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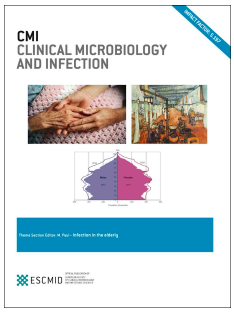

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PII: S1198-743X(21)00407-9

DOI: https://doi.org/10.1016/j.cmi.2021.07.015

Reference: CMI 2622

To appear in: *Clinical Microbiology and Infection*

Received Date: 31 January 2021

Revised Date: 4 July 2021

Accepted Date: 8 July 2021

Please cite this article as: Ruth MM, Raaijmakers J, van den Hombergh E, Aarnoutse R, Svensson EM, Susanto BO, Simonsson US, Wertheim H, Hoefsloot W, van Ingen J, Standard therapy of *Mycobacterium avium* complex pulmonary disease shows limited efficacy in an open source hollow fiber system that simulates human plasma and epithelial lining fluid pharmacokinetics, *Clinical Microbiology and Infection*, https://doi.org/10.1016/j.cmi.2021.07.015.

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Standard therapy of *Mycobacterium avium* complex pulmonary disease shows limited efficacy in an open source hollow fiber system that simulates human plasma and epithelial lining fluid pharmacokinetics

Short running title: MAC-PD therapy has poor activity

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Length of abstract: 242 words. Length of manuscript: 2599 words

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Abstract

Objectives

Mycobacterium avium complex (MAC) bacteria can cause chronic pulmonary disease (PD). Current treatment regimens of azithromycin, ethambutol and rifampicin have culture conversion rates of around 65%. Dynamic, pre-clinical models to assess the efficacy of treatment regimens are important to guide clinical trial development. The hollow fiber system (HFS) has been applied but reports lack experimental details.

Methods

We simulated the human pharmacokinetics of azithromycin, ethambutol and rifampicin both in plasma and epithelial lining fluid (ELF) in a hollow fiber system (HFS), exposing THP-1 cells infected with *M. avium* to the triple-drug regimen for 3 weeks. We accounted for drug-drug interactions and protein-binding and provide all laboratory protocols. We differentiated the effects on the intracellular and extracellular mycobacterial population.

Results

The antibiotic concentrations in the HFS accurately reflected the time to peak concentration (T_{max}), the peak concentration (C_{max}), and half-life of azithromycin, rifampicin and ethambutol in plasma and ELF reported in literature. We find that plasma drug concentrations fail to hold the MAC bacterial load static (ΔLog10 CFU/ml$_{Control:Regimen}$ = 0.66 ± 0.76 and 0.45 ± 0.28 at 3 and 21 days); ELF concentrations do hold the bacterial load static for 3 days and inhibit bacterial growth for the duration of the experiment (ΔLog10 CFU/ml$_{Control:Regimen}$ = 1.1 ± 0.1 and 1.64 ± 0.59 at 3 and 21 days).

Conclusions

In our model, the current therapy against MAC is ineffective, even when accounting for antibiotic accumulation at the site of infection and intracellularly. New treatment regimens need to be

45 developed and be compared to currently recommended regimens in dynamic models prior to clinical
46 evaluation. With the publication of all protocols we aim to open this technology to new users.

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Introduction

Mycobacterium avium complex (MAC) bacteria cause chronic pulmonary disease (MAC-PD) in susceptible patient populations [1]. The recommended antibiotic treatment of MAC-PD is a combination of azithromycin, ethambutol and rifampicin for at least 12 months after negative sputum culture [2]. The cure rates of this regimen are around 65%, with considerable relapse rates [3]. Azithromycin is the active core of the regimen; rifampicin and ethambutol have little bactericidal effect on their own but suppress the emergence of macrolide resistance [2,4].

In a relatively rare disease such as MAC-PD, treated with old generic antibiotics, few clinical trials have been performed [2]. Preclinical models are important screening tools to help select regimens for clinical trials. *In vitro* studies of MAC-PD treatment often expose planktonic bacteria to a multiplicity of the minimum inhibitory concentration of antibiotics, alone or together with companion drugs [5,6] but this approach does not mimic the dynamic drug exposures at the site of infection in the context of a multi-drug regimen. The hollow fiber system model (HFS) is a more sophisticated animal-free model in which intracellular infections can be achieved and pharmacokinetics at the site of infection can be simulated to closely mimic MAC-PD treatment. The HFS has been used to search for new MAC-PD regimens, but previous studies lacked experimental detail and have failed to address four key issues: 1) to simulate the currently recommended azithromycin-ethambutol-rifampicin regimen as a point of reference; 2) to simulate the pharmacokinetics not only in blood plasma but also in lung epithelial lining fluid (ELF) which is more representative of the site of infection; 3) to account for protein binding of the drugs in these matrices [7–10]; and 4) to simulate drug penetration into immune cells such as macrophages.

Here, we describe a HFS experiment modelling MAC-PD and exposure to azithromycin, rifampicin and ethambutol concentrations observed in human plasma as well as those in ELF and measure the antimycobacterial activity of this regimen, with full disclosure of the methodology and calculations.

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Materials & Methods

Antibiotics, bacteria and cells

Azithromycin, ethambutol and rifampicin were purchased from Sigma Aldrich (Zwijndrecht, the Netherlands) *Mycobacterium avium* ATCC 700898 reference strain was acquired from ATCC (Molsheim Cedex, France) and freshly cultured before each experiment; the minimum inhibitory concentrations of study drugs (rifampicin, ethambutol and azithromycin) were determined by broth microdilution according to CLSI guidelines [11]. THP-1 cells were acquired from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany; ACC 16 Lot 32) and freshly cultured before each experiment in RPMI1640 with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Breda, the Netherlands) at 37°C and 5% CO_2.

Pharmacokinetic profiles simulated in the hollow fiber model

We simulated two distinct pharmacokinetic profiles: protein-unbound (free, active) plasma drug concentrations and free ELF drug concentrations versus time after dosing. In plasma, we assumed the following protein bound fractions: rifampicin 90% [12,13] ethambutol 10% [13] and azithromycin 25% [14]. Protein concentrations in the ELF are significantly lower than in plasma [15], and therefore we assumed that protein binding in ELF is negligible [9].

Plasma concentrations, half-lives and time to the highest drug concentration (T_{max}) of rifampicin were simulated after van Ingen *et al.* [16] and Magis-Escurra *et al* cohort of MAC-PD patients [17]. Both sources assessed total (protein-bound + unbound) plasma concentrations and pharmacokinetic data were adjusted for protein binding to achieve protein-unbound pharmacokinetic measures. Half-lives and T_{max} of ethambutol and azithromycin were obtained from Magis-Escurra *et al.* and van Ingen *et al.* As no full pharmacokinetic curves are available for ELF concentrations, we extrapolated these from available data. Rifampicin ELF kinetics were based on the model published by Clewe *et al.* that estimates the distribution from plasma to ELF in tuberculosis patients [18] acknowledging the non-

linear pharmacokinetics and auto-induction of rifampicin in plasma. Based on this, we assumed the free plasma:ELF distribution to be 1:1 assuming again a free fraction of 10% for rifampicin in plasma and negligible protein binding in ELF. Only one study assessed ethambutol ELF concentrations at 4 hours after intake [19], which we used as a baseline for calculations assuming rapid equilibration between plasma and ELF and the same half-life and T_{max} as in plasma, assuming an ELF T_{max} of 3 hours. Azithromycin ELF steady-state concentrations as described in detail by Olsen *et al.* [20] were used as basis for T_{max} and half-life in ELF. For all regimens, we assumed simultaneous, once-daily intake of medication. An overview of simulated pharmacokinetic parameters is shown in table 1.

