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SIMULTANEOUS DETERMINATION OF ALOGLIPTINE, PIOGLITAZONE AND ITS ACTIVE METABOLITE IN HUMAN PLASMA BY LC-MS/MS METHOD AND ITS PHARMACOKINETIC APPLICATION

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ABSTRACT

A simple, rapid and sensitive liquid chromatography-tandem mass spectrometric (LC–MS/ MS) assay method has been developed and fully validated for the simultaneous quantification of Alogliptine, Pioglitazone and Hydroxy Pioglitazone. The analytes were extracted from human plasma via protein precipitation using acetonitrile. The reconstituted samples were chromatographed on a Alltima HP C18 column by using a 60:40 (v/v) mixture of acetonitrile and 10 mM ammonium acetate (pH 3.0) as the mobile phase at a flow rate of 1.1 mL/min. The calibration curves obtained were linear over the concentration range of 3.05-250.29 ng/mL for Alogliptine, 15–2500.50 ng/mL for Pioglitazone and 7–1500 ng/mL for Hydroxy Pioglitazone. The API-4000 LC–MS/MS in multiple reaction monitoring (MRM) mode was used for detection. The results of the intra- and inter-day precision and accuracy studies were well within the acceptable limits. All the analytes were found to be stable in a battery of stability studies. The method is precise and sensitive enough for its intended purpose. The developed assay method was successfully applied to a pharmacokinetic study in human volunteers.

KEYWORDS: Alogliptine, Pioglitazone, Hydroxy Pioglitazone, Human plasma, LC-MS/MS, pharmacokinetic study.

INTRODUCTION

Type 2 diabetes is a complex metabolic disorder with two major biochemical defects, namely impaired insulin secretion and impaired insulin action at the periphery. Chronic hyperglycemia results from these defects. Current American Diabetes Association guidelines suggest that all adults with diabetes should be managed to achieve a low density lipoprotein (LDL) cholesterol less than 100 mg/dl employing statins as first-line therapy.[1]

Alogliptin a member of dipeptidyl peptidase-4 inhibitors is a recent drug developed in 2010 by Takeda Pharmaceutical Company[2], which is used for the treatment of Type 2 diabetes, and it potentiates the effect of incretin hormones through inhibition of their degradation by the dipeptidyl peptidase-4 enzyme.[3]

Pioglitazone hydrochloride, (9/)-5-{4-[2-(5-ethyl-2-pyridyl)ethoxy]benzyl}-2,4-thiazolidinedione hydrochloride salt, is an oral antidiabetic agent that has been shown to affect abnormal glucose and lipid metabolism associated with insulin resistance by enhancing insulin action on peripheral tissues in animal models.[4,5] 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.[6] At steady-state, M-III and M-IV reach serum concentrations equal to or greater than pioglitazone.[6]

As per the literature, several LC–MS/MS methods have been reported for the determination of Alogliptin[7,8], Pioglitazone hydrochloride[9,10,11] individually or with some other drugs in biological samples. To date, no LC–MS/ MS method has been reported for the simultaneous determination of Alogliptin, Pioglitazone hydrochloride and its metabolite in human plasma. To determine the pharmacokinetics of the formulation, a sensitive and specific method that allows simultaneous measurement of Alogliptin, Pioglitazone hydrochloride and its metabolite in human plasma is needed. The application of this assay method to a clinical pharmacokinetic study in human volunteers following oral administration of Alogliptin, Pioglitazone hydrochloride is described.

EXPERIMENTAL

2.1 Materials

The reference samples of Alogliptine benzoate (99.80%), Pioglitazone HCL (99.10), Hydroxy Pioglitazone M-IV (99.80%), Alogliptine 13C D3 (98.50), Pioglitazone

D4(96.64%) and Hydroxy Pioglitazone d4 HCl(97.07%)were purchased from TLC Pharmaceutical Standards Ltd, (Ontario, Canada). Water used for the LC-MS/MS analysis was prepared from Milli Q water purification system procured from Millipore (Bangalore, India). Acetonitrile and methanol were of HPLC grade and purchased from J.T Baker (Phillipsburg, USA). Analytical grade ammonium acetate and acetic acid were purchased from Merck (Mumbai, India). Oasis® HLB 1cc (30 mg) extraction cartridges was purchased from Waters corporation (Milford, Massachuselts, USA). The control K2-EDTA human plasma sample was procured from Deccan Pathological Lab (Hyderabad, India).

