Determinants of pharmacokinetic variability during extracorporeal membrane oxygenation

A roadmap to rational pharmacotherapy in children

Maurice Ahsman

The studies presented in this thesis were done in collaboration between the Pediatric Intensive Care (Sophia Children's Hospital) and the department of Hospital Pharmacy, Erasmus University Medical Center, Rotterdam, the Netherlands.

For more information about drug therapy during ECMO, the reader is referred to the thesis by E.D. Wildschut entitled 'Drug therapies in neonates and children during extra-corporeal membrane oxygenation (ECMO): Keep Your Eyes Open'.

The publication of this thesis was financially supported by Fagron B.V. (Nieuwerkerk a/d IJssel, The Netherlands) and Waters Corporation (Milford, MA, USA)

ISBN: 978-90-8559-023-1

Cover design: Christian Ahsman
Layout and printing: Optima Grafische Communicatie, Rotterdam, the Netherlands

Determinants of Pharmacokinetic Variability during Extracorporeal Membrane Oxygenation

A roadmap to rational pharmacotherapy in children

Determinanten van farmacokinetische variabiliteit tijdens extracorporele membraan oxygenatie

Een routebeschrijving naar rationele farmacotherapie bij kinderen

Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de rector magnificus
Prof.dr. H.G. Schmidt
en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op
Vrijdag 2 juli 2010 om 11.30 uur
door

Maurice Jozef Ahsman

geboren te Amstelveen

ERASMUS UNIVERSITEIT ROTTERDAM

Promotiecommissie

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Chapter 1

Introduction

Introduction

Critically-ill patients admitted to neonatal or pediatric intensive care units often require artificial ventilation to maintain adequate oxygenation. When advanced therapies such as multiple vaso-active drugs, surfactants and nitric oxide inhalation with or without high flow oscillation ventilation no longer suffice, extracorporeal membrane oxygenation (ECMO) can be applied as a last resort. ECMO is a cardiopulmonary bypass technique which provides temporary support of cardiac and/or respiratory function.[1] Over a period of up to 3 weeks, the ECMO system can take care of the patients' oxygenation and/or circulation while the heart and lungs get a chance to recover from life-threatening injury or deformities, on their own or via surgical or pharmacological intervention. The effectiveness of ECMO is based on the extraction of blood from the venous end of the circulation into an artificial circuit which contains a pump, a membrane oxygenator and a heating system (Figure 1). After exchange of oxygen and carbon dioxide in the oxygenator, the oxygenated blood is returned to the patient via a venous or arterial cannula.

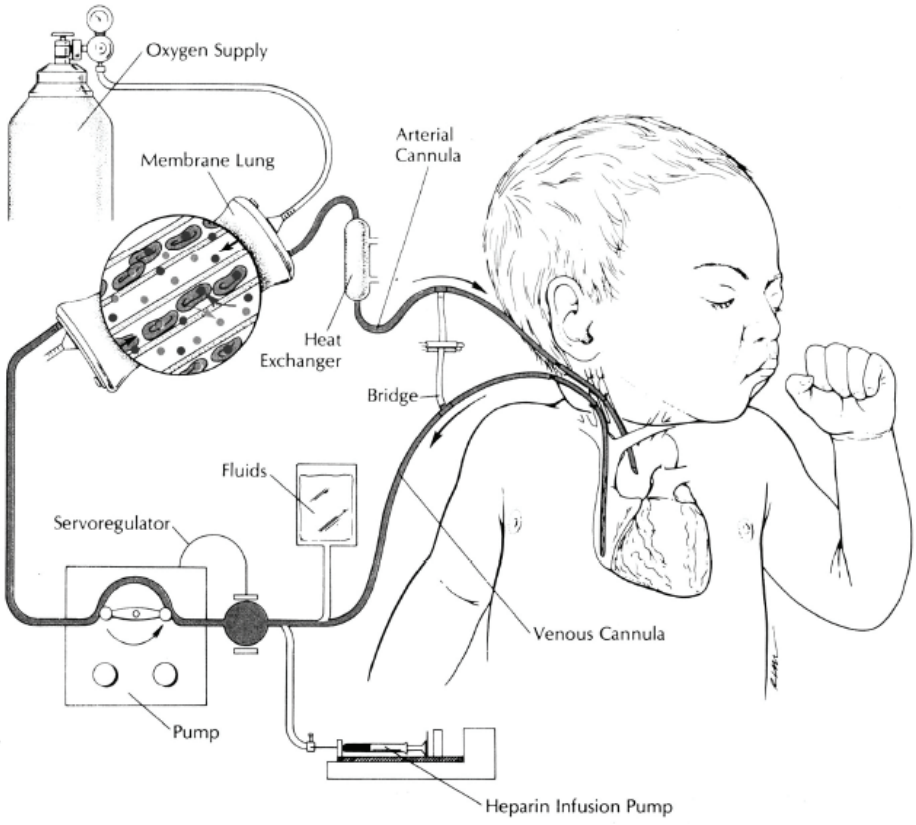

Figure 1. Schematic representation of a venoarterial ECMO circuit, reproduced with permission.[40]

Nowadays, at least in the Erasmus MC-Sophia Children's Hospital, circuits often contain a continuous hemofiltration unit to help maintain proper fluid status and to compensate for reduced renal elimination of endogenous compounds, drugs or their metabolites. [2, 3] ECMO patients receive many drugs, including sedatives to prevent movement and discomfort, analgesics to treat pain, antibiotics to combat bacterial infection, heparin to optimize blood coagulation time and vaso-active drugs to maintain adequate blood pressures. Individual patients often require additional drugs for a specific condition, such as antiepileptic, antifungal or antiviral medication. Neonates with congenital abnormalities or meconium aspiration syndrome often also need sildenafil or bosentan to treat pulmonary hypertension.[4-6] Dosing these drugs can be a challenge: the market authorization file, drug information leaflets and pediatric dosing guidelines do not contain specific dose recommendations. Before 2007, regulatory authorities did not require studies in the pediatric population for registration and market authorization of new drugs, even if there were foreseeable pediatric applications. In essence, the off-label use implies that each time a drug is given, that patient enters an experiment with only one test subject. The number of pediatric studies has increased since 2007, because of new incentive programmes and regulatory requirements issued by the European Medicines Agency (EMA) and the US Food and Drug Administration (FDA).[7, 8] ECMO patients however are not selected to participate in these studies, probably due to their low numbers (30-40 a year in our medical center) and perceived vulnerability, and the potential difficulties in extrapolation of study results to the general pediatric population. The resulting lack of data leads to dose regimens that are based on personal experience, extrapolation from studies in other patients, individual dose titration or an educated guess.

Standard dose regimens: ECMO vs. non-ECMO patients

We know from experience that the standard dose regimens used in non-ECMO patients can be ineffective. The required level of sedation for instance cannot be achieved using standard dose regimens of midazolam, pentobarbital and other drugs [9]; patients often require higher loading doses and continuous infusion rates. This could in part be explained by a lack of appropriate drug exposure at the effector organ or tissues, caused by differences in drug absorption, distribution, metabolism and elimination (collectively termed pharmacokinetics or PK) between ECMO and non-ECMO patients. These changes can be studied by examining drug concentrations in bodily fluids or tissues at various times after drug administration. Such studies have shown that, in general, ECMO patients have a larger volume of distribution and reduced clearance, leading to longer elimination half-lives.[10] This need not be a problem for drugs that can easily be titrated up to the desired effect, but for the remaining drugs (such as antibiotics or antifungals),

this could result in under- or overtreatment. Several contributing factors have been postulated to explain the differences between ECMO and non-ECMO patients:

Adsorption to extracorporeal components

After having observed increased sedative requirements in ECMO patients, several research groups have tested and described the disappearance of sedatives from the lumen of the extracorporeal circuit. *In vitro* tests with small sections of tubing have shown that the loss is caused by adsorption to the polymers that make up the (lining of) the extracorporeal circuits.[11-13] This effect has been described for different drugs, and there are signs that highly lipophilic drugs (such as opiate analgesics, sedatives) are more prone to adsorption than more hydrophilic drugs. Since adsorption is polymer-specific, the type and quality of materials used to create the circuit tubing or oxygenator membranes probably influences adsorption.[14, 15] The size of the membrane surface and tubing diameter in relation to the total circulating volume might cause maximum adsorption capacities to differ between pediatric and neonatal systems, or between different ECMO centers depending on whether they use silicone rubber or polypropylene membranes.

Hemodynamics and fluid balance

ECMO treatment requires supplementation of the circulating volume with albumin, erythrocytes and electrolyte solutions. Direct contact between blood and the foreign material of ECMO tubing generally causes a temporary increase in circulating cytokines, which leads to vasodilation and increased capillary leakage of proteins and leukocytes into surrounding tissues.[16-19] The latter effect will diminish in the first day after cannulation, but the distribution and total volume of circulating fluids remain altered until after decannulation. An increased distribution volume for drugs is to be expected, but altered hemodynamics might affect clearance as well. In venoarterial ECMO, the continuous blood flow provided by the rollerpump might affect renal and hepatic perfusion and subsequently drug clearance.[20] Venovenous ECMO on the other hand carries the risk of recirculation of fluids containing extracorporeally administered drugs, as the expelled blood might partially be sucked back into the inlet when double-lumen catheters are poorly positioned in the vena cava superior or right atrium.[21] The frequency of continuous venovenous hemofiltration (CVVH) among different ECMO centers ranges from incidentally to routine; its main goal is the improvement of fluid balance, but is known to affect the clearance of hydrophilic compounds and drugs with low protein binding.[22]

Recovery from critical illness

Critical illness is correlated with a reduced clearance and an altered volume of distribution, which has been described for several drugs.[23, 24] Once a patient has been placed on ECMO and the underlying critical illness is treated, organ function and drug clearance

should return to normal. This might therefore not only cause differences in volume or clearance between ECMO and non-ECMO patients, but also intra-patient variability from one day to the next. Patients with neurological trauma might require hypothermia to minimize ischemic damage, but this could lead to reduced metabolism and therefore clearance.[25]

Ontogeny

Especially in neonatal patients, metabolic pathways might not have reached their mature level of enzyme expression yet. Combined with the immature renal system and reduced glomerular filtration rate (GFR), the capacity to metabolize and excrete drugs in the early neonatal period might be reduced. Depending on the mechanism(s) involved, weight, gestational age or postnatal age can be important predictors of clearance and volume of distribution.[26-28] Even in ECMO patients, we might see evidence of improving clearance or distribution volume over time due to the developmental status, but considering the short duration of ECMO (10 days on average) and the total impact of other ECMO-related factors, the maximal effect is probably limited.

Construction of ECMO-specific dose regimens

In general, the duration and intensity of a drug's effect is correlated to its concentration or exposure over time. The blood or plasma concentration at each time point is a function of, among other parameters, the volume of distribution and clearance rate. In order to design dose regimens, we need to know these parameters for each drug, preferably with a measure of the inter-individual variation in the ECMO population. Up to now, these have only been available for a limited number of drugs, including midazolam [29], morphine [30, 31], gentamicin [32, 33], vancomycin [34], ranitidine [35] and bumetanide.[36] This means that for a considerable number of commonly used drugs during ECMO, no PK information is available. Therefore, blood concentrations of these compounds should be measured in individual patients. This requires blood to be sampled, assay of drug and metabolite concentrations therein, and calculation of PK parameters, followed by simulation of different dose regimens. Although these studies are necessary to properly dose regularly used drugs, they do not help in selecting the right dose of a new drug for which PK data have not been collected yet. By combining the data from studies with different drugs, we might be able to identify characteristics of drugs, ECMO systems or patients that have predictive value for the changes in clearance or volume of distribution that will occur. To this end, it is vital to do extensive covariate screening and to compare data from different drugs, ECMO systems and patients.

Ethics and feasibility of clinical-pharmacological research in ECMO patients

Classic PK studies require frequent sampling of blood and other body fluids at designated intervals after a dose. After quantification of drugs in these samples, concentration-time curves are plotted from which parameters are estimated for each studied individual. When a target concentration or exposure has been established, these can be used to calculate an appropriate dose regimen. Unfortunately, this approach cannot be used in neonates and infants; the required sampling frequency and volume would result in too much blood being extracted. For ethical reasons, blood withdrawal in neonates is only allowed during occasions at which blood is sampled for clinical reasons as well, for instance for routine clinical chemistry measurements and as long as it remains less than 5% of the circulating volume. From ECMO patients however, blood can be drawn from an extracorporeal port without discomfort or harm to the patient, provided the total volume of blood remains small. A viable alternative to full PK curves is a limited sampling study design with fewer sampling times and smaller volumes of blood, followed by population pharmacokinetic modelling using nonlinear mixed effects modelling (NONMEM) software.[37, 38] NONMEM provides the opportunity to combine samples from all patients to estimate PK parameters for the average patient, followed by back calculation of the individual parameters based on the difference between individual observations and the population average. It also allows sparse sampling strategies without fixed protocol times, making it ideally suited to calculate PK parameters from heterogeneous data acquired during routine care. To reduce the sample burden per patient even further, analytical-chemical methods are required that can be used to quantify drugs selectively and sensitively in small amounts of biological material. Preferably, these methods can be used to quantify multiple drugs or drugs and their metabolites in the least amount of blood or plasma possible. Liquid chromatography in combination with mass spectrometry detection (LC-MS) is particularly suited for this purpose because of its sensitivity and its capacity to selectively detect and quantify multiple analytes in a single sample. [39] By sampling ECMO patients during routine sampling rounds, and by measuring as many drugs as possible in each aliquot of blood, it is possible to evaluate as much data as possible from each patient without harm or additional discomfort to individuals.

Aims and outline of this thesis

For several commonly used drugs, we have no appropriate dosing guidelines for ECMO patients. To get insight into the pharmacokinetics of drugs during ECMO, an observational study was done at the Intensive Care of the Erasmus University Medical Center-Sophia Children's Hospital, in close cooperation with the department of Hospital

Pharmacy. By using blood samples taken during routine care and medication data from the computerized patient data management system (PDMS), drug concentrations were determined and pharmacokinetic models were created to produce dosing recommendations for this specific group of patients. *In vitro* and *ex vivo* tests were done to assess the impact of drug and circuit characteristics on drug adsorption. This thesis describes the results, with the overall objective to optimize drug therapy in ECMO patients.

These studies required blood to be sampled and drugs and metabolites to be assayed therein. The required methods are described in part I of this thesis. Chapter 2 provides an overview of the practical aspects of clinical-pharmacological research in children, with a selection of sampling and assay methods. Chapters 3, 4 and 5 describe assay methods that have been developed to quantify drugs in small aliquots of plasma using LC-MS.

Part II (chapter 6) contains a study with *in vitro* and *ex vivo* ECMO circuits. By injecting known amounts of drugs and measuring concentrations over time, the drug loss due to adsorption or other causes could be quantified. The comparison of used vs. new circuits, pediatric vs. neonatal circuits and centrifugal vs. roller pump circuits provides insight into the mechanisms of drug loss. This could lead to a prediction of adsorption behaviour for new and non-studied drugs.

Part III describes the population pharmacokinetic models created using data from ECMO patients. These models were used to simulate dose regimens, leading to dose recommendations. A PK model was made for cefotaxime and its metabolite desacetylcefotaxime, and used to validate the current dose regimen (Chapter 7). Sildenafil and its metabolite desmethylsildenafil PK were studied in post-ECMO patients (Chapter 8.1) and during ECMO (Chapter 8.2). Chapter 9 contains a PK model for midazolam and its principal metabolites, by which an optimal dose regimen was simulated and the theoretical contribution of each component to overall sedation could be calculated.

In the general discussion (chapter 10), we summarize our findings and discuss the strengths and weaknesses of our methods. By combining our PK models with those previously published, we examine whether patient and drug characteristics can be used to predict (future) drug behaviour during ECMO, so that dosing recommendations can be made. Finally, suggestions are given for future research.

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Part I

Methods for pediatric pharmacological research

Chapter 2

Sample collection, biobanking and drug assay in pediatric clinical pharmacology

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Handb Exp Pharmacol 2010, in press

Abstract

Pediatric pharmacokinetic studies require sampling of biofluids from neonates and children. Limitations on sampling frequency and sample volume complicate the design of these studies. In addition, strict guidelines are in place, designed to guarantee patient safety. This chapter describes the practical implications of sample collection and storage, with special focus on the selection of the appropriate type of biofluid and withdrawal technique. In addition, we describe appropriate measures for storage of these specimens, for example in the context of biobanking, and the requirements on drug assay methods that they pose. Pharmacokinetic studies in children are possible, but they require careful selection of an appropriate sampling method, specimen volume and assay method. The checklist provided could help prospective researchers with the design of an appropriate study protocol and infrastructure.

Introduction

The ICH guidelines on pediatric drug studies emphasize patient safety, which has consequences for the volume of, and methods for, sampling of biofluids.[1, 2] This has consequences for pharmacokinetic studies in children by imposing challenges upon sample collection and drug analysis. The main questions are:

- Which biological specimen can be used and how can this specimen be collected in the intended study population?
- What are the maximum allowed sample volumes per occasion and per study period?
- By what methods can we store samples and acquire reliable drug concentrations?

In this chapter we discuss these questions and aim to provide practical solutions and examples to help those planning pharmacokinetic studies in children.

Sample types and Collection techniques

Blood

For ethical and practical reasons, extensive blood sampling for traditional pharmacokinetic analyses is usually not possible in this population. No official guidelines exist on the maximum amount of blood that can be sampled in pediatric studies. However, several guidelines accept a maximum of 3-5% of the total blood volume per 4 weeks.[3, 4] In the case of simultaneous trials, the recommendation of 3-5% remains the maximum. In addition, repeated blood sampling in children by repeated punctures can be considered non-ethical due to associated pain, anxiety and distress. Safety can be improved by reducing the burden associated with invasive blood sampling and by using methods aimed to reduce the blood volume needed for drug analysis. In children, blood for pharmacokinetic analysis is preferably sampled from indwelling central venous or arterial catheters, already in place for clinical care. Sampling from these catheters is easy, causes minimal or no discomfort and allows for sufficient sample volume collections. Risks associated with the use of these catheters are blood-stream related infections and unintended blood loss. If these catheters are not already in place for clinical purposes, placement of these catheters solely for research purposes is usually not considered acceptable by research ethics committees and/or patients and their family. Alternatively, blood can be sampled from peripherally inserted catheters. Insertion of these catheters solely for research purposes is sometimes acceptable, more specifically in the context of therapeutic drug trials. The main limitation, especially in neonates, is that blood draws are difficult if not impossible from small bore catheters as used in this population. To overcome this problem, blood can be taken from heel prick or venepuncture, the latter being much less painful, preferably when done together with regular blood work. When combining research blood sampling

with regular blood work, the burden may be considered acceptable by children, parents and ethics committees. Disadvantages of heel prick samples are the limitation in the volume, timing of blood samples and painfulness. In general, blood volumes sampled per heel prick are limited to 0.5-0.6 mL, including blood collected for regular clinical blood work. Hence, blood volumes needed for pharmacokinetic analysis should preferably not exceed 0.2-0.3 mL per sample. In addition, timing of sampling is restricted if it needs to coincide with clinical blood sampling. Consequently, extensive and timed sampling for a full pharmacokinetic wash out curve is not possible using this method.

A more detailed and practical guide for different blood sampling methods in children and neonates has been published by the UK Medicine for Children Research Network.[5]

Urine

For renally cleared drugs, urine may provide an alternative to blood for the estimation of pharmacokinetic parameters. Also, urinary excretion of the drug and its metabolites may provide valuable insight in developmental changes in drug metabolism and excretion.[6-8] From a pharmacokinetic point-of-view, the preferred collection method is by urinary catheter or (in older children) direct collection. Using a catheter facilitates complete urine collection over predefined time-periods. Major limitations of the urinary catheters in children are the burden and risks associated with insertion of the catheter, such as pain, infection, urethral restriction (mainly in boys) and displacement. Hence, in general, if a catheter is not already in place for clinical purposes, most research ethics boards will not approve its use for sole research purposes. Adhesive collection bags are also frequently used to collect urine, especially in infants and neonates.[7] In these younger infants, the repeated use of adhesive urinary bags may result in skin abrasion. Skin abrasion is not only painful and causes discomfort; it may also increase the risk of invasive infections in a vulnerable population. The "gauze/cotton ball method" can be used alternatively. A small gauze with cling film (the latter facing the diaper material to prevent urine absorption in the diaper) is put in the diaper and urine is collected by expressing the urine from the gauze.[9] In a similar fashion, non-absorbent diapers can be used.[10] An important limitation of both the bag and gauze/diaper collection methods is that complete urine collection is usually impossible. Urine may leak along the bag into the diaper and not all urine can be expressed from the gauze/diaper. This limitation can be overcome by weighing the diapers to estimate total urine volume and to multiply volume with urine drug concentrations to be able to estimate total urinary drug or metabolite excretion.

Saliva

Saliva can be used as a non-invasive alternative to blood for a significant number of drugs, e.g. caffeine, anti-HIV drugs, anticonvulsants, digoxine and codeine.[11] Saliva can also be used for DNA sampling. It can be collected by simply asking children to spit in

a cup. For DNA sampling, specific cups are available containing anti-DNAse solutions. Understandably, this method is only feasible in older children (>8 yrs of age) who are capable to understand and follow simple instructions.

Younger children can chew on a gauze, cotton 'salivette' or a cotton-cellulose eyespear, from which saliva can be extracted. Citric acid containing products may stimulate saliva production and enhance collection. Several commercially available methods for saliva collection are available.[11] In preterm infants, commercially available products, such as salivettes are difficult to use. The cotton swabs are relatively large compared to mouth size. In addition, the volume of saliva needed to get enough sample after extraction from the cotton is considerably higher than preterms can provide. Before deciding to use one of these methods, it is important to validate the intended method by studying the correlation between blood concentrations and saliva concentrations. Saliva drug concentrations for instance may vary according to whether citric acid is used in the saliva sampling process.[12, 13]

Breath samples

Breath tests using stable or radioactive isotopes are used in the context of drug metabolism studies.[14, 15] Sampling of exhaled, labeled CO_2 is easiest when children can follow instructions to breath into a balloon, from which breath samples can be taken. In younger or critically ill children this approach is obviously not feasible. The collection of respiratory CO_2 used in children, including neonates, was originally done via trapping of CO_2 in sodium hydroxide. This method involves a tight-fitting facemask and passing of the expired air through a condenser containing sodium hydroxide.[16] This is impractical and difficult in neonates. Alternatively, a direct nasopharyngeal sampling technique can be used.[17] This technique allows for direct sampling from the nasopharynx using a gastric tube attached to a syringe, or direct attachment of a syringe to a side-port of the endotracheal tube. During observed expiration, the researcher collects air by pulling the syringe. The collected air is then transferred to a vacuum tube for laboratory analysis.

Meconium

Accumulating from the 12th gestational week until birth, meconium acts as a reservoir for exogenous compounds, such as drugs and metabolites. Drugs are incorporated into meconium through swallowing drug-contaminated amniotic fluid or via biliary excretion. Meconium analysis is thought to detect maternal drug use during the second and third trimesters. Meconium passage occurs in the first 1 to 3 days after birth, but may be prolonged in preterm infants. It can be collected easily by scraping from diapers. 0.5 to 1g of meconium is usually enough for toxicological, quantitative analysis. Contamination of meconium with urine may occur and might obscure the results. Storage at room temperature may reduce the concentrations of drug by degradation. After collection,

meconium can be stored at low temperatures (-20°C). However, due to its complex composition of epithelial cells, swallowed amniotic fluid, bile salts, lipids, other endogenous compounds, and xenobiotics until birth, extraction of drugs is difficult.[18]

Hair

Drugs can be incorporated into hair via blood supply to the hair follicle, by external exposure (e.g. through smoke) or through secretion from sweat and sebum adjacent to the hair follicle.

Hair samples are mainly used for toxicological screening in prenatal alcohol and drug exposure. These samples are best collected from the back of the head. The proximal zone (i.e. the zone which is closer to the root) should be clearly indicated if segmental analysis is to be performed. The sample can then be stored and transported light and moisture protected at room temperature. Since hair grows about 1 cm per month, segmental analysis can be done to estimate the time window of drug exposure.[19]

Leftover material and biobanking

In addition to freshly collected blood samples in the context of a single pharmacokinetic study, the use of leftover or previously stored blood samples should be considered. This may significantly reduce the burden to the individual child participating in a trial. For example, leftover material from regular patient blood work could be used for pharmacokinetic analysis of drugs that the patient is taking therapeutically. As the sample volume available will likely be small, very sensitive analytical techniques, to be described below, are required. This approach has several advantages. First, the pharmacokinetic results will reflect the real life clinical situation, as the drug is studied in the population that actually needs the drug for treatment. Second, the need for additional blood sampling is limited or nonexistent, which can significantly reduce the burden to individual patients. This may even result in a higher informed consent rate from the child and/or his parents to participate in the study.

Blood could be sampled routinely for bio-banking purposes from all consenting children/parents on a specific ward or with a predefined disease for later studies, providing blood sample volumes are within acceptable limits. In this context, biobanking is defined as collection of biological material and the associated data and information stored in an organised system, for a population or a large subset of a population.

This approach is taken in large-scale pharmacogenetic studies in the adult population. Anonymous linking of clinical data may provide researchers with ample opportunity to study multiple research questions. Ethics committees and subjects will generally be amenable to long-term sample storage for future research, provided that there are

sufficient assurances that stringent processes and standards for patient privacy/confidentiality are in place.

When previously collected samples are necessary to perform a new study, it may still be possible to obtain consent from the original participants. However, the consent procedure may vary depending on the source of original data and the intended purpose, see [20, 21] for a discussion on the ethical aspects of using these data. Some have advocated the renewal of consent once former study participants reach adulthood, particularly because the sharing of genetic and phenotypic data could have consequences that were unforeseen at the time of parental consent decades earlier.[22] Although not directly related to biological fluids, the use of *digital* leftover material, i.e. the combination of existing pharmacokinetic data sets from medical literature, may significantly reduce the need for prospective pharmacokinetic trials. This could for instance be used to study the effect of age and other covariates in the pediatric population.[23, 24]

Storage and shipping

According to the Good Clinical Laboratory Practice (GCLP) guidelines issued by the World Health Organization, samples should be kept 'in such a way as to ensure the integrity and accessibility to the material retained'.[25] Good Clinical Practice (GCP) guidelines state that national legislation determines the minimum period during which data records and material should be stored. The samples should be stored to allow (re-) examination, but only for as long as the quality permits evaluation, i.e. for as long as analyte levels can be reliably requantified without excessive degradation. This requires simulation of average and worst-case conditions in sampling, storage and shipping to see the effects on sample integrity. The consequences of different storage and handling protocols for the analytical results of each type of sample is too big a topic to be discussed in this brief overview; the reader is referred to the excellent review by Mehta regarding pre-analytical considerations in drug assays.[26]

The protection of subject confidentiality should be maintained not only in reports of final results but also in the preceding steps, i.e. during storage, shipping and drug assay. This requires storage in coded vials, with access to the original subject data restricted to specific individuals (usually the researchers directly involved in sampling, storage and data extraction). To maintain sample integrity, appropriate measures should be taken to guarantee the right temperature, protection from light, etc. throughout the pre-assay period. These measures might for instance include the use of refrigerators or freezers with continuous temperature registration, and should include standard operating procedures (SOP) describing responsibilities of the individuals involved in sampling and

shipping. For more information on the GCLP guidelines, the reader is referred to the relevant literature.[25, 27] Practical examples of necessary measures are also available.[26, 28-30]

Drug assays

Quantification of analytes in pediatric studies is complicated by the limited availability (both in numbers and volume) of biological specimens. The analytical methods should therefore be sensitive enough to quantify compounds in complex mixtures (such as blood, plasma or cerebrospinal fluid), in sample volumes of 10 to max. 100 µL. Ideally these so-called 'micro-assays' can be used to quantify different analytes of interest in the same sample, e.g. drugs and their metabolites or combinations of co-administered drugs.

Assay methods

The required sensitivity can be reached using mass spectrometric techniques such as liquid (LC-MS) or gas (GC-MS) chromatography-mass spectrometry. These techniques rely on chromatographic separation of analytes from each other and from matrix components, followed by ionization and counting of analytes of a selected molecular mass. Compounds of similar mass can be distinguished via mass filters that allow a single analyte to be selected in the presence of other drugs, metabolites or endogenous compounds. To enhance selectivity even further, the selected compounds can be subsequently fragmented by collision with an inert gas. This leads to molecular fragments that are highly specific for the original drug or metabolite. After selection of one of these fragments via another mass filter, the compound of interest can be quantified. This is called tandem mass spectrometry or MS/MS (Figure 1). The mass spectrometric techniques carry a distinct advantage over other sensitive assays such as enzyme-linked, fluorometric and radioassays. Whereas the latter often suffer from cross-reactivity between structurally related compounds such as drugs and their metabolites or endogenous substrates [31-35], the mass spectrometric methods allow simultaneous quantification of different analytes in a single run by rapidly changing the mass filter settings.[36, 37] Another separation method, that can be used in combination with mass spectrometric detection with small sample volumes (especially for the assay of different enantiomers), is capillary electrophoresis (CE-MS) [38, 39], but due to wide experience and superior sensitivity, LC-MS and GC-MS remain the cornerstones of drug microassays. Whereas urine and serum or plasma are the main biofluids in experimental pharmacology, some biochemical markers and drugs can also be quantified in extracts from dried blood spots. This poses additional requirements on the assay method and its validation, drug or metabolite stability and the availability of reference values for drugs concentrations in whole blood. [40, 41] Nevertheless, the logistic advantages are appealing. Dried bloodspot collection al-

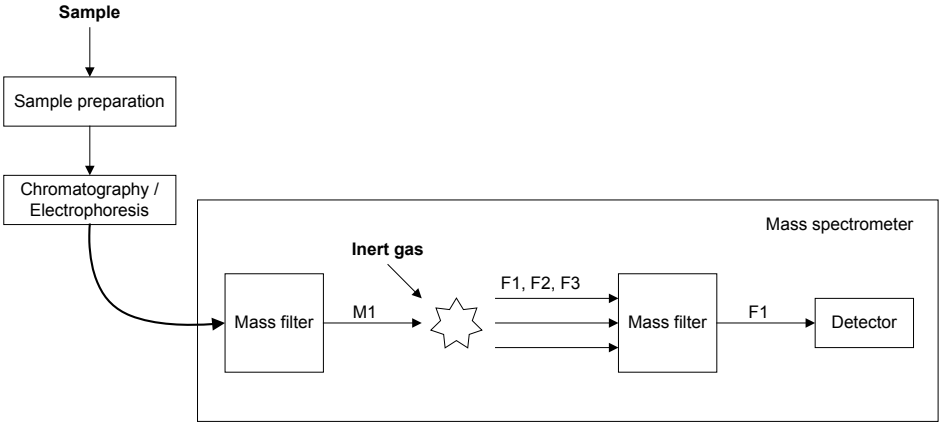

Figure 1. Principle of liquid or gas chomatography with tandem mass spectrometry detection. The sample is cleaned up for chomatographic separation of drugs and metabolites from matrix components. After chomatography or electrophoresis, the effluent enters the mass spectrometer. After selection of molecules of a specific molecular weight (M1) by the first mass filter, the molecules are fragmented with an inert gas. The resulting fragments (F1, F2, F3) are sent though the second filter in which one individual fragment is selected to be quantified at the detector.

lows sampling at remote locations, if necessary even by patients or parents themselves. Once dry, the blood spots can be sent via regular mail services to a central laboratory, without many additional measures to minimize biohazard. Even samples from large multinational studies can then be processed at a single laboratory, which greatly improves efficiency regarding method validation at different study sites, refrigeration or freezing during sample storage and transport, reliability of assay results due to increased experience with the assay and reduction of inter-laboratory variability, etc. Re-assay however is usually not possible, since most methods require the entire dried drop of blood to be processed. So far, there are few published assay methods with dried blood spots but their number seems to be increasing; examples include the quantification of topiramate [42], everolimus [43], tacrolimus [44], metformin [45] and anti-retroviral drugs. [46]

Sample preparation

Endogenous and exogenous components of the biological matrix can interfere with sample preparation or quantification, which compromises accuracy and precision. The mechanisms of these so-called 'matrix effects' are not fully understood, but have been linked to co-elution of different compounds (including inorganic ions and plasma phospholipids) that can interfere with analyte ionization.[47, 48] The degree of signal enhancement or suppression could obviously vary from individual to individual, but also within individuals upon changes in physiological constitution, either due to disease progression or growth and maturation. This implies that validation of the assays should include an evaluation of matrix effects in biological specimens ('blank matrix') from the

intended patient population. The US Food and Drug Administration has issued guidelines on the validation requirements for bioanalytical chromatographic methods [49] without mentioning a specific method to assess matrix effects; current reports on new LC-MS and GC-MS assays often contain a qualitative visual assessment or a quantitative calculation based on work by Matuszewski *et al*.[50, 51]

Samples are cleaned up via solid phase extraction (SPE) or liquid-liquid extraction (LLE) to separate analytes from interfering components and, if possible, to concentrate the analyte in a smaller volume to increase sensitivity.[52, 53] In pediatric and neonatal studies in particular, sample volumes are small. When volumes become too small to reliably be transferred from one vial to another, sample preparation can be a challenge, and pre-assay concentration in a smaller volume impossible. Therefore, microassays often contain minimal sample preparation (i.e. protein precipitation or direct injection, also called 'dilute-and-shoot') and rely heavily on the chromatographic prowess of LC-MS or GC-MS equipment to maintain accuracy and precision without matrix effects. When extensive clean-up cannot be avoided, it is possible to use LLE with minimal amounts of organic solvents or sophisticated and expensive micro-techniques such as 96-wells SPE.[54-56]

Multiple analyte assays

The efficiency of pharmacological studies can sometimes be increased by quantifying multiple analytes in a single sample, since it requires less biological material per patient whilst reducing the total analytical workload. Multiple-analyte assays have been developed for drugs and their main metabolites [57-59] and for drugs from different therapeutic classes that are often co-prescribed in specific patient populations.[60-62] Especially for biobanked samples, these assays can be used to maximize scientific output from limited sample volumes. This requires careful selection of sampling times in relation to the expected dose regimens to allow reliable estimation of pharmacokinetic parameters.

Alternative drug matrices

For studies on fetal drug or pollutant exposure, compounds are assayed in unusual biological matrices such as meconium, hair or cord blood. For these biofluids, it may be even more difficult to find suitable blank material. For meconium in particular, special sample preparation methods may be necessary to prepare solutions that are suitable for LC-MS or GC-MS analysis. See [18, 63-66] for examples of analytical methods that were developed or validated specifically for these matrices.

Conclusion

Pharmacological studies in children are possible, but require careful selection of an appropriate sampling method and sample volume. An assay method should be developed and validated, with special attention to the required sensitivity level, matrix effects and sample preparation. See Table I for a checklist of the main questions that should be addressed when designing a pediatric study.

Table I. Items to be considered before engaging in a pediatric study involving sample collection and drug quantification

Sample collection
- What are the maximum allowed sample volumes per occasion and per study period ?
- Which sampling times are informative (based on population PK study design) and practical?
- Is blood the preferred biological specimen or can alternatives be used?
- How much sample is required, taking into account the intended assay requirements and potential future studies into different analytes with left-over material?
- Which sampling methods are suitable?
- Can these methods be implemented as part of routine clinical procedures and/or do involved staff need extra training?
Biobanking and leftover samples
- Have patients or their guardians given permission for biobanking and use of leftover samples?
- Is there a separate long-term storage facility with temperature control, compliant with GCP guidelines available?
- How are patient data being recorded; is the database suitable for (anonymized) long-term storage and extraction?
- Who decides whether to grant individual researchers access to samples and data, and is there a system that allows tracking of individual samples and researchers that use them?
- Which departments or individuals are responsible for maintenance, logging access rights to samples, etc.?
Shipping and storage
- How stable are the biofluid specimens and analytes at standard storage conditions: ambient temperature (20-25°C), refrigerated (4-7°C), frozen (-20°C), deep-frozen (-80°C)?
- What are the average and worst-case shipping conditions and time?
- Is privacy of study participants guaranteed during shipping and storage?
- What arrangements have been made to allow sampling, storage and processing outside standard working hours, in weekends and on holidays?
- Have roles and responsibilities been agreed upon by the clinical department, logistical services and the laboratory?
- Have standard operating procedures (SOP) containing contact details, storage conditions etc. been agreed upon by and made available to the involved staff?
Assay
- How much sample is required for the assay, taking into account re-assay in case of instrument failure?
- Are the expected concentrations within the assay's calibration range?
- Has the assay been validated for this specific type of sample and analyte?
- Have matrix effects been evaluated in appropriate batches of blank matrix?
(preferably from patients of the intended age, co-medication and disease state)

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Chapter 3

Micro-analysis of β-lactam antibiotics and vancomycin in plasma for pharmacokinetic studies in neonates

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Antimicrob Agents Chemother 2009, 53(1), 75-80

Abstract

Rational dosing of antibiotics in neonates should be based on pharmacokinetic (PK) parameters assessed in specific populations. PK studies in neonates are hampered by the limited total plasma volume, which restricts sample volume and sampling frequency. Available drug assay methods require large sample volumes, are labor-intensive or time-consuming. The objective of this study was to develop a rapid ultra-performance liquid chromatographic method with tandem mass spectrometry detection for simultaneous quantification of amoxicillin, meropenem, cefazolin, cefotaxime, deacetylcefotaxime, ceftriaxone and vancomycin in 50 µL of plasma. Cleanup consisted of protein precipitation with cold acetonitrile (1:4) and solvent evaporation before reversed phase chromatographic separation and detection using electrospray ionization tandem mass spectrometry. Standard curves were prepared over a large dynamic range with adequate limits of quantification. Intra- and inter-run accuracy and precision were within 100±15% and 15%, respectively, with acceptable matrix effects. Coefficients of variation for matrix effects and recovery were less than 10% over six batches of plasma. Stability in plasma and aqueous stocks was generally sufficient, but stability of meropenem and ceftriaxone in extracts could limit autosampler capacity. Instrument runtime was approximately 3.50 min per sample. Method applicability was demonstrated with plasma samples from an ECMO-treated neonate. Different beta-lactam antibiotics can be added to this method with additional ion transitions. Using ultra-performance liquid chromatography mass spectrometry, this method allows simple and reliable quantification of multiple antibiotics in 50 µL of plasma for PK studies in neonates.

Introduction

Successful drug therapy depends on administration of the appropriate dose after the right dose interval, which implies an understanding of the drugs' pharmacokinetic (PK) profile. This is particularly relevant for the treatment of neonatal and pediatric patients, considering potential variation in PK parameters due to an individuals developmental stage or specific morbidity. Therefore, PK parameters should be assessed in the specific populations to which the drugs are given to prevent undertreatment or toxicity. Unfortunately, pharmacokinetic studies in neonates are complicated by the limited amount of biological material (e.g. blood, plasma) available, which poses restrictions on sampling frequency and sample volume.[1] Moreover, the number of patients participating in these studies is often low, especially when studying patients of narrowly defined age groups, with specific (co-) morbidity or during treatment with extracorporeal techniques. Ideally, analytes of interest should be quantified simultaneously in as little sample as possible. This limits the burden on individual patients caused by sampling, while maintaining sufficient data points for reliable data analysis.

For years, drugs have been quantified via high-performance liquid chromatography with ultraviolet detection (HPLC-UV). Many of the published bio-analytical methods require sample volumes of 250 µL or more.[2-6] Some are labor-intensive [2, 3, 7] or require long runtimes [3, 7, 8], potentially leading to poor reproducibility for analytes that may decompose during analysis, such as certain β-lactam antibiotics. Analytes had to be chromatographically separated from the interfering endogenous and exogenous matrix components, and often an elaborate sample preparation was necessary to reach sufficient selectivity and specificity. Co-eluting components would often interfere due to low specificity of UV-detection. With the advent of mass spectrometry (MS), selectivity greatly increased since specific analyte masses could be detected. This led to an even greater specificity when mass spectrometers were set up in sequence (tandem mass spectrometry, or MS/MS): now, not only a specific mass could be identified, but a specific fragmentation pattern could be monitored to differentiate between analytes of the same mass.

Equipment capable of ultra-performance liquid chromatography mass spectrometry (UPLC-MS/MS) has recently become available. With a smaller particle size and higher operating pressures compared to regular HPLC, UPLC provides a shorter runtime and sharper peak shape, which improves sensitivity and reduces potential interference by matrix components.[9-11] UPLC combined with tandem mass spectrometry should therefore allow quantitative analysis of multiple analytes with minimal sample preparation and matrix effects.

Currently, clinical studies in the Sophia Children's Hospital include pharmacokinetic evaluations of multiple antibiotics in patients receiving extracorporeal membrane oxygenation (ECMO) treatment. In order to facilitate these studies, a simple and reliable

method was developed to simultaneously quantify amoxicillin, meropenem, cefazolin, cefotaxime, deacetylcefotaxime, ceftriaxone and vancomycin in 50 µL of plasma. This chapter contains a description of the method, its validation and its applicability using data from an ECMO-treated patient.

Materials and methods

Reagents

LC-MS grade water, liquid chromatography grade methanol and acetonitrile were from Biosolve (Valkenswaard, Netherlands). Formic acid (FA, Sigma, Schnelldorf, Germany) was analytical grade. The following reference standards were purchased from Sigma (FA, Sigma, Schnelldorf, Germany): ceftriaxone (CRO), vancomycin (VAN), cefazolin (CFZ), cefotaxime (CTX) and oxacillin (OXA). Deacetylcefotaxime (DACT) was kindly provided by Sanofi-Aventis (Gouda, Netherlands). Meropenem (MEM) was from Molekula (Wimborne, United Kingdom) and amoxicillin (AMX) from Certa (Braine-l'Alleud, Belgium).

Quality control samples and standard solutions

Standard stock solutions containing β-lactam antibiotics were prepared by dissolving the required amount of antibiotic (calculated as free base) in 25 mL of water. Vancomycin solutions were prepared separately to prevent potential accelerated degradation of other antibiotics.[12, 13] Varying quantities of stock solution were diluted with water, resulting in 8 working standards over the concentration range varying from the lower limit of quantification (LLOQ) to the upper limit of quantification (ULOQ). Calibration standards were prepared by diluting 1 part working standard with 9 parts human plasma. Quality control (QC) samples for intra- and inter-assay comparisons were similarly prepared using a separate stock solution and stored at -80°C; low (L), medium (M) and high (H) controls were prepared at concentrations of respectively 3-4 × LLOQ, 40% of ULOQ and 75% of ULOQ. A stock solution of the internal standard (IS) was prepared by dissolving 10 mg of oxacillin in 50 mL of water. Prior to analysis, 1 part stock solution was added to 99 parts chilled acetonitrile. This precipitant solution was freshly prepared before each analysis.

Sample preparation

To 50 µL of plasma, 200 µL of chilled acetonitrile containing IS was added. The sample was mixed (5°C, 1,250 rpm) for at least 15 min to complete protein precipitation. After centrifugation at 16,000 ×g for 10 min, 200 µL of the supernatant was transferred to a clean vial. The solvent was evaporated to dryness at 40°C under nitrogen gas flow, after which the residue was reconstituted in 100 µL of 0.1% (v/v) aqueous formic acid and left to mix for 30 min (5°C, 1,250 rpm). When cloudy, samples were centrifuged again at

16,000 ×g for 10 min. The supernatant was transferred to a polypropylene autosampler vial and stored at 5°C until analysis by UPLC-MS/MS.

UPLC-MS/MS conditions

The UPLC-MS/MS system consisted of a Waters Acquity Ultra Performance LC coupled to a Quattro Premier XE tandem quadrupole mass spectrometer (Waters Corp., Milford, MA, USA). The analytical column was an Acquity UPLC BEH C18 2.1 mm × 100 mm column with 1.7 µm particle size (Waters Ltd, Dublin, Ireland), to which a 0.2 µm pre-column filter unit was added. The mobile phase was a gradient of solution A (0.1% formic acid in water) and solution B (0.1% formic acid in methanol) with an initial composition of 20% B. Mobile phase composition changed linearly from 20% B at 0.5 min to 40% B at 1.0 min and onward to 100% B at 2.0 min. The composition was switched back to 20% B at 2.5 min and maintained until 3.0 min. The flow rate was 0.4 mL/min with a column temperature of 40°C. Ten µL of each sample was injected onto the column. Analytes were detected via MS with an electrospray ionization (ESI)-interface in positive multiple reaction monitoring (MRM)-mode. Optimized MRM-settings for the individual drugs, including cone voltage and collision energy, are listed in Table I. The acquisition settings were: capillary voltage=3.4 kV; source temperature=120°C; desolvation temperature=300°C; desolvation gas flow=500 L/h; cone gas flow=50 L/h; dwell time=80 ms.

Data analysis

Data were acquired using Masslynx V4.1 software and processed using Quanlynx V4.1 software (Waters Inc.). For all analytes except ceftriaxone, calibration curves were

Table I. Acquisition parameters[a]

	Q1 (*m/z*)	Q3 (*m/z*)	CV (V)	CE (eV)	Dynamic Range (mg/L)	Linear	Rt (min)
Glycopeptide							
VAN	725.3	143.8	25	25	0.7-70	y	0.84
β-Lactam							
AMX	366.1	349.1	18	10	0.2-80	y	0.94
CFZ	455.0	323.0	20	10	0.5-25	y	1.76
					25-250	n	
CRO	554.9	396.0	20	15	2-360	n	1.58
CTX	456.0	324.0	30	12	0.2-100	n	1.70
DACT	414.0	285.1	30	20	0.2-100	n	1.09
MEM	384.2	68.0	20	10	0.2-80	n	1.13
Internal standard							
OXA	402.0	243.1	20	15	-	-	2.35

[a] Q1, parent-ionmass; Q3, daughter-ionmass; CV, cone voltage; CE, collision energy

obtained by plotting the peak area ratios of drug vs. internal standard against the theoretical concentration. Peak height was used for CRO based on superior reproducibility. Standard curves were constructed for each analyte over the calibration range via weighted least squares regression.

Validation

The method was validated based on FDA guidelines for bioanalytical method validation.[14] Vancomycin was added at a later stage in a reduced validation procedure. The full validation procedure included the following parameters:

(a) Specificity and selectivity. Chromatograms from 3 aqueous calibration standards were compared to those of 6 batches of blank plasma and 10 patient samples before and after spiking. Ion traces of each analytes mass transition were checked for interferences at their respective retention times.

(b) Limit of quantification. The lower limit of quantification (LLOQ) was defined as the lowest concentration that could be quantified with accuracy and precision under 20%, as calculated from chromatograms of 6 independent samples.

(c) Standard curves. Curves consisting of eight points were calculated by linear or polynomial regression, each point consisting of independent triplicate measurements. Best fit was selected after exploration of different regression models and weighting factors.

(d) Accuracy and precision. Intra- and inter-run accuracy and precision were calculated for the three quality controls with six duplicate measurements each, or with measurements on six experiments done on different days. Accuracy was defined as a percentual deviation from the theoretical concentration by quantifying QC samples on a freshly prepared calibration curve. Precision was defined as the coefficient of variation (CV=standard deviation/mean of 6 measurements ×100%).

