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INSIGHTS INTO CLOZAPINE'S KINETIC INTERACTIONS: ENZYMATIC INHIBITION OF CYP1A2 BY CIPROFLOXACIN AND NORFLOXACIN

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ABSTRACT. This study aimed to evaluate the kinetic interactions between clozapine (CLZ) and the fluoroquinolone antibiotics ciprofloxacin and norfloxacin using a systematic three-step compartmental modelling approach. Clozapine, primarily metabolized by CYP1A2 and CYP3A4, is known to exhibit altered kinetics when co-administered with fluoroquinolones due to their inhibitory effect on CYP1A2. The proposed models evaluated the absorption, distribution, metabolism, and elimination (ADME) of clozapine and its active metabolite, N-desmethyl clozapine (CLZ-M), under both reference conditions and in the presence of these antibiotics. The selected kinetic models demonstrated a strong correlation between experimental data and predictions ($R^2 > 0.96$), providing robust insights into the mechanisms underlying these interactions. Ciprofloxacin and norfloxacin significantly affected CLZ's presystemic and systemic metabolism, with ciprofloxacin altering relative bioavailability more prominently. These findings emphasize the necessity of dose adjustments for clozapine in clinical practice to mitigate potential adverse effects due to modified drug exposure when co-administered with fluoroquinolones. This study offers a mechanistic framework for understanding complex drug-drug interactions and optimizing dosing strategies in combined therapeutic regimens.

Keywords: *kinetic modelling, drug-drug interaction, preclinical study, clozapine, fluoroquinolone antibiotics.*

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INTRODUCTION

Clozapine (8-chloro-11-(4-methylpiperazin-1-yl)-5H-dibenzo[b,e][1,4]diazepine) is a chemically distinct second-generation antipsychotic. Its unique molecular structure contributes to its wide receptor-binding profile, which includes dopaminergic, serotonergic, and adrenergic receptors, making it particularly effective for managing patients with treatment-resistant schizophrenia. Despite its advantages, clozapine therapy presents significant challenges, including a narrow therapeutic index and a high inter-individual variability in plasma concentration due to its complex kinetics and metabolism [1].

The kinetic behavior of clozapine is mainly directed by its extensive hepatic metabolism mediated by cytochrome P450 enzymes, particularly CYP1A2 and CYP3A4. Other isoforms, such as CYP2C19, and CYP2D6, contribute to a lesser extent to its biotransformation, as supported by kinetic data of expressed enzymes [1-3]. The metabolization processes of clozapine include N-demethylation, hydroxylation, N-oxidation, and conjugation before it is excreted. Clozapine presents two major metabolites, N-desmethylclozapine (or norclozapine), chemically known as 8-chloro-11-(1-methylpiperazin-4-yl)-5H-dibenzo[b,e][1,4]diazepine whose formation is attributed to both CYP1A2 and CYP3A4, and clozapine N-oxide, chemically known as 8-chloro-11-(4-methylpiperazin-1-yl)-5H-dibenzo[b,e][1,4]diazepine N-oxide, whose formation is mainly attributed to CYP3A4 [4-6].

Clozapine presents a rather poor bioavailability after oral administration due to extensive first-pass metabolism, resulting in a low and highly variable systemic bioavailability with an average around 30% [4]. The variability in clozapine metabolism is influenced by factors such as smoking, gender, co-medications, genetic polymorphisms, and drug-drug interactions, particularly those affecting CYP1A2 activity [5].

Ciprofloxacin (1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinolinecarboxylic acid) and norfloxacin (1-ethyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinolinecarboxylic acid) [7,8] are fluoroquinolone antibiotics known to inhibit CYP1A2, the primary enzyme responsible for clozapine metabolism [6]. These interactions can significantly alter the kinetic profile of clozapine, leading to elevated plasma levels, prolonged therapeutic effects, and an increased risk of adverse reactions. From a chemical perspective, these interactions underscore the importance of understanding the molecular mechanisms by which enzyme inhibitors affect the metabolism of clozapine and its metabolites.

Unlike the non-compartmental approach, the compartmental modelling approach provides a valuable tool for describing and quantifying the movement of clozapine and its metabolites within the body [9]. This approach enables the

characterization of CLZ's disposition in the body following oral administration, providing a detailed mechanistic understanding of its absorption, distribution, metabolism, and elimination (ADME) processes [10]. By incorporating kinetic parameters and their modulation by enzyme inhibitors, such as ciprofloxacin and norfloxacin, it is possible to simulate and predict the impact of drug-drug interactions on systemic drug behavior of clozapine.

This study aims to provide a detailed chemical and kinetic analysis of clozapine and its primary metabolite, N-desmethylclozapine, in the presence of ciprofloxacin and norfloxacin. Using experimental data from adult male Wistar rats, a comprehensive kinetic model was developed and used to characterize the interactions at the enzymatic and systemic levels. The results of this study are expected to enhance the understanding of the chemical and molecular insights of these drug-drug interactions and their implications for current practice.

