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Rapid and Stable Liquid Chromatographic Tandem Mass Spectrometric Method for Simultaneous Estimation of Pioglitazone, Keto Pioglitazone and Hydroxy Pioglitazone in Human Plasma: Application to Bioequivalence Study

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High performance liquid chromatographic tandem mass spectrometric method for the determination of pioglitazone, keto pioglitazone (M-III) and hydroxy pioglitazone (M-IV) in human plasma has been developed and validated using pioglitazone-D4, keto pioglitazone-D4 and hydroxy pioglitazone-D5 as internal standards. Solid phase extraction was carried out for sample preparation to extract analyte and internal standard from human plasma. The extracted sample was injected through autosampler in HPLC connected with Hypersil Gold, 100 mm × 4.6 mm, 5 µm using mobile phase consisting of methanol:solution A:solution B - 80:10:10 v/v/v). Pioglitazone, keto pioglitazone (M-III) and hydroxy pioglitazone (M-IV) were chromatographically separated and detected using the MS detector. The best-fit lines using weighting factor (1/concentration^2) linear least square regression analysis were obtained by peak area ratio of pioglitazone, keto pioglitazone (M-III) and hydroxy pioglitazone (M-IV) with their internal standards pioglitazone-D4, keto pioglitazone-D4 and hydroxy pioglitazone-D5, respectively. This report provides the results of various validation parameters including stability studies and extended precision and accuracy which is required for long study samples batch analysis. This analytical method is valid for the determination of pioglitazone in the range of 18.9 ng/mL to 2994.4 ng/mL), keto pioglitazone (M-III) (3.23 ng/mL to 512.60 ng/mL), hydroxy pioglitazone (M-IV) 10.1 ng/mL to 1603.8 ng/mL) and using pioglitazone-D4, keto pioglitazone-D4, hydroxy pioglitazone-D5, respectively as internal standards in human plasma using a Hypersil Gold, 100 mm × 4.6 mm, 5 µm column.

Keywords: Pioglitazone, Keto pioglitazone, Hydroxy pioglitazone, LC/MS, Chromatography.

INTRODUCTION

Pioglitazone is one of the antidiabetic medication, which belongs to the group of thiazolidinedione (TZDs), also called glitazones [1]. The typical mechanism of action of pioglitazone includes the linking to nuclear receptor called as peroxisome proliferator-activated gamma receptor. By decreasing the insulin resistance in liver, muscles, adipose tissues, they act as insulin sensitizers and help in regulating blood sugar level of diabetes mellitus (type-2) patients. Pioglitazone when taken alone does not elevate plasma insulin concentration, as sulfonylureas and meglitinides and hence considered as anti-hyperglycemic drug instead of blood glucose lowering drug [2]. In addition to improving the insulins sensitivity, pioglitazone exhibits encouraging results in metabolism of lipids, maintenance of blood pressure, adiponectin, endothelial function and levels of C-reactive protein, which overall helps in making this glucose control therapy more effective [3].

Pioglitazone is rapidly absorbed after oral administration and unchanged pioglitazone appears in blood after 2 h of oral administration (15-45 mg). The absorption of this drug is not affected by intake of food with an absolute bioavailability of more than 80%. The metabolism of pioglitazone in human body is mainly by liver enzyme, cytochrome P-450, which causes the hydroxylation of aliphatic methylene groups. Metabolism process produces six metabolites which are *i.e.* M-I, M-II, M-III, M-IV, M-V and M-IV (Fig. 1). Out of the 6 metabolites, 3 are active which includes M-II, M-III and M-IV. The protein

