The effects of ethanol on the metabolism of heroin in C57BL/6J mice *in vivo* and *in vitro*

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Preface

This master thesis in toxicology is part of the master degree in biology at the University of Oslo. The work on this master thesis was carried out at the Norwegian Institute of Public Health, Division of Forensic Toxicology and Drug Abuse - Department of Drug Abuse research, during December 2009 to June 2011. Senior scientist researcher Jannike M Andersen, (Norwegian Institute of Public Health) and Professor Ketil Hylland (University of Oslo) supervised me in this work.

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Abstract

Drug overdose is a major cause of premature death among heroin users and there has been a call for studies to identify risk factors for fatal overdoses to improve preventive interventions. The majority of heroin related deaths involves consumption of heroin in combination with other drugs, ethanol being one of the drugs most commonly found. The concomitant use of ethanol and heroin may enhance the risk for a heroin overdose. It has been suggested that ethanol might inhibit the metabolism of heroin, however, the underlying biological mechanisms are poorly understood. This is the first preclinical study examining the effects of ethanol on the heroin metabolism. The aim of this study was to investigate whether ethanol modulates the metabolism of heroin in mice, thereby increasing the concentration of active metabolites (6MAM and/or morphine). A behavior test in mice was applied, where total distance travelled was registered after administration of ethanol in combination with heroin. The stimulating effect of heroin, measured in the locomotor activity test, reflects the concentration of active metabolites in the brain. *In vivo* and *in vitro* kinetic studies in blood and brain from mice were used to examine the concentration vs. time curves of heroin and heroin metabolites with and without the presence of ethanol. LC-MS/MS was used to quantify heroin and heroin metabolites in biological matrices. Our findings indicated that ethanol had small modulating effect on the metabolism of heroin. An elevated concentration of heroin in blood and brain was detected in the presence of ethanol, but no changes in the concentration vs. time curves of 6MAM or morphine in brain were found. These results were reflected in locomotor activity. Ethanol did not increase the maximum response, E_{max}, or result in prolonged locomotor activity.

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Abbreviations

6MAM	6-monoacetylmorphine
AChE	Acetylcholinesterase
ANOVA	Analysis of variance
BuChE	Butyrylcholinesterase
C_{max}	Maximum concentration
E_{max}	Maximal distance travelled
ESI+	Positive electrospray
eV	Collision energy
hCE-1	Liver Carboxylesterase 1
hCE-2	Liver Carboxylesterase 2
HIV	Human immunodeficiency virus
HPLC	High-performance liquid chromatography
HSGC	Head space gas chromatography
kV	Capillary voltage
L/h	Liters per hours
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LOD	Limits of detection
LOQ	Limits of quantitation
M3G	Morphine-3-glucuronide
M6G	Morphine-6-glucuronide
MRM	Multiple reaction monitoring
P.o.	Per os
Rpm	Revolutions per minute
Rt	Retention time
S.c.	Subcutaneous
SEM	Standard error mean
V	Voltage

1. Introduction

1.1 Background

Globally, it is estimated that more than 15 million people, aged 15-64, have consumed illicit opiates (morphine, opium and heroin) over the last 12 months. Of these, a large share use heroin, which is the most lethal drug amongst opiates (UNODC 2010). In Norway there are about 12-15 000 heroin addicts (Berg 2008). Drug overdose is a major cause of premature death among heroin users (Warner-Smith et al. 2001), the mortality rate for heroin addicts being 6-20 times higher compared to the general population of the same age and gender (UNODC 2010). Among illicit narcotics, opiates are the most costly drugs in terms of medical care and treatment. In addition to this, injection of heroin is widespread, which brings about acute and chronic health problems, such as hepatitis C, HIV/aids and blood-borne diseases (UNODC 2010). Several heroin addicts and heroin users die every year due to heroin related overdoses, and there is a growing need for heroin treatment (UNODC 2010).

A report from the World Health Organization has called for studies to identify risk factors for fatal overdoses to improve preventive interventions (WHO 1998). One of the risk factors identified is polydrug use (WHO 1998), a term describing the use of several drugs in combination (Kaufman 1976). Polydrug use is a pattern that has been observed in many drug-using populations (EMCDDA 2009), among them heroin addicts (Darke and Hall 1995). Ethanol is one of the drugs most commonly found in addition to heroin (Rook et al. 2006) and studies have suggested that the intake of ethanol is a risk factor in heroin related deaths (Ruttenber and Luke 1984; Levine et al. 1995; Fugelstad et al. 2003). Interactions between different drugs, such as ethanol and heroin, consumed in combination, could lead to increased toxicity due to additive or potentiating effects, change in pharmacokinetics (EMCDDA 2009) or loss of tolerance (Hickman et al. 2007).

1.2 Heroin

Heroin (figure 1-1), synthesised in 1874 by A.C Wright, was marketed as a less addictive drug than morphine (Sneader 1998). However, heroin is considered to give a more pronounced biological effect and a stronger addiction potential (Way et al. 1960), as well as a more potent reinforcing and analgesic activity, compared to morphine (van Ree et al. 1978; Kaiko et al. 1981; Hubner and Kornetsky 1992). This has led to heroin being one of the drugs among opioids most commonly sold on the illicit drug market (Pichini et al. 1999). In addition to illegal use, heroin are used medically in treatment of severe pain (Sawynok 1986) and also in treatment of heroin addiction (Fischer et al. 2002).

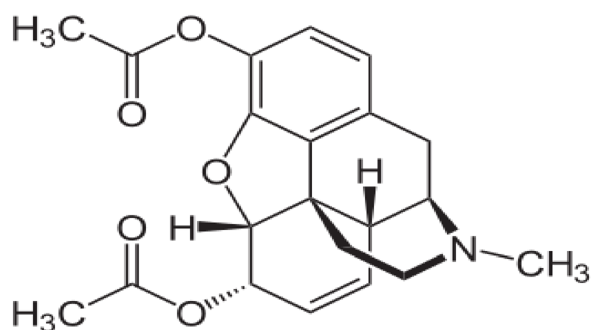


Figure 1-1 The molecular structure of heroin. Figure from http://no.wikipedia.org/wiki/IUPAC

In vivo and *in vitro* kinetic studies of heroin have shown that the rapid metabolism of heroin (figure 1-2) is followed by a quick and strong increase in 6-monoacetylmorphine (6MAM), and a subsequently slower increase in the morphine concentration (Way et al. 1960; Andersen et al. 2009; Boix et al. 2011). Morphine is further metabolized to morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G) in humans (Christrup 1997). Due to different binding affinity to opioid receptors, M6G is considered to be an active metabolite of heroin, while M3G is not (Pasternak et al. 1987).

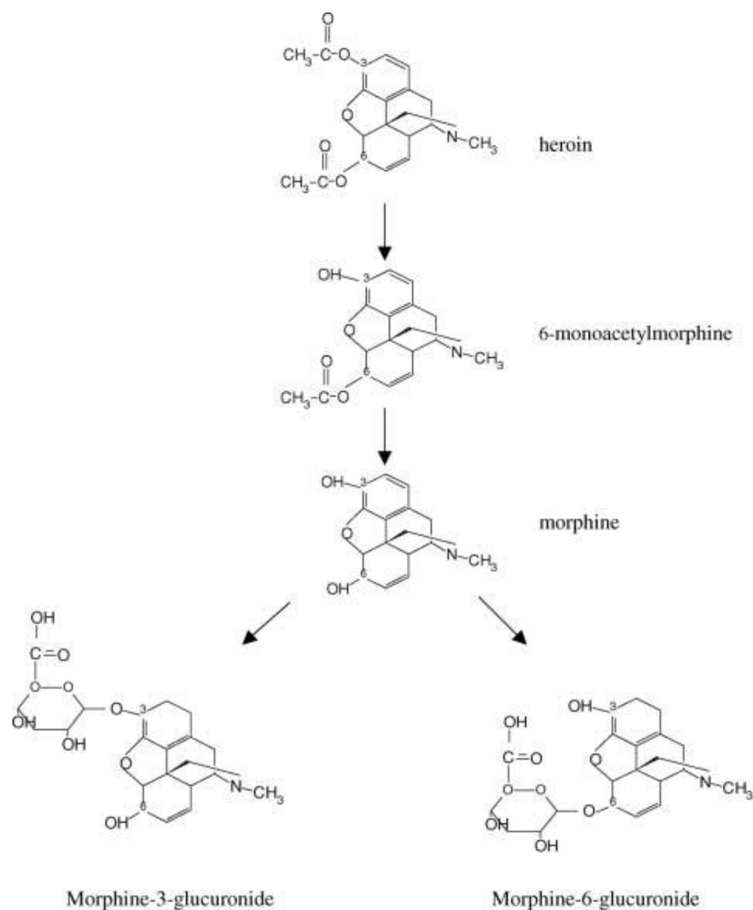


Figure 1-2 The metabolism of heroin to 6MAM, morphine, M3G and M6G (Rook et al. 2006).

The estimated half-life of heroin is short, 2-5 minutes in animals (Way et al. 1960) and 1.3-7.8 minutes in humans (Rook et al. 2006), but because of its active metabolites, the pharmacodynamic action lasts for several hours (Inturrisi et al. 1983). Receptor binding studies have shown that heroin has low affinity to opiate receptors (Inturrisi et al. 1983) and several studies concludes that heroin acts more as a prodrug that mediates its pharmacological effects through the active metabolites 6MAM and morphine (Way et al. 1960; Umans and Inturrisi 1981; Andersen et al. 2009). In a pharmacokinetic study of heroin, it is therefore important to include all the active metabolites of heroin; 6MAM, morphine and M6G. However, in rodents, no or only small traces of M6G is produced (Handal et al. 2002).

The rapid hydrolysis of heroin in blood may both be spontaneous (Kreek et al. 2005; Maurer et al. 2006) or catalyzed by different esterases (Salmon et al. 1999). Esterases are present in the circulatory system and other tissues (Rook et al. 2006). The deacetylation of 6MAM to morphine in blood is slower (Boix et al. 2011). The knowledge on the enzymes involved in the metabolism of heroin, are sparse. However, in humans, erythrocyte acetylcholinesterase

(AChE) and serum butyrylcholinesterase (BuChE) (Salmon et al. 1999), as well as liver carboxylesterase 1 (hCE-1) and liver carboxylesterase 2 (hCE-2) (Maurer et al. 2006), are involved in the deacetylation of heroin to 6MAM. AChE, but not BuChE, is also capable to metabolize 6MAM to morphine in blood (Salmon et al. 1999).

