Rapid, Selective, Rugged and High throughput Simultaneous Method Development and Validation of Pioglitazone and its Metabolites, M III and M IV in Plasma Using 96 well Plate Solid Phase Extraction and Liquid Chromatography Coupled with Tandem Mass Spectrometry

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Abstract- A high throughput, sensitive, selective, and rugged liquid chromatography coupled with mass spectrometry (LC-MS/MS) method for the quantification of Pioglitazone and its active metabolites, M III and M IV, in human plasma was developed and validated. The analytes were extracted from plasma by solid phase extraction technique using Waters Oasis HLB 96 well plate 30 µm (10 mg). Isocratic elution of Pioglitazone and its metabolites were achieved in 5.5 min using Denali, C18, 4.6 X 150mm, 5µ column having a mobile phase of 10mM Ammonium acetate buffer (pH 6.5 ± 0.3): Acetonitrile 50: 50 v/v. The flow rate was 1 mL/min at a column temperature of 35 ± 5°C. Electron spray ionization technique in negative mode was selected to improve the selectivity and sensitivity required for this application. The retention times of Pioglitazone and metabolites M III and M IV were 4.0, 2.35, and 1.80 min, respectively. The method was validated for linearity, precision, accuracy, specificity, sensitivity, matrix effect, dilution integrity, ruggedness, reinjection reproducibility, and stability. The assay produced quadratic calibration curves over the concentration range for Pioglitazone, M III and M IV in the ranges of 20.200–2003.400, 5.010–501.270, and 10.100-1005.6 ng/mL with correlation coefficients greater than 0.9987, 0.9988, and 0.9990, respectively, and by using a 1/x2 weighted least square regression analysis of standard plots associated with nine-point calibration standards. The precision and mean accuracy were within the acceptable limits.

Index Terms- Pioglitazone, M III, M IV, Solid Phase Extraction and Waters Oasis HLB 96 well plate

I. INTRODUCTION

Pioglitazone hydrochloride,(9/)-5-{4-[2-(5-ethyl-2-pyridyl)ethoxy] benzyl}-2,4-hiazolidinedione hydrochloride salt, is prescription oral antidiabetic drug of thiazolidinedione (TZD) class with hypoglycemic action to treat diabetes. It has been shown to affect abnormal glucose and lipid metabolism associated with insulin resistance by enhancing insulin action on peripheral tissues in animal models. Pioglitazone (PIO) is extensively metabolized by hydroxylation and oxidation. Metabolites M-III (keto derivative of pioglitazone) and M-IV (hydroxy derivative of pioglitazone) are pharmacologically active in animal models of type 2 diabetes [1-10]. Analysis of PIO and its metabolites in biological fluids by high performance liquid chromatography (HPLC) with ultraviolet detection and mass spectrometry has appeared in the literature [11-13]. However, these HPLC assays lack specificity, sufficient sensitivity (LLOQ: 50 ng/ml using a sample volume of 0.2 to 0.5 ml) and require long analytical run times (over 20 min), which make the LC-UV method impractical for routine analysis of large numbers of clinical samples. Very few LC-MS/MS methods have been reported using Liquid Liquid extraction techniques for sample extraction [14]. Therefore, this paper describes a sensitive, specific, and rapid LC-MS/MS method for the simultaneous determination of PIO and its two active metabolites (M-III and M-IV) in human plasma using Solid phase extraction technique in 96 well plate format and the validation as per the US FDA guidelines [15].

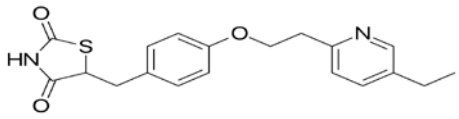

Fig 1: Structure of Pioglitazone

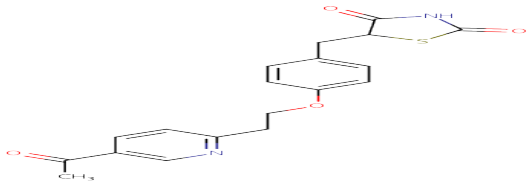

Fig 2: Structure of Pioglitazone M III

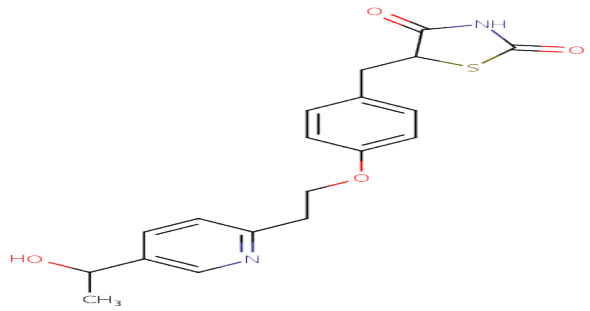

Fig 3: Structure of Pioglitazone M IV

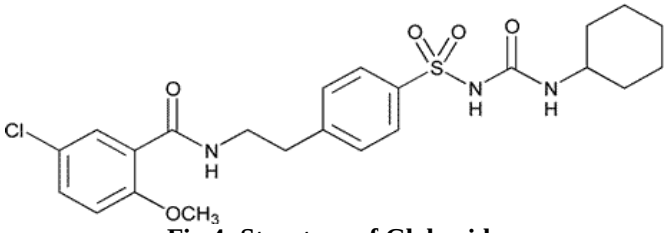

Fig 4: Structure of Glyburide

II. EXPERIMENTAL

Chemicals and reagents

Working standards of Pioglitazone, Pioglitazone M III and Pioglitazone M IV were obtained from Clearsynth. Glyburide was obtained from sigma Aldrich. Waters Oasis HLB 96 well plates 30 µm (10 mg) were purchased from Waters Corporation (Milford, MA, USA). LC–MS grade acetonitrile was purchased from Thermo Fisher Scientific India Pvt. Ltd. (Mumbai, India). GR grade ammonium acetate was pro-cured from Merck Specialties Pvt. Ltd. (Mumbai, India). Formic acid was obtained from Sigma Aldrich. HPLC water was obtained from Milli-Q water purification system (Millipore). Human plasma containing K2 EDTA anticoagulant was obtained from Deccan pathological lab (Hyderabad, India).

Instrumentation

Agilent 1200 Series equipped with a binary pump for solvent delivery was used for the analysis. Mass spectrometric detection was performed on API-4000 triple quadrupole mass spectrometer (MDS SCIEX, Toronto, Canada) equipped with turbo ion spray inter-face. Quantitation was performed in multiple reaction monitoring (MRM) mode and Analyst software version 1.5.1(SCIEX) was used for controlling the hardware and data handling.

