Investigating the critical variables of azithromycin oral absorption using in vitro tests and PBPK Modeling

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Abstract

Azithromycin is an antibiotic listed in the essential list of medicines for adults and pediatrics. Conflicting evidence has been found regarding azithromycin classification according to the Biopharmaceutics classification system (BCS). The purpose of this study was to identify the critical variables that influence the oral absorption of azithromycin in adults and pediatrics.

Azithromycin solubility and dissolution studies (oral suspension) were performed in buffers and biorelevant media simulating the fasted and fed gastrointestinal tract. A PBPK model was developed for azithromycin for healthy adult volunteers and pediatrics (Simcyp® v18.2) informed by *in vitro* solubility and dissolution studies to predict drug performance after administration of azithromycin as an oral suspension.

The developed PBPK model predicted azithromycin plasma concentrations-time profiles after administration of an oral suspension to adults and pediatrics. Sensitivity analysis of solubility *vs* dose suggests that absorption is independent of solubility within the therapeutic dose range in both adults and pediatrics. The developed PBPK model for adults and pediatrics was consistent with the mechanism of permeation through the intestinal membrane (passive and active processes) being the rate-limiting step of azithromycin's absorption.

The physiologically based approach proposed was shown to be useful to determine the factors controlling drug absorption in adults and pediatrics.

Key-words: Pediatric; Absorption; Solubility; Permeability; Biopharmaceutics classification system (BCS); Physiologically Based Pharmacokinetic (PBPK) modeling.

Introduction

The biopharmaceutics classification system (BCS) is a framework that describes the key drivers of *in vivo* drug performance: dose, solubility and permeability [1]. The BCS is a vital part of the drug development process, particularly in the establishment of BCS-based biowaivers [2]. The need for a pediatric biopharmaceutical classification system (pBCS) has been emphasised in the literature [2,3]. Although efforts have been made towards the establishment of a pBCS, uncertainties in the current knowledge of pediatric physiology, heterogeneity of the pediatric population, and other complicating factors still hinder the progress and development of such a tool. Due to the lack of a pBCS, predictions of solubility and permeability classification in pediatrics are often based on the adults' BCS criteria. Caution should be taken with this approach as the pediatric population undergoes developmental changes in anatomy and physiology (such as differences in gastric pH, gastrointestinal (GI) fluid composition, gastric emptying (GE) times, small intestinal transit times (SITT), intestinal transporters, *etc*.), which might alter the drug solubility and permeability classification criteria in the pediatric age groups [3,4]. Consequently, drugs biopharmaceutical properties can be affected as a function of age, and shifts in BCS classification between adults and pediatrics can occur. Unfavourable BCS class shifts can negatively affect drug performance in the pediatric population when compared to drug efficacy and safety in adults [3,5]. Therefore, an extensive evaluation should be performed to assess the risk of BCS classification changes from extending adults' BCS criteria to pediatrics [3,6,7].

The BCS drug solubility criteria can be determined based on three main parameters: the drug's highest dose the initial fasted gastric volume, and the drug solubility across a physiologically relevant pH range. Drugs are classified as highly soluble if the highest therapeutic dose (EMA) or highest strength (FDA) is soluble in 250 mL aqueous liquid at a relevant pH range [8-10].

Current methods for assessing the pediatric relevant luminal volumes are based on the Bodyweight (BW) or Body Surface Area (BSA) extrapolation of adult luminal volumes [2].

The permeability class of a drug is based on the extent of absorption. For a drug to be considered highly permeable, the systemic bioavailability/extent of absorption needs to be greater than 85–90% of the administered dose [9,10]. The preferred methods to determine the permeability class of a drug are pharmacokinetic (PK) studies, such as mass balance or absolute bioavailability studies. Alternative methods can be used, which do not involve human subjects, such as *in vivo* or *in situ* intestinal perfusion in suitable animal models, or *in vitro* permeability experiments such as cultured Caco-2 epithelial cell monolayers derived from human colon adenocarcinoma cell line [9,10]. For pediatrics, permeability values have often been derived from absolute bioavailability data in pediatric patients, however, due to the limited PK data in pediatrics, alternative methods have been suggested. Calculated logP values guided the provisional classification of the drugs included in the World Health Organization (WHO) list of essential drugs for children [3]. The applicability of this method to pediatric groups under 2 years of age remains unknown due to the rapid degree of maturation and growth changes that take place until this age [2]. Recently, a mechanistic PBPK modeling approach has been proposed to detect the sensitivity of the cumulative fraction absorbed to permeability changes in pediatrics, as a method to evaluate the risk of BCS permeability classification changes between adults and pediatrics [11].

Pediatric biopharmaceutic risk assessment should be conducted since the early stages of drug development, along with the adult product development program [7]. A biopharmaceutical risk assessment should involve the coupling of *in vitro* and *in silico* tools to predict the impact of drug solubility, dissolution, and permeability on the drug systemic exposure. The best approach to a successful risk assessment in pediatrics is to apply the current knowledge of pediatric physiology in the development of age-appropriate *in vitro* tools (such as age-appropriate

biorelevant media composition and dissolution conditions) and *in silico* tools (such as age-appropriate PBPK modeling focused on oral drug absorption). The coupling of age-appropriate *in vitro* and *in silico* tools may be a valid strategy to transcend the existing knowledge gaps surrounding each method on its own [2].

In this study, azithromycin was selected as the model compound. Azithromycin is an antibiotic commonly prescribed for respiratory tract infections in adults and pediatrics [12]. The pediatric formulation available in the market is the Zithromax® immediate-release powder for suspension which can be used in children under 45 Kg according to the drug product label [12]. Conflicting evidence has been found regarding the BCS classification of azithromycin. According to the FDA Zithromax® Clinical Pharmacology and Biopharmaceutics review, azithromycin might be either a BCS class I or III compound [12]. On the other hand, WHO has classified azithromycin as BCS class II (poor solubility) or IV (poor solubility and permeability) compound [13]. Additionally, three different studies have attempted to classify azithromycin according to a provisional pBCS. delMoral-Sanchez *et al.* attributed a provisional pBCS class for the oral drugs included in the Essential Medicines List by the World Health Organization (WHO) and compared the pBCS classification with the adults BCS class [6]. For the solubility classification, the dose number was calculated, and a BW-extrapolation was used to scale the pediatric luminal volumes from the reference adult volume [6]. Permeability classification was based on partition coefficient, logP (n-octanol/water partition coefficient), and its relationship with human intestinal permeability. delMoral-Sanchez *et al.* considered azithromycin as a BCS class II in adults, and favourable changes to BCS class I in newborns were predicted based on Fried's Rule for the calculation of the pediatric dose (Fried's rule=[age (months)/150] × adult dose) [6]. Shawahna also proposed a provisional classification system based on a BW-extrapolation to scale the pediatric luminal volumes from adult's initial gastric volumes [14]. Shawahna predicted that azithromycin was as a poorly water-soluble compound in

adults, with no solubility class changes predicted for pediatrics. Predictions for permeability classification changes in pediatrics were not performed in this study. Finally, Gandhi and co-workers used a potential pBCS, where BSA-extrapolation was used to scale pediatric reference volumes from the adult reference volume[7]. Differences in azithromycin's BCS classification between publications could be related to different methods used for the calculation of the dose number (*i.e.* highest dosage strength vs highest single dose), but these were not always reported. For assessment of permeability class changes from adults to pediatrics, the absolute bioavailability in pediatrics was considered. Based on the study findings, azithromycin was classified as BCS class III in adults, and no pediatric BCS class changes were expected for pediatrics [7].

This study aimed to use PBPK modeling informed by *in vitro* and *in silico* biopharmaceutical tools to: (i). clarify the impact of *in vivo* solubility, dissolution, and permeability on the absorption of azithromycin in adults and pediatrics; and (ii). assess the biopharmaceutical risk of age-related changes on oral azithromycin performance. Solubility and dissolution studies were performed in buffers and biorelevant media simulating the fasted and fed state environment of the gastrointestinal tract. A PBPK model was developed for adults and pediatrics in the Simcyp® v18.2 (Certara®, US) simulator, informed by the results obtained in the *in vitro* dissolution and solubility studies to predict drug performance after administration of azithromycin as an oral suspension to adults and pediatrics. Parameter sensitivity analysis (PSA) was performed to generate understanding of the role of critical variables on the oral absorption of azithromycin in adults and pediatrics.

Materials and methods

Materials

Azithromycin analytical standard (90%), dichloromethane, acetonitrile [High-performance liquid chromatography (HPLC) grade], and methanol (HPLC grade) were obtained from Fisher Scientific (UK). Sodium hydroxide, 37% hydrochloric acid, sodium chloride, potassium dihydrogen phosphate, sodium acetate trihydrate were purchased from Fisher Scientific (UK). Water was ultra-pure (Milli-Q) laboratory grade. Ultrafree filter units were obtained from Merck Millipore, USA, and azithromycin dihydrate (98%) was purchased from VWR Chemicals, UK. Zithromax® powder for the suspension (azithromycin dihydrate 209.64 mg/5 ml containing the equivalent of 200 mg azithromycin base per 5 ml , Pfizer Ltd., UK) was obtained from UK pharmacies and Belgium pharmacies. Glass microfiber GF/F and GF/D filters (0.7 and 2.7 µm, respectively) (Whatman®, UK), pepsin (from porcine), were purchased from Sigma Sigma-Aldrich Company Ltd. (UK). Sodium taurocholate (Prodotti Chimici Alimentari S.P.A., Italy), egg lecithin – Lipoid EPCS (Lipoid GmbH, Germany), and Glyceryl monooleate (Biosynth Carbosynth®, UK) were obtained from the specified sources. Ultra-high-temperature treated whole cow's milk standardised to less than 4% fat was acquired from Sainsbury's, UK. Polytetrafluoroethylene (PTFE) filters (13 mm, 0.45 µm) were used in the solubility experiments and were purchased from Fisher Scientific (UK).

