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Tandem Mass Spectrometric Method for the Quantitative Determination of Pioglitazone and their Metabolites in Human Plasma by Using Liquid-Liquid Extraction Technique and its Application to Pharmacokinetic Studies.

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ABSTRACT

There is interest in evaluating the efficacy of lower doses of certain thiazolidinedione family or insulin sensitizers for clinical care. We have developed a **s**ensitive and rapid positive polarity electro-spray ionization with Tandem-mass spectrometry method was validated as per FDA guidelines for the determination of Pioglitazone (PGZ), their metabolites Keto-Pioglitazone (KPGZ) and Hydroxy-Pioglitazone (HPGZ) in human plasma samples. Glyburide was used as an internal standard. The chromatographic separation was achieved by Discovery C_{18} column using isocratic mobile phase consists a mixture of 5mM ammonium acetate (pH 6.4 ± 0.1) and acetonitrile (40:60 v/v). An aliquot of 150 µL plasma was used for the liquid- liquid plasma extraction technique for quantification of the analytes. The molecular ion Q_1, product ion Q_3 transitions for Pioglitazone, Keto-Pioglitazone and Hydroxy-Pioglitazone were found to be 357.2→134.1, 371.3→148.4 and 373.2→150.1 m/z respectively. The proposed method was validated in the concentration range of 10.0-3529.0, 5.0-1209.7 and 5.0-2029.7 ng/mL for PGZ, KPGZ and HPGZ respectively in multiple reaction monitoring mode. The pharmacokinetic parameters for Pioglitazone were found to be T_{max} - 2.5 Hours, C_{max} - 2088.2 ng/mL, $T_{1/2}$ - 6.5 Hours, AUC $_{(0\text{-}T)}$ - 20925.9 ng/mL and AUC $_{(0\text{-}\infty)}$ - 21276.2 ng/mL). The entire results obtained in the study were well within acceptance limits.

Keywords: Determination, Pioglitazone, Metabolites, Plasma and Tandem Mass,

Abbreviations used: PGZ: Pioglitazone; KPGZ: Keto-Pioglitazone; HPGZ: Hydroxy-Pioglitazone; IS: Internal standard and LLE: Liquid-Liquid extraction; AUC: Area under the curve.

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INTRODUCTION

Pioglitazone [1, 2] (PGZ), chemically known as [5-[[4-[2-(5-Ethyl-2-pyridinyl) ethoxy] phenyl] methyl]-2,4-] thiazolidinedione. The structure is shown in fig. 1 (a). It is official in USP [3]. Its empirical formula is $C_{19}H_{20}N_2O_3S$. This was an oral anti diabetic agent [1] belonging to thiazolidinediones which acts mainly by decreasing insulin resistance. PGZ was a peroxisome proliferator-activated receptor-γ (PPARγ) agonist that increases transcription of insulin-responsive genes and there by increases insulin sensitivity. It is used in the treatment of non insulin dependent diabetes Mellitus (Type 2 Diabetes Mellitus [1]). After the oral administration of the pioglitazone, it will be converted into metabolites such as keto and hydroxyl forms of the drug. The structures of the both keto [4] & hydroxy [5] metabolites were shown in figure. 1(b) and figure. 1 (c) respectively. In fasting condition the drug is measurable in plasma within 30 minutes, and reaches the concentration maximum (C_{max}) nearly within two hours of oral administration.

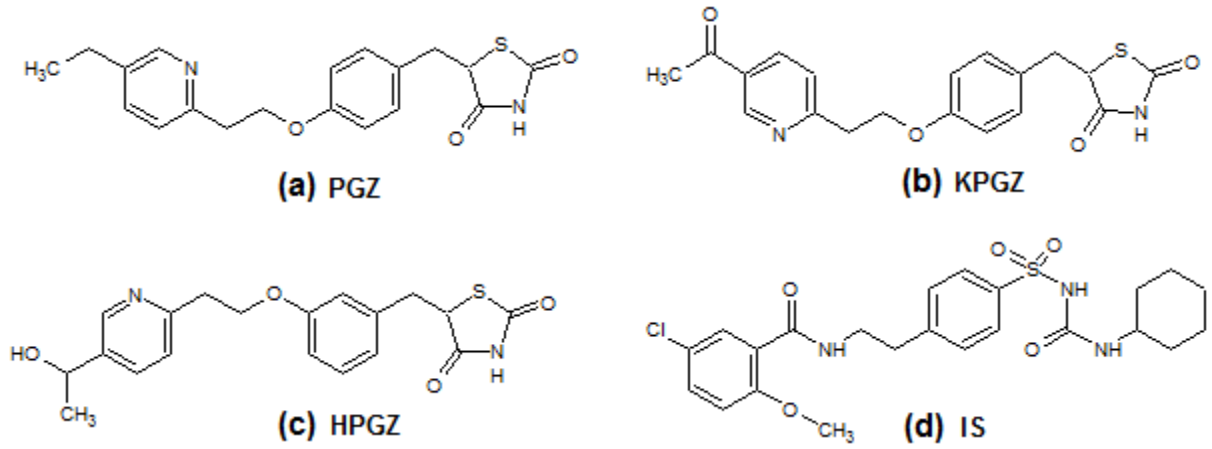

Figure.1 Chemical structures of Pioglitazone (a) and their metabolites (b & c) along with the internal standard glyburide (d).

The C_{max} was delayed up to 3-4 hours with intake of food. More than 99% of the drug bound to plasma proteins. Upon oral administration of the drug, approximately 15% - 30% of the pioglitazone excreted through urine. The drug is excreted primarily as metabolites and their conjugates. Surveys of literature reveals the report of a few methods of determination for Pioglitazone as alone and with its metabolites in pharmaceutical dosage forms [6, 7] , plasma and in other biological fluids based on HPLC [8-13] and Mass Spectrometric [14, 15] are available. The authors now propose a fast, sensitive, accurate and precise tandem mass spectroscopic method for the determination of Pioglitazone and their metabolites in human plasma. The entire results obtained in the present study comply with the acceptance criteria of regulatory requirements [16-18].

