European Journal of Pharmaceutical Sciences 93 (2016) 319–333

Contents lists available at ScienceDirect

European Journal of Pharmaceutical Sciences

journal homepage: www.elsevier.com/locate/ejps

Population pharmacokinetics, optimised design and sample size determination for rifampicin, isoniazid, ethambutol and pyrazinamide in the mouse

Chunli Chen [a,*], Fatima Ortega [b], Laura Alameda [b], Santiago Ferrer [b], Ulrika S.H. Simonsson [a]

[a] Department of Pharmaceutical Biosciences, Uppsala University, Biomedicinskt Centrum (BMC), Box 591, SE-751 24 Uppsala, Sweden
[b] GlaxoSmithKline, Diseases of Developing World (DDW), Medicines Development Campus, Severo Ochoa 2, 28760, Tres Cantos, Madrid, Spain

ARTICLE INFO

Article history:
Received 22 February 2016
Received in revised form 19 June 2016
Accepted 25 July 2016
Available online 26 July 2016

Keywords:
Pharmacokinetics
Design
Rifampicin
Isoniazid
Ethambutol
Pyrazinamide

ABSTRACT

The current first-line therapy for drug-susceptible tuberculosis consists of rifampicin (RIF), isoniazid (INH), pyrazinamide (PZA) and ethambutol (EMB). In this study, we determined the population pharmacokinetics (PopPK) of RIF, INH, EMB and PZA using original experimental sampling designs for single-dose intravenous (IV) and single- and multiple-dose oral administration studies in the mouse model, and used these PopPK models to develop and evaluate new, more informative sampling designs with the aim of reducing the number of animals required for each drug.

The RIF, INH, EMB and PZA blood concentrations after single oral and IV doses and multiple-dose oral administrations based on the original designs were used in the PopPK analysis using NONMEM software. The final PopPK models described the data well. Stochastic simulation and estimation were used to optimise the designs. The relative bias and relative imprecision of each pharmacokinetic parameter for each drug were derived and assessed to choose the final designs. The final single-dose IV and oral designs included up to eight samples per mouse with a total of 24 mice required for RIF and EMB and 33 mice for INH and PZA. In the new multiple-dose (zipper) oral designs, the mice were divided into two groups of three per dose, and four samples were taken from each mouse to cover all seven or eight sampling time points. The final number of mice required for the multiple-dose oral designs was 30 for RIF, INH and EMB, 36 for PZA. The number of mice required in the new designs for RIF, INH and EMB was decreased by up to 7-fold and the relative bias and relative imprecision in the parameter estimates were at least similar to those in the original designs.

1. Introduction

Tuberculosis (TB), an infectious disease caused by *Mycobacterium tuberculosis* (*M. tuberculosis*) which most commonly affects the lungs (pulmonary TB) but can also affect other organs (extra-pulmonary TB), is a major global health problem. The current first-line therapy for drug-susceptible TB consists of rifampicin (RIF), isoniazid (INH), ethambutol (EMB) and pyrazinamide (PZA). The treatment period of 6 months for drug-susceptible TB is associated with a risk of poor compliance, the occurrence of drug resistance, a high risk of relapse and treatment failure. It would therefore be preferable if the treatment duration for new therapies was reduced. Identification of new therapies is dependent on various animal models such as mouse, rabbit and guinea pig. Of these species, the mouse is the primary animal species used for pre-clinical anti-TB drug development because of ease of handling, low costs and susceptibility to *M. tuberculosis*.

Pharmacometrics is an emerging science that quantitatively analyses the pharmacokinetics (PK) and pharmacodynamics (PD) of drugs, i.e. their efficacy and safety. In drug development, it is important to establish exposure-response relationships, preferably for both efficacy and safety, at an early stage. If these relationships are defined early in the process, during pre-clinical animal studies or *in vitro* studies using biomarkers, the information can be used to define the likely dose range for use in Phase II studies in humans. Pharmacokinetic data are essential for defining these relationships, regardless of the stage of the drug in development. Nonlinear mixed effects modelling is employed in the so-called population approach in pharmacometric analysis. It also reliably estimates inter-individual variability (IIV) and inter-occasion variability (IOV), and separates these from residual variability. Pharmacokinetic and pharmacodynamic experimental designs need to be optimised in order to reduce

* Corresponding author.
E-mail address: chunli.chen@farmbio.uu.se (C. Chen).

http://dx.doi.org/10.1016/j.ejps.2016.07.017

Table 1
Summary of original and new study designs for pharmacokinetic characterisation of rifampicin (RIF) in the mouse model. The number of doses and animals, blood-sampling time points, number of samples/animal and number of observations (animals used)/time point for the original designs, the new single-dose intravenous (IV) and oral (PO) designs and the new zipper design for multiple-dose PO administrations are given. The light grey numbers show the identifying number for each animal used in the design to illustrate the time point at which the sample was taken from that animal.

RIF	Original designs									New designs								
Sampling time point (hours post-dose)	Single-dose IV			Single-dose PO			Multiple-dose PO			Single-dose IV			Single-dose PO			Multiple-dose PO (zipper)[a]		
0.08	1	2	3	1	2	3	-	-	-	1	to	9	1	2	3	1	3	5
0.25	4	5	6	4	5	6	-	-	-	-	-	-	-	-	-	-	-	-
0.5	7	8	9	7	8	9	1[b]	2[b]	3[b]	1	to	9	1	2	3	2	4	6
0.75	-	-	-	10	11	12	-	-	-	-	-	-	-	-	-	-	-	-
1	10	11	12	13	14	15	-	-	-	1	to	9	1	2	3	1	3	5
1.5	13	14	15	16	17	18	-	-	-	1	to	9	1	2	3	2	4	6
2	16	17	18	19	20	21	-	-	-	1	to	9	1	2	3	1	3	5
3	19	20	21	22	23	24	-	-	-	1	to	9	1	2	3	2	4	6
4	22	23	24	25	26	27	1[b]	2[b]	3[b]	-	-	-	-	-	-	-	-	-
8	25	26	27	28	29	30	1[b]	2[b]	3[b]	1	to	9	1	2	3	1	3	5
24	-	-	-	-	-	-	1[b]	2[b]	3[b]	1	to	9	1	2	3	2	4	6
Doses	1			5			1			1			5			5		
Animals/dose	27			30			3			9			3			6		
Total no. animals	180									24/30(+9[a])								
Samples/animal/day	1			1			4			8			8			4		
Animals/time point	3			3			3			9			3			3		

[a] Data from the new single-dose IV design were analysed simultaneously with the new multiple-dose PO data to obtain steady-state PK results.
[b] Samples were taken at these times each day for three days.

sample sizes and costs and to make the best use of the information collected. This is preferably done using pharmacometric models and techniques, as shown in this work.

The objectives of this work were to describe the population pharmacokinetics (PopPK) of RIF, INH, EMB and PZA in the mouse using blood concentrations, and to optimise the PK sampling designs for these anti-TB drugs using a PopPK model for each drug, while aiming to reduce the number of animals required in PopPK studies.

2. Material and methods

2.1. Chemicals

All chemicals and reagents were obtained from Sigma-Aldrich (Spain). Water was purified and de-ionised using a Water Purified System (Milli-Q water). The drugs were administered in the following vehicles: RIF in 20% encapsin/80% saline for both the IV and oral routes; INH in Milli-Q water for both the IV and oral routes; EMB and PZA in saline for the IV route and 1% methyl cellulose for the oral route.

2.2. Pharmacokinetic study in mice

Healthy C57BL/6 mice (8–10 weeks old, Harlan Laboratories) were divided into four groups with similar average weights (18.8 g) and each group was administered RIF, INH, EMB or PZA in solution via oral gavage (20 mL/kg) or IV bolus injection (10 mL/kg). Rifampicin was given as a single IV dose (12 mg/kg), a single oral dose (1, 3, 10, 30 or 100 mg/kg) or multiple oral doses (10 mg/kg/day) for three days. Isoniazid was given as a single IV dose (10 mg/kg), a single oral dose (0.2, 0.5, 1, 5 or 25 mg/kg) or multiple oral doses (25 mg/kg/day) for three days.

Table 2
Summary of original and new study designs for pharmacokinetic characterisation of isoniazid (INH) in the mouse model. The number of doses and animals, blood-sampling time points, number of samples/animal and number of observations (animals used)/time point for the original designs, the new single-dose intravenous (IV) and oral (PO) designs and the new zipper design for multiple-dose PO administrations are given. The light grey numbers show the identifying number for each animal used in the design to illustrate the time point at which the sample was taken from that animal.

INH	Original designs									New designs								
Sampling time point (hours post-dose)	Single-dose IV			Single-dose PO			Multiple-dose PO			Single-dose IV			Single-dose PO			Multiple-dose PO (zipper)[a]		
0.08	1	2	3	1	2	3	-	-	-	1	to	18	1	2	3	1	3	5
0.25	4	5	6	4	5	6	-	-	-	1	to	18	1	2	3	2	4	6
0.5	7	8	9	7	8	9	1[b]	2[b]	3[b]	1	to	18	1	2	3	1	3	5
0.75	10	11	12	10	11	12	-	-	-	1	to	18	1	2	3	2	4	6
1	13	14	15	13	14	15	-	-	-	1	to	18	1	2	3	1	3	5
1.5	16	17	18	16	17	18	-	-	-	-	-	-	-	-	-	-	-	-
2	19	20	21	19	20	21	-	-	-	1	to	18	1	2	3	2	4	6
3	22	23	24	22	23	24	-	-	-	-	-	-	-	-	-	-	-	-
4	25	26	27	25	26	27	1[b]	2[b]	3[b]	1	to	18	1	2	3	1	3	5
8	28	29	30	28[c]	29[c]	30[c]	1[b]	2[b]	3[b]	-	-	-	-	-	-	-	-	-
24	-	-	-	-	-	-	1[b]	2[b]	3[b]	1	to	18	1	2	3	2	4	6
Doses	1			5			1			1			5			5		
Animals/dose	30			27/30[c]			3			18			3			6		
Total no. animals	174									33/30(+18[a])								
Samples/animal/day	1			1			4			8			8			4		
Animals/time point	3			3			3			18			3			3		

[a] Data from the new single-dose IV design were analysed simultaneously with the new multiple-dose PO data to obtain steady-state PK results.
[b] Samples were taken at these times each day for three days.
[c] Samples were taken at 8 h post-dose only for the 5 mg/kg and 25 mg/kg doses.

