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Optimization and Validation of HPLC Method for Simultaneous Determination of Vildagliptin, Pioglitazone Hydrochloride and Glimepiride in Bulk and Tablets

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***Abstract**: A new isocratic HPLC method is optimized and validated for simultaneous determination of Vildagliptin (VLD), Pioglitazone Hydrochloride (PIO) and Glimepiride (GLIM) in Bulk and tablets (Gliptus® and Amaglust® tablets). The chromatographic separation was achieved on a reversed-phase analytical column [Hypersilgold® C18 (10µm, 150 x 4.6 mm) column] at ambient temperature. The separation was achieved by applying an isocratic elution system using acetonitrile and 0.05M potassium dihydrogen phosphate buffer, adjusted by orthophosphoric acid to a pH of 3.5 with a ratio of (45:55 v/v) respectively, at a flow rate of 1.5 ml/min. The UV detection was performed at 200 nm, the drugs calibration curves exhibited linear concentration ranges of 5–75, 3–45 and 1–8 µg/ml for Vildagliptin, Pioglitazone and Glimepiride respectively with correlation coefficients not less than 0.9996.*

***Keywords**: Glimepiride, HPLC, Pioglitazone Hydrochloride, Validation, Vildagliptin.*

I. Introduction

Vildagliptin is (S)-1-[N-(3-hydroxy-1-adamantyl)glycyl] pyrrolidine-2- carbonitrile (Fig.1). Vildagliptinis a relatively new anti-diabetic drug used for controlling patient with type 2 diabetes mellitus. It is related to dipeptidyl peptidase-4 inhibitor drugs. Vildagliptinis are used either alone or in combination with other drugs such as metformin (MET), sulfonylurea or thiazolidinedione for better controlling of resistant patients. Combination of Vildagliptin and MET provides a superior HbA1c-lowering effect with a comparable overall tolerability profile and low risk of hypoglycemia [1] and [2]. Several methods have been reported for determination of Vildagliptin either alone or in combination with other drugs .The methods include spectrophotometric and spectrofluorometric methods [3-6], HPLC methods [7-12],UPLC method [13] and HPTLC method [14].

Pioglitazone hydrochloride is (±)-5-(p-[2-(5-Ethyl-2-pyridyl)ethoxy]benzyl)-2,4-thiazolidinedione hydrochloride (Fig.2). Pioglitazone is one of thiazolidinediones group which is a class of oral anti-diabetic drugs that enhance target tissue insulin sensitivity. Pioglitazone has been shown to affect abnormal glucose and lipid metabolism associated with insulin resistance by enhancing insulin action on peripheral tissues [15].The literature review showed many methods for estimation of Pioglitazone hydrochloride which include the official method according to USP 36 [16]and reported methods such as spectrophotometric methods [17-21], HPLC methods [22-24] and HPTLC methods [25 and 26].

Glimepirideis1-((p-[2-(3-Ethyl-4-methyl-2-oxo-3-pyrroline-1-carboxamido)ethyl]phenyl)sulfonyl)-3-(trans-4-methylcyclohexyl)urea (Fig.3). Glimepiride is a sulfonylurea anti-diabetic drug with prolonged effect and moreover, it maintains a more physiological regulation of insulin secretion than Glibenclamide during physical exercise, suggesting that there may be less risk of hypoglycemia with Glimepiride. It acts by increasing the secretion of insulin by β-cells of the pancreas[15].Glimepiride is officially reported in USP 36 [16], and BP 2013 [27]. Several methods have been reported for determination of Glimepiride either alone or in combined dosage forms. The methods include spectrophotometric [28-35], HPLC [22-24] and [36- 37] and HPTLC [38, 39].

