UPLCMS Method Development and Validation of Tablet Dosage form Containing Glimepiride, Metformin and Pioglitazone Using Internal Standard

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Abstract: *A simple, rapid, sensitive and specific Ultra performance liquid chromatography-tandem mass spectrometry method was developed and validated for simultaneous estimation and validation of tablet dosage form containing glimepiride (GLIM), metformin (MET) and pioglitazone (PIO) using Tolzamide (TOLAZ) as an internal standard (IS). The chromatography separation was achieved with Waters ACQUITY HSS C18, 1.8 μm, 2.1X50 mm column with mobile phase containing acetonitrile (A) & 1% ammonium acetate buffer (B) (pH 2.5 adjusted with trifluoro acetic acid) with gradient mode Gradient mode [2 min: 20 A : 80% B, 2-4 min: 70% A : 30% B, 4-5 min,80% A : 20% B, 8-10 min 90%A: 10% B]. The flow rate was 0.4 mL min^{-1} column maintained at $25^{0}C$ and the injection volume was 2 μl. The selected chromatographic condition were found to effectively separate glimepiride, metformin and pioglitazone with retention time of 3.17, 0.425, 2.3 min, respectively. The proposed method was found to be rectilinear over the range of 2-12 ng mL^{-1}, 500-3000 ng mL^{-1} and 15-90 ng mL^{-1} for glimepiride, metformin, and pioglitazone respectively. The signal intensities obtained in both positive and negative ion mode all drugs including internal standard found to be much higher in positive ion mode (M +H)+ parent ions at m/z, 491.11, m/z 129.87, m/z 357.04 and m/z 312.04 respectively in QUATTROZQ full scan mass spectra. The present method was validated as per ICH guidelines with respect to precision, specificity, linearity, limit of detection, limit of quantification, accuracy, and robustness and it can also be used for routine quality control analysis of these drugs in biological samples either alone or in combined pharmaceutical dosage forms.*

I. INTRODUCTION

Diabetes and its abnormalities constitute a major health problem in the modern society [1]. It is characterized by disrupted insulin production, leading to high blood glucose concentration and other complications such as renal dysfunction, neuropathy and cardiopathy [2]. Type II diabetes is a complex metabolic disorder with two major biochemical defects, namely impaired insulin secretion and impaired insulin action at the periphery. Chronic hyperglycemia results from these defects. Many oral antidiabetic drugs with different mechanisms of action have been developed to lower blood sugar and delay the occurrence of serious complications in patients with type II diabetes [2]. For glycemic control in such cases, monotherapy with an oral antidiabetic agent is not adequate to achieve satisfactory blood glucose control [3]. Thus, combination regimens which include drugs with different and complementary mechanisms of action are recommended. The combinational therapy for type II diabetes [4-5] is frequently prescribed when mono therapy fails. The combination of metformin (MET), pioglitazone (PIO), and glimepiride (GLIMP) is approved by FDA for treatment of type II diabetes [6].

Metformin is an orally administered antihyper glycemic drug that lowers glucose by reducing hepatic glucose production and gluconeogenesis and by enhancing peripheral glucose uptake [7].

Pioglitazone is an oral antidiabetic agent used in the treatment of type II diabetes. After administration, pioglitazone decreases insulin resistance in the periphery and liver resulting in increased insulin dependent glucose disposal and decreased hepatic glucose output [8-9]. PIO act through PPARγ, a member of the nuclear receptor superfamily of ligand activated transcription factors [7]. Once activated, PPARγ forms a heterodimer with another nuclear receptor, the retinoid-X receptor. This heterodimer then binds to specific DNA sequences and regulates the transcriptional activity of target genes that play a role in the metabolism of glucose and lipids   [7-9]. Glimepiride is an oral sulfonylurea hypoglycemic agent indicated for the treatment of type II diabetes mellitus. The sulfonylurea glimepiride used widely in the treatment of non-insulin dependent Type II diabetes mellitus [10]. It acts by stimulating insulin secretions from the beta cells of pancreas and is also known to increase peripheral insulin sensitivity thereby decreasing insulin resistance.