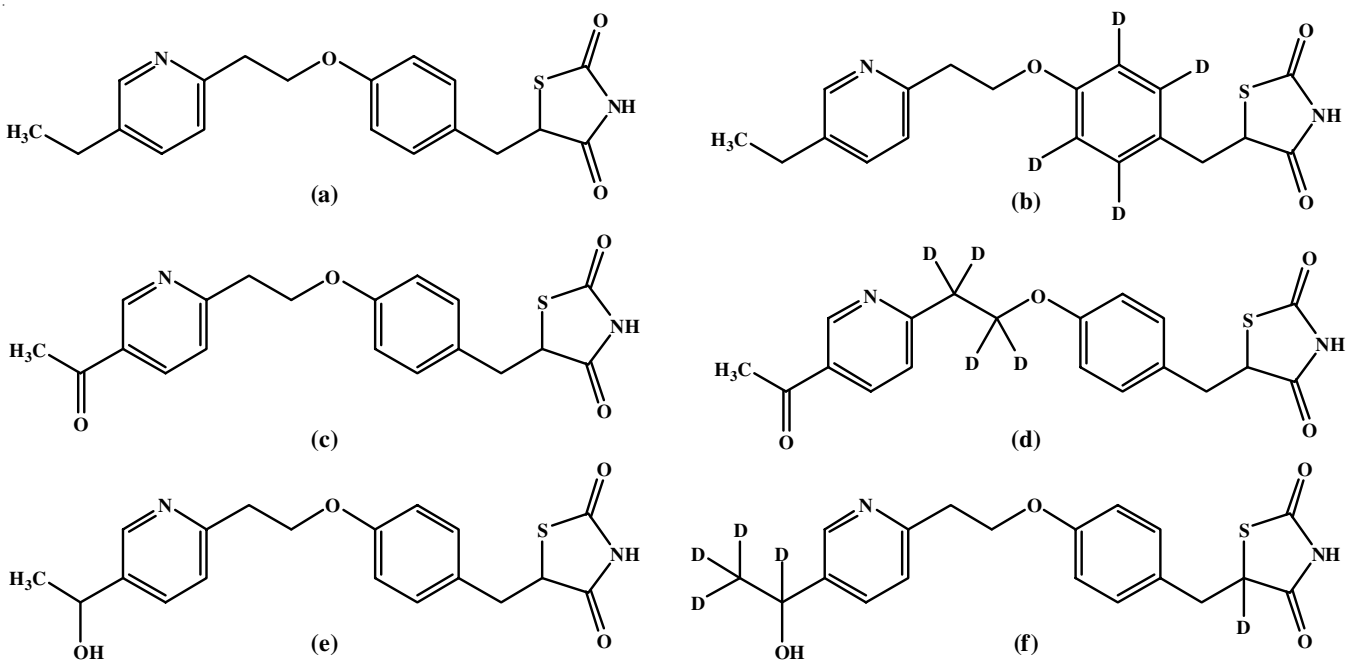

Fig. 1. Chemical structure (a) pioglitazone (b) pioglitazone-D4 (c) keto pioglitazone (M-III) (d) keto pioglitazone-D4 (e) hydroxy pioglitazone (M-IV) (f) hydroxy pioglitazone-D5

binding capability of pioglitazone and its metabolite, M-III, is equal and they also exhibit equivalent efficacy. However for M-IV the efficacy is approximately three-times to that of parent drug, while M-II has the least efficacy. The two metabolites of main drug namely keto pioglitazone (M-III) and hydroxy pioglitazone (M-IV) are therapeutically most active in body and contribute to extended blood sugar reduction. Pioglitazone average elimination half-life in human is around 5 to 6 h, however for the metabolites, which are active it is around 16-23 h. The unchanged pioglitazone is excreted in urine, bile and feces [4]. Post the oral dose of 45 mg pioglitazone mean plasma concentration was reported to be around 1482 ± 499.7 ng/mL, 168 ± 50.7 ng/mL, 639 ± 188.9 ng/mL at t_{max} of 3 (2-5), 16 (8-48), 16 (6-24) h was for pioglitazone, keto pioglitazone (M-III) and hydroxy pioglitazone (M-IV) [5].

Yamashita *et al*. [6] reported a liquid chromatographic method for estimation of five metabolites by setting 269 nm wavelength. Sripalakit *et al.* [7] reported the estimation of pioglitazone by using HPLC with UV detector. To achieve the desired sensitivity required to adequately estimate the drug concentrations of study samples in clinical studies is the major drawback of these method. Zhang *et al.* [8] developed a simultaneous method for analysis of pioglitazone and metformin in beagle dog plasma by LC-MS through single step extraction using protein precipitation but to clean the chromatographic column the runtime of 8 min was used, which is obvious requires longer times to the clean column. Lin *et al.* [9] reported method for bioanalysis of pioglitazone and active metabolites from human plasma by LC-MS using the liquid-liquid extraction. Liquid-liquid extraction is usually tedious and may also result in variability in results during high-throughput study sample analysis particularly when the internal standards are non-deuterated analogues. Xu *et al.* [10] worked on direct injection online solid phase extraction with shorter run times and save the offline extraction procedures but the method was selectively used for analysis of pioglitazone but as per bioanalytical guidelines the analysis of active metabolites. Kawaguchi-Suzuki *et al.* [11] reported the validated LC-MS method for the simultaneous method for the estimation of active metabolites using deuterated standards but the liquid chromatographic method utilized gradient elution to clean the interferences due to the protein precipitation extraction method which increases the run time of the method, the reported run time was 4 min.

It was observed that majority of the bioanalytical methods were either using a longer chromatographic runtime and unclean sample extraction procedures or used non deuterated internal standards for the analysis. It is well known that the bioanalytical methods need to be high throughput with shorter runtime, cleanest sample extraction from the biological matrix and use deuterated internal standard to minimize the variation in the results. Henceforth, in present work, a clean precise and high-throughput bioanalytical method is developled, validated and subsequently use to carry out the pharmacokinetics analysis of bioequivalence study.

EXPERIMENTAL

Working standards solution including pioglitazone (purity 99.65%), keto pioglitazone (M-III) (purity 99.80%) and hydroxy pioglitazone (M-IV) (purity 98.23%) and their deuterated internal standards pioglitazone-d4 (purity 97.76%), keto pioglitazone-D4 (purity 98%) and hydroxy pioglitazone-D5 (purity 98%) were purchase from Vivan Life Sciences, India. All the solvents and reagents of HPLC grade/analytical grade were used for this analysis. Methanol was procured form Sigma Aldrich, India. Formic acid and acetic acid were procured from Rankem (India). Water was taken from Milli-Q system. Oasis HLB 1

cc, 30 mg SPE cartridges used for sample preparation were bought from Waters (India) Pvt. Ltd. Blank plasma containing K2EDTA as anticoagulant was collected from in-house studies, which was stored at ~ -20 ºC.

HPLC system used for this analytical method comprised of Degasser DGU-20A3, Shimadzu, supplying degassed mobile phase to Quaternary Pump LC-20AD, Shimadzu, which was connected to thermostated autosampler SIL-HTC, Shimadzu and Column Oven CTO-20A, Shimadzu, to maintain uniform column temperature. Mobile phase composition of methanol: 0.2% acetic acid:0.2% formic acid (80:10:10 v/v/v) was delivered by quaternary pump isocratically at constant flow of 1.4 mL/min. Automated injection of 10 µL of extracted sample was done through autosampler into the chromatographic column Hypersil Gold 100 mm × 4.6 mm (Thermo), which was kept at 40 ºC in a column oven. All the analytes and internal standards were separated through this chromatographic column within a run time of 1.8 min.

API 3000 triple quadrupole mass spectrometer (AB-SCIEX, Canada) was used in tandem with HPLC by use of multiple reaction monitoring mode (MRM). Ionization mode used for the operation of mass spectrometer was electrospray ionization mode, used in positive polarity with ion spray voltage of 4000 V. The data files were processed using Analyst 1.5 software package, Sciex.

