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Validated Liquid Chromatographic Method for Simultaneous Determination of Metformin, Pioglitazone, Sitagliptin, Repaglinide, Glibenclamide and Gliclazide - Application for Counterfeit Drug Analysis

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Abstract

A rapid, precise and selective RP-LC method was developed for simultaneous determination of the widely used oral antidiabetic; metformin hydrochloride (MTF), with some commonly prescribed oral antidiabetics, namely; sitagliptin phosphate (SIT), pioglitazone hydrochloride (PGZ), gliclazide (GLZ), glibenclamide (GLB) and repaglinide (RPG). The chromatographic separation carried out using gradient elution mode with acetonitrile: 0.05M potassium dihydrogen phosphate (MKP) and 0.01M sodium octane sulphonate (SOS) (pH 3.55) at flow rate 0.85 ml/min on Kromasil 100-C18, (30 × 0.4 cm, 10 µm) at 40°C. UV detection was carried out at 220 nm. The method was validated according to ICH guidelines. Linearity, accuracy and precision were satisfactory over the concentration ranges (µg/ml) of 0.05-205 for MTF, 0.05-100 for PGZ, GLB and SIT, 0.1-100 for RPG and 1-100 µg/ml for GLZ. The correlation coefficients were >0.99 for all analytes. Limits of quantification (LOQs) found were 0.002, 0.003, 0.009, 0.012, 0.007 and 0.024 µg/ml for MTF, SIT, PGZ, GLZ, GLB and RPG respectively. The developed method is specific and accurate for quality control and routine analysis of the cited drugs in their pharmaceutical preparations. It is recommended for application in the quality control of the herbal antidiabetic products to detect possible counterfeits.

Keywords: Anti-diabetic drugs; Counterfeits; Liquid chromatography; Metformin hydrochloride; Pharmaceutical preparations

Abbreviations: AUP: Area under peak; GLB: Glibenclamide; GLZ: Gliclazide; LOD: Limit of detection; LOQ: Limit of quantitation; MKP: Potassium dihydrogen phosphate; MTF: Metformin hydrochloride; PGZ: Pioglitazone hydrochloride; RPG: Repaglinide; SIT: Sitagliptin phosphate; SOS: Sodium octane sulphonate; MRM: Multiple reaction monitoring; DPP-4: Dipeptidyl peptidase 4

Introduction

Repaglinide (RPG), S(+) 2-ethoxy-4(2((3- methyl-1-(2-(1-piperidinyl) phenyl)-butyl) amino)-2-oxoethyl) benzoic acid (Figure 1A), belongs to the meglitinide class of antidiabetics for the treatment of type 2 diabetes. Metformin hydrochloride (MTF), N,N-dimethylimidodicarbonimidic diamide hydrochloride (Figure 1B) [1], is considered to be the main drug in mixed or combination therapies of diabetes. MTF belongs to the biguanide class of oral anti-diabetic [2]. Sitagliptin phosphate (SIT), 1,2,4-triazolo[4,3-a]pyrazine,7-[(3R)-3-amino-1-oxo-4-(2,4,5-trifluorophenyl)butyl]-5,6,7,8- tetrahydro-3-(trifluoromethyl), phosphate (Figure 1C) [3] belongs to dipeptidyl peptidase 4(DPP-4) inhibitors [4]. Pioglitazone hydrochloride (PGZ), ((RS) -5- (4- [2-(5-ethylpyridin-2-yl) ethoxy] benzyl) thiazolidine-2,4-dione (Figure 1D), is one of the thiazolidinedione antidiabetic that is used either alone or in combination with other oral antidiabetic drugs [2]. Glibenclamide (GLB), 5-chloro-N-[2-[4-[[[(cyclohexyl (amino) carbonyl]-amino]sulphonyl] phenyl] ethyl-2- methoxy benzamide (Figure 1E) [5], and gliclazide (GLZ), 1-(3-azabicyclo[3.3.0]oct-3-yl)-3-(p-tolylsulfonyl) urea (Figure 1F) [6] belong to sulfonylurea drugs that are given orally in the treatment of type 2 diabetes mellitus [7].

