Identification and Modulation of Esterases Involved in the Metabolism of Heroin

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Master Thesis in Toxicology
Department of Biosciences
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Trykk: Reprosentralen, Universitetet i Oslo
Preface

This master thesis in toxicology is part of the master’s degree in biology at the University of Oslo. The work presented in this master thesis was carried out at the Norwegian Institute of Public Health, Division for Forensic Sciences - Department of Drug Abuse Research and Method Development from November 2012 to March 2014. The work was supervised by research scientist Inger Lise Bogen (Norwegian Institute of Public Health) and research scientist Jannike M. Andersen (Norwegian Institute of Public Health). Professor Kjetil Hylland was the internal supervisor (University of Oslo).
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Thank you to all my friends and family, for listening to all my rambling about enzymes, heroin and missing metabolites. I would especially like to thank my brother Steinar for all the help I received during the writing process, it was greatly appreciated (even though there were a few discussions concerning the slightly different accuracy of biology compared to chemical engineering 😊). Thanks also to my friend Helene for all the support, as well as not panicking when I told her I was “dying” because I had used the wrong heroin (I do agree that was a misleading expression in that context). Thanks also to my mum and dad for all the support, and for always helping me out with my dogs while I was busy working in the lab.
Abstract

Heroin is the main abused opioid, and is causing most drug use related deaths in the European Union and in the USA. New treatment strategies for heroin addiction are needed, and an alternative could be modulation of the enzymes involved in the heroin metabolism with the objective of reducing the rewarding effects of the drug. Different esterases have been shown to be involved in the metabolism of heroin. However, little is known about the importance of these enzymes in the heroin metabolism in organs other than blood. The aim of this study was to identify the esterases involved in heroin metabolism in liver, lung and brain tissue from rat and investigate whether modulation of these enzymes could decrease the effects of heroin in mice, by reducing the concentration of the active metabolite 6MAM. In vitro heroin metabolism studies were conducted in perfused rat liver, lung and brain tissue in the presence and absence of specific esterase inhibitors. This was followed by in vivo experiments in mice, where the effect of specific esterase inhibitors on the heroin metabolism and the heroin induced behavior were investigated. This was examined by combining a behavioral test and measurements of the heroin metabolites in blood and brain. LC-MS/MS was used to quantify heroin and heroin metabolites in the biological matrices. The results suggest that there are different enzymes metabolizing heroin in liver, lung and brain tissue. In liver and lung tissue mainly carboxylesterase is involved, while in brain mainly acetylcholinesterase and butyrylcholinesterase are involved. Administration of esterase inhibitors to mice did not affect the behavior as a result of reduced levels of 6MAM. Increased knowledge of the enzymes involved in metabolism of heroin is important and could lead to other pharmacokinetic treatment approaches.
## Abbreviations

<table>
<thead>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>6MAM</td>
<td>6-monoacetylmorphine</td>
</tr>
<tr>
<td>AChE</td>
<td>Acetylcholinesterase</td>
</tr>
<tr>
<td>BChE</td>
<td>Butyrylcholinesterase</td>
</tr>
<tr>
<td>Benzylisatin</td>
<td>1-benzyl-1H-indole-2,3-dione</td>
</tr>
<tr>
<td>BNPP</td>
<td>Bis(4-nitrophenyl)phosphate</td>
</tr>
<tr>
<td>Bw284c51</td>
<td>1,5-Bis(4allyldimethylammoniumphenyl)pentan-3-one dibromide</td>
</tr>
<tr>
<td>CE</td>
<td>Carboxylesterase</td>
</tr>
<tr>
<td>Cmax</td>
<td>Maximum concentration</td>
</tr>
<tr>
<td>ESI+</td>
<td>Positive electrospray</td>
</tr>
<tr>
<td>Ethopropazine</td>
<td>10-(2 Diethylaminopropyl)phenothiazine,10-[2-(Diethylamino)propyl]phenothiazine hydrochloride</td>
</tr>
<tr>
<td>eV</td>
<td>Collision energy</td>
</tr>
<tr>
<td>hCE-1</td>
<td>Human liver carboxylesterase 1</td>
</tr>
<tr>
<td>hCE-2</td>
<td>Human liver carboxylesterase 2</td>
</tr>
<tr>
<td>HPLC</td>
<td>High-performance liquid chromatography</td>
</tr>
<tr>
<td>I.p.</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>I.v.</td>
<td>Intravenous</td>
</tr>
<tr>
<td>Iso-OMPA</td>
<td>Tetraisopropyl pyrophosphoramide</td>
</tr>
<tr>
<td>kV</td>
<td>Capillary voltage</td>
</tr>
<tr>
<td>L/h</td>
<td>Liters per hours</td>
</tr>
<tr>
<td>LC-MS/MS</td>
<td>Liquid chromatography tandem mass spectrometry</td>
</tr>
<tr>
<td>LOD</td>
<td>Limits of detection</td>
</tr>
<tr>
<td>LOQ</td>
<td>Limits of quantitation</td>
</tr>
<tr>
<td>M3G</td>
<td>Morphine-3-glucuronide</td>
</tr>
<tr>
<td>M6G</td>
<td>Morphine-6-glucuronide</td>
</tr>
<tr>
<td>MRM</td>
<td>Multiple reaction monitoring</td>
</tr>
<tr>
<td>Neostigmine</td>
<td>3-(N,N-Dimethylcarbamoyloxy)-N,N,N,-trimethylanilinium bromide</td>
</tr>
<tr>
<td>Rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>Rt</td>
<td>Retention time</td>
</tr>
<tr>
<td>S.c.</td>
<td>Subcutaneous</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error mean</td>
</tr>
<tr>
<td>UDPGA</td>
<td>Uridine 5′-diphosphosphate-glucuronic acid</td>
</tr>
<tr>
<td>UGT</td>
<td>Uridine 5′-diphosphate-glucuronosyltransferases</td>
</tr>
<tr>
<td>V</td>
<td>Voltage</td>
</tr>
</tbody>
</table>
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1. Introduction

1.1 Heroin addiction

Opium is the dried latex obtained from the opium poppy (*Papaver somniferum*, figure 1.1). The use of opium is ancient, and through the centuries it has been used in religious rituals as well as for medical purposes [1]. In modern medicine drugs derived from opium are widely used as pain relievers. These drugs are called opioids and include morphine, codeine and oxycodone. In 1874 the English chemist A.C Wright attempted to develop a new drug similar to morphine without its addictive effect [2]. Rather he synthesized heroin, which has an even stronger addiction potential as well as a more potent reinforcing and analgesic activity compared with morphine [3]. This has led heroin to be the main abused opioid, causing most drug use related deaths in the European Union [4] and in the USA [5]. Over the last decade, Norway has had approximately 50 annual deaths per million citizens, of which about 80% are due to heroin overdoses. Thus, Norway is one of the countries in Europe with the highest narcotic related death rate [4, 6, 7].

![Figure 1.1 - Opium poppy (Papaver somniferum)](image)

Heroin addiction is associated with a multitude of health and social problems, often leading to physical dependence and severe withdrawal symptoms. Heroin dependent drug users experience social implications and economic problems which lead to a lower quality of life compared to that of the general population [4]. Injection of heroin increases the risk of fatal overdose and transmission of blood-borne diseases such as HIV/AIDS as well as Hepatitis B and C [5, 8].
Various treatments are available for opioid dependence. Substitution therapy with opioid receptor agonist such as methadone and buprenorphine is currently the predominant alternative in Europe [4]. These pharmaceuticals bind to opioid receptors and decrease the effects of administration of heroin [9, 10]. However, by having the same mode of action as heroin, opioid agonists are themselves susceptible to abuse and overdoses. This typically calls for tight regulation as well as comprehensive administration, as the opioid agonists have limited duration of action [11].

Another class of drugs used in treatment are opioid receptor antagonists, like naltrexone, which successfully block opioid receptors, but have no stimulatory effect [12]. Compliance with antagonist use is generally low [11], which may be caused by their ability to also bind the body’s endogenous opioids causing a negative emotional effect in the patient [13].

1.2 Pharmacology of heroin

Heroin (3,6-diacetylmorphine) is a semi-synthetic opioid synthesized from morphine. The most common routes of administration of heroin are by injection as well as inhalation by vaporization or smoking [4, 14, 15]. The first-pass metabolism of the liver is avoided by both these administration routes [16]. Intravenous injection provides immediately onset of euphoria, while peak effects usually are felt within 10 to 15 minutes when heroin is inhaled [16].

The physical effects of heroin are euphoria and pain relief, and are caused by binding to μ-opioid receptors in the brain [17]. Activation of these opioid receptors increases the levels of the neurotransmitter dopamine by increasing the firing rate of dopaminergic neurons in the nucleus accumbens [18, 19]. This increase in dopamine release is believed to establish and reinforce the habit of drug abuse [20, 21].

Normally endogenous opioid peptides like endorphins and enkephalins bind to μ-opioid receptors and initiate the release of dopamine [22]. The function is to reward behaviors essential for survival and evolutionary success, such as eating, drinking, having social interaction and sex [23]. Heroin acts much more strongly than natural rewards on these neural circuits, and also bypasses the feedback mechanisms normally controlling dopamine release in nucleus accumbens [24, 25]. The excessive release of dopamine caused by repeated drug
Use may lead to several physiological processes, including up and down regulation of numerous genes which eventually leads to neuroadaptations [24]. Addiction to heroin is hence believed to be caused by repeated self-administration of the drug producing enduring adaptations in the neurotransmitter systems of the brain, leaving addicts vulnerable to relapse [23, 26].

