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# TOXICOLOGICAL STUDIES OF OPIATE-RELATED DEATH

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Cover: Advertisement of the opium-based Perry Davis' Vegetable Pain-Killer, 1840.  
"No family should be without it"

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# ABSTRACT

This thesis comprises studies on opiate overdose death, and focuses on evaluation of toxicological characteristics including the possible influence of abstinence and polydrug use for the fatal outcome.

In order to identify and classify drug abusers, a screening method for several drugs of abuse was developed and applied on randomly selected autopsy cases. Out of 75 cases, 26 positive results were found in 16 subjects. In two cases, drug abuse was not previously known by relatives or the police. The method developed includes the detection of the most common illicit drug groups in Sweden, and proved feasible for application in the medicolegal routine casework to confirm or exclude previous drug abuse in deceased subjects. In addition, the method may be applied on hair samples collected from living subjects for the same purpose.

Although the designation heroin overdose gives the impression that these subjects have taken a high dose, or a dose of high purity, blood morphine concentrations vary widely and many victims actually present with fairly low blood morphine levels. To investigate the importance of abstinence in unexpected death at low opiate concentrations, all screening results were confirmed with segmental hair analysis, allowing for a detailed temporal mapping of the previous drug use. Hair samples were collected from 60 deceased addicts of whom 28 were classified as heroin overdose deaths. Segmental hair analysis revealed that 18 of these subjects had recently discontinued opioid use, suggesting a reduced tolerance to opioids. However, their blood morphine levels were similar to those found in the 10 subjects with a continuous opioid use. Further, hair and blood analysis disclosed an extensive use of additional drugs that directly or indirectly may influence the opioid system and contribute to the overdose.

In a larger sample of deceased drug addicts, the possible importance of abstinence and polydrug use in opioid overdose deaths was explored. In 160 of 210 deceased suspected drug addicts, drug abuse was confirmed by hair screening. Of these, 91 were opiate overdose deaths. Hair and blood analysis revealed extensive polydrug use, which was more pronounced among opiate overdose victims than in drug addicts dying of other causes. Blood analysis showed that pure heroin intoxication was very rare; about 60% of the overdose cases had three or more drugs in blood at the time for death. Segmental hair analysis revealed that more than 80% of the opiate overdose cases had not been exposed to opiates during the most recent weeks before their demise. In most cases, the hair opiate concentrations displayed a gradual decrease before the last dose. The toxicological results also showed that opiate overdose death was more likely to occur if opiates were combined with benzodiazepines, but less likely when opiates were combined with stimulants such as amphetamines.

An experimental model was developed to address the issue of tolerance and abstinence in opiate overdose death. Rats given a high dose of morphine or heroin showed a reduced mortality if pre-treated for two weeks with morphine. This protection was reduced if the pre-treatment was followed by one week of opiate abstinence. Delayed deaths, similar to those observed in a proportion of human heroin overdose victims, were also seen in the rats. Toxicological results showed that delayed deaths were not due to accumulation of morphine or morphine glucuronides in either blood or other tissues examined.

In conclusion, these studies provide support for the notion that polydrug use and recent opiate abstinence are important risk factors for opiate overdose death.

## LIST OF PUBLICATIONS

- I. Kronstrand R, Nyström I, Strandberg J, Druid H.  
Screening for drugs of abuse in hair with ion spray LC-MS-MS.  
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- III. Strandberg JJ, Kugelberg FC, Alkass K, Gustavsson A, Zahlén K, Spigset O, Druid H.  
Toxicological analysis in rats subjected to heroin and morphine overdose.  
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- IV. Strandberg JJ, Alkass K, Nyström I, Kugelberg FC, Kronstrand R, Druid H.  
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## LIST OF ABBREVIATIONS

5-HT	5-hydroxytryptamine (serotonin)
6-AM	6-acetylmorphine
7-Flu	7-amino-flunitrazepam
ANOVA	analysis of variance
C-cases	control cases
DRG	dorsal respiratory group
ESI	electrospray ionization
EU	european union
GABA	gamma-aminobutyric acid
GC	gas chromatography
GDP	guanosine diphosphate
GPCR	G protein-coupled receptor
GRK	G protein-coupled receptor kinase
GTP	guanosine triphosphate
GTP $\gamma$ S	guanosine gamma thio-phosphate
i.p.	intraperitoneal
i.v.	intravenous
ICD-10	international classification of diseases 10
ICR	incorporation rate
LC	liquid chromatography
M3G	morphine-3-glucuronide
M6G	morphine-6-glucuronide
M-cases	miscellaneous cases
MDMA	methylenedioxymethamphetamine
MOR	mu-opioid receptor / $\mu$ -opioid-receptor
MS	mass spectrometry
NaCl	sodium chloride (saline)
NMDA	N-methyl-D-aspartic acid
O-cases	opiate overdose cases
S1	segment 1 (most recent hair segment)
VRG	ventral respiratory group
WHO	world health organization



# 1 INTRODUCTION

## 1.1 GENERAL BACKGROUND

Illicit drug use and addiction is an increasingly large problem worldwide. Except for the addicts themselves, others may suffer from consequences like increased violence, sickness and death. Heroin is one of the most dangerous illicit drugs and the drug with highest mortality rate [1].

Approximately 16 million of the world's population between 16 and 64 years old are addicted to opiates, of which 11 million are heroin abusers [2]. Despite extensive efforts to reduce the number of opiate-related deaths, the death toll has continued to rise around the world. In the European Union, opiate abuse account for about 3% of all deaths of people younger than 40 years old. In Sweden, the total number of heavy drug abusers will most likely soon exceed 30 000 and the number of individuals dependent on heroin is estimated at 10 000 [3].

Stemming from the Greek words "*toxicon*" and "*logos*", toxicology today refers to the study of adverse effects of a substance or substances, i.e. poisoning. Paracelsus, considered the father of modern toxicology in the 16<sup>th</sup> century, said "*Alle Dinge sind Gift und nichts ist ohne Gift; allein die Dosis macht, dass ein Ding kein Gift ist.*" In short, "The dose makes the poison" [4]. The truth of this statement for every single substance is debatable, although even apparently innocent chemical compounds such as water and oxygen may be toxic when the exposure is extreme. Furthermore, it is not surprising to most citizens that high doses of many of the drugs used today for medical treatment can be hazardous. The influence of dosage is particularly important in those drugs that cause serious side effects. Opiates, such as morphine, administered in therapeutic doses may efficiently provide pain relief, whereas higher doses can cause respiratory depression and death [5]. Since opioid drugs are currently indispensable in the treatment of severe pain, measures to control dosage and to reduce the risk for respiratory depression are crucial. Regarding the illicit use of opioid drugs, several additional factors related to the drug abuser's situation and condition must be considered in order to better understand the mechanisms behind death resulting from opiate overdose. This thesis deals with toxicological studies on drug abuse death and evaluates the importance of factors that may be involved in such deaths. Throughout this book, the term toxicology refers to "forensic toxicology", i.e. chemical analyses that are designed to aid in medicolegal investigations of death e.g. by drug use.

## 1.2 OPIOIDS

### 1.2.1 Opioid use and abuse

Opium, derived from the opium poppy, *Papaver somniferum*, named and classified by Linnaeus in 1753, has been used as an intoxicant by Sumerians in Mesopotamia since 3400 BC. Opium was probably first used in religious rituals as well as a euphoric

substance before it became a remedy. The Sumerian name for the opium poppy was "*hul gil*" translated as the plant of joy, indicating its primary use. Some hundred years later, documents from the 16<sup>th</sup> – 17<sup>th</sup> century reveal that countries like Turkey, Germany, England, and of course China, all encountered addiction and tolerance problems. One of the most popular medicaments was Thomas Sydenham's Laudanum, a tincture containing opium, sherry wine and herbs. In 1806, morphine, named after the god of dreams, Morpheus, was detected and isolated by Friedrich Sertürner. Merck & Company of Darmstadt (Germany) began commercially manufacturing of morphine in 1827. The research on opium alkaloids continued in an effort to discover the ultimate remedy. The first synthesis of heroin (diacetylmorphine) was accomplished in England by C.R. Wright in 1847, although subsequent experiments of its potency were inconclusive. It was not until the end of 1890s when commercial manufacturing of heroin commenced. At that time Heinrich Dreser, working for the German company Bayer, discovered that diluting morphine with acetyls produced a drug without the side effects of morphine. As a result, Bayer starts the production of diacetylmorphine and coined the name 'heroin' from the Greek word and demigod "*Heros*". [6-8]

After the development of the "god of dreams" in 1806, the production of morphine increased rapidly. Medicaments such as Laudanum entered ordinary households as treatment of the "travails" and "boredom" of Victorian women. Opioid-containing medicines were considered miraculous and the cure of all kinds of diseases. Tinctures were prescribed by most doctors and physicians in treatment of colic, diarrhea, dysmenorrheal and teething in young children [5]. As stated by an advertisement for the opium based Ayer's cherry pectoral: "Cures Colds, Coughs and all Diseases of the Throat and Lungs".