Table 3
Summary of original and new study designs for pharmacokinetic characterisation of ethambutol (EMB) in the mouse model. The number of doses and animals, blood-sampling time points, number of samples/animal and number of observations (animals used)/time point for the original single-dose designs (a multiple-dose study was not performed), the new single-dose intravenous (IV) and oral (PO) designs and the new zipper design for multiple-dose PO administrations are given. The light grey numbers show the identifying number for each animal used in the design to illustrate the time point at which the sample was taken from that animal.

EMB	Original designs									New designs								
Sampling time point (hours post-dose)	Single-dose IV			Single-dose PO			Multiple-dose PO			Single-dose IV			Single-dose PO			Multiple-dose PO (zipper)[a]		
0.08	1	2	3	1	2	3	-	-	-	1	to	9	1	2	3	1	3	5
0.25	4	5	6	4	5	6	-	-	-	1	to	9	1	2	3	2	4	6
0.5	7	8	9	7	8	9	-	-	-	1	to	9	1	2	3	1	3	5
0.75	10	11	12	10	11	12	-	-	-	1	to	9	1	2	3	2	4	6
1	13	14	15	13	14	15	-	-	-	1	to	9	1	2	3	1	3	5
1.5	16	17	18	16	17	18	-	-	-	-	-	-	-	-	-	-	-	-
2	19	20	21	19	20	21	-	-	-	1	to	9	1	2	3	2	4	6
3	22	23	24	22	23	24	-	-	-	-	-	-	-	-	-	-	-	-
4	25	26	27	25	26	27	-	-	-	1	to	9	1	2	3	1	3	5
8	28	29	30	28	29	30	-	-	-	1	to	9	1	2	3	2	4	6
24	-	-	-	31[b]	32[b]	33[b]	-	-	-	-	-	-	-	-	-	-	-	-
Doses	1			5			-			1			5			5		
Animals/dose	30			30/33[b]			-			9			3			6		
Total no. animals	186									24/30(+9[a])								
Samples/animal/day	1			1			-			8			8			4		
Animals/time point	3			3			-			9			3			3		

[a] Data from the new single-dose IV design were analysed simultaneously with the new multiple-dose PO data to obtain steady-state PK results.
[b] Samples were taken at 24 h post-dose only for the 300 mg/kg and 1000 mg/kg doses.

Ethambutol was given as a single IV dose (16 mg/kg) or a single oral dose (10, 30, 100, 300 or 1000 mg/kg). There was no multiple-dose regimen for EMB. Pyrazinamide was given as a single IV dose (25 mg/kg), a single oral dose (15, 25, 50, 150, 400 or 1000 mg/kg) or multiple oral doses (150 mg/kg/day) for four days. All mice received treatment in the fed state.

In the experiments using the original sampling design for single oral and IV administrations of RIF, INH and EMB, only one blood sample was taken from each mouse, at each of nine to ten time points per dose, and three mice were used for each time point. The blood samples were taken by cardiac puncture after the animal had been euthanised by CO_2. For PZA, one sample was taken from each euthanised mouse for the single IV dose, but repeated samples were taken from the lateral tail vein of each mouse at seven or eight time points after each single-dose oral administration. In the original designs for multiple-dose oral administrations, four samples were taken from the tail veins of each mouse each day for three days for RIF and INH and for four days for PZA. All the blood samples were frozen at −70 °C till analysis by ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS). The number of doses, sampling time points, number of animals used per dose, total number of animals used, number of samples taken per animal and number of animals used for sampling at each time point are given under the heading 'Original designs' in Tables 1 to 4.

Table 4
Summary of original and new study designs for pharmacokinetic characterisation of pyrazinamide (PZA) in the mouse model. The number of doses and animals, blood-sampling time points, number of samples/animal and number of observations (animals used)/time point for the original designs, the new single-dose intravenous (IV) and oral (PO) designs and the new zipper design for multiple-dose PO administrations are given. The light grey numbers show the identifying number for each animal used in the design to illustrate the time point at which the sample was taken from that animal.

PZA	Original designs									New designs								
Sampling time point (hours post-dose)	Single-dose IV			Single-dose PO			Multiple-dose PO			Single-dose IV			Single-dose PO			Multiple-dose PO (zipper)[a]		
0.08	1	2	3	-	-	-	-	-	-	1	to	15	1	2	3	1	3	5
0.25	4	5	6	1	2	3	1[b]	2[b]	3[b]	1	to	15	1	2	3	2	4	6
0.5	7	8	9	1	2	3	-	-	-	1	to	15	1	2	3	1	3	5
0.75	10	11	12	1[c]	2[c]	3[c]	-	-	-	1	to	15	1	2	3	2	4	6
1	13	14	15	1	2	3	1[b]	2[b]	3[b]	1	to	15	1	2	3	1	3	5
1.5	16	17	18	-	-	-	-	-	-	-	-	-	-	-	-	2	4	6
2	19	20	21	1	2	3	-	-	-	1	to	15	1	2	3	1	3	5
3	22	23	24	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4	25	26	27	1	2	3	1[b]	2[b]	3[b]	1	to	15	1	2	3	2	4	6
8	28	29	30	1	2	3	1[b]	2[b]	3[b]	1	to	15	-	-	-	-	-	-
24	-	-	-	1[c]	2[c]	3[c]	1[b]	2[b]	3[b]	-	-	-	-	-	-	-	-	-
Doses	1			6			1			1			6			6		
Animals/dose	30			3			3			15			3			6		
Total no. animals	51									33/36(+15[a])								
Samples/animal/day	1			7			5			8			7			4		
Animals/time point	3			3			3			15			3			3		

[a] Data from the new single-dose IV design were analysed simultaneously with the new multiple-dose PO data to obtain steady-state PK results.
[b] Samples were taken at these times each day for four days.
[c] Samples were taken at 0.75 h instead of at 24 h post dose for the 400 mg/kg and 1000 mg/kg doses.

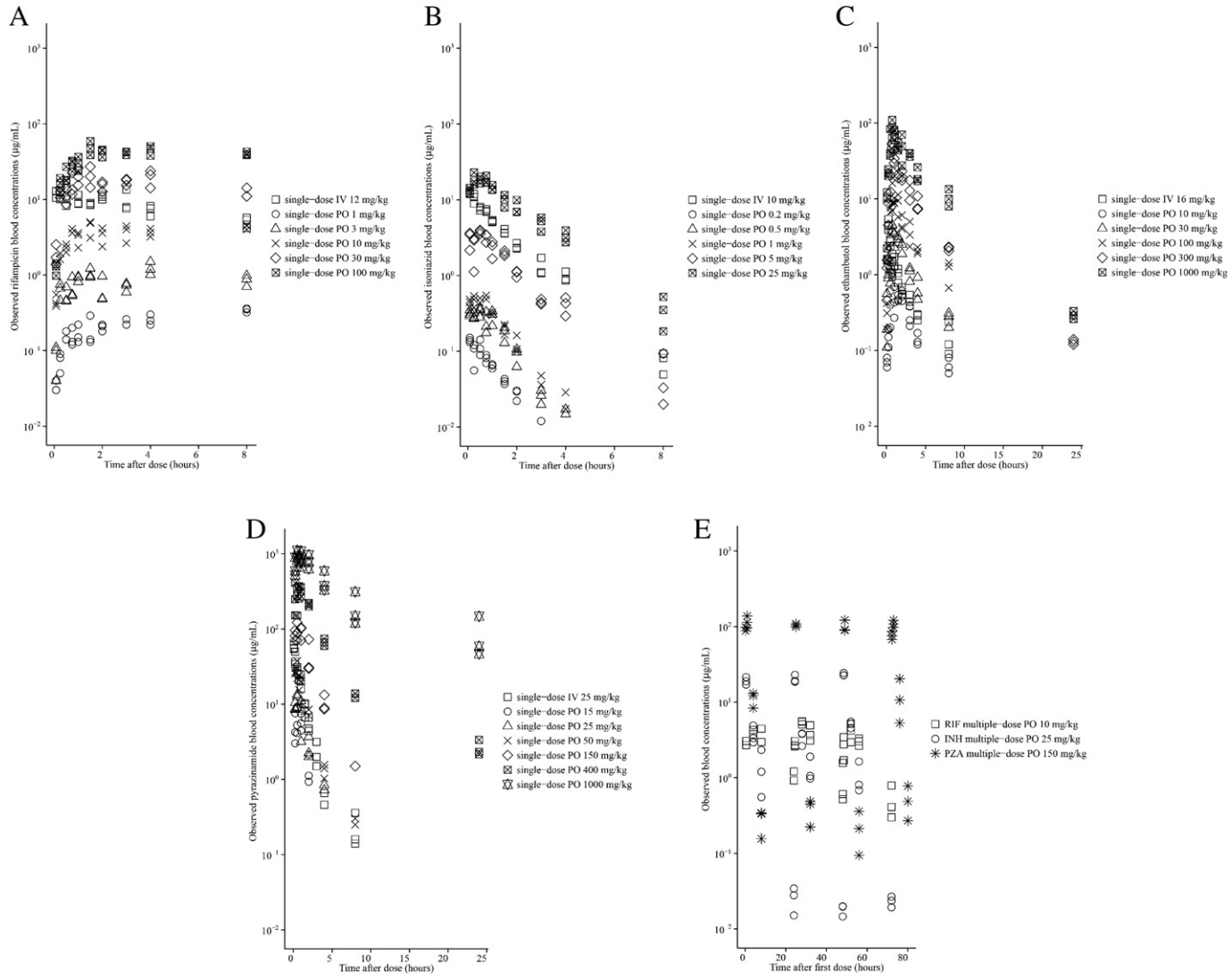

Fig. 1. Observed blood concentrations versus time for the original sampling designs after A) rifampicin (RIF) single-dose intravenous (IV) or single-dose oral (PO), B) isoniazid (INH) single-dose IV or single-dose PO, C) ethambutol (EMB) single-dose IV or single-dose PO, D) pyrazinamide (PZA) single-dose IV or single-dose PO and E) multiple-dose PO of RIF, INH and PZA.

Blood samples were processed by protein precipitation with acetonitrile/methanol (80:20), containing an internal standard, and were analysed by UPLC-MS/MS in positive ion mode with electrospray, using multiple reaction monitoring at the specific mass transitions for RIF, INH, EMB and PZA. The limit of quantification (LOQ) was 10 ng/mL for RIF, 5 ng/mL for INH and EMB, and 100 ng/mL (after IV administration) and 500 ng/mL (after oral administration) for PZA.

The studies were ethically reviewed and carried out in accordance with European Directive 86/609/EEC and the GSK Policy on the Care, Welfare and Treatment of Animals.

