Metabolites of ethanol as markers of alcohol abuse

Glutathione consumption and methionine kinetics in humans

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Dedicated to my family

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Abbreviations

AA	**Acetaldehyde-Adducts**
AcHO	**Acetaldehyde**
ADH	**Alcohol dehydrogenase**
ALDH	**Aldehyde dehydrogenase**
amu	**Atom mass unit (defined as $\frac{1}{12}$ of the mass of a ^{12}C-atom)**
Arg	**Arginine**
AUC	**Area under the curve**
BAC	**Blood alcohol concentration**
BOC	*tert*-**butyloxycarbonyl group (protecting group for amino groups)**
BSTFA	**N,O-bis(trimethylsilyl)trifluoroacetamide**
CDT	**Carbohydrate-deficient transferrin**
CYP	**Cytochrome P 450**
Cys	**Cysteine**
d_5-EtG	**Ethyl-glucuronide where all hydrogen atoms of the ethyl group are replaced by deuterium molecules**
ECD	**Electron-capture detection**
EDTA	**ethylenediamine tetra-acetic acid (used as anticoagulating agent ex vivo)**
ESI	**Electrospray ionisation**
EtG	**Ethyl-glucuronide**
EtLys	**Ethyl-lysine**
EtOAc	**Ethyl acetate**
EtOH	**Ethanol**
FID	**Flame ionisation detection**
GC	**Gas chromatography**
GC-MS	**Gas chromatography - mass spectrometry**
GSH	**Glutathione**
GSSG	**Glutathione disulfide**
γGT	**Gamma-glutamyl-transpeptidase**
Hb	**Haemoglobin**
HPLC	**High performance liquid chromatography**
IS	**Internal standard**

LC-MS	**Liquid chromatography mass spectrometry**
Lys	**Lysine**
m/z	**Mass to charge ratio**
MCV	**Mean corpuscular volume**
MEOS	**Microsomal ethanol oxidising system**
Met	**Methionine**
MS^2	**Mass spectrometry of specified peaks of a mass spectrum**
MTBSTFA	**N-tert-butyldimethylsilyl-N-methyltrifluoroacetamide**
NaCNBH$_3$	**Sodium cyano borohydride**
NAD	**Nicotinamide adenine dinucleotide (oxidised form)**
NADH	**Nicotinamide adenine dinucleotide (reduced form)**
NADPH	**Nicotinamide adenine dinucleotide phosphate (reduced form)**
NAPQI	***N*-acetyl-*p*-benzoquinoneimine**
o.c.	**on column**
PEth	**Phosphatidyl-ethanol**
PFAA	**Pentafluoro propionic acid anhydride**
PITC	**Phenyl-isothiocyanate**
SAH	**S-adenosyl-homocysteine**
SAM	**S-adenosyl-methionine**
SepPak	**Solid phase extraction cartridges (brand name)**
SRM	**Single reaction monitoring**
TBDMS	***tert*-butyl-dimethyl-silyl group**
TFAA	**Trifluoro acetic acid anhydride**
TMCS	**Trimethylchlorosilane**
TMS	**Trimethyl silyl group**

Summary

In the course of the present doctoral studies two projects related to research in alcoholism and relevant for the postgraduate formation of a pharmacist were pursued: one project dealt with the development of analytical tools applicable to the identification of subjects abusing ethanol. The other, more pathophysiologically oriented project, addressed aspects of glutathione turnover of toxicological relevance for alcoholics.

The aims of the first project were to establish analytical tools for the measurement of direct metabolites of alcohol, in particular of ethyl-glucuronide and ethyl-lysine for subsequent clinical studies using these metabolites as markers for excessive alcohol consumption. Several approaches to measure these metabolites were explored and adapted to locally available instruments. As proof of principle the present work demonstrates that ethyl-lysine is detectable by a chromatographic method in animals and humans consuming alcohol. Future studies will have to demonstrate the potential clinical utility of this marker of alcohol exposure for the identification of subjects consuming excessive amounts of alcohol.

The aim of the second project was to estimate the contribution of methionine-derived cysteine to glutathione synthesis in humans. The project originated in the observation that subjects abusing alcohol are more susceptible to the hepatotoxic effects of paracetamol. Since methionine homeostasis is disturbed in chronic alcoholics as evidenced by higher circulating concentrations of methionine and homocysteine and by the impaired activity of several enzymes involved in the methionine cycle, an impaired ability to supply adequate amounts of cysteine generated from methionine via the transsulfuration pathway for the resynthesis of glutathione may be one explanation for the higher susceptibility of alcoholics. Prior to probing the methionine cycle, i.e. the formation of S-adenosylmethionine from methionine, transmethylation and transsulfuration reactions and finally remethylation of homocysteine back to methionine, in alcoholic subjects, a quantitative analysis of the utilization of methionine for glutathione synthesis in healthy subjects under physiological conditions and in situations of a drug-induced stress on the glutathione pool was essential. The pharmacokinetic analysis of labelled methionine in healthy subjects shows that the stress on the glutathione pool stemming from the administration of two grams of paracetamol does not have a measurable effect on the

disposition of methionine. It is therefore unlikely that a potentially impaired utilization of methionine for glutathione synthesis in alcoholic subjects can be demonstrated by the present approach and different options to probe the methionine cycle in alcoholics will have to be explored.

Metabolites of ethanol as markers of alcohol abuse

Introduction

Problem

Alcohol (ethyl-alcohol, ethanol) is together with nicotine one of the most abused, but nevertheless legal, substances. In 1999 each adult person (>15 y) in Switzerland consumed on average 11.45 L of pure alcohol consisting of 28% beer, 56% wine and 16% spirits[139]. In 1998 the direct costs from alcohol consumption were at least 524 million sFr.[31]. The total costs caused by alcohol consumption are estimated to be about 6.5 billion sFr. a year[11]. In Switzerland only about 27% of women and 10% of men are complete teetotallers (all age groups), while 1% of women consume >40 g alcohol/d and 5% of men more than 60 g alcohol/d and are therefore considered to be abusers[39]. The "limits" for alcohol abuse are gender specific because deleterious effects of alcohol tend to occur with a lower rate of consumption in women[34,39].

The illegal substances – the hard drugs like heroin, cocaine and ecstasy - are much more discussed in politics, but are far less used by the general population than alcohol[130].

Classification

The term "alcoholism" is not easily defined. The National Council on Alcoholism and Drug Dependence (USA) has proposed the following definition: "Alcoholism is a primary, chronic disease with genetic, psychosocial, and environmental factors influencing its development and manifestations. The disease is often progressive and fatal. It is characterized by continuous or periodic: impaired control over drinking, preoccupation with the drug alcohol, use of alcohol despite adverse consequences, and distortions in thinking, most notably denial"[79]. Important is, that alcoholism is considered to be a disease that is classified as such as well in the 10th edition of the International Classification of Diseases (ICD 10) of the WHO as in the 4th Diagnostic and Statistical Manual for psychic diseases (DSM-IV) of the American Psychiatric Association[117].

Since there are many reasons for excessive drinking, varying susceptibilities, and different drinking patterns subclassifications have been proposed:

Jellinek distinguished the way of, the dependence and the self-control in drinking. He described 5 different drinking types that experience distinguishable problem patterns[117]. Cloninger proposed a simpler approach, only distinguishing 2 types. One is supposed to be related mostly to environmental factors, the other to genetic / hereditary factors. These types were refined by other authors with relation to the onset of the disease[117].

Consequences of excessive alcohol consumption

A beneficial effect of moderate alcohol consumption (e.g. one glass of wine or beer a day[18,23,97]) has been well documented particularly in regard to cardiovascular function. In contrast, a consumption of >40 g/day in women and of >60 g/day in men over a prolonged period of time is associated with medical, social and psychic problems[34,59,92,104,108,124,135].

Clinical features and effects of alcohol vary with its concentrations. Typical clinical signs of acute alcohol intoxication are summarised in table 1. These signs are dependent on a number of factors including the rate of intake, the degree of individual tolerance and the nutritional status.

Table 1: Clinical features and effects of various alcohol concentrations[111]

Blood alcohol concentration (mg/L)	Clinical effects
2	Euphoria
3	Increased likelihood of having an accident
4	Disinhibited
8	Impaired coordination, legal limit for driving in UK
15	Loss of self-control, slurred speech, drowsiness, amnesia
30	Stupor, coma
50	Coma, death possible
60	Death certain

Medical problems associated with excessive alcohol consumption

As an overview the relative risk attributed to increasing alcohol consumption for selected organs or groups of organs is depicted in figure 1.
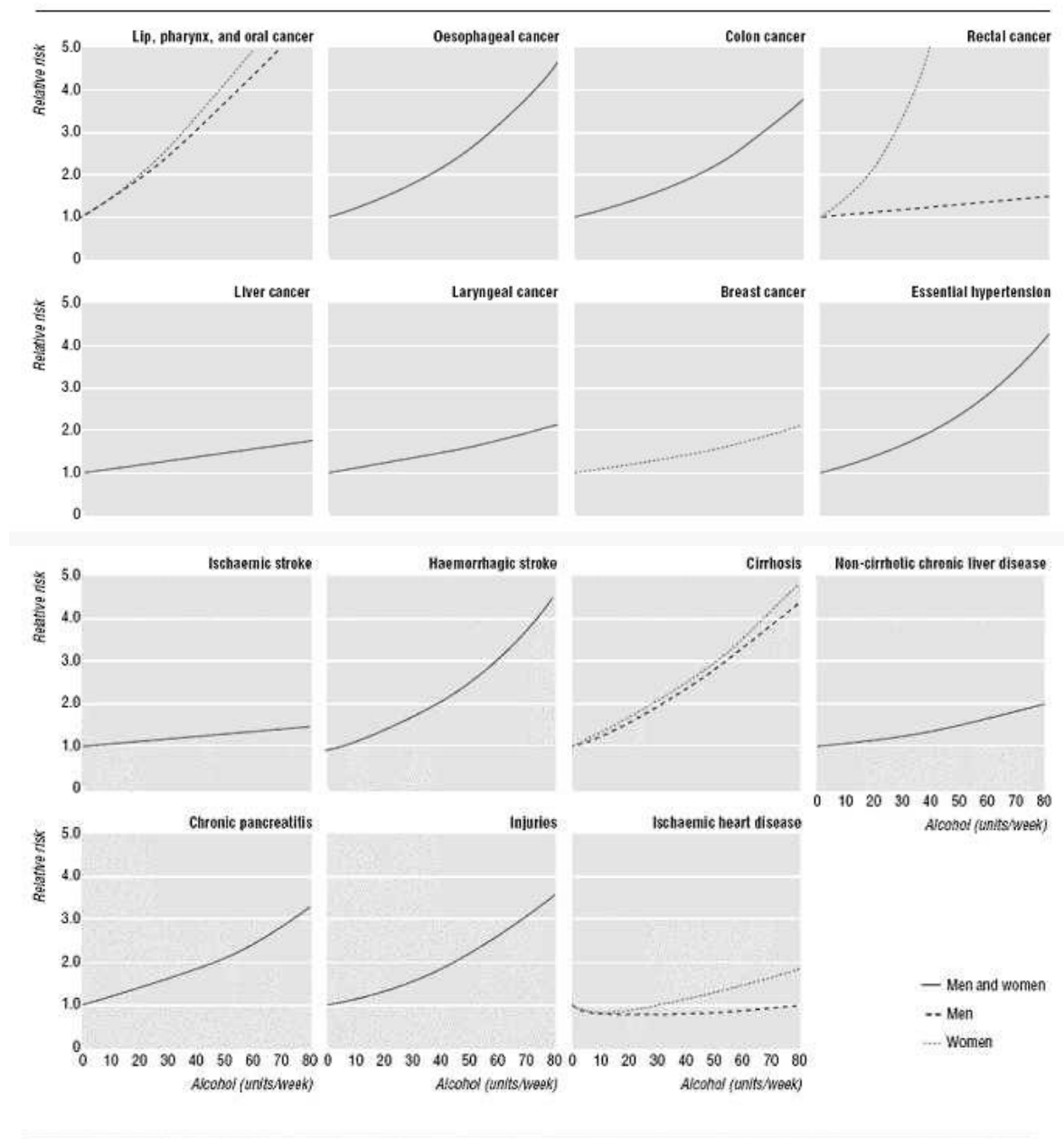

Fig. 1: Cause specific relative risks by alcohol consumption. When men and women are not shown separately, same model is used for both[138]. 1 unit corresponds to 9 g of pure alcohol.

Alcoholic Liver disease[108]

The liver is the major target of adverse effects of excessive alcohol consumption. Following the consumption of moderate amounts of alcohol the liver accumulates triglycerides. The cause for these changes is the disturbed balance of $NAD/NADH_2$. When such a fatty liver is further stressed with alcohol an inflammatory reaction ensues with the development of an alcoholic steatohepatitis. The continuous stimulation of myofibroblasts will lead to the deposition of fibrous tissue and eventually cirrhosis of the liver will occur with all its deleterious consequences (figure 2). Alcoholic hepatitis carries a mortality of between 15 – 25% [108]. Cholestasis quite often aggravates the existing liver disease and worsens the prognosis.

Alcohol

↓

fatty liver ⟶ alcoholic Hepatitis

↓

Cirrhosis
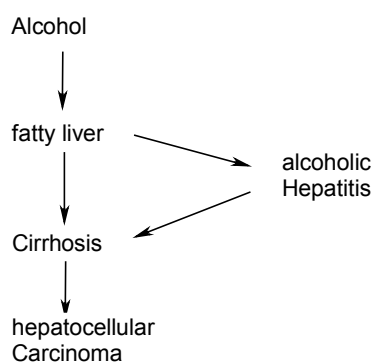
↓

hepatocellular Carcinoma

Fig. 2: scheme of evolving alcoholic liver disease

The relative risk to develop cirrhosis of the liver is – depending on the amount of alcohol consumed – between 1.30 (♀ <20 g/day; ♂ <40 g/day) and 13.00 (♀ >40 g/day; ♂ >60 g/day) [34].

Pancreas

Besides the liver the pancreas is the organ that is most often damaged by alcohol. Alcohol consumption is a frequent cause of acute pancreatitis, and 1-3% of patients with long-time alcohol consumption develop a manifest chronic pancreatitis[16]. Alcoholic pancreatitis develops in three phases: a subclinical phase first which varies in duration between 3 and 20 years, eventually followed by repeated painful attacks of pancreatitis over a period of 5 to 10 years. In the third phase the course is characterized by pancreatic calcifications, pain and irreversible and progressive exocrine and endocrine insufficiency of the gland[16]. 60–80% of all cases of chronic pancreatitis are thought to be related to excessive alcohol consumption[16].

The mechanisms by which the pancreas is damaged by excessive alcohol consumption are not fully understood yet. Studies have been carried out to determine the metabolites of alcohol responsible for the development of pancreatitis. One possibility is a direct toxic effect of alcohol itself[141]. Another possibility is that acetaldehyde (AcHO) is responsible[7,43,47]. Non-oxidative metabolites of alcohol like fatty acid ethyl esters or phosphatidylethanol have also been discussed[60].

Brain[104]

The central nervous system is affected by alcohol consumption. About 7% of all cases of dementia are attributable to excessive alcohol consumption. One possible cause for this could be a lack of thiamine due to alcohol generated malnutrition or malabsorption. Another reason for brain injury may be a dose-dependent direct neurotoxic effect of alcohol on N-methyl-D-aspartate (NMDA) receptors resulting in organic brain syndrome like the Wernicke encephalopathy or the Korsakoff psychosis. Luckily, just a few alcoholics develop such a syndrome. But many suffer from memory deficits and difficulties to concentrate. These symptoms may persist even after withdrawal. A further affection of the brain caused by chronic alcoholism is the markedly increased risk of seizures[34]. Addiction phenomena which are of great importance in the context of alcoholism are also based in the brain.

Heart[34]

The protective effect of moderate alcohol consumption for coronary heart diseases (CHD) is associated with an increase of the high-density lipoprotein (HDL) fraction and changes in the viscosity of the blood[18,97,137]. Depending on the population the protective effect is more or less pronounced. Young people and female gender benefit less than people of older age and males. Mediterranean people tolerate higher amounts of alcohol to still experience lower relative risks for CHD. The pattern of consumption apparently plays an important role: in the Mediterranean people tend to drink alcohol, especially wine, in small amounts on a daily basis, whereas in other populations like the USA or the UK there is a tendency to only consume at weekends, resulting in the same average daily quantity, but with much higher alcohol peak concentrations. The protective effect seems not to be drink-specific. The benefits in the reduction of CHD is a direct alcohol effect and independent from the type of alcoholic beverage[34,58].

On the other hand, even low doses of alcohol have a cardio depressive effect by decreasing the contractility of the heart (negative inotropic effect), eventually leading to alcoholic cardiomyopathy. Alcohol also increases the heart-frequency and arrhythmias may occur. Heavy consumption (70–100 g/d) may lead to systolic hypertension which is due to multifactorial changes e.g. the sympathetic nervous system has an increased activity, there is more antidiuretic hormone released into the blood and the metabolism of electrolytes is disturbed.

Drug Interactions[92]

In social drinkers alcohol dehydrogenase (ADH) is the most important metabolic pathway for the elimination of alcohol. In alcoholic subjects the metabolism of alcohol via the microsomal ethanol oxidising system (MEOS), i.e. cytochrome P450 (CYP) isoenzyme 2E1, is induced and gains more importance[92]. This may lead to a faster elimination of alcohol and increase the tolerance towards alcohol in habitual drinkers[92].

Since some drugs and toxins are metabolised by CYP 2E1 its induction may result in relevant pharmaco- and toxico-kinetic interactions. For example, AcHO, chlorzoxazone, carbon tetrachloride, and paracetamol are substrates of CYP 2E1 as well. Other substances like isoniazid also induce the CYP 2E1. Induction of CYP 2E1 by alcohol may in part be responsible for the increased susceptibility of alcoholics to the hepatotoxicity of paracetamol, whose toxic metabolite is formed by this isozyme. Disulfiram and some antibiotics (metronidazol, chloramphenicol, griseofulvin) inhibit the aldehyde-dehydrogenase (ALDH). When alcohol is consumed in the presence of these compounds the elevated levels of AcHO lead to flushes with red face, weakness and nausea. H_2-blockers and acetylsalicylic acid inhibit the alcohol-dehydrogenase in the stomach resulting in higher blood-alcohol concentrations due to the inhibition of presystemic metabolism.

Pharmacodynamic interactions are common with drugs affecting the central nervous system and may e.g. increase the sedating effect of alcohol itself.

Psycho-Social consequences of excessive alcohol consumption

Alcoholic beverages are often used as soporifics, but they may also disturb the sleep rhythm[59]. A nightly withdrawal can lead to tachycardia and sweating in the second half of

the night. An increased day-sleepiness can result. Alcohol relaxes muscles and this affects also the respiratory tract, leading to snoring and sleep apnoea. An alcoholic experiences a prolonged sleep-latency, reduced sleep-efficiency and –duration and the deep-sleep periods are shortened. Sleep-disorders might persist for months or years after the subject stops drinking.

Alcoholism is a social problem as well. Within the family an alcohol-dependent person may lead to conflicts, violence, financial problems and lesser care-taking for children. All of these problems mentioned may furthermore be passed on over generations because children that grew up under such circumstances are prone to behave like their parents. An alcoholic person may loose employment which leads to additional stress. The subject may be tempted to alleviate the stress by consuming more alcohol so the alcoholic ends up in a vicious circle.

Driving under the influence of alcohol is a risk for the general population and many work-accidents may be related to alcohol consumption.

Biological Markers of excessive alcohol consumption

In clinical practice it would be helpful to identify subjects who consume excessive amounts of alcohol in order to institute measures to prevent the medical, social and psychological problems associated with alcoholism mentioned above. In patients presenting with a medical problem a simple test identifying alcohol as the potential cause would be a big advantage. Thus, there is a need for the development and validation of reliable markers of acute and chronic excessive alcohol consumption.

Basically two types of markers may be considered: state markers that indicate a genetically determined increased vulnerability to alcohol and alcohol related problems; these markers are always present. On the other hand there are trait markers that become manifest when abuse or alcohol related problems occur, and that disappear when the subject reduces the alcohol consumption. For the detection of excessive alcohol consumption a lot of research has been done over the last 40 years[63,64,65,66] but still no single trait marker is established that fulfils all requirements.

Metabolism of alcohol

For the better understanding of potential trait markers a short overview of the metabolism of alcohol is given (figure 3).

Due to its chemical properties (well soluble in water and fat) alcohol is readily absorbed from the stomach and small intestine. Already a few minutes after ingestion alcohol can be detected in blood. About 5-10% of an orally ingested dose are metabolised by ADH of the stomach. In addition to this gastric first pass effect which may vary considerably depending on the nutritional status of the subject, there is an equally variable hepatic first pass effect, so that only a fraction of an ingested amount of alcohol reaches the systemic circulation. Most of the metabolism of alcohol occurs in the liver. Approximately 5-10% are excreted unchanged via the lungs, the kidneys or the skin[45,82,135].

In the liver most of the ingested alcohol is oxidised by ADH to AcHO. AcHO itself is oxidised by ALDH to acetate, which is then coupled to coenzyme A and may undergo further oxidation in the Krebs cycle to yield CO_2 and H_2O or is utilized for the synthesis of fatty acids. All oxidation steps need coenzymes, mainly NAD, which is reduced to $NADH_2$. The resulting shift in the NAD/NADH ratio in part explains the hypoglycaemia, hyperlipidaemia, fatty liver, hyperlactacidaemia and hyperuricaemia that may be the results of alcohol abuse[135].

Approximately 3-8% of the ingested alcohol are metabolised via the MEOS, in particular the CYP 2E1, leading to AcHO as well[82]. As mentioned earlier, this enzyme-system, in contrary to ADH, may get induced when alcohol is consumed chronically[1,82]. Since the induction of the MEOS leads to a faster alcohol oxidation more AcHO is generated which leads to higher blood-AcHO concentrations and therefore more complications due to that toxic substance are expected[92].

The third pathway by which alcohol is oxidised to AcHO is by catalase. This pathway is physiologically unimportant.

The elimination of alcohol from the body follows zero order kinetics due to saturation of the enzymes involved. On average 0.1-0.2‰/h or roughly 4-10 g/h of alcohol are eliminated in men; women have a slightly lower elimination rate.
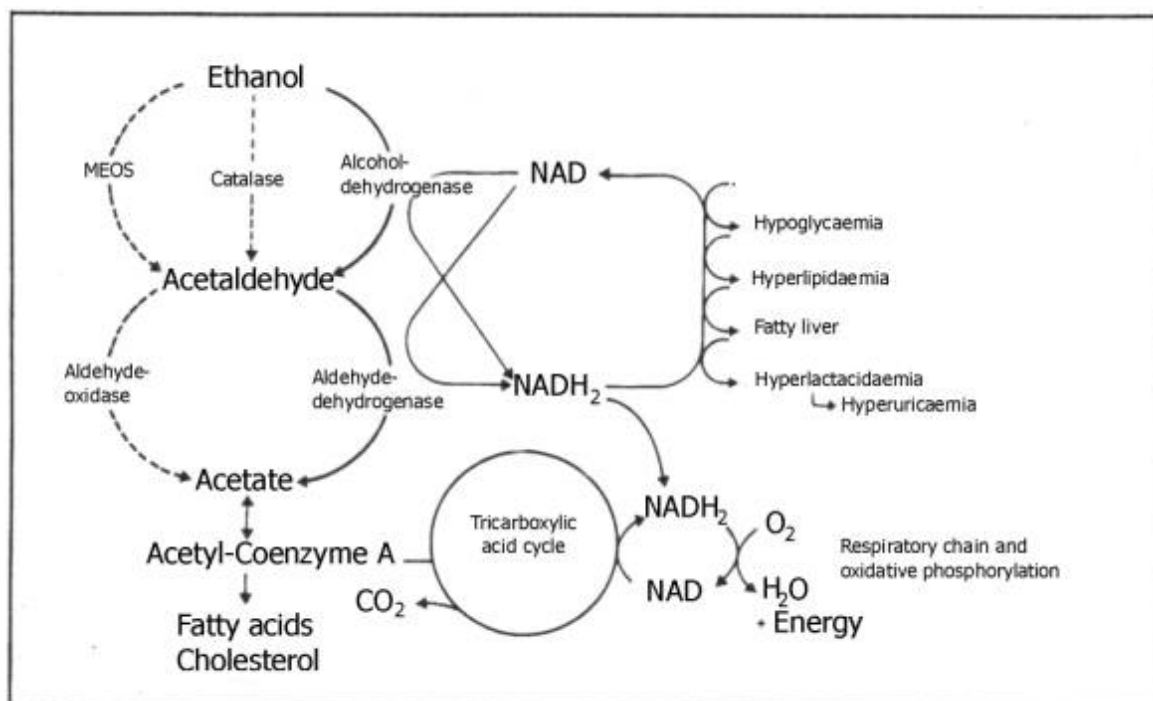
Fig. 3: pathways of alcohol metabolism[135]

Ethanol

After alcohol consumption, alcohol itself is detectable for at most 24 h in expired air, blood or urine. This is a short time interval and reflects only current consumption, but does not distinguish between chronic abuse and a binge. The National Council on Alcoholism (USA)[101] has nonetheless suggested that a blood alcohol concentration (BAC) exceeding 1,5‰ without gross evidence of intoxication or a BAC greater than 3‰ at any time could be used as an indicator for alcohol abuse.

Methanol

The plasma concentration of methanol amounts to 0.5-1.0 mg/L; it may increase by eating fruits or drinking fruit-juices. After drinking alcoholic beverages, the metabolism of methanol is blocked at alcohol concentrations above 0.4 g/L, because both compounds compete for ADH and the affinity of methanol for the enzyme is approximately 10 times lower than that of ethanol[45]. Thus, during the metabolism of ethanol the concentration of endogenously produced methanol increases. Therefore blood methanol levels exceeding 2.2[44] (or 10[49,80]) mg/L might be indicating alcohol abuse.

Congeners

In alcoholic beverages there are congeners other than methanol like propanol, *iso*-propanol, acetone, all forms of butanol and still higher alcohols[80;81]. Acetone and 2-propanol are in a redox-equilibrium. The reduction of acetone to 2-propanol requires NAD and is catalysed by ADH. Thus, a rising level of 2-propanol compared to acetone might be an indicator for alcohol consumption. A cut-off value of the sum of both substances has been proposed as a marker for habitual drinking[80]. Physical activity also results in a rise of blood-acetone levels and so does stress at the dentist[49]. The levels found are much higher than after alcohol consumption, but there is no rise of 2-propanol. In some newly marketed sweetened alcoholic drinks (Alco pops) 2-propanol concentrations were found that exceed 1300 mg/L. As a consequence consumers will test positive for heavy drinking even though only one single drink was imbibed.

The clinical utility of acetone and 2-propanol as alcohol markers is low, because – as stated above – metabolic disorders like stress, hunger or diabetes also influence these parameters.

γ-glutamyl-transpeptidase (γGT)

The activity of the γ-glutamyl-transpeptidase (γGT) is often elevated in serum of alcoholic patients. γGT is a sensitive marker for hepatobiliary diseases[129]. It is also a marker of enzyme induction as for instance in the case of administration of phenytoin[96] where its synthesis increases.

Due to these frequent conditions characterized by an elevated γGT the sensitivity and specificity of γGT as a marker for excessive alcohol consumption are only around 50%. In known chronic alcoholic patients sensitivity and specificity may increase up to 90%[3]. In a study with healthy volunteers who consumed a daily dose of 60 g of alcohol for 3 weeks there was no increase in γGT[111]. If levels are elevated due to alcohol consumption normalisation occurs after 6-8 weeks of abstinence[117]. γGT is rarely elevated in subjects under the age of 30 years and is less sensitive in women[111].

When a drunken driver has an elevated γGT, this is likely due to alcohol abuse. More than 40% of drunken drivers had γGT levels above the upper limit of normal (ULN) of 28 U/L, and 13% had levels even >70 U/L. So this marker has a certain importance in detecting

alcohol problems in drunken drivers[49]. In spite of its drawbacks γGT is considered as the reference biological marker for alcohol abuse.

Mean corpuscular volume (MCV)

The mean corpuscular volume (MCV) of red blood cells is often increased in alcoholics. It correlates with both the amount and frequency of drinking because it reflects a direct toxic effect of alcohol on the bone marrow or erythrocytes. In addition, a deficiency in folic acid, which may be associated with heavy consumption of alcohol, may contribute.
It is a trait marker that rises slowly – at least one month of drinking 60 g of alcohol daily are required - and due to the long half live of the red blood cells remains elevated for a long time (up to 3-4 months). The sensitivity is rather low even in hospital environments (40-50%), but the specificity is high (80-90%), and only a few teetotallers and social drinkers will have elevated MCV values[111]. Since MCV is also affected by a lack of vitamin B_{12}, by liver diseases, reticulocytosis and anticonvulsants[20,101,111] the specificity will be lower in some patient populations. An advantage of this marker is its analysis: it is inexpensive and can be easily determined, which increases its availability. A MCV >98 fL bears a 60% probability that the subject is consuming >450 g of alcohol per week[21,109,117].

In combination, the specificity of MCV and γGT as alcohol markers can be increased. When the cut-off for MCV and γGT were set at >98 fL and >50 U/I, respectively, a high specificity (98%) was achieved, but a very low sensitivity (17%) proved to be a problem[20].

Carbohydrate-deficient transferrin (CDT)

The deficiency in the glucosylation of transferrin first observed in the late seventies by a Swedish group in the cerebrospinal fluid and the serum of alcoholics has been studied world wide (there are more than 200 studies published at present, including some reviews). Transferrin is the major iron transport protein in the body. The protein has two "pockets" where iron – as Fe^{3+} – may be transported. The protein also carries "antennae" of N-glycans that themselves carry sialic acid residues. The most important transferrin form in healthy human is the tetrasialo transferrin (64-80%). In alcoholics the glucosylation is hampered, so they have significantly higher amounts of carbohydrate-deficient forms of transferrin. The term carbohydrate-deficient transferrin (CDT), as it is

used nowadays, includes the three forms of transferrin: asialo, mono- and disialotransferrin.

The pathomechanisms underlying an increased CDT in alcoholics are not fully understood yet. One cause could be that alcohol or even more so AcHO affects N-glycan synthesis in the Golgi apparatus. Another hypothesis is a reduction of mRNA of sialyltransferase, so that there is a lack of enzymes to sialylate the transferrins. Still another idea deals with an increased activity of the sialidase which cleaves the carbohydrates from the protein.

The analysis of CDT is delicate. The concentrations of the CDT-isoforms are low and all the isoforms have very similar chemical and physical properties. However, the isoelectric point (pI) of the isoforms varies not only with the increase in glycosylation, but also with the load of iron of the protein. There is no standard analytical procedure established. So the assessment of CDT as a marker of alcohol abuse has to take into account not only the reported value, but also the cut-off/borderline values and the analytical method used. Although false positive increases in CDT are found in patients with liver disease CDT seems to be a rather specific marker for chronic alcohol abuse, whereas the sensitivity is not very high (γGT has the best sensitivity, but a lack in specificity). A rise in CDT occurs after a daily consumption of 50-80 g of alcohol for at least one week. It remains elevated for about a week upon abstinence [3,8,9,54].

Aminotransferases

The aminotransferases – they are also known as transaminases – are a group of enzymes that catalyse a reversible reaction in which a α-ketoacid is converted into an amino acid. The most important enzymes of this group are the alanine-aminotransferase (ALT) – also known as ALAT or GPT (glutamate-pyruvate-transaminase) – and the aspartate-aminotransferase (AST) – also known as ASAT or GOT (glutamate-oxalate-transaminase). ALT is a more or less liver-specific enzyme with a half-life of approximately 47 h. A rise of its activity in serum generally reflects hepatocellular injury of any origin. AST is a non-liver specific enzyme, that is found in several organs such as muscle (heart and skeletal), kidney, pancreas, brain, lungs and liver. Its activity is high in skeletal muscle and liver. Its half-life in plasma is approximately 17 h. A parallel rise of AST and ALT is an indicator of hepatocellular injury.

These enzymes are clinically helpful in the assessment of alcoholic liver disease. However, in order to identify alcohol abuse these enzymes are of limited value since many alcoholics do not have liver injury and thus elevated transaminases. Only when fatty liver or an alcoholic hepatitis ensues from the consumption of alcohol will the transaminases in serum increase. The two enzymes may be of use in distinguishing alcoholic from non-alcoholic liver disease. An AST/ALT ratio >2 is a strong indicator of an alcoholic origin[101] (sensitivity ~50%; specificity 80%[20,95]) whereas with a ratio of ≤1 it is more likely that the disease is not alcohol related[128].