2.2 LC–MS/MS Instrumentation

An HPLC system (Shimadzu, Kyoto, Japan) consisting of consisting of a Alltima HP C18 HL column (50 mm 4.6 mm, 3 mm; Grace Davison, Deerfield, Ireland) column was used for the study. Aliquots of the processed samples (2 μL) were injected into the column, which was kept at 40 ºC temperature. The isocratic mobile phase, a mixture of acetonitrile and 10mM ammonium acetate (60:40, v/v) was delivered at 0.6 mL/min into the electrospray ionization chamber of the mass spectrometer. Quantitation was achieved with MS-MS detection in positive ion mode for both the analytes and the internal standard. Detection of the ions was carried out in the multiple–reaction monitoring mode (MRM), by monitoring the transition pairs of m/z 340.40 precursor ion to the m/z 115.90 for Alogliptine and m/z 344.40 precursor ion to the m/z 115.90 product ion for the Alogliptine IS, m/z 357.30 precursor ion to the m/z 134.20 for pioglitazone and m/z 361.20 precursor ion to the m/z 134.20 product ion for the pioglitazone IS, m/z 373.20 precursor ion to the m/z 150.20 for Hydroxy pioglitazone and m/z 377.20 precursor ion to the m/z 150.20 product ion for the Hydroxy pioglitazone IS.

2.3 Methods

Preparation of standard solutions

Primary stock solutions of Alogliptine (1000μg/mL), Pioglitazone (1000 μg/mL) and Hydroxy Pioglitazone (1000 μg/mL) for preparation of standard calibration curve and quality control (QC) samples were prepared from separate weighing. These stocks were stored at 2-8 ºC; they were found to be stable for 10 days. From these stock solutions, appropriate dilutions were made using a mixture of methanol and water (60:40, v/v) as a diluent, to produce working standard solutions of Alogliptine, Pioglitazone and Hydroxy Pioglitazone. The primary stock solution of Alogliptine, Pioglitazone and Hydroxy Pioglitazone were prepared

in methanol. A working concentration of mixture internal standards solution was prepared in the diluent (methanol and water, 60:40, v/v).

Preparation of calibration curve standards and quality control samples

Calibration samples were prepared by spiking 950 mL of control human plasma with the appropriate working standard solution of the each analyte. The CC samples were analyzed along with the quality control (QC) samples for each batch of plasma samples. The QC samples were prepared at five different concentration levels of 3.40 (lower limit of quantification, LLOQ), 9.61 (low quality control, LQC), 45.05 (middle quality control, MQC-1), 150.09 (MQC-2) and 249.13 ng/mL (high quality control, HQC) for Alogliptine, 15.8 (LLOQ), 45 (LQC), 450 (MQC-1), 1500 (MQC-2) and 2500 ng/mL (HQC) for Pioglitazone and 6.97 (LLOQ), 11.66 (LQC), 602.05 (MQC-1), 1122.24 (MQC92) and 1498.79 ng/mL (HQC) for Hydroxy pioglitazone in blank plasma.All the prepared plasma samples stored at -70 ^{o}C.

2.4 Sample preparation

All frozen subject samples, calibration standards and quality control samples were thawed and allowed to equilibrate at room temperature prior to analysis. The samples were vortexed to mix for 10 s prior to spiking. 900 mL aliquot of human plasma sample was mixed with 20 mL of the internal standard working solution. To this, 50 mL of the ammonia solution (25%) and 1.0 mL of acetonitrile were added. After vortex-mixing for 30 s and centrifugating at 4000 rpm for 10 min, the supernatant was transferred to another clean test tube and evaporated to dryness at 45 0C under a gentle stream of nitrogen. The residue was reconstituted with 500 mL of the mobile phase and 25 mL was injected into LC–MS/MS system.

