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JENNI VIINAMÄKI

Analysis of Fatal Poisonings Due to Toxic Alcohols and Drugs — Focus on Metabolites

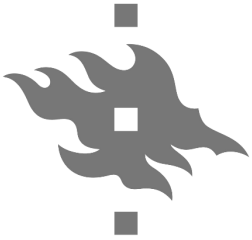
DEPARTMENT OF FORENSIC MEDICINE
FACULTY OF MEDICINE
DOCTORAL PROGRAMME IN POPULATION HEALTH
UNIVERSITY OF HELSINKI

Department of Forensic Medicine
Faculty of Medicine
Doctoral Programme in Population Health
University of Helsinki

ANALYSIS OF FATAL POISONINGS DUE TO TOXIC ALCOHOLS AND DRUGS

Focus on Metabolites

Jenni Viinamäki



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SUPERVISOR:

Professor Ilkka Ojanperä
Department of Forensic Medicine
University of Helsinki, Finland

REVIEWERS:

Dr. Seija Ylijoki-Sørensen
National Institute for Health and Welfare
Forensic Medicine Unit
Helsinki, Finland

Professor Heikki Vuorela
Faculty of Pharmacy
Division of Pharmaceutical Biosciences
University of Helsinki, Finland

OPPONENT:

Dr. Gudrun Høiseth
Norwegian Institute of Public Health
Oslo, Norway

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Nothing in nature is random. A thing appears random only through the incompleteness of our knowledge.

Baruch Spinoza

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following articles, which are referred to in the text by Roman numerals **I-V**

I. Rasanen I, Viinamäki J, Vuori E, Ojanperä I. *Headspace in-tube extraction gas chromatography-mass spectrometry for the analysis of hydroxylic methyl-derivatized and volatile organic compounds in blood and urine*, Journal of Analytical Toxicology **2010:** 34 (3); 113-121.

II. Viinamäki J, Rasanen I, Vuori E, Ojanperä I. *Elevated formic acid concentrations in putrefied post-mortem blood and urine samples*, Forensic Science International **2011:** 208; 42-46.

III. Viinamäki J, Sajantila A, Ojanperä I. *Ethylene Glycol and Metabolite Concentrations in Fatal Ethylene Glycol Poisonings*, Journal of Analytical Toxicology **2015**: 39 (6); 481-485.

IV. Viinamäki J, Ojanperä I. *Photodiode array to charged aerosol detector response ratio enables comprehensive quantitative monitoring of basic drugs in blood by ultra-high performance liquid chromatography*, Analytica Chimica Acta **2015:** 865; 1-7

V. Viinamäki, J. Ojanperä, I. *Concurrent estimation of metabolite concentrations along with parent drug quantification in post-mortem blood*, Forensic Science International **2016**:267; 110-114

AUTHOR'S CONTRIBUTION

Publication I
The author designed the experiments in collaboration with Professor I. Ojanperä and Dr. I. Rasanen. The practical laboratory work was carried out by the author. The results were interpreted and the manuscript was prepared together with the co-authors. In this publication, I. Ojanperä was the corresponding author.

Publication II
The author designed the experiments in collaboration with Professor I. Ojanperä and Dr. I. Rasanen. The practical laboratory work and preliminary interpretation of the results were carried out by the author. The final interpretation of the results and the conclusions about the results were made together with the co-authors. The author drafted the manuscript and it was revised in consultation with the co-authors. The author was the corresponding author in this publication.

Publication III
The author designed the experiments in collaboration with Professor I. Ojanperä. The practical laboratory work and preliminary interpretation of the results were carried out by the author. The final interpretation of the results and the conclusions about the results were made together with the co-authors. The author drafted the manuscript, which was revised in consultation with the co-authors. The author was the corresponding author in this publication.

Publication IV
The author designed the experiments in collaboration with Professor I. Ojanperä. The practical laboratory work and preliminary interpretation of the results were carried out by the author. The final interpretation of the results and the conclusions about the results were made together with the co-author. The author drafted the manuscript, which was revised in consultation with the co-author. The author was the corresponding author in this publication.

Publication V
The author designed the experiments in collaboration with Professor I. Ojanperä. The practical laboratory work and preliminary interpretation of the results were carried out by the author. The final interpretation of the results and the conclusions about the results were made together with the co-author. The author drafted the manuscript, which was revised in consultation with the co-author. The author was the corresponding author in this publication.

ABBREVIATIONS

ADH	Alcohol dehydrogenase
ALDH	Aldehyde dehydrogenase
CAD	Charged aerosol detector
CLND	Chemiluminescent nitrogen detector
CNS	Central nervous system
COM	Calcium oxalate monohydrate
CSI	Captive spray ionization
DAD	Diode array detector
EG	Ethylene glycol
EI	Electron ionization
ELSD	Evaporative light scattering detector
ESI	Electrospray ionization
FA	Formic acid
FID	Flame ionization detector
FTIR	Fourier transformation infrared spectroscopy
GA	Glycolic acid
GC	Gas chromatography
HPLC	High performance liquid chromatography
ITEX	In-tube extraction
LC	Liquid chromatography
LOD	Limit of detection
LOQ	Limit of quantification
MeOH	Methanol
MS	Mass spectrometry
NAD	Oxidized nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide
NSI	Nanospray ionization
OX	Oxalic acid
PLOT	Porous open layer tubular column
PMR	Post-mortem redistribution
RPLC	Reversed-phase liquid chromatography
SI	Similarity index
SIM	Selected ion monitoring
SPME	Solid phase microextraction
TDM	Therapeutic drug monitoring
TFA	Trifluoroacetic acid
UHPLC	Ultra-high performance liquid chromatography
UV	Ultraviolet
V_d	Volume of distribution
VOC	Volatile organic compounds

ABSTRACT

Post-mortem toxicology seeks to determine cases in which death is a consequence of the intake of, or exposure to, acutely toxic compounds, most often drugs or alcohols. In Finland, 800-900 deaths are caused by fatal poisonings due to drugs or alcohols annually, ethanol being the most common individual substance responsible. Other alcohols, mainly methanol and ethylene glycol, cause around ten fatal poisoning cases each year. Drugs, both medicinal and illicit, cause 400-500 fatal poisonings annually. Volatile and semivolatile organic compounds are involved in fewer than ten fatal poisonings annually. Despite the fact that the majority of fatal poisonings are caused by relatively few individual compounds, toxicological laboratories must be capable of identifying and quantifying a wide range of possible toxicants. Small sample volumes, putrefactive changes, and post-mortem redistribution processes impose special demands on both the laboratory investigation and the interpretation of results. The two main objectives of the present thesis are, first, to develop new analytical laboratory methods for toxic alcohols and drugs and, second, to apply these methods to post-mortem toxicology cases in order to generate reference data on lethal concentrations for interpretation purposes. Particular attention is given to the analysis of metabolites and to assessing the significance of metabolite concentrations.

Metabolites of toxic substances constitute an interpretive resource that is far from fully exploited in post-mortem toxicology. The therapeutic, toxic, and lethal concentrations of alcohols and drugs are usually expressed as the concentration of the parent compound in blood. Yet the toxicity of methanol and ethylene glycol, for example, is mainly caused by their toxic metabolites, formic acid and glycolic acid, respectively. A multitude of therapeutic drugs are transformed to metabolites that possess pharmacological activity similar to or greater than that of the parent drug. However, quantification of metabolites may be missed out due to the lack of appropriate analytical methods or the difficulty in acquiring primary reference standards for the metabolites.

A method for the quantitative analysis of ethylene glycol, glycolic acid, and formic acid and for the screening of volatile and hydroxylic organic compounds was developed, using headspace in-tube extraction–gas chromatography-mass spectrometry (ITEX-GC-MS). Due to the fairly low prevalence of both toxic alcohols and volatile organic compounds in fatal poisoning cases, the idea of utilizing the same ITEX-GC-MS instrumentation as two method variants, methyl-derivatized and underivatized, offered a cost-effective way of performing these analyses.

In fatal poisonings by methanol or ethylene glycol, the concentration of the parent alcohol varied significantly, whereas the concentrations of formic acid and glycolic acid in blood were found to be more uniform. Fatal metabolite threshold concentrations were established for those poisoning cases that had not received hospital treatment. Practically no ethanol was detected in these cases as ethanol has a higher affinity for alcohol dehydrogenase and is eliminated first. In cases involving

putrefaction, significant post-mortem production of formic acid was detected, and consequently the concentrations of metabolites should always be interpreted together with those of the parent alcohols.

A comprehensive method for the quantitative monitoring of basic drugs in blood samples was developed utilizing ultra-high performance liquid chromatography coupled with two consecutive detectors: diode array detector and corona charged aerosol detector (UHPLC-DAD-CAD). This method enabled simultaneous identification and quantification of over 150 basic drugs and metabolites in a single run, utilizing historic one-point calibration. The identification power of the method was enhanced by using the response ratio of the two detectors as an identification parameter in addition to retention time and ultraviolet spectrum.

Based on the universal response of CAD, the UHPLC-DAD-CAD method was evaluated for the quantification of drug metabolites using a secondary calibration method with the corresponding parent drug as a calibration reference standard. This approach offers a straightforward way of quantifying metabolites, especially *N*-demethylated metabolites, on a routine basis with an uncertainty comparable to ordinary bioanalysis. This is a clear advantage over mass spectrometry based methods, which usually suffer from highly varying responses between parent drugs and metabolites.

The metabolite concentrations of certain toxicologically relevant drugs were studied in post-mortem blood samples by UHPLC-DAD-CAD using the secondary calibration method. It was found that the metabolite to parent ratios in post-mortem blood samples were generally comparable to the standard ratios defined in clinical therapeutic drug monitoring. With the highest post-mortem parent drug concentrations, the ratios were below the clinical normal ranges, suggesting acute ingestion or poisoning. These findings encourage forensic toxicologists to gather extensive metabolite concentration data and actively to use the metabolite to parent ratio in the interpretation of post-mortem toxicology results.

1. INTRODUCTION

A post-mortem toxicological investigation is performed together with a forensic autopsy if there are suspicions, circumstances, or autopsy findings that suggest substance abuse or poisoning that may have had an influence on the death case. A post-mortem toxicological investigation should also be performed in cases of suspected unnatural deaths, such as homicides, traffic accidents, occupational accidents, and malpractice, when the deceased is a child or a young adult, and when the cause of death remains unknown at autopsy. Deaths of known drug and alcohol abusers are often associated with accidental or suicidal overdoses [1]. In Finland, a forensic pathologist evaluates the significance of the toxicological findings in the death, occasionally after consulting a forensic toxicologist. Autopsy findings and laboratory investigations as well as anamnestic and circumstantial evidence help the forensic pathologist to conclude the cause and manner of death. The causes of death are coded according to the ICD-10 classification of the World Health Organization (WHO). Information from death certificates is then used in the national cause of death database and national mortality statistics. Currently, the medico-legal autopsy rate and the post-mortem toxicology rate from all deaths in Finland are as high as 17% and 12%, respectively, while the medico-legal autopsy rate in many European countries is lower.

Annually 800-900 deaths in Finland are caused by fatal poisoning due to alcohols or drugs. In 2013, there were 861 fatal poisonings of which 312 were alcohol poisonings and 476 poisonings by drugs and medicines. Carbon monoxide caused 62 poisonings, and 11 poisonings were due to miscellaneous substances such as hydrogen cyanide and other volatile compounds. Of the alcohol poisonings, methanol (MeOH) and ethylene glycol (EG) caused seven and four deaths, respectively [2]. In post-mortem toxicology, the main emphasis has traditionally been on detecting and quantifying the toxicants in their unchanged form in the blood, and the cause of death has been determined largely from these concentrations [3]. Furthermore, published therapeutic and toxic reference concentrations are mainly for parent compounds in clinical plasma samples [4]. The importance of metabolites has lately become evident in the interpretation of forensic cases. The toxicity of MeOH and EG is mainly caused by their acidic metabolites, formic acid (FA) and glycolic acid (GA), and many medicinal drugs have metabolites that possess pharmacological activity similar to the parent drug.

Quantification of FA and GA is useful, as the concentrations can be used as criteria for starting hemodialysis [5]. Interestingly, analytical methods for the simultaneous quantification of both the parent alcohols and their metabolites in emergency toxicology have not been presented until very recently [6,7]. In therapeutic drug monitoring (TDM), the importance of metabolite quantification has been emphasized both for drugs that have pharmacologically active metabolites and for inactive metabolites to ascertain patients' compliance and ability to metabolize drugs [8]. Furthermore, normal ranges of metabolite to parent ratios

have been published [9], and ratios deviating from these ranges can indicate problems in drug adherence or altered metabolism caused by genetic variation or drug-drug interaction.

In post-mortem toxicology, the sample volume is often limited, so there is a demand for comprehensive screening methods that can be used for simultaneous quantification of the compounds detected [10]. Gas chromatography-mass spectrometry (GC-MS) has long been a widely recognized technique in comprehensive drug screening [11,12]. In view of the non-volatile and hydrophilic nature of many current drugs, liquid chromatographic (LC) methods, especially coupled to MS, have become increasingly important [12,13]. MS-based quantification methods, however, are calibration-intensive and rely on primary reference standards for each analyte analyzed in the same sequence of runs with the samples. Quantification of drug metabolites is thus often disregarded, because the availability of reference standards is limited and their prices are high.

