ASIAN JOURNAL OF PHARMACEUTICAL AND CLINICAL RESEARCH

INNOVARE ACADEMIC SCIENCES
Knowledge to Innovation

Vol 10, Issue 12, 2017

Online - 2455-3891
Print - 0974-2441

Research Article

A RAPID AND SENSITIVE LIQUID CHROMATOGRAPHY- MASS SPECTROMETRY/MASS SPECTROMETRY METHOD FOR ESTIMATION OF PIOGLITAZONE, KETO PIOGLITAZONE AND HYDROXY PIOGLITAZONE IN HUMAN PLASMA

REVATHI NAGA LAKSHMI PONNURI[1]*, PRAHLAD PRAGALLAPATI[2], RAVINDRA N[3], VENKATA BASAVESWARA RAO MANDAVA[4]

[1]Department of Pharmacy, Faculty Pharmaceutical Sciences, Pharmaceutical Analysis, Krishna University, Machilipatnam - 521 002, Andhra Pradesh, India. [2]Department of Pharmaceutics, Acharya Nagarjuna University, Guntur - 522 510, Andhra Pradesh, India. [3]Department of Pharmaceutical Chemistry, Chilkur Balaji College of Pharmacy, Hyderabad - 500 075, Telangana, India.[4]Department of Chemistry, Faculty of Sciences, Directorate of Admissions, Krishna University, Machilipatnam - 521 002, Andhra Pradesh, India. Email: pharma_revathi@yahoo.com

Received: *27 May 2017,* **Revised and Accepted:** *17 August 2017*

ABSTRACT

Objective: The main objective of the work was to develop a straightforward, fast and selective liquid chromatography/tandem mass spectrometry (LC-MS/MS) assay for determination of pioglitazone (PG), keto pioglitazone (KPG), and hydroxy pioglitazone (HPG) in human plasma and to validate as per recent guidelines.

Methods: Analyte and the internal standard (IS) were extracted from plasma through liquid-liquid extraction and chromatographed on a Xterra RP18, 100×4.6, 5 µ column using methanol: acetonitrile mixture and 10 mM Ammonium formate buffer (70:30, v/v) as the mobile phase at a flow rate of 0.7 mL/min. The API-3200 Q Trap LC-MS/MS instrument in multiple reaction monitoring mode was used for detection. Diphenhydramine was utilized as IS.

Results: The linearity was established in the concentration range of 20.15-1007.58 ng/mL for PG, 20.35-1017.58 ng/mL for KPG, and 19.68-491.22 ng/mL for HPG in human plasma. All the validation parameters were well within the acceptance limits.

Conclusion: A new simple LC-MS/MS method was developed for the determination of PG, KPG, and HPG in human plasma. This method can be easily applied for the estimation of pharmacokinetic parameters of PG, KPG, and HPG.

Keywords: Pioglitazone, Keto pioglitazone, Hydroxy pioglitazone, Human plasma, Method validation, Liquid chromatography/tandem mass spectrometry.

 DOI: http://dx.doi.org/10.22159/ajpcr.2017.v10i12.20284

INTRODUCTION

Pioglitazone (PG) is used for the treatment of diabetes mellitus type 2. Chemical name of PG is (±)-5-[p-[2-(5-ethyl-2-pyridyl)ethoxy]benzyl]-2,4-thiazolidinedione monohydrochloride (Fig. 1) and chemical formula is $C_{19}H_{20}N_2O_3S.HCl$. PG is a thiazolidinedione antidiabetic agent that depends on the presence of insulin for its mechanism of action. PG decreases insulin resistance in the periphery and the liver resulting in increased insulin-dependent glucose disposal and decreased hepatic glucose output. Unlike sulfonylureas, PG is not an insulin secretagogue. PG is a potent and highly selective agonist for peroxisome proliferators - activated receptor - gamma (PPARγ). PPAR receptors are found in tissues important for insulin action such as adipose tissue, skeletal muscle, and liver. Activation of PPARγ nuclear receptors modulates the transcription of a number of insulin responsive genes involved in the control of glucose and lipid metabolism [1]. Keto pioglitazone (KPG), a metabolite of PG with the molecular formula: $C_{19}H_{18}N_2O_4S$ and hydroxy pioglitazone (HPG) is another PG metabolite with the molecular formula: $C_{19}H_{20}N_2O_4S$.

As per the literature, various analytical methods such as membrane sensitive electrode [2], potentiometric [3], ultraviolet-spectrophotometric [4], high-performance liquid chromatographic (HPLC) methods [5-10], ultra-performance liquid chromatograph method [11], and liquid chromatography-mass spectrometry (LCMS) methods [12-17] have been reported for the determination of PG and its metabolites. The particular described methods have to sacrifice time, resolution as well as sensitivity. Today, there exists a prerequisite to develop fast or ultra–fast methods such as LC-MS/MS without any loss of separation efficiency and sensitivity. To date, just one LC-MS/MS method happen to be noted for the determination of PG and its active metabolites KPG and HPG in human plasma samples.

In this paper, the author's details an effective LC-MS/MS assay method for the determination of PG, KPG, and HPG in human plasma using diphenhydramine as an internal standard (IS) to avoid the possible matrix effect related problems and variability in recovery between analytes and the IS. There should be a suitable analytical assay with greater sensitivity to estimate the concentrations at elimination phase of the pharmacokinetic profile. The validated method can be successfully applied to a pharmacokinetic study in humans, and obtained results were authenticated through incurred samples reanalysis. Authors of this paper have also published other simultaneous analytical methods [18,19].

METHODS

Chemicals and reagents

The reference samples PG, KPG, and HPG were obtained from Dr. Reddy's Laboratories Ltd. (Hyderabad, India). Acetonitrile and methanol were of HPLC grade purchased from Rankem. Similarly, analytical grade ammonium acetate was from Merck Ltd (Mumbai, India). Ultra-pure water was prepared using Milli Q water purification (Millipore, Bangalore, India).

