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EFFECT OF DIABETES ON THE PHARMACOKINETICS OF AZITHROMYCIN AND CEFIXINE IN RATS

Amol A Raje[1,2], Vishwottam Kandikere[2], Shanmuganathan Seetharaman[1,3], V Gayathri[1] N Malathi[1*]

Affiliations

[1] Sri Ramachandra Institute of Higher Education and Research (DU), Porur, Chennai 600116, India

[2] Syngene International Limited, Biocon Park SEZ, Bommasandra Industrial Area, Bengaluru 56099, India

[3] Current address: School of Pharmacy, Sri Balaji Vidyapeeth (DU), Puducherry 607402, India

Running Title: Effect of diabetes on pharmacokinetics of antibiotics in rats

***Corresponding author**

Dr. N. Malathi,

ORCID ID: 0000-0003-3001-545X

Department of Oral Pathology, Faculty of Dental Sciences, Sri Ramachandra Institute of Higher Education and Research (DU), Porur, Chennai 600116, India. E-mail: malathi.n@sriramachandra.edu.in; Phone91-44-2476 8027; Ext: 8270

ORCID ID of all the authors

- Amol A Raje: 0000-0002-9948-7028
- Vishwottam Kandikere: 0009-0004-5162-1373
- Shanmuganathan Seetharaman: 0000-0003-3735-1560
- V Gayathri: 0000-0001-9764-0140
- N Malathi: 0000-0003-3001-545X

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Abstract

Many times, diabetes and common/respiratory infection are co-morbid disease conditions. Diabetic patients are more pronefor to developing respiratory infections/pneumonia. The expression and levels of CYP450 and drug transporters are known to modulate in diabetic conditions. Azithromycin and cefixime are widely prescribed medicines for respiratory infections. Therefore,the present study was planned to investigate the effect of diabetes on the pharmacokinetics of azithromycin and cefixime using healthy and dibeticrat as animal model. Azithromycin and cefixime were administered by oral administration to healthy (n=4/group) and diabetic rats (n=4/group). Blood samples were collected from all the animals at predetermined time points. All plasma samples were analyzed by using fit-for-purpose LC-MS/MS method developed for quantification of azithromycin and cefixime in rat plasma.

Azithromycin plasma exposure in healthy and diabetic rats were comparable whereas for cefixime diabetic rats showed lower plasma exposure compared to healthy rats. The lower plasma exposure of cefixime in diabetic rats could be due to increased clearance in diabetic rats. The data suggests that azithromycin can be used at the same dose in diabetic patients with that of non-diabetic patients whereas, clinicians need to monitor the therapeutic effect of cefixime in diabetic patients and titre the dose if required.

KeywordsAzithromycin, Cefixime, Pharmacokinetics, STZ-induced diabetic rats.

1. Introduction

Diabetes is a metabolic disorder resulting in high blood glucose level. Approximately 537 million people across the world from age group 20 to 79 are diabetic. This number is expected as 643 million by year 2030 and 783 million by year 2045[(1)]. Diabetes mellitus is associated with many co-morbid disease conditions like hypertension, hyperlipidemia, obesity etc. Approximatley 40% of diabetic populations has at least 2 or 3 comorbid disease condition[(2)]. Common infection is another increasing comorbidty among the diabetic patients. Diabetic patients are at higher rist for pneumonia, bronchitis, chronic obstructive pulmonary disease, pulmonary fibrosis[(3)].Some studies reported that common and rare infections are more prevalent in diabetic population than the normal population. The data pertaining to this is limited and not consistent[(4)]. The respiratory infections are considered as one of the major infections associated with diabetes. High blood glucose levels and increased protein glycosylation found to be associated with microangipathic changes in the lung[(5)]. It is reported that diabetic patients suffer more from the severe respiratory infections than non-diabetic population (52.3% vs 9.4% of patients)[(6)].