The metabolism of heroin in brain tissue is slower compared to blood (Andersen et al. 2009; Boix et al. 2011). This might be due to lower enzyme activity in the brain. However, the metabolism of heroin and the enzymes involved in the metabolic pathway of heroin in brain and other tissues are poorly understood.

1.3 Ethanol

Intensive ethanol consumption is often a major, but overlooked, component of polydrug use (EMCDDA 2009). Over the last 10 years, ethanol has been the drug most commonly found in blood samples in both mono-intoxications and also polydrug deaths (Jones et al. 2011).

Ethanol can affect the liver, intestine, the central nervous system, the endocrine- and immune system (Linnoila et al. 1979). At low doses, and in the initiating phase, ethanol is considered to be a stimulant drug with suppressing effects on the central inhibitory systems. As the concentration of ethanol increases, sedation, loss of coordination, ataxia and impaired psychomotor performance appear (Holdstock and de Wit 1998).

Acute and chronic intake of ethanol can alter both the pharmacodynamics and pharmacokinetics of other drugs (Sellers and Holloway 1978). Acutely, ethanol can inhibit the binding to δ-opioid receptors, while chronically use of ethanol can increase the density of μ and δ- opioid receptors (Weiss and Porrino 2002). The acute effects of ethanol on drug metabolism is generally inhibitory (Linnoila et al. 1979), while the chronic effects of ethanol may lead to an induction of drug metabolism (Kalant et al. 1976). In low doses, ethanol can accelerate the absorption and bioavailability of other drugs (Linnoila et al. 1979). Ethanol can also be an indirect risk factor, by reducing the capacity to judge the amount of other drugs consumed and by reducing awareness of the loss of tolerance to other drugs (EMCDDA 2009).

1.4 Polydrug use: heroin and ethanol

Heroin addicts are often polydrug users (Darke and Hall 1995). Epidemiological studies have detected ethanol concentrations in blood in addition to heroin metabolites at autopsy (Nakamura 1978; Goldberger et al. 1994; Darke et al. 2000; Fugelstad et al. 2003) and ethanol is considered to be a risk factor in heroin related deaths (Ruttenber and Luke 1984; Levine et al. 1995; Fugelstad et al. 2003). Ruttenber and Luke (1984) found that when ethanol concentration in blood exceeded 1 g/L (1‰), the risk of a fatal heroin overdose increased 22 times. Several studies have detected an inverse correlation between a high ethanol concentration and a low morphine concentration in blood (Ruttenber and Luke 1984; Ruttenber et al. 1990; Fugelstad et al. 2003).

The biological mechanisms behind the increased risk of an overdose when ethanol and heroin are taken simultaneously, are poorly understood (White and Irvine 1999). Several hypotheses have been suggested. Ethanol and morphine are both sedative drugs and studies have indicated that ethanol and morphine can work additively on the respiratory system and increase the risk of respiratory failure (Sporer 1999; Fugelstad et al. 2003). Ethanol and heroin in combination are often found in people who are sporadic users of heroin, which may have a lower tolerance to heroin compared to heroin addicts (Ruttenber et al. 1990). This demonstrates that lower morphine concentrations may cause death when a high blood alcohol concentration is present.

Studies have shown that ethanol might interfere with the metabolism of other drugs (Sellers and Holloway 1978) and it has been suggested that ethanol may interfere with the metabolism of heroin (Polettini et al. 1999). The reduced morphine concentration seen in many epidemiological studies may therefore be explained by a reduced metabolism of 6MAM to morphine. The hypothesis of a kinetic interaction between ethanol and heroin is also supported in other studies. Ethanol inhibits the metabolism of cocaine (Farre et al. 1997), and heroin and cocaine metabolism in humans are mediated through the same metabolic pathway (Kamendulis et al. 1996). This may indicate that ethanol also interferes with the metabolism of heroin. However, to the best of our knowledge, no experimental studies have investigated the pharmacokinetic of heroin in combination with ethanol.

1.5 Animal research and ethics

The research presented in this thesis could not be carried out without the use of laboratory animals. The purpose of using laboratory animals is investigating biological question related to human health (Hem et al. 2001). At the Norwegian Institute of Public Health, all necessary approvals for conducting animal research are fulfilled.

Due to short lifespan, allowing growth of a large number of animals in a short period of time, mice are commonly used in experimental animal studies (Martignoni et al. 2006). All experiments in this thesis, were carried out in C57BL/6J-mice. However, there are species differences between mice and humans such as body size, weight and enzymes. Blood circulation time correlates with total body weight, indicating that smaller animals deliver and excrete drugs more rapidly than larger animals (Lin 1995). There are also differences in expression and activities in drug-metabolizing enzymes (Martignoni et al. 2006). Extrapolating from mice to humans, should therefore be done with care.

1.6 Aim of the study

Epidemiological studies have shown that simultaneous intake of heroin and ethanol may increase the risk of fatal overdose. Several hypotheses have been suggested, but the biological mechanism(s) are poorly understood.

The aim of this study was to investigate one of the suggested hypotheses, i.e. whether ethanol may modulate the metabolism of heroin, thereby changing the concentration of active metabolites (6MAM and/or morphine) in blood and brain. Because of ethical considerations it is difficult to investigate this in humans. A feasible approach is therefore experimental animal studies.

A behaviour test, measuring locomotor activity, combined with *in vivo* and *in vitro* pharmacokinetic studies were used to the answer the following specific research questions:

1. Does ethanol (1 or 2.5 g/kg) increase the E_{max} or result a prolonged locomotor activity in mice after administration of heroin (2.5 or 15 µmol/kg)?

2. Does ethanol (2.5 g/kg) change the total concentration or concentration over time of heroin, 6MAM, morphine or M3G in blood and brain from mice after administration of heroin (15 µmol/kg)?

2. Materials and methods

2.1 Animals

Male C57BL/6J- Bomtac mice (Bomholt, Denmark) and male C57BL/6J Ola Hsd mice (Harlan, Netherlands), 7-8 weeks old, 18-26 g, were used in the experiments. After arrival, the mice were placed 5-8 per cage in the animal facilities at the Norwegian Institute of Public Health with free access to commercial mouse pellets and water. Light-dark cycle was 12:12 hours with light period from 7:00AM to 7:00PM. The air temperature was kept at 22 ± 2˚C and relative humidity ranged from 36 to 44%. All mice were fasted 15-20 hours before the *in vivo* experiments took place. The experiments were conducted during the day, between 8:00AM and 4:00PM. Daily attendance was given and the mice were habituated for at least 4 days before an experiment. The animals were sacrificed immediately after the experiments. All experiments were approved by the Norwegian Review Committee for the Use of Animal Subjects.

2.2 Chemicals

3,6-diacetylmorphine hydrochloride (heroin), mol.wt. 423.9, was delivered from Lipomed AG (Switzerland). NaCl (0.9%) was delivered from B. Braun (Germany). Sodium fluoride (NaF), sodium bicarbonate (NaHCO3) and disodium hydrogen phosphate (Na_2HPO_4) were delivered from Sigma Aldrich (Germany). Ethanol (C_2H_5OH), (1 g/ml), calcium chloride ($CaCl_2$) and potassium chloride (KCl) were delivered from Merck KGaA (Germany). Magnesium chloride ($MgCl_2$) was delivered from Alfa Aeasar (Germany) and glucose ($C_6H_{12}O_6$) was delivered from Koch-light Laboratories (England). Methanol (CH_3OH) and Acetonitrile (CH_3CN) were delivered from Labscan (Poland) and ammonium format (NH_4HCO_2) was delivered from BDH Laboratory Supplies (England). Heparin (100 IU/ml) was delivered from LEO Pharma A/S.

2.3 Methods

2.3.1 Ethanol concentration in blood

Mice were administered ethanol (2.5 g/kg) per os (p.o.). At different time points (20, 40 and 60 min) after ethanol administration, mice were anesthetized with CO_2 and blood samples

were collected by heart puncture. About 500 µl blood per mouse was collected using a syringe containing 80 µL heparin (100 IU/ml) and transferred to Hitachi tubes. Ethanol concentrations in the blood samples were analyzed by the Department of Toxicological Analysis using head space gas chromatography (Kristoffersen et al. 2006).

2.3.2 Locomotor activity

The locomotor activity test was performed as described in (Andersen et al. 2009) with some modifications. Locomotor activity was tested in a VersaMax optical animal activity monitoring system (AccuScan Instruments, Inc., Colombus, OH, USA). The activity chambers were divided into four separate quadrants (20 cm x 20 cm) and two mice were tested simultaneously in each chamber, using nonadjacent quadrants. Locomotor activity was registered with a grid of infrared beams and total distance travelled (cm/5min) was chosen as an expression for locomotor activity. Before injections, each mouse (n = 58) was habituated in its respective activity chamber for 90 min. After habituation, the mice were removed and administered 0.9% saline or ethanol (1 g/kg or 2.5 g/kg, p.o.), and placed back in its home cage. 30 minutes after the first administration, the mice were given 0.9% saline or heroin (2.5 µmol/kg or 15 µmol/kg, s.c.) and immediately placed gently back in its respective activity chamber. Drugs were administered in one room, while the locomotor activity test took place in another. Locomotor activity was measured for 5 hours.