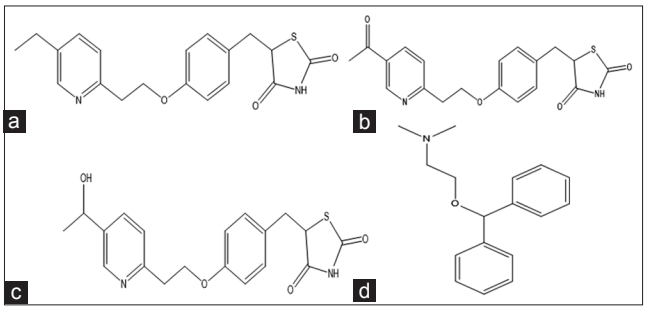

Fig. 1: Chemical structures of (a) pioglitazone, (b) keto pioglitazone, (c) hydroxy pioglitazone, and (d) diphenhydramine

LC-MS/MS instrument and conditions

An HPLC system (Shimadzu, Kyoto, Japan) equipped with a Xterra RP18, 100×4.6 mm, 5 µm column, a binary LC–20AD prominence pump, an auto sampler (SIL–HTc) and a solvent degasser (DGU–$20A_3$) was used for the study. Aliquot of 20 µL of the extracted samples were injected into the column. Column oven temperature was maintained at ambient temperature (40±2°C). Methanol: Acetonitrile (1:1 v/v) mixture and 10 mM Ammonium formate buffer (70:30, v/v) were used as the mobile phase at a flow rate of 0.7 mL/min. API 3200 (Q-Trap) mass spectrometer (Applied Biosystems) equipped with a Turboionspray™ in positive ion mode was used for the quantification of the analyte.

Preparation of spiked plasma samples

Stock solution of PG, KPG, and HPG was prepared in HPLC grade methanol (1 mg/mL). Two separate stock solutions were used for the preparation of calibration curve (CC) standards and quality control (QC) samples. A mixture of methanol and water (50:50, v/v; diluent) was used to prepare naftopidil working solutions. The working concentration of the diphenhydramine (1000 ng/mL) was prepared using the same diluent.

Calibration samples and QC samples were prepared in K_2 EDTA human plasma as a bulk and stored at −70±10°C. Calibrates were prepared at eight concentration levels of PG and KPG ranging from 20.0 to 1000 ng/mL and HPG ranging from 20.0 to 500 ng/mL with interference free K_2 EDTA plasma using final concentration as a single batch at each concentration. Likewise, QC samples at concentrations of PG and KPG ranging from 20.0 to 850 ng/mL and HPG ranging from 20.0 to 850 ng/mL.

Extraction protocol

With draw plasma blank, CC standards, QC samples from deep freezer, and allow them to thaw at room temperature. Vortex the thawed samples to ensure complete mixing of contents.

Extraction procedure: LLE
↓
Aliquot 0.20 mL of plasma sample into a pre labeled RIA vial, add 50 µL of ISTD (1 µg/mL) working stock solution and, vortex.
↓
To the above sample add 2 mL of ethyl acetate
↓
Vortex the sample for 5 min at 2500 rpm for complete mixing
↓
Centrifuge for 5 min at 4500 rpm at 5°C
↓
Remove the supernatant into pre labeled RIA vial and evaporate under
↓
nitrogen gas at 40°C to dryness
↓
Reconstitute with 1.0 mL of mobile phase and vortex the samples for 30 seconds
↓
Load the samples into auto injector according to the batch
↓
Inject 20 µL onto analytical column

Method validation parameters

A thorough and complete method validation of PG, KPG, and HPG in human plasma was carried out as per US FDA [20] and EMEA [21] guidelines. The actual parameters determined were carryover test, selectivity, matrix effect, sensitivity, linearity, precision and accuracy, recovery, dilution integrity, run size evaluation, ruggedness, and stability.

The selectivity of the method was assessed in six different sources of plasma, of which, six were normal K_2 EDTA plasma. Carryover test ended up being conducted to substantiate any carryover associated with analyte as well as the IS actually, which may reveal throughout following goes. The carryover test samples were injected in the following sequence, i.e., MP®AQS ULOQ®MP®Extracted blank®Extracted ULOQ®Extracted blank®MP. Sensitivity was determined by analyzing six replicates of plasma samples spiked with the lowest level of the CC concentrations (lower limit of quantification [LLOQ]). Matrix effect was assessed by comparing the mean area response of post-extraction spiked samples with a mean area of aqueous samples (neat samples) prepared in mobile phase solutions at lower quality control (LQC) and higher quality control (HQC) levels. The overall precision of the matrix factor was expressed as coefficient of variation (CV).

$$\text{Matrix} = \frac{\text{Peak response area ratio in presence of matrix ions}}{\text{Mean peak response area ratio in absence of matrix ions}}$$

The linearity of the proposed method was determined by analysis of standard CCs containing eight non-zero concentrations along with one blank plasma sample and one zero standard (blank sample with the IS). Each CC was analyzed individually by least square weighted ($1/x^2$) linear regression. Intraday precision and accuracy results were determined using six replicates of LLOQ QC, LQC, middle quality control (MQC), and HQC samples analyzed on the same day. Interday precision and accuracy were measured by analyzing five different analytical batches on 3 consecutive days. Extraction recoveries of analyte were determined at LLOQ QC, LQC, MQC, and HQC by comparing the peak area of extracted analyte sample with the peak area of non-extracted standard. Similarly, recovery of IS was determined at a concentration of 1000.00ng/mL. The diluted samples were processed and analyzed using un–diluted CC standards. The ruggedness of the method verified by analyzing one precision and accuracy batch on another instrument of the same make using different column (different batch no.) and processed by another analyst.

The stock solution stability at room temperature and at 2-8°C in the refrigerator was performed by comparing the area response stability samples with the response of the sample prepared from fresh stock solution. The solutions were considered stable if the deviation within ±10% from nominal value. Bench top stability at room temperature (6.3 hrs), processed samples stability (auto sampler stability for 35 hrs, dry extract stability for 24 hrs, and reinjection stability for 52 hrs), freeze-thaw stability were performed at LQC and HQC levels using six replicates at each level. The stability samples were processed and quantified against freshly spiked CC standards along with freshly spiked QC samples. Samples were considered to be stable if assay values were within the acceptable limits of accuracy (±15% standard deviation) and precision (£15% relative standard deviation).