The use of opioids as painkillers in medical treatment increased until the mid 20<sup>th</sup> century. However, due to the increased number of addicts, opioid prescription was strictly regulated by 1940 and inappropriate prescription from physicians was punishable with a suspension of medical license [5]. With the subsequent decrease in opioid prescription due to the regulations and the lack of alternative medicines, pain symptoms became exceedingly under-treated. During the late 20<sup>th</sup> century, opioid therapy regained its unrivaled status in pain relief. Today, the main (legal) use of opioids is to treat severe pain, cough, and to a lesser extent, gastrointestinal disorders. Certain opiates and opioids are also used in substitution therapy.

### **1.2.2 The opioid system**

There are several different types of opioids including endogenous, alkaloids, semi-synthetic and synthetic. Endorphin, dynorphin and enkephalin are the three main types of endogenous opioids. Alkaloids are substances that can be derived directly from the opium poppy e.g. morphine and codeine. Diacetylmorphine (heroin), an ester of morphine, is a semi-synthetic opioid whereas fentanyl and methadone are synthetic. The word "opiate" refers to drugs that are derived from the naturally occurring opium alkaloids or semi-synthetic opioids. Hence, heroin and morphine are true opiates, whereas all other related compounds are designated opioids. In this book, the term 'opiate' is often used to include both opioids and true opiates for the sake of simplicity.

An important breakthrough in opioid research was accomplished in 1973, when the opioid receptors -  $\mu$ ,  $\delta$  and  $\kappa$  - were discovered [9-11]. To date, the  $\mu$ -opioid receptor (MOR) has gained the most attention, as most opioid drugs target this receptor [12].

Morphine, heroin,  $\beta$ -endorphin and enkephalin all have the greatest affinity of for MOR.  $\kappa$ -receptors mainly attract dynorphins whereas  $\delta$ -receptors primarily bind endogenous enkephalins [13].

The opioid receptors are located in the cell membrane and are coupled to heterotrimeric guanine nucleotide (G) proteins of the Gi/Go family [14]. G-protein-coupled receptors (GPCR) have three subunits, alfa, beta and gamma ( $\alpha$ ,  $\beta$  and  $\gamma$ ) which undergo conformational change upon opioid ligand binding. GDP bound to the  $\alpha$ -subunit is exchanged for GTP, which in turn causes dissociation of the G- $\alpha$  subunit. The G- $\beta\gamma$  subunit then couples to the G-protein-coupled receptor kinase (GRK) that then activate several downstream signaling cascades. The effector mechanisms include activation of potassium channels, inhibition of calcium channels and inhibition of adenylyl cyclase [15]. Since the discovery of opioid receptors in the 1970s, almost every opioid ligand has been shown to inhibit cell firing [16]. This inhibition of firing is an important mechanism in opioid induced respiratory depression. MOR, located on respiratory neurons in medulla oblongata can thus suppress neuronal activation and hence slow respiration until it stops completely, resulting in overdose-induced death.

### 1.2.3 Tolerance and dependence

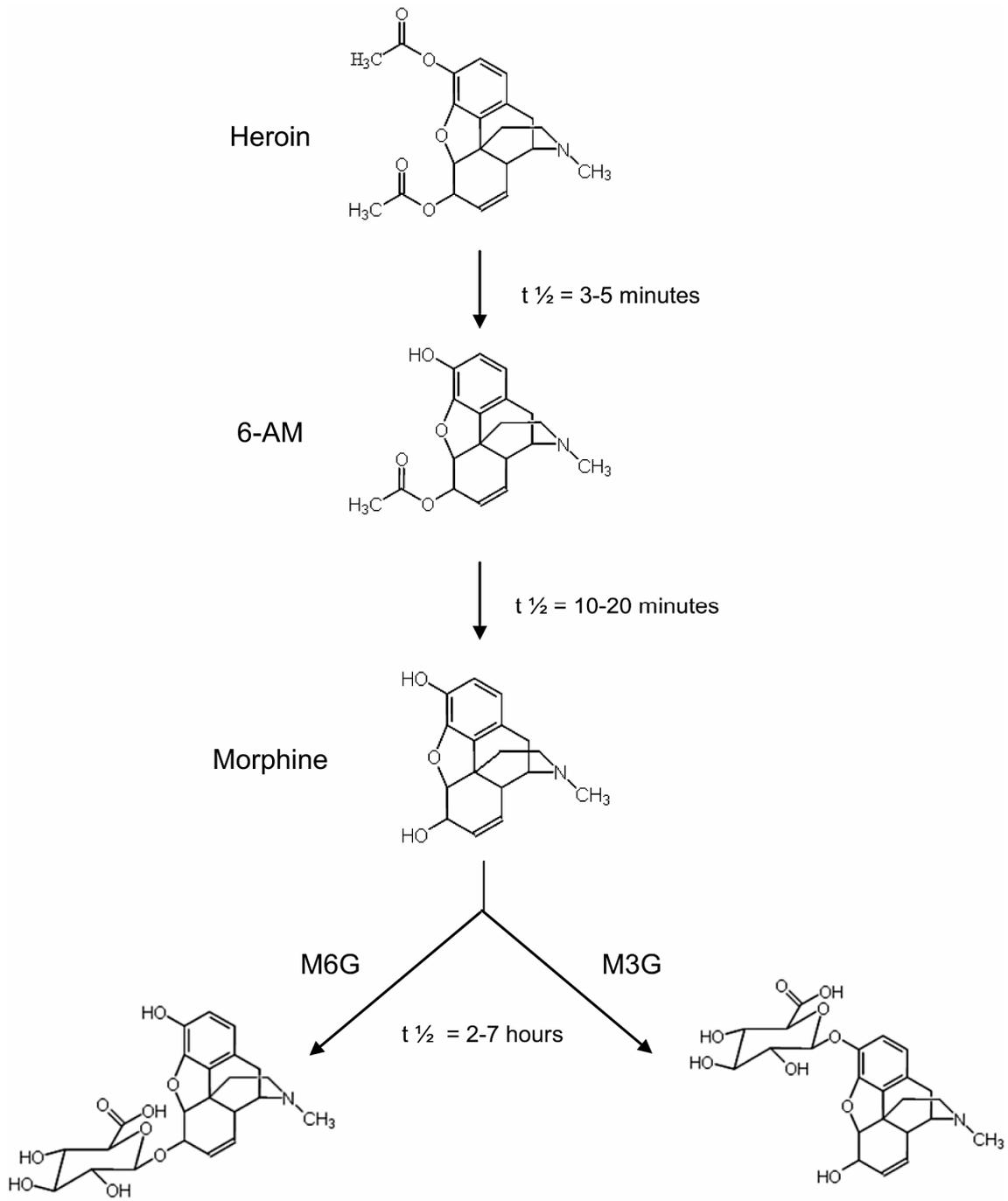
Although opioids are discussed and used in medical treatment, opioids are also extensively mentioned in the context of abuse. The key factors involved in opiate addiction are the development of tolerance and dependence. Tolerance is defined as needing increasingly higher doses of a substance to achieve the original and desired effect. Dependence, as defined by the WHO in the International Classification of Diseases (ICD-10) includes taking a substance repeatedly and having trouble controlling its use. There is also a desire to take the drug despite its harmful consequences, and the craving for the drug decrease efforts to maintain other activities and obligations. The dependence syndrome includes increased tolerance and withdrawal symptoms when the use of the drug has been terminated [17].

Tolerance is not, however, a single distinct entity. The types of tolerance most often related to in addictive behavior are pharmacodynamic and pharmacokinetic tolerance [18]. Pharmacodynamic tolerance is due to adaptive changes within affected cell systems such as down-regulation of receptors and intracellular signaling systems. Pharmacokinetic tolerance is mainly due to an increased metabolism of a drug resulting in lower concentrations in blood and consequently also in target tissues, e.g. by up-regulation of p450 enzyme system in the liver. Other important types of tolerance involved in dependence are innate and learned tolerance. Innate tolerance depends on genetic background (e.g. alcohol sensitivity in Asians) whereas learned tolerance involves environmental cues that are situation-specific i.e. locations usually used when taking a drug [18].

The main dependence-inducing trait of heroin is its profound ability to generate a euphoric effect that surpasses all natural rewards. Other short-term effects include analgesia, sedation, reduced anxiety and respiratory depression. Opiate-related deaths are commonly attributed to the respiratory depressant effects of opioids. It has been suggested that this effect is mediated through MOR within the respiratory centers in the brain stem. However, since overdose strikes unexpectedly even among experienced users, it is likely that moderating mechanisms of this pathway, yet to be characterized, are important.

