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Eco-friendly micellar ultra-performance liquid chromatography (UPLC) method for simultaneous determination of non-communicable diseases drugs in bulk powder, spiked human plasma and in individual dosage forms

Manal M. Fouad[1, 2], Noha S. Rashed [1] and Asmaa I. Hosameldin[1*]

[1]Pharmaceutical Analytical Chemistry Department, Faculty of Pharmacy (Girls), Al-Azhar University, Nasr City, Cairo 11651, Egypt.
[2]Analytical Chemistry Department, Faculty of Pharmacy, October University for Modern Sciences and Arts (MSA), 6th October City, Giza, 11787, Egypt.

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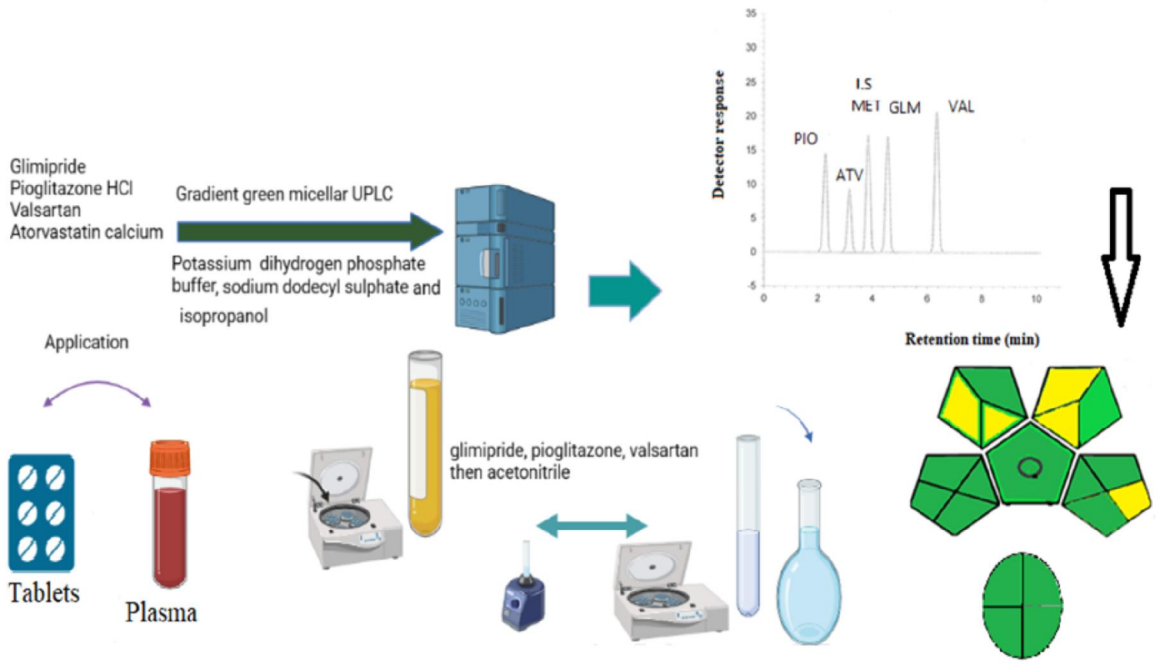
Abstract

Green, simple and accurate micellar UPLC method was established and validated for quantitative determination of glimepiride, pioglitazone HCl, Valsartan and atorvastatin calcium trihydrate in bulk powder, tablets and spiked human plasma. Gradient separation was achieved on Kinetex 1.7µ C_{18} 100A (2.1-mm × 50-mm) at ambient temperature using ecofriendly gradient mobile phase composed of a mixture of 0.05 M potassium dihydrogen phosphate buffer pH 5.00 (A) with 0.10M sodium dodecyl sulfate and isopropanol (15.00-25.00%) (B) at flow rate of 0.20 mL/minute and UV detection at 230.00 nm using metformin as internal standard. The method was linear in the range of 0.50–25.00 µg/mL. The proposed method was effectively applied for the determination of glimepiride, pioglitazone HCl, valsartan and atorvastatin in bulk powder, pharmaceutical dosage forms and human plasma with good accuracy and precision. The results were compared with those of the reported methods and were found to be in good agreement. The proposed method was validated according to ICH guidelines.

Keywords
Glimepiride; pioglitazone HCl; valsartan; atorvastatin calcium trihydrate; UPLC.



*** *Correspondence**: *Asmaa I Hosameldin*
***Tel.:** +2010184568987*
***Email Address:** drasma13687@yahoo.com*

1. Introduction

Recently, noncommunicable diseases sharing similar risk factors represent a prominent threat concerning human health and development. Diabetes, hypertension, and hyperlipidemia have been identified as common modifiable risk factors of cardiovascular disease, frequently occurring together, especially among older people. Accordingly, our work aims at simultaneous determination of drugs combinations used to treat such diseases.