RESULTS AND DISCUSSION

Mass spectrometry

To investigate the pharmacokinetics, tolerability, and safety of newer formulations, one should have an appropriate analytical method for its identification and quantification. Nowadays rapid and highly sensitive

analytical technique such as LC-MS is most widely used in bio-analysis of drugs and metabolites. The proposed LC-MS/MS method is developed with the aim to quantify the PG, KPG, and HPG concentrations. During the method, development stages mass spectrometric and chromatographic conditions critically evaluated to obtain a good and reproducible response with a better resolution from the endogenous components. During tuning of analytes high intense signals were obtained in positive ion mode than the negative mode for the analyte and the IS due to their ability to accept the protons. Furthermore, the adequate and reproducible response was obtained by changing the source dependent parameters. Dwell time was set at 200 ms, at which no cross talk was found. The positive ion spray mass spectrum revealed a protonated molecular by monitoring the transition pairs of m/z 357.20 precursor ion to the m/z 134.20 product ion for PG; m/z 371.00 precursor ion to the m/z 148.20 product ion for KPG; m/z 373.00 precursor ion to the m/z 150.20 product ion for HPG, and m/z 256.30 precursor ion to the m/z 167.30 product ion for the IS. Data acquisition was performed with Analyst Software™ (version 1.6.1) in the multiple reaction monitoring (MRM) mode (Table 1).

Chromatographic conditions

Acetonitrile and methanol are widely used an organic modifier for LC-MS analysis. Hence, during method development, both the solvents were checked in combination with acidic buffers such as ammonium acetate, ammonium formate, formic acid, and acetic acid. The response obtained with methanol and 10 mM ammonium formate as a mobile phase was good; but not reproducible. Moreover, a variety of chromatographic columns such as C_8 and C_{18} of different makes (Kromasil 100-5 C18, 50×4.6 mm, 5 µm; Discovery HS C_{18} 50×4.6 mm, 5 µm; Alltima HP C_{18} 50×4.6, 3 µm; Kromasil 100-5C_{18}, 100×4.6, 5 µm; Zorbax SB C_{18}, 50×4.6, 5 µm; Zorbax XDB–phenyl 75×4.6, 3.5 µm; Ace 3 C_{18} 150×4.6, 3 µm; Hypurity advance 75×4.6, 5 µm) were verified to achieve adequate retention time with short run time, better separation from endogenous components, symmetric peak shape and satisfactory response for the analyte. The best chromatographic conditions were achieved with methanol: Acetonitrile (1:1) and 10 mM ammonium formate (70:30, v/v) as a mobile phase under isocratic conditions. Xterra RP18 100×4.6 mm column gave good peak shape (Figs. 2-4). The mobile phase flow rate was set at 0.7 mL/min, which can produce better acceptable chromatographic peak shape and short run time of 4.0 min (Table 2).

Extraction procedure optimization

The reported procedures have employed solid phase extraction technique for the sample preparation. A sensitive analytical method