RESULTS AND DISCUSSION

Figure 1 presents the mean plasma concentration-time profiles of CLZ and its primary metabolite, CLZ-M. These graphics highlight the extent of drug exposure following the co-administration of CLZ with fluoroquinolone antibiotics, providing valuable insights into the kinetic interactions and their potential implications for CLZ's metabolism and disposition.

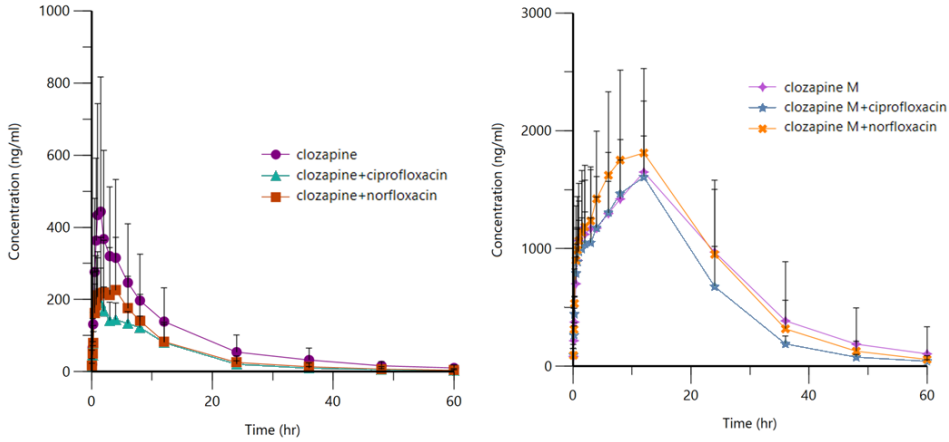

Figure 1. The mean plasma concentration-time profile of clozapine, administered as a single oral dose (20 mg/kg body weight), is depicted under three treatment conditions: as monotherapy (○), after a 6-day pretreatment with ciprofloxacin (15 mg) (Δ), and after a 6-day pretreatment with norfloxacin (30 mg) (□). Data are presented as mean values + standard deviation (left). The corresponding mean plasma concentration-time profile for N-desmethyl clozapine under the same conditions is shown on the right

In the first stage, the focus was on optimizing a model specifically for the parent compound, CLZ, without including the metabolite or potential kinetic interactions. This initial model was aimed and consequently designed to isolate and characterize the kinetic properties of clozapine, establishing a foundational framework for incorporating more complex variables in following stages. A summary of the model parameters evaluated for clozapine kinetics is presented in Table 1.

Table 1. Kinetic models of **clozapine** used in compartmental analysis

Kinetic model	Lag Time	Absorption kinetics	Number of compartments
M1	No	1st order	1
M2	Yes	1st order	1
M3	No	1st order	2
M4	Yes	1st order	2

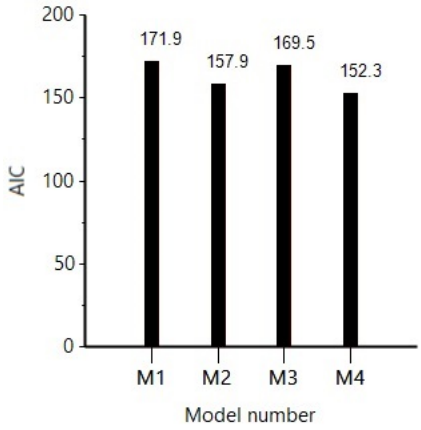

Figure 2. AIC values of M1-4 kinetic models describing the ADME processes of **clozapine** following oral administration

After evaluating multiple models and their respective Akaike Information Criterion (AIC) values, Model 4 (M4) was identified as the most suitable for further analysis. M4 was therefore selected as the baseline for subsequent modelling steps. This model employs first-order absorption kinetics with a lag time and assumes a bi-compartmental distribution for the parent compound, CLZ. The AIC values supporting this selection are illustrated in Figure 2.

Building on M4, four additional models were developed, with their specific differences summarized in Table 2. After re-evaluating AIC values (Figure 3), Model 43 (M43) was determined to be the best representative model.

