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ORIGINAL RESEARCH PAPER

Development of a highly sensitive and eco-friendly high-performance thin-layer chromatography approach for the determination of empagliflozin, pioglitazone, and rosuvastatin simultaneously in pharmaceutical preparations and different biological fluids

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Abstract
A simple, accurate, green and selective high-performance thin-layer chromatography (HPTLC) method has been developed and validated for the simultaneous estimation of empagliflozin, pioglitazone, and rosuvastatin in their synthetic ternary mixture and different biological fluids. These three drugs are used for the treatment of type 2 diabetes mellitus and dyslipidemia and have shown synergistic effects on cardiovascular outcomes. The ternary combination was separated on silica gel TLC plates G60 F_{254}, utilizing a mixture of *n*-hexane−ethyl acetate−methanol−glacial acetic acid in ratio (4.2:4:1.75:0.05, *V/V*) as a developing system using ultraviolet (UV) detection at 230 nm. All experimental parameters were optimized with a linearity range of 5−250 ng per band for each drug, with good sensitivity and low limit of detection values reached, namely 1.72, 1.79, and 1.52 ng per band for empagliflozin, pioglitazone, and rosuvastatin, respectively. The developed method was applied for separation of the studied drugs in their synthetic ternary mixture and different biological fluids, with good recovery results ensuring high efficiency of the proposed approach. Eco scale, green analytical procedure index, and AGREE metric tools were used to evaluate the greenness of the proposed method.

Keywords High-performance thin-layer chromatography (HPTLC) · Empagliflozin · Pioglitazone · Rosuvastatin · Real plasma · Greenness evaluation

1 Introduction

According to the most recent American Diabetes Association guidelines, combining two or more antidiabetic medications significantly lowers blood glucose levels via two different pathways and combining these medications with antihyperlipidemic medications may reduce the cardiovascular issues related to diabetes [1]. Empagliflozin (EMG) is a powerful sodium glucose co-transporter-2 (SGLT2) inhibitor that lowers plasma glucose levels by preventing glucose reabsorption in the renal proximal tubules leading to clinically significant improvements in blood pressure, body weight, and glycemic control [2]. Pioglitazone (PGT) is a drug in the thiazolidinedione class that lowers blood sugar by attaching to peroxisome proliferator activated receptors gamma (PPARγ), which increases the receptors sensitivity to insulin, and is efficient in lowering some indicators of cardiovascular risk and arteriosclerosis [3]. EMG and PGT each has different mechanism in treatment of diabetes and this has a synergistic effect to control the level of glucose in blood. There are two studies showing that this combination reduces the levels of glycated hemoglobin (HbA1c), improves blood pressure, and also improves body weight more than when each drug is alone, with improvement of liver steatosis and fibrosis in patients with non-alcoholic fatty liver disease and type 2 diabetes mellitus (T2DM) [4, 5]. For persons with T2DM who are not sufficiently managed on other drugs, a combination of EMG and PGT is a safe and effective therapy option. Diabetes increases a diabetic patient's risk of cardiovascular disease (CVD) by two to four times compared with non-diabetics. Cardiovascular disorders and atherosclerosis

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are the major factors to early mortality and morbidity in the community. Consequently, lowering cardiovascular risk is essential for patients with T2DM. All patients with T2DM, especially those who also have other cardiovascular risk factors, should be given lipid-lowering medication, according to several guidelines. Numerous recent trials have examined the use of statins medication to treat diabetic dyslipidemia. Additionally, it compares the relative advantages of rosuvastatin (RSV) to other statins in terms of enhancing lipid profiles. It has been demonstrated that RSV reduces levels of low-density lipoprotein cholesterol (LDL-C) by suppressing 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase, a rate-limiting enzyme that converts 3-hydroxy-3-methylglutaryl coenzyme A to mevalonate, a precursor to cholesterol in diabetic patients, to an extent that allows many hypercholesterolemic patients to meet the new LDL-C targets set by the European Union. To reduce cardiovascular issues in patients with diabetes, a combination of oral hypoglycemic medications and statin therapies is useful [6–14]. So, addition of RSV to EMG and PGT lowers plasma cholesterol while also regulating the blood sugar levels, hence controlling both T2DM and the associated dyslipidemia (the structures of the mentioned drugs are shown in Fig. 1). There are no clinically significant medication interactions between the studied drugs EMG, PGT, and RSV [2, 8, 15]; hence, there is no need for dose-adjustment while taking them together. There are several techniques for determining EMG, PGT, and RSV on an individual basis such as high-performance liquid chromatography (HPLC) [16–22], high-performance thin-layer chromatography (HPTLC) [23–25], ultra-performance liquid chromatography (UPLC) [26–28], liquid chromatography−tandem mass spectrometry (LC−MS/MS) [29–32], spectrophotometry [33–35], spectrofluorometry [36–39], and capillary electrophoresis [40, 41]. A survey of the literature found that there is still no known technique for the simultaneous estimation of EMG, PGT, and RSV. Therefore, the goal of this work is to use HPTLC technique for estimating EMG, PGT, and RSV in their synthetic ternary mixture, spiked human plasma, and real rat plasma, simultaneously. The proposed method's greenness characteristics were measured and evaluated using eco-scale assessments (ESA), analytical greenness metric (AGREE), and green analytical procedure index (GAPI) for evaluating greenness. The results were determined to be satisfactory. HPTLC is a diverse, effective, although generally simple, separation technology, as it uses simultaneous multistage analysis to examine numerous samples on a single plate.