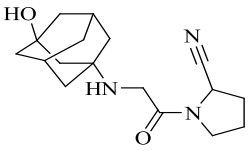

Fig.(1): Vildagliptin

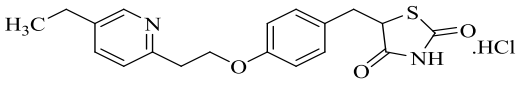

Fig.(2): Pioglitazone hydrochloride

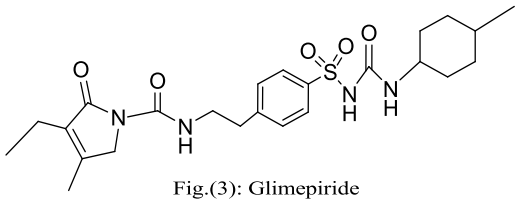

Fig.(3): Glimepiride

II. Experimental

2.1 Materials and reagents

All experiments were performed using pharmaceutical grade authentic reference standards. Standard of Vildagliptin was kindly supplied as a gift samples by Inspire pharmaceutical company (Obour City, Egypt) while as, Pioglitazone hydrochloride and Glimepiride were generously gifted by Medical union pharmaceutical company (MUP), (Abo-sultan, Egypt). These reference standards were certified to contain 99.9, 100.55 and 99.82 % (w/w), of Vildagliptin, Pioglitazone, and Glimepiride respectively.

Acetonitrile (ACN) and methanol (SDS, France) were of HPLC-grade, monobasic potassium dihydrogen phosphate (Winlab, UK); and orthophosphoric acid (≥ 85 %) purity (Riedel-de Haen, Germany) were all of analytical reagent grade. The water for HPLC was prepared in-house by double distillation then filtration through 0.45 µm cellulose nitrate membrane filters (Chem Lab, Spain). All chemicals were used as received without further modification or purification.

The studied pharmaceutical preparations are Gliptus® (batch no. 507551) manufactured by EVA Pharma (Cairo, Egypt). It is Claimed to contain 50 mg of Vildagliptin per tablet and Amaglust® (batch no. 610717) manufactured by Next Pharma(Cairo, Egypt),the pack declaring that a tablet contains 30 and 4 mg of Pioglitazone and Glimepiride respectively were purchased from local drugstores.

2.2 Instrumental and chromatographic conditions

HPLC apparatus is equipped with a Surveyor® quaternary pump with Intel vacuum degasser (Thermo Scientific Co., USA), Surveyor® auto-sampler plus (Thermo Scientific Co., USA), Surveyor® photodiode array detector (PAD) (Thermo Scientific Co., USA). Computer with a software chromo quest 5 (Thermo Scientific Co., USA) for data collection and analysis auto-sampler vials 1.8 ml screw cap (Thermo Scientific Co., USA).The separation and quantitation were made on Hypersil gold® C18 (10um, 150x4.6mm) column (Thermo Scientific Co., USA).

An isocratic mobile phase consisted of acetonitrile and 0.05M potassium dihydrogen phosphate buffer, adjusted by orthophosphoric acid to a pH of 3.5 with a ratio of (45:55 v/v) respectively, at a flow rate of 1.5 ml/min.

Consort P400™ is a digital pH-meter used for the mobile phase pH adjustment. The mobile phase filtered using the vacuum filtration system equipped with 0.45 µm nylon membrane filter and finally degassed for 30 min via an ultrasonic bath and allowed to run for 10 min prior each analysis. The detection wavelength was set at 200 nm. All determinations were performed at ambient temperature (25 °C ± 1).Twenty µl were automatically injected.

2.3 Standard solutions and calibration graphs

Stock standard solutions were prepared separately to give a final concentration of 500µg/ml for Vildagliptin, Pioglitazone and Glimepiride through dissolving an accurately weighed amount (50 mg) in a total of 100 ml ACN for Vildagliptin. Pioglitazone and Glimepiride were dissolved separately in 100 ml methanol. Working solutions for the standard calibration graphs were prepared immediately before usage by further dilutions of the stock solutions with the mobile phase to cover the concentration ranges of 5–75, 3–45, and 1–8 µg/ml for Vildagliptin, Pioglitazone, and Glimepiride respectively. Three replicate each of twenty µl injections for each drug concentration level (simultaneously prepared) were made and directly chromatographed under the specified chromatographic conditions**.**

2.4 Pharmaceutical formulations preparations

The content of 20 tablets of Gliptus® and Amaglust® was weighed and separately grounded to get homogenous powder. A portion of each finely powdered drug equal to one tablet (according to the label claimed), equivalent to 50 mg Vildagliptin, 30 mg Pioglitazone and 4mg Glimepiride was accurately weighed and transferred to a 100 ml capacity volumetric flask. Thirty milliliters methanol and thirty milliliters of ACN were added to the mixture; the mixture was dissolved via ultra-sonication for 30 min at ambient temperature and

then diluted to the mark with the mobile phase. The solutions were filtered through 0.45 µm nylon membrane filter discs [Millipore^{TM}, Milford, MA] before use. Further dilution was carried out using the mobile phase to suit the concentration domain covered by the calibration graphs. The solutions were chromatographed using the HPLC conditions described above and the concentrations of Vildagliptin, Pioglitazone and Glimepiride were calculated**.**

