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Ida Karin Tylleskär

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Pharmacological aspects and dosing in clinical practice

Doctoral thesis

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Nesespray mot overdose

Overdose med heroin og andre opioider gjør at man slutter å puste og dør av hjertestans. Hvert år dør over 250 unge mennesker i Norge og titusenvis i resten av verden av slike overdoser. Utdeling av nesespray med motgiften nalokson er blitt foreslått som et tiltak for å redde liv, og var en hjørnestein i den norske overdosestrategien som ble lansert i 2014. Den gang var motgiften kun tilgjengelig i sprøyteform til injeksjon. Derfor ble improviserte nesesprayer tatt i bruk. Disse var verken testet eller godkjent. Ved NTNU startet vi derfor utvikling og testing av en spesiallaget nesespray med nalokson til kameratredning ved overdose.

Gjennom studier i friske, frivillige deltakere har vi vist at nalokson tas raskt opp i nesen når det gis med vår spesialdesignede nesespray. Vi fant at 50% av medisinen tas opp. Det betyr at dosene må dobles når man gir motgiften i nesen for å virke like godt som motgift med sprøyte. Vi utviklet en modell for å undersøke naloksons evne til å reversere virkningen av opioider. Deltakerne i studiene våre fikk nalokson samtidig som de fikk en målstyrt infusjon med opioidet remifentanil. Dette gav stabil effekt av opioidstoffet slik at vi kunne måle effekten av motgiften.

Til tross for at nalokson har vært brukt i mange tiår er det stort sprik i retningslinjene om hvordan legemidlet skal administreres og hvilke doser som anbefales. Derfor undersøkte vi hvordan nalokson ble brukt i ambulansetjenesten i Oslo. Vi fant at nalokson oftest gis intramuskulært i dosene 0,4-0,8 mg, og at dette er effektivt og trygt. Dette var viktig kunnskap for det videre arbeidet med utviklingen av nesesprayen.

Avhandlingen viktigste bidrag er å vise at nalokson tas raskt opp i blodet når det gis med en spesialtilpasset nesespray, og at opptaket var mye høyere enn for de improviserte nesesprayene. Dette var svært viktig for den videre utviklingen av nesesprayen, som nå er godkjent av legemiddelverkene i 12 europeiske land.

Avhandlingens tittel: *Nalokson ved opioidoverdose - farmakologiske aspekter og dosering i klinisk praksis*
Kandidat: *Ida Karin Tylleskär*
Institutt: *Institutt for sirkulasjon og bildediagnostikk*
Veiledere: *Arne Kristian Skulberg, Ola Dale, Linn Gjersing og Lars Petter Bjørnsen*
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for graden PhD i medisin og helsevitenskap.
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Contents

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First of all, I would like to express my deepest gratitude to my two main supervisors, Ola Dale and Arne Kristian Skulberg. Their dedication and continuous support have been important while working on this project and as my mentors they have always pushed me one step further. Through this eight-year long journey they have guided me in both science and in life, influencing who I am today. It has been inspiring and a joy working with you. I would also like to thank my co-supervisors Linn Gjersing and Lars Petter Bjørnsen for their encouragement, their swift and detailed comments and for always sharing their knowledge.

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Conducting clinical trials of new pharmaceuticals requires knowledge and expertise in many areas. A special thanks to the Clinical Research Facility at St. Olavs hospital and their staff who diligently carried out the studies and always took care of the participants. I am grateful for the assistance with Good Clinical Practice and monitoring by the Unit for Applied Clinical Research at NTNU and the facilities for drug analyses provided by Proteomics and Metabolomics Core Facility, NTNU. Thank you to the Department of Biopharmaceutical Production who produced the nasal sprayers, to the hospital pharmacy at St. Olavs hospital who delivered the comparator and to the Norwegian Medicines Agency providing valuable scientific advice. I am also very grateful for the work done by dne pharma on the spray production which was essential in taking this project one step further.

Last but not least, I thank my family, friends and husband-to-be for always being supportive and cheering me on. I could not have done this without you.

Ida Karin Tylleskär

Trondheim, May 2020

Summary

Deaths from opioid overdoses are increasing and a public health concern. Such overdoses are treated with the antidote naloxone and first aid. For lay people there has been calls for naloxone to be available for administration as a nasal spray. Improvised nasal sprays without scientific documentation have been in wide-spread use. Therefore, NTNU started development of a high-concentration nasal spray in a device delivering a small volume, especially designed for nasal administration. The overall aim of this thesis was to provide an evidence-base for adequate treatment of opioid overdoses using intranasal naloxone.

To describe pharmacokinetic parameters an open-label, randomized, three-way crossover study in healthy volunteers was conducted. Intranasal naloxone 0.8 and 1.6 mg were compared to 1.0 mg intravenous naloxone by measuring the blood concentrations of naloxone. The pharmacokinetic-pharmacodynamic aspects of naloxone was investigated in an explorative study in healthy volunteers administered 1.0 mg intravenous naloxone after steady state opioid agonism was obtained by a target-controlled infusion of remifentanil. Opioid effect was measured by pupillometry and simultaneous arterial and venous blood samples for drug quantification were collected. The current use of naloxone was investigated in a 5-year observational study of pre-hospital naloxone administration by Oslo emergency medical services. Data was linked to the Cause of Death Registry.

The uptake was rapid, and bioavailability of nasal naloxone was 52-54%. The nasal spray was well-tolerated and there were no serious adverse events. The remifentanil infusion provided steady state conditions and the effect of 1.0 mg intravenous naloxone rapidly reversed the opioid effect. In 2215 overdose cases, 92% were treated with intramuscular naloxone, initial doses were 0.4 or 0.8 mg. One-week mortality from drug-related deaths were 4.1/ per 1000 episodes with no deaths from rebound toxicity.

The nasal spray had a rapid, systemic uptake and a higher bioavailability than improvised sprays. This indicates that an optimized nasal spray may deliver a therapeutic dose of naloxone. Using remifentanil TCI to obtain a steady-state opioid agonism may be a useful tool for comparing new naloxone products. Intramuscular naloxone in doses of 0.4 - 0.8 mg were effective and safe in the treatment of prehospital overdoses.

Sammendrag

Dødsfall fra opioidoverdoser er et stort folkehelseproblem og utdeling av nesespray med motgiften nalokson var foreslått som et tiltak for å redde liv. I 2012 fantes det ingen godkjente nesesprayer på markedet. Istedenfor var improviserte, ikke-godkjente sprayer uten vitenskapelig evidens i utstrakt bruk. NTNU startet derfor utviklingen av en slik nesespray. Det overordnede målet med denne doktorgraden var å bidra til evidensbasert behandling av opioidoverdoser med nalokson som nesespray.

Nesesprayens opptak ble undersøkt ved at friske frivillige deltok i en åpen, randomisert, treveis overkrysningsstudie. Nesesprayen ble undersøkt i to ulike doser, 0,8 mg og 1,6 mg, og ble sammenlignet med 1,0 mg nalokson intravenøst ved å se på legemiddel-konsentrasjonen i blodet. For å undersøke effekten av nalokson ble det utviklet en modell hvor friske frivillige fikk 1,0 mg nalokson intravenøst samtidig som de fikk en målstyrt infusjon med opioidet remifentanil. Slik kunne effekten av nalokson måles med endring av pupillestørrelse, samtidig som vi tok både arterielle og venøse blodprøver for å måle konsentrasjonen av legemidlene. Gjennom en 5-års observasjonsstudie i ambulansetjenesten i Oslo undersøkte vi også den nåværende bruken av nalokson ved overdoser, og koblet disse dataene mot dødsårsaksregistret.

Opptaket av legemidlet gikk raskt og biotilgjengeligheten av nesesprayen var 52-54%. Nesesprayen var godt tolerert og det var ingen alvorlige bivirkninger. Remifentanil-infusjonen gav stabile blodkonsentrasjoner gjennom forsøket. Intravenøs nalokson reverserte raskt effekten av opioidet og effekten varte i 118 minutter. Av 2215 overdosepasienter i Oslo fikk 92% intramuskulær nalokson og startdosene var 0,4 og 0,8 mg. En ukes mortalitet for narkotikautløste dødsfall var 4,1/1000 tilfeller. Ingen av dødsfallene var på grunn av reintoksikasjon.

Nesesprayen hadde raskt, systemisk opptak og høyere biotilgjengelighet enn improviserte nesesprayer. Dette indikerer at en optimalisert nesespray kan gi en terapeutisk dose nalokson. En målstyrt remifentanil infusjon gav stabile konsentrasjoner av opioidet i blodet og kan være en nyttig modell for å teste nye naloksonprodukter. I ambulansetjenesten var intramuskulær nalokson i dosene 0,4-0,8 mg effektivt og trygt.

List of papers

Paper I

Tylleskar I, Skulberg AK, Nilsen T, Skarra S, Jansook P, Dale O. *Pharmacokinetics of a new, nasal formulation of naloxone* (European Journal of Clinical Pharmacology, 2017, 73:555–562)

Paper II

Tylleskar I, Skulberg AK, Skarra S, Nilsen T, Dale O. *Pharmacodynamics and arteriovenous difference of intravenous naloxone in healthy volunteers exposed to remifentanil* (European Journal of Clinical Pharmacology, 2018, 74:1547–1553)

Paper III

Tylleskar I, Gjersing L, Bjørnsen LP, Braarud AC, Heyerdahl F, Dale O, Skulberg AK. *Prehospital naloxone administration – what influences choice of dose and route of administration?* (Preprint. Version 1, 29 April 2020) Available at Research Square: https://doi.org/10.21203/rs.3.rs-24941/v1

Declarations of interest

Paper I of this thesis presents data on an Investigational Medicinal Product, an innovation by Professor Ola Dale and Norwegian University of Science and Technology (NTNU). NTNU and its subsidiary Technical Transfer Office (TTO) have after this study was conducted, agreed on a cooperation and licensing agreement with dne pharma as (Oslo, Norway). The agreement regulates the ownership, sale and sharing of any profits from the nasal naloxone formulation. According to this agreement, NTNU remain in ownership of the innovation and dne pharma have the rights to commercialize the nasal spray. Any proceeds will be divided between dne pharma, NTNU, TTO and Ola Dale. NTNU has secured publishing rights for all results from all the studies in the project. I have no financial benefit, direct or in-kind from any proceeds from sale of Ventizolve.

Abbreviations

AD	Anno Domini
AE	Adverse event
AOR	Adjusted odds ratio
AUC_{0-t}	Area under the curve until last sample
$AUC_{0-\infty}$	Area under the curve extrapolated to infinity
CI	95% confidence interval
Cmax	Maximum serum concentration
CO_2	Carbon dioxide
EMA	European Medicines Agency
EMS	Emergency medical services
F	Bioavailability
FDA	US Food and Drug Administration
GCP	Good Clinical Practise guidelines
GCS	Glasgow coma scale
ICH	International Conference on Harmonisation of technical requirements for registration of pharmaceuticals for human use
IM	Intramuscular
IN	Intranasal
IV	Intravenous
ke0	Serum-effect-site equilibration rate constant
Lambda-z / ke	Elimination rate constant
LCMSMS	Liquid chromatography-tandem mass spectrometry
LOQ	Limit of quantitation
NTNU	Norwegian University of Science and Technology
OR	Odds ratio
PK	Pharmacokinetic
PD	Pharmacodynamic
RR	Respiration rate
TCI	Target controlled infusion
THN	Take-home naloxone
Tmax	Time to maximum serum concentration
Tmax50	Time to 50% of maximum serum concentration
Tmax80	Time to 80% of maximum serum concentration
WHO	World Health Organization

Definitions

Agonism	The process where a drug binds to a receptor and activated the receptor to produce a biological response.
Antagonism	Describes a situation where a drug binds to a receptor without activating it, and by doing so prevents the binding of the agonist and causes an action opposite to that of the agonist.
Opioid	Opioids are psychoactive substances derived from the opium poppy, or synthetic analogues with similar effects that bind to opioid receptors.
Opioid overdose	Opioids in high doses can cause respiratory depression and death. An opioid overdose is recognized by a combination of three signs: unconsciousness, respiratory depression and miosis.
Naloxone	An antagonist to the opioid receptor. Reverses opioid effects.
Off-label use of drugs	Use of a marketed medication for other indications, doses or routes of administration than what is specified in the given marketing authorization.
Pharmacokinetics	The science of studying time-course of concentrations of a drug to describe the absorption, distribution, and elimination of drugs.
Pharmacodynamics	The science of measuring biochemical and physiologic effects of pharmaceuticals.
Area under the curve	Description of total systemic exposure of a drug to the body.
Bioavailability	The fraction or percent of administered dose that reaches the systemic circulation intact.
Cmax / Tmax	The highest concentration of a drug after administration, and the time it occurs.

Introduction

Opioid overdoses take young lives and are preventable. Prevention is multi-faceted and take-home naloxone by means of a nasal spray containing the opioid antidote is one such measure. This dissertation is a part of a research and development project that lead to commercialization of such a spray. Characterization of the pharmacology of naloxone was done by studies in healthy volunteers, while the current use of naloxone for opioid overdose by the emergency medical services was also explored.

Opioids

Few drugs have been more influential in the history of man than the opioids. The know-how on processing of opium for pain-relief and euphoria have been known at least since Roman and Greek times and is described in detail in works such as *Materia Medica* published around 70 AD by the Greek physician Dioscorides of Anazarbus (1). Throughout history it has been popular in different forms and used for a variety of medical purposes from pain relief, antitussive, diarrhea treatment, and for sedation and insomnia treatment. It has been important in trade, wars and culture throughout history, always known for its delighting and intriguing abilities, and its deceiving capacity to enslave its users (1). Since the isolation of morphine in early 1800s, it was not long before chemical changes to these molecules were introduced in an effort to try to improve them (1). The pursuit of nonaddictive opioids was a high priority. This drove the development of new compounds and was important for the development of modern chemistry and pharmacology as we know it (1). The goal of a nonaddictive opioid was assumed to be achieved with the 1897 discovery of diacetylmorphine, known as heroin, an assumption that arguably turned out to be one of the largest blunders in the history of pharmacology (1). In 1868, the first laws were passed to regulate the sale of opioids and limit the sale to qualified pharmacists (2), but it has been inherently difficult to limit its use. Today, opioids are still one of the most fascinating drugs in use, superior as an analgesic, and with the strong addictive properties and risk of overdose.

Mechanism of action

Opioids act through binding to the opioid receptors, of which there are three different types; mu (μ), delta (δ) and kappa (κ). The mu receptor is responsible for most analgesic effects (3). Opioid receptors are located throughout the central nervous system in the pain modulating pathways and the respiratory center, as well as peripheral tissues in the rest of the body (4). The opioid receptors are so called G-protein coupled receptors located in the cell membrane. When stimulated they send intracellular signals that reduce the opening of voltage gated calcium channels and stimulate the potassium efflux. The opioids also reduce the production of cyclic adenosine monophosphate (cAMP) that produces a variety of signaling changes (5). These changes effectively prevent neurotransmitter release and excitation of neurons (5). Ligands that act on the opioid receptor are classified according to their ability to initiate the effects from the receptor. Full agonists such as morphine and fentanyl bind effectively to the receptor and activate it, while partial agonists like buprenorphine induces a partial response (6). Antagonist is a drug that binds to a receptor without activating and thereby blocking its effect. Opioids commonly used on medical indications are morphine, fentanyl, oxycodone, buprenorphine and methadone (3). Traditionally, heroin is the opioid most frequently used illegally but there are increasing use of synthetic opioids (7, 8).

Opioid overdose

The three main signs of an opioid overdose are reduced or absent respiration, reduced consciousness and excessive constriction of the pupil (miosis) (9). The opioid overdose physiology is complex and not fully understood but the binding of opioids to the respiratory centers in the brain stem seems to be a central mechanism (5, 10). All opioids that act on the mu opioid receptor can cause this ventilatory depression and the effects are dose dependent. The respiration rate is reduced, and the opioids promote an irregular breathing pattern (11). Larger doses reduce the tidal volume and cause respiratory arrest (5). Insufficient respiration leads to hypercapnia (elevated levels of carbon dioxide, CO_2), hypoxia (low levels of oxygen) and acidosis (11). This deprives the body and vital organs of oxygen and causes organ failure, coma and death (11, 12). Normally, hypercapnia and hypoxia stimulates the ventilatory system but as a part of the intoxication, the

chemoreceptors are less sensitive (5). The magnitude and speed of the ventilatory depressant effect depends on how fast the drug concentration rises at the effect site. Quickly rising concentrations have a higher risk of inducing apnea, than a gradual increase in opioid levels where a slow onset of hypercapnia maintains respiration (5, 13). This might be one of the explanations of the risk related to intravenous injections of opioids, and an explanation of why people who inject opioids have a higher risk of overdose (14). Combining opioids with other sedatives such as benzodiazepines or alcohol, increases the potency and reduce the ventilatory drive more than after opioids alone (12), and they are often found together in both fatal and non-fatal overdoses (11, 15-17).

Overdose from opioids with a potential fatal outcome is a serious problem worldwide (7). The European Monitoring Centre for Drugs and Drug Addiction reports that people who use opioids have a 5-10 times higher risk of dying than their peers, and this increased mortality risk is primarily related to overdose (8). Those who inject heroin or other opioids are considered to have the highest risk for death from overdose. Despite efforts to reduce the number of drug related deaths 8,000-10,000 people die from overdose in Europe each year, and 78% of the overdoses involve opioids (8). The numbers have been relatively stable over time, but several countries report higher overdose rates the last years (8, 11). Norway registered 286 overdose deaths in 2018, of which 82% were related to opioids (18). Norway has one of the highest death rates per capita in Europe (8) and the numbers have been stable at this high level for 15 years, despite public health measures and a national strategy to combat the problem (18, 19). The last few years there has been a change in the pattern of opioid use in Norway from heroin being the main culprit to an increasing numbers of overdoses from synthetic opioids and prescription drugs (18).

The United States has seen a large increase in deaths from opioid overdoses over the last 20 years. In 2017 there were 47,600 deaths related to opioids in a population half the size as Europe and the US opioid epidemic was declared a public health emergency (20, 21). The rise in opioid deaths in United States is assumed to be related to liberal opioid prescribing to patients with non-malignant pain (22, 23), fueled by misconceptions about opioid depot formulations not being addictive and aggressive marketing of opioids (24-

26). The National Survey on Drug Use and Health found that 3.6% of people using nonmedical prescription pain relievers initiated heroin use within 5 years of first use of nonmedical opioids (27). The increase of opioid deaths started slowly 20 years ago with deaths from prescription opioids with recent surges in deaths from of heroin and fentanyl overdoses (7). It has been suggested that introduction of abuse-deterrent opioid formulations and stricter prescribing practices is forcing patients to switch to other substances such as heroin and illegally produced fentanyl (28-30), but it has also been reported that it is due to heroin being cheaper and easier to access (27).

Naloxone

While agonists bind to the receptor and initiate an effect, an antagonist is a ligand that inhibits the effects from the receptor (6). The first pure opioid antagonist that became available for use were naloxone (3). It is a competitive antagonist against all opioid receptors, but with the highest affinity for the mu opioid receptor (3). It displaces the opioids and binds with higher affinity to the same binding sites without activating the receptor (Figure 1). Actually, its specificity for the opioid receptor is so high that radiolabeled naloxone was used when the opioid receptors was first discovered in 1973 (31).

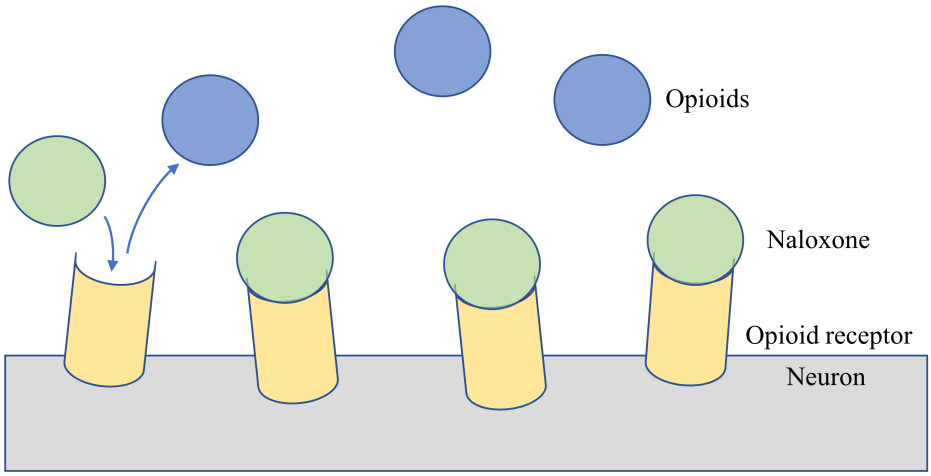

Figure 1. Naloxone competing against the opioids to bind to the opioid receptor.

Naloxone has the chemical structure $C_{19}H_{21}NO_4$, Figure 2. It has a similar structure to oxymorphone, and is derived from thebaine from the opium poppy (32). It normally comes in the form of a salt, either as naloxone hydrochloride anhydride or as naloxone hydrochloride dihydrate (32). Throughout this thesis, the term naloxone will be used and refers to naloxone hydrochloride, unless otherwise specified. Naloxone is metabolized in the liver primarily by glucuronide conjugation and the major metabolite is naloxone-3-glucuronide, which is then excreted in the urine (33, 34). Naloxone has a high first-pass metabolism and a negligible oral uptake (33). It is therefore administered parentally (33). At the start of this project is was available in vials and prefilled syringes for intravenous (IV), intramuscular (IM) and subcutaneous use (9).

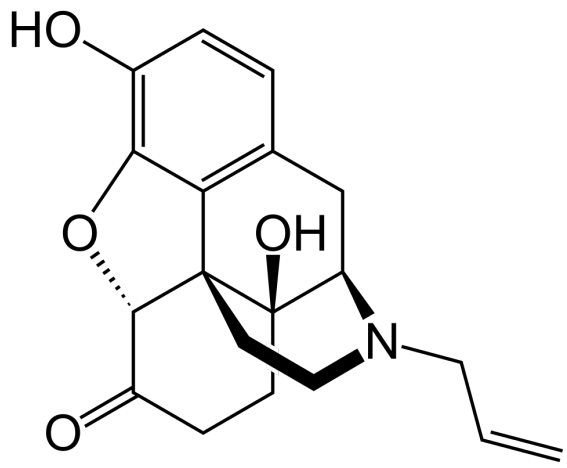

Figure 2. The chemical structure of naloxone.

Naloxone effectively blocks the opioid receptor without producing any physiologic effect itself and thereby counteracting the opioid effect even though the opioids are still in the body. This normalizes breathing and the patient regains consciousness (33). The effect comes within 1-2 minutes after intravenous administration, and within 3-7 minutes after intramuscular or subcutaneous injection (35). The duration of action is somewhat longer after intramuscular than intravenous naloxone, due to the slower absorption. The elimination half-life of naloxone is 1-1.5 hours (33) and the effect of a therapeutic injection may last 2 hours (36). Older studies found that it had a volume of distribution

of 200 L and a total clearance of about 2L/min. However, newer studies reported volumes of distribution of about 320 L and clearances between 3 and 4L/min, two or three times higher than the maximal liver clearance indicating a significant extrahepatic metabolism of naloxone (37).