Since alcohol consumption results in mitochondrial injury, the mitochondrial isoform of AST (mAST) in serum increases more than AST. The two forms can be determined immunologically. The ratio of mAST to total AST has a sensitivity for excessive alcohol consumption of about 90%[20,73,111]. Unfortunately, the ratio does not allow a differentiation of alcoholic and non-alcoholic fatty liver disease[95]. With the probably increasing prevalence of non-alcoholic steatohepatitis the predictive value of the ratio of AST/ALT or mAST/AST is likely to be lower than what has been reported in the literature.

Ethyl-glucuronide (EtG)

Ethyl-glucuronide (EtG) is a minor metabolite of ethanol postulated in 1902[83], first found in rabbits in 1952[55] and then in humans in 1967[51]. EtG is a non-volatile, water-soluble, stable upon storage, direct metabolite of ethanol that can be detected in body fluids and hair samples only after ethanol has been consumed. A minor fraction of a dose of ethanol undergoes conjugation with uridinediphosphate-glucuronic acid to produce EtG (0.5 - 1% in rabbits[55], 0.013 – 0.04% in humans[24,35]). EtG is a marker of alcohol consumption that can be detected in body fluids for an extended time period after complete elimination of alcohol from the body. EtG peaks about 2–3.5 h after the blood alcohol concentration has reached its maximum[107] and is then detectable – depending on the amount of ethanol consumed – for up to 36 h in serum or up to 80 h in urine[5,149]. The terminal half-life is between 2 and 3 h[24,29,107,119].

It is surprising that a hydrophilic carboxylic acid is deposited in hair but EtG is detectable in hair of chronic alcoholics who consume at least 10 g/day over some time[105].

With its specific time frame of detection intermediate between short- and long-term markers, EtG is a promising marker of alcohol consumption in general and of relapse control[147] with a sensitivity of 77% and a specificity of 71%[105].

Acetaldehyde-Adducts

AcHO is a reactive molecule generated by the oxidation of ethanol. The electrophilic carboxy-group of the molecule has the ability to react with amino- and sulfhydril-groups. Identified targets include the ε-amino-group of lysine[46], which is thought to be one of the most important targets[68]; α-amino-groups of terminal amino acids in proteins[46,127], as described for valine in the β-chain of haemoglobin[103], and the sulfhydril-groups of cysteine (Cys) and reduced glutathione (GSH) which serves as scavengers and may inhibit detrimental adduct-formation[27]. The spectrum of chemical reaction products is not fully understood, but investigators have found Schiff bases (unstable to clean-up procedures like acid-precipitation, gel-filtration or dialysis[27]) that could be stabilised into secondary amines like ε-ethyl-lysine (EtLys) through a reduction step[46,127,131]. Also ring formation e.g. with a terminal valine resulting in imidazolidinones[103] has been described. Among the biological structures affected are albumin[27,46,68,78,131,132,140,144], haemoglobin[17,38,50,69,70,84,103,120,122], tubulin, collagen, the family of the cytochrome isoenzymes (especially CYP 2E1[12,70] and CYP 3A4[12,87]), nucleosides, low density lipoproteins and probably most of the plasma proteins and enzymes.

Even if nearly the total dose of ethanol imbibed (46 g $\cong$ 1 mol) is converted to AcHO the blood AcHO concentrations in Caucasians only reach levels of up to 50 µM[38,120]. This is first because the ALDH removes the generated AcHO very rapidly, and on the other hand adduct formation may account for this effect.

A loss of function is expected for all of the affected structures and protein unfolding due to AcHO-adducts (AA) cannot be ruled out[70]. However the oxygen affinity in haemoglobin is not changed[120]. AA might serve as direct, stable and long-lasting markers for excessive alcohol consumption. Depending on the protein affected one could expect varying 'half-lives' of AA, and therefore with one single method alcohol consumption over different time intervals could be traced. In the hope of finding a marker similar to HbA_{1c} which is used to follow diabetic patients haemoglobin adducts with AcHO have been studied extensively[50,69,113].

The analysis of AA is difficult because the amount of adducts generated is likely to be very small. Immunologic methods of detection that may miss some adducts and may have the drawback of cross-reactivity of the antibodies have been used[70,71,142].

Other Markers

β-Hexosaminidase (HEX)

β-Hexosaminidase (HEX) is a lysosomal enzyme that is readily removed from the circulation by non-parenchymal cells in the liver. Its activity in plasma reflects excessive alcohol consumption (more than 60 g of alcohol daily for more than 10 days[111]). It returns to base-line values after 7–10 days of abstinence[95]. HEX is also found in urine, where it is a sensitive marker of renal disease, but elevated urinary HEX levels are also found in about 80% of alcoholics[81]. Although the activity of the enzyme is influenced by hypertension, diabetes, pregnancy, myocardial infarction, and also by using oral contraceptive pills[56,111], HEX may be a useful marker to discriminate between alcoholics and non- or social-drinkers.

Dolichols

Dolichols are long-chain polyprenols containing a α-saturated isoprene unit. They act as glycosyl carrier lipids in the synthesis of N-linked glycoproteins. Elevated concentrations of dolichols in blood and urine have been found in alcoholics[80,81]. It has been postulated that alcohol interferes with their catabolism, because dolichols are also oxidised by ADH. Dolichols may also be elevated in Alzheimer's Disease, ceroid lipofusinosis and advanced age[101]. Formal studies assessing sensitivity and specificity of dolichols as markers for alcohol consumption or alcoholism are not available.

Salsolinol

There is a class of substances (the 6,7-dihydroxy-1,2,3,4-tetrahydroisoquinolines (TIQs)) which are formed by cyclisation of catecholamines with AcHO, the first metabolite of alcohol oxidation. The most important among them is salsolinol which is formed with dopamine. Norsalsolinol is the condensation product of dopamine and formaldehyde which has a much higher propensity to form such ring structures. Salsolinol has been

found in urine, cerebro-spinal fluid, brain homogenates as well as in plasma. Elevated levels of salsolinol are frequently found in alcoholics, but it failed to distinguish alcoholics from non-alcoholics[80]. Furthermore, its analysis is rather complicated and there is only few data on how much alcohol would be needed to get a rise of the marker substance. Near to nothing is known about the influences of dietary conditions that might further affect the result[80,102].

Phosphatidylethanol (PEth)

Phosphatidylethanol (PEth) is an "aberrant" phospholipid of cell membranes (brain, lung, heart, adipose tissue, neutrophils) which is synthesized by phospholipase D in the presence of alcohol. The marker only reacts to a single dose of much more than 60 g of alcohol or a 3 week-load of 750–1000 g alcohol (40–50 g daily). Single doses of 32–47 g alcohol could not be detected. The half-life is about 4 days, so in heavy consumers levels might be detectable up to 3 weeks. No correlation was found with γGT and CDT, and it seems, that PEth is more sensitive in detecting alcohol abuse than CDT[42,80,102]. Since the method of analysis has been improved with the advent of HPLC[134] it is well possible that PEth will prove to be a reliable marker of alcohol consumption. Its sensitivity and specificity still has to be established.

5-OH-Tryptophol (5-HTOL)

A metabolic interaction between alcohol and serotonin might be used for the detection of recent alcohol consumption. Under normal conditions 5-hydroxytryptophol (5-HTOL) is only a minor metabolite of serotonin in urine and serum. After alcohol consumption 5-HTOL increases markedly probably due to inhibition of ALDH or the increase in the NAD/NADH ratio, and the concentration of the normal serotonin metabolite 5-hydroxytryptophol-3-acetic acid (5-HIAA) decreases correspondingly. To exclude variations by diluting or due to dietary sources of serotonin such as bananas, tomatoes or walnuts[80] the ratio of 5-HTOL and 5-HIAA is used. The ratio remains elevated for 6 −15 h after blood alcohol has returned to normal. The marker has a high sensitivity (as little as 20 g/day of alcohol can be detected) and a high specificity for very recent alcohol consumption. This marker is not influenced by heavy long time consumption, but reflects only the alcohol intake of the preceding day (~24 h)[45,81,111]. Since 5-HTOL is not formed in the urine after death this marker distinguishes between alcohol intake before death and alcohol formation after death in forensic practice[80].

Fatty Acid Ethyl Esters (FAEE)

Fatty acid ethyl esters (FAEE) are formed by enzymatic esterification of alcohol with fatty acids. They have been detected in high concentrations in organs commonly damaged by alcohol abuse, in particular the pancreas and the liver[60]. The rate of synthesis of FAEE in these organs proved to be high. FAEE are supposed to contribute to the end organ damage after chronic heavy alcohol consumption. Possible mechanisms involve decreased rate of cell growth and protein synthesis, disruption of oxidative phosphorylation in mitochondria and changes in membrane fluidity, which could account for increased fragility of pancreatic lysosomes[28,61].

FAEE are formed after alcohol ingestion and reach their concentration maximum in a similar time frame as the blood alcohol concentration. Over the first hours their decay parallels that of alcohol, but since they may accumulate in several tissues they have a secondary elimination phase which is much slower than that of alcohol itself. Therefore FAEE are still detectable (>24 h) when alcohol is no longer present in the blood[28,61]. FAEE are accumulated in sebum and so deposited in hair, which allows detection of heavy drinking for as long as 2 months[125]. A positive testing in hair specimens may also be caused by daily use of alcoholic hair lotions as it has been found in an alcohol abstaining hairdresser[10].

Analytically FAEE are now well accessible by GC-MS[150]. FAEE in blood proved to have a high specificity and sensitivity[28].

<u>Questionnaires</u>

For screening purposes there are several questionnaires, some of them with good validation for the use in primary care.

MALT

The Munich Alcoholism Test (MALT) consists of 31 questions that have been selected from over 1000 alcohol related questions. The test reflects amounts consumed, symptoms of alcoholism and biological abnormalities. It is a combination of clinical questions asked by

the physician (MALT-F) and 24 questions concerning alcohol habits filled in by the patient (MALT-S). The rating of the MALT-F answers is higher than for MALT-S answers.

It seems to be a cheap and fast way to identify alcoholism. Sensitivity and specificity are 90% and 85% respectively. One drawback of MALT: it is only available in German[3,117].

CAGE

This is the shortest of all the questionnaires and the name is an acronym for its four questions:

- Have you ever felt you should Cut down on your drinking?
- Have people Annoyed you by criticizing your drinking?
- Have you ever felt bad or Guilty about your drinking?
- Have you ever had a drink first thing in the morning to steady your nerves or to get rid of a hangover (Eye opener)?

In different studies one or two positive answers have been considered as a positive test, giving evidence for alcohol dependence. Already one positive answer requires a more detailed clinical assessment[30,111]. The sensitivity and specificity are in a range of 60-95% and 40-95%, respectively. The T-ACE Test asks two questions differently and is used to detect alcohol consumption during pregnancy. If 2 of the four questions are answered affirmatively (cut-off) 70% of the heavy drinkers in pregnancy are discovered.

MAST

The Michigan Alcoholism Screening Test asks 25 questions that try to discover drinking problems, help seeking behaviour and alcohol related disabilities. The sensitivity and specificity of the questionnaire are reported to be both as high as 85%. A variant of this test has been introduced: The Self-Administered Alcoholism Screening Test (SAAST), which includes 35 questions[111].

AUDIT

The WHO constructed a questionnaire (Alcohol Use Disorders Identification Test = AUDIT) that contains 10 questions that deal with intake, dependence and adverse consequences[4]. With exception of the last two items the questions allude to the previous year, and responses are weighted between 0 and 4 points, generally based on the frequency at which the respective topic in question occurred. The reported sensitivity and

specificity varies considerably depending on the chosen cut-off limits. When a cut-off of 8 points is used the sensitivity is in the range of 83-96% and the corresponding specificity between 66 and 100%.

Other instruments

There are other instruments to determine alcohol abuse such as the Alcohol-Clinical-Index[114]. It consists of 17 clinical symptoms that are closely related to alcohol consumption like spider naevi or palmar erythema, but also of signs that are highly correlated to alcoholism like cigarette burns. In addition, anamnestic information is included like early morning tremor, hallucinations or inability to concentrate. With this Alcohol-Clinical-Index the sensitivity to distinguish alcoholics from social drinkers is 88%[114,117]. In another patient sample the distinction between alcoholics and non-alcoholics was less favourable[6].

Ethyl-Glucuronide (EtG)

Ethyl-glucuronide (EtG) is a known marker of recent alcohol consumption. Its metabolism has not been fully elucidated by now. The original aim of this work was to investigate whether a metabolic activation of EtG could contribute to the toxicity of alcohol. A similar activation to toxic metabolites is known from glucuronides of diclofenac[110] and zomepirac[116]. As a first step a method to analyse EtG in our laboratory was to be set up.

Possible analytical approaches

EtG contains ethanol (EtOH) and analytical methods have been developed for the sensitive and accurate quantification of EtOH in a biological matrix. Thus, a simple approach would be the analysis of EtG via its content of EtOH provided that EtOH can be quantitatively liberated from the glucuronide.

EtG does not absorb any light in the range of wavelengths suitable for analysis by HPLC as shown in figure 4.
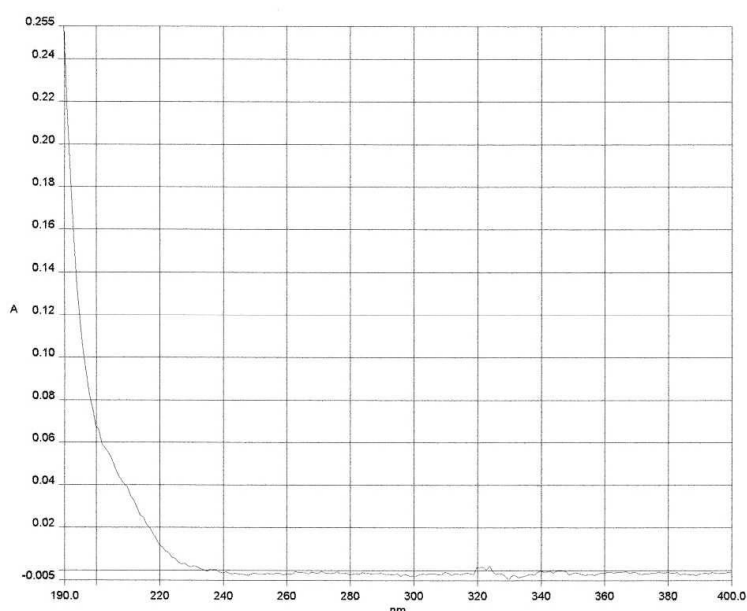
Fig. 4: uv spectrum of a 250 µM aqueous solution of EtG

For HPLC analysis the compound therefore has to be derivatised with a chromophore or a mode of detection such as mass-spectrometry must be used that does not depend on the absorption of light. Few chromophores suitable for the derivatisation of glucuronides and HPLC analysis are available leaving mass-spectrometry as the most promising approach.

Theoretically, EtG should also be amenable to determination by GC if one succeeds in making the compound sufficiently volatile by appropriate derivatisation. Several of the described approaches to the analysis of EtG have been explored as described in the following section.

Hydrolysis of ethyl-glucuronide

In view of the wide availability of gas-chromatographic methods to analyse EtOH the determination of EtG in form of EtOH after its hydrolysis would be an attractive analytical approach.

Method

1000 µL of 1 mM EtG (Medichem Inc. Stuttgart, D) in phosphate buffer (1 M, pH 5.5) was incubated with 20 µL of glusulase (β-glucuronidase EC 3.2.1.31 /arylsulfatase EC 3.1.6.1 from Helix pomatia; Roche Diagnostics, Indianapolis IN, USA) or 20 µL of glucurase (β-glucuronidase solution from bovine liver, Sigma, St. Louis MO, USA) at 37°C for 2 h in an 20 mL ampoule flask . The enzymatic activity was stopped with 100 µL of 60% perchloric acid. 500 µL of *n*-propanol (100 µg/mL $\cong$ 1.67 mM in 0.9% sodium chloride solution) were added as internal standard (IS).

To avoid matrix-effects from enzymes and glucuronic acid residues still present in the reaction mixture a head-space method was applied to analyse the resulting EtOH. Before injection samples were incubated at 60°C for 30 min. in a waterbath Büchi B 465. 1-5 mL of the head-space gas-phase was removed with a plastic syringe and immediately injected into the GC. A Perkin Elmer Sigma 3B Gas Chromatograph equipped with a Tenax 60-80 packed glass column was used for analysis. Gas flows were as follows: nitrogen carrier gas 160 kPa, air 200 kPa and hydrogen 120 kPa. The oven temperature was set at 100°C, the injector at 180°C and the detector at 250°C.

Results

First experiments with liquid injections on GC resulted in chromatograms with many peaks, where the peaks of interest could hardly be seen. With the head-space technique most of the resulting matrix peaks were not present any longer. The EtOH and IS peaks

were well visible at 4.2 min and 11.7 min, respectively, and base-line separated from the few other peaks present.

A calibration curve for the ratio of EtOH and IS is shown in figure 5. Different concentrations of EtOH were incubated at 37°C for 2 h, replacing the enzyme with 20 µL of a sodium chloride solution 0.9%. After the addition of 100 µL of perchloric acid 60% the samples were chromatographed with n-propanol as IS (1.67 mM).
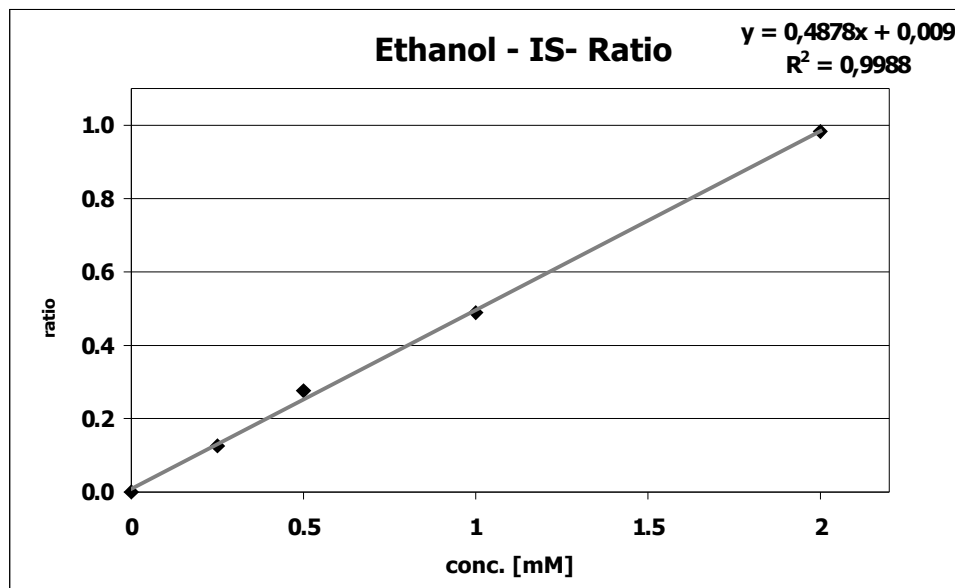

Ethanol - IS- Ratio

$y = 0,4878x + 0,009$
$R^2 = 0,9988$

Fig. 5: calibration curve of EtOH analysed by headspace GC. The EtOH peak area is set in relation to the peak area of the IS

After incubation with glusulase we always recorded a peak after 4.2 min. where EtOH was eluted. In case of EtG or EtOH (as positive control) this was expected, but in case of a blank i.e. water or phosphate buffer incubated instead of EtG no EtOH should be generated. The modification of the GC conditions did not alter the outcome. Therefore the peak exhibiting the same retention time as EtOH was considered as being an artefact of the enzyme mixture used producing either EtOH or a volatile compound with similar retention time during the incubation.

Similar incubation experiments were then carried out with a different β-glucuronidase-preparation. Glucurase, a bovine enzyme, was used. In contrary to glusulase no peaks were found at 4.2 min in any of the incubated samples.

It is possible that the glucuronide-bond of EtG cannot be hydrolysed with glucurase. Thus, the indirect analysis of EtG was not feasible using commercially available hydrolytic enzymes and other techniques with direct determination of EtG were looked at.

LC-MS of ethyl-glucuronide

An advantage of the LC-MS analysis of EtG is that no pre-column derivatisation is needed and that the lack of EtG's uv-absorption may be circumvented with the mass-selective detector. A further advantage is that an optimal internal standard is commercially available in form of deuterated EtG (d_5-EtG)[5]. The available ion-trap mass-spectrometer has the potential to produce spectra up to MS^{10} for the qualitative identification of metabolites. For this purpose, however, the concentrations of the fragments to be further fragmented need to be fairly high. Since our main-focus was to quantify EtG in serum and urine samples we would have preferred a triple-quadrupole mass-spectrometer as is it has been described in the literature[53,119,136,147]. Since no such triple-quadrupole LC-MS system could be used we nonetheless explored HPLC in connection with the ion-trap MS system.

Method

The equipment used for the analysis were a HP 1100 HPLC System with a G1322A Degaser, a G1311A QuatPump, a G1329A ALS and a G1316A ColComp for the separation and a Finnigan LCQ Ion Trap Mass-Spectrometer with electrospray-ionisation (ESI) for the detection of the compounds (spray voltage [kV]: 3.85; spray current [µA]: 2.15; sheath gas flow rate [psi]: 60; aux gas flow rate [psi]: 0; capillary voltage [V]: -4; capillary temp [°C]: 200). More details regarding ion optics and vacuum pump settings may be found in table 2. The column that was used for the separation of the compounds was a MN ET 250 Nucleosil® 5 C_{18} column.

For method development aqueous solutions of EtG and d_5-ethyl-glucuronide (d_5-EtG; Medichem Inc. Stuttgart, D) in different concentrations were used (1.56 − 250 µM). Substances were eluted with aqueous ammonia 0.025% (pH 8.0) and acetonitrile (8:2) at a flow rate of 0.2 or 0.3 mL/min. The run time was 10 minutes. According to the literature[35,53,89,119,145] EtG was measured in negative mode (M-1).

Table 2: LCQ Settings for Analysis of EtG

```
ESI Source
  |Spray Voltage (kV)|:                      3.84
  Spray Current (µA):                        2.15
  Sheath Gas Flow Rate:                     19.14
  Aux Gas Flow Rate:                        -0.38
  Capillary RTD OK:                           Yes
  Capillary Voltage (V):                    -3.95
  Capillary Temp (°C):                     200.10
  8 kV supply at limit:                        No

Vacuum
  Vacuum OK:                                  Yes
  Ion Gauge Pressure OK:                      Yes
  Ion Gauge:                                   On
  Ion Gauge (x10e-5 Torr):                   0.96
  Convectron pressure OK:                     Yes
  Convectron Gauge (Torr):                   0.84

Turbo Pump
  Status:                                 Running
  Life (hours):                             20313
  Speed (rpm):                              60000
  Power (Watts):                               41
  Temperature (°C):                         49.00

Ion Optics
  Multipole Frequency On:                     Yes
  Multipole 1 Offset (V):                    3.36
  Lens Voltage (V):                         37.77
  Multipole 2 Offset (V):                    7.24
  Trap DC Offset (V):                       10.39
  Analyzer Temperature (°C):                26.61

Main RF
  Reference Sine Wave OK:                     Yes
  Standing Wave Ratio OK:                     Yes
  Main RF DAC (steps):                      63.00
  Main RF Detected (V):                      0.01
  RF Detector Temp (°C):                    39.11
  Main RF Modulation (V):                    0.04
  Main RF Amplifier (Vp/p):                  8.74
  RF Generator Temp (°C):                   29.34

Ion Detection System
  Conversion Dynode:                           On
  Multiplier Voltage (V):                 -811.00

Power Supplies
  +5V Supply Voltage (V):                    5.07
  -15V Supply Voltage (V):                 -14.96
  +15V Supply Voltage (V):                  14.91
  +24V Supply Voltage (V):                  23.96
  -28V Supply Voltage (V):                 -28.05
  +28V Supply Voltage (V):                  28.33
  +28V Supply Current (Amps):                0.86
  +35V Supply Voltage (V):                  32.13
  +36V Supply Voltage (V):                  35.89
  -150V Supply Voltage (V):               -151.16
  +150V Supply Voltage (V):                151.00
  -205V Supply Voltage (V):               -200.91
  +205V Supply Voltage (V):                205.95
  Ambient Temp (°C):                        30.27

Instrument Status
  Instrument:                                  On
  Analysis:                                  Idle
  Retention Time (min):                      0.00

Analog Inputs
  External channel 1 (V):                   -0.00
  External channel 2 (V):                   -0.00
  External channel 3 (V):                   -0.00
  External channel 4 (V):                   -0.00

Syringe Pump
  Status:                                   Ready
  Flow Rate (µl/min):                        3.00
  Infused Volume (µl):                       0.00
  Syringe Diameter (mm):                     2.30

Digital Inputs
  READY IN is active:                          No
  START IN is active:                          No
  Divert/Inject valve:                       Load
```

Results

In figures 6 and 7 the LC-MS chromatograms of EtG and d_5-EtG aqueous standard solutions at a concentration of 250 µM (5 nmol o.c.) are shown. The upper part of the figure represents the corresponding mass-trace of EtG or d_5-EtG respectively, while in the lower part of the figure the distribution of masses under the peak is shown; this data includes all masses between 60 and 400 atomic mass units (amu) that were detected by the ion-trap in the total ion current of either EtG or d_5-EtG.

The mass traced in the upper part of figure 6 is the molecular ion of EtG (molecular weight 222) in the negative mode (M-1) with a m/z ratio of 221. The isolation width for this m/z 221 was set to ± 1 amu (m/z 220 − 222). As expected the m/z 221 at its peak maximum was almost the only mass detectable in the total ion current. There was always a background signal present since, besides of the ions produced from our analytes, there was also a constant flow of solvents which already had passed a separation column and there were also some inevitable impurities within the system, which summed up to the total ion current.

The masses that were present at 6.21 min out of the total ion current are shown in the lower part of figure 6. To begin with m/z 221 was the most prominent mass as expected. All masses smaller than 221 could be fragments that were created spontaneously during the analysis or must be considered as background noise from the system. Glucuronic acid without the ethyl group (molecular weight 194 $\rightarrow$ m/z 193) was not detectable. The peak with m/z 173 could be a fragment ion of EtG after the loss of the ethanoyl group and a consequent rearrangement of the molecule including formation of double bonds. The quite prominent peak with m/z 395 is compatible with an adduct of a second glucuronic acid residue to EtG.
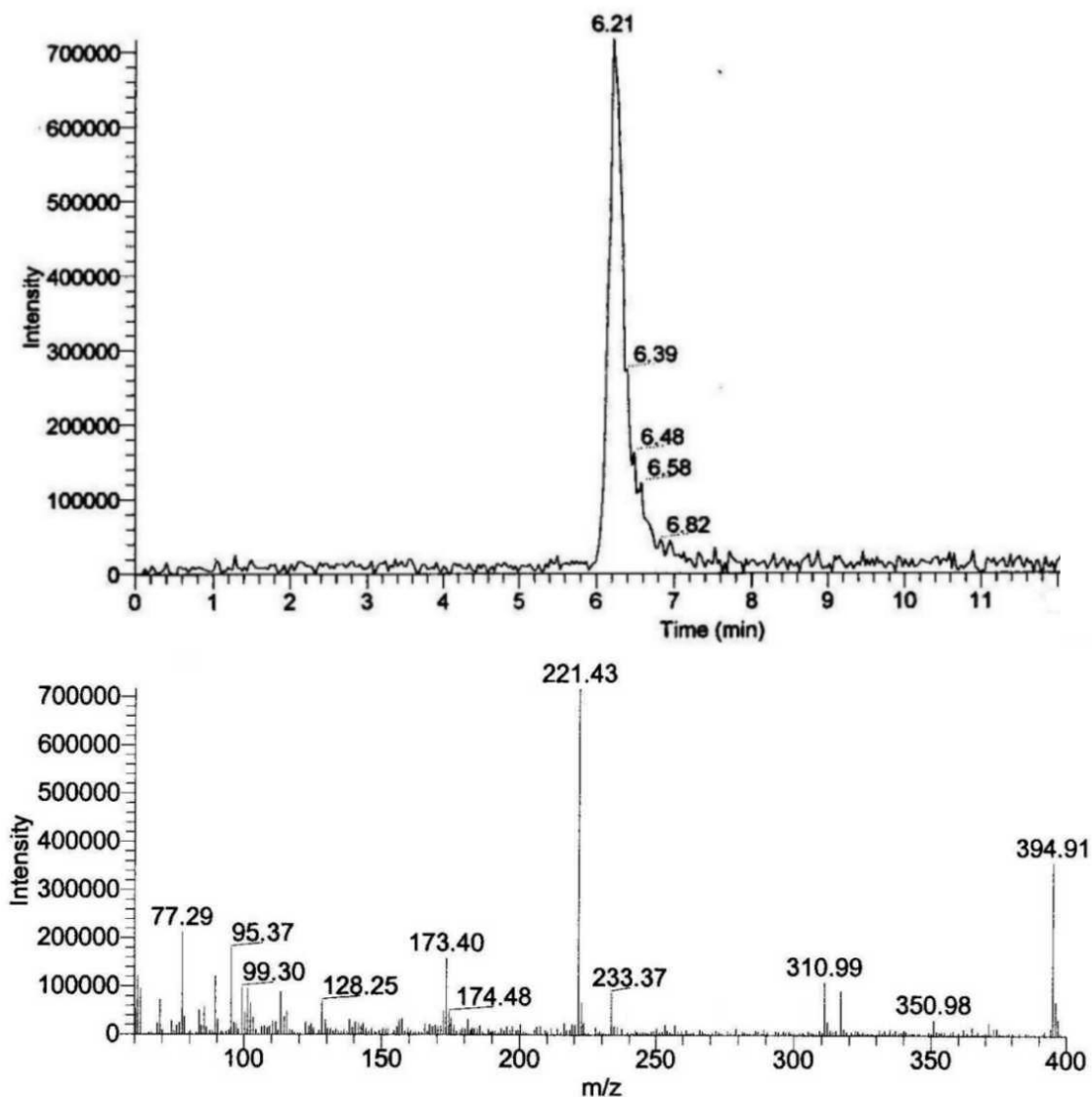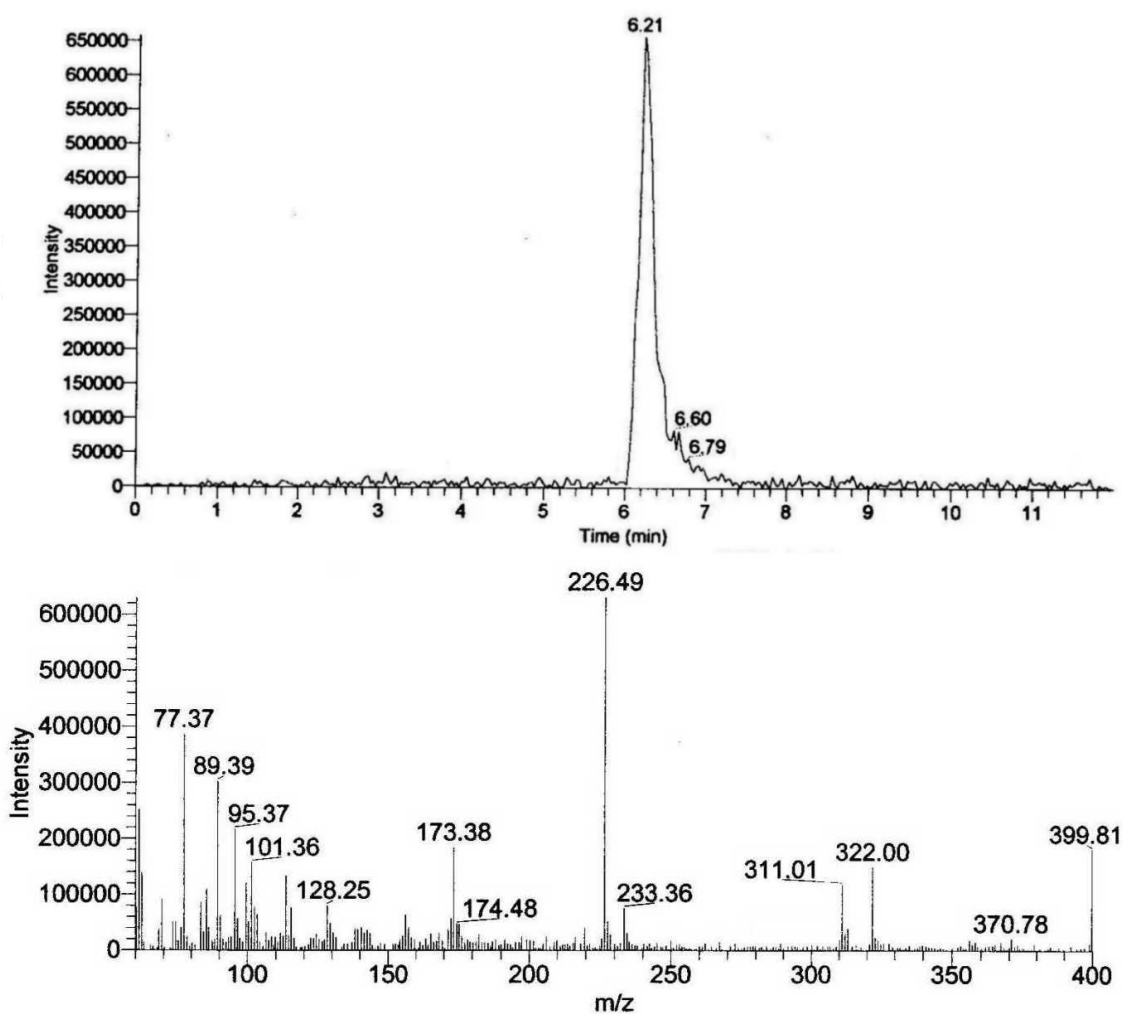
Fig. 6: LC-MS chromatogram of EtG standard 250 µM recorded with a flow-rate of 0.2 mL/min. The mass trace of m/z 221 is shown above and the mass distribution of the total ion current at the peak maximum (6.21 min) is shown below.