III. Results And Discussion

3.1 HPLC method development and optimization

A new reliable, sensitive and accurate RP-HPLC method is developed for simultaneous determination of Vildagliptin, Pioglitazone and Glimepiride in bulk and the method is applied successfully to the pharmaceutical preparations containing these compounds. This is the first time to separate these three drugs.
The chromatographic parameters such as detection wavelength, mobile phase composition, pH value and the flow rate were studied and optimized in order to provide an excellent assessment performance.

The UV absorption spectra of Vildagliptin, Pioglitazone, and Glimepiride prepared in the mobile phase were found to be in a region of 200–300 nm as represented in (Fig.4). It is obvious that each drug does strongly contribute to the overall absorption spectrum of the mixture, resulting in an extensive overlapping between the drugs absorption spectra. Thus, introducing an HPLC technique for the simultaneous analysis of Vildagliptin, Pioglitazone and Glimepiride in ternary mixtures will be more favored than applying any conventional (direct) first derivative or derivative ratio spectrophotometric methods.

3.1.1 The effect of pH value of the buffer and percentage of organic modifier

One of our crucial interests during the method development was the choice of suitable mobile phase components with suitable pH value for better separation of the investigated drugs without affecting their chemical entity. It was noticed that acidic pH values are preferred with Pioglitazone and Glimepiride during their own analyses. However, an analysis at pH value above 7 was to be avoided because it could damage the reversed silica-based analytical columns C18, when they are used frequently. By using pH value of 3.5a good baseline separation was achieved during the experimental study for Vildagliptin, Pioglitazone, and Glimepiride
After several preliminary investigatory chromatographic runs, it was concluded that phosphate buffers gave better peak symmetry than their acetate and citrate counterparts. It was also noticed that peak shapes were improved with increasing the buffer ionic strength with a non-significant change beyond 0.05 M. In order to avoid salt precipitation that can affect the lifespan of the used analytical column. A 0.05 M phosphate buffer was chosen for method validation. An initial mixture of methanol and phosphate buffer was tried in the ratio of (40:60, v/v). Unfortunately, Vildagliptin peak was observed to be forked and showed tailing this together with a delayed elution for Pioglitazone and Glimepiride that reached a (t_R)> 8 min and > 20 min, respectively. When methanol was replaced with acetonitrile at the same ratio a better separation was obtained but with relative tailing and forking for Vildagliptin peak. Increasing the acetonitrile concentration to more than 40 % provided a more symmetrical Vildagliptin peak. Therefore, methanol was excluded again in the dilution process to remove the forking from Vildagliptin peak, a satisfactory separation of the three drugs Vildagliptin, Pioglitazone and Glimepiride was achieved using a mobile phase consisting of acetonitrile and 0.05 M potassium dihydrogen phosphate buffer, pH = 3.5, in a final ratio of (45: 55, v/v).

3.1.2 The effect of flow rate and wavelength - detection

In regard to the flow rate optimization; it was initially set at 1ml/min, causing Glimepiride to be eluted too late at > 18 min. Therefore, the flow rate was gradually increased until it was finally adjusted at 1.5 ml/min to separate Glimepiride at a reasonable time of 8.8 min. This modification allows completing the process of separation of Vildagliptin, Pioglitazone and Glimepiride within ten minutes with better resolution.

Lastly, the wavelength of detection was set regarding the drugs UV absorption spectra and their relative concentrations within the pharmaceutical formulations. Where, 50:30:4 the ratio of Vildagliptin, Pioglitazone and Glimepiride respectively in the dosage form. This is why; an optimum detection wavelength was set at 200 nm during the chromatographic separation, favoring the separation and quantification of Vildagliptin and the quantification of Glimepiride, which represent the less concentrated component of this ternary mixture, achieving adequate signal strengths for the three analytes in respect to each other. In addition, such chosen detection wavelength greatly improves the sensitivity of the proposed method towards the Vildagliptin, since the later possess a high molar absorptive coefficient at 200 nm.