MATERIALS AND METHODS

Chemicals and materials

Reference standards of Pioglitazone, metabolites and internal standard glyburide fig. 1 (d) were purchased from Vivan Life Sciences Ltd (Mumbai, India). HPLC grade methanol and acetonitrile were purchased from J.T Baker (Phillipsburg, USA). Analytical grade ammonium acetate, methyl tertiary butyl ether (MTBE) and formic acid were purchased from Merck Ltd (Mumbai, India). The water used for the mass analysis was prepared by Milli-Q water purification system (Bangalore, India). The blank human plasma samples were purchased from Jeevadhara blood bank (Hyderabad, India).

Instrumentation and optimized chromatographic conditions

The HPLC system (Shimadzu, Kyoto, Japan) comprising of two LC-10AD prominence pump, an auto sampler (SIL-HTc), CTO 10 ASvp column oven, a solvent degasser (DGU-14A) and connected with a Discovery C_{18} column (100 mm X 4.6 mm, 5µm) were used for the chromatographic separation. 10µL of the sample volumes were injected into the column, which was maintained at 35^{o}C in the column oven. The optimized isocratic mobile phase consists a mixture of 5mM ammonium acetate (pH 6.4 ± 0.1) and acetonitrile and with a delivered flow rate of 0.8 mL/min into the ionization chamber of mass spectrometer. The quantitation was achieved with daughter ion (MS/MS) detection in positive polarity with multiple reaction monitoring mode for all the analytes and IS using a MDS Sciex API-4000 mass spectrometer (Foster City, CA, USA) equipped with Turboionspary™ interface at 500^{o}C. The ionspray voltage was set at 5000V. The source parameters viz. the nebulizer gas, curtain gas, auxillary gas and collision gas were set at 45, 40, 50 and 5psi respectively. The compound parameters viz. the de-clustering potential (DP), collision energy (CE), entrance potential (EP) and collision cell exit potential (CXP) were 50.0, 17.0, 10.0 & 6.0 V for PGZ; 50.0, 30.0, 10.0 & 6.0 V for KPGZ; 50.0, 24.0, 10.0 & 6.0 V for HPGZ and 50.0, 25.02, 35.0, 10.0 & 6.0 V for IS. The detection of the ions were carried out by monitoring the transition pairs of m/z 357.2 →134.1 for PGZ, 371.3 →148.4 m/z for KPGZ, 373.2 →150.1 m/z for HPGZ and 494.3 →169.1 m/z for glyburide. Both the quadrupoles Q1 and Q3 were set at unit resolution. The retention times obtained for PGZ, KPGZ, HPGZ and the IS were 2.36 ± 0.3, 1.60 ± 0.3, 1.33 ± 0.3 and 2.13 ± 0.3 respectively, over a total run time of 3 minutes. The analysis data obtained was processed by using Analyst Software™ (Version 1.4.2).

Preparation of Stock solutions of the analytes and IS

Two different sets of primary stock solutions for PGZ, KPGZ and HPGZ were prepared by separate weighing, to get at a concentration of 1mg/mL with methanol and were stored at 2-8^{o}C for their stability. Each set of primary stock solutions of all the analytes were suitably diluted with Methanol: Water (50: 50v/v) for the preparation of working standard solutions of calibration curve (CC) and quality control (QC) samples. A working IS dilution was prepared from the IS stock (methanol) 1mg/mL by using the above diluent. The working standard solutions used for QC and CC of each analyte were prepared individually.

Preparation of calibration curve standards and quality control samples

Calibration samples were prepared by spiking each 10µL working standard solution (for CC) of all three analytes individually in 970 µL of screened blank human plasma. Calibration curve standards consists a set of eight nonzero concentrations ranging from 10.0-3529.0 ng/mL for PGZ, 5.0-1209.7 ng/mL for KPGZ and 5.0-2029.7 ng/mL for HPGZ were prepared. Similarly the samples for the determination of precision and accuracy were prepared by spiking all three analytes in screened blank human plasma. 300 µL of drug spiked plasma sample for both CC standards & QC samples were distributed into different tubes for the bulk storage at -70^{o}C in deep freezer until analysis. The QC samples prepared for each analyte were: for Pioglitazone, 10.1 (lower limit of quantification, LLOQ), 29.4 (low quality control, LQC), 1497.9 (medium quality control, MQC) and 2723.4 ng/mL (high quality control, HQC); for Keto-Pioglitazone, 5.1 (LLOQ), 14.8 (LQC), 502.7 (MQC) and 924.0 ng/mL (HQC) and for Hydroxy-Pioglitazone, 5.1 (LLOQ), 14.7 (LQC), 855.6 (MQC) and 1584.4 ng/mL (HQC).

Extraction process of plasma samples

A 150 µL volume of spiked plasma calibration curve standards and the quality control samples were transferred to a set of pre-labeled polypropylene tubes containing 50 µL of glyburide (50ng/mL) dilution. The tubes were vortexed for few seconds. All the tubes were added 100 µL of 3% formic acid in water for the pre treatment and then vortexed. To each of the tubes 2.5 mL of methyl tertiary butyl ether was added using Dispensette® Organic (GmbH, Wertheim, Germany). The tubes were further vortexed for 10 min at 2500 rpm on a vibramax110 (Heidolph, Germany) shaking unit and then were centrifuged at 4500 rpm for 5 min in a refrigerated centrifuge (Eppendorf® Model 5810 R; USA) at 10^{o}C. From the centrifuged tubes approximately 2.0 mL of the supernatant layer was transferred to new set of pre-labeled glass test tubes and contents of the tubes were evaporated in a stream of nitrogen at 40^{o}C and the residues of the dried tubes were reconstituted with 200 µL of the mobile phase. An aliquot of 10 µL was injected into the HPLC column and then analyzed by Tandem mass spectrometer.

