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Simultaneous determination of sitagliptin and metformin in ternary mixture with sitagliptin acid degradation product

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ABSTRACT

In this work, the acidic degradation product of sitagliptin phosphate monohydrate (STG) was synthesized, separated and its structure was elucidated. Additionally, two reversed-phase liquid chromatographic (RP-LC) methods have been developed for the determination of STG. The first method comprised the determination of STG in binary mixture with sitagliptin acid degradation product (SDP) in laboratory prepared mixtures, in plasma and in dosage form. This method was based on isocratic elution using a mobile phase consisting of potassium dihydrogen phosphate buffer (pH =4.6) - acetonitrile (30:70, *v*:*v*) with fluorometric detection. The fluorometric detector was operated at 267 nm for excitation and 575 nm for emission. In the second method, the simultaneous determination of STG and metformin (MET) in the presence of SDP has been developed. In this method, the ternary mixture of STG, MET and SDP was separated using a mobile phase consisting of potassium dihydrogen phosphate buffer (pH = 4.6) - acetonitrile (15:85, *v*:*v*) with UV detection at 220 nm. Chromatographic separation in the two methods was achieved on a Symmetry® Waters C18 column (150 mm × 4.6 mm, 5 µm). The optimized methods were validated and proved to be specific, robust and accurate for the quality control of the cited drugs in pharmaceutical preparations.

1. Introduction

Sitagliptin (STG), [(2*R*)-1-(2,4,5-trifluorophenyl)-4-oxo-4-[3-(trifluoromethyl)-5,6-dihydro [1,2,4]triazolo [4,3-*a*]pyrazin-7(8*H*)-yl] butan-2-amine] (Figure 1a), is an hypoglycemic drug that belongs to dipeptidyl-peptidase-4 inhibitor class which stimulates glucose-dependent insulin release [1-3]. Metformin (MET), *N*,*N*-dimethylimidodicarbonimidic diamide (Figure 1b), is a biguanide drug that stimulates glycolysis in peripheral tissues [4]. Recently, the combination of the two drugs has been recommended in the treatment of diabetes mellitus to improve glycemic control [5].

A few methods have been described for the determination of STG in pharmaceutical preparations including spectrophotometry [1,6], HPLC [2,3,7] and fluorometry [8]. Besides, some methods have been reported for determination of MET in pharmaceutical preparations including LC/MS/MS [4] and HPLC [9-11].

In a previous work by the same authors of the present work [7], the determination of STG was carried out alone and was also simultaneously determined with MET in the presence of STG alkaline degradation product (SDP). However, the acid degradation of STG could not be separated. So, the aim of the present work presents was to synthesize, separate and study the acidic degradation product of STG and to develop LC methods for the determination of STG either in binary mixture with its acidic degradation product (SDP) or in ternary mixture with MET and SDP. In the first method (LC-fluoro), STG and SDP was determined applying fluorometric detection based on the native fluorescence of the drug and the acidic degradation product. Fluorometric detection was chosen for its better selectivity than UV detection. In the second method (LC-UV), UV detection was applied for the determination of the ternary mixture due to the lack of native fluorescence for MET. SDP was prepared, separated and its structure was elucidated by different spectroscopic techniques and proved to be; 3-amino-4-(2,4,5-trifluorophenyl) butanoic acid, (Figure 1c).

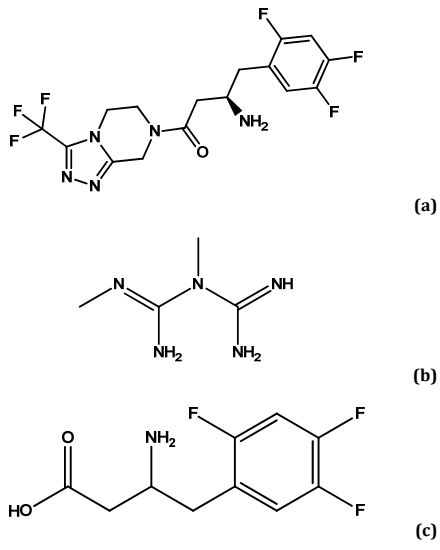

Figure 1. Chemical structures of sitagliptin (a), metformin (b) and sitagliptin acidic degradation product (c).