Chromatographic conditions

Chromatographic separation was performed on Denali, C18, 4.6 X 150mm, 5µ analytical columns. Isocratic mobile phase consisting of 10mM Ammonium acetate buffer (pH 6.5 ± 0.3) and Acetonitrile in 50: 50 v/v ratio was delivered at a flow rate of 1.0 mL/min. The auto sampler was set at $4^0C \pm 2^0C$ and the injection volume was 5 µL. The column oven temperature was set at 35.0 ± 2.0°C.

The retention times of Pioglitazone and metabolites M III and M IV were 4.0, 2.35, and 1.80 min, respectively. The retention time of internal standard (Glyburide) was 3.4 min. The total chromatographic run time was 5.5 min.

Mass spectrometric conditions

Ionization mode: Negative ionization
Resolution: Q1 Unit; Q3 Unit

MRM conditions

Parameters	Q1 (amu)	Q3 (amu)	Dwell Time (msec)	DP (volts)	CE (volts)	CXP (volts)	EP (volts)
Pioglitazone	355.1	312.1	200	-60	-28	-15	-10
Pioglitazone M III	369.0	326.1	200	-60	-25	-15	-10
Pioglitazone M IV	370.9	328.0	200	-60	-25	-15	-10
Glyburide	492.2	169.9	200	-60	-30	-12	-10

Source/ Gas parameters

Parameters	CUR (psi)	GS1 (psi)	GS2 (psi)	IS (Volts)	CAD (psi)	TEMP (°C)
Source/Gas	40	40	40	-4500	4	400

Preparation of calibration standards and quality control samples

Standard stock solutions of Pioglitazone, M III, M IV and internal standard (Glyburide) were prepared by dissolving their accurately weighed amounts in DMSO to give a final concentration of 1mg/mL. Working solutions of analyte (Mixture) were prepared by appropriate dilution of their stock solutions in 50:50 v/v DMSO: Acetonitrile. All the solutions were stored in refrigerator at below 10◦C and were brought to room temperature before use. Working solution of internal

standard (Glyburide, 50 ng/mL) was prepared daily in 50% acetonitrile and was stored at room temperature.

Calibration standards and quality control (QC) samples were prepared by spiking K_2 EDTA human blank plasma with the working solutions (5%) prepared from independent stock weightings. Calibration standards and quality controls were prepared in plasma at following concentrations:

CC standard and QC samples	Pioglitazone (ng/mL)	M-III (ng/mL)	M-IV (ng/mL)
CS9	2003.4	501.270	1005.600
CS8	1600.200	401.250	801.200
CS7	1001.7	250.640	502.800
CS6	801.4	200.510	402.200
CS5	400.8	100.250	201.100
CS4	200.3	50.130	100.600
CS3	100.2	25.060	50.300
CS2	40.4	10.020	20.200
CS1	20.2	5.010	10.100
HQC	1527.100	380.970	30.000
MQC	916.300	228.580	461.400
LQC	59.600	14.860	769.000

Sample Preparation:

Calibration standards, QC's were processed using Ezypress Positive Pressure SPE Manifold by using 100 µL of Plasma Volume

- For CC and QC spike 5 µL of each working solutions into 95 µL of human plasma.
- Add 20 µL of internal standard to each tube except for blank plasma samples.
- Add 20 µL of 50:50v/v MeoH: Water to blank plasma samples
- Add 50 µL of 0.5% OPA solution to each tube and vortex.
- Condition the Waters Oasis HLB 96 well plate 30 µm (10 mg) with 1 mL of methanol followed by 1 mL of Milli Q water.
- Load the samples onto the plate.
- Wash the cartridges with 500 µL of Milli Q Water followed by 500µL of 5% Methanol in water.
- Elute the sample with 800 µL (two aliquots of 400 µL each) of methanol into 96 well collection plate.
- Evaporate the eluent under a gentle stream of nitrogen using a TurboVap 96, at a temperature of approximately 50°C.
- Reconstitute the dried samples with 100 µL of mobile phase and vortex to mix.
- Inject 5 µL of the sample onto the LC-MS/MS system.

Method validation

A complete method validation of Pioglitazone and metabolites M III and M IV was done following the USFDA and EMEA guidelines. Validation runs were performed on different days to evaluate selectivity, sensitivity, linearity, precision, accuracy, recovery, matrix effect, dilution integrity, ruggedness and stability. Each validation run was organized with a set of spiked standard samples, blank (with ISTD and without ISTD) and QC samples as per the validation parameter. Standard samples were analyzed at the beginning of the run and QC samples were distributed consistently throughout the validation runs.

Selectivity of the method toward endogenous and exogenous components of plasma was evaluated in 6 different plasma lots. The blank plasma lots were extracted (without addition of ISTD), and injected for LC–MS/MS detection. Later selectivity in each lot was evaluated by comparing the blank peak responses against the mean peak response observed in plasma spiked LLOQ sample (n = 6).

Linearity of the method was assessed using three calibration curves analyzed on three different days. Each plot was associated with a nine point non-zero concentrations spread over the dynamic range. A quadratic regression analysis with reciprocate of drug concentration as weighing factor (1/X2) was performed on peak area ratios versus analyte concentrations. Peak area ratios for plasma spiked calibration standards were proportional to the concentration of analytes over the established range.

Intra batch (within day) and inter batch (between day) precision and accuracy was evaluated at four distinct concentrations (LLOQ, LQC, MQC, HQC). Precision and accuracy at each concentration level was evaluated in terms of %CV and relative error respectively. The extraction recovery of analytes was determined at LQC, MQC and HQC levels. The relative recoveries were evaluated by comparing the peak areas of extracted samples (spiked before extraction) with that of un-extracted samples (blank extracts spiked after extraction).

The matrix effect was checked at low and high QC level using six different blank plasma lots (including one hemolytic and one lipemic lot). Matrix factor for analytes and internal standard was calculated in each lot by comparing the peak responses of post

extraction samples (blank extracts spiked after extraction) against the peak responses of equivalent aqueous samples prepared in mobile phase. Internal standard normalized matrix factor in each lot was later evaluated by comparing the matrix factor of analyte and internal standard.

Stability of analytes in both solutions and in biological matrix was evaluated after subjecting to different conditions and temperatures that could encounter during regular analysis. Stability in plasma was evaluated in terms of freeze–thaw stability, bench top stability, long-term stability, and extracted sample stability. Freeze–thaw stability was evaluated after seven freeze (at -70^{0}C) thaw (at room temperature) cycles. Bench top stability was assessed at room temperature and the long-term stability was evaluated at both -70^0 C and -20^0 C. Stability of extracted samples was determined after reconstitution (in-injector stability at 4^{0}C). Reinjection reproducibility was proved for 49 Hrs 51 min. All the stability assessments were made at LQC and HQC level by comparing the stability samples against freshly prepared samples.