Naloxone hydrochloride has a wide dosing range that is considered safe, and a review of guidelines found different dosing recommendations ranging from 0.02 mg and up to 20 mg (38). There are different guidelines for in-hospital overdoses and opioid overdoses in the community. In-hospital overdoses occur in a controlled setting related to anesthesia or analgesia where the patients are overdosed with a known opioid at a known dose. This makes reversal easy and controlled, and 0.1-0.2 mg intravenous naloxone is usually recommended, but some advocate for a lower starting doses of 0.02-0.04 mg (38, 39). In community overdoses the doses and the identity of the drugs are often unknown and drugs are often used in combinations which potentiates their effect (9). The setting is often more acute and less organized. This makes reversal more difficult and the recommended starting dose is commonly 0.4-2.0 mg either intravenously or intramuscularly, and titrate to clinical effect to a maximum dose of 10 mg (9, 33). The Norwegian emergency medical services' guidelines for treatment of community overdoses recommend administering 0.4-0.8 mg intramuscular followed by 0.4 mg intravenous naloxone, the IV dose for rapid onset and the IM for presumably longer duration (40, 41).

There have been reports on hypertension, pulmonary oedema and cardiac arrythmias after reversal with naloxone (33), but it is generally considered a safe medication with a wide safety margin. It has few effects when administered to patients not under opioid influence and large doses of 4 mg/kg have been tested in humans, only causing small effects such as a mild increase in heart rate and systolic blood pressure (3). But care must be taken when used in patients currently treated with opioids for pain and in people with opioid dependence. In the first group, the use of naloxone may result in abrupt onset of significant pain (42). In patients with opioid dependence naloxone can cause acute opioid withdrawal syndrome, recognized by physical signs and symptoms like tachycardia, rhinorrhoea, lacrimation, sweating, piloerection, muscle aches, nausea, vomiting, abdominal cramps and diarrhea (43). It can also cause neuropsychiatric symptoms like

restlessness, anxiety, irritability, agitation, intense drug craving and drug seeking behavior (43). Opioid withdrawal is not considered life-threatening. However, withdrawal symptoms are a feared complication among drug users and violent behavior and drug craving are far from trivial and withdrawal may also make patients refuse further necessary follow-up (37, 44). Guidelines and dosing recommendations therefore emphasize the importance of titrating naloxone to clinical response to avoid withdrawal syndrome if possible (9, 37, 38, 45).

The antagonistic effect of naloxone may be followed by recurrent agonism if the opioid is still present in high concentrations as naloxone is eliminated. This is a continued clinical concern as overdose patients are often discharged on site after naloxone treatment by the emergency medical services (EMS). Rebound opioid toxicity has been widely studied, and the conclusion of the reports is that the risk of death is very small, ranging from 0%-0.13% (46-48). Recently, there are reports of an increase of overdoses from other opioids than heroin, such as long-acting agents like methadone, buprenorphine, and tramadol (8, 49). These have been shown to have a higher risk for recurrence of toxicity (50).

With the uncertainty regarding the optimal administration routes, dosing interval, and risk of withdrawal and rebound toxicity, there have been debates on what dose and administration route that should be the benchmark for new naloxone products. The World Health Organization concluded that it was not clear how much naloxone should be carried by lay first responders. They suggested this could be addressed by monitoring the naloxone doses used in the field (9). The continuing changes in the types of opioids used in the community, changes in harm-reduction strategies and addiction treatment may also influence naloxone recommendations. There is therefore a continuous need for updated science in this field.

Naloxone is the antagonist most commonly used for opioid overdose reversal. There are other opioid antagonists as well with somewhat different properties. Nalmefene can be used to treat opioid overdoses but have a slower onset and a longer half-life. Naltrexone can be used to manage alcohol and opioid dependence, while methylnaltrexone, which

do not cross the blood brain barrier, is used to treat opioid induced bowel dysfunction without reversing analgesia (3).

Take-home naloxone

If witnessing an opioid overdose, basic first aid such as securing the airway and performing rescue breaths will provide oxygenation and reduce the risk of brain injury and cardiac arrest in the patient (9). In the last decades there have also been initiatives to make prescription drugs previously in the domain of health professionals available for patients and lay people to treat emergency conditions. New drug formulations have been created, such as the adrenaline-autoinjector for patients with anaphylaxis and buccal midazolam for patients with epilepsy (51, 52). In this vein there has been a growing interest for take-home naloxone (THN) among politicians, medical staff, and caretakers around the world. As many who use opioids report having witnessed overdoses, there is an opportunity to intervene early (9, 53-55). Both peers and family members have been shown to be willing to act as first responders and administer the antidote naloxone (56-58).

The first overdose education and naloxone programs were started as local, grassroot initiatives. In 2010, there were 50 such programs across 15 states in the US. In 2010, they reported having distributed 38,860 naloxone vials over the last year and 10,171 overdose reversals were performed since their start in 1996 (59). Syringe exchange and harm reduction programs were early adopters of these initiatives. The first programs distributed vials of naloxone, syringes and needles for intramuscular administration, but simpler formulations were asked for to further increase availability and expand access. A needle-free naloxone alternative would be favorable, and nasal naloxone was preferred by users (60). No approved products were available, improvised devices with formulations for injection combined with a mucosal atomizer were taken in use for nasal administration. Clinical use indicated that this approach worked, but results were varying (61, 62). A systematic review concluded that the evidence was weak and that there were conflicting results regarding the efficacy of intranasal (IN) naloxone (63). Despite the lack of evidence, there was a widespread use of such off-label intranasal naloxone kits and they accounted for 20.3% of the take-home naloxone provision in US in 2014 (64).

The World Health Organization (WHO) concluded in 2014 that there were few well conducted studies on nasal naloxone (9) and they gave a conditional recommendation to the use of nasal naloxone in THN-programs, despite the lack of licensed products for nasal administration. Regarding dosing of naloxone in community overdoses they stated that questions remained about the optimal dosing and formulation for the intranasal route of administration (9). As a response to the overdose epidemic in the United States, the National Institute of Drug Abuse, the US Food and Drug Administration (FDA) and the US Centers for Disease Control and Prevention initiated development of adequate naloxone nasal sprayers through American pharmaceutical companies (65) and the FDA granted fast track applications to speed up this development. In addition to laypeople administration the development of nasal sprays could impact treatment by the emergency medical services by eliminating the risk of needle stick injuries and shorten time to treatment as venous cannulation of the people who inject drugs may be challenging (63).

Nasal drug administration

The nose is a valuable route of administration of drugs for systemic action as it is easily accessible, has a quick onset of action and avoids the first-pass metabolism of the liver. The reason for this is its anatomy and physiology. The respiratory zone with the turbinates is the main site for drug absorption (Figure 3). It is a highly vascularized and permeable surface, with an area of around 120-150 cm^2 (66). The respiratory epithelium is made up of basal cells, mucus producing goblet cells and ciliated cells. The cilia are continuously "beating" mucus backwards towards nasopharynx where it is swallowed, known as mucociliary transport (66). The olfactory zone is found deep in the nose. It is very small, around 1-5cm^2, but is interesting as it might enable a bypass of the blood brain barrier directly into the central nervous system. Such nose-to-brain uptake of drugs has been proven in animals, but studies have yet to prove that this route exists in humans who have a smaller olfactory region than most mammals (66). If possible, it is a very exciting future treatment option.

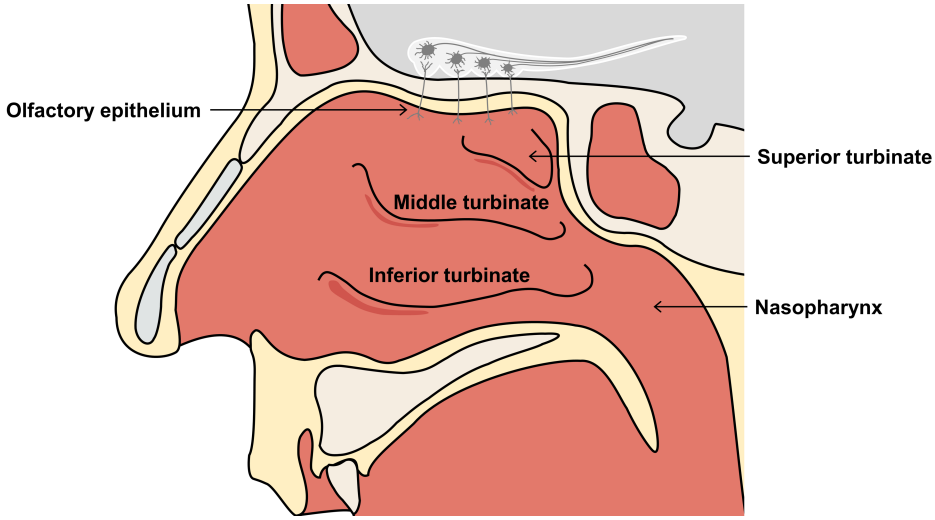

Figure 3. The different areas of the nasal cavity. Drug deposition following intranasal administration mainly occurs in the respiratory zone around the inferior turbinate.

Nasal drugs need to be specially formulated for their purpose. Drugs can be absorbed in the nose for about 15-30 minutes after deposition. Due to mucociliary transport, drugs sprayed into the nose will eventually be swallowed and subsequently be subject to first-pass hepatic metabolism (67). Nasal uptake can be enhanced by prolonging the retention time of the drug in the nose, for example by using thickeners to increase viscosity. A low spray volume is also critical for nasal uptake. Studies have shown that the nose can only retain small volumes without an immediate anterior or posterior run-off from the nose (68-70). Such run off will decrease absorption and generate an undesirable variability. A too large volume is also uncomfortable for the patient. It is therefore commonly recommended that nasal drugs are administered in a volume of 100 – 150 µl per nostril (66, 67, 71, 72). An early pharmacokinetic study of nasal naloxone found 4% bioavailability. In that study, up to 2.5 ml was administered in each nostril, and the participants swallowed a considerable amounts of the drug despite their best efforts not to (73). This stands in contrast to nasal bioavailability of 65 % or more for specially formulated nasal formulations of other drugs (74-76). This understanding raised concerns regarding the use of large volume nasal sprays used in take-home naloxone programs around the world.

In a study of 2 mg naloxone administered nasally as powder, the bioavailability was 30% with a maximum serum concentration (Cmax) of 1.6 ng/ml and a time to maximum serum concentration (Tmax) of 20 min (77). This showed that a higher bioavailability was achieved when the issue of volume was circumvented. On this background we concluded that a well-formulated high-concentration/low-volume naloxone spray could potentially deliver a therapeutic and predictable dose of naloxone through the nose. Suitable ready-to-use devices would also be preferred to deliver the drug in an easy fashion, rather than devices that must be assembled during a stressful situation, as this has been proven difficult even if combined with training (78).

Pharmacokinetics

Pharmacokinetics (PK) is the science of absorption, distribution, and elimination by metabolism and excretion of drugs. Pharmacokinetics describes how the body affects a pharmaceutical product after its administration and is based on the quantification of the actual drug in biological samples, plasma being the most commonly used matrix (79). It can be used to compare different administrations routes of a drug.

Absorption is the process by which a drug passes unchanged from the site of administration to the site of measurement, usually the blood (80). The extent of absorption is decided by the characteristics of a drug and its route of administration. Drugs may be lost in the gut, or metabolized in the liver and never reach the systemic circulation, this is known as first-pass hepatic metabolism (80). Distribution is the process of the drug being transported to and from the blood and other tissues in the body, for examples into the brain and to the liver (80). Blood flow, protein binding and the hydrophobicity of the drug affect is distribution and is described with the parameter distribution volume (80). Elimination happens either by metabolism or excretion and clearance describes the volume of body fluid which the drug cleared from the body per unit of time (80). The metabolism happens mainly in the liver, but there can also be a significant amount of extrahepatic metabolism (80, 81). Drug excretion happen by a number of routes, the most important being the kidneys (80). The processes of absorption, distribution and elimination happen simultaneously, but with time the drug concentration reaches an equilibrium. After this any decline in plasma concentration is due to elimination (82).

Most drugs, including naloxone is eliminated according to a first-order process, meaning that in the elimination phase the drug amount eliminated is directly proportional to the serum drug concentration (82). This give a mono-exponential decline in drug concentration during the elimination phase (82). When this is displayed as a semilogarithmic plot, the elimination phase will become a straight line (Figure 4). The opposite is true where there is an upper limit to the elimination capacity and a constant amount of drug is eliminated per time unit. This is called zero order elimination (80).

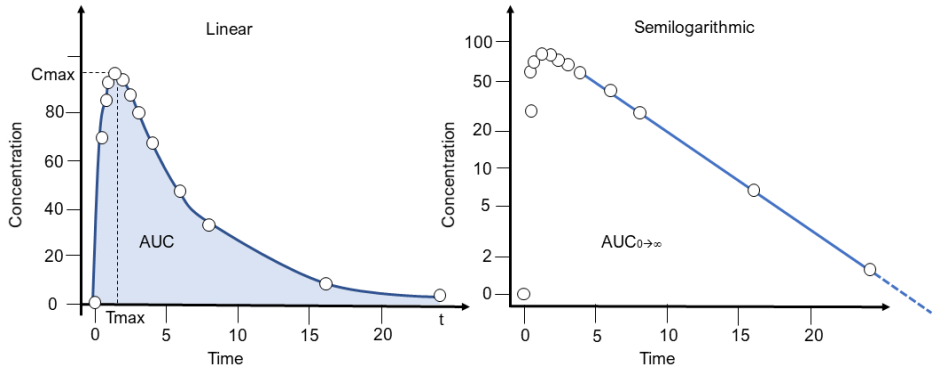

Figure 4. The maximum concentration (Cmax) and time to maximum concentration (Tmax) is illustrated in the panel to the left. The area under the curve until last measurement ($AUC_{0\rightarrow t}$) can be calculated using trapezoidal method (left), while the total area under the curve ($AUC_{0\rightarrow\infty}$) can be estimated by using linear regression on a semilogarithmic plot.

In pharmacokinetics, information on dose of a drug and the following blood concentrations can be combined with mathematical models and equations to describe the drug movement in the body. Non-compartmental methods are commonly used for this purpose. Using this technique, no assumptions are made on the body being made up of different compartments and central pharmacokinetic concepts can be directly interpreted from a concentration-time graph (Figure 4). The results are accurate and acceptable for bioequivalence studies (83).

The highest concentration of a drug after administration is referred to as the maximum concentration (Cmax). The time this occurs at is referred to as the time to maximum concentration (Tmax). They are both dependent on absorption and elimination (79). For many drugs, serum or plasma drug concentration measurements can offer a useful

correlate of response (84). For fast-acting drugs like naloxone, Tmax can be a good approximation of time-to-effect. Venous blood sampling is usually used for these estimations, but for some drugs, there is an arterio-venous difference in plasma drug concentrations early after administration because drugs are distributed to the tissues during perfusion. For fat-soluble drugs this happens to a larger degree, and a significant arteriovenous difference with a shorter arterial Tmax and higher Cmax have been shown for opioids such as fentanyl, heroin and remifentanil (85-87). As arterial blood is supplying the brain the arterial drug concentration is more interesting as the drug exerts its effect in the central nervous system.

In non-compartmental analysis, the total systemic exposure of a drug is estimated by the area under the curve (AUC) (79). The area under the curve until last measurement ($AUC_{0\rightarrow t}$) is calculated, often using linear trapezoidal method (79). The elimination rate constant, ke, also known as lambda-z, can be estimated by applying linear regression on the semilogarithmic time-concentration curve (Figure 4). This can be used to estimate important pharmacological concepts such as clearance, distribution volume and the elimination half-life of the drug (82). It can also be used to estimate the total area under the curve ($AUC_{0-\infty}$) by extrapolation of the curve after the last measurement. A sufficient number of blood samples must be collected to adequately describe the plasma concentration-time profile. Frequent sampling around the expected Tmax is recommended to provide reliable estimates of peak exposure, and at least 3-4 samples collected during the elimination phase to reliably estimate the elimination rate constant (83).

Several factors can affect the systemic exposure. Intravenous drug administration delivers the drug directly to the blood circulation and gives a 100% exposure. By all other administration routes, drug can be lost on its way to systemic circulation (79). The term bioavailability is used to describe the fraction of the administered dose that is intact and absorbed to the systemic circulation. It is calculated by comparing the area under the curve from different administration routes (88). Absolute bioavailability refers to the absorbed fraction of a drug related to the intravenous administration of the same drug.

Relative bioavailability refers to the absorbed fraction related to some other administration form such as intramuscular or peroral route (88).

Another way to analyze pharmacokinetic data is by using compartmental models. In such models one assumes a central compartment representing rapidly equilibrating tissues like blood, kidneys and liver, and one or more compartment(s) representing other parts of the body, such as slower equilibrating tissues like muscles and fat (82). The compartments are hypothetical, but with use of complex differential or poly-exponential equations they can be used to describe drug movements in a more complex fashion (89). Such models are more difficult to develop and validate, and assumptions on the compartmental model that influence the results from the modelling are made. Their advantage is that they can be used to predict the concentration at any given time point, unlike non-compartmental models which are descriptive only.

Pharmacodynamics

Pharmacodynamics (PD) is the science of measuring biochemical and physiologic effects of pharmaceuticals. Pharmaceuticals interact with enzymes, proteins and receptors that produces the effects of the drug (84). Drug effects can be studied alone or can be combined with pharmacokinetics data. For naloxone the site of action is the opioid receptors in the central nervous system. To produce an effect, the drug must therefore cross the blood-brain-barrier. It must bind to the opioid receptors and this must produce intracellular changes that counteracts the opioid effects (89). These additional steps can delay the onset of effect compared to the uptake in the blood (89).

The most prominent effect of naloxone its capacity to counteract the effect of opioids, it produces few measurable effects when administered alone. Therefore, the effect of naloxone must be assessed under influence of opioids. There are several different ways to study the effects of opioids and the subsequent decrease of effect when administering naloxone. Common methods are measuring different aspects of opioid effects such as drug liking, analgesia, respiratory depression or miosis (90-92). Respiration can be by counting the respiration rate or measuring oxygen saturation, carbon dioxide at the end of an exhaled breath (end-tidal CO_2), respiratory inductance plethysmography or by

parasternal intercostal muscle electromyography (93-95). One objective measure that is often used for evaluation of opioid effect is pupillometry. This is possible as the opioids cause miosis and it is an easy and non-invasive procedure (96-100).

The study of the antagonism of opioids are rarer, and the numbers of pharmacodynamic studies of naloxone is very limited. Withdrawal symptoms and pupillary response assessed by photography were used to measure the effect of naloxone in people addicted to opioids in two studies in the early 1990s (101, 102). A study used end-tidal CO_2 measurements to assess the effect of naloxone in healthy volunteers who got either morphine or morphine-6-glucuronide (103). In this study, they refrained from obtaining plasma samples as they observed during their pilot study that frequent sampling had an excitatory effect on the breathing (103). Subjective measurements like analgesia are difficult to measure reliably, and especially pain has many other confounding factors. Heat pain threshold measurement have been tested in one experimental study of naloxone and was proven unreliable (104). In clinical trials of opioid overdose treatment, return of spontaneous respiration and regaining of consciousness measured with Glasgow coma scale (GCS) have been used (61, 105).

Adverse events

Drugs always have several effects, some desired and others defined as adverse. According to the ICH Good Clinical Practice guidelines an adverse event (AE) is “any untoward medical occurrence in a patient or clinical investigation subject administered a pharmaceutical product and which does not necessarily have a causal relationship with this treatment” (106). During a clinical trial, all events are registered as “adverse events” whether or not they are assumed to be related to the drug. They are then investigated to find if they can be classified as an adverse drug reaction, a reaction related to the drug in question. When studying a new formulation of an existing drug, the already known side effects of the drug will normally be present also in the new administration form. It is also possible to have new adverse effects from the new route of administration (72). Nasal spray specific adverse events are odor, taste, dripping, irritation, epistaxis, nasal congestion and urge to sneeze. A high drug concentration is needed to deliver drugs in small volumes. This high drug concentration might cause local irritation. Additives in the

formulation can cause side effects and should only be used if necessary. Bad taste has been reported for some opioid nasal sprays such as pethidine and butorphanol, but not for fentanyl (72). Other opioid sprays, such as methadone, have been reported to give a burning sensation in the nose (74). A nasal formulation of naloxone in clinical use should not be irritating or cause pain or discomfort (72).

Drug development

The development and approval of new drugs is a well-regulated and complex process. Clinical trials of pharmaceuticals are regulated by the Good Clinical Practice guidelines (GCP) from the International Conference on Harmonisation of technical requirements for registration of pharmaceuticals for human use (ICH) (106). It is an international ethical and scientific quality standard of all aspects of clinical trials that involve participation of human subjects, from study design to reporting trials. It provides a unified standard for the European Union, Japan and the United States to facilitate the mutual acceptance of clinical data by regulatory authorities (106). New drugs and new formulations of existing drug must be approved by medicinal authorities such as the European Medicines Agency (EMA) or the U.S. Food and Drug Administration (FDA).

There are several phases in the development of new drugs. The preclinical phase involves in vitro and animal studies of feasibility, efficacy and drug safety data of drugs (107). It can also include device testing such as spray pattern and drug stability and degradation during different conditions. First-in-human studies are often referred to as Phase I trials or human pharmacology trials and usually involve a small number of healthy volunteers. The aim is to study tolerability, safety, and pharmacokinetics and pharmacodynamics of different doses (107, 108). Phase II testing usually involves up to some hundred patients with the condition that the medication is supposed to treat and focuses on the efficacy of the drug and estimating doses for future phase III studies, as well as safety (107, 108). Phase III studies are usually large scale randomized controlled trials aimed at confirming the efficacy and safety of the treatment in the large patient group (107, 108). The Phase IV of drug development is post-marketing surveillance and pharmacovigilance and is designed to detect less common adverse effects. It also evaluates the use of the drug in the general population more than the previous phases (108). For a drug to be approved

documentation regarding chemical stability, degradation products, antimicrobial properties and a range of other chemical and pharmaceutical matters are needed, in addition to evidence on its clinical usefulness.

For new formulations of existing drugs, it is possible to rely on previous knowledge on safety and efficacy, especially if the indication for use is unchanged. Instead of conducting several and large studies, smaller pharmacokinetic studies in healthy volunteers may be used. This approach was recommended by the FDA and the WHO for developing new naloxone products (9, 109). FDA requires that a nasal naloxone product should generate serum concentrations at least comparable to an approved parenteral route of administration (109). To achieve this, the bioavailability of the drug formulation is essential. If bioavailability was only 4% as indicated in the first pharmacokinetic studies (73), it would be difficult to deliver a therapeutic dose with one nasal spray actuation. However, a pilot study of the current high-concentration/low-volume nasal spray the absolute bioavailability was found to be 47% (110). For a drug that treats an emergency condition, the maximum concentration and time to maximum concentration will also be important as the drug needs to start working quickly, and the concentrations in blood is assumed to be closely related to the drug effect.