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2. Experimental

2.1. Instrumentation

The HPLC system consisted of a Shimadzu LC-20 AT Liquid Chromatograph (Japan) using a Symmetry® Waters C18 column (150 mm x 4.6 mm, 5 µm) (Ireland). The system was equipped with a flourometric detector (RF-551, Japan), UV-visible detector (SPD-20A, Japan) and an auto sampler (SIL-20A, Shimadzu, Japan). An Elma S100 ultrasonic processor model KBK 4200 (Germany) was used.

2.2. Reagents and reference samples

Pharmaceutical grade STG, certified to contain 99.80%, Januvia® tablets nominally containing 128.5 mg of STG per tablet (Batch no. S0273) and Janumet® tablets nominally containing 64.25 mg of STG and 1000 mg of MET per tablet (Batch no. 0426570) were all supplied from Merck Sharp and Dohme Co. (Cairo, Egypt). Inactive ingredients of Januvia® tablets include microcrystalline cellulose (E460), calcium hydrogen phosphate, anhydrous (E341), croscarmellose sodium (E468), magnesium stearate (E470b) and sodium stearyl fumarate, polyvinyl alcohol, macrogol 3350, talc (E553b), titanium dioxide (E171), red iron oxide (E172) and yellow iron oxide (E172). Whereas those of Janumet® tablets include microcrystalline cellulose (E460), povidone K29/32 (E1201), sodium lauril sulfate, sodium stearyl fumarate, polyvinyl alcohol, macrogol 3350, talc (E553b), titanium dioxide (E171), iron oxide red (E172) and iron oxide black (E172). Pharmaceutical grade MET, certified to contain 99.79% was kindly supplied by Chemical Industries Development (Cid) Co. (Giza, Egypt). HPLC grade acetonitrile and methanol were purchased from Fisher Scientific (Loughborough, Leicestershire, UK). Potassium dihydrogen phosphate and orthophosphric acid (85%) were purchased from VWR Chemicals (Pool, England). Bi-distilled water was produced in-house (Aquatron Water Still, A4000D, UK). Membrane filters 0.45 µm from Teknokroma (Barcelona, Spain) were used. All other chemicals and reagents used were of analytical grade unless indicated otherwise. Standard stock solutions of each drug (1 mg/mL) were prepared by dissolving 100 mg of the drug in methanol and completing the volume to 100 mL in a volumetric flask and then the required concentrations were prepared by serial dilutions.

2.3. Plasma sample preparation

The spiked plasma samples were extracted after precipitation of proteins using 100 µL of perchloric acid (35%, *w*:*w*). Then, the mixture was vortex-mixed and centrifuged (2 and 3 min, respectively). The supernatant was separated and transferred to another tube and a 25 µL volume was injected into the chromatograph.

2.4. Preparation of the acidic degradation product

An amount of 1 g of STG bulk powder was dissolved in 250 mL of 6 N aqueous hydrochloric acid then the solution was heated for 8 h on a boiling water bath, then cooled and neutralized by 6 N aqueous sodium hydroxide. The formed precipitate was filtered, washed several times and dried. Complete degradation was confirmed using TLC plates and its structure was then elucidated by different spectroscopic techniques.

2.5. Chromatographic conditions

2.5.1. LC-fluoro method

Chromatographic separation was achieved on a Symmetry® Waters C18 column (150 mm × 4.6 mm, 5 µm) applying an isocratic elution based on potassium dihydrogen phosphate buffer (pH = 4.6) - acetonitrile (30:70, *v*:*v*) as a mobile phase. The fluorometric detector was operated at 267 nm for excitation and 575 nm for emission. The buffer solution was filtered through 0.45 µm membrane filter and degassed for 30 min in an ultrasonic bath prior to its use. The mobile phase was pumped through the column at a flow rate of 1 mL/min. Analyses were performed at ambient temperature and the injection volume was 25 µL.

2.5.2. LC-UV method

Chromatographic separation was achieved on a Symmetry® Waters C18 column (150 mm × 4.6 mm, 5 µm) applying an isocratic elution based on potassium dihydrogen phosphate buffer (pH = 4.6) - acetonitrile (15:85, *v*:*v*) as a mobile phase. The UV detector was operated at 220 nm. The buffer solution was filtered through 0.45µm membrane filter and degassed for 30 min in an ultrasonic bath prior to its use. The mobile phase was pumped through the column at a flow rate of 1 mL/min. Analyses were performed at ambient temperature and the injection volume was 25 µL.