Literature survey revealed that MTF and RPG have been simultaneously determined using HPLC methods in rabbit plasma [8] and pharmaceutical formulations [1,9-11]. They have been also simultaneously determined by LC-tandem MS in human plasma [12] and by LC-MS/MS-ESI in rat plasma [13]. Spectrophotometric methods have been reported for the simultaneous estimation of MTF and RPG [14,15]. MTF and SIT have been simultaneously determined using HPLC [4,16-20] and spectrophotometric techniques [3,21-24] and together with degradation products by spectrophotometric and spectrofluorimetric methods [25]. MTF has been simultaneously determined with PGZ by spectrophotometric methods [26,27] and by HPLC in binary mixture [28-31] and with other components by HPLC [32-35]. GLZ has been simultaneously determined with MTF by spectrophotometric methods [36-38] and by LC in dosage forms [39-43] and in human plasma [44,45]. MTF and GLB have been simultaneously determined by spectrophotometry [46,47] and by LC in dosage forms [48-50] and in human plasma [51]. It is worth noting that many mixtures composed of more than two drugs of the present mixture were simultaneously determined using RP-HPLC technique in dosage forms [52,53], in plasma [54] and in both-dosage forms and human plasma or serum [55,56]. Spectrophotometric determinations have been reported for mixtures composed of more than two drugs [57-59]. To the best of our knowledge, no method has been reported for the simultaneous determination of all six components in the mixture of the proposed LC method. Thus, the aim of this method was to develop and validate an analytical method using the most

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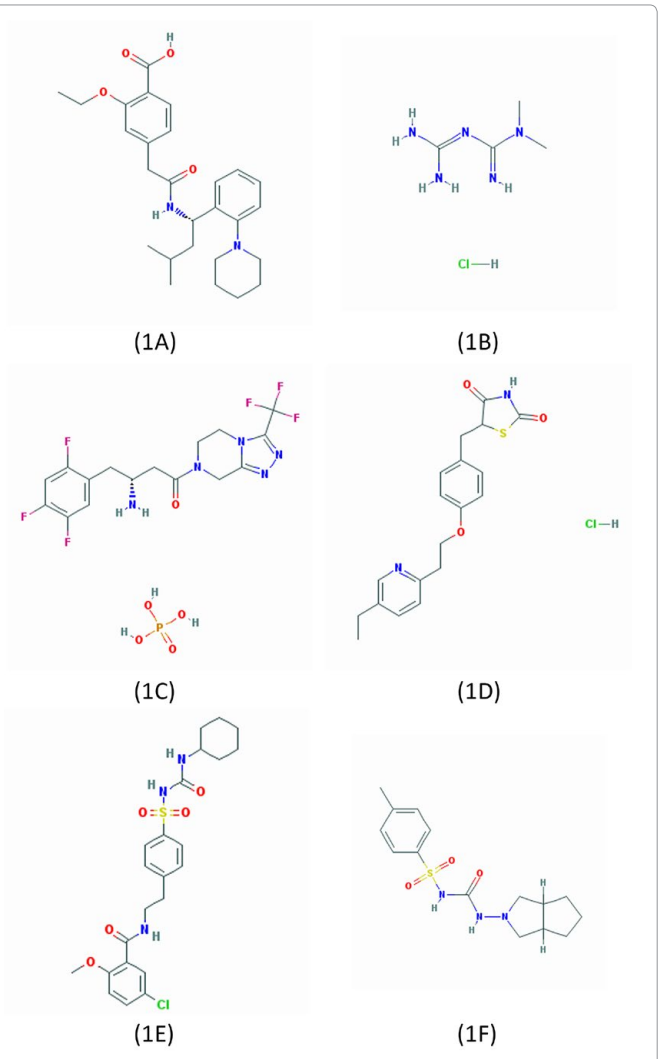