Glimepiride (GLM) is 1-[[p-[2-[3-ethyl-4-methyl-2-oxo-3-pyrrolinepyrroline-1-carboxamido] ethyl]-phenyl]-sulfonyl]-3-[trans-4-methylcyclohexyl] urea]. It is an oral anti-diabetic drug of sulfonylurea class, effective at low doses in patients with non-insulin-dependent diabetes mellitus [1]. Pioglitazone hydrochloride (PIO) is [[±]-5-[[4-[2-[5-ethyl-2-pyridinyl] ethoxy] phenyl] methyl]-2, 4-] thiazolidine-dione monohydrochloride. It is an oral anti-hyperglycemic agent that decrease insulin resistance, used in treatment of type-II diabetes mellitus [1]. Valsartan (VAL) is chemically "2S-3-methyl-2-[N-([1-tetrazol-5-yl)phenyl]phenyl]methyl)pentanamido] butanoic acid" . It is a potent anti-hypertensive drug classified as an angiotensin II receptor blocker that blocks the effect of angiotensin II in the rennin–angiotensin system and furthermore delaying the progress of chronic heart failure [2]. Atorvastatin calcium trihydrate (ATV); [(3R,5R)-7- [3-(phenyl carbamoyl)-5-(4-fluorophenyl)-2-isopropyl 4-phenyl-1H-pyrrol-1-yl]- 3,5-dihydroxyheptanoic acid, calcium salt] is an anti-hyperlipidemic drug acting by inhibiting the enzyme (HMG-co A) reductase [3]. **Figure 1**.

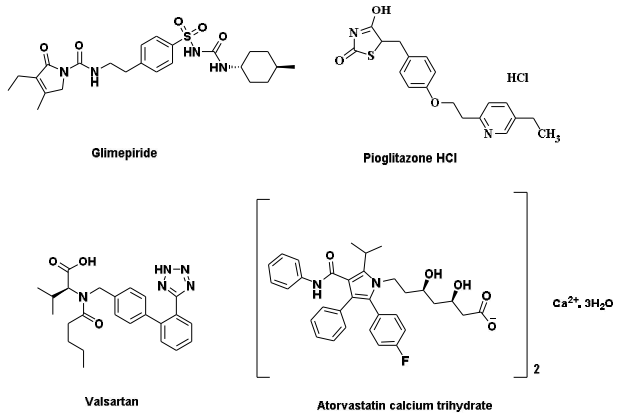


Figure 1. Chemical structure of glimepiride, pioglitazone HCl, valsartan and atorvastatin calcium trihydrate.

Different analytical methods have been published for the determination of GLM and PIO either alone or in combination including spectrophotometric [4-7], spectrofluorometric[8], HPLC [1,9-15], TLC [1,16,17]. Several methods have been used for determination of VAL [2,18-27], whereas ATV was determined by spectrophotometric [3,28], spectrofluorometric [29], HPLC [24,30-32], TLC [33]and electrochemical [34] methods.

UPLC technology takes full advantages of chromatographic principles to run separations. As, compared to HPLC, the decrease of the length of the column, reduces solvent consumption and saves time. So nowadays in industrial area UPLC refers for some of the most recent work field mixtures [35]. The advantages obtainable by micellar mobile phases, include the smaller concentration of organic solvent in the mobile phase which lead to low cost, low toxicity, low volatility, the ability to distinct various hydrophobic and hydrophilic drugs, the high solubilizing action of micelles and the ability to be applied for complex sample matrices as biological fluids [36].

A literature survey revealed that no method has been used for the simultaneous determination of GLM, PIO, VAL and ATV.

In the present work, the proposed method utilized the power of green micellar UPLC technique in determination of GLM, PIO, VAL and ATV in tablets and in plasma.

2. Experimental

2.1. Pure samples

GLM pure sample was kindly obtained from SEDICO Pharmaceutical Company 1st. industrial zone, 6th of October City, Cairo, Egypt. The purity was 99.96% as mentioned by the supplier.

PIO , VAL and metformin (MET) (used as internal standard) pure samples were kindly supplied by Amoun Pharmaceutical Industries Company 1st industrial zone Elobour City, Cairo, Egypt. Their purity were 99.95%, 99.97% and 98.97as stated by the supplier.

ATV pure sample was kindly supplied by Egyptian International Pharmaceutical Industries Co. (EIPICO), 10th of Ramadan City, Cairo, Egypt with a purity of 99.95% as stated by the supplier.

2.2. Pharmaceutical preparations

All pharmaceutical formulations were purchased from local market.

Amaryl® 2 mg (Batch No. BEG024) each tablet claimed to contain 2.00 mg of GLM manufactured by Sanofi aventis, 1st industrial zone, Elobour city, Cairo, Egypt.

Diabitonorm®45 mg (Batch No. B05820521) each tablet claimed to contain 45.00 mg of PIO manufactured by October pharma S.A.E.6 October City, Egypt.

Amaglust®30 /4 (Batch No. 2009678), each tablet contain 4.00 mg of GLM and 30.00 mg of PIO, manufactured by EVA pharma for pharmaceutical and medical appliances for Next pharma, Cairo, Egypt.

Valsatens® 80 mg (Batch No. 200709) each tablet claimed to contain 80.00 mg of VAL manufactured by Amoun Pharmaceutical Industries Company 1st industrial zone, Elobour city, Cairo, Egypt.

Ator® 40 mg (Batch No. 2100035) each tablet claimed to contain 40.00 mg ATV product of Egyptian International Pharmaceutical Industries Co. 10th of Ramadan City, Cairo, Egypt.

2.3. Standard solutions

Stock solutions (0.10 mg/mL) were prepared by dissolving 10.00 mg of each drug in 25 mL methanol and completed to 100 mL with methanol.

2.4. Chemicals and reagents

All reagents used were of analytical grade and solvents were of HPLC grade.