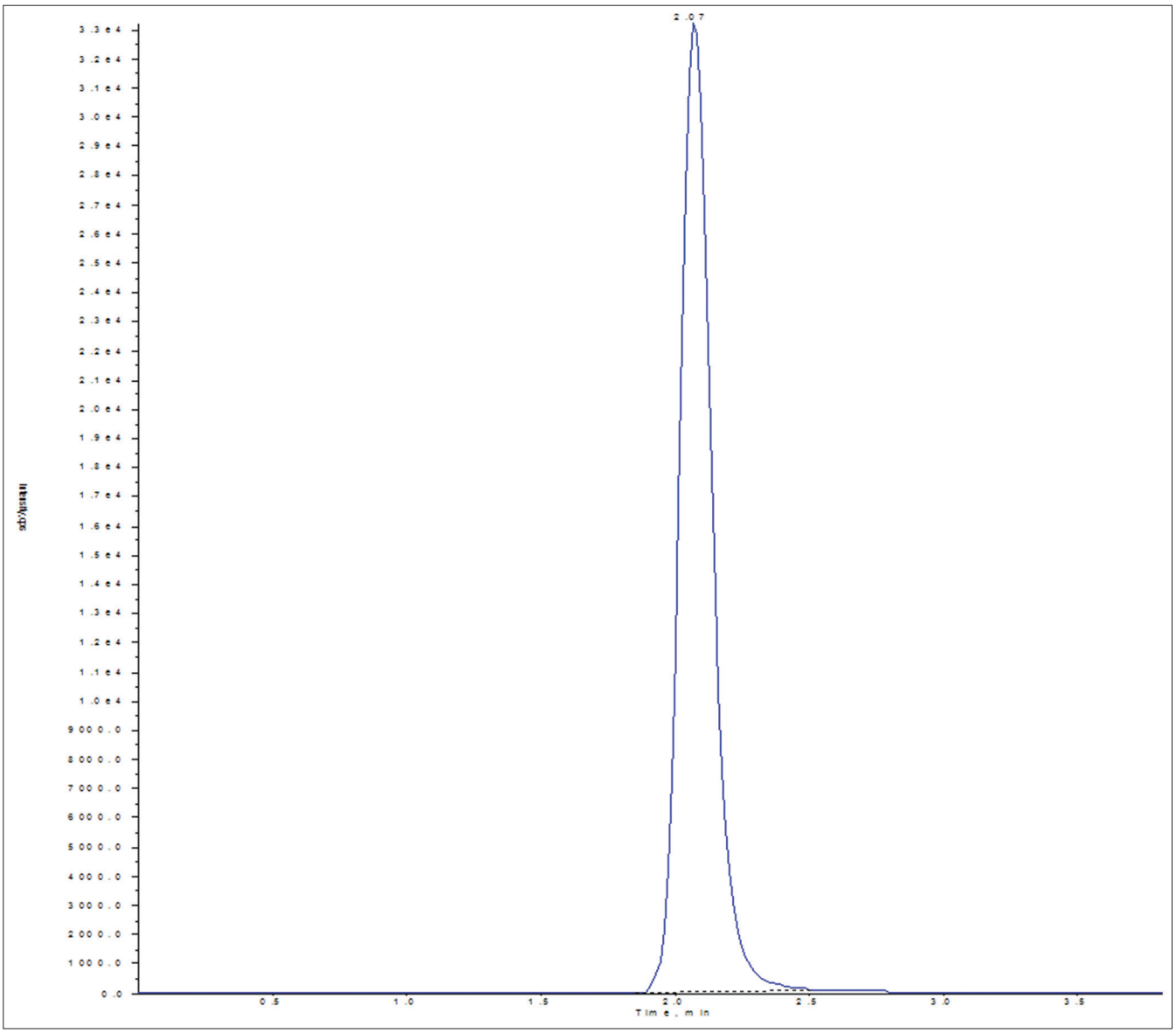

Fig. 2: Representative chromatogram of zero standard sample of pioglitazone

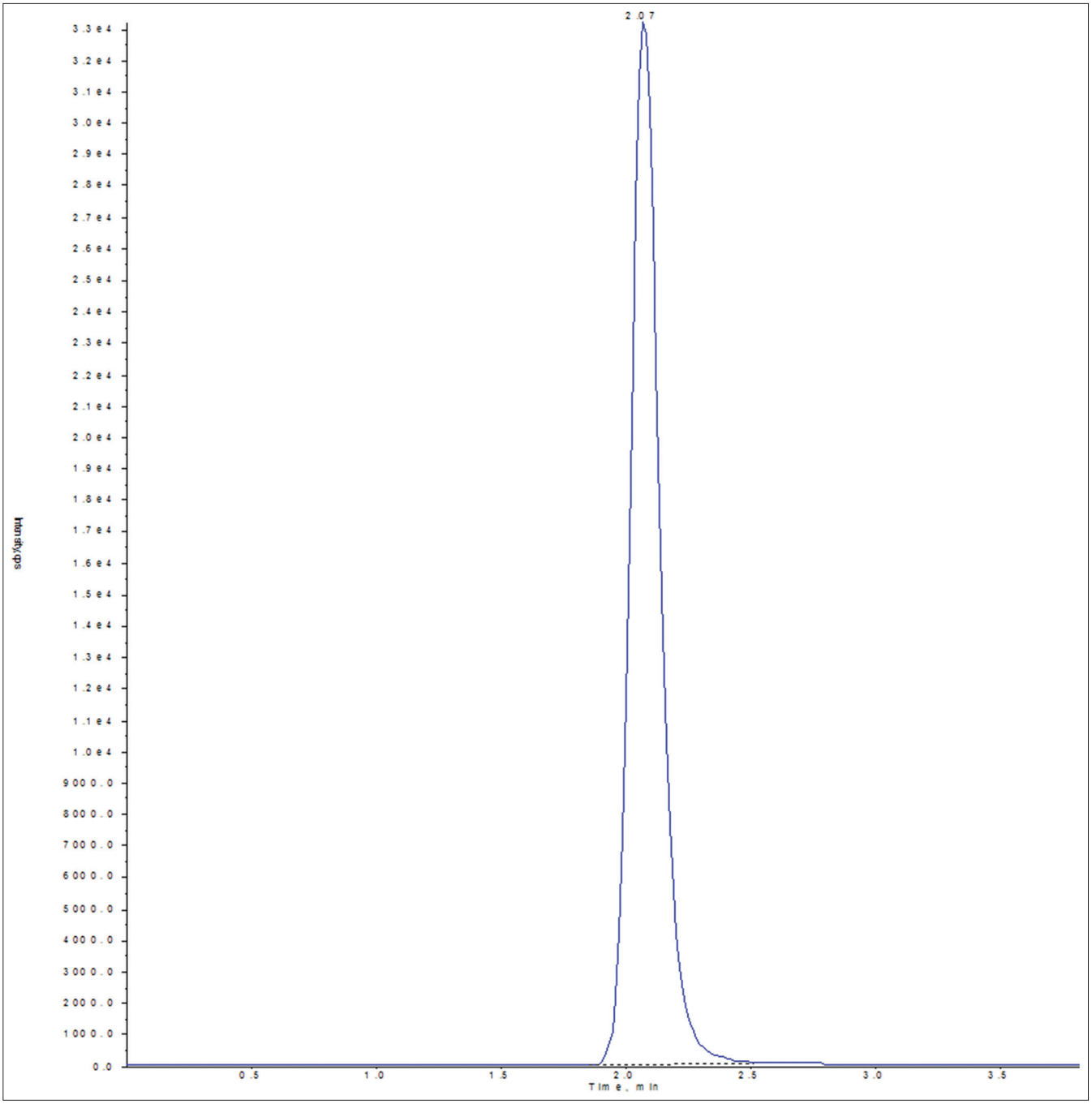

Fig. 3: Representative chromatogram of zero standard sample of keto pioglitazone

in biological samples requires a judicious selection of extraction procedure which can harvest high recovery with negligible or no matrix effect. Therefore, locally linear embedding (LLE) was tried.

Weighting factor of regression method

To determine whether to fit the data for the CCs by weighted or unweighted linear regression, the functional dependence of the standard deviation of the PG/diphenhydramine, KPG/diphenhydramine, and HPG/diphenhydramine and area ratio on sample concentration is evaluated. It was found that best fit and weighting is linear with offset $1/X^2$.

Autosampler carry over

Carryover test was performed in the following sequence. MP®AQS ULOQ®MP®Extracted blank®Extracted ULOQ®Extracted blank®MP. No significant injector carryover is observed.

Selectivity

Selected blank human K_2EDTA plasma sources was carried through the extraction procedure and chromatographed to determine the extent to which endogenous human K_2EDTA plasma components may contribute to chromatographic interference with the PG, KPG, and HPG, and the IS. No significant interference is observed in six different lots of human K_2EDTA plasma samples.

Sensitivity

Six LLOQ samples were prepared independent of CC standards. All the results were found within the limits.

