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Liquid chromatographic determination of sitagliptin either alone or in ternary mixture with metformin and sitagliptin degradation product

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ABSTRACT

Two reversed-phase liquid chromatographic (RP-LC) methods have been developed for the determination of sitagliptin phosphate monohydrate (STG). The first method comprised the determination of STG alone in bulk and plasma; and in its pharmaceutical preparation. This method was based on isocratic elution of STG using a mobile phase consisting of potassium dihydrogen phosphate buffer pH (7.8)–acetonitrile (70:30, v/v) at a flow rate of 1 mL min^{-1} with flourometric detection. The flourometric 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 sitagliptin alkaline degradation product (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–methanol (30:50:20, v/v/v) at a flow rate of 1 mL min^{-1} 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). Linearity, accuracy and precision were found to be acceptable over the concentration ranges of 0.25–200 μg mL^{-1} for STG with the first method and 5–160 μg mL^{-1}, 25–800 μg mL^{-1} for STG and MET, respectively with the second method. 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] (Fig. 1a) is a novel hypoglycemic drug that belongs to dipeptidyl-peptidase-4 inhibitor class which stimulates glucose-dependent insulin release [1–3]. Metformin (MET), *N*,*N*-dimethylimidodicarbonimidic diamide (Fig. 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]. This combination proved to be effective in controlling the metabolic syndrome and resulted in significant weight loss, reversal of insulin resistance, islet and adipocyte hypertrophy and alleviated hepatic steatosis [6].

Few methods have been described for the determination of STG in pharmaceutical preparations or biological fluids including spectrophotometry [1] and HPLC [2,3]. Besides, some methods have been reported for determination of MET in pharmaceutical preparations and biological fluids including LC/MS/MS [4] and HPLC [7–9].

The present work presents two RP-LC methods for the determination of STG alone and for the simultaneous determination of STG and MET in the presence of STG alkaline degradation product (SDP) (stability indicating assay for STG). In the first method (LC-flouro), STG was determined in plasma and pharmaceutical preparation applying flourometric detection based on the native fluorescence of the drug. Flourometric detection was applied due to its high sensitivity and selectivity [10]. This was the first LC method to apply LC-fluorometric detection for the determination of STG in bulk, plasma and pharmaceutical preparations with the highest sensitivity. The second method (LC-UV) comprised the first LC method (LC-UV) for the simultaneous determination of STG and MET in the presence of SDP, 3-(trifluoromethyl)-5,6,7,8-tetrahydro-[1,2,4]triazolo[4,3-a]pyrazine (Fig. 1c). SDP was prepared by alkaline hydrolysis of STG and its structure was elucidated by different spectroscopic techniques. SDP is also the synthetic intermediate of STG [11] and the active metabolite of the drug [12]. However, UV detection was applied for the determination of the ternary mixture due to the lack of native fluorescence for MET and SDP.

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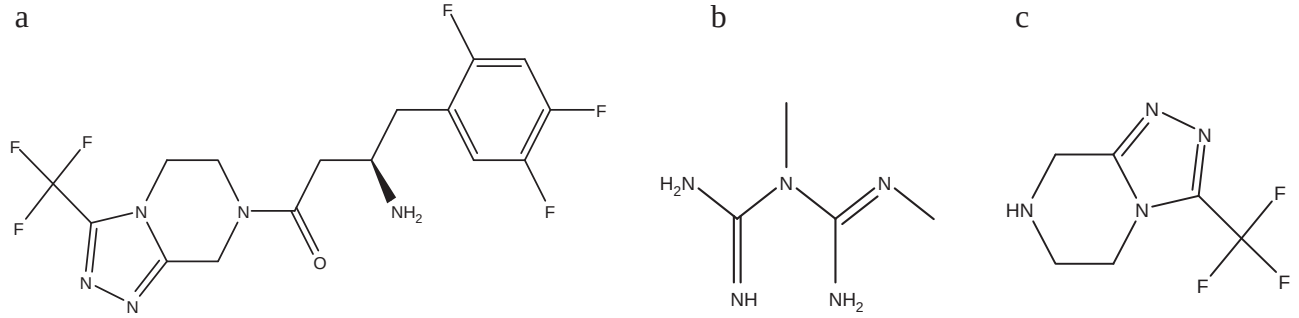

Fig. 1. Chemical structures of sitagliptin (a), metformin (b) and sitagliptin alkaline degradation product (c).