1.3 Metabolism of heroin

After administration heroin is rapidly deacetylated to 6-monoacetylmorphine (6MAM), then further metabolized into morphine, morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G) [3]. This reaction is showed in figure 1.2.

![Figure 1.2 - The metabolism of heroin to 6MAM, morphine, M3G and M6G](image)
The metabolism of heroin to 6MAM occurs primarily in the periphery [3, 27, 28]. This causes high blood levels of 6MAM which readily crosses the blood-brain barrier and binds to the \(\mu\)-opioid receptors in the brain. Opioid receptors are stereo-specific, and heroin shows a lower \(\mu\)-opioid receptor affinity than its metabolites [17, 29]. Hence heroin is behaving as a pro-drug, and 6MAM mediates its early acute behavioral effects, at least in mice [27]. Additionally morphine contributes to these effects, but to a lesser degree due to lower levels and slower accumulation in brain [30].

Heroin has a short half-life, between 1.3 and 7.8 minutes in human blood [28] and between 2 and 5 minutes in mice blood [3]. 6MAM has a somewhat longer half-life than heroin, estimates ranging from 5.4 to 52 minutes in human blood [28].

In the body the hydrolysis of heroin and 6MAM is thought to be catalyzed by different types of esterases as well as non-enzymatically [28, 31]. Morphine is further metabolized mainly in the liver by glucuronidation to the inactive metabolite M3G and the active metabolite M6G [32]. However, in rodents, no or only small traces of M6G is produced [33]. The glucuronidation to the 3- and 6-positions of morphine is catalyzed by the enzyme uridine 5’-diphosphate-glucuronosyltransferases (UGT) [34].

1.4 Esterases capable of heroin metabolism

Enzymes belonging to two distinct families within higher eukaryotes are known to metabolize heroin: cholinesterases and carboxylesterases [28]. Both families are phase-I drug metabolizing enzymes [35, 36].

Cholinesterases are esterases that catalyze the hydrolysis of esters of choline. There are two known cholinesterases; acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) [37]. Both enzymes are capable of hydrolyzing heroin [31].

The main function of AChE (EC 3.1.1.7) is to catalyze the hydrolysis of the neurotransmitter acetylcholine into choline and acetic acid, a reaction necessary to allow a cholinergic neuron to return to its resting state after activation [38]. In addition the enzyme have some non-catalytic functions, such as involvement in cell adhesion and proliferation [39]. AChE is mainly found in neuromuscular junctions and in neural synapses, but also in the blood on red
blood cell membranes [40]. The enzyme is one of the fastest and most efficient enzymes known [41], each molecule of AChE degrades about 25000 molecules of acetylcholine per second [42]. This reaction is under normal conditions limited solely by the rate of acetylcholine diffusion into the gorge of the enzyme [39].

BChE (EC 3.1.1.8) is produced in the liver and primarily found in the plasma [43]. BChE is also present in the brain, where it accounts for about 10% of total brain cholinesterase activity, whereas AChE accounts for the residue [44]. BChE is less specific than AChE and capable of hydrolyzing a broad spectrum of structurally different substrates [37]. The function of this enzyme is therefore thought to be metabolism of various xenobiotics [45]. BChE also acts as a scavenger of many natural and synthetic anticholinesterase compounds, eliminating them before they reach AChE where they could cause serious neural dysfunction [46]. Humans lacking functional BChE appear healthy, which suggests that this enzyme is not essential for normal physiological functions [47].

Comparison of the structure of AChE and BChE shows extensive similarities in protein sequences and in molecular forms. AChE and BChE have more than 50% identical amino acids, have almost the same backbone structure as well as similar protein folding [48].

The third enzyme known to metabolize heroin is carboxylesterase (CE; EC 3.1.1.1). CE catalyze the hydrolysis of many clinically useful drugs as well as the narcotic cocaine [49], and is considered to be an important detoxification enzyme in mammals [50]. CE is mostly expressed in tissues likely to be exposed to xenobiotics, including the liver, lung, small intestine and kidney [51]. In humans, two CEs, hCE1 and hCE2, are important mediators of drug metabolism. Both are expressed in human liver, but the amount of hCE1 greatly exceeds hCE2 [52].

1.5 Inhibition of esterases

The overall structure and catalytic activity of the two cholinesterases and CE are quite similar [53]. However, the details of the structure of the functional sub-domains differ. These differences affect the enzymes affinity to different substrates as well as their sensitivity against inhibitors [35, 39]. The use of enzyme inhibitors can therefore be utilized as a diagnostic tool to identify the different enzymes involved in a specific enzymatic reaction,
e.g. the deacetylation of heroin. Identification of the enzymes present in a tissue is commonly studied with techniques like immunohistochemistry or Western blotting. By the use of inhibitors, the importance of a specific enzyme in an enzyme reaction can be investigated at the same time.

To understand how various inhibitors affect the metabolic capacity of esterases, it is necessary to know how these enzymes hydrolyze their substrates. Each subunit of these esterases has their active site located in a gorge that penetrates approximately halfway into the enzyme. Kinetic and structural studies have shown that the different esterases have similar functional sub-domains within this gorge [45, 48, 54]. A peripheral anionic site is located at the entry of the gorge. When a substrate binds to this site, it triggers a conformational change of the active site, allowing the substrate to slide down the gorge. There the substrate binds to the active site, called acyl binding site, and is then hydrolyzed in a two-step reaction. The product then disassociates, and the enzyme is ready to catalyze a new reaction [45, 54, 55]. In figure 1.3 the structure of the active site of BChE is illustrated.

![Diagram of the active site of BChE](image)

*Figure 1.3 - The structure of the active site of BChE [45]*
Enzyme inhibitors affect enzyme reactions by binding to the enzymes and decreasing their activity. A large number of esterase inhibitors have been discovered as well as synthesized. In the following section the enzyme inhibitors used in this master thesis will be presented. These inhibitors were chosen based on their documented use in tissue localization of cholinesterases [56-59] and CE [60] and their known tolerance in animal in vivo experiments [61-63].

The general cholinesterase inhibitor neostigmine (figure 1.4.) is a carbamate [64, 65]. Multiple studies show that it does not affect CE at concentrations up to at least 1 mM [66, 67]. Neostigmine is used as a therapeutic agent, including in anesthesia, to reverse the neuromuscular blockade produced by neuromuscular blockers [68]. Neostigmine is not lipid soluble, hence it does not cross the blood-brain barrier [69]. Neostigmine binds to the active site of the enzyme, and due to its structure it has great binding strength and is therefore resistant to hydrolysis [64].

![Figure 1.4 – Neostigmine](image)

BW284c51 (figure 1.5) is a bisquaternary ammonium compound and a highly specific inhibitor of AChE [70, 71]. The inhibitor has been shown to have approximately 347-fold more selectivity towards AChE than BChE in vitro [39]. The preference of BW284c51 towards AChE is due to the presence of specific amino-acid residues in the active catalytic site and anionic site of the enzyme [72, 73].

![Figure 1.5 - BW284c51](image)
Ethopropazine (figure 1.6) is a phenothiazine derivative and a specific BChE inhibitor, which has shown an approximately 1800-fold preference for BChE compared to AChE [73]. Ethopropazine has been in clinical use in the treatment of Parkinson’s disease [74]. Due to different amino acids in the active gorge of AChE and BChE, the volume of the gorge of BChE is larger compared to AChE. This causes the specificity of ethopropazine towards BChE [75-77].

![Figure 1.6 - Ethopropazine HCl](image)

Iso-OMPA (figure 1.7) is an organophosphate and is found to inhibit BChE more effectively than AChE [78]. It has been shown to be approximately 2700-fold more selective towards BChE than AChE in vitro [39]. The larger active gorge of the enzyme is the cause of the specificity of iso-OMPA towards BChE. Even though iso-OMPA often is considered being a specific BChE inhibitor [58, 79, 80], it has shown some activity towards CEs as well [81-84].

![Figure 1.7 - Iso-OMPA](image)

BNPP (figure 1.8) is an organophosphate and has been widely used as a specific CE inhibitor [60, 85]. However, some studies claim that due to the structure homology between CE and other esterases, organophosphates like BNPP may inhibit many different enzymes, including CEs, AChE and BChE [49, 86].
Benzylisatin (figure 1.9) is a recently synthesized inhibitor of mammalian CEs [49]. This inhibitor is based on the structure of benzil (diphenylethane-1,2-dione), a known potent inhibitor of hCE1 and hCE2 [87]. Benzylisatin has not shown activity towards cholinesterases up to 100 µM. It is possible that benzylisatin will be better tolerated \textit{in vivo} than benzil because it has a more similar structure to drugs that are already in clinical use [49].

The inhibition mechanism of these six inhibitors is mainly competitive, and is caused by blockade of the active gorge. The different inhibitors accomplish this by binding to amino acids that line and/or form the active gorge of AChE, BChE and CE [35, 39]. Neostigmine, BW284c51, ethopropazine and benzylisatin inhibit the enzyme reversible, reducing the reaction rate to minutes rather than microseconds [49, 64, 73, 77]. Iso-Ompa and BNPP are irreversible inhibitors, which means that the enzyme is permanently inactivated. These inhibitors are both organophosphates and acts by phosphorylation of the active site of the enzyme [88, 89].

