Use of ethyl glucuronide and ethyl sulphate in forensic toxicology

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Abbreviations

EtG Ethyl glucuronide

EtS Ethyl sulphate

BAC Blood alcohol concentration

EDI Estimated daily intake of ethanol

LOD Limit of detection

LOQ Limit of quantification

LC-MS Liquid chromatography-mass spectrometry

GC-MS Gas chromatography-mass spectrometry

UGT Uridine diphosphate glucuronosyl transferase

SULT Sulfotransferase

C_{max} Maximum blood concentration

T_{max} Time to maximum blood concentration

AUC Area under the curve

C_0 Initial concentration

V_d Volume of distribution

DUI Driving under the influence

List of papers

I. Høiseth G, Karinen R, Christophersen AS, Olsen L, Normann PT, Mørland J (2007) A study of ethyl glucuronide in post-mortem blood as a marker of ante-mortem ingestion of alcohol. Forensic Sci Int 165:41-45

II. Høiseth G, Karinen R, Johnsen L, Normann PT, Christophersen AS, Mørland J (2008) Disappearance of ethyl glucuronide during heavy putrefaction. For Sci Int. 176:147-51

III. Høiseth G, Kristoffersen L, Larssen B, Arnestad M, Hermansen NO, Mørland J (2008) In vitro formation of ethanol in autopsy samples containing fluoride ions. Int J Leg Med. 122:63-66

IV. Høiseth G, Bernard JP, Karinen R, Johnsen L, Helander A, Christophersen AS, Mørland J (2007) A pharmacokinetic study of ethyl glucuronide in blood and urine: Applications to forensic toxicology. Forensic Sci Int 172:119-24

V. Høiseth G, Morini L, Polettini A, Christophersen AS, Mørland J (2009) Pharmacokinetics of ethyl glucuronide and ethyl sulphate in heavy drinkers during alcohol detoxification. Forensic Sci Int. In press. doi:10.1016/j.forsciint.2009.03.017

VI. Høiseth G, Morini L, Polettini A, Christophersen AS, Johnsen L, Karinen R, Mørland J (2009) Serum/whole blood concentration ratio for ethyl glucuronide and ethyl sulphate. J Anal Toxicol 33:227-230

1. Introduction and background

1.1 The importance of ethanol in forensic toxicology

Ethanol has a long history as the most important substance within the field of forensic toxicology. In living humans, this molecule is present at low endogenous levels, but it can also be ingested in doses large enough to cause intoxication and criminal behaviour such as drunk driving.

The endogenous blood concentrations of ethanol in living subjects are too low to be detected by regular toxicological analyses. However, because of glucose fermentation due to micro-organisms, these levels might rise considerably after death, leading to endogenous ethanol levels that are difficult to differentiate from those caused by ingestion.

Oxidative metabolism, which is the main pathway for ethanol, has been subject to a large amount of research, but non-oxidative pathways have been studied much less. This thesis concerns this minor ethanol metabolism, and the use of two non-oxidative products; ethyl glucuronide and ethyl sulphate, within forensic toxicology.

1.2 Oxidative metabolism of ethanol

The first to present a mathematical approach to the kinetics of ethanol was Widmark in the 1930s (1,2), but ethanol absorption and elimination had been studied even earlier (3). During the decades that followed, ethanol metabolism was investigated in great detail (4-8). Ethanol is most readily absorbed from the small intestine (9) and has a variable bioavailability, according to prandial state and size of the dose ingested (9-15). It can reach its maximum concentration (C_{max}) in blood 30 minutes or less after ingestion on an empty stomach whereas absorption can take as long as 2 hours or more if it is administered with food (16-20).

Most ethanol is metabolised in the oxidative pathways, and around 90% of the ingested dose is metabolised by the enzyme alcohol dehydrogenase (ADH) to acetaldehyde and further to acetate by the enzyme acetaldehyde dehydrogenase (ALDH) (21). ADH is also present in the stomach and this is considered to be one mechanism for the lower bioavailability if the ethanol remains in the stomach for some time before absorption (22-24). Some of the

ingested ethanol is also transferred to acetaldehyde by the cytochrome P450 system, mainly by the isoform 2E1 (25). In heavy drinkers, this enzyme is induced, which is one of the mechanisms for accelerated ethanol metabolism in these subjects (26). A very minor amount of ingested ethanol, 2-5%, is excreted directly in sweat, breath, urine or saliva (21).

1.3 Non-oxidative metabolism of ethanol; ethyl glucuronide and ethyl sulphate

This work deals with the non-oxidative metabolism of ethanol. A very small amount (<0.2%) of the ingested ethanol undergoes phase II reactions leading to formation of the conjugated metabolites ethyl glucuronide (EtG) (27,28) and ethyl sulphate (EtS) (29-31). The formation of EtG is a result of the reaction between ethanol and glucuronic acid, catalysed by the enzyme uridine diphosphate glucuronosyl transferase (UGT). UGT is located in the endoplasmatic reticulum, mostly in the liver (32), but animal studies have indicated that this glucuronidation also occurs in lung tissue (33). EtS is formed after conjugation with sulphate (3′-phosfoadenosine 5′-phosfosulphate) catalyzed by the cytosolic sulphotransferase (SULT), also present in the liver and to a smaller degree in the pulmonary cells, according to animal studies (29).

EtG was first described in 1901 and verified in 1952 (28) while EtS was first described in the literature in the 1960s (30). In the last few decades, numerous articles have been published describing the analyses of these metabolites. EtG and EtS have gained popularity and interest because of their high sensitivity and specificity as biomarkers for alcohol intake (34-40).

Research up to the year 2000 only concerned EtG. After this, EtS was included in the analytical repertoire of many laboratories and, since 2006, most studies have included both metabolites. In this thesis, only EtG will be mentioned in discussion of earlier research, while both EtG and EtS are mentioned together in discussion of the more recent studies.

EtG and EtS have mostly been used as relapse markers for alcohol ingestion, measured in urine (41-61) and rarely in other matrices (62,63). Also, EtG has recently been suggested as a marker of chronic high alcohol intake, measured in hair (64-76). According to the research so far, the sensitivity and specificity exceeds that of the traditional markers for chronic high

alcohol intake, like the liver enzymes aspartate aminotranserase (AST), alanine aminotransferase (ALT) and gamma-glutamyl transferase (GGT) as well as carbohydrate-deficient transferrin (CDT) (34,35,77-80).

On the other hand, EtG and EtS have nearly never been mentioned in the context of forensic toxicology (40,81). The aim of this thesis was to study two aspects of EtG and EtS; their presence in post mortem cases and their blood kinetics in living subjects. The intention was to apply this knowledge to the use of these metabolites in forensic toxicology. To do this, knowledge about the serum/whole blood ratio for EtG and EtS would also be needed.

1.4 Presence of EtG and EtS post mortem

1.4.1 Determination of post mortem ethanol origin

Post mortem formation of ethanol is a common problem in forensic toxicology (82-88). Usually low levels (<0.5 g/L) are seen, and findings are not, therefore, relevant to cause of death, but they may be important for other reasons, such as legal and insurance related issues linked to car accidents. Occasionally, levels of ethanol can reach significant concentrations (>1.5 g/L) (89-92). There are a large number of examples, especially from traumatic accidents, where high ethanol concentrations are considered to be false positives. For example, one of the corpses from the explosion on the American battle ship USS Iowa had a BAC of 1.9 g/L post mortem (91). From American aircraft accidents, very likely or possible false positive ethanol findings were concluded in 27% and 46% of the ethanol positive cases, respectively (82,89,90). The findings of 1.75 g/L ethanol in the blood of Princess Diana's driver in the fatal car accident has been questioned (88) as well as the Austrian politician, Jørg Haider's, BAC of 1.8 g/L (93).

Although post mortem formation of ethanol can be prevented by refrigeration of the corpse (83) and addition of fluoride ions to blood samples (94-97), formation still might occur in a significant number of cases. It has been suggested that approximately 50% of the cases with ethanol at a level of 0.1 g/L could be explained by endogenous formation, with lower frequencies at higher BACs (98). The substrate used for post mortem ethanol synthesis is most often glucose (99), and bacteria are responsible for the formation (97,100). For this reason, more severe formation would be expected after trauma, especially abdominal (83,101). Different criteria have been used to determine whether detected ethanol is post

mortem or ante mortem in origin. The case history, of course, provides important information, but the degree of putrefaction of the corpse (85,87,102) has also been decisive. The distribution of ethanol between different body fluids has been used to distinguish exogenous ethanol from that formed post mortem (103-105), as ethanol was thought to be most easily formed in blood. However, this method is not reliable, as formation in urine and even in vitreous humour has occurred (85,106). For more specific markers of ante mortem alcohol ingestion, the altered concentration ratio between the serotonin metabolites 5-hydroxytryptophol (5-HTOL) and 5-hydroxyindole-3-acetic acid (5-HIAA) in urine has briefly been suggested (107-110), as this rises after ethanol ingestion (111), but its use as a marker of ante mortem alcohol ingestion has never been studied in detail. We wanted to know whether EtG could be used for this purpose.

