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Autopsy rates and findings with emphasis on codeine

Doctoral thesis

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Trondheim, May 2016

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Rettstoksikologi i Midt-Norge: Obduksjonsfrekvens og funn med hovedvekt på kodein

Obduksjon kan gi informasjon om dødsårsaker, særlig ved uklare og mistenkelige dødsfall, og kan være et korrektiv til klinisk stilte diagnoser og dødsårsaker. Rettslige obduksjonsaker undersøkes rutinemessig for forekomst av alkoholer, medikamenter og andre substanser, funn som med stor sannsynlighet ikke blir erkjent uten gjennomføring av obduksjon. Frekvensen av obduksjon har falt i mange land de siste tiårene, til bekymring for rettsmedisinere verden over.

Blant toksikologiske funn i obduksjonssaker er opioider (morfinliknende stoffer) en av de stoffklassene som hyppigst knyttes til forgiftningsdødsfall. Kodein har i en årrekke vært det mest forskrevne smertestillende legemidlet i denne stoffklassen i Norge. Kodein har liten farmakologisk effekt i seg selv, og må ved terapeutiske doser omdannes til morfin for å utøve vesentlige effekter. Denne omdanningen avhenger av leverenzymet cytokrom P450 2D6 (CYP2D6), som er gjenstand for betydelig genetisk variasjon. Betydningen av dette ved kodeinforgiftninger er fortsatt uavklart.

Denne avhandlingen består av fire delstudier basert på kliniske opplysninger og toksikologiske analyser i rettslige obduksjonssaker fra Midt-Norge i perioden 2003-2012, som belyser ulike aspekter ved disse problemstillingene.

Studie I kartlegger regional praksis vedrørende begjæring av rettslig obduksjon og generelle toksikologifunn i vårt nedslagsfelt, og viser at obduksjonsfrekvensen kan variere betydelig med dødsmåte, politidistrikt, kjønn og alder. Studien avdekket store regionale forskjeller i obduksjonsfrekvenser mellom Sør- og Nord-Trøndelag fylke på tross av nasjonal lovgivning vedrørende begjæring av rettslig obduksjon. Toksikologifunnene i denne studien indikerer at alkohol, rus- og legemidler utgjør en vesentlig faktor ved plutselig, uventet død, og at en grundig og omfattende toksikologisk analyse er påkrevd ved undersøkelse av slike dødsfall. Studien er en av svært få systematiske undersøkelser av frekvensen av rettslig obduksjon.

Studie II presenterer blodkonsentrasjoner av kodein, morfin og morfinmetabolittene morfin-3-glukuronid (M3G) og morfin-6-glukuronid (M6G), samt genotyping av den metabolske kapasiteten av CYP2D6 i en serie av 34 kodein-relaterte dødsfall. Studien viste en stor spredning i de målte nivåene av morfin etter kodeininntak, og demonstrerte at nivået av morfin ikke kan forutsies fra kodeinkonsentrasjonen i slike obduksjonssaker, selv når CYP2D6-genotypen er kjent. Studien illustrerer like fullt viktigheten av å medbestemme nivåene av morfin og morfinmetabolitter i kodein-relaterte dødsfall.

I studie III presenteres en nyutviklet, validert væskekromatografi-massespektrometri (LC-MS)-metode for spesifikk mengdebestemmelse av kodein, kodeinmetabolittene kodein-6-glukuronid (K6G), norkodein og morfin, samt morfinmetabolittene M3G og M6G i postmortalt blod, øyevæske, muskel-, fett- og hjernevev. Metoden er anvendt i studie IV.

Studie IV presenterer samtidige nivåer av kodein, K6G, norkodein, morfin, M3G og M6G i blod, øyevæske, muskel-, fett- og hjernevev, samt CYP2D6-genotype i en serie av 23 kodein-relaterte dødsfall. Denne studien viste også stor spredning i de målte nivåene av kodeinmetabolitter i forhold til kodeinkonsentrasjonen, tilsynelatende uavhengig av CYP2D6-genotype. Studien viste generelt lavere konsentrasjoner i de andre prøvematrisene enn i blod, og spesielt i fett- og hjernevev. Et interessant funn var at de antatt farmakologisk aktive analyttene morfin og M6G i et flertall av sakene var under analysemetodens påvisningsgrense i hjerne.

Resultatene fra studie II, III og IV tilfører fagområdet et validert analytisk verktøy og ny innsikt i kodeins postmortale toksikologi, og er slik av relevans for rettstoksikologisk analyse- og sakkyndighetsarbeid.

Joachim Frost

Institutt for laboratoriemedisin, barne- og kvinnesykdommer (LBK)

Veiledere: Professor Lars Slørdal og professor Ivar Skjåk Nordrum

Finansiering: LBK, NTNU og St. Olavs Hospitals forskningsfond

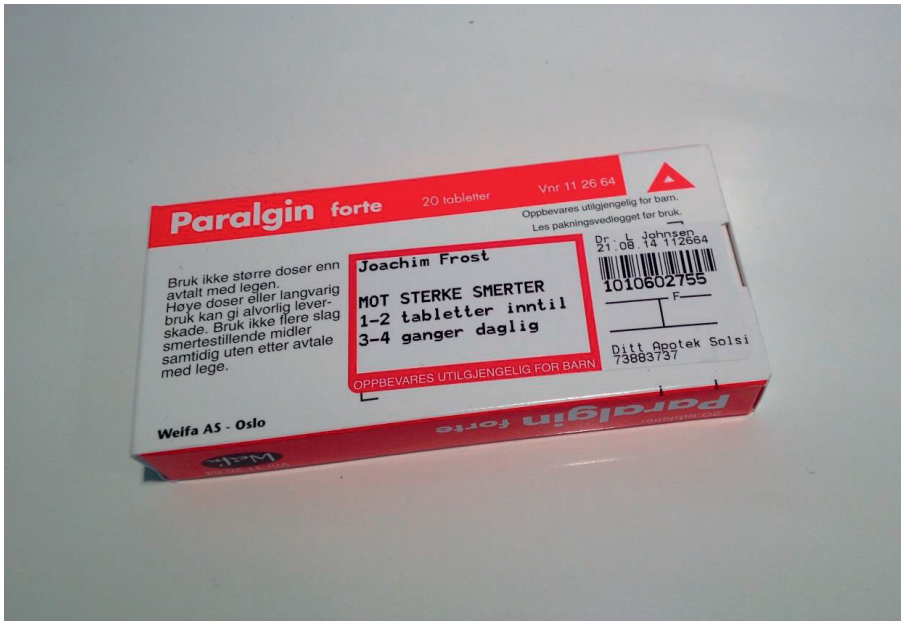
Ovennevnte avhandling er funnet verdig til å forsvares offentlig for graden philosophiae doctor (ph.d.) i klinisk medisin. Disputas finner sted i Auditoriet (MTA), Medisinsk teknisk forskningssenter, onsdag 11.05.2016 kl. 12:15.

But we can learn so much from the people we've lost.

If there's something we missed, we'd like to know, and we'd also like to know when we are right.

Lisa Salberg, chief executive of the Hypertrophic Cardiomyopathy Association, US

Barbara Crain, director of the autopsy service at Johns Hopkins Medicine, Baltimore, US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Oppbevares utilgjengelig for barn.
Les pakningsvedlegget før bruk.
Bruk ikke større doser enn avtalt med legen. Høye doser eller langvarig bruk kan gi alvorlig leverskade. Bruk ikke flere slag smertestillende midler samtidig uten etter avtale med lege.
Joachim Frost
MOT STERKE SMERTER
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Papers

Acknowledgements

The work presented in this thesis was carried out in the period from 2010 to 2015 at the Department of Laboratory Medicine, Children's and Womens's Health, Faculty of Medicine, Norwegian University of Science and Technology (NTNU), and at the Department of Clinical Pharmacology, St. Olavs Hospital, Trondheim University Hospital. I am grateful to the Faculty of Medicine and the Research Department at St. Olavs Hospital for financial support and to the Department of Clinical Pharmacology for providing laboratory facilities and resources.

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Finally, I wish to express my greatest gratitude to my lovely wife Helene and my wonderful children Leonard, Peter and Johanne for reminding me every day what matters most in life. Without you, this work would not have been worthwhile!

Trondheim, December 2015

Joachim Frost

Abbreviations

CYP2D6	Cytochrome P450 isoenzyme 2D6
CYP3A4	Cytochrome P450 isoenzyme 3A4
C6G	Codeine-6-glucuronide
C6G/C-ratio	Codeine-6-glucuronide to codeine concentration ratio
DDD	Defined daily dose
EM	Extensive metabolizer
ForTox	The Forensic Toxicology Project
GC-MS	Gas chromatography mass-spectrometry
GHB	Gamma-hydroxybutyrate
HOD	Norwegian Ministry of Health and Care Services
ICD-10	International Classification of Diseases
IM	Intermediate metabolizer
LC-MS	Liquid chromatography mass-spectrometry
LLOQ	Lower limit of quantification
M/C-ratio	Morphine to codeine concentration ratio
ME	Matrix effects
MPLA	Multiplex ligation-dependent probe amplification
M3G	Morphine-3-glucuronide
M6G	Morphine-6-glucuronide
N6G	Norcodeine-6-glucuronide
NC/C-ratio	Norcodeine to codeine concentration ratio
NPS	New psychoactive substances

PCR	Polymerase chain reaction
PM	Poor metabolizer
REK	Regional Committee for Medical and Health Research Ethics
TIAFT	The International Association of Forensic Toxicologists
ULOQ	Upper limit of quantification
UM	Ultrarapid metabolizer
V_d	Volume of distribution
WHO	World Health Organization
6-MAM	6-Monoacetylmorphine

List of papers

Paper I:

Joachim Frost, Lars Slørdal, Åshild Vege, Ivar S. Nordrum

Forensic autopsies in a naturalistic setting in Norway: Autopsy rates and toxicological findings

Forensic Science International 223 (2012) 353-358

Paper II:

Joachim Frost, Arne Helland, Ivar S. Nordrum, Lars Slørdal

Investigation of morphine and morphine glucuronide levels and cytochrome P450 isoenzyme 2D6 genotype in codeine-related deaths

Forensic Science International 220 (2012) 6-11

Paper III:

Joachim Frost, Trine N. Løkken, Wenche R. Brede, Solfrid Hegstad, Ivar S. Nordrum, Lars Slørdal

A validated method for simultaneous determination of codeine, codeine-6-glucuronide, norcodeine, morphine, morphine-3-glucuronide and morphine-6-glucuronide in post-mortem blood, vitreous fluid, muscle, fat and brain tissue by LC-MS

Journal of Analytical Toxicology 39 (2015) 203-212

Paper IV:

Joachim Frost, Trine N. Løkken, Arne Helland, Ivar S. Nordrum, Lars Slørdal

Post-mortem levels and tissue distribution of codeine, codeine-6-glucuronide, norcodeine, morphine and morphine glucuronides in a series of codeine-related deaths

Submitted

1. Introduction

1.1 The forensic autopsy

An autopsy can provide documentation of external or internal injuries, diseases and the presence of alcohols, drugs and other substances in body fluids and tissues. Autopsies can thus give valuable information about the cause of death to health care professionals and the next of kin. The autopsy also has an important place in medical education and the acquisition of medical knowledge. Furthermore, the autopsy is of significance for the acquisition of reliable mortality data, which in turn are essential for valid cause of death statistics and proper monitoring of changes in the incidence of specific causes of death (e.g. violent deaths, diseases and lethal poisonings). National registries of causes of death are generated using the World Health Organization (WHO) International Classification of Diseases (ICD-10) codes [1] based on information given in death certificates issued by physicians, and reports from clinical and forensic autopsies. The value of autopsies as a corrective for the information given in death certificates has been demonstrated in several studies [2-7]. Compromised standards for autopsy requests and performance may thus have a negative impact on cause of death statistics, and hamper proper knowledge about the status and trends in causes of death. This might in turn have implications for the planning of health services and interventions in the population.

According to law and provision in Norway, the police is commonly recommended to request a forensic autopsy under certain given circumstances, e.g. sudden unexpected deaths and suspected accidents [8]. In cases of suspected homicide, unidentified corpse and unexplained death in children (< 18 years) the police is obliged to request a forensic autopsy [8]. Forensic autopsies may thus shed light on the circumstances of death in ambiguous and potentially criminal cases. Moreover, quantitative post-mortem chemical and toxicological analysis, essential in the determination of alcohol- and drug-related deaths [9, 10], is in our region rarely performed outside the forensic autopsy setting. The forensic autopsy thus stands out as a pivotal investigative tool both for the legal system and the health services.

In many countries autopsy rates have declined continuously over the last decades, particularly for clinical autopsies [11-15]. This phenomenon has concerned pathologists worldwide [15-20]. There has also been raised concern about shifts in autopsy patterns towards an increasing percentage of autopsies among external cause deaths and younger persons, possibly affecting the cause of death determination for important disease conditions in the higher age groups. Finland has seen exceedingly high forensic autopsy rates compared to other countries after the implementation of a national authority for medico-

legal affairs [21]. In Norway, no similar initiatives have been implemented. In our country the clinical autopsy rate has declined from about 5% to 3% from 2001 through 2012 [22, 23], whereas the forensic autopsy rate has remained relatively constant at approximately 4% over the same period [22, 23]. Systematic knowledge of how forensic autopsy rates relate to manner of death and demographical variables over time is, however, limited.

1.2 Post-mortem toxicology

1.2.1 General

Results from qualitative and quantitative analyses of alcohols, medicinal and illicit substances in body fluids are central and often pivotal factors when deciding the underlying, immediate and contributory causes of death in forensic autopsy cases. Analyses of both ante-mortem and post-mortem samples may have important criminal and civil legal implications. For practical, legal and ethical reasons toxicological analysis in most forensic autopsy cases is limited to post-mortem specimens, usually blood, urine and vitreous humor. Both the analysis of post-mortem specimens and the interpretation of findings present a range of challenges for forensic toxicologists.

1.2.2 Preanalytical and analytical aspects

In forensic toxicology a number of preanalytical and analytical variables must be taken into account, e.g. sampling procedures, specimen quality and composition, storage stability of relevant analytes and the quality and validity of analytical methods [24-27]. Some of these issues can be monitored by establishing a secure specimen chain of custody, i.e. procedures that account for the integrity, identification, and security of each specimen by tracking its handling and storage from point of collection to final disposition [28]. Precautions must be taken to avoid contamination of collected specimens with other biological materials like urine, tissue fluids and gastric/bowel contents, cleaning and disinfection solutions, etc. Optimal storage conditions, especially with regard to temperature, must be maintained, and preservation of specimens is sometimes advantageous. However, autolytic and putrefactive alterations occurring before the collection of specimens cannot be eliminated. A systematic and harmonized performance of screening and specific analyses is also required for cases to be comparable from one jurisdiction to another, and methods intended for routine use must be fully validated to objectively demonstrate their applicability for the intended use [29].

1.2.3 Interpretational challenges

The interpretation of results from quantitative analysis offers another set of challenges that may prove difficult, and sometimes impossible, to manage. The impact of a drug detected in post-mortem specimens is not simply a matter of concentration, but also has to be interpreted in light of the circumstances of death. Possibly contributing factors such as individual vulnerability (e.g. age, relevant diseases/comorbidity, increased sensitivity to drugs, etc.) and drug tolerance must be considered. Similarly, the role of individual drugs and potential drug interactions must be evaluated when multiple drugs or poisons are detected. Another important aspect is the time from ingestion to death. In delayed poisoning deaths, for instance, one or more toxic moieties might be present in a toxic concentration inducing coma and respiratory depression, but keeping the patient alive long enough to allow the substance(s) to be metabolized or excreted to a concentration normally not associated with toxicity.

Information about all these factors is potentially assessable from the clinical history, which is an essential part of forensic toxicology casework. Assurance of comprehensive clinical information depends not only on the death investigation by the police, but requires proper information exchange between police authorities, pathologists, toxicologists and other involved parties.

1.2.4 Therapeutic and toxic concentrations

The therapeutic and toxicological monitoring in medical practice is usually based on analyses in serum samples from living subjects. A considerable body of knowledge about drug dose/concentration and effect relationships has been established for many drugs, constituting an important basis for clinical decision making. In the context of post-mortem toxicology, however, these data have several limitations. Firstly, they are to a large part generated from pharmacokinetic studies with healthy volunteers that for ethical reasons have been given relatively low, non-toxic doses of the drug in question, or from experimental studies with animal models that not necessarily apply to humans. Secondly, many drugs show an uneven distribution between whole blood, serum and cellular phase [30], which limits the applicability of reference data from serum samples in the assessment of post-mortem whole blood concentrations. Thirdly, post-mortem changes in biological material make extrapolation from pharmacological parameters in live subjects inaccurate or even erroneous. Post-mortem alterations may also limit the selection and utility of relevant specimens. Finally, reliable pharmacokinetic and pharmacodynamic information is generally less available for illicit substances than for therapeutic drugs. This has both analytical and interpretational implications, and is at present

particularly relevant when dealing with unknown substances or new psychoactive substances (NPS), e.g. synthetic cannabinoids and substituted cathinones.

Naturalistic studies of fatal poisonings or deaths where high concentration of a particular drug is detected in post-mortem specimens are usually limited to case reports or at best case series with a limited number of comparable cases. Still, several compilations of therapeutic, toxic and fatal concentrations of drugs have been established based upon the existing literature regarding individual drugs [31-35]. These reference data are primarily elaborated for blood, and corresponding data for various tissue specimens are generally scarce. Such elaborations are naturally limited by the quality of the data on which they are based. Indeed, there is a lack of adequate studies of the true relationship between fatal events and the concentrations that can be subsequently measured [36]. Consequently, toxicological reference data serve merely as a guiding tool, which always should be applied with careful consideration of the clinical history and circumstances of death. As pointed out by Druid and Holmgren [35] every approach to the compilation of fatal and toxic drug concentrations may also be susceptible to some degree of circular reasoning, emphasizing the importance of continuous international monitoring and publication of toxicology results, ideally on the basis of uniform and consistent systematic toxicological analysis.

1.2.5 Degradation, formation and redistribution of drugs post-mortem

More than 50 years ago Curry and Sunshine [37] observed differences in the concentrations of barbiturates in liver and femoral blood specimens from autopsy cases. 30 years later, Pounder and Jones [38] described the presently acknowledged phenomenon of post-mortem drug redistribution as a toxicological nightmare, creating major difficulties for interpretation and undermining the reference value of data bases where the site of origin of post-mortem blood samples is unknown. Over the last decades there have been substantial improvements in our understanding of the significance of post-mortem toxicology [39]. It has been shown that some drugs may be subject to post-mortem biological degradation/metabolism, exemplified by nitrobenzodiazepines (e.g. nitrazepam, flunitrazepam and clonazepam) that may be metabolized to their respective 7-amino metabolites both *in vivo* and post-mortem [40, 41], and morphine glucuronides that have been shown to undergo post-mortem hydrolysis to yield elevated morphine levels in specimens stored above -20°C [42, 43]. Other substances, such as ethanol [44-47] and gamma-hydroxybutyrate (GHB) [48, 49], can be formed in clinically significant levels after death. GHB may cause further interpretational problems because of its endogenous nature and wide distribution of endogenous concentrations [48, 49]. The nature of post-mortem changes and site dependent variability in drug concentrations have been thoroughly described and reviewed in the literature [36, 39]. Among others, Hilberg and co-workers have performed several experimental

animal studies looking into the possible mechanisms behind post-mortem drug concentrations shifts [50-55]. These studies demonstrated post-mortem changes in drug concentrations similar to those described in humans, and suggested that drug reservoirs in the gastrointestinal or respiratory tract (the latter after aspiration of gastric contents) may diffuse into surrounding tissues and blood after death, and that highly tissue-bound drugs (e.g. in the lungs) may dissociate from binding sites and redistribute as the autolytic processes commence. Illustratively, a rapid increase in heart blood concentration within the first 2 hours post-mortem was demonstrated for the tricyclic antidepressant amitriptyline [50]. Hilberg and co-workers [55] also emphasized that analyses of several samples of blood and tissues are preferable for a reliable interpretation of toxicological findings, although this is often limited by the general scarcity of reference values for drug concentrations in tissues.

Based on current knowledge the apparent volume of distribution (V_d) and the ratio of central to peripheral blood concentrations (in practice the heart blood/femoral blood concentration ratio) of a drug are often used as predictors of the drug's liability to undergo post-mortem redistribution. It is generally acknowledged that the effect of post-mortem redistribution on measured concentrations can be reduced by collecting blood specimens from peripheral rather than central sites, and by minimizing the post-mortem interval prior to sampling. However, a recent study [56] comparing drug concentrations in blood specimens collected upon mortuary admission and blood samples taken at autopsy indicated that significant redistribution can occur for some drugs even when taking peripheral specimens, irrespective of the delay in the post-mortem interval.

Despite experimental and analytical efforts, biological processes occurring post-mortem are still in many instances poorly characterized and understood. Detailed information with regard to post-mortem drug redistribution phenomena is still scarce for many substances, and published data are often conflicting. There is also a continuing need for better understanding of the effects of storage conditions and storage time on the analytical outcomes.

Together, this illustrates the challenges associated with establishing a reliable and robust connection between concentration at the time of death and concentrations measured post-mortem, and emphasizes caution when assessing the contribution of drugs to the cause of death in cases where only post-mortem drug concentrations are available, especially if analyzed specimens are few or of poor quality.

1.3 Codeine

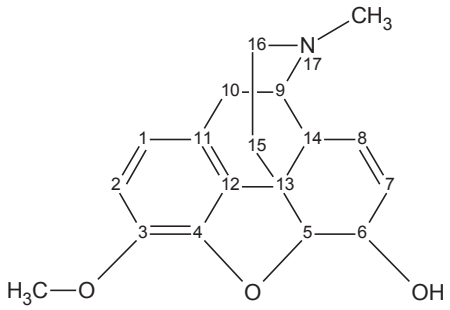

Fig. 1. Structural formula of codeine

1.3.1 Historical background

Codeine, or 3-methylmorphine (Fig. 1), is an opioid alkaloid occurring naturally in opium, the dried latex obtained from the opium poppy (Papaver somniferum) [31]. The cultivation and use of opium for consumption, both for food, anaesthesia and ritual purposes, have been known for millennia [57]. Morphine was the first opium alkaloid to be isolated from the poppy in the early 1800s [57]. Codeine was first isolated from opium several decades later [31]. Today, codeine is usually produced commercially by 3-O-methylation of morphine, which is far more abundant in the juice of the poppy plant [31].

Codeine is widely used as an analgesic throughout the world, often in combination formulations with paracetamol. Combinations with other analgesics or miscellaneous drugs such as acetylsalicylic acid, ibuprofen, carisoprodol, caffeine, barbiturates and sedative antihistamines are available in some countries. Codeine is also used as a cough suppressant, either alone or in combination with other antitussive drugs. In Norway, codeine (in fixed combination formulations with paracetamol) has been the number one selling opioid for several years, accounting for 50% of the total opioid sales and 16% of the total sales of analgesics in 2014 [58]. In 2014 the total consumption of codeine and paracetamol combinations in Norway was approximately 10 defined daily doses (DDD) per 1,000 inhabitants per day [58].

1.3.2 Pharmacokinetics

Codeine is usually administered orally, but can also be administered rectally or as a subcutaneous or intramuscular injection. Single doses in adults are normally from 15 to 60 mg, and the total daily dose may range from 60 to 240 mg.

Basic pharmacokinetic data of codeine [59-63] are shown in Table 1. When administrated orally the absorption of codeine is nearly complete. This is at odds with the relatively low oral bioavailability, presumably due to first-pass metabolism. The oral bioavailability also displays large interindividual variability both within and between different studies, which may be explained by differences in individual hepatic drug-metabolizing capacity and the analytical methods applied in the studies.

Table 1. Pharmacokinetic data of codeine

Oral bioavailability	40-80%
Average time to maximum plasma concentration (t_{max})	1.5 h
Elimination half-life ($t_{1/2}$)	2-6 h
Protein binding in plasma	7-25%
Volume of distribution (V_d)	2.2-3.5 l/kg
Clearance	10-15 ml/min/kg

One study comparing rectal and oral administration of codeine reported similar plasma concentration profiles with similar interindividual variability and no essential difference in bioavailability [64]. Another comparative study reported retarded peak plasma concentration after rectal administration, but rectal absorption resulted in bioavailabilities nearly as large as seen after oral absorption [62].

Codeine is lipophilic and penetrates the blood-brain barrier well, leading to a rapid distributional equilibrium between unbound concentrations in blood and brain [65, 66].

Codeine is mainly metabolized in the liver, although some intestinal and CNS metabolism occurs. The principal pathways are outlined in Fig. 2.

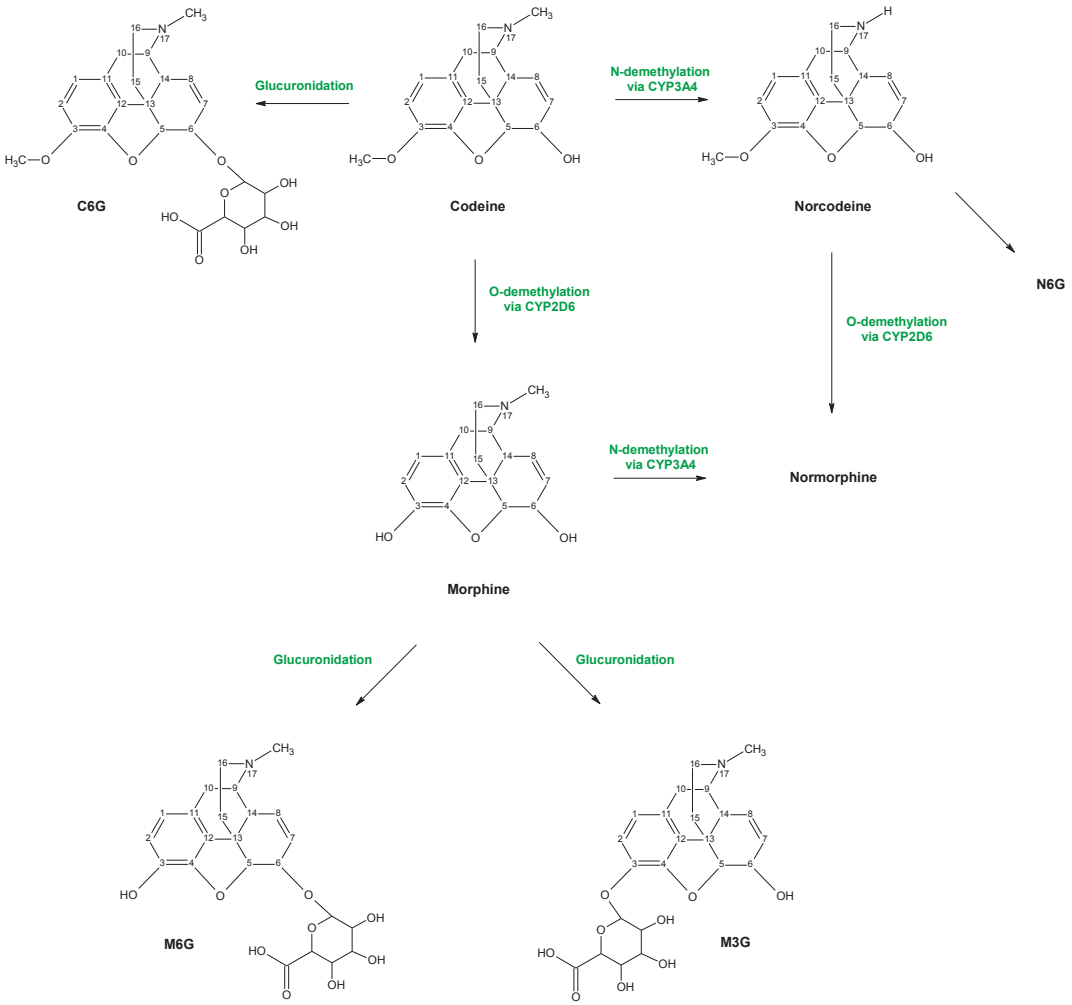

Fig. 2. Principal pathways for codeine metabolism in man. C6G = Codeine-6-glucuronide; N6G = Norcodeine-6-glucuronide; M3G = Morphine-3-glucuronide; M6G = Morphine-6-glucuronide; CYP2D6 = cytochrome P450 isoenzyme 2D6; CYP3A4 = cytochrome P450 isoenzyme 3A4.

A major part (50-70%) of a codeine dose is glucuronidated to codeine-6-glucuronide (C6G), while 10-15% is N-demethylated to norcodeine via the cytochrome P450 isoenzyme 3A4 (CYP3A4) [67]. Norcodeine is in turn glucuronidated to norcodeine-6-glucuronide (N6G), and a minor part is O-demethylated to normorphine [68, 69]. Of an ingested codeine dose, 0-15% is O-demethylated to morphine by the polymorphic cytochrome P450 isoenzyme 2D6 (CYP2D6), and further glucuronidated to the inactive metabolite morphine-3-glucuronide (M3G; approximately 60% of morphine formed) and the active metabolite morphine-6-glucuronide (M6G; 5-10% of morphine formed) [67]. A minor part of morphine is N-demethylated to normorphine [68, 69]. All metabolites are excreted almost entirely by the kidneys.

Due to the dominant role of the liver and kidneys in codeine metabolism and excretion, respectively, concomitant liver and/or kidney disease may alter the pharmacokinetics significantly, thus having clinical implications with regard to analgesia, adverse effects and toxicity.

1.3.3 Pharmacodynamics

Opioids generally exert their effects through binding to three types of opioid receptors (μ, δ and κ) located in the central and peripheral nervous system and in the gastrointestinal tract, producing analgesia, euphoria/dysphoria, sedation, respiratory depression, pupil constriction, reduced gastrointestinal motility and physical dependence [70]. The μ opioid receptors are believed to be responsible for most of the analgesic effects and major side effects of opioids. The δ opioid receptors seem to be more important in the periphery, but may also contribute to analgesia. The κ opioid receptors contribute to analgesia at the spinal level and may produce sedation and dysphoria.