In figure 7 the chromatograms for d_5-EtG are shown. Since in the ethyl-group all of the hydrogen atoms have been replaced with deuterium atoms the molecular weight of d_5-EtG is increased by 5 amu compared to EtG. Therefore the molecular weight is 227 amu and the corresponding negatively charged molecular ion had a m/z of 226. Here again the isolation width was set to ± 1 amu. The retention time of the deuterated form of EtG does not differ from that of ordinary EtG because the chemical and physical properties of both substances are identical except for the mass-difference. Regarding the mass-spectrum in the lower part of figure 7 from the total ion current there are mainly the same ions present as it has been observed for EtG. Only two mass peaks present have

different masses. The expected molecular peak at m/z 226 and the proposed adduct of a second glucuronic acid residue which by now has a m/z of 400. The finding of this latter mass-peak which is indeed similar to the one found in the case of EtG (m/z 395) does support the explanation given for its nature. Also the origin of the m/z 173 peak remains plausible since with the ethanoyl group removed the deuterated part of the molecule has been cleaved away and the remaining fragment should undergo the same rearrangements under identical conditions as undeuterated EtG.

Fig. 7: LC-MS chromatogram of d5-EtG standard 250 μM recorded with a flow-rate of 0.2 mL/min. The mass trace of m/z 226 is shown above and the mass distribution of the total ion current at the peak maximum (6.21 min) is shown below.

For the quantification of EtG the internal standard d_5-EtG was chromatographed together with EtG and the ratio of their peak areas was calculated. Sample chromatograms for EtG in concentrations from 1.56 μM to 250 μM (15.6 to 2500 pmol o.c.), each with 50 μM of d_5-EtG (500 pmol o.c.) are shown below (figure 8).
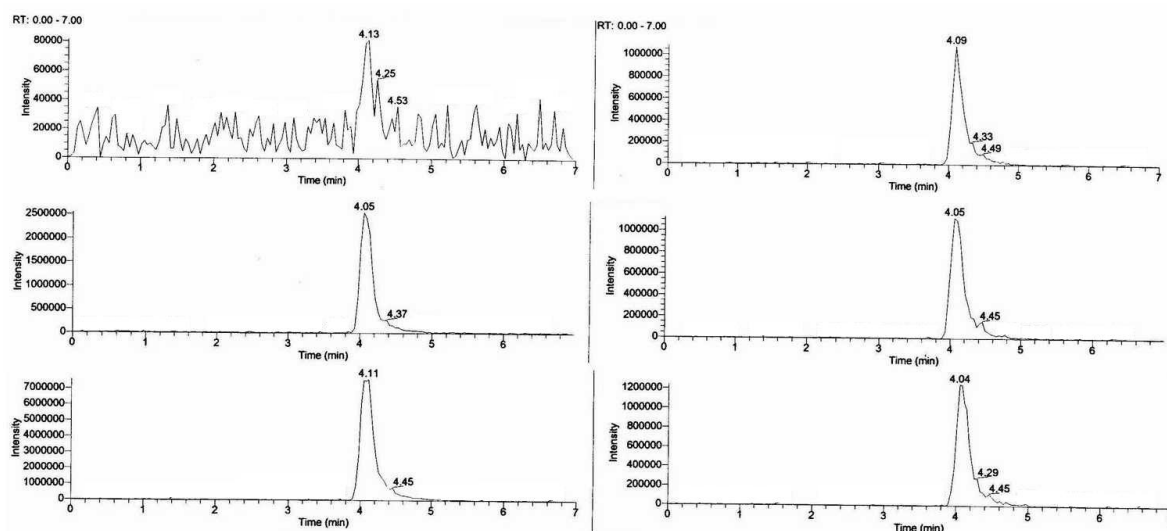
Fig. 8: Comparison of three concentrations of EtG chromatographed with 50 µM of d$_5$-EtG. The traces on the left side are those of EtG (m/z 221) in concentration (from above) 1.56 µM, 50 µM and 250 µM, the traces on the right are from the internal standard d$_5$-EtG (m/z 226) in a concentration of always 50 µM. (flow rate 0.3 mL/min)

The quantification of EtG as the ratio of m/z 221 and m/z 226 provided a linear correlation between 0.4 and 250 µM (figure 9). A similar range for the linear quantification of EtG was reported by Nishikawa et al[89].
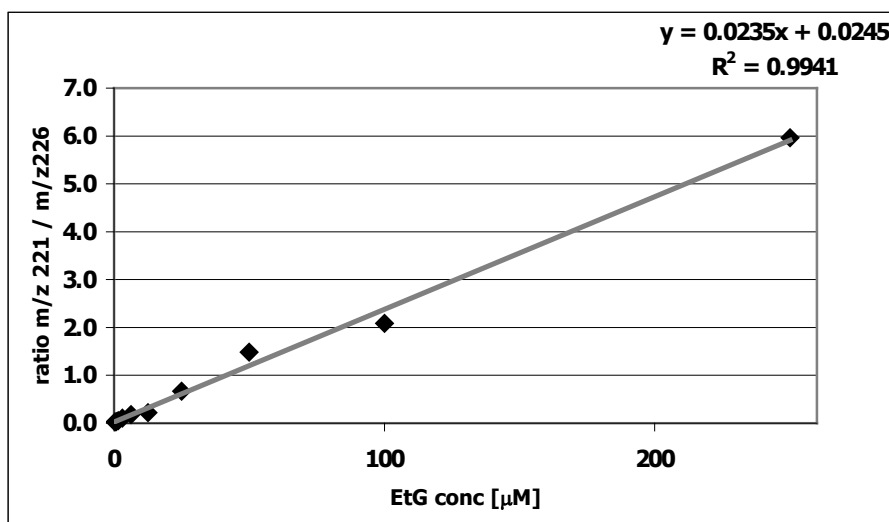
Fig. 9: Plot of the ratio of m/z 221/226 against the concentration of EtG [µM]

In a biological matrix like serum or urine other co-eluting ions with identical m/z-ratios as EtG (221) or d$_5$-EtG (226) must be expected. To ascertain that the ions with m/z-ratios of

221 and 226 are really EtG or d_5-EtG, respectively, we wanted to quantify their specific fragments.

In principle there are two different ways of producing fragments. Either one specifies which mass should be fragmented and obtains the mass-spectrum of these fragments (MS^2) or both, the mass to be isolated and fragmented and the resulting fragment one is interested in (single reaction monitoring, SRM), can be specified. The MS^2 technique allows the monitoring of all fragments generated from the peak of interest so that the relative abundances of the single fragments may be taken into account when judging the origin of a fragment peak. The SRM technique allows a more sensitive monitoring of highly specific fragments that may appear in small quantities only. Since they are readily isolated out of the bulk of the other fragments they might be more significant for the quantification of the fragments. The fragmentation pattern in any case is strongly influenced by the amount of energy applied on the selected ions. A good setting for this purpose would leave part of the molecular ion unaffected and therefore still visible when MS^2 spectra are recorded.

Our ion-trap system requires a defined method (scan-event) for every ion that has to be fragmented. This way, for the complete analysis of one sample of EtG with internal standard three scan events need to be specified: one for the full scan mode where information about the (parent) ions m/z 221 and m/z 226 is collected and one each for the fragmentation of EtG and d_5-EtG respectively.

The ion-trap mass-analyser works on a discontinuous mode of action. Ions are collected by the trap for some time (dependent on the settings, but usually up to 200 ms) and then the inlet is closed until the desired scan event (full scan, MS^n (n = 2–10), single ion monitoring (SIM), selected reaction monitoring (SRM), consecutive reaction monitoring (CRM)) has been carried out and is finished (takes about 200 µs). After that the ion-trap is refilled again and the next scan-event is carried out. If more than one scan event is selected, these are run consecutively resulting in gaps in the data-collection for the single scan-event. In our setting one turn of three scan events lasted about 2.4 seconds (0.8 sec/scan-event).

Based on an article of Wurst and co-workers[146] the transition from m/z 221 to m/z 75 in a SRM method seems to be typical for EtG. Others have adapted this method for the identification of EtG[53,119,136,145,147,148].

The MS^2 fragmentation pattern of EtG is shown in figure 10. The parent ion (m/z 221) is still present in this setting (with 25 "energy units" used for the fragmentation). The resulting m/z 203 may derive from the loss of water and m/z 159 from a subsequent loss of carbon-dioxide. This m/z 159 may further lose an ethyl group (m/z 129) or an ethanoyl group (m/z 113). A loss of water from m/z 113 would result in a fragment with m/z 95, whereas a loss of carbon monoxide would leave a fragment with m/z 85.

The fragment of interest (m/z 75) is the product of a different fragmentation cascade and is supposed to have either of the structures shown in figure 11. Of course there might be other possible structures, but these two are the most probable ones.
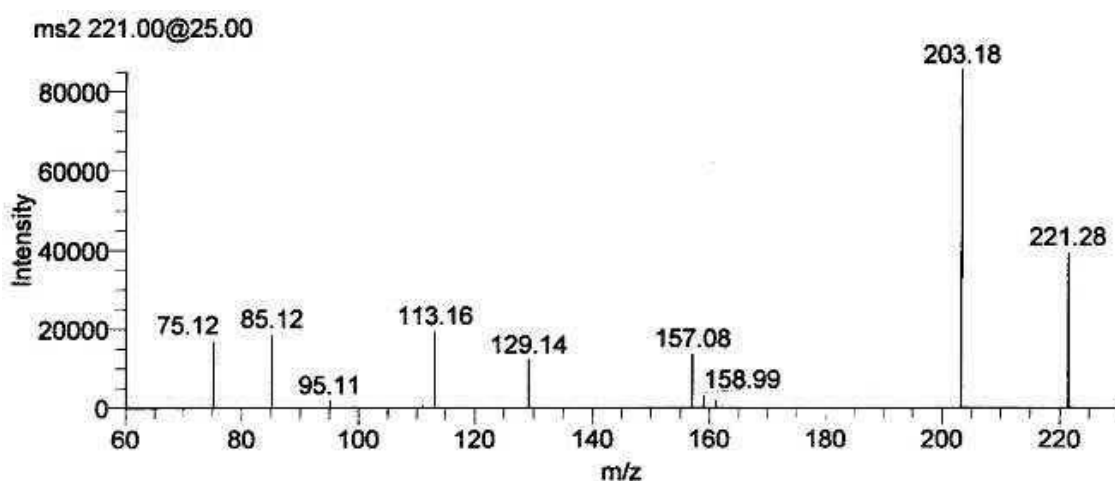
Fig. 10: MS^2 spectrum of EtG; the main fragments are addressed in the text (see above). The concentration of EtG was 125 µM (1.25 nmol o.c.)
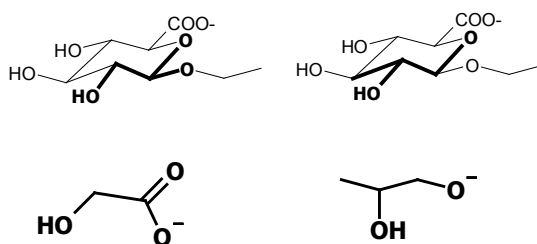
Fig. 11: possible structures for the m/z 75 fragment from EtG (or d$_5$-EtG) with their origin highlighted in the structure of the parent ion.

The fragmentation pattern of d$_5$-EtG is very similar to that of EtG (figure 12). As long as the deuterated ethanoyl group is present in the molecule the corresponding peaks have a m/z-ratio that is 5 amu higher than in EtG (m/z 226, m/z 208, m/z 164). After its removal the m/z ratios are the same (m/z 129, m/z 113, m/z 95, m/z 85, m/z 75) as in EtG.
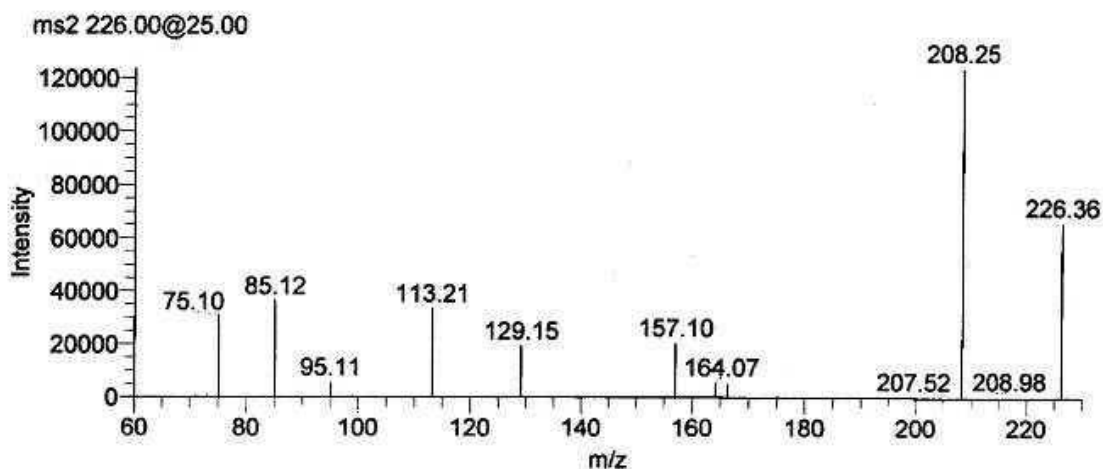
Fig. 12: MS^2 spectrum of d_5-EtG; the main fragments are addressed in the text (see above). The concentration of d_5-EtG was 125 µM (1.25 nmol o.c.)

These fragmentation patterns were similar to those others had found earlier[53,136].

We planned to establish an automatic integration method for the recorded fragment peaks of the m/z 75 fragments of m/z 221 and m/z 226 for the quantification of EtG by the software of the MS. When recording MS^2 chromatograms for the potential quantification of m/z 75 fragments reasonably shaped peaks were produced (figure 13).
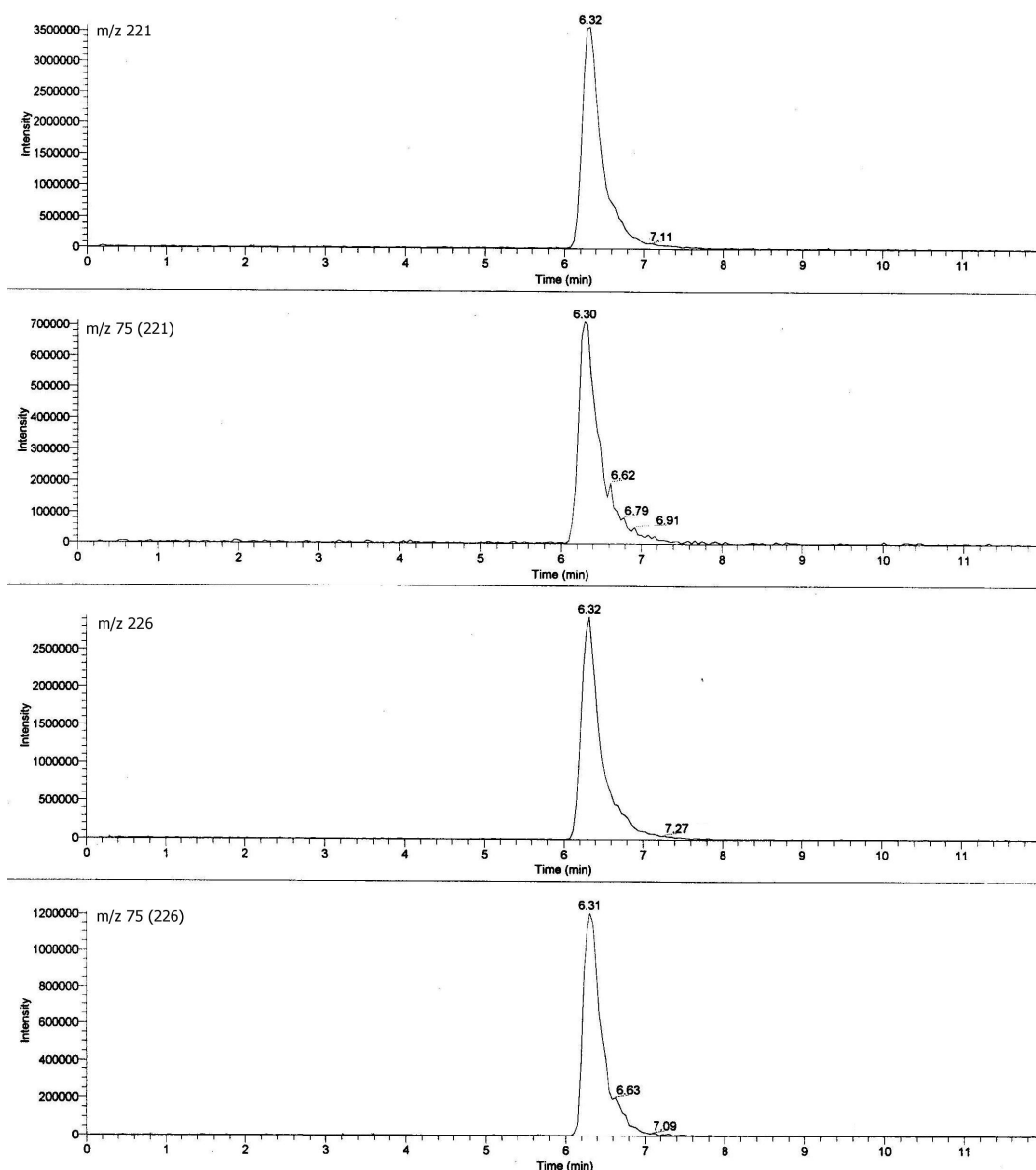
Fig. 13: traces of m/z 221 and the corresponding m/z 75; of m/z 226 and its corresponding m/z 75. The m/z 221 and 226 traces are isolated from the TIC whereas the m/z 75 were recorded in MS^2 mode as separate scan events by isolation of m/z 221 or m/z 226 ions, respectively (flow rate 0.2 mL/min). The concentrations of EtG and d$_5$-EtG were 125 µM (1.25 nmol o.c.)

A similar situation as shown above for the MS^2 fragmentation was found when a SRM fragmentation was applied (figure 14). After SRM the fragment peaks had less favourable shapes for automatic integration by the software of the MS, compared to the MS^2-method. This might be due to the discontinuous data collection by the MS when more than one scan event is carried out.

Such fragment peaks of irregular shape and width may have peak areas that are not correlated to the amount of EtG in a direct linear mode. For the quantitative analysis of

EtG in urine or plasma samples peaks like those of the m/z 75 tracings in figure 14 are problematic for quantification.
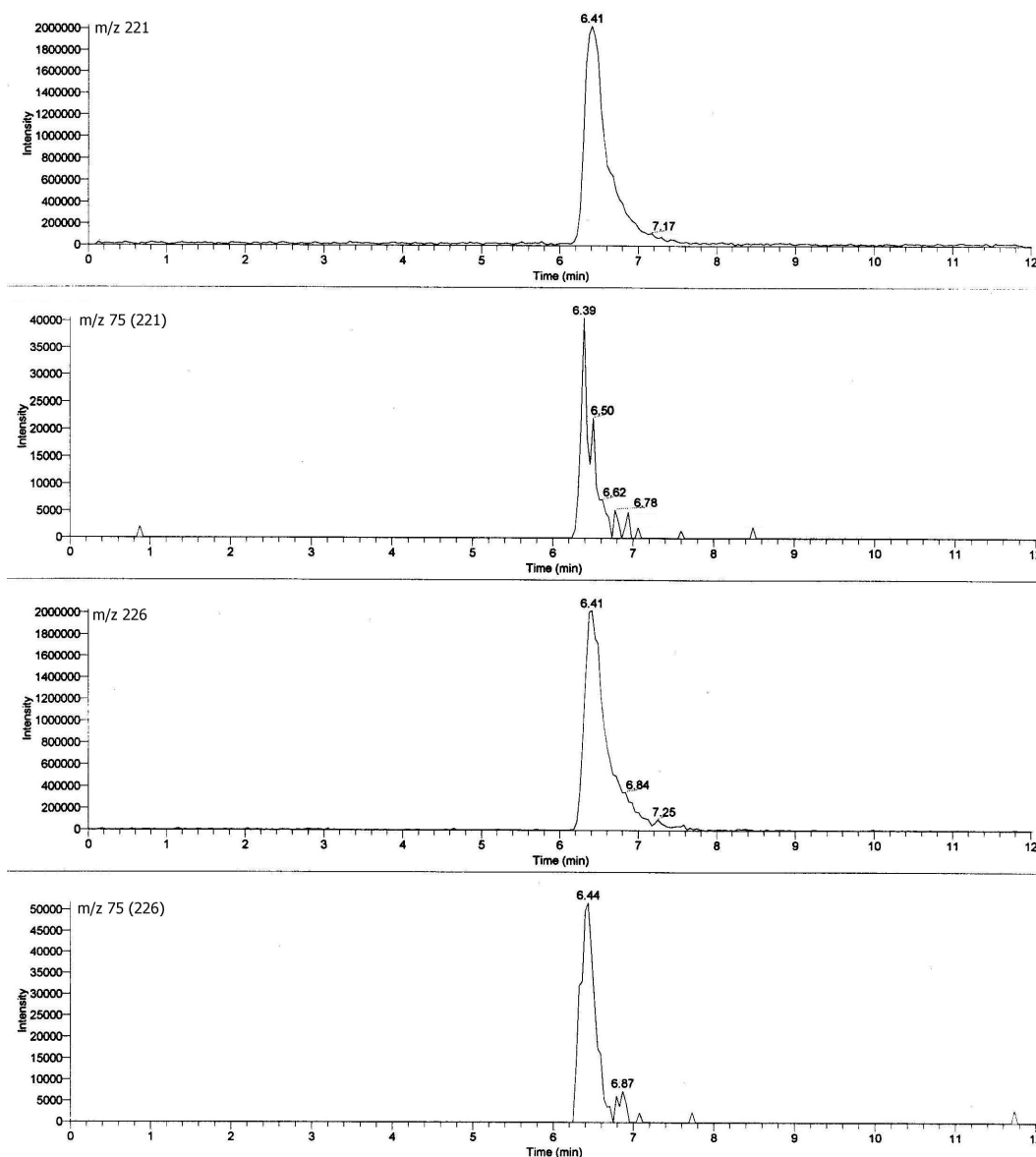
Fig. 14: traces of m/z 221 and the corresponding m/z 75; of m/z 226 and its corresponding m/z 75. The m/z 75 fragments result of a SRM procedure isolating m/z 221 or m/z 226 ions, respectively, and recording the resulting fragments (flow rate 0.2 mL/min). The concentrations of EtG and d_5-EtG were 125 µM (1.25 nmol o.c.)

A series of diluted urine samples were analysed without any further sample pre-treatment prior to the analysis. The small amounts of EtG that must have been present in urine samples after the consumption of 1.2 L beer (one sample was collected 90 min. after the beer had been drunk, a second 5 h later and a third one after 12 h) could hardly be

detected by tracing the m/z 221 (EtG; figure 15). By tracing the m/z 75 fragment (after MS^2) the presence of EtG could be confirmed.

A different, shorter separation column was used for these chromatograms to avoid the contamination of the original column used in the other experiments (MN EC 125/2 Nucleosil$^®$ 100-C$_{18}$ HD instead of MN ET 250 Nucleosil$^®$ 5 C$_{18}$). Therefore EtG was already eluted between 1.8 and 1.9 min as shown in figure 15.

The following samples were analysed: an aqueous standard solution of EtG (50 µM), an urine blank and a sample of the urine 5 h after drinking 48 g of alcohol. The m/z 221 tracing of EtG consists of a single peak at 1.86 min. and the MS^2-spectrum provides the same fragments as seen before in figure 10. The m/z 221 tracing of the urine blank sample consists of many peaks and so does the one of the urine sample after drinking. To demonstrate the presence of EtG in the 5 h urine sample its m/z 75 tracing and the mass-spectrum of m/z 221 after MS^2 at 1.92 min. is shown at the bottom of figure 15. The EtG specific fragments with m/z 75, 113 and 203 are present in both of the spectra of EtG standard and the urine specimen after drinking indicating that EtG has been formed.
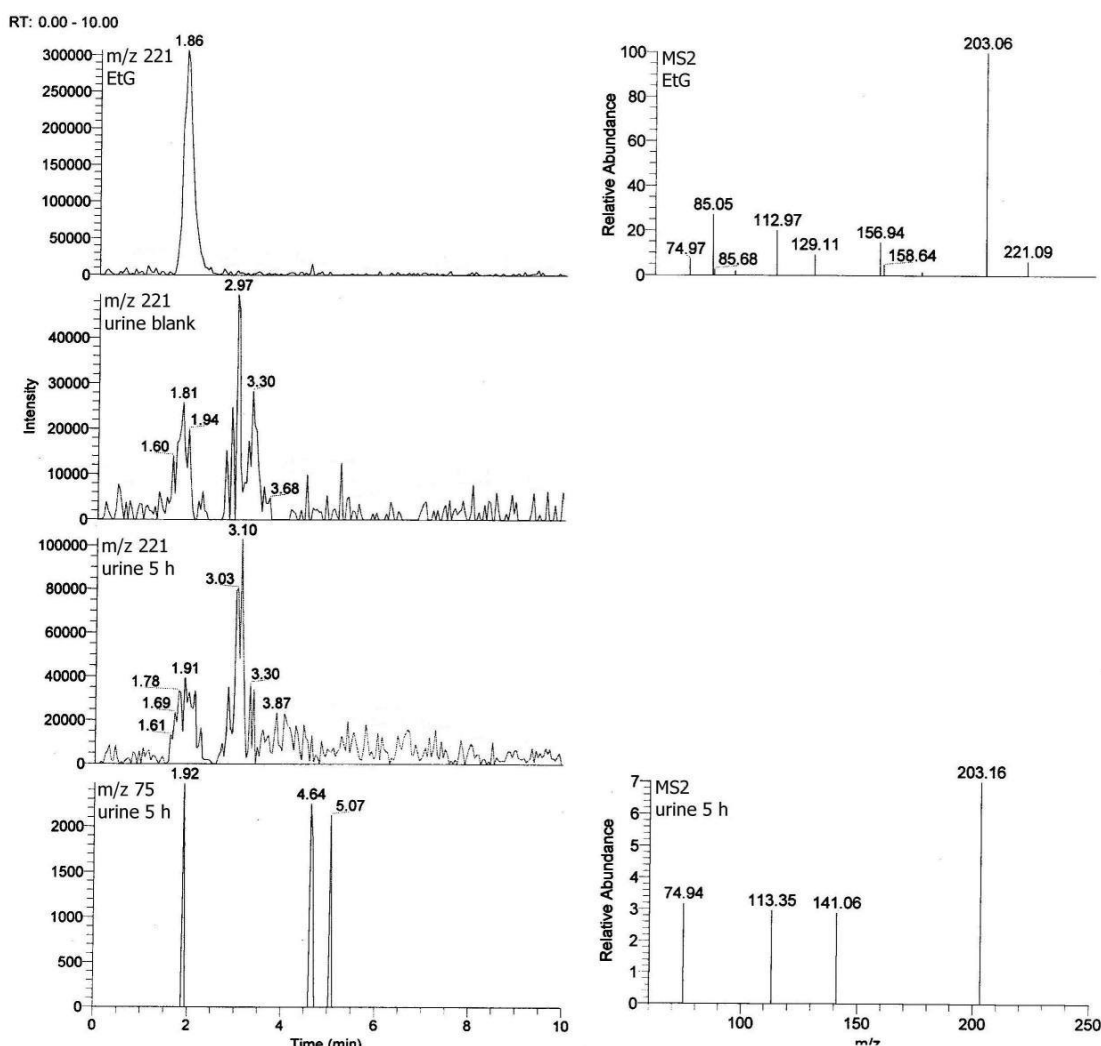
Fig. 15: the chromatograms (left) and MS²-spectra (right) of (from top) EtG 50 µM, urine blank and urine 5 h after consumption of 48 g of alcohol. The bottom line shows the m/z 75 tracing of the 5 h urine sample with the corresponding MS²-spectrum of m/z 221 at 1.92 min.

To be able to judge the presence of EtG from the m/z 221 tracing already the urine samples need to be cleaned-up prior to analysis. To further improve the specific identification of EtG with the m/z 75 fragments and the MS²-spectra the samples should also be concentrated. Such chromatograms would then be comparable to results from previously published methods.

Discussion

A first difficulty of this LC-MS analysis was the fact that EtG does not absorb any light in the range of wavelengths suitable for analysis by HPLC and that we were therefore not able to optimise the HPLC system prior to the use of the MS so that we were confronted with the improvement of two systems at once.

One reason for the analytical difficulties may have been the generation of negatively charged ions from the HPLC-output for the analysis in the MS. The liquid phase that is present after the separation of the compounds on the HPLC system needs to be converted into a ionic gas-phase. This had been compared to the impossible union of a fish with a bird (figure 16).
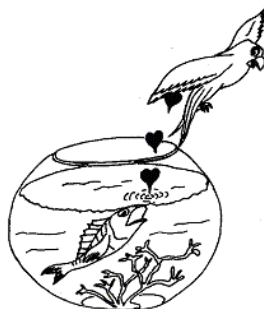
Fig. 16: the „impossible" connection of HPLC and MS, like the union of a fish and a bird

This problem has been largely solved nowadays. The electrospray-ionisation (ESI) that was used to produce the ions for the analysis in the MS allows the production of both positively and negatively charged ions depending on the settings of the polarity between the injection needle and the heat-capillary (figure 17). The entering liquid is dispersed at the edge of the needle into small droplets. These droplets need to lose the solvent and to be ionised.
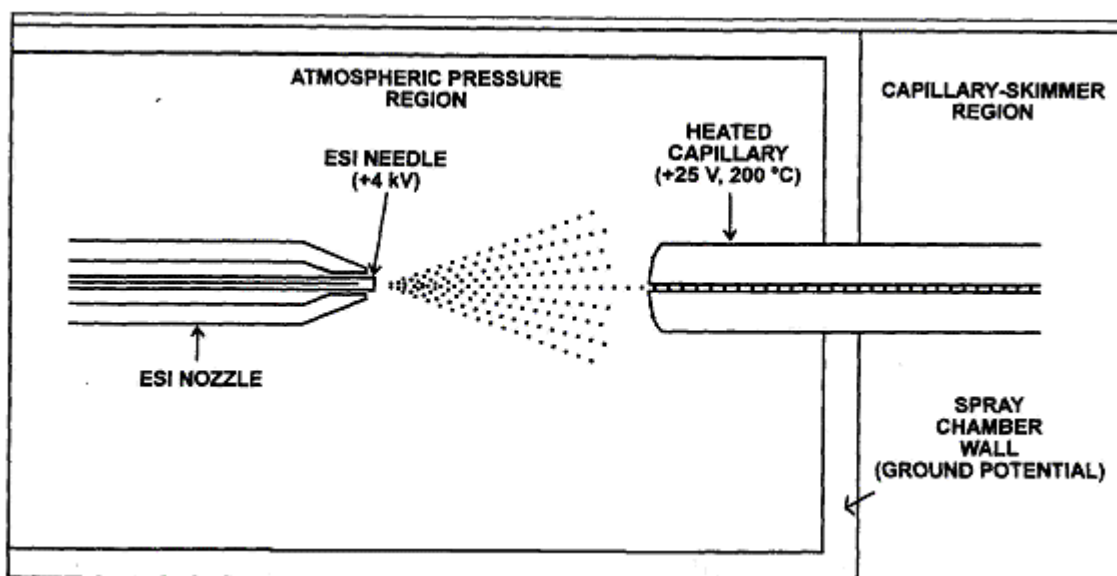
Fig. 17: schematic drawing of the ESI ionisation (here with the polarity set for positive ions).