Methods

Preparation of media

USP simulated gastric fluid sine pepsin (SGF*sp*) pH 1.2, acetate buffer pH 4.5, and phosphate buffer pH 6.8 were prepared following the USP [15]. Adult and pediatric biorelevant media were freshly prepared for each experiment, as described by Maharaj *et al.* [16]. Adult standard biorelevant media used consisted of fasted state simulated gastric fluid (FaSSGF), fasted state

simulated intestinal fluid (FaSSIF-V2), fed state gastric simulated fluid (FeSSGF) or fed state intestinal simulated fluid (FeSSIF-V2) [16]. The infant biorelevant media used was infant fasted state simulated gastric fluid (Pi-FaSSGF) and infant fed state simulated intestinal fluid (Pi-FeSSIF) [16]. For the two-stage dissolution testing (performed in the inForm platform), double concentrated FaSSIF-V2 was prepared with an additional amount of sodium hydroxide to achieve the final composition of FaSSIF-V2 (pH 6.5) after its addition to the gastric phase.

Solubility studies

Solubility measurements

Solubility experiments were performed using the shake flask method in a shaking water bath (model Grant SS40-2, Grant Instruments, UK) (37 °C, 200 strokes/minute). All solubility assessments were performed in triplicate. An excess of the solid drug was used to saturate the volume of medium used. Experiments were conducted with 2 mL of medium in centrifuge tubes. Aqueous-based samples were collected at 24 h, filtered and appropriately diluted in methanol. Filter adsorption studies were performed prior to the experiment. No adsorption to the filters used was observed. For samples containing milk-based products, the undissolved drug was removed by centrifugation (Eppendorf Heraeus Fresco 17 centrifuge, Thermo Electron LED GmbH, Germany) at 8000 rpm for 15 min at 4 °C. The supernatant was then treated with methanol (1000 µL of methanol were added to 500 µL of the sample), vortexed for 1 min, centrifuged (8000 rpm, 15 min, 4°C) and the resulting supernatant was filtered through a 0.45 µm PTFE filter and injected into the HPLC.

Drug Solubility Classification

Azithromycin solubility classification was determined by the calculation of dose number for pediatrics and adults. The dose number (D_0) was estimated across the different pediatric age groups using the following equation:

$$D_0 = \frac{Dose}{S \times V_0} \qquad \textbf{Equation 1}$$

where V_0 is the initial gastric volume and S is the drug (azithromycin) aqueous solubility. A dose number > 1 indicates a low solubility compound, and a dose number < 1 indicates the opposite [3,6,7].

For pediatrics, the initial gastric volume (V_0) was calculated by a BW-extrapolation or BSA-extrapolation according to **Equations 1** and **2**, respectively.

$$V_0 = \frac{BW \times 0.4\ mL/Kg}{37.1\ mL} \times 250\ mL \qquad \textbf{Equation 2}$$

In **Equation 2**, BW stands for the age-specific pediatric bodyweight. The BW values were the 50th percentile values in the Centers for Disease Control and Prevention (CDC) growth charts, where for each age the average of boy and girl BW value was used [17]. The 0.4 mL/Kg and 37.1 mL are estimates of fasted gastric fluid volumes in pediatrics and adults, respectively, and 250 mL is the reference volume used in the BCS [2,9,10,13].

$$V_0 = \frac{BSA}{1.73\ \mathrm{m}^2} \times 250\ mL \qquad \textbf{Equation 3}$$

In **Equation 3**, BSA stands for the specific pediatric body surface area. BSA was calculated according to Mosteller formula, where the average of boy and girl BW and body height (BH) used for each specific age, corresponded to the mean of boys and girls 50 percentile values in the CDC growth charts [17]. The adult BSA (1.73 m^2) is the reference value for a 70 Kg fasted adult [7] and 250 mL is the standard adult reference volume used in the BCS [2,9,10,13].

The doses used in the calculations were the highest azithromycin dose for each age and its BW, as indicated in the drug label [12]. For pediatric patients up to 15 Kg a 10 mg/Kg was used for calculations. For children above 15 Kg the dose was (i). 200 mg dose for children 15 -25 Kg; (ii). 300 mg for children 26 to 35 Kg; (iii). 400 mg for adolescents 35 to 45 Kg. The dose used for adult dose number calculations was 500 mg.

Dissolution studies

USP 4 apparatus dissolution studies

Dissolution studies were conducted in an Erweka® flow-through dissolution tester (USP, 2009) (DFZ720, Erweka GmbH, Germany) equipped with Ø 22.6 mm cells that were maintained at 37 ± 0.5 °C and connected to an Erweka® Piston Pump (model HKP720). A 5 mm – size glass bead was positioned in the tip of the cell, and glass beads of 1 mm-size were added to the conical part of each cell. On the top of the cell Whatman® glass fiber filters were used (a GF/F (0.7 µm) followed by a GF/D (2.7 µm)). The azithromycin oral suspension (Zithromax® immediate-release (IR) powder for oral suspension (200 mg/5mL)) was prepared by reconstitution with the appropriate amount of water, as per the drug label [12]. During the experiment, the exact volume of suspension was measured with a syringe and placed on top of the glass beads. Residence times in the USP 4 apparatus were appropriately selected to mimic the fasted and fed state gastrointestinal volumes and transit times in each subpopulation (**Table 1**) [2]. Flow-rates were selected to achieve a balance between the duration of the exposure of the drug product to the various simulating media and total fluid volumes, taking into account the lack of radial water flux *in vitro* [18]. Experiments were run in open-mode (with sequential media change from gastric to intestinal medium), with fresh media continuously passing through the cell containing the dosage form [19]. Fluid samples were collected in volumetric cylinders, which

were exchanged every 15 min after the start of the experiment. The drug content in the samples was assayed by HPLC. Experiments were run in triplicate.

inForm platform dissolution studies

Dissolution studies were performed in the inForm platform (Pion® Inc., UK) using a two-stage approach. The azithromycin oral suspension was prepared as in subsection 5.2.2.3.1. The clinical dose (*i.e.* infants 130 mg, children 300 mg and adults 500 mg) was down-scaled according to age-appropriate volumes (*i.e.* infants 150 mL, children 200 mL and adults 500 mL) (**Table 1**) according to a final dissolution volume of 80 mL. A two-stage approach was followed: fasted gastric condition were simulated for 30 min (40 mL), followed by intestinal simulated conditions (40 mL of two-fold concentrated FaSSIF-V2 was added leading to a final volume of 80 mL). Sample collection (0.5 mL) took place at 5, 15, 30, 45, 60, 75, 90, 120, 180 and 240 min. After collection, samples were filtered through an ultrafree filtering unit 0.45 µm PTFE and centrifuged (Eppendorf mini spin plus, Germany) at 8000 rpm for 2 min at room temperature. The filtrate was diluted with methanol and injected into the HPLC. Experiments were performed in triplicate.

Please insert Table 1 here

Chromatographic conditions for drug quantification

The chromatographic method used for quantification of azithromycin was a modification of the method by Baxevanis *et al.* [20]. Quantification of azithromycin was performed using HPLC coupled with ultraviolet (UV) detector [Waters HPLC system (Saint-Quentin-en-Yvelines, France) and Agilent HPLC system 1100/1200 series (Agilent Technologies, USA)] using a C18 column (X-Bridge C18 column 150 × 4.6 3.5 µm) at 40 °C. The injection volume was 20 µL and the detection was carried out by UV at 210 nm. The analytical method used an isocratic

mobile phase composed of a mixture of 10 mM potassium phosphate buffer (pH 7.5) and acetonitrile (45:55 v/v) at a flow rate of 1 mL/min. Quantification of montelukast in samples was performed with calibration curves of freshly prepared standard solutions (calibration curve range: 3–100 µg/mL). Standards were prepared in the medium of interest for each experiment by appropriate dilution of a 1 mg/mL stock solution of azithromycin analytical standard in methanol. The limit of detection (LOD) and the limit of quantification (LOQ) were 0.90 and 2.5 µg/mL, respectively.

PBPK model development

Pharmacokinetic Data Collection and Data treatment

PK studies of azithromycin, administered intravenously or orally as an oral suspension, in adults and pediatrics, were obtained from the literature. The plasma concentration-time profiles, and trial design information were extracted from the published reports and used for the PBPK model building and validation (**Table S1** and **Table S2**). One study reported single dose administrations of IV infusion of azithromycin (1000 mg to 4000 mg doses) in adults [21]. Three studies reported single dose administration of azithromycin oral suspension (500 mg) to adults in the fasted state [22-24] and one in the fed state [22]. One pediatric study reported IV infusion administration (10 mg/Kg) in infants (0.5 to 2 years), children (2 to 6 years), old children (6 to 12 years) and adolescents (12 to 16) [25]. Finally, two PK studies reported oral administration of azithromycin suspension in a 5-day oral regimen (10 mg/Kg on the 1st day and 5 mg/Kg day 2 to 5) to pediatric patients: infants and young children (0.5 to 5 years) and old children and adolescents (6 to 15 years) [26,27]. The observed PK profiles that were found in the literature were digitalised with WebPlotDigitalizer® v4.1 software [28]. PK data analysis was performed with PKSolver® add-in program for Microsoft Excel® [29]. Non-compartmental (NCA) analysis was

used to calculate the observed PK parameters, which are reported in the supplementary materials (**Table S1** and **Table S2**).

Adult PBPK model

PBPK modeling and simulations were performed using the Simcyp® Simulator (V18.2; Certara, UK). The PBPK modeling strategy followed the workflow presented in **Figure 1**. The relevant input parameters for the development of the PBPK models and simulations performed are summarised in **Table 2.** Azithromycin-specific information obtained from the literature consisted of its physicochemical properties including molecular weight (MW), octanol: water partition coefficient ($logP_{o:w}$), fraction unbound in plasma (f_u) and blood to plasma ratio (B:P) [30-32].

The distribution model was described using a minimal PBPK model with a Single Adjusting Compartment (SAC), a non-physiological compartment that represents a cluster of tissues (excluding liver and portal vein). The parameters that defined the distribution were: first-order rate constants [h^{-1}] of the drug into (k_{in}) and out (k_{out}) of the SAC, V_{SAC} (apparent volume associated with the SAC) and V_{ss} (steady-state volume of distribution). The disposition parameters were initially calculated based on a 2 compartmental fit to plasma concentration-time profiles obtained after an IV infusion administration of 1000 mg of azithromycin [21]. These initial distribution parameters were optimised to accurately describe the IV infusion administration of 1000 mg clinical data, and the simulations were externally validated for the IV infusion administration of 2000 and 4000 mg doses [21]. The intravenous clearance of azithromycin ranges between 31 - 46.5 L/h in healthy adults [12,32-34]. Azithromycin is mainly eliminated unchanged in the faeces via biliary excretion (more than 50%) with the rest being attributed to renal clearance (less than 20% eliminated unchanged in urine) [21,32,33]. Azithromycin PK profiles do not show evidence of enterohepatic recirculation, therefore this process was not investigated (assumed 0% available for re-absorption in the elimination tab).