Codeine is commonly considered to be a weak μ opioid receptor agonist, but it was early hypothesized that the analgesic effects of codeine come from its conversion to morphine [59, 71]. Receptor affinity studies have now demonstrated a 100-600-fold greater μ opioid receptor affinity for morphine and M6G than for codeine and its main metabolites C6G and norcodeine/N6G [72-74]. Normorphine has about four times weaker μ opioid receptor affinity than morphine [72] and is produced in small amounts after codeine ingestion [69]. The analgesic effect of codeine thus appear to be largely dependent on metabolic conversion to morphine by CYP2D6. This has also been demonstrated in clinical studies [75-78]. In contrast to the CYP2D6 phenotype-related differences in the analgesic effect, reported differences in adverse effects of codeine in relation to CYP2D6 phenotype are more equivocal [76, 79-83]. Interestingly, studies investigating the effect of incapacitating CYP2D6 on

codeine dependence and abuse liability have yielded inconclusive results [84-87]. Some studies imply morphine-independent central nervous effects of codeine, presumably mediated by codeine itself or its CYP2D6-independent metabolites C6G and norcodeine [77, 88-93], and some investigators have even proposed analgesic synergy between codeine and morphine [94]. However, all these studies have their shortcomings with regard to providing direct evidence for such mechanisms.

Local conversion of codeine to morphine in brain tissue has been demonstrated in rats [95], and it has been proposed that O-demethylation to morphine within the CNS, supposedly also by CYP2D6, may be of importance for the clinical effects of codeine, and even be of greater relevance for codeine analgesia than bioconversion and circulating levels in blood [61].

M6G has been shown to possess μ opioid receptor affinity and analgesic potency similar to or greater than morphine in animal studies [72, 73, 96]. A marked increase in M6G potency has been reported as administration of M6G is shifted from systemic to intrathecal sites in rats and mice [96]. Human studies have indicated that formation of M6G may take place within the central nervous system, but also that M6G, although to a small extent, may be able to cross the blood-brain barrier [96-98]. However, the source of M6G detected in brain tissue, and its potency and contribution to morphine and codeine effects in humans, remain unsettled [96].

1.3.4 CYP2D6 polymorphism

CYP2D6 and CYP3A4 belong to the cytochrome P450 superfamily of enzymes, a large and heterogeneous group of enzymes whose function primarily is to catalyze the oxidation of organic substances, including various xenobiotic substances such as drugs and other potentially toxic chemicals.

CYP2D6 is subject to significant and well-described genetic polymorphisms, causing large individual variability in the enzyme activity (Fig. 3). Approximately 7-10 % of the European population lack functional CYP2D6 enzyme due to homozygous inactivating mutations in the CYP2D6-encoding gene (poor metabolizers (PM)) [99], and are thus unable to convert codeine to morphine. It has been demonstrated in several studies that such individuals do not obtain pain relief from codeine [75-78, 83]. Diminished O-demethylation of codeine to morphine has also been associated with a significant weakening of objective opioid effects, e.g. respiratory, psychomotor and pupillary effects [100].

Conversely, 1-10 % of the European population (with the highest frequency in Southern Europe) possesses allele duplications in the CYP2D6-encoding gene (ultrarapid metabolizers (UM)) [101]. These individuals will metabolize a larger proportion of ingested codeine to morphine [81, 102-104], thus increasing the risk of toxic effects [105-110]. Individuals with "normal" CYP2D6 activity are commonly classified as extensive metabolizers (EM). EM are principally either homozygous for the normal wild-type allele or heterozygous with one wild-type and one inactivated allele. Consequently, homozygous EM are capable of metabolizing more codeine to morphine than heterozygous EM. The distribution of PM, EM and UM differ significantly between ethnic groups [111]. Moreover, environmental factors such as inhibitory interactions from other drugs (e.g. certain antidepressants and antipsychotics) may influence CYP2D6 activity. Interestingly, intracerebroventricular injections of CYP2D6 inhibitors in rats have been shown to yield lower codeine-induced analgesia measured with tail-flick test, lower morphine levels in brain, but not in plasma, and lower CYP2D6 activity in brain membranes, but not in liver microsomes [112]. These findings indicate that variations in brain CYP2D6 activity not reflected in plasma concentrations may influence the response to codeine. Together, this results in a large and unpredictable intra- and interindividual variability in the amount of morphine produced after ingestion of a given codeine dose.

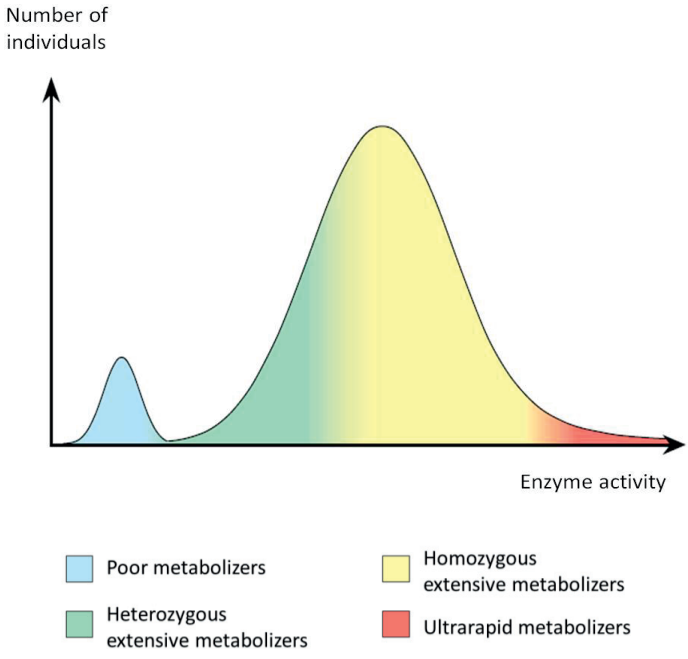

Fig. 3. Distribution of the activity of the polymorphic cytochrome P450 isoenzyme 2D6 (CYP2D6) in the European population. (From "Cytokrom P-450-systemet" by O. Spigset, Tidsskr Nor Laegeforen 121 (2001) 3296-3298. Adapted with permission.)

From a pharmacodynamic perspective the widespread use of codeine as an analgesic is thus encumbered with uncertainty regarding both efficacy and safety. On this basis, clinical practice guidelines for CYP2D6 genotype and codeine therapy have issued strong recommendations to avoid codeine use in PM and UM [113, 114].

Pharmacokinetic studies of codeine and its metabolites in Caucasian healthy volunteers after a single oral dose (50 mg) of codeine have demonstrated a significantly higher urinary recovery of norcodeine in PM compared to EM [80, 115], taken as an indication of compensatory metabolism via N-demethylation in PM [80].

1.3.5 Toxicity

In overdose, codeine is associated with depressive effects on the central nervous system. Death from respiratory arrest may occur with large overdoses. In the reference literature, the minimum lethal oral dose for an adult is estimated at 0.5-1.0 g, i.e. 17-34 pills containing 30 mg codeine each [31]. According to The International Association of Forensic Toxicologists (TIAFT), serum codeine concentrations above 0.30 mg/L have been associated with toxicity, whereas concentrations above 1.6 mg/L are considered possibly lethal [32]. Other compilations of toxic and fatal concentrations of drugs in blood indicate codeine concentrations of 0.5-1.0 mg/L as potentially toxic [34] and concentrations of 0.6-2.1 mg/L as potentially lethal [31, 33-35, 116]. In comparison, TIAFT has listed therapeutic serum levels of codeine as 0.01-0.05 mg/L (trough) and 0.05-0.25 mg/L (peak) [31]. The mechanism behind codeine toxicity is not fully understood, and toxic effects of codeine itself or any of its non-O-demethylated metabolites in overdose cannot be excluded. In particular, high concentrations may to some extent overcome low receptor affinity. However, μ opioid receptor affinity studies [72-74] indicate that this is plausible only in cases of massive overdose. The time between codeine ingestion and death will usually be sufficient for some codeine to be metabolized, particularly in prolonged death, which is commonly observed in overdose cases. In most subjects, this will allow for a certain amount of morphine and M6G to be formed. Establishing and interpreting toxic and fatal levels for codeine alone without the consideration of simultaneous concentrations of active metabolites may thus be problematic.

The existing literature on codeine-implicated deaths has limitations with regard to simultaneous concentrations of codeine and codeine metabolites in post-mortem specimens, particularly in biological matrices other than blood and urine, where even reports of codeine levels are scarce.

Published data (Table 2) derive from various case reports and series [10, 31, 35, 108, 109, 116-128], of which five [35, 116, 118, 126, 128] are large autopsy series. In just one of these larger materials, CYP2D6 genotype was investigated [128]. However, in this study no individual assessments of codeine and morphine levels, CYP2D6 genotype and clinical findings were presented. Published post-mortem blood and tissue concentrations of other codeine metabolites than morphine, including main metabolites C6G and norcodeine and morphine metabolites M6G and M3G, are limited to a case report of fatal and severe codeine intoxication in 3-year old twins [125] and a method application on 31 unspecified autopsy blood samples [129], although one study reported codeine levels in both hydrolyzed and unhydrolyzed blood and tissue specimens in a single codeine fatality [120] and one study reported free and total codeine and morphine in blood from 3 codeine-associated pediatric deaths [127]. In these studies, CYP2D6 genotype was determined in just two of the fatal cases [125, 127].

Table 2. Other published studies of toxicological findings in codeine-implicated deaths

Study	Ref.	Codeine implication	n	Specimen(s)	Analyte(s)
Wright et al. (1975)	[117]	Fatalities associated with codeine	9	Post-mortem blood and urine	Codeine, morphine
Nakamura et al. (1976)	[118]	Deaths involving codeine	45	Post-mortem blood, bile, liver, kidney and urine	Codeine, morphine
Peat and Sengupta (1977)	[119]	Fatal mixed intoxication with codeine and alcohol	2	Ante-mortem blood and gastric contents. Post-mortem liver blood, peripheral blood, urine and gastric contents	Codeine
Pearson et al. (1979)	[120]	A fatality due to ingestion of codeine	1	Post-mortem blood, bile, brain, liver, kidney and gastric contents	Codeine (hydrolyzed and unhydrolyzed)
Havier and Lin (1985)	[121]	Deaths attributed to the combination of codeine and glutethimide	16	Post-mortem blood, bile, vitreous fluid, brain, liver, kidney, spleen, urine and gastric contents	Codeine, morphine
Bailey and Shaw (1985)	[122]	Fatal intoxications involving glutethimide and codeine	12	Post-mortem blood	Codeine
Klys and Brandys (1988)	[123]	Codeine-related death in a 5-year old	1	Post-mortem blood and liver	Codeine

Table 2 *(Continued)*

Study	Ref.	Codeine implication	n	Specimen(s)	Analyte(s)
Kintz et al. (1991)	[124]	Fatal ingestion of codeine	1	Post-mortem blood, bile, brain, liver, kidney, heart, urine and gastric contents	Codeine, morphine
Baselt and Cravey (1993)	[31]	Fatal overdose with codeine/paracetamol	17	Post-mortem blood	Codeine
Gerostamoulos et al. (1996)	[116]	Codeine-related deaths	107	Post-mortem blood	Codeine, morphine
Druid and Holmgren (1997)	[35]	Codeine-related deaths	46	Post-mortem blood	Codeine
Al-Asmari and Anderson (2007)	[129]	Not specified	31	Post-mortem blood	Codeine, C6G, norcodeine, morphine, M3G, M6G and normorphine
Ferreiros et al. (2009)	[125]	Fatal and severe codeine intoxication in 3-year old twins	2	Serum, urine and cerebrospinal fluid/ brain tissue	Codeine, C6G, norcodeine, morphine, M3G, M6G and normorphine
Ciszkowski et al. (2009)	[108]	A fatal case of a toddler receiving codeine after adenotonsillectomy for obstructive sleep apnea syndrome	1	Post-mortem blood	Codeine, morphine
Kelly et al. (2012)	[109]	Fatal and life-threatening codeine toxicity in children after tonsillectomy	3	Post-mortem blood and serum	Codeine, morphine
Langlois et al. (2012)	[10]	Codeine/opioid toxicity	3	Post-mortem blood	Codeine, morphine
Häkkinen et al. (2012)	[126]	Codeine-related deaths	146	Post-mortem blood	Codeine, morphine
Friedrichsdorf et al. (2013)	[127]	Codeine-associated pediatric deaths	3	Post-mortem blood	Codeine (free and total), morphine (free and total)
Lam et al. (2014)	[128]	Codeine-related deaths	68	Post-mortem blood	Codeine, morphine

Published data indicate that codeine and morphine may exhibit post-mortem redistribution, but the results are inconsistent, particularly for morphine [31, 53, 130-137]. One study of the post-mortem redistribution of M3G and M6G as well as morphine concluded that significant post-mortem

redistribution of morphine and its glucuronide metabolites seems unlikely. Post-mortem hydrolysis of morphine glucuronides has been shown to yield elevated morphine levels during specimen storage above -20°C [42, 43]. Accordingly, measured concentrations of codeine and its metabolites are potentially subject to post-mortem changes, both in terms of post-mortem drug redistribution (all substances) and post-mortem deglucuronidation (C6G, M3G and M6G).

1.3.6 Abuse potential

The abuse of and addiction to opioids, including prescription opioid analgesics, is a global problem that affects the health and the social and economic welfare of all societies [138]. It is estimated that between 26.4 million and 36 million people abuse opioids worldwide [138, 139]. In the US, where the total number of prescribed opioid analgesics has soared over the last decades, an estimated 2.1 million people suffered from substance use disorders related to prescription opioid analgesics in 2012, and the number of unintentional overdose deaths from prescription pain relievers has increased dramatically, more than quadrupling since 1999 [138]. Opioid analgesics are now responsible for more deaths than both suicides and motor vehicle accidents in the US [140]. In Europe, the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) has estimated an average annual prevalence of problem opioid use among adults (15-64 years) at around 0.4 %, corresponding to 1.3 million problem opioid users in the European countries in 2012 [141]. These data, however, include heroin, which constitutes the main opioid used. At the national level the European prevalence estimates of problem opioid use vary, with reported data from Norway stating about 2-4 problem opioid users per 1,000 inhabitants aged 15-64 [141]. Data from the Norwegian Cause of Death Registry denote 311 deaths attributed to "addictive substances, drug abuse and poisonings" from a total of 40,344 deaths in Norway in 2014 [142]. The impact of prescription opioid analgesics, and specifically codeine, in these deaths, is not clear. However, in a one-year observational study of fatal poisonings in Oslo, opioids caused most deaths, accounting for 65% of cases [143]. Most of these cases were evaluated as accidental deaths.

Several pharmacoepidemiological studies of opioid use in Norway are available. One study reported that about 13% of the adult population were dispensed opioids in Norwegian pharmacies in 2007, of which only 10% of dispensed opioids were prescribed for cancer pain [144]. In this study, 4% of users were considered daily users for chronic non-cancer pain. The same study observed a 9 % increase from 2004 to 2007 in the number of persons receiving opioids and the number of dispensions, whereas opioid types, doses, and indications appeared stable. A study focusing on prescription patterns of codeine reported that about 10% of adults in Norway were dispensed codeine in 2005, but a majority (58%) received codeine only once, most likely for acute pain, and it was concluded that only

a small minority (0.5%) had a prescription pattern indicating problematic opioid use [145]. Another study investigating the use of codeine analgesics in individual patients in Norway, with special emphasis on high-consumers, also found that codeine use was mainly sporadic, but that a relatively large sub-group of users were dispensed repeated prescriptions of the drug in combination with other potential drugs of abuse, in particular benzodiazepines [146]. A different study exploring problematic opioid use in a cohort of opioid-naïve users after they had started treatment with weak opioids, reported prescription patterns indicative of persistent and problematic opioid use in 0.3% and 0.08%, respectively [147]. Problematic opioid use may thus seem to be of relatively low prevalence in the general population of opioid-prescribed patients in Norway (including persons receiving opioids for acute and cancer-related pain). However, considering high recent estimates of chronic pain prevalence in Norway and increasing focus on alleged undertreatment of chronic pain [148], alongside the American epidemic of opioid abuse and addiction after liberalization of practices and laws regarding opioid prescription in chronic non-cancer pain [140], caution and vigilance seem warranted with regard to the use of opioids in this group of patients. A recent investigation of trends and characteristics of codeine overdose deaths in Australia found high rates of chronic pain, substance use and comorbid mental health problems in these deaths, and concluded that codeine-related deaths (both with and without the involvement of other toxic drugs) are increasing as the consumption of codeine-based products increases [149]. Another recent study of codeine intoxication experiences amongst recreational drug users, as posted on public internet forums, identified and investigated almost 100 'trip reports' and more than 150 thread discussions relating to the sole use of codeine, and found, among other things, that codeine's appeal centered on its availability via family medicine cabinets, prescribers and pharmacies [150]. Recent searches in the Norwegian Prescription Database show that codeine continues to be, by far, the number one selling opioid analgesic in Norway [151]. Codeine is thus an opioid requiring attention in our country.

1.4 The Regional Biobank of Central Norway

The Regional Biobank of Central Norway (Biobank1) is a large regional research facility containing a collection of human biological material (e.g. blood, tissue and urine samples) and a database with relevant information about the samples and the patients they originate from, as well as information obtained from analysis of the material [152]. The materials are collected in connection with medical examinations, diagnosis or treatment. Biobank1 was established in 2003 in collaboration between the Central Norwegian Regional Health Authority and the Faculty of Medicine at the Norwegian University of Science and Technology (NTNU), and is owned and operated by official authorities on a nonprofit basis.

1.5 The Forensic Toxicology Project

The Forensic Toxicology Project (ForTox) [153] is a forensic research project established in late 2006 by Professors Ivar Skjåk Nordrum, Lars Slørdal and Åshild Vege at the Department of Laboratory Medicine, Children's and Women's Health, NTNU. The ForTox project involves the integration of clinical data and post-mortem toxicological specimens from forensic autopsy cases in Central Norway into Biobank1.

The main goal of the ForTox project is to contribute to improved knowledge among forensic scientists and health authorities through studies of

- prevalence and impact of drugs and alcohol (ethanol) in forensic autopsy cases in Central Norway
- distribution and post-mortem changes of therapeutic and illicit drugs in different body fluids and tissues
- effects of storage conditions on specimen concentrations

Searches in the biobank database provide rapid overview of cases of particular interest, e.g. deaths where a selected drug is implicated, and information about the availability and location of specimens from relevant cases. If available, specimens may be collected from the storage repositories and analyzed for substances of interest.

The toxicological analyses are performed at the Department of Clinical Pharmacology, St. Olavs Hospital, Trondheim University Hospital.

2. Aims of the thesis

By creating and utilizing an expanding biobank encompassing clinical information and specimen data from forensic autopsy cases in Central Norway we aimed to

- assess forensic autopsy rates in this region, addressing the need for information on current autopsy practices (Paper I).

- evaluate the impact of alcohol and drugs in these forensic autopsy cases, surveying toxicological findings possibly undisclosed without the performance of an autopsy (Paper I).

Using specific analytical methodology (liquid chromatography-mass spectrometry (LC-MS) and gas chromatography-mass spectrometry (GC-MS) methods) together with clinical data and autopsy specimens from the biobank we aimed to

- provide detailed quantitative toxicological assessment of codeine-related deaths, enabling elucidation of the toxicity profile, distribution, putative redistribution and metabolic pattern of codeine (Papers II, III and IV).

In the investigations of codeine-related deaths we aimed to

- perform an assessment of post-mortem blood levels of morphine and morphine glucuronides, in relation to CYP2D6 genotype (Paper II).

- develop an efficient and validated method for the determination of codeine and codeine metabolites in post-mortem tissues (Paper III).

- provide a comprehensive assessment of the distribution of codeine and codeine metabolites in post-mortem tissues, in relation to CYP2D6 genotype (Paper IV).

3. Materials and methods

3.1 Design

Papers I, II and IV are clinical studies, whereas paper III is a quantitative method paper. Papers I, II and IV are all descriptive, observational studies. Whilst paper I has a cross-sectional study design, papers II and IV are consecutive case series.

3.2 Data and sample collection

The materials in this thesis are based on the forensic medical service in Central Norway (Fig. 3), provided by the Department of Pathology and Medical Genetics and the Department of Clinical Pharmacology at St. Olavs Hospital, Trondheim University Hospital in Trondheim, Norway. These departments provide forensic medical service to the two counties Sør- and Nord-Trøndelag, the region Nordmøre and Romsdal in the county Møre and Romsdal, and the region Helgeland in the county Nordland.

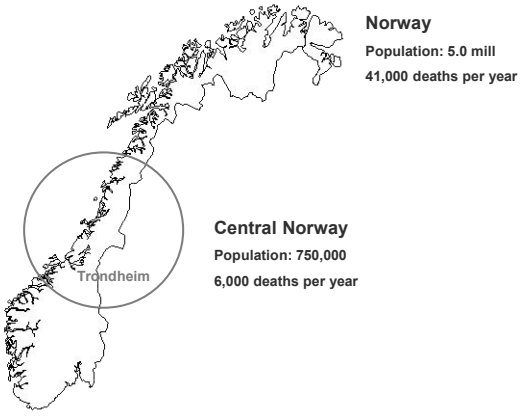

Fig. 3. Central Norway

Forensic autopsy cases from this region are subjected to comprehensive analysis of toxicological specimens on a routinely basis by the Department of Clinical Pharmacology. The routine toxicological analyses are usually performed in post-mortem specimens of blood, urine and vitreous humor,

depending on availability, and the toxicology results are later accessible both from the autopsy reports and the archives at the Department of Clinical Pharmacology.

As part of the ForTox project a separate set of toxicological specimens has been continuously collected at the forensic autopsies during the study period (2006-2012). When available, duplicate specimens of femoral blood, heart blood, urine, vitreous fluid, muscular tissue (right psoas muscle), liver tissue (right and left lobe), kidney tissue (right kidney), fat tissue (suprapubic fat) and brain tissue (right frontal cortex), and a single specimen of gastric contents from individual cases have been collected, labeled and stored in ultra-low temperature freezers (-80°C). The sample collection and handling have been performed by specially instructed autopsy technicians according to a standardized protocol, ensuring a uniform sampling procedure in efforts to minimize the risk of errors, contamination and further decomposition of the samples.

Specimen information (e.g. specimen number, type of matrix and storage location) and relevant clinical information from individual cases (e.g. demographical data, circumstances of death, comorbidity, results from routine toxicological analysis and pathologist's conclusion as to the cause of death) have been obtained through review of forensic autopsy reports, and registered in the database of Biobank1.

In paper I all forensic autopsy cases from the two counties Sør- and Nord-Trøndelag (total population approximately 425,000) during the period 2007-2009 (n = 364) were reviewed and assessed with regard to manner of death, demographical data (sex, age, county) and toxicological findings. Data on all deaths in the two counties over the same period (n = 10,862) was collected from the Norwegian Cause of Death Registry. From these two datasets, forensic autopsy rates by manner of death, sex and age were determined, and toxicological findings assessed.

In paper II all forensic autopsy cases in Central Norway from January 2003 to January 2011 (n = 1,444) were reviewed, identifying and investigating cases with codeine concentrations in femoral blood exceeding the TIAFT toxicity threshold of 0.30 mg/L (n = 34) (Fig. 4). All toxicological information was collected from the results of the routine forensic autopsy analyses.

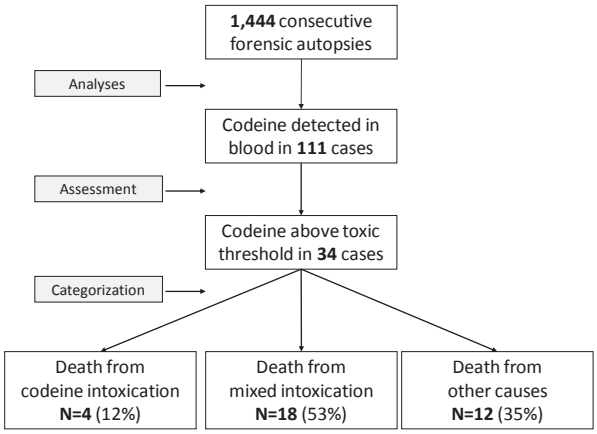

Fig. 4. Study flow chart from paper II

In paper IV all forensic autopsy cases in Central Norway from September 2006 through December 2012 (n = 1,120) were reviewed, identifying and investigating cases with codeine concentrations in femoral blood exceeding the TIAFT toxicity threshold (n = 23) (Fig. 5). Available material in the ForTox biobank from the identified cases was subjected to new quantitative analyses of codeine, C6G, norcodeine, morphine, M3G and M6G with the analytical method described in paper III. All other toxicological information discussed in paper IV was collected from the results of the routine forensic autopsy analyses.

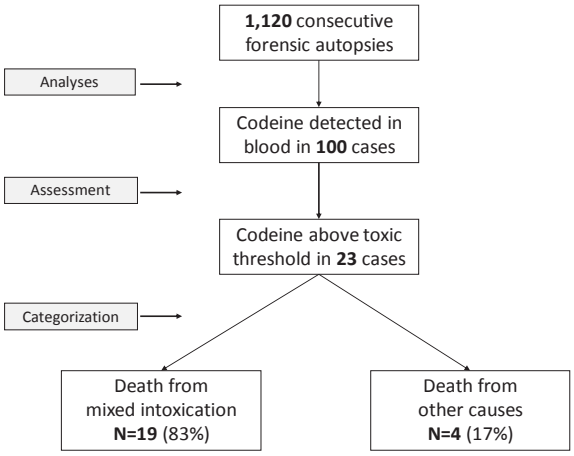

Fig. 5. Study flow chart from paper IV

In paper II we investigated levels of codeine, morphine, M3G and M6G in peripheral blood only. Since these results were already available from the routine forensic autopsy analyses, no utilization of the ForTox biobank material was necessary. In this study, we were thus able to include cases prior to the establishment of the ForTox project in late 2006. Hence, included cases cover a longer time interval in paper II than in paper IV.

All post-mortem specimens used for the development and validation of the analytical method in paper III were sampled from the ForTox biobank material. Blank ante-mortem blood specimens for the same purpose were sampled from a pool of anonymous blood bank specimens available at The Department of Clinical Pharmacology.

3.3 Toxicological analysis

The routine toxicology findings from forensic autopsies applied in this material were determined by LC-MS and GC-MS methods. Post-mortem blood specimens from the autopsies were routinely subjected to specific analyses for alcohols (ethanol, methanol, isopropanol, acetone) using a headspace GC-MS method, and specific analyses for benzodiazepines (diazepam, desmethyldiazepam, oxazepam, nitrazepam, 7-aminonitrazepam, flunitrazepam, desmethylflunitrazepam, 7-aminoflunitrazepam, clonazepam, 7-aminoclonazepam, alprazolam, midazolam), opioids (morphine, codeine, ethylmorphine, oxycodone, M3G, M6G) and amphetamines (amphetamine, methamphetamine, MDMA and MDA) using LC-MS methods. Routine blood specimens were also screened against comprehensive drug libraries (National Institute of Standards and Technology Mass Spectral Library, Forensic Toxicology Retention Time Locking Database/Library and Pfleger/Maurer/Weber Drugs and Pesticides Library for Toxicology) with a GC-MS method. When available, urine was screened for drugs of abuse as well, using LC-MS methods. Positive screening results and explicit information about drug use in the case histories were confirmed by specific analyses in blood using LC-MS or GC-MS methods. In cases where blood was not available, e.g. due to extensive bleeding or putrefaction, toxicological screening and specific analyses were performed with homogenates of suitable tissue samples, usually spleen, liver and/or muscle tissue, using the same methodology. Three such cases with positive toxicology were described in paper 1, whereas no such cases occurred among the cases constituting papers II and IV.

All quantitative analyses of codeine, C6G, norcodeine, morphine, M3G and M6G were performed with the LC-MS method described in paper III. This method had originally been developed at the

Department of Clinical Pharmacology for analysis of codeine, morphine, M3G and M6G in serum and whole blood. To enable the analyses required in paper IV, the method was further developed and validated for analysis of the additional metabolites C6G and norcodeine, and for application in vitreous fluid, muscle, fat and brain tissue, as described in paper III.

The Department of Clinical Pharmacology participates in international interlaboratory comparisons and proficiency testing programs, and is accredited by Norwegian Accreditation, the Norwegian body for accreditation of laboratories, sampling organizations, etc.

3.4 CYP2D6 genotyping

When possible, CYP2D6 genotyping was performed in cases meeting the inclusion criteria of papers II and IV. In some cases, CYP2D6 genotyping had already been performed as part of the routine toxicology investigations. All genotyping was performed at the Department of Pathology and Medical Genetics, St. Olavs Hospital, Trondheim University Hospital.

The CYP2D6 genotyping was performed in post-mortem blood samples by isolating genomic DNA from peripheral leukocytes using either an iPrep™ Purification Instrument with iPrep™PureLink™ gDNA Blood Kit (Invitrogen Corporation, Carlsbad, USA) or a BioRobot® EZ1 with EZ1 DNA Blood 200 µl Kit (Qiagen, Hilden, Germany), according to the manufacturers' guidelines. In a majority of cases the inactivating alleles *3, *4, *6, *7 and *8 were determined by allele-specific polymerase chain reaction (PCR) analysis and the PCR product was analyzed directly by agarose gel electrophoresis. These samples were also tested by long-PCR for the deletion of CYP2D6 (the inactivating allele *5) and for the duplicated/multiduplicated gene (the *2Xn mutation). In later cases the inactivating alleles *3, *4, *5, *6, *7, *8 and the *2Xn mutation were determined by Sanger sequencing and multiplex ligation-dependent probe amplification (MLPA), due to change of methodology at the Department of Pathology and Medical Genetics.

Alleles in which none of the described variants were found were classified as *1 (wild-type) alleles. Cases with two functional (wild-type) alleles (homozygous EM) were assigned an EM phenotype, indicative of a metabolizing capacity in the normal range, whereas cases with any of the inactivating alleles alongside one functional allele (heterozygous EM) were assigned an intermediate metabolizer (IM) phenotype, indicative of a metabolizing capacity in the lower normal range. Cases with two

inactivating alleles were assigned a PM phenotype, indicative of a very low metabolizing capacity. Other allele combinations, as well as the detection of strong CYP2D6 inhibitors (e.g. paroxetine and levomepromazine) in blood, were also taken into account when assigning metabolizer phenotypes. Weaker CYP2D6 inhibitors (e.g. citalopram and methadone) were not considered significant in relation to phenotype.