There are two proposed models for the production of ions in Electrospray Ionisation: The Ion Evaporation Model (IEM) proposes the droplets to shrink due to the solvent evaporation, charges to move towards the surface of the droplet and the resulting charge density on the surface of the droplet to force ions out of it (figure 18).
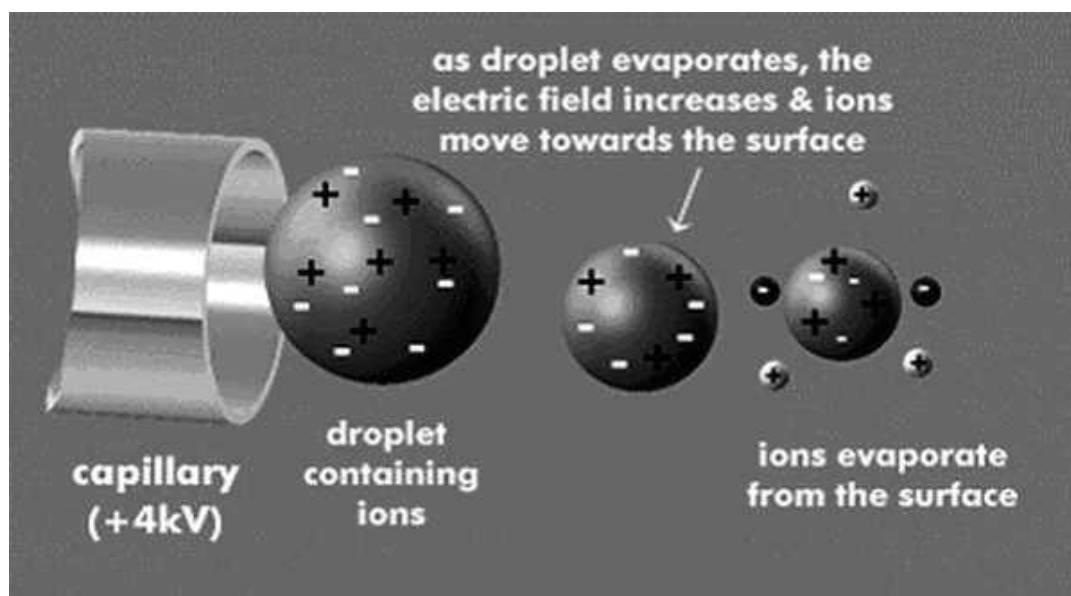
Fig. 18: schematic drawing of the Ion Evaporation Model for the generation of ions in electrospray-ionisation

The Charged Residue Model (CRM) proposes the droplets to shrink and then break up due to coulombic forces creating micro-droplets containing one analyte ion only that – by further drying – will set free a completely desolvated ion (figure 19)
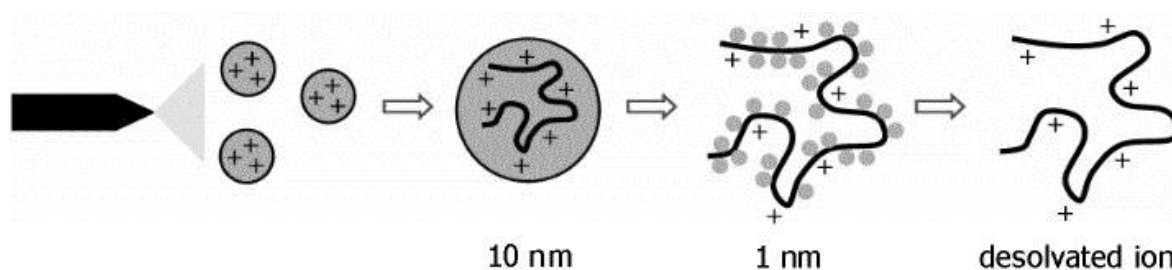
Fig. 19: schematic drawing of the Charged Residue Model (CRM) for the generation of ions in electrospray-ionisation

In ESI the building of positive ions is preferred over the negative ones. The ions in negative mode are supposed to present structures like [M-H]⁻, which might be thought of as a loss of "counter-cations" (Na^+, K^+, H^+). The negative ions have a greater tendency of getting discharged[88]. ESI is the preferred method for (LC-) MS of macromolecules like proteins because near to no destruction and fragmentation of the ions is observed. The EtG in contrast to proteins is very small and it might therefore be more difficult to ionise these tiny particles.

In order to improve the formation of the ions, the spray performance and the setting of the ion-optics the system was re-tuned several times. Only little improvement could be achieved this away.

A further reason for these analytical difficulties might be the method of MS analysis. The ion-trap system is probably a sub-optimal choice for this kind of analysis. It is likely that a triple-quadrupole MS – that was described in the literature – would have performed better.

If only the molecular ions (i.e. m/z 221 and m/z 226) were taken into account the method was comparable to other methods published. Our limit of detection (LOD), defined as a signal to noise ratio of 3, was about 200 µg/L (1 µM; 10 pmol o.c.; cf. figure 8) and the limit of quantification (LOQ; signal to noise ratio 10) 600 µg/L (~ 3 µM; 30 pmol o.c.). Janda et al reported a LOD (with a GC-MS) of 37 µg/L (~ 5 pmol o.c.) in serum and 168 µg/L in urine[52], whereas Schmitt et al reported a LOD of 100 µg/L in both serum and urine[106]. The previously published cut-off for considered abuse of alcohol – 5 mg/L in serum[105,107] – would probably be detectable with our method; further analyses in urine (or serum) samples instead of the used aqueous standard solutions would have to prove this assumption.

GC of ethyl-glucuronide

A different method to quantify EtG was necessary to investigate its metabolism in blood, urine and stool. Since the LC(-MS) approach was not as successful as expected a GC method was explored. EtG itself is a non volatile substance. A precolumn derivatisation was therefore needed. An overview of some of the possible procedures is depicted in figure 20.
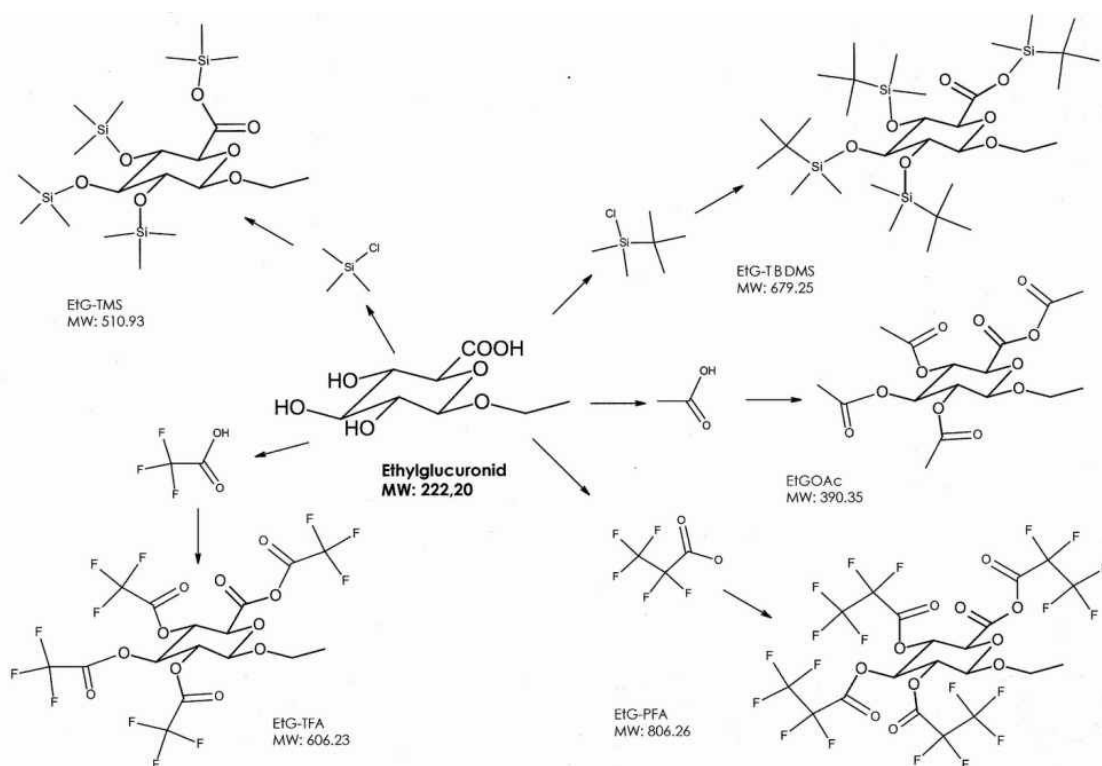

Fig. 20: overview of possible derivatives of EtG for GC analysis

Acetylation of ethyl-glucuronide[106,107]

Method

The acetylation of EtG with acetic acid anhydride as it has been described in the literature[106] was thought to be a good method for the analysis of EtG.

100 µL of an aqueous solution of EtG in concentrations ranging from 125 to 1000 µM were dried under a stream of air in a 10 mL Sovirel®-vial. 300 µL of acetic acid anhydride were added and the solution was incubated at 80°C for 30 min. on a heat-block (intern.

Laborat. App. GmbH, D- Dottingen; M22/1). The solution was air-dried again, the residue dissolved in 100 µL of methanol and one µL thereof was injected for analysis by a HP 5890 series II gas chromatograph with flame ionisation detection (FID). A MN Optima 1 10 m x 0.2 mm; 0.35 µm GC-column was used for the separation of the compounds. Gas flows were as follows: nitrogen carrier gas 207 kPa, air 283 kPa and hydrogen 124 kPa. The total gas-flow was between 90 and 100 mL/min. The oven temperature was kept at 170°C (isotherm), the injector was set at 200°C and the detector at 240°C.

Results of the acetylation

In figure 21 the chromatogram of a reagent blank is compared to the chromatogram of a 250 µM aqueous solution of EtG. The blank was prepared by drying 100 µL of water instead of an aliquot of dissolved EtG. The peak corresponding to the acetylated form of EtG (EtGOAc) is detected after 3.5 min. The peak corresponding to the acetylated form of EtG could also be detected in an urine sample spiked with EtG prior to derivatisation (figure 22).
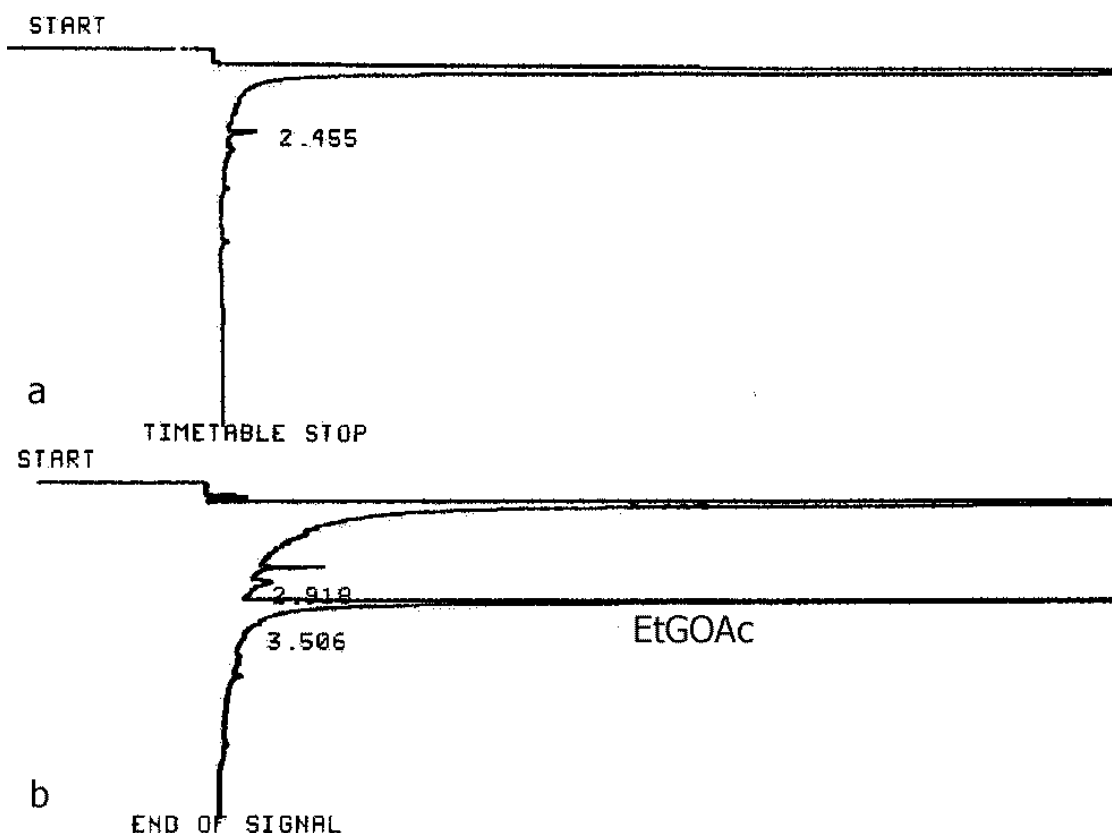
Fig. 21: GC chromatogram a) of a blank showing no peak at 3.5 min and b) of acetylated EtG in a concentration of 1 mM with its peak at 3.5 min.

In figure 22 the chromatogram of an urine blank is compared to an urine sample spiked with EtG. For the blank 100 µL of urine from a teetotaller were dried and derivatised afterwards as described above. The spiked sample was prepared by adding 50 µL of a 500 µM aqueous EtG solution to 100 µL of an other aliquot of the same urine and evaporating the mixture to dryness before derivatisation.
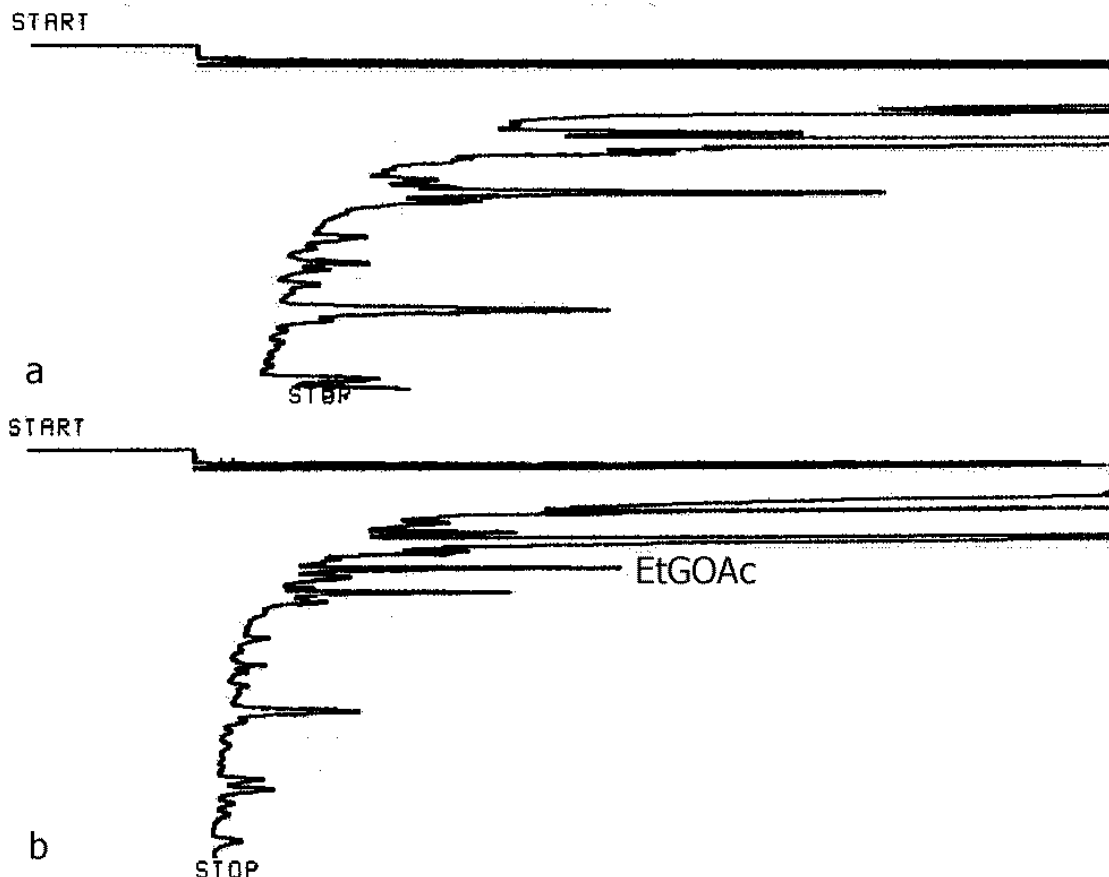

Fig. 22: a) acetylated urine sample without EtG; b) the same urine sample spiked with 500 µM EtG. EtG peak at 3.5 min.

The EtGOAc-peak appeared in a section of the chromatogram where no other disturbing peaks were present in the blank urine.

The lowest concentrations we were able to detect with this derivatisation method were more than 20 times higher than the concentrations we expected to be present in urine or plasma. Because of these facts and bearing in mind that other derivatisation procedures might provide better results the further evaluation of the method was ceased.

Halogenation of ethyl-glucuronide[121]

Method

To improve the GC sensitivity of the derivatised EtG we chose halogenation for the derivatisation and electron-capture detection (ECD) for the detection of the resulting products. Compounds containing halogens like chlorine or fluor should be detected with a far higher sensitivity by ECD than by the rather unspecific flame-ionisation detection (FID).

100 µL of a methanolic solution of EtG in concentrations from 1.56 µM to 400 µM were dried under a stream of air. 250 µL of ethyl acetate (EtOAc) and 25 µL of halogenation reagent (trifluoroacetic acid anhydride (TFAA) or pentafluoropropionic acid anhydride (PFAA); Fluka, Buchs CH) were added and the solution incubated at 60°C for 30 min. The solution was air-dried and the residue redissolved in 50 µL of EtOAc. One µL thereof was injected for analysis by a HP 5890 series II gas chromatograph with electron capture detection (ECD). A MN Optima 17 25 m x 0.25 m; 0.25 µm GC column was used. The total gas-flow was set to 57 mL/min. For the analysis of TFA-esters of EtG the oven was set at 100°C, the injector at 180° and the detector at 250°C. For the analysis of PFA-esters of EtG the oven was set at 120°C, the injector at 200°C and the detector at 250°C.

Results of the halogenation

The products after derivatisation with TFAA did not provide peaks that could be assigned to EtG derivatives with certainty. Since no such derivatives could be found by using TFAA the higher fluoridated PFAA was explored (figure 23). These PFA-esters yielded peaks for the EtG derivatives but the reproducibility of this derivatisation was far from being satisfying. We observed that peak areas of one single concentration of EtG after derivatisation with PFAA differed by more than a factor 10, indicating strongly that these derivatives were unstable. Therefore the reproducibility of this approach was not sufficiently reliable although with this halogenation method we were able to detect EtG derivatives at lower concentrations than with the acetylation procedure described above. Nonetheless the non-linearity of the concentration to signal ratio as well as the poor reproducibility of this method did not allow us to base all of the further planned experiments on this method.
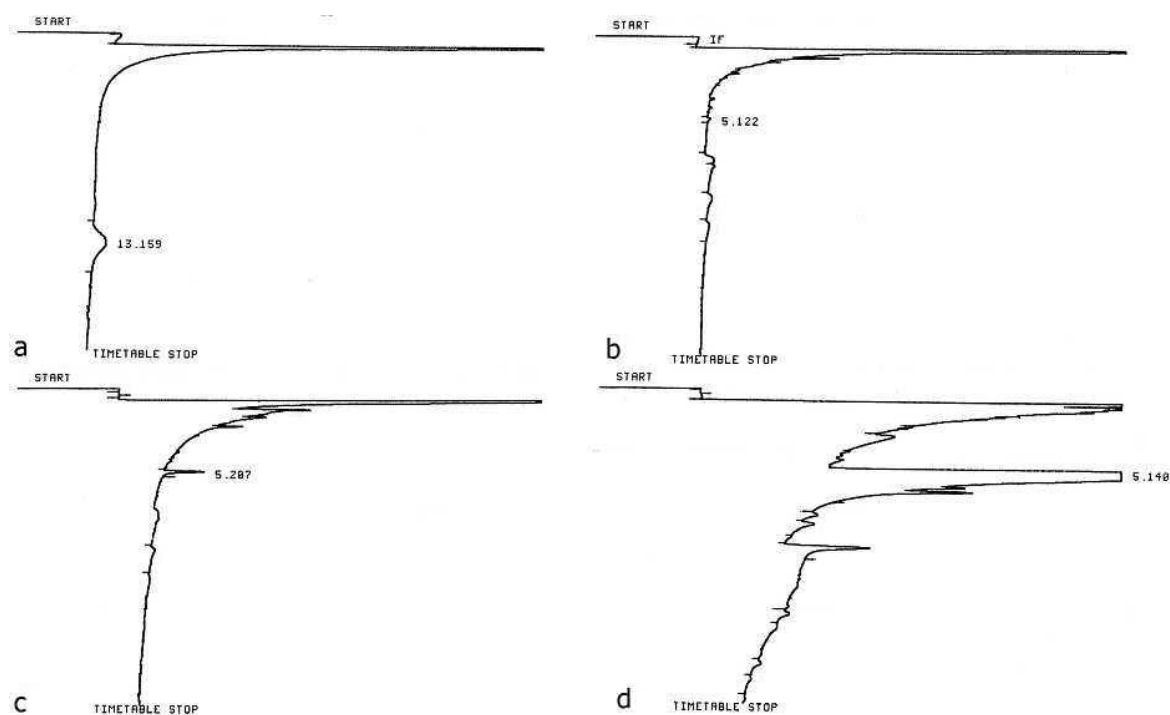
Fig. 23: Chromatograms of EtG pentafluoro-propionic acid anhydride (PFAA) derivatives; the EtG-PFA-ester peak has a retention time of about 5.2 min. a) only the solvent EtOAc, b) 1.56 µM EtG-PFA, c) 25 µM EtG-PFA, d) 400 µM EtG-PFA

Silylation of ethyl-glucuronide

One last approach to the analysis of EtG by GC or GC-MS was the silylation of the hydroxyl-groups as a simple and effective derivatisation procedure[52,146]. The EtG contains four hydroxyl-groups in the glucuronic acid residue which may all be silylated. Thus highly volatile derivatives should be produced and a good sensitivity for their analysis can be presumed.

Method

100 μL of an aqueous solution of EtG in concentrations ranging from 125 to 1000 μM were dried under a stream of air in a 10 mL Sovirel®-vial. 40 μL of acetonitrile and 10 μL of N-tert-butyldimethylsilyl-N-methyltrifluoroacetamide (MTBSTFA) were added, and the solution was incubated at 80°C for 30 min. on a heat-block (intern. Laborat. App. GmbH, 7801 Dottingen; M22/1). One μL thereof was injected for analysis by a HP 5890 series II gas chromatograph with flame ionisation detection (FID). A MN Optima 1 10 m x 0.2 mm; 0.35 μm GC-column was used for the separation of the compounds. Gas flows were as follows: nitrogen carrier gas 207 kPa, air 283 kPa and hydrogen 124 kPa. Total gas-flow was between 90 and 100 mL/min. For the analysis of these *tert*-butyldimethylsilyl (TBDMS)-derivatives the oven temperature was set at 110°C, the injector at 200°C and the detector at 240°C.

For the GC-MS we used a HP 5890 series II gas chromatograph connected to a HP 5871 mass-spectrometer operated in electron impact mode (Hewlett-Packard, Palo Alto, CA). Gas chromatographic separation was accomplished on a MN Optima 1 12 m x 0.2 mm; 0.35 μm GC-column. The injector temperature was set at 260°C and the detector at 300°C. Helium was used as the carrier gas with a total flow of 0.9 mL/min. For the GC separation of the compounds the oven temperature was increased after two minutes at 110°C at a rate of 25°C/min to 250°C where it was kept constant for 3 minutes.

Results of the silylation

The derivatisation products of EtG with MTBSTFA (EtG-TBDMS) were found with good reproducibility at 4.6 min. with the GC-FID method. Since the derivatives proved to be quite unstable against any humidity we started looking for a suitable internal standard. Out of the substances tried out for this purpose none could be used because either its TBDMS-derivative got lost in the solvent front or co-eluted with EtG-TBDMS.

A sample chromatogram of EtG-TBDMS in a concentration of 250 µM (0.5 nmol o.c.) is shown in figure 24.
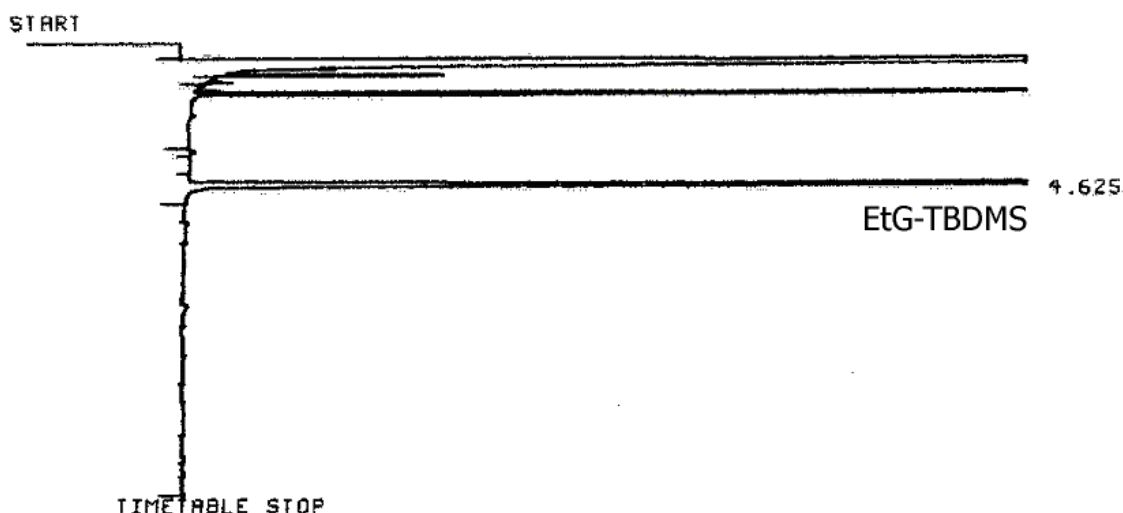

Fig. 24: chromatogram of EtG-TBDMS in a concentration of 250 µM, with the corresponding peak at 4.6 min.

Even tough the chromatograms looked promising we were experiencing problems with the peak of interest at 4.6 min. Probably caused by the instability of the silylated products series of identical concentrations of EtG derivatives produced peak areas in the chromatograms that differed by up to a factor 5. Even blank chromatograms where the EtG solution had been replaced by pure water produced (small) peaks at 4.6 min.

Due to the size of EtG-TBDMS derivatisation of EtG with BSTFA would likely provide derivatives that are better suited for GC-MS. Aqueous standard solutions resulted in well detectable peaks and the mass difference of EtG and d_5-EtG was seen in some of the fragments (difference of 5 amu). Figure 25 shows a GC-MS chromatogram with the corresponding mass spectrum of EtG in scan-mode whereas figure 26 shows the respective d_5-EtG chromatogram and spectrum. In these spectra the same fragments were found as was reported in the literature[5,100].
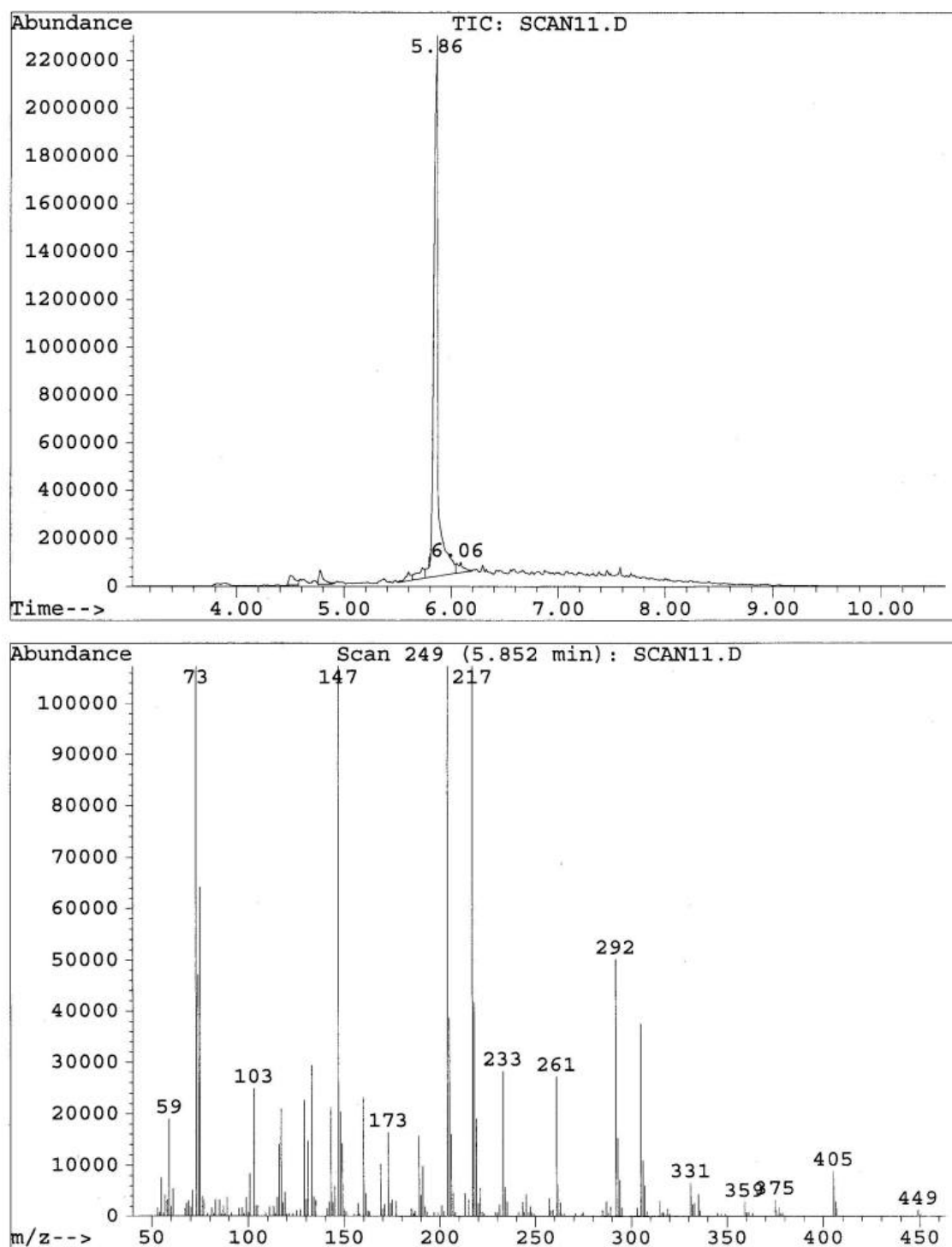
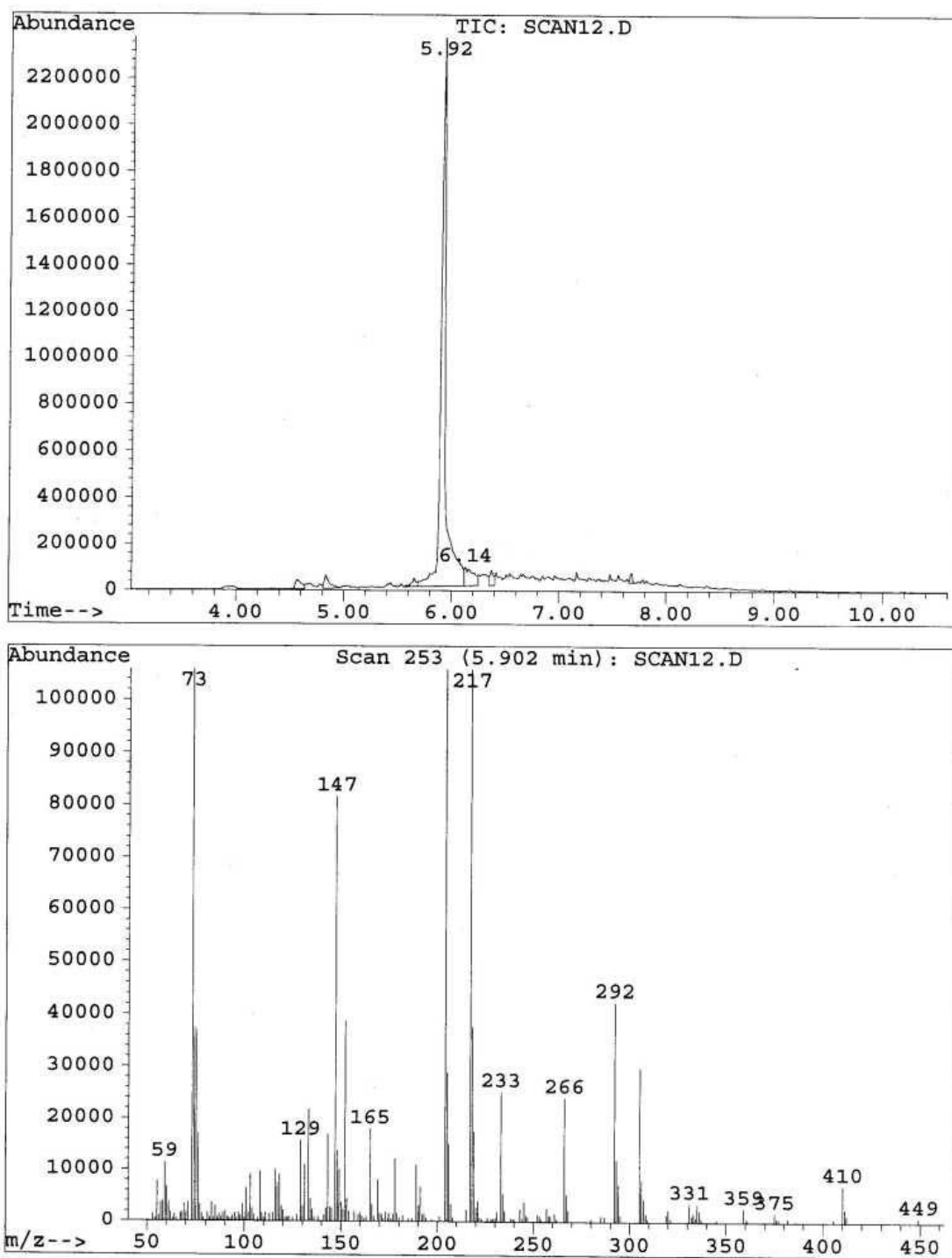**Fig. 25: Chromatogram of silylated EtG and the corresponding mass-spectrum at the peak-maximum**

Fig. 26: Chromatogram of silylated d_5-EtG and the corresponding mass-spectrum at the peak-maximum

Alt and coworkers[5] used the fragments with m/z 160, 261 and 405 for the identification of EtG and the m/z pair 405 and 410 for the quantification of EtG.

