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Development and validation of a reversed-phase column liquid chromatographic method for simultaneous determination of two novel gliptins in their binary mixtures with Metformin

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ABSTRACT

A new, simple, accurate, and precise liquid chromatographic method has been developed and validated for the determination of two novel dipeptidylpeptidase-4 (DPP-4) inhibitors; namely vildagliptin (VLG) and saxagliptin HCl (SXG) simultaneously in their binary mixtures with metformin HCl (MET). Chromatographic separation was achieved on an Inertsil® CN-3 column (250 mm x 4.6 mm, 5 µm). Isocratic elution using a mobile phase of potassium dihydrogen phosphate buffer pH (4.6) - acetonitrile (15:85, v:v) at a flow rate of 1 mL/min with UV detection at 208 nm was performed. The liquid chromatographic method was used for the simultaneous determination of either VLG, SXG and MET in the range of 5-200, 0.5-20 and 50-2000 µg/mL, respectively. The methods developed were satisfactorily applied to the analysis of the pharmaceutical formulations and proved to be specific and accurate for the quality control of the cited drugs in pharmaceutical dosage forms.

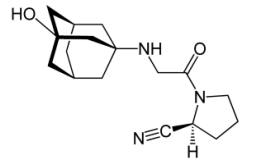
1. Introduction

Vildagliptin (VDG), *S*-1-[*N*-(3-hydroxy-1-adamantyl) glycyl] pyrrolidine-2-carbonitrile (Figure 1), is an oral hypoglycemic drug of the dipeptidylpeptidase-4 (DPP-4) inhibitor class [1]. DPP-4 inhibitors represent a new therapeutic approach to the treatment of type 2 diabetes [2]. Literature survey reveals that only one spectrophotometric method [3] and one chromatographic method was reported for the determination of VDG in the presence of its synthetic intermediate [4].

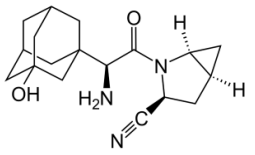
Saxagliptin (SXG), (1*S*,3*S*,5*S*)-2-[(2*S*)-2-amino-2-(3-hydroxy -1-adamantyl)acetyl]- 2-azabicyclo [3.1.0] hexane-3-carbonitrile (Figure 1), is another new dipeptidylpeptidase-4 (DPP-4) inhibitor [5]. SXG was recently approved for the treatment of type 2 diabetes mellitus [6]. Literature survey reveals that only one LC-MS/MS [7] has been reported for SXG. Besides, another LC method with UV detection [8] and a spectrophotometric method [9] have been reported for the determination of the drug.

Metformin hydrochloride (MET), *N*,*N*-dimethylimidodicarbonimidic diamide (Figure 1), is a biguanide hypoglycemic drug that is regarded as the main drug in mixed therapies of oral hypoglycemics. Literature survey reveals that some methods have been reported for determination of MET in mixtures including LC/MS/MS [10] and HPLC [11-14].

Due to the lack of reported LC methods describing determination of the mixtures under investigation, it was deemed useful to develop simple, sensitive and selective LC method that could be useful for the simultaneous determination of VDG and MET or the simultaneous determination of SXG and MET. The proposed method was designed to be suitable for the quality assessment of these mixtures in pharmaceutical preparations.



(a)



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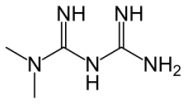

(c)

Figure 1. Chemical structures of vildagliptin (a), saxagliptin (b) and metformin (c).

2. Experimental

2.1. Instrumentation

The HPLC system consisted of a Schimadzu LC-20 AT Liquid Chromatograph (Japan) using an Inertsil® CN-3 column (250 mm x 4.6 mm, 5 µm). The system was equipped with a UV-visible detector (SPD-20A, Japan) and an autosampler (SIL-20A,

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Schimadzu, Japan). An Elma S100 ultrasonic processor model KBK 4200 (Germany) was used for the degassing of the mobile phases.

2.2. Reagents and reference samples

Pharmaceutical grade VDG, certified to contain 99.70% and Eucreas® tablets, nominally containing 50 mg VDG and 500 mg of MET per tablet, were supplied from Novartis Europharm limited company (London, United Kingdom). Pharmaceutical grade SXG, certified to contain 99.85% and Kombiglyze® tablets, nominally containing 5.58 mg of SXG and 500 mg of MET per tablet, were supplied by Bristol-Myers Squibb/ AstraZeneca EEIG (United Kingdom). Pharmaceutical grade MET, certified to contain 99.79% was supplied by Chemical Industries Development (CID) Co. (Giza, Egypt). Methanol (HiPerSolv for HPLC), acetonitrile (HiPerSolv), potassium dihydrogen phosphate and orthophosphoric acid (85%) were obtained 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 in a 100 mL volumetric flask and then completed to volume with methanol. Then required concentrations were prepared by serial dilutions with methanol of these stock solutions.