As per the literature, various methods are available for the estimation of these three drugs individually or in combination of two drugs in a pharmaceutical dosage form and also from biological samples. Very few methods are available for simultaneous estimation of all the three drugs together in a tablet dosage form [10] This paper describes a simple, precise, and accurate UPLCMS method for simultaneous estimation of metformin, glimepiride and pioglitazone.
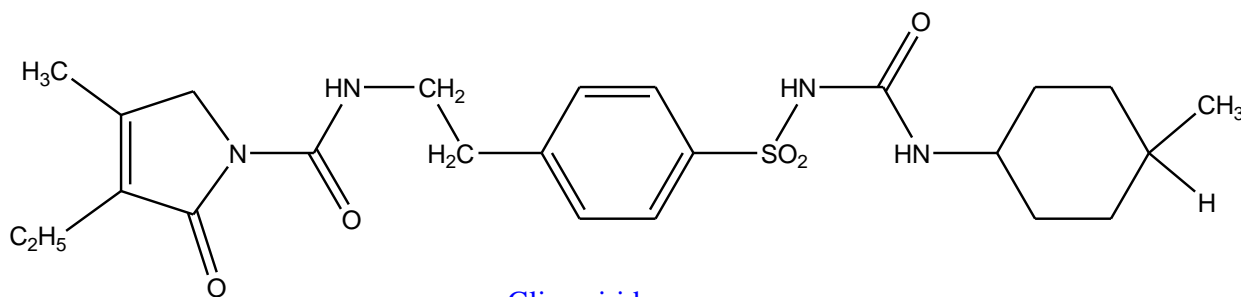
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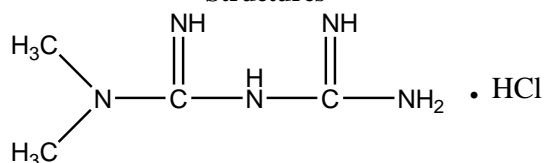
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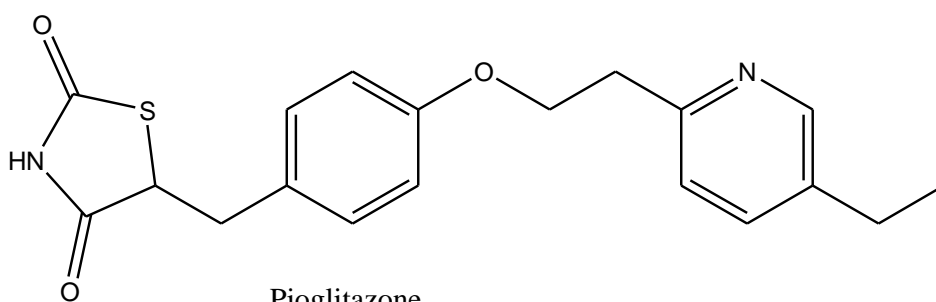
Pioglitazone

Glimepiride

II. EXPERIMENTAL

2. a. Materials and reagents

Working standards, Glimepiride (GLIMP), Metformin (MET) and Pioglitazone (PIO) were procured from Ranbaxy laboratories, New Delhi, India. Ammonium acetate AR grade, HPLC grade Acetonitril, Methanol and water from Ranchem, Mumbai. The pharmaceutical dosage form containing GLIMP 2 mg, MET 500 mg & PIO 15 mg Geoglit GM (Cadila pharmaceuticals Ltd.), 20 tablets purchased from a local drug store. Tolazamide which was employed as an internal standard (IS) was obtained from Ranbaxy Laboratories, New Delhi.

Instrumentation

UPLC Condition

The development and validation of the assay was performed on a Waters Aquity UPLC Micromass Quattro Micro API Mass spectrometer (Waters Corp., MS, USA), equipped with a binary solvent delivery system, auto sampler, photodiode array and ELSD detector. The chromatography was performed on a

7

ACQUITY HSS C18, 1.8 μm, 2.1X50 column (Waters Corp., Ireland) with mobile phase containing acetonitrile (A) & 1% ammonium acetate (B) buffer pH 2.5 adjusted with trifluoro acetic acid, [Gradient mode (2 min: 20 A : 80% B, 2-4 min: 70% A : 30% B, 4-5 min,80% A : 20% B, 8-10 min 90% A: 10% B)]. The flow rate was 0.4 mL min^{-1} column maintained at 25^{0}C and the injection volume was 2 μl.