2.3. Population pharmacokinetic modelling and evaluation

All modelling was conducted using NONMEM 7.2 software (Icon Development Solutions, Ellicott City, MD) and the first-order conditional estimation method. Perl-speaks-NONMEM (PsN) (version 4.2.0) (http://psn.sourceforge.net/; Keizer et al., 2013) was used to create a prediction-corrected visual predictive check (pcVPC) (Bergstrand et al., 2011). Rstudio (version 2.14.2) was used for graphical analysis and data management. Xpose (version 4.4.1) (http://xpose.sourceforge.net/; Keizer et al., 2013) was used for visualisation of data and results. Run record was produced with Pirana software (version 2.8.0) (http://www.pirana-software.com/; Keizer et al., 2011). Proportional, additive and combined error models were evaluated in order to describe residual variability.

Model selection was based on the change in the objective function value (OFV). A decrease of 3.84 after addition of a single parameter for nested models was considered significant, corresponding to a 5% significance level with one degree of freedom. Scientific plausibility, parameter precision, goodness-of-fit plots and pcVPC were also considered in the process of model selection.

All the blood concentrations of each drug (obtained using the original sampling designs) were modelled simultaneously using a nonlinear mixed effects approach. Initially, one- and two-compartmental structural PK models, with first-order absorption and elimination, were evaluated. For PZA, a three-compartmental model was also evaluated due to data-driven observations. Dose dependence and time dependence were also tested for RIF, INH and PZA. Only dose dependence was evaluated for EMB since there was no PK information available from multiple-

Table 5
Final pharmacokinetic parameter estimates for rifampicin (RIF), isoniazid (INH), ethambutol (EMB) and pyrazinamide (PZA) in the mouse, based on blood concentrations and the original sampling designs.

Parameters	RIF		INH		EMB		PZA	
	Typical value	RSE (%)	Typical value	RSE (%)	Typical value	RSE (%)	Typical value	RSE (%)
k_a (h^{-1})	1.02	15.2	12.6	12.1	0.869	9.9	2.84	11.2
CL (mL/h/kg)[a]	79.3	11.4	855	8.2	2560	5.9	515	5.3
V (mL/kg)	1250	4.3	989	4.2	2180	12.8	532	5.8
Q (mL/h/kg)	–	–	–	–	1760	13.5	43.8	23.5
V_2 (mL/kg)	–	–	–	–	4910	7.6	709	18.9
F (%)	65.6	6.4	84.3	4.8	64.0	6.7	55.9	8
T_{lag} (h^{-1})	–	–	–	–	0.0577	11.1	–	–
$V_{lowest\ dose}$ (mL/kg)	2280	8.4	437	13	–	–	–	–
$CL_{highest\ dose}$ (mL/h/kg)	–	–	–	–	–	–	95	17.1
CL_{MAX} (%)	–	–	75.2	25.9	–	–	–	–
CL_{50} (μg/kg)	–	–	17,500	67.4	–	–	–	–
CL at Day 3 (mL/h/kg)	132	9.5	–	–	–	–	–	–
IIV in *CL* (%)	15.9	23.3	14.6	16.5	–	–	27.8	14.7
IIV in k_a (%)	55.6	22	–	–	–	–	–	–
IIV in *V* (%)	21.8	23.6	–	–	–	–	–	–
IIV in *Q* (%)	–	–	–	–	–	–	118.7	19.5
IOV in *F* (%)							15.6	20.8

CL = clearance; $CL_{highest\ dose} = CL$ at the highest dose level; CL_{MAX} = the maximum percentage decrease in *CL* of isoniazid; CL_{50} = the dose level at which *CL* reached 50% of CL_{MAX} for isoniazid; *F* = bioavailability; *IIV* = inter-individual variability expressed as the coefficient of variation and as a percentage of the parameter estimate; *IOV* = inter-occasional variability expressed as the coefficient of variation and as a percentage of the parameter estimate; k_a = absorption rate constant; *Q* = intercompartmental rate; RSE = relative standard error reported on the approximate standard deviation scale; T_{lag} = absorption lag time; *V* = volume of distribution; $V_{lowest\ dose} = V$ at lowest oral dose.

[a] Rifampicin clearance during Days 1 and 2.

dose oral administrations. The IIV and IOV (Karlsson and Sheiner, 1993) were evaluated on all fixed effects using log-normal distribution:

$$P_i = P \cdot exp(\eta_i)$$

where P_i is the value of the parameter in individual *i*, *P* is the typical value of the parameter in a population and η_i is the normally distributed IIV with a mean 0 and variance ω^2.

The percentage of data below the LOQ for PZA was 18.8%. There were no RIF, INH and EMB blood concentration observations below the LOQ. Attempts were made to use the M3 method in NONMEM for modelling PZA PK but this was not successful, because there was no improvement in OFV. Therefore, data below the LOQ was handled by setting the first LOQ observation to half of the LOQ value.

2.4. Design optimisation and sample size determination

A stochastic simulation and estimation (SSE) approach was utilized for the developed PopPK models for each of the four drugs in order to optimise the sampling designs and decrease the number of animals required. Different sampling schemes and numbers of mice were evaluated for each drug with the aim of reducing the total number of animals required while retaining at least the same relative imprecision and relative bias (*rBias*) in the fixed and random effects parameters. The new designs for each drug were optimised for both single-dose and multiple-dose experiments with the PK data obtained on one occasion (the multiple-dose designs used single-dose IV design plus single-dose oral design at steady-state). For the single-dose designs, the number of samples per animal was increased to eight, which was judged to be practical and ethically justified. This allowed the capture of more PK information from each animal while reducing the total number of animals in comparison with the original design from which the PK data were obtained to build the PopPK models. For the single-dose design, other scenarios explored included extending the sampling period to 24 h post-dose, varying the total number of animals used and varying the sampling time points. The zipper design allowed the sampling scheme within each animal to be reduced in the new multiple-dose design compared with the new single-dose design. This was thought to be a more practical option for multiple-dose experiments where PD information such as colony forming units or other biomarkers is sampled within the same time frame as the PK. For the multiple-dose oral zipper design, three or four samples per animal were evaluated but samples from at least one animal were also collected at each of the other optimal sampling time points identified in the evaluation of the single-dose design, for each drug. In this way, data from different animal groups receiving the same dosage but sampled at different times were zipped together to contribute to the full PK profile (Tables 1 to 4; Fig. 3). Data from the single-dose IV experiment were included in the estimation of the new single- and multiple-dose oral designs. Since the auto-induction of RIF in the PopPK model was described using a separate clearance (*CL*) value only on Day 3 rather than for the full time-course of RIF auto-induction, the multiple-dose oral zipper design only included simulation of the PK following a single dose but with the zipper PK sampling scheme. As such, the RIF *CL* on Day 3 was not estimated in the SSE. Because the half-lives of RIF, INH, EMB and PZA were short, the PK for the multiple-dose experiments were obtained following a single dose, mimicking a PK occasion at any time during a PKPD experiment. The last samples for the new designs for each drug and route of administration were dependent on the LOQ for the observed data used for building the PopPK models.

One thousand replicates were simulated for each design, and each of the simulated datasets was analysed using the final PopPK model for each drug, which was also used for the simulations. The designs were assessed by estimating the *rBias* and relative imprecision. The latter was expressed as the relative root mean square error (*rRMSE*). The *rBias* and *rRMSE* were derived as:

$$rBias = 100\% \cdot \frac{1}{N} \cdot \sum_i \frac{estimation_i - true_i}{true_i}$$

$$rRMSE = 100\% \cdot \sqrt{\frac{1}{N} \cdot \sum_i \frac{(estimation_i - true_i)^2}{true_i^2}}$$

where $estimation_i$ denotes the estimated parameter *i* value, $true_i$ is the true parameter *i* value used in the initial simulations and *N* is the number of simulations for each set of $true_i$ ($N = 1000$).

3. Results

3.1. Rifampicin population pharmacokinetics

The observed blood concentrations of RIF after single-dose IV and single- and multiple-dose oral administrations using the original design are shown in Fig. 1. A one-compartment model with first-order absorption and elimination provided the best fit for the RIF PK data. The volume of distribution at the lowest dose of RIF ($V_{lowest\ dose}$) was significantly higher than that at higher doses (*V*). Neither a nonlinear nor a linear relationship describing the change in *V* with dose was supported by the data. Due to auto-induction of RIF, clearance (*CL*) on Day 3 (132 mL/h/kg) was statistically significantly higher than that on Days 1 and 2 (79.3 mL/h/kg). The bioavailability (*F*) was estimated as 65.6%. The IIV of the absorption rate constant (k_a), $V/V_{lowest\ dose}$ and *CL* were estimated as 55.6%, 21.8% and 15.9%, respectively. The data did not

