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# Optimization of Colistin Dosage in the Treatment of Multiresistant Gram-negative Infections

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ACTA  
UNIVERSITATIS  
UPSALIENSIS  
UPPSALA  
2013

ISSN 1651-6206  
ISBN 978-91-554-8640-2  
urn:nbn:se:uu:diva-197724

Dissertation presented at Uppsala University to be publicly examined in Hörsalen, Klinisk mikrobiologi, Dag Hammarskjölds väg 17, Uppsala, Monday, May 20, 2013 at 17:52 for the degree of Doctor of Philosophy (Faculty of Medicine). The examination will be conducted in English.

### **Abstract**

Karvanen, M. 2013. Optimization of Colistin Dosage in the Treatment of Multiresistant Gram-negative Infections. Acta Universitatis Upsaliensis. *Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine* 889. 56 pp. Uppsala. ISBN 978-91-554-8640-2.

As multidrug resistance in Gram-negative bacilli increases, the old antibiotic colistin has rapidly gained attention as one of few last line treatment options in the form of colistin methanesulfonate (CMS), which is hydrolyzed to colistin both in vitro and in vivo. There is a dearth of knowledge on fundamental aspects of colistin, including pharmacokinetics and optimal dosing regimens. The aim of this thesis was to improve the basis for optimal colistin therapy.

To be able to study colistin, an LC-MS/MS assay method was developed which is sensitive, specific and useful in both in vivo and in vitro studies. Using this method we detected a significant loss of colistin during standard laboratory procedures. This loss was characterized and quantified, the hypothesis being that the loss is mainly caused by adsorption to labware.

The pharmacokinetics of colistin was studied in two populations of critically ill patients, one with normal renal function and one with renal replacement therapy. Plasma concentrations were assayed with the method above, and population modeling was employed to describe the data. The results include a previously unseen, long elimination half-life of colistin. The data from the population on renal replacement therapy was described without modeling, and showed that both CMS and colistin are cleared by hemodiafiltration.

Combination therapy is an approach that is often used when treating patients infected with multidrug-resistant pathogens. The thesis discusses how the joint effect of antibiotics can be measured using colistin and meropenem as a model, and proposes a method for testing antibiotic combinations. Furthermore, a PKPD model was adapted to describe the pharmacodynamics of the combination.

In conclusion, a specific and sensitive method for analysis of colistin was developed and the adsorption of colistin to materials was described. The assay method has been well accepted internationally. The pharmacokinetics of colistin and CMS was described in two important patient populations, partly with surprising results that have influenced dosages of colistin worldwide. The pharmacodynamics of combination therapy was investigated and quantified, and the methods applied could be further developed into clinically useful tools for selection of antibiotic combinations.

**Keywords:** Colistin, CMS, Pharmacology, Pharmacokinetics, PKPD, Antibiotics, Combination therapy, Pharmacometrics, Dosing regimens, Antibiotic resistance

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ISSN 1651-6206

ISBN 978-91-554-8640-2

urn:nbn:se:uu:diva-197724 (<http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-197724>)

*My family*



# List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

- I Jansson B, **Karvanen M**, Cars O, Plachouras D, Friberg LE. (2009) Quantitative analysis of colistin A and colistin B in plasma and culture medium using a simple precipitation step followed by LC/MS/MS. *J Pharm Biomed Anal.* 5;49(3):760-7.
- II **Karvanen M**, Malmberg C, Mohamed A, Lagerbäck P, Friberg LE, Cars O. (2013) Colistin is Extensively Lost during Normal Experimental Conditions. *Manuscript*.
- III Plachouras D, **Karvanen M**, Friberg LE, Papadomichelakis E, Antoniadou A, Tsangaris I, Karaikos I, Poulakou G, Kontopidou F, Armaganidis A, Cars O, Giamarellou H. (2009) Population pharmacokinetic analysis of colistin methanesulfonate and colistin after intravenous administration in critically ill patients with infections caused by gram-negative bacteria. *Antimicrob Agents Chemother.* 53(8):3430-6.
- IV **Karvanen M**, Plachouras D, Friberg LE, Paramythiotou E, Papadomichelakis E, Karaikos I, Tsangaris I, Armaganidis A, Cars O, Giamarellou H. (2013) Colistin methanesulfonate and colistin pharmacokinetics in critically ill patients receiving continuous venovenous hemodiafiltration. *Antimicrob Agents Chemother.* 57(1):668-71.
- V **Karvanen M**, Odenholt I, Friberg LE, Cars O. (2013) Describing and Measuring Effects of Colistin and Meropenem Combination Treatment. *Manuscript*.
- VI Mohamed A, **Karvanen M**, Cars O, Friberg LE. (2013) Interaction of Colistin and Meropenem on a Wild-type and a Resistant strain of *Pseudomonas aeruginosa* In-vitro as Quantified in a Mechanism-based Model. *Manuscript*.

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# Contents

Introduction.....	11
Colistin.....	12
Chemistry .....	12
Toxicity .....	13
Mechanism of action .....	14
Susceptibility and resistance .....	15
Clinical use and dosage regimens .....	16
Assay methods.....	17
Pharmacokinetics (PK) .....	19
Population Modeling.....	20
Protein binding .....	21
Pharmacodynamics, PD .....	22
Minimal inhibitory concentration (MIC) .....	22
Time-kill experiments .....	23
PD endpoints .....	23
Integration of pharmacokinetics and pharmacodynamics, PK/PD .....	24
PK/PD indices .....	24
$fAUC/MIC$ .....	24
$fC_{max}/MIC$ .....	24
$fT_{>MIC}$ .....	25
Modeling of PKPD.....	25
Combination therapies .....	26
Synergy .....	26
Methods for determination of joint effects .....	27
Aims.....	29
Paper I.....	30
Materials and Methods .....	30
Results .....	31
Conclusions .....	31

Paper II.....	32
Materials and methods .....	32
Results .....	32
Conclusions .....	33
Paper III .....	34
Materials and methods .....	34
Results .....	34
Conclusions .....	36
Paper IV .....	37
Methods .....	37
Results .....	37
Conclusions .....	38
Paper V .....	39
Materials and methods .....	39
Results .....	39
Conclusions .....	40
Paper VI .....	41
Materials and methods .....	41
Results .....	41
Conclusions .....	42
Conclusions.....	43
General discussion and future perspectives .....	44
Colistin and analysis methods .....	44
Dosing regimens.....	44
Combination therapies.....	45
Sammanfattning på svenska.....	47
Acknowledgments.....	49
References .....	51



# Abbreviations

GNB	Gram-negative bacilli
MDR	Multidrug resistant
XDR	Extensively drug resistant
PDR	Pan-drug resistant
CMS	Colistimethate
IU	International Unit
CBA	Colistin Base Activity
LC-MS/MS	Liquid chromatography-Tandem mass spectrometry
AUC	Area under the curve
MIC	Minimal inhibitory concentration
AUBC	Area under the bactericidal curve
PK	Pharmacokinetics
PD	Pharmacodynamics
PK/PD	Pharmacokinetics/harmacodynamics
$T_{>MIC}$	Time above MIC
AUC/MIC	AUC divided by MIC
$C_{max}/MIC$	$C_{max}$ (peak concentration) divided by MIC



# Introduction

Antibiotic resistance is an increasing threat to patients and healthcare throughout the whole world. Much of the medical progress achieved during the last century is reliant on access to effective antibiotic treatment and without antibiotics, many medical procedures, such as transplantations, major surgery and the care of prematurely born babies are at stake.

In the last two decades, resistance development has increased dramatically. As some bacterial strains have acquired resistance to several different classes of antibiotics, there has been a need to classify different degrees of resistance. Multi-drug resistant (MDR) bacteria are defined as resistance to at least three classes of antibiotics. The development of resistance has necessitated further classification into extensively drug resistant (XDR) bacteria and pan-drug resistant bacteria (PDR) (1). When such bacteria have emerged physicians are forced to turn to old drugs, shelved decades ago, such as the polymyxins, or they are faced with bacteria resistant to all available drugs.

The most serious situation currently is with the Gram-negative bacteria (GNB). The most common such species are *Escherichia coli* and *Klebsiella pneumoniae*, although *Pseudomonas aeruginosa* and *Acinetobacter baumannii* are notorious for being difficult to treat. They are mainly opportunistic pathogens, but their great capacity to adapt to different environments has caused them to be true problem organisms. Apart from causing infections in cystic fibrosis patients, these bacteria mainly affect critically ill patients; they are causative agents in ventilator associated pneumonias, trauma patients and patients with severe burns. Some strains circulate within intensive care unit (ICU) wards, and as the use of antibiotics in ICUs is abundant these circulating strains develop resistance towards many different antibiotics (2).

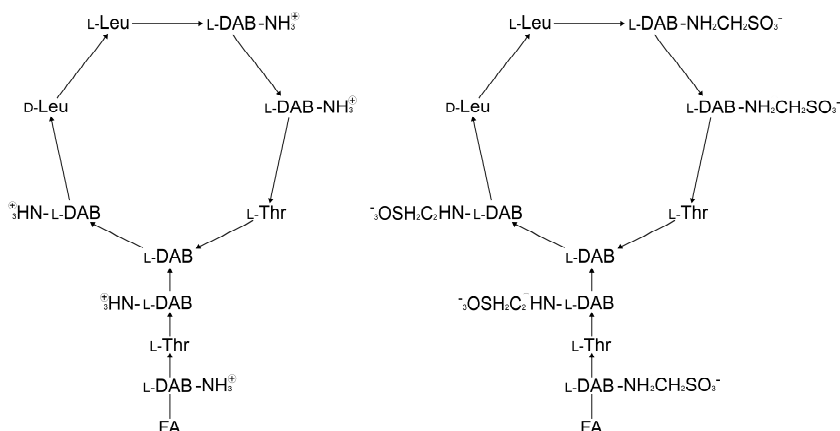
As MDR Gram-negative bacteria become more common, there is an increasing need for new antibiotics. These new drugs need to be able to penetrate the bacterial membranes and have novel targets or mechanisms of action to overcome known resistance mechanisms. This poses a serious problem for drug discovery, and the consequence is that, according to a recent analysis of the antibiotic pipeline, only two new drugs are currently in clinical development (3). This entails that, for the foreseeable future, healthcare will rely on the drugs that are currently available and that physicians will continue to turn to old, abandoned drugs such as colistin when treating potentially life-threatening infections.