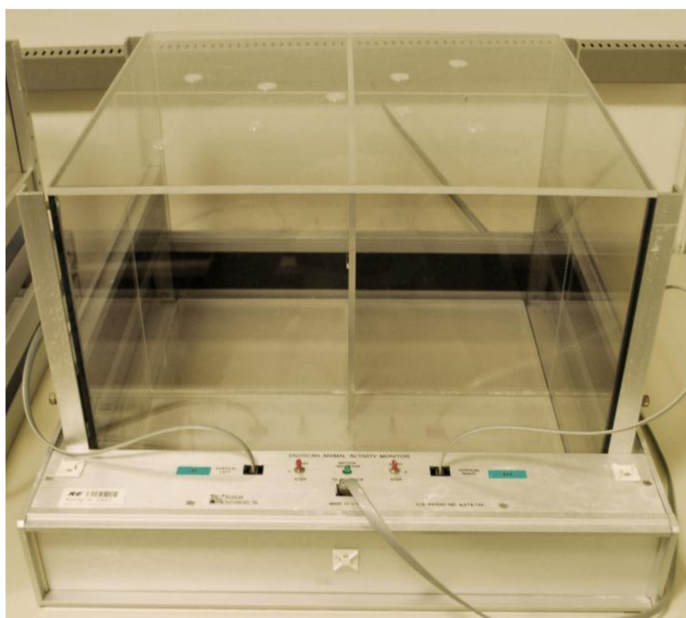


Figure 2-1 Locomotor activity chamber

2.3.3 *In vivo* kinetics

The *in vivo* pharmacokinetic studies were performed as described in (Andersen et al. 2009) with some modifications. Mice (n = 100) were administered saline or ethanol (2.5 g/kg, p.o.) at t = 0, and placed back in their home cage. At t = 30 min, mice were administered heroin (15 µmol/kg, s.c.). At different time points (1, 3, 5, 10, 15, 30, 60 or 120 min) after the heroin injection, the mice were anesthetized with CO_2. About 500 µl blood per mouse was collected by heart puncture with a syringe containing sodium fluoride (final concentration of 4 mg/ml) dissolved in heparin (100 IU/ml). Sodium fluoride was used to inhibit esterase activity thereby preventing metabolism of heroin (Brogan et al. 1992). 100 µl blood was placed in micro centrifuge tubes, diluted 1:1 with ice-cold 5 mM ammonium format buffer, pH 3.1, and quickly frozen in liquid N_2. Immediately after blood sampling, the mice were sacrificed and the brain, except the cerebellum, was quickly removed. The brains were blotted and homogenized (1:2) in 5 mM ice-cold ammonium format buffer, pH 3.1. Brain homogenates were immediately frozen in liquid N_2. Ice-cold ammonium format buffer, pH 3.1, was used because an earlier study (Barrett et al. 1992) has shown that heroin is most stabile at low temperature and low pH. Blood- and brain samples were stored at -80˚C until analyzed. Heroin is rapidly metabolized in biological tissues (Goldberger et al. 1994), especially in blood (Boerner et al. 1975). Blood samples were therefore analyzed the same day, or the day after an experiment, and brain samples within 3 days after an experiment took place.

In all the *in vivo* experiments, ethanol/saline was administered per os (p.o.) in a total volume of 0.02 ml/g, while heroin/saline was injected subcutaneous (s.c.) in a total volume 0.01 ml/g.

2.3.4 *In vitro* kinetics

The *in vitro* pharmacokinetic studies were performed as described in (Boix et al. 2011) with some modifications. Mice (n = 40) were anesthetized with CO_2. About 500 µl blood per animal was collected using heart puncture with a syringe containing heparin (100 IU/ml) to prevent coagulation of the blood. The blood was transferred directly to a tube placed on ice. Right after blood sampling, mice were sacrificed and brains were removed, blotted in ice-cold 0.9% saline and transferred to a plastic tube placed on ice. Brains were added Tris-Krebs-buffer (1:2) (10 mM Tris, 140 mM NaCl, 5 mM KCl, 5mM $NaHCO_3$, 1 mM $MgCl_2$, 1.2 mM Na_2HPO_4, 10 mM glucose, 1.2 mM $CaCl_2$, pH 7.4) and homogenized with a Teflon/glass

homogenizator. Blood and brain homogenate (100 µl) were transferred to separate plastic-tubes kept in a water bath, at 37˚C. 10 µl 0.9% saline (in blood samples) or 10 µl Tris-Krebs-buffer (in brain homogenate) or 10 µl ethanol (final concentration 2‰) was added to each plastic tube and mixed well. After 30 sec, 12 µl heroin solution (final concentration 0.4 µM) was added to each tube. The tubes were mixed and placed back in the water bath. At given time points (3, 5, 10, 20, 30, 60, 120, 300 or 600 seconds), 78 µl ice-cold 5 mM ammonium format buffer with sodium fluoride (final concentration 2 mg/ml), pH 4, was added to stop the enzymatic reaction. The tubes were immediately mixed and quickly frozen in liquid N_2. Blood and brain samples were placed in a deep-freezer at -80˚C. Blood samples were analyzed the next day, while brain samples were analyzed 2 days after the experiment took place.

In all experiments, heroin and ethanol were dissolved in 0.9% saline the day before the experiment or the same day as the experiment took place.

2.3.5 Preparation of blood and brain samples

Preparation of blood and brain samples was performed as described in (Karinen et al. 2009) with some modifications. 100 µl standards (ranging from 0.005-5 µM for heroin and 0.005-10 µM for 6MAM, morphine and M3G) and controls were added to separate plastic tubes, and placed on ice. 100 µl human blood (preparation of blood) or 100 µl brain tissue homogenate from mice (preparation of brain samples) were added to all standards and controls. Blood and brain samples from the experiments were gently thawed on ice. 50 µl internal standard mixture was added to all tubes and mixed on a vortex mixer. Thereafter, 500 µl ice-cold acetonitrile/methanol (85:15) was added and mixed well for 10-15 sec. All samples were capped and placed in the deep-freezer (-20˚C) for minimum 10 min before centrifuged (4750 rpm, 4˚C, 10 min) (Turbovap, Zymark Corporation, Hopkinton, MA, USA). The organic phase was transferred to 5 ml glass tubes and evaporated to dryness in a water bath under a stream of nitrogen (7 bar, 40˚C, 60 min). 100 µl cold 5 mM ammonium format buffer with acetonitrile (97:3), pH 3.1, was added to all tubes before they were centrifuged (4750 rpm, 4˚C, 10 min) and transferred to 0.3 ml auto sampler vials.

2.3.6 LC-MS/MS Analysis

The presence of heroin and the heroin metabolites; 6MAM, morphine and M3G, in the blood- and brain samples from mice were analyzed using liquid chromatography tandem mass spectrometry (LC-MS/MS). This method combines the separation power of HPLC and the detection power of MS, and is selective and sensitive analysis method well established at the Division of Forensic Toxicology and Drug Abuse at the Norwegian Institute of Public Health (Karinen et al. 2009).

Reversed phase chromatography with an acidic mobile phase combined with positive electrospray (ESI+) detection was used. For more detailed information on the LC-MS/MS conditions, see chapter 8, Appendix.

2.4 Statistics

Data were analyzed using SPSS Statistics 17.0; (SPSS Inc., Chicago, IL. USA). Data are presented as mean ± SEM unless otherwise stated.

The locomotor activity data and data from the *in vitro* tests were analyzed using a General Linear Model (GLM) with repeated measures (Field 2009). The high (15 µmol/kg) and low (2.5 µmol/kg) doses of heroin were analyzed separately, with treatment (1 or 2.5 g/kg ethanol vs. saline) as fixed factor and time as repeated factor. Due to > 3 variables, sphericity was met (Field 2009). Sheffè test was used as a post-hoc test (Field 2009).

In the *in vivo* pharmacokinetic study each time point represents the mean concentration from 4-9 mice. The intention was to use a two-way ANOVA test to analyse the data with treatment and time as factors. Due to clear trends in the residuals, also following log-transformation, the treatments were compared separately for each time point using t-test (Field 2009).

P-values ≤ 0.05 were considered statistical significant.

3. Results

3.1 Ethanol concentration in blood

The blood ethanol concentration, presented in figure 3-1, was stable at approximately 2‰ 20 to 60 minutes after administration of ethanol (2.5 g/kg, p.o.). The particular time points (20, 40 and 60 min) were chosen to assure that the presence of ethanol was stabile under the subsequent *in vivo* and *in vitro* experiments.

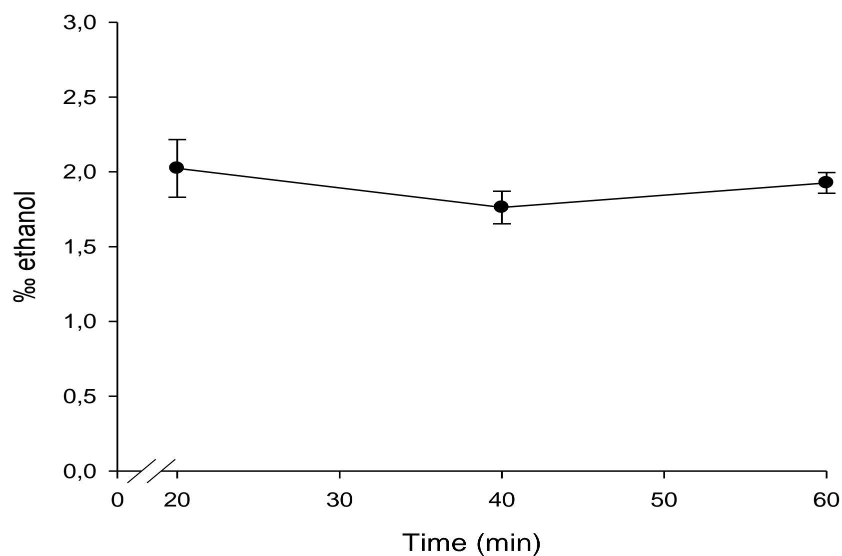
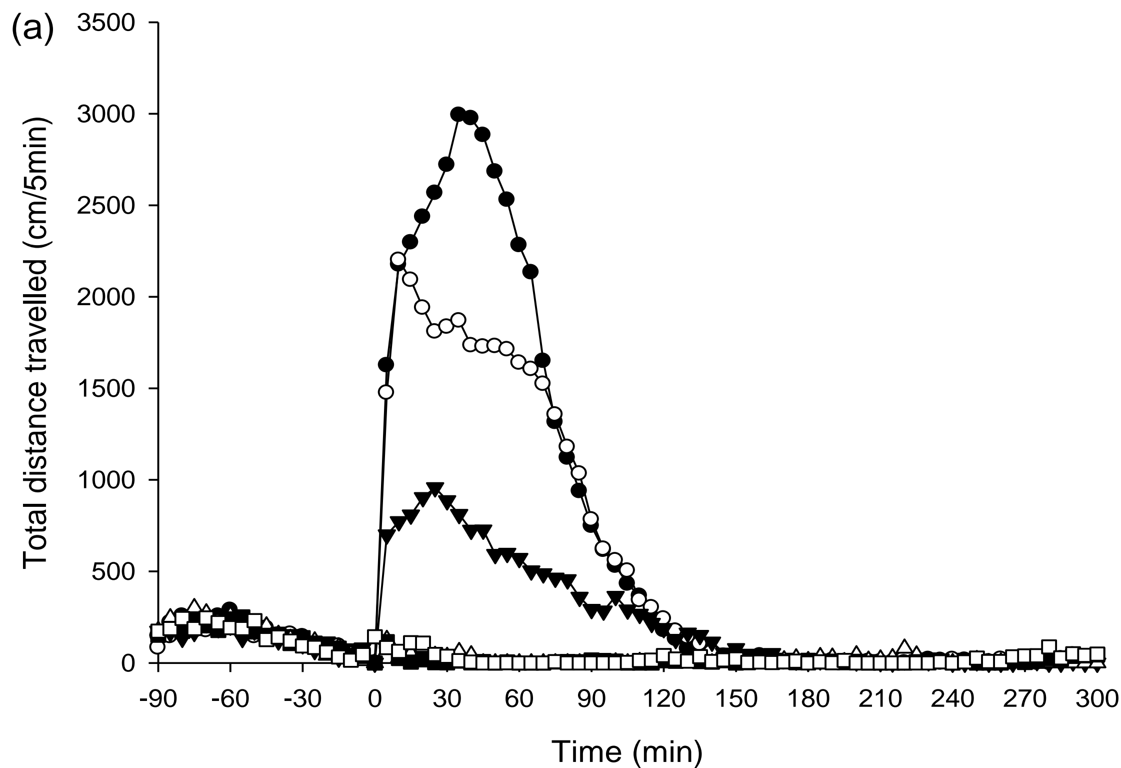