When using enzyme inhibitors to study the importance of different enzymes in a specific enzymatic reaction, the inhibitor used should be highly specific. This means that it should show significantly higher activity towards the enzyme of interest compared to other enzymes. In this case this is difficult to achieve, due to the fact that these esterases demonstrate
considerable amino acid and structural homology. Compounds that are marketed and have been used and described in numerous studies as specific esterase inhibitors may inhibit several esterases [49]. The specificity of the described esterase inhibitors is dependent on their concentration, since they are mainly competitive inhibitors [39]. Hence a lower binding affinity could be overcome by higher inhibitor concentration.

1.6 Animal research and ethics
Animal models are often used to predict the metabolic behavior of compounds in humans, and mice and rats are among the most commonly used test species. This is a consequence of the many practical advantages that these species possesses, as well as similar physiology to humans [90, 91]. Important physiological parameters, such as body temperature, hematocrit and serum albumin concentrations, are relatively conserved among rats, mice and humans [92]. Functional counterparts of almost all human genes also exist within the murine genome [93].

While significant advances in our understanding of drug metabolism have been derived from studies in rodent models, there are some major limitations to the use of laboratory animals as models for human drug metabolism. Body size and weight affect the pharmacokinetic parameters of xenobiotics across species. As a consequence of differences in body size and weight, the relative amount of hepatic enzymes is higher in small animals than in humans [90]. Blood circulation time correlates with total body weight, indicating that smaller animals eliminate drugs more rapidly than humans [94]. There are also interspecies variations in the expression and activities of drug metabolizing enzymes [90]. Extrapolation of data across species should therefore be done with care.

The C57BL/6 mouse strain and the Sprague-Dawley rat strain used for the studies in this master thesis were selected for their known sensitivity for heroin, and for comparison to previous studies performed in the same strains at the Norwegian Institute of Public Health [27, 30, 33, 95]

The research presented in this master thesis could not have been conducted without the use of animals. Animal research in Norway is regulated by the animal rights law given by the Ministry of Agriculture and Food. When using animals for research, alternatives must always
be considered and used if possible. The number of animals should also be minimized to the absolute requirement. The possible suffering of the animals must be weighed against the probable gain in each study, and the level of pain must be minimized. At the Norwegian Institute of Public Health all the necessary approvals for conducting animal research were obtained.

1.7 Aim of the study

Various esterases have been shown to be involved in the metabolism of heroin [28, 31, 96, 97]. Studies concerning heroin metabolism in blood have been carried out previously [31, 98, 99], but there is little knowledge about the importance these esterases have in the heroin metabolism in other tissues. Three organs are of special interest due to their relevance in heroin metabolism: the brain, the liver and the lungs. The effect of heroin is exerted in the brain, the liver has a key role in the general xenobiotic metabolism, while the lungs are of interest because inhalation is a common administration route of heroin.

The hypothesis of this master thesis was that there are distinct enzymes metabolizing heroin in brain, liver and lung. The first objective was therefore to identify the esterases involved in the heroin metabolism in isolated rat organs. The second objective was to test whether it would be possible to decrease the enzymatic conversion of heroin to 6MAM in a mice model, and thereby reduce the physiological effects of the drug. These objectives were investigated by doing metabolism studies in vitro, inhibitor studies in vitro and in vivo and a behavior test in mice.

A more detailed understanding of heroin metabolism could be utilized to develop new treatment strategies for heroin dependence. This is needed, since all present treatment of heroin addiction is associated with frequent relapses and is often unsuccessful [11].
2. Materials and methods

2.1 Chemicals

Table 2.1 - Chemicals

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Specifications</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>BNPP</td>
<td>Bis(4-nitrophenyl)phosphate</td>
<td>Sigma Aldrich, Germany</td>
</tr>
<tr>
<td>BW284c51</td>
<td>1,5-Bis(4allyldimethylammoniumphenyl)pentan-3-one dibromide</td>
<td>Sigma Aldrich, Germany</td>
</tr>
<tr>
<td>Ethopropazine hydrochloride</td>
<td>10-(2 Diethylaminopropyl)phenothiazine, 10-[2-(Diethylamino)propyl]phenothiazine hydrochloride</td>
<td>Sigma Aldrich, Germany</td>
</tr>
<tr>
<td>Heroin</td>
<td>3,6-diaceylmorphine hydrochloride</td>
<td>Lipomed AG, Switzerland.</td>
</tr>
<tr>
<td>Iso-OMPA</td>
<td>Tetraisopropyl pyrophosphoramide</td>
<td>Sigma Aldrich, Germany</td>
</tr>
<tr>
<td>Ketalar®</td>
<td>Ketamine, 10 mg/ml</td>
<td>Pfizer, Norway</td>
</tr>
<tr>
<td>N-Benzylisatin</td>
<td>1-benzyl-1H-indole-2,3-dione</td>
<td>Sigma Aldrich, Germany</td>
</tr>
<tr>
<td>Neostigmine bromide</td>
<td>3-(N,N-Dimethylcarbamoyloxy)-N,N,N,trimethylanilinium bromide</td>
<td>Sigma Aldrich, Germany</td>
</tr>
<tr>
<td>Rompun®</td>
<td>Xylazine hydrochloride, 20 mg/ml</td>
<td>Bayer Healthcare, Norway</td>
</tr>
<tr>
<td>Titriplex® III</td>
<td>Ethylenedinitrilotetraacetic acid disodium salt dihydrat</td>
<td>Merck Millipore, Germany</td>
</tr>
</tbody>
</table>

All other chemicals were supplied by standard commercial suppliers.
2.2 Animals

Male Sprague-Dawley rats from Taconic (Bomholt, Ejby, Denmark), age 8-10 weeks and weight about 200-250 g, were used in the in vitro experiments. 26 male C57BL/6J-Bom mice from Taconic (Bomholt, Ejby, Denmark), age 7-8 weeks and weight between 21.7 to 27.0 g, were used in the in vivo experiments. The animals were kept in the animal facilities at the Norwegian Institute of Public Health at 22 ± 1°C, 50 ± 10% humidity with light period 7:00 AM–7:00 PM. Rats were housed two per cage, mice were housed seven to eight per cage. Animals arrived at least 5 days before the experiments. Commercial rat/mice pellets and water were available ad libitum.

The Animal Research Authority for the use of animal subjects approved the experimental protocols of the studies. Procedures and handling of all animals used in these experiments were carried out in agreement with the Norwegian Animal Welfare Act.

2.3 Preparation of rat organs for in vitro experiments

**Non-perfused liver:** Rats were sacrificed by decapitation. Liver was collected, rinsed in NaCl and immediately placed on ice. The liver was homogenized in Tris/Krebs-buffer (10 mM Tris, 140 mM NaCl, 5 mM KCl, 5mM NaHCO\(_3\), 1 mM MgCl\(_2\), 1.2 mM Na2HPO\(_4\), 10 mM glucose, 1.2 mM CaCl\(_2\), pH 7.4) with a teflon/glass homogenizer (2 ml/g tissue). The homogenate was then diluted 1:1 in Tris/Krebs-buffer. For experiments on fresh tissue the tissue material was kept on ice and used within 2 hours after harvest. The rest of the tissue was frozen in liquid N\(_2\) and stored at -80°C. The non-perfused livers were used for comparing heroin metabolism in frozen and fresh tissue.

**Perfused organs:** Rats were anaesthetized using a mixture containing Ketalar\(^®\) (100 mg/kg) and Rompun\(^®\) (10 mg/kg) administered intraperitoneally (10 ml/kg). While being ventilated through a tracheal catheter, the rats were perfused (30-32 ml/min) via the left heart chamber with a PBS buffer (0.01 M PO\(_4^{3-}\), 0.0027 M KCl and 0.137 M NaCl, pH 7.2 - 7.6). The organs were perfused for 10-15 min. Brain, liver and lungs were removed and homogenized (2 ml/g tissue) in ice-cold PBS buffer using a teflon/glass homogenizer. The lungs were minced with scissors and blotted on filter paper before homogenization. The tissues were frozen in liquid N\(_2\) and then stored at -80°C. The perfused organs were used in the heroin metabolism
experiments and enzyme inhibitor experiments. The perfusion procedure was done in cooperation with the animal department at the Norwegian Institute of Public Health.

2.4 In vitro heroin metabolism in rat organs
The heroin used in the in vitro experiments was dissolved in 0.9% NaCl maximum 7 days prior to the experiments and stored in refrigerator at 4°C. Experiments were conducted showing that heroin is stable in this solution for at least 7 days (unpublished results). All incubation times, concentrations and conditions for the in vitro experiments were chosen based on earlier published studies [27, 30] and pilot experiments conducted as a part of this master thesis.