3.5 Data analysis

All results were managed using the established ForTox database in Biobank1 and the statistical software Microsoft Excel 2010-2013 and SPSS 16.0.

3.6 Ethical considerations

All studies comprising this thesis were performed within the framework of the ForTox project. ForTox is approved by the Regional Committee for Medical and Health Research Ethics (REK) (approval 007-06) and the Director of Public Prosecutions in Norway. Biobank1 is approved by REK and the Norwegian Data Protection Authority, and all information in the biobank is handled according to the guidelines of the Norwegian Data Protection Authority.

All presented data originate from deceased individuals. Thus, ethical considerations regarding potential health-related harms and adverse outcomes for the study subjects are not considered relevant. All collected data and materials were stored and presented anonymously, so that no data, samples or published results reveal the identity or compromise the integrity and privacy of these individuals. The collection of clinical information from the forensic autopsy reports, however, could not be undertaken without consulting individual records. A waiver of confidentiality regarding this information was granted by the Director of Public Prosecutions. Because of the security issues involved, this part of the data collection was performed by a single investigator (Joachim Frost).

When the ForTox project was established in 2006, REK approved that the collection of biological samples during the forensic autopsies was performed without prior or subsequent consent by the deceased or next of kin. This was based on the presumption that forensic autopsy cases, which originate from circumstances involving legal authorities requesting the procedure, are of a character that may introduce ethical dilemmas as to the appropriateness of delegating decisions on behalf of the

deceased to the next of kin. Correspondingly, the next of kin cannot deny a forensic autopsy in Norway if the legal authorities find it necessary.

At that point, the laws and provisions regulating research on biological material from deceased individuals in Norway did not provide specific directives with respect to forensic autopsies, and the application and approval regarding consent and reservations were handled by REK. However, in 2014, the Norwegian Ministry of Health and Care Services (HOD) issued a specification in these regulations by stating that research on materials from forensic autopsies henceforth would require that the next of kin had been informed and given the opportunity to abstain from inclusion in research projects. This generated practical and ethical challenges regarding previously collected materials, which were not addressed in the statement from HOD. Upon request, REK subsequently stated that such retrospective reservation by the next of kin was problematic and possibly unethical in itself, and was waivered for ForTox materials collected prior to the initiative from HOD. This decision applies to all presented data.

Adequate quality assurance of forensic services may be of major importance for preventive medicine, public health and security under the law. There are firm historical precedents demonstrating that research based on forensic autopsy materials is important. It should, however, be recognized that this is a sensitive issue, affecting both the general population and individuals and their families in profound, and sometimes conflicting, ways. The regulation and enforcement of these procedures thus require transparency and accessibility, but at the same time professionalism and determination. The laws and provisions regulating research on forensic autopsy materials remain obscure, even after the initiative from HOD, and revision and clarification of these regulations are warranted.

4. Results

4.1 Summary of paper I

Forensic autopsies in a naturalistic setting in Norway: Autopsy rates and toxicological findings

Joachim Frost, Lars Slørdal, Åshild Vege, Ivar S. Nordrum

Forensic Science International 223 (2012) 353-358

To address the need for information on current autopsy practices, forensic autopsy rates in two adjacent counties in Central Norway over the period 2007-2009 were assessed. To investigate toxicological findings that could possibly remain undisclosed without the performance of an autopsy, the impact of alcohol and drugs in forensic autopsy cases from this material was evaluated.

The total forensic autopsy rate in this material was 3%. The forensic autopsy rates were low for natural deaths (1%), accidental falls (12%) and the heterogeneous category "other accidents" (21%), relatively high for accidental poisonings (84%), and less than adequate for road traffic accidents (57%). For suicides the forensic autopsy rate was 63%, and for recognized homicides 100%. The total forensic autopsy rate was higher for men than for women (5% vs. 2%), and decreased with age, being 38% in the age group < 30 years, 23% in the age group 30-59 years, and 1% in the age group > 59 years. Despite that Norwegian legislation and regulations regarding forensic autopsy requests are national, the forensic autopsy rates were generally lower in the county of Nord-Trøndelag than in Sør-Trøndelag, with most striking differences in suicide deaths (11% vs. 91%) and road traffic accidents (46% vs. 67%). This illustrates how autopsy rates, and possibly cause of death registries, might be susceptible to the influence of regional variations in law enforcement, with possible consequences for the quality and validity of cause of death statistics.

Of the forensic autopsy cases where toxicological analysis was performed (361 of 364 cases) a total of 71% had positive toxicology results; 12% were positive for alcohol only, 44% were positive for drugs only, and 15% were positive for both alcohol and drugs. The toxicology results suggest that alcohol and drugs are important factors in sudden unexpected deaths, and that a thorough and comprehensive toxicological analysis is called for when investigating these deaths. Mean BAC in alcohol positive forensic autopsy cases was 1.7‰ (median 1.6‰, range 0.29-4.1‰). The average number of substances

detected in toxicology positive cases was 2.6 (median 2, range 1-10). The by far most frequently detected classes of substances were (1) benzodiazepines, (2) opioids and (3) alcohol.

4.2 Summary of paper II

Investigation of morphine and morphine glucuronide levels and cytochrome P450 isoenzyme 2D6 genotype in codeine-related deaths

Joachim Frost, Arne Helland, Ivar S. Nordrum, Lars Slørdal

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This paper presents a case series of codeine-related deaths where concentrations of morphine, M6G and morphine-3-glucuronide (M3G), as well as CYP2D6 genotype, are taken into account. Post-mortem toxicological specimens from a total of 1,444 consecutive forensic autopsy cases in Central Norway were analyzed. Among these, 111 cases with detectable amounts of codeine in femoral blood were identified, of which 34 had femoral blood concentrations exceeding the TIAFT toxicity threshold of 0.30 mg/L. Autopsy records of these 34 cases were retrieved and reviewed (Fig. 4).

In the 34 reviewed cases, there was a large variability in individual morphine to codeine concentration ratios (M/C-ratios), and morphine levels could not be predicted from codeine concentrations, even when CYP2D6 genotype was known. 13 cases had codeine concentrations exceeding the TIAFT threshold for possibly lethal serum concentrations (1.6 mg/L). Among these, 8 individuals had morphine concentrations below the toxic threshold according to TIAFT (0.15 mg/L). In one case, morphine as well as M6G and M3G concentrations were below the limit of detection.

A comprehensive investigation of codeine-related fatalities should, in addition to a detailed case history, include quantification of morphine and morphine metabolites. CYP2D6 genotyping may be of interest in cases with unexpectedly high or low M/C-ratios.

4.3 Summary of paper III

A validated method for simultaneous determination of codeine, codeine-6-glucuronide, norcodeine, morphine, morphine-3-glucuronide and morphine-6-glucuronide in post-mortem blood, vitreous fluid, muscle, fat and brain tissue by LC-MS

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This article presents a validated method for simultaneous determination of codeine, codeine metabolites C6G, norcodeine and morphine, and morphine metabolites M3G and M6G in post-mortem whole blood, vitreous fluid, muscle, fat and brain tissue by high-performance liquid chromatography mass spectrometry. Samples were prepared by solid-phase extraction.

The validated ranges were 1.5-300 ng/mL for codeine, norcodeine and morphine, and 23-4600 ng/mL for C6G, M3G and M6G, with exceptions for norcodeine in muscle (3-300 ng/mL), morphine in muscle, fat and brain (3-300 ng/mL) and M6G in fat (46-4600 ng/mL). Within-run and between-run accuracy (88.1-114.1%) and precision (CV 0.6-12.7%), matrix effects (CV 0.3-13.5%) and recovery (57.8-94.1%) were validated at two concentration levels; 3 and 150 ng/mL for codeine, norcodeine and morphine, and 46 and 2300 ng/mL for C6G, M3G and M6G. Freeze/thaw and long-term stability (6 months at -80ºC) was assessed, showing no significant changes in analyte concentrations (-12-+8%).

The method was applied in two authentic forensic autopsy cases implicating codeine in both therapeutic and presumably lethal concentration levels.

4.4 Summary of paper IV

Post-mortem levels and tissue distribution of codeine, codeine-6-glucuronide, norcodeine, morphine and morphine glucuronides in a series of codeine-related deaths

Joachim Frost, Trine N. Løkken, Arne Helland, Ivar S. Nordrum, Lars Slørdal

Submitted

This article presents levels and tissue distribution of codeine, C6G, norcodeine, morphine and the morphine metabolites M3G and M6G in post-mortem blood (peripheral and heart blood), vitreous fluid, muscle, fat and brain tissue in a series of 23 codeine-related fatalities. CYP2D6 genotype is also determined and taken into account. Quantification of codeine, C6G, norcodeine, morphine, M3G and M6G was performed with the validated solid phase extraction LC-MS method presented in paper III. The series comprises 19 deaths (83%) attributed to mixed drug intoxication, 4 deaths (17%) attributed to other causes of death, and no cases of unambiguous monointoxication with codeine (Fig. 5). The typical peripheral blood concentration pattern in individual cases was C6G>>codeine>>norcodeine>morphine, and M3G>M6G>morphine. In matrices other than blood, the concentration pattern was similar, although in a less systematic fashion. Measured concentrations were generally lower in matrices other than blood, especially in brain and fat, and in particular for the glucuronides (C6G, M3G and M6G) and, to some extent, morphine. In brain tissue, the presumed active moieties morphine and M6G were both below the LLOQ (0.0080 mg/L and 0.058 mg/L, respectively) in a majority of cases. In general, there was a large variability in both measured concentrations and calculated blood/tissue concentration ratios. There was also a large variability in calculated ratios of morphine to codeine, C6G to codeine and norcodeine to codeine in all matrices, and CYP2D6 genotype was not a reliable predictor of these ratios. The different blood/tissue concentration ratios showed no systematic relationship with the post-mortem interval. No coherent degradation or formation patterns for codeine, morphine, M3G and M6G were observed upon reanalysis in peripheral blood after storage.

5. Discussion

5.1 Paper I – autopsy rates and general toxicological findings

In paper I we investigated forensic autopsy rates and post-mortem toxicological findings systematically with regard to manner of death and demographical data. Although many of the findings in paper I may be recognizable for forensic pathologists and toxicologists, this study provides needed systematic documentation of important trends in autopsy rates and substance use. In particular, it seems likely that the issues of low and regionally different autopsy rates, with or without rational motives, are current both in other regions of Norway and other parts of the world. Indeed, it has been a general impression among forensic pathologists in Norway that the practice regarding autopsy requests and performance vary from region to region [154], and that there is a need for more systematic and determined practices [155]. As one of very few studies investigating autopsy rates in a systematic manner, paper I materializes these current opinions. Hence, it is our belief that descriptive studies such as this may provide documentation of interest for forensic scientists and health authorities, and may contribute to improved knowledge among the decision makers.

To our knowledge, there are few comparable studies in the existing literature. In particular, at the time of publication (2012) we had not identified any studies presenting autopsy rates and toxicological findings in the same material. Available studies of blood alcohol concentrations in forensic autopsy cases other than those cited in the article were published more than 20 years ago and thus considered outdated for relevant comparisons. Although there are several publications of toxicological findings in selected manners of death, particularly in materials of killed motor vehicle drivers and deceased drug addicts, we had not identified any studies from outside Scandinavia presenting non-alcohol toxicological findings in a comparable way, e.g. reporting detection frequencies of the same substance classes for all manners of death. Comparative review of all aspects of these findings in each individual manner of death would require extensive elaboration of the manuscript, and was omitted to sharpen focus on particular toxicological findings in this material and other important observations, such as the generally low autopsy rates for violent deaths and the striking regional differences in autopsy rates between the adjacent counties Sør- and Nord-Trøndelag. Recently, however, a similar study investigating forensic autopsy practices in rural areas of Korea was published [156]. This study also presented forensic autopsy rates and toxicological findings in the same material, although toxicology results other than alcohol were not specified as to which drugs and chemicals were detected. The autopsy rates were calculated for several areas and according to road distance between the relevant police station and site of autopsy, showing that autopsy rates decreased significantly as the road distance increased. This phenomenon was also described in a previous study from Austria [157], cited

in paper I. As discussed briefly in paper I, direct comparisons of such studies may not be advisable, due to national differences in the practical and legislative aspects of autopsy requests, demographics, disease panorama, etc. However, while this observation may have many possible explanations, it should be noted that increasing rates of fatal injury and prehospital death with increasing degree of rurality have been observed, at least in our country [158-160]. Thus, the potential benefit of sustaining adequate autopsy practices for public health and preventive medicine does not seem to be less relevant in rural areas. Since the publication of paper I, some interesting studies investigating various aspects of the differences in autopsy rates between Denmark and Finland have also been published [161-163]. These studies documented systematically lower autopsy rates in Denmark than in Finland, particularly for suicides [161], and linked this to a much higher frequency of ill-defined and unknown cause of death codes in the Danish death statistics compared to the Finnish [162]. However, in a cost-consequence analysis of the cause of death investigations in the two countries, the same group of researchers stated that the implementation of an alternative practice increasing the autopsy rates in Denmark comes at a price, with costs per 10,000 deaths estimated to increase by about 50% compared to current practice [163].

The cause of death data retrieved from the Norwegian Cause of Death Registry is generated according to the rules of the ICD-10, which sorts undetermined deaths as a subcategory of natural deaths. Since the actual cause of death in undetermined deaths principally may be both natural and unnatural, this coding practice may represent a potential source of error in the calculation and interpretation of the autopsy rates. Unfortunately, information about the number of undetermined deaths was not available in the material from the Norwegian Cause of Death Registry, but the number of undetermined deaths is probably minor, and the impact of these deaths on the presented data likely negligible. It is emphasized that the Norwegian Cause of Death Registry data, like all registry data, is generally susceptible to shortcomings in the information on which it is based, i.e. errors in death certificates, discontinuities in coding practices, etc. Interestingly, a recent study comparing data from the Norwegian Cause of Death Registry and the Norwegian Patient Register found that the benefit of using data from the Norwegian Patient Register for quality assurance efforts of the Cause of Death Registry was limited among deceased persons with an unknown or unspecified cause of death [164]. This finding substantiates the importance of maintaining adequate autopsy practices for this purpose.

Paper I addressed that the prevalence of non-forensic autopsies in cases where forensic autopsy was not performed is unknown in this material, and it was stated that the performance of clinical autopsies probably does not account for much of the observed discrepancies in autopsy rates. A study of clinical autopsies after deaths outside hospital in Eastern Norway [165] published after paper I depicts several

aspects regarding this. Firstly, the number of clinical autopsies after deaths outside hospital increased during the observation period, which was linked to an increase in the total number of such deaths. Secondly, more clinical autopsies were performed in the second half of the year, taken as an indication that restricted police budgets may have caused clinical autopsies to be requested in place of forensic autopsies. The latter was also supported by the observation that a large proportion of these cases was considered to be unnatural deaths prior to autopsy, and thus were obvious candidates for a forensic autopsy. How this relates to our study remains unsettled, but it is possible that the impact of clinical autopsies on the forensic autopsy rates may have been underestimated. It seems that with more deaths occurring outside hospital the requirement of autopsies in general, and forensic autopsies in particular, may become increasingly important, warranting the attention of authorities in both the health services and the legal system. Although it is hard to find valid arguments for sustaining low autopsy rates for certain manners of death, e.g. homicides, accidents and drug-related deaths, the best approach to this issue is not necessarily to perform more forensic autopsies *per se*. It seems prudent, however, to pursue consistent and determined practices regarding autopsy requests and performance, and to secure the quality and status of the autopsy. The mentioned study [165] pointed out toxicological analysis as a key element of the investigation of deaths outside hospital. In Norway comprehensive post-mortem toxicological analysis is at present only performed routinely in the forensic autopsy setting. This implies that the utilization of toxicological analyses is an integral part of the establishment of a good autopsy practice.

As discussed in paper I, the toxicology results in this material of forensic autopsy cases are prone to selection bias. Nevertheless, they may give indications of the prevalence and pattern of use/abuse of detected substances in the general population, as well as the impact of these substances in deaths where toxicological analysis is waived. The high detection rates for substances with abuse potential, particularly benzodiazepines, opioids and alcohol, exhibit substance abuse as a public health issue and emphasize the need for continuous and vigilant drug monitoring.

In 20% of the forensic autopsy cases death was attributed to poisoning (18% accidental, 2% suicidal). Considering the relatively high autopsy rate for accidental poisoning deaths, their high prevalence in this material likely reflects that the outer circumstances (i.e. uncertain cause of death, no witness, etc.) make these deaths susceptible to police involvement and forensic autopsy requests. Still, this observation, together with a considerable prevalence of positive toxicology in accidental deaths in general, substantiates the significance of drugs and alcohol in accidental, and potentially preventable, deaths.

No findings of NPS were made in this material. This may be because the presented material, which originates from 2007-2009, dates from a time before NPS became prevalent in drug seizures and biological samples [166]. However, all toxicological screening in the presented material was performed with GC-MS and LC-MS methodology prior to the introduction of more powerful screening instruments utilizing LC-QTOF-MS in our laboratory, increasing the possibility that unknown substances, such as many NPS, may have passed undetected.

Of the 255 toxicology positive cases in this material, 39 cases (15%) had detectable amounts of codeine in post-mortem blood. In 11 of the 39 codeine positive cases (28%) the codeine blood concentration exceeded the TIAFT toxicity threshold of 0.30 mg/L. Practically all poisoning deaths were due to drugs and/or alcohol. In two thirds of the accidental poisonings opioids were detected, with codeine appearing in 45% of these cases. In one third of the suicidal poisonings one or more opioids were implicated, with codeine among the detected opioids in each case. In total, codeine accounted for 32% of all opioid findings in this material, being the second most frequently detected opioid after morphine/heroin. These findings highlight the importance of codeine as a possible cause of injury or death in our country, and motivate further investigations of codeine-related deaths.

5.2 Paper II – blood levels of codeine, morphine and morphine glucuronides

In paper II, post-mortem blood concentrations of codeine, morphine, M6G and M3G, as well as CYP2D6 genotype, were assessed in a series of codeine-related deaths. To our knowledge, this is the first comparatively large case series where morphine, morphine glucuronides and CYP2D6 genotype is systematically co-determined alongside codeine concentrations in codeine-implicated deaths.

In this paper (and in paper IV) the TIAFT toxicity threshold for codeine of 0.30 mg/L was set as the central inclusion criterium. This was done to introduce an objective parameter quantifying the degree of codeine involvement that was considered to best accommodate to the existing literature. The decision, however, might be debatable both in light of our assertion that the assessment of toxic codeine concentrations without simultaneous evaluation of morphine and M6G levels may represent a limitation in previously published studies, and the mentioned risk of circular reasoning when basing results on established reference values. Accordingly, it cannot be excluded that relevant cases might have been excluded from the series through this strategy. On the other hand, minimal reported fatal values should be employed with caution, as such "low extremes" might be a result of hypersensitivity or idiosyncratic reactions, not representative of regular poisoning deaths [35]. A thorough review of

the clinical history and circumstances of death may to some extent shed light on this, but available information is often deficient and/or ambiguous. Still, paper II may provide a more detailed and comprehensive interpretation of post-mortem codeine findings than some previously published results.

In paper II we cited two large autopsy series reporting codeine concentrations in post-mortem blood samples obtained from cases of lethal codeine overdoses [35, 116], and stated that only one of these sources reports morphine concentrations [116], and that neither report concentrations of morphine metabolites or CYP2D6 genotype. Several previously published case reports [31, 108, 117-125] were not cited. In particular, the case series by Nakamura et al. [118] and Wright et al. [117], which presented both codeine and morphine concentrations in post-mortem blood, may have been mentioned in this context. The findings in these studies, however, do not affect the main conclusions in our paper. The large autopsy series by Häkkinen et al. [126] and Lam et al. [128] were published after paper II.

As a relatively small number of cases of codeine-related fatalities from a selected material of forensic autopsy cases is presented, the results are susceptible to confounding by selection bias, potentially hampering their general validity. However, this is a problem inherent to all such investigations.

One of the most conspicuous findings in this study of post-mortem blood findings is that morphine levels could not be reliably predicted from codeine concentrations, even when CYP2D6 genotype was known. This likely reflects that post-mortem blood concentrations of codeine and morphine (as well as other codeine metabolites) are dependent on other factors than the metabolizing capacity, i.e. the time from codeine ingestion to death, the post-mortem interval, etc. This observation may also be due to inherent properties of the post-mortem material possibly effacing the impact of genotype. Indeed, it has been shown that CYP2D6 genotyping alone is no guarantee for certain prediction of morphine formation from codeine even under controlled conditions [104]. These considerations were further actualized in the extended study presented in paper IV.

5.3 Paper III – analytical aspects

Several methods for quantitative analysis of codeine and various codeine metabolites in post-mortem specimens are previously published [129, 167-174]. These methods, however, either apply to analysis of few analytes in multiple biological matrices, or analysis of multiple analytes in selected matrices. To our knowledge, paper III presents the first validated method for simultaneous determination of

codeine, C6G, norcodeine, morphine, M3G and M6G in blood, vitreous fluid, muscle, fat and brain tissue, thus tailored to pursue the objective of paper IV; comprehensive determination and assessment of the concentrations and tissue distribution of codeine and metabolites in codeine-related deaths.

It is emphasized that because ~0.4 g muscle, fat, and brain tissue was used to prepare 1 mL tissue homogenate in order to achieve optimal analytical performance, the method's actual LLOQ in the tissue analyses were 4 ng/mL for codeine, norcodeine and morphine, and 58 ng/mL for C6G, M3G and M6G, with exceptions for norcodeine in muscle (8 ng/mL), morphine in muscle, fat and brain (8 ng/mL) and M6G in fat (115 ng/mL). Based on measured concentrations and frequent absence of detectable amounts of the presumed active moieties morphine and M6G in brain, it was stated in paper IV that further investigations of opioid levels within the CNS in opioid intoxication victims with more sensitive methods are warranted. With LLOQs in brain of 4-8 ng/mL for codeine, norcodeine and morphine, and 58 ng/mL for C6G, M3G and M6G concentrations below the LLOQ may be considered relatively low, at least for codeine, norcodeine and morphine. However, considering that the potency of M6G may be significantly higher than that of morphine, and the event that very low concentrations of the active moieties within the CNS are sufficient to cause toxic opioid effects, the sensitivity of this method may not be commensurate with application in brain tissue, particularly with regard to M6G. This is also discussed in paper IV.

The upper limits of quantification (ULOQ) were adequate for determination of norcodeine, morphine and morphine glucuronides in non-diluted samples of all matrices in paper IV. For C6G the ULOQ of 4.6 mg/L was adequate in all matrices except peripheral blood, where a considerable number of samples required dilution to obtain concentrations within the validated range. For codeine, the ULOQ of 0.30 mg/L necessitated quantification in diluted samples in a large proportion of cases, especially in peripheral blood. Considering that the inclusion criterion for cases in paper IV was a peripheral blood concentration above the TIAFT toxicity threshold of 0.30 mg/L, this was to be expected. The frequent need for dilution in the quantifications of codeine by this method is not optimal. Controlled experiments investigating dilution effects, however, yielded precision and accuracy within ±20%, and mostly within ±15%. Inclusion of higher standards yielded inadequate calibration curves, and was abolished in favor of lower LLOQs.

Stability experiments conducted in whole blood as part of the validation of this method showed no significant concentration changes for any of the analytes. This was in concordance with recent studies [174, 175], and provided useful information for the assessment of peripheral blood concentration

changes from the analyses performed in connection with the forensic autopsy and reanalysis in paper IV.

5.4 Paper IV – extended assessment of codeine and metabolites

In paper IV, the perspectives of paper II were extended by adding the metabolites C6G and norcodeine to the analytical repertoire, and assessing levels and distribution of the investigated analytes in multiple matrices. Again, CYP2D6 genotype was taken into account. The case series of codeine-related deaths assessed in paper IV was different from the series described in paper II, primarily due to limited availability of post-mortem materials for further analyses. When eligible, however, cases were included in both studies.

The intention of this study was to shed light on some of the pending issues regarding codeine toxicity through the assessment of metabolism, distribution and redistribution of codeine and its metabolites in codeine-related deaths. In particular, concentrations in brain tissue were anticipated, as they presumably would provide the best representation of drug levels at the site of action. However, large variability and lack of appreciable patterns in the data confined the basis for conclusive interpretations and detailed discussion in relation to the questions raised. That being said, the generally low brain concentrations of all metabolites and frequent absence of detectable amounts of the presumably active moieties morphine and M6G in brain remain a finding of possible importance and validity in this paper. Srinivasan made some interesting observations with regard to this in his studies of rats administered codeine, C6G and morphine, respectively [176]. Firstly, after intracerebroventricular administration of C6G, which was reported to produce analgesic responses in the rats, M6G was detected in brain in concentrations ranging 43-78 ng/g. This shows that M6G may be formed from C6G within the CNS in rats. Considering that M6G may have greater analgesic potency than morphine, particularly after intrathecal administration, this finding may indicate that relatively low concentrations of M6G in brain are sufficient to induce significant opioid effects, as suggested in paper IV. It also makes it difficult to assess the individual contribution of C6G to the opioid effects with any kind of certainty. Secondly, after intracerebroventricular administration of codeine, also reported to produce analgesic responses in the rats, codeine and morphine (but no glucuronides) were detected in considerable amounts. Correspondingly, this illustrates that morphine may be formed from codeine within the CNS, making it difficult to assess the individual opioid effects of codeine. Interestingly, none of these observations upon intracerebroventricular administration were reflected in plasma concentrations.

It is recognized that the material in paper IV contained no unambiguous monointoxications with codeine, but mostly cases with significant additional toxicological findings, obscuring the toxicological significance of codeine intake and metabolism to active metabolites in these deaths. Ideally, concentrations should be assessed in a material comprising well-defined cases of codeine monointoxications or mixed intoxications where codeine clearly constitutes the main intoxicant. However, other investigators have also reported that a majority of codeine-related deaths are the result of multiple drug toxicity [128, 149], and this may be a general feature of these deaths. A more sensitive analytical method and quantitative analyses within different parts of the CNS may also have been favourable. Specifically, a more sensitive method and clearly defined cases may have enabled better assessment of the impact of the post-mortem interval and CYP2D6 genotype. This may be required in order to clarify the unsettled issues regarding the distribution and metabolic fate of opiates and their glucuronides on both sides of the blood-brain barrier in humans, e.g. the source of M6G detected in brain tissue. This may also facilitate interpretations of other biological variations within the material, such as interindividual differences in the formation of M3G and M6G, differences between acute and chronic users, users with or without renal impairment, etc. Nevertheless, the inherent and largely intractable factors encumbered with post-mortem materials remain. Indeed, an extensive review of post-mortem clinical pharmacology has stated that deductions from morphine metabolite ratios in post-mortem samples cannot be made with certainty [36]. In this context, the results from a pharmacogenomic model-based analysis of the influence of CYP2D6, CYP3A4 and glucuronidation activities and renal impairment on the effects of codeine [177] provide some interesting observations. Even this model-based analysis predicted a wide range of morphine levels after codeine administration. The analysis also reproduced a dominant role of CYP2D6 activity for morphine exposure, demonstrated only minor influence of mild and moderate renal impairment and co-administration of CYP3A4 inhibitors, and predicted that increased glucuronidation activity is associated with a decrease in presumed active moieties. While these observations obviously depend on the validity of the presumptions and specifications of the applied model, this study captures the large and complex variability in codeine pharmacokinetics and -dynamics. Considering that CYP2D6 genotyping alone does not provide certain prediction of morphine formation from codeine even under controlled conditions [104], matters become even more obscure.

The lack of appreciable patterns in the data may also in part be due to the small sample size. In particular, there were few cases in the IM and PM phenotype groups. However, from theoretical considerations one might assume that all PM would present with invariably low concentrations of CYP2D6-dependent metabolites. Indeed, previous investigators have pointed out a need for larger sample sizes to correlate EM and IM phenotypes with codeine effects, whilst reporting codeine effects consistent with CYP2D6 genotype in the genotypes at either extremity (PM and UM) [83]. Even with

more clearly defined cases, a larger sample size would be advantageous. Still, a larger sample size does not necessarily facilitate a clear association between CYP2D6 genotype and M/C-ratio in post-mortem measurements [128].

Due to the small sample sizes, no statistical tests of the observed differences in M/C-ratio, C6G/C-ratio and NC/C-ratio between the assigned phenotypes were included in paper IV, as this was not considered to provide reliable information. Correlations between concentration changes and time between original analysis and reanalysis, and correlations between different tissue concentration ratios and the post-mortem interval were investigated, without revealing any clear correlations or systematic patterns. This was merely stated in paper IV. In light of the quality and size of the material emphasis was placed on presenting the data in a descriptive manner. In this regard, detailed display of the correlations was considered excessive, and was thus not included in the paper.

The omission of normorphine in this study might be debatable, as receptor affinity studies have indicated that normorphine has higher μ opioid receptor affinity than the included analytes C6G and norcodeine [72]. Both metabolic pathways from codeine to normorphine are CYP2D6-dependent, and the formation of normorphine has been shown to be significantly lower in PM compared to EM [80, 115]. Accordingly, all O-demethylated active metabolites (morphine, M6G and normorphine) may be formed in significantly higher concentrations in UM compared to EM. Regardless, it seems likely that the formation of normorphine in any event is too minor relative to the μ opioid receptor affinities and simultaneous concentrations of morphine and M6G for normorphine to have an important role in the therapeutic and toxic effects of codeine. This has also been argued by others [178], although Yue et al. [115] suggested that normorphine might play a significant part in codeine analgesia.

6. Conclusions

This material from Central Norway shows that autopsy rates can vary with manner of death, sex and age, and may be susceptible to the influence of regional variations in law enforcement, with possible consequences for the quality and validity of cause of death statistics. The general toxicology results suggest that alcohol and drugs are important factors in sudden unexpected deaths, and that a thorough and comprehensive toxicological analysis is called for when investigating these deaths.

