Decreasing clinical burden of neonatal sepsis through pharmacometric optimization of antibiotic monitoring

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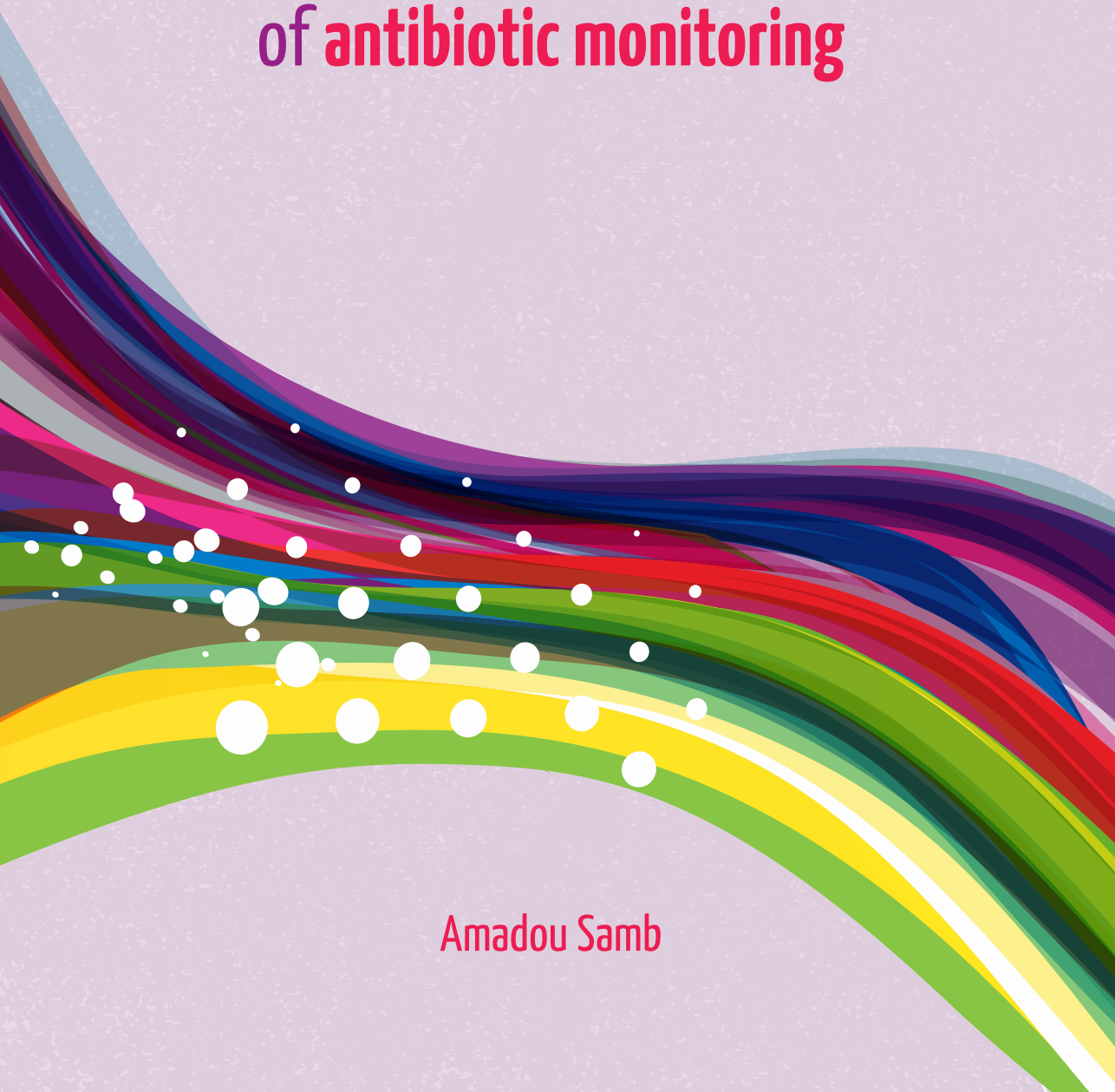

Amadou Samb

Decreasing clinical burden of **neonatal sepsis** through pharmacometric optimization of **antibiotic monitoring**

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Decreasing clinical burden of neonatal sepsis through pharmacometric optimization of antibiotic monitoring

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Faculteit der Geneeskunde

Contents

General introduction and thesis outline

Chapter 1

General introduction

Neonatal sepsis

Sepsis is a well-known clinical syndrome that was originally described in ancient Greece by Hippocrates, "the father of medicine". Nowadays sepsis is defined as a systemic inflammatory response syndrome (SIRS) due to a primary infection with ensuing acute (multi) organ dysfunction [1,2]. Neonatal sepsis manifests itself within the first 28 days of life and is generally classified as either early onset sepsis (EOS) or late onset sepsis (LOS). It is one of the leading causes of mortality and morbidity in neonates worldwide with a potentially severe impact on long-term development [3]. The initial clinical signs and symptoms of neonatal sepsis are non-specific and may include apnea, tachycardia or bradycardia, changes in body temperature, hypoglycemia and seizures. Cardio-respiratory compromise may develop within hours following these initial nonspecific symptoms. In the context of this thesis the term neonatal sepsis indicates bacterial neonatal sepsis with bacteremia.

Early onset sepsis

EOS presents within the first 72 hours of life and is mostly contracted during (or shortly before) delivery as a result of vertical transmission of commensal or pathogenic bacteria present in the amniotic fluid, maternal colon and/or - urogenital tract [3]. Important maternal risk factors for EOS are premature delivery, prolonged rupture of membranes (PROM), chorioamnionitis, maternal fever and *Streptococcus Agalactiae* (Group-B-Streptococci; GBS) colonization [4]. GBS colonization in particular is an important risk factor for EOS, since GBS is a prominent pathogen for EOS and up to 30% of pregnant women carry GBS in the genital tract [5]. GBS and *Escherichia Coli* are the most frequently encountered pathogens in blood cultures from patients with EOS, accounting for roughly 34% and 17% of EOS cases respectively [6,7]. The mortality of EOS is estimated at approximately 16% [8].

Late onset sepsis

Clinical signs and symptoms of LOS present after the first 72 hours of life [3]. It is generally considered to be contracted through invasive medical procedures during hospital admission, such as endotracheal intubation and insertion/manipulation of venous and/or arterial catheters [3]. Risk factors for LOS are therefore indwelling central venous - or arterial lines or drains; the need for prolonged parenteral feeding; extreme premature birth and very low birth weight (VLBW). Pathogens that are most commonly associated with LOS are coagulase negative staphylococci (CoNS) (75%) and *Staphylococcus Aureus* (13%) [9,10]. Severe cases of LOS can be seen due to anaerobic species or gram negative bacteria during LOS in necrotizing enterocolitis. Overall mortality due to LOS is estimated at approximately 9% [8].

Antimicrobial treatment

Standard first line practice in cases of (suspected) neonatal sepsis is immediate treatment with broad-spectrum antibiotics, typically an aminoglycoside and a penicillin derivative in EOS [3]. For LOS antibiotic treatment is generally aimed at gram negative coverage and staphylococcal coverage [3]. Antibiotic policy may vary widely between hospitals and neonatal departments [11]. Prior to any antibiotic treatment, blood cultures (and if indicated lumbar

puncture for CSF culture) are performed for diagnosis and pathogen identification, which can take up to 24 to 48 hours [12]. If blood cultures are found positive, pathogen-specific therapy is initiated based on their microorganism sensitivity profile. In the Netherlands, the Dutch Children's Formulary is used as guideline for antibiotic dosing [13].

Antimicrobial prophylaxis and treatment in early-onset sepsis

Since EOS has a maternal origin, intrapartum antibiotic prophylaxis may be considered if maternal risk factors for EOS are present during delivery. In cases of (suspected) EOS, newborns are treated with intravenous gentamicin and a penicillin derivative for 7 days or until negative blood culture. Intrapartum benzylpenicillin every 4 hours is common practice for GBS prophylaxis, though amoxicillin or ampicillin may be used as an alternative [14]. The dose of gentamicin is based on gestational age (GA) and the recommended starting doses in the Netherlands are 5 mg/kg/48h for extremely premature neonates (GA < 32 weeks), 5 mg/kg/36h for preterm neonates (GA ≥ 32 weeks - 37 weeks), 4 mg/kg/24h for term neonates (GA > 37 weeks), or 5 mg/kg/36h for neonates GA≥36 undergoing controlled hypothermia [15][16].

Antimicrobial treatment in late-onset sepsis

Empirical amikacin combined with a β-lactam antibiotic (i.e. flucloxacillin) is initiated for neonates with a postnatal age (PNA) more than 3 days that present with clinical signs of LOS. As with gentamicin, amikacin doses are based on GA and weight with suggested starting doses in the Netherlands of 16 mg/ kg/ 48h for premature neonates of PNA <14 days and <1,200 grams; 15 mg/kg/36h for premature neonates and 1,200 – 2,800 grams; 15 mg/kg/24h for premature neonates of PNA <14 days and ≥ 2,800 grams; 20 mg/kg/36h for premature neonates of PNA 14 – 31 days and <1,200 grams; 18 mg/kg/24h for premature neonates of PNA 14 – 31 days and ≥1,200 grams; and 15 mg/kg/24h for term neonates [17–19]. If blood culture results indicate infection with CoNS, a common pathogen for LOS, vancomycin is added with a dose of in 20-60 mg/kg/day intravenous divided over 2-4 doses for 7 days, depending on GA, PNA and body weight [20–22].

Therapeutic drug monitoring

Many antibiotic drugs commonly used in neonatal sepsis, most notably gentamicin, amikacin and vancomycin have narrow therapeutic indexes [18,21,23]. Main toxic effects of aminoglycosides are nephrotoxicity and ototoxicity, whereas for vancomycin this is limited to nephrotoxicity [24,25].

Due to their high inter-individual variability (IIV) in pharmacokinetics (PK) in neonates, therapeutic drug monitoring (TDM) of these drugs is strongly recommended to personalize dosing to ensure effective and safe drug concentrations in plasma [21,24]. TDM is typically performed through quantification of antimicrobial concentrations in 1-2 plasma samples collected within the dose interval (i.e. a peak concentration 1 hour after the dose and a trough concentration 0.5 hours before the following dose for aminoglycosides). Preferably through model informed precision dosing (MIPD) methods, dose magnitude and intervals can be adjusted after evaluation of concentration and target attainment [26].

Pharmacokinetics of antimicrobials in term- and premature neonates

As stated before, PK of antibiotics have considerable variability in the neonatal population. Differences in demographics, physiological, clinical and anthropomorphic characteristics such as GA, PNA, body weight, kidney function, disease state and body temperature all have a substantial impact on the distribution and elimination of drugs [27,28]. Moreover, rapid changes in postnatal body composition and fluid status combined with continuing organ maturation all influence the individual PK properties over time [26,29–31]. For instance as neonates grow older, kidney function improves resulting in an increased elimination rate and changes in clearance (CL) can be observed as early as the first week of life.

Vancomycin, gentamicin and amikacin are relatively hydrophilic drugs and therefore volume of distribution (V_d) is highly dependent on body weight due to differences in extravascular water [24,31]. Moreover, these drugs are mainly eliminated renally and thus differences in kidney maturation, often expressed as a function of age, have a considerable effect on CL rate [24,30].

The PK profile of benzylpenicillin during intrapartum GBS prophylaxis is dependent on both maternal- and fetal characteristics. As an example, GA may influence drug distribution in maternal tissue as well as fetal tissue due to changes in body composition, placental function and fetal renal development [32]. Moreover, compromised placental function by chorioamnionitis may influence the transplacental passage of benzylpenicillin [33]. Scarce evidence exists on the fetal PK profiles of commonly used antibiotics during pregnancy [14,34].

Non-invasive salivary therapeutic drug monitoring

As stated before, TDM is necessary for safe and effective dosing in neonates and is clinical routine. However, the collection of multiple plasma samples through the manipulation of venous or arterial catheters or heel lance procedures introduces considerable discomfort, pain and infection risk to patients [35]. Also, the use of excessive plasma collection may increase the risk of neonatal anemia since the total plasma volume of neonates is low (approximately 75 ml/kg) [36,37]. Hence, an effective non-invasive TDM method using saliva samples could be highly beneficial in minimizing pain and stress, while maintaining optimized dose regimens for individual patients and possibly decreasing the risk of anemia and infection. Saliva is a readily available body fluid that is produced and secreted in abundance.

Pharmacodynamic endpoints

Pharmacokinetic-pharmacodynamic indexes

Pharmacological effects of antibiotics are generally expressed as pharmacokinetic-pharmacodynamic (PK-PD) endpoints, which are based on PK derived metrics and minimal inhibitory concentrations (MIC) [38]. These PK-PD endpoints may be regarded as surrogate markers. True PD endpoints such as bacterial colony forming units in plasma are commonly not evaluated as these are difficult to assess clinically [39].

Typical efficacy PK-PD indexes that are evaluated are I) the percentage of time during the dose interval that the unbound drug concentration remains above the MIC (penicillins), II) maximum concentration (C_{max}) over MIC (aminoglycosides) and III) area under the curve (AUC_{0-24h}) over MIC (vancomycin) [24,40,41]. Trough concentrations of aminoglycosides and vancomycin are generally assumed to be indicative of toxicity risk [24,25].

Clinical response and antibiotic effect on biomarkers

Although the currently advised PK-PD indexes are mainly based on documented MICs per species [42] PK-PD targets that are based on clinical response and/or validated biomarkers are not studied in the clinical setting, to our best knowledge. Whilst MIC based PK-PD indexes are indicative of antimicrobial effect, these indexes are relatively dichotomous, i.e. antimicrobial effect is present when drug concentrations are above the MIC, whereas it is absent when the measured plasma concentration is below the MIC [38,39,43]. In reality, the antibiotic effect is a continuous parameter. Any change in antimicrobial effect at increasing antibiotic plasma concentrations is gradual, or "dynamic" as the measure of treatment effect will always be a sum of the antibiotic effect and the bacterial multiplication or growth speed. Consequently, when studying the efficacy of antibiotics a PKPD model should be used that takes the continuous character of the effect into account. With such a model true PK-PD targets can be identified and this should ideally result in optimized antibiotic treatment.

Ideally treatment efficacy based on PK-PD results should be available at short notice. However, since treatment success after antibiotic treatment is currently confirmed by means of a negative repeat blood culture of which results may take up to 48 hours, there is a considerable risk of unnecessary antibiotic exposure or possible delay in necessary dose adjustment.

Lastly, exposure to antibiotics should be prudent and justified in order to prevent the development of antibiotic resistance, which is currently a threat to global health [44].

Thesis objective

The objectives of this thesis were to:

I) Assess the feasibility of non-invasive salivary TDM of antibiotics in comparison to plasma TDM.
II) To determine the applicability of the bacterial DNA load as a measure of antibiotic effect during neonatal sepsis.
III) To assess the effectiveness of benzylpenicillin prophylaxis in GBS positive pregnant women during delivery by analyzing antibiotic concentrations in umbilical cord blood samples.

Thesis outline

Chapter 1 (this chapter) contains a general introduction to the subject matter as well as the thesis objective and outline.

Chapter 2 describes the development and validation of an analytical method using tandem-liquid-chromatography coupled with mass-spectrometry to measure antibiotic concentrations in saliva.

Chapter 3 provides a description of the development of an integrated PK model for gentamicin in plasma and saliva, as well as the evaluation of the performance of saliva TDM by simulation.

Chapter 4 elaborates on the applicability of saliva sampling for TDM of amikacin through the development of a PK model in plasma and saliva, supported by a simulated target attainment evaluation.

In **chapter 5** the applicability of bacterial DNA loads as a PD marker for vancomycin treatment effect in CoNS-positive LOS is investigated.

Chapter 6 describes benzylpenicillin concentrations in umbilical cord blood and neonatal plasma following intrapartum doses and evaluates doses that deviate from current national guidelines.

Chapter 7 discusses the results of this thesis in a general context and provides future aspects that support clinical implementation of the developed methods.

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Part

Development of bioanalytical methods in plasma and saliva

Chapter 2

Determination and validation of amikacin and flucloxacillin in human newborn plasma and saliva by liquid chromatography-mass spectrometry and its clinical application

Fleur. S. Sinkeler*, **Amadou Samb***,
Timo R. de Haan, Dennis van der Laan,
Marcel Pistorius, Yuma A. Bijleveld, Ron A. A. Mathôt

**Shared first authorship*

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Abstract

Background

Amikacin and flucloxacillin are used as antibiotic treatment for late-onset sepsis in neonates. Therapeutic drug monitoring is used to optimize therapy and improve clinical outcomes. Drawing plasma samples causes considerable burden in this vulnerable population. Using saliva as an alternative may be beneficial. This study presented the development and validation of a highly sensitive method for determination of amikacin and flucloxacillin in plasma and saliva using tandem liquid chromatography tandem mass spectrometry (LC-MS/MS).

Methods

In 10 µl plasma, proteins were precipitated with methanol/formic acid containing penicillin V as internal standard. Calibration standards were prepared over a range of 10 – 50000 ng/mL. The chromatographic runtime was 6.5 minutes.

Results

For determination of amikacin in plasma, accuracy, within-run and between-run imprecision were between 95% and 113% and <14%, respectively; respective results for saliva were between 95% and 97% and <4%. For determination of flucloxacillin in plasma, accuracy, within-run and between-run imprecision were between 101% and 105% and <15%. For saliva values were between 103% and 107% and <6%. Samples were stable at different temperatures examined. For flucloxacillin, ultrafiltration efficiencies did not meet the criteria due to adsorption on the filter. Quantification of amikacin and flucloxacillin in hemolytic, icteric and lipemic plasma samples was accurate. The applicability of the method was shown by analyzing amikacin plasma and saliva samples from 2 neonatal patients.

Conclusion

An accurate and precise LC-MS/MS method was developed for the simultaneous quantification of amikacin and flucloxacillin in a small volume (10 µL) of plasma and saliva.

Background

Neonatal sepsis is identified as the third major cause of neonatal mortality [1]. Late onset sepsis, occurring at or after 72 hours post-partum, is usually caused by Gram-positive bacteria (*Staphylococcus aureus,* coagulase-negative *Staphylococcus, Streptococci* spp) or Gram-negative bacteria (*Escherichia coli, Enterococci* spp) [2]. The primary antibiotic regimen for neonatal late-onset sepsis in our hospital consists of the combination of amikacin and flucloxacillin.

Amikacin is an aminoglycoside antibiotic widely used in the treatment of infections caused by aerobic Gram-negative bacteria. Aminoglycosides display a concentration-dependent bactericidal activity and therefore an C_{max} (maximum drug concentration) to MIC (minimum inhibitory concentration) ratio ≥ 8 is associated with improved clinical outcomes in neonates [3].

Flucloxacillin is a β-lactam antibiotic and frequently used in the treatment of infections caused by Gram-positive bacteria. Flucloxacillin is highly protein bound and it is the unbound antibiotic concentration that is pharmacologically active and responsible for the antibacterial effect [4]. The time that the unbound concentration in plasma exceeds the MIC (%Tf>MIC) should be at least 40 to 50% of the dosing interval in neonates [5].

Therapeutic drug monitoring (TDM), measuring drug concentrations to individualize dosing, can be used to optimize therapy and improve clinical outcomes in this vulnerable population [6]. Amikacin shows high inter-individual and residual variability, together with a narrow therapeutic index and therefore TDM is performed on a routine basis [3]. TDM of flucloxacillin is not performed on a routine basis but might be considered when the therapy seems ineffective or in case of *S. aureus* infections, due to higher MICs. In our institution, two plasma samples (peak and trough level) are drawn after starting amikacin therapy, which causes a considerable burden for the neonate. TDM on basis of saliva concentrations may therefore be beneficial.

To date, no tandem liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for the determination of amikacin or flucloxacillin in saliva has been reported. Several studies have determined amikacin and flucloxacillin concentrations by using LC-MS/MS in various matrices, except for saliva [7-12]. The aim of the present study was the development and validation of a sensitive LC-MS/MS assay for the simultaneous quantification of amikacin and total and unbound flucloxacillin concentrations in both plasma and saliva. Finally, the possible clinical applicability of this method is shown by presenting the amikacin concentration time profiles of 2 neonatal patients.

Methods

Chemicals and reagents

Amikacin was purchased from Toronto Research Chemicals (Toronto, Canada). Flucloxacillin and penicillin V were both purchased from Sigma-Aldrich (St. Louis, USA). Penicillin V was used as an internal standard (IS) in a concentration of 0.04 mg/L in methanol/formic acid. Structures are shown in **Figure 1**. Purified and deionized water was obtained using an ELGA Purelab DV-25 (Veolia Water Technologies, St. Maurice, France). Human plasma was obtained from Sanquin (Amsterdam, The Netherlands) and saliva was donated by healthy volunteers from the Amsterdam UMC, location AMC (Amsterdam, The Netherlands). LC-MS/MS grade methanol and acetonitrile were obtained from Merck (New Jersey, USA). SalivaBio Infant Swabs were used for saliva collection (Salimetrics, Carlsbad, USA).

LC-MS/MS Instrumentation and conditions

Samples were analyzed using a LC-30 Nexera system (Shimadzu, Kyoto, Japan) coupled to a 5500 QTrap tandem mass spectrometer (AB Sciex, Framingham, USA). Analyst software version 1.7.1. was used for data processing and quantification (Applied Biosystems, Foster City, USA).

The compounds were analyzed on a Hypersil GOLD aQ (50 x 2.1 mm, 1.9µm) column (Thermo Fisher Scientific, Waltham, USA) at a flow rate of 0,4 mL/min. Mobile phase A (A) comprised perfluoropentanoic acid 3.2% (w/w) with ammonium acetate 1% (w/v) in ultra-pure water and phase B (B) comprised acetonitrile with perfluoropentanoic acid 3.2% with ammonium acetate 1%. The LC gradient elution was performed starting at 95% A/5% B, then inverted in 5 minutes to 5% A/95% B, stayed 5% A/95% B for 1 minute and back to 95% A/ 5% B for the remainder of the run. The autosampler temperature was set at 5˚C and the column oven was set at 30˚C. The compounds were detected in positive ion mode using multiple reaction monitoring with ion transitions 586.2 to 163.1 m/z for amikacin, 350.95 to 160.00 m/z for penicillin V and 454.05 to 160.00 m/z for flucloxacillin. Total run time was set at 6.5 minutes, with a retention time of 2.85 minutes for penicillin V, 2.88 minutes for amikacin and 3.25 minutes for flucloxacillin.

Preparation of stock solutions, calibration standards and quality control standards

Standard stock solutions (1.0 mg/mL) were prepared by separately dissolving 5.0 mg amikacin and 5.0 mg flucloxacillin in purified water. Two work solutions of amikacin and flucloxacillin each (5.0 and 50.0 µg/mL) were prepared by dissolving them in water/methanol (1:1). The work solutions were diluted with blank human plasma to produce calibration standards at concentrations of 10, 20, 50, 100, 500, 1 000, 5 000, 10 000 and 50 000 ng/mL.

Quality control (QC) samples for determination of accuracy and imprecision were prepared at concentrations of 10 ng/mL (lower limit of quantification, LLOQ), 30 ng/mL (low level of quantification, LOW), 150 ng/mL (middle level of quantification, MLQ) and 37 500 ng/mL (upper

limit of quantification, HLQ). For the determination of amikacin and flucloxacillin concentrations in saliva, the LOW and HLQ were prepared using saliva. All solutions were stored at -80˚C until analysis.

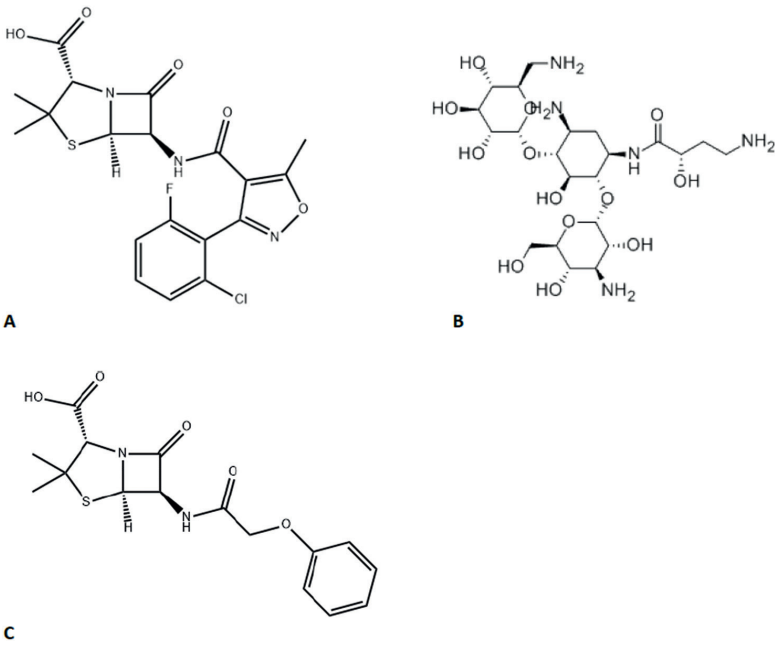

Figure 1. Chemical structure of (A) flucloxacillin, (B) amikacin and (C) penicillin V.

Preparation of samples

The saliva swabs were cut in half, after which the saturated end was placed in a collection tube and refrigerated (2 - 8˚C) before being centrifuged within 12 hours after sampling. Blood was collected in standard EDTA tubes. The EDTA tubes were centrifuged and the plasma was analyzed within two hours after sampling.

Before processing, all samples were shortly vortexed. Ten µL of each sample was pipetted into a HPLC vial with insert, 25 µL of acetic acid 30% (v/v) was added and the vial was shortly vortexed. Subsequently, 5 µL of ammonia 10% (v/v) solution was added and the samples were shortly vortexed again. For protein precipitation, 150 µL of IS solution was used. After equilibration at room temperature for 10 minutes, the samples were vortexed for 5 minutes and centrifuged at 2 750 x *g* for 5 minutes.

Quantification

Calibration curves were constructed by plotting peak area ratio of analyte over IS versus concentration. Patient and QC samples were back-calculated using the calibration curve by their corresponding ratio of analyte/IS MS response.

Method validation

The method was validated according to the European Medicines Agency guideline for bioanalytical method validation [13].

Selectivity

To assess selectivity and specificity blank plasma samples from six different patients not receiving amikacin or flucloxacillin, were used. These were prepared as described above, but without addition of the IS. The mean peak area of the interfering components in the blank samples should not be more than 20% of the average response of amikacin and flucloxacillin of the LLOQ and not more than 5% of the IS response.

Also, the IS was injected (n=3). The mean peak area of the interfering components in the IS samples should not be more than 20% of the average response of amikacin and flucloxacillin of the LLOQ.

Calibration curve

A total of six calibration lines consisting of nine different concentrations between LLOQ and ULOQ were prepared in plasma and analyzed in six runs. Calibration curves were obtained by fitting the peak area ratio to a weighted (1/x2) least squared regression model. An average correlation coefficient of $R2 > 0.990$ was acceptable for linearity. The acceptance criteria for the calibration curve is that 75% of the calibration standards should be within the 15% of the nominal value, 20% for the LLOQ. However, if the LLOQ does not comply with this criteria, it should be rejected. In addition, a minimum of six calibration standards should meet these criteria.

Accuracy and imprecision

The accuracy and imprecision were assessed for the LLOQ, LOW, MLQ and HLQ during six consecutive runs. For the within-run imprecision all QC samples were analyzed in six-fold in the first run. For the between-run imprecision a single sample of each of the QC concentrations was analyzed in the following five runs. In addition, the accuracy and imprecision of 5-fold diluted human plasma samples at the level of HLQ and 5x ULOQ were analyzed in six-fold in one run for amikacin. For flucloxacillin, the accuracy and imprecision of 10-fold diluted human plasma samples at the level of HLQ were analyzed in six-fold in one run.

For the determination of the accuracy and imprecision of saliva samples, LOW and HLQ were analyzed in three consecutive runs.

The mean accuracy and imprecision should be within 85 – 115% for LOW, MLQ and HLQ, and within 80 – 120% for LLOQ.

Matrix effects

QC LOW and HLQ were 10-fold diluted in methanol/water (1:1). Then the following samples were prepared to evaluate the matrix effects: 10x QC LOW and HLQ in blank plasma (n=1), in blank patient plasma samples (n=5) and in ultrapure water (n=6). The matrix factor (MF) was calculated by the ratio of the peak area in samples spiked with matrix to the peak area in the aqueous samples. The IS normalized MF was calculated by dividing the MF of the analyte by the MF of the IS. The relative standard deviation (RSD) should be ≤ 15% and the IS-corrected MF should be around 1.

Stability

The stability of amikacin and flucloxacillin in plasma and saliva was evaluated for LOW and HLQ during three freeze-thaw cycles and in the freezer for 22 months at -80˚C. The autosampler stability was validated for 48h and 120h at 5˚C, by reinjecting a run after a set period. These samples were compared with freshly prepared QC samples and quantified with a freshly prepared calibration curve. Samples were considered stable in stock and working solutions if on average 85 – 115% of the reference concentration was recovered.

Carry-over effects

Carry-over was evaluated by injection of a blank plasma sample directly after injection of ULOQ (n=6). Carry-over should be < 20% of the mean peak area of the LLOQ and no more than 5% of the mean peak area of the IS (see Accuracy and imprecision).

Plasma stress test

Two levels (QC LOW and HLQ) in hemolytic, lipemic and icteric plasma samples were analyzed. Plasma samples with a hemolysis index of 500 or containing bilirubin >1000 µmol/L or triglyceride >20 mmol/L were used as hemolytic, icteric and lipemic samples, respectively. The accuracy and imprecision should be within 85 – 115%.

Determination of the unbound flucloxacillin and amikacin concentration

Ultrafiltrate was obtained by centrifuging the blank plasma over the filter (Amicon ultra 0.5 ml 30K, Merck Millipore, Burlington, USA) to determine the ultrafiltration efficiency. Six samples of QC LOW and six samples of QC HLQ were prepared in ultrafiltrate and of each level, three samples were centrifuged over the filter. Ultrafiltration efficiency (%) was calculated as the proportion of the ratio of filtered analyte/IS and the ratio of non-filtered analyte/IS. The ultrafiltration efficiency should be between 85 – 115% with a RSD <15%. If the ultrafiltration efficiency is <85% or >115%, but the RSD is <15%, the results may be corrected.

Recovery saliva samples

The recovery for saliva samples was determined for levels LOW and ULOQ, measured in triplicate. The recovery should be within 85 – 115%.

Collection of plasma and saliva samples from patients

The developed and validated method was applied to plasma and saliva samples from 2 patients receiving amikacin and flucloxacillin for late-onset sepsis in clinical practice. Two plasma samples were collected as peak and trough samples as standard-of-care, the additional samples were obtained from residual material from diagnostic blood samples and were therefore collected randomly in the dosing period. Saliva samples were collected by letting the patients suck on a SalivaBio Infant Swab for 1 minute. Ethical approval for the study was obtained from the local ethics committee.

Results

Chromatography

Figure 2 shows the chromatograms of the amikacin and flucloxacillin LOW and HLQ plasma samples, the internal standard and blank human plasma sample. All chromatograms show a small symmetrical peak with a baseline peak less than 10 seconds. The total run time was 6.5 minutes, with retention times of 2.85 for penicillin V, 2.88 min for amikacin and 3.25 min for flucloxacillin.

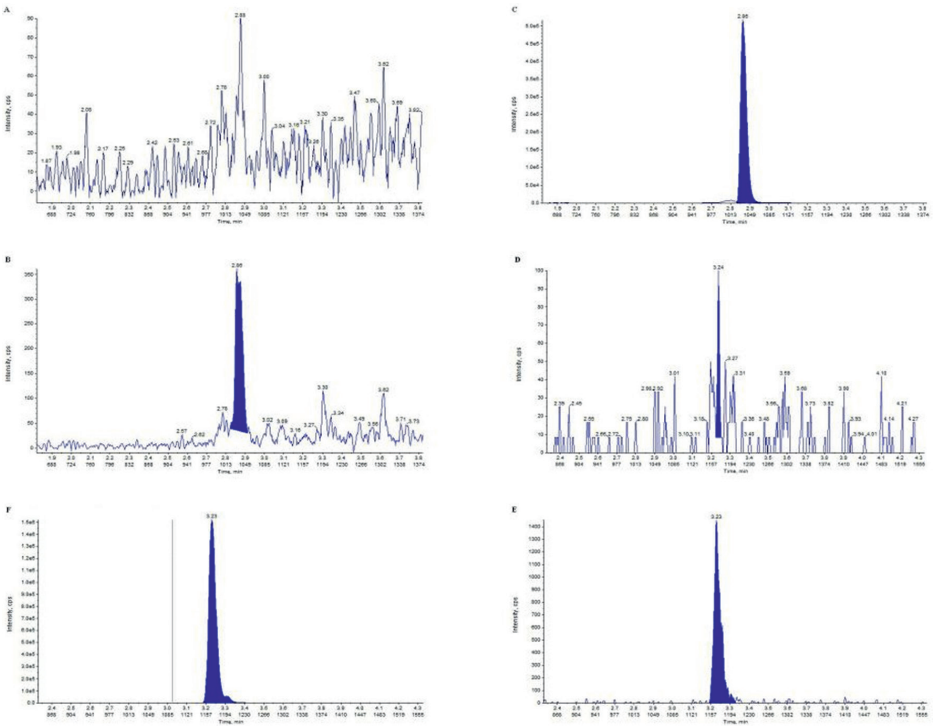

Figure 2. Chromatograms of (A) blank amikacin, (B) amikacin QC LOW, (C) amikacin QC HLQ, (D) blank flucloxacillin, (E) flucloxacillin QC LOW and (F) flucloxacillin QC HLQ.

Selectivity

No interfering components were observed. The mean peak (endogenous components) of the blank plasma samples was < 20% of the LLOQ peak area for both amikacin and flucloxacillin, and < 5% of the mean peak area of the IS.

Calibration curve

The calibration curves showed a quadratic response. The calculated value of each calibration standard for amikacin and flucloxacillin was within ± 15% of the nominal value. The mean correlation coefficient (R^2) of the calibration curves was 0.998 (range 0.996-0.999) for amikacin and 0.997 (range 0.995-0.999, n=6) for flucloxacillin and therefore above the accepted limit of 0.990.

Accuracy and imprecision

The mean accuracy ranged from 95% – 113% for amikacin and from 101% – 105% for flucloxacillin, and therefore the acceptance criteria of 85% – 115% was met for all QC levels in plasma. For both amikacin and flucloxacillin, within-run and between-run imprecision were within the criteria for all QC levels (**Table 1**).