2.6. Sample preparation

Twenty tablets of Januvia® and Janumet® were separately weighed. An accurately weighed amount of the finely powdered Januvia® tablets equivalent to 100 mg of STG and a quantity of the powdered Janumet® tablets equivalent to (6.425 mg) STG and (100 mg) MET were separately made up to 100 mL with methanol and sonicated to dissolve. The solutions were filtered followed by serial dilutions to the required concentrations for each experiment.

2.7. Procedure

2.7.1. Linearity and repeatability

2.7.1.1. LC-fluoro method

Accurately measured aliquots of STG stock solution equivalent to 50-2000 µg were transferred into a series of 10 mL volumetric flasks and then completed to volume with methanol. A volume of 25 µL of each solution was injected into the chromatograph. The conditions including the mobile phase at a flow rate 1 mL/min, fluorometric detection (λ_{ex}: 267 nm, λ_{em}: 575 nm) and run time program for 10 min were adjusted. A calibration curve was obtained by plotting area under the peak (AUP) against concentration (C). The repeatability of the method was assessed by analyzing a mixture containing 30 and 100 µg/mL of SDP and STG, respectively (n = 6). The precision (%R.S.D.) values of peak areas and retention times were calculated, Table 1.

2.7.1.1.1. LC-fluoro method in plasma

Accurately measured aliquots of plasma samples equivalent to 0.25-8.00 µg STG were prepared after its extraction as mentioned under Section 2.3 with the same conditions under Section 2.7.1.1. A calibration curve was obtained by plotting area under the peak (AUP) against concentration (C). The repeatability of the method was assessed by analyzing a mixture containing 1.5 and 5.0 µg/mL of SDP and STG, respectively (n = 6). The precision (%R.S.D.) was calculated, Table 1.

Table 1. System suitability tests for LC-fluoro method for the determination of sitagliptin in binary mixture with its acidic degradation product.

Item *	In laboratory mix		In plasma	
	STG	SDP	STG	SDP
N	860	1024	784	2578
R	6.29	6.29	7.6	7.6
T	1.14	1.19	1.02	1.05
%R.S.D. of 6 injections				
Peak area	0.38	0.43	0.48	0.36
Retention time	0.27	0.44	0.57	0.22

* N (number of theoretical plates), R (peak resolution factor), T (tailing of chromatographic peak), and repeatability as %R.S.D. of peak area for six injections and reproducibility of retention as %R.S.D. of retention time.

Table 2. System suitability tests for LC-UV method for the simultaneous determination of sitagliptin and metformin in the presence of sitagliptin acidic degradation product.

Item *	STG degradation product	STG	MET
N	1022	3136	1733
R	6.5	2.1	2.1
T	1.09	1.01	1.05
%R.S.D. of 6 injections			
Peak area	0.46	0.59	0.47
Retention time	0.66	0.22	0.28

* N (number of theoretical plates), R (peak resolution factor), T (tailing of chromatographic peak), and repeatability as %R.S.D. of peak area for six injections and reproducibility of retention as %R.S.D. of retention time.

2.7.1.2. LC-UV method

Accurately measured aliquots of stock solutions equivalent to 50-1600 µg STG and 250-8000 µg MET were separately transferred into two series of 10 mL volumetric flasks and then completed to volume with methanol. A volume of 25 µL of each solution was injected into the chromatograph. The conditions including the mobile phase at a flow rate 1 mL/min, detection at 220 nm and run time program for 10 min were adjusted. A calibration curve for each compound was obtained by plotting area under the peak (AUP) against concentration (C).

The repeatability of the method was assessed by analyzing a mixture containing 25.7, 400.0 and 7.7 µg/mL of STG, MET and SDP, respectively (n = 6). This mixture was considered as the working standard solution. The precision (%R.S.D.) values of peak areas and retention times were calculated for each compound, Table 2.

2.7.2. Assay of laboratory prepared mixtures, plasma and Januvia® tablets

The procedure mentioned under Section 2.7.1.1 was repeated using concentrations equivalent to 10-185 µg/mL STG and 3-55 µg/mL SDP (10% to 30% of STG, *w*:*w*) and equivalent to 1.0-7.5 µg/mL STG and 0.3-2.0 µg/mL SDP (10% to 30% of STG, *w*:*w*). For the determination of STG in Januvia® tablets, the sample solution prepared under Section 2.6 was serially diluted and then injected in triplicates. The concentrations of STG were calculated using calibration equation.