Human participants: This work was carried out in accordance with the basic principles specified in National Ethical Guidelines for Biomedical and Health Research involving Human Participants, notified by ICMR, India. The subjects gave written informed consent to participate in the study. A total of thirty four subjects (either male or female) were enrolled into the study as per the defined inclusion and exclusion criteria. The mean ± standard deviation age, height, weight and BMI of the study subjects were 27.8 ± 5.81 years (range 18-39), 165.6 ± 5.50 cm (range 155-182), 60.2 ± 8.80 Kg (47-84) and 21.90 ± 2.79 kg/m^2 (range 18.51-27.74), respectively.

Extraction procedure: Required number of calibration curve standards and QC samples were withdrawn from cold storage maintained at -20 ºC and defrosted at ambient room temperature. The standards and QC samples were then vortexed for roughly 1 to 2 min for uniform mixing of contents. Sample (200 µL) was pipetted into micro-centrifuge tubes, then 50 µL of IS dilution containing 5000 ng/mL of pioglitazone-D4 (IS), 1250 ng/mL of keto pioglitazone-D4 (IS) and 5000 ng/mL of hydroxy pioglitazone-D5 (IS) were added. Water (200 µL) was added and again vortexed to ensure the uniform mixing. The positive-pressure solid phase extraction machine was loaded with the required numbers of HLB 1cc, 30 mg extraction cartridges for the sample extraction. The HLB cartridges were conditioned with 1 mL of methanol followed by washing with 1 mL of water. The vortexed contents were pipetted in the extraction cartridges and then allowed to pass through cartridges under a constant pressure. Water (1 mL) was added to wash the contents of cartridge and final elution of sample was done by using 1 mL of methanol. These samples were further dried at 50 ºC, 20 psi on nitrogen evaporator and reconstituted with 1 mL mobile phase. A 10 µL of this was injected into system for analysis.

Calibration curve of standards and quality control samples: Stock solutions of 1 mg/mL were separately prepared by using methanol. The potency and amount of the working standard weighted were considered to calculate the actual and final concentration. The stock solutions and working solutions were kept in refrigerator at 2-10 ºC.

Preparation of internal standard dilution was done by the dilution of stock solutions in 50:50 v/v methanol:water solution to get approximately 5000 ng/mL of pioglitazone-D4, 1250 ng/mL of keto pioglitazone-D4 and 5000 ng/mL of hydroxy pioglitazone-D5.

Before spiking, standard stock dilutions of pioglitazone, keto pioglitazone and hydroxy pioglitazone were prepared by using methanol:water 50:50 v/v solution. A 200 µL of serial dilutions prepared were spiked in 10 mL of blank plasma in a volumetric flask and then volume made up with plasma to achieve the concentrations range of 18.9-2994.4 ng/mL for pioglitazone, 10.1-1603.8 ng/mL for hydroxy pioglitazone & 3.23-512.60 ng/mL for keto pioglitazone. The QC samples ranges were prepared at concentration of 19.0, 54.5, 1212.0, 2424.1 ng/mL, respectively for pioglitazone, 3.26, 9.37, 208.12, 416.24 ng/mL for keto pioglitazone and 10.2, 29.3, 651.3, 1302.6 ng/mL, respectively for hydroxy pioglitazone, respectively, for LOQQC, LQC, MQC and HQC. All the QC samples were stored at -20 ºC.

Calibration curve: Accuracy and precision of the method was analyzed in different batches. Each batch comprised of one complete calibration curve consisted of one blank plasma, one blank plasma with internal standard, eight different concentration samples and six sets of quality control samples each at level of LOQQC, LQC, MQC and HQC. The mass spectrometric signals were acquired and suitably integrated in software provided by the respective LC/MS manufacturer. The back calculated concentration of samples was calculated using regression line analysis of calibration curve by applying suitable weighting factor.

$$y = m(x) + b$$

where, y = peak area ratio of analyte and internal standard, m = slope of the calibration curve, x = concentration of analyte, b = y-axis intercept of the calibration curve.

Calibration curve was considered acceptable, if correlation coefficient was above 0.99 and respective back calculated concentrations of the calibration curve standards was within ± 15% deviation of the nominal value with an exception to LOQ samples for which the acceptance limit of was considered to be within ± 20% as per USFDA guidelines [12].

LC-MS/MS method development: Reversed phase liquid mode was chosen for chromatography which is most commonly available and used in laboratories. The mobile phase included organic solvents like methanol along with volatile buffers were selected as starting materials to set the chromatographic method using a C18 chromatographic column. Lower particle size and short columns helped to elute all the analytes in a short period of time. High pressure liquid chromatographic system selected for analysis helped to deliver a higher flow rate of mobile phase, which further helped to reduce the run time.

Mass spectrophotometer was operated in the electrospray ionization mode in positive polarity as all the analytes and the internal standards have the tendency for protonation to form M+H ions. Source parameters were optimized and set as nebulizer = 14.00, collision gas = 4.00, curtain gas = 12.00, ion spray voltage = 4000.00 and temperature = 500.00. The parent and daughter ions in positive polarity were observed as *m/z* 357.02/134.10, 371.03/148.20, 373.00/150.20, 361.06/138.00, 374.99/152.20 and 377.91/154.20 for pioglitazone, keto pioglitazone (M-III) and hydroxy pioglitazone (M-IV), pioglitazone D4, keto pioglitazone D4 and hydroxy pioglitazone D5, respectively. The parameters of all the analytes and internal standards were optimized automatically through using Analyst software. Source parameters including nebulizer, ion spray voltage, *etc.* were manually optimized to get the best ionization and to make sure that the entire mobile phase is suitably evaporated in source before entering the mass spectrometer so that the MS does not get contaminated and the method performance is stable over extended batch analysis. Dwell time of 100 ms was selected to yield good number of data points for all analytes, internal standards and ideal chromatographic pattern.