For amikacin, the mean accuracy of 5-fold dilution at QC HLQ and 5x QC ULOQ level ranged from 98 – 99% and within-run imprecision from 3% – 4%. For flucloxacillin, the mean accuracy of 10-fold HLQ was 85% and within-run imprecision 3%. Therefore, both were within the criteria. For both saliva LOW and HLQ samples, mean accuracy and within-run impression met the acceptance criteria for both QC levels. The mean accuracy ranged from 95% – 97% for amikacin and from 103% – 107% for flucloxacillin (**Table 1**).

Matrix effects

No significant matrix effects were seen. Results for the IS-corrected MF for amikacin and flucloxacillin are shown in **Table 1**. The IS-corrected MF for amikacin is not around 1. However, for both amikacin and flucloxacillin, the RSD is ≤15%.

Stability

Table 1 depicts the stability results; data are presented as mean (n=4). Three freeze-thaw cycles did not affect stability of the samples. Autosampler stability was set at 120 hours at 5˚C. Freezer stability was set at 22 months at -80˚C.

Carry-over effects

For flucloxacillin, the mean peak area of the blank samples was < 20% of the mean peak area of the LLOQ and < 5% of the mean peak area of the internal standard and therefore met the requirements.

For amikacin, the peak area of the blank sample injected after ULOQ was 104% of the peak area of the LLOQ, although <5% of the mean peak area of the internal standard. To reduce

the high carry-over of the analyte, 5 instead of 1 blank samples were injected after ULOQ. This resulted in a carry-over <20% after the 6th blank injection. If a patient sample is >10 000 ng/mL, the subsequent sample will be reanalyzed.

Table 1. Summary of the validation results for multiple performance-indicating parameters.

Parameter	QC level	Amikacin	Flucloxacillin
Accuracy plasma (%)	LLOQ	113	105
	LOW	99	101
	MLQ	103	101
	HLQ	95	101
Within-run imprecision plasma (%)	LLOQ	9	14
	LOW	10	8
	MLQ	12	6
	HLQ	8	4
Between-run imprecision plasma (%)	LLOQ	7	15
	LOW	14	14
	MLQ	9	8
	HLQ	3	6
Accuracy saliva (%)	LOW	95	107
	HLQ	97	103
Within- run imprecision saliva (%)	LOW	4	6
	HLQ	2	3
MEs ± RSD (%)	LOW	0.72 ± 13.7	0.97 ± 7.3
	HLQ	0.58 ± 9.7	1.21 ± 5.1
Stability freeze/thaw 3x (%)	LOW	92	106
	HLQ	101	99
Stability freezer (-80˚C) 22 months (%)	LOW	114	98
	HLQ	97	89
Stability autosampler (5˚C) 120 hours (%)	LOW	99	100
	HLQ	100	102
Plasma stress test (accuracy ± RSD (%))	LOW	100.4 ± 3.4	98.3 ± 12.6
- Hemolytic sample	HLQ	98.6 ± 7.7	99.9 ± 7.4
	LOW	114.2 ± 7.2	96.9 ± 1.1
- Icteric sample	HLQ	109.7 ± 2.1	98.0 ± 5.0
	LOW	98.8 ± 9.2	96.7 ± 2.1
- Lipemic sample	HLQ	101.9 ± 2.8	97.5 ± 3.2
Ultrafiltration efficiency (recovery ± RSD (%))	LOW	119 ± 8.3	20 ± 8.8
	HLQ	111 ± 2.7	68 ± 1.7

ME, matrix effect; RSD, relative standard deviation

Plasma stress test

The results are shown in **Table 1**. For both amikacin and flucloxacillin, the accuracy and imprecision are all < 15%.

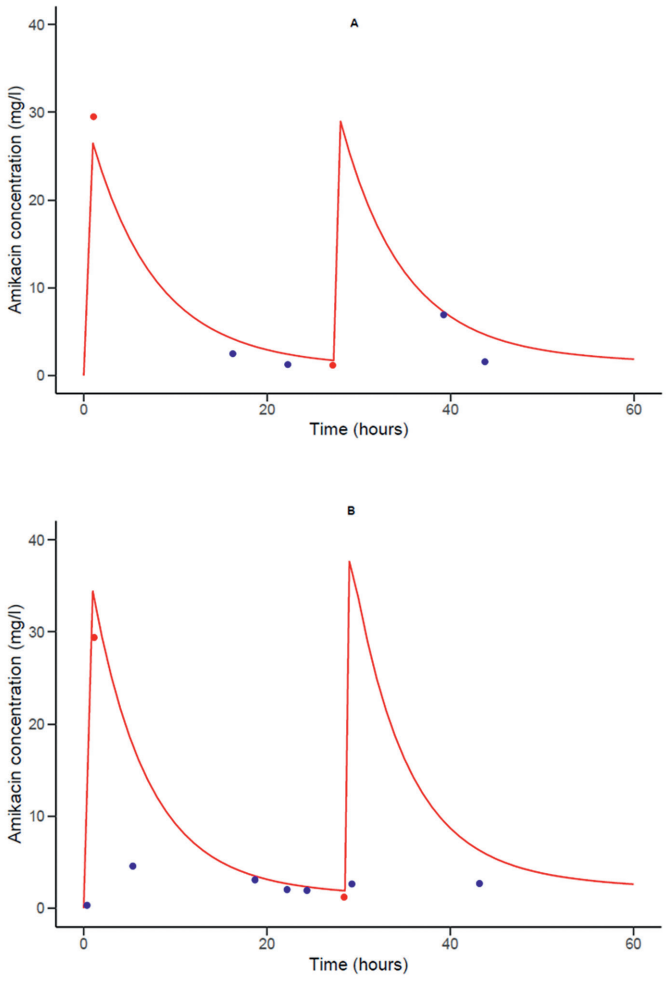

Figure 3. Preliminary results of the amikacin concentration in plasma (red dots and solid line) and saliva (blue dots). On the x-axis is the time after dosing (hours) and on the y-axis the total concentration (mg/L).

Determination of the unbound flucloxacillin and amikacin concentration

For flucloxacillin, the ultrafiltration efficiencies did not meet the criteria of 85% - 115%, as these were 20% and 68% for QC LOW and HLQ, respectively. For amikacin, the mean ultrafiltration efficiency did meet the criteria for QC HLQ (111%), but not for LOW (119%).

Recovery saliva samples

The criteria for recovery of the saliva samples was met for both amikacin and flucloxacillin. For amikacin levels LOW and ULOQ was 104.6% and 74.5%, respectively. For flucloxacillin levels LOW and ULOQ this was 98.4% and 96.2%.

Clinical application – plasma and saliva samples of patients

With the developed method amikacin and flucloxacillin plasma and saliva samples drawn from neonates were analyzed. The concentration-time curves of 2 patients for amikacin plasma and saliva samples are presented in **Figure 3**. The concentration curve of saliva samples seems to follow the plasma time profile.

Discussion

In this study, a LC-MS/MS method has been developed and validated for the simultaneous quantification of amikacin and flucloxacillin concentrations in human plasma and saliva. The method has proven to be sensitive, accurate and precise. This is the first study describing a highly sensitive method to simultaneously determine amikacin and flucloxacillin in saliva. Analytical methods for the determination of amikacin have been published for various matrices, including bronchial epithelial lining fluid, urine, and (newborn) plasma [14-16]. However, there is only one study describing the determination of amikacin in saliva, using an immunoassay [17]. The LLOQ in this study (2000 ng/mL) was 200 times higher than the LLOQ of our method. Probably that is why the authors reported undetectable amikacin levels in the measured patient saliva samples.

General principles of drug distribution apply to the salivary distribution of drugs. The physico-chemical properties of the compound, such as molecular size, lipid solubility, pKa and protein binding are important factors for transfer of the drug from the blood into saliva. In addition, the salivary pH, flow rate and existing pathophysiology of the oral cavity are contributing factors as well [18]. Amikacin is a relatively large (Mw 585.6 g/mol), hydrophilic molecule with a basic pKa of 8.1 [19]. The physiological pH of saliva can range from 5.8 to 7.8 [20]. Therefore, amikacin is predominately positively charged at physiological pH. The protein binding of amikacin ranges from 0% – 11% [21], which is favorable for the distribution in saliva. The preliminary data of our study of the 2 patients confirmed that amikacin can be found in saliva of neonates.

In plasma, a matrix effect was observed for amikacin as the IS-corrected MF was lower than 1. However, this was a constant effect, resulting in a RSD <15% and thus was accepted. Ideally, a stable isotope labeled internal standard may have corrected for matrix effects, but was not implemented due to limited commercial availability of labeled isotopomers.

The unbound concentration of flucloxacillin in plasma could not be determined as a substantial amount of flucloxacillin was lost during the filtration process due to adsorption on the filter. Flucloxacillin is 75-95% plasma protein bound, which is highly relevant as it is the unbound

fraction that is pharmacologically active. In neonates, plasma protein binding seems to be particularly variable ranging from 34.3 – 89.7% [4]. Flucloxacillin protein binding in neonates showed a significant correlation with gestational age, body weight and postconceptional age [4,18]. Because of the many covariates affecting protein binding, the constantly changing plasma composition in neonates may affect protein binding as well [18]. Therefore, if TDM of flucloxacillin will become common practice to optimize individual dosing, measuring the unbound concentration would be valuable and the validation for unbound flucloxacillin in this population should be further explored.

The developed LC-MS/MS method was used to analyze the plasma and saliva samples of 2 patients and was deemed suitable for the simultaneous quantification of both amikacin and flucloxacillin in only 10 µL of plasma and saliva. This small volume of plasma is extremely valuable in this vulnerable population, where blood sampling is often difficult. Another advantage of the present method is the accurate quantification of amikacin and flucloxacillin in hemolytic, lipemic and icteric plasma samples. Hemolysis, icterus and lipemia can cause interference with the measurements of various analytes [22]. In some circumstances, such as newborn jaundice, samples without interference are not available and rejection of the samples could hinder treatment.

Conclusion

In conclusion, this study presented a sensitive LC-MS/MS method for the simultaneous quantification of amikacin and flucloxacillin in plasma and saliva. The application of this method on patient material seems to reveal a reliable relationship between saliva and plasma amikacin samples. Therefore, this method will be used in a new study to further investigate the use of saliva as a sampling method for TDM of amikacin and flucloxacillin in this vulnerable population.

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Part II

Pharmacokinetics and therapeutic drug monitoring of aminoglycosides

Chapter 3

Saliva as a sampling matrix for therapeutic drug monitoring of gentamicin in neonates: A prospective population pharmacokinetic and simulation study

Amadou Samb*, Matthijs Kruizinga*, Younes Tallahi, Michiel van Esdonk, Willemijn van Heel, Gertjan Driessen, Yuma Bijleveld, Rik Stuurman, Adam Cohen, Anton van Kaam, Timo de Haan, Ron Mathôt

** Shared first authorship*

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Abstract

Introduction

Therapeutic drug monitoring (TDM) of gentamicin in neonates is recommended for safe and effective dosing and is currently performed by plasma sampling, which is an invasive and painful procedure. In this study, feasibility of a non-invasive gentamicin TDM strategy using saliva was investigated.

Methods

This was a multicenter, prospective, observational cohort study including 54 neonates. Any neonate treated with intravenous gentamicin was eligible for the study. Up to 8 saliva samples were collected per patient at different time-points. Gentamicin levels in saliva were determined with liquid chromatography tandem mass-spectrometry. A population pharmacokinetic (PK) model was developed using Nonlinear Mixed-Effects Modeling (NONMEM) to describe the relation between gentamicin concentrations in saliva and plasma. Monte Carlo simulations with a representative virtual cohort (N=3,000) were performed to evaluate the probability of target attainment with saliva- versus plasma TDM.

Results

Plasma PK was adequately described with an earlier published model. An additional saliva compartment describing the salivary gentamicin concentrations was appended to the model with first-order input (k_{13} 0.023 h^{-1}) and first-order elimination (k_{30} 0.169 h^{-1}). Inter-individual variability of k_{30} was 38%. Postmenstrual age (PMA) correlated negatively with both k_{13} and k_{30}. Simulations demonstrated that TDM with 4 saliva samples was accurate in 81% of the simulated cases versus 94% when performed with 2 plasma samples and 87% when performed with 1 plasma sample.

Conclusion

TDM of gentamicin using saliva is feasible and the difference in precision between saliva and plasma TDM may not be clinically relevant, especially for premature neonates.

Introduction

Neonates admitted to the neonatal intensive care unit (NICU) have a high risk for bacteremia or sepsis due to premature birth, low birth weight and indwelling central venous lines [1]. Intravenous treatment with the aminoglycoside gentamicin provides gram-negative coverage and is part of the first line antibiotic treatment protocols for early and late onset sepsis in premature and term neonates.

Gentamicin has a narrow therapeutic index, with oto- and nephrotoxicity as its possible concentration-dependent adverse drug events (ADE), for which neonates are especially vulnerable [2]. Furthermore, dosing is complicated by high variability in body composition, kidney function and organ maturation of neonates [3]. Gentamicin concentrations can therefore be unpredictable and therapeutic drug monitoring (TDM) is necessary to ensure adequate dosing regimens. TDM requires repeated plasma sampling from central venous lines and via heel lance, which is invasive, painful and may contribute to clinical anemia or infection [4]. As a result, TDM by plasma sampling is complicated in neonates [5], possibly leading to suboptimal individual gentamicin doses and thereby causing a decrease in therapeutic efficacy and an increased risk of ADE.

Non-invasive TDM methods in neonates would allow for a decreased burden of plasma collection, an increased sampling frequency and safer and more efficacious dosing. Moreover, saliva collection is easy, cheap, saliva is readily available and other than oromucosal inflammations and lesions there are no contraindications for saliva collection [6]. Previous studies have shown that the use of saliva as a matrix for TDM is feasible for several anti-epileptic drugs and caffeine [6,7]. Analyses of salivary gentamicin concentrations and other aminoglycosides during intravenous treatment of children and adults have been published with varying results. Some studies reported a good correlation between gentamicin saliva and plasma concentrations, while others reported undetectable aminoglycoside concentrations in saliva [8–11]. To date, no such studies have been performed in a neonatal population.

The aim of this study was to determine the feasibility of a non-invasive gentamicin TDM strategy by measuring salivary gentamicin concentrations and relating these to gentamicin concentrations in routinely drawn plasma samples in neonates.

Materials and methods

Study design

This was a multicenter, prospective, observational pharmacokinetic (PK) study conducted in the Emma children's hospital (Amsterdam UMC, Amsterdam, the Netherlands) and the Juliana children's hospital (Haga Hospital, The Hague, the Netherlands). Gentamicin concentrations were measured in saliva and compared with plasma concentrations obtained as part of routine TDM. The local ethics committee of the Amsterdam UMC approved this study

(number 2018_193). Local feasibility was tested and approved for the Haga hospital. The study was registered in the Dutch Trial Registry (NTR, NL7211).

Subjects

Inclusion of subjects took place between October 8th 2018 and March 4th 2020. Any neonate that was treated with gentamicin according to local clinical guidelines was eligible for the study. Patients were included in this study after signed informed consent of both parents was given. For the analysis, three distinct subgroups based on gestational age (GA) were pre-specified and treated with 0.5h intravenous gentamicin infusion according to local dosing protocols: 1) Neonates with GA < 32 weeks (5 mg/kg/48h); 2) neonates with GA ≥ 32 weeks - 37 weeks (5 mg/kg/36h); and 3) neonates with GA ≥ 37 weeks (4 mg/kg/24h at Emma Children's hospital and 5 mg/kg/36h at Juliana Children's Hospital). Clinical data were obtained from the digital medical files of the patients (sex, GA, postnatal age (PNA), postmenstrual age (PMA), birth weight (BW), current body weight (WT), perinatal asphyxia, therapeutic hypothermia and concomitant medication).

No formal sample size calculations were performed. A total of 60 patients (20 patients per group) were scheduled to be enrolled into the study, since 20 patients per subgroup are deemed sufficient for NONMEM analysis [12].

Sample collection

Saliva samples were collected using SalivaBio Infant's Swabs (Salimetrics, Carlsbad, CA, USA). Swabs were placed in the cheek pouch of the patient for approximately 90 seconds, according to the manufacturer's instructions [13]. Nursing staff and researchers received training in sample collection before study initiation. After collection, swabs were centrifuged at 4,000 RPM for 5 minutes and extracted saliva was stored at -80° C until analysis for a maximum of three months. Per patient a maximum of 8 saliva samples were collected up to 48 hours after the last gentamicin dose following a predetermined sampling schedule. However, deviation from the sampling schedule due to clinical practice was allowed. Any adsorption of gentamicin to the swab was assessed through recovery tests prior to analysis. Adsorption of less than 15% was deemed acceptable, as this is a commonly used boundary value for the precision and accuracy of quantitative analytical laboratory techniques. Gentamicin concentrations in plasma were collected from 2 routine TDM measurements, 1h after the first dose and 12-48h after the first dose. Additional plasma levels were determined in residual material, when available.

Bio-analytical assay

The major components of gentamicin (C1, C1a and C2) were quantified in saliva samples using a previously published validated LC-MS/MS method [14]. This method has been validated for saliva samples for the purposes of this study. In short, the accuracy and within run imprecision at the lowest level of quantification (LLOQ) were 118% and 10.2%, respectively. The accuracy and imprecision were 98.4% and 3.3%, respectively, at the middle level of quantification

(MLQ). At the upper limit of quantification (ULOQ), accuracy was 98.7% and imprecision was 3.2%. The LLOQ was 0.056 mg/L and minimal sample volume was 10 µl.

Pharmacokinetic analysis

Data handling, data visualization and descriptive statistics were performed using R statistics version 4.0.2 [15]. A population PK (POP-PK) model was developed using nonlinear mixed-effects modeling, as implemented in NONMEM version 7.4.0 (ICON Development Solutions, Dublin, Ireland). Gentamicin concentrations in plasma and saliva were logarithmically transformed.

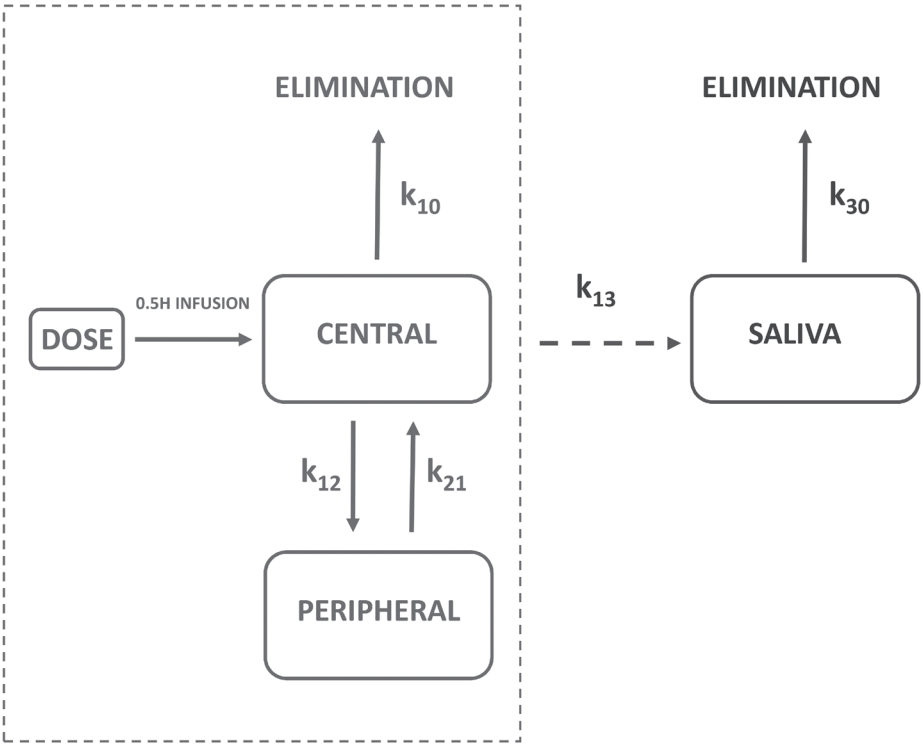

Figure 1. Conceptual model for gentamicin PK in plasma and saliva. Within dashed lines: Gentamicin in plasma. Dose is administered as a 0.5h IV infusion to the central compartment. k_{12}: Transport rate from central to peripheral compartment. k_{21}: Transport rate from peripheral compartment to central compartment. k_{10}: Elimination rate from the central compartment. Outside dashed lines: gentamicin PK in saliva. k_{13}: Transport rate from central compartment to saliva compartment. The dashed arrow signifies that gentamicin loss from the central compartment is assumed to be negligible. k_{30}: : Elimination rate from saliva.

An integrated model describing gentamicin in plasma and saliva was developed using a stepwise modeling approach. First, plasma PK data was described using a previously published model by Fuchs *et al.* [16], fixing the PK parameters. The control stream for this model was provided by the authors. This is a 2-compartment model with inter-individual variability (IIV) on clearance (CL) and central volume of distribution (V_c). Further specifications for the

plasma model are depicted in **Supplementary Table S1**. Model performance was evaluated through the assessment of goodness-of-fit (GOF) plots and visual predictive checks (VPCs).

An additional compartment describing the salivary gentamicin concentrations was appended to the model. The conceptual model for gentamicin in plasma and saliva is depicted in **Figure 1**. The first-order transport rate from the central (plasma) compartment to the saliva compartment was expressed as k_{13}, whilst the first-order rate of gentamicin elimination from the saliva compartment was expressed as k_{30}. No transport from the saliva compartment back to the central and peripheral compartments was modeled, since the oral bioavailability of gentamicin is negligible [17]. Central gentamicin mass decrease due to transport from the central compartment to the saliva compartment was assumed to be negligible, as this was expected to be proportionally diminutive compared to the total amount of gentamicin in the central compartment, similar to a hypothetical effect compartment model [18]. Both fixed and random effects of rate constants k_{13} and k_{30} were estimated using the ADVAN6 subroutine in NONMEM. Model parameters were evaluated by assessing changes in the objective function value (OFV), relative standard error (RSE) assessment and diagnostic plots. A ΔOFV of -3.81 corresponds with p = 0.05, which was the significance level for inclusion of any parameter. Gentamicin concentrations in saliva below LLOQ were accounted for with the M3-method [19]. First, the structural model was estimated, describing the relations between parameters, as well as estimation of IIV on the parameters. Thereafter, the error model was developed, describing the residual error structure in the model. Finally, the covariate model explains part of the variability based on covariates.

GA, PNA, PMA, BW, WT, sex, perinatal asphyxia, therapeutic hypothermia and concomitant drugs were evaluated as covariates on the saliva distribution parameters for this model. Covariate analysis was performed with stepwise forward inclusion (α=0.05) and backwards elimination (α=0.01). Continuous covariates were included in the model as a power equation function (**Eq.1**).

(1) $$p = \theta_p * \frac{cov}{median}^{\theta_{cov}}$$

Parameter p was calculated from typical parameter θ_p, multiplied with the fractional deviation from the median value of the covariate. The magnitude of the covariate effect was estimated as θ_{cov}. Dichotomous covariates were coded in NONMEM as shown in **Eq.2**.

(2) $$p = \theta_p + cov * \theta_{cov}$$

Dichotomous covariates could take the value of either 0 or 1. Reference parameter value θ_p was estimated and the parameter difference between covariate parameters was estimated as θ_{cov} to calculate parameter p.

Assessments of diagnostic tools such as GOF plots, RSE, η-shrinkage and ε-shrinkage were used for model evaluation during all steps. Non-parametric bootstrap analyses (n=1,000), as well as the simulation based prediction-corrected VPCs (pcVPC) were employed for assessment of the model robustness and internal validation of the final model [20].

TDM performance simulation

R version 4.02 and the mrgsolve [21] package were used for Monte Carlo simulations. A simulation cohort (n = 3,000) with a uniform distribution of GA and corresponding WT (**Supplementary Figure S2**) [22] was prepared and a single administration of 5 mg/kg/48h (GA < 32 weeks), 5 mg/kg/36h (GA ≥ 32-37 weeks) or 4 mg/kg/24h (GA ≥ 37 weeks) was simulated for each subject in accordance with Dutch dosing guidelines.

For plasma and saliva TDM different sampling schedules were simulated with measurements at different time-points after the first dose. First, a schedule with a single intermediate (14h post-dose) sample was simulated and the performance of this schedule in the context of TDM was appraised. Second, a two-sample schedule with a peak- (1h for plasma and 3h for saliva post-dose samples) and trough (0.5h before next dose) sample was evaluated. Next, the combination of peak-, intermediate- and trough samples was evaluated. Finally, schedules were evaluated in which samples were added (at 7h post-dose; at 7 – 18h post-dose; at 1h pre-dose and 7 – 18h post-dose). Bayesian maximum *a posteriori* (MAP) optimization was used to estimate the empirical Bayes estimates of the individual CL, V_c and k_{30} for each subject based on the simulated concentrations [23]. Based on the estimated CL and V_c, true peak- and trough plasma concentrations were estimated for each subject, who then entered a basic decision rule optimizing the dose to reach a targeted peak plasma concentration between 9-11 mg/L and trough concentration < 0.8 mg/L after the third dose. Target ranges were deliberately set stricter than clinical guidelines (peak 8-12 mg/L and trough < 1 mg/L) to account for residual error in the estimations. For each subject, two additional dose intervals of gentamicin were simulated after dose adjustment. Finally, the proportions of subjects with true peak- and trough concentrations within clinical guideline reference ranges (target attainment) after the third dose were calculated.

Simulations were performed for plasma TDM (1-6 samples), saliva TDM (1-6 samples), model-based dose optimization ('M' samples) and 'no TDM' (standard dosing, 0 samples). Model-based dosing was performed using the typical PK parameter estimates based on the covariates included in the population model published by Fuchs *et al* [16]. The proportion of subjects with target attainment after each simulated scenario was calculated and compared in order to appraise the added value of saliva and plasma TDM.

Results

Demographic characteristics

Table 1 depicts the demographic characteristics of the included patients. In total 54 of the planned 60 neonates were enrolled in this study. The SARS-CoV-2 pandemic resulted in an early termination of the study. A total of 267 saliva samples were collected during the study. Seventy-three samples (27.3%) could not be analyzed, either due to low sample volumes (23.5%) or blood contamination of samples (3.8%).

Table 1. Demographic characteristics of the study population.

Demographic	Value
Enrolled patients - N	54
Males - N (%)	31 (57.4)
GA in weeks - median (range)	34.8 (24.3 - 41.7)
< 32 weeks - N (%)	21 (38.9)
32 - 37 weeks - N (%)	13 (24.1)
≥ 37 weeks - N (%)	20 (37.0)
PMA in days - median (range)	244.2 (170.5 - 294.2)
PNA in days - median (range)	1.5 (0.3 -6.8)
Birth weight in kg - median (range)	2.4 (0.7 - 4.5)
Actual weight in kg - median (range)	2.4 (0.7 - 4.3)
Total saliva samples - N (%)	267 (100)
Analyzed - N (%)	194 (72.7)
Failed - N (%)	73 (27.3)
Analyzed saliva samples per patient - median (range)	3 (1 - 8)
Plasma samples - N	99
Peak samples - N	43
Trough samples -N	56
Plasma samples per patient - median (range)	2 (1 - 4)
Oro-esophageal congenital anomalies - N	1
Controlled hypothermia - N	3
Perinatal asphyxia - N	3

GA: Gestational age. PNA: Postnatal age. PMA: Postmenstrual age.

Swab adsorption

Adsorption of gentamicin to the swab was found to be less than 3.1% at the low concentration level and 8.2% at the high concentration level and therefore below the predetermined acceptable percentage of 15%.

Gentamicin pharmacokinetics in plasma

Model diagnostic figures indicated that the model provided by Fuchs *et al.* could adequately describe the plasma PK data of the study population, based on 97 plasma TDM concentrations. It seems that the model had a slight bias towards underprediction at the low concentration range, though upon inspection of all diagnostic plots, the performance of the model was deemed acceptable (**Supplementary Figure S3**). The model was used to estimate individual plasma PK and served as a basis for the construction of the saliva model.

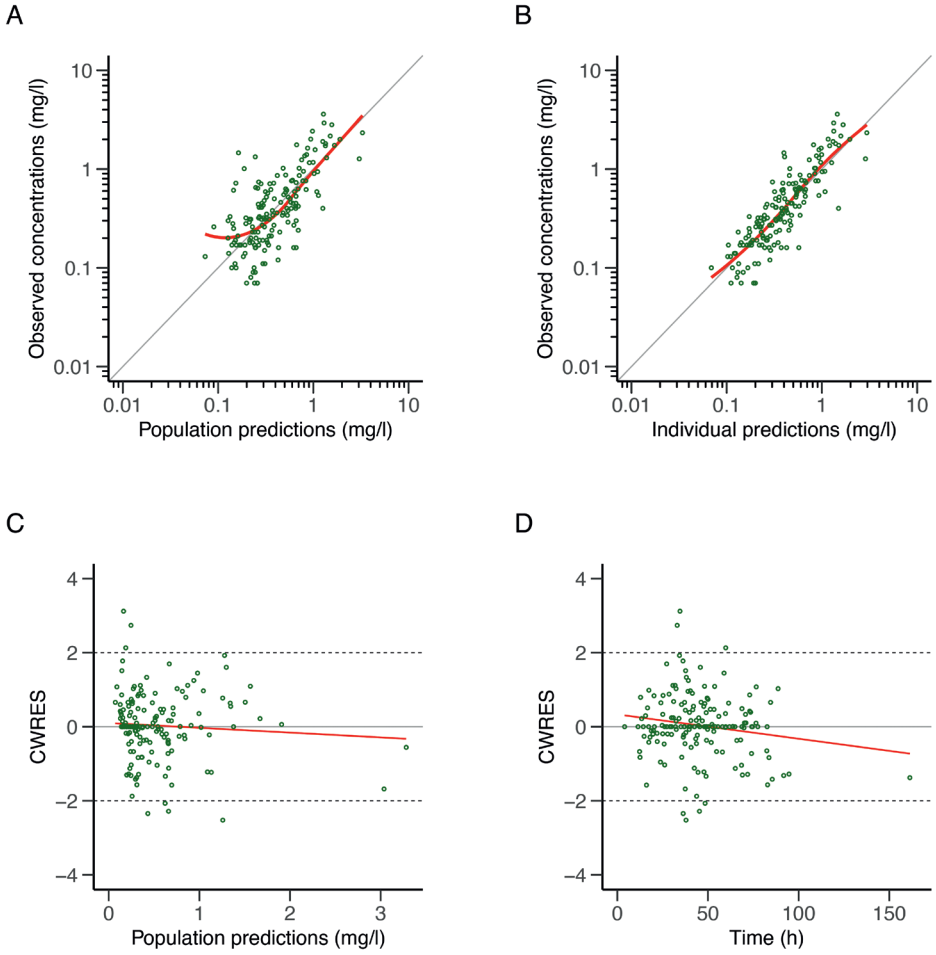

Figure 2. Goodness of fit plots of the final model. A: Population predictions versus observed concentrations in saliva. B: Individual predictions versus observed concentrations. C: Population predictions versus conditional weighted residuals (CWRES). D: Time versus CWRES.

Gentamicin pharmacokinetics in saliva

The salivary PK of gentamicin was described by adding a saliva compartment to the plasma model (**Figure 1**). For the structural model a k_{13} of 0.036 h^{-1} and k_{30} of 0.267 h^{-1} were estimated,

as well as IIV on k_{30} (63.6%) (**Table 2**). The estimate of IIV on k_{30} had an acceptable η-shrinkage and ε-shrinkage of 26.2% and 0.1%, respectively. A logarithmic proportional error model was used to describe the residual error (58.4%). Twenty-seven (14%) of all analyzed saliva samples were below the LLOQ and these measurements were accounted for with the M3 method [19]. Inclusion of additional transit compartments to account for lag in saliva uptake did not improve the model fit; neither did 1st order transport from the peripheral compartment to the salivary compartment. Though it was also possible to successfully fit a model with estimations for both IIV on k_{30} and k_{13}, η-shrinkage on these parameters was 56% and 34%, respectively. These levels of η-shrinkage were unacceptable and therefore that model was rejected [24].

Table 2. Population PK parameters and bootstrap results.

Parameter	Structural model OFV = 877.3		Final model OFV = 738.7		Bootstrap results (N=1000)		
	Estimate	RSE (%)	Estimate	RSE (%)	Median	2.5th %	97.5th %
θ_{k13} (h^{-1})	0.036	79	0.023	16	0.023	0.016	0.033
θ_{k30} (h^{-1})	0.267	70	0.169	15	0.171	0.123	0.239
$\theta_{PMA\ K13}$	-	-	-8.8	16	-8.7	-11.7	-5.7
$\theta_{PMA\ K30}$	-	-	-5.1	28	-4.9	-8.1	-2.0
σ_{prop} (%)	58.4	9	49.7	7	49.0	40.8	56.4
IIV_{k30} (%)	63.6	12	38.0	17	37.3	30.5	43.8

θ_{k13}: 1st order rate constant from central plasma compartment to saliva compartment. θ_{k30}: 1st order elimination rate constant from saliva compartment. $\theta_{PMA\ K13}$: Power equation exponent PMA on k_{13}. $\theta_{PMA\ K30}$: power equation exponent PMA on k_{30}. σ_{prop}: Proportional error. IIV_{k30}: Inter-individual variability of k_{30}.

$$k_{13} = \theta_{k_{13}} * \left(\frac{PMA}{244.2}\right)^{\theta_{PMA\ k13}}$$

$$k_{30} = \theta_{k_{30}} * \left(\frac{PMA}{244.2}\right)^{\theta_{PMA\ k30}}$$

Stepwise forward inclusion of PMA as a power function covariate on k_{13} led to the largest decrease in OFV (ΔOFV = -61.33). PMA was also included as a covariate on k_{30} as a power function (ΔOFV = -17.25). None of the other tested covariates improved the model; controlled hypothermia/perinatal asphyxia was not tested due to a lack of power (N=3). The parameter estimates of the final model are shown in **Table 2**. Final estimates for k_{13} and k_{30} were 0.023 h^{-1} and 0.169 h^{-1}, respectively. IIV of k_{30} was 38% in the final model, whereas proportional residual error was 49.7%. The exponents of PMA as a covariate on k_{13} and k_{30} respectively were -8.8 and -5.1. This describes a negative correlation between PMA and both the transport and elimination rate of gentamicin in saliva, indicating that gentamicin is more readily available in the saliva of patients of low PMA, such as premature neonates. Evaluation of the GOF plots of the final model demonstrated a good description of the observed gentamicin concentrations in saliva (**Figure 2**). For demonstrative purposes, observations and model predictions have been plotted for 1 representative patient per GA group (**Figure 3**).

