European Journal of Chemistry 4 (4) (2013) 444-449

European Journal of Chemistry

Journal homepage: www.eurjchem.com

Development and validation of a reversed phase liquid chromatographic method for the determination of three Gliptins and Metformin in the presence of Metformin impurity (1-cyanoguanidine)

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ARTICLE INFORMATION

Received: 04 June 2013
Received in revised form: 26 June 2013
Accepted: 18 August 2013
Online: 31 December 2013

KEYWORDS

Sitagliptin
Metformin
Saxagliptin
Vildagliptin
Liquid chromatography
Stability indicating assay

ABSTRACT

A simple and precise liquid chromatographic method has been developed and validated for the determination of either sitagliptin (STG), vildagliptin (VLG) or saxagliptin HCL (SXG) and metformin HCL (MET) in the presence of metformin degradation product, 1-cyanoguanidine (CGN). Chromatographic separation was achieved on a Symmetry® cyanide column (150 mm × 4.6 mm, 5 µm). Isocratic elution using a mobile phase of potassium dihydrogen phosphate buffer (pH = 4.6) - acetonitrile (30:70, *v:v*) at a flow rate of 1 mL/min with UV detection at 210 nm was performed. The LC method was used for the simultaneous determination of STG, VLG, SXG and MET in the ranges of 5-200, 5-200, 0.5-80.0 and 20-800 µg/mL, respectively. The results were statistically compared with the reference method for each drug using one-way analysis of variance (ANOVA). The method developed was 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

Sitagliptin (Figure 1a), vildagliptin, (Figure 1b), and saxagliptin (Figure 1c) are oral hypoglycemic drugs of the dipeptidyl peptidase-4 (DPP-4) inhibitors class. DPP-4 inhibitors represent a new therapeutic approach to the treatment of type-2 diabetes that functions to stimulate glucose-dependent insulin release and reduce glucagons levels. This is done through inhibition of the inactivation of incretins, particularly glucagon-like peptide-1 (GLP-1) and gastric inhibitory polypeptide (GIP) [1].

Few methods have been described for the determination of STG in pharmaceutical preparations or biological fluids including spectrophotometry [2] and high performance liquid chromatography (HPLC) [1,3]. A liquid chromatographic (LC) method was reported for the determination of STG in ternary mixture with MET and sitagliptin degradation product [4]. For VDG, literature survey reveals that only one spectroscopic method and one LC method were reported for its determination [5,6]. SXG is recently approved for the treatment of type-2 diabetes mellitus [7]. Literature survey reveals that only one LC-MS/MS [8] and one spectrophotometric method have been reported for its determination [9].

Metformin (Figure 1d), is a biguanide hypoglycemic drug that is regarded as the main compound in mixed therapies of oral hypoglycemics. Literature survey reveals some methods for the determination of MET in mixtures including LC/MS/MS [10] and HPLC [11-14]. 1-Cyanoganidine (CGN) is a potential impurity of metformin hydrochloride, which is reported in USP and BP (Figure 1e) [15,16]. Only one LC method has been reported for the determination of MET in the presence of CGN [16].