# Colistin

Colistin belongs to a class of naturally occurring surface active antibiotics, the polymyxins, produced by *Bacillus polymyxa*. They were first discovered in 1947, whereas colistin was discovered in 1949 (4). The main groups in clinical use are polymyxin B and polymyxin E (colistin).

## Chemistry

The polymyxins are cyclic decapeptides with a fatty acid tail (*Figure 1*). Five amino acid residues present free amine groups, which are cationic in physiological solutions. The polymyxins have differences in amino acid composition and the fatty acid tail. Each subgroup of polymyxins contains of several molecules with small differences, e.g. polymyxin E1 and E2, also called colistin A and B, respectively (5). The peptide parts are hydrophilic, whereas the fatty acid tails are hydrophobic, making the molecules amphipathic.



*Figure 1.* Structure of colistin (left) and CMS (right). DAB=diaminobutyric acid, FA=fatty acid.

More than 30 different variants of colistin have been identified, but the main components of commercial preparations are colistin A and B, (6). Colistin powder preparations are a sulphuric acid precipitate, colistin sulphate.

Colistin was early associated with toxic side effects, mainly nephro- and neurotoxicity, so efforts were made to modify the molecule to reduce toxicity. In 1959, colistin methane sulphonate (CMS or colistimethate) was presented as a less toxic derivative. CMS was produced by allowing colistin to react with formaldehyde and sodium bisulphate. The reaction adds a  $-\text{CH}_2\text{SO}_3^-$  -group to all free amines, making the molecule anionic (7). CMS was considered to be approximately four times less toxic than colistin, albeit with a cost of reduced efficacy. Thus, CMS rapidly became the preferred drug for intravenous use. However, the problem of toxicity still remained, and when new antibiotics that were considered less toxic were released (e.g. the aminoglycosides) colistin/CMS was shelved (8, 9). In Sweden, CMS was withdrawn from the market in 1977.

With the uprise of MDR Gram-negative bacteria and use of colistin/CMS, research efforts have been directed to learn more about this drug. One of the most significant findings of modern colistin research is that CMS is, in fact, an inactive prodrug of colistin (10). When dissolved in aqueous media, CMS is spontaneously hydrolyzed by removal of the  $-\text{CH}_2\text{SO}_3^-$  -groups from the amines, rendering at first a mixture of partially sulphomethylated derivatives of CMS, as well as free colistin base. The inactivity of CMS has been elegantly shown by Bergen et al (10), by comparing antibacterial killing kinetics of CMS and colistin and relating the data to concentrations of colistin and CMS. Killing by CMS was delayed by approximately 0.5 hours compared to colistin, and the commencement of killing was spatially connected to the formation of free colistin base in the culture medium. This conclusion is also plausible with regard to the mechanism of action of colistin, which is discussed below.

## Toxicity

Nephrotoxicity of colistin is found at rates of approximately 14-24% in recent studies, which is lower compared to 20-50% in older studies (11, 12). This difference can be explained by more pure drug formulations as well as better general care and monitoring. Nephrotoxicity is mostly manifested as elevated serum creatinin values, indicating acute tubular necrosis, within four days after treatment is started. After discontinuation of colistin therapy, serum creatinin values stay elevated for up to 2 weeks, and return to normal within 3-9 weeks (11).

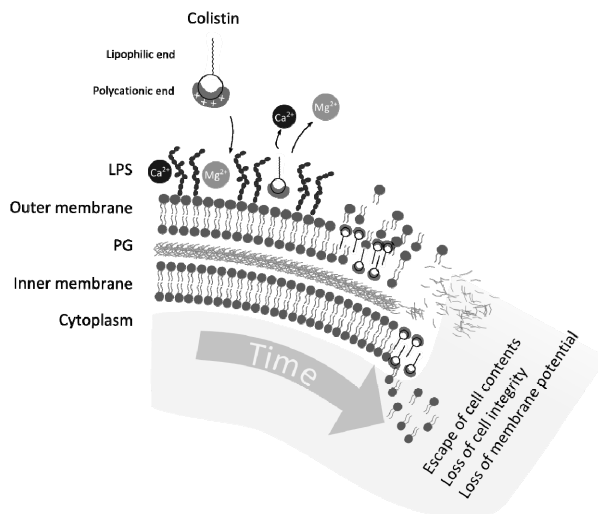
The risk of developing renal impairment is elevated in patients with known kidney diseases, and when patients are treated with other nephrotoxic drugs. As most patients receiving colistin are critically ill and receive many

concomitant medications, the role of colistin in development of renal impairment is difficult to determine.

In newer assessments of the toxicity of colistin/CMS, neurotoxicity has been found in 7% of the patients and the adverse effects are mostly mild and reversible (11). The most common adverse effect is paresthesias (numbness), but isolated cases of neuromuscular blockade (paralysis), apnea (stopped breathing), and rhabdomyolysis (breakdown of skeletal muscle tissue) have been reported. Local side effects include vascular irritation at the site of infusion, and inhalation therapy can cause "chest tightness", bronchial hyper-responsiveness and bronchospasm (11).

## Mechanism of action

Colistin acts through a two-stage detergent-like mechanism (*Figure 2*). The first stage targets the lipopolysaccharide (LPS) layer in the bacterium's outer membrane. LPS consists of a lipid moiety and a polysaccharide moiety, where the lipid part is anchored to the lipid bilayer, and the long, negatively charged chains are facing outwards tightly packed and stabilized by divalent cations, in particular  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ . Colistin, which has five positively charged amines can competitively replace  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  in LPS and the bulky ring structure disrupts the compact LPS structure. This diminishes membrane integrity, allowing colistin and potentially other substances into the periplasmic space (13).



*Figure 2.* Mechanism of action of colistin

In the second stage, colistin attacks the plasma membrane and increases the permeability of polar and charged molecules. This is achieved by the large, positively charged peptide part interacting with negatively charged membrane phospholipids and the lipid moiety being incorporated into the membrane. The large positive charge reduces the effect of stabilizing positive ions on the negative surface of the membrane, and the insertion of the cone shaped colistin in the membrane results in that the structure of the plasma membrane layer is compromised. Disruption of the plasma membrane leads to increased permeability of ions and polar compounds, causing failure in cell respiration and membrane integrity ultimately leading to cell death and lysis (13).

CMS, being anionic, would be less prone to interact with negatively charged membrane structures, but would rather be expected to be repelled. It is therefore no surprise that CMS is inactive, as shown by Bergen et al (10).

## Susceptibility and resistance

Most species of Gram-negative bacilli are susceptible to colistin, namely *Pseudomonas aeruginosa*, *Acinetobacter* spp, *Escherichia coli*, *Klebsiella* spp, *Enterobacter* spp, *Salmonella* spp, *Shigella* sp, *Haemophilus influenzae*, *Bordetella pertussis* and *Legionella* spp. *Stenotrophomonas maltophilia* is less susceptible. Gram-negative genera that are non-susceptible to colistin include *Proteus*, *Morganella*, *Serratia* och *Providencia*. Gram-positive bacteria, Gram-negative cocci and anaerobic bacteria are also non-susceptible. The European Committee on Antimicrobial Susceptibility Testing (EUCAST) has defined clinical MIC breakpoints for colistin; in the case of *Pseudomonas*, MIC breakpoints are  $S \leq 2$  mg/L and  $R > 4$  mg/L, whereas the breakpoints for other species are  $S \leq 2$  mg/L and  $R > 2$  mg/L (14).

The development of resistance to colistin has been low, the most probable cause being that the drug has not been used extensively. Unfortunately, the increased use of colistin since the 1990s has been followed by several reports of emergence of resistance. Colistin resistant strains have been detected in many species, including *P. aeruginosa*, *A. baumannii*, *E. coli* and *K. pneumoniae*.

The first descriptions of resistance mechanisms against polymyxins included two types of changes in the LPS. Firstly, there were changes in the lipid A –section, in which the charged phosphate groups were replaced with uncharged groups (15, 16). The change leads to weaker binding of polymyxin to lipid A. The second LPS-change occurs in the outer core sugar structure, reducing the negative charge of LPS (17), and thus the need for cations as stabilizers. The system can be induced by low concentrations of  $Ca^{2+}$  and  $Mg^{2+}$  and low levels of polymyxin. More recently, the changes have been connected to the *pmrAB*-pathway(18, 19).

Another proposed resistance mechanism involves oprH, an outer membrane protein in *P. aeruginosa*. Increased expression of the protein has been associated with resistance to the aminoglycosides and polymyxins (20, 21). The proposed mechanism of resistance is that oprH, which is a basic polycation, replaces the membrane  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  as a stabilizer. Since oprH is firmly anchored in the membrane, the polymyxins are incapable to sufficiently destabilize LPS.

A phenomenon that has gained much attention is the existence of colistin resistance mainly found in *A. baumannii*, often referred to as heteroresistance. The hypothesis is that the strains in question either contain pre-existing subpopulations with resistance to colistin, or that these strains are highly adaptable and can develop resistant phenotypes rapidly when confronted with colistin. Regardless of the mechanism, is true, the problem is that conventional MIC testing will recognize these strains as susceptible. However, when antibiotic therapy kills the susceptible population, the “resistant” subpopulations can grow and cause treatment failure.

## Clinical use and dosage regimens

Currently, intravenous colistin is mainly used as salvage therapy against MDR *Pseudomonas* and *Acinetobacter*, although ESBL-carrying *E. coli* and *Klebsiellae* are also becoming common targets. Colistin can be administered intravenously or intramuscularly or be nebulized for inhalation when the infection is limited to the lungs. Less common routes of administration are intrathecal or intraventricular injections in central nervous infections, oral syrup for bowel decontamination and ointments for topical use in e.g. external otitis and burns. In cystic fibrosis, colistin is used nebulized against *P. aeruginosa* colonization (9). In clinical use, “colistin” is often used interchangeably for both CMS and colistin base, and the terminology is often confusing. The parenteral formulations of colistin, e.g. Colimycin®, Promixin® and Tadim®, which are used both intravenously and nebulized for inhalation, contain CMS, whereas the topical ointments and syrup (not used in Sweden) contain colistin base.