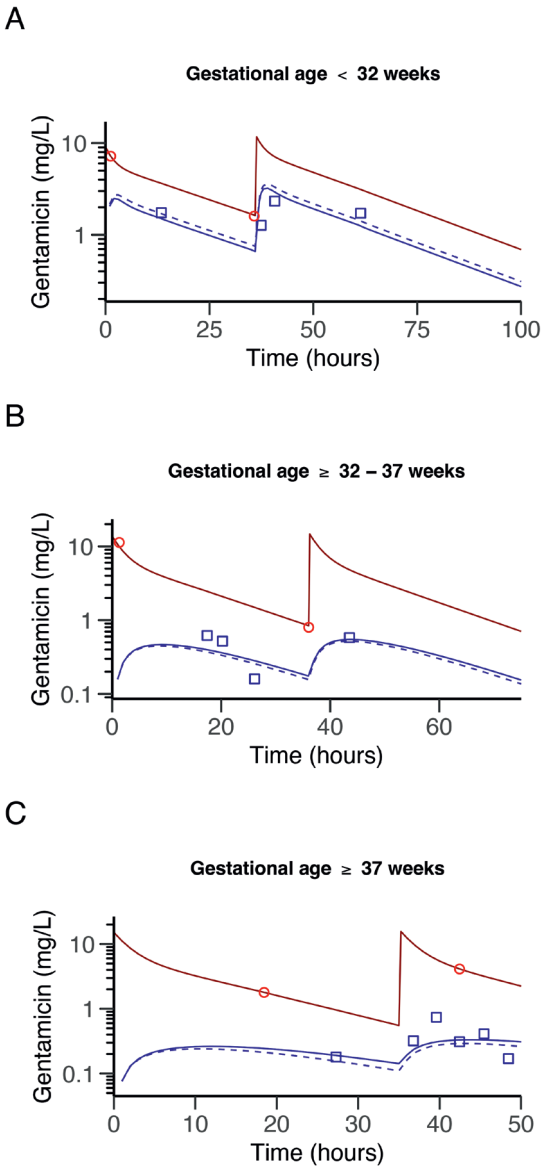

Figure 3. Individual pharmacokinetic profiles of gentamicin in plasma and saliva for typical patients of each GA group. A: Individual patient of GA < 32 weeks; B: Individual patient of GA ≥ 32 – 37 weeks; C: Individual patient of GA ≥ 37 weeks. black circles: observed plasma concentrations; gray squares: observed saliva concentrations; solid black line: individual predicted plasma concentrations; solid gray line: individual predicted saliva concentrations; dashed gray line: population predicted saliva concentrations; black crosses: observed saliva concentrations < LLOQ.

Bootstrap and internal model validation

The robustness of the final model was evaluated using a bootstrap procedure (n=1,000) and results are summarized in **Table 2**. Of the bootstrap runs, 98.3% were successful and the results indicated that the model was robust. For internal validation a pcVPC (n=1,000 samples) of the final model was evaluated (**Figure 4**). Most of the 10th, 50th and 90th percentiles of the observed values lie within the 95% confidence intervals of the 10th, 50th and 90th percentiles of the simulated values for all bins.

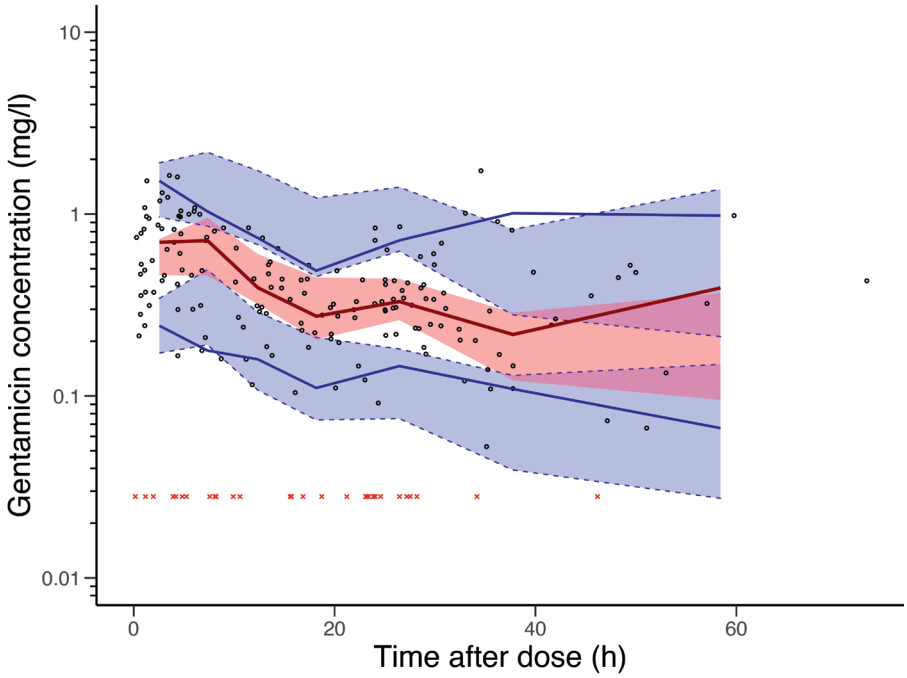

Figure 4. Prediction-corrected visual predictive check of the saliva model (n=1000). Black circles: Observed gentamicin concentrations; thick black line: median observed concentrations; thin black lines: 80% interval of the observed concentrations; dark gray field: 95% confidence interval of the median prediction; light gray fields with dashed border: 95% confidence intervals of the 10th and 90th percentiles of the predictions; red crosses: observations below LLOQ (0.056 mg/l).

Simulations

The simulated proportion of subjects with peak- and trough levels within the target range are displayed in **Figure 5**. Applying TDM using saliva led to a higher percentage of subjects reaching target attainment compared to no TDM (>75% vs 48%, respectively). However, saliva TDM led to a lower percentage of target attainment compared to plasma TDM. Obtaining more than four samples for saliva TDM did not result in increased TDM performance. On the contrary, obtaining additional saliva samples at 18h and 1h pre-dose led to a slightly decreased performance (-3% and -4%, respectively) compared to the strategy using four samples. Examples of individual TDM simulations are depicted in **Supplementary Figure S4**.

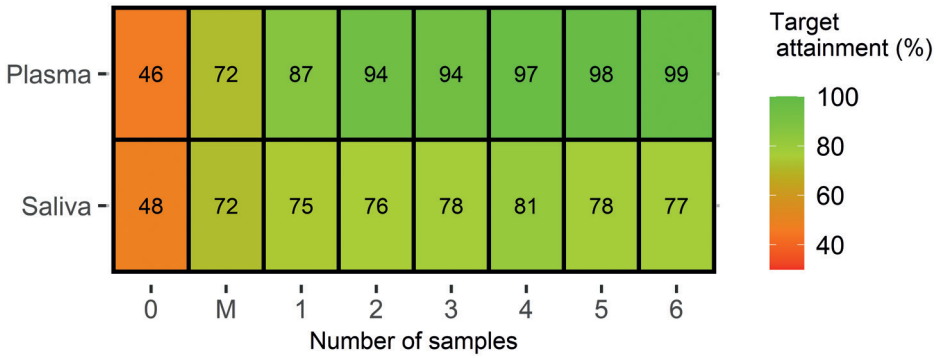

Figure 5. Heat map displaying the simulated proportion of subjects who reach target attainment of gentamicin after plasma- and saliva TDM using an increasing number of samples. Time-points where samples were simulated: 0: standard dosing according to guidelines without dose optimization; M: à priori tailored dosing without samples; 1: sample (14h); 2: peak sample (3h for saliva or 1h for plasma) and trough sample (0.5h pre-dose); 3: samples at peak, 14h and trough; 4: samples at peak, 7h, 14h and trough; 5: samples at peak, 7h, 14h, 18h and trough; 6: samples at peak, 7h, 14h, 18h, 1h pre-dose and trough.

Discussion

In this study, we have quantified the PK of salivary gentamicin concentrations in neonates and demonstrated the feasibility of monitoring gentamicin concentrations in saliva. Concentration-time profiles in both plasma and saliva were described with an integrated PK model. The potential use of salivary concentrations in the context of TDM was assessed through Monte Carlo simulations and MAP estimations. Simulations predicted a target attainment of up to 81% for TDM with 4 saliva samples versus 94% when performed with 2 plasma samples.

In the past, several investigators have assessed the use of saliva for TDM of aminoglycosides with varying results [6,8–11]. Berkovitch *et al.* reported a good correlation between plasma and saliva concentrations of gentamicin for a once daily dosing regimen in children [8]. Other investigators reported that aminoglycosides did not penetrate into saliva of children with cystic fibrosis or tuberculosis [10,11]. Work regarding saliva TDM in neonates has covered multiple drugs, including caffeine, morphine and antiepileptic drugs [6]. Interestingly, all studies focused on linear correlations. Incorporating saliva concentrations in nonlinear mixed effect models may allow for more flexibility to account for delayed penetration, delayed elimination and variability in saliva/plasma ratio (S/P). To our best knowledge, this POP-PK model is the first to apply this principle for gentamicin in saliva and there are only few published models which incorporate this methodology to describe saliva concentrations for other drugs [25,26].

The model developed during this study was constructed by appending a plasma PK model for gentamicin with a saliva compartment. Initially, a 2-compartment model by Bijleveld *et al.* [27] was used to fit the plasma PK of the study population. This model was chosen since it was developed with data from patients that were admitted to the same NICU as the present

study, thereby accurately reflecting the study population. Parameters in this model were allometrically scaled for BW, included IIV on CL and V_c and used PMA as a covariate for CL [27]. Though the model could adequately describe the plasma PK, the full saliva model could not accurately predict V_c through Bayesian MAP estimation using saliva samples during simulation. Therefore, the model by Fuchs *et al*. [16] (**Supplementary Table S1**) was used to describe the plasma PK of the study population (**Supplementary Figure S3**). Though this model was highly similar to the model by Bijleveld *et al*., the added benefit was that a stronger correlation between CL and V_c was included, allowing for more accurate predictions during Bayesian MAP estimations using the full saliva model. Constructing a new plasma PK model with the study data did not result in a better fit.

Gentamicin concentrations in saliva could best be described with drug transport from the central compartment (**Figure 1**). The final model could accurately describe the PK of gentamicin in plasma and saliva. However, in **Figure 4**, a slight model misspecification can be seen in later times after the last dose. It should be noted that there were few samples drawn beyond 50 hours post-dose and therefore there was little information regarding this period. Also, in current clinical settings the dose interval does not exceed 48 hours. Moreover, this misspecification was not present in other diagnostic tools (**Figure 2**) and therefore this finding was deemed of limited clinical relevance and the model was accepted. Models incorporating drug transport from the peripheral compartment to saliva were evaluated but did not accurately describe the data. Two separate rate constants were estimated for the saliva model. A 1st order rate constant k_{13} of 0.023h^{-1} and an elimination rate k_{30} of 0.169 h^{-1} were estimated. As k_{13} was much lower than k_{30}, transport from the central plasma compartment to the saliva compartment is the rate-limiting step determining the concentration-time profile in saliva [28]. Oromucosal reabsorption of gentamicin was not included in the model, since the contribution of oral absorption cannot be quantified after IV administration of a drug if bioavailability is low, as is the case with gentamicin [17]. Therefore elimination of gentamicin from saliva was best described with a single elimination constant (k_{30}). Moreover, models with zero-order salivary elimination did not result in adequate fits. When predicting gentamicin concentrations in plasma and saliva in typical patients (**Figure 3**, **Supplementary Figure S4**) it seems that the S/P ratio stabilizes hours after the last dose is administered. During this phase, the concentration-time curve of saliva is perpendicular to plasma, indicating that the salivary gentamicin elimination rate is linear to the plasma concentration and therefore is dependent on k_{13}. In a separate population analysis we could not identify any demographic descriptors to accurately predict individual S/P ratios. Due to the inter-individual variability in the S/P ratio it is not feasible to use a simple algorithm or guideline to convert saliva concentrations to plasma concentrations.

Considerable IIV was detected. Part of this was accounted for by taking PMA into consideration. It was estimated that IIV on k_{30} was 38% in the final model. PMA had a large influence on the salivary PK profile of gentamicin. Inclusion of PMA as a covariate on both k_{30} and k_{13} significantly improved the model. The exponents of the power equation functions were -5.5

and -8.8 for k_{30} and k_{13} respectively, demonstrating a strong age dependency of gentamicin disposition in saliva. With increasing PMA, k_{13} and k_{30} decrease by a large margin. Indeed, it was observed that salivary gentamicin levels were generally much lower in term neonates, compared to premature neonates. Furthermore, 75% of samples below the LLOQ were from older neonates (PMA>260 days). Though the model did not contain a parameter describing the IIV in k_{13}, inclusion of PMA as a covariate on k_{13} significantly improved model fit, decreased RSE on all parameters and decreased residual error. It was quite notable that gentamicin was more freely distributed in saliva of premature neonates. In a development study in rats, it was suggested that tight junctions of the submandibular saliva glands are immature at late gestation [29]. This might result in more permeable saliva glands due to increased paracellular transport of compounds. These findings may be indicative that salivary TDM could be more efficacious and possibly more accurate in premature neonates.

TDM performance was assessed through simulation in a fictional cohort of 3,000 neonates with a uniform distribution of GA and corresponding distribution of WT (**Supplementary Figure S2**) [22]. By applying Bayesian MAP during simulation one can use information obtained from multiple samples to estimate the peak- and trough concentrations which reduces the prediction error in the process. Additionally, the optimization process prevents that outlier saliva concentrations are extrapolated to extreme plasma concentrations on which dose adaptations are then falsely made. Results from this simulation may be optimistic, since each virtual subject was subjected to a rigid dose decision rule for dose optimization and inter-occasion variability was not accounted for. In practice, time-dependent factors such as changes in CL are considered during TDM. However, the simulations give an indication of the expected reliability of TDM with saliva samples versus plasma samples, as well as the comparative performance of several sampling schedules and can be used as a proof of concept.

Simulations indicated that a target attainment of 81% is possible with saliva TDM. Obtaining the necessary 4 saliva samples at 3h, 7h, 14h post-dose and 0.5h pre-dose is logistically feasible in this scenario. Interestingly, using more than 4 saliva samples seemed to decrease the accuracy of saliva TDM. However, sampling times during MAP estimation were selected rather arbitrarily and were equal for all dose regimens. A more thorough evaluation of optimal sampling times for MAP estimation was not performed during this study, given its explorative nature. Target attainment following TDM with 2 plasma samples (94%) was higher than with 4 saliva samples. This difference in performance for saliva and plasma TDM can be explained by the large difference in residual error between the two matrices. The uncertainty in the Bayesian optimization process introduced by these parameters was too large to address the precision difference in saliva and plasma TDM with additional sampling or different sampling schedules. Moreover, assessed saliva sampling schedules were equal for all dosing regimens, therefore the evaluated additional samples may have had limited value for dosing regimens of 36 or 48 hours. Plasma TDM performs better in settings where collection of 2 plasma samples is protocol. However, in many clinical settings TDM protocols require a single intermediate concentration sample. In that case, plasma TDM has a predicted target attainment of 87%

(**Figure 5**). This difference with saliva TDM is substantially smaller. Taken together with the uncertainties of the simulations, TDM with 4 saliva samples may be a suitable alternative to plasma TDM with a single intermediate concentration sample. Moreover, since the same sampling strategies were employed for all dose regimens during simulation, the difference in predicted target attainment may not be clinically relevant for all GA groups. This may especially be true for premature neonates in which gentamicin was more readily available in the saliva. Coincidentally, premature neonates could benefit most from a non-invasive TDM method.

This study has several limitations. First, there was a large proportion of saliva samples with insufficient volumes for analysis. This is unlikely the result of mechanical ventilation or use of anticholinergic drugs, since patients in the study cohort had a nasopharyngeal tube placement not interfering in any way with the oral cavity and anticholinergic drugs were not given. However, the low sample volumes may be due to inadequate sampling technique or insufficient saliva production by subjects, especially with premature neonates. Future studies may employ a different sampling strategy to ensure that an adequate volume of saliva is drawn, such as use of a different swab or cutting the saturated end of the swab [30,31]. Saliva secretion was not stimulated with citric acid as it substantially increases the burden of saliva collection. Currently no standardized method for the collection of saliva from neonates exists. It is important that a standardized saliva collection method is developed in the future, to ensure accurate saliva yields and that saliva collection is comparable between hospitals. Moreover a small number of samples could not be used due to contamination with blood therefore did not represent saliva concentrations of gentamicin. However, this occurred rarely (3.8% of all samples were contaminated and all contaminated samples were from 2 patients). The blood that contaminated the saliva originated from preexisting lesions as a result from clinical procedures such as intubation or suctioning, rather than being a side-effect of our used sampling method. Due to the delicate method of saliva sampling, blood contamination is highly unlikely. In a clinical setting, blood contamination of samples is immediately observed due to the strong red discoloration of the swabs. If this is encountered, subsequent saliva samples are likely to be contaminated as well. For these few patients, saliva sampling is not viable for this purpose and plasma samples should be used for TDM. Nonetheless, a large number of samples was available for model development, thus we do not expect this has influenced the parameter estimates. Second, due to the low volumes of the collected samples it was not possible to determine pH of the collected samples. Saliva pH has been proposed to influence salivary distribution of drugs [32]. Though little has been published regarding saliva pH of neonates we expect that fluctuations in saliva pH have little influence on the protonated fraction of gentamicin since the strongest basic pKa is 10.18 [33]. Third, assumptions made during simulation, such as the underlying covariate distribution and sampling strategies, have an influence on the proportion of subjects reaching target attainment. However, considering that the goal of the simulation was to compare saliva and plasma TDM, the comparative differences found in these simulation scenarios should be independent of these assumptions. Finally, the final saliva model contained a large proportional residual error of 49.7%. High residual

variability in the saliva compartment complicates the predictive power of the model. However, to compensate for this and to obtain reliable predictions, more saliva samples are required. As was found in the simulation study, more samples were required for saliva TDM of gentamicin than for plasma TDM.

Strengths of this study are the use of POP-PK, allowing for the description of nonlinear relations between plasma and saliva gentamicin concentrations with both fixed- and random effects. In addition, a relatively large cohort of neonates of different GA receiving varying dosing regimens originating from both a peripheral pediatric ward and NICU, improved the generalizability of the model. Moreover, use of highly sensitive LC-MS/MS allowed for determination of low gentamicin concentrations in small sample volumes with an LLOQ of 0.056 mg/l, which was substantially lower than earlier publications investigating gentamicin in saliva [9–11]. POP-PK modeling allowed for deviation from scheduled sampling times and identification of covariates. Collected saliva samples were evenly distributed, providing information for all time-points of the dose intervals. The TDM simulations of a wide range of sampling strategies give an adequate overview of the expected performance of saliva TDM in different scenarios. Moreover, since a large cohort was simulated (N=3,000), it can be assumed that estimations were accurate and standard error was low. Confidence intervals of the target attainments were therefore not calculated, since it was of little added value and repeated calculations would be overtly laborious and computationally intensive with a sample size this large.

This study is the first to demonstrate that TDM of gentamicin saliva of an exclusively neonatal population is feasible. A target attainment of 81% was found based on explorative simulations with 4 saliva samples and performance is close to plasma TDM with 1 intermediate sample. In the future, the real-life performance of saliva TDM employing an improved sampling technique should be investigated prospectively in premature neonates as gentamicin appears more readily in the saliva of premature neonates and these most fragile infants may benefit most from non-invasive TDM.

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Supplementary material

Supplementary Table S1. Population pharmacokinetic model of gentamicin in plasma by Fuchs *et al*.

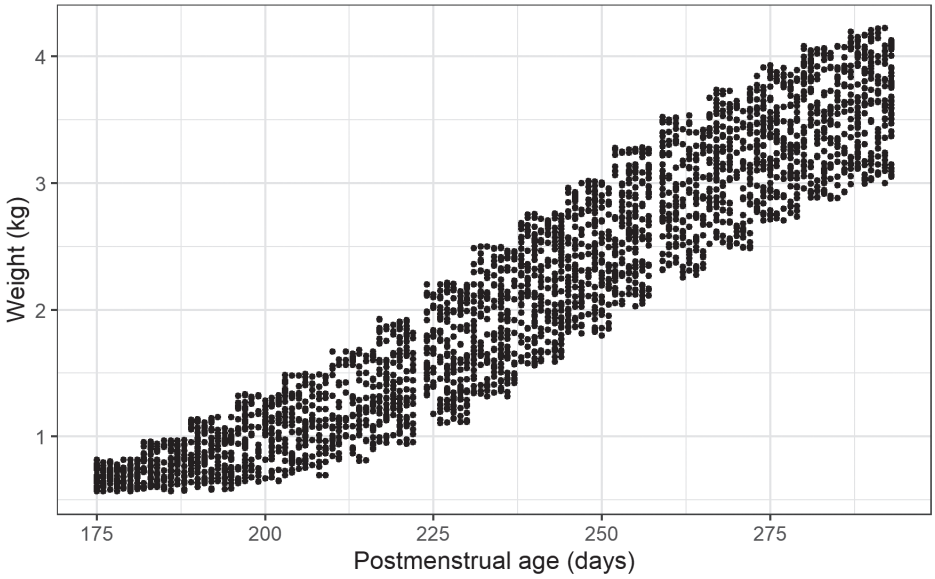
Parameter	value
θ_{CL}	0.089
θ_{CLWT}	0.75
θ_{CLGA}	1.87
θ_{CLPNA}	0.054
θ_{CLDOPA}	−0.120
θ_{Vc}	0.908
θ_{VcWT}	1
θ_{VcGA}	−0.922
θ_{Q}	0.157
θ_{QWT}	0.75
θ_{Vp}	0.56
θ_{VpWT}	1
IIV CL (%)	28
IIV V_c (%)	18
Correlation CL-Vc (%)	87
Additive residual error (mg l^{-1})	0.1
Proportional residual error (%)	18

$$TVCL = \theta_{CL} * \left(\frac{WT}{2170}\right)^{\theta_{CLWT}} * \left[1 + \theta_{CLGA} * \left(\frac{GA - 34}{34}\right)\right] * \left[1 + \theta_{CLPNA} * \left(\frac{PNA - 1}{1}\right)\right] * (1 + \theta_{CLDOPA})$$

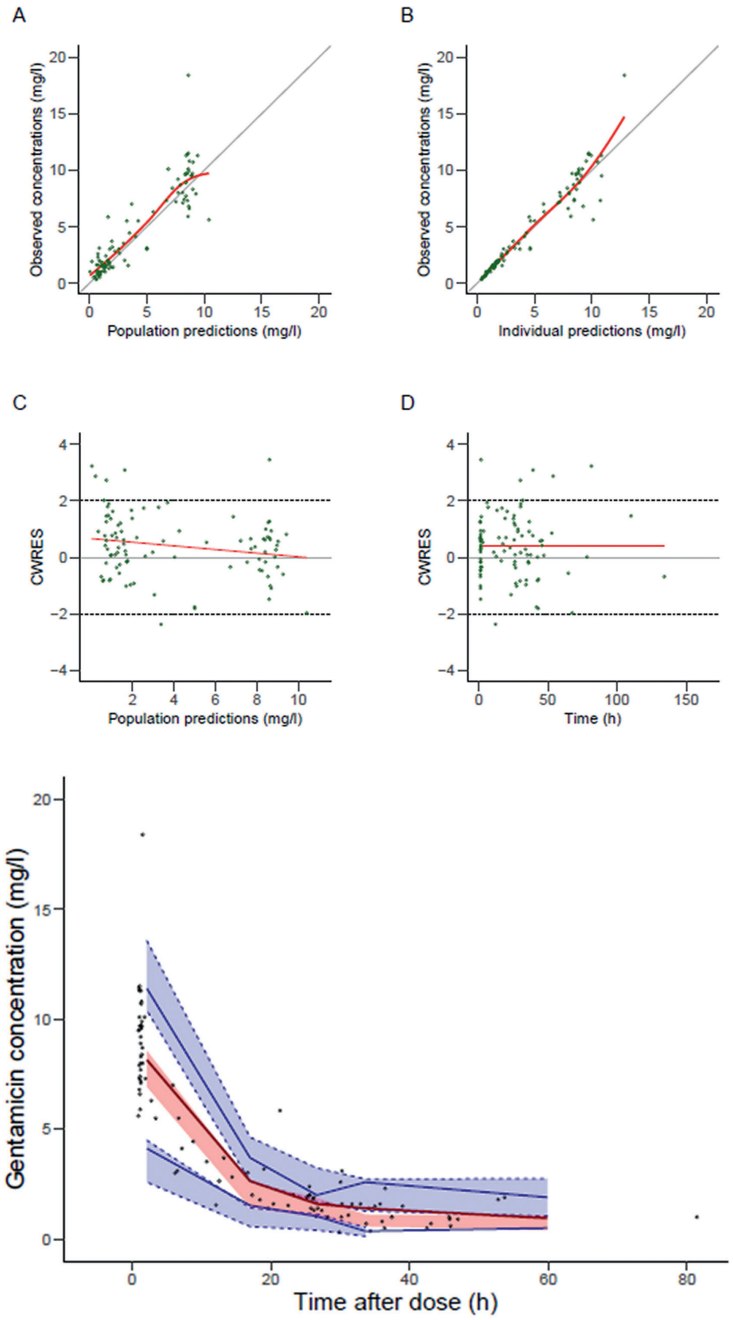
$$TVV_C = \theta_{V_C} * \left(\frac{WT}{2170}\right)^{\theta_{V_C WT}} * \left[1 + \theta_{V_C GA} * \left(\frac{GA - 34}{34}\right)\right]$$

$$Q = \theta_Q * \left(\frac{WT}{2170}\right)^{\theta_{Q_{WT}}}$$

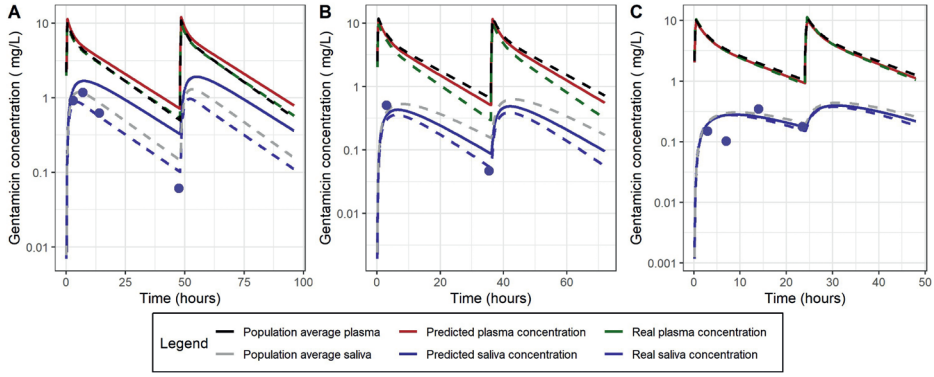
$$V_p = \theta_{V_C} * \left(\frac{WT}{2170}\right)^{\theta_{pWT}}$$



Supplementary Figure S2. Distribution of PMA and weight in the simulation dataset.



Supplementary Figure S3. Diagnostic plots plasma model. Plot A & B: Predictions are distributed around the unity line without systemic bias, indicating that predictions acurate. Plot C & D: No slope in CWRES based on population predictions or time, majority of predictions within ±2, no predictions outside ±4. Plot E: VPC shows that median observed concentrations are within the 95%CI of the simulated median. The same is true for the 10th and 90th percentile of the observation.



Supplementary Figure S4. Examples of simulated individual TDM concentration-time curves. Examples of individual TDM (left: GA < 32 wk, 4 samples; middle: GA 32-37 wk, 2 samples; right: GA > 37 wk, 4 samples) using Bayesian MAP methodology. Dark blue dots represent simulated TDM samples. Dotted gray and black lines represent the population average concentration-time profile for saliva and plasma, respectively. The dark blue and red lines represent the predicted saliva and corresponding plasma concentration-time profile, which is calculated based on the simulated TDM samples. The dotted light blue and green lines represent the 'true' concentration-time profile of these individuals. Ideally, the predicted and 'true' concentration-time profiles overlap completely. Residual or between-subject variability leads to predictions closer to the population average and further from the true concentration-time profile.

Chapter 4

Therapeutic drug monitoring of amikacin in preterm and term neonates with late-onset sepsis. Can saliva samples replace plasma samples?

Amadou Samb*, Fleur Sinkeler*, Yuma A. Bijleveld, Anton van Kaam, Timo R. de Haan, Ron Mathôt

** Shared first authorship*

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Abstract

Background

Amikacin is an aminoglycoside antibiotic that is frequently used for the treatment of neonatal late-onset sepsis (LOS), for which therapeutic drug monitoring (TDM) is advised. In order to decrease the TDM associated burden of plasma sampling, a non-invasive TDM method using saliva samples was investigated.

Methods

A prospective single-center, observational feasibility study with 23 premature and term neonates from which up to 8 saliva samples were collected, together with residual plasma from clinical routine. Amikacin concentrations in saliva and plasma were quantified with liquid chromatography tandem mass-spectrometry. A population pharmacokinetic (PK) analysis was performed to develop an integrated PK model of amikacin in plasma and saliva and for the identification of covariates. TDM performance of different sampling regimens was evaluated using Monte Carlo simulations in a fictional cohort of representative neonates (n=10,000).

Results

Amikacin could be detected in all saliva samples and a saliva compartment was appended to a 2-compartment plasma model. First-order absorption (k_{13}) of the saliva compartment was 0.0345 h^{-1} with an inter-individual variability of 45.3%. The rate of first-order elimination (k_{30}) was 0.176 h^{-1}. Post-menstrual age had a significant negative covariate effect on k_{13}, with an exponent of -4.3. Target attainment increased from 77.6% to 79.2% and from 79.9% to 83.2% using 1 up to 5 saliva samples or 1 up to 5 plasma samples, respectively.

Conclusion

TDM of amikacin using saliva samples results in target attainment comparable to plasma samples and may be beneficial for (premature) neonates with LOS.

Introduction

Intravenous amikacin in combination with flucloxacillin is often used as an empirical therapy in cases of (suspected) neonatal late onset sepsis (LOS), providing broad spectrum antimicrobial coverage. LOS presents after 72 hours post-partum during hospital admission and is one of the leading causes of neonatal mortality [1]. Amikacin is an aminoglycoside antibiotic with a narrow therapeutic index and there is a high interpatient variability in amikacin pharmacokinetics (PK) in neonates due to ongoing changes in organ maturation and body composition [2]. Therefore, therapeutic drug monitoring (TDM) is performed during amikacin treatment in the neonatal intensive care unit (NICU). TDM for amikacin is performed by evaluating the amikacin peak plasma level collected 1h post-dose (C_{max}) and a trough plasma level collected 0.5h pre-dose. To ensure bactericidal efficacy, a C_{max}/ MIC ratio of at least 8 is advised, typically corresponding to plasma concentrations of 15-30 mg/l [3]. To prevent concentration dependent nephrotoxicity and ototoxicity, trough concentrations should be below 5 mg/l [4]. Blood sampling is required for TDM in these small fragile patients, which is invasive, painful, stress inducing and associated with an increased risk for infection and anemia [5,6]. Therefore TDM of amikacin is complicated in neonates, stressing the need for a non-invasive TDM strategy that could decrease this patient burden and simultaneously guarantee optimal individualized amikacin dosing regimens during the NICU admission.

TDM using saliva samples is desirable since it decreases patient burden, allows for more (non-invasive) samples and may result in individual dosing regimens. Moreover, saliva sampling is safe, fast, low-cost and requires little training [6]. We recently found that saliva could potentially serve as a sampling matrix for the TDM of gentamicin, another aminoglycoside [7]. Though a number of studies investigating salivary TDM of aminoglycosides in different populations have been conducted with varying results, no studies specifically investigating salivary amikacin TDM in the NICU setting have been performed [8–11].

The main objectives of this study were to describe the PK profile of amikacin in saliva and plasma samples of neonates admitted in the NICU and to evaluate the feasibility of amikacin TDM using saliva.

Materials and methods

Study design

This was a single-center, prospective observational feasibility study conducted at the neonatology department of the Emma children's hospital (Amsterdam UMC, Amsterdam, the Netherlands). The study was approved by the local ethics committee of the Amsterdam UMC prior to patient inclusion (study number 2020_064).

Any neonate treated with amikacin for (suspected) LOS was eligible for the study. According to local protocol based on national dosing guidelines [12], 15mg/kg/36h or 15mg/kg/24h amikacin

was administered to neonates with a postmenstrual age (PMA) of < 30 weeks or ≥ 30 weeks, respectively. Clinical and anthropomorphic data were collected from the electronic patient records, i.e. gestational age (GA), postnatal age (PNA), PMA, current body weight (WT), birth weight (BW), sex, concomitant treatment with inotropic medication (dopamine, adrenalin, noradrenalin, dobutamine, isoprenalin), controlled hypothermia and serum creatinine (S_{cr}).

A total of 30 patients were planned for inclusion, based on our prior experience in a comparable study involving TDM of gentamicin using saliva in the same patient population [7]. Moreover a sample size of at least 20 patients is generally deemed sufficient for population-PK (POPPK) analysis [13].

Sample collection and bioanalytical assay

Following an opportunistic sampling schedule, during multiple dose intervals a maximum of 8 saliva samples were collected per patient by placing SalivaBio Infant's Swabs (Salimetrics, Carlsbad, CA, USA) in the cheek pouch for 90 seconds according to manufacturer instructions [14]. Thereafter, the swabs were placed in collection tubes with the saturated side downwards while the unsaturated side was cut off and discarded. The collection tubes were immediately refrigerated at 2 – 8 °C for a maximum of 12 hours until saliva extraction. For the extraction of saliva the collection tubes were centrifuged at 2,750 x g, after which the samples were stored at -80 °C until analysis. Any saliva samples that were contaminated with blood, as assessed via visual inspection, were excluded from analysis. Amikacin concentrations in plasma were collected from routine TDM measurements. Residual plasma samples saved from clinical diagnostic routine sampling were also collected to determine amikacin concentrations.

Tandem liquid chromatography coupled with mass-spectrometry (LC-MS/MS) was used for the quantification of amikacin in saliva [submitted, 2023]. In short, the accuracy for amikacin was acceptable (acceptance criteria: 85% - 115%) at all quality control (QC) levels with ranges of 94.7% – 113.4% and 100.5% - 104.6%, respectively. Within-day- and between-day imprecision were below the acceptance criteria of 15%. The minimal sample volume required was 10 µl and the quantification limit was 0.010 mg/l.