Rationale

Deaths from opioid overdoses are an increasing public health concern. As these deaths are preventable, it was recommended that the opioid antidote naloxone was made available to people likely to witness overdoses. New administration routes were suggested for this provision of naloxone. Intranasal naloxone seemed to be a viable route and was preferred among the target groups for the intervention. When this research project started in 2010, there was a widespread use of dilute solutions of naloxone and improvised devices. There were indications that intranasal naloxone could be useful to reverse opioid overdoses, but no high-concentration/low-volume naloxone formulation was available for intranasal administration. The World Health Organization concluded in 2014 that questions remained about the optimal dosing and formulation for intranasal naloxone (9). They suggested it could be addressed by a pharmacokinetic study or a randomized clinical trial (9). They also pointed out that it was not clear how much naloxone should be carried

by lay people and suggested this could be addressed by monitoring the naloxone doses used in the field (9). On this background, our group at NTNU commenced a research and development program of a high-concentration/low-volume nasal naloxone formulation. The results in a pilot study of the spray were promising and indicated an uptake of around 50% (110).

Aim and research questions

The overall aim of this thesis work was to provide an evidence-base for adequate treatment of opioid overdoses using intranasal naloxone. We had the following research questions:

1. Can a single nasal spray actuation deliver a therapeutic dose of naloxone for opioid overdose? (Paper I)

2. Is it possible to develop a model for studying the effect of naloxone in healthy volunteers? (Paper II)

3. What doses and administration routes of naloxone for opioid overdose are used in clinical practice in Oslo, and are these safe in the prehospital setting? (Paper III)

Methods

This thesis is based on two clinical intervention studies and one observational study (Table **1**). The first was a clinical study of nasal naloxone in healthy volunteers. The second study was an explorative study of blood concentrations and effect of naloxone during an opioid infusion, also in healthy volunteers. The third study was an observational study in patients treated with naloxone by emergency medical services in Oslo, with linking of data to the National Cause of Death Registry.

Table 1. An overview over the methods used in the different papers

Paper	Design	Data collection method	Data analysis methods
I	Randomized, open-label, three-way crossover trial. A pharmacokinetic study in 12 healthy volunteers	One and two doses of naloxone nasal spray were compared to intravenous naloxone with blood sampling over 6 hours	- Non-compartmental pharmacokinetic analysis - Mixed model with subject specific random intercepts
II	Explorative, pharmacokinetic-pharmacodynamic study in 12 healthy volunteers	Remifentanil infusion induced a state of opioid agonism that was then reversed with intravenous naloxone. Pupillometry was used to measure the effect. Simultaneous arterial and venous blood sampling over 2 hours	- Non-compartmental pharmacokinetic analysis - Paired sample t-test with and without Bonferroni correction
III	A 5-year observational study of patients treated with naloxone by Oslo emergency medical services. n=2215	Data on sex, age, naloxone doses and administration routes, place of attendance, transfer rates and clinical variables as respiration rate and consciousness measured with GCS score were extracted from medical records and linked to the National Cause of Death Registry	- Descriptive statistics - Univariate and multivariable logistic regression analyses

The nasal naloxone formulation

The solution for intranasal delivery was formulated by using naloxone hydrochloride dihydrate ($C_{19}H_{21}NO_4 \cdot HCl \cdot 2H_2O$, CAS number: 51481-60-8). The naloxone concentration was 8 mg/ml and contained well-known excipients such as glycerine (12 mg/ml) as humectant and isotonic adjustment agent, polyvinyl pyrrolidone (1.0 mg/ml) as viscosity adjuster and absorption enhancer, sodium edetate (0.5 mg/ml) as absorption enhancer and benzalkonium chloride (0.2 mg/ml) as preservative and penetration enhancer. Citric acid-sodium citrate buffer (2.0 and 2.8 mg/ml, respectively) was used to maintain the formulation's pH of 4.3. The formulation was created as contractual work for NTNU by Phatsawee Jansook, PharmD, PhD, Chulalongkorn University, Thailand under support from professor Thorsteinn Loftsson, University of Iceland, Reykjavík, Iceland. A bidose disposable nasal spray device from Aptar Pharma (Louveciennes, France) was used (Figure 5). They deliver 0.1 ml of liquid per actuation. The total dose was 0.8 mg or 1.6 mg of naloxone hydrochloride for one and two sprays, respectively. The formulation was produced, and the device was assembled by the Department of Biopharmaceutical Production, Norwegian Institute of Public Health (FHI), Oslo, Norway. The production complied with Good Manufacturing Practice. The formulation is not patented, but Norwegian University of Science and Technology have a licensing agreement with dne pharma as (Oslo, Norway) regarding the spray formulation giving them the rights to commercialize the nasal spray.

Figure 5. The Aptar Bidose was used for delivering the nasal spray.

Study design of the clinical intervention studies (Paper I-II)

The first study was a randomized, open-label, three-way crossover trial in human, healthy volunteers. The primary outcome in **paper I** was to determine the absolute bioavailability of nasal naloxone, and the secondary outcomes were to investigate maximum concentration, time to maximum concentration and the safety of the formulation.

Thirteen healthy men and women aged 18–45 consented to participation and fulfilled inclusion criteria. They had hemoglobin, creatinine, aspartate transaminase, alanine transaminase, and gamma-glutamyl transferase within reference values and a normal electrocardiogram. Regular use of medications, including herbal medicines, was not allowed. A negative pregnancy test and use of high efficacy contraception were required for women, and they could not be breastfeeding during the study period. Subjects with known drug allergy, drug addiction or previous nasal surgery were excluded. One participant was excluded during the study as the subject no longer met the study criteria.

The subjects participated on three different occasions. They were treated with nasal naloxone on two occasions and intravenous naloxone on one occasion. Blood samples for analysis of naloxone were drawn over a period of six hours. Nasal naloxone was administered as one and two sprays, 0.8 mg and 1.6 mg, respectively. The 1.6 mg dose was administered as 2 x 0.8 mg with one actuation in each nostril at time 0. Norwegian treatment guidelines for the prehospital services recommended starting doses of 0.4-0.8 mg naloxone by injection (40). The doses used in the current study were chosen to match these as a pilot study had indicated a bioavailability of the nasal spray of around 50% (110). The comparator dose was 1.0 mg intravenous naloxone. This was chosen as this is in the middle of the recommended dosing range for the first dose of naloxone in the Summary of product characteristics (33).

Treatment sequences were decided by concealed randomization through a webserver at Unit for Applied Clinical Research, NTNU, Trondheim, Norway. The system does block randomization with varying block size. After drug administration, blood samples for analysis of naloxone were collected over 6 hours. There was a three-day washout period between the treatments. Within four weeks after the last study day, a follow-up interview

was conducted. The study was conducted at the Clinical Research Facility at St. Olavs hospital, Trondheim University Hospital, Norway.

Paper II describe an explorative, open-label study in twelve, healthy volunteers. The aim was to develop a model for studying the effects of naloxone using an opioid infusion. We also examined if there was a significant arterio-venous difference in the pharmacokinetic profile of naloxone under opioid influence. The time-course of arterial and venous concentrations of remifentanil was also explored.

The inclusion/exclusion criteria were similar as in study I, but excluded participants with history of drug abuse, professional access to drugs with abuse potential or prolonged used of opioid analgesics. Participants were screened for drug and alcohol problems using the CAGE AID questionnaire (111, 112). They also had to pass Allen's test of collateral circulation of the hand.

An arterial cannula was placed in the radial artery for the collection of blood samples, and all the participants received local anaesthetics before cannulation to minimize pain. Venous blood samples were drawn from an IV cannula placed in the antecubital fossa. A state of opioid influence was created by a remifentanil infusion and its effect was measured by pupillometry. After 12 minutes of remifentanil infusion the opioid state was considered stable and was then reversed with 1.0 mg intravenous naloxone. The time to, and the effect of, the reversal was measured with pupillometry. Simultaneous arterial and venous blood samples were collected for the analysis of naloxone and remifentanil. The remifentanil infusion was continued for 90 minutes after naloxone administration, a total of 102 minutes of remifentanil for each participant. The study session took three hours. Within four weeks after the last study day, a follow-up interview was conducted. The study was conducted at the Intensive Care Unit with staff from the Clinical Research Facility at St. Olavs hospital, Trondheim University Hospital, Norway.

Acquisition and analyses of biological samples

Blood sampling

In study I venous blood samples were drawn at baseline and at 2, 5, 10, 15, 20, 25, 30, 35, 45, 60, 90, 120, 240 and 360 minutes after naloxone administration. In study II, simultaneous arterial and venous blood sampling for analysis of naloxone were done at baseline and at 2, 5, 10, 15, 20, 25, 30, 35, 45, 60, 90 and 120 minutes, relative to naloxone administration. Blood for naloxone analysis in both studies were collected in Vacuette® tubes (Greiner Bio-One, Austria) and left to coagulate for 30 minutes. Samples were then centrifuged, and 2 ml serum was frozen in cryotubes at −80 °C until analyzed. In study II, whole blood samples for analysis of remifentanil were collected simultaneously from the arterial and venous sampling site. The samples were taken at baseline and at -9.5, -7, - 2, 30, 60 and 90 min relative to the naloxone administration. Blood for remifentanil analysis were collected in sodium heparin blood collection tubes (Vacuette®, Greiner Bio-One, Austria). The tubes were prefilled with 50 % citric acid (weight/volume) solution to prevent hydrolysis of remifentanil through pH-control (113). After vigorous mixing, the blood samples were immediately put on ice and frozen at −20 °C within 10 minutes. Samples were moved to a −80 °C freezer by the end of the day.

Serum concentration analyses by liquid chromatography-tandem mass spectrometry

Quantification of naloxone and remifentanil for studies I and II was conducted using a validated high-performance liquid chromatography-tandem mass spectrometry (LCMSMS) method. The liquid chromatography is used to separate the chemical compounds in the sample before the mass spectrometry is used to identify and quantify them. Selecting substances based on their molecule weight LCMSMS allow for very accurate quantification of analytes and is a commonly accepted method for quantifying drugs in biological samples. It is important to have an accurate and reliable method that is validated and has a low enough limit of quantitation (83). One of the early studies on naloxone pharmacokinetics used an insensitive method for naloxone quantification, introducing a serious limitation to their research (73). The analytical methods for both naloxone and remifentanil were fully validated by assessing linearity, accuracy, precision,

sensitivity, specificity/selectivity, in process and storage stability, dilution integrity and assay ruggedness according to acknowledged principles (114, 115). The calibration range for the naloxone method was 0.02 – 45 ng/ml with nine calibration standards, and a limit of quantitation (LOQ) of 0.02 ng/ml. For the remifentanil method the calibration range was 0.01 – 5.0 ng/ml with eight calibration standards, and a limit of quantitation of 0.01 ng/ml. The analyses were conducted at the Proteomics and Metabolomics Core Facility (PROMEC), Faculty of Medicine, NTNU, Norway. Further details on the analyses are available in appendix 1 and 2.

Pharmacodynamic model

Remifentanil administration

To assess the effect of the opioid antagonist naloxone, it must be given to subjects who are under the influence of an opioid. In study II, this was achieved by an infusion of remifentanil hydrochloride (Ultiva, GlaxoSmithKline, Brentford, United Kingdom). Remifentanil is an opioid binding to the mu opioid receptors and induces the classic opioid symptoms like analgesia, miosis, respiratory depression and reduced consciousness. Remifentanil is a highly potent opioid with a very short half-life of 3-10 minutes as it is metabolized by unspecific esterases in blood and tissue (116). It can quickly reach steady state, and response to discontinuation is rapid with a return to baseline within 10 minutes (116). The drug has previously been used in Norwegian research to assess opioid effects in healthy volunteers (117). It is also commonly used in anesthesia, together with sedative drugs like propofol. Remifentanil was administered by Target Controlled Infusion (TCI) plasma control Minto model (118) delivered with Alaris PK Guardrail syringe pumps (CareFusion Cooperation, UK). TCI is a computerized infusion system designed to rapidly achieve a steady state plasma concentration using a multi-compartment pharmacokinetic model. The dosing regimen consists of a bolus injection, followed by frequent changes in the infusion rate to maintain the targeted plasma drug concentration (119). The Minto model uses the variables age and sex-specific lean body mass to adjust the drug administration (118, 119). The system was set to a plasma target of 1.3 ng/ml. Intravenous naloxone was administered after 12 minutes of

remifentanil infusion. The infusion was continued for another 90 minutes with a total duration of the infusion of 102 minutes.

Pupil measurements

The pharmacodynamics, the opioid effect and its reversal, was estimated by measuring size of the pupils in study II. Pupillometry was chosen as it is a non-invasive measurement, and pupil size is a well-recognized measure of opioid effect (96). It has been used in previous studies of opioids (74, 90) and naloxone (101, 102). Moderate and stable ambient lighting in all study sessions was ensured using a luxometer. Accommodation was controlled by having the participant focusing on a distant point in the room. The measurements were conducted using a Neuroptics VIP 200 Pupillometer (Neuroptics, Irvine, CA, USA). The device has a digital camera that captures several photos and computes the pupil average size based on the photos and reports it in millimeters with standard deviation. The pupillometer was placed over the eye and the position adjusted until the eye was correctly aligned in the screen of the pupillometer. The measurement takes less than 10 seconds. Pupillary measurements were conducted at −20, −17, −14, −3, −1, 1, 4, 7, 9, 12, 14, 17, 19, 24, 29, 34, 39, 44, 49, 59, 69, 79, 89, 99, 109 and 119 min relative to the naloxone administration at timepoint 0. The pupillometer used had a high inter-observer agreement and repeatability of the measurements (120) and the same observer conducted all pupil measurements during each study session.

Safety

In our protocol we used remifentanil. This minimizes risk and increases safety for the participants compared to other opioid agonists. Participants were required to fast, with intake of no solid food six hours prior to and no liquids two hours prior to start (121). The co-administration of naloxone also increases safety and reduce discomfort for the participants. They were monitored by continuous oxygen saturation, three lead electrocardiogram and invasive blood pressure throughout remifentanil infusion. For safety and to avoid adverse events from remifentanil, metoclopramide 10 mg intravenous once, ondansetron 4 mg intravenous once, ephedrine 10 mg intravenous once and oxygen on nasal prongs (max 2L/minute) were allowed as concomitant medications in our study. Additional intravenous naloxone was available as rescue medicine in case of any safety

concerns. The study was conducted at the intensive care unit and a trained anesthesiologist was present at all times during the administration of remifentanil. With all these measures taken it was considered safe to conduct the study.

Pharmacokinetic calculations and statistics

Pharmacokinetic calculations were done in studies I and II using non-compartmental techniques. Variables such as area under the curve, terminal elimination half-life, maximum serum concentration and time to maximum serum concentration were calculated by use of computerized curve fitting using Win-Nonlin (Pharsight Corporation, NJ, USA). $AUC_{0\text{-}t}$ was calculated using linear trapezoidal method. The calculation of $AUC_{0\text{-}\infty}$ and half-life was based on the estimation of the elimination rate constant, ke. The program's best-fit method uses linear regression on a semilogarithmic plot to decide the number of data points to use in the calculation of ke (122). It starts with three points on the regression line, and adding more one by one, aiming to maximizing the value of the adjusted R^2 (122). The estimated slope of the line is equal to -ke. The slope will be negative, but the elimination rate constant is a positive number.

Measurements below LOQ were not used in the analysis. Outlier points of the serum concentration profile that deviated more than twice, or less than half, of the expected value were taken out of the analysis. Missing data were not imputed.

In study I, absolute bioavailability (F) was calculated according to this formula, where AUC is area under the curve and D is dose.

$$F = \frac{[AUC_{IN}] \times D_{IV}}{[AUC_{IV}] \times D_{IN}}$$

Dose-corrected values for $AUC_{0\text{-}t}$ and Cmax for 0.8 and 1.6 mg IN doses were compared with paired *t* test. A p value < 0.05 was considered significant. Within- and between-subject variability of bioavailability and Cmax were examined using mixed models with subject specific random intercepts. Time to 50 and 80% of maximum concentration (Tmax50, Tmax80) were also calculated.

Data was described by mean with 95% confidence intervals if not specified otherwise. SPSS (IBM, NY, USA) was employed for descriptive statistics, while Stata version 14.1 (StataCorp, TX, USA) was used for the mixed models. In study II, comparison of changes in pupil size was performed by paired sample *t* test. Linear regression was applied on pupillary data from 19-89 minutes to estimate the duration of the naloxone effect. Comparisons between arterial and venous samples were done with paired *t* tests and Bonferroni correction for multiple testing.

Study design of the observational study (Paper III)

Paper III report an observational study of patients treated with naloxone by the Oslo City Center Emergency Medical Services during 2014-2018. We examined the naloxone administration routes, dosage, and use of multiple doses. We also examined associations between initial naloxone dose and clinical and demographic variables, as well as the associations between multiple naloxone doses and clinical and demographic variables. Finally, we examined transfer rates following EMS treatment and the one-week mortality after EMS attendance.

Patients were included if they were 18 years or older. Patients treated with naloxone were prospectively included between June 1st, 2014 and December 31st, 2018. They were given information about the study and could withdraw from registration. They were followed through the National Cause of Death Registry until December 31st, 2018. Data from January 1st, 2014 to May 31st, 2014 were collected retrospectively and registered anonymously with no possibility of matching against the National Cause of Death Registry.

Data from medical records for included patients were manually entered into a database. Sex, age, place of attendance, transfer rates, naloxone doses and their routes of administration were registered. Clinical variables such as respiration rate (RR) and consciousness reported as Glasgow Coma Scale (GCS) before and after naloxone were also registered. National identity numbers were used to link episodes and for linking against the National Cause of Death Registry. Prior to analysis, key data were verified by two researchers against the original medical records.

Statistical methods

Statistical analyses were conducted in Stata 15.1 (StataCorp, TX, USA). Descriptive statistics were used to examine data on the route of naloxone administration, naloxone dosages and the number of doses administered during EMS attendance. Univariate and multivariable logistic regression analyses were used to examine the associations 1) between naloxone dose and patient sex, patient age, place of attendance and vital signs and 2) between multiple naloxone doses (≥2) during an EMS attendance and patient sex, patient age, place of attendance, vital signs and initial naloxone dose. The regression analyses included the cases with a valid national identity number, as this allowed for accounting of repeated events by including identity as a cluster variable in the model. Results are reported as odds ratios (OR) and adjusted odds ratios (AOR) with 95% confidence intervals (CI). Transfer rates following EMS treatment were reported. These rates included being left at the scene or transferred to a hospital, a primary care accident and emergency outpatient clinic, or other places such as home or addiction treatment facilities. Univariate and multivariable logistic regression were used to examine whether initial naloxone dose and multiple naloxone doses were associated with transfer rates. Data on deaths was retrieved from the National Cause of Death Registry and the one-week mortality after EMS treatment was examined. Deaths registered on the same date as EMS treatment were defined as “day 0” and deaths the following date as “day 1”. To estimate one-week mortality, we used deaths that occurred on day 0 through day 7.

Measures

The dependent variable in the first logistic regression (Model 1) was IM naloxone at doses of 0.4 and 0.8 mg, and 0.4 mg naloxone was the reference category. Only 3.6% received naloxone in other dosages and 7.4% via other routes; therefore, we excluded these from the analysis. In Model 1, the following explanatory variables were included: patient sex, patient age, GCS score and respiration rate at presentation to the EMS and if the overdose was attended at the safe injection facility. Low GCS score and respiration rate are part of the classic opioid overdose triad and have been shown to influence the choice of naloxone dose (123, 124). Other patient characteristics, such as sex, have also been found to influence the choice of naloxone dose in one study (124). Age and treatment at the safe injection facility were included as part of an exploratory analysis.

The dependent variable in the second logistic regression model (Model 2) was multiple doses of naloxone (≥2 doses). The reference category was a single dose only. In Model 2, the following explanatory variables were included: patient sex, patient age, GCS and respiration rate at first evaluation, if the overdose occurred at the safe injection facility and the initial naloxone dose. To ensure that missing data were not deleted listwise in both the logistic regression analyses, a category for missing responses for variables with incomplete recordings (no valid reports) was included.

Adverse events

Any symptoms in studies I and II were registered as adverse events and seriousness classified according to the ICH Good Clinical Practice guidelines (106). Serious adverse events are any adverse event that results in death, disability, birth defect, is life-threatening or requires hospitalization or prolongation of existing hospitalization (106). Furthermore, adverse events can be classified as adverse drug reactions if the events are assumed to be related to the drug. Adverse event severity was classified according to the Common Terminology Criteria for Adverse Events (CTCAE v 4.0). The system divides symptoms in organ system categories with subcategories and their specific parameters relating to the organ system involved. Toxicity is graded as mild (Grade 1), moderate (Grade 2), severe (Grade 3), life-threatening (Grade 4) and death (Grade 5). The scale was developed by The National Cancer Institute, U.S. Department of Health and Human Services, to standardize the reporting in oncology trials, but are also used for non-cancer drug trials (125). There was a system in place for expedited reports from the principal investigator to sponsor in case of serious adverse events and from sponsor to regulatory authorities in case of serious and unexpected adverse drug reactions (106). There were no structured data collection on adverse events in the observational study.

Ethics, approvals and grants

All procedures performed in the studies involving human participants were in accordance with the ethical standards of the Helsinki declaration (126). The studies were approved by The Regional Committees of Medical and Health Research Ethics in Norway who also approved the written participant information. Studies I and II were also approved by the Norwegian Medicines Agency, followed the ICH Good Clinical Practice guidelines (106)

and were preregistered in www.clinicaltrials.gov. In these studies, written informed consent were collected prior to inclusion. The participant compensation were 1500 Norwegian kroner (equivalent to 160 EUR at that time) for each visit in study I, and 1000 Norwegian kroner (110 EUR at that time) for participation in study II. The participants were insured through the Drug Liability Association, Norway, during the trials. In study III, patients included after June 1st, 2014, were given oral and written information about the study and were given the opportunity to withdraw. Patients included retrospectively before June 1st, 2014 were registered anonymously, in accordance with the approval from the ethics committee. There was no compensation for participation in study III.

In all studies, registration and storage of participant data were carried out in accordance with national legislation and regulations on medical research and privacy issues. The subjects were identified by participant number that was used in case report forms or in the database. An identifier with full names and national identity number were stored separately.

The studies have been supported by grants from public sources and non-profit foundations. Grants have been received from the Lærdal Foundation for Acute Medicine, Unimed Innovation, Felles Forskningsutvalg, St. Olavs hospital Trondheim University Hospital, The Norwegian University of Science and Technology (NTNU), The Liaison Committee for Education, Research and Innovation in Central Norway, Norwegian Center for Addiction Research at University of Oslo and Oslo University Hospital.

Results

Paper I

Twelve subjects completed the study, 10 men and two women with mean age 24.5 years and body mass index 23.5kg/m^2. Fifteen blood samples were taken over a period of six hours, and the serum was analyzed for naloxone content. The bioavailability of the nasal formulation was 0.54 or 54% (0.45–0.63) for the 0.8 mg and 0.52 or 52% (0.37–0.67) for the 1.6 mg intranasal formulation, respectively. The time-course for the serum concentrations is illustrated in Figure 6.