Distilled water used was recently prepared.

Methanol and isopropanol were of HPLC grade (Fisher HPLC grade,UK).
Potassium dihydrogen phosphate (Fisher, UK).
Sodium dodecyl sulfate (SDS) was from Fluka, Buchs, Switzerland.
Blank human plasma samples were friendly obtained from Alazhar University Hospitals and kept frozen at -5°C until use after gentle thawing.

2.5. Instruments

Agilent 1100 Ultra HPLC with binary pump and UV detector (USA).
Kinetex C_{18} column (2.1×50 mm, 1.7 µm) Merck, Germany.
Vortex mixer (IVM-300p, Taiwan) used for plasma sample preparation.
TDL-60B Centrifuge (Anke, Taiwan)
pH meter 3510 (Jenway, USA).
BHA-180T ultrasonic bath (Abbotta, USA).

2.6. Procedures

Chromatographic conditions

Gradient separation was done on Kinetex 1.7µ C_{18} 100A (2.1-mm × 50-mm) at ambient temperature using micellar mobile phase consists of filtered and degassed mixture of 0.05 M potassium dihydrogen phosphate buffer with 0.10M (SDS) of pH 5.00 (A) and isopropanol (B). The mobile phase was filtered through 0.45 µm Millipore membrane filter and degassed by sonicator before use.

The mobile phase was pumped at flow rate of 0.20 mL/minute and UV- detection was carried out at 230.00 nm using MET as internal standard (IS).
Gradient programmed composition consisted of 0-3 min 15% mobile phase B; 3-10 min gradient up to 25% mobile phase B.

Method validation

Linearity

Volumes of standard solutions (100.00 µg/mL) covering (5.00–250.00 µg) of each drug were transferred separately into a series of 10- mL volumetric flasks, 2.50 mL of MET stock solution (100.00 µg/mL) was added as an IS to each flask and adjusted to volume with methanol to obtain final concentrations of 0.50-25.00 µg/ mL for each drug. One µL of each solution were injected into the column under described conditions. Calibration graphs were constructed by plotting the peak area ratio (drug / IS) versus the equivalent drug concentration in µg/mL and corresponding regression equation was derived.

Selectivity

Selectivity of the proposed method was assured by the application of laboratory prepared mixtures of the cited drugs at different concentrations within the linearity range (0.50-25.00 µg/mL). Recovery % of each drug was calculated.

Application to pharmaceutical preparations

Ten Amaryl® 2 tablets each labeled to contain 2.00 mg GLM or Ten Diabitonorm®45 mg tablets each labeled to contain 45.00 mg of PIO were accurately weighed, crushed and mixed well. An exact amount of each powder corresponding to 10.00 mg of the drugs was transferred into separate 100-mL volumetric flasks and the volume was completed to the mark with methanol then sonicated for 15.00 minutes before filtration. The clear filtrate which contain 100.00 µg/mL of each drug was examined by the suggested UPLC method and the drug concentrations were determined.
Ten Amaglust ® tablets each labeled to contain 4.00 mg GLM and 30.00 mg PIO were accurately weighed, crushed and mixed well. An exact amount of the powder equal to 60.00 mg PIO together with 8.00 mg GLM was transmitted into a 100-mL volumetric flask and the volume was made to the mark with methanol. The flask was sonicated for 15 minutes then filtered. The clear filtrate which contain 80.00 µg/mL of GLM and 600.00 µg/mL of PIO was analyzed by the proposed UPLC method to determine the drugs concentrations.
Ten Valsatens® tablets each labeled to contain 80.00 mg of VAL or ten Ator ® tablets each labeled to contain 40.00 mg ATV were precisely weighed, ground and mixed well. Quantity of the powders equivalent to one tablet was introduced into two distinct 100-mL volumetric flasks and the volume was completed to the mark with methanol. The flasks was sonicated for 15 minutes then filtered. The clear filtrates claimed to contain 800.00 µg/mL VAL or 400.00 µg/mL ATV were further diluted with methanol to obtain solutions labeled to contain 100.00 µg/mL of each drug which were analyzed by the proposed UPLC method as mentioned above and the drugs concentrations were obtained from the corresponding regression equation.

Analysis of spiked human plasma

1.00 mL aliquots of human plasma were transmitted into a series of 10-mL centrifugation tube, spiked with (3.50-5.30 µg) for GLM, (2.50–12.00 µg) for PIO or (3.50-35.00 µg) for VAL followed by 3.00 mL acetonitrile. The tubes were mixed for 1.00 min using a vortex mixer followed by centrifugation for 30.00 min. at 4000 rpm. The obtained supernatant was vaporized under vacuum and then accurately transmitted into a series of 10-mL volumetric flasks. To each flask, 0.30 mL of MET (IS) standard solution (100.00µg/mL) was added and completed to the volume with methanol. Blank samples were prepared similarly. The prepared samples were analyzed following the procedure described before.