Figure 3-1. Ethanol concentration measured in mice blood 20, 40 and 60 minutes after administration (2.5 g/kg, p.o.). Values are presented as mean ± SEM, n = 2-3.

3.2 Locomotor activity

The results from the locomotor activity test are presented in figure 3-2. SEM bars are omitted for clarity. Injection of saline (0.9%) did not induce stimulation of locomotor activity in the mice.

The maximum distance travelled (E_{max}), and the duration time of the locomotor activity were related to the heroin dose. The inhibiting effect of ethanol on the locomotor activity was also dose dependent. Administration of ethanol in combination with the high (a) and the low (b) dose of heroin gave significant lower locomotor activity than mice administered saline and heroin, respectively [$F(2,15) = 35.729$; $p < 0.05$] and [$F(2,13) = 30.884$; $p < 0.05$]. For the high dose of heroin (a), the post-hoc tests showed that both doses of ethanol reduced locomotor activity significantly ($p < 0.05$), while for the low dose of heroin (b), only the high dose of ethanol gave a significant reduction in locomotor activity ($p < 0.05$).
Neither the high nor the low dose of ethanol changed the duration time of the total distance travelled. Independent of the pre-treatment (high or low dose of ethanol or saline), the locomotor activity was approximately 150 minutes for the high dose of heroin (a) and approximately 45 minutes for the low dose of heroin (b).



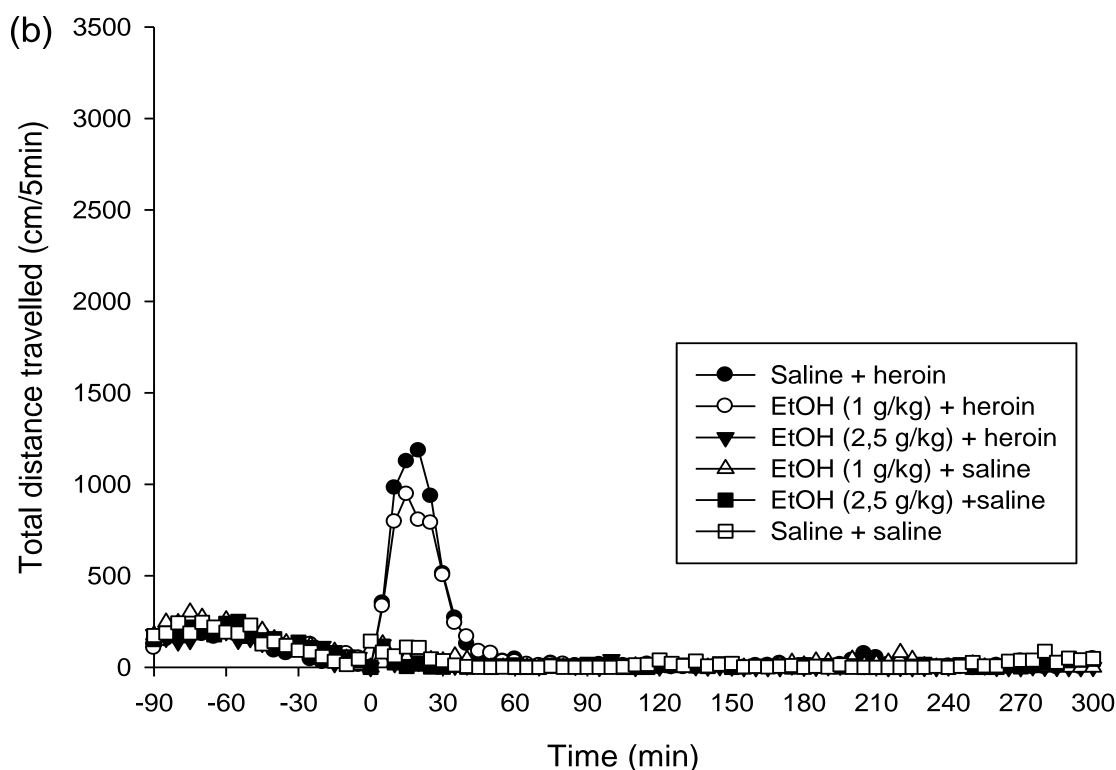


Figure 3-2. Locomotor activity expressed as total distance travelled (cm) per 5 minutes, after administration (s.c.) of 15 µmol/kg (a), or 2.5 µmol/kg (b) heroin to mice pre-treated (p.o.) with saline (0.9%) or ethanol (1 or 2.5 g/kg). Values are presented as mean, n = 4-7. SEM bars are omitted for clarity.

3.3 *In vivo* kinetics

The metabolism of heroin in blood and brain in mice pre-treated with saline (0.9%) or ethanol (2.5 g/kg) are presented in figure 3-3. Significant differences are marked with stars.

At 3, 10 and 15 minutes a significant difference was observed between the ethanol and saline group in the concentration of heroin in blood, respectively [$F(1,13) = 9.234$; $p < 0.05$], [$F(1,14) = 9.358$; $p < 0.05$], and [$F(1,12) = 31.015$; $p < 0.05$]. In brain, there was a significant difference between ethanol and saline at 5 and 10 minutes, respectively [$F(1,13) = 5.809$; $p < 0.05$] and [$F(1.14) = 5.820$; $p < 0.05$]. No significant differences were observed between the ethanol and saline group in the concentration of 6MAM or morphine in blood or brain ($p > 0.05$ for each time point). At 10 minutes there was a significant difference in M3G in blood between the ethanol and saline group [$F(1,14) = 19.755$; $p < 0.05$]. No differences were seen in M3G in brain ($p > 0.05$ for each time point).

The curve profiles for heroin, 6MAM and morphine in brain reflected the curve profiles seen in blood. The concentration of heroin was low in both blood and brain and the rapid disappearance of heroin was accompanied by a rapid increase in the 6MAM concentration. 6MAM was present at high concentrations in both blood and brain, reached maximum concentration (C_{max}) around 20 minutes and disappeared within 120 minutes. The metabolism of 6MAM to morphine was slower. Morphine was present at lower concentrations than 6MAM in both blood and brain and disappeared slower than heroin and 6MAM. M3G was abundant in blood, while present at very low concentration in brain, which indicates a slow uptake of M3G to the brain. M6G was not detected in blood or brain homogenate.