Table 2. Kinetic models of **clozapine** (CLZ) and **N-desmethyl clozapine** (CLZ-M) assessed during the compartmental modelling approach

Kinetic model	Number of compartments for CLZ-M	Presystemic metabolism	Systemic metabolism	Other elimination routes from central compartment for CLZ
M41	1	No	Yes	Yes
M42	1	Yes	Yes	Yes
M43	2	No	Yes	Yes
M44	2	Yes	Yes	Yes

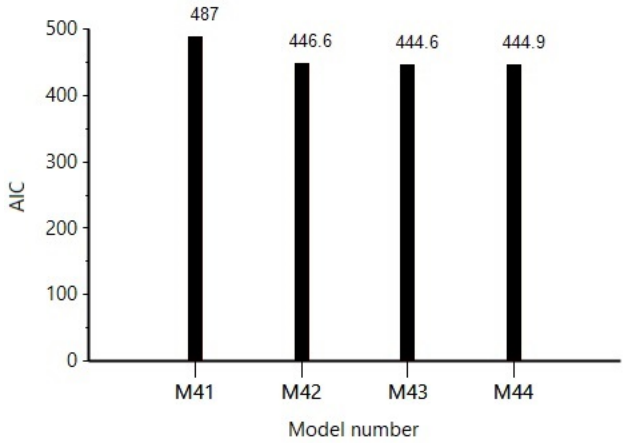

Figure 3. Akaike Index Criteria (AIC) results for the four models describing the kinetics of **N-desmethyl clozapine** after oral administration of clozapine

M43 is characterized by first-order absorption kinetics with a lag time, bi-compartmental distribution for CLZ, no presystemic metabolism, and a bi-compartmental distribution for its primary metabolite, N-desmethyl clozapine (CLZ-M). This preference for M43 over M42 aligns with the fact that most medications exhibit bi-compartmental rather than mono-compartmental distribution patterns.

In the third stage, two parallel models were developed to explore the interaction between CLZ and the selected fluoroquinolones, ciprofloxacin and norfloxacin. The specific differences between these models are detailed in Table 3.

Table 3. Kinetic models of clozapine (CLZ) and N-desmethyl clozapine (CLZ-M) used in compartmental analysis of the interactions with both fluoroquinolone antibiotics

Kinetic model	Number of compartments for CLZ and CLZ-M	Presystemic metabolism	Other elimination routes for CLZ	Relative bioavailability
M431	2	No	No	Same
M432	2	No	No	Different

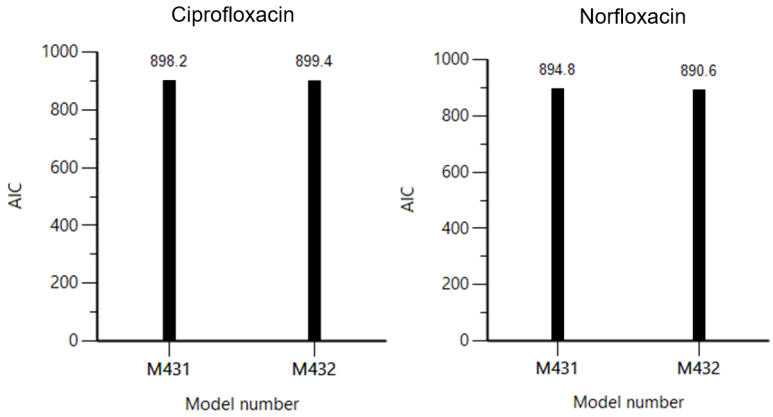

Figure 4. Akaike Index Criteria (AIC) results for the compartmental kinetic modelling of drug-drug interactions between **clozapine** and ciprofloxacin and norfloxacin

Based on the AIC scores (Figure 4), Model 431 (M431) was the best fit for the interaction between CLZ and ciprofloxacin, while Model 432 (M432) proved optimal for CLZ-norfloxacin interaction. Both models assumed first-order absorption kinetics with a lag time for CLZ, bi-compartmental distribution for CLZ and CLZ-M, and the elimination of CLZ exclusively via metabolism to CLZ-M. The distinct feature of M432 is the assumption of a relative bioavailability different than 100%.

Figure 5 illustrates the kinetic processes described by the M43 model, depicting the distribution of CLZ and CLZ-M between compartments, along with their associated kinetic processes and rate constants. This comprehensive framework provides valuable insights into the kinetic behaviours of CLZ and its interactions with the selected fluoroquinolones. Additionally, it offers important details about the processes of absorption, distribution, metabolism, and elimination for both CLZ and CLZ-M.