Pharmacokinetic analysis

After data extraction, all data handling, descriptive statistics and visualization was performed with R Version 4.1.0 [15]. Following logarithmic transformation of concentration data, POPPK analysis was performed using the NONMEM version 7.4 (ICON Development Solutions, Dublin, Ireland).

POPPK implements nonlinear mixed-effects modeling for all patients simultaneously, allowing estimation of individual parameters for individuals with sparse samples through Bayesian post-hoc analysis. POPPK modeling estimates population parameters θ (fixed effects) as well as inter-individual variability η (IIV) in parameter estimates. Any remaining prediction error is accounted for by including residual error ε in the PK model. Both IIV and residual error

are random effects following a normal distribution expressed as $\eta \sim N(0,\omega^2)$ and $\varepsilon \sim N(0,\sigma^2)$, respectively. Part of the variability may be explained by including covariates in the model.

For POPPK model development firstly four published PK models for amikacin in similar populations were evaluated with the current study data [3,16–18]. The model that could best describe the observed amikacin plasma concentrations was selected as base plasma model based on precision and bias, defined as mean error (*me*) distribution and rooted mean squared error (*rmse*). In addition, goodness-of-fit (GOF) plots as well as prediction-corrected visual predictive checks (pcVPC) were examined for visual inspection of model performance. Following model selection, individual plasma PK parameters were estimated using the POSTHOC option in NONMEM.

Next, a saliva compartment was attached to the selected PK model with the ADVAN6 subroutine (**Figure 1**) to construct the final saliva model. Similar to the PK model of gentamicin [7], absorption rate constant k_{13} and elimination rate constant k_{30} were estimated for the salivary PK of amikacin, evaluating multiple absorption models (i.e. zero-order, 1st order, transit compartments). It was assumed that the influence of k_{13} on plasma amikacin was negligible and that no oral reabsorption of amikacin took place, as shown in **Figure 1**. After an adequate structural model had been developed, the influence of demographic characteristics on salivary PK of amikacin was evaluated through the inclusion of covariates. Covariate analysis was performed with stepwise forward inclusion followed by backwards elimination. The level of significance for covariate inclusion was $p \leq 0.05$, whereas the significance level for covariate elimination was set at $p \leq 0.01$. Continuous covariates were evaluated as either a linear function (**Eq.1**) or as a power function (**Eq.2**). Binary covariates were coded in NONMEM according to **Eq.3**.

(1) $$p = \theta_p * \left(1 + \theta_{cov} * \frac{COV - COV_{median}}{COV_{median}}\right)$$

(2) $$p = \theta_p * \frac{cov}{cov_{median}}^{\theta_{cov}}$$

(3) $$p = \theta_p + cov * \theta_{cov}$$

In the equations above, parameter p is dependent on the typical parameter value θ_p, covariate *cov* and covariate effect θ_{cov}. Continuous covariates were scaled for the median value covmedian. Covariates that were evaluated included GA, PNA, PMA, WT, BW, sex, inotropic medication use and controlled hypothermia.

During model development, parameters were included by assessing the change in the objective function value (OFV). The OFV is chi-squared distributed and a ΔOFV of -3.84 corresponds to a p value of 0.05 with one degree of freedom for nested models. Furthermore, model fit was assessed by evaluating GOF plots, relative standard error (RSE), η distribution and shrinkage. Internal model validation was performed with pcVPCs (n=1,000) and model robustness as well as parameter certainty was assessed with model bootstraps (n=1,000).

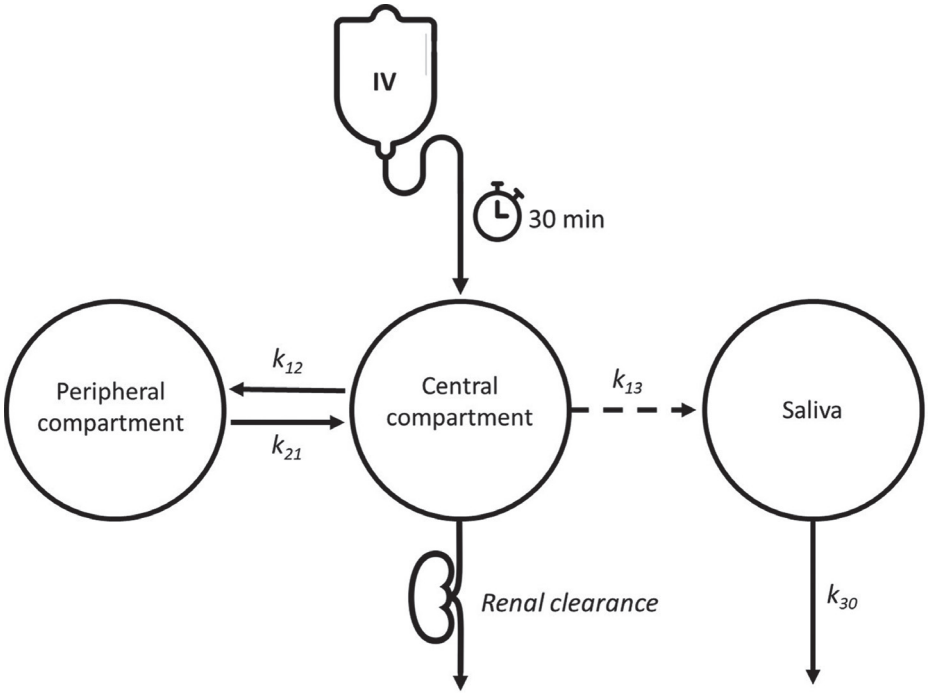

Figure 1. Compartment model of amikacin pharmacokinetics in plasma and saliva. After intravenous infusion to the central plasma compartment, amikacin is transported to and from peripheral compartment with first-order rates k_{12} and k_{21}, respectively. Amikacin is transported from the central compartment to the saliva compartment with a first-order rate k_{13} and eliminated from the saliva with rate k_{30}.

Simulated TDM evaluation

Monte Carlo simulations were performed for the final model with the mrgsolve package for Rstatistics V 4.1.0 [15,19]. A simulation dataset (n=1,000) was created using randomly sampled GAs and PNAs from normal distributions similar to the distributions of the study data, truncated at the ranges found included patients. Body weights corresponding to ages were added to the dataset [20]. PK profiles following amikacin doses (15 mg/kg/24h) were simulated for each individual within the simulation dataset. No distinction in dose regimen was made between neonates with a PMA < 30 weeks versus ≥ 30 weeks, since this would likely overcomplicate the simulations, whilst the comparative nature of the evaluations remains unchanged.

TDM performance was evaluated using the final saliva model and the base plasma model. Inter-individual variability in central volume of distribution during simulation was empirically set to 10% for more realistic C_{max} levels. Saliva and plasma samples were simulated based on different sampling regimens consisting of 1-5 samples and used for TDM evaluation. In short, a fixed dose regimen (0 samples and no dose adjustments), a population dose regimen (dose adjustments based on population predictions of the patient) and dose regimens using individual predictions based on 1, 2, 3, 4 or 5 plasma- or saliva samples were evaluated. Samples were simulated at 1, 3, 6, 9, 12, 18 or 23.5 hours post-dose and sampling regimens were planned according to

Supplementary table S1. Current practice for TDM of amikacin in the neonatology ward of the Amsterdam UMC is a plasma sample at 1 and a plasma sample at 23.5 hours. Maximum à posteriori (MAP) optimization was performed with the simulated samples to obtain empirical Bayes estimates (EBE) of the individual parameters of the simulated cohort [21]. The EBEs were used to predict the full individual concentration-time profiles in plasma and it was assessed whether steady-state concentrations were within the target range (peak 24-35 mg/l, trough <5 mg/l) [16,17]. If peak concentrations were outside the target range, the third and subsequent simulated doses were automatically adjusted by a factor of $30/C_{max}$. This correction factor was chosen since it is in the middle of the target range for peak concentrations (24-35 mg/l). If trough levels exceeded 5 mg/l the dose interval was extended to 36 hours. The true PK profiles for the (un)adjusted doses were predicted for 6 consecutive doses and steady state peak- and trough plasma levels were evaluated for target attainment (pTA). The proportion of patients with peak- and trough plasma concentrations within target ranges was calculated for each sampling regimen in plasma and saliva and TDM performances were compared.

Results

Demographic characteristics

The study was conducted between December 3rd 2020 and April 11th 2022. A total of 23 patients with (suspected) LOS and intravenous amikacin treatment were included. This was less than the predetermined number of patients (n=30)as an interim analysis indicated that data of 23 patients would be sufficient to describe amikacin POPPK, reducing patient burden.

Table 1. Demographic characteristics of the study population.

Characteristic	value
patients - n	23
females - n(%)	12 (52.2 %)
GA (weeks) - median (IQR)	28 (25.7 - 29.51)
PNA (days) - median (IQR)	8.1 (4.8 - 14.1)
PMA (days) - median (IQR)	206.8 (198.5 - 220.7)
WT (kg) - median (IQR)	1.03 (0.837 - 1.485)
BW (kg) - median (IQR)	0.86 (0.68 - 1.247)
Inotropic comedication - n(%)	3 (13 %)
Saliva samples - n	127
per patient - median (range)	7 (1 - 8)
contaminated	9 (14.1 %)
Plasma samples - n	63
per patient - median (range)	2 (2 - 8)

GA: gestational age, PNA: postnatal age, PMA: postmenstrual age, WT: current body weight, BW: birth weight

An overview of the demographic characteristics of the study population is depicted in **Table 1**. The majority of patients was premature, with a median GA of 28 weeks. A total of 63 plasma samples were available, whereas 127 saliva samples were collected, averaging at 5.5 saliva samples per patient. However, 9 saliva samples were excluded from analysis due to blood contamination. Amikacin concentrations in the uncontaminated saliva samples ranged between 0.044 mg/l and 12.1 mg/l. No salivary amikacin levels were below the lower quantification limit.

Pharmacokinetic model

For development of an integrated PK model as presented in **Figure 1**, PK profiles in plasma were described using a published model. Out of the 5 evaluated models, the model by the Cock *et al*. (**Supplementary Table S2**, [16]) could best describe the study data. The model was unbiased (*me* 95%CI: -0.12– 0.23 mg/l) and precision was acceptable (*rmse*: 0.67 mg/l). A pcVPC of the base plasma model performance has been depicted in **Supplementary Figure S3**.

Following selection of the base plasma model, a salivary compartment was connected to the model (**Table 2**). A 1st order salivary absorption rate k_{13} of 0.0239 h^{-1} with 69.6% IIV was estimated. First-order salivary amikacin elimination rate k_{30} was 0.164 h^{-1}, though IIV on k_{30} could not be estimated for the model. Residual error in the structural saliva model was described as a proportional error of 57.9%. Other residual error models were less accurate in describing the data. Following covariate analysis, PMA was included as a covariate on k_{13} as a power function with an exponent of -4.3. Moreover, IIV in k_{13} was decreased from 69.6% to 45.3% (**Table 2**). Basic goodness-of-fit plots for the final model are depicted in **Supplementary Figure S4**, indicating an adequate model fit. Model predicted time profiles of amikacin in plasma in saliva of 2 typical patients have been depicted in **Figure 2**. Salivary amikacin levels in premature neonates are shown to be notably higher compared to term neonates.

Table 2. Population PK parameters of amikacin in saliva.

	structural model OFV = 145.21		**Final model** OFV = 131.22		**Bootstrap (N=1000)** Successful runs: 960	
Parameter	**Estimate**	**RSE**	**Estimate**	**RSE**	**Median estimate**	**95% CI**
θ_{k13} (h^{-1})	0.0239	22%	0.0345	31%	0.0343	0.0203 – 0.0548
θ_{k30} (h-1)	0.164	15%	0.176	32%	0.176	0.112 – 0.238
θ_{PMA}	-	-	-4.3	31%	-4.3	-6.1 – -2.2
ω_{k13} [shrinkage]	69.6% [10%]	15%	45.3% [19%]	36%	42.0%	16.2 – 58.5%
σ_{prop} [shrinkage]	57.9% [9%]	11%	58.3% [8%]	16%	57.7%	48.0 – 68.7%

θ_{k13}: population parameter for first-order salivary amikacin absorption k_{13} of a typical patient with a PMA of 206.8 days. θ_{k30}: population parameter for first-order salivary amikacin elimination k_{30}, θ_{PMA}: exponent of the post-menstrual age covariate function. ω_{k13}: inter-individual variability in k_{13}. σ_{prop}: proportional residual error.

$$k_{13} = \theta_{k_13} * \frac{PMA}{PMA_{median}}^{\theta_{PMA}}$$

Ninety-six percent of the nonparametric bootstrap runs (N=1,000) were successful and calculated 95% confidence intervals provided good coverage of the final parameter estimates (**Table 2**). Therefore, model robustness and parameter certainty were acceptable. The saliva model was internally validated using a pcVPC (**Figure 3**). The 10th, 50th and 90th percentiles of the observed amikacin concentrations in saliva were all contained within the 95% confidence intervals of the corresponding percentiles of the simulated salivary amikacin concentrations. Thus, the simulation-based evaluation of model performance was deemed satisfactory.

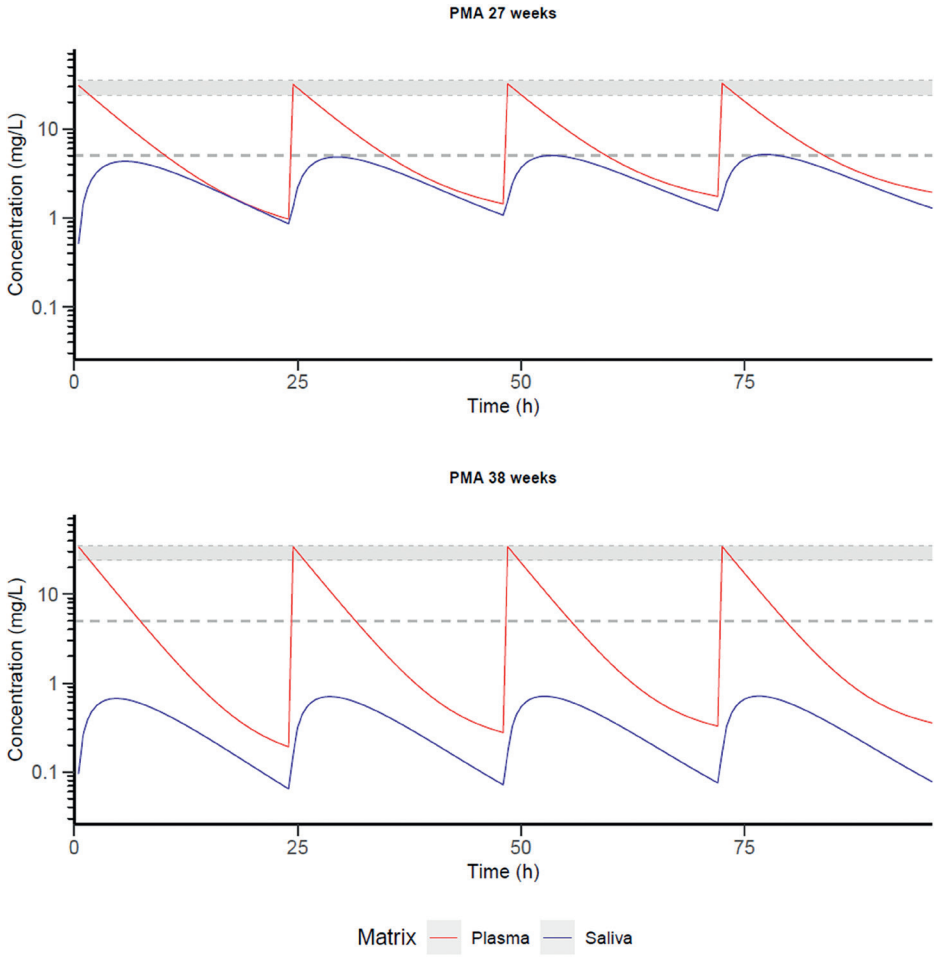

Figure 2. Individual concentration-time profiles of amikacin concentrations in 2 typical patients. Red lines: amikacin concentrations in plasma. Blue lines: amikacin concentrations in saliva. Shaded grey area: peak concentration target range (24-35 mg/l). Dashed grey line: trough target threshold (<5 mg/l). Top figure: PK profile of a premature neonate with a PMA of 27 weeks. Bottom figure: PK profile of a term neonate with a PMA of 38 weeks.

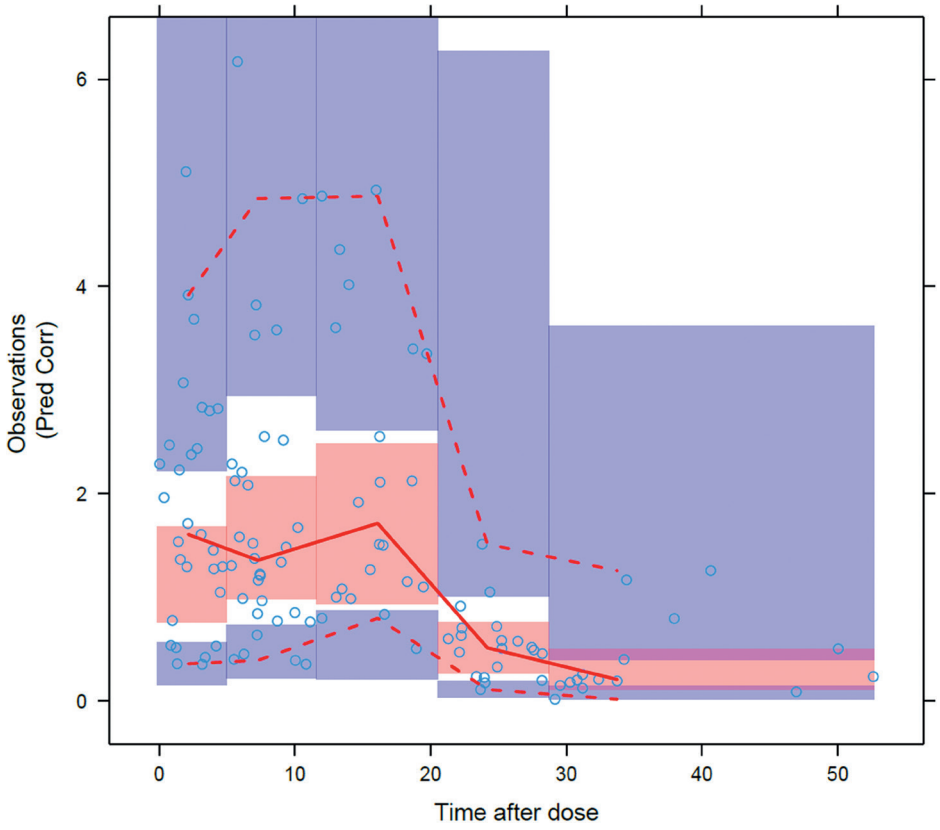

Figure 3. Prediction corrected visual predictive check of the final saliva model. Blue circles: observed amikacin concentrations. Dashed red lines: 10th and 90th percentiles of the observed amikacin concentrations. Solid red line: 50th percentile of the observed amikacin concentrations. Blue ribbons: 95% confidence intervals of the 10th and 90th percentiles of the simulated amikacin concentrations. Red ribbon: 95% confidence interval of the 50th percentile of the simulated amikacin concentrations.

	Fixed dose	Population predictions	1 sample	2 samples	3 samples	4 samples	5 samples
plasma pTA	73.1%	77.6%	79.9%	80.3%	81.7%	82.6%	83.2%
saliva pTA	73.1%	77.6%	77.6%	78.1%	78.5%	78.9%	79.2%

Figure 4. Heat map of target attainment for multiple TDM regimens. Plasma pTA: target attainment using plasma TDM. Saliva pTA: target attainment using saliva TDM. Fixed dosing: starting dose regimen dose adjustments. Population predictions: dose regimen based on population PK and covariates, without samples. 1, 2, 3, 4, 5 samples: number of samples drawn at 1, 3, 6, 9, 12, 18 and 23.5 hours post-dose.

TDM evaluation

Amikacin concentrations in plasma and saliva were simulated in a fictional cohort of neonates. The simulated PK profiles in plasma and saliva of patients receiving an (un)adjusted dose of 15mg/kg/24h have been depicted in **Supplementary Figure S5**. Compared to a fixed dose regimen of 15mg/kg/24h, PK guided dose adjustments using population predictions resulted in the largest improvement in pTA from 73.1% to 77.6% (**Figure 4**). pTA improved if more saliva samples were collected, reaching a maximum of 79.2% with 5 saliva samples. pTA for plasma TDM was slightly higher than saliva TDM for any number of samples.

Discussion

Based on the results from the present study, amikacin reaches quantifiable levels in saliva of term and preterm neonates with late-onset sepsis during amikacin treatment. In addition, salivary distribution and excretion of amikacin could be described in a pop-PK model and the performance of saliva TDM was evaluated. After dose adjustment following a TDM strategy using 1 to 5 saliva samples, 77.6 to 79.2% of simulated patients achieved targeted amikacin levels in saliva. Using a fixed dose amikacin treatment resulted in a pTA of 73.1%. The differences in pTA between saliva and plasma TDM are small and may not be clinically relevant (**Figure 4**).

To our best knowledge, this is the first report describing salivary amikacin PK and applicability of TDM using saliva samples in neonates with LOS admitted to the NICU. So far, little knowledge on the application of saliva samples for TDM of amikacin has been shared in literature. A single study reported unquantifiable amikacin levels in pediatric cystic fibrosis (CF) patients [11]. In addition, other aminoglycosides did not reach quantifiable levels in saliva of children with CF [9,10]. However, it is unlikely that these results are representative for a neonatal population with LOS regarding the obvious differences in development and body composition. Moreover, for all of the aforementioned studies concentrations were measured using immunoassays with relatively high lower quantification limit of 0.18 – 2.0 mg/l [9–11]. It has been reported that gentamicin levels were detectable in saliva of children treated with once-daily gentamicin for urinary tract infections, with good correlations between saliva and plasma levels [8]. Whilst the detection limit of the used immunoassay was not reported, concentrations as low as 0.09 mg/l were included in the final analysis [8]. Thus, it seems that low detection limits of the quantification method are essential for the development of a saliva TDM method of aminoglycosides. We have previously investigated and reported that gentamicin is distributed in the saliva of NICU admitted neonates with (suspected) early-onset sepsis [7]. Moreover, gentamicin PK in plasma and saliva was described and TDM using 4 saliva samples was shown to be feasible, with a pTA of 81%, similar to our findings for amikacin as reported in the present study.

As the plasma data collected during this study was too scarce for model development, amikacin plasma PK were described using models that had already been developed, validated and published in literature. Based on *me* distribution and *rmse* as well as visual inspection of

predictions and observations, it was found that the PK model by de Cock *et al*. [16] best described the current study data and was thus selected as the foundation for the integrated PK model for amikacin in plasma and saliva. It was found that salivary absorption of amikacin from the central plasma compartment could be described as a 1st order rate k_{13} of 0.0345 h^{-1} with 45.3% inter-individual variability (**Table 2**). The estimated 1st order amikacin elimination rate k_{30} was 0.176 h^{-1}. Since the salivary elimination rate greatly exceeded absorption, salivary distribution of amikacin is predominately determined by k_{13}. PMA had a significant effect on k_{13} when included as a covariate using a power function normalized for median PMA. A negative exponent of -4.3 was estimated, indicating that amikacin is distributed more freely in the saliva of premature neonates. A This finding is evident when comparing the salivary amikacin PK profile of premature– and term neonates, as depicted in **Figure 2**. The aforementioned findings demonstrate high similarities to our previous study with gentamicin, as the final model structure is near identical [7]. Moreover, results from that study also suggested that higher salivary gentamicin levels are present in premature neonates, when compared to term neonates, possibly reflecting differences in blood / saliva barrier development. Such a finding was presented by Shimono *et al*., where less tight junctions were observed in the submandibular glands of developing rats, possibly facilitating increased paracellular transport of drugs during the early maturation stage of the salivary glands [22].

Performance of the final model in the context of TDM with multiple sampling strategies was evaluated and compared to plasma TDM using simulated PK profiles in a fictional cohort of 10,000 neonates. Interestingly, high pTAs of 73.1% and 78.9% were achieved in the fixed dose regimen and in the population prediction regimen. This is likely due to the inability of the model to simulate high variability in peak concentrations, as no IIV in V_c was included in the used plasma model [16]. Whilst 10% IIV in V_c was included in the model for simulation purposes, this did not result in PK profiles with peak concentrations outside the target ranges.

There were a number of strengths to this study. First, salivary amikacin concentrations could be reliably measured in the saliva of neonates, in part due to the sensitive LC-MS/MS method used. As indicated above, previous studies reported that amikacin did not achieve quantifiable levels in saliva. Moreover, it is broadly assumed that molecules need to be lipophilic, small, uncharged at physiological pH and have a low protein-bound fraction in order to be freely distributed in saliva [23]. The protein-bound fraction of amikacin is very low (<10%) and thus has a large fraction of unbound amikacin that could freely pass the plasma/saliva barrier, in theory [24]. However, amikacin is a relatively large molecule of 585.6 Da, is positively charged at physiological pH and thus hydrophilic [25]. Nonetheless, we demonstrated that amikacin is transported to neonatal saliva.

Another strength is that the time-dependent PK of amikacin in plasma and saliva could be described using a single integrated model. Contrary to popular conventions that explore linear correlations between plasma and saliva concentrations and report universal saliva-to-plasma ratios (s/p ratios), a time-dependent relationship between salivary and plasma amikacin

concentrations was found in this study. Furthermore, the pop-PK approach used allowed for the quantification of IIV and the influence of PMA on salivary absorption of amikacin.

A third considerable strength of this study was that the developed pop-PK model could be used to simulate and predict salivary amikacin distributions within the population. This allows for the assessment of dose adequacy or for the evaluation and optimization of multiple TDM sampling strategies. The calculated pTAs (**Figure 4**) provide a comparative overview for different sampling strategies using saliva or plasma for amikacin TDM. The results from the simulation based TDM performance analysis could serve as a framework for the investigation of the real-life performance of salivary TDM of amikacin.

A limitation of the study was that currently all publicly available PK models for amikacin in neonates are flawed to some extent. The model developed by de Cock ***et al***. could adequately describe the plasma concentrations but did not include any IIV in volume of distribution [16]. This would indicate that variability in V_d could be fully explained by WT and thus that the first dose based on WT would always result in optimal peak concentrations during simulation, which is not representative for the clinical situation. Another limitation of this study was that the residual proportional error of the final saliva model was 58.3%. High residual error adversely impacted the predictive power of the saliva model, which is demonstrated by the limited added benefit of additional saliva samples on the pTA (**Figure 4**). More adequate plasma PK models for amikacin should be developed for improved TDM performance using either plasma and saliva samples.

Insights from this study indicate that amikacin can be measured and modeled using saliva from a neonatal population and that it performs reasonably well during TDM. Salivary concentrations of amikacin seem to reach higher concentrations in extremely premature neonates and a prospective study investigating real-world performance of a salivary TDM strategy should be performed in the near future.

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Supplementary material

Supplementary Table S1. simulated sample times for TDM evaluation.

TDM method	Sampling times (hours after dose)		Dose adjustment
	Saliva	Plasma	
Fixed dose	-	-	None
Population	-	-	At third dose, based on expected peak- and trough levels of population predictions
1 sample	12	12	At third dose, based on projected peak- and trough levels following EBE
2 samples	12, 18	1, 23.5	At third dose, based on projected peak- and trough levels following EBE
3 samples	9, 12, 18	1, 12, 23.5	At third dose, based on projected peak- and trough levels following EBE
4 samples	9, 12, 9, 18, 23.5	1, 12, 18, 23.5	At third dose, based on projected peak- and trough levels following EBE
5 samples	3, 9, 12, 18, 23.5	1, 6, 12, 18, 23.5	At third dose, based on projected peak- and trough levels following EBE

Supplementary Table S2. Parameter estimates of the plasma model by de Cock *et al* [16].

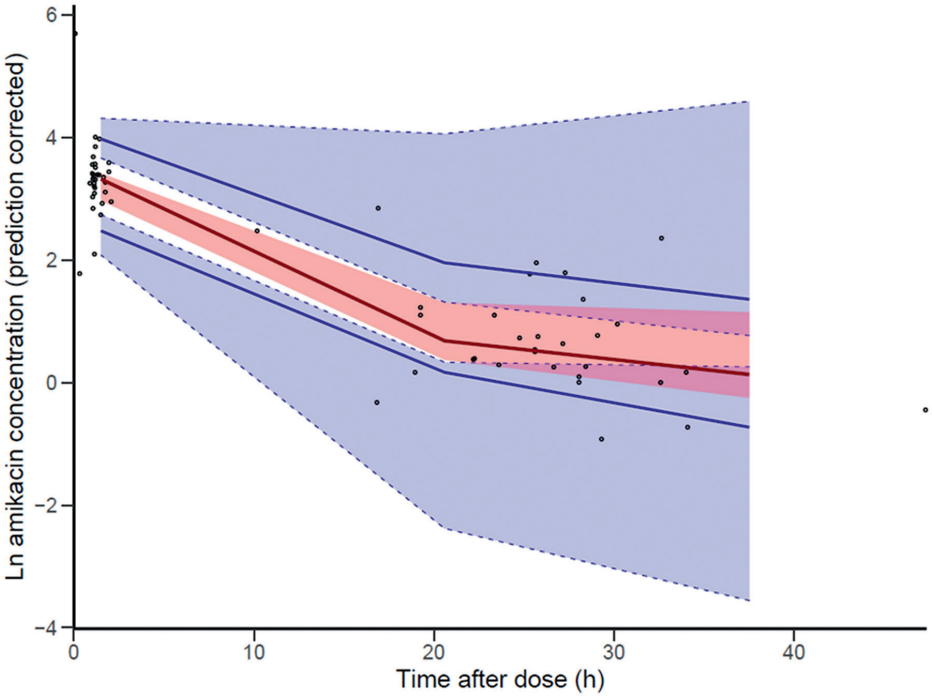
Parameter	Estimate
θ_{CL}	0.0493 L/h
θ_{fQ}	0.415
θ_{BW-CL}	1.34
θ_{PNA-CL}	0.213
θ_{IBU}	0.838
$\theta_{V1} = \theta_{V2}$	0.833 L
θ_{WT-V}	0.919
ω_{CL}	30%
σ_{prop}	24.8%
σ_{add}	0.517 mg/L

θ_{CL}: typical population clearance, θ_{fQ}: inter-compartmental clearance (fraction of θ_{CL}). θ_{BW-CL}: exponent of covariate function of birth weight on CL, normalized for a median birth weight of 1.75 kg. θ_{PNA-CL}: slope for covariate function of postnatal age on CL, normalized for a median postnatal age of 2 days. θ_{IBU}: Change in CL for concomitant ibuprofen use. θ_{V1}: typical central volume of distribution. θ_{V2}: typical peripheral volume of distribution. θ_{WT-V}: exponent of covariate function of current body weight on V1, normalized for a median current body weight of 1.760 kg. ω_{CL}: IIV in clearance. σ_{prop}: proportional residual error. σ_{add}: additive residual error.

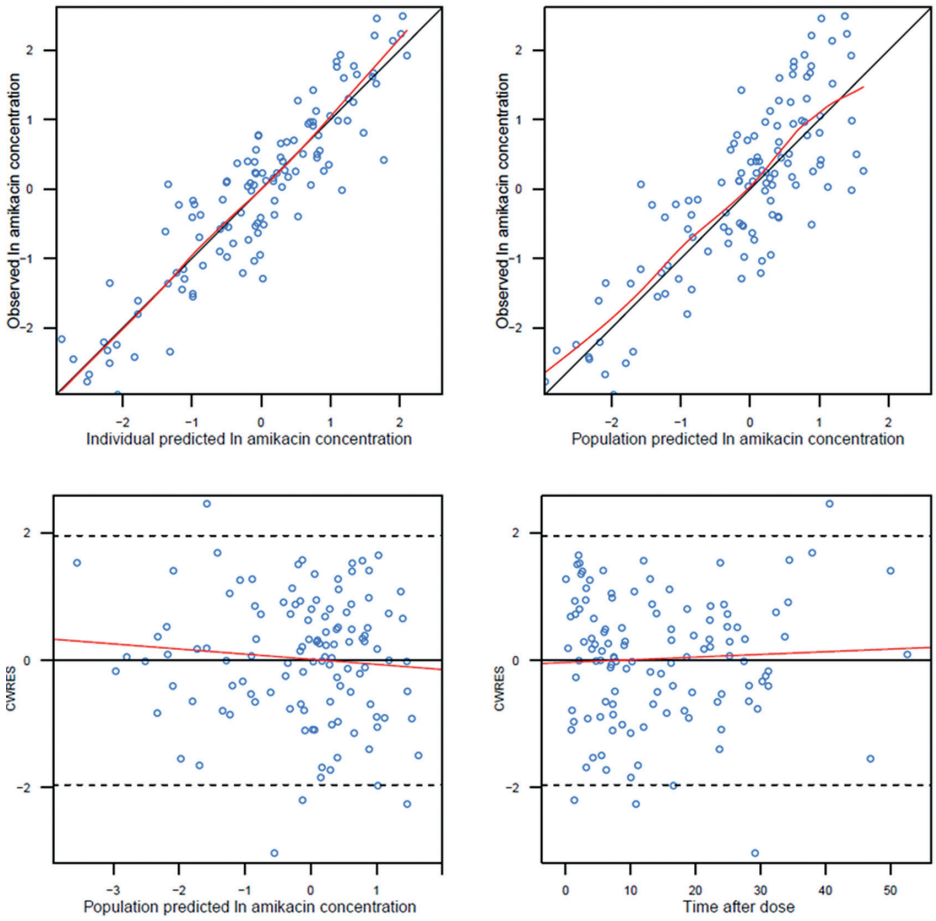
$$CL = \theta_{CL} * \left(\frac{BW}{1.75}\right)^{\theta_{BW-CL}} * \left(1 + \theta_{PNA-CL} * \left(\frac{PNA}{2}\right)\right) * \theta(IBU)$$

$$V1 = \theta_{V1} * \left(\frac{WT}{1.76}\right)^{\theta_{WT-V1}}$$

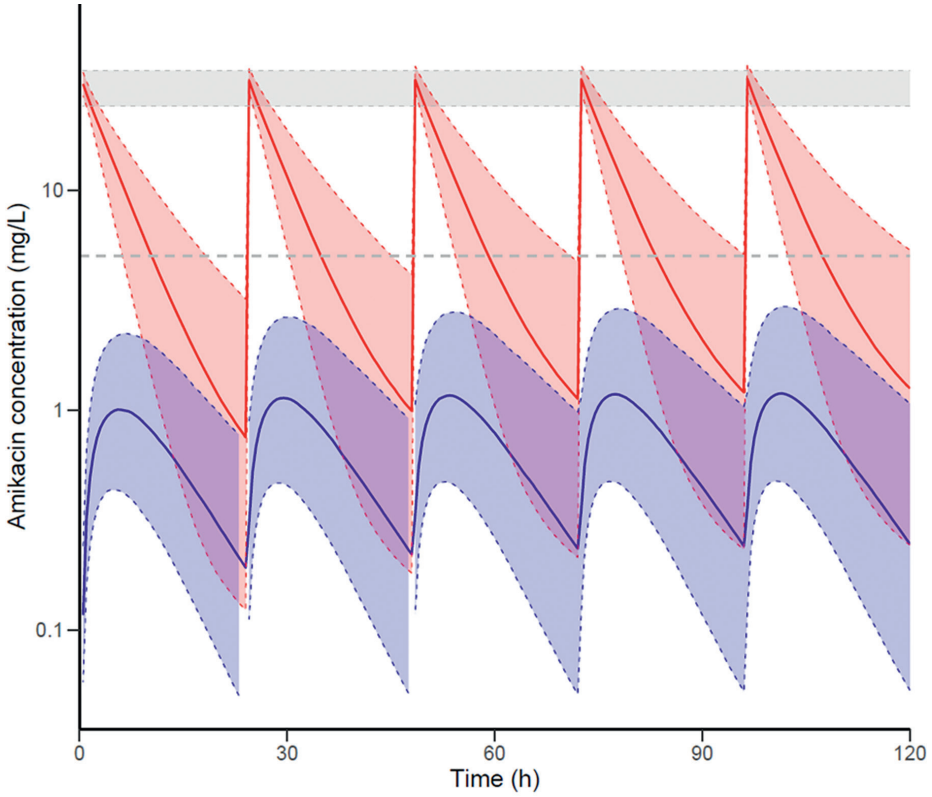
$$Q = \theta_{fQ} * \theta_{CL}$$



Supplementary Figure S3. Prediction-corrected visual predictive check of the log transformed data with the plasma model by de Cock *et al* [16].