There has been a lack of dosage guidelines, by and large pertaining to the deficiency of pharmacokinetic and pharmacodynamic data as well as confusions regarding the amount of CMS in different preparations (Table 1.)(22). Globally, no manufacturer of colistin/CMS express vial contents as “n mg CMS”, but rather use units related to potency. In Europe, vial contents are expressed in International Units (IU, or U), whereas Northern American and Australian products are labeled in Colistin Base Activity (CBA). The International Unit is defined as the amount of the CMS preparation that has the same activity as 0.00007874 mg of the international reference preparation (23), and vials generally contain 1 million IU. The CBA is also a compara-



tive unit. A vial containing e.g. 150 mg CBA contains the amount of CMS powder required to reach the same activity as 150 mg of colistin “base”. Conversions between units is that 1 million IU equals approximately 80 mg CMS, and 150 mg CBA equals approximately 400 mg CMS (9, 22). 150 mg CBA is thus approximately 5 million IU, and thereby 1 million IU is approximately 30 mg CBA. Also worth noting is that 1 IU CMS does not equal 1 IU colistin base in weight, they merely have the same activity.

Table 1. *Amount of CMS in the European and North American preparations.*

Trade name	IU	mg CBA	mg CMS
Tadim®	1000000	≈30	≈80
Coly-Mycin M ®	≈5000000	150	≈400

Current dosage recommendations from European manufacturers are 1-2 million IU q8h for patients weighing more than 60 kg, and 50000 IU/kg as daily doses, divided in three equal doses q8h, for patients weighing less than 60 kg. However, clinical experience from e.g. Greece had locally caused a *de facto* elevation of dosages to 3 million IU q8h on a purely empiric basis during the last decades (D. Plachouras, personal communication).

## Assay methods

Over the years several assays for colistin have been developed, from industrial scale HPLC and TLC methods to more simple microbiological assays (24-27). For an assay to be useful in clinical pharmacokinetics, it needs to:

1. be specific for colistin, i.e. be able to discern between the analyte and other substances present (including prodrugs or metabolites),
2. be sensitive, i.e. be able to quantify low concentrations in a sample and
3. have work up and run conditions that do not affect the analyte.

Colistin assays entail problems in all three areas. First, it is administered to patients as a prodrug (CMS), which hydrolyzes spontaneously in aqueous solutions. This hydrolysis is temperature and pH dependent (28-30). Second, colistin has low native fluorescence and absorbance, so it needs to be derivatized in order to use fluorescence or absorbance for low level detection (28). Third, colistin binds easily to labware, both plastics and glass, causing loss of the analyte (paper II).

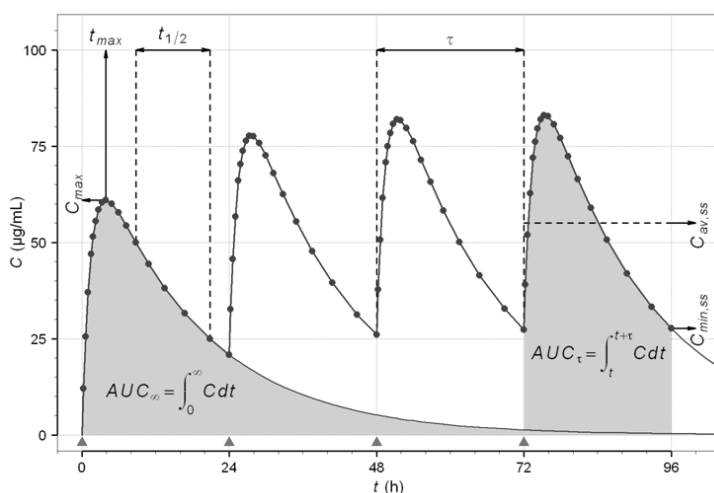
In the case of the earlier TLC and HPLC methods, specificity is relatively good as well as the running conditions. However, sensitivity is low. Microbiological methods (24) on the other hand have low specificity and run conditions that cause hydrolysis of the prodrug (CMS) thus increasing concentrations of the analyte. The HPLC-based method of Reed et al (31) is spe-

cific and sensitive, but the run conditions are such that CMS is hydrolyzed to colistin during sample workup. The more recent methods by Li et al (28) and Le Brun et al (32), use Fmoc and OPA derivatization, respectively, to increase fluorescence. The derivatization is performed on solid-phase extraction cartridges, a process which is expensive, laborious and under conditions that cause a 5% CMS hydrolysis. The more recent LC-MS/MS method developed by Ma et al (33) also requires extensive sample purification, with the same problem of CMS hydrolysis.

# Pharmacokinetics (PK)

Pharmacokinetics is the study of what the body does with the drug the main processes being absorption, distribution, metabolism and excretion (ADME). Typically when a drug is given orally, there is an absorption phase, when the drug is transported into the circulatory system, a distribution phase during which the drug spreads from the blood into the different organs of the body, and an elimination phase during which the drug is removed from the body. When a drug is given intravenously, the absorption phase is lacking. The processes occur simultaneously rather than sequentially, as e.g. elimination starts directly when the drug reaches the organ that eliminates it, and the phases are characterized by which process dominates the phase.

In the breakpoint between distribution and elimination phases, the drug reaches its maximum plasma concentration,  $C_{\max}$  (peak concentration). The time it takes for the drug to reach this concentration after administration is  $t_{\max}$ . The total exposure to the drug is described by the area under the concentration-time curve, AUC. When a drug is given in repeated doses, the drug concentration just before the next dose is called  $C_{\min}$ , or trough concentration (*Figure 3*).



*Figure 3.* Pharmacokinetic parameters shown on a simulated concentration-time curve. © User:Alfie66 / Wikimedia Commons / CC-BY3.0

The main pathways of elimination of drugs are renal excretion, hepatic metabolism and biliary excretion. The measure for describing the elimination of drugs is clearance, CL, expressed as e.g. L/h, which is the volume of blood or plasma from which all drug would be removed within a unit of time. The (apparent) volume of distribution,  $V_D$ , is the theoretical volume in which the total amount of drug would need to be uniformly distributed to produce the observed concentration of the drug. The half-life,  $t_{1/2}$ , which is the time required to lower the concentration to half of the current concentration, is dependent on the elimination rate constant,  $k_e$ , which is  $CL/V_D$ . In some cases, a non-negligible amount of drug remains in the patients' circulation at the time of the next dose, causing an accumulation of drug in the system until steady-state, where the amount given in a dose is equal to the amount eliminated during the dosage interval, is achieved, i.e. the input rate is equal to the output rate. The time required to reach steady-state is determined by the half-life, and is approximately  $4-5 \times t_{1/2}$ .

In the case of colistin, only two pharmacokinetic studies were been performed between 1990 and 2008 (31, 34), of which the former has been criticized due to flaws in the assay (35). As discussed above, the assay methods prior to the 1990s were unreliable due to hydrolysis of CMS during the assay. In the one more reliable study on the pharmacokinetics of colistin, mean values for colistin (base) at steady state were  $C_{max} \sim 2\text{mg/L}$ ,  $t_{max}$  60-120min,  $t_{1/2}$  251min, and CL was not calculated (34). Elimination of CMS is believed to mainly be through renal routes, whereas a part is hydrolyzed to colistin. Colistin on the other hand does not seem to be eliminated renally to any great extent, but is mainly believed to be eliminated by hydrolysis (9).

## Population Modeling

The pharmacokinetic parameters above can be calculated using the standard equations which define them. A more informative way to approach pharmacokinetics is population modeling. In population modeling, typical values for the above parameters are estimated and the variability between and within individuals in the population can be quantified. This gives the investigator a possibility to search for covariates, i.e. factors that can explain the variation.

Once a pharmacokinetic model is constructed, it is a powerful tool for predictions and simulations of concentration-time profiles. Using the model, the investigator can by simulation evaluate the effects of different dosing regimens by varying the interval or size of doses *in silico* in order to find a dosage regimen that will reach a pharmacokinetic target, e.g. a certain  $C_{max}$ , in a portion of the population.

## Protein binding

All drugs interact with a variety of components of blood, the most notable of these being plasma proteins which bind a multitude of drugs to various degrees. This protein binding can be chemically described as an equilibrium reaction,  $[D] + [P] \rightleftharpoons [DP]$ , where D is drug and P is protein, with its corresponding equilibrium constant  $K = [DP]/[D][P]$ . However, protein binding is more commonly characterized as a fraction of the total concentration,  $f_u = C_u/C_{tot}$ , where  $f_u$  is the fraction of unbound drug,  $C_u$  is the concentration of unbound drug in plasma and  $C_{tot}$  is the total drug concentration in plasma. It is commonly accepted that only the unbound fraction of drug can exert an effect. Depending on the drug and the concentrations investigated, the protein binding can be linear i.e. the  $f_u$  is constant, as is the case for meropenem with  $f_u$  98% (36) and colistin B with  $f_u$  43% (37), or concentration dependent i.e. the  $f_u$  is varies with the concentration. This is the case with colistin A, where the maximum  $f_u$  was 31%. The PK model in that study predicted that the total  $f_u$  in the concentration range 0.01-2.5 mg/L would range from 26% to 41%. The most common proteins associated with protein binding are serum albumin and acidic  $\alpha$ -aminoglycoprotein (AAG).

# Pharmacodynamics, PD

Pharmacodynamics is the study of what the drug does to the body or, in the case of antibiotic therapy, what the drug does to the bacterium. The effect could either be inhibition of bacterial growth or killing of the bacteria.