2.5. Method validation

The validation of the above method was carried out as per US FDA guidelines.[12] The parameters determined were selectivity, sensitivity, matrix effect, linearity, precision, accuracy, recovery, stability and dilution integrity. Selectivity was assessed by comparing the chromatograms of six different batches of blank plasma obtained from six different sources including one lipemic and one hemolyzed plasma. Sensitivity was determined by analyzing six replicates of plasma samples spiked with the lowest level of the calibration curve concentrations. Matrix effect was checked with six different lots of K2-EDTA plasma. Three replicate samples each of LQC and HQC were prepared from different lots of plasma (36 QC

samples in total). For checking the linearity standard calibration curves containing at least nine points (non-zero standards) was plotted. In addition, blank plasma samples were also analyzed to confirm the absence of direct interferences. Intraday precision and accuracy were determined by analyzing six replicates at five different QC levels on two different days. Inter-day precision and accuracy were determined by analyzing six replicates at five different QC levels of five different runs. Recoveries were determined by comparing the peak area of extracted analyte standard with the peak area of non-extracted standard. Dilution integrity was performed to extend the upper concentration limit with acceptable precision and accuracy. Six replicates each at a concentration of about 1.6 times of the uppermost calibration standard were diluted 2- and 4-fold with blank plasma. The diluted samples were processed and analyzed. Stability tests were conducted to evaluate the analyte stability in stock solutions and in plasma samples under different conditions. The stock solution stability at room temperature and refrigerated conditions (2–8 ^{o}C) was performed by comparing the area response of the analytes (stability samples) with the response of the sample prepared from fresh stock solution. Bench top stability (12 h), processed samples stability (autosampler stability for 51 h, wet extract stability for 27 h and reinjection stability for 44 h), freeze-thaw stability (5 cycles), long-term stability (68 days) were performed at LQC and HQC levels using six replicates at each level. Samples were considered to be stable if assay values were within the acceptable limits of accuracy (85–115%) and precision (r15% RSD).

2.6. Pharmacokinetic study design

A pharmacokinetic study was performed in healthy subjects. The ethics committee approved the protocol and the volunteers were provided with informed written consent. Blood samples were collected following oral administration of of Alogliptine (25mg) and Pioglitazone (45mg) at pre-dose and 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 7, 8, 10, 12, 16, 24, 48, 72 h, in K2-EDTA vacutainer collection tubes (BD, Franklin, NJ, USA). The tubes were centrifuged at 3200 rpm for 10 min and the plasma was collected. The collected plasma samples were stored at 70 ^{0}C till their use. Plasma samples were spiked with the IS and processed as per the extraction procedure described earlier. Along with the clinical samples, the QC samples at low, middle 1, middle 2 and high concentration levels were also assayed in triplicate. Plasma concentration–time profile of Alogliptine, Pioglitazone and hydroxy Pioglitazone was analyzed by non-compartmental method using WinNonlin Version 5.1.

3. RESULTS AND DISCUSSION

3.1. Mass spectrometry

MS parameters were optimized by infusing the standard analyte solution of 100 ng/mL into the mass spectrometer using electrospray as the ionization source and operating in the MRM mode. The signal intensities obtained in positive mode were much higher than those in negative ion mode since the analytes and IS have the ability to accept protons. Protonated form of each analyte and IS, [MþH]þ ion was the parent ion in the Q1 spectrum and was used as the precursor ion to obtain Q3 product ion spectra. The most sensitive mass transition was monitored from m/z 340.40 to 115.90 for Alogliptine, Alogliptine and m/z 344.40 precursor ion to the m/z 115.90 product ion for the Alogliptine IS, m/z 357.30 precursor ion to the m/z 134.20 for pioglitazone and m/z 361.20 precursor ion to the m/z 134.20 product ion for the pioglitazone IS, m/z 373.20 precursor ion to the m/z 150.20 for Hydroxy pioglitazone M IV and m/z 377.20 precursor ion to the m/z 150.20 product ion for the Hydroxy pioglitazone M IV IS. LC-MRM is a very powerful technique for pharmacokinetic studies since it provides sensitivity and selectivity requirements for analytical methods. Thus, the MRM technique was chosen for the assay development.

