Bulletin of Faculty of Pharmacy, Cairo University 55 (2017) 311–317

Contents lists available at ScienceDirect

Bulletin of Faculty of Pharmacy, Cairo University

journal homepage: www.sciencedirect.com

Original Article

Chromatographic methods for the simultaneous determination of binary mixture of Saxagliptin HCl and Metformin HCl

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ARTICLE INFO

Article history:
Received 18 December 2016
Received in revised form 3 April 2017
Accepted 18 April 2017
Available online 26 April 2017

Keywords:
Saxagliptin HCl
Metformin HCl
RP-HPLC
TLC

ABSTRACT

Two chromatographic methods were suggested for the simultaneous determination of a binary mixture containing Saxagliptin HCl (SAG) and Metformin HCl (MET). First method was RP-HPLC method. Chromatographic separation was done on Kinetex™ column–C_{18} (4.6 × 150 mm, 2.6 μm) using mobile phase consisted of acetonitrile:phosphate buffer pH = 4.5 ± 0.1 adjusted with orthophosphoric acid (13:87, v/v). Isocratic elution at a flow rate 1.5 mL/min and UV detection at 220.0 nm was performed. Second method was spectro-densitometric method. Chromatographic separation was done on precoated silica gel aluminium plates 60 F_{254} as a stationary phase and developing system consisting of chloroform: methanol:formic acid (80:20:0.3, by volume). The density of the separated bands was measured by UV detector at 210.0 nm. The proposed methods were validated as per the ICH guidelines parameters like Linearity, precision, accuracy, selectivity, limit of detection and limit of quantitation. Statistical comparison was done between the obtained results and those obtained by the reported methods, showing no significant difference with respect to accuracy and precision.

1. Introduction

Saxagliptin HCl (SAG), (Fig. 1a) is chemically named as (1S,3S,5 S)-2-[(2S)-2-amino-2-(3-hydroxy-1-adamantyl)acetyl]-2-azabicyclo[3.1.0]hexane-3-carbonitrile hydrochloride [1].

Saxagliptin HCl (SAG) is an oral hypoglycemic (anti-diabetic drug) of the dipeptidyl peptidase-4 (DPP-4) inhibitor class of drugs. SAG is used as monotherapy or in combination with other drugs for the treatment of type II diabetes. The drug works to competitively inhibit a protein/enzyme, dipeptidyl peptidase 4 (DPP-4), that results in an increased amount of active incretins; Gastric inhibitory polypeptide (GIP) and glucagon-like peptide-1 (GLP-1), reduced amount of release of glucagon and increased release of insulin [2,3].

Metformin hydrochloride (MET), (Fig. 1b) is chemically designated as 1,1-Dimethylbiguanide hydrochloride [1]. Metformin hydrochloride (MET) is a biguanide antidiabetic. It is given orally in the treatment of type II diabetes mellitus; a disease characterized by defects in both insulin secretion and insulin sensitivity, and is the drug of first choice in overweight patients [2].

SAG is not an official drug in any pharmacopeia. While MET was determined by British Pharmacopoeia (BP) [4] and United States Pharmacopeia (USP) [5], both suggest a non aqueous titration method for the assay of MET using anhydrous formic acid as a solvent and 0.1 M perchloric acid as a titrant. End point is determined potentiometrically.

Literature survey represented that SAG or MET can be determined in combination with other drugs by several methods including; spectrophotometry [6–8], TLC [9,10], HPLC [11–20], and UPLC [21,22].

Simultaneous determination of SAG and MET in combination was done using; spectrophotometric [23], TLC [24] and HPLC methods [25–35].

2. Experimental

2.1. Apparatus

(a) Camage TLC Scanner 3 S/N 130319 with winCATS software (Muttenz, Switzerland), Camage Linomat v autosampler (Muttenz, Switzerland), Precoated silica gel aluminium plates 60 F_{254} 20 × 20 cm with 0.25 mm thickness made by Merck KgaA (Damstad, Germany), UV lamp with short wavelength 254 nm manufac-

Peer review under responsibility of Faculty of Pharmacy, Cairo University.
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http://dx.doi.org/10.1016/j.bfopcu.2017.04.002
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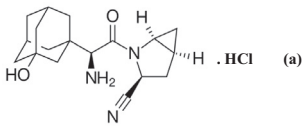

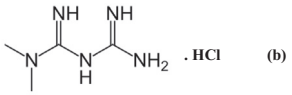

Fig. 1. Chemical structures of (a) Saxagliptin HCl (SAG) and (b) Metformin HCl (MET).

tured by Camag (Muttenz, Switzerland), SonixTV s-series ultrasonicator manufactured by Covaris (Woburn, MA).