MS Condition:

Ion polarity: ES+, Ion source type: Atmospheric Pressure Electrospray Ionisation, Capillary voltage (kv): 3.50-3.51, Cone voltage(v): 30.00-33.09, Source temperature (°c): 120-119, Desolvation gas flow (l/h): 1000-998, Cone gas flow (L/Hr): 50-50, Desolvation gas temperature (°c): 500-498, A mass spectrum was over a scan range of *m/z:* 50 Da to 2048 Da, Extractor: 3.00-3.79, Ion Energy: 1-0.5.

Standard Preparation

Separate stock solutions of 10,000 ng mL^{-1} of glimepiride, metformin, pioglitazone and tolazamide were prepared by dissolving in 25 mL HPLC grade Methanol and diluted to 100 mL with acetonitrile, then 10 mL of stock solution into a 100 mL of standard volumetric flask and diluted with acetonitrile (stock solution contains 1000 ng mL^{-1}). The prepared stock solutions were stored at 25^{0}C protected from light.

Preparation of Calibration Plot

Standard solutions were freshly prepared from the stock solution by diluting with acetonitrile as 2, 4, 6, 8, 10 & 12 ng mL^{-1} glimepiride, 500, 1000, 1500, 2000, 2500 & 3000 ng mL^{-1} of metformin and 15, 30, 45, 60, 75 & 90 ng mL^{-1} pioglitazone respectively. Each solution was injected in triplicate and chromatographed under the chromatographic conditions specified above. Tolazamide (20 ng mL^{-1}) was used as internal standard for determination of mixtures of glimepiride, metformin & pioglitazone with tolazamide. Linear relationships were obtained when drug to internal standard peak-area ratios were plotted against the corresponding concentrations for each drug.

Sample Preparation

Average weight was calculated by weighing 20 tablets. The tablets were crushed into homogenous powder. A quantity of powder equivalent to one tablet containing 2 mg of glimepiride, 500 mg of metformin and 15 mg of pioglitazone was transferred into a 100 mL volumetric flask. To this flask, 25 mL of methanol were added, and the solution was sonicated for 30 min with intermittent shaking. The solution was cooled to ambient temperature. Then the volume was made up with acetonitrile and centrifuged at 10,000 rpm for 10 min. The centrifuged solution filtered through a 0.45 μm Nylon filters (Millipore, Milford, MA, USA). From the filtered solution, aliquots of appropriate volume were transferred to 10 mL volumetric flasks and diluted to volume with acetonitrile to furnish the concentration range listed in **Table - 1.**

Method Validation

❖ **Linearity & Range**

The linearity of the method was evaluated by analyzing different concentration of the drugs. According to ICH recommendations, at least five concentrations must be used. In this study six concentrations were chosen, in the ranges 2, 4, 6, 8, 10 & 12 ng mL^{-1} glimepiride, 500, 1000, 1500, 2000, 2500 & 3000 ng mL^{-1} of metformin and 15, 30, 45, 60, 75 & 90 ng mL^{-1} pioglitazone respectively.

❖ **Accuracy and precision**

The accuracy of the method was determined by recovery experiments using the standard addition method. Each solution was injected in triplicate and percentage recovery was calculated. The precision of the method was assessed by studying intra-day and inter-day variation. In the intra-day studies, standard and sample solutions were analysed in triplicate on the same day and percentage RSD was calculated. In the inter-day studies, standard and sample solutions were analysed in triplicate on three consecutive days and percentage RSD were calculated.