For the mechanistic absorption modeling, the advanced dissolution absorption and metabolism (ADAM™) model was used [35]. The ADAM™ model was used to investigate the impact of passive and active permeability (*i.e.* Caco-2 *vs* MechPeff) and the impact of formulation input (*i.e.* biorelevant solubility inputted into SimCYP® Diffusion Layer Model (DLM) *vs* direct input of biorelevant *in vitro* dissolution) on azithromycin performance.

Azithromycin has been shown to be a P-gP substrate [36], efflux transport in the intestine was implemented in the PBPK model in two ways. In option 1: the effect of active transport in SimCYP® was assessed directly by using data from *in vitro* cell systems. By performing a comparison of Caco-2 data that is treated/non-treated with P-gP inhibitors it is possible to assess the limiting effects of P-gP on the passage of drug across the monolayer. Caco-2 data treated with an inhibitor of P-gP were entered as 'Passive' and without inhibitor of P-gP were inputted as 'Passive & Active'. In option 2: the influence of transporter was also investigated in SimCYP® by activating the MechPeff model. Since information of regional absorption to further validate the predicted regional permeability were not available, the predicted $P_{eff,man}$ was applied to all segments of the gastrointestinal tract to achieve a better description of the PK profiles. The intestinal P-gP *in vitro* transporter intrinsic clearance $CL_{int,T}$ (µL/min) was fitted to observed plasma concentration-time profiles after administration of azithromycin oral suspension in the fasted and fed state in adults published by Foulds *et al* [22].

Two levels of different activity of P-gP were assumed for fasted and fed state to simulate the inhibition of P-gP substrate by food (**Table 2**). The interaction of drug transporters with food has been discussed in the literature by several authors [37-39], where a high-fat meal is suspected to be able to inhibit P-gP [38].

The impact of azithromycin *in vitro* dissolution and solubility on drug absorption was evaluated with the PBPK model following two approaches: (i). application of the diffusion layer model (DLM), using the measured biorelevant solubility data as input; and (ii). direct entry of the *in*

vitro dissolution data [Dissolution Profile Model (DPM)] as discrete dissolution profiles. For the fasted state DPM model, the impact of the dissolution on the performance of azithromycin was evaluated by comparison of the observed and simulated plasma concentration-time profiles when inputting the dissolution profiles from the USP 4 apparatus *vs* the inForm ones. Precipitation was not explored, as the dissolution studies indicated no precipitation over 4 h.

In terms of physiology, all adult simulations were run using the healthy volunteer population library of the Simcyp® simulator. Mean gastric residence time (MGRT) in the fasted state was adjusted to 0.50 h to better describe the adult fasted *in vivo* data (adjusted against the Foulds *et al.* 1996 dataset and validated with the remaining fasted state adult datasets) [22-24]. For the remaining physiological parameters, default software values were assumed.

Please insert Figure 1 here

Please insert Table 2 here

Pediatric PBPK model

The Simcyp® pediatric population library was used. The population file gathers information on pediatric demography (age, body height, bodyweight and body surface area) and developmental physiology (liver size, renal function, liver blood flow *etc*.) [40]. In pediatrics, the adult SAC parameters, k_{in} (1/h), k_{out} (1/h), are allometrically scaled by the software according to the pediatric BW and an adult BW of 70 Kg, with exponents of -0.25 [41]. Since biliary elimination appears to be reasonably well developed reaching adult levels at birth or in the first few months of postnatal age no scaling was applied to biliary clearance [32]. Scaling of the renal function was captured in the Simcyp® pediatric module as previously described [42]. For both the fasted and fed state, the pediatric ADAM™ model contains information of developmental

changes as a function of age on: size of the gastrointestinal tract, gastric emptying time, gastrointestinal volumes, pH, *etc.* [40,43]. The fasted MGRT in pediatrics was assumed to be the same as adults (*i.e.* 0.5h). Remaining physiological and anatomical values were maintained at default values.

Trial design information

All simulations were performed with 10 trials of 10 subjects in each trial. The trial design was performed using the 'Virtual population' option in Simcyp®. The maximum and minimum age, as well as the proportion of females was adjusted according to the population of the PK study used for the validation of the model. Study details are presented in the supplementary materials (**Table S1** and **Table S2**)

PBPK model validation

The PBPK model was validated by comparison of the simulated azithromycin plasma concentration-time profiles, and the relevant PK parameters, against the clinically observed data. The mean predicted plasma concentration-time profiles were assessed by the average fold error (AFE) and validated with the absolute average fold error (AAFE) (**Equations 5.4** and **5.5**, respectively) [44].

$$\mathrm{AFE} = 10^{\frac{1}{n}\sum \log(\frac{predicted_i}{observed_i})} \qquad \textbf{Equation 5.4}$$

$$\mathrm{AAFE} = 10^{\frac{1}{n}\sum \left|\log(\frac{predicted_i}{observed_i})\right|} \qquad \textbf{Equation 5.5}$$

where n denotes the number of observed sampling points, $\mathrm{predicted}_i$ and $\mathrm{observed}_i$ denote the predicted and observed plasma concentration at the sampling time point i, respectively.

AFE values indicates whether the simulated data underpredicts (AFE < 1) or overpredicts (AFE > 1) the observed plasma concentrations, while an AAFE value close to unity represents the precision of the simulations. An AAFE ≤ 2 indicates an acceptable prediction [45]. The relative accuracy of the mean predicted PK parameters describing drug exposure [area under the plasma concentration-time curve (AUC), the maximum concentration (C_{max}), and time to reach the maximum concentration (T_{max})] was assessed against the mean observed PK parameters using the fold error (FE) (**Equation 5.6**). A FE within a 2-fold range (FE values between 0.5 and 2) indicates an acceptable prediction.

$$\mathrm{FE} = \frac{predicted}{observed} \qquad \textbf{Equation 5.6}$$

Parameter sensitivity analysis

Parameter sensitivity analysis (PSA) was conducted to identify the limiting steps of absorption and their impact on the *in vivo* drug performance (*e.g.* C_{max}, AUC). Sensitivity analysis was performed for two types of parameters. The first type was related to drug properties and included parameters such as drug solubility in the duodenum (range: 1 to 27 mg/mL) *vs* dose (range for adults: 250 to 600 mg; infants and young children: 40 to 220 mg; children and adolescents: 120 to 500 mg), effective permeability ($P_{eff,man}$ range: 0.1 to 3 x 10^{-4} cm/s) and intrinsic clearance of intestinal P-gP transporter (P-gP $Cl_{int,T}$ range: 10 to 300 µL/min). The dose ranges were based on the maximum and minimum observed doses in PK studies in adults and pediatrics. For the permeability, the range was based on the calculated P_{eff} from the Caco-2 method and calculated P_{eff} with the MechPeff model (described in Adult PBPK section). Finally, the investigated range of $Cl_{int,T}$ was based on the values used in the fasted and fed state models (**Table 2**). The second type of parameters was related to physiology, with MGRT being selected due to potential age-related differences in food types and feeding cycles between

adults and pediatrics. As the basis for the PSA, the simulations after oral administration of azithromycin in the fasted state for adults and pediatrics were run with MechPeff as permeability input and the DLM-based ADAM™ model. For the interpretation of the PSA results, predicted PK parameters were compared to the values used in the developed PBPK model (*i.e*. baseline simulation).

Results

Solubility studies

Solubility measurements

The mean azithromycin solubility in buffers and biorelevant media is presented in **Figure 2**. Azithromycin solubility ranged from 2 mg/mL to 13 mg/mL in all media tested. Higher solubility was observed in acidic media (pH 1.2 - 1.6) in comparison to more basic media (pH 6.5 - 6.8) as expected based on ionization properties of azithromycin (weak base, pKa = 8.6). Comparison between azithromycin solubility in buffers and in biorelevant media showed that drug solubility was sensitive to bile salts concentrations in the media. The azithromycin solubility in the SGF*sp* pH 1.2 was approximately 9 mg/mL and in the fasted gastric fluid (FaSSGF) its solubility increases (slightly) by 1.4 fold. The highest solubility of azithromycin was obtained in FaSSGF biorelevant media at pH 1.6. The solubility of azithromycin in infant fasted gastric simulating fluid (Pi-FaSSGF) was lower than the one in the respective adult FaSSGF. In intestinal simulated fluids, azithromycin presented the lowest solubility in the fasted intestinal simulating fluid (FaSSIF-V2) and the highest solubility in the fed intestinal simulating fluid (FeSSIF-V2), suggesting that buffer capacity and ionic strength affects its solubility. Drug solubility was similar in the adult and the infant fed intestinal simulating fluid (FeSSIF-V2 and Pi-FeSSIF, respectively).