The investigations of post-mortem blood concentrations of codeine, morphine, M3G and M6G in codeine-related deaths show a large variability in the measured amounts of morphine after codeine intake, and demonstrate that morphine levels cannot be reliably predicted from codeine concentrations in forensic autopsy cases, even when CYP2D6 genotype is known. Still, it seems prudent that investigations of codeine-related deaths should include quantification of morphine and morphine metabolites in order to achieve a comprehensive interpretation of post-mortem codeine findings, and CYP2D6 genotyping may be of interest in cases with unexpectedly high or low M/C-ratios.

A simple and reliable solid phase extraction LC-MS method for simultaneous determination of codeine, codeine metabolites C6G, norcodeine and morphine, and morphine metabolites M3G and M6G in post-mortem whole blood, vitreous fluid, muscle, fat and brain tissue was developed and validated. The method was applicable in forensic autopsy cases implicating codeine in both therapeutic and presumably lethal concentration levels.

The investigations of levels and tissue distribution of codeine, C6G, norcodeine, morphine, M3G and M6G in post-mortem blood, vitreous fluid, muscle, fat and brain tissue in codeine-related deaths indicate generally lower concentrations of the investigated analytes in matrices other than blood post-mortem. Especially, this seems to be the case in brain and fat, and in particular for the glucuronides (C6G, M3G and M6G) and, to some extent, morphine. The observation that the presumed active moieties morphine and M6G both are below the LLOQ in brain in a majority of the studied cases is of particular interest, and warrants further investigations of opioid levels within the CNS in victims of opioid toxicity with more sensitive methods. Measured brain concentrations in this material seem to provide little support for the conception of C6G and/or norcodeine as mediators of codeine toxicity. No systematic patterns indicating post-mortem formation, degradation or redistribution of any of the investigated analytes could be established.

The generally large variability and lack of appreciable patterns in the analytical results from this case series may be due to inherent properties of the post-mortem material, possibly effacing the impact of other parameters, such as the post-mortem interval and CYP2D6 genotype. It may also be due to variable toxicological significance of codeine intake and metabolism to active metabolites.

7. Future perspectives

The systematic investigations of autopsy rates carried out in paper I should be repeated in other parts of Norway and other countries to reproduce and corroborate these findings. In the wake of such studies the need for national and international systematization of autopsy practices may be evaluated.

The generally large variability and lack of appreciable patterns in the levels and distribution of codeine and metabolites in papers II and IV, presumably due to inherent properties of the post-mortem material and variable toxicological significance of codeine intake within the material, call for further investigations. This should preferably be performed in well-characterized materials where codeine is the main or only ingested intoxicant. The relative scarcity of such cases in the current material suggests that such an investigation may only be feasible as a collaborative effort including multiple centers. Furthermore, such investigations will likely benefit from more advanced and sensitive analytical methods. In this context, the prospect of mass spectrometry imaging as a tool for spatial localization and quantification of drugs and their metabolites in whole tissue sections are intriguing [179-182], and should be pursued. When possible, future investigations should include quantitative analyses in different parts of the investigated tissues, in particular within the CNS. The inclusion of normorphine to the investigated analytes may also be considered.

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Forensic autopsies in a naturalistic setting in Norway: Autopsy rates and toxicological findings

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ABSTRACT

Autopsies can give valuable information about the cause of death, and represent an important tool for obtaining valid cause of death statistics. In particular, they may shed light on the circumstances of death in ambiguous and criminal cases. To address the need for information on current autopsy practices, forensic autopsy rates in two counties in Central Norway over the period 2007–2009 were assessed. To investigate toxicological findings that could possibly remain undisclosed without the performance of an autopsy, the impact of alcohol and drugs in forensic autopsy cases from this material was evaluated.

The total forensic autopsy rate in this material was 3%. The forensic autopsy rates were low for natural deaths (1%), accidental falls (12%) and the heterogeneous category "other accidents" (21%), relatively high for accidental poisonings (84%), and less than adequate for road traffic accidents (57%). For suicides the forensic autopsy rate was 63%, and for recognized homicides 100%. The total forensic autopsy rate was higher for men than for women (5% vs. 2%), and decreased with age, being 38% in the age group <30 years, 23% in the age group 30–59 years, and 1% in the age group >59 years. Despite that Norwegian legislation and regulations regarding forensic autopsy requests are national, the forensic autopsy rates were generally lower in the county of Nord-Trøndelag than in Sør-Trøndelag, with most striking differences in suicide deaths (11% vs. 91%) and road traffic accidents (46% vs. 67%). This illustrates how autopsy rates, and possibly cause of death registries, might be susceptible to the influence of regional variations in law enforcement, with possible consequences for the quality and validity of cause of death statistics.

Of the forensic autopsy cases where toxicological analysis was performed (361 of 364 cases) a total of 71% had positive toxicology results; 12% were positive for alcohol only, 44% were positive for drugs only, and 15% were positive for both alcohol and drugs. The toxicology results suggest that alcohol and drugs are important factors in sudden unexpected deaths, and that a thorough and comprehensive toxicological analysis is called for when investigating these deaths. Mean BAC in alcohol positive forensic autopsy cases was 1.7‰ (median 1.6‰, range 0.29-4.1‰). The average number of substances detected in toxicology positive cases was 2.6 (median 2, range 1–10). The by far most frequently detected classes of substances were (1) benzodiazepines, (2) opioids and (3) alcohol.

1. Introduction

Autopsies can provide documentation of external or internal injuries, diseases and the presence of alcohols, drugs and other substances in body fluids and tissues. The autopsy can thus give valuable information about the cause of death to relevant health care professionals and the next of kin. Moreover, the autopsy is an important tool in the acquisition of reliable mortality data, which in turn is essential for valid cause of death statistics. National registries of causes of death are generated using the World Health Organization (WHO) International Classification of Diseases (ICD-10) codes [1] based on information given in death certificates issued by physicians, and reports from clinical and forensic autopsies. The value of autopsies as an important corrective for the determination of causes of death has been demonstrated in several studies [2–7].

Forensic autopsies can, according to law and provision in Norway, be requested by the police under given circumstances, e.g.

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suspected accidents, drug-related deaths, suicides and sudden unexpected deaths [8]. In cases of suspected homicide or unidentified corpse the police is obliged to request a forensic autopsy [8]. Forensic autopsies may shed light on the circumstances of death in ambiguous and potentially criminal cases, providing essential information to police authorities and courts of law as well as medical examiners, and thus contributing to quality assurance in both the legal system and the health services. Furthermore, clear assessment of alcohol- and drug-related deaths requires quantitative post-mortem chemical and toxicological analysis [9], which in our region rarely is performed outside the forensic autopsy setting.

Autopsy rates are affected by many factors, such as manner of death, sex, age, region and legislation [10,11]. Low autopsy rates may reduce the quality and validity of cause of death statistics, and thus compromise proper knowledge about the status and trends in causes of death. In individual cases absence of autopsy data may increase the possibility of incorrect conclusions as to the cause and manner of death and, in the worst instance, failure to detect homicide [12,13]. Autopsy rates, particularly for clinical autopsies, have declined continuously over the last decades in many countries [14–19], to the concern of pathologists worldwide [18,20–23]. There has also been raised concern about shifts in autopsy patterns resulting in an increasing percentage of autopsies in external cause deaths and younger persons, possibly affecting the cause of death determination for important disease conditions in the higher age groups [19]. Finland has exceedingly high forensic autopsy rates compared to other countries after the implementation of a national authority for medico-legal affairs [24]. In Norway, no similar initiatives have been implemented. In our country the combined clinical and forensic autopsy rate has declined from about 9% to 7% from 2002 to 2010 [25,26]. The observed decline is explained by a decrease in the clinical autopsy rate from about 5% to 3%, as the forensic autopsy rate has remained relatively constant at just below 4% over this period [25,26]. Our knowledge of how the frequency of forensic autopsies relates to manner of death and demographical variables over time is limited.

To address the need for information on current autopsy practices, this article presents an investigation of forensic autopsy rates in two counties in Central Norway over the period 2007–2009. To survey toxicological findings possibly undisclosed without the performance of an autopsy, the impact of alcohol and drugs in forensic autopsy cases from this material is evaluated.

2. Materials and methods

All forensic autopsy cases ($n = 364$) from the two counties Sør- and Nord-Trøndelag in Central Norway (total population approximately 425,000) during the period 2007–2009 were reviewed and assessed with regard to manner of death, demographical data (sex, age, county) and toxicological findings. The data was registered in a database at The Regional Biobank of Central Norway, and managed using the same database and statistical software (Microsoft Excel 2007 and SPSS 16.0). Data on all deaths in the two counties over the same period was collected from the Norwegian Cause of Death Registry ($n = 10,862$). From these two data sets forensic autopsy rates by manner of death, sex and age were determined.

Manner of death is defined as the fashion or circumstances that result in death, and mainly corresponds to the underlying cause of death. The underlying cause of death is defined by the WHO as "the disease or injury which initiated the train of morbid events leading directly to death, or the circumstances of the accident or violence which produced the fatal injury" in accordance with the rules of the ICD-10 [27]. Manner of death is commonly categorized as natural, accidental, suicidal, homicidal or undetermined. This categorization is also applied throughout this article, but since none of the forensic autopsy cases in this material were assigned an undetermined manner of death this category has been omitted.

Natural death comprises those dying of diseases, sudden infant death syndrome (SIDS) and cases of preserved bodies with unknown cause of death. Accidents are subcategorized into accidental poisonings (typically unintentional overdoses/intoxications and therapeutic misadventures), road traffic accidents (drivers and passengers of motor vehicles, pedestrians and bicyclists killed in accidents on public roads involving a motor vehicle), accidental falls (including both occupational fall accidents and fragile, elderly individuals dying in the lapse of

Table 1
Number of deaths and forensic autopsy cases in Central Norway 2007–2009, by manner of death, sex and age.

	Sør-Trøndelag			Nord-Trøndelag			Total		
	Deaths	Forensic autopsy cases	Autopsy rate	Deaths	Forensic autopsy cases	Autopsy rate	Deaths	Forensic autopsy cases	Autopsy rate
	n	*n*	%	*n*	*n*	%	*n*	*n*	%
Manner of death									
Natural death	6844	107	2	3419	20	1	10,263	127	1
Accidents	354	124	35	128	36	28	482	160	33
Accidental poisoning	60	50	83	17	15	88	77	65	84
Road traffic accident	30	20	67	24	11	46	54	31	57
Accidental fall	77	8	10	24	4	17	101	12	12
Other accidents	187	46	25	63	6	10	250	52	21
Suicide	70	64	91	38	4	11	108	68	63
Homicide	7	7	100	2	2	100	9	9	100
Sex									
Male	3447	227	7	1733	44	3	5180	271	5
Natural death	3200	81	3	1633	16	1	4833	97	2
Unnatural death	247	146	59	100	28	28	347	174	50
Female	3828	75	2	1854	18	1	5682	93	2
Natural death	3644	26	1	1786	4	0.2	5430	30	1
Unnatural death	184	49	27	68	14	21	252	63	25
Age									
<30 years	160	62	39	45	15	33	205	77	38
Natural death	105	13	12	22	6	27	127	19	15
Unnatural death	55	49	89	23	9	39	78	58	74
30–59 years	586	151	26	244	36	15	830	187	23
Natural death	470	54	11	195	7	4	665	61	9
Unnatural death	116	97	84	49	29	59	165	126	76
>59 years	6529	89	1	3298	11	0.3	9827	100	1
Natural death	6269	40	1	3202	7	0.2	9471	47	0.5
Unnatural death	260	49	19	96	4	4	356	53	15
Total	7275	302	4	3587	62	2	10,862	364	3

low-energy falls, e.g. causing hip fractures) and other accidents (i.e. fatal fire and high-voltage accidents, boat accidents, accidental drowning or suffocation, fatal accidental hypothermia, fatal accidental gunshot wounds, fatal occupational accidents other than fall, etc.). Fire accidents comprise both death from burn injuries and carbon monoxide poisoning caused by fire. Accordingly, accidental fire-related carbon monoxide poisonings are not classified as accidental poisonings. Cases of accidental non-fire related carbon monoxide poisonings, however, are classified as accidental poisonings. Suicides are categorized into suicidal poisonings and other deaths by suicide. In the case of suicide, both fire and non-fire related carbon monoxide poisonings are classified as suicidal poisonings.

Toxicological analysis of the forensic autopsy specimens was performed routinely by the Department of Clinical Pharmacology at St. Olavs Hospital – Trondheim University Hospital in Trondheim, Norway. When available, post-mortem blood specimens from the autopsies were subjected to specific analyses for alcohols (ethanol, methanol, isopropanol, acetone) using a headspace gas chromatography–mass spectrometry (GC–MS) method, and specific analyses for benzodiazepines (diazepam, desmethyldiazepam, oxazepam, nitrazepam, flunitrazepam, desmethylflunitrazepam, clonazepam, alprazolam, midazolam), opiates (morphine, codeine, ethylmorphine, oxycodone, morphine-3-glucuronide, morphine-6-glucuronide) and amphetamines (amphetamine, methamphetamine, 3,4-methylenedioxy-N-methylamphetamine (MDMA) and 3,4-methylenedioxyamphetamine (MDA)) using liquid chromatography–mass spectrometry (LC–MS) methods. Blood specimens were also screened against comprehensive drug libraries (National Institute of Standards and Technology Mass Spectral Library, Forensic Toxicology Retention Time Locking Database/Library and Pfleger/Maurer/Weber Drugs and Pesticides Library for Toxicology) with a GC–MS method. If available, urine was screened for drugs of abuse as well, using LC–MS methods. Positive screening results and explicit information about drug use in the case histories were confirmed by specific analyses in blood using LC–MS or GC–MS methods. In cases where blood was not available, e.g. due to extensive bleeding or putrefaction, toxicological screening and specific analyses were performed with homogenates of suitable tissue samples, preferably spleen, liver and/or muscle tissues, using the same methodology. In three cases toxicological analysis was not performed due to particular circumstances ($n = 1$) or lack of sample material ($n = 2$). These cases are excluded from the presentation of the toxicology results.

The toxicology results are categorized into alcohol (A+/−) and drug (D+/−) findings. Positive alcohol findings correspond to the detection of ethanol in a concentration ≥0.2‰, whereas positive drug findings comprise detection of all other exogenous drugs and poisons in concentrations above the limit of quantification for the respective specific analyses, including other alcohols such as methanol and ethylene glycol. However, the detection of abnormal levels of carbon monoxide in post-mortem specimens is not included.

Blood alcohol concentrations (BAC) are reported in per mill (‰). One per mill (1.0‰) equals 100 mg ethanol per 100 ml blood or 21.7 mmol/L. In Norway, it is prohibited by law to operate a motor vehicle with a BAC exceeding 0.2‰.

This study is part of an ongoing research project started in late 2006, which involves the establishment of a regional research biobank coupling post-mortem toxicological specimens and clinical data from forensic autopsy cases in Central Norway. The project has been approved by the Regional Committee for Medical and Health Research Ethics (approval 007-06) and the Director General of Public Prosecution.

3. Results

Forensic autopsy rates by manner of death, sex, age and county are presented in Table 1.

Alcohol and drug findings (+/−) in post-mortem blood from the forensic autopsy cases are summarized by manner of death, sex and age in Table 2. In three of the cases where blood was not available positive toxicological findings were made in alternative biological matrices other than urine. In one of these cases a positive alcohol finding in vitreous humor was the only toxicological finding, and death was attributed to natural causes. In the other two cases drugs (but not alcohol) were detected in spleen tissue homogenate and pleural fluid, and death was attributed to accidental poisoning and suicidal poisoning, respectively. It should be emphasized that reference data for toxic and fatal concentrations of drugs primarily are elaborated for blood, and that corresponding data for various tissue specimens generally are scarce.

The distribution of post-mortem BAC in the forensic autopsy cases subjected to toxicological analysis is presented by manner of death in Table 3. Mean BAC in alcohol positive cases was 1.7‰ (median 1.6‰, range 0.29–4.1‰). In one case the reported alcohol concentration was based on analysis of vitreous humor (marked footnote in Table 3). There were no other cases with positive alcohol findings consistent with ante-mortem intake.

Table 2
Alcohol (A+/−) and drug (D+/−) findings in post-mortem blood or tissue specimens in forensic autopsy cases subjected to toxicological analysis in Central Norway 2007–2009 ($n = 361$), by manner of death, sex and age.

Manner of death	Sex		Age			Total
	Male	Female	<30 yrs	30–59 yrs	>59 yrs	
	n	*n*	*n*	*n*	*n*	*n*
Natural death						
A−/D−	34	10	9	21	14	44
A+/D−	11	2	0	6	7	13
A−/D+	43	18	9	29	23	61
A+/D+	7	0	0	5	2	7
Total	95	30	18	61	46	125
Accidental poisoning						
A−/D−	1[a]	0	1[a]	0	0	1[a]
A+/D−	3	1	0	3	1	4
A−/D+	34	9	9	31	3	43
A+/D+	13	4	1	14	2	17
Total	51	14	11	48	6	65
Road traffic accident						
A−/D−	11	7	5	7	6	18
A+/D−	4	0	1	2	1	4
A−/D+	3	3	2	1	3	6
A+/D+	1	1	2	0	0	2
Total	19	11	10	10	10	30
Accidental fall						
A−/D-	2	1	0	1	2	3
A+/D−	2	1	0	2	1	3
A−/D+	4	1	0	3	2	5
A+/D+	1	0	0	1	0	1
Total	9	3	0	7	5	12
Other accidents						
A−/D−	14	2	2	7	7	16
A+/D−	6	2	4	2	2	8
A−/D+	7	7	6	1	7	14
A+/D+	10	4	1	12	1	14
Total	37	15	13	22	17	52
Suicidal poisoning						
A−/D−	1[b]	0	0	1[b]	0	1[b]
A+/D−	0	1	0	1	0	1
A−/D+	2	2	1	3	0	4
A+/D+	0	3	1	1	1	3
Total	3	6	2	6	1	9
Other suicides						
A−/D−	17	2	6	10	3	19
A+/D−	7	2	7	2	0	9
A−/D+	19	4	2	13	8	23
A+/D+	6	2	3	5	0	8
Total	49	10	18	30	11	59
Homicide						
A−/D−	2	2	2	1	1	4
A+/D−	1	0	0	0	1	1
A−/D+	1	2	1	2	0	3
A+/D+	1	0	0	0	1	1
Total	5	4	3	3	3	9
Total						
A−/D−	82	24	25	48	33	106
A+/D−	34	9	12	18	13	43
A−/D+	113	46	30	83	46	159
A+/D+	39	14	8	38	7	53
Total	268	93	75	187	99	361

[a] Accidental carbon monoxide poisoning not classified as fire accident (i.e. other accidents).
[b] Suspected suicide by insulin overdose.

Table 3
Forensic autopsy cases in Central Norway 2007–2009 with measured post-mortem blood alcohol concentrations (BAC) ≥0.2‰ (n = 96), by manner of death.

Manner of death	BAC ≥ 0.2‰		BAC (‰)				
	n	%	0.20–0.49	0.50–0.99	1.0–1.9	2.0–2.9	≥3.0
			n	n	n	n	n
Natural death	20	16	7	4	4	5[a]	0
Accidental poisoning	21	32	0	1	7	3	10
Road traffic accident	6	20	0	1	2	2	1
Accidental fall	4	33	1	0	0	3	0
Other accidents	22	42	2	2	8	10	0
Suicidal poisoning	4	44	0	2	2	0	0
Other suicides	17	29	1	5	7	4	0
Homicide	2	22	0	0	1	1	0
Total	96	27	11	15	31	28	11

[a] In one of these cases the alcohol concentration in vitreous humor is reported.

Number of substances (including alcohol) detected in post-mortem blood in the toxicology positive forensic autopsy cases are presented by manner of death in Table 4. The average number of substances detected in positive cases was 2.6 (median 2, range 1–10). The detection frequency of alcohol and various drugs and poisons in post-mortem blood are shown in Table 5. The by far most frequently detected classes of substances were (1) benzodiazepines, (2) opioids and (3) alcohol. The three cases where positive toxicological findings were made in alternative biological matrices are indicated by marked footnotes in Tables 4 and 5. In these cases one (alcohol), one (methadone) and two (valproate, lithium) substances were detected, respectively.

4. Discussion

The forensic autopsy rates in this material are low for natural deaths, accidental falls and the heterogeneous category "other accidents" (Table 1). The autopsy rates are relatively high for accidental poisonings, and less than adequate for road traffic accidents, particularly in the county of Nord-Trøndelag. All recognized homicides were subject to forensic autopsy in both counties. In Sør-Trøndelag forensic autopsy was performed in nearly all suicide deaths, whereas the autopsy rate for suicides is very low in Nord-Trøndelag.

Generally, the forensic autopsy rates are lower in Nord-Trøndelag than in Sør-Trøndelag. The most striking differences between the two counties are seen in suicide deaths and, to a certain extent, in road traffic accidents. For suicide deaths there is a remarkable 8-fold difference in the forensic autopsy rates (11% vs. 91%). The characteristics of the unautopsied suicides in Nord-Trøndelag are unknown, but even if the local police authorities may have regarded these deaths to be resolved in the absence of an autopsy, this practice is contradicted by the fact that autopsy rates have been shown to affect the validity of suicide mortality statistics [28]. Considering that Norwegian legislation and regulations regarding forensic autopsy requests are national, the observed discrepancies may represent differences in the interpretation and practice of law, possibly attributable to economic or other non-medical incentives. This illustrates how autopsy rates, and possibly cause of death registries, might be susceptible to the influence of regional variations in law enforcement. In this material, however, the circumstances of death are not known in cases where a forensic autopsy was not performed. In particular, it is not known whether or not the police was notified about the death and thus had the opportunity to consider the requirement of a forensic autopsy. Neither is it known whether any of the missing cases were subject to a non-forensic autopsy, although it is unlikely that this explains much of the discrepancies.

The reason for the strikingly low forensic autopsy rates for accidental falls and other accidents is not clear. Unfortunately, the

Table 4
Number of substances (including alcohol) detected in post-mortem blood in toxicology positive forensic autopsy cases in Central Norway 2007–2009 (n = 255), by manner of death.

Manner of death	Positive toxicology		Number of substances			
	n	%	1	2–3	4–6	>6
			n	n	n	n
Natural death	81	65	42[a]	26	11	2
Accidental poisoning	64	98	10[b]	21	24	9
Road traffic accident	12	40	9	2	1	0
Accidental fall	9	75	4	5	0	0
Other accidents	36	69	17	14	4	1
Suicidal poisoning	8	89	2	2[c]	4	0
Other suicides	40	68	16	15	8	1
Homicide	5	56	3	1	1	0
Total	255	71	103	86	53	13

[a] In one of these cases toxicological results were based on analysis of vitreous humor.
[b] In one of these cases toxicological results were based on analysis of spleen tissue.
[c] In one of these cases toxicological results were based on analysis of pleural fluid and spleen tissue.

Table 5
Detection frequency of alcohol and various drugs and poisons in post-mortem blood in forensic autopsy cases in Central Norway 2007–2009.

Substance	Detection frequency
	n
Alcohol	96[a]
Benzodiazepines	140
Opioids	122[b]
Z-hypnotics	25
THC	16
Carisoprodol/meprobamate	6
Amphetamine/metamphetamine	17
Cocaine/benzoylecgonine	2
Paracetamol	76
Antidepressants	53
Antipsychotics	29
Antiepileptics	19[c]
Sedating antihistamines	8
Lithium	4[c]
Barbiturates	1
Methanol	3
Ethylene glycol	1

[a] In one of these cases toxicological results were based on analysis of vitreous humor.
[b] In one of these cases toxicological results were based on analysis of spleen tissue.
[c] In one of these cases toxicological results were based on analysis of pleural fluid and spleen tissue.

data on other accidents from the Norwegian Cause of Death Registry were not stratified with regard to type of accident, making forensic autopsy rates for the different subtypes of accidents within this category unavailable. After the introduction of Automated Classification of Medical Entities (ACME) in 2005, deaths from specified accidental falls are classified as accidental falls in the Norwegian Cause of Death Registry, whereas falls not otherwise specified are classified as other accidents. Both categories might presumably include a considerable number of elderly and fragile individuals dying in the lapse of low-energy falls, which may be part of the reason why the autopsy rates for accidental falls and other accidents are low. Considering that these categories essentially comprise violent deaths, and that the forensic autopsy rates for road traffic accidents are suboptimal, autopsy rates for violent deaths are generally low in this material. In our opinion, this raises concern about the possible loss of precise knowledge of death and injury mechanisms in these violent accidents, which is important for preventive endeavors.

The observed decline in total autopsy rate with age is due to more individuals dying of natural causes with age (Table 1), particularly in the age group >59 years, which makes it less likely that a forensic autopsy is requested. The higher total autopsy rate for men compared to women, on the other hand, cannot be ascribed entirely to more women dying of natural causes and a higher incidence of unnatural deaths among men, as a considerably larger fraction of men than women was subjected to forensic autopsy both among those dying of natural causes and those succumbing to unnatural causes (Tables 1 and 2). This systematic gender-related difference might, however, be a function of the circumstances of death. Moreover, in this material men constitute the majority of deaths by accidental poisoning and suicide (data not shown); manners of deaths with a substantial impact on the total autopsy rates.

Whenever an autopsy is performed in Norway, a summary of the autopsy report containing the cause of death formula, additional findings and a conclusion are routinely sent to the Norwegian Cause of Death Registry, which registers the underlying cause of death and a maximum of five other causes of death in each case. In general, the cause of death statement in the autopsy report overrides the formal death certificate in case of inconsistencies. Accordingly, it should be kept in mind that the data set from the Norwegian Cause of Death Registry is based on different diagnostic foundations in cases where an autopsy is performed and cases where it is not, and generally is susceptible to discontinuities and errors in coding practices, e.g. underreporting of suicides.

To our knowledge, no previously published studies from outside Scandinavia have investigated forensic autopsy rates in the same systematic manner. However, a recent study from Japan comparing forensic autopsy practises in areas with and without a medical examiner system [29], reported differences in forensic autopsy rates for natural deaths (18% and 4%), accidental deaths (36% and 8%) and accidental poisonings (91% and 60%). This study showed both lower autopsy rates and a higher frequency of death certificate errors in the area without a medical examiner system. A survey of autopsy rates covering all German pathological and medicolegal institutes [30] reported a total forensic autopsy rate of 2%, with up to 5-fold differences between the various states of the country. Analogously, a study from Austria [31] demonstrated a significant decrease in autopsy rates with increasing distance between the location of death and the site of post-mortem examination. Due to important national differences not only in the practical and legislative aspects of autopsy requests, but also in demographics, disease panorama, etc., direct comparisons of such studies may not be advisable. Nevertheless, the challenge of sustaining adequate and homogenous autopsy rates seems to be ubiquitous.

For all manners of death combined more forensic autopsy cases sort to the drug positive and alcohol negative category than any other category of toxicological findings (Table 2). Interestingly, this is also the case when looking solely at natural deaths. This illustrates how this crude categorization of toxicological findings have limitations in reflecting the true impact of drugs and alcohol, as it does not distinguish between findings that play a central role in the death mechanism and findings that are insignificant or irrelevant with regard to the cause of death. Nevertheless, the observation that a minimum of 70% of all forensic autopsy cases in this material have positive toxicology results suggests that alcohol and drugs are important factors in deaths subjected to forensic autopsy, particularly sudden unexpected deaths, and that a thorough and comprehensive toxicological analysis is called for when investigating these deaths. Another interesting observation is that the lowest fraction of toxicology positive cases is seen in road traffic accidents (Tables 2 and 4). In this category 6 of 30 cases were positive for alcohol, with or without accompanying drug findings. It is emphasized that this category comprises not only motor vehicle drivers, but also passengers, cyclists and pedestrians killed in road traffic accidents.

Table 3 reveals that alcohol is frequently involved in forensic autopsy cases in this material. Not surprisingly, BAC $\geq$ 0.2‰ was detected more often in unnatural deaths (32%) than in natural deaths (16%), with suicidal poisonings (44%) and other accidents (42%) being most conspicuous. Although these findings are based on a limited material, and thus should be interpreted with caution, they do not seem to overestimate the prevalence of alcohol compared to previous studies of forensic autopsy cases from Scandinavia [32,33]. The majority of alcohol positive cases had BAC $\geq$ 1.0‰. In practically all cases with BAC $\geq$ 3.0‰ death was attributed to accidental poisoning. A forensic autopsy study from Scotland published in 1997 [34] reported relatively similar detection frequencies of alcohol, whereas more contemporary autopsy studies from Ireland [35] and Slovenia [36] found generally higher prevalences of alcohol in violent deaths, i.e. accidental deaths, suicides and homicides, compared to our study, possibly reflecting a higher level of alcohol consumption and high-risk drinking in these countries. However, variations in manner of death categorization and cut-off level for alcohol positive cases may hamper comparisons between these studies.

Among the forensic autopsy cases with positive toxicology, one single substance was the most common finding (40%) (Table 4). In a majority of cases where >6 substances were detected, death was ascribed to accidental poisoning. In most accidental poisoning deaths 4–6 substances were detected. The latter was also observed in suicidal poisonings, suggesting a substantial element of mixed intoxications in poisoning deaths in this material. However, the number of cases is low, particularly for suicidal poisonings.

The by far most frequently detected classes of substances in post-mortem blood were (1) benzodiazepines, (2) opioids, and (3) alcohol (Table 5). It is noteworthy that there were no benzodiazepine-induced deaths among these cases, whereas a considerable number of fatal opioid and alcohol poisonings were observed. Benzodiazepines thus appear to be contributory factors rather than primary substances with regard to the cause of death in this material of forensic autopsy cases. The comparatively low detection frequency of tetrahydrocannabinol (THC) possibly reflects the short detection time of THC in blood. For cocaine and its main metabolite benzoylecgonine the low detection frequency most likely reflects low use of the drug in this population. Correspondingly, the detection frequency of carisoprodol and its active metabolite meprobamate is likely affected by the events of carisoprodol being upgraded to the highest scheduling level (Class A) from 1 August 2007 and withdrawn from the market in Norway on 1 May 2008 [37].