3.2. Method development

Alogliptine, Pioglitazone and Hydroxy Pioglitazone have different physicochemical properties; it was difficult to set chromatographic conditions that produced sharp peak shape and adequate response. The method development includes mobile phase selection, flow rate, column type and injection volume. Methanol and acetonitrile were tried in different ratio with buffers like ammonium acetate, ammonium formate as well as acid additives like formic acid and acetic acid in varying strength. It was observed that acetonitrile and 10 mM ammonium acetate (pH 3.0 ±0.05) (60:40, v/v) as the mobile phase was most appropriate to give best sensitivity, efficiency and peak shape. Acidic buffer helped to improve the peak shape and spectral response. The use of a short chromatography column Alltima HP C18 HL (50 mm 4.6 mm, 3 mm) helped in the separation and elution of all three compounds in a very short time. Initially the extraction liquid–liquid extraction (LLE) was tried using different organic solvents like ethyl acetate, hexane, dichloromethane, diethyl ether and methyl tert-butyl ether (MTBE) for LLE. The recovery results obtained were consistent for alogliptine with negligible matrix effect but not for pioglitazone. But as the purpose was to develop a simple, quick and inexpensive method, protein precipitation (PP) was tested. The extracts were clear but the recovery was in the range of 70–90% for all the solvents but not reproducible.

Addition of ammonia solution to the plasma samples in different volume ratios helped in obtaining consistent and reproducible response. Precipitation with acetonitrile containing ammonia solution caused the lowest matrix effect with better peak shape compared to other organic solvents. When direct residue of the protein precipitant was injected, the peak shape of alogliptine was unacceptable at lower concentration levels and also matrix effect was high. Hence supernatant was evaporated and the residue was reconstituted with the mobile phase. The method gave clear extracts with minimum matrix effect and quantitative extraction was possible for all the analytes and IS. The mean recoveries were good and reproducible. Moreover, the validation results and subject sample analysis study support this extraction methodology and hence it was accepted in the present study. It is necessary to use an internal standard to obtain high accuracy when HPLC is equipped with MS as the detector. For LC-MS/MS analysis, use of stable isotope-labeled drugs as internal standards proves to be helpful when a significant matrix effect is possible.

3.3. Selectivity

The degree of interference by endogenous plasma constituents with the analytes and IS was assessed by inspection of chromatograms derived from processed blank plasma sample. As shown in Fig. 2, no significant direct interference in the blank plasma traces was observed from endogenous substances in drug-free plasma at the retention time of the analytes.

3.4. Sensitivity

The lowest limit of reliable quantification for the analytes was set at the concentration of the LLOQ. The accuracy at LLOQ concentration were found to be 101.56%, 100.25% and 101.54% for Alogliptine, Pioglitazone and Hydroxy Pioglitazone respectively.

3.5. Extraction efficiency

A simple protein precipitation with acetonitrile proved to be robust and provided cleanest samples. The recoveries of the analytes and the IS were good and reproducible. The mean overall recoveries of Alogliptine, Pioglitazone and Hydroxy Pioglitazone were 98.74 - 105.68%, 100.25-103.76% and101.54 -103.58% respectively.

3.6. Linearity

Nine-point calibration curve was found to be linear over the concentration range of 3.4-250.13 ng/mL for Alogliptine, 15.8-2500.08 ng/mL for Pioglitazone and 6.97-1500 ng/mL for Hydroxy Pioglitazone. After comparing the two weighting models (1/x and 1/x2), a

regression equation with a weighting factor of 1/x2 of the drug to the IS concentration was found to produce the best fit for the concentration–detector response relationship for both the analytes in human plasma.

3.7. Precision and accuracy

The results for intra-day and inter-day precision and accuracy in plasma quality control samples are summarized in Table 1. The intra-day and inter-day precision deviation values were all within 15% of the relative standard deviation (RSD) at low, middle 1, middle 2 and high quality control levels, whereas within 20% at LLOQ QCs level. The intra-day and inter-day accuracy deviation values were all within 100 ± 15% of the actual values at low, middle 1, middle 2 and high quality control level, whereas within 100±20% at LLOQ QCs level. The results revealed good precision and accuracy.

3.8. Stability Studies

In the different stability experiments carried out viz. bench top stability (12 h), autosampler stability (51 h), repeated freeze-thaw cycles (six cycles), reinjection stability (44 h), wet extract stability and long-term stability at -70 0C for 68 days the mean % nominal values of the analytes were found to be within ± 15% of the predicted concentrations for the analytes at their LQC and HQC levels (Table 2,3,4). Thus, the results were found to be within the acceptable limits during the entire validation.

3.9. Pharmacokinetic Study Results

In order to verify the sensitivity and selectivity of this method in a real-time situation, the present method was used to test for Alogliptine, Pioglitazone and Hydroxy Pioglitazone concentrations in human plasma samples collected from healthy volunteers (n=6). The mean plasma concentrations vs time profile of Alogliptine, Pioglitazone and Hydroxy Pioglitazone are shown in Fig. 5. The pharmacokinetic parameters estimated are shown in Table 5.