#### 1.2.4 Heroin pharmacokinetics

In the body, heroin is rapidly degraded to 6-acetylmorphine (6-AM), which in turn is further de-acetylated to morphine (see Figure 1). This process takes only about 15 minutes, whereas the metabolism of morphine to morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G) may take several hours [19]. Even though heroin enters the brain very rapidly, the main pharmacological effects are considered to be conferred mainly by the metabolites morphine and M6G [20]. Approximately 10% of the morphine is converted to the active metabolite M6G and about 50% to M3G, which is considered to lack agonistic properties at opioid receptors either *in vivo* or at the cell level [21, 22]. However, a few studies have shown that M3G can block the binding of other opioid metabolites to the opioid receptors [23]. In a recent study, Handal and colleagues [24] reported that M3G decreased the locomotor effects in mice administered morphine but enhanced the locomotor effects in mice given M6G. Whereas the effects of M3G in humans remain to be further characterized, M6G possesses agonistic properties similar to morphine. However, since substantial time is needed for M6G to be formed in functional amounts after morphine or heroin exposure, M6G is not likely to be involved in rapid opiate overdose deaths.



**Figure 1.** Heroin metabolism.

### 1.2.5 Toxicology

In general, a high dose of virtually any substance may cause side effects. Such side effects are typically dose-dependent and related to the pharmacological effects of the particular substance. For most pharmaceutical drugs, a relationship can be established between the dose and the serum, plasma, or whole blood concentration [25]. For postmortem material, reference data exist regarding concentrations of various drugs in fatal poisonings, and to some extent for postmortem controls [26-28]. Fatal poisonings of pharmaceutical drugs usually show high blood drug concentrations and these toxicological results often prove very valuable for the diagnosis. However, there are some pitfalls that should be considered.

After death, cells disintegrate and fluid is exchanged between different compartments, i.e. a post-mortem redistribution occurs. The impact of this phenomenon on blood drug levels may be substantial regarding certain drugs, particularly those which show a very uneven antemortem tissue distribution. For example, tricyclic antidepressant drugs have a high affinity to lung tissue, and after death, the diffusion processes may cause a significant rise in the concentration in heart blood [29-31]. Drugs, including alcohol may also be present in the ventricle and/or intestines at the time of death, and may contribute to increased central blood levels due to direct diffusion through the disintegrated membranes and vessel walls [32-34].

Additional interpretational problems include blood loss, which could cause changes in the blood levels of drugs due to physiological compensatory mechanisms [35-37] and poor metabolic capacity [38, 39]. Neither of these factors are likely to cause serious interpretation problems regarding the toxicity and cause of death, but may be important for the elucidation of the circumstances surrounding death, and hence, for the determination of the manner of death.

Although post-mortem influences on drug concentrations generally should be considered, morphine has been reported to be less subjected to such post-mortem redistribution [40, 41]. In order to reduce the influence of post-mortem redistribution effects, analysis of peripheral blood is recommended.

Regarding opioids, a greater problem is that the concentrations vary widely among certified or suspected opiate overdose death. Due to the large variation in blood morphine concentration between subjects it is not possible to use presence of morphine alone to diagnose opiate overdose death [42-44]. In addition, the most common street heroin in Sweden, brown heroin, often contains acetylcodeine. Since this substance is metabolized to morphine and can be used (abused) separately, it is somewhat risky to use positive co-detections of codeine and morphine to verify heroin intake. However, if morphine to codeine concentration ratio exceeds 1, it is likely that the results are due to heroin intake [45]. Therefore, a careful medicolegal examination including assessment of information from the police and relatives as well as a comprehensive toxicological analysis must be performed to ascertain opiate overdose death.

In a recent study, femoral blood morphine concentration in 2048 opioid intoxications ranged from 0.005 to 14.7  $\mu\text{g/g}$ , with a median of 0.155  $\mu\text{g/g}$  (Kugelberg & Druid, TIAFT meeting, Ljubljana 2006, Table 1). A large overlap was seen with morphine concentration in hangings (0.002-0.98  $\mu\text{g/g}$ , median 0.04  $\mu\text{g/g}$ , n=53) and traumatic deaths (0.005-5.5  $\mu\text{g/g}$ , median 0.07  $\mu\text{g/g}$ , n=572). Although these numbers may be

skewed by the inclusion of morphine values derived from intake of morphine rather than heroin, this extreme overlap in concentration makes conclusions based on toxicological data in isolation unreliable.

**Table 1.** Femoral blood concentration ( $\mu\text{g/g}$ ) of morphine.

	Overdose (n=1582)	Hanging (n=24)	Trauma (n=154)	Fatal burns (n=19)
Median	0.155	0.04	0.07	0.96
Range	0.005 – 14.7	0.002 – 0.98	0.005 – 5.5	0.005 – 5.3

Few studies have investigated correlation between blood morphine concentration and morphine concentration in brain tissue. In brain sections from 21 suspected heroin addicts, Pare et al. [46] found brain morphine concentration to be constantly higher than  $0.2 \mu\text{g/g}$ , whereas blood concentration only exceeded  $0.2 \mu\text{g/g}$  in five cases. Apparently, more data are needed to determine if toxicological analysis of select brain regions may improve the diagnosis of opioid overdose death.

### 1.2.6 Opiate-related death

Opiate abusers have an increased mortality rate of approximately 13 times compared to non-abusing peers [47, 48]. It has been estimated that heroin abusers loose about 18 years of potential life due to premature death before the age of 65 [49].

In the EU, opiate abuse result in approximately 8000 deaths each year [50]. Forensic records estimate that about 300 to 400 drug related deaths occur each year in Sweden and that half of them are attributed to opiates [51].

In addition, a high proportion of opiate abusers experience non-fatal overdose episodes. The prevalence of having experienced a non-fatal overdose among heroin users has been reported to be 29% during lifetime [52] and 22% within the last year [53]. However, in a recent study Dietze et al. [54], reported that 75% of individuals treated for a non-fatal overdose had experienced at least one additional non-fatal overdose.

The word "overdose" is an unfortunate misnomer, suggesting that an excessive dose was taken. Consequently, a high concentration of heroin metabolites would be expected in these subjects. However, there is little support in the literature that the intake of an excess of heroin is the main reason for overdose. In fact, many overdose victims present with morphine concentrations in the lower range compared to living individuals [55-57]. In addition, abusers having experienced a non-fatal overdose do not rank the amount of drug consumed as important as other factors, such as opiate abstinence or the concomitant intake of multiple drugs, i.e. polydrug use [58]. It is somewhat difficult to explain overdoses among experienced abusers having survived periods with intake of high amounts of heroin, and hence could be regarded as very tolerant and most likely cautious in their drug use behavior. The reasons for such deaths may lie in neuropharmacological changes yet to be determined, e.g. loss of neurons essential for respiratory function [59, 60].

Other hypotheses that have been proposed to explain overdose deaths include: low tolerance or abstinence [20, 55, 61], delayed death, during which the drug concentration will gradually decrease [62, 63], and co-ingestion of other drugs (i.e. polydrug use), reducing the opiate concentration required to become toxic [64].

In Table 2, examples of proposed risk factors are given.

**Table 2.** Risk factors implicated in opiate overdose.

Gender [65, 66]	Polydrug	Amount [69, 79]
Age [67, 68]	– Alcohol [56, 76, 77]	Purity [80, 81]
Environmental cues [69-71]	– Benzodiazepines [67, 78]	Injecting [64, 65, 82]
Tolerance/Abstinence [67, 72, 73]	– Amphetamine [53]	Suicide [83-85]
– after prison [74, 75]		

However, measuring tolerance or other factors involved in opiate overdose is, unfortunately, rather complicated. Today, there is no pharmacological test for estimation of the degree of opiate tolerance that could be applied on either dead or living subjects. Subjective estimates can be obtained by interviewing living addicts, but the reliability in self-reports may be questioned. However, hair analysis can provide objective data about previous drug use and disclose periods of abstinence [74, 75, 86].

## 1.3 HAIR

### 1.3.1 Reason for hair analysis

Several different tissues and fluids can be collected and used to detect and quantify drugs. In forensic toxicology, analysis of blood, urine and vitreous humor can provide information about an acute intake. To obtain information about prior exposure, analysis of hair or nails can be carried out. Whereas nail growth is rather complicated, and the mechanisms for incorporation of drugs poorly studied, many studies have been published about hair growth, and about the drug incorporation mechanisms. Once incorporated, drugs remain very stable in hair, which allows for detection of substances up to several months after intake. By cutting hair into segments, drug intake at different time points can be disclosed. The shorter the segments and the more care taken during sampling, the more accurate the temporal mapping becomes.

Due to the problems with interpretation of post-mortem blood morphine concentration, the significance of a particular blood concentration can be expected to be improved if reliable information about previous, particularly recent, intake can be demonstrated.

### 1.3.2 History of hair analysis

In 1858, Hoppe published what seems to be the first study of poison in human hair in Caspers 'Praktisches Handbuch der Gerichtlichen Medizin', a handbook of legal medicine, where he studied arsenic levels in an exhumed body [87]. There is also a

swedish example of how arsenic could be detected even after hundreds of years. The Swedish king, Erik XIV (1533-1577) is thought to have been poisoned by arsenic in his pea soup. Hair analysis 400 years later confirmed the presence of arsenic, although the importance of this finding is debatable, since arsenic was frequently used during that time period [88].