3. Results and discussion

The proposed method was concentrating chiefly on using green mobile phase for UPLC analysis for the simultaneous determination of GLM, PIO, VAL and ATV in bulk powder, spiked human plasma and in individual dosage forms.
In micellar LC, the main changes in the observed chromatographic performance are due to the adsorption of surfactant monomers on the stationary phase. The migration order of analytes can be interpreted in terms of the electrostatic interaction between analytes and the SDS monomers adsorbed on the stationary phase [36].
Gradient separation was carried out on Kinetex C_{18} column using a mobile phase composed of mixture of 0.05M potassium dihydrogen phosphate buffer with (0.10M SDS) at pH 5.00 (mobile phase A) and isopropanol (mobile phase B) at flow rate 0.20 mL/ min and UV detection at 230.00 nm.
Different conditions affecting the chromatographic separation were optimized after taking in consideration the resolution between the four drugs. Isocratic separation was used first but long time was needed so gradient separation was carried out. Different organic modifiers were tested including methanol, ethanol and isopropanol. The best one was isopropanol showing acceptable resolution and efficiency within a small run time (less than 7.00 min). The outcome of change of isopropanol

concentration on the separation of the analytes was summarized in **Table 1**. Different wavelengths (210.00-250.00 nm, at 10 nm interval) were tried to select the most suitable one where 230.00 nm showed the highest sensitivity. The pH of the mobile phase was altered in the range of 4.50–5.50, pH 5.00 was found to be the optimum pH showing well-resolved symmetrical peaks with the greatest number of theoretical plates and maximum resolution within a small run time. The effect of the change of SDS concentrations (0.05–0.15 M) was studied, 0.10 M SDS was sufficient for giving the greatest number of theoretical plates and best resolution. A flow rate was optimized at 0.20 mL/min due to the highest efficiency, higher resolution and highest number of theoretical plates, **Table 1**. System suitability test parameters were done throughout the method development to confirm that the system is functioning successfully during the analysis. The test was performed by injecting the standard drug solution in five replicate and the parameters were calculated as reported by the ICH guidelines [37]. The final System suitability test parameters including tailing factor (T), column efficiency (number of theoretical plates, N), Resolution (Rs) and Selectivity factors (α) are summarized **in Table 1**.

Figure 2 demonstrates a typical chromatogram for the prepared mixture of GLM, PIO, VAL and ATV under the optimum conditions showing good-separated symmetrical peaks.

Parameters	GLM					PIO					VAL					ATV				
Robustness	**K**	**N**	**R**	**ά**	**T**	**K**	**N**	**R**	**ά**	**T**	**K**	**N**	**R**	**ά**	**T**	**K**	**N**	**R**	**ά**	**T**
Isopropanol																				
(13-23)%	3.49	5012	9.02	8.07	0.82	2.66	5541	6.33	7.85	0.83	2.88	6944	8.20	7.61	0.90	3.02	6551	7.07	7.30	0.88
(15-25)%	3.54	5128	9.12	8.11	0.83	2.79	5633	6.46	7.91	0.88	2.93	7065	8.22	7.65	0.92	3.13	6689	7.11	7.33	0.90
(17-27)%	3.50	4998	9.04	8.03	0.85	2.61	5434	6.38	7.89	0.86	2.86	6991	8.18	7.57	0.88	3.05	6508	7.09	7.28	0.86
pH																				
4.50	3.48	4973	8.99	8.02	0.81	2.70	5364	6.36	7.82	0.81	2.84	6921	8.19	7.56	0.89	3.04	6469	7.06	7.23	0.82
5.00	3.54	5128	9.12	8.11	0.86	2.79	5633	6.46	7.91	0.88	2.93	7065	8.22	7.65	0.92	3.13	6689	7.11	7.33	0.90
5.50	3.44	5011	9.01	7.99	0.79	2.67	5429	6.31	7.84	0.84	2.81	6899	8.16	7.59	0.87	3.01	6499	7.02	7.27	0.84
Flow rate																				
0.18	3.50	5067	9.10	8.08	0.83	.722	5512	6.40	7.88	0.85	2.89	6954	8.20	7.63	0.90	3.06	6582	7.08	7.29	0.87
0.20	3.54	5128	9.12	8.11	0.85	2.79	5633	6.46	7.91	0.88	2.93	7065	8.22	7.65	0.92	3.13	6689	7.11	7.33	0.90
0.22	3.52	5009	9.09	8.10	0.85	2.74	5499	6.43	7.86	0.87	2.91	6982	8.18	7.60	0.91	3.09	6604	7.10	7.31	0.89
SDS concentration (M)																				
0.05	3.48	4973	8.99	8.02	0.81	2.71	5362	6.36	7.82	0.81	2.84	6921	8.19	7.56	0.89	3.04	6469	7.06	7.23	0.82
0.10	3.52	5135	9.15	8.12	0.86	2.78	5629	6.46	7.91	0.88	2.93	7065	8.22	7.65	0.92	3.13	6689	7.11	7.33	0.90
0.15	3.50	5009	9.01	8.05	0.85	2.72	5491	6.42	7.86	0.85	2.90	6981	8.17	7.60	0.90	3.09	6604	7.10	7.31	0.89

Table 1. Robustness results of GLM, PIO, VAL and ATV by the proposed UPLC method.