Method Validation

The entire validation of the method was carried out as per FDA guidelines (Bioanalytical Method Validation published in May 2001). The method was validated for various parameters like selectivity, sensitivity, matrix effect, linearity, precision, accuracy, recovery, dilution integrity and stability. Selectivity of the method was assessed by analyzing eight different lots of blank human plasma matrix samples which includes one hemolyzed plasma lot and one lipemic plasma lot. The area responses of the interfering substances or noise at the retention times of the PGZ, KPGZ and HPGZ were acceptable, if the % interference was less than 20% of the mean response of the lowest standard in calibration curve point or LLOQ (n = 6). The area responses of the interfering substances or noise at the retention time of internal standard were acceptable, if the % interference was less than 5% mean response of the internal standard areas in the 6 LLOQ samples.

Sensitivity was carried out by the noise or the response from six spiked LLOQ samples. The six replicates should have a precision of ≤ 20% and with an accuracy of ±20% of the nominal value of the LLOQ. The matrix effect was calculated to make sure that precision, selectivity and sensitivity were not affected by the matrix. This parameter was calculated by taking eight different lots of human plasma, which includes one hemolyzed plasma lot and one lipemic plasma lot. Three replicates samples each of HQC and LQC were prepared individually from each plasma lot. Totally 48 QC samples were analyzed from the above said different lots, for the estimation of matrix effect.

The linearity of the method was determined for PGZ, KPGZ and HPGZ in the concentration range of 10.0-3529.0, 5.0-1209.7 and 5.0-2029.7 ng/mL respectively. As per the guidelines for the determination of linearity, a standard calibration curve should at least contain eight nonzero standards. In addition to the CC standards, blank plasma samples were also analyzed to confirm the absence of any interference. These processed blank plasma sample were not used in the calibration plot and they called as zero standards. The acceptance limit of accuracy for each of the back calculated concentration was ±15% of their nominal values except LLOQ which it was ±20% of the LLOQ concentration. For a calibration curve to be accepted at least 67% of nonzero standards should pass including the lower and the upper limit of quantification (LLOQ & ULOQ) concentration. Even two consecutive standards should not fail. Otherwise the calibration curve was rejected. Four replicate analyses were performed on each calibration standard curve.

Intraday precision and accuracy were determined by analyzing six replicates at four different QC levels in the day. Inter day precision and accuracy were determined by analyzing six replicates at

four different QC levels on four different analytical batch runs in between the days. The acceptance criteria for accuracy should within ±15% standard deviation to their nominal concentrations except for LLOQ, it should be ±20 and precision should within ≤15% relative standard deviation (RSD) except for LLOQ, it should be ≤20%.

The recovery of the drug and IS from the plasma extraction method was calculated by comparing the peak areas of the respective analyte in spiked plasma samples (each six of LQC, MQC and HQC) with that of the aqueous equivalents, which were having the same concentration of the QC samples prepared in mobile phase without any extraction procedure. Similarly, the recovery of the IS was determined by comparing the mean peak areas of the extracted QC samples for their IS area with the aqueous equivalent's IS area. The acceptance for the recovery was the RSD of the areas obtained for extracted and aqueous equivalents should be ≤15% individually at each level of QC samples and the RSD of the % recoveries for all of QC levels should be ≤15%. The recovery should be <115% at any level of concentration.

The dilution integrity (DI) was determined the objective of validating the dilution test to be carried out for a higher analyte concentrations above the calibration level (ULOQ) during the analysis of subject samples. DI was processed at 1.8 times of the ULOQ concentration level for all the analytes present. Six replicates each of half and quarter concentrations were prepared and their concentrations were calculated by multiplying with dilution factors of 2 and 4 respectively.

Stability experiments were conducted to evaluate the analyte stability in stock solution and in plasma matrix at different conditions. The stock solution stability at room temperature and refrigerated conditions 2-8^{o}C was determined by comparing the peak area of the analytes (stability samples) with the response of the samples prepared from fresh stock solution (comparison samples). Bench top stability for 10.5 Hours, processed sample stability or auto sampler stability for 36.0 Hours, dry extract stability for 34.5 hours, reinjection stability for 42.5 Hours, freeze thaw stability for 5 cycles and long term stability for 39.5 days were determined at six replicates of LQC & HQC levels. Samples were considered stable if assay values were within the acceptance limits of accuracy should be ±15% of their nominal concentrations and precision should be ≤15 RSD.

Pharmacokinetic study design

The study was performed in screened male healthy volunteers (n = 6). The ethics committee approved the protocol and the volunteers provided with informed written consent. The 45 mg of Pioglitazone was orally administered. Blood samples were collected after oral administration at a fixed time points of pre dose (0.00), 0.33, 0.67, 1.00, 1.50, 2.00, 2.50, 3.00, 3.50, 4.00, 4.50,

5.00, 6.00, 8.00, 10.00, 12.00, 14.00, 16.00, 20.00, 24.00, 36.00, 48.00, 72.00, 96.00, 120.00 and 144.00 hours in K_2EDTA vacutainer collection tubes (BD Vacutainer®, NJ, USA). The tubes were centrifuged for 20 min at 3000 rpm on a refrigerated centrifuge (Eppendorf® Model 5810 R; USA) at 4^{o}C and the plasma was collected and were stored at -70^{o}C until their use. These subject samples were analyzed along with CC and QC samples. The LQC, MQC and HQC were well distributed along between the subject samples in the analytical batch run; individually 50% at each QC level and overall 67% of all the QC samples utilized in the analytical batch should pass. The plasma concentration Vs time profile of each analyte was analyzed by non-compartmental method using WinNonlin® Version 5.2 (Pharsight Corporation, Mountain View CA, USA).