(b) HPLC Agilent 1290 Infinity Series Liquid chromatograph, Waldbornn, Germany. It consists of:

- Binary pump-model G 4200 B.
- Variable wavelength detector (VWD)-model G 1314 E.
- Autosampler-model G 4226 A.
- Column: Kinetex™ C_{18} column- (4.6 × 150 mm), particle size (2.6 μm), USA.
- Column heater-model G 1316 A.

2.2. Pure samples

Saxagliptin HCl (SAG): It was kindly supplied by Bristol-Myers Squibb/AstraZeneca EEIG (United Kingdom), its purity was found to be 100.18 ± 1.102 according to the reported method [36]. Metformin HCl (MET): It was kindly supplied by National Organization for Drug Control and Research (NODCAR), Cairo, Egypt, its purity was found to be 99.02 ± 1.006 according to the reported method [37].

2.3. Pharmaceutical formulations

Kombiglyze XR® tablets (batch number 1E6008PA), manufactured by Bristol-Myers Squibb, from Vogue Pharmaceuticals Inc., Calgary, Canada. Each tablet was labeled to contain 5.58 mg SAG and 1000 mg MET.

2.4. Reagents

Methanol (Sigma-Aldrich, St. Louis, USA), Chloroform, acetone, ethyl acetate, ammonia, glacial acetic acid and formic acid (EL-NASR Pharmaceutical Chemicals Co., Abu-Zaabal, Cairo, Egypt), Acetonitrile HPLC grade (SDS, Peypin, France), Orthophosphoric acid (EL-NASR Pharmaceutical Chemicals Co., Abu-Zaabal, Cairo, Egypt), Deionised water (SEDICO Pharmaceutical Co., Cairo, Egypt), Phosphate buffer (pH 4.5 ± 0.1, prepared by dissolving 3.40 g potassium dihydrogen orthophosphate in 1000-mL volumetric flask and completing the volume with deionised water, then the pH was adjusted using orthophosphoric acid. [38]

2.5. Standard stock and working solutions

2.5.1. For TLC method

- SAG and MET stock solutions: 1 mg/mL of each in methanol.

2.5.2. For HPLC method

- SAG stock solution: 1 mg/mL in mobile phase consisting of acetonitrile:phosphate buffer pH = 4.5 ± 0.1 (13:87, v/v).
- SAG working solution: 0.05 mg/mL in mobile phase consisting of acetonitrile:phosphate buffer pH = 4.5 ± 0.1 (13:87, v/v).
- MET stock solution: 1.25 mg/mL in mobile phase consisting of acetonitrile:phosphate buffer pH = 4.5 ± 0.1 (13:87, v/v).

2.6. Chromatographic conditions

2.6.1. For TLC method

The bands were spaced 9.4 mm apart from each other, 15 mm from the bottom edge of the plate with a band length of 6 mm using Camage Linomat v autosampler. The plates were developed in a chromatographic tank previously saturated with the mobile phase of chloroform:methanol:formic acid (80:20:0.3, by volume), by ascending chromatography to a distance of 7.5 cm from the spotting line at room temperature. The plates were left to dry then the separated bands were visualized under UV lamp (210.0 nm) and the plates were scanned under the following conditions:

- Source of radiation: Deuterium lamp.
- Scan mode: Absorption mode.
- Slit dimension: 3.00 mm × 0.45 mm.
- Result output: Chromatogram and integrated peak area.
- Scanning speed: 20 mm/s.
- Wavelength: 210.0 nm.