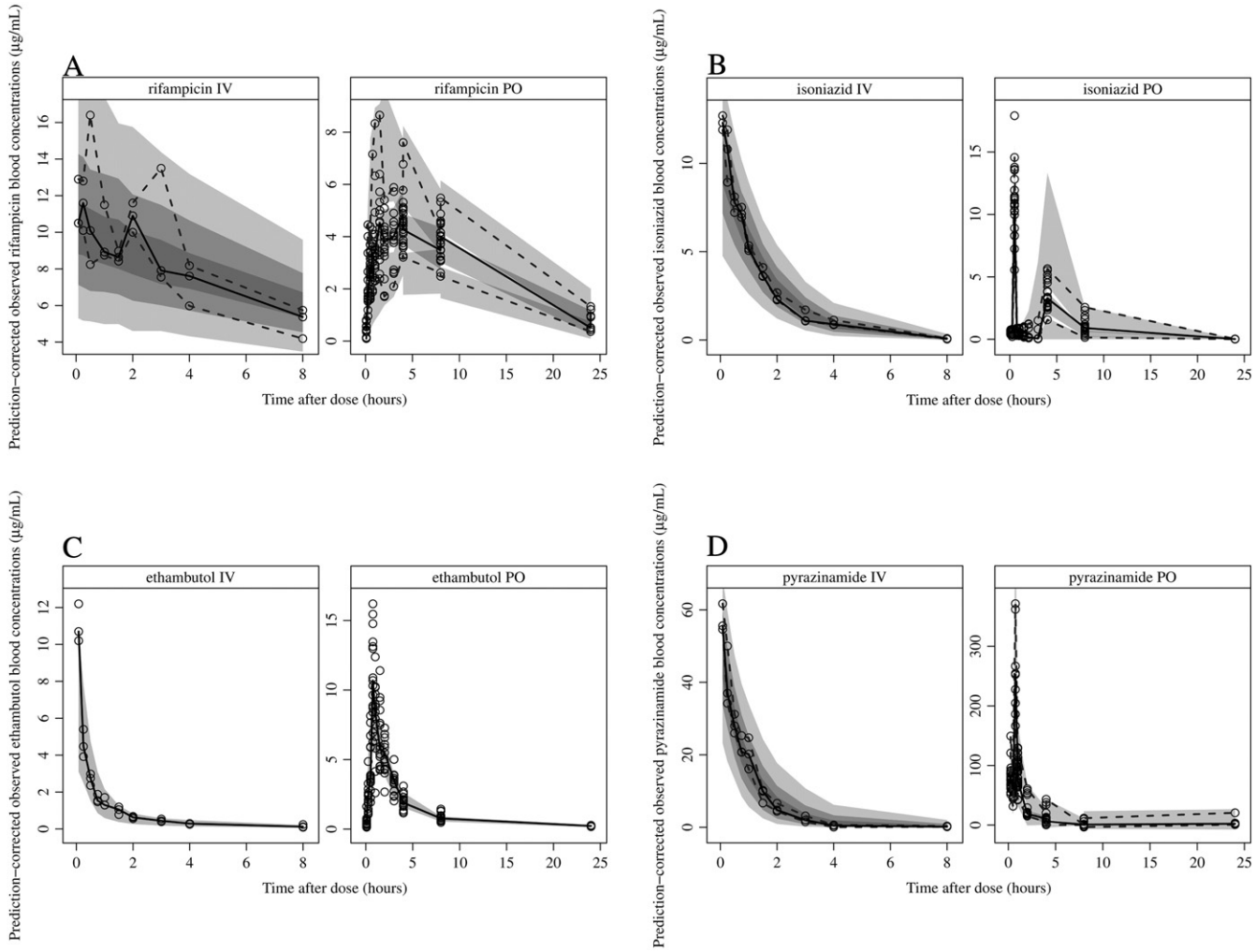

Fig. 2. Prediction-corrected visual predictive checks (pcVPC) of A) rifampicin, B) isoniazid, C) ethambutol and D) pyrazinamide final population pharmacokinetic models in the mouse based on blood concentrations and the original experimental designs. The left panel shows the intravenous (IV) data, and the oral (PO) data are in the right panel. The black circles are the prediction-corrected observed blood concentrations. The black solid lines are the medians of prediction-corrected observed data. The dark grey shaded areas are the simulation-based 95% confidence intervals for the medians. The observed 5% and 95% percentiles are presented as dashed grey lines and the 95% confidence intervals for the corresponding model-predicted percentiles are shown as light grey shaded areas.

support inclusion of IOV in any of the PK parameters. The final PopPK parameter estimates for RIF are presented in Table 5.

3.2. Isoniazid population pharmacokinetics

The observed blood concentrations of INH after single-dose IV and single- and multiple-dose oral administrations using the original design are shown in Fig. 1. Isoniazid blood PK was best described using a one-compartment PK model with dose-dependent *V* and *CL*. The *CL* decreased with increasing dose levels according to:

$$CL = TVCL \cdot \left(1 - \frac{CL_{MAX} \cdot (dose - 0.2)}{CL_{50} + (dose - 0.2)}\right)$$

where *TVCL* is the typical clearance at a dose of 0.2 mg/kg, CL_{MAX} is the maximum percentage decrease in *CL*, and CL_{50} is the dose needed to obtain 50% of CL_{MAX}. The volume of distribution at a dose of 0.2 mg/kg ($V_{lowest\ dose}$) was 1.8-fold lower than the *V* at higher doses. The IIV of *CL* was estimated as 14.6%. The *F* of INH was estimated as 84.3%. The final PopPK parameter estimates for INH are presented in Table 5.

3.3. Ethambutol population pharmacokinetics

The observed blood concentrations of EMB after single-dose IV and single-dose oral administrations using the original design are shown in Fig. 1. Ethambutol blood PK was described well by a two-compartment model with an absorption lag time (T_{lag}) of 3.5 min and an *F* of 64%. The volumes of distribution of the central and peripheral compartments (*V* and V_2) were 2180 mL/kg and 4910 mL/kg, respectively. The IIV was not estimated in the PK of EMB because of the very sparse original design using only one sample per mouse. The final PopPK parameter estimates for EMB are presented in Table 5.

3.4. Pyrazinamide population pharmacokinetics

The observed blood concentrations of PZA after single-dose IV and single- and multiple-dose oral administrations using the original design are shown in Fig. 1. Pyrazinamide blood PK was best described using a two-compartment PK model. The PZA *CL* was statistically lower at the highest PZA dose ($CL_{highest\ dose}$) than at lower doses. The IIV of *CL* and the inter-compartmental rate (*Q*) were estimated as 27.8% and

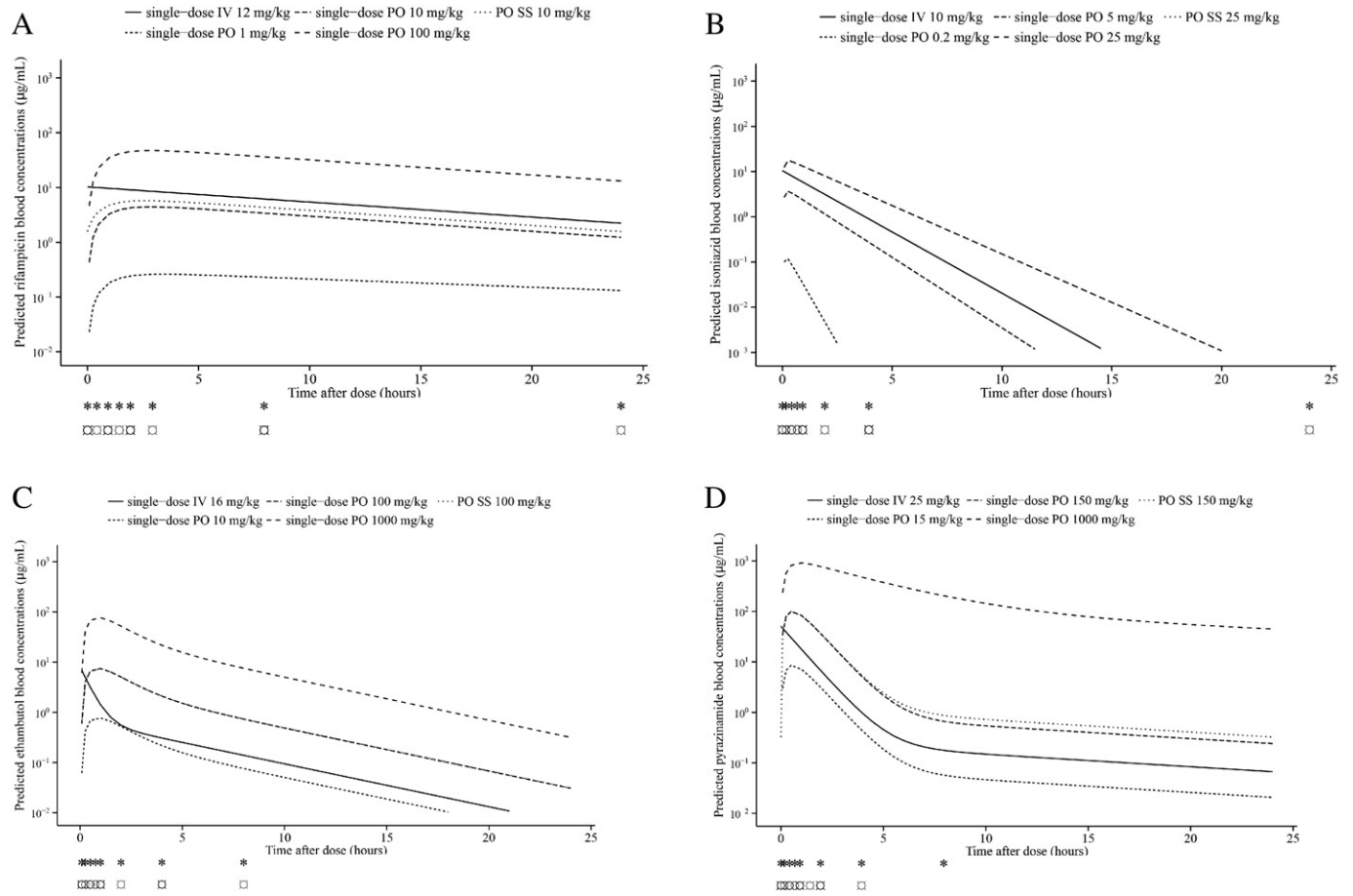

Fig. 3. Predicted blood concentrations versus time based on the final population pharmacokinetic models after single-dose intravenous (IV) or single-dose oral (PO) or PO steady-state (SS) administration of A) rifampicin, B) isoniazid, C) ethambutol and D) pyrazinamide. The sampling time points in the new designs for single-dose administration are indicated by * in each plot. For the multiple-dose (zipper) oral administration designs, half of the animals were sampled according to ¤ in black and the other half according to ¤ in grey. For detailed information about sampling time points, please see Tables 1–4.

118.7%, respectively, in the final PZA PopPK model. The *F* of PZA was estimated as 55.9%. The final PopPK parameter estimates for PZA are presented in Table 5.

3.5. Prediction-corrected visual predictive checks

The pcVPCs for RIF, INH, EMB and PZA PK, stratified by routes of administrations, are shown in Fig. 2.

3.6. Optimisation of sampling designs

The last PK sample for each drug in the new designs was dependent on the respective half-life and the LOQ of the drug, as the designs targeted data above the LOQ. A sample was also taken at 24 h in the new single-dose oral and IV designs for RIF and INH, since the *rRMSE* values for some parameters were lower than those without a 24-hour sample (data not shown). For the new single-dose oral design for PZA, the last sampling time was 4 h post-dose, since most of the observations from the new simulated data were below the LOQ after the lower doses at 4 h post-dose (data not shown). Pyrazinamide blood samples following oral and IV administrations were analysed using assays with two different LOQ values. The LOQ for PZA blood concentrations was 100 ng/mL after IV administration and 500 ng/mL after oral administration. Therefore, the PK sampling times after PZA oral and IV administrations were ended at 4 and 8 h post-dose, respectively. Due to the short half-life of EMB, the 24-hour sample was deleted from the original design. The sampling time points for the single-dose designs were assessed after evaluation of the last sample taken for each drug. Several time points were omitted from the original designs for each drug in order to limit the designs to up to 8 samples from each mouse, covering the whole experimental period up to the last sampling time point. For the new single-dose IV and oral designs, the sampling time points of 0.25, 0.75 and 4 h post-dose were deleted from the original RIF design, those of 1.5, 3 and 8 h post-dose were deleted from the original INH design, and those of 1.5, 3 and 24 h post-dose were deleted from the original EMB design. In the new designs for PZA, the 3-hour post-dose sampling time point was omitted from the original single-dose IV design and sample taking at 8 and 24 h post-dose was excluded from the single-dose oral design. A new sampling time point was added at 0.08 h post-dose for the single-dose oral design and multiple-dose oral zipper design, and another at 1.5 h post-dose was added to the oral zipper design. For RIF, INH and EMB new single-dose designs, the sampling time points were identical for the IV and oral studies. The sampling time points for the investigated drugs and designs, and the predicted concentrations, are shown in Fig. 3.