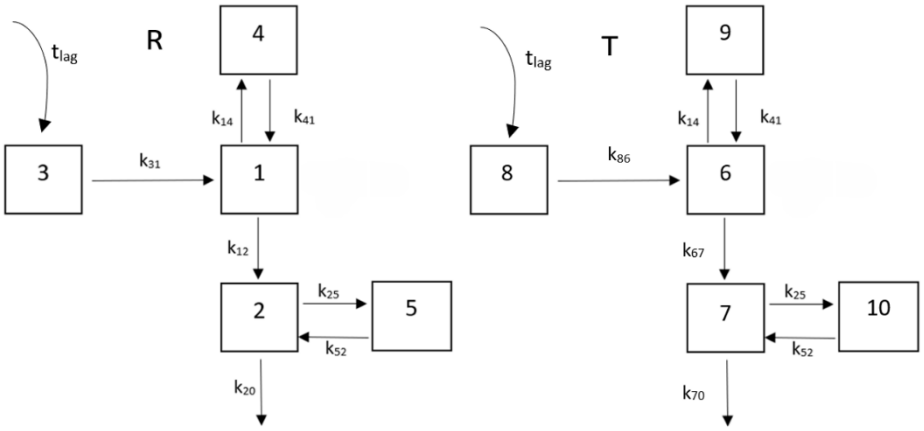

Figure 5. Schematic representation of the kinetic processes in model M43. On the left: "3" denotes the extravascular absorption site; "1" and "2" represent the central compartments for clozapine and N-desmethyl clozapine, respectively, while "4" and "5" are their peripheral distribution compartments. On the right: "8" represents the extravascular absorption site; "6" and "7" correspond to the central compartments for clozapine and N-desmethyl clozapine during the reference period, with "9" and "10" being their respective peripheral compartments during the test periods. The absorption latency time is represented as t_{lag}, and the absorption rate constant of clozapine is k_{31}. Distribution rate constants are k_{14} and k_{41} for the reference period, and k_{25} and k_{52} for the test periods. The systemic metabolization rate constant from clozapine to its metabolite is k_{12}. The elimination rate constants for clozapine (non-metabolic) and N-desmethyl clozapine are k_{20} and k_{70}, respectively, across both reference and test periods.

In this kinetic modelling study, compartmental models were employed to describe the disposition of CLZ and its primary metabolite, N-desmethyl clozapine (CLZ-M), between central and peripheral compartments over time [11,12]. These models are described by partial differential equations that quantify the rate of change in parent drug and metabolite concentration or amount within each compartment, reflecting the kinetic processes of absorption, distribution, metabolism, and elimination (ADME). The equations are based on the principle of mass balance, which mentions that the rate of change within a compartment is determined by the net balance of drug entering and leaving that compartment [13,14].

Rate constants incorporated into these equations provide the mathematical foundation for depicting the time-dependent kinetics of CLZ and CLZ-M across compartments. Specifically, the equations for the M43 kinetic model, used to describe the kinetics of CLZ and CLZ-M during both

the reference period (absence of inhibitors) and test periods (presence of CYP1A2 inhibitors ciprofloxacin or norfloxacin), were formulated using the general mass balance equation:

$$\frac{\partial Ai}{\partial t} = (Rate\ of\ drug\ entering\ the\ compartment) - (Rate\ of\ drug\ leaving\ the\ compartment)$$

where *Ai* denotes the amount of drug in compartment *i*, and *t* represents time.

The complete set of equations underlying the M43 model is presented in Figure 6, offering a detailed depiction of the kinetic processes governing CLZ and CLZ-M under the respective experimental conditions. The results of the kinetic analysis are summarized in Table 4.

$$M43\begin{cases}
\frac{\partial QC_{c1}}{\partial t} = k_{31} * QC_{abs3} * (1 - f_1) - k_{12} * QC_{c1} - k_{14} * QC_{c1} + k_{41} * QC_{p4} \\
\frac{\partial QN_{c2}}{\partial t} = k_{12} * QC_{c1} * 0.997 - k_{20} * QN_{c2} - k_{25} * QC_{c2} + k_{52} * QC_{p5} + k_{31} * f_1 * QC_{abs3} * 0.997 \\
\frac{\partial QC_{abs3}}{\partial t} = -k_{31} * QC_{abs3} \\
\frac{\partial QC_{p4}}{\partial t} = k_{14} * QC_{c1} - k_{41} * QC_{p4} \\
\frac{\partial QN_{p5}}{\partial t} = k_{25} * QN_{c2} - k_{52} * QN_{p5} \\
\frac{\partial QC_{c6}}{\partial t} = k_{31} * QC_{abs8} * (1 - f_2) - k_{67} * QC_{c6} - k_{14} * QC_{c6} + k_{41} * QC_{p9} \\
\frac{\partial QN_{c7}}{\partial t} = k_{67} * QC_{c6} * 0.997 - k_{70} * QN_{c7} - k_{25} * QN_{c7} + k_{52} * QN_{p10} + k_{31} * f_2 * QC_{abs8} * 0.997 \\
\frac{\partial QC_{abs8}}{\partial t} = -k_{31} * QC_{abs8} \\
\frac{\partial QC_{p9}}{\partial t} = k_{14} * QC_{c6} - k_{41} * QC_{p9} \\
\frac{\partial QN_{p10}}{\partial t} = k_{25} * QN_{c7} - k_{52} * QN_{p10}
\end{cases}$$

Figure 6. Mathematical equations for the kinetic model M43 in which $QC_{c1/6}$ and $QC_{p4/9}$ represent the amounts of clozapine in the central and peripheral compartments, respectively, while $QN_{c2/7}$ and $QN_{p5/10}$ denote the amounts of the metabolite (N-desmethyl clozapine) in its central and peripheral compartments. A molar ratio of 0.997 between clozapine and N-desmethyl clozapine was calculated and used as a conversion factor for translating molar units to mass units in the metabolic processes. Additional parameters for the model are provided in the legend of Figure 5.