Azithromycin and cefiximeare widely prescribed anbacterialsby physicians for the treatment of respiratory infectioins in India. Azithromycin is from macrolide class of antibacterials and inhibits some gram positive and gram negative bacterias[(7)]. Azithromycin has been useful for various infections like middle ear infection, step throat, pneumonia, intestinal infection, skin infection. Azithromycin has high

tissue distribution property and its long elimination half life allows a single large dose once in a day and still maintains the adequate concentrations in the infected tissues for several days[8]. Cefixime is an antibacterial agent from cephalosporin class and is prescribed for various infections like pneumonia, step throat, urinary tract infection, gonorrhea. Both of these medicines are also prescribed for respiratory infections in diabetic patientws. The inflammatory components involved in pathophysiology of diabetes are reported to modulate the functional activity of CYP450 enzymes in an isoform-specific manner[9]. The modulation of the expression and functional activity of CYP450 enzymes and drug transporters (uptake and efflux) in the disease state leads to higher inter-individual variability in drug disposition further causing variability in glycemic control and incidences of adverse drug events in diabetic patients receiving the same treatment options[10]. For effective control and management of infection in diabetic patients, the azithromycin and cefixime doses needs to be accurate and precise. To the best of our knowledge, there were no reports available in the literature about the influence of diabetes on the pharmacokinetics of azithromycin and cefixime. The current study was planned with an objective to evaluate the effect of diabetes on the pharmacokinetics of azithromycin and cefixime in diabetic rats. Rat is preferred animal model used in drug discovery research, we used the same preclinical speicesfor investigations.We belive the STZ induced diabetic rats will mimic the the clinical situation. Hence the outcome of the current study will help physicians to titrate the dose of azithromycin and cefixime in the diabetic patient population.

2. Materials and methods

2.1. Materials

Streptozotocin, Cefixime and azithromycinwere purchased fromBioOrganics, Bengaluru, India. Methylcellulose (400 cP), Verapamil, Tween 80®and formic acid (MS grade), were purchased from Sigma-Aldrich Chemicals Limited, Bangalore, India. HPLC-grade acetonitrileand dimethyl sulphoxide were procured from JT Baker (USA). Isoflurane was procured from Piramal Life Sciences, India.

2.2. Animals

Male Sprague Dawley rats (275±25 g body weight) were obtained from the approved animal vendor. Upon arrival at the facility, rats were quarantined for one week for health monitoring. The rats were maintained in the laboratory for one week before initiation of experimentsunder standard environmental conditions (12h light/dark cycle). All rats received rodent chow and filtered water*ad libitum.* Animals were overnight fasted (10-12h fasting) before dose administration. Feed was offered 4-hours post dose administration. During the fasting period, animals were suppliedwith *ad libitum*water. All animal experiments were approved by the Institutional Animal Ethics Committee(SYNGENE/IAEC/1183-08-2020) and in accordance with the Committee for Control and Supervision of Experiments on Animals (CCSEA), Ministry of Fisheries, Animal Husbandry and Dairying, Government of India. The clinical All the animals were monitored by veterinarian twice daily for presence of signs of distress, pain and adverse clinical signs. Animals with signs of pain and distress were supposed to discontinue from the study.

2.3. Induction of Diabetes

Diabetes mellitus was induced by a single intravenous bolus (50 mg/kg) administration of Streptozotocin (STZ)[11]. The dosing solution was prepared in 0.1 M citrate buffer (pH 4.5) at 25 mg/mL concentration and administered at the dose volume of 2 mL/kg. The blood glucose levels were checked at periodic intervals post-5th Day of STZ administration. The rats showing consistent fasting blood glucose ≥ 200

mg/dL were considered diabetic and selected for the study. The study was performed post-20th Day of STZ administration.

2.4. Pharmacokinetics of Azithromycin and Cefixime in healthy and STZ induced diabetic rats

The study consist of total four groups each containing 4 rats. Group-1 and 2 rats were normal healthy rats whereas Group-3 and 4 rats were STZ-induced diabetic rats. Two days prior to the study, jugular vein cannulation was performed in each rat using the method reported in the literature[(12)]. On the day of the study, rats from Group-1 and 3 rats received Azithromycin (50 mg/kg) by oral administration. Whereas Group-2 and 4 animals received Cefixime (20 mg/kg) by oral administration. The dose volume for all the groups was 10 mL/kg. The formulation vehicle comprised Tween 80®(1% v/v) and methylcellulose (0.5 % w/v) in water q.s. The blood samples (~150 µL) were collected from jugular vein cannula at 0.25, 0.5, 1, 2, 4, 8, 12 and 24 hours post dose administration from all the animals. An equal volume of saline was administered at each blood collection to compensate the volume loss. The blood samples were centrifuged within 15 min of collection and separated plasma was separated into labelled eppendorff tubes and kept below -70 ºC until bioanalysis.