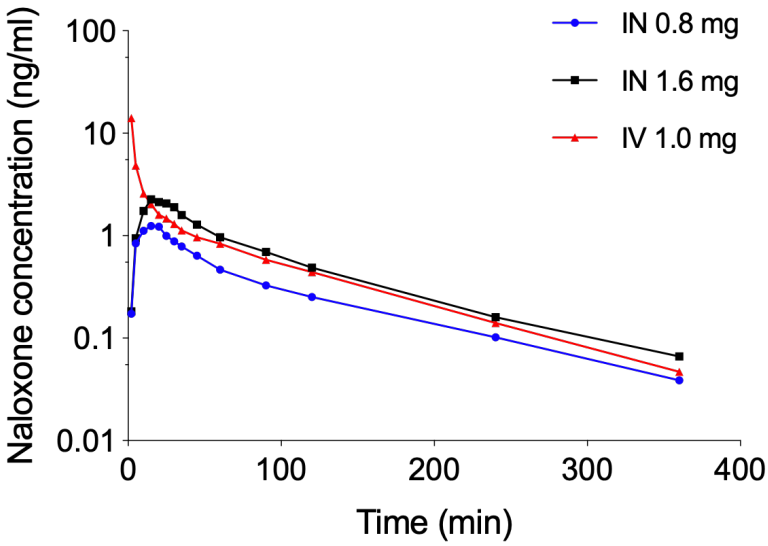

Figure 6. Serum concentrations after administration of 0.8 and 1.6 mg intranasal naloxone compared to 1.0 mg intravenous naloxone. The 1.6 mg intranasal serum concentrations surpassed the intravenous serum concentrations at 15 minutes and stayed above for the rest of the examined period. The serum concentrations after 0.8 mg intranasal naloxone never reached the concentration levels after intravenous naloxone.

The mean maximum serum concentrations were 1.45 ng/ml for 0.8 mg and 2.57 ng/ml for 1.6 mg nasal spray, respectively. The respective dose-corrected values were 1.72 and 1.61 ($p = 0.674$). Time to maximum concentration was reached at 17.9 min and 18.6 min for the 0.8 mg and the 1.6 mg doses, respectively (Table 2).

Table 2. Pharmacokinetic variables in healthy volunteers after intranasal and intravenous administration of naloxone in an open, randomized three-way crossover trial

Naloxone dose	Cmax (ng/ml)	Tmax (min)	Tmax50 (min)	Tmax80 (min)
0.8 mg IN	1.45 (1.07-1.84)	17.9 (11.4-24.5)	8.34 (7.62-9.07)	12.1 (10.9-13.3)
1.6 mg IN	2.57 (1.49-3.66)	18.6 (14.4-22.9)	10.5 (9.74-11.2)	16.8 (15.7-17.9)
1.0 mg IV	14.2 (9.13-19.2)	2.25 (1.70-2.80)		

Data are presented as mean values (95% confidence intervals). IN = intranasal, IV = intravenous, Cmax = maximum concentration, Tmax = time to Cmax, Tmax50 = time to 50% of Cmax, Tmax80 = time to 80% of Cmax.

A close up of the time-course of the serum concentrations in the early phase is illustrated on a linear scale in Figure 7. Overall, there were considerable variation between the individuals and the different treatments. The within-subject variability was smaller than the between-subject variability. For bioavailability it was 0.012 vs 0.035 respectively, and for Cmax it was 0.387 vs 0.607.

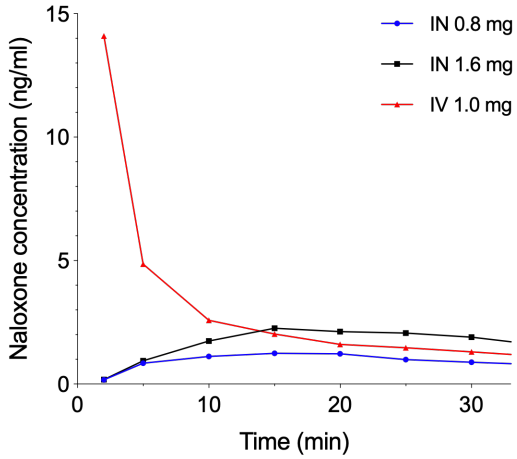

Figure 7. The naloxone concentrations the first 30 minutes after intranasal and intravenous administration of naloxone, illustrated with a linear y-axis.

There were no serious adverse events. Taste sensation of the nasal spray was common and was reported in 50% of the visits. One participant was excluded during the trial due to a nasal cauterization after a spontaneous nosebleed of the contralateral nostril to where naloxone had been administered.

Paper II

Six men and six women completed the study. Their mean age was 23.0 years and body mass index 22.4kg/m^2. Venous sampling failed for one subject. The participants had large pupil diameter at the start of the trial (mean 7.36 mm). The remifentanil infusion was started at -12 minutes relative to naloxone administration, and induced miosis that reached a mean nadir of 3.55 mm. A dose of 1.0 mg intravenous naloxone quickly and completely reversed the effect of a 1.3 ng/ml TCI remifentanil infusion, with maximum effect 4 minutes after administration (

Figure 8). One of the aims with the study was to study the serum-effect-site equilibration rate constant (ke0) and its half-life (t1/2ke0), but due to the complete reversal achieved within a few minutes, this gave us too few observations to reliably estimate these variables.

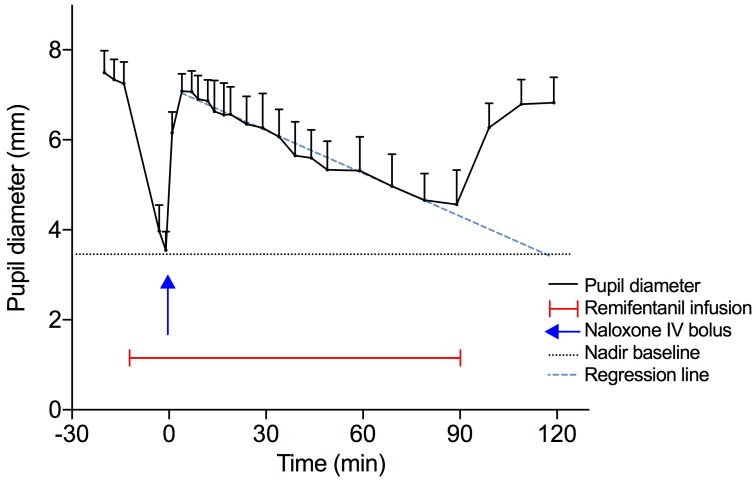

Figure 8. Pupil diameter during the course of the trial. Miosis was induced by remifentanil started at -12 minutes relative to naloxone. It was rapidly reversed after naloxone bolus that was given at t=0. n=12, mean (95% confidence interval). Using linear regression, the effect of naloxone was estimated to last for 118 minutes.

The duration of the opioid effect reversal lasted at least 90 minutes after the naloxone administration, although with diminishing effect. After the infusion was terminated the pupils rapidly increase in size to a mean of 6.83 mm. A regression line (f (x) = − 0.0292x

+ 6.9924) based on the period 19–89 min crossed the nadir line at 118 minutes. The mean venous concentration of naloxone at this time (120 min) was 0.51 ng/ml.

The arterial and venous concentrations of naloxone were similar and almost completely overlapping the first 30 minutes (Figure 9a). After 30 minutes there was a tendency for the venous samples to have a slightly higher concentration. The arterial $AUC_{0\text{-}t}$ was 94% of the venous $AUC_{0\text{-}t}$. The opioid model produced a steady state arterial concentration of remifentanil during the trial as seen in Figure 9b. The concentration was on average 1.15 ng/ml, 12% lower than the expected 1.3 ng/ml. There was a clear arterio-venous difference of remifentanil.

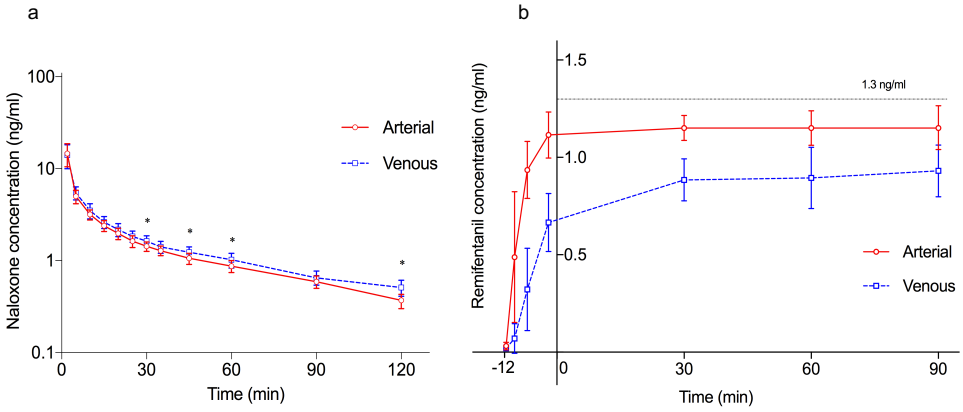

Figure 9a) The time-course of the arterial and venous serum concentrations of naloxone. The samples marked with an asterisk (*) had a statically significant difference. b) Time-course of arterial and venous concentrations of remifentanil during a Minto model target-controlled infusion with plasma concentration target of 1.3 ng/ml. The achieved concentration was 1.15 ng/ml. a and b) Data presented as mean with 95% confidence intervals, n=11.

There were no serious adverse events. Two subjects experienced mild nausea that resolved without use of medication were classified as having a possible relationship to the test drug.

Paper III

Overall, 2,215 cases treated with naloxone were included and 1,720 of these cases had a valid national identity number. The mean age of the patients was 38.3 years, and 77.1% were men. Patients were attended in public places (50.1%), at the safe injection facility (33.5%), private homes (7.3%), shelters and other facilities for people using drugs (6.5%), and other places such as hotels and public transport (2.6%).

The majority (91.9%) of cases were administered intramuscular (IM) naloxone as their initial treatment. A minority of the cases were treated with intravenous (IV) naloxone; 1.9% were treated with IV alone, and 3.8% were administered IV naloxone after the administration of an IM dose. Other administration routes were used for 2.5% of the cases. Among those treated with IM naloxone (n=2035), the initial naloxone dose was 0.8 mg for 56.5% of the cases and 0.4 mg for 39.9% of the cases. Only 3.6% received IM naloxone in other doses. 15% of all cases (n=2215) received multiple doses of naloxone. The total administered naloxone dose including initial and subsequent doses of titration was 0.4 mg for 33.0% of the cases, 0.8 mg for 51.2% of the cases and more than 0.8 mg for 12.7% of the cases. 3.1 % received other doses less than 0.8 mg. Only 1.0% of patients received ≥ 2 mg naloxone in total, and the maximum dose used was 3.0 mg. The mean total dose of naloxone in patients with respiratory arrest and/or cyanosis was 0.8 mg.

The naloxone dose model (Model 1, Table 3) on treatment with either 0.4 mg or 0.8 mg IM naloxone in patients with a valid national identity number (n=1530) showed that unconscious patients with GCS scores of 3/15 or 4 - 9/15 were seven- and four-times more likely to be administered 0.8 mg naloxone than those who were awake (GCS 15). Compared to patients with a respiratory rate of ≥9 breaths/minute, those with respiratory arrest or a respiratory rate of 1-8 breaths per minute were three- and two-times as likely to be treated with 0.8 mg naloxone, respectively. Furthermore, men were more than twice as likely as women to be administered a dose of 0.8 mg. Those attended at the safe injection facility were 40% less likely to receive 0.8 mg naloxone than patients treated at other locations.

Table 3. The putative associations between intramuscular naloxone dose (0.4 mg vs 0.8 mg) and sex, age, vital signs and place of attendance (n=1530)

	0.4 mg 100% (n=657)	0.8 mg 100% (n=873)	Unadjusted OR (95% CI)	Adjusted OR (95% CI)
Sex				
Women	30.8 (202)	18.6 (162)	ref	ref
Men	69.3 (455)	81.4 (711)	2.0*** (1.5, 2.5)	2.2*** (1.7, 2.9)
Age (years)				
< 30	24.2 (159)	23.1 (202)	ref	ref
30-49	58.5 (384)	59.7 (521)	1.1 (0.8, 1.4)	1.2 (0.9, 1.5)
≥50	17.4 (114)	17.2 (150)	1.0 (0.7, 1.5)	1.3 (0.9, 1.8)
Glasgow Coma Scale				
3/15	27.7 (182)	56.5 (493)	9.1*** (5.2, 16.2)	7.1*** (3.8, 13.1)
4-9/15	21.6 (142)	19.8 (173)	4.1*** (2.2, 7.5)	4.0*** (2.1, 7.5)
10-14/15	33.8 (222)	13.8 (120)	1.8* (1.0, 3.2)	1.8 (1.0, 3.2)
15/15	8.2 (54)	1.8 (16)	ref	ref
No valid report	8.7 (57)	8.1 (71)	4.2*** (2.2, 8.0)	3.8*** (2.0, 7.4)
Respiration rate				
0/minute	7.2 (47)	20.6 (180)	5.1*** (3.5, 7.6)	3.4*** (2.2, 5.3)
1-8/minute	35.5 (233)	43.0 (375)	2.2*** (1.7, 2.7)	1.7*** (1.3, 2.2)
≥9/minute	44.9 (295)	25.2 (220)	ref	ref
No valid report	12.5 (82)	11.2 (98)	1.6** (1.1, 2.3)	1.6* (1.1, 2.3)
Place of attendance				
Safe injection facility	41.1 (270)	37.5 (327)	0.9 (0.7, 1.1)	0.6*** (0.5, 0.8)
All other locations	58.9 (387)	62.5 (546)	ref	ref

Logistic regression analysis was used, and identity was included as a cluster variable to account for the possibility that an individual had repeated events. OR = odds ratio, 95% CI = 95% confidence interval. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

A second model on use of multiple doses (≥2) of naloxone during one EMS attendance (Model 2, Table 4) showed that unconscious patients with GCS scores of 3/15 or 4 - 9/15 were seventeen- and eight-times more likely to be administered multiple doses than those who were awake. Compared to patients with a respiratory rate of ≥9 breaths/minute, patients with respiratory arrest were twice as likely to be treated with multiple doses. Furthermore, men were almost twice as likely as women to receive multiple doses. Those attended at the safe injection facility were 80% less likely to be treated with multiple doses than patients treated at other locations. Finally, those treated with an initial

naloxone dose of 0.8 mg were 60% less likely to receive multiple doses than patients treated with an initial dose of 0.4 mg naloxone.

Table 4. The likelihood of multiple-dose administration of naloxone during a single EMS attendance as a function of sex, age, vital signs, place of attendance and dose (n=1530)

	Single dose 100% (n=1303)	Multiple doses 100% (n=227)	Unadjusted OR (95% CI)	Adjusted OR (95% CI)
Sex				
Women	24.7 (322)	18.5 (42)	ref	ref
Men	75.3 (981)	81.5 (185)	1.5 (1.0, 2.2)	1.8** (1.2, 2.6)
Age (years)				
<30	23.0 (300)	26.9 (61)	ref	ref
30-49	59.5 (775)	57.3 (130)	0.8 (0.6, 1.2)	1.0 (0.7, 1.5)
≥50	17.5 (228)	15.9 (36)	0.8 (0.5, 1.3)	1.1 (0.6, 1.9)
Glasgow Coma Scale				
3/15	40.5 (528)	64.8 (147)	9.5*** (2.3, 39.2)	17.1***(3.9, 75.0)
4-9/15	21.2 (276)	17.2 (39)	4.8* (1.1, 20.5)	7.8** (1.8, 34.4)
10-14/15	24.6 (321)	9.3 (21)	2.2 (0.5, 9.7)	2.7 (0.6, 11.9)
15/15	5.2 (68)	0.9 (2)	ref	ref
No valid report	8.4 (110)	7.9 (18)	5.6* (1.2, 24.9)	7.9** (1.7, 36.9)
Respiration rate				
0/minute	13.8 (180)	20.7 (47)	1.6* (1.1, 2.5)	1.9* (1.2, 3.2)
1-8/minute	39.6 (516)	40.5 (92)	1.1 (0.8, 1.6)	1.0 (0.7, 1.5)
≥9/minute	34.1 (444)	31.3 (71)	ref	ref
No valid report	12.5 (163)	7.5 (17)	0.7 (0.4, 1.1)	0.8 (0.4, 1.4)
Place of attendance				
Safe injection facility	43.1 (562)	15.4 (35)	0.2*** (0.2, 0.4)	0.2*** (0.1, 0.3)
All other locations	56.9 (741)	84.6 (192)	ref	ref
Initial naloxone dose				
0.4 mg IM	42.1 (549)	47.6 (108)	ref	ref
0.8 mg IM	57.9 (754)	52.4 (119)	0.8 (0.6, 1.1)	0.4*** (0.3, 0.5)

Logistic regression analysis was used, and identity was included as a cluster variable to account for the possibility that an individual had repeated events. IM = intramuscular, EMS = emergency medical service, OR= odds ratio, 95% CI = 95% confidence interval. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

The majority (57.1%) of the 2215 cases were left at the scene. A total of 28.1% of the cases were transferred to the Oslo Accident and Emergency Outpatient Clinic, 12.9% were hospitalized and 1.9% were transferred to other places. One patient was in cardiac arrest and died despite treatment with advanced cardiac life support. This patient was treated with naloxone and was therefore included in the study. Whether the patients were transferred from the scene following treatment was not significantly associated with the initial dose either in the univariate logistic regression analysis (OR 1.1, 95% CI 0.9-1.3),

or after adjusting for individual characteristics and vital signs (AOR 1.1, 95% CI 0.9-1.5). However, patients transferred following treatment were 70% more likely to have been treated with multiple doses of naloxone both in unadjusted analysis and after adjusting for individual characteristics and vital signs (AOR 1.7, 95% CI 1.2 – 2.3).

Among the 1,720 cases with a valid national identity number, there were 10 deaths within the first week after EMS treatment. Seven deaths were drug-related deaths, six of which were classified as unintended poisoning and one as a suicide by way of heroin. Three patients died from natural causes. Those who died from overdose or suicide had all been left at the scene. The overall one-week mortality rate for drug-related deaths was 4.1 per 1000 episodes and 5.5 per 1000 episodes for patients left at the scene by the EMS. There were no deaths due to rebound toxicity.

Discussion

Methodological considerations

This thesis comprises two experimental studies and one observational study. The results from these studies presented in this thesis must be interpreted in view of their limitations and strengths discussed in the following sections.

Study designs

The pharmacokinetic study (Study I)

The first study was an open, randomized crossover study in healthy volunteers. In a crossover study the participants are allocated to a sequence of two or more interventions, and all participants receive all interventions (127). The crossover design is preferred if possible because the subjects act as their own controls. This reduces the number of subjects needed and the study gains precision as treatment is compared within, rather than between participants (127). It is a suitable study design if the treatment effect is fairly quick, the treatment effect is reversed when the treatment is removed, and the condition is stable (127). These prerequisites are often met in pharmacokinetic studies and this study design is therefore commonly used in such studies (128).

The main issue with crossover studies is carry-over effects between treatments. This is a situation where the first treatment lasts into the second and affect the measurements from the second treatment (127). In pharmacokinetic studies such carry-over effects are unlikely to occur if the washout period is sufficiently long (128). To achieve this, we applied a three-day washout period between treatments. This was considered sufficient as the half-life of naloxone is around 60-90 minutes (33) and a wash-out period of five half-lives or more is recommended for the drug to be sufficiently eliminated (83). Furthermore, serum concentrations of naloxone were also measured at baseline in all sessions to eliminate the possibility of naloxone being present, as suggested by the ICH (128).

This study was a randomized crossover study. Randomization is a method of experimental control where random sequences are used to decide treatment allocation. This is to reduce accidental bias and to ensure comparable study groups (129). Use of randomization is considered gold standard in clinical research and is the strongest measure we can use to equalize the study groups and reduce the systematic bias (130). In our study, the order of treatments was decided with randomization. The allocation was done after the participants were approved for inclusion in the study, having consented and fulfilled inclusion and exclusion criteria. This is an important principle, so the treatment allocation does not influence the participants willingness to participate in the study (129). The randomization was done in a concealed fashion with a digital solution and was independent of the study staff, to avoid the staff of influencing the results (129). The digital solution used block-randomization, which ensures equal group sizes even in small samples (129).

In addition, this randomized crossover study had an open-label design. By open-label means that the identity of the treatment is known to both participants and study staff (128). This is often used in phase I pharmacokinetic studies (107) and it is recognized by the ICH guidelines that phase I studies may be open-label (108). For studies in later phases, double-blinding is recommended to reduce the potential for observations bias for both subject and investigators (128). Both conscious and unconscious bias can be introduced by subjects and investigators knowing what treatment is used, and this risk is higher when subjective measurements are used (128). If an open-label study design is used, ICH recommend that every effort is taken to minimize the known sources of bias and that the primary variables should be as objective as possible (128). In our study, the primary endpoints were objective, like drug concentration measurements. This minimizes the risk of observation bias.

The potential for this bias in our study could have been reduced by using a double-dummy design, where a nasal spray and an injection had been given simultaneously, one with active substance and one with placebo (131). In this manner, the participant and examiner would not know if the participant was treated with intranasal or intravenous naloxone, a so called double-blinded study. There were some substantial challenges in applying a

double-blind study design in the current study. Producing placebo nasal sprayers would have doubled the already high costs of the nasal spray production. Finding identical intravenous comparators would have been extremely difficult, and the production costs would have been even higher than for intranasal sprays. An open-label study without masking was therefore chosen for the current study, but a double-blinded, double-dummy design were chosen for a later phase III clinical trial of the nasal spray.

The treatment in study I was one and two doses of the nasal spray. One spray delivered 0.8 mg naloxone, and two sprays gave 1.6 mg naloxone in total. The dose selection was an important part of the study design. With an estimated bioavailability for the nasal spray of around 50% (110), a doubling of the standard doses is needed to achieve a similar systemic exposure. We based our dose selection on the guidelines for Norwegian prehospital emergency services, that suggest intravenous and intramuscular doses in the range of 0.4 - 0.8 mg (40, 41). To compare with this, we chose 0.8 mg as our standard dose. As titration of naloxone treatment is recommended (40, 41, 45), we tested both one and two doses to check for dose linearity. Intravenous naloxone 1.0 mg was used as comparator, in line with the European guidelines recommending use of an intravenous comparator in pharmacokinetic studies (132).

The pharmacokinetic-pharmacodynamic study (Study II)

The second study was an open-label study with an explorative endpoint. In this study, the aim was to develop a model for studying the effects of naloxone using an opioid infusion. As there were only one treatment, there were no randomization to treatment and the study was not blinded. As there were no comparison between different treatments and the primary endpoints were objective, this is assumed to be of less importance for the results.

Remifentanil target-controlled infusion was chosen as it provides the possibility to study naloxone under steady state conditions. There have previously been different strategies to studying the effect of opioid antagonism in healthy volunteers. Bolus injections of morphine and per oral alfentanil have been used together with naloxone, and per oral tramadol was used in a study of naltrexone (103, 133, 134). A bolus of intravenous remifentanil was employed in one study of the effects of the mu-opioid receptor antagonist samidorphan (135). These models are confounded by the absorption,

distribution and elimination of the opioid as they fail to create a steady state model. To study the effects of naloxone without introducing this confounding, a steady state model is superior.