Use of deuterated internal standards for all analytes *i.e.*, pioglitazone, keto pioglitazone, hydroxy pioglitazone yielded lower RSDs (less than 5%) for area ratios in LCMS analysis and avoided any deviation during routine sample extractions procedures errors, which did not impact the results.

Volatile ammonium salts like ammonium acetate, formate, acetic acid and formic acid were tried with the aim to have the best ionization. Buffers like acetic acid gave better ionization as compared to ammonium acetate and ammonium format. The final composition of acetic acid was fixed to 1% which yielded the maximum ionization. Addition of formic acid helped to clear the column from some late eluting peaks and also helped to reduce the run time and improved the peak shape. Finally, 1% formic acid was used along with 1% acetic acid along with methanol in mobile phase. Hypersil column 100 × 4.6 mm, 5 µ resulted in best symmetric peak shapes for all analytes and internal standard.

Sample extraction method development: To have a short run time and a clean sample extraction method was the need of the hour. We selected solid phase extraction technique for extraction of analytes and internal standards from biological matrix. Due to selective retention of analytes on the solid phase extraction bed, the biological matrix components were easily washed during multiple wash cycles of organic solvents like methanol followed by water. Only 200 µL of biological matrix was used for analysis to get the desired sensitivity using this bioanalytical method. The extraction recovery was calculated during sample preparation optimization. The final method yielded reproducible extraction recoveries less than 15% which is as per regulatory requirements. The final elute was dried on nitrogen evaporator till complete dryness. The reconstitution of dried residues deposited on test tubes were done in mobile phase to maintain similar solvents strength mobile phase and sample to be injected, which resulted in the better peak shapes of the extracted samples. By using the final optimized sample extraction procedure, the reproducible results and the excellent chromatographic curves were able to obtain from the clean samples. It helped to avoid multiple cleaning of columns, analytical instrument and also helped in the long batch analysis with desired accuracy and precision.

RESULTS AND DISCUSSION

Chromatography: The illustrative chromatograms of reference mix in solvent solution, extracted blank plasma, extracted blank sample containing internal standard, calibration curve of representative regression analysis for pioglitazone, keto pioglitazone (M-III) and hydroxy pioglitazone (M-IV),QC samples (LOQQC, LQC, MQC and HQC), respectively, are shown in Figs. 2-4.

Selectivity: Ten different batches of plasma (including lipemic and hemolyzed plasma) with dipotassium ethylene diamine tetraacetate (K2EDTA) were processed and analyzed. None of the batch exhibited any significant interference at the retention time of pioglitazone, keto pioglitazone (M-III), hydroxy pioglitazone (M-IV), pioglitazone-D4 (IS), keto pioglitazone-D4 and hydroxy pioglitazone-D5 (IS).

Linearity: The linearity of pioglitazone, keto pioglitazone (M-III) and hydroxy pioglitazone (M-IV) were determined by weighed least square regression analysis plotted by using eight calibration curve standards, respectively. The coefficient of determination was found to be 0.9942, 0.9974 and 0.9956 for pioglitazone, keto pioglitazone (M-III) and hydroxy pioglitazone (M-IV), respectively.

Sensitivity: Lower limit of quantitation for pioglitazone, keto pioglitazone (M-III) and hydroxy pioglitazone (M-IV) was 18.9 ng/mL, 3.23 ng/mL and10.1 ng/mL, respectively. Between and within batch precision and accuracy was calculated by using the peak area ratio of analyte to respective internal standard. Between batch precision and accuracy at level of LOQQC concentration were 2.6% and 94.9%, 8.4% and 95.3%, 6.4% and 91.6% for pioglitazone, keto pioglitazone (M-III) and hydroxy pioglitazone (M-IV), respectively.

Accuracy: Accuracy was calculated as ratio of back-calculated average values of QC samples and their respective nominal values. The within batch accuracy of the method ranged from 90.5% to 99.2% for pioglitazone, 89.7% to 100.4% for keto pioglitazone (M-III) and 88.4% to 102.1% for hydroxy pioglitazone (M-IV). The between batch accuracy of the method ranged from 91.5% to 98.5% for pioglitazone, 95.3% to 97.6% for keto pioglitazone (M-III) and 91.6% to 99.6% for hydroxy pioglitazone (M-IV) as presented in Table-1.

Precision: Precision was calculated by determining the % coefficient of variation of back calculated concentrations of the replicates samples analyzed across the concentration levels of LOQQC, LQC, MQC and HQC quality control samples of pioglitazone, keto pioglitazone (M-III) and hydroxy pioglitazone (M-IV). Within batch precision of the method ranged from 1.0% to 3.1% for pioglitazone, 1.6% to 8.4% for keto pioglitazone (M-III) and 1.3% to 6.4% for hydroxy pioglitazone (M-IV) and between batch precision of the method ranged from 1.5% to 2.6% for pioglitazone, 2.0% to 8.4% for keto pioglitazone (M-III) and 4.7% to 6.4% for hydroxy pioglitazone (M-IV) (Table-1).