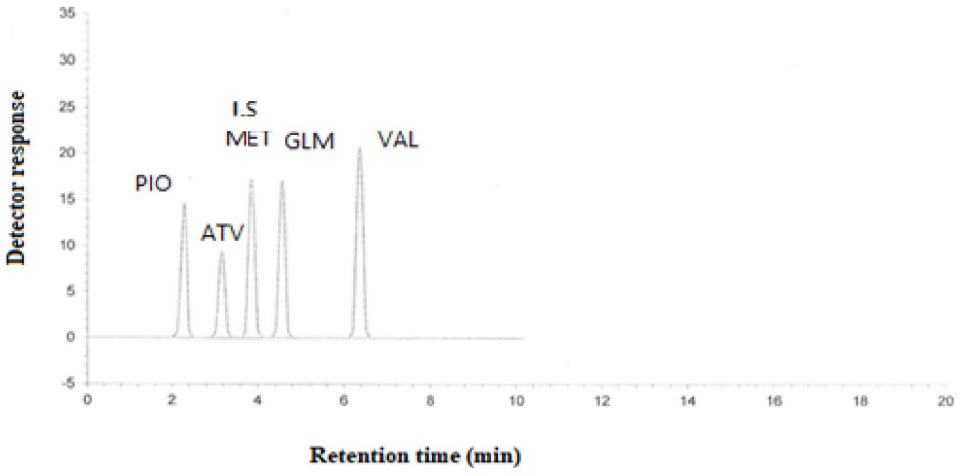


Figure 2. UPLC chromatogram of a prepared mixture of GLM, PIO, VAL and ATV (15.00µg/mL) of each using 25.00 µg/mL of MET as IS

3.1. Method validation

The proposed method was validated according to ICH guidelines [37].

Linearity

The peak area ratios (drug/IS) were plotted against the corresponding drug concentration under the prescribed conditions, good linear relationships were obtained over the range of 0.50–25.00 µg/mL for all drugs; Data were summarized in **Table 2**.

Limit of detection (LOD) and limit of quantitation (LOQ)

LOD and LOQ were determined using the following equations:
$LOD = 3.3\ \sigma / S$
$LOQ = 10\ \sigma / S$
Where σ is the residual standard deviation of a regression line and S is the slope of the calibration curve. The results are summarized in **Table 2**. The small values of LOD and LOQ indicate good sensitivity.

Accuracy

Accuracy was assessed as % recovery of drug concentrations. The obtained values are summarized in **Table 2**; the good R% confirms excellent accuracy.

Precision

Three different concentrations of each drug were determined in the same day and in three successive days to estimate intraday and inter-day precision. The precision as percent relative standard deviation (RSD %) were estimated as shown in **Table 2**, the small RSD % confirms the precision of the method.

Selectivity

Selectivity of the proposed method was assessed by the analysis of different synthetic laboratory prepared mixtures containing different ratios of the studied drugs within their linearity ranges. Satisfactory results were obtained and shown in **Table 3**.

Robustness

It was assessed by studying the capacity of the developed method to continue unaffected by minor but deliberate deviations in a definite method parameters as change of pH of the mobile phase (5.00 ± 0.10), isopropanol concentration (± 0.50%v/v) and SDS concentration (0.10± 0.01 M). These variations did not cause important change of the peak area of drugs confirming robustness of the procedure.

3.2. Applications

3.2.1. Application to dosage forms

The suggested method was effectively used for GLM, PIO, VAL and ATV analysis in their tablets; **Figure (3)**. **Table 4** showing good consistent of the obtained results with those obtained by the reported method. [10, 26]. Statistical study of the results obtained using Student's *t*-test and variance ratio *F*-test [37] indicated no important change between both of them with regard to accuracy and precision, respectively. The validity of the suggested method was further evaluated by the standard addition technique where acceptable results were attained **Table 5**.

Parameters	GLM	PIO	VAL	ATV
Wavelength (nm)		230.00		
Linearity range (µg/mL)		0.50-25.00		
Regression parameters				
- Slope± (S_Y)	0.3332±0.001	0.4101±0.001	0.3288±0.0004	0.1201±0.0007
- Intercept±(SX)	-0.0063±0.019	0.0049±0.017	-0.0017±0.008	0.004±0.013
-SD of residual(S_{YX})	0.011	0.010	0.011	0.006
-Correlation coefficient (r)	0.9999	0.9999	0.9999	0.9999
LOD (µg/ mL)	0.10	0.08	0.10	0.16
LOQ (µg/ mL)	0.33	0.24	0.33	0.50
Accuracy (% recovery)	100.17	99.81	100.62	100.11
Precision (%RSD):				
- Repeatability*	1.22	0.90	0.97	0.79
- Intermediate precision**	1.40	1.11	1.12	0.84

**The intraday (n=3), average of three responses of different three concentrations repeated three times at the same day.*
***The interday (n=3), average of three responses of different three concentrations repeated three times at three successive days.*

Table 2. Analytical performance data for the determination of GLM, PIO, VAL and ATV by the proposed method