The specificity of this HPLC method is illustrated at the typical chromatograms (Fig.5), where complete separation of the drugs was noticed. The average retention time ± R.S.D. (%) of Vildagliptin, Pioglitazone and Glimepiride were found to be; 1.268 ± 0.159, 2.71 ± 0.322 and 8.87 ± 0.211 min, respectively, for five replicates. The obtained peaks were sharp and had clear baseline separation.

IV. Method Validation

4.1 Linearity and range (calibration curve)

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are either, directly or through mathematical transformation proportional to the concentration of the analyte. This proposed HPLC method was assessed by least-squares linear regression analysis of the calibration curve [40]. According to the ICH guidelines [41], at least five concentrations must be used. In this presented study, five different concentrations, of each studied drug were chosen and prepared as previously discussed. Each concentration was injected in triplicate and the mean value of the peak areas was imputed into a Microsoft Excel® spreadsheet program for the calibration curve plotting. The repeated runs were genuine repeats and not just repetitions at the same reading in which three replicate samples of each concentration level were prepared; this in order to provide information on the variation of the peak area between samples of the same concentration. The regression analyses revealed satisfactory correlations (r = 0.9996 –0.9997), this, indicating a good linearity of the calibration graphs**(**Fig.5, 6&7).Characteristic parameters for the regression equations of the HPLC method obtained by least-squares treatment of results were given in table (1).

4.2 Precision

The precision of the proposed HPLC analysis was evaluated as repeatability and reproducibility levels; using three independent concentrations of each drug. The repeatability (intra-day precision) studies were performed on the same day, whereas, that of the intermediate precision (inter-day precision) were checked by repeating these studies on three consecutive days. Every sample was injected in triplicates and both the retention times (t_R) and peak areas were determined. Within the examined time range, the peak area results presented in table (2) and show excellent precision for the method both during one analytical run and between different runs, with an intra-day and inter-day R.S.D. (%), the range was 0.202–1.305 and 0.262–1.327, respectively.

4.3 Detection and quantitation limits

The limit of detection (LOD) for an HPLC method is the lowest drug concentration that produces a response detectable above the noise level of the system, typically taken as three times. The limit of quantification (LOQ) is the lowest level of the drug that can be accurately measured, and it is often evaluated as ten times the noise level. Both quantities were evaluated regarding the International Conference on Harmonization (ICH) guidelines [41]. Both the values of LOD and LOQ were assessed practically and given in table (1).

4.4 Accuracy

The accuracy of the proposed method, which is defined as the closeness or the nearness of the true and found values, was evaluated by measuring the drug recoveries by using the standard addition technique. The standard addition analysis involves the addition of three concentration levels of each drug standard solution (covering the linearity range and higher than LOQ*)* to pre-analyzed pharmaceutical samples containing; 25, 15 and 2 µg mL^{-1} of Vildagliptin, Pioglitazone and Glimepiride respectively**.** Each set of addition was repeated five times, and the results obtained were compared with those expected from the calibration curve, table (3).

4.5 Selectivity

The selectivity of the proposed method was checked by preparing five laboratory-prepared mixtures of the studied drugs at various concentrations within their linearity range. The laboratory-prepared mixtures were analyzed according to the previous procedure described under the proposed method. Satisfactory results were obtained as listed in table (4) indicating the high selectivity of the proposed method for simultaneous determination of the studied drugs.

4.6 Robustness

Robustness relates to the capacity of the method to remain unaffected by small but deliberate variations introduced into the method critical parameters. For this, four chromatographic parameters such as; buffer pH, organic composition of the mobile phase, elution flow rate and the detection wavelength were varied around the value set in the proposed method in order to reflect changes likely to arise in different testing environments. The pH was varied in the range 3.4–3.6 (± 0.1 units), while as the mobile phase organic strength was varied through ± 1 % (v/v) alteration of the acetonitrile compositing proportions along the chromatographic separation. On the other hand, the influence of the flow rate was evaluated between 1.45–1.55 ml/min, while as the detection wavelength was evaluated over ± 2 nm alterations. During the robustness study, only one factor was changed at a time and the analyses were performed in replicate injections (n = 3). The results, presented in table (5), confirm the method robustness since the observed variations were less than 1.401 %.