Table 4. The kinetic parameters of clozapine and **N-desmethyl clozapine** determined with models M431 and M432

Parameter	Unit	M431 – T1	Standard error	M432 – T2	Standard error
f_{rel}	-	-	-	1.2945	0.2559
k_{12}	hr^{-1}	3.7684	1.3288	3.7684	1.0440
k_{14}	hr^{-1}	3.2326	2.2127	4.1156	1.8304
k_{20}	hr^{-1}	1.1346	0.3606	1.2576	0.2186
k_{25}	hr^{-1}	3.4353	0.8887	3.7578	0.8570
k_{31}	hr^{-1}	0.1292	0.0550	0.1292	0.0373
k_{41}	hr^{-1}	0.2437	0.2471	0.3155	0.2361
k_{52}	hr^{-1}	0.3993	0.0720	0.4212	0.0667
k_{67}	hr^{-1}	8.2529	3.1144	6.6553	2.3678
k_{70}	hr^{-1}	0.8666	0.3758	0.7659	0.4752
k_{86}	hr^{-1}	0.1053	0.0317	0.0931	0.0311
t_{lag}	hr	0.0690	0.0170	0.0715	0.0144
V_F	L	1.0434	0.4097	1.1709	0.3100

To model the drug-drug kinetic interaction of CLZ with ciprofloxacin, the best-fitting model selected based on AIC values (M431) assumed no change in CLZ's bioavailability following co-administration with the inhibitor. In this scenario, the extent and rate of absorption, as reflected by the parameters k_{31} and k_{86}, were affected by the interaction (0.1292 hr^{-1} vs. 0.1053 hr^{-1} for the interaction of CLZ with ciprofloxacin; 0.1292 hr^{-1} vs. 0.0931 hr^{-1} for the interaction of CLZ with norfloxacin), as the effect of ciprofloxacin occurred primarily at the metabolic level, impacting both presystemic and systemic metabolism without interfering with the absorption dynamics.

On the other hand, model M432, used to assess the interaction with norfloxacin, assumed a relative bioavailability (f_{rel}) different from 100%, compared to the reference period (monotherapy). Specifically, during the test period with norfloxacin co-administration, f_{rel} was 30% higher than in the reference period (f_{rel}=1.2945). This increase was attributed predominantly to hepatic inhibition of CYP1A2, the primary enzyme involved in CLZ metabolism, and potentially to intestinal inhibition of CYP3A4 during the absorption phase, which plays a lesser but still relevant role. This altered presystemic metabolism during the absorption phase modified CLZ's oral bioavailability. Additionally, the extent and rate of absorption (k_{31} and k_{86}) were slightly impacted (0.1292 hr^{-1} vs. 0.0931 hr^{-1}) due to metabolic-level interactions during presystemic and systemic metabolism.

Throughout the reference and test periods, extensive metabolism of CLZ to CLZ-M was observed, even under enzymatic inhibition of CYP1A2. This is evidenced by supra-unitary values for systemic metabolization constants (k_{12}=3.7684 hr^{-1} during the reference period, k_{67}=8.2529 hr^{-1} for co-administration with ciprofloxacin, and k_{67}=6.6553 hr^{-1} for co-administration with norfloxacin). Notably, the magnitude of interaction was greater with ciprofloxacin than norfloxacin, as indicated by the higher k_{67} value for ciprofloxacin.

Further analysis of the metabolization rate constants for CLZ-M between study periods (k_{20} for the reference period and k_{70} for the test periods) revealed decreased elimination of CLZ-M during the test periods (k_{20}=1.1346 hr^{-1} vs. k_{70}=0.8666 hr^{-1} for ciprofloxacin and k_{20}=1.2576 hr^{-1} vs. k70 = 0.7659 hr^{-1} for norfloxacin co-administration). This reduction is likely due to the involvement of a metabolic pathway in CLZ-M elimination, which becomes saturated at higher metabolite concentrations during the test periods. This assumption is further supported by alterations in metabolism rate constants.

The graphical outputs from fitting model M431 for the interaction between CLZ and ciprofloxacin, and model M432 for the interaction with norfloxacin, are presented in Figures 7 and 8, respectively. These figures highlight the dynamics of absorption, distribution, and metabolism, providing a visual representation of the kinetic interactions under each test condition.