Figure 1: The chemical structures of repaglinide (A), metformin HCl (B), sitagliptin phosphate (C), pioglitazone HCl (D), glibenclamide (E) and gliclazide (F).

preferable RP-LC technique for the simultaneous determination of the well-known oral anti-diabetic metformin hydrochloride, along with commonly prescribed oral antidiabetic drugs from different pharmacological classes as mentioned before, namely; sitagliptin phosphate, pioglitazone hydrochloride, gliclazide, glibenclamide and repaglinide. Examples of pharmaceutical products include, but not limited to, Glucovance 500/5, Januvia 100 (or 50), Janumet 50/500 (or 50/1000) Actos 30, Diamedizen 60, Prandimet 1/500 (or 2/500) and Novonorm 0.5 (or 2). Besides, the proposed method is applied to the detection of counterfeits in herbal antidiabetic therapy.

Materials and Methods

Materials and reagents

All the chemicals used were of analytical reagent grade, and the solvents were of HPLC grade. Elnasr Pharmaceutical Company supplied MTF (certified to contain 99.9%). Nutra Specialties Private limited supplied SIT (certified to contain 99.8%). Mepaco Pharmaceutical Company supplied PGZ (certified to contain 99.2%). Zhejang Jiuzhou supplied GLZ (certified to contain 100.2%). Amrya Pharmaceutical Company supplied GLB (certified to contain 99.5%). Marcyrl Pharmaceutical Company supplied RPG (certified to contain 100.2%). Sodium octane sulphonate (SOS) was obtained from Sd Fine Chemical Limited - India. Acetonitrile was obtained from Poch limited-Product of Poland. Actos® 30 mg tablets; (Takeda Pharmaceutical Company) each labeled to contain 30 mg PGZ, Glucovance® 500/5 mg tablets; (Merck Serono Pharmaceutical Company) each labeled to contain 500 mg MTF and 5 mg GLB, Novonorm® 0.5 mg tablets; (Novonordisk Pharmaceutical Company) each labeled to contain 0.5 mg RPG, Januvia® 100 mg tablets; (MSD Pharmaceutical Company) each labeled to contain 123.9 mg SIT and Diamedizen® 60 mg tablets each labeled to contain 60 mg GLZ; were purchased from commercial sources in local market. Potassium dihydrogen phosphate (MKP), sodium hydroxide and o-Phosphoric acid 85% (Sigma-Aldrich) were used.

Instrumentation

The LC system used was Agilent HPLC system 1100 series equipped with G1311A quaternary pump, G1314A variable wavelength detector and Agilent 1260 Infinity G1322A degasser. Agilent Chemstation PC program was used for the instrument control, data acquisition and analysis. Separation was achieved on Teknokroma; Kromasil 100-=C18, 10 µm, 30 × 0.4 cm. Hanna instruments® pH-meter-Romania was used for pH adjustment of the buffer. Water was purified for HPLC analysis using Purelab-flex water system-UK. Mobile phase was mixed and degassed using Crest® sonicator-USA and filtered through 0.2 µm Sartorius AG® membrane filter.

Chromatographic conditions

Chromatographic separation of the six oral antidiabetic drugs (MTF, SIT, PGZ, GLZ, GLB and RPG) was achieved on Agilent 1100 HPLC system using Teknokroma; Kromasil 100-C18 (30 × 0.4 cm, 10 µm). The mobile phase consisted of acetonitrile (solution A) and buffer (solution B); the latter was composed of 0.05 M MKP and 0.01M SOS, pH adjusted to 3.55 by 85% ortho-phosphoric acid. The mobile phase flow was pumped at 0.85 ml/min through the column adjusted at temperature 40°C on a gradient program as follows; Time/Buffer %: 0/50, 3/35, 7/30, 15/30, 20/50. The injection volume was 20 µl. The effluent was detected at 220 nm. The run time for each injection was 20 minutes. Post run time for equilibration was 4 minutes. Data acquisition was performed on Agilent LC Chemstation software. The retention times were 2.24, 3.13, 6.3, 7.41, 8.41 and 14.32 for MTF, SIT, PGZ, GLZ, GLB and RPG (Figure 2).