Table 1: Precision and accuracy of the method for determining Alogliptine, Pioglitazone and Hydroxy Pioglitazone in plasma samples.

Analyte	QC(Concentration added ng/mL)	Intra-batch		Inter-day	
		Mean concentration found(ng/ml)	Accuracy (%)	Mean concentration found(ng/ml)	Accuracy (%)
ALO	3.41	3.50	101.56	3.55	102.13
	9.61	10.01	105.68	10.23	106.24
	45.03	47.23	104.25	46.25	102.87
	150.93	152.36	100.03	150.26	98.24
	249.74	251.47	98.74	252.87	99.47
PIO	15.51	15.67	100.25	14.58	99.27
	44.76	44.94	100.67	45.69	101.57
	449.58	451.24	103.49	452.18	104.28
	1498.53	1500.47	102.59	1499.37	101.72
	2497.58	2499.54	103.76	2498.28	102.45
OH PIO	6.97	7.87	101.54	7.14	100.83
	11.66	12.58	101.78	13.25	102.53
	602.58	604.58	102.89	603.47	101.58
	1122.54	1124.78	102.36	1125.36	103.57
	1498.24	1501.26	103.58	1499.54	102.41

Table 2: Stability samples result for Alogliptine.

Stability test	Alogliptine			
	QC (concentration added (ng/mL)	Mean (ng/mL)	Stability (%)	Precision (% CV)
Aautosampler stability (at 10°C for 54 h)	3.41 249.74	3.54 250.36	100.25 101.05	0.45 1.87
Wet extract stability (at room temperature for 49 h)	3.41 249.74	3.47 250.87	100.17 101.58	2.25 1.49
Bench top stability (19 h)	3.41 249.74	3.87 250.14	102.54 100.85	3.17 1.84
Freeze-thaw stability (five cycles)	3.41 249.74	3.67 251.69	100.18 103.05	2.24 1.51
Reinjection stability (125 h)	3.41 249.74	3.56 251.47	100.54 102.85	1.20 0.41
Long-term Stability (at –70 °C for 30 days)	3.41 249.74	3.45 248.35	100.12 99.84	2.87 1.09

Table 3: Stability samples result for Pioglitazone.

Stability test	Pioglitazone			
	QC (Nominal concentration added (ng/mL)	Mean (ng/mL)	Stability (%)	Precision (% CV)
Aautosampler stability (at 10°C for 54 h)	15.51 2497.58	16.54 2499.36	101.24 101.78	1.41 2.01
Wet extract stability (at room temperature for 49 h)	15.51 2497.58	16.12 2498.57	101.14 101.59	1.12 1.52
Bench top stability (19 h)	15.51 2497.58	15.01 2498.24	100.58 100.41	1.14 2.08
Freeze-thaw stability (five cycles)	15.51 2497.58	15.25 2499.14	100.87 101.57	1.25 1.42
Reinjection stability (125 h)	15.51 2497.58	14.98 2496.15	100.27 100.47	0.45 1.42
Long-term Stability (at –70 °C for 30 days)	15.51 2497.58	15.47 2499.25	101.57 101.64	1.43 1.12

Table 4: Stability samples result for Hydroxy Pioglitazone.

Stability test	Hydroxy Pioglitazone			
	QC (Nominal concentration added (ng/mL)	Mean (ng/mL)	Stabilit y (%)	Precision (% CV)
Aautosampler stability (at 10°C for 54 h)	6.97 1498.24	7.02 1499.36	101.47 100.25	0.47 2.41
Wet extract stability (at room temperature for 49 h)	6.97 1498.24	6.85 1496.24	99.54 98.14	0.11 2.41
Bench top stability (19 h)	6.97 1498.24	6.45 1496.25	98.12 97.12	1.36 1.54
Freeze-thaw stability (five cycles)	6.97 1498.24	7.25 1499.25	102.54 100.14	1.47 1.46
Reinjection stability (125 h)	6.97 1498.24	6.13 1496.25	98.03 98.24	0.64 1.34
Long-term Stability (at –70 °C for 30 days)	6.97 1498.24	7.12 1501.24	101.84 102.41	2.10 1.35

Table 5: Pharmacokinetic parameters of Alogliptine, Pioglitazone and Hydroxy Pioglitazone.