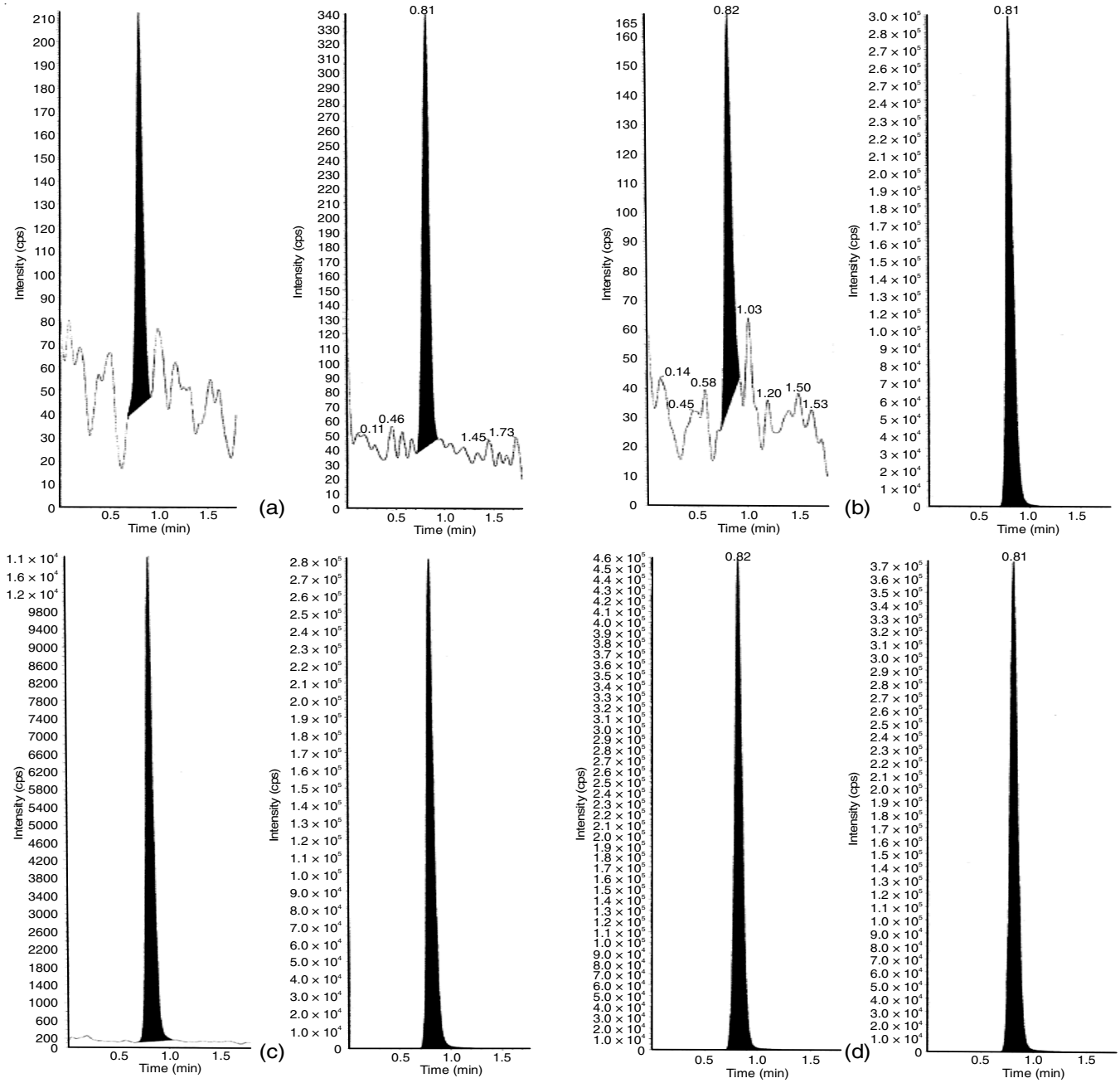

Fig. 2. Chromatograms (a) pioglitazone & pioglitazone D4 in blank plasma (b) pioglitazone & pioglitazone D4 in blank plasma spiked with pioglitazone D4 (c) pioglitazone & pioglitazone D4 in plasma at LOQ level (d) pioglitazone & pioglitazone D4 in reference sample

TABLE-1
PRECISION & ACCURACY DATA

Parameter	Pioglitazone	Keto Pioglitazone (M-III)	Hydroxy Pioglitazone (M-IV)
Precision & accuracy			
Within batch accuracy (%)	90.5-99.2%	89.7-100.4%	88.4-102.1%
Between batch accuracy (%)	91.5-98.5%	95.3-97.6%	91.6-99.6%
Within batch precision (% CV)	1.0-3.1	1.6-8.4	1.3-6.4
Between batch precision (% CV)	1.5-2.6	2.0-8.4	4.7-6.4
Extended precision & accuracy			
Within batch accuracy (%)	92.2-98.7	96.6-97.4	94.3-98.1
Within batch precision (% CV)	2.0-2.8	1.5-3.0	1.8-3.5
Ruggedness			
Within batch accuracy (%)	91.7-103.8	87.5-98.3	95.0-98.7
Within batch precision (% CV)	1.3-2.4	2.7-13.0	1.1-4.2