One of the first studies of drugs in hair was made by Goldblum et al. in 1954 [89], where the incorporation of barbiturates in rat hair was examined. Since then, hair analysis has become an ever more popular matrix for the study of different markers, e.g. toxic metals during the 1960s and 70s. In 1979, Baumgartner and colleagues [90] made the first discovery of morphine in hair using a radioimmunoassay method. During the 1980s and 1990s, additional methods for hair analysis have been developed, including efficient chromatographic methods coupled to sensitive detection systems. The applications have also become wider, and today hair analysis is extensively used for e.g. workplace testing.

### **1.3.3 Physiology and anatomy of hair**

A brief and comprehensive review of hair physiology and anatomy is Harkeys' article published in 1993 [91]. Hair consists of 65-95% proteins (mostly keratin), 15-35% water, 1-9% lipids and <1% minerals. The hair follicle has three different layers: cuticle, cortex and medulla (figure 2a). The outer membrane (cuticle) offers protection and shine to the hair. The cortex contains melanin, which gives hair its color and provides some stretching ability.

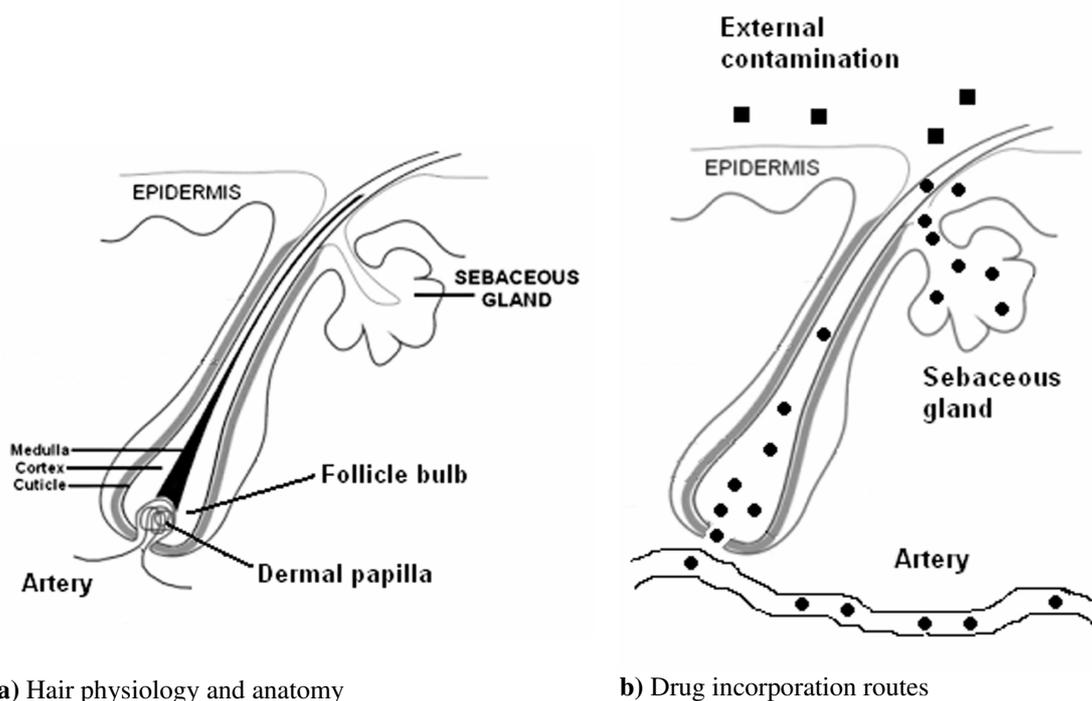
Hair growth starts in the hair follicle and occurs in cycles called anagen, catagen and telogen [92]. Every hair follicle has its own cycle independent of the neighboring cells. At any given time, about 85% of the hair strands are in the anagen phase while the remaining hair resides in the catagen and anagen phases pending the next growth phase [91].

During the anagen (growth) phase the hair follicle is produced and the hair cells differentiate into cuticle, cortex and medulla. The hair generally remains in this stage for 7 to 94 weeks, depending on hair type, anatomical location, race, sex and age. Throughout the catagen phase, the hair cell activity decreases and in the telogen phase the hair is at rest, growth has completely stopped and the hair bulb has diminished.

### **1.3.4 Drug incorporation and binding in hair**

The exact mechanism by which drugs are incorporated and bind to different structures in the hair is not completely known, but has been investigated by several groups. The main route of drug incorporation is believed to occur through passive diffusion from blood [93-96]. Many capillaries surround the hair follicle and substances circulating in the blood system will eventually be delivered to the hair follicle. Other routes that have been investigated include incorporation of drugs present in excretion from sweat glands, sebaceous glands, and deposition of drugs from the environment (Figure 2b). External contamination is not an incorporation route per se, but considered important as external

deposition of drugs on the hair surface can produce false positive results if not taken into proper consideration during preparation, analysis and interpretation.



**Figure 2.** Simplified histological structure of hair and drug incorporation routes.

Studies of drug binding to hair have mainly focused on the hair pigment melanin, although there are some studies of drug binding to keratin, the main constituent of the hair matrix [97, 98]. Potts [99] was the first to demonstrate that drugs bind to melanin, and since then this interaction has been made known to be one of the strongest retention mechanisms in human body [100]. The physiological importance of this binding is not fully understood but theories implicate melanin as a local regulator, releasing and binding endogenous and exogenous substances. Melanin could also function as a protective chemical filter, a reason for its presence in sensitive tissues such as the eye, ear and brain [100].

Melanin affinity is one of three main factors that determine the amount of drug incorporated. The other two are the lipophilicity and the basicity of the substance [101-104]. It has been shown that basic drugs have a stronger incorporation to melanin containing hair compared to non-pigmented. By studying the incorporation of cocaine in rat hair, Nakahara did some pioneer work in early 1990s and coined the concept of incorporation rate (ICR). ICR is defined as the drug concentration in hair divided by the area under the concentration-time curve for plasma. Briefly, it is a quantitative measure of how efficiently drugs are incorporated in hair. A high ICR drug is more easily deposited in hair compared to a low ICR drug. Cocaine has been placed in the high group and morphine is in the low ICR group whereas amphetamines and MDMA belong to the medium ICR group. ICR ranges from 3.6 for cocaine to 0.21 for 6-AM and down to 0.03 for morphine [105].

### 1.3.5 Analysis of opiates in hair

The relationship between opiate dose and concentration in hair is an important issue that has been studied by several groups in different settings [74, 90, 106, 107]. Musshoff et al [108] found a correlation between dose and total opiate concentration in a controlled heroin administration setting, whereas Kintz and colleagues studied hair samples from heroin abusers enrolled in a clinical trial with heroin hydrochloride and found no correlation between the accumulated dose and total opioid concentration in hair [109]. Neither was a correlation detected between heroin dose and 6-AM concentration in hair in another study [110]. The poor correlation between dose-hair concentrations may not be a surprise given the poor correlation between dose-blood concentrations and the many confounders in human studies. In contrast, *in vivo* experiments with animals, where it is possible to control many of the factors that influence drug incorporation, generally show good correlation [101, 111-113].

The inter-individual variation in drug concentration is believed to primarily depend on differences in melanin content [114]. The existence of different types of melanin including eumelanins (black-brown) and pheomelanins (yellow-red) complicates such comparisons even further [115].

Tagliaro et al, Kronstrand et al and Darke et al, have investigated the concentration of heroin metabolites in hair in fatal overdoses [74, 75, 86]. All concluded that absence or low concentrations of opiates in hair was due to abstinence and hence may have been the cause of death owing to reduced tolerance. Both Darke and Tagliaro compared the findings in hair from deceased drug users to those from living subjects. However, as discussed above, the concentrations vary substantially depending on many other factors in addition to the dosing and comparisons between individuals require serious caution.

As opposed to comparison of drug hair concentrations *between* individuals, comparisons between concentrations in different hair segments of the same individual is possible, since the conditions for drug incorporation is constant from time to time and the drugs remain stable in the hair once incorporated. Depending on the length of the segments used, it is possible to estimate abstinence, drugs abuse pattern and changes in drug taking within weeks prior to death. A hair segment of 5 mm corresponds to approximately 10-15 days of growth and in particular, the drug content of the innermost 5 mm segment should be very valuable for the interpretation of the degree of tolerance, since it seems likely that tolerance should be reduced if opiate drug use has been discontinued for more than 10 days.

Hair analysis can therefore be used to both detect specific drugs and investigate patterns of abuse. In addition, toxicological analysis of opioids in hair segments will provide information about abstinence in opiate overdose victims and thus assist in the interpretation of other toxicological findings in each individual case.

## 2 AIMS OF THE THESIS

The main objectives of the work included in this thesis were to develop strategies using toxicological analyses to investigate opiate overdose deaths and factors involved.