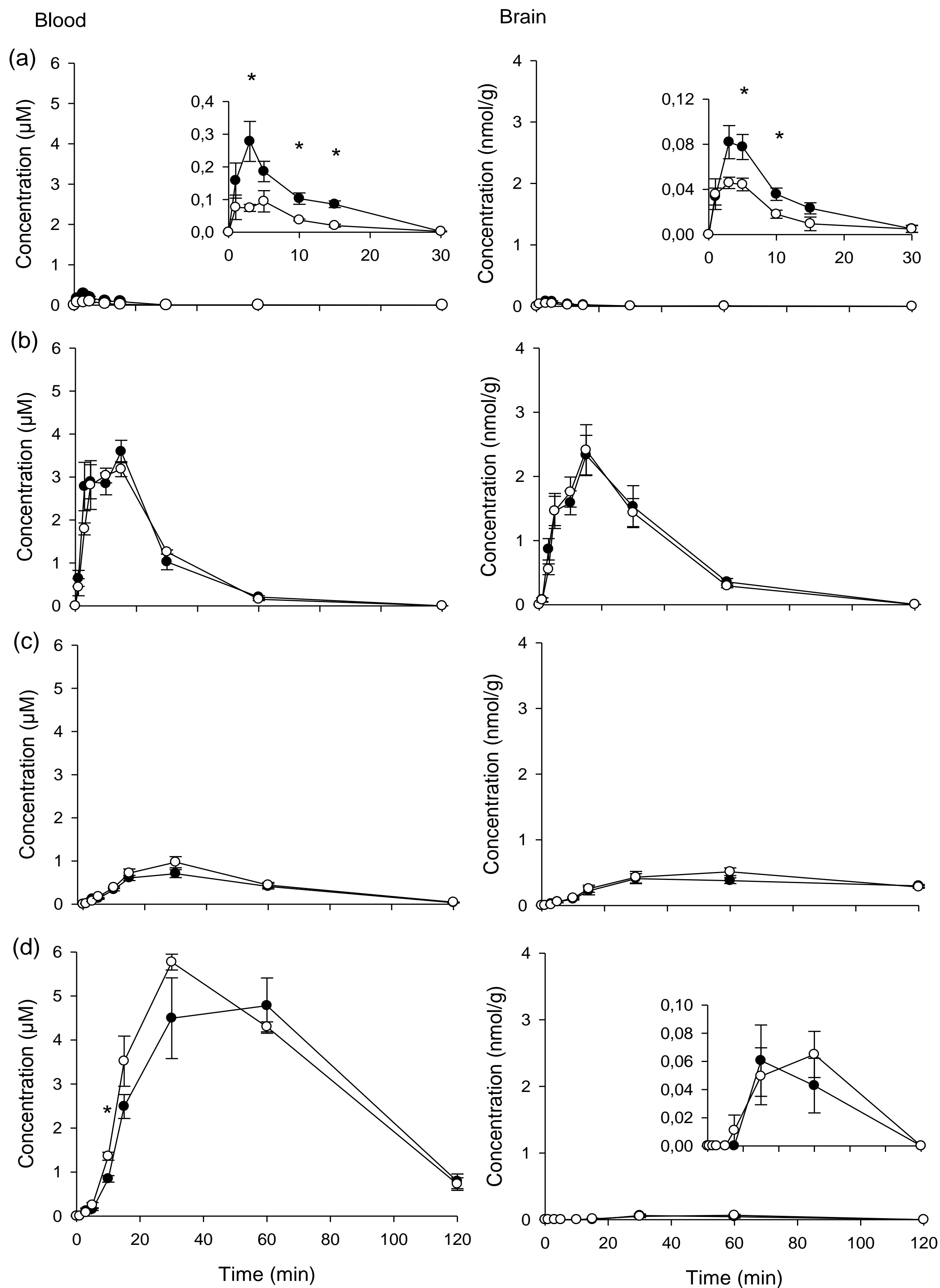


Figure 3-3. In vivo concentration curves of heroin (a), 6MAM (b), morphine (c) and M3G (d) in blood (µM) and brain (nmol/g) as function of time (min) after administration of heroin (15 µmol/kg, s.c.) to mice pre-treated with saline (0.9%, p.o.) or ethanol (2.5 g/kg, p.o.). ● ethanol + heroin, ○ saline + heroin. Values are presented as mean ± SEM, n = 4-9. Significant differences ($p < 0.05$) between saline and ethanol are marked with stars in the figure.

3.4 *In vitro* kinetics

The metabolism in blood and brain homogenate from mice after addition of heroin to samples with saline or ethanol is presented in figure 3-4.

The concentration of heroin (a) in blood was significant higher when ethanol was present [$F(1,6) = 8.618$; $p < 0.05$], while there was no significant difference in the brain homogenate [$F(1,4) = 0.19$; $p > 0.05$]. The concentration of 6MAM (b) in the presence of ethanol were not significantly different from the saline group either in blood [$F(1,6) = 0.189$; $p > 0.05$] or in brain homogenate [$F(1,4) = 0.914$; $p > 0.05$].

Heroin disappeared quickly in blood accompanied with a fast appearance of 6MAM. In blood, 6MAM reached C_{max} after about 120 seconds and was stabile up to 600 seconds. The metabolism of heroin in brain homogenate was slower. In brain, heroin was still present at 600 seconds, with a corresponding slower increase in 6MAM concentration. At 600 seconds, the concentration of 6MAM in the brain was still ascending. Morphine, which is not shown, appeared at very low concentrations after approximately 3 minutes in blood and after approximately 5 minutes in brain homogenate. M3G and M6G were not detected in blood or brain homogenate.

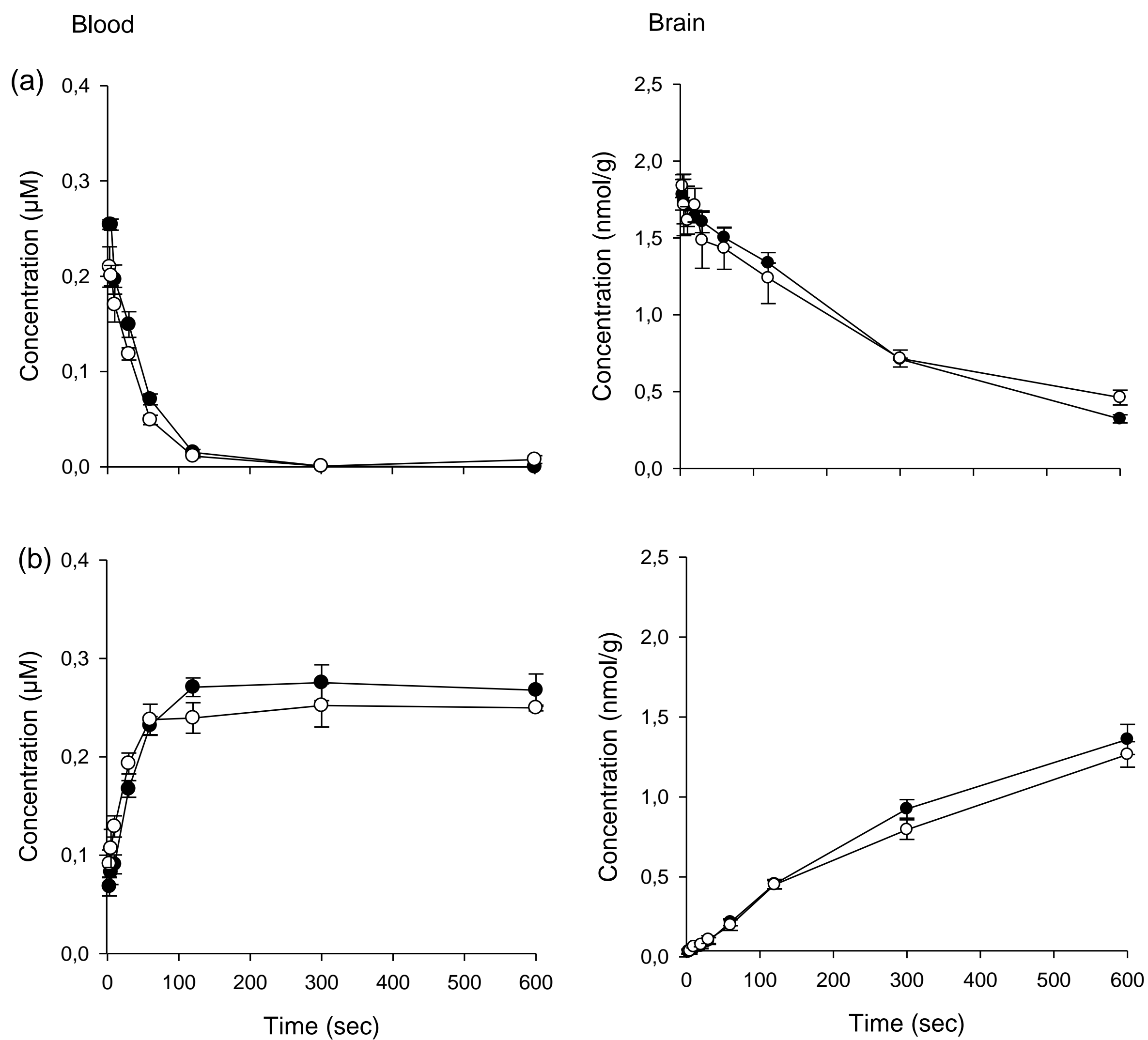


Figure 3-4. In vitro concentration curves of heroin (a) and 6MAM (b) in blood and brain homogenate from mice as function of time (sec) after addition of heroin (0.4 µM) to samples with saline (blood) or Tris-Krebs buffer (brain homogenate) or ethanol (2‰).
● ethanol + heroin, ○ saline + heroin. Values are presented as mean ± SEM, n = 3-4.

4. Discussion

4.1 Locomotor activity

Our results show, in accordance with earlier studies (Schlussman et al. 2008; Andersen et al. 2009), that the control group (given only saline) had almost no locomotor activity and that heroin produced an immediate dose-dependent increase in locomotor activity. Heroin in combination with ethanol gave a reduction in locomotor activity, the highest dose of ethanol giving the greatest reduction. Previously, it has been shown that ethanol in combination with heroin will reduce the locomotor activity compared to mice given only heroin (Castellano et al. 1976).

By visual observation, mice administered the high dose of ethanol (2.5 g/kg) were more sedated than mice administered saline or the low dose of ethanol (1 g/kg), but because of the low activity in mice administered saline, the sedating effects of ethanol was hard to show in the model. Earlier studies have shown that ethanol may affect the locomotor activity differently in different strains of mice (Tambour et al. 2006). Studies have shown that ethanol have a monophasic depressant effect in C57BL/6-mice (Frye and Breese 1981; Phillips and Dudek 1991; Tritto and Dudek 1994), but biphasic effects have been found (Lopez et al. 2003). Heroin has also been shown to have a biphasic effect in C57BL6J-mice, resulting in stimulating effects at lower doses and stultifying effects at very higher doses (Andersen et al. 2009).

Ethanol in combination with the high dose of heroin gave highly intoxicated mice, which resulted in incoherent running and in total reduced locomotor activity. If ethanol had inhibited the metabolism of heroin, with a following increase in the concentration of active metabolites, we would have expected an increase in E_{max} and/or prolonged locomotor activity, which were not seen. Interference between the sedative effects of ethanol and the high dose of heroin, may therefore explain the reduced locomotor activity.

The low dose of ethanol had no effect on the locomotor activity after administration of the low dose of heroin. The high dose of ethanol in combination with the low dose of heroin resulted in no locomotor activity. This may be explained by the sedative effects of ethanol exceeding the stimulating effects of heroin. However, in mice administered the high dose of

heroin, the sedative effects of ethanol was not able to inhibit the locomotor activity completely, probably because the stimulating effects of the heroin were too strong.