2.4.1 Heroin metabolism comparing fresh and frozen liver
Non-perfused liver homogenate (110 μl) was transferred to plastic-tubes kept in a water bath at 37°C and pre-incubated for 15-30 min. 12 μl heroin solution was added to each tube (final concentration 0.4 μM). The tubes were briefly mixed and placed back in the water bath. The samples were incubated for a pre-specified time (0.05, 0.25, 0.5, 1, 3, 5, 10 and 30 min). Then 78 μl ice-cold 13 mM ammonium format buffer (pH 3.1; final concentration 5 mM) with sodium fluoride (final concentration 2 mg/ml) was added to stop the enzymatic reaction. The tubes were immediately mixed and quickly frozen in liquid N₂. The tissue samples were placed in a deep-freezer at -80°C before sample preparation and analyses by LC-MS/MS the same day (see section 2.7-2.8).

2.4.2 Heroin metabolism in liver, lung and brain
The studies of in vitro heroin metabolism in perfused liver, lung and brain were conducted as described in section 2.4.1 with some modifications. Tissue homogenate was diluted 1:1 in PBS buffer and then 100 μl and 10 μl PBS were added to the tubes. The incubation time was 0.05, 0.25, 0.5, 1.5, 5, 10 and 30 min for liver homogenate and 0.05, 0.5, 1, 3, 5, 10 and 30 min for lung and brain homogenates. The total protein concentration in each of the samples was measured as described in section 2.9 to be 0.454 mg ± 0.029 in liver, 0.138 mg ± 0,014 in lung and 0,346 mg ± 0,033 in brain (mean ±SEM).
2.4.3 Inhibition of heroin metabolism in liver, lung and brain

The enzyme inhibitor experiments in perfused liver, lung and brain were conducted as described in 2.4.1, with some modifications. The tissue homogenate was first diluted 1:1 in Tris/Krebs-buffer and then 1100 µl tissue homogenate was added to 1300 µl Tris/Krebs-buffer. Then 60 µl tissue homogenate (liver, lung, brain) was added to the tubes. Before adding heroin, 40 µl NaCl and 10 µl enzyme inhibitor were added to each plastic-tube to a final concentration of 10 µM or 100 µM (see table 2.2). Then the samples were pre-incubated for 15-30 min. The incubation times after addition of heroin were 3 min for liver, 60 min for lungs and 30 min for brain. These time points were chosen based on the data from the time curves from the in vitro heroin metabolism in perfused organs. The total protein concentration in the samples was measured as described in section 2.9 to be 0.245± 0.015 in liver, 0.75± 0.008 in lung and 0.187± 0.018 in brain (mean ± SEM).

Table 2.2 – Enzyme inhibitor concentrations

<table>
<thead>
<tr>
<th>Enzyme inhibitor</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW284c51</td>
<td>10 µM and 100 µM (dissolved in NaCl 0.9%)</td>
</tr>
<tr>
<td>Iso-OMPA</td>
<td>10 µM and 100 µM (dissolved in NaCl 0.9%)</td>
</tr>
<tr>
<td>BNPP</td>
<td>10 µM and 100 µM (dissolved in NaCl 0.9%)</td>
</tr>
<tr>
<td>Neostigmine</td>
<td>10 µM 100 µM (dissolved in NaCl 0.9%)</td>
</tr>
<tr>
<td>Ethopropazine</td>
<td>10 µM 100 µM (dissolved in dH2O)</td>
</tr>
<tr>
<td>Benzylisatin</td>
<td>10 µM 100 µM (dissolved in ethanol)</td>
</tr>
</tbody>
</table>

2.5 In vitro heroin metabolism and sample preparation in microsomes, cytosol and s9-fraction

Different liver fractions, s9-fraction, microsomes and cytosol from male Sprague-Dawley rats were purchased from Life Technologies (Frederick, MD, USA). See table 2.3 for further explanation of the different fractions.
Table 2.3 – Explanation of the different liver fractions used [100]

<table>
<thead>
<tr>
<th>Liver fraction</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>S9</td>
<td>A supernatant fraction obtained from liver homogenate by centrifuging liver homogenate at 10,000g. Contains both microsomes and cytosol.</td>
</tr>
<tr>
<td>Microsomes</td>
<td>Further centrifugation of the s9 fraction at 100,000g yields the endoplasmic reticulum-derived microsomes.</td>
</tr>
<tr>
<td>Cytosol</td>
<td>The cytosol is the portion of the cytoplasm not contained within membrane-bound organelles.</td>
</tr>
</tbody>
</table>

130 µl NADPH regeneration solution (1.3 mM NADP⁺, 3.3 mM glucose 6-phosphate, 0.4U/ml glucose 6-phosphate dehydrogenase, 3.3 mM MgCl₂) was added to separate plastic-tubes kept in a water bath, at 37°C. 10 µl heroin was added (final concentration 0.5 µM) and the samples were mixed well. 10 µl s9, microsomes or cytosol fraction (protein concentration 20 mg/ml) were added and the samples were incubated for a given time (0, 1, 3, 5, 10, 30 and 60 min). The reaction was then terminated by adding 20 µl 1 M formic acid to each tube and vortexing for 1 min. 30 µl internal standard was added before the samples were centrifuged for 5 min at 14 500 rpm (11 500g, 4°C, 5 min). The supernatants were transferred to autosampler vials. The standards and controls were prepared by mixing 100 µl standard/control, 70 µl distilled water and 30 µl internal standard.

2.6 Enzyme inhibitor studies in vivo

2.6.1 In vivo exposure

The mice (n=26) were injected intraperitoneally (i.p.) with saline or an enzyme inhibitor (all dissolved in 0.9% NaCl): BNPP (100 mg/kg), BW284c51 (1 mg/kg) or Iso-OMPA (10 mg/kg). The injection volume was 0.1 ml/10 g mouse for all solutions. Heroin (2.5 µmol/kg) was injected subcutaneous (s.c.) or intravenously (i.v.) in a volume of 0.05 ml/10 g mouse. The experiment was done in cooperation with supervisors at the Norwegian Institute of Public Health who injected the mice.
2.6.2 Locomotor studies

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. Based on previous experience in our lab [27, 101], distance travelled (cm/5min) was used as an expression for locomotor activity. The mice were injected with enzyme inhibitor and then habituated in their respective activity chamber for 1 hour. Then the mice were injected with heroin (2.5 µmol/kg) s.c. or i.v. The heroin was dissolved in 0.9% NaCl the same day as the experiment. The mice were immediately returned to their respective locomotor chambers where the activity was measured for 20 min. The locomotor activity test was performed as described in Andersen et al. [27] with some modifications. Drugs were administered in one room, while the locomotor activity test took place in another.

2.6.3 Harvesting of mice blood and brain

Immediately after measurement of locomotor activity, the mice were anaesthetized with isoflurane before blood samples (~500 µl) were obtained by heart puncture (t=25 min), using a syringe containing 80 µl of sodium fluoride (final concentration 4mg/ml) dissolved in heparin (100 IU/ml).

The blood was diluted 1:1 in ice-cold 5 mM ammonium format buffer, pH 3.1, and immediately frozen in liquid N₂. After blood sampling, the brain (except cerebellum) was quickly removed, washed in ice-cold 0.9% NaCl and blotted on a filter paper. Then the brain was homogenized (2 ml/g tissue) in 5mM ammonium format buffer, pH 3.1 with sodium fluoride (4mg/ml) and diluted 1:1 in ice-cold 5 mM ammonium format buffer before being frozen in liquid N₂. Ice-cold acidic buffer was used to dilute the blood samples and to homogenize brain tissue, since heroin has been shown to be most stable at low temperatures and low pH (Barrett et al., 1992). Sodium fluoride (final concentration 2 mg/ml) was used to inhibit plasma esterase activity, thereby stabilizing the amount of heroin and 6-MAM (Brogan et al., 1992). All samples were stored at -80°C until analyzed by LC-MS/MS. Heroin is rapidly metabolized in biological tissues [102], especially in blood [103], and therefore the blood samples were analyzed the same day, and the brain samples were analyzed the following day.
2.7 Preparation of samples from rats and mice prior to LC-MS/MS

Preparation of samples was performed as described in Karinen et al. (2009) with modifications as described below. 100 μl standards and controls were added to separate plastic tubes and placed on ice. Then 100 μl tissue homogenate/blood was added to all standards and controls. For in vitro heroin metabolism comparing fresh and frozen liver rat liver was used in standards and controls. For in vitro pharmacokinetics and enzyme inhibitor experiments human blood was used in the standards and controls. For the in vivo experiments rat brain and rat blood were used in the standards and controls for the respective organ.

Samples from the experiments were gently thawed on ice. 50 μl internal standard mixture was added to all tubes and mixed well. Thereafter 500 μl ice-cold acetonitrile/methanol (85:15) was added and the tubes were mixed well for 10-15 sec. All samples were capped and placed in the deep-freezer (-20°C) for minimum 10 min before being centrifuged at 4500 rpm (4700g) at 4°C for 10 min. 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, 0 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 at 4500 rpm (3800g) at 4°C for 10 min. The supernatant was transferred to 0.3 ml autosampler vials.

2.8 LC-MS/MS analysis

The presence of heroin and the heroin metabolites 6MAM, morphine and M3G in the samples were analyzed using liquid chromatography tandem mass spectrometry (LC-MS/MS). Reversed phase chromatography in a XTerra ® MS C18 column (Waters Corp., Milford, MA, USA) with an acidic mobile phase combined with positive electrospray (ESI+) detection was used with a flow rate of 0.2 ml/min. This method combines the separation capabilities of HPLC with the detection capabilities of MS and is a selective and sensitive analysis method well established at the “Division for Forensic Sciences” at the Norwegian Institute of Public Health [104]. For more detailed information on the LC-MS/MS conditions, see appendix section 7.2.
2.9 Determination of protein levels

The amount of protein in the different tissue homogenates was determined as described by Lowry et al. [105]. In this method a color reaction between phosphomolybdic ions and proteins will appear if the proteins are pre-exposed to copper ions (Cu$^{2+}$) in an alkaline environment.