❖ **Limits of Detection (LOD) and Limit of Quantitation (LOQ)**

In accordance with ICH recommendations the approach based on the standard deviation of the response and the slope of the calibration plots was used to determine detection and quantification limits. LOD and LOQ values were estimated as [(standard deviation of repeatability)/(Slope of the regression equation)] by multiplying with 3.3 and 10 respectively. The values obtained are given in **Table - 4.**

❖ **Selectivity**

The selectivity of the method was evaluated by assessing whether excipients present in the pharmaceutical formulations interfered with the analysis. A placebo for each tablet was prepared by mixing the respective excipients, and solutions were prepared by following the procedure described in the section on sample preparation. The commonly used tablet excipients did not interfere with the method.

❖ **Robustness**

Robustness is a measure of capacity of analytical methods to remain unaffected by small but deliberate variation of the operating conditions. This was tested by studying the effect of changing mobile phase pH by ± 0.2, the amount of buffer in the mobile phase by $\pm 2\%$, and detector wavelength by ± 2 nm

III. RESULTS AND DISCUSSION

It is important to optimize the UPLC conditions and develop UPLCMS method and validate in pharmaceutical formulations, preliminary tests were performed with the objective of selecting optimum chromatographic conditions. The separation was tried using different columns described previously in the literature or alternative stationary phases. The main problems encountered during these investigations were lack of resolution between glimepiride, metformin and pioglitazone with IS. To solve these problems, three columns, HSS C18, BEH C8, and BEH Phenyl, BEH C18 were used for simultaneous determination of the drugs. The best resolution and peak shape, without excessive tailing, were obtained by use of the HSS C18 column. The effect of mobile phase composition, flow rate and pH were also studied. The best resolution with reasonable retention time was obtained with mobile phase containing acetonitrile and 1% ammonium acetate buffer pH adjusted 2.5 with trifluoroacetic acid in gradient elution mode with 0.5 mL min^{-1} flow rate. A major reason for using a concentration of 1% was to achieve maximum sensitivity of PDA detection at low wavelengths. The specificity of the method is illustrated in Fig. 1, which indicates separation of the compounds was complete. Average retention times $\pm$ standard deviation for IS, GLIMP, MET and PIO were 3.165$\pm$0.027, 0.425$\pm$0.005 and 2.3$\pm$0.025min, respectively, for six replicate analyses. In determination of accuracy and precision, recovery was 99.95 $\pm$ 2%, which indicates the method is accurate, and intra-day and inter-day variation, as RSD, were no more than 0.482%, indicating the method is precise. In determination of the robustness of the method, slight variation of mobile phase pH, amount of buffer, in the mobile phase, and detector wavelength had no significant effect on chromatographic resolution.

Mass spectrometry parameters to develop and validate a selective and rapid assay method for simultaneously determination of glimepiride, metformin and pioglitazone in pharmaceutical dosage form. MS parameters were optimized by infusing standard analyte solution of 500 ng mL^{-1} into the mass spectrometer having atmospheric pressure electrospray as the ionization source. The signal intensities obtained in ion mode for glimepiride, metformin and pioglitazone was found to be much higher positive ion mode (M+)- parent ion at m/z, 491.11, 129.87 and 357.04 and tolazamide (internal standard) were higher in positive ion mode (M +H)+ parent ions at m/z 312.04, respectively in QUATTROZQ full scan mass spectra. Fragmentation was initiated using sufficient nitrogen for collision-activated dissociation and by applying 8V collision energy to break the parent ions. It was observed that higher nebuliser gas pressure (70 psi) had a better impact on spectral response. The intensity was further enhanced after acidifying the solution, as it increases the ionization (protonation) resulting in high response in positive ion mode. Cone voltage, capillary voltage Source temperature and desolvation gas temperature did not have much impact on behavior of compounds and were maintained at 3.50, 30.00, 120 °C and 350 °C, respectively. There was no cross talk between the MRMs of analytes and IS. Fine tuning of gas 1 (nebuliser gas), gas 2 (heater gas) and CAD gas was done to get a consistent and stable response with high signal to noise ratio. Figs. 1–3 show the mass spectra of parent and product ions for analytes and IS, respectively. An APEI single probe was selected as the ionization source as it gave high spectral response for both the analytes and the regression curves obtained were linear. The APEI source provided reliable data on method validation and for quantitation of drugs in pharmaceutical dosage form. Since glimepiride, metformin, pioglitazone and tolazamide have different physicochemical properties, it was difficult to set chromatographic conditions that produced sharp peak shape and adequate response. This included mobile phase selection, flow rate, column type and injection volume. Acetonitrile and methanol were tried in different ratio with buffers like ammonium acetate, ammonium formate as well as acid additive like acetic acid in varying strength. It was observed that 1% ammonium acetate (pH 2.5 with trifluoro acetic acid): acetonitrile as the mobile phase in gradient mode was most appropriate to give best sensitivity, efficiency and peak shape. Acidic buffer helped to improve the peak shape and spectral response. 50% aqueous part was adequate to retain the polar compound glimepiride, metformin and pioglitazone. The use of a short chromatography column Waters ACQUITY HSS C18, 1.8 μm, 2.1X50 mm helped in the separation and elution of all four compounds including internal in a very short time. The total chromatographic run time was 5 min for each run. Simultaneous recovery of the