2.3. Chromatographic conditions

Chromatographic separation was achieved on an Inertsil® CN-3 column (250 mm x 4.6 mm, 5 µm). Isocratic elution using a mobile phase consisting of potassium dihydrogen phosphate buffer pH (4.6) - acetonitrile (15:85, v:v) with UV detection at 208 nm was performed. The buffer solution was filtered through 0.45 µm membrane filter and degassed for 30 min in an ultrasonic bath prior to 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.

2.4. Samples' preparation

Twenty tablets of each mixture were weighed. An accurately weighed amount of the finely powdered Eucreas® tablets equivalent to 100 mg of VDG and 1000 mg of MET was made up to 100 mL with methanol. An accurately weighed amount of the finely powdered Kombiglyze® tablets equivalent to 11.16 mg of SXG and 1000 mg of MET were made up to 100 mL with methanol. The solutions were filtered followed by serial dilution to the required concentrations for each experiment.

2.5. Procedure

2.5.1. Linearity and repeatability

Accurately measured aliquots of working standard solutions equivalent to 50-2000 µg VLG, 5.0-200.0 µg SXG and 0.5-20.0 mg 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 chromatographic conditions mentioned in Section 2.3. including the mobile phase at a flow rate 1 mL/min, detection at 208 nm and run time program for 12.5 min were adjusted. A calibration curve for each drug was obtained by plotting area under the peak (AUP) against concentration (C). The repeatability of the method was assessed by analyzing a mixture containing 100 µg/mL of VDG and 1000 µg/mL of MET, and another mixture containing 11.16 µg/mL of SXG and 1000 µg/mL of MET (n = 6). The % R.S.D. for the peak area and retention time was calculated (Table 1 and 2).

Table 1. System suitability test for the proposed LC method for the determination of vildagliptin in binary mixture with metformin.

Item	VDG	MET
N	2135	2975
R	3.1	3.1
T	1.02	1.10
RSD% of Peak area (6 injections)	0.57	0.69
RSD% of Retention time (6 injections)	0.16	0.28

Table 2. System suitability test for the proposed LC method for the determination of saxagliptin in binary mixture with metformin.

Item	SXG	MET
N	1811	2835
R	3.9	3.9
T	1.00	1.09
RSD% of Peak area (6 injections)	0.71	0.62
RSD% of Retention time (6 injections)	0.28	0.19

2.5.2. Assay of drugs in laboratory prepared mixtures and in pharmaceutical preparations

The procedure mentioned in Section 2.5.1 was repeated using two sets of laboratory prepared mixtures equivalent to 100-1450 µg/mL MET with 10-145 µg/mL VLG and 2-13 µg/mL SXG in different concentration ratios. For the determination of the examined drugs in Eucreas® and Kombiglyze® tablets, the samples' solutions prepared in Section 2.4 were serially diluted to prepare solutions equivalent to 15-75 and 150-750 µg/mL of VLD and MET, respectively; and equivalent to 1.67-8.37 and 150-750 µg/mL of SXD and MET, respectively; and then injected in triplicates. The concentrations of the examined drugs were calculated by the calibration equations (Table 3).

3. Results and discussion

The combinations of VDG or SXG with MET are new to the market. No previous method was reported for the LC determination of VDG or SXG in their binary mixture with MET. Thus, the aim of this work was to develop a simple, accurate and reproducible LC method that could be applied to the simultaneous determination of VDG or SXG with MET in their laboratory prepared mixtures or pharmaceutical preparations of these drugs.

3.1. Method development

During the optimization cycle, several chromatographic conditions were attempted using an Inertsil® CN-3 column (250 mm x 4.6 mm, 5 µm). Recently, cyano columns have been used for the separation and quantitation of drugs [15-16]. Various mobile phase compositions containing different ratios of organic and aqueous phases were tried in an isocratic mode. 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 drugs. Due to the poorly absorbing chromophores in the three drugs examined, detection was carried out at 208 nm to obtain sufficient peak intensity for these drugs. The retention times were 7.2, 6.3 and 8.7 min for VLD, SXG and MET, respectively; as presented in Figure 2 and 3.