Stability of analytes in stock solutions and in working solutions was assessed at room temperature (short-term stability) and at 2-8^{0}C (long-term stability). All comparisons were made against freshly prepared stock solutions or working solutions. Before each analytical run, system suitability was evaluated by injecting six replicates of MQC sample to check the system precision and chromatography. System suitability was considered acceptable when the coefficient of variation for response ratios was less than 4.0%.

III. Results and Discussion

Method development

For consistent and reliable estimation of analytes it was necessary to give equal importance for optimization of extraction procedure along with chromatographic and mass spectrometric conditions. Analytes and ISTD were tuned in negative polarity mode using electrospray ionization technique. The Q1 and the MSMS scans were made in infusion mode and further compound and gas parameters were optimized in flow injection analysis. The [M-H] peaks were observed at m/z of 355.1, 369.0, 370.9, 492.2 for Pioglitazone, M III, M IV and Glyburide respectively. Most abundant product ions were found at m/z of 312.1, 326.1, 328.0, 169.9 for Pioglitazone, M III, M IV and Glyburide respectively (Fig. 5 to 8) by applying sufficient collision activated dissociation gas and collision energy. Increase in source temperature beyond 400^{0}C augmented the intensity. A 5% change in ionspray voltage and gas parameters did not affect the signal intensity.