The observational study (Study III)

Paper III describes a five-year observational study of patients treated with naloxone by the Oslo City Center EMS. In an observational study, the data is collected as they naturally exist and there are no interventions (136). Observational studies allow for empiric investigations where it is not possible to conduct experiments (137). Without random assignment the study groups may not be comparable, and there is a risk of bias due to pretreatment differences (137). Pretreatment differences that have been accurately measured can be adjusted for, but there can also be differences not recorded, introducing hidden biases (137). It is important that there is a coherent pattern of associations in such studies and it is important to remember the fact that association does not imply causation (137). An observational study design was chosen as it was an efficient way of gaining knowledge on the current use of naloxone. The underlying assumption, and the basis for drawing conclusions on naloxone dosing from this study, is that today's treatment is safe and effective. These assumptions were tested by investigating transfer rates after treatment and by linking the data to the National Cause of Death Registry. This allowed for investigation of one-week mortality after EMS attendance for opioid overdose.

Sample and selection bias

In research, we collect a sample of individuals and study those in detail. The aim is to generalize the conclusions from the sample to a larger population (138). We must assume that the sample is representative to make valid generalizations from the study results (138). The sampling procedure is therefore important, as sampling or selection bias can occur when the individuals selected over- or underrepresent certain population attributes that are related to the phenomenon under investigation (138). Such bias can be conscious and intentional, like choosing healthy volunteers for a study, or it can be unintentional and related to the recruitment process or dropouts happening not at random (138).

A common way to reduce random error and increase precision in studies is to increase the sample size, but practical constraints on resources inevitably limits study size (139). Prior to the investigation, it is therefore important to evaluate the number of subjects needed to answer the study aims. This is essential to reduce the risk of conducting type I errors where the study hypothesis is incorrectly rejected, and type II errors where the study hypothesis is false, but not rejected (139). To have sufficient statistical power to find significant differences the sample size should be calculated prior to study start (140). However, in these studies no formal sample size calculation was conducted. Prior knowledge on the topic under investigation is needed to calculate the sample size. As the clinical trials were exploratory, there were little available information to use in such a calculation. Twelve subjects are often used and generally an acceptable number of participants for exploring pharmacokinetic and pharmacodynamic studies, and usually provides adequate data for estimates on inter-individual variations. The European regulatory authorities has supported this and recommends no less than 12 subjects for such studies (83). Study I also gain precision as the nasal spray is administered twice to the same individuals. Due to the exploratory nature of the studies and the lack of sample size calculations, the data from these studies cannot be the sole basis of a choice between two alternatives, like approve of not approve the new drug formulation. For such studies, a thorough sample size calculation is necessary during the planning phase of the study. The ICH acknowledges the fact that data from exploratory trials are needed to support the choices done in confirmatory trials (128). The results generated form the studies presented here were later used in sample size calculations for a confirmatory trial. In the observational study it was not deemed necessary to conduct a formal sample size calculation because this was an observational study for a predefined time period. Five years was assumed to be sufficient for the analysis conducted in this trial.

In the two clinical experimental studies (Study I and II), we used healthy volunteers. Healthy volunteers are often used in these kinds of studies and this practice is recommended by the European Medicines Agency and by the ICH (83, 108). Healthy volunteers may bias the results as strict selection criteria regarding liver and kidney function, body size and age are normally used. All these factors can influence the processes of absorption, distribution and elimination. Patients receiving treatment for

overdose will by definition be under the influence of opioids, and there may be interactions between opioids and the drugs tested in the study which are not conveyed in healthy volunteers. Patients may also use vasoconstrictive nasal drugs that affect the nasal uptake of naloxone. An alternative is to conduct the study in a group of patients. It was assumed to be difficult to recruit opioid users to a study of naloxone, as they are commonly afraid of opioid withdrawal (44). It would also introduce a problem with dropouts and possible attrition bias. Starting the pharmaceutical development by testing on patients with overdose was not considered ethical in early stages. Drug development is ideally a step-wise procedure in which information from small early studies are used to support and plan later, more definitive studies (108). A phase III trial of nasal naloxone in patients with opioid overdose were therefore planned as a part of our drug development plan.

The observational study (Study III)

Selection bias might have been introduced in the observational study because it was a single center study only including patients from the Oslo City Center ambulance station. This ambulance station covers 67% of the overdose emergencies in the city, but the selection might be skewed towards opioid overdoses occurring in people who inject drugs as many drug user services and a safe injection facility is localized close to this ambulance station. Other opioid use, for example use of prescription drugs may be more evenly spread throughout the city. Safe injection facilities or supervised drug consumption facilities exists in 51 cities in Europe (8) and this increase the generalizability of the results to other countries and settings that have such facilities. There are debates on the optimal naloxone doses to use in regions with illicit manufactured fentanyl is common. One study indicated an increased use of multiple naloxone doses while another found no increase in naloxone doses despite a large increase in fentanyl deaths during in the investigated time period (37). As there have been few recorded deaths from overdoses with fentanyl or other synthetic opioids in Norway (18), the results from this study cannot be used to enlighten the debate on naloxone doses in settings where fentanyl is more frequent.

Misclassification is another additional source of bias. It may be introduced if subjects are misclassified as overdoses when they have another diagnosis (136). There is no international uniform definition of a non-fatal opioid overdose. WHO defined overdose as "the use of any drug in such an amount that acute adverse physical or mental effects are produced" (9), some studies use toxicology screens to verify the diagnosis (141) and the American Center for Disease Control utilizes a long list of different ICD-codes (the International Classification of Diseases) and combinations of chief complaints to identify cases of non-fatal overdose (142). In the current study, suspected opioid overdoses are defined as any patient treated with naloxone. This definition can introduce some misclassification, as patients that were misdiagnosed as opioid overdoses were not removed from the material. This is suspected in an unconscious patient that was assumed to have a head injury and in cases where the patients were assumed to have overdosed on gamma-hydroxybutyric acid. These patients are not expected to recover from naloxone, but the antidote was used as a diagnostic tool to rule out that they suffered from an opioid overdose. As we did not have access to in-hospital records or toxicology screening, it was not possible to exclude patients that got another diagnosis than opioid overdose. This may underestimate the effect of naloxone for opioid overdose and overestimate the need for multiple naloxone doses. On the other hand, this is also a strength, as it reflects how naloxone is used in real life, and this is also how take-home naloxone is intended to be used.

National identity numbers were missing for patients included before 1st of June 2014 due to late application and approval of the study from the ethical board. After this date, 312 out of 1720 patients did not disclose their identity to the emergency medical services. This has also been reported in other studies of prehospital intoxication treatment (46, 143). There might be a systematic difference in who discloses their identity to the EMS, and some of the cases might be the same individual. They could therefore not be included in the logistic regression analysis and could not be matched against the National Cause of Death Registry. They were still included in the study to get a complete sample during the time period on the number of cases, the amount of naloxone used and the transfer rates to hospital and other facilities offering follow-up.

There might also be a selection bias because the inclusion criteria also require that the emergency medical services were alerted. During the study period, Oslo rolled out a large-scale take-home naloxone program. Patients may therefore have been treated with naloxone outside the emergency medical services (144).

Information bias

In epidemiology, information bias refers to bias in estimating an effect caused by measurement errors in the needed information (145). Information bias occurs when any information used in a study is either measured or recorded inaccurately. Missing data is one such bias and may produce systematic errors in the data material. There were missing data on baseline recordings for GCS and respiratory rate which appeared to be missing at random. This could therefore be compensated by including missing as a variable in the models described in paper III to avoid listwise deletion of these subjects. However, for the consecutive measurements of respiration and consciousness, data seemed to be missing not at random. This means that the probability of an observation being missing depends on unobserved measurements. Patients who were left on site are assumed to have responded to treatment with adequate GCS increase and sufficient respiration, but they had more often missing data on these variables. With data missing not at random, techniques such as analysis on complete cases, last-observation-carried forward and multiple imputation all can produced bias results, and it is difficult to adjust for this in our models. We therefore refrained from reporting data on the effect of naloxone on respiration and consciousness as these were assumed to be biased. Instead, multiple naloxone doses and transfer rates were used as estimates of treatment effect. As this was a prospective study, data collection could have been standardized by using a case report form. Targeted data collection would have improved data on the effect of the treatment. This would have been especially important for getting reliable data on adverse events, as there are indications that is not adequately investigated in observational studies (37, 146).

There can be an element of the phenomenon known as Hawthorne effect in our study, which is a tendency of participants performing better when knowing they are being observed (130). The staff in the study were closely monitored over the years of the study and knew that their medical records were subject to scrutiny. There were a lot of focus on

naloxone and overdose treatment during the study period and a large randomized controlled trial of intranasal naloxone commenced at the study site in June 2018. All this can have affected both the practice and the medical recording. One observation is that fewer and fewer patients are treated with intravenous naloxone during the study period. We can only speculate in the reasons for this, but one possible explanation is that this is related to the ongoing study and its focus on naloxone treatment.

Confounding factors

A confounder is a variable that is associated both with the independent variables and the outcome and is not a part of the casual link between the exposure and the outcome (147). Confounders can introduce bias and misinterpretations of the results. The experimental design in the clinical studies is important to reduce the risk of confounding, and the randomization is an important tool to control for unrelated factors by having them evenly distributed in the data material.

Confounding is often an issue in observational studies, because other factors than those observed may contribute to the observed relationships. In the observational study (Study III) approximately half of the cases in identified individuals were in patients that appeared several times in the register. To account for patients being treated several times, identity was included in the analysis. Age and sex are often considered potential confounders because of their common association with disease and disability, as often related to the presence of many exposures (147). We therefore included age and sex in the models to adjust for this possible confounding. Another common confounder is weight (147), and the Oslo EMS guidelines recommend naloxone dosages based on the patient's weight (40). Accurate weight of patients was not available to EMS staff and not recorded in the medical records. It could therefore not be included in the analyses. However, we believe that this factor was partly adjusted by the inclusion of sex in the model. Regarding naloxone dosing, a possible confounder is the study staff and their experience and preferences, which may affect both how they perceive the situation and what treatment they choose. The emergency medical teams could have been included as a factor however we did not have access to such data.

Internal and external validity

Internal validity refers to the extent of which a study design and conduct of a study are likely to have prevented bias. More rigorously designed, better quality studies are more likely to yield results that are closer to the truth (148). External validity refers to the extent to which the study results can be generalized beyond the population and settings in the study (130, 149).

The pharmacokinetic study (Study I)

Randomized controlled trials and experiments often have a high internal validity due to the rigorous design of the experiments and the strict selection criteria for study inclusion. In the first study, all the treatments were supervised so there were no compliance issues. The study sessions were conducted in a similar fashion, and objective measurements such as drug concentrations was used to evaluate the treatments. Data collection was done through a clinical research facility with study nurses familiar with research, and the data collection was meticulously done. There were no dropouts, but one subject was excluded after one study session due to violation of inclusion and exclusion criteria. Ideally, all treated subjects should be included in the pharmacokinetic analysis. However, if subjects in a crossover study do not provide data from both the test and the reference product it is difficult to include them in the pharmacokinetic analysis (83). The subject was therefore excluded from the pharmacokinetic analysis but was included in the safety analysis.

Essential for the internal validity and the value of a pharmacokinetic study is the blood sampling schedule and the analytical method used. Frequent sampling at the start of the first study gave a sound estimation of Cmax and Tmax after treatment with the nasal spray. To give a reliable estimate of the extent of exposure, it is necessary that the sampling schedule (AUC_{0-t}) should cover at least 80% of $AUC_{0-\infty}$ (83). In study I, the sampling schedule covered 95% of the serum concentration curve and on average 5.3 samples were used to estimate the elimination rate constant, which gave reliable estimations. The method used to analyze the samples with liquid chromatography-tandem mass spectrometry allows for very accurate quantification of analytes. The method used to quantify the amount of naloxone had a limit of quantitation of 0.02 ng/ml which is lower than the requirement of 1/20 of Cmax set by European Medicines Agency (83).

The method was validated according to acknowledged principles and produced reliable results (114, 115).

A threat to the internal validity was that almost all spray devices delivered a lower volume than predicted. The sprays should give 0.100 and 0.200 ml but gave on average 0.093 ml and 0.187 ml for one and two doses. Therefore, after the study was completed, concerns regarding the production and performance of the spray devices were raised. The devices had not been specifically tested with the naloxone formulation, but the devices were approved for this kind of use. The filling volume was therefore controlled in additional tests. These showed that average filling weight volumes were 0.013 ml less than specified from the device producer. This is assumed to explain the overall lower than expected spray delivery. This was managed by using calculated doses, rather than nominal doses which is normally used in studies with a regulatory purpose. Secondly, there was one spray that deviated significantly from the others by delivering a volume of 0.162 ml, far less than the anticipated 0.200 ml. The low performance of this spray indicated a leakage, and the study-session in which it had been used was excluded from the pharmacokinetic analysis. Later assembly faults in the production facility was uncovered, so for the following trials, the device was replaced, and the production was adjusted accordingly.

People who use drugs have numerous health conditions and will differ from the healthy volunteers in many ways that may reduce the external validity of the results from a pharmacokinetic study. Data must be interpreted with caution when extrapolating from studies in healthy volunteers to patients. The absorption in the nose may be different in patients. One study (conference abstract only) reports that vasoconstrictive nasal spray 30 minutes prior to use of a dilute naloxone nasal spray may impair the systemic uptake of naloxone (150). It is possible that other vasoconstricting drugs such as cocaine could produce a similar effect and reduce the uptake of nasal naloxone in patients. The bioavailability could also be influenced by the state the patients are in. A recent study of nasal naloxone in volunteers receiving an opioid infusion showed a bioavailability of 75%, compared to 40-50% in studies where nasal naloxone are tested without concomitant opioids (37, 104). This may indicate an interaction between opioids and nasal naloxone, which is important as the indication for nasal naloxone is opioid overdose.

This can reduce the external validity of study I as the volunteers here were not under opioid influence. If such an interaction is confirmed, it increases the exposure to naloxone in patients compared to volunteers, but it still raises questions about the approval of new nasal naloxone formulations based on studies in healthy volunteers without opioid exposure.

The pharmacokinetic-pharmacodynamic study (Study II)

As for study I, the experimental design conducted in the same environment and by the same study staff reduced systematic errors. This increase internal validity of this study. By using a steady state model of opioid agonism, combined with pupillometry in stable light conditions, the effect measured on pupil size can be attributed to naloxone only. The remifentanil TCI model was assumed to provide steady state conditions. This assumption was controlled by analyzing blood concentrations of remifentanil during the study. In addition, the lumination was controlled during the experiments to ensure similar light exposure throughout the study. The method used to quantify the amount of naloxone and remifentanil allowed for accurate quantification of analytes and were validated according to acknowledged principles (109, 110). Furthermore, the outcomes were objective measurements such as drug concentrations and pupillometry were used to evaluate the treatment.

The advanced experimental set up and the exploratory nature of study II means that there are several factors to take into account that limits the direct applicability of the results to the clinical setting. The steady state model of remifentanil was chosen as it gives the opportunity to isolate the effects of naloxone in the experiment. However, in overdose treatment the patients will never be under steady state opioid influence. For patients under influence of short acting opioids the concentrations will fall during the time naloxone exerts its effect, while for patients influenced by longer acting opioids, the concentrations can continue to rise after naloxone administration.

In this study only one opioid was studied. In reality, a broad specter of opioids is used recreationally and may cause overdoses. Different opioids will have somewhat different properties regarding receptor association-dissociation kinetics that can influence the rate and magnitude of naloxone reversal (103, 151). This is well-known for buprenorphine,

which larger naloxone doses are needed to reverse (152) and there are debates on the naloxone doses needed to reverse fentanyl overdoses (34, 37). Remifentanil is rarely used outside of hospital and has unique properties due to the elimination by unspecific esterases in the blood and very short half-life. Its receptor interactions with naloxone is not known, but one study in healthy volunteers showed possibly a smaller reversal of remifentanil induced respiratory depression with naloxone, than what was seen during an infusion with alfentanil (153). How these opioids compare to other opioids such as heroin, morphine or fentanyl is not known. In addition, interactions in community overdoses will be affected by other drugs such as benzodiazepines.

The use of pupillometry as pharmacodynamic measurement can also be discussed. Reversal of respiratory depression would have been a more clinically relevant endpoint. However, experimental research on respiration is very complicated. The simple measurements like respiration rate and oxygen saturation are insensitive and more complicated methods such as end tidal carbon dioxide measurements, respiratory inductance plethysmography or by parasternal intercostal muscle electromyography require competence and equipment (93-95). The results can also very easily be influenced by stimulating the participants, just talking or touching the participants stimulate their respiratory drive (95). This limits the possibility for studying respiration as well as collecting blood samples during the study (103). The strengths of using pupillometry is that it is a well-recognized and objective measure on opioid effect (96). It has also successfully been used to measure the antagonism of opioid effect in several studies (101, 102, 133, 154, 155) and it also produced clear results in the current study.

The observational study (Study III)

All the medical records in study III were digitalized by the same person throughout the study period. This strengthens the internal validity and avoids bias that could have been introduced by several individuals digitalizing the records. The medical record layout was changed once during the study period which could have influence recording practices. On the other hand, no major changes introduced. Recording practices are therefore assumed to have been similar throughout the study period and was not evaluated further.

Clinical observations on respiration and consciousness are reported in standardized fashion in the emergency medical services using respiration rate and Glasgow coma scale. Reliable scoring is fundamental to the use of such scores, and interrater variability can be an issue. There are several studies on the interrater variability of the Glasgow coma scale, but many of the studies have been of poor quality (156). Adequate reliability has been found in good quality studies, but the scoring is influenced by education, training, the level of consciousness in the patient and the type of stimuli used (156). There is therefore a possibility of scoring differences between the EMS staff. On the other hand, the EMS staff are well acquainted with the score and use it on all their callouts. Furthermore, all EMS staff follow the same training program and guidelines, and this reduces the reporting error. This possible reporting error were handled by grouping the score in categories rather than as a continuous scale from 3-15 in the models.

Reproducibility of study results across countries and in different settings strengthens the confidence in the study findings. We find that patients with a lower respiration rate and lower level of consciousness get higher naloxone doses. This is also found in a similar study in Australian emergency medical service which strengthens confidence in the results (124). The analysis on the influence of the safe injection facility were exploratory, and as these facilities not exist in all cities and they are run in different way, this is not necessarily generalizable to all other settings. The results also must be interpreted knowing that illicit manufactured fentanyl analogues were rarely used in Oslo during this time period (18).

Ethical considerations

All procedures performed in the studies involving human participants were in accordance with the ethical standards of the Helsinki declaration. The studies were approved by The Regional Committees of Medical and Health Research Ethics in Norway. Study I and II were also approved by the Norwegian Medicines Agency. Both these protocols were registered in clinicaltrials.gov prior to inclusion of participants. The ICH Good Clinical Practice guidelines were followed for the clinical studies, which provides public assurance that the rights, safety and well-being of the subjects are protected and consistent with the principles of the Helsinki declaration (106). Elements of these guidelines were

also used in study III even though this was not required, to improve the scientific quality of the work.

The pharmacokinetic study (Study I)

The Declaration of Helsinki recognizes that research ultimately must include studies involving human subjects, if we should achieve medical progress (126). Whom to include in clinical trials is a continuous discussion, we have chosen to include healthy subjects. This is often done in phase I trials, unless the drugs studied have significant toxicity, such as cytotoxic drugs that would rather be studied in patients (108). Even when studying new compounds, the risk for participants in phase I trials is in general considered to be low (157). In study I, we did not test a new compound, but a new formulation of an existing drug. The drug is well-known and has been used for decades. It is considered to be a safe medication, and has essentially no pharmacologic activity in the absence of opioids, even when given in doses ten times the usual therapeutic dose (33). Few adverse events were therefore expected, and it was considered safe to use healthy volunteers.

Medical research involving human subjects may only be conducted if the importance of the objective outweighs the risks and burden to the research subjects (126). Developing a naloxone nasal spray would be beneficial for future patients, but not for the study subjects. To secure the participants autonomy, they were given both written and verbal information about the nature, purpose, possible risk, and benefit of the study before they consented to participation. They were informed about the strict confidentiality of their participant data, and that we did not access hospital medical records. It was emphasized that the participation was voluntary and that they without consequence might terminate their study participation at any time and all participants signed a written consent before inclusion. In all, the risks were small, participants well informed and consented and therefore the study could be conducted.

The participants were compensated with 1500 NOK for a study day of 7 hours. It is common to compensate subjects for their participation in phase I studies as they have no benefit of the study, but it is important that the compensation is not too large, so people accept a greater risk than they elsewise had done. This could be a problem, as students with small economies are normally recruited to such studies. The compensation was set

to a level that would have compared to one day's work, and this magnitude was considered to be a fair amount and deemed not to be coercive. The ethical board approved the size of the compensation. The participants were also informed that they would be compensated for their participation, even if they decided to withdraw before the study was finished.

The pharmacokinetic-pharmacodynamic study (Study II)

In study II, healthy individuals were given a controlled substance, with abuse potential and safety concerns during its administration. The aim was to measure the effect of the antidote naloxone, and to measure the effect of the opioid antagonist, an opioid agonist must be present. In this study, this was achieved by administered a target-controlled infusion of remifentanil to healthy volunteers. An alternative approach would have been to include a population already using opioids. People who use drugs are generally considered to be a vulnerable population, and according to the Helsinki declaration they should receive specifically considered protection (126). Medical research in vulnerable group is only justified if the research addresses the health needs or priorities of this group and that the research cannot be carried out with a non-vulnerable group (126). This raised ethical concerns as there is a high risk that intravenous naloxone would cause withdrawal symptoms in patients who use opioids regularly, especially if it is not needed as a part of their treatment.

For the same reason patients in the hospital receiving opioids for pain management after surgery were not considered as a viable alternative. Patients coming for elective surgery and receiving remifentanil could have been an option. This carried the risk of naloxone acting longer than the study, and into the planned surgery and had also required large amounts of organizational work. The exposure would have been similar to exposing otherwise healthy individuals to the intervention and it was therefore considered a better solution to use healthy participants. In studies of abuse potential of drugs non-dependent recreational drug users are sometimes included (91). However, this is also debatable as this may help to aggravate their drug use. Studies in which healthy volunteers are administered controlled substances have been conducted both internationally and in

Norway. Balancing all these considerations, including healthy volunteers and design the study as safe as possible was found to be the best solution.

To ensure the safety of the participants during the study, several measures were taken. The study was conducted at an intensive care unit and a trained anesthetist was always present during the remifentanil infusion. Remifentanil was administered at a low dose compared to standard doses used in anesthesia, and without sedatives or other drugs that could interact with remifentanil. The subjects were required to fast before the study intervention according to standard protocols, in case intubation should be necessary (121). Remifentanil has known side effects such as respiratory depression, low blood pressure, chest wall rigidity, itching and nausea. Any side-effects were expected to be greatly reduced when naloxone was administered after 12 minutes. For safety and to avoid discomfort for the participants, the protocol allowed for use of anti-emetics, ephedrine and extra oxygen as concomitant medications in our study. Additional naloxone was available as rescue medicine in case of any safety concerns. With all these measures, the immediate safety of the participants during the study was considered well taken care of. This was confirmed during the study by a low rate of adverse events and no additional interventions such as intubation, discontinuation of remifentanil or administration of concomitant medications were needed during the study.