The frequent detection of antidepressant, antipsychotic and antiepileptic drugs is not surprising considering that this material comprises a large number of sudden unexpected deaths, and that individuals with psychiatric disorders or epilepsy have an increased risk of such deaths [38–42]. When relating the detection frequency of these classes of drugs to their respective prescription rates in this region over the same period [43], antiepileptics and particularly antipsychotics occur more frequently than antidepressants. Interestingly, a similar comparison of benzodiazepines and the two Z-hypnotics marketed in Norway (zopiclone and zolpidem), shows that Z-hypnotics, despite being prescribed approximately twice as much as benzodiazepines [43], were detected far less frequently than benzodiazepines in this material. No cases with zopiclone or zolpidem poisoning as the underlying cause of death were identified. To what extent these observations are related to the characteristics of the individuals being prescribed these drugs or the effects of the drugs themselves is difficult to assess.

The autopsy rates presented in this article are calculated from a limited material, both quantitatively and geographically. The toxicology results originate from a selected material of forensic autopsy cases. Accordingly, the findings of this study are not necessarily representative for the general population of Norway, and should be interpreted with caution. They do, however, provide information about recent trends in forensic autopsy requests and the pattern of substance use and abuse in this population. In that sense, the results have relevance beyond the study population.

5. Conclusions

This material from Central Norway shows that autopsy rates can vary with manner of death, sex and age, and may be susceptible to the influence of regional variations in law enforcement, with possible consequences for the quality and validity of cause of death statistics. The toxicology results suggest that alcohol and drugs are important factors in sudden unexpected deaths, and that a thorough and comprehensive toxicological analysis is called for when investigating these deaths.

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Paper II

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Investigation of morphine and morphine glucuronide levels and cytochrome P450 isoenzyme 2D6 genotype in codeine-related deaths

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ABSTRACT

Compared to morphine and morphine-6-glucuronide (M6G), codeine and its other major metabolites codeine-6-glucuronide and norcodeine have weak affinity to opioid μ-receptors. Analgesic effects of codeine are thus largely dependent on metabolic conversion to morphine by the polymorphic cytochrome P450 isoenzyme 2D6 (CYP2D6). How this relates to toxicity and post-mortem whole blood levels is not known. This paper presents a case series of codeine-related deaths where concentrations of morphine, M6G and morphine-3-glucuronide (M3G), as well as CYP2D6 genotype, are taken into account. Post-mortem toxicological specimens from a total of 1444 consecutive forensic autopsy cases in Central Norway were analyzed. Among these, 111 cases with detectable amounts of codeine in femoral blood were identified, of which 34 had femoral blood concentrations exceeding the TIAFT toxicity threshold of 0.3 mg/L. Autopsy records of these 34 cases were retrieved and reviewed. In the 34 reviewed cases, there was a large variability in individual morphine to codeine concentration ratios (M/C ratios), and morphine levels could not be predicted from codeine concentrations, even when CYP2D6 genotype was known. 13 cases had codeine concentrations exceeding the TIAFT threshold for possibly lethal serum concentrations (1.6 mg/L). Among these, 8 individuals had morphine concentrations below the toxic threshold according to TIAFT (0.15 mg/L). In one case, morphine as well as M6G and M3G concentrations were below the limit of detection. A comprehensive investigation of codeine-related fatalities should, in addition to a detailed case history, include quantification of morphine and morphine metabolites. CYP2D6 genotyping may be of interest in cases with unexpectedly high or low M/C ratios.

1. Introduction

Codeine is an opiate analgesic that is widely used, often in combination formulations with paracetamol (acetaminophen). Combinations with other analgesics or miscellaneous drugs such as acetylsalicylic acid, ibuprofen, carisoprodol, caffeine, barbiturates and sedative antihistamines are available in some countries. Codeine is also used as a cough suppressant, either alone or in combination with other antitussive drugs.

Codeine is mainly metabolized in the liver, although some intestinal and CNS metabolism occurs. The principal pathways are outlined in Fig. 1. A major part (50–70%) of a codeine dose is glucuronidated to codeine-6-glucuronide (C6G), while 10–15% is N-demethylated to norcodeine via the cytochrome P450 isoenzyme 3A4 (CYP3A4) [1]. Norcodeine is in turn glucuronidated to norcodeine-6-glucuronide (N6G), and a minor part is O-demethylated to normorphine [2,3]. Of an ingested codeine dose, 0–15% is O-demethylated to morphine by the polymorphic cytochrome P450 isoenzyme 2D6 (CYP2D6), and further glucuronidated to the inactive metabolite morphine-3-glucuronide (M3G; approximately 60% of morphine formed) and the active metabolite morphine-6-glucuronide (M6G; 5–10% of morphine formed) [1]. A minor part of morphine is N-demethylated to normorphine [2,3]. Compared to morphine and M6G, codeine and its main metabolites C6G and norcodeine/N6G have weak affinity to opioid μ-receptors [4–6]. Normorphine has about four times weaker opioid μ-receptor affinity than morphine [4] and is produced in small amounts [3]. Accordingly, the analgesic effects of codeine are largely dependent on metabolic conversion to morphine by CYP2D6.

Approximately 7–10% of the European population lack functional CYP2D6 enzyme due to a genetic polymorphism (''poor metabolizers'') [7], and are thus unable to convert codeine to morphine. It has been demonstrated in several studies that such individuals do not obtain pain relief from codeine [8–11]. Diminished O-demethylation of codeine to morphine has also

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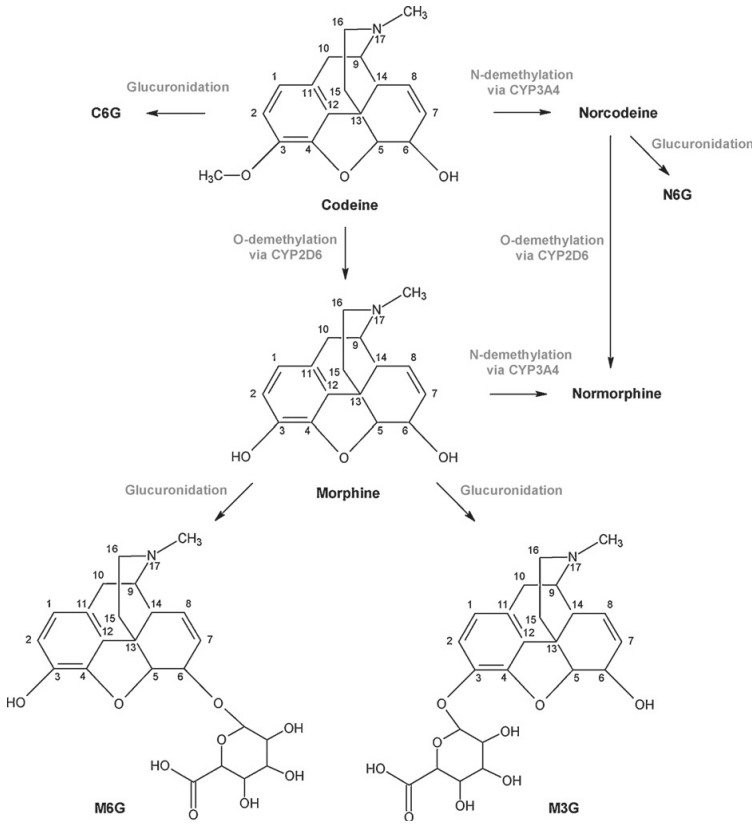

Fig. 1. Principal pathways for codeine metabolism in man. C6G, codeine-6-glucuronide; N6G, norcodeine-6-glucuronide; M3G, morphine-3-glucuronide; M6G, morphine-6-glucuronide; CYP2D6, cytochrome P450 isoenzyme 2D6; CYP3A4, cytochrome P450 isoenzyme 3A4.

been associated with a significant weakening of objective opioid effects, e.g. respiratory, psychomotor and pupillary effects [12]. Conversely, 1–10% of the European population (with the highest frequency in Southern Europe) possesses allele duplications in the CYP2D6-encoding gene (“ultrarapid metabolizers”) [13]. These individuals will metabolize a larger proportion of ingested codeine to morphine [14], thus increasing their vulnerability to toxic effects [15–18]. The distribution of poor, extensive and ultrarapid metabolizers differ significantly between ethnic groups [19]. Also, environmental factors such as inhibitory interactions from other drugs (e.g. certain antidepressants and antipsychotics) influence CYP2D6 activity. Together, this results in a large and unpredictable intra- and interindividual variability in the amount of morphine produced after ingestion of a given codeine dose.

In overdose, codeine is associated with depressive effects on the central nervous system. Death from respiratory arrest may occur with large overdoses. In the reference literature, the minimum lethal oral dose for an adult is estimated at 0.5–1.0 g, i.e. 17–34 pills containing 30 mg codeine each [20]. According to The International Association of Forensic Toxicologists (TIAFT), serum codeine concentrations above 0.3 mg/L have been associated with toxicity, whereas concentrations above 1.6 mg/L are considered possibly lethal [21]. Other compilations of toxic and fatal concentrations of drugs in blood indicate codeine concentrations of 0.5–1.0 mg/L as potentially toxic [22] and concentrations of 0.6–2.1 mg/L as potentially lethal [20,22–25]. The mechanism behind codeine toxicity is not fully understood, and toxic effects mediated by codeine itself or any of its non-O-demethylated metabolites cannot be excluded, although opioid μ-receptor affinity studies [4–6] do not support this.

In fatal poisonings with morphine *per se*, post-mortem blood concentrations of conjugated and total morphine (after acid hydrolysis and silylation) as low as 0.07 mg/L and 0.2 mg/L, respectively, have been reported [26,27]. The TIAFT list indicates that toxic effects of morphine have occurred at serum morphine concentrations above 0.15 mg/L [21].

Two large autopsy series report codeine concentrations in post-mortem blood samples obtained from cases of lethal codeine overdoses [23,24]. Only one of these sources reports morphine concentrations [24], and neither report concentrations of morphine metabolites or CYP2D6 genotype. Provided that conversion to morphine is a central mechanism behind codeine toxicity, as has been demonstrated for the analgesic effects of codeine, this might represent a limitation in the existing literature.

In this article a case series of codeine-related deaths is presented, where the concentrations of morphine, M6G and M3G, as well as the CYP2D6 genotype of the deceased, are taken into account, providing a more detailed and comprehensive interpretation of post-mortem codeine findings.

2. Materials and methods

All forensic autopsy cases in four counties in Central Norway (total population approximately 750,000) from January 2003 to January 2011 were reviewed. In

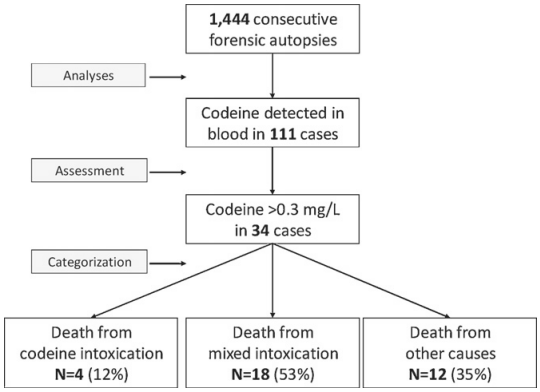

Fig. 2. Forensic autopsy cases involving codeine in Central Norway from January 2003 to January 2011. Analyses included screening against a comprehensive drug library and quantitative liquid chromatography–mass spectrometry (LC–MS) and gas chromatography–mass spectrometry (GC–MS) methods. Assessment was based on the TIAFT toxicity threshold (0.3 mg/L), and categorization on the forensic pathologist's conclusion as to the cause of death.

Norway a forensic autopsy can, according to law and provision, be requested by the police under given circumstances, including sudden unexpected deaths, suspected homicides and suicides, other violent deaths, suspected drug-related deaths and unidentified corpses. The combined clinical and forensic autopsy rate in Norway has been about 10% in recent years, of which approximately 40% are forensic autopsies. In our region the annual number of forensic autopsies varies from 160 to 190. From this material, post-mortem toxicological specimens from a total of 1444 autopsies were received and analyzed. Among these, 111 cases with detectable amounts of codeine in femoral blood were identified, of which 34 had femoral blood concentrations exceeding 0.3 mg/L. Autopsy records of these 34 cases were retrieved and reviewed. Cases were categorized into three groups according to cause of death assessed by the forensic pathologists performing the autopsies; (1) death from codeine intoxication, (2) death from intoxication with multiple substances including codeine, and (3) death from other causes. A study flow chart is presented in Fig. 2. CYP2D6 genotyping was performed in 32 cases, whereas two subjects could not be genotyped due to lack of sample material.

All blood specimens were subjected to specific analyses for alcohols (ethanol, methanol, isopropanol, acetone) using a headspace gas chromatography–mass spectrometry (GC–MS) method, and specific analyses for benzodiazepines (diazepam, desmethyldiazepam, oxazepam, nitrazepam, flunitrazepam, desmethylflunitrazepam, clonazepam, alprazolam, midazolam), opiates (morphine, codeine, ethylmorphine, oxycodone, M3G, M6G) and amphetamines (amphetamine, methamphetamine, MDMA and MDA) using liquid chromatography–mass spectrometry (LC–MS) methods. In addition, blood specimens were screened against comprehensive drug libraries (National Institute of Standards and Technology Mass Spectral Library, Forensic Toxicology Retention Time Locking Database/Library and Pfleger/Maurer/Weber Drugs and Pesticides Library for Toxicology) with a GC–MS method. When available, urine was also screened for drugs of abuse using LC–MS methods. Positive screening results, as well as explicit information about drug use in the case histories, were confirmed by specific analyses in blood using LC–MS or GC–MS methods.

In the method used for quantification of opiates, 1 mL blood and 50 μL d3-morphine and d3-codeine (internal standards) were mixed with 2.0 mL 10 mM ammonium carbonate. The mixture was eluted through a SPE Chromabond C18 column preconditioned with methanol, water and 10 mM ammonium carbonate. After washing with 3.0 mL 10 mM ammonium carbonate the column was eluted with 0.5 mL methanol:0.5 M acetic acid (9:1). After evaporation of the eluent, the residue was reconstituted in 50 μL 50 mM ammonium carbonate pH 7.0, transferred to vials and injected on an Agilent MSD 1100 LC–MS system (Agilent, Palo Alto, CA). The LC–MS system consisted of G1379A degasser, G1311A quaternary pump, G1313A autosampler, G1316A column oven and G1946A mass spectrometer. Separation was performed on Zorbax Eclipse XDB-C8 (4.6 mm × 150 mm) column with gradient elution, with a mobile phase initially consisting of 6% 25 mM formic acid, 3% methanol and 91% 50 mM ammonium acetate, then 2.5% 25 mM formic acid, 60% methanol and 37.5% 50 mM ammonium acetate after 2.5 min, and finally 6% 25 mM formic acid, 3% methanol and 91% 50 mM ammonium acetate after 2.85 min. Total runtime was approximately 6 min. Morphine (unconjugated), codeine (unconjugated), M3G and M6G were monitored after positive electrospray ionization at m/z 286.1, 300.3, 462.3 and 462.3, respectively, while the internal standards d3-morphine, d3-codeine and d3-M3G were monitored at m/z 289.1, 303.3 and 465.3, respectively. To account for and reduce matrix effects, specific standard curves constructed from spiked blood samples were used. The calibrated ranges were 0–0.30 mg/L for morphine and codeine, and 0–4.6 mg/L for M3G and M6G. Linearity was demonstrated over this interval. Three quality control samples covering the range from 0.0030 mg/L to 0.15 mg/L for morphine and codeine and the range from 0.046 mg/L to 2.3 mg/L for M3G and M6G were analyzed with every batch of unknown samples. Samples with concentrations above the calibrated ranges were diluted and reanalyzed. For morphine and codeine the between-day coefficient of variation calculated from quality control samples was 3.2% and 9.0%, respectively, at 0.0030 mg/L, 3.6% and 7.0% at 0.015 mg/L, and 2.7% and 5.8% at 0.15 mg/L. For M3G and M6G the between-day coefficient of variation was 4.7% and 8.9%, respectively, at 0.046 mg/L, 2.8% and 5.8% at 0.46 mg/L, and 2.7% and 4.6% at 2.3 mg/L. The limit of quantitation was 0.0015 mg/L for morphine and codeine, and 0.023 mg/L for M3G and M6G. The limit of detection was 0.00057 mg/L for morphine, 0.00030 mg/L for codeine, and 0.0046 mg/L for M3G and M6G.

CYP2D6 genotyping was performed by isolating genomic DNA from peripheral leukocytes using either an iPrep™ Purification Instrument with iPrep™PureLink™ gDNA Blood Kit (Invitrogen Corporation, Carlsbad, USA) or a BioRobot® EZ1 with EZ1 DNA Blood 200 μL Kit (Qiagen, Hilden, Germany), according to the manufacturers' guidelines. The inactivating alleles *3, *4, *6, *7 and *8 were determined by allele-specific polymerase chain reaction (PCR) analysis and the PCR product was analyzed directly by agarose gel electrophoresis. The sample was also tested by long-PCR for the deletion of CYP2D6 (the inactivating allele *5) and for the duplicated/multiduplicated gene (the *2 × n mutation). Alleles in which none of these variants were found were classified as *1 (wild-type) alleles. Cases with two functional (wild-type) alleles were designated an extensive metabolizer phenotype, cases with any of the inactivating alleles alongside one functional allele were designated an intermediate metabolizer phenotype, and cases with two inactivating alleles were designated a poor metabolizer phenotype. Particular cases with functional allele duplication alongside any of the inactivating alleles were designated an extensive metabolizer phenotype.

3. Results

Individual post-mortem femoral blood concentrations of codeine, morphine, M6G and M3G in the 34 reviewed cases are presented in Table 1. Codeine concentrations ranged from 0.30 to 15.0 mg/L, whereas morphine concentrations ranged from not detectable (n/d) to 3.1 mg/L. M6G and M3G were measured in concentrations ranging from n/d to 0.55 mg/L and 1.5 mg/L, respectively. Individual morphine to codeine concentration ratios (M/C ratios), CYP2D6 genotype and predicted phenotype are shown in Table 1. M/C ratios averaged 0.054 and ranged from 0 (morphine n/d) to 0.39 (SD 0.087). All cases where genotyping was performed fell into three phenotype categories: (1) extensive metabolizer, indicative of a metabolizing capacity in the normal range (N = 18; 56%), (2) intermediate metabolizer, indicative of a metabolizing capacity in the lower normal range (N = 12; 38%), and (3) poor metabolizer, indicative of a very low metabolizing capacity (N = 2; 6%). Within these categories, mean M/C ratios were 0.058 (range 0–0.39, SD 0.093), 0.043 (range 0–0.22, SD 0.077) and 0.025 (range 0.0054–0.044, SD 0.027), respectively.

Various other drugs and/or ethanol were detected in blood in all 34 cases. In 32 cases ≥3 different drugs were found in addition to codeine, and in 5 cases ≥6 different additional drugs were found. Paracetamol was found in 33 of 34 cases, indicating ingestion of paracetamol–codeine combinations. In the one case where paracetamol was not detected, a codeine concentration of 0.30 mg/L was measured, and the only additional finding was a low concentration of the non-steroidal anti-inflammatory drug ibuprofen. Opioids other than codeine and morphine were detected in 8 cases. Other frequently detected drugs were benzodiazepines (N = 25), ethanol (N = 10), carisoprodol and its active metabolite meprobamate (N = 9), and zopiclone (N = 5).

From the 34 reviewed cases, 4 deaths (12%) were attributed to codeine intoxication, 18 deaths (53%) to intoxication with multiple substances including codeine, and 12 deaths (35%) to other causes (Fig. 2). These conclusions were made by the attending pathologist after a review of all available data. In the 13 cases with possibly lethal codeine concentrations (>1.6 mg/L), 3 deaths were attributed to codeine intoxication, 8 to intoxication with multiple

Table 1
Case characteristics; sex, age, individual post-mortem blood concentrations (mg/L) of codeine, morphine, morphine-6-glucuronide (M6G) and morphine-3-glucuronide (M3G), morphine to codeine concentration ratios (M/C ratios), cytochrome P450 isoenzyme 2D6 (CYP2D6) genotype and predicted phenotype, and the forensic pathologist's conclusion as to the cause of death.

Case#	Sex	Age	Codeine [mg/L]	Morphine [mg/L]	M6G [mg/L]	M3G [mg/L]	M/C ratio	CYP2D6 genotype and predicted phenotype	Pathologist's conclusion
1	F	31	9.82	0.49	0.07	0.30	0.05	*1/*1, EM	Codeine intoxication
2	M	55	8.38	3.14	0.55	0.83	0.39	*1/*1, EM	Codeine intoxication
3	F	64	6.02	0.28	0.32	1.20	0.05	*1/*1, EM	Codeine intoxication
4	F	38	1.52	0.09	0.02	0.19	0.06	*4/*2 × 2, EM	Codeine intoxication
5	F	36	14.97	0.31	0.05	0.51	0.02	*1/*4, IM	Mixed intoxication
6	M	38	8.83	0.14	0.00	0.00	0.02	*1/*4, IM	Mixed intoxication
7	M	59	4.19	0.01	0.00	0.03	0.00	*1/*4, IM	Mixed intoxication
8	M	52	3.89	0.09	0.14	1.15	0.02	*1/*1, EM	Mixed intoxication
9	F	66	2.99	0.00	0.00	0.00	0.00	*1/*4, IM[a]	Mixed intoxication
10	F	65	2.75	0.01	0.00	0.00	0.01	*4/*4, PM	Mixed intoxication
11	M	29	2.25	0.02	0.04	0.26	0.01	*1/*4, IM	Mixed intoxication
12	F	44	1.93	0.01	0.01	0.05	0.01	*1/*4, IM	Mixed intoxication
13	M	38	1.03	0.00	0.00	0.00	0.00	*1/*1, EM[b]	Mixed intoxication
14	M	52	0.78	0.02	0.00	0.05	0.02	*1/*1, EM	Mixed intoxication
15	F	54	0.78	0.01	0.03	0.17	0.02	*4/*2 × 2, EM	Mixed intoxication
16	F	40	0.72	0.01	0.00	0.03	0.01	*1/*1, EM	Mixed intoxication
17	F	50	0.57	0.11	0.02	0.15	0.19	*1/*4, IM	Mixed intoxication
18	M	50	0.48	0.02	0.02	0.11	0.04	*4/*5, PM	Mixed intoxication
19	F	21	0.48	0.01	0.00	0.00	0.03	*1/*1, EM	Mixed intoxication
20	M	48	0.39	0.00	0.02	0.13	0.01	*1/*1, EM	Mixed intoxication
21	F	63	0.39	0.00	0.01	0.04	0.01	*1/*4, IM	Mixed intoxication
22	F	47	0.36	0.01	0.11	0.31	0.03	*1/*1, EM	Mixed intoxication
23	M	32	3.29	0.46	0.34	1.52	0.15	*1/*1, EM	Other cause of death
24	F	33	1.65	0.03	0.05	0.15	0.02	Not available	Other cause of death
25	M	54	1.47	0.00	0.00	0.02	0.00	*1/*1, EM	Other cause of death
26	F	73	0.72	0.02	0.04	0.22	0.03	*1/*1, EM	Other cause of death
27	F	97	0.58	0.00	0.00	0.00	0.00	*1/*3, IM	Other cause of death
28	M	53	0.57	0.02	0.00	0.07	0.04	*1/*5, IM	Other cause of death
29	M	55	0.48	0.00	0.00	0.00	0.00	*1/*4, IM	Other cause of death
30	F	49	0.40	0.01	0.02	0.09	0.03	*1/*1, EM	Other cause of death
31	M	61	0.36	0.05	0.07	0.45	0.13	*1/*1, EM	Other cause of death
32	M	69	0.36	0.07	0.04	0.18	0.22	*1/*4, IM	Other cause of death
33	M	51	0.34	0.00	0.01	0.02	0.00	*1/*1, EM	Other cause of death
34	M	45	0.30	0.07	0.00	0.00	0.23	Not available	Other cause of death

EM, extensive metabolizer, indicative of a metabolizing capacity in the normal range; IM, intermediate metabolizer, indicative of a metabolizing capacity in the lower normal range; PM, poor metabolizer, indicative of a very low metabolizing capacity.
[a] Possibly PM phenotype due to interaction with paroxetine.
[b] Possibly PM phenotype due to interaction with levomepromazine.

substances including codeine, and 2 to other causes. Among these cases, 8 individuals had morphine concentrations below 0.15 mg/L, and in one case the morphine concentration as well as M6G and M3G concentrations were below the limit of detection. In 13 of the 18 deaths attributed to mixed intoxication, morphine was not detected (2 cases) or found in low concentrations usually not associated with toxic effects (11 cases).

In all cases categorized as fatal codeine intoxication (cases 1–4) codeine appeared to originate from an overdose of an oral combination analgesic containing paracetamol. In case 1, a 31-year-old woman found dead in her home, toxicological findings in addition to codeine and its metabolites (Table 1) in blood were paracetamol (489 mg/L), ethanol (0.06 g/dL), diazepam (0.60 mg/L), desmethyldiazepam (0.60 mg/L), oxazepam (0.29 mg/L), nitrazepam (0.025 mg/L) and 7-aminonitrazepam (0.34 mg/L). No signs of injury or disease were revealed by autopsy. Case 2 was a 55-year-old man found dead in his home. Autopsy results showed putrefaction, coronary atherosclerosis and signs of heart failure, but no certain signs of injury. Additional toxicological findings consisted of paracetamol (591 mg/L), ethylmorphine (0.04 mg/L), diazepam (0.22 mg/L), desmethyldiazepam (0.51 mg/L), oxazepam (0.34 mg/L), carbamazepine (3.5 mg/L) and carbamazepine-10,11-epoxide (0.88 mg/L). Case 3 was a 64-year-old woman with advanced multiple sclerosis found dead in her bed. Autopsy revealed steatosis, fibrosis and mild inflammation of the liver, moderate to severe coronary atherosclerosis and signs of chronic ischemic heart disease. Toxicological findings in addition to codeine and its metabolites were paracetamol (571 mg/L), diazepam (0.68 mg/L), desmethyldiazepam (0.11 mg/L), nitrazepam (0.079 mg/L), 7-aminonitrazepam (1.0 mg/L), zolpidem (0.27 mg/L), citalopram (0.42 mg/L), mianserin (0.064 mg/L), desmethylmianserin (0.028 mg/L), amitriptyline (0.10 mg/L) and nortriptyline (0.12 mg/L). In case 4, a 38-year-old woman found dead in her home, autopsy results did not show any signs of injuries or disease. Additional toxicological findings in blood were paracetamol (149 mg/L), carisoprodol (7.5 mg/L) and meprobamate (11.8 mg/L).

4. Discussion

This study shows that in post-mortem blood samples, high concentrations of codeine do not necessarily correspond to high concentrations of the active metabolites morphine and M6G, and that these relations cannot be reliably predicted by genotyping. In general, there was a weak correlation between codeine and morphine concentrations, illustrated in Fig. 3. The relatively large variation in observed M/C-ratios in individual cases could be due to differences in survival times from codeine intake to death and varying CYP2D6 metabolic capacity. All but two of the genotyped cases (10 and 18) had a genotype indicative of a metabolizing capacity within the normal range, demonstrating a considerable variation in M/C-ratio within this group. The exclusion of two cases (9 and 13) that possibly had a poor metabolizer phenotype caused by CYP2D6 inhibition from paroxetine and levomepromazine,

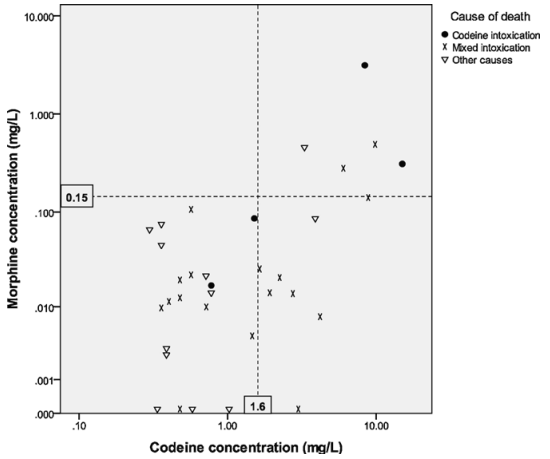

Fig. 3. Scatter plot of cases ($N = 34$) showing the relation between codeine and morphine blood concentrations. Note that the scales are logarithmic. Dotted lines show thresholds according to the TIAFT list for possibly lethal serum concentrations of codeine (1.6 mg/L) and toxic serum concentrations of morphine (0.15 mg/L). The correlation between the codeine and morphine concentrations is weak ($R^2 = 0.21$).

respectively, did not change this general impression. Besides CYP2D6 genotyping and possible CYP2D6 interaction in these two cases, there was no available information highlighting the role of inter-individual factors possibly influencing drug response, e.g. drug tolerance and diseases, in our material. In particular, no indications of additional relevant drug–drug interactions were found. However, the possible influence of inhibitory drugs no longer detectable in biological specimens cannot be excluded. The complex interplay of genetic and epigenetic factors for the clinical pharmacokinetics and toxicity of codeine is well illustrated in a case report by Gasche et al. [16].

Co-ingestion of morphine or heroin cannot be ruled out as a contributing source to measured morphine and morphine glucuronide concentrations in individual cases. In particular, co-ingestion of morphine was suspected in case 2 (Table 1). Genotyping in this case was indicative of a metabolizing capacity in the normal range (homozygous extensive metabolizer). Similar M/C-ratios like the one measured in case 2 (0.39) has been reported in extensive metabolizers [28]. However, this was observed after a single oral dose of 30 mg codeine, and no earlier than 24 h after codeine intake. In our case the survival time from codeine intake to death is unknown, and the ingested dose was clearly supratherapeutic. The heroin-specific metabolite 6-monoacetylmorphine was not detected in post-mortem blood or urine in any of the 34 cases.