(e) Robustness. Variations in analytical conditions were mimicked based on observations of unexpected performance changes during method development: (a) signal intensity and 24 h autosampler stability of extracts (from low and high QC samples in duplicate) reconstituted in aqueous 0.1% FA were compared to those observed in samples reconstituted in water; (b) retention time and signal intensity in medium QC samples (in duplicate) at a column temp of 40°C were compared to those observed at a column temp of 30°C; and (c) signal intensities of medium QC samples (in duplicate) were compared to those of medium QC samples that were diluted pre-precipitation (1:9 with water), to see whether dilution improved analyte recovery.

(f) Matrix effects. Plasma and eluent components in the ionization chamber cause batch-specific ion suppression or enhancement, leading to inter-patient and intra-patient signal variability.[15, 16] These matrix effects were evaluated in two ways. First, extracts of six batches of blank plasma were injected whilst analytes were continuously infused into the mass spectrometer. Ion traces were recorded for each compound over the entire

runtime. Signal stability at the relevant retention time was visually assessed for each analyte over the six batches of blank plasma. Second, matrix effects were quantified as proposed by Matuszewski *et al.*[17] In short, chromatograms were recorded of plasma that was spiked pre-extraction, plasma spiked post-extraction and spiked aqueous eluent. In total, six batches of blank plasma were spiked with low and high concentrations of each analyte in duplicate. Recovery (RE) was defined as the relative signal of samples spiked post-extraction vs. pre-extraction. Matrix effects (ME) were similarly defined as the relative signal of post-extraction spiked plasma samples vs. spiked aqueous samples. Process efficiency (PE) was defined as the product of RE and ME, i.e. the overall signal of spiked plasma vs. an aqueous standard solution. Average values and coefficients of variation of RE, ME and PE were calculated over the six plasma batches.

(g) Sample stability. Storage conditions and periods were chosen to mimic those at blood collection, during long-term storage of stock solutions and plasma, during freeze-thaw cycles, at table-top during processing and in the autosampler awaiting analysis. QC samples were tested for stability over time (a) in aqueous stock solution and plasma at -80°C (¼-1-2 months), (b) in aqueous stock solutions and plasma at 5 and 20 °C and EDTA-decoagulated whole blood at 5 °C (6-18-24-48-144 hr), (c) in extracts at -80, 5 and 20 °C (6-18-24-48 hr) and (d) in extracts after three freeze-thaw cycles. Maximum storage periods were estimated, based on an allowed concentration drop of max 10%.

Method applicability: plasma levels in a neonatal ECMO patient

Patients receiving ECMO treatment were included after written parental consent. The study protocol was approved by the Institutional Ethics Committee. We present data from a term neonate with persistent pulmonary hypertension (PPHN) after meconium aspiration. Antimicrobial treatment was given in accordance with the departmental protocol and included cefotaxime 50 mg/kg b.i.d. and amoxicillin 25 mg/kg q.i.d. for suspected sepsis and a single bolus injection of vancomycin 20 mg/kg in preparation of decannulation. In total, 11 samples were taken from a pre-oxygenator access point on the circuit during the 84 hr ECMO-run. After this period, the patient was successfully decannulated and transferred to the referring NICU on conventional ventilation. Plasma levels of cefotaxime, deacetylcefotaxime, amoxicillin and vancomycin were simultaneously measured in 50 µL of plasma. Individual pharmacokinetic curves were constructed for CTX, AMX and VAN by fitting measured plasma levels to previously reported pharmacokinetic parameters using MW\Pharm software (MW\PHARM 3.58, Mediware, The Netherlands). Cefotaxime was modelled on a one-compartment model derived from data in non-ECMO neonates [18] using iterative Bayesian fitting. Amoxicillin was modelled on a one-compartment model derived from non-ECMO neonates [19] using iterative Bayesian fitting. Vancomycin was modelled on a two-compartment model derived from ECMO-neonates [20] using non-Bayesian fitting.

Results

Selectivity was achieved by the independent separation mechanisms of chromatography and tandem mass spectrometry. None of the aqueous standards, plasma standards and spiked patient samples contained interfering components. Representative ion traces can be seen in Figure 1. A minor interference in the CTX-ion trace was probably caused by (*m/z* +1) isotopes of CFZ, but CTX and the interference were chromatographically sepa-

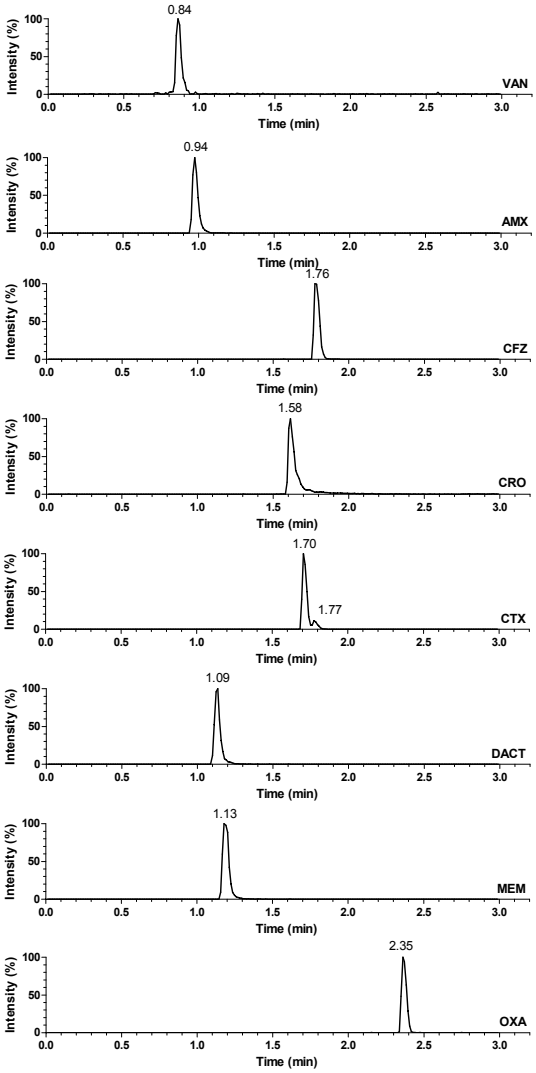

Figure 1. Representative chomatograms for a mixture of analytes in plasma, with an individual ion trace for each analyte. Most analytes have good peak shapes, with the exception of CTX (which has a peak at the retention time of CFZ) and CRO (which shows some tailing).

rated with retention times of 1.67 vs. 1.74 min. An alternative mass transition for CFZ and CTX was rejected based on loss of signal intensity. Standard curves were prepared with a weighting factor of 1/*x*. Most of the standard curves were best described via nonlinear regression. The CFZ calibration curve could be divided in a linear and a nonlinear segment, resulting in a better fit. A linear calibration curve is generally more desirable and can be applied to samples with low to medium concentrations of CFZ. We tested whether high QC samples could be diluted tenfold with blank plasma before extraction. This led to a tenfold drop in concentration while maintaining accuracy and precision (results not shown), but led to a proportional increase in the LLOQ as well. Coefficients of determination (R^2) varied from 0.994 (VAN) up to 0.998 (CTX & DACT). See Table I for the dynamic range for each analyte. The low end of the dynamic range was considered to be the LLOQ; accuracy and precision for all analytes were within 100 ± 20% and CV <20%, respectively. See Table II for intra-run accuracy and precision. Inter-run accuracy and precision were similar, with accuracy and precision of within 100 ± 15% and CV <15% for all analytes.

Table II. Intra-run accuracy and precision (n=6 for each concentration)

	Accuracy			Precision		
	L	M	H	L	M	H
VAN	95.3%	103.6%	101.9%	9.9%	7.6%	6.7%
AMX	107.4%	101.2%	106.5%	8.4%	5.4%	4.7%
CFZ	105%	104.8%	105.2%	11.3%	9.2%	7.3%
CRO	117.2%	99.4%	99.9%	8.0%	10.1%	7.6%
CTX	107.7%	95.3%	99.5%	4.8%	7.5%	4.9%
DACT[a]	-	-	-	-	-	-
MEM	99.3%	93.0%	95.0%	7.9%	6.0%	10.5%

[a] not determined, assumed to be similar to CTX

Robustness

Reconstitution with water instead of 0.1% FA improved signal intensity for CFZ (+8%), DACT (+10%) and CRO (+300%); signal intensity of the other analytes was unaffected.24 h degradation (autosampler, 5°C) however was considerably worse in water for MEM (-67 vs. -40%), AMX (-50 vs. -7%), CTX (-45 vs. -19%) and DACT (-38 vs. -20%). A decrease in column temperature to 30°C led to longer retention times without a deterioration of signal intensity, peak shape and resolution. Tenfold sample dilution led to a correspondingly decreased signal intensity for all analytes except VAN; dilution of samples containing VAN likely improved sample clean-up resulting in relatively high signal intensity.

Matrix effects

Visual inspection of chromatograms of plasma injected during T-infusion revealed a signal loss of roughly 30-50% due to matrix components. Inter-plasmabatch variability appeared

small and there were no sudden signal loss or peaks around the respective retention times. ME, RE and PE were similar for low and high QC-samples, see Figure 2 for the high QC samples. PE varied between 20% (VAN) and 75% (CRO) with notable ME and RE for each analyte. CRO was the only analyte with ion enhancement due to matrix components, as opposed to the ion suppression seen with the other analytes. ME and RE coefficients of variation over the six different plasma batches were under 10% for each analyte.

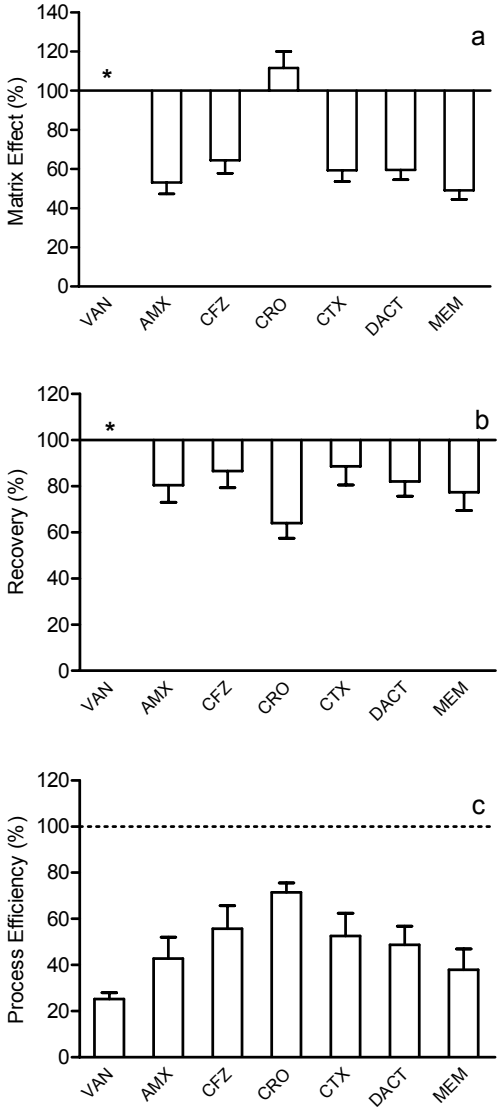

Figure 2. Matrix effects (a), recovery (b) and overall process efficiency (c) of analytes in high QC samples. Values are averages with their corresponding 95%-confidence intervals. For vancomycin, only process efficiency was tested.

Sample stability

Analytes were stable for at least 2 months in water and plasma at -80°C. Maximum storage periods of aqueous solutions, plasma, whole blood and extracts (Table III) are based on a maximum degradation of 10%. After three freeze-thaw cycles (n=2 for both low and high QC concentrations), average remaining concentrations in extracts were at least 90% of the initial concentration, except for MEM (79%).

Table III. Maximum in-process and autosampler storage period (in h)

	Stock		Plasma		Blood	Extract		
	5°C[a] (n=3)	20°C[b] (n=3)	5°C[a] (n=3)	20°C[b] (n=3)	5°C[b] (n=2)	-80°C[b] (n=2)	5°C[b] (n=2)	20°C[b] (n=2)
AMX	144	48	36	24	24	48	24	24
CFZ	144	48	144	48	48	48	12	12
CRO	144	36	24	24	12	48	8	4
CTX	144	48	48	12	6	48	18	18
DACT[c]	144	48	144	12	6	48	18	18
MEM	144	48	36	6	18	48	6	3
OXA	144	48	144	48	48	48	48	48

[a] Maximum testing period was 144 h
[b] Maximum testing period was 48 h
[c] Corrected for CTX-degradation

Measurement of plasma levels in a neonatal ECMO patient

Plasma levels of CTX, DACT, AMX and VAN were measured simultaneously in the 50 µL plasma samples and successfully fitted to the previously reported models. Figure 3 contains individual concentration-time curves and measured concentrations. Individual parameters were as follows: elimination rate constant (k_{el})=0.197 hr^{-1} and volume of distribution (V)=0.912 L/kg for CTX; k_{el}=0.330 hr^{-1} and V=0.713 L/kg for AMX. Clearance=7.63 L/h/1.85m^2, V_1=1.03 L/kg, k_{12}=1.5 hr^{-1}, k_{21}=2.634 hr^{-1} for VAN.

Discussion

We report the development of a fast UPLC-MS/MS method with an analytical performance meeting FDA specifications. The required plasma volume is 50 µL, which is sufficient to allow reinjection. If the latter is not deemed necessary, the sample volume could probably even be reduced to 20 µL.

This method can be expanded for the quantification of other β-lactam antibiotics by scanning additional mass transitions. We have for instance tested ceftazidime and cefuroxime with sufficient retention, signal intensity and peak shape. From the presented group of antibiotics, only CRO had a potentially problematic peak shape, but accuracy and preci-

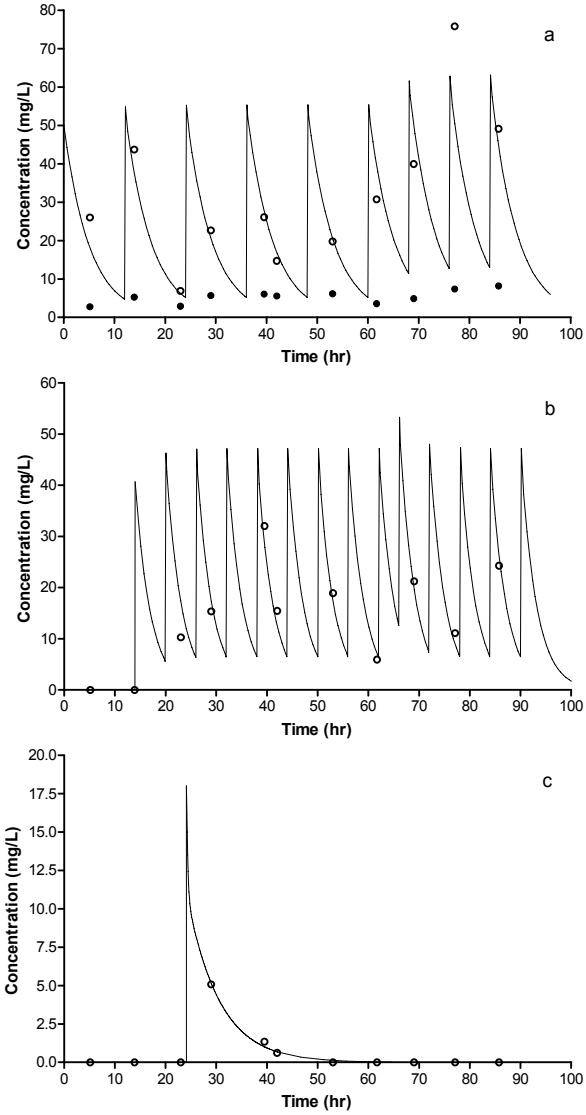

Figure 3. Measured concentrations (open circles) and individually fitted curves (lines) for CTX (a), AMX (b) and VAN (c) in an ECMO-treated neonate. DACT-concentrations (closed circles) were not fitted.

sion were adequate. We tested an adjusted gradient, but were unsuccessful in removing CRO tailing while maintaining resolution between CTX and the CFZ-isotope peak.

Limited availability of a reference standard complicated a full validation of the DACT assay. Considering the structural, chromatographic and mass spectrometric similarities between DACT and CTX, we assumed that their analytical performances would be similar and limited the validation procedure to the critical aspects of matrix effects and sample stability.

Initially, this method was designed for beta-lactam antibiotics only. Vancomycin was later added with a reduced validation procedure. Since vancomycin stability has been demonstrated before [4], we did not include stability testing.

Many reported LC-MS methods contain an elaborate clean-up procedure such as liquid-liquid extracton (LLE) or solid-phase extraction (SPE) to provide sufficient response without interfering matrix effects. Our method shows that a simple protein precipitation with acetonitrile in combination with the narrow peakshape provided by UPLC-MS/MS can be used to quantify antibiotics with acceptable accuracy and precision, despite matrix effects. At least 80% of most analytes was recovered after protein precipitation. This could imply that either a small fraction of analyte was not displaced from protein binding sites, or that the analytes does not readily dissolve into the high-organic solvent. Despite incomplete recovery and matrix effects, method accuracy and precision comply with pre-defined specifications.

The limited stability of certain β-lactam antibiotics potentially limits analysis of large sample runs for the least stable antibiotics meropenem and ceftriaxone. Total analysis time is however less than 4 min per sample, which allows large sample runs for meropenem and cetriaxone as well, provided that samples are processed and analyzed without delay.

We measured cefotaxime, deacetylcefotaxime, amoxicillin and vancomycin simultaneously in 50 µL samples taken from an ECMO-treated neonate. Compared to non-ECMO-treated neonates [18], cefotaxime showed similar clearance with a twofold increase in volume of distribution, which can be explained by the added volume of the ECMO circuit and edema. Amoxicillin clearance did not differ from the clearance found in non-ECMO-treated neonates [19]; volume of distribution was also slightly increased but this may have been underestimated because of the few amoxicillin concentrations directly following injection. Vancomycin clearance and volume of distribution were similar to those reported previously in ECMO-treated neonates [20], although clearance is higher in our patient.

With pharmacokinetic software, we were able to construct concentration-time curves and calculate individual pharmacokinetic parameters for this neonate using existing models. The high sampling frequency during classic pharmacokinetic studies can be problematic in neonates. We expect to be able to compute population pharmacokinetic parameters for this specific population using nonlinear mixed effects modelling (NONMEM) software, combining sparse and randomly sampled concentration data from multiple patients. This complements the microanalysis method, making maximum use of as little and as few sample(s) as possible.

This UPLC-MS/MS method for quantification of amoxicillin, meropenem, cefazolin, cefotaxime, deacetylcefotaxime, ceftriaxone and vancomycin in 50 µL of plasma provides reliable concentation data. In combination with pharmacokinetic modelling software, this enables efficient pharmacokinetic studies in neonates.

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Chapter 4

Simultaneous assay of sildenafil and desmethylsildenafil in neonatal plasma by ultra performance liquid chromatography-tandem mass spectrometry

Bregje C.M. Witjes, Maurice J. Ahsman, Bart C. van der Nagel, Dick Tibboel, Ron A.A. Mathot

Biomed Chromatogr 2010, 24(2), 180-5

Abstract

Sildenafil is used to treat pulmonary hypertension in neonatal and pediatric patients. Pharmacokinetic studies in these patients are complicated by the limited sample volume. We present the validation results of an assay method to quantitate sildenafil and desmethylsildenafil simultaneously in 50 µL of plasma. Deuterated sildenafil was used as an internal standard. After liquid-liquid extraction, analytes were separated on an ultra performance liquid chromatography (UPLC)-column and quantified via tandem mass spectrometry. The calibration curve was linear, with acceptable accuracy and a precision of <15% for both compounds over the full range. The lower limits of quantification were 1 ng/mL. Matrix effects were present, but inter-plasmabatch variability was under 12%. The method was successfully applied to samples from a pharmacokinetic study of sildenafil in neonates, making maximum use of the limited number and amount of plasma samples available.

Introduction

Sildenafil is a potent phosphodiesterase (PDE-5) inhibitor of vascular smooth muscle cells and has been licensed under the trade name Revatio® for the treatment of pulmonary hypertension (PH) in adults. In pediatrics, PH treatment options include the off-label use of sildenafil, but so far, only one pharmacokinetic study has identified a suitable dosage regimen in a subset of pediatric patients.[1] More studies are needed to define the optimal dose for individual neonatal and pediatric patients.[2, 3] These studies are complicated by the limited amount of biological material (e.g. blood, plasma) available, which poses restrictions on sampling frequency and sample volume. Ideally, analytes of interest should be quantified simultaneously in as little sample as possible. This limits the burden on individual patients, while maintaining sufficient data points for reliable data analysis.

A sensitive method to quantify analytes in low volumes of complex matrices is ultra-performance liquid chromatography with tandem mass spectrometry detection (UPLC-MS/MS). With a smaller particle size and higher operating pressures compared to regular HPLC, UPLC provides a shorter runtime and sharper peak shape, which improves sensitivity and reduces potential interference by matrix components.[4-6] UPLC-MS/MS should therefore allow quantitative analysis of multiple analytes with minimal sample preparation and matrix effects.

Several reports on the assay of sildenafil and its active metabolite desmethylsildenafil (Figure 1) are available, but none describes the combination of UPLC-MS/MS with a low-sample volume and its application to neonatal or pediatric pharmacokinetic studies.[7-11] We present the validation results of a method for simultaneous assay of sildenafil and desmethylsildenafil in human plasma after liquid-liquid extraction, using ultra-performance liquid chromatography and tandem mass spectrometry. Method applicability is demonstrated with pharmacokinetic curves of sildenafil and desmethylsildenafil in a neonate with PH.

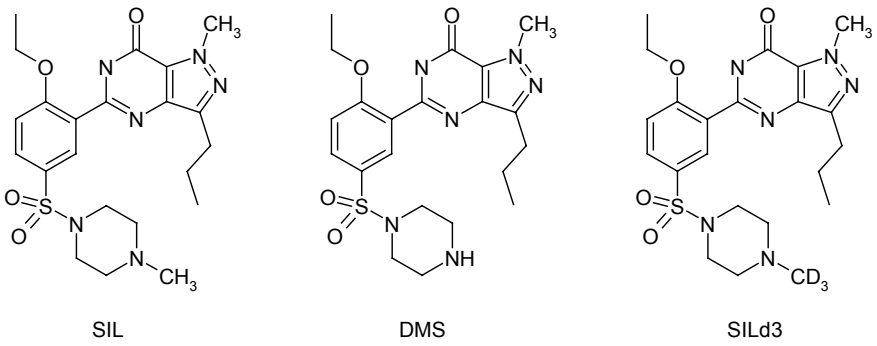

Figure 1. Chemical structures of sildenafil (SIL), desmethylsildenafil (DMS) and internal standard sildenafil-d_3 (SILd$_3$).

Experimental

Reagents

LC-MS grade water and liquid chromatography grade methanol were from Biosolve (Valkenswaard, Netherlands). Formic acid (FA, Sigma, Schnelldorf, Germany) was analytical grade. Analytical grade acetic acid, ethyl acetate and sodium hydroxide were from Merck Co. (Darmstadt, Germany). Sildenafil citrate was kindly provided by Pfizer Inc. (Groton, CT, USA). N-Desmethylsildenafil citrate was purchased from Bio-Connect (Huissen, Netherlands). Sildenafil-d_3 was from C/D/N Isotopes Inc (Pointe-Claire, Quebec, Canada).

Quality control samples and standard solutions

Standard stock solutions containing either sildenafil citrate (SIL) or desmethylsildenafil (DMS) citrate (100 and 50 µg/mL respectively, calculated as free base) were prepared in water. These stock solutions were serially diluted and added to blank water or human plasma (ratio 1:9), to produce final concentrations of 1, 3, 10, 20, 70, 100, 300, 600 and 1000 ng/mL for SIL, and 1, 3, 10, 20, 50, 70, 100, 300 and 500 ng/mL for DMS, respectively. Calibration standards were prepared along with quality control (QC) samples for assessment of intra- and interassay variability, which were similarly prepared using a separate stock solution. Low (L), medium (M) and high (H) controls were prepared in plasma at concentrations of 5, 400 and 800 ng/mL for SIL and 5, 200 and 400 ng/mL for DMS. All stock and working standard solutions were stored at -80°C. The internal standard (IS) sildenafil-d_3 was dissolved in water to a concentration of 100 µg/mL and diluted with water to prepare a final concentration of 200 ng/mL.

Sample preparation

To 50 µL of plasma, 20 µL of 0.02 M aqueous sodium hydroxide, 20 µL IS solution and 500 µL of ethyl acetate were added in a glass tube. The sample was vortexed for 20 seconds. After centrifugation at 16,000 ×g for 10 min, the supernatant organic phase was transferred to a clean glass vial. The solvent was evaporated to dryness at 35°C under nitrogen gas flow, after which the residue was reconstituted in 50 µL of methanol/water/acetic acid solution (40/59/1 v/v/v). When cloudy, samples were centrifuged again at 16,000 ×g for 10 min. The supernatant was transferred to a polypropylene autosampler vial with glass insert and stored at 5°C until assay with UPLC-MS/MS.

UPLC-MS/MS conditions

The UPLC-MS/MS system consisted of a Waters Acquity Ultra Performance LC coupled to a Quattro Premier XE tandem quadrupole mass spectrometer (Waters Corp., Milford, MA, USA). The analytical column was an Acquity UPLC BEH C18 2.1 mm × 100 mm column with 1.7 µm particle size (Waters Ltd, Dublin, Ireland), to which a 0.2 µm pre-column

filter unit was added. The mobile phase was a gradient of solution A (0.1% formic acid in water) and solution B (0.1% formic acid in methanol) with an initial composition of 60% B. Mobile phase composition changed linearly from 60% B to 90% B over the course of 1 minute. The composition was switched back to 60% B and left to equilibrate. Total runtime was 2 minutes. The flow rate was 0.3 mL/min with a column temperature of 30°C and an injection volume of 10 µL. Analytes were detected via MS/MS with an electrospray ionization (ESI)-interface in positive multiple reaction monitoring (MRM)-mode. A solvent delay function was used to divert flow to waste whenever possible. Optimized MRM-settings for the individual drugs, including cone voltage and collision energy, are listed in Table I. The acquisition settings were: capillary voltage=3.4 kV; source temperature=120°C; desolvation temperature=300°C; desolvation gas flow=500 L/h; cone gas flow=50 L/h. Data were acquired using Masslynx V4.1 software and processed using Quanlynx V4.1 (Waters Inc.).

Table I. UPLC-MS/MS acquisition parameters[a] for SIL and DMS

	Q1 (*m/z*)	Q3 (*m/z*)	CV (V)	Dwell time (s)	CE (eV)	Rt (min)
SIL	475.1	99.7	45	0.200	28	1.20
DMS	461.1	283.0	45	0.200	36	1.22
IS (SIL-d_3)	478.2	311.2	50	0.200	30	1.21

[a] Q1, parent-ion mass; Q3, daughter-ion mass; CV, cone voltage; CE, collision energy; Rt, retention time

Validation procedure

The validation procedure included specificity, sensitivity, linearity, accuracy, precision, matrix effects, recovery and autosampler stability.

(a) Specificity and selectivity. Chromatograms from aqueous calibration standards were compared to those of 5 batches of blank plasma before and after spiking with SIL, DMS and IS. Ion traces of each analyte's mass transition were checked for interferences at the respective retention times.

(b) Limit of quantification. The lower limit of quantification (LLOQ) was defined as the lowest concentration that could be quantified with accuracy and precision within ± 20%, as calculated from chromatograms of the 5 lowest calibration samples.

(c) Standard curves. Curves consisting of nine concentrations were constructed for each analyte, with five independent measurements for each calibration standard. Best fit was selected after exploration of different regression models and weighting factors: the final curves were constructed by weighted (1/*x*) least square linear regression with a fixed origin.

(d) Accuracy and precision. Intra- and interday accuracy and precision were calculated for the three quality controls with five measurements each, and in duplicate on six different days. respectively. Accuracy was defined as a percentual deviation from the

nominal concentration by quantifying QC samples on a freshly prepared calibration curve. Precision was defined as the coefficient of variation (CV=standard deviation/mean ×100%).

(e) Matrix effects and recovery. Plasma and solvent components in the ionization chamber cause batch specific ion suppression or enhancement, leading to inter-patient and intra-patient signal variability.[12, 13] Matrix effects were quantified as previously described.[14] In short, chromatograms were recorded of plasma that was spiked pre-extraction, plasma spiked post-extraction and spiked aqueous eluens. In total, six batches of blank plasma were spiked with low and high concentrations of each analyte in duplicate. Recovery (RE) was defined as the relative signal of samples spiked post-extraction vs. pre-extraction. Matrix effects (ME) were similarly defined as the relative signal of post-extraction spiked plasma samples vs. spiked aqueous samples. Process efficiency (PE) was defined as the product of RE and ME, i.e. the overall signal of spiked plasma vs. an aqueous standard solution. Average values and coefficients of variation of RE, ME and PE were calculated over the six plasma batches.

(f) Sample stability. Stock stability was tested in QC samples at -20°C (1 month) and compared to reference samples stored at -80°C. Autosampler stability was tested in 2 QC-samples whose extracts were stored at 4°C for up to 3 days. We chose to forego stability testing in plasma and whole blood, since these have been described extensively before. SIL and DMS have been proven to be stable in plasma for at least 3 months at -70 and -20°C, after five freeze-thaw cycles, and for 48 h at room temperature.[7-9, 15] After 7 days at 4°C, >85% of SIL and DMS can be recovered from whole blood.[16]

Clinical application

We present data from a term neonate with pulmonary hypertension after extracorporeal membrane oxygenation (ECMO) treatment. This was part of a larger study into population pharmacokinetics during ECMO at the Sophia Children's Hospital; the study protocol was approved by our Institutional Ethics Committee. She received sildenafil treatment starting from a postnatal age of 121 days, with a total body weight of 4.8 kg. Sildenafil therapy (0.5 mg/kg q.i.d. via a nasogastric tube) was started while on ECMO, the dose was incrementally increased based on perceived efficacy up to a total of 28 mg/day. Blood (between 100 and 500 µL) was sampled from an arterial line after ECMO decannulation and consisted of a five point curve at t=0, 1, 2, 4 and 6 hrs after a dose. In addition, samples were taken at random times, three times daily for as long as an arterial line was available. After centrifugation, plasma was stored at -80°C until analysis. Plasma levels of SIL and DMS were measured in 50 µL. Individual pharmacokinetic curves were constructed by fitting measured plasma levels to a 1-compartment population pharmacokinetic model of 11 patients treated with sildenafil (9 female, median postnatal age 20 days, median weight 4.0 kg) using nonlinear mixed effects modelling (NONMEM, Globomax LLC, Ellicott City, MD, USA).

Results and discussion

The mass transitions of sildenafil (*m/z* 475.1 → 99.7), desmethylsildenafil (*m/z* 461.1 → 283.0) and sildenafil-d_3 (*m/z* 478.2 → 311.2) were selected for electrospray ionization based on their stability and high intensity. Interestingly, SIL's daughter ion of 99.7 provided better stability and intensity than the daughter ion at 311.2, which was selected for its deuterated counterpart. The internal standard and both analytes showed similar chromatographic behaviour, which is reflected in the retention times (Figure 2). There were no discernable interfering compounds in plasma judging from a comparison between spiked plasma, spiked aqueous standards and plasma blanks. The patient samples containing SIL and DMS did not show additional peaks in their total ion currents. From this, we concluded that the combination of liquid-liquid extraction, UPLC and MS/MS detection via multiple reaction monitoring provides sufficient specificity and selectivity. The runtime of 2 min is probably suitable for high sample throughput and could potentially be reduced even further, but this was deemed unnecessary as the total analysis time was mainly dependent on the liquid extraction procedure.

The calibration curves provided reliable linear responses from 1 to 1000 ng/mL for SIL and 1 to 500 ng/mL for DMS, respectively. Mean coefficients of determination (R^2) of the 1/*x*-weighted calibration curves were 0.9990 (range 0.9986-0.9999, n=5) for SIL and 0.9963 (range 0.9936-0.9969, n=5) for DMS, respectively. Plots of weighted residuals vs. concentrations did not indicate nonlinearity. Despite the low sample volume, sensitiv-

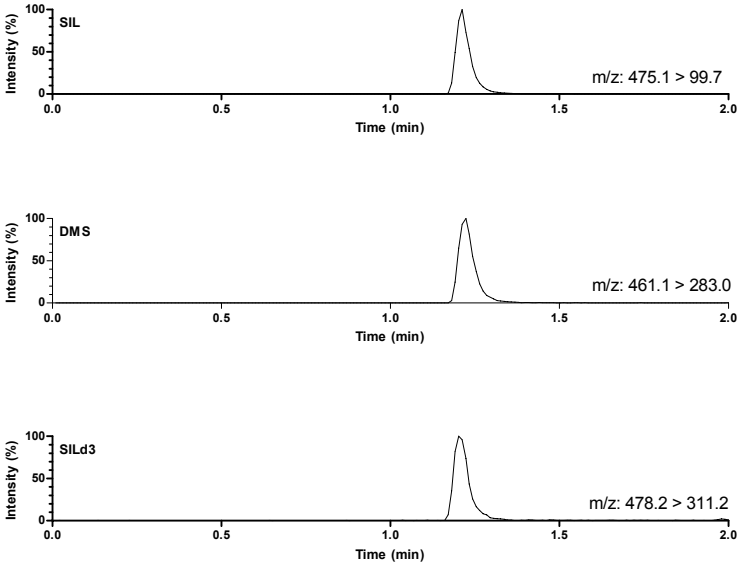

Figure 2. Chomatogram of sildenafil (SIL), desmethylsildenafil (DMS) and internal standard sildenafil-d_3 (SILd_3). Each curve is the ion trace of a mass transition specific for each analyte.

ity is sufficient with an LLOQ of only 1 ng/mL in plasma for both compounds, which is reflected in the precision and accuracy results (Table II). Intra- and interday precision is under 15% for L and H QC samples for both SIL and DMS. Accuracy is between 90 and

Table II. Intra- and inter-run precision and accuracy data for SIL and DMS

	Concentration (ng/mL)	Precision		Accuracy (%)
		Measured (ng/mL)	CV[a] (%)	
Intra-day (n=5)				
SIL	4.99	4.84	3.46	97.0
	400	409	3.11	102
	799	769	3.47	96.3
DMS	5.01	5.20	8.60	104
	200	206	10.8	103
	400	418	3.47	104
Inter-day (n=12)				
SIL	4.99	4.85	13.4	97.2
	400	383	9.95	96.0
	799	813	7.07	102
DMS	5.01	5.34	10.7	107
	200	232	13.9	116
	400	501	13.6	125

[a] CV, coefficient of variation

110% for all samples, with the exception of interday DMS samples. We have no explanation for these aberrant results, but considering SIL pharmacokinetics is our main focus, we chose to forego extensive efforts into method improvement for DMS and accept the deviations in accuracy. Since we expect DMS plasma concentrations to be in the lower to medium range of the calibration curve, the implications of a reduced accuracy are probably mild. Perhaps a deuterated internal standard for DMS could remedy this problem, but at great financial expense.

Matrix effects were extensively evaluated with low and high QC-dilutions in 6 batches of blank plasma (Table III). Although plasma components appear to cause significant signal loss (on average 52% for SIL and 28% for DMS) and recovery is below 65%, the liquid extraction and UPLC-separation appear to lead to good reproducibility: interbatch

Table III. Matrix effects (ME), recovery (RE) and process efficiency (PE) with interbatch coefficients of variation (CV) for the assay of SIL and DMS in 6 batches of blank human plasma

	Sildenafil		Desmethylsildenafil	
	L	H	L	H
ME (CV)	0.50 (3.3%)	0.47 (1.3%)	0.81 (6.9%)	0.64 (2.5%)
RE (CV)	0.44 (10.5%)	0.64 (10.4%)	0.23 (7.8%)	0.32 (5.2%)
PE (CV)	0.22 (11.0%)	0.30 (10.5%)	0.18 (10.4%)	0.21 (5.7%)

variability for ME, RE and PE is under 15% for both analytes. This might be a result of the use of a deuterated internal standard vs. a structural analogue.

We evaluated autosampler stability to estimate the potential autosampler storage period between sample processing and UPLC-MS/MS injection. After three days at 4°C, signal intensity of processed L, M and H QC samples was within 90-110% of the reference value, indicating that samples can be processed beforehand as long as they are stored at 4°C. Stock stability was satisfactory as well: after one month of storage at either -80°C or -20°C, we could identify no degradation of SIL or DMS.

This method was applied to a pharmacokinetic study of sildenafil in neonatal patients with pulmonary hypertension. We successfully modelled SIL and DMS plasma concentrations on a one-compartment model for each compound, with oral absorption and first-order elimination. Using the population model, individual Bayesian pharmacokinetic parameters were calculated for a neonatal PH patient. Concentration-time curves were constructed using the dose history, see Figure 3. Parameter estimates for the population

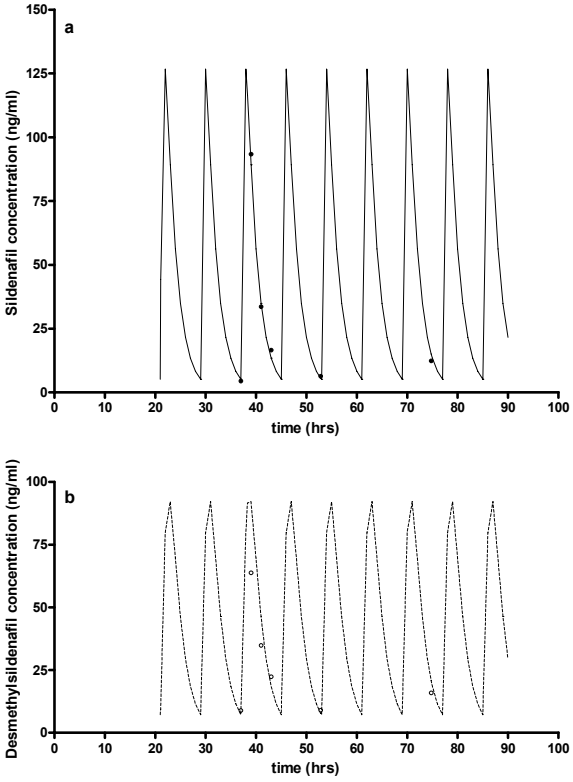

Figure 3. Concentration-time curves of sildenafil (a) and desmethylsildenafil (b) in a neonate with pulmonary hypertension. Circles are measured concentrations; the curves were constructed using the recorded dose regimen and the individual Bayesian predicted PK parameters derived from a population model of 11 individuals.

and the selected individual are given in Table IV. Inter-patient variability in clearance and distribution volume estimates was large (from 62% for CL_{DMS} up to 94% for V_{SIL}), which explains the difference between the population parameters and the selected individual. These data show that the LC-MS method can be used in combination with PK modelling software to deduce PK parameter estimates using sparse sampling designs with few samples per individual.

Table IV. Pharmacokinetic parameter estimates[a]

	Population estimates	Individual estimates
Sildenafil		
k_a (h^{-1})	2.4 ± 4.2 %	2.4
V (L)	34 ± 5.0 %	38
CL (L/h)	7.3 ± 17 %	18
Desmethylsildenafil		
V (L)	14 ± 35 %	14
CL (L/h)	9.7 ± 19 %	20

[a] k_a, absorption rate constant; V, distribution volume; CL, clearance. Population estimates are given as medians±their corresponding standard deviations. V and CL estimates are displayed assuming a biological availability of 100% and a SIL to DMS conversion fraction of 100%

Conclusion

We present a sensitive, simple UPLC-MS/MS method for simultaneous quantification of sildenafil and its main metabolite in 50 µL of plasma. With an LLOQ of 1 ng/mL for both analytes and an instrument runtime of only 2 min, this method allows high sample throughput. In combination with pharmacokinetic modelling software, the low sample requirements make it particularly suitable for pharmacokinetic studies in neonates, making maximum use of the limited number and amount of plasma samples available.

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Chapter 5

Quantification of midazolam, morphine and metabolites in plasma using 96-well solid phase extraction and ultra-performance liquid chromatography-tandem mass spectrometry

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Biomed Chromatogr 2010, in press

Abstract

Currently, PK-PD studies of sedatives and analgesics are performed in neonates and children to find suitable dose regimens. As a result, sensitive assays using only small volumes of blood are necessary to determine drug and metabolite concentrations. We developed an ultra-performance liquid chromatographic method with tandem mass spectrometry detection for quantification of midazolam, 1-hydroxymidazolam, hydroxymidazolam-glucuronide, morphine, morphine-3-glucuronide and morphine-6-glucuronide in 100 µL of plasma. Cleanup consisted of 96 wells micro-solid phase extraction, before reversed phase chromatographic separation (ultra-performance liquid chromatography, UPLC) and selective detection using electrospray ionization tandem mass spectrometry. Separate SPE methods were necessary to quantify morphine, midazolam and their metabolites because of each groups physicochemical properties. Standard curves were linear over a large dynamic range with adequate limits of quantitation. Intra- and inter-run accuracy and precision were within 85-115% (of nominal concentration using a fresh calibration curve) and 15% (coefficient of variation, CV) respectively. Recoveries were >80% for all analytes, with interbatch CV's (as a measure of matrix effects) of less than 15% over six batches of plasma. Stability in plasma and extracts was sufficient, allowing large autosampler loads. Runtime was 3.00 min per sample for each method. The combination of 96-well micro-SPE and UPLC-MS/MS allows reliable quantification of morphine, midazolam and their major metabolites in 100 µL of plasma.

Introduction

Sedatives and analgesics (both opioid and non-opioid) are routinely used in the treatment of critically ill children. In order to minimize drug dependence and unnecessarily long hospital admissions while maintaining adequate control of pain and anxiety or excitement, a growing number of pharmacokinetic and pharmacodynamic (PK/PD) studies are performed in patients of different age groups, varying from neonates to adolescents.[1-5] These studies require the quantification of drugs and their metabolites in a limited sample volume. In addition, the large numbers of samples in certain studies make high-throughput sample preparation and analysis desirable. Unfortunately, physicochemical properties can vary greatly, especially between lipophilic parent drugs and their hydrophilic metabolites. Therefore, current assay methods are usually optimized to quantify a single compound, sometimes in combination with some of its metabolites (hydroxymidazolamglucuronide, the morphine-glucuronides).[6-8]

To assay drugs and their metabolites in a limited volume of plasma with sufficient selectivity and sensitivity, ultra-performance liquid chromatography (UPLC-MS) seems ideal. With a smaller particle size and higher operating pressures compared to regular HPLC, UPLC provides a shorter runtime and sharper peak shape, which improves sensitivity and reduces potential interference by matrix components.[9-11] Moreover, multiple drugs and their metabolites can be assayed within a single run by monitoring their respective ion fragmentation pattern, which provides high selectivity. UPLC-MS/MS should therefore allow quantitative analysis of multiple analgesics or metabolites with minimal sample preparation and matrix effects. The efficiency and speed of sample clean-up can be increased using 96-well micro-format solid phase extraction (SPE), especially for pharmacokinetic studies with a large number of samples.

We present the development and validation of an assay method for midazolam, morphine and their main metabolites (1-hydroxymidazolam, hydroxymidazolamglucuronide, morphine-3- and morphine-6-glucuronide) in 100 µL of plasma, using two 96-well micro-SPE methods and UPLC-MS/MS, to facilitate pharmacokinetic studies in neonatal and pediatric clinical pharmacology. Method applicability is demonstrated with plasma data from neonatal patients who received midazolam and morphine during extracorporeal membrane oxygenation (ECMO).

Experimental

Chemicals and reagents

LC-MS grade water, liquid chromatography grade methanol, acetonitrile and isopropanol were from Biosolve BV (Valkenswaard, Netherlands). Formic acid and ammonium carbon-

ate (Sigma-Aldrich Co, Schnelldorf, Germany) were analytical grade. Morphine-3-β-D-glucuronide (M3G, purity 98%) and morphine-6-β-D-glucuronide (M6G, purity 98%) were also purchased from Sigma-Aldrich Co. Midazolam (MDZ, purity 100.2%) hydrochloride and morphine (MOR, purity 99.3%) sulphate were from Bufa BV (Uitgeest, Netherlands). Hydroxymidazolam (OHM, purity 98%) was kindly donated by Hoffmann-La Roche Ltd (Basel, Switzerland). The deuterated internal standards midazolam-d_4 and morphine-d_3 (MDZd4 and MORd3, with purity ≥ 98%), were from Cerilliant Corp. (Round Rock, TX, USA). Hydroxymidazolamglucuronide (HMG) was not commercially available. Instead, we collected urine from a patient who received high midazolam doses and extracted HMG using HLB solid phase extraction cartridges, via an SPE-method based on one previously used in sample preparation.[12] See the appendix for the extraction protocol. The extract's HMG concentration was measured via HPLC-UV as 1-hydroxymidazolam after enzymatic deglucuronidation and liquid-liquid extraction.[1] This HPLC-UV method was also used to confirm the absence of unconjugated MDZ and OHM in the extract. Blank plasma was obtained from the institutional blood bank supply, derived from healthy adult volunteers.

Instrumentation

Solid phase extraction was done on a Waters Extraction Plate Manifold with Oasis Hydrophilic-Lipophilic-Balanced (HLB) µElution plates in 96-well format (Waters Corp., Milford, MA, USA). All reagents were added manually using repeater pipettes (Eppendorf AG, Hamburg, Germany). The UPLC-MS/MS system consisted of a Waters Acquity Ultra Performance LC System coupled to a Quattro Premier XE tandem quadrupole mass spectrometer. Data were acquired using Masslynx V4.1 software and processed using Quanlynx V4.1 software (Waters Corp., Milford, MA, USA).

Liquid chromatography

The analytical column was an Acquity UPLC BEH C18 2.1 × 100 mm column with 1.7 µm particle size, to which a 0.2 µm pre-column filter unit was added (Waters Ltd, Dublin, Ireland). The mobile phase was a gradient of solution A (0.1% formic acid in water) and solution B (0.1% formic acid in methanol) with an initial composition of 10% B. Mobile phase composition changed from 10% B to 30% B in 0.7 min, after which the composition was gradually changed to 90% B over a period of 1.30 min. After 0.5 min at 90% B, it was switched back to 10% B and left to equilibrate for another 0.5 min, leading to a total runtime of 3.00 min. The flow rate was 0.4 mL/min with a constant column temperature of 40°C.

Mass spectrometry

Analytes were detected via tandem MS with an electrospray ionization (ESI)-interface in positive multiple reaction monitoring (MRM)-mode. Optimized MRM-settings for the

individual drugs, including cone voltage and collision energy, are listed in Table I. The acquisition settings were: capillary voltage=3.4 kV; source temperature=120°C; desolvation temperature=300°C; desolvation gas flow=500 L/h; cone gas flow=50 L/h; dwell time=80 ms.