2.5. Analysis of the plasma samples

A rapid, selective, specific discovery grade analytical method was developed on LC-MS/MS for the quantification of azithromycin and cefixime in the rat plasma samples.The analytical system consistedof the NexeraHigh-Performance Liquid Chromatography (HPLC) system (Shimadzu, Japan) connected toan API4500 mass spectrometer (Applied Biosystem/MDS SCIEX Ontario, Canada). The analytical conditions were optimized for gettingadequate resolution, sensitivity, and symmetric peak shapesfor the analytes. For azithromycin, the mobile phase consists of 5 mM ammounium acetate buffer (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B). Synergi polar column (75 × 2.0 mm, 4 µm) was used for the analysis. The gradient analytical method (mobile phase B starting from 5 % to 95 % in 0.8 min withholdtill1.6 min and back to 5 %) with 2.3 minutes run timewas used for analysis. For cefixime, the mobile phase consists of 0.1% Formic acid in water (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B). Phenomenex Kinetex C18 100A° column (100 × 2.1 mm, 5 µm) was used for the analysis. The gradient analytical method (mobile phase B starting from 10 % to 45 % in 1.0 min withhold till 1.3 min, 95% at 1.5 min withhold till 2.5 min, 10% at 2.7 minwith run time with 3.5 minutes run time was used for analysis.The mass spectrometric parameters are given in Table 1. Multiple reaction monitoring (MRM) mode with electrospray ionization with the positive mode of detection was used for quantification of the analyte and internal standard. Peak area ratios of analyte to internal standard from calibration curve samples (CC) were plotted against nominal concentrations of analyte. Chromatograms of drug-free plasma samples, drug-free plasma plus internal standard and lower limit of quantification (LLOQ), and upper limit of quantification (ULOQ) samples from the calibration curve are shown in Figure 1and Figure 2 for azithromycian and cefixime, respectively.

2.6. Sample Preparation

Working dilutions (acetonitrile: water, 1:1 v/v) were prepared from 1 mg/mL DMSO stock solution. The CC and quality control (QC) standards were prepared by spiking working dilutions in plasma samples. The protein precipitation technique was used for extraction. The samples (CC, QCs, and study samples)were extracted with 15-fold volumes of ice-cold acetonitrile containing internal standards (verapamil, 50 ng/mL). After the addition of acetonitrile, all the samples were thoroughly mixed using a

vortexer and centrifuged at 13000 rpm for 5 min at 4 °C. After centrifugation, the supernatant was transferred into a 96-well plate and injected (5 µL) into LC-MS/MS for analysis.

2.7. Pharmacokinetic data analysis

Plasma pharmacokinetic parameters of azithromycin and cefixime from individual animals were calculated by using non-compartmental analysis module of Phoenix®WinNonlin® version 8.3 (Certara Inc, USA). The data is reported as the mean ± SD. A maximum plasma concentration (C_{max}) and time to reach maximum plasma concentration (T_{max}) were reported as observed values. A linear-log trapezoidal method was used for the calculation of the area under the plasma concentration versus the time curve (AUC_{0-t}). The elimination half-life was calculated by using the formula 0.693/Ke. Where Ke is the elimination rate constant calculated by using threetime points in the regression line of the elimination phase.

2.8. Statistical analysis

GraphPad Prism 9 for Windows (GraphPad Software, Inc. San Diego, CA, USA) was used for statistical analysis. The plasma concentration-time profile and pharmacokinetics parameters obtained from healthy and STZ induced diabetic rats were analyzed by using paired t test. For both statistical analyses, the p-value ($p < 0.05$) was considered statistically significant.

3. Results

3.1. Development of fit-for-purpose LC-MS/MS method for quantification of azithromycin and cefixime in rat plasma.

The calibration curve of azithromycin and cefixime in the rat plasma samples was found to be linear from 1 ng/mL to 5000 ng/mL and 2 to 10000 ng/mL respectively. The correlation coefficient (r) was >0.9952 and 0.9951 respectively. A linear regression ($1/x^2$) weighting was used for curve fitting.

