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DEVELOPMENT OF A LC/MS-MS METHOD FOR THE STUDY OF THE RATIOS BETWEEN MORPHINE, MORPHINE-3-β-D-GLUCURONIDE AND MORPHINE-6-β-D-GLUCURONIDE IN BLOOD SAMPLES FROM HEROIN FATALITIES

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Abstract

In heroin fatalities the diagnosis of the cause of death may be particularly difficult because of several reasons, such as the relationship between lethal dose and current individual tolerance, the complexity of heroin metabolism, the presence of systemic dysfunction, and the contemporary use of other drugs of abuse. Thus, a wide variability is present in post-mortem blood concentration of morphine (MOR), the main metabolite of heroin, which is usually the most important analytical result for the interpretation of the cause of death. Recently, increasing interest has grown towards the role of the metabolites morphine-3-β-D-glucuronide (M3G) and morphine-6-β-D-glucuronide (M6G) in mediating heroin effects. The aim of this PhD study has been the development of a LC/MS-MS method for the determination of MOR, M3G and M6G in autopsy blood samples. An ESI-QqQ Mass Spectrometer, operating in positive ionisation and MRM mode, was used. Chromatographic separation was achieved thanks to a Reversed-Phase method, using a C_{18} column and a gradient elution with a binary mobile phase. SPE technique was employed to extract the analytes from biological samples. After validation, the method was applied to twenty-five blood specimens collected from cases of suspected fatal heroin overdose. The concentrations and the molar ratios of MOR, M3G and M6G were investigated. The influence of some risk factors, such as the contemporary use of alcohol, methadone or cocaine, was also studied.

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1. Introduction

1.1 HEROIN: PAST, PRESENT AND FUTURE

1.1.1 THE DISCOVERY AND THE MEDICAL USE

Heroin (diacetylmorphine) was first marketed by Bayer in 1898 as an antitussive drug for patients with asthma and tuberculosis, with the idea that it would make a valuable contribution to medicine. The name of the drug reflects this belief, since the German term “heros” refers to an ancient Greek hero who was honoured as a demigod because of his deeds.

Fig.1: Bayer advertisement for heroin and other drugs, 1900.

However, heroin was not an original discovery by the Bayer team. When, in 1862, Augustus Matthiessen was appointed as lecturer in chemistry at St Mary’s Hospital Medical School in London, his research focused on the opium alkaloids. Later, he was joined by Charles Alder Wright who, after Matthiessen’s death, synthesized several morphine esters, including acetylcodeine, acetylmorphine, and diacetylmorphine (in 1874). The initial pharmacological tests of the hydrochlorides of these new esters were inconclusive, so that these molecules were not immediately developed as drugs.

Some years later, the Bayer team started working to acetylated derivatives from different compounds, such as tannic and salicylic acids, in order to develop molecules without the common side effects of nausea

and vomiting. In this context, Felix Hoffmann synthesized diacetylmorphine on August 21th 1897, as recorded in his laboratory notebook, two weeks after he had synthesized acetylsalicylic acid. To assess heroin value as a substitute for codeine in severe coughing, studies were carried out in rabbits and in humans too. The Bayer team found that this new molecule was able not only to relieve cough, but also to both slow and deepen respiration, having therefore a stimulant action on the respiratory system.

Heroin was therefore marketed in June 1898, with the name of *acetomorphine*, as a cough suppressant to assist breathing in severe lung diseases, but it was soon used in neurological and gynaecological illnesses and for pain too. The new compound acquired the reputation of being no more addictive than codeine, since it was used in smaller doses and in chronic diseases, thereby hiding withdrawal symptoms. This belief and the idea that heroin had a stimulant action on respiratory system were confuted by other studies within few years, but in the meanwhile several pharmaceutical preparations were developed and sold over the counter, so that heroin became one of the most common drugs. After few years heroin revealed however heavy collateral effects, such as phenomena of strong habituation and addiction, and tolerance(see paragraph 1.2.7). In 1912 Phillips published a report about cases of heroin addiction among people who sniffed the drug. This fact, coupled with a widespread anti-German sentiment, led the USA Congress to introduce federal narcotic controls and to limit the maximum amount of heroin in proprietary preparations to less than 10 mg per g of product (Harrison Act, Dec 1914). Finally, USA banned the medical use of heroin in 1924. Many other countries withdrew heroin from the market, but not the United Kingdom. In that country, the epidemic of heroin abuse recorded in the USA during the early years of the XX century did not occur. As a consequence, no outright ban of this drug was introduced, even if more and more warnings about the risk of addiction were issued in the *British Pharmaceutical Codex* (Sneader, 1998).

Nowadays, heroin is still employed in some countries, with the name of *diamorphine*, as analgesic drug (United Kingdom) or in treatment

programs for heavily dependent narcotic addicts (Belgium, Denmark, Germany, the Netherlands, Spain, Switzerland, United Kingdom). On the contrary, from 1957 heroin is no more included in Italian Pharmacopeia, being conversely present in the list of illicit drugs (Table I, DPR 309/90, modified by the L.49/06).

1.1.2 CURRENT EPIDEMIOLOGY OF HEROIN ABUSE

Although the medical use of heroin was dismissed in most countries some decades ago, this substance has kept a worldwide diffusion up to now. Unfortunately, abuse and illicit traffic have supported this use, which has been closely associated with public health and social problems at first in the USA, then in Europe (since the 1970s) and in other continents.

According to the Annual Report 2011 of the EMCDDA (European Monitoring Centre for Drugs and Drug Addiction), heroin is still responsible for the greatest share of morbidity and mortality related to drug use in the European Union. After two decades (from late 1970s to early 1990s) of mostly growing heroin problems, the use of this drug and the associated harm have decreased in Europe during the late 1990s and the early years of the present century. Since 2003-2004, however, the trend has become less clearly defined, with indicators suggesting a more stable or mixed picture. Heroin consumed in Europe is produced primarily in Afghanistan, which accounts for most of the global illicit opium output. The other producing countries are Burma/Myanmar, which mainly supplies markets in east and south-east Asia, Pakistan and Laos, followed by Mexico and Colombia, which are considered the largest suppliers of heroin to the USA (World Drug Report 2011, United Nations Office on Drugs and Crime-UNODC). Global opium production has decreased from 2007, essentially because of a decline in Afghanistan production. Heroin arrives in Europe by two trafficking routes. The historically important Balkan route brings heroin produced in Afghanistan through Pakistan, Iran and Turkey, and then towards other transit or destination countries, mainly in western and southern Europe. Heroin is also trafficked via the ‘silk route’ through central Asia and towards Russia. To a limited extent,

this heroin is then smuggled through Belarus, Poland and Ukraine to other destinations such as Scandinavian countries via Lithuania. Within the European Union, the Netherlands and, to a lesser extent, Belgium play a key role as secondary distribution hubs.

EMCDDA defined “problem drug use” as injecting drug use or long duration/regular use of opioids (i.e. heroin or other molecules with similar pharmacological activity; see also paragraph 1.2.5), cocaine and/or amphetamine. In most countries, this phenomenon is more common in urban areas and among socially excluded groups, and heroin misuse accounts for the greater part of this abuse. Nonetheless, it must be noticed that problem drug users are mostly polydrug users. In Europe, the overall prevalence of problem drug use is reported to range from 2 to 10 cases per 1000 population aged 15-64, with wide differences among the countries. Such estimates may have large uncertainty ranges and specific limitations, since they come from statistical extrapolations of law enforcement and drug treatment data.

Several studies have indicated that the characteristics of opioid users have changed in the last years. This population has become older on average, the proportion of injectors has decreased and the proportion of users of opioids other than heroin and of polydrug users has increased. However, injecting is still the usual mode of administration of heroin. This behaviour is associated with a high risk of experiencing health problems, such as blood-borne infections (e.g. HIV/AIDS, hepatitis) or drug overdoses. In addition to active injectors, there is a large number of former injecting drug users, who may suffer from health problems because of their past drug use (OEDT, 2011).

1.1.3 HEROIN-RELATED DEATHS

Drug use is one of the major causes of health problems and mortality among young people in Europe, and can account for a considerable proportion of all deaths among adults. In detail, studies have found that between 10% and 23% of mortality among those aged 15-49 could be attributed to opioid use. Overall mortality related to heroin comprises drug-induced deaths and those indirectly caused, such as through the

transmission of infectious diseases, chronic health problems, traffic accidents, violence and suicide. Deaths indirectly related to drug use are difficult to quantify, but their impact on public health can be considerable. Such deaths are mainly concentrated among problem drug users, although some (e.g. traffic accidents) occur among occasional users (OEDT, 2011). Opioids, primarily heroin, are the main cause of drug-induced deaths. In early 1990s fatal opioid overdose emerged as a major public health problem internationally, with a tripling of the overdose rate within few years in Australia, USA, Spain, Italy, Austria, England and Wales. On the other hand, drug-related sudden deaths have decreased in France, Belgium and Germany since 1991, and appeared to have stabilized or declined since 1995 in the Netherlands, Portugal, Finland, Italy, Luxembourg, France, Switzerland and Spain (Warner-Smith *et al.*, 2001). In Italy a further decrease have occurred in the last years, being fatal drug overdoses 1002 in 1999 and 374 in 2010 (Fig.2; font: Relazione annuale al Parlamento 2011, Dipartimento delle Politiche Antidroga).

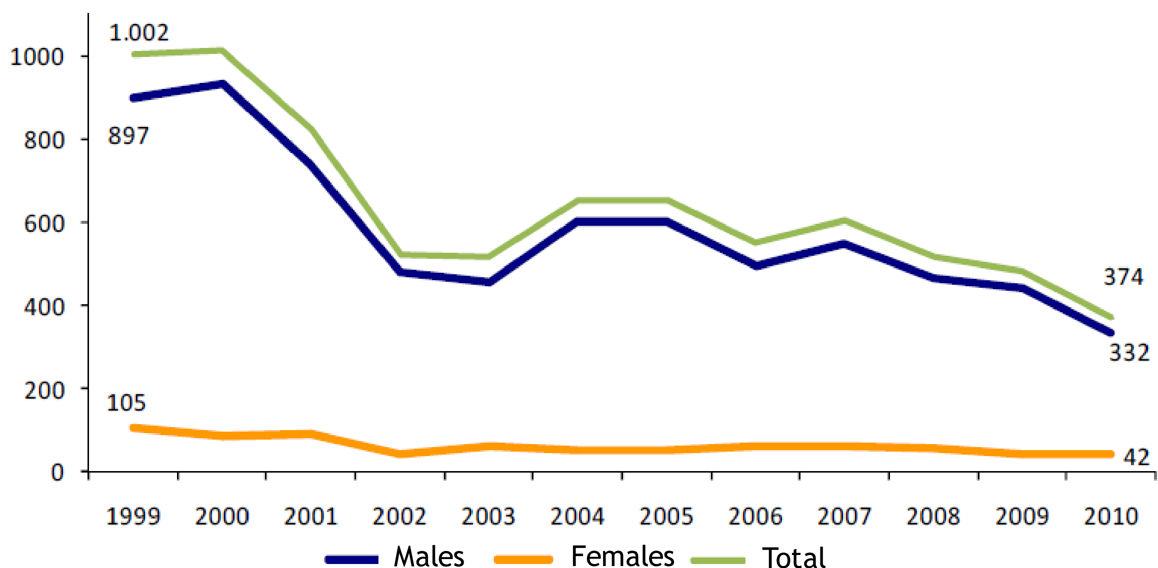

Fig.2: Trend of fatal drug overdoses in Italy from 1999 to 2010.

In most European countries the average age of people dying of heroin overdoses is the mid-30s (37 years in Italy), and in many cases it is increasing. This suggests a possible stabilisation or decrease in the number of young heroin users, and an ageing cohort of problem opioid

users. Only 12% of overdose deaths reported in Europe occur among those aged under 25 years. Moreover, men account for most heroin overdose deaths in Europe (81%).

A number of factors are associated with fatal and non-fatal heroin overdoses (see paragraph 1.3.2). These include injection and simultaneous use of other substances, in particular alcohol, benzodiazepines and some antidepressants. Other factors linked with overdoses are binge drug use, co-morbidity, homelessness, poor mental health (e.g. depression and intentional poisoning), not being in assistance treatment, previous experience of overdose and being alone at the time of overdose. The time immediately after release from prison or discharge from drug treatment is a particularly risky period for overdoses, as illustrated by several studies (Sporer, 1999; OEDT, 2011).

1.1.4 HARM REDUCTION AND ASSISTANCE PROGRAMS

Beside social-educational programs to prevent drug abuse, reducing the mortality and morbidity related to drug use is a central issue of drug policies. The principles of harm reduction have been applied to heroin users through needle-exchange programs to reduce HIV infection. Education about risk factors, such as the polydrug use and the loss of tolerance after a withdrawal period, as well as encouragement of use of the emergency system, may help reduce heroin overdoses. The concept of "take home" naloxone (an opioid antagonist used as antidote in opioid overdose; see paragraph 1.2.6) as a method of preventing heroin overdose–related deaths was discussed, but its use raised several ethical issues and practical complications. The availability of naloxone may remove the deterrent effect of heroin dosing, thus inadvertently increasing the number of overdoses. On the other hand, the withdrawal symptoms (see paragraph 1.2.7) caused by naloxone may make drug addicts reluctant to use it even if available. Moreover, patients may be unwilling to go to a hospital for observation after successful resuscitation at home; as a result, patients with pulmonary and other complications will not receive timely medical care. Finally, the occasional seizure and other complications of naloxone use will occur in a less controlled environment (Sporer, 1999).

Nonetheless, the potential opportunity to prevent thousands of heroin overdoses has been investigated. Nowadays, some countries report the existence of community-based programs that prescribe naloxone to drug users at risk of opioid overdose. Naloxone prescribing is accompanied by compulsory training in recognising overdoses, providing basic life-support techniques (e.g. rescue breathing, recovery position) and correctly administering naloxone. This intervention targets drug users, their families and peers, and aims to help them to take effective action in overdose situations, while awaiting the arrival of emergency services. In Europe, the distribution of naloxone to drug users is reported by some countries, such as Italy, Germany, United Kingdom (England and Wales), Bulgaria, Denmark and Portugal. In Scotland, provision of 'take-home naloxone' to all at-risk individuals leaving prison was nationally introduced in 2010.

In Europe, heroin is the primary drug for entering assistance treatment, even if considerable differences exist across the countries. Assistance treatment is usually necessary to give up heroin use, since this drug causes a severe psychological and physical dependence (see paragraph 1.2.7). In most cases the use of a secondary drug, often alcohol, cannabis, cocaine or other stimulants, often occurs, as previously described. The combination of heroin and cocaine (including crack) is quite common among clients, either injected together or used separately.

Both drug-free and substitution treatment for opioid users are available in all European Union Member States, Croatia, Turkey and Norway. In most countries, treatment is conducted in outpatient settings, which may include specialised centres, general practitioners' surgeries and low-threshold facilities. In a few countries, specialist inpatient communities play an important role in the treatment of opioid dependence. As previously mentioned, a small number of countries (Belgium, Denmark, Germany, the Netherlands, Spain, Switzerland, United Kingdom) offer heroin-assisted treatment for a selected group of chronic heroin users.

For opioid users, treatment is generally preceded by a detoxification program, which provides them pharmaceutical assistance to manage the physical withdrawal symptoms (see paragraph 1.2.7). This therapeutic approach generally requires individuals to abstain from all substances, including substitution medication. Patients participate in daily activities and receive intensive psychological support. Substitution treatment, generally integrated with psychosocial care, is typically provided at specialist outpatient centres. It is estimated that about half of problem opioid users have access to substitution treatment in the European Union, a level that is comparable to those reported for Australia and the USA, though higher than that reported for Canada. China reports much lower levels, while Russia, despite having the highest estimated number of problem opioid users, has not introduced this type of treatment yet.

In Europe, methadone (an opioid agonist; see paragraph 1.2.6) is the most commonly prescribed substitution medication, received by up to three quarters of clients. Buprenorphine (an opioid partial agonist) is prescribed to up to a quarter of European substitution clients, and is the principal substitution drug in the Czech Republic, France, Cyprus, Finland, Sweden and Croatia. The combination buprenorphine/naloxone is available in some countries. Treatments with slow-release oral morphine, codeine (Germany, Cyprus) and diacetylmorphine (Belgium, Denmark, Germany, Spain, Netherlands, United Kingdom) represent a small proportion of all treatments (OEDT, 2011). Opioid substitution treatment, combined with psychosocial interventions, is considered to be the most effective treatment option for opioid dependence. In comparison with detoxification or no treatment at all, both methadone and high dosage buprenorphine treatments show better rates of retention in treatment and significantly better outcomes for drug use, criminal activity, risk behaviours and HIV-transmission, overdoses and overall mortality (World Health Organisation-WHO, 2009).

The opioid receptor antagonist naltrexone has also been proposed to prevent relapse to opioid use. However, there is a theoretical risk of hypersensitivity to overdose following naltrexone maintenance. This,

combined with the well-recognized risk of overdose due to normal loss of tolerance after periods of abstinence, suggests that naltrexone maintenance therapy may in fact be a risk factor for overdose (Warner-Smith *et al.*, 2001).

1.2 HEROIN: CHEMISTRY AND PHARMACOLOGY

1.2.1 CHEMICAL PROPERTIES

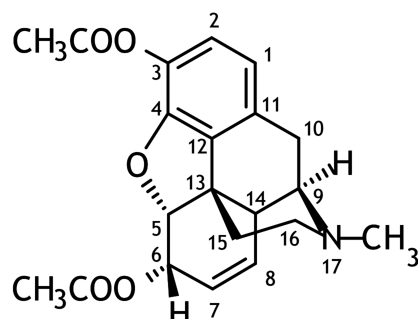

Fig.3: Chemical structure of heroin.

(5α,6α-7,8-Didehydro-4,5-epoxy-17-methylmorphinan-3,6-diol diacetate ester)

Heroin ($C_{21}H_{23}NO_5$=369.42; diacetylmorphine, *acetomorphine*, *diamorphine*) is a white crystalline powder, with a melting point (m.p.) of 173°C, a partition coefficient (octanol/water) (LogP) of 1.58, and a solubility of 1/1700 in water, 1/31 in ethanol, 1/1.5 in chloroform, and 1/100 in ether. In most cases, pharmaceutical or illicit heroin is in the form of heroin hydrochloride ($C_{21}H_{23}NO_5 \cdot HCl \cdot H_2O$=423.87), an almost white crystalline powder, which has a m.p. of 229-233° and is soluble 1/2 in water, 1/11 in ethanol, 1/1.6 in chloroform, and insoluble in ether.

The groups at the 3- and 6- positions make heroin much more lipophilic than morphine, being the ionisation constant (pK_a) of heroin 7.6 instead of a value of 9.4 for morphine. As a consequence, on average 40% of heroin will be in a non-ionised form at physiological pH, being accessible for membrane-transport especially through the Blood Brain Barrier (BBB) (Rook *et al.*, 2006). Heroin ester bonds are rapidly hydrolysed in aqueous solution or in plasma at room temperature, while their stability is improved at pH 3.5-5.2 and at temperatures below 4°C.

1.2.2 "STREET HEROIN"

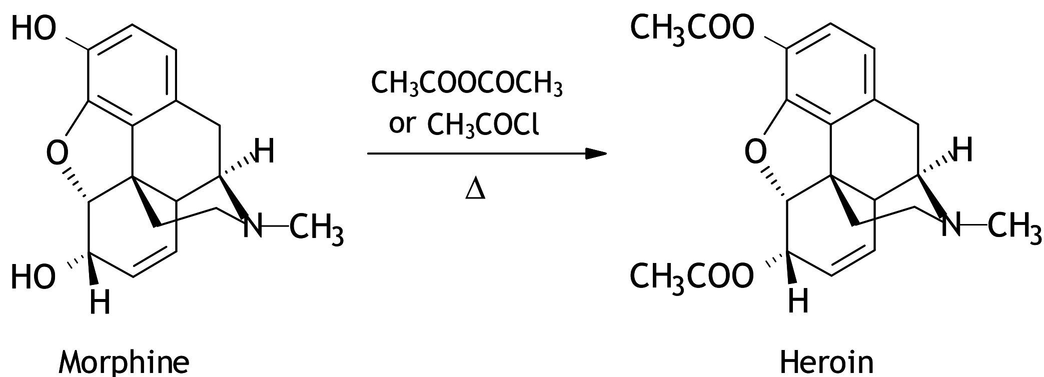

Fig.4: Synthesis of heroin from morphine.

Heroin is a semi-synthetic derivative of morphine, the main alkaloid of opium, a naturally occurring product of the *Papaver somniferum* L. (opium poppy). The green seed heads of this plant are cut to release a milky latex, which dries on the seed head to form a gum and is then collected and bulked as raw opium. Raw opium is treated to extract morphine, which is then acetylated at high temperatures with acetic anhydride (or acetyl chloride) to produce heroin (Fig.4). Acetylation initially occurs at position 3, the phenolic-hydroxyl group of the morphine molecule, and subsequently at position 6, the alcoholic-hydroxyl group. The mixture is boiled for three-four hours and, after cooling, water and Na_2CO_3 are added to precipitate heroin base. Once filtered and dried, heroin base is converted to heroin chloride. This synthesis requires neither complex equipments nor wide knowledge in chemistry, so that it is easily carried out in clandestine laboratories.

Bulk shipments of heroin may be packaged as rectangular blocks or in other configuration and street-level heroin may be a quite rough powder of different colours (Fig.5) according to the purity of the precursor morphine, with a particular sharp smell due to acetic acid used in its synthesis. The content in heroin varies according to the amount of cutting substances added. Heroin coming from the clandestine laboratories has usually a purity of 55-60%, since it contains some minor by-products from origin and/or manufacturing, such as 3-monoacetylmorphine,

acetylcodeine, noscapine, narcotine, papaverine and thebaine. Finding these substances may be important to have information about where the illicit drug has been manufactured, and to compare material from different seizures. Both inert substances, such as sugars, sodium bicarbonate, talcum, caolin, and drugs, especially acting on Central Nervous System (CNS) (adulterants), like caffeine, paracetamol, procaine, lidocaine and strychnine, are then added to heroin. Finally, also coffee, tea, chamomile or other aromatic compounds can be used. The goal of this process is to reduce the content of heroin to increase the gain, but also to elicit drug effects thanks to adulterants.

Fig.5: “Street heroin” powders.

The so-known “Chinese Heroin”, which usually comes from south-east Asia, is a white powder since it is formed by heroin hydrochloride and minor amounts of other opium alkaloids and adulterants. Therefore, this kind of heroin is ideally suited for injection. Conversely, heroin from south-west Asia is a much cruder product, typically seen as a brown powder, which contains diacetylmorphine base with variable amounts of other opium-derived alkaloids and adulterants (e.g. caffeine, paracetamol, dextromethorfan) (Fig.5; Moffat *et al.*, 2004).

1.2.3 ROUTES OF ADMINISTRATION

The main route of administration of heroin is intravenous injection, but also snorting, smoking and intramuscular injection often occur. Intravenous injection is the most common mode of administration, because about a half-dose is required compared to intramuscular

injection, and moreover heroin reaches more quickly the CNS (Savini *et al.*, 2006). The immediate effect of intravenous heroin is often described by heroin dependents as a "flash" or a "rush", a warm and intensively pleasant sensation. The flash is followed by an euphoric, benumbed state, which may be more related to heroin metabolites (Rook *et al.*, 2006). Pharmacokinetics parameters of heroin and its metabolites following different routes of administration have been determined in studies involving patients with chronic pain or cancer carried out in the United Kingdom, where heroin (as diacetylmorphine or *diamorphine*) is still registered for therapeutic purposes, or in heroin-assisted treatment programs in countries where its use for this purpose is allowed (see paragraph 1.1.4). Some of these studies have shown that considerable peak plasma concentrations of heroin and a fast absorption rate occur after intranasal application (snorting) or inhalation in the lung, with maximal heroin concentrations within 2-5 min and half-lives comparable to intravenous administration. As a matter of fact, heroin is rapidly absorbed through the mucous membranes thanks to its lipophilicity and low ionisation grade at physiological pH. Furthermore, the intranasal mucosa and the lung are well-perfused organs, and in particular the alveolar-capillary bed of the lung forms a very large area for absorption (approximately 100 m^2 in healthy male adults). It must also be considered that the first-pass effect by the liver is avoided in these routes of administration. In some studies on heroin snorters, plasma concentration-time profiles demonstrated a second peak, indicating that the heroin dose was partly swallowed and later absorbed from the gastro-intestinal tract (Rook *et al.*, 2006).

In a heroin-assisted trial for the treatment of heroin addiction in The Netherlands, pharmaceutically prepared heroin base was administered by smoking the agent from aluminium foil ("*chasing the dragon*"). In this technique, the heroin smoker keeps a lighter under a piece of aluminium foil filled with heroin base and the sublimated heroin fumes are inhaled by a straw in the mouth. The bioavailability of smoking heroin by this procedure was estimated between 38-53%, plasma peaks of heroin were 2-

4 times lower than after equivalent intravenous doses, but the “flash” effect was achieved. In contrast, when cigarettes containing both tobacco and heroin were smoked, a low recovery of 14% was found, probably because of disintegration of heroin due to high temperatures (Rook *et al.*, 2006). After intramuscular administration, peak plasma concentrations of heroin are significantly lower than after intravenous injection. However, heroin is not rapidly metabolised in the muscle tissue, and it is slowly released from this tissue into the circulation, so that its effects are protracted (Rook *et al.*, 2006). Oral and rectal administration of heroin were studied as non-invasive alternative for the long-term application within heroin-assisted treatment programs. Appropriate oral and rectal doses and dosage intervals were demonstrated adequate to produce “flash” and high effects without any cardiovascular and respiratory side-effects nor withdrawal symptoms (Gyr *et al.*, 2000). Higher doses are required for oral administration, because of the first-pass effect by esterases in the liver and the hydrolysis of heroin into morphine under the alkaline conditions of the duodenum and colon before absorption. After application of a heroin suppository, the first-pass mechanism by the liver is avoided to a high extent, resulting in scores on euphoria significantly higher than after an equal oral heroin dose (Rook *et al.*, 2006).

1.2.4 METABOLISM AND PHARMACOKINETICS

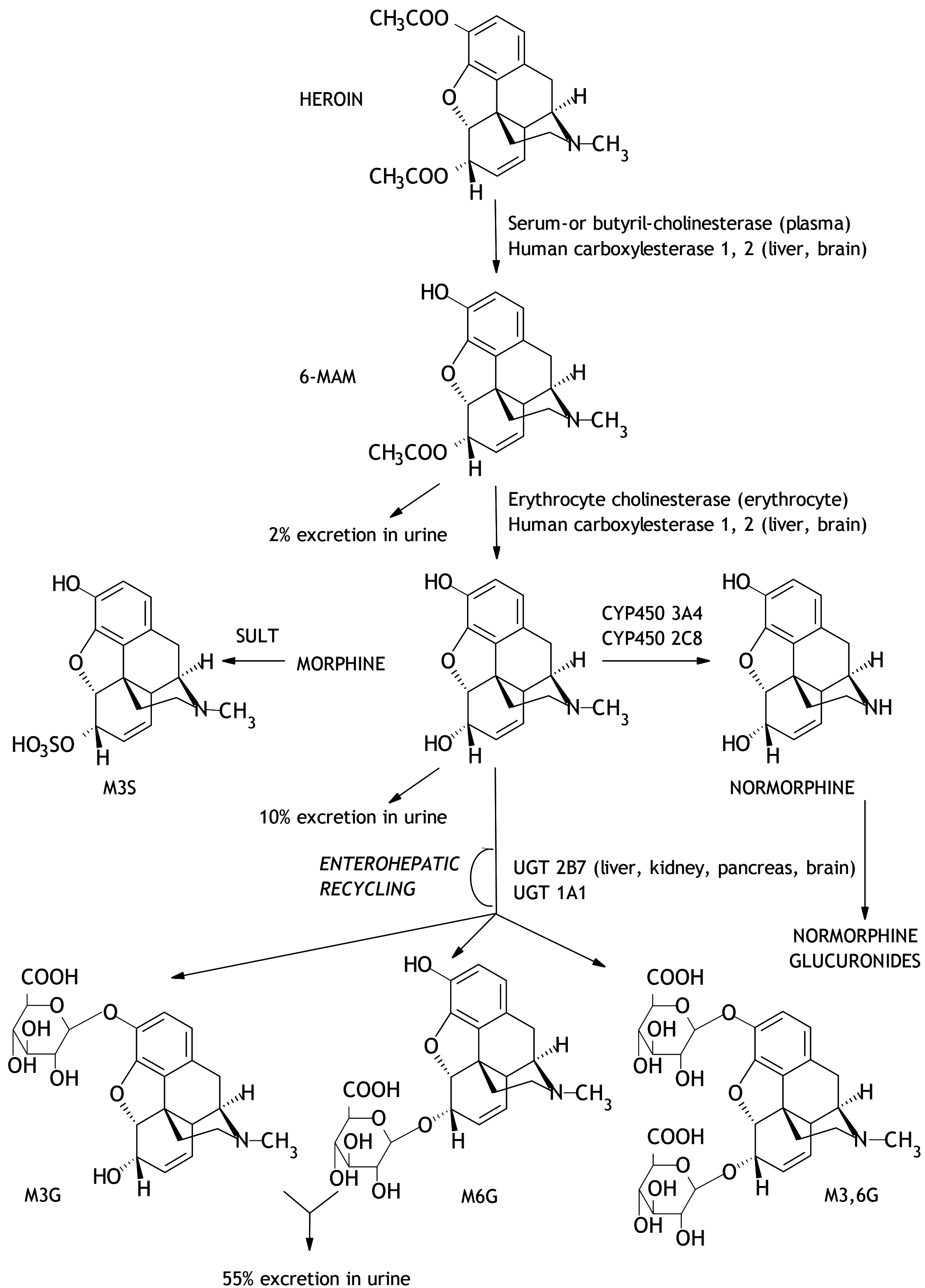

Fig.6: Overview of heroin metabolism.

In human plasma heroin is rapidly hydrolysed to 6-monoacetylmorphine (6-MAM) and finally to morphine, which are both

active molecules and therefore partly responsible for the pharmacological action. Morphine is in turn metabolised (98%) via conjugation with glucuronic acid at its 3- and 6-positions to form morphine-3-β-D-glucuronide (M3G) and morphine-6-β-D-glucuronide (M6G), with a M6G/M3G ratio of approximately 0.15. Morphine glucuronides are hydrophilic compounds, which are mainly excreted in urine, and to a lesser extent in bile. Other minor metabolites are normorphine, morphine-3-β-D,6-β-D-diglucuronide (M3,6G) and morphine-3-ethersulphate (M3S). After intravenous administration, about 70% of the total heroin dose is recovered in the form of its metabolites in urine, for the most part as morphine glucuronides (55%) (Fig.6). Heroin isn't usually found in urine, suggesting that this drug is virtually fully converted into its metabolites before renal excretion (Rook *et al.*, 2006).

The hydrolysis (phase I metabolism) of heroin and 6-monoacetylmorphine is catalysed by different esterases (Fig. 6), which are abundantly present in plasma and in tissues. Glucuronidation (phase II metabolism) occurs mainly in the liver, but it is also significant in brain, kidney and intestine. This reaction is catalysed by uridine-5'-diphosphate-glucuronosyl-transferases (UGT), primarily by the UGT 2B7 subtype and, to a lesser extent, by the UGT 1A1 subtype. As previously mentioned, M3G is normally more abundant than M6G, probably because of physico-chemical and steric issues that affect the binding of morphine to the phase II enzyme (Rook *et al.*, 2006). However, several studies have demonstrated that the M6G/M3G ratio can vary after exposure to "street heroin" or other substances (see paragraph 1.3.2), with consequences on the pharmacological action. *N*-Demethylation of morphine into the minor metabolite normorphine is mediated by cytochrome P450 enzymes 3A4 and 2C8.

Heroin binding to serum albumin or erythrocytes is comparable to that of morphine (20-40%); both molecules rapidly quit the circulation to accumulate in organs such as lung, kidney, liver, spleen and muscle tissue. However, heroin is able to pass the Blood-Brain-Barrier (BBB) much faster than morphine thanks to its lipophilicity, so that it gives rise

to a more intense and immediate effect. Heroin blood levels decline very rapidly after intravenous drug administration, becoming undetectable after 10-40 minutes. After intravenous administration, the half-life of heroin is about 1.3-3.8 minutes, while after intramuscular administration it can reach a value of about 8 minutes. Some studies have shown that heroin volume of distribution is around 60-100 L, while its clearance value could vary from 128 to 1939 L/h. The high clearance of heroin from plasma is due to the rapid elimination by esterases, the spontaneous hydrolysis of heroin at physiological pH and the extensive distribution (Rook *et al.*, 2006).

After heroin injection, 6-monoacetylmorphine reaches its maximal concentration at 0.7-2.7 minutes, having a mean half-life of about 30-40 minutes, and being detectable in plasma for 1-3 hours. According to some studies, 6-MAM has a clearance value of 564-607 L/h, following different routes of administration. This metabolite is in turn rapidly hydrolysed into morphine, which shows its maximal concentration between 3.6-8.0 minutes after heroin administration, and has a half-life of 100-280 minutes. These data are comparable with those from pharmacokinetic studies after morphine administration, suggesting that the formation of morphine from its precursor heroin is not the rate-limiting step in heroin metabolism. Finally, after heroin administration, the half-lives of morphine glucuronides (M3G and M6G) range from 2.0 to 6.4 h, and T_{max} varied from 0.7 to 5.1 h, which are results similar to those of morphine pharmacokinetic studies. The long circulation time and the high bioavailability of morphine and its glucuronides is probably maintained by enterohepatic recycling: morphine glucuronides are hydrolised into morphine by α-glucuronidase enzymes of the colon flora in the digestive tract, and morphine is again available for re-absorption into the circulation.

In a study with high intravenous heroin doses by Rentsch *et al.*, heroin and its major metabolites were measured in arterial and venous blood. Initially, the arterial plasma concentrations of heroin, 6-monoacetylmorphine and morphine were considerably higher than venous

plasma values; equilibrium between the arterial and venous compartments was however reached within 4-6 minutes. M3G and M6G exhibited no arterio-venous differences. In the same study, it was shown that peripheral tissues contribute to heroin, 6-MAM and morphine metabolism. Moreover, pharmacokinetics of heroin appeared linear up to high doses, supporting its use as a safe drug in assistance treatment.

"Street heroin" usually contains traces of acetylcodeine coming from acetylation of codeine, an impurity of the raw morphine used for the production of heroin. In the body acetylcodeine is hydrolysed by esterases into codeine, so that traces of this substance can be found in biological specimens. Codeine is in turn metabolised via conjugation with glucuronic acid to form codeine-6-β-D-glucuronide (C6G), which represents the main metabolite in urine and bile. In detail, after codeine administration, 80% of codeine is conjugated with glucuronic acid to form codeine-6β-D-glucuronide, while only 5% is *O*-demethylated by CYP450 2D6 to morphine, which is rapidly conjugated and excreted by the kidneys.

It must be considered that heroin metabolism and pharmacokinetics, and consequently its pharmacological and toxic effects, can be affected by several factors. First of all, the pharmacokinetic profiles of heroin and its metabolites depend on the route of administration, as previously described (see paragraph 1.2.3). P-glycoprotein (P-gp) and Organic Anion Transporting Polypeptides (OATPs) have also a role in heroin pharmacokinetics. P-gp is an efflux pump that protects the body against xenobiotic compounds, promoting the excretion into urine and limiting the absorption from the gastro-intestinal tract and the entrance to the brain through the BBB. Several *in vitro* studies have demonstrated that both morphine and morphine glucuronides are P-gp substrates (Rook *et al.*, 2006), even if the transfer capacity of P-gp for these molecules was relatively small compared to other compounds (e.g. paclitaxel) and opioids (e.g. loperamide). Moreover, Aquilante *et al.* (2000) demonstrated that chronic exposure to morphine increased P-gp density in rat brains, contributing to the development of tolerance (see paragraph 1.2.7). Whether heroin itself is subject to P-gp-mediated transport remains to be

studied. OATPs are also protective efflux transporters in brain, liver and kidney. Several experiments with probenecid, the specific OATPs inhibitor, have shown that morphine, M3G and to a lesser extent M6G are substrates of OATPs-mediated transport.

Drug-drug interactions must also be taken into account.

Co-medication	***Interaction***	***Type of Study, Results***		***Clinical Relevance***
Hydrolisis of heroin and 6-MAM				
Cocaine	Inhibition	*In vitro*	Competitive inhibition	Unknown
Ethanol	Inhibition	*Post-mortem*	Increase in 6-MAM levels	Enhanced risk for overdose
Glucuronidation to Morphine-3-β-D-glucuronide and Morphine-6-β-D-glucuronide				
Paracetamol	Induction	*In vivo*	Observational study: morphine treated patients	Unknown
Benzodia-zepines	Inhibition	*In vitro*	Competitive inhibition M3G formation relatively more inhibited by oxazepam	Unknown
		In vivo	Reduction of M3G/morphine serum ratio in morphine treated patients	Unknown
Chloram-phenicol	Inhibition	*In vitro*	*Competitive inhibition*	*Unknown*
		In vivo	Increase in morphine bioavailability in rodents	Unknown
Ethanol	Inhibition	*In vitro*	Dose dependent	Unknown
Ranitidine	Inhibition	*In vitro*	M6G formation relatively spared	↑ opioid effect
		In vivo	In healthy volunteers	
Amitriptyline nortriptyline fluoxetine	Inhibition	*In vitro*	Competitive and non-competitive inhibition	Unknown
Transporting enzymes (morphine substrates)				
Quinidine	P-gp blocker	*In vivo*	In healthy volunteers, increase in morphine oral bioavailability	Insignificant for iv assumption
Valspodar	P-gp blocker	*In vivo*	In healthy volunteers, increase in M3G plasma concentration after morphine iv assumption	Insignificant pharmacodynamic effect
Rifampin	P-gp induction	*In vitro*	-	Reduction in analgesic effect
		In vivo	In healthy volunteers, reduction in morphine oral bioavailability	
Probenecid	OATPs blocker	*In vitro*	-	Unknown
		In vivo	Increase in antinociception in rodents	

Table 1: Some drug interactions of heroin and its metabolites.

This table summarises some demonstrated or suggested interactions between heroin or its metabolites and other drugs (adapted from Rook *et al.*, 2006), which may influence the metabolism, and consequently the pharmacological effects of heroin, acting on different levels.

For example, UGT 2B7 is the primary enzyme for morphine metabolism, but it is also responsible for the metabolism of several endogenous and exogenous compounds, such as steroid hormones, ranitidine, naltrexone, naloxone, and ethanol. As a consequence, these compounds could interfere with the production of morphine metabolites *in vivo* (Witter and Kern, 2006). Interactions with drugs of abuse are discussed in paragraph 1.3.2, for their possible role in contributing to fatal heroin overdose.