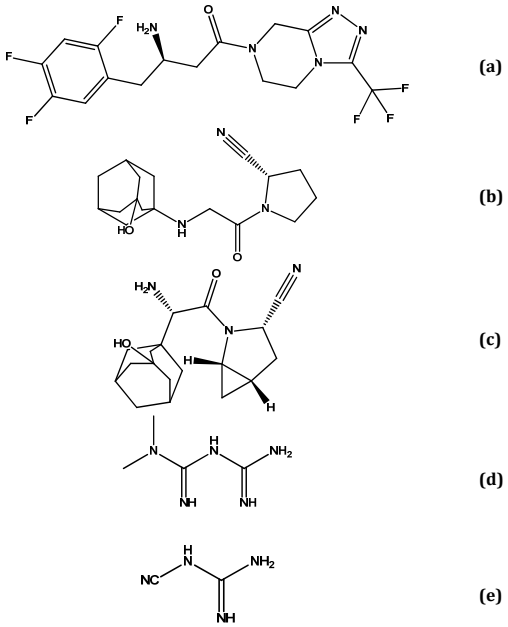

Figure 1. Chemical structures of the drugs; (a) Sitagliptin, [(2*R*)-1-(2,4,5-trifluorophenyl)-4-oxo-4-[3-(trifluoromethyl)-5,6-dihydro [1,2,4] triazolo [4,3-*a*] pyrazin-7(8*H*)-yl] butan-2-amine]; (b) Vildagliptin, *S*-1-[*N*-(3-hydroxy-1-adamantyl) glycyl] pyrrolidine-2-carbonitrile; (c) Saxagliptin, (1S,3S,5S)-2-[(2S)-2-amino-2-(3-hydroxy-1-adamantyl)acetyl]-2-azabicyclo[3.1.0] hexane-3-carbonitrile; (d) Metformin, *N,N*-dimethylimidodicarbon imidic diamide ; (e) Cyanoguanidine.

ISSN 2153-2249 (Print) / ISSN 2153-2257 (Online)
DOI:10.5155/eurjchem.4.4.444-449.844

The aim of the present work was to develop and validate one simple reversed phase liquid chromatographic (RP-LC) method that could be applied for the simultaneous determination of each of the three commonly used Gliptins; STG, VDG and SXG and MET in their ternary mixture with MET degradation product, CGN (stability indicating assay for MET).

2. Experimental

2.1. Instrumentation

The HPLC system consisted of a Shimadzu LC-20 AT Liquid Chromatograph (Japan) using a Symmetry® cyanide column (150 mm × 4.6 mm, 5 µm) (Ireland). The system was equipped with a 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 for the degassing of the mobile phases.

2.2. Reagents and reference samples

Pharmaceutical grade STG monohydrate, certified to contain 99.80%, Janumet® tablets nominally containing 64.25 mg of STG and 1000 mg of MET per tablet were all kindly supplied from Merck Sharp and Dohme Co. (Cairo, Egypt). Pharmaceutical grade VDG, certified to contain 99.70% and Eucreas® tablets nominally containing 50 mg VDG and 500 mg of MET per tablet were kindly supplied from Novartis Europharm Limited Company (London, U.K.). 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 kindly supplied by Bristol-Myers Squibb/Astra Zeneca EEIG (United Kingdom). Pharmaceutical grade MET, certified to contain 99.79% was kindly supplied by Chemical Industries Development (CID) Co. (Giza, Egypt). CGN was brought from Fluka (Steinheim, Germany). Methanol (HiPerSolv for HPLC), acetonitrile (HiPerSolv) were purchased from Fisher Scientific (Loughborough, Leicestershire, UK). Potassium dihydrogen phosphate and orthophosphric 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 a Symmetry® Cyanide column (150 mm × 4.6 mm, 5 µm). Isocratic elution using a mobile phase consisting of potassium dihydrogen phosphate buffer (pH = 4.6) - acetonitrile (30:70, *v*:*v*) with UV detection at 210 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 pharmaceutical preparation were weighed. An accurately weighed amount of the finely powdered Janumet®, Eucreas® and Kombiglyze® tablets equivalent to (64.25 mg of STG and 1000 mg of MET), (equivalent to 100 mg of VDG and 1000 mg of MET) and (equivalent to 11.16 mg of SXG and 1000 mg of MET) respectively were made up to 100 mL with methanol. The solutions were sonicated for 15 min and 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 STG, 50-2000 µg VLG, 5.0-800 µg SXG and 0.2-8.0 mg MET were separately transferred into four 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 210 nm and run time program for 10 min were adjusted. The calibration curves were obtained by plotting area under the peaks (AUP) against concentrations (C). The repeatability of the method was assessed by analyzing a mixture containing 32.1 µg/mL of STG, 150 µg/mL of CGN and 500 µg/mL of MET (n = 6). The precision (% R.S.D.) was calculated (Table 1) and analyzing a mixture containing 50 µg/mL of VDG, 150 µg/mL of CGN and 500 µg/mL of MET (n = 6). The precision (%R.S.D.) was calculated (Table 2). And analyzing a mixture containing 5.6 µg/mL of SXG, 150 µg/mL of CGN and 500 µg/mL of MET (n = 6). The precision (%R.S.D.) was calculated (Table 3).