2. Experimental

2.1. Instrumentation

The HPLC system consisted of a Schimadzu LC-20 AT Liquid Chromatograph (Japan) using a Symmetry® Waters C18 column (150 mm × 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 autosampler (SIL-20A, Schimadzu, Japan). An Elma S100 ultrasonic processor model KBK 4200 (Germany) was used.

2.2. Reagents and reference samples

Pharmaceutical grade sitagliptin phosphate monohydrate, certified to contain 99.80%, Januvia® tablets nominally containing 128.5 mg of sitagliptin phosphate monohydrate per tablet (batch no. S0273) and Janumet® tablets nominally containing 64.25 mg of sitagliptin phosphate monohydrate and 1000 mg of metformin per tablet (batch no. 0426570) were all kindly supplied by 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), 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 metformin hydrochloride, 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^{-1}) 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 aqueous phase was separated and transferred to another tube and a 25 μL volume was injected into the chromatograph.

2.4. Preparation of alkaline degradation product

An amount of 1 g of STG bulk powder was dissolved in 250 mL of 5 N aqueous sodium hydroxide then the solution was refluxed for 6 h on a boiling water bath, cooled and neutralized by 5 N aqueous hydrochloric acid. 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-flouro method

Chromatographic separation was achieved on a Symmetry® Waters C18 column (150 mm × 4.6 mm, 5 μm) applying an isocratic elution (Figs. 2–5) based on potassium dihydrogen phosphate buffer pH (7.8)–acetonitrile (70:30, v/v) as a mobile phase. The flourometric detector was operated at 267 nm for excitation and

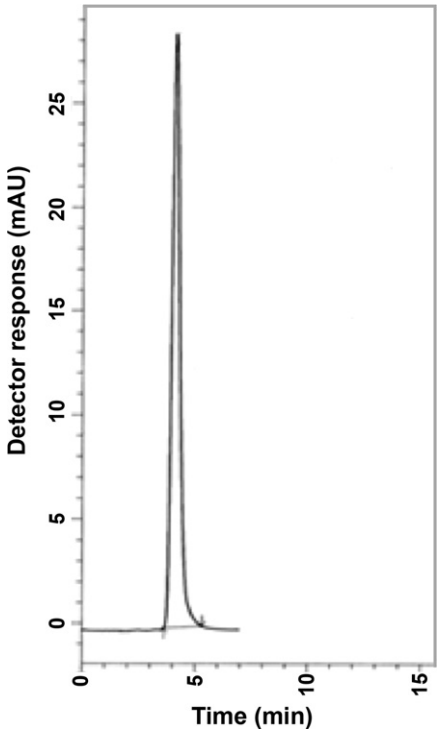

Fig. 2. A typical LC chromatogram of 25 μL injector of Januvia® sample solution (30 μg mL^{-1}) by LC-flouro method.

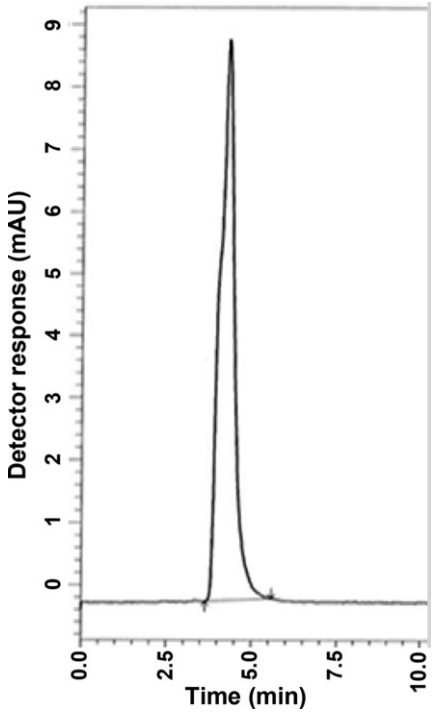

Fig. 3. A typical LC chromatogram of 25 μL injector of sitagliptin in plasma (5 μg mL^{-1}) by LC-flouro method.