4 µl tissue homogenate (0.17 g/ml) was mixed with 196 µl dH$_2$O. The reaction was initiated by adding 1 ml application solution (2% Na$_2$CO$_3$ in 0.1 M NaOH, 0.5% CuSO$_4$·5H$_2$O, 1% K(Na)tartrate; 98:1:1). Each sample was mixed well.

After 10 min the reaction was stopped by adding 100 µl “stop reagent” (Folin-Ciocalteus reagent, dH$_2$O; 1:1) and mixed well. Three parallels were made of each sample. The samples were placed on the bench for a minimum of 30 min before the absorbance was read at 750 nm by a spectrophotometer (Lambda 3; Perkin Elmer, Waltham, MA, USA). The concentrations of the standards for determination of protein levels are shown in table 2.4.

<table>
<thead>
<tr>
<th>Standard</th>
<th>Concentration of BSA (dissolved in dH$_2$O)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blind</td>
<td>0 µg</td>
</tr>
<tr>
<td>Standard 1</td>
<td>1 µg</td>
</tr>
<tr>
<td>Standard 2</td>
<td>2.5 µg</td>
</tr>
<tr>
<td>Standard 3</td>
<td>5 µg</td>
</tr>
<tr>
<td>Standard 4</td>
<td>10 µg</td>
</tr>
<tr>
<td>Standard 5</td>
<td>15 µg</td>
</tr>
<tr>
<td>Standard 6</td>
<td>20 µg</td>
</tr>
<tr>
<td>Standard 7</td>
<td>25 µg</td>
</tr>
</tbody>
</table>
2.10 Data analysis

Data were analysed using SPSS Statistics 20.0; (SPSS Inc., Chicago, IL, USA). Data are presented as mean ± SEM unless otherwise stated. The pharmacokinetic software Kinetica v.5.1 (Thermo Fisher Scientific Inc., Waltham, MA, USA) was used to analyse the data from comparison of heroin metabolism in fresh and frozen liver and heroin metabolism in perfused organs. The mean values were calculated for each time point measured and the resulting data fed to Kinetica using a simple model for linear pharmacokinetics with three compartments (heroin -> 6-MAM -> morphine) and bolus as heroin administration method. The rate constants $k \text{ (min}^{-1}\text{)}$ were estimated by Kinetica by fitting the first order differential equations generated by the model to the experimental data. Half-life ($t_{1/2}$) was calculated from the corresponding parameters. For the comparison of heroin metabolism in fresh and frozen liver the calculated rate constant for heroin and 6MAM was tested separately for significance, comparing fresh and frozen liver by performing a t-test. For the heroin metabolism in perfused organs the calculated rate constant for heroin and 6MAM was analyzed separately and a comparison between the tissues was done by a univariate General Linear Model, followed by a Tukey’s post-hoc test.

The opioid concentration data from the in vitro and in vivo enzyme inhibitor study were analyzed using a univariate General Linear Model. Heroin, 6MAM, morphine and M3G were analyzed separately, with inhibitors as a fixed factor. For the data from the in vitro inhibitor study, Tukey’s test was used as a post-hoc test. For the in vivo inhibitor study, a two-sided Dunnett's test was used as post-hoc test. The data from the locomotion studies was tested for significance by performing t-tests for each time point.

P-values ≤ 0.05 were considered statistically significant.
3. Results

3.1 Comparison of heroin metabolism in frozen and fresh liver in vitro

The heroin metabolism in fresh and frozen liver tissue was compared to evaluate whether frozen tissue could be used in metabolism studies. The metabolism of heroin (0.4 μM) in frozen and fresh liver homogenate from rats is presented in figure 3.1.

The transition from heroin to 6MAM in frozen tissue was not significantly different from fresh tissue \[ t(5)=1.179; p>0.05 \]. However, the transition from 6MAM to morphine was significantly slower in frozen tissue compared with fresh tissue \[ t=6.619; p=0.01 \]. It was concluded that this difference was so minor that frozen tissue could be used in further experiments as discussed in the discussion section 4.1.
Figure 3.1. In vitro concentration of heroin, 6MAM and morphine in fresh and frozen liver homogenate from rats as function of time (min) after addition of heroin (0.4 μM). All values are presented as mean ± SEM, n=3-4.
3.2 Heroin metabolism in liver, lung and brain tissue \textit{in vitro}

The heroin metabolism in perfused liver, lung and brain tissue was measured and is presented in figure 3.2-3.4. The metabolism was measured for 30 minutes based on pilot experiments which showed that within this time the concentration of heroin was approaching zero in all tissues.

The heroin metabolism in liver homogenate after addition of heroin (0.4 μM) is presented in figure 3.2. Heroin concentrations decreased rapidly in liver while 6MAM concentrations increased fast. The concentration of heroin reached zero after 5 min. At about 1.5 min 6MAM reached maximal concentration (Cmax), and then started descending reaching 0.31 nmol/g at 30 minutes. At approximately 7.5 minutes, the concentration of morphine exceeded the concentration of 6MAM and continued rising throughout the experiment (30 min).

![Liver concentration curves](image)

\textbf{Figure 3.2.} \textit{In vitro concentration curves of heroin, 6MAM and morphine in rat liver homogenate as function of time (min) after addition of heroin (0.4 μM). All values are presented as mean ± SEM, n=4.}

The metabolism of heroin in lung homogenate is presented in figure 3.3 and in brain homogenate in figure 3.4 respectively. In lung the heroin concentration was measured to be 0.12 nmol/g at 30 minutes. The concentration of 6MAM continued to increase up to 2.41 nmol/g at 30 minutes. In brain tissue the concentration of heroin reached zero at 30 minutes. Cmax of 6MAM was achieved after approximately 10 minutes and were stable up to 30 minutes at approximately 2.26 nmol/g. In both tissues morphine increased slowly, reaching a concentration of 0.3 nmol/g in brain tissue and 0.13 nmol/g in lung tissue at 30 minutes.
There was found a significant difference in the reaction rate between the tissues in the transition from heroin to 6MAM \([F(2,9)=85.693; p<0.001]\) and in the transition from 6MAM to morphine \([F(2,9)=179.198; p<0.001]\). Liver had a significant faster reaction rate compared to brain \((p<0.001)\) and lung \((p<0.001)\) in the conversion from heroin to 6MAM. Also in the conversion from 6MAM to morphine liver had a significant faster reaction rate compared to
brain (p<0.001) and lung (p<0.001). No significant differences in reaction rates were found between brain and lung (p>0.05). The half-life of heroin was calculated to be 1 min (±0.09) in liver, 4 min (±0.15) in brain and 6.5 min (±0.33) in lung (mean±SEM). 6MAM was calculated to have a half-life of 7.5 min (±0.54) in liver, 106 min (±9.62) in brain and 154 min (±27.04) in lung (mean±SEM). The morphine metabolites M3G and M6G were not detected in liver, brain or lung homogenate.

3.3 Inhibition of heroin metabolism in liver, lung and brain tissue in vitro

The effect of cholinesterases and CE inhibitors on heroin metabolism was tested in liver, brain and lung homogenate. Due to the solubility of the inhibitors, different controls were used for the different inhibitors; this is described in table 2.2 in the method section 2.4.3. The controls were not significant different from each other; hence solely the saline control is shown in the diagrams 3.5 and 3.6.

3.3.1 Effect of cholinesterase inhibitors

The effect on the heroin metabolism by the cholinesterase inhibitors BW284c51, neostigmine, ethopropazine and iso-OMPA was tested separately in vitro in liver, brain and lung homogenate. The results are shown in figure 3.5.

In liver and lung homogenate the presence of 100 µM iso-OMPA significantly increased the concentration of heroin by 275% and 216% respectively compared to the control. Pre-incubation with 100 µM iso-OMPA significantly decreased the concentration of 6MAM by 16% in liver homogenate and 35% in lung homogenate compared to the control. BW284c51, neostigmine, ethopropazine and 10 µM of iso-OMPA had no significant effect on the concentration of heroin or heroin metabolites in liver or lung homogenate.
Figure 3.5. Concentrations of heroin, 6MAM and morphine in liver- brain- and lung homogenate after addition of heroin (0.4 μM) in vitro in the presence of one of the cholinesterase inhibitors (10 μM or 100 μM); BW (BW284c51), ETZ (ethopropazine), Iso (iso-OMPA) and Neo (neostigmine). The incubation time was 3 min for liver tissue, 60 min for lung tissue and 30 min for brain tissue. Values significantly different from the control are indicated with asterisks (*p<0.05, **p<0.01, ***p<0.001). All values are presented as mean ± SEM, n=3-4.
In brain homogenate the concentration of heroin and morphine was significant different from the control in the presence of 10 µM and 100 µM BW284c51, neostigmine and ethopropazine. The heroin concentrations were increased by 23-69% while the morphine concentrations were decreased by 7-38% compared to control. Pre-incubation with iso-OMPA showed no significant effect on the concentration of heroin or heroin metabolites in brain homogenate.