## Minimal inhibitory concentration (MIC)

The most common measure of effect of antibiotics *in vitro* is the minimal inhibitory concentration, MIC. The MIC is defined as the lowest concentration that inhibits visible growth of the bacterium after  $18 \pm 2$  h incubation. There are many methods for determining a MIC, and the two most common techniques being broth microdilution and Etest. MIC is used to define susceptibility of a bacterial strain to antibiotics, with European breakpoints set by EUCAST.

### Broth microdilution

Broth microdilution is a method that is most commonly performed in 96-well microtiter plates. A two-fold dilution series of the antibiotic in culture broth is constructed in the well rows (or columns) of the plate with a final volume of 100  $\mu\text{L}$ . The wells are then inoculated with the bacterium to a concentration of  $5 \times 10^5$  cfu/mL.

Plates are incubated for  $18 \pm 2$  hours in  $37^\circ\text{C}$  before reading. Plates are read visually, and the lowest concentration that inhibits growth is the MIC (38).

### Etest

The Etest is a simpler method for determining the MIC. It consists of a plastic strip with a predefined, dry gradient of the antibiotic on one side, and a printed scale on the other. A standardized suspension of the bacterial strain is spread evenly over an agar plate, to yield confluent growth over the surface. After spreading the bacteria, the Etest strip is applied to the surface of the agar and the plate is incubated as above. The antibiotic is transferred to the agar surface, and inhibits the growth of the bacteria along the strip.

The plates are read visually by following the inhibition isobole to where it intersects the strip. The MIC is the concentration on the printed scale closest to the intersect.

## Time-kill experiments

A more informative way of examining the pharmacodynamics of antibiotics is by time-kill experiments. In these experiments, the bacterium is exposed to the antibiotic in an *in vitro* culture, which is sampled repeatedly for counting of viable cells. The samples are diluted and spread onto agar plates, which are incubated overnight. After incubation, the colonies on the plates are counted. One colony is assumed to originate from one single bacterium, and can thus be used to calculate the concentration of viable bacteria in the experiment.

One limitation with the classic time-kill experiment is that the bacteria are exposed to a constant, or static, concentration of the antibiotic, whereas the antibiotic concentrations *in vivo* fluctuate according to the PK profile of the drug. In an attempt to simulate the *in vivo* situation better, a number of kinetic time-kill models have been developed that can expose bacteria to concentrations that simulate human kinetics. Sampling and viable counts are performed as in the static time-kill experiments.

## PD endpoints

As the main effect of antibiotics is to inhibit growth of or kill bacteria, the endpoints used differ from other fields of pharmacology. MIC is the most common way of expressing antimicrobial activity, but inherits several limitations and problems, such as static concentrations and evaluation of effect at one time point.

Second to the MIC, the most commonly used endpoint is the difference between the cell counts at 0 h and 24 h, in this thesis called the net killing effect or  $\text{kill}_{\text{net}}$ . Other possible endpoints are the maximum killing effect ( $\text{kill}_{\text{max}}$ ), initial killing rate ( $k_{\text{kill}}$ ) or the area under the bactericidal curve (AUBC).  $\text{Kill}_{\text{max}}$  is defined as the difference between the cell counts at 0 h and at the time when the lowest bacterial counts are obtained. Initial killing rate is defined as the difference in cell counts at 0 h and e.g. 1 h divided with the time interval. These three parameters are point-based, looking at one point of the whole time-kill experiment, and ignoring other events during the experiment. As time-kill experiments of this kind generate much data and the killing kinetics change over time, much data and information are wasted if the endpoint is relying on only one or two time points.

Last, the area under the bactericidal curve, AUBC, is a total compressed measure of effect over a time course, analogous to AUC. However, as bacterial counts are commonly presented on a logarithmic scale, it is mathematically nonsensical to calculate the AUBC on this data. A derivative is the area between the bactericidal curve and limit of detection (LOD),  $\text{AUBC} > \text{LOD}$ .

# Integration of pharmacokinetics and pharmacodynamics, PK/PD

Given that the concentration of antibiotics in the body fluctuates with time (PK), and that the effect of antibiotics depends on the concentration (PD), an integration of these two is needed in order to understand the time-course of drug effect and to determine the appropriate dosage of antibiotics. One difficulty in this is that the most common measure of efficacy, the MIC, is measured at static concentrations over a period of 16-20 hours. Another problem is that the effect of many antibiotics is dependent on the magnitude of the drug exposure, whereas others, mainly the  $\beta$ -lactams, are more dependent of the duration of the exposure (39).

In order to evaluate the antibacterial effect of different concentration profiles a series of studies (40-42) used an infection model where antibiotics were administered at a range of different dosages to mice infected with bacteria with different susceptibilities. With this approach, the currently often used PK/PD indices were developed (43).

## PK/PD indices

### $fAUC/MIC$

Antibiotics that are dependent on the magnitude of the exposure (i.e. large AUC), fall into this category. These drugs are often called concentration dependent, as an increase of the concentration increases the effect. AUC/MIC is defined as the 24 hour AUC of the antibiotic at steady state divided with the MIC (44, 45).

### $fC_{max}/MIC$

The antibiotics in this group are also concentration dependent, but the main parameter driving the effect is not the exposure *per se*, but rather the maximum concentration reached, or peak concentration. The index, sometimes written as peak/MIC, is the ratio of the peak concentration and MIC (44, 45).



$fT_{>MIC}$

For the antibiotics in this group, the most important feature is the duration of the exposure. After reaching a concentration of approximately  $5 \times MIC$ , further increase of the concentration does not increase the effect. The index states that the percentage of time during a dosage interval or 24 h that the antibiotic concentration stays above the MIC is predictive of the effect (44, 45).

## Modeling of PKPD

The PK/PD indices described above are summary endpoints, and while they are useful simplifications they also have limitations. In addition to that the understanding of the rate of bacterial killing and potential resistance development is lost, the PK/PD indices are sensitive for the uncertainty inherent in MICs, and different PK profiles in different patient populations may also affect which index is most predictive of effect (46).

A method to optimize dosages which is less sensitive to these errors is development of mathematical PK/PD models that describes the time-course of effect. PK/PD models have the advantage that they can be extrapolated to other subpopulations of patients such as children or patients with renal impairment. Since PK/PD models take the whole time span into account rather than 24 hour bacterial load they may give less bias than the PK/PD indices.

# Combination therapies

There are several reasons to consider combination regimens when using colistin:

1. To increase efficacy.
2. To counteract resistance development.
3. To widen antibacterial spectrum when there are multiple pathogens or the pathogen is unknown.

In the first case the rationale is that the highest dose that may be given with acceptable toxicity an antibiotic given separately may not be sufficiently efficacious to clear an infection. In the second case, the combination of antibiotics may minimize the risk of selecting preexisting subpopulations of resistant bacteria, as well as those arising by *de novo* mutations if one of the drugs reaches the pharmacodynamic target of those bacterial populations. A special situation is when the resistance mechanism renders the bacterium more susceptible to other drugs (21). The third case is becoming increasingly important in healthcare settings, especially because of the lack of rapid point of care microbiological diagnostic tools and the fact that inappropriate empiric antibiotic therapy is a well identified risk factor for increased mortality in severely ill patients.

In view of the increasing demand of combination therapy, there is a need for reliable methods for determining joint effects of antibiotics.

## Synergy

When discussing antimicrobial combinations, the concept of synergy along with the related terms additivity, indifference and antagonism are mostly used. The concept is treacherous in its simplicity, and its clinical relevance in antibacterial therapy is questionable. Furthermore, the meaning of synergy in the medical literature is not always clearly defined, and there are differences in definitions between investigators, also with respect to other term in this context, i.e. additivity, indifference and antagonism.

Bliss proposed, in 1939 (47), a theory for the effect of drug combinations, stating that two agents do not interact with one and another, but rather work independently towards a common goal, i.e. that effects are additive. According to the theory, when the effect of the combination equals the sum of the

effects of the single drugs, additivity is present. Any effect greater than this is synergy, and any effect lower is antagonism (47). These definitions may result in a situation where a substantial increase in the antibacterial effect of a combination will be classified as antagonism.

In the clinical situation, synergy is of secondary importance regardless of how it is defined. For the treating physician, the important question is whether or not a combination therapeutic regimen will increase chances of bacterial eradication and cure of a patient. A clinician's main concern would thus be an enhancement of effect by adding another drug, as in some cases the effect of a combination may be equal to or even worse than the one of a single drug.

## Methods for determination of joint effects

### Checkerboard

In a more practical sense, there are two main methods to investigate combination effects, checkerboard and time-kill experiments (38). The checkerboard method produces a two-dimensional grid with concentration gradients of the two investigated drugs crossing each other. Each cell in the assay contains a unique combination of drug concentrations. The cells are then inoculated and incubated, which will result in an inhibition pattern. From that pattern, a fractional inhibition concentration index can be calculated. The formula used is  $FICI = (IC_{A+B}/IC_A) + (IC_{A+B}/IC_B)$ , where IC is inhibitory concentration for drug A, B or the combination A+B. Interpretation criteria may vary, but the most common are  $FICI \leq 0.5$  for synergy,  $>0.5 - 4.0$  for indifference and  $\geq 4$  for antagonism. Some studies divide the indifference criterion to indifference and additivity, with the latter defined as  $FICI >0.5 - 2$ . Checkerboard assays are quite widely used, although they are often heavily criticized (48) for the use of fixed concentrations, concentration ranges potentially way outside those that are clinically relevant and that the FICI breakpoints are more or less arbitrary.