Supplementary Figure S4. Basic goodness-of-fit plots of the final saliva model. Top left: observed individual amikacin concentrations vs predicted individual amikacin concentrations. Top right: observed population amikacin concentrations vs predicted population amikacin concentrations. Bottom left: Conditional weighted residuals vs predicted population amikacin concentrations. Bottom right: Conditional weighted residuals vs time after dose (hours).



Supplementary Figure S5. Amikacin concentrations of the simulated cohort. Red line: median concentration in plasma. Red area: 90% prediction interval of the plasma concentrations. Blue line: median concentration in saliva. Blue area: 90% prediction interval of the saliva concentrations. Grey area: target range for peak plasma concentrations. Dashed grey line: upper threshold for trough plasma concentrations.

Part III

Pharmacodynamic aspects of antimicrobial therapy in neonatal sepsis

Chapter 5

Predicting Treatment Response to Vancomycin Using Bacterial DNA Load as a Pharmacodynamic Marker in Premature and Very Low Birth Weight Neonates: A Population PKPD Study

Amadou Samb, Rimke de Kroon, Koos Dijkstra, Marre van den Brand, Martine Bos, Frank van den Dungen, Agnes Veldkamp, Bram Wilhelm, Timo R. de Haan, Yuma Bijleveld, Marceline Tutu van Furth, Paul Savelkoul, Noortje Swart, Ron Mathot, Mirjam van Weissenbruch

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Abstract

Background

Whilst positive blood cultures are the gold standard for late-onset sepsis (LOS) diagnosis in premature and very low birth weight (VLBW) newborns, these results can take days and early markers of possible treatment efficacy are lacking. The objective of the present study was to investigate whether the response to vancomycin could be quantified using bacterial DNA loads (BDL) determined by real-time quantitative polymerase chain reaction (RT-qPCR).

Methods

VLBW and premature neonates with suspected LOS were included in a prospective observational study. Serial blood samples were collected to measure BDL and vancomycin concentrations. BDL were measured with RT-qPCR, whereas vancomycin concentrations were measured by LC-MS/MS. Population pharmacokinetic-pharmacodynamic modeling was performed with NONMEM.

Results

Twenty-eight patients with LOS treated with vancomycin were included. A one-compartment model with post-menstrual age (PMA) and weight as covariates was used to describe the time PK-profile of vancomycin concentrations. In 16 of these patients time profiles of BDL could be described with a pharmacodynamic turnover model. The relationship between vancomycin concentration and first-order BDL elimination was described with a linear effect model. Slope *S* increased with rising PMA. In 12 patients no decrease in BDL over time was observed, which corresponded with clinical non-response.

Discussion

BDLs determined through RT-qPCR were adequately described with the developed population PKPD model and treatment response to vancomycin using BDL in LOS can be assessed as early as 8 hours after treatment initiation.

Introduction

Late onset sepsis (LOS) or nosocomial sepsis is a severe infectious neonatal condition characterized by bacteremia and a systemic inflammatory response that clinically manifests after the first 72 hours of life and typically originates from indwelling central lines or catheters during hospital admission [1]. Premature and/or very low birth weight (VLBW) neonates are at an increased risk for LOS, due to an immature immune system and frequent exposure to invasive procedures in neonatal intensive care units (NICU) [2]. Coagulase negative staphylococci (CoNS) are the predominant pathogen in LOS [3]. Approximately 21% of VLBW infants experience at least one episode of culture-proven LOS [4]. LOS has a high morbidity and mortality rate in premature and/or small for gestational age infants [2]. In cases of (suspected) LOS the current standard of care is initiating treatment with broad-spectrum antibiotics (an aminoglycoside and a penicillin derivative) until the pathogen is identified by blood culture, which can take up to 48 hours. Thereafter, narrow-spectrum antibiotic treatment targeting the isolated pathogen is initiated.

Intravenous (i.v.) vancomycin is given for 7 days in neonates with CoNS-positive LOS. Standardized loading doses of vancomycin are initiated and subsequent doses may be corrected and individualized by means of therapeutic drug monitoring (TDM). However, bactericidal efficacy of vancomycin is currently not assessed by readily accessible or reliable pharmacodynamic (PD) markers. Current vancomycin dosing regimens rely on indexed pharmacokinetic (PK) efficacy targets, based on the ratio of area under the curve ($AUC_{0\text{-}24h}$) and minimal inhibitory concentrations (MIC) [5–7]. As these PK/PD indices rely heavily on MIC, these may be inadequate as MICs are highly variable between patients, bacterial strains and over time [8].

There is a need for PD markers that accurately describe the early and gradual bactericidal action of vancomycin in case of CoNS-positive LOS. The bacterial DNA load (BDL), determined through real-time quantitative PCR (RT-qPCR), is a marker that may be used for this purpose. The method has a short turnover time, is sensitive, biologically accurate and most of all requires a small volume of blood [9,10]. The objective of this study was to assess changes in BDL to characterize the early response to vancomycin treatment in CoNS-positive LOS. A population-PK/PD model was developed quantifying the effect of vancomycin on BDL in CoNS-positive LOS. In addition, potential covariates that influence the measure of the bactericidal effect were explored. Using this model we aimed to gain insight on the vancomycin bactericidal effect over time and evaluate PD and clinical responses within the first hours of vancomycin treatment. A quantified vancomycin effect on BDL could support the accuracy of TDM.

Materials and Methods

Study design

This was a single center, prospective, observational study during a consisting intervention in the Amsterdam University Medical Center (division of neonatology, pediatrics department, location

VU Medical Center, Amsterdam, The Netherlands). Serial blood samples were collected for the determination of antibiotic concentrations, BDL and inflammatory markers. The study was approved by the local ethics committee (METC Vrije Universiteit Medisch Centrum, Amsterdam, The Netherlands) prior to inclusion of patients and recorded in the Dutch CCMO registry (file number: NL22434.029.08).

Study population

Inclusion of subjects took place between February 1st 2009 and November 13th 2014. Neonates with suspected LOS and/or meningitis that were either premature or VLBW were eligible for inclusion. For the identification of LOS, the definitions by van der Zwet ***et al.*** were adhered [11]. Prematurity was defined as a gestational age (GA) < 32 weeks and VLBW was defined as a birth weight below 1,500 grams. Patients with syndromal or chromosomal abnormalities, as well as congenital metabolic disease were excluded from the study. Oral and written informed consent was a prerequisite for study participation. Following clinical practice at that time, the first-in-line treatment for LOS was intravenous amikacin (12 mg/kg/day) combined with intravenous benzylpenicillin (200,000 IU/kg/day). After initial blood culture results were known, treatment was switched to a targeted antibiotic. For the research presented in this article, only the studied patients with culture proven CoNS infection and vancomycin treatment (20-48 mg/kg/day) in 1-hourly intravenous infusions were included in the analysis.

Sample collection

Whole blood samples were collected from newly inserted peripheral venous cannulas and (if available) from the central (umbilical) venous catheters. Blood samples (0.2 mL) for BDL measurement were collected at t = 0h, t = 4h, t = 24h and t = 48h. The samples at t = 0h were collected before the first vancomycin dose. Plasma samples (0.1 mL) were collected at t = 1h, t = 2h, t = 4h and t = 12h for the measurement of vancomycin concentrations. After collection, samples were stored at -20 °C until analysis.

Additional clinical and anthropomorphic data such as dose information, GA, postmenstrual age (PMA), postnatal age (PNA), birth weight (BW), current weight (WT), length (LT), concomitant medication, c-reactive protein (CRP) and serum creatinine (SCr) were collected from the patient electronic medical files.

Real-time quantitative PCR

The design, validation and evaluation of the used RT-qPCR method has been described in earlier publications [9,10]. In short, 200 µL of EDTA anticoagulated whole blood samples were treated with TTE (1% Triton X-100, 20mM Tris-HCl pH 8.3, 1 mM EDTA) twice for hemolysis and removal of hemoglobin. Next, samples were incubated for 10 min in 200 µL bacterial lysis buffer (Biocartis, Mechelen, Belgium) at 95 °C whilst shaking at 800 rpm. 20 µL of neutralization buffer (Biocartis, Mechelen, Belgium) was added to and DNA was purified with the NucliSENS EasyMag device (bioMérieux, Zaltbommel, The Netherlands). Samples were spiked with Phocine Herpesvirus 1 as an internal control.

PCR was performed on a LightCycler 480II device (Roche Diagnostics, Almere, The Netherlands). 12.5 µL 2x LightCycler 490 Probes Master, 2.5 µL primers and probes and 10 µL purified DNA sample were used as reaction mixtures. Samples were screened for the eight most common pathogens of LOS in a multiplex assay [9]. Cycling conditions were 10 min at 95 °C, followed by 45 cycles of 15s at 95 °C and 1 min at 60 °C. If amplification was detected, the sample was then evaluated in a monoplex assay for quantitative analysis. BDLs were determined using a standard curve of serial dilutions of cloned PCR amplicons. The BDL was expressed in colony forming units equivalents per ml (CFU eq/ml) by correcting for blood volumes and the number of PCR target copies per genome. The lower limit of quantification (LLOQ) for CoNS was 55 CFU eq/ml.

Liquid chromatography coupled to tandem mass-spectrometry (LC-MS/MS)

Vancomycin concentrations were measured in plasma samples using LC-MS/MS. In short, an Acquity TQD Tandem Quadrupole UPLC/MS/MS system (Waters, Milford, USA) was used and the method had a within-run accuracy and an imprecision of at least 94.5% and at most 5.3%. Between-run accuracy and imprecision were at least 104.5% and at most 8.7%, respectively. The LLOQ of the used method was 0.22 mg/L [12].

Bactericidal responses and clinical record evaluation

Changes in BDL over time following the initial vancomycin dose were plotted for all individuals in the study population. Thereafter, clinical records and treatment response patterns of patients were evaluated by a neonatologist of our research team for correspondence to BDL profiles.

Pharmacokinetic-pharmacodynamic data analysis

The BDL responses were modeled for all patients demonstrating a bactericidal response. Nonlinear mixed-effects population PKPD (pop-PKPD) modeling was performed to describe and evaluate the relationship between vancomycin dose, concentration, and BDL and used to estimate pop-PKPD parameters. The model was developed using NONMEM version 7.4.0 software Icon Development Solutions, Ellicott City, MD, USA). All data handling, data visualization and descriptive statistics were performed using R statistics version 4.1.0. [13]. Model validation and evaluation steps were performed with PsN version 5.3.0.

In a pop-PKPD analysis data from all patients are analyzed simultaneously, allowing the analysis of patients for which only sparse samples are available. With sparse sampling individual parameter estimates may be obtained by post-hoc (Bayesian) analysis. Both “fixed effects” (typical parameters), as well as “random effects” (inter-individual variability (IIV), residual variability) are estimated in pop-PKPD modeling. Thus, parameters such as clearance (CL) as well as the IIV in CL are embedded in the model. Part of the IIV may be explained by including covariates such as age in the model. Any remaining inaccuracies in predictions are included in the model as residual error. Parameter estimations were evaluated by assessing the objective function value (OFV), which is a maximum likelihood estimation based approach. For parameter

inclusion in nested models, a change in OFV of -3.84 corresponds with a p-value = 0.05 given 1 degree of freedom, which was deemed significant for parameter inclusion in this study. After each modeling step, model accuracy was assessed by evaluating goodness-of-fit (GOF) plots, parameter relative standard error (RSE) and changes in OFV. Models were evaluated and internally validated using visual predictive checks (VPC, N=1,000) or prediction-corrected VPCs (pc-VPC, N=1,000), a simulation based diagnostic to evaluate predictive performance of the model. Model robustness and parameter certainty was assessed by a sampling importance resampling (SIR) procedure [14,15]. Using the covariance matrix as the initial proposal distribution, 5 iterations with 1000, 1000, 1000, 2000, 2000 samples (M) and 200, 400, 500, 1000, 1000 resamples (m) were performed during SIR.

The integrated PKPD model was developed using a sequential estimation approach. First, a vancomycin PK model found in literature was used to describe the data. Seven different published PK models were screened, selected on model evaluation and validation and similarities in study population [16–22]. The model with the highest precision, defined as the root mean squared error (*rmse*) was selected. It was a prerequisite for model performance to be unbiased, which was assessed using the distribution of the mean error (*me*). This was further supported by evaluation of GOF and VPCs. Finally the model that most accurately described the data was used as the foundation for the final model and used to reliably estimate the individual CL and volumes of distribution (V_d) of the study population using the POSTHOC setting in NONMEM.

An empirical turnover PD model was appended to the PK model (**Figure 1**). In this PD model, it was assumed that there is both bacterial growth and bacterial decay in absence of vancomycin, which rates were respectively parameterized as k_{growth} and k_{death} in the model. Effect *E* of vancomycin was modeled to augment the effect of k_{death} in the model, as shown in differential **Eq.1**.

(1) $$\frac{\delta N}{\delta t} = k_{growth} * N - E * k_{death} * N$$

In this equation, k_{growth} is the first order multiplication rate h^{-1}, k_{death} is the first order decay rate of in h^{-1}, N is the BDL in CFU eq/ml and *E* is the stimulatory effect of vancomycin on k_{death}. If simultaneous estimation of k_{growth}, k_{death} and effect parameters was not possible due to insufficient data, k_{growth} was defined as a zero order constant (**Eq.2**), changing the PD model to **Eq.3**:

(2) $$k_{growth} = \frac{BDL_0}{k_{death}}$$

k_{growth} is the zero order multiplication rate of BDL in CFU eq*ml^{-1}*h^{-1}, k_{death} is the first order decay rate in h^{-1} and BDL_0 is the estimated BDL in CFU eq/ml at t=0h.

(3) $$\frac{\delta N}{\delta t} = k_{growth} - E * k_{death} * N$$

Effect *E* was parameterized as either a linear effect model (**Eq.4**), or a sigmoidal E_{max} model (**Eq.5**):

(4) $$E = 1 + S*C$$

(5) $$E = 1 + \frac{E_{max}*C^{\gamma}}{EC_{50}^{\gamma} + C^{\gamma}}$$

Eq.4, Effect *E* is the product of the vancomycin concentration *C* and slope *S*. For the sigmoidal E_{max} model (**Eq.5**), effect *E* is described by maximum effect E_{max}, vancomycin concentration *C*, the vancomycin concentration where 50% of the maximum effect is achieved (EC_{50}) and a hill constant γ that describes the steepness of the effect.

Stepwise covariate analysis was performed, with a significance threshold of p=0.05 for forward inclusion and p=0.01 for backwards elimination. GA, PMA, PNA, WT, BW, baseline and CRP were evaluated as potential covariates. Continuous covariates were modeled as a linear or power function (**Eq.6** and **Eq.7**).

(6) $$P = \theta_p * \left(1 + \theta_{cov} * \frac{COV - COV_{median}}{COV_{median}}\right)$$

(7) $$P = \theta_p * \left(\frac{COV}{COV_{median}}\right)^{\theta_{cov}}$$

Where parameter *P* is expressed by typical parameter θ_p, which is affected by deviation of covariate *COV* from the median covariate value COV_{median} with an effect of magnitude θ_{cov}.

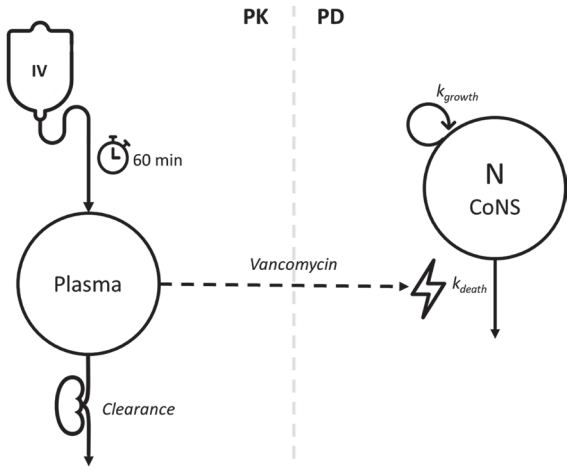

Figure 1. Conceptual pharmacokinetic-pharmacodynamic model for vancomycin effect on Bacterial DNA load. Left side of the dashed line: One-compartment PK model. Vancomycin is infused intravenously for 1 hour and is renally cleared from the body. Right side of the dashed line: Empirical turnover PD model. The number of CoNS (N) has a natural in vivo multiplication rate of kgrowth and natural cell death rate of kdeath. The vancomycin concentration in plasma has a bactericidal action by stimulation of kdeath, depicted by the dashed arrow connecting the left and right sections of the figure.

Results

Demographic characteristics

A total of 28 patients with CoNS-positive LOS and vancomycin treatment were included for analysis. The demographic characteristics of these patients are depicted in **Table 1**. A total of 94 vancomycin concentrations and 132 BDLs were available for analysis.

Table 1. Demographic characteristics of the study population.

Characteristic	
Patients *- N*	28
Vancomycin samples *- N*	94
BDL samples *- N*	132
Female *- N (%)*	9 (32.1%)
GA (weeks) – *median (IQR)*	28.4 (26.7 - 29.9)
PNA (days) – *median (IQR)*	11 (8 - 14)
PMA (days) – *median (IQR)*	214 (200 - 220)
WT (grams) – *median (IQR)*	1110 (955 - 1279)
BW (grams) – *median (IQR)*	1150 (971 - 1235)
LT (cm) – *median (IQR)*	37.5 (35.0 - 39.6)
BLT (cm) – *median (IQR)*	38.0 (35.0 - 39.1)
SCr (µmol/l) – *median (IQR)*	49.5 (44.8 - 55.3)

GA: Gestational age; PNA: Postnatal age; PMA: Postmenstrual age; WT: Body weight at inclusion; BW: Birth weight; LT: Length at inclusion; BLT: Birth length; SCr: Serum creatinine

Bacterial DNA load response

Pooled BDL responses during vancomycin treatment are depicted in **Figure 2**. A bactericidal BDL response, defined as a decrease in BDL in 48 hours was observed in 16 patients, combined with clinical efficacy (**Figure 2A**). Six patients demonstrated persisting septic BDL profiles (**Figure 2B**). Five of these patients had complicated central line infections in which lines were not removed during BDL measurements. This probably explains the lack of clinical and BDL response, since clinical response was observed upon line removal. The two erratic profiles (**Figure 2C**) could be explained by patients with presence of an infected thrombus and an infected line obstructed by a pustule, respectively. Four patients had near unquantifiable BDL profiles (**Figure 2D**). However, in three of these patients amikacin susceptible CoNS were isolated and were therefore cured due to the empirical pretreatment with amikacin. The persisting septic, erratic and unquantifiable profiles were pooled as 'non-response' (n=12).

Vancomycin pharmacokinetic-pharmacodynamic model

Seven published PK models for vancomycin in pediatric/neonatal populations were evaluated to fit the study data. The one-compartment model by Marqués-Miñana *et al.* (**Table 2**) was

most accurate in describing the vancomycin levels of our study population [19]. This was decided based on precision, as defined by the lowest *rmse* and bias which was evaluated using the confidence interval of the *me* [23]. An *rmse* of 2.959 mg/l was found, which was lowest out of all tested models. The *me* was 0.147 mg/l (-0.453 - 0.747 mg/l), indicating that model performance was unbiased. This was supported GOF plots and pc-VPC (**Supplementary Figure S1**). Whilst the model included comedication with spironolactone and amoxicillin as covariates, no patients in the study received any of these drugs during the study period. The PK model was used to estimate the individual CL and V_d of the study population.

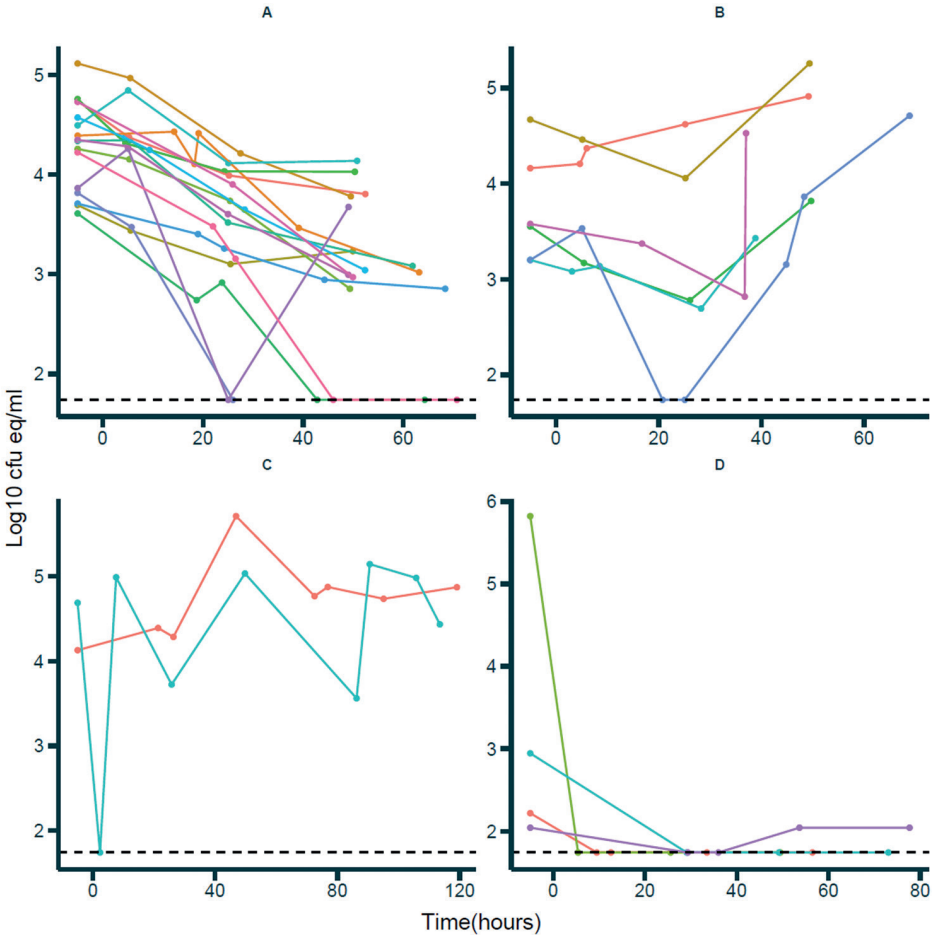

Figure 2. Bacterial DNA load (BDL)-time profiles during vancomycin treatment. X-axis: Time in hours; Y-axis: BDL in log10 cfu eq/ml. Dots and lines of the same color within a plot indicate observations for individual patients. **A:** Bactericidal responses; individuals demonstrating a negative trend in BDL over time. **B:** Persisting septic responses; individuals demonstrating a constant or increasing trend in BDL over time. **C:** Erratic responses; individuals demonstrating an erratic BDL over time following no specific pattern. **D:** unquantifiable response; individuals with very low BDL and BDL below quantification limit since vancomycin treatment.

A population PK-PD model was developed using only the data of the patients demonstrating a bactericidal response to vancomycin (n=16; **Figure 2A**). It was attempted to develop a model including all patients, though no model could be fit due to data limitations. Pharmacodynamics were described with an empirical turnover model (**Figure 1**). Parameter estimates of the model are summarized in **Table 2**. The natural BDL decay was described in the structural model with the first-order rate constant k_{death} of 0.0033 h^{-1}. The stimulatory effect *E* of vancomycin on k_{death} was expressed as a linear effect model (**Eq.4**), with a slope *S* of 0.862. IIV of the slope was 64.1%. Due to the limited data, the natural growth constant k_{growth} could not be estimated as an independent parameter. Therefore, k_{growth} was expressed as **Eq.2**, and was a zero-order rate constant dependent on the initial BDL_0 at t=0h and k_{death}. BDL_0 was estimated and last measured BDL before vancomycin treatment (BL) was included as a linear structural covariate. IIV in BDL_0 was 72% and was slightly correlated with IIV of *S*, as indicated by an off-diagonal of 30.1% in the omega matrix. Proportional residual error was 16.3%.

Table 2: Structural model, final model and SIR results for the plasma and saliva model.

	structural model OFV = 81.361		**Final model** OFV = 69.883		**SIR results (5 iterations)** M=1000, 1000, 1000, 2000, 2000 m=200,400,500,1000,1000	
Parameter	**Estimate**	**RSE**	**Estimate**	**RSE**	**Mean estimate (95% CI)**	**RSE**
Plasma model [19]						
θCL (l* h^{-1} *kg^{-1} *wk^{-1})	0.00192	-	0.00192	-	-	-
θV (l kg^{-1})	0.572	-	0.572	-	-	-
θAMX-CL	0.65	-	0.65	-	-	-
θSPI-V	0.344	-	0.344	-	-	-
ωCL	35.6%	-	35.6%	-	-	-
ωV	19.3%	-	19.3%	-	-	-
σ_{add} (mg l^{-1})	2.69	-	2.69	-	-	-
BDL model						
θ_{kdeath} (h^{-1})	0.0033	31%	0.0035	31%	0.0037 (0.0016-0.0065)	37%
θ_S	0.862	36%	0.833	32%	0.968 (0.503-1.893)	37%
θ_{PMA}	-	-	8.23	31%	7.76 (3.23-13.27)	33%
θ_{BDL-0}	10600	24%	10400	22%	11213 (7815-15717)	18%
θ_{BDL-S}	1.13	25%	1.02	20%	1.17 (0.974-1.327)	8%
ω_S [shrinkage]	64.1% [24%]	71%	58.8% [20%]	36%	45% (12.6%-72.5%)	40%
ω_{BDL-s} [shrinkage]	72.0% [11%]	18%	75.8% [11%]	27%	60% (34.6%-83.1%)	23%
σ_{prop} [shrinkage]	0.163 [27%]	16%	16.3% [27%]	16%	17.7% (13.0%-24.8%)	16%

θ_{cl}: clearance. PMA: Post-menstrual age. WT: Bodyweight. AMX: amoxicillin comedication. θ_{AMX-CL}: amoxicillin effect on clearance. θ_V: volume of distribution. SPI: Spironolactone use. θ_{SPI-V}: spironolactone effect on volume of distribution θ_{kdeath}: 1st order rate constant for natural bacterial death. θ_S: Slope of the

linear effect model. θ_{PMA}: Power equation exponent of PMA on θ_S. θ_{BDL0}: typical BDL_0. $\theta_{BDL0\text{-}S}$: slope of linear BDL_0 function. ωS: Inter-individual variability in slope S, $\omega_{BDL0\text{-}S}$: inter-individual variability in typical BDL_0. σ_{prop}: Proportional residual error. OFV: objective function value. The BDL_0 was scaled with the median BDL_0 of 14520.

$$CL = \theta_{CL} * PMA * WT * (1 + AMX * \theta_{AMX-CL})$$
$$V = \theta_V * WT * (1 - SPI * \theta_{SPI-V})$$
$$k_{death} = \theta_{kdeath}$$
$$S = \theta_S * \left(\frac{PMA}{213}\right)^{\theta PMA}$$
$$BDL_0 = \theta_{BDL0} * \left(1 + \theta_{BDL0-s} * \frac{BL - 14520}{14520}\right)$$

In the covariate analysis, PMA was significantly associated with slope *S*. Using a power model (**Eq.7**) an exponent of 8.2 was estimated, thereby decreasing IIV of the *S* from 64.1% to 58.8% (**Table 2**). The correlation between the IIV in *S* and BDL in the off-diagonal of the omega matrix was increased to 72.3% for the final model. Relative standard errors of all parameters were within the acceptable range. GOF plots of the final model indicated an adequate model fit to the data (**Figure 3**). The bactericidal effect of vancomycin for individual patients has been depicted in **Figure 4**.

Model performance was evaluated using a pc-VPC (N=1,000) (**Figure 3**). The upper, median and lower percentiles of the observations were within the 95% CIs of the corresponding simulated percentiles, indicating that the model could adequately predict the BDL levels of patients responding to treatment as a function of vancomycin PK. To assess model robustness and parameter uncertainty, SIR was performed. The SIR results of the final model are shown in **Table 2** and **Supplementary Figure S2**. The proposal parameter distribution was above the reference distribution. After the final iteration, the dOFV plots showed a Chi-squared distribution with less degrees of freedom than the number of parameters in the model. No temporal trends were observed and therefore the SIR results were accepted. There was full coverage of the final parameters.

Discussion

In this study, bacterial DNA loads were used as a PD marker to evaluate the bactericidal effect of vancomycin in premature and/or VLBW neonates with CoNS-positive late onset sepsis. In patients that demonstrated a bactericidal response, the time profile of the BDL could be quantified with an empirical PK/PD model. Using this model the time course and measure of the bactericidal effect of vancomycin could be described. (**Figure 4**).

To our knowledge the present study is the first to quantify the bactericidal effect of vancomycin in premature and VLBW neonates with CoNS-positive LOS through the assessment of time profiles of BDL using RT-qPCR.

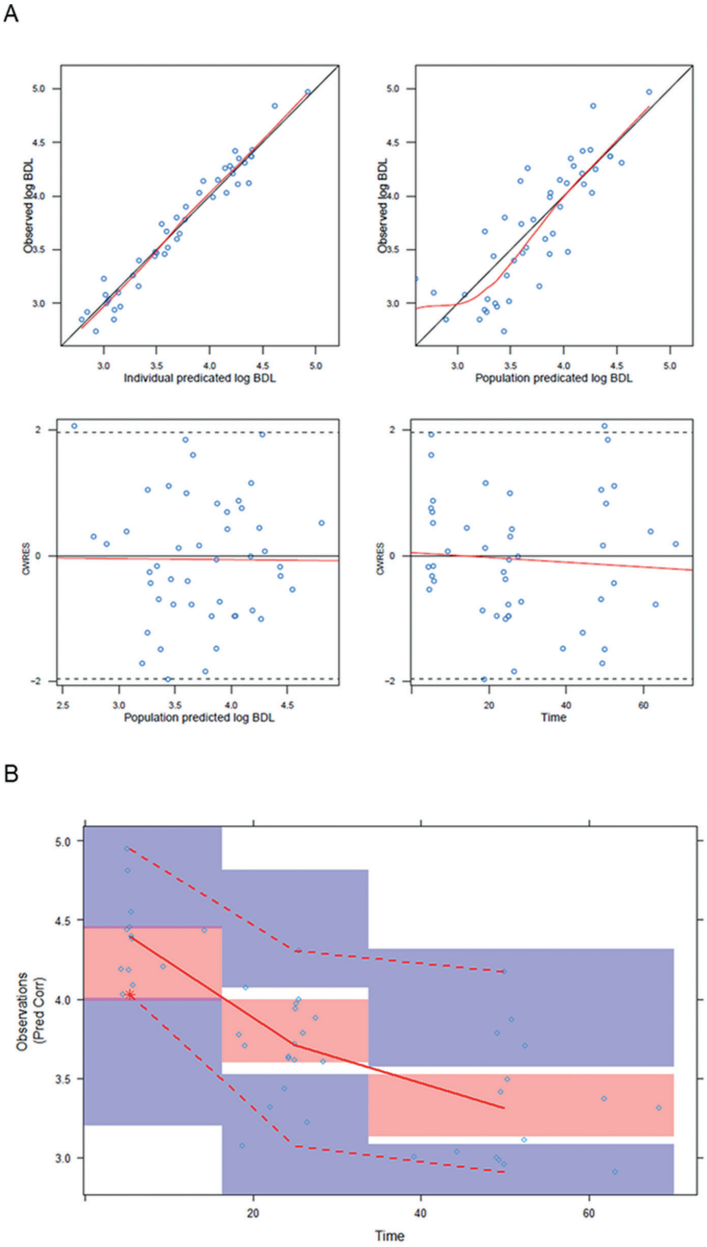

Figure 3. Goodness of fit plots and prediction-corrected VPC of the final model: A- Goodness of fit plots for the PD model. Top left; individual log10 BDL predictions vs log10 BDL observations. Top right; population log10 BDL predictions vs log10 BDL observations. Bottom left; population BDL log10 predictions vs CWRES. Bottom right; Time after last dose vs CWRES. CWRES: conditional weighted residuals. B- Prediction-corrected VPC of the PD model (n=1000). X-axis: time after last dose; Y-axis, prediction-corrected Log10 BDL. Black dots: observations. Blue lines 10th and 90th percentiles of observations. Red line: median of observations. Blue shaded areas: 95% CIs of simulated 10th and 90th percentiles. Red shaded area: 95% CI of simulated median.

Bacterial PCR has been investigated in the past as a potential diagnostic tool and for antimicrobial susceptibility screening in the context of LOS and varying results have been reported with some studies suggesting BDL measured through PCR methods could serve as a surrogate for blood culture in the diagnosis of some infectious diseases [9,12], while others demonstrated that PCR-based diagnostics were inferior to conventional blood culture [25,26]. All studies seem to indicate that PCR-based diagnostics are feasible, however.