Standard stock solutions preparation

Accurately weighed 25 mg of MTF and 10 mg of each of RPG, PGZ, GLZ, GLB and SIT; were separately transferred into 100 ml volumetric flasks, dissolved in and completed to volume with solvent mixture C which consists of equal volumes of acetonitrile and buffer (0.05 M MKP+ 0.01 M SOS, pH: 3.55). (0.25 mg/ml for MTF and 0.1 mg/ml for SIT, PGZ, GLZ, GLB and RPG).

Construction of calibration graphs

Accurately measured aliquots were transferred from the standard stock solutions of MTF, SIT, PGZ, GLZ, GLB and RPG; into separate sets of 10 ml volumetric flasks to prepare concentrations equivalent to 0.05-205 µg/ml for MTF, 0.05-100 µg/ml for SIT, PGZ and GLB, 0.1-100 µg/ml for RPG and 1-100 µg/ml for GLZ. Solvent mixture C was used to complete each flask to mark. The calibration graphs were obtained by plotting the area under the peak versus the concentration of each drug in µg/ml and the corresponding regression equations were derived.

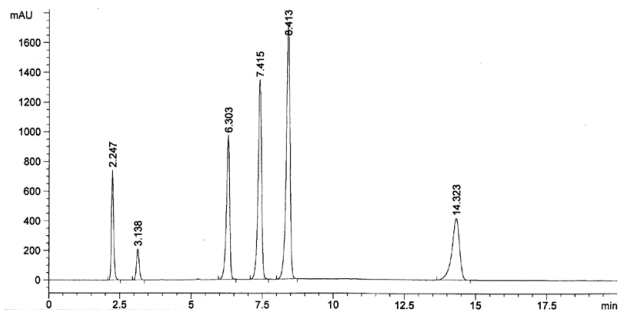

Figure 2: Typical chromatogram of a laboratory prepared mixture of MTF (2.247 min), SIT (3.138 min), PGZ (6.303 min), GLZ (7.415 min), GLB (8.413 min) and RPG (14.323 min) under the described chromatographic conditions.

Assay of cited drugs in bulk

The procedure described under "Construction of Calibration Graphs" was repeated for concentrations equivalent to 15-90 µg/ml for each of MTF, SIT, PGZ, GLB and RPG and 30-90 µg/ml for GLZ. The mean recovery values were calculated using the corresponding regression equations.

Preparation of the laboratory prepared synthetic mixtures

Laboratory prepared mixtures of the six drugs were prepared by mixing aliquots taken from stock standard solutions in the ratio of 100: 24.8 (and 12.4): 6: 12: 1: 0.4 (and 0.1) for MTF, SIT, PGZ, GLZ, GLB and RPG respectively. The volumes were taken into a series of 10 ml volumetric flasks to obtain concentrations of (130-202 µg/ml), (20-50 µg/ml), (7.7-12 µg/ml), (16-25 µg/ml), (1.3-2.2 µg/ml) and (0.5-0.8 µg/ml) for MTF, SIT, PGZ, GLZ, GLB and RPG respectively. Concentrations of the six drugs were calculated using the corresponding regression equations.

Pharmaceutical samples preparation

Ten tablets of each of Glucovance, Januvia, Actos 30, Diamedizen 60 and Novonorm 0.5 were separately weighed, powdered using mortar and pestle to fine particle size. An amount of the powder equivalent to 500 mg, 123.9 mg, 30 mg, 60 mg, 5 mg and 0.5 mg for MTF, SIT, PGZ, GLZ, GLB and RPG; respectively were separately weighed and transferred into a set of 100 ml volumetric flasks. Approximately 50 ml of solvent mixture C were added, sonicated for 10 minutes with frequent shaking. Solutions were diluted to volume with solvent mixture C and were filtered through 0.45 µm filter paper. The first 10 ml of the filtrates were discarded then the filtrates were diluted to the appropriate concentrations (µg/ml) (10-180), (5-96), (29-90), (30-95), (5-75) and (1.5-72) for MTF, SIT, PGZ, GLZ, GLB and RPG respectively. Standard addition technique was also carried out. The mean recovery values of the pharmaceutical products and added standards were calculated using the corresponding regression equations.