Table 1: Overview of pharmacokinetic parameters simulated in the hollow fiber system

Free plasma concentration					Free ELF Concentration				
Drug	C_{max} (mg/L)	T_{max} (h)	Half-Life (h)	Reference	Drug	C_{max} (mg/L)	T_{max} (h)	Half-Life (h)	Reference
Azithromycin	0.16	5	11	19; 20	Azithromycin	3.0	10	20	23
Ethambutol	2.4	3	10	19; 20	Ethambutol	3.0	3	10	22
Rifampicin	1.5	2	2	19; 20	Rifampicin	1.5	2	2	21

Hollow Fiber System

All experiments were performed in triplicate and in RPMI1640 (BioWest, Haarlem, the Netherlands) with 2% FBS broth medium. Hollow fiber systems were incubated at 37°C and 5% CO_2. Hollow fiber Cellulosic cartridges were acquired form FiberCell Systems (3008; FiberCell Systems). Each drug was added using computerized syringe pumps and routed into the central reservoir. The total volume of the system was 344mL for plasma and 322mL for ELF experiments.

Diluent medium also consisting of RPMI640 with 2% FBS was prepared in 4L PETG bottles capped with 38mm caps (FiberCell Systems; A1006) extended with platinum cured silicone tubing to reach the bottom of the media bottle, fixated with a zip tie. Medium was pumped into the system using peristaltic pumps with a speed of 2.1mL/min for plasma and 1.9mL/min for ELF experiments. A general description of the full set-up and maintenance is given in the Supplementary material section Set-up and Maintenance. A schematic overview of the whole system is shown in Supplementary Figure 1.

THP-1 cells, used to represent human macrophages, concentrated to a density of 1.5 x 10^6 cells/mL and resuspended in fresh RPMI1640+10%FBS. *M. avium* brought to 0.5 McFarland turbidity was added in a multiplicity of infection of 1:3 bacteria:cells and incubated at 37°C and 5% CO_2 overnight. The next day, the cells were washed and re-suspended in the same volume RPMI1640+10%FBS, and inoculated into the HFS at 20mL/system using BD Plastipak syringes (BD Bioscience, Erembodegem, Belgium). The systems were allowed to equilibrate for 24 hours before the first drug was added and day 0 bacterial counts from the system were enumerated.

An overview of equations used and their implementation is shown in the supplementary material section “Calculations”. The used calculation sheet can be assessed as Supplementary datasheet 1. To account for natural fluctuations in inflow, outflow and drug distribution, we added 10% on top of the calculated syringe concentration and rounded to a convenient concentration. An overview of used syringe concentrations and injection regimens is shown in *Supplementary Table 1*.

Study design

We conducted two distinct sets of experiments, simulating plasma and ELF drug concentrations separately. Each set had a control (drug-free) arm and an experimental arm. The duration of each experiment was 21 days. At each bacterial sample point, intracellular and extracellular bacteria were enumerated. From this, the proportion of intracellular and extracellular bacteria was calculated. THP-1 cells were also enumerated at each sample point. From the intracellular bacteria and the THP-1 cell

count, the multiplicity of infection (MOI) was calculated as a measurement for the average amount of bacteria per cell. PK measurements were done twice, once at day 0 for initial drug concentrations, and once at day 16 for steady state drug concentrations.

Bacterial & THP-1 enumeration

At day 0, 3, 7, 14 and 21, the intracellular and extracellular bacterial load, as well as the THP-1 cell counts, were assessed. The cartridges were vigorously mixed using two 20ml syringes, and 2mL suspension was removed from the cartridge. 1mL of this sample was spun down for 10min at 1500rpm, and supernatant was removed for extracellular bacterial enumeration. The pellet was resuspended in 1mL demineralized water with 0.05% Tween80 for THP-1 cell lysis, vortexed and enumerated, as previously described [21]. Samples were quantified using a 10-fold serial dilution with triplicate spot-plating of each well on M7H10 agar plates. Plates were incubated at 37°C for 7 days.

THP-1 cells were stained 1:1 with tryptophan blue to exclude dead cells. 10ul suspension was pipetted in plastic counting slides (KOVA international, CA, USA) were and counted according to the manufacturers' high density table, from which a final cellular concentration was calculated.

Pharmacokinetic measurements

Samples from the HFS were taken at 0, 1, 2, 3, 5, 8, 10, 12, 14, 16, 22.5 and 23.5 hours form the central reservoir, capturing peak drug concentrations as well as trough levels, in order to verify that pharmacokinetic parameters were correctly simulated. Samples were processed immediately. Penetration of the antibiotics into the extra capillary space of the cartridge was verified in a preliminary experiment using the same protocols (Supplementary table 2).

Processed samples were analyzed for rifampicin, ethambutol and azithromycin content using an ultra high performance liquid-chromatography tandem mass spectrometer (XEVO TQ-S micro triple quadrupole mass spectrometer, Waters). Columns used were an Acquity UPLC BEH C18 1.7µm 2,1x100mm for rifampicin and ethambutol; and a Xbridge C18 3.5µm 2.1x50mm for azithromycin. The

159 transitions for precursor to product ions were, for rifampicin, ethambutol and azithromycin
160 respectively, 832.2299 m/z to 791.4200 m/z; 205.0319 m/z to 115.9701 m/z; and 479.457 m/z to
161 116.02 m/z. All runs had a labelled internal standard present for each compound (Supplementary
162 material 3).

163 Accuracies for azithromycin, rifampicin and ethambutol ranged from 91.8 to 98.5% . Intra- and inter-
164 day precision were 4.42-8.69 and 2.85-8.69% for azithromycin, 1.63-4.34% and 0.97-2.62% for
165 rifampicin, and 1.01-1.22% and 0.43-3.42% for ethambutol. Ranges of the methods were 0.015-1.5
166 mg/L, 0.15-50 mg/L and 0.05-15 mg/L respectively. If the expected concentration of a sample fell out
167 of the linear measurement range, samples were pre-diluted in RPMI1640+2% FBS before processing.
168 Set-up and validation are outlined in the supplemental material sections “Pharmacokinetic
169 measurement protocols”.

170 *Calculations & Statistics*

171 Plotting and calculations performed on primary data was done using GraphPad Prism version 5.03
172 (GraphPad Software Inc., La Jolla, CA, USA) or R version 3.1.2 (R Foundation for Statistical Computing,
173 Vienna, Austria; https://www.R-project.org/). Error bars show are the standard error of the mean.

174 *Ethics Declaration*

175 Donors of human macrophages gave their informed consent for use of their blood for medical
176 research.

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178 **Results**

179 The outcomes of the plasma- concentration mimicking hollow-fiber experiment are shown in figure 1.
180 The treatment regimen could not hold the bacterial population static, but rather slowed its growth for
181 the duration of the experiment, holding the bacterial load lower than the growth control (figure 1a,
182 1b). All systems had a comparable proportion of extracellular bacteria (figure 1a). Both initial (figure
183 1c) and steady-state (figure 1d) drug concentrations mirrored our planned exposures. All systems
184 reached a plateau of THP-1 concentrations of around $1.5x10^7$ cells/mL around day 14 with
185 considerable inter-system variation (figure 1e). The multiplicity of infection (MOI) started at around
186 0,03 bacteria per cell, and ended at around 100 bacteria per cell, with a considerably higher MOI for
187 the treatment regimen from day 3 to 14 (figure 1f).

188 The outcome of the ELF-concentration mimicking hollow-fiber experiment are shown in figure 2. The
189 regimen could hold the inoculum static for 3 days to an overall lower bacterial load than in the plasma-
190 mimicking HFS (figure 2a, 2b). The antibiotic-treated HFM systems had a higher proportion of
191 extracellular bacteria than the control; both treated regimens had a bacterial burden lower than the
192 growth control (figure 1a, 2a). Due to the longer half-life and ELF accumulation of azithromycin, the
193 drug concentrations were higher at day 16 than at day 0 (accumulation, figure 2c, d), and trough
194 concentrations were comparable between days (figure 2d). All systems reached a plateau of THP-1
195 concentrations of around $1.0x10^7$ cells/mL around day 14 with low inter-system variation (figure 2e).
196 The MOI over time of the ELF-regimen was overall lower than the plasma-regimen, not going above
197 10 (figure 2f).