1.4.2 Earlier studies of EtG post mortem

Wurst et al introduced the idea of using EtG in post mortem material in 1999 (112) when they measured EtG in post mortem serum and urine in 3 alcoholics and 2 abstainers (113). They also analysed EtG in fat, liver, brain and cerebrospinal fluid in some of these subjects.

After the start of our work, one research group published an LC-MS/MS method, modified from an earlier urine method which they used for post mortem blood in 12 cases (114). They also studied stability of EtG in liver and skeletal tissue (n=3) (115). Recently, more case reports where EtG was used post mortem and a stability study have been published (116-119).

1.5 Blood kinetics of EtG and EtS in living subjects

1.5.1 Determination of time of alcohol ingestion

Forensic toxicologists are often asked to determine, by the use of blood ethanol measurements, at what time, related to an incident, a living suspect ingested alcohol. This is especially frequent in cases of drunk driving. A person suspected of driving under the influence (DUI) might claim that alcohol was ingested after the incident and that he/she had been abstinent for a certain period before driving (the hip flask defence) (120). One single ethanol result yields no information about time of ingestion, but the police often draw two subsequent blood samples for ethanol analysis, separated by ~30 minutes. These samples

are often collected ~1.5-2 h after the incident (121). The toxicologist is then able to determine if the suspect was in absorptive or post absorptive phase. If the person is in the absorptive phase (had increasing levels of ethanol in two subsequent samples), this strengthens his/her explanation of drinking after the incident. On the other hand, if the suspect is in post absorptive phase, this does not necessarily exclude the explanation, because maximum ethanol values might be reached as early as 30 minutes after end of drinking (16,18), and most blood samples are collected after this (121,122). Some researchers have also used of the urine/blood ratio for ethanol (17,123,124) and measurements of congener alcohols (125-129) to determine time of alcohol ingestion in such cases. We hypothesised that EtG and EtS could be used for this purpose.

1.5.2 Earlier studies of EtG and EtS blood kinetics

Most research on EtG and EtS has been performed in urine and there are far fewer publications regarding blood. The analysis in serum using both GC-MS (53) and LC-MS (130) was published around 1995. For whole blood, there are no earlier publications we have been able to find.

One single study of EtG kinetics in serum had been published prior to our work. Ten volunteers were administered 44-80 g of ethanol and EtG and ethanol were measured in serum until breath ethanol was negative, in most subjects ~8 h after drinking. Therefore, the terminal elimination of EtG was missed. The published study protocol was not described in detail and information on the type of alcohol, fasting state and food ingestion during the study was missing (131,132). A case report describing infusion of ethanol reaching 2.7 g/L in blood, including EtG concentration measurements had also been published (133).

After our work, another kinetic study of EtG and EtS in the serum of social drinkers was carried out (134) and an LC/MS method for EtG in plasma (135) and an LC-MS/MS method for serum (136) as well as for dried blood (137) have been published. Also, EtG and EtS were measured simultaneously in serum with LC-MS/MS (134,138).

1.6 Use of whole blood in forensic toxicology

Most clinical laboratories perform their analyses in serum, obtained from whole blood by centrifuging the sample after collection and discharge the solid layer, which represents

blood cells, mostly erythrocytes. The distribution of a molecule between the plasma water and the erythrocyte fraction of the blood will differ according to the molecule's size, water solubility, degree of plasma protein binding and other factors (139,140). Forensic toxicology uses whole blood as a medium and the serum/whole blood ratios are therefore important when results are compared to those from clinical laboratories. For instance, the concentration of tetrahydrocannabinol has been found to be almost twice as high when measured in serum rather than in whole blood (141). This ratio is also well known for ethanol (142-145), but no such data existed on EtG and EtS.

1.7 Summary

As described in section 1.4, there were no definite criteria to determine the origin of a post mortem ethanol result, and a marker of ante mortem alcohol ingestion was needed. We wanted to investigate the use of EtG for this purpose systematically, as this idea had only been mentioned briefly in the literature.

As explained in section 1.5, only limited methods existed to determine time of alcohol ingestion in living subjects and additional methods were needed. The use of EtG for this purpose had been mentioned briefly (132). We wanted to study the blood kinetics of EtG and EtS and hypothesised that if the blood kinetics of EtG and EtS were well known, the measurement of these ethanol metabolites could be helpful.

As described in section 1.6, the serum/whole blood ratio for EtG and EtS had never been published. If EtG and EtS were to be used in forensic toxicology, making analyses in whole blood, the serum/whole blood ratios would be important knowledge.

2. General and specific aims

2.1 General aim

As lined out in the introduction, the general aim of this thesis was to study the ethanol metabolites EtG and EtS and to apply this knowledge within forensic toxicology.

2.2 Specific aims

On the basis of the summary of the introduction, the studies below were carried out with the following, more detailed, aims:

1. To investigate the use of EtG as a marker of ante-mortem alcohol ingestion in post mortem cases (paper I, II and III).

2. To study the stability of EtG, and hence the possibility of false negative or false positive results, in post mortem cases (paper II and III).

3. To describe the blood kinetics of EtG in social drinkers after ingestion of low dose of ethanol (paper IV).

4. To describe the blood elimination of EtG and EtS in heavy drinkers after ingestion of large and repeated doses of ethanol (paper V).

5. To use EtG and EtS kinetics in addition to ethanol in determining time of ethanol ingestion in, for instance, cases of drunken driving (paper IV and V).

6. To determine a serum/whole blood ratio for EtG and EtS (paper VI).

3. Summaries of the results

3.1 Paper I

A study of ethyl glucuronide in post-mortem blood as a marker of ante mortem ingestion of alcohol

In this study, we wanted to evaluate the sensitivity and specificity of EtG in blood as a marker of ante mortem alcohol ingestion. Forensic autopsy cases were divided into groups with and without ante mortem alcohol ingestion, according to strict inclusion criteria. The criteria for alcohol ingestion were: anamnestic history of alcohol ingestion before death, equal or higher level of ethanol in urine or vitreous humour, no trauma prior to death, no other putrefactive products detected (n-propanol, isopropanol) and no reported putrefaction of the corpse. In 93 cases (group 1a-1c) which fulfilled the criteria for ante-mortem alcohol ingestion, EtG was detected in blood in all cases, even when levels of ethanol were low (group 1c). In another 53 cases (group 2a and 2b) where there were no indications of ante mortem alcohol intake, EtG could not be detected in blood in a single case, including 11 cases in which ethanol was detected and considered to be most probably formed post mortem (group 2b).

Group	Description	n	No. of cases with EtG detected	Blood EtG concentrations; Range, mg/L	Blood EtG concentrations; Median, mg/L
1a	Alcohol ingested, high BAC	41	41	1,2 - 40	4,80
1b	Alcohol ingested, intermediate BAC	40	40	0,5 - 56	3,60
1c	Alcohol ingested, low BAC	12	12	0,11 - 4,0	0,77
2a	Alcohol not ingested, no endogenous ethanol	42	0	<LOQ	<LOQ
2b	Alcohol not ingested, endogenous ethanol	11	0	<LOQ	<LOQ

Table 1.
Description of groups, number of cases positive for EtG, and concentration levels (median, range).

In addition, 4 ethanol positive problem cases were presented, in which endogenous formation of ethanol was suspected. In three of these, no EtG was detected in blood, and this strengthened the assumption of post mortem ethanol formation. In the fourth case, a 17 year old male who died in a car accident, EtG was detected in blood in a concentration of 1.3 mg/L, which strongly suggested ante mortem ingestion of alcohol. In conclusion, this study showed that ante mortem alcohol ingestion led to detection of EtG post mortem, while no EtG was detected if alcohol was not ingested ante mortem.

3.2 Paper II

Disappearance of ethyl glucuronide during heavy putrefaction

In paper I, we found a high sensitivity and specificity of EtG as a marker of ante mortem alcohol ingestion. Putrefied cases were, however, deliberately excluded from this study and the stability of EtG during putrefaction was unknown. The aim of paper II was to investigate this stability and the possibility of false negative and false positive EtG results. For this, we used an in vitro study. Further, we used the information from the in vitro study on real cases to get an impression of the practical problems of degradation or formation of EtG.