One explanation how the fragment with m/z 405 (for EtG) or 410 (d_5-EtG) can be generated is the loss of a trimethylsilyl group including the adjacent oxygen atom plus a

single methyl group. The further loss of the carbonyl group (with the TMS group) and part of the ring structure leads to one of many possible m/z 261 (m/z 266) fragments. After the loss of a further TMS group and an oxygen and a carbon atom a fragment with m/z 160 (m/z 165) remains. The structures of such fragments are shown in figure 27.

m/z 510

m/z 405

m/z 261

m/z 160
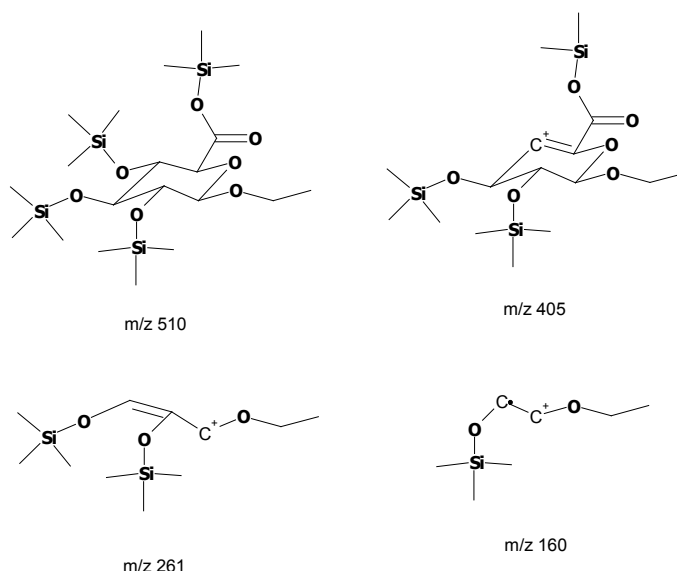
Fig. 27: structure of the molecular ion after derivatisation with BSTFA and possible structures of the fragments monitored by Alt and coworkers[5]. Since the ethanoyl group is present in all those fragments depicted, the structures may also explain the corresponding fragments of d_5-EtG.

These three fragments are those that are different in EtG and d_5-EtG. All the other main fragments have the same m/z ratios for EtG and d_5-EtG indicating that the ethanoyl group is easily cleaved during fragmentation.

In spite of several attempts to clean up and concentrate EtG the necessary sensitivity to quantitate EtG by GC-MS could not be achieved.

Discussion of ethyl-glucuronide analysis

The analysis of EtG has been described in several publications and it may be introduced in routine laboratory in the near future. The method of choice is probably a LC-MS method, because not only the peak might be found but it can also be identified with certainty. We have tried to adapt the existing methods for our needs to be able to investigate further a possible enterohepatic circulation of EtG and to find out whether there would be a source

of microbially generated EtOH metabolites. In addition this would have enabled us to also focus on the role of EtG in the toxicity of alcohol.

To our great disappointment the adaptation of existing methods to our presently available laboratory equipment in a reproducible and sensitive fashion was much more time consuming than expected. Thus, the analysis of EtG, which would have been the basis for future research, could not be continued any further.

Ethyl-Lysine

Ethyl-lysine (EtLys), one of the products of the reaction of acetaldehyde (AcHO) with amino groups, has been detected with antibodies directed against AcHO-modified proteins in tissue and plasma of subjects abusing alcohol[17,68,85,86,132,140,143]. The wide variety of different chemical forms of AcHO-adducts (AA; cf. introduction) which provoke many different antibodies[70] proved to be a problem for the comparison of the results between laboratories. Furthermore the cross reactivity of the antibodies with structures similar to EtLys modified proteins could possibly provide false positive results.

The elucidation of the different structures of these adducts has been of major interest. As an intermediate product the structure of a Schiff base was expected. Schiff bases present themselves as an equilibrium between an imine form (with a double bond between the carbon and the nitrogen atoms) and an enamine form (where the double bond is situated between two carbon atoms; figure 28)
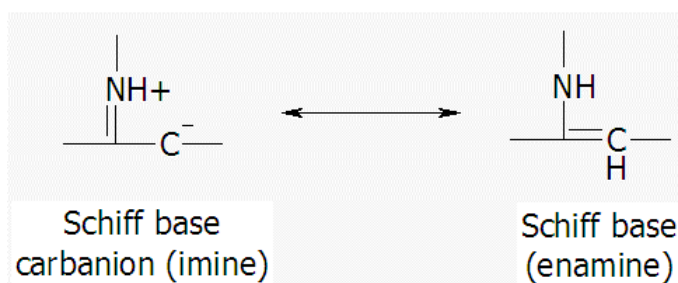

Fig. 28: The Schiff base is an equilibrium between a carbanion (imine) and an enamine

The Schiff bases are not stable structures and it is not possible to isolate them but they may be stabilised by reduction. In *in vitro* experiments sodium-borohydride ($NaBH_4$) or sodium-cyano borohydride ($NaCNBH_3$) was used for this purpose. Tuma et al. have also employed ascorbic acid[131,132]; this could be one way how the reduction is effected also *in vivo*.

From these data we concluded that in order to form stable EtLys a reduction step is needed to stabilize the putative Schiff bases formed between free amino groups and the carbonyl group of AcHO (figure 30). The proposed reaction scheme for the synthesis of ε-EtLys, as it would be formed in proteins, is shown below in figures 29 - 31. To ascertain the exclusive formation of ε-EtLys α-*tert*-butyloxycarbonyl (BOC)-Lys was used as starting reagent. The reduction of the Schiff base was achieved with $NaCNBH_3$ and hydrolysis with

hydrochloric acid was used to remove the protecting α-BOC-group and thus liberating the synthesised ε-EtLys.
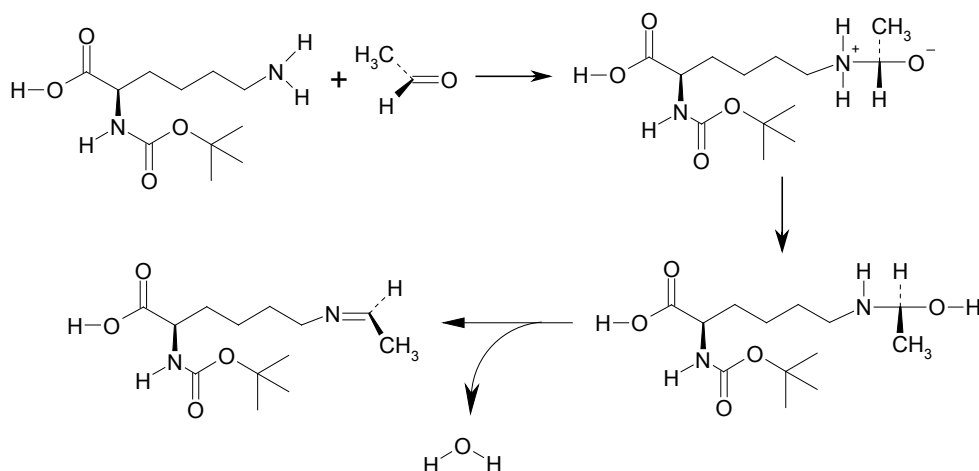

Fig. 29: reaction of α-BOC-Lys with AcHO resulting in a Schiff base
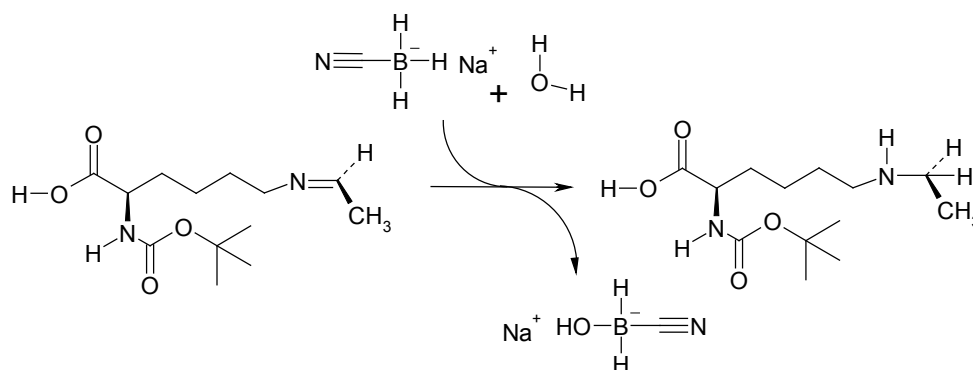

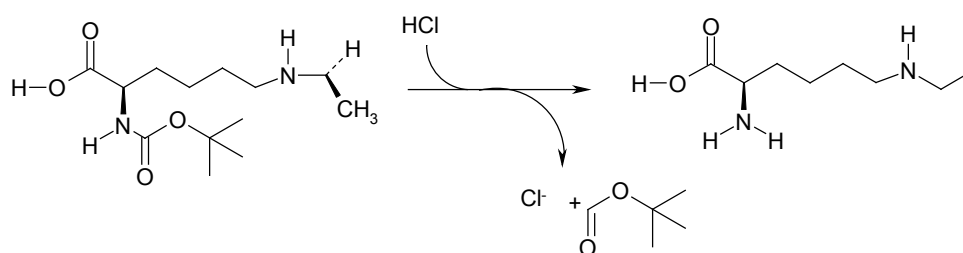Fig. 30: reduction of the Schiff base with sodium-cyanoborohydride

Fig. 31: acidic removal of the BOC protecting group resulting in ε-EtLys

To avoid the drawbacks of the immunologic approaches the aim of the present study was therefore to explore the possibility of analysing and quantifying EtLys in circulating proteins by chromatographic means. EtLys in circulating albumin which has a half-life of 14-20 days in man, but only approximately 2-3 days in rats[33,36,57,94], could provide a

marker for increased consumption of alcohol. Similar to glycosylated haemoglobin in patients with diabetes mellitus an ethylated form of haemoglobin has been proposed[120] as a marker for alcohol abuse with a half-life of around 120 days.

Our first aim was to develop a GC-MS method for the identification and quantification of EtLys in proteins. Based on our previous experience with the gaschromatographic-massspectrometric analysis of amino acids[99] the possibility of using a chromatographic analysis of silylated EtLys was first explored. Subsequently additional derivatisation procedures were investigated.

GC (-MS)

Method

Silylation

100 μL of an aqueous solution of EtLys (N6-ethyl-D,L-lysine hydrobromide; Toronto Research Chemicals, North York, Ontario, Canada) in concentrations from 50 to 500 μM were dried under a stream of air in Reacti-vials. 40 μL of acetonitrile and 10 μL of silylation reagent (BSTFA + 1% TMCS or MTBSTFA) were added and the tubes were incubated at 80°C for 30 min. After cooling one μL thereof was injected into the gas-chromatograph.

Halogenation

100 μL of a methanolic solution of EtLys in concentrations from 125 to 500 μM were dried under a stream of air. 250 μL of ethyl acetate (EtOAc) and 25 μL of halogenation reagent (TFAA or PFAA) were added and the solution was incubated at 60°C for 30 min. The solution was air-dried and redissolved in 50 μL EtOAc. After cooling one μL thereof was injected into the gas-chromatograph.

Chromatographic analysis

In order to determine the optimal chromatographic conditions the derivatised EtLys was first analysed using a HP 5890 series II gas chromatograph with flame ionisation detection (FID). A MN Optima 1 10 m x 0.2 mm; 0.35 μm GC-column was used for the separation of the compounds. Gas flows were as follows: nitrogen carrier gas 207 kPa, air 283 kPa and hydrogen 124 kPa. Total gas-flow was between 90 and 100 mL/min. The

column was heated according to the following program: 110°C for 2 min, then increment of the temperature at a rate of 15°C/min up to 250°C where the temperature was kept for 8 min. The temperature of the injector was set at 240°C and the temperature of the FID-detector at 270°C.

For the analysis of tri- and penta-fluoro-acetylated EtLys the oven temperature started at 70°C for 2 min. It then increased at a rate of 15°C/min. up to 250°C where it was kept for 4 min. The injector was set at 240°C and the ECD Detector at 280°C.

The chromatographic conditions for GC-MS were as follows: a HP 5890 series II gas chromatograph connected to a HP 5971 mass spectrometer operated in the electron impact mode (Hewlett-Packard, Palo Alto, CA). Gas chromatographic separation was accomplished on a MN Optima 1 12 m x 0.2 mm, 0.35 µm GC-column. Injector temperature was set at 260°C and detector temperature was 300°C. Helium was used as the carrier gas with a total flow of 0.9 mL/min. The oven temperature increased from 110°C (2 min) to 250°C (3 min) at a rate of 25°C/min.

Results

Figure 32 depicts a gas-chromatographic tracing of the BSTFA derivative of lysine (Lys) using FID. With GC-MS the silylated Lys and EtLys were detectable (figures 33 and 34). However, the concentrations required for an adequate, quantifiable signal were much higher than we expected them to be in plasma samples.

Therefore a procedure to concentrate the samples was necessary because we aimed to detect EtLys in plasma, where a high background of amino acids and other compounds would complicate the analysis. In order to isolate and concentrate EtLys we used a SepPak extraction based on anion exchange chromatography prior to derivatisation, but the recovery of the compounds of interest was so low that the sensitivity of GC-MS was judged to be inadequate to analyse EtLys in protein hydrolysates.
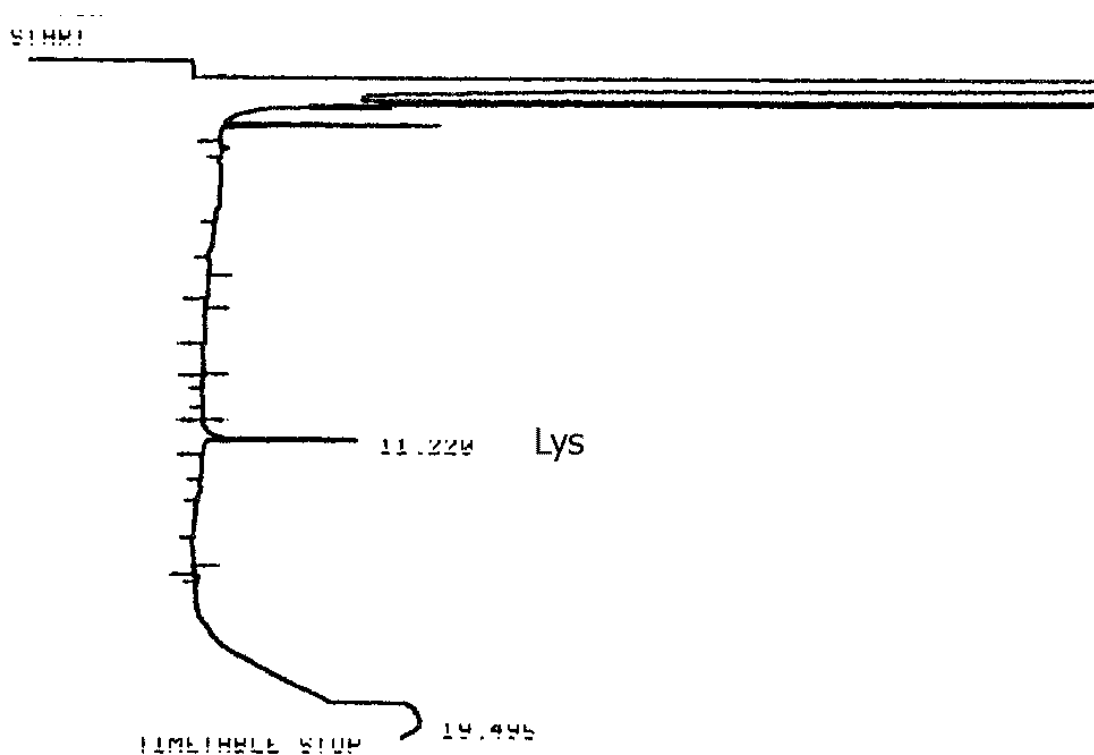
Fig. 32: BSTFA-derivative of Lys 500 µM; detection by GC-FID

In order to potentially increase the sensitivity halogenation and subsequent detection by ECD was explored. No reaction product of Lys and EtLys with either TFAA or PFAA was found.
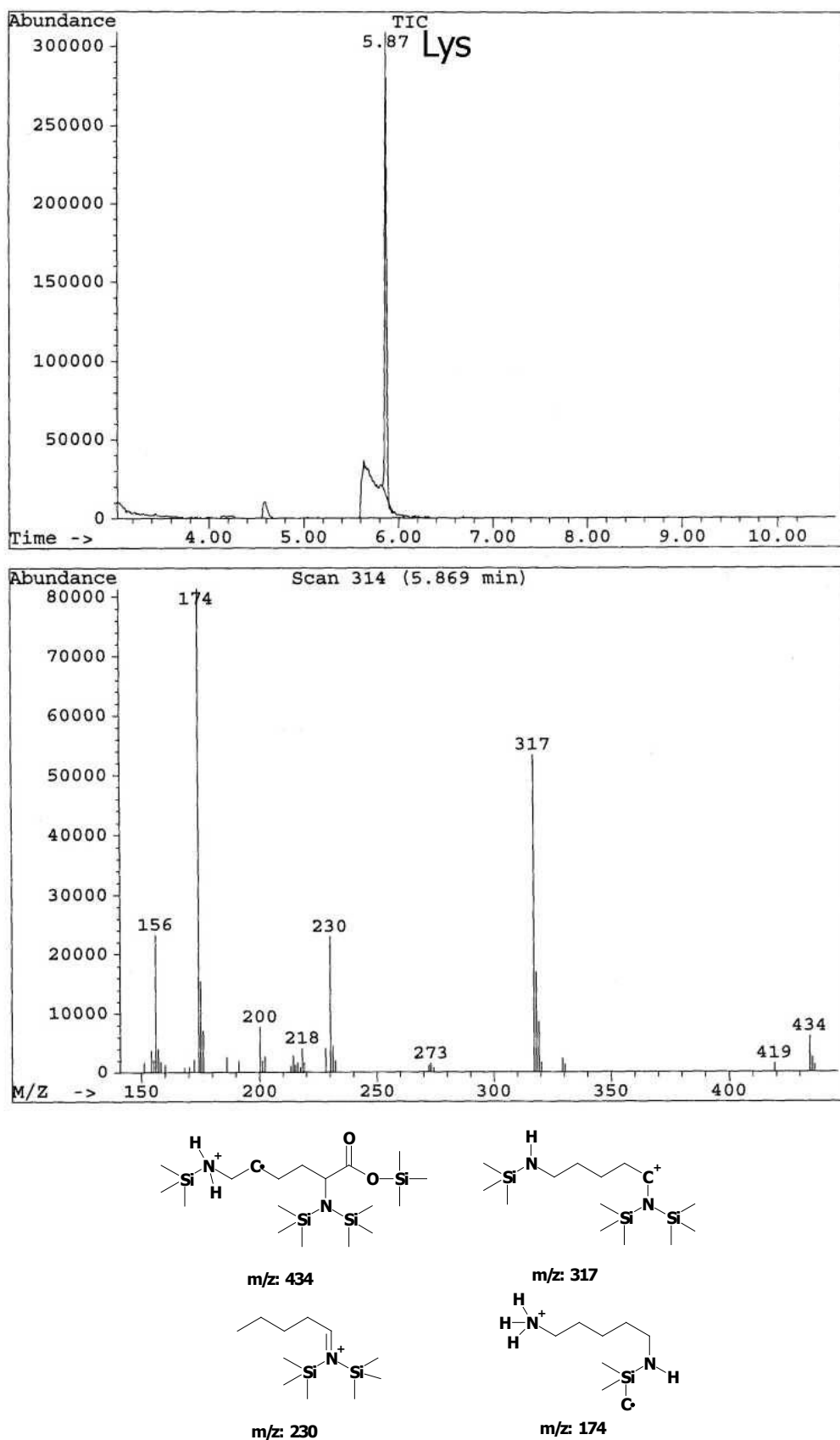
Fig. 33: GC-MS chromatogram of Lys 500 µM (derivatised with BSTFA + 1% TMCS, in acetonitrile) and the resulting fragments. Below possible structures of the major fragments.
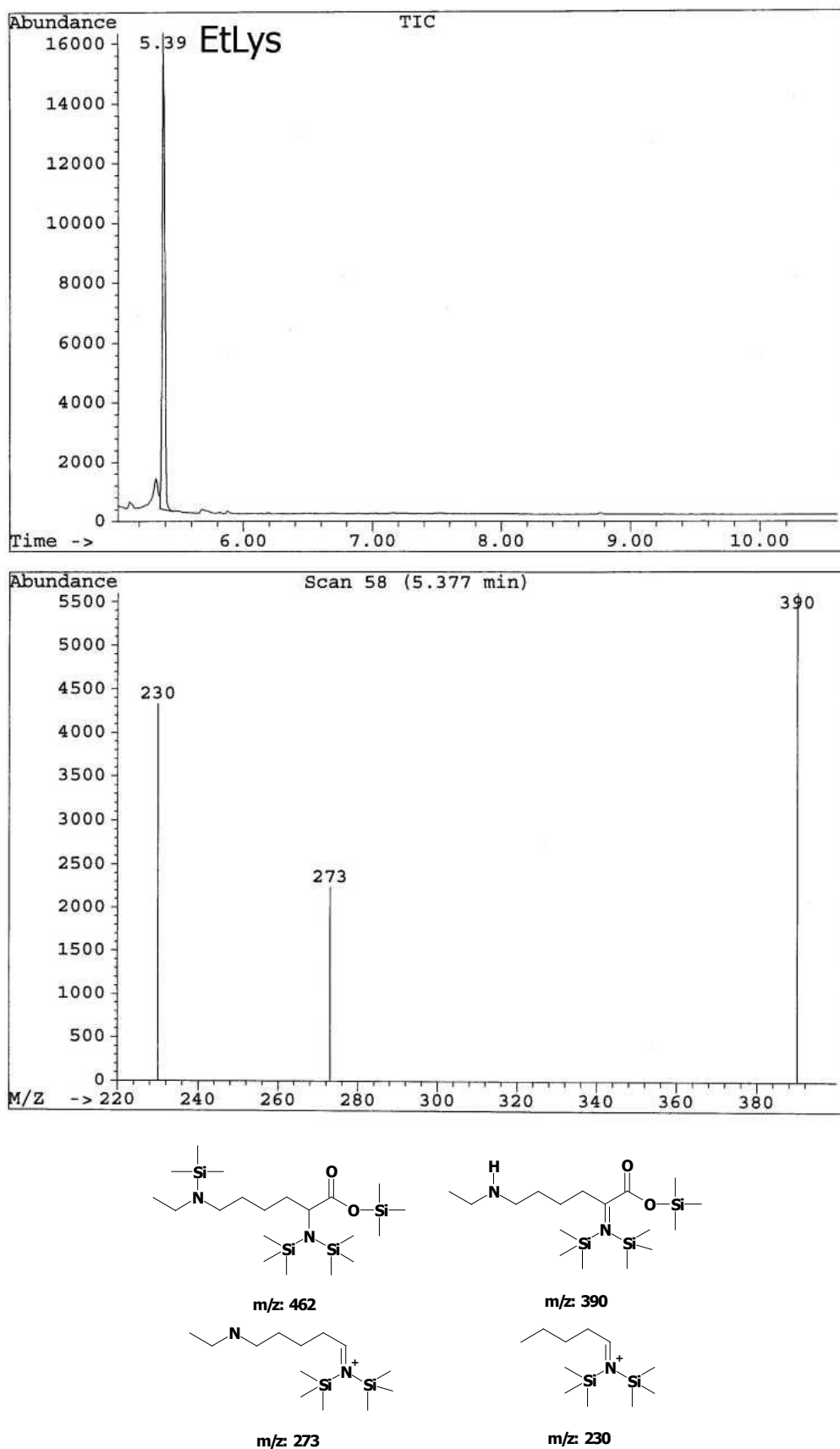
Fig. 34: GC-MS chromatogram of EtLys 500 μM (derivatised with BSTFA + 1% TMCS, in acetonitrile) and the resulting fragments. Below possible structures of the major fragments.

LC-MS

Analysis of EtLys using LC-MS was the method we explored next. The expected advantages were that no derivatisation was needed and that a high specificity would be achieved.

Method

For LC-MS analysis aqueous solutions of standards were directly used for method-development. The equipment used for this analysis was a HP 1100 HPLC System with a G1322A Degaser, a G1311A QuatPump, a G1329A ALS and a G1316A ColComp. For the separation of the compounds a MN ET 250/4 Nucleosil® 100-5 SA column was used.

Substances were eluted with aqueous sodium hydroxide 0,001% (pH 7,4) and acetonitrile (1:1) with a flow rate of 0.4 mL/min and a runtime of 10 min. Detection: Finnigan LCQ Ion Trap Mass-Spectrometer with electrospray-ionisation (ESI). Spray voltage [kV]: 3.54; spray current [µA]: 15.82; sheath gas flow rate [psi]: 60; aux gas flow rate [psi]: 0; capillary voltage [V]: 17.42; capillary temp [°C]: 250. More details regarding ion optics and vacuum pump may be found in table 3.

Table 3: LCQ Settings for Analysis of ethyl-lysine

```
ESI Source
  |Spray Voltage (kV)|:                    3.54
  Spray Current (µA):                     15.82
  Sheath Gas Flow Rate:                   59.42
  Aux Gas Flow Rate:                      -0.22
  Capillary RTD OK:                         Yes
  Capillary Voltage (V):                  17.42
  Capillary Temp (°C):                   250.10
  8 kV supply at limit:                      No

Vacuum
  Vacuum OK:                                Yes
  Ion Gauge Pressure OK:                    Yes
  Ion Gauge:                                 On
  Ion Gauge (x10e-5 Torr):                 1.02
  Convectron pressure OK:                   Yes
  Convectron Gauge (Torr):                 0.80

Turbo Pump
  Status:                               Running
  Life (hours):                           29021
  Speed (rpm):                            59000
  Power (Watts):                             41
  Temperature (°C):                       51.00

Ion Optics
  Multipole Frequency On:                   Yes
  Multipole 1 Offset (V):                 -1.91
  Lens Voltage (V):                      -23.61
  Multipole 2 Offset (V):                 -6.08
  Trap DC Offset (V):                     -9.96
  Analyzer Temperature (°C):              29.25

Main RF
  Reference Sine Wave OK:                   Yes
  Standing Wave Ratio OK:                   Yes
  Main RF DAC (steps):                    31.00
  Main RF Detected (V):                   -0.00
  RF Detector Temp (°C):                  41.80
  Main RF Modulation (V):                  0.04
  Main RF Amplifier (Vp/p):                8.97
  RF Generator Temp (°C):                 34.42

Ion Detection System
  Conversion Dynode:                         On
  Multiplier Voltage (V):               -815.69

Power Supplies
  +5V Supply Voltage (V):                  5.07
  -15V Supply Voltage (V):               -14.96
  +15V Supply Voltage (V):                14.91
  +24V Supply Voltage (V):                23.89
  -28V Supply Voltage (V):               -28.07
  +28V Supply Voltage (V):                28.31
  +28V Supply Current (Amps):              0.98
  +35V Supply Voltage (V):                31.80
  +36V Supply Voltage (V):                35.85
  -150V Supply Voltage (V):             -151.59
  +150V Supply Voltage (V):              150.86
  -205V Supply Voltage (V):             -202.14
  +205V Supply Voltage (V):              207.93
  Ambient Temp (°C):                      33.20

Instrument Status
  Instrument:                                On
  Analysis:                                Idle
  Retention Time (min):                    0.00

Analog Inputs
  External channel 1 (V):                 -0.00
  External channel 2 (V):                 -0.00
  External channel 3 (V):                 -0.00
  External channel 4 (V):                 -0.00

Syringe Pump
  Status:                               Running
  Flow Rate (µl/min):                     30.00
  Infused Volume (µl):                   182.00
  Syringe Diameter (mm):                   2.30

Digital Inputs
  READY IN is active:                        No
  START IN is active:                        No
  Divert/Inject valve:                   Inject
```

Results

EtLys has a molecular mass of 174.2 and a m/z ratio of 175.2 in the expected ionised state. Figure 35 shows the corresponding ion tracing of EtLys and figure 36 the fragmentation pattern of the peak at 4.55 min. The m/z ratios of 175.2, 168.8 and 158.2 are likely to represent the molecular ion, a possible loss of seven hydrogen atoms and a loss of an amino- or hydroxyl-group, respectively. The m/z ratios of 176.2 and 176.8 are suspected to result from the natural abundance of stable isotopes of C, H, N and O (^{13}C, ^{2}H, ^{15}N, (^{17}O), ^{18}O).
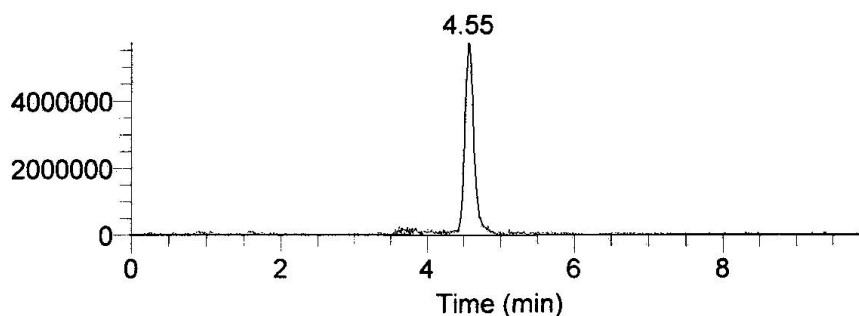
Fig. 35: Mass trace of m/z 175.2, the aqueous standard solution of EtLys, from the total ion current
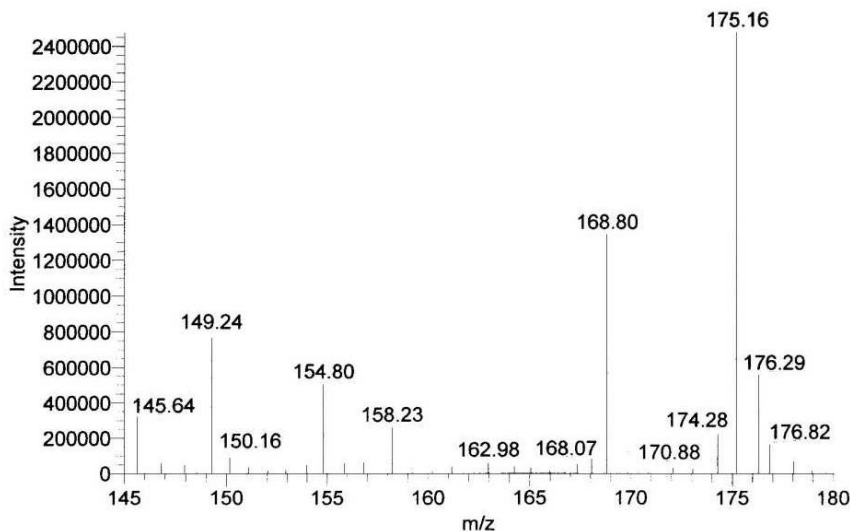
Fig. 36: fragmentation pattern of EtLys at 4.55 min (cf. figure 35)