198 An overview of observed pharmacokinetic parameters of both systems is shown in table 2.

199 **Table 2:** Comparison of simulated and achieved pharmacokinetic parameters

		Plasma		ELF	

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Parameter	Drug	Target	Actual	Target	Actual
Half-Life (h)	Azithromycin	11	8.7	20	20.37
	Ethambutol	10	8.6	10	10.82
	Rifampicin	2	3.6	2	3.2
T_{max} (h)	Azithromycin	5.25	5	10	10
	Ethambutol	3.1	3	3	3
	Rifampicin	2	2	2	2
C_{max} (Steady State; mg/L)	Azithromycin	0.16	0.16 ± 0.01	3	3.1 ± 0.07
	Ethambutol	2.4	2.43 ± 0.24	3	3.1 ± 0.11
	Rifampicin	1.5	1.44 ± 0.15	1.5	1.51 ± 0.09

200

201 The minimum inhibitory concentrations of azithromycin , ethambutol and rifampicin

202 against *M. avium* ATCC 700898 were 8, 4 and 4 mg/L.

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Discussion

Hollow fiber model experiments of MAC-PD treatment, informed by real-life pharmacokinetic data, and accounting for site-specific properties including drug protein binding and intracellular localisation of bacteria, show very poor efficacy of the currently recommended rifampicin-ethambutol-azithromycin regimen both in plasma and -to a lesser extent- in ELF. We recorded considerably less killing than Deshpande *et al.* who investigated an azithromycin – rifabutin- ethambutol regimen [22]. However, Deshpande *et al.* did not explicitly report the origin of their pharmacokinetic data, whether ELF or plasma was modelled and whether or not protein-binding was accounted for [21,23]. Especially in the case of the *in vitro* more active rifabutin [24], modelling total rather than free C_{max} and AUC might significantly influence mycobacterial killing in the HFS, as its protein binding is 71% [25]. The limited efficacy of the azithromycin-ethambutol-rifampicin regimen in our model is more negative than the initial clinical observation that 65% of patients attain prolonged culture conversion [3]; of which 40% of the patients with good initial outcomes experience a recurrence and 25% of these are true relapses suggestive of failure to clear the infection [26]. The hollow-fiber model focuses on the antibiotic-bacterium interaction and negates the influence of the immune system and additional measures to reduce bacterial such as surgical intervention and airway clearance techniques on treatment outcome. It has become increasingly clear that non-tuberculous pulmonary disease (NTM-PD) patients have subtle immunodeficiencies and these immunodeficiencies likely influence treatment outcome of MAC-PD [27]. Though THP-1 cells take up mycobacteria and are generally regarded a suitable alternative to human monocyte derived macrophages (hMDMs) [28], it is unknown whether or not they can also kill engulfed mycobacteria. *In vitro*, the killing of MAC by hMDMs is negligible [29]. Thus, our ELF-HFS reflects treatment of MAC-PD in absence of a functional immune system and other adjunctive treatments. We could show that intracellular bacterial load is alike for hMDMs and THP-1 cells, though mycobacteria might escape THP-1 cells at an increased rate (Supplementary Figure 2). Drug accumulation and effect is thus comparable between the two cell lines.

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An important distinction is the difference between intracellular and extracellular MAC in the HFS in our study. Because of natural cell death, and a general homeostasis between extracellular and intracellular bacteria, some bacteria will escape the THP-1 cells and will be exposed to extracellular drug concentrations [21]. Especially in the ELF-HFS, the intracellular population is markedly lower than the extracellular population, whereas in the plasma-HFS, the two populations are more alike, which might be an indication of concentration-dependent drug, specifically azithromycin, accumulation in the cells, as previously reported *in vivo* (Supplementary Figure 2) [30]. However, when isolating intracellular from extracellular CFU counts, bacteria stuck to the cell surface of the THP-1 cells might be isolated together with them, and enumerated as intracellular bacteria. How large their contribution is, is not known.

We evaluated the standard MAC-PD treatment regimen in the HFS, studied drug concentrations in ELF alongside plasma, considered protein binding of drugs, and evaluated an intracellular HFS, all to best mimic the clinical conditions. There are, however, significant limitations to our study. Although protein binding in RPMI1640 itself is negligible, the extra-capillary space where THP-1 cells and mycobacteria reside has a significant protein concentration owing to cell lysis. It is reasonable to assume that a proportion of drug binds to dead cells, or other proteins present in that space. However, fresh drug was added continuously, so the extent of the drug binding is likely limited. Also, though likely unimportant for our C_{max} and overall drug exposure of the bacteria, we did not account for hysteresis in our experiments, which describes a delayed T_{max} in the ELF compared to plasma, but rather assumed a 1:1 translation between ELF and plasma. Also, our simulated rifampicin concentration might be too low, as we applied 90% protein binding and a 1:1 plasma:ELF ratio, whereas 80% protein-binding and a plasma:ELF ratio 1:2.6 has been used by others [18]. Also, the rifampicin half-life is longer than anticipated, but would still fall within parameters observable in a clinical population. However, rifampicin itself adds little to the bactericidal activity of the azithromycin-ethambutol-rifampicin regimen and is primarily included to prevent macrolide resistance [26].

Concluding, while the three-drug standard regimen performs better with simulated ELF concentrations, both regimens fail to reduce the bacterial load below inoculum. Drug therapy alone seems unable to cure MAC-PD. A new truly bactericidal regimen is needed to improve treatment outcomes. The described model and regimen can serve as the benchmark to evaluate new regimens prior to moving into clinical trials. We provide full disclosure of the methodology to enable its wider use.

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Funding

Jakko van Ingen is supported by a personal grant from the Netherlands Organization for Scientific Research (NWO/ZonMW grant Veni 016.176.024).

Transparency Declarations

The authors have no conflicts of interest to declare.

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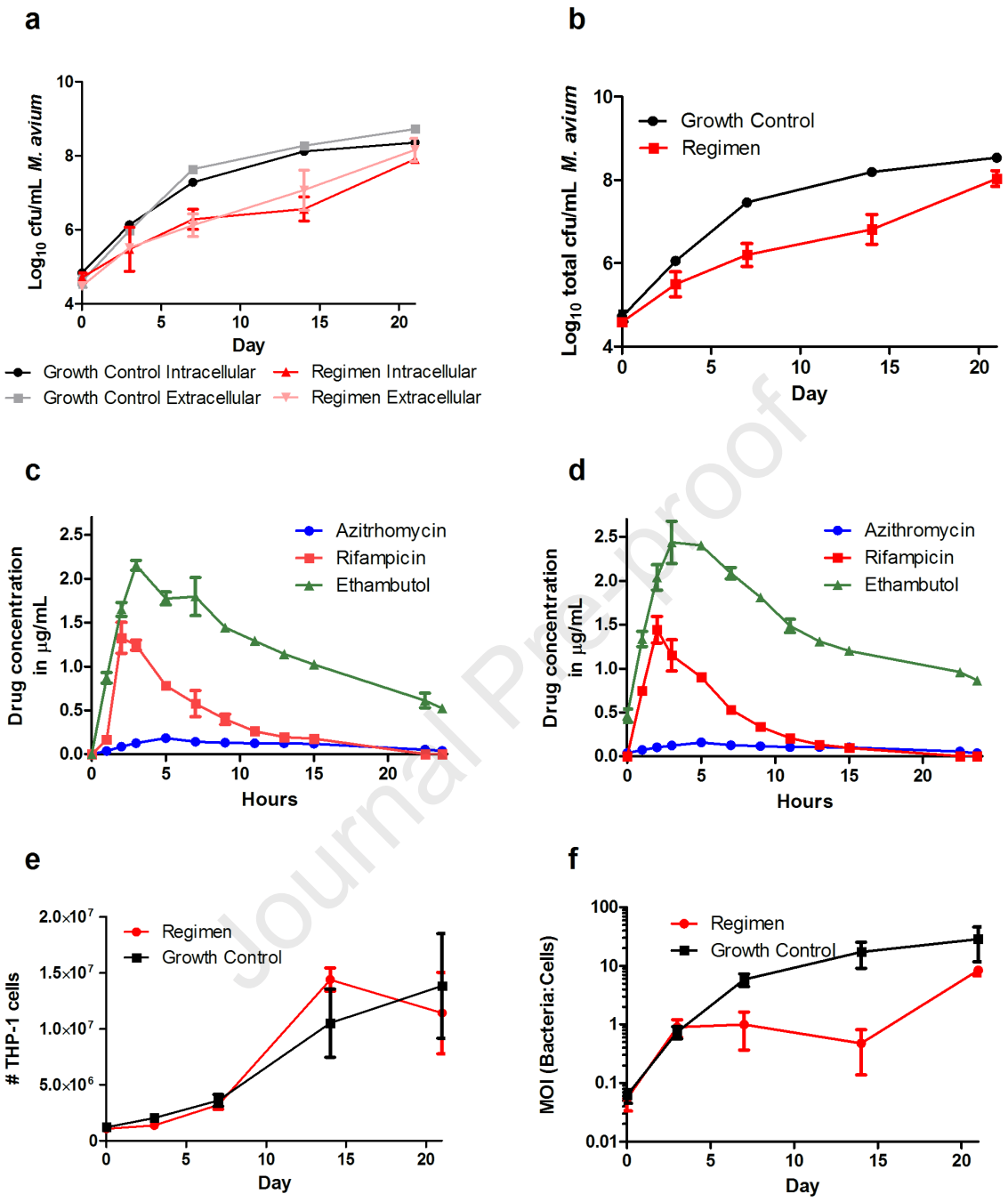
364 **Figure 1:** Pharmacokinetic and pharmacodynamic measurements in the plasma free-drug mimicking
365 hollow fiber system. **a:** Intracellular and extracellular CFU counts. **b:** Total CFU counts. **c:**
366 pharmacokinetic curve of day 0. **d:** Steady-state pharmacokinetics at day 16. **e:** THP-1 counts in the
367 extracapilary compartment. **f:** Multiplicity of infection (Bacteria:Cells) over time.