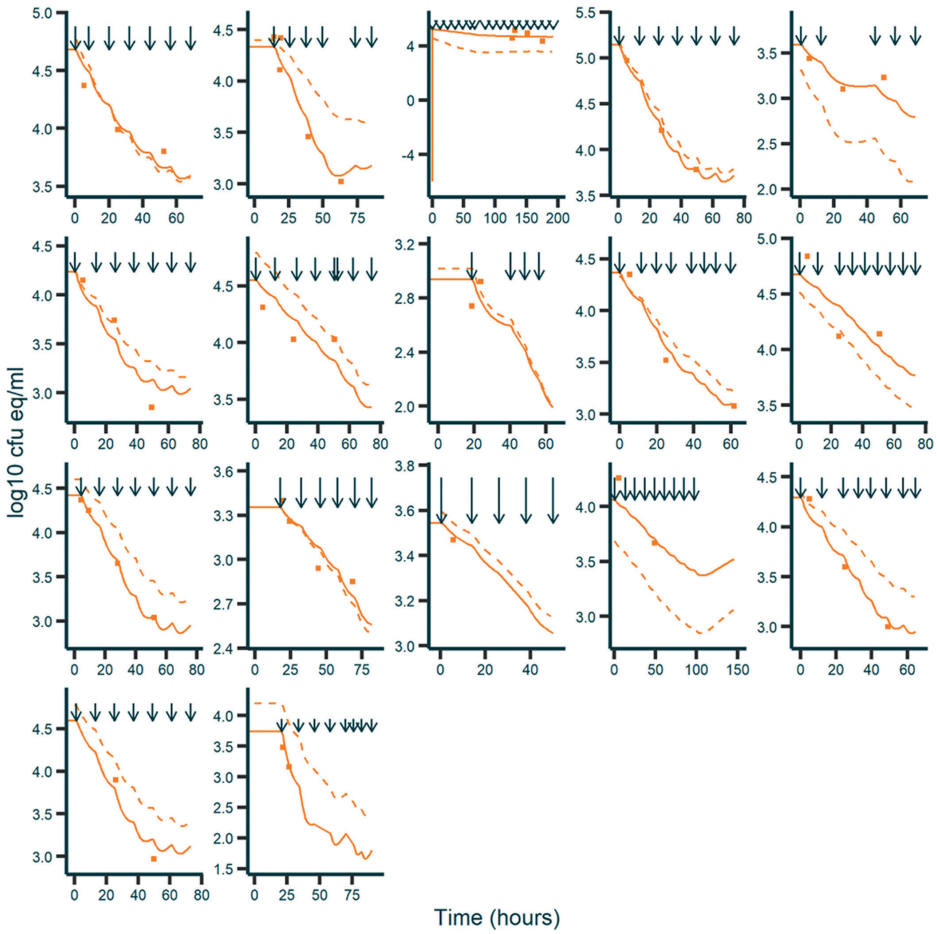

Figure 4. Fitted and observed BDL over time for individual responders. X-axis: Time in hours. Y-axis: BDL in log10 cfu eq/ml. Dark blue arrows: Vancomycin dosing times. Orange squares: observations. Solid orange line: individual predictions. Dashed orange line: population predictions.

After assessing previously published population PK models for vancomycin the model by Marqués-Miñana *et al.* was found to best describe the observed vancomycin levels of our study population [16–22]. The selected model was developed using data from 70 neonates admitted to a NICU and treated with vancomycin, with a large proportion of patients that were

comparable to our study population [19]. The model was internally and externally validated by the original authors.

For patients responding to vancomycin treatment time profiles of BDL were described using a turnover model (**Figure 1**). There was a positive relation between slope *S* and PMA, when PMA was included as a power function with an estimated exponent of 8.23. This indicates that older patients demonstrated a larger bactericidal effect than younger patients at equal vancomycin concentrations. However, it is questionable whether this perceived age dependency in bactericidal activity of vancomycin is truly the underlying process for the increased BDL decline for patients of higher PMA. Innate- and adaptive immunity is immature for premature neonates and it is possible that increased bactericidal action at higher PMA is a product of higher immune activity, rather than an increased effect of vancomycin [27]. However, the data did not support estimation of IIV on k_{death}, nor could PMA be estimated as a covariate on k_{death}. Nonetheless, including PMA in the model as an exponential function on slope *S* significantly improved model fit and explained 5.3% of the IIV in slope *S*. Still, the remaining IIV in slope *S* in the final model was 58.8% and could be considered very high for classical PKPD models. This high variability is likely the result due to oversimplification of the model. A true mechanistic antimicrobial PKPD model incorporates a complicated system of true bacterial growth, decay and sigmoidal E_{max} effects, as well as resistance mechanisms, ideally with multiple levels of IIV. Oversimplification of these underlying mechanisms has results in a model where all these types of variability have been combined in a single, large IIV for slope. As for high IIV in BDL_0, this is strongly supported by the data since the included patients demonstrated enormous variability in BDL at the first vancomycin dose.

A number of mechanistic PKPD models for antimicrobials have been published, however these models have been exclusively applied in *in vitro* and animal studies. No such models could be found using clinical PD data. An overview of published antimicrobial PKPD models has been provided in a database by Minichmayr *et al*. [28]. A single similar publication was found that modeled the effect of vancomycin on CoNS colonization in central line associated LOS using an *in vitro* hollow fiber infection model and a rabbit model [29]. The authors found that, based on a translational model using their preclinical data, currently accepted dosing guidelines of AUC/MIC ≥ 400 were potentially too low for neonates ≤ 29 weeks GA and argued that efforts should be made for the development of more efficacious dosing regimens in central line associated LOS, optimizing bactericidal efficacy, minimizing toxicity and preventing drug resistance.

Since the analyzed population was small, bootstrapping methods were deemed unsuitable for the evaluation of parameter uncertainty and model stability [14]. Thus, SIR was used for this purpose. SIR converged after 5 iterations (**Supplementary Figure S2**) and reliable RSE and 95% confidence intervals were obtained (**Table 2**). Based on the SIR results, the model was deemed accurate.

Simulation based model evaluation using pc-VPCs (N=1,000) indicated that the final model could adequately predict the observed BDLs in the study population. Therefore, the final constructed model was deemed suitable for the purposes of this study and provided sufficient insight that vancomycin effect on BDLs as determined through RT-qPCR can be described in a population-PKPD model.

Six patients demonstrated an increasing or constant BDL, as demonstrated in **Figure 2B**. These 'persisting septic' BDL responses were evaluated by a neonatologist of our research team using the recorded clinical response of the patient. Indeed, five of these patients had clinically persistent CoNS bacteremia during the vancomycin therapy. In these patients, the primary source of LOS was CoNS colonization of the central venous line (CVL) in situ and removal of the CVL resulted in clinical improvements and negative blood cultures. Although CVL infections are a frequent source of nosocomial sepsis, there seems to be little consensus whether CVL removal is beneficial in CoNS bacteremia [30–32]. It has been found that in over 70% of CoNS CVL infections, line retention does not interfere with antimicrobial efficacy [32]. However, Benjamin *et al*. state that CVL removal should be considered for patients with persistent sepsis, identified as 4 consecutive blood cultures positive for CoNS [30]. Two of our patients presented erratic BDL profiles measurements, showing no specific pattern in BDL change over time (**Figure 2C**). In one of these two patients, CoNS bacteremia was secondary to an infected thrombus. Presence of an infected thrombus has been implied as a risk factor for persistent or recurrent staphylococcal sepsis in multiple case reports [33,34]. Incremental degradation of the colonized thrombus due to shearing stress, releasing CoNS infected debris into the bloodstream at irregular time intervals could explain the observed erratic BDL profile. The other patient suffered from an infected peripheral venous line complicated by the presence of a pustule at the ankle. Likewise, irregular mechanical stress at the primary site of infection could release high loads of infected material into the bloodstream at random intervals. For some patients, most measured BDL were below or near the quantification limit following vancomycin treatment (**Figure 2D**). In these cases, the isolated CoNS culture was susceptible to amikacin. During this study, empirical amikacin and benzylpenicillin treatment was clinical routine until diagnostic blood culture results. Therefore, susceptible CoNS exposed to 48 hours of amikacin is expected to demonstrate substantial bacterial killing, explaining the absence of CoNS BDL during subsequent vancomycin therapy.

There were some limitations to this study. First, since RT-qPCR quantifies the total bacterial DNA in the study sample, it could not distinguish between DNA from living or dead bacteria. Whilst it is known that circulating free DNA has a half-life of 1-2 hours and is cleared through macrophage-mediated phagocytosis and enzymatic degradation in the spleen and liver, to our best knowledge the rate at which dead bacteria are cleared from the neonatal bloodstream is unknown [35]. To account for the time delay between vancomycin dosing and BDL decrease in the model, a lag time (T_{lag}) was attempted to be estimated. However, including a T_{lag} in the model did not increase model fit and overcomplicated the model. Using total BDL allowed for a comprehensive approximation of bactericidal activity. Another limitation of the study

was the risk of sample contamination. Not only are CoNS the most frequent pathogen in LOS in developed countries, CoNS are also the predominant contaminating micro-organism in blood samples [36]. Therefore, efforts should be made to minimalize the contamination risk. For instance, assessment of bacterial density could be considered or multiple sample sources could be used [37]. This was not performed during this study, as this would result in an unacceptable burden due to increased blood sampling. Regardless, culture based assessments should be combined with careful clinical examination of patients, to minimize risk of unnecessary treatment due to sample contamination. The final limitation of the study is that no BDL profiles in absence of antibiotic treatment were available. Therefore, it was difficult to distinguish between natural bacterial growth and treatment and thus the estimate of k_{growth}, k_{death} and slope *S* as separate parameters. Unfortunately, no values of k_{growth} or k_{death} of CoNS, either as initial estimates or fixed parameters, could be found in the literature. A single study was identified that investigated the mechanistic PKPD relations between vancomycin and CoNS based on *in vitro* and animal data, though no parameter estimates were published in the model [29]. In our final model it was assumed that k_{growth} was a zero-order constant dependent on BDL at T=0 and k_{death}. As cellular multiplication relies on cell doubling, it is most certainly a 1st order process. Moreover, relating k_{growth} to BDL at T=0 results in a function in which the BDL cannot exceed this value, at the cost of model accuracy. By estimating the BDL with IIV, the model could more accurately predict BDLs that were above the last BDL before the first vancomycin dose.

There were a number of strengths to this study and its implications. First, an empirical model was developed to describe the bactericidal action of vancomycin in CoNS-positive LOS. CoNS are the predominant infective pathogen in LOS, accounting for approximately 53.2%–77.9% of all culture-proven LOS cases in developed countries [3]. Vancomycin is the first-in-line antibiotic in CoNS-positive LOS and dosing guidelines in neonatology are currently based on an $AUC_{0\text{-}24h}$/MIC index, in which a target of at least 400 is generally associated with efficacy [5]. However, PK/PD indices heavily rely on MIC, which is associated with considerable variability between bacterial strains, patients and occasions [8]. Moreover, these indices treat bactericidal action as a binary “all-or-nothing” response. This implies that bacterial killing is only active at concentrations above MIC and inactive at levels below MIC, whereas in reality bacterial killing changes dynamically with concentration. The method proposed in this study incorporates gradual bacterial killing as a function of vancomycin concentration and provides a more nuanced insight in bactericidal dynamics, independent of MIC. This could be of particular benefit in the context of TDM, as improved concentration targets could be identified. Second, a relatively large number of drug concentrations and BDLs were available for each enrolled patient. Blood sampling in neonatology comes with considerable risk and the number of samples collected per patient is hampered in the research context [38]. Therefore studies with a large amount of samples per patient are infrequent and valuable in this population. The relatively large amount of measurements per patient in our study allowed for more accurate depictions of the underlying PK and PD principles. A third strength of this study was that bacterial blood colonization was determined through multiplex RT-qPCR. The method used

was validated and evaluated in the clinical setting and allowed for the quantification of CFU eq/ml by adjusting measured DNA load for sample volume and CoNS genome load [9,10]. Thus, a surrogate marker for blood colonization that could be quantified within 8 hours provided information on the bactericidal action of vancomycin in this study. Lastly, BDL profiles that indicated treatment non-response (**Figure 2B, Figure 2C, Figure 2D**) were compared with the corresponding clinical records by a neonatologist to assess whether these patients did not respond clinically. By doing so, the assumption to create the PKPD model based only on data of patients demonstrating a decrease in BDL was confirmed.

This study demonstrates that a decrease in BDL in CoNS positive LOS can be quantified and predicted as a function of vancomycin concentration over time for patients that respond to vancomycin therapy. If developed further combining preclinical data with clinical data, this would allow for more nuanced and precise dosing regimens, as compared to the currently used “all-or-nothing” dosing guidelines based on MIC targets. Moreover, it is expected that the applicability and accuracy of TDM could significantly improve if more evidence based targets are identified.

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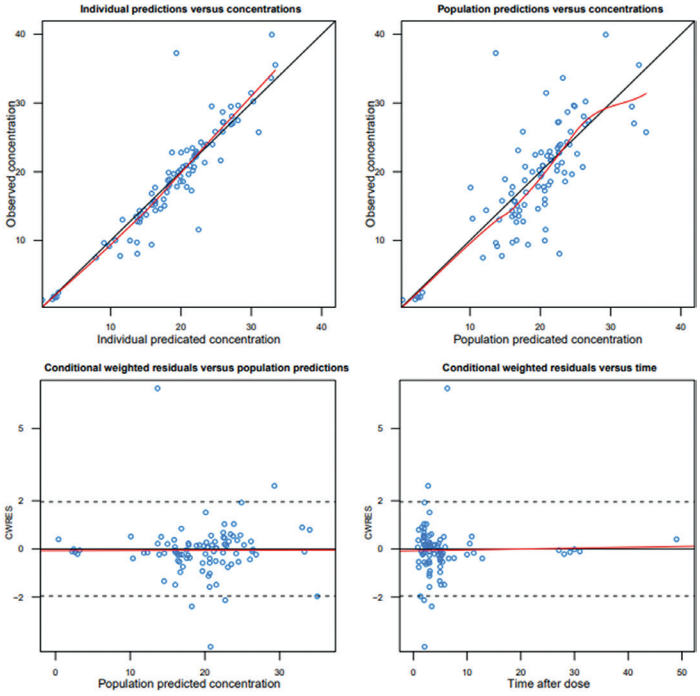
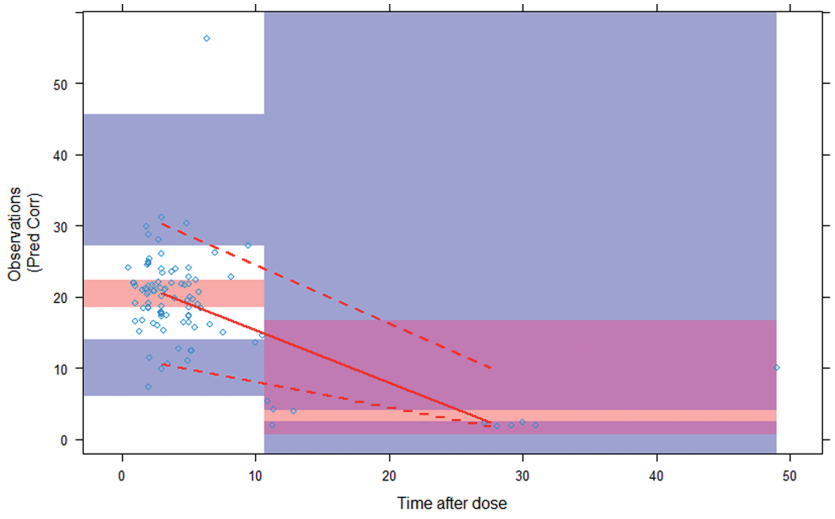
Supplementary material

A: Goodness of fit plots

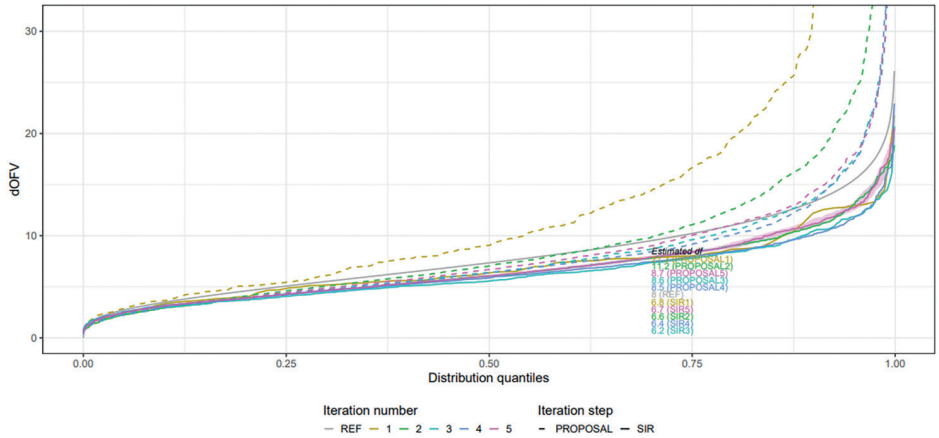


B: (Prediction corrected) visual predictive check

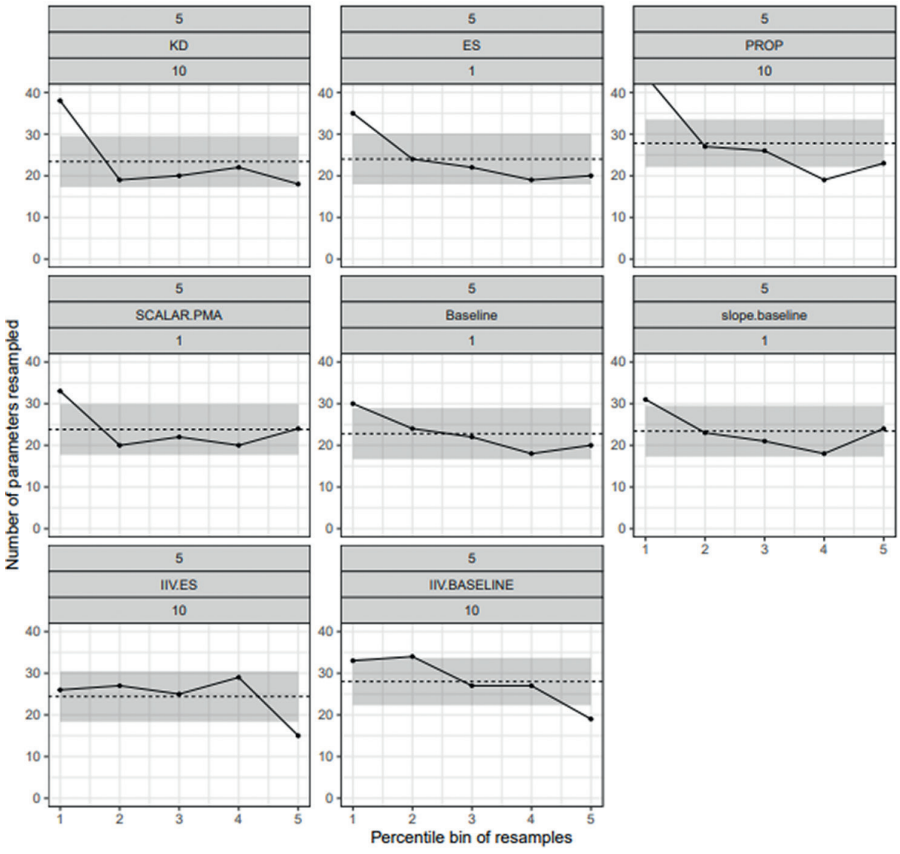


Supplementary Figure S1. Graphical model evaluation of the PK model by Marqués-Miñana *et al.* [19]

A: dOFV distributions



B: Temporal trends of 5th iteration



Supplementary Figure S2. SIR results.

Chapter 6

Benzylpenicillin concentrations in umbilical cord blood and plasma of premature neonates following intrapartum doses for group B streptococcal prophylaxis

Amadou Samb, Thomas H. Dierikx, Yuma A. Bijleveld, Timo R. de Haan, Caspar J. Hodiamont, Elisabeth van Leeuwen, Anton H. L. C. van Kaam, Ron A. A. Mathôt, Douwe H. Visser

Matern Health Neonatol Perinatol. 2023 Jul 1;9(1):9.

Abstract

Background & method

Dutch obstetrics guideline suggest an initial maternal benzylpenicillin dose of 2,000,000 IU followed by 1,000,000 IU every 4 hours for group-B-streptococci (GBS) prophylaxis. The objective of this study was to evaluate whether concentrations of benzylpenicillin reached concentrations above the minimal inhibitory concentrations (MIC) in umbilical cord blood (UCB) and neonatal plasma following the Dutch guideline.

Results

Forty-six neonates were included. A total of 46 UCB samples and 18 neonatal plasma samples were available for analysis. Nineteen neonates had mothers that received intrapartum benzylpenicillin. Benzylpenicillin in UCB corresponded to concentrations in plasma drawn directly postpartum ($R2=0.88$, $p<0.01$). A log-linear regression suggested that benzylpenicillin concentrations in neonates remained above the MIC threshold 0.125 mg/L up to 13.0 hours after the last intrapartum dose.

Conclusions

Dutch intrapartum benzylpenicillin doses result in neonatal concentrations above the MIC of GBS.

Background

Early-onset neonatal sepsis (EOS) is a severe infectious disease with signs of bacteremia resulting from vertical transmission of pathogens originating in the maternal genital tract or amniotic fluid [1]. EOS is contracted before or during labor and typically presents with signs and symptoms within the first 72 hours of life. It is associated with both a high neonatal mortality and morbidity [2]. Group B streptococcus (GBS), *Streptococcus agalactiae*, is one of the leading pathogens for EOS and 10-30% of pregnant women carry GBS in the genital tract, allowing for vertical transmission during delivery [3].

In the last 30 years the incidence of GBS positive EOS has declined drastically from 1.7 to 0.4 cases per 1,000 live births due to the implementation of widespread and systematic screening for maternal risk factors for EOS, such as GBS colonization and/or chorioamnionitis. Some countries have implemented a screen all strategy around 35-37 weeks' gestation, and give prophylaxis to all screen positives while other countries follow a risk based strategy, where screening only takes place for instance in preterm delivery or prolonged rupture of membranes. In the Netherlands, if risk factors are present mothers are treated with targeted intrapartum antibiotic prophylaxis until delivery [3,4]. Maternal intravenous benzylpenicillin prophylaxis within at least 4 hours before delivery is considered adequate for the prevention of EOS. Benzylpenicillin concentrations assessed in fetal serum and amniotic fluid typically exceed the minimal inhibitory concentrations (MIC) of GBS at delivery [3,5].

Several studies have evaluated benzylpenicillin concentrations in umbilical cord blood (UCB) and fetal blood in term pregnancies with maternal benzylpenicillin loading doses of 5,000,000 IU, followed by 2,500,000 IU every 4 hours intrapartum [4–6]. However, the current Dutch obstetric guideline advices lower doses, i.e. a loading dose of 2,000,000 IU followed by 1,000,000 IU every 4 hours [7], but evidence for the latter dose is lacking and it is unknown whether this results in adequate benzylpenicillin concentrations in the neonate. Furthermore, the magnitude of fetal exposure to benzylpenicillin following intrapartum prophylaxis before 37 weeks' gestation is unknown. This evidence is needed because trimester-dependent changes in maternal body composition, placental function and fetal organ maturation may result in different pharmacokinetic (PK) properties [8].

The primary aim of this study was to assess whether maternal intrapartum intravenously administered benzylpenicillin at doses according to the current Dutch obstetric guideline is sufficient to produce adequate therapeutic benzylpenicillin concentrations in neonates. As a secondary objective, additional intrapartum antibiotic (amoxicillin, cefazolin and gentamicin) concentrations in UCB were investigated.

Methods

Study design and population

Neonatal plasma and UCB samples collected as part of a previous multicenter prospective observational cohort study were used (The Diagnostic Accuracy of Presepsin in Early-Onset Neonatal Sepsis: a Prospective Cohort Study, submitted). That study was conducted between August 2018 and June 2021. Neonates admitted to the neonatology ward of the Amsterdam UMC (Amsterdam, the Netherlands) or OLVG East and West (Amsterdam, the Netherlands), undergoing sepsis work up and receiving antibiotics according to the Dutch early onset neonatal sepsis guideline [9] were eligible for inclusion. Infants were excluded in case of a confirmed congenital infection (toxoplasmosis, rubella, cytomegalovirus infection, syphilis and herpes). For the current study documented maternal intrapartum antibiotic treatment was an additional entry criterion for inclusion and analysis.

The original study, as well as the amendment for the current study was approved by the medical ethics committee (WO 18.020). Written and informed consent was required from both parents or legal guardians for study participation and use of blood samples and clinical data.

Data collection

Immediately after delivery, 1 mL UCB was collected in EDTA tubes, which was assumed to represent fetal blood. If possible, 0.2 mL neonatal plasma was drawn during initial sepsis work up directly postpartum before initiation of neonatal antibiotic treatment. All samples were centrifuged at 2,000 x g within 4 hours after collection and the plasma was stored at -80 °C until analysis.

Benzylpenicillin, amoxicillin, gentamicin and cefazolin concentrations were measured in UCB and plasma samples using tandem liquid chromatography-mass spectrometry (LC-MS/MS). The accuracy was acceptable at all quality control (QC) levels (acceptance for QClow: 80% - 120%; QCmiddle & QChigh: 85% - 115%) for benzylpenicillin, amoxicillin, gentamicin and cefazolin with ranges between 98.3% - 101.1%, 97.5% - 99.3%, 96.6% - 118% and 96.3% - 110.0%, respectively. Within-day- and between-day imprecision were below the acceptance criteria of 15%.

Additional clinical, demographic and anthropomorphic data were collected from the digital medical patient files (EPIC, Verona, Wisconsin, USA). The following maternal data were collected: benzylpenicillin, cefazolin, gentamicin and amoxicillin maintenance dose (2,000 mg, 5 mg/kg and 1,000 mg respectively), time of dose, weight, length, presence of chorioamnionitis, gestational age at delivery, concomitant medication, GBS colonization status, multiple pregnancy, gravidity and parity. Neonatal data collected were: Time of dose, concomitant medication, birth weight, gestational age, sex, presence of perinatal asphyxia.

Statistical analysis

Descriptive statistics, data handling and data visualization were performed with R [10]. If the measured UCB concentrations of the antibiotics were not normally distributed, median concentrations and interquartile range (IQR) were reported. Correlation between benzylpenicillin in UCB and neonatal plasma was tested to assess interchangeability. Tests of correlation as well as log-linear regression methods were performed to relate intrapartum benzylpenicillin dosing times to UCB benzylpenicillin concentrations. This relationship was then used to evaluate MIC target attainment. The target MIC used for benzylpenicillin was 0.125 mg/L, which is the highest MIC in the wild type distribution and the clinical breakpoint of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) for GBS meningitis [11,12]. For cefazolin and amoxicillin the EUCAST clinical breakpoint MICs for *E. coli* and GBS, two frequently isolated pathogens in EOS, were used. If no clinical breakpoint was defined by EUCAST, the highest MIC in the wild type distribution was used as target MIC [11]. For *E. coli*, the target MICs used for cefazolin and amoxicillin were 4 mg/L and 8 mg/L, respectively [12]. For GBS, the target MIC of amoxicillin was 0.125 mg/L, which is the highest MIC in the wild type distribution [11] For GBS, there is no clinical breakpoint for cefazolin, nor is there a known wild type distribution in the EUCAST database of antimicrobial wild type distributions. No target MICs for gentamicin were included, since gentamicin effect is dependent on maximum concentrations (C_{max}), which is not represented by the measured drug concentrations. The two-tailed level of acceptance for the rejection of the null-hypotheses was $p \leq 0.05$.

Results

A total of 333 neonates were included in the study between July 30th 2018 and July 23rd 2021 of whom 46 mothers were treated with intrapartum antibiotics and UCB material was available. Nineteen of these mothers received intrapartum EOS prophylaxis with benzylpenicillin in a dose of 2,000,000 IU followed by 1,000,000 IU every 4 hours. The demographic characteristics of the included infants and their mothers have been depicted in **Table 1**. The median gestational age for the benzylpenicillin group was 29.3 weeks. All infants were premature, except for one (GA=42 weeks). The median maternal age during delivery was 30.2 years. Nine neonatal plasma samples drawn directly postpartum were available for comparison to UCB benzylpenicillin concentrations (**Table 1**).

The median concentration of benzylpenicillin in UCB was 1.69 (0.90 – 2.94) mg/L. Median concentrations in UCB and neonatal plasma at birth were similar (**Figure 1**) and there was a strong correlation between benzylpenicillin concentrations in UCB and neonatal plasma at birth ($R^2 = 0.88$, $p < 0.01$), indicating that UCB concentrations could be interpreted as fetal or neonatal concentrations. As expected, benzylpenicillin concentrations in UCB were dependent on the time after last intrapartum administration (**Figure 2**). A clear decrease in UCB benzylpenicillin concentration is present. All but one observation were above the MIC of 0.125 mg/L for GBS, well past the 4 hour dose interval. There was a significant log-linear relation ($p < 0.01$) between benzylpenicillin concentration and time after dose, indicating a moderate correlation ($R^2 = 0.59$, **Figure 2**).

Table 1. Basic charactersitics of the study population stratified for benzylpenicillin and other antibiotics.

Characteristic			
Intrapartum antibiotic		**Benzylpenicillin**	**Other antibiotics**
Total patients - n		19	27
percentage female infants - %		52.6	56.7
maternal age (years) - median (IQR)		30.2 (28.0 - 33.0)	32.5 (29.0 - 35.0)
mode of delivery			
	vaginal	18	9
	caesarian	1	18
membrane rupture duration (hours) - median (IQR)		3.5 (0 - 47.3)	12.5 (0 - 74.8)
Maternal GBS colonization - n			
	positive	6	4
	negative	13	10
	unknown	-	13
multiparous pregnancies - n		4	3
Gestational age (weeks) - median (IQR)		29.3 (27.1 - 30.8)	31.0 (28.2 - 36.0)
Infant birth weight (kg) - median (IQR)		1.15 (0.93 - 1.67)	1.52 (1.19 - 2.56)
Intrapartum antibiotic - n			
	amoxicillin	-	14
	gentamicin	-	3
	cefazolin	-	13
umbilical cord blood samples - n		19	27
neonatal blood antibiotic concentrations - n		145	

Based on the linear regression line, benzylpenicillin concentrations in UCB were above the MIC threshold of 0.125 mg/L for 13.0 hours after the last intrapartum dose. When also taking the 95% confidence interval of the regression line into account, it seems more likely that concentrations reach subtherapeutic concentrations after 10 hours. The slope of the regression line can be interpreted as an elimination constant k_e, thus the elimination half-life can be calculated as $t_{\frac{1}{2}} = \frac{\log(2)}{0.1176} = 2.6\,hours$. No covariate effect of GA on the relation between time after last intrapartum dose and benzylpenicillin concentration was found when included as an additional independent variable in the log-linear regression, since the slope of GA was not significant (p=0.7).

The concentrations of amoxicillin, gentamicin and cefazolin were also measured in UCB. For amoxicillin the median concentration (n= 14) was 4.61 (2.53 - 6.32) mg/L, well above the GBS MIC threshold of 0.125 mg/L, though below the *E. coli* MIC of 8 mg/L. Cefazolin concentrations (n=13) were also above the *E. coli* MIC threshold of 4 mg/L, with a median concentration of 17.5 (6.4 - 23.2) mg/L. Only 3 gentamicin concentrations were measured (below the lower limit of quantification, 1.33 mg/L and 3.50 mg/L).

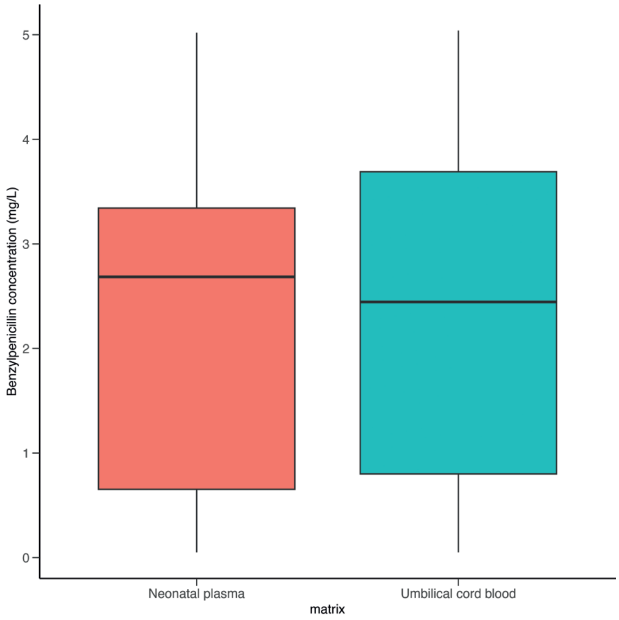


Figure 1. Boxplots of benzylpenicillin concentrations (mg/L) in umbilical cord blood (UCB) and neonatal plasma at birth (N=9). The left boxplot depicts neonatal plasma concentrations. The right boxplot depicts UCB concentrations.

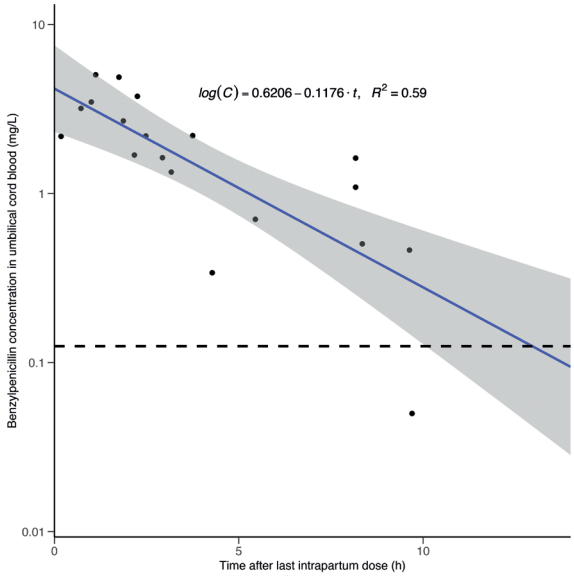


Figure 2. Benzylpenicillin concentrations in umbilical cord blood (mg/L) versus time after last intrapartum dose (hours). Points are measured concentrations. The blue line depicts the log-linear regression of the benzylpenicillin concentrations versus time after last intrapartum dose. The shaded area displays the 95% confidence interval of the regression line. The dashed horizontal line depicts the MIC threshold of 0.125 mg/L.