The new optimised total number of animals required for the single-dose designs were 24, 24, 33 and 33 for RIF, EMB, INH and PZA, respectively. For RIF, INH and EMB, the numbers of animals required for the new single-dose designs were up to 7-fold lower than in the original designs (Tables 1–4). The number of animals required for PZA only decreased by 35% because the original sampling design already included more than one sample per mouse.

The multiple-dose oral zipper designs were based on the sampling time points that had been optimised for the new single-dose oral

designs for RIF, INH, EMB and PZA. However, only four samples per mouse were required for the zipper designs, compared to eight for the single-dose designs. The re-estimation of data in the SSE for the zipper design analysis used PK information from the single-dose IV and single-dose oral experiments in order to include information about the bioavailability and to use the richer sampling from the single-dose IV experiment. The new optimised total numbers of animals required for the multiple-dose oral zipper designs were 30, 30, 30 and 36 for RIF, EMB, INH and PZA, respectively. For RIF, INH and EMB, these numbers were almost 5-fold lower in the oral zipper designs than in the original designs, which included up to 186 animals for each drug (Tables 1–4). The required number of animals for PZA was similar to that in the original design because more than one sample per mouse was also taken in the original design. The *rBias* and *rRMSE* for each parameter, based on the oral zipper designs (which included the new single-dose IV design and eight samples per animal), were at least as low as those from the new single-dose design for each drug. As shown in Figs. 4 and 5, the *rBias* and *rRMSE* for each parameter in the new designs for each drug, i.e. the oral zipper design with four samples per animal for multiple-dose oral administration and the single-dose IV or oral design with up to eight samples per animal, were similar or lower than those for the original designs, except for the *rBias* in IIV for the *CL* of PZA, which was 2% higher in the zipper design than in the original design. The *rBias* in the INH CL_{50} was also 6% and 3% higher in the new single-dose and zipper designs than in the original design.

4. Discussion

This study describes the PopPK of RIF, INH, PZA and EMB in the mouse, using blood concentrations and nonlinear mixed effects modelling (Table 5). In addition, the sampling designs were optimised to reduce the number of animals required. Careful design is important for animal experiments from an ethical perspective as well as in order to save money and increase the information content of a study.

Before a PK-PD experiment is done for a new compound, the PK after a single dose needs to be defined. We therefore explored an optimised single-dose design for the four chosen anti-TB drugs. The most important design aspect was to increase the number of samples taken from each animal to up to eight samples per mouse, a number which was judged to be practical and ethical. Using the final PopPK models, we performed simulations using an SSE approach in order to derive optimal PK sampling designs and total population sizes for each of the four anti-TB drugs. In an SSE approach, data is simulated based on a model and then re-estimated based on different designs of interest in order to investigate the bias and imprecision in parameter estimates. We found that changing the design from one sample per animal to up to eight samples

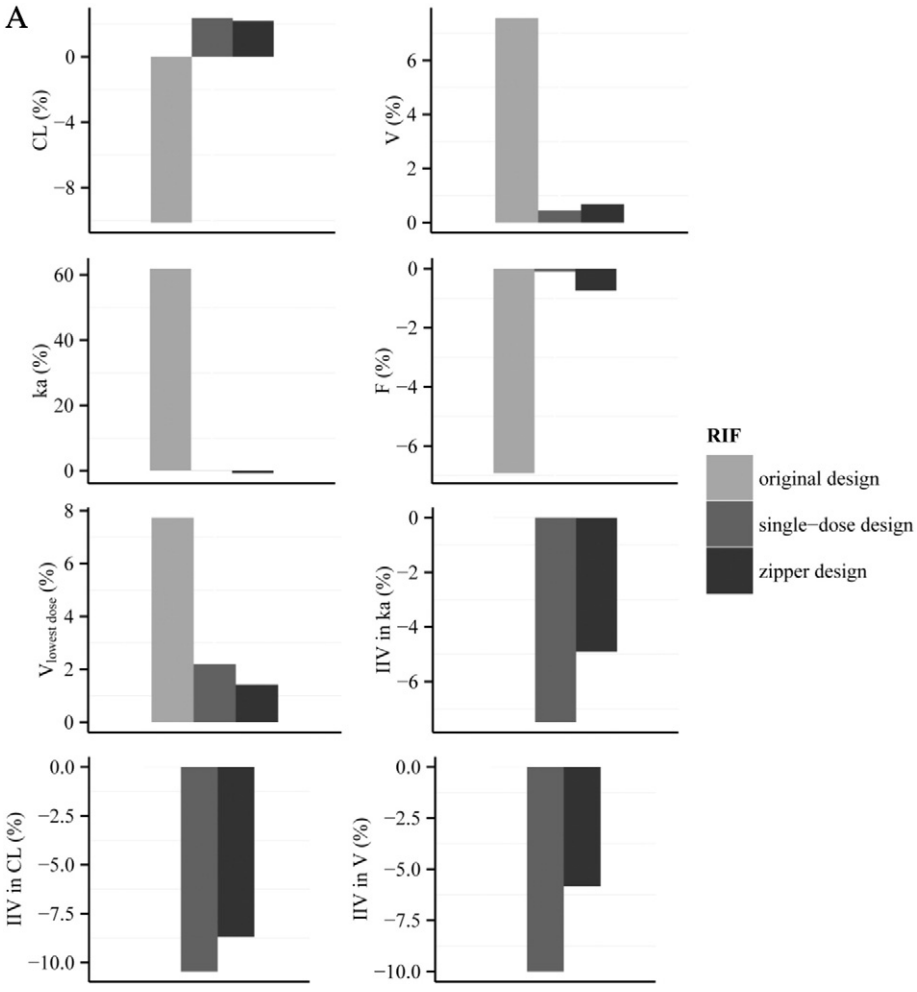

Fig. 4. Relative bias [*rBias* (%)] of pharmacokinetic parameter estimates of A) rifampicin (RIF), B) isoniazid (INH), C) ethambutol (EMB) and D) pyrazinamide (PZA) for the new single-dose designs (dark grey bars), the new multiple-dose zipper designs (black bars), compared with the original designs (light grey bars). The results were obtained using stochastic simulation and estimation and the new population pharmacokinetic models. Inter-individual variability (IIV) was not re-estimated in the original designs for RIF and INH as the designs only included one sample per animal. The final population pharmacokinetic model for EMB did not include IIV.

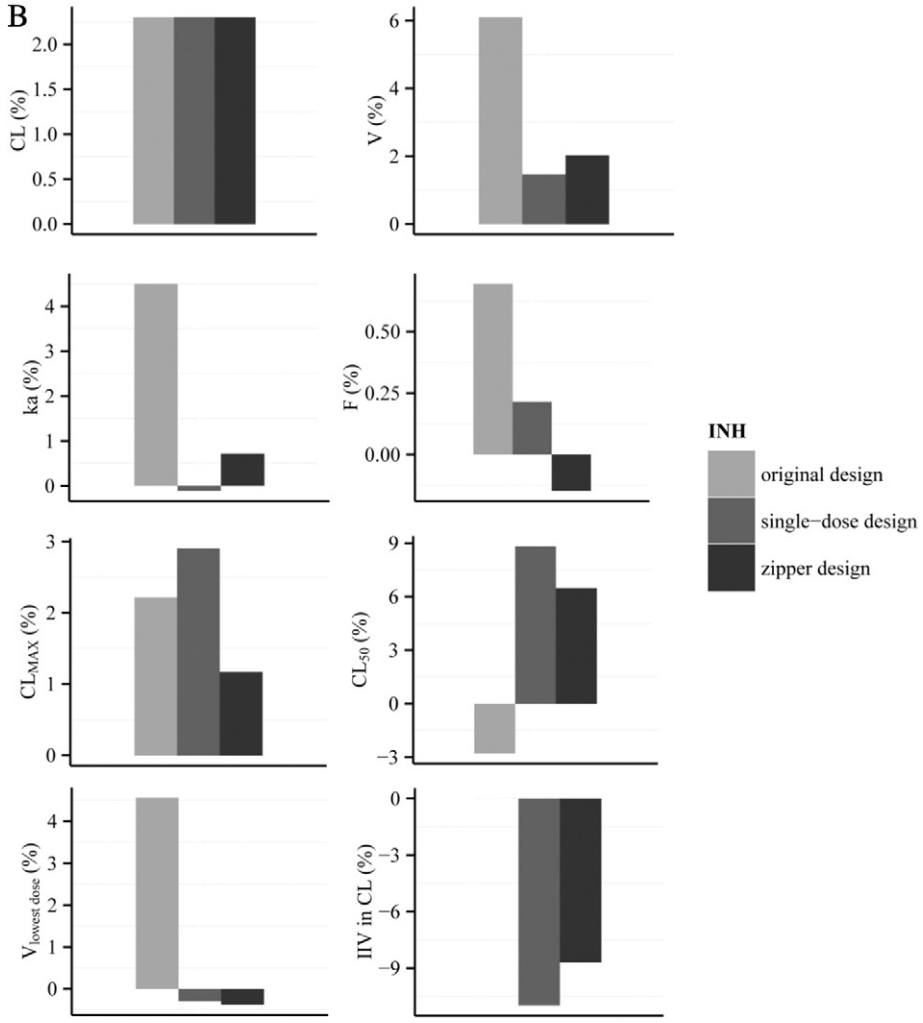

Fig. 4 (*continued*).

per animal reduced the number of required animals by up to 7-fold without compromising the precision of the PK parameters in the single-dose designs.