glimepiride, metformin and pioglitazone were 98.50%-99.88%, 98.91%-99.95% & 97.5%-99.9% respectively. Overall, this method is fast and simple in terms of chromatography and sample preparation, respectively, which helped in giving a high turn around for routine sample analysis. The developed method was validated in accordance with ICH guidelines. Since all three drugs viz glimepiride, metformin and pioglitazone and tolazamide (internal standard) had similar chromatographic behavior. Moreover, there was no significant matrix effect of IS on the analytes. Also, the validation results obtained from this UPLC–MS/MS methodology encouraged its selection as an IS for the present study.

Method Validation
❖ *System Suitability*
The RSD values of peak area and retention time for drugs and IS are within 2% indicating the suitability of the system (**Table - 2**)

❖ *Linearity*
The calibration curves were prepared by plotting the peak areas of the drug to IS which were linear in the range of 2-12 ng mL^{-1}, 500-3000 ng mL^{-1} and 15-90 ng mL^{-1} of GLIMP, MET, and PIO respectively. Peak area ratios and concentrations were subjected to least square linear regression analysis to calculate the calibration equations and correlation coefficients. The mean regression equations were found as y = 0.0066x R^2 = 0.9987 (n= 6), y = 0.0014x - 0.0084 R^2 = 0.9988 (n= 6) and y = 0.0031x R^2 = 0.9976 (n= 6) for GLIMP, MET, and PIO respectively. y= ax+b where "y" is the peak area ratio of drugs, "a" is the slope, "b" is the intercept and "x" is the concentration of the measured solution in ng mL^{-1}. The result shows that there is an excellent correlation between the peak area ratios and the concentrations of drugs in the range tested.

❖ *LOD and LOQ*
The LOD was 0.5 ng mL^{-1} for GLIMP, 1.0 ng mL^{-1} for MET and 1.0 ng mL^{-1} for PIO at a signal to noise ratio of 3.3. The limit of quantification was determined as 1 ng mL^{-1} for GLIMP, 5 ng mL^{-1} MET, and 5 ng mL^{-1} for PIO at a signal to noise ratio of 10.

❖ *Precision*
Intra-day precision was performed by relative standard deviation of six repeated assays of samples at the three concentration levels. Inter-day precision was determined by analyzing the same set of samples of five different days. The RSD values were found to be 1.106-1.922% for GLIMP, 0.013-0.043% for MET and 0.083-0.712% for PIO respectively, indicating good precision (**Table - 2**).

❖ *Recovery*
To examine the accuracy of the method, recovery studies were carried out by standard addition method. The percent recovery of the added standard to the assay samples was calculated from:

$$\text{Recovery \%} = [(C_1-C_u)/C_a] \times 100$$

Were C_1 is the total concentration of analyte found; C_u is the concentration analyte present in the formulation; and C_a is the concentration added to the formulation. The average percent recoveries recoveries obtained as 98.95-99.87% indicate good accuracy of the method (**Table- 3**)

❖ *Specificity*
The specificity of the UPLCMS method was determined by the complete separation of GLIMP, MET, and PIO and IS as show in (**Figure: 1**) with parameters like retention time (t_R), resolution (R_s) and tailing factor (T). The peaks obtained for GLIMP, MET, and PIO and IS were sharp and have a clear baseline separation.

