q2R1 2005). ANOVA showed a non-significant linearity deviation (p>0.05). Lange et al. (2012) used concentrations of 70.0 to 130.0 μg mL^{-1} for linearity of STG, Peraman et al. (2013) studied in the ranges 50- 450 μg mL^{-1} for metformin and 10-150 μg mL^{-1} for STG. The new methodology developed used smaller concentrations with satisfactory results and suitable for the proposed objective. Results are in agreement with other methodologies used to quantify STG and other drugs (El-Bagary et al. 2011; Lange et al. 2012; Peraman et al. 2013).

RSD for STG sample assay in the method repeatability study was 2.14% and RSD for the area of impurities were 1.21% and 2.46% for impurity 1 and 2, respectively. Assay RSD obtained in intermediate precision study are reported in Table 2. These results confirmed that the method was precise and demonstrated that the two data analyses were in agreement. These findings show that they are in accordance with studies analyzing STG using different analytical methods (El-Bagary et al. 2011; Lange et al. 2012; Peraman et al. 2013).

Recovery of STG ranged from 101 to 103% and from 97% to 99% for impurities 1 and 2, respectively. In the equation used the results were obtained by theoretical concentration and area of standard. The deviation from the theoretical recovery is small and the condition for mean recovery in this validation is met. The results obtained in other studies were similar to this research and although the limit for impurities can be higher for the small concentration used, the findings were similar (El-Bagary et al. 2011; Lange et al. 2012; Peraman et al. 2013).

Table 2: Intraday and interday precision and data on accuracy

Intraday (n = 6)			
	Sitagliptin (%)	Impurity 1 (%)	Impurity 2 (%)
Mean	100.60	98.4	100.88
RSD	2.14	1.21	2.46
Interday (n = 18)			
	Sitagliptin (%)	Impurity 1 (%)	Impurity 2 (%)
Mean	99.9	98.57	98.43
RSD	2.03	1.69	3.2
Accuracy			
	Taken (μg mL^{-1})	Found (μg mL^{-1})	Recovery (%)
Sitagliptin	10.0	10.29	102.85
	20.0	20.38	101.90
	30.0	30.80	102.66
Impurity 1	3.0	2.96	98.83
	5.0	4.89	97.76
	10.0	9.99	99.87
Impurity 2	3.0	2.97	98.86
	5.0	4.88	97.52
	10.0	9.79	97.94

Robustness of an analytical method is a dimension of its capability to remain unchanged by small, but deliberate changes in method parameters and provides an indication of its reliability during normal usage (Cavazutti 2013). Temperature, flow rate, and percentage of ACN in mobile phase were selected and small changes performed. The effect of those changes on STG and impurities 1 and 2 assay, area, and area RSD was assessed. Results are presented as coefficient plots that show the significance of factors evaluated by DoE. Whenever the confidence interval bar crosses zero, the factor is considered non significant for the parameter assessed.

Currently, several researches have used the Box Behnken experimental design to evaluate the robustness and it proved to be effective for the proposed objective (Ragonese et al., 2002; Beg et al., 2012; Hasnain et al., 2013). Figure 4 demonstrates that modifications of chromatographic factors do not significantly alter the method capacity to quantify STG, impurity 1 and impurity 2 accordingly.

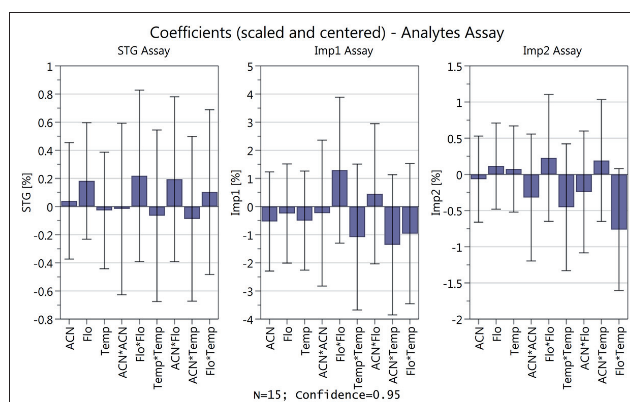

Fig. 4: Effect of variations on ACN %, flow and temperature of sitagliptine and impurities assay. Uncertainty bar crossing y=0 represents non significant factors (α=0.05).

RSD assay values for STG and impurities 1 and 2 were assessed throughout the Box Behnken experimental design. Figure 5 shows that there was no significant variation in RSD responses for quantification of STG, impurity 1 and impurity 2. Applying Box-Behnken to evaluate method robustness it was found that "Flow rate" is the factor with a very significant effect on area values for STG and impurities 1 and 2 (Fig. 6). These findings are in accordance with chromatography theories, since the increase in mobile phase flow rate causes a reduction in the area value because peak width is reduced and absorptivity remains the same.