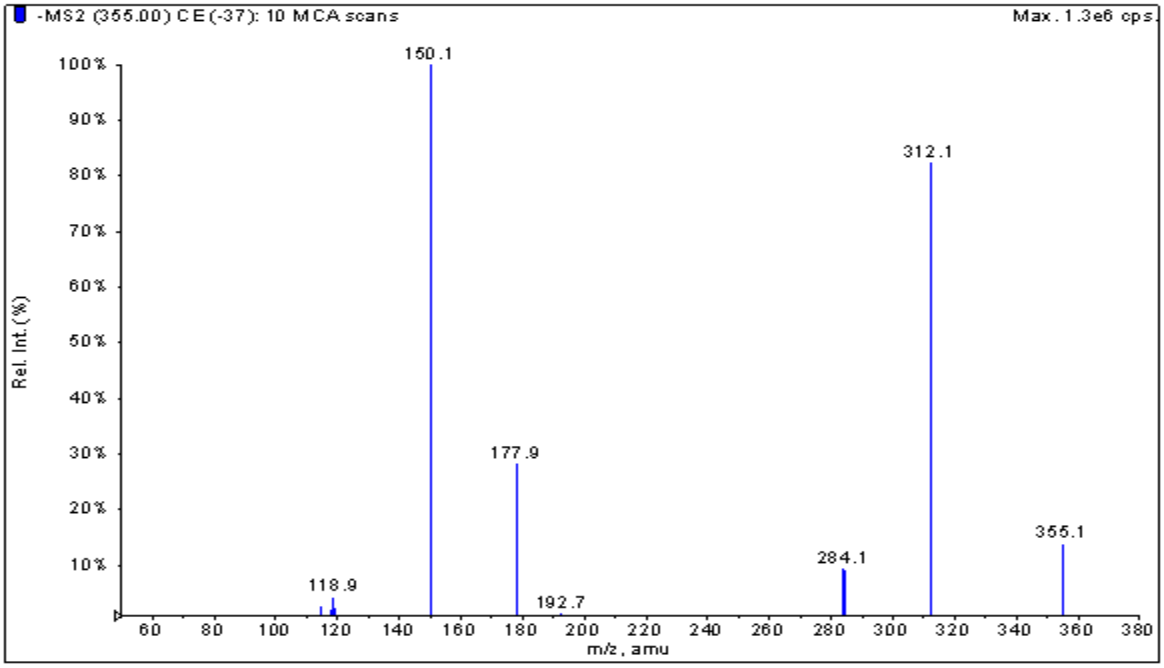

Fig 5: Pioglitazone MS/MS SCAN

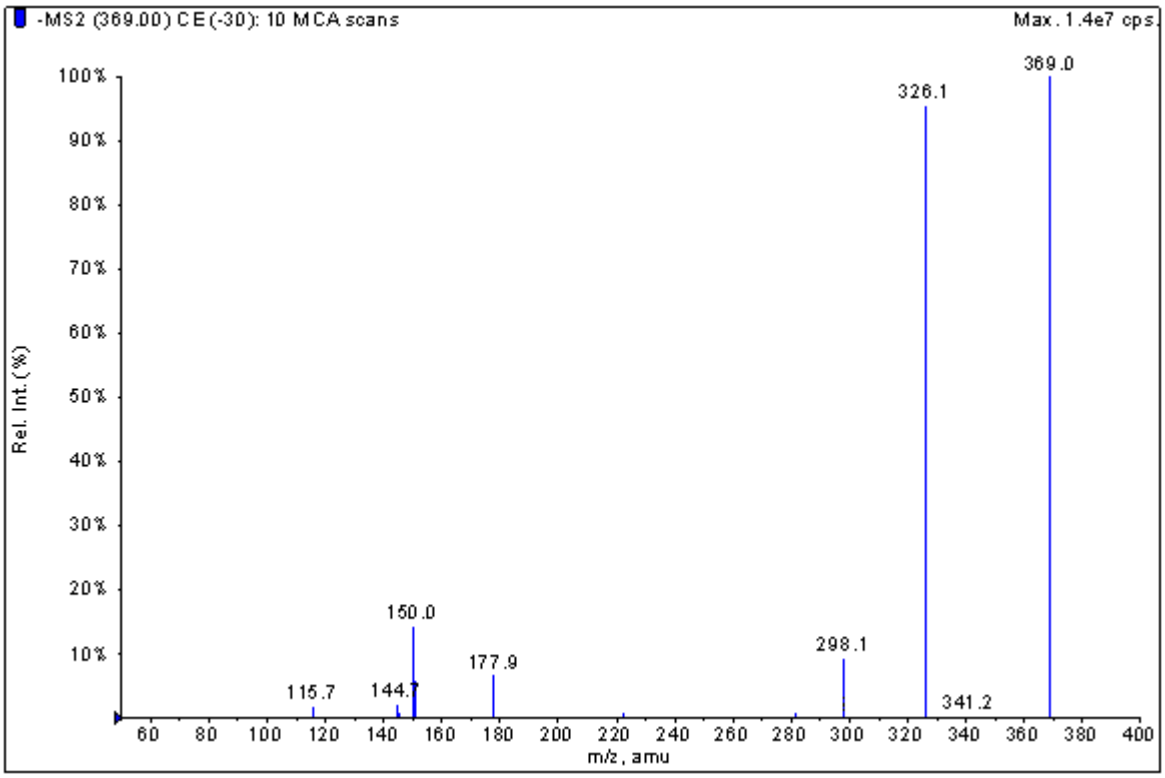

Fig 6: Pioglitazone M III MS/MS SCAN

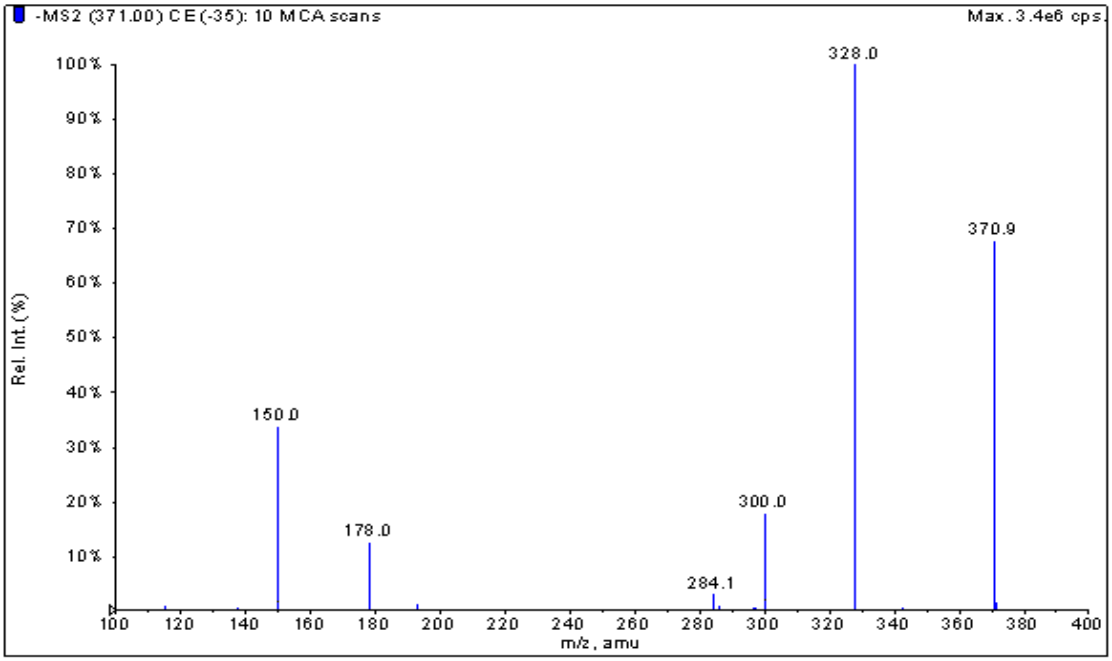

Fig 7: Pioglitazone M IV MS/MS SCAN

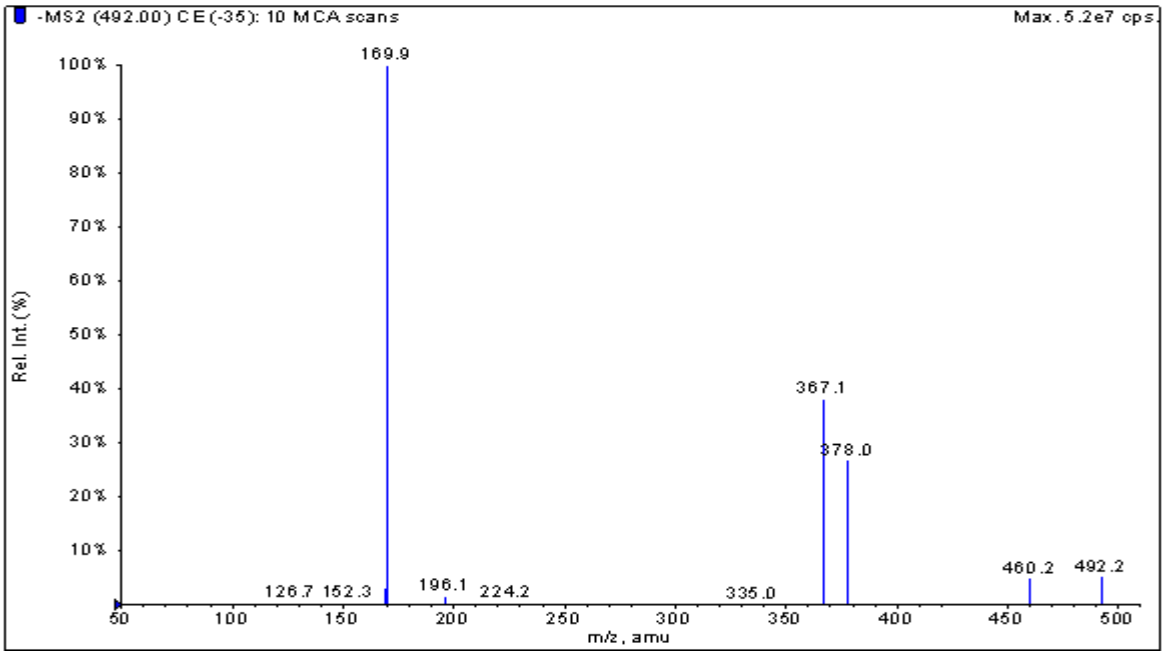

Fig 8: Glyburide MS/MS SCAN

In the optimization of chromatographic conditions, isocratic mode was selected as no cross talk was observed between analytes and ISTD. Use of acetonitrile over methanol in the mobile phase has shown significant improvement in the signal intensities. Replacement of milli-Q water with 10 mM ammonium acetate buffer in mobile phase gave good chromatographic peak shapes and further increase in the buffer concentration was resulted in loss of response. A flow rate of 1.0 mL/min was used to minimize the run time.

Solid Phase extraction extraction was initiated with individual cartridges. Later on the method was shifted to 96 well plate format. Impact of different solutions and their concentration on recovery of analytes was monitored and the final optimized conditions are depicted in Section 2.6. During the optimization of chromatographic conditions and extraction procedure, more emphasis was given to improve the sensitivity and recovery. No significant matrix effects were observed with the proposed chromatographic and extraction conditions.