2.6.2. For HPLC method

The method was carried out at ambient temperature on Kinetex™ column – C_{18} (4.6 × 150 mm, 2.6 μm). The mobile phase consisted of acetonitrile:phosphate buffer pH = 4.5 ± 0.1 adjusted with orthophosphoric acid (13:87, v/v). The mobile phase was filtered using 0.45 μm membrane filters and degassed by ultrasonic vibrations for 30 min prior to use. It was delivered at a flow rate of 1.5 mL/min and the injection volume was 5.0 μL. The column was conditioned for at least 30 min. UV detection was achieved at 220.0 nm.

2.7. Procedures

2.7.1. Linearity

2.7.1.1. For TLC method. Accurately measured aliquots of each of SAG and MET stock standard solution (1 mg/mL) were separately spotted onto two different TLC plates, using Camag Linomat autosampler with microsyringe (100 μL). TLC plates were chromatographed as under the previously mentioned chromatographic conditions. Calibration curves relating the integrated peak area versus the corresponding concentration of each SAG (2–22 μg/band) and MET (1–20 μg/band) were constructed and the regression equations were computed.

2.7.1.2. For HPLC method. Aliquots of SAG and MET were separately and accurately transferred from either working solution (0.05 mg/mL), in case of SAG or stock solution (1.25 mg/mL), in case of MET, into two separate sets of 10-mL volumetric flasks and the volume was completed to the mark with the mobile phase to prepare solutions having concentration in the range of 1.5–25 μg/mL and 25–1000 μg/mL, for SAG and MET, respectively. A volume of 5.0 μL of each solution was injected in triplicates into the liquid chromatograph under the previously mentioned chromatographic conditions. Linear calibration curves was obtained for each of SAG and MET relating the relative peak area (using internal standard (IS) of 10 μg/mL of hydrochlorothiazide (HZ)) to the corresponding concentrations and the regression equations were computed.

2.7.2. Application of the proposed methods for the determination of SAG and MET in Kombiglyze XR® tablets

2.7.2.1. For TLC method. Ten tablets of Kombiglyze XR® formulation were accurately weighted. The average weight of two tablets was calculated and then the tablets were finely powdered. An amount of the powder equivalent to the average weight of two tablets (11.16 mg SAG and 2000 mg MET) was weighted, dissolved in enough volume of methanol, by shaking in ultrasonic bath for 30 min. The solution was accurately transferred to a 100-mL volumetric flask, the volume was completed with methanol and was finally filtered. Volumes (20 and 1.0 μL) equivalent to (2.232 and 20.0 μg), for SAG and MET, respectively, of the prepared solution were applied in triplicates using Linomat applicator onto a TLC plate and the procedure was completed as described under linearity.

2.7.2.2. For HPLC method. Ten tablets of Kombiglyze XR® formulation were accurately weighted. The average weight of one tablet was calculated and then the tablets were finely powdered. An amount of the powder equivalent to the average weight of one tablet (5.58 mg SAG and 1000 mg MET) was weighted, dissolved in enough volume of the mobile phase of acetonitrile:phosphate buffer pH = 4.5 ± 0.1 (13:87, v/v), by shaking in ultrasonic bath for 30 min. The solution was accurately transferred into a 100-mL volumetric flask, and the volume was completed with the same solvent, and was finally filtered. An appropriate dilution was made using mobile phase to prepare the working solution of final concentration 5.58 μg/mL and 1000 μg/mL for of each SAG and MET, respectively. The procedure was then completed as described under linearity.