Hepatic and renal impairment may influence heroin metabolism, too. Liver enzymes participate to heroin hydrolysis, but esterases are also abundantly present in blood and other organs. Similarly, even if the liver is the main organ involved in glucuronidation, morphine metabolism is relatively normal in patients with severe liver cirrhosis, probably because glucuronidation is taken over by other organs. Moreover, liver diseases can cause low albumin serum concentrations, but this is not relevant, since the binding of heroin and morphine to albumin is moderate. Therefore, hepatic impairment seems to have no great influence on the pharmacokinetics of heroin, even if bioavailability of oral heroin increases in patients with serious liver disease, because of the loss of the first-pass effect (Rook *et al.*, 2006).

As far as kidneys are concerned, these organs are primarily involved in the excretion of morphine and morphine glucuronides following heroin administration. Morphine is subject to glomerular filtration, active secretion in proximal tubules and probably re-absorption, resulting in a net tubular secretion, while morphine glucuronides are partly reabsorbed in the kidney, but active excretion of the morphine glucuronides in urine doesn’t occur. Accumulation of morphine glucuronides can be considerable in patients with serious renal impairment, but it seems not clinically relevant in milder cases. Although pathologic-anatomical

abnormalities of the kidney are commonly found in post-mortem samples belonging to intravenous heroin users, renal impairment is probably relatively mild in most heroin dependents. Checking of the creatinine clearance value during morphine or heroin-assisted treatment is however advised, as some lethal cases occurred (Rook *et al.*, 2006; Lagas *et al.*, 2010).

On the other hand, the decreased renal function with age would also result in lower systemic clearance of both morphine glucuronides, leading to longer accumulation of M6G and a potentially extended effect. As a matter of fact, researchers have reported that also gender and age contribute to differences in the pharmacokinetics of heroin and its metabolites that will ultimately affect pharmacologic effect. Results from a study showed that elderly women had higher levels of morphine metabolites compared to elderly men, and that their clearance of the metabolites had decreased. Further analysis revealed that progesterone levels may affect the clearance rates mainly of M3G, and this could contribute to its accumulation in elderly women who are on chronic opioid therapy (Wittwer and Kern, 2006).

Finally, the role of genetics must also be considered. Lötsch *et al.* (2004) published an extensive review on this topic. Briefly, different *in vitro* studies have demonstrated a large variability in phenotypes and genotypes of human esterases, but to what extent genetic differences in expressing esterase activity are responsible for variability in heroin metabolism *in vivo* has not been widely studied. Some authors have stated that UGT 2B7 or UGT 1A1 polymorphisms did not contribute significantly to the variability in the morphine/morphine glucuronides ratio. On the contrary, some researchers have suggested that individual variability in UGT activity may help to explain cases of sudden death among heroin users (Fugelstad *et al.*, 2003). Genetic differences may also trigger or modify drug interactions, which in turn can alter the clinical response to opioid therapy, as shown in several *in vitro* and *in vivo* experiments. Furthermore, variants in the gene that encodes for the μ opioid receptor (see paragraph 1.2.5) have been linked to clinically measurable differences

in the analgesic action, but not in the respiratory effects, of M6G (Wittwer and Kern, 2006), so that pharmacogenetics may facilitate the optimisation of an individualised opioid therapy. Finally, a review by Yuferov *et al.* (2010) has summarised recent progress in studies of association of gene variants with vulnerability to develop opioid and cocaine addictions, focusing primarily on genes of the opioid and monoaminergic systems.

1.2.5 MECHANISM OF ACTION

Heroin exerts its action by a specific interaction with some subclasses of receptors related to antinociceptive control and to the genesis of emotions, whose endogenous binding molecules are neuropeptides (opiopeptins or endogenous opioid peptides) stored in neurons of specific brain areas. The term “opioid” usually describes natural and semi-synthetic alkaloids prepared from opium, as well as synthetic surrogates whose pharmacological effects, rather than structure, mimic those of morphine, and also the endogenous neuropeptides mentioned above. Although often used as a synonym for “opioid”, the term “opiate” is properly limited to the natural alkaloids found in the latex of the opium poppy. In some definitions, the semi-synthetic substances that are directly derived from the opium poppy alkaloids are considered to be “opiates” as well, while in other classification systems these compounds are simply referred to as “semi-synthetic opioids”. All these molecules interact with one or more subclasses of opioid receptors, acting as agonists, antagonists or partial agonists.

Opioid receptors have been classified into three major families, designated as μ (mu), δ (delta), κ (kappa), which in turn can be divided into the subtypes μ_1, μ_2 and μ_3, δ_1 and δ_2, κ_1, κ_2 and κ_3 respectively. Since only one gene for each of the μ, δ, and κ receptor families has been isolated and characterised thus far, receptor subtypes may probably arise from alternate splice variants of a common gene. Similarly to their receptors, endogenous opioid peptides can be classified into three groups, called endorphins, enkephalins (pentapeptides methionine-enkephalin and leucine-enkephalin), and dynorphins, which come from different precursor proteins: the Pre-Pro-Opio-Melanocortin (POMC), the preproenkephalin

(proenkephalin A), and the preprodynorphin (proenkephalin B). Each receptor family shows a preferential binding to one of the endogenous peptide class, as shown in Table 2 (adapted from Katzung, 2006). This table shows also the main functions of the three receptor families.

Family	Functions	Endogenous Opioid Peptide Affinity
μ (mu)	Supraspinal (μ_1) and spinal (μ_2) analgesia; sedation; respiratory depression; euphoria; hypothermia; miosis; bradycardia; modulation of hormones and neurotransmitters release; physical tolerance and dependence; slowed gastrointestinal (GI) transit	Endorphins>enkephalins>dynorphins
δ (delta)	Supraspinal and spinal analgesia; respiratory depression; dysphoria; delusions; hallucinations; cognitive functions; modulation of hormones and neurotransmitters release; vasomotor stimulation; GI motility	Enkephalins>endorphins and dynorphins
κ (kappa)	Supraspinal and spinal analgesia; sedation; mild respiratory depression; dysphoria; hypothermia; miosis; diuresis; food-intake regulation; neuroendocrine secretion; slowed GI transit	Dynorphins>>endorphins and enkephalins

Table 2: Opioid receptor subtypes, their functions, and their endogenous peptide affinities.

The opioid system is involved in two major physiological processes: pain transmission and reinforcement-reward system. In addition, endogenous peptides play a role in regulating body temperature, respiratory function, diuresis, intake of food, drinking, sexual activity and memory processes. They seem to interact with the endocrine and immune systems, too.

The opioid system is still under investigation and new receptor families and endogenous ligands have been recently described. For example, it has been stated that opioids may also bind to σ receptors, a minor opioid receptor family, to produce central excitation, resulting in tachycardia, hypertension, tachypnea, mydriasis, and hallucinations (Levine, 2003). Besides, the endogenous peptides endomorphin-1 and

endomorphin-2 have been found to possess some properties of opioid peptides, notably analgesia and high-affinity binding to the μ receptor. Furthermore, a novel receptor-ligand system homologous to the opioid one has been found. The main receptor for this system is the G protein-coupled orphanin opioid receptor-like subtype 1 (ORL_1) or OP4, whose endogenous ligand has been termed nociceptin by one group of investigators and orphanin FQ by another group. This ligand-receptor system is currently known as the N/OFQ system. Nociceptin is structurally similar to dynorphin except for the absence of an *N*-terminal tyrosine, but a study in mice showed that it caused hyperalgesia, acting only at the ORL_1 receptor. Moreover, this system seems to be able to modulate drug reward, reinforcement, learning, and memory processes (Katzung, 2006), so that agonists and antagonists have been studied for various clinical applications. A fascinating field is also the study of endogenous morphine. Since the 1980s, different laboratories have been able to characterise morphine and morphine precursors in animal tissues, suggesting the role of these molecules as endocrine and neuroendocrine mediators (Stefano *et al.*, 2000; Glattard *et al.*, 2006).

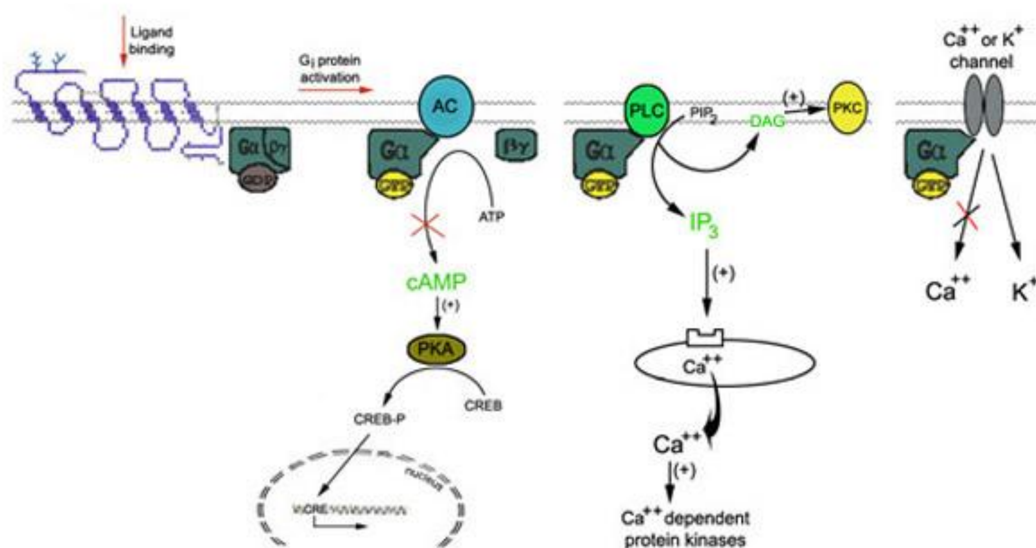

Fig.7: Signal transduction of opioid receptors.

Opioid receptors are members of the $G_{i/o}$ protein-coupled family of receptors, and show significant amino acid sequence homologies. Their

activation leads to inhibition of adenylate cyclase, resulting in a decreased level of cAMP, which in turn affects ion channel gating, modulates intracellular Ca^{2+} disposition, and alters protein phosphorylation (Fig.7; font: medscience.us).

Opioid receptor binding sites have been localized autoradiographically with high-affinity radioligands and antibodies. A study by Maurer *et al.* showed that opioid-binding sites were generally predominant in grey matter and almost absent in white matter. A very high density of μ opioid receptor was found in the brainstem (area tegmentalis ventralis, grisum central mesencephali, inferior colliculus and nucleus interpeduncularis); a moderate density was found in the molecular layer of the cerebellar cortex (also a part of the brainstem) and the neocortex (laminae I-V) and hippocampus (gyrus dentatus) of the telencephalon. More recently, quantitative radiography detected μ opioid receptors in the telencephalon, with high density in the laminae I-III of the neocortex, and the nucleus caudatus; moderate density in the laminae III-IV of the neocortex, the nucleus basalis of Meynert, and the corpus amigdaloideum; low density in the laminae V-VI of the neocortex and the claustrum. Moderate density was also found in the thalamus (diencephalon) and the cerebellum (brainstem). Low density was found in the hypothalamus (diencephalon). Interestingly, Gabilondo *et al.* found that no apparent alterations in the densities and affinities of μ opioid receptors in various brain regions could be observed in post-mortem brain of heroin addicts. Brain α_2-adrenoreceptor densities, however, appeared to be down-regulated during opioid dependence in humans.

The three major opioid receptor families are present, although in different concentrations, in some peripheral organs, such as pancreas, lung, heart, liver and gastrointestinal tract. However, as mentioned above, these receptors are primarily present in brain and spinal cord regions involved in the transmission and modulation of pain. For example, they are located in high concentrations in the dorsal horn of the spinal cord, where they have two actions (Fig.8; font: Katzung, 2006):

-they close voltage-gated Ca^{2+} channels on pre-synaptic nerve terminals and thereby reduce transmitter (e.g. glutamate, acetylcholine, norepinephrine, serotonin, and substance P) release;

-they hyperpolarise, and thus inhibit post-synaptic neurons by opening K^+ channels.

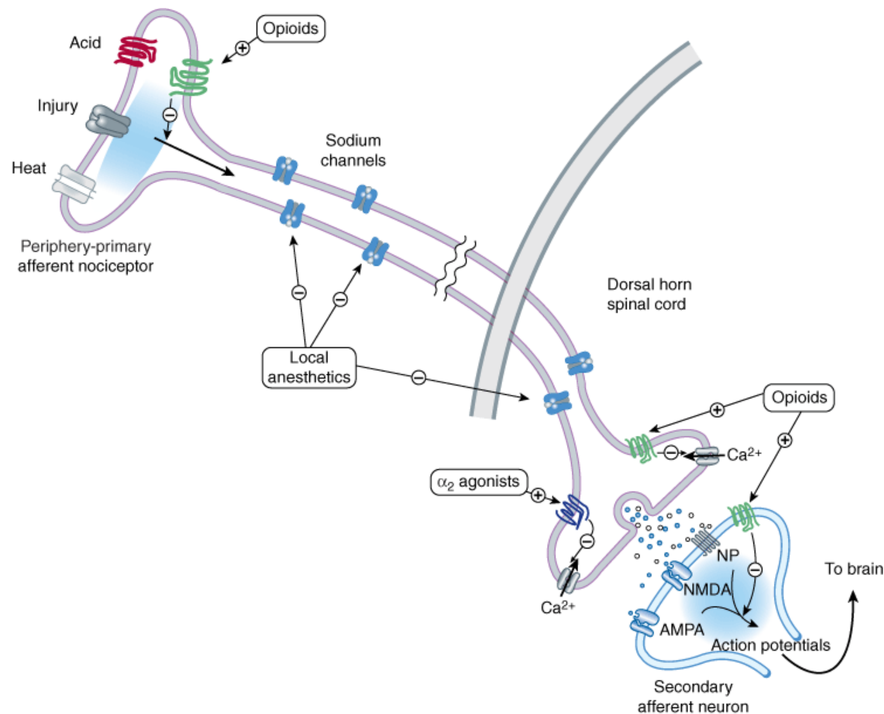

Fig.8: Opioids actions on nociception in the spinal cord.

As a consequence, opioid drugs may exert a powerful analgesic effect directly on the spinal cord. This spinal action has been exploited clinically by direct application of opioid agonists to the spinal cord, which provides a regional analgesic effect, while reducing the unwanted respiratory depression, nausea and vomiting and sedation which may occur from the supraspinal actions of systemic administration.

Nonetheless, under most circumstances opioid drugs are given systemically and so act simultaneously at multiple sites, inhibiting the transduction of painful stimuli from peripheral sensory terminals, but also acting on descending (modulatory) pathways. Moreover, part of the pain-relieving action of exogenous opioids involves the release of endogenous opioid peptides. For example, morphine acts primarily and directly at the

μ receptor, but this action may evoke the release of endogenous opioids that additionally act at δ and κ receptors, triggering a complex sequence of events which involves multiple synapses, transmitters, and receptor types.

Opioid binding sites are widely present also in the mesolimbic dopamine system, which is formed by brain areas primarily responsible for the arousal of emotion in humans. As a consequence, activation of opioid receptors prevents the recognition of painful sensations, inhibits the negative emotional component of pain, and can produce euphoria in some instances. This is true for μ receptors, which inhibit GABA (Gamma-Amino-Butyric-Acid) neurons in the Ventral Tegmental Area (VTA), but not for κ receptors, which are expressed on and inhibit dopamine neurons, thus causing disphoria. This action on limbic system is also connected with the dependence which usually occurs when assuming opioid drugs (Katzung, 2006).

Respiratory depression is the major side-effect related to opioid medical use and heroin misuse. Brain respiratory centres are localized in the brain stem, even if also the cortex and other brain regions may influence this physiological process. Two major groups of neurons have been identified in the medulla oblongata: the dorsal respiratory group and the ventral respiratory group. Important inputs come also from the pons. The respiratory rhythm is generated by activation and inhibition of neurons, which are mainly mediated by excitatory amino acids and GABA, respectively. Other neurotransmitters may modulate the respiratory rhythm. For example, it was found that opioid peptides are able to decrease activation via a reduction in glutamate-induced excitation (White and Irvine, 1999).

When opioid receptors subtypes were initially studied, it was stated that respiratory depression was mediated by μ_2 receptor subtype. This could have important implications, as it suggested that opioids without respiratory depressant effects could be developed. However, considerable controversy is now present about a clear separation of the effects between these receptor subtypes. It can therefore be stated that opioid drugs act at several sites involved in respiratory control, by depressing neuronal

activity. At chemoreceptors, the inhibitory activity appears to be mediated mainly by μ opioid receptors, and results in decreased sensitivity to changes in oxygen and carbon dioxide outside normal concentration ranges. Besides, both μ and δ receptors are located in central respiratory centres, suggesting that opioid peptides may have a role in respiration, even if this action hasn't been elucidated yet. On the contrary, the effects of exogenous opioids on respiration have been widely studied. At low concentrations these drugs appear to have effects mainly on tidal volume (i.e. normal volume of air displaced between normal inspiration and expiration when extra-effort is not applied), whereas at higher concentrations both tidal volume and respiratory frequency may be affected. It has been suggested that these two different parameters may be modulated by different opioid receptors, with μ opioid receptors mediating the depression in tidal volume and δ opioid receptors the depression of frequency, but this has not been unequivocally demonstrated yet (White and Irvine, 1999).

Opioid-induced respiratory depression remains one of the most difficult clinical challenges in the treatment of severe pain. A small to moderate decrease in respiratory function, may be well tolerated in the patient without prior respiratory impairment. However, in individuals with increased intracranial pressure, asthma, or chronic obstructive pulmonary disease, this decrease in respiratory function may not be tolerated.

As opioid receptors have different roles and an opioid drug may act as an agonist, partial agonist, or antagonist at more than one receptor class or subtype, these ligands may potentially give rise to various pharmacological effects. Therefore, the receptor binding profile of each exogenous opioid has been widely studied. Heroin was shown to have agonist effects at the μ and δ receptors, while morphine has affinity primarily to the μ receptor and, to a lesser degree, to the κ and δ receptors. The presence of different μ receptors subtypes, with specific affinity for heroin and morphine respectively, has been recently demonstrated (Pan, 2005; Pan *et al.*, 2007). This finding may explain the different effects of

heroin, compared to morphine, even as an analgesic, despite its rapid degradation to its metabolites. 6-Monoacetylmorphine is also an active molecule, which may have a higher receptor affinity than its precursor heroin, as indicated by some studies.

In the last forty years, also morphine-3-β-D-glucuronide (M6G) and morphine-6-β-D-glucuronide (M3G) have been extensively studied for their contribution to pharmacological effects following administration of opioid analgesics or assumption of “street heroin”. Nonetheless, their contribution to both the desired and the side effects are the subject of clinical controversy (Andersen *et al.*, 2003; Wittwer and Kern, 2006). In the past, these metabolites were believed not able to cross the BBB to a significant extent because of their high hydrophilicity. Moreover, the evidence of their rapid excretion by the kidneys seemed to confirm that they were simply the way through which morphine was eliminated by the body. On the contrary, in the late 1960s, M6G was found to exert antinociceptive effects, and then more and more researchers have demonstrated that this molecule is a potent agonist to μ opioid receptors (Rossi *et al.*, 1995; Rossi *et al.*, 1996; Pasternak, 2001).

On the other hand, in an *in vitro* study M3G showed a weak agonist activity at opioid receptors (Ulens et al., 2001), while other studies demonstrated that this molecule has no opioid activity in humans. Several experiments in animal models suggested that M3G has opposite effects compared to morphine and M6G, that is, anti-analgesic and neuroexcitatory effects, which appear not to be mediated by opioid receptors. It has been proposed that this molecule may be the cause of allodynia, which sometimes occurs under morphine therapy, and also of the development of tolerance (see paragraph 1.2.7). On the contrary, other studies demonstrated that this metabolite had no anti-morphine effects. For example, after direct administration to healthy volunteers, it showed neither influence on morphine effects nor other actions (Penson *et al.*, 2000). M3G accumulate in patients with renal failure, as M6G, but in that case the toxic effects seem to be related to sedative/depressing action

typical of opioids, and thus connected with morphine and M6G rather than M3G (Lötsch, 2005).

Unlike M3G, M6G is a potent μ opioid agonist. The first experiments were performed in rats and mice, where M6G was injected subcutaneously or intracerebroventricularly, resulting in antinociceptive effects. Intracerebroventricular infusion of M6G was found to cause respiratory depression in dogs. In these studies, M6G showed a greater potency than morphine to produce antinociception or respiratory depression, with a relative potency of M6G to morphine varying between 2:1 and 678:1, depending on the experimental model and the species studied (Lötsch, 2005). Further studies indicated that this molecule binds to a distinct opioid receptor compared to morphine, and seems able to reach CNS concentrations high enough to contribute to the overall effects associated with morphine administration (Rossi *et al.*, 1996; Lötsch and Geisslinger, 2001; Pasternak, 2001; Ulens *et al.*, 2001; Mantione *et al.*, 2005). Much attention and effort have been directed at investigating the properties of M6G, since this derivative could be an alternative to morphine in the treatment of pain. Some clinical studies have demonstrated that M6G exhibits increased potency, but the possibility of a better side-effect profile compared with morphine is still under investigation (Lötsch, 2004; Binning *et al.*, 2010). Moreover, M6G may participate to heroin pharmacological profile, prolonging the short last effects of the drug (Antonilli *et al.*, 2003, see also paragraph 1.3.2). Thus differences in the pharmacological effects of heroin and morphine may also depend on the rate of M6G formation.

As far as codeine is concerned, its analgesic activity has been attributed to morphine for a long time. Nonetheless, its main metabolite codeine-6-β-D-glucuronide is an active molecule, in analogy with morphine-6–β-D-glucuronide. Some studies have demonstrated that codeine-6-β-D-glucuronide has an analgesic activity, with less immunosuppressive effects than codeine *in vitro* (Srinnivasan *et al.*, 1996), is relatively stable *in vivo* (in rats) and has a receptor affinity similar to that of codeine (Srinnivasan *et al.*, 1997). This molecule may therefore

show clinical benefit in the treatment of pain. Moreover, some authors have recently stated that the analgesic activity of codeine is due to codeine-6-β-D-glucuronide rather than to morphine, since a population of "poor metabolizers of codeine", those lacking the CYP450 2D6 isoenzyme for the *O*-demethylation to morphine, experienced analgesia from codeine (Vree *et al.*, 2000). Conversely, other studies demonstrated that some genetic mutations of CYP450 2D6 make codeine ineffective for the lack of morphine formation (Lötsch *et al.*, 2004). Further investigation is needed to explain these, maybe only apparently, contradictory findings.

1.2.6 PHARMACOLOGICAL EFFECTS

The effects of each endogenous or exogenous opioid is related to its pharmacological profile, i.e. its receptor binding profile. The main effects of heroin and other opioids with major affinity for μ receptors are on the CNS; the most important ones include analgesia, euphoria, sedation, and respiratory depression. Repeated use causes a high degree of tolerance (see paragraph 1.2.7), even if the rate of development of this phenomenon is not the same for all the effects.

Acute overdose may occur after administration of 20 mg of heroin in non-addicted people, or of greater doses in heroin addicts according to their tolerance. Symptoms are respiratory depression, followed by a drop in blood pressure, miosis, and decreased temperature. The treatment begins with assessment of adequacy of ventilation, followed by assisted ventilation and naloxone-therapy if necessary. Naloxone is a potent antagonist at μ, κ and δ receptors, which is rapidly absorbed intravenously, intramuscularly, and via endotracheal tube. In the body, it rapidly enters the CNS owing to its lipophilicity, so that its action occurs in 1-2 minutes after intravenous injection. When a fatal overdose occurs, death seems to be due to a severe respiratory depression, till coma, pulmonary oedema and cardio-circulatory arrest, even if the exact mechanism is still unclear (see paragraph 1.3.2).

A brief list and description of the pharmacological effects of a μ opioid agonist (e.g. heroin/morphine) is given below (adapted from Katzung, 2006).

Effects on Central Nervous System

Analgesia

As previously described, opioids reduce both sensory and affective (emotional) components of the pain experience, acting at supraspinal (via activation of μ_1, κ_3, δ_1 and δ_2 receptor subtypes) and spinal (via activation of μ_2, κ_2, δ_1 receptor subtypes) level.

Euphoria

Typically, a pleasant sensation with reduced anxiety and distress is felt after opioid agonists administration. However, dysphoria, an unpleasant state characterized by restlessness and malaise, may sometimes occur.

Sedation

Drowsiness and clouding of mentation are common effects of opioid analgesics. However, morphine causes little or no amnesia and a milder sedation than other central depressant drugs. Besides, this drug produces anxiolysis, probably acting on μ receptors located in the nucleus coeruleus.

Respiratory Depression

This side effect is dose-related and remains one of the most difficult clinical challenges in the treatment of severe pain, especially in patients with previous pulmonary diseases. Research to overcome this problem is focused on δ receptor pharmacology and serotonin signalling pathways in the brainstem respiratory control centres.

Cough Suppression

Heroin was first marketed as cough suppressant agent, since opioids are able to suppress cough reflex acting on the cough centre in the brainstem.

Miosis

Constriction of the pupils is seen with virtually all opioid agonists and is mediated by parasympathetic pathways, i.e. inhibition at the third cranial nerve.

Nausea and Vomiting

The opioid analgesics can activate the brainstem chemoreceptor trigger zone to produce nausea and vomiting. Since ambulation seems to increase the incidence of nausea and vomiting, a vestibular component may be also present.

Temperature

Endogenous opioid peptides are involved in homeostatic regulation of body temperature, so that the administration of most opioids causes an impairment of this physiological function.

Neuroendocrine

Acting on different receptor subtypes in the hypothalamus, opioid analgesics stimulate the release of prolactin (μ_1), Growth Hormone (GH) (μ_2 or δ) and Gonadotropin-releasing Hormone (GrH) (μ_3). The results are decreased libido, amenorrhea or impotence.

Truncal Rigidity

Some opioids cause an intensification of tone in the large trunk muscles, which interferes with ventilation. The concomitant use of neuromuscular blocking agents can prevent this phenomenon.

Peripheral effects

Cardiovascular System

Most opioids have no significant direct effects on heart and blood pressure, even if in some cases hypotension may occur probably as a consequence of peripheral arterial and venous dilation, which has been attributed to central depression of vasomotor-stabilizing mechanisms and release of histamine.

Gastrointestinal Tract

Opioid receptors are widely present in the gastrointestinal tract, and the typical constipating effects are mediated through an action on the enteric nervous system as well as the CNS. In the stomach, opioids cause a decrease in the secretion of hydrochloric acid and in the motility, but an increase in the tone. Small intestine resting tone is increased, with periodic spasms, but the amplitude of non-propulsive contractions is

decreased. In the large intestine, propulsive peristaltic waves are diminished and tone is increased. The large bowel actions are the basis for the use of opioids derivatives in the management of diarrhea. Interestingly, tolerance does not develop to opioid-induced constipation.

Biliary Tract

Opioids enhance the contraction of biliary smooth muscle, which may result in biliary colic. Moreover, they may cause the reflux of biliary and pancreatic secretions, resulting into elevated plasma amylase and lipase levels.

Kidneys

Opioids may affect renal function acting at central and peripheral sites. For example, these drugs decrease renal plasma flow, enhance renal tubular sodium reabsorption and increase ureteral and bladder tone, while their influence on AntiDiuretic Hormone (ADH) release is still controversial.

Uterus

Both peripheral and central actions of the opioids can reduce uterine tone, so that these drugs may prolong labor.

Pruritus

Opioid analgesics may produce flushing and warming of the skin, together with sweating and itching, probably because of CNS effects and peripheral histamine release. These side effects are more frequent when intravenous administration is used.

Miscellaneous

The opioids have effects on lymphocyte proliferation, antibody production, and chemotaxis. For example, natural-killer cell cytolytic activity and lymphocyte proliferative responses to mitogens are usually inhibited by opioids.

1.2.7 DRUG DEPENDENCE AND TOLERANCE

"Addiction" is a term of long-standing and variable usage. According to the World Health Organization (WHO), it describes "the repeated use of

a psychoactive substance or substances, to the extent that the user (referred to as an addict) is periodically or chronically intoxicated, shows a compulsion to take the preferred substance (or substances), has great difficulty in voluntarily ceasing or modifying substance use, and exhibits determination to obtain psychoactive substances by almost any means". The life of the addict may be dominated by substance use till the virtual exclusion of all other activities and responsibilities. The term "addiction" also conveys the sense that such substance use has a detrimental effect on society, as well as on the individual. "Addiction" is also defined as "a discrete disease entity, a debilitating disorder rooted in the pharmacological effects of the drug, which is remorselessly progressive".

From the 1920s to the 1960s attempts were made to differentiate between "addiction" and "habituation", which is a less severe form of psychological adaptation, characterised by "absence of physical dependence, desire rather than compulsion to take the drug, and little or no tendency to increase the dose" (WHO Expert Committee, 1957). In 1964 the WHO replaced both terms in favour of "dependence", which can exist in various degrees of severity. Addiction is not a diagnostic term in ICD-10 (International Classification of Diseases-tenth revision), but continues to be very widely employed by professionals and the general public alike.

According to the World Health Organization (WHO), the "dependence", as a general term, is "the state of needing or depending on something or someone for support or to function or survive. As applied to alcohol and other drugs, the term implies a need for repeated doses of the drug to feel good or to avoid feeling bad". In DSM-IIIR (revision of the Diagnostic and Statistical Manual of Mental Disorders III, 1987), "dependence" is defined as "a cluster of cognitive, behavioural and physiologic symptoms that indicate a person has impaired control of psychoactive substance use and continues use of the substance despite adverse consequences". It is roughly equivalent to the "dependence syndrome", which is defined as "a cluster of behavioural, cognitive, and physiological phenomena that may develop after repeated substance use. Typically, these phenomena include

a strong desire to take the drug, impaired control over its use, persistent use despite harmful consequences, a higher priority given to drug use than to other activities and obligations, increased tolerance, and a physical withdrawal reaction when drug use is discontinued". In ICD-10, the diagnosis of dependence syndrome is made if three or more of six specified criteria have been experienced within a year.

The term "dependence" can be used generally with reference to the whole range of psychoactive drugs (drug dependence, chemical dependence, substance use dependence), or with specific reference to a particular drug or class of drugs (e.g. alcohol dependence, opioid dependence). However, it is important to underline that different drugs may cause different dependence symptoms. In unqualified form, "dependence" refers to both physical and psychological elements. "Psychological or psychic dependence" refers to the experience of impaired control over drinking or drug use, while "physiological or physical dependence" is connected with metabolic and functional changes especially in the CNS (neuroadaption). In biologically-oriented discussion and in a narrower sense, "dependence" is used to refer only to "physical dependence".

Both "psychological dependence" and "physical dependence" characterise heroin chronic intoxication. In detail, opioid drugs, and especially heroin, cause a strong "physical dependence" after a short time. This phenomenon increases in intensity and is mediated by "tolerance" to drug effects (see below).

If a substance causes "physical dependence", the ceasing or reduction of the use causes the so known "withdrawal syndrome", i.e. "a group of symptoms of variable clustering and degree of severity, which may be accompanied by signs of physiological disturbance". The onset and course of the "withdrawal syndrome" are time-limited and are related to the type of substance and dose being taken immediately before the cessation or reduction of the use, to the degree of dependence, to the health and psychological conditions of the addict. Typically, its features are the opposite of those of acute intoxication. Opioid withdrawal is

accompanied by rhinorrea (running nose), lacrimation (excessive tear formation), aching muscles, chills, gooseflesh, sweating, restless sleep and, after 24-48 hours, mydriasis, anorexia, irritability, tremor, muscle and abdominal cramps. In heroin withdrawal syndrome, these symptoms reach the maximum after 48-72 hours. The physical symptoms typically disappear within 5-10 days, while psychological side effects continue and include anxiety, depression and drug-seeking, being responsible for the high frequency of relapses.

Finally, the term "tolerance" describes "a decrease in response to a drug dose that occurs with continued use". As a consequence, increased doses of alcohol or other drugs are required to achieve the effects originally produced by lower doses. Both physiological and psychosocial factors may contribute to the development of tolerance, which may be physical, behavioural, or psychological. With respect to physiological factors, both "metabolic and/or functional tolerance" may develop. By increasing the rate of metabolism of the substance, the body may be able to eliminate the substance more readily. "Functional tolerance" is defined as a decrease in sensitivity of the CNS to the substance (neuroadaption). "Behavioural tolerance" is a change in the effect of a drug as a result of learning or alteration of environmental constraints. These three kinds of "tolerance" seem all to occur in heroin addiction. The WHO defined also other types of "tolerance", which are not usually associated with opioid misuse. "Acute tolerance" is rapid, temporary accommodation to the effect of a substance following a single dose. "Reverse tolerance", also known as sensitization, refers to a condition in which the response to a substance increases with repeated use.

Interestingly, variation has been found in the acquisition of tolerance to the different effects of opioids. Tolerance to the respiratory depressant effects of opioids may be incomplete and may develop more slowly than tolerance to the euphoric effects. As a result, long-term heroin users may be at higher risk of overdose as the result of a reduction in the difference between the dose required to achieve the desired effects and the dose sufficient for lethal respiratory depression (Sporer, 1999; see also

paragraph 1.3.2). Such variation is not surprising when the cellular bases of the development of tolerance are considered. The mechanisms of functional tolerance to drugs involve many of the components in the receptor-transduction system, including changes in gene expression, uncoupling of G-proteins, changes in adenylate cyclase and in protein kinase activities. These variations can differ from cell to cell and from one brain region to another. On the contrary, pharmacokinetic tolerance is unlikely responsible for differences in the rate of tolerance development across different opioid effects. It is reasonable to suspect that the rate of loss of tolerance may also vary between effects, with consequences on the risk of overdose (see paragraph 1.3.2).

1.3 POST-MORTEM TOXICOLOGY

1.3.1 GENERAL REMARKS

The cause of death is always established by a medical examiner or coroner, who may be also asked to assess the identity of the deceased, and/or estimate the time of death, and/or determine the additive effect of trauma or pre-existing conditions, and/or distinguish homicide from suicide, inferring the type of weapon used if a violent death is suspected. In this regard, the aim of an autopsy is to observe and make a permanent legal record, as soon as possible, of the macroscopic and minute anatomical peculiarities of a recently discovered dead body. Clinical, or microscopic, examination of organ parts is often necessary to further support the forensic pathologist's conclusions, although such examination would be impossible in an exhumation case or if the family opposed it. Information about the surrounding circumstances in which death occurred, the clinical and/or addiction history are also evaluated.

Whenever unnatural, sudden, violent or unexpected deaths occurred or is suspected, toxicological analysis is required to support the medical examiner’s task. Post-mortem toxicology is used to determine whether alcohol, drugs or other poisons may have caused or contributed to the death of a person. It differs from clinical toxicology not only for the complexity of the matrices, especially post-mortem blood and tissues, but also for the greater difficulty in interpreting the results (Skopp, 2010; Moffat *et al.*, 2011). In most of these cases, a legal proceeding is implied, so that a strict procedure must be followed. To this purpose, laboratory guidelines and a quality assurance program are fundamental. In some countries, such as the United Kingdom, there are severe rules on sampling and storage of human tissues and this extends to post-mortem specimens. Post-mortem toxicological analysis is requested via the coroner and all samples are officially under his or her jurisdiction, so that samples can be handled after having obtained his or her permission.

The main steps of post-mortem toxicology are described in the following paragraphs, while heroin fatalities are discussed in detail in paragraph 1.3.2.

Samples Collection

First of all, a proper collection of the samples during autopsy, followed by appropriate storage and transport, is necessary. The term "sample" covers the fluid and tissue and its primary container, and the sampling procedure starts with the selection of appropriate samples and ends with the correct disposal of the materials. The forensic pathologist should select and collect appropriate specimens, which are a representative part of the whole and suitable to perform certain kinds of experiments (Skopp, 2010). Up to now, a coordinated protocol for sampling suspected poisoning or drug-related deaths has not been established, even if some recommendations were published in some countries. The selection and volume or amount of specimens may vary from case to case, depending on requests, legal aspects and availability. However, two blood samples, including at least one from a peripheral site, and urine and gastric contents should be collected as a minimum set of specimens. A list of the recommended post-mortem specimens and corresponding volumes is shown in Table 3 (Font: Skopp, 2010).

In severely decomposed cases found outdoors or in cases of exsanguinations and burns, skeletal muscle, pleural effusions, hair, bone or bone marrow and entomological specimens may be the only specimens available. Insect eggs, larva or pupae can be used to estimate the post-mortem interval, to indicate movement of the corpse and to perform toxicological analysis. Post-mortem samples from a patient who died in hospital several days after a poisoning episode are likely to be negative. In these cases, specimens obtained soon after admission to hospital should preferably be investigated. Non-biological material found at the scene, including spoons, syringes, and mugs or glasses containing drug residues, household products, solvents or pesticides, may provide additional information to assist and focus toxicological analyses. In cases where poisoning by volatiles or gas is suspected, a specimen should be collected directly at the scene, for example an aerosol container, or other sources (Skopp, 2010).

Specimen	Volume/amount	Use/comment
Blood from the femoral or subclavian veins	10-20 mL	Quantitative data, acute impairment or poisoning
Heart blood	50 mL or all available	General unknown analysis, concentration may be increased due to post-mortem redistribution
Urine	50 mL or all available	Standard samples for drug screening, general unknown analysis, organophosphates, aromatic hydrocarbons (metabolites)
Gastric contents	50 mL or all available	Result should be referred to the total amount; tablets, herbal remains, etc. placed in individual containers
Tissues (brain, liver, lungs, kidneys, muscle, subcutaneous fat)	10-50 g	Body load may help to interpret post-mortem blood data. Lungs, brain: inhalant poisoning
Gall bladder fluid	All available	Drug screening, accumulation of drugs undergoing enterohepatic recycling
Hair sample from the scalp or the body, or nails as an alternative	Pencil-like tuft	Exposure data for weeks or months before death, tolerance
Vitreous humour	All available	Alcohol, cardiac glycosides, diabetes
Cerebrospinal fluid	All available	General unknown analysis, devoid of enzymes and proteins
Skin and subcutaneous fat	Approx 2x2x1 cm^2	Skin exposure, injection marks (insulin, intravenous drug abuse), anesthetic-related incident
Contents of large and small intestine	All available and fractionated, if applicable	Suspicion of drug exposure by the rectal route, poisoning by plants or mushrooms
Pericardial fluid	50 mL or all available	In putrefied cases
Pleural fluid	50 mL	In putrefied cases
Entomological specimens species	As available	In putrefied cases, should immediately be frozen
Bone, bone marrow	Piece of 3-5 cm, > 1 g	Advanced putrefaction, extensively burnt bodies
Swabs (intranasal, rectal, vaginal)	≥ 2 swabs	Route of administration or exposure

Table 3: List of recommended post-mortem specimens for routine toxicology examination.