2 Experimental

2.1 Apparatus

Semi-automatic sample application is provided by a CAMAG (Muttenz, Switzerland) HPTLC system that includes the Linomat 5 applicator and a TLC Scanner 3 with visionCAT software. On aluminum plates covered with silica gel 60 F_{254} (20 cm × 5 cm), the sample was applied in bands (band size of 4 mm) using a CAMAG 100 µL sample syringe while being sprayed with nitrogen. We used a TLC tank (standard type, 27.0 cm width × 26.5 cm height × 7.0 cm diameter, Sigma-Aldrich Co, St. Louis, MO, USA), an ultraviolet (UV) lamp (short wavelength 254 nm, Vilber Louranate 220 V 50 Hz, Marne-la-Vallee Cedex, France),

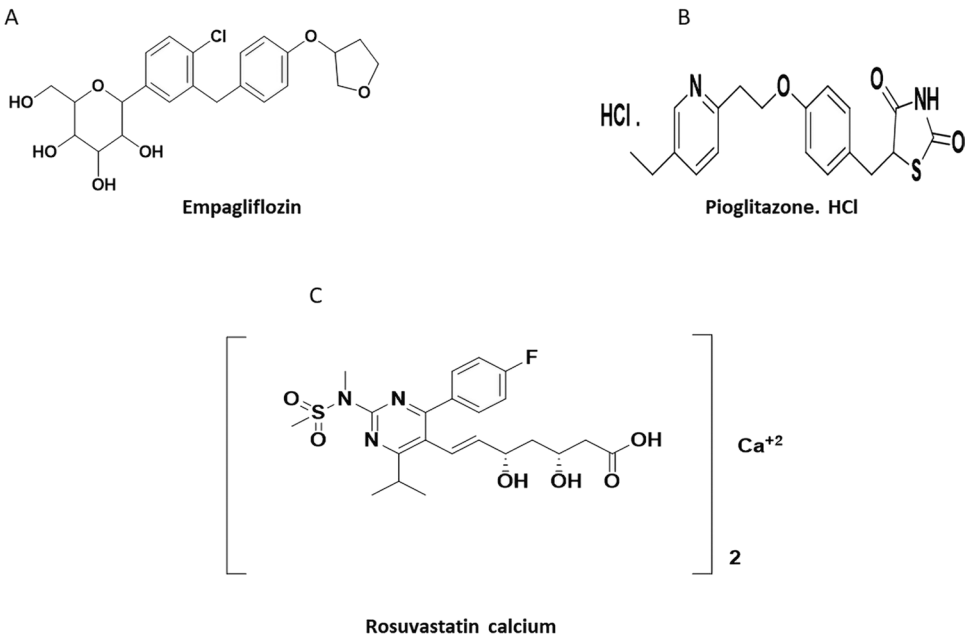


Fig. 1 The chemical structures of **A** empagliflozin (EMG), **B** pioglitazone HCl (PGT), and **C** rosuvastatin calcium (RSV)

a Mettler Toledo MS204S Newclassic MS Analytical Balance (Greifensee, Switzerland), and an ultrasonic cleaner WiseClean.

2.2 Active pharmaceutical ingredients and chemical reagents

EMG (purity, 99.00%), PGT HCl (purity, 98.67%), and RSV calcium (purity, 98.70%) were provided by Global Nabi Pharmaceuticals, EPICO, and Amoun (all Cairo, Egypt), respectively. Methanol was purchased from Sigma Aldrich (Cairo, Egypt), ethyl acetate and *n*-hexane were purchased from Piochem (Cairo, Egypt), and glacial acetic acid was purchased from El-Nasr Pharmaceutical Chemicals Co. (Cairo, Egypt). Dose form Empacoza® (containing 10 mg of EMG per tablet from Pharmaglob, Egypt), Crestolip® (containing 10 mg of RSV per tablet from Global Nabi Pharmaceuticals, Egypt), and Actozone® (containing 30 mg of PGT HCl from Amoun Pharmaceutical Company, Egypt) were provided from the local market. Human plasma was supplied from Sohag University Hospital's blood bank (Sohag, Egypt). Sigma-Aldrich Canada Co. (Oakville, Canada) supplied the TLC aluminum sheets precoated with silica gel 60 F_{254} plates (20 cm × 20 cm).

2.3 Preparation of standard solutions for EMG, PGT and RSV

Due to poor solubility of each of the investigated drugs in water and free solubility in methanol, standard stock solutions of each drug were obtained by dissolving 10 mg of drug powder in 10 mL of methanol; however, PGT and RSV needed to be sonicated at 35 °C for 5 min for complete solubility [42–44]. The concentration of the resulting solution was 1000 µg/mL for each drug. Using methanol, the proper dilutions were made to provide working standard solutions for each drug, where 5 µL injection volume was used on the TLC plates.

2.4 Chromatographic conditions

The Linomat 5 automatic sample applicator was used to apply 5 µL of standard and/or sample solutions as spots on 20 cm × 5 cm TLC plates (10 mm at start line). For each chromatographic run, a mixture of *n*-hexane−ethyl acetate−methanol−glacial acetic acid (4.2:4:1.75:0.05, *V/V*) was used as the mobile phase. The plate was dried before being developed in TLC tank, filled with specific amount of the mentioned mobile phase after saturation time 20 min. Ascending development was finished up to a migration distance of 40 mm from the origin. The plate was removed and allowed to dry. Then, scanning absorbance was assessed at 230 nm. To develop the calibration curves, the working solutions of the target ternary mixture were detected on the TLC plate along with various concentrations (5−250 ng per band, for each drug) of the investigated medications. Following the previous stated analysis, the resulting peak areas were plotted *versus* drug concentrations.

2.5 Procedure for tablets

Ten tablets of each of the following dosage forms: Empacoza® (10 mg EMG/tablet), Actozone® (30 mg PGT/tablet), and Crestolip® (10 mg RSV/tablet) were separately weighed, grinded, and mixed. Each tablet powder was accurately weighed to equal 50 mg of the medication in a 50 mL calibrated flask before being filled to the proper level with methanol. After 15 min of sonication, filtration was conducted. To get sample solutions of the EMG, PGT, and RSV mixture, additional dilutions were accomplished. The suggested HPTLC/UV detection method was then used as described above.

2.6 Procedure for spiked human plasma samples

Human plasma samples were provided from Sohag University Hospital, and kept frozen at −20 °C. Before analysis, plasma samples were left to thaw at room temperature. After thawing, 900 µL of plasma was spiked with 100 µL of different concentrations of the investigated drugs, then centrifuged for 5 min at 10,000 rpm [45]. These plasma samples were processed on Cleanert™ ODS C_{18} cartridges for solid-phase extraction (SPE). These cartridges were preconditioned with 1 mL of methanol before the samples were loaded, and they were then equilibrated with 1 mL of double-distilled water. The cartridges were first rinsed with 1 mL of 5% (*V/V*) methanol in double distilled water, followed by 1 mL of double distilled water, after the sample had been loaded. With the aid of 1 mL of methanol, the analyte was extracted from the cartridge and collected in prelabeled vials. Following that, 5.0 µL of the eluate was put onto TLC plates and quantified as previously mentioned.