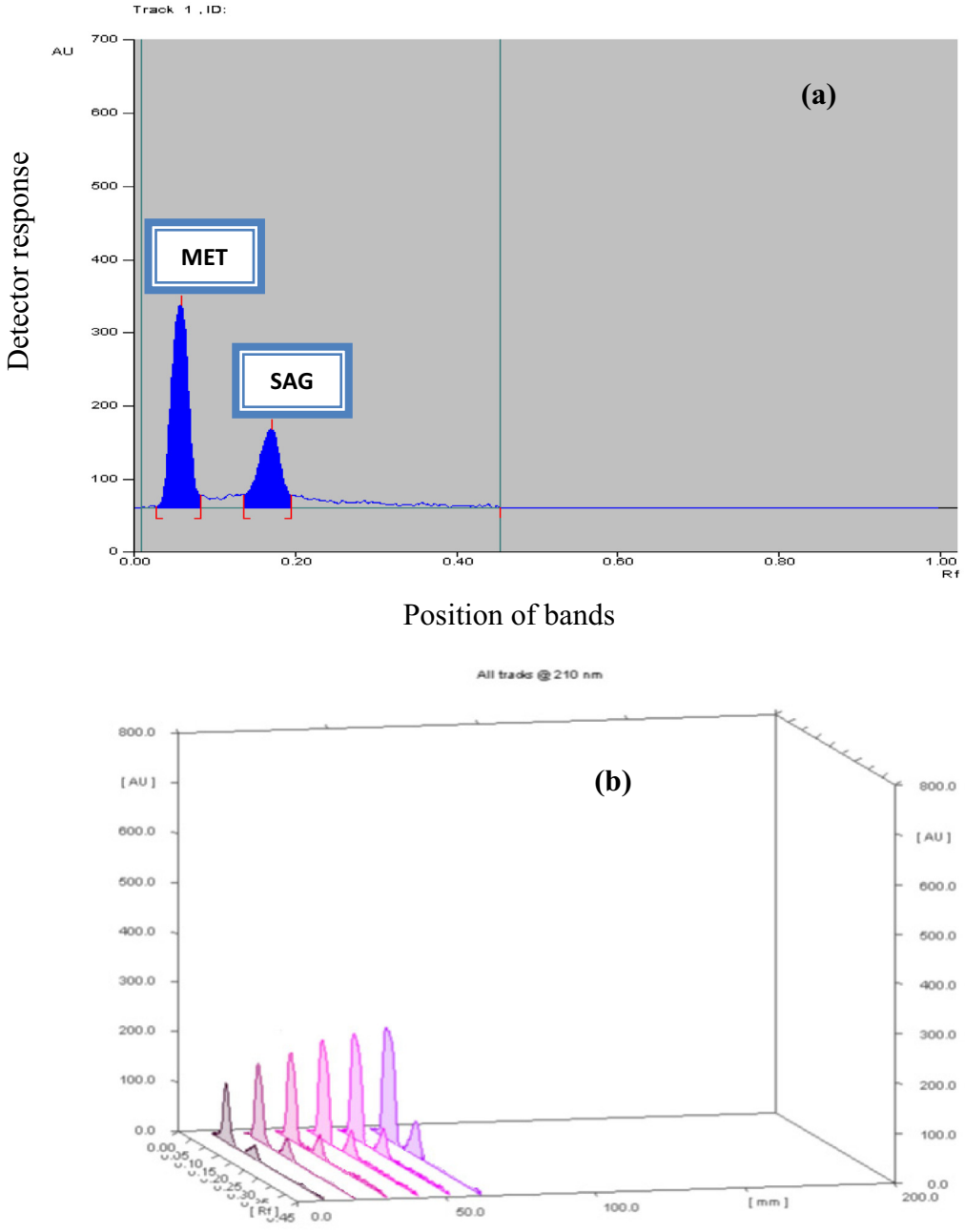

Fig. 2. (a) TLC chromatogram (2D) of a resolved mixture of MET (10 μg/band, R_f = 0.06) and SAG (2 μg/band, R_f = 0.17) (b) TLC chromatogram (3D) of mixtures of SAG and MET at 210 nm using chloroform:methanol:formic acid (80:20:0.3, by volume) as a developing system.

The concentrations of the cited drugs were obtained from the corresponding computed regression equations and the validity of the proposed TLC and HPLC methods was further assessed by applying the standard addition technique.

3. Results and discussion

Chromatographic methods have made a great progress and attained a wide acceptance as a major analytical tool for qualitative and quantitative methods of analysis for drugs in mixtures, stability indicating pharmacokinetics and enantiomeric purity [39–41]. Therefore the aim of this work is to develop simple, accurate, precise and rapid chromatographic methods. The aim of this work was to develop simple, accurate and precise TLC – densitometric method for the determination of SAG and MET in presence of each other without prior separation. Several trials were carried out to obtain good and optimum separation of SAG and MET. Firstly, the reported mobile phase [24] was tried for separation of SAG and MET but the spot of MET showed tailing therefore, the quantitative determination of both drugs cannot be done. Therefore different mobile phases compositions with different ratios were tried such as chloroform:methanol:ammonia (5:5:0.1, by volume), chloroform:ethyl acetate:glacial acetic acid (8:2:0.1, by volume) and chloroform:methanol:acetone:formic acid (80:15:5:0.3, by volume), but no complete separation was achieved. Satisfactory separation was obtained upon using chloroform:methanol:formic acid (80:20:0.3, by volume) and R_f were found to be 0.06 and 0.17 for MET and SAG, respectively. This separation allows the determination of the two components at 210.0 nm without any interference with each other, (Fig. 2).