3.3.2 Effect of CE inhibitors
The effect on the heroin metabolism by the CE inhibitors benzylisatin and BNPP was tested separately in vitro in liver, brain and lung homogenate. The results are shown in figure 3.6. In liver homogenate the presence of 100 µM benzylisatin significantly increased the heroin concentration by 295% and significantly decreased 6MAM concentration by 12% compared to the control. In the presence of 10 µM and 100 µM BNPP the concentration of heroin significantly increased by 267% and 592% respectively, while 6MAM significantly decreased by 23% and 45% respectively compared to the control. In addition the presence of 100 µM BNPP decreased the concentration of morphine significantly by 69%.

In lung homogenate the presence of 100 µM of benzylisatin significantly increased the heroin concentration by 178% and significantly decreased 6MAM concentration by 28% compared to the control. The heroin concentration was also significantly increased by 125% compared to the control in the presence of 100 µM BNPP.

The two CE inhibitors did not affect the concentration of heroin or heroin metabolism in brain homogenate.
Figure 3.6. Concentrations of heroin, 6MAM and morphine in rat liver-, brain- and lung homogenate after addition of heroin (0.4 μM) in vitro in the presence of one of the CE inhibitors (10 μM or 100 μM); Bn-isatin (benzylisatin) and BNPP. The incubation time was 3 min for liver tissue, 60 min for lung tissue and 30 min for brain tissue. Values significantly different from the control are indicated with asterisks (*p <0.05, **p<0.01, ***p<0.001). All values are presented as mean ± SEM, n =3-4.
3.4 Heroin metabolism in different liver fractions

The metabolism of heroin was investigated in different liver fractions from rat; s9, microsomes and cytosol bought commercially. The aim was to investigate the cellular localization of the enzyme responsible for the heroin metabolism. The results are presented in figure 3.7.

The heroin concentration was zero after about 5 minutes in the microsomes, 10 min in the s9 and 60 min in the cytosol. 6MAM reached Cmax at 1 min in microsomes and then declined to zero at approximately 30 min. In s9 6MAM reached Cmax at 5 min and declined to approximately zero at 60 min. In cytosol the 6MAM concentration increased slowly, reaching a concentration of 0.41 µM at 60 min.
Figure 3.7. Concentrations of heroin, 6MAM and morphine in rat microsomes, cytosol and s9 fraction as function of time (min) after addition of heroin (0.4 μM). For time points 0-30 min in microsomes and 0-10 min in cytosol and s9, n= 2. These values are presented as mean ± SEM. For the time point 60 min in microsomes and 30 and 60 minutes in s9 and cytosol, n=1.
In the initial experiments, it was found that the total concentration of heroin and heroin metabolites (6MAM and morphine) in microsomes and s9 was severely reduced over time, as shown in figure 3.8. In both fractions the reduction was approximately 60% at 60 min. In comparison the total concentration of metabolites in cytosol was reduced approximately 7% at 60 min. The morphine metabolites M3G and M6G were not detected. As a result of this finding, only a few experiments were conducted. Several control experiments were performed to try to find the cause of the reduction of total metabolites. This is further discussed in the discussion section 4.3.

Figure 3.8. Total concentration of heroin and heroin metabolites (6MAM and morphine) in rat microsomes, cytosol and s9 fraction at different time points (min) after addition of heroin (0.4 μM). For time points 0-30 min in microsomes and 0-10 min in cytosol and s9, n= 2. These values are presented as mean. For the time point 60 min in microsomes and 30 and 60 minutes in s9 and cytosol, n=1.
3.5 The effects of inhibition of heroin metabolism in vivo

The effects on heroin metabolism and locomotor activity by pre-treatment with three esterase inhibitors were tested in vivo. Mice were pre-treated with either NaCl (0.9 %), BNPP (100 mg/kg), BW284c51 (1 mg/kg) or iso-OMPA (10 mg/kg) prior to an injection with heroin (2.5 μmol/kg) as described in method section 2.6.1. The locomotor activity of the mice was then tested to assess the effect of the inhibitors on the heroin induced behavioral response. Additionally, the concentration of heroin metabolites was measured in the blood and brain of the mice. The results are described in sections 3.5.1 and 3.5.2.

3.5.1 Effects on locomotor activity

The results from the locomotor activity test showed that injection of heroin stimulated locomotor activity after both s.c. administration (figure 3.9) and i.v. administration (figure 3.10). The mice pre-treated with NaCl reached maximal response at 15 min and 10 min after s.c. and i.v. administration of heroin respectively. When heroin was administered s.c. pre-treatment with BW284c51 reduced the locomotor activity significantly at 10 min and 15 min, respectively [t=(4)5.429;p=0.006] and [t=(4)4.056;p=0.015]. However, the mice pre-treated with BW284c51 showed signs of cholinergic syndrome (whole body tremors and prostration), which affected the locomotor activity. This is further discussed in the discussion section 4.4. Pre-treatment with BW284c51 did not significantly affect the locomotor activity when heroin was administered i.v. (p >0.05). Pre-treatment with BNPP or iso-OMPA did not significantly affect the locomotor activity either for s.c. or i.v. heroin administration (p >0.05).
Figure 3.9. Locomotor activity expressed as distance travelled (cm) per 5 min, after administration of heroin (2.5 μmol/kg, s.c.) to mice pre-treated (i.p) with Iso-OMPA (10 mg/kg), BW284c51 (1 mg/kg), BNPP (100 mg/kg) or 0.9% NaCl. Values significantly different from the control are indicated with asterisks (*p<0.05, **p<0.01, ***p<0.001). All values are presented as mean ± SEM, n = 3.

Figure 3.10. Locomotor activity expressed as distance travelled (cm) per 5 min, after administration of heroin (2.5 μmol/kg, i.v.) to mice pre-treated (i.p) with Iso-OMPA (10 mg/kg), BW284c51 (1 mg/kg), BNPP (100 mg/kg) or 0.9% NaCl. All values are presented as mean ± SEM, n=3-4.
3.5.2 Effects on concentrations of heroin metabolites in blood and brain

The concentration of heroin metabolites in blood and brain is presented in figure 3.11 and 3.12. The concentration of heroin was under the detection limit in both blood and brain. 6MAM and morphine were present in both tissues. M3G was abundant in blood, while present at much lower concentration in brain, which indicates a slow uptake of M3G to the brain.

Figure 3.11 shows that after s.c. injection of heroin (2.5 μmol/kg) mice pre-treated with iso-OMPA had a significant higher 6MAM concentration in both blood and brain compared to the control mice, respectively [F(3,8)=5.098;p=0.029, post hoc test;p=0.013] and [F(3,8)=5.538;p=0.024, post hoc test;p=0.011]. Compared to the control the increase in brain concentration was 68%, and the increase in blood concentration 124%. After i.v. injection of heroin (2.5 μmol/kg) presented in figure 3.12, pre-treatment with BNPP and iso-OMPA showed significant higher blood concentrations of 6MAM compared to the control, respectively [F(3,10)=19.617;p<0.001, post hoc test;p<0.001] and [F(3,10)=19.617;p<0.000, post hoc test;p=0.010]. Compared to the control the increase in blood concentration was 92%, for BNPP and 86% for iso-OMPA. Pre-treatment with BW284c51 did not have significant effects on the concentration of 6MAM, morphine or M3G in blood or brain (p >0.05).
Figure 3.11 Concentration of 6MAM, morphine and M3G in brain and blood after s.c. administration of heroin (2.5 µmol/kg) to mice pre-treated (i.p.) with Iso-OMPA (10 mg/kg), BW284c51 (1 mg/kg), BNPP (100 mg/kg) or 0.9% NaCl. Values significantly different from the control are indicated with asterisks (*p<0.05). All values are presented as mean ± SEM, n=3.

Figure 3.12 Concentration of 6MAM, morphine and M3G in brain and blood after i.v. administration of heroin (2.5 µmol/kg) to mice pre-treated (i.p.) with Iso-OMPA (10 mg/kg), BW284c51 (1 mg/kg), BNPP (100 mg/kg) or 0.9% NaCl. Values significantly different from the control are indicated with asterisks (**p<0.01, ***p<0.001). All values are presented as mean ± SEM, n=3-4.
4. Discussion

4.1 Comparison of heroin metabolism in frozen and fresh tissue

Rat blood is known to contain esterases [96], and to be able to identify the esterase enzymes involved in metabolism of heroin in different organs, it is necessary to reduce the contribution of blood to this metabolism. Therefore the animals were perfused, and due to this being a comprehensive procedure, it was desirable to use frozen tissue in the experiments. Cold denaturation has been documented for several proteins [106, 107] which could be caused by critical stressors to which proteins are exposed to during freezing, for example low temperature and the formation of ice. This could lead to conformational changes and loss of biological activity [108]. How esterases in liver and other tissues are affected by freezing/thawing is not well studied. The observed effects seem to differ between species [109-112] and one study conducted in human liver samples showed decreased activity of CE after freezing [113]. Research on the specific enzyme of interest appears to be needed before a conclusion may be drawn. Consequently, heroin metabolism in frozen liver was compared with fresh liver tissue. The results showed that there was no difference in the transition from heroin to 6MAM, but that the metabolism from 6MAM to morphine was somewhat slower in frozen liver compared with fresh liver. However, the reduction of enzyme activity was limited in extent and relatively constant from one sample to the next. This indicates that the use of frozen tissue was appropriate, since the focus in this thesis was on comparative rather than quantitative heroin metabolism. It was therefore decided that the use of frozen tissue was acceptable for further studies. However, a wide variety of chemically diverse compounds is available that can provide cryoprotection to proteins [108], and this approach could be considered for future studies.