When standard mixtures of amino acids were analysed using the described chromatographic method it turned out that arginine (Arg), another dibasic amino acid, had the same retention time. It also had the identical mass and fragmentation pattern (figures 37 and 38).
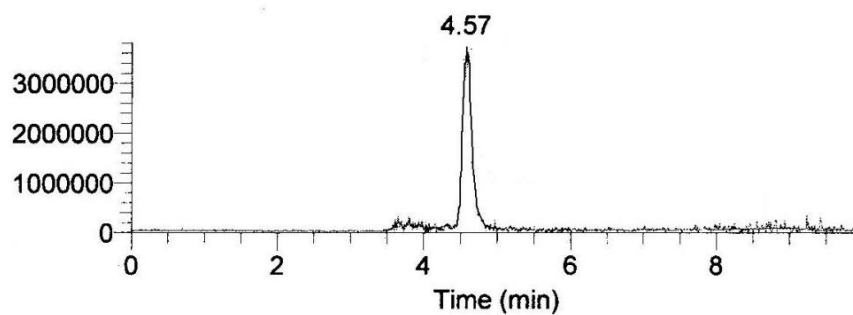
Fig. 37: Mass trace of m/z 175.2, the aqueous standard solution of Arg, from the total ion current
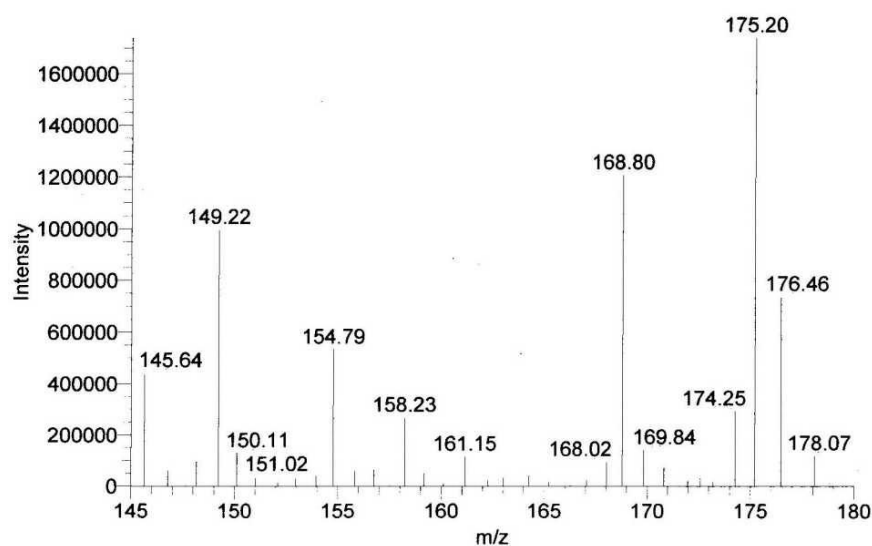
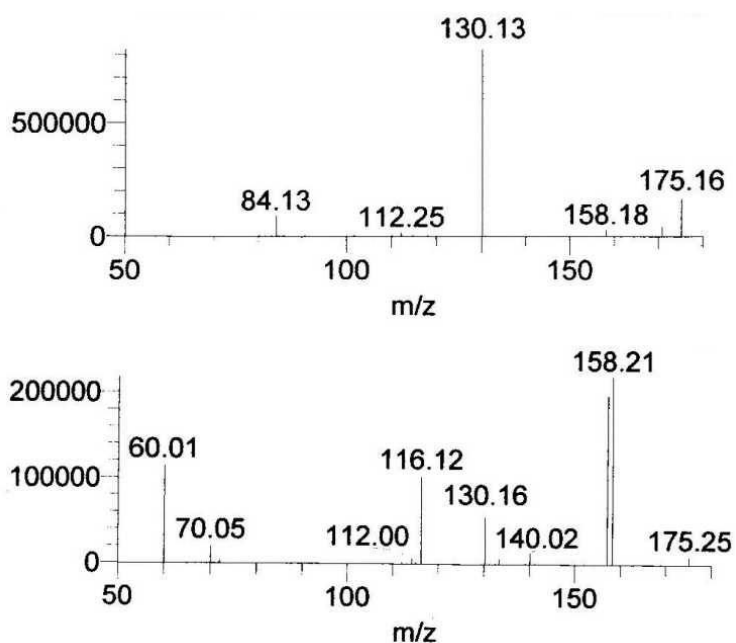Fig. 38: fragmentation pattern of Arg at 4.57 min. (cf. figure 37)

Fig. 39: MS^2-mass-spectra of EtLys and Arg at 4.55 and 4.57 min. respectively (cf. figures 35 and 37).

The fragments of the MS^2-spectra of the two amino acids were qualitatively similar, but the relative abundance of selected masses was different (figure 39). The molecular ions (175.2) are present. There is probably a NH$_2$-group that is more easily removed in Arg than in EtLys resulting in a more abundant m/z 158.2 fragment (figure 40). The loss of the N-ethyl-group (including N) results in the leading fragment with m/z 130.1 from EtLys (figure 41). There is nonetheless also a fragment with m/z 158 in EtLys and a fragment with m/z 130 in Arg. Consequently, the percentage of EtLys cannot be calculated from the MS^2-spectra of a mixture of Arg and EtLys (as it is present in plasma samples).
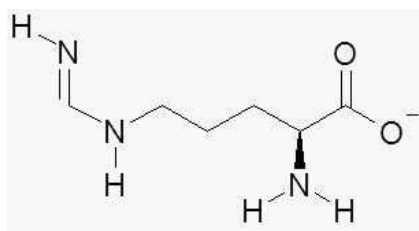
Fig. 40: possible fragment with a m/z ratio of 158.2 from Arg
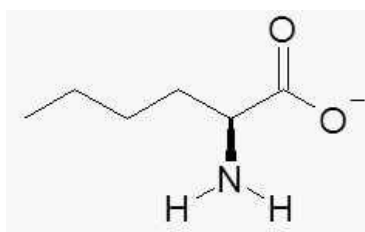
Fig. 41: possible fragment with a m/z ratio of 130.1 from EtLys

Attempts to separate the two amino acids by varying the conditions of the chromatographic analysis were unsuccessful due to the limited choice of solvents that can be used in LC/MS.

HPLC

To overcome the limitations of solvents from LC-MS, a HPLC method with uv detection was adapted to our needs. For this purpose amino acids had to be derivatised. We have applied a method with phenyl-isothiocyanate (PITC) to obtain compounds suitable for uv detection at 254 nm.

Method

50 µL of a solution of amino acids were dried under a gentle stream of air at room temperature. The residue was dissolved in 10 µL of coupling buffer A (100 µL of methanol + 100 µL of water + 50 µL of triethylamine), well mixed and air dried. The resulting residue was then dissolved in 10 µL of coupling buffer B (420 µL of methanol + 60 µL of water + 60 µL of triethylamine + 6 µL of PITC) mixed and incubated for 20 min. at room temperature. The solution was then dried on a SpeedVac (Maxi Dry Plus, Kleiner AG, Wohlen, CH) for 45 min at 35–40°C. The residue was dissolved in 100 µL of water and 10 to 20 µL thereof were subsequently analysed by HPLC.

A gradient system with potassium phosphate buffer 50 mM (pH 6.2) and acetonitrile was used to separate the PITC-amino acids. The solvent system of ammonium acetate and acetonitrile mentioned in the literature[132] had to be abandoned due to incompatibilities with the HPLC-pumps.

The following gradient was used (table 4): Solvent A = potassium phosphate buffer 50 mM (pH 6.2), solvent B = acetonitrile; flow rate: 0.5 mL/min. Detection at 254 nm.

Table 4: HPLC gradient used for the separation of PITC derivatised amino acids

Time [min.]	% A	% B
0.00	90	10
25.00	70	30
35.00	60	40
40.00	60	40
40.01	90	10

Results

A chromatogram of a standard solution of 15 amino acids is shown below (figure 42). The compounds of interest, namely Lys and EtLys, were clearly separated. Other dibasic amino acids, in particular Arg, did not interfere with the analysis. When the amino acids Lys and EtLys were injected in the same concentrations the ratio of their peak areas was in the range of 0.97–1.03 and they were detected with higher sensitivity than all the other amino acids at the same concentration (figure 43). We could measure EtLys down to 4 µM (figure 44).
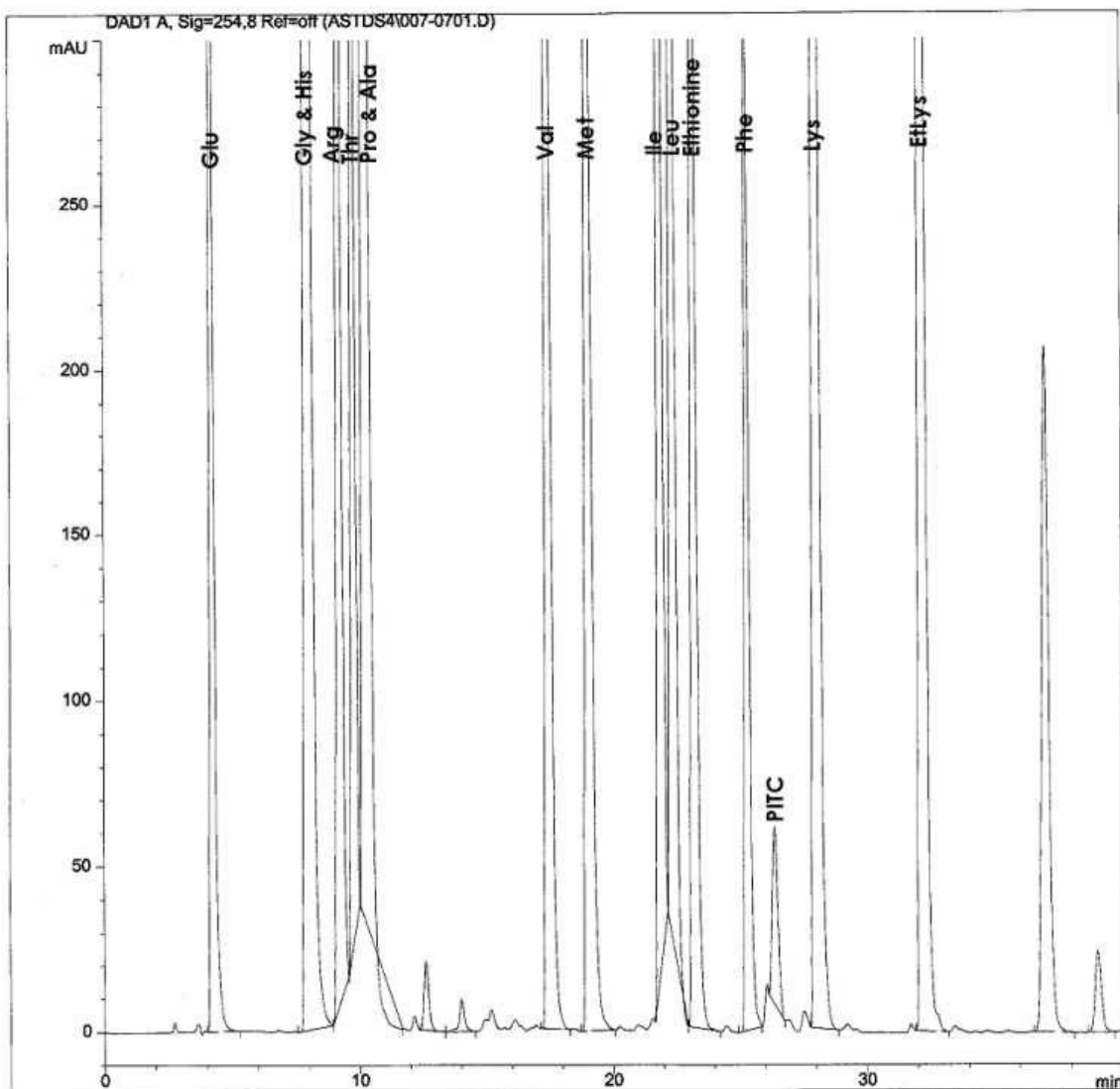
Fig. 42: A mixture of 15 amino acids, including alphabetically (3 letter code where applicable) Ala, Arg, ethionine, EtLys, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Thr and Val, all in a concentration of 500 µM (5 nmol o.c.). This mixture was used as a reference for peak identification and the system's performance and stability.
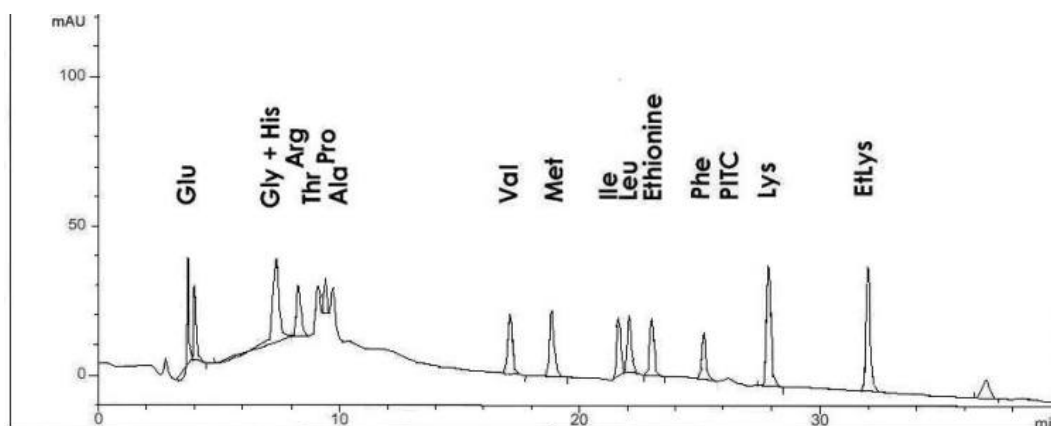

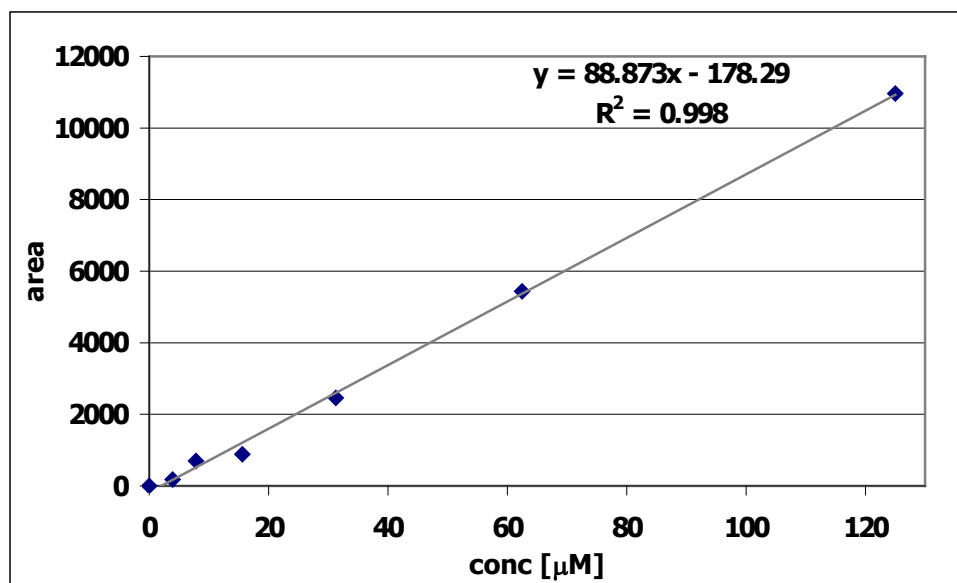**Fig. 43: the mixture from figure 42, but the concentration of the amino acids was only 15 µM (156 pmol o.c.)**

Fig. 44: calibration curve of EtLys by HPLC

Analysis of Ethyl-Lysine in proteins

Method

Blood sample preparation

In order to detect EtLys in circulating proteins 100 µL of plasma, serum or erythrocytes were mixed with 100 µL of phosphate buffer 0.1 M (pH 7.4) and 200 µL of sodium-cyano borohydride 1 mM (in H_2O) and incubated for one hour at room temperature for the reduction of unstable AcHO adducts i.e. Schiff bases. The proteins were precipitated by addition of 100 µL of ice-cold 15% sulfosalicylic acid. After centrifugation the supernatant was discarded. The residue was then hydrolysed for two hours at 150°C after the addition of 1000 µL of 6 M hydrochloric acid. This procedure hydrolyses proteins to its constituent amino acids[98].

After cooling the mixture to room temperature it was dried under a gentle stream of air at 40°C. The residue was dissolved in 1000 µL of water, mixed well and centrifuged in an Eppendorf Centrifuge 5415 C at 14000 rpm for 5 minutes. 50 µL of the supernatant were derivatised for amino acid analysis as described above.

Generation of ethyl-lysine in proteins

Human albumin (4, 6 and 40 mg/mL) was incubated with AcHO (10 µM up to 5 mM) for up to 10 days at 37°C. Identical incubations were done with α-BOC-Lys (0.2, 4 and 6 mg/mL) instead of albumin. After incubation the α-BOC-group can be removed by acidic hydrolysis releasing ϵ-EtLys and unreacted Lys.

In order to find the optimal incubation time 475 µL of α-BOC-Lys (4 mg/mL) or albumin (40 mg/mL) were mixed with 475 µL of 2 mM AcHO and 50 µL of water or 50 µL of 10 mM NaCNBH$_3$.

In order to find out whether rising concentrations of AcHO would result in a linear rise of EtLys, 475 µL of albumin (40 mg/mL) were incubated with 475 µL of AcHO 0, 10, 20, 50, 100, 200, 500 µM or 1, 2, 5 mM. Again, 50 µL of 10 µM NaCNBH$_3$ was added.

Results

Generation of ethyl-lysine in the presence of acetaldehyde

Albumin and α-BOC-Lys were incubated over a period of 10 days with AcHO at a final concentration of 1 mM (figures 45 - 47). Half of the samples were incubated with NaCNBH$_3$ under reducing conditions, and half of the samples were incubated without reducing agent but were reduced with NaCNBH$_3$ after the incubation with AcHO for 1 h at room-temperature. Aliquots were withdrawn every second day to determine the ratio of EtLys to Lys and thus the optimal incubation time.

Samples that were not reduced during the incubation did not yield any stable EtLys, even after reduction for 1 h at the end of the incubation period. Samples incubated in the presence of NaCNBH$_3$ that were further reduced after the incubation did not show a further increase in EtLys formation.

When incubating α-BOC-Lys there was no plateau yet after 10 days. Incubation of albumin did not yield the same amounts of EtLys. This might be due to the fact, that α-BOC-Lys was free in solution, whereas the Lys residues of albumin are bound in the protein and therefore less accessible to AcHO (steric hindrance).
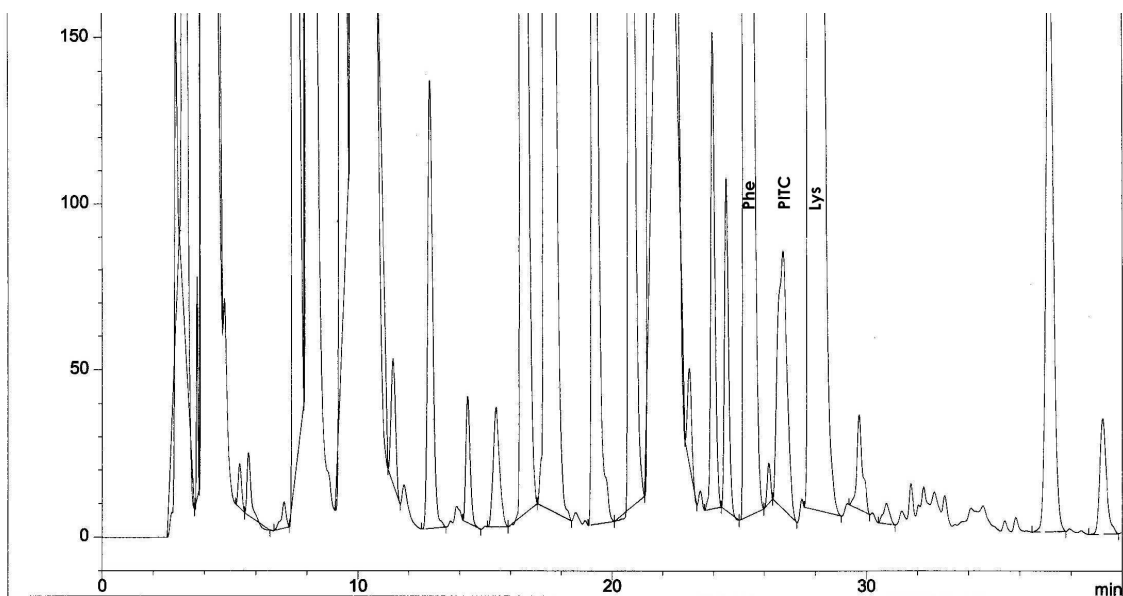
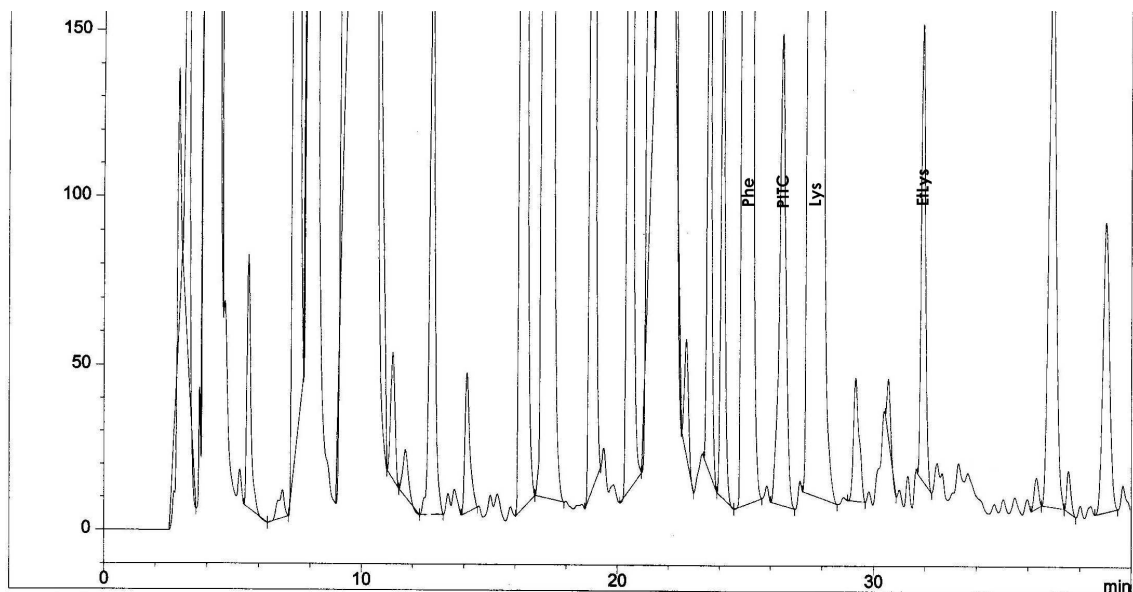**Fig. 45: Sample chromatogram of hydrolysed and reduced albumin blank**

Fig. 46: Albumin after incubation with 1 mM AcHO (under reducing conditions) for 10 days and subsequent hydrolysis and derivatisation.
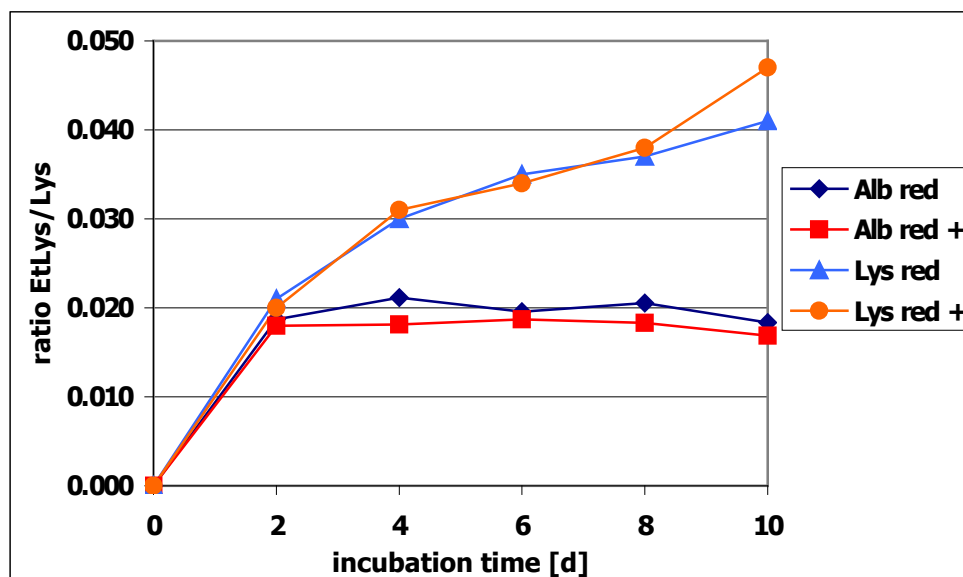
Fig. 47: Generation of EtLys from α-BOC-Lys and albumin when incubated with 1 mM AcHO. Alb red = albumin, reduced during incubation time only; Alb red + = albumin, reduced during incubation and after incubation; Lys red = α-BOC-Lys, reduced during incubation time only; Lys red + = α-BOC-Lys, reduced during incubation and after incubation.

In a second series of experiments the incubation time was kept constant and the concentration of AcHO was varied from 10 µM to 5 mM. The lowest AcHO concentrations did not result in any measurable EtLys and the ratio of EtLys and Lys was, therefore, not different from the blank (where no AcHO was added). Concentrations greater than 50 µM resulted in a concentration-dependent increase in the EtLys:Lys ratio up to a concentration of AcHO of 5 mM (figure 48).
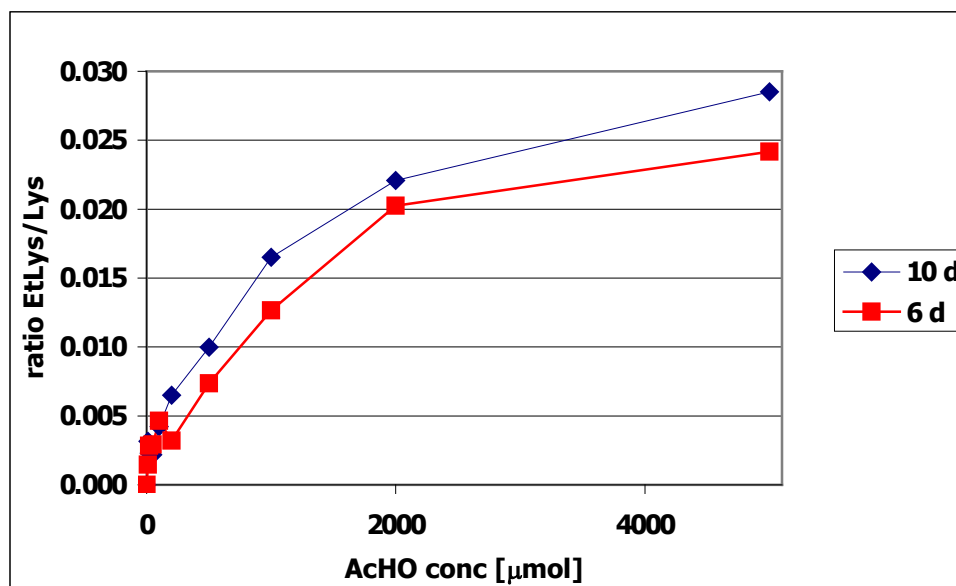
Fig. 48: Generation of EtLys from albumin incubated with different concentrations of AcHO. Samples were incubated for 6 or 10 days.

The amount of Lys that was ethylated by AcHO yielding EtLys was only a few percents as it is reflected by their ratio.

Plasma AcHO levels in vivo (Caucasians) only reach levels of about 50 µM[38,120]. After the consumption of 1 g of alcohol per kg of body weight within two hours AcHO levels were still below detection limit, whereas an oriental subject who consumed only 0.1 g/kg of alcohol had AcHO levels of 15 µM[84]. The intracellular AcHO concentration is likely to be higher because that is the compartment where the metabolism of EtOH takes place. Most of the generated AcHO is readily further metabolised to acetate by the aldehyde-dehydrogenase. Since AcHO is a highly reactive molecule it will react with intracellular structures like proteins and DNA. All proteins that are synthesised in alcohol metabolising cells – mainly hepatocytes – may be a target of free AcHO thus preventing it to reach the plasma.

Ethyl-lysine in ethanol-fed rats

Animals

Male alcohol naïve rats (Wistar) of about 200 g body weight at start were used. After an initial blood sample had been obtained from the retro orbital plexus in slight anaesthesia as base line, the animals were fed an alcohol diet (see table 5 for details) for 4 weeks. After 4 weeks on the alcohol diet the rats were maintained on a regular, alcohol-free diet.

Further blood samples were taken every week for 6 weeks to determine the concentrations of blood alcohol and plasma EtLys. The protocol was approved by the local animal welfare committee.

Table 5: composition of the alcohol-containing liquid diet for rats

Resource Meritene®	87 g
Thistle oil	5 g
Plain milk	400 ml
Supradyn® effervescent	4.7 g
Ethanol	63.3 ml

Determination of Blood Alcohol Concentration

Blood alcohol concentrations of the rats were measured after the third week on the alcohol diet.

A photometric assay, based on the measurement of NADH, which had been modified from a method for an automated analysis, was used. 320 µL of water was mixed with 500 µL of buffer pH 9.0 (300 mM 1,3-diamino-2-propanol (Sigma-Aldrich, St. Louis MO, USA) adjusted with hydrochloric acid) and 10 µL of sample were added. The reaction was started by addition of 150 µL of a mixture of 36 mM NAD (free acid, crystallized; Boehringer Mannheim GmbH, D) and 2000 µcat/L ADH (from yeast, lyophilised; Boehringer Mannheim GmbH, D) in 50 mM sodium citrate buffer. The reaction was followed on the spectrophotometer by measuring the samples every 10 seconds for 5 min. at 378 nm. Noise at 409 nm was subtracted for better performance.