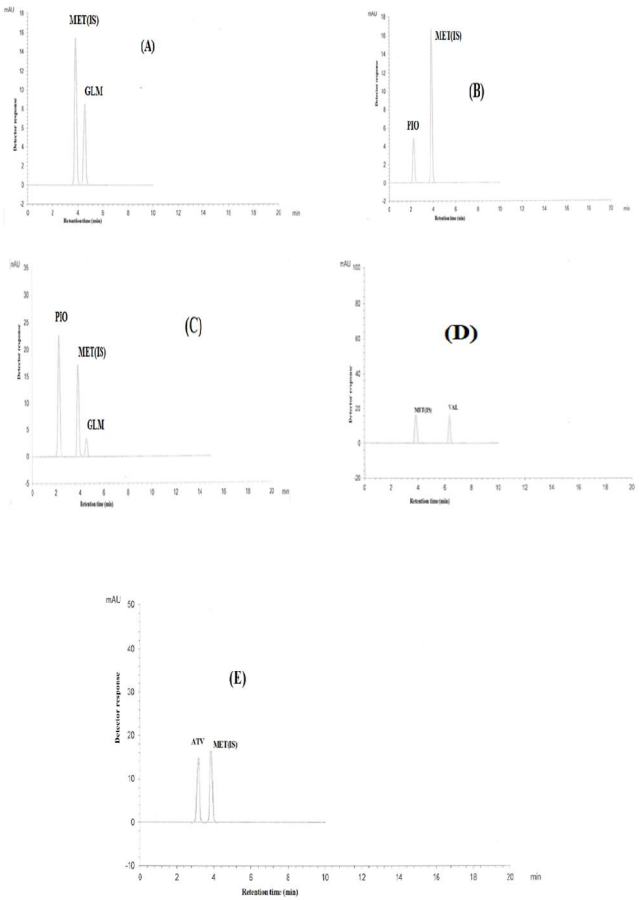


Figure 3. Chromatograms resulted from the application of the developed method to the analysis of tablets (A): GLM 5.00 µg/mL, (B): PIO 3.00 µg/mL, (C): 2.00 µg/mL of GLM and 15.00 µg/mL of PIO, (D): VAL 8.00 µg/mL, (E): ATV (16.00 µg/mL) using 25.00 µg/mL of MET as IS.

3.2.2. Application to spiked human plasma

GLM is reported to be completely absorbed from the gastrointestinal tract after oral administration, time to reach maximum plasma concentration (T_{max}) is attained at 2–3 hours and its maximum plasma concentration is 532.50 ng/ mL. Protein binding was greater than 99.5% [38] . PIO peak plasma concentration is observed within two hours after being absorbed from the gastrointestinal tract, it is rapidly absorbed within an hour with peak plasma concentration (C_{max}) of 2.265 µg/ mL achieved at 2- 3 hours. It is highly bound to plasma proteins (>99%) primarily to serum albumin [39]. VAL is rapidly absorbed after oral administration with plasma peak levels (C_{max}) of 3.46 mg/L occurred 2-4 hours after oral administration. The drug is extensively bound to plasma proteins (85 to 99%) [40]. Atorvastatin is readily absorbed after the oral administration. Multiple daily dosages produce a maximum steady state concentration (C_{max}) of 27.00-66.00 ng/ mL within 1–2 hours. It is highly bound to plasma proteins [41]. Regarding all the above findings, the developed method was applied successfully for the quantitative determination of GLM, PIO and VAL only in spiked human plasma; **Figure (4)**.

Under the above described experimental conditions, a linear relationship was established by plotting the peak area ratio against the drug concentration in µg/mL giving the following equations:

P =-0.0025+1.1164C(*r*=0.9993) for GLM
P=0.0020+0.8438C(*r*=0.9996) for PIO
P=-0.0351+ 1.1257C(*r*=0.9996) for VAL

Where P is the peak area ratio, C is the concentration of the drug in µg/mL and *r* is the correlation coefficient. **Table 6**.

GLM	PIO	VAL	ATV	GLM	PIO	VAL	ATV	GLM	PIO	VAL	ATV
Taken(µg/mL)				Found(µg/mL)				Recovery%			
10.00	10.00	10.00	10.00	10.11	9.98	10.10	10.17	101.11	99.80	101.02	101.70
2.00	15.00	—	—	1.98	15.14	—	—	99.00	100.93	—	—
15.00	—	15.00	15.00	15.03	—	15.01	14.97	100.20	—	100.07	99.80
7.00	—	7.00	—	7.03	—	6.96	—	100.43	—	99.43	—
25.00	—	—	25.00	24.95	—	—	24.97	99.80	—	—	99.88
—	5.00	5.00	—	—	5.01	5.03	—	—	100.20	100.60	—
—	20.00	—	20.00	—	19.90	—	19.90	—	99.50	—	99.50
—	8.00	8.00	8.00	—	8.01	7.94	7.99	—	100.13	99.25	99.88
—	—	12.00	12.00	—	—	12.16	12.03	—	—	101.33	100.25
Mean % ± SD								**100.11±0.781**	**100.11±0.536**	**100.28±0.846**	**100.17±0.788**

Table 3. Determination of GLM, PIO, VAL and ATV in laboratory prepared mixtures by the proposed UPLC method

Parameters	Amaryl® 2 tablet	Diabitonorm® tablet	Amaglust®30/4 tablets		Reported method [1]		Valsatens® tablet	Ator® tablet	Reported method [24]	
	GLM	PIO	GLM	PIO	GLM	PIO	VAL	ATV	VAL	ATV
Linearity range µg/mL	0.50-25.00				5.00-30.00	5.00-175.00	0.30-30.00	0.30-30.00	16.00-96.00	4.00-24.00
n	3	3	3	3	3	3	3	3	3	3
Mean %	99.79	99.39	99.32	100.54	101.07	99.74	100.79	101.22	100.35	99.89
SD	1.17	1.41	1.29	1.66	0.86	1.27	1.11	1.82	1.81	1.33
Variance	1.369	1.988	1.664	2.756	0.740	1.613	1.232	3.12	3.276	1.769
Student's t-test	1.537 (2.776)	0.322(2.776)	1.961(2.776)	0.663(2.776)	——	——	0.356 (2.776)	1.019 (2.776)	——	——
F -value	1.873 (19)	1.242 (19)	2.274 (19)	1.719 (19)	——	——	2.639 (19)	1.875 (19)	——	——