Stock and working solutions

Stock solutions were prepared in 5% methanol for each analyte. Separate stock solutions were prepared for calibration standards and QC samples. Working solutions were prepared using fixed volumes of each stock solution which were diluted with water to obtain 10 mL of standard solution. Seven standards were prepared over the calibration range, see Table I for the resulting plasma concentration ranges for each analyte (calculated as free base). QC working solutions (L, M, H) were prepared at 1.5%, 20% and 80% of the highest calibration standard concentration. Internal standards were diluted in methanol to prepare a stock solution with concentrations of 1000 ng/mL (MDZd4 and MORd3). A working internal standard solution was prepared by diluting 1 part IS-stock with 99 parts water. All stock and working solutions were stored at -20°C.

Preparation of standards and QC samples

In polypropylene vials, 50 µL of each working standard solution was added to 450 µL of blank plasma to obtain calibration standard samples. QC samples were prepared in a similar manner and stored in 50 µL aliquots at -80°C. For blanks, the 50 µL of working or QC standard solution was substituted with 50 µL of water.

Sample preparation

Due to the divergent physicochemical characteristics of the studied drugs and their metabolites, two separate SPE methods were necessary to achieve sufficient recovery. See Table II for a description of both methods. Of each sample, calibration standard or QC, 50 µL was processed via both methods. After elution into 96-well collection plates, 10 µL was injected into the UPLC-system.

Validation procedure

The method validation was based on FDA guidelines for bioanalytical method validation [13] and included the following parameters:

(a) Linearity and LLOQ. Calibration curves of seven concentrations (*n*=6) were analyzed and constructed using linear or polynomial regression. Best fit was selected after exploration of different regression models and weighting factors and assessed using the correlation coefficient and potential trends in the weighted residuals vs. concentration plots. The lower limit of quantification (LLOQ) was defined as the lowest concentration that could be quantified with accuracy of 85-115% and preci-

sion <20% (n=6). Accuracy was defined as a percentual deviation from the nominal concentration after quantification on a freshly prepared calibration curve. Precision was defined as the coefficient of variation (CV,%).

(b) Intra- and inter-day accuracy and precision. Accuracy and precision were calculated using QC samples (L, M and H) on the same day (intra-day CV; n=6) and over 6 days (inter-day CV; n=5).

(c) Stability. Tabletop and autosampler stability of processed QC samples (L, M and H; n=2) were tested for 24 h at room temperature (20°C) and for 48 h in the autosampler (5°C). Freeze-thaw stability (3 cycles) was tested with QC samples (L, M and H; n=2). A minimal remaining signal intensity of 85% was considered acceptable. Stability in plasma was not studied; the high stability of these analytes, both during long-term storage (-20°C and -80°C) and processing (4-7°C and 20-22°C) have been described extensively, the reader is referred to the literature regarding MDZ/OHM [7, 14] and MOR/M3G/M6G.[6, 15].

(d) Recovery and matrix effects. Recovery was quantified (average and CV) by calculating the ratio of the absolute signal of each analyte in spiked plasma (L, M and H, n=6) over that of an academic aqueous standard. Plasma and eluent components in the ionization chamber can cause batch-specific ion suppression or enhancement, leading to inter-patient and intra-patient signal variability.[16] These matrix effects were visualized with a tee-infusion experiment. Extracts of six batches of blank plasma were injected onto the column while analytes were continuously infused into the mass spectrometer. Ion traces were recorded for each compound over the full runtime. Signal suppression or enhancement at its respective retention time was visually assessed for each analyte in the six batches of blank plasma.

(e) Clinical application. We present plasma concentrations from a pediatric patient (6 years old, 22kg, placed on ECMO for acute respiratory distress resulting from pneumonia) who received intravenous midazolam for sedation, and morphine for analgesia during ECMO treatment. These data are from a larger study into population pharmacokinetics during ECMO at the Pediatric Intensive Care Unit (PICU) of the Sophia Children's Hospital. The study protocol was approved by the Institutional Ethics Committee and the parents gave consent for blood withdrawal and use of the medication history. Medication was given intravenously as required, based on validated sedation and pain scores. Besides bolus injections of fentanyl, no other opiates or benzodiazepines were given. Midazolam was given intravenously via continuous infusion (300 µg/kg/h), combined with bolus doses of 4 mg to prevent distress during medical procedures. Morphine was continuously infused over a period of 30 hours to provide constant pain relief (23 µg/kg/h). Blood samples (n=12, 500 µL each) were collected three times daily from extracorporeal access ports. Plasma was separated from EDTA-decoagulated samples by centrifugation (6 min, 16.000 ×g) and stored at −80°C until analysis.

Results and discussion

Method development

See Figure 1 for the analytes' structural formulae. In MS/MS optimization, the protonated molecular precursor ions $[M+H]^+$ were selected for fragmentation. Respective fragment ions were selected in selective reaction monitoring (SRM) mode upon continuous infusion of each analyte, based on sufficient intensity and stability of the resulting MS signal.

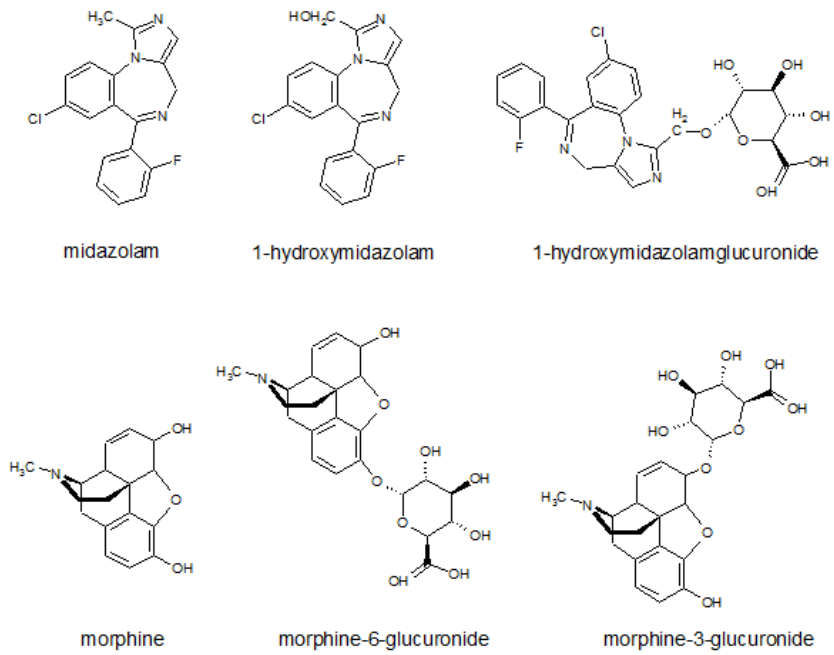

Figure 1. Structural formula for each analyte.

The optimal ion transition (Table I) was the same for each of the morphine glucuronides (M3G and M6G), which implied that they had to be separated chromatographically to allow reliable quantification. With an ion transition of *m/z* 462.1 → 286.1, the ester bond appears to break first, causing the most intense fragment ion to be morphine. Without complete chromatographic separation, fragmentation of the M3G could add to the MOR signal. Similarly, HMG might add to the OHM signal: the ion transitions appear to differ (*m/z* 518.1 → 324.2 for HMG and 342.0 → 202.8 for OHM), but the HMG fragment is probably a dehydrated version of OHM (with a $\Delta_{m/z}$ of approximately 18) and could therefore provide similar fragments. The absence of these interferences was therefore confirmed by injecting an extracted QC plasma sample (H) containing only M3G or HMG, respectively; no OHM or MOR signal could be detected at their respective retention times. An MRM method was created using the optimized SRM's for each analyte.

Table I. Acquisition parameters[a]

	Q1 (*m/z*)	Q3 (*m/z*)	CV (V)	CE (eV)	Rt (min)	Dynamic range in plasma (ng/mL)	IS
MDZ	326.1	290.9	40	25	1.99	5-1000	MDZd4
OHM	342.0	202.8	35	25	2.04	3-600	MDZd4
HMG	518.1	324.2	50	25	2.09	7-1400	MDZd4
MOR	286.1	152.0	45	55	0.98	2.5-500	MORd3
M3G	462.1	286.1	45	30	0.74	1.25-250	MORd3
M6G	462.1	286.1	45	30	0.93	2.5-500	MORd3
MDZd4	330.1	295.2	40	25	1.99	-	-
MORd3	289.1	152.0	45	55	0.98	-	-

[a] Q1, parent-ionmass ($[M+H]^+$); Q3, fragment-ionmass ($[M+H]^+$); CV, cone voltage; CE, collision energy ; IS, internal standard

We initially tried to quantify all analytes in a single run using a simple protein precipitation with acetonitrile or methanol, but were unable to reach sufficient sensitivity, chromatographic separation and peak shape of hydrophilic components (M3G, M6G, MOR, HMG). A generic SPE-method (Table II, method 1) showed that most analytes could be adequately retained and recovered. However, M3G and M6G recoveries and peak shapes were still unsatisfactory, due to the low pH of the load solution and high organic content of the injected extract, respectively. A separate method (method 2, based on previously published methods [17, 18]) provided good chromatographic separation of morphine and its metabolites. Further optimization experiments indicated that MDZ requires acidic load conditions and elution with a strong eluent (methanol) to be successfully extracted from plasma. The irreconcilable requirements for simultaneous quantification of hydro-

Table II. Solid-phase extraction methods[a]

	1	2
Analytes	MDZ, OHM, HMG	MOR, M3G, M6G
Conditioning	200 µl MeOH	200 µl MeOH
Equilibration	200 µl water 200 µl water + 1% FA	200 µl water 200 µl 10mM $(NH_4)_2CO_3$
Load	200 µl water + 1% FA + 50 µl sample + 50 µl IS-solution	200 µl 10mM $(NH_4)_2CO_3$ + 50 µl sample + 50 µl IS-solution
Wash	200 µl 10mM $(NH_4)_2CO_3$	200 µl 10mM $(NH_4)_2CO_3$
Elution	20 µl MeOH + 1% FA 20 µl MeOH + 1% FA	20 µl ACN/IPA + 1% FA 20 µl ACN/IPA + 1% FA
Dilution (though column)	150 µl water	150 µl water

[a] MeOH, methanol; FA, formic acid; 10mM $(NH_4)_2CO_3$, 10mM ammonium carbonate buffer pH 8.8; IS, internal standard; ACN/IPA, mixture of acetonitrile/isopropanol (40:60 *v/v*)

philic and lipophilic components necessitated separate SPE-methods for MDZ/OHM/HMG and MOR/M3G/M6G. Repeated elution with the same total volume of eluent led to superior recoveries over a single elution step. Through-column dilution with water (to flush out remaining organic eluent caught in the micro-SPE tip) improved recovery while maintaining good peak shapes, chromatographic retention and sensitivity. M3G and M6G maintained resolution over all the calibration standards; peaks were completely resolved at baseline even at the highest concentrations. See Figure 2 for individual ion traces of a sample at the LLOQ level using the final methods.

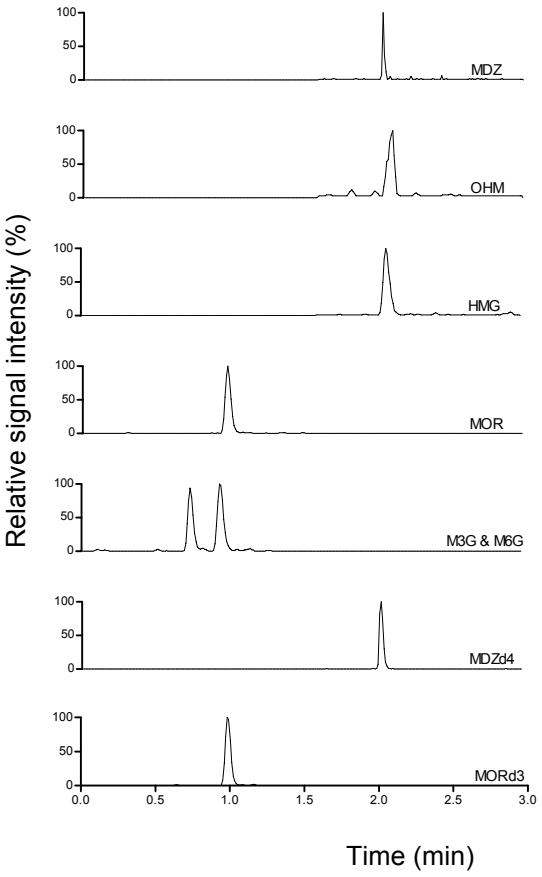

Figure 2. Representative chomatograms of a spiked blank plasma sample at the LLOQ level. MDZ, OHM and HMG were extracted with SPE-method 1; MOR, M3G and M6G with SPE-method 2.

Validation

(a) Linearity and LLOQ. Calibration curves were linear for all analytes. Weighted residuals (1/*x*) after linear regression were evenly distributed. Correlation coefficients (r^2)

and LLOQ for each analyte are in Table III. See Figure 2 for a chromatogram of each analyte at the LLOQ concentration.

(b) Intra- and inter-day accuracy and precision. Precision and accuracy for low, medium and high quality controls are shown in Table III. Accuracy was between 91.9 and 110.9% for all analytes. Intra- and interday precision were <20% (at LLOQ) and <15% (L, M, H) for all analytes.

(c) Stability. All analytes were stable (≥ 85% of nominal concentration) in extracts stored in 96-well collection plates for at least 24 h at room temperature (20°C), at least 48 h in the autosampler (5°C) and after three freeze-thaw cycles (-20°C).

Table III. Validation results

	Correlat.-coeff. (r^2)	Conc.	Intra-day		Inter-day		Recovery and matrix effects	
	Range	(ng/mL)	Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)	(%)	Interbatch CV (%)
MDZ	0.995-0.999	5.1	100.2	18.2	(LLOQ)			
		25.1	105.4	9.5	106.6	6.7	96.2	14.1
		400.7	105.2	8.1	101.0	5.8	87.3	2.8
		801.5	108.9	3.7	101.8	6.1	92.9	2.2
OHM	0.997-0.999	3.1	110.1	16.0	(LLOQ)			
		15.6	93.3	10.8	108.9	5.9	110.4	7.2
		250	109.1	8.3	104.7	4.1	102.3	7.0
		500	100.7	3.4	104.4	4.5	99.1	4.0
HMG	0.990-0.998	7.2	104.6	19.9	(LLOQ)			
		35.9	92.2	5.6	88.2	8.8	277.4	11.4
		287	91.9	10.8	93.6	7.1	284.9	6.5
		1149	106.7	5.2	102.7	4.7	274.9	7.0
MOR	0.997-0.999	2.4	103.6	14.3	(LLOQ)			
		12.0	106.4	9.5	109.4	8.7	120.7	15.4
		192	97.6	7.8	99.0	11.0	104.2	6.0
		384	105.4	3.3	101.5	5.5	104.2	2.8
M3G	0.993-0.998	1.2	110.9	20.0	(LLOQ)			
		6.0	93.1	14.1	105.2	14.1	90.1	14.3
		96.2	96.9	7.2	96.1	8.0	92.0	3.4
		192	107.4	3.3	99.9	7.2	83.6	4.1
M6G	0.995-0.998	2.5	100.7	16.2	(LLOQ)			
		12.5	101.5	11.6	108.3	14.7	107.7	13.4
		200	108.7	6.2	96.5	12.5	101.1	6.6
		400	102.0	3.4	99.3	6.2	96.9	5.1

(d) Recovery and matrix effects. Recoveries and interbatch CV (%) are in Table III. Recoveries were over 80% for all analytes, with marked ion enhancement for HMG (appr. 280%), which might be caused by a difference in formic acid concentration: the academic standards were prepared in eluent A (0.1% aqueous FA) whereas the extracts contain approximately 0.25% FA after dilution. This would indicate that the ionization of HMG is heavily dependent on the availability of protons in the ionization chamber. In tuning experiments, higher FA concentrations increased the HMG signal. To visually evaluate matrix effects, extracts of six batches of blank plasma were injected onto the column during continuous infusion of analytes directly into the mass spectrometer. The MS signal was recorded for each individual analyte. There was no apparent inter-plasmabatch variability in signal intensity for each of the analytes. In addition, the signal was stable within each plasma batch at the respective retention time of each analyte. See Figure 3 for one of the composite total ion currents during a plasma extract run for each of the SPE-methods.

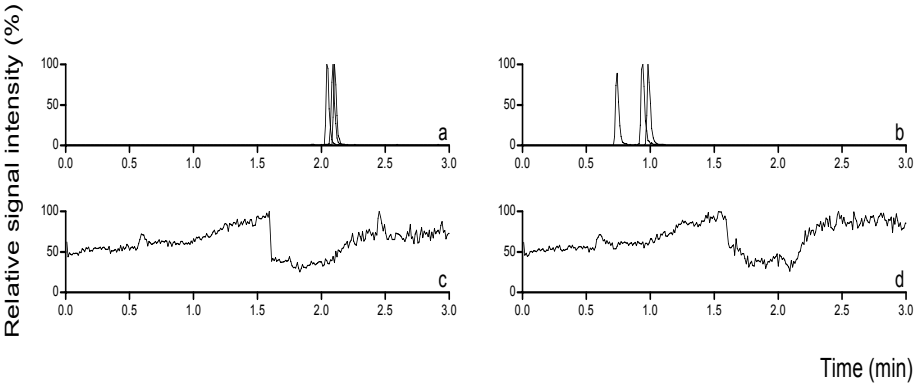

Figure 3. Composite chomatograms of medium QC samples with SPE-method 1 (a) and method 2 (b). Upon injection of blank plasma extracts, directly infused analytes provide a constant signal at their respective retention times, as can be seen from the ion currents during plasma infusion (c, method 1; and d, method 2).

Clinical application

MDZ, MOR and metabolites were successfully quantified (singly) in plasma samples from a pediatric ECMO patient, see Figure 4 for the concentration-time curves. Midazolam concentrations were relatively constant over the observation period (around 700 ng/mL). HMG accumulated up to a maximum of 2000 ng/mL. Relevant samples was diluted with an equal volume of blank plasma to quantify HMG-levels above the dynamic range. Assuming a relative potency of around 6% compared to MDZ [19], this implies that HMG potentially provides a significant contribution to the overall sedative effect of midazolam. Until renal function is restored, the HMG levels could extend the sedative effect until long

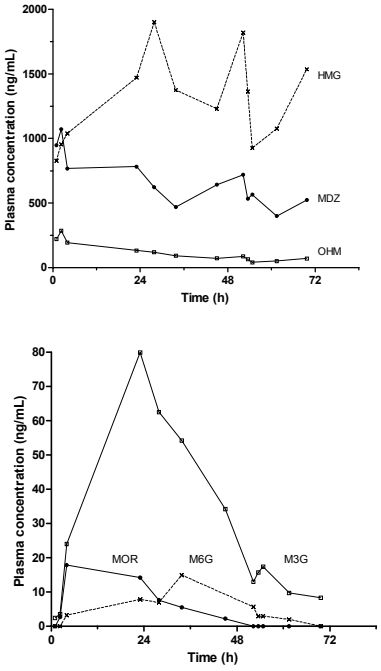

Figure 4. Concentration-time curves for each analyte in a pediatric patient (22 kg). Midazolam was given continuously at 300 μg/kg/h, with bolus injections of 4 mg at 0, 13 and 45 h. Morphine was given at 23 μg/kg/h for approximately 24 h.

after MDZ drops below its minimal effective concentration, which has previously been described for adults with impaired renal function.[8] MOR and its glucuronides were present, but were largely eliminated over a period of 48 hours after the end of morphine infusion.

Method performance

Until now, midazolam, morphine and their metabolites have been successfully quantified in more than 1400 patient samples. These analytical runs showed good linearity of the calibration curve (containing five concentrations in duplicate), with QC concentrations (L, M and H before and after patient samples) within 15% of their nominal concentrations. The calibration curve was reduced to five standards to save slots on the 96-wells plates, which allowed the most efficient thawing, processing and refreezing of stored patient samples. One of the advantages of this method is the quantification of HMG without deglucuronidation, which reduces processing time and minimizes sample volume. HMG quantification is hardly mentioned in previously published methods.[7, 14, 20, 21] The combined analysis of multiple drugs in itself reduces the required sample volume as well, especially in combination with a micro-SPE manifold that allows samples of 50 μL or less with minimal elution volumes. Most published analytical methods for these analytes require more sample, varying from 200 μL to quantify MDZ and OHM [20]

up to 700 µL or more for MOR, M3G and M6G.[22] Recently, an LC-MS method has been published using 100 µL, but without quantification of all analytes.[23] This SPE method can be used with 96-well plates in max 1¼ hours per plate. Unfortunately, all analytes could not be quantified using a single method, which means that extra plasma, time and resources have to be spent to quantify all analytes. The extra runtime could be reduced by dividing the LC gradient into two separate gradients. This way, we estimate that the runtime could probably be reduced to between 1½ and 2 min per sample; we deemed this unnecessary since our maximal assay capacity is not limited by LC-MS runtime but by the time required for thawing of samples and SPE. Calculated per sample, the cost of micro-SPE is similar to that of ordinary (1-3 mL) SPE cartridges of the same sorbent material. We were able to reuse the disposable SPE-plates up to 7 times without detrimental effects on reproducibility or signal intensity. A lower LLOQ might be possible, considering the signal intensities at LLOQ presented in Figure 2 and the absence of sample concentration in our current SPE method, but sensitivity was sufficient for the expected concentrations in our patients.[1, 2, 24]

Conclusion

This LC-MS method with 96-well solid-phase extraction provides efficient quantification of midazolam, morphine and their main metabolites in 100 µL of plasma, with acceptable accuracy, precision, recovery and LLOQ. This facilitates pharmacokinetic studies and therapeutic drug monitoring of commonly used sedatives and analgesics in neonatal and pediatric clinical pharmacology.

Acknowledgements

The authors thank Enno Wildschut and Prof. Dick Tibboel for their advice and support in the preparation of this manuscript.

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Part II

Extracorporeal membrane oxygenation: Drug adsorption

Chapter 6

Determinants of drug adsorption in different ECMO circuits

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Submitted

Abstract

The aim of this *in vitro* study was to evaluate potential determinants of drug loss in different ECMO circuits. Midazolam, morphine, fentanyl, acetaminophen, cefazolin, meropenem and vancomycin were injected into 3 neonatal rollerpump, 2 pediatric rollerpump and 2 clinically used neonatal rollerpump circuits, all with silicone membranes, and 2 neonatal centrifugal pump circuits with polypropylene hollow fibre membranes. Serial blood samples were taken from a post-oxygenator site. Drug recovery was calculated as the ratio between the determined and the theoretical maximum concentration. The latter was obtained by dividing dose by theoretical circuit volume. Average drug recoveries at 180 minutes in 3 neonatal silicone membrane rollerpump circuits were; midazolam 0.62%, morphine 23.9% fentanyl 0.35%, acetaminophen 34%, cefazoline 84.3%, meropenem 82.9% and vancomycin 67.8%. There was a significant correlation between the lipophilicity of the drug expressed as log P and the extent of drug adsorption; $p<0.001$. The recovery for midazolam and fentanyl in centrifugal pump circuits with hollow-fibre membrane oxygenator was significantly higher compared to neonatal rollerpump circuits with silicone membranes; midazolam 0.62% vs. 63.4%, fentanyl 0.35% vs. 33.8%, $p<0.001$. Oxygenator size, and use do not significantly affect drug losses. Significant adsorption of drugs occurs in the ECMO circuit, correlating with increased lipophilicity of the drug. Centrifugal pump circuits with hollow-fibre membrane oxygenators show less adsorption for all drugs, especially lipophilic ones. Low plasma levels should be expected when dosing lipophilic drugs on ECMO.

Introduction

Extracorporeal membrane oxygenation (ECMO) is a form of prolonged temporary cardiopulmonary bypass for patients with cardiac or respiratory failure that is unresponsive to other treatment. In general, patients on ECMO receive more than 10 drugs during ECMO for sedation, analgesia and treatment of underlying or concomitant conditions.[1] Altered pharmacokinetics of several drugs such as morphine, midazolam, vancomycin and gentamicin have been observed during ECMO; the volume of distribution is generally increased whereas clearance is decreased.[2-5] Evidence based dosing regimes on ECMO are sparse, with only a few clinical-pharmacological trials.[6] Adsorption of medication in ECMO circuits appears to be one of the reasons for the increased volume of distribution. The studies on this subject have left important questions unanswered.[7-11] Levels of drug adsorption by polymers, silicone rubber and other materials have been linked to the drugs' lipophilicity. Assuming this holds for ECMO membranes and tubing as well, there might be a correlation between drug loss and lipophilicity, expressed as log P (or the partition coefficient between 1-octanol and water). Other system-related factors that could affect drug loss include the type of pump and circuit; reduced adsorption by hollow-fiber membranes; shorter tubing and circuits with a centrifugal pump or roller pump systems. As the total absorptive capacity is linked to the surface area, pediatric systems, with their larger membrane oxygenators and longer tubing, are expected to show larger adsorption, although this has yet to be established in trial settings. Finally, if adsorption is maximized by saturation of the surface, one would expect clinically used ECMO circuits to show less adsorption than freshly blood-primed ECMO circuits, but reports are contradictory.[9, 11]

To evaluate these aspects, we tested drug loss in different ECMO circuits. The drugs studied are some of the most frequently used sedatives (midazolam), analgesics (fentanyl, morphine, acetaminophen) and antibiotics (vancomycin, meropenem, cefazolin) in ECMO patients treated in the two ICUs participating in this study. To assess the effect of lipophilicity on adsorption these drugs were also chosen to reflect a wide range of log P values.

Materials and Methods

The study was conducted at the intensive care unit of the Sophia Children's Hospital, Erasmus University Medical Center, Rotterdam, the Netherlands and the neonatal intensive care unit, University Hospitals Leuven, Belgium. The same investigators conducted the experiments at both sites. Drug behavior was studied in three different ECMO circuits (Table I).

Table I. Description of tested circuits[a]

Description	Neonatal	Neonatal	Pediatric	Neonatal (used)
	Roller pump	Centrifugal pump	Roller pump	Roller pump
Priming Volume	350ml	200ml	900ml	350ml
Tubing	Medtronic® Sh. 70 USP class VI ¼ x 1/16 superTygon®	Intercept® CLASS VI ¼ x 1/16	Medtronic Sh. 70 USP class VI 3/8 x 3/32 superTygon®	Medtronic Sh. 70 USP class VI ¼ x 1/16 superTygon®
Oxygenator	Medtronic® 1,5 m^2 silicone membrane, Pediatric Extended Capacity Membrane Oxygenator	MEDOS HILITE® 800LT RHEOPARIN® coated polypropylene microporous hollow fibre	Medtronic® I-2500-2A 2,5m^2 silicone Surgical Membrane Oxygenator	Medtronic® 1,5 m^2 silicone membrane, Pediatric Extended Capacity Membrane Oxygenator
Heat Exchanger	Medtronic® Heat Exchanger Monitoring adapter and Luer-lock	n/a	Medtronic® Heat Exchanger Monitoring adapter and Luer-lock	Medtronic® Heat Exchanger Monitoring adapter and Luer-lock
Hemofilter	Hospal Multiflow 100	n/a	Hospal Multiflow 100	Hospal Multiflow 100
Remarks	Freshly primed, reference group	Freshly primed	Freshly primed	Clinically used for at least 48h before experiment

[a] Manufacturers: Medtronic, Minneapolis, USA; Medos Medizintechnik AG, Stolberg, Germany; Hospal, Lyon, France. n/a, not applicable

ECMO circuits

New ECMO circuits were primed according to hospital-based protocols; the only exception was the age of erythrocytes used for priming: leftover erythrocytes over 1 week old were used. ECMO circuits were primed with carbon dioxide, ringers lactate solution, albumin, tris(hydroxymethyl)aminomethane, sodium bicarbonate and erythrocytes. Drug losses in three freshly primed neonatal roller pump circuits were used as reference values for comparison with two freshly primed neonatal centrifugal circuits, two freshly primed pediatric circuits, and two used neonatal circuits. Used circuits were tested within 6 hours after decannulation, without replacement of its contents. Medication prior to decannulation consisted of continuous midazolam (150-200 µg/kg/h) and morphine (15-25 µg/kg/h) infusions for more than 12 hours, cefotaxime (50 mg/kg b.i.d) and amoxicillin (50 mg/kg t.i.d), magnesium sulphate (50 mg/kg q.i.d) , hydrocortisone (1 mg/kg t.i.d) and vancomycin (15 mg/kg). Temperature, hematocrit and pH were maintained within normal range.

ECMO circuits were made continuous via an incorporated bridge connection. As the centrifugal circuits lacked a bridge connection, these circuits were made continuous with the use of a reservoir bag containing 50 mL of priming fluid. The ECMO circuit was filled to maximal capacity with pre-oxygenator pressures of 250 mmHg. Before injection of the drugs an equal volume of fluid was subtracted from the ECMO circuit. The

volume of the neonatal roller pump circuit was estimated at 350 mL, that of the pediatric circuit volume at 900 mL and that of the centrifugal circuit volume (including reservoir) at 200 mL. Flow rates were set at 350 mL/min for neonatal circuits and 1000 mL/min for pediatric circuits.

Drug administration

Drugs were injected at 5 second intervals into a pre-bladder injection site simulating actual drug administration in patients. The line was flushed with at least 3 mL of physiological saline solution (0.9%) in between injection of the different drugs to avoid crystallization or depot effects. Drugs were dosed according to a standardized weight for a newborn (3 kg) and for an older child (15 kg). The order of drug injection was the following (neonatal/pediatric): fentanyl 15 µg/75 µg (FEN), morphine 0.6 mg/3 mg (MOR), midazolam 0.6 mg/3 mg (MDZ), acetaminophen 45 mg/250 mg (AAP), cefazolin 150 mg/750 mg (CFZ), meropenem 60 mg/300 mg (MEM), vancomycin 30 mg/250 mg (VAN).

Samples

Samples were taken from a post-oxygenator line before injection and 2, 4, 6, 8, 10, 30, 60 and 180 minutes after injection. Whole blood was collected in polypropylene tubes containing ethylenediaminetetraacetic acid (EDTA) and chilled to 4°C until further processing. The blood samples were centrifuged (6 min at 3000 ×g) after which the plasma supernatant was transferred to polypropylene cryogenic vials with polyethylene screw caps (Nalgene Labware, Rochester, NY, USA). Samples were stored at -80°C until analysis. Absence of drug loss caused by pipettes used to transfer samples (PVC, glass and polypropylene) was confirmed by testing the recovery of fentanyl morphine and midazolam from an aqueous standard after having been in a pipette tip for 3 min.

Quantification of analytes in plasma

Drugs were quantified via ultra-performance liquid chromatography with tandem mass spectrometry detection (UPLC-MS/MS). The UPLC-MS/MS circuit consisted of a Waters Acquity Ultra Performance LC coupled to a Quattro Premier XE tandem quadrupole mass spectrometer (Waters Corp., Milford, MA, USA). The analytical column was an Acquity UPLC BEH C18 2.1 mm × 100 mm column with 1.7 µm particle size (Waters Ltd, Dublin, Ireland), to which a 0.2 µm pre-column filter unit was added. Analytes were detected via MS with an electrospray ionization (ESI)-interface in positive multiple reaction monitoring (MRM)-mode. Optimized MRM-settings for the individual drugs, including cone voltage and collision energy, are listed in Table II. Data were acquired using Masslynx V4.1 software and processed using Quanlynx V4.1 software (Waters Inc.). Precision and accuracy was <15% for all analytes. Samples were diluted with plasma to stay within the

dynamic linear assay range for each analyte. Antibiotics and analgesics/sedatives were quantified via two similar methods:

(a) Antibiotics. See the method published by Ahsman *et al.*[12] The limits of quantification (LOQ) were 0.2 ng/mL (CFZ, MEM), and 0.7 ng/mL (VAN).

(b) Analgesics/sedatives. To 50 µL of plasma, 200 µL of chilled acetonitrile containing internal standard (midazolam-d_4, morphine-d_3, acetaminophen-d_4, fentanyl-d_5) was added. The sample was mixed (5°C, 1,250 rpm) for at least 15 min to complete protein precipitation. After centrifugation at 16,000 ×g for 10 min, 200 µL of the supernatant was transferred to a clean vial. The solvent was evaporated to dryness at 40°C under nitrogen gas flow, after which the residue was reconstituted in 100 µL of 0.1% (v/v) aqueous formic acid and left to mix for 30 min (5°C, 1,250 rpm). The supernatant was transferred to a glass insert autosampler vial and stored at 5°C until analysis by UPLC-MS/MS. The mobile phase was a gradient of solution A (0.1% formic acid in water) and solution B (0.1% formic acid in methanol) with an initial composition of 10% B, increasing to 65% B at 2.2 min. The flow rate was 0.4 mL/min with a column temperature of 40°C. Of each sample, 10 µL was injected onto the column. See Table II for the monitored ion transitions and MS-settings. The acquisition settings were: capillary voltage=3.4 kV; source temperature=120°C; desolvation temperature=300°C; desolvation gas flow=500 L/h; cone gas flow=50 L/h; dwell time=80 ms. The limits of quantification (LOQ) were 0.5 ng/mL (fentanyl), 5 ng/mL (midazolam), 2.5 ng/mL (morphine) and 1 µg/mL (acetaminophen).

Table II. Mass-spectrometric acquisition parameters[a]

	Q1 (*m/z*)	Q3 (*m/z*)	CV (V)	CE (eV)
MDZ	326.1	290.9	40	30
MOR	286.1	152.0	45	55
PAR	152.0	110.0	20	15
FEN	337.2	187.9	40	25
MDZ-d_4	330.1	295.2	40	30
MOR-d_3	289.1	152.0	45	55
PAR-d_4	156.0	114.0	20	15
FEN-d_5	342.2	188.1	40	25

[a] Q1, parent-ionmass; Q3, fragment-ionmass; CV, cone voltage; CE, collision energy

Ratio of whole blood/plasma concentrations

Theoretical maximum concentration in blood was calculated by dividing the administered dose by the volume of the ECMO circuit. However, since drug assays were performed in plasma, the ratio between the plasma and the blood concentration had to be determined to calculate drug recovery. A sample of prime solution (including

erythrocytes and albumin, 37°C) was divided into two aliquots. One of these aliquots was centrifuged for 6 min at 3000 ×g to obtain the supernatant plasma. 450 µL of this plasma was spiked with 50 µL of an aqueous solution containing the 7 drugs and vortexed, resulting in concentrations comparable to those in the pediatric *in vitro* circuits after drug infusion. These samples are considered reference samples.

The second aliquot of whole blood was spiked in fourfold with the same spike solution (50 µL per 450 µL of whole blood) and vortexed. Separate aliquots of these samples were stored at 37°C for 5, 30 and 180 minutes before being centrifuged, after which the plasma was transferred to polypropylene vials. Samples were stored at -80°C until analysis via UPLC-MS/MS. The blood plasma ratio was calculated as the average of the concentration in the whole blood samples divided by the spiked plasma samples. This was done in triplicate, the mean value was used to calculate whole blood concentrations from measured plasma concentrations.

Data analysis

Data were plotted and analysed with Graphpad Prism v4.03 (Graphpad Software Inc., La Jolla, CA, USA). In the used circuits, pre-existing drug levels (as assessed from the samples taken at 0 min) were subtracted from subsequent measurements. Concentrations were converted from plasma to the corresponding whole blood concentrations using the blood plasma ratio. To calculate the percentage of drug recovered from the circuit, the drug concentration 180 min after infusion was divided by the theoretical concentration, as calculated from the administered amount of drug divided by the estimated circuit volume. Log P values for the individual drugs are derived from the University of Alberta Drugbank website.[13] A student's t-test ($p<0.05$) was used to assess statistical significance of a difference in recoveries in neonatal vs. pediatric and used vs. new circuits. A nonlinear sigmoidal curve fit was applied to plot the recovery vs. log P, based on theoretical binding kinetics. Correlation between log P values and recovery rates was calculated using a two-sided Spearman test.

Results

Ratio of whole blood/plasma

The average ratio of whole blood/plasma for each drug was: 1.41 (MEM), 1.23 (VAN), 1.21 (CFZ), 0.94 (MOR), 0.90 (AAP), 0.77 (FEN) and 0.75 (MDZ). There was no discernible trend in ratio vs. incubation time. The relative standard deviation over all whole blood samples was ≤10%.

Drug loss

Table III lists the average recoveries after 180 min. and range for each category.

Table III. Recovery in % (range) of drugs after 180 minutes of circulation.

System	Neonatal Roller pump (n=3)	Neonatal Centrifugal pump (n=2)	Pediatric Roller pump (n=2)	Neonatal (used) Roller pump (n=2)
MDZ	0.62 (0.47-0.73)	63.4 (61.6-65.2)[a]	0.74 (0.66-0.81)	-0.06 (-0.93-0.81)
MOR	23.9 (14.6-35.8)	32.1 (31.9-32.3)	30.5 (28.6-32.4)	29.8 (17.1-42.6)
FEN	0.35 (0.15-0.50)	33.8 (32.4-35.3)[a]	0.28 (0.18-0.37)	0.54 (0.36-0.72)
PAR	34.0 (29.4-41.8)	44.2 (40.8-47.6)	44.9 (44.3-45.4)	47.3 (42.4-52.3)
CFZ	84.3 (72.4-100.8)	97.9 (92.5-103.3)	49.4 (44.7-54.1)	76.7 (65.7-87.6)
MEM	82.9 (69.1-101.4)	89.1 (76.4-101.7)	58.1 (54.4-61.9)	72.9 (60.1-85.7)
VAN	67.8 (49.2-95.3)	67.1 (61.6-72.6)	54.4 (43.4-65.3)	53.8 (47.4-60.3)

[a] statistically significant deviation (group averages, two-tailed, $p<0.001$) from new neonatal system with roller pump

Neonatal roller pump circuits

In the three neonatal roller pump circuits, FEN, MDZ, MOR and AAP showed significant drug loss within the first 2 minutes (Figure 1a). While MOR and AAP reached an apparent steady state within 10 minutes, MDZ and FEN did not: loss appeared to continue for at least 120 min. Losses after 2 minutes (100% minus recovered percentage of drug) for FEN, MDZ, MOR and AAP were 98.7%, 92.5%, 73% and 59.3%, respectively. After 180 minutes, these losses had increased to 99.6%, 99.4%, 76% and 65.9%, respectively.

Antibiotic loss was much lower (Figure 1b). After 2 minutes, 13% of CFZ, 18% of MEM and 25% of VAN was lost. After 180 min, an apparent steady-state had been reached at losses of 15%, 17% and 32%, respectively. For most drugs, especially the lipophilic drugs (MDZ, MOR), a large fraction of the administered dose appears to have been lost within two minutes of circulation. Despite having been injected first, FEN started to appear only after 4 minutes, indicating strong pooling or adsorption in the early phase of the experiment.

Correlation between recovery and log P

Since there were no statistically significant differences in recovery between all of the rollerpump circuits, these systems were all combined (n=8) to assess the correlation between recovery and lipophilicity. A sigmoidal curve best described the data, without apparent patterns in residuals vs. log P values (Figure 2). There was a significant correlation between log P and recovery ($df=53$, $r_{spearman=}0.807$, $p<0.001$).

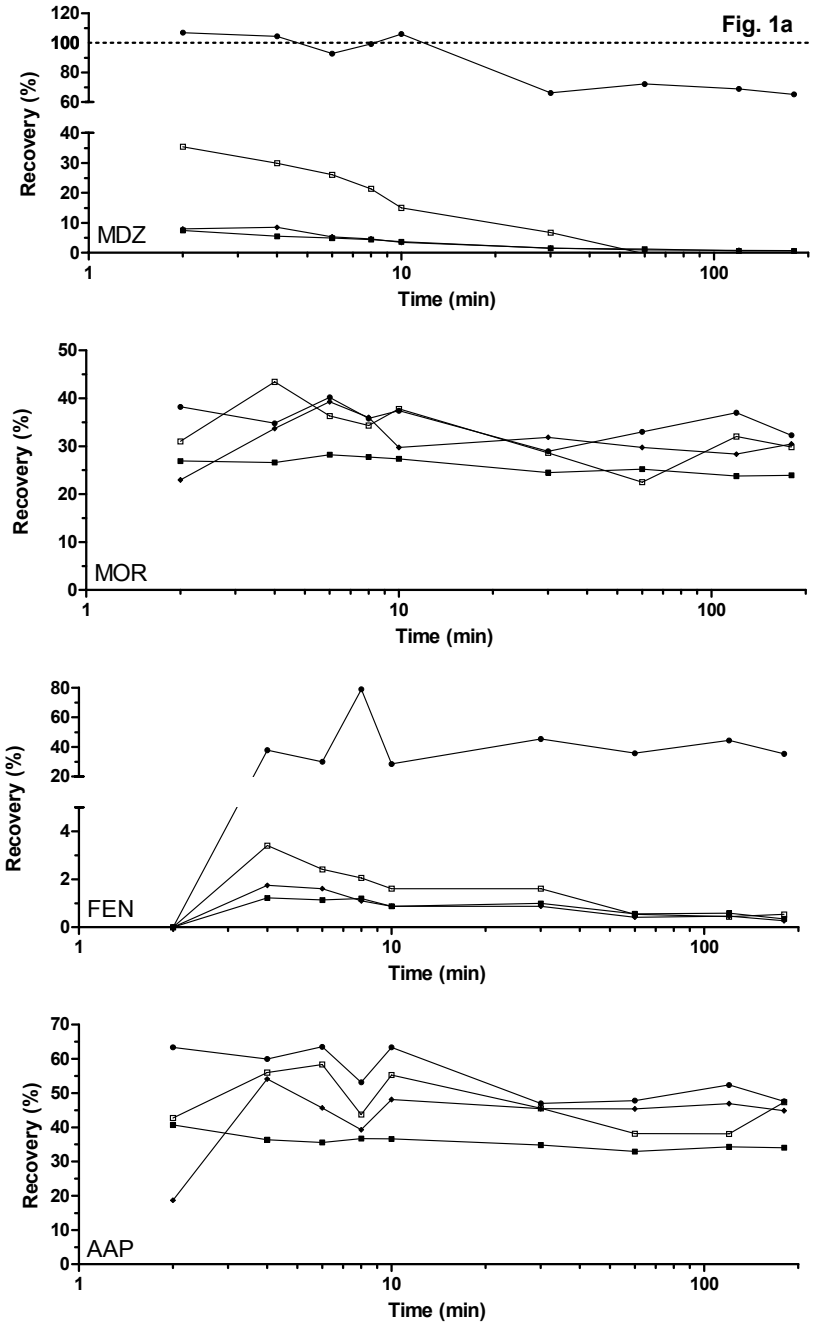

Figure 1. Average recovery vs. time for each drug. Displayed are the average recoveries for the neonatal roller pump circuits (filled squares), neonatal centrifugal pump circuits (filled circles), pediatric neonatal roller pump circuits (filled diamonds) and neonatal used roller pump circuits (open squares). Analgesics and sedatives are in fig. 1a, antibiotics in fig. 1b.

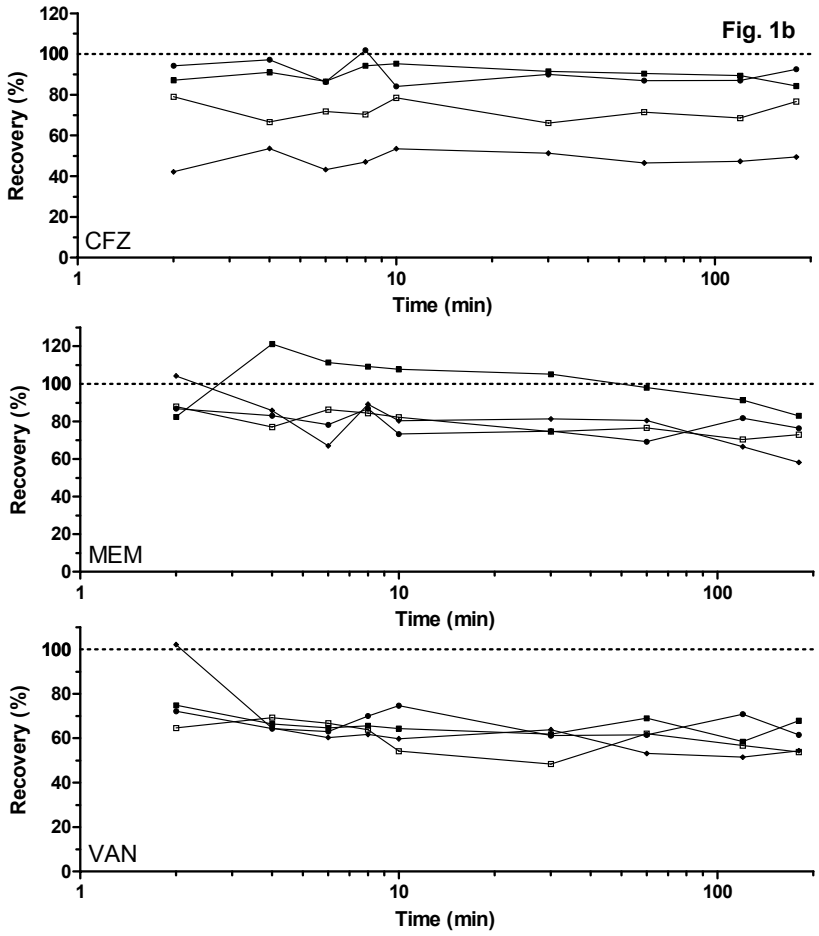

Figure 1. Average recovery vs. time for each drug. Displayed are the average recoveries for the neonatal roller pump circuits (filled squares), neonatal centrifugal pump circuits (filled circles), pediatric neonatal roller pump circuits (filled diamonds) and neonatal used roller pump circuits (open squares). Analgesics and sedatives are in fig. 1a, antibiotics in fig. 1b.

Centrifugal vs. roller pump circuits

There was a significant difference in drug recovery between the roller pump and centrifugal pump circuits for MDZ (0.6% vs. 63%, $p<0.001$) and FEN (0.4% vs. 34%, $p<0.001$). MOR recovery appeared higher in the centrifugal circuits, but without statistical significance (24.0% vs. 32.2%, $p=0.38$). Drug recovery was comparable in both circuits for AAP, VAN, MEM and CFZ after 180 min.

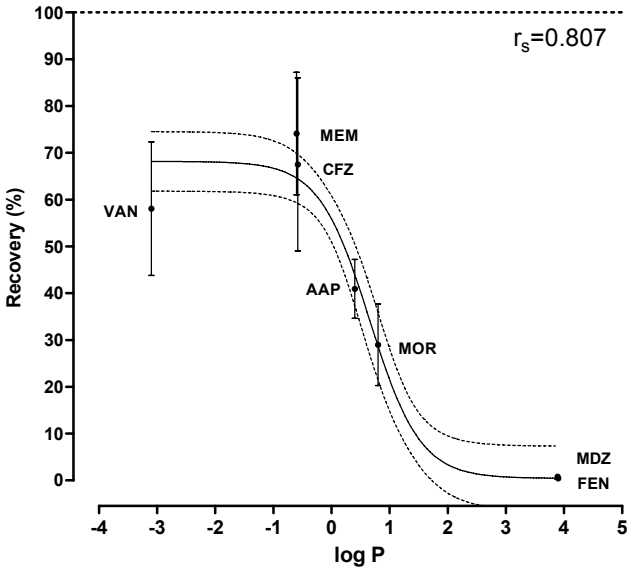

Figure 2. Recovery of drugs in roller pump circuits (n=8) vs. their lipophilicity, expressed as log P values. Displayed are the means and 95% confidence intervals for each drug, with a non-linear sigmoid curve fit (solid line) and its 95% confidence interval (dotted lines).