Discussion

UCB concentrations of benzylpenicillin, which is frequently used for prophylaxis in GBS-positive pregnancies, were above the suggested MIC threshold of 0.125 mg/L [12]. Although the internationally suggested dose of 5,000,000 IU followed by 2,500,000 IU every 4 hours results in satisfactory fetal exposure [4,5], this also seems the case for the lower doses of 2,000,000 IU, followed by 1,000,000 IU every 4 hours, as suggested by the Dutch obstetric guideline [7]. As deducted by linear regression, benzylpenicillin concentrations in UCB remained above the MIC threshold of 0.125 mg/L up to 13.0 hours after the last intrapartum dose. No relationship between GA and benzylpenicillin concentrations in UCB was found, though it is likely that data were insufficient for the detection of such a relationship. Furthermore, 18 of the 19 neonates that were exposed to intrapartum benzylpenicillin were premature with a GA range of 25 – 32 weeks, and the single term neonate had could be potentially seen as an outlier, with a GA of 42. Though it was evaluated whether exclusion of the patient of 42 weeks impacted the concentration-time profile, the slope of the log-linear regression did not change by a large margin. Moreover, exclusion of the patient of 42 weeks is unjustified, since it is part of the true population for GBS prophylaxis. Nonetheless these results mostly apply to the specific age group of premature neonates of GA 25 - 32 weeks. Thisis acceptable, since prematurity is a major risk factor that is considered for GBS prophylaxis. Benzylpenicillin $T_{1/2}$ in UCB was 2.6 hours, which is shorter than the reported $T_{1/2}$ in term and preterm neonates (approximately 3.5 hours) [13]. This could be the result of a shared clearance mechanism with the mother, since benzylpenicillin $T_{1/2}$ is only 30 minutes in adults [14].

As the Dutch recommended benzylpenicillin dosing regimen results in therapeutic UCB concentrations, the international community may consider lower benzylpenicillin doses for intrapartum GBS prophylaxis. Benzylpenicillin is a time-dependent antimicrobial and the bactericidal effect is expressed as the fraction of time above the MIC (*f*T>MIC), rather than concentration dependent (i.e. C_{max}/MIC or AUC/MIC) [15]. Thus, no increased effect is expected for higher concentrations relative to the MIC. Whilst increasing concentrations may overcome drug resistance in some situations, specific alterations in drug target sites may be unaffected by increased concentrations [16]. Antibiotic stewardship through precise dosing to combat the propagation of antibiotic resistance should be considered, since this phenomenon is a growing concern in modern medicine. In addition, substantially lower doses per patient are more cost-efficient and could save money in the long term.

Also, it was investigated whether amoxicillin and cefazolin reached therapeutic concentrations in UCB. Most of these antibiotics too seem to exceed their corresponding MICs for GBS and/or *E.coli* [12] with the doses prescribed. The amoxicillin concentrations in UCB exceeded the MIC for GBS, however, were below the MIC for *E. coli* [12]. Since amoxicillin is prescribed as an alternative to benzylpenicillin for GBS prophylaxis in this context, the measured concentrations are satisfactory. For cefazolin, no target MIC for GBS could be defined [11,12]. Nonetheless, cefazolin concentrations exceeded the MIC for *E. coli*. Since intrapartum cefazolin is prescribed

for prophylaxis of maternal indications during caesarian sections rather than GBS prophylaxis, it seems that there is an added benefit of neonatal protection from *E. coli*. Gentamicin concentrations were not evaluated with respect to MIC, since gentamicin bactericidal effect is related to C_{max}/MIC and concentrations in UCB did not represent C_{max}. Gentamicin concentrations were above 1 mg/L in two of the three measurements in UCB. Such concentrations should be considered to prevent errors in therapeutic drug monitoring during subsequent gentamicin dosing of the neonate. It should be noted however that only 3 gentamicin concentrations were measured in UCB so any conclusions would be underpowered.

This is the first study analyzing the PK results of the Dutch obstetric benzylpenicillin protocol for GBS prophylaxis by assessing both UCB and neonatal plasma samples [7]. To our best knowledge, no evidence is publicly available describing therapeutic benzylpenicillin concentration target attainment in fetal/neonatal plasma following an intrapartum starting dose of 2,000,000 IU followed by 1,000,000 IU every 4h. The use of a the log-linear regression line enabled the extrapolation of benzylpenicillin concentrations in UCB with limited data.

Limitations to the study were the limited number of samples. This severely hampered the applicability of gold standard statistical approaches such as population PK analyses. In addition, the absence of maternal PK blood samples complicated the applicability of population PK modeling and though physiology-based pharmacokinetics (PBPK) was considered as a potential method to assess and simulate the PK of benzylpenicillin, this was complicated due to the absence of basic demographic maternal data, such as maternal weight.

Conclusions

Benzylpenicillin was detectable in therapeutic concentrations in UCB and equal to neonatal plasma concentrations. The current Dutch dosing protocol for benzylpenicillin dosing for intrapartum GBS prophylaxis is adequate even though it recommends lower doses as compared to international literature. A future study, combining maternal, UCB and neonatal plasma benzylpenicillin concentrations in a PBPK model should be conducted to provide a detailed assessment of prophylactic intrapartum benzylpenicillin treatment to prevent EOS. It may be beneficial to revise international benzylpenicillin dose recommendations in GBS prophylaxis to reduce unnecessary drug overexposure.

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General discussion and summaries

Chapter 7

General discussion and summaries

General discussion

Aminoglycosides and vancomycin have a high inter-individual variability (IIV) in pharmacokinetics (PK) and a narrow therapeutic index. Therefore, therapeutic drug monitoring (TDM) is a valuable tool for individualizing doses of these drugs to improve efficacy and prevent toxicity especially in critically ill patients [1]. In current medical practice, antibiotic concentrations are measured in plasma samples and evaluated using population PK models and Bayesian forecasting to ensure that doses are adequate to reach target concentrations that are efficacious and non-toxic. For instance, peak and trough plasma samples are collected for the monitoring of aminoglycosides and doses are adjusted after model informed evaluation of target attainment. TDM is currently state of the art medical practice in both adult and pediatric populations.

Whilst the practice of TDM is known to be beneficial [1], collection of plasma samples is performed by an invasive procedure that poses discomfort and risks to the (premature) neonate, the most fragile group of the pediatric population. Although the concrete risk and burden of TDM-related plasma samples is not defined in the landscape of NICU admission, it is generally accepted that any reduction in plasma sampling is beneficial to the infant [2]. Thus, a non-invasive TDM method for antibiotics using saliva samples could improve patient comfort, whilst maintaining adequate drug exposure and safety. However, since salivary volumes and salivary antibiotic concentrations are low, it is of importance that bioanalytical assays are precise, specific and require low volumes. Such a bioanalytical method, when developed, should be available in all hospitals admitting neonatal patients for widespread implementation. In addition, the validity of commonly used PK targets needs to be established and it could potentially be beneficial to develop disease specific biomarkers and PD outcomes during TDM. Current PK targets rely on the minimal inhibitory concentration (MIC), such as specified targets based on area under the curve (AUC) over MIC (AUC/MIC; i.e. vancomycin), maximum concentration (C_{max}) over MIC (C_{max}/MIC; e.g. aminoglycosides) or the time of the unbound fraction over the MIC (*f*T>MIC; e.g. β-lactams). The current clinically used biomarkers indicating adequate antibiotic treatment, such as a decrease in C-Reactive Protein (CRP) values or negative blood cultures, may not truly reflect therapeutic efficacy in clinical sepsis and cannot be used to evaluate a direct concentration-dependent response. Furthermore, an optimized sepsis prophylaxis strategy for pregnant women colonized with Group-B-Streptococci (GBS) could decrease the use of postnatal antibiotics and thus indirectly reduce the need for TDM during NICU admission and the related burden as a consequence. The results of the studies presented in this thesis are discussed below, elaborating upon the requirements for clinical implementation of saliva as a novel TDM medium and thus the reduction of clinical burden experienced by NICU admitted neonates due to antimicrobial therapy. Furthermore, a novel parameter for therapeutic efficacy during neonatal antibiotic treatment is discussed and the current antibiotic prophylactic dose schedule for GBS colonized pregnant women was evaluated.

Part I: Development of bioanalytical methods in plasma and saliva

A highly sensitive bioanalytical method is a prerequisite for saliva TDM, since salivary drug concentrations and sample volumes are low. Whilst a sensitive quantification method was available for gentamicin, no such method was available for amikacin and flucloxacillin. Therefore, a method for the simultaneous quantification of amikacin and flucloxacillin using tandem liquid chromatography coupled to mass spectrometry (LC-MS/MS) was developed, as described in **Chapter 2**.

For amikacin and flucloxacillin, accuracy and imprecision were in accordance with the acceptance criteria. Thus, we were able to accurately quantify both amikacin and flucloxacillin concentrations as low as 10 µg/l in plasma and saliva samples with volumes as low as 10 µl. Previous studies investigating aminoglycoside concentrations in samples using immunoassays did not detect any quantifiable aminoglycoside levels in saliva [3–5]. The LLOQs reported in those studies were between 0.18 – 2.0 mg/l and thus this newly developed method can detect amikacin and flucloxacillin levels that are at least 10-fold lower than previously published methods, at concentration ranges that are to be expected in neonatal saliva.

Flucloxacillin is highly protein bound and only the unbound fraction is pharmacologically active. Moreover, protein binding is relevant for the salivary distribution of drugs, since only the unbound fraction can freely pass the plasma/saliva barrier [6]. However, the unbound fraction of flucloxacillin could not be determined due to loss during the ultrafiltration process. This is problematic since plasma protein binding of flucloxacillin is high and highly variable in the neonatal population, varying between 34.3% and 89.7% dependent on gestational age (GA), postnatal age (PNA) and body weight [7,8]. As a result serious improvements in the ultrafiltration methods need to be realized before the proposed method can be applied in pharmacologically relevant studies for flucloxacillin.

The developed method could accurately quantify amikacin concentrations in plasma and saliva, at the µg/l range in samples of at least 10 µl. Though this method was used in **Chapter 4** and could potentially be used for TDM, not all hospital pharmacy laboratories have access to LC-MS/MS equipment. However all NICU's are located in academic hospitals that usually can perform LC-MS/MS analysis of drugs. The additional costs of LC-MS/MS analyses should ideally outweigh the cost of multiple plasma analyses procedures and e.g. the need for blood transfusions in these often critically ill neonates.

Part II: Pharmacokinetics and therapeutic drug monitoring of aminoglycosides

Pharmacokinetics of amikacin and gentamicin in neonates

PK of amikacin and gentamicin in neonates are known to be highly variable. Whilst starting doses are calculated based on body weight, GA and PNA, still large differences are observed in plasma drug concentrations due to considerable IIV in PK parameters such as clearance (CL)

and volume of distribution (V_d). V_d and CL of gentamicin have been found to range between 0.45 - 0.75 l/kg and 0.53 – 1.72 ml min^{-1} kg^{-1}, respectively [9]. Similarly, amikacin V_d and CL range from 0.34 – 0.72 l/kg and 0.36 - 1.71 ml min^{-1} kg^{-1}, respectively. Since oral bioavailability is negligible for both drugs, gentamicin and amikacin are commonly administered via intravenous infusion over 30 minutes. Both drugs exhibit a short distribution phase and an extended 1st – order elimination phase. Due to large population differences in body composition (i.e. extracellular water) and differences in organ maturation during the postnatal time period (i.e. kidney function), PK data from the adult and general pediatric population cannot simply be extrapolated to (premature) neonates. Therefore, specialized and targeted PK studies in neonates have been performed for many antibiotics [10–32]. Since usually sparse samples are available in these studies due to ethical considerations and clinical burden, nonlinear mixed-effects modeling (NONMEM) is frequently applied for population PK analysis in the neonatal population. In the context of PK analysis, this method allows for modeling of nonlinear relations between the dependent variables (drug or biomarker concentrations) model parameters (i.e. CL, V_d) and the independent variable (time). In addition, estimation of pharmacodynamic (PD) parameters such as maximum effect (E_{max}) and the concentration at which 50% of the maximum effect is achieved (EC_{50}) can be estimated for the development of an integrated PKPD model. Whereas fixed effects constitute average parameter estimates for typical patients, random effects are stochastic parameters that indicate IIV and residual variability. IIV parameters describe the deviation from the individual parameter value from the fixed parameter value. Residual variability, or residual error, quantifies the differences between observations and individual predictions by the model [33]. Thus, a mixed effects model includes both fixed- and random effects for a specific population. Inclusion of covariates in the model, such as weight, gestational age and creatinine CL is a crucial part of constructing a nonlinear mixed-effects model, explaining part of the variability.

Since the aminoglycoside antibiotic gentamicin is part of the first-line antibiotic treatment regimen for suspected and confirmed EOS in many NICU's and often subject to TDM, population PK of gentamicin in neonates have been extensively explored in the past [12–15,31,32,34]. Most recent models used two compartments to describe gentamicin PK in plasma [12,14,15,32]. Nonetheless, some investigators have described gentamicin PK in neonates with a three-compartment model [31,34]. It seems, however, that empirical two-compartment models are equally accurate in predicting peak- and trough concentrations when compared to more complex three-compartment models [35]. Thus, for the evaluation of gentamicin TDM performance a two-compartment model would suffice. Age, either defined as (a combination of) GA, PNA or postmenstrual age (PMA), was included as a covariate on CL in most models, except for the model by de Cock ***et al*** [14]. Current body weight or birth weight was generally included as a structural allometric scaling factor on all parameters [15,31,32,34]. The models by Fuchs ***et al***. and de Cock ***et al***. included weight as an estimated covariate effect on V_d and/or CL [12,14]. Whilst some models included serum creatinine as a covariate on CL [14,15,31], the validity of creatinine CL on PK models for LOS is doubtful since creatinine levels are generally assumed to be representative of the maternal levels during the first days of life [36]. Concomitant dopamine use was included as

a significant covariate on CL in the model by Fuchs *et al*., which can be supported by the inotropic effect of dopamine, which may increase renal perfusion [12]. Indeed, it was found that the model by Fuchs *et al*. could best describe the peak- and trough plasma concentrations observed in our study (**Chapter 3**).

As of yet no data existed investigating the PK relationship between plasma and saliva aminoglycoside levels in neonatal cases. Gentamicin concentrations were quantifiable in neonatal saliva samples due to the sensitivity of our LC-MS/MS method (**Chapter 3**). When investigating the relationship between gentamicin concentrations in plasma and saliva, we expanded the plasma model by Fuchs *et al*. with a single saliva compartment with both first-order absorption and elimination. The absorption rate decreased with the rise of postmenstrual age, whereas elimination rates were more or less stable. Thus, gentamicin concentrations in saliva were much higher in premature neonates compared to term neonates. Most likely this is the result of the maturation status of the salivary glands and the blood saliva gland barrier, which are both immature for premature neonates and therefore more permeable for paracellular transport [37]. The higher salivary concentrations found in premature neonates could indicate that TDM using saliva is more accurate for this population that would, coincidentally, also benefit most from a non-invasive TDM strategy as premature neonates are generally more vulnerable than term neonates and more frequently suffer episodes of sepsis. In the integrated plasma/saliva PK model it was assumed that gentamicin transport to saliva did not impact the plasma kinetics, since the amount of gentamicin transported from plasma to saliva was negligible. The proportional error of the saliva model was 49.7%, which is unfortunate since it is associated with less accurate parameter estimations whilst using empirical Bayesian estimation (EBE). Accuracy of parameter estimations could be improved through the collection of more samples when performing TDM, which is feasible due to the non-invasive nature of salvia sampling.

Though some plasma PK models for amikacin have been published, model accuracy seems suboptimal for premature neonates since the models have generally been developed for dose finding purposes rather than TDM [16–19]. In general, there seems to be consensus between the models that weight is a covariate on CL and V_d. Age seems a predictor for CL, though the models by de Cock *et al*. and Illamola *et al*. only included PNA and no effect of GA was included in the models [17,18]. Concomitant NSAID therapy was included as a covariate on CL in the model by Allegaert *et al* [16]. Similarly, the model by de Cock *et al*. described ibuprofen use as a CL covariate [17]. When fitting the study data, the model by de Cock *et al*. could best describe the observed concentrations. There were some major caveats to the model however, since only PNA was included as covariate and there was no effect of GA included in the model. Furthermore there was no IIV in central V_d, which is an important parameter for peak concentration prediction. Regardless, the model by de Cock *et al*. performed best with the study data, possibly due to the large proportional residual error of 50% that was present in the model by Illamola *et al*.

Amikacin concentrations were quantifiable in all samples due to our highly sensitive LC-MS/MS method (**Chapter 2**), which is an interesting finding since earlier studies could not detect amikacin in saliva due to a less sensitive biochemical analysis method [4]. Using the plasma PK model by de Cock *et al*. as a basis, an additional compartment was appended for the description of the salivary amikacin concentrations (**Chapter 4**). Following a similar model structure as for gentamicin, a single saliva compartment was used to describe salivary amikacin concentrations. The salivary absorption rate constant was found to decrease with increasing PMA. A high proportional error of 58.3% was estimated, again indicating that a higher number of samples are required for salivary TDM.

Whilst the salivary PK models for gentamicin and amikacin both were developed from the ground up, testing multiple structural models and covariates, the final models demonstrated many similarities. The overall model structure was the same for both models and k_{30} greatly exceeded k_{13} indicating absorption dependent kinetics. Moreover, PMA was a strong covariate in both the gentamicin and amikacin models, indicating that the drugs are more freely distributed in the saliva of premature neonates. As mentioned earlier, premature neonates are the most fragile patients and have the lowest total blood volume and thus would benefit most from a non-invasive TDM strategy using saliva samples.

Salivary TDM of gentamicin and amikacin

To our best knowledge, we are the first to demonstrate that salivary TDM of aminoglycosides is feasible for neonates. Earlier studies were unable to quantify aminoglycosides in the saliva of children, including neonates, and had the assumption of constant saliva to plasma ratio [4,5,38]. However, we found that the saliva to plasma ratio is variable over time and there is a delay in C_{max} in saliva compared to the C_{max} in plasma. Thus a model-based approach is of added value in the context of salivary TDM of aminoglycosides in saliva.

As reported in **Chapter 3**, the simulated probability of target attainment (pTA) for gentamicin following TDM with saliva sampling regimens was higher compared to fixed weight based dose without TDM (48%) and dose regimens based on *a priori* tailored dosing without TDM (72%). However, the difference in pTA between a single saliva sample versus *a priori* predictions seems minimal and may be a result of variability (75% versus 72%, respectively). Nonetheless, by increasing the sampling frequency a maximum pTA of 81% can be achieved with 4 saliva samples, after which pTA no longer increases with additional samples. Given the large proportional error of 49.7% in the saliva model, more samples are required for accurate EBEs. However, the fictional cohort represented both premature and term neonates. As was seen in the measured samples and the final PK model, salivary gentamicin concentrations are much lower in older neonates. The benefit of plasma TDM of gentamicin was evident, resulting in a pTA of 87% for a single mid interval sample and 94% for a peak- and trough sampling regimen, which is clinical practice. This finding can further be supported by the lower proportional error in the plasma model of 18% [12]. It should be reported that the calculated pTAs are not representative for real life situations, since demographic population distributions and variability in

dosing times were simplified in the fictional cohort. Furthermore, a maximum pTA of 99% (6 plasma samples) is unlikely, in part due to inter-occasion variability that was not accounted for in the model. Nonetheless, the calculated pTAs are of much comparative value and it seems that TDM with 4 saliva samples is feasible for gentamicin using our PK developed model, albeit less accurate than the current practice of peak- and trough plasma TDM (81% versus 94% respectively).

Target attainment for the amikacin model was also evaluated in a simulated cohort (**Chapter 4**). During sample simulation an IIV in V_c of 10% was empirically included in the plasma model since the original model did not include any variability in V_c, which is unrealistic for TDM simulation [17]. The pTA for the fixed weight based dose regimen without TDM was 73.1% and pTA was higher for all saliva TDM regimens, reaching a maximum of 79.2% for 5 samples. Doses that were adjusted *a priori* demonstrated a pTA of 77.6% and was equal to the pTA for a single saliva sample. Plasma TDM increased the pTA to 79.9% for a single sample regime and 80.3% when using a peak- and trough sample. It is of particular interest that the pTA was already high for the fixed dose regimen and the *a priori* regimen, whereas only minor increases in pTA were observed for both saliva (5 samples; 79.2%) and plasma TDM (2 samples; 80.3%). This could be due to the severe limitations in the plasma model, since no IIV in V_c was included. This resulted in the fact that most simulated peak concentrations were within target ranges leading to an overestimation of pTA compared to the real life setting. One could argue that both saliva sampling and plasma sampling are not clinically relevant compared to *a priori* based dosing. However, it has been demonstrated that TDM of amikacin is beneficial for the attainment of concentrations targets [16]. Since all available plasma PK models for amikacin in neonates are flawed, no representative profiles could be simulated [16–19]. Therefore it is of upmost importance that more accurate plasma PK models for amikacin are developed. This is beneficial for the quality of plasma TDM, as well as saliva TDM since an accurate plasma component is required for an adequate saliva model. For instance, a PK model with a small residual error and IIV on both CL and V_c is likely to better represent the true PK profiles. Although the PK model by Sherwin *et al*. fulfills those criteria, it could not accurately describe the observed concentrations [19]. Although it is of utmost importance that accurate POP-PK models are used during Monte Carlo simulations for a reliable representation of the accuracy of TDM, it was found that the simulated saliva TDM performance was similar to plasma TDM, with pTAs of respectively 79.2% versus 80.3%.

Regardless of the limitations in the simulation study, it seems that saliva TDM is feasible for gentamicin and amikacin, though some hurdles have to be overcome for clinical implementation (**Chapter 7**, *future perspectives).* Target attainment for gentamicin and amikacin was optimal with 4 and 5 saliva samples, respectively. Due to the non-invasive nature of saliva TDM, collection of 4 or 5 saliva samples does not increase clinical burden. Nonetheless, there was high residual variability in both developed saliva PK models, possibly due to differences in salivary pH, enteral feeding times and the absence of a standardized sampling method [2]. Furthermore, it seems that salivary PK can be more accurately described in premature

neonates, since salivary concentrations are much higher than in term neonates. The results discussed in this thesis emphasize the need for a study investigating the real-life performance of saliva TDM using improved models in a premature population, rather than using simulation-based pTAs.

Part III: Pharmacodynamic aspects of antimicrobial therapy in neonatal sepsis

Antimicrobial effect of vancomycin on coagulase-negative staphylococci

Currently antibiotic doses are determined based on pre-specified PK targets related to the causative micro-organism's MIC. The MIC is a pathogen-specific antibiotic concentration at which no bacterial growth is observed in a serial dilution test. Whilst it is theoretically possible to determine MICs ad hoc, this process is time-consuming and generally breakpoints recorded in databases such as EUCAST are used [39].

Therapeutic effect during treatment of clinical sepsis is often evaluated by subjective assessments of clinical recovery, decrease in inflammatory parameters (e.g. CRP) or negative follow-up blood cultures. In current clinical practice, trough plasma samples of vancomycin are used for TDM evaluating the target attainment of an AUC/MIC ≥ 400 (assuming an MIC of 1 mg/l) and adjusting doses accordingly [40]. Since MICs are variable between bacterial strains and patients, insight in the direct pharmacological responses would be beneficial for tailor-made dosing regimens and monitoring [41]. No such direct or measurable clinical PD responses to antibiotic concentrations over time are currently available.

The antimicrobial effect of vancomycin on Coagulase-negative staphylococci (CoNS) was investigated in **Chapter 5**, using the bacterial DNA loads (BDL) in blood as a direct biomarker of therapy efficacy. A real-time quantitative polymerase chain reaction (RT-qPCR) method was developed and validated for the simultaneous identification and quantification of common pathogens related to LOS, including CoNS [42,43]. This method allows for the expression of BDL in units equal to CFU/ml (CFU eq/ml) and requires blood volumes that are much lower than the blood volumes required for a traditional blood culture. As our data demonstrated, a decrease in CFU eq/ml corresponded to microbial decay and thus antibiotic plasma concentrations could be related to CFU eq/ml for the quantification of antimicrobial effect. CoNS infections represent between 53.2%–77.9% of all cases of culture proven LOS in developed countries [44]. Given that vancomycin is the antibiotic of choice for the treatment of CoNS-positive LOS, quantification of the PKPD relation between vancomycin concentration and CFU eq/ml could be highly beneficial to optimize treatment of LOS.

A one-compartment PK model was used to describe the vancomycin concentrations in the study population [27]. An empirical PKPD model was estimated, relating vancomycin concentrations to BDL, estimating a 1st-order bacterial decay (k_{death}), BDL before vancomycin treatment and a linear concentration dependent bactericidal effect. Ideally, natural bacterial growth (k_{growth})

and k_{death} should be estimated as independent parameters with IIV in both rate constants [41,45]. However, the study data was too sparse and BDL data in patients without vancomycin treatment were not collected due to ethical considerations. Thus, bacterial growth was expressed as a function of bacterial decay and BDL at t = 0 hours. Since vancomycin is a bactericidal drug rather than bacteriostatic, the influence of vancomycin was modeled as a stimulatory effect on k_{death}. The effect of vancomycin concentration on k_{death} was estimated using a linear effect model. Whilst a sigmoidal E_{max} model would be a more accurate representation of the underlying mechanics, the data were too sparse to estimate that many parameters. Postmenstrual age positively affected the bactericidal effect of vancomycin in the model, indicating that bacterial killing was enhanced for older neonates at equal antibiotic concentrations. An explanation for this phenomenon might be that the increased effect for older neonates is the combined result of the antibiotic and an enhanced immune activity due to maturation of the innate and adaptive immune system [46].

We were able to quantify the PKPD of vancomycin in the clinical setting, though some major assumptions and abstractions were made. If further explored in a subsequent study, novel concentration targets independent of MIC should be defined for TDM of vancomycin. For example, vancomycin PK outcomes associated with maximum BDL decrease could be used as targets during TDM. Furthermore, routinely determined BDLs in conjunction with vancomycin concentrations could be evaluated during TDM using an integrated PKPD model for dose optimization. Arguably the best approach is the development of a semi-mechanistic PKPD model, though the study design and data density did not support such a model. Semi-mechanistic PKPD models describe the natural growth and decay of bacteria in absence of antibiotic concentrations and assess the effect of drugs on the overall bacterial killing [41]. Moreover, semi-mechanistic PKPD models can be used to describe the effect of the immune system and antimicrobial resistance mechanisms and therefore provide a mechanistically accurate representation of antimicrobial pharmacodynamics. Notwithstanding, our model demonstrated that the bactericidal effect of vancomycin can be expressed as a dynamic process. This is in contrast with current MIC based efficacy measures that indicate a binary response based on a single threshold value. Furthermore, the patients that demonstrated no decrease in BDL were identifiable as early as 8 hours after the first vancomycin dose and this corresponded with clinical examinations and symptoms of non-response. RT-qPCR is a well-known technique that is applicable and feasible in clinical routine.

For patients that did not respond to vancomycin therapy and had an increasing BDL had primary infections of the central venous line (CVL) that were retained during therapy. Whilst CVL retention was found not to interfere with vancomycin efficacy in 70% of cases, there is clinical consensus that line removal is beneficial in cases of persistent sepsis [47,48]. Erratic BDL profiles potentially resulted from a diagnosed infected thrombus and a persistent abscess. Infected thrombi have been implied as a risk factor for recurrent staphylococcus aureus infection [49,50]. From a mechanistic point of view, it could be that the irregularities in BDL were a consequence of the release of infected debris of the thrombus in the blood stream due to

shearing stress, increasing the BDL at irregular intervals. The same mechanism could be at play for infected pustules, though no such data is available. For patients in which BDLs were below the quantification limit before vancomycin therapy was initiated, it was found that the bacteria were sensitive to amikacin, which was used as a first line antibiotic treatment regimen before blood culture based vancomycin was started. This finding further supported the notion that BDL could be a useful biomarker for infection state.

Efficacy measures for vertically transmitted antibiotics: pregnancy, GBS and prophylaxis

Whilst antimicrobial treatment for EOS is frequently applied in the NICU setting, it is current practice to prophylactically treat pregnant women that present risk factors for neonates with EOS. Optimized prophylaxis of EOS can decrease both the incidence and disease severity in neonates. As a result, intrapartum EOS prophylaxis in pregnant women will also decrease the burden of antimicrobial therapy during the NICU admission of their child.

Intrapartum antibiotics are indicated for GBS prophylaxis. GBS are a major pathogen in EOS and are carried in the genital tract of 10-30% of healthy pregnant women [51]. Following the development of international guidelines for intrapartum GBS prophylaxis in the 1990s, the incidence of GBS-positive EOS was decreased with 80% worldwide [52–55]. Current international guidelines advise 2,500,000 IU intrapartum benzylpenicillin every 4 hours following a loading dose of 5,000,000 IU [52,56,57]. However, the Dutch obstetric dosing guidelines advise a lower dose of 1,000,000 IU intrapartum benzylpenicillin every 4 hours after an intrapartum loading dose of 2,000,000 IU without evidence of efficacy [58]. Amoxicillin or ampicillin can be used for GBS prophylaxis as an alternative to benzylpenicillin [58]. Another reason for prophylactic antibiotic treatment in pregnant women is to prevent maternal infection following caesarian section. In these cases 2,000 mg cefazolin intravenously is advised at the start of the operative procedure. In addition, a clinically proven chorioamnionitis or clinical intra uterine infection is often treated with 5 mg/kg gentamicin intravenously to prevent maternal / fetal complications and to treat the current infection.

It is of clinical importance to know how effective this maternal antibiotic prophylactic treatment is in protecting the fetus for serious infection. Thus we investigated whether the administered doses of intrapartum antibiotics attained efficacious levels in neonatal plasma and umbilical cord blood (UCB) in **Chapter 6**. Most importantly, intrapartum prophylactic benzylpenicillin doses in the Netherlands are less than 50% of internationally advised doses and it was evaluated whether these lower doses resulted in sufficient antimicrobial coverage. For this purpose benzylpenicillin, amoxicillin, cefazolin and gentamicin levels were analyzed in residual material from an earlier study [59].

Benzylpenicillin concentrations in UCB were equal to the concentrations observed in neonatal plasma before sepsis treatment and therefore UCB concentrations were interpreted as adequate. The decline of the pooled UCB concentrations could be described with an exponential function

yielding an elimination half-life ($T_{1/2}$) of 2.6 hours. Though $T_{1/2}$ of benzylpenicillin in neonates is approximately 3.5 hours, CL could have been enhanced due to the maternal contribution of benzylpenicillin CL [60,61]. Benzylpenicillin $T_{1/2}$ in adults is approximately 0.5 hours, and since fetuses and mothers have a shared CL, it is likely that the apparent fetal CL is higher than neonatal CL. Neonatal benzylpenicillin levels were above the GBS MIC of 0.125 mg/l up to 13.0 hours after the last intrapartum dose. Since plasma protein binding of benzylpenicillin is less than 40% [62], it seems highly plausible that the unbound fraction was above the MIC for GBS when adhering to the current 4 hour dosing interval, given that the median benzylpenicillin concentration in UCB was 2.2 mg/l [63]. We believe that the pharmacological efficacy of the Dutch obstetric benzylpenicillin dosing guidelines for GBS prophylaxis is adequate based on these results. Ideally a PBPK model should be used for quantification of maternal-fetal drug exposure, however, no maternal weights were recorded in the original study nor were these reported in the patient records. Body weight is a crucial determinant for the calculation of organ sizes and body fluid volumes in such models [64]. Furthermore, a standard PK model could not be developed, since no maternal blood samples were available for further analysis. It is of global interest whether the doses suggested by international guidelines are unnecessarily high and can possibly be lowered. Cefazolin, amoxicillin and gentamicin all reached clinically relevant levels in UCB. Cefazolin achieved concentrations above the MIC for *E. coli* in UCB, another major pathogen in EOS. However, cefazolin is prescribed for prophylaxis of maternal complications rather than neonatal complications. For amoxicillin, concentrations above the MIC for GBS and E.coli were achieved in all UCB samples. Gentamicin reached concentrations in UCB that potentially could interfere with TDM of subsequent neonatal doses, though only 3 samples from patients with intrapartum gentamicin therapy were available for analysis.

Future perspectives

TDM of gentamicin and amikacin using saliva samples

The results presented in this thesis provide a springboard for the potential renaissance of abandoned scientific- and clinical hypotheses on salivary TDM. Salivary TDM in pediatrics was of particular interest during the 1970s and 1980s. Whilst salivary TDM of aminoglycosides was further investigated in the 1990s and the early 2000s, it was generally assumed that aminoglycosides were not distributed into saliva [3,5,38,65,66]. However, the laboratory methods used during those studies are now outdated and proven insensitive. For instance, immunofluorescence assays were used with quantification limits that were above the aminoglycoside concentrations typically encountered in saliva of newborns [3–5]. Furthermore, earlier studies generally investigated linear correlations between aminoglycoside concentrations in plasma and saliva samples in trough samples, which is an implausible assumption since the ratio between aminoglycoside concentrations between plasma and saliva is not constant. Though feasibility of salivary TDM of gentamicin and amikacin was proven in this thesis, large and preferably multicenter studies are required for further model improvement, external model validation, sampling standardization and real-life TDM evaluation.

First, improved plasma PK models are required. The publicly available plasma and serum models are inadequate (**Chapter 4**) and since it serves as a foundation for the saliva PK analyses, the plasma model needs to be accurate. A large multicenter cohort study in which multiple plasma and saliva samples are collected from patients treated with amikacin and gentamicin should provide the data for the development of an accurate PK model for amikacin in saliva. Many unexplored covariates remain due to our small sample sizes and therefore the residual error in both models was very high (**Chapter 3** & **Chapter 4**). For instance, the influence of pH on saliva concentration distribution needs to be addressed, as it affects the protonation and thus the charge of basic drugs [6]. With pKa's of 10.12 and 9.79 for gentamicin and amikacin, respectively, both drugs are protonated and thus positively charged at physiological pH [67,68]. However, variability in salivary pH between individuals and occasions may consequentially impact the protonated fraction and salivary distribution. Since aminoglycosides appear to be more readily available in the saliva of premature infants of a lower gestational age, subsequent studies should focus on feasibility and applicability in prematurely born infants. External validation of the model with an external dataset should be performed as external validation of PK models is essential/important for model optimization [69]. Potentially PBPK modeling could support the use of saliva samples for TDM. PBPK simulates drugs concentrations in different tissues using physicochemical properties of drugs and physiological- and anthropomorphic data [64]. This allows for inclusion of mechanisms that have not been assessed in our study (**Chapter 5**). Evaluation of the collected saliva concentrations with the simulated PK profiles would provide further insight in to the influence of processes that impact PK that are not included in traditional PK models.