4.2 *In vivo* kinetics

The results from the *in vivo* pharmacokinetic study show that in the presence of ethanol there was an elevated concentration of heroin at some time points, both in blood and brain. However, heroin works only as a pro-drug, not mediating the biological response (Way et al. 1960; Umans and Inturrisi 1981; Andersen et al. 2009). The elevated concentration of heroin did not affect the concentration of 6MAM, which is assumed to be the most important heroin metabolite concerning acute effects. There were no significant differences in the 6MAM or morphine concentration in blood or brain between the ethanol and saline group. Due to the short half-life of heroin (Way et al. 1960), heroin concentration in blood and brain disappeared rapidly, and were not detected after approximately 30 minutes. In accordance with previous studies (Salmon et al. 1999; Boix et al. 2011), the deacetylation of 6MAM in blood and brain was slower. This resulted in an accumulation of 6MAM with a following low morphine concentration. The quick disappearance and the low C_{max} of heroin, compared with the accumulation and the high C_{max} of 6MAM, might explain why the elevated heroin concentration in the presence of ethanol was not reflected in the 6MAM concentration. It might also be that the presence of ethanol resulted in an increased distribution of heroin to other tissues. At one time point (10 minutes), the concentration of M3G in blood was lower in the presence of ethanol compared to saline. Since M3G is considered to be an inactive metabolite (Pasternak et al. 1987), and the difference was seen only at one point in the time curve, this difference is of low biological relevance.

The biological response of a heroin is mediated through the presence of active metabolites in the brain (Inturrisi et al. 1983). No differences were seen in the brain concentrations of 6MAM or morphine between the ethanol group and the saline group, which indicates that ethanol, has minimal effect on the biological response of heroin. The elevated concentration of heroin when ethanol was present might be due to other factors than a change in the metabolism. Ethanol might affect the absorption, distribution and bioavailability of other drugs (Linnoila et al. 1979). Heroin was administered subcutaneously and ethanol might have accelerated the uptake of heroin into the circulation system and the brain.

4.3 *In vitro* kinetics

The results from the *in vitro* kinetic study confirmed the main finding from the *in vivo* kinetic study. In blood, there was a significantly higher concentration of heroin in the presence of ethanol compared to saline, but no significant difference in 6MAM concentration. The rapid disappearance of heroin in blood was followed by a rapid increase in 6MAM concentration. This is in accordance with earlier studies (Nakamura et al. 1975; Boix et al. 2011). 6MAM concentration in blood was stable from 2-10 minutes, indicating a slow deacetylation of 6MAM *in vitro*.

No differences were seen between the ethanol and saline group in the concentration of heroin or 6MAM in the brain homogenate. In accordance with previous studies (Boix et al. 2011), the metabolism of heroin to 6MAM in brain homogenate appeared to be slower than in blood. This was also reflected in the subsequent slow increase in 6MAM concentration, which was still ascending at 600 seconds. These findings may indicate that there is lower enzyme activity in the brain compared to blood.

4.4 Locomotor activity and heroin kinetics – do the results correspond?

In C57BL/6J-mice, the immediate rise in locomotor activity after heroin administration is mediated by 6MAM, while morphine is of importance later in the response curve (Andersen et al. 2009). Administration of ethanol in combination with heroin resulted in reduced locomotor activity compared to mice administered saline and heroin. Our results from the pharmacokinetic studies do not support that the reduced locomotor activity seen in mice pre-treated with ethanol was due to a change in the concentration of 6MAM or morphine in the brain. With our results from the kinetic studies, there should theoretically be no change in the locomotor activity in the presence of ethanol. The reduction in locomotor activity in mice administered ethanol and heroin may reflect the sedative properties of ethanol suppressing the stimulating effect of heroin. The results from the locomotor activity test are difficult to interpret when testing two drugs with opposite effects.

4.5 Agreement with previous findings?

Due to the inverse correlation between a high ethanol concentration and a low morphine concentration detected in blood post-mortem in heroin-related deaths, it has been suggested that ethanol might interact with the metabolism of heroin (Polettini et al. 1999; Rook et al. 2006). Polettini (1999) found a correlation between ethanol and 6MAM/morphine concentrations in plasma post-mortem, and indicated that the hydrolysis of 6MAM to morphine had been delayed by ethanol use. Ethanol has been shown to inhibit the metabolism of cocaine *in vitro* (Roberts et al. 1993; Farre et al. 1997) and *in vivo* (Dean et al. 1992) and studies have shown that heroin and cocaine metabolism in humans are catalyzed by the same enzymes (Kamendulis et al. 1996; Satoh et al. 2002). In the light of these findings, ethanol might also inhibit the metabolism of heroin. It has been suggested that there might be an interaction between ethanol and heroin at the degree of glucuronidation of morphine (Fugelstad et al. 2003). One study, investigating the chronic effects of ethanol on the morphine metabolism, showed that ethanol increased the rate of morphine glucuronidation (Narayan et al. 1991). If this is true also after heroin administration, this might explain the lower blood morphine concentration in the presence of ethanol detected post-mortem in epidemiological studies. However, another study investigating the acute effects of ethanol showed that ethanol inhibited the morphine metabolism in a dose-dependent manner *in vitro* (Bodd et al. 1986; Aasmundstad et al. 1996).

Our results do not indicate that ethanol inhibited the metabolism of heroin. Ethanol did not affect the concentration or half-life of 6MAM or morphine in blood or brain, which indicates that ethanol, had minimal effect on the biological response of heroin. However, an elevated concentration of heroin in blood and brain was detected in the presence of ethanol. This could be explained by ethanol`s ability to increase the absorption and bioavailability of other drugs (Linnoila et al. 1979).

Epidemiological studies have suggested alternative hypotheses to explain the elevated risk of overdose when combining heroin and ethanol. Given that both ethanol and benzodiazepines often are detected post-mortem in heroin related deaths, and both drugs are central nervous system depressants, it is likely that there might be an additive effect on the respiratory system when these drugs are taken simultaneously with heroin (Darke and Hall 2003). Ethanol may augment the respiratory depressant effect of morphine (Darke et al. 1997; White and Irvine

1999). Hence, in the presence of ethanol, a lower heroin dose may be responsible for a fatal overdose (Darke et al. 1997; Hickman et al. 2007), which again could explain the lower blood morphine concentration detected post-mortem in epidemiological studies.

Ruttenber and Luke (1984) indicated that ethanol consumption was more widespread among sporadic heroin users, which may have a lower tolerance to heroin compared to heroin addicts. It is also found that users who are decreasing their heroin intake, thereby reducing their tolerance, may drink more (White and Irvine 1999). Hence, ethanol administration in combination with a reduction in heroin tolerance, might explain the increased risk of overdose. Ethanol intake may also impair the users judgment of their tolerance to heroin (Hickman et al. 2007).

In epidemiological studies, 6MAM, but most often morphine, is used as indicator of heroin intake. The literature of the morphine concentrations detected in heroin related deaths, show that different analytic methods are used, as well as reporting of both free morphine (unconjugated) and total morphine (Fugelstad et al. 2003). Considering the morphine concentration detected in blood post-mortem, the time passing between the death and the sampling, is of significant importance. Post-mortem formation, degradation and redistribution of morphine may occur, and morphine may be formed from morphine glucuronides (Fugelstad et al. 2003). This may result in great differences in morphine concentrations, which make epidemiological studies difficult to interpret.

Few experimental studies have investigated the effects of ethanol on the metabolism of heroin. The fact that the metabolism of heroin in biological tissue is rapid (Inturrisi et al. 1983) makes heroin a drug difficult to work with. Moreover, heroin demands specific conditions, such as low pH and low temperature, to be stabile (Barrett et al. 1992). Heroin is often detected in very low concentration (if detected at all), hence sensitive methods are needed to analyze heroin. This may explain part of the reason why there are relatively few experimental studies done on heroin.

4.6 Mice models in drug abuse research

Compared to epidemiological studies, experimental studies are easier to control, and therefore of value when investigating specific biological mechanisms. However, due to species differences, extrapolating from mice to humans should be done with care. As mentioned earlier, body weight and size, as well as drug metabolizing enzymes might differ between species. Compared to humans, mice deliver and excrete drugs more rapidly (Lin et al 1995).

The locomotor activity test is a well-known and well established model for investigating the acute stimulating effects of different drugs.

Heroin administration, with a following increase in dopamine release in nucleus accumbens, induces locomotor activity in some mice strains (Dichiara and Imperato 1988). In C57BL/6-mice, the immediate rise in locomotor activity after heroin administration is mediated by 6MAM and later morphine (Andersen et al. 2009). Studies have shown that ethanol administration to C57BL/6-mice results in depressant sedative effects (Frye and Breese 1981; Phillips and Dudek 1991; Tritto and Dudek 1994; Tambour et al. 2006). Our results showed that ethanol and heroin in combination resulted in total reduced locomotor activity compared to mice administered saline and heroin. Due to the sedative effects of ethanol and the stimulating effects of heroin, our results are hard to interpret. Therefore, investigating the effect of ethanol on the metabolism of heroin might be less suitable in this model. This model is better fitted when investigating the acute effects of drugs separately.

The methods used for investigating and detecting heroin and heroin metabolites both *in vivo* and *in vitro*, was based on previous studies (Andersen et al. 2009; Karinen et al. 2009; Boix et al. 2011). This is a good method for investigating the potential modulating effect of ethanol on the metabolism of heroin in blood and brain in mice.

Heroin is metabolized to 6MAM and further to morphine in both humans and mice (Way et al. 1960; Rook et al. 2006). In humans, morphine is conjugated to M3G and M6G, while in mice M6G is not detected (Handal et al. 2002). However, the enzymes involved in the metabolic pathway of heroin in mice and humans are not well described. Nonetheless, studies have shown that erythrocyte AChE and serum BuChE (Lockridge et al. 1980; Salmon et al. 1999), as well as hCE-1 and hCE-2 (Maurer et al. 2006) are involved in the metabolism of

heroin to 6MAM in humans. Species differences in esterase activity have been observed between mice and humans (Berry et al. 2009). Both humans and mice have butyl cholinesterases in plasma (Berry et al. 2009), but mice have, in contrast to humans, high levels of carboxylesterases in blood (Li et al. 2005). Due to insufficient knowledge on the enzymes involved in the metabolism of heroin in mice and humans, more research is needed, and precautions should be taken, when extrapolating studies in mice to humans.