Pediatric vs. neonatal circuits

Sedatives and analgesics losses were similar in neonatal and pediatric circuits at 180 minutes. MEM and CFZ losses were higher in the pediatric circuits than in neonatal circuits, but without statistical significance (Table III).

Used vs. new circuits

The average pre-existing concentrations in the used circuits were: MDZ 344 ng/mL, MOR 16 ng/mL, FEN <0.5 ng/mL, AAP <1 µg/mL, CFZ <0.2 ng/mL, MEM <0.2 ng/mL, VAN 10.5 ng/mL. MDZ loss in the first 10 minutes in used circuits was lower than in new ECMO circuits; 4.1% vs. 26.1%, p=0.0004. This difference had disappeared after 180 minutes (Table I). There were no other significant differences in drug loss between used and freshly primed neonatal roller pump circuits.

Discussion

This comprehensive *in vitro* study enabled us to answer several questions regarding adsorption of drugs in ECMO circuits. First of all, drug loss is correlated to the individual drug's log P values and adsorption might therefore be predicted. More lipophilic drugs

such as fentanyl and midazolam disappeared almost completely, whereas the less lipophilic antibiotics showed much lower loss (10-35%).

Second, midazolam and fentanyl recovery was significantly higher in the centrifugal ECMO circuits. For lipophilic drugs, circuit size and/or type of oxygenator seem to influence adsorption. Others reported a marked difference in drug loss between polypropylene microporous or tubular membranes and silicone based CPB membranes.[14] Our study was not designed to localize the site of drug loss and therefore only general conclusions may be drawn. Less PVC tubing, different oxygenators and inclusion of a hemofilter may all contribute to the differences found. Due to technical difficulties, temperatures of the centrifugal circuits were maintained at 29°C and not at 35-37°C. This discrepancy may have contributed to the differences found. On the other hand, Skacel *et al.* showed no clear differences in drug losses between circuits maintained at low temperatures (24-25°C) and normal temperatures (37°C).[15]

Third, we found no significant difference between our pediatric and neonatal roller pump circuits; perhaps the effects of an increased dose and the increased polymer surface, combined with a relatively larger circulating volume, cancel each other out. Finally, contrary to previous reports [9, 11] we found no significant difference in drug loss between freshly primed and used ECMO circuits after 180 minutes.

In the present study concentrations of most drugs declined within the first minutes of ECMO after which an apparent steady state was reached. This was not the case for midazolam and fentanyl: concentrations of these drugs declined continuously during the three hour period. This suggests the presence of a greater amount of binding sites for midazolam and fentanyl than for the other compounds. As alternative explanations, the loading dose could have been below the saturation level; or steady state is reached after our last observation. This might explain the findings for the freshly primed circuits, but does not explain why similar adsorption patterns were found in the used ECMO circuits. An alternative explanation, degradation by enzymes or other causes, is unlikely; since both drugs are usually metabolized in the liver as opposed to circulating hydrolytic enzymes. *In vitro* experiments simulating continuous infusions or multiple dosing may help to clarify this issue.

Increased adsorption could be a cause of the increased dose requirements seen for midazolam.[16] The increase, however, is not tenfold as the >90% loss suggests; apparently other factors affect drug adsorption in the *in vivo* setting. Fentanyl shows the lowest recovery and takes long to appear at the other end of the circuit, which suggests pooling or a strong tendency to bind to any available binding site. This phenomenon has major clinical implications: since fentanyl and other lipophilic drugs will not be as effective when administered at a pre-oxygenator line, they should be given directly to the patient instead. Morphine is absorbed to a lower extent, and is therefore the preferred opioid for ECMO patients.

Midazolam, fentanyl, morphine and vancomycin losses in the roller pump circuits were higher than those previously reported.[7-8, 11, 17] Several factors may explain this discrepancy. Most studies are based on one to three ECMO circuits and we found substantial variability between individual circuits. Although study design and sampling errors may in part explain these variations, pooling of medication in the pressure-monitoring bladder and intrinsic qualities of the ECMO circuit may have an effect on recovery rates and release from the circuit components. The variability makes it difficult to compare the results from different studies. Mehta *et al.* reported stable fentanyl concentrations in blood primed circuits for up to 3 hours, although in their wet primed circuit fentanyl loss at 3 hours was 78%.[8] After 24 hours, fentanyl was no longer detectable indicating ongoing drug loss in the ECMO circuit, similar to our observations for midazolam and fentanyl. Both studies tested rollerpump circuits with a Medtronic® silicone membrane, but experimental methods were distinctly different. Mehta *et al.* used a reservoir bag pre-primed with medication before connecting this to the ECMO circuit; altered distribution within the ECMO circuit or reservoir bag could result in different time-dependent elimination curves. Another potential cause of variation is the presence of a hemofilter in the ECMO circuit, which we now use routinely to manage the fluid balance and improve caloric intake.[18-20] There was no dialysis flow during the *in vitro* trial but drug loss by the hemofilter membrane might have occurred. We tested several drugs simultaneously to simulate actual medication administration in ECMO patients. Although we cannot completely rule out drug-drug interactions, we consider this experimental approach to accurately reflect daily clinical practice.

The goal of this *in vitro* study was to evaluate potential determinants of drug loss in different ECMO circuits. In this study we tried to mimic the clinical situation in which solutions of routinely used drugs are injected into blood-primed circuits with short intervals. Previous studies were done in aqueous media[7, 17] or a spiked bag of blood to represent a patient.[8] The use of a whole blood system with assays in plasma required us to determine a ratio of whole blood concentration and plasma concentration and the estimation of the total volume, but the resulting experimental setup is close to the clinical situation. Without the use of a reservoir, baseline concentrations could not be measured. Instead, theoretical concentrations were estimated from dose and estimated volume of the ECMO circuits. This may have led to over- or under estimation of percentual drug losses. Priming volumes of all three circuits are known, however, and any error in estimated volume should not exceed 5%, and equally affects all drugs. The general trend therefore is clear: sedatives and analgesics are lost due to adsorption by membranes or tubing whereas antibiotics remain largely unaffected. This confirms observations done in studies of cardiopulmonary bypass circuits.[10, 21-22]

Conclusion

Significant uptake of drugs occurs in the ECMO circuit, which could lead to unexpectedly low plasma concentrations and higher dosage requirements for lipophilic drugs in particular. The log P value may be used to predict drug loss for roller pump circuits. Application of centrifugal pump circuits with hollow-fibre membrane oxygenators limits adsorption for all drugs, notably lipophilic drugs. Oxygenator size, and previous use of a circuit do not significantly affect drug losses. In combination with the inter-patient variability that is inherent to critically ill children, these drug losses likely contribute to the altered pharmacokinetics observed in patients on ECMO.

Acknowledgements

Special thanks to the ECMO specialists Addie Koole and José Groenewold of the Sophia Children's Hospital, Erasmus MC, Rotterdam and Leen Vercaemst, perfusionist in the University Hospitals, Louvain, Belgium, for their support and cooperation.

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Part III

Extracorporeal membrane oxygenation: Population pharmacokinetics

Chapter 7

Pharmacokinetics of cefotaxime and desacetylcefotaxime in infants during extracorporeal membrane oxygenation

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Antimicrob Agents Chemother 2010, in press

Abstract

Extracorporeal membrane oxygenation (ECMO) is used to temporarily sustain cardiac and respiratory function in critically ill infants, but can cause pharmacokinetic changes that necessitate dose modifications. Cefotaxime (CTX) is used to prevent and treat infections during ECMO, but the current dose regimen is based on pharmacokinetic data in non-ECMO patients. The objective of this study was to validate the standard dose regimen of 50 mg/kg b.i.d. (postnatal age (PNA) <1 wk), 50 mg/kg t.i.d. (PNA 1-4 wks) and 37.5 mg/kg q.i.d. (PNA>4 wks). We included 37 neonates on ECMO, with a median PNA (range) of 3.3 days (0.67-199) and a body weight of 3.5 kg (2.0-6.2) at onset of ECMO. Median (range) ECMO duration was 108 h (16-374). Plasma samples were taken during routine care and pharmacokinetic analysis of CTX and its active metabolite desacetylcefotaxime (DACT) was done using nonlinear mixed-effects modelling (NONMEM). A 1-compartment pharmacokinetic model for CTX and DACT adequately described the data. During ECMO, CL_{CTX} was 0.36 L/h (range 0.19-0.75), V_{CTX} was 1.82 L (0.73-3.02), CL_{DACT} was 1.46 L/h (0.48-5.93) and V_{DACT} was 11.0 L (2.32-28.0). Elimination half-lives for CTX and DACT were 3.5 h (1.6-6.8) and 5.4 h (0.8-14). Peak CTX concentration was 98.0 mg/L (33.2-286). DACT concentration varied between 0 and 38.2 mg/L, with a median of 10 mg/L in the first 12 h post-dose. Overall, CTX concentrations were above a minimal inhibitory concentration of 8 mg/L over the entire dose interval. Only 1 out of the 37 patients had a sub-MIC concentration for over 50% of the dose interval. In conclusion, the standard cefotaxime dose regimen provides sufficiently long periods of supra-MIC concentrations to provide adequate treatment of infants on ECMO.

Introduction

Extracorporeal Membrane Oxygenation (ECMO) is used as a standardized last resort to support critically ill infants who can no longer maintain sufficient cardiac and respiratory function with conventional life support techniques.[1, 2] Over a period of up to max. 3 weeks, blood flow is continuously diverted via a venous cannula into an extracorporeal circuit, oxygenated via a membrane and returned to the general circulation via a venous or arterial cannula. A hemofiltration unit can be added to the circuit to supplement insufficient renal function. Standard pharmacological treatment includes high doses of antibiotics for the treatment of pre-existing or nosocomial infections, which are facilitated by the direct microbial access to the patients general circulation via cannulas and circuit components.[3] One of the antibiotics commonly used in neonates on ECMO is cefotaxime (CTX), which possesses antimicrobial activity against many of the pathogens commonly involved in neonatal and ECMO-related infections, such as E. coli, Klebsiella pneumoniae, Enterobacter and Staphylococcus spp.[4] In adults, cefotaxime can be excreted unchanged via the renal system, but also after hepatic conversion into its active metabolite desacetylcefotaxime (DACT, for 15-25% of a dose).[5] There appears to be an inverse correlation between renal function and elimination half-life, particularly for DACT.[6]

In the absence of specific pharmacokinetic data, our current cefotaxime dose regimen is the same for both ECMO and non-ECMO patients. In general however, ECMO is associated with altered pharmacokinetics for a variety of drugs, probably due to an increase in circulatory volume, a disease-related clearance reduction or adsorption of drugs to membranes and other circuit components.[7] We designed this study to evaluate the pharmacokinetics of cefotaxime and desacetylcefotaxime during ECMO and validate our dose regimen.

Materials and Methods

All neonates about to receive ECMO treatment at the Sophia Children's Hospital (Erasmus University Medical Center) from December 2006 to June 2009 were eligible. The local institutional ethics review board approved this study. Parental informed consent was obtained for blood sampling and use of clinical data. Criteria for ECMO treatment were: gestational age >34 weeks, birth weight >2.0 kg, mechanical ventilation <7 days, an alveolar arterial oxygen difference more than 600 mmHg, and an oxygenation index >25. Concomitant drugs were given in accordance with the departmental treatment protocol and doses were adapted to each neonate's clinical condition. The most recent weight available prior to ECMO was used for dose calculation and pharmacokinetic analysis. Drug administrations, laboratory results and real-time parameters such as ECMO flow were recorded in a Patient Data Management System.

ECMO

The ECMO circuit consisted of extracorporeal cannulae (Medtronic®, Kerkrade, the Netherlands), PVC tubing (Bentley Bypass 70 tubing, Baxter, The Netherlands), a silicone rubber membrane oxygenator (Pediatric Extended Membrane Oxygenator, Medtronic®), and Heat Exchanger (Heat Exchanger Monitoring adapter and Luer-lock, Medtronic®). Priming volume ranged between 300 and 350 mL. A continuous venovenous hemofiltration (CVVH)-filter (Multiflow 60, Hospal, Lyon, France) was placed parallel to the ECMO circuit, distal to the ECMO roller pump. Pressure was measured proximal and distal to the filter; the difference was kept constant at 40 mmHg.

Cefotaxime administration

Cefotaxime was given intravenously as a bolus injection over max. 3 minutes. Dose regimens have been standardized hospital-wide to vary with postnatal age from 50 mg/kg b.i.d. (PNA <1 wk) and 50 mg/kg t.i.d. (PNA 1-4 wks) to 37.5 mg/kg q.i.d. (PNA >4 wks) [8] for ECMO and non-ECMO patients alike, but doctors could deviate from protocol at their own discretion. Doses were rounded off to the nearest 5 mg to allow reliable administration of prescribed CTX doses. Nurses validated physician-prescribed medication orders and recorded actual injection times in the data management system as part of their standard care routine. CTX was administered via an extracorporeal line after the oxygenator, just before blood was returned to the patients circulation.

Blood sampling and assay

Blood was collected during routine laboratory rounds three times daily. When possible, additional samples were taken 1 h before and 0, 1 and 3 h after cannulation to characterize early pharmacokinetic changes. Sampling continued for max. 24 h after decannulation. Blood (max. 1 mL) was taken from a venous pre-oxygenator access point dedicated to sample withdrawal on the ECMO circuit and collected in ethylenediaminetetraacetic acid (EDTA)-decoagulation vials, which were stored at 4-7°C until further processing. After centrifugation (5 min, 4000 ×g), the supernatant serum was stored at -80°C until assay. Sampling times and duration of storage at 4-7°C were recorded. CTX and DACT concentrations were quantified via liquid chromatography-mass spectrometry (LC-MS) as previously described.[9] Limits of quantification were 0.2 mg/L for both CTX and DACT. Intra- and inter-assay coefficients of variation were <15%.

Blood culture

Blood cultures are performed daily at our institution. Samples were taken from a venous access port and sent in for microbiological surveillance.

PK model development

CTX and DACT models were developed sequentially using nonlinear mixed-effects modelling software (NONMEM VI 2.0, Globomax LLC, Ellicott City, MD). NONMEM allows the estimation of typical population pharmacokinetic parameters, and their respective inter- and intra-individual variability in combination with residual random variability. The first-order conditional estimation (FOCE) method, with interaction between the inter-individual and random effects, was used throughout method development. Differential equations were used with NONMEM's ADVAN 6 subroutine to describe the population PK of CTX and DACT. After selection of an appropriate base model, inter-individual random effects were evaluated on clearance (CL) and volumes of distribution (V) with an exponential model. Covariance between CL and V was modelled using an omega block function. Residual variability was described with a proportional error model; the proportional variance coefficient was separately estimated for samples taken within 1 h post-dose to account for expected variable discrepancies between the actual and the recorded dose time. Post-sampling degradation was incorporated into the error model by calculating the concentration at the time of sampling using the degradation rate constant in EDTA-decoagulated whole blood from literature (k_{deg}=0.0132, $t_{½}$=52 h)[9]; the median correction of observed CTX concentrations was +15.7%. Covariate effects on CL or V were incorporated into the model as previously described [10] and their statistical significance was assessed in a stepwise inclusion and exclusion procedure.[11] The tested covariates include gestational age (GA), postnatal age (PNA), body weight (WT), time after dose (t_{DOSE}), time after start or end of extracorporeal circulation (t_{EC} and t_{END}), ECMO on/off, ECMO-flow (Q_{ECMO}), CVVH-flow (Q_{CVVH}), indication, the number of ECMO runs, ECMO-modality (venovenous or venoarterial), sex, body temperature, urine output, fluid balance, serum albumin, serum creatinine and concomitant use of vasopressive medication (norepinephrine, dopamine, dobutamine or epinephine). After selection of appropriate covariates, remaining inter-occasion variability was tested on CL and V for CTX and DACT in which occasions were defined as t_{EC} periods of 48 h; pre- and post-ECMO observations were considered separate occasions.

PK model performance

Evaluation of models was based on improvements in the minimum value of objective function (OFV), standard error of parameter estimates and goodness-of-fit plots generated via the Xpose software package (v 4.0.4, Dr. M. Karlsson, University of Uppsala, Sweden) [12] within R (v 2.8.1, The R Foundation for statistical computing, www.R-project.org). Additional plots were prepared using GraphPad Prism 4.03 (GraphPad Software Inc, La Jolla, CA). Goodness-of-fit plots included, among others, plots of measured drug concentrations vs. population (PRED) or individual (IPRED) predictions, conditional weighted residuals (CWRES) [13] vs. time or other covariates and plots of observed concentrations

(dependent variable or DV), PRED and IPRED vs. time. Bayesian IPRED concentrations were obtained via NONMEM's *posthoc* option. Statistical significance of a potential model improvement was determined via the log-likelihood ratio test for nested models, using the OFV produced by NONMEM. A decrease in OFV of 3.84 (p=0.05, χ^2 distribution, 1 degree of freedom) was considered statistically significant. A stricter criterion (p=0.01, ΔOFV=6.63) was used in the backward elimination procedure for covariate effects: if deletion of a covariate did not result in a significant worsening of the objective function, the covariate was removed from the model. The resulting model was considered the final model. Shrinkage was calculated to assess whether the estimated η and ε parameter distributions matched those of the original data assuming normal distribution.[14] Stability and performance of the final model were checked using an internal validation procedure via the bootstrap resampling technique, in which 1200 bootstrap data sets were generated by random sampling with replacement.[15] We used the Wings for NONMEM software package (v6.12 March 2007, Dr N. Holford, Auckland, New Zealand). Model validity was assessed by calculating median values and the 2.5th and 97.5th percentiles of parameter distribution generated by the bootstrap, and comparing them with the original estimates. The bootstrap was also used to calculate standard errors for each estimate.

Dose regimen evaluation

The fraction of a dose interval during which the cefotaxime concentration exceeds the minimal inhibitory concentration of susceptible micro-organisms ($t_{>MIC}$, as % of dose interval over 24 h) is considered an appropriate measure of efficacy.[16, 17] Based on bacteriological screening results of our ECMO patients and literature on pathogens involved in pediatric meningitis [4], the main pathogens include Escherichia, Staphylococcus, Klebsiella, Serratia and Enterobacter species. Reported MIC values (MIC distributions of wild type microorganisms, via www.Eucast.org) are at or below 4 µg/mL (S. aureus). Assuming a worst case scenario of up to 40% protein binding [18], the maximal MIC value in plasma is around 8 µg/mL. Using the individual parameter estimates derived from the final PK model, concentration-time curves were constructed for each individual by simulating the predicted concentration over intervals of 0.2 h. We calculated $t_{>MIC}$ over 24 h for each individual patient and compared the median values for each dose regimen; we considered the antimicrobial effect to be optimal at a $t_{>MIC}$ of at least 50%.[16]

Results

Data

We included 37 patients with a total of 392 samples (median per patient: 10, range 1-17). Pre-ECMO samples were available for 8 individuals (1 each); post-ECMO samples were

available for 13 individuals (on average 2.1 each). See Table I for patient characteristics. CTX and DACT were successfully quantified in all samples, with 4 (CTX, 1.0%) and 3 (DACT, 0.8%) concentrations below the quantification limit (BQL). DACT concentrations were converted to CTX equivalents using a molecular weight ratio of 455.5/413.4 (Mw_{CTX}/Mw_{DACT}).

Table I. Patient characteristics[a]

General	
Sex	18 M / 19 F
Primary Diagnosis	Meconium aspiration syndrome, n=17 (46%) Congenital diaphragmatic hernia, n=8 (22%) Pulmonary hypertension (other causes), n=5 (14%) Congenital heart defects, n=4 (11%) Other (sepsis, viral infections, etc.), n=3 (7%)
Body weight (kg)	3.5 (2.0-6.2)
Gestation (weeks)	37 (34-42)
Postnatal age at start ECMO (days)	3.3 (0.67-199)
Survival	25 (68%)
Cefotaxime	
Dose (i.v.)	50 mg/kg b.i.d., n=24 (65%) 50 mg/kg t.i.d., n=7 (19%) 37.5 mg/kg q.i.d., n=3 (8%) 25 mg/kg b.i.d., n=2 (5%) 37.5 mg/kg t.i.d., n=1 (3%)
Serum chemistry	
Albumin (g/L)	31 (21-40)
Serum creatinine (μmol/L)	32 (19-69)
ASAT (IU/L)	44 (14-369)
ALAT (IU/L)	10 (0.5-40)
ECMO	
ECMO modality	Venovenous (VV), n=22 (54%) Venoarterial (VA), n=19 (46%) Four patients had 2 ECMO runs each: 3 VV + VA, 1 VA + VV
Median ECMO flow (mL/kg/min)	308 (50-530)
Duration of ECMO (h)	108 (16-374)
Continuous venovenous haemofiltration	30 Y / 7 N
CVVH flow (mL/min)	193 (100-350)
Body temperature	2 hypothermic (24°C) / 35 normothermic (36°C)

[a] Parameters expressed as median (range) or n (%). ASAT=aspartate amino transferase; ALAT=alanine aminotransferase; CVVH=continuous venovenous hemofiltration.

Blood culture

34 patients had negative blood cultures throughout their ECMO run during CTX administration. Two patients had one positive culture at day 8 and 10 of ECMO respectively,

but both had negative cultures beforehand and at least two days thereafter; it is unclear whether these were false-positive cultures or transient infections. One patient had positive cultures at days 11 and 13, in which an enterococcus could be isolated.

PK model development

A 1-compartment model with first-order elimination for both CTX and DACT best fit the data; additional compartments improved goodness-of-fit plots nor the OFV. BQL concentrations were removed from the dataset; deletion did not change CL and V parameter estimates for the base model. Proportional residual error terms improved the model whereas an additional error did not. There was a structural deviation in CWRES vs. t_{DOSE} plots indicating lower than expected concentrations in the first hour after CTX infusion. A separate proportional residual error for samples with t_{DOSE} <1 h reduced this deviation. Alternatively, first-order absorption and lag-time models were tested but they did not significantly improve fit, probably because only a fraction of the concentrations was overpredicted. No other covariates were correlated with this deviation. Inter-individual variability was successfully estimated for CL and V for both compounds. Covariance between CL and V significantly improved minimization and stability; correlation varied from 70.6% (CL_{DACT}~V_{DACT}) to 90.8% (V_{CTX}~V_{DACT}). Inter-occasion variability (occasions of 48 h) was tested only after trends with t_{EC} or other time-varying covariates proved non-significant and improved fit with a significant ($p<0.001$) reduction in OFV. An increase in CL_{CTX} and CL_{DACT} upon cannulation, which could be seen in 8 patients based on one pre-ECMO sample each, could not be modelled with statistical significance. Allometric scaling [19] was tested before other covariates, but this did not reduce the OFV. The covariate inclusion procedure suggested that the following covariates might be correlated to V or CL and improve the OFV upon inclusion ($p<0.05$): GA, Q_{CVVH}, WT, PNA, vasopressor use and t_{END} (CL_{CTX}); fluid balance and serum creatinine (V_{CTX}); sex, duration of pregnancy, WT, Q_{ECMO}, t_{END} and Q_{CVVH} (CL_{DACT}); t_{END} (V_{DACT}). After stepwise exclusion, the only significant remaining effects were WT (CL_{CTX}), Q_{CVVH} (CL_{DACT}) and t_{END} (CL_{CTX} and CL_{DACT}), but drops in unexplained inter-individual variability were small: -2.7% (WT~CL_{CTX}), -8.1% (Q_{CVVH}~CL_{DACT}), -0.5% (t_{END}~CL_{CTX}), -4.2% (t_{END}~CL_{DACT}). None of the covariates reduced inter-individual variability for V_{CTX} or V_{DACT}. See Table II for parameter estimates of the final model. See Appendix 1 for the differential equations used in the final model, including covariate effects.

PK model performance

See Figure 1 for the goodness-of-fit plots. In certain individuals, DACT was structurally underestimated (see Figure 1c) but there was no significant trend with any covariate; inter-individual variability on PK parameters corrected this pattern (Figure 1d). There was no trend in CWRES vs. t_{EC}. All parameter estimates were within the 95% confidence

Table II. Parameter estimates[a]

	Unit	CTX		DACT		Remarks
		Estimate (CV %)	Bootstrap median (95% CI)	Estimate (CV %)	Bootstrap median (95% CI)	
Population parameters						
V	L	1.82 (8.2%)	1.86 (1.60-2.20)	11.0 (14.0%)	11.0 (7.90-14.0)	
CL	L/h	0.36 (7.9%)	0.36 (0.30-0.41)	1.46 (11.5%)	1.42 (1.10-1.77)	
Covariate effects						
θ_{WT}	-	0.56 (43.7%)	0.55 (0.02-1.00)	-	-	$CL = CL_{pop} \times (WT/3.5)^{\theta_{WT}}$
θ_{CVVH}	-	-	-	0.72 (35.8%)	0.69 (0.10-1.10)	$CL = CL_{pop} \times (Q_{CVVH}/193)^{\theta_{CVVH}}$ without CVVH, CL=CL_{pop}
$\theta_{t_{END}}$	-	0.16 (80.8%)	0.16 (0.002-0.48)	0.53 (53.7%)	0.51 (0.18-1.20)	$CL = CL_{pop} \times (t_{END}/100)^{\theta_{t_{END}}}$ when t_{END}=0, CL=CL_{pop}
Interindividual variability						
V	%	35.4 (24.2%)	35.9 (16.7-51.5)	59.8 (19.7%)	60.8 (39.4-84.2)	
CL	%	36.1 (21.5%)	34.8 (24.1-53.1)	51.4 (18.6%)	53.3 (39.7-76.1)	
Interoccasion variability						
V	%	25.0 (20.7%)	24.5 (15.7-35.8)	25.0 (20.7%)	24.5 (15.7-35.8)	Calculated over periods of 48 h on ECMO
CL	%	25.0 (20.7%)	24.5 (15.7-35.8)	25.0 (20.7%)	24.5 (15.7-35.8)	Calculated over periods of 48 h on ECMO
Residual variability						
Proportional (t_{dose}<1 h)	%	69.4 (25.4%)	68.3 (44.9-90.7)	69.4 (25.4%)	68.3 (44.9-90.7)	
Proportional (t_{dose}>1 h)	%	32.7 (8.2%)	32.3 (27.4-37.6)	32.7 (8.2%)	32.3 (27.4-37.6)	

[a] CTX=cefotaxime; DACT=desacetylcefotaxime; CV=coefficient of variation; V=volume of distribution; CL=clearance; WT=body weight in kg; Q_{CVVH}=CVVH flow; t_{END}=time after decannulation in h; t_{dose}=time after last dose. CL and V estimates for DACT were calculated assuming a conversion fraction ($F_{DACT/CTX}$) of 1

interval calculated using bootstrap data (Table II). The higher coefficients of variation for the covariate effects show that their estimation is difficult in this dataset, probably due to the small sample size and high residual variability. Shrinkage was calculated for inter-individual variability (η) on CL_{CTX} (5.2%), V_{CTX} (4.7%), CL_{DACT} (6.4%), V_{DACT} (4.4%) and the residual variability (ε, 2.2%) using Perl-speaks-NONMEM.[20]

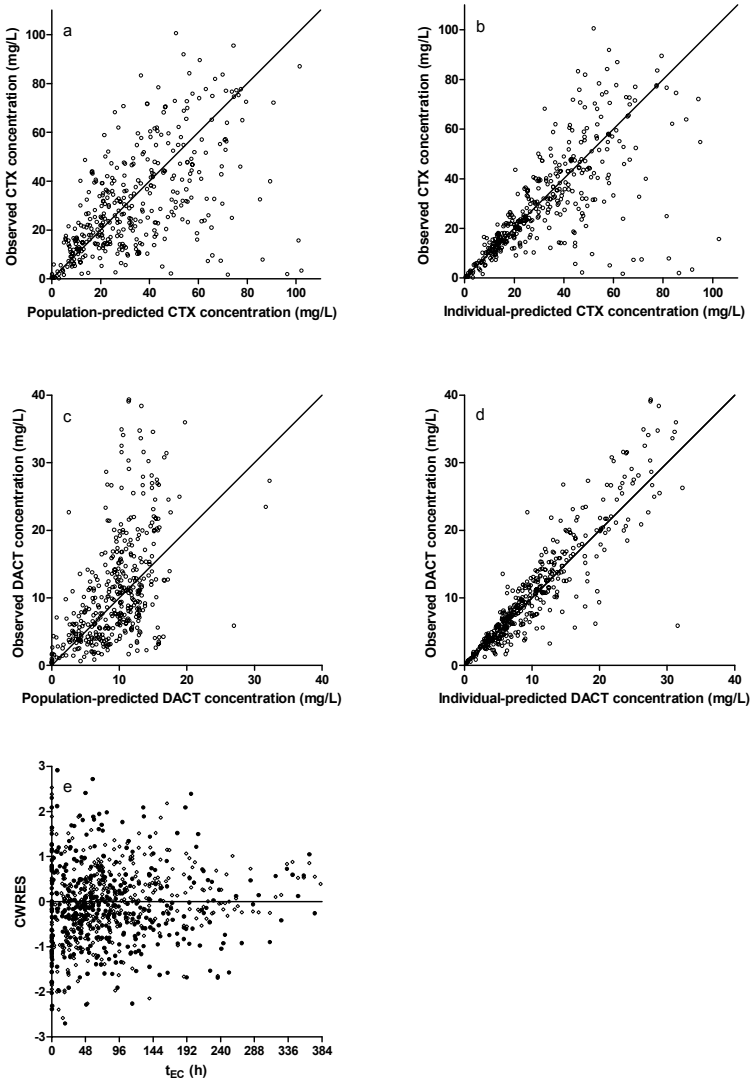

Figure 1. Goodness-of-fit plots for the final model. Observed cefotaxime (CTX) concentration vs. population predicted (a) and individual-predicted (b) concentration. Similar plots are displayed for desacetylcefotaxime (DACT) (c and d). There is no apparent pattern in conditional weighted residuals (CWRES) vs. time after start of ECMO (t_{EC}) for CTX (closed circles) or DACT (open circles, e).

CTX and DACT pharmacokinetics

See Table II for parameter estimates. During ECMO, median CL_{CTX}=0.36 L/h (0.19-0.75), V_{CTX}=1.82 L (0.73-3.02), CL_{DACT}=1.46 L/h (0.48-5.93) and V_{DACT}=11.0 L (2.32-28.0). Over the weight range of 2-6.2 kg, median CL_{CTX} varies from 0.26-0.50 L/h. The elimination half-life is 3.5 h (CTX, 1.6-6.8) and 5.4 h (DACT, 0.8-14). In the individuals for which pre or post-ECMO samples are available, CTX and DACT clearance appear to increase upon cannula-

tion (median CL_{CTX}=0.30 to 0.36 L/h, CL_{DACT}=1.37 to 1.46 L/h). After decannulation, CL_{CTX} and CL_{DACT} drop almost instantaneously but recover steadily over the following 72 h (from 0.22 to 0.40 L/h and from 0.18 to 1.38 L/h). See Figure 2 for plasma concentrations and clearance estimates for one of the studied individuals.

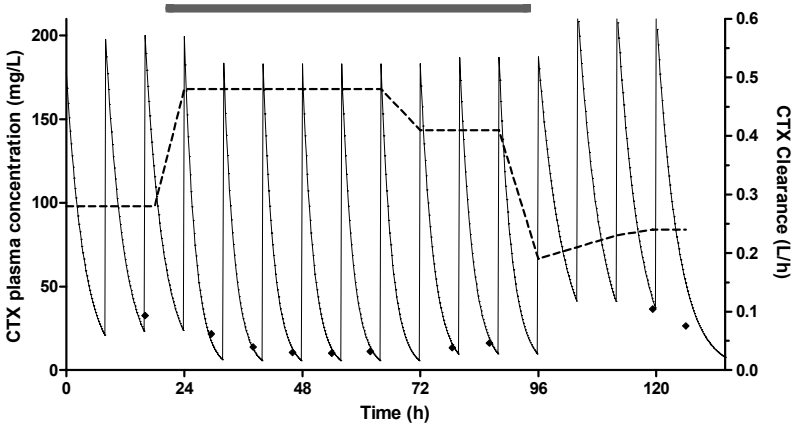

Figure 2. Characteristic concentration-time curve for one of the subjects (with a dose of 50 mg/kg t.i.d.) with a number of samples pre- and post-ECMO. Displayed are the Bayesian estimated CTX plasma concentration profile (continuous curve) with the observed concentrations (diamonds, both left axis) and CTX clearance (intermittent curve, right axis). The duration of ECMO-treatment is indicated by the grey box.

Dose regimen

Individual *posthoc* estimates of CTAX plasma concentration at intervals of 0.2 h over the entire observation period were used to calculate the $t_{>MIC}$ for each patient. Peak CTX concentrations were 98.0 mg/L (33.2-286). DACT concentrations varied between 0 and 38.2 mg/L, with a median of 10 mg/L in the first 12 h postdose. The median $t_{>MIC}$ (calculated for CTX only) was 100%. 36 out of 37 patients had a $t_{>MIC}$ over 50% for all their CTX doses. The remaining patient (PNA <1 wk) had declining plasma concentrations even after a new dose; it is possible that one or more doses were skipped due to medical procedures at dose time, inadvertent dose registration without actually having given the dose, or other unknown reasons. This caused this individuals $t_{>MIC}$ to drop to 49%. See Figure 3 for the individual-predicted CTX and DACT concentrations over a dose interval of 12 h. With the exception of the aforementioned patient, concentrations in all three age categories (PNA <1 wk with n=26; 1-4 wks with n=7, and >4 wks with n=4) were above the MIC over a period of at least 6 h. In general, the patients with a PNA of 1-4 wks were at the bottom of the concentration-time curve, but their dose interval is only 8 h.

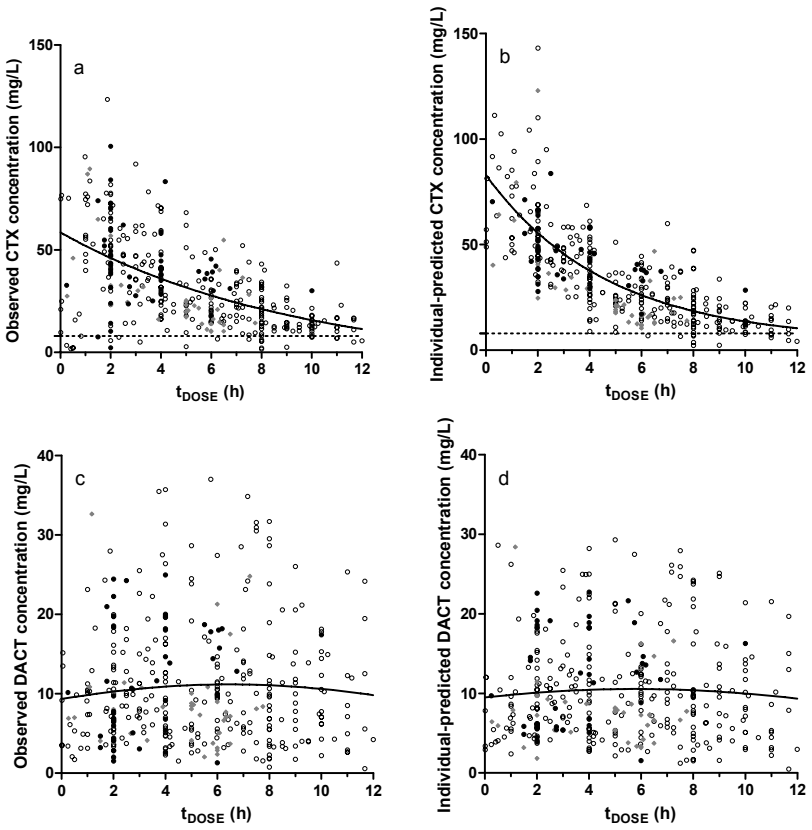

Figure 3. Observed and individual-predicted concentrations versus dose-time for cefotaxime (CTX, a & b) and desacetylcefotaxime (DACT, c & d). In plots a and b the target MIC is indicated by the intermittent line. Data points are marked to stratify data by postnatal age (PNA): < 1 wk (open circles), 1-4 wks (grey diamonds), > 4 wks (closed circles). The solid lines represent a naive pooling fit of all data for CTX (nonlinear first-order decline curve) and DACT (coarse LOWESS curve).

Discussion

In the present study, the standard dose regimens provided sufficient $t_{>MIC}$ values for antibiotic efficacy during ECMO, which is reflected in the low number of positive blood cultures. The patient with the lowest $t_{>MIC}$ (49%) had negative cultures throughout his ECMO run while the patients with positive cultures had $t_{>MIC}$ of 90% or higher, but this could be caused by resistance or lack of efficacy of other concomitant antibiotics. The CTX clearance we estimated for ECMO patients (0.36 L/h) is similar to that in non-ECMO treated full-term neonates, which varies from 0.20-0.55 L/h.[21-23] The distribution volume however is larger than in non-ECMO patients (1.82 L vs. 0.68-1.14 L) [22, 23], which could be caused by hemodilution or capillary leakage of protein-bound drug into the extravascular compartment, especially in the early phase of ECMO (24-36 h after cannulation). This increase is

consistent with studies on the pharmacokinetics of vancomycin [24] and theofylline [25] during ECMO. There were no signs of the rapid increase of V following cannulation that has been described for midazolam.[10, 26] Unfortunately we only had few samples before and after ECMO, but patients for whom we do have some samples show an interesting clearance pattern upon which we might formulate a hypothesis on the physiological processes involved. It would seem that these critically ill patients have a reduced clearance before cannulation. Many of them use vasopressor drugs with prolonged periods of circulatory shock and profound effects on renal function. As soon as ECMO is initiated, clearance rises to that of a non-ECMO treated patient, possibly due to the continuous hemofiltration and improved organ perfusion the extracorporeal circulation provides. After decannulation, clearance drops again (as the patient is still critically ill) but slowly increases due to maturation or improved disease state. This pattern is visible for both CTX and DACT.

$T_{>MIC}$ was sufficiently high despite the increased distribution volume, which suggests that cefotaxime is dosed higher than strictly necessary in non-ECMO patients. This need not be a problem with drugs that are as safe as cefalosporins are considered to be.[27, 28] Our standard dose regimen is based on studies in neonatal and pediatric patients that have identified the influence of gestational age [29], body weight [29], postnatal age [21] and renal function [30] on CTX pharmacokinetics. Although creatinine clearance is a clinically relevant predictor of renal CTX clearance in non-ECMO patients [30], we had no measure of creatinine clearance due to the young age of most patients and the underlying disease state.[31] Serum creatinine was measured, but there was no correlation with CTX clearance after body weight had been added to the model. Interestingly, gestational age and postnatal age did not predict CL or V; other factors such as disease state, protein binding, organ perfusion, etc. might be responsible. A study in 107 neonates [21] showed that clearance increases dramatically with PNA during the first week after birth, but there was no sign of this development in our dataset. It's possible that critical illness in our ECMO patients, with the use of drugs influencing renal perfusion (i.e. high doses of norepinephrin and dopamine) has lead to a low baseline renal clearance that is artificially supplemented by CVVH; the median Q_{CVVH} per individual did not vary much. Although we were able to identify several variables with a statistically significant effect on CTX and DACT pharmacokinetics, the percentage of variability explained is max. 8.1%, which illustrates our limited understanding of ECMO-related sources of PK variability. Considering the sufficiently high $t_{>MIC}$ values in all patients, we probably do not need to adjust the dosage based on these covariates.

DACT concentrations are highly variable as indicated by Figure 3c and 3d. The contribution to the antibacterial effect varies with the microbial species involved, which makes it difficult to make a general assessment of efficacy.[32] DACT concentrations are similar to those in other studies [21, 33]; there does not seem to be an increased risk of DACT accumulation, as has been suggested for hydrophilic metabolites during ECMO.[10]

The concentrations may have been slightly overestimated because of the increased CTX hydrolysis that can occur following hemolysis caused by contact with circuit surfaces or storage in plasma tubes.[32]

Since most samples were taken during routine care, the dataset contained a large number of samples for each patient, spread out over the full duration of ECMO. This allows a reliable characterization of time-effects on PK parameters. A potential drawback of this method, as opposed to dose and sample registration by dedicated researchers or their assistants, is additional variability due to inter-observer differences in registration. We expected a maximum discrepancy of 30 min between actual and recorded dose times based on a comparison of observed work routines of individual nurses. A high residual variability in the first hour post-dose is probably caused by inter-nurse variability in the time between CTX injection and medication order validation. Since this phenomenon appeared to be randomly distributed over individuals, doses, t_{EC}, etc, we estimated a separate residual variability, which in effect entails less influence on the final model compared to the samples taken at later dose-times. This also affects the median curve of individual predictions compared to the same curve in the original observations (Figure 3a vs. 3b). Data that were recorded during standard clinical practice should therefore be used with caution, but a balanced dataset without blood withdrawal at non-routine sampling times offers important advantages.

Conclusion

The standard cefotaxime dose regimen provides sufficiently high $t_{>MIC}$ in ECMO infants. The CTX distribution volume is higher in ECMO vs. non-ECMO patients (1.82 vs. 0.68-1.14 L), whereas CTX clearance is similar. A dose regimen of 50 mg/kg b.i.d. (PNA <1 wk), 50 mg/kg t.i.d. (PNA 1-4 wks) or 37.5 mg/kg q.i.d. (PNA >4 wks) can be used to effectively treat these patients.

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Appendix 1 Equations final PK model cefotaxime and desacetylcefotaxime

Cefotaxime (CTX):

$$CL_{CTX,ij} = \left(CL_{CTX,pop} \times \left(\frac{WT}{3.5} \right)^{\theta_{WT}} \times \left(\frac{t_{END}}{100} \right)^{\theta_{T_{END}}} \right) \times e^{(\eta_{IIV,i} + \eta_{IOV,j})} \quad \text{(Eq. A1)}$$

in which $CL_{CTX,ij}$ is the CTX clearance for individual i at the jth occasion, $CL_{CTX,pop}$ is the population average CL for patients with a median weight (3.5 kg), WT is body weight, t_{END} is time after ECMO-decannulation, $\eta_{IIV,i}$ is the inter-individual variability for individual i, and $\eta_{IOV,j}$ is the accompanying inter-occasion variability (in periods of 48 h during ECMO). When t_{END}=0 (i.e. before and during ECMO), the accompanying covariate effect is removed from the equation.

$$V_{CTX,ij} = V_{CTX,pop} \times e^{\eta_{IIV,i}} \quad \text{(Eq. A2)}$$

in which $V_{CTX,ij}$ is the CTX distribution volume for individual i at the jth occasion, $V_{CTX,pop}$ is the population average and $\eta_{IIV,i}$ is the inter-individual variability for individual i.

Desacetylcefotaxime (DACT):

$$CL_{DACT,ij} = \left(CL_{DACT,pop} \times \left(\frac{t_{END}}{100} \right)^{\theta_{T_{END}}} \times \left(\frac{Q_{CVVH}}{193} \right)^{\theta_{CVVH}} \right) \times e^{(\eta_{IIV,i} + \eta_{IOV,j})} \quad \text{(Eq. A3)}$$

in which $CL_{DACT,ij}$ is the DACT clearance for individual i at the jth occasion, $CL_{DACT,pop}$ is the population average, t_{END} is time after ECMO-decannulation, Q_{CVVH} is the CVVH flow, $\eta_{IIV,i}$ is the inter-individual variability for individual i, and $\eta_{IOV,j}$ is the accompanying inter-occasion variability (in periods of 48 h during ECMO). When $t_{END=}$0 or Q_{CVVH}=0, the accompanying covariate effects are removed from the equation.

$$V_{DACT,ij} = V_{DACT,pop} \times e^{\eta_{IIV,i}} \quad \text{(Eq. A4)}$$

in which $V_{DACT,ij}$ is the DACT distribution volume for individual i at the jth occasion, $V_{DACT,pop}$ is the population average and $\eta_{IIV,i}$ is the inter-individual variability for individual i.

Differential Equations:

$$\frac{dCTX}{dt} = D - \frac{CL_{CTX}}{V_{CTX}} \times AMT_{CMT1} \quad \text{(Eq. A5)}$$

in which dCTX/dt is the rate of CTX transit, D is the administered dose, CL_{CTX} is CTX clearance, V_{CTX} is the apparent distribution volume and AMT_{CMT1} is the amount of CTX present in compartment 1 at any one time.

$$\frac{dDACT}{dt} = \left(\frac{CL_{CTX}}{V_{CTX}} \times AMT_{CMT\,1} \right) - \left(\frac{CL_{DACT}}{V_{DACT}} \times AMT_{CMT\,2} \right) \quad \textbf{(Eq. A6)}$$

in which dDACT/dt is the rate of DACT transit, CL_{CTX} is CTX clearance, V_{CTX} is the apparent distribution volume, CL_{DACT} is DACT clearance, V_{DACT} is the apparent distribution volume, AMT_{CMT1} is the amount of CTX present in compartment 1 and AMT_{CMT2} is the amount of DACT present in compartment 2 at any one time, assuming that all CTX is converted to DACT.

Chapter 8.1

Sildenafil exposure in neonates with pulmonary hypertension after administration via a nasogastric tube

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Arch Dis Child Fetal Neonatal Ed 2010, 95(2), F109-14

Abstract

This study was set up to describe the pharmacokinetics and exposure of oral sildenafil in neonates (2-5 kg) with pulmonary hypertension. We included eleven neonates (body weight 2-5 kg, PNA 2-121 days) who received sildenafil and ECMO-treatment for pulmonary hypertension. Sildenafil capsules were given via a nasogastric tube. Blood samples were collected via a pre-existing arterial line to quantify sildenafil and metabolite plasma levels (219 samples). Nonlinear mixed effects modelling was used to describe sildenafil (SIL) and desmethylsildenafil (DMS) pharmacokinetics. A one-compartment model was suitable for both SIL and DMS. Inter- and intra-patient variability for clearance at 100% bioavailability were 87% and 27% (SIL) and 62% and 26% (DMS). Patient weight, postnatal age and post-ECMO time did not explain variability. Concomitant fluconazole use was associated with a 47% reduction in sildenafil clearance. The exposure expressed as $AUC_{24\ (SIL+DMS)}$ ranged from 625 to 13579 ng*h/mL. An oral dose of 4.2 mg/kg/24h would lead to a median $AUC_{24\ (SIL+DMS)}$ of 2650 ng*h/mL equivalent to 20 mg t.i.d. in adults. Inter-patient variability was large, with a simulated $AUC_{24\ (SIL+DMS)}$ range (10^{th} and 90^{th} percentiles) of 1000-8000 ng*h/mL.