Non-MS detection in chromatography offers a more stable response with less calibration work. The emergence of universal detectors, notably the corona charged aerosol detector (CAD), shows potential for quantification without primary reference standards. As the CAD response is basically independent of the chemical structure of the analyte, quantification might be performed using a secondary calibration standard.

In the absence of post-mortem reference values for potentially toxic substances, and especially for their metabolites, the reference values obtained from living subjects are commonly used to interpret post-mortem toxicological results. However, due to post-mortem redistribution (PMR), the concentrations of drugs may be significantly different between ante-mortem and post-mortem samples [14]. On the other hand, the metabolite to parent ratios of antidepressants, for example, have shown to remain constant even when the absolute concentrations are affected by post-mortem changes [15,16].

These considerations call for improved laboratory methods for the analysis of metabolites along with the parent toxicants in post-mortem samples, and further, the routine utilization of these methods in casework to accumulate concentration data for interpretation purposes.

2. REVIEW OF THE LITERATURE

2.1 Toxic alcohols and drugs

2.1.1 Toxic alcohols and volatile compounds

Toxic alcohols, mainly methanol (MeOH) and ethylene glycol (EG), are used in products such as windshield washing fluids and antifreeze agents available in petrol stations and stores. They have intoxicating effects similar to those of ethanol, but their toxicity is much higher. The lethal dose of both MeOH and EG for an adult is approximately 100 mL, or 1–2 mL/kg for MeOH and 1.4–1.6 mL/kg for EG [17-19]. With MeOH, permanent visual damage may occur with as little as 0.1 mL/kg [20]. Both substances cause fatal poisonings, either accidental when the toxic alcohol is confused with ethanol or when contaminated ethanol is consumed, or intentional in cases of suicide or homicide. EG poisonings are usually individual cases, but MeOH poisonings occur both as individual events and as epidemics [21]. MeOH poisoning epidemics occur world-wide and are usually related to contaminated beverages sold as ethanol [22-26]. Simultaneous ingestion of MeOH and EG is rare, and only one fatal case has been reported so far [27].

The metabolisms of MeOH and EG are illustrated in Fig. 1. MeOH and EG are metabolized in the liver similarly to ethanol, first by alcohol dehydrogenase (ADH) to formaldehyde and glycoaldehyde, respectively, and further by aldehyde dehydrogenase (ALDH) to formic acid (FA) and glycolic acid (GA), respectively [28,29]. GA is further metabolized to glyoxylic acid and oxalic acid (OX). OX combines with calcium to form the poorly soluble calcium oxalate monohydrate (COM) and dihydrate crystals that can be detected in urine [30]. In addition, approximately 20% of EG is excreted unchanged via the kidneys. Even though formaldehyde and glycoaldehyde are toxic compounds, they have a minor impact in the toxicity of MeOH and EG as they are rapidly converted to FA and GA. Glycoaldehyde has not been detected in acute EG poisonings and the glyoxylate concentrations have been <0.2 mM [30,31]. Compared to other phases of the metabolism, the conversion of FA to carbon dioxide and conversion of GA to glyoxylic acid is slow, which causes their accumulation and makes them responsible for the toxic effects.

FA, and especially OX, are present in mammals in small amounts [32-37], mainly due to degradation of amino acids and external sources like diet. Small amounts of MeOH are also present in alcoholic beverages, and accumulation of FA has been detected in the brains of chronic alcohol abusers [38].

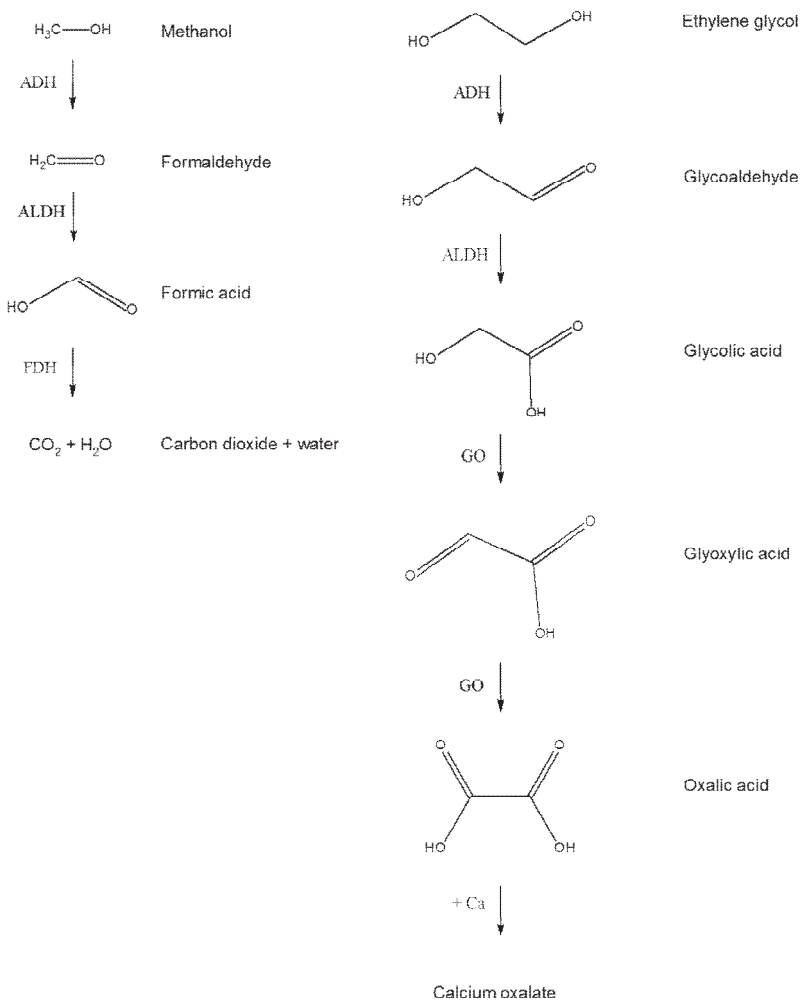


Figure 1. Metabolisms of methanol and ethylene glycol. ADH: alcohol dehydrogenase, ALDH: aldehyde dehydrogenase, GO: glycolate oxidase, FDH: formate dehydrogenase.

After ingestion of MeOH or EG, there is a latent period before symptoms appear, approximately 12–24 h for MeOH and 6–12 h for EG [21]. Simultaneous ingestion of ethanol delays the symptoms, as ADH's affinity for ethanol is 10-20 times higher than for MeOH and some 100 times higher than for EG, and thus ethanol is metabolized first [17,39,40]. The symptoms of MeOH poisoning include visual disturbances, central nervous system (CNS) depression and metabolic acidosis [19,20]. These symptoms are mainly caused by FA, which inhibits the mitochondrial cytochrome oxidase leading to cellular hypoxia [41,42], damages the myelin sheaths of the optic nerves leading to visual damage [43-46], and causes necrotic damage in the basal ganglia leading to CNS depression [47]. Metabolic acidosis is also caused

by FA, both directly and indirectly [48]. FA increases the ratio between reduced and oxidized nicotinamide adenine dinucleotide (NADH/NAD ratio), forcing the conversion of pyruvate to lactate [41,49]. Both lactic acid and FA contribute to the anion gap and acidosis, FA in the early stages of MeOH poisoning, lactic acid in the later stages.

Symptoms of EG poisoning include CNS depression with seizures, cardiopulmonary complications, acute renal failure and delayed neurological sequelae [50,51]. Like FA, GA increases the NADH/NAD ratio and leads to increased levels of lactic acid. The metabolic acidosis in EG poisonings is due to the joint effect of GA, OX, and lactic acid [19]. The renal outcome is caused by COM crystals that deposit in the renal tubules and cause necrotic cell death [30,52,53]. The CNS alterations in EG poisonings are partly caused by the acidic metabolites and metabolic acidosis, partly by deposition of COM crystals in the cerebral vessels [19,54].

Diagnosing toxic alcohol intoxication as early as possible is critical in order to begin treatment, but it may be challenging as the early stages of MeOH and EG intoxications present the same kind of symptoms as intoxication with ethanol [55]. Furthermore, the infrared breath alcohol analyzers used to estimate blood ethanol concentrations can falsely report MeOH as ethanol even in cases where no ethanol has been consumed [56]. This can lead to delayed diagnosis or misdiagnosis and adverse outcome or even death. The gold standard for diagnosis is direct measurement of MeOH or EG in blood or urine samples with gas chromatographic (GC) methods [17,55,57]. As GC instrumentation is only available in specialized laboratories and in major hospital laboratories, enzymatic methods have been developed for MeOH [58], FA [59] EG [60,61] and GA [62]. These methods, however, suffer from false-positive results caused by less toxic alcohols like ethanol [63], propylene glycol, and endogenous compounds [64], and usually require confirmation analysis with chromatographic methods. Other diagnostic tools include measurement of the osmolal gap, anion gap and acidosis [55,57,65]. However, these indirect tests are unspecific, and abnormal results can be due to causes other than ingestion of the toxic alcohols. Their value has been questioned, especially with EG poisonings [66]. If EG intoxication is suspected, urine samples can be screened for calcium oxalate crystals. This test can also produce both false-positive and false-negative results, as calcium oxalate crystals are not always present in intoxication cases [55,67], while OX from dietary sources can cause formation of crystals [35,55].

Treatment of toxic alcohol poisonings has two important goals: to inhibit the metabolism of MeOH or EG using either ethanol or fomepizole, and to remove toxic metabolites by hemodialysis [28,29,48,68-70]. With MeOH poisonings, the treatment can also include enhancement of FA metabolism with folic acid. Measurement of the serum concentration of FA or GA is recommended as an aid to evaluating whether hemodialysis should be started [5,26,71-73].

Despite the generally well known fact that toxicity of MeOH and EG is directly correlated with the FA or GA concentration in blood [28,72,74], many laboratories still screen only for the parent alcohols, and clinical criteria for initiating or

terminating hemodialysis are based on the concentration of MeOH or EG. Recently, chromatographic methods utilizing GC with flame ionization detection (FID) and GC coupled with mass spectrometry (GC-MS) have been presented for fast and efficient quantitative screening of EG, GA and 1,2-propylene glycol [6,7]. These methods are suitable for emergency toxicology, as the turnaround time is about 30 minutes and no specialized equipment is required. A combination of quick colorimetric enzymatic tests that can differentiate between ethanol, MeOH, EG and diethylene glycol has been presented [75]. This method utilizes saliva samples and can be performed outside clinical laboratories. In addition, a bedside test for FA was recently presented [73].

A few papers have reported the concentrations of MeOH, EG, and their acidic metabolites in post-mortem blood samples [76-79], as well as the distribution of FA in fatal MeOH poisoning [80]. Plasma FA concentrations in healthy subjects have also been reported [32-34]. However, in these previous studies the number of cases has been fairly low. In particular, post-mortem reference concentrations of FA in blood or urinary concentrations of EG and GA in fatal poisonings have not been available.

Volatile organic compounds (VOC) are a group of miscellaneous chemically divergent compounds, including aliphatic and aromatic hydrocarbons, oxygenated compounds such as alcohols, ethers, nitrites, and halogenated compounds like inhalation anesthetics. Because of their low price and wide availability they are abused to achieve euphoria and intoxication [81]. Even though the prevalence in VOC abuse has decreased in recent decades [82,83], these compounds still cause fatalities, especially among young people [81]. VOC are readily absorbed via the lungs, and due to the extensive capillary surface area in the lungs the peak concentration in blood is reached rapidly. Due to their generally high lipophilic properties, VOC are distributed to tissues with high lipid content, such as the CNS, liver and kidneys. Most deaths associated with VOC abuse are caused by direct toxicity of the compounds inhaled [84], and the most common cause of mortality is acute cardiac toxicity [83]. For this reason, the main attention in post-mortem toxicology is given to the parent compounds detected in blood as they provide a direct link to toxicity [81]. If there is a need to evaluate recent exposure or continuous environmental or occupational exposure, screening urine samples for the metabolites of VOC can be used [85,86].

When interpreting the results of VOC analysis, care is needed as volatile compounds can easily evaporate if the sample containers are not properly closed. Furthermore, VOC are formed as a consequence of post-mortem changes from degrading tissues. Putrefaction is a complicated process that is greatly affected by the circumstances during the post-mortem period, such as humidity, temperature and microorganisms [87,88]. There are studies concerning the VOC forming during putrefaction [89-93], but these reports mainly focus on the compounds released from decomposed cadavers or carcasses, not compounds detected in blood.