368

369 **Figure 2:** Pharmacokinetic and pharmacodynamic measurements in the ELF free-drug mimicking
370 hollow fiber system. **a:** Intracellular and extracellular CFU counts. **b:** Total CFU counts. **c:**
371 pharmacokinetic curve of day 0. **d:** Steady-state pharmacokinetics at day 16. **e:** THP-1 counts in the
372 extracapilary compartment. **f:** Multiplicity of infection (Bacteria:Cells) over time.

Journal Pre-proof



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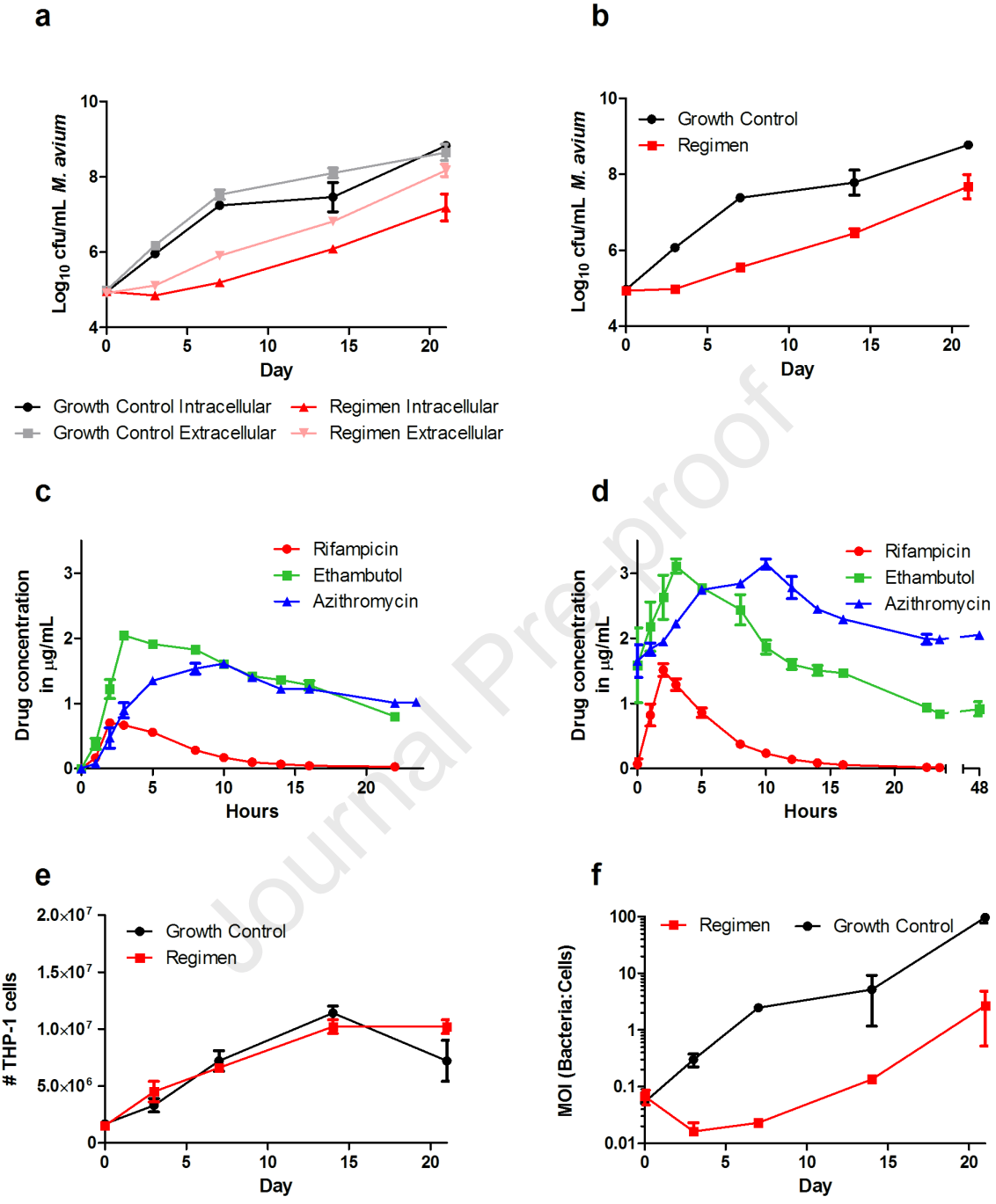


Journal Pre-proof

Supplementary data

Supplementary material –Set-Up and Maintenance

Materials

The central reservoir was built from 500ml PETG bottles and pre-filled with 300ml RPMI1640 with 2% FBS and capped with a PK/PD reservoir cap (FiberCell Systems Inc. New Market, Md, USA; A1007). Hollow fiber Cellulosic cartridges were acquired form FiberCell Systems (3008; FiberCell Systems). Between reservoir and cartridge, a 0.22µm polyethersulfone (PES; Sartorius, Nieuwegein, the Netherlands) filtered louer-fit clave connector (ICU Medical Inc, San Clemente, CA, USA) was installed for pharmacokinetic measurement sample collection. Each drug was added via an individual Braun 3-way stopcock (B. Braun, Meselungen, Germany). Medium from the diluent media bottles was pre-filtered using autoclaved Culture Guard 0.22 µm filters (Repligen, Waltham, MA, USA). Tubing to and from the system was Pumpsil 1.6mm Bore x 1.6mm wall thickness platinum cured silicone tubing (Watson Marlow, Barendrecht, NL) fitted with 3/32 ID luer fittings. Peristaltic pumps used were Watson Marlow 530S with 313x pump head extensions (Barendrecht, the Netherlands). Computerized syringe pumps were Legato 210 Infuse/Withdraw syringe pump with Legato 200 series small syringe multi-racks (KD Scientific Inc., Hollistion, MA, USA).

When taking pharmacokinetic samples, we first flushed the filtered clave connector by removing 2mL medium through it, and then taking a 1mL sample, ensuring only fresh medium was sampled.