4.7 System suitability test

System suitability tests (SST) are based on the concept that the equipment, electronics, analytical operations and samples to be analyzed constitute an integral system that can be evaluated as such. These tests were performed in accordance with the BP guidelines to ensure adequate performance of both the chromatographic system and the equipment, for the analysis to be performed. The repeatability test was carried out by injecting six separated injections at concentrations 50, 30, and 4 µg/ml regarding VILD, Pioglitazone,

and Glimepiride. The observed R.S.D. (%), of the retention times regarding these repetitive injections, was considered satisfactory, meeting the BP recommendation (R.S.D. (%) < 1.0). Other chromatographic parameters were calculated from experimental data, such as; capacity factor (*k'*) also known as mass distribution ratio (D_m), tailing factor (T_f) also known as peak asymmetry factor (A_s) and the apparent number of theoretical plates (N). All of these parameters are usually employed in assessing the performance of the column. Results obtained from system suitability tests are presented in table (6). Good agreement was found when results were compared with recommended values.

4.8 Analytical solutions stability

The solutions were stored in tightly capped volumetric flasks and wrapped with aluminum foil under reduced light conditions. It was found that Vildagliptin and Pioglitazone analytical solutions exhibited no changes for at least 10 days when stored refrigerated at 4˚C and for 24 hours when kept at room temperature. Glimepiride analytical solution in methanol exhibited no changes for 7 days when stored refrigerated at 4˚C and for 18 hours when kept at room temperature. Solutions of the studied compounds in the mobile phase exhibited no changes for 8 hours when kept at room temperature.

4.9 Analysis of pharmaceutical products

The validated HPLC method was applied for the determination of Vildagliptin, Pioglitazone and Glimepiride in pharmaceutical preparation using Gliptus® and Amaglust® tablets (Fig. 9). Three replicate determinations were performed at each concentration level. Satisfactory results were obtained for each compound in good agreement with label claims table (7). The obtained results were compared statistically by Student's *t*-test (for accuracy) and variance ratio F-test (for repeatability) with the reported method [7] for Vildagliptin & [42] for Pioglitazone and Glimepiride. The results showed that the calculated t and F values were smaller than the critical values at 95% confidence limit indicating that there is no significant difference between the proposed and reported methods, table (7).

V. Conclusion

The proposed HPLC method provides simple, accurate and reproducible quantitative analysis for the simultaneous determination of pharmaceutical products containing Vildagliptin, Pioglitazone, and Glimepiride without any interference from excipients. The study showed that this chromatographic method is suitable for routine analysis of the studied compounds in their pharmaceutical preparations; the proposed RP-HPLC method is the first study for the simultaneous determination of Vildagliptin, Pioglitazone and Glimepiride as multi-drug pharmaceutical formulations providing a collective chromatographic profile regarding these three drugs. This, in turn, will be suitable as a guideline for the routine and QC analyses.

Acknowledgement

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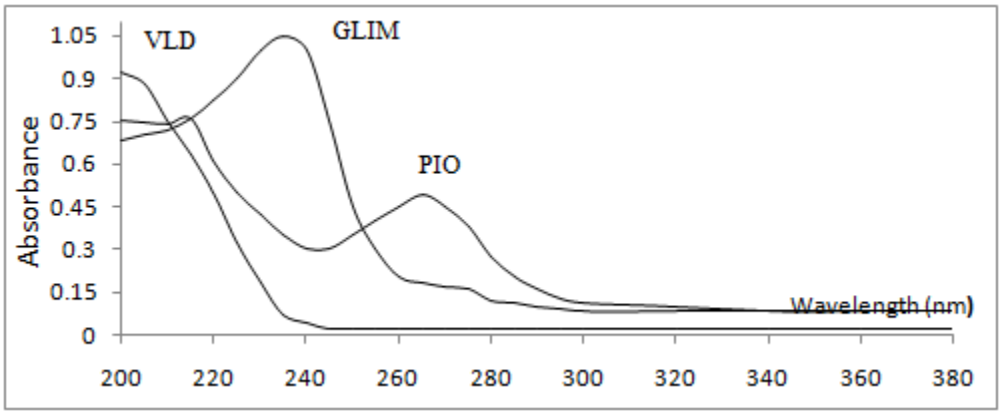

Fig.4: UV absorption spectra of 50 µg /ml Vildagliptin, Pioglitazone and Glimepiride in the mobile phase