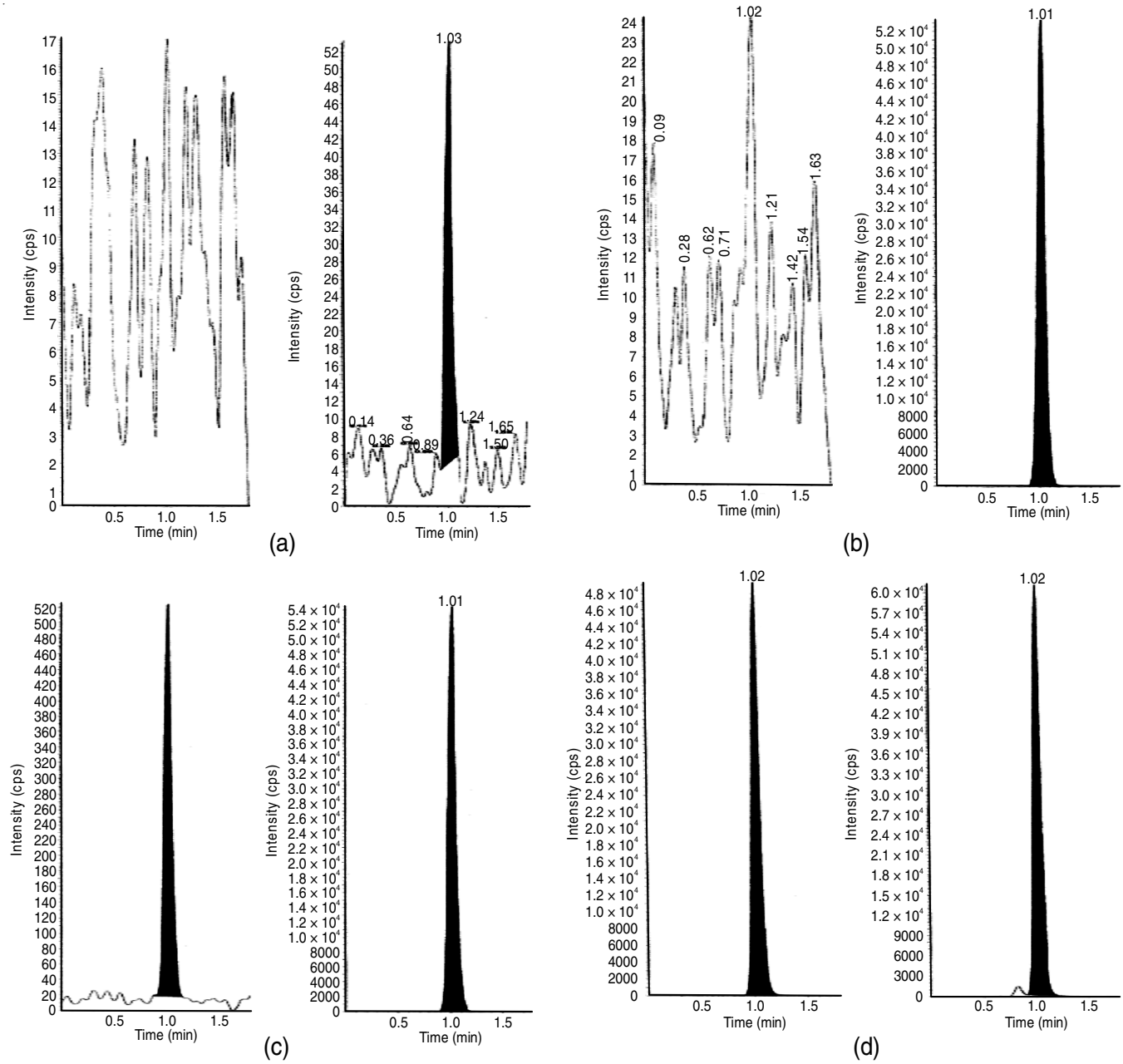

Fig. 3. Chromatograms (a) keto pioglitazone (M-III) & keto pioglitazone D4 in blank plasma (b) keto pioglitazone (M-III) & keto pioglitazone D4 in blank plasma spiked with keto pioglitazone D4 (c) keto pioglitazone (M-III) & keto pioglitazone D4 in plasma at LOQ level (d) keto pioglitazone (M-III) & keto pioglitazone D4 in reference sample

Extraction recovery: Recovery percentage of pioglitazone, keto pioglitazone (M-III) and hydroxy pioglitazone (M-IV) was calculated as a ratio of area response of extracted quality control samples at concentration levels of LQC, MQC and HQC against peak area response of aqueous quality control samples at equivalent concentration levels of LQC, MQC and HQC. The mean percentage recovery of pioglitazone, keto pioglitazone and hydroxy pioglitazone was 64.90%, 82.63% and 81.47%, respectively.

Similarly, recovery percentage of pioglitazone-D4 (IS), keto pioglitazone-D4 (IS) and hydroxy pioglitazone-D5 (IS) was calculated as a ratio of peak area response of spiked quality control samples at concentration levels of MQC against area response of aqueous internal solution (IS) dilution of same concentration. The percentage recovery of pioglitazone-D4 (IS), keto pioglitazone-D4 (IS) and hydroxy pioglitazone-D5 (IS) was 68.4%, 97.3% and 74.4%, respectively (Table-2).

Matrix effect: Matrix effect was analyzed at the concentration level of LQC and HQC samples in duplicate into six different batches of matrix each and evaluated against mean area response of six replicate aqueous injections at level of LQC and HQC. The matrix factor calculated as percentage coefficient of variation was found to be 1.9% and 2.9% for pioglitazone, 3.4% and 2.4% for keto pioglitazone (M-III) and 2.0% and 2.0% for hydroxy pioglitazone (M-IV) at level of LQC and HQC (Table-3).