CCs

CCs were found to be consistently accurate and precise over the range of 20.15-1007.58 ng/mL for PG, 20.35-1017.58 for KPG, and 19.68-491.22

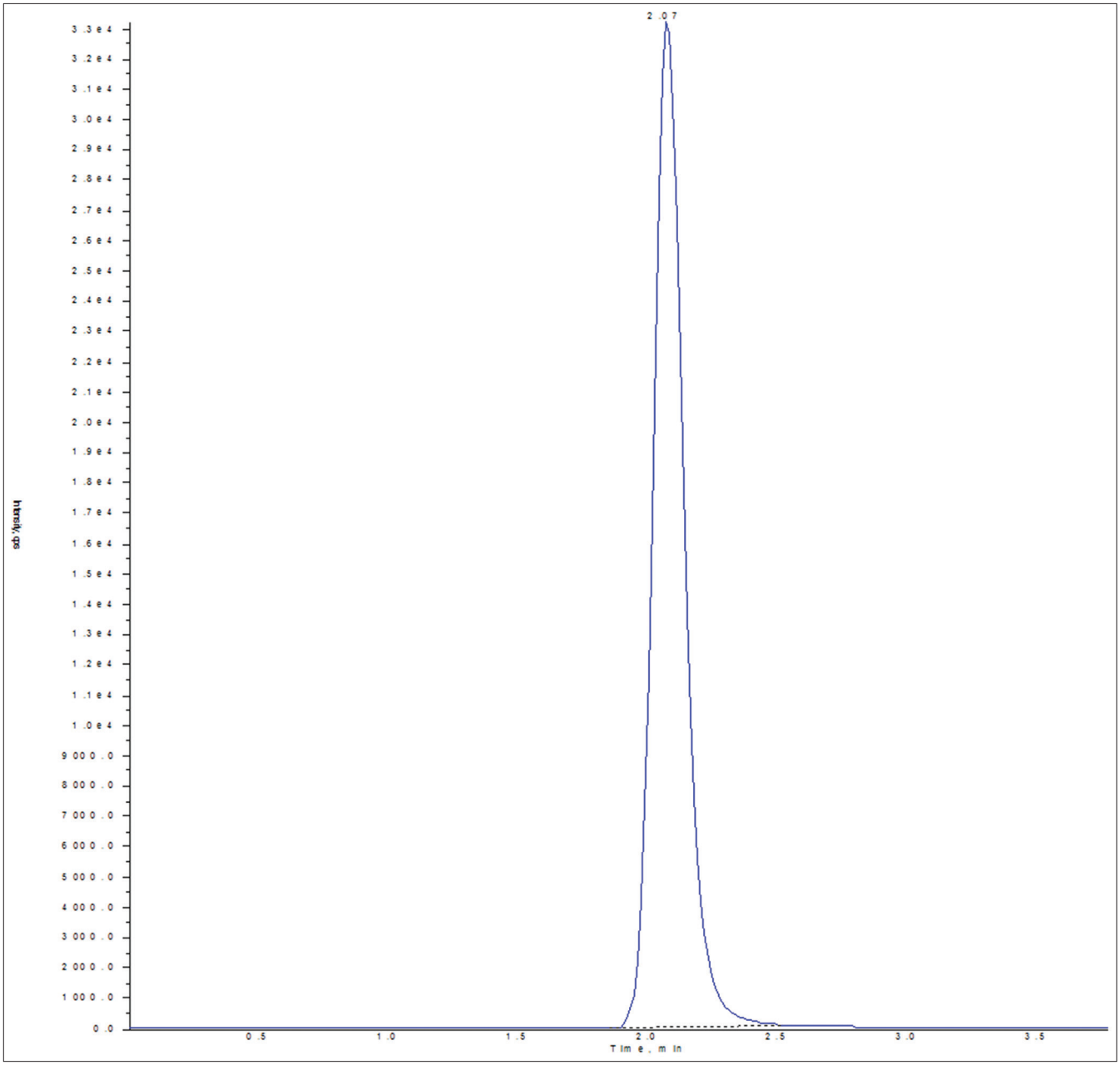

Fig. 4: Representative chromatogram of zero standard sample of hydroxy pioglitazone

for HPG. The regression coefficient (r) is ≥0.9800. Back calculations were made from the CCs to determine PG, KPG, and HPG concentrations of each calibration standard.

Interday accuracy and precision

The interday accuracy and precision evaluation were assessed by the repeated analysis of human K_2EDTA plasma samples containing different concentrations of PG, KPG, and HPG on separate occasions. A single run consisted of a CC, six replicates of LLOQ, LQC, MQC, and HQC samples.

The interday coefficients of variation ranged from 2.64 to 6.15, 5.34 to 6.05, and 3.15 to 7.17 for PG, KPG, and HPG, respectively. The interday percentage of nominal value ranged from 85.61 to 114.69, 85.61 to 111.72, and 84.18 to 114.78 for PG, KPG, and HPG, respectively. Results are presented in Tables 3 for PG, KPG, and HPG, respectively.

Intraday accuracy and precision

Analyze replicate concentrations of PG in human K_2 EDTA plasma performed intraday accuracy and precision evaluations. The run consisted of a CC plus a total of 24 spiked samples; six replicates each of LLOQ, LQC, MQC, and HQC samples.

The intraday CV ranged from 1.60 to 7.88, 1.96 to 8.82, and 1.17 to 7.05 for PG, KPG, and HPG, respectively. The intraday percentage of nominal value ranged from 92.81 to 114.69, 87.82 to 111.72, and 93.66 to 114.26 for PG, KPG, and HPG. Results are presented in Table 4, respectively.

Dilution integrity

Dilution QC is diluted 5th and 10th in human K_2EDTA plasma. Before extraction, six samples each of 5^{th} and 10^{th} diluted samples were processed and analyzed with freshly processed calibration samples. The calculated concentrations, including the dilution factor for $1/5^{th}$ and

Table 1: Mass spectrometric conditions

Name	Condition
Curtain gas (CUR)	25 psi
Collision gas (CAD)	Medium
Ion spray voltage (IS)	5500 V
Turbo probe temperature	400°C
Nebulizer gas (Gas 1)	35 psi
Heater gas (Gas 2)	40 psi
EP	10 V
Dwell time	200 ms
Resolution	Q1: Unit
	Q3: Unit

EP: Entrance potential

Table 2: Summary of the chromatographic conditions

Name	Values	
Analytical column	Xterra RP18 100×4.6 mm	
Mobile phase	10 mM ammonium formate: methanol [1]:acetonitrile[1] - 30:70, v/v	
Injection volume	20 µL	
Flow rate	0.7 mL/min	
Run time	4.00 min	
Column oven temperature	40°C	
Auto sampler temperature	10°C	
Retention times (min)		
Analyte	Pioglitazone	2.75
	Keto pioglitazone	2.10
	Hydroxy pioglitazone	1.90
Internal standard	Diphenhydramine	2.10

1/10th yielded CV for PG, KPG, and HPG are 1.58-3.79, 2.16-3.17, and 3.07-3.74, respectively. Percentages of nominal values for 1/5th and 1/10th dilution for PG, KPG, and HPG are 96.71-112.37, 89.04-90.93, and 97.43-101.76, respectively.