In particular, the aims were:

- to establish a strategy for investigation of suspected drug overdose death using hair analysis (Paper I)
- to use segmental hair analysis to map previous drug use and to disclose recent drug intake (Paper II)
- to explore the importance of polydrug use for opiate overdose death (Paper IV), and
- to establish an experimental model of opiate overdose and to assess the impact of tolerance and abstinence on the outcome after an acute, high dose of opiates (Paper III)

## **3 METHODS**

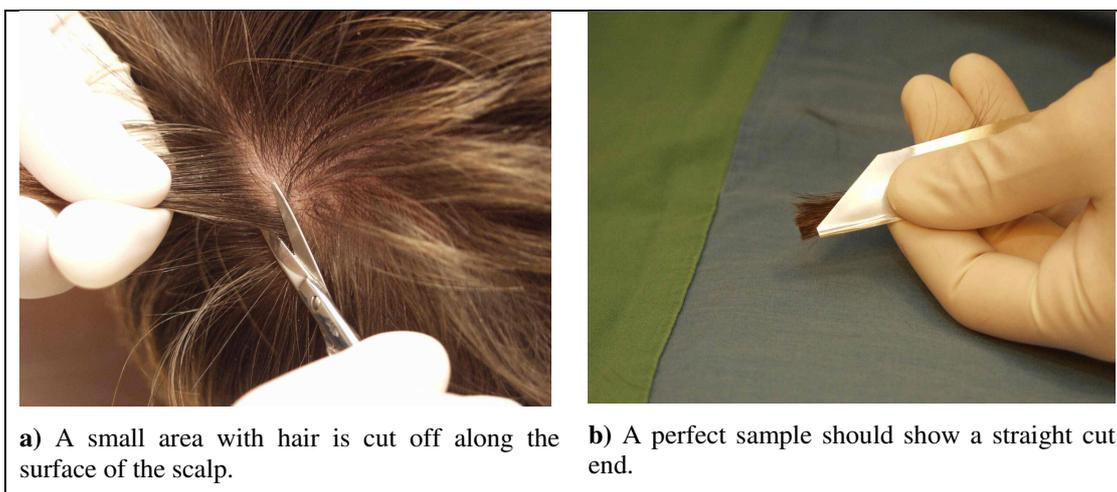
### **3.1 ETHICAL CONSIDERATIONS**

At forensic examinations, several different samples are collected for further toxicological, chemical and histopathological analyses. The selection of samples collected and used in the forensic evaluation depends on the circumstances and findings in each case. In Paper I, II and IV, results of analyses of blood and hair samples, collected at routine examination, have been compiled together with other results from the medicolegal investigation, e.g. police report and medical history in order to investigate drug use and contributing circumstances in opiate overdose death. All data used for the compilation and evaluation have been anonymized and handled according to Swedish laws and regulations. Hence, no person-identifiable information can be derived from these data. The animal studies have been approved by the local animal ethical committee at the Karolinska Institute.

### **3.2 POST-MORTEM STUDIES**

#### **3.2.1 Case selection and sampling procedures**

In Paper I, hair was collected from 75 consecutive autopsy cases at the Department of Forensic Medicine in Stockholm during May 2003. In Papers II and IV, hair samples were collected from suspected drug abusers during 2003-2006 at the Department of Forensic Medicine in Stockholm. Selection criteria in Papers II (n=60) and IV (n=166) included drug abuse history based on information from police and relatives as well as findings suggesting drug abuse during the external and internal examination at the autopsy. In Paper II, hair and blood samples were collected from 60 deceased drug abusers, classified as 'tolerant' or 'abstinent' according to the results of hair confirmation analysis. The 166 cases in Paper IV were further classified by an experienced forensic pathologist as 'overdose victims' (O-cases), 'control cases' (C-cases) or 'miscellaneous cases' (M-cases). The C-cases comprised victims with a history of drug abuse, which died from another cause of death than overdose, and were obviously not incapacitated by drugs at the time for their demise. The M-cases did not die of an overdose either, although incapacitation could not be excluded. Victims with shaved heads were not included in either study. In all cases a full autopsy with extensive toxicological testing and microscopic examination was performed. All samples for toxicological analysis were collected according to standardized procedures established by the Swedish National Board of Forensic Medicine [26]. Femoral blood was collected by cutting off the iliac vein and pressing blood into a 20mL plastic tube containing 1% potassium fluoride. To minimize the effects of variations in hair growth rate, hair samples were always collected from posterior vertex as close to the scalp as possible, aligned, wrapped in aluminum foil and stored at room temperature in an envelope (Paper II, Figure 3 a and b).



**Figure 3.** Hair collecting procedure.

### 3.2.2 Hair analysis strategy

Hair analysis was performed in two steps:

#### 1) *Screening analysis*

In all cases a screening of drugs of abuse was performed on one unwashed hair sample. One of the three hair samples collected was cut in small pieces (1–5 mm), weighed and put into a 10 mL screw-capped glass tube and analyzed with LC–MS–MS. The remaining hair samples were stored at room temperature pending confirmation analysis if a positive result was obtained at screening.

#### 2) *Confirmation analysis*

To investigate recent abstinence or tolerance in a screening-positive case, another set of analyses was performed on several washed hair segments, usually 3 proximal 5-mm segments followed by 2 10-mm segments, together corresponding to approximately 3 months of hair growth (drug history). The washing and segmentation of the hair allowed us to evaluate recent abstinence from certain drugs. Assuming a hair growth of 0.44 mm/day [91], a positive detection in the most recent segment, S1, should imply exposure within approximately 10-15 days before death, considering differences in hair growth. An opioid-negative result in S1 was interpreted as reduced tolerance to opioids keeping in mind that loss of tolerance is a gradual process.

This strategy allows for the identification of the previous drug use pattern, pinpointing the exact drugs used, and at what times before death, and also provides information of possible periods of abstinence.

### 3.2.3 Screening for drugs of abuse in hair

To 10–50 mg of hair were added 0.5 mL of acetonitrile:methanol:20 mM formate buffer pH 3.0 (10:10:80,) and 25  $\mu$ L of internal standard (2.0  $\mu$ g/mL of d3-morphine, d3-cocaine, d5-amphetamine and d5-diazepam) and the sample was incubated in a water bath

with vigorous orbital shaking at 37°C during 18 h. A 150- $\mu$ L aliquot was transferred to an autosampler vial and 10  $\mu$ L were injected into the chromatographic system. Daily calibration was performed by addition of standard solutions to 20 mg of drug-free hair prior to incubation. Final concentrations were 0.5, 1.0, 1.5, 3.0, 5.0 and 7.5 ng/mg. In Paper IV, the drug screen was modified to also include the MOR agonists fentanyl, buprenorphine, methadone, propoxyphene, tramadol and ketobemidone.

### **3.2.4 Confirmation of drugs of abuse in hair**

A set of hair samples was carefully aligned and sectioned into short segments; 5 mm long for the most recent segments (S1-S3), and 10 mm long for the outer segments (S4-S5). The hair segments were separately washed for 15 min with 2 mL 2-propanol, 3 x 30 min with 2 mL 0.01 M phosphate buffer (pH 6) and finally again with 2 mL 2-propanol as proposed by Baumgartner and Hill (Baumgartner and Hill, 1993). After drying the hair at room temperature, aliquots were weighed and transferred to 10-mL screw-capped tubes for target analysis with GC-MS for opiates and amphetamines [74] or LC-MS-MS for benzodiazepines [116].

### **3.2.5 Drug analysis in femoral blood**

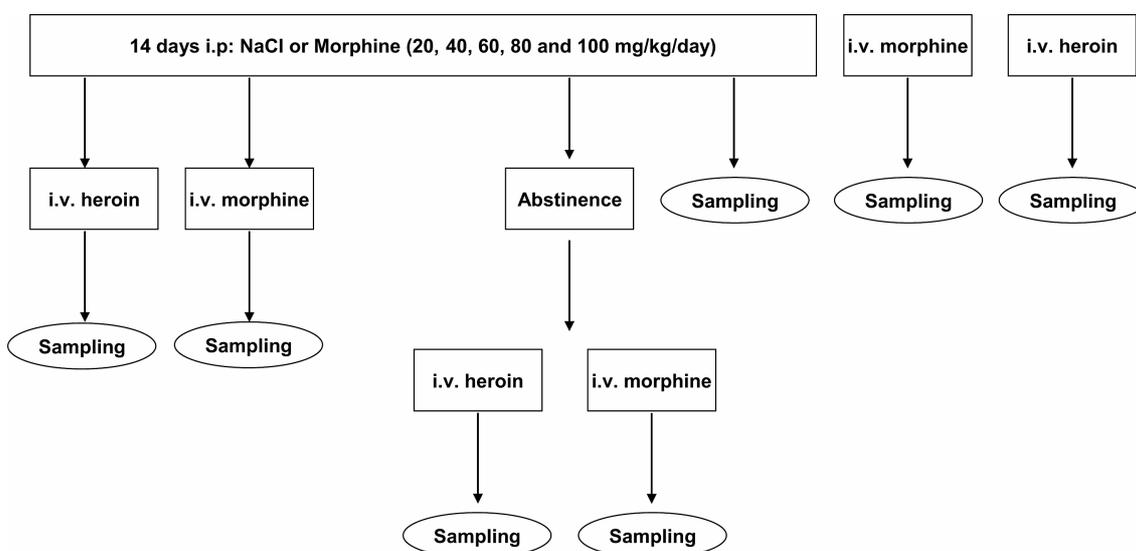
Opiates and cocaine were analyzed with gas chromatography-mass spectrometry (GC-MS) after solid phase extraction, whereas amphetamines were subject to liquid/liquid extraction before GC-MS detection [74]. Hypnotics, tranquilizers, antidepressants and narcotics such as methadone, tramadol, propoxyphene and ketobemidone were isolated by liquid/liquid extraction. Each case was extracted twice, under neutral and basic conditions (pH 7 and pH 11) and the extracts were then analyzed separately with gas chromatography using NP-detection as previously described [26]. Ethanol was analyzed with head-space gas chromatography accordingly to a previously described method [117]. Fentanyl was analyzed with GC-MS according to Kronstrand et al. [118] and buprenorphine was analyzed using LC-MS-MS with a procedure modified from Kronstrand et al [119].