2.7 Procedure for real rat plasma samples

All study components complied with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. According to the Office International des Epizooties (OIE) guidelines for using animals in research, the study proposal was approved by the Faculty of Veterinary Medicine's Ethical Committee in Assiut, Egypt (permission number: 06/2023/0064). Five male Wistar rats, 8 weeks old and weighing 180−220 g, were used. The rats were kept in plastic cages and kept at 25 °C with an alternating light–dark cycle and free access to food and drink. Blood samples were obtained from the lateral tail vein following administration

of the three co-administered drugs EMG (10 mg/kg), PGT (3 mg/kg), and RSV (200 mg/kg). A total of 3 mL of blood sample was collected after the reported T_{max} of each drug in rat plasma [46–48]. The blood samples were drawn into heparinized tubes and centrifuged at 10,000 rpm for 5 min, then 1 mL of the plasma sample will be taken and done in the same way as mentioned above in spiked plasma.

3 Results and discussion

3.1 Spectral analysis

3.1.1 Optimization of chromatographic conditions

The UV-absorption spectra of the three medications under investigation show that for EMG, PGT, and RSV, their maximal absorbance wavelengths were at 230, 232, and 240 nm, respectively. After the substances under investigation had been completely separated on TLC plates, it was discovered that the wavelength 230 nm serves as an appropriate wavelength for the detection of the selected drugs as shown in Fig. 2.

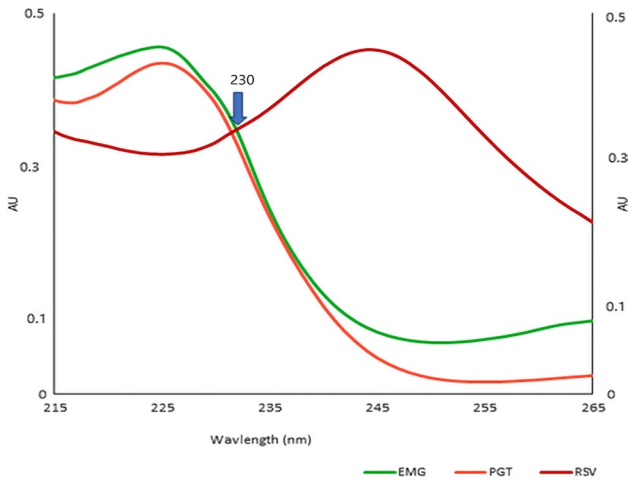


Fig. 2 Absorption spectra of EMG, PGT, and RSV

3.2 Method optimization

3.2.1 Mobile phase and saturation time effects

Trials for the selection of the mobile phase composition were based on the difference in polarity between EMG, PGT, and RSV. First, a mobile phase composed of 1,4-dioxane−phosphate buffer (pH 4) was used with composition (5.0:5.0, *V/V*); however, 1,4-dioxane was a nonpolar solvent and therefore PGT split off to the opposite side of the polar silica gel plate while EMG and RSV traveled fast along the solvent front zone. After changing the ratio of the previous mobile phase to be of 3.0:7.0 (*V/V*) composition, no separation occurred. Another mobile phase system based on the utilization of ethyl acetate−methanol was used with composition of 5.0:0.1 (*V/V*); however, no separation occurred. By adding toluene (nonpolar solvent) to the previous system, the system became toluene−ethyl acetate−methanol (4.0:4.0:2.0, *V/V*); EMG and RSV both had the same R_F but PGT had a R_F far from them, and by replacing toluene with *n*-hexane, as it is more nonpolar solvent than toluene, the three mentioned drugs were separated well from each other, but the RSV spot was very broad and so, by adding a modifier (glacial acetic acid) with a very small quantity (0.05 µL), the separation occurred very well and the broadness disappeared. The trials' outcomes are reported in Table 1. So, *n*-hexane−ethyl acetate−methanol−acetic acid (4.2:4:1.75:0.05, *V/V*) was found to be the ideal mobile phase composition that provided excellent R_F values, acceptable peak shapes, and satisfactory resolution. Figure 3A depicts the 2D densitogram produced by this mobile phase system.

To determine the ideal saturation period, experiments were conducted with various saturation times ranging from 10 to 30 min. The ideal saturation period was determined to be 20 min. The best saturation time was chosen based on the shortest amount of time that produced the best separation between two bands and the most reliable findings for R_F.

Table 1 The impact of utilizing various mobile phases for separating EMG, PGT, and RSV using the developed HPTLC method

Mobile phase composition	Ratio (*V/V*)	Retardation factor (R_F)		
		EMG	PGT	RSV
1,4-Dioxane−phosphate buffer pH (4)	5.0:5.0	0.9	0.69	0.85
	3.0:7.0	0.82	0.80	0.81
Ethyl acetate−methanol	5.0:0.1	0.76	0.81	0.81
Toluene−ethyl acetate−methanol	4.0:4.0:2.0	0.51	0.68	0.51
n-Hexane−ethyl acetate−methanol	4.0:4.0:2.0	0.45	0.70	0.52
n-Hexane−ethyl acetate−methanol−acetic acid	4.2:4.0:1.75:0.05	0.37	0.73	0.52