Table 3. Results obtained by LC method for the determination of metformin in binary mixture with vildagliptin or saxagliptin.

Item	Metformin	Vildagliptin	Saxagliptin
Retention time (min)	8.7	7.2	6.3
Wavelength of detection	208 nm	208 nm	208 nm
Range of linearity	50-2000 µg/mL	5-200 µg/mL	0.5-20 µg/mL
Regression equation	Area x 10^{-6} = 0.0528 $C_{\mu g/mL}$ + 0.0231	Area x 10^{-5} = 0.1126 $C_{\mu g/mL}$ + 0.4040	Area x 10^{-5} = 0.2415 $C_{\mu g/mL}$ + 0.5112
Regression coefficient (r^2)	0.9999	0.9996	0.9968
LOD µg/mL	19.36	0.53	0.29
LOQ µg/mL	46.54	1.78	0.48
S_b	2.1 x 10^{-4}	1.1 x 10^{-3}	6.7 x 10^{-2}
S_a	2.7 x 10^{-2}	0.15	0.58
Confidence limit of the slope	0.0528 ± 0.14 x 10^{-2}	0.1126 ± 0.02	0.2415 ± 0.14
Confidence limit of the intercept	0.0231 ± 0.05 x 10^{-4}	0.4040 ± 0.44 x 10^{-3}	0.5112 ± 3.43 x 10^{-2}
Standard error of the estimation	0.341	0.186	0.489
Intraday % R.S.D.	0.38-0.87	0.37-0.68	0.33-0.57
Interday % R.S.D.	0.33-1.22	0.41-1.51	0.54-1.18
Drug in lab. prepared mixture	100.12 ± 0.99	99.49 ± 0.90	100.30 ± 1.25
Drug in dosage form	100.42 ± 0.92 (Eucreas®) 99.87 ± 0.81 (Kombiglyze®)	100.11 ± 0.67	99.47 ± 1.15
Drug added	100.83 ± 1.30 (Eucreas®) 100.17 ± 0.86 (Kombiglyze®)	100.53 ± 0.89	100.64 ± 1.40

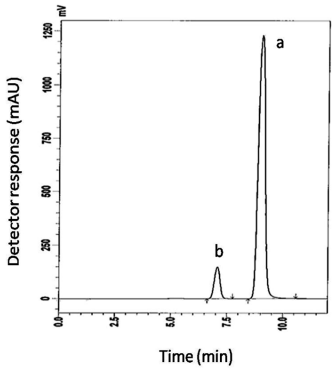

Figure 2. A typical LC chromatogram of 25 µL injector of Eucreas® sample solution containing (a) metformin hydrochloride (400 µg/mL) and (b) vildagliptin (40 µg/mL).

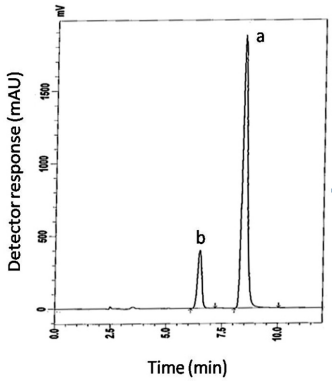

Figure 3. A typical LC chromatogram of 25 µL injector of Kombiglyze® sample solution containing (a) metformin hydrochloride (500 µg/mL) and (b) saxagliptin hydrochloride (5.58 µg/mL).

3.2. System suitability tests

According to the United States Pharmacopoeia, 2007 [17], system suitability tests are an integral part of liquid chromatographic methods in the course of optimizing the conditions of the proposed method. In the proposed LC method, system suitability tests are used to verify that resolution and reproducibility were adequate for analysis performed. Different parameters affecting the chromatographic separation were studied. The parameters of this test are column efficiency (number of theoretical plates), tailing of chromatographic peak, peak resolution factor, and repeatability as %R.S.D. of peak areas for six injections and reproducibility of retention times. The results of these tests are listed in Table 1 and 2.

3.3. Method validation

3.3.1. Linearity

Linearity was studied for VDG, SXG and MET. A linear relationship between area under the peak (AUP) and component concentration (C) was obtained. The regression equations were also computed. 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 3.

3.3.2. Accuracy

Accuracy of the results was calculated by % recovery of five different samples of the laboratory prepared mixtures of VLD and SXG in their binary mixture with MET and also by standard addition technique for Eucreas® and Kombiglyze® tablets. The results obtained including the mean of the recovery and relative standard deviation are displayed in Table 3.