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^{-1}. 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–methanol (30:50:20, v/v/v) as a mobile phase. The UV detector was operated at 220 nm. 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^{-1}. Analyses were performed at ambient temperature and the injection volume was 25 μL.

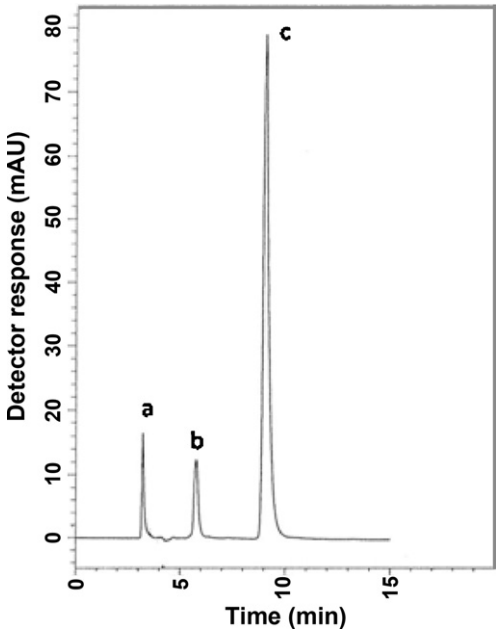

Fig. 4. A typical LC chromatogram of 25 μL injector of synthetic ternary mixture of sitagliptin degradation product (7.7 μg mL^{-1}) (a), sitagliptin (25.7 μg mL^{-1}) (b), and metformin (400 μg mL^{-1}) (c) by LC-UV method.

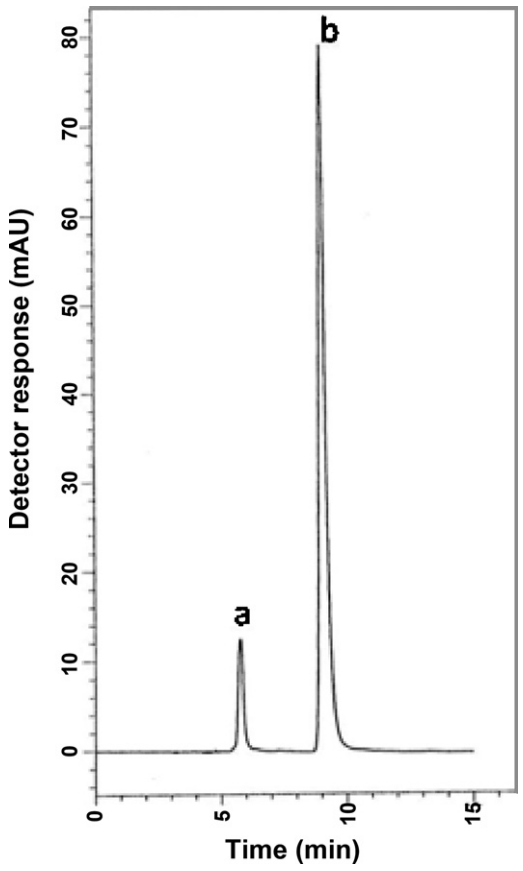

Fig. 5. A typical LC chromatogram of 25 μL injector of Janumet® sample solution, sitagliptin (25.7 μg mL^{-1}) (a) and metformin (400 μg mL^{-1}) (b) by LC-UV method.

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. 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-flouro method. Accurately measured aliquots of STG stock solution equivalent to 2.5–2000 μg were transferred into a series of 10 mL volumetric flasks. The volumes were completed 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^{-1}, flourometric 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

Table 1
System suitability tests for LC-flouro method for the determination of sitagliptin in bulk and in plasma.