The other safety concern was causing or worsening addiction in susceptible individuals. Therefore, all participants were screened using the CAGE AID questionnaire (111, 112) and care were taken not to include people with history of drug abuse, prolonged used of opioid analgesics or professional access to drugs with abuse potential. Remifentanil was chosen because it reaches steady state quickly when given as a TCI so time under opioid influence could be minimized. It had also shown smaller abuse potential than other opioids in one study (158) and has also been used for research in similar protocols with no proof of addiction or misuse problems for the participants (104, 117, 159). The fact that naloxone was co-administered with the opioid also reduced these concerns, as the time under full opioid influence essentially was reduced to 12 minutes. It also decreases the risk that opioid users seek inclusion, as they would be very reluctant to join a study where they receive naloxone.

An arterial cannula was placed during the study. The risk of complications with this was small, but to reduce the risk even further, the participants were screened with Allen's test of collateral circulation of the hand. To minimise discomfort and pain, all the participants received local anaesthetics before cannulation.

The observational study (Study III)

In the observational study, medical records from patients 18 years or older that were treated with naloxone for opioid overdose were included. There was no intervention. Data on naloxone doses could have been collected in an anonymous database without consent. However, it was important to register patient identity to link subsequent episodes in the same individual and for the linking the data to the National Cause of Death Registry. Patient autonomy are one of the central principles in research and is usually achieved through informed consent (126). In this case, as the amount of data collected was limited and the only new data collected were linking to the registry. A solution where patients were informed about the project and given the opportunity to withdraw from registration was therefore chosen. The patients were informed about the strict confidentiality of their data, and that we did not access medical records other than the EMS record from the current episode, and that this was linked to the National Cause of Death Registry. This consent procedure safeguarded patients' rights and that their integrity was respected. Eight patients did not consent to the study. Patients included retrospectively were registered anonymously as they had not been given the possibility to withdraw from registration. With all these measures, the autonomy of the patients was respected and the balance between risk and burdens to the patients were considered acceptable. The study was approved by the Regional Committees of Medical and Health Research Ethics in Norway, who also approved the written participant information.

General discussion

The aim of this thesis work was to provide an evidence-base for adequate treatment of opioid overdoses using intranasal naloxone. More specifically, it investigated the pharmacokinetics of a high-concentration/low-volume nasal naloxone formulation, investigate the pharmacodynamics and arteriovenous difference of naloxone and examine what doses and routes of administration of naloxone are used in clinical practice.

1. Can a single nasal spray actuation deliver a therapeutic dose of naloxone for opioid overdose? (Paper I)

Take-home naloxone has been suggested since the late 1990s (160), but there were no approved pharmaceutical products for this purpose. Consequently, there has been a widespread use of improvised kits for intramuscular and intranasal administration of naloxone. As a response to this, NTNU started the development of a naloxone nasal spray in 2010. The overall aim was to produce a nasal spray in a ready-to-use device, that could deliver a therapeutic dose of naloxone in one spray actuation.

The shortest way for approval of a new formulation of an existing drug is to rely on previous findings of safety and effectiveness of the already approved product. To do this, knowledge about the new and existing products must be bridged. For example, by showing that the blood concentrations achieved with the alternative administration route is comparable to the already approved formulation. Bioequivalence drug trials are common for this purpose. In such trials with a drug administered by a single dose, AUC_{0-t} and Cmax are the most important variables. Formulations are bioequivalent when the 90% confidence interval for the ratio of the geometric means of AUC and Cmax (after log transformation) for the test and the reference product are within the 80-125% interval (83). The advice from the FDA regarding nasal naloxone formulations was to investigate the bioavailability and make the dose selection based on this knowledge, and then compare the new drug to a parenteral dose of at least 0.4 mg naloxone and aim for a similar or greater exposure from the new drug (109). To investigate the potential for the new nasal spray, its bioavailability and maximum concentration will be important. The time to maximum concentration is not a part of the bioequivalence criteria, but as

naloxone is used to reverse respiratory depression the speed of onset is crucial. Tmax will therefore be an important characteristic of a nasal formulation. As we compare two different routes of administration the exact bioequivalence criteria may not be met, but the new formulation may still be approved. This is unlike comparing drug with same route of administration where the AUC 90% confidence interval must be within the above set limits. Study I was designed following many of the same principles as a bioequivalence studies, but without bioequivalence as a primary endpoint.

Most take-home naloxone programs employed improvised devices with prefilled syringes containing 2 mg/2 ml naloxone formulation. The syringe was connected to a nasal mucosal atomizer device. There have been many reports on successful reversal with these kits (37). At the same time, they have been debated as these dilute formulations have not been properly investigated or approved (161). Before the start of our project, the only available study on intranasal naloxone suggested an absolute bioavailability of 4% only (73). In this study large volumes (2.5 ml per nostril) of naloxone had been administered nasally, and despite best efforts the subjects swallowed a great amount of the drug. The drug assay also had low sensitivity. However, this 0.4 mg/ml solution was never used much for nasal administration in clinical practice.

Until recently there were no studies published on the pharmacokinetics of the 2 mg/ 2ml formulation, but an examination of data in patent reports indicated that its relative bioavailability was 10% to intramuscular naloxone (162). Later a more thorough investigation estimated its relative bioavailability to be about 19-23% (163). Administering 2 mg of naloxone with an improvised nasal spray solution with 10-20% bioavailability is equivalent to a 0.2-0.4 mg injected naloxone dose. This is outside or in the lower end of the recommended dosing range for naloxone. Increasing the volume might not increase the exposure to the drug, as the bioavailability might be reduced even further when higher nasal volumes are employed (73). Thus, the safety margin of these improvised devices may be unsatisfactory.

In contrast, by comparing $AUC_{0\text{-}t}$ for the different treatment options in study I we found an absolute bioavailability of 52-54% for nasal naloxone. This was far higher than previously shown for any nasal naloxone spray at that time. Follow-up studies of our

nasal spray formulation confirmed absolute bioavailability of about 50%, later also shown for other approved naloxone nasal sprays (164, 165). The relative bioavailability compared to intramuscular administration was reported to be about 44-55% both for the NTNU spray, and for other approved formulations (164-166). All the approved nasal naloxone sprays currently in the market are delivered in a volume of 0.1 ml (37). However, it should be noted that the relative bioavailability of the NTNU spray to intramuscular naloxone under remifentanil infusion was as high as 75% (104). Based on the results from study I, and other work at NTNU a dose of 1.4 mg/0.1 ml intranasal naloxone was chosen as the formulation to further studies aiming at market authorization (164).

Comparing data on the maximum concentration for nasal naloxone found in this study with available data from the public domain showed that the reported Cmax in this study was much higher than the 0.5 ng/ml reported after the 2 mg/2 ml improvised nasal spray (162). The 0.8 mg IN dose aimed for a concentration close to a 0.4 mg IM injection, and the 1.6 mg dose aimed for a concentration close to a 0.8 mg IM injection. The Cmax found after 0.8 mg IN in this study was higher than the Cmax (1.1-1.2 ng/ml) reported after 0.4 mg naloxone IM, and the 1.6 IN gave higher concentrations than 2 x 0.4 mg IM that gave a Cmax of 2.2 ng/ml (167). This indicated that our nasal spray achieved clinically relevant concentrations and subsequently that a therapeutic dose could be provided in one single spray actuation.

A later review has found that dose-corrected Cmax for nasal naloxone in high-concentration/low-volume formulations varies from 1.3-2.0 ng/ml (37), which coincides well with our dose-corrected Cmax of 1.6-1.7 ng/ml. We also found a dose-serum concentration relationship indicating that there was no saturation of the uptake at the doses investigated here. This has also been reported elsewhere and for a wider dosing range (37). In this study, the two sprays were given in different nostrils at the same time. In a following trial, two actuations (1.4 mg x 2) were given in the same nostril 3 minutes apart (164). In this study, dose linearity was also confirmed. This is important for titration and repeated administration.

Intravenous naloxone 1.0 mg was used as comparator to the nasal spray in study I. This provided an instantly high naloxone concentration measured at 14.2 ng/ml 2 minutes after administration. The maximum concentration for the nasal spray was achieved after 18 minutes and were 1.5 and 2.6 ng/ml for the 0.8 and 1.6 mg doses, respectively. Since we used intravenous naloxone as comparator, the bioequivalence criteria could not be met (83). In intravenous administration Tmax is immediately achieved and the maximum serum concentration are many times higher for the intravenous administration than for intranasal naloxone (Figure 6, Figure 7). Bioequivalence was not an objective in this study, but for future studies intramuscular naloxone should be considered as a comparator as this might have characteristics more similar to IN naloxone.

In relation to intravenous naloxone our nasal spray provided a lower Cmax (1.5-2.6 ng/ml), than the initial concentrations measured after intravenous administration (14.2 ng/ml 2 minutes after administration) which may explain the high risk for withdrawal symptoms after IV naloxone. The 1.6 mg IN also maintained a higher concentration after 15 min (Figure 6). This may indicate a lower risk for precipitation of withdrawal symptoms combined with a possibly longer duration of action for IN naloxone compared to IV naloxone. This allows for the titration to clinical response that is highly recommended. This could maximize the effect and minimize the occurrence of withdrawal reactions (45).

The time to maximum concentration were 18 minutes for the nasal spray in study I. This confirmed the findings of our pilot study where Tmax was estimated to 16 minutes (110). It also agrees with available reports of a Tmax of 15-20 minutes for intramuscular naloxone (167) and 20-30 minutes for intranasal naloxone (166). Tmax50 was between 8-10 minutes. This was encouraging, and lead to the use of intramuscular naloxone as comparator in other studies from our research group. A recent review on take-home naloxone have found that among the intranasal naloxone formulations available, the Tmax is reported between 15-30 minutes, somewhat slower than for intramuscular naloxone that has a reported Tmax of 8-24 minutes (37).

There were no serious adverse events during the study. Mild local reactions such as the taste of the nasal spray was commonly reported. One participant suffered a spontaneous

nosebleed during the study to the contralateral nostril to where naloxone had been administered and was excluded after a nasal cauterization as he no longer fulfilled the inclusion criteria. No definitive conclusions regarding the safety of the spray can be drawn from a study in 12 subjects, but it seems to be well tolerated.

Overall, our nasal spray has a satisfactory bioavailability and achieved clinically relevant maximum concentrations within a reasonable time when compared with available data from other studies. The nasal spray was well-tolerated and there were no serious adverse events. Based on this study it was probable that a single naloxone nasal spray can deliver a therapeutic dose of naloxone for opioid overdose, but the dose must be adjusted and compared with intramuscular naloxone.

2. Is it possible to develop a model for studying the effect of naloxone in healthy volunteers? (Paper II)

To measure the effect of naloxone, the subjects must be given an opioid. Previous studies have failed to create a model where the opioid effect is stable throughout the trial (103, 133-135). Naloxone pharmacology was our primary objective, and therefore we made efforts to avoid the confounding processes of absorption, distribution and elimination of the opioid. This was achieved by a steady state model of opioid agonism in study II. We applied a model in which the subjects received a plasma target-controlled infusion of remifentanil. This computerized infusion uses a multi-compartment pharmacokinetic model system to deliver remifentanil to rapidly achieve and maintain a set blood concentration (119).

As seen in Figure 9b steady state of remifentanil in arterial blood was reached after 12 minutes, which was maintained throughout the infusion period. The observed arterial concentrations of remifentanil were 12% lower than predicted by the TCI system. One explanation for this is that the target used were lower than in clinical practice, and lower than what the infusion-algorithm were developed for. This was assumed to be of less relevance for the study findings, as the steady state were confirmed. There was a clear arteriovenous difference for remifentanil. This complies with previous reports (87).

The plasma target for remifentanil infusion was 1.3 ng/ml. Another trial by our research group used remifentanil TCI at targets of 2.5, 1.3 and 1.0 ng/ml in combination with 0.8 mg intranasal and 0.8 mg intramuscular naloxone had seen slower and much less pronounced changes in pupil size, especially at the 2.5 ng/ml target (104). On this basis the choice of 1.3 ng/ml for study II was made. Study II also had a higher naloxone dose and intravenous administration that produced a strong effect on pupil size, indeed so fast we did not meet our end point on the modelling of the serum-effect-site equilibration rate constant and its half-life.

The participants were kept in a room with dim ambient light, so the pupil size was large (7.4 mm) at the beginning of the trial. The procedure gave clear results as the remifentanil infusion reduced pupil size to 3.6 mm and this was rapidly and completely reversed by 1.0 mg of intravenous naloxone (
Figure 8) during the remifentanil infusion. The effect of naloxone was clearly visible, but the naloxone dose might have been too large compared to the dose of remifentanil so we may have seen a ceiling effect. It is possible that with a higher opioid level, the maximum effect (Emax) of 4 minutes had arrived a little later. One of the aims was to study the serum-effect-site equilibration rate constant and its half-life, but due to the complete reversal achieved within few minutes and only two pupillary measurements within this time period this gave us too few observations to reliably estimate these variables.

In addition to opioid agonism, remifentanil infusion may produce interactions with naloxone. As mentioned above, a remifentanil infusion may increase the relative bioavailability of intranasal to intramuscular naloxone to 75% (104). So far, we are not able to explain the mechanism behind this observation. The summary of product characteristics of naloxone and remifentanil does not indicate any pharmacokinetic interactions between the drugs (33, 116). However, opioid influence in general, or remifentanil in particular, may produce physiologic changes that affect naloxone pharmacokinetics.

As in study I, 1.0 mg intravenous naloxone was administered to the volunteers. Comparing these results with our findings in a similar study on intranasal and intramuscular naloxone during remifentanil TCI (104), the differences between the

administration pathways are clearly visualized. The effect of intravenous naloxone kicks in far quicker and more profoundly than both intramuscular and intranasal naloxone. This can be explained by the much higher initial serum concentrations after intravenous naloxone than after IM and IN naloxone. This may in some deeply intoxicated subjects be beneficial, but for those suffering a less severe intoxication, this may cause withdrawal symptoms (44, 45).

The remifentanil infusion lasted for 90 minutes after naloxone administration. After these 90 minutes, the naloxone effect was still visible on the pupil size. As the effect on pupil size were gradually reduced in a linear pattern, it was possible to estimate the duration of the opioid reversal effect. It was found to be 118 minutes (
Figure 8). This is similar to what was found in a study of opioid dependent subjects that were given intravenous naloxone where the maximum level of withdrawal symptoms was found after 5 minutes, and the effect lasted for 90-180 minutes (101). This also corresponds to other reports of naloxone effect lasting between 45 minutes and 180 minutes (33). The estimated minimum effective concentration of naloxone was 0.5 ng/ml. This was estimated during steady state in the elimination phase for naloxone and is not necessarily applicable in the initial uptake phase. Further investigations into the minimum effective naloxone concentration in clinically relevant outcomes such as respiration on opioids used in the community would be of great interest. One way this could have been achieved would have been to include patients in treatment for example in heroin clinics and combine this with measurements of respiration depression (93). Such a study could take the opportunity to evaluate the possible interaction between nasal naloxone administration and concomitant use of opioids.

Opioids such as heroin, fentanyl and remifentanil has been shown to have an arterio-venous difference in the first minutes to the first hour after administration (85-87). If this was true for naloxone, only studying the pharmacokinetics of naloxone in venous samples could have impaired the understanding of the drugs pharmacokinetics. The arterial blood concentration is the most important for naloxone where the time to onset is crucial as the arterial blood is supplying the brain. In study II, we investigated the arterio-venous difference of naloxone (Figure 9a) and found no obvious indication that there is such a

difference. The statistical analysis showed that there might be a higher naloxone concentration in venous samples after 30 minutes, but the difference was very small and clinically irrelevant as the arterial AUC_{0-t} was 93% of the venous AUC_{0-t}. For the early phase after intravenous administration there were no arterio-venous difference in naloxone concentrations. This is important, as venous blood concentration then can be used for modelling purposes allowing us to combine data from this study with data from previous studies in a pharmacokinetic-pharmacodynamic population model.

To summarize, knowledge on physiological response is important as there can be significant delays between serum concentrations and response. Pharmacokinetic-pharmacodynamic studies may bridge the gap between healthy volunteers and real patients. The model produced a steady state of opioid influence and allowed for studying the effect of naloxone in healthy volunteers. One mg naloxone IV resulted in a rapid and complete reversal of the remifentanil effect. The model could be used to estimate the duration of effect, which was 2 hours. The model could be used to visualize dose-response effects and differences regarding time to maximum effect between the IV administration in the current study and IN and IM treatment routes used in another study. A similar study on patients treated with commonly used opioids would have been valuable supplement. The concentrations of naloxone in arterial and venous blood was for practical purposes similar. Thus, venous concentrations can be used in future PK-PD modelling.

3. What doses and administration routes of naloxone for opioid overdose are used in clinical practice in Oslo, and are these safe in the prehospital setting? (Paper III)

In our work with developing a naloxone nasal spray the choice of comparator administration route and dose to the spray was paramount. Intravenous administration of naloxone is recommended if possible in the summary of product characteristics for the drug (33), but many emergency medical services also in Norway had guidelines recommending use of intramuscular naloxone as first line treatment (40). The dosing guidelines are wide and often recommend starting dose between 0.4 mg and 2.0 mg naloxone for suspected opioid overdose in the community (33). Internationally, there are different policies regarding dosages and routes of administration across services and countries (9) and there is no agreement on the optimal route or dose of administration of

naloxone. This has also been pointed out by the WHO (9). To have a sound scientific rationale behind the choice of comparator for our nasal spray we conducted study III where we investigated the doses, administration routes and some safety aspects of naloxone that were used in one of the largest emergency medical services in Norway.

During study III, a total of 2215 cases were included over 5 years. There were 77% were men in the sample. The overrepresentation of men among patients treated for overdose is well-known and our findings compares well with reports between 70-80% men among patients treated for overdose in both Norway and other countries (143, 146, 168-170). The mean age in the study was 38.3 years. Internationally, the mean age of people treated for overdose varies from 33-40 years (168-170). However, in a previous study of use of naloxone in Oslo, Norway, conducted in 1998-99, it was found a mean age of 32.6 years (146). This might indicate that the population are older now than 20 years ago. But in general, the key demographic variables in the study could be compared with previous reports and indicates a representative sample.

Most of the cases (92%) were treated with intramuscular naloxone only as the first treatment. Few were treated with intravenous naloxone. The guideline for the Oslo EMS recommends intramuscular naloxone followed by intravenous naloxone, but this regime was used in only 4 % of the cases. Over the last 20 years there is a reduction in the mean total naloxone dose in Oslo from 1.2 mg (146) to 0.8 mg naloxone for patients with respiratory arrest and cyanosis. This reduction is largely explained by a change in clinical practice over the last 20 years where fewer patients are administered IV naloxone immediately following an IM injection like the guideline outlines (40, 146). There are many possible explanations to this change. One is the time it takes to establish IV access. In a study comparing time from arrival of EMS staff to the effect of naloxone, there is no difference between use of intravenous and subcutaneous naloxone (171). The intravenous naloxone was found to have a faster onset from administration, but time won in onset were lost in time used to place the IV cannula (171). Intravenous naloxone also has a high rate of adverse events from withdrawal (146) and anecdotally, EMS staff report reduced opioid withdrawal and increased cooperation of the patients after reversal with antidote through IM injections alone. This was also reported in the study that compared

intravenous and subcutaneous naloxone (171). A survey of 25 guidelines on naloxone dosing found that a majority of the resources surveyed recommended initial naloxone doses an order of magnitude lower than that suggested in the early 1990s and argued for the decreased risk of opioid withdrawal and the ability to re-dose as needed (38). This might indicate that there may be an international trend towards more careful and lower naloxone dosing.

Among those treated with intramuscular naloxone, the most common starting doses were 0.4 and 0.8 mg. We found an inverse relationship between level of consciousness and naloxone dose, and the same was found for respiratory rate (Table 3). Patients with a lower respiratory rate and lower GCS scores were more often treated with the larger dose, 0.8 mg naloxone as the starting dose. It seems reasonable that initial clinical presentation influences the choice of initial naloxone dose and indicate that emergency medical staff uses clinical judgement in their dose selection. In a previous study the same relationship was found (124). Interestingly, in that study the naloxone doses were higher, and 1.6-2.0 mg naloxone were considered the "standard dose". In our study, men were more likely to be treated with the higher dose than women. This was also found in the Australian study (124), but this could also be related to the Norwegian guideline that suggest to consider body size when deciding the initial dose (40).

One or more repeated doses were used in 15% of the cases. The need for multiple naloxone doses was associated with unconsciousness and respiratory arrest, the same factors as those associated with the initial dose (Table 4). Furthermore, an initial dose of 0.8 mg reduced the probability of the administration of multiple dosages by 60%. This indicates that EMS staff use their clinical judgment to titrate naloxone dosing according to clinical presentation and treatment response.

Interestingly, patients at the safe injection facility were often treated with the lower naloxone dose (0.4 mg) and were less likely to receive a second dose than patients at other locations. This even though they often presented with deep coma and respiratory arrest (172). The staff at the facility does not administer naloxone but manages patients with bag-mask ventilation until the arrival of the EMS. The lower naloxone dose may be a consequence of patients being attended by staff from the safe injection facility while

waiting for the EMS and therefore becoming less hypoxic. The facility is also a well-organized work environment and allows the EMS to start lower in their titration of dosages and give this lower dose time to work. Patients treated in the safe injection facility were also more likely to be left at the premises, probably due to the facility offering post-overdose monitoring and counseling (172).

The risk of death by rebound intoxication after leaving the patient on scene after naloxone administration is considered to be low (173). In our study, a large proportion of the patients declined transfer to further care after naloxone treatment by the EMS. This has previously been reported both in Norway and in other countries (146, 173). The number of patients left on site after naloxone treatment in Oslo have decreased from 85% to 57% the last 20 years (146). Three patients died of overdose on day 1 after they had been treated by EMS with naloxone. These patients were alive longer than the expected duration of action of the naloxone administered by the EMS and are therefore unlikely to be rebound opioid intoxications. This shows that the naloxone dosing regimens used, combined with an average observation time of 32 minutes, are safe in terms of immediate mortality. These findings are in keeping with previous studies on discharging patients on site after naloxone treatment (46, 173-175).

Even though the risk from rebound intoxication is low, the one-week mortality from drug-related deaths after being left at the scene was 5.5 per 1000 episodes. This is much higher than the 0.8 per 1000 episodes reported as the risk for death by rebound opioid toxicity after naloxone treatment for patients left on the scene in a recently published review (173). Most of the patients in our study died of a new overdose. The same was found in a study of 2241 patients discharged after naloxone treatment, the 48-hour mortality was reported to be 5.8 per 1000 episodes when counting all overdose-related deaths, not just those attributed to rebound opioid toxicity (46). This might indicate a need to widen the perspective beyond solely focusing on reversing the current overdose and risk of rebound toxicity for patients treated with naloxone, but also taking into account their risk of death by new overdoses. Opioid overdoses are known risk factors for early death (176) and deaths from new overdoses must be considered preventable events. The emergency

medical services may play an important part in providing better follow-up care to this patient group.