An HPLC method was also described for the simultaneous determination of SAG and MET. In order to optimize the proposed HPLC method, all the experimental conditions were investigated. Several trials were carried out to obtain good and optimum separation of SAG and MET. The choice of the stationary phase was studied, the reversed – phase separation was preferred due to the drawbacks of the normal – phase mode; for example, hydration of silica with water, which causes peak tailing. Different composition of mobile phases with different ratios were tried such as methanol: phosphate buffer pH = 3.5 (65:35, v/v), methanol:phosphate buffer pH = 4.5 (50:50, v/v) and acetonitrile:phosphate buffer pH = 4.5 (20:80, v/v), the best resolution was obtained upon using acetonitrile:phosphate buffer pH = 4.5 ± 0.1 (13:87, v/v) with a flow rate of 1.5 mL/min. The respective t_R values were found to be 1.283 and 2.644 for MET and SAG, respectively. It's noteworthy that the development of HPLC method with Kinetex™ C_{18} (150 mm × 4.6 i.d.) column with particle size (2.6 μm) enabled significant reduction in separation time, solvent consumption and improved peak symmetry as compared to using C_{18} (250 mm × 4.6 i.d.) column with particle size (5 μm) (Fig. 3).

System suitability is used to ensure the maximum performance of the system before and during the analysis of drugs. System suitability was checked by calculating different parameters for HPLC such as capacity factor, tailing factor, column efficiency (N), selectivity and resolution factors, where the system was found to be suitable relative to the reference values [5], or by calculating selectivity and resolution for TLC. The system was found to be suitable relative to the reference values [42] as shown in (Tables 1 and 2).

Table 1
System suitability parameters of the proposed TLC – densitometric method.

Parameters	SAG	MET	Reference values [42]
R_f	0.17	0.06	–
Peak width ($W_{0.05}$)	0.048	0.056	–
Resolution (R_s)	2.11		Rs > 1.5
Asymmetry factor (T)	1.14	0.92	T = 1, for a typical symmetrical peak
Selectivity factor (α)	2.88		α > 1

Table 2
System suitability parameters of the proposed HPLC method.

Parameters	SAG	MET	Reference values [5]
Retention time (t_R)	2.644	1.283	–
Resolution (R_s)	9.51		Rs > 1.5
Tailing factor (T)	1.0	0.9	T = 1, for a typical symmetrical peak
Capacity factor (K′)	4.28	1.56	1 < K′ < 10
Selectivity factor (α)	2.74		α > 1
Column efficiency (N)	11,185	1488.9	Increase with the efficiency of the separation
Height equivalent to theoretical plate (HETP) (mm)	0.0100	0.0013	The smaller the value the higher the column efficiency