Item	In bulk	In plasma
N	1094	609
T	1.05	1.09
%R.S.D. of 6 injections of		
Peak area	0.42	0.62
Retention time	0.27	0.41

assessed by analyzing 6 injections of 100 μg mL^{-1} STG. The precision (%R.S.D.) was calculated, Table 1.

2.7.1.1.1. LC-flouro method in plasma. Accurately measured aliquots of plasma samples equivalent to 0.25–8.0 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 6 injections of 5 μg mL^{-1} STG in plasma. The precision (%R.S.D.) was calculated, Table 1.

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. The volumes were completed 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^{-1}, detection at 220 nm and run time program for 15 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 and 7.7 μg mL^{-1} of STG, MET and SDP, respectively ($n = 6$). This mixture was considered as the working standard solution. The precision (%R.S.D.) for each compound was calculated, Table 2.

2.7.2. Assay of STG in bulk, plasma and Januvia® tablets

The procedure mentioned under Section 2.7.1.1 was repeated using concentrations equivalent to 2–125 μg mL^{-1} STG in bulk and equivalent to 1–7.5 μg mL^{-1} in plasma samples. 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^{-1} STG, 155.6–700.4 μg mL^{-1} MET and 3–13.5 μg mL^{-1} SDP (10–30% 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^{-1} of STG and MET, respectively; and then injected in triplicates. The concentrations of the examined drugs were calculated using calibration equations.

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

Item	SDP	STG	MET
N	4145	5936	5233
R	–	7.22	6.53
T	1.01	1.00	1.03
%R.S.D. of 6 injections of			
Peak area	0.75	0.62	0.59
Retention time	0.94	0.15	0.25

Fig. 6. UV spectrum of the alkaline degradation product of sitagliptin phosphate.

3. Results and discussion

HPLC greatly reduces the analysis time and allows for the determination of many individual components in a mixture using one single procedure [13]. So, the development of RP-LC methods for the determination of STG alone utilizing fluorometric detection and also for the simultaneous determination of STG and MET in the presence of SDP was of interest as no such methods have been reported. SDP was prepared after alkaline 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 UV, infra-red, mass and ^{1}H NMR spectroscopic techniques. UV spectroscopy showed the absence of the characteristic maximum of intact STG at 267 nm (Fig. 6). I.R. spectrum showed the absence of the characteristic peak of the carbonyl group at 1639 cm^{-1} (Fig. 7). Besides, mass spectroscopy confirmed the complete hydrolysis of STG showing the molecular weight of the obtained SDP at 192 (Fig. 8). Finally, ^{1}H NMR showed the absence of aromatic hydrogens at 7–8 ppm (Fig. 9).

3.1. Methods development

3.1.1. LC-flouro 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 (7.8)–acetonitrile (70:30, v/v) was applied as STG was eluted in a reasonable time and good peak shape with this mobile phase at a flow rate 1 mL min^{-1}. The flourometric detector was operated at 267 nm for excitation and 575 nm for emission where high detector sensitivity was achieved at these wavelengths. The retention time of STG was 4.2 min as presented in Fig. 2.

3.1.2. LC-UV method

Various reversed-phase columns, isocratic mobile phase systems and different pH values were attempted to separate the ternary mixture of STG, MET and SDP. Isocratic elution based on potassium dihydrogen phosphate buffer, pH (4.6)–acetonitrile–methanol (30:50:20, v/v/v) was applied due to better resolution and sensitivity obtained with this mobile phase. Minimum retention times were obtained at a flow rate 1 mL min^{-1}. The UV detector was operated at 220 nm where high detector sensitivity was achieved at this wavelength. The retention times were 3.22, 5.78 and 9.04 min for SDP, STG and MET, respectively.