Both degradation and formation of EtG was investigated. In the in vitro experiment, EtG was very unstable, and the concentrations decreased in blood samples at 30/40˚C without preservatives. This experiment was designed to simulate putrefaction in the corpse. On the other hand, EtG was stable with potassium fluoride at room temperature. This was designed to simulate storage in the laboratory. There was no formation of EtG either at 30/40˚C without preservatives, or at room temperature with potassium fluoride.

To illustrate the practical problems of degradation or formation of EtG, we used real autopsy samples analysed for EtG in blood. A case series of 39 post mortem ethanol positive cases sent routinely to the Norwegian Institute of Public Health for toxicological analysis were described. In 19 of these, EtG was positive in blood, with a median concentration of 1.6 mg/L (range 0.2-34.9). In the other 20 cases, EtG was negative in blood and the ethanol detected was, therefore, suspected to be of post-mortem origin. In 15 of these, a urine sample was available. We hypothesised that since concentrations are often higher in urine, there would still be traces of EtG left in this medium if post-mortem degradation was the reason for the negative result in blood. The results of analysis of EtG in urine demonstrated that 6 of the 15 cases were positive for EtG in urine, suggesting that the negative EtG in blood was a false negative. The other 9 showed no trace of EtG in urine, making the assumption of post mortem alcohol formation more reliable.

In conclusion, this study indicated that false negative results caused by degradation could occur, but we found no indication of false positive results caused by formation.

3.3 Paper III

In vitro formation of ethanol in autopsy samples containing fluoride ions

This is a case report describing substantial post mortem formation of ethanol despite the presence of preservative. In a post mortem blood sample from a diabetic patient, the concentration of ethanol increased from 0.4 g/L to 3.5 g/L during 8 days of normal storage in the laboratory (4ºC). The concentration in urine increased from 0 to 0.6 g/L. After 30 days, there was no trace of EtG in blood or urine. This indicated that no ethanol was ingested before death and also showed that no EtG was formed during this period despite massive post mortem formation of ethanol.

Days after autopsy	Ethanol blood (g/L)	Ethanol urine (g/L)	EtG blood (mg/L)	EtG urine (mg/L)
1	-	0	-	-
2	0.4	-	-	-
7	2.1	-	-	-
10	3.5	0.6	-	-
30	-	-	0	0

Table 2.
Levels of ethanol and EtG in blood and urine.

The samples were collected using standard equipment for post mortem cases at the Norwegian Institute of Public Health. These contain potassium fluoride as a preservative. The presence of fluoride in the blood and urine samples was verified by analysis. The concentrations were somewhat lower than expected, but this could be caused by the analytical method used. Microbiological analysis of the blood sample revealed the presence of *E. coli* as well as the anaerobic bacteria *Clostridium*, *Bacteroides* and *Prevotella species.*

3.4 Paper IV

A pharmacokinetic study of ethyl glucuronide in blood and urine: Applications to forensic toxicology

10 healthy male volunteers ingested 0.5 g ethanol per kg body weight in a fasted state. Median age was 24 years (range 21-46) and median body mass index was 23.9 kg/m^2 (range 20.1-28.3). They were all social drinkers with a median use of 32.5 standard drinks/month (range 10-60), and had abstained from alcohol during the week preceding the study, according to self-report. Blood and urine samples were collected for 14 h and 45-50 h after drinking, respectively.

Maximum concentrations (C_{max}) of EtG were reached after median 4 h (range 3.5-5), a median of 3 h (range 2-4.5) after C_{max} for ethanol. Maximum concentrations of EtG in blood ranged between 0.27 and 0.50 mg/L, with a median value of 0.32 mg/L. The maximum concentrations of ethanol in blood ranged between 0.49 and 0.78 g/L, with a median value of 0.58 g/L. The ethanol-to-EtG ratios in blood (ethanol in g/L, EtG in mg/L) were >1 only for the first median 3.5 h (range 2.5-3.5) after drinking. EtG elimination occurred with a median half-life of 2.2 h (range 1.7-3.1 h), and the renal clearance was 8.32 L/h (median, range 5.25-20.86). Total detection time was median 10 h (range 10-14) for EtG in blood.

The concentrations of EtG were always much higher in urine than in blood. The maximum concentrations of EtG in urine ranged between 41 and 73 mg/L, with a median value of 47 mg/L. The total amount of EtG excreted in the urine was median 30 mg (range 21.5-39.7), representing 0.017% (median, range 0.013-0.022) of the ethanol given, on a molar basis. Total detection time was median 30 h (25-35) for EtG in urine.

In conclusion, this study described in detail the blood kinetics of EtG and indicated that if alcohol ingestion occurred only a few hours before blood sampling, low and increasing levels of EtG would be expected.

3.5 Paper V

Pharmacokinetics of ethyl glucuronide and ethyl sulphate in heavy drinkers

16 chronic heavy drinkers who stopped drinking <24 h earlier were included directly after admission to an alcohol withdrawal clinic. Each patient was thoroughly interviewed about their alcohol consumption, time of last ingestion, use of other drugs or medicines and relevant diseases. On admission, and repeated at varying intervals for the next 20-43 h, 3-5 blood samples were drawn. The median age was 46 years (range 39-61) and the median body mass index was 26 kg/m^2 (range 19-39). The median EDI the last 3 months was 172 g (range 60-564). All subjects, therefore, fulfilled the criteria for heavy drinking.

The first blood sample was collected median 2.5 h after end of drinking (range 0.5-23.5). Two patients had levels of EtG and EtS below LOQ (0.09 mg/L) in all samples, the first collected 19.25 and 23.5 h after cessation of drinking, respectively. Of the remaining 14 patients, one subject, suffering from both renal and hepatic disease, showed concentrations of EtG and EtS substantially higher than the rest of the patients. This patient's initial value of EtG was 17.9 mg/L and of EtS 5.9 mg/L, with terminal elimination half lives of 11.9 h for EtG and 12.5 h for EtS. Among the remaining 13 patients, the initial median values were 0.7 g/L (range 0-3.7) for ethanol, 1.7 mg/L (range 0.1-5.9) for EtG and 0.9 mg/L (range 0.1-1.9) for EtS. Elimination occurred with a median half-life of 3.3 h for EtG (range 2.6-4.3) and 3.6 h for EtS (range 2.7-5.4). Concentrations of EtG and EtS returned to below or just above LOQ after median 23.5 h (range 7-40).

In conclusion, this study indicated that the blood kinetics of EtG and EtS did not differ considerably between social and heavy drinkers. One important exception was the subject suffering from kidney disease, but this condition was not necessarily related to alcohol abuse.

3.6 Paper VI

Serum/whole blood concentration ratio for ethyl glucuronide and ethyl sulphate

Serum/whole blood ratios for EtG and EtS were determined by withdrawing two blood samples simultaneously, one for analysis in serum and one for analysis in whole blood, from 13 subjects at admission to an alcohol detoxification unit. EtG and EtS were analyzed in serum and whole blood with calibration standards and controls prepared both in serum and in whole blood. The median serum/whole blood value for EtG was 1.69 and the range was 1.33-1.90. For EtS, the median serum/whole blood ratio was 1.30 and the range was 1.08-1.47. The serum/whole blood ratio was significantly lower for EtS than for EtG ($p<0.001$).

In conclusion, this study showed that concentrations of EtG and EtS were higher when measured in serum compared to whole blood.

4. Methodological considerations

4.1 Analysis of EtG and EtS in blood and urine

The analytical methods used to determine EtG and EtS in whole blood and urine samples in the papers included in this thesis were fully validated LC-MS methods comprising the use of deuterated internal standards for both compounds. Until 2006, we only analysed EtG. EtS was later included in the EtG and EtS blood and urine methods, respectively.

The LOQ for EtG and EtS in blood was 0.058 mg/L and 0.021 mg/L, respectively and the LOQ for EtG and EtS in urine was 0.19 mg/L and 0.12 mg/L, respectively. These values were considered satisfactory for the purpose of this thesis.