Parameter	Alogliptine	Pioglitazone	Hydroxy Pioglitazone
t_{max} (h)	11.67 ±0.26	13.51±1.45	24.17±0.71
C_{max} (ng/mL)	57.85±8.65	824.127±522.13	628.38±300.02
$t_{1/2}$ (h)	15.01±2.69	7.54±1.87	30.42±6.29
Kel (h^{-1})	0.04±0.01	0.09±0.01	0.02±0.01

Legends to Figures

Figure 1.

Chemical structures of Alogliptine, Pioglitazone and Hydroxy pioglitazone.

Figure 2.

Chromatograms of Alogliptine (A) an LLOQ sample (B) ULOQ sample.

Figure 3.

Chromatograms of Pioglitazone (A) LLOQ sample (B) ULOQ sample.

Figure 4.

Chromatograms of Hydroxy pioglitazone (A) an LLOQ sample (B) ULOQ sample.

Figure 5.

(A) Plasma concentration–time profile of Alogliptine

(B) Plasma concentration–time profile of Pioglitazone

(C) Plasma concentration–time profile of Hydroxy Pioglitazone

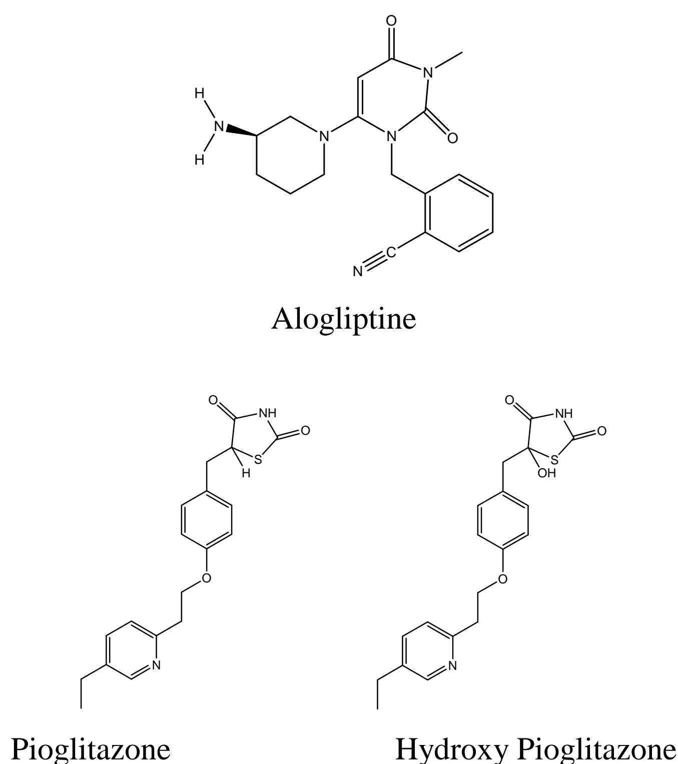
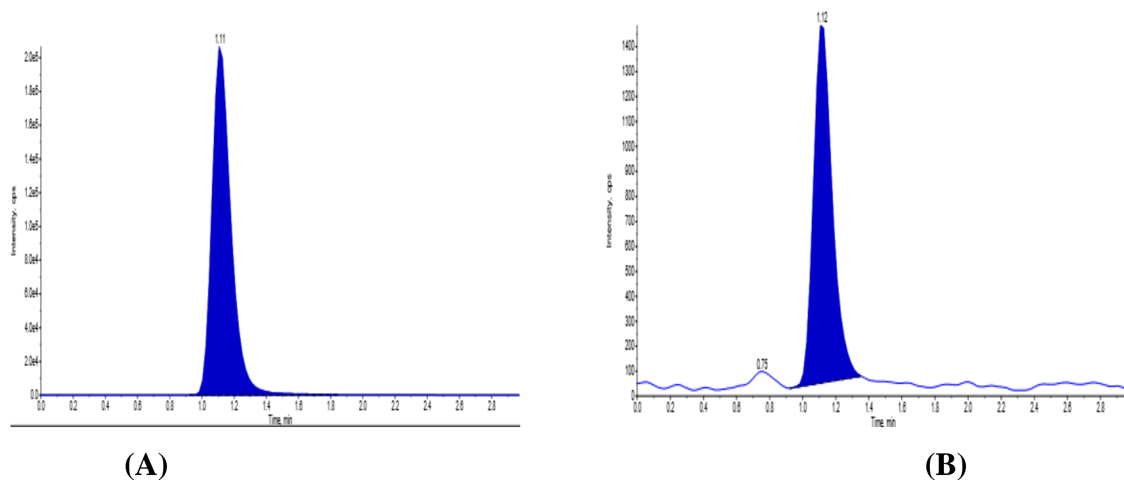
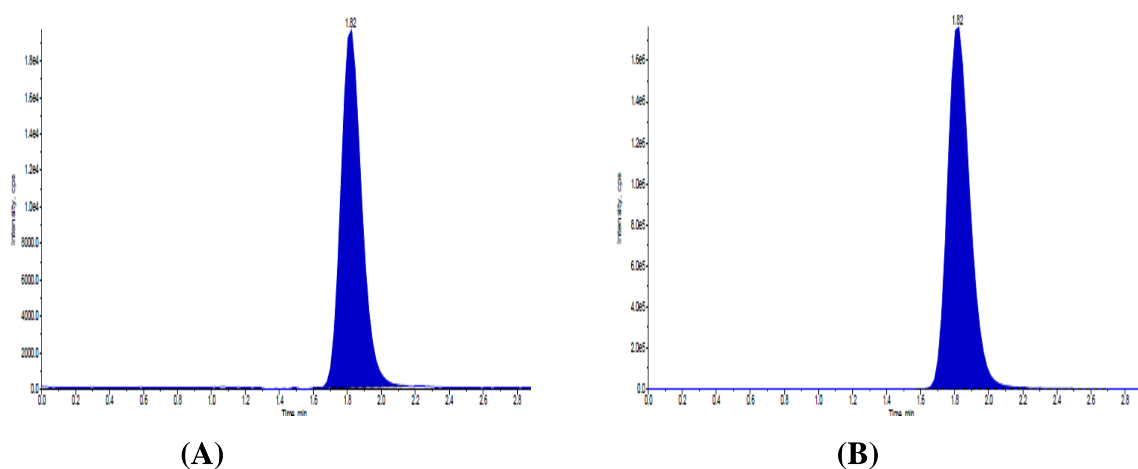