### 3.3 ANIMAL STUDY PROTOCOL

In Paper III we used male Sprague-Dawley rats (Scanbur BK AB, Sollentuna, Sweden) weighing between 216 and 376 g. All rats were housed in groups of four in a temperature-controlled environment and allowed food and water ad libitum. Lights were on only between 7.00 am and 7.00 pm. All experiments were performed in strict accordance with guidelines at Karolinska Institutet and the consent of the Animal Ethics Committee of Northern Stockholm. Heroin hydrochloride was purchased from Macfarlan Smith Ltd., Edinburgh, UK and morphine hydrochloride was purchased from the Swedish Pharmacy (Apoteket AB). Both drugs were dissolved in 0.9% NaCl and slightly heated to 40-50°C if not dissolved.

#### 3.3.1 Experimental procedure

Rats were randomly allocated to seven groups, each comprising between 6 and 14 rats. Drug-naïve rats were injected i.v. with heroin, morphine or NaCl and sacrificed after eight hours if not dead. Additional groups of rats were pre-treated with ip morphine for 14 days (with or without one week of subsequent abstinence) followed by i.v. heroin and morphine (figure 4).



**Figure 4.** Simplified scheme of animal groups in Paper III. i.v.=intravenous injection, i.p.=intraperitoneal injections.

Using the up-and-down method described by Dixon and Bruce [120, 121] we established a dose causing 60-80% mortality in naïve rats. In the rat strain used, this resulted in a dose of 21.5 mg/kg for heroin and 223 mg/kg for morphine. All ip injections were given once daily between 10-12 am, using a 0.6x25 mm needle. The i.v. injections were administered early in the morning with volumes ranging from 0.31 to 0.56 mL for i.p. injections and from 0.42 to 0.46 mL for i.v. injections. The schedule for

the ip injections was: 20 mg/kg (day 1-3), 40 mg/kg (day 4-6), 60 mg/kg (day 7-9), 80 mg/kg (day 10-12) and 100 mg/kg (day 13-14). Immediately after injection, rats were placed in separate cages and observed for a maximum of 8 hours. Rats that died spontaneously during this period were decapitated and tissue collected. Rats that survived were quickly anesthetized with isoflurane (Forene®; Abbott Scandinavia AB, Solna, Sweden) and decapitated 8 hours after the i.v. injection. After decapitation, blood was drawn from the heart and mixed with sodium fluoride to prevent degradation of drugs. The brain was quickly removed and the occipital lobe and the cervical spinal cord were dissected free and collected for toxicological analysis. The rest of the brain was rapidly frozen in isopentane and dry ice at -35°C for neuropharmacological analysis. The lungs were removed and the right lung was frozen in isopentane and dry ice and the right lung was kept for toxicological analysis. The blood, brain and lung samples were stored at -80°C until analysis.

### **3.3.2 Analysis of 6-AM, morphine, M3G, and M6G in rat tissues and fluids**

Analysis of 6-AM, morphine, M3G and M6G in blood and tissues was performed by electrospray liquid chromatography-mass spectrometry (ESI-LC-MS-MS). The samples were analyzed at the Department of Clinical Pharmacology, St. Olav University Hospital, Trondheim, Norway. Tissue homogenates (from occipital lobe, cervical spinal cord and lung tissue) and diluted blood samples were extracted according to Bogusz et al [122] with slight modifications as described in detail in Paper III.

## **3.4 STATISTICAL METHODS**

Difference between groups in Paper III was analyzed with ANOVA, and if significant, Fisher's protected least significant difference (PLSD) post hoc test was applied. In Paper IV difference between groups were analyzed with a two-tailed Student's *t*-test for unpaired observations. In all analyses a *p* value < 0.05 was considered statistically significant. Statistical analysis was performed using StatView® for Windows Version 5.0 (SAS® Institute Inc., Cary, NC, USA) or STATISTICA 7.1 (StatSoft Inc. 2005, Tulsa, OK).

## 4 RESULTS AND DISCUSSION

### 4.1 Drug screening in hair

Several reports on hair testing on samples from deceased drug addicts have been published. However, most of these publications relate to individual cases, i.e. case reports or a limited number of cases, where the purpose has been to provide supplementary information about that case. In addition, many methods for hair analysis cover only a few drugs or just one or two groups of drugs. To our knowledge, systematic application on deceased, suspected drug abusers of a screening method that encompasses the majority of drugs of abuse is not routine anywhere.

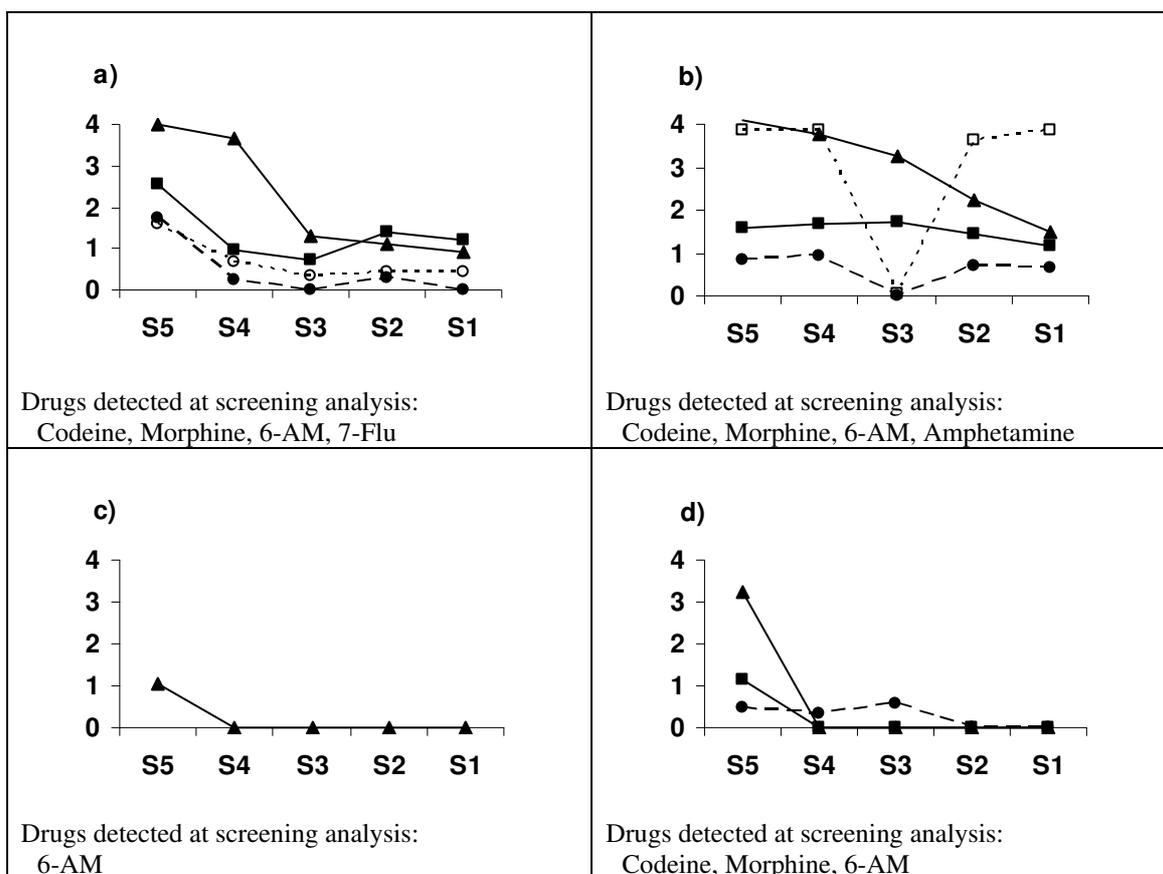
Various methods for drug analysis in hair have been developed, primarily for workplace testing purposes. Most screening methods are based on immunological reactions, i.e. antibodies targeting characteristic structures on selected groups of drugs. Although extensively used, these methods suffer from cross-reactions and do not allow for any quantification of individual drugs [123].