The method's precision and accuracy was determined during the method validation and is included in papers I, IV, V and VI. In 2006, 131 urine samples were analysed for EtG using our methods at the Norwegian Institute of Public Health (NIPH). Three months later, the same samples were reanalysed for EtG at Karolinska University Hospital (KUH), Stockholm, Sweden, using a fully validated, published method (44) (table 3, unpublished results). The samples were collected from healthy volunteers in the drinking experiment (paper I) using Sterilin tubes without preservatives and stored at 4ºC. Comparing the results showed slightly lower values from the NIPH compared to KUH, with only 13 of 131 samples having a NIPH/KUH ratio above 1. This strengthened the assumption that there was no instability of EtG during the 3 months storage. 45 of the samples were negative for EtG, 86 were positive. Of these, a mean NIPH/KUH ratio of 0.92 was found (SD 0.15) (table 3). The calibration curve differed between the two methods, including higher concentrations at the KUH (highest standard 1500 mg/L vs 40 mg/L). Only very few samples contained more EtG than 40 mg/L, but among these, the results from KUH would be the most reliable. The results were considered satisfactorily similar and analytical variations are therefore unlikely to have biased the results in this thesis.

No	Result NIPH (EtG, mg/L)	Result, KUH (EtG, mg/L)	Ratio NIPH /KUH	No	Result NIPH (EtG, mg/L)	Result, KUH (EtG, mg/L)	Ratio NIPH /KUH	No	Result NIPH (EtG, mg/L)	Result, KUH (EtG, mg/L)	Ratio NIPH /KUH
1	4.96	6.75	0.73	30	6.98	6.74	1.04	59	36.76	44.3	0.83
2	6.29	8.32	0.76	31	1.6	1.49	1.07	60	21.83	24.3	0.90
3	34.89	50.9	0.69	32	0.18	0.16	1.13	61	2.49	2.77	0.90
4	36.64	47.6	0.77	33	0.15	0.1	1.50	62	2.09	2.37	0.88
5	52.73	72.5	0.73	34	19.16	18.4	1.00	63	1.57	1.6	0.98
6	17.33	19	0.91	35	6.68	6.68	1.00	64	0.47	0.55	0.85
7	3.2	3.52	0.91	36	6.84	6.94	0.99	65	4.78	5.65	0.85
8	0.33	0.4	0.83	37	9.2	9.86	0.93	66	32.46	38.4	0.85
9	0.31	0.46	0.67	38	21.01	23.9	0.88	67	63.13	90.2	0.70
10	0.15	0.18	0.83	39	5.2	5	1.04	68	23.34	24.9	0.94
11	26.56	29.7	0.89	40	2.62	2.86	0.92	69	10.67	12.2	0.87
12	47.23	63.4	0.74	41	0.81	0.73	1.11	70	12.26	14.7	0.83
13	24.85	29.9	0.83	42	1.42	1.71	0.83	71	2.51	2.81	0.89
14	28.71	33.6	0.85	43	10.72	6.33	1.69	72	2.36	2.76	0.86
15	24.17	29.1	0.83	44	10.67	13.3	0.80	73	1.16	1.31	0.89
16	9.78	9.93	0.98	45	17.45	10.8	1.62	74	0.27	0.29	0.93
17	6.25	6.48	0.96	46	3.52	4.05	0.87	75	0.13	0.08	1.63
18	2.03	2.55	0.80	47	1.73	1.7	1.02	76	0.2	0.19	1.05
19	0.68	0.79	0.86	48	0.31	0.32	0.97	77	16.03	17.4	0.92
20	0.22	0.33	0.67	49	4.95	5.51	0.90	78	43.13	50.2	0.86
21	0.13	0.35	0.37	50	16.34	28.2	0.58	79	6.34	6.49	0.98
22	10.38	11.9	0.87	51	14.48	15.4	0.94	80	3.11	3.24	0.96
23	62.81	83.9	0.75	52	33.28	39	0.85	81	2.35	2.63	0.89
24	20.86	26.2	0.80	53	8.94	8.81	1.01	82	33.12	41.7	0.79
25	6.96	8.23	0.84	54	1.18	1.46	0.81	83	53.85	72.3	0.74
26	9.87	12.8	0.77	55	1.07	1.35	0.79	84	7.96	9.08	0.88
27	2.77	2.84	0.98	56	0.17	0.19	0.89	85	17.11	18.7	0.91
28	1	1.11	0.90	57	19.56	18.8	1.04	86	38.46	54.6	0.70
29	0.58	0.59	0.98	58	5.49	6.01	0.91				

Table 3. Results of the analyses of the same positive urine samples (n=86) at the Norwegian Institute of Public Health (NIPH) and at Karolinska University Hospital (KUH).

4.2 Post mortem presence of EtG

Paper I had methodological weaknesses. First, there was a problem with the classification of cases into “alcohol ingested before death” or “no alcohol ingested before death”. To be able to determine the sensitivity and specificity of EtG as a marker of ante mortem alcohol ingestion in post mortem cases, a gold standard was needed. This was complicated as there was no reliable criterion for post mortem alcohol formation. We, therefore, had to use all available criteria together to minimize the chance of misclassifying cases. The cases included in this paper were those in which classification of ethanol origin would be easiest, as all criteria for alcohol ingestion were fulfilled in group 1 (table 1, page 15), and all criteria for post mortem formation were fulfilled in group 2. The most problematic criterion was the absence of putrefaction of the corpse. We used this as a criterion for the ethanol detected being caused by ingestion before death, as it minimized the chance of endogenous formation of ethanol. A case in which the corpse was putrefied was, therefore, excluded from group 1. In group 1a and 1b, where blood alcohol levels were high or intermediate, this

was a smaller problem, but in group 1c, where BAC, and expected EtG, was low, inclusion of putrefied cases could have led to false negatives due to degradation of EtG. This would have lowered the sensitivity. Correspondingly, the group 2b, in which endogenous formation of ethanol was assumed because of presence of putrefaction, this could be misclassified and the negative EtG could be caused by degradation. On the other hand, the fulfilment of all the other criteria made this less probable. Paper I investigated EtG as a marker of ante mortem alcohol ingestion, but this study did not include complicated cases. Unfortunately, these complicated cases are those where the classification of ethanol as endogenous or exogenous is most difficult to assess, and where EtG is most needed.

Second, the work in paper I depended on correct information from forensic pathologists on case history and putrefaction of the corpse as we had no way to check that information.

Third, as the cases were included retrospectively, the samples used were up to 5 years old. This was necessary to obtain a sufficient number of cases. During this period, the samples were stored at the laboratory at -20ºC and they did contain potassium fluoride as a preservative. Considering the good stability of EtG during storage, this was assumed not to represent a large methodological problem.

The in vitro study in paper II also had obvious weaknesses. The part of the study where blood samples without preservatives were incubated at 30/40ºC, aimed to simulate the putrefaction occurring in a corpse, before sample collection. The ideal model of this would be to store a corpse under different conditions and collect blood samples before and immediately after death and then at varying intervals. This model was considered unethical, and other ways to use an intact corpse, for example animal models, were considered less representative. We, therefore, had to use samples of post mortem blood and these might have behaved differently from a whole corpse, where, for example, continual degradation would keep adding micro-organisms to the blood.

Also, in a better design we would have studied a number of blood aliquots drawn from the same corpse, to exclude analytical and other errors by verifying that the EtG in identical samples behaved similarly. Since the amount of peripheral blood available from a corpse was limited, we chose to study one large blood aliquot from each corpse instead of several

smaller ones. This was to prevent the withdrawal of blood for analysis from changing the volume significantly and altering the conditions for further degradations.

The part of paper II with blood samples containing potassium fluoride was aimed to simulate sample storage in the laboratory and represented fewer methodological problems. We used samples sent to the Norwegian Institute of Public Health routinely for toxicological analysis, but selected the samples directly after arrival. The stability in these samples was studied for the same time, 3 weeks, and storage at 4/-21ºC for a longer time period was not investigated.

In papers I and II, we assumed that the samples contained fluoride when standard equipment was used and the presence of fluoride ions was not verified. Of course, it would have been better to verify this by analysis of fluoride ions in every single sample. However, the markedly improved stability in the samples with fluoride (paper II) made the absence of fluoride highly unlikely. In paper III, presence of fluoride was verified. The concentration (corresponding to 0.21% w/v potassium fluoride in blood and 0.25% w/v in urine) was lower than the recommended 1% w/v. There are two possible explanations for this: First, the analytical method detected only free fluoride ions and the amount bound to calcium, proteins and other compounds in blood could constitute a significant part. Second, the amount of potassium fluoride, added manually, might have been too small in these particular two vials. It would be easier to interpret the findings if a series of samples were analysed and the results compared. We realised this after we received the results from the first sample. The analysis of fluoride in whole blood was complicated, and unfortunately analysis of more samples was impossible.