3.2. Pharmacokinetics of azithromycin in healthy and STZ induced diabetic rats

All animals tolerated the azithromycin dose levels and did not show any adverse clinical signs. All animals were active and did not show signs of pain and distress during the study period. After oral gavage administration of azithromycin at 50 mg/kg dose in healthy and STZ-induced diabetic rats,the mean plasma C_{max} of azithromycin in healthy and diabetic rats was 955 ± 106 and 1120 ± 73.1ng/mL respectively and the mean plasma AUC_{last} values were 7180 ± 325, and 7110 ± 948h*ng/mL respectively. The median time to achieve maximum plasma concentrations (T_{max}) was around 2hourin healthy rats whereas it was around 0.25 hours in diabetic rats. The volume of distribution (Vd/f), clearance (Cl/f) and elimination half life ($t_{1/2}$) of azithromycin in healthy rats were 96.4 ± 5.93 L/kg, 83.8 ± 3.62 mL/min/kg and 13.3 ± 0.637 h respectively, whereas in STZ induced diabetic rats, the corresponding parameters were 92.6 ± 29.9 L/kg, 95.1 ± 12 mL/min/kg and 11.3 ± 3.18 h respectively(Table 2).The azithromycin concentrations were quantifiable till 24 h post-dose in both groups (Figure 3). The pharmacokinetic parameters of azithromycin in healthy and diabetic rats were comparable ($p < 0.05$).

3.3. Pharmacokinetics of cefixime in healthy and STZ induced diabetic rats

All animals tolerated the cefixime dose levels and did not show any adverse clinical signs. All animals were active and did not show signs of pain and distress during the study period. After oral gavage administration of cefixime at 20 mg/kg dose in healthy and STZ-induced diabetic rats, the mean plasma C_{max} of cefixime in healthy and diabetic rats was 13000 ± 1610 and 14000 ± 901ng/mL respectively and the mean plasma AUC_{last} values were 63800 ± 3270, and 49400 ± 2610 h*ng/mL respectively. The

median time to achieve maximum plasma concentrations (T_{max}) was around 2 hour in healthy and diabetic rats. The volume of distribution (Vd/f), clearance (Cl/f) and elimination half life ($t_{1/2}$) of azithromycin in healthy rats were 1.43 ± 0.42 L/kg, 5.22 ± 0.25 mL/min/kg and 3.15 ± 0.79 h respectively, whereas in STZ induced diabetic rats, the corresponding parameters were 1.56 ± 0.24 L/kg, 6.77 ± 0.37 mL/min/kg and 2.67 ± 0.41 h respectively (Table 2). The azithromycin concentrations were quantifiable till 24 h post-dose in both groups (Figure 4). Although the plasma Cmax of cefixime in healthy and diabetic rats was comparable, the plasma AUClast, and clearance values in diabetic rats were approximately 1.3-fold lower compared to healthy rats ($p<0.01$).

4. Discussion

It is reported that in disease condition, the pharmacokinetic profile of many drugs is modulated leading to adverse drug reactions (ADRs) or suboptimal therapeutic effect[13]. The diabetic patients may havebronchitis, pneumonia, ear, throat, and sexually transmitted diseases. Azithromycin and cefixime are preferred antibacterials by many physicians to treat these disease conditions. Therefore, in the current study, we investigated the pharmacokinetic of azithromycin and cefixime in healthy and diabetic rats. The plasma exposure of Azithromycin was comparable between healthy and diabetic rats suggesting diabetes did not modulate the pharmacokinetic profile of azithromycin in diabetic rats. The volume of distribution of azithromycin in healthy and diabetic rats in the current study is approximately 130-fold higher than total body water in rats. This suggests azithromycin is highly distributed into the tissues. The comparable data between healthy and diabetic rats indicates that diabetic condition did not modulate the high tissue distribution of azithromycin. The azithromycin PK parameters in healthy rats are comparable with that of reported in the literature[14]. Clarithromycin, another macrolide antibiotics, following intravenous and oral administration in alloxan and streptozotocin induced diabetic rats, showed lower plasma exposure than control rats. The lower plasma exposure of clarithromycin in both types of diabetic rats could be because of increased metabolism associated with increased expression and mRNA level of CYP3A23 in diabetic rats[15]. Kim et al reported that the expression and mRNA levels of drug metabolizing enzymes CYP1A2, 2B1/2, 2E1, and 3A23 increased approximately 2 to 3-folds in diabetic rats induced by alloxan and streptozotocin compared to controls[16]. The anotherinvestigational drug- cefixime evaluated in this study showed comparable plasma Cmax in healthy and diabetic rats, however the plasma exposure (AUClast and AUCinf) was approximately 1.3-fold lower in diabetic rats compared to healthy rats. This marginal decrease in plasma exposure could be because of marginal increase in plasma clearance of cefixime in diabetic rats. The pharmacokinetic parameters of cefixime obtained in healthy rats in the current study were comparable with that reported earlier in the literature[17].