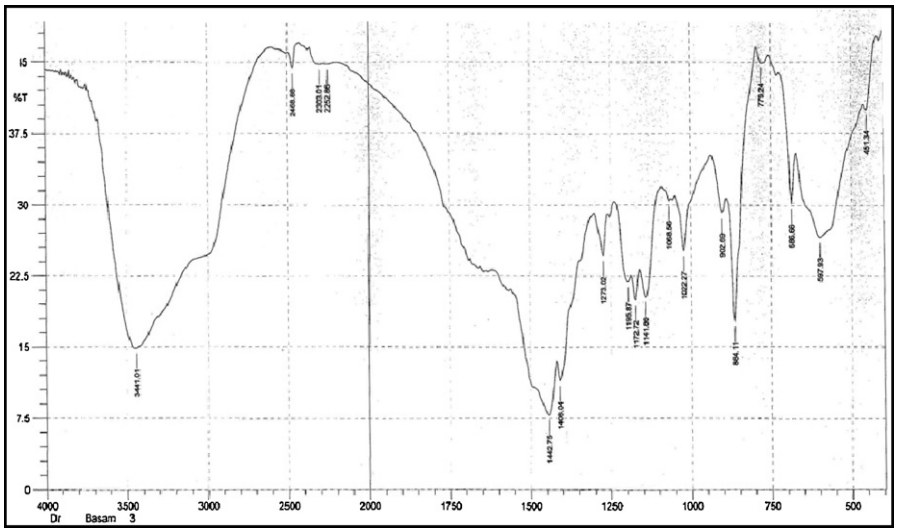

Fig. 7. IR spectrum of the alkaline degradation product of sitagliptin phosphate.

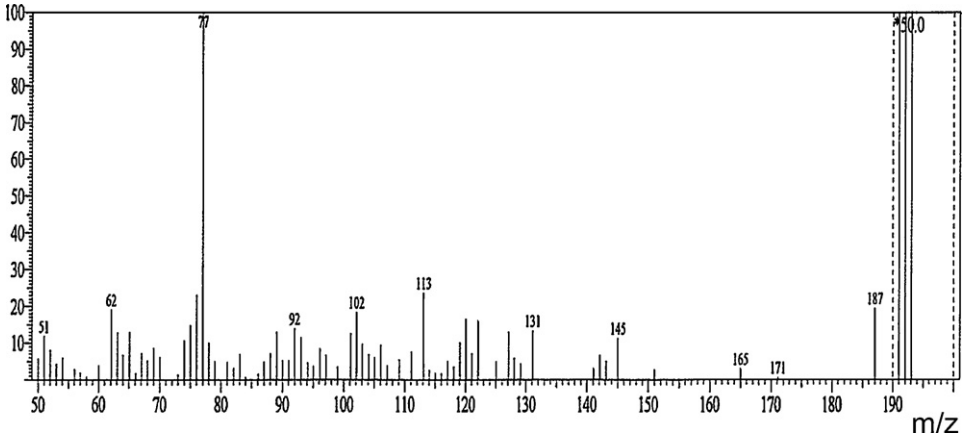

Fig. 8. MS spectrum of the alkaline degradation product of sitagliptin phosphate.