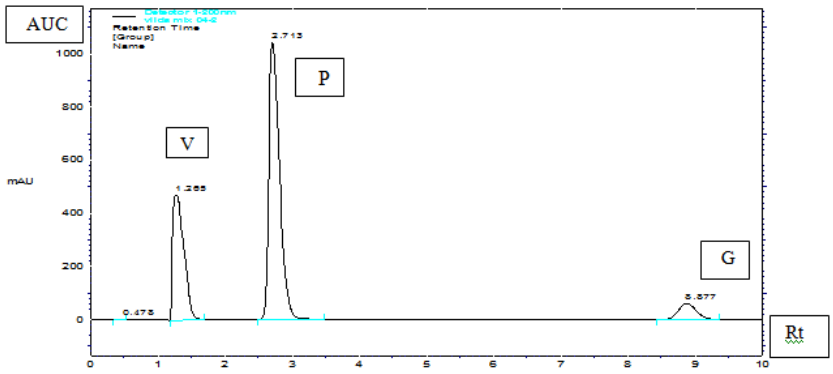

Fig. 5: HPLC Chromatogram of authentic mixture of Vildagliptin (V) (50 µg/ml), Pioglitazone HCl (P)(30 µg/ml) and Glimepiride (G) (4 µg/ml) using the optimal conditions.

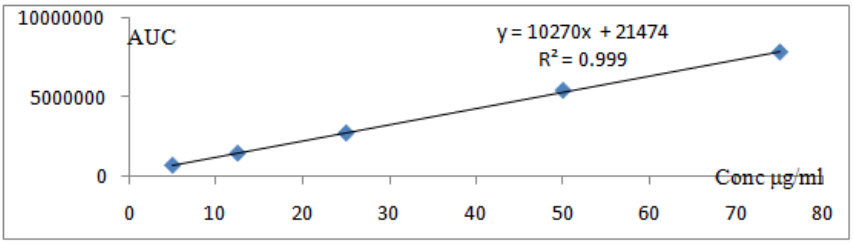

Fig.6: Calibration curve of Vildagliptin using the proposed HPLC method

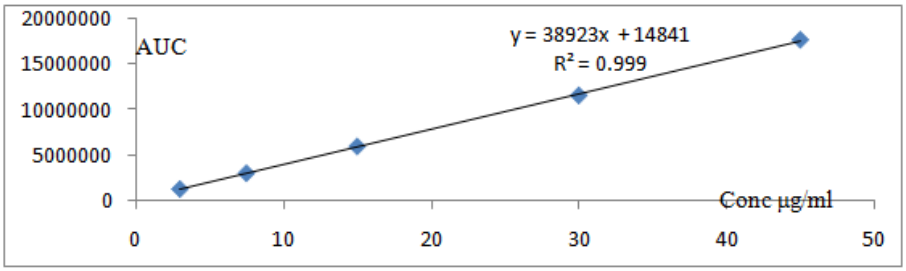

Fig.7: Calibration curve of Pioglitazone hydrochloride using the proposed HPLC method

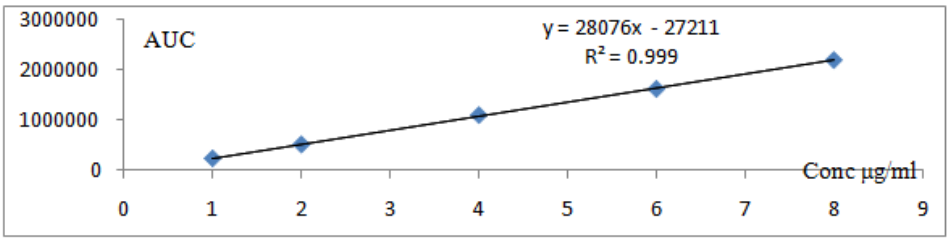

Fig.8: Calibration curve of Glimepiride using the proposed HPLC method

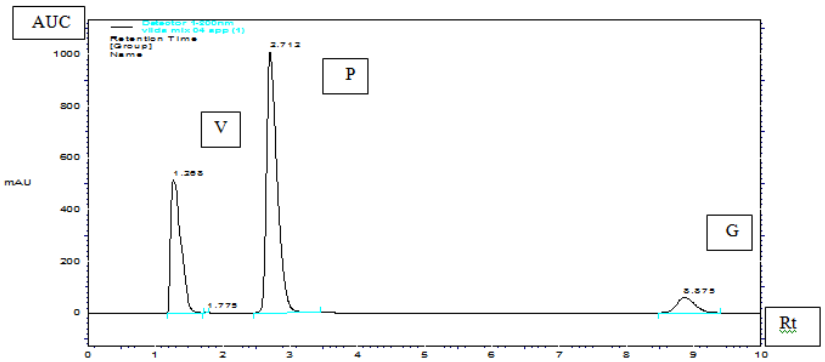

Fig.9: HPLC Chromatogram of the analysis of Gliptus® and Amaglust® tablets.