Sildenafil pharmacokinetics are highly variable in post-ECMO neonates and infants. In a median patient, the current dose regimen of 0.5-2.0 mg/kg q.i.d. leads to an exposure comparable to the recommended adult dose of 20 mg t.i.d. Careful dose titration, based on efficacy and the occurrence of hypotension, remains necessary. Follow-up research should include appropriate pharmacodynamic endpoints, with a population PK/PD analysis to assign a suitable exposure window or target concentration.

Introduction

Pulmonary hypertension (PH) is an important cause of cardio-respiratory failure in the newborn.[1] Treatment options include, amongst others and in addition to nitric oxide inhalation (iNO) and extracorporeal membrane oxygenation (ECMO), the potent phosphodiesterase (PDE-5) inhibitor sildenafil. The latter has been licensed under the trade name Revatio® for the treatment of pulmonary hypertension (PH) in adults and is used off-label in neonates. A dose of 0.5-2.0 mg/kg four times daily is considered appropriate to treat PH in newborns [2], but supporting evidence is limited to case reports and small pilot studies with varying dose regimens.[3-5]

Recently, Mukherjee *et al.* published the first population pharmacokinetic study in which they characterised the pharmacokinetics of intravenous sildenafil via continuous infusion in term neonates up to 7 days of age, postulating rapid maturation of the CYP3A-mediated clearance of sildenafil.[6] The CYP3A-mediated metabolism also makes sildenafil prone to drug-drug interactions with commonly used comedication such as bosentan and fluconazole.[7] Considering this maturation and the remaining substantial inter-patient variability of clearance and volume of distribution, an adequate sildenafil dose would vary substantially from one patient to another and within a patient over the course of a couple of days. Since the intravenous formulation is not available in the Netherlands, we treat our patients orally by administering the contents of extemporaneously prepared capsules via a nasogastric tube. In patients of 7 days and older, sildenafil clearance and exposure have not been studied in detail, especially in the post-ECMO period.

Goals of this study were to characterise the pharmacokinetics of sildenafil and its metabolite desmethylsildenafil in post-ECMO neonates after nasogastric administration, to explain variability by age, weight and other covariates, and to simulate the effects of different dose regimens on sildenafil exposure.

Methods

Patients

This study was approved by our institutional ethics review board as part of a larger study into pharmacokinetics during extracorporeal membrane oxygenation (ECMO). After verification via repeated cardiac ultrasounds, patients were initially treated with inhaled nitric oxide according to the standard departmental protocol, but eventually had to be placed on venoarterial ECMO to maintain sufficient oxygenation. Criteria for ECMO in our institution have been published in prior publications describing different aspects of pharmacotherapy during ECMO.[8-10] After written parental consent, we included 11 former ECMO patients with pulmonary hypertension who had an arterial line during

their stay at the ICU. Sildenafil therapy had started on ECMO, based on persistent PH, either via an echocardiogram or the inability to wean from ECMO, and was continued after decannulation. Sildenafil capsules (1, 2, 5 and 10 mg) were prepared extemporaneously by the hospital pharmacy from commercial Viagra® tablets, with pharmaceutical grade lactose as filler and only excipient. Intermediate doses were given by combining multiple capsules. Production facilities and procedures were in accordance with the Good Manufacturing Practice guidelines for hospital pharmacy production as issued by the Netherlands Association of Hospital Pharmacists (NVZA). Content uniformity was assessed in each individual batch as described in the European Pharmacopoeia, 6th edition. Sildenafil content was within 90-110% of the specified amount for each batch of capsules. Capsules were opened and their contents dispersed in a syringe filled with water before administration via the nasogastric tube. The syringe was flushed with water and afterwards the tube was flushed with a final aliquot of water. Initially, 0.5 mg/kg of sildenafil three or four times daily was given via a nasogastric tube. The dose was titrated up to a maximum of 10 mg/kg/24h to reach adequate pre- and postductal saturation, while maintaining adequate systemic blood pressure. Concomitant drug therapy consisted of inotropics, diuretics, sedatives, analgesics, bosentan, fluconazole and antibiotics as required, adjusted to the individual needs of each patient. Sildenafil and concomitant medication were recorded, as well as patient characteristics, clinical parameters, lab results and ECMO and ventilation settings.

Blood sampling

Arterial EDTA-decoagulated blood samples (100-200 µL) were taken from an existing line. Sampling took place between disconnection from ECMO and discharge from the intensive care unit for as long as a line was present; the first sample was taken once the parents had given written consent. On the first day of sampling, a PK curve of 5 points was taken at 0, 1, 2, 4 and 6 hours after a sildenafil dose. The following days, max. three blood samples were taken per day at varying sampling times for as long as an arterial line was present, with a median of 13 samples per patient (range 7-55). For newborns cannulated within max 3 days after birth, the last sample was taken max. 4 weeks after decannulation. On average, patients were followed for 228 h (range 54-528 h). Plasma was separated via centrifugation and stored at -80°C until analysis. Sildenafil and desmethylsildenafil were quantified in 50 µL of plasma with a validated LC-MS/MS method after liquid-liquid extraction.[11] Accuracy, intra- and interday precision were within 13% for both compounds. The lower limits of quantification were 1 ng/mL. Matrix effects were present, but inter-plasmabatch variability in recovery was under 12%.

Pharmacokinetic analysis

In total, 219 SIL and 219 DMS concentration-time points were analyzed simultaneously using nonlinear mixed-effects modelling software (NONMEM VI 2.0, Globomax LLC, Ellicott City, MD, USA), with the first-order conditional estimation (FOCE) method, which allows interaction between structural and residual variance components. Models were parameterised in terms of volume of distribution and clearance, as if the absolute bioavailability (F) and conversion ratio of DMS/SIL (Fc) equaled unity. Using NONMEM, we can estimate average pharmacokinetic parameters for the population, as well as inter- and intra-individual variability and a residual error. Model fit was assessed using plots of population and individual predicted concentrations vs. observed concentrations and weighted residuals over time. Models were compared using the minimum value of objective function (MVOF) via the log likelihood ratio test for nested models. An MVOF-drop of 3.84 ($p<0.05$) was considered statistically significant, following a χ^2 distribution with 1 degree of freedom. Inter-individual variability was modelled as a proportional deviation from the population parameter values. Intra-individual variability was modelled as separate variance terms for periods of 2 days in a row, allowing clearance and volume of distribution to vary over time for each individual. Residual error was estimated with a proportional error model. The influence of demographics, clinical parameters and coadministered drugs on clearance and volume of distribution were analyzed via univariate analysis of each covariate. Statistically significant covariate effects ($p<0.05$) were included in an intermediate multivariate model. Subsequently, covariates were deleted stepwise and removed from the model if the MVOF did not significantly increase. The resulting model was considered the final model. The tested covariates included the use of all coadministered drugs (including bosentan, inotropics fluconazole), PNA, body weight, gender, blood pressure, saturation, liver function, time after ECMO-decannulation, heart rate and the sildenafil dose in mg/kg/24h.

Using NONMEM, the average plasma concentration area under the curve over 24 hours ($AUC_{24\,(SIL+DMS)}$) was calculated as a measure of drug exposure. Since DMS possesses activity itself (50% as potent as SIL [12]) 50% of $AUC_{24\,(DMS)}$ was added to $AUC_{24\,(SIL)}$. To correct for differences in dose frequency, AUCs were calculated over 24 hour periods. The model was validated with the bootstrap resampling method, in which the model is repeatedly fitted on a reduced dataset. 1250 Resampled data sets were used to calculate the median parameter estimates and 95% confidence interval. The model was considered valid if the original parameter estimates fell within this interval. Plasma concentration time profiles of SIL and DMS were simulated with NONMEM using the final parameter estimates in 800 patients, replicated from the original dataset, with dose regimens of 2, 3, 5 and 7 mg/kg/24h.

Results

Table I contains demographic data of the study population and the calculated median $AUC_{24\ (SIL+DMS)}$ for each patient. The high pharmacokinetic variability was visible in both the calculated $AUC_{24\ (SIL+DMS)}$ and the original plasma concentrations; see Figure 1 for the

Table I. Patient characteristics and calculated SIL and DMS exposure

Patient	Sex	PNA (days)[a]	Time after ECMO (days)[a]	Body weight (kg)[a]	Primary diagnosis[b]	Sildenafil dose (mg/kg)[c]	CYP3A comedication[d]	Average $AUC_{24\ (SIL+DMS)}$ (ng*h/mL)[e]	Survived
1	F	121	> 100	4.8	VSD	2.0 (t.i.d.)	-	1598	N
2	F	22	0.3	4.0	PA	2.5 (t.i.d.)	BOS	8284	Y
3	M	2	0.1	2.3	CDH	2.0 (q.i.d.)	-	19514	Y
4	M	19	2.5	4.0	TGA	2.5 (q.i.d.)	BOS, FLU	13579	Y
5	F	17	1.4	2.0	CDH	1.5 (t.i.d.)	FLU	625	Y
6	F	22	10	4.1	ASD, VSD	1.5 (t.i.d.)	-	8297	Y
7	F	11	6.5	4.3	PPHN	0.5 (q.i.d.)	-	939	N
8	F	11	0.5	2.7	PPHN	2.25 (t.i.d.)	-	3935	Y
9	F	20	4.5	3.0	CDH	0.33 (q.i.d.)	BOS, FLU	1618	Y
10	F	22	8.4	3.0	CDH	2.5 (t.i.d.)	-	4232	Y
11	F	101	93	5.1	CDH	3.0 (t.i.d.)	-	2404	Y

[a] at inclusion; [b] ASD, atrial septal defect; CDH, congenital diaphagmatic hernia; PA, pulmonary atresia; PPHN, persistent pulmonary hypertension of the newborn; TGA, transposition of the great arteries; VSD, ventricular septal defect; [c] average dose after titration, given thee (t.i.d.) or four (q.i.d.) times daily; [d] bosentan (BOS) or fluconazole (FLU) that was used in conjunction with SIL for any length of time during the study period ; [e] calculated over a period of 24 h, as a combination of SIL and DMS assuming a DMS potency of 50%

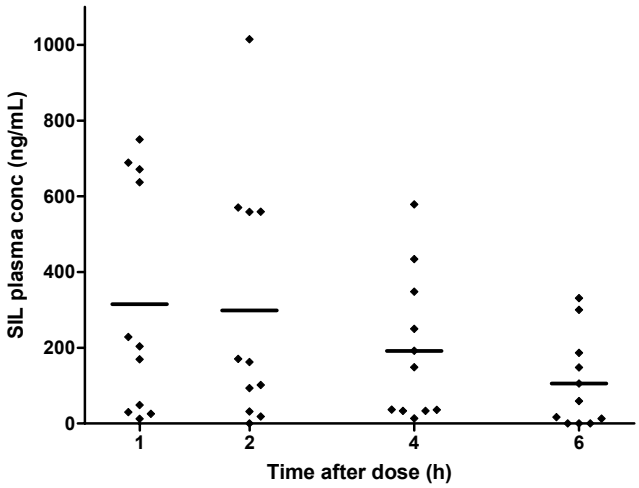

Figure 1. Sildenafil plasma concentrations for each individual at sampling times of a full PK curve (1, 2, 4 and 6 hours post-dose). Lines indicate the mean concentration at each time point.

measured sildenafil concentrations at 1, 2, 4 and 6 hours post-dose. None of the patients required renal replacement therapies during the observation period. Median ECMO-duration (range) was 148 h (42-292). Pharmacokinetics could be described adequately with a sequential one-compartment model for SIL and DMS with first-order absorption (Figure 2). Pharmacokinetic parameter estimates, together with their standard error

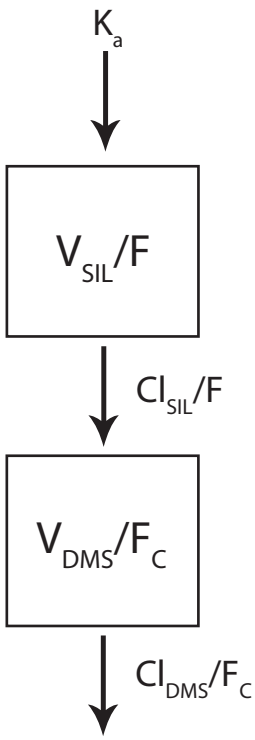

Figure 2. Sequential one-compartment model describing sildenafil (SIL) and desmethylsildenafil (DMS) pharmacokinetics. K_a=absorption rate constant, V_{SIL}/F=apparent sildenafil volume of distribution, CL_{SIL}/F=apparent sildenafil clearance, V_{DMS}/F_C=apparent desmethylsildenafil volume of distribution, CL_{DMS}/F_c=apparent desmethylsildenafil clearance. True pharmacokinetic parameters require correction for bioavailability (F) and the DMS/SIL conversion ratio (F_c), respectively.

estimates generated by NONMEM's covariance option, are given in Table II. Inclusion of inter- and intra-patient variability improved the model, as can be seen in the plots of population predictions and individual predictions vs. observed concentrations (Figure 3). The residuals vs. time plot revealed no structural deviation from zero. Residual variability was modelled with a proportional error model for each analyte. Shrinkage of variance terms, which has the potential to distort covariate effects and induce model misspecification with sparse sampling study designs.[13-15] was acceptable at 5.7% (ε) and max 7.0% (η's). In a forward-inclusion procedure, postnatal age, heart rate, bosentan

Table II. Pharmacokinetic parameter estimates[a]

	Sildenafil		Desmethylsildenafil	
	Model estimate (CV %)	Bootstrap median (5^{th}-95^{th} percentile)	Model estimate (CV %)	Bootstrap median (5^{th}-95^{th} percentile)
Population Average				
k_a (h^{-1})	2.4 (4.2)	2.5 (2.0-5.0)	-	-
V/F (L)[b]	34 (5.0)	34 (25-55)	14 (35)	14 (9.3-24)
CL/F (L/h)[b]	7.3 (17)	7.3 (5.0-12)	9.7 (19)	9.7 (6.9-13)
CL-reduction with fluconazole use (%)	47 (28)	47 (21-69)	-	-
Intra-patient variability				
CL (%)	27 (56)	24 (10-35)	26 (29)	25 (17-31)
Inter-patient variability				
V (%)	94 (54)	92 (64-114)	-	-
CL (%)	87 (42)	90 (71-106)	62 (46)	76 (59-90)
Residual variability				
Proportional (%)	38 (5.3)	37 (32-41)	30 (17)	30 (24-39)

[a] CV, coefficient of variation generated by NONMEM's covariance option

[b] apparent values, divided by F to correct for bioavailability (sildenafil) and F_c to correct for the metabolic conversion ratio of SIL to DMS (desmethylsildenafil).

use and fluconazole use were identified as potential covariates ($p<0.05$), but only fluconazole remained in the final model after the stricter backward covariate exclusion ($p<0.001$). Concomitant fluconazole use led to a 47% drop in clearance, see Figure 4. Inter-individual variability on SIL clearance (CL_{SIL}), DMS clearance (CL_{DMS}) and SIL volume of distribution (V_{SIL}) significantly improved fit. Intra-patient variability explained 4.5% (SIL) and 6.2% (DMS) of variability in clearance. Bootstrap validation results are shown in Table II. All estimates were within the 5^{th} and 95^{th} percentile rangeThe median $AUC_{24\ (SIL+DMS)}$ was 3935 ng*h/mL (Table I: range 625-13579 ng*h/mL). The AUC ratio of DMS vs. SIL (AUC_{DMS}/AUC_{SIL}) was on average 0.94 (range 0.14-2.16). Of the 11 patients, one patient died of sepsis (28 mg SIL/day, $AUC_{24\ (SIL+DMS)}$=939 ng*h/mL) and one died of progressive cardiac failure caused by therapy-resistant PH (8 mg SIL/day, $AUC_{24\ (SIL+DMS)}$=1598 ng*h/mL). The correlation coefficient between weight-normalised sildenafil dose and $AUC_{24\ (SIL+DMS)}$ was only 0.36 due to large remaining inter-patient variability in SIL and DMS clearance (87 and 62%, respectively). The correlation between $AUC_{24\ (SIL+DMS)}$ and simulated dose regimens of 2, 3, 5 and 7 mg/kg/24h is displayed in Figure 5. A dose of 4.2 mg/kg/24h corresponds to an $AUC_{24\ (SIL+DMS)}$ of 2650 ng*h/mL, with large variability, as indicated by the 10^{th} and 90^{th} percentiles (1000 and 8000 ng*h/mL, respectively).

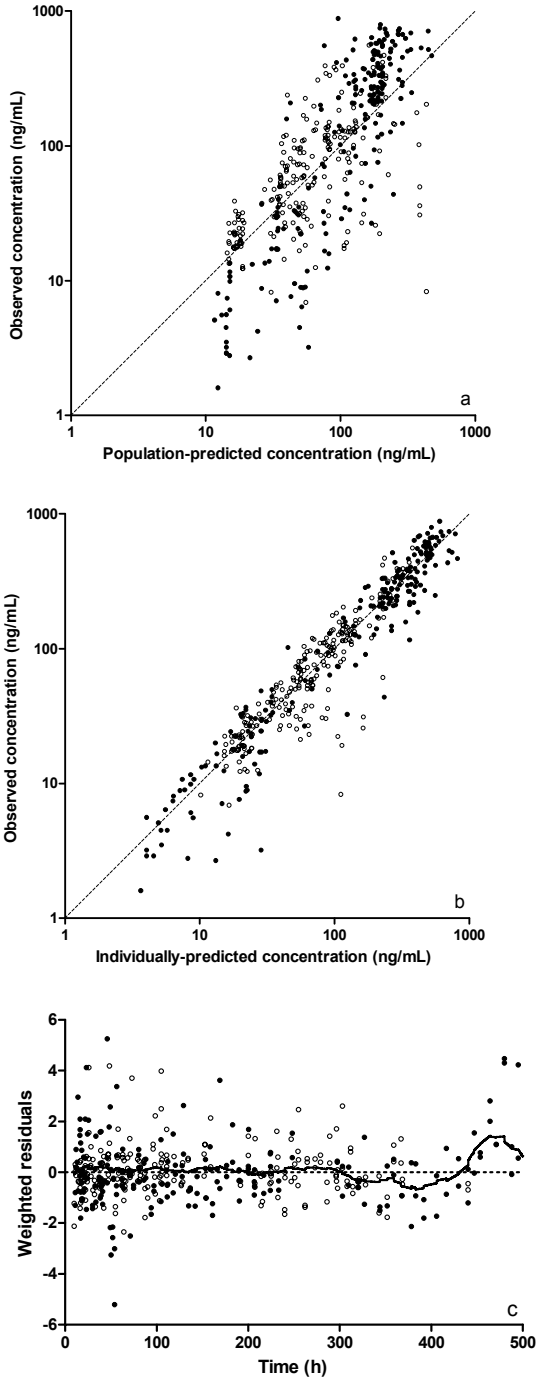

Figure 3. Goodness-of-fit plots of sildenafil (closed) and desmethylsildenafil (open) for the final model. Population predicted concentrations (a) vs. observed concentrations; Individual predicted concentrations (b)vs. observed concentrations; weighted residuals (c) vs. time with trend line.

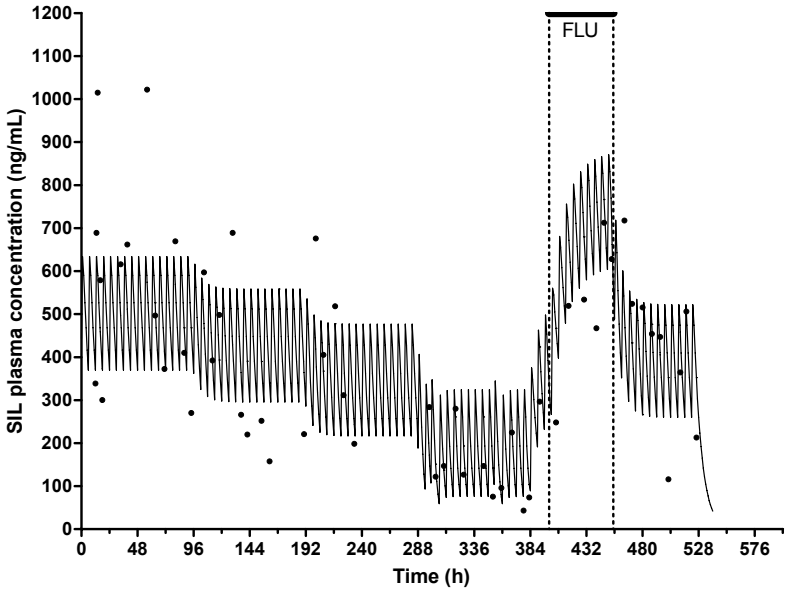

Figure 4. Plasma concentration vs. time plot for a patient who received thee doses of fluconazole (6 mg/kg) during sildenafil therapy (10 mg/kg/24h). The dots are measured sildenafil concentrations, the solid line is the individual. Bayesian prediction of sildenafil concentrations based on the final model. Sildenafil concentrations are higher during fluconazole treatment (between 400 and 455 h). After the fluconazole treatment has ended, sildenafil pharmacokinetics return to normal.

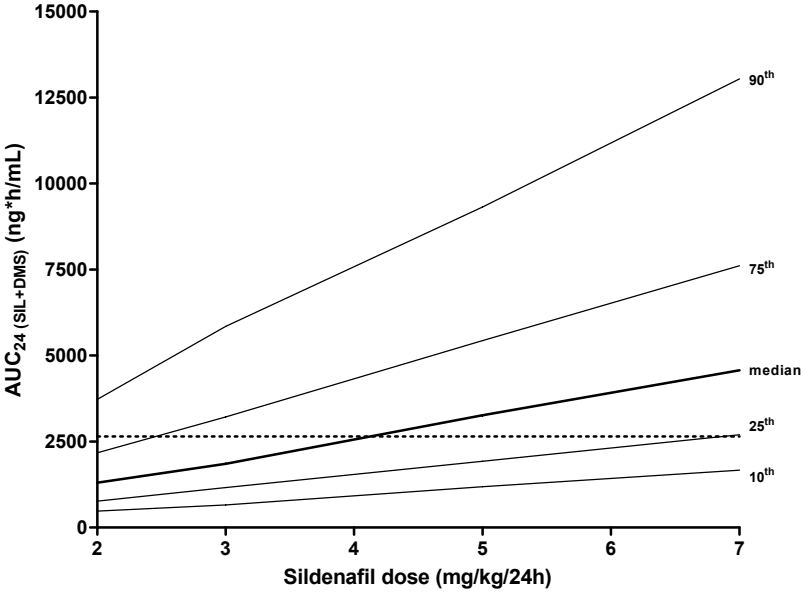

Figure 5. Simulated area-under-the-curve ($AUC_{24\,(SIL+DMS)}$) for sildenafil and desmethylsildenafil with dose regimens of 2, 3, 5 and 7 mg/kg/day. Curves represent the 10th, 25th, 50th (median), 75th and 90th percentiles of AUC distribution.

Discussion

Both the calculated exposure and the measured plasma concentrations of SIL and DMS were highly variable between one patient and the next, which might lead to inadvertent under- or overdosing upon administration of a standard dose. The underlying variation in PK parameters could not be explained by age, body weight, time after ECMO-decannulation or other covariates, which might have been caused by the relatively narrow age and weight range: most patients were between 10 and 22 days old. Variable gut absorption might in part be responsible, which has thus far been described for enteral feeding only.[16] Another explanation could be flow-limited hepatic clearance in combination with hemodynamic changes, even though sildenafil is considered to have an intermediate extraction ratio in healthy adults.[17] Unfortunately, we did not have reliable markers for hepatic blood flow for these patients. There was no evidence of hepatic dysfunction; median (range) ALAT and ASAT blood levels were 14 (10-16) and 29 (15-40) IU/L. The vasopressor score, as a composite marker of overall hemodynamic instability (via quantification of the required amount of inotropic support) [18, 19], was not correlated with sildenafil or metabolite clearance.

These patients had an average CL_{SIL}/F of 7.3 L/h, equivalent to an adult value of 62 L/h/70kg after allometric scaling with a ¾ power exponent [20], which is higher than the clearance found in adults treated for PH after oral administration (30.7 L/h).[21] The high clearance is offset by an increased volume of distribution: this could in part be a remnant of the preceding ECMO-treatment with its associated higher distribution volumes, an effect that has been described for a multitude of drugs.[22] After intravenous administration, Mukherjee *et al.* found an increased SIL clearance from a PNA of 1 day (0.84 L/h, or 8.1 L/h/70kg) up to 7 days (2.6 L/h, or 25 L/h/70kg) and attributed this to maturing CYP3A-metabolism.[6] The apparent trend was probably captured in our patients by modelling intra-patient variability. Co-administration of fluconazole was associated with a decreased CL_{SIL} (-47%), but data from more individuals are needed to conclusively determine covariate effects.

When we compare our CL_{SIL}/F of 62 L/h/70kg to a clearance of 25 L/h/70kg after intravenous administration (which is the value Mukherjee found in 7-day old patients), we can calculate a biological availability of 40%. This could imply that the bioavailability of our sildenafil capsules is similar to that of the commercial tablets in adults.[21] Enteral feeding might have an effect on bioavailability, but this would have been reflected in statistically significant differences between CL or V for patients of different indications. However, the oral administration could provide an extra source of variability compared to intravenous administration, related to variable absorption capacity which might be linked to specific disease states, diarrhoea or vomiting. Unfortunately, these aspects are difficult to model quantitatively and were not present in our dataset. A direct

comparison between oral and intravenous sildenafil could be used to find out whether variability in absorption is an explanation for the high inter-patient variability we have seen in our study. Mukherjee estimated inter-patient variability on CL_{SIL} and V_{SIL} to be 55 and 43% respectively. This is much smaller than the variability we found in our dataset (87 and 94%) which would indicate less variability with the i.v. formulation. This could in part be caused by our smaller number of patients which makes it difficult to accurately estimate inter-patient variability and statistically significant covariate effects. In general however, it would seem that the intravenous formulation might lead to a more predictable clearance and volume of distribution, making it desirable over the oral capsules. In the absence of accepted efficacy parameters in this population, it is difficult to assign a suitable dose regimen. As a surrogate efficacy parameter, we used a corresponding efficacious exposure in adults after oral administration. In adults with PH, a dose of 20 mg t.i.d. is considered effective with acceptable side effects.[23] Assuming a ratio of AUC_{DMS}/AUC_{SIL} of 0.74 and a CL_{SIL}/F of 30.7 L/h in adults with PH [7], the corresponding $AUC_{24\ (SIL+DMS)}$ for a dose of 20 mg t.i.d.is 2650 ng*h/mL (Equation 1).

$$AUC_{24\ (SIL+DMS)}=(Dose_{24}/(CL_{SIL}/F))*(1+0.5*0.74) \quad \text{(Eq. 1)}$$

All of the patients with $AUC_{24\ (SIL+DMS)}$ >2650 ng*h/mL survived. Of our 4 patients with AUCs <2650 ng*h/mL, one died of cardiac failure, which may indicate undertreatment. Unfortunately, we do not know why her dose was not titrated upward; she received 0.67 mg/kg t.i.d. from the onset of treatment. She experienced hypotension, but it started abruptly and only after having received sildenafil for thee consecutive days, which makes sildenafil an unlikely cause. Several patients reached higher AUCs (up to 13579 ng*h/mL, equivalent to an adult dose of 100 mg t.i.d.). We could not detect an increased chance of hypotensive episodes in patients with higher drug exposure. To reach a median $AUC_{24\ (SIL+DMS)}$ of 2650 ng*h/mL in our patients, sildenafil should be dosed orally at 4.2 mg/kg/24h. This is equivalent to a dose of 1.0 mg/kg given four times daily, but even with a dose as high as 7 mg/kg/24h a quarter of patients will have an average $AUC_{24\ (SIL+DMS)}$ under 2650 ng*h/mL (Figure 4). This high variability in exposure implies that careful dose titration is necessary.

There is an ongoing debate on the best marker of PH severity in neonates, comparable with the 6 min walking test in adults.[23-25] In the absence of a generally accepted endpoint, current dose titration is based on a subjective perception of efficacy which includes the difference between pre- and postductal saturation, predetermined vascular parameters obtained by echocardiogram, a decreased tension following sildenafil administration and/or the ability to wean from ECMO, in addition to acute occurrence of hypotension as a dose-limiting side effect. Unfortunately, all of these parameters are affected by concomitant medication (e.g. inotropes) and underlying illness (e.g. con-

genital cardiac deformities), which confound assessment of efficacy. To confirm a target concentration or exposure window in these specific patients, we need valid, generally accepted pharmacodynamic endpoints and a larger study to define their correlation with exposure. Recently, the National Heart, Lung and Blood Institute (NHLBI) Working group on Pediatric Respiratory Diseases Research published a detailed overview of the areas in pediatric pulmonology requiring additional research.[26] This strategic plan explicitly states the need for research into, among others, appropriate diagnostic markers and pharmacodynamic endpoints, differences in drug efficacy between adults and children, age-dependent patterns of pharmacokinetics and dose optimization for the individual patient. We concur and would like to stress the important contribution that population pharmacokinetic modelling can make to the optimization of pharmacotherapy: by quantifying inter-patient variability, by evaluating the effects of age and other covariates on pharmacokinetics, and by making maximum use of sparsely sampled plasma concentrations, thereby facilitating studies in populations of vulnerable patients.

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Chapter 8.2

Population pharmacokinetics of oral sildenafil during and after extracorporeal membrane oxygenation in neonates

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Abstract

The aim of this study was to describe the pharmacokinetics and exposure of sildenafil and desmethylsildenafil in neonates with pulmonary hypertension during and after extracorporeal membrane oxygenation (ECMO), following oral administration of sildenafil. We included 23 neonates who received sildenafil and ECMO-treatment. Sildenafil was administered via a nasogastric tube. Blood samples were collected via the ECMO circuit or an arterial line to quantify sildenafil and metabolite plasma concentrations (397 samples during ECMO in 23 individuals, with 96 samples post-ECMO in 12 individuals). Nonlinear mixed effects modelling was used to describe sildenafil (SIL) and desmethylsildenafil (DMS) pharmacokinetics. A one-compartment model was suitable for both SIL and DMS. The median (range) parameter estimates for clearance (CL) and volume of distribution (V) during ECMO (F=100%) were: CL_{SIL}=8.6 L/h (1.4-2987), V_{SIL}=120 L (15-325), CL_{DMS}=19 L/h (1.5-50) and V_{DMS}=18 L (0.91-105). After decannulation, CL and V dropped to the following values: CL_{SIL}=3.3 L/h (1.3-18), V_{SIL}=29 L (15-338), CL_{DMS}=7.6 L/h (1.2-28) and V_{DMS}=18 L (0.91-105). Inter-patient variability was large (as coefficient of variation): 88.5%, 91.4%, 80.4% and 166% respectively. There was a gradual nonlinear increase of CL_{SIL}, V_{SIL} and CL_{DMS} over the course of an ECMO-run, with a return to pre-ECMO levels upon decannulation. For a 6 day-ECMO run, CL_{SIL} increased from 1.0 to 6.7 L/h, V_{SIL} increased from 50 to 116 L, CL_{DMS} increased from 7.6 to 21 L/h. Post-ECMO, a dose of 3-5 mg/kg/24h lead to a combined SIL and DMS exposure equivalent to that of an adult treated with 3 dd 20 mg p.o. During ECMO, a dose of between 5 and 7 mg/kg/24h provides the same exposure. In conclusion, SIL and DMS parameters progressively increase after the start of ECMO. As a result of the return of PK parameters to non-ECMO values, the total exposure calculated as $AUC_{24\ (SIL+DMS)}$ increases at decannulation, unless the dose is reduced by approximately 50%. Clinicians should therefore be aware of a potential increase in efficacy or side effects after the end of ECMO therapy. However, additional studies are needed to define a target exposure window in neonates with pulmonary hypertension, using appropriate pharmacodynamic endpoints.

Introduction

Persistent pulmonary hypertension of the newborn (PPHN) is a life-threatening disease that requires mechanical ventilation, cardiac support and pharmacological interventions.[1] In severe or therapy-resistant PPHN that is unresponsive to conventional treatment, extracorporeal membrane oxygenation (ECMO) is used to allow the pulmonary vascular resistance to decrease while maintaining sufficient cardial and respiratory function.[2] Inhaled nitric oxide is generally considered the drug of first choice in patients with PPHN. In case of failure and/or absent response, sildenafil, a potent phosphodiesterase (PDE-5) inhibitor, is considered either to prevent the institution of ECMO or as additional therapy to ease weaning off ECMO. Sildenafil has been licensed for the treatment of pulmonary hypertension (PH) under the tradename Revatio®, but is used off-label in pediatric populations.[3] Mukherjee *et al.* studied the pharmacokinetics of sildenafil with a newly developed intravenous formulation in term neonates.[4] Unfortunately, the intravenous formulation is not available in the Netherlands, which has lead to treatment via a nasogastric tube, with sildenafil capsules prepared extemporaneously from tablets. An oral dose of 0.5-2.0 mg/kg four times daily leads to an appropriate sildenafil exposure to treat pulmonary hypertension in newborns [5], assuming similar pharmacodynamics between adults and neonates. The addition of the ECMO circuit might alter sildenafil requirements: ECMO-related pharmacokinetic changes have been described for a multitude of drugs. [6-10] In general, the distribution volume of drugs during ECMO appears to be increased, whereas ECMO-related effects on clearance vary, due to reduced protein binding, increased perfusion, organ damage or drug absorption. [11, 12] In the absence of ECMO specific pharmacokinetic data, standard dose regimens might therefore lead to over- or undertreatment. At present, sildenafil doses are titrated upward during ECMO; we do not yet know what happens to concentrations of sildenafil and its active metabolite desmethylsildenafil after decannulation. Via covariate analysis, determinants of pharmacokinetic differences between patients and between the ECMO and post-ECMO period might be identified using nonlinear mixed effects modelling (NONMEM). This would allow sildenafil and metabolite concentrations to be simulated, to find a dose regimen that provides a sufficiently high and constant exposure during and after ECMO. This study was set up to characterize the pharmacokinetics of sildenafil and desmethylsildenafil in neonates during and after ECMO.

Methods

Patients

This study was approved by our institutional ethics review board. After verification of pulmonary hypertension via repeated cardiac ultrasounds, patients were initially treated with inhaled nitric oxide according to the standard departmental protocol, but eventually had to be placed on ECMO to maintain sufficient oxygenation. Criteria for ECMO in our institution have been published in prior publications describing different aspects of pharmacotherapy during ECMO.[7, 13, 14] Written parental consent was obtained for blood sampling and use of clinical data from the patient data management system (PDMS), which included fluid balance, medication, physiological and real-time ECMO parameters.

Medication

Sildenafil capsules (1, 2, 5 and 10 mg) were prepared extemporaneously by the hospital pharmacy from commercial Viagra® tablets, with pharmaceutical grade lactose as filler and only excipient. Intermediate doses were given by combining multiple capsules. Production facilities and procedures were in accordance with the Good Manufacturing Practice guidelines for hospital pharmacy production as issued by the Netherlands Association of Hospital Pharmacists (NVZA). Content uniformity was assessed in each individual batch as described in the European Pharmacopoeia, 6th edition. Sildenafil content was within 90-110% of the specified amount for each batch of capsules. Sildenafil therapy had started on ECMO, based on persistent PH, either via an echocardiogram or the inability to wean from ECMO, and was continued after decannulation. Capsules were opened and their contents dispersed in a syringe filled with water before administration via the nasogastric tube. Afterwards, the syringe and tube were flushed with at least two aliquots of water. Initially, sildenafil was dosed at 0.5 mg/kg three or four times daily. The dose was titrated to reach adequate pre- and postductal saturation, while maintaining adequate systemic blood pressure. Concomitant drug therapy consisted of inotropics, diuretics, sedatives, analgesics, bosentan, fluconazole and antibiotics as required, and was adjusted according to the needs of individual patients. Sildenafil doses were recorded, as well as patient characteristics, clinical parameters, lab results and ECMO and ventilator settings.

Blood sampling

Blood sampling and observations started on the first day of ECMO. Actual doses and sampling times were entered into the patient data management system (PDMS) by trained nurses. During ECMO, blood (1.0-1.5 mL) was sampled from a pre-oxygenator access port on the ECMO circuit. No medication was administered via this port. Blood was sampled at

routine clinical chemistry rounds, three times daily, and processed in ethylenediaminetetraaceticacid (EDTA-) decoagulation tubes. After decannulation, sampling continued for as long as an arterial line was present and the patient was in the ICU. These arterial blood samples (100-200 µL) were taken from an existing line. On the first day of post-ECMO sampling, a PK curve of 5 points was taken at 0, 1, 2, 4 and 6 hours after a sildenafil dose. The following days, max. three blood samples were taken per day at varying sampling times. For newborns cannulated within 3 days after birth, the last sample was taken max. 4 weeks after decannulation. Plasma was separated via centrifugation and stored at -80°C until analysis. Sildenafil (SIL) and desmethylsildenafil (DMS) were quantified in 50 µL of plasma with a validated LC-MS/MS assay after protein precipitation [15] for samples during ECMO. A similar method with liquid-liquid extraction was used for post-ECMO samples.[16] Accuracy, intra- and interday precision were within 13% for both compounds and assays. The associated lower limits of quantification were 1 ng/mL.

Pharmacokinetic analysis

SIL and DMS concentration-time points were analyzed simultaneously using nonlinear mixed-effects modelling software (NONMEM VI 2.0, Globomax LLC, Ellicott City, MD, USA), with the first-order conditional estimation (FOCE) method, which allows interaction between structural and residual variance components. Using NONMEM, we can estimate average pharmacokinetic parameters for the population, as well as inter- and intra-individual variability and a residual error. Differential equations were used with NONMEM's ADVAN 6 subroutine to describe the population PK of SIL and DMS. Models were parameterized in terms of volume of distribution (V) and clearance (CL), as if the bioavailability (F) and conversion ratio of DMS/SIL (Fc) equaled unity. All DMS concentrations were converted to their molar equivalents of sildenafil (M_w SIL=474.58 g/mol, M_w DMS=460.55 g/mol). The population model was built stepwise. A specific assumption was tested at each step, at which model adequacy was assessed through examination of goodness-of-fit plots, generated by the Xpose software package (v 3.2, Dr. M. Karlsson, University of Uppsala, Sweden) [17] and R.[18] Goodness-of-fit plots consisted of measured drug concentrations vs. population (PRED) or individual (IPRED) predictions, and conditional weighted residuals (CWRES) vs. time plots.[19] Bayesian IPRED concentrations were obtained via NONMEM's *posthoc* option. Models were also compared using the minimum value of objective function (MVOF) via the log likelihood ratio test for nested models. An MVOF-drop of 3.84 ($p<0.05$) was considered statistically significant, following a χ^2 distribution with 1 degree of freedom. Inter-individual variability was modelled as a proportional deviation from the population parameter values. Residual error was estimated with a proportional error model, in which the difference between the two drug assays was included. Covariate effects on CL or V were incorporated into the model as previously described [5] and their statistical significance was assessed in a stepwise

inclusion and exclusion procedure.[20] Continuous covariates, such as postnatal age, were modelled in an exponential manner as shown in equation 1:

$$CL_i = \theta_{pop} \times \left(\frac{PNA}{9.4} \right)^{\theta_{PNA}} \quad \textbf{(Eq. 1)}$$

in which θ_{pop} is the clearance in individuals with the median PNA of the population (9.4 d), and θ_{PNA} is an exponent describing the correlation function. Time-dependent covariate effects were also tested in an additive model as described in equation 2:

$$CL = CL_{base} + \theta * t_{EC} \quad \textbf{(Eq. 2)}$$

in which CL equals clearance, CL_{base} is the baseline clearance before cannulation, θ is the slope of the regression function, and t_{EC} is the time after cannulation.
Categorical covariates, such as ECMO, were modelled as shown in equation 3:

$$CL_i = \theta_{pop} \times \left(\theta_{ECMO} \right)^{ECMO} \quad \textbf{(Eq. 3)}$$

in which θ_{pop} is the population value for clearance without an ECMO-effect and θ_{ECMO} is the fractional difference between clearance in the ECMO (ECMO=1) and non-ECMO period (ECMO=0). The tested covariates include sildenafil dose, gestational age (GA), postnatal age (PNA), body weight (WT), duration of pregnancy, time after start or end of extracorporeal circulation (t_{EC} and t_{END}), ECMO on/off, ECMO-flow (Q_{ECMO}), CVVH-flow (Q_{CV-VH}), indication, the number of ECMO runs, ECMO-modality (venovenous or venoarterial), gender, body temperature, urine output, liver function (ASAT, ALAT) and concomitant use of fluconazole [5], bosentan [21, 22] or vasopressive medication (as a categorical covariate: norepinephrine, dopamine, dobutamine or epinephrine). An MVOF drop of 3.84 (p=0.05, χ^2 distribution, 1 degree of freedom) was considered statistically significant. A stricter criterion (p=0.01, ΔOFV=6.63) was used in the backward elimination procedure for covariate effects: if deletion of a covariate did not result in a significant worsening of the objective function, the covariate was removed from the model. The resulting model was considered the final model. Shrinkage was calculated to assess whether the estimated η and ε parameter distributions match those of the original data assuming normal distribution.[23] Stability and performance of the final model were checked using an internal validation procedure via the bootstrap resampling technique, in which 1000 bootstrap data sets were generated by random sampling with replacement [24] via the Wings for NONMEM software package (v6.12 March 2007, Dr N. Holford, Auckland, New Zealand). Model validity was assessed by calculating median values and the 2.5th and 97.5th percentiles of parameter distribution generated by the bootstrap, and comparing

them with the original estimates. The bootstrap was also used to calculate standard errors for each estimate. Shrinkage in inter-individual and random variability estimates was calculated using Perl-speaks-NONMEM.[25]

To visualize the effect ECMO and PNA have on SIL and DMS exposure, concentration time profiles of SIL and DMS were simulated with NONMEM using the final parameter estimates.

Simulation

Concentration-time profiles were simulated for a period of 10 days on ECMO and 10 days after decannulation in a dataset with 90 fictitious patients in three age categories, in which observations started at a PNA of 1, 30 and 90 days respectively. The doses simulated for each age group were 2, 3, 5 and 7 mg/kg/24h, divided over three daily doses. Using NONMEM, the average plasma concentration area under the curve over 24 hours ($AUC_{24\ (SIL+DMS)}$) was calculated as a measure of drug exposure. To correct for differences in dose frequency, AUCs were calculated over 24 hour periods. Since DMS possesses activity itself (50% as potent as SIL [26]) 50% of $AUC_{24\ (DMS)}$ was added to $AUC_{24\ (SIL)}$ to calculate the total exposure $AUC_{24\ (SIL+DMS)}$.

Results

Data

We included 23 patients with pulmonary hypertension and an ECMO indication; see Table I for the patient characteristics. In total, 493 SIL and 492 DMS concentrations were successfully assayed and used to create the PK model. Five (SIL) and six (DMS) concentrations were below the quantification limit and removed from the dataset without changes in CL or V in the base model. Post-ECMO samples were available for 12 out of 23 individuals, with a median of 8 samples per patient (range 1-54). These patients were followed for a median of 97 h post-ECMO (range 14-586 h).

PK model development

A 1-compartment base model for SIL and DMS best described the data. A 2-compartment model was tested for both compounds, but rejected based on poor minimization and inflated standard errors, despite a significantly improved MVOF ($p<0.05$). Additive and proportional residual error terms improved the model. Two different assays were used for ECMO and post-ECMO samples; a separately estimated proportional error term for each method significantly improved fit. See Table II for an overview of the main modelling steps. Inter-individual variability on CL and V was successfully estimated for both compounds. Covariance between CL and V significantly improved minimization

Table I. Patient characteristics[a]

General	
Sex	11 M / 12 F
Primary Diagnosis	Pulmonary hypertension, n=11 (48%) Congenital diaphagmatic hernia, n=6 (26%) Meconium aspiration syndrome, n=3 (13%) Other (congenital heart defects, resp. failure), n=3 (13%)
Body weight (kg)	3.3 (2.0-11)
Gestation (weeks)	38 (37-42)
Postnatal age (days)	9.6 (1.4-1644)
Survival	16 (70%)
Sildenafil	
Dose (after titration during ECMO)	7.4 mg/kg per 24 h (1.3-25), given p.o.: in thee doses, n=8 (35%) in four doses, n=15 (65%)
Relevant comedication	Milrinone, n=18 (78%) Bosentan, n=9 (39%) Fluconazole, n=7 (30%) Clarithomycin, n=1 (4%)
Serum chemistry	
Albumin (g/L)	33 (4-56)
Serum creatinine (μmol/L)	27 (11-165)
ASAT (IU/L)	39 (6-1861)
ALAT (IU/L)	16 (0-558)
GGT (IU/L)	47 (4-308)
LD (IU/L)	941 (139-5798)
ECMO	
ECMO modality	Venovenous (VV), n=6 (23%) Venoarterial (VA), n=20 (77%) Thee patients had 2 ECMO runs each: 2 VV + VA, 1 VA + VV
Median ECMO flow (mL/min)	350 (50-1690)
Duration of ECMO (h)	192 (27-424)
Continuous venovenous haemofiltration	16 Y / 7 N
CVVH flow (mL/min)	300 (30-515)
Body temperature (°C)	2 hypothermic (24-27°C) / 21 normothermic

[a] Parameters expressed as median (range) or n (%). Comedication was defined as having received at least one dose of a specific drug during the observation period. ASAT=aspartate amino transferase; ALAT=alanine aminotransferase; CVVH=continuous venovenous hemofiltration; GGT=gamma-glutamyl transpeptidase; LD=lactate dehydrogenase

and stability; correlation varied from 23% (CL_{SIL}~V_{SIL}) to 85% (CL_{DMS}~CL_{SIL}). A lag time was added, which improved goodness-of-fit and minimization; inter-individual variability on this parameter did not improve fit. CWRES vs. time plots showed a higher CL_{SIL}, V_{SIL} and CL_{DMS} during ECMO (not shown). After inclusion of ECMO as a binary covariate (Equation

Table II. Model components[a]

			Interindividual variab. (% CV)			
	OFV		CL_{SIL}	V_{SIL}	CL_{DMS}	V_{DMS}
Model without covariates, SIL & DMS 1-compartment	8971.5	n/a	68.9	73.7	61.2	150
+ Covariance CL_{SIL}, V_{SIL}, CL_{DMS}, V_{DMS}	8957.5	n/a	70.4	65.8	62.1	162
+ Lag time	8930.3	p<0.001	68.6	86.4	60.7	195
+ Increased CL_{SIL} with t_{EC}	8891.9	p<0.001	70.1	82.9	60.7	195
+ Increased V_{SIL} with t_{EC}	8842.1	p<0.001	77.8	85.9	73.9	209
+ Increased CL_{DMS} with t_{EC}	8702.7	p<0.001	90.8	81.5	82.9	150
+ Increased CL_{SIL} with PNA = FINAL MODEL	8607.1	p<0.001	88.5	91.4	80.4	166

[a] CL=clearance; CV=coefficient of variation; DMS=desmethylsildenafil; OFV=minimal objective function value, which equals -2 log likelihood; PNA=postnatal age; SIL=sildenafil; t_{EC}=time after cannulation; V=volume of distribution; n/a=not applicable: the likelihood ratio test is only applicable when comparing nested models with a difference of 1 degree of freedom

3), there was still a time-related CL_{SIL} increase for ECMO and post-ECMO samples alike. PNA was the covariate that best improved goodness-of-fit; in non-ECMO periods, there was no correlation of CL or V with weight, time after start of ECMO or postmenstrual age after inclusion of PNA. During ECMO however, CWRES vs. time plots showed a remaining gradual increase in CL_{SIL}, V_{SIL} and CL_{DMS} over time. After exploration of different covariate structures, including an instantaneous increase in CL_{SIL}, V_{SIL} and CL_{DMS}, an additive increase after cannulation was selected over other models based on goodness-of-fit, MVOF and CWRES vs. time plots. See Table III for the equations for clearance and volume of distribution in the final model. A combined instantaneous increase after cannulation and linear increase over time improved MVOF even more, but with poor minimization and large standard errors, and was therefore rejected. No other covariates had any statistically significant effect in a forward inclusion procedure. In backwards exclusion, all covariate effects proved significant; the full model was therefore considered the final model.