Figure 1.



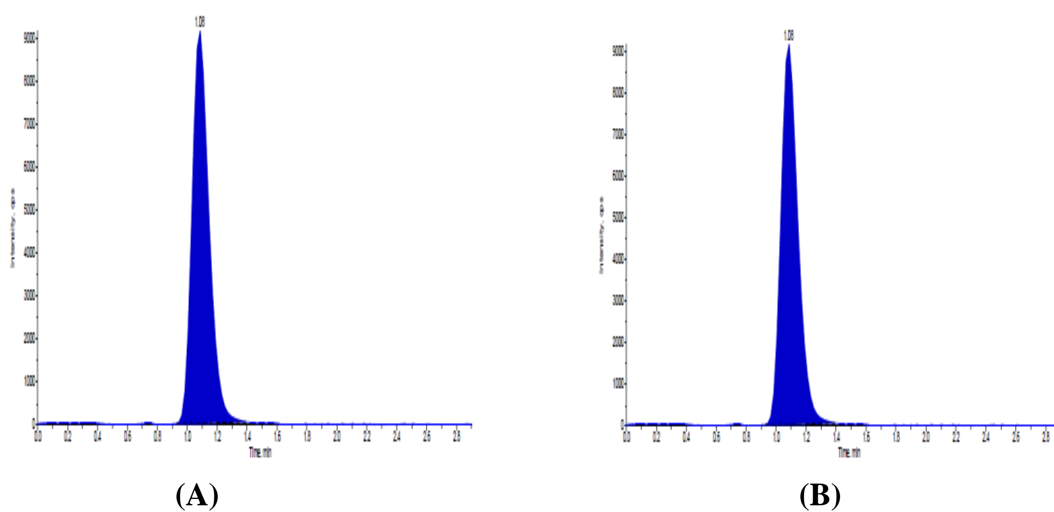
(A) **(B)**

Figure 2.