2.1.2 Medicinal drugs and their metabolites

In many countries, illicit and licit drugs constitute the largest portion of fatal poisonings. In Finland, the majority of fatal drug poisonings are caused by medicinal drugs [2]. Table 1 lists the 25 most prevalent single drugs as the principal finding in fatal poisonings in 2013 along with the number of fatal poisonings caused by these drugs. Interestingly, the majority of these drugs are therapeutic substances that generally originate from treatment. Buprenorphine involves a considerable amount of smuggling, and the amphetamines group contains mainly illicit drugs. Approximately two-thirds of these fatal poisonings were caused by concomitant use of one or more drugs and/or alcohol. Opioids were found in 30–40% of all fatal drug poisoning cases.

Table 1. *The 25 most prevalent single drugs as principle finding in fatal poisonings in Finland in 2013 with number of cases in parenthesis [2].*

Opioids	**Antidepressants**	**Sedatives**	**Antipsychotics**	**Miscellaneous**
Buprenorphine (50)	Amitriptyline (22)	Pregabalin (26)	Quetiapine (21)	Paracetamol (17)
Tramadol (40)	Venlafaxine (17)	Temazepam (13)	Levomepromazine (15)	Insulins (17)
Codeine (22)	Bupropion (15)	Zopiclone (13)	Chlorprothixene (9)	Amphetamines* (10)
Oxycodone (22)	Doxepin (9)	Alprazolam (10)	Olanzapine (8)	Amlodipine (9)
Methadone (12)	Clozapine (8)			Metformin (8)
Fentanyl (11)				Propranolol (7)

* Includes all amphetamine-like stimulants

Therapeutic concentrations of drugs are conventionally presented as the concentration of parent drug in a serum or plasma sample at the steady state, and extensive lists of therapeutic and toxic plasma concentrations are available [4,94]. However, many medicinal drugs, such as antidepressants, antipsychotics, and opioids, have pharmacologically active metabolites that possess pharmacological activity similar to the parent drug. There is a recommendation within therapeutic drug monitoring (TDM) to use the combined concentrations of the parent drug and the metabolite for certain drugs with active metabolites [8,9]. These pairs include for example amitriptyline and nortriptyline, clomipramine and norclomipramine, doxepin and nordoxepin, risperidone and 9-hydroxyrisperidone, and venlafaxine and *O*-desmethylvenlafaxine. Use of the combined concentration has, however, been criticized [95], as the affinity for target protein between parent drugs and metabolite can be significantly different. For example, amitriptyline and nortriptyline have opposite affinities for serotonin and norepinephrine transporters, which

complicates the assessment of their activity [96]. Doxepin acts as an antihistamine and sedative, whereas nordoxepin has effects similar to those of tricyclic antidepressants [96].

In addition to quantifying the active metabolites, measurement of the concentrations of less active metabolites is also recommended in TDM in order to gain information on the patient's compliance and ability to metabolize drugs. To aid the interpretation of metabolite concentrations, ranges of normal metabolite to parent ratios for some antidepressants and antipsychotics have been suggested [9]. Deviation from these ranges can indicate altered metabolism due to altered liver function, polymorphism on the liver cytochrome P450 (CYP) enzymes, or drug-drug interactions. In particular, changes in CYP2D6 and CYP2C19 affect the metabolism of several drugs with pharmacologically active metabolites [97].

In the past, the therapeutic and toxic ranges defined with living subjects have also been applied in post-mortem toxicology due to the lack of post-mortem reference values. However, these clinical reference concentrations should be applied with caution, as post-mortem redistribution (PMR) can significantly alter the drug concentrations in blood samples taken several days after death [98-103]. Particularly in the case of those lipophilic drugs with a high volume of distribution (V_d), the concentrations measured in post-mortem blood samples can be significantly higher, even when there is no reason to suspect overdose [99,101]. For other drugs, such as some benzodiazepines and cocaine, the concentration in post-mortem blood can be lowered by bacterial metabolism after death [104,105] or instability of the compound in the biological matrix [106]. Furthermore, water-soluble drugs and drugs with high protein binding and blood to plasma ratio below unity, such as some anti-epileptics, have been shown to possess lower post-mortem concentrations [107,108].

Currently there are a few compilations of post-mortem blood drug reference concentrations available, but attention has mainly been given to the parent drugs and data on metabolite concentrations is scarce [108-111]. A positive exception is the article by Reis et al. [16] which reports drug and metabolite concentrations and metabolite to parent ratios for 15 antidepressants divided into groups as follows: cases of fatal poisoning by a single drug, cases of fatal poisoning where other drugs are also involved, cases with other causes of death, and clinical TDM cases. Other available data on post-mortem concentrations of drug metabolites consists mainly of case studies including individual cases or case series [112-115] or studies focusing on the genotype's effect on the parent and metabolite concentrations [116-122].

2.2 Analytical laboratory methods

2.2.1 Analysis of hydroxylic and volatile compounds

Ethanol is the most commonly analyzed substance in both clinical and forensic toxicology, and several methods for ethanol determination are available. In clinical settings methods based on enzymatic oxidation, mainly ADH, are used [123]. ADH oxidizes ethanol to acetaldehyde using the coenzyme NAD+, which at the same time is reduced to NADH. After reduction, NADH is measured spectrophotometrically. Enzymatic methods are fast, sensitive, and simple to use and generally meet all analytical requirements except specificity [124]. However, as there is a need to distinguish ethanol from other alcohols and volatile compounds, chromatographic methods are considered superior in alcohol analysis.

Using chromatographic methods enables the detection and quantification of volatile alcohols such as MeOH and isopropyl alcohol, and volatile compounds used as denaturation agents, such as acetone and methyl ethyl ketone, simultaneously with ethanol. The most widely used chromatographic method for alcohol analysis in toxicology laboratories worldwide is GC-FID with static headspace sampling [125]. In headspace-GC-FID analysis the sample is diluted with an aqueous solution of the internal standard, usually n-propanol or t-butanol, and heated in an air-tight glass vial. A sample of the gaseous phase is then injected into the GC and analyzed under isothermal conditions with a run time of a few minutes. The main advantages of headspace-GC-FID are its ease of automation, sensitivity, accuracy, and specificity, especially when two columns with different polarities are used [126]. The precision and accuracy of headspace-GC-FID methods have proved to be very good, generally the coefficients of variation are 3–5% [127-129]. Furthermore, headspace sampling has been shown to extend column life and prevent injector contamination, as the gaseous phase contains fewer components than the biological matrix [125,130]. GC-FID methods with direct injection [131-133] as well as headspace-solid phase microextraction (SPME) [134,135] have been presented with the aims of reducing the sample volume required and increasing the sensitivity of alcohol analysis. The accuracy of the SPME method, however, has proved to be poorer than that of the static headspace method [136]. GC-MS methods designed to achieve more unequivocal identification of other volatile compounds simultaneously with ethanol quantification have been published [126,137,138]. However, these methods are not practical in routine alcohol analysis because of their much longer turnaround time.

Because of its high boiling point, EG cannot be determined as such with headspace-GC methods, and consequently it is seldom included in routine forensic alcohol analysis. A few chromatographic methods for simultaneous determination of volatile alcohols and EG have been published, but without including FA and GA [139-141]. These methods utilize direct injection into the GC-FID after deproteinization by ultrafiltration or protein precipitation with acetonitrile. Traditionally EG has been derivatized with phenylboronic acid to form its phenylboronate ester followed by GC-FID analysis [142-144]. This method,

however, does not enable the determination of GA. Adding isobutyl chloroformate as a second derivatizing agent enables the determination of 1,2-propylene glycol and GA along with EG by either GC-FID [6] or GC-MS [7] using direct injection. Using silylation [145-147] or derivatization with heptafluorobutyric acid [148] enables simultaneous determination of GA by GC-FID [145] or GC-MS [146-148] using direct injection. FA is usually determined as a methyl formate ester following methylation with MeOH in sulfuric acid using headspace-GC-FID [77,78,149]. No headspace methods for the analysis of EG and GA have been published prior to the present thesis.

The diversity of VOC poses a challenge to toxicological analysis. Most published methods focus on identification or quantification of a group of chemically similar compounds such as lighter gas components [150], aromatic hydrocarbons [151-153], petroleum products [154], inhaled solvents [155], and propellant gases used in different aerosol products [156]. Due to their highly volatile nature, analysis of VOC has traditionally been performed with headspace-GC coupled with either FID [155,157-159], MS [137,138,150] or Fourier transform infrared spectroscopy (FTIR) [160]. In addition to static headspace methods, dynamic headspace methods such as headspace-SPME [152,161] and purge-and-trap sampling [151,160,162] have also been presented. The chromatographic separation can be performed with commonly used capillary GC columns, but using a column with a greater film thickness results in better separation of VOC [81]. Even though FID has good sensitivity for hydrocarbons, identification of VOC is simplified when spectral detectors like MS or FTIR are used. For low molecular weight compounds, the MS spectra are less specific and FTIR can provide more information [81]. The sensitivity of FTIR, however, is lower than that of MS, and most current methods utilize MS detection [137,150,152,156].

2.2.2 In-tube extraction (ITEX)

In-tube extraction (ITEX) is a dynamic headspace microextraction technique that has been commercially available since 2006 [163]. As with SPME, the equipment consists of a gastight syringe, a needle filled with sorbent material and an external heater. The extraction procedure has four stages: sample conditioning, extraction, injection by thermal desorption, and trap cleaning. Unlike with SPME, the traps can be used multiple times and sampling is fully automated. The extraction procedure is illustrated in Fig. 2. In the first phase, the sample is heated and mixed, resulting in equilibrium of the analytes in the sample and the gas phase above the sample. Once equilibrium has been reached, the gas phase is aspirated through the sorbent-packed needle several times, enriching the analytes in the sorbent and resulting in a new equilibrium between the sample and the gas phase after each extraction stroke. After the extraction, the sorbent is flash heated and the analytes are injected into the GC injector by thermal desorption. In the cleaning phase, the trap is flushed with inert gas to prevent carryover.

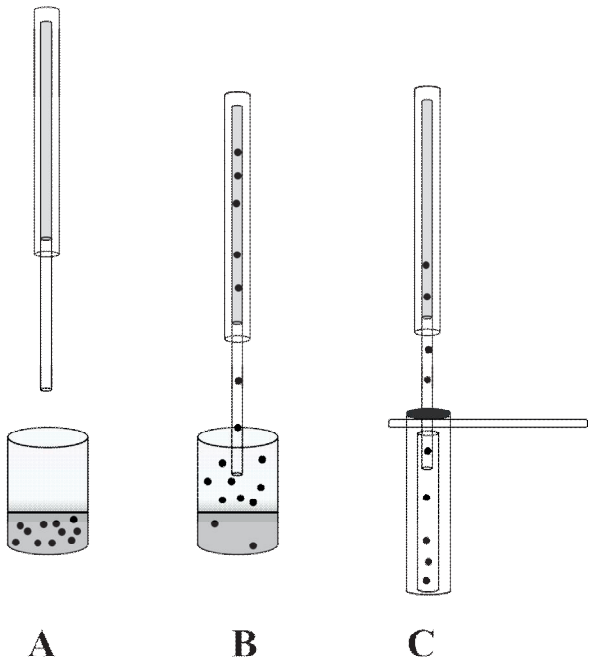


Figure 2. Principle of in-tube extraction (ITEX). A: Sample is heated and mixed until equilibrium is reached. B: Gas phase (headspace) is repeatedly pumped through a heated needle containing Tenax trap. C: Trap is flash heated and analytes are desorbed into GC injector.

Several factors affect the extraction efficiency, the most notable being the sorbent material, the temperatures of the sorbent and the sample, the number of extraction strokes, and the extraction speed [164,165]. Each extraction stroke increases the sensitivity, and for most compounds maximum sensitivity cannot be reached even after 50 strokes [165]. As each cycle increases the analysis time, a compromise has to be reached between sensitivity and throughput of the method.

ITEX-GC-MS has been utilized in the analysis of volatile organic hydrocarbons from aqueous samples [165,166], volatile aroma compounds in alcoholic and non-alcoholic beverages [167-170] and plants [163], aliphatic hydrocarbons in petroleum source rocks [171], and for metabolic profiling of oxidized lipid-derived volatiles in blood [172]. No ITEX applications have been published for the analysis of toxic alcohols and their metabolites within clinical or post-mortem toxicology prior to the present thesis.

2.2.3 Liquid chromatography-diode array detection (LC-DAD)

Electron ionization (EI) GC-MS in the full scan mode is a standard technique for comprehensive drug screening due to its sensitivity and specificity and the excellent reproducibility of mass spectra, which enables the use of large spectral libraries [173]. In the selected ion monitoring (SIM) mode, the technique has established its position in the confirmation analysis of target drugs. However, only thermally stable and sufficiently volatile drugs can be detected in their native form, while others require derivatization [174,175]. Derivatization can lead to formation of artifacts from drugs or metabolites, complicating identification. The hydrophilic and nonvolatile nature of many drugs makes reversed-phase liquid chromatography (RPLC) a suitable method for toxicological analysis as no derivatization is needed [176,177]. Furthermore, of the toxicologically interesting compounds, some 90% have significant ultraviolet (UV) absorption, making DAD a useful general detector [173,175,178]. The high long-term stability of DAD detection compared to MS techniques is a clear advantage and enables quantification using historic calibration [173,179].