PK model performance

See Figure 1 for the goodness-of-fit plots. There was some overestimation of SIL and DMS concentrations (Figure 1b), but in general, the predictions were accurate. The covariate inclusion removed most of the time-related deviations in CWRES (Figure 1c-f), but there was some structural deviation in the first 2 days after cannulation and decannulation, which indicates that there is an instantaneous physiological change which could not be captured in the model. All parameter estimates were within the 95% confidence interval calculated using bootstrap data (Table III). Shrinkage was calculated for inter-individual variability (η) on CL_{SIL} (3.7%), V_{SIL} (5.3%), CL_{DMS} (3.7%), V_{DMS} (15%) and the residual variability (ε, 2.2%).

Table III. Parameter estimates[a]

	Unit	SIL		DMS		Remarks
		Estimate (CV %)	Bootstrap median (95% CI)	Estimate (CV %)	Bootstrap median (95% CI)	
PK parameters						
Lagtime	h	0.25 (30)	0.25 (0.07-0.50)	-	-	
k_a	h^{-1}	4.0 (110)	3.4 (0.79-18)	-	-	
V/F	**L**					$V=\theta_1 \times (\theta_2 + (t_{EC}/192)^{\theta tEC}$
θ_1	-	86 (47)	88 (30-233)	7.6 (67)	11 (2.2-32)	
θ_2	-	0.58 (56)	0.45 (0.26-1.8)	-		
θ_{tEC}	-	0.91 (64)	1.00 (0.04-2.6)	-		
CL/F	**L/h**					$CL=\theta_1 \times (PNA/9.6)^{\theta PNA} \times (\theta_2 + (t_{EC}/192)^{\theta tEC}$
θ_1	-	6.1 (42)	6.2 (4.4-18)	15 (21)	14 (7.0-19)	
θ_2	-	0.46 (34)	0.48 (0.15-0.90)	0.51 (21)	0.53 (0.23-0.74)	
θ_{PNA}	-	0.45 (38)	0.43 (0.19-0.92)	-	-	
θ_{tEC}	-	0.78 (31)	0.93 (0.60-1.9)	0.50 (24)	0.44 (0.28-0.66)	
Interindividual variability						
V	%	91 (35)	78 (37-179)	166 (40)	101 (37-184)	
CL	%	89 (31)	93 (60-186)	80 (20)	76 (34-98)	
Residual variability						
Additive	ng/mL	0.60 (42)	1.2 (0.30-2.8)	1.6 (39)	1.7 (0.10-2.2)	
Prop. post-ECMO ($\sigma_{prop,non\text{-}ECMO}$)	%	66 (6.1)	63 (57-72)	49 (7.1)	46 (40-53)	
$\theta_{prop,ECMO}$	-	1.5 (13)	1.4 (0.97-1.7)	1.5 (13)	1.4 (0.97-1.7)	$\sigma_{prop,ECMO} = (\sigma_{prop,\ non\text{-}ECMO})^{\theta prop,ECMO}$

[a] CL=clearance; CV=coefficient of variation; DMS=desmethylsildenafil; F=fractional bioavailability compared to intravenous administration; k_a=absorption rate constant; PNA=postnatal age in days; σ=residual error; SIL=sildenafil; t_{EC}=time after cannulation in h; θ=fixed effect parameter; V=volume of distribution. CL and V estimates for DMS were calculated assuming a conversion fraction ($F_{DMS/SIL}$) of 1.

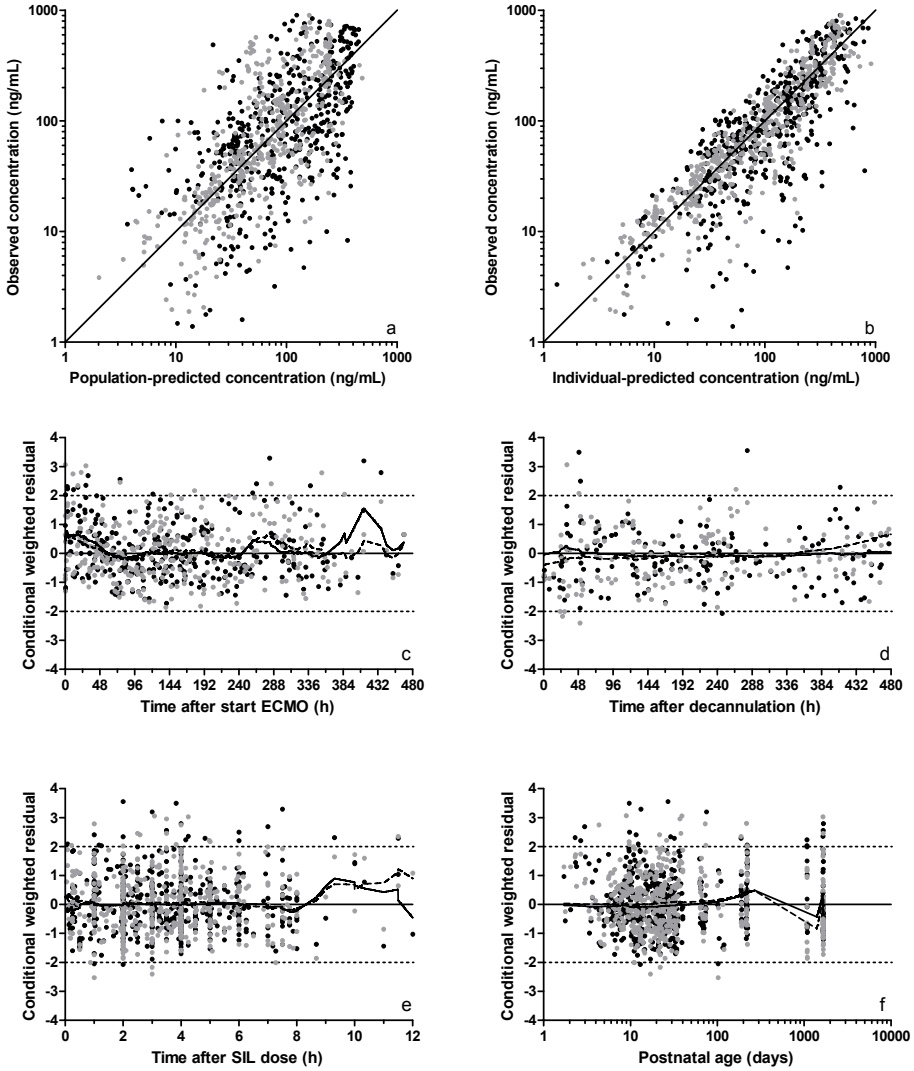

Figure 1. Goodness-of-fit plots for the final model. Observed SIL (black) and DMS (grey) concentration vs. population predicted (a) and individual-predicted (b) concentration. Conditional weighted residuals are displayed vs. t_{EC} (c), t_{END} (d), dose time (e) and postnatal age (f). LOWESS splines were calculated for SIL (continuous curve) and DMS (interrupted curve).

SIL and DMS pharmacokinetics

See Table II for parameter estimates. After a short lag time (0.25 h), there was rapid absorption of sildenafil. The median (range) parameter estimates during ECMO (F=100%) were: CL_{SIL}=8.6 L/h (1.4-2987), V_{SIL}=120 L (15-325), CL_{DMS}=19 L/h (1.5-50) and V_{DMS}=18 L (0.91-105). After decannulation, CL and V dropped to the following values: CL_{SIL}=3.3 L/h (1.3-18), V_{SIL}=29 L (15-338), CL_{DMS}=7.6 L/h (1.2-28) and V_{DMS}=18 L (0.91-105). There was a

gradual nonlinear increase of CL_{SIL}, V_{SIL} and CL_{DMS} over the course of an ECMO-run, with a return to pre-ECMO levels upon decannulation. For a 6 day-ECMO run, CL_{SIL} increased from 1.0 to 6.7 L/h, V_{SIL} increased from 50 to 116 L, CL_{DMS} increased from 7.6 to 21 L/h. See Figure 2 for sildenafil clearance in a median patient. The median (range) ratio of $AUC_{24\ (DMS)}$ over $AUC_{24\ (SIL)}$ was 0.75 (0.14-2.1).

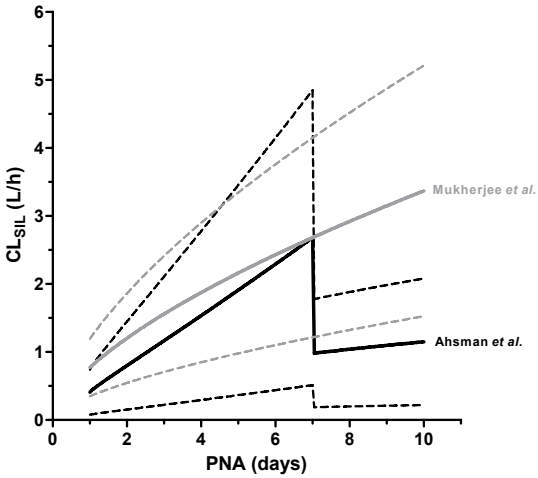

Figure 2. Sildenafil clearance (CL_{SIL}) vs. postnatal age (PNA) according to models in this paper (black) and from literature in a mixed set on ECMO and non-ECMO neonates with pulmonary hypertension (grey).[2] Displayed are the median clearance (continuous curve), plus or minus the standard deviation (interrupted curves).

Simulation

Simulation of four different dose regimens showed that the total SIL and DMS exposure increased by ca. 200% at decannulation, after which a plateau was reached (Figure 3). For post-ECMO children, a sildenafil dose between 3 and 5 mg/kg/24h lead to an exposure equivalent to that of an adult with pulmonary hypertension treated with 3dd 20mg p.o.[5] During ECMO, a dose of between 5 and 7 mg/kg/24h lead to a similar median exposure. Inter-patient variability however was very large, as indicated by the 10th and 90th percentiles of distribution. There was no significant difference ($p<0.05$) between the median exposures in the three age groups (with a PNA of 1, 30 and 90 days at cannulation).

Discussion

By combining concentration-time data during ECMO with those post-ECMO, we were able to model PK changes for SIL and DMS and predict the resulting changes in drug ex-

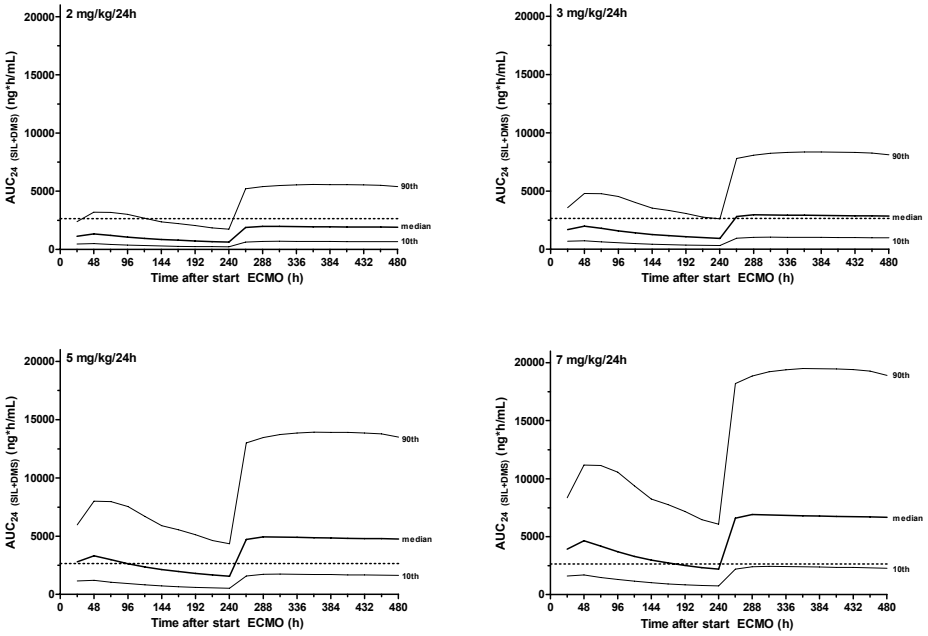

Figure 3. Simulated area-under-the-curve ($AUC_{24\ (SIL+DMS)}$) for sildenafil and desmethylsildenafil with dose regimens of 2, 3, 5 and 7 mg/kg/day, with ECMO applied from 24 to 240 hours. Curves represent the 10th, 50th (median) and 90th percentiles of AUC distribution.

posure. The sparse sampling design and sensitive drug assays allowed the estimation of PK parameters with few samples per patient, but there are some limitations. The absorption phase for instance is difficult to characterize, since this requires multiple sampling within the first 0.5 to 1 hour after drug administration; a model with a lag time and a high absorption rate however was successful in estimating even the high concentrations around the expected time of peak concentrations. PPHN often results from a congenital diaphragmatic hernia, a condition which is associated with a reduced and unpredictable gastrointestinal motility that could influence sildenafil PK. However, inter-individual variability in the lag time could not be modelled with statistical significance.

The sparse sampling also makes distribution volumes more difficult to estimate. An ECMO-effect on V_{DMS} for instance could not be modelled, but this is to be expected in the absence of data after pure metabolite administration. Without rich data during ECMO, it is also more difficult to estimate time-dependent clearance increases; this might be one of the reasons why we were unsuccessful in estimating CL_{SIL} with an instantaneous increase upon cannulation in combination with an additive t_{EC}-effect, despite goodness-of-fit plots that pointed in this direction. Since there is some structural deviation in the CWRES vs. t_{EC} and t_{END} plots just after cannulation and decannulation, it seems that there is an acute change in physiology at those events; it would probably take rich data and a greater understanding of the changes in physiology surrounding ECMO to prepare

a mechanistic model that will also properly describe pharmacokinetics in the first two days after (de-)cannulation.

In practice however, a mild underestimation of clearance in the first days on ECMO is probably not relevant: patients are still in the titration phase and are on ECMO support. Moreover, a reversal of pulmonary resistance is merely the effect of venoarterial ECMO (in case of high flows) or the presence of oxygen-rich blood in the pulmonary vasculature (in case of venovenous ECMO). Problems are expected to occur directly after decannulation, when concentrations will increase due to a sudden drop in clearance. As a consequence, sildenafil doses would have to be decreased after decannulation, from 5-7 mg/kg/24h during ECMO to 3-5 mg/kg/24h in the post-decannulation period (Figure 3). This is close to the 4.2 mg/kg/h we recommended after modelling concentrations in post-ECMO patients [5], which was based on data in 11 individuals. In that model, we found a statistically significant 47% reduction in CL_{SIL} with use of fluconazole (a known CYP3A4-inhibitor [27]) which we did not see this in our dataset. An alternate clearance route during ECMO could explain this difference, such as hemofiltration. It is unlikely when we assume a protein binding of >95% (in adults [28]), but protein binding could be altered in ECMO patients, which potentially has a large effect on CVVH clearance.[29, 30]

Simulations showed that the mg/kg dose regimen leads to similar exposures for patients with a PNA of 1, 30 and 90 days. A weight-based regimen therefore seems appropriate for median patients, despite the PNA-related clearance pattern. It is unclear whether we can extrapolate the post-decannulation PK parameters (12 out of 23 patients) to all former ECMO patients.

This study was done using extemporaneously prepared capsules, before an appropriate sildenafil suspension had been designed and introduced in clinical practice. A new study might identify differences in bioavailability or in the absorption profile between the old and new formulation, which would require modification of the presented PK parameters. An i.v. formulation would provide more reliable administration to the general circulation, but this is only available as compassionate use in selected ECMO centers. In addition, bioavailability is not necessarily constant throughout infancy. It would be interesting to combine this dataset with that of Mukherjee *et al.* to model the combined influence of PNA, formulation and ECMO on SIL and DMS pharmacokinetics.

The increase in clearance and volume of distribution at start of ECMO and a return to pre-ECMO values at decannulation are similar to those seen in cefotaxime during ECMO. [10] These critically ill patients have a low clearance before cannulation. Many of them receive inotropic drugs with prolonged periods of circulatory changes and possibly reduced hepatic or renal function. As soon as ECMO is initiated, clearance rises, possibly due to the continuous hemofiltration and improved organ perfusion the extracorporeal circulation provides. After decannulation, clearance drops again (as the patient is still critically ill) but slowly increases due to maturation or improved disease state. In ad-

dition, an underlying maturation process is likely, which would explain the correlation between CL_{SIL} and PNA seen in both our dataset and in that of Mukherjee *et al.*[4] In the latter study, an increasing CL_{SIL} over time was modelled in a mixed population of young ECMO- and non-ECMO patients (PNA <13 days); no influence of decannulation was described. When we compare the estimates to our own (Figure 2) there is a large overlap during ECMO assuming a bioavailability of 40% after oral administration.[31] Our dataset probably contained more post-ECMO data points over a larger age range (PNA 1.4-1644 days, median 9.4), which would allow better characterization of the non-ECMO clearance vs. PNA pattern.

As was discussed in a previous paper, we could not identify a correlation between drug concentrations and an increased occurrence of hypotensive episodes in the post-ECMO period.[5] This could be the result of a large therapeutic window or the masking of side effects by vasopressor use, ECMO-flow (during venoarterial ECMO) or other therapeutic interventions. On the other hand it could also mean that sildenafil is relatively ineffective in this patient population, with only a minor influence on blood pressure and pulmonary vascular resistance. In our original dataset for instance, sildenafil doses were not reduced after decannulation, which would be expected if there was an increased incidence of hypotension. Evidence is limited to case reports in older children [32] and appropriate efficacy studies have not been done before, which is why some believe that routine off-label use should be withheld until such evidence has been generated. [33] Unfortunately, we do not have sufficiently selective PD endpoints to assess efficacy in these patients.

In conclusion, sildenafil and desmethylsildenafil clearance, and sildenafil volume of distribution, progressively increase after the start of ECMO. As a result of a return of PK parameters to non-ECMO values, the total exposure calculated as $AUC_{24\ (SIL+DMS)}$ increases at decannulation. Clinicians should therefore be aware of a potential increase in efficacy or side effects after the end of ECMO therapy. Assuming an ECMO-run of 10 days, a dose of 5-7 mg/kg/24h during ECMO, and 3-5 mg/kg/24h in the post-decannulation period provides an exposure equivalent to that in adults treated with 20 mg p.o. three times daily. However, additional studies are needed to define a target exposure window in neonates with pulmonary hypertension, using appropriate pharmacodynamic endpoints.

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Chapter 9

Population pharmacokinetics of midazolam and metabolites during venoarterial extracorporeal membrane oxygenation in neonates

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Clin Pharmacokinet 2010, in press

Abstract

Midazolam is used to sedate children during extracorporeal membrane oxygenation (ECMO). Pharmacokinetic changes are expected due to extracorporeal circulation and maturation. We present a population pharmacokinetic model for midazolam and major metabolites in neonates during venoarterial ECMO. We included 20 neonates on veno-arterial ECMO, with a median postnatal age (range) of 0.79 (0.17-5.8) days and a body weight of 3.0 (2.7-3.9) kg at onset of ECMO. Median (range) ECMO duration was 124 (70-275) h. Plasma concentrations were measured at introduction and discontinuation of midazolam infusion (100-300 µg/kg/h). Analysis of midazolam (MDZ), 1-hydroxymi-dazolam (OHM) and its glucuronide (HMG) concentrations were performed using nonlinear mixed-effects modelling (NONMEM). A 2-compartment model for MDZ and 1-compartment model for the metabolites OHM and HMG adequately described the data, with allometric scaling of all parameters.

Following the start of ECMO the volume of distribution for MDZ increases from 4.29 to 14.6 L/3kg with a half-life of 1.85 h. Median MDZ and OHM clearance increase 3-fold within the first 5 days (up to 1.38 and 5.31 L/h/3kg, respectively), whereas HMG clear-ance remains constant at 0.18 L/h/3kg. Inter-patient variability estimates on MDZ, OHM and HMG clearance and MDZ and HMG volume of distribution vary between 87% and 129%. Concomitant inotropic infusion increases HMG clearance by 23%.

After allometric scaling, MDZ and OHM clearance increase as a result of maturation or recovery from critical illness. In ECMO patients of 2.7-3.9 kg, a continuously infused midazolam dose of 300 µg/kg/h for 6 h, and 150 µg/kg/h thereafter, provides adequate plasma concentrations for sedation. The dose will have to be increased substantially after 5-7 days. HMG accumulates during ECMO, providing an increased proportion of the overall effect, up to 34% after 7 days. Large unexplained inter-patient variability warrants careful titration on sedation and side effects.

Introduction

Extracorporeal membrane oxygenation (ECMO) is a cardiopulmonary bypass technique designed to temporarily support respiratory or cardiac function in critically ill patients. Adequate sedation is necessary to alleviate physical and psychological distress, but also to prevent complications related to cannula displacement and/or obstruction. Excessive movement could also cause complete cannula dislodgement, leading to life-theatening blood loss. Midazolam (MDZ) is the sedative of choice for critically ill neonates because of its rapid elimination compared to other benzodiazepines.[1] However, the perceived short half-life may not hold true for neonates during ECMO-treatment. In general, elimination clearance is reduced and distribution volume increased while on ECMO.[2, 3] There is ample evidence for the sequestration of midazolam by polymeric components of ECMO circuits, potentially leading to low bioavailability.[4-6] Several years ago, Mulla *et al.* studied midazolam pharmacokinetics in these patients. They concluded that the distribution volume increases over the course of an ECMO-run and that the apparent elimination half-life from onset to steady-state is prolonged fivefold.[7] In addition to the ECMO-treatment, physiological changes during the first weeks of life might lead to altered pharmacokinetic parameters. The main active metabolite 1-hydroxymidazolam (OHM) is formed though CYP3A4/5/7-mediated hydroxylation and is subsequently glucuronidated via UGT1A4 and UGT2B4/7 into hydroxymidazolamglucuronide (HMG).[8, 9] In general, the metabolic capacity via these enzymatic pathways increases with age.[10] While pharmacologically less potent, HMG could accumulate when renal function is compromised, thereby adding to the depth of sedation.[11, 12] Unfortunately, the pharmacokinetic profile of hydroxymidazolamglucuronide and its contribution to the total depth of sedation in ECMO patients is unknown.

In order to construct an appropriate dosing regimen, it would be beneficial to assess the pharmacokinetics of midazolam and its major metabolites, taking into account the time-dependent pharmacokinetic changes caused by ECMO-treatment, maturation and size-dependent metabolic differences between individual neonates.

The aim of this study was to describe the pharmacokinetics of midazolam and its major metabolites 1-hydroxymidazolam and hydroxymidazolamglucuronide in neonates during ECMO. Inter-patient variability was estimated and explained by patient weight, age and other covariates. Potential dose regimens were evaluated though dose simulations.

Methods

ECMO neonates

Approval for this study was obtained from the Erasmus MC institutional review ethics board. Parental informed consent was obtained for blood sampling, effect observations and use of clinical data. We included neonates with a postnatal age <7 days who were treated with venoarterial extracorporeal membrane oxygenation. Criteria for ECMO treatment were: gestational age >34 weeks, birth weight >2.0 kg, mechanical ventilation <7 days, an alveolar arterial oxygen difference less than 80 kPa, and an oxygenation index >25. Isovolumetric exchange transfusion was applied to correct electrolyte, protein and hematocrit imbalances while preventing fluid overload. Concomitant drugs were given in accordance with the departmental treatment protocol and doses were adapted to each neonate's clinical condition. The most recent weight available prior to ECMO was used for dose calculation and pharmacokinetic analysis. This was either the birth weight or the latest estimate of the body weight, depending on the time between birth and cannulation. This dataset was part of a study into the feasibility and desirability of drug interruptions to prevent adverse effects of and tolerance to sedatives.

Procedure

In accordance with standard procedure, 0.2 mg/kg/h of midazolam was given before cannulation. Upon discomfort, midazolam was introduced as a continuous infusion with an infusion rate of 0.1 mg/kg/h. The midazolam dose was incrementally adjusted (with steps of 0.1 mg/kg/h) based on the required level of sedation, which was objectified with the validated COMFORT behavior scale.[13, 14] Once fully sedated, midazolam infusion was interrupted until COMFORT scores indicated that additional sedation was necessary. Drug infusion rates, bolus doses and ECMO flow were recorded every 2 h from the onset of ECMO. Other recorded parameters included heart rate, respiratory rate, withdrawn volumes of blood, urinary output and serum chemistry: bilirubin, albumin, creatinine, urea, alanine (ALT), and aspartate (AST) aminotransferase, gamma-glutamyltranspeptidase (GGT). Blood was collected at designated intervals of 24 h. Samples were taken 0.5-1-3-6-12 h after discontinuation of midazolam infusion and 0-0.17-0.5-1-3-6-12-18-21 h after (re-) introduction of midazolam. Blood samples (500 µL) were taken from a venous access port on the ECMO circuit and collected in heparinized tubes. After centrifugation (5 min, 4000 ×g), the supernatant serum was stored at -80 °C until analysis.

ECMO System

The ECMO circuit consisted of extracorporeal cannulae (Medtronic®, USA), PVC tubing (Bentley Bypass 70 tubing, Baxter, The Netherlands), a silicone rubber membrane oxygenator (Pediatric Extended Membrane Oxygenator, Medtronic®), and Heat Exchanger

(Heat Exchanger Monitoring Adapter and Luer-lock, Medtronic®). The priming volume of the system ranged between 300 and 350 mL.

Assay method

Midazolam (MDZ), 1-hydroxymidazolam (OHM) and hydroxymidazolamglucuronide (HMG) concentrations in serum were measured in each sample using high-performance liquid chomatography (HPLC-UV) as previously described.[15] MDZ and OHM were quantified after a liquid-liquid extraction with dichloromethane. HMG was measured as OHM after enzymatic deglucuronidation. The limits of quantification (LOQ) were 11 and 6 µg/L for MDZ and OHM respectively, which corresponds to 10 µg/L for HMG. Intra- and interassay coefficients of variation were less than 8% (MDZ & OHM) and 13% (MDZ & OHM), respectively.

Pharmacokinetic analysis

Data from all patients were analyzed simultaneously using nonlinear mixed-effects modelling software (NONMEM VI 2.0, Globomax LLC, Ellicott City, MD). NONMEM modelling allows the estimation of typical population pharmacokinetic parameters, and their respective inter- and intra-individual variability in combination with the estimation of residual random variability. The first-order conditional estimation (FOCE) method, with interaction between the inter-individual and random effects, was used throughout method development. The final model was constructed with a convergence criterion of six significant digits. All doses and concentrations were converted to their molar equivalents of midazolam (Mw_{MDZ}=325.76 g/mol, Mw_{OHM}=341.77 g/mol, Mw_{HMG}=517.87 g/mol).

The population model was built stepwise. A specific assumption was tested at each step, at which model adequacy was assessed though examination of goodness-of-fit plots, generated by the Xpose software package (v 3.2, Dr. M. Karlsson, University of Uppsala, Sweden) [16] and S-Plus (v 6.2.1, professional edition, Insightful Corp., Seattle, WA). Goodness-of-fit plots consisted of measured drug concentrations vs. population (PRED) or individual (IPRED) predictions, and weighted residuals (WRES) vs. time plots. Bayesian IPRED concentrations were obtained via NONMEM's *posthoc* option.

(a) Basic model. Differential equations were used with NONMEM's ADVAN6 subroutine to describe the population PK of midazolam and its metabolites, expressed as clearances and volumes of distribution. The conversion fraction (Fc) of MDZ to OHM and OHM to HMG could not be determined with this study design; parameter estimates are reported with Fc fixed to unity. Between-patient variability in volumes of distribution and clearances was characterized with exponential models. For example, the elimination clearance for the i^{th} individual (CL_i) was estimated using equation 1:

$$CL_i = \theta_{pop} \times e^{\eta_i} \quad \textbf{(Eq. 1)}$$

in which θ_{pop} is the population value for clearance, and η represents the between-patient variability with mean 0 and variance ω^2. Covariance between two elements of η (for instance between clearance and volume of distribution) was incorporated into the model. The error model that described residual (unexplained) variability consisted of a proportional and an additive term (equation 2):

$$C_{obs,ik} = C_{pred,ik} \times \varepsilon_{prop,ik} + \varepsilon_{add,ik} \quad \textbf{(Eq. 2)}$$

in which $C_{obs,ik}$ is the k^{th} observed plasma concentration for the i^{th} individual, $C_{pred,ik}$ is the corresponding model predicted concentration and $\varepsilon_{prop,ik}$ and $\varepsilon_{add,ik}$ are residual random errors with mean 0 and variance σ^2. The proportional term $\varepsilon_{prop,ik}$ was estimated for MDZ, OHM and HMG independently. In the elimination phase after continuous infusion had ended, a first sub-LOQ concentration was included in the dataset at a value of half the LOQ; subsequent sub-LOQ observations were deleted. The additive term $\varepsilon_{add,ik}$ was fixed at a value of $(½ \times LOQ)^2$ for each compound to allow the predicted concentration to vary roughly between 0 and the LOQ.

(b) Covariate analysis. To explain pharmacokinetic variability between and within patients, correlations between pharmacokinetic parameters and patient characteristics were evaluated by stepwise inclusion in the model.[17] To account for variability in pharmacokinetic parameters due to the varying sizes of individual neonates, the parameter values were standardized to a median bodyweight of 3 kg using an allometric power model (equation 3).[18]

$$P_i = P_{3kg} \times \left(\frac{WT_i}{3} \right)^{\theta_P} \quad \textbf{(Eq. 3)}$$

in which P_i is the parameter for the i^{th} individual, WT_i is the body weight for the i^{th} individual, P_{3kg} is the parameter in a neonate with a standardized weight of 3 kg and θ_p is the power exponent fixed at 0.75 for clearances and 1 for volumes of distribution. This approach also allows comparison of the estimated pharmacokinetic parameters with those from older children and adults. However, allometric scaling does not explain clearance variability caused by ECMO-related shifts in organ function or a developmental shift in the expression of drug-metabolizing enzymes.[19, 20] We expected time-dependent changes in clearance and volume of distribution based on maturation, disease progression and drug absorption. A significant degree of collinearity between PNA, PMA and time after cannulation (t_{EC}, i.e. time after the start of Extracorporeal Circulation) is to be expected. We decided to test the effect of each of these temporal covariates individually. After having selected the one covariate that best improved model fit (which we assessed using goodness-of-fit plots and the objective function value, OFV), we tested a potential additional effect of each

remaining temporal covariate. Other covariate effects were tested after appropriate temporal covariates had been included in the model.

We also anticipated an increase in the central volumes of distribution upon connection of the ECMO circuit [7] (equation 4):

$$V = V_{\max} - (V_{\max} - V_{base}) \times e^{\left(\frac{-t_{EC} \times 0.693}{t_{1/2}}\right)} \quad \textbf{(Eq. 4)}$$

in which V is the central volume of distribution at a specific time after cannulation, V_{max} is the maximal volume of distribution, V_{base} is the baseline volume of distribution before cannulation, t_{EC} is the time after start of extracorporeal circulation and $t_{½}$ is the half-life of the time-dependent increase in volume of distribution. Equation 4 was also tested with postnatal and postmenstrual age (PNA and PMA) instead of t_{EC}. Clearance changes (whether maturation or disease related) were modelled with an additive linear function (equation 5):

$$CL = CL_{base} + \theta * PNA \quad \textbf{(Eq. 5)}$$

in which CL equals clearance, CL_{base} is the baseline clearance at PNA=0, θ is the slope of the regression function, and PNA is the postnatal age (in days). t_{EC} and PMA were modelled in a similar manner.

After the temporal covariates, we tested the effect of the following characteristics on pharmacokinetic parameters: gender, ECMO duration, serum chemistry, ECMO flow, heart rate, respiratory rate, erythomycin use (CYP3A inhibitor), inotrope use and vasopressor score [21], phenobarbital use (CYP3A inductor), frequency and volume of exchange transfusions and urinary excretion rate (in mL/h, calculated over intervals of 24 h). Continuous covariates, such as plasma albumin concentrations, were modelled in an exponential manner as shown in equation 6:

$$CL_i = \theta_{pop} \times \left(\frac{ALB}{29}\right)^{\theta_{ALB}} \quad \textbf{(Eq. 6)}$$

in which θ_{pop} is the clearance in individuals with the median ALB of the population (29 g/L), and θ_{ALB} is an exponent describing the correlation function.

Categorical covariates, such as gender or concomitant drug use, were modelled as shown in equation 7:

$$CL_i = \theta_{pop} \times \left(\theta_{gender}\right)^{GNDR} \quad \textbf{(Eq. 7)}$$

in which θ_{pop} is the population value for clearance of females and θ_{gender} is the fractional difference between males (GNDR=1) and females (GNDR=0).

Exchange transfusion could be considered an extra clearance route for hydrophilic or extensively protein-bound drugs, as a substantial percentage of the circulating volume is replaced with fresh erythocytes or albumin solution. A model was tested in which an exchanged volume of 50 mL or more opened an additional clearance route for MDZ, OHM or HMG. This was done by defining an additional clearance from the central compartment, that would only be available for a duration of one hour after the start of each exchange transfusion, using NONMEM's MTIME (event time) option.

(c) Statistical analysis. Statistical significance of a potential covariate effect was determined via the log-likelihood ratio test for two hierarchical models, using the objective function value (OFV) produced by NONMEM. A decrease in OFV of 3.84 (p=0.05, 1 degree of freedom) was considered statistically significant. All covariates selected during the first stage were included in an intermediate model. These covariates, including temporal covariates and weight, were subsequently excluded in a backward elimination procedure. If deletion of a covariate did not result in a significant worsening of the objective function (ΔOFV <3.84), the covariate was removed from the model. The resulting model was considered the final model.

Model validation

Stability and performance of the final model were checked using an internal validation procedure via the bootstrap resampling technique [22], in which 1000 bootstrap data sets were generated by random sampling with replacement. We used the Wings for NONMEM software package (v6.12 March 2007, Dr N. Holford, Auckland, New Zealand). Model validity was assessed by calculating median values and the 2.5th and 97.5th percentiles of parameter distribution generated by the bootstrap, and comparing them to the original estimates. The bootstrap was also used to calculate standard errors of parameter estimates.

Dose simulation

Dose regimens were simulated with 500 fictitious patients (with an ECMO-run of 1 week). Plasma concentrations of MDZ, OHM and HMG were estimated. To account for the contribution of OHM and HMG to the sedative effect, a composite plasma level of MDZ-equivalents was calculated, assuming a sedative potency of 80% for OHM [11] and 6% for HMG relative to MDZ. HMG potency was calculated assuming an *in vitro* receptor affinity of 8.8% and a molar weight ratio of 160% compared to MDZ.[12]

Results

Twenty neonates were included, see Table I for patient demographics. In total, 293 blood samples were collected. The medication regimens included antibiotics (dependent on patient weight and antimicrobial sensitivity of suspected and cultured micro-organisms: tobramycin, amoxicillin, penicillin, meropenem, cefotaxime, ceftazidim or erythomycin), atropine, vecuronium, fentanyl, furosemide, vaso-active drugs (dobutamine, dopamine, norepinephrine), magnesium sulphate, phenobarbital, morphine, nystatin, and hydrocortisone. Plasma concentrations of MDZ, OHM and HMG were successfully determined in 291, 284 and 281 samples each, with sub-LOQ values in 1.4, 13 and 3.2% of observa-

Table I. Patient characteristics[a]

General	
Male/Female	10 / 10 (50% / 50%)
Primary Diagnosis	Meconium aspiration syndrome, n=10 (50%) Congenital diaphagmatic hernia, n=7 (35%) Therapy-resistant respiratory insufficiency, n=2 (10%) Sepsis, n=1 (5%)
Body weight (kg)	3.0 (2.7-3.9)
Gestational age at start ECMO (weeks)	40.4 (37.0-42.1)
Postnatal age at start ECMO (days)	0.79 (0.17-5.6)
Duration of ECMO (h)	124 (70-275)
Midazolam	
Continuous infusion rate (mg/kg/h)	0.15 (0.10-0.30)
Number of additional injections	4 (0-9)
Bolus dose (mg/kg)	0.2 (0.10-0.30)
Serum chemistry	
Albumin (g/L)	29 (23-33)
Urea (mmol/L)	3.2 (1.9-4.8)
Creatinine (μmol/L)	50 (31-63)
Bilirubin (μmol/L)	69 (22-101)
ASAT (IU/L)	39 (17-59)
ALAT (IU/L)	9.0 (2.9-23)
GGT (IU/L)	17 (7.5-97)
Other	
Heart rate (per min)	152 (123-191)
Respiratory rate (per min)	50 (43-70)
Urine production (mL/h)	6.5 (6.0-20)
Survival	15 (75%)

[a] Parameters expressed as median (range) or n (%). ASAT=aspartate amino transferase; ALAT=alanine aminotransferase; GGT=glutamyl transpeptidase

tions, respectively. See Figure 1 for the individual MDZ concentration vs. time profiles. The median midazolam plasma concentration was just under 400 ng/mL. None of the patients required hemofiltration during the ECMO-run.

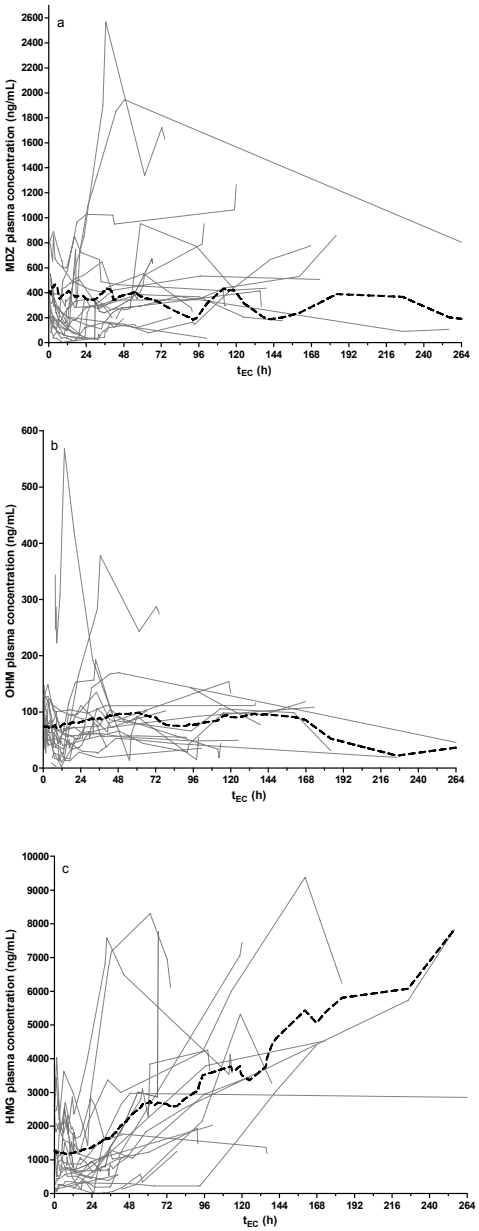

Figure 1. Individual curves of midazolam (a), 1-hydroxymidazolam (b) and hydroxymidazolamglucuronide (c) plasma concentration vs. time after cannulation (t_{EC}), with a trendline (dotted, Lowess curve of 10 points per window).

Model development and performance

The data best fit a two compartment model (central and peripheral) for MDZ, and two sequential compartments representing OHM and HMG (Table II and Figure 2). Inter-individual variability was successfully estimated on the clearance of MDZ, OHM and HMG and the (central) volume of distribution of MDZ and HMG. Allometric scaling of all clearances and volumes of distribution explained between 19% (CL_{OHM}) and 71% ($V_{MDZ,C}$) of inter-patient variability in pharmacokinetic parameters (Table II). Estimation of the exponents is complicated by the narrow body weight distribution.

Table II. Model development[a]

			Interindividual variability (CV, %)				
	OFV		$V_{MDZ,C}$	CL_{MDZ}	CL_{OHM}	V_{HMG}	CL_{HMG}
No covariates, MDZ=1-compartment	17628	n/a	87	103	96	101	233
No covariates, MDZ=2-compartments	10695	p<0.001	240	139	137	253	237
+ Allometric scaling	10663	n/a	69	80	111	93	102
+ Increased V_{MDZ} with t_{EC}	10633	p<0.001	70	74	109	96	102
+ Increased CL_{MDZ} with t_{EC}	10406	p<0.001	86	77	104	103	135
+ Increased CL_{OHM} with PNA	10298	p<0.001	83	83	84	103	144
+ Increased CL_{OHM} with t_{EC}	10292	p<0.05	89	85	90	105	142
+ Increased $CL_{HMG}$with INO = FINAL MODEL	10286	p<0.05	87	86	91	104	129

[a] OFV=minimal objective function value, which equals -2 log likelihood; CV=coefficient of variation; V=volume of distribution; CL=clearance; MDZ=midazolam (central compartment); OHM=1-hydroxymidazolam; HMG=hydroxymidazolamglucuronide; t_{EC}=time after cannulation; PNA=postnatal age; INO=concomitant inotrope drugs; n/a=not applicable: the likelihood ratio test is only applicable when comparing nested models with a difference of 1 degree of freedom

$V_{MDZ,C}$ rapidly increased from 4.29 to 14.6 L/3kg after start of ECMO-treatment, with a half-life <2 hours. This increase was not significantly correlated with PMA or PNA and was fixed in subsequent runs to prevent minimization errors. One patient presented with impaired liver function (AST 163, ALT 162, BILI and GGT normal). Although his MDZ clearance appeared unimpaired, the OHM and HMG concentrations were significantly lower. This deviation was modelled with a separate conversion fraction ($F_{OHM/MDZ}$) for this individual; the resulting estimate showed a 97% reduction in $F_{OHM/MDZ}$. Individual PK curves showed an increasing overprediction of concentrations with time. To model an increased clearance, we first tested maturation with either t_{EC}, PMA or PNA using equation 5. T_{EC} was the best predictor of CL_{MDZ} with an OFV drop of 227 points (p<0.001) and markedly improved goodness-of-fit plots. PNA and PMA did not improve the OFV as much as t_{EC}. On top of t_{EC}, we tested an additional effect of PNA or PMA, but neither led to an improvement above the p=0.05 significance level. Next, we applied a similar

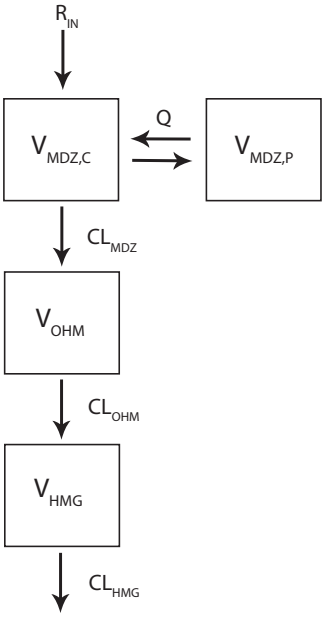

Figure 2. Schematic representation of the structural model that best-fitted the midazolam and metabolite data. $R_{in=}$Rate of midazolam infusion; $V_{MDZ,C}$=volume of distribution for midazolam (central compartment); $V_{MDZ,P=}$volume of distribution for midazolam (peripheral compartment); V_{OHM}=volume of distribution for 1-hydroxymidazolam; V_{HMG}=volume of distribution for 1-hydroxymidazolamglucuronide; Q=intercompartmental midazolam clearance; CL_{MDZ}=midazolam clearance; CL_{OHM}=1- hydroxymidazolam clearance; CL_{HMG}=hydroxymidazolamglucuronide clearance.

procedure to OHM clearance, which showed that PNA as a single covariate best improved clearance estimates (with an OFV drop of 108 points, $p<0.001$) and that on top of PNA, t_{EC} improved the OFV and goodness-of-fit even further whereas PMA did not (an additional drop of 6.4 points, $p<0.05$). See Figure 3 for the MDZ and OHM clearance maturation for each patient, and a simulated patient of median weight and PNA. There was no significant improvement of goodness-of-fit when changes in V_{OHM}, V_{HMG} or CL_{HMG} were modelled with either t_{EC}, PNA or PMA. Covariance between clearance and volume of distribution parameters improved minimalization and model stability; estimates ranged from 0.30 to 0.68. After the temporal covariates had been modelled, there was no remaining pattern in the weighted residuals vs. time plot.