RESULTS AND DISCUSSION

Mass spectrometry

Mass parameters were tuned in both +Ve and –Ve ionization polarity modes for the analytes. The more reproducible area was achieved in positive polarity mode. The data from MRM mode was considered to obtain better selectivity. The protonated $[M + H]^+$ form of each analyte and IS was the parent ion in the Q1spectrum. The same was used as the precursor ion to obtain Q3 product ion spectra. The most sensitive mass transitions were monitored from m/z 357.2 to 134.1 for PGZ, 371.3 to 148.4 m/z for KPGZ, 373.2 to 150.1 m/z for HPGZ and 494.3 to 169.1 m/z for IS. The details of fragmentation pattern for the PGZ, KPGZ, HPGZ and IS were shown in figure.2 to figure.5 respectively.

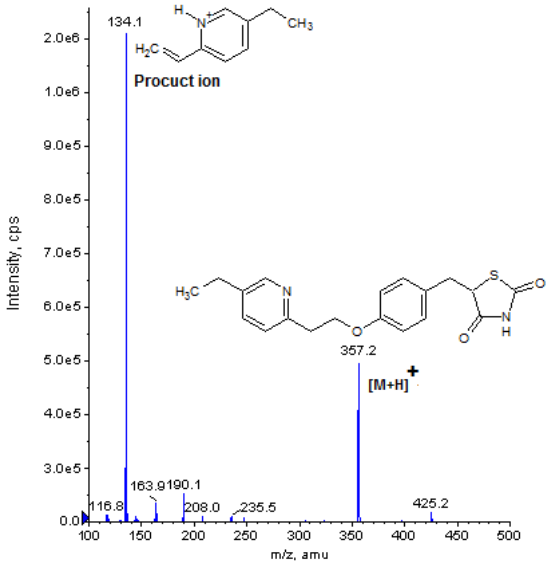

Figure 2. Positive MRM scan mass spectra of Pioglitazone for precursor and product ions.

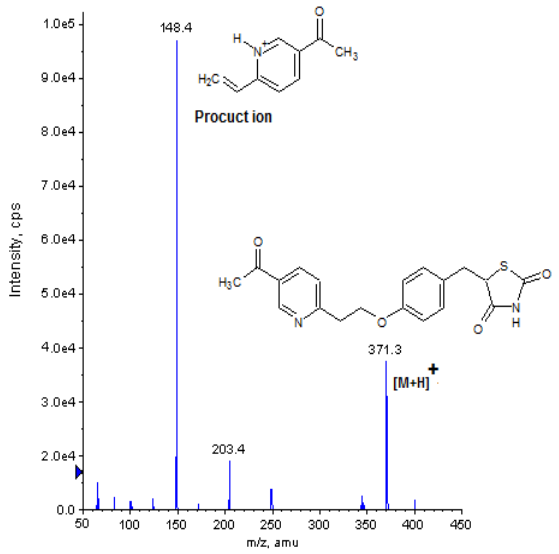

Figure 3. Positive MRM scan mass spectra of Keto-Pioglitazone for precursor and product ions.

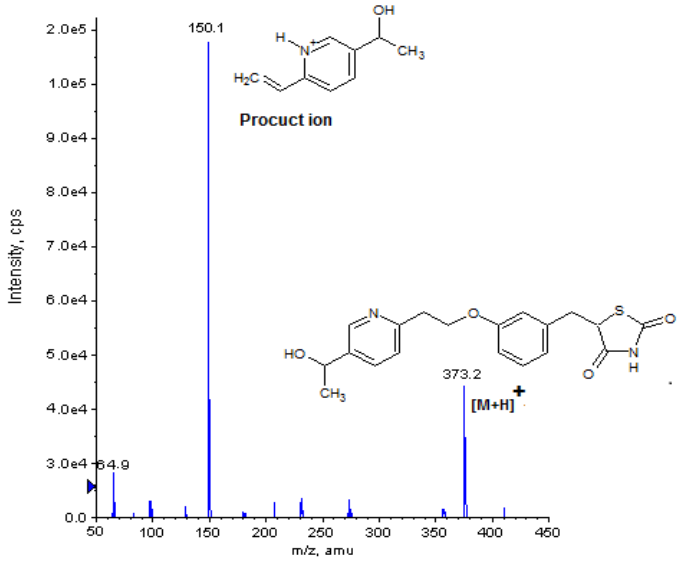

Figure 4. Positive MRM scan mass spectra of Hydroxy-Pioglitazone for precursor and product ions.

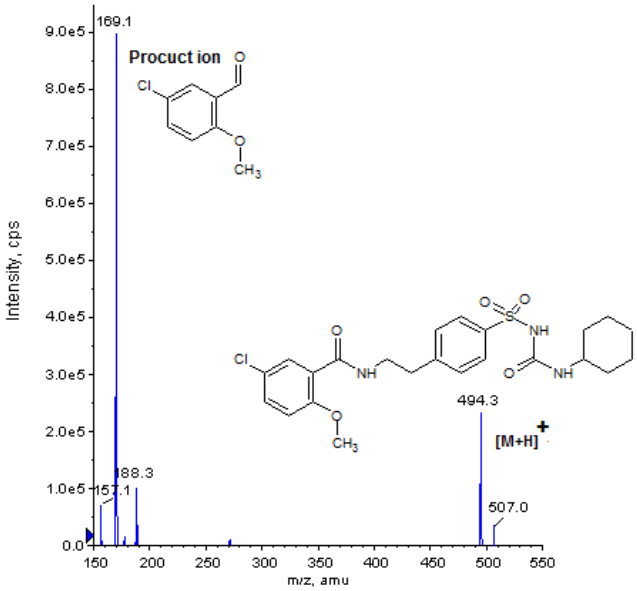

Figure 5. Positive MRM scan mass spectra of glyburide (IS) for precursor and product ions.

Method development

Separation was attempted using various combinations of acetonitrile and buffer with varying contents of each component on different columns; C_8 and C_{18} of different makes like Grace Hypersil, Highpurity Advance, Zorbax, Kromosil, Ace Intersil and Discovery. The use of 5mM ammonium acetate buffer (pH 6.4 ± 0.1) was useful in achieving optimum reproducible response. Mobile phase consisting of acetonitrile and 5mM ammonium acetate buffer (pH 6.4 ±

0.1) was found to be suitable, at which the analytes were protonated and well separated. Discovery C18 (Supelco; 100 mm X 4.6 mm, 5µm) give a good peak shape and response even at LLOQ level for all the analytes and IS. The mobile phase drawn at a flow rate of 0.8 mL/min gave shorter run time of the chromatography.