In paper III, EtG could have been analysed on arrival and on multiple occasions thereafter. Unfortunately, EtG was not analysed until day 30, when the case came to our attention. We cannot absolutely exclude the possibility that the negative EtG was caused by degradation but this appears unlikely, considering the low ethanol value in the first analysis as well as the negative result in urine.

In all papers investigating EtG post mortem (papers I, II and III), simultaneous determination of EtS would have been valuable, but unfortunately, no analytical method for EtS existed at that time. As a control, some of the cases presented in paper II were analysed

for EtS when the analytical method was fully validated, three years after the first study was carried out. Nine of these ethanol positive cases were negative for EtG in both blood and urine. We cannot totally exclude the possibility that they were false negatives caused by degradation in both media, but the analysis of EtS could answer this question, as recent research has found superior stability of EtS (119,146-148). The analyses of EtS showed that total loss of EtG also in urine probably did not occur, because we found that none of the nine urine samples negative for EtG contained EtS. The ethanol detected could therefore with great certainty be considered to be formed post mortem, given the negative EtG and EtS in both blood and urine. Also, the case described in paper III was reanalysed for EtG and EtS, and also in this, there were no traces of EtS in blood or urine. This further strengthened the assumption that no alcohol was ingested before death. There were, of course, methodological problems in reanalysis several years later and more studies of EtS in post mortem material are needed.

4.3 Blood kinetics of EtG and EtS in living subjects

We first performed a kinetic study in healthy, male social drinkers, without medical illness or use of any drugs (paper IV). A single, low dose of ethanol was administered in a fasted state and only water and a simple diet was ingested after this, at controlled intervals. The idea of performing this study in such a homogenous group of individuals and under such strictly controlled conditions might sound strange as the transferability to forensic material is uncertain. However, the aim of this study was to start a systematic investigation of EtG kinetics, and this required a good, controlled study as a basis. Where the results are used to give recommendations on determining time of alcohol ingestion, these are valid only in equally controlled conditions, which are almost never found in forensic toxicology.

A kinetic study of a different population with ingestion of higher doses of ethanol was therefore desirable, but it was then difficult to use an experimental design. The study of EtG kinetics in heavy drinkers (paper V) therefore had methodological weaknesses, the most obvious being the lack of data during ethanol absorption phase and the amount of alcohol ingestion based on self report. Regarding the missing data during ethanol absorption phase, this was regrettable, but impossible to overcome. On the other hand, 10 out of the 16 patients were included within 3 h after the end of drinking. Efforts were made to minimize bias in the self declarations of consumption. A thorough interview was carried out and all

subjects, except one, provided concise information. Misreporting will always be a problem but any over or under estimating of alcohol intake would not bias the results of the study particularly. The information about other diseases and use of drugs or medicines was also based on self report because the approved protocol for the study did not allow us to collect information from the patient journals. Twelve of the 16 subjects used different medicinal drugs. The interaction with glucuronidation of ethanol for some of these might be hypothesised and could explain some of the larger variations in concentration levels compared to paper IV. We did not make any analyses to quantify the extent of these interactions; as such problems would also be present in forensic material.

Also, because of limitations from the ethics committee and the clinical condition of the patients, only 3-5 samples were collected per patient at varying intervals. On the other hand, this design was, in our opinion, the best way to study a situation comparable to for instance drunk driving.

4.4 Serum/whole blood ratios for EtG and EtS

For the reliability of these results, it was important that the serum and whole blood samples were collected at exactly the same time. We chose to use the same needle insertion and the sample for analysis in blood was then drawn prior to the sample for analysis in serum. As there were only a very few seconds between them, we considered this satisfactory.

We had to exclude the possibility that analytical differences between the two matrices were the reason for different results. The whole blood calculations were performed using calibrations and controls in whole blood, while calibrations and controls were prepared in serum for the determination of concentrations in serum samples. Also, as an extra control, we calculated EtG and EtS concentrations in the blood and serum samples from calibrations and controls prepared in both types of matrices. The almost identical results in both situations strengthened our findings. Also, the use of deuterated internal standards for both compounds improved this method.

5. General discussion

This thesis has explored some important, but previously unknown aspects of EtG and EtS; presence in post mortem cases and kinetics in blood of living subjects. We studied the presence in post mortem cases in order to use EtG and EtS as a marker of ante mortem alcohol ingestion. Our main finding was that EtG could serve as a marker of ante mortem alcohol ingestion, but false negative results could occur as a result of putrefaction of the corpse. We studied the blood kinetics in living subjects in order to find out whether EtG and EtS could be used to determine time of alcohol ingestion in, for example, cases of drunk driving. Our main finding was that high levels of EtG and EtS attenuate an explanation of BAC caused by very recent alcohol ingestion. We also determined the serum/whole blood ratios and found that concentrations of EtG and EtS were higher in serum than in whole blood.

5.1 Use of EtG and EtS as a marker of ante mortem alcohol ingestion in post mortem cases

We showed that ethanol ingestion ante mortem did lead to the presence of EtG in post mortem samples, but that this EtG might be lost during putrefaction of the corpse. We also showed that no EtG was present in post mortem samples if alcohol was not ingested ante mortem, even if endogenous ethanol was formed.

To date, no systematic studies of EtG in forensic material have been published by others, but stability has been studied quite thoroughly in recent years. Both our study (paper II) and others have documented that EtG is unstable in the presence of bacteria, both in post mortem material (115,119) and in clinical urine samples (146,147). The largest decrease in concentrations was seen in a study by Helander and Dahl, where a nearly complete loss of 39.3 mg/L EtG occurred during 5 days at room temperature. The smallest decrease was seen in the early study of Schloegl et al, with ~30% decrease in EtG concentrations (n=3) during 30 days at room temperature (115). These samples did not contain any preservatives (W. Weinmann, personal information). We found that refrigeration and the presence of preservatives made EtG stable (paper II), results that are also supported by other publications (146,148). It is also well documented that EtS is stable in the presence of bacteria, both in clinical urine samples and post mortem material (119,146,147), although a very recent publication showed some instability under extreme conditions, also of EtS

(149). According to the available research, the stability of EtS must however be assumed to be much superior to that of EtG. The degradation of EtG was probably caused by ß-glucuronidase, which occurred in 3 of 11 bacteria tested (146), and 5 of 20 bacteria tested (119), respectively. In both studies, ß-glucuronidase activity was found in E. coli. Since E.coli is such a frequent micro-organism both in urinary tract infections and in post mortem material (150), this is likely to constitute a significant problem. Degradation of EtS would occur as a result of hydrolysis by sulphatase, but this was not found in any of the bacteria tested and also, commercially prepared sulphatases did not hydrolyze EtS (146). We could not exclude the possibility that such bacteria exists (151), but the lack of sulphatase in the most common micro-organisms makes a practical problem of EtS degradation unlikely.

Regarding the possibility of formation of EtG, the results are more insecure. Both our work (papers II and III) and other studies (115,138) showed that no EtG was formed, while another work (147) showed formation of EtG in clinical urine samples containing bacteria. No formation of EtS was found (138,147). The only sign of EtS instability was the strange phenomenon occurring in the kinetic study of Halter et al, where a compliant subject suddenly increased urine EtS, but not EtG, 42 h after ingestion (134). There was no explanation for this, but error in the analysis could not be excluded.

If no post mortem formation of ethanol has occurred, one could still expect a very low level of ethanol in these cases, since living subjects have endogenous blood ethanol physiologically (152-154). Considering that an expected EtG level of about 1/1000 of normal endogenous ethanol levels (~0.0015 g/L) would not exceed the analytical lower limits of detection for EtG, this would not be expected to cause false positive results. The negative EtG in all of the 42 post mortem cases regarding children in our study (paper I), as well as measurements in living abstainers (131) support this assumption.

From the research so far, it therefore looks like EtG and EtS together are reliable markers for alcohol ingestion in post mortem cases. They can be determined in problem cases, but there are few reports of the practical use of this. A small number of case reports have been published in recent years. One publication described a deceased child with BAC 2.0 g/L at autopsy. EtG was only analysed in liver, where it was <LOD, and no EtS was analysed. The negative EtG could therefore be caused by degradation. On the other hand, the reason for the large post mortem formation of ethanol was assumed to be an intra venous infusion of

glucose prior to death, and there was no putrefaction of the corpse (118). Another publication described accidental alcohol ingestion in a child, resulting in death and post mortem ethanol concentrations of 4 and 5 g/L in blood and urine, respectively. In this case, the presence of EtG in this child's hair (46 and 54 pg/mg) indicated chronic exposure to large amounts of alcohol (117). Also, in a corpse of an alcoholic exhumed 27 years after death, EtG and EtS were analysed. There was no information about ethanol results, but low levels of EtG and EtS were found in liver, kidney and blood (116). This was the only report of EtS post mortem, and it indicated that total degradation of EtG and EtS did not occur during such an extremely long time period. It is difficult to exclude the possibility that the presence of EtG was caused by formation after death, but the positive EtS made this less probable. On the other hand, studies on the fate of EtS during such extreme conditions have never been published before. Interestingly, the EtS/EtG ratio post mortem was much higher than in living subjects (61,134,155), maybe because EtS has superior stability compared to EtG.