As far as a collection of samples is concerned, separate disposable or clean devices or instruments should be used for each sample to avoid contamination. Body fluids are usually taken by needle aspiration using a hypodermic syringe or by a pipette, whereas a spoon or ladle is more appropriate for viscous materials. Swabs should be taken using cotton

pads, and tissue specimens can be cut out with disposable scalpels, knives or scissors. Whenever volatile compounds are suspected, specimens must be promptly collected and sealed in a container. Specimen preservation is not necessary, except for blood sample, which ideally should be divided between an unpreserved and a preserved tube, containing 1–5% of sodium fluoride and sometimes potassium oxalate. Generally, preservation of specimens with sodium fluoride is recommended in ethanol, GHB, cocaine and carbon monoxide analyses, whereas fluoride preservation must not be used when organophosphorous chemicals are involved. Ascorbic acid may be used to stabilise drugs such as olanzapine and apomorphine.

Tubes containing liquids should be filled to minimise the evaporation of volatiles and oxidative degradation of drugs. Nonetheless, a small headspace of about 20% should be left if they are likely to be frozen. Most types of plastic containers (polycarbonate, polyethylene or polypropylene copolymers) are suitable for the collection of body fluids and tissues in drug-related fatalities. A glass container must be used, however, if solvent abuse or an anesthetic death is suspected. Samples should be sealed in such a way that tampering should be evident.

When these specimens are submitted to the laboratory, also a post-mortem toxicology request should be given. This document should indicate the identity of the deceased, demographic information and history (e.g. circumstances of death, relevant medical history, autopsy findings), the submitted samples with directions about packaging, transport and storage, and the required analysis. Moreover, it identifies the submitter and serves as chain-of-custody document. The extent of information may be reduced if the samples are transferred within the same department. Besides, each specimen must be labelled uniquely to identify the deceased, the type of sample and the date of collection. When these specimens arrive in the laboratory, they must be checked in order to verify the correct transport and also the correspondence with labels and documentation. The toxicological analysis laboratory may add a further label with a new code, if an internal classification is applied. When more

than one specimen of the same matrix is submitted, each container should be labelled uniquely (e.g. A, B, C, etc.). Post-mortem specimens are indeed not homogeneous and different containers of the same specimen type (e.g. blood) can sometimes have different drug concentrations. Moreover, the date of the arrival must be noticed, beside the approximate volume or mass, the type of container and any abnormal appearance of the specimen (e.g. decomposed, heat denatured, bloody urine, broken tubes, etc.).

As previously mentioned, a chain of custody must be assured. The laboratory should document what was received, from whom, by what means (by hand, courier, mail) and when. Storage of the specimens should be secure, and access to specimens and case documentation should be limited to authorised people. Freezing of specimens is highly recommended except for hair and a portion of the blood sample, which should be kept at ambient temperature or refrigerated. If samples are analysed within a few days after collection, freezing may not be necessary. The date of the analysis and the aliquot of each specimen should be recorded, as well as the date on which residual specimens are discarded or returned to the submitter. The length of retention of tissues by the laboratory may be a set period of time, or the time required to complete any legal proceedings (Moffat *et al.*, 2011).

Post-mortem specimens

Toxicological analysis can theoretically be performed on almost any specimen, but it is usually limited to the most common matrices in routine cases. For establishing the cause of death, the most important sample is usually blood, since the presence of a drug in this fluid shows that the person had used it shortly before dying. This compound could therefore cause or contribute to the death.

In post-mortem toxicology whole blood is used, as separation of red blood cells from serum is usually not possible. Post-mortem blood is however different from the whole blood collected from a living person. In addition to haemolysis and a fall in the pH, blood coagulates in the early post-mortem period, and then becomes fluid again. The extent of these

two processes will determine whether post-mortem blood is clotted, fluid or partially clotted and partially fluid. There is also a wide variation in the water content of post-mortem blood, ranging from 59 to 89%. All these changes may affect the original blood drug levels. Moreover, a few studies have demonstrated that this matrix is not homogenous, and that the concentrations of many drugs may vary from site to site of collection (see later). Autopsy blood is a very complex matrix to analyse, also because of its content in fatty acids, cholesterol and other steroids. A suitable sample preparation is therefore necessary to perform a chromatographic analysis. Furthermore, quantitative results must be correctly interpreted by taking into account the complexity of the matrix and the distribution phenomena which may have occurred.

Urine is also a useful fluid in post-mortem toxicology, even if some disadvantages are present. First of all, urine is available in about 50% of deaths, as it is quite common for the bladder to be voided during the dying process. Besides, urine often contain metabolites, whereas the parent drug may be not detected, or present at a very low concentration. A further and fundamental disadvantage is that urinary concentrations of most drugs are difficult, if not impossible, to interpret. Indeed, the concentrations of drugs and metabolites in urine depend on the time of urine formation in relation to sampling and drug ingestion. From an analytical point of view, urine contains relatively few endogenous substances that interfere with chromatography or immunoassay tests, since it is usually formed by more than 95% of water. As a consequence, most screening tests are performed on urine samples, when available.

Bile is a traditional post-mortem specimen, because it contains high concentrations of drugs and of their corresponding conjugates, especially if enterohepatic recycling occurs. Detection of morphine and many other drugs (e.g. benzodiazepines, colchicine and buprenorphine) is therefore more likely in this fluid than in blood, in which concentrations may be as much as 1000 times lower. This is the reason why bile was analysed in the past, when the sensitivity of the analytical method could be limited. The analysis of this matrix is still useful if urine samples are not available.

Moreover, a great amount of a drug or its metabolites in bile has been interpreted as a consequence of repeated use, which would lead to accumulation into this fluid. Nonetheless, bile is a waste fluid and the correlation between blood and bile concentrations of drugs is generally poor. Bile is a quite complex fluid, but a small volume is usually analysed because of the high concentrations of the analytes. As a consequence, this fluid doesn't create any particular analytical problems.

Stomach (or gastric) contents can be also analysed, especially if an oral overdose is suspected. Indeed, in that cases drug concentrations in the stomach may be quite high, even if a great amount of the drug has been already absorbed. Besides, stomach contents usually contain drugs rather than their metabolites. The disadvantage of stomach contents is its composition, which varies from a thin watery fluid to a semi-solid, depending on the amount and type of food present. The interpretative value of stomach contents is usually in confirming the consumption of an oral overdose, but the detection of a drug or metabolite in the stomach contents does not necessarily mean that the drug was taken orally. As a matter of fact, gastric juice is constantly secreted into the stomach, which in turn is formed from extracellular fluid; this latter may contain significant amounts of basic drugs and metabolites circulating in the blood. Moreover, gastric juice may have been contaminated with bile from retching or vomiting. If the total amount of drug detected in the stomach contents is significantly greater than the prescribed dose, the possibility of drug abuse or an overdose should be considered. Nonetheless, stomach contents are rarely homogeneous, and therefore it is difficult to measure accurately the representative concentration of drug in the volume of stomach contents received, unless the contents are homogenised. Unfortunately, the total stomach contents are often not given to the laboratory, so that results should be reported as the amount of drug present in the volume or mass of stomach contents received.

Sometimes, the distribution of a drug or its metabolites among the tissues can be useful to establish the cause of death. To this purpose, liver, brain, kidney, spleen and lung have been analysed in most cases.

Brain shows some advantages compared to other organs, because it is a relatively isolated organ, endowed with the cranium, with lower metabolic activity, resulting in slower decomposition and delayed process of putrefaction. Besides, brain should be unaffected by trauma to the abdomen and chest, and it may be an important matrix to analyse whenever psychoactive drugs are investigated. Different concentrations of drugs from one region of the brain to another should be considered anyway. Drug concentrations in kidney and spleen have little intrinsic significance, other than as part of the overall assessment of the body distribution of a drug. Kidney has been found to be useful in determining heavy metal concentrations, while spleen has been used as a secondary specimen for compounds, such as carbon monoxide and cyanide, which bind to haemoglobin. An appropriate sample preparation is one of the most important pre-requisite for the successful identification and quantification of drugs in body tissues. These samples are indeed rich in interfering species, such as proteins and lipids, which should be eliminated to achieve good analytical results.

Beside these samples, vitreous humour, hair, nasal swabs, injection sites, syringes or other items may be analysed sometimes. Finally, ante-mortem specimens may be available, in most cases blood, and sometimes urine and gastric contents, collected as part of the medical evaluation and treatment. Analysis of these specimens may be very useful in interpreting the cause of death.

Analytical Toxicology

For taking into account all the substances which may have played a role in the cause of death, a Systematic Toxicological Analysis (STA) is usually applied to post-mortem samples. STA refers to an adequate analytical strategy for the detection and identification of as many as possible potentially toxic compounds and their metabolites.

As far as non-volatile compounds analysis is concerned, a screening test is first carried out in most cases. This very simple and rapid test, based on an immunoassay, shows the positivity for some classes of drugs, but not for a specific compound. A sample is considered “positive” if it

contains a drug and/or its metabolites above a reference concentration, known as “cut-off”. In some cases, a semi-quantitative result is also given. Several types of screening tests and instrumentation have been developed and usually applied to urine samples. Blood screening tests have been recently introduced.

The second step is represented by confirmatory testing, through which quantitative determination of a drug and/or its metabolites is given. Quantification is necessary to state whether a drug has caused, prevented or contributed to the death. The choice of the analytical technique depends on what kind of molecule(s) must be investigated. Traditionally, Gas-Chromatography coupled with Mass Spectrometry is employed in most cases. This technique allows a so-known “generic investigation”, since unknown compounds may be identified by comparison with mass spectra libraries. Nonetheless, more and more Liquid Chromatography–Mass Spectrometry (LC/MS) or tandem mass spectrometry (LC/MS-MS) methods have been developed in the last years.

A chromatographic analysis can be performed after an adequate sample preparation, which consists usually of a Liquid-Liquid extraction (L-L) or a Solid-Phase Extraction (SPE). Sample preparation methods must be optimised according to the analytes and also to the matrix to extract. For example, tissues must be usually submitted to homogenisation and deproteination steps before the extraction.

Specific analysis are then carried out whenever a particular poisoning (e.g. carbon monoxide, cyanide) is suspected on the basis of circumstances of death and autopsy findings.

Finally, the Blood Alcohol Concentration (BAC) is usually determined. When available, the femoral or iliac blood is used, because it is less subject to post-mortem diffusion phenomena which may alter ethanol level. This kind of analysis is mostly performed by means of a Head-Space-GasChromatography (HS-GC) instrument coupled to a Flame-Ionisation-Detector (FID).

Interpretation of the Results

The interpretation of analytical results is the most challenging task in post-mortem toxicology, and can derive only from a complete knowledge of the case history, including autopsy findings, information from the scene and relevant medical history. Experienced post-mortem toxicologists rely first on their own case experience, and on published case reports. Indeed, it is not possible to simply refer to tables of therapeutic, toxic and fatal concentrations.

As previously said, blood is usually the most important sample to establish the cause of death. However, substantial changes may occur in blood drug concentrations during the interval between the agonal phases of death and autopsy, so that post-mortem drug concentrations do not necessarily reflect concentrations at the time of death. It must be also considered that the concentrations of drugs in post-mortem blood are site-dependent, as demonstrated by some studies (Logan and Smirnow; 1996; Bogusz, 1996; Skopp, 2010). Higher concentrations are usually found in central vessels and heart, since these compartments are more subject to post-mortem increase. This was observed for barbiturates, antidepressants, benzodiazepines, cocaine, non-opioid analgesics and, for morphine, M3G and M6G. Moreover, it was shown that, at peripheral sites, femoral and iliac blood morphine concentrations were well correlated with each other, making both vessels an appropriate site to collect peripheral blood for toxicological testing.

All mechanisms and processes causing artefactual increases or decreases in drug concentration during the post-mortem period can be included under the generic term of “post-mortem redistribution”. These phenomena occur mainly during the first 24 h after death. Post-mortem distribution seems to be different from one drug to another, according to some characteristics, such as physico-chemical and pharmacokinetic properties of the drug (size, shape, charge, pK_a-value, partition coefficient, apparent volume of distribution, binding to proteins, blood cells and/or tissues, initial concentration, residual enzyme activity in the early post-mortem period) or environmental conditions (pH value, orientation of

solute-flux, temperature, time, blood coagulation and hypostasis, blood movement due to fluidity changes and pressure, position of the corpse, lysosomal enzyme activities, bacterial invasion). Lipophilic drugs, with an apparent distribution volume >3 L/Kg and central/peripheral blood concentration ratio >1, seem to be candidates for a wider post-mortem redistribution (Skopp, 2010).

Site-dependent differences can derive from incomplete distribution of a drug at the time of death, release from the binding sites and/or passive diffusion through blood vessels or from the lumen of a body cavity into the surrounding organs. These phenomena mainly occur in organs like the intestines, stomach, liver, lungs and myocardium, through the blood vessels, but also pleural and peritoneal fluids. Diffusion of ethanol from the stomach to the heart is common, so that BAC is usually determined in femoral blood, as previously described. Drug concentrations may change during agonal phases, when hypoxia reduces intracellular pH, with a consequent accumulation of basic drugs into cells. After death has occurred, acidification up to a pH value of 5.5 and changes in ionic strength may damage lysosomal membranes, and subsequently cause the enzymatic digestion of the cell membrane and components. As the permeability of membranes increases, drugs are redistributed into the extracellular space, and haemolysis occurs. Moreover, the disintegration of physiological and anatomical barriers causes a rapid progress in post-mortem redistribution processes.

It is evident that these changes are not identifiable by post-mortem sampling and toxicological analysis, making the interpretation of the results a critical issue. In this regard, it may be important to determine brain concentration of drugs of abuse. Indeed, these molecules exert their effects mainly via the CNS, and moreover brain is less subject to post-mortem distribution phenomena. Thus, it can be assumed that the encephalic concentration of these drugs, measured in post-mortem specimens, is close or equal to their peri-mortem concentration at their site of action.

1.3.2 HEROIN FATALITIES

Heroin fatal overdose is still a public health problem in most countries, as described in paragraph 1.1.3. Several studies have therefore focused on this topic, by investigating epidemiology and post-mortem findings in order to highlight risk factors and consequently prevention strategies. The exact mechanism of fatal heroin overdose is still unclear. Death derives from the administration of a dose beyond the current tolerance of the person. This amount of drug induces respiratory depression till coma and cardio-pulmonary arrest, but for example the role of pulmonary oedema is problematic. Indeed, it can be of relatively rapid onset and thus contribute to death, or it may gradually develop with no evident role (White and Irvine, 1999).

Most heroin fatalities occur when other people are present, and medical help is not sought or is sought too late. Instant death from heroin injection does not seem to be the norm; on the contrary, most decedents are estimated to have died 1 to 3 hours after injection, a time interval that would allow intervention. Epidemiology is interesting and problematic at the same time. Only a minority of these deaths occur among novice users. Most victims of fatal heroin overdose are older (around 35 years old) and experienced (5-10 years) users, who would have high levels of tolerance and be skill in calculating and administering safe doses. Nonetheless, these “hard” heroin users often suffer from pathologies, such as liver and kidney diseases, which may influence drug metabolism and consequently increase toxicity. Loss of tolerance due to a withdrawal period must also be considered.

The contemporary use of therapeutic agents, especially methadone and benzodiazepines, and/or other drugs of abuse, such as cocaine and alcohol, may have a role in causing death, by acting at pharmacokinetic and/or pharmacodynamic level (Bertol *et al.*, 1997; Polettini *et al.*, 1999; Sporer, 1999; White and Irvine, 1999; Warner-Smith *et al.*, 2001; Fugelstad *et al.*, 2003; Polettini *et al.*, 2005; Al-Asmari and Anderson, 2007). The interpretation of heroin fatalities may be not immediately supported by analytical data, since a large proportion of these deaths

show blood concentrations of heroin metabolites below, or similar to, those of living intoxicated heroin users, or of heroin users who died of causes other than overdose (Warner-Smith *et al.*, 2001). The cause of death must therefore be established after considering the overall case history, and thus all the elements previously mentioned and described in detail in the following paragraphs.

Loss of tolerance

A number of fatal or non fatal heroin overdoses appears to occur after periods of reduced use (White and Irvine, 1999; Fugelstad *et al.*, 2003), for example a short time after release from prison. This increased risk is likely to be related to abstinence or infrequent use in prison resulting in reduced tolerance. As a consequence, a person administering a dose that previously produced euphoric effects with minimal respiratory depression may experience pronounced respiratory impairment. These findings were supported by studies performed on hair samples, which provide information on the use of illicit or therapeutic drugs in the last months. Morphine concentrations in hair samples of fatal overdose cases were found to be significantly lower than those of current users (Tagliaro and De Battesti, 1999). This was confirmed by further studies, and suggested that loss of opioid tolerance in this group is at least partially responsible for their fatal overdose (Warner-Smith *et al.*, 2001).

Some authors stated that long-term heroin users may cut down heroin consumption, with consequent reduction of their degree of tolerance. This may be a partial explanation for the age patterns observed in heroin-related fatalities. Paradoxically, it is even possible that repeated heroin use may increase the risk of overdose, because of the different rate of tolerance development among the drug effects (see also paragraph 1.2.7). A model that predicts such a change is shown in Fig.9 (font: White and Irvine, 1999), which describes time reduction in the ratio of doses that produce intoxicating versus lethal effects. Because of the relatively incomplete tolerance to respiratory depression, this ratio becomes smaller, and fatal overdose may consequently be more frequent. On the other hand, the rates of loss of tolerance may vary between effects. Thus, long-

term users who have recently reduced their consumption may be at greater risk of overdose as their tolerance to the respiratory depressant effects may have diminished more rapidly than their tolerance to the desired psychotropic effects.

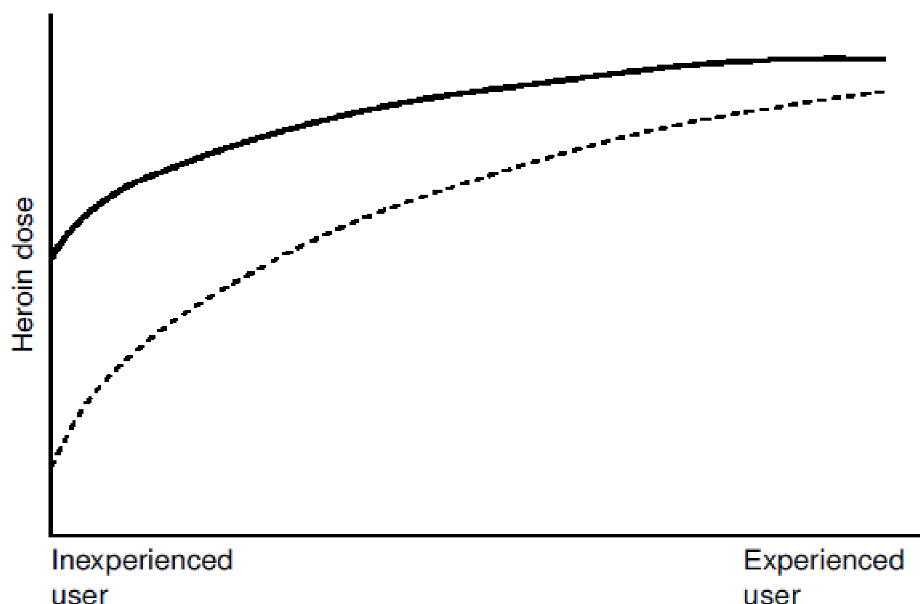

Fig.9: Hypothetical model of tolerance to intoxicating (---) and lethal (—) effects of opioids following repeated administration.

Drug interactions

In several heroin fatalities, death is ruled as a consequence of the combined effects of heroin and other drugs. The contemporary use of alcohol, benzodiazepines, and other illicit drugs is common among heroin addicts (see also paragraph 1.1.2), as well as smoking. These substances are active molecules, which have their own pharmacological and toxic effects. As a consequence, there may be a synergic action with heroin, but also an interaction at pharmacokinetics level. Besides, the use or abuse of therapeutic or illicit drugs can contribute to systemic diseases, especially kidneys, lung and liver dysfunctions. A lot of *in vitro*, *in vivo* and epidemiologic studies have investigated the influence of these substances on heroin action. In post-mortem toxicology, the role of each substance should be interpreted by considering, beside blood concentrations, also the autopsy findings, and the medical history.

Benzodiazepines and alcohol are themselves relatively weak respiratory depressants, but when combined with a potent respiratory depressant, such as heroin, they can enhance the effects of the latter drug. The potential for such interactions can be better understood by considering the actions of these compounds in the central respiratory regions. Benzodiazepines produce their effects by action at the benzodiazepine receptor site on the $GABA_A$ receptor complex, which play a major role within the respiratory control centres. Alcohol also acts via the $GABA_A$ receptor, binding to a distinct receptor site. In addition, alcohol acts at the NMDA receptor as a non-competitive antagonist, by reducing the effect of excitatory amino acids. The effects of alcohol and benzodiazepines should be at least additive with those of μ receptors agonists. Thus, in the presence of these substances, a "normal" or usual dose of heroin may be fatal (Bertol *et al.*, 1997). Moreover, it is important to underline that when respiratory depression is due to the combined actions of an opioid and a non-opioid drug, specific opioid antagonists such as naloxone will block or reverse only that component of respiratory depression due to the opioid (White and Irvine, 1999).

On the contrary, stimulants can act as functional or physiological antagonists of opioids and may therefore minimise the respiratory depressant effects of opioids. For example, caffeine, which is a common adulterant in "street heroin" , acts as antagonist at adenosine receptors in the CNS, modulating many opioid effects. This compound could therefore stimulate respiration depressed by opioid administration. Up to now, this effect hasn't been specifically evaluated (White and Irvine, 1999).

Alcohol may enhance the risk for a heroin overdose also for its action at pharmacokinetic level. The hydrolysis of 6-monoacetylmorphine into morphine seems to be delayed by alcohol use, as well as the formation of morphine glucuronides and their excretion (Polettini *et al.* 1999). Besides, ethanol inhibits the glucuronidation of morphine dose-dependently *in vitro* (Rook *et al.*, 2006; see also paragraph 1.2.4).

Tobacco smoking is typically highly prevalent among heroin users. Older heroin users are likely to have a history of daily tobacco use dating

back 10–15 years. A significant degree of tobacco-induced pulmonary disease among these population is highly probable. Besides, the smoking and "chasing" of heroin appear to be widespread in many countries and may have an adverse effect on lung function.

Similarly, it is well recognised that smoking crack cocaine can impair lung function. Epidemiologic studies have found that this behaviour is quite common among heroin users in some countries (see also paragraph 1.1.2). Moreover, drug addicts often use heroin and cocaine at the same time ("speed-balling" or "speed basing"). Since both heroin and cocaine are metabolised by carboxylesterases, a competitive inhibition of heroin metabolism by cocaine may occur, as already demonstrated in *in vitro* experiments (Rook *et al.*, 2006; see also paragraph 1.2.4). On the other hand, some authors suggested that a pharmacological interaction may prevail, as the glucuronidation and the total excretion didn't change in heroin-cocaine fatalities (Polettini *et al.*, 2005).

In heroin fatalities, it is not unusual to find also methadone and its metabolites. This drug is the most common therapeutic agent for heroin dependence treatment in some countries, such as Italy (see also paragraph 1.1.4). Being an opioid agonist, methadone has synergic effects, and may therefore play a role in causing the fatal respiratory depression. Moreover, treatment with methadone implies the withdrawal of heroin use, so that the individual degree of tolerance may decrease. This must be considered when interpreting analytical results from post-mortem specimens.

Recently, the influence of other substances on morphine glucuronides has been also studied for its possible influence on morphine or heroin action and toxicity. As mentioned in paragraph 1.2.4, morphine glucuronidation can be modulated by xenobiotics, resulting in increased concentrations of M6G and consequently in an enhancement of the pharmacological effects. Long-term exposure to morphine is not able to alter the rate of morphine glucuronides formation (Faura *et al.*, 1998). On the contrary, some studies have demonstrated that heroin may influence this process. Heroin addicts are usually exposed not only to a wide array

of drugs, but also to chemical impurities of “street heroin” which may influence the metabolic pathways. For example, among the heavy metals which are present in illicitly marketed heroin, cadmium and copper selectively inhibit M3G formation by guinea pig liver microsomes (Lawrence *et al.*, 1992). Interestingly, cadmium is present also in tobacco leaves and Intravenous Heroin Users (IHU) are often heavy smokers, as previously mentioned. In this respect, Antonilli *et al.* have systematically studied the effects of chronic exposure to heroin and to contaminants of “street heroin” on morphine glucuronidation. First of all (Antonilli *et al.*, 2003a), these authors found that long-term IHU showed higher levels of M6G compared to control groups. The changes in morphine glucuronidation were not directly dependent on the length of heroin exposure. However, IHU involved in the study reported at least two years of heroin assumption, so that it may be assumed that this time was sufficient to express all the possible effects of “street heroin” on glucuronidation. The authors suggested that also the subclinical nephropathy typical of IHU may cause a change in synthesis and excretion of morphine glucuronides, even if controversies are present on this topic (see also paragraph 1.2.4). This observational study was performed on a small group of subjects and had several limitations, but it confirmed data obtained by other researchers in *in vitro*, *ex vivo* and *in vivo* studies performed on animals. The same authors observed that, in rats, repeated administration of cadmium inhibited M3G without enhancing M6G production, while repeated exposure to heroin but not to morphine decreased the synthesis of M3G and stimulated the formation of M6G, which is usually almost undetectable in rats. These studies were performed *in vivo* and *ex vivo* (i.e. microsomal preparation obtained from rats treated *in vivo*) and the effects were reversible after few days of drug abstinence. According to the authors, while cadmium action may be attributed to non-competitive inhibition via covalent binding to UGT enzymes, heroin influence could be due to modulation of gene expression and/or direct enzymatic inhibition (Antonilli *et al.*, 2003b).

In a further study, these researchers investigated the role of μ opioid receptor in mediating the action of heroin on morphine glucuronidation, by studying the effects of methadone and naltrexone on the same process. The role of alcohol was also evaluated. The study, performed in liver microsomes obtained from treated rats (*ex vivo*), showed that μ opioid receptor does not seem to mediate heroin effects on the formation of morphine glucuronides. Moreover, methadone and alcohol didn't share this action, while naltrexone increased the synthesis of M3G (Antonilli *et al.*, 2005).

These results were partly confirmed by an *in vitro* study which showed that, in rat hepatocytes, heroin inhibited M3G synthesis and induced the formation of M6G; morphine produced similar effects but to a lesser extent, while methadone didn't share the same activity. Moreover, heroin effects were not counteracted by naltrexone, so that the authors could confirm that this drug is able to affect morphine glucuronidation via non-opioid actions (Graziani *et al.*, 2008).

In order to investigate the enzyme subtype responsible for the altered synthesis of M3G and M6G, the influence of heroin and naltrexone on glucuronidation of estradiol-3-glucuronide and estradiol-17-glucuronide was studied in rats. These hormone derivatives are selectively formed by UGT 1A1 and UGT 2B1, respectively. As a consequence, this study allowed to state that heroin and naltrexone specifically interact with UGT 1A1 subtype, being the synthesis of estradiol-17-glucuronide not affected by both opioids. Anyway, neither UGT 1A1 nor UGT 2B1 expression was changed in comparison with control group (Antonilli *et al.*, 2008).

Recently, the same authors investigated whether M6G synthesis can be induced by intravenous heroin self-administration in the rat, both *in vivo* and *ex vivo*. Once again, heroin induction of the synthesis of M6G was confirmed and a correlation between *in vitro* V_{max} for M6G and plasma levels of this metabolite was observed (Meringolo *et al.*,2011).

Further studies performed on heroin addicts are necessary to investigate if these phenomena occur also in humans. This may have implications in post-mortem toxicology (as partly already shown by some

studies; see paragraph "Analysis of post-mortem specimens"), as well as in the understanding of individual propensity to develop addiction.

Systemic dysfunction and organ diseases

As previously mentioned, long-term misuse of heroin can cause systemic diseases, especially kidneys, lung and liver dysfunctions. These pathological conditions may in turn influence heroin action and toxicity, thus being a risk factor for fatal overdose. Even if hepatic impairment seems not to be clinically meaningful in patients receiving morphine (see paragraph 1.2.4), this condition together with a general metabolic impairment may affect heroin pharmacokinetics and pharmacodynamics in drug addicts. Hepatic impairment is mainly due to hepatitis C, which is very common in IHU. Prevalence of simultaneous infection with multiple strains of hepatitis is also high among this population. The abuse of alcohol contributes also to liver diseases. Acute liver damage or liver function abnormalities are usually found during autopsy in heroin fatalities cases. However, it is unlikely that liver disease *per se* could precipitate fatality from overdose; on the contrary, reduced metabolism of opioids may prolong the period of heavy intoxication, thus increasing the probability of overdosing. Nonetheless, further research is required to establish whether an association exists between hepatic disease and overdose, and the nature and extent of this association.

Some studies have suggested that mortality from heroin overdose may be associated with pulmonary dysfunction, since the mechanism of death is respiratory arrest. In this regard, heroin users are likely to suffer from impaired pulmonary function as a result of smoking (mainly tobacco, but also heroin and other drugs), complications of previous non fatal overdose, and increased susceptibility to infection. Very few epidemiological data on this topic are available anyway. It must be considered that heroin users may also be at greater risk of infective diseases, because of their poor state of health and life-style. Malnutrition and poor hygiene are prerequisites for opportunistic acute respiratory infections, and are common among heroin users. As a result, these people may be more likely to suffer from both chronic and acute respiratory

infections. Undiagnosed pneumonia is frequently seen at autopsy in heroin overdose fatalities (Warner-Smith *et al.*, 2001).

Pathologic-anatomical abnormalities of the kidney and of the heart are also commonly found in post-mortem samples belonging to IHU, even if renal and cardiac impairment is probably relatively mild in most heroin dependents. No connections between heart pathologies (e.g. myocarditis and focal myocardial fibrosis) and risk for overdose were observed in a study by Fugelstad *et al.* (2003).

Analysis of post-mortem samples

The interpretation of the cause of death in heroin fatalities may be particularly difficult, because of the several contributing factors previously described. Heroin metabolism is also to be considered, since it gives rise to active metabolites, such as 6-MAM, morphine and M6G, which play a role in fatal overdose mechanism. These molecules are also the analytes which are detected by post-mortem analysis, since heroin can't be usually found because of its extremely short half-life.

In suspected fatal heroin overdose biological samples are suitably extracted, and subsequently Gas Chromatography (GC) methods are traditionally applied to determine morphine, which is the main metabolite, and 6-MAM, when detectable. Beside these molecules, also the impurities and the cutting agents which are present in "street heroin" may be found, for example acetylcodeine and its metabolite codeine, paracetamol and caffeine. On the other hand, GC can't detect M3G and M6G because of their lipophilicity.

The concentration of morphine in blood has frequently been regarded as a measure of heroin action and toxicity. However, a number of overdose fatalities show relatively low blood concentrations of morphine, i.e. below, or similar to, those of living intoxicated heroin users, or of heroin users who died of causes other than overdose (Warner-Smith *et al.*, 2001). This is due to several factors, as previously described, and above all to the relationship between fatal overdose and current individual degree of tolerance. That's why the cause of death must be established by considering the overall case history, from the circumstances of death to

demographic and medical information, from autopsy findings to analytical results.

Some authors have studied the ratio of "free" to "total" morphine, beside the presence of 6-MAM, in order to correlate this value with the time of death or the influence of other misused drugs (especially alcohol or cocaine; see also paragraph "Drug Interactions"). "Free" morphine stands for un-conjugated morphine, while "total" morphine refers to the amount of morphine which is determined after application of a hydrolysis step to the sample. In this way, morphine glucuronides, but also 6-MAM and other minor metabolites, are converted to morphine. In a study performed in various biological matrices (Goldberger *et al.*, 1994), the analytical results of rapid or delayed heroin fatalities were investigated. Death was classified as rapid or delayed according to known circumstances of death. Compared with delayed deaths, rapid deaths were characterised by the following trends: higher mean concentrations of 6-MAM, "free" and "total" morphine in blood; a higher ratio of "free" morphine concentrations to "total" morphine concentrations in blood; lower mean concentrations of 6-MAM and morphine in urine; greater likelihood of 6-MAM detection in blood; and lesser likelihood of heroin detection in urine. This study included the analysis of multiple tissue specimens from two subjects who died of heroin intoxication. Heroin was identified in urine and injection-site tissue. Concentrations of 6-MAM in cerebrospinal fluid, spleen, and brain were substantially higher than in blood, liver, lung, and kidney. All specimens were positive for morphine. Heroin metabolites were detected in hair specimens. The identification of heroin and 6-MAM in biological tissues effectively established the presence of heroin in cases of acute narcotic intoxication, even if heroin is not usually detected. Indeed, this was a designed experiment, in which samples were recovered and stored in the best conditions. In "real" cases, a very rapid hydrolysis probably occurs in post-mortem fluids and tissues, especially if autopsy is not performed within a short time. As a result, heroin is not present or is present below the Limit of Detection (LOD, see paragraph 5.8.3) of the applied methods.

Another study investigated the distribution of “free” and “total” morphine in body fluids and tissues in a fatal heroin overdose, in order to assess the stability of morphine conjugated. It was found that non-specific hydrolysis of conjugated morphine to “free” morphine would not occur in corpses at least for a few days after death. Moreover, it was proposed that femoral muscle may be a specimen of choice for roughly predicting the ratio of “free” to “total” morphine in blood, because it is relatively spared of both post-mortem diffusion of drugs and bacterial invasion (Moriya and Hashimoto, 1997).

As far as other tissues are concerned, a number of studies showed that brain morphine concentrations correlate very well with blood concentration in most cases, and thus this matrix could be used as indicator of recent heroin use when blood is not available (Baselt, 2000). Similarly to blood data, brain morphine levels show a wide variability (Sporer, 1999).

Finally, in the last years, increasing interest has grown towards the role of morphine-3-β-D-glucuronide (M3G) and especially morphine-6-β-D-glucuronide (M6G) in mediating heroin or morphine action. In order to directly determine these metabolites, some Liquid Chromatography (LC) methods were developed, using ultraviolet (UV) spectrometric, electrochemical or fluorescence (Aderjan *et al.*, 1995) detection or combined detection techniques. Up to now, the best results have been reached with LC coupled to a Mass-Spectrometry (MS) detector, since this technique allows a higher sensitivity and selectivity (Zuccaro *et al.*, 1997; Pichini *et al.*, 1999). As a consequence, an array of LC/MS methods for the detection of heroin or morphine metabolites with clinical (Binning *et al.*, 2010, Sakurada *et al.*, 2010; Hammoud *et al.*, 2011) and forensic applications (Bogusz *et al.*, 1997 a and b; Dienes-Nagy *et al.*, 1999; Svensson *et al.*, 2007; Stout *et al.*, 2009; Taylor and Elliott, 2009) have been developed in the last twenty years.

In post-mortem toxicology, a LC/MS method was applied to further evaluate the stability of morphine and its glucuronides in spiked fresh blood and plasma from living individuals as well as in four authentic post-

mortem blood specimens for a time interval of up to six months (Skopp *et al.*, 2001). Morphine and its glucuronides were found to be stable in both blood and plasma at 4°C for the whole observation period. In post-mortem blood the analytes were stable only when stored at -20°C, because of post-mortem hydrolysis of the glucuronides to morphine. These are obviously very important findings, which should be taken into account by the analytical toxicologists.

Besides, M3G and M6G have been studied in an attempt to highlight a level and/or a trend in their concentrations, which would allow to distinguish between "therapeutic" and "toxic" effects, and consequently support a better interpretation of heroin fatalities. Aderjan *et al.* (1995) developed a method to investigate the concentrations of morphine and its glucuronides in serum samples of heroin consumers and heroin-related deaths. Data showed that the ratios of the concentrations of M6G or M3G to morphine, and of M6G to M3G depended on the time elapsed since the last administration of morphine or heroin. Moreover, M6G values were found to be higher in cases of death than in the living addicts. These authors suggested therefore that fatal heroin overdose could be better assessed by measuring also M6G and M3G concentrations and ratios to morphine. Another study (Bogusz *et al.*, 1997a) performed on blood, urine, vitreous humour and cerebrospinal fluid belonging to heroin victims, confirmed the importance of determining these metabolites in post-mortem specimens. According to this study, the ratio of M6G to M3G in blood seemed to be higher in heroin fatalities than during oral morphine therapy. Indeed, these findings are in agreement with data from *in vitro* and *in vivo* experiments, which have showed a variation in morphine glucuronidation after exposure to heroin and/or other substances (Lawrence *et al.*, 1992; Antonilli *et al.*, 2003a; Antonilli *et al.*, 2003b; Antonilli *et al.*, 2005; Antonilli *et al.*, 2008; Graziani *et al.*, 2008; Meringolo *et al.*, 2011; see paragraph "Drug interaction").

Therefore, the study of M3G and M6G could represent the right direction to follow in deepening post-mortem toxicological investigations.

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2. Aim of the study

The PhD study focused on heroin fatalities, which are often particularly difficult to interpret because of several reasons, as previously described (see paragraph 1.3.2).

The “Laboratorio di Analisi Chimico-tossicologica” where I worked usually analyses post-mortem samples submitted mainly by the “Istituto di Medicina Legale e delle Assicurazioni” of the “Università degli Studi di Palermo”. These specimens are collected from people whose death can’t be immediately related to a natural cause. Thus, after evaluating information about circumstances of death, history of drug addiction or diseases, autopsy and histological examination, the medical examiner may ask toxicological analysis.

In our laboratory post-mortem samples are subjected to a Systematic Toxicological Analysis (STA) in order to highlight the presence of unknown exogenous compounds (e.g. drugs of abuse) which may have played a role in the mechanism of death. If circumstances or other elements suggest that a compound could be involved, the medical examiner requires only a targeted analysis. In most cases, a STA is however carried out. Among the post-mortem matrices, blood is usually the key one, because it provides information about exposure which had occurred shortly before death. Nonetheless, as described in the introduction (paragraph 1.3.1 and 1.3.2), the interpretation of analytical results derives only from a complete knowledge of the case history, being not possible to simply refer to tables of therapeutic, toxic and fatal concentrations.

Whenever a case of suspected heroin fatal overdose is submitted, a screening test and subsequently a Solid Phase Extraction (SPE) followed by GC/MS analysis is performed. These methods are usually applied to blood, urine and bile specimens, when available, and allow to determine the main metabolites of heroin, i.e. morphine (MOR) and, when detectable, 6-monoacetylmorphine (6-MAM). Beside these molecules, also the impurities and the cutting agents which are present in “street heroin” may be found, for example acetylcodeine and its metabolite codeine, paracetamol and caffeine. Heroin is usually not detected because of its

extremely short half-life. Moreover, the amount of “total” morphine (and “total” codeine) in urine and bile samples is determined, by applying a chemical hydrolysis before SPE. Thanks to this step, the conjugated derivatives of morphine (and codeine) are converted into the corresponding molecule, thus being detectable by GC/MS technique. Blood samples can’t be hydrolysed in the applied conditions, so that only “free” morphine (and “free” codeine) is determined.