Herbal samples preparation

Four Samples of herbal bulk powders and tea bags (Figure 3A-3D) that were under suspicion of adulteration with undeclared synthetic drugs were purchased from local herbal druggists and retail outlets. According to the labels on the boxes, the products contain herbal ingredients and were declared as anti-diabetic and anti-obesity formulations. These products were mainly from suburbs of Cairo.

One teaspoonful from each of the 3 herbal bulk powders and one teabag from the fourth sample were each transferred into a 100 ml volumetric flask, extracted in 50 ml solvent mixture C by sonication for 10 minutes with frequent shaking, then diluted to volume with the same solvent, mixed and filtered through 0.45 µm filter paper. The first 10 ml of the filtrates were discarded then an aliquot of 2 ml was transferred from each preparation to a series of 10 ml volumetric flasks and diluted to volume with solvent mixture C. Another series of corresponding dilutions that were spiked with 10 µg/ml MTF, SIT, PGZ, GLZ, GLB and RPG were also prepared.

Results and Discussion

The main concern of this work was to develop a fast and validated LC method suitable for the determination of some well-known antidiabetic MTF, along with commonly prescribed oral antidiabetic drugs from different pharmacological classes. Besides, the proposed method will be useful for the detection of counterfeits in antidiabetic therapy.

Optimization of chromatographic conditions

During the optimization cycle, different columns with different lengths and internal diameters were tried namely, Waters symmetry C18 (30 × 0.4 cm, 10 µm) and Supelco supelcosil C18 (25 × 0.46 cm, 5 µm) but finally satisfactory separation was obtained on Teknokroma kromasil 100-C18 (30 × 0.4 cm, 10 µm) column. Methanol and acetonitrile were examined individually and simultaneously as organic modifiers and acetonitrile was found to be more suitable, individually, as it allowed better separation of the six analytes under investigation. Isocratic mode of elution with different ratios of organic to aqueous phases was tried and it was found unsuitable so gradient elution mode was attempted. Different gradient elution modes were tested in order to achieve proper separation of the cited analytes in a reasonable run time. The described gradient elution mode stated in section 2.3 was selected. The use of 0.05M MKP buffer was necessary in this method in order to influence the ionization of the analytes and to help in their co-elution. Also, it kept the pH constant as each of MTF, PGZ, GLZ, GLB and RPG is obviously affected by the mobile phase composition and pH. The effect of pH on the separation of the analytes was studied. It was found that pH higher than 4.59 was not suitable as due to improper separation of the analyzed compounds. pH was adjusted at 3.55 for the best separation of the six analytes in a reasonable run time (<15 min) and with good resolution between all peaks (Figure 2). Different flow rates were studied and flow rate of 0.85 mL min^{-1} was found to be optimum. Quantitation was achieved with UV-detection at 220 nm. Many trials were done to enhance symmetry and sharpness of peaks and hence improve the resolution of the cited drugs. Addition of 10mM sodium octane sulphonate, as ion pairing agent, produced significant improvement. The use of optimized mobile phase containing acetonitrile and buffer (0.05 M MKP + 0.01 M SOS, pH: 3.55) (50:50 *v/v*) instead of methanol as a solvent led to good peaks` symmetry, sharpness and best resolution between the cited analytes (Figure 2). The retention times for MTF, SIT, PGZ, GLZ, GLB and RPG were 2.24, 3.13, 6.30, 7.41, 8.41 and 14.32 min respectively. The system suitability tests were used to verify that the conditions of the chromatographic system were adequate for the separation and hence for the analysis [60] (Table 1).