The conclusion as to the cause of death in individual cases was made by different forensic pathologists at their discretion, and the conclusions were not adjusted or altered after subsequent scrutiny. In particular, the distinction between codeine intoxications and mixed intoxications may be subject to differences in opinion. Indeed, in all 4 cases attributed to codeine intoxication additional toxicological findings with potentially contributory effects were present. It is noteworthy that 65% of the 34 cases with blood codeine concentrations exceeding 0.3 mg/L were considered to have died of poisoning. Knowing that multiple toxicological findings in addition to codeine (and morphine) were made in all of these poisoning deaths, this illustrates both the prevalence of mixed intoxications in this material and the possible contributory actions of codeine in these intoxications.

Interestingly, in many cases morphine was either found at concentrations not usually associated with toxic effects or below the limit of detection, even when measured codeine concentrations were high and at levels associated with potentially lethal effects (>1.6 mg/L). A similar observation was made by Gerostamoulos et al. in a summary of toxicological findings in 6 drug-related deaths with codeine as the principal toxicological agent [24], where morphine was reported in 2 of 6 cases. Some studies have suggested morphine-independent central nervous effects of codeine, presumably mediated through codeine itself or its CYP2D6-independent metabolites C6G and norcodeine [29–31], but direct evidence for such a mechanism is still lacking. Receptor affinity studies have demonstrated a 100–600-fold greater opioid μ-receptor affinity for morphine and M6G than for codeine, C6G and norcodeine [4–6]. M6G has been shown to possess opioid μ-receptor affinity and analgesic potency similar to or greater than morphine in animal studies [4,5,32]. However, a marked increase in M6G potency has been observed in animals when administration of M6G is shifted from systemic to intrathecal sites [32]. Reasoning strictly from opioid μ-receptor affinities does not explain why plasma morphine concentrations have been shown to be lower after codeine administration than after morphine administration in presumably equianalgetic doses [9,33]. A possible explanation of this discrepancy could be that codeine exerts much of its effects through local conversion to morphine (and possibly M6G) within the central nervous system, and that plasma/blood concentrations consequently do not adequately reflect concentrations at the site of action. When investigating codeine-related deaths, co-analysis of morphine, M6G and the inactive M3G can also contribute to the estimation of survival times from codeine intake to death, particularly in cases of acute overdose where measured concentrations do not reflect steady state levels. Even though the precise mechanism behind codeine toxicity is not known, it thus seems prudent to include analysis of morphine and its glucuronide metabolites when interpreting post-mortem codeine findings. Although CYP2D6 genotyping proved to be of little value in predicting M/C ratios in this material, genotyping in general may, in concert with drug/metabolite ratios, provide additional information pertinent for the determination of time from drug ingestion to death. Moreover, CYP2D6 genotyping may help identifying unintentional overdoses by therapeutic misadventure in cases with ultrarapid metabolizer phenotype. In our material there were no ultrarapid metabolizers to illustrate this.

In all 4 deaths attributed to codeine intoxication, measured blood morphine concentrations were in themselves above potentially lethal levels for opioid-naïve individuals. However, in 3 of these cases, codeine was measured in concentrations 50–200 times greater than what is normally measured in plasma after ingestion of standard therapeutic doses of codeine [17,34]. In such cases, it is possible that codeine itself and/or CYP2D6-independent metabolites may exert additive effects, and that high concentrations to some extent may overcome low receptor affinity.

All analyses were performed in post-mortem blood. Accordingly, measured concentrations are potentially subject to post-mortem changes, both in terms of post-mortem drug redistribution (all substances) and post-mortem deglucuronidation (M6G, M3G and C6G). Published data indicate that codeine and morphine may exhibit post-mortem redistribution, but the results are inconsistent, particularly for morphine [20,35–40]. One study of the post-mortem redistribution of M6G and M3G as well as morphine concluded that significant post-mortem redistribution of morphine and its glucuronide metabolites seems unlikely [35]. In our study blood specimens were collected from the femoral artery, which lessens the potential for post-mortem changes due to redistribution. Post-mortem hydrolysis of morphine glucuronides

has been shown to yield elevated morphine levels during specimen storage above −20 °C [41,42].

5. Conclusions

Our findings demonstrate a large variability in the M/C-ratio after codeine intake in forensic autopsy cases, and show that morphine levels cannot be predicted from codeine concentrations. Whereas a significant amount of data suggests that bioconversion of codeine to morphine is essential for codeine-induced analgesia, the precise mechanism behind codeine toxicity is not known. Nevertheless, investigations of codeine-related deaths should include quantification of morphine and morphine metabolites together with a detailed case history, in order to achieve a comprehensive interpretation of post-mortem codeine findings. CYP2D6 genotyping may be of interest in cases with unexpectedly high or low M/C ratios.

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Paper III

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Article

A Validated Method for Simultaneous Determination of Codeine, Codeine-6-Glucuronide, Norcodeine, Morphine, Morphine-3-Glucuronide and Morphine-6-Glucuronide in Post-Mortem Blood, Vitreous Fluid, Muscle, Fat and Brain Tissue by LC–MS

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The toxicodynamics and, to a lesser degree, toxicokinetics of the widely used opiate codeine remain a matter of controversy. To address this issue, analytical methods capable of providing reliable quantification of codeine metabolites alongside codeine concentrations are required. This article presents a validated method for simultaneous determination of codeine, codeine metabolites codeine-6-glucuronide (C6G), norcodeine and morphine, and morphine metabolites morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G) in post-mortem whole blood, vitreous fluid, muscle, fat and brain tissue by high-performance liquid chromatography mass spectrometry. Samples were prepared by solid-phase extraction. The validated ranges were 1.5–300 ng/mL for codeine, norcodeine and morphine, and 23–4,600 ng/mL for C6G, M3G and M6G, with exceptions for norcodeine in muscle (3–300 ng/mL), morphine in muscle, fat and brain (3–300 ng/mL) and M6G in fat (46–4,600 ng/mL). Within-run and between-run accuracy (88.1–114.1%) and precision (CV 0.6–12.7%), matrix effects (CV 0.3–13.5%) and recovery (57.8–94.1%) were validated at two concentration levels; 3 and 150 ng/mL for codeine, norcodeine and morphine, and 46 and 2,300 ng/mL for C6G, M3G and M6G. Freeze–thaw and long-term stability (6 months at −80°C) was assessed, showing no significant changes in analyte concentrations (−12 to +8%). The method was applied in two authentic forensic autopsy cases implicating codeine in both therapeutic and presumably lethal concentration levels.

Introduction

The opiate codeine is widely used. A substantial body of evidence from receptor-affinity studies (1–3) and clinical studies (4–8) have indicated that the analgesic effects, as well as the dose-limiting side effects, of codeine are dependent on its metabolism to morphine by the polymorphic cytochrome P450 isoenzyme 2D6 (CYP2D6). This is, however, not reflected in the toxicological literature, where excessive effects of codeine are presented as a function of the parent compound concentration without additional information about the levels of active metabolites (9–12).

The principal metabolic pathways of codeine in man are outlined in Figure 1. A major fraction (50–70%) of a codeine dose is glucuronidated to codeine-6-glucuronide (C6G), while 10–15% is N-demethylated to norcodeine via the cytochrome P450 isoenzyme 3A4 (CYP3A4) (13). Norcodeine is in turn glucuronidated to norcodeine-6-glucuronide (N6G), and a minor part is O-demethylated to normorphine (14, 15). Of an ingested codeine dose, 0–15% is O-demethylated to morphine by CYP2D6, and further glucuronidated to the inactive metabolite morphine-3-glucuronide (M3G; ~60% of morphine formed) and the active metabolite morphine-6-glucuronide (M6G; 5–10% of morphine formed) (13). A minor part of morphine is N-demethylated to normorphine (14, 15).

Receptor-affinity studies have demonstrated a 100- to 600-fold greater opioid μ-receptor affinity for morphine and M6G than for codeine, C6G and norcodeine (1–3). Still, some investigators have suggested codeine, C6G and norcodeine as putative mediators of codeine toxicity (16–18). Quantitative analysis of all these moieties in toxicological cases implicating codeine may shed light on this controversy.

Furthermore, existing literature suggests that codeine and its metabolites may exhibit post-mortem redistribution, but published data are limited and inconsistent (19–25). Determination of the concentration and tissue distribution of codeine and its metabolites in codeine-related fatalities may provide further information about this phenomenon.

Several methods for quantitative analysis of codeine and various codeine metabolites in post-mortem specimens have been published (26–34). These methods, however, either apply to analysis of few analytes in multiple biological matrices, or analysis of multiple analytes in selected matrices. The aim of this work was to develop and validate a method for simultaneous determination of codeine, C6G, norcodeine, morphine, M3G and M6G in post-mortem whole blood, vitreous fluid, muscle, fat and brain tissue, using high-performance liquid chromatography mass spectrometry (LC–MS).

Experimental

Reagents and standards

Methanol (HPLC grade) was purchased from Merck (Darmstadt, Germany). Ammonium carbonate and formic acid (both analytical grade) were supplied by Acros Organics (Morris Plains, NJ, USA). Acetic acid and ammonium acetate (both analytical grade) were obtained from VWR International (Leuven, Belgium).

Norcodeine, morphine, M3G, morphine-d_3, codeine-d_3 and M3G-d_3 were obtained from Sigma-Aldrich (St. Louis, MO, USA). Codeine was purchased from Norwegian Medical Depot (Oslo, Norway), and C6G from Lipomed (Arlesheim, Switzerland). M6G was supplied by Chiron (Trondheim, Norway).

Preparation of solutions

Stock solutions were prepared in methanol at a concentration of 3 mg/mL for codeine, norcodeine and morphine, and in methanol : water (1 : 1) at a concentration of 1.15 mg/mL for C6G, M3G

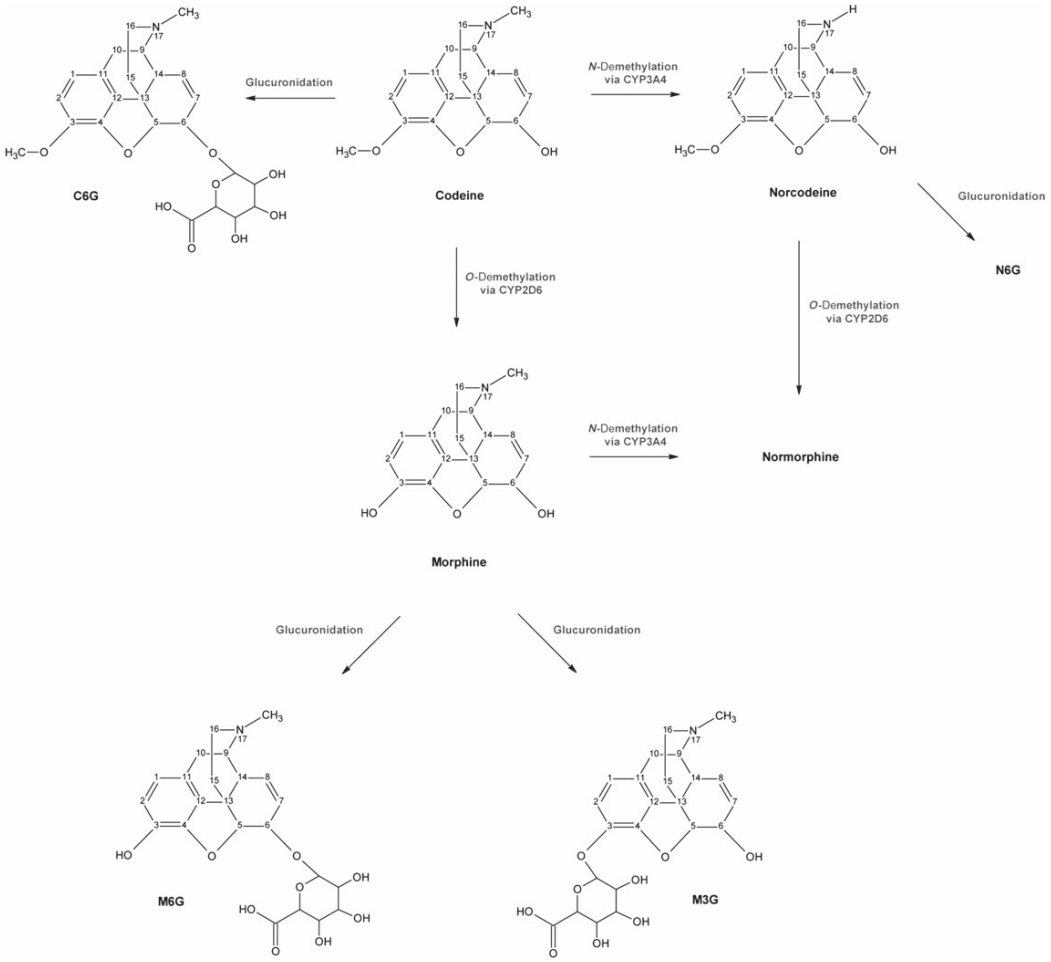

Figure 1. Principal pathways for codeine metabolism in man. C6G, codeine-6-glucuronide; N6G, norcodeine-6-glucuronide; M3G, morphine-3-glucuronide; M6G, morphine-6-glucuronide; CYP2D6, cytochrome P450 isoenzyme 2D6; CYP3A4, cytochrome P450 isoenzyme 3A4.

and M6G. Working solutions for calibrator and quality control (QC) samples were prepared by diluting stock solutions with methanol to concentrations of 0.15, 0.30, 1.5, 3, 15 and 30 μg/mL for codeine, norcodeine and morphine, and with water to concentrations of 2.3, 4.6, 23, 46, 230 and 460 μg/mL for C6G, M3G and M6G. Working solutions were used to prepare calibration solutions in whole blood, water, muscle, fat and brain tissue to final concentrations of 1.5, 3, 15, 30, 150 and 300 ng/mL for codeine, norcodeine and morphine, and 23, 46, 230, 460, 2,300 and 4,600 ng/mL for C6G, M3G and M6G. Similarly, QC solutions in whole blood, water, muscle, fat and brain tissue were prepared to final concentrations of 3, 15 and 150 ng/mL for codeine, norcodeine and morphine, and 46, 460 and 2,300 ng/mL for C6G, M3G and M6G. Internal standards (ISs) codeine-d_3 and morphine-d_3 were prepared in methanol at concentrations of 0.7 and 10 μg/mL, respectively, whereas IS M3G-d_3 was prepared in water at a concentration of 2 μg/mL. Stock, working and IS solutions were stored at 4°C. Calibration and QC solutions in matrix were stored at −80°C.

Sample extraction

Sample pretreatment for whole blood and vitreous fluid

One milliliter of blood or vitreous fluid and 50 μL IS were mixed with 2 mL 10 mM ammonium carbonate.

Sample pretreatment for muscle, fat and brain tissue

Approximately 0.4 g tissue was added 1 mL 10 mM ammonium carbonate and 50 μL IS, and homogenized with an Omni TH-02 homogenizer with Omni Tips™ Hard Tissue Disposable Rotor Stator Generator Probes (30750H). The tissue homogenate was mixed with 2 mL 10 mM ammonium carbonate.

Solid-phase extraction

Pretreated samples were extracted using an Agilent Bond Elut-C18 column (Part No. 52,102,024) preconditioned with 1 mL methanol, 1 mL water and 2 mL 10 mM ammonium carbonate, pH 9.0. After washing with 3 mL 10 mM ammonium carbonate and vacuum drying for 5 min the column was eluted with 0.5 mL methanol : 0.5 M acetic acid (9 : 1). After evaporation (dry, compressed air, 40°C) of the eluent, the residue was reconstituted in 50 μL 50 mM ammonium acetate, pH 7.0, transferred to vials and injected on the LC–MS.

LC–MS

An Agilent MSD 1,100 LC–MS system (Agilent, Palo Alto, CA, USA) consisting of G1379A degasser, G1311A quaternary pump, G1313A autosampler, G1316A column oven and G1946A mass spectrometer was used. Separation was performed on a Supelguard™ Discovery® 18 (2 cm × 4.0 mm, 5 μm) column (Supelco/Sigma-Aldrich, Bellefonte, PA, USA) with gradient elution, with a mobile phase initially consisting of 6% 25 mM formic acid, 3% methanol and 91% 50 mM ammonium acetate, then 2.5% 25 mM formic acid, 60% methanol and 37.5% 50 mM ammonium acetate after 2.5 min, and finally 6% 25 mM formic acid, 3% methanol and 91% 50 mM ammonium acetate after 2.85 min. The column temperature was kept constant at 30°C. Injection volumes were 2 μL for blood and vitreous fluid and 5 μL for muscle, fat and brain samples. Total runtime was 4.0 min. Codeine, norcodeine, morphine, C6G, M3G and M6G were monitored after positive electrospray ionization at m/z 300.3 (fragmentor voltage (FV) 200 V), 286.1 (FV 175 V), 286.1 (FV 175 V), 476.0 (FV 225 V), 462.3 (FV 125 V) and 462.3 (FV 125 V), respectively, while IS codeine-d_3, morphine-d_3 and M3G-d_3 were monitored at m/z 303.2 (FV 100 V), 289.2 (FV 175 V) and 465.3 (FV 125 V), respectively. Acquisition was made in the selected ion monitoring (SIM) mode. Capillary voltage was set to 3.5 kV, drying gas temperature to 350°C, drying gas flow to 9.0 L/min and nebulizer pressure to 40 psig. All vials were kept at 4°C during LC–MS analysis and between runs.

Method validation

Matrices

Validation experiments were generally performed with human ante-mortem whole blood, water and forensic autopsy specimens of muscle, fat and brain tissue, with the exception of accuracy/precision and matrix effect experiments. Ante-mortem whole blood and water was used due to limited availability of post-mortem blood and vitreous fluid.

Selectivity/specificity

Chromatograms for blank samples of ante-mortem whole blood, water and forensic autopsy specimens of muscle, fat and brain tissue from six different sources were examined with regard to endogenous interference. Exogenous interference was evaluated by direct injection on the LC–MS of various drugs and metabolites with molecular mass similar to the analytes (difference < 10 g/mol) and likely to occur in real samples (7-aminoclonazepam, 7-aminoflunitrazepam, alprazolam, amitriptyline, benzoylecgonine, buprenorphine, diazepam, doxepin, fluoxetine, imipramine, methadone, nitrazepam, norflunitrazepam, norfluoxetine, oxazepam, pethidine, promethazine, propranolol, trimeprazine, trimipramine, venlafaxine and zolpidem). Analyses of blank samples with IS only and blank samples fortified with all analytes, but no IS, were performed at two concentration levels (3 and 150 ng/mL for codeine, norcodeine and morphine, and 46 and 2,300 ng/mL for C6G, M3G

Table I
Main Validation Parameters for Whole Blood

	Codeine	C6G	Norcodeine	Morphine	M3G	M6G
Calibrated range (ng/mL)	1.5–300	23–4,600	1.5–300	1.5–300	23–4,600	23–4,600
Correlation coefficient (R^2)	0.9994	0.9989	0.9999	0.9999	0.9998	0.9995
LOD (ng/mL)	0.30	2.3	0.75	0.75	2.3	2.3
LLOQ (ng/mL)	0.75	2.3	1.5	1.5	2.3	23
Accuracy for QC (%)						
Within-run ($n = 6$)						
3/46 ng/mL	102.2	110.8	92.1	105.9	105.5	104.5
150/2,300 ng/mL	99.1	101.7	105.9	96.0	96.9	103.9
Between-run ($n = 5$)						
3/46 ng/mL	98.2	99.7	100.8	101.8	101.7	101.9
150/2,300 ng/mL	96.1	98.5	103.4	92.9	100.0	105.9
Precision for QC, CV (%)						
Within-run ($n = 6$)						
3/46 ng/mL	3.8	5.8	11.0	2.4	1.9	3.9
150/2,300 ng/mL	2.0	3.7	4.9	2.8	2.7	3.4
Between-run ($n = 5$)						
3/46 ng/mL	7.0	9.3	5.5	4.5	3.2	1.4
150/2,300 ng/mL	2.9	5.7	5.3	3.4	1.6	4.0
Matrix effects						
Theoretical concentration (ng/mL)	3/150	46/2,300	3/150	3/150	46/2,300	46/2,300
Matrix effect (%)	88.5/86.7	75.1/87.8	67.1/93.3	74.7/77.5	57.8/76.2	54.8/79.4
Relative matrix effect, CV (%)	4.7/4.2	8.7/5.2	13.9/4.6	8.7/9.9	29.2/14.9	30.7/16.4
Matrix effect corrected with IS (%)	107.8/106.6	91.2/108.0	81.4/114.7	95.8/93.7	94.6/100.1	89.6/104.5
Relative matrix effect to IS, CV (%)	7.6/5.3	4.7/6.8	11.6/6.2	8.5/3.9	4.8/4.5	13.5/7.1
Recovery (%) for QC ($n = 6$)						
3/46 ng/mL	82.0	94.1	81.1	80.9	82.5	94.1
150/2,300 ng/mL	85.6	84.8	89.0	84.9	78.3	79.9

and M6G) to assess interference from the ISs and analyte interference with the internal standard detection, respectively.

Calibration model

Six-point calibration curves with three replicates of each standard were made for each analyte in all matrices (spiked blank samples of ante-mortem whole blood, water and post-mortem muscle, fat and brain tissue). The calibration curves were based on peak area ratios of analyte relative to IS using weighted ($1/x^2$) quadratic models excluding the origin. The calibrated ranges are given in Tables I–V.

Limit of detection and quantification

The upper limit of quantification was set as the highest calibrator level. The limit of detection (LOD) and lower limit of quantification (LLOQ) were evaluated based on signal-to-noise ratio (S/N), with S/N criteria ≥ 3 and ≥ 10, respectively. LOD and LLOQ were assessed for each matrix by analysis of extracted dilutions of the lowest calibrator spiked in blank samples of ante-mortem whole blood, water and forensic autopsy specimens of muscle, fat and brain tissue from six different sources.

Accuracy and precision

Within-run accuracy and precision were estimated in ante-mortem whole blood and post-mortem vitreous fluid, muscle, fat and brain tissue by analysis of six replicates of QC samples at two concentration levels (3 and 150 ng/mL for codeine, norcodeine and morphine, and 46 and 2,300 ng/mL for C6G, M3G and M6G) in a single assay. Between-run accuracy and precision were determined in the same matrices by analysis of two replicates of QC samples at two concentration levels (3 and 150 ng/mL for codeine, norcodeine and morphine, and 46 and 2,300 ng/mL for C6G, M3G and M6G) on five different days.

Matrix effects

Matrix effects (ME) were evaluated in post-mortem whole blood, muscle, fat and brain tissue by the method proposed by Matuszewski *et al.* (35). For each analyte the peak area of blank matrix samples spiked after extraction was compared with the peak area of spiked neat standards prepared in mobile phase, with and without IS correction. ME experiments were performed at two concentration levels (3 and 150 ng/mL for codeine, norcodeine and morphine, and 46 and 2,300 ng/mL for C6G, M3G and M6G) with five replicates of spiked neat standards and five replicates of spiked blank matrix extracts from six different forensic autopsy cases.

Recovery

Recovery experiments were performed with six replicates of blank samples of ante-mortem whole blood, water and post-mortem muscle, fat and brain tissue spiked with all analytes at two concentration levels (3 and 150 ng/mL for codeine, norcodeine and morphine, and 46 and 2,300 ng/mL for C6G, M3G and M6G). Recovery was estimated by comparing peak area ratios of analyte relative to IS in blank matrix samples spiked prior to extraction and blank matrix extracts spiked after extraction.

Stability

Freeze–thaw and long-term stability was evaluated by comparing quantitative results of five replicate samples of ante-mortem whole blood freshly spiked with all analytes with the results of five replicates of the same samples after three freeze–thaw cycles and 6 months under long-term storage conditions (-80°C), respectively. The stability of processed samples was

Table II
Main Validation Parameters for Vitreous Fluid

	Codeine	C6G	Norcodeine	Morphine	M3G	M6G
Calibrated range (ng/mL)	1.5–300	23–4,600	1.5–300	1.5–300	23–4,600	23–4,600
Correlation coefficient (R^2)	0.9999	0.9994	0.9998	0.9994	0.9998	0.9986
LOD (ng/mL)	0.15	2.3	0.30	0.30	2.3	11.5
LLOQ (ng/mL)	1.5	2.3	0.75	1.5	11.5	23
Accuracy for QC (%)						
Within-run ($n = 6$)						
3/46 ng/mL	101.3	92.6	97.7	97.9	99.9	101.4
150/2,300 ng/mL	93.5	99.8	114.1	95.2	97.9	104.3
Between-run ($n = 5$)						
3/46 ng/mL	100.4	98.6	105.0	102.9	100.7	99.8
150/2,300 ng/mL	93.5	95.1	102.2	93.2	98.8	107.5
Precision for QC, CV (%)						
Within-run ($n = 6$)						
3/46 ng/mL	1.5	1.8	3.7	2.9	1.4	3.3
150/2,300 ng/mL	0.8	4.0	4.4	2.2	1.7	5.2
Between-run ($n = 5$)						
3/46 ng/mL	4.6	4.8	6.6	1.7	1.5	3.5
150/2,300 ng/mL	1.1	2.0	3.8	4.0	2.2	3.2
Matrix effects						
Theoretical concentration (ng/mL)	ND*	ND	ND	ND	ND	ND
Matrix effect (%)	ND	ND	ND	ND	ND	ND
Relative matrix effect, CV (%)	ND	ND	ND	ND	ND	ND
Matrix effect corrected with IS (%)	ND	ND	ND	ND	ND	ND
Relative matrix effect to IS, CV (%)	ND	ND	ND	ND	ND	ND
Recovery (%) for QC ($n = 6$)						
3/46 ng/mL	79.1	90.3	84.6	64.2	94.1	69.2
150/2,300 ng/mL	87.2	93.2	91.0	86.8	92.1	91.2

*ND, not determined.

Table III
Main Validation Parameters for Muscle

	Codeine	C6G	Norcodeine	Morphine	M3G	M6G
Calibrated range (ng/mL)	1.5–300	23–4,600	3–300	3–300	23–4,600	23–4,600
Correlation coefficient (R^2)	0.9998	0.9996	0.9992	0.9998	0.9982	0.9999
LOD (ng/mL)	0.15	2.3	0.15	0.75	2.3	2.3
LLOQ (ng/mL)	0.75	23	3	3	2.3	23
Accuracy for QC (%)						
Within-run ($n = 6$)						
3/46 ng/mL	95.8	89.3	112.3	102.3	103.7	92.8
150/2,300 ng/mL	94.5	95.4	99.8	94.2	97.7	96.4
Between-run ($n = 5$)						
3/46 ng/mL	99.7	103.4	113.6	106.6	103.5	102.5
150/2,300 ng/mL	90.5	95.0	96.2	92.5	96.7	105.6
Precision for QC, CV (%)						
Within-run ($n = 6$)						
3/46 ng/mL	3.1	8.6	5.0	3.1	1.6	3.2
150/2,300 ng/mL	0.8	5.2	1.4	2.2	1.9	3.5
Between-run ($n = 5$)						
3/46 ng/mL	1.5	7.5	7.0	5.5	3.3	4.7
150/2,300 ng/mL	4.6	5.7	7.2	5.2	5.3	4.8
Matrix effects						
Theoretical concentration (ng/mL)	3/150	46/2,300	3/150	3/150	46/2,300	46/2,300
Matrix effect (%)	83.0/86.4	85.4/90.5	73.8/88.6	66.4/78.5	60.1/85.0	53.8/80.5
Relative matrix effect, CV (%)	2.9/4.1	4.0/4.0	4.6/4.4	10.4/8.3	21.3/8.4	25.4/10.0
Matrix effect corrected with (%)	104.0/104.8	107.0/109.8	92.3/107.5	94.9/101.9	100.0/106.4	88.7/100.7
Relative matrix effect tolS, CV (%)	5.4/0.8	5.4/1.8	3.7/1.9	4.7/3.9	4.2/2.2	9.9/4.3
Recovery (%) for QC ($n = 6$)						
3/46 ng/mL	62.7	74.6	68.0	67.9	69.6	68.8
150/2,300 ng/mL	66.0	89.3	67.3	65.2	69.8	66.3

Table IV
Main Validation Parameters for Fat

	Codeine	C6G	Norcodeine	Morphine	M3G	M6G
Calibrated range (ng/mL)	1.5–300	23–4,600	1.5–300	3–300	23–4,600	46–4,600
Correlation coefficient (R^2)	0.9998	0.9988	0.9989	0.9908	0.9992	0.9930
LOD (ng/mL)	0.15	2.3	0.15	3	2.3	11.5
LLOQ (ng/mL)	0.75	2.3	0.30	3	2.3	46
Accuracy for QC (%)						
Within-run ($n = 6$)						
3/46 ng/mL	100.3	105.8	113.5	102.8	102.0	99.8
150/2,300 ng/mL	91.8	95.8	100.4	95.1	97.1	99.9
Between-run ($n = 5$)						
3/46 ng/mL	99.0	98.2	98.8	100.1	100.9	100.8
150/2,300 ng/mL	94.4	97.3	106.6	95.5	97.3	103.0
Precision for QC, CV (%)						
Within-run ($n = 6$)						
3/46 ng/mL	1.2	4.9	1.6	1.3	2.4	4.6
150/2,300 ng/mL	3.0	5.8	3.6	1.1	5.0	2.9
Between-run ($n = 5$)						
3/46 ng/mL	5.4	11.0	11.0	6.8	1.8	3.8
150/2,300 ng/mL	2.1	2.9	5.8	2.2	3.6	3.1
Matrix effects						
Theoretical concentration (ng/mL)	3/150	46/2,300	3/150	3/150	46/2,300	46/2,300
Matrix effect (%)	83.4/92.5	88.1/96.1	82.2/95.1	88.9/91.8	86.3/93.3	88.2/93.9
Relative matrix effect, CV (%)	5.0/4.0	1.3/1.9	7.2/5.0	4.3/2.2	6.8/4.0	3.4/2.3
Matrix effect corrected with IS (%)	99.5/105.5	105.2/109.6	97.9/108.3	101.7/99.3	99.7/102.7	102.2/103.5
Relative matrix effect tolS, CV (%)	1.5/1.2	4.9/3.0	3.8/2.6	3.1/2.0	2.3/1.2	5.5/2.9
Recovery (%) for QC ($n = 6$)						
3/46 ng/mL	69.3	76.0	79.0	76.3	63.9	67.5
150/2,300 ng/mL	72.9	86.2	80.3	82.0	80.8	71.6

assessed by comparing peak area ratios of analyte relative to IS in prepared samples of ante-mortem whole blood, water and post-mortem muscle, fat and brain tissue after injection–reinjection on the LC–MS at 0, 8, 24, 48 and 72 h. These experiments were performed with five replicates of spiked blank matrix samples, each from five different sources. Samples were kept at 4°C during LC–MS analysis and between runs. Performance at room temperature was not evaluated.