In Paper I, we present a method for screening for drugs of abuse in hair samples. This method is based on liquid chromatography combined with mass spectrometry (LC-MS-MS). A decade ago, this method was not an option since the interface between the chromatographic and detector systems suffered from technical problems. Thanks to innovations regarding the interface, solutions like the electrospray and atmospheric pressure chemical ionization have been developed. Today, this instrumentation allows for the simultaneous analysis of compounds with different physicochemical properties. The method that we developed includes all groups of illicit drugs used by the Swedish addicts, except for cannabinoids (which are not regarded as pharmacologically hazardous). This method was evaluated using hair samples from 75 consecutive autopsy cases at the Department of Forensic Medicine in Stockholm, Sweden. Some of the subjects that tested positive were not known to be drug abusers by the police or the relatives. One of the opiate overdose deaths was an apparent naïve user, who used heroin for the first time. These findings would have gone undetected if hair analysis had not been carried out.

The method described in Paper I proved successful as a way to objectively disclose previous drug use as well as being easy to apply and cost-effective. However, it does not discriminate between recent use and intake several weeks-months back, depending on length of the hair strand. Hair samples collected and used for screening purpose have variable lengths, depending on subject, availability and other conditions. Most of the samples analyzed have been 1-4 cm long, and hence the relative contribution from a recent exposure to the positive result is impossible to establish. Both Tagliaro [75] and Darke [86] used hair samples of considerable length when concluding that deceased heroin addicts had lower levels of morphine in hair than living abusers. Thus from such data, it can only be concluded that the average levels in hair were lower among the deceased overdose subjects, since the hair analysis was not accurate enough to tell if subjects *recently* had been using opiates or not.

## 4.2 Segmental hair analysis

In order to temporally map the previous exposure pattern and obtain information of recent drug intake, we analyzed the presence of various drugs in short hair segments. The purpose for choosing short segments was to reveal recent abstinence, particularly regarding opioid drugs. Hence, in Papers II and IV, the innermost three segments were only 5 mm long. Figure 5 show the drug use pattern over time in four cases. In all these cases, the most proximal 3.5 cm of the hair was used for analysis in five segments. The result of the initial drug screening is listed below each case, and when these detections are compared with the drug concentration pattern in the corresponding graph, the advantage of such a detailed segmental mapping should be easily appreciated.



**Figure 5.** Four examples of confirmation analysis of drugs of abuse in hair segments (S1-S5) from opiate overdose victims. S1 is the most recent hair segment, representing 10-15 days before death. Below each case are listed the drugs detected at hair screening analysis. Drug concentrations are given in ng/mg hair. ■ = morphine, ▲ = 6-acetylmorphine (6-AM), ● = codeine, ○ = 7-amino-flunitrazepam (7-Flu), □ = amphetamine.

## 4.3 Abstinence

Hypothetically, experienced heroin addicts would be able to tolerate both high doses of heroin and several additional drugs due to a regular exposure and practice. However, drug addicts may change drug use pattern over time if imprisoned or upon entering a treatment

program. Hence, it has been suggested that a substantial proportion of fatal overdoses is caused by reduced tolerance after a period of abstinence [53, 55, 73]. As of today, the only means is to estimate the tolerance by documentation of previous, particularly recent, drug exposure. Such information may be obtained by interviewing the addicts about their previous drug use [54, 58]. However, if dead, the addicts (and nobody else) will not talk. An alternative is therefore to carry out drug analysis on hair to find out if a subject had previously used drugs and, if so, which drugs.

Evaluation of the influence of abstinence in opiate related deaths revealed that reduced drug use preceding last intake was very common. In Paper II we found that a majority (18/28) of the heroin overdose victims had had a period of opiate abstinence prior to death. In Paper IV, we extended the material to also include all forms of opiate overdose deaths, although most of them apparently had used heroin. In the latter study, we found an even more striking proportion of subjects with recent opiate abstinence (75/91).

Several other research groups have investigated and demonstrated the importance of abstinence in overdose deaths [20, 55, 61]. It is therefore somewhat unexpected to find that drug addicts having experienced a non-fatal overdose rank reduced tolerance as less important than other risk factors, such as heroin quantity and polydrug use [58]. Of the opiate overdose victims that were not abstinent in Paper IV, many had a recent decrease in intake of opiates, suggesting reduced tolerance.

In Paper III, we found that two weeks of pre-treatment with increasing doses of morphine reduced the mortality significantly after a high i.v. dose of morphine, whereas it was less protective against a high i.v. dose of heroin. The mortality rate increased if the pre-treatment was followed by one week of abstinence. In fact, rats given a high i.v. dose of heroin displayed the same mortality as opiate-naïve rats. In a study by Siegel et al [124] pretreatment of Wistar rats reduced mortality with 64%. These findings lend additional support to the notion that abstinence during a fairly short period can influence the sensitivity of the opioid system to a new challenge. Even if the effect of abstinence probably is species-related, it seems likely that a gradual loss of tolerance in humans occurs with a few weeks, which also was the reason for using short hair segments in Papers II and IV.

In summary, our studies provide strong support to the notion that recent opiate abstinence is risk factor for opiate overdose death.

#### **4.4 Blood morphine concentration**

Several other studies have found blood morphine concentrations to be extremely variable and be fairly low as compared to living abusers [56, 57].

In Paper II, one main purpose was to explore the blood morphine concentrations in heroin overdose subjects. As mentioned previously, blood morphine concentrations vary substantially among these victims, and many of them present with fairly low concentrations. It has therefore repeatedly been hypothesized that a period of abstinence will make the heroin addict more vulnerable and even die when taking a "regular" dose.

This would explain why some victims show very low blood morphine concentrations. By conducting segmental hair analysis, we have differentiated between victims with evidence of a recent drug free period from those showing a continuous opiate use prior to death. Surprisingly, the blood morphine levels in both groups were very similar. Similar results were also obtained in Paper IV. One explanation for this finding may be that the deaths occur at variable times after intake. Detailed information about time of intake, and time of death was difficult to obtain, except for cases where the overdose was witnessed, and/or where rapid intervention by paramedics was documented.

In order to more closely address the pharmacokinetic pattern in an overdose setting (i.e. toxicokinetics), we developed an experimental model using Sprague-Dawley rats (Paper III). To investigate the impact of previous drug use on blood morphine concentration, rats were made tolerant using pre-treatment with morphine. Subsequently, a high dose of either morphine or heroin was administered intravenously. In rats dying rapidly (within 10 minutes), we saw a substantial variation in morphine concentration in blood, lung tissue and investigated brain regions. Hence, even during very controlled conditions, it seems impossible to obtain concordance between blood morphine concentrations or to establish a fatal blood morphine concentration for heroin or morphine exposure. Surviving rats that were sacrificed 8 hours after i.v. injection all displayed similar blood morphine concentrations. Apparently, even under highly controlled conditions, blood morphine concentration per se is a very poor indicator of morphine or heroine overdose death.

Having discussed the possible reasons for generally variable blood morphine concentrations in both tolerant and abstinent victims, the reason for overdose remains to be explained. One explanation may be that many of the tolerant subjects had taken their "normal" dose, although that these individuals at that occasion were more vulnerable to an opioid drug challenge for some reason [69]. Regarding the abstinent subjects, one reason for overdose could be that their MOR system had been up-regulated during a drug free period.

In contrast to the morphine concentration in blood, the morphine concentration found in cervical medulla in rats was fairly uniform (Paper III). Hence, the determination of morphine concentration in the cervical medulla might serve as a more reliable measure of toxicity compared to morphine concentration in either blood or other parts of the brain. Since the central respiratory centre in the rat is located in medulla oblongata [125, 126], it would have been logical to examine the opioid concentrations in this region. However, in this study, we also wanted to study possible neuropharmacological changes in this area, hence it was not available for toxicology.

## 4.5 Delayed death

As discussed above, reasons for variable blood morphine concentration could be due to the time after intake to death occurs. In contrary to general beliefs, death from opiate overdose does not always happen directly after drug intake. Instead, there are a substantial number of deaths that occurs a considerable amount of time after injection [56, 127, 128]. The time to death after heroin injection has been found to range between a few to several hours. Nakamura [129] found 48% of overdose victims to have died after two hours.

We found evidence of delayed death in about 40% of the opioid overdose victims in Paper IV. Most of the victims with delayed death had become ill soon after intake or fallen asleep after more than half an hour and subsequently become numb. When reviewing the toxicological findings, these subjects showed no obvious difference in recent or acute drug use pattern as compared to overdose victims dying rapidly.