4.2 Esterases metabolizing heroin in different organs

Heroin metabolism in liver, brain and lung tissue was investigated in vitro to establish concentration-versus-time graphs and to identify the enzymes involved by the use of inhibitors.

In liver and lung tissue the results showed that the specific CE inhibitors benzylisatin [49]and BNPP [60] significantly inhibited the hydrolysis of heroin. Cholinesterase inhibitors showed less effect. This suggests the involvement of CE. 100 µM but not 10 µM of iso-OMPA gave also a significant effect in liver and lung. Even though often considered a specific BChE
inhibitor [58, 79, 80], iso-OMPA has also shown activity towards CE [81-84]. One study found that 10 µM iso-OMPA inhibited a negligible amount of CE (5%) while 100 µM iso-OMPA inhibited 50% of CE in perfused rat liver [114]. It thus seems reasonable to infer that the result in liver and lung could be caused by inhibition of CE activity. In agreement with these findings, Berry et al. [96] found that the activity of CE in rat liver was high while cholinesterase activity was not detectable.

In brain tissue, the results showed that the specific AChE inhibitor Bw284c51 [70, 71], the specific BChE inhibitor ethopropazine [77, 115] and the general cholinesterase inhibitor neostigmine [65, 67] significantly inhibited heroin metabolism. There were no significant results for CE inhibitors. This suggests the involvement of cholinesterases, and that both AChE and BChE are involved in the heroin metabolism in rat brain.

To investigate the tissue specific contribution to heroin metabolism in vivo, it is relevant to compare the in vitro results from liver, lung and brain tissue with heroin metabolism in blood. Bogen et al. have studied heroin metabolism in rat blood using the same methods as used in this thesis. These results can be found in the appendix 7.1 (unpublished data) and show a significant inhibition of heroin metabolism by CE inhibitors and iso-OMPA, suggesting that it is mainly CE which metabolize heroin in rat blood. This is in accordance with Berry et al. [96] who found that CE are the esterase enzyme with highest activity in rat blood.

The results from these inhibitor studies show that different enzymes are responsible for metabolizing heroin in different tissues. Additionally the heroin metabolism concentration-versus-time graphs presented in section 3.2 showed that the metabolism in liver was significantly faster compared to brain and lung. Boix et al [30] have previously found that the rate of heroin metabolism is slower in brain compared to blood. Consequently, it seems as CE in blood and liver is the major contributor to hydrolysis of heroin in vivo and is central in the metabolism of heroin in rat. However, hydrolysis in tissues other than blood may have different degrees of importance in vivo depending on the route of administration.
4.3 Localization of esterases metabolizing heroin in liver fractions

The cellular localization of the enzymes responsible for heroin metabolism in rat liver was investigated. This was done by using three different liver fractions: microsomes which are derived from the endoplasmic reticulum, cytosol which is the intracellular fluid and s9 which contain both cytosol and microsomes. To our knowledge the use of liver fractions to study heroin metabolism has not been done previously. As rat and human liver fractions can be bought commercially, this is a convenient way of investigating the metabolism of drugs. This study was thus also an attempt to establish a new method of studying the metabolism of heroin in vitro.

The results indicated that all the liver fractions studied were capable of hydrolyzing heroin to 6MAM and further to morphine to some extent. However, in microsomes heroin was completely hydrolyzed to 6MAM twice as fast as compared to s9 and twelve times faster compared to cytosol. This indicates that esterases located in the microsomal fraction of the liver are more abundant and/or more efficient in hydrolyzing heroin. This observation is in agreement with previous work [116, 117] where liver CE has been found to be localized on the luminal surface of the endoplasmic reticulum membrane.

However, establishing this method was associated with some challenges. The total level of heroin and heroin metabolites in microsomes and s9 was severely reduced (approximately 60%) during the experiment. This happened to a lesser extent in cytosol where only a reduction of approximately 7% was observed. Several control experiments were carried out to investigate whether this could be caused by metabolites binding to the plastic in the experimental tubes or being captured in the pellet, or whether the metabolites were degraded by the precipitation method. All these hypotheses were negative. It was then speculated if 6MAM and/or morphine was converted to a metabolite that was not included in the chosen experimental setup. Consequently, a time-of-flight mass spectrometry was carried out to analyze all molecules present in the sample simultaneously. The result showed the presence of nor-morphine, which may explain the loss of metabolites, as morphine can be N-demethylated by hepatic CYP3A4 and CYP2C8 to nor-morphine [32]. In microsomes and s9 these enzymes are abundant [118]. This may lead to a larger degree of CYP metabolism of morphine in microsomes and s9 compared to cytosol where these enzymes are not present. Additionally, UDPGA is a cofactor necessary for optimal glucuronidation by UGT [119, 120], the enzyme
responsible for metabolizing morphine [32]. This cofactor lacks in the microsomal and s9 fractions [121] possibly causing morphine metabolism by UGT to be unable to compete with the CYP enzyme pathway. The lack of UDPGA could explain why a similar reduction in metabolites was not seen in crude liver homogenate. Unfortunately, it was not possible within the time frame of this thesis to establish a method to quantify the nor-morphine content of the samples. For further studies UDPGA could be added to the enzyme assay for microsomes and s9 to achieve optimal UGT activity and hence resemble physiological conditions.

4.4 Inhibition of heroin metabolism in vivo

A behavioral test measuring locomotor activity, combined with measurements of heroin metabolites in blood and brain tissue were used to assess the effect of esterase inhibitors on the heroin metabolism in vivo. The aim was to reduce the brain levels of the potent heroin metabolite 6MAM.

The locomotor activity test is a well-established model for investigating the acute stimulating effects of different drugs [122, 123]. Administration of heroin, with a following increase in dopamine release in the nucleus accumbens, is known to produce an immediate dose-dependent increase in locomotor activity [18]. In C57BL/6-mice, the immediate rise in locomotor activity after heroin administration is mediated by 6MAM, while morphine is involved later in the response [27, 30].

In the first experiment, heroin was administrated s.c. which has been the main administration method of choice in previous studies of heroin effects [27, 124, 125]. The results from this experiment showed significantly higher concentrations of 6MAM in both blood and brain after pre-treatment with the inhibitor iso-OMPA. However, there were no measured changes in locomotor activity for these animals. Since the aim was to inhibit the enzymatic reaction from heroin to 6MAM, with the objective to reduce 6MAM levels in the brain the result was opposite of what was expected. This could be caused by the s.c. administration of heroin, which could subject the drug to increased peripheral metabolism at the injection site [126] so that heroin already was metabolized to 6MAM before entering the bloodstream and reacting with the enzyme inhibitor. Hence, the inhibitor seems to have acted on the second step of the heroin metabolism, impeding the transition of 6MAM to morphine. To circumvent the peripheral metabolization, the administration route of heroin was changed to i.v. injection in
the following experiment. This resulted in no significant differences in concentration of heroin metabolites in the brain for any of the pre-treatments with inhibitors, only in blood.

Some of the mice administered the AChE inhibitor Bw284c51 displayed a significant reduction in locomotor activity. The mice showed typical signs of cholinergic syndrome, including whole body tremors and prostration. This is a condition caused by accumulation of excessive levels of acetylcholine [127], and was probably caused by the inhibitor.

Since the effect of heroin is mediated through the presence of active metabolites in the brain [29], the results showed that the esterase inhibitors had no clear effect on the biological response of heroin in vivo. This could be caused by the mainly competitive mechanism of the enzyme inhibitors [39]. Because the substrate and inhibitor compete for binding to the catalytic site of the enzyme, this type of inhibition can be overcome by a high substrate concentration. It is also possible that the esterases have a greater affinity for heroin than the inhibitors, which would further favor hydrolysis of heroin. Therefore, it might not be possible to reach concentrations high enough to affect heroin metabolism in vivo with these enzyme inhibitors, at least not without having toxic side effects. A non-competitive inhibitor could possible work better to decrease the concentration of 6MAM, since such an inhibition is not affected by substrate concentration.

Other studies using iso-OMPA [62, 82, 84] and BNPP [128, 129] in the same or lower concentrations in vivo have successfully measured significant inhibition of the activity of esterase enzymes in blood and/or brain. However, even though the experimental conditions are quite similar, they are different in one or more important factors such as the time intervals following pre-treatment with inhibitor, administration method of inhibitor and/or different experimental species. For BW284c51 the literature is even less conclusive about doses measured to inhibit AChE in mice in vivo. Therefore, for further studies the enzyme activity could be determined by sampling blood from mice to assess whether the esterase inhibition is successful. This can be done by for example microtiter plate-based assay [114] for CE and the Ellman method for BChE and AChE [130] which are both established methods.
4.5 Species differences in heroin metabolism

Some of the advantages of the use of rodents as a model for drug development are a metabolic pathway similar to humans and numerous similar anatomical and physiological characteristics [91]. However, both the rate of drug metabolism and the metabolites formed may differ between animal species. The main reasons for these discrepancies include different metabolic capacity and different enzyme activity [131]. It is thus important to identify these factors when using rodents to predict the metabolism of a drug in humans.