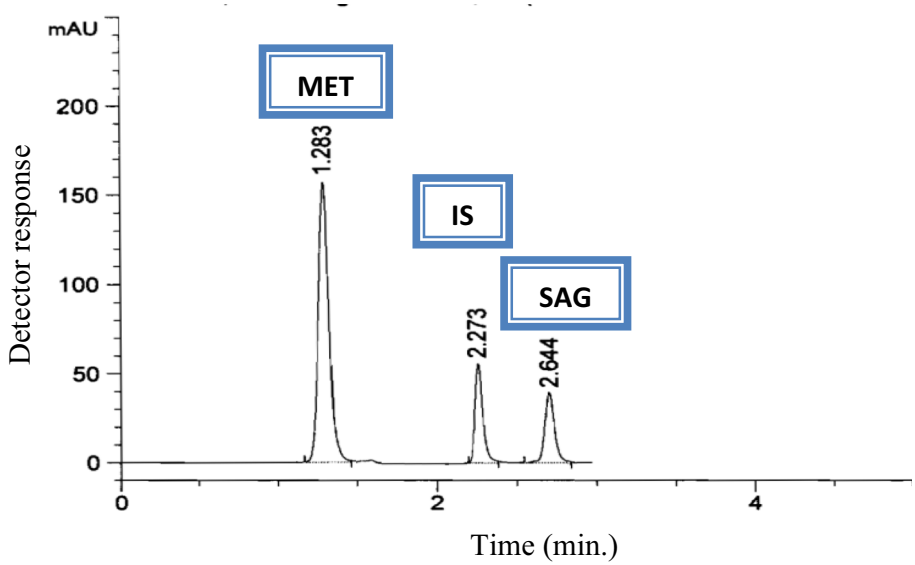

Fig. 3. HPLC chromatogram of a resolved mixture of MET (500 μg/mL, t_R = 1.283), HZ (IS) (10 μg/mL, t_R = 2.273) and SAG (2.5 μg/mL, t_R = 2.644) using mobile phase of acetonitrile:phosphate buffer pH = 4.5 ± 0.1 (13:87, v/v) and Kinetex™ C_{18} column – (4.6 × 150 mm), particle size (2.6 μm).

By comparison between the reported HPLC method which used for determination of SAG and MET in pharmaceutical dosage form [13] and the suggested HPLC method we found that, our HPLC has much lower run time equal to 3 min (10 min for the reported HPLC) in addition to using lower amount of acetonitrile equal to13 mL (58 mL for the reported HPLC). This allows saving of solvent and give advantages to our method to be more economic and more eco-friendly to environment. In addition to using an internal standard (hydrochlorothiazide) that renders the method applicable in biological fluids.

Linear relationships for HPLC method were obtained for SAG and MET relating the relative peak areas at the selected wavelength (220.0 nm) to the corresponding concentration ranges of 1.5–25 μg/mL and 25–1000 μg/mL for SAG and MET, respectively. While the linear relationships for TLC were found to exist between the integrated peak area of the separated bands at the selected wavelength (210.0 nm) and the corresponding concentration of the two drugs in the range of (2–22 μg/band) for SAG and (1–20 μg/band) for MET.

Regression and validation parameters for the determination of pure SAG and MET samples by applying the proposed HPLC and TLC methods were determined and listed in Table 3.

The proposed HPLC and TLC methods have been successfully applied for the determination of SAG and MET in Kombiglyze XR® tablets and the validity of the methods was further assessed by applying the standard addition technique, (Table 4).

The results obtained for the analysis of SAG and MET in their pure form by the proposed methods were statistically compared with those obtained by applying the reported methods for SAG [36] and MET [37]. The calculated t and F values were less than

Table 3
Regression and validation parameters for the determination of pure SAG and MET samples by applying the proposed TLC and HPLC methods.

Parameters	TLC method		HPLC method	
	SAG	MET	SAG	MET
Range	2–22 (μg/band)	1–20 (μg/band)	1.5–25 (μg/mL)	25–1000 (μg/mL)
Slope[a]	0.6812	1.3831	0.2620	0.0115
Intercept[a]	2.1259	3.4614	−0.0760	1.4911
SE of the slope	0.0042	0.0067	0.0018	0.0001
SE of the intercept	0.0576	0.0798	0.0261	0.01538
Correlation coefficient (r)	0.9997	0.9998	0.9998	0.9999
LOD[a]	0.409	0.313	0.478	8.178
LOQ[a]	1.241	0.949	1.447	24.782
Accuracy				
Mean ± SD	99.85 ± 1.188	99.98 ± 0.762	99.92 ± 1.158	100.17 ± 0.996
RSD%	1.190	0.762	1.159	0.989
Precision (RSD%)				
Repeatability[b]	1.017	1.008	1.015	1.046
Intermediate precision[c]	1.105	1.099	1.009	0.902

[a] Average of three determinations.
[b] The intraday (n = 3 × 3) relative standard deviation of concentrations (6, 12, 24 μg/mL) of SAG and (150, 300, 600 μg/mL) of MET for HPLC method or (5.5, 10.5 and 15.5 μg/band) of each SAG and MET for TLC repeated three times within the same day.
[c] The interday (n = 3 × 3) relative standard deviation of concentrations (6, 12, 24 μg/mL) of SAG and (150, 300, 600 μg/mL) of MET for HPLC or (5.5, 10.5 and 15.5 μg/band) of each SAG and MET for TLC repeated three times in three successive days.