Table 1. System suitability tests of the proposed LC method for the simultaneous determination of sitagliptin, metformin and metformin degradation product.

Item	MET degradation product	STG	MET
N	4096	1984	1296
R	4.8	4.1	4.1
T	1.01	1.00	1.05
%R.S.D. of 6 injections			
Peak area	0.62	0.23	0.54
Retention time	0.82	0.19	0.33

* N: Number of theoretical plates, R: Peak resolution factor, T: Ttailing of chromatographic peak, and repeatability as percent relative standard deviation %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 of the proposed LC method for the simultaneous determination of vildagliptin, metformin and metformin degradation product.

Item	MET degradation product	VLG	MET
N	4096	2175	1296
R	6.7	2.5	2.5
T	1.00	1.02	1.05
%R.S.D. of 6 injections			
Peak area	0.55	0.72	0.44
Retention time	0.49	0.11	0.25

* 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 3. System suitability tests of the proposed LC method for the simultaneous determination of saxagliptin, metformin and metformin degradation product.

Item	MET degradation product	SXG	MET
N	4096	2704	1296
R	5.3	3.2	3.2
T	1.00	1.04	1.05
%R.S.D. of 6 injections			
Peak area	0.48	0.51	0.88
Retention time	0.76	0.27	0.38

* 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.

2.5.2. Assay of drugs in laboratory prepared mixtures and in pharmaceutical dosage forms

The procedure mentioned in Section 2.5.1 was repeated using three sets of laboratory prepared mixtures equivalent to either 13-48 µg/mL STG, 20-75 µg/mL VLG or 2.0-8.5 µg/mL

SXG and 200-750 µg/mL MET. For the determination of the examined drugs in tablets, the sample solutions prepared under Section 2.4 were serially diluted to prepare solutions equivalent to 6.5-32.0 and 100-500 µg/mL of STG and MET, respectively; and equivalent to 10-50 and 100-500 µg/mL of VLD and MET, respectively; and equivalent to 1.0-5.5 and 100-500 µg/mL of SXG and MET, respectively; and then injected in triplicates. The concentrations of the examined drugs were calculated by the corresponding calibration equations.

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 [17]. No previous method was reported for the LC determination of either STG, VDG or SXG in their ternary mixture with MET and CGN. CGN is a known impurity with a limit of not more than 0.02%, it is also called dicyandiamide, is the dimer of cyanamide. Efficient control of these unwanted compounds in a formulation is necessary [15].

3.1. Method development

Different chromatographic systems including different C_{18} columns and different mobile phases at different pH values were attempted. Unfortunately, simultaneous elution of the ternary mixtures at reasonable time could not be achieved. Recently, cyano columns have been used for the separation and quantitation of drugs [18-21]. Using a mobile phase of acidic pH value, C_{18} column was consequently replaced with a cyano column in order to separate mixture components. For the cyano-bonded phase column, the weak polar cyano groups, together with the short hydrocarbon chains allow a mixed mechanism of separation [22], and hence give reasonable retention times for the three components under investigation in every mixture when operating under optimum conditions. Isocratic elution based on potassium dihydrogen phosphate buffer (pH = 4.6) - acetonitrile (30:70, *v*:*v*) was applied. Minimum retention times were obtained at a flow rate 1 mL/min. The UV detector was operated at 210 nm where good detector sensitivity was achieved for all the examined drugs. The retention times were 6.0, 7.1, 6.5, 9.1 and 4.1 min for STG, VLD, SXG, MET and CGN, respectively; as presented in Figure 2-7.