### Time-kill assay

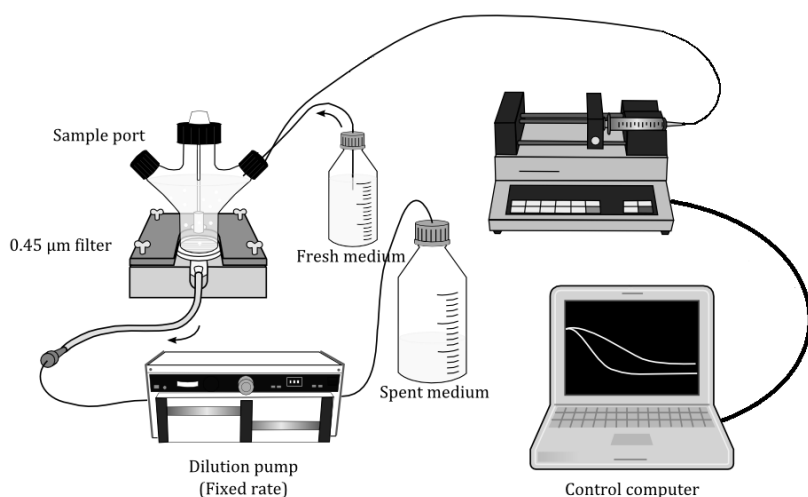
Time-kill combination assays are mostly static systems, where the bacterium is exposed to the drugs of choice both individually and in combination. Killing kinetics are monitored by viable counts, but mostly the data analysis only takes the endpoint data into account. Synergy in these experiments is defined as a  $>2 \log$  CFU/ml decrease in viable bacteria after 24 hours exposure compared to the most active single drug. Worth noting is that the definition requires that the bacterial strain is resistant to at least one of the drugs (49). However, this requirement is often disregarded. These methods have also been criticized, although being better than checkerboard. One point of criticism is the use of fixed concentrations and 24 hour endpoint, which do not

reflect the clinical situation with drug concentrations changing over time and drugs being administered twice or thrice daily (48). Also, the definition of synergy lacks a mathematical basis, and does not take the dynamics of killing into account at all. It simply wastes all data from between 0 h and 24 h.

### Kinetic/dynamic in vitro models

In an effort to bridge the gap between the lab benchtop and the clinics, several dynamic in vitro models have been developed (50-52). Common for all these methods is that human pharmacokinetics can be simulated in the lab by diluting the culture medium.

The dynamic in vitro work in this thesis was performed in a model developed in our laboratory (50, 51, 53) which consists of an air-tight, open bottomed spinner flask and a pump (*Figure 4*). The pump draws medium from the flask through a sterile filter at a rate corresponding to the half-life of the investigated drug. The withdrawn medium is replaced with fresh, keeping the volume constant and efficiently diluting the drug in the flask. The sterile filter prevents dilution of the bacteria, which is a problem in many other models (50). The model has also been modified to accommodate to combination therapies, and is described in detail in paper V.



*Figure 4.* The in vitro kinetic model with syringe pump for two different pharmacokinetic profiles.

# Aims

The general aim of this thesis was to contribute to the improved use of colistin by increasing knowledge on its pharmacokinetics and pharmacodynamics and the effect of combination therapy.

The specific aims were:

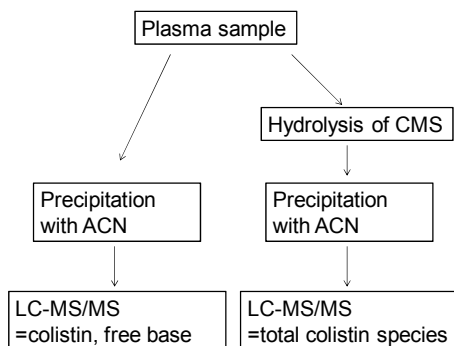
- To develop a specific and sensitive method for assaying plasma and culture medium (Paper I).
- To characterize the rate, extent and the causes of the loss of colistin during a normal laboratory experiment (paper II).
- To describe the pharmacokinetics of CMS and colistin in critically ill patients (Papers III & IV).
- To describe the pharmacodynamics of colistin alone and in combination with meropenem (paper V)
- To compare different ways of measuring the joint effect of combination regimens (paper V).
- To develop a PKPD model that describes the in vitro joint effect of colistin and meropenem alone and to predict combination dosages of colistin and meropenem that would result in efficacious bacterial killing and overcoming resistance (paper VI)

# Paper I

To be able to study the optimal dosage of colistin, a sensitive and specific assay was needed. Since efforts to replicate the method developed by Li et al (28) was unsuccessful in our hands, we decided to develop a method that was inspired by some of the earlier described methods, but with some new components and refinement.

## Materials and Methods

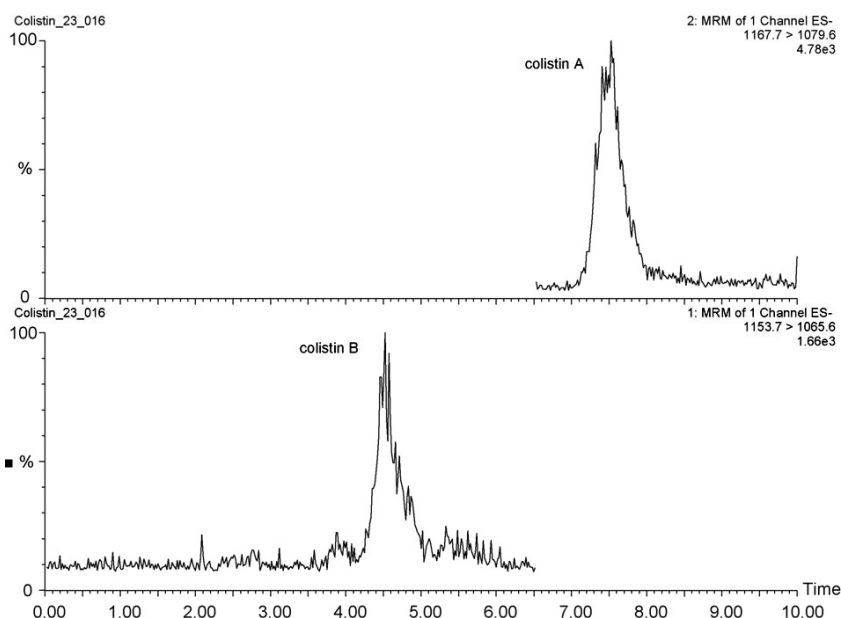
We used liquid chromatography (LC), coupled with tandem mass spectrometry to separate and detect the main components of colistin preparations, colistin A and colistin B. The use of mass spectrometry abolishes the need for derivatization, and only a simple precipitation step with acetonitrile and trifluoroacetic acid was needed prior to LC. Due to adsorption of colistin to plastics, samples of culture media were mixed with an aliquot of drug-free plasma. For quantitation of CMS, an aliquot was hydrolyzed to colistin by addition of sulphuric acid. The difference in concentration between the hydrolyzed and not hydrolyzed aliquots is the concentration of CMS and partially sulphomethylated colistin species in the sample (*Figure 5*).



*Figure 5.* Schematic description of the work-flow of the assay.

## Results

Working in negative ionization mode, daughter molecules of colistin A and B were detected with  $m/z$  1079.6 and 1065.6, respectively (*Figure 6*). The combined lower limit of quantification for colistin A and B was  $0.03\mu\text{g/l}$  in plasma and  $0.04\mu\text{g/l}$  in medium. CV was  $< 12\%$  and accuracy  $\pm 13\%$ . CMS could not be ionized, so the CMS assay is indirect, as are the other published methods (28, 33). Tests of colistin and CMS stability in assay conditions revealed that both colistin and CMS are stable in the solutions used for several hours, when stored refrigerated.



*Figure 6.* Chromatograms showing the peaks of colistin A (top) and B (down).

## Conclusions

The described method is sensitive and selective for the analysis of colistin in plasma as well as for culture medium. Only 100L plasma is required and the simple and rapid sample preparation method makes it possible to analyze samples from clinical studies without getting falsely high colistin from hydrolysis of CMS.

The fact that colistin is stable for at least 3 h at  $37\text{ }^{\circ}\text{C}$  allows for in vitro studies at physiological temperature. Important factors to take into account are the acid and thermo instability of CMS and the adsorption of colistin in water solutions to different materials used in the laboratory.

## Paper II

During the work with paper I, it became obvious that colistin is adsorbed to laboratory utensils, such as test tubes. Binding of colistin to laboratory materials has been mentioned in the literature, but no systematic studies seem to have been performed to describe this binding in detail.

### Materials and methods

The study was designed to mimic a normal *in vitro* experiment with static concentrations of colistin prepared in different common laboratory materials. The impact of two different dilution methods, a serial and an incremental, was studied by measuring the concentrations of colistin in each dilution step.

In the main experiments, three types of commonly used large test tubes were studied: soda-lime glass, polypropylene and polystyrene. In addition polystyrene microplates commonly used for MIC assays and microtubes specifically developed for low binding of proteins and peptides were studied. The tested concentration range was 0.125-8 µg/mL colistin base. The resulting concentration-time profiles were analyzed by fitting exponential one phase and two phase functions to the data. The adsorption of colistin per surface area of material in the large tubes was modeled by fitting to the Langmuir adsorption model. The Langmuir model is similar to the Hill equation, but describes the adsorption of peptides to surfaces. The main parameters are  $L_{\max}$ , which is a measure of the binding capacity of the material, and  $K$ , the equilibrium constant for the equilibrium  $[P] + [S] \rightleftharpoons [PS]$ , where P is peptide and S is surface.  $K$  is thus a measure of the binding strength between the peptide and the surface.

### Results

There is a marked loss already during the dilutions of colistin, and the loss was more prominent in the serial dilution. In the large tubes, the measured start concentrations ranged between 44%-101% (*Figure 7*) of the concentrations expected on the basis of measured concentrations in the stock solutions. The rate constants for the loss ranged between 0.06-0.80 s<sup>-1</sup>. The maximum binding capacity of the three materials ranged between 0.33-1.08 µg/cm<sup>2</sup>, and



the equilibrium constants (binding strength) ranged between 0.09-0.54 mL/ $\mu$ g.

The loss of colistin was most notable in the microplates, where 96-100% was lost already in the start samples. Least loss was found in the low protein binding microtubes, with 10%-45% lost at the start, and loss during the experiment was markedly lower.

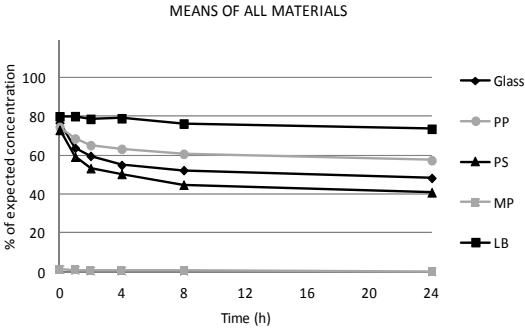


Figure 7. Mean recovery of colistin from all materials. Recovery is adjusted to the measured concentrations in the stock solutions. Glass=soda-lime glass, PP=polypropylene, PS=polystyrene, MP=polystyrene microplate, LB=low protein binding microtube.