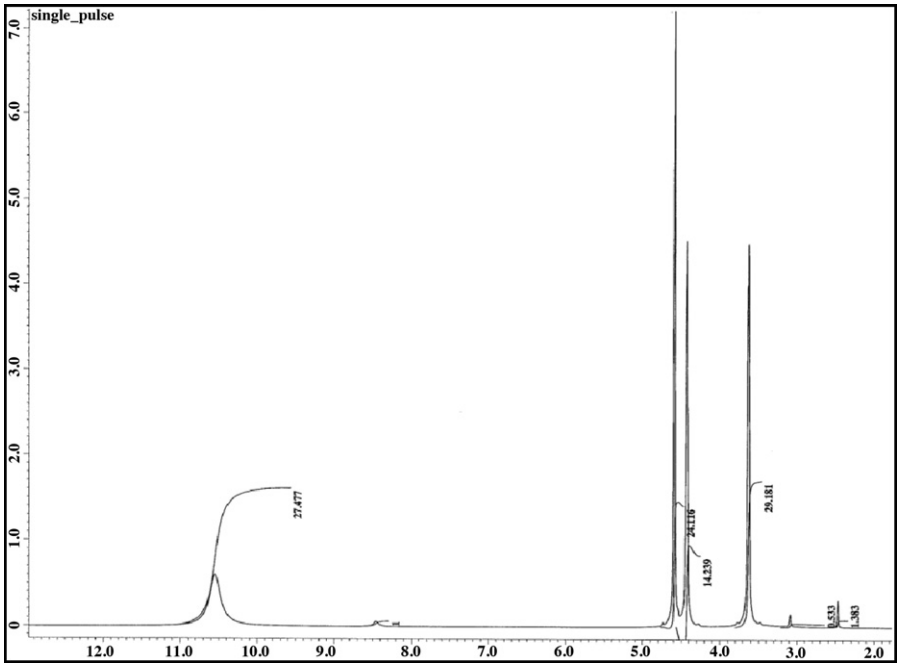

Fig. 9. NMR spectrum of the alkaline degradation product of sitagliptin phosphate.

Table 3
Results obtained by LC-flouro method for the determination of sitagliptin in bulk and in plasma.

Item	In bulk	In plasma
Retention time	4.12	4.29
Excitation wavelength of detection	267 nm	267 nm
Emission wavelength of detection	575 nm	575 nm
Linearity		
Range of linearity	0.25–200 μg mL^{-1}	0.25–8 μg mL^{-1}
Regression equation	Area $\times 10^{-5} = 0.3912\, C_{\mu g\,mL}{}^{-1} + 0.0397$	Area $\times 10^{-4} = 3.3173\, C_{\mu g\,mL}{}^{-1} - 0.5711$
Regression coefficient (r^2)	0.9999	0.9996
LOD ng ml^{-1}	24.5	80
LOQ ng ml^{-1}	75	240
S_b	2.8×10^{-3}	4.56×10^{-3}
S_a	0.31×10^{-2}	1.5×10^{-2}
Confidence limit of the slope	$0.3912 \pm 0.12 \times 10^{-2}$	$3.3173 \pm 4.98 \times 10^{-2}$
Confidence limit of the intercept	$0.0397 \pm 0.11 \times 10^{-3}$	$-0.5711 \pm 2.60 \times 10^{-3}$
Standard error of the estimation	0.489	0.222
Precision		
Intraday		
%R.S.D.	0.08–0.68	0.12–0.79
Interday		
%R.S.D.	0.93–1.62	0.68–1.78
Drug in dosage form	100.98 ± 1.00	
Accuracy		
Drug in bulk	99.95 ± 1.61	99.42 ± 1.65
Drug added	99.46 ± 1.02	–

3.1.3. System suitability tests

According to USP 2007 [15], system suitability tests are an integral part of liquid chromatographic methods in the course of optimizing the conditions of the proposed method. System suitability tests were 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.

3.2. Methods validation

3.2.1. Linearity

3.2.1.1. LC-flouro 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. The analytical data of the calibration curve including standard deviations for the slope and intercept (S_b, S_a) are summarized in Table 3. The LC-fluoro method was found appropriate for the determination of STG in human plasma with the range starting from 0.25 to 8 μg mL^{-1} that covers the range used in the pharmacokinetic study of the drug [16].

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 was 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-flouro method. Accuracy of the results was calculated by % recovery of 5 different concentrations of STG and also by standard

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

Item	Sitagliptin	Metformin
Retention time	5.78	9.04
Wavelength of detection	220 nm	220 nm
Linearity		
Range of linearity	5–160 μg mL^{-1}	25–800 μg mL^{-1}
Regression equation	Area $\times 10^{-5} = 0.2430\, C_{\mu g\,mL}{}^{-1} - 0.0760$	Area $\times 10^{-6} = 0.2064\, C_{\mu g\,mL}{}^{-1} + 0.4626$
Regression coefficient (r^2)	1.0	1.0
LOD μg ml^{-1}	1.32	3.66
LOQ μg ml^{-1}	4.39	12.2
S_b	8.1×10^{-4}	3.8×10^{-4}
S_a	3.46×10^{-3}	1.4×10^{-2}
Confidence limit of the slope	$0.2430 \pm 0.84 \times 10^{-3}$	$0.2064 \pm 2.9 \times 10^{-3}$
Confidence limit of the intercept	$-0.0760 \pm 0.62 \times 10^{-4}$	$0.4626 \pm 1.76 \times 10^{-4}$
Standard error of the estimation	0.107	0.252
Precision		
Intraday		
%R.S.D.	0.12–0.45	0.09–0.78
Interday		
%R.S.D.	0.23–1.25	0.34–1.53
Drug in dosage form	100.44 ± 0.81	99.98 ± 0.81
Accuracy		
Drug in laboratory mixture	99.64 ± 1.47	99.62 ± 1.35
Drug added	100.22 ± 1.54	99.78 ± 1.04