Calculations

Pump rates in both drug syringes and media pumps were calculated using standard pharmacokinetic equations. To mimic the half-lives of the different drugs, the pumps were set to eliminate the drug with the shortest half-life accurately. To achieve distinct other half-lives, we then calculated how much additional drug would need to be injected per time unit of the other drugs to artificially prolong their apparent half-lives.

The elimination rate constant k was calculated with $k = \ln(2)/t_{1/2}$, where $t_{1/2}$ is the half-life of the drug with the shortest *in vivo* half-life. The clearance CL per hour was calculated using $CL = k * V_D$, where V_D was the total volume of the system. During infusion, we calculated the drug concentration C using $C = k_0/CL * (1- e^{-ke*t})$, where k_0 is the infusion rate and k is the elimination rate constant calculated before. The concentration C of drug at time t after infusion was calculated using $C_t = C_0 * e^{-kt}$ where C_0 is the concentration at T_{max} and k is the elimination rate constant. We assumed that after 5 x half-lives drug concentrations are undetectably low.

Set-up

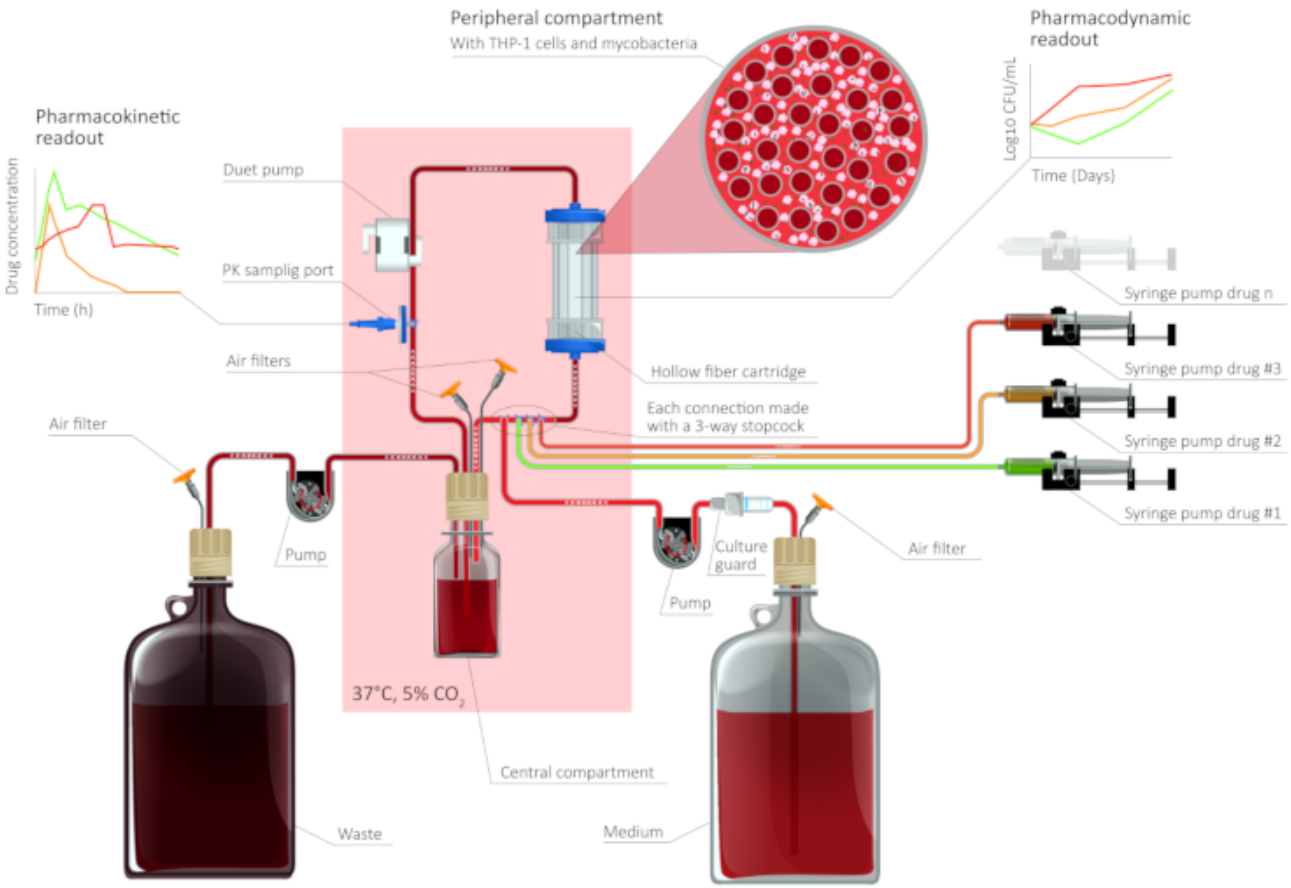
Hollow fiber systems without bacteria or cells present were set-up without drug inflow 2 days before the start of the experiment. Fresh diluent medium was added at 0.3 ml/h, and pumped out at 0,6 ml/h for 24h. 1 day before the start of the experiment, MAC infected THP-1 cells were added to the system by first pulling a vacuum in the cartridges and then carefully injecting the cell suspension in the cartridge. This allows for equilibration of the suspension and compensates for an initial drop in bacterial counts by changing their environment. Drug ports were only opened just before the first injection cycle, day 0 samples were taken before that to acquire a baseline cell and bacterial count.

Maintenance

Because of potential clogging, Culture Guards were replaced weekly, or whenever visible buildup was present. Media inflow was kept at 0.3mL/min in control systems. Duet pumps were kept at arbitrary medium to high speeds to allow for rapid distribution and equilibration of media and drug. Waste media was removed from the system by peristaltic pumps set to double the inflow speed and routed into 4L amber glass jugs filled with 10mL of Terralin (Schülke & Mayr Benelux B.V., Haarlem, the Netherlands) solution. Tubing in the peristaltic pumps was moved weekly to ensure tubing integrity.

In the case of a tube break, the outside of the tube around the break was sterilized with 70% ethanol, cut around the break and re-connected by two pre-autoclaved interlocked Luer Locks.

Supplementary Figure 1- Schematic overview of the hollow fiber system.



Supplementary Figure 2 – THP1 and Macrophage comparison

Methods

The study drugs clofazimine, amikacin, clarithromycin, azithromycin, rifampicin and ethambutol were purchased from Sigma-Aldrich; bedaquiline was kindly provided by Janssen Pharmaceuticals (Beerse, Belgium). Minimum inhibitory concentrations (MICs) to the study drugs were determined by broth microdilution in cation-adjusted Muller Hinton broth per CLSI guidelines (11).

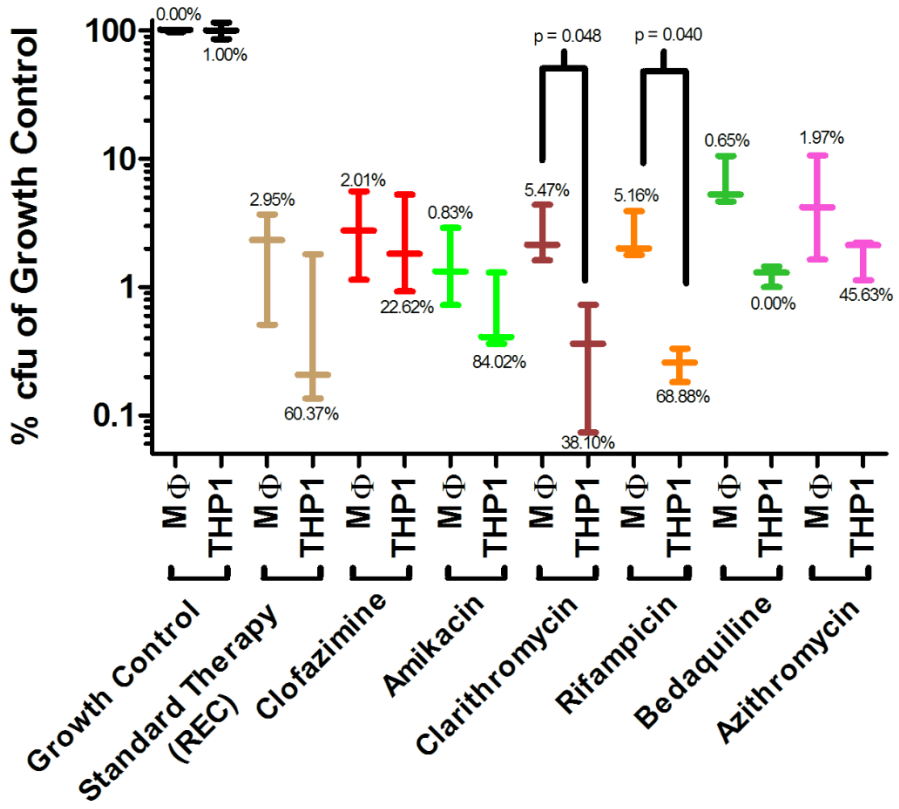
Peripheral blood mononuclear cells were obtained from three healthy volunteers (Sanquin Bloodbank, Nijmegen, NL). Informed consent was obtained for use of their blood for scientific purposes, as approved by the Ethics Committee of Radboud University Medical Centre (Nijmegen, NL). The isolation and differentiation protocol was executed as described before (23). THP-1 cells (ATCC TIB-202) were cultured in RPMI 1640 supplemented with 10% fetal bovine serum. Cells were seeded at a density of 1×10^5 cells/well and made adherent with 25 ng/mL phorbol myristate acetate for 24 h.