5. Conclusions

Ethanol does not seem to have a modulating effect on the metabolism of heroin in blood or brain from mice. There was no change in the blood or brain concentration of active heroin metabolites (6MAM and morphine) in the presence of ethanol either *in vivo* or *in vitro,* even though an elevated concentration of heroin was seen in the presence of ethanol. The pharmacokinetic results were in agreement with the findings in the locomotor activity study. Ethanol did not increase E_{max} or result in a prolonged locomotor activity. The sedative effects of ethanol might have affected the locomotor activity by reducing the stimulating effect of heroin, resulting in a total reduction in locomotor activity.

In the light of our findings in mice, the increased risk of overdose due to simultaneously intake of ethanol and heroin does not seem to be due to a modulation in heroin metabolism with a following increase in active heroin metabolites.

6. Future studies

Few experimental studies have investigated the interaction between ethanol and heroin, and more studies in this field are needed to increase the insufficient knowledge.

- The *in vitro* kinetic study of heroin with and without ethanol present in mice blood could also be conducted in human blood to look at similarities and differences in the metabolism and enzymes between experimental animals and humans.

- There is a need to investigate the effects of long-term and sporadic administration of heroin, in combination with acute or chronic ethanol administration. This might give more knowledge concerning heroin tolerance.

- Investigation of the respiratory depressant effects of ethanol and morphine, would give us more knowledge of the potential additive effect of ethanol and morphine.

- It would be of interest to investigate the effects of ethanol on the uptake and excretion of heroin since our *in vivo* kinetic results indicate an elevated concentration of heroin in the presence of ethanol.

- Finally, there is a need to investigate further the effects of ethanol on the heroin metabolism. More studies are needed in mice as well as in other laboratory animals. Due to the different responses in different strains of mice, similar experiments could be done in other mice strains.

7. References

Aasmundstad, T. A., Lillekjendlie, B. and Morland, J. (1996). Ethanol interference with morphine metabolism in isolated guinea pig hepatocytes. *Pharmacol Toxicol*. 79, 114-119.

Andersen, J. M., Ripel, A., Boix, F., Normann, P. T. and Morland, J. (2009). Increased locomotor activity induced by heroin in mice: pharmacokinetic demonstration of heroin acting as a prodrug for the mediator 6-monoacetylmorphine in vivo. *J Pharmacol Exp Ther*. 331, 153-161.

Barrett, D. A., Dyssegaard, A. L. and Shaw, P. N. (1992). The effect of temperature and pH on the deacetylation of diamorphine in aqueous solution and in human plasma. *J Pharm Pharmacol*. 44, 606-608.

Berg, M. M. (2008). *Skremt av all heroinen.* TV2 Nettavisen. TV2 AS, Bergen. http://www.tv2nyhetene.no/skremt-av-all-heroinen-2441667.html (downloaded 12.04.2011).

Berry, L. M., Wollenberg, L. and Zhao, Z. (2009). Esterase activities in the blood, liver and intestine of several preclinical species and humans. *Drug Metab Lett*. 3, 70-77.

Bodd, E., Gadeholt, G., Christensson, P. I. and Morland, J. (1986). Mechanisms behind the inhibitory effect of ethanol on the conjugation of morphine in rat hepatocytes. *J Pharmacol Exp Ther*. 239, 887-890.

Boerner, U., Abbott, S. and Roe, R. L. (1975). Metabolism of morphine and heroin in man. *Drug Metab Rev*. 4, 39-73.

Boix, F., Andersen, J. M. and Morland, J. (2011). Pharmacokinetic modeling of subcutaneous heroin and its metabolites in blood and brain of mice. *Addict Biol*. (Epub 2011/04/13).

Brogan, W. C., III, Kemp, P. M., Bost, R. O., Glamann, D. B., Lange, R. A. and Hills, L. D. (1992). Collection and handling of clinical blood samples to assure the accurate measurement of cocaine concentration. *J Anal Toxicol*. 16, 152-154.

Castellano, C., Filibeck, U. and Oliverio, A. (1976). Effects of Heroin, Alone or in Combination with Other Drugs, on Locomotor Activity in 2 Inbred Strains of Mice. *Psychopharmacology*. 49, 29-31.

Christrup, L. L. (1997). Morphine metabolites. *Acta Anaesth Scand*. 41, 116-122.

Darke, S. and Hall, W. (1995). Levels and correlates of polydrug use among heroin users and regular amphetamine users. *Drug Alcohol Depend*. 39, 231-235.

Darke, S. and Hall, W. (2003). Heroin overdose: Research and evidence-based intervention. *J Urban Health*. 80, 189-200.

Darke, S., Ross, J., Zador, D. and Sunjic, S. (2000). Heroin-related deaths in New South Wales, Australia, 1992-1996. *Drug Alcohol Depend*. 60, 141-150.

Darke, S., Sunjic, S., Zador, D. and Prolov, T. (1997). A comparison of blood toxicology of heroin-related deaths and current heroin users in Sydney, Australia. *Drug Alcohol Depend*. 47, 45-53.

Dean, R. A., Harper, E. T., Dumaual, N., Stoeckel, D. A. and Bosron, W. F. (1992). Effects of ethanol on cocaine metabolism - formation of cocaethylene and norcocaethylene. *Toxicol Appl Pharmacol*. 117, 1-8.

Dichiara, G. and Imperato, A. (1988). Drugs abused by humans preferentially increase synaptic dopamine concentrations in the mesolimbic system of freely moving rats *Proc Natl Acad Sci U. S. A*. 85, 5274-5278.

EMCDDA (2009). *Polydrug: Patterns and Responses*. Office for Official Publications of the European Communities, Luxemburg.

Farre, M., DeLaTorre, R., Gonzalez, M. L., Teran, M. T., Roset, P. N., Menoyo, E. and Cami, J. (1997). Cocaine and alcohol interactions in humans: Neuroendocrine effects and cocaethylene metabolism. *J Pharmacol Exp Ther*. 283, 164-176.

Field, A. (2009). *Discovering statistics using SPSS*. 3rd ed., London, SAGE Publication Ltd.

Fischer, B., Rehm, J., Kirst, M., Casas, M., Hall, W., Krausz, M., Metrebian, N., Reggers, J., Uchtenhagen, A., Van Den Brink, W. and Van Ree, J. M. (2002). Heroin-assisted treatment as a response to the public health problem of opiate dependence. *Eur J Pub Health*. 12, 228-234.

Frye, G. D. and Breese, G. R. (1981). An evaluation of the locomotor stimulating action of ethanol in rats and mice. *Psychopharmacology*. 75, 372-379.

Fugelstad, A., Ahlner, J., Brandt, L., Ceder, G., Eksborg, S., Rajs, J. and Beck, O. (2003). Use of morphine and 6-monoacetylmorphine in blood for the evaluation of possible risk factors for sudden death in 192 heroin users. *Addiction*. 98, 463-470.

Goldberger, B. A., Cone, E. J., Grant, T. M., Caplan, Y. H., Levine, B. S. and Smialek, J. E. (1994). Disposition of Heroin and Its Metabolites in Heroin-Related Deaths. *J Anal Toxicol*. 18, 22-28.

Handal, M., Grung, M., Skurtveit, S., Ripel, A. and Morland, J. (2002). Pharmacokinetic differences of morphine and morphine-glucuronides are reflected in locomotor activity. *Pharmacol Biochem Behav*. 73, 883-892.

Hem, A. L., Eide, D. M., Engh, E. and Smith, A. (2001). *Kompendium i forsøksdyrlære.* Norges Veterinærhøgskole.

Hickman, M., Lingford-Hughes, A., Bailey, C., Macleod, J., Nutt, D. and Hendersen, G. (2007). Does alcohol increase the risk of overdose death: the need for a translational approach. *Addiction*. 103, 1060-1062.

Holdstock, L. and de Wit, H. (1998). Individual differences in the biphasic effects of ethanol. *Alcohol Clin Exp Res*. 22, 1903-1911.

Hubner, C. B. and Kornetsky, C. (1992). Heroin, 6-acetylmorphine and morphine effects on treshold for rewarding and aversive brain-stimulation. *J Pharmacol Exp Ther*. 260, 562-567.

Inturrisi, C. E., Schultz, M., Shin, S., Umans, J. G., Angel, L. and Simon, E. J. (1983). Evidence from opiate binding-studies that heroin acts through its metabolites. *Life Sci*. 33, 773-776.

Jones, A. W., Kugelberg, F. C., Holmgren, A. and Ahlner, J. (2011). Drug poisoning deaths in Sweden show a predominance of ethanol in mono-intoxications, adverse drug-alcohol interactions and poly-drug use. *Forensic Sci Int*. 206, 43-51.

Kaiko, R. F., Wallenstein, S. L., Rogers, A. G., Grabinski, P. Y. and Houde, R. W. (1981). Analgesic and mood effects of heroin and morphine in cancer patients with post operative pain. *N Eng J Med*. 304, 1501-1505.

Kalant, H., Khanna, J. M., Lin, G. Y. and Chung, S. (1976). Ethanol - direct inducer of drug-metabolism. *Biochem Pharmacol*. 25, 337-342.

Kamendulis, L. M., Brzezinski, M. R., Pindel, E. V., Bosron, W. F. and Dean, R. A. (1996). Metabolism of cocaine and heroin is catalyzed by the same human liver carboxylesterases. *J Pharmacol Exp Ther*. 279, 713-717.

Karinen, R., Andersen, J. M., Ripel, A., Hasvold, I., Hopen, A. B., Morland, J. and Christophersen, A. S. (2009). Determination of Heroin and Its Main Metabolites in Small Sample Volumes of Whole Blood and Brain Tissue by Reversed-Phase Liquid Chromatography-Tandem Mass Spectrometry. *J Anal Toxicol*. 33, 345-350.

Kaufman, E. (1976). Abuse of multiple drugs. 1. Definition, classification, and extent of problem. *Am J Drug Alcohol Abuse*. 3, 279-292.

Kreek, M. J., Bart, G., Lilly, C., Laforge, K. S. and Nielsen, D. A. (2005). Pharmacogenetics and human molecular genetics of opiate and cocaine addictions and their treatments. *Pharmacol Rev*. 57, 1-26.

Kristoffersen, L., Stormyhr, L. E. and Smith-Kielland, A. (2006). Headspace gas chromatographic determination of ethanol: The use of factorial design to study effects of blood storage and headspace conditions on ethanol stability and acetaldehyde formation in whole blood and plasma. *Forensic Sci Int*. 161, 151-157.