Traditionally, PK is determined in satellite experiments and the data are then used as fixed PK input in PK-PD models for TB (Chen et al., 2014). The design would, of course, be more informative if the PK and PD are obtained in the same animal. With such a design in mind, we developed the oral zipper design which obtains the PK of the drug after one dose in a multiple-dose experiment. In this design, only four samples are taken per animal but all the samples from all the animals are zipped together to cover the entire sampling period. Similarly, for the multiple-dose oral zipper design, changing the sampling design from one sample per animal to four samples per animal decreased the number of animals required by up to 5-fold. The four drugs that we have studied are part of the standard clinical treatment of drug-susceptible TB and are often used as a control in PD experiments. As such, the suggested optimised PK designs can be used in future PK-PD experiments with these drugs as controls. For new compounds, the lessons learnt from this work are the advantages of using serial sampling instead of single sample designs. However, if the PK is not known for a new compound, there is no standard sampling series that can fit for drugs with both short and long half-lives. We therefore suggest that a small pilot experiment is done with a zipper design where the samples are taken in order to collect some information. Based on the information from the initial work, the design can be optimised as done in this study. This is also in agreement with the learning-confirming paradigm (Sheiner, 1997).

It is important to notice that the PK in this study was based on the blood concentrations of the four anti-TB drugs. The data in PK are dependent on whether blood, plasma, saliva, etc. is sampled, and PK data based on plasma samples would potentially be different from those based on blood samples. In the original data/design, single-dose administrations of RIF and INH did not include information on IIV, since only one sample from each mouse was taken. However, it was possible to estimate IIV due to the simultaneous fit of single- and multiple-dose data and multiple samples were taken from mice after multiple-dose oral administrations of RIF and INH. However, no repeated within-animal sampling was obtained for EMB. Therefore the final PopPK model for EMB did not include IIV. All original data/designs for PZA included repeated within-animal sampling and the final PopPK model for PZA therefore included estimation of IIV.

The PopPK of RIF, INH, PZA and EMB was established in healthy mice in this work. It is not known if the PK would be different in mice infected with *M. tuberculosis* but it cannot be ruled out. For instance, the terminal half-life of RIF was reported to be 4.4 h (de Steenwinkel et al., 2013) and 12 h (Jayaram et al., 2003) in the BALB/c mouse, and 7.6 h in the Swiss mouse (Ji et al., 1993). This should be compared to a half-life of approximately 6.6 h in our study using the C57BL/6 mouse. The PK of RIF

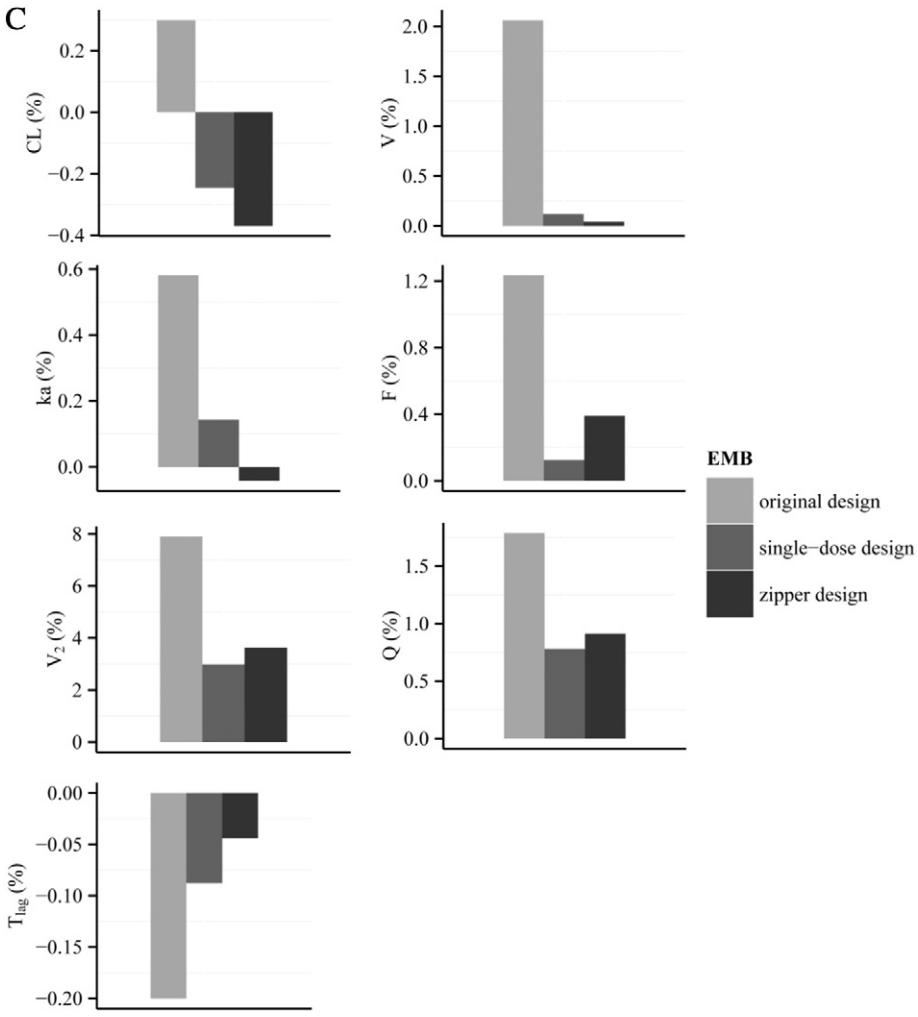

Fig. 4 (*continued*).

undergoes auto-induction in the mouse. Following once daily oral administration of 10 mg/kg RIF to the BALB/c mouse for five days, there was a reduction in plasma exposure on Day 5 compare to Day 1 (Hosagrahara et al., 2013). We also found in this work that *CL* of RIF in blood on Day 3 (132 mL/h/kg) was significantly higher than that on Days 1 and 2 (79.3 mL/h/kg). Isoniazid was found to display dose-dependent *V* and *CL* in a dose range of 0.2–25 mg/kg in our study. An earlier study showed linear PK for INH in the dose range of 0.1–120 mg/kg using the BALB/c mouse (Jayaram et al., 2004). The terminal half-life of 25 mg/kg of INH in serum using the Swiss mouse has been reported to be 1.7 ± 0.17 h (Grosset et al., 1992), and approximately 1.4 h in blood using the C57BL/6 mouse in our study. The maximum concentration of 25 mg/kg of INH in serum was 22 μg/mL using the BALB/c mouse (Almeida et al., 2009), and approximately 21 μg/mL in blood in our study. The peak concentration of radioactive EMB ^{14}C labelled occurred at 2 h after 32 mg/kg (Kelly et al., 1981) using Charles River mouse. The peak concentration of 30 mg/kg EMB in this work occurred at approximately 0.75 h after dose. Pyrazinamide was found to display dose-dependent *CL* in a dose range of 15–1000 mg/kg in our study using the C57BL/6 mouse where *CL* at 15–400 mg/kg was estimated to approximately 515 mL/h/kg and 95 mL/h/kg at 1000 mg/kg, whereas it was estimated to 460 mL/h/kg in the BALB/c mouse and to 410 mL/h/kg in the C3HeB/FeJ mouse (Irwin et al., 2016) at 150 mg/kg. Since efficacy studies in the mouse model involve chronic dosing, it is essential to understand the PK behaviour of anti-TB compounds following repeated oral administration. The observed systemic exposure after a single dose may be different from that after multiple-dose administrations if the compound has the potential to induce or inhibit clearance or absorption mechanisms, leading to dose- or time-dependent PK. In humans, RIF is a known inducer of cytochrome P450 (Combalbert et al., 1989) and P-glycoprotein through activation of the nuclear pregnane X receptor (Geick et al., 2001), which causes potential for drug-drug interactions. Full steady-state RIF auto-induction in humans is reached after 40 days of oral dosage (Smythe et al., 2012). In this study, RIF *CL* on Day 3 was predicted to be 66.4% higher than on Days 1 and 2, which indicates that RIF is auto-induced not only in humans, but also in the mouse. However, a more mechanistic model estimating the enzyme turn-over could not be quantified because of the short PK sampling period. More research is required in order to evaluate the turnover of RIF auto-induction in the mouse. It is very important to notice that humans may differ from animals with regard to expression and catalytic activities of drug metabolizing enzymes. For instance, cytochrome P450 2B (CYP2B) was not detectable in the human intestine (Kaminsky and Zhang, 2003), but expressed in the intestine of mouse (Martignoni et al., 2005). Although animal models are not expected to always predict drug exposure in human, they serve as valuable tools for quantifying exposure-response relationships and ideally predict response at certain dose levels in human.

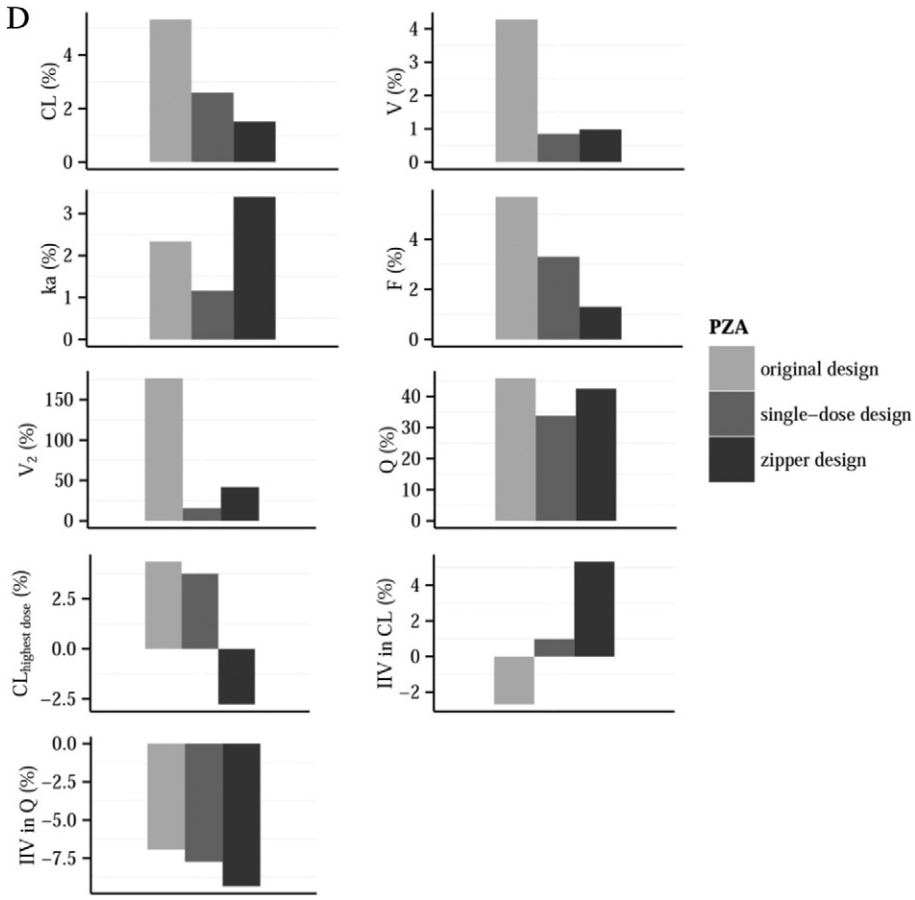

Fig. 4 (*continued*).