On the day of infection, the medium was removed and *M. avium* ATCC700898 was added at a ratio of 5:1 (bacteria:cells) for 1 h at 37°C to allow phagocytosis before medium was changed to RPMI 1640 supplemented with 10% human pooled serum. Clofazimine, amikacin, clarithromycin, rifampicin, bedaquiline, azithromycin or the standard rifampicin-ethambutol-clarithromycin regimen was added, all in 2xMIC concentration, after the 1 h incubation and a washing step, and remained for the rest of the experiment. After six days, the amount of extracellular and intracellular bacteria was quantified as previously described (23). Total cfu counts (extracellular + intracellular) of individual antibiotics were normalized on, and reported as a percentage of, their respective growth control. All assays were performed in triplicate measurements. The Mann-Whitney U test was used to assess the difference in extracellular cfu counts between macrophages and THP-1 cells. A factorial ANOVA with subsequent post-hoc Tukey range-test was used to assess differences in total bacterial load between macrophages

and THP-1 cells for individual antibiotics. Calculations were performed in R version 3.5.2 (R Foundation for Statistical Computing, Vienna, Austria; https://www.R-project.org/).

Results

The percentage of total cfu normalized on the respective growth control is shown in supplementary figure 2. Assays with THP-1 cells had significantly more extracellular bacteria (p=0.026), indicating that researchers should asses both the extracellular and intracellular *M. avium* populations in HFM experiments. The relative total cfu count in THP-1 cells after treatment was significantly lower than in macrophages exposed to rifampicin (p=0.040) and clarithromycin (p=0.048), two key anti-*M. avium* drugs. Surprisingly in this context, azithromycin, another commonly used macrolide in NTM therapy, did not show a significant difference between cell types. There was no significant difference in total cfu counts for other antibiotics, generally confirming THP-1 cells as a suitable model for *M. avium* infections in the HFM.



Supplementary Figure 2: Percentage of total cfu counts in human monocyte-derived macrophages (Mϕ) and THP-1 cells of different treatment conditions relative to their respective growth control. Percentages under and above the bars indicate the average fraction of the total cfu count that was localized extracellularly. REC refers to the Rifampicin-Ethambutol-Clarithromycin standard therapy. Error bars indicate standard error of the mean.

Supplementary table 1– Syringe pump settings for daily infusions

Plasma Concentrations			
Drug	Infusion Volume (mL/h)	Time (hours:minutes)	Syringe concentration (mg/L)
Azithromycin	1.2	05:05	25
	0.6	11:55	
	0.4	03:00	
	Delay	04:00	
Ethambutol	2.5	03:05	250
	1	04:55	
	0.6	10:00	
	0.25	04:00	
Rifampicin	0.9	02:20	500
	Delay	21:40	
System parameters			
Pump inflow	2.1 mL/h	Distribution Volume	344 mL
Epithelial Lining Fluid Concentrations			
Drug	Infusion Volume (mL/h)	Time (hours:minutes)	Syringe concentration (mg/L)
Azithromycin	1.4	10:05	250
	0.72	09:55	
	0.15	04:00	
Ethambutol	1.2	03:05	500
	0.5	04:55	
	0.35	12:00	
	0.15	04:00	
Rifampicin	1.3	02:20	400
	Delay	21:40	
System parameters			
Pump inflow	1.9 mL/h	Distribution Volume	322 mL

Supplementary table 2– Penetration of Rif, Emb and Azt into the extra capillary space. Replicate 1 / Replicate 2. N/A refers to samples lost in preparation.

Timepoint	Rif outside	Rif Inside	Emb outside	Emb inside	Azt outside	Azt inside
2h 10min	13.3/20.0	10.6/14.4	1.74/1.89	1.66/2.03	0.16/0.16	0.13/0.13
4h	10.73/7.6	8.5/10.3	2.51/2.24	2.63/2.44	0.23/0.17	0.2/0.17
8h	2.0/2.2	1.9/2.5	1.8/1.8	2.0/2.0	0.22/0.19	0.19/NA
12h	0.7/0.9	0.6/0.6	1.2/1.2	1.6/1.3	Na/ 0.18	0.19/0.19
15h	0.2/0.3	0.3/0.34	1.2/1.0	Na/1.4	0.19/0.20	0.18/0.19

Pharmacokinetic Measurement protocols

Azithromycin Pharmacokinetic Sample measurement protocol

Reference materials

- Azithromycin European Pharmacopoeia (EP) Reference Standard (Sigma-Aldrich, Zwijndrecht, The Netherlands)
- Azithromycin [^{13}C, $^{2}H_3$] (Alsachim, Illkirch-Graffenstaden, France)

Chemicals and solvents

- Acetonitrile, Hypergrade for LC-MS (Merck, Darmstadt, Germany)
- Methanol, Absolute ULC/MS (Biosolve, Valkenswaard, The Netherlands)
- Formic acid, 98-100% for LC-MS (Merck, Darmstadt, Germany)
- RPMI Medium 1640 (Thermo Fisher Scientific, Breda, The Netherlands)
- Fetal bovine serum (Thermo Fisher Scientific, Breda, NL)

Stock and work solutions

Three independent stock solutions (for calibrators, quality controls, and a reserve), were prepared and stored at -40 °C. Azithromycin (AZT) stock 1mg/ml was prepared in methanol.
The labeled internal standard stock is made by dissolving the compound in the same manner as the unlabeled compound stock, at 1 mg/ml.
From the labeled internal stock solutions, a protein precipitation (PP) solution is made with a concentration of 0.1 mg/L in Acetonitrile. All stocks and solutions were stored at -40°C.

Calibration and quality control solutions

For the preparation of the calibration curve, one of the stock solutions was used and diluted with acetonitrile to achieve seven calibration solutions 0.30–0.54–1.95–3.75–7.5–15-30 mg/L AZT.
Quality control (QC) solutions are made at three levels (low, medium and high) from a different stock than the calibration solutions at 0,4–6-20 mg/L. During sample preparations these working solutions are further diluted (factor 20) in RPMI 1640+2%
All solutions were stored at −80 °C until analysis and are stable for at least 7 months.