Bringing the patient to the hospital after an overdose is not in itself sufficient. An Australian study of 3921 overdoses reported 11 deaths from new overdoses within one week after EMS treatment (175). Nine of those who died had been brought to the hospital. Three of them had even self-discharged and died within 24 hours of EMS attendance (175). Being brought to a hospital or a healthcare facility is therefore not necessarily protective but probably depends on what further treatments are offered during hospitalization. Medical observation is important to catch if the patient deteriorates and need more naloxone, but further measures are probably needed to prevent future overdoses such as engagement in addiction treatment (177).

The study was also used to provide evidence for what comparator dose to use in future studies. We found that the total doses of naloxone administered including initial and subsequent doses of titration was 0.4 mg for 33% of the cases, 0.8 mg for 51% of the cases and more than 0.8 mg for 13% of the cases. We wanted to find an optimized dose that would provide sufficient reversal without unnecessary causing withdrawal symptoms in patients. It is a limitation with the study that we do not know if patients could have been treated with a smaller dose than the initial dose. Even though the reliability of the improvised sprays are questioned due to their low uptake (37, 162) there have been many reports on successful reversals with the improvised dilute naloxone spray (56, 144, 178). This raises questions regarding the doses needed for reversal of an overdose and might indicate that it is possible to use lower doses what we are used to today. This would not have been discovered with the present study design as the underlying assumption in this study is that today's treatment is optimal. A clinical study of naloxone titration, starting dose at around 0.1-0.2 mg, with new doses every 2-3 minutes until sufficient reversal could have enlightened this question.

It is important to strike a balance between quickly reversing the respiratory depression cause by the overdose and to avoid withdrawal symptoms if possible (45). In our study, 87% of the cases were treated with 0.8 mg or less. Aiming at a dose similar to this would provide sufficient reversal for a large part of the population. The higher dose will also

more quickly rise above the concentration needed to reverse the overdose and provide a quicker onset which could be important if nasal naloxone has a somewhat slower Tmax that intramuscular naloxone. A previous publication on the first two years of this data collection described the patients clinical status based on their location, and found that patients that are treated in private homes are those with lowest GCS score and respiration rate (172). These are the patients who will be treated with naloxone spray as a part of the take-home naloxone programs. As we have showed here, they are also more likely to be treated with 0.8 mg naloxone IM. As lay people are supposed to administer take-home naloxone, the dose should have a high safety margin.

This has been recognized within the regulatory and scientific community (179). After discussions within the FDA Anesthetic and Analgesic Product Advisory Committee, the committee narrowly voted to increase the minimum recommended naloxone exposure for novel products entering the market from 0.4 mg without specifying an acceptable dose (179). At the same time, FDA have approved take-home naloxone products delivering 2 mg intramuscular and 4 mg intranasal naloxone (166, 167). In our study, only 1% of patients were treated with 2 mg naloxone or more. A 2 mg dose by injection (IM/IV) would have overtreated almost the whole population in our study with a larger risk of causing withdrawal symptoms. In a report of community use of the 4 mg nasal spray, 37.8% of the patients had adverse events that may be related to opioid withdrawal (180).

There are also debates on the need for higher naloxone doses due to the increasing amount of overdoses from fentanyl, and the use of multiple naloxone doses by EMS in United States are reported to have increased from about 15% in 2012 to 18% in 2015 (181). At the same time, naloxone doses of up to 0.8 mg has been found to be sufficient in the community setting where illicitly manufactured fentanyl circulates, and for use by EMS when treating fentanyl overdoses (141, 182). There is also a range of other reports that support that 0.8 mg naloxone will be sufficient for most patients (183). Doses surpassing 0.8 mg are more likely to precipitate significant withdrawal symptoms (9). Many argue that lay people cannot titrate naloxone. However, there are many reports from studies of non-medical personnel using naloxone in regimes with titrating and the overall impression is that this has worked well (34).

In balancing these considerations, we chose a comparator of 0.8 mg intramuscular naloxone for our future studies and plan that the nasal spray will be delivered with two sprays per pack. This will reverse a large proportion of the overdoses with the first spray, and a second dose can be administered if the first is unsuccessful.

To conclude, 87% of the cases were treated with 0.8 mg naloxone or less, and intravenous naloxone was seldom required. Thus, intramuscular naloxone in initial doses of 0.4 to 0.8 mg appear effective and safe for the treatment of prehospital opioid overdoses. The data indicated that the emergency medical staff titrates naloxone based on clinical presentation and effect, and GCS and respiratory rate stand out as strong predictors for dosing choices by EMS. Even though the risk of rebound opioid toxicity was low, the population in this study had an alarmingly high one-week mortality rate, which was much higher than previously reported. We chose 0.8 mg intramuscular naloxone as comparator for our nasal spray in future studies based on these results.

Conclusion

With regards to the research questions, the following conclusions can be drawn.

1. Can a single nasal spray actuation deliver a therapeutic dose of naloxone for opioid overdose?

Yes. The high-concentration/low-volume naloxone nasal spray had a satisfactory bioavailability and achieved clinically relevant maximum concentrations within a reasonable time when compared with available data from other studies. The nasal spray was well-tolerated and there were no serious adverse events. Based on these results, it was probable that a naloxone nasal spray can deliver a therapeutic dose of naloxone for opioid overdose in one spray.

2. Is it possible to develop a model for studying the effect of naloxone in healthy volunteers?

Yes. The employed remifentanil TCI model produced proven steady state conditions, allowing for studying the pharmacodynamic effects of naloxone only in healthy volunteers. One mg naloxone IV resulted in a rapid and complete reversal of the remifentanil effect lasting for 2 hours. The concentrations of naloxone in arterial and venous blood was for practical purposes similar. Thus, venous concentrations can be used in future PK-PD modelling.

3. What doses and administration routes of naloxone for opioid overdose are used in clinical practice in Oslo, and are these safe in the prehospital setting?

It was found that 87% of the cases were treated with 0.8 mg naloxone or less, and intravenous naloxone was seldom required. Thus, intramuscular naloxone with initial doses of 0.4-0.8 mg naloxone appear effective and safe for the treatment of prehospital opioid overdoses. The staff titrates naloxone based on clinical presentation. The risk of rebound opioid toxicity was low, but the population one-week mortality rate was high.

Future perspectives

The results from the study of the naloxone nasal spray was promising and initiated a process towards a commercialization. Through industry collaboration with dne pharma as, NTNU has conducted a regulatory pharmacokinetic study in 22 healthy volunteers. This has resulted in the marketing authorization of Ventizolve 1.26 mg naloxone, corresponding to 1.4 mg naloxone hydrochloride, in 12 European countries. This product is planned available in the market from August 2020.

Further knowledge on the pharmacokinetics and pharmacodynamics can be achieved by utilizing data from the studies conducted and combining them in a pharmacokinetic-pharmacodynamic population model.

Although studies in healthy volunteers can lead to marketing authorization, the safety must be proved in post marketing surveillance or adequately conducted randomized clinical trials. To provide further evidence for the clinical use of naloxone nasal spray NTNU are currently conducting a double-blinded, double-dummy, randomized controlled trial in 200 patients treated for opioid overdose by emergency medical services in Oslo and Trondheim. Based on the results from study III, 0.8 mg intramuscular naloxone have been used as a comparator in both the regulatory pharmacokinetic study and the randomized controlled trial.

As new approved formulations of intranasal naloxone are available, the time has come to replace improvised solution with evidence-based and approved treatment options. Further efforts in the field of opioid overdose, such as nalmefene nasal spray, may improve treatment for overdoses with long-acting opioids. The effort over the last few years to create proper high-concentration/low-volume formulations of naloxone should be extended to other drugs where off-label nasal use is prevalent. Any off-label use of nasal drugs should be considered experimental and be discouraged except as a part of a scientific process to provide evidence for efficacy and safety.

Intranasal naloxone is only one aspect in prevention programs for opioid overdose. As shown in study III, the risk of repeated overdose and death is high. This must be

recognized, and measures put in place to also prevent the next overdose. This can be done by strengthening the “chain of survival”, a concept in emergency medicine first developed for cardiac arrest. It is a framework to coordinate efforts from different contributors with the aim of increasing survival. A chain of survival in opioid overdose start at bystanders who must recognize overdose early, alert medical services, perform rescue breaths and administer take-home naloxone. For the emergency medical services, it would emphasize swift dispatch of an ambulance that assess the patient, provide basic life support and titrate more naloxone if needed. The last very important link for the chain to be successful is direct transfer to a unit providing evidence-based post-overdose follow-up care.

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I

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II

Paper 2: Tylleskär, Ida; Skulberg, Arne Kristian; Skarra, Sissel; Nilsen, Turid; Dale, Ola. Pharmacodynamics and arteriovenous difference of intravenous naloxone in healthy volunteers exposed to remifentanil. European Journal of Clinical Pharmacology 2018 ;Volum 74.(12) s. 1547-1553.

III

Tylleskär, Ida; Gjersing, Linn; Bjørnsen, Lars Petter Bache-Wiig; Braarud, Anne-Cathrine; Heyerdahl, Fridtjof; Dale, Ola; Skulberg, Arne Kristian.
Prehospital naloxone administration – what influences choice of dose and route of administration?.

Prehospital naloxone administration – what influences choice of dose and route of administration?

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Abstract

Introduction:

Amidst the ongoing opioid crisis there are debates regarding the optimal route of administration and dosages of naloxone. This applies both for lay people administration and emergency medical services, and in the development of new naloxone products.

We examined the characteristics of naloxone administration, including predictors of dosages and multiple doses during patient treatment by emergency medical service staff in order enlighten this debate.

Methods:

This was a prospective observational study of patients administered naloxone by the Oslo City Center emergency medical service, Norway (2014-2018). Cases were linked to The National Cause of Death Registry. We investigated the route of administration and dosage of naloxone, clinical and demographic variables relating to initial naloxone dose and use of multiple naloxone doses and one-week mortality.

Results:

Overall, 2,215 cases were included, and the majority (91.9%) were administered intramuscular naloxone. Initial doses were 0.4 or 0.8 mg, and 15% of patients received multiple dosages. Unconscious patients or those in respiratory arrest were more likely to be treated with 0.8 mg naloxone and to receive multiple doses. The one-week mortality from drug-related deaths was 4.1 per 1000 episodes, with no deaths due to rebound toxicity.

Conclusions:

Intramuscular naloxone doses of 0.4 and 0.8 mg were effective and safe in the treatment of opioid overdose in the prehospital setting. Emergency medical staff appear to titrate naloxone based on clinical presentation.

Background

There has been an ongoing rise in deaths from opioids(1), in 2017, the U.S. Department of Health and Human services declared this a public health emergency(2). In response to the opioid overdose epidemic, take-home naloxone programs and new naloxone formulations for opioid overdose reversal have been developed(1, 3, 4). There is no agreement on the optimal route of administration or dosages, leaving no established best practices when naloxone is administered in the community. After discussions within the U.S. Food and Drug Administration (FDA), the agency narrowly voted to increase the minimum recommended naloxone exposure of 0.4 mg for novel products entering the market without specifying an acceptable dose(5).

Importance

When investigating new formulations such as nasal naloxone, one needs to know what doses and routes new formulations should be compared to. It is not only take-home naloxone programs that lack uniform guidelines and best practices. Naloxone has been available to emergency medical services (EMS) since the 1970s, the recommended initial dosage range is wide, ranging from 0.4 to 2 mg naloxone hydrochloride(1, 6), and the optimal route and dosages have not been scientifically established. Traditionally, naloxone has been administered both intravenously (IV), intramuscularly (IM) and subcutaneously, with different policies regarding dosages and routes of administration across services and countries(1). In the treatment of respiratory arrest, rapid restoration of the patient's own breathing is vital, but the price to pay for aggressive naloxone treatment is eliciting opioid withdrawal symptoms(7). This should not be ignored as a minor issue. Withdrawal symptoms can lead to further drug seeking and may make patients refuse further necessary follow-up(8). Consequently, there is a need for more evidence on the most effective route of administration and dosages that do not induce opioid withdrawal symptoms but that also ensure no rebound toxicity.

Goal of this investigation

We examined characteristics of naloxone administration among patients attended by the largest EMS in Norway between 2014 and 2018, including a) route of administration, b) dosage and c) number of doses administered at each EMS attendance. We estimated the putative associations between naloxone dose and sex, age, place of attendance and vital signs. We estimated the likelihood of administration of multiple naloxone dosages in a single EMS attendance as a function of initial dose, sex, age, place of attendance and vital signs. We examined transfer rates following EMS treatment and the one-week mortality rate to provide safety data for clinical practice.

Methods

Study design

This was a 5-year observational study of patients treated with naloxone by the Oslo City Center EMS. Participants were prospectively included between June 1st, 2014 and December 31st, 2018 and were thereafter followed through the National Cause of Death Registry until December 31st, 2018. Data from January 1st, 2014 to May 31st, 2014, were collected retrospectively and registered anonymously with no matching against the death registry.

Setting

Norway has a population of 5.3 million people(9) with a high rate of fatal opioid overdoses(10). Oslo has 690,000 inhabitants(11). Oslo City Center EMS is the largest service and attends the majority (67%) of the city's overdose cases. The most commonly used illicit opioid is heroin. Although a range of other opioids are misused, fentanyl plays a minor role in the current drug market(10). The recommended local management of suspected opioid overdose is assisted ventilation and naloxone administration. The suggested therapy is the administration of 0.4 mg to 0.8 mg IM naloxone followed by 0.4 mg IV. Dosing should be based on clinical presentation, and a suggestion is made to consider 0.8 mg IM for patients weighing more than 70 kg. Further titration with 0.4 mg IV up to a total dose of 2 mg is recommended if respiration and consciousness are not restored(12). The EMS administers naloxone hydrochloride in formulations for injection of 0.4 mg/mL.

Participants

Patients were included if they were 18 years or older and naloxone was administered by the Oslo City Center EMS. Patients with opioid-induced cardiac arrest were not included, as they are not administered naloxone during advanced life support(6). Patients received oral and written information about the study. Participants were given the opportunity to withdraw from the study on site or later by phone.

Data sources

The Oslo EMS uses paper-based medical records. Records for included patients were copied and filed in a separate system. A trained research nurse manually entered data from the records into a database. Patient sex, patient age, place of attendance, and naloxone doses and their routes of administration were registered. Clinical variables such as respiration rate (RR) and consciousness reported as a Glasgow Coma Scale (GCS) score, both before and after EMS treatment with naloxone, were also recorded. Prior to analysis, the key data were verified by two researchers against the original records. Missing data were not imputed. The data management system used was VieDoc version 4 (PCG Solutions, Uppsala, Sweden). For each patient, the first event after June 1st, 2014, was defined as the index episode, and all subsequent episodes were classified as "repeated episodes". National identity numbers were used to link episodes. The date and cause of death were retrieved from the National Cause of Death Registry.

Statistics

Statistical analyses were conducted in STATA 15.1. Descriptive statistics were used to describe the route of naloxone administration, naloxone dosages and the number of doses administered during EMS attendance. We used univariate and multivariable logistic regression analyses to examine 1) the associations between naloxone dose and patient sex, patient age, place of attendance and vital signs and 2) the associations between multiple naloxone doses (≥ 2) during an EMS attendance and patient sex, patient age, place of attendance, vital signs and initial naloxone dose. The regression analyses only included cases with a valid national identity number, as this allowed for accounting of repeated events by including identity as a cluster variable in the models. Odds ratios (ORs) and adjusted ORs (AORs) with 95% confidence intervals (CIs) are reported.

We reported transfer rates following EMS treatment. These rates included being left at the scene or transferred to a hospital, a primary care accident and emergency outpatient clinic, or other places such as home or addiction treatment facilities. We examined one-week mortality after treatment. The date of death was retrieved from the National Cause of Death Registry. The time of death was not available. Deaths registered on the same date as EMS treatment were defined as "day 0" and deaths the following date as "day 1". To estimate one-week mortality, we used deaths that occurred on day 0 through day 7.

Measures

The dependent variable in the first logistic regression (Model 1) was IM naloxone at doses of 0.4 and 0.8 mg, and 0.4 mg naloxone was the reference category. Only 3.6% received naloxone by other dosages and 7.4% via other routes; therefore, we excluded these from the analysis. In Model 1, the following explanatory variables were included: patient sex, patient age, GCS and respiration rate at presentation to the EMS and if the overdose was attended at the safe injection facility. Low GCS score and respiration rate are part of the classic opioid overdose triad and have been shown to influence the choice of naloxone dose(13, 14). Other patient characteristics, such as sex, have also been found to influence the choice of naloxone dose in one study(14). Age and treatment at the safe injection facility were included as part of an exploratory analysis.

The dependent variable in the second logistic regression model (Model 2) was multiple doses of naloxone (≥ 2 doses). The reference category was a single dose only. In Model 2, the following explanatory variables were included: initial naloxone dose, patient sex, patient age, GCS and respiration rate at first evaluation and if the overdose occurred at the safe injection facility. To ensure that missing data were not deleted listwise in the logistic regression analysis, a category for missing responses for variables with incomplete recordings (no valid reports) was included.

Results

Between 2014 and 2018, 2,215 cases were treated with naloxone by the Oslo City Center EMS (Figure 1). Eight patients declined participation. Twenty-nine patients were excluded because they were administered naloxone by others prior to EMS attendance, and no further naloxone administration was needed.

The mean age of the patients was 38.3 years, and 77.1% were men (Table 1). The median GCS was 4, and the median respiratory rate was 7 breaths/minute. As shown in Table 1, the safe injection facility was the place of attendance in 33.5% of the patients. The remaining cases (not shown) were attended in public places (50.1%), private homes (7.3%), shelters/other facilities for people using drugs (6.5%), and other places such as hotels and public transport (2.6%).

----------Insert figure 1 here----------

----------Insert table 1 here----------

Intramuscular injection was the most common route of naloxone administration (Table 2), as 91.9% (n=2,035) of the 2,215 cases received this as their initial treatment. Only a minority of patients were treated with IV naloxone; 1.9% (n=41) were treated with IV alone, and 3.8% (n=84) were administered IV naloxone after the administration of an IM dose. A minority of patients (2.5%) were administered naloxone by other routes, such as intranasal or subcutaneous routes. The use of IV naloxone as the initial treatment became less frequent during the study period, decreasing from 50 cases in 2014 to only two cases in 2018.

Among those treated with IM naloxone (n=2035), the most common dose was 0.8 mg (56.5%), followed by 0.4 mg (39.9%). Only 3.6% (n=74) received IM naloxone in other doses.

Overall, only 15.0% (n=332) of the 2,215 cases were administered a second or third dose of naloxone. The majority (82.0%) of these 332 cases were treated with only one additional dose. Among those administered multiple doses (≥2), 51.5% received IV and 48.5% received IM naloxone.

Among the 2,215 cases, the total administered naloxone dose was 0.4 mg for 33.0% of the patients, 0.8 mg for 51.2% of the patients and more than 0.8 mg for 12.7% of the patients. 3.1% received other doses less than 0.8 mg.

This included the initial and subsequent doses of titration. Only 1.0% of patients received ≥ 2 mg naloxone in total, and the maximum dose used was 3.0 mg. The mean total dose of naloxone in patients with respiratory arrest or cyanosis was 0.8 mg.

----------Insert table 2 here----------

Naloxone dose and its associations with clinical variables

Of the 2,215 cases, 1,720 cases had a valid national identity number (Table 1). This subgroup comprised 869 individuals; 76.3% were men, and the mean age was 38.6 years. The majority of these individuals (66.0%, n=574) were only attended once. Two attendances were registered in 15.4% of these patients (n=134), while 18.5% of these patients (n=161) were attended three times or more, with a maximum of 27 attendances in the same individual.

The majority (89.0%, n=1,530) of the 1,720 patients with a valid national identity number were treated with either 0.4 mg or 0.8 mg IM naloxone. Among these patients (Model 1, Table 3), unconscious patients with GCS scores of 3 or 4 to 9 were seven- and four-times more likely to be administered 0.8 mg naloxone than those who were awake (GCS 15). Compared to patients with a respiratory rate of ≥9 breaths/minute, those with respiratory arrest or a respiratory rate of 1-8 breaths per minute were three- and two-times as likely to be treated with 0.8 mg naloxone, respectively. Furthermore, men were more than twice as likely as women to be administered a dose of 0.8 mg. Those attended at the safe injection facility were 40% less likely to receive 0.8 mg naloxone than patients treated at other locations.

----------Insert table 3 here----------

Multiple naloxone dosages and their associations with clinical variables

Overall, multiple doses (≥2) of naloxone during one EMS attendance were administered in 14.8% (n=227) of the 1,530 patients with a valid national identity number who received either 0.4 mg or 0.8 mg IM naloxone. Among these cases (Model 2, Table 4), unconscious patients with GCS scores of 3 or 4 to 9 were seventeen- and eight-times more likely to be administered multiple doses than those who were awake. Compared to patients with a respiratory rate of ≥9 breaths/minute, patients with respiratory arrest were twice as likely to be treated with multiple doses. Furthermore, men were almost twice as likely as women to receive multiple dosages. Those attended at the safe injection facility were 80% less likely to be treated with multiple dosages than patients treated at other locations. Finally, those treated with an initial naloxone dose of 0.8 mg were 60% less likely to receive multiple doses than patients treated with an initial dose of 0.4 mg naloxone.

----------Insert table 4 here----------

Transfer rates

The majority (57.1%) of the 2,215 patients were left at the scene (Table 5), 28.1% were taken to the Oslo Accident and Emergency Outpatient Clinic, 12.9% were hospitalized and 1.9% were transferred to other places. One patient was in cardiac arrest and died despite treatment with advanced cardiac life support. This patient was treated with naloxone and was therefore included in the study. In the subsample of patients left on the scene (n=1264), 50.4% were left without medical supervision, while 49.6% were left at the safe injection facility or other health services such as nursing homes. For patients left on the scene, the average time for EMS attendance was 32.7 minutes. Whether the patient was transferred from the scene following treatment was not significantly associated with the initial dose either in the univariate logistic regression analysis (OR 1.1, 95% CI 0.9-1.3), or after adjusting for individual characteristics and vital signs (AOR 1.1, 95% CI 0.9-1.5). However, patients transferred following treatment were 70% more likely to have been treated with multiple doses of naloxone both in unadjusted analysis and after adjusting for individual characteristics and vital signs (AOR 1.7, 95% CI 1.2 – 2.3).

----------Insert table 5 here----------

One-week mortality

Among the 1,720 episodes between June 1st, 2014 and December 31st, 2018 with a valid national identity number, there were 10 deaths within the first week after EMS treatment. The crude one-week mortality rate was 5.8 per 1000 episodes. None of the patients died on day 0. However, three died on day 1, another three died on day 2, and four more deaths occurred during the following five days, between days 3 and 7.

Overall, ten patients died. Seven deaths were drug-related deaths, six of which were classified as unintended poisoning and one as a suicide by way of heroin. Three patients died from natural causes: one 96-year-old nursing home patient, one patient in palliative care, and a 62-year-old complex medical patient in home care. Those who died from overdose or suicide had all been left at the scene. The overall one-week mortality rate for drug-related deaths was 4.1 per 1000 episodes and 5.5 per 1000 episodes for patients left at the scene by the EMS.