In accordance with literature, a wide variability in blood concentrations of morphine (and codeine) was present among the submitted cases, and it was sometimes difficult to explain the death as “a fatal heroin overdose” on the basis of the analytical results. Certainly, the interpretation of the cause of death comes from the evaluation not only of toxicological data, but also of all the other available elements, as previously described. Nonetheless, a deeper comprehension of the role of heroin metabolites could be important to support the diagnosis of the cause of death in heroin fatalities. In the last years it has been proposed that the metabolites morphine-3-β-D-glucuronide (M3G) and especially morphine-6-β-D-glucuronide (M6G) could mediate heroin or morphine action (see also paragraph 1.2.5 and 1.3.2). Since GC/MS technique can’t determine these highly hydrophilic molecules, an array of LC methods has been developed in the last twenty years, and applied to the clinical (Binning *et al.*, 2010, Sakurada *et al.*, 2010; Hammoud *et al.*, 2011) and the forensic (Aderjan *et al.*, 1995; Bogusz *et al.*, 1997 a and b; Dienes-Nagy *et al.*, 1999) fields.

The aim of this PhD work has been the development of a LC/MS method for the determination of morphine (MOR) morphine-3-β-D-glucuronide (M3G) and morphine-6-β-D-glucuronide (M6G) in blood samples from heroin fatalities. The method would be applied to blood samples belonging to cases of suspected fatal heroin overdose, in order to evaluate if the concentrations of M3G and M6G, beside MOR, might better explain the cause of death, as suggested by some authors (Aderjan *et al.*, 1995; Bogusz *et al.*, 1997 a). Biological specimens were provided by the groups of Prof. Rino Froldi and Prof. Paolo Procaccianti, who are the

directors of the “Istituto di Medicina Legale e delle Assicurazioni” of Macerata and of Palermo, respectively, and who contributed also to the interpretation of the results. The influence of some risk factors, such as the contemporary use of alcohol, methadone or cocaine, would be also studied. Indeed, it has been demonstrated that morphine glucuronidation can be modulated by xenobiotics, such as heroin itself, and this may cause an increase in M6G concentrations and consequently an enhancement of the pharmacological effects (Antonilli *et al.*, 2003 a and b; Antonilli *et al.*, 2005; Antonilli *et al.*, 2008; Graziani *et al.* 2008; Meringolo *et al.*, 2011). The ratio between the concentration of M6G and M3G may therefore vary in drug addicts, resulting in a higher M6G level, which could play a key role in fatal heroin overdose mechanism.

3. Systematic Toxicological Analysis

As mentioned in the previous Chapter, the “Laboratorio di Analisi Chimico-tossicologica” where I developed my PhD work usually analyses post-mortem samples for which a toxicological analysis has been required by the medical examiner. These specimens come from people who died under circumstances which seem not to be related to a natural death but, on the contrary, need to be investigated, by establishing if the use of exogenous substances (especially drugs of abuse) had occurred.

To this purpose, a Systematic Toxicological Analysis (STA) is carried out (see also 1.3.2). When urine is available, a screening test is first performed, by applying a small volume of specimen onto a commercial device (Testcard®). This very simple test provides a qualitative screen for the presence of four classes of compounds, which are related to the following ones:

- Methamphetamine (cut-off: 500 ng/mL)
- Cocaine (cut-off: 300 ng/mL)
- Morphine (cut-off: 300 ng/mL)
- THC (Δ9-Tetrahydrocannabinol) (cut-off: 50 ng/mL)

All the available biological fluids (blood, urine, bile) samples are then analysed by GasChromatography/MassSpectrometry (GC/MS), after a suitable Solid-Phase-Extraction and derivatisation, in order to detect a wide array of substances and their metabolites. Thus, a so-known “general investigation” of most drugs of abuse is achieved. This method is applied for a qualitative purpose, i.e. detecting molecules thanks to known Retention Time (R.T.) and mass spectra or comparing unknown spectra with libraries of mass spectra (NIST MS Search 2.0, Wiley 275, PMW_Tox3). If a drug of abuse and/or its metabolites and/or other exogenous compounds are detected, a further GC/MS analysis is performed to quantitative purposes. Sample preparation (e.g. initial volume of matrix) and instrument settings are targeted to the compounds to quantify, and Working Standard samples (WSs), i.e. “blank” biological fluid spiked with standard solutions, are analysed together with the unknown specimens. The quantification is usually applied to methadone,

and its main metabolites EMDP (2-Ethyl-5-methyl-3,3-diphenylpyrroline) and EDDP (2-Ethyl-1,5-dimethyl-3,3-diphenyl-pyrrolidine), using proadifen as Internal Standard (IS); amphetamine, methamphetamine, 3,4-Methylendioxymethamphetamine (MDMA), using a variety of IS; cocaine and benzoylecgonine, using scopolamine as IS; codeine, morphine, 6-MAM and acetylcodeine using nalorphine as IS. In some specific cases, also other molecules are quantified. If morphine and/or codeine are present, urine and bile specimens are also subjected to chemical hydrolysis, followed by SPE and GC/MS analysis in the same conditions of STA. This procedure allows to determine "total" morphine and/or "total" codeine in urine and bile samples, but it can't be applied to blood specimens. A Head Space-GasChromatography/Flame Ionisation Detector (HS-GC/FID) analysis is also performed in order to determine ethanol concentration in blood samples. When available, the femoral blood is used, because it is less subject to post-mortem diffusion phenomena which may alter ethanol level (see also paragraph 1.3.1). Whenever unknown peaks are found in the HS-GC/FID chromatogram, a HS-GC/Mass Spectrometer (MS) analysis is also carried out.

Sometimes, gastric contents and tissues are analysed by applying the previously mentioned GC/MS method, after a suitable and quite complex sample preparation. When available, hair samples are also tested, in order to provide information about exposure occurred during the last months before death. Besides, THC and its metabolites (11-Nor-9-carboxy-Δ9-THC, 11-hydroxy-THC) are investigated in some cases, by applying a suitable Liquid-Liquid (L-L) extraction followed by GC/MS analysis in targeted conditions.

Finally, specific analysis may be required whenever a particular poisoning (e.g. carbon monoxide, cyanide) is suspected on the basis of circumstances of death and autopsy findings.

The "Laboratorio di Analisi Chimico-tossicologica" is a quality certified laboratory (ISO 9001), and thus each routine method is applied following a Standard Operative Procedure (SOP) and registered in the corresponding form (date, operator, results,...).

All the analytical data are communicated to the medical examiner, who will interpret them taking into account also other available information. The diagnosis of the cause of death will come from an overall evaluation of all the elements previously described.

An overview of the analytical results of the period 2008-2011 is given in the following paragraph. These data were useful to select blood samples for the PhD study, and also to interpret LC/MS-MS results.

3.1 SAMPLES ANALYSIS AND RESULTS

In the period from January 2008 to October 2011, our laboratory analysed autopsy samples coming from seventy-two cases for which toxicological analysis had been asked. Among them, fifty people had assumed drugs of abuse just before dying, as shown by the presence of these substances or their metabolites in autopsy specimens. As described in Chapter 1, it is important to find out the presence and blood levels of these compounds because they may have played a role in the cause of death.

When samples arrive in our laboratory, they are registered as described by the SOP. Every sample belonging to a case is named and labelled with a number referred to the year, followed by the letter B (as they are biological specimens), a progressive number, and finally a letter for each individual specimen. For example, 08/B01/A indicates the blood (A) sample belonging to the first case (B01) of 2008 (08). This registration is applied also to biological samples coming from living people.

A document is usually sent with the samples, where some information about the case history is given, for example demographic data (name, age, gender), the date of death and of the autopsy, type of samples collected, circumstances of the death, information about drug-addiction and/or methadone assistance therapy for heroin dependence. Table 4 shows age, gender and relevant circumstances of the fifty cases in which drugs of abuse or their metabolites were detected. Most people (48) were men, while the average age was 35 years old (range: 19-52).

CASE No.	AGE	GENDER	RELEVANT CIRCUMSTANCES
08/B03	45	M	First injection? Family denies a history of drug dependence
08/B04	47	M	Sudden death after a presumed sexual intercourse
08/B08	34	M	Found dead at home with a syringe nearby; injection sites at autopsy
08/B09	35	M	Occasional use of illicit drugs reported; injection sites at autopsy
08/B13	23	M	Found dead in his bed; injection sites at autopsy
08/B17	41	M	Found at home with a syringe nearby; he died at the hospital
08/B18	35	M	Found dead in a street; strong smell of alcohol
08/B21	40	M	Found dead at home; history of drug dependence
08/B22	45	M	In treatment with methadone; alcohol ingestion reported
09/B04	24	M	Found in his bed; no signs of injection; white foam and blood at the mouth and at the nose
09/B05	30	M	Found in his bed; no signs of injection; white foam and blood at the mouth and at the nose
09/B11	42	M	Found dead at home; history of drug addiction; injection sites at autopsy
09/B12	45	M	Found dead in a street with a syringe nearby
09/B17	36	M	Found dead in a street; injection sites at autopsy
09/B18	22	M	Led to the hospital; a double dose of heroin was reported
09/B19	41	M	Found dead in his car
09/B20	35	M	In treatment with methadone for heroin dependence
09/B21	45	M	Found dead in a toilet with a syringe nearby
09/B22	44	M	Found dead at home with a syringe in the arm
09/B23	31	M	Found dead in a toilet with a syringe in the arm
09/B27	30	M	Found dead at home in front of TV; history of drug dependence and methadone treatment; empty bottle found in a room
09/B30	uk	M	He died in prison; suspected contemporary use of methadone and buprenorphine
09/B31	32	M	Found dead at home; syringe and spoon found in the toilet; history of drug dependence
10/B01	45	M	He died after a downfall from scaffolding; in treatment with methadone
10/B02	21	M	Arrived dead at the hospital
10/B06	36	M	Found unconscious at home, he died shortly after; suspected drug addict; injection sites at autopsy

10/B07	32	M	Found in a street after a probable malaise; at the hospital his relatives reported a history of drug dependence and a suspected HIV positivity; death probably due to hemorrhagic shock because of splenic rupture
10/B08	32	M	Found dead in a car with two syringes and drug powder
10/B10	25	M	Found dead under a bridge
10/B19	21	M	Found dead in a toilet; syringe and spoon nearby; no signs of injection
10/B20	52	M	Found dead in his car, in which he lived; history of drug dependence and currently of alcohol abuse
10/B21	26	M	Found dead with a syringe nearby
10/B22	34	M	Found dead in his car; injection sites at autopsy
10/B26	37	M	Found in a car; in assistance treatment
10/B27	uk	M	uk
10/B36	39	F	Found dead at home with some syringes
10/B37	22	M	Found dead in a street
10/B38	39	M	Found dead at home with a syringe nearby
10/B39	22	M	Found dead at home with a syringe nearby
10/B40	44	M	Found dead in his car; in treatment with buprenorphine/naloxone
10/B41	22	M	Found dead at home with suspected “brown-sugar”
11/B01	32	M	History of drug dependence; injection sites at autopsy
11/B02	37	M	History of drug dependence; in treatment with methadone; injection sites at autopsy
11/B03	43	M	Found dead with a syringe nearby
11/B07	42	M	Found dead at his home; history of drug dependence
11/B08	35	M	Found dead in a toilet with a syringe nearby
11/B13	50	M	Found dead in a public place
11/B14	45	M	Found dead in a public place
11/B15	25	M	Found dead in his bed; history of drug dependence; in treatment with methadone
11/B16	19	F	Found dead in a public place; ingestion of drug tablets was reported
Avg. age = 35			
Gender distribution =2F/49M			

Table 4: Overview of demographic information and circumstances of death of the fifty cases in which drugs of abuse and/or their metabolites were found (uk =unknown).

For each case, all the available biological fluids (blood, urine, bile) were analysed by applying the routine procedure previously described. Blood specimen is usually the most important for establishing the cause of death, thus it was always analysed. In few cases, kidney or bladder wash was submitted, and therefore analysed, instead of urine, while gastric contents analysis was sometimes required. In some heroin fatalities, urine and/or bile volume was very low, so that hydrolysis was directly applied in order to determine "total" morphine and/or codeine. In some cases, also molecules other than the usual ones were quantified (e.g. flurazepam). A number of specimens showed a positive or uncertain result for THC at the screening test, but this was never confirmed by GC/MS, so that the result of this analysis is omitted.

The following tables (Table 5 a,b,c,d) show the results of the samples belonging to the fifty cases in which drugs of abuse or their metabolites were detected. For the screening test, +/-/± = positive, negative, uncertain result, respectively; for Blood Alcohol Concentration (BAC), n.p. = not present, i.e. at a concentration below 0.5 g/L; for GC/MS analysis, - = not present, i.e. below the Limit of Detection (LOD), n.q. = not quantified, because of lack of standard or sample, traces = above the LOD, but below the Lower Limit of Quantitation (LLOQ); empty box indicates that no analysis was carried out.

2008

CASE No.	RESULTS							
	BAC (g/L)	Screening	GC/MS (ng/mL)					
08/B03	n.p.	MOR + COC - MET - THC -	compound	BLOOD	URINE	HYDR. URINE	BILE	HYDR. BILE
			codeine	90		3089	-	-
			morphine	420		34451	8650	19850
08/B04	2.8	MOR + COC + MET - THC -	compound	BLOOD	URINE	HYDR. URINE	BILE	HYDR. BILE
			cocaine	16	24			
			BZE	-	764			
			codeine	65	-	-		
			morphine	596	-	15		

08/B08	n.p.	**MOR +** **COC +** MET - THC -	**compound**	**BLOOD**	**URINE**	**HYDR. URINE**	**BILE**	**HYDR. BILE**
			cocaine	184	63523			
			BZE	5443	142019			
			codeine	18	-	675		353
			morphine	86	-	16091		71347
08/B09	n.p.	**MOR +** **COC +** MET - THC -	**compound**	**BLOOD**	**URINE**	**HYDR. URINE**	**BILE**	**HYDR. BILE**
			cocaine	20	4156			
			BZE	2544	117772			
			codeine	129	-	-		-
			morphine	614	-	266		1842
08/B13	n.p.	**MOR +** COC - MET - THC -	**compound**	**BLOOD**	**URINE**	**HYDR. URINE**	**BILE**	**HYDR. BILE**
			cocaine	20	419		756	
			BZE	10	124		-	
			codeine	10	1055	3974	-	255
			morphine	33	7055	25947	-	22211
08/B17	n.p.	**MOR +** **COC +** MET - THC -	**compound**	**BLOOD**	**URINE**	**HYDR. URINE**	**BILE**	**HYDR. BILE**
			cocaine	10	39			
			BZE	50	1379			
			codeine	45	54	1814		
			morphine	187	1936	2141		
08/B18	1.2	MOR - COC - MET - THC -	**compound**	**BLOOD**	**URINE**	**HYDR. URINE**	**BILE**	**HYDR. BILE**
			methadone	717	574			
08/B21	1.5	**MOR ±** COC - MET - THC -	**compound**	**BLOOD**	**URINE**	**HYDR. URINE**	**BILE**	**HYDR. BILE**
			desmethyl-diazepam	1497	20	-		-
			codeine	60	-	-		-
			morphine	97	-	58		200
08/B22	1.7	MOR - COC - MET - THC -	**compound**	**BLOOD**	**URINE**	**HYDR. URINE**	**BILE**	**HYDR. BILE**
			methadone	2284	4002			

Table 5a.

2009

CASE No.	RESULTS							
	BAC (g/L)	Screening	GC/MS (ng/mL)					
09/B04	n.p.	MOR + COC + MET - THC -	**compound**	**BLOOD**	**URINE**	**HYDR. URINE**	**BILE**	**HYDR. BILE**
			BZE	-	479			
			codeine	73	294	646		-
			morphine	315	1275	7602		91235
			acetylcodeine	-	78			
09/B05	n.p.	MOR + COC + MET - THC -	**compound**	**BLOOD**	**URINE**	**HYDR. URINE**	**BILE**	**HYDR. BILE**
			BZE	-	283			
			codeine	87	1192	4267		-
			morphine	498	8275	75691		1690732
			acetylcodeine	-	99			
09/B11	n.p.		**compound**	**BLOOD**	***KIDNEY***	**HYDR. URINE**	**BILE**	**HYDR. BILE**
			codeine	64	-			
			morphine	338	*1089 ng/g*			
09/B12	0.6	MOR + COC + MET - THC +	**compound**	**BLOOD**	**URINE**	**HYDR. URINE**	**BILE**	**HYDR. BILE**
			cocaine	-	54			
			BZE	-	8000			
			codeine	150	-	-		44
			morphine	688	68	99		3765
09/B17	2.0	MOR + COC - MET - THC +	**compound**	**BLOOD**	**URINE**	**HYDR. URINE**	**BILE**	**HYDR. BILE**
			codeine	193	-	22		
			morphine	615	71	161		
09/B18	n.p.		**compound**	**BLOOD**	**URINE**	**HYDR. URINE**	**BILE**	**HYDR. BILE**
			codeine	17				705
			morphine	412				197885
09/B19	n.p.	MOR + COC + MET - THC -	**compound**	**BLOOD**	**URINE**	**HYDR. URINE**	**BILE**	**HYDR. BILE**
			cocaine	75	37798			
			BZE	1847	455896			
			codeine	23	426	1744		122
			morphine	533	4083	35935		43786

			compound	BLOOD	URINE	HYDR. URINE	BILE	HYDR. BILE
09/B20	0.5	**MOR +** COC - MET - THC -	EMDP	621	2470			
			EDDP	719	1810			
			methadone	890	1395			
			codeine	traces	210	472		
			morphine	77	378	12505		
09/B21	n.p.	**MOR +** COC - MET - THC -	**compound**	**BLOOD**	**URINE**	**HYDR. URINE**	**BILE**	**HYDR. BILE**
			codeine	25	-	-		-
			morphine	228	417	848		571
09/B22	n.p.	**MOR +** COC - MET - THC -	**compound**	**BLOOD**	**URINE**	**HYDR. URINE**	**BILE**	**HYDR. BILE**
			codeine	72	-	-		-
			morphine	313	51	740		6638
09/B23	n.p.	**MOR +** COC - MET - THC -	**compound**	**BLOOD**	**URINE**	**HYDR. URINE**	**BILE**	**HYDR. BILE**
			codeine	20		75		-
			morphine	441		1986		5559
			6-MAM	n.q.				
09/B27	n.p.	MOR - COC - MET - THC -	**compound**	**BLOOD**	**URINE**	**HYDR. URINE**	**BILE**	**HYDR. BILE**
			EMDP	78	1256		13171	
			EDDP	traces	n.q.		n.q.	
			methadone	1421	9105		24609	
09/B30	n.p.	MOR - COC - MET - THC -	**compound**	**BLOOD**	**URINE**	**HYDR. URINE**	**BILE**	**HYDR. BILE**
			EMDP	-	traces		106	
			EDDP	-	7708		39774	
			methadone	582	1198		543	
			flurazepam	1303	959		3819	
09/B31	n.p.	**MOR +** COC - MET - THC -	**compound**	**BLOOD**	**URINE**	**HYDR. URINE**	**BILE**	**HYDR. BILE**
			codeine	657	41	201		
			morphine	330	490	3861		

Table 5b.

2010

CASE No.	RESULTS							
	BAC (g/L)	Screening	GC/MS (ng/mL)					
10/B01	n.p.	MOR - COC - MET - **THC +**	**compound**	**BLOOD**	**URINE**	**HYDR. URINE**	**BILE**	**HYDR. BILE**
			EMDP	-	85			
			EDDP	-	21301			
			methadone	267	7460			
10/B02	n.p.	**MOR +** COC - MET - **THC ±**	**compound**	**BLOOD**	**URINE**	**HYDR. URINE**	**BILE**	**HYDR. BILE**
			codeine	220	103,00	229	-	199
			morphine	234	1534	6486	4574	62853
			Tryptamine-like substance found in blood sample					
10/B06	*0.32mg/g**	**MOR +** COC - MET - **THC ±**	**compound**	***BLOOD****	**URINE**	**HYDR. URINE**	**BILE**	**HYDR. BILE**
			codeine	-	49	234	42	109
			morphine	-	817	5527	5025	24254
				** SOLID*				
10/B07	n.p.	**MOR +** COC - MET - THC -	**compound**	**BLOOD**	**URINE**	**HYDR. URINE**	**BILE**	**HYDR. BILE**
			EMDP	-	34		389	
			EDDP	-	31177		n.q.	
			methadone	105	1182		903	
			codeine	146	682	2000	-	106
			morphine	281	10425	76651	3234	33422
10/B08	0.9	**MOR +** COC - MET - THC -	**compound**	**BLOOD**	**URINE**	**HYDR. URINE**	**BILE**	**HYDR. BILE**
			codeine	141	-	12	-	-
			morphine	368	200	231	2400	3605
10/B10	1.1	**MOR +** COC - MET - THC -	**compound**	**BLOOD**	**URINE**	**HYDR. URINE**	**BILE**	**HYDR. BILE**
			codeine	133	18	23	-	-
			morphine	309	248	330	365	692
10/B19	1.3	**MOR +** COC - MET - THC -	**compound**	**BLOOD**	**URINE**	**HYDR. URINE**	**BILE**	**HYDR. BILE**
			codeine	10	12	20	-	44
			morphine	191	87	231	523	9240
10/B20	n.p.	MOR - COC - MET - THC -	**compound**	**BLOOD**	**URINE**	**HYDR. URINE**	**BILE**	**HYDR. BILE**
			EMDP	traces	traces		traces	
			EDDP	traces	traces		traces	
			methadone	613	3076		3390	

			compound	BLOOD	URINE	HYDR. URINE	BILE	HYDR. BILE
10/B21	n.p.	**MOR +** COC - MET - **THC +**	codeine	40	274	1935	90	250
			morphine	542	2508	28519	6976	177808
10/B22	n.p.	**MOR ±** COC - MET - THC -	**compound**	**BLOOD**	**URINE**	**HYDR. URINE**	**BILE**	**HYDR. BILE**
			codeine	traces	-	11	140	223
			morphine	88	traces	105	3359	36820
10/B26	n.p.	MOR - COC - MET - THC -	**compound**	**BLOOD**	**URINE**	**HYDR. URINE**	**BILE**	**HYDR. BILE**
			EMDP	-	-		137	
			EDDP	-	8120		-	
			methadone	497	1431		2730	
10/B27	3.0	MOR - **COC +** MET - THC -	**compound**	**BLOOD**	**URINE**	**HYDR. URINE**	**BILE**	**HYDR. BILE**
			cocaine	57	7215		2235	
			BZE	1541	14034		3697	
			Other cocaine metabolites (cocaethylene and ecgonine methyl ester) = n.q.					
10/B36	n.p.		**compound**	**BLOOD**	**URINE**	**HYDR. URINE**	**BILE**	**HYDR. BILE**
			EMDP	-			n.q.	
			EDDP	traces			n.q.	
			methadone	832			5344	
			cocaine	30			646	
			BZE	499			2614	
			morphine	-			4510	
10/B37	n.p.	**MOR +** **COC +** MET - THC -	**compound**	**BLOOD**	**URINE**	**HYDR. URINE**	**BILE**	**HYDR. BILE**
			EMDP	traces	-			
			EDDP	-	n.q.			
			methadone	888	4700			
			cocaine	traces	2387			
			BZE	62	3737			
			codeine	traces	470	858		
			morphine	257	2688	15550		
			6-MAM	-	n.q.			
10/B38	0.7	**MOR +** COC - MET - THC -	**compound**	**BLOOD**	**URINE**	**HYDR. URINE**	**BILE**	**HYDR. BILE**
			codeine	80	-	22	-	40
			morphine	847	83	688	768	5500
			6-MAM	-	traces		-	

			compound	BLOOD	URINE	HYDR. URINE	BILE	HYDR. BILE
10/B39	n.p.	**MOR +** **COC +** MET - **THC +**	EDDP	-	n.q.			
			methadone	115	1218			
			cocaine	traces	3175			
			BZE	traces	11103			
			codeine	-	870			
			morphine	77	4678	4304		
			6-MAM	-	n.q.	51040		
10/B40	n.p.	MOR - **COC +** MET - THC -	**compound**	**BLOOD**	**URINE**	**HYDR. URINE**	**BILE**	**HYDR. BILE**
			cocaine	-	9526			
			BZE	7664	28457			
			buprenorphine	-	10			
10/B41	0.5	MOR - COC - MET - THC -	**compound**	**BLOOD**	**URINE**	**HYDR. URINE**	**BILE**	**HYDR. BILE**
			codeine	63	traces	11		
			morphine	1070	80	160		
			6-MAM	-	n.q.			

Table 5c.

2011

CASE No.	RESULTS							
	BAC (g/L)	Screening	GC/MS (ng/mL)					
11/B01	0.7	**MOR +** COC - MET - THC -	**compound**	**BLOOD**	**URINE**	**HYDR. URINE**	**BILE**	**HYDR. BILE**
			codeine	36	18	292	-	156
			morphine	2116	520	11240	6984	73825
			6-MAM	-	18		-	
11/B02	0.6	MOR - COC - MET - THC -	**compound**	**BLOOD**	**URINE**	**HYDR. URINE**	**BILE**	**HYDR. BILE**
			cocaine	44	22			
			BZE	1995	147			
11/B03	0.5	**MOR +** **COC +** MET - THC -	**compound**	**BLOOD**	**URINE**	**HYDR. URINE**	**BILE**	**HYDR. BILE**
			cocaine	44	41206		4136	
			BZE	4376	68200		10560	
			codeine	12	331	1162	-	97
			morphine	570	1754	13844	4608	38223
			6-MAM	-	594		-	

11/B07	2.2	**MOR +** COC - MET - THC -	**compound**	**BLOOD**	**URINE**	**HYDR. URINE**	**BILE**	**HYDR. BILE**
			codeine	traces	10	50	-	-
			morphine	130	120	860	71	620
11/B08	1.2	**MOR +** COC - MET - THC -	**compound**	**BLOOD**	**URINE**	**HYDR. URINE**	**BILE**	**HYDR. BILE**
			codeine	traces	traces	20	-	300
			morphine	171	250	140	282	860
			6-MAM	-	n.q.	-	-	-
11/B13	n.p.	MOR - **COC ±** MET - THC -	**compound**	**BLOOD**	***Bladder wash***	**HYDR. URINE**	**BILE**	**HYDR. BILE**
			BZE	5672	*509*			
			cocaine	39	-			
11/B14	n.p.		**compound**	**BLOOD**	**URINE**	**HYDR. URINE**	**BILE**	**HYDR. BILE**
			EMDP	-			6284	
			EDDP	-			n.q.	
			methadone	166			15080	
			BZE	276			396	
			cocaine	32			-	
11/B15	n.p.	MOR - COC - MET - THC -	**compound**	**BLOOD**	**URINE**	**HYDR. URINE**	**BILE**	**HYDR. BILE**
			EMDP	26	769			
			methadone	1020	13396			
			EDDP	-	n.q.			
11/B16	n.p.	MOR - COC - MET - THC -	**compound**	**BLOOD**	**URINE**	***Gastric cont.***	**BILE**	**HYDR. BILE**
			EMDP	traces	traces	*116*	-	
			EDDP	n.d.	22	*640*	36330	
			methadone	268	982	*1511*	2170	

Table 5d.

Thanks to the applied STA, heroin fatalities could be identified to later apply the developed PhD method. In detail, heroin had been assumed in thirty-six cases (72% of the fifty cases), as indicated by the presence of morphine, 6-MAM (found in urine specimens in some cases), acetylcodeine ("street heroin" impurity, which was detected only in two cases, 09/B04 and 09/B05) and codeine (metabolite of acetylcodeine, usually present). The contemporary use of other drugs of abuse, especially ethanol or cocaine, had often occurred. In some cases, people were in assistance treatment with methadone, so that fatal overdose could be related to loss of tolerance (Table 6).

	n	%
Heroin	10	**28**
Heroin + ethanol	9	**25**
Heroin + cocaine	7	**19**
Heroin + cocaine + ethanol	3	**8**
Heroin + methadone + cocaine	3	**8**
Heroin + methadone + ethanol	1	**3**
Heroin + methadone	1	**3**
Heroin + benzodiazepine+ ethanol	1	**3**
Heroin + other compounds	1	**3**

Table 6: Per cent distribution of heroin fatalities among the fifty cases in which the use of drugs of abuse had occurred.

4. LC/MS method development

4.1 CHOICE OF THE ANALYTICAL TECHNIQUE

As previously described, GC/MS technique doesn't allow the direct determination of morphine-3-β-D-glucuronide and morphine-6-β-D-glucuronide because of their high hydrophilicity. In the last years, Liquid Chromatography (LC) methods have been therefore developed, using ultraviolet (UV), Diode-Array (DAD), electrochemical, fluorescence, and especially Mass-Spectrometry (MS) detection (Pichini *et al.*, 1999; see also 1.3.2). The application of Mass-Spectrometry has been achieved thanks to the development of LC-MS interfaces, which have overcome the initial coupling problems between the LC flow and the MS vacuum.

The PhD method for the determination of morphine (MOR), morphine-3-β-D-glucuronide (M3G) and morphine-6-β-D-glucuronide (M6G) was developed using an LC coupled to API-ESI-QqQ instrument, which is available in our laboratory.

The ESI (Electro-Spray Ionisation) system is an API (Atmospheric Pressure Ionisation) interface, where LC eluate is sprayed from a conductive needle, to which a high voltage is applied, so that a spray of charged droplets is formed. This process is assisted by a nebulising gas (air or nitrogen), which flows concentrically around the needle. The electrospray needle has a high potential difference applied to it (typically in the range from 2.5 to 5.5 kV) with respect to a counter electrode. As a consequence, the droplets are repelled from the needle towards the Mass Spectrometry Ion source cone on the counter electrode. A heated desolvation/drying gas (nitrogen) flows around the spray exiting the probe, evaporating the solvent from the surface of the droplets. At the end of this process, analyte ions reach the surface and, when the charge density increases, are expelled from the droplets as gas-phase ions ("ion evaporation" process). An alternate theory, called "droplet fission", states that coulombic repulsion on the surface of the droplets increases as the solvent evaporates, causing them to explode until single ions are produced. In this way, only gas-phase ions reach the counter electrode and, after having crossed a capillary, the MS detector, where high vacuum is kept (Fig.10, adapted from lamondlab.com).

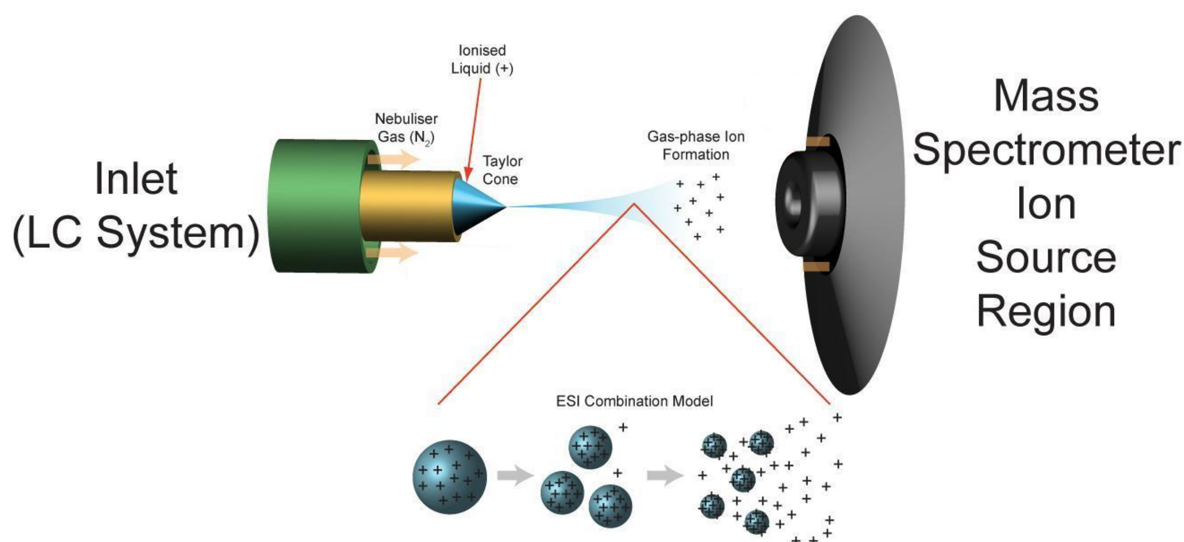


Fig.10: Overview of Electro-Spray Ionisation (ESI).

Positive (ESI^+) or negative ionisation (ESI^-) mode is possible, depending on the nature of the analytes. The first one is produced when a positive ion is added to a molecule (e.g. H^+, NH_4^+, Na^+); in the second one, a H^+ is removed from a molecule or a negative ion, such as Cl^-, is added. The mobile phase is usually chosen taking into account the ionisation mode, in order to enhance it. For example, formic acid is often employed when positive ionisation mode is applied. The carboxylic group of the glucuronic acid allows a negative ionisation to M3G and M6G, as well as the phenolic group of morphine and M6G (Appendix 1). Nonetheless, the positive ionisation mode was preferred, since it gave the best results and could be applied to a wide array of “street heroin” metabolites. In the positive ionisation mode, the basicity of the aliphatic amino-group was exploited.

In the ESI^+ source, morphine, M3G and M6G are ionised to mainly form their corresponding Molecular Ions $[M+H]^+$, which reach the MS Ion counter electrode and are detected by the analyser (quadrupole). MS-MS analysis was performed to enhance selectivity, reducing the problems of the possible matrix interference and of the lack of any structural information due to the soft ionisation in the ESI interfacing system. This technique increases also the sensitivity of the method. In detail, the triple

quadrupole mass spectrometer (QqQ) was set to work in MRM (Multiple Reaction Monitoring) mode. This approach is widely used for quantitative purposes on QqQ and is based on the filtration of the Molecular (Precursor) ion of the investigated compound in the first quadrupole (Q_1), its fragmentation (CID = Collision-Induced Dissociation) in an Argon (Collision Gas) enriched atmosphere in the second quadrupole (Q_2), and finally on the filtration of one of the product ions, which is then detected, in the third one (Q_3) (Fig.11, adapted from srmatlas.org).

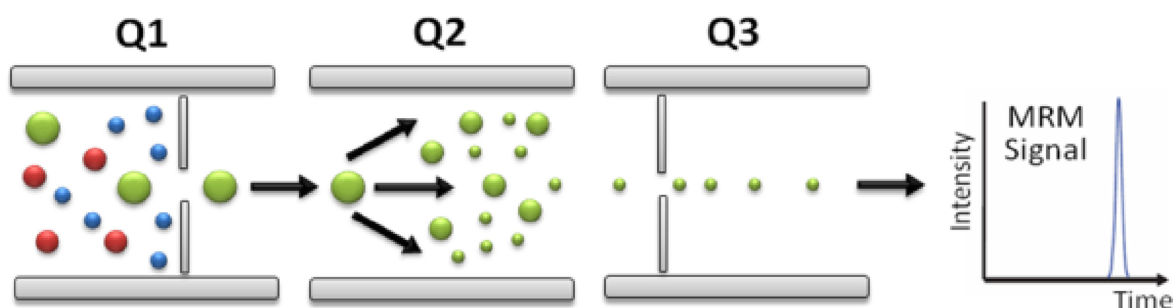


Fig.11: Schematic representation of a MRM experiment in a triple quadrupole instrument: Q_1 = selection of a Precursor Ion; Q_2 = collision gas-mediated fragmentation of the Precursor Ion; Q_3 = selection of a single (or some) Product Ion(s).

As far as chromatography is concerned, it was necessary to develop a LC method which would be able to separate the analytes, and also to be coupled to the ESI-QqQ detector.

The development of the LC/MS-MS method is described in the following paragraphs, while the final settings are shown in Chapter 5.

4.2 MASS SPECTROMETER PARAMETERS

First of all, morphine, morphine-3-β-D-glucuronide (M3G) and morphine-6-β-D-glucuronide (M6G) were characterised by direct infusion of a solution (1 ppm = 1 μg/mL in methanol) in the ESI-QqQ mass spectrometer.

As previously described, positive ionisation mode was used, and consequently the corresponding Molecular Ion $[M+H]^+$ of each molecule was first monitored by the Q_1. The optimisation of the ESI parameters was achieved thanks to both manual set-up and to instrument software tools. Ionisation parameters, such as needle voltage, counter electrode (shield) voltage, capillary voltage, nebulising gas (nitrogen) pressure, drying gas (nitrogen) pressure and temperature, were studied and finally set at their optimised values. The detection parameters, like Q_0 offset, L_4 offset and Q_1 Ion guide, were also optimised.

During the initial experiments, 3mM ammonium formate buffer and methanol with 0.1% formic acid were used; these solvents assisted ionisation by transferring H^+ ions to the analytes.

Among the MS-MS modes, the MRM one was applied, using Argon at a pressure of 2.00 mTorr as CID (Collision-Induced Dissociation) Gas. To this purpose, the pattern of fragmentation of each analyte at different collision energies was studied and two specific transitions from Molecular (Precursor) ion to Product ions were selected for each molecule (Table 16 in Chapter 5). One of these MRM transitions was used for quantification (quantifier MRM), while the other one for identification (qualifier MRM). For all the analytes, the most abundant MRM transition was the "Molecular" one, that is to say the MRM transition from Molecular ion $[M+H]^+$ (filtered in Q_1) to Molecular ion $[M+H]^+$ (detected in Q_3) at low collision energy (usually 5-6 V). In this case, the advantage of MS-MS in comparison to MS mode is the reduction of the background noise, resulting in a higher sensitivity.

Electromultiplier voltage was set at 1750V, in accordance with the last instrument calibration.

4.3 CHROMATOGRAPHIC SEPARATION

When MRM mode is applied, it is possible to identify a molecule thanks to the presence of all its specific MRM transitions, being the selected transitions of other analytes not present. A complete chromatographic separation may be therefore not fundamental, even if the possible interference of other molecules and/or matrix components must be evaluated. This is not the case of our molecules, since M3G and M6G are characterised by the same MRM transitions, so that it is necessary to chromatographically separate them.

In choosing the mobile phase, also the coupling to ESI interface had to be taken into account. Only volatile buffer solutions can be used, and moreover the mobile phase should be able to enhance the ionisation process. As previously mentioned, 3mM ammonium formate buffer and methanol with 0.1% formic acid were initially employed, since this was a commonly used binary mobile phase in positive ionisation mode.

Different Reversed-Phase (RP) columns were tested, such as diphenyl and C_{18} columns. Good results were achieved with a C_{18} Kinetex column, which is filled by 2.6 μm not-fully porous particles, with a solid silica core and a homogenous and porous shell. This innovative technology increases resolution, throughput, and sensitivity.