In addition to possible instability, there are some relatively minor problems one must be aware of when using EtG and EtS to exclude post mortem alcohol formation. First, the lag time of EtG formation compared to alcohol could lead to false negative results. If death occurred immediately after alcohol ingestion, it is possible to imagine that BAC would be positive, but there has not been enough time to form EtG and EtS. This time gap is, however, short, <30 minutes (paper I), and would not normally constitute a big practical problem.

Second, the longer detection time for EtG and EtS compared to ethanol could lead to false positive results. For example, if a person died 12 h after alcohol ingestion, alcohol could be completely eliminated from the body, but EtG or EtS would still be detectable in blood. If ethanol was detected, this might be formed post mortem, but the positive EtG and EtS would lead to the opposite conclusion. This difference in detection times for ethanol and EtG are however quite short in blood, ~10 h, according to our results (paper I and IV) and others (134) and the problem would be more pronounced if EtG and EtS were only analyzed in urine, in which this difference might be up to several days (45,61). This shows how the analysis in blood and urine complement each other; the risk of false negatives caused by instability is highest in blood and the risk of false positives from previous ethanol ingestions is highest in urine.

Third, if EtG and EtS were detected together with alcohol, it is difficult to exclude the possibility that some of the ethanol was formed post mortem. The level of EtG and EtS compared to ethanol could give an indication, but this would also vary according to where in the time course of alcohol metabolism the subject was at time of death. Also, some of the ethanol detected might have diffused from the stomach into the blood stream after death (156,157), if death occurred too early after ingestion to allow absorption to be completed. According to the road traffic legislations in Norway, a measured BAC above 0.2 g/L, or an amount consumed that would later lead to such a BAC, defines the limit for drunk driving. However, in other cases, this could be a problem.

The discussion about the sensitivity of EtG alone might sound unnecessary as most laboratories perform simultaneous determination of EtS. On the other hand, some laboratories have started to use the immunological screening, which only detects EtG (41,52,158). I would conclude that negative EtG results in post mortem cases in which endogenous formation of ethanol was suspected, obtained for instance by immunological screening, should be confirmed by simultaneous EtG and EtS analysis.

5.2 Implications; Use of EtG and EtS as markers of ante mortem alcohol ingestion in post mortem cases

Because of the research in papers I, II and III, as well as other progress in the field, EtG and EtS were routinely analyzed at the Norwegian Institute of Public Health in autopsy cases where post mortem formation of ethanol was suspected, according to the traditional criteria. From the method for EtS analysis validated in June 2007 until December 2008, 36 such cases were identified. In 19 of these, EtG and EtS were positive in the media analyzed (blood and/or urine). This strongly suggested that alcohol was ingested before death. In 16 cases, EtG and EtS were negative in the media analyzed, strongly suggesting that the ethanol detected was formed post mortem (table 4).

Group according to EtG and EtS results	Et blood, mean (SD)	Et urine, mean (SD)	EtG blood, mean (SD)	EtS blood, mean (SD)	EtG urine, mean (SD)	EtS urine, mean (SD)
EtG and EtS positive (n=19)	0.15 (0.22)	0.44 (0.58)	2.5 (5.5)	1.3 (1.8)	65.2 (67.0)	17.0 (24.5)
EtG and EtS negative (n=16)	0.32 (0.34)	0.25 (0.24)	0 (0)	0 (0)	0 (0)	0 (0)

Table 4. Levels of ethanol, EtG and EtS in blood and urine in 19 cases where EtG and EtS were positive as well as 16 cases where EtG and EtS were negative. Et=Ethanol. Ethanol in g/L, EtG and EtS in mg/L.

In the remaining case, there was inconsistency between the results of EtG and EtS (table 5). In this case, EtG and EtS were both positive in urine, EtS was also positive in blood, while EtG was negative. One possible explanation is degradation of the probably low level of EtG that was present in blood. The ethanol detected was, therefore, most likely ingested, and this case showed the practical problem of EtG instability.

Case nr.	Ethanol blood g/L	Ethanol urine, g/L	EtG blood, mg/L	EtS blood, mg/L	EtG urine, mg/L	EtS urine, mg/L
1	0.3	0.4	0	0.1	12.1	3.2

Table 5. One case in which there were inconsistency between EtG and EtS results. Et=Ethanol. Ethanol in g/L, EtG and EtS in mg/L.

These results show that EtG and EtS together could lead to conclusions on the origin of ethanol in post mortem cases. These ethanol metabolites still cannot be used as a gold standard, but if combining EtG and EtS, one would assume a very high sensitivity and specificity to detect ante mortem alcohol ingestion, according to all available knowledge. It is probable that many of the questionable ethanol findings in the literature, for instance from aviation accidents and the USS Iowa disaster, could have been answered by post mortem analysis of EtG and EtS.

5.3 Blood kinetics of EtG and EtS in living subjects

To date, two studies of EtG kinetics in serum have been published, in addition to ours. These were controlled drinking experiments in healthy volunteers with low to moderate doses of ethanol ingested. We showed that in healthy volunteers after ingestion of low doses of ethanol, the C_{max} varied relatively little between subjects (paper IV). Such relatively small inter individual variations were also seen in the second kinetic study (134). In

contrast, a much larger inter individual variation was seen in the third publication. This could only partly be explained by different doses, and other factors must also have contributed, such as age, diseases, ingestion of food and drink and others. The very high EtG was reached in the only female participant (131), but there is no evidence of enhanced glucuronidation in females compared to males, in fact the opposite is mentioned in the literature (159,160). The other kinetic study (134) included both men and women but after my own calculations based on the authors' results (corrected for dose of ethanol), there were no statistically significant differences in the AUC for EtG between men and women ($p=0.069$). In our study of heavy drinkers (paper V), the four female subjects did not show different EtG/ethanol ratios from the male participants.

In our kinetic study, EtG peaked median 3 h after ethanol and the level of EtG (mg/L) x 10^3 was lower than that of ethanol (g/L) for the first 3.5 h after ingestion. This was in accordance with both the other kinetic studies (131,134). In our study, EtG was eliminated with a terminal half life of 2.2 h. Terminal half life for EtG has only been reported in one other study, and the results were in accordance with ours, although they calculated half lives based on data from only two subjects (131). In the data from a case report infusing a high dose of ethanol, the EtG levels reached a plateau during the continuous supply of ethanol, started to decrease some hours after end of infusion and was eliminated with a somewhat longer half life than in to our data (133).

An interesting issue is the ratio between EtG and EtS. We studied levels of EtS in urine (not included in this thesis) (61). Levels of EtS in blood have been reported in one other low dose experiment (134) as well as in our study of heavy drinkers (paper V). Both our studies and other studies of EtG and EtS in blood and urine indicated that levels of EtG were higher than the corresponding levels of EtS (61,155), also when corrected for different molecular weight, with molar EtG/EtS ratios reported as 1.7. Only one study in urine showed higher concentrations of EtS than EtG (161), and similar molar concentrations of EtG and EtS were seen in serum in the kinetic study by the same group (134), although the AUC was higher for EtG. The reason for the higher EtS concentrations is unknown, but direct ingestion of EtS could be hypothesised. Also, the measurement in serum would be expected to cause even lower concentrations for EtS compared to EtG, according to our results in paper VI. It is documented that certain alcoholic wines contain EtS (up to 40 mg/L) and to a much smaller extent EtG (up to 4 mg/L) (162), as fungi and yeasts can carry out phase two

reactions (163) during the production process. If such beverages were ingested in the experiment, this could lead to higher concentrations of EtS. On the other hand, there is no knowledge about the bioavailability of EtG and EtS. The fact that EtG and especially EtS could be formed from ethanol in alcoholic beverages could theoretically strengthen the idea that EtG and EtS could form in biological matrices. On the other hand, there is quite good evidence suggesting that no formation of EtS occurs. The amount of micro bacteria present during the production of alcoholic beverages is probably much higher, and the species could be different from that present in biological matrices.