## Conclusions

The dilution procedure can be optimized by selecting materials with low binding properties and minimizing dilution steps, especially at low concentrations. None of the materials performed very well, but the best performing material was low protein binding polypropylene, followed by standard polypropylene. Worst performance was with polystyrene microplates. Use of low protein binding polypropylene or standard polypropylene in applications where it is possible can be encouraged, and the use of standard polystyrene discouraged in order to minimize the effect of colistin loss due to adsorption. However, as none of the materials performed well enough, this study recommends measuring the colistin concentrations in experimental settings.

## Paper III

From an international perspective, the antibiotic resistance situation in Sweden is still favorable and the use of colistin is still rarely needed. However, in some countries in Europe, especially in Greece, multiresistant Gram-negative bacteria are a major problem and colistin use is common. In order to improve the knowledge of colistin PK using the method in paper I, collaboration was initiated with Attikon University hospital in Athens.

### Materials and methods

Eighteen critically ill patients given colistin as part of their standard care were included in this study. Plasma samples for colistin assay were drawn before the start of infusion and at 0.25, 0.5, 1, 1.5, 2, 4, and 7.75 h after the start of infusion. Sampling was performed after the first dose and after the fourth dose, when steady state was assumed to be reached. The samples were frozen and shipped to Uppsala on dry ice for analysis. The resulting concentration data was used to develop a population PK model in NONMEM v.6.

### Results

Concentration time data from individual patients are shown in *Figure 8*. The developed model used a two compartment model for CMS, whereas a one compartment model was sufficient for colistin. No significant covariates were identified. Due to that CMS needs to be hydrolyzed *in vivo*,  $t_{\max}$  was around 6 h. Furthermore, the half-life of colistin was very long, 14.4 h, which causes steady state to be achieved after approximately 2-3 days. As time is critical, especially in ICU patients, up to three days to reach steady state is a very long time.

The model predicts (*Figure 9*) that, for a typical patient receiving 3 million IU CMS q8h, colistin  $C_{\max,ss}$  is 2.3 mg/L,  $CL/f_m$  is 9.09 L/h and  $V/f_m$  is 189 L. The alternative dosing regimens with loading doses reach steady-state levels within the first 24 h. The variability between individuals is large as well as the variability between sampling occasions in one individual.

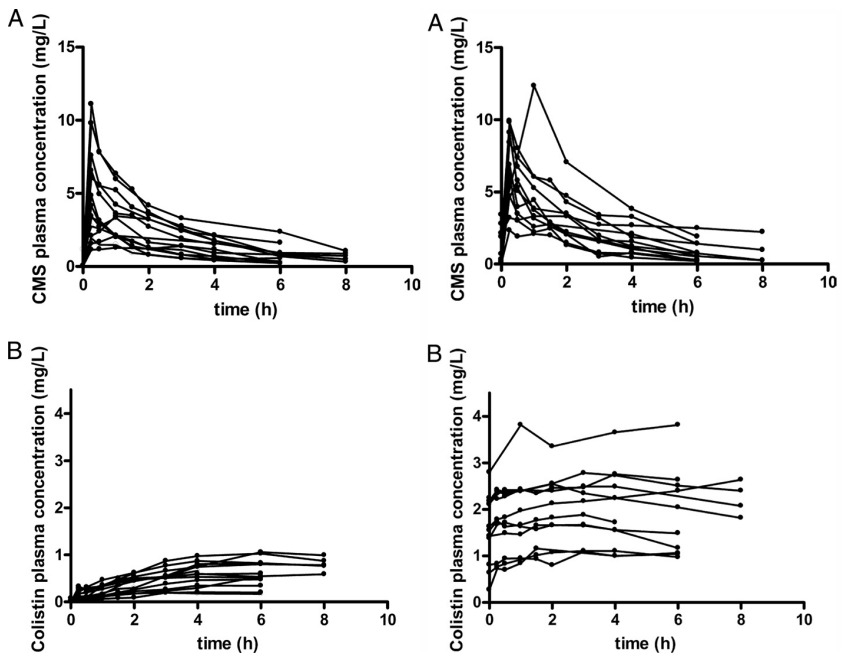


Figure 8. Concentrations of CMS and colistin in individual patients. Left panel is after the first dose, right panel after fourth dose.

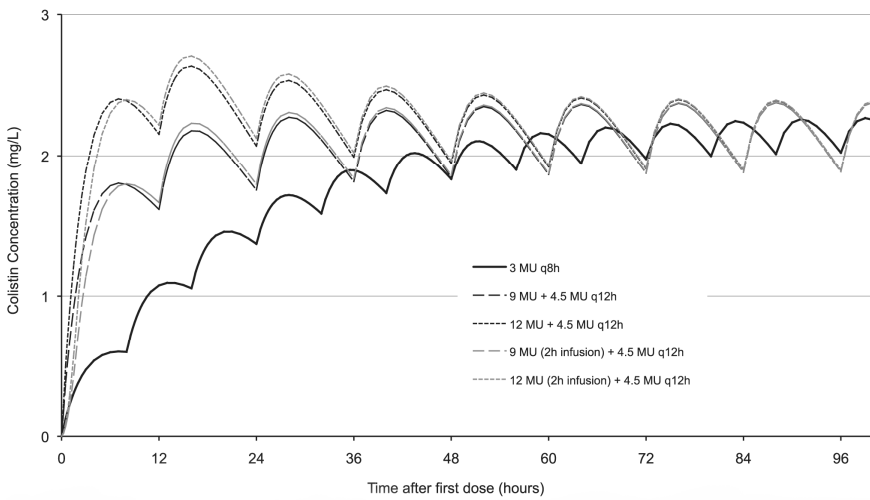


Figure 9. Model prediction for a typical individual with standard (3 MU q8h) dosage and alternative dosage regimens.

## Conclusions

The main findings in the paper were that because colistin is being formed from CMS and colistin has a long half-life, there is a slow increase of the active drug in plasma. We therefore suggest the use of a loading dose to shorten the time to steady-state, and the PK profiles of a few candidate regimens were simulated.

Both the interindividual variation and the interoccasion variation are very large in the studied population. For a typical patient, colistin  $C_{\max, ss}$  was 2.3 mg/L. Taking the large variation and protein binding (37) into account it is obvious that the achieved concentrations are low in comparison to the defined MIC breakpoints.

## Paper IV

Many critically ill patients suffer from renal impairment, and previous small studies have indicated that CMS/colistin dosage regimens are inadequate. As CMS is excreted renally and there has been a lack of knowledge about dosage in patients receiving continuous renal replacement therapy, many physicians have lowered the doses of colistin due to concerns of toxicity. The patients in this study are a subset excluded from the above study (paper III).

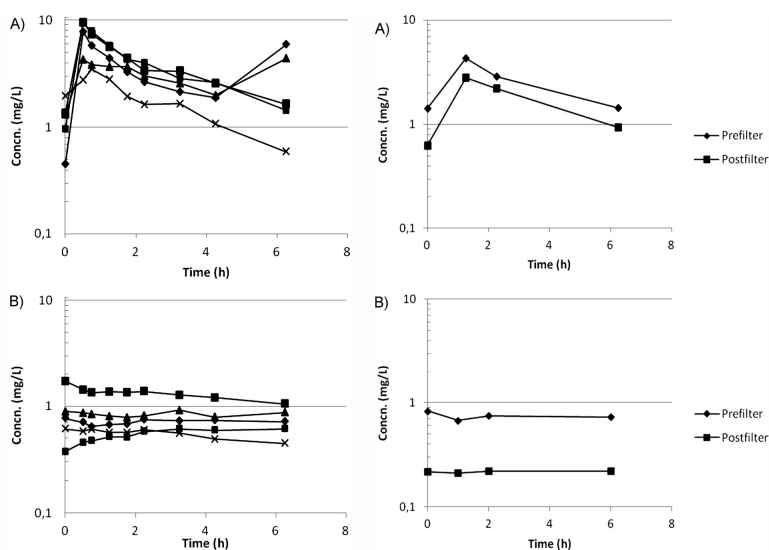
### Methods

Five critically ill patients given colistin as part of their standard care were included in this study. Plasma samples for colistin assay were drawn before the start of infusion and at 0.25, 0.5, 1, 1.5, 2, 4, and 7.75 h after the start of infusion. Sampling was performed after the first dose and after the fourth dose, when steady state was assumed to be reached. The samples were frozen and shipped to Uppsala on dry ice for analysis.

The resulting concentration data was analyzed by calculating key PK parameters for CMS and colistin in the patients, and by estimating the efficacy of CVVHDF in clearing the drugs.

### Results

Concentration time data from individual patients are shown in *Figure 10*. For CMS, the mean  $C_{\max}$  after fourth dose was 6.92 mg/L and total CL 8.23 L/h. The terminal half-life was 3.3 h. CMS was removed by the filter with an extraction ratio of 0.3, corresponding to a filter clearance of 1.9 L/h. For colistin, the mean concentration was 0.92 mg/L and  $CL/f_m$  18.91 L/h. Colistin was removed by the filter with an extraction ratio of 0.68, corresponding to a filter clearance of 4.3 L/h.



*Figure 10.* Individual plasma concentrations of CMS and colistin (left) and median plasma concentrations of CMS and colistin in afferent and efferent blood (right).

## Conclusions

Both CMS and colistin were cleared by CVVHDF. Colistin concentrations were below the current MIC breakpoints, and  $fAUC/MIC$  was lower than recommended, suggesting that a dosage regimen of 160 mg CMS q8h is inadequate.