Equipment and settings

Pump settings (ACQUITY UPLC H-Class QSM; Waters, Milford, MA, USA)			
Time (min)	**Flowrate (ml/min)**	**% A** Water + 0.1% formic acid	**% B** Acetonitrile + 0.1% formic acid
0	0,3	98	2
1.00	0,3	30	70
2.00	0.6	30	70
3.00	0,1	30	2
3.25	0,3	98	2
5.50	0,3	98	2
6.00	0.3	98	2
Seal wash: 90/10 water/acetonitrile			
Seal wash period: 5 min			

Autosampler and column oven (ACQUITY UPLC H-Class SM-FTN; Waters, Milford, MA, USA)
Purge solvent: 95/5 water/acetonitrile + 0.1% formic acid
Wash solvent: 40/60 water/acetonitrile + 1% formic acid
Injection volume: 1 ul
Pre-inject wash time: 5 sec
Post-inject wash time: 5 sec
Needle placement: 4mm
Column Temperature 25 ±1°C
Column: Xbridge C18 3.5um 2,1x50mm
Temp. tray: 10°C

Ionspray source
Capillary: 2 kV
Polarity positive ion mode
Desolvation temperature: 500 °C
Desolvation gas flow: 950 L/hr
Cone flow: 10 L/hr

MS settings (XEVO TQS-micro; Waters, Milford, MA, USA)					
Compound Name	**Parent (m/z)**	**Daughter (m/z)**	**Dwell (s)**	**Cone (V)**	**Collision (V)**
Azithromycin	749.5	116.0	0.1	16	48
Azithromycin [^{13}C, $^{2}H_3$]	753.4	158.1	0.1	46	36
Ionspray source					
Capillary: 2 kV					
Polarity: positive ion mode					
Desolvation temperature: 500 °C					
Desolvation gas flow: 950 L/hr					
Cone flow: 10 L/hr					

Accessories and disposables

Name
Finnpipette® Air displacement pipette 50-200 µl (ThermoFisher Scientific, Breda, The Netherlands)
Finnpipette® Air displacement pipette 5 - 40 µl (ThermoFisher Scientific, Breda, The Netherlands)
Finnpipette® Pipette tips 250 µl (ThermoFisher Scientific, Breda, The Netherlands)
HandyStep ® Repeater pipette (Brand, Wertheim, Germany)
HandyStep ® Repeater pipette tip 5 ml (Brand, Wertheim, Germany)
Autosampler vials: TPX micro-vial ND9 insert int. 0.2 ml TPX clair 32x11.6mm (VWR, Amsterdam, The Netherlands)
Autosampler caps: PP Screwcap 9mm pre-slit septum (VWR, Amsterdam, The Netherlands)
Safe-Lock tube 1,5 ml (Eppendorf, Nijmegen, The Netherlands)
DVX-2500 Multi-Tube Vortexer (VWR, Amsterdam, The Netherlands)

Sample preparation

1. Thaw and mix the (pre) created calibrators and qc's
2. In safe-lock tubes, dilute the calibrators and qc's a factor 20 in RPMI 1640+2% FBS (e.g. 30ul in 570 ul RPMI 1640+2% FBS)
3. Mix the diluted calibrators and qc's with a multi-tube vortex

4. Pipette in a safe-lock tube: 50 µl sample, diluted QC or calibrator and add 150 µl PP solution
5. Mix with a multi-tube vortex 20 sec. speed 2500 rpm
6. Centrifuge 5 min.(18.620 g)

7. Close the autosampler vial with a pre-slit cap
8. Inject the sample(s) on the chromatographic separation system

Calculations

Acquired data was processed using Waters TargetLynx software (version 4.1). The calibration curves were linear fitted as ratio signal response compound peak area and internal standard peak area versus concentration, also a $1/x^2$ weighing factor was used.

Chromatogram

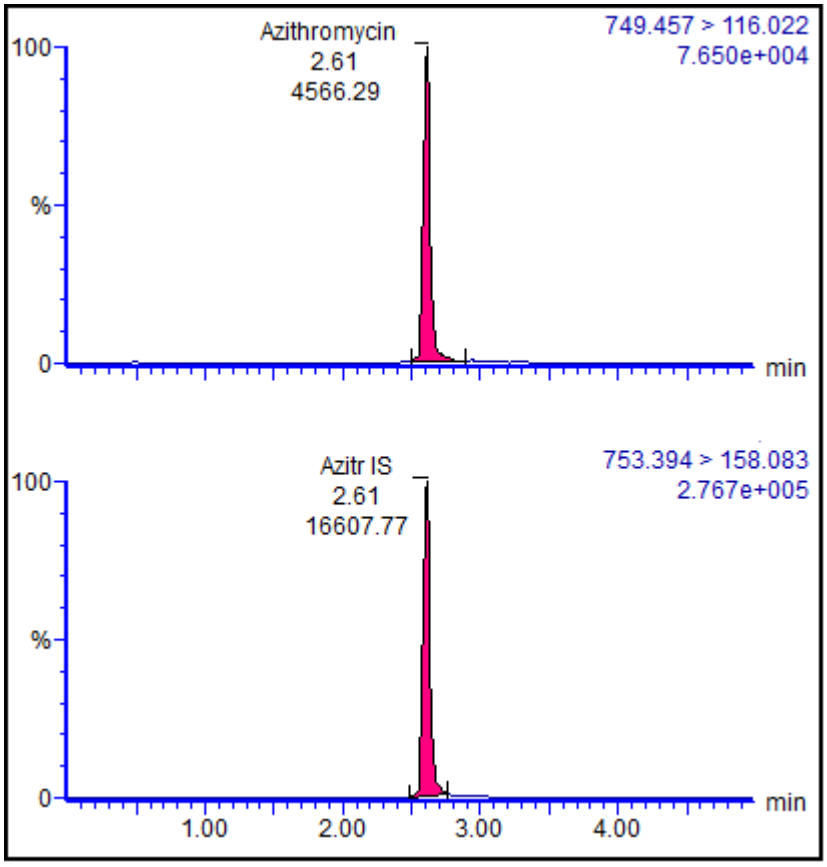


Figure 1: Chromatogram of the lowest calibrator with internal standard

Validation results

	Conc.	Within run (n=5)		Between run (n=15)	
	(mg/l)	Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)
AZIT	0.0150	97.9	8.69	99.1	8.69
	0.0190	91.8	4.42	94.3	3.88
	0.301	94.5	5.43	98.1	3.36
	1.004	93.4	7.61	97.6	2.85
	1.507	95.9	8.12	98.5	8.12

Supplementary material – Rifampicin & Ethambutol pharmacokinetic Sample measurement protocol

Reference materials

- Ethambutol dihydrochloride salt, antimycobacterial (Sigma-Aldrich, Zwijndrecht, The Netherlands)
- Ethambutol $^{2}H_{4}$ (Alsachim, Illkirch-Graffenstaden, France)
- Rifampicine >97% powder (Sigma-Aldrich, Zwijndrecht, The Netherlands)
- Rifampicine $^{2}H_{8}$ (Alsachim, Illkirch-Graffenstaden, France)

Chemicals and solvents

- Acetonitrile, Hypergrade for LC-MS (Merck, Darmstadt, Germany)
- Methanol, Absolute ULC/MS (Biosolve, Valkenswaard, The Netherlands)
- Ascorbic acid, Ph.Eur. (Spruyt-Hillen, IJselstein, The Netherlands)
- Formic acid, 98-100% for LC-MS (Merck, Darmstadt, Germany)
- Ammonium formate, Eluent additive for LC-MS (Sigma Aldrich, Zwijndrecht, The Netherlands)
- RPMI Medium 1640 (Thermo Fisher Scientific, Breda, The Netherlands)
- Fetal bovine serum (Thermo Fisher Scientific, Breda, NL)

Stock and work solutions

Three independent stock solutions (for calibrators, quality controls, and a reserve), were prepared and stored at -40 °C. Ethambutol (EMB) stock 1mg/ml was prepared in water and rifampicin (RIF) stock 2 mg/ml in methanol/water 80/20 v/v with 0.16mg/ml ascorbic acid.
The labeled internal standard stocks are made by dissolving the compounds in the same manner as the unlabeled compounds stocks, only at 1 mg/ml.
From the labeled internal stock solutions, a protein precipitation (PP) solution is made with 0.1 mg/L EMB and 0.1 mg/L RIFA in acetonitrile/methanol 75/25 v/v. All stocks and solutions were stored at -40°C.