❖ *Robustness*
To ensure the insensitivity of the HPLC method to minor changes in the experimental conditions it is important to demonstrate robustness of the method. None of the modifications caused a significant change in the resolution between the drugs and IS, peak area RSD, USP tailing factor, peak width or theoretical plates.

IV.   CONCLUSION

A simple, rapid, and reliable UPLCMS method has been established for simultaneous determination of GLIMP, MET, and PIO either alone or in their ternary formulations. The method has several advantages, including rapid analysis, a simple mobile phase, simple sample preparation, and improved sensitivity. It is suitable for analysis of these antidiabetic agents in their ternary formulations in a single isocratic run, in contrast with previous methods. This makes the method suitable for routine analysis in quality-control laboratories.

Acknowledgement

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Table - 1. Results of system suitability study

	Glimepiride		Metformin		Pioglitazone		Tolazamide (IS)	
	Retention time (min)	Peak area	Retention time (min)	Peak area	Retention time (min)	Peak area	Retention time (min)	Peak area
Mean	3.17	2439	0.425	133977	2.3	1823	2.8	63575
SD	0.027	17.352	0.005	1920.70	0.025	18.76	0.009	374.278
RSD	0.865	0.712	1.289	1.434	1.073	1.029	0.321	0.589

Table - 2. Intra-day and inter-day precision and accuracy of GLIMP, MET and PIO

Name of the drug	Actual concentration (ng mL^{-1})	Intra-day			Inter-day		
		Found concentration (ng mL^{-1}) ± SD	RSD (%)	RME (%)	Found concentration (ng mL^{-1}) ± SD	RSD (%)	RME (%)
GLIMP	6	5.84± 0.112	1.922	0.860	5.761± 0.082	1.428	0.639
	8	7.723±0.147	1.913	0.855	7.753± 0.148	1.912	0.855
	10	9.898± 0.109	1.106	0.495	9.768± 0.146	1.495	0.669
MET	1500	1499.760±0.202	0.013	0.006	1499.323±0.648	0.043	0.019
	2000	1999.390± 0.437	0.022	0.010	1999.035± 0.859	0.043	0.019
	2500	2499.338± 0.701	0.028	0.013	2499.135± 0.727	0.029	0.013
PIO	45	44.835± 0.129	0.287	0.128	44.703± 0.224	0.502	0.224
	60	59.890±0.079	0.131	0.059	59.550± 0.424	0.712	0.319
	75	74.815± 0.062	0.083	0.037	74.607± 0.467	0.626	0.280

Table - 3. Results of recovery studies by standard addition method

Name of the drug	Amount of drug in tablet (ng)[a]	Amount of pure drug added (ng)	Total found (ng)[b] (Mean±SD)	RSD (%)	Recovery of pure drug added (%)
GLIMP	5.24	4	9.198± 0.044	0.483	98.95
	5.24	6	11.19± 0.042	0.377	99.23
	5.24	8	13.18± 0.051	0.391	99.25
MET	1497.62	1000	2496.29± 0.958	0.038	99.87
	1497.62	1500	2992.28± 6.866	0.229	99.60
	1497.62	2000	3483.72± 12.779	0.367	99.30
PIO	44.85	30	74.558± 0.279	0.374	99.0
	44.85	45	89.49± 0.431	0.482	99.20
	44.85	60	104.26± 0.359	0.345	99.02

Table - 4.