Table 4. Results obtained by the proposed UPLC method compared with a reported method (1, 26)for determination of GLM, PIO, VAL and ATV in pharmaceutical preparations

Standard addition												
Pharmaceutical preparation	Amaglust ® tablets						Valsatens® tablets			Ator® tablets		
	GLM			PIO			VAL			ATV		
	Claimed taken (µg/mL)	Added (µg/mL)	Recovery % of added	Claimed taken (µg/ mL)	Added (µg/ mL)	Recovery % of added	Claimed taken (µg/ mL)	Added (µg/ mL)	Recovery % of added	Claimed taken (µg/ mL)	Added (µg/ mL)	Recovery % of added
	2.00	1.00	98.01	15.00	1.00	101.66	2.00	5.00	100.13	2.00	5.00	98.11
	2.00	2.00	97.55	15.00	2.00	99.89	2.00	10.00	99.98	2.00	10.00	99.05
	2.00	3.00	100.63	15.00	3.00	99.73	2.00	15.00	99.29	2.00	15.00	99.39
Mean % ± SD	98.73±1.66			100.43±1.07			99.80±0.448			98.85±0.663		

Table 5. Application of standard addition technique for the determination of GLM, PIO, VAL and ATV in pharmaceutical preparations by the proposed UPLC method.

Parameters	GLM			PIO			VAL		
	Amount taken (µg/mL)	Amount found (µg/mL)	%Recovery*	Amount taken (µg/mL)	Amount found (µg/mL)	%Recovery*	Amount taken (µg/mL)	Amount found (µg/mL)	%Recovery*
	0.35	0.35	100.00	0.25	0.25	100.00	0.35	0.33	94.29
	0.40	0.39	97.50	0.30	0.30	100.00	1.00	0.98	98.00
	0.45	0.44	97.78	0.60	0.61	101.67	2.00	1.99	99.50
	0.50	0.51	102.00	0.90	0.89	98.89	3.00	2.98	99.33
	0.53	0.52	98.11	1.20	1.21	100.83	3.50	3.36	96.00
Mean %			99.08			100.28			97.42
SD			1.90			1.04			2.24
% RSD			1.92			1.04			2.30

Each result is the average of three separate determinations.

Table 6: Assay results for the determination of GLM, PIO and VAL in spiked human plasma by the proposed method.

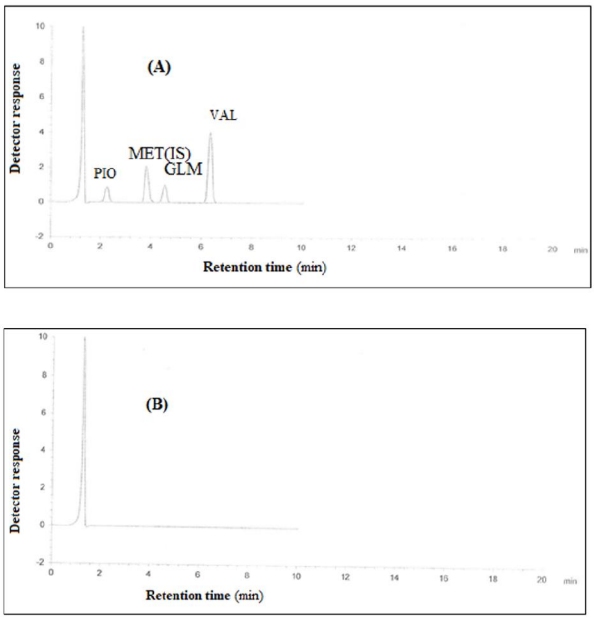
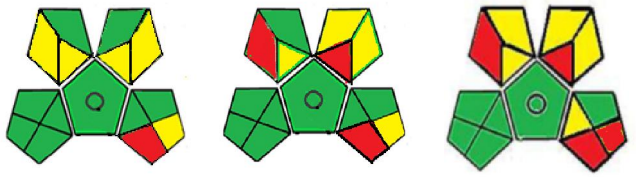


Figure 4. Representative chromatograms of :(A) (0.45-0.60-2.00) GLM, PIO and VAL in human plasma using 3.00 µg/ mL MET as IS under the described chromatographic condition. (B)Blank human

Assessment of greenness of the proposed method

The evaluation of the greenness of the analytical procedures becomes important step in recent times [42]. To examine the greenness of the suggested micellar UPLC method, a NEMI tool has been applied [43]. **Table 7** compares the suggested and reported methods using NEMI profile; it was clear that the developed micellar UPLC had smart green profiles with four green-shaded quadrants, while reported methods have only two green-shaded quadrants. Consequently, the developed method achieves the four criteria of the greenness profile. Additionally, the GAPI is a new tool for the evaluation of the greenness of the whole method has been applied[42,43]. The GAPI profile for the suggested method together with other published methods are existing in **Figure 5**, which showed the highest greenness of the developed method. Moreover, analytical Eco-scale [45] was applied to evaluate the greenness of the suggested method. The Eco-scale assessment technique is depended on penalty points deducted from a base of 100. The suggested method and other reported methods were evaluated for greenness by calculating penalty points, including instruments, reagents and waste, it was established that the proposed method scored the highest Eco-scale of 83, as shown in **Table 8** confirming that it is an excellent green method of analysis. It is concluded from all assessment tools that the developed micellar UPLC method was considered as environmentally friendly.