Please insert Figure 2 here

Drug Solubility Classification

The dose numbers calculated for adults and pediatrics are presented in **Figure 3.** Azithromycin displays dose number lower than 1 for adults based on the drug solubility measured in USP buffers. For pediatrics, dose number was lower than 1 when calculated with USP buffers

446 solubility, whether pediatric V_0 was calculated through BW or BSA-based extrapolation, which
447 indicates that azithromycin is classified as a highly soluble compound in all age groups.
448 For pediatrics, dose numbers calculated based on azithromycin solubility in the fasted state
449 simulated intestinal fluid (FaSSIF-V2) and the BW-extrapolation method for the calculation of
450 pediatric V_0 show that azithromycin displays a dose number higher than 1 in the pediatric
451 population but not in adults. When the BSA-extrapolation method is used of extrapolation of
452 initial gastric volume, the calculated dose number was below or equal to one. The results
453 demonstrate that the BW-extrapolation leads to a more strict solubility classification, due to
454 the estimation of higher V_0, as previously reported by delMoral Sanchez *et al.* [46]. The
455 differences between dose numbers calculated with BW- and BSA-based extrapolation reinforce
456 the need for a standardised pediatric Biopharmaceutics Classification System (pBCS).
457
458 ***Please insert Figure 3 here***
459
460 **Dissolution studies**
461 **USP 4 apparatus studies**
462 *In vitro* dissolution studies of oral suspension in the USP 4 apparatus in the fasted and fed state
463 are presented in **Figure 4**.
464 In the fasted state the dissolution from azithromycin oral suspension was complete in all set-
465 ups. Dissolution was very rapid dissolution (> 85% within 15 min) in the adults' set-up. In the
466 children and infants dissolution set-ups, dissolution was rapid (> 85% within 30 min). All set-
467 ups tested more than 85% of azithromycin dissolved in the simulated gastric fluids, which is in
468 agreement with the azithromycin solubility in acidic conditions (**Figure 4**). Due to high
469 similarity of dissolution from azithromycin oral suspension between all age groups in the fasted
470 state, in the fed state, dissolution was only tested in the adults and infants set-ups. Under fed
471 state conditions, dissolution of azithromycin oral suspension with the adults set-up under the

fed state was very rapid (>85% within 5 min). A slightly slower azithromycin dissolution from the oral suspension was observed in the infants set-up under the fed state conditions (>85% within 45 min) compared to the one under the fasted state conditions. The (small) differences observed in the azithromycin dissolution rate under the fasted and fed state between the different age groups are attributed to the different *in vitro* hydrodynamics in the USP 4 apparatus (*i.e.* differences in flow-rates which reflect age-related changes in GI volumes and GI fluids composition.

Please insert Figure 4 here

inForm platform dissolution studies

In vitro fasted state dissolution studies of Zithromax® oral suspension performed in the inForm platform are presented in **Figure 4.** For all dissolution set-ups tested in the inForm platform, the dissolution rate was slower in the gastric phase and increased once the intestinal medium was added. Within one hour, approximately 80% azithromycin was dissolved in all the set-ups tested. The dissolution rate appears to be slightly faster initially in the first 30 min in the children set-up (when compared to the adult and infant dissolution profiles) and at the end of the 4h a higher dissolution extent was observed in the adult dissolution set-up. A comparison with the results of the USP 4 apparatus shows that the dissolution rate was slower in all age groups set-ups when tested in the inForm platform. Dissolution profiles obtained in the inForm platform present a high variability (coefficient of variation (CV) ranged 8 to 43 %). The inForm platform can only run one replicate at a time, each replicate had to be performed on different days, which appears to have contributed to the high CV % of the results. As azithromycin is a weak base, the dissolution studies with this set-up were performed to investigate precipitation *in vitro*. As expected from the results obtained in the solubility studies, azithromycin precipitation was not observed during the dissolution experiments.

Adult PBPK model

Intravenous Administration

The results of the simulations of the IV administration of azithromycin in adults are presented in the supplementary materials (**Figure S1**). The simulations after azithromycin IV administration were verified against the observed PK studies after IV administration of 1000, 2000, and 4000 mg doses. The predicted plasma concentration-time profiles were in good agreement with the observed plasma concentration-time profiles measured after the IV infusion administration of a wide range of azithromycin doses with all AAFE values below 1.50 (**Table S3**).

Oral Administration

Simulated plasma concentration-time profiles after oral administration of azithromycin suspension to adults in the fasted and fed state are presented in **Figure 5**. The FE, AFE and AAFE for all simulations are presented in the supplementary materials (**Table S4**). Independently of the permeability or formulation input, the simulations of the administration of azithromycin in the fasted state were able to meet the validation criteria with all AAFE values ≤ 1.60.

For the fasted state simulations, the simulation performance was very similar independently of the permeability inputs tested (AAFE for Caco-2 and MechPeff + P-gP input ranged from 1.14 and 1.52). The simulations with Caco-2 data as input predicted better the mean observed T_{max} (FE = 1.09) in the fasted state in comparison to the simulations with MechPeff + P-gP $Cl_{int,T}$ as permeability input (FE = 0.72). The simulations with MechPeff + P-gP $Cl_{int,T}$ as permeability input predicted a higher variability, reflected by larger 5^{th} and 95^{th} percentiles of the simulations when compared to simulations from the simulations with Caco-2 data. As individual PK profiles were not available in the literature, it is not possible to comment on which model is

more representative of the *in vivo* variability; the models (**Figure 5**) appear to overestimate the *in vivo* variability (predicted Cmax CV% ranged from 100-125% in comparison to an observed Cmax CV% from the Najib *et al* of approximately 38%; predicted AUC CV% was approximately 40% in comparison to the observed AUC CV% from Najib *et al* of approximately 32%)[24]. When testing the model formulation input for the fasted state (the type of dissolution or solubility data), the simulations performed very similarly on predicting the observed data (AAFE ranged from 1.31 to 1.60). No differences were observed in the predicted T_{max} (FE = 0.72) when comparing the simulation inputs of solubility (DLM-based PBPK model) and dissolution (direct entry of dissolution profiles with USP 4 apparatus and inForm platform).

Two different levels of P-gP $Cl_{int,T}$ were set between fasted and fed state, in order to simulate the inhibition of P-gP substrate by food (**Table 2**). For the fed state simulations with Caco-2 data, the *in vitro* Caco-2 data with inhibited P-gP was able to predict the observed data better than if the Caco-2 data with non-inhibited P-gP (data not shown). In the PBPK model developed with the MechPeff + P-gP $Cl_{int,T}$ data as permeability input, by fitting a new intrinsic clearance of P-gP ($Cl_{int,T}$) in the fed state, compared to the $Cl_{int,T}$ used in the fasted state, we assume a lower P-gP $Cl_{int,T}$ in the fed state (due to inhibition by food). Simulation results of oral administration of azithromycin suspension in the fed state show that both approaches for adding the permeability input in the PBPK model predicted well the observed data; all the predictions in the fed state met the validation criteria (AAFE values: 1.32 – 1.33).

Please insert Figure 5 here

Pediatric PBPK model

Intravenous Administration

Simulation results of IV infusion administration of azithromycin to pediatrics are presented in the supplementary materials (**Figure S2**). Simulations were verified against observed PK studies after IV infusion administration of azithromycin at a dose of 10 mg/Kg to infants (0.5 to 2 years), young children (2 to 5 years), old children (5 to 12 years) and adolescents (12 to 15 years). A good agreement between the simulated (obtained from the PBPK model) and the observed plasma concentration-time profiles was observed with AAFE values ≤ 1.37 (**Table S5**).

Oral Administration

Simulated and observed plasma concentration-time profiles in pediatrics after oral administration of azithromycin suspension in the fasted state are presented in **Figure 6**. The AAFE and AFE for all the simulations are presented in the supplementary materials [**Table S6** (infants and young children) and **Table S7** (old children and adolescents)]. AAFE values for all simulations in the investigated pediatric age groups are less than 1.49, indicating successful predictions. The levels of expression of intestinal P-gP from birth to adulthood have been reported to be similar to those in adults [43,47]. Therefore, the permeability input of Caco-2 data or MechPeff + P-gP $Cl_{int,T}$ data in the pediatric PBPK model were maintained at the same values as in the adult PBPK models (**Table 2**). Both simulations with Caco-2 and MechPeff + P-gP $Cl_{int,T}$ as permeability inputs successfully predicted the plasma concentration-time profiles in both pediatric age groups (AAFE values ≤ 1.49). Similarly to what was observed in the PBPK models for adults (section 3.3.2.), the best prediction of T_{max} was obtained when using the Caco-2 data as permeability input in the model. For all simulation of oral administration of azithromycin suspension, underprediction of the observed C_{max} in old

children and adolescents data was observed (FE ranged from 0.51 to 0.83). Overall, the simulations with the MechPeff + P-gP $Cl_{int,T}$ as permeability input and the solubility input (PBPK DLM-based model) provide the best prediction of the observed C_{max} in old children and adolescents (FE = 0.83).

Please insert Figure 6 here

Since pediatric patients were fasted overnight before receiving the final dose on day 5, therefore the observed PK data (obtained from the 5th day of the multiple-dose regimen) is representative of the fasted state [26,27]. After validation of the fasted state PBPK models (comparison of observed *vs* simulated PK data), prediction of the fed state was performed with the MechPeff + P-gP $Cl_{int,T}$ based input and the solubility input (DLM-based PBPK model). The fed state simulated results are presented in **Table 3**. The food effect was predicted based on the ratio of the PK parameters ($AUC_{0\text{-}inf}$ and C_{max}) of the fed state simulated plasma concentration-time profile and the PK parameters ($AUC_{0\text{-}inf}$ and C_{max}) of the observed fasted state data. Fed state simulations in pediatrics show that the C_{max} and $AUC_{0\text{-}inf}$ are within a 2-fold range when compared to the fasted state observed ones, which indicates that azithromycin food-effect in pediatrics is also not clinically significant, as it was observed in adults; a slight increase in predicted C_{max} was observed but $AUC_{0\text{-}inf}$ remained unchanged for both age groups.

Please insert Table 3 here

Parameter sensitivity analysis

The results of the PSA performed are presented in **Figure 7**. The investigated changes in gastric residence time in the range of 0.10 to 2 h did not show a substantial impact on the predicted

AUC in all the investigated populations. An increase in MGRT from 0.50 to 2 h led to a prolonged T_{max} of approximately 3 h and a decreased C_{max} by approximately 20% when compared to simulation results with baseline values.

The results of the sensitivity analysis of the passive permeability ($P_{eff,man}$) and active transport (Pg-P $Cl_{int,T}$) are presented in **Figure 7a,b.** In adults, the reduction of $P_{eff,man}$ from 1.33 x 10^{-4} cm/s (baseline value) to 0.10 x 10^{-4} cm/s resulted in a decrease of C_{max} by approximately 90% and of AUC by 76%, when compared to the simulation results using the baseline values. An increase in $P_{eff,man}$ from the baseline value (1.33 x 10^{-4} cm/s) to 3.00 x 10^{-4} cm/s led to an increase in C_{max} by 79% and a 12% increase in AUC. A reduction in P-gP $Cl_{int,T}$ from 200 µL/min (baseline value for the fasted state) to 10 µL/min resulted in an increase of predicted C_{max} by approximately 60% in adults. Similar trends are observed in both of the pediatric groups investigated for $P_{eff,man}$ and P-gP $Cl_{int,T}$. Overall, the PSA results show that the AUC and the C_{max} are influenced the most by permeation through the intestinal membrane, and both passive and active transport control the absorption of azithromycin in both adults and pediatrics.