Validation of the method

Validation of the proposed RP-HPLC method was performed with respect to linearity, limit of detection (LOD), limit of quantitation (LOQ), accuracy, precision and robustness according to ICH guidelines [60].

Linearity: Linearity was assessed for the six oral antidiabetic drugs at concentration ranges 0.05-205 µg/ml for MTF, 0.05-100 µg/ml for SIT, PGZ and GLB, 0.1-100 µg/ml for RPG and at 1-100 µg/ml for GLZ. A linear relationship was established at these ranges between Area under the peak (AUP) and concentration. Good linearity was proved by high values of coefficient of determinations. The calibration data are shown in Table 2.

Limit of detection (LOD) and Limit of quantitation (LOQ): According to ICH Q2 (R1) recommendations [60], LOD was considered as the minimum concentration with a signal to noise ratio of 3, while LOQ was taken as a minimum concentration with signal to noise ratio of 10. LOD and LOQ were calculated experimentally. Results are shown in Table 2.

Accuracy and precision: Accuracy of the method was proved by recovery study of each of MTF, SIT, PGZ, GLZ, GLB and RPG in bulk, in their synthetic mixtures of their medicinally recommended ratios and by standard addition technique. Results of recovery tests are summarized in Table 2. Repeatability (intra-day) and intermediate precision (inter-day) were assessed using three concentrations and three replicates of each concentration. The relative standard deviations were found to be small indicating reasonable repeatability and intermediate precision of the proposed method (Table 2).

Robustness: Robustness test was performed by applying small deliberate changes to the chromatographic conditions. The most important parameters studied under this test were flow rate (changed by ± 0.1), mobile phase composition-acetonitrile percent (changed by ± 2 in all levels of the gradient program) and pH value of the buffer component of the mobile phase (changed by ± 0.1). Each of the previous parameters were changed while keeping the other parameters constant. The resolution factors between each two successive peaks were calculated for each change. The resolution factors under different parameters of robustness are summarized in Table 1. Robustness test revealed good values of resolution that were not significantly changed by minor changes in the chromatographic conditions, which indicates the good robustness of the proposed LC method.

Application to detection of counterfeits: By comparing the chromatograms of the herbal products (Figure 3A-3D) with the chromatogram of the standard solution of the cited antidiabetics (Figure 3E), the presence of a peak at the retention times of MTF in herbal products (1-3) was observed. Besides, the presence of a small peak at the retention time of SIT was also observed in the chromatogram of herbal products (1, 3, and 4). MTF is relatively cheap so the adulteration with MTF maybe suspected and further investigation is suggested to assure the adulteration with MTF and SIT with different spectroscopic techniques as MS, NMR and IR spectroscopy. Besides, the investigated herbal products were spiked with MTF and SIT among the other components; PGZ, GLZ, GLB and RPG which increased the height of MTF peak at 2.2 min for herbal products (1-3) and increased the height of SIT peak at 3.1 min for herbal products (1,3,4). Spiking the herbal extracts with the standard materials confirmed suitability of detection of the investigated antidiabetics in the present study in the herbal matrix. Herbal products (1-3) were subjected to mass spectrometric analysis to confirm or deny adulteration with SIT and MTF that were suspected due to the presence of peaks at their respective retention times in HPLC-UV analysis. Adulteration in herbal products 1 and 2 was denied by the mass spectrometric analysis. Meanwhile, adulteration of herbal product 3 with MTF was confirmed due to the presence of an intense peak of metformin in the multiple reaction monitoring (MRM) chart (Figure 3F). Quantitation was carried out by a pre-developed method for MTF. It was calculated that each 1 g of herbal product 3 is equivalent to ca. 40 mg of MTF. As the matrix of the herbal product was not available, selectivity study of the developed method in the herbal matrices was not feasible.