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

Statistical term	Reference method**	LC-flouro in bulk	LC-flouro in plasma	LC-UV in bulk
Mean	100.5	99.95	99.42	99.64
S.D.±	1.39	1.61	1.65	1.47
S.E.±	0.62	0.72	0.74	0.66
%R.S.D.	1.38	1.61	1.66	1.48
n	5	5	5	5
V	1.93	2.59	2.72	2.16
t (2.306*)		0.58	1.12	0.95
F (6.39*)		0.75	0.71	0.89
F-value	0.07			
p-value	0.976 > 0.05***			

* Figures in parentheses are the theoretical *t* and *F* values at ($p = 0.05$).
** 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 at $p < 0.05$.

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 5 different concentrations of the laboratory prepared mixtures of the two drugs analyzed by the proposed methods 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-flouro method. Precision was estimated by repeatability. The repeatability was assessed by analyzing a solution of 100 μg mL^{-1} STG ($n = 6$). The values of the precision (%R.S.D.) of repeatability along with 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, respectively.

3.2.3.2. LC-UV method. The repeatability was assessed by analyzing a mixture containing 25.7, 400 and 7.7 μg mL^{-1} of STG, MET and SDP, respectively ($n = 6$). The values of the precision (%R.S.D.) of repeatability for STG and MET peaks along with intra-day and inter-day precision (using 3 different concentrations in triplicates for three consecutive days) are displayed, Tables 2 and 4, respectively.

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. In the present work, 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 drugs (Figs. 2 and 4). In addition, the chromatogram of each drug 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 of LC-UV method

Robustness was performed by deliberately changing the chromatographic conditions. 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^{-1} to 0.8 mL min^{-1} and 1.2 mL min^{-1}, where resolution factors obtained were (6.53, 7.22), (6.38, 7.01) and (6.40, 7.61), respectively. The ratio of methanol was changed from 20% to 22% and 18%, where resolution factors obtained were (6.53, 7.22), (6.43, 7.35), (6.74, 7.52), respectively. Besides, the ratio of acetonitrile was changed from 50% to 52% and 48%, where resolution factors obtained were (6.53, 7.22), (5.92, 6.89), (6.12, 6.73), 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 (6.53, 7.22), (6.21, 6.78) and (6.11, 6.69), 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.

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

Statistical term	Reference method*	HPLC method
Mean	100.4	99.62
S.D.±	0.28	1.35
S.E.±	0.13	0.6
%R.S.D.	0.28	1.36
n	5	5
V	0.08	1.82
t (2.306)**		1.27
F (6.39**)		0.04
F-value	1.19	
p-value	0.265 > 0.05***	

* 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 [14].
** Figures in parentheses are the theoretical *t* and *F* values at ($p = 0.05$).
*** No significant difference between groups by using one way ANOVA at $p < 0.05$.

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 have the advantages of simplicity, precision, accuracy and convenience for the separation and quantification of STG either alone or in combination with MET. The LC-flouro method is more sensitive and suitable for the quantification of STG in bulk, plasma and tablets. The proposed LC-UV method is capable of the simultaneous determination of STG and MET in the

presence of SDP (stability indicating assay for STG) in laboratory prepared mixtures and tablets. Hence, the proposed LC methods can be used for the quality control of the cited drugs in ordinary laboratories.

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