The impact of solubility in intestinal fluids on azithromycin PK was further investigated through PSA (**Figure 8** and **Figure S3**). Similar results were observed for PSA of solubility in the gastric fluids and its impact of PK (data not shown). Based on the PSA results, it can be concluded that azithromycin's solubility in the gastrointestinal fluids is not the rate-limiting step for its absorption for the investigated therapeutic dose range for adults and pediatrics. This finding sheds light on the BCS classification of azithromycin in terms of solubility, suggesting that it is indeed a highly-soluble compound.

Please insert Figure 7 here

622 ***Please insert Figure 8 here***

623

624 **Discussion**

625 PBPK absorption models have the potential to perform exploratory analysis to clarify the
626 critical parameters of drug absorption in the different pediatric age groups [11]. In this study, the
627 impact of critical variables on azithromycin's oral absorption were evaluated by coupling *in*
628 *vitro* (age-biorelevant *in vitro* solubility and dissolution) and *in silico* methods. A general
629 schematic outlining the strategy followed in this manuscript for the biopharmaceutic risk
630 assessment of azithromycin critical parameters for absorption is presented in **Figure 9.**
631 Based on the solubility measurements a change in the solubility classification from adults to
632 pediatrics was seen when the dose number was calculated with biorelevant solubility in
633 FaSSIF-V2 and initial pediatric gastric volume calculated with the BW-extrapolation approach.
634 BW-based extrapolation and BSA-based extrapolation of initial gastric volumes can lead to
635 differences in the solubility classification of a drug. This demonstrates that standardization is
636 needed in the future on the best strategy to assess age-related changes of the solubility
637 classification of drugs. Although useful in theory, the establishment of a pBCS is not
638 straightforward since it is unlikely that the necessary scrutiny between the pediatric groups is
639 achieved while still maintaining the desirable simplistic character as observed for the adult
640 BCS. The need to take into account multiple doses for more than one age group can lead to
641 multiple classifications across the pediatric age range. The necessary differentiation of age
642 groups cannot be achieved without a certain degree of complexity, in which case, a strategy
643 coupling mechanistic PBPK absorption modeling with *in vitro* data can become a more
644 powerful tool. Therefore, to further clarify/assess the risk of age-related changes in the
645 solubility classification of azithromycin, an adult and pediatric PBPK model was built.
646 In adults, an increase of short duration (persists for less than 4 h) in C_{max} (1.6-fold) in
647 comparison to the observed fasted state C_{max} is observed [22]. In this study, we hypothesised that

this increase in C_{max} after oral administration of azithromycin in the fed state could be attributed to the inhibition of P-gP (an efflux transporter). As a P-gP substrate, azithromycin permeates through the gut wall and is partially pumped back into the gastrointestinal lumen. If P-gP is inhibited, an increase in the net permeability is seen and therefore an increase in the extent of absorption would be observed [48]. The interaction of transporters with food has been previously discussed in the literature [37-39], and a high-fat meal is suspected to inhibit P-gP [38]. Evidence to support this scientific statement lays in several studies which showed that food components present in gastrointestinal fluids as a result of the ingestion of high-fat meals affected the *in vitro* transport of P-gP substrates [49-51]. Konishi and co-workers have shown that a variety of monoglycerides and fatty acids were capable of inhibiting P-gP mediated efflux of rhodamine-123 and daunomycin in Caco-2 cells [49,50]. The prediction of food effects that are caused by the interaction of food components with intestinal transporters (influx or efflux) is complex due to the inability to accurately measure the inhibitory effect caused by the food component [37].

Please insert Figure 9 here

Simulation results of oral administration of azithromycin suspension in the fed state showed that both investigated permeability inputs [Caco-2 data (with P-gP inhibitor), or MechPeff + P-gP $Cl_{int,T}$ permeability] in the PBPK model resulted in a good prediction of the small increase in the *in vivo* observed C_{max} in adults. When performing pediatric simulations, the 'assumption' of food-inhibited P-gP was also applied to the simulated pediatric age groups. The translation of this mechanism to the pediatric simulations predicted a small increase in C_{max} (below 2-fold ratio when compared to the fasted state-observed C_{max}) and AUC remained unchanged. However, fed state data was not available in pediatric age groups to further validate these predictions.

673 Our hypothesis that the increase in Cmax after oral administration of azithromycin in the fed
674 state could be attributed to the inhibition of P-gP (an efflux transporter), was investigated by a
675 simple approach and the P-gp CLin,T was fitted in the model. It was beyond the scope of this
676 study to fully validate this hypothesis using DDI and/or polymorphism data. The contribution
677 of P-gP in the absorption of azithromycin should be further investigated in the future; since
678 azithromycin is a weak base with high lipophilicity (logP = 4[30]), some of its absorption features
679 could potentially be explained by other mechanisms such as lysosomal trapping.
680 After the PBPK model was built and validated in the populations of interest, PSA were
681 performed to identify the critical parameters influencing absorption of azithromycin.
682 Sensitivity analysis showed that azithromycin's solubility in the gastrointestinal fluids was not
683 the rate-limiting step for its absorption for adults and pediatrics in the investigated therapeutic
684 dose range. This finding sheds light on the BCS classification of azithromycin, which suggests
685 that azithromycin is indeed a highly-soluble compound in adults, and therefore belongs to
686 either BCS class I or III. Since sensitivity analysis in pediatrics also showed that azithromycin's
687 solubility in the gastrointestinal fluids was not the rate-limiting step for its absorption, there is
688 a low risk of changes in solubility class when extrapolating the solubility class from adults to
689 pediatrics. The results of the PSA showed that the C_{max} and the AUC were influenced by
690 permeation through the intestinal membrane, and both passive and active transport control the
691 absorption of azithromycin in adults and pediatrics. These results suggest that azithromycin
692 belongs to BCS class III in adults in the investigated dose range [52].
693 Consensus regarding the maturation of permeability in younger pediatric subgroups (especially
694 newborns) is still lacking [2,4,43]. As a result, it is still unclear at which age adult permeability
695 values are reached [43,47]. Permeability changes as a function of age could occur as a consequence
696 of the morphological development of villi and microvilli in the small intestine, which results
697 in a reduced surface area for absorption in younger pediatric patients [2,4,43]. Literature suggests

698 that passive permeability is developed by the age of 4 months, with general agreement of full
699 maturity by the age of 2 years old [2,4,43]. Although some contradictory information is available
700 on P-gP after birth, it is generally accepted that the levels of expression of intestinal P-gP from
701 birth to adulthood are likely to be similar to adults [2,4,43]. In the developed PBPK model,
702 azithromycin $P_{eff,man}$ or P-gP $Cl_{int,T}$ values were not changed when the adult model was
703 translated to the pediatrics. A similar degree of sensitivity to permeability-related parameters
704 was observed for the investigated pediatric age groups and adults. These results indicate that if
705 the adult permeability classification criterion is still applicable then no changes in permeability
706 classification criteria are expected between adults and pediatrics. Since a pBCS is not currently
707 available, a pediatrics biopharmaceutics classification of azithromycin is not feasible.
708 However, results of the PBPK model and PSA (of the impact of solubility and permeation
709 parameters on the PK of azithromycin) show that there is a low biopharmaceutics risk
710 associated with the extrapolation of the adult BCS class III of azithromycin to pediatrics in the
711 investigated dose range. Future studies could explore higher therapeutic doses in adults and
712 pediatrics, since adult doses for certain complications can be as high as 2 g and for pediatrics
713 doses higher than 10 mg/Kg are described in the British National Formulary for Children
714 (BNF-C). It should be noted that no clinical data was found for the administration of the higher
715 doses of azithromycin suspension in adults and/or pediatrics [53].
716 Regulatory authorities have recognised the potential of PBPK modeling for predictions in
717 pediatrics [47,54,55]. PBPK modeling coupled with *in vitro* data (solubility/dissolution studies) has
718 a huge potential to support the development of pediatric medicines, therefore, continuous
719 improvement of these pediatric biopharmaceutic tools with high-quality physiological and
720 clinical data is essential.
721

Conclusions

In this study, PBPK modeling and *in vitro* tools (solubility and dissolution studies) were used for identifying the critical variables affecting the oral absorption of azithromycin in adults and pediatrics.

Differences were observed in the solubility classification of azithromycin when using buffers and biorelevant media and according to two extrapolation methods of pediatric initial gastric volume, which reinforces the need for a standardised strategy that can be used during pediatric drug development to understand age-related changes in oral drug absorption. A mechanistic investigation of the oral absorption of azithromycin with PBPK modeling was able to clarify that azithromycin solubility is not a limiting step for adults and the investigated pediatric age groups, for the therapeutic dose range used in each group. The PBPK modeling approach revealed that permeation through the gut wall is the key driver of azithromycin oral absorption in both adults and pediatrics. The absorption process is similar in the pediatric age groups investigated in this study compared to adults as passive and transport mediated processes are likely to be similar in these populations.

Overall, the PBPK modeling approach followed can be used to increase the understanding of the critical parameters of absorption in pediatrics, especially when coupled with age-relevant *in vitro* methods.

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List of Tables

Table 1 Dissolution conditions used in the experiments performed in the USP 4 apparatus and the inForm platform.