2.7.3. Assay of laboratory prepared mixtures and Janumet® tablets

The procedure mentioned under Section 2.7.1.2 was repeated for the determination of laboratory prepared mixtures equivalent to 10-45 µg/mL STG, 155.6-700.4 µg/mL MET and 3.0-13.5 µg/mL SDP (10% to 30% of STG, *w*:*w*). For the determination of the examined drugs in Janumet® tablets, the sample solution prepared under Section 2.6 was serially diluted to prepare solutions equivalent to 10-45, 155.6-700.4 µg/mL of STG and MET, respectively; and then injected in triplicates. The concentrations of the examined drugs were calculated using calibration equations.

3. Results and discussion

SDP was prepared after acidic hydrolysis of the amide bond of the intact drug. Complete degradation was confirmed using TLC plates. Structure elucidation of the obtained secondary amine was confirmed using infra-red, mass and ^{1}H NMR spectroscopic techniques and proved to be: 3-amino-4-(2,4,5-trifluorophenyl) butanoic acid. It is worth noting that the other hydrolysis product which is: 3-(trifluoromethyl)-5,6,7,8-tetra hydro-[1,2,4]triazolo[4,3-a]pyrazine could not be separated from the reaction medium with the acid but it was studied in a previous work by the same authors of this work [7]. The IR spectrum of SDP showed the shift of the characteristic peak of the carbonyl group of the amide bond at 1639 to 1697 cm^{-1} which is the characteristic peak of the carbonyl group of the carboxylic acid in addition to the appearance of the characteristic broad peak of the OH of the carboxylic group. The characteristic forked band of the amino group appears at about 3100 cm^{-1} due to the effect of hydrogen bonding with the neighboring fluoride atom, which is a strong hydrogen bond acceptor. Besides, mass spectroscopy confirmed the complete hydrolysis of STG showing the molecular weight of the obtained SDP at 233 *m/z*. Finally, ^{1}H NMR showed the appearance of the H at 12.4 ppm which confirmed the acid product and also showed the absence of the heterocyclic hydrogens which ensure the degradation.

3.1. Methods development

3.1.1. LC-fluoro method

Various reversed-phase columns, isocratic mobile phase systems and different pH values of the buffer were attempted. Isocratic elution based on potassium dihydrogen phosphate buffer (pH = 4.6) - acetonitrile (30:70, *v*:*v*) was applied as STG and SDP were eluted in a reasonable time and good peak shape with this mobile phase at a flow rate 1 mL/min. The fluorometric detector was operated at 267 nm for excitation and 575 nm for emission where high detector sensitivity was achieved at these wavelengths. The retention times were 3.9 and 6.1 min for SDP and STG, respectively as shown in Figure 2 and the retention times were 3.8 and 6.3 min for SDP and STG, respectively in plasma as shown in Figures 3 and 4.

3.1.2. LC-UV method

During the optimization cycle, several chromatographic conditions were attempted using Symmetry® Waters C18 column (150 mm x 4.6 mm, 5 µm). The C18 column was chosen for its availability in most quality control laboratories. Various mobile phase compositions containing different ratios of organic and aqueous phases were tried in an isocratic mode.

Table 3. Results obtained by LC-flouro method for the determination of sitagliptin in binary mixture with its acidic degradation product.

Item *	STG	STG in plasma
Retention time	6.1	6.3
Excitation wavelength of detection	267 nm	267 nm
Emission wavelength of detection	575 nm	575 nm
Range of linearity	5-200 µg/mL	0.25-8 µg/mL
Regression equation	Area×10^{-3} = 0.6710 C (µg/mL)- 0.2104	Area × 10^{-4} = 2.7479 C (µg/mL) + 0.0155
Regression coefficient (r^2)	1.0	0.9998
LOD	1.04 µg/mL	0.05 µg/mL
LOQ	3.48 µg/mL	0.16 µg/mL
S_b	1.39 × 10^{-3}	6.3×10^{-2}
S_a	0.19	0.29
Confidence limit of the slope	0.6710±0.13	2.7479±0.80
Confidence limit of the intercept	-0.2104±0.29×10^{-3}	0.0155±0.10×10^{-2}
Standard error of the estimation	0.24	0.4165
Precision		
Intra-day %R.S.D.	0.12-0.58	0.24-0.61
Inter-day %R.S.D.	0.83-1.53	0.72-1.21
Drug in dosage form	100.39±1.43	
Accuracy		
Drug in laboratory prepared mixture	99.65±1.0	100.81±1.65
Drug added	99.62±1.56	

* %R.S.D.: Percent relative standard deviation, LOD: Limit of detection, LOQ: Limit of quantification.