Because the half-life of PZA is short, the concentrations fall quite quickly below the LOQ. Although samples below the LOQ can be included in a PopPK analysis using the M3 method, it is preferable to sample concentrations above the LOQ. In our studies, different LOQ values, arising from different mass spectrometry analyses used in the animal studies, were used for IV (100 ng/mL) and oral (500 ng/mL) PZA concentrations. Since the different LOQ values used for oral and IV administrations of PZA could have had an impact on the study design, we made the last sampling time points for IV and oral administration at 8 and 4 h post-dose, respectively.

The $V_{lowest\ dose}$ for RIF was higher than the *V* for the other doses. The $V_{lowest\ dose}$ for INH was also different but, in contrast to RIF, it was lower than V for the other doses. Dose-dependent *CL* was identified for INH and PZA. The mechanisms behind the dose-dependent PK identified in this work remain unknown. Mechanistic information is important but, from a PK-PD modelling perspective, empirical models as used in the final PopPK models presented here will be predictive within the dose ranges studied and will be able to provide useful exposure input for PK-PD modelling exercises.

5. Conclusions

The new single-dose design with enrichened individual sampling was more informative than the original design in which only one sample was taken from each mouse. The proposed oral zipper design allows informative PK sampling in a multiple-dose administration scenario for characterising PK-PD relationships. Simulations showed that the suggested improved designs resulted in similar or lower biases and imprecisions in parameter estimates, and decreased the total number of animals required by up to 7-fold compared to the original designs where only one sample was taken from each animal.

Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted. There were no human participants in the studies described in this article.

Conflict of interest

GlaxoSmithKline contributed financially to the conduct of the experimental work. The authors Fatima Ortega, Laura Alameda and Santiago Ferrer are employees of GlaxoSmithKline. There is no conflict of interest associated with the authors Ulrika Simonsson and Chunli Chen.

Acknowledgements

The research was funded by the Swedish Research Council, the Innovative Medicines Initiative Joint Undertaking (www.imi.europa.eu) under grant agreement n°115337, resources of which are composed of financial contribution from the European Union's Seventh Framework Programme (FP7/2007–2013) and EFPIA

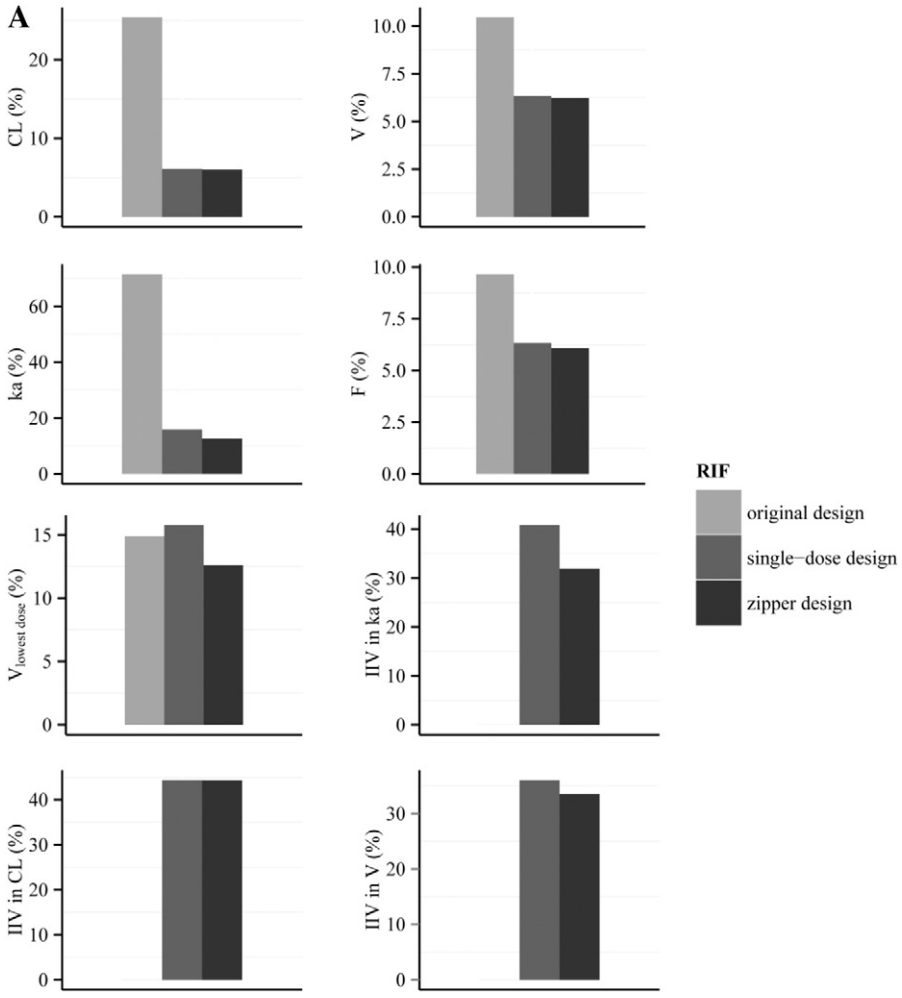

Fig. 5. Relative imprecision [*rRMSE* (%)] of pharmacokinetic parameter estimates of A) rifampicin (RIF), B) isoniazid (INH), C) ethambutol (EMB) and D) pyrazinamide (PZA) for the new single-dose designs (dark grey bars) and the new multiple-dose zipper designs (black bars), compared with the original designs (light grey bars). The results were obtained using stochastic simulation and estimation and the new population pharmacokinetic models. Inter-individual variability (IIV) was not re-estimated in the original designs for RIF and INH as the designs only included one sample per animal. The final population pharmacokinetic model for EMB did not include IIV.

companies' in kind contribution and the Chinese Scholarship Council. The funding parties were not involved in the study design, the analysis or interpretation of data or the writing of this article.

The experimental work was financed by GlaxoSmithKline. The authors Fatima Ortega, Laura Alameda and Santiago Ferrer are employees of GlaxoSmithKline.

References

Almeida, D., Nuermberger, E., Tasneen, R., Rosenthal, I., Tyagi, S., Williams, K., Peloquin, C., Grosset, J., 2009. Paradoxical effect of isoniazid on the activity of rifampin-pyrazinamide combination in a mouse model of tuberculosis. Antimicrob. Agents Chemother. 53, 4178–4184.

Bergstrand, M., Hooker, A.C., Wallin, J.E., Karlsson, M.O., 2011. Prediction-corrected visual predictive checks for diagnosing nonlinear mixed-effects models. AAPS J. 13, 143–151.

Chen, C., Ortega, F., Rullas-Trincado, J., Alameda, L., Angulo-Barturen, I., Ferrer, S., Simonsson, U.S.H., 2014. Semi-mechanism pharmacokinetic-pharmacodynamic modeling of rifampicin treatment response in a tuberculosis mouse model. 54th Interscience Conference on Antimicrobial Agents and Chemotherapy (abstract).

Combalbert, J., Fabre, I., Fabre, G., Dalet, I., Derancourt, J., Cano, J.P., Maurel, P., 1989. Metabolism of cyclosporin A. IV. Purification and identification of the rifampicin-inducible human liver cytochrome P-450 (cyclosporin A oxidase) as a product of P450IIIA gene subfamily. Drug Metab. Dispos. 17, 197–207.

de Steenwinkel, J.E.M., Aarnoutse, R.E., de Knegt, G.J., ten Kate, M.T., Teulen, M., Verbrugh, H.A., Boeree, M.J., van Soolingen, D., Bakker-Woudenberg, I.A.J.M., 2013. Optimization of the rifampin dosage to improve the therapeutic efficacy in tuberculosis treatment using a murine model. Am. J. Respir. Crit. Care Med. 187, 1127–1134.

Geick, A., Eichelbaum, M., Burk, O., 2001. Nuclear receptor response elements mediate induction of intestinal MDR1 by rifampin. J. Biol. Chem. 276, 14581–14587.

Grosset, J., Truffot-Pernot, C., Lacroix, C., Ji, B., 1992. Antagonism between isoniazid and the combination pyrazinamide-rifampin against tuberculosis infection in mice. Antimicrob. Agents Chemother. 36, 548–551.

Hosagrahara, V., Reddy, J., Ganguly, S., Panduga, V., Ahuja, V., Parab, M., Giridhar, J., 2013. Effect of repeated dosing on rifampin exposure in BALB/c mice. Eur. J. Pharm. Sci. Off. J. Eur. Fed. Pharm. Sci 49, 33–38.

Irwin, S.M., Prideaux, B., Lyon, E.R., Zimmerman, M.D., Brooks, E.J., Schrupp, C.A., Chen, C., Reichlen, M.J., Asay, B.C., Voskuil, M.I., Nuermberger, E.L., Andries, K., Lyons, M.A., Dartois, V., Lenaerts, A.J., 2016. Bedaquiline and pyrazinamide treatment responses are affected by pulmonary lesion heterogeneity in mycobacterium tuberculosis infected C3HeB/FeJ mice. ACS Infect. Dis 2, 251–267.

Jayaram, R., Gaonkar, S., Kaur, P., Suresh, B.L., Mahesh, B.N., Jayashree, R., Nandi, V., Bharat, S., Shandil, R.K., Kantharaj, E., Balasubramanian, V., 2003. Pharmacokinetics-pharmacodynamics of rifampin in an aerosol infection model of tuberculosis. Antimicrob. Agents Chemother. 47, 2118–2124.

Jayaram, R., Shandil, R.K., Gaonkar, S., Kaur, P., Suresh, B.L., Mahesh, B.N., Jayashree, R., Nandi, V., Bharath, S., Kantharaj, E., Balasubramanian, V., 2004. Isoniazid pharmacokinetics-pharmacodynamics in an aerosol infection model of tuberculosis. Antimicrob. Agents Chemother. 48, 2951–2957.

Ji, B., Truffot-Pernot, C., Lacroix, C., Raviglione, M.C., O'Brien, R.J., Olliaro, P., Roscigno, G., Grosset, J., 1993. Effectiveness of rifampin, rifabutin, and rifapentine for preventive therapy of tuberculosis in mice. Am. Rev. Respir. Dis. 148, 1541–1546.