As the log partition coefficient (log P) value was higher [I] for the drug, LLE would be one of the best extraction techniques. The employed LLE technique in this work helped in producing a clean sample for the analysis there by reducing interferences into the mass spectroscopy. As it was LLE method, it minimizes the experimental cost. Clean sample are necessary for minimizing the ion suppression and matrix effect in mass spectrometry. Among all the extraction solvents checked individually and in combination with other solvents. MTBE was found to be the best. It produces a clean chromatogram for plasma blank and yields a good recovery for all the analytes. As the drug was more than 99% protein bounded, the addition of formic acid provides free drug from the plasma.

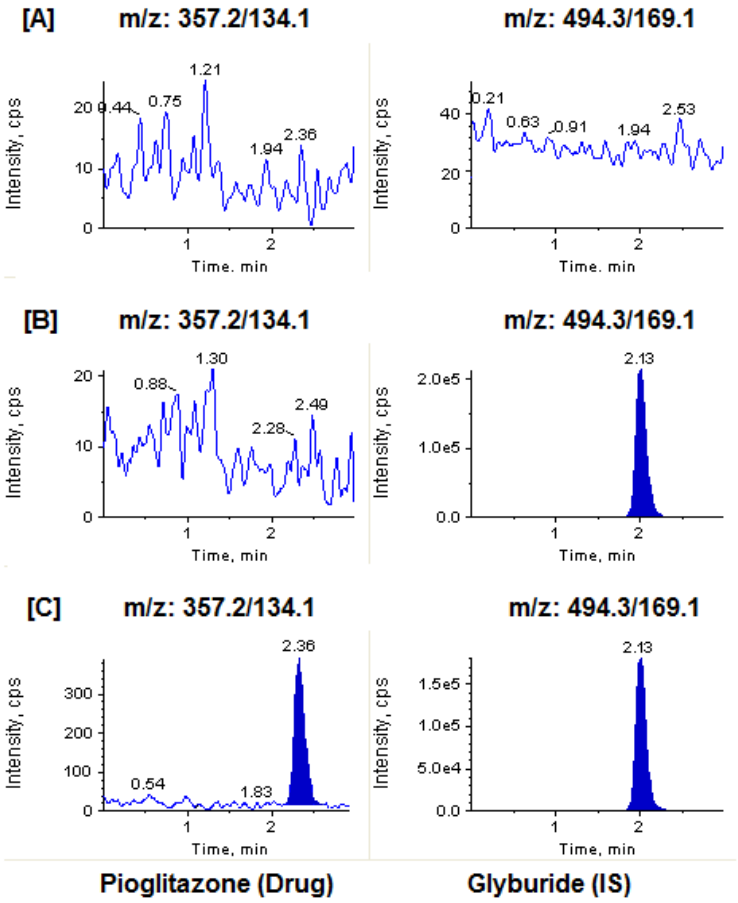

Figure 6. Typical chromatogram of Pioglitazone (left panel) and IS (right panel) in human blank plasma [A], plasma spiked with internal standard [B] and lower limit of quantification sample along with IS [C].

A suitable internal standard must mimic the analyte during extraction and compensate for any analyte on the column. Generally use of stable deuterated compound of the drug as an IS was helpful, when a significant matrix effect is possible. There was no significant matrix effect for all the analytes and IS. After checking with several available compounds, finally glyburide was found to be the best to serve as an internal standard.

Selectivity

The degree of interference by endogenous plasma constituents with the analytes and IS was assessed by inspection of chromatograms derived from a processed blank plasma sample (pure blank & IS added blank). As shown in Figure.6 to 8 for individual analytes, no significant interference in the processed blank plasma samples were observed at the retention times of the analytes and internal standard.

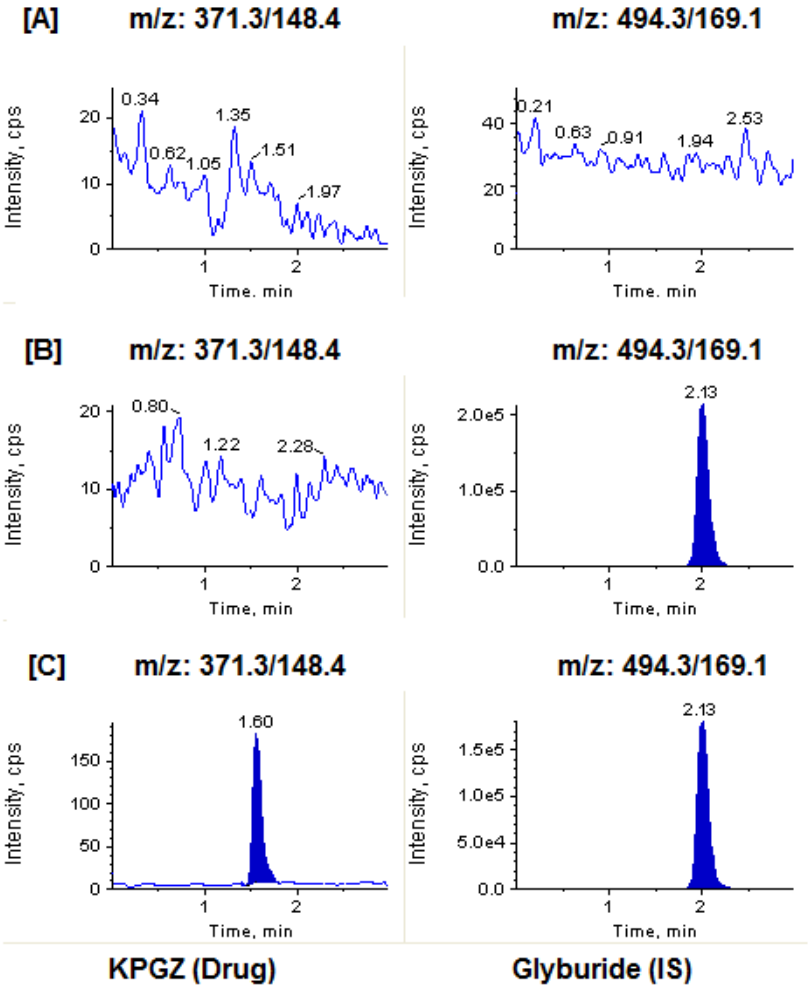

Figure 7. Typical chromatogram of Keto-Pioglitazone (left panel) and IS (right panel) in human blank plasma [A], plasma spiked with internal standard [B] and lower limit of quantification sample along with IS [C]