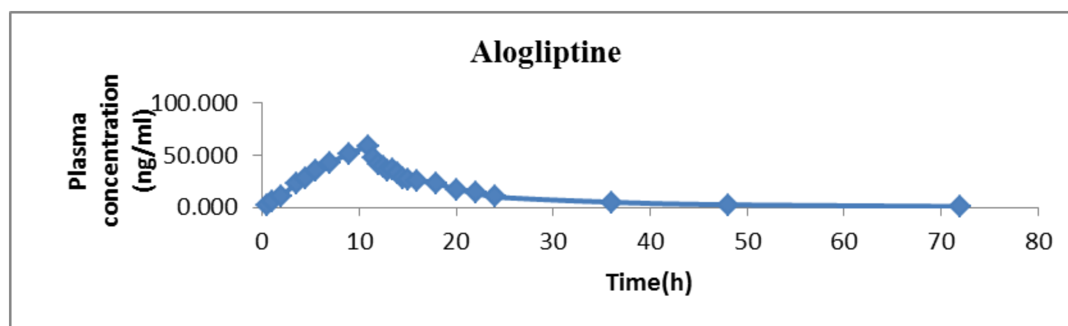
(A) **(B)**

Figure 3.

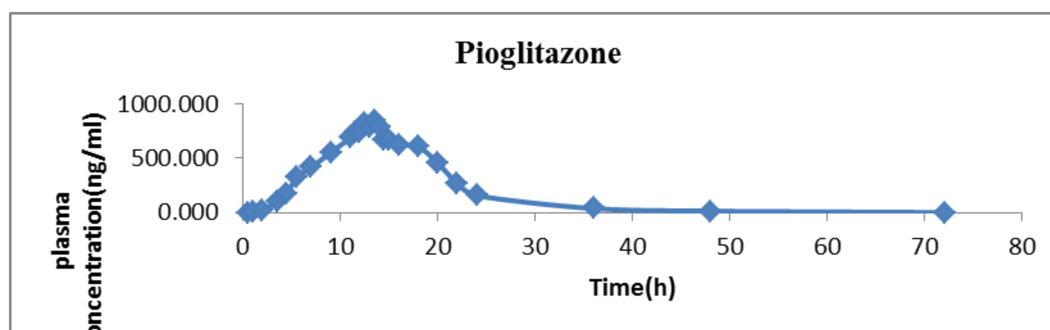


(A) **(B)**

Figure 4.



(A)



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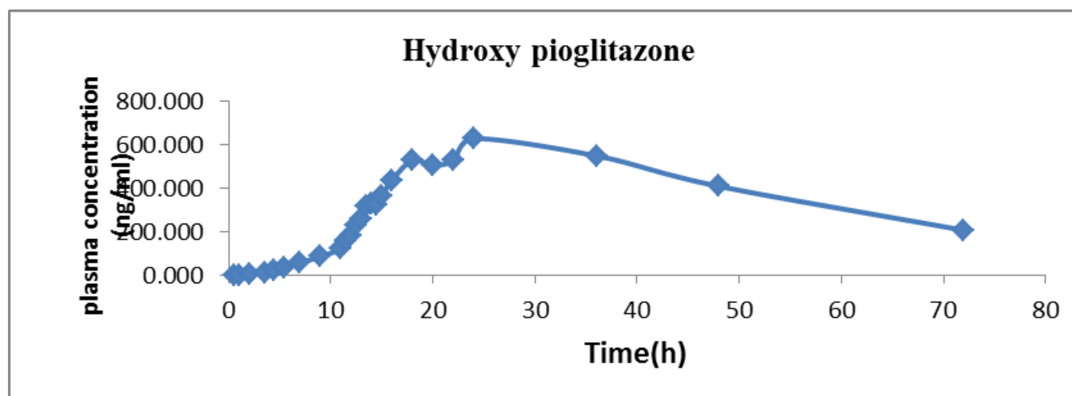

(C)

Figure 5.

CONCLUSIONS

1. Till now no reports are available for the simultaneous quantification of Alogliptine, Pioglitazone and Hydroxy Pioglitazone. Validated methods are essential for the determination of Alogliptine, Pioglitazone and Hydroxy Pioglitazone in human plasma for pharmacokinetic studies.
2. A simple LC-MS/MS method is developed for simultaneous quantification of Alogliptine, Pioglitazone and Hydroxy Pioglitazone in human plasma and validation is performed in according to FDA guidelines.

3. The developed method can be successfully applied to pharmacokinetic studies in healthy subjects with the desired precision and accuracy.

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