Table 1: Characteristic parameters for the calibration equations of the proposed HPLC method for the simultaneous determination of Vildagliptin, Pioglitazone and Glimepiride

Parameters	**Vildagliptin**	**Pioglitazone HCl**	**Glimepiride**
Linearity range (µg/ml)	5–75	3–45	1–8
Detection limit (µg/ml)	9.27×10^{-2}	2.44×10^{-2}	3.39×10^{-2}
Quantitation limit (µg/ml)	30.9×10^{-2}	8.15×10^{-2}	11.30×10^{-2}
Regression equation [a]			
Slope (*b*)	1.02702×10^{5}	3.89232×10^{5}	2.80762×10^{5}
Intercept (*a*)	2.14744×10^{5}	1.48417×10^{5}	2.7211×10^{4}
Correlation coefficient (r)	0.9996	0.9997	0.9997

[a] $Y = a + bC$, where C is the concentration of the reference standard substance(µg/ml) and Y is the peak area.

Table 2: Results of the intra-day and inter-day precision in the assay of Vildagliptin, Pioglitazone and Glimepiride using the proposed HPLC method

Drug	**Concentration taken µg/ml**	**Intra-day precision**		**Inter-day precision**	
		Found µg/ml	**Recovery (%) ± S.D.; R.S.D. [a] (%)**	**Found µg/ml**	**Recovery (%) ± S.D.; R.S.D. [b] (%)**
VLD	12.5	12.59	100.76 ± 1.315 ; 1.305	12.51	100.07 ± 0.579 ; 0.578
	50	49.69	99.39 ± 0.833 ; 0.838	49.68	99.36 ± 0.861 ; 0.866
	75	75.38	100.50 ± 0.649 ; 0.645	75.45	100.60 ± 0.743 ; 0.739
PIO	7.5	7.51	100.19 ± 0.565 ; 0.564	7.56	100.88 ± 0.264 ; 0.262
	30	30.12	100.42 ± 1.03 ; 1.02	30.39	101.31± 0.524; 0.518
	45	45.47	101.04 ± 0.204 ; 0.202	45.23	100.53 ± 0.511 ; 0.509
GLIM	1	0.997	99.77 ± 0.656 ; 0.657	0.992	99.26 ± 0.550 ; 0.554
	4	4.01	100.29 ± 0.561 ; 0.559	4.03	100.69 ± 0.856 ; 0.851
	6	6.01	100.17 ± 0.807 ; 0.806	6.00	100.02 ± 1.328 ; 1.327

[a] Means, S.D. and R.S.D. (%), of three replicates on same day. [b] Means, S.D. and R.S.D. (%), of three replicates on three consecutive days.

Table 3: Results of the accuracy studies by standard addition technique in the assay of Vildagliptin, Pioglitazone and Glimepiride using the proposed HPLC method (n=5)

Drug	**Concentration (µg/ml)**				**Recovery (%)**	**R.S.D. (%)**	**Relative error E_r(%)**
	Initial tablet sample	**Authentic amount added**	**Claimed total amount**	**Total amount found ± S.D. [a]**			
VLD	25	15	40	40.08 ± 0.906	100.52	0.901	0.08
	25	30	55	54.85 ± 0.278	99.42	0.280	-0.0027
	25	50	75	74.82 ± 1.196	99.76	1.199	-0.0024
PIO	15	10	25	25.07 ± 0.157	100.27	0.156	0.0028
	15	20	35	35.17 ±0.970	100.48	0.965	0.0048
	15	30	45	44.76 ± 1.476	99.46	1.484	-0.0053
GLIM	2	2	4	4.01 ± 1.064	100.34	1.061	0.0025
	2	4	6	6.04 ± 1.180	100.76	1.172	0.0066
	2	6	8	8.02 ± 1.696	100.30	1.691	0.0025

Table 4: Determination of Vildagliptin, Pioglitazone and Glimepiride in laboratory prepared mixtures using the proposed HPLC method