Proposed micellar UPLC method **Reported method [1, 24]** **Reported method [31]**

Figure 5. GAPI profile for the suggested method in comparison with the reported method.

Drugs	Method	Mobile phase	Run time (min.)	Flow rate (mL/min.)	Waste* (g/run)	Greenness profile**
GLM and PIO	Reported method [1]	phosphate buffer Methanol Acetonitrile Triethylamine	10.00	1.00 mL/min	10.00	
	Reported method [14]	Acetonitrile KH2PO4 buffer Ortho-phosphoric acid	10.00	1.50 mL/min	15.00	
	Reported method [15]	K2HPO4 buffer Acetonitrile Tetrahydrofuran	8.00	1.70mL/min	13.60	
VAL	Reported method [22]	Methanol Acetonitrile Water Isopropylalcohol Triethylamine	12.00	1.00 mL/min	12.00	
VAL and ATV	Reported method [24]	Acetonitrile Ammonium acetate buffer	7.00	1.50 mL/min	7.50	
ATV	Reported method [31]	Acetonitrile Ammonium acetate buffer Tetrahydrofuran (THF)	60.00	1 .00ml/min	60.00	
ATV	Reported method [46]	Water Acetonitrile Ortho-phosphoric acid	10.00	1.50 ml/min	15.00	
GLM , PIO, VAL and ATV	Proposed UPLC method	Phosphate buffer Isopropanol Sodium dodecyl sulphate	10.00	0.20	2.00	

** Run time x flow rate [47]* *** Four key terms are referred to PBT (persistent, bio-accumulative, and toxic), Hazardous, Corrosive, and Waste.*

Table 7. Greenness profile of the developed and reported HPLC methods [43]

GLM and PIO							
Proposed UPLC method		**Reported method [1]**		**Reported method [14]**		**Reported method [15]**	
Reagents	**PPs**	**Reagents**	**PPs**	**Reagents**	**PPs**	**Reagents**	**PPs**
Methanol	6	phosphate buffer	0	Methanol	6	K_2HPO_4 buffer	0
KH_2PO_4 buffer	0	Methanol	6	Acetonitrile	8	Acetonitrile	8
Isopropanol	4	Acetonitrile	8	KH_2PO_4 buffer	0	Tetrahydrofuran	12
SDS	0	Triethylamine	4	Ortho-phosphoric acid	2		
IS	4						
Instrument		**Instrument**		**Instrument**		**Instrument**	
UPLC-UV	0	HPLC-UV	2	HPLC-PAD	2	HPLC-UV	2
Occupational hazard	0	Occupational hazard	0	Occupational hazard	0	Occupational hazard	0
Waste	3	Waste	5	Waste	5	Waste	5
Total PPs	Σ17		Σ25	Total PPs	Σ23	Total PPs	Σ27
Eco-Scale	83		75	Eco-Scale	77	Eco-Scale	73
VAL		**VAL and ATV**		**ATV**		**ATV**	
Reported method [22]		**Reported method [24]**		**Reported method [31]**		**Reported method [46]**	
Reagents	**PPs**	**Reagents**	**PPs**	**Reagents**	**PPs**	**Reagents**	**PPs**
Methanol	6	Methanol	6	Methanol	6	Methanol	6
Acetonitrile	8	Acetonitrile	8	Acetonitrile	8	Water	0
Water	0	ammonium acetate buffer	2	Ammonium acetate buffer	2	Acetonitrile	8
Isopropanol	4			Tetrahydrofuran	12	Ortho-phosphoric acid	2
Triethylamine	4					IS	4
Instrument		**Instrument**		**Instrument**		**Instrument**	
HPLC-UV	2	HPLC-PDA	2	HPLC-UV	2	HPLC-UV	2
Occupational hazard	0	Occupational hazard	0	Occupational hazard	0	Occupational hazard	0
Waste	5	Waste	5	Waste	5	Waste	5
Total PPs	**Σ29**	**Total PPs**	**Σ23**	**Total PPs**	**Σ35**	**Total PPs**	**Σ27**
Eco-Scale	**69**		**77**	**Eco-Scale**	**65**	**Eco-Scale**	**73**

Table 8. Eco-scale approach [45] for the suggested and the reported methods

5. Conclusion

A new green simple, accurate and sensitive chromatographic method was estimated for the simultaneous determination of GLM, PIO VAL, and ATV. The method allowed the separation of the four drugs with high resolution factor and in a time less than 7.00 min. It could be useful for the analysis of the studied drugs in their single dosage forms, in addition to simultaneous determination of GLM and PIO in tablet dosage form. The proposed method also extended for determination of GLM, PIO and VAL in plasma with simple pretreatment procedure.

Conflict of interests

All authors declare that there is no conflict of interest regarding the publication of this work.

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Author contribution

Authors Manal M Fouad and Noha S Rashed are the supervision of the work designed the study and the protocol. Author Asmaa I Hosameldin finished the experimental study and statistical analysis, wrote the first draft and managed literature searches. All authors agreed the final manuscript.

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