The activity of CE in the liver is similar in rodents and humans [96]. In agreement with our results from rat liver, human hCE-1 and in part hCE-2 is present in the human liver and is capable of deacylating heroin to 6-MAM [97, 132, 133]. However, our results indicated that AChE was taking part in the metabolism of heroin in rat brain, while Salmon et. al (41) found that the human brain synaptic form of AChE was not capable to hydrolyze heroin and 6MAM to morphine. Rats and mice are also known to have high activity of CE in plasma [96, 134, 135] and this enzyme is thought to hydrolyze heroin to 6MAM. Human plasma contains mostly BChE [96, 136] and in human blood the hydrolysis of heroin is thought to be catalyzed by erythrocyte AChE and serum BChE [31]. Blood erythrocyte AChE is found capable of further hydrolyzing 6MAM to morphine, while BChE is not [31].

4.6 Potential pharmacokinetic treatment strategies for heroin addiction

When looking for pharmacokinetic treatment options for heroin addiction, the aim is to reduce the rapid and high brain concentration of the first heroin metabolite 6MAM. This is challenging due to the extremely fast conversion from heroin to 6MAM, and then the much slower conversion of 6MAM to morphine. This renders high blood levels of the dominant active metabolite 6MAM to cross the blood-brain barrier and exhibit its strong rewarding and addictive effect in the brain [27]. By hindering the rewarding effect of the drug, it may be possible to support a change in behavior which could lead to recovery. In this thesis it was investigated if a reduced 6MAM concentration could be achieved by inhibiting the hydrolysis from heroin to 6MAM. The results indicate that this is associated with some challenges. However, there are several alternative pharmacokinetic approaches to reduce the levels of 6MAM in the brain. One possible alternative is to act directly on 6MAM itself by blocking its actions or to speed up the clearance of 6MAM from the body. The first possibility is already being studied by developing antibodies which binds to 6MAM and
thereby restrict its entry into the brain [137-140]. Another possibility is to increase the metabolism of 6MAM to morphine by adding enzymes with increased catalytic efficiency. In human blood AChE is found to hydrolyze 6MAM to morphine at the low rate of 0.1 nmol/min (Salmon et al. 1999). Increasing this metabolism could hinder the accumulation of high levels of 6MAM. This option is already being studied in cocaine treatment with mutant enzymes [141-143]. The fact that cocaine, heroin and probably 6MAM are metabolized by the same esterase enzymes [144] indicates that such a pharmacokinetic approach could also be a possible strategy for the treatment of heroin addiction.

5. Conclusions

Heroin metabolism appears to be catalyzed mainly by AChE and BChE in rat brain and by CE in rat lung and liver. The calculated half-lives show that brain and lung tissue has significantly less capacity to hydrolyze heroin compared to liver. Several esterase inhibitors produced high grades of inhibition of the heroin metabolism in vitro; however these results were not reproduced in vivo. This could be because higher concentrations of the inhibitors are needed to significantly inhibit the enzymes. At this initial stage enzyme inhibition as a strategy to reduce the levels of the potent metabolite 6MAM seems challenging. Increased knowledge of the enzymes involved in metabolism of heroin is important and could thus lead to other pharmacokinetic treatment approaches, such as increased heroin metabolism by addition of mutant enzymes.
6. References


Tsuijita, T. and H. Okuda, Acetylcholinesterase knockout mouse.

Alston, T., 1969.


7. Appendix

7.1 Inhibitor studies of heroin metabolism in rat blood

Bogen et al. have studied heroin metabolism in absence and presence of esterase inhibitors in rat blood (unpublished results) using the same methods as used in this master thesis. These results are included to be able to compare heroin metabolism in blood with liver, lung and brain tissue. The enzyme inhibitor experiments in rat blood were conducted as described in materials and methods section 2.4.4 with some modifications: The blood was fresh and was diluted 1:3 in NaCl and the incubation time was 3 min. The results are shown in figure 7.1 and 7.2.

**Figure 7.1.** Concentrations of heroin, 6MAM and morphine in rat blood after addition of heroin (0.4 μM) in vitro in the presence of the cholinesterase inhibitors (10 μM or 100 μM); BW (BW284c51), ETZ (ethopropazine), Iso (iso-OMPA) and Neo (neostigmine). The incubation time was 3 min. Values significantly different from the control are indicated with asterisks (*p value<0.05, **p value<0.01, ***p value<0.001). All values are presented as mean ± SEM, n =3-6. Tested for significance by univariate General Linear Model. Each metabolite analyzed separately. Tukey’s test was used as a post-hoc test.
Figure 7.2. Concentrations of heroin, 6MAM and morphine in rat blood after addition of heroin (0.4 μM) in vitro in the presence of the CE inhibitors (10 μM or 100 μM): Bn-isatin (benzylisatin) and BNPP. The incubation time was 3 min. Values significantly different from the control are indicated with asterisks (*p value<0.05, **p value<0.01, ***p value<0.001). All values are presented as mean ± SEM, n =3-4. Tested for significance by univariate General Linear Model. Each metabolite analyzed separately. Tukey’s test was used as a post-hoc test.
7.2 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 150 mm)
Flow rate: 0.2 ml/min
Column temperature: 50°C
Run time: 16 min
Software: Mass Lynx

Table 7.1 - HPLC pump gradient timetable

<table>
<thead>
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<th>Time (min)</th>
<th>A %</th>
<th>B %</th>
<th>Flow</th>
</tr>
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<tr>
<td>0.00</td>
<td>3.0</td>
<td>97.0</td>
<td>0.200</td>
</tr>
<tr>
<td>8.00</td>
<td>60.0</td>
<td>40.0</td>
<td>0.200</td>
</tr>
<tr>
<td>10.00</td>
<td>60.0</td>
<td>40.0</td>
<td>0.200</td>
</tr>
<tr>
<td>10.00</td>
<td>3.0</td>
<td>97.0</td>
<td>0.300</td>
</tr>
<tr>
<td>16.00</td>
<td>3.0</td>
<td>97.0</td>
<td>0.300</td>
</tr>
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</table>

**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 reactions monitoring (MRM) was used for quantification. The source block temperature was 120°C and the capillary voltage was 2 kV. The cone gas (N2) was heated up to 400 °C and the flow was set to 50 L/h. The desolvation gas (N2) was delivered at flow 900 L/h.
Table 7.2 - LC-MS/MS method

<table>
<thead>
<tr>
<th>Substance</th>
<th>Rt (min)</th>
<th>MRM 1 (m/z)</th>
<th>MRM 2 (m/z)</th>
<th>Cone voltage (V)</th>
<th>Collision energy (eV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M3G</td>
<td>2.7</td>
<td>462.0 &gt;286.0</td>
<td>462.0 &gt;268.0</td>
<td>45</td>
<td>30/30</td>
</tr>
<tr>
<td>Morphine</td>
<td>4.6</td>
<td>286.0 &gt;201.0</td>
<td></td>
<td>45</td>
<td>20</td>
</tr>
<tr>
<td>6MAM</td>
<td>8.1</td>
<td>328.0 &gt;211.0</td>
<td></td>
<td>45</td>
<td>25</td>
</tr>
<tr>
<td>Heroin</td>
<td>9.6</td>
<td>370.0 &gt;268.0</td>
<td></td>
<td>50</td>
<td>30</td>
</tr>
</tbody>
</table>

**Internal standard**

<table>
<thead>
<tr>
<th>Substance</th>
<th>Rt (min)</th>
<th>MRM 1 (m/z)</th>
<th>Cone voltage (V)</th>
<th>Collision energy (eV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M3G-d3</td>
<td>2.7</td>
<td>465.0 &gt; 289.0</td>
<td>50</td>
<td>30</td>
</tr>
<tr>
<td>Morphine-d6</td>
<td>4.6</td>
<td>292.0 &gt; 201.0</td>
<td>45</td>
<td>25</td>
</tr>
<tr>
<td>6MAM-d6</td>
<td>8.1</td>
<td>334.0 &gt; 211.0</td>
<td>45</td>
<td>25</td>
</tr>
<tr>
<td>Heroin-d9</td>
<td>9.6</td>
<td>379.0 &gt; 272.0</td>
<td>45</td>
<td>28</td>
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</table>

Table 7.3 – The limits of detection (LOD) and the limits of quantitation (LOQ)

<table>
<thead>
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<th>6MAM</th>
<th>Morphine</th>
<th>M3G</th>
</tr>
</thead>
<tbody>
<tr>
<td>LOD Blood (mg/L)</td>
<td>0.00096</td>
<td>0.00033</td>
<td>0.00049</td>
<td>0.0065</td>
</tr>
<tr>
<td>LOQ Blood (mg/L)</td>
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<td>0.00065</td>
<td>0.0012</td>
<td>0.019</td>
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<tr>
<td>LOD Brain tissue (μg/g)</td>
<td>0.0029</td>
<td>0.0010</td>
<td>0.0015</td>
<td>0.020</td>
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<tr>
<td>LOQ Brain tissue (μg/g)</td>
<td>0.0077</td>
<td>0.0022</td>
<td>0.0036</td>
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