Selectivity

Selectivity of the method in human K2 EDTA plasma was evaluated in six individual matrix lots along with one lipemic and one hemolytic lot. Peak responses in blank lots were compared against the response of spiked LLOQ and negligible interference was observed at the retention time of analytes and ISTD. Figs. 9 to 11 demonstrate the selectivity of the method with the chromatograms of blank plasma and LLOQ sample respectively.

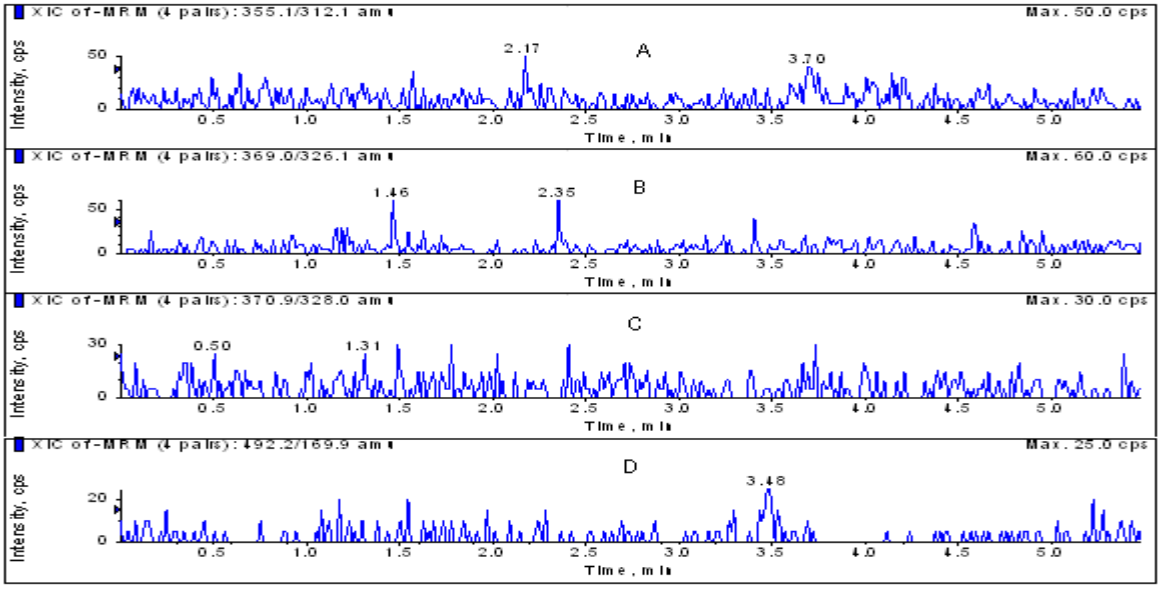

Fig 9: Blank Plasma

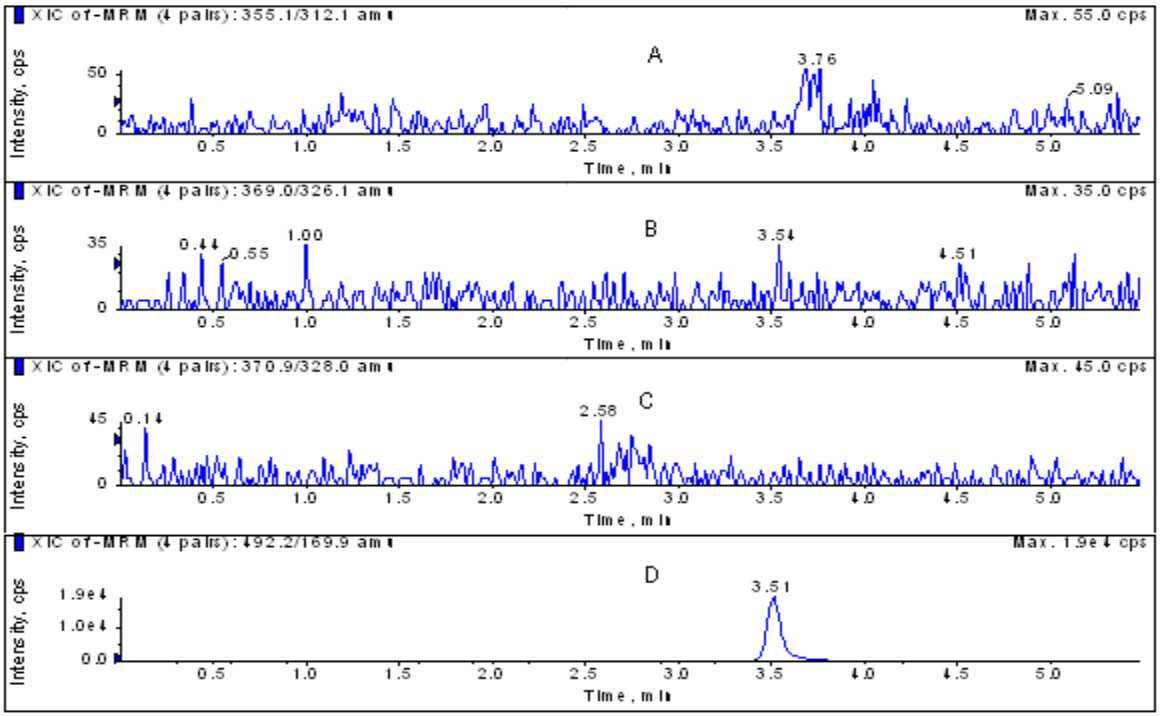

Fig 10: Zero Standard (Blk + IS)