Kaminsky, L.S., Zhang, Q.Y., 2003. The small intestine as a xenobiotic-metabolizing organ. Drug Metab. Dispos. Biol. Fate Chem 31, 1520–1525.

Karlsson, M.O., Sheiner, L.B., 1993. The importance of modeling interoccasion variability in population pharmacokinetic analyses. J. Pharmacokinet. Biopharm. 21, 735–750.

Keizer, R.J., van Benten, M., Beijnen, J.H., Schellens, J.H.M., Huitema, A.D.R., 2011. Piraña and PCluster: a modeling environment and cluster infrastructure for NONMEM. Comput. Methods Prog. Biomed. 101, 72–79.

Keizer, R.J., Karlsson, M.O., Hooker, A., 2013. Modeling and simulation workbench for NONMEM: tutorial on Pirana, PsN, and Xpose. CPT Pharmacomet. Syst. Pharmacol 2, e50.

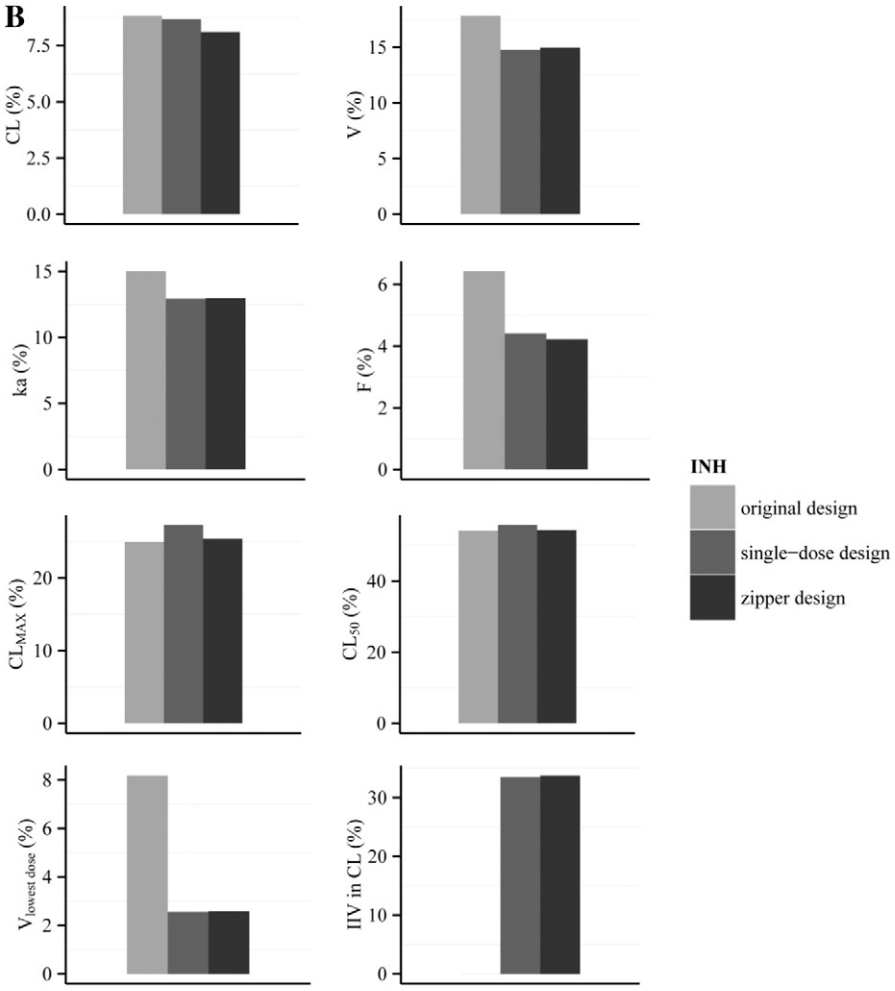

Fig. 5 (*continued*).

Kelly, R.G., Kaleita, E., Eisner, H.J., 1981. Tissue distribution of [14C]ethambutol in mice. Am. Rev. Respir. Dis. 123, 689–690.

Martignoni, M., de Kanter, R., Moscone, A., Grossi, P., Monshouwer, M., 2005. Lack of strain-related differences in drug metabolism and efflux transporter characteristics between CD-1 and athymic nude mice. Cancer Chemother. Pharmacol. 55, 129–135.

Sheiner, L.B., 1997. Learning versus confirming in clinical drug development. Clin. Pharmacol. Ther. 61, 275–291.

Smythe, W., Khandelwal, A., Merle, C., Rustomjee, R., Gninafon, M., Lo, M.B., Sow, O.B., Olliaro, P.L., Lienhardt, C., Horton, J., Smith, P., McIlleron, H., Simonsson, U.S.H., 2012. A semimechanistic pharmacokinetic-enzyme turnover model for rifampin autoinduction in adult tuberculosis patients. Antimicrob. Agents Chemother. 56, 2091–2098.

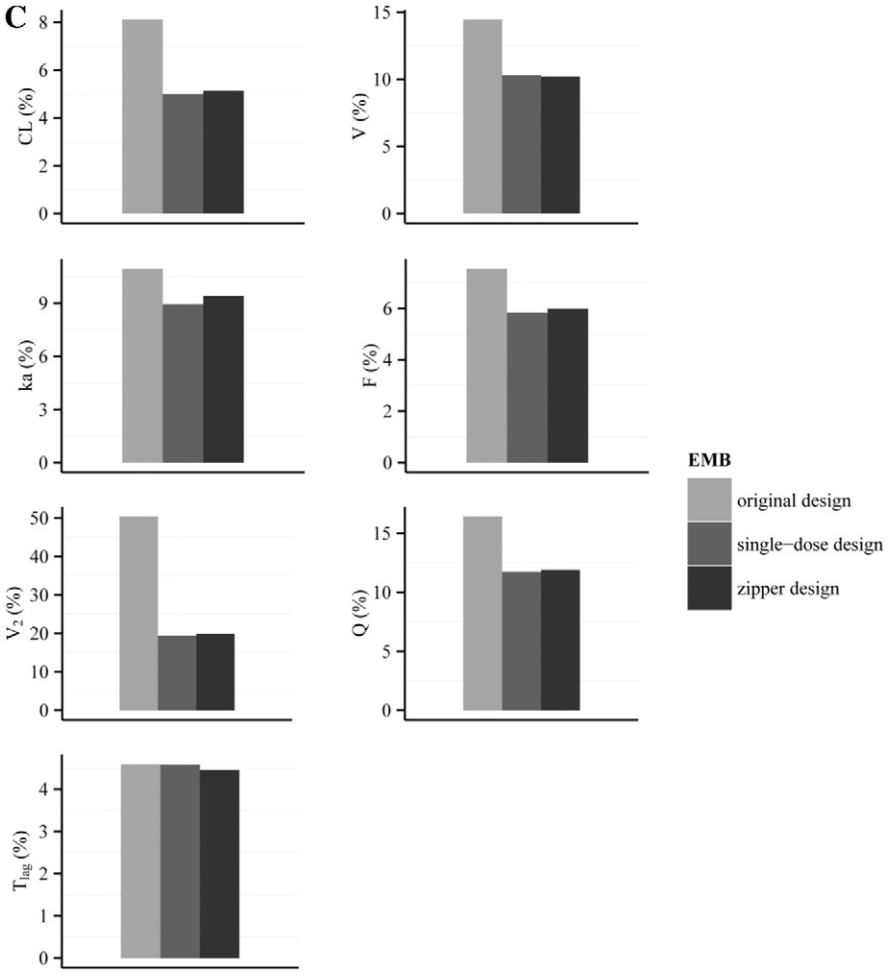

Fig. 5 (*continued*).

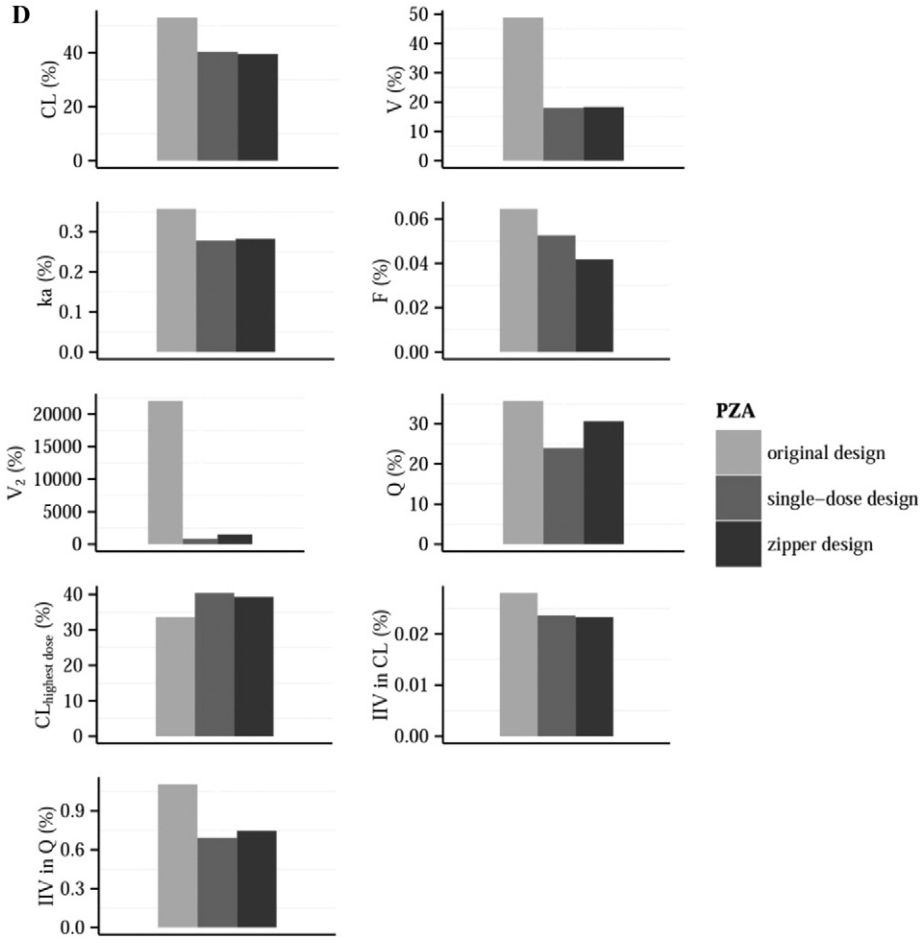

Fig. 5 (*continued*).