USP 4 apparatus – fasted state									
Age groups	**Clinical Dose (mg)**	**Gastric residence time (min)**	**Gastric flow-rate (ml/min)**	**Volume (mL)**	**Biorelevant media gastric compartment**	**Intestinal residence time (min)**	**Intestinal flow-rate (ml/min)**	**Volume (mL)**	**Biorelevant media intestinal compartment**
Infants	120	0-30	4	120	Pi-FaSSGF	30-270	3	720	FaSSIF-V2
Children	120	0-30	8	240	FaSSGF	30-270	4	960	FaSSIF-V2
Adults	120	0-30	12	360	FaSSGF	30-270	4	960	FaSSIF-V2
USP 4 apparatus – fed state									
Age groups	**Clinical Dose (mg)**	**Gastric residence time (min)**	**Gastric flow-rate (ml/min)**	**Volume (mL)**	**Biorelevant media gastric compartment**	**Intestinal residence time(min)**	**Intestinal flow-rate (ml/min)**	**Volume (mL)**	**Biorelevant media intestinal compartment**
Infants	120	0-120	3	360	FeSSGF	120-360-	4	960	Pi-FeSSIF
Adults	120	0-90	8	720	FeSSGF	90-330	6	1440	FeSSIF-V2
inForm platform– fasted state									
Age groups	**Clinical Dose (mg)**	**Down-scaled Dose (mg)**	**Gastric residence time (min)**	**Volume (mL)**	**Biorelevant media gastric compartment**	**Intestinal residence time (min)**	**Volume (mL)**		**Biorelevant media intestinal compartment**
Infants	130	69	0-30	40	Pi-FaSSGF	30-270	80		FaSSIF-V2
Children	300	120	0-30	40	FaSSGF	30-270	80		FaSSIF-V2
Adults	500	80	0-30	40	FaSSGF	30-270	80		FaSSIF-V2

915 **Table 2.** Summary of azithromycin input parameters used in the Simcyp® simulator.

Input parameter	Value	Reference
Physicochemical Properties and Blood Binding		
Molecular weight (g/mol)	749	[30]
logP (experimental)	4	[30]
Compound type	Weak Base	[30]
pKa_1	8.6	[31]
Blood: plasma ratio	1	[32]
$f_{u,p}$	0.69	[32]
Distribution		
Model	Minimal PBPK	
K_{in} (L/h)	0.93	[21]
K_{out} (L/h)	0.50	[21]
V_{sac} (L/Kg)	23	[21]
V_{ss} (L/Kg)	32	[21]
Elimination		
CL_{IV} (L/h)	46.5	[32]
Cl_{int} (bile) (µL/min/10^6)	9.25	[32]
Cl_{renal}(L/h)	8.67	[32]
Absorption		
Model	ADAM™	
	MechPeff+ P-gP $Cl_{int,T}$ input	
$P_{eff,man}$ [10^{-4} cm/s]	1.33	Predicted by Simcyp® Mech Peff model, used in all compartments [predicted $P_{trans,0}$ = 1100]
$Cl_{int,T}$ (µL/min) - Fasted state	200	Assumed - user selected after performing sensitivity analysis [against data from Foulds *et al*[22]]
$Cl_{int,T}$ (µL/min) - Fed state	10	
	Caco-2 input	
$P_{eff,man}$ [10^{-4} cm/s], fasted state	0.29	Calculated from Caco-2 data (7.4:7.4) untreated with inhibitor, inputted as 'Passive & Active' (P_{app} 2.2 nm/s [36])
$P_{eff,man}$ [10^{-4} cm/s], fed state	0.71	Calculated from Caco-2 data (7.4:7.4) treated with a P-gP inhibitor, inputted as 'Passive' (P_{app} 16.7 nm/s [36])
Formulation	azithromycin oral suspension	NA
Diffusion Layer Model (DLM)	Segmental solubility option with age-appropriate biorelevant solubility in fasted/fed state	NA
Solubility fasted state in adults, and children and adolescents (mg/mL)	13 (gastric); 2 (intestinal)	
Solubility fasted state in infants and young children (mg/mL)	12 (gastric); 2 (intestinal)	

Solubility fed state in all age-groups (mg/mL)	10 (gastric); 6 (intestinal)	
Dissolution Profile Model (DPM)	Age-appropriate dissolution profiles measured with USP 4 apparatus and inForm platform, entered as discrete profiles	NA

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Table 3. Simulated mean PK parameters of azithromycin in pediatrics in the fed and fasted state and predicted food effect (by comparison of predicted fed state PK parameters with fasted state-observed PK parameters).

Age group	PK Parameter	Observed Value*	Fasted state Predicted Value*	Fed state Predicted Value*	Food effect [Ratio (Pred/Obs)]
Older Children and Adolescents	T_{max} (h)	2.00	1.20	1.80	0.90
	C_{max} (ng/mL)	360	298	305	0.85
	$AUC_{0\text{-}inf}$ (ng/mL.h)	4564	4218	4495	0.98
Infants and Young Children	T_{max} (h)	2.00	1.20	1.80	0.90
	C_{max} (ng/mL)	200	231	265	1.32
	$AUC_{0\text{-}inf}$ (ng/mL.h)	3438	3485	3772	1.10

* after the administration of the last dose on the 5th day of a 5-day oral regimen (10 mg/Kg on the 1st day and 5 mg/Kg day 2 to 5).

Figure Captions

Figure 1. Schematic representation of the workflow describing the PBPK development for azithromycin. Abbreviations: Parameter Estimation (PE), Parameter sensitivity analysis (PSA), Dissolution Profile Model (DPM) and Diffusion Layer Model (DLM).

Figure 2. Mean solubility (± Standard Deviation (SD)) in buffers (white bars) and biorelevant media (grey bars) at 37 °C for 24 h.

Figure 3. Adult (> 18 years) and Pediatric (0.1 to 14 years) dose numbers calculated for azithromycin according to solubility in buffers and biorelevant media. In pediatrics, initial gastric volumes (V_0) were extrapolated with a BW- or a BSA-based calculation. The horizontal line indicates a dose number = 1. A dose number > 1 indicates a low solubility compound, and a dose number < 1 indicates the opposite.

Figure 4. Mean % azithromycin dissolved (± SD) from Zithromax® oral suspension. Straight vertical lines represent the time for media change in the fasted state in all age groups set-ups ; dashed and dotted vertical lines represent the time for media change in the fed state in adults and infants set-up, respectively.

Figure 5. Simulated azithromycin plasma concentration-time profiles (solid line: population mean; dashed lines: 5^{th} and 95^{th} percentile of the population) in healthy adult subjects after administration of a single dose of 500 mg of azithromycin oral suspension in the fasted and the fed state against observed data [22-24] [simulations were performed with MechPeff + P-gP $Cl_{int,T}$ or Caco-2 data as permeability input; for the formulation input, simulations were performed either with Diffusion Layer Model (DLM) using solubility as input, or direct input of dissolution with the dissolution profile model (DPM) (input of age-appropriate dissolution profiles that were obtained with USP 4 apparatus or inForm platform)].

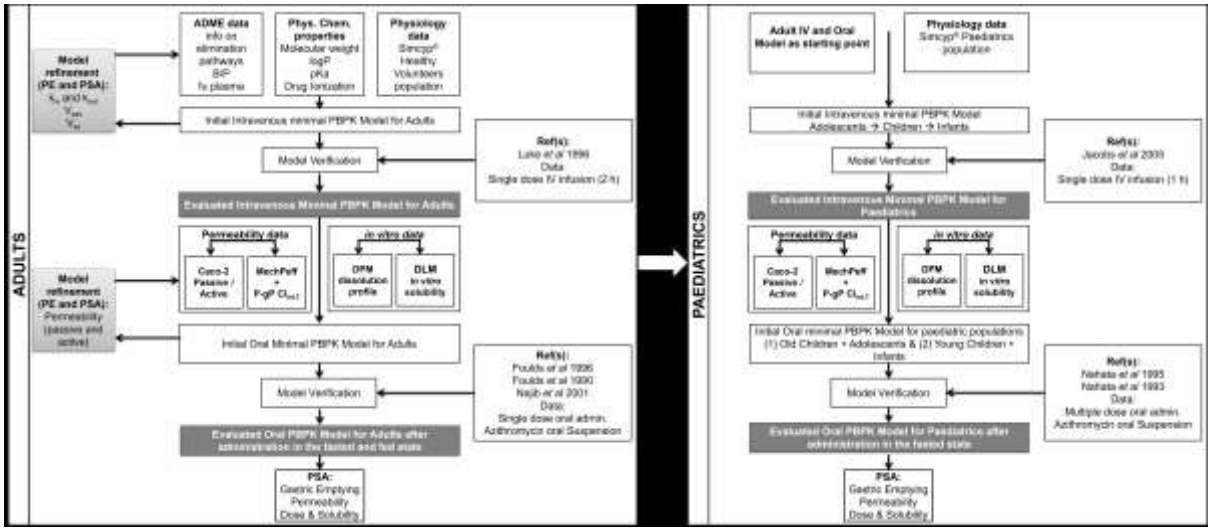
Figure 6. Simulated azithromycin plasma concentrations-time profiles (solid line: population mean; dashed lines: 5^{th} and 95^{th} percentile of the population) on the 5^{th} day after oral administration of azithromycin as an oral suspension in a multiple-dose regimen of 10 mg/Kg (day 1) and 5 mg/Kg (day 2 to 5) to infants and young children (0.5 to 5 years) and old children and adolescents (6 to 15 years). The final dose was administered after an overnight fast [26,27] [simulations were performed with MechPeff + P-gP $Cl_{int,T}$ or Caco-2 data as permeability input. For the formulation input, simulations were performed either with Diffusion Layer Model (DLM) using solubility as input, or direct input of dissolution with the dissolution profile model (DPM) (input of age-appropriate dissolution profiles that were obtained with USP 4 apparatus or inForm platform)].

Figure 7. Parameter sensitivity analysis for azithromycin after administration of an oral suspension for C_{max} and AUC [(a) and (b), respectively] as a function of effective permeability ($P_{eff,man}$) and P-gP $Cl_{int,T}$; and (c) sensitivity analysis of C_{max} and AUC to mean gastric residence time (MGRT). Values used in the PBPK model are shown in red (*i.e.* baseline simulation).

Figure 8. Parameter sensitivity analysis for the C_{max} and AUC obtained after administration of azithromycin oral suspension to adults and infants and young children as a function of dose (adults – 250 to 600 mg; pediatrics – 40 to 220 mg) and duodenal solubility (range from 1 to 27 mg/mL).

Figure 9. Schematic summarizing the steps followed to perform the risk assessment investigation of the critical variables of azithromycin's oral absorption.