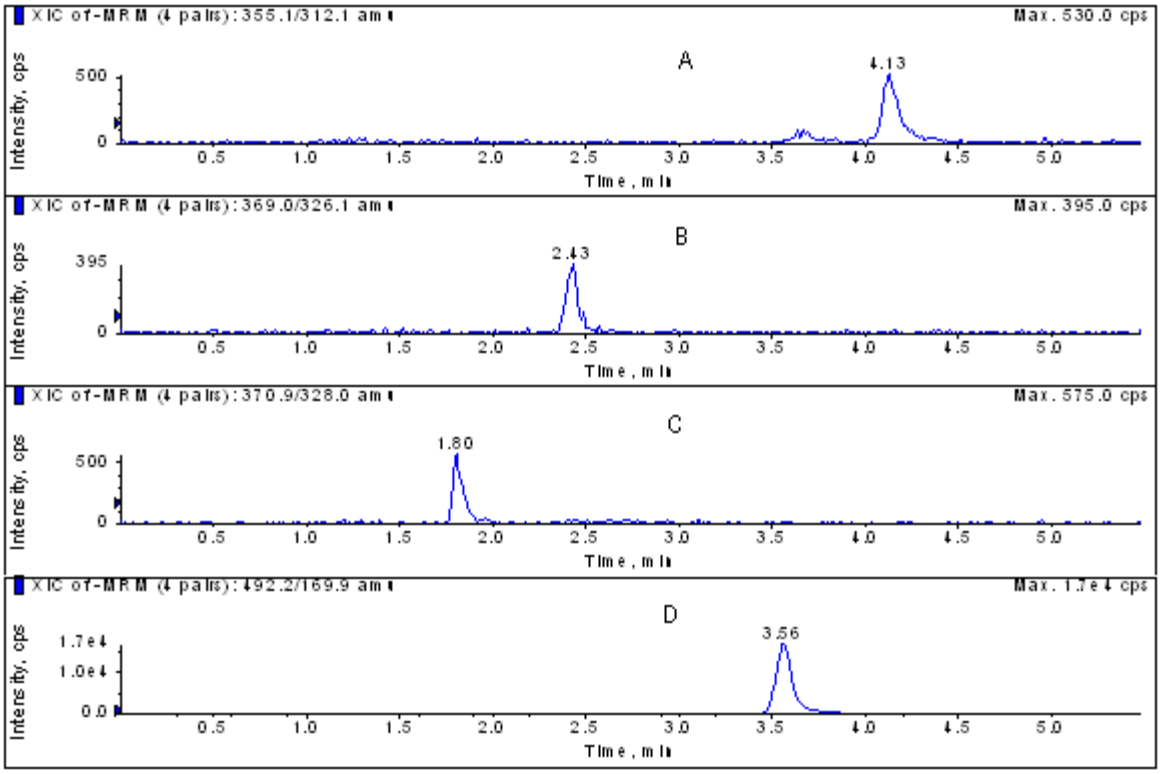

Fig 11: LLOQ

Linearity and sensitivity

The linearity of each calibration curve was determined by plotting the peak area ratio (y) of analytes to ISTD versus the nominal concentration (x) of analyte. Calibration curves were linear in the range of 20.200–2003.400, 5.010–501.270, and 10.100-1005.6 ng/mL for Pioglitazone, M III and M IV respectively. The r values were more than 0.9987, 0.9988, and 0.9990 for Pioglitazone, M III and M IV respectively. The r values were calculated from three intra and inter day calibration curves using weighted (1/X2) quadratic regression analysis. The observed mean back calculated concentrations with accuracy (% Nominal) and precision (%CV) are presented in Table 1. The lower limit of quantitation (LLOQ) for determination of analytes was found to be 20.2, 5.010 and 10.100 ng/mL respectively for Pioglitazone, M III and M IV. At LLOQ (n = 6) accuracy (% Nominal) was 106.7%, 104.9% and 97.9% with a %CV of 4.5%, 3.2% and 7.8% respectively for Pioglitazone, M III and M IV.

Table 1: Summary of Calibration Standards

Analyte	Nominal (ng/mL)	Mean (ng/mL)	%CV	% Nominal
Pioglitazone	20.200	19.9509	1.2	98.8
	40.400	42.2315	2.4	104.5
	100.200	97.4612	1.3	97.3
	200.300	182.8071	0.4	91.3
	400.800	408.6824	1.7	102.0
	801.400	830.2504	0.6	103.6
	1001.700	1045.4409	1.8	104.4
	1600.200	1585.2648	1.9	99.1
	2003.400	1979.3592	2.0	98.8
Pioglitazone M III	5.010	4.9365	1.6	98.5
	10.020	10.4475	3.2	104.3
	25.060	24.5775	0.8	98.1
	50.130	46.7379	0.4	93.2

	100.250	104.0261	0.5	103.8
	200.510	207.3942	0.8	103.4
	250.640	232.5939	0.7	92.8
	401.250	375.3025	1.1	93.5
	501.270	473.7002	1.0	94.5
Pioglitazone M IV	10.100	10.1539	1.9	100.5
	20.200	20.4087	4.9	101.0
	50.300	49.4449	6.4	98.3
	100.600	97.0119	2.3	96.4
	201.100	204.9879	1.9	101.9
	402.200	425.3935	2.8	105.8
	502.800	493.5820	3.2	98.2
	801.200	759.0035	2.1	94.7
	1005.600	945.2640	3.4	94.0

%CV, percent coefficient of variation;

a Mean of 3 replicates at each concentration

Precision and accuracy

Precision and accuracy was evaluated using three intra and inter day precision and accuracy runs, with each batch consisting of six replicates of quality control samples at four concentration levels (LLOQ, LQC, MQC and HQC). The intra batch precision was between 1.3 to 5.9 %, 2.5 to 3.9 %, and 2.7 to 7.8 % with % Nominal between 100.1 to 106.7, 94.1 to 104.9, and 95.9 to 98.5 for Pioglitazone, M III and M IV respectively. The inter batch precision was between 1.3 to 5.6 %, 2.3 to 3.7 % and 3.5 to 5.3 % with % Nominal between 99.6 to 101.3, 94.7 to 103.5 and 95.7 to 100.2 for Pioglitazone, M III and M IV respectively. Results of precision and accuracy are presented in Table 2.

Table 2: Intra batch and inter batch precision and accuracy

Analyte Name	QC level	Nominal conc. (ng/mL)	Intra Batch [a]			Inter Batch[b]		
			Mean Conc Found (ng/mL)	% CV	% Nominal	Mean Conc Found (ng/mL)	% CV	% Nominal
Pioglitazone	LLOQ	20.200	21.5467	4.5	106.7	20.4422	4.2	101.2
	LQC	59.600	60.8814	5.9	102.2	60.3582	5.6	101.3
	MQC	916.300	926.3793	1.4	101.1	924.1395	2.3	100.9
	HQC	1527.100	1528.8816	1.3	100.1	1521.2461	1.3	99.6
Pioglitazone M III	LLOQ	5.010	5.2563	3.2	104.9	5.1865	3.7	103.5
	LQC	14.860	15.1745	3.9	102.1	15.0259	3.4	101.1
	MQC	228.580	215.0176	2.5	94.1	216.4907	2.3	94.7
	HQC	380.970	360.0167	2.7	94.5	366.2392	3.1	96.1
Pioglitazone M IV	LLOQ	10.100	9.8913	7.8	97.9	9.8851	5.0	97.9
	LQC	30.000	28.7650	4.5	95.9	28.6983	3.5	95.7
	MQC	461.400	454.3252	2.7	98.5	462.3228	4.8	100.2
	HQC	769.000	744.2638	3.1	96.8	765.7531	5.3	99.6

%CV, percent coefficient of variation. Conc., Concentration

a 6 replicates at each concentration.

b 18 replicates at each concentration

Matrix effect

Co-eluting matrix components can suppress or enhance the ion- ization but might not result in a detectable response in matrix blanks due to selectivity of the MS detection, however they can affect the precision and accuracy of the assay. Therefore the potential for variable matrix related ion suppression was evaluated in six independent sources (containing one hemolytic and one lipemic lot) of human plasma, by calculating the IS normalized matrix factor. The mean IS normalized matrix factor between all the analytes was ranged between 0.9727 and 1.1545 with a %CV of 1.5 to 6.6 as shown in Table 3 to 5.