It was found that 85% of organic modifier was needed to elute all peaks within 10 min. Acetonitrile was found optimum for the elution. Besides, different buffers at different pH values were attempted along with acetonitrile. Therefore, a mobile phase consisting of potassium dihydrogen phosphate buffer (pH = 4.6) - acetonitrile (15:85, *v*:*v*) and pumped at a flow rate of 1.0 mL/min, in an isocratic mode, gave good separation of the three compounds. Detection was carried out at 220 nm to obtain sufficient peak intensity for both drugs and SDG. The retention times were 3.0, 5.6 and 6.5 min for SDP, STG and MET, respectively as in Figures 5 and 6.

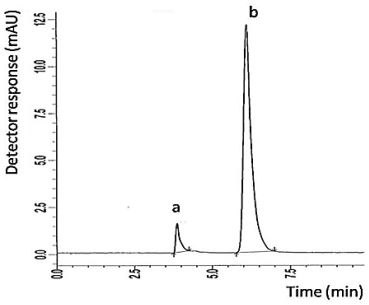

Figure 2. A typical LC chromatogram with fluorometric detection of 25 µL injector of synthetic binary mixture of sitagliptin acidic degradation product (30 µg/mL) (a) and sitagliptin (100 µg/mL) (b).

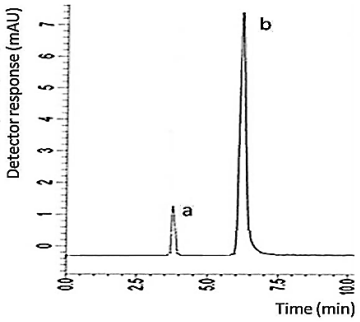

Figure 3. A typical LC chromatogram with fluorometric detection of 25 µL injector of synthetic binary mixture of sitagliptin acidic degradation product (1.5 µg/mL) (a) and sitagliptin (5 µg/mL) (b) in plasma.

3.1.3. System suitability tests

System suitability tests are used to verify that the resolution and reproducibility were adequate for the analysis performed. The parameters of these tests are column efficiency (number of theoretical plates), tailing of chromatographic peak, peak resolution factor, and repeatability as %R.S.D. of peak area for six injections and reproducibility of retention as %R.S.D. of retention time. The results of these tests for the two proposed methods are listed in Tables 1 and 2.

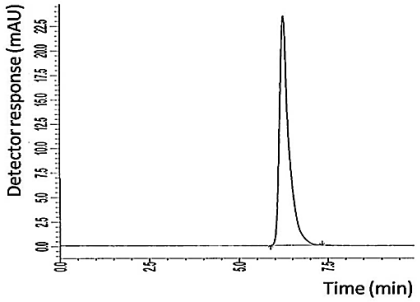

Figure 4. A typical LC chromatogram with fluorometric detection of 25 µL injector of Januvia® sample solution (30 µg/mL).

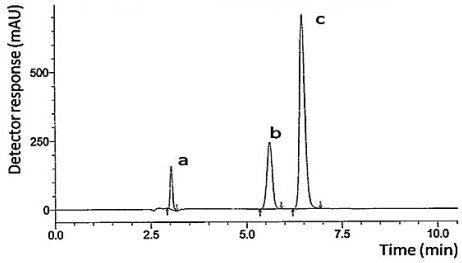

Figure 5. A typical LC chromatogram of 25 µL injector of synthetic ternary mixture of sitagliptin acidic degradation product (7.7 µg/mL) (a), sitagliptin (25.7 µg/mL) (b), and metformin (400 µg/mL) (c).

3.2. Methods validation

3.2.1. Linearity

3.2.1.1. LC-fluoro method

A linear relationship between area under the peak (AUP) and STG concentration (C) was obtained. The regression equation was computed, Table 3. The linearity of the calibration curve was validated by the high value of correlation coefficient.

Table 4. Results obtained by LC-UV method for the simultaneous determination of sitagliptin and metformin in the presence of sitagliptin acidic degradation product.