Figure 1


ADULTS
ADME data
info on elimination pathways
BP
fu plasma
Phys. Chem. properties
Molecular weight
logP
pKa
Drug formulation
Physiology data
Simcyp® Healthy Volunteers population
Model refinement (PE and PSA):
Initial Intravenous minimal PBPK Model for Adults
Model Verification
Ref(s):
Luke et al 1999
Data
Single dose IV infusion (2 h)
Evaluated Intravenous Minimal PBPK Model for Adults
Permeability data
in vitro data
Caco-2 Passive / Active
MechPeff + P-gP CLint
DPM dissolution profile
DLM in vitro solubility
Model refinement (PE and PSA):
Permeability (passive and active)
Initial Oral Minimal PBPK Model for Adults
Model Verification
Ref(s):
Foulds et al 1996
Foulds et al 1999
Najib et al 2001
Data:
Single dose oral admin. Azithromycin oral Suspension
Evaluated Oral PBPK Model for Adults after administration in the fasted and fed state
PSA:
Gastric Emptying
Permeability
Dose & Solubility
PAEDIATRICS
Adult IV and Oral Model as starting point
Physiology data
Simcyp® Paediatrics population
Initial Intravenous minimal PBPK Model Adolescents → Children → Infants
Model Verification
Ref(s):
Jacobs et al 2000
Data:
Single dose IV infusion (1 h)
Evaluated Intravenous Minimal PBPK Model for Paediatrics
Permeability data
in vitro data
Caco-2 Passive / Active
MechPeff + P-gP CLint
DPM dissolution profile
DLM in vitro solubility
Initial Oral minimal PBPK Model for paediatric populations (1) Old Children + Adolescents & (2) Young Children + Infants
Model Verification
Ref(s):
Nahata et al 1995
Nahata et al 1993
Data:
Multiple dose oral admin. Azithromycin oral Suspension
Evaluated Oral PBPK Model for Paediatrics after administration in the fasted state
PSA:
Gastric Emptying
Permeability
Dose & Solubility

967 **Figure 2**

968

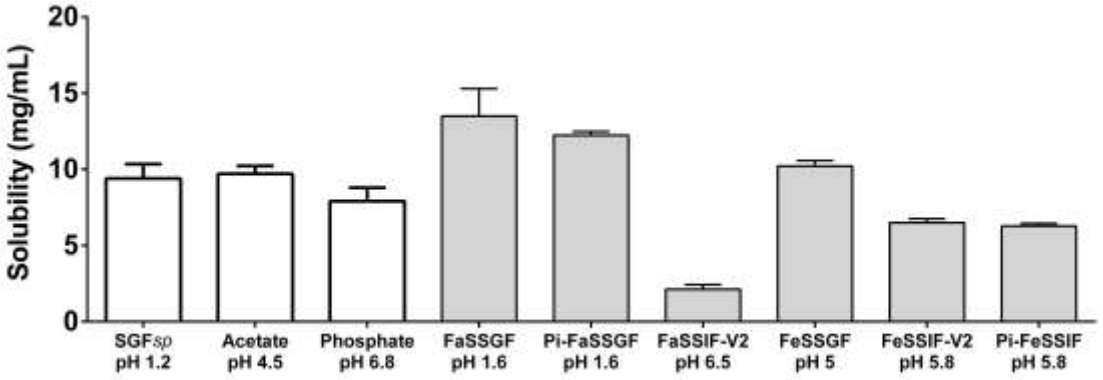


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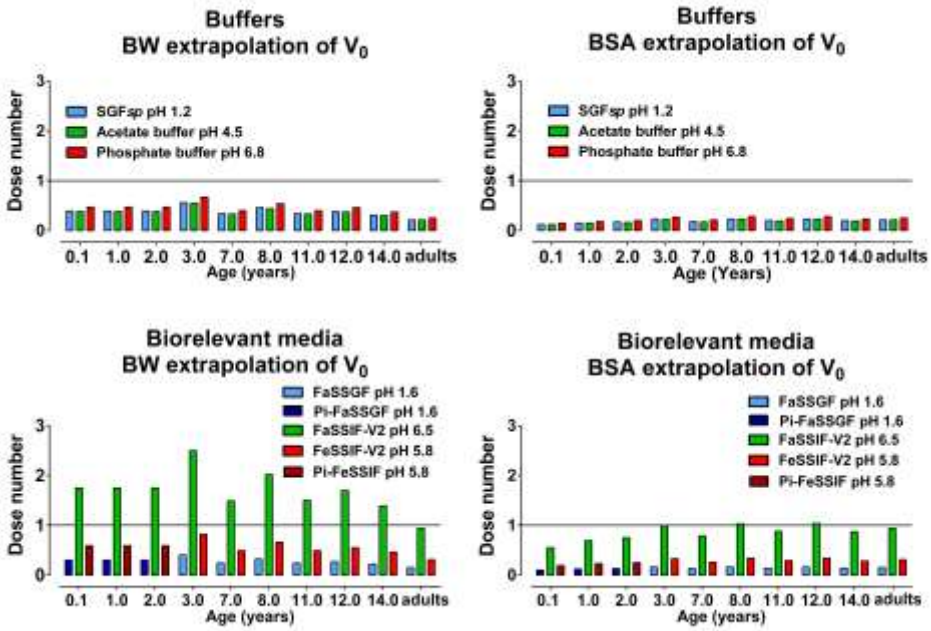
Figure 3


Buffers
BW extrapolation of V0
SGFsp pH 1.2
Acetate buffer pH 4.5
Phosphate buffer pH 6.8
Dose number
0.1 1.0 2.0 3.0 7.0 8.0 11.0 12.0 14.0 adults
Age (years)
Buffers
BSA extrapolation of V0
SGFsp pH 1.2
Acetate buffer pH 4.5
Phosphate buffer pH 6.8
Dose number
0.1 1.0 2.0 3.0 7.0 8.0 11.0 12.0 14.0 adults
Age (Years)
Biorelevant media
BW extrapolation of V0
FaSSGF pH 1.6
Pi-FaSSGF pH 1.6
FaSSIF-V2 pH 6.5
FeSSIF-V2 pH 5.8
Pi-FeSSIF pH 5.8
Dose number
0.1 1.0 2.0 3.0 7.0 8.0 11.0 12.0 14.0 adults
Age (years)
Biorelevant media
BSA extrapolation of V0
FaSSGF pH 1.6
Pi-FaSSGF pH 1.6
FaSSIF-V2 pH 6.5
FeSSIF-V2 pH 5.8
Pi-FeSSIF pH 5.8
Dose number
0.1 1.0 2.0 3.0 7.0 8.0 11.0 12.0 14.0 adults
Age (years)

977 **Figure 4**

978

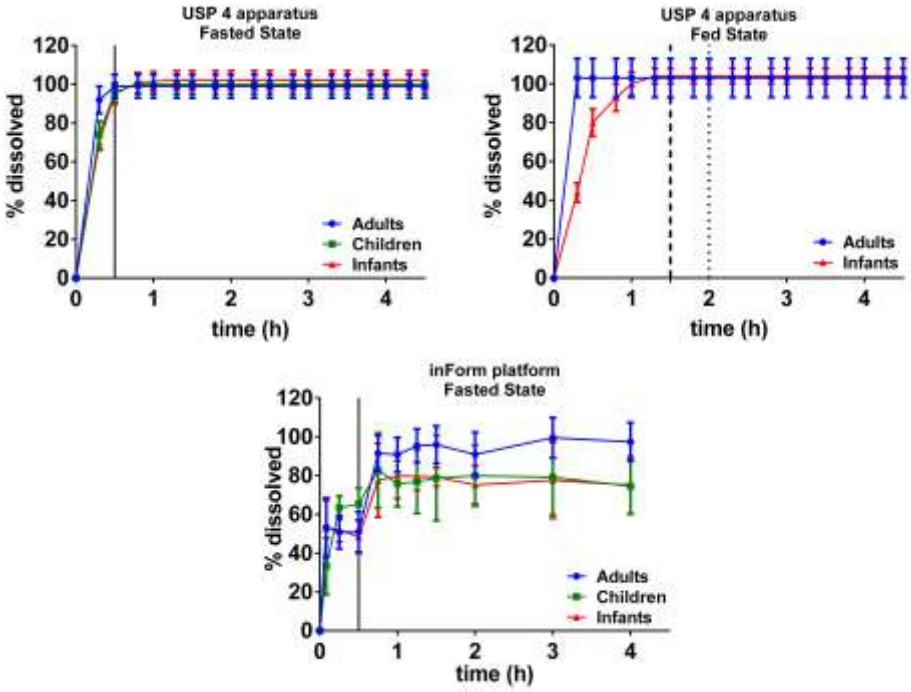


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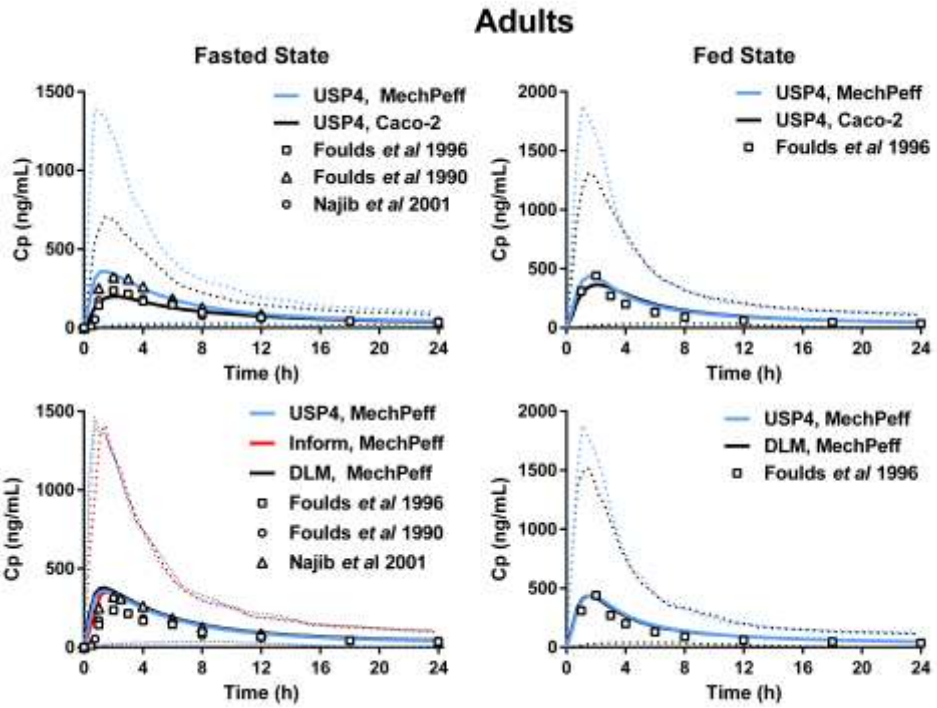
Figure 5