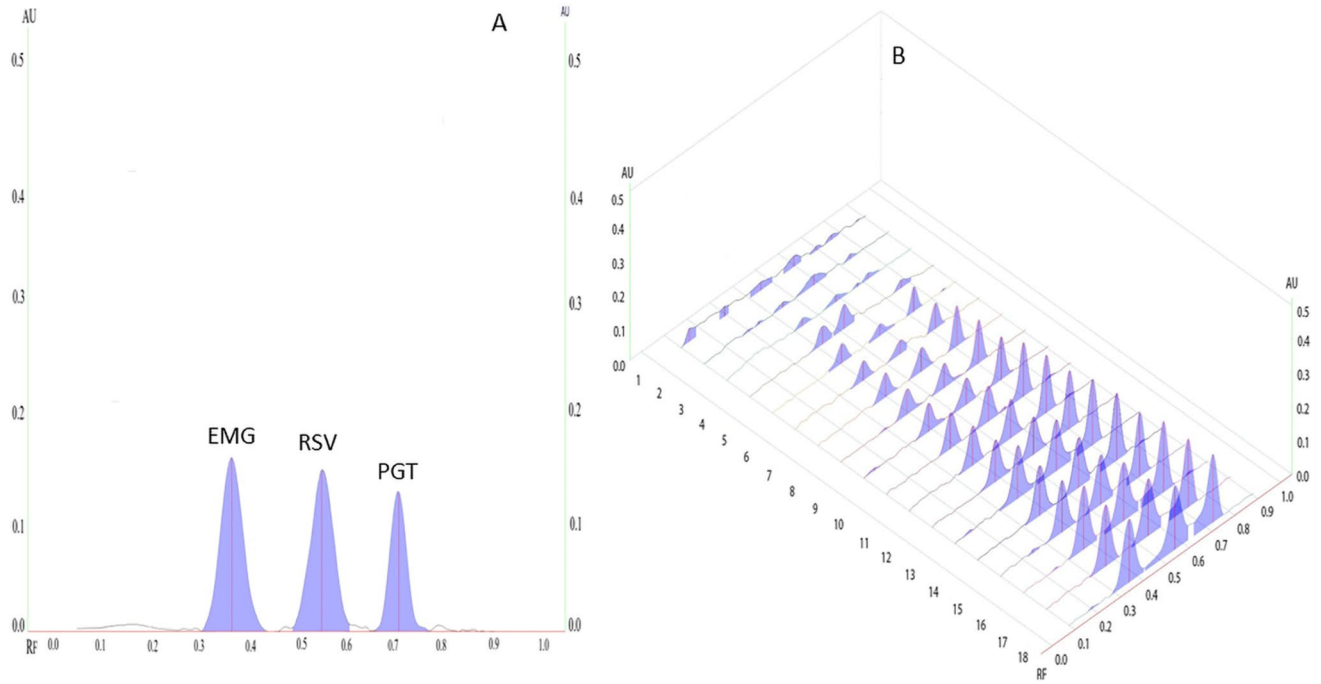


Fig. 3 **A** Two-dimensional and **B** three-dimensional HPTLC densitograms of a mixture containing EMG, PGT, and RSV (each 100 ng/band) at 230 nm using *n*-hexane−ethyl acetate−methanol−glacial acetic acid (4.2:4:1.75:0.05, *V/V*)

3.3 Method validation

In accordance with the International Council for Harmonisation (ICH) criteria for the validation of analytical procedures, the suggested method was validated [49].

3.3.1 Calibration curve

The linearity of the method was evaluated by two tests: the lack-of-fit test and Mandel's fitting test [50] (Table 2). The lack-of-fit test is a statistical method that checks how well a linear regression model fits the data. The lack-of-fit test showed that the linear regression model was good for all drugs. Mandel's fitting test is another statistical method that measures the linearity of the calibration curve. The Mandel's fitting test proved that the linear regression model worked well for the drugs EMG and PGT, but not for the drug RSV. The quadratic regression model had very slightly higher R^2 and lower standard error values than the linear regression model for two drugs [50]. This means that both the linear and quadratic regression models fit the data of these drugs very well. Table 3 shows the

Table 2 Summary of the linear regression and validation findings in pure form of the investigated drugs simultaneously

Parameters	EMG	PGT	RSV
R_F	0.36 ± 0.01	0.73 ± 0.01	0.53 ± 0.01
Linearity range (ng per band)	5–250	5–250	5–250
Slope ± SD[a]	$2.73\times10^{-5}\pm1.32\times10^{-6}$	$2.15\times10^{-5}\pm1.52\times10^{-6}$	$6.55\times10^{-5}\pm2.08\times10^{-6}$
Intercept ± SD[a]	$1.21\times10^{-4}\pm1.42\times10^{-5}$	$7.86\times10^{-5}\pm1.16\times10^{-5}$	$3.22\times10^{-4}\pm3.03\times10^{-5}$
Correlation coefficient (*r*)	0.9991	0.9990	0.9992
Limit of detection (LOD)	1.72	1.79	1.52
Limit of quantification (LOQ)	5.23	5.42	4.61
Lack of fit test (*f* calculated)[b]	1.46	5.49	2.81
Mandel's test (*f* calculated)[b]	4.22	1.02	19.66

SD standard deviation, R_F retardation factor

[a]Average of four replicates

[b]Tabulated *f* value at $p = 0.05$ is 7.71 at ($df1 = 1$) and ($df2 = 4$)

comparison between the linear and quadratic regression parameters for the three drugs.

Linear regression is a simpler, more robust, and more versatile model than quadratic regression. It is easier to use, faster to compute, and more resistant to overfitting. It can also handle outliers better. Quadratic regression should only be used if there is a strong theoretical or empirical basis to believe that the relationship between the independent and dependent variables is quadratic [50].

3.3.2 Linearity, quantification, and detection limits

Peak areas were created following simultaneous analysis of the standard drugs (EMG, PGT, and RSV synthetic mixture) using the suggested approach under ideal chromatographic conditions, where good linearities (5−250 ng per band) for each drug with correlation coefficient values ranging from 0.9990 to 0.9992 were obtained. According to statistical estimates, the formulae for limit of detection (LOD) = 3.3 × standard deviation (SD) of intercept/slope and limit of quantitation (LOQ) = 10 × SD of intercept/slope (SD is the standard deviation). LOD and LOQ values (1.72−5.23, 1.79−5.42, and 1.52−4.61 ng per band) were provided for the standard drugs: EMG, PGT, and RSV, respectively. The three-dimensional chromatograms and linearity plots are displayed in Fig. 3B. Table 2 displays the significant quantitative parameters; the statistical values show that the approach has good sensitivity for identifying the medications under investigation.

3.3.3 Accuracy

The closeness of agreement between the values regarded as a conventional true value or an approved reference value and the value found expresses the accuracy of an analytical method. As shown in Table 4, good recovery values and low percent relative standard deviation (%RSD) values demonstrated the suggested approaches' accuracy. Six duplicates of each of three distinct concentrations; 50, 150, and 250 ng per band for EMG, PGT, and RSV were used to examine the accuracy of the proposed method.