The method was optimised by changing the mobile phase composition and the gradient elution. In detail, acetonitrile with 0.1% formic acid provided better results in comparison to methanol with 0.1% formic acid. 3mM Ammonium formate buffer was prepared by dissolving 3 mmol/L of ammonium formate salt in ultra-pure water in an ultrasonic bath (15 minutes). After adding 0.1% of formic acid and mixing, the pH was checked with a pHmeter and led to a value of 3 (± 0.1) with some further drops of formic acid. Some attempts were initially made by working with different percentage compositions of the binary mobile phase in isocratic conditions. A high percentage of aqueous phase (95%) gave the best results. This may be due to the high hydrophilicity of morphine and, to a greater extent, of M3G and M6G, especially if they are in the protonated form as in the applied acidic conditions. Among the three analytes, M3G

was first eluted, while the separation between M and M6G was found to be particularly critical. MRM mode assured distinct identification, and moreover a fairly good separation was achieved thanks to the application of a slight gradient (Fig.12) and a constant column temperature (40°C) (Appendix 2).

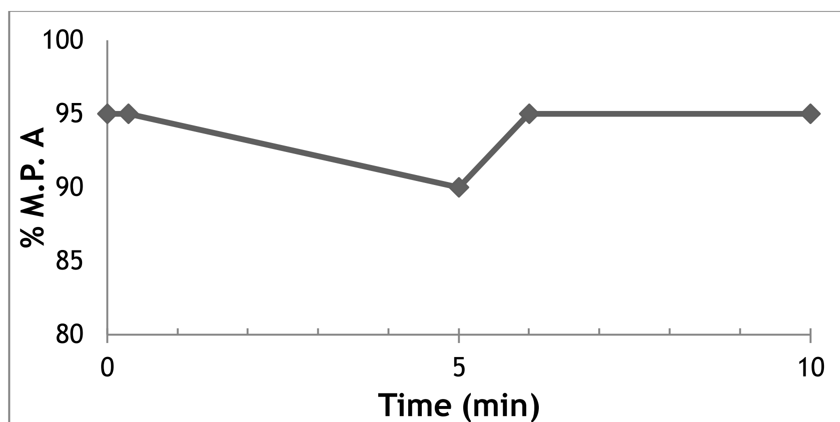


Fig.12: Diagram of the applied gradient elution
(% M.P.A = percentage of mobile phase A).

The chromatographic method was further modified in order to apply it to biological samples extracts and also to the analysis of other molecules (see paragraph 4.7). A guard-column was installed in front of the analytical column to prevent column damage, especially when analysing biological samples extracts.

4.4 SAMPLE EXTRACTION

The LC/MS-MS method could be applied to biological specimens only after an adequate pre-treatment, in order to remove interfering matrix components which might reduce the life of the column and/or affect analytes separation and detection.

Solid Phase Extraction (SPE) is the most widely used sample preparation technique in the analysis of drugs or their metabolites at ng/mL levels in biological fluids. SPE is commonly applied in our laboratory for the purification of a high number of samples, by means of a cheap, simple and manual apparatus which allows the contemporary extraction of twenty samples. This technique is based on dissolution (repartition) or binding (adsorption) of the target compounds into/to a stationary phase contained in a small cartridge, from which the molecules are finally recovered by an elution solvent/mixture. In detail, a traditional SPE extraction is divided into the following steps (Fig.13, adapted from services.leatherheadfood.com):

1) Conditioning: the cartridge is flushed with specific solvents, in order to prepare the stationary phase to interact with the target molecules. This step is fundamental to activate the stationary phase, for example by stretching the C_8 lipophilic chains.
2) Sample loading: The aqueous sample is loaded on the cartridge and eluted at low speed (1 mL/min or less) in order to allow the repartition or adsorption phenomena. If the sample is solid it must be solved, while if it is too viscous it must be diluted. A buffer solution is usually used, because a correct pH value is fundamental to assure the interactions between the target molecules and the stationary phase.
3) Washing: After loading the sample, the cartridge must be washed to eliminate all the matrix interfering

	components which could be weakly bounded to the stationary phase.
4) Drying:	Residual water is eliminated from the stationary phase by drying it under air flow at high vacuum.
5) Elution:	The cartridge is finally flushed with a solvent which is able to recover the compounds of interest.

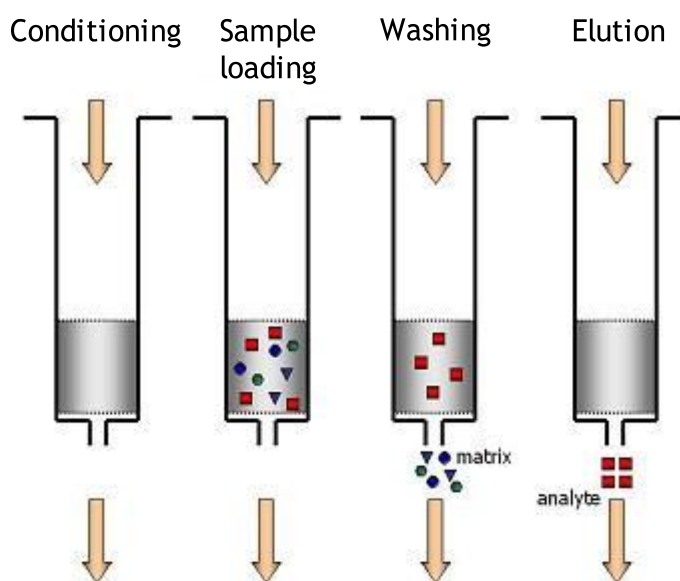


Fig.13: Schematic representation of SPE steps.

The stationary phase of the cartridges, the solvents and the buffer solutions are chosen according to the target compounds. After SPE, the extracts are usually dried under a stream of nitrogen and the residue is dissolved in an adequate solvent to be analysed.

In the PhD work, the routine SPE method for morphine of our laboratory was first evaluated. As expected, it allowed a good recovery of morphine but not of M3G and M6G. This SPE method was therefore modified, keeping the same mixed-mode sorbent cartridges (Bond Elut Certify™) and changing the wash and elution steps. To this purpose, literature was a useful starting point, even if the authors used different types of column cartridges, sometimes openly stating the unsuitability of Bond Elut Certify (Al-Asmari and Anderson, 2007).

Bond Elut Cerify cartridges contain a mixed-mode bonded silica gel, which consists of hydrophobic (C_8) and strong cation exchanger (benzene sulfonic acid) functional groups. These columns have been successfully applied to the extraction of various classes of drugs from biological fluids, and are particularly suitable for morphine because of its amphoteric nature. This stationary phase seemed to be suitable also for M3G and M6G, since these molecules are structurally similar to morphine. On the contrary, the higher hydrophilicity of M3G and M6G could be responsible for the lack of recovery of these molecules with the routine elution mixture (dichloromethane:isopropyl alcohol:ammonium hydroxide-80:20:2). For these reasons, the extraction method was initially changed by washing with pH 9 buffer solution and eluting with methanol, as indicated by Al-Asmari and Anderson (2007).

SPE step	
1) Conditioning	2.0 mL methanol, 2.0 mL pH 9 buffer solution
2) Sample loading	
3) Washing	4.0 mL pH 9 buffer solution
4) Drying	10 minutes under high vacuum
5) Elution	3.0 mL methanol

Table 7: Initial SPE conditions.

These conditions (Table 7) led to a good recovery of all the three analytes, but the extracts took a long time to be dried under a stream of nitrogen at 40°C. Moreover, when applied to biological samples, the corresponding final samples couldn’t be directly analysed; on the contrary, they needed to be submitted to centrifugation (urine and bile) or filtration (blood).

The SPE process was further optimised focusing on autopsy blood extraction, since this is the most important matrix in post-mortem toxicology. As described in Chapter 1, autopsy blood is a very complex matrix to analyse, because it contains a lot of interfering compounds and often some clots. Besides, post-mortem blood samples can be very different one another, depending on the time elapsed between death and

collection, the type and circumstances of death and other factors. It was therefore fundamental, but also not so immediate, to develop an extraction procedure which would assure a clean extract.

As mentioned before, the initially developed SPE required a final filtration of the blood extracts with a syringe 0.2 μm filter; this step was time-consuming, expensive and potentially dangerous for the operator, so that several attempts were made to avoid it. First of all, an acetonitrile:water (70:30) elution mixture was used instead of methanol. The extracts contained less interfering compounds, but it was still necessary to filter, and moreover the recovery was much lower.

Some deproteination techniques were then tested, in order to eliminate matrix interfering components before SPE. Sample preparation was initially achieved by adding 3 mL of water and 2 mL of pH 9 buffer solution to 1 mL of blood sample, in order to dilute it and stabilise the pH. Nalorphine, the GC/MS method Internal Standard (IS), was also added in order to better assess the SPE efficiency during the several experiments. According to the literature (Chen *et al.*, 1993) and some previous GC/MS experiments, the best pre-treatment is achieved by using an ultrasonic bath, while chemical deproteination agents usually cause a reduction in the recovery of the analytes. Nonetheless, a deproteination experiment with acetonitrile was carried out, with the aim of verifying if the higher purification of the extract could balance the loss in the recovery. To 1 mL "blank" autopsy blood spiked with 100 ng of MOR, M3G and M6G and 100 ng of nalorphine, a volume of 2 mL of acetonitrile was added. After gently vortex mixing and centrifugation (4000 rpm, 30 min), the supernatant was separated and evaporated to dryness. The residue was then dissolved with 3 mL of water and 2 mL of pH 9 buffer solution, and SPE was performed. The corresponding extract was cleaner, but it was still necessary to filter, because thin particles were present after dissolution of the dried residue into the mobile phase. Moreover, the reduction in the SPE efficiency was confirmed. Ultrasonic bath was also considered, but this step didn't prove useful.

Some other mixed-mode cartridges were tested, applying the same conditions (Table 7), but results were similar or sometimes worse in terms of recovery and purification of the extracts. The application of the extracts to some purification columns and filters was also evaluated, without a real improvement.

An attempt to change the pH of extraction was also made. Morphine and its glucuronides are characterised by a basic moiety, and consequently protonated at acidic pH, so that they can interact with the cationic groups of the stationary phase. As shown in some papers (Chen *et al.*, 1993; Rook *et al.*, 2005), good results were achieved with mixed-mode cartridges (apolar-cation exchange) by working at pH 3. At this pH value, basic compounds should be bound to the stationary phase, while acidic ones (such as fatty acids of the matrix) could be easily eliminated by washing the cartridge with an organic solvent. The applied conditions are shown in Table 9, while sample preparation was the same of the previous method unless the use of pH 3, instead of pH 9, buffer solution.

SPE step	
1) Conditioning	2.0 mL methanol, 2.0 mL pH 3 buffer solution
2) Sample loading	
3) Washing+Drying	2.0 mL water, 3.0 mL acetic acid 1M; 10 minutes under high vacuum
4) Washing+Drying	0.5 mL hexane;1-2 minutes under high vacuum
5) Elution	3.0 mL methanol with 0.5% ammonium hydroxide

Table 8: Conditions of the pH 3 SPE method.

This method was first applied to urine samples, with the aim of initially assessing the recovery of the analytes without wasting “blank” autopsy blood. Very good results were reached in terms of purification and especially of recovery of the M6G, but also of the other molecules. Surprisingly, the application of the same method to blood samples didn’t allow the recovery of M3G and M6G. Several experiments were performed to explain these findings. First of all, the good results of this new method on urine specimens were confirmed, so that it seemed to be a problem

related to the matrix. In detail, if the blood specimen was prepared at pH 9 and the same buffer solution was used in the condition step, but the new washing and elution conditions were applied, the glucuronides were not recovered. The pH of the samples to extract was then assessed. Two Working Standard blood samples were contemporary prepared. The first one was added with pH 9 buffer solution and the pH value was about 8.0; it was extracted following the initial conditions (see Table 7). For the second sample, pH 3 buffer solution was used and measured pH was around 5.0; a volume of 150 μL of acetic acid was added to reach a pH of 3.3 before carrying out SPE in the new conditions (Table 8). At this acidic pH, deproteination also occurred, as demonstrated by a change in sample colour and by partial precipitation. In this experiment, good results in the recovery of morphine glucuronides were achieved. The pH 3 method gave no evident advantages in terms of recovery, even though it was characterised by a slight improvement in sample purification. Moreover, the pH had to be checked and set around 3 for each sample, with risk of contamination among the samples during the pHmeter measurements. This method was therefore dismissed and other experiments were performed.

Keeping the conditions of the previous steps, a further washing with 3.0 mL of hexane:ethyl acetate (80:2) was added to eliminate lipid interfering compounds. In this way, the final sample could be simply submitted to centrifugation (6000 rpm, 15 min), separating and analyzing the supernatant.

Finally, it was found that an elution volume of 2.0 mL gave a recovery similar to the previous elution volume of 3.0 mL. As a consequence, the first one was used in the final method (see also Chapter 5).

As far as sample is concerned, a starting volume of 0.5 mL was chosen, because it was usually available for all the unknown samples, and it contained an amount of analytes above the Lower Limit of Quantitation (see also 5.4) in most cases.

4.5 QUALI-QUANTITATIVE ANALYSIS

In the LC/MS-MS method, the qualitative analysis of each molecule derived from the presence of all its MRM transitions at the corresponding Relative Retention Time (R.R.T.).

As mentioned in paragraph 4.1, MS-MS analysis was performed to enhance selectivity, reducing the problems of the possible matrix interference and of the poor fragmentation due to the soft ionisation in the ESI interfacing system. Among the MS-MS mode, MRM was chosen because it was based on a selection of both the Precursor Ion and the Product Ion. As a consequence, no interference was observed among the structurally similar molecules which were analysed.

Nonetheless, the identification was supported by the R.R.T, primarily in the case of M3G and M6G which are characterised by the same MRM transitions. It was necessary to take into account the R.R.T. rather than the absolute Retention Time (R.T.) because of changes of the R.T. among the chromatographic runs. The R.R.T. of each analyte was calculated by dividing the R.T. of that compound by the R.T. of the corresponding Internal Standard (IS, see below).

Beside the previous described advantages, MRM mode allowed also to increase the sensitivity of the method. In detail, the peak area of a specific MRM transition for each analyte was considered for quantification. For morphine, the MRM transition from Molecular ion $[M+H]^+$ to Molecular Ion $[M+H]^+$ (286.3-286.3; 6.0 V) was chosen as quantifier MRM transition, while for M3G and M6G the MRM transition from 462.2 to 286.1 at 27.5 V was used for quantification.

Moreover, the use of different IS was evaluated (Appendix 3). Deuterated IS are usually the first choice when working in LC/MS, so that morphine-D3, morphine-3-β-D-glucuronide-D3 (M3G-D3) and morphine-6-β-D-glucuronide-D3 (M6G-D3) were used. Nevertheless, nalorphine (NAL), the routine GC/MS internal standard, was also investigated. All these molecules were characterised by direct infusion in the mass spectrometer, optimising the ionisation and detection parameters and studying the fragmentation pattern in the MRM mode (Table 9).

Compound	Q1 first mass	Q3 first mass	Capillary (V)	Collision Energy (V)
MOR-D3	289.1	289.1	80.0	5.5
	289.1	228.8	80.0	12.5
	289.1	200.9	80.0	17.0
M-3-G-D3	465.5	465.5	80.0	5.0
	465.5	289.0	80.0	24.0
M-6-G-D3	465.5	465.5	80.0	5.0
	465.5	289.0	80.0	24.0
NAL	312.3	270.2	64.1	13.5
	312.3	201.3	64.1	18.5
	312.3	165.2	64.1	33.5

Table 9: MRM transitions of the tested internal standards.

Five Working Standard solutions were then prepared in the following way: 100 µL of NAL (1 µg/mL in methanol), 100 µL of MOR-D3 (1 µg/mL in methanol), 100 µL of M3G-D3 (1 µg/mL in methanol), 100 µL of M6G-D3 (1 µg/mL in methanol), were added to 1.0 mL of water spiked with increasing concentrations (5, 10, 50, 100, 200 ng) of MOR, M3G and M6G. After adding 2.0 mL of pH=9 buffer solution and mixing, these samples underwent SPE following the initial conditions previously described (Table 7) and the final specimens were analysed by LC/MS-MS. The MRM transition from Molecular ion $[M+H]^+$ to Molecular Ion (289.1-289.1; 5.5 V) was chosen as quantifier MRM transition for MOR-D3, the MRM transition from 465.5 to 289.1 at 25.5V for M3G-D3 and M6G-D3, and the MRM transition from 312.3 to 201.3 at -18.5 V for NAL. Starting from the data obtained for each molecule, regression lines were calculated by the method of least squares, plotting the signals as a function of analytes content and forcing the curve by the O (0,0) point. In detail, regression lines equations were expressed as follows:

$$y = a\,x + b$$

where: $y = \text{Area}_{\text{analyte}}/\text{Area}_{\text{IS}}$;

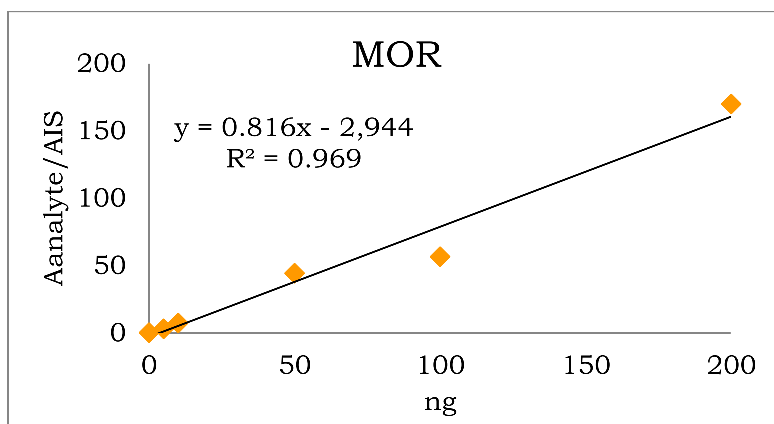
a = slope of the regression line;

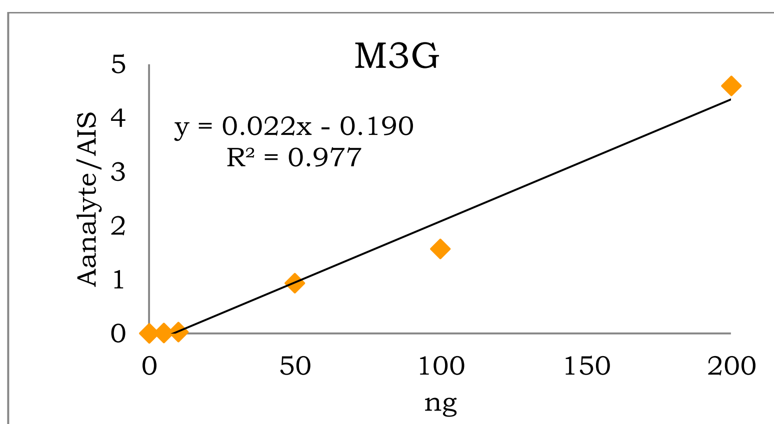
x = ng of each analyte;

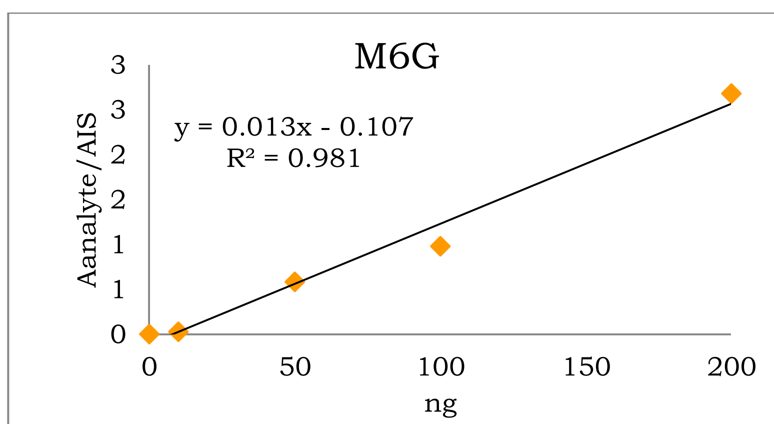
b = y-intercept of the regression line.

For each analyte, two regression lines were calculated: in the first one NAL was used as IS, while in the second one the corresponding IS-D3 was considered. The square of the correlation coefficient (R^2) was also calculated. The plots with the equations and the R^2 values of the calibration curves of each compound are shown below.

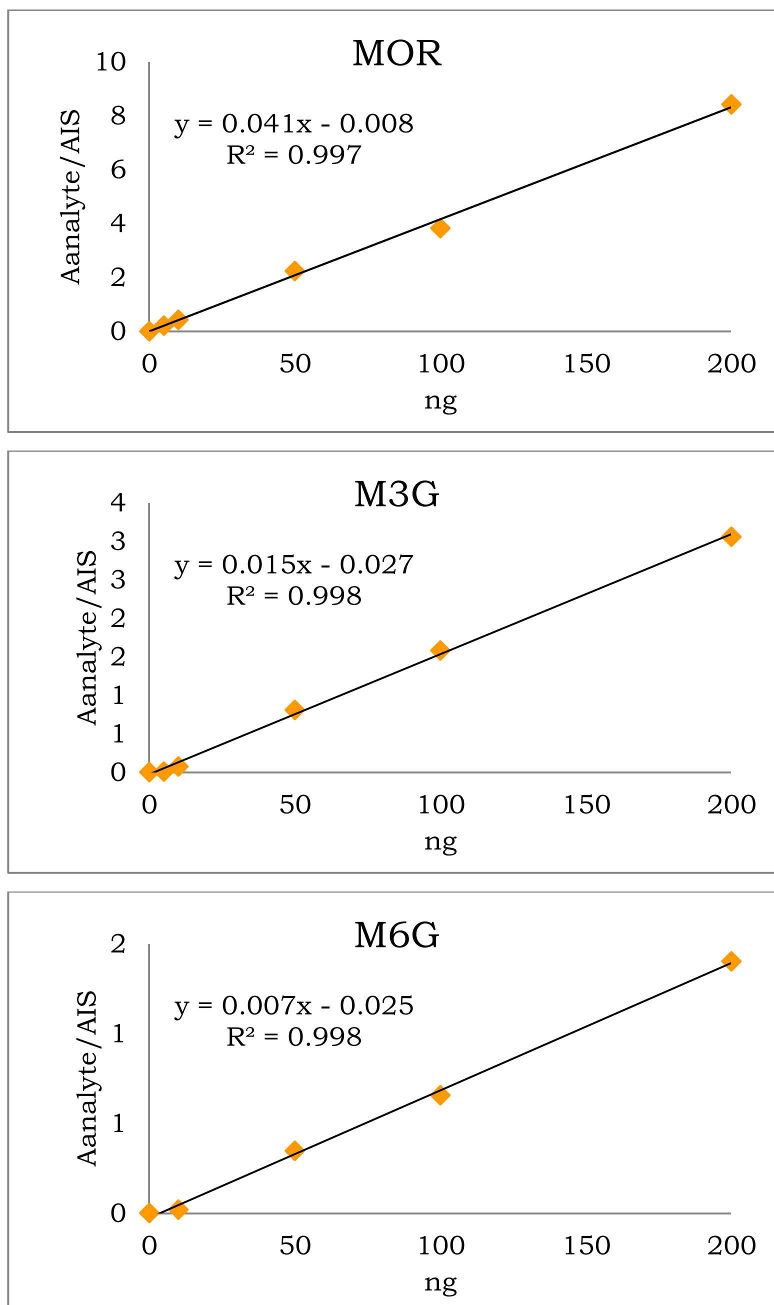
Regression Lines (IS=Nalorphine):







Regression Lines (IS=morphine-D3, morphine-3-β-D-glucuronide-D3 and morphine-6-β-D-glucuronide-D3, respectively):



It must be noticed that the first Working Standard sample (5 ng) was too low for M6G, so that only four points were used for the regression curves. R^2 values above 0.99 were found for all the analytes when using the corresponding IS-D3, indicating a good linearity of the method. On the contrary, NAL led to regression lines with lower R^2 values.

Further data were obtained by calculating the % RSD (Per cent Relative Standard Deviation, see paragraph 5.8.5) of the RF (Response

Factor, see paragraph 5.7) for each Working Standard solution. This precision index confirmed the results of the regression curves study (Table 10).

Compound	% RSD w NAL	% RSD w IS-D3
MOR	20,81	5,55
M3G	19,21	7,65
M6G	15,56	3,50

Table 10: Comparison of the intra-assay precision using NAL or deuterated MOR,M3G and M6G (IS-D3) as internal standard
(% RSD = Per cent Relative Standard Deviation)

The following experiments were therefore carried out using the corresponding deuterated IS of each analyte.

In order to assess the reliability of the method, a validation procedure was performed. The results and the details of this process are given in Chapter 5, while some considerations on the choices are discussed below.

First of all, as for the GC/MS method, the level of the Working Standard samples was expressed in absolute quantity (ng); this made easier to perform quantitative analysis when different volumes of unknown samples were used.

For the study of linearity and on each working day, five Working Standard samples at increasing concentrations (10, 25, 50, 100, 200 ng) were prepared. This concentrations range was chosen because preliminary experiments and, for morphine, GC/MS data showed that most unknown samples contained an amount of analytes within this limits, taking into account that we started from 0.5 mL of blood. The lowest concentration was chosen according to the Lower Limit of Quantitation (LLOQ) of the analytes (see also Chapter 5). For morphine, lower levels could be reached but this was beyond the purpose of the study, as demonstrated by the higher concentrations detected in all the unknown samples. The upper concentration was chosen according to the most common concentrations of morphine which had been determined by means of the GC/MS method; moreover, a too great amount of the analytes could cause saturation at ionisation and/or detection level, and consequently inaccurate

quantification. However, in some unknown specimens, morphine level went above the upper concentration of the standard curve. When this occurred, the analysis should be repeated starting from a lower blood volume, if still available. Nonetheless, the three analytes could show very different concentrations one another in the same unknown sample, so that it was impossible to find a blood volume to extract which would guarantee a correct quantification of all the three analytes. That's why it was decided to analyse 0.5 mL of each unknown blood and to quantify all the analytes starting from these data. In most cases, results were within the range of the curve. When higher concentrations or concentrations lower than the LLOQ (but above the LOD) were found, quantification was not so accurate, but still acceptable for the purpose of the study.

Finally, it must be said that quantitative analysis was based on RF method, as in the routine GC/MS method. On each working day, the RF value of the WSs was calculated, and the average value was used for quantification of unknown samples (see also paragraph 5.7).

4.6 MATRIX EFFECTS AND SPE RECOVERY EVALUATION

Matrix effect is a phenomenon which can affect LC/MS-MS analysis. This term refers to any changes in the ionisation process of the analyte due to a co-eluting compound, resulting in ion suppression or ion enhancement. Some authors have suggested to directly use “signal suppression or enhancement” instead of matrix effect, since the co-eluting compounds responsible for this phenomenon can be also other substances than matrix components (Verplaetse and Tytgat, 2011). Matrix effects mainly occur at the beginning of the chromatographic run, and in that case are due to all polar and non-retained substances eluted close to the solvent front. This phenomenon depends on the polarity of the compounds and on the matrix, being greater if the sample is complex. Moreover, the ionisation source must be considered. As far as ESI is concerned, four mechanisms have been proposed to explain matrix effects (see also paragraph 4.1 and Fig.10 for the ESI ionisation steps). According to the first one, in samples containing interfering compounds, saturation of the ionisation process is reached, and thus the analyte of interest and other substances have to compete for ionisation. Ion suppression is caused by a limited number of charges on the droplet surface or by analytes which can’t reach the surface of the droplets to form gas-phase ions. In the second mechanism, non-volatile compounds may precipitate with the analyte resulting in solid formation. Otherwise, interfering substances can change the viscosity and the surface tension of the droplets, causing a reduction of solvent evaporation and preventing the analytes to reach the gas-phase. Finally, droplets can be contaminated with compounds which may evaporate as neutrals and have a higher gas-phase proton affinity, so that protons could be transferred from the analyte to these interfering species (Verplaetse and Tytgat, 2011).

Matrix effects may affect precision, accuracy and sensitivity of a LC/MS-MS method. If possible, strategies to reduce them should be applied, for example improving sample preparation.

It was particularly important to investigate this phenomenon because the three analytes of interest elute at the beginning of the run, when matrix effects are more likely to occur. Moreover, post-mortem blood specimens are very rich in interfering compounds which may cause ion suppression/enhancement.

Several methods have been described to study matrix effects (Matuszewski *et al.*, 2003; Al-Asmari and Anderson, 2007; Karinen *et al.*, 2009; Verplaetse and Tytgat, 2011). For the purpose of the PhD method, the following procedure was applied to evaluate SPE recovery and matrix effects. Three Working Standard samples 50 ng were prepared from "blank" blood and extracted by SPE (see paragraphs 5.4 and 5.5) together with three "blank" blood samples, which were fortified after SPE to become post-extraction Working Standard samples 50 ng. On the same day, three unextracted Working Standard samples 50 ng were prepared in 3 mM ammonium formate buffer (mobile phase A).

Per cent extraction recovery (% E.R.) of each analyte and corresponding IS-D3 were calculated by dividing the average corresponding peak area in the three extracted Working Standard blood samples by the average corresponding peak area in the post-extraction Working Standard blood samples, and calculating the per cent value.

Matrix effects for each analyte were expressed by a % value, calculated in the following way:

Matrix effect (%) = B/A x 100

where A is the average peak area of the unextracted standards and B is the average peak area of the post-extraction Working Standard blood samples.

As shown in Table 11, extraction recovery was good for MOR and MOR-D3, while it was low for M3G and M6G and for the corresponding IS-D3. Nonetheless, the accuracy of the method was not affected, as indicated by a validation study (% REC; see also paragraph 5.8.6). Moreover, all the analytes but especially M3G and M3G-D3 showed a large ion suppression (% M.E.). Nonetheless, when the average peak area ratio of each analyte to the corresponding internal standard was considered,

good results were achieved, as shown by Corrected Matrix Effects (% C.M.E.) values (Karinen *et al.*, 2009; Taylor and Elliott, 2009). As a result, the LC/MS-MS method showed a good precision and accuracy, as shown by validation studies (see Chapter 5).

Compound	% E.R.	% M.E.	% C.M.E.
MOR	**91.0**	**69.0**	**118.8**
MOR-D3	87.8	58.1	-
M3G	**62.0**	**40.4**	**96.1**
M3G-D3	64.5	42.0	-
M6G	**62.4**	**72.2**	**88.9**
M6G-D3	60.4	81.2	-

Table 11: Per cent extraction recovery (% E.R.), matrix effects (% M.E.) and corrected matrix effects (% C.M.E.) of WSs 50 ng (n = 3).

4.7 APPLICATION TO OTHER MOLECULES

Some experiments were performed in order to extend the LC/MS-MS method to other molecules, which may be present in blood samples coming from suspected heroin fatalities. Other metabolites of "street heroin", such as 6-monoacetylmorphine, codeine and acetylcodeine, were first evaluated (Appendix 4). Methadone and its main metabolites EDDP and EMDP were then considered (Appendix 5).

As previously described (paragraph 4.2), also these compounds were characterised by direct infusion in the mass spectrometer, optimising the ionisation and detection parameters and studying the fragmentation pattern in the MRM mode (Table 12).

Compound	Q1 first mass	Q3 first mass	Capillary (V)	Collision Energy (V)
6-MAM	328.4	328.4	75.0	5.0
	328.4	165.1	75.0	31.5
Codeine	300.1	300.1	67.3	6.5
	300.1	215.2	67.3	18.0
	300.1	165.2	67.3	35.0
Acetylcodeine	342.1	342.1	75.0	6.0
	342.1	225.1	75.0	21.0
Methadone	310.1	310.1	64.6	4.5
	310.1	265.1	64.6	9.5
	310.1	105.0	64.6	20.0
EDDP	278.1	278.1	65.0	7.0
	278.1	249.1	65.0	20.0
	278.1	234.1	65.0	27.0
EMDP	264.1	264.1	69.0	6.5
	264.1	235.1	69.0	19.0
	264.1	220.1	69.0	26.5

Table 12: MRM transitions of each analyte.

The chromatographic method was modified in order to achieve a good separation and resolution of all the compounds. If the previous gradient elution was applied, just extending the final isocratic elution (see Fig.12, paragraph 4.3), the R.T. of these molecules was above 5 minutes and

partly overlapped, even if the specific MRM transitions of each compound allow to distinguish them. Thus, the first part of the gradient elution was kept, as it assured a good separation of the three main analytes, i.e. morphine, M3G and M6G. On the other hand, some changes were applied to the second part of the elution gradient in order to have a good separation also of the other molecules. The final chromatographic conditions are shown in Fig.14.

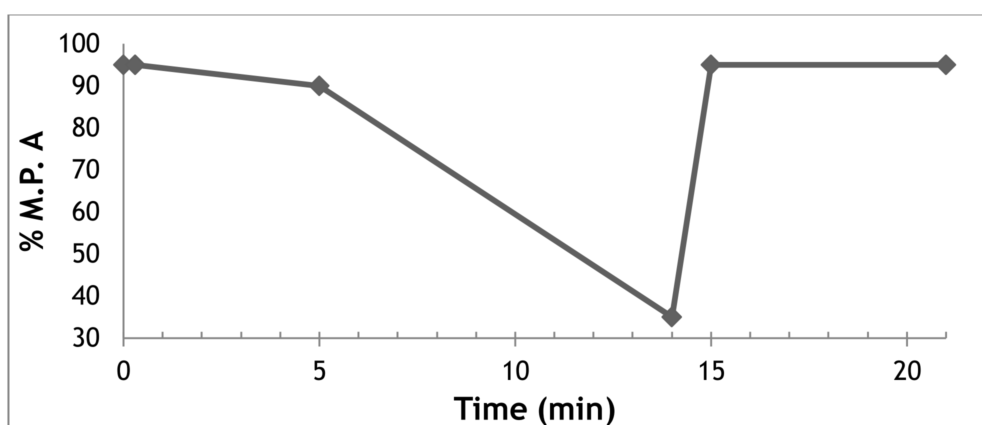


Fig.14: Diagram of the final gradient elution
(% M.P.A = percentage of mobile phase A).

Because of the increased number of analytes, it was necessary to create acquisition segments to keep a good resolution of each molecule. Three acquisition segments were therefore created, and only two MRM transitions were kept for each analyte (Table 13 a,b,c; Appendix 6). A comparison between the initial method and the method with three acquisition segments is shown in Appendix 7 and 8.

Compound	Q_1 first mass	Q_3 first mass	Capillary (V)	Collision Energy (V)
MOR	286.3	286.3	70.9	6.0
	286.3	201.2	70.9	15.0
M3G, M6G	462.2	462.2	32.9	5.0
	462.2	286.1	32.9	27.5
MOR-D3	289.1	289.1	80.0	5.5
	289.1	200.9	80.0	17.0
M3G-D3, M6G-D3	465.5	465.5	32.9	5.0
	465.5	289.1	32.9	25.5

Table 13a: Molecules and corresponding MRM transitions acquired in the first segment (0-6 min).

6-MAM	328.4	328.4	75.0	5.0
	328.4	165.1	75.0	31.5
Codeine	300.1	300.1	67.3	6.5
	300.1	215.2	67.3	18.0
Acetylcodeine	342.1	342.1	75.0	6.0
	342.1	225.1	75.0	21.0
Nalorphine	312.3	270.2	64.1	13.5
	312.3	201.3	64.1	18.5

Table 13b: Molecules and corresponding MRM transitions acquired in the second segment (6-11 min).

Methadone	310,1	310,1	64,6	4,5
	310,1	265,1	64,6	9,5
	310,1	105,0	64,6	20,0
EDDP	278,1	278,1	65,0	7,0
	278,1	249,1	65,0	20,0
	278,1	234,1	65,0	27,0
EMDP	264,1	264,1	69,0	6,5
	264,1	235,1	69,0	19,0
	264,1	220,1	69,0	26,5

Table 13c: Molecules and corresponding MRM transitions acquired in the third segment (11-21 min).

Some SPE experiments were then carried out. The method which had been optimised for morphine, M3G and M6G allowed the extraction also of

the other analytes when starting from water spiked with standard solutions or urine. Nonetheless, some problems were found for 6-MAM and acetylcodeine when applying the method to blood samples, probably because of interfering components which may affect both the extraction and the analysis. Further investigation and experiments are therefore needed to completely extend the method to these molecules.

5. LC/MS-MS method

5.1 CHEMICALS AND REAGENTS

- Morphine base (S.A.L.A.R.S., Como, I)
- Morphine-3-β-D-glucuronide 1 mg/mL in methanol with 0.05% NaOH (Cerilliant, Round Rock, T-U.S.A.)
- Morphine-6-β-D-glucuronide 0.1 mg/mL in methanol:H_2O (1:1) (Cerilliant, Round Rock, T-U.S.A.)
- Morphine-D3 1 mg/mL in methanol (S.A.L.A.R.S., Como, I)
- Morphine-3-β-D-glucuronide-D3 100 μg/mL in methanol with 0.05% NaOH (Cerilliant, Round Rock, T-U.S.A.)
- Morphine-6-β-D-glucuronide-D3 100 μg/mL in methanol:H_2O (1:1) (Cerilliant, Round Rock, T-U.S.A.)
- Nalorphine HCl (S.A.L.A.R.S., Como, I)
- Codeine base (S.A.L.A.R.S., Como, I)
- 6-Monoacetylmorphine 1 mg/mL in acetonitrile (Cerilliant, Round Rock, T-U.S.A.)
- Acetylcodeine base (S.A.L.A.R.S., Como, I)
- Methadone HCl (Alltech, Deerfield, I-U.S.A.)
- EMDP (2-Ethyl-5-methyl-3,3-diphenylpyrroline) HCl (Alltech, Deerfield, I-U.S.A.)
- EDDP (2-Ethyl-1,5-dimethyl-3,3-diphenyl-pyrrolidine) HI (Alltech, Deerfield, I-U.S.A.)
- Water (18.2 mΩ/cm) was obtained with Milly-Q System (Millipore, F)
- Sodium tetraborate/hydrochloric acid pH 9 buffer solution (Fluka, Seelze, D)
- Formic acid (Fluka, Seelze, D)
- Ammonium formate (Fluka, Seelze, D)
- Methanol (Sigma-Aldrich , Steinheim am Albuch, D)
- Hexane(BDH Prolabo, VWR International srl, Milano, I)
- Ethyl acetate (BDH Prolabo, VWR International srl, Milano, I)
- Acetonitrile (Carlo Erba, Milano, I)

All reagents are of analytical grade and are stored as required by the manufacturer.