# Paper V

Combinations of antibiotics are becoming more important as resistance increases. As several studies indicate that the joint effect is strain specific, there is a need for reliable and clinically relevant tools for quantification of joint effects. The purpose of this study was to explore the *in vitro* pharmacodynamics of colistin and meropenem in combination therapy against *P. aeruginosa* and *A. baumannii*, and to find an approach to quantify and compare the single versus joint effects.

## Materials and methods

Four strains of each species, with and without meropenem resistance, were exposed to clinically relevant, lower range concentrations of the antibiotics simulating human pharmacokinetics. Experiments were run for 8 hours, i.e. one dosage interval for both drugs, in the kinetic model developed by Löwdin et al (50). The model has been further developed to be able to simulate several different PK profiles by adding computer-controlled syringe pumps to continuously adjust the concentrations of the antibiotics with the shorter half-lives. Included endpoints were initial kill rate, maximum killing effect, net killing effect and area under the bactericidal curve (AUBC).

## Results

In three of the strains, including one resistant to meropenem, the combination yielded an increased effect with respect to all endpoints (*Figure 11*). The AUBC was significantly (paired t-test,  $p < 0.05$ ) lowered in all three strains. Synergy, as defined as a 2  $\log_{10}$  reduction of the combination compared with the most active single drug at 8 h, could not be shown in any of the strains.

# Conclusions

As time-kill experiments of this kind generate much data and the killing kinetics change over time, much data and information are wasted if the end-point is relying on only one or two time points. The AUBC was assumed to

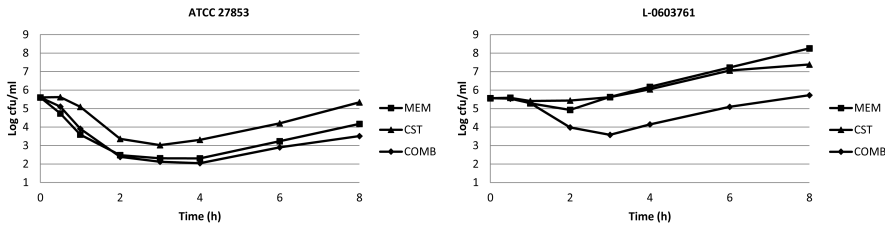


Figure 11. Time-kill curves of two of the strains investigated.

be the most informative measure of these, as it contains information about the time course, although compressed into one figure.

The combination of meropenem and colistin is a useful option for treatment of multidrug resistant infections, even if the strain is resistant to meropenem. A simple method towards quantifying joint effects is to perform a statistical test for difference between paired samples (students' t-test) using the AUBC as measure of effect. The paper suggests that this area-based combination effect (ACE) is a plausible analysis of joint effect in a future clinically useful test for combination therapy.



# Paper VI

Mechanism-based pharmacokinetic-pharmacodynamic (PK/PD) models that describe the *in vitro* bacterial time-kill curves of a combination of antibiotics may be useful as tools to determine the joint effect of a combination. The purpose of this study was to develop a mechanism-based pharmacokinetic-pharmacodynamic (PK/PD) model that describes the *in vitro* bacterial time-kill curves of colistin and meropenem alone and in combination.

## Materials and methods

*In vitro* time-kill curve experiments were conducted for 24 h on two strains of *P. aeruginosa*; wild-type and a meropenem resistant type. Antibiotics were added to result in static concentrations of 0.25-16×MIC for single experiments and 0.25- 6×MIC in combination experiments. Mechanism-based PKPD models describing resistance development were fitted to the observed bacterial counts in NONMEM. Predictions assuming additive killing effects on the bacterial killing were first made, thereafter all parameters were re-estimated, including an interaction term. Validation was conducted by visual predictive checks (VPC). The final model was combined with PK models of colistin and meropenem to predict the bacterial killing for the meropenem-resistant strain with different combination dosages of colistin and meropenem.

## Results

A model with compartments for growing and resting bacteria, with a function allowing the maximal bacterial killing to reduce with exposure, characterized both the bactericidal effect and resistance development for both antibiotics (*Figure 12*). The estimated interaction term between the killing effects for the two drugs in combination identified no benefit of combining the two drugs for the sensitive strain and a higher than expected (synergistic) effect for the resistant strain. Even though the combination was considered synergistic for the resistant strain, only high doses of the antibiotics yielded a 3 Log<sub>10</sub> killing effect (*Figure 13*).

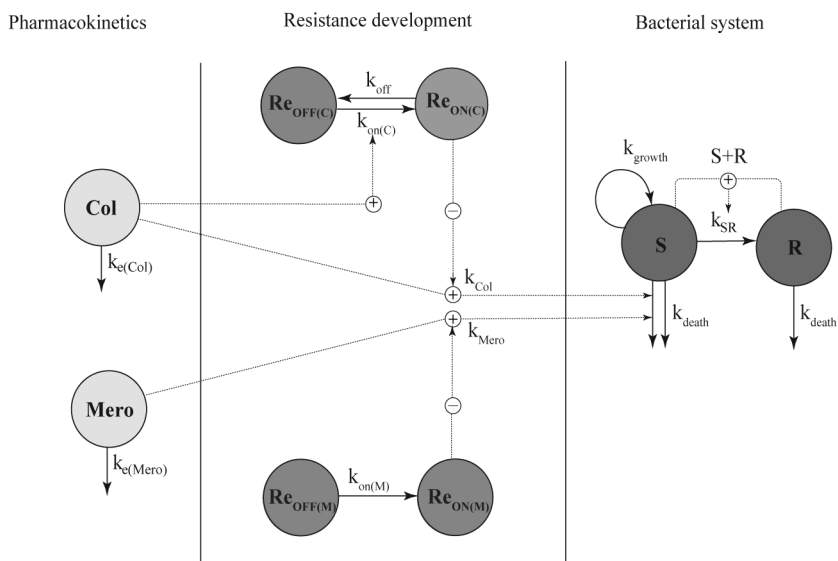


Figure 12. Schematic description of the model structure.

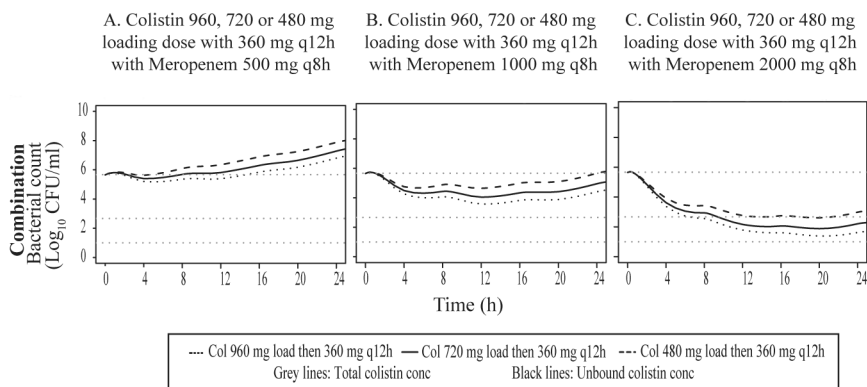


Figure 13. Predictions of bacterial kill with the combination of colistin and meropenem at different dosages.

## Conclusions

The developed PKPD model successfully described the effect of colistin and meropenem and regrowth in both wild-type and resistant *P. aeruginosa* in single drug and combination experiments. For the resistant *P. aeruginosa* strain, the study supports that a high dose combination therapy with the two drugs is needed to overcome the resistance, despite the presence of synergism.

# Conclusions

- The developed assay method for colistin was sensitive and selective for the analysis of colistin in plasma as well as for culture medium. The simple and rapid sample preparation makes it possible to analyze samples from clinical studies without getting falsely high colistin concentrations from hydrolysis of CMS. The inter-day variability of the QC samples for plasma was low, which shows that the method is reliable and robust.
- Colistin is extensively lost during normal experimental conditions in a strong concentration and material-dependent manner. It is important to carefully monitor colistin concentrations during an experiment, and to use materials with low adsorption of colistin.
- In the first report of a population pharmacokinetic analysis of colistin after intravenous administration in critically ill patients, we showed that the concentrations of colistin were very low and showed great inter-individual variability, and that the half-life was long. A reevaluation of the CMS dosage appears to be warranted. Use of a loading dose and longer dosing intervals deserves further study.
- In critically ill patients on CVVHDF, both CMS and colistin are cleared by this renal replacement technique. The dosage regimen used, 2 million IU q8h, together with the increased clearance of colistin, results in colistin concentrations that are approximately half of the corresponding concentrations in patients not undergoing CVVHDF given 240 mg CMS q8h. The resulting drug exposure raises serious concerns regarding the optimal dosage and the need of dosage adjustment in patients receiving CVVHDF.
- We have identified a way to quantify joint effects of antibiotics, Area-based Combination Effect (ACE), which we believe could be developed further and become a more useful and clinically relevant tool than checkerboard assays. We also found that the combination of meropenem and colistin may increase the joint antibacterial effect even in strains that are resistant to meropenem.
- The developed PKPD model successfully describes the effect and regrowth of colistin and meropenem in combination on both wild-type and resistant *P. aeruginosa*. The model supports combination therapy with the two drugs to overcome resistance and the model was shown to have potential to be useful in the development of drug combination regimens.

# General discussion and future perspectives

## Colistin and analysis methods

Colistin is rapidly emerging as a last resort antibiotic for the treatment of MDR Gram-negative bacteria. The fact that retail preparations of colistin may contain up to 30 different variants of colistin has hampered the understanding of the pharmacokinetics and pharmacodynamics of colistin. Currently, 6 HPLC-based analysis methods for colistin have been published, and all have been criticized for one or several limitations and weaknesses. An external validation study, comparing the results of the different methods is clearly justified. Most methods are laborious and time-consuming, and thus not well suited for therapeutic drug monitoring (TDM) (c.f. page 16, 45).

The introduction of CMS minimized toxicity, and made colistin available in active concentrations in the urine, but also created new problems. CMS cannot be measured directly by any available method, and can thus only be measured indirectly after hydrolysis to colistin. The spontaneous hydrolysis of CMS along with the different variants of colistin mixed in the preparations are critical factors that have the potential to ruin a otherwise good method.