Method application

The method was applied in two authentic forensic autopsy cases implicating codeine.

Three QC samples covering the range 3–150 ng/mL for codeine, norcodeine and morphine and the range 46–2,300 ng/mL for C6G, M3G and M6G were analyzed with each batch of authentic samples. Samples with concentrations above the calibrated ranges were diluted and reanalyzed.

Table V
Main Validation Parameters for Brain

	Codeine	C6G	Norcodeine	Morphine	M3G	M6G
Calibrated range (ng/mL)	1.5–300	23–4,600	1.5–300	3–300	23–4,600	23–4,600
Correlation coefficient (R^2)	0.9996	0.9981	0.9994	0.9999	0.9991	0.9998
LOD (ng/mL)	0.75	2.3	0.15	0.30	2.3	2.3
LLOQ (ng/mL)	0.75	2.3	0.15	3	2.3	4.6
Accuracy for QC (%)						
Within-run ($n = 6$)						
3/46 ng/mL	98.9	111.2	105.4	88.1	98.3	95.3
150/2,300 ng/mL	98.7	101.1	111.3	91.9	96.9	103.4
Between-run ($n = 5$)						
3/46 ng/mL	102.0	103.4	111.2	102.8	100.2	99.0
150/2,300 ng/mL	98.7	103.9	112.4	98.1	97.9	107.4
Precision for QC, CV (%)						
Within-run ($n = 6$)						
3/46 ng/mL	2.4	10.4	4.2	2.0	2.6	0.6
150/2,300 ng/mL	2.1	1.5	8.6	3.2	3.3	3.7
Between-run ($n = 5$)						
3/46 ng/mL	5.9	7.7	10.1	9.4	2.4	4.2
150/2,300 ng/mL	2.5	10.4	12.7	6.1	3.9	3.8
Matrix effects						
Theoretical concentration (ng/mL)	3/150	46/2,300	3/150	3/150	46/2,300	46/2,300
Matrix effect (%)	91.2/93.6	82.2/88.3	79.0/93.3	79.5/84.5	77.1/83.7	72.1/84.6
Relative matrix effect, CV (%)	4.5/2.1	5.0/2.7	7.7/2.5	6.5/1.5	5.2/0.8	6.3/2.2
Matrix effect corrected with IS (%)	104.7/105.6	94.3/99.6	90.6/105.2	99.7/100.4	96.6/103.5	90.2/104.6
Relative matrix effect toIS, CV (%)	1.1/1.6	4.2/2.9	5.2/1.3	2.3/0.3	2.5/2.7	3.8/3.0
Recovery (%) for QC ($n = 6$)						
3/46 ng/mL	63.7	68.4	67.5	57.8	66.1	61.8
150/2,300 ng/mL	67.2	71.8	67.7	66.2	72.4	69.7

All post-mortem specimens were sampled from the Regional Biobank of Central Norway (36), after approval by the Regional Committee for Medical and Health Research Ethics (approval 007–06) and the Director General of Public Prosecution.

Results and discussion

Method development and validation

The method produced sharp, symmetrical and well-separated peaks for codeine, C6G, norcodeine, morphine, M3G, M6G and IS codeine-d_3, morphine-d_3 and M3G-d_3 with practical retention times of 2.2, 1.9, 2.0, 1.6, 0.6, 1.0, 2.2, 1.5 and 0.5 min, respectively. SIM-chromatograms from an authentic forensic autopsy case are shown in Figure 2.

The validated ranges were 1.5–300 ng/mL for codeine, norcodeine and morphine, and 23–4,600 ng/mL for C6G, M3G and M6G, with exceptions for norcodeine in muscle (3–300 ng/mL), morphine in muscle, fat and brain (3–300 ng/mL) and M6G in fat (46–4,600 ng/mL). Estimated LLOQs based on S/N $\geq$ 10 were in the 0.15–3 ng/mL range for codeine, norcodeine and morphine, and in the 2.3–46 ng/mL range for C6G, M3G and M6G. Acceptable values for within-run and between-run accuracy (88.1–114.1%) and precision (CV 0.6–12.7%), ME (CV 0.3–13.5%, corrected with IS) and recovery (57.8–94.1%) were obtained at both concentration levels. Main validation parameters are presented in Tables I–V.

ME experiments showed consistent and significant ion suppression for most analytes, particularly at low theoretical concentrations (Tables I–V). However, this was effectively corrected with the applied IS. Assessment of ME also revealed high variability of ME for M3G and M6G at low theoretical concentrations in blood and muscle (CV 21.3–30.7%), but applied IS (M3G-d_3) compensated effectively for this variability. For all other analytes variability of ME was acceptable in all assessed matrices, also without IS correction (CV 1.3–13.9%).

Stability experiments showed no significant changes in the whole blood concentrations of the analytes. Concentration changes ranged from −1 to +8% (freeze–thaw) and from −12 to +7% (long term). The largest concentration changes were observed for codeine (−12%) and morphine (−10%) after long-term storage. This is in concordance with recent studies investigating the stability of opiates in whole blood under long-term storage at −20°C (34, 37). Processed samples showed good reproducibility for all analytes up to 48 h after sample preparation. Analysis >48 h after sample extraction, however, is not recommended as reinjection of processed samples at 72 h yielded divergent results for some of the analytes, and in particular for C6G.

Injection volumes of 2 and 5 μL were tested for all matrices on the LC–MS. For prepared samples of blood and fat injection of 2 μL resulted in slightly better reproducibility, whereas 5 μL was preferable for muscle and brain extracts. For fat extracts, however, LOD and LLOQ improved with higher injection volume, particularly for morphine and M6G. For vitreous fluid method performance did not differ with injection volume. Consequently, 2 μL was chosen for blood and vitreous fluid, and 5 μL for muscle, fat and brain.

To our knowledge, this article presents the first validated method for simultaneous determination of codeine, C6G, norcodeine, morphine, M3G and M6G in blood, vitreous fluid, muscle, fat and brain tissue. The validation parameters show that SPE/LC–MS methods still may serve as robust and reliable low-cost tools for demanding routine analyses.

Method application

The method was applied in two authentic forensic autopsy cases where codeine was implicated (Table VI); one with therapeutic

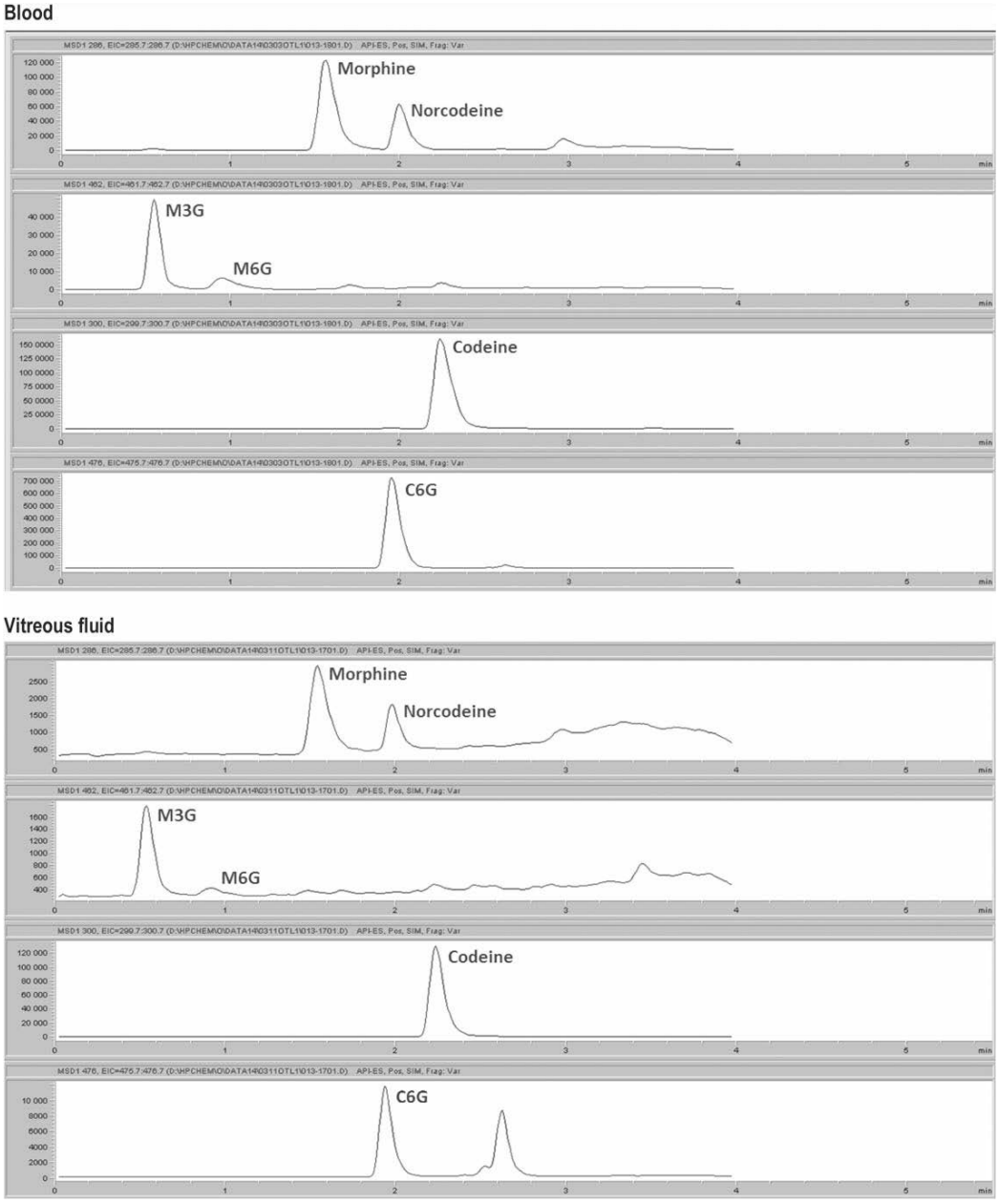

Figure 2. SIM-chromatograms for codeine, codeine-6-glucuronide (C6G), norcodeine, morphine, morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G) in post-mortem blood, vitreous fluid, muscle, fat and brain tissue from an authentic forensic autopsy case implicating codeine (case B).

concentrations of codeine, morphine and metabolites (case A), and one with potentially lethal concentrations of morphine and M6G following codeine ingestion (case B) (9). Both scenarios are frequently encountered in forensic toxicology casework. In either case urine specimens from the autopsy had been screened for the heroin-specific metabolite 6-monoacetylmorphine with negative result.

The measured concentrations of codeine and its metabolites show high variability between the different matrices in both cases. The results also show that relatively low concentrations

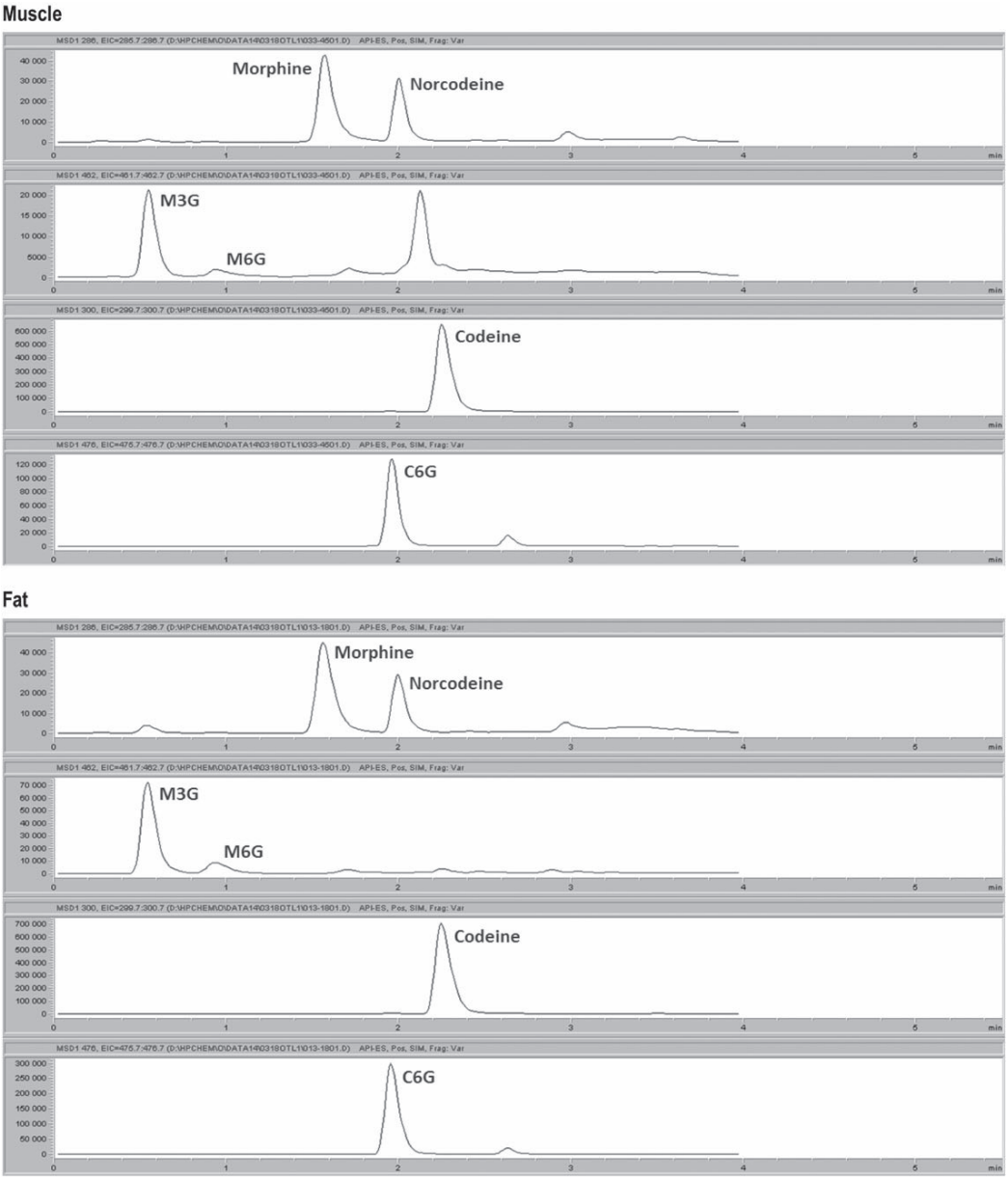

Fig. 2 *Continued*

of active metabolites (morphine and M6G) may occur even in cases with high codeine concentrations. These observations illustrate some of the problems that require attention in further studies of codeine-implicated poisonings, exceeding the scope of this article.

Conclusion

A simple and reliable LC–MS method for simultaneous determination of codeine, codeine metabolites C6G, norcodeine and morphine, and morphine metabolites M3G and M6G in postmortem whole blood, vitreous fluid, muscle, fat and brain tissue

Brain

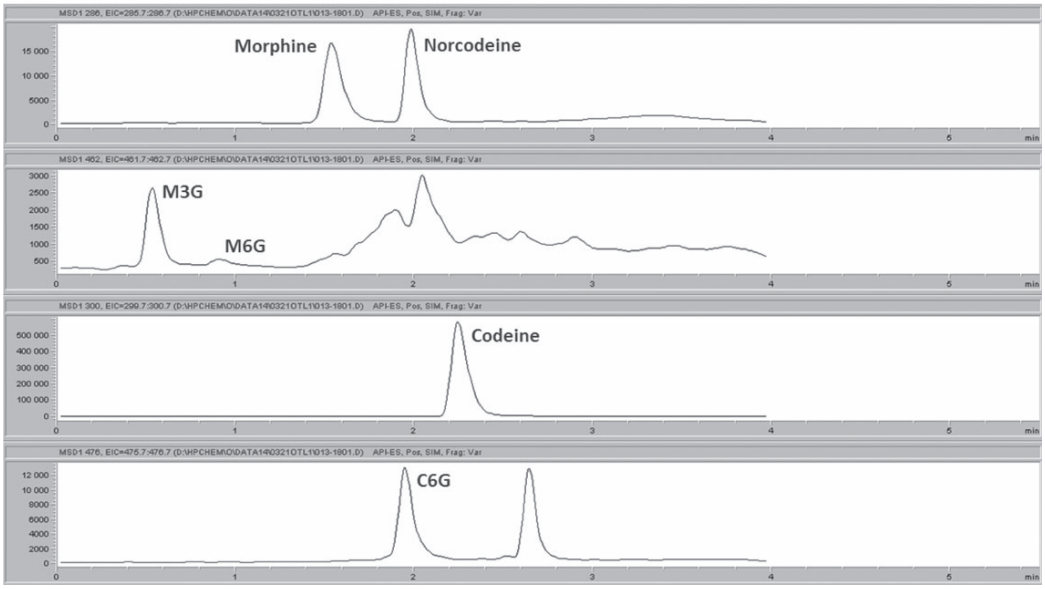

Fig. 2 *Continued*

Table VI

Concentrations (ng/mL or ng/g) of Codeine, Codeine-6-Glucuronide (C6G), Norcodeine, Morphine, Morphine-3-Glucuronide (M3G) and Morphine-6-Glucuronide (M6G) in Post-Mortem Blood, Vitreous Fluid, Muscle, Fat and Brain Tissue from Two Authentic Forensic Autopsy Cases

	Codeine	C6G	Norcodeine	Morphine	M3G	M6G
Case A						
Peripheral blood	221	3,525	17	3	125	<23
Heart blood	223	2,174	19	3	111	<23
Vitreous fluid	279	185	9	2	<23	0
Muscle	165	447	33	<3	<23	0
Fat	53	256	0	0	0	0
Brain	278	286	12	<3	<23	0
Case B						
Peripheral blood	8,770	17,121	500	521	1,855	606
Heart blood	1,578	2,179	73	114	328	75
Vitreous fluid	1,181	1,230	25	34	161	57
Muscle	1,870	1,938	171	141	385	91
Fat	2,130	5,660	121	106	775	213
Brain	1,899	380	139	69	<23	<23

was developed and validated. The method was applied in authentic forensic autopsy cases implicating codeine in both therapeutic and presumably lethal concentration levels.

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Paper IV

Post-mortem levels and tissue distribution of codeine, codeine-6-glucuronide, norcodeine, morphine and morphine glucuronides in a series of codeine-related deaths

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Abstract

This article presents levels and tissue distribution of codeine, codeine-6-glucuronide (C6G), norcodeine, morphine and the morphine metabolites morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G) in post-mortem blood (peripheral and heart blood), vitreous fluid, muscle, fat and brain tissue in a series of 23 codeine-related fatalities. CYP2D6 genotype is also determined and taken into account. Quantification of codeine, C6G, norcodeine, morphine, M3G and M6G was performed with a validated solid phase extraction LC-MS method. The series comprise 19 deaths (83%) attributed to mixed drug intoxication, 4 deaths (17%) attributed to other causes of death, and no cases of unambiguous monointoxication with codeine. The typical peripheral blood concentration pattern in individual cases was C6G>>codeine>>norcodeine>morphine, and M3G>M6G>morphine. In matrices other than blood, the concentration pattern was similar, although in a less systematic fashion. Measured concentrations were generally lower in matrices other than blood, especially in brain and fat, and in particular for the glucuronides (C6G, M3G and M6G) and, to some extent, morphine. In brain tissue, the presumed active moieties morphine and M6G were both below the LLOQ (0.0080 mg/L and 0.058 mg/L, respectively) in a majority of cases. In general, there was a large variability in both measured concentrations and calculated blood/tissue concentration ratios. There was also a large variability in calculated ratios of morphine to codeine, C6G to codeine and norcodeine to codeine in all matrices, and CYP2D6 genotype was not a reliable predictor of these ratios. The different blood/tissue concentration ratios showed no systematic relationship with the post-mortem interval. No coherent degradation or formation patterns for codeine, morphine, M3G and M6G were observed upon reanalysis in peripheral blood after storage.

Keywords: Forensic toxicology; codeine; toxicity; post-mortem; tissue distribution

1. Introduction

The opiate codeine is widely used in many countries as an analgesic and cough suppressant, either alone or in combination with other drugs, and is a frequent and often important finding in post-mortem forensic toxicology.

Codeine is mainly metabolized in the liver, although some intestinal and CNS metabolism probably occurs. The principal metabolic pathways are outlined in Fig. 1. A major part (50-70%) of a codeine dose is glucuronidated to codeine-6-glucuronide (C6G), while 10-15% is N-demethylated to norcodeine via the cytochrome P450 isoenzyme 3A4 (CYP3A4) [1]. Norcodeine is in turn glucuronidated to norcodeine-6-glucuronide (N6G), and a minor part is O-demethylated to normorphine [2, 3]. Of an ingested codeine dose, 0-15% is O-demethylated to morphine by the polymorphic cytochrome P450 isoenzyme 2D6 (CYP2D6), and further glucuronidated to the inactive metabolite morphine-3-glucuronide (M3G; approximately 60% of morphine formed) and the active metabolite morphine-6-glucuronide (M6G; 5-10% of morphine formed) [1]. A minor part of morphine is N-demethylated to normorphine [2, 3]. CYP2D6 activity may be significantly influenced by genetic polymorphisms and environmental factors such as inhibitory interactions from other drugs, which results in a large and unpredictable intra- and interindividual variability in the amount of morphine produced after ingestion of codeine [4-10].

Compared to morphine and M6G, codeine and its main metabolites C6G and norcodeine have weak affinity to opioid μ-receptors [11-13]. Normorphine has about one fourth of the μ opioid receptor affinity of morphine, and is produced in small amounts [3, 11]. Accordingly, the analgesic effects of codeine appear to be largely dependent on metabolic conversion to morphine by CYP2D6 [6, 14-16]. Whether this also applies to the toxicity of codeine,

however, remains a matter of controversy. Although unsubstantiated by receptor affinity studies [11-13], some investigators have suggested codeine, C6G and norcodeine as putative mediators of codeine toxicity [17-20].

Previous studies of codeine-related deaths have reported a limited array of codeine metabolites in biological specimens, particularly in matrices other than blood and urine. Published data derive from various case reports and series [5, 21-37], of which five [23, 30, 31, 35, 37] are large autopsy series. Published post-mortem concentrations of other codeine metabolites than morphine are limited to 2 cases of fatal and severe codeine intoxication [32], and a series of 31 unspecified autopsy blood samples [38]. Furthermore, published data regarding post-mortem redistribution of codeine and its metabolites are limited and inconsistent [21, 39-47].

In a previous study, we investigated simultaneous concentrations of codeine, morphine and morphine glucuronides in codeine-related deaths in relation to CYP2D6 genotype and assigned phenotype [48]. This study, however, was limited to post-mortem blood concentrations, and did not assess the main metabolites C6G and norcodeine.

To address these limitations in the literature, further investigations of the concentrations and tissue distribution of codeine and its metabolites in codeine-implicated deaths are warranted. This article presents levels and tissue distribution of codeine, C6G, norcodeine, morphine, M3G and M6G in post-mortem blood (peripheral and heart blood), vitreous fluid, muscle, fat and brain tissue in a series of 23 codeine-related fatalities where CYP2D6 genotype is taken into account.

2. Materials and methods

Forensic autopsy cases

All forensic autopsy cases in four counties in Central Norway (total population approximately 750,000) from September 2006 through December 2012 were reviewed. In Norway a forensic autopsy can, according to law and provision, be requested by the police under given circumstances, e.g. suspected accidents, drug-related deaths, suicides and sudden unexpected deaths [49]. In cases of suspected homicide or uncertain identity the police is obliged to request a forensic autopsy [49]. The combined clinical and forensic autopsy rate in Norway has been about 10 % in recent years, of which approximately 40 % are forensic autopsies [50, 51]. In our region the annual number of forensic autopsies is approximately 200. From this material, post-mortem toxicological specimens from 1,120 autopsies were analyzed. Among these, 100 cases with detectable amounts of codeine in post-mortem blood were identified, of which 23 had femoral blood concentrations exceeding the TIAFT toxicity threshold of 0.3 mg/L in the original toxicological analyses. Autopsy records from these 23 cases were reviewed and classified according to cause of death as determined by the forensic pathologists performing the autopsy. A study flow chart is presented in Fig. 2. In 18 of the 23 included cases additional specimens of post-mortem peripheral (femoral) blood, heart blood, vitreous fluid, muscular tissue (right psoas muscle), fat tissue (suprapubic fat) and brain tissue (right frontal cortex) were available for further analyses. In the other 5 cases, femoral blood was unavailable for further analyses in 2 cases, vitreous fluid unavailable in 2 cases, and both femoral and heart blood were missing in 1 case. All analytical results from available matrices in the 23 cases were included in the study. Reanalyses in femoral blood were performed with different femoral blood samples than those used in the original analyses. All samples were collected simultaneously at autopsy, by the same personnel, and in accordance with an

established procedure minimizing the risk of contamination, systematic and random errors. Samples were quickly and continuously stored at -80ºC.

Toxicological analysis

In the original toxicological analyses performed in connection with the forensic autopsy peripheral blood specimens were subjected to specific analyses for alcohols (ethanol, methanol, isopropanol, acetone) using a headspace gas chromatography-mass spectrometry (GC-MS) method, and specific analyses for benzodiazepines (diazepam, desmethyldiazepam, oxazepam, nitrazepam, 7-aminonitrazepam, flunitrazepam, desmethylflunitrazepam, 7-aminoflunitrazepam, clonazepam, 7-aminoclonazepam, alprazolam, midazolam), opioids (morphine, codeine, ethylmorphine, oxycodone, M3G, M6G) and amphetamines (amphetamine, methamphetamine, MDMA and MDA) using liquid chromatography-mass spectrometry (LC-MS) methods. In addition, blood specimens were screened against comprehensive drug libraries (National Institute of Standards and Technology Mass Spectral Library, Forensic Toxicology Retention Time Locking Database/Library and Pfleger/Maurer/Weber Drugs and Pesticides Library for Toxicology) with a GC-MS method. When available, urine was also screened for drugs of abuse using LC-MS methods. Positive screening results, as well as explicit information about drug use in the case histories, were confirmed by specific analyses in blood using LC-MS or GC-MS methods.

All quantitative analyses of codeine, C6G, norcodeine, morphine, M3G and M6G were performed with a validated and previously published solid phase extraction LC-MS method [52]. The validated ranges were 0.0015-0.30 mg/L for codeine, norcodeine and morphine, and 0.023-4.6 mg/L for C6G, M3G and M6G, with exceptions for norcodeine in muscle (0.0030-0.30 mg/L), morphine in muscle, fat and brain (0.0030-0.30 mg/L) and M6G in fat (0.046-4.6

mg/L). Because ~0.4 g muscle/fat/brain tissue was used to prepare 1 mL tissue homogenate, the actual lower limit of quantification (LLOQ) in the tissue analyses were 0.0040 mg/L for codeine, norcodeine and morphine, and 0.058 mg/L for C6G, M3G and M6G, with exceptions for norcodeine in muscle (0.0080 mg/L), morphine in muscle, fat and brain (0.0080 mg/L) and M6G in fat (0.12 mg/L). Samples with concentrations below the LLOQs were reported as negative. Samples with concentrations above the validated ranges were diluted and reanalyzed.

CYP2D6 genotyping

CYP2D6 genotyping was performed in all cases by isolating genomic DNA from peripheral leukocytes using either an iPrep™ Purification Instrument with iPrep™PureLink™ gDNA Blood Kit (Invitrogen Corporation, Carlsbad, USA) or a BioRobot® EZ1 with EZ1 DNA Blood 200 µl Kit (Qiagen, Hilden, Germany), according to the manufacturers' guidelines. In a majority of cases (N=15) the inactivating alleles *3, *4, *6, *7 and *8 were determined by allele-specific polymerase chain reaction (PCR) analysis and the PCR product was analyzed directly by agarose gel electrophoresis. These samples were also tested by long-PCR for the deletion of CYP2D6 (the inactivating allele *5) and for the duplicated/multiduplicated gene (the *2Xn mutation). In the remaining cases (N=8) the inactivating alleles *3, *4, *5, *6, *7, *8 and the *2Xn mutation were determined by Sanger sequencing and multiplex ligation-dependent probe amplification (MLPA). Alleles in which none of these variants were found were classified as *1 (wild-type) alleles. Cases with two functional (wild-type) alleles were assigned an extensive metabolizer phenotype, cases with any of the inactivating alleles alongside one functional allele were assigned an intermediate metabolizer phenotype, and cases with two inactivating alleles were assigned a poor metabolizer phenotype. Other allele

combinations, as well as the detection of strong CYP2D6 inhibitors, were also taken into account when assigning metabolizer phenotypes.

Ethics

This study is part of an ongoing research project which involves the establishment of a regional research biobank coupling post-mortem toxicological specimens and clinical data from forensic autopsy cases in Central Norway [53, 54]. The project has been approved by the Regional Committee for Medical and Health Research Ethics (approval 007-06) and the Director General of Public Prosecution.

3. Results

The presented series comprises 19 deaths (83%) attributed to drug intoxication (with multiple substances including codeine) and 4 deaths (17%) attributed to other causes of death (Fig. 2). No cases of death due to monointoxication with codeine occurred in this material. In all cases, at least one central nervous system depressant in addition to codeine and morphine (i.e. other opioids, benzodiazepines, Z-hypnotics, carisoprodol/meprobamate, pregabalin or ethanol) was detected in blood. In 19 of the 23 cases, two or more of these substances were found. Opioids other than codeine and morphine were detected in blood in 7 cases. Central nervous system stimulants (methamphetamine/amphetamine and/or cocaine/benzoylecgonine) were detected in blood in 3 cases. Paracetamol was found in 22 of the 23 cases, probably reflecting intake of paracetamol-codeine combinations.