Item *	Sitagliptin	Metformin
Retention time	5.6	6.5
Wavelength of detection	220 nm	220 nm
Range of linearity	5-160 µg/mL	25-800 µg/mL
Regression equation	Area × 10^{-5} = 0.1285 C (µg/mL) - 0.0098	Area × 10^{-6} = 0.1191 C (µg/mL) + 0.6991
Regression coefficient (r^2)	1.0	0.9999
LOD	1.39 µg/mL	5.78 µg/mL
LOQ	4.63 µg/mL	19.26 µg/mL
S_b	4.48×10^{-4}	5.9×10^{-4}
S_a	0.04	0.27
Confidence limit of the slope	$0.1285\pm5.14\times10^{-3}$	$0.1191\pm3.2\times10^{-2}$
Confidence limit of the intercept	$-0.0098\pm0.04\times10^{-4}$	$0.6991\pm4.12\times10^{-4}$
Standard error of the estimation	0.0035	0.39
Precision		
Intra-day %R.S.D.	0.14-0.47	0.08-0.57
Inter-day %R.S.D.	0.21-1.35	0.24-1.13
Drug in dosage form	100.24±1.63	100.55±1.31
Accuracy		
Drug in laboratory mixture	99.72±1.50	99.70±1.74
Drug added	100.50±1.61	100.13±1.63

* %R.S.D.: Percent relative standard deviation, LOD: Limit of detection, LOQ: Limit of quantification.

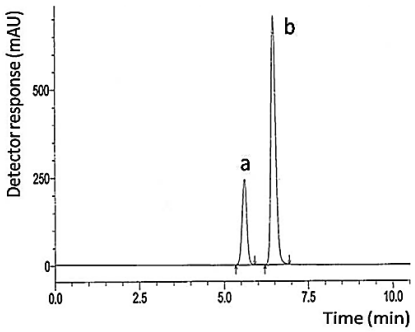

Figure 6. A typical LC chromatogram of 25 µL injector of Janumet® sample solution, sitagliptin (25.7 µg/mL) (a) and metformin (400 µg/mL) (b).

The analytical data of the calibration curve including standard deviations for the slope and intercept (S_b, S_a) are summarized in Table 3.

3.2.1.2. LC-UV method

Linearity was studied for STG and MET. A linear relationship between area under the peak (AUP) and components' concentrations (C) was obtained. The regression equation for each drug was also computed, Table 4. The linearity of the calibration curves were validated by the high value of correlation coefficients. The analytical data of the calibration curves including standard deviations for the slope and intercept (S_b, S_a) are summarized in Table 4.

3.2.2. Accuracy

3.2.2.1. LC-fluoro method

Accuracy of the results was calculated by % recovery of 5 different concentrations of STG in laboratory-prepared mixtures with SDP (10 to 30% of STG, *w*:*w*) and also by standard addition technique applied for Januvia® tablets, all carried out in triplicates. The results obtained including the mean of the recovery and standard deviation are displayed in Table 3.

3.2.2.2. LC-UV method

Accuracy of the results was calculated by %Recovery of five different concentrations of the laboratory prepared mixtures of the two drugs with SDP and also by standard addition technique applied for Janumet® tablets, all carried out in triplicates. The results obtained including the mean of the recovery and standard deviation are displayed in Table 4.

3.2.3. Precision

3.2.3.1. LC-fluoro method

Precision was estimated by repeatability. The repeatability was assessed by analyzing a mixture containing 30 and 100 µg/mL of SPD and STG, respectively (n = 6). The values of repeatability, intra-day and inter-day precision (using 3 different concentrations in triplicates for three consecutive days) for STG are displayed in Tables 1 and 3.

3.2.3.2. LC-UV method

The repeatability was assessed by analyzing a mixture containing 25.7, 400.0 and 7.7 µg/mL of STG, MET and SDP, respectively (n = 6). The values of repeatability for STG and MET peaks, intra-day and inter-day precision (using 3 different concentrations in triplicates for three consecutive days) are displayed, Tables 2 and 4.

3.2.4. Specificity

Specificity is the ability of the analytical method to measure the analyte response in the presence of interferences including degradation products and related substances. The proposed methods were used for the determination of STG alone or the mixture of STG and MET in the presence of SDP; the acid degradation of STG. Besides, the chromatograms of the samples were checked for the appearance of any extra peaks. No chromatographic interference from any of the excipients was found at the retention times of the examined compounds. In addition, the chromatogram of each compound in the sample solution was found identical to the chromatogram received by the standard solution at the wavelengths applied. These results demonstrate the absence of interference from other materials in the pharmaceutical formulations and therefore confirm the specificity of the two proposed methods.