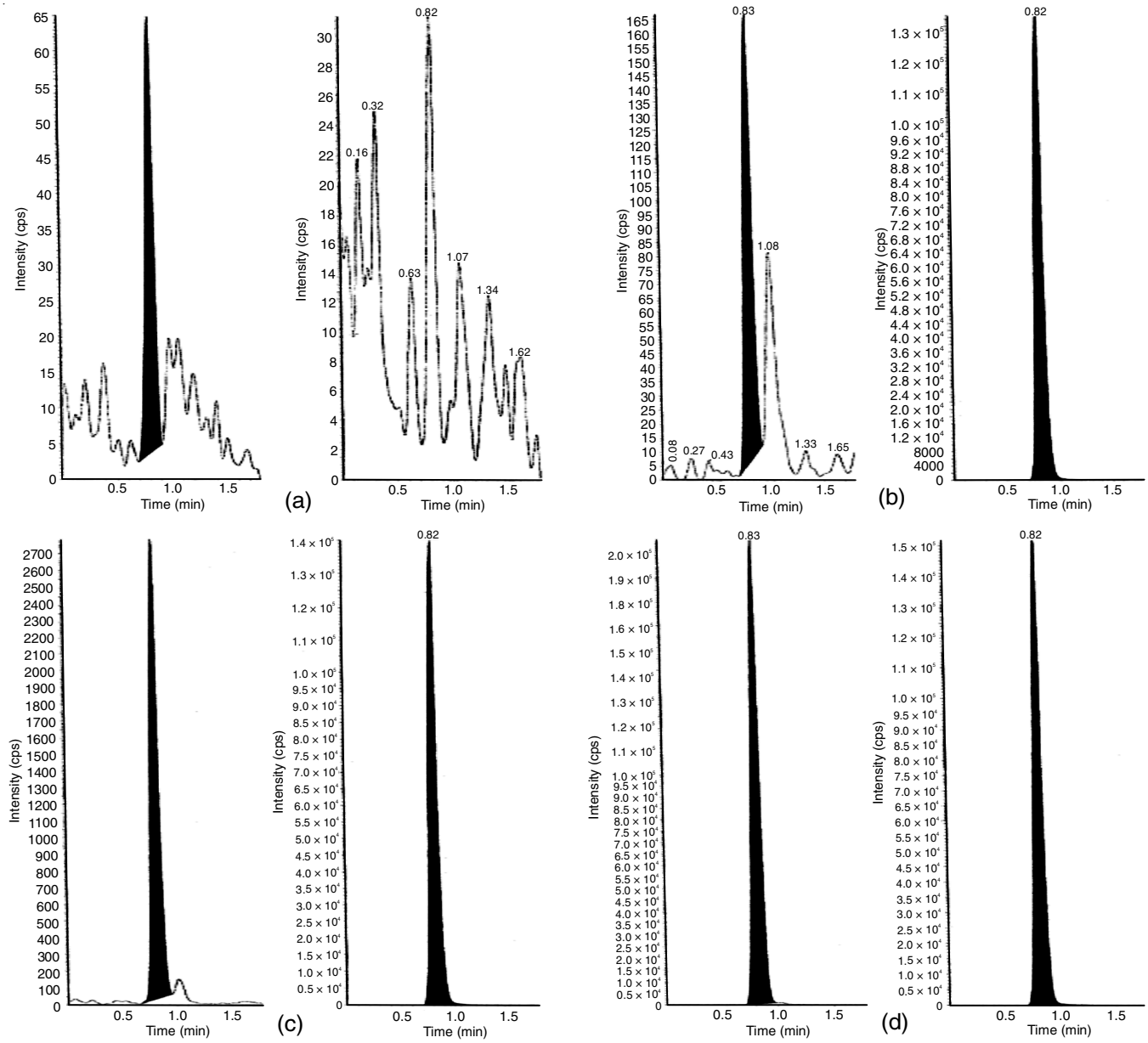

Fig. 4. Chromatograms (a) hydroxy pioglitazone (M-IV) & hydroxy pioglitazone D5 in blank plasma (b) hydroxy pioglitazone (M-IV) & hydroxy pioglitazone D5 in blank plasma spiked with hydroxy pioglitazone D5 (c) hydroxy pioglitazone (M-IV) & hydroxy pioglitazone D5 in plasma at LOQ level (d) hydroxy pioglitazone (M-IV) & hydroxy pioglitazone D5 in reference sample

TABLE-2
EXTRACTION RECOVERY DATA

QC Level	Pioglitazone	Pioglitazone-D4 (IS)	Keto Pioglitazone (M-III)	Keto Pioglitazone-D4 (IS)	Hydroxy Pioglitazone (M-IV)	Hydroxy Pioglitazone-D5 (IS)
LQC	62.0	–	81.6	–	79.2	–
MQC	67.4	68.4	86.6	97.3	82.5	74.4
HQC	65.3	–	79.7	–	82.7	–
% Mean	64.90	68.4	82.63	97.3	81.47	74.4
% CV	4.2		4.3		2.4	

Ruggedness: Method ruggedness to ensure the reproducibility of extraction process along with chromatographic technique was evaluated by analyzing six sets of QC samples at concentration level of LOQQC, LQC, MQC, HQC against a set of eight calibration curve standards by another chemist using different column of same make. The within batch precision varied from 1.3% to 2.4% for pioglitazone, 2.7% to 13.0% for keto pioglitazone (M-III) and 1.1% to 4.2% for hydroxy pioglitazone (M-IV). The within batch accuracy ranged from 91.7% to 103.8% for pioglitazone, 87.5% to 98.3% for keto piogli-

TABLE-3
OTHER METHOD VALIDATION EXPERIMENTS DATA

Parameter	Pioglitazone	Keto Pioglitazone (M-III)	Hydroxy Pioglitazone (M-IV)
Matrix effect			
LQC	1.9	3.4	2.0
HQC	2.9	2.4	2.0
1:2 Dilution integrity			
Accuracy (%)	96.1	97.8	95.3
Precision (% CV)	1.7	1.2	1.9
1:4 Dilution integrity			
Accuracy (%)	100.0	102.1	97.8
Precision (% CV)	3.1	3.4	2.0
Stability studies			
Bench top stability (for 7.42 h)	99.8-101.3	97.5-99.5	100.4-101.6
In-injector stability (for 70.60 h)	99.4-100.5	100.5-107.3	100.3-101.2
Freeze thaw stability (after 3 cycles)	99.7-101.2	95.4-99.8	100.6-105.2
Stock solution stability (27.83 h)	100.3	93.8	100.2

tazone (M-III) and 95.0% to 98.7% for hydroxy pioglitazone (M-IV) (Table-1).

Dilution integrity: Dilution integrity evaluation was performed by analyzing four replicate QCs which were spiked about twice the upper limit of quantification concentration, which were induced to multiple cycles of freeze and thaw to simulate actual subject sample analysis. The respective QC samples were first frozen for minimum 24 h at below -20 ºC, after which they were allowed to thaw completely in wet ice bath. After this, the samples were again frozen for minimum of 12 h at > -20 ºC. This was repeated additionally for at least two times and finally samples were diluted 1:2 and 1:4 before extraction using the blank plasma and assayed against freshly prepared and spiked calibration standards (Table-3).