Table 4
Results obtained for the determination of SAG and MET in Kombiglyze XR® tablets by the proposed TLC and HPLC methods and results of application of standard addition technique.

Method	Drug	Found (%)* ± SD	Standard addition technique		
			Pure added (μg/band)	Found (μg/band)	Recovery %
TLC	SAG	98.83 ± 0.860	2.5	2.47	98.80
			5	4.97	99.40
			10	9.99	99.90
	Mean ± SD				99.37 ± 0.551
	MET	100.45 ± 0.925	2.5	2.47	98.80
			5	5.00	100.00
			10	9.84	98.40
	Mean ± SD				99.07 ± 0.833
Method	Drug	Found (%)* ± SD	Standard addition technique		
			Pure added (μg/mL)	Found (μg/mL)	Recovery %
HPLC		100.74 ± 1.196	2.5	2.53	101.20
			5	5.05	101.00
			10	9.94	99.40
	Mean ± SD				100.53 ± 0.987
	MET	100.25 ± 1.079	50	50.48	100.97
			100	100.62	100.62
			200	203.03	101.52
	Mean ± SD				101.03 ± 0.454

Claimed amount taken for TLC was 2.79 and 10 μg/band of SAG and MET, respectively.
Claimed amount taken for HPLC was 11.16 and 500 μg/mL of SAG and MET, respectively.
* Average of three determinations of Kombiglyze XR® tablets (claimed to contain 5.58 mg SAG and 1000 mg MET) B. N. 1E6008PA.

Table 5
Statistical comparison of the results obtained by the proposed TLC and HPLC methods and the reported methods for the determination of SAG [36] and MET [37] in their pure form.

Item	TLC method		HPLC method		Reported method [36]	Reported method [37]
	SAG	MET	SAG	MET	SAG[a]	MET[b]
Mean	99.85	99.98	99.92	100.17	100.18	99.02
SD	1.188	0.762	1.158	0.996	1.102	1.006
Variance	1.411	0.872	1.341	0.992	1.214	1.012
n	11	11	7	8	5	5
Student's *t* test*	0.526 (2.145)	1.865 (2.145)	0.390 (2.228)	2.019 (2.201)		
F value*	1.16 (5.96)	1.16 (3.48)	1.10 (6.16)	1.02 (4.12)		

* These values represent the corresponding tabulated values of t and F at $p = 0.05$.
[a] Direct UV spectrophotometry at $\lambda_{max} = 208.0$ nm.
[b] HPLC method; Octadecylsilyl silica gel column (5 μm) (250 mm × 4 mm i.d.), mobile phase: methanol:water (30:70, v/v), flow rate: 0.5 mL/min, UV detection: 233.0 nm.

the tabulated ones, which revealed that there is no significant difference between the two methods with respect to accuracy and precision, (Table 5).

4. Conclusion

The proposed chromatographic methods are highly selective, fast, accurate and reproducible methods for the quantitative analysis of SAG and MET. The developed TLC – densitometric method is highly sensitive and has the advantage over the reported method [24] of short run time, large sample capacity and the use of minimal solvent volumes. Inspite of being more expensive, compared to spectrophotometric methods, the proposed HPLC method gives a good resolution between SAG and MET, in the presence of internal standard (HZ) within suitable analysis time. Unlike the reported HPLC methods for the analysis of the cited drugs, the proposed method has the advantage of employing a good resolution, better peak symmetry and nearly halftime of analysis than other HPLC systems using 250 nm (with 5 μm particle size) analytical columns with compromise on over all separation, therefore, it is more time and cost saving compared to the reported ones. The use of internal standard makes the method suitable for the determination of both drugs in biological fluids.

The good recoveries obtained in both cases, as well as the reliable agreement with the reported methods, proved that the proposed chromatographic methods could be applied efficiently for determination of this binary mixture with quite satisfactory precision and could be easily applied in quality control laboratories for their analysis in pharmaceutical formulation or in pure form.

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