5.2 INSTRUMENTATION

- Laboratory balance with sensibility ± 0.01 mg (Sartorius, Goettingen, D)
- Analog Vortex Mixer (VWR, Briare, F)
- Benchtop centrifuge EBA 20 (Hettich, Beverly, MA-U.S.A)
- Thermoblock Digitale ® (Falc, Bergamo, I)
- Screw-cap tubes (Kartell, Milano, I)
- Bond Elut Certify™ 10 mL cartridges (Varian, Palo Alto, CA-U.S.A)
- Vacuum manifold Vac Elut 20® for SPE extraction (Varian, Palo Alto, CA-U.S.A)
- Fixed volume micropipettes with 50 μL, 100 μL and 200 μL capacity and variable volume micropipette with 50-50 μL capacity (Socorex®, Ecublens, CH)
- Variable volume micropipette with 20-200 μL capacity (Transferpette®, BrandTech Scientific, Essex, GB)
- Volumetric glass pipettes (AS class) with 5 ± 0.015 mL, 2 ± 0.01 mL, 1 ± 0.007 mL capacity
- Graduated glass pipettes with 10 ± 0.05 mL, 5 ± 0.03 mL, 2 ± 0.01 mL, 1 ± 0.007 mL, 0,5 ± 0.005 mL capacity (AS class)
- Graduated disposable pipettes with 1 mL, 5 mL and 10 mL capacity
- Volumetric glass flasks (A class) with capacity of 50 ± 0.06 mL, 20 ± 0.04 mL e 10 ± 0.04 mL
- Volumetric glass cylinder with 50 ± 1.0 mL, 100 ± 0.75 mL, 500 ± 3.75 mL capacity
- 2 mL glass crimp autosampler vials with rubber/teflon® caps
- 500 μL glass microvials
- 2 mL glass autosampler vials hand crimper
- Pasteur glass pipettes
- 10 mL high recovery glass tubes.

5.3 STANDARD SOLUTIONS AND MOBILE PHASE PREPARATION

All the standard solutions and mixtures are stored in screw-cap tubes at -20°C.

- **Standard Solution of morphine 1 mg/mL (SS-1000 of MOR)**

10.00 mg of morphine base are exactly weighted and dissolved in methanol in a 10 mL volumetric flask.

- **Standard Solution of morphine 100 μg/mL (SS-100 of MOR)**

1.000 mL of SS-1000 of morphine is diluted with methanol in a 10 mL volumetric flask. The final concentration is 100 μg/mL.

- **Standard Solution of morphine 10 μg/mL (SS-10 of MOR)**

1.000 mL of SS-100 of morphine is diluted with methanol in a 10 mL volumetric flask. The final concentration is 10 μg/mL.

- **Standard Solution of morphine-D3 100 μg/mL (SS-100 of MOR-D3)**

1.000 mL of morphine-D3 1 mg/mL is diluted with methanol in a 10 mL volumetric flask. The final concentration is 100 μg/mL.

- **Standard Solution of morphine-3-β-D-glucuronide 100 μg/mL (SS-100 of M3G)**

0.500 mL of morphine-3-β-D-glucuronide 1 mg/mL is diluted with methanol in a 5 mL volumetric flask. The final concentration is 100 μg/mL.

- **Standard Solution of morphine-3-β-D-glucuronide 10 μg/mL (SS-10 of M3G)**

1.000 mL of SS-100 of morphine-3-β-D-glucuronide is diluted with methanol in a 10 mL volumetric flask. The final concentration is 10 μg/mL.

- **Standard Solution of morphine-6-β-D-glucuronide 10 μg/mL (SS-10 of M6G)**

0.500 mL of morphine-6-β-D-glucuronide 0.1 mg/mL is diluted with methanol in a 5 mL volumetric flask. The final concentration is 10 μg/mL.

- **Morphine, morphine-3-β-D-glucuronide, morphine-6-β-D-glucuronide Standard Mixture 2 μg/mL (MIX 2)**

200 μL of SS-100 of morphine, 200 μL of SS-100 of morphine-3-β-D-glucuronide and 200 μL of morphine-6-β-D-glucuronide 0.1 mg/mL are transferred into a 10 mL volumetric flask and diluted with methanol (1:50), to get a final concentration of 2 μg/mL.

- **Morphine, morphine-3-β-D-glucuronide, morphine-6-β-D-glucuronide Standard Mixture 1 μg/mL (MIX 1)**

1.000 mL of SS-10 of morphine, 1.000 mL of SS-10 of morphine-3-β-D-glucuronide and 1.000 mL of SS-10 of morphine-6-β-D-glucuronide are transferred into a 10 mL volumetric flask and diluted with methanol (1:10), to get a final concentration of 1 μg/mL.

- **Morphine, morphine-3-β-D-glucuronide, morphine-6-β-D-glucuronide Standard Mixture 0.5 μg/mL (MIX 0.5)**

0.500 mL of SS-10 of morphine, 0.500 mL of SS-10 of morphine-3-β-D-glucuronide and 0.500 mL of SS-10 of morphine-6-β-D-glucuronide are transferred into a 10 mL volumetric flask and diluted with methanol (1:20), to get a final concentration of 0.5 μg/mL.

- **Morphine, morphine-3-β-D-glucuronide, morphine-6-β-D-glucuronide Standard Mixture 0.25 μg/mL (MIX 0.25)**

0.250 mL of SS-10 of morphine, 0.250 mL of SS-10 of morphine-3-β-D-glucuronide and 0.250 mL of SS-10 of morphine-6-β-D-glucuronide are transferred into a 10 mL volumetric flask and diluted with methanol (1:40), to get a final concentration of 0.25 μg/mL.

- **Morphine, morphine-3-β-D-glucuronide, morphine-6-β-D-glucuronide Standard Mixture 0.1 μg/mL (MIX 0.1)**

100 μL of SS-10 of morphine, 100 μL of SS-10 of morphine-3-β-D-glucuronide and 100 μL of SS-10 of morphine-6-β-D-glucuronide are transferred into a 10 mL volumetric flask and diluted with methanol (1:100), to get a final concentration of 0.1 μg/mL.

- **Internal Standard Mixture 1 μg/mL (ISM-D3)**

100 μL of SS-100 of morphine-D3, 100 μL of morphine-3-β-D-glucuronide-D3 100 μg/mL and 100 μL of morphine-6-β-D-glucuronide-D3 100 μg/mL are transferred into a 10 mL volumetric flask and diluted with methanol (1:100), to get a final concentration of 1 μg/mL.

- **3 mM Ammonium formate buffer (pH = 3)**

About 95 mg of ammonium formate (M_w=63.06 g/mol) are weighted and dissolved into 500.0 mL of ultra-pure water in an ultrasonic bath (15 minutes). After adding 0.500 mL of formic acid and mixing, the pH is checked with a pHmeter and some further drops of formic acid are added till a pH value of 3 (±0.1).

5.4 SAMPLE PREPARATION

For each working day, the following samples are prepared and analysed:

- "Blank" solvent Working sample;
- "Blank" blood Working sample;
- Working Standard samples 10, 25, 50, 100 and 200 ng (WSs 10, 25, 50, 100 and 200 ng);
- Unknown Working samples.

0.5 mL of water, of "blank" blood and of each unknown blood sample are transferred into a tube. 2.0 mL of water, 50 μL of ISM-D3 and 2.0 mL of pH 9 buffer solution are then added. After vortex mixing and centrifugation (4000 rpm, 10 min), the supernatant is extracted as described in paragraph 5.5.

The five Working Standard samples, containing increasing concentrations (10, 25, 50, 100 and 200 ng) of morphine, morphine-3-β-D-glucuronide and morphine-6-β-D-glucuronide, are prepared by transferring 100 μL of the MIX (0.1-0.25-0.5-1-2) in five tubes and evaporating the solvent to dryness under a stream of nitrogen at 40°C. 0.5 mL of "blank" blood is then added to each tube and, after vortex mixing, the WSs are handled as the unknown and "blank" specimens.

5.5 SOLID-PHASE EXTRACTION (SPE)

Samples are extracted with the SPE technique using Varian Bond Elut Certify™ cartridges.

For this purpose, the cartridge is conditioned with 2.0 mL of methanol and 2.0 mL of pH 9 buffer solution, not allowing the cartridge to dry during this phase. After loading the sample, keeping the flow at around 1 mL/min, the cartridge is washed with 4.0 mL of pH 9 buffer solution, and subsequently dried under air flow for around ten minutes. A final wash with 3.0 mL of a mixture hexane:ethyl acetate (8:2) is applied and the cartridge is dried again under air flow for one minute. The compounds of interest are finally eluted with 2.0 mL of methanol, collecting the eluate in a high recovery glass tube.

The extract is partially dried under a nitrogen stream at 40°C, transferred in a 2 mL vial and then completely dried. The residue is dissolved with 250 μL of mobile phase A (see paragraph 5.6.2). After vortex mixing and centrifugation (6000 rpm, 15 min), the supernatant is transferred into a 500 μL microvial and analysed by LC/MS-MS.

5.6 LC/MS-MS ANALYSIS

5.6.1 APPARATUS

The analysis were performed on a Varian LC-320 triple quadrupole mass spectrometer, equipped with two Varian 212 LC chromatographic pumps and a Varian 410 tray cooled autosampler. The system is managed by Varian Workstation software (Version 6.9.2).

5.6.2 CHROMATOGRAPHIC CONDITIONS AND INSTRUMENT SET-UP

Column: Kinetex C_{18} 2.6 μm x 50mm (Phenomenex) with Security Guard column C_{18} 4 x 2.0mm (Phenomenex).

Mobile phase:

- A: 3mM ammonium formate buffer (pH = 3.0)
- B: acetonitrile with 0.1% formic acid

under the following conditions (Table 14):

Time (min)	% A	% B	Flow rate (μL/min)
0.00	95.0	5.0	200
0.30	95.0	5.0	200
5.00	90.0	10.0	200
14.00	35.0	65.0	200
15.00	95.0	5.0	200
21.00	95.0	5.0	200

Table 14: Details of the applied gradient elution.

Manifold temperature: 42°C.

Housing temperature: 50°C.

Column temperature: 40°C.

Injection volume: 10 μL.

Injection mode: partial loopfill.

Syringe washing solvent: isopropyl alcohol.

Table 15 shows the R.T. (preceded by ~ because they may vary, as mentioned in the previous Chapter) and the Relative Retention Time (R.R.T.) of each analyte.

Compound	R.T. (min)	R.R.T.(min)
MOR	~ 2.7	1.04
MOR-D3	=	-
M3G	~ 1.8	1.03
M3G-D3	=	-
M6G	~ 2.9	1.02
M6G-D3	=	-

Table 15: Retention Time (R.T.) and Relative Retention time (R.R.T.) of each molecule.

5.6.3 MS-MS PARAMETERS

Analysis are performed in positive ionisation mode, applying the following conditions:

Needle Voltage: + 5000 V.

Shield Voltage: + 600 V.

Nebulizing Gas (N_2) Pressure: 40.00 psi.

Drying Gas (N_2) Pressure: 40.00 psi.

Drying Gas (N_2) Temperature: 200°C.

Q_0 Offset: + 3.261 V.

L_4 Offset: + 2.000 V.

CID Gas (Ar) Pressure: 2.00 mTorr.

Dwell time: 0.100 msec.

Electromultiplier: 1750 V.

As described in Chapter 4, Multiple Reaction Monitoring (MRM) mode was used. The MRM transitions selected for each analyte and its corresponding deuterated Internal Standard (IS) are shown in Table 16.

Compound	Q_1 first mass	Q_3 first mass	Capillary (V)	Collision Energy (V)
MOR	**286.3**	**286.3**	**70.9**	**6.0**
	286.3	201.2	70.9	15.0
M3G, M6G	462.2	462.2	32.9	5.0
	462.2	**286.1**	**32.9**	**27.5**
MOR-D3	**289.1**	**289.1**	**80.0**	**5.5**
	289.1	200.9	80.0	17.0
M3G-D3, M6G-D3	465.5	465.5	32.9	5.0
	465.5	**289.1**	**32.9**	**25.5**

Table 16: MRM transitions of each analyte and corresponding IS (in bold: MRM used for quantitative analysis).

5.7 QUALI-QUANTITATIVE ANALYSIS

As described in Chapter 4, the LC/MS-MS qualitative analysis is based on the presence of all specific MRM (Multiple Reaction Monitoring) transitions of each compound at the corresponding R.R.T. (Relative Retention Time).

For quantification, the peak area of a specific transition for each analyte is considered (see Table 16 at the end of the previous paragraph), applying the Response Factor (RF) method, where RF is defined as:

$$RF = \frac{A_{analyte} / A_{IS}}{C_t}$$

where $A_{analyte}$ and A_{IS} are the peak area of the quantifier MRM transition of the analyte and the peak area of the quantifier MRM transition of the corresponding deuterated IS, respectively, and C_t is the analyte concentration in the Working Standard sample expressed as total ng of analyte. On each working day, five different WSs were prepared and analysed, as previously described (paragraphs 5.4-5.6). The RF is calculated for each Working Standard sample and the average of the five (RF_{medium}) is considered for the quantification of all the compounds in the unknown samples, applying the following formula:

$$C_x = \frac{A_{analyte} / A_{IS}}{RF_{medium}}$$

where $A_{analyte}$ and A_{IS} are the peak area of the quantifier MRM transition of the analyte and the peak area of the quantifier MRM transition of the IS, respectively, and C_x is the analyte concentration in the unknown specimen expressed as total ng of analyte. This value is finally divided by the unknown sample volume (0.5 mL) to get the concentration as ng/mL (of blood).

5.8 VALIDATION

The LC/MS-MS method for the quantification of morphine (MOR), morphine-3-β-D-glucuronide (M3G) and morphine-6-β-D-glucuronide (M6G) in post-mortem blood samples was then validated. In detail, specificity, linearity, Limit Of Detection (LOD), Lower Limit of Quantitation (LLOQ), repeatability, intermediate precision and accuracy were evaluated. Because of the complexity and limited availability of "blank" matrices, a simplified validation procedure and a higher variability and tolerance towards the results were applied compared to the pharmaceutical field, as it often happens in post-mortem toxicology.

5.8.1 SPECIFICITY

Specificity of a method is defined by ICH (International Conference on Harmonisation) as its "ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix, etc".

Specificity of the LC/MS-MS was evaluated by repeatedly extract and analyse 0.5 mL of "blank" solvent and 0.5 mL of "blank" blood spiked with 50 μL of the ISM-D3, checking the presence of interfering peaks for each MRM at the R.R.T. of the investigated compounds. Appendix 9 and 10 show the specificity of the method for our purposes.

5.8.2 LINEARITY

Linearity of an analytical procedure is defined as "the ability of the method to obtain, within a given concentration range of the compound of interest, test results directly proportional to the concentration (amount) of the analyte in the sample".

To study this parameter, three calibration curves have been prepared and analysed in three different working days, by spiking 0.5 mL "blank" blood samples with five increasing concentration levels (WSs 10, 25, 50, 100, 200 ng respectively) of MOR, M3G and M6G. Each WSs is prepared, extracted and analysed as previously described (paragraphs 5.4-5.6). Starting from the data obtained for each working day, three regression lines were calculated by the method of least squares, plotting the signals

as a function of analytes content and forcing the curve by the O (0,0) point.

Regression lines equations are expressed as follows:

y= a x + b

where: y = Area $_{analyte}$/Area $_{IS}$;

a = slope of the regression line;

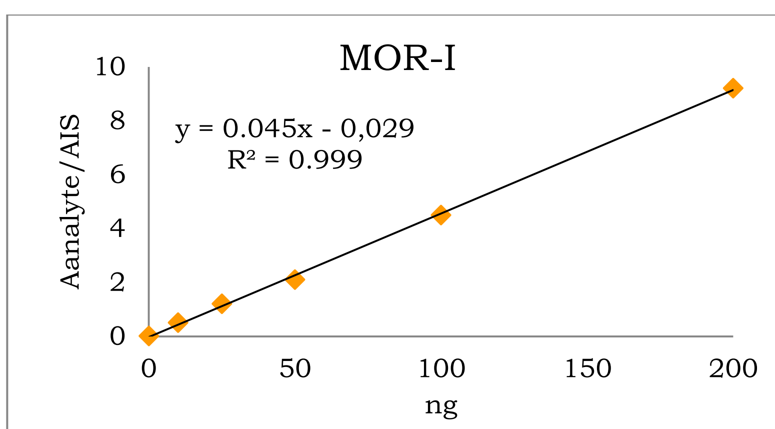
x = ng of each analyte;

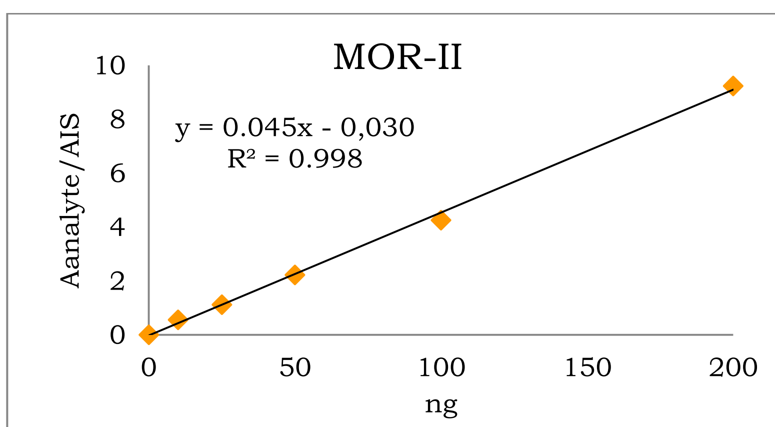
b = y-intercept of the regression line.

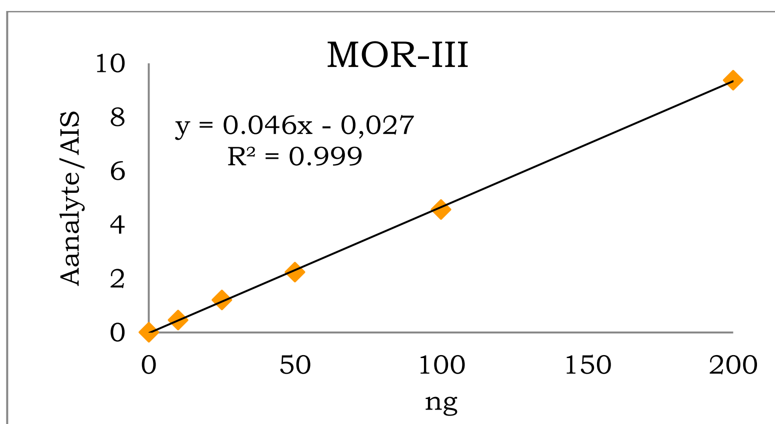
The square of the correlation coefficient (R^2) was also calculated. R^2 values above 0.99 were found for all the analytes, indicating a good linearity of the method. Experimental data, equations and plots of the calibration curves of each compound are shown in the following pages (Table 17 a,b,c and corresponding graphs).

MOR	$A_{analyte}/A_{IS}$		
ng	**I**	**II**	**III**
10	0.4945	0.5553	0.4549
25	1.1942	1.1179	1.2010
50	2.0919	2.2225	2.2359
100	4.4876	4.2542	4.5618
200	9.1959	9.2352	9.3675

Table 17a: Morphine (MOR) peak area ratios of the five WSs analysed on three working days (I,II,III) and used for the study of linearity.
Below: plots and equations of the corresponding regression lines.

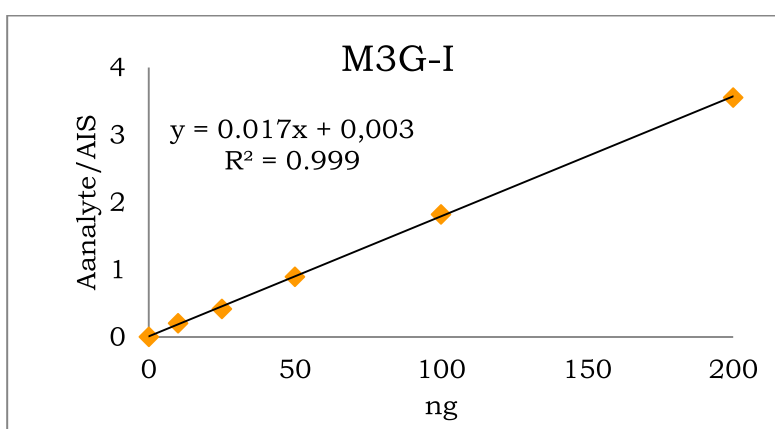


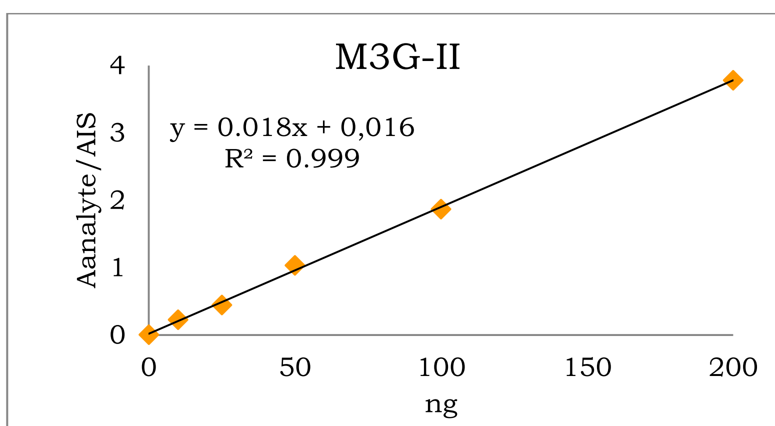


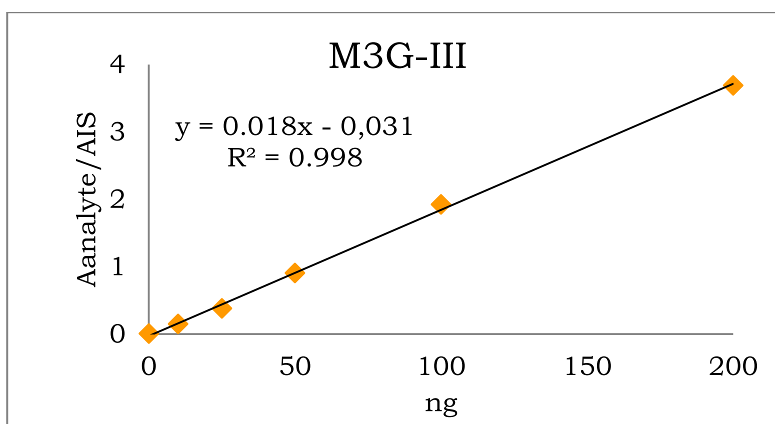


M3G	$A_{analyte}/A_{IS}$		
ng	I	II	III
10	0.2017	0.2223	0.1428
25	0.4142	0.4407	0.3738
50	0.8913	1.0294	0.8992
100	1.8176	1.8640	1.9174
200	3.5522	3.7772	3.6839

Table 17b: Morphine-3-β-D-glucuronide (M3G) peak area ratios of the five WSs analysed on three working days (I,II,III) and used for the study of linearity. Below: plots and equations of the corresponding regression lines.

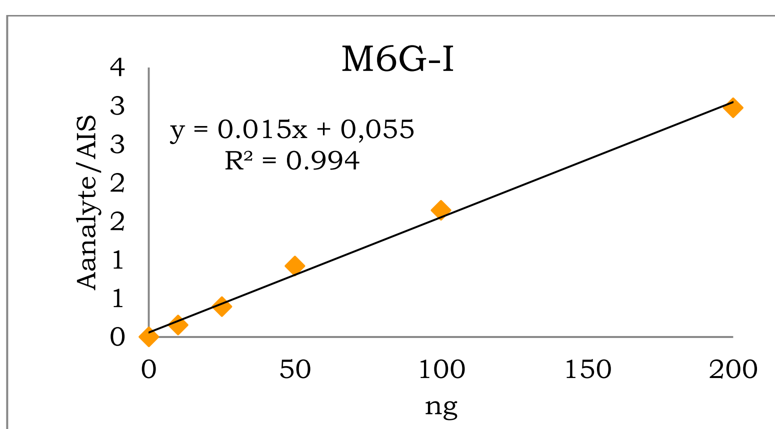


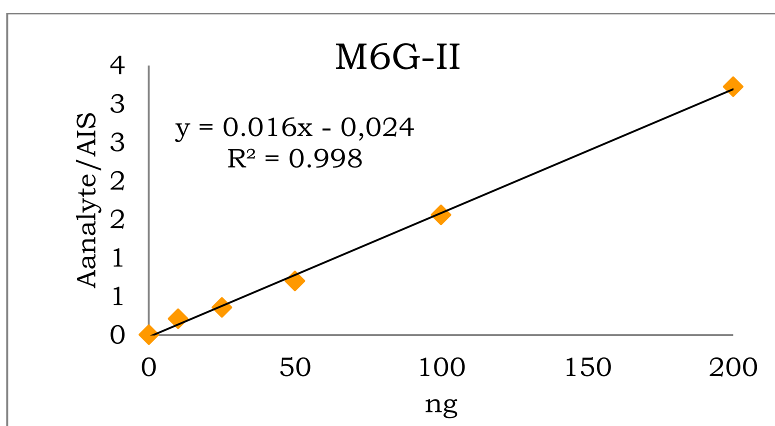


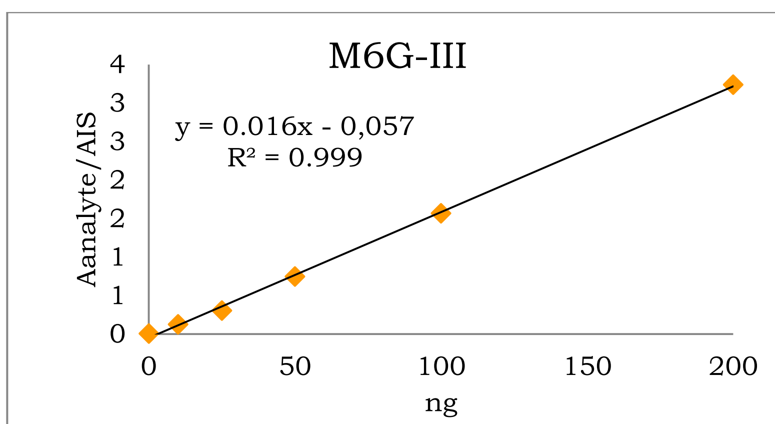


M6G	$A_{analyte}/A_{IS}$		
ng	I	II	III
10	0.1537	0.2074	0.1195
25	0.3926	0.3545	0.2983
50	0.9201	0.6981	0.7411
100	1.6442	1.5569	1.5621
200	2.9761	3.2220	3.2331

Table 17c: Morphine-6-β-D-glucuronide (M6G) peak area ratios of the five WSs analysed on three working days (I,II,III) and used for the study of linearity. Below: plots and equations of the corresponding regression lines.







5.8.3 LIMIT OF DETECTION (LOD)

The detection limit of an individual analytical procedure is "the lowest amount of analyte in a sample which can be detected but not necessarily quantified as an exact value".

To this purpose, WSs 1 ng and WSs 5ng were prepared by spiking 0.5 mL of "blank" blood with 10 μL and 50 μL, respectively, of MIX 0.1. Subsequently, 50 μL ISM-D3, 2.0 of mL water and 2.0 mL of pH=9 buffer solution were added and, after vortex mixing and centrifugation, the supernatant was extracted and then analysed as previously described.

On the basis of the results, the LOD of the three analytes was set at 1 ng. Indeed, at this concentration peaks of Molecular MRM transition of both M3G and M6G was not detectable because of high background noise; nonetheless, the presence of these molecules could be confirmed by the other MRM transition (from 462.2 to 286.1 at 27.5 V) at the same R.T. of IS-D3 signals (Appendix 11).

5.8.4 LOWER LIMIT OF QUANTITATION (LLOQ)

The Lower Limit of Quantitation of an individual analytical procedure is "the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy".

This parameter was evaluated on the basis of the % RSD and % REC values of the lowest Working Standard samples (WSs 10 ng) of the three regression lines calculated for the assessment of linearity.

Good results were obtained on each working day, so that the LOQ was set at 10 ng for all the molecules. A lower LOQ could be evaluated for morphine, but a 10 ng value was enough for the purposes of our method (Appendix 12).

5.8.5 PRECISION

The precision of an analytical procedure expresses "the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions". Precision was considered at two levels, repeatability and intermediate precision. Repeatability expresses the

precision under the same operating conditions over a short interval of time (and is also termed intra-assay precision), while intermediate precision expresses within-laboratories variations, i.e. results obtained on different days or in different environmental conditions.

Repeatability was evaluated by calculating the ± SD (Standard Deviation) and the % RSD (% Relative Standard Deviation) of the RF of the five WSs prepared on three different days for the study of linearity. The RF was calculated as described in paragraph 5.7. The calculation of the ± SD and the % RSD of the RF of all the fifteen WSs of the study of linearity was used for the assessment of intermediate precision (Table 18 a,b,c).

MOR					
ng	**RF I**	**RF II**	**RF III**		
10	0.0495	0.0555	0.0455		
25	0.0478	0.0447	0.0480		
50	0.0418	0.0444	0.0477		
100	0.0449	0.0425	0.0456		
200	0.0460	0.0462	0.0468		
RF_{medium}	*0.0460*	*0.0467*	*0.0460*	**$RF_{mediumTOT}$**	**0.0463**
± SD	*0.0003*	*0.0051*	*0.0012*	**± SD_{TOT}**	**0.0032**
% RSD	*6.3*	*10.9*	*2.6*	**% RSD_{TOT}**	**7.0**

Table 18a: Repeatability (*% RSD*; n = 5;increasing concentration WSs) on three different days (I,II,II) and intermediate precision (% RSD_{TOT}; n = 15) of MOR.

M3G					
ng	**RF I**	**RF II**	**RF III**		
10	0.0202	0.0222	0.0143		
25	0.0166	0.0176	0.0150		
50	0.0178	0.0206	0.0180		
100	0.0182	0.0186	0.0192		
200	0.0178	0.0189	0.0184		
RF_{medium}	*0.0181*	*0.0196*	*0.0170*	**$RF_{mediumTOT}$**	**0.0182**
± SD	*0.0013*	*0.0018*	*0.0022*	**± SD_{TOT}**	**0.0020**
% RSD	*7.2*	*9.3*	*12.9*	**% RSD_{TOT}**	**11.0**

Table 18b: Repeatability (*% RSD*; n = 5;increasing concentration WSs) on three different days (I,II,II) and intermediate precision (% RSD_{TOT}; n = 15) of M3G.

M6G					
ng	RF I	RF II	RF III		
10	0.0154	0.0207	0.0120		
25	0.0157	0.0142	0.0119		
50	0.0184	0.0140	0.0148		
100	0.0164	0.0156	0.0156		
200	0.0149	0.0161	0.0162		
RF_{medium}	*0.0162*	*0.0161*	*0.0141*	$RF_{mediumTOT}$	**0.0154**
± SD	*0.0014*	*0.0027*	*0.0020*	± SD_{TOT}	**0.0023**
% RSD	*8.5*	*17.0*	*14.4*	% RSD_{TOT}	**14.8**

Table 18c: Repeatability (*% RSD*; n = 5;increasing concentration WSs) on three different days (I,II,II) and intermediate precision (% RSD_{TOT}; n = 15) of M6G.

5.8.6 ACCURACY

The accuracy of an analytical procedure expresses the "closeness of agreement between an accepted reference value and the value found". It is sometimes termed trueness.

For the evaluation of the accuracy, each WSs of the linearity study was quantified using the $RF_{mediumTOT}$ calculated for the study of intermediate precision (Table 18 a,b,c). The results (ng_{exp}) were then compared to the theoretical values (ng_{theor}) in order to calculate the corresponding % Recovery (% REC) of the method, by applying the following formula:

$$\% \text{ REC} = \frac{ng_{exp}}{ng_{theor}} \times 100$$

where

ng_{exp} = ng calculated for each WSs starting from the $RF_{mediumTOT}$ calculated for the study of Repeatability (Table 18 a,b,c);

ng_{theor} = theoretical amount of analyte added to the WSs.

Starting from the results of each working day, the average value of % REC, ± SD and % RSD were calculated (Table 19 a,b,c). Although matrix effects (see paragraph 4.6), good results were obtained for all the compounds, as shown by the following tables.

MOR				
ng_{theor}	ng_{exp}	% REC	± SD	% RSD
10.0	10.8	108.4	1.1	1.0
25.0	25.3	101.2	1.0	1.0
50.0	47.2	94.4	1.7	1.8
100.0	95.8	95.8	3.5	3.6
200.0	200.3	100.1	1.9	1.9
		% REC_{TOT}	± SD_{TOT}	% RSD_{TOT}
		100.0	7.0	7.0

Table 19a: Mean accuracy (% REC), ± SD and % RSD for each WSs concentration level (n = 3) and for all the WSs (% REC_{TOT}; ± SD_{TOT} and % RSD_{TOT}; n=15) of MOR.

M3G				
ng_{theor}	ng_{exp}	% REC	± SD	% RSD
10.0	10.4	103.7	2.3	2.2
25.0	22.5	89.9	1.8	2.1
50.0	51.6	103.2	4.3	4.1
100.0	102.4	102.4	2.7	2.7
200.0	201.5	100.8	6.2	6.2
		% REC_{TOT}	± SD_{TOT}	% RSD_{TOT}
		100.0	11.1	11.1

Table 19b: Mean accuracy (% REC), ± SD and % RSD for each WSs concentration level (n = 3) and for all the WSs (% REC_{TOT}; ± SD_{TOT} and % RSD_{TOT}; n=15) of M3G.

M6G				
ng_{theor}	ng_{exp}	% REC	± SD	% RSD
10.0	10.4	104.0	2.9	2.8
25.0	22.6	90.5	3.1	3.4
50.0	51.0	102.1	7.6	7.5
100.0	103.1	103.0	3.2	3.1
200.0	204.1	102.0	9.4	9.2
		% REC_{TOT}	± SD_{TOT}	% RSD_{TOT}
		100.3	14.3	14.3

Table 19c: Mean accuracy (% REC), ± SD and % RSD for each WSs concentration level (n = 3) and for all the WSs (% RECTOT; ± SDTOT and % RSDTOT; n=15) of M6G.

5.9 SAMPLES ANALYSIS AND RESULTS

Up to now, the LC/MS-MS method has been applied to the analysis of twenty-five blood samples coming from people who had assumed heroin just before dying, as shown by the results of the previous GC/MS analysis. Among these specimens, eighteen belonged to the fifty cases which had been previously analysed in our laboratory (see Chapter 3), while seven blood samples were provided by the "Istituto di Medicina Legale e delle Assicurazioni" of Macerata, where a STA analysis had been performed by applying methods similar to our laboratory ones.

To the specific purpose of this work, the twenty-five cases were classified into five classes on the basis of STA results. In detail the five classes and the corresponding cases are:

- **I** = Heroin: case No.1-13.
- **II** = Heroin and ethanol (BAC ≥ 0.5 g/L): case No.14-19;
- **III** = Heroin, methadone and ethanol (BAC ≥ 0.5 g/L): case No.20;
- **IV** = Heroin and cocaine: case No.21-23;
- **V** = Heroin, cocaine and ethanol (BAC ≥ 0.5 g/L): case No.24-25.

An overview of classification, demographic information and circumstances of death of the twenty-five cases is given in Appendix 13.

Each unknown sample was prepared, extracted, analysed and quantified as described in paragraphs 5.4-5.7. The chromatograms of three cases are shown in Appendix 14, 15, 16, while a table with the quantitative results of all the cases is given in Appendix 17. Beside MOR, M3G and M6G, codeine (COD) was found in most cases. Although the method hasn't been validated for COD, a quantitative result was calculated also for this analyte, using nalorphine or morphine-D3 as IS. To this purpose, the specific MRM transitions 300.1/300.1 (-6.5V) and 312.3/201.3 (-18.5V) or 289.1/289.1 (-5.5 V), for the analyte and the IS respectively, were considered.

Because of the low volume of blood, only one working specimen could be prepared in most cases. In this regard, some preliminary tests had been performed. A working specimen of case No.3 was prepared and

analysed in duplicate on the same day and a third one on a different day, in order to assess the reproducibility of the method on a real sample.

CASE No.3

	ng/mL			
Compound	I	II	mean	± SD
MOR	178	180	179	1.41
M3G	218	229	224	7.78
M6G	49	59	54	7.07
COD	16	17	17	0.71

	ng/mL					
Compound	I	II	III	mean	± SD	% RSD
MOR	178	180	166	175	8	4.3
M3G	218	229	250	232	16	7.0
M6G	49	59	60	56	6	10.9
COD	16	17	14	16	2	9.8

Table 20: Results of three Working samples of the same case (No.3).

As shown in Table 20, good results were obtained for MOR, M3G and COD, both within the same day and between two different days. Worse results were reached for M6G, but a % RSD lesser than 15.0% is acceptable anyway, especially if a low amount of analyte is present.

Starting from LC/MS-MS results, some other values were calculated to better interpret each case. In detail, the total amount of morphine, morphine-3-β-D-glucuronide and morphine-6-β-D-glucuronide (MOR+M3G+M6G) was calculated. The sum of MOR and M6G (MOR+M6G), which are the main active metabolites of heroin, was also considered. In these calculations, the stoichiometric ratio was taken into account, by relating M3G and M6G concentrations to the MOR corresponding one (morphine M_w=285.34; M3G and M6G M_w=461.47). Finally, the molar ratios between the concentrations of morphine-6-β-D-glucuronide and morphine-3-β-D-glucuronide (M6G/M3G), morphine-3-β-D-glucuronide and morphine-6-β-D-glucuronide (M3G/M6G), morphine-3-β-D-glucuronide and morphine (M3G/MOR), morphine-6-β-D-glucuronide and morphine (M6G/MOR), morphine and M+M3G+M6G (MOR/MOR+

M3G+M6G) were evaluated. All the calculated sums and molar ratios are shown in Appendix 18.

In order to highlight a relationship between the blood concentrations of the analytes and the presence of risk factors, such as the contemporary use of ethanol and/or cocaine, the cases were divided as previously described and for each class some statistical data were calculated (Appendix 19a and 19b). No further calculations were done for class III, because it included only one case.

Finally, the influence of the risk factor "ethanol" was evaluated by studying the relationship between the concentrations and/or the molar ratios of the analytes and blood ethanol level (Appendix 20).

6. Discussion and Conclusions

As previously discussed, the interpretation of analytical results is the most challenging task in post-mortem toxicology. As far as the cause of death is concerned, a complete knowledge of the case history is fundamental. Thus, for each case analytical results of the developed LC/MS-MS should be interpreted taking into account also autopsy findings, information from the scene and relevant medical history. This kind of evaluation belongs to the medical field, and moreover is useful to solve the individual case. On the contrary, I will limit the discussion to general considerations, with some references to specific cases under study.

First of all, codeine (COD) was also found besides morphine (MOR), morphine-3-β-D-glucuronide (M3G) and morphine-6-β-D-glucuronide (M6G), thus confirming the use of "street heroin" (see also Chapter 1). In two cases (case No.9 and 11), this molecule was not detected, so that this conclusion can't be drawn on the basis of the only analytical results. COD is an active molecule, which could contribute to "street heroin" overall action. Nonetheless, since COD concentration was usually low and the PhD work focused originally on the study of MOR, M3G and M6G, no other considerations were made about this compound.