Second, no standardized method for saliva sampling currently exists [2]. Like the studies discussed in this thesis (**Chapter 3** & **Chapter 4**), saliva sampling is generally performed using the SalivaBio infant Swab, though vacuum aspiration and portable suction have been reported as well [70]. Whilst vacuum aspiration and portable suction seem to yield higher sample volumes, the techniques take 5 to 10 minutes compared to 90 seconds and would be more burdening to neonates and cumbersome to perform in the clinical setting. To promote generalizability and clinical implementation, an optimal sampling method needs to be investigated and validated. Training of nursing staff on saliva sampling could substantially decrease any inaccuracies and residual error in the measured concentrations and PK models, respectively. Furthermore, sampling times should be coordinated with clinical routine, i.e. before enteral feeding, rather than after enteral feeding. Also the use and need for citric acid for the stimulation of salivation needs to be addressed since this could improve sample yield, though this could potentially interfere with PK.

Finally, the accuracy of TDM using saliva samples needs to be evaluated in a real life setting. Whilst the performed simulations in this thesis provide early insight in the TDM performance, it contains many assumptions that are not in line with the true settings, such as dosing- and sampling times. Additionally, the simulated cohort is not a true representation of the population and does not account for demographic characteristics that may be present in reality.

Efficacy of vancomycin on bacterial DNA loads

Up until the start of this thesis, the effect of vancomycin on BDL had not been explored. Results of our study provided a proof of concept that BDLs could be used as a predictor for response to vancomycin therapy in CoNS-positive LOS (**Chapter 5**). Nonetheless the developed model, whilst acceptable, was fundamentally flawed in its assumptions. Ideally, a semi-mechanistic PKPD model is developed to describe PKPD properties of antibiotics [41,45]. These models simultaneously describe natural bacterial growth, - decay and drug effect and thus are a more accurate representation of antimicrobial pharmacology compared to MIC based PK target evaluation. For such a model to be developed, a combination of *in vitro*-, animal- and clinical data is required. For the estimation of a good bacterial model, *in vitro*- and animal studies could provide data of the BDL profile of CoNS in absence of vancomycin. Inoculation of bacterial strains to growth culture and animals and subsequent BDL measurement from serial samples at different time points could be used to express the natural bacterial growth- and decay profiles. In addition, animal data could be used to assess the contribution of the immune system on CoNS proliferation. Combined with a rich sampling scheme in a large population of neonates, a full model could be developed for both responders and non-responders. Whereas the current PK index for vancomycin is an AUC/MIC of at least 400, an accurate representation in a PKPD model using BDL could be used to not only evaluate the correctness of the PK target, but also has potential for the identification of novel non-MIC based concentration targets. Though the current technique provides a rough overview of the clinical antimicrobial action of vancomycin, it is too crude to be clinically implemented in its current state. It is important that a more accurate PKPD model is developed that could also predict treatment response and includes non-responders to vancomycin therapy, since the current model can only be used for neonates that have been found to respond to vancomycin.

Intrapartum GBS prophylaxis

In the future, it should be reevaluated on a national and possibly international scale, whether the intrapartum benzylpenicillin doses for GBS prophylaxis advised by international guidelines are currently too high, as we found that 50% lower doses reached adequate concentrations in UCB and thus in neonatal plasma (**Chapter 6**). However, a thorough prospective examination of the PK is required, describing benzylpenicillin concentrations in mother and child. For instance, a PBPK model could be of value in such an investigation [64]. In addition more 'traditional' population PK models have been developed for amikacin and cefazolin following intrapartum therapy [71–73]. A similar methodology could be employed for benzylpenicillin. Lower international benzylpenicillin doses could be of value for the preservation of the commensal flora, whilst maintaining prophylactic- or therapeutic effect.

Concluding remarks

In conclusion, we believe that the results of the studies described in this thesis demonstrate the feasibility of non-invasive TDM of aminoglycosides for neonatal sepsis. Additionally, we conclude that the collective knowledge is one step closer in understanding the clinical effect

of vancomycin on bacterial loads in LOS. Furthermore, the adequacy of intrapartum GBS prophylaxis with benzylpenicillin using lower doses compared to internationally accepted guidelines has been demonstrated, indirectly assisting in the global fight against antimicrobial resistance. Though many challenges, such as the development of a standardized saliva sampling method, more accurate PK models and clinical TDM evaluation studies have to be performed before clinical implementation, we strongly recommend that the findings in this thesis are used for further research. In the end, our findings support the notion that the clinical burden associated with neonatal sepsis can be decreased with an improved clinical outcome, thanks to non-invasive TDM and more accurate dosing.

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Summary

In this thesis the personalized antimicrobial treatment and prophylaxis of neonatal sepsis were explored together with novel assessment methods for therapeutic drug monitoring (TDM). In **Part I** the development and validation of a highly sensitive bioanalytical method for the determination of amikacin and flucloxacillin concentrations in plasma and saliva are described and discussed. This method was used in **Part II**, which focusses on the pharmacokinetics (PK) of the aminoglycoside antibiotics amikacin and gentamicin in plasma and saliva. Additionally, the applicability of TDM of aminoglycosides based on saliva samples was evaluated. In **Part III** the clinical efficacy of several current antibiotic dose regimes was evaluated. First, the effect of the antibiotic vancomycin on bacterial DNA loads (BDLs) was quantified using an integrated pharmacokinetic-pharmacodynamic (PK-PD) model. Second, it was assessed whether the dose of currently administered intrapartum antibiotics for early-onset sepsis prophylaxis resulted in adequate concentrations in the fetus and neonate.

General introduction

Chapter 1 provides a general introduction to the classification, epidemiology and current treatment options for neonatal sepsis. Moreover, the basic principles of TDM and the outline of this thesis are discussed in detail in this chapter.

Neonatal sepsis is a frequently encountered severe clinical bacterial infectious disease of the bloodstream with an incidence of 2,824 cases per 100,000 live births and a high mortality of approximately 17%. Neonatal sepsis can be classified as either early-onset sepsis (EOS) when contracted within the first 3 days of life or late-onset sepsis (LOS) when contracted after the first 3 days of life. Neonatal sepsis is commonly treated with an intravenous administration of aminoglycoside antibiotics (gentamicin or amikacin) combined with benzylpenicillin or flucloxacillin. Whereas gentamicin is the aminoglycoside of choice to treat EOS, amikacin is the preferred aminoglycoside for the treatment of LOS. In cases of LOS caused by coagulase-negative staphylococci (CoNS), antimicrobial therapy is switched to vancomycin treatment. Furthermore, clinicians prescribe intrapartum benzylpenicillin for the prophylaxis of EOS caused by GBS in colonized pregnant women. Intrapartum benzylpenicillin prophylaxis significantly decreases the incidence of EOS and is thus a staple for antimicrobial management during the neonatal period.

Given that the PK of aminoglycosides and vancomycin is highly variable in the neonatal population and these drug have a narrow therapeutic index, plasma concentrations are routinely monitored by hospital pharmacists for the evaluation of concentration target attainment and subsequent dose adjustments, if necessary. This practice, called therapeutic drug monitoring (TDM), is necessary to ensure safe and efficacious doses, though the collection of the plasma samples required for TDM is painful and potentially harmful for the neonate. Thus, we investigated whether saliva samples could be used for the purpose of TDM of aminoglycosides.

Part I | Development of bioanalytical methods in plasma and saliva

Chapter 2 elaborates upon the development and evaluation of a sensitive bioanalytical method using state-of-the-art liquid-chromatography coupled to tandem mass-spectrometry (LC-MS/MS) for the simultaneous quantification of amikacin and flucloxacillin in saliva and plasma samples. For both amikacin and flucloxacillin, accuracy and imprecision at the lowest limit of quantification (LLOQ), low quantification level (LLQ), middle level of quantification (MLQ) and upper quantification limit (ULOQ) were within the acceptable range of 85–115%. Thus, we were able to accurately quantify amikacin and flucloxacillin in the µg/l range in samples with volumes as low as 10 µl. LLOQs reported in earlier studies were between 0.18 – 2.0 mg/l. This newly developed method can detect amikacin levels that are at least 10-fold lower than previous published methods, at concentration ranges that are to be expected in neonatal saliva. However, the ultrafiltration efficiencies for the determination of the unbound fraction of flucloxacillin did not meet the criteria of 85-115% at the LLQ and ULOQ, meaning that this method is suitable for the quantification of total (plasma protein bound plus unbound) flucloxacillin only.

Part II | Pharmacokinetics and therapeutic drug monitoring of aminoglycosides

In **Chapter 3**, a population PK (POP-PK) model was developed for the description of time profiles of gentamicin concentration in plasma and saliva of term and premature neonates with EOS. An allometrically scaled two-compartment model was constructed with post-natal age (PNA), gestational age (GA) and dopamine use as covariates on clearance (CL) and GA as a covariate on central volume of distribution (V_c). A single saliva compartment was appended to the plasma compartment, describing a salivary absorption rate (k_{13}) and salivary elimination rate (k_{30}) of 0.023 h^{-1} and 0.169 h^{-1}, respectively. Post-menstrual age (PMA) had a negative effect on both k_{13} and k_{30} when included as a power function, though the negative effect was stronger on k_{13} than k_{30}, as indicated by the respective exponents of -8.8 and -5.1. This finding demonstrates that gentamicin was more readily distributed in the saliva of premature neonates compared to term neonates. The final PK model was used in Monte-Carlo simulations in a fictional cohort of neonates to evaluate the probability of target attainment (pTA) in multiple TDM scenarios using plasma or saliva samples. It was found that a maximum pTA of 81% was achievable when performing TDM using only saliva samples, compared to 94% when using a peak- and trough plasma sample and 87% when using a mid-interval plasma sample. These findings support the feasibility of salivary TDM, though a future study investigating the real-life performance of salivary- and plasma TDM is necessary to evaluate non-inferiority.

In **Chapter 4** the newly developed LC-MS/MS method (Chapter 2) is described which was used to quantify amikacin concentrations in plasma and saliva samples collected during a prospective observational study with 24 neonates. A POP-PK analysis was performed to develop an integrated PK model of amikacin in plasma and saliva and for the identification of covariates. A single saliva compartment was appended to an existing 2-compartment plasma model. The first-order absorption rate constant k_{13} of the saliva compartment was

0.0345 h^{-1} with an inter-individual variability (IIV) of 45.3%. The rate of first-order elimination rate constant k_{30} was 0.176 h^{-1}. PMA had a significant negative covariate effect on k_{13} with an exponent of -4.3, demonstrating the higher salivary uptake of amikacin in preterm neonates when compared to term neonates. When TDM scenarios were evaluated in a fictional cohort using Monte-Carlo simulations, saliva TDM reached a maximum pTA of 79.2% using 5 saliva samples, which was similar to the pTA of current amikacin TDM protocols using plasma samples. Whilst this exploratory study provides a solid basis for the feasibility of salivary TDM of amikacin, a future study is required for clinical implementation.

Part III | Pharmacodynamic aspects of antimicrobial therapy in neonatal sepsis

Chapter 5 describes the development of a PK-PD model describing the effect of vancomycin on bacterial DNA loads (BDL) of coagulase-negative staphylococci (CoNS) measured using a real-time quantitative polymerase chain reaction (RT-qPCR) method. A total of 28 patients with LOS treated with vancomycin were included. A one-compartment model with PMA and weight as covariates was used to describe the PK profile of vancomycin concentrations. In patients that responded to therapy (n=16), time profiles of BDL could be described with a PD turnover model. The relationship between vancomycin concentration and first-order BDL elimination was described with a linear-effect model; in this model the slope increased with increasing PMA. This indicates that, at equal drug concentrations, the bactericidal effect of vancomycin was stronger in older neonates than in younger neonates. It is likely, however, that this increased effect represent a more mature immune response. In 12 patients, no decrease in BDL over time was observed, which corresponded with clinical non-response. Causes for clinical and BDL non-response included primary central venous line infections with line retention and infected thrombi. It seems plausible that the developed PK-PD could be used for the evaluation of bactericidal activity and treatment response.

In **Chapter 6** the target attainment of antibiotic concentrations in umbilical cord blood (UCB) following intrapartum antimicrobial prophylaxis was evaluated. Dutch obstetric guidelines suggest an initial maternal benzylpenicillin dose of 2,000,000 IU followed by 1,000,000 IU every 4 hours for group-B-streptococci (GBS) prophylaxis, which 50% lower compare to the doses suggested in international guidelines. Out of 46 neonates that were included in the study, a total of 46 UCB samples and 18 neonatal plasma samples were available for analysis. Nineteen neonates had mothers that received intrapartum benzylpenicillin. Benzylpenicillin in UCB corresponded to concentrations in plasma drawn directly from the neonates postpartum (R^2=0.88, $p<0.01$). A log-linear regression suggested that benzylpenicillin concentrations in neonates remained above the MIC threshold 0.125 mg/L up to 13.0 hours after the last intrapartum dose. Furthermore, intrapartum dosage of amoxicillin, cefazolin and gentamicin all resulted in pharmacologically active concentrations in UCB for the most common pathogens for EOS. It was found that Dutch intrapartum benzylpenicillin doses recommendations result in neonatal concentrations above the MIC for GBS.

Part IV| General discussion

General discussion

A general discussion on the findings in this thesis is provided in **Chapter 7.**

Conclusions

The results of the studies described in this thesis demonstrate that the use of saliva as a medium for TDM is feasible in neonates as compared to the use of plasma sampling. When applied, this may hopefully decrease the clinical burden associated with more invasive TDM methods. The results of this thesis further demonstrated that the decrease in bacterial DNA load can be used as a possible measure of therapeutic effect during vancomycin treatment in CoNS-positive LOS. The results of UCB studies demonstrated that the Dutch intrapartum benzylpenicillin dose recommendations result in neonatal concentrations above the MIC for GBS.

Samenvatting

Algemene introductie

Neonatale sepsis en antibioticabeleid

Sepsis is een veel voorkomend klinisch beeld dat wordt gekenmerkt door de aanwezigheid van bacteriën in de bloedbaan en ernstige klinische symptomen zoals lage bloeddruk en beademingsbehoefte. Wanneer sepsis optreedt gedurende de eerste 30 levensdagen van een pasgeborene, ofwel neonaat, spreekt men van neonatale sepsis. Wereldwijd ondergaan bijna 3,000 per 100,000 neonaten minimaal één episode van sepsis, waarvan ongeveer 17% een fatale afloop heeft. Neonatale sepsis wordt in Nederland behandeld met een combinatie van 2 soorten antibiotica; één antibioticum van de aminoglycosideklasse en één antibioticum uit de β-lactamklasse. Neonatale sepsis wordt verdeeld in twee categorieën afhankelijk van de start van de sepsis episode, namelijk *early-onset* sepsis (EOS) en *late-onset* sepsis (LOS).

EOS wordt gekarakteriseerd door een sepsis die optreedt bij neonaten jonger dan 3 dagen. EOS wordt doorgaans veroorzaakt door bacteriën die zich bevinden in het geboortekanaal van de moeder. In geval van EOS wordt een combinatie van gentamicine (aminoglycoside) met benzylpenicilline (β-lactam) gegeven en er worden bloedkweken afgenomen om de ziekteverwekker te identificeren. Ook wordt er volgens het klinisch protocol voor gekozen om moeders met een bekende bacteriële aanwezigheid, gedurende de bevalling profylactisch (preventief) te behandelen met benzylpenicilline, zodat een episode van EOS na geboorte kan worden voorkomen. Redenen voor deze profylaxe zijn o.a. maternale kolonisatie met groep-B-streptokokken (GBS) of EOS in de voorgeschiedenis. Sinds de invoering van dit profylactische beleid in de jaren ’90 is de incidentie van EOS met 70% afgenomen; dit is dus een zeer effectieve strategie.

Sepsis die optreedt bij neonaten ouder dan 3 dagen wordt gedefinieerd als LOS. De oorzaak van LOS ligt voornamelijk bij invasieve ziekenhuisingrepen, zoals het hebben van een centrale lijn, centrale drain of het herhaald afnemen van bloed. LOS wordt behandeld met amikacine (aminoglycoside) en flucloxacilline (β-lactam), en er worden bloedkweken afgenomen om de ziekteverwekker te identificeren. Bij LOS episodes die zijn verwerkt door coagulase-negatieve stafylokokken (CoNS) wordt overgestapt naar vancomycine monotherapie.

Klinische farmacologie en therapeutic drug monitoring

Klinische farmacologie is een wetenschap waarin de werking van geneesmiddelen bij mensen wordt bestudeerd. In de relatie tussen de dosering en het effect van een geneesmiddel zijn twee aspecten belangrijk, farmacokinetiek (PK) en farmacodynamiek (PD). PK brengt in kaart *wat het lichaam doet met het geneesmiddel*. In het kort beschrijft PK de opname van het geneesmiddel (absorptie), verdeling over het plasma en weefsel (distributie), afbraak van geneesmiddelen (metabolisme) en de uitscheiding van het geneesmiddel en de afvalproducten daarvan (excretie). Om hier een goed beeld van te krijgen worden na toediening

concentraties van het geneesmiddel in het plasma gemeten op verschillende tijdstippen en wordt gekeken naar het verloop van de concentratie van het geneesmiddel gedurende de tijd na toediening (het concentratie-tijd profiel). Hieruit kunnen verschillende PK parameters worden berekend die het concentratie-tijd profiel bepalen. De PK parameter klaring (CL) kwantificeert de snelheid van eliminatie en de parameter verdelingsvolume (V_d) beschrijft de distributie over het lichaam. PD beschrijft *wat het geneesmiddel met het lichaam doet*. Het beschrijft de relatie tussen de concentratie van het geneesmiddel en een effect dat optreedt. Dit effect kan zowel het gewenste (therapeutische) effect zijn als een ongewenst effect (bijwerking/ toxiciteit). Bij bestudering van de relatie tussen dosis, concentratie en effect moeten zowel de PK als PD bestudeerd worden. Geneesmiddelconcentraties worden bepaald door de PK en beïnvloeden vervolgens de mate van het effect (PD). In de klinische farmacologie wordt vaak gesproken over de PKPD relatie.

Bij een gelijke dosering kunnen er verschillen zijn in het effect en bijwerkingen tussen individuele patiënten. Een reden voor deze verschillen kan zijn dat geneesmiddelenconcentraties in plasma verschillen, omdat patiënten niet even groot en zwaar zijn. Daarom worden de doseringen vaak gecorrigeerd op basis van het lichaamsgewicht. Echter, soms blijkt dat deze correctie onvoldoende is en er nog steeds verschillen in de concentratie-tijd profielen zijn. In andere woorden, er is veel variabiliteit in de PK tussen patiënten. Dit wordt de interindividuele variabiliteit (IIV) genoemd. Hetzelfde doet zich voor bij PD; bij een gelijke geneesmiddelconcentratie in het plasma kan het effect tussen patiënten nog steeds verschillen. Om ervoor te zorgen dat de doseringen per individu geoptimaliseerd kunnen worden, is het van belang de relatie tussen de dosering, concentratie en het effect voor de individuele patiënt te karakteriseren. Hierbij wordt vaak een populatie-PK (POP-PK) analyse toegepast. Deze wiskundige POP-PK analyse wordt uitgevoerd door middel van *non-linear mixed-effects modeling*. In een POP-PK model wordt de (non-lineaire) relatie tussen de onafhankelijke variabele (tijd), PK parameters (CL, V_d) en de afhankelijke variabele (geneesmiddelconcentratie) gemodelleerd. In het POP-PK beschrijven *fixed effects* de gemiddelde waarde van een PK parameter (CL, V), terwijl *random effects* de willekeurige afwijking van de gemiddelde parameterwaarde per individu (IIV) beschrijven. *Fixed effects* en *random effects* worden tezamen *mixed effects* genoemd. Binnen een POP-PK model is het mogelijk om IIV tussen patiënten te onderzoeken en verklaren. Relaties tussen de patiëntfactoren (gewicht, leeftijd, nierfunctie) en PK parameters kunnen worden onderzocht en gekwantificeerd als zogenaamde covariaten.

De PK van gentamicine, amikacine en vancomycine kenmerkt zich door een grote IIV in de neonatale populatie. Daarnaast is de marge tussen een effectieve en toxische concentratie klein. Om toxiciteit te voorkomen worden hierom in de klinische praktijk plasmamonsters afgenomen om de concentratie te meten. Deze concentraties worden met behulp van een POP-PK model geëvalueerd door een ziekenhuisapotheker, waarna eventueel de dosis wordt aangepast om de beoogde concentraties te behalen. Deze evaluatie, *therapeutic drug monitoring* (TDM) genoemd, is noodzakelijk om veilige en effectieve doseringen te waarborgen. Nadeel van deze methode is dat bloed moet worden afgenomen. Dit is pijnlijk en het kan mogelijk leiden tot

gevaarlijke complicaties voor neonaten. Om de belasting voor de pasgeborene te verminderen is in dit proefschrift onderzocht of het mogelijk is om speekselmonsters te gebruiken in plaats van plasmamonsters voor de TDM van aminoglycosiden, omdat speekselafname pijnloos en niet invasief is. Ook is er onderzocht wat de PD relatie is tussen de vancomycine concentratie in plasma en de bacteriële DNA concentratie (als maat voor effect) aangezien dit mogelijk leidt tot een betere dosering van dit antibioticum. Tot slot is onderzocht of aan de moeder toegediende profylactische benzylpenicillinedoseringen rondom de geboorte leidden tot effectieve en beschermende concentraties in de neonaat. Dit werd in het navelstrengbloed en het bloed van het kind na de geboorte onderzocht.

Deel I | de ontwikkeling van een bio-analytische methode in plasma en speeksel

Hoofdstuk 2 beschrijft de ontwikkeling en validatie van een gevoelige bio-analytische methode waarbij gebruik wordt gemaakt van vloeistofchromatografie met massaspectrometrische detectie (LC-MS/MS). M.b.v. deze methode kunnen de concentratie van amikacine en flucloxacilline in speeksel en plasma worden bepaald. Met deze gevoelige methode kunnen zeer lage concentraties in kleine monstervolumes gemeten worden, een vereiste voor TDM bij neonaten. Voor zowel amikacine als flucloxacilline waren de juistheid en precisie bij de onderste kwantificatielimiet (LLOQ), het lage kwantificatieniveau (LOQ), het middelste kwantificatieniveau (MLQ) en de hoogste kwantificatielimiet (ULOQ) binnen de acceptatiecriteria van 85-115%. Daarmee konden we amikacine en flucloxacilline betrouwbaar bepalen vanaf lage 10 µg/L in slechts 10 µL plasma of speeksel. In eerdere studies naar aminoglycoside concentraties in speeksel was de LLOQ van de analysemethode tussen 0.18 en 2.0 mg/L. Onze nieuw ontwikkelde methode kan dus amikacineconcentraties bepalen met een meetgrens die meer dan een factor 10 lager ligt dan de eerder gepubliceerde methoden.

Deel II | Farmacokinetiek en TDM van aminoglycosiden

In **Hoofdstuk 3** werd een POP-PK model ontwikkeld voor de beschrijving van de concentratie-tijd-profielen van gentamicine in plasma en speeksel voor neonaten met EOS. Voor het beschrijven van de plasmaconcentraties werd gebruik gemaakt van een twee-compartimentsmodel met lichaamsgewicht, postnatale leeftijd (PNA), zwangerschapsduur (GA) en dopaminetherapie als covariaten op CL. GA was een covariaat op het centrale verdelingsvolume. Alle parameters waren geschaald op geboortegewicht. Een speekselcompartiment werd toegevoegd aan de centrale plasmacomponent. Transport van plasma naar speeksel en eliminatie vanuit speeksel werden beschreven met de eerste-orde snelheidsconstantes k_{13} en k_{30} met geschatte waardes van 0.023 h^{-1} en respectievelijk 0.169 h^{-1}. Tussen de post-menstruele leeftijd (PMA = GA + PNA) enerzijds en zowel k_{13} en k_{30} anderzijds bestond een negatieve correlatie. Uit deze correlatie blijkt dat gentamicine sneller in speeksel van premature neonaten wordt opgenomen dan het geval is in oudere, à terme neonaten. Het finale POP-PK model werd gebruikt voor Monte-Carlo simulaties in een virtueel cohort van neonaten. Hierbij werd geëvalueerd of met behulp van TDM op basis van gentamicine concentraties gemeten in speeksel of plasma de dosering juist werd aangepast om werkzame en veilige concentraties te bereiken. In plasma werd in

94% van de virtuele patiënten adequate gentamicine concentraties bereikt met het meten van alleen een top- en een dal plasma concentratie. In het geval van speeksel werd in 81% van de virtuele patiënten adequate gentamicine concentraties in plasma behaald op basis van 4 speekselmonsters. Deze bevindingen laten zien dat het mogelijk is TDM uit te voeren op basis van speekselconcentraties. Echter, een studie met klinische patiënten is vereist om de werkelijke geschiktheid van TDM op basis van speeksel aan te tonen.

In **Hoofdstuk 4** werd onze nieuwe LC-MS/MS methode (Hoofdstuk 2) gebruikt om de amikacineconcentraties te bepalen in plasma- en speekselmonsters die waren verzameld in een prospectieve observationele studie met 24 neonaten met LOS. Een POP-PK analyse werd uitgevoerd om een PK model voor amikacine in plasma en speeksel te ontwikkelen en voor de identificatie van covariaten die van invloed waren op de verschillende PK parameters. Een speekselcompartiment werd toegevoegd aan een eerder gepubliceerd 2-compartimentsmodel voor plasma. De eerste-orde absorptieconstante k_{13} van het speekselcompartiment was 0.034 h^{-1} met een IIV van 45.3%. De eerste-orde eliminatieconstante k_{30} was 0.176 h^{-1}. PMA werd geïncludeerd als een covariaat op k_{13} met een exponent van -4.3. Vergelijkbaar met onze bevindingen voor gentamicine wordt amikacine ook sneller opgenomen in het speeksel van premature neonaten vergeleken met à terme neonaten. Bij toepassing van TDM op basis van 5 speekselmonsters werd in 79.2% van de patiënten adequate plasma concentraties bereikt, bleek uit Monte Carlo simulaties in een virtueel cohort met neonaten. Dit percentage was vergelijkbaar met het percentage gevonden na TDM met 2 plasmamonsters, zoals dat in de huidige praktijk plaatsvindt. Deze studie toont aan dat TDM van amikacine met speekselmonsters haalbaar is, maar een toekomstige prospectieve studie is een vereiste voor eventuele klinische implementatie.

Deel III | Farmacodynamische aspecten van antimicrobiële therapie voor neonatale sepsis

In **Hoofdstuk 5** is een PKPD model ontwikkeld dat de concentratie van het antibioticum vancomycine relateert aan de bacteriële DNA hoeveelheid (*load*) (*Bacterial DNA Load*; BDL) van CoNS in het bloed, gemeten met een *real-time* kwantitatieve polymerase *chain reaction* (RT-qPCR) methode. In totaal werden 28 patiënten met CoNS-positieve LOS in de studie geïncludeerd die werden behandeld met vancomycine. Een 1-compartimentsmodel met PMA en gewicht als covariaten werd gebruikt om het PK-profiel van vancomycine te beschrijven. Van de 16 patiënten die reageerden op vancomycinetherapie werd de afname van BDL beschreven met een PD model. De relatie tussen de vancomycine concentratie en eerste-orde BDL eliminatie werd omschreven met een lineair effect model. Er was een positieve correlatie tussen de helling van het lineaire effect model en PMA. Dit betekent dat bij gelijke concentraties vancomycine bij een oudere neonaat een groter antimicrobieel effect bereikt wordt dan bij een jongere neonaat. Dit zou mogelijk verklaard kunnen worden door een sterkere immuunrespons bij oudere neonaten. Er waren 12 patiënten die niet reageerden op de vancomycinetherapie zoals vastgesteld op basis van zowel het klinisch beloop en niet-dalende BDL profielen. Oorzaken voor deze klinische- en BDL non-respons waren een

primaire kolonisatie van de aanwezige centraal veneuze katheter die niet verwijderd kon worden, een secondaire huidinfectie en de aanwezigheid van een geïnfecteerde trombus in de bloedbaan. Met het ontwikkelde PKPD model was het mogelijk de relatie tussen dosering, concentratie en de bactericide activiteit van vancomycine te kwantificeren. Dit maakt het mogelijk om doseringen verder te optimaliseren en streefwaarden voor de plasmaconcentratie verder te onderbouwen.

De preventie van EOS middels maternaal toegediende antibiotica gedurende de bevalling (intrapartum profylaxe) is onderzocht in **Hoofdstuk 6**. Er werd onderzocht of de gemeten antibioticaconcentraties in navelstrengbloed (UCB) zich boven de MIC (minimale inhiberende concentratie) bevonden. Voor GBS profylaxe wordt in de Nederlandse verloskundige richtlijnen een initiële intrapartum benzylpenicillinedosering van 2,000,000 internationale eenheden (IE) geadviseerd, gevolgd door 1,000,000 IE elke 4 uur. Deze doseringen zijn meer dan 50% lager dan de internationaal geadviseerde doseringen van 5,000,000 IE gevolgd door 2,500,000 IE elke 4 uur. Er was tot op heden geen goede onderbouwing voor deze lagere doseringen in Nederland. Van de 46 neonaten die waren geïncludeerd in de studie waren totaal 46 UCB monsters en 18 monsters neonataal plasma direct na geboorte beschikbaar. Van 19 neonaten was de moeder behandeld met intrapartum benzylpenicilline. De benzylpenicillineconcentraties in UCB waren goed gecorreleerd met de concentraties in het neonataal plasma (R^2=0.88, p<0.01) en dus kon geconstateerd worden dat concentraties in UCB representatief zijn voor de concentraties in neonataal plasma. Uit een log-lineaire regressie werd gevonden dat de benzylpenicillineconcentraties in neonataal plasma zich tot 13 uur na de laatste intrapartum dosering boven de MIC grenswaarde van 0.125 mg/L bevonden. Daarnaast werd gevonden dat de concentraties van amoxicilline, cefazoline en gentamicine allemaal hoger waren dan de MIC waardes voor de meest voorkomende verwekkers van EOS. De studie wijst erop dat de Nederlandse intrapartum benzylpenicilline doseringen voor GBS profylaxe voldoende hoog zijn voor antibacterieel effect en dus de preventie van EOS.

Deel IV | Algemene discussie

Algemene discussie

Een algemene discussie over de bevindingen in dit proefschrift is beschreven in **Hoofdstuk 7**.

Conclusies

De resultaten van de onderzoeken die zijn beschreven in dit proefschrift laten zien dat het mogelijk is om speekselmonsters te gebruiken voor de TDM van gentamicine en amikacine. Indien speeksel monitoring wordt toegepast in de klinische praktijk zal dit de belasting en risico's die gepaard gaan bij afname van bloed bij neonaten verminderen. Bovendien hebben we aangetoond dat de afname in BDL een geschikt PD eindpunt is om het therapeutisch effect van vancomycine in patiënten met CoNS-positieve LOS te kwantificeren en optimaliseren. De resultaten van de studies met het navelstrengbloed toonden aan dat de benzylpeni-

cilline dosering die volgens de Nederlandse richtlijn aan de moeder tijdens de bevalling wordt gegeven resulteert in adequate plasma concentraties voor de profylaxe van een GBS infectie.

Appendices

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Zonder de academische en emotionele steun van de anderen had de totstandkoming van dit proefschrift nooit gerealiseerd kunnen worden. Ik wil deze pagina's daarom gebruiken om iedereen daarvoor te bedanken.

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Portfolio

Name PhD student:	A. Samb
PhD period:	January 2020 – January 2023
Names PhD supervisors	Prof. Dr. R.A.A. Mathôt
	Prof. Dr. A.H.L.C. van Kaam
Name PhD co-supervisors:	Dr. T.R. de Haan
	Dr. Y.A. Bijleveld

1. PhD training

	Year	ECTS
General courses		
- E-BROK ('Basiscursus Regelgeving Klinisch Onderzoek')	2020	1.5
- Advanced Topics in Biostatistics	2021	2,1
- Project management	2020	0.6
- AMC World of Science	2020	0.7
- Research data management	2020	0.4
- Present like a boss links and PPT	2021	0.4
(Inter)national conferences		
- Pharmacometrics Benelux Network meeting	2022	0.5
- ESPID	2020	0.5
- ESDPPP	2022	1.5
- Voorjaarsdag Nederlandse Vereniging voor Klinische Farmacologie en Biofarmacie	2022	0.5
- FIGON Dutch Medicine Days	2021	0.5
Poster presentations		
- Predicting treatment response to vancomycin using bacterial DNA load as a pharmacodynamic marker in premature and very low birth weight neonates: A population PKPD study	2022	0.5
Oral presentations		
- Predicting treatment response to vancomycin using bacterial DNA load as a pharmacodynamic marker in premature and very low birth weight neonates: A population PKPD study	2022	0.5
- Saliva as a sampling matrix for therapeutic drug monitoring of gentamicin in neonates: A prospective population pharmacokinetic and simulation study	2021	0.5
Journal Clubs		
- NONMEM Journal club	2020-2023	3.0

2. Teaching

	Year	ECTS
Lecturing		
- Clinical lessons Neonatology	2022	0.5

3. Parameters of Estee

	Year
Grants	
- Janivo fonds (2019378)	2019
- Vaillant fonds	2019
- Stichting kinderziekenhuizen van Oranje	2021

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Samb A, Kruizinga M, Tallahi Y, van Esdonk M, van Heel W, Driessen G, et al. Saliva as a sampling matrix for therapeutic drug monitoring of gentamicin in neonates: A prospective population pharmacokinetic and simulation study. Br J Clin Pharmacol. 2022 Feb;88(4):1845-1855. doi: 10.1111/bcp.15105.

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About the author

Amadou Samb was born on February 24th 1992 in de Bilt, the Netherlands. He attended secondary school at the Heemlanden College in Houten, where he received his Atheneum degree in 2010. In 2011 he started with his Bachelor's in pharmacy and he received his Master's degree in Drug Innovation in 2019. During his studies he developed a particular interest in perinatal pharmacology and pharmacometrics. In January 2020 he started his PhD research at the Amsterdam UMC – location AMC under supervision of prof. dr. R.A.A. Mathôt, prof. dr. A.H.L.C. van Kaam, dr. T.R. de Haan and dr. Y.A. Bijleveld, which has resulted in this thesis. Following the completion of his PhD, he will continue to pursue a professional career in clinical drug development and quantitative pharmacology.