Table 3: Matrix Effect of Pioglitazone

	LQC			HQC		
Lot #	MF of Analyte	MF of ISTD	ISTD Normalized Factor	MF of Analyte	MF of ISTD	ISTD Normalized Factor
1	1.032	0.994	1.038	0.925	0.955	0.968
2	0.989	0.970	1.020	0.923	0.957	0.964
3	1.013	1.028	0.985	0.915	0.924	0.990
4	0.892	0.964	0.925	0.936	0.944	0.991
5	1.033	1.045	0.988	0.919	0.958	0.959
6	0.979	0.957	1.023	0.950	0.986	0.963
Mean	-		0.9965	-		0.9727
SD			0.04068			0.01421
% CV			4.1			1.5
N			6			6

MF: Matrix Factor

Table 4: Matrix Effect of Pioglitazone M III

	LQC			HQC		
Lot #	MF of Analyte	MF of ISTD	ISTD Normalized Factor	MF of Analyte	MF of ISTD	ISTD Normalized Factor
1	0.997	1.000	0.997	1.069	0.965	1.108
2	0.962	0.992	0.969	1.053	0.988	1.066
3	1.068	1.091	0.979	0.998	0.905	1.103
4	1.057	1.111	0.951	1.073	0.995	1.078
5	1.207	1.180	1.022	1.029	0.927	1.110
6	1.075	1.148	0.936	1.108	1.022	1.084
Mean	-		0.9757	-		1.0915
SD			0.03111			0.01789
% CV			3.2			1.6
N			6			6

MF: Matrix Factor

Table 5: Matrix Effect of Pioglitazone M IV

	LQC			HQC		
Lot #	MF of Analyte	MF of ISTD	ISTD Normalized Factor	MF of Analyte	MF of ISTD	ISTD Normalized Factor
1	1.036	1.004	1.032	1.029	0.885	1.162
2	0.960	0.950	1.011	1.042	0.886	1.176
3	1.039	1.035	1.003	0.951	0.800	1.189
4	1.020	0.985	1.035	1.005	0.881	1.141
5	1.171	1.199	0.977	0.963	0.845	1.139
6	0.992	0.846	1.172	1.040	0.929	1.120
Mean	-		1.0382	-		1.1545

SD		0.06892		0.02589
% CV		6.6		2.2
N		6		6

MF: Matrix Factor

Extraction recovery and dilution integrity

The extraction recovery of analytes from EDTA plasma was determined by comparing the peak responses of plasma samples (n= 6) spiked before extraction with that of plasma samples spiked after extraction. The mean recovery was found to be 62.77%, 68.91% and 65.52% with %CV of 5.2%, 5.4% and 6.0% for Pioglitazone, M III and M IV respectively as shown in Table 6. For Internal standard the recovery was found to be 73.1%.

Dilution integrity experiment was carried out at 3 times the ULOQ concentration. After 1/5, 1/10 and 1/50 dilution the mean back calculated concentration for dilution QC samples was within 85–115% of nominal value with a %CV of ≤2.2 as shown in Table 7.

Table 6: Recovery

Analyte	QC Level	A	B	% Recovery	Mean Recovery	% CV
Pioglitazone	LQC	8235.0	12525.3	65.9	62.77	5.2
	MQC	1389710.3	2342445.5	59.3		
	HQC	2400865.3	3796604.3	63.2		
Pioglitazone M III	LQC	38214.2	55743.8	68.6	68.91	5.4
	MQC	6147017.5	9402048.7	65.4		
	HQC	10009873.5	13749403.2	72.8		
Pioglitazone M IV	LQC	25663.8	41143.8	62.4	65.52	6.0
	MQC	4372339.0	6802800.3	64.3		
	HQC	7123993.2	10191008.2	69.9		
Glyburide		133005.5	181889.5	73.1	-	

A: Mean Peak response of Extracted Samples

B: Mean Peak response of un Extracted Samples

Table 7: Dilution Integrity

Analyte	Dilution Factor[a]	% Nominal	% CV
Pioglitazone	1/5	104.9	1.5
	1/10	108.1	1.6
	1/50	113.6	1.9
Pioglitazone M III	1/5	106.5	1.0
	1/10	109.5	2.1
	1/50	114.3	1.0
Pioglitazone M IV	1/5	104.3	1.9
	1/10	111.0	2.2
	1/50	113.5	1.2

a: Six replicates at each dilution factor

Ruggedness

Six samples each of LLOQ, LQC, MQC and HQC in human plasma were analyzed along with CC samples. The ruggedness was assessed by changing analyst, solvent lot and different column of same make. Results are summarized in Table 8. The data obtained were within acceptance criteria.

Table 8: Ruggedness

Analyte	QC Level	% Nominal	% CV
Pioglitazone	LLOQ QC	104.4	5.8
	LQC	96.8	2.6
	MQC	101.7	4.8
	HQC	95.8	3.0
Pioglitazone M III	LLOQ QC	103.6	3.5
	LQC	99.3	4.6
	MQC	96.1	3.8
	HQC	97.0	2.3
Pioglitazone M IV	LLOQ QC	97.1	3.0
	LQC	93.8	1.5
	MQC	100.0	4.2
	HQC	99.3	4.8

N = 6 at each level

Extended Precision and Accuracy Batch

The Extended Precision and Accuracy batch was assessed by processing and analyzing CC samples along with twenty five samples each of LQC,

MQC and HQC in human plasma. Calibration standards were used to determine the accuracy of quality control samples. The batch of 75 samples analysed was found to acceptable for the maximum no of sample that can be processed during routine sample analysis. Results are summarized in Table 29. The data obtained were within acceptance criteria.