Levine, B., Green, D. and Smialek, J. E. (1995). The Role of Ethanol in Heroin Deaths. *J Forensic Sci*. 40, 808-810.

Li, B., Sedlacek, M., Manoharan, I., Boopathy, R., Duysen, E. G., Masson, P. and Lockridge, O. (2005). Butyrylcholinesterase, paraoxonase, and albumin esterase, but not carboxylesterase, are present in human plasma. *Biochem Pharmacol*. 70, 1673-1684.

Lin, J. H. (1995). Species similarities and differences in pharmacokinetic. *Drug Metab Disp*. 23, 1008-1021.

Linnoila, M., Mattila, M. J. and Kitchell, B. S. (1979). Drug-interactions with alcohol. *Drugs*. 18, 299-311.

Lockridge, O., Mottershaw-Jackson, N., Eckerson, H. W. and Ladu, B. N. (1980). Hydrolysis of diacetylmorphine (heroin) by human-serum cholinesterase. *J Pharmacol Exp Ther*. 215, 1-8.

Lopez, M., Simpson, D., White, N. and Randall, C. (2003). Age- and sex-related differences in alcohol and nicotine effects in C57BL/6J mice. *Addict Biol*. 8, 419-427.

Martignoni, M., Groothuis, G. M. M. and de Kanter, R. (2006). Species differences between mouse, rat, dog, monkey and human CYP-mediated drug metabolism, inhibition and induction. *Expert Opin Drug Metab Toxicol*. 2, 875-894.

Maurer, H. H., Sauer, C. and Theobald, D. S. (2006). Toxicokinetics of drugs of abuse: Current knowledge of the isoenzymes involved in the human metabolism of tetrahydrocannabinol, cocaine, heroin, morphine, and codeine. *Ther Drug Monit*. 28, 447-453.

Nakamura, G. R. (1978). Toxicologic assessments in acute heroin fatalities. *Clin Toxicol*. 13, 75-87.

Nakamura, G. R., Thornton, J. I. and Noguchi, T. T. (1975). Kinetics of heroin deacetylation in aqueous alkaline-solution and in human-serum and whole-blood. *J Chromatogr*. 110, 81-89.

Narayan, S. S., Hayton, W. L. and Yost, G. S. (1991). Chronic ethanol-consumption causes increased glucuronidation of morphine in rabbits. *Xenobiotica*. 21, 515-524.

Pasternak, G. W., Bodnar, R. J., Clark, J. A. and Inturrisi, C. E. (1987). Morphine-6-glucuronide, a potent mu agonist. *Life Sci*. 41, 2845-2849.

Phillips, T. J. and Dudek, B. C. (1991). Locomotor-activity responses to ethanol in selectively bred long-sleep and short-sleep mice, 2 inbred mouse strains, and their F1-hybrids. *Alcohol Clin Exp Res*. 15, 255-261.

Pichini, S., Altieri, I., Pellegrini, M., Zuccaro, P. and Pacifici, R. (1999). The role of liquid chromatography-mass spectrometry in the determination of heroin and related opioids in biological fluids. *Mass Spectrom Rev*. 18, 119-130.

Polettini, A., Groppi, A. and Montagna, M. (1999). The role of alcohol abuse in the etiology of heroin-related deaths. Evidence for pharmacokinetic interactions between heroin and alcohol. *J Anal Toxicol*. 23, 570-576.

Roberts, S. M., Harbison, R. D. and James, R. C. (1993). Inhibition by ethanol of the metabolism of cocaine to benzoylecgonine and ecgonine methyl-ester in mouse and human liver. *Drug Metab Dispos*. 21, 537-541.

Rook, E. J., Huitema, A. D. R., van den Brink, W., van Ree, J. M. and Bejinen, J. H. (2006). Pharmacokinetics and Pharmacokinetic Variability of Heroin and its Metabolites: Review of the Literature. *Curr Clin Pharmacol*. 1, 109-118.

Rook, E. J., van Ree, J. M., van den Brink, W., Hillebrand, M. J. X., Huitema, A. D. R., Hendriks, V. M. and Beijnen, J. H. (2006). Pharmacokinetics and pharmacodynamics of high doses of pharmaceutically prepared heroin, by intravenous or by inhalation route in opioid-dependent patients. *Basic Clin Pharmacol Toxicol*. 98, 86-96.

Ruttenber, A. J., Kalter, H. D. and Santinga, P. (1990). The role of ethanol abuse in the etiology of heroin-related death. *J Forensic Sci*. 35, 891-900.

Ruttenber, A. J. and Luke, J. L. (1984). Heroin-related deaths - new epidemiologic insights. *Science*. 226, 14-20.

Salmon, A. Y., Goren, Z., Avissar, Y. and Soreq, H. (1999). Human erythrocyte but not brain acetylcholinesterase hydrolyses heroin to morphine. *Clin Exp Pharmacol Physiol*. 26, 596-600.

Satoh, T., Taylor, P., Bosron, W. F., Sanghani, S. P., Hosokawa, M. and La Du, B. N. (2002). Current progress on esterases: From molecular structure to function. *Drug Metab Dispos*. 30, 488-493.

Sawynok, J. (1986). The therapeutic use of heroin - a review of the pharmacological literature. *Can J Physiol Pharmacol*. 64, 1-6.

Schlussman, S. D., Zhang, Y., Hsu, N. M., Allen, J. M., Ho, A. and Kreek, M. J. (2008). Heroin-induced locomotor activity and conditioned place preference in C57BL/6J and 129P3/J mice. *Neurosci Lett*. 440, 284-288.

Sellers, E. M. and Holloway, M. R. (1978). Drug kinetics and alcohol ingestion. *Clin Pharmacokinet*. 3, 440-452.

Sneader, W. (1998). The discovery of heroin. *Lancet*. 352, 1697-1699.

Sporer, K. A. (1999). Acute heroin overdose. *Ann Intern Med*. 130, 584-590.

Tambour, S., Didone, V., Tirelli, E. and Quertemont, E. (2006). Locomotor effects of ethanol and acetaldehyde after peripheral and intraventricular injections in Swiss and C57BL/6J mice. *Behav Brain Res*. 172, 145-154.

Tritto, T. and Dudek, B. C. (1994). Differential activating effects of ethanol in C57BL/6ABG and DBA/2ABG mice. *Alcohol*. 11, 133-139.

Umans, J. G. and Inturrisi, C. E. (1981). Pharmacodynamics of subcutaneously administered diacetylmorphine, 6-acetylmorphine and morphine in mice. *J Pharmacol Exp Ther*. 218, 409-415.

UNODC (2010). *World Drug Report 2010*. United Nations Publication.

van Ree, J. M., Slangen, J. L. and de Wied, D. (1978). Intravenous self-administration of drugs in rats. *J Pharmacol Exp Ther*. 204, 547-557.

Warner-Smith, M., Darke, S., Lynskey, M. and Hall, W. (2001). Heroin overdose: causes and consequences. *Addiction*. 96, 1113-1125.

Way, E. L., Kemp, J. W., Young, J. M. and Grassetti, D. R. (1960). The pharmacologic effects of heroin in relationship to its rate of biotransformation. *J Pharmacol Exp Ther*. 129, 144-154.

Weiss, F. and Porrino, L. J. (2002). Behavioral neurobiology of alcohol addiction: Recent advances and challenges. *J Neurosci*. 22, 3332-3337.

White, J. M. and Irvine, R. J. (1999). Mechanisms of fatal opioid overdose. *Addiction*. 94, 961-972.

WHO (1998). *Opioid overdose trends, risk factors, interventions and priorities of action*. World Health Organization, Substance Abuse Department, Geneve.

8. Appendix

8.1 LC-MS/MS conditions

HPLC conditions

Water alliance 2695 HPLC Pump Conditions:

Injection volume: 10 µl

Mobile phase: methanol (A)

5 mM ammonium formate buffer, pH 3.1 (B)

Column: Xterra® MS C18 (2.1 mm x 150mm)

Flow rate: 0.2 ml/min

Column temperature: 50˚C

Run time: 16 minutes

Soft ware: Mass Lynx

Table 8-1 showing HPLC pump gradient timetable

Time (min)	A %	B %	Flow
0.00	3.0	97.0	0.200
8.00	60.0	40.0	0.200
10.00	60.0	40.0	0.200
10.00	3.0	97.0	0.300
16.00	3.0	97.0	0.300

MS/MS-conditions

MS detection was performed on a Quattro Premier XE tandem quadrupole MS. Ionization was achieved using electrospray in the positive mode (ESI+) and multiple reaction monitoring (MRM) was used for quantification. The source block temperature was 120˚C and the capillary voltage was 2 kV. The cone gas (N_2) was heated up to 400 ˚C and the flow was set to 49 L/h. The desolvation gas (N_2) was delivered at flow 1097 L/h.

Table 8-2 LC-MS/MS method

Substance	**Rt (min)**	**MRM 1 (m/z)**	**MRM 2 (m/z)**	**Cone voltage (V)**	**Collision energy (eV)**
M3G	2.7	462.0 > 286.0	462.0 > 286.0	45	30/30
Morphine	4.6	286.0 > 201.0	286.0 > 152.0	45	20/40
6MAM	8.1	328.0 > 211.0	328.0 > 165.0	45	25/40
Heroin	9.6	370.0 > 268.0	370.0 > 165.0	50	30/40
Internal standard					
M3G-d3	2.7	465.0 > 289.0		50	30
Morphine-d6	4.6	292.0 > 201.0		45	25
6MAM-d6	8.1	334.0 > 211.0		45	25
Heroin-d9	9.6	379.0 > 272.0		50	28

Table 8-3 showing the limits of detection (LOD) and limits of quantitation (LOQ)

	Heroin	**6MAM**	**Morphine**	**M3G**
LOD blood (mg/L)	0.00096	0.00033	0.00049	0.0065
LOQ Blood (mg/L)	0.0025	0.00065	0.0012	0.019
LOD brain tissue (µg/g)	0.0029	0.0010	0.0015	0.020
LOQ Brain tissue (µg/g)	0.0077	0.0022	0.0036	0.059