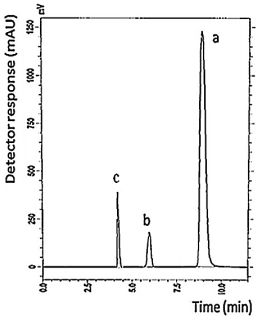

Figure 2. A typical LC chromatogram of 25 µL injector of the synthetic ternary mixture, (a) metformin hydrochloride (500 µg/mL), (b) sitagliptin (32.1 µg/mL) and (c) cyanoguanidine (150 µg/mL).

3.2. System suitability tests

According to United States pharmacopeia (USP) [23], 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 Tables 1-3.

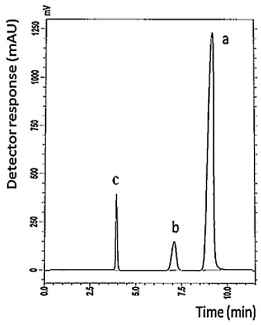

Figure 3. A typical LC chromatogram of 25 µL injector of the synthetic ternary mixture, (a) metformin hydrochloride (500 µg/mL), (b) vildagliptin (50 µg/mL) and (c) cyanoguanidine (150 µg/mL).

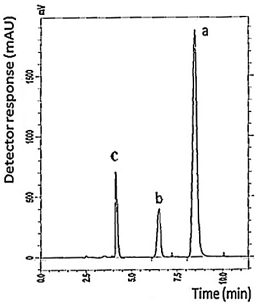

Figure 4. A typical LC chromatogram of 25 µL injector of the synthetic ternary mixture, (a) metformin hydrochloride (500 µg/mL), (b) saxagliptin hydrochloride (5.6 µg/mL) and (c) cyanoguanidine (150 µg/mL).

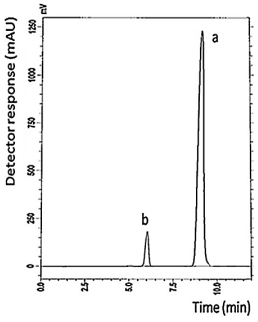

Figure 5. A typical LC chromatogram of 25 µL injector of Janumet® sample solution, (a) metformin hydrochloride (500 µg/mL) and (b) sitagliptin (32.1 µg/mL).

3.3. Method validation

3.3.1. Linearity

Linearity was studied for STG, 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 Tables 4 and 5.

Table 4. Results obtained by LC method for the determination of metformin in binary mixture with sitagliptin or vildagliptin or saxagliptin.

Item *	Metformin
Retention time	9.1
Wavelength of detection, nm	210
Range of linearity, µg/mL	20-800
Regression equation	Area × 10^{-6} = 0.0630 $C_{\mu g/mL}$ + 0.4827
Regression coefficient (r^2)	0.9998
LOD, µg/mL	12.26
LOQ, µg/mL	19.7
S_b	1.6×10^{-3}
S_a	1.4×10^{-1}
Confidence limit of the slope	$0.0630\pm0.09\times10^{-1}$
Confidence limit of the intercept	$0.4827\pm0.77\times10^{-3}$
Standard error of the estimation	0.22
Intraday %R.S.D.	0.29-0.67
Interday %R.S.D.	0.13-1.1
Drug in laboratory mixture	100.29±1.65 with STG
	100.57±0.90 with VLG
	100.23±0.91 with SXG
Drug in dosage form	99.71±1.68 (Janumet®)
	99.95±1.20 (Eucreas®)
	100.14±1.19 (Kombiglyze®)
Drug added	100.26±1.48 (Janumet®)
	99.99±1.01 (Eucreas®)
	99.73±1.24 (Kombiglyze®)

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

Table 5. Results obtained by LC method for the determination of sitagliptin, vildagliptin and saxagliptin.