In RPLC, chromatographic separation of analytes is performed on a nonpolar stationary phase with an aqueous, moderately polar mobile phase [177]. DAD detection is based on UV absorption caused by the analyte chromophores, generally the conjugated π-electrons and the free electron pairs of heteroatoms [173]. Two molecules sharing the same absorption system can usually be distinguished because of the electronic or steric effects caused by other parts of the molecule. If a molecule has more than one chromophore, the UV spectrum is approximately the sum of the spectra caused by each chromophore [173].

The applicability of LC-DAD methods in forensic toxicology casework was presented already in the 1990s [180]. The evolution of software and detectors has enabled the use of LC-DAD in routine analysis [173,181-185]. Advances in detector technology and software made it possible to collect and process the whole UV spectrum, which enables the monitoring of peak purity, detecting co-eluting peaks [186,187], and comparison with spectral libraries [173]. In similarity comparison, the two spectra are described as vectors in an n-dimensional space, where n is the number of absorbance-wavelength pairs. The length of the vector is only proportional to the concentration of the compound. Similarity between two spectra is expressed as the angle between the two vectors, θ, or as cosine θ, called similarity index (SI) [173,188,189]. The smaller the θ, or the closer to 1 the SI is, the more similar the spectra are. SI>0.9990 (or $\theta<2.56$) is considered positive identification. Between SI=0.9900 and SI=0.9990 ($2.6<\theta<8.1$), the spectra are very similar, but differences may occur [173].

Since the introduction of LC coupled to tandem mass spectrometry (LC-MS/MS), LC-DAD methods have been held in lower esteem, as the UV spectra display only a limited number of maxima and minima, and there is no simple correlation to the chemical structure [178]. However, the identification power of LC-DAD was proved to be comparable to GC-MS as long ago as the 1990s, when spectral data was used in conjunction with retention indices [181]. In the analysis of

61 toxicologically relevant drugs, an accuracy rate of 96.7% was achieved when both the UV spectra and relative retention times were used for identification [190]. These results are comparable to those obtained with conventional GC-MS methods. In a study of over 2,500 drugs, 84.2% of analytes were distinguishable from each other when UV spectra and relative retention time were used [178].

However, the limitations of LC-DAD methods include lower sensitivity compared to MS-based methods [185] and problems in detecting compounds with little or no UV absorption [176]. Furthermore, the shape of the UV spectrum is dependent on the mobile phase pH, which makes the use of commercial UV spectral libraries difficult [178]. Spectral libraries have been made available, but if these are to be used the analytes should be measured under similar conditions, i.e. with the same kind of aqueous buffer at the same pH as that used to build the library [173].

2.2.4 Ultra-high performance liquid chromatography (UHPLC)

The weaknesses of high performance liquid chromatography (HPLC) were the moderately low separation efficiency and long analysis times, which limit its use in the separation of complex mixtures [191]. It was shown long ago that substantially better separation efficiencies and shorter analysis times can be achieved with smaller stationary phase particles [192]. However, decreasing the column particle size increased the backpressure above the allowed HPLC pressure limit of usually 6 000 psi, unless the column length was reduced at the same time [193]. Increasing the column temperature lowers the viscosity of the mobile phase and hence the backpressure, but, especially in the analysis of pharmaceutical compounds, the column temperature is typically limited to < 60°C to avoid on-column degradation of analytes [193]. For this reason, 250 mm × 4.6 mm i.d. columns packed with 5 µm particles were mostly used in HPLC separations. The first commercial UHPLC instrument with a system pressure limit of 15 000 psi was introduced in 2004, and after improvements in detector technology, applications utilizing UHPLC have increased [194]. The UHPLC columns are typically 100–150 mm long with an i.d. of 2.1 mm, packed with pH-stable, sub-2 µm particles [191]. The introduction of UHPLC has considerably improved the performance of LC systems in terms of resolution, speed and reproducibility [177]. However, LC-MS systems rarely utilize the full power of UHPLC due to restrictions in the mobile phase composition.

2.2.5 Corona charged aerosol detector (CAD)

The idea of universal aerosol detection for LC was first introduced in 2002 [195] and the detector was made commercially available in 2004. The operating principle of CAD is illustrated in Fig. 3. The principle is similar to that of atmospheric pressure chemical ionization (APCI). The LC mobile phase is first nebulized to droplets using a nitrogen flow, and the resulting aerosol is transported through a drift tube where the remaining solvent and volatile components are evaporated off. Analyte particles

are then collided with a stream of positively charged nitrogen and the charge is transferred to the particles. The charged particles are directed to the collector where the charge is measured using a sensitive electrometer [196,197]. The response of the detector is independent of the chemical or physical properties of the analyte and directly proportional to the weight of the analyte present. However, as with MS and evaporative light scattering detection (ELSD), the composition of the mobile phase has an effect on the response [198]. The peak areas have been shown to increase nearly five-fold when the proportion of acetonitrile was increased from 10% to 90%. This effect can be corrected using an inverse gradient. The response of CAD is not absolutely linear, but over a range of two orders of magnitude a linear response has been achieved [197,199].

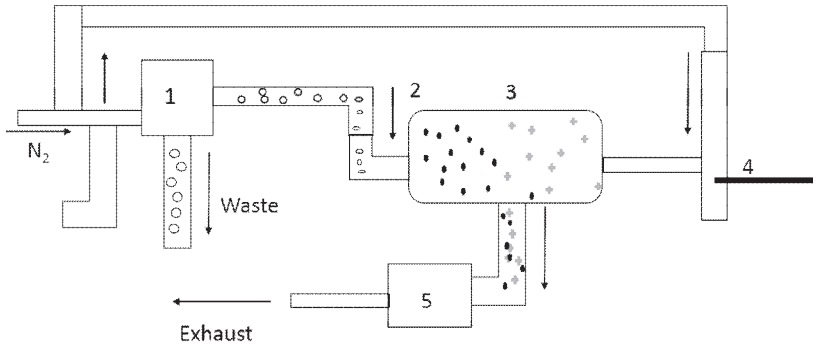


Figure 3. Operating principle of the corona charged aerosol detector (CAD). Mobile phase from LC is nebulized into droplets in the nebulizer (1) and large droplets are removed. The droplets are dried into particles in the drying tube (2). In the charge transfer chamber (3) the particles are collided with nitrogen gas charged using a corona discharge needle (4) and the charge is transferred to the particles. Charged particles are transferred to a collector (5) where charge is measured with an electrometer.

Compared with other universal detectors, CAD has several advantages. Unlike chemiluminescent nitrogen detector (CLND) and UV detectors, it is truly universal for non-volatile compounds. Analytes lacking chromophores and nitrogen can also be detected. Depending on the analyte structure, it has also been shown to be more sensitive than UV or ELSD [196,197,200]. In addition, the linearity of CAD has proven to be better than that of ELSD [196].

CAD has been used in the analysis of pharmaceutical products, both for detecting impurities [201-206] and for quantifying active pharmaceutical ingredients [197,200,207-210]. The detector has also been used in the analysis of antibiotics in biological samples [211], pollutants in water [212] and food additives in food supplies and beverages [213-215]. No CAD applications have been published for the analysis of drugs and metabolites within clinical or post-mortem toxicology prior to the present thesis.

2.3 Quantification of drug metabolites without reference standards

There is a constant need for the quantitative analysis of drug metabolites in many disciplines, including drug development and toxicology. However, the availability of synthetic primary reference standards is limited or their price can be high. To overcome this challenge, several approaches have been presented. In drug discovery and development as well as in preclinical research, radiolabeled drugs and radiometric detection have traditionally been used for the evaluation of metabolite concentrations [216,217]. Other techniques include utilization of modified LC-MS methods, nuclear magnetic resonance detectors (NMR), and universal detectors.

LC-MS methods are widely used in drug analysis because of their selectivity, sensitivity, and speed. However, the response of LC-MS with electrospray ionization (ESI) to analytes is non-uniform because of the different ionization efficiencies caused by different chemical structures and LC gradients [218-220], and consequently calibration with the primary reference standard is necessitated in every sequence of runs [221]. Several attempts to equalize the MS responses have been presented, for example by using a parallel detector that provides equimolar responses [218,222,223], or by using a calibration factor obtained with a different ionization technique, such as nanospray ionization (NSI) [219,224-228] or captive spray ionization (CSI) [229,230].

Semi-equimolar responses for several structurally distinct drugs and their metabolites have been achieved when a very low flow rate LC-NSI-MS technique was used [224]. Even more uniform responses were achieved when an inverse gradient was used with LC-NSI-MS [226]. LC-NSI-MS techniques have not, however, gained wide use because of the difficulties associated with their maintenance, operation, and reproducibility [229]. To overcome these issues, commercially available LC-CSI-MS has been tested in the quantification of drug metabolites [229,230]. The suitability of LC-CSI-MS for quantification without primary reference standards has remained controversial and the technique has not been widely adopted. While good results have been reported [229], others have reported statistically significant differences in the calibration curves of drugs and their metabolites [230].

Correcting the LC-ESI-MS responses with universal detectors such as CLND [218] and UV detectors has also been tested [222,223]. When the LC-ESI-MS response was corrected with a calibration factor gained from CLND, the quantitative results for benzodiazepines using a secondary calibration were comparable to those obtained with conventional calibration [218]. UV correction has yielded good results with those drug metabolites that share the same chromophores, and thus have a similar UV spectrum with the parent drug [223]. However, this approach should be treated with caution as even with apparently similar UV spectra, quantification can be erroneous if the chemical changes take place in the vicinity of a chromophore. For example, the quantitative results for buspirone metabolites were enhanced by UV correction, but a three-fold difference was nevertheless detected [222].

NMR has traditionally been used as a reference technique for elucidating the structures of drug metabolites in the pharmaceutical industry. The emergence of Fourier transform NMR spectrometers along with improvements in both software and hardware has increased the applicability of NMR also in quantitative analysis [223,231].

The response of the universal detectors is fairly independent of the chemical structure of the analyte, and hence these detectors may enable single-calibrant quantification. CLND provides an equimolar response to nearly all nitrogen-containing compounds and has been successfully applied in the quantification of several drugs using caffeine for calibration [232-235]. In addition, LC-CLND quantification of drugs in plasma and blood has been demonstrated when a universal correction factor was used to compensate for the differences in extraction recoveries [236,237]. The ELSD response depends on the mobile phase composition of the LC gradient [238] but the differences in responses can be corrected with an inverse LC gradient [239], while using chemically similar compounds for calibration enables quantification without primary reference standards [240,241]. However, CLND and ELSD are less sensitive than DAD for most drug analytes, making these detectors less suitable for single-calibrant quantification of drugs in toxicological samples [240,242].

3. AIMS OF THE STUDY

The first aim of this thesis was to develop new analytical laboratory methods for the analysis of toxic alcohols, medicinal drugs, and their metabolites in post-mortem toxicology samples. The second aim was to gather metabolite concentration data by these methods and apply the data to the interpretation of toxicological results.

The specific aims of the individual publications were

- To develop a qualitative screening method for volatile and semivolatile compounds in blood samples by in-tube extraction-gas chromatography-mass spectrometry (ITEX-GC-MS) (**I**)

- To develop a quantitative analysis method for ethylene glycol (EG), glycolic acid (GA) and formic acid (FA) in blood and urine samples by ITEX-GC-MS (**I**)

- To evaluate the concentrations of methanol (MeOH), EG and their acidic metabolites in blood and urine samples from post-mortem cases (**II**, **III**)

- To develop a robust non-MS method based on ultra-high performance liquid chromatography coupled with diode array detection and charged aerosol detection (UHPLC-DAD-CAD) for quantitative monitoring of toxicologically relevant basic drugs in blood samples (**IV**)

- To utilize UHPLC-DAD-CAD for quantitative monitoring of drug metabolites without primary reference standards (**V**)

- To assess the meaning of combined parent drug – metabolite concentrations and metabolite to parent ratios obtained by UHPLC-DAD-CAD in post-mortem blood samples (**V**)

4. MATERIALS AND METHODS

4.1 Materials

4.1.1 Chemicals and reagents

All reference standards for drugs were purchased from pharmaceutical companies and were of pharmaceutical purity. Deuterated internal standards were purchased from Aldrich (Steinheim, Germany), Isotec (Miamisburg, OH, USA), and Cambridge Isotope Laboratories (Andover, MA, USA). All other chemicals were pro analysis, liquid chromatography (LC), or LC-mass spectrometry (MS) grade and purchased from various suppliers.

4.1.2 Post-mortem blood and urine samples

Post-mortem blood and urine samples were taken at medico-legal autopsies by forensic pathologists for toxicological analysis and were stored refrigerated with a preservative (1% sodium fluoride) prior to analysis. Blood samples were femoral venous blood.