Conclusion

A quick, selective, specific and validated RP-LC method for the simultaneous determination of six commonly used oral antidiabetics, namely; metformin hydrochloride, sitagliptin phosphate, pioglitazone hydrochloride, gliclazide, glibenclamide and repaglinide. This method is characterized by simplicity, accuracy, preciseness besides its wide range of applications and it can be used for routine analysis and quality control of the cited drugs separately or in combinations in many dosage forms. The developed method proved suitability for the detection of counterfeit drugs.

Parameter			Components/Data					
			MTF	SIT	PGZ	GLZ	GLB	RPG
N			5790	6217	16037	20063	21138	15655
Tailing factor (T)			1.449	1.234	1.04	1.057	1.014	0.934
% RSD of 6 injections			0.0157	0.298	0.531	0.7480	0.1262	0.333
R	Normal		-	6.46	16.2	6.23	4.41	17.45
	pH of buffer (± 0.1)	3.45	-	6.28	15.67	6.97	4.45	16.91
		3.65	-	6.46	16.79	5.59	4.49	17.79
	Flow rate (± 0.1)	0.75	-	6.79	16.22	6.26	4.39	17.88
		0.95	-	6.14	16	6.11	4.45	17.11
	Acetonitrile % (± 2)	52%	-	5.46	15.99	5.66	3.87	16.87
		48%	-	7.26	15.84	6.69	4.88	17.8

(N: Number of theoretical plates; R: Resolution factor; T: Tailing factor; RSD: Relative standard deviation, Flow rate (ml/min))

Table 1: System suitability and Robustness tests for the proposed LC method.