Table 3 Comparison between the linear and quadratic equation of the studied drugs

Drug	Calibration curve	R^2	SE	Model equation
EMG	Linear	0.9991	1.2×10^{-4}	$y=1.21\times10^{-4}+2.73\times10^{-5}x$
	Quadratic	0.9995	9.9×10^{-5}	$y=2.23\times10^{-4}+2.46\times10^{-5}x^2+1.06\times10^{-8}x$
PGT	Linear	0.9990	9.8×10^{-5}	$y=7.86\times10^{-5}+2.15\times10^{-5}x$
	Quadratic	0.9990	1.1×10^{-4}	$y=7.06\times10^{-5}+2.18\times10^{-5}x^2-1\times10^{-9}x$
RSV	Linear	0.9992	2.4×10^{-4}	$y=3.22\times10^{-4}+6.55\times10^{-5}x$
	Quadratic	0.9998	1.2×10^{-4}	$y=8.97\times10^{-4}+5.69\times10^{-5}x^2+2.99\times10^{-8}x$

The equations for linear and quadratic calibration models are $y=a+bx$ and $y=a+bx^2+cx$, respectively (a is the intercept; b and c represent the slopes values)

Table 4 Accuracy and precision of the proposed approach

Drug	Concentration (ng per band)	Accuracy Recovery ± SD[a]	Precision Intra-day recovery[b] ± RSD (%)	Precision Inter-day recovery[c] ± RSD (%)
EMG	50	100.65 ± 1.05	99.65 ± 1.05	99.67 ± 1.42
	150	101.75 ± 1.17	97.75 ± 0.38	97.82 ± 0.80
	250	101.25 ± 0.49	102.04 ± 1.17	101.61 ± 0.74
PGT	50	99.27 ± 1.24	101.31 ± 1.97	101.20 ± 1.10
	150	100.08 ± 1.26	100.31 ± 1.12	99.32 ± 1.60
	250	100.13 ± 0.39	100.19 ± 1.02	100.08 ± 1.24
RSV	50	96.45 ± 1.21	96.61 ± 1.21	96.50 ± 0.36
	150	100.82 ± 0.59	101.01 ± 0.91	100.92 ± 1.12
	250	100.16 ± 1.56	100.19 ± 0.72	100.90 ± 0.49

[a]Six determinations were averaged

[b]Three determinations were averaged

[c]Nine determinations were averaged in 3 days

3.3.4 Precision

Intra-day and inter-day precision studies for EMG, PGT, and RSV were used to demonstrate the precision of the suggested analytical procedure. Six duplicates of each of three distinct concentrations: 50, 150, and 250 ng per band for each of the mentioned drugs were used to perform intra-day precision. For 3 days in a row, the same three concentrations, 50, 150, and 250 ng per band were used to measure inter-day precision. To identify any intra-day and inter-day variability, the %RSD value was calculated as shown in Table 4. The results showed that the %RSD did not exceed 3%, confirming the great precision of the suggested approach.

3.3.5 Selectivity and specificity

The analytical method's selectivity refers to its capacity to identify analytes without interference from other matrix elements. The method's selectivity was investigated by analyzing EMG, PGT, and RSV mixture spiked with human plasma sample on the TLC plate as seen in Fig. 4A and B showing that there were no interfering plasma component peaks and that the R_F values of the examined medicines in human plasma were identical to those in the reference combination. The combined spectra of their individual tablet extracts, along with the superimposed spectra of the EMG, PGT, and RSV standard mixtures, confirm the suggested method's high specificity and strong correlation. Figure 5 displays spectral comparison

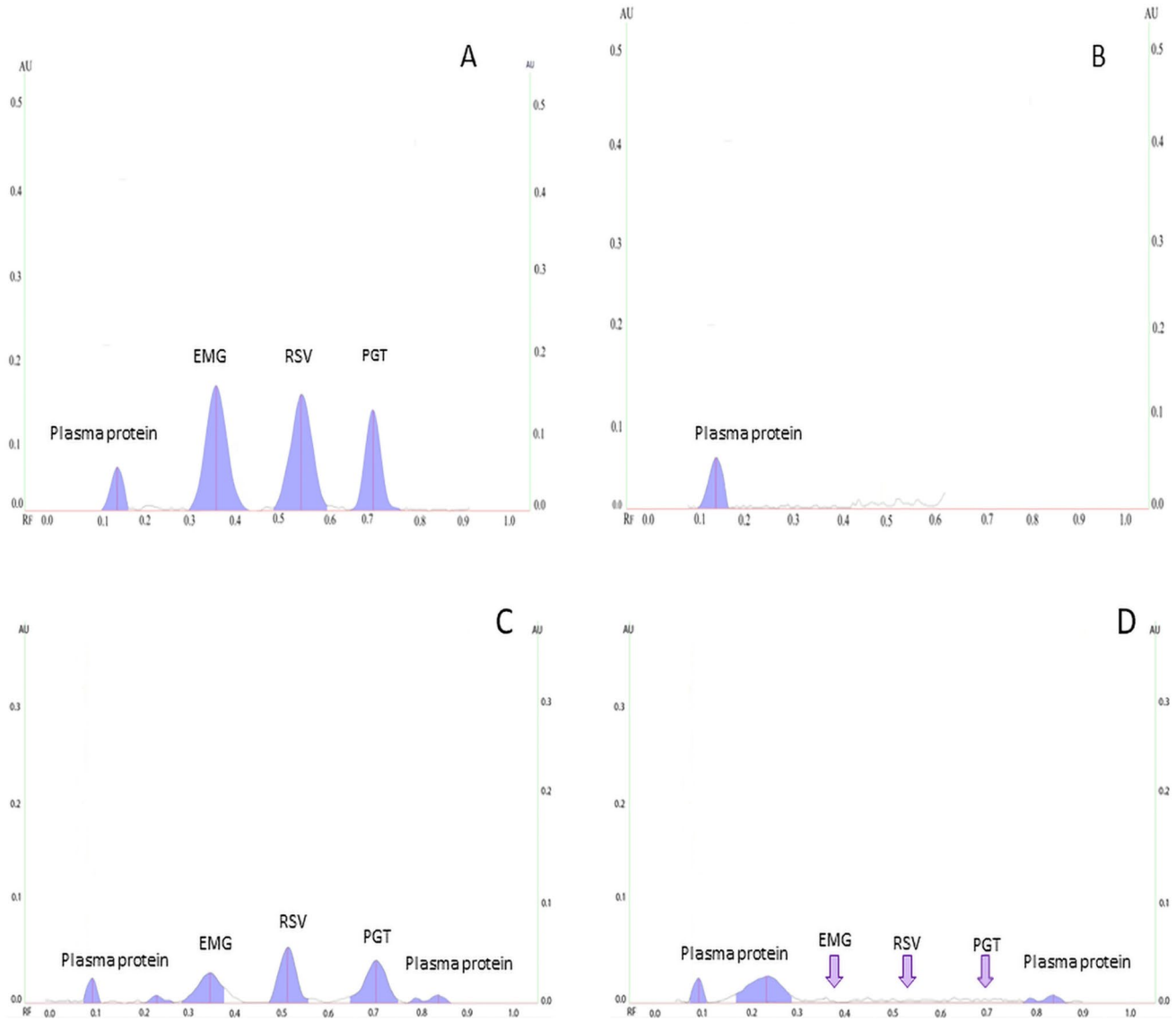


Fig. 4 Two-dimensional HPTLC densitogram of **A** human plasma sample spiked with EMG, PGT, and RSV (each 100 ng per band), **B** blank plasma sample, **C** real rat plasma following administration of single oral dose of EMG (10 mg/kg), PGT (3 mg/kg), and RSV (200 mg/kg) after their reported T_{max}, and **D** blank rat plasma