A total of 47 fatal methanol (MeOH) or ethylene glycol (EG) poisonings were studied (**II**, **III**). Of these 23 were positive for MeOH, 21 were positive for EG and in three cases both MeOH and EG were detected. The MeOH positive cases covered all cases within a one-year period in which MeOH was found with headspace-gas chromatography-flame ionization detection (headspace-GC-FID) in either blood or urine samples or both (**II**). The EG positive cases represent a five-year period (**III**). To study the post-mortem formation of formic acid (FA), 30 putrefied cases were analyzed along with 59 non-putrefied cases where no MeOH was detected. The criterion for putrefaction was either a mention of decomposition in the covering note from the forensic pathologist, or a long post-mortem interval and visual inspection of the sample (**II**). To evaluate the usefulness of urinary oxalic acid (OX) in screening for EG poisonings, 100 previously analyzed, non-EG positive urine samples were re-analyzed for OX (**III**). The selection of cases for toxic alcohols is presented in Fig. 4 (**II**, **III**).

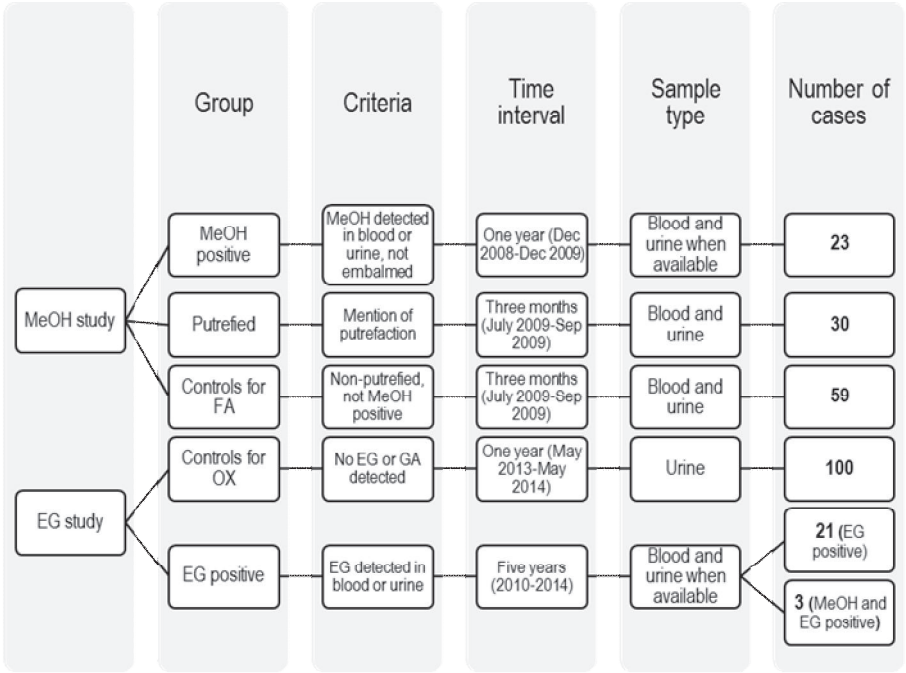


Figure 4. Selection of cases for analysis of toxic alcohols and their metabolites (**II**, **III**). MeOH: methanol, FA: formic acid, EG: ethylene glycol, GA: glycolic acid, OX: oxalic acid.

To study the metabolite to parent ratios of therapeutic drugs, all previously analyzed post-mortem blood samples within one year were examined. Samples containing analytes of interest were retrospectively reprocessed with the secondary calibration method utilizing the parent drug as calibration standard. From the samples in which the parent and the metabolite concentrations exceeded the limit of quantification (LOQ), the six most frequently occurring drugs were included in the study (**V**). The selection of samples is presented in Fig. 5. Due to the frequency of polydrug use, one sample could contain more than one drug. The selected cases represented all causes of death and were not sorted according to the cause or manner of death or to any other attribute. The selected cases were divided into quartile groups based on the combined concentration of the parent drug and the metabolite.

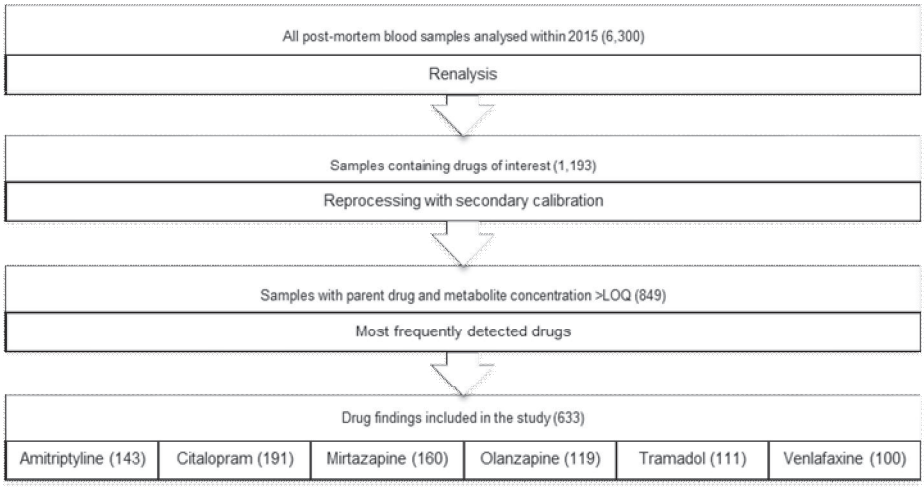


Figure 5. Selection of cases for studying metabolite to parent ratios of medicinal drugs with number of cases in parenthesis (**V**). LOQ: limit of quantification.

4.2 Sample preparation

4.2.1 Determination of formic acid, ethylene glycol, glycolic acid, and volatile compounds

For the analysis of volatile organic compounds (VOC), the samples were diluted with water and internal standard was added. For the determination of EG, glycolic acid (GA), and FA, in-vial methylation with dimethylsulfate was performed for urine samples and protein-precipitated blood samples (**I**-**III**).

4.2.2 Analysis of basic drugs and their metabolites

For the quantitative monitoring of basic drugs and their metabolites blood samples were extracted under basic conditions with a mixture of ethyl acetate and butyl acetate (**IV**, **V**). The organic layer was evaporated to dryness and reconstituted with 100 µL of 0.1% aqueous trifluoroacetic acid (TFA):MeOH (80:20, v:v). For calibration and control samples, 50 µl of the standard or control mixture was added to sheep whole blood prior to extraction.

4.3 Instrumentation and analytical methods

4.3.1 ITEX-gas chromatography-mass spectrometry (ITEX-GC-MS)

GC-MS was performed with a 5975B VL mass selective detector coupled with a 6890N gas chromatograph (Agilent Technologies, Santa Clara, CA, USA). The GC was equipped with a CTC CombiPal autosampler with ITEX (CTC Analytics, Zwingen, Switzerland). The column was a CP PoraPLOT Q HT capillary column of 25 m (and 2.5 m particle trap) x 0.32 mm i.d., with 10 μm film thickness (Varian, Lake Forest, CA, USA). A 2.5 ml headspace syringe was used (Hamilton, Bonaduz, Switzerland). A Tenax TA (80/100mesh) trap was used in the syringe needle. The GC-MS was operated with ChemStation software with integrated CTC Control software.

Analyte separation was performed with a thermal gradient from 40°C to 250°C (15°C/min) with an initial isothermal period of 2 min. The isothermal period at the end was 4 min for the determination of EG, GA, and FA and 10 min for the VOC. The mass detector was operated in electron ionization (EI) and full scan mode in the range m/z 15–550. The target and qualifier ions for EG, GA, FA, OX, and the deuterated internal standards are presented in Table 2.

Table 2. *Target and qualifier ions used for quantification of ethylene glycol, glycolic acid, formic acid, oxalic acid, and deuterated internal standards by ITEX-GC-MS (**I-III**). ME: methyl ester, 2ME: dimethyl ester*

Derivative	Target ion (*m/z*)	Q1 (*m/z*)	Q2 (*m/z*)
Ethylene-d4 glycol-2ME	94	64	47
Ethylene glycol-2ME	90	60	45
Glycolic-d2 acid-2ME	76	47	
Glycolic acid-2ME	74	45	
Oxalic acid-2ME	59	45	29
Formic-d acid-ME	61	32	
Formic acid-ME	60	31	

In both methods, the ITEX incubation time was 5 min, extraction volume 1500 μL, extraction speed 150 μL/min, needle temperature 55°C, desorption temperature 230°C, desorption speed 20 μL and injection volume 2000 μL. The incubation temperature was 60°C for the VOC and 90°C for EG, GA, and FA. The number of extraction strokes was 30 for the VOC and 10 for EG, GA, and FA.

4.3.2 UHPLC-DAD-CAD

The chromatography instrumentation consisted of an Acquity UPLC® binary solvent manager, sample manager and column manager (Waters Corporation, Milford, MA, USA). Detection involved two consecutive detectors in series, the first being an Acquity PDA detector (Waters) and the second a Corona Ultra CAD (ESA Biosciences, part of Thermo Scientific, Sunnyvale, CA, USA). Empower software (Waters) was used for data processing (version 2 in **IV** and version 3 in **V**), chromatographic optimization was performed with Fusion AE (S-Matrix, Eureka, CA, USA), and SPSS statistical software (SPSS Inc. Chicago, IL, USA) was used for statistical analysis (version 20 in **IV** and version 23 in **V**).

UHPLC separation was performed with a HSS C18 column of 2.1 mm × 150 mm equipped with a HSS C18 precolumn of 2.1 mm × 5.0 mm, both with a particle size of 1.8 µm (Waters) using a mobile phase gradient consisting of 0.1% aqueous TFA (mobile phase A) and MeOH (mobile phase B). The mobile phase was initially 5% B (0–3 min), followed by a linear gradient from 5% B to 95% B (3–18 min) and held at 95% B (18–19 min). To clean the column, a linear gradient from 95% B to 99% B (19–19.5 min) followed and was held for 4 min. The equilibrium time between samples was 4 min. The flow rate was 0.4 mL/min and injection volume 5 µl. Column temperature was 60°C. Ultraviolet (UV) spectra were collected over the range 210–400 nm at collection speed of 20 point/sec and the quantification wavelength was 230 nm. The CAD was operated using a nitrogen pressure of 35 psi.

The identification was based on retention time, UV spectrum, and the response ratio of DAD (230 nm) to CAD. An in-house UV library was created for the wavelength range 210–400 nm with 1.2 nm resolution using standard solutions of the drugs. Spectral matching was performed using Empower software by spectral contrast angle (match angle). In this comparison method, spectra at the peak apex are converted to vectors in n-dimensional space, the vectors are moved into a two-dimensional plane and the angle between them is measured using linear algebra. The criterion for identification by UV spectrum was match angle ≤ 2.0° (SI=0.9994). The detector response ratio was calculated by dividing analyte peak area on the DAD by the corresponding peak area on the CAD. For each compound a reference value was determined individually. The acceptance criterion for the response ratio was set at ±30% tolerance from the reference value.

One-point calibration was performed with both detectors individually using dibenzepin as internal standard. In the analysis of metabolite to parent ratios (**V**), the historic calibrations of the parent drugs were used to quantify both the parent drugs and the metabolites.

5. RESULTS AND DISCUSSION

5.1 Hydroxylic and volatile compounds

5.1.1 Analytical laboratory methods

The headspace in-tube extraction gas chromatography-mass spectrometry (ITEX-GC-MS) approach was developed for comprehensive analysis of low molecular weight organic compounds in blood and urine. One aliquot was analyzed after in-vial derivatization with dimethyl sulfate for ethylene glycol (EG), glycolic acid (GA), formic acid (FA), and other hydroxylic compounds, and another aliquot for underivatized volatile organic compounds (VOC).

In the case of FA, EG, and GA determinations by ITEX-GC-MS, derivatization is needed to increase the volatility of these hydroxylic compounds. The basic methods for derivatization of hydroxyl groups for GC analysis include acylation, alkylation, esterification, and silylation [243]. Most derivatization agents require that the analytes are extracted into an organic phase prior to derivatization. Dimethyl sulfate has the distinct advantage that analytes can be transformed to the corresponding methyl esters in aqueous matrices such as water and urine [244,245]. Previously, *in situ* derivatization with dimethyl sulfate has been used for the analysis of organic acids and phenols in water [244-249] or urine samples [250] prior to GC-MS analysis with headspace sampling [246], headspace-solid phase microextraction (SPME) [245,247,250], or large-volume on-column injection [248].

The method for hydroxylic compounds enables the detection and identification of many alcohols, glycols, phenolic compounds, and organic acids, including high concentrations of γ-hydroxybutyric acid. Due to the decomposition of the dimethyl sulfate reagent to methanol (MeOH) and sulfuric acid during the derivatization process, simultaneous determination of MeOH was not possible with this method. The linear concentration ranges obtained for FA, GA, and EG in blood and urine cover the toxicologically relevant range. The limits of detection (LOD) and quantification (LOQ) for EG and GA are similar to those obtained by GC-flame ionization detection (FID) or GC-MS using derivatization with phenylboronic acid or silylation [251], but not as low as those obtained with direct injection into GC-MS following derivatization with bis-*N*,*O*-trimethylsilyl trifluoroacetamide [147] or isotope dilution GC-MS following derivatization with heptafluorobutyric anhydride [148]. For FA, the LOD could not be determined in the present study because of the endogenous FA found in all post-mortem samples. The LOQ of 0.05 g/L obtained here is lower than that reported for GC-FID following derivatization with MeOH and sulfuric acid [77,78].