One additional major problem with the analysis of colistin is its adsorption to plastic materials (paper II). This may significantly underestimate the true concentrations of colistin in fluids with no or low concentrations of protein, e.g. fluids from bronchoalveolar lavage, dialysate, synovial fluid etc. This problem clearly also relates to the measurements of MICs in vitro, where the activity of colistin could be significantly underestimated (and the MICs overestimated) due to binding to the labware. This is a significant problem that must be further studied and could be addressed either by mathematical corrections or preferably by using materials in which the binding of colistin to the surfaces is minimized. If MICs of colistin are significantly overestimated, this will in turn influence the PK/PD relationships and optimal dosing regimens.

## Dosing regimens

When the thesis work was initiated, knowledge of colistin PK was sparse. Most of the earlier pharmacokinetic studies were not reliable due to the

problems discussed above. The very slow elimination (long half-life), low steady-state concentrations and large inter-individual variation in ICU-patients were surprising. The results have been confirmed by other researchers, and according to a recent survey of colistin use, loading doses are used in many countries (54).

In addition to critically ill patients, there are many other patient populations that need to be studied with regard to colistin dosage. In e.g. patients with renal failure receiving continuous veno-venous hemodiafiltration, colistin is removed via the hemodiafiltration procedure, and the proper dosage is probably close the normal dosage. Other patient populations that should be studied for further characterization include children (55, 56) and elderly. Alternative routes of administration, such as intraventricular injections (57-59), should also be studied better.

Even though a loading dose reduces the time to reach steady-state concentrations, it does not elevate the concentration at steady-state. Currently, there is data available, from both *in vivo* animal models and *in vitro* models that suggest that the PD-index driving efficacy is  $fAUC/MIC$  (60-62). After adjusting for protein binding (37), it becomes obvious that the achieved concentrations are suboptimal (paper IV) in comparison to the clinical breakpoints set by EUCAST (14).

As clinical studies do indicate that colistin is an effective antibiotic in spite of these limitations, there seems to be a translational problem between PK/PD and the clinic. One possible solution to the dilemma is if the adsorption of colistin to labware (paper II) causes a systematic overestimation of the MIC, the PK/PD indices will necessarily follow.

Due to the lack of data and recommendations regarding proper dosage of colistin as well as the large inter-individual variation, there is an increasing demand for TDM. However, the large inter-occasion variability causes TDM to be of limited use (63).

## Combination therapies

In the face of the globally increasing problem of multidrug resistance, combination therapies have gained much attention. Colistin has been of special interest for investigators, and a multitude of different combinations of antibiotics have been tested in both *in vitro* and *in vivo* animal models. The results imply that the joint effect is strain specific, as one combination is effective against some strains but not others. Interestingly, antagonism, defined as a decreased effect, is rarely found.

A common problem with describing joint effects is that the methods for determining or measuring joint effects are focusing on synergy, a concept that is constantly debated and of uncertain clinical relevance. The work put

forth in this thesis is an effort to improve both the methods and the measures of joint effects with a clinical perspective.

Blaser (48) has provided a detailed critique of checkerboard assays, proposing that kinetic time kill experiments should become standard. However, no robust method for quantification of the joint effect in kinetic time-kill models has been proposed. In this thesis, an approach is described where AUBC, a summary measure of antimicrobial effect, instead of point-based measures. As the concept of synergy is problematic, and from a clinical point of view irrelevant, that concept is bypassed and focus is set on enhancing the total antimicrobial effect. The definition of what constitutes an enhanced effect can e.g. be a statistical significance as in the proposed ACE or a cut-off value for clinical significance.

Another possible route is by using PK/PD modeling. As is discussed in paper V, a model can be developed from simple, static time-kill experiments. The model can then be combined with PK-models predicting concentration-time profiles that drive the PK/PD. As was shown in paper VI this approach is able to characterize the bacterial killing and regrowth of two *P. aeruginosa* strains in the in vitro kinetic model from paper V.

In order to achieve a more rational approach to combination therapies, much effort is needed. Faster methods for determining combination effects are needed, from simple screening methods to more precise tests or mathematical models that are validated in animal models or clinical trials. To help physicians in selection of empirical combination therapies, databases of combinomes of local pathogens could be constructed. So much work, so little time...

# Sammanfattning på svenska

I och med att multiresistens bland Gram-negativa bakterier har ökat lavinartat under senare år har kolistin snabbt fått ökad uppmärksamhet som ett av de sista behandlingsalternativen. Kolistin är ett peptidantibiotikum, som introducerades på 1950-talet men slutade användas på 1970-talet när antibiotika med mindre biverkningar blev tillgängliga på marknaden. Kolistin ges oftast intravenöst till svårt sjuka patienter i form av kolistimetat (CMS), som hydrolyseras till kolistin och ett antal mellanformer både *in vitro* och *in vivo*. Det finns fortfarande stora kunskapsbrister om grundläggande aspekter av kolistin, inklusive grundläggande farmakologi, farmakokinetik, farmakodynamik och optimal dosering. Syftet med denna avhandling var att förbättra grunden för optimerad kolistinanvändning.

För att över huvud taget kunna studera kolistin, har vi utvecklat en LC-MS/MS analysmetod som är känslig, specifik och användbar i både *in vivo* och *in vitro*-studier. Med denna metod har vi upptäckt en betydande förlust av kolistin under normala laboratorieförhållanden. Denna förlust karakteriserades och kvantifierades; hypotesen var att förlusten främst beror på adsorption till laboratoriematerial. Detta problem kan innebära en allvarlig felkälla, bl.a. när det gäller att bedöma bakteriers känslighet för kolistin och vi föreslår hur denna felkälla skall kunna hanteras.

Farmakokinetiken för kolistin studerades i två grupper av svårt sjuka patienter, vårdade i Grekland. I en patientgrupp låg njurfunktionen i det normala intervallet. Den andra gruppen hade en mycket nedsatt njurfunktion som krävde dialysvård. Plasmakoncentrationer analyserades med metoden ovan, och populationsmodellering användes för att beskriva data. Ett av de viktigaste resultaten från studien är att kolistin utsöndras mycket långsamt med en tidigare okänd, mycket lång halveringstid. Slutsatsen av detta är att en laddningsdos bör användas för att snabbt komma upp i jämviktskoncentration. Resultaten från den mindre studien av patienter som behandlades med dialys (kontinuerlig hemodiafiltration) visade att både CMS och kolistin extraheras med hemodiafiltration. Resultaten tyder på att dosjustering på grund av njursvikt inte skall göras hos dessa patienter.

Kombinationsbehandling med flera antibiotika används allt oftare vid behandling av med infektioner orsakade av multiresistenta bakterier. Kolistin blir allt vanligare i sådana kombinationer och i avhandlingen diskuteras hur den kombinerade effekten av antibiotika kan mätas. Vi har studerat kombinationsterapier med kolistin och meropenem som modell, och föreslår en

metod för att testa antibiotikakombinationer i en kinetisk *in vitro* modell. Modellen simulerar human farmakokinetik, även för läkemedel med olika halveringstider, och måttet på effekt är arean under den baktericida kurvan. Vidare har en farmakokinetisk/farmakodynamisk modell anpassats för att beskriva farmakodynamiken av kombinationen. Dessa studier visade att kombinationen av kolistin och meropenem kan vara användbar även när bakterien är resistent mot meropenem.

Sammanfattningsvis har en specifik och känslig metod för analys av kolistin utvecklats och en viktig felkälla beskrivits (adsorption till laboratoriematerial) som kan påverka tolkningen av studier av kolistinets antibakteriella effekter. Den specifika analysmetoden användes sedan i alla studier i denna avhandling och har blivit väl accepterad internationellt. Farmakokinetiken för kolistin och CMS beskrevs i två viktiga patientgrupper, delvis med överraskande resultat som redan har påverkat doseringen av kolistin i många delar av världen. Farmakodynamiken av kombinationsterapi undersöktes och kvantifierades, och de metoder som använts kan vidareutvecklas till kliniskt användbara verktyg för val av antibiotikakombinationer.



# Acknowledgments

**Otto Cars**, my main supervisor, for believing I could pull this off when few others would have. Your trust, patience, kindness and support have been unprecedented, and you've always been there when it mattered. I've learned a lot during these years.

**Lena Friberg**, my cosupervisor. Whenever I've had a question about pharmacokinetics or modeling, you've been there. Almost like an encyclopedia, but better. Always reading drafts and tirelessly correcting errors... I'm glad we're on the same side.

**Pernilla Lagerbäck** and **Patricia Komp-Lindgren**, the two P's. For all the discussions about all possible things; kids, pets, diseases... and occasionally work.

**Christer Malmberg**, for all kinds of help, tech stuff, lab work, curve fits, images (image credit for Figures 2 & 4)...

**Lotta, Juliana** and **Anita**, our present and former "lab rats", for helping out whenever needed.

**Thomas Tängden** I've enjoyed our lectures, just too bad that the clinical trial died. The people at farmbio:

**Britt Jansson**, without you this thesis wouldn't exist. Without the tools and expertise you provided at least four out of six studies would have been impossible to perform. As simple as that.

**Ami Mohamed**, for helping me out with understanding modeling, doing some fun labwork and always having a smile on your face.

**Elisabeth Nielsen**, for the discussions, travel company and shopping for the kids.

**David Khan**, **Anders Kristoffersson** and **Salim Bouchene**. Everything is more fun when one is not alone.

My **Greek** coauthors and collaborators, I'll mention no names as I always forget them - except **Diamatis Plachouras**.

**Colleagues** and **senior researchers** at the the department. I know I'm a bit odd, but you're always just as kind. Special thanks to **Carl Pålson** and **Björn Herrmann**, you've always listened, helped, answered questions and alike.

My family, **Tanja, Nelly, Lovisa** and **Elsa**. Lets even that girl power out with Cooper the welsh, Casper "Turbotassu" and, well Pinja is female again... Do rugrats count?? Without you I wouldn't be half the man I am today. Tanja, I gave you a promise almost 16 years ago, and I'm still going to hold on to it.

Last, but surely not least, **God Almighty**, *Pantokrator*, to whom I owe everything.

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