We calculated that 0.013-0.022% (n=10) of the ingested dose of ethanol was excreted in urine as EtG (paper IV). This was in accordance with an earlier study after the same dose of ethanol (n=6) (49), but lower than another study at a lower dose (n=7) (55). We further showed that somewhat less of the ingested ethanol was excreted as EtS (61) (not included in this thesis). The amount excreted as EtG was significantly higher than EtS ($p<0.001$). This was lower than in an earlier single case which reported results for EtS (161). However, this was the only study to show higher values of EtS than EtG in urine.

Another interesting question is whether a different proportion of the ingested ethanol is converted to EtG at higher doses. An early, preclinical study indicated that more ethanol was converted to EtG at higher doses in rabbits (28). The method of administration was not reported. In humans, this should be studied by administering different doses and collecting all urine voided until the end of EtG elimination. One study administered 3-25 g of ethanol to 4 subjects, and showed a dose-response relationship, with a larger % of the ethanol excreted as EtG at higher doses (56). When comparing this to our results (paper IV) and those by Dahl et al (49), where larger doses were ingested, this further strengthens the picture of a dose-response curve. In our study of heavy drinkers (paper V), where very large doses were ingested, the EtG/ethanol ratios were higher, but this could also be caused by accumulation of conjugated metabolites over a longer time period. Although the terminal half life for EtG and EtS is only ~3 hours, accumulation could still occur, as some subjects ingest alcohol in frequent doses (paper V). All these results could be explained by saturation of the oxidative ethanol metabolism, driving more ethanol in the non-oxidative direction. ADH is saturated at approximately 0.2 g/L ethanol, and this is in accordance with the findings of glucuronidation, which showed increasing activity after ingestion of doses higher than 0.15 g/kg, resulting in blood alcohol concentrations around 0.2 g/L (56). An

alternative explanation is the lower bioavailability of ethanol when ingested in low doses. The pre-systemic gastric metabolism of ethanol is more pronounced after ingestion of low doses, and a smaller percentage of the ingested ethanol would be available for glucuronidation. The possibility can, therefore, not be excluded that the same amount of available ethanol is metabolised to EtG and EtS at different doses, even if concentrations of these metabolites were higher after higher doses ingested orally. To answer this question, intra venous ethanol must be administered. Also, our post mortem material (paper I) and results from urines collected from drunk drivers (48) showed the opposite results with a lower ratio between EtG and ethanol at higher BACs. These results were less reliable as they were not controlled experiments, but only randomly collected single samples from each subject. I suggest that these results were caused by the fact that lower BACs occur mainly in the later phase of ethanol metabolism, where the levels of EtG have reached higher concentrations. The explanation was, therefore, not necessarily a limited capacity of the glucuronidation process, as we originally suggested in paper I. Also, all kinetic studies showed a tendency towards higher levels of EtG in blood or urine at higher BAC. The research so far could therefore suggest that there is no saturation of the enzymes responsible for EtG formation, at least in the ethanol doses tested, and that the non oxidative metabolism possibly increases as the oxidative pathways are saturated.

There are no reports in the literature of extra renal excretion of EtG or EtS, but this cannot be excluded theoretically, considering that the glucuronides of morphine, for example, are partly excreted in bile and eventually in faeces (164,165). In paper IV, we calculated a renal clearance for EtG, based on the AUC in blood and the urinary excretion during the same time interval. We then calculated a total clearance, by using a calculated V_d for EtG. That calculation was inaccurate and only gave an indication, as it was based on a theoretically calculated C_0, representing the ingested “dose” of EtG. However, a much higher value for total clearance compared to renal clearance would indicate extra renal excretion. Our values for total clearance were in the same order of magnitude as the renal clearance, actually slightly higher, indicating inaccurate calculations, but probably no significant extra renal excretion. As these aspects were not studied by others, we can not relate our results to other research.

The up or down regulation of UGT and SULT during chronic heavy alcohol use is another interesting question. Preclinical studies have indicated both increased (166-168) and

unchanged (168,169) glucuronidation and sulphatation after chronic ethanol feeding. Also, decreased activity was shown (170), but this was due to reduced availability of required co-factors, not lower enzyme activity. The results are therefore quite confusing, and clinical evidence is missing. Our kinetic study of heavy drinkers (paper V), which is the only publication on a sample from this population, gave a picture of the difference in kinetics between social and heavy drinkers. Our interesting finding was that the terminal half lives, with the exception of one subject, were comparable in the two populations, at 2-3 hours. This may indicate that there is no up-regulation of enzyme activity, but it also described the excretion of EtG and EtS. The concentrations of EtG and EtS returned to a low level around 24 h after end of dinking. This was in accordance with studies of EtG in plasma in patients with alcohol use disorders, where EtG was not detected median 20 h after end of drinking (136). One single subject in our study of heavy drinkers had kinetics of EtG and EtS which differed completely from the rest of the material with four times longer half lives. He suffered from kidney disease, which could cause delayed excretion and therefore considerably lengthen the half lives of the conjugated ethanol metabolites. This effect of kidney disease is described for excretion of other glucuronides, for example, those of morphine (171-173). This individual also suffered from hepatic disease (hepatitis B and C), but this should not, in theory, cause prolonged half lives. Also, his ethanol metabolism was normal, and the two other patients suffering from hepatitis had normal kinetics of EtG and EtS. He still showed a high level of EtG and EtS 31 h after end of drinking and if using his half life of 12 h, the calculated detection times for EtG and EtS in blood would be about 100 h after end of drinking. One previous study found EtG in plasma in 4 of 81 patients >48 h after self reported end of drinking. This was interpreted as misreporting, but could also represent subjects with delayed excretion caused by renal failure or other factors (135). Also, in an abstract from 1995, it was reported that half lives for EtG in heavy drinkers ranged between 2 and 12 h and that “some subjects showed longer half lives than social drinkers” (174). No further information about the study was available.

The maximum levels of EtG and EtS in heavy drinkers were higher than in social drinkers, but this was caused by higher doses ingested and accumulation of EtG and EtS, not necessarily by up regulation of the enzymes. To answer the question of effect of chronic heavy alcohol use on UGT and SULT satisfactorily would therefore require different study designs from ours, with measurements of total amount excreted both in social and heavy drinkers in controlled experiments.

I conclude that the kinetics of EtG and EtS are comparable in social and heavy drinkers. We found no evidence that the effect on the liver frequently seen after heavy alcohol use would affect the kinetics of EtG and EtS. We showed that the presence of renal disease probably causes substantially prolonged half lives. This clinical status is not necessarily related to heavy alcohol use. We can not exclude other factors that may also result in delayed renal excretion, for example, interactions with drugs like probenecid. This drug inhibits the excretion of substances which are secreted in the kidney by tubular secretion (175), which is the well known excretion route for both glucuronic acid and sulphate conjugates (176). Diseases affecting other organs would be less likely to affect the half lives of EtG and EtS given that these conjugated ethanol metabolites are almost solely excreted renally.

5.4 Use of EtG and EtS to determine time of alcohol ingestion

As described in the introduction (section 1.5), there were only limited methods to determine time of alcohol ingestion in a living subject and one aim of this thesis was to use measurements of EtG and EtS in blood samples for this purpose.

Our controlled low dose kinetic study (paper IV) as well as two other kinetic EtG studies (131,134), indicated that concentrations of EtG could provide information about time of alcohol ingestion. According to all three studies, EtG was formed with a delay compared to ethanol and EtG levels should be increasing in two subsequent samples if alcohol was ingested in the 2-4 hours before sample collection. Also, if ingestion was within the 2-3 h before sampling, the levels of EtG x 10^3 should be below that of ethanol (mg/L and g/L, respectively).

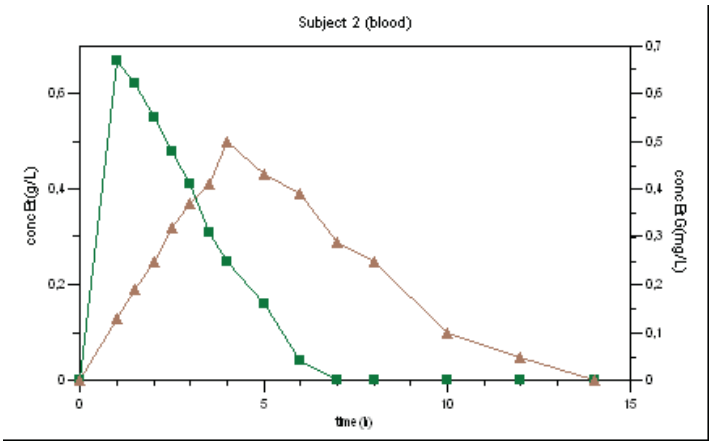

Figure 1. The time after ingestion of ethanol determines the level of EtG (brown line) compared to ethanol (green line).