3.2.5. Robustness

3.2.5.1. LC-fluoro method

Robustness was performed by deliberately changing the chromatographic conditions.

Table 5. Statistical comparison between the results of proposed methods and the reference method for the determination of sitagliptin.

Statistical term [a]	Reference method [b]	LC-fluoro	LC-fluoro plasma	LC-UV
Mean	100.5	99.65	100.81	99.72
±S.D.	1.39	1.00	1.65	1.50
±S.E.	0.62	0.45	0.74	0.67
%R.S.D.	1.38	1.00	1.64	1.50
n	5	5	5	5
V	1.93	1.00	2.72	2.25
t (2.306) [c]	-	1.10	0.32	0.85

[a] S.D.: Standard deviation, S.E.: Standard error, %R.S.D.: Relative standard deviation, n: Number of samples, V: Variance.
[b] Reference method: Aliquots of standard solutions in distilled water containing 2-10 µg/mL STG were measured at 220 nm using water as a blank [1]. No significant difference between groups by using one way ANOVA with F = 0.84 and p = 0.49.
[c] Figure in parentheses are the theoretical t value at (p = 0.05).

Table 6. Statistical comparison between the proposed method and the reference method for the determination of metformin.

Statistical term [a]	Reference Method [b]	HPLC method
Mean	100.4	99.70
±S.D.	0.28	1.74
±S.E.	0.13	0.78
%R.S.D.	0.28	1.75
n	5	5
V	0.08	3.03
t (2.306) [c]	-	0.89

[a] S.D.: Standard deviation, S.E.: Standard error, %R.S.D.: Relative standard deviation, n: Number of samples, V: Variance.
[b] Reference method: Aliquots of standard solutions in distilled water containing 2-12 µg/mL MET were measured at 232 nm using water as a blank [12]. No significant difference between groups by using one way ANOVA with F = 0.79 and p = 0.4.
[c] Figure in parentheses are the theoretical t value at (p = 0.05).

The most important parameter to be studied was the resolution factor between the two peaks of SDP and STG. The flow rate of the mobile phase was changed from 1 mL/min to 0.8 mL/min and 1.2 mL/min, where resolution factors obtained were 6.29, 6.14 and 6.08, respectively. The ratio of acetonitrile was changed from 70% to 72% and 68%, where resolution factors obtained were 6.29, 6.34 and 6.07, respectively. Finally, the value of pH of the phosphate buffer was varied from 4.6 to 4.5 and 4.7, where resolution factors obtained were 6.29, 6.39 and 6.48, respectively. As can be seen from these results, good values of the resolution factor were obtained for all these variations, indicating good robustness of the proposed LC method.

3.2.5.2. LC-UV method

The most important parameter to be studied was the resolution factor between the two peaks of SDP and STG and also between the two peaks of STG and MET. The flow rate of the mobile phase was changed from 1 mL/min to 0.8 mL/min and 1.2 mL/min, where resolution factors obtained were (6.5, 2.1), (6.36, 2.14) and (6.44, 1.95), respectively. The ratio of acetonitrile was changed from 85% to 87% and 83%, where resolution factors obtained were (6.5, 2.1), (5.98, 1.89), (6.66, 1.91), respectively. Finally, the value of pH of the phosphate buffer was varied from 4.6 to 4.5 and 4.7, where resolution factors obtained were (6.5, 2.1), (6.33, 2.12) and (6.18, 2.16), respectively. As can be seen from these results, good values of the resolution factor were obtained for all these variations, indicating good robustness of the proposed LC method.

3.2.6. Limit of detection and limit of quantification

Limit of detection (LOD) which represents the concentration of analyte at S/N ratio of 3 and limit of quantification (LOQ) at which S/N is 10 were determined experimentally for the proposed methods and results are given in Tables 3 and 4.

3.2.7. Statistical analysis

A statistical analysis of the results obtained by the proposed methods and the reference methods was carried out by "SPSS statistical package version 11". The significant difference between groups was tested by one way ANOVA (F-test) at p = 0.05 as shown in Tables 5 and 6. The test ascertained that there was no significant difference among the methods.

4. Conclusion

The two proposed LC methods proved to be simple, accurate and reproducible for the determination of STG either alone or in combination with MET in the presence of STG acidic degradation product in reasonable run times. The two methods were validated showing satisfactory data for all the method validation parameters tested. The developed methods are stability indicating and can be conveniently used by quality control laboratories.

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