Co-ingestion of morphine or heroin could not be ruled out as a contributing source to measured morphine and morphine glucuronide concentrations. In particular, co-ingestion of morphine was suspected in one case. Genotyping in this case was indicative of a CYP2D6

metabolizing capacity in the normal range (extensive metabolizer phenotype). The heroin-specific metabolite 6-monoacetylmorphine, however, was not detected in blood or urine in any of the cases. Ethylmorphine, which is partially metabolized to morphine by CYP2D6 similarly to codeine, was detected in blood in one case, but at a very low concentration (0.011 mg/L). In this case, CYP2D6 genotyping was indicative of a very low metabolizing capacity (poor metabolizer phenotype).

In just one case an immediate cause of death that was not drug intoxication could be ascertained (a case of carbon monoxide poisoning due to fire). In the remaining cases assigned other causes of death the significance of drug intake and inebriation was unclear. In one case the deceased was found with a plastic bag over the head fastened with tape around the neck, and death was attributed to suffocation in combination with drug intoxication. Another case involved a woman found dead at home shortly after a surgical procedure, and was presumed to be a case of sudden cardiac death based on the findings of coronary atherosclerosis. The last case involved a woman found dead on the seashore at wintertime, where the presumed cause of death was drowning, although typical findings ascribed to drowning were not present at autopsy. Because drug intake and inebriation cannot be ruled out as a contributory factor in the deaths attributed to other causes, the results in this study are presented for all cases combined.

Reliable information about the time of death was available in all cases. In no instance were the deceased found dead more than a few days after last being seen alive. Accordingly, there were no cases with extensive putrefaction in this material.

All cases in the series were genotyped and fell into three assigned phenotype categories: (1) extensive metabolizer (EM), indicative of a metabolizing capacity in the normal range (N=15; 65%), (2) intermediate metabolizer (IM), indicative of a metabolizing capacity in the lower normal range (N=5; 22%), and (3) poor metabolizer (PM), indicative of a very low metabolizing capacity (N=3; 13%). One of the cases assigned an EM phenotype had functional allele duplication alongside one inactivating allele. Two of the three PM cases had a genotype that normally would have been assigned an IM phenotype, but were assigned a PM phenotype due to simultaneous presence of a strong CYP2D6 inhibitor (paroxetine and levomepromazine, respectively) in blood. Weaker CYP2D6 inhibitors, such as citalopram and methadone were also detected in some cases, but were not considered significant in relation to phenotype. No findings of metabolic inductors (e.g. phenobarbital, carbamazepine, phenytoin) or CYP3A4 inhibitors were made. No cases with an ultrarapid metabolizer phenotype were identified in this material.

Measured levels of codeine, C6G, norcodeine, morphine, M3G and M6G are given in Table 1. The tissue distribution of the analytes is presented as ratios of concentrations in heart blood, vitreous fluid, muscle, fat and brain tissue relative to peripheral blood in Table 2. Only cases with concentrations above the LLOQ of the analytical method in both of the compared matrices are included in Table 2.

Concentration ratios of morphine to codeine (M/C-ratio), C6G to codeine (C6G/C-ratio) and norcodeine to codeine (NC/C-ratio) categorized by assigned CYP2D6 phenotype are given in Tables 3-5 for peripheral blood, heart blood, vitreous fluid, muscle, fat and brain tissue, respectively. Cases with concentrations of morphine, C6G or norcodeine below the LLOQ are

also included with assigned ratio 0. Cases with morphine, C6G or norcodeine concentrations above the LLOQ, but calculated ratio <0.005, are also assigned ratio 0.

Concentrations measured at the time of autopsy were compared to those measured at reanalysis in relation to this study. Concentration changes (%) for codeine, morphine, M3G and M6G in peripheral blood are displayed in Table 6, categorized by number of years between analyses. One case showing extreme increases in the peripheral blood concentrations of both codeine, morphine, M3G and M6G (+800%, +120%, +560% and +940%, respectively) is excluded from the table and discussed separately. No comparative values for C6G and norcodeine or concentrations in other matrices than peripheral blood were available.

4. Discussion

In general, there was a large variability in the measured levels and calculated concentration ratios in this material (Tables 1-2), and clear patterns in the distribution of analytes in the investigated matrices could not be distinguished. However, some tendencies were apparent: Firstly, concentrations tended to be lower in matrices other than blood for all analytes, especially in brain and fat. In particular, this seemed to be the case for the glucuronides (C6G, M3G and M6G) and, to some extent, morphine. It is emphasized that in a majority of cases where concentration ratios were missing, morphine, M3G and M6G were below the LLOQ in the non-blood matrix, and not in peripheral blood. Secondly, C6G and norcodeine were below the LLOQ far less frequently than morphine in matrices other than blood. This reflects that C6G and norcodeine generally were detected in higher concentrations than morphine in matrices other than blood, which becomes more apparent when assessing individual concentrations from case to case (data not shown). The typical peripheral blood distribution pattern in individual cases in this material was C6G>>codeine>>norcodeine>morphine, and

M3G>M6G>morphine. In matrices other than blood, the distribution pattern was similar, but in a less systematic fashion.

There was also a large variability in calculated M/C-ratios, C6G/C-ratios and NC/C-ratios, and assigned CYP2D6 phenotype was not a reliable predictor of the degree of morphine formation and possibly related variations in formed amounts of C6G and norcodeine in this material (Tables 3-5). No clear differences in M/C-ratio were observed between EMs and IMs, and the M/C-ratios were only slightly lower among PMs than EMs and IMs. With a possible exception for muscle, where the M/C-ratios appeared to be slightly higher, M/C-ratios were of a similar magnitude in the different matrices. Again, because of large spread in the data, the validity of this observation is uncertain. Indeed, significant disparity between skeletal muscle and blood concentrations of morphine with a lack of predictability has been described by others [55]. C6G/C-ratios showed a tendency towards lower values in matrices other than blood, but again the variability in the data was large and consistent patterns were lacking. While there was a tendency for C6G/C-ratios to increase in concert with presumed CYP2D6 metabolic capacity, NC/C-ratios did not show any distinctive patterns in relation to assigned CYP2D6 phenotype or type of matrix. A previous study assessing the relationship between genetic polymorphisms and drug interactions on codeine and morphine blood concentrations in 68 codeine-related deaths found no clear association between CYP2D6 genotype and M/C-ratio [37].

According to The International Association of Forensic Toxicologists (TIAFT), serum codeine concentrations above 0.3 mg/L have been associated with toxicity, whereas concentrations above 1.6 mg/L are considered possibly lethal [56]. Other compilations of toxic and fatal concentrations of drugs in blood indicate codeine concentrations of 0.5-1.0

mg/L as potentially toxic [57] and concentrations of 0.6-2.1 mg/L as potentially lethal [21, 30, 31, 57, 58]. The TIAFT list indicate that toxic effects of morphine have occurred at serum morphine concentrations above 0.15 mg/L [56]. For other codeine metabolites no such limits are established. All cases in this study where peripheral blood was available for reanalysis had a peripheral blood codeine concentration above 0.3 mg/L at reanalysis. In 7 of the cases (35%) the peripheral blood concentration of codeine was above 1.6 mg/L. In none of these cases M6G was above the LLOQ in brain, whereas morphine was above the LLOQ in brain in just 2 cases. Brain concentrations were also relatively low for C6G (below 0.6 mg/L) and norcodeine (below 0.2 mg/L) in these cases. Codeine brain concentrations were considerably lower than peripheral blood concentrations in a majority of cases, but showed large variability in an unpredictable way.

Receptor affinity studies have demonstrated a 100-600-fold greater μ opioid receptor affinity for morphine and M6G than for codeine, C6G and norcodeine [11-13]. M6G has been shown to possess μ opioid receptor affinity and analgesic potency similar to or greater than morphine in animal studies [11, 12, 59]. A marked increase in M6G potency has been reported as administration of M6G is shifted from systemic to intrathecal sites in rats and mice [59]. Still, the potency of M6G compared to morphine and its exact role and contribution to morphine and codeine effects in humans remain unsettled [59]. The generally low concentrations and frequent absence of detectable amounts of morphine and M6G in brain are thus of particular interest, as these moieties from a pharmacodynamic perspective may best represent the active principle. This observation might indicate that very low concentrations relative to the levels measured in blood is sufficient at the site of action to induce toxic opioid effects, or, alternatively, that codeine intake did not contribute significantly to the deaths. This matter remains unsolved, and may warrant detailed mapping of opioid levels in different parts of the

brain (and in cerebrospinal fluid) from victims of opioid toxicity with more sensitive methods than the one used presently. Indeed, considering that the potency of M6G may be significantly higher than that of morphine, and the event that very low concentrations of the active moieties within the CNS are sufficient to cause toxic opioid effects, the sensitivity of the applied analytical method may have been too low to fully assess the impact of M6G, and perhaps also of morphine. In this context, it should be noted that measured brain concentrations of C6G and norcodeine in general were low as well, both compared to peripheral blood concentrations of C6G and norcodeine, and brain concentrations of codeine (Table 1). In light of the low μ receptor affinities of these metabolites, this seems to provide little support for the conception that C6G and/or norcodeine are responsible for the toxic effects of codeine, or that high concentrations of C6G/norcodeine at the site of action have overcome low receptor affinity in these deaths. The latter, however, cannot be ruled out for codeine in some of the cases. The concentrations in brain tissue should be interpreted with caution, as opiate concentrations have been shown to vary significantly from one brain region to another in the same subject, although homogenously distributed within investigated brain segments [60]. The analyzed brain specimens in this study were collected from the right frontal cortex. Even though μ opioid receptors are located in several layers of the cerebral cortex it is difficult to assess to what extent the measured concentrations in this area of the brain reflect relevant concentrations at the site of action, e.g. without knowing the density and distribution of opioid receptors in the sampled tissue, differences in regional blood flow, etc. Interestingly, a previous autopsy study of 'lingering deaths' after heroin overdose found higher concentrations of morphine, codeine and 6-monoacetylmorphine in the medulla oblongata (site for cardiac and respiratory control) than in femoral blood [61]. A study of rats subjected to heroin and morphine overdose, on the other hand, found no support for brain accumulation of morphine as an explanation for delayed deaths or formation of toxic concentrations of

M6G [62]. Results from studies of morphine metabolism in mice and rats, however, do not necessarily apply to humans, as some of these studies have indicated that M6G is not formed in measurable quantities in these species [63-65].

A recent study evaluating the relationship between post-mortem and ante-mortem concentrations of codeine and morphine in whole blood concluded that factors such as metabolism and post-mortem interval may affect post-mortem concentrations in an unpredictable manner, and that both codeine and morphine appeared to be affected by post-mortem redistribution in a majority of cases [45]. Investigations of the relation of the different concentration ratios to the post-mortem interval in the present material did not show any clear correlations or systematic patterns (data not shown).

Investigations of the peripheral blood concentration changes from the original autopsy analysis to the reanalysis in this study provided no coherent degradation or formation patterns for codeine, morphine, M3G and M6G in relation to the time between analyses (Table 6). In particular, there were no clear indications of post-mortem deglucuronidation of M3G and/or M6G to morphine, which has been described in a study where whole blood samples were incubated for 2 weeks at 37°C [66]. The differences in measured concentrations were well above ±15% in many instances. In particular, considerable decreases in morphine and increases in morphine glucuronide concentrations were observed in individual cases, but again there was a large spread in the data, and no consistent changes in concentrations over time was seen. It should be noted that all reanalyses in peripheral blood in relation to this study were performed with different blood samples than those used in the original analyses in connection with the forensic autopsy, and that this, to some extent, might account for differences in measured concentrations. However, all samples were collected simultaneously

during autopsy, and analyzed with the same LC-MS method. A particular case showing extreme increases in peripheral blood concentrations in all analytes was excluded from Table 6. In this case neither reanalysis of the study blood sample, nor review of the original analyses could provide any explanation for the large discrepancies. This sequence of concentration changes, however, appear biologically implausible.

All samples were collected by the same personnel, and in accordance with an established procedure minimizing the risk of contamination and errors. Samples were quickly and continuously stored at -80ºC. The applied analytical method was fully validated. Repeated analyses with and without dilution in particular cases yielded acceptable reproducibility. Thus, the large variability and lack of appreciable patterns in the data in this material is largely believed to be due to inherent properties of the post-mortem material. These inherent factors possibly efface the impact of other parameters, such as the post-mortem interval and CYP2D6 genotype.

Even though all included cases had peripheral blood concentrations above the TIAFT toxicity threshold of 0.3 mg/L this material contains no monointoxications with codeine. Indeed, in many cases there were additional toxicological findings of presumably greater toxicological significance. The toxicological significance of codeine intake and metabolism to active metabolites in individual cases is thus largely unknown and likely to vary from case to case.

5. Conclusions

This material indicates generally lower concentrations of the investigated analytes in matrices other than blood post-mortem, especially in brain and fat, and in particular for the glucuronides (C6G, M3G and M6G) and, to some extent, morphine. The observation that the

presumed active moieties morphine and M6G both were below the LLOQ in brain in a majority of cases is of particular interest, and warrants further investigations of opioid levels within the CNS in victims of opioid toxicity with more sensitive methods than the one used presently. Measured brain concentrations in this study seem to provide little support for the conception of C6G/norcodeine as mediators of codeine toxicity. No systematic patterns indicating post-mortem formation, degradation or redistribution of any of the investigated analytes could be established in this material. The generally large variability and lack of appreciable patterns in the data may be due to inherent properties of the post-mortem material, possibly effacing the impact of other parameters, such as the post-mortem interval and CYP2D6 genotype. It may also possibly be due to variable toxicological significance of codeine intake and bioconversion to active metabolites within this material.

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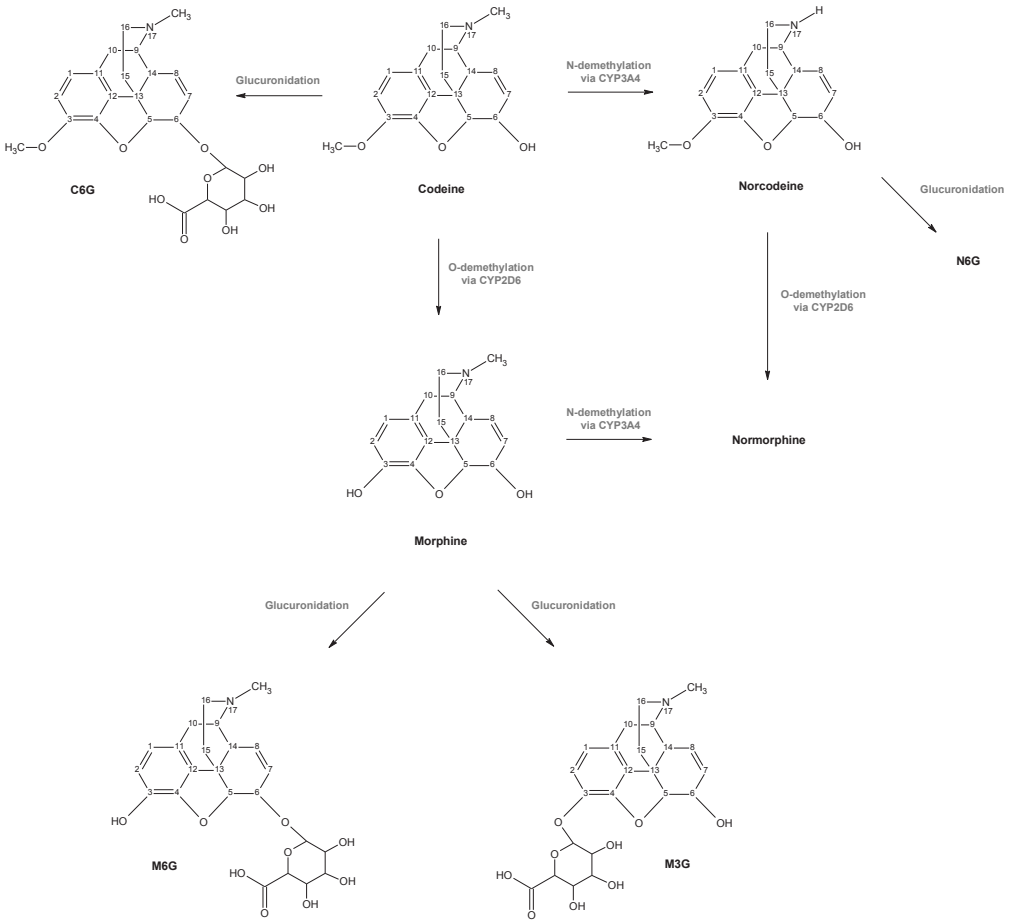

Fig. 1. Principal pathways for codeine metabolism in man. C6G = Codeine-6-glucuronide; N6G = Norcodeine-6-glucuronide; M3G = Morphine-3-glucuronide; M6G = Morphine-6-glucuronide; CYP2D6 = cytochrome P450 isoenzyme 2D6; CYP3A4 = cytochrome P450 isoenzyme 3A4.

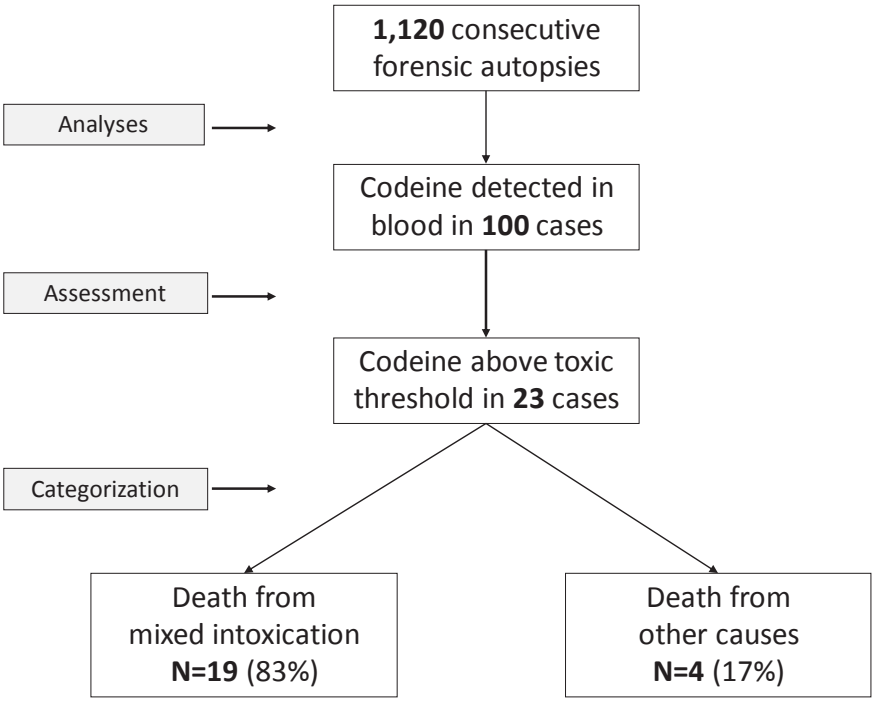

Fig. 2. Forensic autopsy cases involving codeine in Central Norway from September 2006 through December 2012. Analyses included screening against a comprehensive drug library and quantitative liquid chromatography-mass spectrometry (LC-MS) and gas chromatography-mass spectrometry (GC-MS) methods. Assessment was based on the TIAFT toxicity threshold (0.3 mg/L), and categorization on the forensic pathologist's conclusion as to the cause of death.

Table 1
Measured levels (mg/L) of codeine, codeine-6-glucuronide (C6G), norcodeine, morphine, morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G) in peripheral blood, heart blood, vitreous fluid, muscle, fat and brain tissue. N = number of cases with available data; SD = standard deviation of the mean.

	Codeine				C6G				Norcodeine			
	N	Mean	Range	SD	N	Mean	Range	SD	N	Mean	Range	SD
Peripheral blood	20	1.8	0.32 - 8.8	2.1	20	14	0.31 - 124	28	20	0.27	0.018 - 1.2	0.34
Heart blood	22	0.94	0.007 - 3.7	1.1	22	1.6	0.029 - 9.1	2.0	22	0.078	0.004 - 0.36	0.083
Vitreous fluid	21	0.93	0.016 - 4.9	1.2	21	1.0	0.071 - 8.8	1.8	21	0.040	0.002 - 0.13	0.036
Muscle	23	0.85	0.019 - 4.4	1.1	23	0.94	0 - 5.7	1.3	23	0.12	0 - 0.59	0.15
Fat	23	0.49	0.006 - 3.1	0.75	23	1.7	0 - 11	2.6	23	0.089	0 - 1.4	0.28
Brain	23	0.78	0.010 - 4.9	1.1	23	0.25	0 - 0.91	0.25	23	0.053	0 - 0.23	0.057
	Morphine				**M3G**				**M6G**			
	N	Mean	Range	SD	N	Mean	Range	SD	N	Mean	Range	SD
Peripheral blood	20	0.075	0.002 - 0.52	0.13	20	0.89	0 - 6.9	1.6	20	0.19	0 - 1.8	0.40
Heart blood	22	0.031	0 - 0.31	0.065	22	0.11	0 - 0.33	0.10	22	0.019	0 - 0.084	0.029
Vitreous fluid	21	0.018	0 - 0.20	0.042	21	0.048	0 - 0.25	0.071	21	0.008	0 - 0.058	0.019
Muscle	23	0.057	0 - 0.46	0.11	23	0.11	0 - 1.2	0.26	23	0.014	0 - 0.16	0.040
Fat	23	0.016	0 - 0.12	0.034	23	0.12	0 - 0.77	0.23	23	0.016	0 - 0.21	0.053
Brain	23	0.013	0 - 0.11	0.027	23	0	0 - 0	-	23	0	0 - 0	-

Table 2
Concentration ratios of codeine, codeine-6-glucuronide (C6G), norcodeine, morphine, morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G) in heart blood, vitreous fluid, muscle, fat and brain tissue relative to peripheral blood. N = number of cases with available data; SD = standard deviation of the mean.

	Heart blood/ Peripheral blood				Vitreous fluid/ Peripheral blood				Muscle tissue/ Peripheral blood				Fat tissue/ Peripheral blood				Brain tissue/ Peripheral blood			
	N	Mean	Range	SD	N	Mean	Range	SD	N	Mean	Range	SD	N	Mean	Range	SD	N	Mean	Range	SD
Codeine	20	1.0	0.002 - 4.2	1.2	18	0.7	0.01 - 2.2	0.6	20	0.6	0.006 - 2.7	0.8	20	0.2	0.001 - 2.1	0.5	20	0.6	0.001 - 2.5	0.8
C6G	20	0.6	0.001 - 2.9	0.7	18	0.5	0.003 - 5.5	1.2	19	0.1	0.003 - 0.7	0.2	18	0.3	0.0005 - 2.5	0.6	15	0.1	0.0006 - 0.5	0.1
Norcodeine	20	0.9	0.01 - 5.0	1.2	18	0.3	0.01 - 1.4	0.4	19	0.6	0.01 - 2.6	0.7	14	0.3	0.01 - 3.1	0.8	18	0.3	0.004 - 1.1	0.3
Morphine	18	1.1	0.08 - 6.1	1.3	12	0.3	0.03 -1.4	0.4	10	0.6	0.02 - 1.5	0.4	6	0.3	0.04 - 0.8	0.3	7	0.1	0.04 - 0.2	0.1
M3G	14	0.6	0.02 - 2.0	0.6	8	0.3	0.008 - 0.8	0.3	6	0.5	0.007 - 2.6	0.9	6	0.4	0.02 - 1.0	0.4	0	-	-	-
M6G	8	0.7	0.01 - 3.0	0.9	2	0.5	0.04 - 0.9	0.4	1	0.1	-	-	2	0.7	0.1 - 1.2	0.5	0	-	-	-

Table 3
Morphine to codeine ratios (M/C-ratio) in peripheral blood, heart blood, vitreous fluid, muscle, fat and brain tissue by assigned phenotype of the cytochrome P450 isoenzyme 2D6 metabolizing capacity. EM = extensive metabolizer, indicative of a metabolizing capacity in the normal range; IM = intermediate metabolizer, indicative of a metabolizing capacity in the lower normal range; PM = poor metabolizer, indicative of a very low metabolizing capacity; N = number of cases with available data; SD = standard deviation of the mean.

M/C-ratio

	EM				IM				PM			
	N	Mean	Range	SD	N	Mean	Range	SD	N	Mean	Range	SD
Peripheral blood	12	0.05	0.00 - 0.25	0.07	5	0.07	0.01 - 0.30	0.11	3	0.02	0.00 - 0.04	0.02
Heart blood	14	0.07	0.00 - 0.45	0.11	5	0.04	0.00 - 0.10	0.03	3	0.02	0.00 - 0.03	0.01
Vitreous fluid	14	0.02	0.00 - 0.14	0.03	4	0.02	0.00 - 0.04	0.02	3	0.00	0.00 - 0.01	0.01
Muscle	15	0.11	0.00 - 0.69	0.19	5	0.33	0.00 - 1.49	0.59	3	0.01	0.00 - 0.02	0.01
Fat	15	0.02	0.00 - 0.20	0.05	5	0.02	0.00 - 0.06	0.02	3	0.03	0.00 - 0.09	0.04
Brain	15	0.03	0.00 - 0.29	0.07	5	0.02	0.00 - 0.09	0.03	3	0.00	0.00 - 0.00	0.00

Table 4
Codeine-6-glucuronide to codeine ratios (C6G/C-ratio) in peripheral blood, heart blood, vitreous fluid, muscle, fat and brain tissue by assigned phenotype of the cytochrome P450 isoenzyme 2D6 metabolizing capacity. EM = extensive metabolizer, indicative of a metabolizing capacity in the normal range; IM = intermediate metabolizer, indicative of a metabolizing capacity in the lower normal range; PM = poor metabolizer, indicative of a very low metabolizing capacity; N = number of cases with available data; SD = standard deviation of the mean.

C6G/C-ratio

	EM				IM				PM			
	N	Mean	Range	SD	N	Mean	Range	SD	N	Mean	Range	SD
Peripheral blood	12	5.9	0.50 - 26	7.1	5	2.1	0.67 - 4.0	1.5	3	2.3	0.44 - 4.7	1.8
Heart blood	14	2.6	0.14 - 12	3.0	5	1.9	0.60 - 4.2	1.2	3	0.85	0.09 - 1.6	0.60
Vitreous fluid	14	1.8	0.05 - 11	3.0	4	1.1	0.44 - 1.9	0.52	3	0.29	0.15 - 0.41	0.11
Muscle	15	3.1	0.04 - 21	5.3	5	0.91	0.00 - 2.3	0.85	3	0.68	0.12 - 1.7	0.74
Fat	15	4.1	0.10 - 16	4.1	5	2.9	0.00 - 7.8	2.7	3	1.4	0.00 - 3.7	1.6
Brain	15	1.3	0.00 - 12	3.0	5	0.44	0.00 - 0.93	0.34	3	0.02	0.00 - 0.04	0.02

Table 5
Norcodeine to codeine ratios (NC/C-ratio) in peripheral blood, heart blood, vitreous fluid, muscle, fat and brain tissue by assigned phenotype of the cytochrome P450 isoenzyme 2D6 metabolizing capacity. EM = extensive metabolizer, indicative of a metabolizing capacity in the normal range; IM = intermediate metabolizer, indicative of a metabolizing capacity in the lower normal range; PM = poor metabolizer, indicative of a very low metabolizing capacity; N = number of cases with available data; SD = standard deviation of the mean.

NC/C-ratio

	EM				IM				PM			
	N	Mean	Range	SD	N	Mean	Range	SD	N	Mean	Range	SD
Peripheral blood	12	0.12	0.02 - 0.40	0.10	5	0.27	0.08 - 0.49	0.15	3	0.15	0.05 - 0.32	0.12
Heart blood	14	0.24	0.02 - 1.8	0.43	5	0.26	0.12 - 0.47	0.13	3	0.12	0.04 - 0.23	0.08
Vitreous fluid	14	0.08	0.01 - 0.45	0.12	4	0.09	0.06 - 0.12	0.02	3	0.06	0.03 - 0.12	0.04
Muscle	15	0.20	0.00 - 0.87	0.27	5	0.59	0.05 - 1.9	0.68	3	0.20	0.03 - 0.51	0.22
Fat	15	0.10	0.00 - 0.40	0.11	5	0.16	0.00 - 0.28	0.11	3	0.17	0.00 - 0.48	0.22
Brain	15	0.18	0.00 - 1.1	0.29	5	0.16	0.04 - 0.33	0.10	3	0.09	0.02 - 0.23	0.10

Table 6
Concentration changes (%) of codeine, morphine, morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G) in peripheral blood from time of autopsy to time of reanalysis, categorized by number of years between analyses. N = number of cases with quantitative results from both analyses.

Time (years)	Codeine			Morphine			M3G			M6G		
	N	Mean	Range	N	Mean	Range	N	Mean	Range	N	Mean	Range
2	1	+8	-	1	-28	-	1	-13	-	1	-33	-
3	3	-1	-43 – +36	3	-16	-46 – +13	3	-8	-52 – +35	1	-17	-
4*	3	-15	-14 – -15	3	-25	-37 – -11	2	-20	-24 – -16	0	-	-
5	5	-14	-35 – -4	5	-26	-36 – -17	5	-2	-25 – +49	3	-8	-26 – +19
6	2	+10	+6 – +14	2	-33	-38 – -28	2	+19	+6 – +32	1	+17	-
7	3	+6	-11 – +31	2	-31	-39 – -23	3	+74	+21 – +136	3	+93	-29 – +233
8	1	-11	-	1	+32	-	1	+42	-	1	+92	-
Total	18	-4	-43 – +36	17	-22	-46 – +32	17	+13	-52 – +136	10	+31	-33 – +233

*One case showing extreme concentration increases of both codeine, morphine, M3G and M6G (+800%, +120%, +560% and +940%, respectively) is excluded from the table and discussed separately