We tested the remaining covariates in a forward inclusion procedure. Of the potential covariates, only weight (all parameters), PNA (CL_{OHM}), t_{EC} (CL_{MDZ} and CL_{OHM}) and concomitant inotrope use (CL_{HMG}) significantly improved both the goodness-of-fit and OFV and were therefore included in the intermediate model. Concomitant administration of norepinephrine, dobutamine or dopamine increased HMG clearance by 23%. Exchange transfusions did not temporarily increase clearance. The selected covariates were removed from the intermediate model in a backward elimination procedure, in

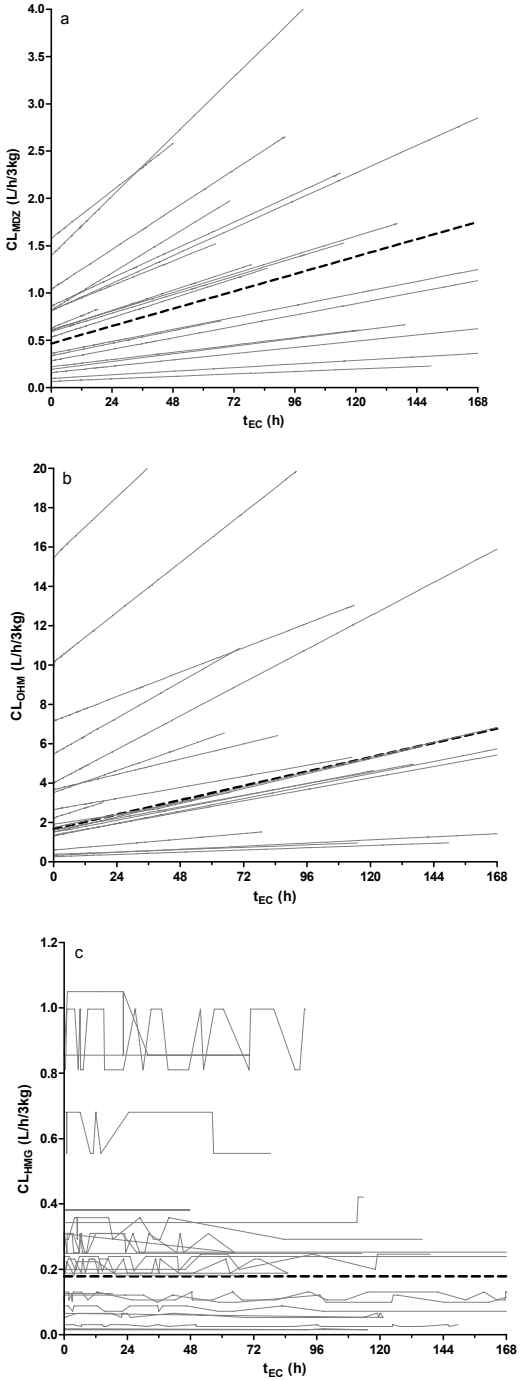

Figure 3. Individual Bayesian estimates of clearance values for midazolam (a), 1-hydroxymidazolam (b) and hydroxymidazolamglucuronide (c), with the population-derived clearance in a median patient represented by the dotted line.

which allometric scaling (body weight) was considered a single covariate. Removal led to significant increases in OFV for each covariate, and consequently, the intermediate model was considered the final model. Goodness-of-fit plots for the final model are in Figure 4. Population estimates were within the 95% confidence range generated with 1000 bootstrap replicates (Table III).

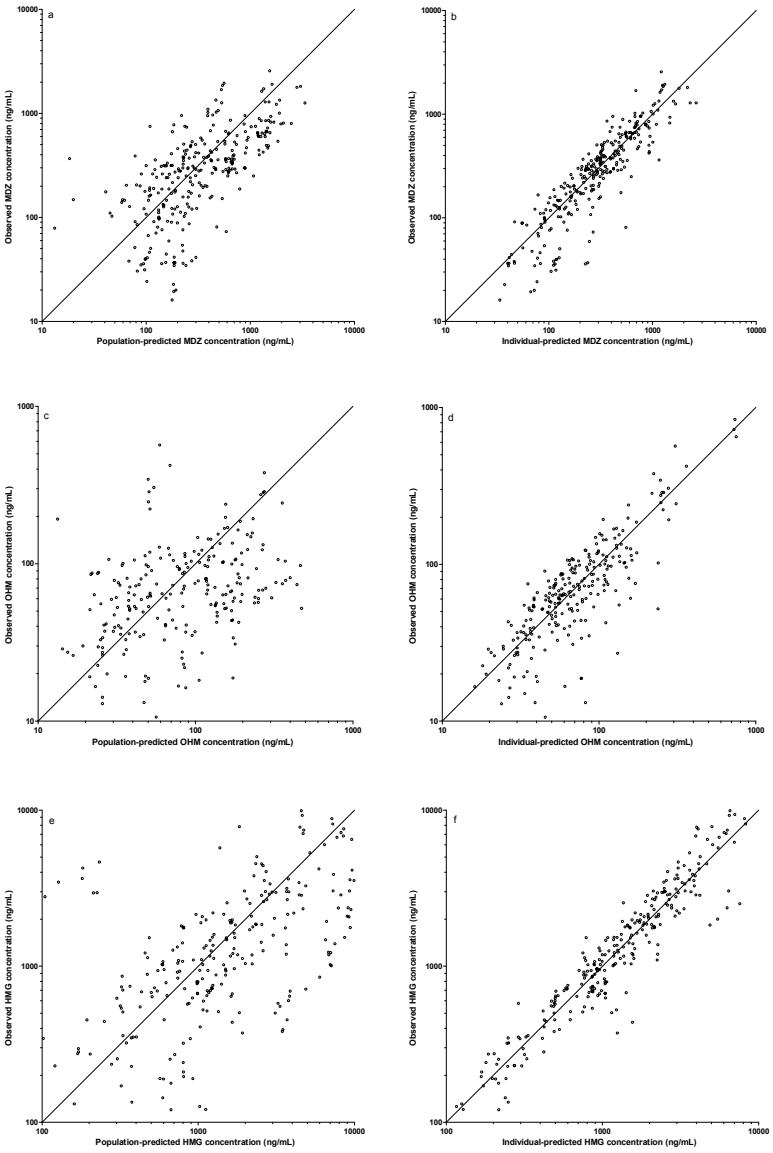

Figure 4. Goodness-of-fit plots for the final model. Observed midazolam (MDZ) concentration vs. population predicted (a) and individual-predicted (b) concentration. Similar plots are displayed for 1-hydroxymidazolam (OHM) (c and d) and hydroxymidazolamglucuronide (HMG) (e and f).

Table III. Parameter estimates[a]

	Unit	MDZ		OHM		HMG	
		Estimate (CV %)	Bootstrap median (95% CI)	Estimate (CV %)	Bootstrap median (95% CI)	Estimate (CV %)	Bootstrap median (95% CI)
Parameter estimates							
V	L/3kg	-	-	10.2 (33.7%)	9.86 (2.73-17.0)	1.21 (48.6%)	1.03 (0.35-2.48)
V_{base}	L/3kg	4.29 FIX	-	-	-	-	-
V_{max}	L/3kg	14.6 FIX	-	-	-	-	-
V_p	L/3kg	13.3 (96.3%)	12.8 (5.36-39.5)	-	-	-	-
CL	L/h/3kg	0.47 (28.0%)	0.47 (0.26-0.77)	1.38 (30.7%)	1.38 (0.75-2.43)	0.18 (24.4%)	0.20 (0.12-0.32)
Q	L/h/3kg	0.51 (104%)	0.51 (0.21-2.76)	-	-	-	-
Covariate effects							
t_{EC} on V ($t_{½}$)	h	1.85 FIX	-	-	-	-	-
t_{EC} on CL	L/h^2	0.00765 (29.3%)	0.00750 (0.00422-0.0129)	0.015 (61.7%)	0.00174 (0.0001-0.0427)	-	-
PNA on CL	$L/(24h^2)$	-	-	0.368 (40.3%)	0.388 (0.110-0.753)	-	-
INO on CL	-	-	-	-	-	1.23 (11.9%)	1.26 (1.02-1.61)
Interindividual variability							
V	%	87.0 (27.2%)	83.4 (48.4-140)	-	-	129 (26.7%)	137 (64.4-210)
CL	%	85.6 (18.1%)	83.0 (52.3-112)	90.8 (19.0%)	83.8 (53.2-115)	104 (19.4%)	83.6 (53.0-114)
Residual variability							
Additional	ng/mL	30 fixed	-	9 fixed	-	25 fixed	-
Proportional	%	37.2 (10.3%)	36.5 (28.8-43.6)	34.9 (13.8%)	39.3 (29.5-50.2)	32.6 (8.30%)	32.1 (27.3-38.0)

[a] MDZ=midazolam; OHM=1-hydroxymidazolam; HMG=hydroxymidazolamglucuronide; CV=coefficient of variation; V=volume of distribution; V_{base}=baseline volume (MDZ); V_{max}=maximal volume (MDZ); $t_{½}$=half life of V increase (MDZ); CL=clearance; Q=peripheral MDZ-clearance; PNA=postnatal age in days; t_{EC}=time after cannulation in h; INO=inotrope infusion; R=correlation coefficient. CL and V estimates for OHM and HMG were calculated assuming a conversion fraction ($F_{OHM/MDZ}$ and $F_{HMG/OHM}$) of 1

Representative parameter estimates

In a simulated neonate with a median weight of 3 kg and postnatal age of 0.8 days, V_{MDZ} increases from 4.29 L at the onset of ECMO-treatment to 14.6 L on day 14, while V_{OHM} and V_{HMG} remain constant at 10.2 and 1.2 L. MDZ and OHM clearance increase 6-fold within the first two weeks, whereas HMG clearance remains constant (Figure 3).

Dose simulation

When we assume that all patients were successfully titrated to the required level of sedation, we can use the median midazolam concentration of 400 ng/mL as a target. Several dose regimens were tested including the base regimen (bolus injection of 200 µg/kg with continuous infusion of 100 µg/kg/h) and the regimen proposed by Mulla *et al.* (continuous infusion of 350 µg/kg/h for 6h, followed by 50 µg/kg/h). As shown in Figure 5a, neither regimen leads to stable midazolam plasma concentrations around 400 ng/mL. Our patients required additional bolus doses and a median continuous infusion rate of 150 µg/kg/h, which leads to simulated concentrations in the target range (curve not shown). To quickly reach stable plasma concentrations of 400 ng/mL, the optimal dose regimen would be a continuous infusion of 300 µg/kg/h for the first 6 h, after which the infusion rate should be reduced to 150 µg/kg/h. After 5 days, the infusion rate should be increased to 200 µg/kg/h to compensate for the continually increasing MDZ and HMG clearance (Figure 5b), which could also be observed in the original dataset. With this proposed regimen. median OHM and HMG plasma concentrations are 88.6 ng/mL and 1420 ng/mL, respectively. The individual contributions of each metabolite to the total sedation, calculated as MDZ-equivalents, are 11.2 and 13.5%. The contribution of HMG to sedation increases over time whereas OHM concentrations remain constant (Figure 5c).

Discussion

There is some controversy in the modelling community on the choice between fixed-power allometric scaling vs. estimation of a fixed-effect parameter in which body weight is considered a regular continuous covariate.[23-25] We chose to scale upfront (which led to a significant reduction in the objective function), after which we included further age- or time-dependent changes of CL or V, modelled using equation 4 or 5. This allowed us to correct for differences in size based on the estimated body weight at t_{EC}=0. Because most patients are placed on ECMO in a short time frame (within the first 2 days after birth), and because only neonates with a gestational age of at least 34 weeks are eligible for ECMO, collinearity between postnatal age, postmenstrual age and the time after cannulation (t_{EC}) is likely. We selected an appropriate combination of temporal covariates based on the best improvement in goodness-of-fit and statistical significance at the 95% confidence level. This led to a model in which CL_{MDZ} increased with t_{EC}, and CL_{OHM} with t_{EC} & PNA. The increased clearance with age could explain why there were increased dose requirements over the course of an ECMO-run in the original dataset.

Burtin *et al.* found no significant correlation between PNA and MDZ pharmacokinetics in non-ECMO neonates (both term and preterm) with a PNA of under 2 weeks, but found significant correlations with birth weight and gestational age.[26] Over a larger age range,

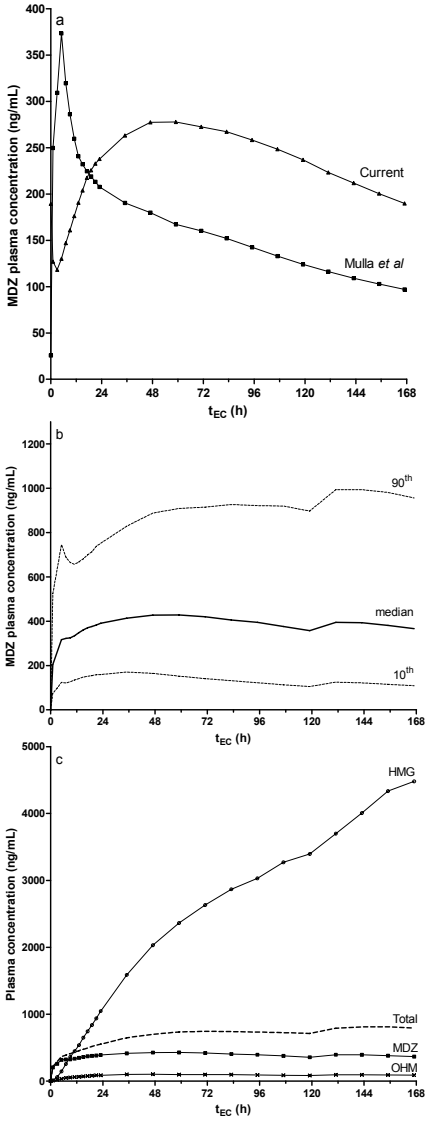

Figure 5. **Concentration-time profiles obtained after dose simulation with the final model. Plot (a) describes the plasma midazolam concentration profiles in a median patient of 3 kg with our current dose regimen (200 μg/kg by bolus injection followed by 100 μg/kg/h) versus the regimen previously suggested by Mulla et al (350 μg/kg/h for 6h, followed by 50 μg/kg/h). Plot (b): A continuous infusion of 300 μg/kg/h for 6 h, followed by 150 μg/kg/h onward and an increase to 200 μg/kg/h after 120 h, leads to sufficient midazolam concentrations in the first week. Interpatient variability remains high, as can be seen from the median (solid line) and 10th and 90th percentiles of distribution (dotted lines), of the midazolam plasma concentration-time profile simulated for 500 patients replicated from the original dataset. Plot (c): Contribution of midazolam (MDZ), 1-hydroxymidazolam (OHM) and hydroxymidazolamglucuronide (HMG) to a total sedative concentration expressed as MDZ equivalents, based on a relative potency of 80% for OHM and 6% for HMG (dotted line). All concentrations are plotted against the time after cannulation (t_{EC}).**

midazolam clearance in critically-ill (non-ECMO) patients does increase with postnatal age, probably due to CYP3A4-maturation: clearance reports vary from 1.8 and 2.2 mL/kg/min at a PNA of 0-11 days, via 5.0 mL/kg/min at 3 months, to 5.8-13.6 mL/kg/min between 1 month and 5 years.[26-29] In this study, a clearance of 2.6 mL/kg/min increases to 7.6 mL/kg/min over the course of 5 days for an ECMO patient of 3 kg. Over the narrow age range of our patients, metabolic maturation is an unlikely explanation for the increased clearance because the CYP3A4 pathway matures slowly over the course of several weeks [19] with negligible CYP3A7 activity.[30-32] Another explanation could be stabilization of hepatic blood flow-which is suspected to partly explain reduced clearance in critically-ill vs. healthy patients [15, 33]-as a result of the circulatory support that venoarterial ECMO provides. The individual contribution of maturation, disease progression and other factors to pharmacokinetic changes could be modelled in a combined dataset of ECMO and non-ECMO treated neonates.[34]

In a similar group of critically neonates on ECMO, Mulla *et al.* did not report a correlation between clearance and time or postnatal age.[7] Instead, they found a constant clearance of 1.4 mL/kg/min. The only apparent differences between our two datasets are the type of ECMO applied (venovenous vs. venoarterial), and possibly the manufacturer of circuits and membranes; a rollerpump is used in both our institutions. The physicochemical properties of drug and polymer can influence the speed and extent of drug absorption [2, 35-37]; it is possible that our circuits were more prone to midazolam absorption. The midazolam recovery *in vitro* (Chapter 6) with freshly primed and used ECMO circuits was under 10%, which is substantially lower than the recovery reported by Mulla *et al.* in their PVC circuit with Avecor membrane oxygenator (over 25% after 30 min).[4] As ECMO circuits and membranes are subjected to the continuous blood flow, the surface might get damaged over time, leading to higher midazolam losses, but to our knowledge, this effect has not been described before.

V_{MDZ} rapidly increases to 4.9 L/kg with a $t_{½}$ of 1.85 h, which is similar to the maximum of 4.1 L/kg reported by Mulla *et al.*, with a $t_{½}$ of 3.6 h. The few samples in the early observation period meant that V_{MDZ} had to be fixed rather than estimated; a sensitivity analysis showed that other values did not significantly improve model fit and OFV.

We found no significant effect of serum creatinine on PK parameters; although its use as a marker of renal function in newborns is in dispute [38, 39], it was the only marker of renal function available. We also tested known CYP3A4-inhibitors and -inducers, but found no effect on clearance of midazolam or metabolites, which contradicts earlier findings. [28] This may have been caused by the low incidence of erythomycin and phenobarbital use in combination with high residual variability. Catecholamine infusion was significantly correlated with a 23% increase in CL_{HMG}, which might be the result of improved renal perfusion. Even with inclusion of covariates, inter-patient variability remains high,

reflecting the heterogeneous nature of the patient population. There was no discernible pattern between the primary indication for ECMO and individual parameter estimates.

Plasma concentrations were simulated for different dose regimens. Our target concentration was based on the average plasma concentration of midazolam reached in our dataset (400 ng/mL), which is similar to median concentrations reported for children under 12 months old.[40] However, it is difficult to assign a (minimal) effective concentration because of varying levels of concomitant medication with analgesic or sedative properties. Another confounding factor could be the reluctance of hospital staff to reduce infusion rates in patients with low COMFORT-scores, for fear of future undersedation. These problems notwithstanding, simulations show that a continuous infusion of 300 µg/kg/h for the first 6 h and 150 µg/kg/h thereafter leads to midazolam concentrations of around 400 ng/mL (Figure 5c). After one week, the increased clearance will cause plasma concentrations to drop below 400 ng/mL, requiring a higher infusion rate, as was visible in the original data set as well. The CL_{HMG} remains more or less constant, leading to increasing concentrations and a greater contribution to the overall effect: while the median contribution of 13.5% to the total effect seems modest, it increases from 12% after 1 day to 34% after 7 days, as becomes apparent in Figure 5c. In comparison, OHM's contribution to overall sedation never rises above 13% and reaches a plateau of 10% after 3 days. After the patient is disconnected from ECMO, there will most likely be a sedative aftereffect until HMG is effectively eliminated renally. This is similar to the enhanced sedation seen in adult critically-ill patients with impaired renal function.[12] HMG clearance in our patients was highly variable, but we could not attribute this to creatinine clearance due to the discrepancy between neonatal serum creatinine levels and renal function.[38] Apart from its contribution to sedation, high HMG levels might be a cause of adverse events in heavily sedated patients. More studies are needed to address this safety concern.

Conclusion

CL_{MDZ} and CL_{OHM} increase approximately 3-fold within the first 5 days of neonatal ECMO, up to 1.38 and 5.31 L/h/3kg, respectively. V_{MDZ} increases to a maximum of 14.6 L/3kg within a couple of hours. OHM provides a constant 10% of the total sedative effect, whereas HMG accumulates, providing a rising contribution from 12% to 34% of sedation over the course of 7 days. A continuous infusion of 300 µg/kg/h for 6 h, and 150 µg/kg/h thereafter, provides adequate plasma concentrations for sedation of ECMO patients (2.7-3.9 kg). After 5-7 days, the infusion rate will have to be increased substantially to compensate for the increased MDZ and OHM clearance, despite higher HMG levels. Doses have to be titrated carefully, based on validated sedation scores and observed side effects: large residual and inter-patient variability thwart accurate individual predictions.

Acknowledgements

The authors thank Saskia N. de Wildt for her critical evaluation of this manuscript.

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Chapter 10

Discussion

Introduction

Despite a renewed interest in pediatric pharmacology, physicians still have to dose many drugs during ECMO based on dose regimens from non-ECMO patients. A limited number of studies during ECMO indicated that, as a rule of thumb, the volume of distribution of the drugs is increased and that the elimination half-life is lengthened. For drugs that have not been studied during ECMO the question remains whether this rule-of-thumb provides safe and efficacious dose regimens in clinical practice.

In order to provide practical dose advice for some of the most commonly used drugs in the therapeutic arsenal, we studied the PK profile for three of them representing groups of commonly used drugs being antibiotics, vaso-active drugs and analgesics/sedatives: cefotaxime, sildenafil and midazolam. These analyses were based on datasets containing information on drug dosage, patient characteristics and plasma concentrations of drugs and their metabolites. As discussed in chapter 2, the design and practical execution of clinical-pharmacological studies in this population are challenging. We were able to overcome many of the obstacles with a sparse sampling study design, drug assay in micro-volumes of plasma and nonlinear mixed effects modelling (NONMEM).

Methodology

Research into the determinants of differing dose requirements between ECMO and non-ECMO patients has been scarce. Limiting factors may have been the small number of patients at individual medical centers, the absence of a research infrastructure, and the practical issues of sampling and drug assay in small amounts of biological fluid. Another potential cause is the assumption that clinical-pharmacological research in infants is by definition legally unapproved unless there is a direct benefit to the individual participant, because we are dealing with incapacitated patients who are unable to give informed consent. This past decade however, doctors and researchers have come to realize that not studying drugs means that each patient is by definition an *in vivo* experiment, subjected to treatment without proper scientific rationale or evidence. [1, 2] This could lead to inappropriate drugs being given, undertreatment leading to therapeutic failure or overtreatment leading to side effects or an increased mortality. Recently, this insight has also reached the Dutch political community via a report by the Doek-committee which advocates a less restrictive approach to pediatric studies, accompanied by a draft revision of the Medical Research (Human Subjects) Act.[3] In the studies presented in this thesis, we obtained blood samples during routine rounds, developed efficient drug assay methods and applied population-based PK modelling. Consequently, we were able

to develop dose regimens for three commonly used drugs representative of different classes of drugs, without apparent harm or burden to patients.

Drug assay can be difficult in pediatric studies due to constraints on the sample volume, but the liquid chromatography mass spectrometric (LC-MS) methods we designed were successful in getting results with just a little blood per individual (50-100 µL of plasma per multi-analyte assay). The sparse sampling design and NONMEM-analysis has contributed to a further reduction in samples per patient [4]: it allowed the assay of different drugs in a single sample, as was done for the antibiotics assay described in chapter 3. The simultaneous assay of metabolites with their parent compounds reduces volume requirements even further, which was put into practice in the drug assays of sildenafil with desmethylsildenafil (chapter 5) and midazolam and morphine with their respective (partially) active metabolites (chapter 6). Limits of detection and reproducibility were appropriate for all assays despite the low volumes and limited sample preparation (for antibiotics in particular). Although protein precipitation worked in the case of antibiotics, solid-phase or liquid-phase extraction is still preferred due to the cleanliness of the samples, leading to fewer problems with matrix effects and robustness.[5] The tandem mass spectrometric detection allows the assay of different metabolites at the same time as their parents, which makes it easy to quantify their contribution to overall drug exposure. Now that these assays have been designed, they can be used for a multitude of studies in children and neonates: their application is not limited to studies in just ECMO or intensive care patients.

In our study, routine sampling has lead to a large biobank of nearly 2000 plasma samples of ca. 90 individuals in which drugs and their metabolites can be quantified. Since the drug assays required only minimal amounts of plasma, there is enough remaining plasma to quantify different drugs. This has potential for future studies, even regarding drugs that are not used as often. The problem with routine data however is that it can contain an extra source of variability due to differing attitudes among nurses, ranging from 'patient first' to research first'. Nevertheless, the use of routine data in this study has led to an extensive dataset and useful PK models for a number of drugs. The sparse sampling method relies on a good distribution of measured concentrations across a dose interval. For this we depended on the practical routine at the intensive care: lab rounds were sometimes brought forward or pushed back depending on how busy the ward was at the time, which was appropriate as long as the exact time of sampling was recorded. In later stages of the study, nurses appeared to get used to the sample regimen and started to take the samples exactly at the time at which the patient data management system instructed them to. In our case this was not a problem since dose intervals were usually different from the lab round intervals, but researchers should be aware of this phenomenon when designing studies with routine sampling designs.

NONMEM is an essential tool to handle these sparse data and was successful in estimating average and individual PK parameters. Shrinkage of variability estimates was small in all models, and bootstraps showed good confidence intervals for all major PK parameters. The volume of distribution is difficult to estimate in sparse sampling designs, but for most intravenously administered drugs (midazolam, cefotaxime) this is not a problem. Collinearity was a challenge: covariates such as weight, age, time on ECMO, ECMO-flow, etc. are often correlated to each other and it can be difficult to unravel these patterns.[6] In ECMO patients however, weight is estimated beforehand and is considered constant over the short period in which ECMO is applied. This means that age/weight collinearity is rarely a problem when modelling time-dependent changes in PK parameters during ECMO.[7] The remaining time-varying covariates (such as time after cannulation and post-natal age) are very closely correlated because most newborns are put on ECMO within a short time-frame after birth. This implies that their effects are difficult to interpret and model separately, unless there is a wide range of ECMO-durations in the dataset. For sildenafil in particular, we also had data on the days after ECMO, which made it possible to separately describe age-related and ECMO-related increases in clearance. Ideally, the dataset should be expanded with data from non-ECMO patients to allow a description of the maturation of clearance. This way, the non-ECMO children provide additional power for the estimation of baseline PK parameters in ECMO patients as well, leaving more room to investigate ECMO-related effects.[8] Unfortunately, such datasets are hard to come by for most drugs. This will change as more and more studies are done in pediatric populations and hopefully, the sparse sampling, drug assay and NONMEM methodology described in this thesis can facilitate this development. Over the years, a solid infrastructure to perform these studies has been built based on the strong collaboration between the Intensive Care and Department of Hospital Pharmacy in our medical center.

Pharmacokinetics of individual drugs

Using the methods described above, we were successful in preparing PK models for some commonly used drugs. These models do not only allow simulations to find appropriate dose regimens, but may also provide some insight into possible mechanisms involved in PK changes during ECMO.

Cefotaxime

The PK model in chapter 7 showed that cefotaxime (CTX) clearance is similar to that in non-ECMO patients (0.36 L/h vs. 0.20-0.55 L/h), but the volume of distribution is increased by approximately 100% (1.82 L vs. 0.68-1.14 L). Despite the increased volume of distribution compared to non-ECMO neonates, the percentage of time above the minimal inhibi-

tion concentration ($t_{>MIC}$) was at an effective level for all patients. In this particular case, the increased volume of distribution therefore has little effect on the dose regimen; the standard dose regimen is appropriate for ECMO patients as well. The only covariates with a statistically significant correlation were body weight and time after decannulation (CTX clearance), and hemofiltration flow and time after decannulation (DACT clearance); these do not offer us predictive determinants or new clues into mechanisms of PK changes, especially considering the large unexplained inter-patient variability. In a few individuals for which samples were available pre- and post-ECMO, we could see a temporarily increased clearance leading to lower plasma concentrations. We were unable to model this increase with statistical significance, but it indicates that ECMO-therapy temporarily improves metabolism and excretion. The instantaneous improvement at the time of cannulation suggests that improved perfusion or absorption could be the underlying mechanisms. This is supported by the sudden clearance drop after decannulation, since this entails cessation of artificially improved organ perfusion and oxygenation. The volume of distribution did not change upon cannulation, but its estimation is notoriously difficult in a sparse sampling design with few samples in the distribution phase, especially when a steady-state has been reached because of multiple doses of cefotaxime prior to cannulation. The active metabolite desacetylcefotaxime (DACT) largely followed its parent compound. There were no signs of accumulation due to a reduced clearance in comparison with non-ECMO patients, possibly due to routine use of CVVH. DACT concentrations were similar to those in the reference group.[9, 10] In conclusion, the standardized dose regimens for cefotaxime appear suitable for ECMO patients as well, but this is based on the large therapeutic window of cefotaxime and not on the absence of ECMO-induced PK changes: the volume of distribution is increased while clearance remains unaffected.

Sildenafil

An effective concentration or exposure window has not been defined, but we can measure the combined area-under-the-curve of SIL and DMS as a measure of exposure, under the assumption that DMS is half as potent a vasodilator as SIL. In a set of 11 post-ECMO neonates (chapter 8.1), a dose of ca. 1.0 mg/kg q.i.d. was shown to lead to an exposure of sildenafil (SIL) and its active metabolite desmethylsildenafil (DMS) that is equivalent to 20 mg t.i.d. in adults, a dose often used to treat pulmonary hypertension in adults. The study described in chapter 8.2 combined dose and concentration data during ECMO with those after decannulation, which showed a temporarily increased clearance (+161%) and volume of distribution (+313%) for SIL during ECMO. DMS clearance was increased as well (+150%). Unfortunately, there are no specific non-ECMO reference data in this age range to compare post-ECMO PK parameters with; the only available PK data are of a mixed group of neonates (ECMO and non-ECMO) in which no PK differences were described between treatment groups.[11] The covariate screen-

ing showed an extra time-related increase in clearance on top of postnatal age (PNA), which suggests an improvement in metabolic (probably hepatic) function during ECMO. Another option might be increased drug loss due to adsorption, unless this process is maximized due to saturation of binding sites.[12] DMS follows the SIL pattern as well, with an increased clearance during ECMO, but without an additional PNA-related clearance increase. Simulations show that, as a result of the PK changes, the sildenafil dose has to be decreased after decannulation to prevent accumulation. A sildenafil dose of 3-5 mg/kg/24h is sufficient after decannulation, whereas 5-7 mg/kg/24h is required during ECMO to reach a similar exposure. In conclusion, sildenafil clearance and the volume of distribution are higher during ECMO and rise faster than one would expect based on PNA alone; after decannulation the PK parameters drop again and resume their normal PNA-related increase. In future, patients with congenital diaphragmatic hernia (CDH) may serve as a good model since in a number of European centers (CDH EURO-Consortium), therapy is fully standardized regarding drug therapy for pulmonary hypertension.[13]

Midazolam

The required doses of midazolam (MDZ) are much higher in ECMO patients when compared to non-ECMO patients: Mulla *et al.* showed that the MDZ plasma concentration in the first 24 hours after cannulation is lower than expected, based on PK parameters in non-ECMO neonates, indicating an altered distribution and/or sequestration of drugs in the circuit.[14] In the referred study after 48 hours, maintenance doses had to be reduced, which indicates a saturable process, supporting the hypothesis that MDZ is lost due to sequestration. This has been confirmed in *in vitro* studies that indicate a loss of 60-95% in the first hours after cannulation.[15, 16] Our model showed an increase in the distribution volume after cannulation, which confirms results from a previous PK study in 19 neonates on venoarterial ECMO.[17] (Table I). The baseline volume of distribution in both ECMO studies was similar to non-ECMO treated critically ill neonates (Table I). The difference in clearance can in part be

Table I. Comparison of midazolam PK parameters between critically ill neonates, 19 venoarterial ECMO patients and our set of 20 venoarterial ECMO patients[a]

	Critically ill neonates	ECMO (n=19)	ECMO (n=20)
	[17]	[15]	Chapter 9
Volume of distribution (L/kg)	1.0	0.8 to 4.1, $t_{½}$=3.6 h	1.4 to 4.9, $t_{½}$=1.9 h
Interpatient variability V (% CV)	65%	53%	87%
Clearance (mL/kg/min)	1.8-2.2	1.4	2.6 (day 1) 7.6 (day 5)
Interpatient variability clearance (% CV)	85%	73%	86%

[a] CV=coefficient of variation

explained by larger drug losses in our circuits, as was seen in *in vitro* studies (> 90% vs. 60-95%) [15, 18], but there is also large inter-patient variability in all studies. In our patients, MDZ clearance improves over time; we cannot deduce from these data whether this is caused by a maturation of MDZ clearance (hepatic, via CYP3A4 and CYP3A5), temporary support of hepatic blood flow, or an improvement in underlying illness. Alpha-hydroxymidazolam (OHM) is easily cleared and does not accumulate, but the clearance of hydroxymidazolam-glucuronide (HMG) seems to lag behind. This could lead to active concentrations after even a couple of days on ECMO, causing prolonged sedation. Both OHM and HMG clearance are influenced by concomitant use of inotropic drugs. Similar to cefotaxime and sildenafil PK models, there are few ECMO parameters, clinical chemistry results or patient characteristics that are correlated with inter-patient and intra-patient variability. Most variability remains unexplained, which underlines our limited understanding of physiology, organ function and their effects on pediatric PK during critical illness and ECMO. In conclusion, volume of distribution and clearance of MDZ are higher during ECMO, and absorptive drug loss could be a cause of higher dose requirements.

Determinants of ECMO-related pharmacokinetic changes

The data from CTX, SIL and MDZ show that the PK effects of ECMO vary greatly between drugs, and that the tested covariates explained only a minor fraction (if any) of inter-patient variability. By combining data presented in this thesis with published data on different drugs, determinants of PK changes might be identified and confirmed in specific experiments such as the *in vitro* experiment in chapter 6. Based on PK data in literature and of our own, PK differences between ECMO and non-ECMO patients might be correlated to the specifications of the ECMO circuit, patient parameters or drug characteristics.

ECMO circuit

Drug loss due to binding to polymers and other materials is a well-described process. [20, 21] The upper limit of adsorption is related to the area of the exposed surface, which could in theory cause differences between neonatal and pediatric circuits, but these were not statistically significant in our *in vitro* tests. In theory, adsorption would lead to an artificially increased volume of distribution upon cannulation, which we could see for instance in the PK of MDZ in chapter 9. Hollow-fiber membranes are less prone to drug loss than silicone membranes, which was confirmed in our *in vitro* experiment in chapter 6: this should lead to a smaller increase in volume of distribution compared to silicone rubber. This could be a result of the smaller contact area between blood and the oxygenator, or an inherently lower adsorption tendency of polypropylene compared to silicone rubber. The lower drug loss in hollow-fiber membranes is encouraging and

indicates that technological advances could improve drug therapy in the intensive care via a reduction of PK fluctuations at cannulation or decannulation. It also implies that PK data from patients treated with silicone rubber and hollow-fiber membranes are not interchangeable and therefore require separate PK studies and a separate set of dose regimens. Circuit material, size and priming fluid composition could affect the increase in volume of distribution and clearance upon cannulation; the ongoing loss of drugs in used circuits we saw in chapter 6 suggests a lasting decrease of biological availability. [12, 15, 22-24] Clearance can also be affected by the use of hemofiltration units (CVVH), which has become standard practice in a number of centers over the past decade to correct fluid imbalance and limit the negative consequences of 'cytokine storm'.[25-27] Unfortunately, the combination of CVVH and ECMO might lead to increased hemolysis.[28] The resulting increase in serum creatinine makes it difficult to reliably assess renal function and therefore to be used as a marker for renal drug filtration. Finally, the type of ECMO (venovenous or venoarterial) might affect pharmacokinetics due to differences in organ perfusion, but the ECMO-type has never been identified as a significant covariate in any of the models in chapters 7-9, nor in literature.

Patient

In healthy children, PK varies from one individual to the next, due to factors such as age, weight, body composition, genetic factors, etc. Maturation of metabolism can affect PK [29, 30], but it is unlikely that this maturational pattern is different between ECMO and non-ECMO patients. In fact, the sildenafil model shows that after decannulation, the normal PNA-related increase in clearance is resumed. The level of illness might be a determinant for impaired organ function and therefore clearance.[31-33] A temporary relief from these effects could be one of the reasons why clearance increases directly after cannulation. An increased clearance during ECMO might reflect an improved organ function on top of drug losses or CVVH-mediated clearance. This would also explain a correlation with the time after cannulation, since patients generally become less ill during ECMO; in fact, they can often be discharged from the Intensive Care after only two extra days of mechanical ventilation. The indication for ECMO treatment could be a marker, but we observed no significant differences in PK parameters patients with a congenital diaphragmatic hernia (CDH) and meconium aspiration syndrome (MAS) for CTX, SIL or MDZ. Even patients with birth asphyxia, who receive hypothermic treatment known to slow down hepatic function [34], do not clear hepatically eliminated drugs such as sildenafil at a lower rate (chapter 8.2). The distribution of disease types in our datasets might have obscured these relationships: most patients either suffer from CDH or MAS, with only a few patients treated for septic shock, congenital cardiac abnormalities or birth asphyxia. Variability in clearance within the groups was very large. An alternative to stratification by indication is a scoring system of organ function, which is part of scores used in predic-

tion of outcome in the critically ill, such as PELOD[35], SNAP [36] or PRISM. [37] Unfortunately, there is no such scoring system that has been validated for ECMO patients; both SNAP and PRISM are predictive tests for mortality validated for evaluation in the first 24 hours after admission. The potential individual components of such a score (such as liver function parameters, renal function, venovenous vs. venoarterial ECMO, etc.) were included in covariate analyses for CTX, SIL and MDZ, but rarely had a statistically significant covariate effect by themselves. The same goes for concomitant drug use that could act as a marker for severity of illness, such as vasopressor or antifungal medication.

Drug

In vitro studies have focussed on drug characteristics that influence drug loss, many of them have shown larger losses for lipophilic drugs (benzodiazepines, opioids) than hydrophilic drugs (antibiotics, epinephrine).[38] The log P value, a measure of the distribution of a drug over an organic and aqueous phase, might therefore be correlated with drug loss and ultimately the apparent volume of distribution. The *in vitro* study described in chapter 6 confirmed the assumption that lipophilicity is linked to drug loss, particularly in circuits with silicone membrane oxygenators. We have tried to mimic the clinical situation as much as possible by using a blood-primed circuit with injection of drugs at their clinical concentrations, whereas experiments based on water-filled or plasma-filled circuits might have been easier but less realistic. As a consequence, theoretical reference concentrations in whole blood had to be converted to plasma concentrations and calculated based on an estimated circuit volume. This brings additional sources of variability, but by using an experimentally verified correction ratio and the protocolized circuit volume we have attempted to reduce this to acceptable levels.

Strongly protein bound, hydrophilic drugs are probably more susceptible to ECMO-induced and disease-related fluid shifts, i.e. an increase in extracellular volume.[39] This could imply that clearance and volume of distribution of these drugs can change rapidly in the case of cannulation or (nosocomial) infection. The elimination pathway could also be a predictor of ECMO-related PK changes. For instance, high renal elimination via glomerular filtration could imply that hemofiltration increases clearance during ECMO, whereas hepatically eliminated drugs are much more dependent on maturation of metabolic enzymes or improved hepatic blood flow (depending on the extraction ratio of the individual drug). This could explain the different covariate models we saw between drugs and their more hydrophilic metabolites. Finally, the route and site of drug administration could influence drug behaviour. In venovenous ECMO for instance, recirculation or pooling could occur if susceptible drugs are injected into the circuit [40], which leads to an artificially increased volume of distribution. Injection directly into the patient however might lead to lower drug loss due to good peripheral distribution (for lipophilic drugs in particular) and therefore cause less problems.

Perspectives

The PK models we presented in chapter 7, 8 and 9 have led to new dose regimens; these should be tested to see whether they lead to improved pharmacotherapy and patient care, such as a lower incidence of withdrawal symptoms (midazolam) or improved pulmonary perfusion (sildenafil). Adequate dose regimens are still lacking for many regularly used drugs, such as bosentan, milrinone, clonidine, pentobarbital, thiopental, amoxicillin, acyclovir and phenytoin. By analyzing samples in our biobank, we might be able to get enough samples to model these drugs and their metabolites as well, so that proper dose regimens can be constructed. Perhaps the number of samples might be increased in future by using leftover material from routine clinical chemistry measurements, but the structural use of these samples requires informed consent and approval from medical-ethical boards. Using LC-MS, we would be able to put the small amounts to good use.

So far, the influence of pharmacogenetics has not been assessed, but genetic variation in the enzymes involved in metabolism and elimination might explain some of the variability we observed. DNA-sampling is difficult however: blood sampling should occur before cannulation since patients receive blood from donors to prime the circuit and correct hematocrit levels. On the other hand, in case of congenital anomalies, DNA is harvested routinely from blood samples and/or fibroblast cultures resulting in immortalized cell lines.

The power of PK studies in these patients could be enhanced by incorporating data from critically ill and relatively healthy non-ECMO patients, but only for drugs that are used in different patient categories. For drugs like midazolam (for which the influence of illness and maturation are being studied by combining different datasets in non-ECMO patients [41]), the ECMO data might be included in the model. To help identify factors that underlie PK changes, studies into fluid dynamics, organ perfusion, capillary function and microcirculation might be useful, but it is still a long way before we might use them in (mechanistic) population PK analyses.

Dosing drugs on ECMO can be a challenge, particularly for drugs of which no PK data is available. Via the methodology we used and described in this thesis, we could construct new dose regimens and confirm old ones. Because of the large variability in PK parameters, titration of drugs up to the desired effect is preferable to extrapolation from other populations. Therapeutic drug monitoring (TDM) could help concentrations reach a predefined therapeutic window by concentration guided dose adjustments.[42-44] However, when titration or TDM cannot be done and the physician or pharmacist has no idea how to dose this drug, one might prepare a dose regimen based on literature values in patients of an appropriate age and weight. A model for ECMO-induced PK changes that incorporates patient, ECMO and drug characteristics would therefore be a great asset, but we are a long way from preparing one. As an exploratory analysis, we did a literature

search for any study or case report that might contain PK data for ECMO patients. For every drug, a reference study was selected for a population of patients of the same postnatal age, weight and administration route. When more than one reference study was available, the study with most patients was selected. By comparing PK parameters in both groups (Table II), a percentual difference in volume of distribution and clearance was calculated. This shows that the differences in volume of distribution and clearance between ECMO and non-ECMO patients are highly variable. The volume of distribution is increased (median ΔV is +40%, range -9 to +400%, whereas clearance is decreased (median ΔCL is -36%, range -79 to +42%). On average, this leads to an increased elimination half-life (median $\Delta t_{½}$ is +168%, range +27 to +1048%). See Figures 2 and 3 for the increase and median reference (non-ECMO) value of volume of distribution and clearance for each drug. The studies with ECMO circuits containing hollow-fibre membranes do not show an increase in volume of distribution, but these were case reports for adult patients whose increase in circulatory volume is relatively small compared to neonates or infants. Table II also contains data on the extent of protein binding, the route of elimination and the log P value of the drug. There is no correlation between the change in clearance or volume of distribution and protein binding, the route of elimination or PK parameters in the reference patients. A potential correlation exists between log P values and the ΔV (Figure 1), for which there might be a theoretical basis: between log P values of -1 to 3, the increase of the volume of distribution is small. Drugs with a low log P value often have a small volume of distribution, since they prefer to remain in the aqueous plasma compartment. When the circuit is connected to the general circulation, this causes a relatively large increase in the volume of this compartment. This effect is not to be expected for lipophilic drugs which tend to distribute in peripheral tissues; their volumes of distribution are largely unaffected by the circulatory increase. They do however have a higher tendency to bind to membranes and tubing (chapter 6), which could cause their apparent volume of distribution to increase. This rise in Figure 3 is largely caused by midazolam; we need multiple data on drugs at the higher end of the log P scale to confirm this theory. Figure 2 shows that for some drugs, clearance appears increased during ECMO, but there is no prognostic factor that links these drugs and separates them from the rest. For most drugs, clearance is lower in ECMO patients compared to their non-ECMO counterparts. However, in the PK studies presented in this thesis CTX, SIL and MDZ clearance instantaneously increase within ECMO patients upon cannulation. Apparently, even before cannulation or after decannulation, ECMO patients are physiologically different from other critically ill patients. The choice of reference group is therefore very important to the validity of your prediction of PK during ECMO. This poses an additional problem to physicians when treating their patients with drugs for which PK studies have not been done yet.

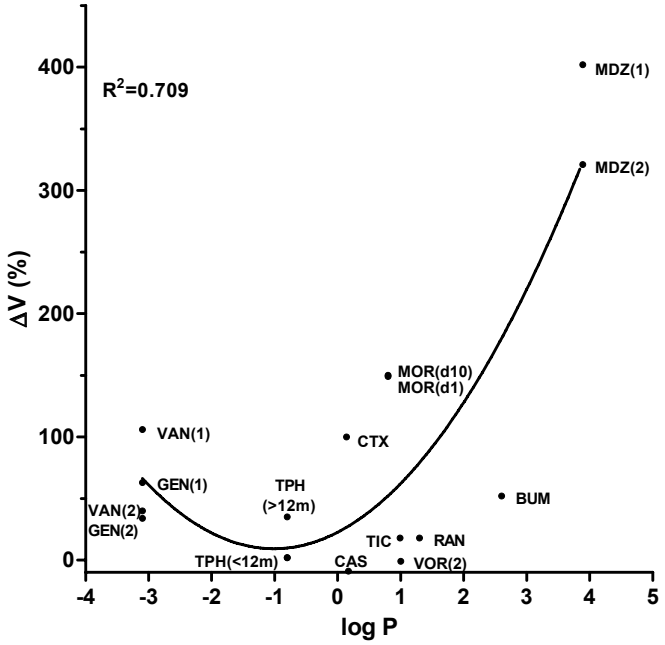

Figure 1. Correlation between log P and the increase in volume of distribution (ΔV) for the drugs from table II, with a parabolic nonlinear curve fit.

The research presented in this thesis has focussed entirely on PK so far, but it is possible that patients respond differently because of pharmacodynamic (PD) differences as well. This requires appropriate PD endpoints, many of which have not been developed or validated yet. However, studies into the efficacy of drug treatment have started; in combination with PK analyses, we can then identify drugs for which an unexpected or lack of response is not a result of inadequate exposure. The reader is referred to the thesis of E.D. Wildschut entitled 'Drug therapies in neonates and children during extracorporeal membrane oxygenation (ECMO): Keep Your Eyes Open' for an overview of studies into the effectiveness of treatment of infections with antibiotics, of anuresis with furosemide (in combination with CVVH) and movement or agitation with sedatives. Interestingly, the latter showed that sedative requirements are higher during ECMO despite effective concentrations; altered PD might be a crucial cause of this. Similarly, 24% of patients suffered from an ongoing sepsis despite antibiotic treatment. If this is valid for patients treated with cefotaxime (for which the level of exposure has been confirmed in chapter 7), it could imply a specific pattern of resistance with consequences for the choice of antibiotics. Despite this renewed effort, additional research into new PD endpoints and their practical measurement in the ICU would be welcome. The need for these studies is especially high for drugs that lack a properly defined exposure window such as sildenafil for the treatment of pulmonary hypertension.

Conclusions

The combination of routine sparse sampling, drug assay via LC-MS and a PK analysis using NONMEM allow the study of drug behaviour in neonates and children during ECMO without harm to the individual subject. In these vulnerable patients PK models and dose regimens were successfully constructed for cefotaxime, sildenafil and midazolam. *In vitro* experiments showed that drug loss in ECMO circuits is correlated to the lipophilicity of the individual drug, expressed as the log P value. Hollow-fiber membranes appear to cause less drug loss than those made of silicone rubber. Drug loss in clinically used circuits and pediatric circuits was no different from freshly prepared neonatal circuits. A drugs log P value is weakly correlated with the increase in volume of distribution. Our understanding of ECMO-induced PK-altering mechanisms is insufficient to prepare a predictive model. However, we were able to provide dosing recommendations for a number of drugs. In addition, the methodology presented in this thesis can help the design and execution of other studies in pediatric clinical pharmacology. The combination of a guaranteed infrastructure with solid cooperation between pediatricians, pharmacists and clinical pharmacologists, leads to more evidence-based dose regimens for pediatric patients, in particular the most vulnerable ones: the critically ill newborn and child.