Calibration and quality control solutions

For the preparation of the calibration curve, one of the stock solutions was used and diluted with methanol/water 50/50 v/v + 0.16 mg/ml ascorbic acid to achieve seven calibration solutions 0.50–0.75–4.0–11–38–75-150 mg/L EMB and 1.6–2.5–12.5–38–125–250-500 mg/l RIF.
Quality control (QC) samples are made at three levels (QC Low, medium and high) from a different stock than the calibration solutions at 1.1–11-120 mg/L EMB and 4–40-400 mg/l RIF
All solutions were stored at −40 °C until analysis and are stable for at least 6 months.

Equipment and settings

Pump settings (ACQUITY UPLC H-Class QSM; Waters, Milford, MA, USA)			
Time (min)	**Flowrate (ml/min)**	**% A** 10mM ammoniumformate buffer in water pH 4,5	**% B** 10mM ammoniumformate buffer in methanol pH 4,5
0	0,3	98	2
2.05	0,3	70	30
4.00	0,3	10	90
5.00	0,3	10	90
5.10	0,3	98	2
10.00	0,3	98	2
Seal wash: 90/10 water/acetonitrile			

Seal wash period: 5 min

Autosampler and column oven (ACQUITY UPLC H-Class SM-FTN; Waters, Milford, MA, USA)
Purge solvent: 95/5 water/acetonitrile + 0.1% formic acid
Wash solvent: 80/20 water/methanol
Injection volume: 1 ul
Pre-inject wash time: 5 sec
Post-inject wash time: 5 sec
Needle placement: 4mm
Column Temperature 25 ±1°C
Column: Acquity UPLC BEH C18 1.7um 2,1x100mm + assay frit 0.2um 2,1mm
Temp. tray: 10°C

Ionspray source
Capillary: 2 kV
Polarity positive ion mode
Desolvation temperature: 500 °C
Desolvation gas flow: 950 L/hr
Cone flow: 10 L/hr

MS settings (XEVO TQS-micro; Waters, Milford, MA, USA)					
Compound Name	**Parent (m/z)**	**Daughter (m/z)**	**Dwell (s)**	**Cone (V)**	**Collision (V)**
Ethambutol	205.0	116.0	0.05	26	14
Ethambutol $^{2}H_{4}$	209.2	120.0	0.05	26	14
Rifampicine	823.2	791.4	0.05	18	16
Rifampicine $^{2}H_{8}$	831.2	799.4	0.05	18	16
Ionspray source					
Capillary: 2 kV					
Polarity: positive ion mode					
Desolvation temperature: 500 °C					
Desolvation gas flow: 950 L/hr					
Cone flow: 10 L/hr					

Accessories and disposables

Name
Finnpipette® Air displacement pipette 50-200 µl (ThermoFisher Scientific, Breda, The Netherlands)
Finnpipette® Air displacement pipette 5 - 40 µl (ThermoFisher Scientific, Breda, The Netherlands)
Finnpipette® Pipette tips 250 µl (ThermoFisher Scientific, Breda, The Netherlands)
HandyStep ® Repeater pipette (Brand, Wertheim, Germany)
HandyStep ® Repeater pipette tip 5 ml (Brand, Wertheim, Germany)
Autosampler vials: TPX micro-vial ND9 TPX clair 0.3ml 32x11.6mm (VWR, Amsterdam, The Netherlands)
Autosampler caps: PP Screwcap 9mm pre-slit septum (VWR, Amsterdam, The Netherlands)
Safe-Lock tube 1,5 ml (Eppendorf, Nijmegen, The Netherlands)
DVX-2500 Multi-Tube Vortexer (VWR, Amsterdam, The Netherlands)

Sample preparation

9. Thaw and mix the (pre) created calibrators and qc's
10. In safe-lock tubes, dilute the calibrators and qc's a factor 10 in RPMI 1640+2% FBS (e.g. 40ul in 360 ul RPMI 1640+2% FBS)
11. Mix the diluted calibrators and qc's with a multi-tube vortex

12. Pipette in a safe-lock tube: 50 µl sample, diluted QC or calibrator and add 150 µl PP solution
13. Mix with a multi-tube vortex 20 sec. speed 2500 rpm
14. Centrifuge 5 min.(18.620 g)

15. Add to the autosampler vials: 95 µl ascorbic acid 0,9 mg/ml and 75 µl supernatant
16. Close the autosampler vial with a pre-slit cap
17. Mix the autosampler vials with a multi-tube vortex 20 sec. speed 2500 rpm
18. Inject the sample(s) on the chromatographic separation system

Calculations

Acquired data was processed using Waters TargetLynx software (version 4.1). The calibration curves were linear plotted as log ratio signal response compound peak area and internal standard peak area versus log concentration.

Chromatogram

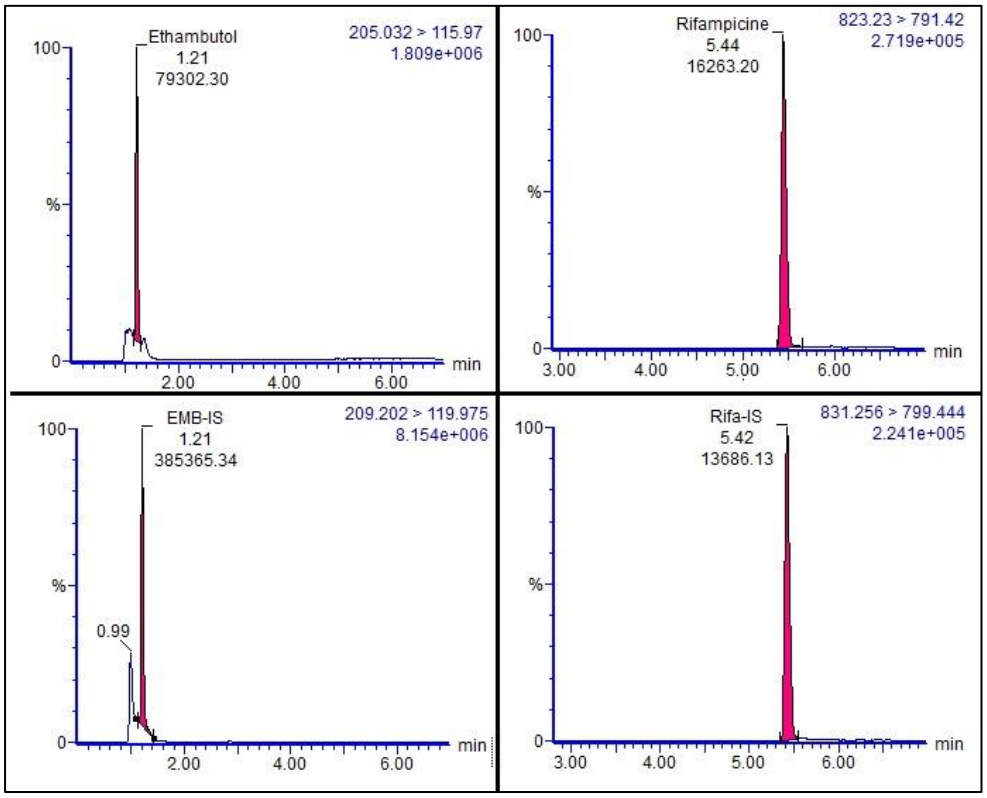


Figure 2: Chromatogram of the lowest calibrator with internal standards

Validation results

	Conc.	Within run (n=5)		Between run (n=15)	
	(mg/l)	Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)
EMB	0.0480	98.0	1.22	98.8	0.94
	0.113	97.5	1.02	97.9	0.43
	1.130	97.9	1.37	98.2	1.37
	11.30	95.1	1.25	98.9	3.42
	15.18	96.3	1.01	98.5	1.90
RIFA	0.158	97.1	4.34	98.6	2.62
	0.401	95.6	3.89	98.2	2.00
	4.006	98.5	1.63	99.5	0.97
	40.06	96.0	3.99	99.6	2.99
	49.85	96.2	2.93	97.8	1.28