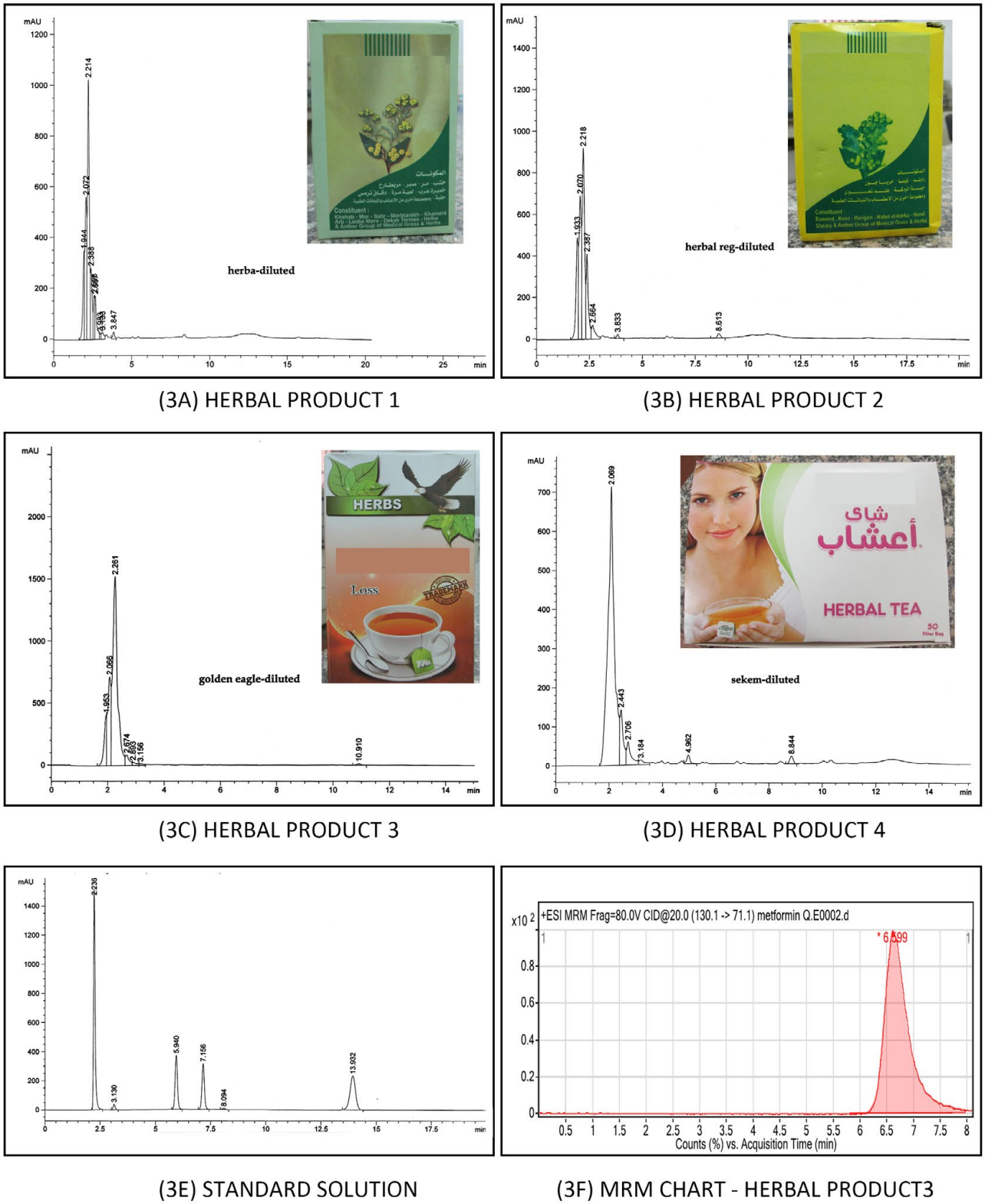

Figure 3: LC chromatograms of the studied herbal products; (A) herbal product 1, (B) herbal product 2, (C) herbal product 3, (D) herbal product 4, (E) standard solution, (F) MRM chart for herbal product 3.

Parameter		Components/Data					
		MTF	SIT	PGZ	GLZ	GLB	RPG
Linearity range*		0.05-205	0.05-100	0.05-100	1-100	0.05-100	0.1-100
Retention time		2.24	3.13	6.30	7.41	8.41	14.32
Wavelength of detection		220 nm					
Regression equation		AUP_{MTF}=64.2C*+33.34	AUP_{SIT}=5.77C+0.44	AUP_{PGZ}=47.17C-19.55	AUP_{GLZ}=39.13C-29.4	AUP_{GLB}=69.23C-1.41	AUP_{RPG}=52.89C-3.41
Correlation coefficient (R^2)		0.9996	0.9999	0.9993	0.9997	0.9999	0.9999
LOD*		0.001	0.001	0.003	0.004	0.002	0.007
LOQ*		0.002	0.003	0.009	0.012	0.007	0.024
Sb*		0.457	0.022	0.427	0.282	0.175	0.198
Sa*		21.463	1.046	20.089	15.867	8.23	9.816
Precision	Intraday R.S.D %	0.011-0.092	0.036-0.64	0.05-0.22	0.035-0.23	0.077-0.36	0.03-0.45
	Interday R.S.D%	0.32 -0.71	0.5-2.0	0.7-1.8	0.5-1.6	0.18-1.9	0.2-1.6
Accuracy	Drug in bulk	100.9 ± 0.737	99.9 ± 0.921	100 ± 1.969	101.5 ± 0.621	100.1 ± 1.27	101 ± 0.364
	Drug in lab prepared mixture	101.2 ± 0.643	100.1 ± 0.75	100.9 ± 0.932	98.7 ± 0.385	100.4 ± 1.501	100.2 ± 1.578
	Drug in dosage form*	102% ± 0.033	91.7% ± 0.024	95.2 ± 3.118	100.1% ± 0.013	104% ± 0.009	101.3% ± 0.033
	Drug added	100.9% ± 1.481	99.5% ± 1.11	98.9% ± 0.008	100.3% ± 1.343	99.7% ±1.246	98.9% ± 1.218

(Linearity range (µg/ml); LOD (µg/ml); LOQ (µg/ml)), C: Concentration (µg/ml), Sa: Standard error of intercept, Sb: Standard error of slope; Dosage form: mentioned for each component in section 2.1 and for preparation technique in section 2.8

Table 2: Characteristics and Results of the Proposed RP-LC Method.

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