Limitations and strengths

Data collection was based on paper records, which limited the number of variables. National identity numbers were not available in 22.3% of patients. Data on clinical evaluations after naloxone administration, such as GCS scores and respiratory rate, were missing in a large proportion of the records, which made it difficult to reliably estimate the efficacy of treatment. Local guidelines recommend naloxone dosages based on the patients' weight which was not recorded in the medical records and could not be included in analyses. There were few recorded overdoses with fentanyl or other strong synthetic opioids in Norway, and the results are therefore not necessarily generalizable to settings where fentanyl is more frequent. Linking of data with other national registers and better data on follow-up would have improved the study.

A strength of the study was the long observation period of five years. Key demographic variables in the study could be compared with previous reports in Oslo and other countries(15). The issues with missing data were handled by including missing data as a variable in the models to avoid observations being deleted listwise. Norway has unique national identity numbers, which made it possible to link the data to the National Cause of Death Registry.

Discussion

The majority of included patients were administered IM naloxone injections of 0.4 or 0.8 mg. Multiple doses (≥ 2) were administered in 15% of cases. Patients who were unconscious or in respiratory arrest were more likely to be treated with 0.8 mg naloxone than 0.4 mg naloxone and more likely to receive multiple doses. Patients who were attended at the safe injection facility were less likely to be treated with 0.8 mg naloxone and less likely to be treated with multiple doses. An initial dose of 0.8 mg naloxone reduced the likelihood of multiple doses by 60%. Patients were left on the scene in more than half of the cases. The one-week mortality rate for drug-related deaths for patients was 4.1 per 1000 episodes. None of the deaths were due to rebound toxicity.

The naloxone dosage observed in the present study was similar to what was found in an Austrian study from 2000(13). We observed a dose reduction in Oslo from 1.2 mg 20 years ago to 0.8 mg today in overdoses with respiratory arrest or cyanosis(16). This reduction is explained by a change in clinical practice, where fewer patients are administered IV naloxone (16). During our 5-year observation period, only 3.8% of the cases received this treatment. We speculate that the reduced use of IV naloxone is related to the time it takes for establishing IV access(17) and its high rate of adverse events(16). Anecdotally, staff report reduced opioid withdrawal and increased cooperation of the patients after reversal with antidote through IM injections alone.

In line with a previous study(14), there was an inverse relationship between level of consciousness or respiratory rate and naloxone dosage. Men were twice as likely to be treated with the higher dose than women. This has also been shown previously(14) and might be related to guidelines emphasizing dosing depending on body size(12). The need for multiple dosages was associated with the same factors as those associated with the initial dose, and an initial dose of 0.8 mg reduced the probability of the administration of multiple dosages by 60%. This indicates that EMS staff use their clinical judgment to titrate naloxone dosing according to clinical presentation and treatment response.

Interestingly, patients at the safe injection facility were often treated with the lower naloxone dosage (0.4 mg) and were less likely to receive a second dose than patients at other locations, despite presenting in deep coma and respiratory arrest. The staff at the facility does not administer naloxone but manages patients with bag-mask ventilation. The lower dose may be a consequence of patients being ventilated while waiting for the EMS and therefore becoming less hypoxic. The facility is also a well-organized work environment and allows the EMS to start lower in their titration of dosages and give this dose time to work. Patients treated in the safe injection facility were also more likely to be left at the premises, probably due to the facility offering post-overdose monitoring and counseling(18).

A large proportion of the patients declined transfer to further care after naloxone treatment by the EMS. This is a recognized challenge world wide(15, 16). Three patients died of overdose on day 1 after they had received EMS naloxone. These patients were alive longer than the expected duration of action of the naloxone and are therefore unlikely to be rebound opioid intoxications. This shows that the naloxone dosing regimens used, combined with an average observation time of 32 minutes, are safe in terms of immediate mortality. These findings are in keeping with previous studies on discharging patients on site after naloxone treatment(15, 19–21).

Opioid overdoses are known risk factors for early death(22). Repeated overdose prevention and addiction treatment should therefore be a priority. In this study, the one-week mortality rate after being left at the scene was 5.5 per 1000 episodes. This is higher than the 0.8 per 1000 episodes previously reported in a review of the risk for rebound opioid toxicity after naloxone treatment for patients left on the scene(15). On the other hand, in a study of 2241 patients discharged after naloxone treatment, the 48-hour mortality was reported to be 5.8 per 1000 episodes when counting all overdose-related deaths, not just those attributed to rebound opioid toxicity(20). This might indicate a need to widen the perspective beyond solely focusing on rebound toxicity but also on the risk of death by repeated overdoses for patients being left on the scene after treatment with naloxone. Deaths from new overdoses must be considered preventable events, and efforts must be made to provide appropriate interventions. An Australian study of 3921 overdoses reported 11 deaths from new overdoses within one week after EMS treatment. Nine had been brought to the hospital, of which three self-discharged and died within 24 hours of EMS attendance(21). Being brought to a hospital or a healthcare facility is therefore not necessarily protective, but probably depends on what further treatments are offered during hospitalization.

A dose of naloxone of up to 0.8 mg has been found to be sufficient in the community setting where illicitly manufactured fentanyl circulates and for use by EMS when treating fentanyl overdoses(23, 24). For patients with a higher level of consciousness and higher respiratory frequency, a dose of 0.4 mg could be a safe alternative. These findings are relevant in the discussions around dosages administered through take-home naloxone regimens and for new naloxone formulations.

Conclusion

Initial doses of 0.4 to 0.8 mg of IM naloxone appear effective and safe for the treatment of prehospital opioid overdoses. The data support that the emergency medical staff titrates naloxone based on clinical presentation and effect. GCS and respiratory rate stand out as strong predictors for dosing choices by the EMS in Oslo. Even though the risk of rebound opioid toxicity was low, the population in this study had an alarmingly high one-week mortality rate, much higher than previously reported.

Declarations

Ethics approval and consent to participate

The study was approved by The Regional Committee for Medical and Health Research Ethics (REK 2014/140) and was conducted in accordance with the Declaration of Helsinki. Patients included after June 1st, 2014, were given oral and written information about the study and were given the opportunity to withdraw at any time.

Consent for publication

Not applicable

Availability of data and materials

Due to the nature of this research on a vulnerable patient group participants of this study did not agree for their data to be shared publicly, so supporting data is not available.

Competing interests

NTNU and its subsidiary Technical Transfer Office have signed cooperation and licensing contracts with dne pharma as/farmaholding to seek commercialization of a nasal naloxone formulation. This regulates potential royalties for Ola Dale through NTNU. Dne pharma as has compensated OD for business travel from Trondheim to Oslo and to Lisbon. Arne Kristian Skulberg spoke at a seminar arranged by dne pharma as in Lisbon October 2019 without honorarium or other compensation. The other authors declare no conflicts of interest. dne pharma as/farmaholding have no role in the study design, data collection, data analysis, data interpretation or writing of this article. Oslo University Hospital has full ownership of the data and results generated in this study.

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Authors' contributions

AKS, ACB, FH and OD conceived the study, designed the trial, and obtained funding. AKS and ACB supervised the conduct of the trial and data collection. IT and AKS managed the data, including quality control. IT, LG and LPB provided statistical advice on study design and analyzed the data. IT drafted the manuscript, and all authors contributed substantially to its revision. All authors have seen and approved the final draft for submission.

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Tables

Table 1. Cases in which naloxone was administered by Oslo City Center emergency medical services between 1 January 2014 and 31 December 2018

	Total 100% (n=2215)	No valid report % (n)
Known national identity number (% (n))	77.6 (1720)	22.3 (495)
Men (% (n))	77.1 (1707)	0.7 (15)
Age (mean (SD))	38.3 (11.2)	13.5 (298)
Glasgow Coma Scale (median (min-max))	4/15 (3-15)	8.5 (188)
Respiration rate/minute (median (min-max))	7 (0-40)	12.5 (276)
Attended in safe injection facility (% (n))	33.5 (743)	0 (0)

Table 2. Routes of administration and dose of naloxone in 2215 suspected cases of opioid overdose and subsequent administration of naloxone after the initial dose

Initial naloxone treatment	% (n)		Subsequent naloxone administration, % (n)	
Total	100 (2215)		15.0 (332)	
IM only	91.9 (2035)		15.6 (318)	
0.4 mg		39.9 (811)		16.5 (134)
0.8 mg		56.5 (1150)		15.0 (172)
Other doses <0.8 mg		3.5 (72)		
Other doses >0.8 mg		0.1 (2)		
IV only	1.9 (41)		9.8 (4)	
0.4 mg		75.6 (31)		
0.8 mg		17.1 (7)		
Other doses <0.8 mg		7.3 (3)		
IM and IV	3.8 (84)		2.4 (2)	
0.4 IM + 0.4 IV		17.9 (15)		
0.8 IM + 0.4 IV		65.5 (55)		
0.8 IM + 0.8 IV		10.7 (9)		
Other doses >0.8 mg		6.0 (5)		
Other	2.5 (55)		14.6 (8)	

IM=intramuscular, IV=intravenous

Table 3. The putative associations between intramuscular naloxone dose (0.4 mg vs. 0.8 mg) and sex, age, vital signs and place of attendance (n=1530)

	0.4 mg 100% (n=657)	0.8 mg 100% (n=873)	Unadjusted OR (95% CI)	Adjusted OR (95% CI)
Sex				
Women	30.8 (202)	18.6 (162)	ref	ref
Men	69.3 (455)	81.4 (711)	2.0*** [1.5, 2.5]	2.2*** [1.7, 2.9]
Age (years)				
< 30	24.2 (159)	23.1 (202)	ref	ref
30-49	58.5 (384)	59.7 (521)	1.1 [0.8, 1.4]	1.2 [0.9, 1.5]
≥50	17.4 (114)	17.2 (150)	1.0 [0.7, 1.5]	1.3 [0.9, 1.8]
Glasgow Coma Scale				
3/15	27.7 (182)	56.5 (493)	9.1*** [5.2, 16.2]	7.1*** [3.8, 13.1]
4-9/15	21.6 (142)	19.8 (173)	4.1*** [2.2, 7.5]	4.0*** [2.1, 7.5]
10-14/15	33.8 (222)	13.8 (120)	1.8* [1.0, 3.2]	1.8 [1.0, 3.2]
15/15	8.2 (54)	1.8 (16)	ref	ref
No valid report	8.7 (57)	8.1 (71)	4.2*** [2.2, 8.0]	3.8*** [2.0, 7.4]
Respiration rate				
0/minute	7.2 (47)	20.6 (180)	5.1*** [3.5, 7.6]	3.4*** [2.2, 5.3]
1-8/minute	35.5 (233)	43.0 (375)	2.2*** [1.7, 2.7]	1.7*** [1.3, 2.2]
≥9/minute	44.9 (295)	25.2 (220)	ref	ref
No valid report	12.5 (82)	11.2 (98)	1.6** [1.1, 2.3]	1.6* [1.1, 2.3]
Place of attendance				
Safe injection facility	41.1 (270)	37.5 (327)	0.9 [0.7, 1.1]	0.6*** [0.5, 0.8]
All other locations	58.9 (387)	62.5 (546)	ref	ref

Logistic regression analysis was used, and identity was included as a cluster variable to account for the possibility that an individual had repeated events. OR= odds ratio, 95 CI = 95 confidence interval. * p < 0.05, ** p < 0.01, *** p < 0.001

Table 4. The likelihood of multiple-dose administration of naloxone during a single EMS attendance as a function of sex, age, vital signs, place of attendance and dose (n=1530)

	Single dose 100% (n=1303)	Multiple doses 100% (n=227)	Unadjusted OR (95% CI)	Adjusted OR (95% CI)
Sex				
Women	24.7 (322)	18.5 (42)	ref	ref
Men	75.3 (981)	81.5 (185)	1.5 [1.0, 2.2]	1.8** [1.2, 2.6]
Age (years)				
<30	23.0 (300)	26.9 (61)	ref	ref
30-49	59.5 (775)	57.3 (130)	0.8 [0.6, 1.2]	1.0 [0.7, 1.5]
≥50	17.5 (228)	15.9 (36)	0.8 [0.5, 1.3]	1.1 [0.6, 1.9]
Glasgow Coma Scale				
3/15	40.5 (528)	64.8 (147)	9.5*** [2.3, 39.2]	17.1*** [3.9, 75.0]
4-9/15	21.2 (276)	17.2 (39)	4.8* [1.1, 20.5]	7.8** [1.8, 34.4]
10-14/15	24.6 (321)	9.3 (21)	2.2 [0.5, 9.7]	2.7 [0.6, 11.9]
15/15	5.2 (68)	0.9 (2)	ref	ref
No valid report	8.4 (110)	7.9 (18)	5.6* [1.2, 24.9]	7.9** [1.7, 36.9]
Respiration rate				
0/minute	13.8 (180)	20.7 (47)	1.6* [1.1, 2.5]	1.9* [1.2, 3.2]
1-8/minute	39.6 (516)	40.5 (92)	1.1 [0.8, 1.6]	1.0 [0.7, 1.5]
≥9/minute	34.1 (444)	31.3 (71)	ref	ref
No valid report	12.5 (163)	7.5 (17)	0.7 [0.4, 1.1]	0.8 [0.4, 1.4]
Place of attendance				
Safe injection facility	43.1 (562)	15.4 (35)	0.2*** [0.2, 0.4]	0.2*** [0.1, 0.3]
All other locations	56.9 (741)	84.6 (192)	ref	ref
Initial naloxone dose				
0.4 mg IM	42.1 (549)	47.6 (108)	ref	ref
0.8 mg IM	57.9 (754)	52.4 (119)	0.8 [0.6, 1.1]	0.4*** [0.3, 0.5]

Logistic regression analysis was used, and identity was included as a cluster variable to account for the possibility that an individual had repeated events. IM = intramuscular naloxone. EMS= emergency medical service, OR= odds ratio, 95 CI =95 confidence interval. * p < 0.05, ** p < 0.01, *** p < 0.001

Table 5. Transfer rates after naloxone treatment

Information on transfer	100% (n=2215)	
Left at the scene	57.1 (1264)	
Safe injection facility or health service		49.6 (627)
Public place, homes, shelters and other places		50.4 (637)
Accident and Emergency Outpatient Clinic	28.1 (623)	
Hospitalized	12.9 (286)	
Transported home, to addiction treatment facilities or other places	1.9 (41)	
Died	0.05 (1)	

Figures

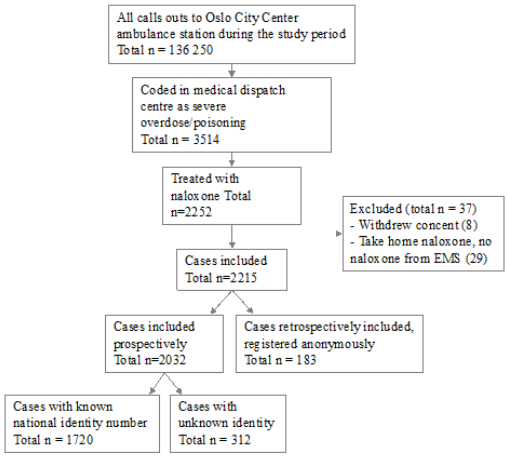

Figure 1

Flowchart of inclusion-exclusion criteria in the study

Annex I

Supplementary file 1

Pharmacokinetics of a new, nasal formulation of naloxone

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Naloxone Analysis

Naloxone was analyzed by a validated high performance liquid chromatography tandem mass spectrometry method at the Proteomics and Metabolomics Core Facility (PROMEC), NTNU, Norway. The analytical method was validated according to Dadgar et al [1] and Shah et al [2].

Naloxone hydrochloride dihydrate ($C_{19}H_{21}NO_4$ HCl $2H_2O$, CAS number: 51481-60-8) and deuterated naloxone-d5 solution ($C_{19}H_{16}NO_4D_5$, CAS number: 1261079-38-2) were used as reference material (Sigma-Aldrich, St. Louis, MO, USA), and acetonitrile (HPLC-grade) was from Lab-Scan Analytical Sciences (Gliwice, Poland). The calibration standards and quality controls were prepared with plasma from blood donors (St Olav's University Hospital, Trondheim, Norway).

The analytical preparation procedure was essentially as for the method described by Edwards et al [3]. Standards, quality controls and samples (200 µl) were spiked with the internal standard deuterated naloxone-d5 (20 µl, 50 ng/ml). Plasma proteins were precipitated with acetonitrile (0.9 ml), vortexed, and after 30 minutes (4ºC) centrifuged for 10 minutes at 12000 x g (10ºC). Supernatants were evaporated to dryness in a MiVac concentrator and reconstituted in 50 µl mobile phase (mobile phase = 20% acetonitrile in 0.1% formic acid). The reconstituted samples were injected (3 µl) in the mobile phase (flow = 300 µl/min) by a Shimadzu auto injector (20AC) to a Zorbax SB-C18 column (5 µm, 2.1 x 150 mm) and further introduced to the Applied Biosystems API 5500 triple quadrupole by an Turbo V^{TM} Ion Source operating in positive ion mode. Ion pairs were 328.2/268.2 and 333.2/273.2 for naloxone and the internal standard, respectively. Sample analysis were performed by multiple reaction mode. The turbo ion-spray probe temperature was set to 625°C, nebulizer and curtain gas flow rates of 70 psi and 30 psi. The ion-spray voltage was 5500 V, while the declustering and entrance potentials were set to 126 V and 10 V. The collision cell energy was 37 V using a collision activated dissociation (CAD) set at 9, the collision cell exit potential was 22 V.

Calibration range was 0.02 – 45 ng/ml (9 calibration standards). The correlation coefficient (r^2) was > 0.9986 for all the calibration curves. The limit of quantitation (LOQ) was 0.02 ng/ml, with the coefficient of variation (CV) < 15.9 % and inaccuracy < 1.1 % (n = 16). The quality controls (QC 1, 2, 3) were in the lower (0.05 ng/ml), middle (15 ng/ml) and upper (30 ng/ml) calibration range. In the pre-run validation (n = 18) CV and inaccuracy were found to be < 10.7 %, 4.2 % (QC 1), < 3.9 %, 5.9 % (QC 2) and < 4.2 %, 2.8 % (QC 3) respectively. During in-run validation CV and inaccuracy for the quality controls (n = 35) were < 6.6 %, 1.1 % (QC 1), < 4.4 %, 8.3 % (QC 2) and < 2.5 %, 4.6 % (QC 3).

Stability tests were performed prior to analyses: Auto sampler stability (24 hours), freeze/thaw stability (three times), long terms stability (12 months). Stability data was within limits given [1,2,4] and all samples were analysed within 2 months.

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Annex II

Supplementary file 2 –

Pharmacodynamics and arteriovenous difference of intravenous naloxone in healthy volunteers exposed to remifentanil

Authors

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Quantitation of remifentanil in human blood by liquid chromatography ion-spray tandem mass-spectrometry (LC-MS/MS)

Remifentanil (R) was analysed by a high performance liquid chromatography tandem mass spectrometry method and fully validated according to Shah et al. [1] and Dadgar et al. [2].

The reference material, remifentanil hydrochloride $C_{20}H_{28}N_2O_5$ HCl (mw 412.9) for injection (5mg ampoule) lot no: J518, was from Ultiva® Glaxo-Smith-Kline Inc, Research Triangle Park, NC Lots 6Z PO653 and fentanyl $C_{22}H_{28}N_2O$ (Art.no Fen-622-FB) was from Lipomed, Arlesheim Switzerland.

The LC system was from Shimadzu Kyoto, Kyoto Prefecture, Japan. The API 5500 Triple Quad MS/MS and the Quantitation program Analyst version 1.5.1 was from Applied Biosystems SCIEX Instruments, (Foster City, CA. USA).

Samples for remifentanil quantification were collected in VACUETTE® NH Sodium Heparin Blood Collection Tubes (Greiner Bio One GmbH, Austria). The tubes were prefilled with 50 % citric acid (weight/volume) solution to prevent hydrolysis of remifentanil through pH-control [3]. After strictly mixing, the blood samples were immediately put on ice and frozen at -20 °C within 10 minutes. Blood from healthy volunteers was handled similarly and then spiked with remifentanil for quality controls and calibration standards. Samples, quality controls and calibrators were stored in a -80 °C freezer until analysis. Remifentanil stock solutions were prepared in 1.0 mM HCl.

The extraction procedure was essentially according to Bender et al. [3]. After thawing in a refrigerator overnight, 0.5 ml blood (samples, quality controls and calibration standards) was transferred to glass tubes and mixed with 50 µl fentanyl (100 ng/ml H_2O) as internal standard (IS). To enhance extraction efficiency pH was readjusted by adding 0.5 ml 0.1 M phosphate buffer pH 7.4. Extraction was conducted by adding 2.0 ml of dichloromethane (DCM) and vigorously vortex mixed for about 10 seconds until a homogeneous sample was obtained.

Then rotation (Rotamix) for 10 minutes followed by centrifugation at 3000 g (4 °C) in further 10 minutes. The lower DCM layer was transferred to conical glass tubes, and evaporated to dryness at 40 °C under a stream of nitrogen (N_2).

Since fentanyl was used as the internal standard (IS), chromatographic separation was performed according to Bjelland et al. [4], using an Eclipse XDB-C8 (4.6 x 150 mm, 5 µm) column with an Eclipse XDB-C8 (4.6 x 12.5 mm, 5 µm) pre-column (Agilent Technologies) and a gradient elution. The samples were reconstituted by adding 50 µl mobile phase (mobile phase = 65 % MeOH with 0.1 % formic acid), vortex mixed, transferred to vials and injected (3 µl) to the LC-MS/MS with a flow rate of 0.7 ml/min.

The samples were introduced to the triple quadrupole by a Turbo V™ Ion Source operating in positive ion mode. Ion pairs were 377.2/228.0 for remifentanil and 337.2/146.0 for fentanyl (IS). Sample analysis was performed by multiple reaction mode (MRM). The turbo-ion spray probe temperature was set to 625 °C, nebulizer and curtain gas flow rates to 70 psi and 30 psi. The ion spray voltage was 5500 V, while the declustering and entrance potentials were set to 60 V and 10 V. The collision cell energy was 30 V (R) and 40 V (IS), using a collision activated dissociation (CAD) set at 9. The collision cell exit potential (CXP) was 18 V.

Calibration range was 0.01 – 5.0 ng/ml (8 calibration standards). The limit of quantitation (LOQ) was 0.01 ng/ml, with the coefficient of variation (CV) < 2.5 % and inaccuracy < 1.8 % (n = 18). The quality controls (QC1, 2, 3) were in the lower (0.03 ng/ml), middle (1.75 ng/ml) and upper (3.75 ng/ml) calibration range. In the pre-run validation (n = 18) CV and inaccuracy were found to be < 3.4 %, 5.7 % (QC1), < 3.0 %, 0.6 % (QC2) and < 3.5 %, 3.8 % (QC3) respectively. During in-run validation CV and inaccuracy for the quality controls were < 6.6 %, 1.1 % (QC1, n = 23), < 4.4 %, 8.3 % (QC2, n = 24) and < 2.5 %, 4.6 % (QC3, n = 24).

Stability tests were performed prior to analyses essentially according to the references [1, 2, 5]. Auto sampler stability (24 hours), freeze/thaw stability (three times), on bench stability (5 and 15 hours).

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