In addition, we also found delayed deaths among rats (Paper III) given high i.v. dose of morphine, but not among those given high i.v. dose heroin. When the dose was adjusted, rats administered lower doses survived with minimal signs of respiratory depression, and those administered higher doses than 21.5 mg died rapidly. Rats administered a high dose of morphine had a time to death between 2 and 262 minutes. In a similar study, Borron et al found a median time to death of 2.5 hours and that time to death increased significantly when combining morphine and flunitrazepam [130].

The finding of delayed death is of importance since many drug abusers inject in pairs to be able to assist if one experience an overdose. However, if the time to death is increased by several hours, many addicts would probably be alone and dozed off at the time the respiratory depression is initiated. The mechanisms and factors influencing delayed death are still unknown, but regarding heroin, this phenomenon might be favored by multiple drug use since we did not see any delayed deaths in rats injected with a high dose of heroin.

## 4.6 Polydrug use

Another main finding in Paper II was that almost all overdose victims had several other drugs in hair and blood. This finding, together with the increased mortality risk of multiple drug use [76, 127, 131] initiated further investigations of this phenomenon. Of the 91 opiate overdose victims examined in Paper IV an overwhelming majority (80%) had more than three drugs present in hair and 85% victims had at least two drugs present in blood - even after excluding alcohol. 135 subjects out of 166 had more than one drug in hair whereas 94/166 had more than one drug in blood. In Table 3 the most common substances in hair and blood are listed for comparison.

**Table 3.** The most common drugs detected in hair and blood in Paper IV.

<b>a) Substance</b>	O-cases n (%), (n=91)		C-cases n (%), (n=30)		M-cases n (%), (n=45)	
	Hair	Blood	Hair	Blood	Hair	Blood
Amphetamine	56 (62)	17 (19)	19 (63)	10 (33)	30 (67)	17 (38)
6-Acetylmorphine	54 (59)	21 (23)	3 (10)	1 (3)	10 (22)	0 (0)
Morphine	51 (56)	61 (67)	4 (13)	2 (7)	7 (16)	1 (2)
Codeine	41 (45)	47 (52)	11 (37)	4 (13)	6 (13)	1 (2)
Diazepam	31 (34)	31 (34)	5 (17)	4 (13)	7 (16)	2 (4)
Methamphetamine	26 (29)	2 (2)	12 (40)	2 (7)	19 (42)	1 (2)
Tramadol	22 (24)	6 (7)	4 (13)	0 (0)	11 (24)	1 (2)
Benzoylgonine	17 (19)	4 (4)	7 (23)	1 (3)	6 (13)	2 (4)
Cocaine	17 (19)	2 (2)	7 (23)	0 (0)	6 (13)	2 (4)
MDMA	15 (16)	1 (1)	2 (7)	1 (3)	3 (7)	0 (0)
<b>b) Drug group</b>						
Opiates	83 (91)	84 (92)	16 (53)	4 (13)	24 (53)	4 (9)
Amph	63 (69)	19 (12)	21 (70)	10 (33)	32 (71)	18 (40)
Benzos	36 (40)	41 (45)	5 (17)	4 (13)	9 (20)	3 (7)
Coc	17 (19)	4 (4)	7 (23)	1 (3)	6 (13)	2 (4)
<b>c) Drug group combination</b>						
Opiates + Amph	29 (32)	7 (8)	5 (17)	2 (7)	12 (27)	0 (0)
Opiates + Amph + Benzos	18 (20)	11 (12)	3 (10)	0 (0)	4 (9)	1 (2)
Opiates	10 (11)	36 (40)	3 (10)	0 (0)	5 (11)	3 (7)
Opiates + Benzos	9 (10)	26 (29)	1 (3)	2 (7)	1 (2)	0 (0)
Opiates + Amph + Coc	6 (7)	0 (0)	3 (10)	0 (0)	1 (2)	0 (0)

Amph=amphetamines. Benzos=benzodiazepines. Coc=cocaine and/or benzoylgonine.

An intriguing finding in Paper IV was the presence of benzodiazepines and absence of amphetamine in blood of opiate overdose victims. Amphetamine was the most frequently detected substance in hair of all groups, but not at all as common in blood of opiate overdose victims. Drug users have apparently survived previous abuse when amphetamine was present in blood. It is thus tempting to speculate that amphetamine may confer some protection against opiate overdose.

Conversely, benzodiazepines are reported in several studies to have a more hazardous influence [132, 133]. In our sample, 45% of opiate overdose victims had benzodiazepines present in blood, compared to the 12% that had amphetamine blood. Mixing opiates and ethanol has also been implicated as a risk factor for overdose [77, 128, 134]. However, in our sample, only 29% of the opiate overdose victims were positive for ethanol in blood, and in most cases at fairly low levels, suggesting that other factors are more important. In addition, we found no significant difference in blood morphine concentration between ethanol-negative and ethanol-positive cases.

Borron et al [130] showed that addition of flunitrazepam to rats administered different opiates caused variable effects both in terms of mortality and in time to death. It is apparently difficult to predict the outcome of a particular drug combination by a theoretical consideration of each drug's pharmacological characteristics. Unfortunately, there are only a limited number of studies where combination effects of illicit drugs have been studied in a controlled setting. Some studies have reported an increased reward when combining cocaine and heroin [135], or methamphetamine and heroin [136] but no difference in reward when combining MDMA and heroin [137]. The paucity of toxicological data regarding pharmacodynamic interactions of illicit drugs is unfortunate. Further studies are needed, especially since polydrug use apparently is becoming more and more common.

#### **4.7 Pathophysiological mechanisms in opiate overdose death & Future studies**

The pharmacodynamic basis of opiate toxicity is not completely known. However, ample evidence suggests that the fatal effect is related to respiratory depression [20, 127]. Apnoea is a complication frequently reported after opiate intake, a reason why opiate addicts typically operate in pairs, where one can resuscitate the other. The typical autopsy finding in opiate overdose victims is a massive lung oedema, strongly indicating cessation of breathing prior to cardiac arrest. Respiration is dependent on neuronal input from the central nervous system to the diaphragm, and the muscles in the thoracic wall. Two major groups of respiratory neurons have been identified in both the medulla and the brain stem, the dorsal respiratory group (DRG) and the ventral respiratory group (VRG) [138, 139].

Most research regarding central respiratory mechanisms has been conducted in animals in various models, often *in vitro* preparations of isolated rat brain stem and spinal cord. In the rat, lesions of the pre-Bötzinger complex, located in the rostral VRG, almost completely diminish breathing [140]. This is believed to result from the elimination of specific pacemaker neurons, which are essential for normal respiratory function. However, the human respiratory centre remains elusive.

The experiments in Paper III were designed to examine both toxicological and neuropharmacological changes within respiratory sensitive areas. Hence, medulla oblongata was frozen in isopentane-dry ice within a minute after death, and subsequently cut in 14  $\mu\text{m}$  thick sections. The sections were then stained with an antibody for topographical identification of neurons (Neu-N), pending agonist-stimulated [ $^{35}\text{S}$ ]GTP $\gamma\text{S}$  for determination of the density of activated MOR as described by Sim et al [141]. At this moment, these studies are not completed. The aim is to investigate the relationship between opioid concentrations in the brain and the degree of MOR activation in the respiratory centres of the brain stem.

Finally, there is also a possibility that chronic exposure to opiates eventually results in a cellular exhaustion and necrotic or apoptotic cell death. In particular, repeated non-fatal opiate overdoses may result in hypoxic damage to select brain areas including those involved in respiratory control, and hence reduce the number of functional neurons. Such a reduction of respiratory neurons in key areas would make a subject more sensitive to

noxious influences. Further studies will focus on the status of the opioid system in pre-Bötzing complex, but the possible influence of other systems should also be explored when experiments with additional drugs are conducted. The recent observation by Manzke et al [142] of a reversal of the respiratory depressant effect of the MOR agonist fentanyl by a 5-HT<sub>4</sub> agonist, without reduction of the nociceptive effect serves as an example of findings that should encourage more studies.

## 5 CONCLUSIONS

### **Paper I**

The LC-MS-MS method for screening of drugs of abuse in hair proved very helpful, sensitive, and as specific as the confirmation analysis. Screening for the most common drugs can detect previous unknown abuse as well as provide guidance for further analysis.

### **Paper II**

Abstinent opiate overdose victims, as shown by segmental hair analysis, present with variable blood morphine concentrations. However, these concentrations do not differ from those in addicts with a continuous use prior to death. Abstinent and tolerant opiate abusers may die from equal heroin doses.

### **Paper III**

Morphine pre-treatment significantly reduces mortality after acute high dose of morphine or heroin, but this protection is reduced if pre-treatment is followed by a period of abstinence. Delayed death is not related to accumulation of opiates in blood or brain.

### **Paper IV**

Opiate overdose victims are polydrug users and their abuse pattern is very inconsistent. Amphetamine and benzodiazepines are the most common drugs combined with opiates. Abstinence is very common among opiate related deaths.

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