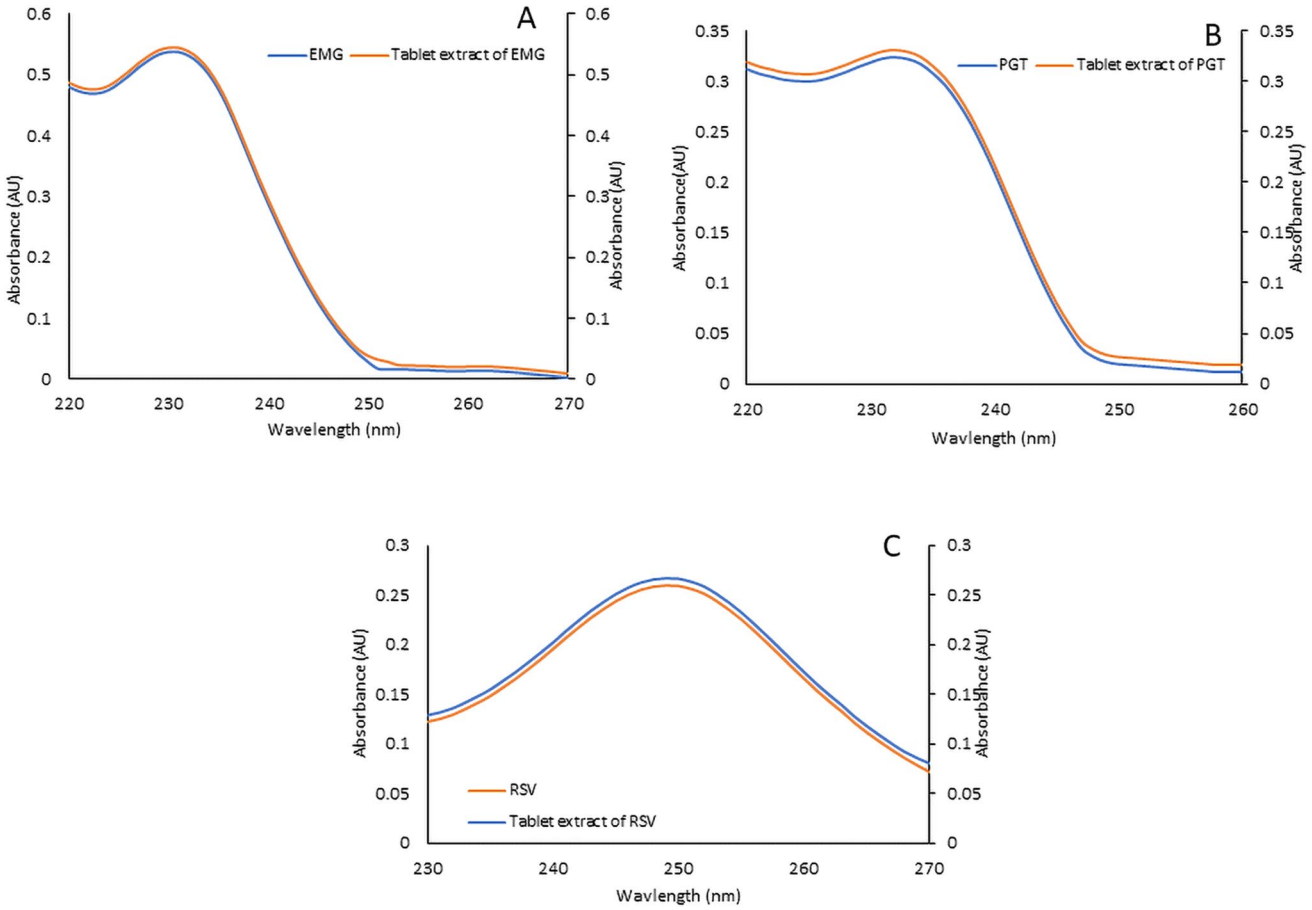


Fig. 5 Spectra comparison of **A** standard solution containing 150 ng per band of (EMG) and tablet extract sample solution 150 ng per band of EMG, **B** standard solution containing 150 ng per band of (PGT) and tablet extract sample solution 150 ng per band of PGT, and **C** standard solution containing 150 ng per band of (RSV) and tablet extract sample solution 150 ng per band of RSV

between tablets and standard mixture solutions, proving the purity and identity of the peaks.

3.3.6 Robustness

Robustness is a measure of method capacity to persist unaltered after minor but intended variations in the parameters of an analytical process. Saturation duration, development distance and ratios of the mobile phase (*n*-hexane−ethyl acetate−methanol−glacial acetic acid) underwent minor alterations that were evaluated. The robustness of the approach illustrated in Table 5 showed good recoveries and low %RSD values.

3.4 Applications of the suggested approach

3.4.1 Pharmaceutical formulations

The proposed HPTLC method was successfully used to identify EMG, PGT, and RSV in the locally produced tablets. The average recovery rates ± RSD for the manufactured tablets for EMG, PGT, and RSV, respectively, were (99.66 ± 1.10), (100.93 ± 0.34), and (101.41 ± 1.71), according to the procedure described in the study. The results of the proposed methods and the outcomes of reported methods [51–53] were compared statistically. The simultaneous study of EMG, PGT, and RSV in their dose forms utilizing the suggested protocol, as given in Table 6, was carried out with good accuracy and precision because no significant differences were detected using applications of *t* test and *F* test at 95% confidence level.