Ruggedness

Ruggedness was performed by the different analyst using new or different column. The run consisted of a CC and a total of 24 spiked samples; six replicate each of the LLOQ, LQC, MQC, and HQC samples. The within-run % coefficients of variation ranged from 1.95 to 3.86, 2.32 –to 6.78, and 2.39 –to 3.01 for PG, KPG, and HPG, respectively. The percentage of nominal values ranged from 92.59 to 111.38, 85.61 –to 104.85, and 92.61 –to 113.01 for PG, KPG, and HPG, respectively.

Recovery

Recovery of PG, KPG, and HPG was evaluated by comparing mean analyte responses of six extracted samples of LQC, MQC, and HQC samples to mean analyte response of six replicates injection of unextracted QC samples.

For PG mean recovery values are 83.95%, 82.35%, and 80.98% at the HQC, MQC, and LQC samples, respectively. For KPG mean recovery values are 83.50%, 82.84%, and 79.52% at the HQC, MQC, and LQC samples, respectively. For HPG mean recovery values are 70.03%, 73.82%, and 69.89% at the HQC, MQC, and LQC samples, respectively. Results are presented in Tables 5 for HQC, MQC, and LQC levels of PG, KPG, and HPG, respectively.

For the IS mean IS responses of 18 extracted samples were compared to the mean IS responses of 18 injections (6 replicates injections from each LQC, MQC, and HQC) of unextracted IS samples.

Mean recovery value for the IS diphenhydramine is 78.63%. Results are presented in Tables 6.

Table 3: Interday accuracy and precision

Statistical parameters	LLOQ			LQC			MQC			HQC		
	PG	KPG	HPG	PG	KPG	HPG	PG	KPG	HPG	PG	KPG	HPG
Actual concentration	20.18 ng/mL	20.38 ng/mL	19.72 ng/mL	59.01 ng/mL	59.59 ng/mL	57.67 ng/mL	453.92 ng/mL	458.42 ng/mL	221.81 ng/mL	856.45 ng/mL	864.95 ng/mL	418.52 ng/mL
N	24	24	24	24	24	24	24	24	24	24	24	24
Mean±SD	19.48±1.198	20.61±1.105	18.84±1.3500	64.27±1.7285	59.08±3.223	61.32±2.6107	479.47±27.2612	461.34±27.92	236.83±13.2938	909.11±23.975	869.42±46.44	451.13±14.2077
CV%	6.15	5.36	7.17	2.69	5.46	4.26	5.69	6.05	5.61	2.64	5.34	3.15
Minimum	96.55	101.15	95.54	108.91	99.15	106.34	105.63	100.64	106.77	106.15	100.52	107.79
Maximum	17.28	18.49	16.60	60.82	51.01	53.54	421.30	402.57	205.13	863.78	782.19	413.45
% Nominal	21.98	22.77	22.53	67.68	64.46	65.06	520.36	504.89	254.59	958.59	924.15	469.68

SD: Standard deviation, CV: Coefficient of variation, LLOQ: Lower limit of quantification, LQC: Lower quality control, MQC: Middle quality control, HQC: Higher quality control, PG: Pioglitazone, KPG: Keto pioglitazone, HPG: Hydroxy pioglitazone

Table 4: Intraday accuracy and precision

Statistical parameters	LLOQ			LQC			MQC			HQC		
	PG	KPG	HPG	PG	KPG	HPG	PG	KPG	HPG	PG	KPG	HPG
Actual concentration	20.18 ng/mL	20.38 ng/mL	19.72 ng/mL	59.01 ng/mL	59.59 ng/mL	57.67 ng/mL	453.92 ng/mL	458.42 ng/mL	221.81 ng/mL	856.45 ng/mL	864.95 ng/mL	418.52 ng/mL
N	6	6	6	6	6	6	6	6	6	6	6	6
Mean±SD	20.43±0.8501	21.44±1.1684	20.26±1.139	65.19±1.8808	60.46±1.782	62.92±1.654	473.78±37.3545	454.71±40.118	230.36±16.229	940.91±15.0863	902.51±17.677	460.57±5.390
CV%	4.16	5.45	5.620	2.89	2.95	2.628	7.88	8.82	7.045	1.60	1.96	1.170
Minimum	19.48	19.78	19.44	63.71	57.91	61.55	421.30	402.57	207.75	924.97	882.99	454.59
Maximum	21.98	22.77	22.53	67.68	62.38	65.06	515.92	504.89	250.35	958.59	924.15	465.64
%Nominal	101.24	105.20	102.74	110.47	101.45	109.11	104.38	99.19	103.85	109.86	104.34	110.05

SD: Standard deviation, CV: Coefficient of variation, LLOQ: Lower limit of quantification, LQC: Lower quality control, MQC: Middle quality control, HQC: Higher quality control, PG: Pioglitazone, KPG: Keto pioglitazone, HPG: Hydroxy pioglitazone

Table 5: Recovery

S. No	PG			KPG			HPG		
	HQC	MQC	LQC	HQC	MQC	LQC	HQC	MQC	LQC
1	85.21	85.08	77.94	87.55	84.32	76.34	81.13	77.58	67.12
2	83.02	84.24	80.89	79.64	82.17	81.16	46.28	73.94	71.71
3	83.25	80.98	81.39	87.16	83.57	78.73	74.00	70.47	67.36
4	84.17	81.31	82.87	83.74	82.41	80.24	73.74	74.76	69.14
5	85.51	80.89	81.95	82.32	83.26	77.18	75.12	74.30	73.54
6	82.56	81.63	80.83	80.58	81.31	83.50	69.91	71.88	70.48
Mean±SD	83.95±1.2110	82.35±1.8248	80.98±1.6720	83.50±3.33	82.84±0.88	79.52±2.02	70.03±12.1869	73.82±2.4611	69.89±2.5149
CV%	1.44	2.22	2.06	3.99	1.06	2.54	17.40	3.33	3.60
%Nominal	83.95	82.35	80.98	83.50	82.84	79.52	70.03	73.82	69.89