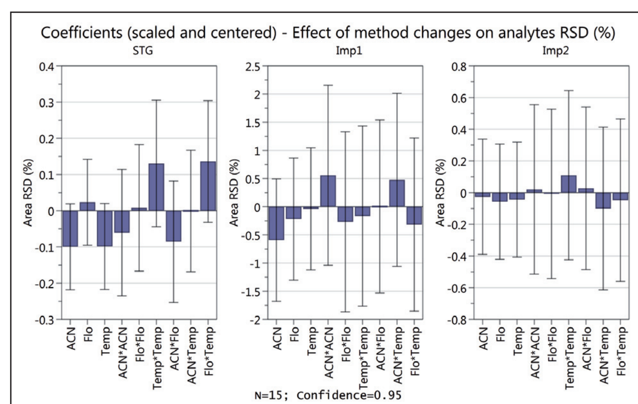

Fig. 5: Evaluation of the effect of variations on the analytical method for the sitagliptin and impurities assay. Uncertainty bar crossing y=0 represents non significant factors (α=0.05).

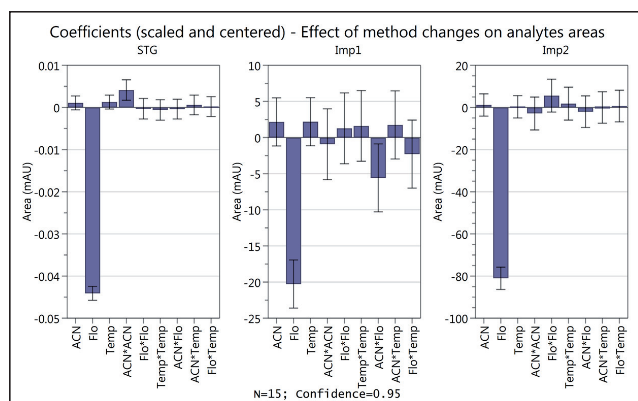

Fig. 6: Evaluation of the effect of pre-defined variations on the analytical method.

According to the statistical analysis of results of the Box-Behnken design, it could be ensured that some parameters such as temperature, flow, and percentage of organic solvent (acetonitrile), under the observed range, are not statistically significant for the accurate detection and quantification of STG and its impurities of synthesis (1 and 2). The Box-Behnken model design was evaluated by ANOVA for regression and residual analysis. It was found statistically significant and with no lack of fit (α=0.05) for all checked parameters, being suitable to assess method robustness.

Analysis of system suitability shows that assessed parameters (Table 3) were within the acceptable range, indicating that the method developed is suitable for the intended analysis.

Table 3: Data of system suitability

Parameters	Sitagliptin	Impurity 1	Impurity 2
Retention time (min)	7.35	3.86	4.89
Resolution	8.96	6.33	4.91
Theoretical plates	6366	7729	8969
Asymmetry	1.13	0.97	0.81
Peak capacity factor	1.86	0.49	0.86

The present study was able to detect and quantify STG synthesis impurities at low levels, which is very important when analyzing small amounts of new drug substances. The isocratic HPLC method developed for quantitative analysis of STG and its main impurities is precise, accurate, linear, robust and specific. This method exhibited an excellent performance in terms of sensitivity and speed, even though it used small concentration of impurities. For the robustness test none of the changes performed significantly altered the expected results. According to official guidelines, drugs such as STG and its main synthetic products, an analytical study is required to evaluate the raw material and the finished product in order to supply the demands of quality control in the pharmaceutical industries. The isocratic HPLC method is suitable and can be allocated for routine analysis of sample production and to check the quality of STG.

3. Experimental

3.1. Chemicals

STG phosphate reference standard (99.5%) and 3-(trifluoromethyl)-5,6,7,8-tetrahydro-[1,2,4]triazolo[4,3-a]pyrazine-HCl (99,3%) (impurity 1) were supplied by Sequoia Research Products (Oxford, UK). O-Benzylhydroxylamine hydrochloride (99%) (impurity 2) was supplied by Sigma-Aldrich (Brazil). Januvia® tablets, STG 50 mg (Merck Sharp & Dohme) were purchased from the local market. Inactive ingredients are microcrystalline cellulose, magnesium stearate, lactose and sodium starch glycolate. All chemical reagents were pharmaceutical or analytical grade. Purified water was obtained by Millipore® Direct-Q 3UV (Molsheim, France). Mobile phase was filtered through 0.45 µm cellulose acetate membranes (Sartorius Stedim Biotech, Germany) and the solutions for injection were filtered through 0.45 µm nylon membranes (Vertical Chromatography, Jatujak, Bangkok, Thailand) before use.

3.2. Instrumentation and analytical conditions

An Agilent liquid chromatograph (Santa Clara, CA, USA) model LC- 1220 series, equipped with a Q 1311A quaternary pump, ALS-G1329A auto sampler, TCC-G1316A column oven, G1315B photodiode array detector, G1322 A degasser and ChemStation manager system software was used in the analytical scale studies. Separations were obtained in an XBridge™ Phenyl column (250 mm X 4.6 mm d.i., 5 µm). The mobile phase consisted of isocratic elution based on acetonitrile: formic acid (0.05% aqueous solution, pH 3.0) mixture (40:60, v/v). The flow rate was 1.0 mL min^{-1}, time run 8 minutes and in the presence of the main degradation product the time run was 12 minutes, injection volume 20 µL, detection wavelength 207 nm.