3.4.2 Spiked human plasma

Three medications, EMG, PGT, and RSV, can be detected simultaneously in spiked human plasma using the solid-phase extraction (SPE). Advantages of SPE are low intrinsic costs, little solvent usage, and being a less complex processing method [54]. Calibration curves for the three investigated drugs spiked with human plasma were established.

Table 5 Robustness findings of the proposed TLC method for determining EMG, PGT, and RSV, simultaneously

Parameters	Retardation factor (R_F)			% Recovery		
Traveling distance (4.5 cm ± 0.3 cm)	EMG	PGT	RSV	EMG	PGT	RSV
4.2 cm	0.37	0.75	0.55	97.42	102.06	102.00
4.5 cm	0.36	0.73	0.53	99.45	100.97	101.13
4.8 cm	0.34	0.70	0.51	96.27	101.76	99.67
% Mean recovery				97.71	101.59	100.93
SD				1.61	0.56	1.18
%RSD				1.64	0.55	1.17
Composition of the mobile phase (*n*-hexane−ethyl acetate−methanol−acetic acid) (4.2:4:1.75:0.05, *V/V*)	EMG	PGT	RSV	EMG	PGT	RSV
(4.1:3.9:1.65:0.04)	0.37	0.74	0.56	100.92	98.48	97.67
(4.2:4:1.75:0.05)	0.36	0.73	0.53	100.54	99.87	98.04
(4.3:4.1:1.85:0.06)	0.33	0.69	0.50	98.67	101.54	99.47
% Mean recovery				100.04	99.96	98.39
SD				1.20	1.53	0.95
%RSD				1.20	1.53	0.97
Saturation time of the mobile phase (20 ± 3 min)	EMG	PGT	RSV	EMG	PGT	RSV
17 min	0.35	0.74	0.51	100.98	100.08	97.99
20 min	0.36	0.73	0.53	98.97	101.24	98.73
23 min	0.34	0.69	0.49	101.09	102.35	98.34
% Mean recovery				100.34	101.22	98.35
SD				1.19	1.135	0.37
%RSD				1.18	1.121	0.38

Table 6 Using the proposed method and the reported method, EMP, PGZ ,and ROS dose forms were analyzed ($n = 6$) [51–53]

Tablets	% Recovery ± %RSD[a]		*t* test[b]	*F* test[b]
	Proposed method	Reported method		
Empacoza®	99.66 ± 1.10	101.18 ± 1.02	2.01	3.37
Actozone®	100.93 ± 0.34	102.35 ± 0.73	2.01	2.59
Crestolip®	101.41 ± 1.71	101.72 ± 0.51	2.13	5.46

[a]Average of six determinations

[b]At a 95% confidence level, the theoretical values for the *t* and *F* tests were 2.57 and 9.27, respectively

A linear relationship was found between the integrated peak area and the concentrations of EMG, PGT, and RSV spiked with human plasma over the concentration range of 5−250 ng per band. The LOD and LOQ results were found to be (1.29−3.93), (1.68−5.10), and (1.12−3.38) ng per band for EMG, PGT, and RSV, respectively. Table 7 lists all regression analysis information obtained from spiked human plasma. After examining human plasma samples at three different concentration levels, good recovery percentages and low %RSD values were discovered (Table 8). By showing a high level of drug extraction efficiency from plasma without interference from intrinsic plasma constituents, these results illustrated the selectivity of the method (Fig. 4A and 4B). The results showed that the suggested procedure may be used on real plasma.

3.4.3 Real rat plasma

By measuring the amount of EMG, PGT, and RSV simultaneously in real rat plasma, the effectiveness of the suggested approach was further assessed. After giving a single oral dose of EMG 10 mg/kg, PGT 3 mg/kg, and RSV 200 mg/kg to five male Wistar rats, blood samples were taken. From reported studies of the EMG, PGT, and RSV in rat plasma, EMG (3 mg/kg by oral route) was metabolized by glucuronidation, its C_{max} was 0.167 ± 0.829 µg/mL after T_{max} 2 h and excreted in feces mainly. PGT (3 mg/kg by oral route) was metabolized by hydroxylation and oxidation via liver, its C_{max} was 3.87 ± 0.15 µg/mL after T_{max} 2.67 ± 0.52 h and approximately 15−30% of a PGT dose was removed in the urine as metabolites, with the remainder excreted either unaltered or as metabolites in the bile. RSV (200 mg/kg by oral route) had N-desmethylrosuvastatin, a less potent primary metabolite, its C_{max} was 3.04 µg/mL after T_{max} 0.87 ± 0.29 h and excreted in feces mainly (90%) [46–48]. It was found that the resolution and peak shape of the real samples were comparable to those obtained when utilizing the

Table 7 An overview of the results of linear regression in spiked human plasma and recovery of mentioned drugs in real rat plasma

Parameters	EMG	PGT	RSV
Calibration data in plasma			
Linearity range, ng per band	5–250	5–250	5–250
Slope (b) ± SD	$2.53\times10^{-5}\pm1.34\times10^{-6}$	$1.96\times10^{-5}\pm1.48\times10^{-5}$	$6.14\times10^{-5}\pm1.75\times10^{-5}$
Intercept (a) ± SD	$8.99\times10^{-5}\pm1\times10^{-5}$	$9.05\times10^{-5}\pm1\times10^{-5}$	$1.07\times10^{-4}\pm2.08\times10^{-5}$
Correlation coefficient (*r*)	0.9989	0.9991	0.9981
Limit of detection, ng per band	1.29	1.68	1.11
Analysis of real plasma			
Concentration claimed (ng per band)	6.87	7.43	6.76
Concentration found (ng per band) after T_{max}	6.35	7.03	6.15
% Recovery ± SD	92.57 ± 2.82	94.61 ± 2.12	90.97 ± 3.13

Table 8 Results of the proposed method in spiked human plasma

Drug	Concentration taken (ng per band)	Concentration found (ng per band)	% Amount found (mean ± SD)	%RSD
EMG	50	45.56	91.12 ± 0.32	0.35
	150	138.76	92.51 ± 1.06	1.17
	250	234.67	93.87 ± 1.27	1.39
PGT	50	46.61	93.21 ± 1.13	1.25
	150	138.09	92.06 ± 1.73	1.92
	250	234.35	93.74 ± 1.55	1.70
RSV	50	46.04	92.08 ± 2.15	2.38
	150	140.47	93.65 ± 1.31	1.44
	250	226.57	90.63 ± 1.23	1.35

reference samples, and there were no interference peaks seen (Figs. 4C and 4D). The recovery percentage obtained by dividing the response of samples by that of standards using the same concentration level for both sample and standard was in the range of 90.97 ± 3.13 to 94.64 ± 2.12 (Table 7).