Item	Sitagliptin	Vildagliptin	Saxagliptin
Retention time	6.0	7.1	6.5
Wavelength of detection, nm	210	210	210
Range of linearity, µg/mL	5-200	5-200	0.5-80
Regression equation	Area×10^{-5} = 0.1013 $C_{\mu g/mL}$+ 0.0736	Area×10^{-5} = 0.1126 $C_{\mu g/mL}$ + 0.4040	Area×10^{-5} = 0.1495 $C_{\mu g/mL}$ + 0.0114
Regression coefficient (r^2)	0.9998	0.9996	0.9987
LOD, µg/mL	1.48	0.53	0.10
LOQ, µg/mL	4.94	1.78	0.42
S_b	1.16×10^{-4}	1.6×10^{-4}	1.8×10^{-4}
S_a	0.17	0.22	0.25
Confidence limit of the slope	0.1013±0.02	0.1126±0.02	0.1495±0.04
Confidence limit of the intercept	$0.0736\pm0.09\times10^{-4}$	$0.4040\pm0.65\times10^{-4}$	$0.0114\pm0.02\times10^{-4}$
Standard error of the estimation	0.19	0.27	0.31
Intraday %R.S.D.	0.29-0.58	0.21-0.42	0.43-0.68
Interday %R.S.D.	0.31-1.14	0.23-0.92	0.56-1.31
Drug in laboratory mixture	100.32±1.44	100.17±1.41	100.01±1.51
Drug in dosage form	100.13±1.15	99.95±1.60	99.85±1.68
Drug added	100.52±1.21	100.24±1.65	99.53±1.46

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

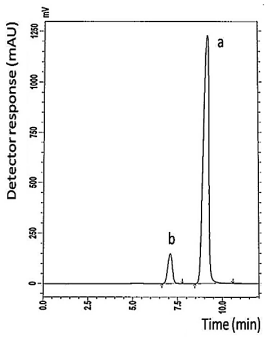

Figure 6. A typical LC chromatogram of 25 µL injector of Eucreas® sample solution, (a) metformin hydrochloride (500 µg/mL) and (b) vildagliptin (50 µg/mL).

3.3.2. Accuracy

Accuracy of the results was calculated by % recovery of five different solutions of the laboratory prepared mixtures of STG, VLD and SXG in their ternary mixture with MET and CGN and also by standard addition technique for Janumet®, Eucreas® and Kombiglyze® tablets. The results obtained including the mean of the recovery and standard deviation are displayed in Tables 4 and 5.

3.3.3. Precision

The repeatability of the method was assessed by six determinations for each of the three concentrations of the laboratory prepared mixture of STG (25.7, 32.1, and 38.5 µg/mL) with MET (400, 500, and 600 µg/mL) and CGN (120, 150, and 180 µg/mL) representing 80, 100 and 120%, respectively, and three concentrations of the laboratory prepared mixture of VLG (40, 50, and 60 µg/mL) with MET (400, 500, and 600 µg/mL) and CGN (120, 150, and 180 µg/mL) representing 80, 100, and 120%, respectively, and three concentrations of the laboratory prepared mixture of SXG (4.5, 5.6, and 6.7 µg/mL) with MET (400, 500, and 600 µg/mL) and CGN (120, 150, and 180 µg/mL) representing 80, 100, 120%, respectively. The repeatability of sample application and measurement of peak area for active compound were expressed in terms of percentage relative standard deviation (%R.S.D.) and found to be less than 1% in the three concentrations. Results for the determination of precision are displayed in Tables 1-5.

3.3.4. 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.