Table 9: Extended PA Batch

Analyte	QC Level	% Nominal	% CV
Pioglitazone	LQC	98.6	8.4
	MQC	103.7	4.5
	HQC	99.9	3.1
Pioglitazone M III	LQC	96.3	4.9
	MQC	104.5	5.3
	HQC	102.6	5.4
Pioglitazone M IV	LQC	95.1	6.3
	MQC	101.8	5.7
	HQC	99.6	4.2

N = 25 at each level

Reinjection Reproducibility

Reinjection reproducibility was performed by injecting the previously passed precision and accuracy batch after a period of 49 hr 51 min. The reinjected quality control samples concentrations were back calculated against initially injected CC curve. The % CV of back calculated concentrations for all quality control samples of LQC, MQC and HQC concentration levels ranged from 1.7 to 4.3, 1.9 to 3.0, and 2.0 to 2.9, respectively, for Pioglitazone, Pioglitazone M III and Pioglitazone M IV, which are within the acceptance limit of 15.00%. The % mean accuracy of back calculated concentrations for all quality control samples at LQC, MQC and HQC concentration levels were ranged from 101.0 to 106.2, 94.5 to 95.4, and 92.6 to 98.2, respectively, for Pioglitazone, Pioglitazone M III and Pioglitazone M IV, which is within acceptance limit 85.00–115.00%.

Haemolysis Effect

Six samples each of blank matrix, LLOQ, LQC and HQC in 2% and 100% haemolyzed plasma were analyzed along with CC samples (Un-haemolyzed plasma). The overall % nominal were 89.4 to 111.9% with % CV of 1.0 to 7.3%. The data obtained were within acceptance criteria.

Stability

Stability evaluations were performed in both aqueous and matrix based samples. The stock and working solutions were stable for a period of 7 h at room temperature. Stability evaluations in matrix were performed against freshly spiked calibration standards using freshly prepared quality control samples (comparison samples). The analyte was stable up to 28 h on bench top at room

temperature and over 8 freeze-thaw cycles. The processed samples were stable up to 47 h min in autosampler at 4^0C. Reinjection reproducibility is done for 49 h 51 min. The long-term matrix stability was evaluated at both -20^0C and -50^0C over a period of 121 days. The % change shows that there was no significant degradation of analytes was observed over the stability duration and conditions. The stability results presented in Table 6 were within 85-115%.

Table 8: Stability Data

Stability	Analyte	QC	A	%CV	B	%CV	% Change
Bench Top Stability at room Temperature (28 hrs)	Pioglitazone	LQC	54.6433	2.2	55.3088	2.7	-1.2
		HQC	1405.1865	1.9	1398.3146	1.1	0.5
	Pioglitazone M III	LQC	13.2601	1.4	13.5028	3.1	-1.8
		HQC	346.6827	2.3	340.0792	1.3	1.9
	Pioglitazone M IV	LQC	26.5550	2.1	28.2350	5.0	-6.0
		HQC	699.5337	2.7	705.3012	2.4	-0.8
Freeze-thaw (after 8 cycle)	Pioglitazone	LQC	55.0108	5.3	55.3088	2.7	-0.5
		HQC	1404.6775	1.5	1398.3146	1.1	0.5
	Pioglitazone M III	LQC	14.1913	2.9	13.5028	3.1	5.1
		HQC	344.5239	2.2	340.0792	1.3	1.3
	Pioglitazone M IV	LQC	26.8250	3.6	28.2350	5.0	-5.0
		HQC	698.1238	2.2	705.3012	2.4	-1.0
Auto sampler stability (47 hrs)	Pioglitazone	LQC	56.7789	3.9	55.3088	2.7	2.7
		HQC	1452.7811	3.1	1398.3146	1.1	3.9
	Pioglitazone M III	LQC	14.6396	5.0	13.5028	3.1	8.4
		HQC	383.8908	2.2	340.0792	1.3	12.9
	Pioglitazone M IV	LQC	28.2050	5.3	28.2350	5.0	-0.1
		HQC	791.5573	1.4	705.3012	2.4	12.2
Long term stability for 121 days (below -50^{0}C)	Pioglitazone	LQC	58.9941	4.2	61.8648	3.3	-4.6
		HQC	1576.2217	3.1	1541.0984	1.7	2.3
	Pioglitazone M III	LQC	14.7436	2.8	14.2062	3.1	3.8
		HQC	374.3030	1.3	378.8747	0.6	-1.2
	Pioglitazone M IV	LQC	30.4450	5.6	28.8100	3.4	5.7
		HQC	771.6915	1.9	767.5902	1.3	0.5
Long term stability for 121 days (below -20^{0}C)	Pioglitazone	LQC	60.2655	5.7	61.8648	3.3	-2.6
		HQC	1568.8407	1.5	1541.0984	1.7	1.8

	Pioglitazone M III	LQC	14.2359	4.3	14.2062	3.1	0.2
		HQC	365.4137	2.2	378.8747	0.6	-3.6
	Pioglitazone M IV	LQC	29.9250	5.0	28.8100	3.4	3.9
		HQC	749.7750	2.5	767.5902	1.3	-2.3

A: Mean concentration of stability samples B: Mean concentration of Comparison samples

IV. Conclusion

The experiments performed during the validation concluded that the method is validated for the simultaneous quantitation of Pioglitazone, Pioglitazone M III and Pioglitazone M IV in human plasma over the concentration range of 20.200–2003.400, 5.010–501.270 and 10.100-1005.6 ng/mL respectively, using glyburide as an internal standard. The precision and mean accuracy are within the acceptable limits. Consistent recoveries were observed for LQC, MQC and HQC. The method is specific enough in the presence of K2EDTA anticoagulant. The method is precise and accurate enough to dilute samples, if necessary. The stability experiments were performed during the validation concluded that the intended analyte and metabolites were stable at different conditions like autosampler (47 hrs) bench top stability (28 hrs), autosampler stability (47 hrs), and eight freeze and thaw cycles. The analyte, metabolites and ISTD stock solutions were stable at room temperature for 7 hr. Reinjection reproducibility was proved for 49 hr 51 min. The method was proved to be rugged by different column. The extended Precision and accuracy batch was proved with 75 QC samples.

A rapid, sensitive, high throughput and accurate liquid chromatography with electrospray ionization tandem mass spectrometry method was developed for determination of Pioglitazone, Pioglitazone M III and Pioglitazone M IV in human plasma. The extraction method utilizes a low sample volume of 100µL and shown consistent and reproducible recoveries for analyte and ISTD with minimum plasma interference and matrix effect. The validated method can be successfully used to a clinical and tox studies. The method was validated and demonstrated to be robust with high precision and accuracy. The high throughput method can reduce overall processing time and allowing to process and analyze more number of samples in short duration.

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