SD: Standard deviation, CV: Coefficient of variation, LLOQ: Lower limit of quantification, LQC: Lower quality control, MQC: Middle quality control, HQC: Higher quality control, PG: Pioglitazone, KPG: Keto pioglitazone, HPG: Hydroxy pioglitazone

Table 6: Recovery of IS

S. No	AQ IS area	Extracted IS area	% Recovery
1	436166	351089	80.49
2	441244	349938	79.31
3	449645	351481	78.17
4	451643	344104	76.19
5	442404	350604	79.25
6	447495	337328	75.38
7	417311	329016	78.84
8	441002	340280	77.16
9	439305	346389	78.85
10	445262	349456	78.48
11	448832	341730	76.14
12	446844	358121	80.14
13	439493	358613	81.60
14	443280	343225	77.43
15	443942	353001	79.52
16	438181	343411	78.37
17	452417	361610	79.93
18	441984	353801	80.05
Mean±SD	442580.56±7835.4874	347955.39±8145.9950	78.63±1.6559
CV%	1.77	2.34	2.11
Mean recovery			78.63

SD: Standard deviation, CV: Coefficient of variation, IS: Internal standard

Stability

Short-term stock solution stability

Two portions of solutions for analytes (PG, KPG, and HPG) and ISs (diphenhydramine) from the stock solutions taken. The solutions kept on the bench as such at room temperature and in the deep freezer for approximately 7.00 hrs. Fresh stock solution (comparison samples) for analytes (PG, KPG, and HPG) and ISs (diphenhydramine) prepared. Then, six vials (each from bench top, deep freezer, and freshly prepared for both drug and ISs) at SSMQC level prepared. Give six replicates injection from each vial and use area response to determine % change over time.

PG, KPG, and HPG, and ISs (diphenhydramine) are found to be stable in reconstitution solution, on the bench at room temperature and in the deep freezer for approximately 6.00 hrs.

For PG, KPG, and HPG the % change found are −3.26, −0.63, and 1.77 on bench top −1.72, −1.89, and 1.45 in deep freezer, respectively. For ISs diphenhydramine, the % change found are 0.16 on bench top −1.04 in deep freezer, respectively.

Bench top stability

Six samples each of LQC and HQC (stability samples) was kept on the bench at room temperature for approximately 6.30 hrs. Stability samples were processed and analyzed along with freshly processed calibration and comparison samples (six samples each of LQC and HQC). Concentrations were calculated to determine % change over time.

PG, KPG, and HPG are found to be stable for 6.30 hrs at room temperature. The % change of PG, KPG, and HPG for LQC is −2.58, −1.86, and −3.67, respectively, and for HQC is 2.95, 6.25, and 1.06, respectively.

Freeze-thaw stability

Six samples each of low and high QC were retrieved from −70±5°C after 24 hrs of storage of samples. After thawing the stability, samples were restored for at least 12 hrs, and again the same samples were retrieved and kept on the bench at room temperature for thaw. The samples were restored, and after at least 12 hrs again retrieve and thawed. Six stability samples (after three cycles) and six comparison samples at each level (LQC and HQC) were processed and analyzed along with freshly processed calibration samples. Concentrations were calculated to determine % change over time.

PG, KPG, and HPG are found to be stable for three freeze-thaw cycles at −70±5°C. The % change of PG, KPG, and HPG for LQC is 6.84, −0.66, and −0.31, respectively, and for HQC is 0.14, −3.59, and −4.02, respectively.

Autosampler stability

Six samples (stability samples) each of LQC and HQC were processed and kept in an auto sampler at 10°C for approximately 35.00 hrs. The stability samples were analyzed along with freshly processed calibration and comparison samples (six samples each of LQC and HQC). Concentrations were calculated to determine % change over time. PG, KPG, and HPG are found to be stable for 35.00 hrs at auto sampler 10°C. The % change of PG, KPG, and HPG for LQC is −1.80, 4.58, and 2.71, respectively, and for HQC is −2.59, −1.62, and −0.85, respectively.

Dry extract stability

Six samples each of LQC and HQC samples were processed up to dry extract stage and stored in a deep freezer maintained at −70±5°C for approximately 24.00 hrs (stability samples). Stability samples were reconstituted and analyzed along with freshly processed CC and six replicates of LQC and HQC samples (comparison samples). Concentrations were calculated to determine % change over time.

PG, KPG, and HPG are found to be stable for 24.00 hrs at room temperature.

The % change of PG, KPG, and HPG for LQC is −0.58, 8.04, and −0.91, respectively, and for HQC is 7.12, 8.60, and 3.96, respectively.

Reconstitution solution stability

Six samples (stability samples) each of LQC and HQC level and keep it on bench top at room temperature for at least six hrs. The stability samples analyzed along with freshly processed calibration, LQC and HQC control samples (comparison samples). Calculate concentrations to determine % change over time. PG, KPG, and HPG are found to be stable for approximately 6.00 hrs at room temperature.

The % change of PG, KPG, and HPG for LQC is −9.83, −8.05, and −3.67, respectively, and for HQC is −8.63, −9.33, and −6.09, respectively.

Matrix effect

Eighteen blank matrix samples from six different lots of matrix are processed. The reconstituted blank samples spiked with HQC and LQC level (from each lot one blank, one HQC and one LQC sample)

and compared against corresponding aqueous HQC and LQC sample injected in six replicates. No effect of quantitation for PG, KPG, and HPG, and IS (diphenhydramine) was observed.

Reinjection reproducibility

After the analysis of any precision and accuracy batch, re inject the QC samples from the last three sets (LQC and HQC) and calculate the concentration. The back calculated concentrations of the HQC and LQC samples using the CC of the same precision and accuracy batch. The % change of PG, KPG, and HPG for LQC is 2.96, 2.11, and 2.03, respectively, and for HQC is −0.79, 5.02, and −0.55, respectively.