3.5 Greenness evaluation of the developed HPTLC technique and comparison with the previously reported methods

To assess the environmental impact of analytical procedures, additional metrics have been developed in recent years. To compare and evaluate various analytical methods more effectively in terms of their green characteristics, it is often advised to use a variety of color evaluation tools. Three new techniques have been established to evaluate greenness in our research: the analytical eco-scale [55], the green analytical procedure index (GAPI) [56], and the new analytical greenness metric (AGREE) [57].

The analytical eco-scale [55] has the advantage of being semi-quantitative and having a straightforward scoring system. The eco-scale score is determined by deducting from

Table 9 Eco-scale penalty points for the developed HPTLC method for determining EMG, PGT, and RSV simultaneously

Reagents	Penalty points
Solvents	
Methanol	6
Ethyl-acetate	4
n-Hexane	8
Acetic acid	2
Instruments	
Energy used (UV Scanner) 1 (< 1.5 kWh per sample)	1
Occupational hazard	0
Waste	3
Total penalty points	24
Analytical eco-scale score	76, excellent green method

Waste: the volume of the mobile phase/no of spots per TLC plate

100 the number of penalty points awarded to each component (reagent amount and nature, occupational hazard, energy consumption, and amount of created waste) that does not adhere to the ideal green analysis standards. The scores above 75 indicate a very good quality green analysis, the values between 50 and 75 indicate a good green analysis, and the scores below 50 indicate a poor green analysis. The developed methods received an analytical eco-scale score of 76, which makes it a very good green analysis with no adverse environmental impact (Table 9).

The new GAPI index [56] has the advantage of covering the entire analytical process when compared with the analytical eco-scale [55]. There are five pentagrams, each representing a step in the analytical process, such as collecting the samples, preparing the samples, using reactive and solvents in the apparatus, and the objective of the analytical process. GAPI has three color coding systems: red denotes a high environmental risk, whereas yellow and green denote a lower risk and greater greenness. Three other previously

mentioned analytical separation methods [17–19] have been compared with the newly developed HPTLC method. Table 10 displays the GAPI index for the suggested method evaluation along with three published analytical techniques for the examination of the three substances under investigation. All of these methods can identify two of the studied medications; however, our method can identify all three of the studied medications simultaneously. According to Table 10a, the suggested green HTPLC method's GAPI index has nine green, four yellow, and two red pentagrams. The methods shown in Table 10b–d at this time have three red pentagrams. The red areas show a big ecological impact, the yellow areas show a smaller ecological impact, and the green areas show a greater ecological impact.

An additional assessment of greenness was made utilizing the innovative, more inclusive, and informative AGREE tool [57] to produce the most accurate and logical rating possible. The 12 green analytical chemistry (GAC) principles are taken into account by this tool, which is an advantage. As a result, a thorough assessment of each method's green color was made, and a thorough distinction between the developed and reported methods' green characteristics was established. The AGREE tool demonstrated the superiority of our created HPTLC method, which received the best analytical score (0.81) from AGREE while using only 1 mL per sample of waste.

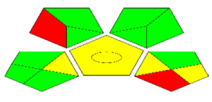
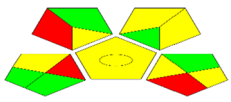
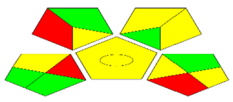
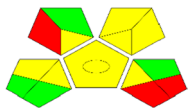
AGREE scores and waste amounts of the mentioned reported methods were 0.67 and 9 mL, 0.66 and 2 mL, and 0.58 and 11.8 mL [17–19] for the reported methods presented in Table 10b–d. All of the pictograms for the developed and reported methods are included in Table 10. Finally, after comparing the three different methods, we found that our method HPTLC is more environmentally friendly than the other methods mentioned.

Furthermore, our developed method is the most sensitive method and used to detect the quantities of the drugs mentioned earlier in spiked human plasma and real rat plasma as the mentioned reported methods have very low sensitivity and all applied in dosage form only.

4 Conclusion

A green, sensitive, and selective HPTLC method coupled with UV detection was developed for the first time to separate and determine the three co-administered drugs: EMG, PGT, and RSV in their ternary synthtic mixture, spiked human plasma samples and real rat plasma with high precision and accuracy results. This mentioned combination lowers plasma cholesterol while also lowering blood sugar levels, effectively managing both diabetes and the resulting dyslipidemia. Using *n*-hexane–ethyl acetate–methanol–acetic acid (4.2:4:1.75:0.05, *V/V*) as the mobile phase and UV detection at 230 nm, the ternary mixture was separated on precoated silica gel TLC plates G60 F254. All of the experimental parameters were optimized for linearity ranges of 5–250 ng per band for EMG, PGT, and RSV and good sensitivity with low limit of detection values. The suggested method offers a number of benefits, including a quick analysis time, a high sample capacity per run, and minimum solvent usage; it also demonstrated excellent sensitivity. The new HPTLC method is green and environmentally safe,

Table 10 Comparison of the developed HPTLC method with some reported separation methods

Variable	Developed method (a)	[17] (b)	[18] (c)	[19] (d)
Technique used	HPTLC-UV	HPLC–UV	HPLC–UV	HPLC–UV
Mobile phase	*n*-Hexane–ethyl acetate–methanol–glacial acetic (4.2:4:1.75:0.05, *V/V*)	Methanol–water (90 : 10, *V/V*)	Orthophosphoric acid buffer–acetonitrile (30:70, *V/V*) pH 2.7	Acetonitrile–0.01% (*V/V*) formic acid buffer adjusted with acetic acid
Linearity range µg/mL	0.005–0.250	5–50	20–250	30–210 for EMG and 50–350 for PGT
Analytes similarity	EMG, PGT and RSV	PGT and RSV	EMG and PGT	EMG and PGT
Application	Dose form, spiked human plasma and real rat plasma	Dose form	Dose form	Dose form
GAPI assessment				
AGREE assessment				

according to the results of the three separate methods used to test greenness.

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Declarations

Conflict of interest The authors declare that they do not have any conflict of interest.

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