The present screening method for volatile organic compounds allowed identification of over 100 VOC, including lighter gas, gasoline additives, inhalation anesthetics and miscellaneous solvents. Compared with previously published

comprehensive screening methods [158,160], a wider range of VOC could be identified in a single run. Compounds could even be tentatively identified without primary reference standards based on their mass spectrum, and confirmed later using the appropriate standard. The identification limits obtained for most VOC were superior to those with static headspace techniques. The identification limits were higher than those from purge and trap GC-MS [252], but comparable to those of other methods used for screening volatile compounds in blood [81,253].

Choosing a porous open layer tubular (PLOT) column enabled the detection and separation of very volatile gases such as propane and butane above ambient temperature. The chromatographic peak shape was generally good, but could be improved for early eluting, very volatile compounds by using cryofocusing. However, as a result of the poor peak shape, the limits of identification for lower alcohols without derivatization were fairly high, and consequently ethanol and MeOH are better determined using standard headspace GC techniques. Quantification of VOC was tested with a single internal standard, diethyl ketone. This enabled semiquantitative analysis, but for precise quantitative results individual internal standards should be used. However, quantification of volatile compounds is futile [254], as their concentration in the blood sample can be diminished both before sampling and after sampling if the sample container is not full or is opened and closed before the analysis.

5.1.2 Concentrations of toxic alcohols and their metabolites in post-mortem samples

The mean and median concentrations and concentration ranges of MeOH, FA, EG, and GA in fatal MeOH or EG poisoning cases are presented in Table 3. There was a large variability in the urine concentrations of all the compounds, as well as in the blood concentrations of MeOH and EG. However, there was a significant correlation between the blood and urine concentrations of MeOH and EG, the Pearson correlations being r^2=0.928 for MeOH and r^2=0.896 for EG. This is consistent with previous studies of ethanol and MeOH [77,255]. The correlation between the blood and urine concentrations of both FA and GA was poor. Even though FA is a minor metabolite of EG, in the present material elevated FA concentrations were not seen in EG poisoning cases, which is consistent with a previous study with monkeys [31]. In all cases, the urine concentrations of MeOH, EG, FA, and GA were higher than those in blood. This warrants the use of urine for screening purposes, whenever this sample is available. In clinical settings, screening of FA and GA in blood or serum samples along with MeOH and EG is recommendable if the patient is acidotic or suffers from oliguria or anuria.

Table 3. *Mean and median concentrations and concentration ranges of methanol (MeOH), formic acid (FA), ethylene glycol (EG), and glycolic acid (GA) in fatal poisoning cases (**II**, **III**).*

Compound	Blood			Urine		
	Mean	Median	Range	Mean	Median	Range
MeOH	3.0 ‰	3.0 ‰	1.1–6.0 ‰	4.4 ‰	4.7 ‰	1.6–8.7 ‰
FA	0.80 g/L	0.88 g/L	0.19–1.0 g/L	3.4 g/L	3.3 g/L	1.7–5.6 g/L
EG	1.5 g/L	0.87 g/L	< LOQ–5.6 g/L	5.7 g/L	4.3 g/L	1.6–18 g/L
GA	1.6 g/L	1.6 g/L	0.69–2.3 g/L	5.2 g/L	5.4 g/L	2.6–8.3 g/L

Fig. 6 shows the blood FA and GA concentrations in fatal toxic alcohol poisoning cases plotted against the concentration of MeOH and EG. As can be seen from Fig. 6, there is no correlation between the concentration of the parent alcohol and the acidic metabolite. Rather, the concentrations of FA and GA in blood samples seem to be very uniform, suggesting that there is a fatal threshold for the metabolite concentration in blood in nonhospitalized toxic alcohol ingestion cases. For FA this concentration is approximately 0.8–1.0 g/L and for GA approximately 1.6–2.0 g/L. These findings are consistent with previous studies of fatal MeOH and EG poisonings [77,79], but previous authors have not discovered the fatal threshold blood concentrations in their material. The fatal threshold concentrations are roughly twice the clinical blood concentration associated with poor outcome, as the admittance concentration of FA > 0.5 g/L has been shown to cause permanent visual damage or death [76,77] and a GA concentration of 0.8 g/L to cause renal injuries [28,71,256].

Other toxicologically relevant findings in the fatal MeOH or EG poisonings were scarce. Ethanol was detected in only two urine samples and in one blood sample. As ethanol has the highest affinity for alcohol dehydrogenase (ADH), it is metabolized before MeOH and EG. Therefore, it is difficult to estimate the role of ethanol in fatalities including toxic alcohols. The most common concomitant findings in the fatal toxic alcohol poisoning cases were benzodiazepines. They were found in therapeutic concentrations in one-third of the cases. Benzodiazepines are commonly used in the treatment of alcohol abusers [257] and as part of intensive care. As many of the fatal MeOH or EG poisoning victims had a history of alcohol abuse and as about one-fourth of them died in a healthcare unit, this result is reasonable. In two of the mixed MeOH/EG poisoning cases, the GA concentrations in both blood and urine were very low or not detected at all, whereas the MeOH and FA concentrations were high. This suggests that MeOH was consumed prior to, or simultaneously with, EG, as MeOH has a higher affinity for ADH than EG. In the third case, MeOH and FA concentrations were substantially lower and GA was present along with EG. In this case, EG was presumably ingested prior to MeOH.

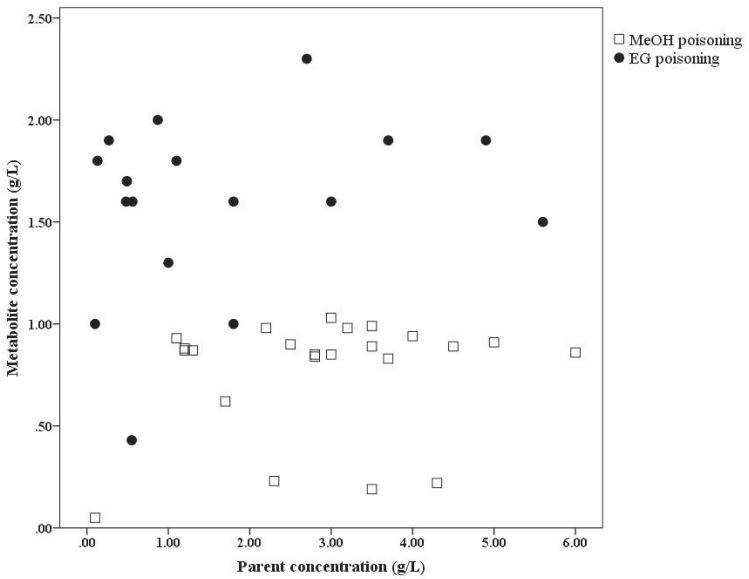


Figure 6. Distribution of FA and GA concentrations in blood in fatal MeOH and EG poisoning cases.

Calcium oxalate monohydrate (COM) crystals are one of the factors contributing to EG toxicity, and the crystals are detected in the kidneys in the majority of fatal EG poisonings cases [79]. Of the EG poisoning cases studied in the present thesis, one presented considerable EG and GA concentrations in blood and urine, but no visible COM crystals were found in the kidneys. This suggests that death had occurred before formation of COM crystals due to the toxicity of EG and GA, as their concentrations were among the highest detected in the study. However, the lack of COM crystals can complicate the cause of death investigation, as EG and GA are not routinely screened in many forensic laboratories, and quantification of EG is often performed only after COM crystals have been detected.

The urinary oxalic acid (OX) concentration was found to provide very little additional information for the post-mortem diagnosis of EG poisoning. Even though OX was not detected or the concentration was <0.10 g/L in 79% of the EG-negative urine samples, there were individual cases in which the urinary OX concentrations were even higher than those detected in the EG poisoning cases. This is probably due to OX from dietary and endogenous sources [35].

The mean (median) concentration of FA in non-putrefied, non-MeOH positive cases was 0.04 g/L (0.04 g/L) in blood and 0.06 g/L (0.04 g/L) in urine, never exceeding 0.2 g/L in blood or 0.3 g/L in urine. These results are consistent with the previous studies of endogenous FA [32-34,258,259]. In putrefied samples, the FA concentrations were significantly higher: the mean (median) FA concentration being 0.24 g/L (0.22 g/L) in blood and 0.25 g/L (0.15 g/L) in urine. The FA

concentrations ranged from below LOD to 0.77 g/L in blood and from 0.02 to 1.4 g/L in urine. In three cases the blood FA concentration exceeded 0.5 g/L, a level suggesting fatal MeOH poisoning. In the re-analysis of ten putrefied blood and urine samples, the FA concentrations were within ± 25% of the original results, which suggests that the formation of FA took place before sampling, not during storage. A probable explanation for these high FA concentrations is bacterial breakdown of lipids, carbohydrates and proteins [88]. It has been demonstrated that FA concentration can increase substantially in urine samples stored at room temperature due to bacteria [258]. FA has also been found in decomposing pig carcasses [91]. Even though post-mortem production of EG has been reported [260], in this study no EG or GA was detected in post-mortem cases that were not connected to EG ingestion.

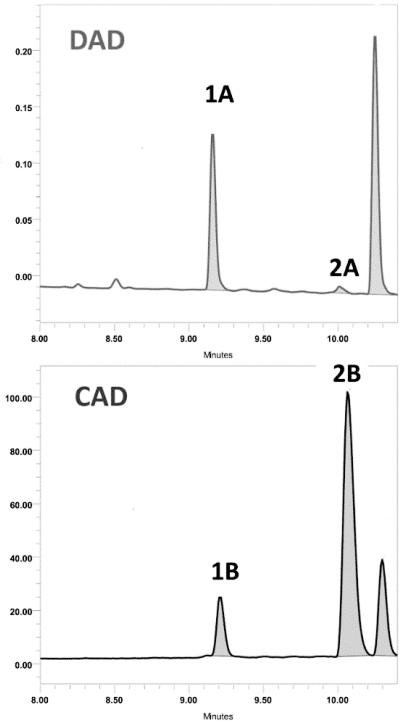
A large proportion of deaths caused by toxic alcohols took place in a healthcare unit. As many of the deceased had a history of ethanol abuse and as breath alcohol analyzers may falsely interpret MeOH as ethanol [56], there is a chance of misdiagnosis. Diagnosing intoxication caused by toxic alcohols rather than ethanol still remains a difficult task, as the symptoms of the intoxication are similar to those caused by ethanol. Furthermore, hospital laboratories seldom have appropriate analytical tools to detect MeOH or EG, let alone their toxic metabolites. In Ireland, only 2.6% of hospital laboratories had assays for determining MeOH or EG [261]. The situation is similar in Finland, as only the biggest hospital laboratories have GC methods for toxic alcohols. In a clinical setting, detecting the parent alcohols or classic MeOH or EG poisoning symptoms may be sufficient to initiate treatment, but quantification of the acidic metabolites aids the interpretation of the case in post-mortem toxicology.

5.2 Medicinal drugs and their metabolites

5.2.1 Quantitative monitoring of basic drugs

A quantitative drug screening method for blood samples was developed based on ultra-high performance liquid chromatography (UHPLC) with two consecutive detectors: diode array detector (DAD) and charged aerosol detector (CAD). Chromatography was based on sub-2 μm C18 bonded phase sorbent and the mobile phase consisted of 0.1% trifluoroacetic acid (TFA)/MeOH in gradient mode. Identification of analytes was based on the precise retention time, ultraviolet (UV) spectra, and the response ratio of DAD to CAD. Calibration was based on historic one-point calibration. The response ratio was introduced to enhance the identification power of the method. Fig. 7 shows an example of the calculation of the DAD to CAD response ratio, and Fig. 8 presents the response ratios of 154 drugs across the retention time axis. As can be seen from Fig. 8, there is great divergence between the response ratios even with closely eluting compounds. With the criteria used for analyte identification, there was only one pair of drugs, citalopram and

norcitalopram, that were undistinguishable. However, these compounds are generally detected together and can be distinguished by their elution order. Furthermore, the response ratios were repeatable over two orders of magnitude with a median relative standard deviation of 12%. Based on these results, a stricter criterion for the response ratio could be applied in order to increase the reliability of the identification. Analysis of 20 negative blood samples gave no false positive results for basic drugs. The stability of retention times over a one-month period was excellent and comparable to that obtained with GC using retention time locking [262], as the median intermediate precision was 0.04% and the median absolute difference in retention time was 0.01 min. The high precision was largely due to the favorable chromatography of basic drugs as TFA ion-pairs [263]. In liquid chromatography-MS (LC-MS) methods similar chromatographic precision has been reported mostly in intra-assay experiments [264].



$$\mathbf{RR(1)}=\frac{\mathbf{Area\ 1A}}{\mathbf{Area\ 1B}}=\mathbf{4.8}$$

$$\mathbf{RR(2)}=\frac{\mathbf{Area\ 2A}}{\mathbf{Area\ 2B}}=\mathbf{0.04}$$

Figure 7. Example of diode array detector (DAD) to corona charged aerosol detector (CAD) response ratio (RR) calculation for Compound 1 and Compound 2 in a calibration sample: peak area from DAD is divided by peak area from CAD. Response ratio is compound-dependent because the response of DAD is dependent on the UV spectrum and the response of CAD is independent of structure.