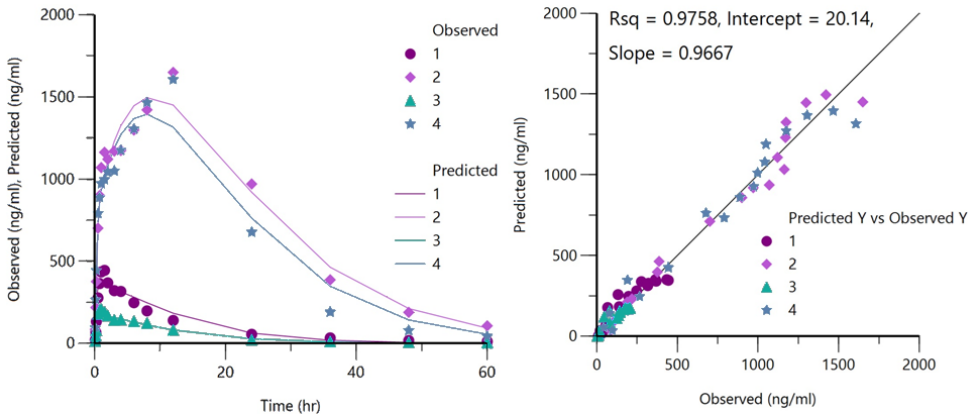

Figure 7. The kinetic fitting of model M431 during the first test period is shown on the left, along with the correlation between experimental and fitted values on the right. In the model, "1" represents **clozapine** in the central compartment during the reference period, "2" denotes **N-desmethyl clozapine** in the central compartment during the reference period, "3" represents **clozapine** in the central compartment during the first test period (with ciprofloxacin), and "4" corresponds to **N-desmethyl clozapine** in the central compartment during the first test period.

The experimental data align closely with the model predictions, as evidenced by the high correlation coefficients (R^2). For the CLZ-alone and CLZ-ciprofloxacin dataset, the R^2 value is 0.9667 (Figure 7), while for the CLZ-alone and CLZ-norfloxacin dataset, the R^2 value is 0.9725 (Figure 8). These values reflect a high level of correlation between the experimental observations and the model's predictions, underscoring the robustness and reliability of the kinetic model.

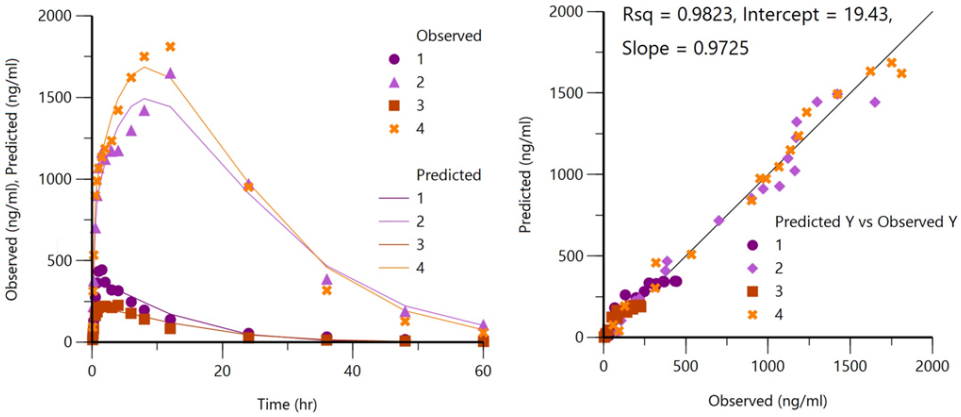

Figure 8. The kinetic fitting of model M432 during the second test period is displayed on the left, alongside the correlation between experimental and fitted values on the right. In this model, "1" represents **clozapine** in the central compartment during the reference period, "2" denotes **N-desmethyl clozapine** in the central compartment during the reference period, "3" represents clozapine in the central compartment during the second test period (with norfloxacin), and "4" corresponds to **N-desmethyl clozapine** in the central compartment during the second test period.

CONCLUSIONS

This study presents a comprehensive kinetic modelling framework for clozapine (CLZ) and its primary metabolite, N-desmethyl clozapine (CLZ-M), focusing on the interactions between CLZ and the fluoroquinolone antibiotics ciprofloxacin and norfloxacin. By employing a systematic three-tier modelling approach, the kinetic behaviour of CLZ and CLZ-M was elucidated across reference and test conditions, incorporating key processes such as absorption, distribution, metabolism, and excretion (ADME).

In the first step, compartmental modelling focused exclusively on the parent compound, clozapine, as its kinetics influence the kinetics of its metabolite, but not vice versa. This stage established the foundational kinetics of CLZ using a bi-compartmental model with first-order absorption and lag time.

In the second step, the metabolite was incorporated into the model, enabling simultaneous fitting of two datasets (clozapine and its metabolite) while accounting for additional parameters such as metabolite distribution and the effects of presystemic and systemic metabolism.