Results

Each rat consumed approximately 1.85 g of ethanol per 100 g body-weight per day (figure 49). One of the five rats died after one week on the alcohol diet. During the experiment the animals put on weight after a slight decrease at the beginning of the alcohol feeding period (figure 50). The four rats had blood alcohol levels in a range of 3.5 up to 30.0 mM in the early morning (figure 51). The large interindividual difference is most likely due to the fact that they were consuming their diet at different time points during the night. Since they all gained weight during the preceding three weeks the animals most likely consumed approximately the same quantity of alcohol. Concentrations of around 14 mM are found in humans 30 minutes after consumption of 0.5 g ethanol per

kg body weight. At baseline and also after six weeks no EtLys was found in any sample. Already after one week EtLys was detectable in circulating proteins. The ratio remained elevated for two more weeks, before it started to drop during the last week of the alcohol diet. Upon removal of EtOH from the diet the EtLys disappeared within two weeks (figure 52).
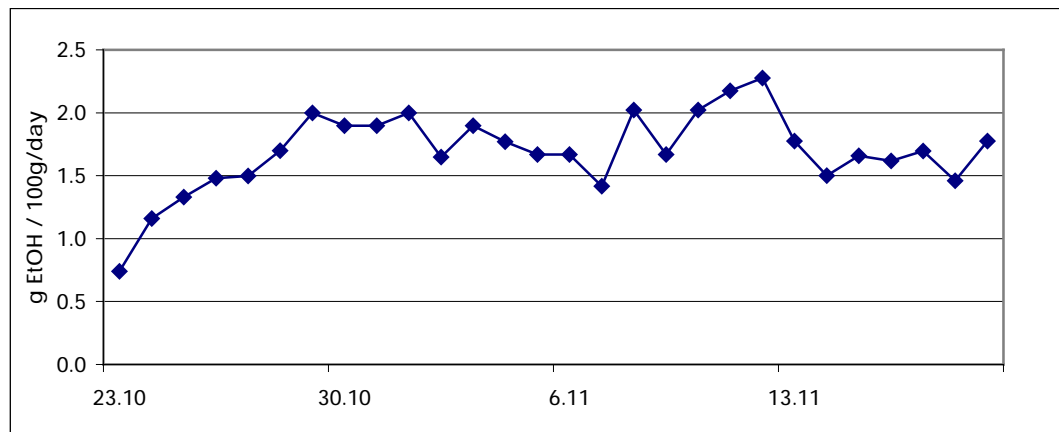

Fig. 49: Mean daily alcohol consumption per animal plotted as grams of ethanol per 100 g of body weight. This was calculated from the amount of food prepared and from what remained the following day.
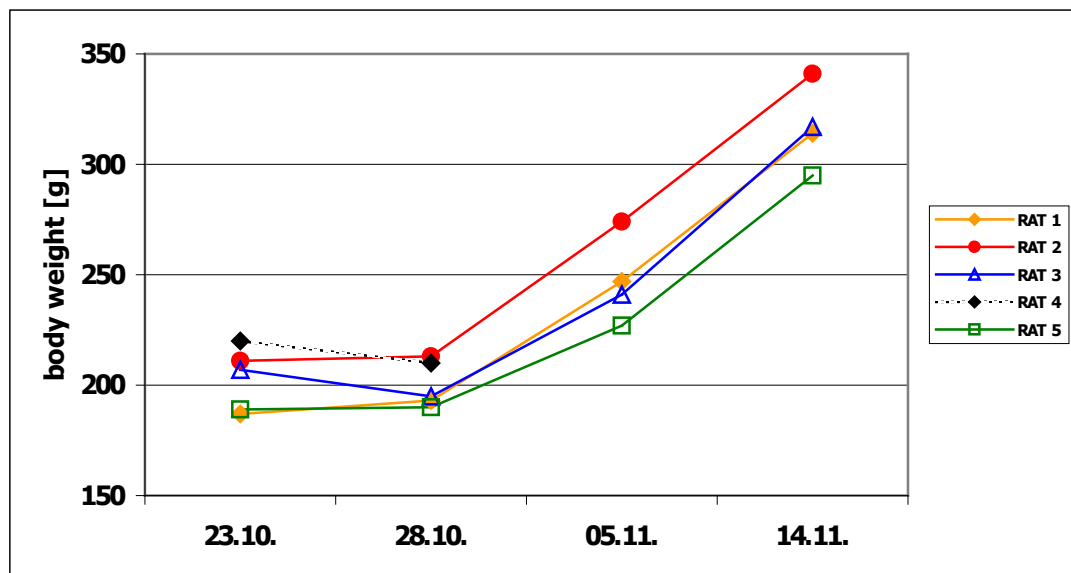

Fig. 50: Weight development of the animals during the alcohol diet period; animal no 4 died after the first week on the diet
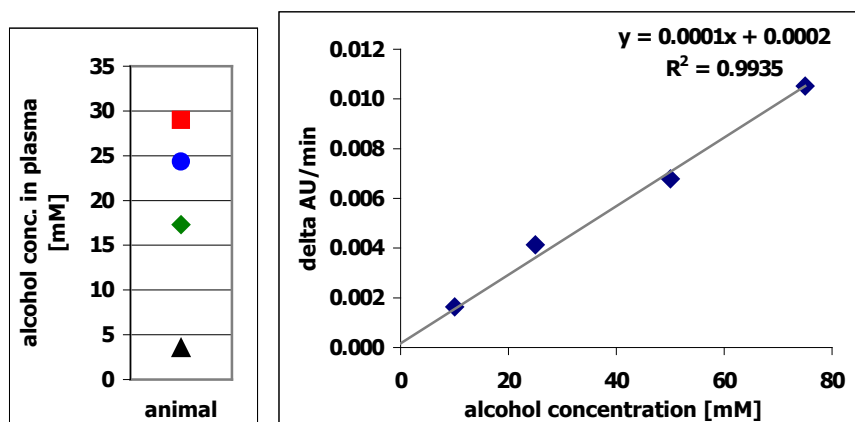
Fig. 51: Concentration of ethanol [mM] in rat plasma early in the morning (after 3rd week on alcohol diet) and the calibration curve for the measurement of ethanol.
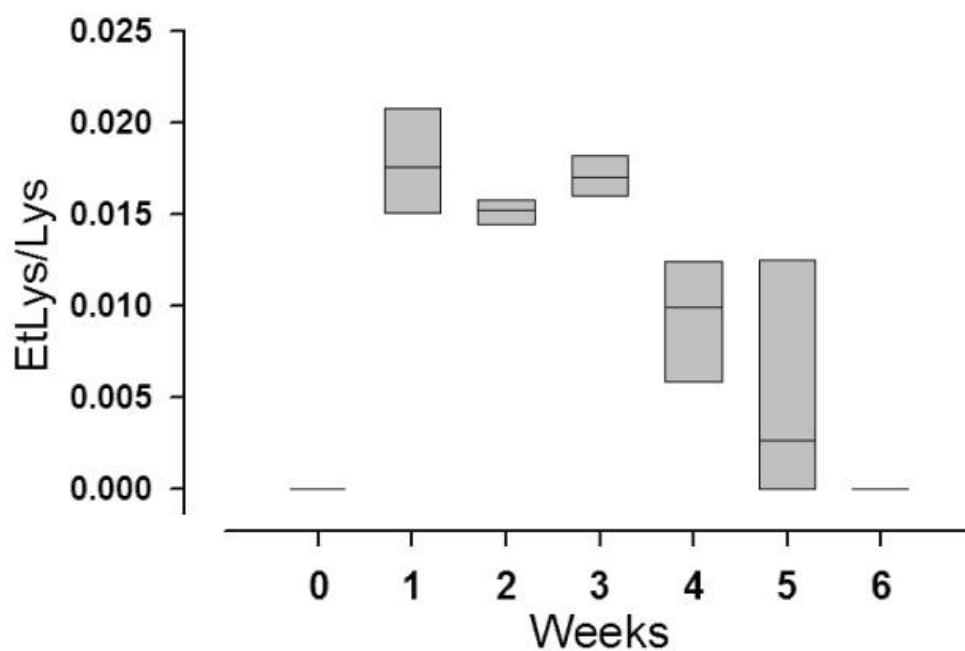

Fig. 52: plot of the ratio of EtLys to Lys in the plasma proteins of rats, shown as box plots with median and range, n=5 for the weeks 0 and 1, n=4 for the rest of the study period.

Ethyl-lysine in Humans

Method

Blood samples from 6 patients, known as alcoholics and under treatment for alcoholic liver disease, were received from our hepatological outpatient clinic. The patients were asked during a follow-up visit to donate an additional blood sample (besides the samples needed for the check-up) for the purpose of this analysis. From all patients heparinised blood samples were obtained. These were centrifuged immediately and plasma was removed. The volume of plasma removed was substituted with sterile isotonic sodium chloride solution 0.9‰. After mixing this "recombinant blood" was used to test for EtLys in erythrocytes following the procedure described above.

As a control blood samples of one subject were analysed after a three week period during which an average of 50 g of alcohol was consumed daily and after a washout period of one month during which no alcohol at all was imbibed.

EtLys was determined in plasma proteins (after their hydrolysis) and in erythrocytes after their haemolysis and precipitation of erythrocyte proteins with sulfosalicylic acid as described for plasma proteins.

Results

There exists in fact a relation between alcohol consumption and the presence of EtLys in the plasma proteins of humans as it was seen in our control subject. After the three week drinking period of an average of 50 g of pure EtOH daily (in form of beer or wine) a well detectable EtLys peak was found in the plasma proteins as shown in figure 53. To make sure the suspected peak was EtLys indeed, the sample was spiked with 50 µM of EtLys and reanalysed (figure 54).
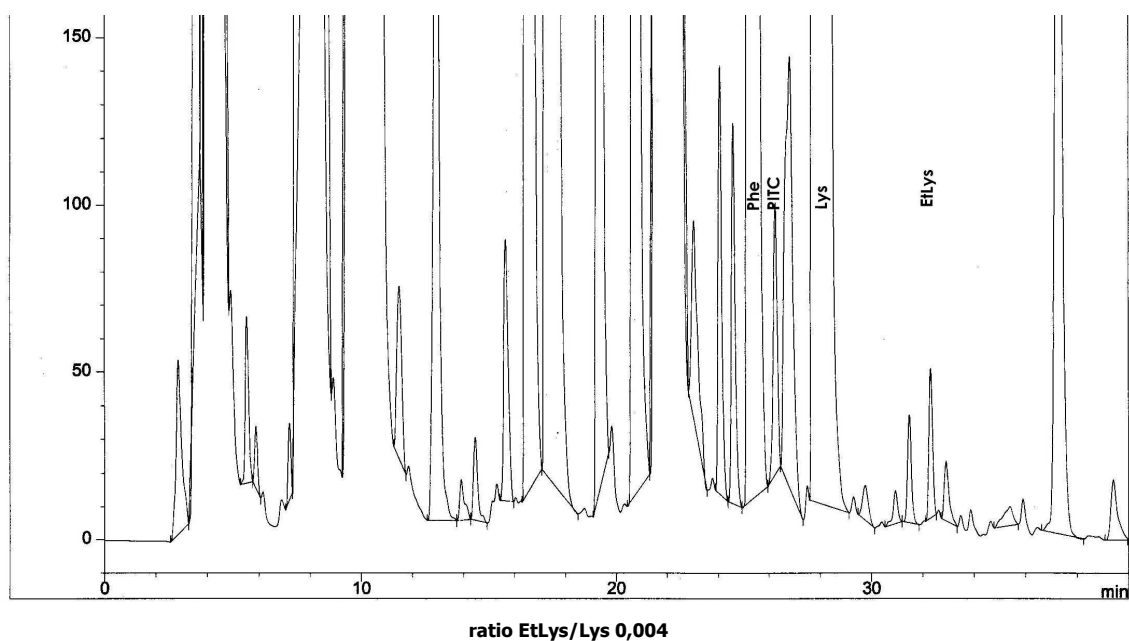
ratio EtLys/Lys 0,004

Fig. 53: Chromatogram of a control subject after 3 weeks of a daily consumption of 50 g of alcohol on average.
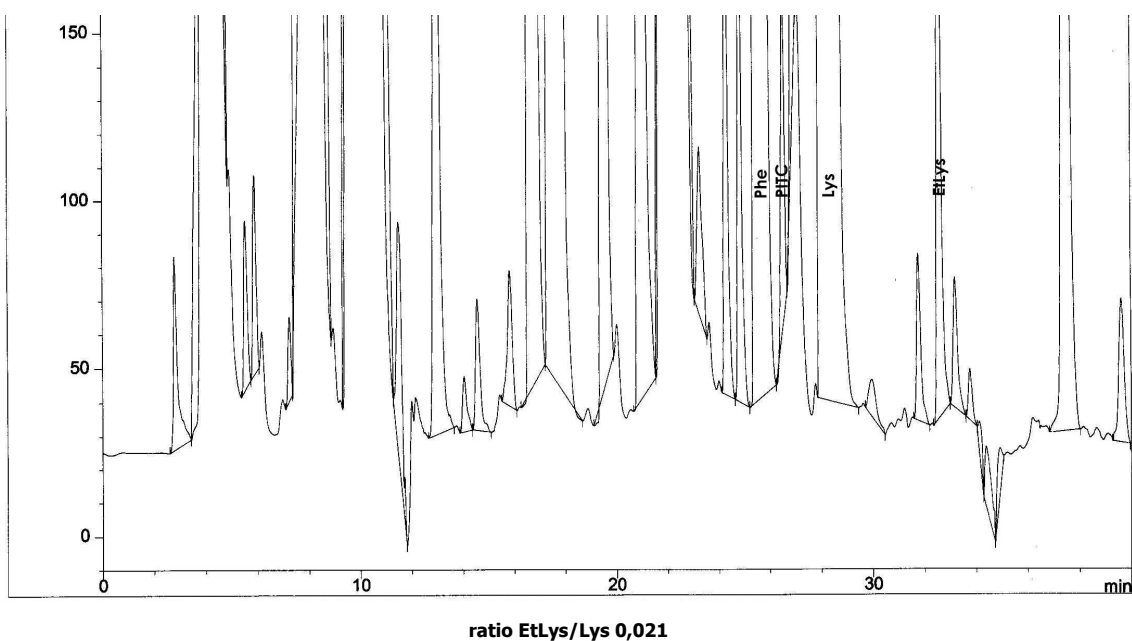

ratio EtLys/Lys 0,021

Fig. 54: The sample from figure 53 spiked with 50 µM EtLys

After the washout period during which the subject did not consume any alcohol at all an other blood sample was analysed. The amount of EtLys was hardly detectable (figure 55) and again this finding was secured by spiking the sample with EtLys (figure 56).
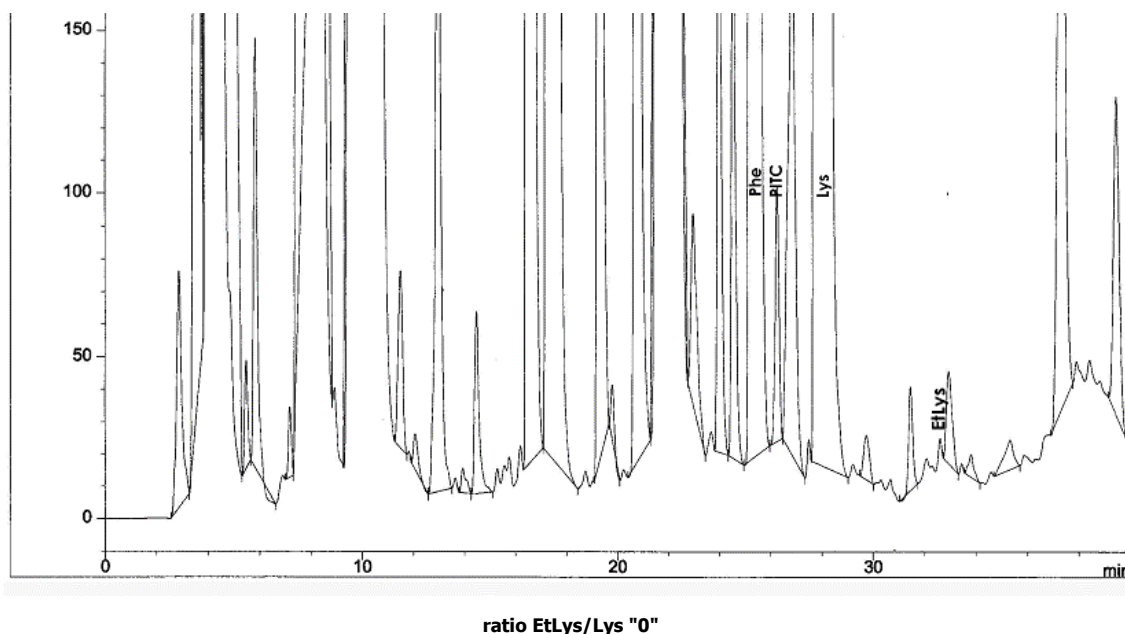
ratio EtLys/Lys "0"

Fig. 55: Chromatogram of a control subject after an abstinence period of one month. An EtLys peak is still visible, but it is below the limit of quantification.

ratio EtLys/Lys 0,019
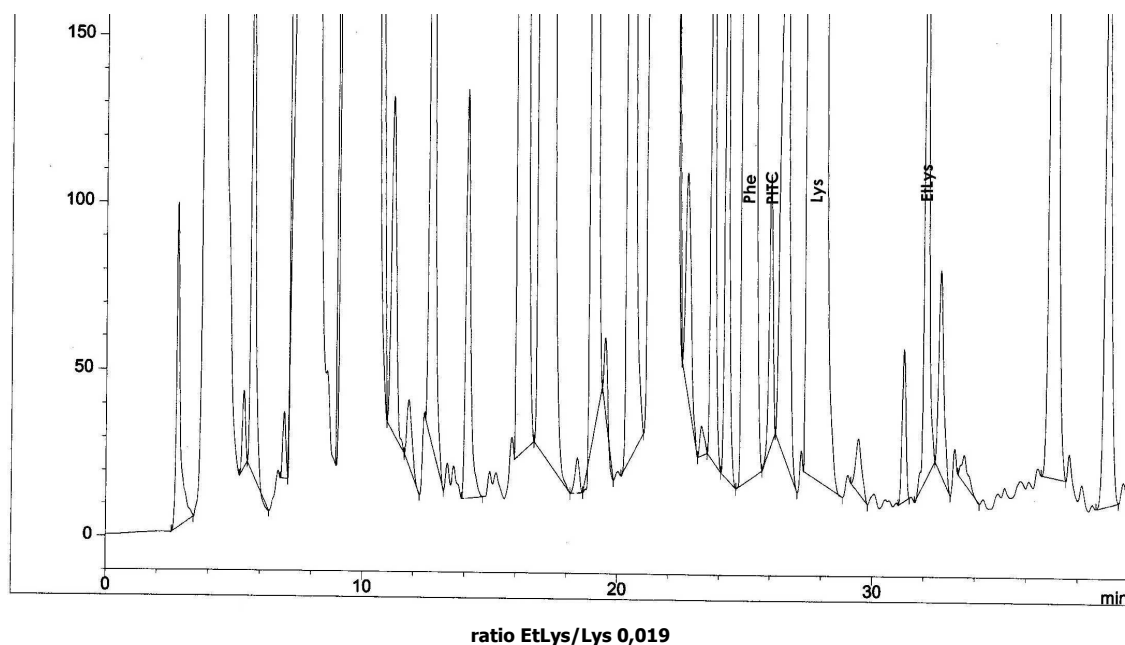
Fig. 56: The chromatogram from figure 55 reanalysed after spiking it with 50 µM EtLys

The measured EtLys/Lys ratios in the samples of the alcoholic patients was in a range of between 0.01% and 0.06% (table 6). As shown in figure 57 alcohol consumption on a daily basis results in an elevated ratio of EtLys and Lys. Except for the sample of MG it seems that there might be a tendency towards higher ratios when the imbibed dose is augmented.

Table 6: Resulting EtLys/Lys ratios of the 6 alcoholic patients and the controls

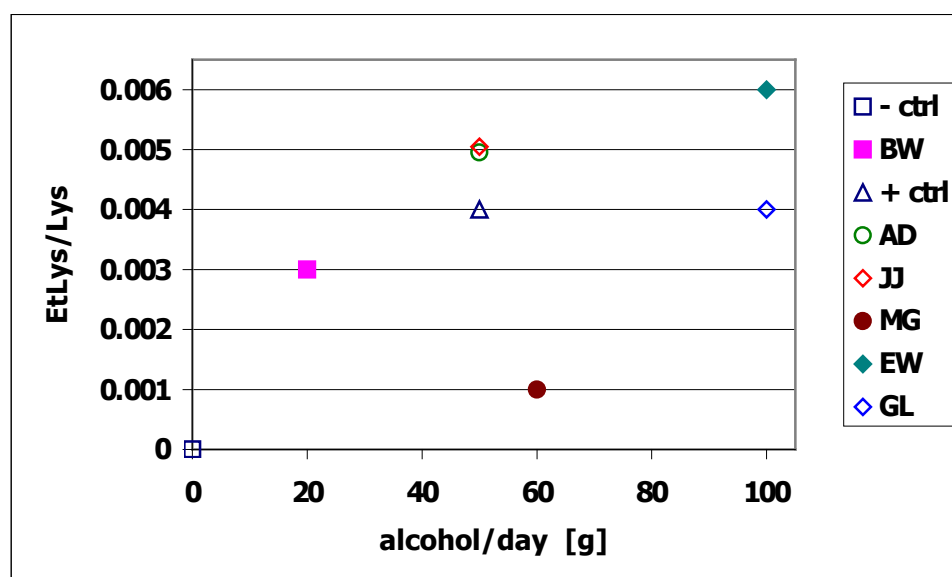| Name	Sex	Alcohol/d [g]	Lys_{area}	$EtLys_{area}$	EtLys/Lys
AD	♂	50	112148	536	0.005
MG	♂	60	107617	149	0.001
JJ	♂	50	110961	521	0.005
GL	♂	100	108211	385	0.004
EW	♀	100	111101	616	0.006
BW	♂	20	109935	343	0.003
+ ctrl	♂	50	110320	440	0.004
- ctrl	♂	0	105754	n/a	0

Fig. 57: The correlation of the amount of alcohol consumed daily and the EtLys/Lys ratio.

No EtLys was found in any of the samples derived from erythrocytes after their lysis and the subsequent hydrolysis of the proteins (figure 58). One reason for this finding could be that only up to 0.2% of the total haemoglobin is modified by AcHO[69]. Besides of that AcHO modifies mainly N-terminal valine residues of the β-chain[103] probably because of their free accessibility. There are less Lys residues in haemoglobin (α-chain 7.8%, β-chain 6.8%) than in albumin (9.9%). Modified Lys residues which only account for a minor fraction of AA in haemoglobin may therefore not reach detectable concentrations.
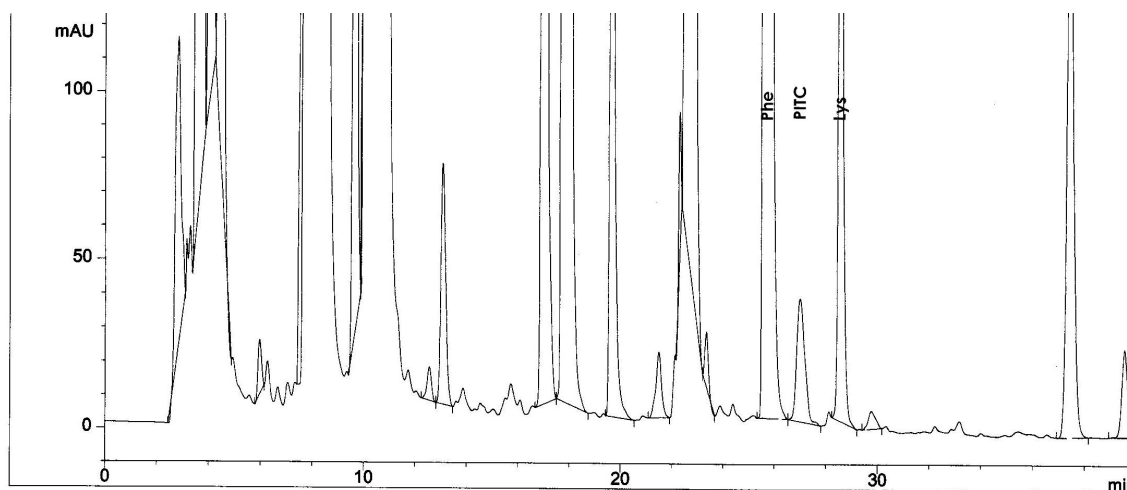
Fig. 58: amino acid pattern derived from lysed erythrocytes and subsequent hydrolysis of the proteins present. No EtLys could be detected (~ 32 min.)

Discussion

The present work demonstrates that the first metabolite of alcohol in the body – AcHO – forms EtLys *in vitro* and *in vivo*. This has been demonstrated by incubation of α-BOC-Lys and albumin with AcHO and by detection of EtLys-species in alcohol fed rats, as well as in subjects consuming alcohol. A direct comparison with results from other investigators who detected AA *in vivo* by immunologic means was not possible because we were the first to determine the ratio of EtLys/Lys as a possible marker of alcohol consumption in plasma proteins. Our *in vitro* results (incubation of albumin with AcHO) generated similar amounts of EtLys as others have found before[46,131] using radioactive AcHO. The AcHO concentrations used for the *in vitro* experiments were much higher than they can be found in plasma. As mentioned above most of the detrimental action of AcHO happens inside of the cells and there is no information so far concerning the intracellular AcHO concentration. Therefore it can only be speculated whether the AcHO concentrations used for the incubation were far from being physiological or not.

The analysis of EtLys proved to be quite a challenge. The broad spectrum of GC methods applied for its detection did not yield results sensitive enough for further analysis in plasma proteins where naturally occurring amino acids are present in much higher concentrations.

The LC-MS approach was unsuccessful because of the basic amino acid Arg which in our method could not be separated chromatographically from EtLys. Unfortunately EtLys and Arg which have identical masses and similar fragmentation patterns co-eluted. Their

separation would probably have needed a special HPLC-column which would have tolerated separations at pH-values around 11. The MS, which was used as the detector, restricted the choice of eluents to organic solvents and water, which in consequence did not allow us to isolate and quantify EtLys with the available LC-system.

The HPLC-method which was finally used was able to solve some of the problems stated above. We were able to isolate EtLys from the other amino acids, including basic amino acids, and we achieved a better sensitivity.

The kinetics of EtLys in proteins *in vivo* remains to be elucidated. In the rats the concentration of EtLys decreased promptly after the feeding of ethanol stopped. There was a tendency for the concentration of EtLys in plasma proteins to decrease even before the cessation of alcohol feeding. This may be a chance occurrence or may represent an adaptive response. Further studies will have to provide an answer to this question.

The samples of the alcoholic subjects and the control subject who drunk under controlled circumstances contained small amounts of EtLys. The ratio of EtLys and Lys amounted to approximately 0.01, indicating that about 1% of all Lys residues of plasma proteins had been altered by AcHO to EtLys. After the control subject quit drinking we could demonstrate that EtLys indeed disappeared.

The chromatographic analysis of EtLys by HPLC is a reproducible method that bears the potential to open up an area for the development of a new marker of (recent) alcohol consumption.

Glutathione consumption and methionine kinetics in humans

Numerous functions of glutathione (GSH), the tripeptide consisting of glycine, glutamic acid and cysteine (Cys), have been established (for reviews see[41,91]). Best known is its function as a nucleophile that reacts with electrophilic metabolites of endo- and xeno- biotics to form glutathione adducts. These are excreted after further metabolism to form the corresponding cysteine adduct and, in many cases, the N-acetylcysteine adduct (or so called mercapturic acid). The conjugation reaction can occur spontaneously but is often catalysed by one of the glutathione S-transferases. These enzymes markedly speed up the conjugation of electrophiles with GSH and thereby prevent the reaction of potentially toxic metabolites with vitally important macromolecules. This is the major mechanism by which GSH protects against the hepatotoxic metabolite of paracetamol: GSH traps the toxic metabolite N-acetyl-p-benzoquinoneimine (NAPQI). When intracellular GSH is depleted, the metabolite binds to critical macromolecules and a process ultimately leading to necrosis and apoptosis is set in motion. Although the synthesis of GSH is stimulated when its concentration decreases, the availability of precursor amino acids, particularly the availability of Cys, is not sufficient in the case of a paracetamol intoxication to sustain intracellular GSH.

GSH not only traps electrophilic metabolites, it is also a versatile metal binding ligand and plays an important role in the transport of metals, their storage and their excretion. GSH is the most abundant intracellular non-protein thiol and as such is critical for the maintenance of the intracellular redox homeostasis[26]. Cells – and particularly their mitochondria – are continuously exposed to reactive oxygen species. The reduction and detoxification of hydrogen peroxide and other peroxides by GSH is catalysed by glutathione peroxidase. The resulting glutathione disulfide (GSSG) is rapidly reduced back to GSH by glutathione reductase with nicotinamide adenine dinucleotide phosphate (NADPH) utilized as the reducing equivalent. By maintaining the proper redox status of the cell and protecting it from reactive oxygen species, GSH plays a central role in the delicate balance between cell death and cell survival. Recent studies have shown that depletion of GSH sensitises cells to pro-apoptotic stimuli and activates programmed cell death[40]. Conversely, heat shock proteins that are induced during stress responses increase the intracellular concentration of GSH and facilitate the inhibition of apoptosis[76].

GSH also modulates gene expression and the activation of transcription factors and thereby plays an important role in organ regeneration[37].

Finally, GSH appears to regulate the activity of some transporters of organic solutes and ion channels. Since many drugs, in particular chemotherapeutic agents used in oncology, are exported by such pumps, GSH depletion could inhibit their excretion and potentially enhance their toxicity[72].

The maintenance of adequate intracellular concentrations of GSH is therefore crucial for cellular function, and the organism must have the capacity to replenish GSH stores when the tripeptide is depleted. Cys is the rate limiting amino acid in the synthesis of GSH. The intracellular pool of free Cys, however, is one order of magnitude smaller than the pool of GSH. Therefore, the Cys required for the synthesis of GSH must be provided by increased uptake of circulating Cys, protein catabolism or from methionine (Met) via the transsulfuration-pathway. In rats, the major source of Cys utilized for the synthesis of GSH following its depletion by a large dose of paracetamol originates from Met[22,118,126]. The source of Cys in a similar situation in humans is not known. The aim of the present study was to estimate the contribution of Met as a source of Cys for GSH synthesis in humans in a situation where the synthesis of GSH is stimulated.

Met is one of nine essential amino acids for humans, i.e. the body is not able to synthesise it. The amino acid is needed for the maintenance of the biochemical equilibrium and therefore has to be provided with food. The free Met pool in the body has a concentration of 20-25 µmol/kg[48]. Met is needed for the synthesis of proteins – every newly synthesised protein starts with a Met – and it is important for methylating reactions in the body (via S-adenosyl-methionine (SAM)). A small fraction of Met may also undergo transamination. The pool of Met is maintained by protein breakdown, by remethylation of homocysteine in the methionine cycle and by dietary intake.

The first step in the methionine cycle is a condensation of Met with adenosine triphosphate (ATP) catalysed by methionine-adenosyl-transferase leading to SAM. This is an important methyl-donating molecule for many reactions. The utilization of SAM for transmethylation reactions yields S-adenosyl-homocysteine (SAH) and, following the loss of the adenosyl-group, homocysteine. The cycle is completed by remethylation of homocysteine to Met (figure 59). This reaction may occur via methionine-synthase that uses the methyl-group of 5-methlytetrahydrofolate or by the demethylation of betaine. The latter reaction occurs only in the liver whereas the former one occurs in all tissues[2].
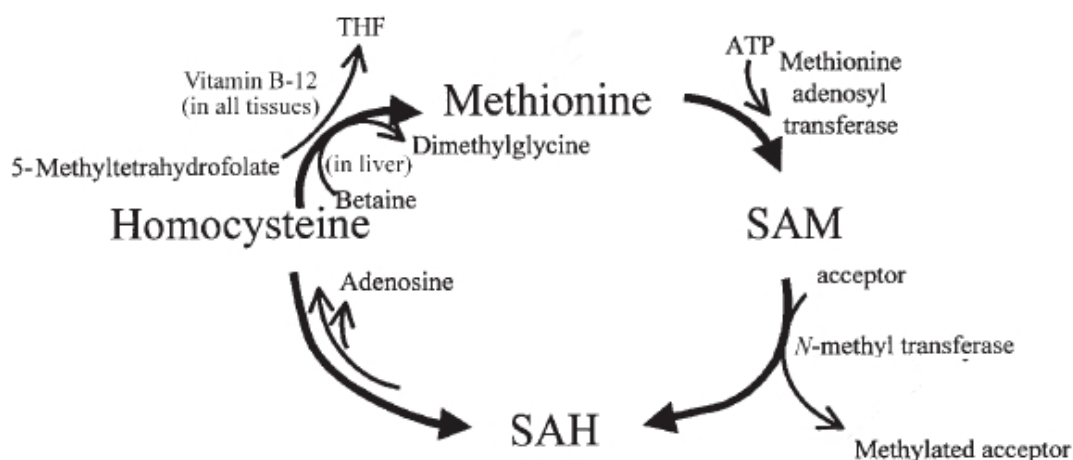
Fig. 59: The methionine cycle. SAH = S-adenosyl-homocysteine, SAM = S-adenosyl-methionine, THF = tetrahydrofolate (from [67])

Approximately 45% of homocysteine originating from the breakdown of SAH are remethylated to yield Met[112]. The remaining 55% enter the transsulfuration-pathway where Cys, the rate limiting amino acid in the synthesis of GSH, is one of the products (figure 60).
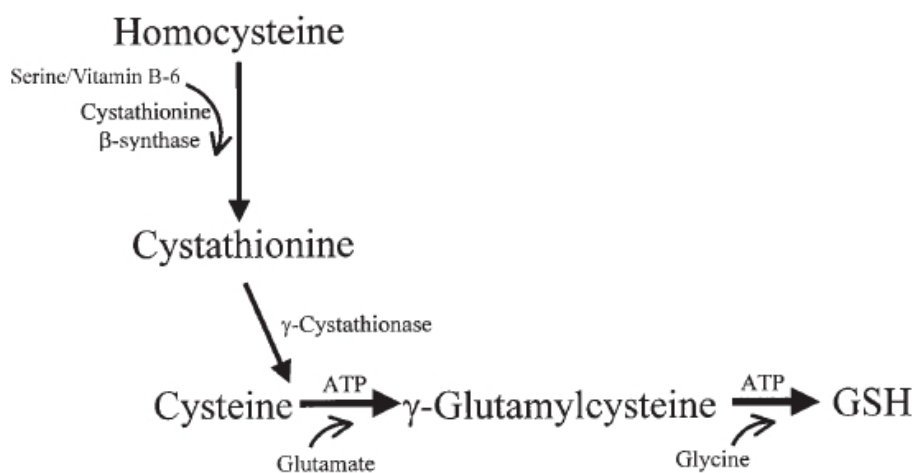
Fig. 60: The transsulfuration pathway of homocysteine. GSH = glutathione (from [67])

The study of GSH metabolism in humans is complex since the tripeptide is mainly intracellular and cells of interest, such as hepatocytes, are not readily accessible. Moreover, humans can not be exposed to any risks by excessive depletion of their GSH stores. One way to safely stimulate GSH turnover and thereby study some of the regulatory mechanisms in humans is the administration of therapeutic doses of paracetamol.