3.3.3. Precision

The repeatability of the method was assessed by analyzing 100 µg/mL of VLG, 11.16 µg/mL of SXG and 1000 µg/mL of MET (n = 6). The values of the precision (%R.S.D.) of repeatability, inter-day and intra-day precision (using three different concentrations in triplicates for three days) are displayed in Table 1-3.

3.3.4. Specificity

Specificity is the ability of the analytical method to measure the analyte response in the presence of interferences. Due to lack of the degradation profile of the studied gliptins, interferences due to the degradation products could not be studied. In the present work, specificity was checked by analyzing VDG with MET and SXG with MET in laboratory prepared mixtures. Good resolution and absence of interference between drugs being analyzed are shown in Figure 2 and 3. Besides, the chromatograms of the pharmaceutical formulation 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 (Figure 2 and 3).

Table 4. Statistical comparison between the results of the proposed LC method and the reference method for the determination of metformin.

Statistical term	Reference method[b]	Proposed method
Mean	100.4	100.12
S.D. ±	0.28	0.99
S.E. ±	0.13	0.44
% R.S.D.	0.28	0.99
n	5	5
V	0.08	0.98
t ([a] 2.306)	-	0.61
F ([a] 6.39)	-	0.08

[a] Figures in parentheses are the theoretical t and F values at (p=0.05).
[b] Reference method for the spectrophotometric determination of metformin in the Indian pharmacopeia [18].

Table 5. Statistical comparison between the results of the LC method and the reference methods for the determination of vildagliptin and saxagliptin.

Statistical term	Reference method for vildagliptin [b]	HPLC method	Reference method for saxagliptin[c]	HPLC method
Mean	100.01	99.49	100.20	100.3
S.D. ±	0.99	0.90	1.10	1.25
S.E. ±	0.44	0.40	0.49	0.56
%RSD	0.99	0.90	1.1	1.25
n	5	5	5	5
V	0.98	0.81	1.21	1.56
t ([a] 2.306)	-	0.87	-	0.13
F ([a] 6.39)	-	1.21	-	0.78

[a] Figures in parentheses are the theoretical t and F values at (p=0.05).
[b] Reference method: aliquots of standard solutions in distilled water containing 5-25 µg/mL VDG were measured at 208 nm using water as a blank [3].
[c] Reference method: aliquots of standard solutions in distilled water containing 5-40 µg/mL SXG were measured at 208 nm using methanol as a blank [8].

In addition, the chromatograms of the drugs in the samples' solutions were found identical to the chromatograms received by the standard solutions at the wavelengths applied. Moreover, good results were obtained for the determination of the cited drugs in the two dosage forms, Table 3. These results confirm the absence of interference from other materials in the pharmaceutical formulations and therefore confirm the specificity of the two proposed methods.

3.3.5. Robustness

Robustness was performed by deliberately changing the chromatographic conditions. The most important parameter to be studied was the resolution factor between the peaks of VLG and MET and also between the peaks of SXG and MET. The flow rate of the mobile phase was changed from 1.0 mL/min to 0.8 mL/min and 1.2 mL/min, where resolution factors obtained were (3.1-3.9), (3.25-4.11) and (3.15-3.87), respectively. The organic strength was changed by ±%2, where resolution factors obtained were (3.1-3.9), (3.22-3.78) and (3.1-4.0) respectively. Finally, a value of pH of the phosphate buffer was varied from 4.6 to 4.5 and 4.7, where resolution factors obtained were (3.1-3.9), (3.05-4.25) and (2.95-3.18), respectively. As it 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.

3.3.6. 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 Table 3.

3.3.7. Statistical analysis

Statistical analysis of the results obtained by the proposed methods and the reference methods for each drug were carried out by "SPSS statistical package version 11". The significant difference between the reference methods and the described methods was tested by one way ANOVA (F-test) at p = 0.05 as shown in Table 4 and 5. The test ascertained that there was no significant difference among the methods.

4. Conclusion

A single LC method can be applied to the simultaneous determination of VDG and SXG with metformin. The proposed LC method has the advantages of simplicity, precision, accuracy and convenience for the separation and quantization of VDG or SXG in combination with MET. The method can be applied for the determination of the cited drugs in tablets without interference from the tablets' inactive ingredients. The method was validated showing satisfactory data for all the method validation parameters tested. Thus, the developed method can be conveniently used by quality control laboratories.

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