Adults
Fasted State
Fed State
USP4, MechPeff
USP4, Caco-2
Foulds et al 1996
Foulds et al 1990
Najib et al 2001
Inform, MechPeff
DLM, MechPeff
Cp (ng/mL)
Time (h)

985 **Figure 6**

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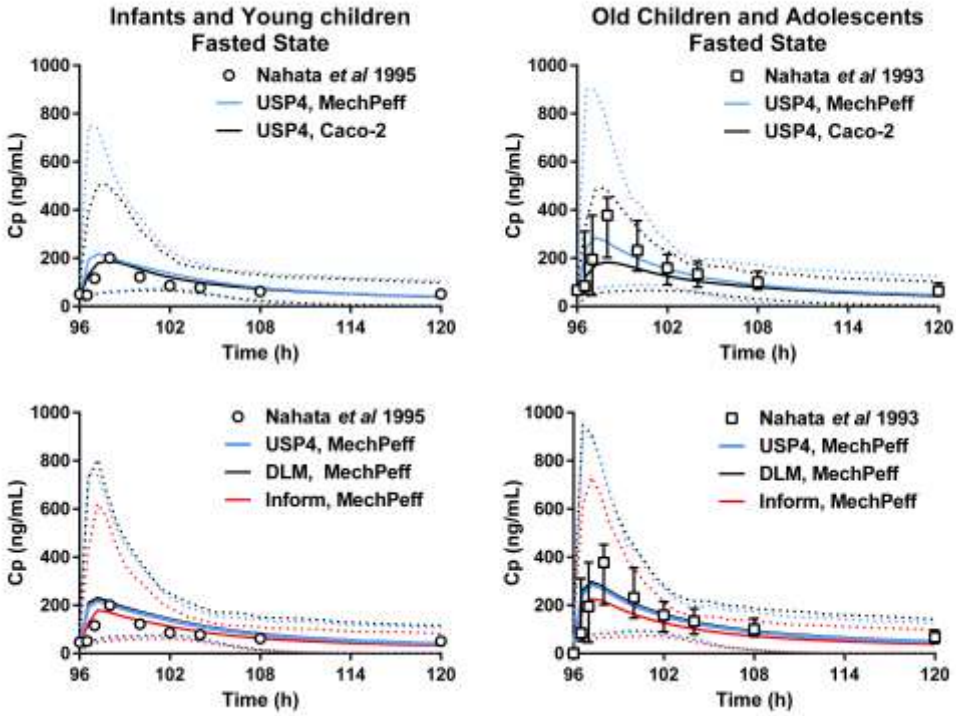
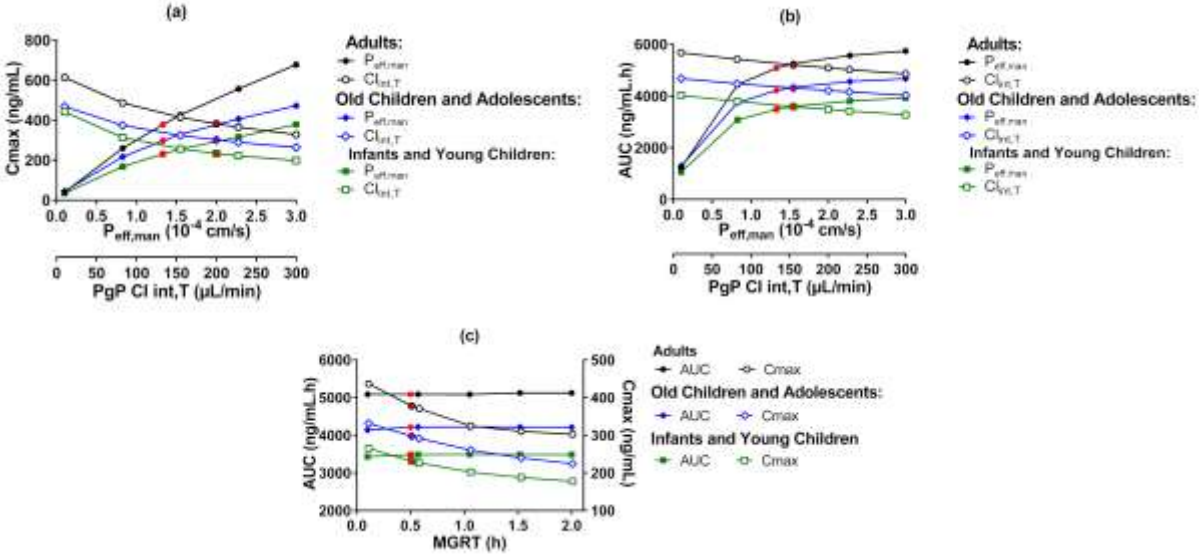


987

988

Figure 7


(a)
Cmax (ng/mL)
Peff,man (10-4 cm/s)
PgP Cl int,T (µL/min)
Adults:
Old Children and Adolescents:
Infants and Young Children:
(b)
AUC (ng/mL.h)
Peff,man (10-4 cm/s)
PgP Cl int,T (µL/min)
Adults:
Old Children and Adolescents:
Infants and Young Children:
(c)
AUC (ng/mL.h)
Cmax (ng/mL)
MGRT (h)
Adults
AUC
Cmax
Old Children and Adolescents:
Infants and Young Children

994 **Figure 8**

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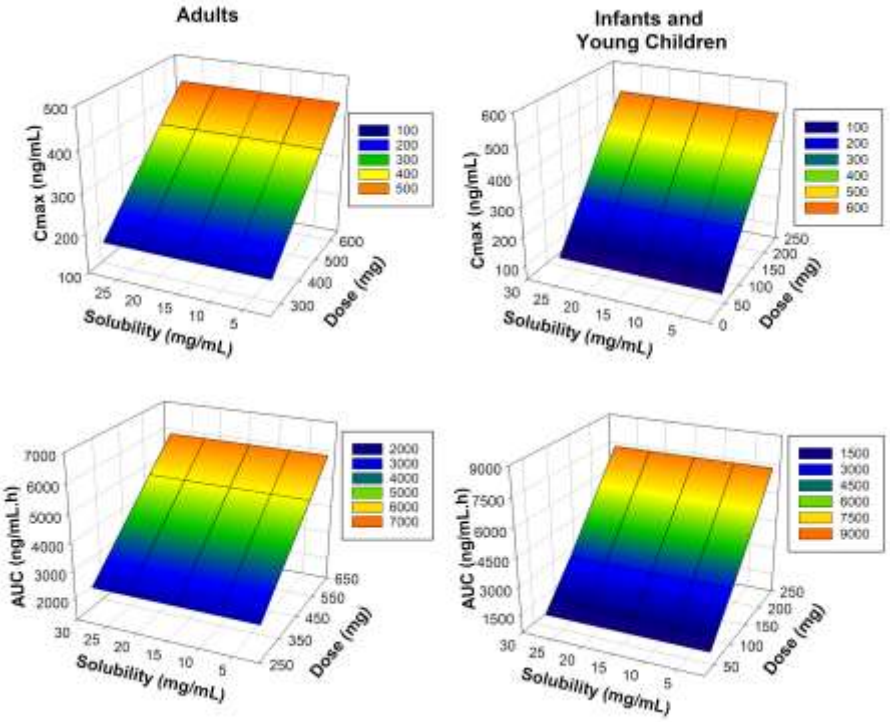

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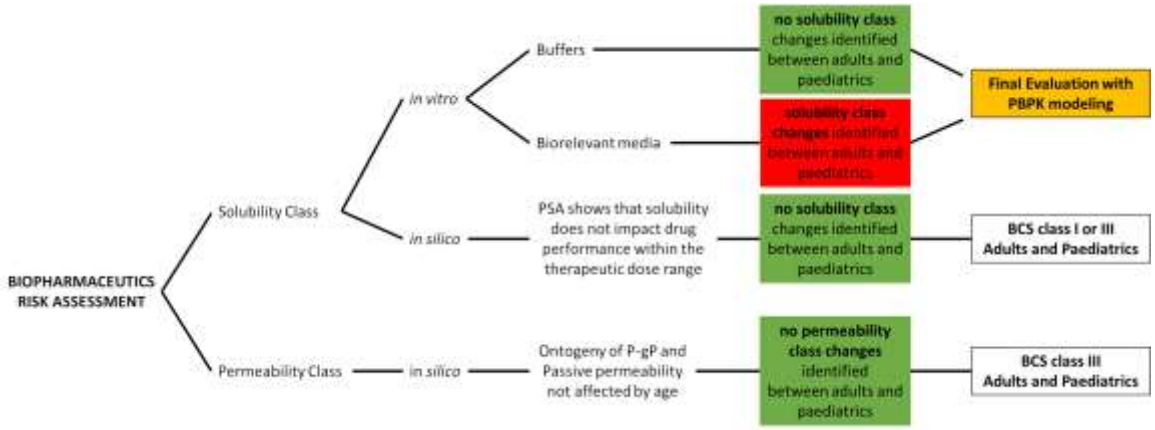
Figure 9


BIOPHARMACEUTICS RISK ASSESSMENT
Solubility Class
Permeability Class
in vitro
in silico
in silico
Buffers
Biorelevant media
PSA shows that solubility does not impact drug performance within the therapeutic dose range
Ontogeny of P-gP and Passive permeability not affected by age
no solubility class changes identified between adults and paediatrics
solubility class changes identified between adults and paediatrics
no solubility class changes identified between adults and paediatrics
no permeability class changes identified between adults and paediatrics
Final Evaluation with PBPK modeling
BCS class I or III Adults and Paediatrics
BCS class III Adults and Paediatrics