Name of the drug	Linearity range (ng mL^{-1})	LOD (ng mL^{-1})	LOQ (ng mL^{-1})	Regression equation
GLIMP	2-12	0.5	1.0	y = 0.0066x R² = 0.9987 (n= 6)
MET	500-3000	1.0	4.0	y = 0.0014x - 0.0084 R² = 0.9988 (n= 6)
PIO	15-90	1.0	5.0	y = 0.0031x R² = 0.9976 (n= 6)

UPLC Chromatograms
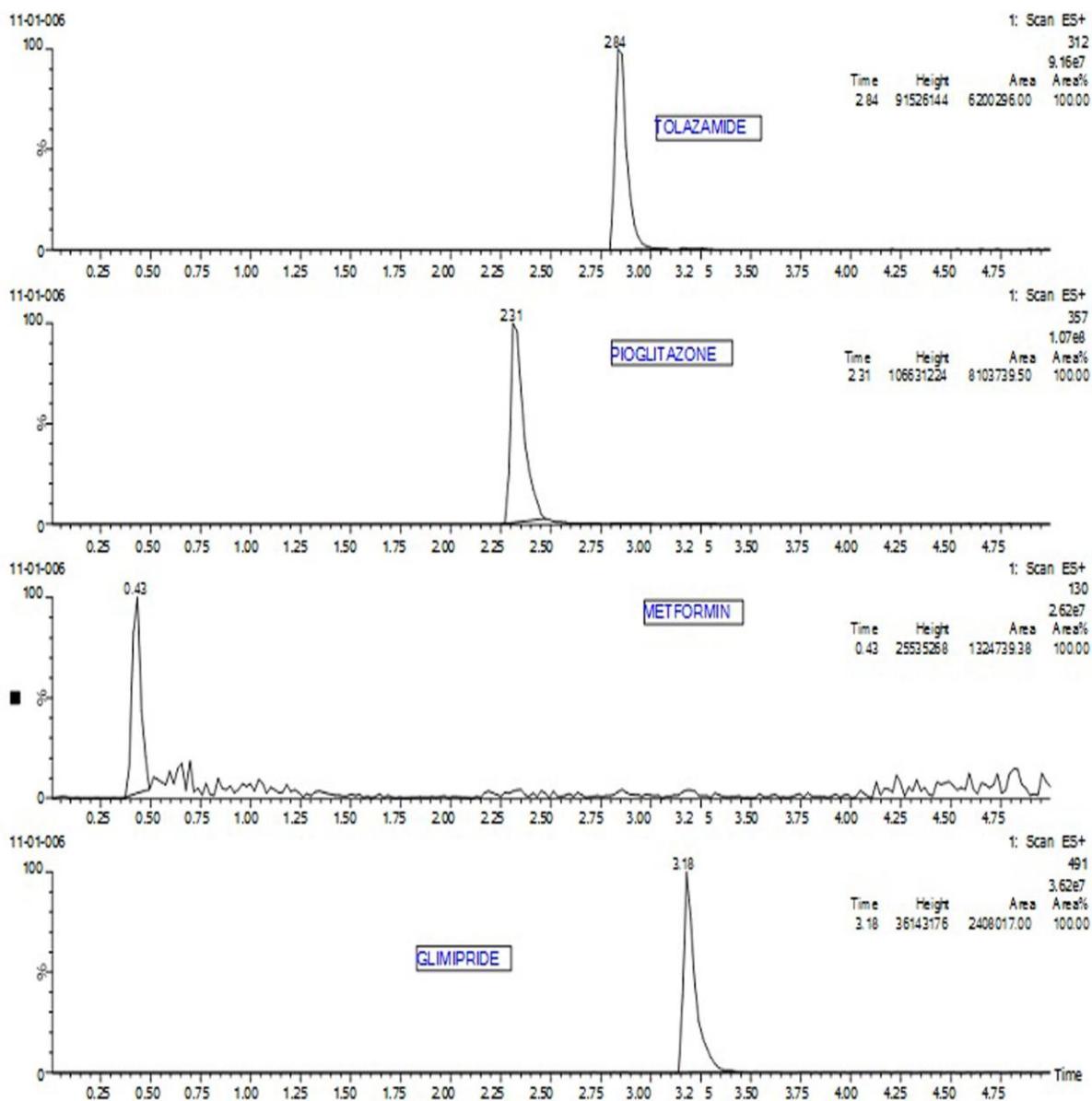
Figure: 1

MS spectra

Figure: 2

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Figure: 3

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