CONCLUSION

The particular LC-MS/MS assay shown with this report is simple, fast and sensitive pertaining to the determination of PG, KPG, and HPG in human plasma. This method is fully validated as per the recent FDA guidelines. This process makes use of Diphenhydramine as IS intended for quantification avoiding possible matrix influence associated difficulties in addition to variability in extraction efficiency between the analyte and the IS. The straightforward LLE technique is consistent and also reproducible recoveries for the analyte along with the IS from plasma. Furthermore, the whole analysis time period (extraction in addition to chromatography) would be the smallest compared to most of these reported procedures. The method furnished beneficial linearity and being trusted to guide pharmacokinetic study in humans. In the outcomes of all the agreement parameters, the proposed assay can be useful regarding bioavailability in addition to bioequivalence studies and therapeutic drug monitoring while using the ideal perfection in addition to reliability.

ACKNOWLEDGMENTS

The authors gratefully acknowledge Src Laboratories Private Limited (Hyderabad, India), for providing essential services to carry out this specific operate.

REFERENCES

1. Al-Majed A, Bakheit AH, Abdel Aziz HA, Alharbi H, Al-Jenoobi FI. Pioglitazone. Profiles Drug Subst Excip Relat Methodol 2016;41:379-438.
2. El-Ghobashy M, Yehia A, Mostafa A. Application of membrane-selective electrodes for the determination of pioglitazone HCl in the presence of its acid degradant or metformin HCl in tablets and plasma. Anal Lett 2009;42:123-40.
3. Faridbod F, Ganjali MR, Nasli-Esfahani E, Larijani B, Riahi S, Norouzi P, et al. Potentiometric sensor for quantitative analysis of pioglitazone hydrochloride in tablets based on theoretical studies. Int J Electrochem Sci 2010;5:880-94.
4. Ali MY, Swamy PV, Borgaonkar P, Raju SA. UV-spectrophotometric determination of pioglitazone in pharmaceutical dosage forms. Int J Chem Sci 2008;6:2062-5.
5. Saber AM. Determination of pioglitazone hydrochloride in tablets by high-performance liquid chromatography. Pak J Anal Environ Chem 2008;9:118-21.
6. Radhakrishna T, Sreenivas Rao D, Om Reddy G. Determination of pioglitazone hydrochloride in bulk and pharmaceutical formulations by HPLC and MEKC methods. J Pharm Biomed Anal 2002;29(4):593-607.
7. Abdelmonem AA, Ragab GH, Hashem HA, Bahgat EA. High performance liquid chromatographic and spectrophotometric determinations of pioglitazone-HCl either alone or in combination with metformine-HCl. J Liq Chromatogr Relat Technol 2012;35:2706-23.
8. Zhong WZ, Lakings DB. Determination of pioglitazone in dog serum using solid-phase extraction and high-performance liquid chromatography with ultraviolet (229 nm) detection. J Chromatogr 1989;490(2):377-85.
9. Yamashita K, Murakami H, Okuda T, Motohashi M. High-performance liquid chromatographic determination of pioglitazone and its metabolites in human serum and urine. J Chromatogr B Biomed Appl 1996;677(1):141-6.
10. Zhong WZ, Williams MG. Simultaneous quantitation of pioglitazone and its metabolites in human serum by liquid chromatography and solid phase extraction. J Pharm Biomed Anal 1996;14(4):465-73.
11. Lakshmi NY, Barhate VD. Development and validation of stability indicating UPLC method for the simultaneous determination of anti-diabetic drugs in pharmaceutical dosage forms. J Pharm Res 2010;3(12):3081-7.
12. Karra VK, Pilli NR, Inamadugu JK, Rao JS. Simultaneous determination of pioglitazone and candesartan in human plasma by LC-MS/MS and its application to a human pharmacokinetic study. J Pharm Anal 2012;2(3):167-73.
13. Lin ZJ, Ji W, Desai-Krieger D, Shum L. Simultaneous determination of pioglitazone and its two active metabolites in human plasma by LC-MS/MS. J Pharm Biomed Anal 2003;33(1):101-8.
14. Sripalakit P, Neamhom P, Saraphanchotiwitthaya A. High-performance liquid chromatographic method for the determination of pioglitazone in human plasma using ultraviolet detection and its application to a pharmacokinetic study. J Chromatogr B Analyt Technol Biomed Life Sci 2006;843(2):164-9.
15. Tahmasebi E, Yamini Y, Saleh A. Extraction of trace amounts of pioglitazone as an anti-diabetic drug with hollow fiber liquid phase microextraction and determination by high-performance liquid chromatography-ultraviolet detection in biological fluids. J Chromatogr B Analyt Technol Biomed Life Sci 2009;877(20-21):1923-9.
16. Chinnalalaiah R, Pigili R, Avanapu SR. Liquid chromatography and tandem mass spectrometry method for quantitative determination of pioglitazone and its metabolite 5-hydroxy pioglitazone in human plasma. Ann Pharm Fr 2017;75(2):105-11.
17. Jagadeesh B, Bharathi DV, Pankaj C, Narayana VS, Venkateswarulu V. Development and validation of highly selective and robust method for simultaneous estimation of pioglitazone, hydroxypioglitazone and metformin in human plasma by LC-MS/MS: Application to a pharmacokinetic study. J Chromatogr B Analyt Technol Biomed Life Sci 2013;930:136-45.
18. Lakshmi PR, Prahlad P, Mastanamma SK, Ravindra N, Rao MV. UPLC separation analysis of emtricitabine, tenofovir, cobicistat and elvitegravir from their degradation products. Int J Pharm Pharm Sci 2016;8(4):362-9.
19. Lakshmi PR, Prahlad P, Mastanamma SK, Ravindra N, Rao MV. Development and validation of a stability indicating reverse phase high performance liquid chromatography method for simultaneous determination of clindamycin, metronidazole, and clotrimazole in pharmaceutical combined dosage forms. Asian J Pharm Clin Res 2017;10(1):1-7.
20. US DHHS, FDA, CDER. Guidance for Industry: Bioanalytical Method Validation. Rockville, MD: US Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research and Center for Veterinary Medicine; 2001.
21. European Medicines Agency. Guideline on Bioanalytical Method Validation, Science and Medicinal Health. European Medicines Agency (EMEA), EMEA, CHMP, EWP/192217; 2009.