5. Conclusions

In the current study, we used healthy and diabetic rats for pharmacokinetic evaluation of widely prescribed antibiotics under the same experimental conditions. The azithromycin plasma exposure were comparable between healthy and diabetic rats. The cefixime plasma exposure in diabetic rats were marginally lower in diabetic rats compared to healthy rats. The data indicates that in diabetic condition, the dose of azithromycin can be similar to that of non-diabetic patients whereas for cefixime, the physicians need to monitore the dose based on the the desired therapeutic effect.

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Tables

Table1: Optimized mass spectrometry parameters of azithromycin and cefixime

Compounds	**Analytes**		**Internal standards**
	Azithromycin	**Cefixime**	**Verapamil**
Precursor ion (m/z) Q1	749.103	453.9	455.3
Product ion (m/z) Q2	591.211	284.9	165.1
Ionization/Polarity	ESI/ Positive	ESI/Positive	ESI/Positive
Dwell time (msec)	50	50	50
Declustering Potential (DP)	90	96	80
Entrance Potential (EP)	10	10	10
Collision Energy (CE)	35	21	24
Cell exit Potential (CXP)	8	10	10
Collision gas (CAD) psi	10	10	10
Curtain gas (CUR) psi	40	40	45
Ion source gas 1 (GS1) psi	55	55	45
Ion source gas 2 (GS2)	50	50	50
TurboIon Spray voltage	5500	5500	5500
Temperature (TEM) ºC	550	550	500

Table 2: Summary of pharmacokinetics parameters of azithromycin following oral gavage administration at 50 mg/kg into healthy and STZ-induced diabetic Sprague Dawley rats.

Groups	**Healthy Normal control**	**Diabetic control**
T_{max} (h)	2 (2.0-2.0)	0.25 (0.25-0.25)
C_{max} (ng/mL)	955±106	1120 ± 73.1^{NS}
AUC_{last} (h*ng/mL)	7180±325	7110 ± 948^{NS}
AUC_{INF} (h*ng/mL)	9990±457	8950 ± 1280^{NS}
Vd/f (L/kg)	96.4±5.93	92.6 ± 29.9^{NS}
Cl/f (mL/min/kg)	83.8±3.62	95.1 ± 12^{NS}
$T_{1/2}$ (h)	13.3±0.637	11.3 ± 3.18^{NS}
T_{last} (h)	24.0 (24.0 -24.0)	24.0 (24.0 -24.0)

Data is represented as Mean ± SD of n=4 animals/group except, T_{max} and T_{last}, where median value is reported with lowest to highest values in parenthesis. Paired t test was applied. NS- Non-significant compared to normal control group,.

Table 3: Summary of pharmacokinetics parameters of cefixime following oral gavage administration at 20 mg/kg into healthy and STZ-induced diabetic Sprague Dawley rats.

Groups	**Healthy Normal control**	**Diabetic control**
T_{max} (h)	2.0 (2.0 – 2.0)	2.0 (2.0 -2.0)
C_{max} (ng/mL)	13000 ± 1610	14000 ± 901^{NS}

AUC_{last} (h*ng/mL)	63800 ± 3270	49400 ± 2610**
AUC_{INF} (h*ng/mL)	64000 ± 3140	49400 ± 2620**
Vd/f (L/kg)	1.43 ± 0.426	1.56 ± 0.24NS
Cl/f (mL/min/kg)	5.22 ± 0.256	6.77 ± 0.372*
$T_{1/2}$ (h)	3.15 ± 0.796	2.67 ± 0.411NS
T_{last} (h)	24.0 (24.0 – 24.0)	24.0 (24.0 – 24.0)

Data is represented as Mean ± SD of n=4 animals/group except, T_{max} and T_{last}, where median value is reported with lowest to highest values in parenthesis. Paired t test was applied. *P<0.05, **P<0.001 compared to healthy normal control group, whereas NS- Non-significant compared to healthy normal control group.,.