3.3. Preparation of reference standards and sample solutions

3.3.1. Reference standard solution

Stock standard solutions of STG, impurity 1 and impurity 2 were prepared by accurately weighing 10 mg, transferred to a 20 mL volumetric flask and diluted to volume with mobile phase, in order to obtain 500 µg mL^{-1} of STG, impurity 1 and impurity 2. Stock solutions were stored at 2-8 °C protected from light.

3.3.2. Sample solution

Tablets containing 50 mg of STG were accurately weighed and crushed to thin powder. Appropriate amounts were transferred to a 20 mL volumetric flask. Then, 15 mL of methanol were added, followed by 30 minutes in UltraSonic Cleaner USC-2850, methanol were added to final volume. The solution was filtered through a 0.45 µm membrane filter. For analysis, an appropriate aliquot from STG stock solution (100 µg mL^{-1}) was diluted to appropriate concentration in mobile phase.

3.4. Method validation

The chromatographic method was validated considering the following parameters: specificity, linearity, limits of detection (LOD) and quantification (LOQ), precision, accuracy and robustness. Parameters were analyzed in this study according to international guidelines (ICH Q2R1 2005; ICH Q3A 2006).

3.4.1. Specificity

This parameter was evaluated by analysing the interference of formulation inactive ingredients with the STG, impurities 1 and 2 by comparing the chromatograms obtained from STG and impurities 1 and 2 reference standards and simulated sample of excipients.

Sample solutions of STG (1.0 mg mL^{-1}) dissolved in methanol were placed in closed 1cm^3 quartz cells and placed in a UV chamber (100 x 18 x 17cm) with internal mirrors and CSR F30W T8 UV fluorescent lamp emitting radiation at 254 nm for 4 h. Before the final study, samples were diluted to 100.0 µg mL^{-1} with mobile phase and analyzed by HPLC.

3.4.2. Limits of detection (LOD) and quantification (LOQ)

LOD and LOQ for impurities were determined at a signal-to-noise ratio of 3:1 and 10:1, respectively, by injecting a series of diluted solutions with known concentrations. The precision study was also carried out at the LOQ level by injecting six (n = 6) individual preparations and calculating the area RSD.

3.4.3. Linearity

Standard curves were prepared at seven concentration levels of STG (5, 15, 25, 35, 45, 55, 65 µg mL^{-1}), impurity 1 (3, 5, 10, 15, 20, 25, 30 µg mL^{-1}) and impurity 2 (2.5, 5, 10, 15, 20, 25, 30 µg mL^{-1}). Linearity was evaluated by linear regression analysis and ANOVA (analysis of variance) ($\alpha = 0.05$) for compliance of the linear model.

3.4.4. Precision

Method precision was verified by repeatability and by intermediate precision. Repeatability was checked by injecting six individual preparations of STG (50.0 µg mL^{-1}) spiked with its two impurities (15.0 µg mL^{-1}) and placebo. The detected concentration for each impurity was calculated. Intermediate precision of the method was also evaluated using different analysts and performing the analysis on three different days.

3.4.5. Accuracy

Assay method accuracy was evaluated in triplicate for 5.0 µg mL^{-1} of STG standard spiked with 5.0, 15.0 and 25.0 µg mL^{-1} of the drug product. Drug product recovery was calculated for each added concentration.
For impurities, solutions with placebo and impurities 1 and 2 at 3.0, 5.0 and 10.0 µg mL^{-1} were prepared. Each solution was prepared in triplicate and the values expressed as the percentage ratio of mean experimental concentration and theoretical concentration.

3.4.6. Robustness

Box-Behnken was the experimental design chosen to evaluate the effect of small chromatographic variations on parameters related to analyte quantification and system suitability. Temperature, flow rate, and percentage of ACN were selected as critical factors for method robustness assessment. The center points of the two level response surface design were the optimized values for each factor, further -1 and +1 levels were as follows: ACN (38 and 42 %); flow rate (0.9 and 1.1 mL/min); temperature (24 and 26 °C). DoE was modeled and results processed using MODDE Pro 11 statistical software (MKS Umetrics, Sweden).

3.4.7. System suitability

System suitability tests are used to check if the chromatographic system is able to perform the intended analysis and if it is capable of providing acceptable, good quality results. It verifies the availability and suitability of the chromatographic system (Wahlich and Carr 1990; USP 34 2011).
The parameters measured were: resolution (R), theoretical plates (N), and retention time. It can be determined experimentally from the result of one or more experiments (Heyden et al. 2011).

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Compliance with ethical standards: This research has no conflict of interest and does not contain any studies with human participants or animals performed by any of the authors.

Conflicts of interest: None declared.

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