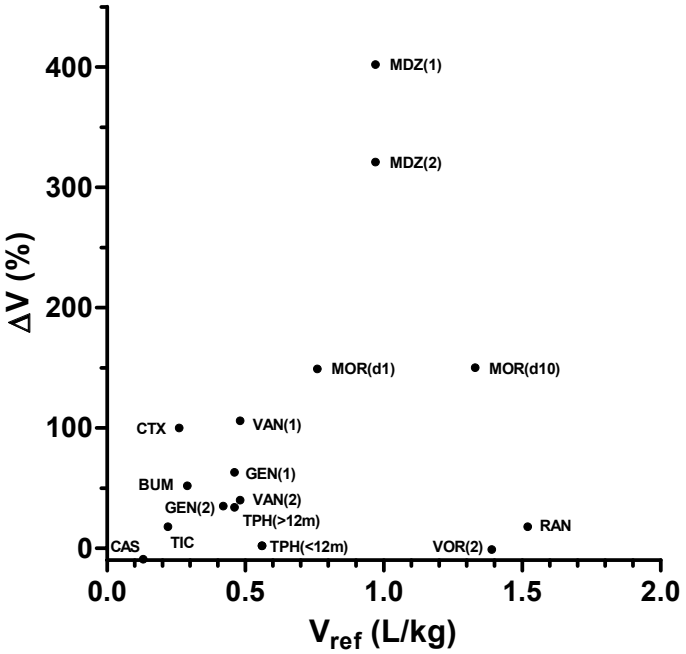

Figure 2. Increase in volume of distribution (ΔV) during ECMO vs. the median reference value in non-ECMO patients (V_{ref}).

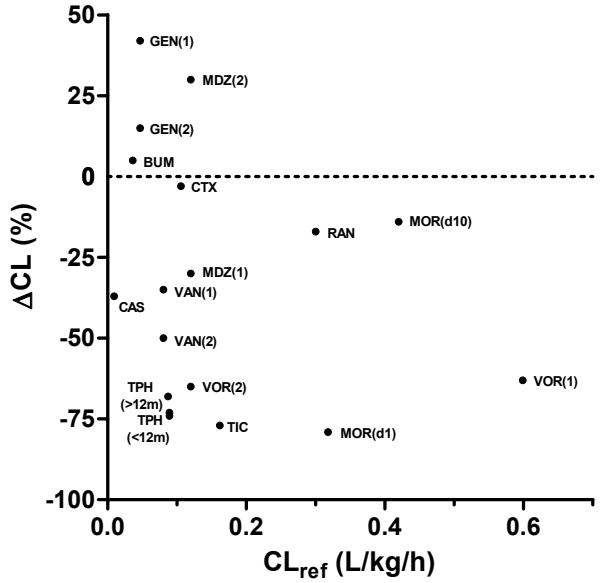

Figure 3. Clearance change (ΔCL) during ECMO vs. the median reference value in non-ECMO patients (CL_{ref}).

Table II. Comparison of PK data during ECMO with literature values in non-ECMO patients

Drug	Code	ECMO patients							Reference group		
		Ref	n	PNA[a,b]	WT[b,c] (kg)	Oxygenator	CL (L/kg/h)	V (L/kg)	Ref	n	PNA[a,b]
Bumetanide	BUM	[45]	11	1-7 d	3.8	Silicone membrane (Avecor 0800)	0.038	0.44	[46]	14	0-7 d
Caspofungin	CAS	[47]	2	17 & 41 y	-	Hollow-fiber PE (MEDOS Hilite 8000 LT)	0.0059	0.12	[48]	36	-
Cefotaxime	CTX	Ch 7	37	3.3 d	3.5	Silicone membrane (Medtronic)	0.10	0.52	[49, 50]	7, 15	<1 w, 3.5 d
Gentamicin	GEN (1)	[51]	6	-	3.6	-	0.066	0.75	[52]	58	0-10 d
	GEN (2)	[18, 53-55]	72	-	-	-	0.054	0.62	[52]	58	0-10 d
Midazolam	MDZ (1)	[17]	20	3.8 d	3.4	Silicone membrane (Avecor 0800)	0.084	4.1	[19]	187	0-10 d
	MDZ (2)	Ch 9	20	0.8 d	3	Silicone membrane (Medtronic)	0.16	4.9	[19]	187	0-10 d
Morphine	MOR (d 1)	[8] [56]	14	3 d	3.2	Silicone membrane (Medtronic)	0.066	1.9	[57]	184	1-580 d
	MOR (d 10)	[8] [56]	14	13 d	3.2	Silicone membrane (Medtronic)	0.36	3.3	[57]	184	1-580 d
Ranitidine	RAN	[58]	13	< 1 m	-	Silicone membrane (Avecor 0800)	0.25	1.8	[59]	27	0-1 d
Ribavirin	RIB	[60]	1	14 d	3.3	Silicone membrane (Avecor 0800)	0.046	1.4	-	-	-
Sildenafil	SIL	Ch 8	23	1 d - 4 y	3.3	Silicone membrane (Medtronic)	1.6[g]	19[g]	-	-	-

Reference group				Drug characteristics			ΔPK		Remarks
WT[b] (kg)	Description	CL (L/kg/h)	V (L/kg)	Main CL route[d]	log P[e]	PB[e] (%)	ΔCL[f] (%)	ΔV[f] (%)	
-	Neonates, GA 26-40 w	0.036	0.29	R	2.6	97	5	52	
-	Healthy volunteers	0.0094	0.13	O	0.17	97	-37	-9	
-	Neonates with suspected sepsis or confirmed gram-negative infections	0.11	0.26	R + H	0.14	40	-3	100	
3.1	Neonates with suspected sepsis	0.047	0.46	R	-3.1	15	42	63	
3.1	Neonates with suspected sepsis	0.047	0.46	R	-3.1	15	15	34	
0.7-5.2	Critically ill neonates	0.12	0.97	H	3.89	97	-30	321	
0.7-5.2	Critically ill neonates	0.12	0.97	H	3.89	97	30	402	
4.2	Postoperative neonates and children without renal or hepatic dysfunction	0.32	0.76	H	0.8	35	-79	149	
4.2	Postoperative neonates and children without renal or hepatic dysfunction	0.42	1.33	H	0.8	35	-14	150	
-	Neonates vomiting blood without renal or hepatic dysfunction	0.3	1.5	H	1.3	15	-17	18	
-	-	-	-	-	-	-	-	-	No reference group available with comparable age
-	-	-	-	-	-	-	-	-	No purely non-ECMO reference group available

Table 1 continued

Drug	Code	ECMO patients							Reference group		
		Ref	n	PNA[a,b]	WT[b,c] (kg)	Oxygenator	CL (L/kg/h)	V (L/kg)	Ref	n	PNA[a,b]
Theophylline	TPH (0-1 m)	[40]	38	8.4 d	3.3	Silicone membrane (Avecor 0800)	0.023	0.57	[61]	13	4-18 m
	TPH (1-12 m)	[40]	14	122 d	4.8	Silicone membrane (Avecor 0800)	0.024	0.57	[61]	13	4-18 m
	TPH (>12 m)	[40]	23	2236 d	25	Silicone membrane (Avecor 0800)	0.028	0.57	[62]	30	6-17 y
Ticarcillin	TIC	[63]	2	-	-	-	0.038	0.26	[64]	22	1-108 m
Vancomycin	VAN (1)	[65]	12	1.5 d	3.3	Silicone membrane (Avecor 0800)	0.052	0.99	[66]	17	4-8 w
	VAN (2)	[67]	15	8.2 d	3.5	-	0.040	0.67	[66]	17	4-8 w
Voriconazole	VOR (1)	[68]	1	5 y	-	-	0.22	-	[69]	82	2-12 y
	VOR (2)	[47]	2	17 & 41 y	-	Hollow-fiber PE (MEDOS Hilite 8000 LT)	0.042	1.4	[70]	-	-

[a] d, days; w, weeks; m, months; y, years; CL, clearance; GA, gestational age; PB, protein binding; PNA, postnatal age; V, volume of distribution; WT, weight. [b] Median values or range depending on data in original publication; [c] Estimated before ECMO; [d] R, renal; H, hepatic; O, other (hydrolysis), based on adult data [71]; [e] Based on adult data [71]; [f] Compared to reference values; [g] Values as V/F and CL/F in which F is the bioavailability; data are after gastric administration

Reference group				Drug characteristics			ΔPK		Remarks
WT[b] (kg)	Description	CL (L/kg/h)	V (L/kg)	Main CL route[d]	log P[e]	PB[e] (%)	ΔCL[f] (%)	ΔV[f] (%)	
3.8	Non-critically ill infants, 4-18 months	0.089	0.56	H	-0.8	40	-74	2	Studies in this age range are all in ex-premature patients
3.8	Non-critically ill infants, 4-18 months	0.089	0.56	H	-0.8	40	-73	2	
-	children with asthma	0.087	0.42	H	-0.8	40	-68	35	
-	Children with suspected infection	0.16	0.22	-	0.99	45	-77	18	
-	Small, critically ill children	0.080	0.48	R	-3.1	55	-35	106	
-	Small, critically ill children	0.080	0.48	R	-3.1	55	-50	40	
23	Pediatric aspergillosis	0.60	0.81	H	1	58	-63	-	
-	Healthy volunteers	0.12	1.4	H	1	58	-65	-1	

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Appendices

Summary

Critically-ill infants often require cardiopulmonary support in the form of vaso-active drugs or ventilation to maintain adequate oxygenation. When these no longer suffice, extracorporeal membrane oxygenation (ECMO) can be applied as a last resort. The accompanying medication (including antibiotics, sedatives, analgesics and vasodilators) is essential to successful completion of an ECMO-run and ICU stay. Pharmacokinetic data are often lacking for this particular population, which leads to dose regimens that are based on personal experience or extrapolation from studies in other patients. However, drugs behave differently during ECMO: a reduced clearance rate and higher volume of distribution could lead to treatment failure (undertreatment), or excess toxicity (overtreatment). Therefore, it is necessary to conduct observational studies in ECMO patients to estimate pharmacokinetic parameters and design appropriate dose regimens. This thesis aims to add to the understanding of determinants of drug behavior during ECMO, and to provide dose recommendations for commonly used drugs.

The introduction (Chapter 1) presents the rationale for pharmacokinetic studies in ECMO patients, with an overview of factors that might contribute to the differences seen between ECMO and non-ECMO patients. Legal, ethical and practical restrictions can complicate clinical-pharmacological studies in children. These are discussed to clarify the need for sparse sampling study designs and sensitive drug assays to reduce the sample burden for individual patients.

Part I addresses practical problems that are encountered in pediatric studies. Chapter 2 provides an overview of sampling methods, types of biological material that can be used to measure drug exposure, assay methods and regulatory obligations. This chapter also contains a checklist of items to be considered in the design of a new study. Sensitive drug assays are particularly important to minimize the volume of blood that has to be sampled to reliably measure drug concentrations. Ultra-performance liquid chromatography with mass spectrometry detection (UPLC-MS) is particularly suited for this. UPLC-MS assays for samples of 100 µL or less were developed for antibiotics (cefazoline, cefotaxime, ceftriaxone, amoxicillin, meropenem, vancomycin, Chapter 3), sildenafil and desmethylsildenafil (Chapter 4) and midazolam, morphine and their main metabolites (Chapter 5).

Drug adsorption by the circuit tubing or the membrane oxygenator contributes to pharmacokinetic changes. In an *in vitro* experiment (part II, chapter 6), we tested potential determinants of drug adsorption. The oxygenator size (pediatric vs. neonatal) or previous use of circuits have little influence on drug loss. Drug adsorption is correlated to the lipophilicity (log P value) of individual drugs. This effect is strongest for circuits

with a silicone membrane oxygenator; a sigmoidal function adequately describes the correlation between log P value and drug recovery. Drug loss is smaller in circuits with a centrifugal pump, probably due to shorter tubing length and the polypropylene hollow-fibre membrane, which is especially poignant for lipophilic drugs such as midazolam or fentanyl. These drug losses can partly explain an increase in volume of distribution that is commonly seen during ECMO. As a consequence, dose recommendations for lipophilic drugs based on studies with one type of oxygenator are probably not valid for another. In addition, drugs should preferably be injected into patients instead of the extracorporeal circuit. Due to its lower drug loss and faster equilibration, morphine is the preferred opioid over fentanyl.

Part III describes the results from studies in ECMO patients of whom a small volume of blood was sampled during routine care. Drug concentrations were measured via micro-assays and pharmacokinetic models constructed to produce dosing recommendations for commonly used drugs.

The nonlinear mixed-effects (NONMEM) model in chapter 7 describes the pharmacokinetics of cefotaxime and desacetylcefotaxime during ECMO. Despite a similar clearance and an enlarged volume of distribution (+100%) compared to non-ECMO neonates, standard dose regimens based on non-ECMO patients provide an exposure that is sufficient for antimicrobial efficacy. With the standard dose regimen of 50 mg/kg b.i.d. (postnatal age <1 wk), 50 mg/kg t.i.d. (PNA 1-4 wks) and 37.5 mg/kg q.i.d. (PNA >4 wks), the time above MIC ($t_{>MIC}$) is above 50% of a dose interval, which is considered to be the optimal $t_{>MIC}$ for antimicrobial efficacy, for all but one patient. Apparently, non-ECMO patients receive a dose that is higher than strictly necessary, which inadvertently leads to an appropriate dose for ECMO patients. Within a patient, cefotaxime clearance increases after cannulation and decreases after decannulation, which suggests an improved metabolism or renal function during extracorporeal support.

Sildenafil is widely used to treat pulmonary hypertension. The intravenous formulation is not available in our country, which is why capsules are produced extemporaneously. After administration via a nasogastric tube, sildenafil is readily absorbed; a dose of 4.2 mg/kg/24h leads to an exposure equivalent to that in adults with pulmonary hypertension treated with 20 mg t.i.d. (Chapter 8.1). During ECMO, sildenafil clearance and volume of distribution show an additive increase, with a return to pre-ECMO values after decannulation (Chapter 8.2). With a constant maintenance dose, this leads to an increase in exposure after decannulation. In most patients, a dose of 3-5 mg/kg/24h leads to an appropriate exposure post-ECMO, whereas 5-7 mg/kg/24h is required during ECMO.

However, large inter-patient variability necessitates individual dose titration based on efficacy and side effects.

Midazolam is widely used to sedate patients of all ages and sizes. Its principal metabolites alpha-hydroxymidazolam and hydroxymidazolamglucuronide also have a sedative effect, albeit less than the parent drug. Midazolam requirements are higher in ECMO patients. Chapter 9 describes a NONMEM-model that was created to investigate the effects of ECMO, size and maturation on midazolam pharmacokinetics. Following the start of ECMO, the volume of distribution for midazolam increases asymptotically from 4.3 to a maximum of 15 L/3kg with a half-life of 1.9 h. Median midazolam and alpha-hydroxymidazolam clearance increase 3-fold as a result of maturation or recovery from critical illness within the first 5 days (up to 1.4 and 5.3 L/h/3kg, respectively), whereas the glucuronide clearance remains constant at 0.18 L/h/3kg. Concomitant infusion of inotropic drugs increases glucuronide clearance by 23%, which suggests an increased excretion due to improved renal perfusion. In ECMO patients of 2.7-3.9 kg, a continuously infused midazolam dose of 300 µg/kg/h for 6 h, and 150 µg/kg/h thereafter, provides adequate plasma concentrations for sedation. The dose will have to be increased by 25% after 5-7 days. The glucuronide accumulates during ECMO, providing an increased proportion of the overall effect, up to 34% after 7 days.

Finally, we discuss in Chapter 10 what the main determinants of ECMO-related pharmacokinetic changes are and whether we can predict these changes when dosing a new drug.

In general, ECMO patients have a slower metabolism and excretion than non-ECMO patients, and a higher volume of distribution. This combination would require doses to be increased, and the dose interval lengthened. In contrast, within patients the period between cannulation and decannulation shows an increased clearance compared to pre- and post-ECMO, which suggests drug adsorption by the circuit and membrane oxygenator, CVVH-clearance or improved hepatic and renal perfusion. The poor predictability of these effects leads to the conclusion that individual drugs should be studied in ECMO patients to come to produce pharmacokinetic models and dose recommendations. The methodology we present in this thesis, which consists of sparse sampling during routine lab rounds, drug assay via UPLC-MS methods and pharmacokinetic modelling via NONMEM, is an effective tool to support such studies.

Samenvatting

Ernstig zieke kinderen die op de intensive care (IC) van een (academisch) kinderziekenhuis worden opgenomen, hebben vaak een slechte bloedsomloop, of kunnen zelfstandig onvoldoende ademhalen. Daarom worden geneesmiddelen toegediend om de bloeddruk en hartslag op peil te houden. Daarnaast wordt beademingsapparatuur gebruikt om hun longen goed te laten werken, en zo de organen en weefsels van voldoende zuurstof te voorzien. Soms is dit niet voldoende; patiënten kunnen ondanks deze behandelingen steeds zieker worden en uiteindelijk overlijden. Wanneer dit dreigt te gebeuren, is extracorporele membraan oxygenatie (ECMO) een laatste redmiddel. Deze hart-longmachine is in staat om tijdelijk (gedurende maximaal twee tot drie weken) de bloedsomloop en ademhaling over te nemen, zodat het lichaam de kans krijgt te herstellen. De behandeling vindt uitsluitend plaats in gespecialiseerde ziekenhuizen: in Nederland wordt dit gedaan in Nijmegen (UMCN St. Radboud, alleen voor pasgeborenen) en in Rotterdam (Erasmus MC Sophia Kinderziekenhuis, voor alle kinderen van 0 tot 18 jaar); dit betekent dat kinderen vaak met spoed moeten worden overgeplaatst. Patiënten krijgen na aankomst op de IC een of twee buisjes in bloedvaten in de nek geplaatst, waarmee de bloedstroom wordt omgeleid. Na via een pomp door een kunstlong en vaak een kunstnier te zijn gegaan, wordt het bloed opgewarmd en teruggegeven aan de patiënt. Dit is een ingrijpende gebeurtenis: er is een verhoogde kans op infecties, de onderliggende ziekte of stoornis moet goed worden behandeld en er bestaat het risico op losschieten van de buisjes, met een bloeding tot gevolg. Daarom krijgen ECMO-kinderen veel geneesmiddelen, waaronder antibiotica om het infectiegevaar te verminderen, vaatverwijders om de zuurstof-toevoer te verbeteren, en pijnstillers en slaapmiddelen om te voorkomen dat patiënten pijn hebben, onrustig worden of teveel bewegen.

Helaas is er weinig onderzoek gedaan naar de juiste dosering van geneesmiddelen tijdens ECMO. Op dit moment worden geneesmiddelen gedoseerd op basis van persoonlijke ervaring van de artsen, of op basis van onderzoeken in patiënten zonder ECMO. We weten echter dat geneesmiddelen zich anders gedragen tijdens ECMO: de afbraaksnelheid en de concentratie na inname van een enkele dosis blijken lager te zijn dan in niet-ECMO patiënten. Hierdoor bestaat er een kans op onderbehandeling (waarbij onvoldoende effect wordt bereikt) of overbehandeling (waarbij bijwerkingen ontstaan). We moeten dus onderzoeken doen in ECMO patiënten om de juiste dosis te vinden voor veelgebruikte geneesmiddelen. Het doel van dit proefschrift is:

- uit te zoeken welke factoren bepalen hoe een geneesmiddel zich tijdens ECMO gedraagt.
- doseringen te vinden voor enkele veelgebruikte geneesmiddelen.

Voor het opstellen van een doseeradvies, moet de farmacokinetiek van een geneesmiddel bekend zijn, oftewel 'wat doet het lichaam met het geneesmiddel'. Farmacokinetische kenmerken beschrijven hoe het geneesmiddel wordt opgenomen, hoe het zich verspreid in het lichaam en hoe het uiteindelijk wordt afgebroken en uitgescheiden. De belangrijkste maten zijn het verdelingsvolume en de klaring. Het verdelingsvolume is een maat voor hoe sterk het geneesmiddel zich verdeelt over verschillende weefsels en organen. De klaring is een maat voor de snelheid waarmee het lichaam een geneesmiddel afbreekt en uitscheidt. Voor farmacokinetisch onderzoek wordt op verschillende tijdstippen na inname van een geneesmiddel bloed afgenomen zodat de concentratie hierin kan worden bepaald en een concentratie-tijd curve kan worden getekend. Aan de hand hiervan kunnen verdelingsvolume en klaring worden berekend, en een doseeradvies worden gegeven. Het zou ook zo kunnen zijn dat een geneesmiddel bij dezelfde concentratie in het bloed een ander effect heeft in ECMO patiënten dan niet-ECMO patiënten. Dit zou het gevolg kunnen zijn van een andere farmacodynamiek: 'wat doet het geneesmiddel met het lichaam'. Dat hebben we echter niet onderzocht in dit proefschrift. Voorbeelden van onderzoeken hiernaar zijn te vinden in het proefschrift van E.D. Wildschut, getiteld 'Medicamenteuze therapie in neonaten en kinderen gedurende extracorporele membraan oxygenatie (ECMO): Houd uw ogen open'.

In de introductie (hoofdstuk 1) staat beschreven waarom farmacokinetische onderzoeken bij deze patiënten zijn gedaan, met vermelding van een aantal factoren waarvan we verwachten dat ze het verschil in farmacokinetiek tussen ECMO en niet-ECMO patiënten kunnen verklaren. Wettelijke, ethische en praktische problemen bij het doen van geneesmiddelonderzoek in kinderen worden besproken, om aan te geven dat studies bij kinderen en pasgeborenen alleen kunnen worden gedaan met zo min mogelijk bloedafnames en een zo klein mogelijke hoeveelheid bloed per afname.

In deel I is geprobeerd oplossingen te vinden voor praktische problemen die men tegenkomt bij het doen van farmacologisch onderzoek bij kinderen. In hoofdstuk 2 staat een overzicht van verschillende manieren om bloed, urine en andere lichaamsmaterialen af te nemen en hier concentraties van geneesmiddelen in te meten. Ook is een lijst gemaakt van de factoren waar men aan moet denken bij het opstellen van een nieuw onderzoeksplan. Om deze onderzoeken te kunnen doen zijn methoden nodig waarmee concentraties van geneesmiddelen en hun werkzame afbraakproducten in bloed kunnen worden gemeten. Een manier is ultrahoge druk vloeistofchromatografie-massa spectrometrie (UPLC-MS), een techniek waarmee in honderdsten tot tienden van milliliters betrouwbaar geneesmiddelconcentraties kunnen worden gemeten. Er zijn methoden opgezet voor de bepaling van verschillende veelgebruikte antibiotica (hoofdstuk 3), de vaatverwijder sildenafil en zijn werkzame afbraakproduct desmethyl-

sildenafil (hoofdstuk 4) en slaapmiddel midazolam met pijnstiller morfine en enkele van hun afbraakproducten (hoofdstuk 5).

Geneesmiddelen kunnen blijven plakken aan de kunststof onderdelen van de ECMO-buizen en de kunstlong. In een laboratoriumopstelling is uitgetest hoe groot het verlies is en door welke factoren dit wordt beïnvloed (deel II, hoofdstuk 6). De doseringen zijn van tevoren al verhoogd bij gebruik van een groter ECMO circuit. De grootte van het ECMO circuit en de kunstlong blijken geen invloed te hebben; een even groot percentage van de dosis blijkt te verdwijnen en verdere aanpassing van de dosering is dus niet nodig. Wanneer circuits eenmaal gebruikt zijn blijkt er evenveel geneesmiddel aan de wanden te plakken als bij de nieuwe circuits. De vetoplosbare geneesmiddelen plakken meer dan de wateroplosbare geneesmiddelen. Er is een verband tussen de log P waarde (een maat voor de vetoplosbaarheid van een molecuul) en de hoeveelheid geneesmiddel die we terugvinden in het bloed. We verliezen bovendien minder geneesmiddel in een circuit met een zogenaamde 'hollow-fiber' kunstlong (gemaakt van polypropyleen) dan bij een kunstlong van siliconenrubber. De circuits met een 'hollow-fiber' long hebben daarnaast een korter buizenstelsel en een ander type pomp, wat hieraan bij zou kunnen hebben gedragen. Tijdens ECMO is de benodigde dosis vooral hoger bij midazolam en fentanyl; dit komt overeen met onze resultaten: beiden zijn zeer vetoplosbaar en plakken dus sterk aan buizen en kunstlong. Doordat er verschil is tussen de verschillende ECMO circuits zijn doseeradviezen uit onderzoeken met één type circuit niet automatisch geldig voor patiënten die met het andere circuit worden behandeld. Daarnaast moeten geneesmiddelen worden geïnjecteerd in de patiënt in plaats van het circuit, om verlies van werkzaamheid zoveel mogelijk te verminderen. Wegens een lager verlies en het snel bereiken van een stabiele bloedconcentratie, heeft morfine als pijnstiller de voorkeur boven fentanyl.

Deel III beschrijft de resultaten van farmacokinetische studies in ECMO patiënten. Hiervoor is tijdens labrondes op de IC een klein beetje extra bloed afgenomen, waarin geneesmiddelconcentraties zijn gemeten. Met behulp van een computerprogramma genaamd NONMEM zijn de concentratie van alle patiënten samengebundeld en is berekend hoe groot het verdelingsvolume en de klaring waren in de gemiddelde patiënt. Daarnaast is geschat hoe groot de variatie tussen de patiënten was, en zijn verschillende doseringen uitgeprobeerd op het computermodel om te voorspellen welke dosering de meest geschikte bloedconcentraties op zou leveren.

Er is een NONMEM-model gemaakt voor het antibioticum cefotaxim (CTX) en het werkzame afbraakproduct deacetylcefotaxim (DACT) in hoofdstuk 7. De standaarddosering die ook bij niet-ECMO patiënten wordt gebruikt (twee maal daags 50 mg/kg bij een leeftijd <1 week, 50 mg/kg drie maal daags bij 1-4 weken en 37.5 mg/kg vier maal daags >4

weken), blijkt voldoende werkzame concentraties op te leveren tijdens ECMO. Doordat CTX een zeer veilig geneesmiddel is, wordt bij niet-ECMO patiënten aan de hoge kant gedoseerd. Zelf met de verhoging van het verdelingsvolume (+100%) wordt hierdoor tijdens ECMO een voldoende hoge concentratie gehaald. Binnen ECMO patiënten lijkt de klaring tijdens ECMO hoger te zijn dan ervoor en erna; dit zou kunnen komen doordat de organen door een betere doorbloeding tijdens ECMO beter werken, of doordat de kunstnir van het ECMO-circuit CTX uit de bloedsomloop verwijdert.

Ook voor sildenafil (SIL) en het werkzame afbraakproduct desmethylsildenafil (DMS) is een NONMEM-model gemaakt. Sildenafil wordt gebruikt om hoge bloeddruk te verminderen, door bloedvaten in de longen te verwijden. Een injectie was niet beschikbaar in ons land, daarom zijn uit tabletten, die door volwassenen worden gebruikt, capsules gemaakt in doseringen die geschikt zijn voor jonge ECMO patiënten. Wanneer deze worden gegeven via een maagsonde, blijkt 4.3 mg/kg per 24 uur een gelijke blootstelling te geven als in volwassen ECMO patiënten die met een standaarddosis (3 maal daags 20 mg) worden behandeld (hoofdstuk 8.1). We zien dat tijdens ECMO de klaring en het verdelingsvolume toenemen (hoofdstuk 8.2). Na het einde van ECMO gaan klaring en verdelingsvolume weer snel naar beneden. Dit heeft een groot effect op de dosis die nodig is om een goede blootstelling te halen: tijdens ECMO is 5-7 mg/kg per 24 uur nodig, daarna is dat slechts 3-5 mg/kg. We zien echter ook dat er tussen individuele patiënten grote verschillend zijn; het is dus verstandig om langzaam de dosis op te hogen totdat voldoende effect wordt behaald zonder teveel bijwerkingen.

Midazolam (MDZ) wordt gebruikt om patiënten rustig te houden en in slaap te brengen. De werkzame afbraakproducten alfa-hydroxymidazolam (OHM) en hydroxymidazolamglucuronide (HMG) zijn minder actief dan MDZ. We weten uit ervaring dat tijdens ECMO meer midazolam nodig is om hetzelfde effect te bereiken. Daarom is ook hiervan een NONMEM-model gemaakt (hoofdstuk 9). Na de start van ECMO blijkt het verdelingsvolume snel toe te nemen van 4.3 naar 15 L voor een patiënt van 3 kg. De klaring van MDZ en OHM neemt met 200% toe binnen de eerste 5 dagen. Wanneer bloeddrukondersteunende geneesmiddelen (bv. noradrenaline of dopamine) worden gegeven, gaat de klaring van HMG omhoog met 23%, wat een effect kan zijn van verbeterde doorbloeding van de nieren. In de gemiddelde ECMO patiënt zal een dosis van 300 µg/kg per uur een goede bloedspiegel geven. Na 6 uur moet deze verlaagd worden naar 150 µg/kg per uur. Na 5-7 dagen zal de dosis weer moeten worden opgehoogd om dezelfde concentratie te behouden. HMG zal stapelen in het lichaam; na 7 dagen wordt het verantwoordelijk voor 34% van het effect.

In de discussie (hoofdstuk 10) staat beschreven welke factoren de farmacokinetiek tijdens ECMO beïnvloeden en of we deze effecten kunnen voorspellen wanneer er nog geen informatie uit dit soort onderzoeken beschikbaar is. In het algemeen blijken ECMO patiënten een langzamere afbraak en uitscheiding te hebben dan niet-ECMO patiënten, en een hoger verdelingsvolume. Hierdoor zou de dosering moeten worden verhoogd en het interval tussen doseringen verlengd om een zelfde concentratie in het bloed te bereiken. Maar, binnen patiënten blijkt de klaring juist tijdelijk hoger te worden na de start van ECMO, wat een gevolg zou kunnen zijn van het plakken van geneesmiddelen aan het ECMO circuit, de kunstnier of verbeterde doorbloeding van organen. Doordat deze effecten slecht voorspelbaar zijn, is het nodig alle individuele geneesmiddelen te bestuderen tijdens ECMO, om zo modellen en doseeradviezen te kunnen ontwerpen. De technieken die in dit proefschrift worden beschreven (afname tijdens labrondes, gevoelige meting van concentraties in kleine hoeveelheden bloed en NONMEM-modellen) kunnen bijdragen aan deze onderzoeken, en zo de zoektocht naar geschikte doseringen vergemakkelijken.

Abbreviations

AAP	Acetaminophen (Eur. paracetamol)
AMX	Amoxicillin
ASD	Atrial septal defect
AUC	Area under the (concentration-time) curve
b.i.d.	Twice daily (L. bis in die)
BOS	Bosentan
BQL	Below the lower limit of quantification
CDH	Congenital diaphragmatic hernia
CFZ	Cefazolin
CL	Clearance
CPB	Cardiopulmonary bypass
CRO	Ceftriaxone
CTX	Cefotaxime
CV	Coefficient of variation
CVVH	Continuous venovenous hemofiltration
CWRES	Conditional weighted residual
CYP	Cytochrome-P-450 enzyme
DACT	Desacetylcefotaxime
DMS	Desmethylsildenafil
DV	Dependent variable
ECMO	Extracorporeal membrane oxygenation
EDTA	Ethylenediaminetetraacetic acid
ESI	Electro-spray ionization
F	Bio-availability
FA	Formic acid
F_c	Parent-to-metabolite conversion ratio
FDA	Food and drug administration
FLU	Fluconazole
FO	First-order approximation
FOCE	First-order conditional estimation
GA	Gestational age
GC(L)P	Good clinical (laboratory) practice
GC-MS	Gas chromatography-mass spectrometry

HMG	Hydroxymidazolamglucuronide
HPLC	High-performance liquid chromatography
ICU	Intensive care unit
iNO	Inhaled nitric oxide
INO	Inotropic medication (norepinephrine, dopamine, dobutamine, epinephrine)
IPRED	Individually predicted (Bayesian) concentration
IS	Internal standard
LC-MS	Liquid chromatography-mass spectrometry
(L/U) LOQ	(lower, upper) Limit of quantification
M3G	Morphine-3-glucuronide
M6G	Morphine-6-glucuronide
MAS	Meconium aspiration syndrome
MDZ	Midazolam
MDZd4	Deuterated midazolam (4 substituted protons)
ME	Matrix effects
MEM	Meropenem
MIC	Minimal inhibitory concentration
MOR	Morphine
MORd3	Deuterated morphine (3 substituted protons)
MRM	Multiple reaction monitoring
MS(/MS)	(Tandem) Mass spectrometry
MVOF	Minimum value of objective function
NONMEM	Nonlinear mixed effects modelling
OHM	Alpha-hydroxymidazolam
OXA	Oxacillin
PA	Pulmonary atresia
PD	Pharmacodynamics
PDE-5	Phosphodiesterase isoform 5
PE	Polyethylene
PE	Process efficiency
PELOD	Pediatric logistic organ dysfunction (score)
PH	Pulmonary hypertension
PK	Pharmacokinetics
PMA	Postmenstrual age

PNA	Postnatal age
PP	Polypropylene
PPHN	Persistent pulmonary hypertension of the newborn
PRED	Population predicted concentration
PRISM	Pediatric risk of mortality (score)
PVC	Polyvinylchloride
Q	Blood flow
Q	Intercompartmental clearance
QC	Quality control
q.i.d.	Four times daily (L. quater in die)
RE	Recovery
R_{in}	Rate of infusion
SIL	Sildenafil
SILd3	Deuterated sildenafil (3 substituted protons)
SNAP	Score for neonatal acute physiology
SOP	Standard operating procedure
SPE	Solid phase extraction
SRM	Selective reaction monitoring
$t_{>MIC}$	Percentage of time during which the concentration surpasses the MIC, calculated per 24 h
t_{DOSE}	Time after last dose
t_{EC}	Time after start of extracorporeal circulation
t_{END}	Time after decannulation (end of extracorporeal circulation)
TGA	Transposition of the great arteries
t.i.d.	Three times daily (L. ter in die)
UGT	Uridine 5′-diphospho-glucuronosyltransferase
UPLC	Ultra-performance liquid chromatography
V	Volume of distribution
VAN	Vancomycin
VSD	Ventricular septal defect
WT	Body weight
WRES	Weighted residual

Dankwoord

De ironie van een proefschrift zit hem in het feit dat de tekst die aan het eind en in de haast geschreven wordt, het meest gelezen wordt. Het venijn zit hem normaal gesproken in de staart, maar geheel tegen de aard van de apotheker in wordt ditmaal geen venijn uitgedeeld. Integendeel, dit proefschrift zou niet tot stand zijn gekomen zonder de steun en hulp van velen, welke ik bij deze graag bedank:

Dr. Mathôt, beste Ron, toen ik nog in de collegebanken concentratie-tijd curves moest ontleden, dacht ik altijd dat farmacokinetiek een abstracte bezigheid met weinig klinische relevantie was: een hobby voor vakidioten. Doordat jij me hebt geïntroduceerd in de wereld van de klinische farmacologie en populatiekinetiek heb ik dat beeld drastisch moeten bijstellen: dit is een van de weinige disciplines waarin apothekers met hun unieke combinatie van basale en klinische kennis daadwerkelijk farmaceutische patientenzorg leveren, in samenspraak met behandelaars en met wetenschappelijke onderbouwing. En hoe zeldzaam is het niet in de moderne wereld om als 'gezel' het vak van een 'meester' te mogen leren? Ik hoop in de toekomst nog vele artikelen van jouw hand (en die van mijn opvolger) te kunnen lezen. En vice versa natuurlijk! Bedankt voor alles.

Prof.dr. Tibboel, beste Dick, op zoek naar inspiratie, nieuwe ideeën en concrete oplossingen was een tochtje naar het eind van die gang op de 3e verdieping altijd een goed idee. Ik hoop dat ik ook ooit in staat zal zijn duizend-en-één ideeën te genereren, ze gestructureerd over te brengen en in concrete daden om te zetten. Bedankt voor de prettige samenwerking en het feit dat je mij als promotor wilde begeleiden.

Enno, dokter (en binnenkort doctor) Wildschut, ik denk met veel plezier terug aan onze avonturen. Van bloed aan het plafond bij de *in vitro* experimenten tot een tochtje richting Leuven, inclusief onbedoelde sightseeing tour van de binnenstad. Maar bovenal met veel uitwisseling van inzicht, kennis en ideeën. Ik vind dat het resultaat er mag zijn en afgaande op het voornemen van onze begeleiders om meer arts- en apotheker-promovendi te koppelen, denk ik dat ik daar niet alleen in sta. Veel succes in je verdere carrière en met je gezin, we gaan elkaar ongetwijfeld nog tegenkomen.

Geachte commissieleden, ik prijs me gelukkig dat u in mijn commissie plaats hebt willen nemen. Prof.dr. Vulto, beste Arnold, ik herinner me nog goed hoe we elkaar tegenkwamen in de fietsenstalling na mijn sollicitatie naar deze promotieplek in 2006. Ik kan me vergissen, maar als ik me goed herinner zei je 'Goed gedaan jochie, dit was een van de prettigste sollicitatiegesprekken die ik me kan herinneren. Maar ik kan me nog steeds niet voorstellen dat dit onderzoek succesvol zal verlopen'. Hopelijk heb ik je wat dit

laatste betreft op andere gedachten kunnen brengen; ik heb in ieder geval veel van je geleerd de afgelopen vijf jaar. Je enthousiasme werkt nog steeds aanstekelijk. Prof.dr. Allegaert en prof.dr. van den Anker, beste Karel en John, ik stel uw aanwezigheid in deze commissie zeer op prijs, vooral gezien de afstanden die hiervoor overbrugd dienden te worden. Dit geldt uiteraard ook voor de grote commissie: prof.dr. Danhof en prof.dr. Knibbe, bedankt voor de bereidheid als opponent op te treden. Dr. Saskia de Wildt en dr. Teun van Gelder, het was de afgelopen jaren een plezier met jullie samen te werken en ik ben dan ook verheugd dat jullie mij beiden zullen ondervragen tijdens de verdediging. Al kijk ik er natuurlijk nog meer naar uit jullie na afloop met een borrel aan te treffen.

Dit project zou direct zijn gestrand zonder de medewerking en hulp van alle (ECMO-) verpleegkundigen, ECMO coördinatoren, arts-assistenten en specialisten van de ICK. Jullie hulp bij inclusie en bloedafnames was onmisbaar. José Groenewold en Addie Koole, bedankt voor de bereidheid om een simpele apotheker de ins en outs van ECMO bij te brengen. Manon Hanekamp, bedankt dat we je dataset mochten lenen; het heeft geleid tot een mooie publicatie. Marjolein Augustus en Saskia de Reus, bedankt voor het beschikbaar stellen van jullie tijd en PDMS/Business Objects vaardigheden.

Wanneer je lief en leed met elkaar deelt wordt de groep hechter. Ik denk dat dit zeker van toepassing is op de 'jonge' apothekers en farmakundige waarmee ik de afgelopen jaren heb mogen optrekken. Bedankt voor alle gezellige borrels, etentjes, Swirls, weekendjes weg, koffiepauzes en therapeutische kamergesprekjes: Anna, Anouk, Asmar, Bart, Brenda, Bregje, Carolien, Delia, Ferdi, Jan-Dietert, Laureen, Liselotte, Lyonne, Maarten, Maren, Matthijs, Mila, Monique, Rachida, Reinier, Ryan, Satu, Savita, Tessa, Vincent en Yves. En natuurlijk alle andere apothekers in het Erasmus MC: ik kijk met plezier terug op onze samenwerking de afgelopen vijf jaar; succes met al jullie persoonlijke en professionele plannen, dichtbij of ver weg. Er zijn een paar mensen aan wie ik graag een paar extra woorden wijd:

Brenda, ik ken niemand die zo nonchalant kan overkomen terwijl ze de meest briljante modellen en algebraïsche vergelijkingen bedenkt, een benijdenswaardig proefschrift schrijft en 'drinking buddy' wordt van de grootheden in haar vakgebied. Ik hoop en verwacht dat we elkaar nog regelmatig zullen opzoeken, waar ook ter wereld.

Ferdi en Reinier, ik weet zeker dat jullie een hoop moois kunnen en zullen bereiken in de ziekenhuisfarmacie. Het was een eer kamergenoten met jullie te zijn geweest. Heleen, onder jouw hoede te mogen starten in zo'n grote organisatie raad ik iedereen aan; zonder de warmte en humor die jij brengt had ik waarschijnlijk voortijdig de handdoek in de ring gegooid. Bregje, veel succes in Amsterdam; ik koester die foto van jou in vol ornaat bij een van onze ECMO patiënten. Nauras en Jaap, bedankt voor jullie inzet tijdens jullie bijvak-projecten. Rachida, als jongste loot aan de boom is het aan jou om

de tradities van de onderzoekerskamer voort te zetten. Ongetwijfeld gaat dat lukken; ik kijk uit naar je proefschrift.

De afgelopen jaren heb ik met teveel mensen uit de apotheek samengewerkt om iedereen persoonlijk te noemen, maar bij deze wil ik jullie allemaal bedanken voor een prettige tijd. In het bijzonder natuurlijk mijn oud-collega's van het laboratorium, Medicator-team, logistiek, KDV, en in het Sophia: sorry dat ik jullie beperkte ruimte regelmatig misbruikte als parkeerhaven voor mijn step. Bart v.d. Nagel, het was een genoegen je als LC-MS sparringpartner en vraagbaak te hebben. En tot slot de dames van het secretariaat, met name Wassima en Martha: zonder jullie draait de machine een stuk minder soepel; jullie mogen van mij daarvoor wel eens expliciet worden genoemd.

Het voordeel van zo'n interdisciplinair project is dat je in contact komt met zoveel verschillende interessante onderzoeken, patientengroepen en bovenal personen. Ik doel natuurlijk op de 'motley crew' van de Farma-onderzoekers in het Sophia. Ik heb altijd met veel plezier met jullie gediscussieerd, gepresenteerd, geborreld, gegeten, bloed/urine/liquor vervoerd en 'een Doppio gedaan': Alexandra, Anniek, Bram, Erik, Gerbrich, Iba, Ilona, Ilse, Joke, Lieke en Nienke. En natuurlijk Geert, Heleen, Matthijs en Monique, aangevuld met Elke, Maurice en Rifka. Veel succes met de mooie projecten, ongetwijfeld gaan daar prachtige proefschriften, fellowships, grants en (wie weet) leerstoelen uit voortkomen.

Satu en Jolanda: niks 'zwakke geslacht', ik heb bij voorbaat al medelijden met welke opponent dan ook. Bedankt voor jullie hulp en steun de afgelopen jaren. Een promovendus kan zich geen betere paranimfen wensen.

Collega's in het Vlietland en Ruwaard van Putten Ziekenhuis, ik heb er vertrouwen in dat we zelfs in onze korte tijd samen een hoop kunnen bereiken. Bedankt voor jullie gastvrije onthaal.

Vrienden en familieleden, die paar pagina's zijn altijd te kort om iedereen met naam en toenaam te bedanken. Dit doet echter niets af aan mijn waardering voor de ondersteuning, motivatie en vooral afleiding die jullie hebben geboden. Toty, Maria, Thijs, Opa & Oma Booms, het is weer tijd om te proeven of de 'Absolut' nog niet over de datum is.

Wanneer je broer de kaft ontwerpt terwijl hij zich eigenlijk zou moeten voorbereiden op zijn reis naar Australië dan is dat een grote eer. Chris, ik hoop in de toekomst nog veel van je ontwerpen, televisieprogramma's en andere projecten te mogen genieten.

Jos en Henny, zonder jullie opvoeding en onvoorwaardelijke liefde en steun was ik nooit zover gekomen Daar ben ik nog iedere dag dankbaar voor. Misschien zeg ik dit niet vaak genoeg, maar jullie zijn een grote inspiratiebron; dit boekje is dan ook een klein beetje van jullie.

Querido Itamarzinho, este livro marca o fim de um período de estresse e finais de semana perdidos. Eu não teria feito isso sem contar com o seu apoio e compreensão. Agora é o momento de pegar a lista dos destinos de viagem novamente. Eu te amo e obrigado acima de tudo. Um abraço,

Maurice

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Ahsman MJ, Hanekamp M, Wildschut ED, Tibboel D, Mathot RA. Population pharmacokinetics of midazolam and metabolites during venoarterial extracorporeal membrane oxygenation in neonates. Clin Pharmacokinet, 2010. *In press*

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PhD Portfolio

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Courses		
Pharmacokinetic simulation in R, Jan Freijer, Amsterdam, the Netherlands	2009	8
Fisher/Shafer Course: PK and PD Analysis with NONMEM, San Francisco, USA	2008	28
Advanced Course in LC-MS, Avans Professional School, Breda, the Netherlands	2007	28
Erasmus Summer Programme: Introduction to Data-Analysis, Erasmus University, Rotterdam, the Netherlands	2007	20
Erasmus Summer Programme: Regression Analysis, Erasmus University, Rotterdam, the Netherlands	2007	40
Erasmus Winter Programme: Pediatric Drug Research, Erasmus University, Rotterdam, the Netherlands	2007	28
Seminars and workshops		
Pharmacological Research Meetings, Pediatric Intensive Care Erasmus MC Sophia Children's Hospital, Rotterdam, the Netherlands	2008-2010	50
Pharmacogenetics: from Epidemiology to Bedside, Erasmus MC, Rotterdam, the Netherlands	2009	3
Pain in Children, Erasmus MC Pain Knowledge Centre, Rotterdam, the Netherlands	2009	3
ESDP Education Day, Erasmus MC, Rotterdam, the Netherlands	2008	8
Tailor-made Drug Therapy for Children, Top Institute Pharma, Leiden, the Netherlands	2007	8
Pharmacogenetics in Practice, Erasmus MC, Rotterdam, the Netherlands	2007	4

	Year	Work-load (hours)
Conferences and presentations		
European Association for Clinical Pharmacology and Therapeutics, Edinburgh, Scotland (UK) – *Sildenafil exposure in neonates after extracorporeal membrane oxygenation for treatment of pulmonary hypertension (oral)*	2009	40
Population Approach Group Europe, St. Petersburg, Russia – *Population PK of Midazolam and Metabolites during Venoarterial ECMO in Neonates (poster)*	2009	40
Netherlands Association for Hospital Pharmacists, Leiden, the Netherlands – *Midazolam pharmacokinetics during Extracorporeal Membrane Oxygenation (poster)* – *Microanalysis of antibiotics in neonatal plasma using ultra-performance liquid chromatography-mass spectrometry (poster)*	2007-2008	20
11th Biannual European Society of Developmental, Perinatal and Pediatric Pharmacology, Rotterdam, the Netherlands – *Microanalysis of antibiotics in neonatal plasma using ultra-performance liquid chromatography-mass spectrometry (poster)*	2008	30
Dutch Society of Clinical Pharmacology and Biopharmacy, Lunteren, the Netherlands – *Microanalysis of antibiotics in neonatal plasma using ultra-performance liquid chromatography-mass spectrometry (poster)*	2008	10
Teaching activities		
Prescription and Pharmacotherapy (medical students)	2006-2009	120
Training in Computerized Prescription Order Entry (medical residents)	2006-2009	24
Supervising Master's theses in Pharmaceutical Science (J. Kerkvliet)	2008-2009	40
Supervising Master's theses in Pharmaceutical Science (N. Shuker)	2007-2008	40