The third step involved simultaneous fitting of four datasets, considering the kinetic interactions between clozapine and ciprofloxacin or norfloxacin. This step addressed changes in presystemic and systemic metabolism mediated by CYP1A2 enzymatic inhibition, as well as variations in the relative bioavailability of clozapine between the reference and test periods due to changes in the extent of absorption. This final step also integrated the metabolite's kinetics, also altered by the assessed drug-drug interactions.

The selected models demonstrated excellent predictive accuracy, with high correlation coefficients ($R^2 > 0.96$) between experimental data and model predictions, underscoring the robustness of the proposed approach.

Key findings highlight the significant impact of ciprofloxacin and norfloxacin on CLZ kinetics, including altered presystemic and systemic metabolism and changes in bioavailability. These results provide critical insights into the molecular and kinetic interplay between CLZ and fluoroquinolones, offering valuable guidance for optimizing dosing strategies in clinical settings where these drugs are co-administered.

This rational and mechanistic modelling framework not only advances the understanding of CLZ-fluoroquinolone interactions but also sets a precedent for investigating complex kinetic scenarios involving metabolic inhibitors. The findings have significant implications for both clinical practice and future research into drug-drug interactions.

EXPERIMENTAL SECTION

Chemical and reagents

Clozapine (Leponex®) for animal administration was purchased from Mylan (Hatfield, Hertfordshire, UK), while analytical standards for clozapine and N-desmethyl clozapine used in LC-MS analysis were purchased from Sigma-Aldrich/Merck Group (Darmstadt, Germany). Ciprofloxacin (Ciprinol®) and norfloxacin (Nolicin®) were acquired from KRKA (Novo Mesto, Slovenia). For animal anesthesia, ketamine (Vetased®) was purchased from Farmavet (Romania), xylazine (XylazinBio®) from Bioveta (Czech Republic), and diazepam from Terapia (Cluj-Napoca, Romania). Heparin sodium (5000 IU/mL) was obtained from Belmedpreparaty (Minsk, Belarus). Analytical-grade formic acid and methanol were purchased from Merck (Darmstadt, Germany), and carboxymethyl cellulose was acquired from Sigma-Aldrich (Taufkirchen, Germany).

Study design

This study was approved by the local Ethics Committee of the "Iuliu Haţieganu" University of Medicine and Pharmacy, Cluj-Napoca, Romania, and the National Sanitary Veterinary and Food Safety Authority, in compliance with Romanian Law 43/2014, which governs the protection of animals used for scientific purposes. The legislation aligns with Directive 2010/63/EU of the European Parliament and Council, dated 22 September 2010, as published in the Official Journal of the European Union. Ethics approval was granted under reference number 313, dated 20 May 2022. The study was conducted at the Centre for Experimental Medicine and Practical Skills, Cluj-Napoca, Romania, using an open-label, three-period sequential design.

The experimental design involved three periods and involved the use of 16 white male Wistar rats, for each study period, weighing between 240 and 415 g. In the initial reference period, the rats received a single oral dose of clozapine (20 mg/kg body weight, b.w.). In the first test period, the animals were pretreated with 15 mg of ciprofloxacin administered orally for five days in order to reach the steady-state plasma concentration for this enzymatic inhibitor, followed by a combination of ciprofloxacin and clozapine (20 mg/kg b.w.) on the sixth day. In the second test period, the same protocol was followed, replacing ciprofloxacin with a 30 mg dose of norfloxacin. Both clozapine and norfloxacin were suspended in 1% carboxymethylcellulose and vortexed for 5 minutes before oral administration by intragastric gavage.

Sample preparation

To determine the plasma concentration of each analyte, namely the parent drug clozapine (CLZ) and its metabolite (CLZ-M), venous blood samples were collected from each rat during each study period. Cannulation of the left femoral vein was performed one day before clozapine administration under general anesthesia induced by a combination of ketamine, xylazine, and diazepam (1:1:1) via intramuscular injection. This procedure was done for each rat and allowed for automated blood sampling using the BASi Culex ABC® system (BASi Research Products, West Lafayette, IN, USA), ensuring consistency and reducing variability.

Blood samples (200 µL each) were collected at multiple time points: 5, 10, 15, 30, and 45 minutes; 1, 1.5, 2, 3, 4, 6, 8, 12, 24, 36, 48, and 60 hours after clozapine administration. Samples were stored at -20°C until analysis. Sample preparation involved mixing 60 µL of blood with 180 µL of methanol, vortexing for 10 seconds, and centrifuging at 10,000 rpm for 8 minutes. The resulting supernatant was transferred to autosampler vials for quantification via HPLC-MS.