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

Statistical term [a]	Reference method [b]	Proposed method
Mean	100.4	100.36
S.D.	0.28	1.15
S.E.	0.13	0.51
%R.S.D.	0.28	1.15
n	5	5
V	0.08	1.32
t (2.306) [c]	-	0.08

[a] S.D.: Standard deviation, S.E.: Standard error, %R.S.D.: Relative standard deviation, n: Number of samples, V: Variance.
[b] Reference method for the spectrophotometric determination of metformin in the Indian Pharmacopeia [24].
[c] Figures in parentheses are the theoretical t value at (p = 0.05). No significant difference between groups by using one way ANOVA with F = 0.01 and p = 0.94.

Table 7. Statistical comparison between the results of the LC method and the reference methods for the determination of sitagliptin, vildagliptin and saxagliptin.

Statistical Term	Reference Method for sitagliptin [b]	HPLC method	Reference Method for vildagliptin [c]	HPLC method	Reference Method for saxagliptin [d]	HPLC method
Mean	100.50	100.32	100.01	100.17	100.20	100.01
S.D.	1.39	1.44	0.99	1.41	1.10	1.51
S.E.	0.62	0.64	0.44	0.63	0.49	0.68
%R.S.D.	1.38	1.44	0.99	1.41	1.1	1.51
n	5	5	5	5	5	5
V	1.93	2.1	0.98	1.98	1.21	2.28
t (2.306) [a]		0.20		0.21		0.23

[a] Figure in parentheses are the theoretical t value at (p = 0.05).
[b] Reference method: aliquots of standard solutions in distilled water containing 2-10 µg/mL STG were measured using methanol as a blank [5]. No significant difference between groups of sitagliptin by using one way ANOVA with F = 0.04 and p = 0.85.
[c] Reference method: aliquots of standard solutions in distilled water containing 5-25 µg/mL VDG were measured using water as a blank [5]. No significant difference between groups of vildagliptin by using one way ANOVA with F = 0.04 and p = 0.84.
[d] Reference method: aliquots of standard solutions in distilled water containing 5-40 µg/mL SXG were measured using methanol as a blank [9]. No significant difference between groups of saxagliptin by using one way ANOVA with F = 0.05 and p = 0.83.

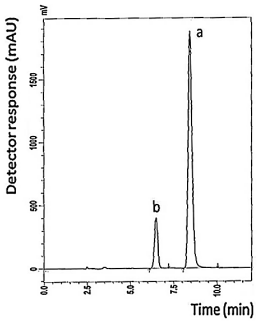

Figure 7. A typical LC chromatogram of 25 µL injector of Kombiglyze® sample solution, (a) metformin hydrochloride (500 µg/mL) and (b) saxagliptin hydrochloride (5.6 µg/mL).

In the present work, specificity was checked by analyzing STG with MET, VDG with MET and SXG with MET in laboratory prepared mixtures with CGN. Good resolution and absence of interference between drugs being analyzed are shown in Figure 2-7. 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 5-7). 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, Tables 4 and 5. 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

The most important parameter to be studied was the resolution factor between the two peaks of STG and MET, VLG and MET and also between the two peaks of SXG and MET. Besides, any interference from the peak of CGN was checked visually. 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 (4.1, 2.5, and 3.2), (4.2, 2.45, and 3.11) and (4.15, 2.6, and 3.2), respectively. The organic strength was changed by %±2 where resolution factors obtained were (4.1, 2.5, and 3.2), (4.25, 2.5, and 3.28) and (4.12, 2.6, and 3.35), 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 (4.1, 2.5, and 3.2), (3.95, 2.62, and 3.21) and (3.9, 2.65, and 3.31), respectively. As can also 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. No interference was observed from the peak of CGN in all the previously mentioned conditions.

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 Tables 4 and 5.

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 Tables 6 and 7. The test ascertained that there was no significant difference among the methods.

4. Conclusion

The proposed LC method has the advantages of simplicity, precision, accuracy and convenience for the separation and quantization of STG, VDG or SXG in combination with MET in the presence of CGN. The method can be applied for the determination of the cited drugs in pharmaceutical dosage forms. The method was validated showing satisfactory data for

all the method validation parameters tested. The developed method can be conveniently used by quality control laboratories.

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