A high variability in MOR blood concentrations can be noticed, in accordance with our GC/MS data and literature. A wide distribution of M3G and M6G concentrations is also present (Table 21, Fig.15 a,b,c and Appendix 17).

	MOR	M3G	M6G*
Mean	329	643	177
± SD	336	639	209
min	30	3	/
MAX	1349	2307	821

Table 21: Mean, Standard Deviation (± SD) and range (min, MAX) of each analyte among the twenty-five cases (twenty-four cases for M6G, since this analyte was not detected in case No.11).

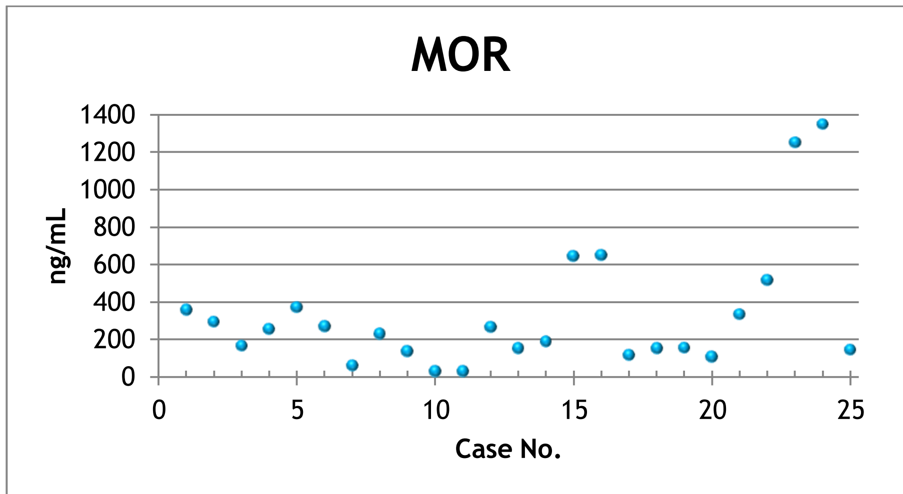


Fig.15a: Morphine (MOR) concentrations in the twenty-five cases.

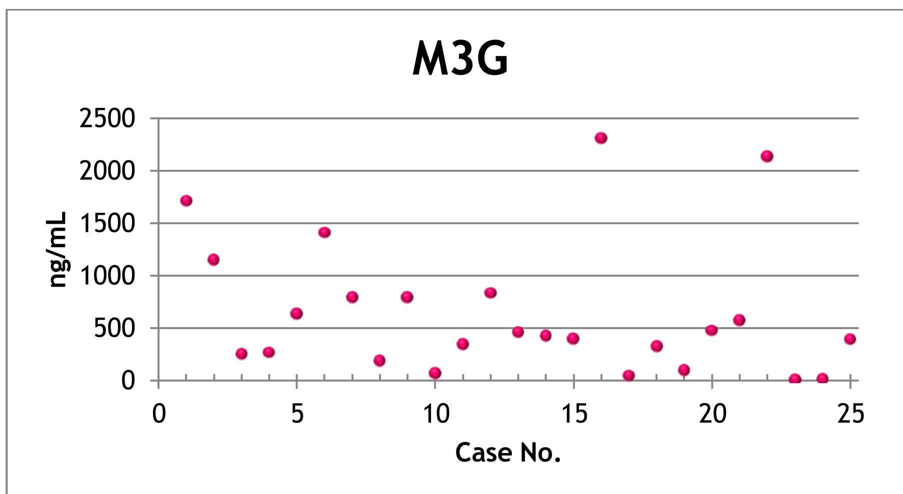


Fig.15b: Morphine-3-β-D-glucuronide (M3G) concentrations in the twenty-five cases.

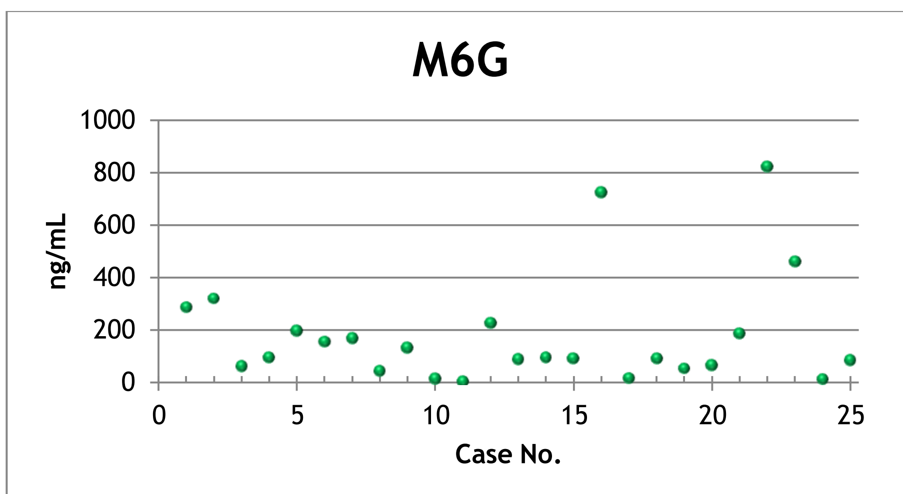
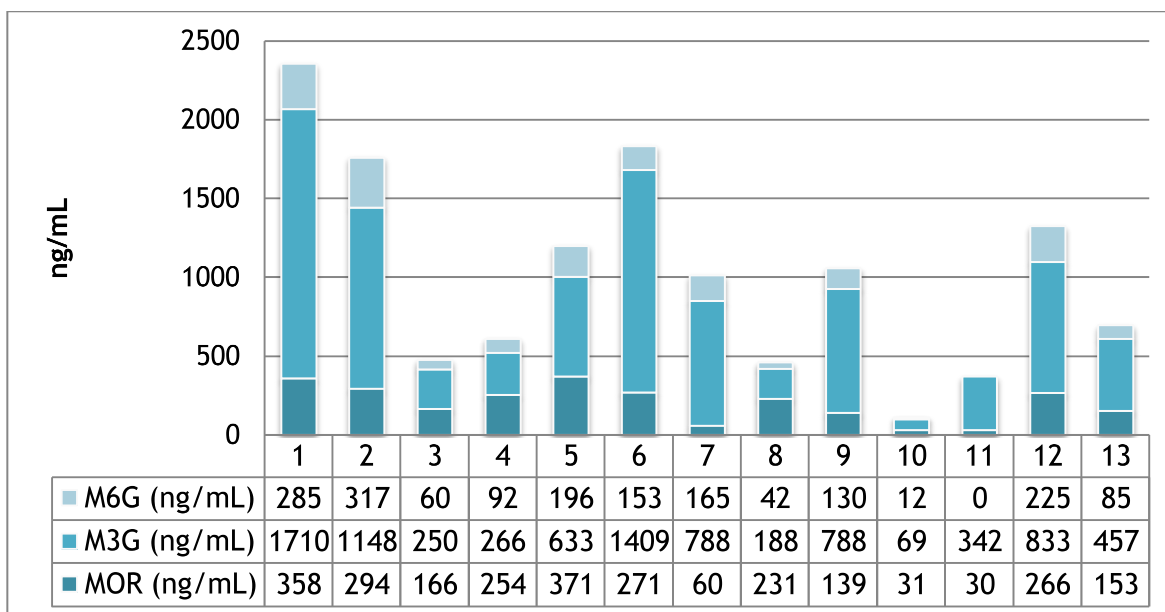


Fig.15c: Morphine-6-β-D-glucuronide (M6G) concentrations in twenty-four cases.

If only the thirteen cases belonging to the class I are considered, this variability is still present (Appendix 19a and Fig.16). This class includes cases in which only heroin metabolites was found by GC/MS analysis, being therefore no evident risk factors present.



	1	2	3	4	5	6	7	8	9	10	11	12	13
M6G (ng/mL)	285	317	60	92	196	153	165	42	130	12	0	225	85
M3G (ng/mL)	1710	1148	250	266	633	1409	788	188	788	69	342	833	457
MOR (ng/mL)	358	294	166	254	371	271	60	231	139	31	30	266	153

Fig.16: Overview of MOR, M3G and M6G concentrations in class I.

For each case, also the total amount of morphine, morphine-3-β-D-glucuronide and morphine-6-β-D-glucuronide (MOR+M3G+M6G) and the sum of MOR and M6G (MOR+M6G) were calculated (Appendix 18). This latter value may be particularly useful, as MOR and M6G are active metabolites, responsible for the more protracted effects of heroin. Thus, considering MOR+M6G concentration could be better than referring only to MOR in order to explain the cause of death. Also for MOR+M6G, very different results can be found anyway.

As suggested by some authors (Aderjan *et al.*, 1995; Bogusz *et al.*, 1997), the molar ratios of the concentrations of morphine-6-β-D-glucuronide to morphine-3-β-D-glucuronide (M6G/M3G) or of morphine-3-β-D-glucuronide to morphine-6-β-D-glucuronide (M3G/M6G), of morphine-3-β-D-glucuronide to morphine (M3G/MOR), of morphine-6-β-D-glucuronide to morphine (M6G/MOR) and of morphine to the sum of all the analytes (MOR/MOR+M3G+M6G) were also evaluated (Appendix 18). Thus, the relationship between two concentrations may be investigated.

For case No.11 it was not possible to calculate all the ratios, because M6G hadn't been detected. Looking at the ratios of all the other cases, it can be noticed that variability is still present, but within a narrower range especially if M6G/M3G (or M3G/M6G, since they have the same meaning in terms of data interpretation) is considered. Only case No.24 and especially case No.23 are characterised by very different values compared to all the other ones, so that they were not considered in the calculation of mean values (Table 22).

	M6G/M3G	M3G/M6G	M3G/MOR	M6G/MOR	MOR/MOR+M3G+M6G
Mean	0.25	4.47	2.15	0.43	0.37
± SD	0.09	1.69	1.96	0.36	0.18
min	0.11	1.94	0.24	0.07	0.09
MAX	0.52	9.21	8.10	1.70	0.76

Table 22: : Mean, Standard Deviation (± SD) and range (min, MAX) of the ratios of analytes concentrations.

In detail, case No.23 (chromatogram is given in Appendix 16) shows a M6G/M3G value far more different from the others (152.67 against a mean value of 0.25 ± 0.09). This could be explained by the contemporary use of cocaine, as indicated by the previous GC/MS analysis. Cocaine might have affected heroin metabolism, causing an increase in M6G formation and/or a reduction in M3G production. Nonetheless, this statement should be confirmed by a larger number of concordant data, and moreover the result seems to be contradicted by data of case No.21 (chromatogram is given in Appendix 15) and 22. Indeed, those cases belong to the same class, i.e. contemporary use of cocaine occurred; the ratio of M6G to M3G is however close to the mean one (0.33 and 0.38, respectively). It must be noticed that case No.23 has a very high MOR concentration compared to case No.21 and 22, and this might have been also significant in the overall metabolic process.

As previously mentioned, some authors have studied the influence of xenobiotics on morphine glucuronidation, and have demonstrated that heroin itself is able to affect this process (Antonilli *et al.*, 2003 a and b; Antonilli *et al.*, 2005; Antonilli *et al.*, 2008; Graziani *et al.* 2008; Meringolo

et al., 2011). As a result, drug addicts may have an increased M6G level. In this regard, M6G/M3G of case No.1 should be lower than the other ones, since family denied an history of drug addiction of the dead. The value is instead not statistically different from the mean one (0.17).

The relationship between analytical results and time of death was also considered, as suggested by some authors (Goldberger *et al.*, 1994; Bogusz *et al.*, 1997; Al-Asmari and Anderson, 2007). Unfortunately, no great information about time of death was available. Nonetheless, in case No.4 and No.5 people were found with a syringe still in the arm, indicating that a quite immediate death had occurred. The ratio of M6G to M3G is however quite close to the mean and to the other ones in both cases (0.35 and 0.31, respectively).

As described in Chapter 1, heroin metabolism can be affected by several factors, for example the contemporary use of other psychoactive drugs. These substances may change blood concentrations of heroin metabolites and/or contributing to pharmacological and toxic effects. The twenty-five cases were therefore divided into five classes according to the presence of other drugs of abuse, as shown by STA analysis (for the classification, see also Chapter 5). Mean values, ± SD and range were calculated for each class, but no significant differences both in absolute concentrations and ratios can be found, except for ethanol (Appendix 19a and 19b). Indeed, a greater number of samples should be considered to fully represent classes III, IV and V, and consequently to evaluate the role of methadone (and loss of tolerance), cocaine and of cocaine and ethanol together.

As far as ethanol is concerned, the cases were further classified into three groups according to BAC (Blood Alcohol Concentration, g/L): the first group included case No.1-13 (i.e. class I), where BAC was <0.5 g/L; in the second group, BAC was between 0.5 and 1.0 and case No.14-17 were included; the third group was formed by cases No. 18 and 19, which had a BAC ≥0.1 g/L. Cases No. 20-25 were not considered because also other “risk factors” were present. We found that no significant differences among the three groups are present (Appendix 20). The mean value

MOR/MOR+M3G+M6G shows a moderate correlation with BAC. This was in accordance with literature (Polettini *et al.*, 1999; Fugelstad *et al.*, 2003), and may be explained by the role of ethanol as enzymatic inhibitor. Indeed, the second and the third group included a too low number of cases (n= 4 and 5, respectively), so that further analysis is necessary to get a significant result and thus fully evaluate this influence (Fig.17).

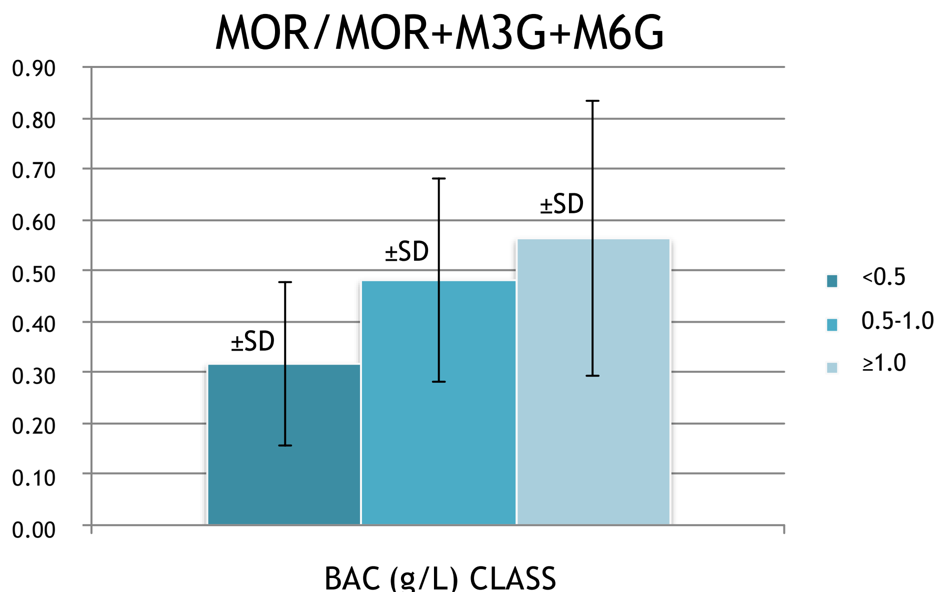


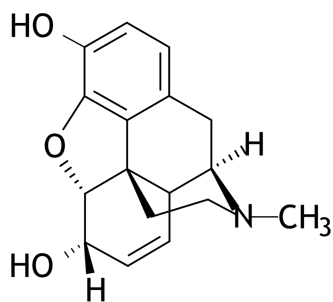
Fig.17: Mean ratios (± SD) of MOR to MOR+M3G+M6G of the three groups with different BAC values.

Moreover, it would be interesting to study the role of methadone, both for its synergic effects with heroin and for the relationship to the degree of tolerance. Unfortunately, only one case (No.20) was available, and therefore neither general considerations nor statistical calculations could be made. Indeed, case No.20 shows a low M6G/M3G (0.13) which could indicate a lesser exposure to heroin in the last months before death, and consequently a lower production of M6G. A similar value (0.11) was however present in case No.6.

Finally, it must be underlined that a wide variability of the ratios of morphine metabolites has been described also in the clinical field, when morphine treatment is involved. In this regard, several authors have demonstrated a lack of relationship between this value and long-term or post-operative therapy outcomes (Andersen *et al.*, 2002; Hammoud *et al.*,

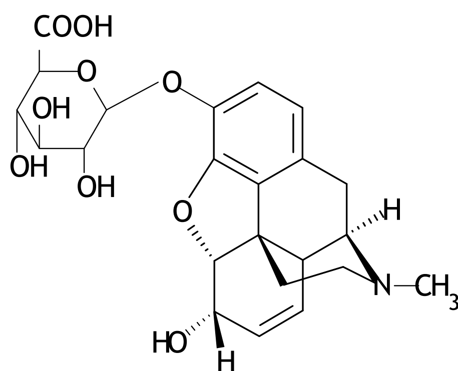
2010). In this PhD work, a higher mean M6G/M3G (0.25 ± 0.09) compared to the one reported by clinical trials (Andersen *et al.*, 2002: mean or median value of M3G/M6G from 5.0 to 8.5, i.e. M6G/M3G from 0.12 to 0.20, in several studies) was found. This finding is in accordance with other published data (Bogusz *et al.*, 1997a) and seems to confirm that morphine glucuronidation may change in drug addicts, as demonstrated by *in vitro* results and outcomes from animals studies (Antonilli *et al.*, 2003 a and b; Antonilli *et al.*, 2005; Antonilli *et al.*, 2008; Graziani *et al.* 2008; Meringolo *et al.*, 2011). Certainly, differences between morphine therapy and "street heroin" use, as well as the overall health of patients and addicts, and also all the difficulties of post-mortem toxicology, must be considered. Nonetheless, in heroin fatalities M6G/M3G should be further investigated, beside other concentrations and/or ratios, in order to study the role of some risk factors, such as the contemporary use of ethanol and cocaine or the loss of tolerance. To this purpose, a larger number of blood samples will be analysed, when available.

Appendices



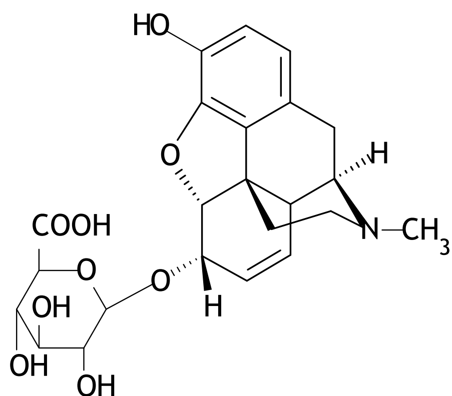
Morphine (MOR)

Mw = 285.34



Morphine-3-β-D-glucuronide (M3G)

Mw = 461.47

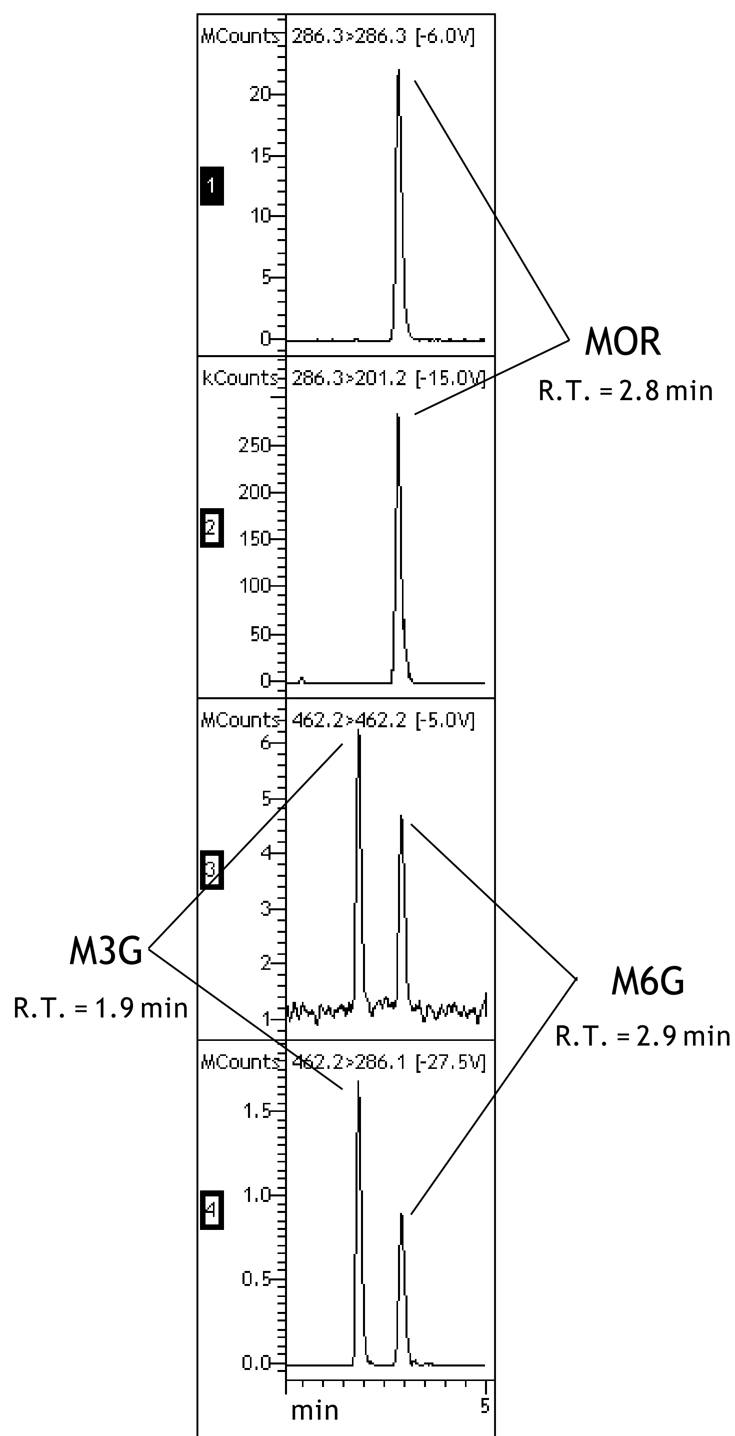


Morphine-6-β-D-glucuronide (M6G)

Mw = 461.47

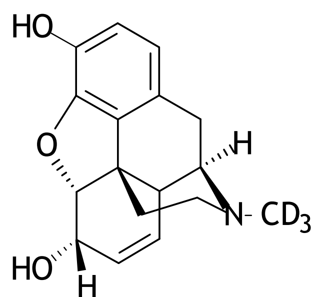
Chemical structure and Molecular Weight of the three molecules of interest.

Appendix 1



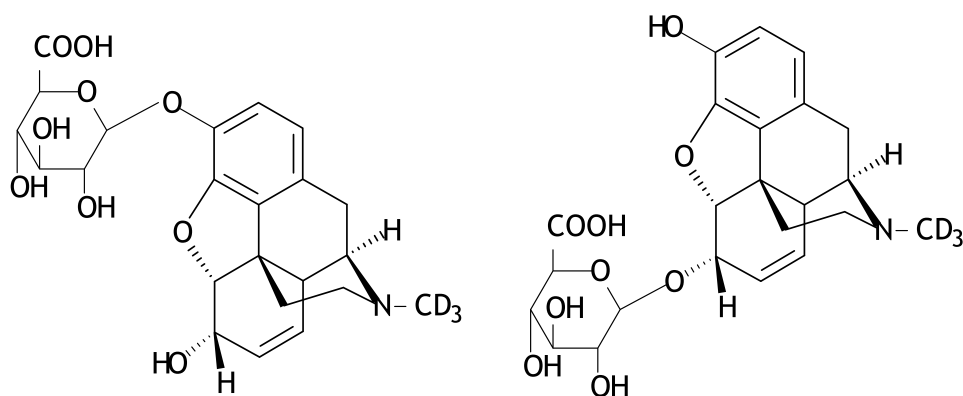
Selected MRM transitions of morphine (MOR), morphine-3-β-D-glucuronide (M3G) and morphine-6-β-D-glucuronide (M6G) (R.T. = Retention Time).

Appendix 2



Morphine-D3 (M-D3)

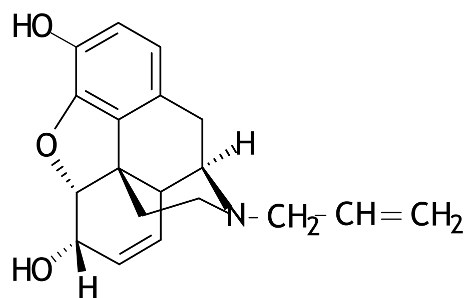
Mw = 288.32



Morphine-3-β-D-glucuronide-D3 (M3G-D3)

and morphine-6-β-D-glucuronide-D3 (M6G-D3)

Mw = 464.44

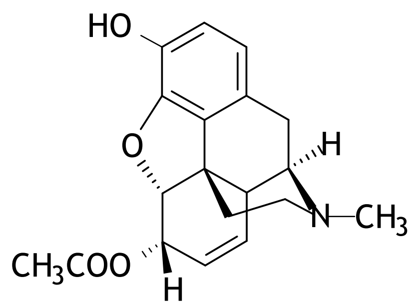


Nalorphine (NAL)

Mw = 311.39

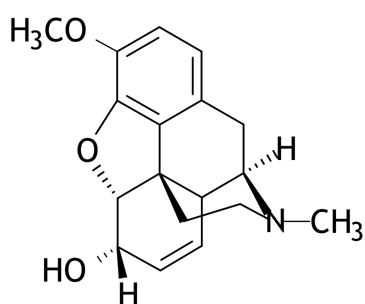
Chemical structure and Molecular Weight of Internal Standards (IS).

Appendix 3



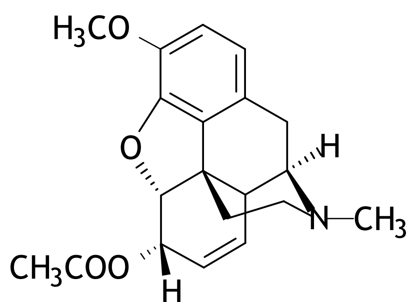
6-Monoacetylmorphine (6-MAM)

Mw = 327.38



Codeine (COD)

Mw = 299.37

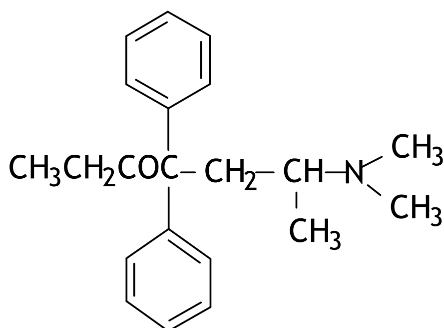


Acetylcodeine (ACOD)

Mw = 341.40

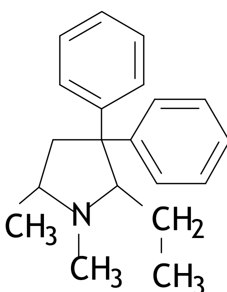
Chemical structure and Molecular Weight of other molecules analysed by LC/MS.

Appendix 4



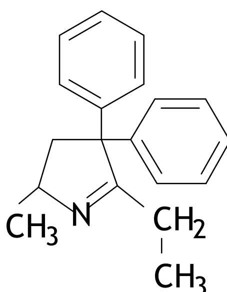
Methadone (MET)

Mw = 309.49



EDDP (2-Ethyl-1,5-dimethyl-3,3-diphenyl-pyrrolidine)

Mw = 278.46

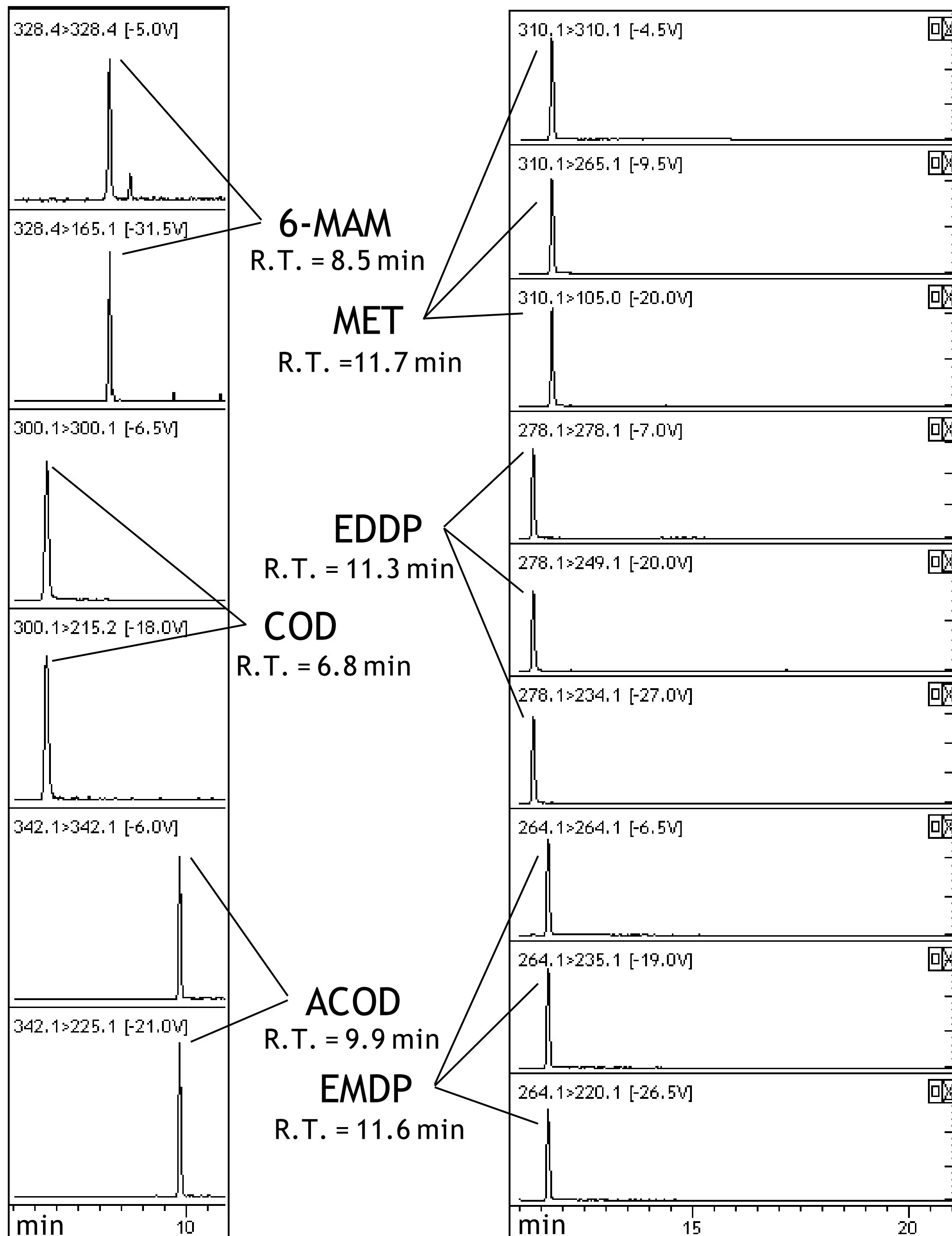


EMDP (2-Ethyl-5-methyl-3,3-diphenylpyrroline)

Mw = 264.39

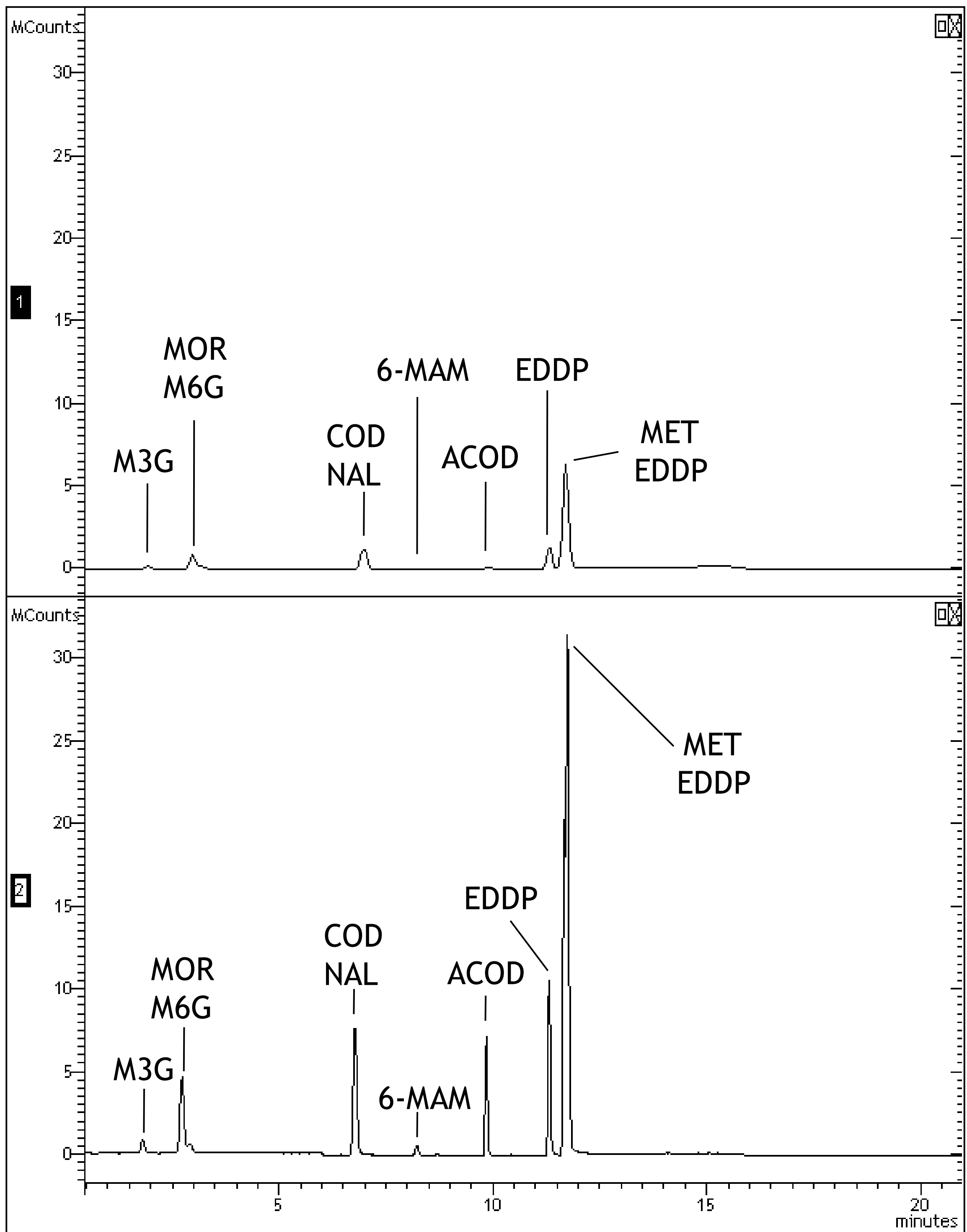
Chemical structure and Molecular Weight of other molecules analysed by LC/MS.

Appendix 5



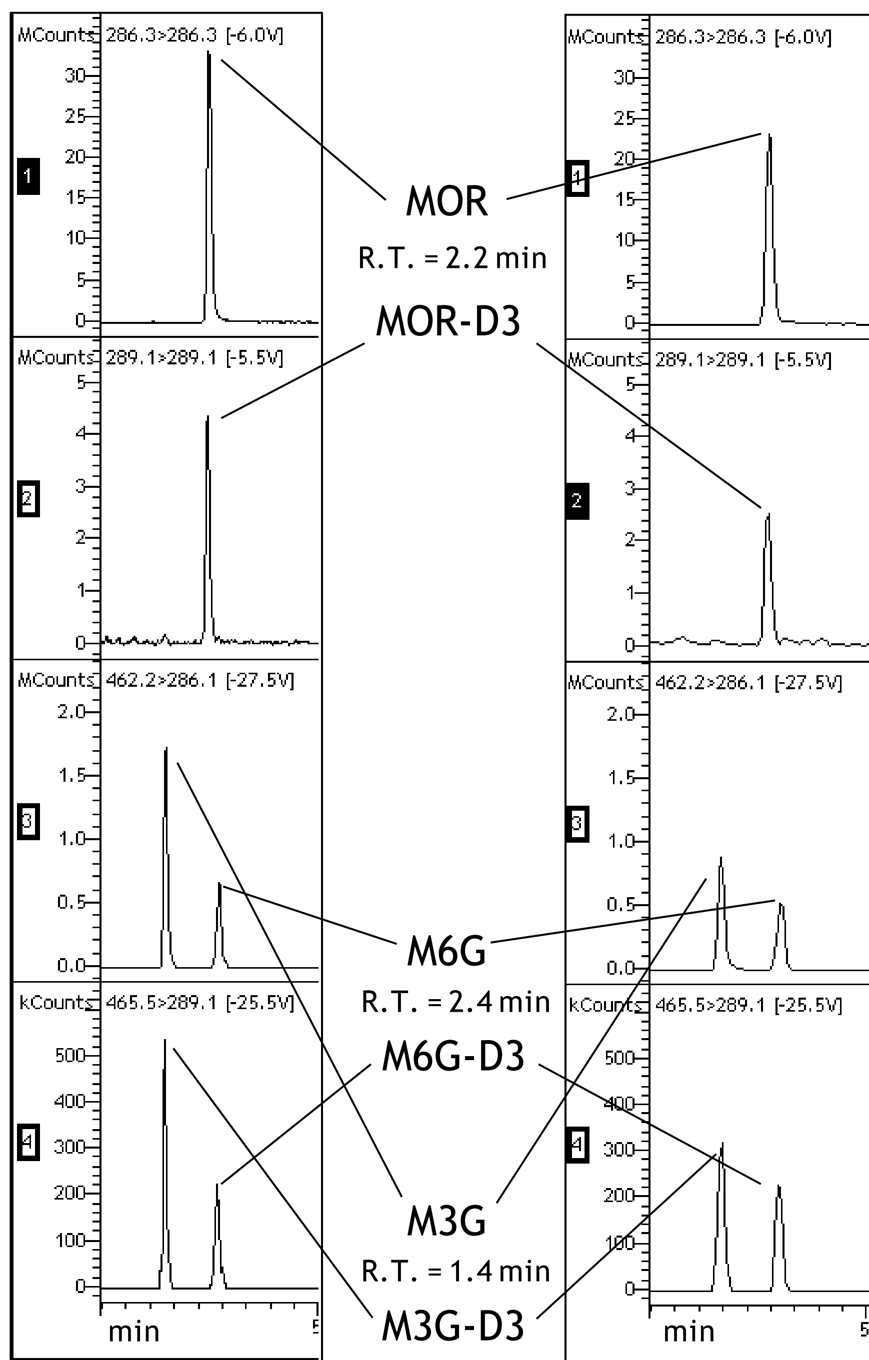
Selected MRM transitions of 6-monoacetylmorphine (6-MAM), codeine (COD), acetylcodeine (ACOD), methadone (MET) and its main metabolites EDDP (2-Ethyl-1,5-dimethyl-3,3-diphenyl-pyrrolidine) and EMDP (2-Ethyl-5-methyl-3,3-diphenylpyrroline) (R.T. = Retention Time).