Extended precision and accuracy batch: Extended precision and accuracy batch analysis were performed to evaluate the extended stable performance of bioanalytical method to cover the expected run time of the complete batch of study samples in biostudy. The batch accuracy of the method varied from 92.2% to 98.7% for pioglitazone, 96.6% to 97.4% for keto pioglitazone (M-III) and 94.3% to 98.1% for hydroxy pioglitazone (M-IV). The batch precision of the method varied from 2.0% to 2.8% for pioglitazone, 1.5% to 3.0% for keto pioglitazone (M-III) and 1.8% to 3.5% for hydroxy pioglitazone (M-IV) (Table-1).

Stability studies: Stability of the spiked samples at various storage conditions like Benchtop, during freeze thaw, in-injector duration was calculated by analyzing four replicate of QC samples at level of LQC and HQC and evaluating the mean back calculated values of the QC samples against freshly spiked at same levels. Room temperature stock solution stability was performed by storing pioglitazone, keto pioglitazone (M-III), hydroxy pioglitazone (M-IV), pioglitazone-D4 (IS), keto pioglitazone-D4 (IS) and hydroxy pioglitazone-D5 (IS) stock solutions at storage conditions of ambient laboratory temperature. The evaluation of stability was done by analyzing 6 replicate injections of stock dilution prepared from fresh stock solutions of pioglitazone, keto pioglitazone (M-III), hydroxy pioglitazone (M-IV), pioglitazone-D4 (IS), keto pioglitazone-D4 (IS) and hydroxy pioglitazone-D5 (IS), respectively against 6 replicate injections of dilution prepared from stored aliquots of pioglitazone, keto pioglitazone (M-III), hydroxy pioglitazone (M-IV), pioglitazone-D4 (IS), keto pioglitazone-D4 (IS) and hydroxy pioglitazone-D5 (IS) stock solutions, respectively. It was found that the samples were stable during the stipulated timeframes and the data is presented in Table-3.

Application of the method in human volunteers: In a single dose, cross-over, fasting condition, bioequivalence study of pioglitazone 45 mg in 34 healthy human subjects, 50 blood samples (1 × 6 mL) were collected from each subject at different time points (pre-dose, 0.25, 0.50, 0.75,1.00, 1.25, 1.50, 1.75, 2.00, 2.50, 3.00, 3.50, 4.00, 6.00, 8.00, 10.00, 12.00, 14.00, 16.00, 20.00, 24.00, 36.00, 48.00, 72.00, 96.00 h after dosing in each period) to draw the pharmacokinetic profiles. Plasma was separated and collected by centrifuging the collected blood samples. These samples were analyzed for estimation of pioglitazone and its active metabolites using this analytical method and the pharmacokinetic profiles are shown in Fig. 5, while the parameters are given in Table-4.

Conclusion

The analytical method described in this study was validated for the determination of pioglitazone (over a range of 18.9 ng/mL to 2994.4 ng/mL), hydroxy pioglitazone (M-IV) (over a

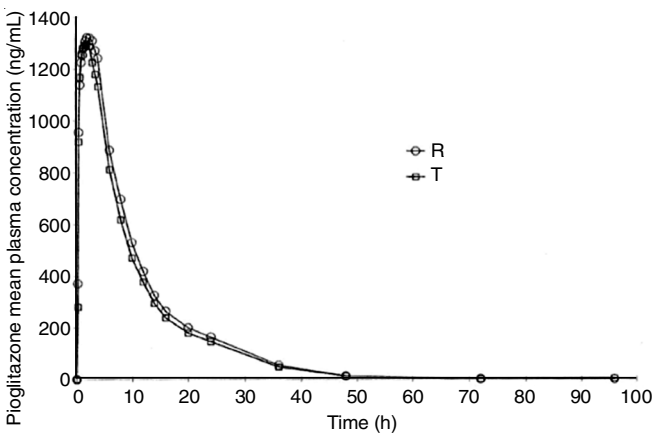

Fig. 5. Mean plasma concentration time profile of pioglitazone test *vs.* reference after 45 mg of single oral dose in 34 healthy volunteers

TABLE-4
PHARMACOKINETIC PARAMETERS OF PIOGLITAZONE IN 34 HEALTHY, ADULT HUMAN SUBJECTS

Parameters	C_{max} (ng/mL)	AUC_{0-t} (ng h/mL)	$AUC_{0-\infty}$ (ng h/mL)
Geometric least square mean			
Reference product (R)	1448.15	14180.4077	14575.4942
N	34	34	34
Test product (T)	1342.74	12878.7273	13481.4492
N	34	34	34
Ratio of LSM			
T/R (%)	92.72	90.82	93.31
90% Confidence interval			
Lower limit	81.36	83.14	86.48
Upper limit	105.67	99.22	100.68
P-Value			
Period	0.4045	0.389	0.0714
Treatment	0.3349	0.0743	0.1394
Sequence	0.8495	0.2078	0.3807
Power (%)	87.99	99.27	99.87
Intrasubject CV (%)	32.7	21.8	18.1
Intersubject CV (%)	36.8	33.1	31.8

range of 10.1 ng/mL to 1603.8 ng/mL) and keto pioglitazone (M-III) (over a range of 3.23 ng/mL to 512.60 ng/mL) using pioglitazone-D4, hydroxy pioglitazone-D5 and keto pioglitazone D4, respectively as internal standards in human plasma using Hypersil Gold, 100 mm × 4.6 mm, 5 µm column. The method was successfully applied for accurate determination of the pharmacokinetic parameters to establish bioequivalence of pioglitazone 45 mg tablets test and reference products under fasting conditions.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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