Drug analysis from plasma samples

Clozapine and its active metabolite concentrations in rat plasma samples were simultaneously determined using a validated liquid chromatography-tandem mass spectrometry (LC-MS/MS) method. The HPLC system used was an Agilent 1100 series, featuring a binary pump, autosampler, and thermostat (Agilent Technologies, Santa Clara, CA, USA), coupled to an Agilent Ion Trap 1100 SL mass spectrometer. Chromatographic separation was achieved on a Zorbax SB-C18 column (100 mm x 3.0 mm i.d., 3.5 µm) (Agilent Technologies, Santa Clara, CA, USA). The mobile phase consisted of 0.3% formic acid in water (v/v) and methanol in a 68:32 ratio, with isocratic elution maintained for 3 minutes. The injection volume was set to 3 µL, with a flow rate of 1 mL/min, and the column temperature was kept at 45°C. Under these chromatographic settings, the retention times were 2.4 min for clozapine and 1.9 min for its metabolite. Mass spectrometric detection utilized multiple reaction monitoring (MRM) mode with an electrospray ionization source in positive ion mode. The monitored mass transitions were m/z 270 from m/z 328 for clozapine and m/z (253, 270) from m/z 314 for its metabolite. Calibration curves for both clozapine and its metabolite were linear within the concentration range of 5 to 1000 ng/mL.

Kinetic and statistical analysis of data

A three-tiered modelling strategy was utilized to manage the extensive number of variables, which would otherwise result in numerous model combinations, making computation infeasible.

In the first stage, the focus was on optimizing a model specifically for the parent compound, CLZ, without including the metabolite or potential kinetic interactions. This initial model was aimed and consequently designed to isolate and characterize the kinetic properties of clozapine, establishing a foundational framework for incorporating more complex variables in following stages. A summary of the model parameters evaluated for clozapine kinetics was presented in Table 1.

Figure 9 depicts the three-tier modelling approach used to further analyze the kinetics of clozapine's primary metabolite, N-desmethyl clozapine (CLZ-M), as well as the combined kinetics of CLZ and CLZ-M in the context of drug-drug interactions with ciprofloxacin and norfloxacin.

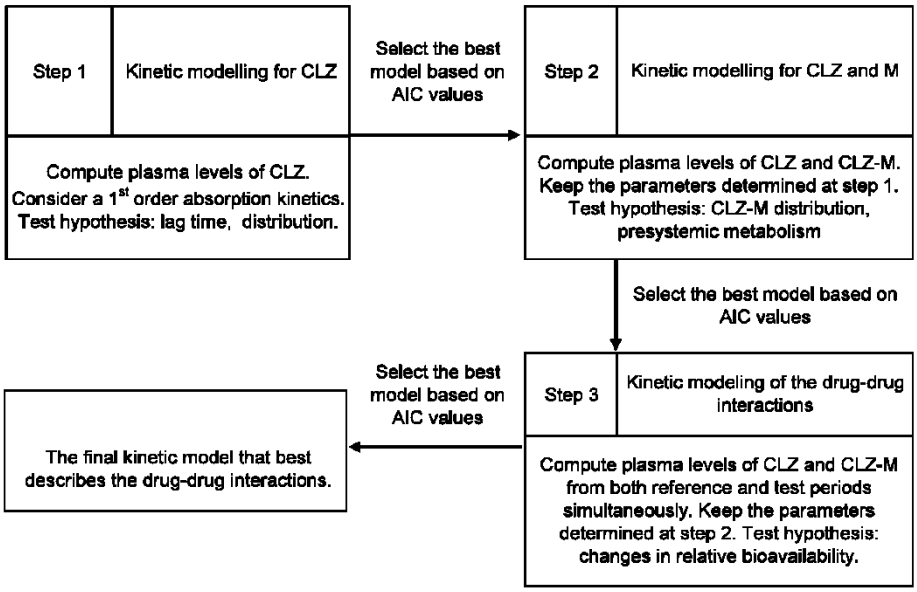

Figure 9. The three-tier framework utilized for kinetic modelling of clozapine (CLZ), its primary metabolite (N-desmethyl clozapine, CLZ-M), and the drug-drug interactions involving clozapine and the fluoroquinolone antibiotics ciprofloxacin and norfloxacin.

The Akaike Information Criterion (AIC) was employed as the primary method for model selection and evaluation to determine the kinetic model that best fit the experimental data [15]. AIC considers the number of observations, the quality of the fit, and model complexity. It is calculated using the formula:

$$AIC = m * \ln(WSSR) + 2 * p,$$

where m represents the number of observations, WSSR is the weighted sum of squares of residuals, and p is the number of structural parameters in the model [11-14,16]. A lower WSSR value indicates an improved fit, which results in a lower AIC score and a better overall fit to the data [16].

The AIC disadvantages models with more parameters, as its final value increases proportionally with p. Therefore, when comparing two models with similar fit quality (e.g., identical WSSR values), the model with fewer parameters will yield a lower AIC and be considered the better choice. Overall, a lower AIC value signifies a superior fit, provided the same data and error assumptions apply across the models [11,14].

CONFLICT OF INTEREST

The authors declare no conflict of interest regarding the publication of this research article.

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