Appendix 6



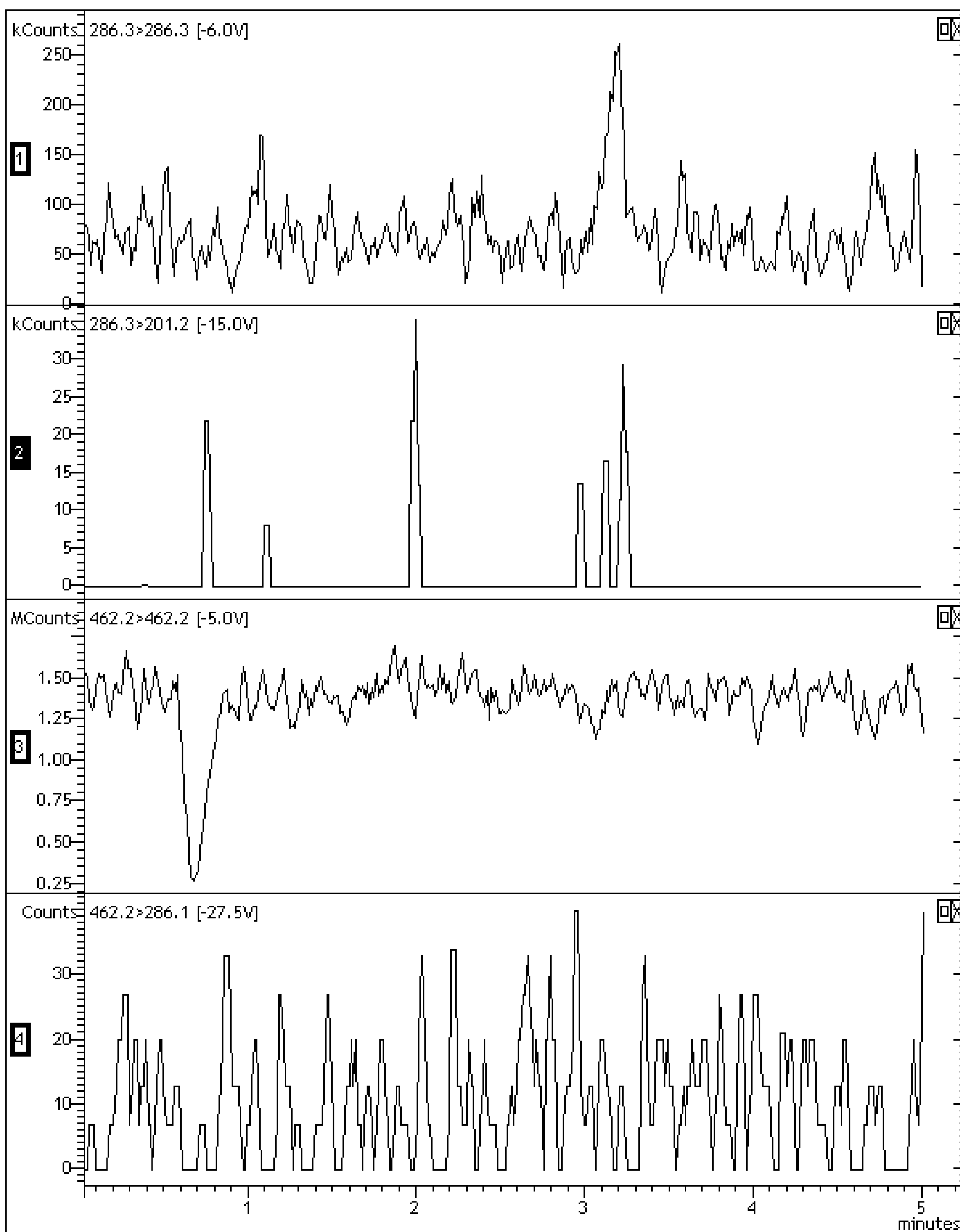
TIC (Total Ion Chromatogram, i.e. sum of all MRM transitions signals) of an extracted WSs 200 ng analysed by applying initial method (1) and the method with three acquisition segments (2).

Appendix 7



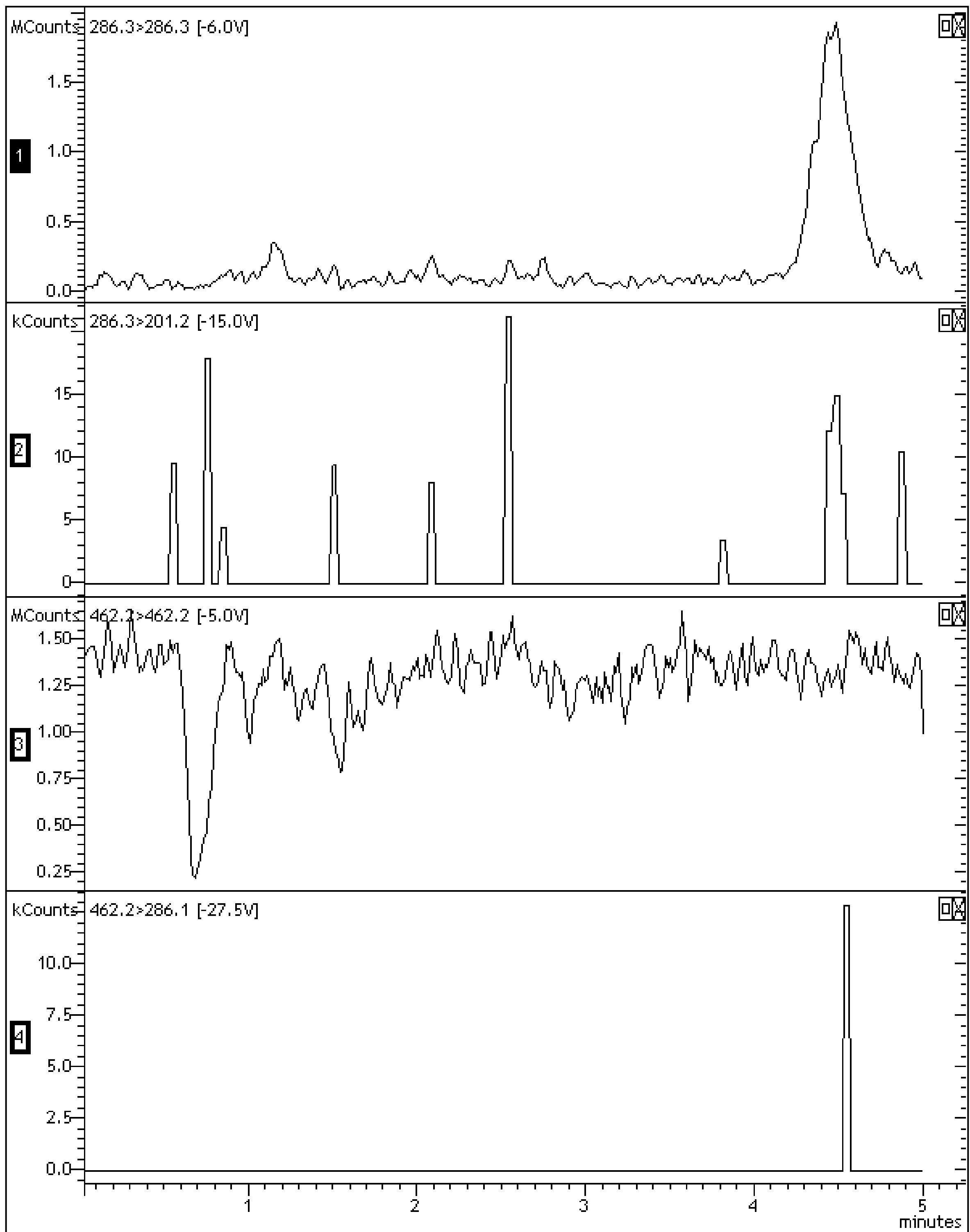
Quantifier MRM transition of MOR, M3G and M6G and of the corresponding IS-D3 of an extracted WSs 200 ng analysed by applying the initial method (right) and the method with three acquisition segments (left) (R.T. = Retention Time).

Appendix 8



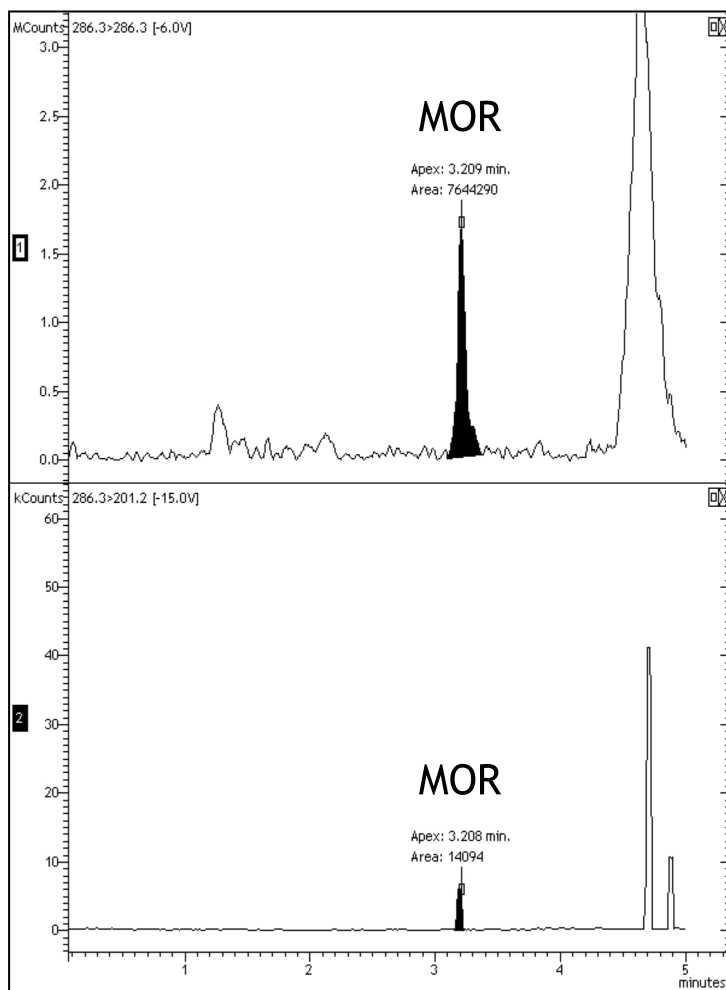
Chromatogram of a “blank” solvent Working sample (panel 1 and 2: MRM transitions of MOR; panel 3 and 4: MRM transitions of M3G and M6G).

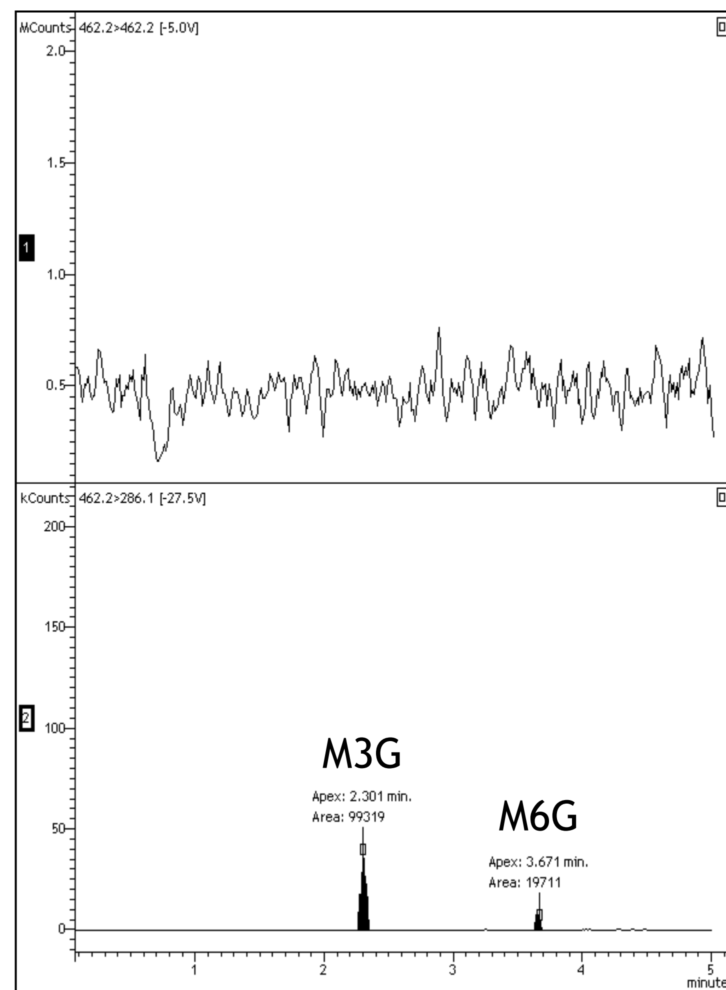
Appendix 9



Chromatogram of a “blank” blood Working sample (panel 1 and 2: MRM transitions of MOR; panel 3 and 4: MRM transitions of M3G and M6G).

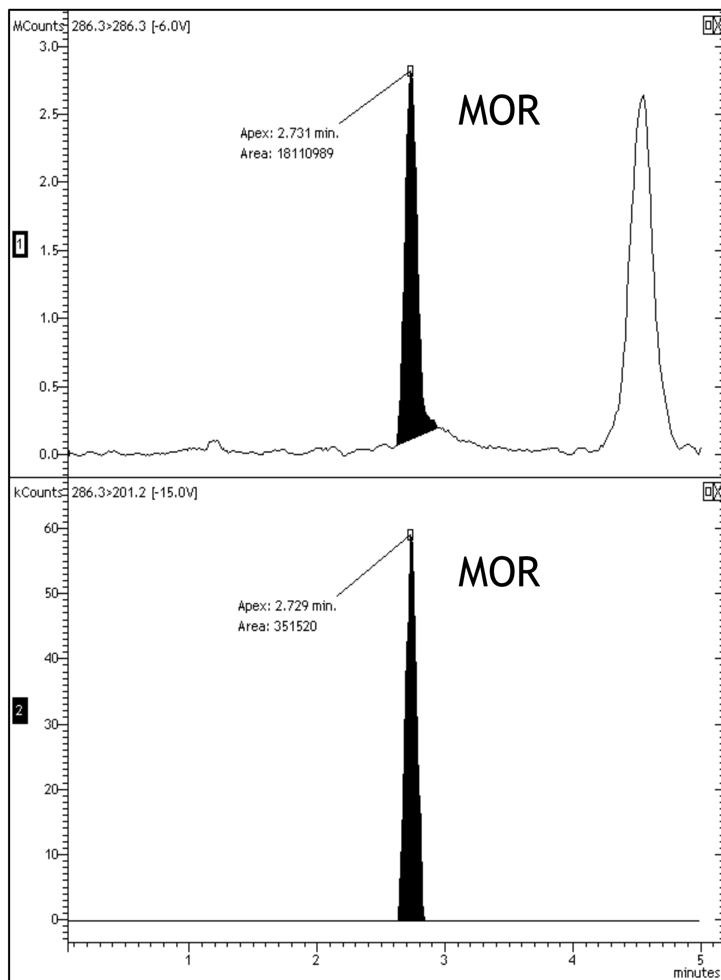
Appendix 10

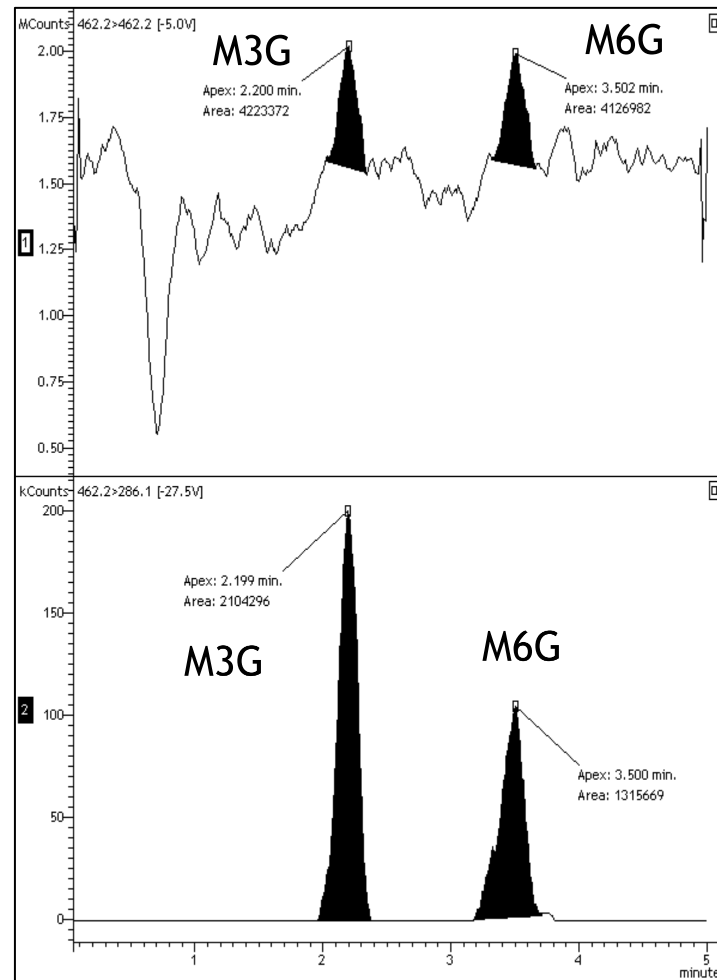




MRM transitions of MOR, M3G and M6G of a Wss 1 ng (LOD) (Apex = Retention Time).

Appendix 11





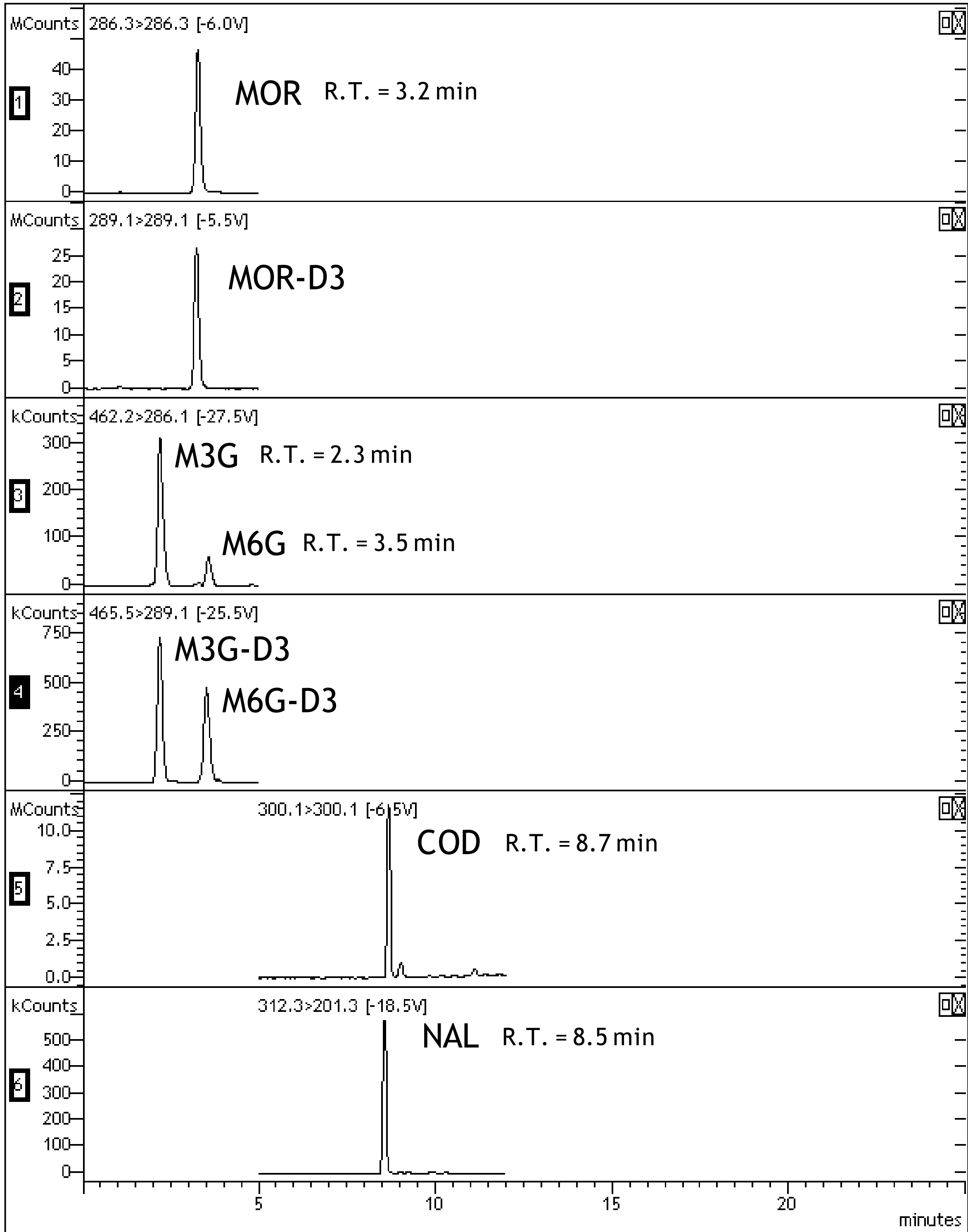
MRM transitions of MOR, M3G and M6G of a Wss 10 ng (LLOQ) (Apex = Retention Time).

Appendix 12

CLASS	CASE No.	AGE	GENDER	BAC (g/L)	RELEVANT CIRCUMSTANCES
I	1	45	M	0.1	First injection?! Family denies a history of drug dependence
	2	22	M	n.p.	led to the hospital; reported a double dose of heroin
	3	45	M	n.p.	Found dead in a toilet with a syringe nearby
	4	44	M	0.3	Found dead at home with a syringe in the arm
	5	31	M	n.p.	Found dead in a toilet with a syringe in the arm
	6	26	M	n.p.	Found dead with a syringe nearby
	7	34	M	0.4	Found dead in his car; injection sites at autopsy
	8	uk	M	n.p.	uk
	9	uk	M	n.p.	uk
	10	uk	M	n.p.	uk
	11	uk	M	n.p.	uk
	12	uk	M	n.p.	uk
	13	uk	M	n.p.	uk
II	14	39	M	0.7	Found dead at home with a syringe nearby
	15	22	M	0.5	Found dead at home with suspected “brown-sugar”
	16	39	M	0.7	History of drug dependence; injection sites at autopsy
	17	42	M	2.2	History of drug dependence; found dead at his home
	18	35	M	1.3	Found dead in a toilet with a syringe nearby
	19	uk	M	1.0	uk
III	20	35	M	0.5	In treatment for heroin dependence
IV	21	24	M	0.2	Found in his bed; no signs of injection; white foam and blood at the mouth and at the nose
	22	30	M	0.1	Found in his bed; no signs of injection; white foam and blood at the mouth and at the nose
	23	41	M	n.p.	Found dead in his car
V	24	45	M	0.6	Found dead in a street with a syringe nearby
	25	43	M	0.5	Found dead with a syringe nearby

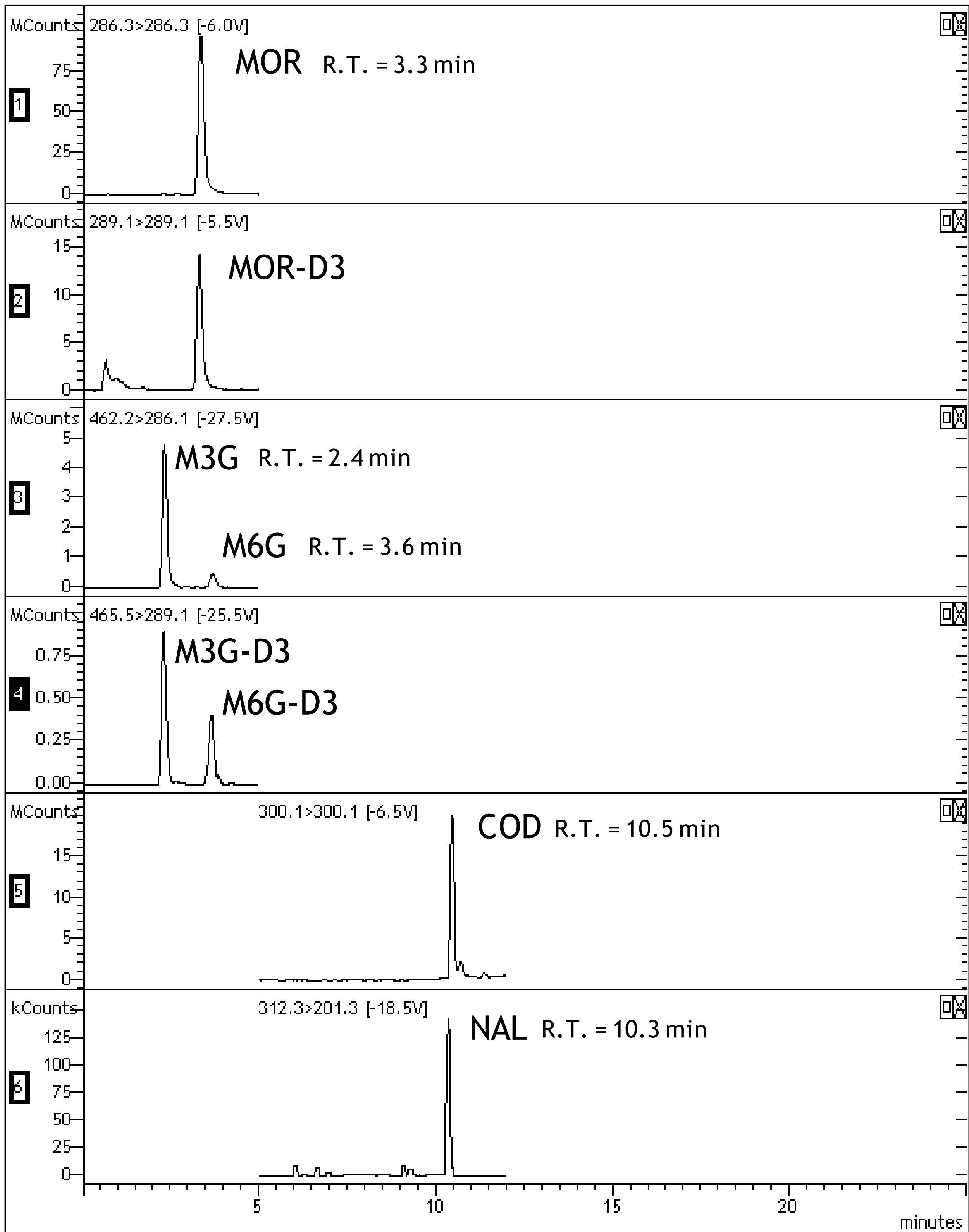
Classification, age, gender and blood ethanol of the twenty-five cases analysed by LC/MS-MS (BAC = Blood Alcohol Concentration; n.p. = not present, i.e. <0.5 g/L; M = male; uk = unknown).

Appendix 13



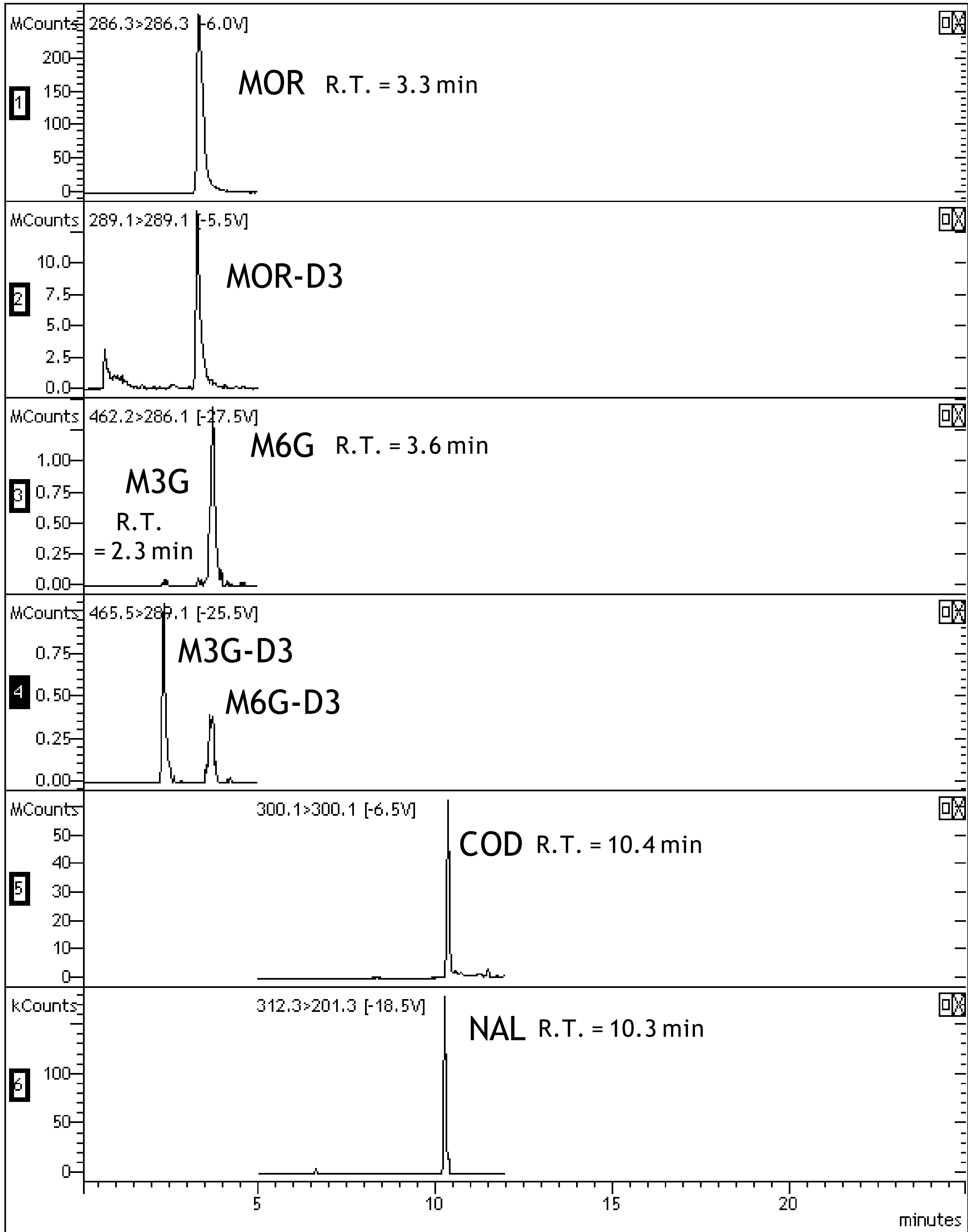
Case No.17: quantifier MRM transition of MOR, M3G, M6G, their corresponding IS-D3, COD and NAL.

Appendix 14



Case No.21: quantifier MRM transition of MOR, M3G, M6G, their corresponding IS-D3, COD and NAL.

Appendix 15



Case No.23: quantifier MRM transition of MOR, M3G, M6G, their corresponding IS-D3, COD and NAL.

Appendix 16

CLASS	CASE No.	MOR (ng/mL)	M3G (ng/mL)	M6G (ng/mL)	COD (ng/mL)
I	1	358	1710	285	59
	2	294	1148	317	20
	3	166	250	60	14
	4	254	266	92	33
	5	371	633	196	41
	6	271	1409	153	30
	7	60	788	165	8
	8	231	188	42	28
	9	139	788	130	n.d.
	10	31	69	12	3
	11	30	342	n.d.	n.d.
	12	266	833	225	18
	13	153	457	85	18
II	14	187	427	92	32
	15	646	398	90	61
	16	649	2307	721	89
	17	117	46	14	11
	18	152	322	88	16
	19	156	97	50	26
III	20	108	474	63	18
IV	21	334	569	185	31
	22	515	2138	821	66
	23	1252	3	458	76
V	24	1349	11	10	271
	25	145	394	82	9

Concentrations of Morphine (MOR), M3G (Morphine-3-β-D-glucuronide), M6G (Morphine-3-β-D-glucuronide) and Codeine (COD) of the twenty-five blood samples analysed by LC/MS-MS (n.d.= not detected).

Appendix 17

CASE No.	MOR	M3G	M6G	COD	MOR +M3G +M6G	MOR +M6G	M6G/ M3G	M3G/ M6G	M3G/ MOR	M6G/ MOR	MOR/ MOR +M3G +M6G
1	358	1710	285	59	1591	534	0.17	6.00	2.95	0.49	0.22
2	294	1148	317	20	1200	490	0.28	3.62	2.41	0.67	0.25
3	166	250	60	14	358	203	0.24	4.17	0.93	0.22	0.46
4	254	266	92	33	475	311	0.35	2.89	0.65	0.22	0.53
5	371	633	196	41	884	492	0.31	2.23	1.05	0.33	0.42
6	271	1409	153	30	1237	366	0.11	9.21	3.21	0.35	0.22
7	60	788	165	8	649	162	0.21	4.78	8.10	0.70	0.09
8	231	188	42	28	373	257	0.22	4.48	0.50	0.11	0.62
9	139	788	130	n.d.	707	219	0.17	6.06	3.50	0.58	0.20
10	31	69	12	3	81	38	0.17	5.75	1.37	0.24	0.38
11	30	342	n.d.	n.d.	241	30	/	/	7.03	/	0.12
12	266	833	225	18	920	405	0.27	3.70	1.93	0.52	0.29
13	153	457	85	18	488	206	0.19	5.38	1.84	0.34	0.31
14	187	427	92	32	508	244	0.22	4.64	1.41	0.30	0.37
15	646	398	90	61	948	702	0.23	4.42	0.38	0.09	0.68
16	649	2307	721	89	2521	1095	0.31	3.20	2.19	0.69	0.26
17	117	46	14	11	154	126	0.30	3.29	0.24	0.07	0.76
18	152	322	88	16	405	206	0.27	3.66	1.31	0.36	0.37
19	156	97	50	26	247	187	0.52	1.94	0.38	0.20	0.63
20	108	474	63	18	440	147	0.13	7.52	2.71	0.36	0.25
21	334	569	185	31	800	448	0.33	3.08	1.05	0.34	0.42
22	515	2138	821	66	2344	1023	0.38	2.60	2.56	0.98	0.22
23	1252	3	458	76	1537	1535	152.67	0.01	0.001	0.23	0.81
24	1349	11	10	271	1362	1355	0.91	1.10	0.01	0.005	0.99
25	145	394	82	9	439	196	0.21	4.80	1.68	0.35	0.33

Sums and ratios of analytes concentrations in the twenty-five cases analysed by LC/MS-MS (n.d.= not detected; / = not calculated).

Appendix 18

Case No. CLASS 1 - 13 I		MOR	M3G	M6G	C	MOR +M3G +M6G	MOR +M6G	M6G/M3G	M3G/M6G	M3G/MOR	M6G/MOR	MOR/ M+M3G+M6G
	MEAN	202	683	147	25	708	307	0.22	4.94	2.73	0.48	0.32
	± SD	116	500	96	16	441	153	0.07	1.72	2.36	0.42	0.16
	RANGE	30-371	69-1710	/-317	/-59	81-1592	30-534	/-0.35	0-9.21	0.50-8.10	/-1.70	0.09-0.62

Case No. CLASS 14 - 19 II		MOR	M3G	M6G	C	MOR +M3G +M6G	MOR +M6G	M6G/M3G	M3G/M6G	M3G/MOR	M6G/MOR	MOR/ M+M3G+M6G
	MEAN	318	600	176	39	797	427	0.31	3.52	0.99	0.28	0.51
	± SD	256	851	269	30	889	388	0.11	0.97	0.78	0.23	0.20
	RANGE	117-649	46-2307	14-721	11-89	154-2521	126-1095	0.22-0.52	1.94-4.64	0.24-2.19	0.07-0.69	0.26-0.76

Mean, Standard Deviation (± SD) and range of analytes concentrations and ratios of class I and II (/ = not calculated).

Appendix 19a

Case No. CLASS		MOR	M3G	M6G	C	MOR +M3G +M6G	MOR +M6G	M6G/M3G	M3G/M6G	M3G/MOR	M6G/MOR	MOR/ M+M3G+M6G
21 - 23 IV	MEAN	700	903	488	58	1561	1002	51.13	1.90	1.20	0.52	0.48
	± SD	486	1106	319	24	772	544	87.94	1.65	1.29	0.41	0.30
	RANGE	334-1252	3-2138	185-821	31-76	800-2345	448-1535	0.33-152.67	0.01-3.08	0.001-2.56	0.23-0.98	0.22-0.81

Case No. CLASS		MOR	M3G	M6G	C	MOR +M3G +M6G	MOR +M6G	M6G/M3G	M3G/M6G	M3G/MOR	M6G/MOR	MOR/ M+M3G+M6G
24 - 25 V	MEAN	747	203	46	140	901	775	0.56	2.95	0.84	0.18	0.66
	± SD	851	271	51	185	652	820	0.50	2.62	1.18	0.24	0.47
	RANGE	145-1349	11-394	10-82	9-271	439-1362	196-1355	0.21-0.91	1.10-4.80	0.01-1.68	0.005-0.35	0.33-0.99

Mean, Standard Deviation (± SD) and range of analytes concentrations and ratios of class IV and V.

Appendix 19b

CLASS Case No.	n	BAC (g/L)		MOR	M3G	M6G	C	MOR +M3G +M6G	MOR +M6G	M6G/M3G	M3G/M6G	M3G/MOR	M6G/MOR	MOR/ M+M3G+M6G
I 1-13	13	<0.5	MEAN	202	683	147	25	708	307	0.22	4.94	2.73	0.48	0.32
			± SD	116	500	96	16	441	153	0.07	1.72	2.36	0.42	0.16
			RANGE	30-371	69-1710	/-317	/-59	81-1592	30-534	/-0.35	0-9.21	0.50-8.10	/-1.70	0.09-0.62
II 14-17	4	0.5-1.0	MEAN	410	807	238	52	1056	557	0.32	3.55	1.09	0.32	0.48
			± SD	275	1011	322	29	1019	426	0.14	1.25	0.88	0.26	0.20
			RANGE	156-649	97-2307	50-721	26-89	247-2521	287-1095	0.22-0.52	1.94-4.64	0.38-2.19	0.09-0.69	0.26-0.68
III 18-19	2	≥1.0	MEAN	135	184	51	14	280	166	0.29	3.44	0.77	0.22	0.57
			± SD	25	195	52	4	178	57	0.02	0.26	0.75	0.20	0.27
			RANGE	117-152	46-322	14-88	11-16	154-405	126-206	0.27-0.30	3.29-3.66	0.24-1.31	0.07-0.36	0.37-0.76

Mean, Standard Deviation (± SD) and range of analytes concentrations and ratios according to Blood Alcohol Concentration (BAC) (/ = not calculated).

Appendix 20

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