Figures

Fig. 1 Representative ion chromatogram of **A)**drug-free plasma**B**) drug-free plasma + Internal standard (verapamil), **C)**lower limit of quantification (LLOQ) of azithromycin, and **D)**upper limit of quantification (ULOQ) of azithromycin from calibration curve samples.

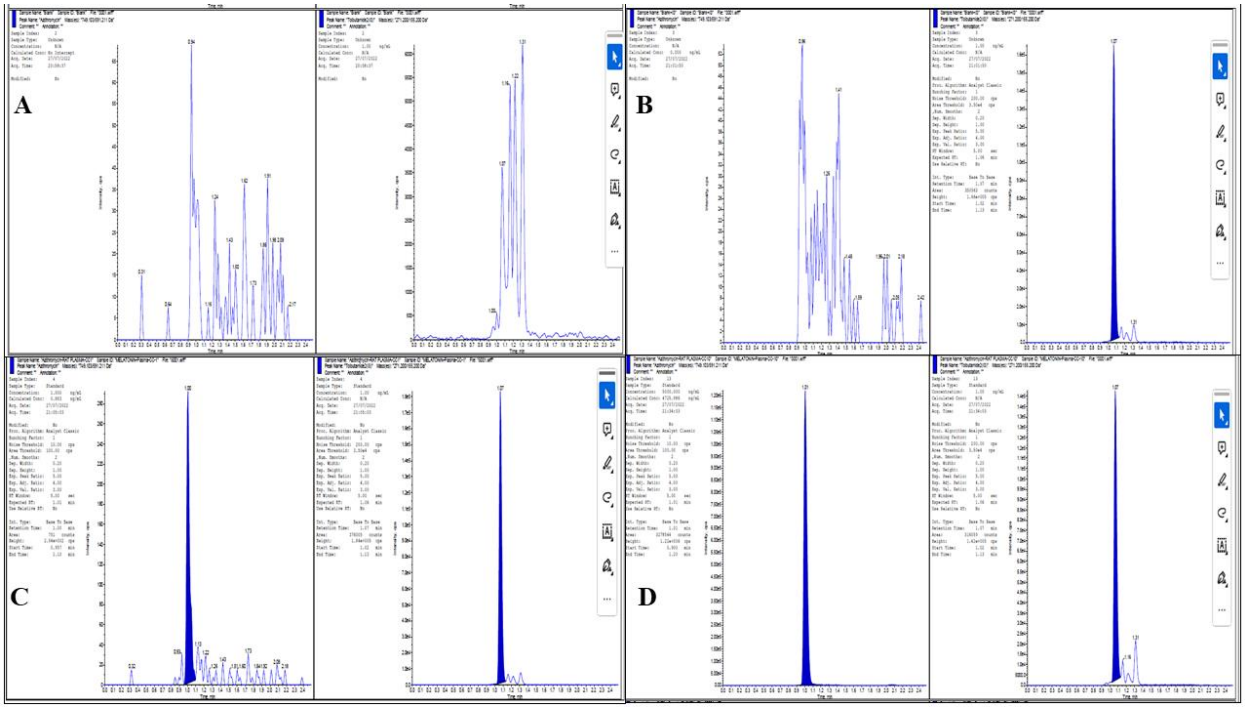

Fig. 2 Representative ion chromatogram of **A)** drug-free plasma **B**) drug-free plasma + Internal standard (verapamil), **C)** lower limit of quantification (LLOQ) of cefixime, and **D)** upper limit of quantification (ULOQ) of cefixime from calibration curve samples.