The linear range of the method was generally 0.05–5 mg/L, the lowest point equalling the LOQ. In two previous studies, the response of CAD has been reported to be non-linear over a wide concentration range [198,199], but in the present study the calibration curves were linear over two orders of magnitude ($r^2 > 0.99$). Similar linearity has been reported in previous studies in which a corresponding concentration range was used [201,265]. The LOQ was within the therapeutic range for most compounds, but drugs with a low therapeutic concentration, such as some antipsychotics, some adrenergic beta-blocking drugs, cannabinoids, and buprenorphine, cannot be detected at their therapeutic or recreational concentrations.

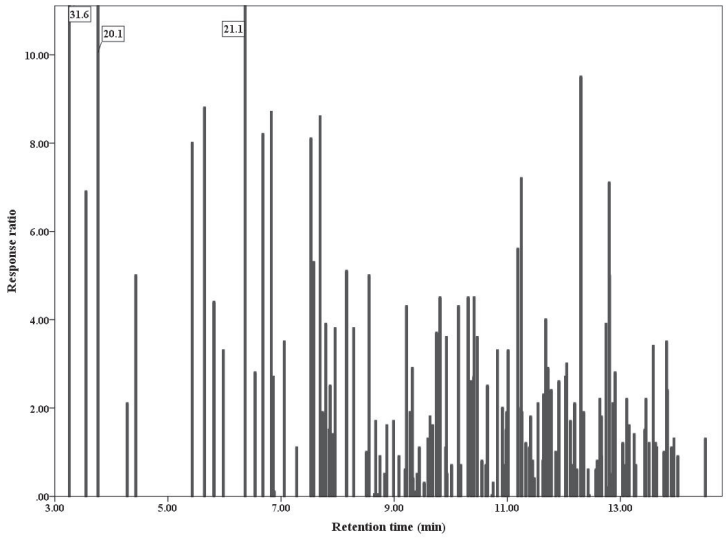


Figure 8. Distribution of diode array detector (DAD) to corona charged aerosol detector (CAD) response ratios plotted against UHPLC retention time for 154 drug analytes.

The long-term stability of historic one-point calibration was found to be appropriate for the purpose of quantitative monitoring. After one month, the median bias from the initial result was 6.8%, and for most compounds the bias was < 10%. For some compounds, however, more frequent calibration is needed because of the limited stability of calibration. The results of a proficiency testing blood sample containing codeine, tramadol, citalopram, methadone, and amitriptyline were very good (z-score ≤ 1) when the quantification was performed with historic one-point calibration. The method has been accredited by the Finnish Accreditation Service FINAS.

Small-scale liquid-liquid extraction was selected for sample preparation because of its broad scope and simplicity of operation. Several solvents, including dichloromethane, ethyl acetate, butyl acetate and their mixtures were tested. Extraction with dichloromethane, as suggested earlier [173], yielded good recoveries but turned out to be impractical in routine analysis. A mixture of ethyl acetate and

butyl acetate suggested earlier [266] also yielded good results, but a mixture of 50:50 generated non-soluble particles when the residue was reconstituted with the injection solvent, and the proportion of ethyl acetate was therefore reduced to 25% to avoid column blocking. The 20% MeOH content in the injection solvent was necessary to dissolve the residue, including the lipophilic drugs, prior to chromatography.

To ensure high throughput and reliable identification, the analytes were divided up into five distinct data processing methods that were used concurrently. This enabled reliable identification of close eluting compounds by presenting a maximum of five drug candidates for each chromatographic peak based on the retention time. The correct finding was then manually selected among the drug candidates based on the UV spectrum and the DAD to CAD response ratio.

5.2.2 Quantification of drug metabolites employing parent calibration

Quantification of drug metabolites involving the use of a secondary calibration with the parent drug generally yielded an accuracy comparable to ordinary drug bioanalysis. The differences between primary and secondary calibration with each detector for the 23 metabolites tested are presented in Fig. 9. The *N*-dealkylated metabolites eluted in close proximity to their corresponding parent drugs and, in addition, their UV spectra were very similar to those of the parent drugs. This enabled the use of secondary calibration with the parent drug, the average difference between primary and secondary calibration being 12% and 15% for CAD and DAD, respectively. The quantitative results obtained with secondary calibration were comparable to those obtained with LC-MS methods utilizing nanospray ionization (NSI) [224]. However, unlike with LC-NSI-MS methods, no specialized instrumentation was required for this level of accuracy in quantification. For the *O*-demethylated metabolites the accuracy was poorer, due to differences in retention time and UV spectra between parent drug and metabolite. The CAD results for *O*-demethylated metabolites could be improved by using an inverse mobile phase gradient, but the overall loss in detection sensitivity would outweigh the advantages gained.

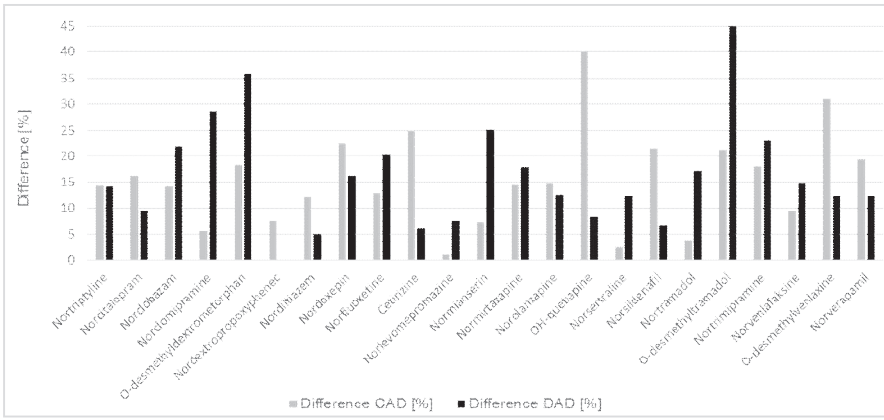


Figure 9. Relative differences in metabolite quantification between primary and secondary calibration by UHPLC-DAD-CAD in post-mortem blood. Cetirizine is a metabolite of hydroxyzine.

5.2.3 Metabolite to parent ratios of medicinal drugs in post-mortem blood samples

The UHPLC-DAD-CAD data from 633 previously analyzed post-mortem blood samples representing all causes of death was retrospectively reprocessed using the secondary calibration method (**V**). The concentrations of six toxicologically relevant drugs, amitriptyline, citalopram, mirtazapine, olanzapine, tramadol, and venlafaxine, were ranked according to the combined parent drug-metabolite concentration. From previous studies it could be assumed that approximately 10–20% of the cases were fatal poisonings caused by the drugs studied [108]. Three of the drugs included in this study, amitriptyline, tramadol and venlafaxine, are among the 20 most common drugs causing fatal poisonings in Finland [2]. Only those cases for which both the parent drug and the metabolite concentration were above LOQ were included in the study. Fig. 10 shows the distribution of the metabolite to parent ratios in the three quartile groups of combined parent-metabolite concentration.

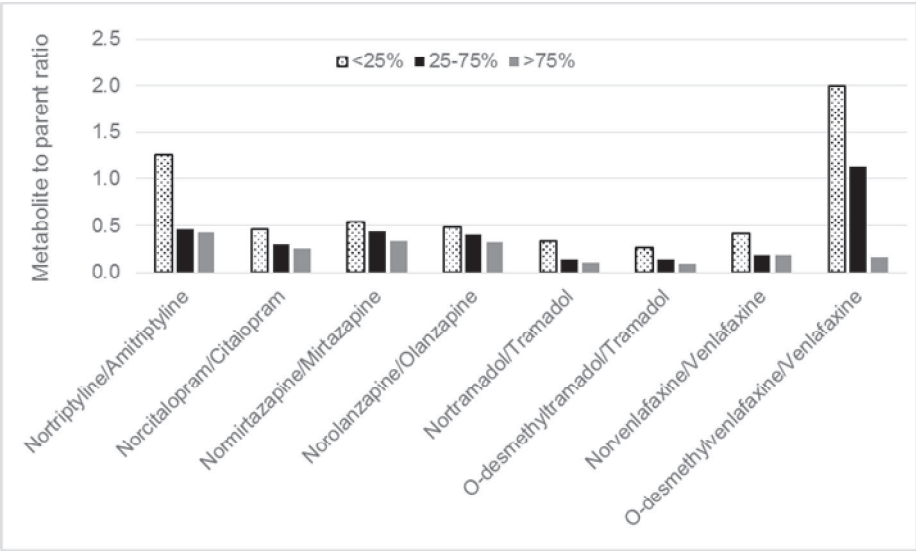


Figure 10. Distribution of metabolite to parent drug concentration ratios in quartile groups of combined parent-metabolite concentration in post-mortem blood.

In nearly 60% of these cases the concentration of the parent drug was above the clinical therapeutic range in plasma, and in 27% of the cases the concentration was at least twice as high as the median concentration for post-mortem cases reported previously [108]. The median combined parent drug-metabolite concentrations were 29–80% higher than the median parent drug concentrations, the average being 46%. The metabolite to parent ratios fell within the normal ranges defined in clinical therapeutic drug monitoring (TDM) [9], even when the parent drug concentration exceeded the therapeutic range. Only with the highest combined concentrations were the ratios below the normal ranges. In the latter cases, the parent concentrations were generally significantly above the clinical therapeutic range and higher than the median post-mortem concentrations, suggesting acute overdose. Furthermore, the present results are in agreement with the metabolite to parent ratios of antidepressants measured in fatal poisoning cases and in other post-mortem samples [16].

Fig. 11 illustrates the distribution of the metabolite to parent drug concentration ratios in post-mortem blood positive for amitriptyline and venlafaxine. For each drug, the horizontal lines correspond to the upper and lower limits of the normal clinical ratio defined for TDM [9], and the vertical line represents the post-mortem median concentration according to a large database [108]. Fig. 11 shows that even with fairly high amitriptyline concentrations, the metabolite to parent ratio falls within the normal range, whereas in the case of venlafaxine, the ratio is already below the normal range with moderate venlafaxine concentrations. One possible explanation is the difference in post-mortem redistribution (PMR) behavior between amitriptyline and venlafaxine. Amitriptyline has been reported to exhibit

considerable PMR [267], whereas venlafaxine has been found to be less prone to PMR with therapeutic concentrations in post-mortem samples close to those defined in TDM [100,268]. It has been shown that in acute venlafaxine poisoning cases the *O*-desmethylvenlafaxine to venlafaxine ratio is significantly lower, approximately 0.1, than in other post-mortem cases, where the ratio is between 1 and 4 [16,268]. Hence, slightly elevated venlafaxine concentrations along with significant concentrations of *O*-desmethylvenlafaxine are more likely to indicate therapeutic use of venlafaxine than overdose [268].

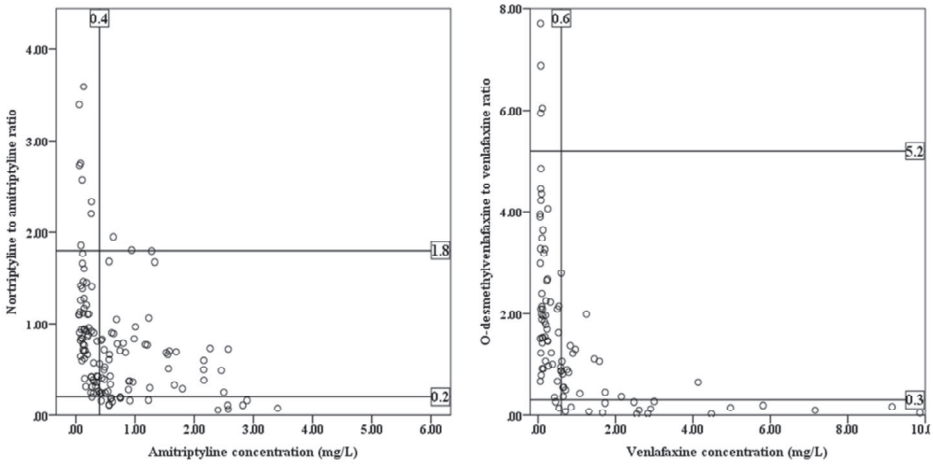


Figure 11. Distribution of metabolite to parent ratios in amitriptyline and venlafaxine positive cases. Horizontal lines correspond to normal ratio [9] and vertical lines represents post-mortem median concentration of amitriptyline and venlafaxine [108].