This would only be valid if ethanol was ingested in the same pattern as in the experiments; one single, low to moderate dose ingested by a social drinker, otherwise healthy. This scenario is hardly ever the case in drunk driving. If high or decreasing levels of EtG are found, it could then be concluded that those levels did not originate from a single, recent ingestion, and alcohol was also drunk before driving. The question is how much earlier could drinking occur that would lead to such high levels of EtG? Could an individual drink the night before and still have high EtG concentrations even if the alcohol was eliminated a long time ago? The explanation of drinking after driving as the only source of impairment could therefore be correct, but the EtG levels had no connection with that ingestion.

That was what we showed in our study of heavy drinkers (paper V). The results indicated that after a period of very heavy drinking, the terminal half lives for EtG and EtS were ~3 h and the levels of EtG and EtS in blood would return to below LOQ after approximately 24 h, ~10 h after ethanol. High levels of EtG and EtS could therefore not be explained by drinking the day before blood sampling.

If a suspect claims to have drunk alcohol after an incident, it would be a relatively quick ingestion of a single dose of alcohol. This situation resembles that of the controlled, kinetic studies of social drinkers. As we also showed that kinetics in heavy drinkers did not significantly differ from the social drinkers, the data between the populations could be transferrable.

We could therefore suggest that high and/or decreasing EtG and EtS levels weakens an explanation of recent drinking, while low and/or increasing levels of EtG and EtS strengthens such an explanation.

We must not forget that there was one important exception in our study of heavy drinkers. The patient suffering from kidney disease had considerably prolonged half lives for these ethanol metabolites. Such a condition must therefore be excluded before using measurements of EtG and EtS in the way described above. An indication of the subject's individual half life for EtG and EtS might be obtained from calculations if two subsequent blood samples show decreasing values.

It must also be noted that low levels of EtG would not be a definite proof of recent drinking, as some subjects show very minor glucuronidation of ethanol. This was the case for one of the 51 subjects studied in kinetic experiments (131). This was not surprising, considering the high prevalence of Gilbert's disease. This condition is a hereditary defect in the isoenzyme 1A1 of the UGT family, the enzyme predominantly responsible for ethanol glucuronidation (32). The disease is present in 5-10% of the population and glucuronidation in these subjects is approximately 30% of normal (177-179). The levels of EtG could therefore be low, but not necessarily absent. There are also more seldom and severe conditions affecting glucuronidation, such as the very rare Crigler-Najjar syndrome type 1, which shows no UGT1A1 expression at all (180).

There are no well known conditions affecting the sulphatation of substances in an equivalent way, but one of the subjects in our kinetic studies showed no EtS in serum, although he had EtS in urine (134). The reason for this was unknown. However, conditions affecting both systems are less likely, and this shows the advantage of testing for both these ethanol metabolites. If a suspect has a high BAC e.g. 2 h after the incidence and 1 h after alleged time of drinking, low or increasing levels of both EtG and EtS very much support this explanation.

5.5 Implications; use of EtG and EtS to determine time of alcohol ingestion

As described above, measurements of EtG and EtS could be very helpful in determining the time of ethanol ingestion, although they give no definite proof. As a result of the research in papers IV and V, EtG was analysed in selected routine cases of drunk driving at the Norwegian Institute of Public Health. One such case is described below and shown in figure 2 as an example:

A 50 year old male was involved in a car accident at 2.30 p.m. Blood samples collected at 5.15 p.m. and 5.45 p.m showed ethanol concentrations of 2.3 and 2.2 g/L, respectively. The suspect explained that he had consumed 150 g alcohol after the incident, at 3.00 p.m. Before this he had been abstinent for about 20 h. The level of EtG would therefore be expected to be very low. In fact, very high levels of EtG were found. According to the results from papers IV and V, this could not be explained by a single ingestion 2.5 h earlier at 3pm or by consumption more than 20 hours earlier. High doses of alcohol must therefore have been consumed during a longer, continuous interval before sampling.

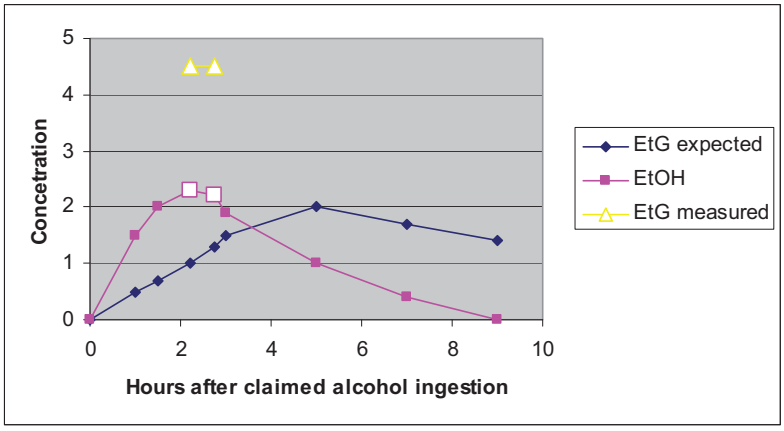

Figure 2.
Concentrations of ethanol and EtG according to measured values (largest points, unfilled), otherwise simulated. From information about a single ingestion of 150 of ethanol 2.25 and 2.75 h before sample collection, values in the area of the "EtG expected" should be seen. The measured values of EtG were much higher than expected and therefore weaken the explanation given. Ethanol in g/L, EtG in mg/L.

5.6 Serum/whole blood ratios

Concentrations of both EtG and EtS were higher when measured in serum than in whole blood, according to our results in paper VI. This difference was more pronounced for EtG

than for EtS. There were and still are no other publications addressing this question. The chemical properties of these molecules were not investigated, but from the structural formula, there are some qualities that could explain our findings. First, they are much larger than their parent molecule ethanol and would be expected to have problems penetrating the cell membrane to enter the red blood cells. The smaller size of EtS compared to EtG could possibly explain the lower serum/whole blood ratio for EtS. Second, their water solubility and corresponding lack of lipofilicity makes penetration into red blood cells difficult.

Bindings to serum proteins for EtG and EtS are not reported in the literature. We cannot, therefore, exclude this as a possibility, and it could explain some of the higher values in serum than in whole blood.

The difference between serum and whole blood concentrations of EtG and EtS could be of practical importance. Although there are both inter and intra individual variations in EtG and EtS levels obtained from ingestion of a certain ethanol dose, a 70% higher result when measuring EtG in serum than in whole blood is important. One could imagine a couple of examples. First, when comparing publications where similar ethanol levels lead to different EtG and EtS results, the serum/whole blood ratio should be considered as one possible explanation. Second, if wider use of EtG and EtS is evolved, a serum sample for clinical analysis and a whole blood sample for forensic analysis could be obtained from the same patient within the same period and analysed for EtG and EtS. This currently happens for ethanol. It would then be relevant to have a simple explanation for different results.

In conclusion, the comparison between serum and whole blood concentrations could be necessary in different situations, and this thesis revealed the expected difference in results between these two media.

6. Conclusions

1. EtG can be used as a marker of ante mortem alcohol ingestion in post mortem cases.

2. Due to stability problems, false negative results could occur and a negative blood EtG result in heavily putrefied cases should be interpreted with caution. Analysis of another medium might raise the sensitivity. We found no formation of EtG during putrefaction. There appears, therefore, to be a low chance of false positive results.

3. In social drinkers after a low dose of ethanol, EtG reaches its C_{max} 2-3 h after C_{max} for ethanol and is eliminated with a terminal half life of 2-3 h. The total amount of EtG excreted in the urine was median 0.017% of the ingested ethanol.

4. The elimination of EtG and EtS occurs at the same rate in heavy drinkers after ingestion of high and repeated doses of ethanol with a terminal half life in blood of ~3 h for EtG and ~3.5 h for EtS. One important exception is the presence of kidney disease.

5. If ingestion of alcohol took place ~3 h or less before blood sampling, levels of EtG should be below that of ethanol (ethanol in g/L, EtG in mg/L). If two subsequent blood samples are drawn, increasing values of EtG should be seen up to ~3 h after intake. If high and/or decreasing levels of EtG are seen, this supports an explanation of recent drinking. Drinking much earlier, eg, the night before is less likely to explain high levels of EtG.

a. The concentrations of EtG and EtS are higher in serum than in blood with serum/whole blood ratios of ~1.7 for EtG and 1.3 for EtS.

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