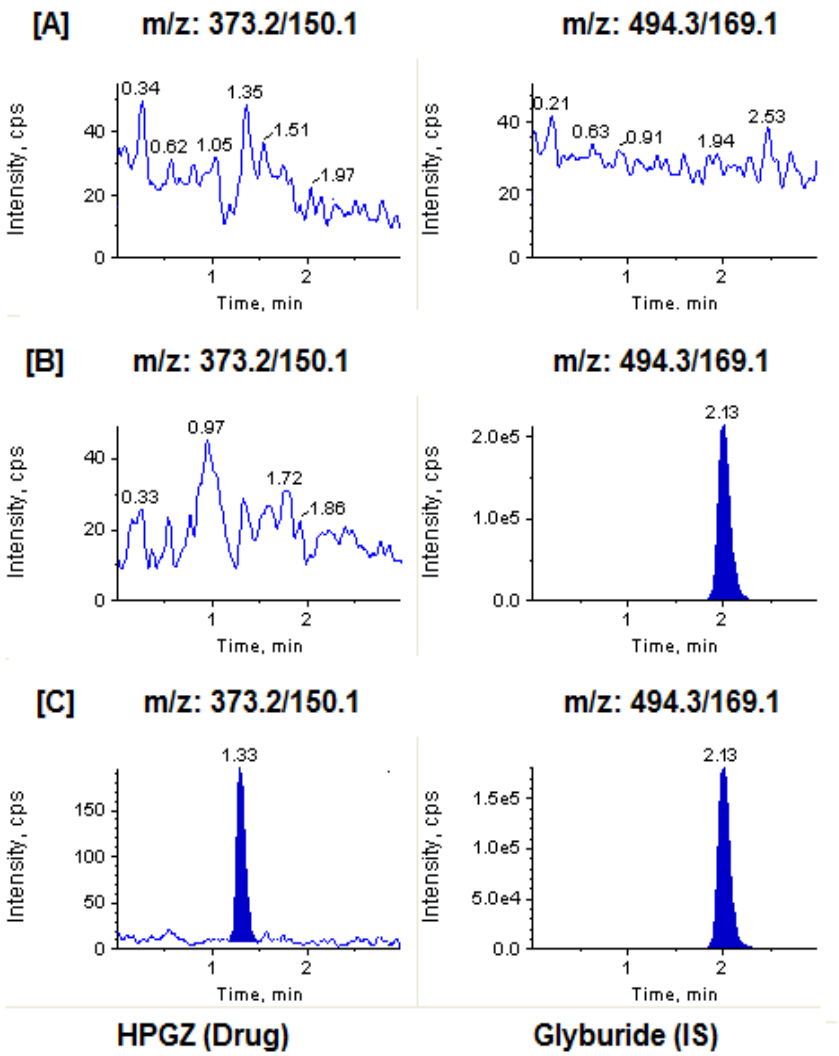

Figure 8. Typical chromatogram of Hydroxy-Pioglitazone (left panel) and IS (right panel) in human blank plasma [A], plasma spiked with internal standard [B] and lower limit of quantification sample along with IS [C].

Sensitivity

The lowest limit of reliable quantification for the analytes was set at the concentration of the LLOQ. The precision and accuracy at LLOQ concentration were found to be 10.1 & 98.0, 5.1 & 102.4 and 5.1 & 104.1 for PGZ, KPGZ and HPGZ respectively.

Extraction efficiency

A simple LLE with MTBE as an extraction solvent proved to be rugged and provided the cleanest samples. The recoveries of the analytes and IS were good and reproducible. The mean over all recoveries with precision range of PGZ, KPGZ and HPGZ were presented in Table.1.

Table 1. Mean overall recoveries of Pioglitazone, Keto Pioglitazone, Hydroxy Pioglitazone and glyburide (IS).

Analyte name	Sample conc.ng/mL	Response unextracted (mean ± SD)	Response Extracted (mean ± SD)	Recovery (%)	Mean (±SD) recovery
Pioglitazone	29.4	5187.5 ± 129.94	4969.0 ± 86.00	95.8	94.61 (± 1.47)
	1497.9	289496.3 ± 2727.14	275266.8 ± 4686.59	95.1	% CV 1.6
	2723.4	509602.2 ± 7476.30	473695.2 ± 8684.30	93.0	
Keto Pioglitazone	14.8	5187.5 ± 129.94	4548.3 ± 96.69	91.0	99.11 (± 1.21)
	502.7	178195.0 ± 471.90	161426.8 ± 3118.94	90.6	% CV 1.3
	924.0	328937.7 ± 8179.01	291862.8 ± 7313.60	88.7	
Hydroxy Pioglitazone	14.7	4188.8 ± 87.88	3833.0 ± 62.39	91.5	90.94 (± 1.69)
	855.6	178564.5 ± 4641.70	158972.0 ± 375.08	89.0	% CV 1.9
	1584.4	367090.7 ± 6906.44	338747.7 ± 744.13	92.3	
Glyburide IS	500.0	412973.7 ± 133.18	379847.8 ± 6057.14	92.0	

Matrix effect

There was no significant matrix effect was observed in all the eight lots of human plasma for the analytes at low and high QC level concentrations. The precision and accuracy for PGZ, KPGZ and HPGZ at LQC were 4.1% & 104.3 %, 4.1% & 104.3 % and 4.1% & 104.3 % respectively. Similarly at HQC were 4.1% & 104.3 %, 4.1% & 104.3 % and 4.1% & 104.3 % respectively.