VLD*			**PIO***			**GLIM***		
Taken µg/ml	**Area under peak**	**Recovery %**	**Taken µg/ml**	**Area under peak**	**Recovery %**	**Taken µg/ml**	**Area under Peak**	**Recovery %**
5	721973	98.77	3	1341968	102.21	1	249238	98.46
12.5	1486838	99.09	7.5	3077022	100.32	2	529647	99.17
25	2757208	99.02	15	6030616	100.74	4	1117143	101.89
50	5446751	101.88	30	11642159	98.43	6	1643848	99.19
75	7863473	99.29	45	17768165	100.59	8	2220075	100.05
Mean		99.615			100.46			99.75
±SD		1.283			1.352			1.322
±RSD		1.288			1.346			1.325
±SE		0.485			0.511			0.50009
Variance		1.647			1.828			1.749

* Average of five independent procedures.

Table 5: Results of the robustness evaluation in the assay of Vildagliptin, Pioglitazone and Glimepiride using the proposed HPLC method (n = 3)

Parameters		**VLD (50 µg/ml)**	**PIO (30 µg/ml)**	**GLIM(4 µg/ml)**
	Value	**Mean recovery [a](%) ± R.S.D. (%)**	**Mean recovery [a](%) ± R.S.D. (%)**	**Mean recovery [a](%) ± R.S.D. (%)**
Flow rate	1.45	99.33± 0.291	100.73 ± 0.800	99.80 ± 0.787
	1.55	100.59 ± 0.130	101.34 ± 0.455	100.75± 0.483
ACN: Buffer	44:56	99.78 ± 0.196	100.47 ± 0.152	99.51 ± 0.109
	46:54	100.35 ± 1.179	99.71 ± 0.873	100.07 ± 0.510
pH	3.4	99.58 ± 1.191	100.66 ± 0.857	100.22 ± 0.934
	3.6	99.45 ± 0.772	99.89± 0.413	100.40 ± 1.07
Wavelength(nm)	202	99.14 ± 0.679	99.98 ± 1.401	100.31 ± 0.944

[a] Each mean value was compared with the mean value obtained under optimum conditions

Table 6: Results of the statistical analysis parameters required for system suitability testing using the proposed HPLC method

Parameter	**VILD**	**PIO**	**GLIM**	**Recommended values**
Retention time (t_R)(min)	1.27	2.71	8.87	–
Tailing factor (T_f)	1.33	0.92	1.16	$0.8 < T_f \leq 1.5$
Capacity factor (k')	0.66	1.51	2.95	$0.5 < k' < 10$
Theoretical plates No. (N)	257	1331	5095	The more plates, the better separation efficiency
R.S.D. (%)for six separate injections	0.773	0.454	0.283	≤ 1

Table 7: Statistical comparison between the proposed HPLC method and reported methods for the determination of Vildagliptin, Pioglitazone and Glimepiride in Gliptus® and Amaglust® tablets

Analyte	Amount taken µg /ml	Proposed method		Reportedmethods [b]		*t*-test (2.31)*	*F*-test (6.39)*
		Recovery (%) ± S.D. [a]	R.S.D. [a] (%)	Recovery (%) ± S.D. [a]	R.S.D. [a] (%)		
VLD	12.5	100.42 ± 0.437	0.435	99.56 ± 1.303	1.309	0.143	0.057
	25	100.59 ± 0.901	0.896	100.00 ± 1.078	1.078	0.377	0.108
	50	99.47 ± 0.273	0.275	99.96 ± 1.275	1.276	0.446	0.011
PIO	7.5	99.34 ± 0.831	0.837	100.46 ± 1.871	1.865	0.290	0.127
	15	99.96± 0.387	0.387	101.02 ± 1.429	1.415	0.179	0.032
	30	98.22 ± 0.195	0.198	100.38 ± 1.385	1.379	0.0236	0.039
GLIM	1	99.30 ± 0.530	0.534	100.13 ± 1.387	1.385	0.316	0.108
	2	99.68 ± 0.546	0.548	99.79 ± 1.49	1.50	0.887	0. 129
	4	100.46 ± 1.060	1.055	101.11 ± 1.465	1.449	0.458	0.622

a. Average of five determinations.
b. Reported method [7] for Vildagliptin and [42] for Pioglitazone and Glimepiride respectively.
* Tabulated t and F values at 95 % confidence limit

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