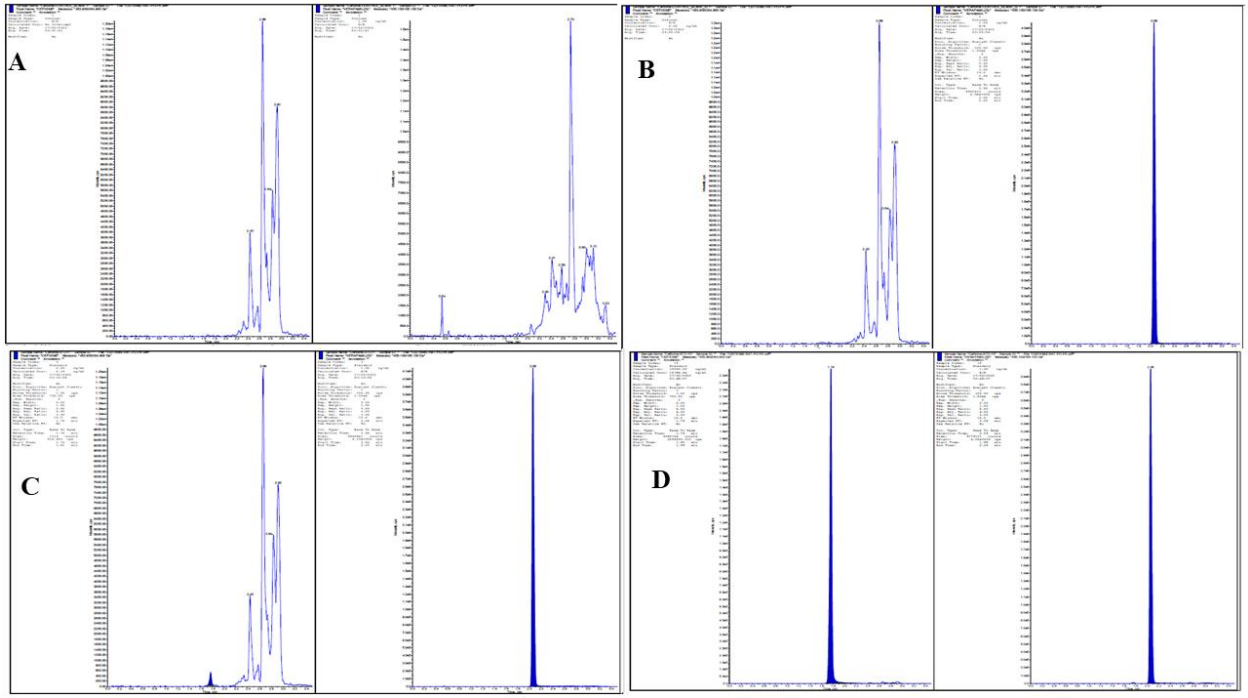

Fig.3 Plasma concentration-time profile of azithromycin following oral administration (50 mg/kg) in healthy and STZ-induced male Sprague Dawley rats.

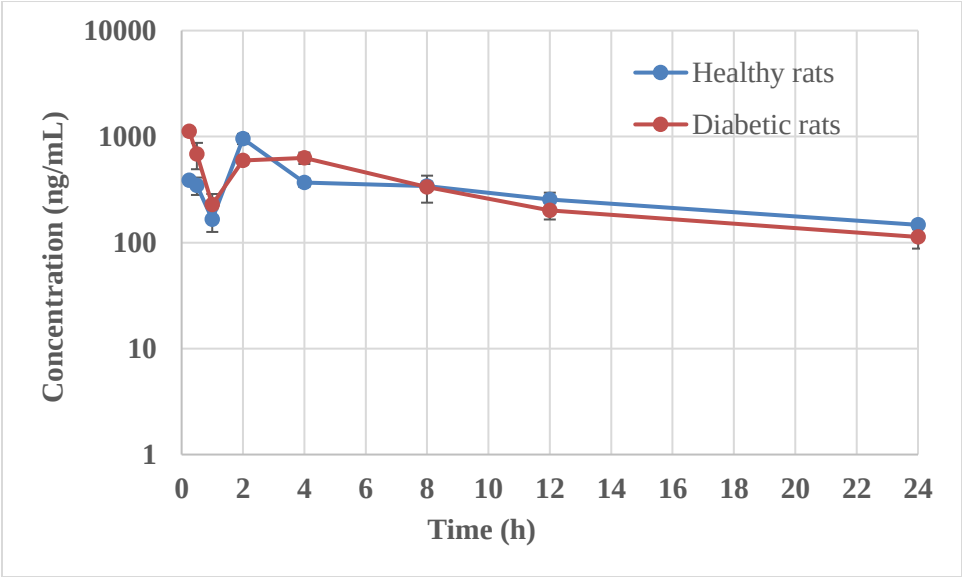

Fig. 4 Plasma concentration-time profile of cefixime following oral administration (20 mg/kg) in healthy and STZ-induced male Sprague Dawley rats.