Linearity

The eight-point calibration curve was found to be linear over the concentration range of 10.0-3529.0, 5-1209.7 and 5-2029.7 ng/mL for PGZ, KPGZ and HPGZ respectively. After comparing the weighting factor models at none, 1/X and $1/X^2$, the linear regression equation with weighting factor $1/X^2$ of the analytes to the internal standard concentration was found to be the best fit in plasma samples. The mean (n = 4) correlation coefficient of the calibration curves generated in the validation was 0.99, 0.991 and 0.993 for PGZ, KPGZ and HPGZ respectively.

Precision and accuracy

Precision and accuracy data for intra, inter day samples for all the analytes were presented in Table 2. The results obtained in both the criteria were well within acceptance limits.

Table 2. Precision and accuracy of the method for determining Pioglitazone, Keto Pioglitazone, Hydroxy Pioglitazone

		Intra-day precision and accuracy (n = 12) six from each batch			Inter-day precision and accuracy (n = 24) six from each batch		
Analyte	**Concentration ng/mL**	**Conc. found (Mean; ng/mL)**	**Precision (% CV)**	**Accuracy (%)**	**Conc. found (Mean; ng/mL)**	**Precision (% CV)**	**Accuracy (%)**
PGZ	10.1	9.77	6.2	96.7	9.72	7.0	96.2
	29.4	29.78	4.0	101.3	28.74	4.5	97.8
	1497.9	1546.88	3.5	103.3	1548.50	3.5	103.4
	2723.4	2834.72	2.5	104.1	2808.27	3.3	103.1
KPGZ	5.1	5.18	6.6	101.6	5.08	9.0	99.7
	14.8	14.43	5.4	97.5	14.91	6.2	100.7
	502.7	497.13	2.6	98.9	507.06	5.4	100.9
	924.0	923.33	1.9	99.9	927.63	3.5	100.4
HPGZ	5.1	4.73	8.6	92.8	5.23	9.0	102.5
	14.7	15.37	4.0	104.5	15.07	4.2	102.5
	855.6	883.80	1.7	103.3	846.90	4.3	99.0
	1584.4	1583.60	2.1	99.9	1560.38	5.3	98.5

Dilution integrity

The ULOQ concentration was extend to 6352.2, 217.2 & 3652.2 ng/mL for PGZ, KPGZ and HPGZ half (1/2) and quarter (1/4) dilution were done with screened blank human plasma. The mean back calculated concentrations for the dilution samples were within 85-115% of their nominal values. The coefficient of variation (CV) for the dilution samples≤8 for all the analytes.

Stability studies

The different stability experiments carried out, viz. bench-top stability for 10.5 hours, auto-sampler stability for 36.0 hours, repeated freeze-thaw cycles for 5 cycles, re-injection stability for 42.5 hours, dry extract stability for 34.5 hours at 1-10^{o}C, and long-term stability at -70 ^{o}C for 39.5 days. The mean percentage nominal values of the analytes were found to be within ± 15% of the predicted concentrations for the analytes at their LQC and HQC levels. Thus, the results were found to be within the acceptable limits during the entire validation Table.3.

Table 3. Stability data of the Pioglitazone, Keto Pioglitazone, Hydroxy Pioglitazone

Analyte	**Stabilities**	**QC Conc. (ng/mL)**	**Mean ± SD**	**Precision (% CV)**	**Stability (%)**
PGZ	Auto-sampler (40Hr)	29.4 (LQC)	28.98 ± 1.57	5.42	98.5
			2784.50 ± 266.94	9.51	102.2
	Dry extract (36.5Hr)	2723.4	29.61 ± 1.37	4.62	100.7
		(HQC)	2979.16 ± 87.98	2.94	109.8

	Bench top (10.25Hr)		29.38 ± 1.01	3.51	99.9
			3074.83 ± 230.40	7.45	112.3
	Freeze-thaw (5Cycle)		30.15 ± 0.91	3.03	102.5
			2973.12 ± 93.32	3.13	109.1
	Re injection (42.5Hr)		29.96 ± 1.19	4.01	101.9
			2968.51 ± 92.23	3.01	108.9
	Long term (39.5Days)		29.36 ± 2.12	7.21	99.8
			3026.14 ± 110.26	3.64	111.1
KPGZ	Auto-sampler (40Hr)	14.8	15.08 ± 0.60	4.02	101.9
		(LQC)	995.66 ± 51.58	5.18	107.7
	Dry extract (36.5Hr)		15.61 ± 0.84	5.40	105.4
		924.0	1012.83 ± 44.16	4.36	109.6
	Bench top (10.25Hr)	(HQC)	16.31 ± 0.61	3.73	110.2
			972.16 ± 49.98	5.14	105.2
	Freeze-thaw (5Cycle)		15.83 ± 0.71	3.98	106.2
			1005.16 ± 36.67	3.65	108.7
	Re injection (42.5Hr)		14.74 ± 1.54	10.47	99.6
			1008.66 ± 70.71	7.01	109.1
	Long term (39.5Days)		15.58 ± 0.85	5.47	105.2
			1030.51 ± 35.27	3.42	111.5
HPGZ	Auto-sampler (40Hr)	14.7	15.05 ±1.17	7.78	102.4
		(LQC)	1543.66 ± 116.98	7.57	97.4
	Dry extract (36.5Hr)		14.48 ± 1.59	10.96	98.5
		1584.4	1588.41 ± 58.36	3.67	100.2
	Bench top (10.25Hr)	(HQC)	15.75 ± 1.21	7.74	107.1
			1613.51 ± 109.26	6.77	101.8
	Freeze-thaw (5Cycle)		14.93 ± 1.55	10.32	101.5
			1626.33 ± 97.26	5.99	102.6
	Re injection (42.5Hr)		15.18 ± 1.15	7.59	103.2
			1650.66 ± 92.53	5.60	104.7
	Long term (39.5Days)		14.96 ± 0.88	5.91	101.2
			1735.16 ± 67.22	3.88	109.2