These observations encourage the use of the metabolite to parent ratios in the interpretation of toxicological results in cause of death investigations. Ratios outside the normal ranges can indicate altered metabolism due to drug-drug interactions, altered liver function, or genetic variation in the liver CYP enzymes, especially CYP2D6 or CYP2C19. Low ratios and high combined concentrations can indicate acute ingestion or poisoning. However, more data on metabolite concentrations and metabolite to parent ratios in post-mortem blood should be acquired and connected with cause of death information. Furthermore, drawing conclusions from these results is complicated by the high pharmacological activity of the *O*-demethylated metabolites of tramadol and venlafaxine in particular [96].

6. GENERAL DISCUSSION

In toxicological analyses, there is a constant need to identify and quantify a wide range of drugs in complex biological samples [10], and the use of two complementary sample matrices is recommended [183,269]. For a long time, gas chromatography-mass spectrometry (GC-MS) has been the gold standard for comprehensive drug screening, and several applications have been recently presented [270-273]. GC-MS has good sensitivity and specificity and, thanks to the high repeatability of electron ionization (EI), large mass spectral libraries are available. However, only thermally stable and sufficiently volatile drugs can be detected in their native form, others requiring derivatization [174,175]. Following the emergence of ultra-high performance liquid chromatography (UHPLC) and a range of advanced liquid chromatography (LC)-MS techniques, GC based methods are today mostly used for volatile and semivolatile compounds.

Hydroxylic compounds and volatile organic compounds (VOC) represent an important and diverse group of toxicologically relevant substances, but the prevalence of fatal poisoning cases by individual substances is low. In Finland, methanol (MeOH) and ethylene glycol (EG) accounted for 1.7% of fatal poisonings during 2011–2013, while the figure for VOC was 0.39% [2]. Consequently, utilizing the same instrumentation enables a cost-effective way to analyze samples for all these substances. The in-tube extraction (ITEX)-GC-MS method presented here is the first that enables quantification of EG and glycolic acid (GA) along with other compounds by headspace sampling instead of direct injection. The commonly used direct injection technique is much more vulnerable to matrix effects and the resulting false positive or negative findings. Using the two variants of the ITEX-GC-MS method in parallel, i.e. derivatized and non-derivatized, permits comprehensive screening of volatile and semivolatile compounds, including hydroxylic compounds. In addition, the porous open layer tubular (PLOT) column used provides high chromatographic retention for very volatile compounds such as nitrous oxide and hydrogen cyanide, allowing the determination of these compounds with only minimal alterations to the current method [274,275]. In view of the high prevalence of the recreational use of nitrous oxide by inhalation [276], associated with possible accidental fatalities caused by asphyxiation, screening for this compound has become more important.

The toxicity of MeOH and EG is mainly caused by their acidic metabolites, formic acid (FA) and glycolic acid (GA), respectively, and there is a clear correlation between the concentration of the acidic metabolite and the severity of the symptoms [71,72,277]. No such correlation has been detected between the concentration of the unchanged alcohol and the symptoms [278]. The present results concerning fatal MeOH and EG poisonings clearly support this understanding. Interestingly, in nonhospitalized MeOH and EG poisoning cases, there seems to be a fatal threshold concentration of FA and GA in blood. This result emphasizes the importance of screening the acidic metabolites along with the parent alcohols in cases where

ingestion of MeOH or EG is suspected. In post-mortem toxicology, the metabolite concentration can verify that death was caused by ingestion of toxic alcohol.

In a clinical context, the screening of MeOH and EG usually covers only the unchanged alcohols, and these concentrations along with clinical symptoms are used as the criteria for hemodialysis [48,68,279]. Furthermore, because of the low prevalence of poisonings caused by toxic alcohols, analytical methods even for MeOH and EG are not available in all healthcare units [261]. There are recently published chromatographic methods also covering the acidic metabolites that are suitable for emergency toxicology [6,7]. A bedside test for FA has also been made available [73]. These improved diagnostic tools will obviously aid the recognition of intoxications caused by toxic alcohols and reduce the number of fatal poisonings caused by misdiagnosis or delayed treatment.

Another way to reduce fatalities caused by toxic alcohols is to limit their availability. After Finland joined the European Union in 1995, MeOH-based products appeared on the market. This led to an enormous increase in MeOH poisonings, with 20–30 fatal cases each year [280]. In 2011, the availability of products containing MeOH and EG was restricted by a government decree. Since 2011, the number of fatal MeOH and EG poisonings has decreased [2] but the long-term effect of the new decree remains to be seen.

The majority of deaths associated with VOC are caused by direct toxicity of the compounds concerned [84], and with the most commonly abused substances, such as butane, cardiac arrest occurs soon after inhalation [83,281]. Analysis of VOC metabolites is less relevant in post-mortem toxicology as the presence of the parent compound in blood is usually sufficient to prove the substance use [81]. However, the half-life of many VOC is very short, and detection of their metabolites could extend the window of detection in suspected VOC abuse cases [86]. For example, the half-life of alkyl nitrates is only a few minutes [282] and the toxicity of these compounds is mainly caused by methemoglobinemia. Following metabolic transformation, alkyl nitrates can be easily detected as the corresponding alcohols, such as isopentyl alcohol [283]. In cases like this, the ability to detect and identify both parent compound and metabolites can aid interpretation of toxicological results. VOC metabolites may also help in assessment of the post-mortem interval (PMI) [284-286]. Estimation of the time of death still remains one of the biggest challenges in forensic medicine [287], and several recent studies have focused on the usefulness of metabolomics in post-mortem biochemistry [285-287]. Many of the compounds identified in the metabolic studies concerning biochemical changes after death can be determined with the methods presented in (**I**), including glyoxylic acid and oxalic acid (OX). Using ITEX-GC-MS in the selected ion monitoring (SIM) mode instead of full scan mode would increase the sensitivity, and selection of appropriate internal standards would enable quantification of these metabolites. However, more research is still needed to evaluate the value of metabolites in PMI investigations.

LC-MS enables the analysis of a broad range of toxicologically relevant substances without derivatization [288]. LC-MS methods have become increasingly important in recent years [12,13], and several screening methods are available [289-

296]. These methods are fast, sensitive and selective, and the advances in accurate mass measurement have enabled tentative identification of compounds even without reference standards [297]. However, quantification by LC-MS with electrospray ionization (LC-ESI-MS) is prone to matrix effects. To ensure reliable quantitative results, matrix effects should be carefully studied during method validation, and calibration needs to be performed frequently [221,298-300]. Furthermore, due to the inter-instrument variation in ionization, use of spectral libraries in LC-MS is more limited than in GC-MS [301,302].

Before LC-MS methods became dominant in toxicological screening, high performance liquid chromatography-diode array detection (HPLC-DAD) was a standard technique for large-scale drug screening, and several methods are available [173,178,303]. Compared to MS methods, LC-DAD is inexpensive, adequately selective, and requires little instrument maintenance [177]. In addition, the long-term stability of LC-DAD methods enables utilization of historic calibration [175]. LC-DAD still has a strong position in quantification, especially in the pharmaceutical industry, but also in toxicological laboratories [304,305]. LC-MS, GC-MS and LC-DAD techniques should be considered complementary to each other, as none of these techniques is sufficient to detect and quantify all toxicologically relevant compounds in a rational manner [304]. MS-based methods are invaluable in the quantification of substances that have low therapeutic concentrations in blood to ensure their appropriate detection [185]. MS-based techniques are also indispensable in the identification and structural elucidation of unknown compounds [305]

As DAD is not a destructive detector, it can be connected in series with another detector, such as a corona charged aerosol detector (CAD) to gain more information and enhance sensitivity for compounds with poor ultraviolet (UV) absorption. Using the response ratio of DAD to CAD as an identification parameter is a novel approach to increase the reliability of identification. Because of the variable UV response and constant CAD response, the response ratios between basic drugs possess the desired variation.

Assessing whether a drug concentration measured in a post-mortem blood sample is on the fatal level is complicated by post-mortem changes, mainly post-mortem redistribution (PMR) [98-100,306]. For several drugs, the post-mortem concentrations are significantly increased due to PMR, and as a general principle drugs with volume of distribution (V_d) >3-4 L/kg have been considered to be more prone to PMR. However, it has been demonstrated that the metabolite to parent ratios may not be affected by PMR, as the concentrations of the metabolites of amitriptyline, citalopram and fluoxetine, for example, increased in the same proportion as the parent drug concentration [15,267,307]. However, this cannot be generalized to all drugs. For example, the concentrations of amiodarone (V_d 66 L/kg) do not change due to PMR, but the concentration of its active metabolite, desmethylamiodarone, increases up to three-fold. Furthermore, the interpretation of post-mortem concentrations is complicated by tolerance. For example with opioids, concentrations measured in regular opioid users can be fatal to naive users [308].

The metabolite to parent ratio of tricyclic antidepressants has been used to estimate the time of ingestion [15,309]. A very low ratio can be associated with acute intoxication, while ratios within the normal range may be attributed to therapeutic use. This assumption was further supported by a large study of concentrations of antidepressants in post-mortem blood samples [16], showing that in fatal poisoning cases, the ratios were lower than in cases where the cause of death did not relate to poisoning. Furthermore, these ratios are comparable to those used in clinical therapeutic drug monitoring (TDM) [9]. However, more data on the metabolite concentrations and metabolite to parent ratios in post-mortem samples is still needed.

Quantification of metabolites in post-mortem samples is also important in finding cases where altered metabolism may have caused adverse effects. For example clobazam, an anticolvunsant often used with pediatric patients, has caused several poisonings via its active metabolite norclobazam [116,310]. Both clobazam and norclobazam are metabolized through CYP2C19, a polymorphic enzyme. However, the half-life of norclobazam is much longer than that of clobazam, and in poor metabolizers this causes accumulation of norclobazam at therapeutic doses. Other drugs associated with altered concentrations of the active metabolite due to genetic polymorphism include amitriptyline [311], doxepin [312], tramadol [313], and venlafaxine [120].

7. CONCLUSIONS

In general, methods used for the determination of methanol (MeOH) and ethylene glycol (EG) only detect the parent alcohol in blood or urine samples. However, quantification of the acidic metabolites formic acid (FA) and glycolic acid (GA) is necessary for the diagnosis of toxic alcohol ingestion and for cause of death determination. The novel in-tube extraction-gas chromatography-mass spectrometry (ITEX-GC-MS) method enabled facile quantification of EG, GA, and FA following derivatization in biological samples. The parallel method was feasible in the qualitative screening of volatile organic compounds (VOC), including fuel gases, gasoline, alcohols, solvents, glues, anesthetic agents, and organic nitrites. Using full-scan MS data acquisition allows detection of practically any low molecular weight organic compound that is sufficiently volatile or can be volatilized by methylation with the method developed here.

The ITEX-GC-MS method was used to study the concentrations of FA, EG, and GA in fatal poisoning cases due to toxic alcohols (**II**, **III**). The study revealed that although the concentrations of MeOH and EG in blood samples are largely unpredictable, the fatal concentrations of FA and GA seem to settle above a threshold value. For FA, the threshold value is 0.8–1.0 g/L and for GA 1.6–2.0 g/L. These concentrations are approximately twice as high as those associated with poor outcome in the medical treatment of poisoning. It was shown that FA is formed during putrefaction, presumably due to bacterial action and the decomposition of lipids, carbohydrates and proteins (**II**). Thus, post-mortem FA concentration alone cannot be used to reveal MeOH poisonings. It was also shown that the urinary oxalic acid (OX) concentration does not provide much additional information in suspected EG intoxication cases as the concentrations found are low and cannot be distinguished from non-poisoning cases (**III**).

In post-mortem toxicology, both comprehensive screening methods and accurate quantification methods are needed. GC-MS and liquid chromatography (LC)-MS techniques can be used to quantify volatile and low-dose compounds, respectively, but they require extensive calibration with primary reference standards to produce reliable quantitative results. The ultra-high performance liquid chromatography-diode array detection-corona charged aerosol detection (UHPLC-DAD-CAD) method developed is a non-MS technique that enables quantitative monitoring of 161 basic drugs in a single run (**IV**). The method provides excellent linearity and stability based on historic one-point calibration on a monthly basis, reducing the calibration workload compared to methods based on LC-MS. Furthermore, UHPLC-DAD-CAD shows no ionization-related matrix effects associated with LC-MS methods with electrospray ionization (ESI). The DAD to CAD response ratio increases the reliability of identification compared to traditional DAD methods.

The UHPLC-DAD-CAD method enables the quantification of *N*-dealkylated drug metabolites without primary reference standards by employing secondary calibration with the parent drug (**V**). Limited reference data is available for

metabolite concentrations in post-mortem toxicology, and consequently the method developed can be used to accumulate this data on a routine basis. It is anticipated that the combined parent drug – metabolite concentrations and the metabolite to parent ratios, when connected to cause of death information and anamnesis, will provide a more useful reference for the interpretation of toxicological results than the concentration of the parent drug alone. Utilizing the metabolite concentrations and the metabolite to parent ratios in interpretation of toxicological results requires close co-operation between the forensic pathologist and the forensic toxicologist to gain the full advantage of these results in the cause of death determination.

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