Pharmacokinetic study

In order to verify the sensitivity and selectivity of this method in a real-time analysis, the present method was used to test for pioglitazone in human plasma samples collected from healthy male volunteers (n=6) in between the age 18-45 years. Institutional review board approval was obtained before study start, and all subjects given written informed consent before participation. Each subject received single oral dose of Pioglitazone 45 mg tablets and plasma samples obtained were analysed for pioglitazone, keto pioglitazone and hydroxy pioglitazone. The mean plasma concentration verses time profiles of Pioglitazone, Keto Pioglitazone and Hydroxy Pioglitazone are shown in Figure 9. The plasma concentration–time data were analyzed by non-compartmental analysis method using the WinNonlin® Software (Version 5.0.1 from Pharsight Corporation, USA) for estimation of pharmacokinetic parameters. The results obtained were shown in Table.4 and matching with the published data[19-21].

Table 4. Pharmacokinetic data for Pioglitazone

Parameter	Piogltazone (PGZ)	Keto PGZ	Hydroxy PGZ
C_{max} in ng/mL	2088.2 ± 550	656.9 ± 320	1275.3 ± 436
T_{max} in hrs	2.5 ± 2.0	10.0 ± 3.2	14.0 ± 3.5
AUC $_{(0\text{-}T)}$ in ng.hrs/mL	20925.9 ± 4254	26450.0 ± 3756	62692.8 ± 6538
AUC $_{(0\text{-}\infty)}$ in ng.hrs/mL	21276.2 ± 3975	27012.1 ± 3863	64456.2 ± 6079
$t_{1/2}$ in hrs	6.5	20.4	25.3

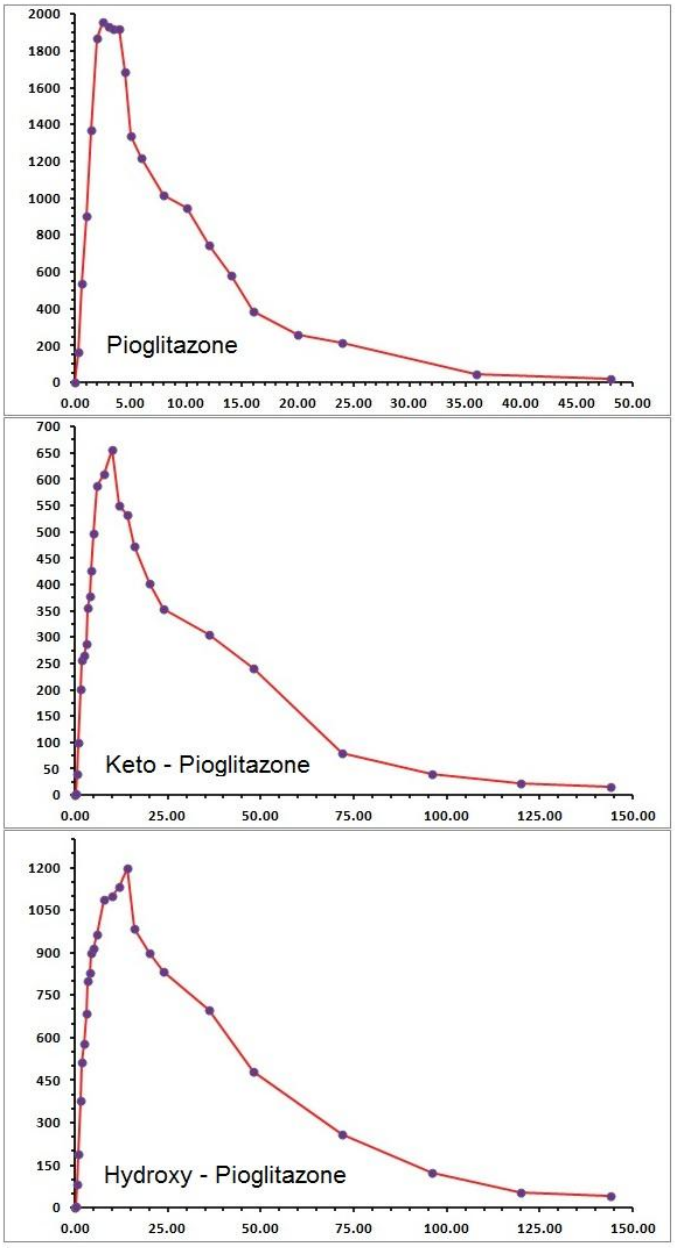

Figure 9. Mean concentration - Time curve of the 6 volunteers for Pioglitazone and its metabolites.

Validated methods are essential for the determination of PGZ, KPGZ and HPGZ concentrations in human plasma for bioequivalence studies. To the best of our knowledge, this is the first time

all three analytes have been estimated simultaneously in any of the matrix without compromising on the reported sensitivity for each analyte using a single IS with a simple LLE technique using methyl tertiary butyl ether with a short run time of 3 minutes for each sample analysis with a good sensitivity (LLOQ 10.1, 5.1 & 5.1 ng/mL for PGZ, KPGZ & HPGZ respectively).

CONCLUSION

In summary, we have developed and validated a rapid, sensitive, specific, reproducible and high-throughput LC-MS/MS method to quantify PGZ, KPGZ and HPGZ simultaneously using a single IS. The cost –effectiveness, simplicity of the assay , using liquid-liquid extraction, and the sample turnover rate less than 3.0 minutes per sample, make it an attractive procedure for high-throughput bio-analysis of PGZ, KPGZ and HPGZ. From the results of all the validation parameters, we can conclude that the developed method can be useful for bioavailability studies and routine therapeutic drug monitoring with the desired precision and accuracy.

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