Approximately 5% of a therapeutic dose of paracetamol are normally activated by the cytochrome P450 isoenzyme 2E1 to the toxic metabolite NAPQI which is detoxified by GSH and subsequently excreted as thiol metabolites of paracetamol[25,77]. When large amounts of NAPQI are formed and the available GSH is consumed, the rapid resynthesis of GSH is essential to prevent binding of NAPQI to cellular proteins and thereby affecting their function.

The turn-over of the methionine cycle can be monitored by labelling Met with stable isotopes. In our case the S-methyl-group was ^{13}C-labelled. Labelled Met is incorporated into SAM and the labelled methyl-group is transferred to an acceptor in the course of the methionine cycle. The resulting methylated acceptor is degraded over several steps to ^{13}C enriched CO_2, which was monitored as a surrogate marker for the use of Met when there is an increased demand for homocysteine. For the generation of Cys via the transsulfuration pathway the methionine cycle is stimulated resulting in an increased formation of CO_2.

In order to determine the reaction of the body to an increased consumption of GSH we carried out an open cross-over study. In a first period the methionine metabolism was monitored in each participant after oral administration of 2 mg/kg body weight of L-[S-^{13}C]methionine. Blood and breath samples were collected for 2 and 3 hours respectively after Met ingestion. In a second period 2 g of paracetamol were given orally (Dafalgan Odis®) 30 minutes before the administration of the labelled Met.

Using a similar approach but with double-labelled stable Met isotopes (L-[S-^{2}H$_3$-1-^{13}C]methionine) we have recently shown a reduction of the remethylation in patients with alcoholic liver disease[99] in whom intracellular GSH is often decreased.

Our aim was to investigate the reaction of the methionine cycle under high therapeutic doses of paracetamol by determination of the enrichment of $^{13}CO_2$ in breath and by quantifying the metabolism of Met and of thiols like homocysteine, Cys and GSH in plasma.

Material and Methods

Subjects

Six healthy male volunteers aged between 29 and 59 years (median 32 years) participated in the study which was carried out in the Clinical Investigation Unit (CIU) of the university hospital in Bern. They did not take any medication regularly and none drank

more than 30 g of alcohol per week. All participants were carefully informed of the nature, purpose and possible risks of the study before they gave written consent to participate. The local ethics committee approved the protocol and consent form. In three additional subjects the same protocol was followed but only breath samples were collected.

Study Protocol

The subjects were studied on two occasion one week apart. After fasting overnight, the subjects were admitted to the unit in the early morning, and an intravenous catheter was inserted into a forearm or hand vein. After plasma and breath samples for baseline measurements had been obtained, the subjects ingested 2 mg/kg (13 µmol/kg) of L-[S-^{13}C]methionine (MassTrace, Woburn, MA) dissolved in 30 mL of water. Further blood and breath samples were then obtained at intervals over the following 2 hours while the subjects were at rest.

Blood samples were drawn into precooled 5-mL ethylenediaminetetra-acetic acid (EDTA)-coated containers (S-Monovette, Sarstedt, Nümbrecht, D) and were immediately centrifuged; the separated plasma samples were either kept on ice until further analysis or were immediately derivatised for the determination of free thiols. Breath samples were collected through a straw into glass vials that were immediately sealed airtight. After obtaining the last sample, plasma samples were stored at −20°C and breath samples at room temperature until further analysis.

The study was repeated following the ingestion of 2 grams of paracetamol (Dafalgan Odis®) 30 minutes prior to the administration of the labelled Met.

Analytical Methods

Methionine

Met was isolated from plasma by anion-exchange chromatography. To 500 µL of plasma 20 µL of ice-cold 15% sulfosalicylic acid were added for deproteinisation, and the mixture was centrifuged for 10 minutes at 14000 rpm in an Eppendorf 5415 C centrifuge. To 300 µL of the supernatant 20 µL of ethionine (500 µM in water) which served as internal standard, 1 mL of water and 100 µL of 1 M sodium hydroxide were added. The sample was then applied to an anion-exchange column (AG 1-X8, 100-200 mesh, chloride form; bed size: 2 mL; BioRad, Hercules, CA), and the column was washed with 7 mL of water

and then with 2 mL of methanol. The analytes were eluted with 6 mL of 2 M acetic acid in methanol and dried under a stream of air at 40°C. The sample was taken up in 100 µL of water and an aliquot of 30 µL was dried under air at 40°C, 30 µL of acetonitrile and 10 µL of N-[tert-butyldimethylsilyl]-N-methyltrifluoro-acetamide (MTBSTFA) were added for derivatisation and the vial was kept tightly closed for 30 min. at 80°C.

Gas Chromatography

The *tert*-butyldimethylsilyl (TBDMS) derivatives of Met were measured by gas chromatography-mass spectrometry (GC-MS) with a HP 5890 series II gas chromatograph connected to a HP 5971 mass spectrometer operated in the electron impact mode (Hewlett-Packard, Palo Alto, CA). Gas chromatographic separation was accomplished on a MN Optima 1 12 m x 0.2 mm, 0.35 µm GC-column. The temperature increased from 110°C (2 min.) to 250°C (3 min.) at a rate of 25°C/min. Injector temperature was set at 260°C and detector temperature was 300°C. Helium was used as a carrier gas with a total flow of 0.9 mL/min. One µL of the derivatives was injected for GC-MS. Met eluted at 5.4 min. and ethionine at 5.7 min. The TBDMS-derivatives of unlabeled Met, ^{13}C-methionine and ethionine were monitored in single-ion monitoring mode at m/z 320, m/z 321 and m/z 334, respectively. The monitored masses correspond to the derivates following the loss of a *tert*-butyl group (M -57). The concentrations of endogenous Met was calculated from the ratio of the peak areas of TBDMS-Met and TBDMS-ethionine. A typical calibration curve of this ratio of 'Met' (m/z 320) and 'ethionine' (m/z 334) is shown in figure 61. Concentrations of ^{13}C-Met were calculated from the enrichment and the endogenous Met concentration (figure 62).
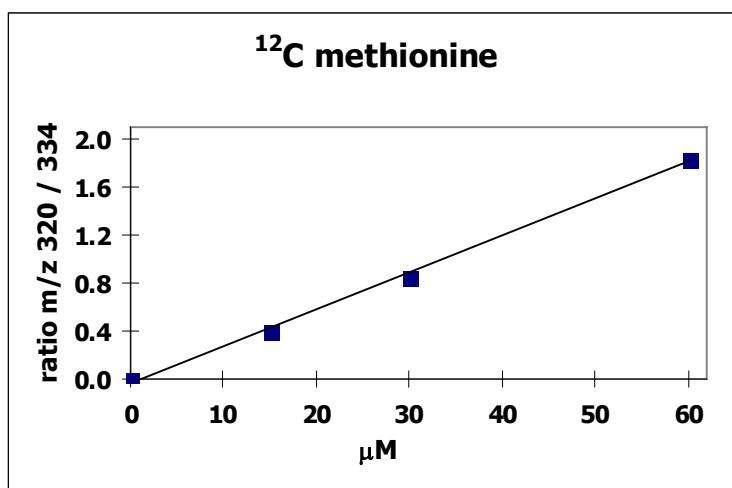

Fig. 61: typical calibration curve for Met based on the ratio of 'Met' (m/z 320) to ethionine (m/z 334)
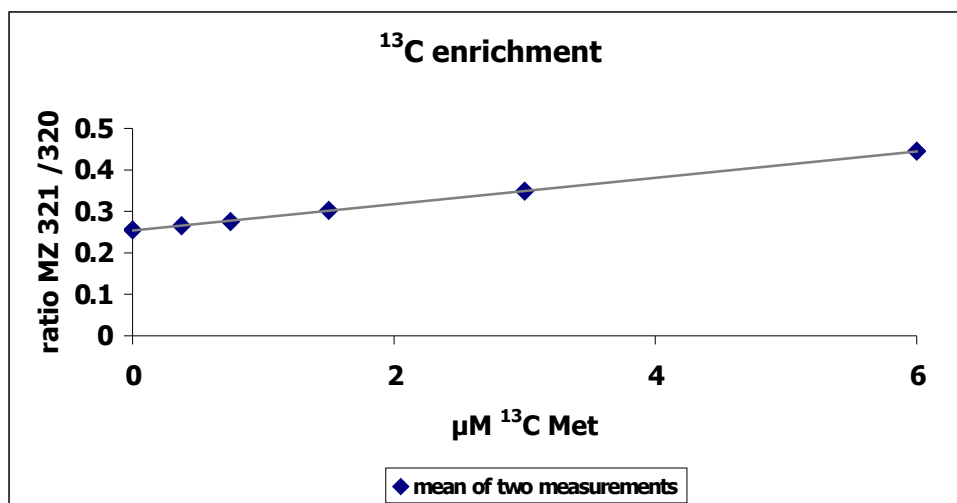
Fig. 62: calibration curve for the enrichment of ^{13}C-methionine (m/z 321) to ^{12}C-methionine (m/z 320) in µM. 30 µM of ^{12}C Met were mixed with increasing amounts of ^{13}C Met.

$^{13}CO_2$ determination

$^{13}CO_2$ in breath samples was measured by isotope ratio mass spectrometry with an Automated Breath ^{13}Carbon Analyzer (Europa Scientific, Cheshire, United Kingdom).

Determination of thiols in plasma

Free thiols

EDTA-coated containers (S-Monovette, Sarstedt, Nümbrecht, D) were prepared by adding 160 µL of a L-serine-borate solution before a blood sample of 4 mL was obtained. The L-serine-borate solution was prepared by dissolving 0.11 g of L-serine and 0.38 g of *di*-sodiumtetraborate decahydrate in 20 mL of 0.5 M phosphate-buffer pH 6.2. L-serine-borate inhibits γGT which could destroy GSH ex vivo. The blood was mixed well and centrifuged at 3000 rpm for exactly 3 minutes. Immediately afterwards 50 µL of the plasma were added to an Eppendorf tube containing 50 µL of Tris-buffer, 20 µL of 10 mM monobromobimane (MBB)-solution and 10 µL of internal standard and mixed well. After 5 minutes of incubation at room-temperature the reaction was stopped by adding 20 µL of perchloric acid 20%. The samples were stored at –80°C until their analysis by HPLC.

The Tris-buffer was prepared by dissolving Tris-HCl (24.23 g/L) and sodium-EDTA (1.871 g/L) and adjusting of the solution to pH 8,5. The MBB was dissolved in acetonitrile. For the internal standard 3.73 mg of penicillinamine were dissolved in 10 mL of water. 20 µL thereof were mixed with 480 µL of Tris-buffer and 20 µL of 10 mM MBB-solution and

incubated for 5 minutes at room-temperature before stopping the reaction by adding 20 µL of perchloric acid 20%.

Two Waters series 510 pumps with a Waters Automated Gradient Controller, a Waters 717plus Autosampler and a Merck/Hitachi F 1000 Fluorescence Spectrophotometer were used for HPLC analysis. For the separation of the MBB derivatives a Macherey Nagel EC 250/4 Nucleosil® 100-7 C_{18} column was used. Eluent A was acetonitrile and eluent B a mixture of 1 g/L octanesulfonic acid and 10 mL/L acetic acid. The gradient used is shown in table 7.

Table 7: HPLC-gradient used for the determination of free thiols

Time [min.]	% A	% B	Curve	Flow [mL/min.]
Initial	9	91	*	1,0
35	15	85	8	1,0
40	30	70	6	1,0
42	50	50	6	1,0
43	9	91	11	1,0

The run-length was 47 minutes but only every 52 minutes 10 µL of a sample were injected for better equilibration of the system prior to the next run. The excitation wave-length was set at 385 nm and the emission was measured at 490 nm.

Total thiols

For the total thiols 100 µL of L-serine-borate (see above) were added to the EDTA-containers (S-Monovette, Sarstedt, Nümbrecht, D). Blood samples were immediately centrifuged at 3000 rpm for 10 minutes and plasma was stored at −20° C until derivatisation.

To 30 µL of plasma 30 µL of freshly prepared 9 mM bis(2-mercaptoethyl)sulfone (BMS; 1.68 mg/mL dissolved in 0.2 M Tris-HCl buffer pH 8.5) were added and incubated at room-temperature for 40 minutes. After the addition of 20 µL of 20 mM MBB-solution the mixture was incubated for a further 5 minutes at room-temperature before stopping the reaction by addition of 50 µL of perchloric acid 20%. These samples were centrifuged for 10 minutes at 14000 rpm at 4°C. These supernatants were stable for 3 weeks at −20°C.

40 µL of the supernatant were mixed with 10 µL of internal standard and 15 µL thereof were injected for analysis by HPLC. For the internal standard 3.73 mg of penicillinamine

were dissolved in 10 mL of water. 20 µL thereof were mixed with 480 µL of Tris-buffer and 20 µL of 20 mM MBB-solution and incubated for 5 minutes at room-temperature before stopping the reaction by adding 20 µL of perchloric acid 20%.

A Waters 600 series HPLC pump was used together with a Waters 600 E system controller, a Waters 717plus Autosampler and a Merck/Hitachi F 1000 Fluorescence Spectrophotometer for HPLC analysis. For the separation of the reduced thiols a MN ET 250/4 Nucleosil® 100-5 C_{18} column was used. A gradient of eluent A (acetonitrile) and eluent B (1 g/L octanesulfonic acid and 10 mL/L acetic acid) was used as shown in table 8.

Table 8: HPLC-gradient used for the determination of total thiols

Time [min.]	% A	% B	Curve	Flow [mL/min.]
Initial	9	91	*	1,0
35	13	87	9	1,0
45	30	70	6	1,0
52	45	55	6	1,0
57	9	91	11	1,0

The run-length for the analysis of total thiols was 65 minutes and samples were injected every 70 minutes. The excitation wave-length was set at 385 nm and the emission was measured at 490 nm.

Paracetamol in plasma

50 µL of plasma were mixed with 30 µL of internal standard, 120 µL of perchloric acid 10% and 100 µL of water. After mixing well the samples were centrifuged for 10 min. at 4°C and 14000 rpm in a Eppendorf 5415 C centrifuge.

The internal standard consisted of 3-aminophenol. 2.5 mg thereof were dissolved in 2.5 mL of perchloric acid 10% and diluted to a final concentration of 200 µg/mL with perchloric acid 10%.

For the HPLC a Waters 600 series pump, a Waters WISP 712 injector, and a Spectroflow 773 detector was used. The separation was carried out on a MN CC 150/4.6 Hypersil 10-3 ODS column. A 50 mM KH_2PO_4 solution containing 5% of methanol was used as eluent. The flow was 0.7 mL/min. The detection wavelength was 242 nm. Each run lasted for 22 min.

Calculations

Pharmacokinetic analysis

The exhaled $^{13}CO_2$ was calculated from the measured enrichment and assuming a constant endogenous CO_2 production of 0.14 mmol/kg·min[115] using standard formula. The area under the plasma concentration time curve of ^{13}C-Met was calculated using the trapezoidal rule. Mean transit time (MTT) was calculated as area under the moment curve, AUMC, divided by AUC of ^{13}C-Met (m/z 321). If the mean appearance time of tracer in the circulation, i.e. the rate of absorption, is constant, MTT provides an estimate of the rate of elimination. Oral clearance was calculated as dose divided by AUC. 'Breath clearance', i.e. the amount of Met metabolised to CO_2 and exhaled in breath relative to its plasma concentration was calculated as the amount of $^{13}CO_2$ appearing in breath divided by the AUC of ^{13}C-Met in plasma.

Statistical Methods

All data are presented as mean $\pm$ standard error (SE) unless stated otherwise. Student's paired t-test was used to assess the statistical significance of differences between the period with and without paracetamol. A p value <0.05 was considered statistically significant. Statistical analyses were performed with the aid of the software program Sigma Stat (Jandel Scientific Co., San Rafael, CA, USA).

Results

The concentration of endogenous i.e. unlabelled Met in plasma during the two study periods is shown in figure 63. The plasma concentrations did not vary significantly following the administration of labelled Met with and without paracetamol.
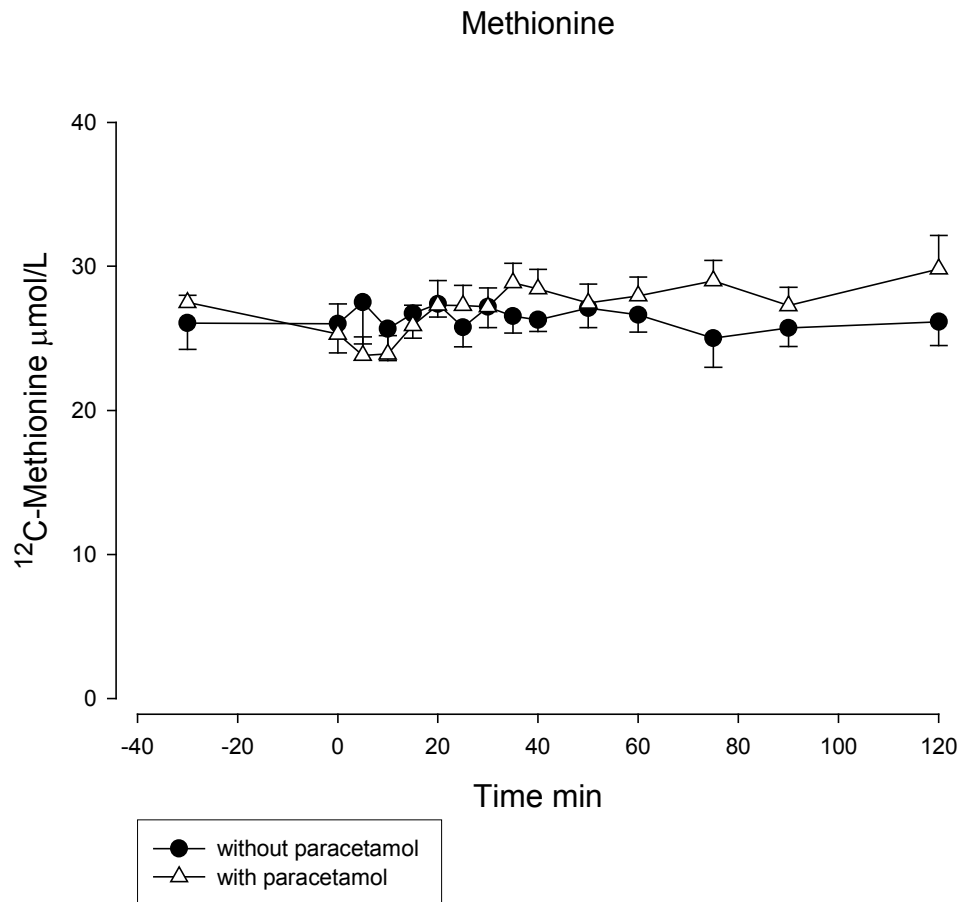
Methionine

Fig. 63: concentration of ^{12}C Met in the presence (triangles) and absence (circles) of paracetamol. Mean±SE, n=6

The time course of the plasma concentration of ^{13}C-Met in the presence and absence of paracetamol is shown in figure 64. There was no statistically significant difference at any time point between the two study periods. The oral clearance of labelled Met and its mean residence time were not different with and without paracetamol (figure 65).
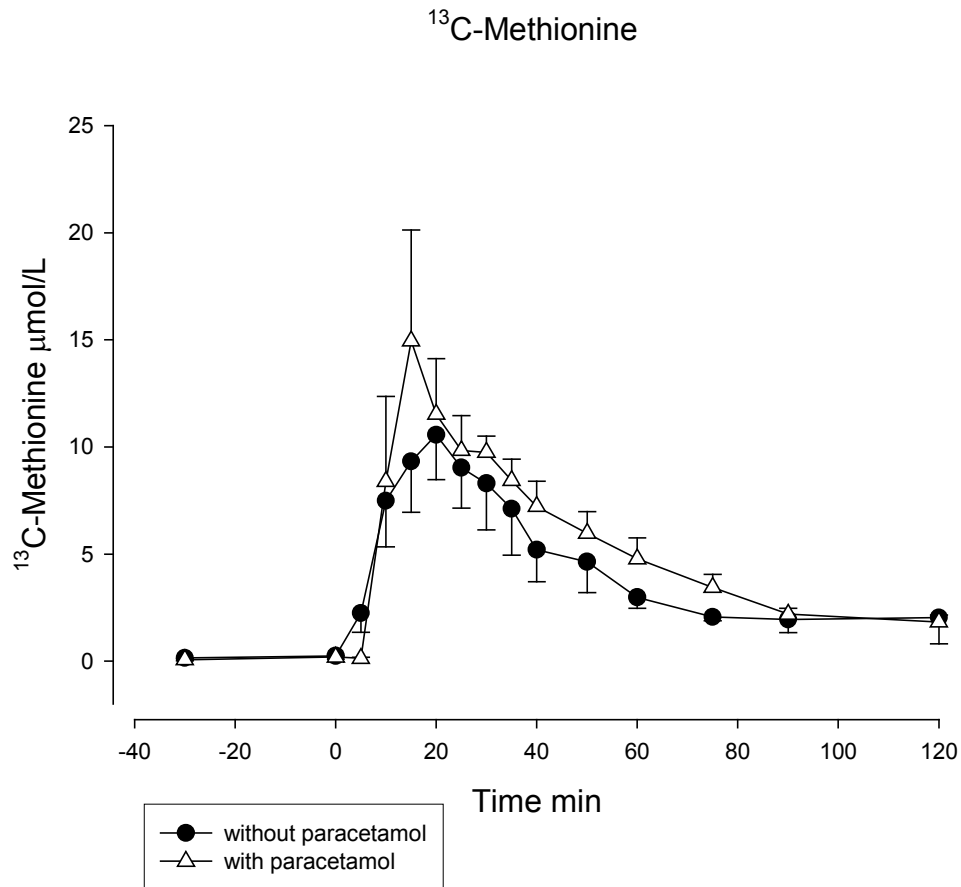
¹³C-Methionine

Fig. 64: concentrations of ¹³C Met in the presence (triangles) and absence (circles) of paracetamol. Mean±SE, n=6

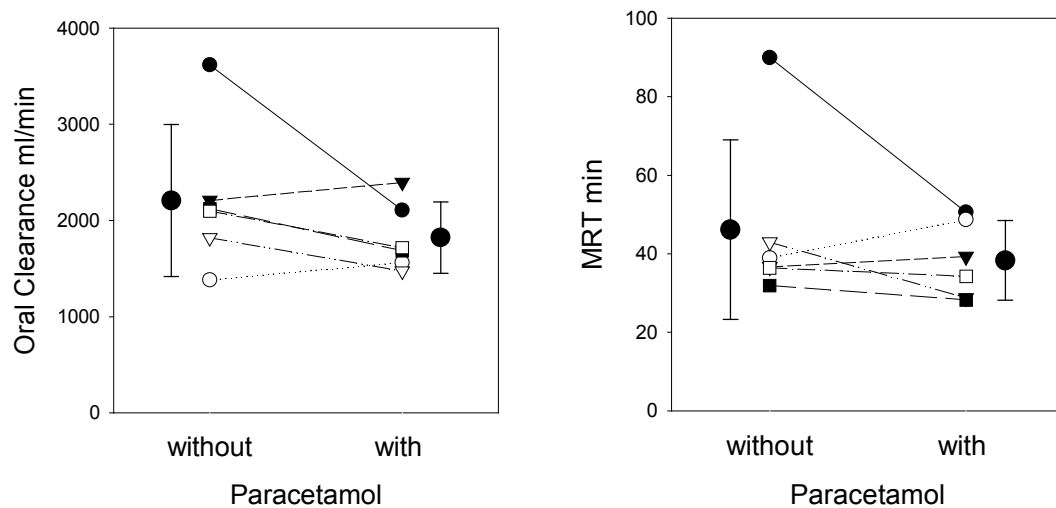**Fig. 65: oral clearance of ¹³C-Met and its mean residence time (MRT) with and without paracetamol. The graphs show the values of the six individuals and the mean with its standard error.**

The time course of the exhalation of $^{13}CO_2$ is shown in figure 66. Again, the time courses with and without paracetamol were essentially the same. Consequently, the fraction of

the administered dose exhaled as CO_2 within 2 hours and the breath clearance were identical in the two study periods (figures 67 and 68).

Exhalation of $^{13}CO_2$
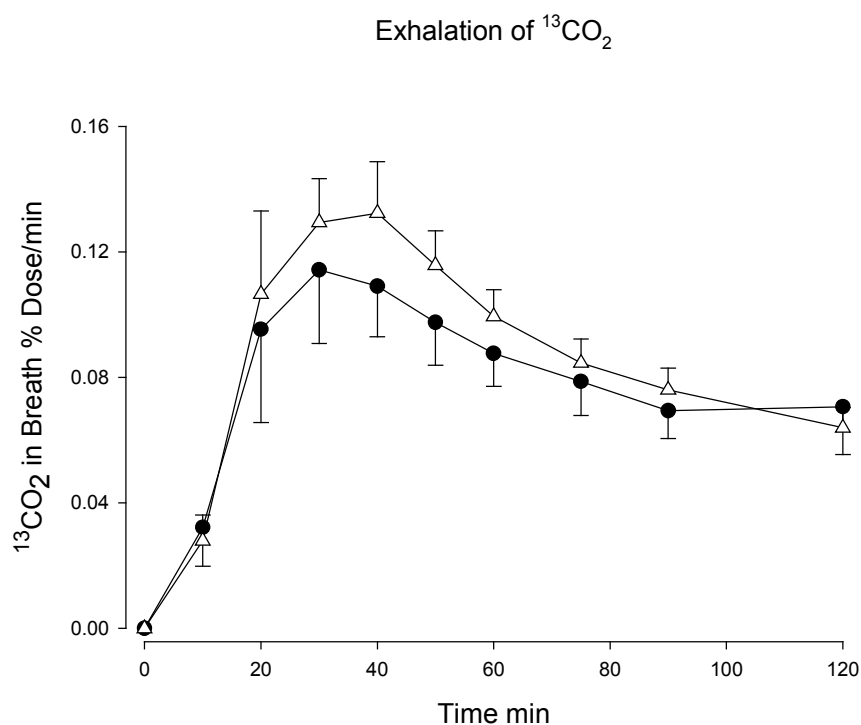
Fig. 66: time course of $^{13}CO_2$ exhalation with (triangles) and without (circles) paracetamol over two hours shown as mean ± SE. n = 6
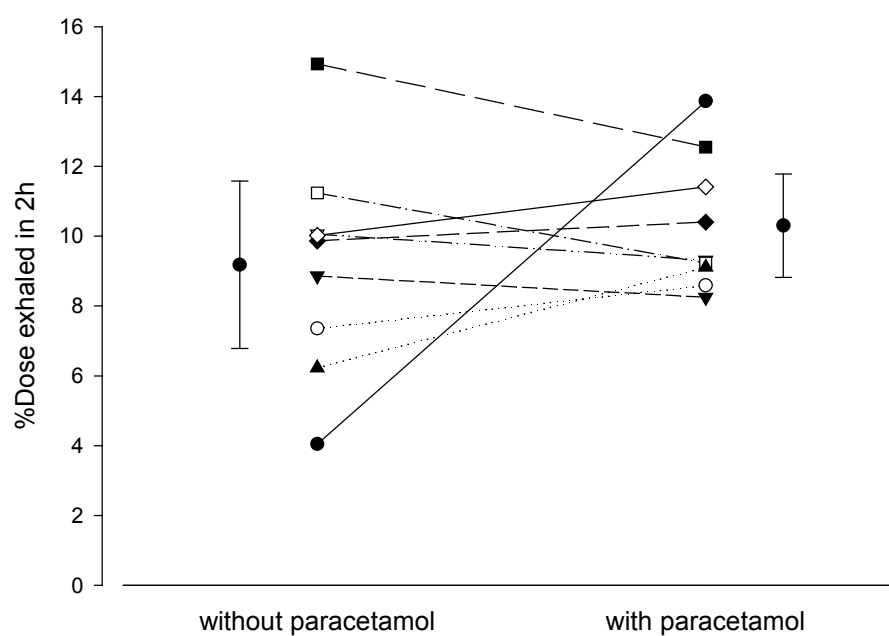
Fig. 67: the percentage of the dose of ^{13}C-Met exhaled as $^{13}CO_2$ of the nine individuals with and without paracetamol (mean ± SE)

Fig. 68: the breath clearance of ^{13}C-Met of the six individuals with and without paracetamol (mean ± SE)

The time courses of free and total GSH, Cys and homocysteine in plasma are shown in figures 69 and 70. No free homocysteine was detectable.
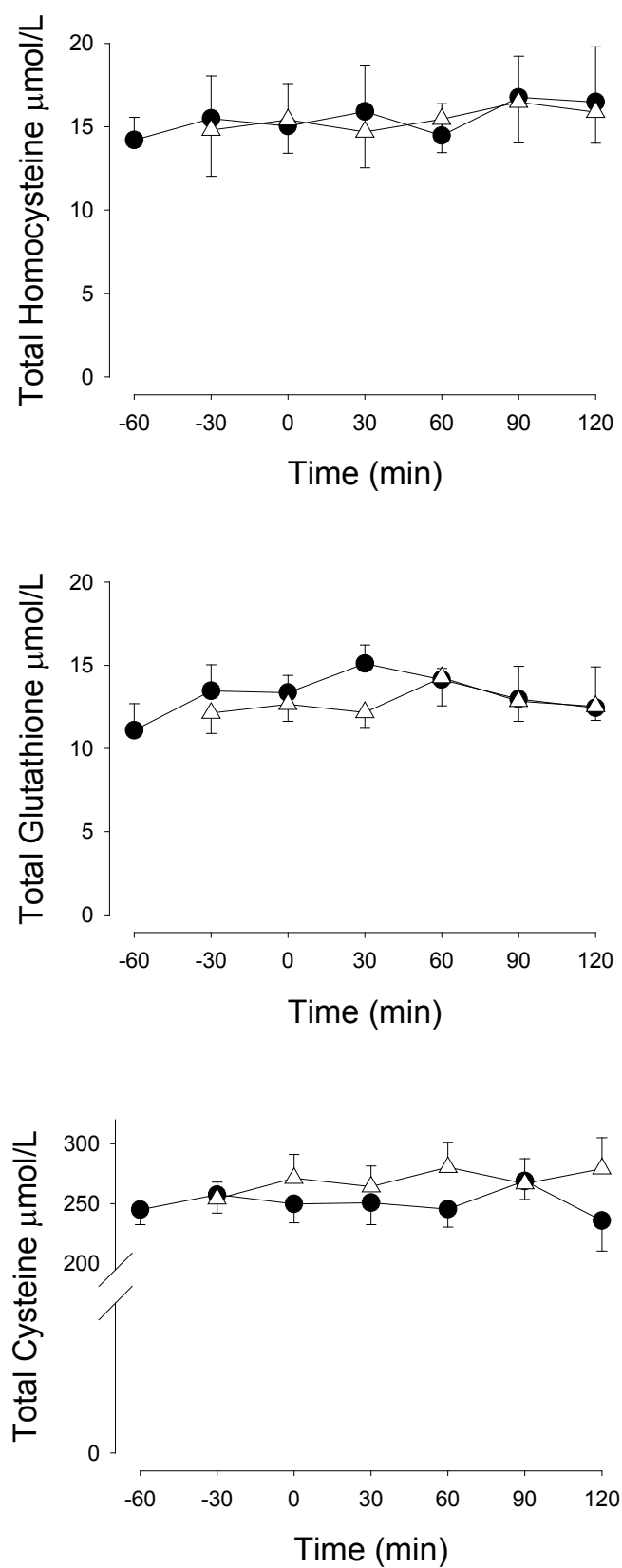
Fig. 69: total thiols as mean ± SE with (triangles) and without (circles) paracetamol
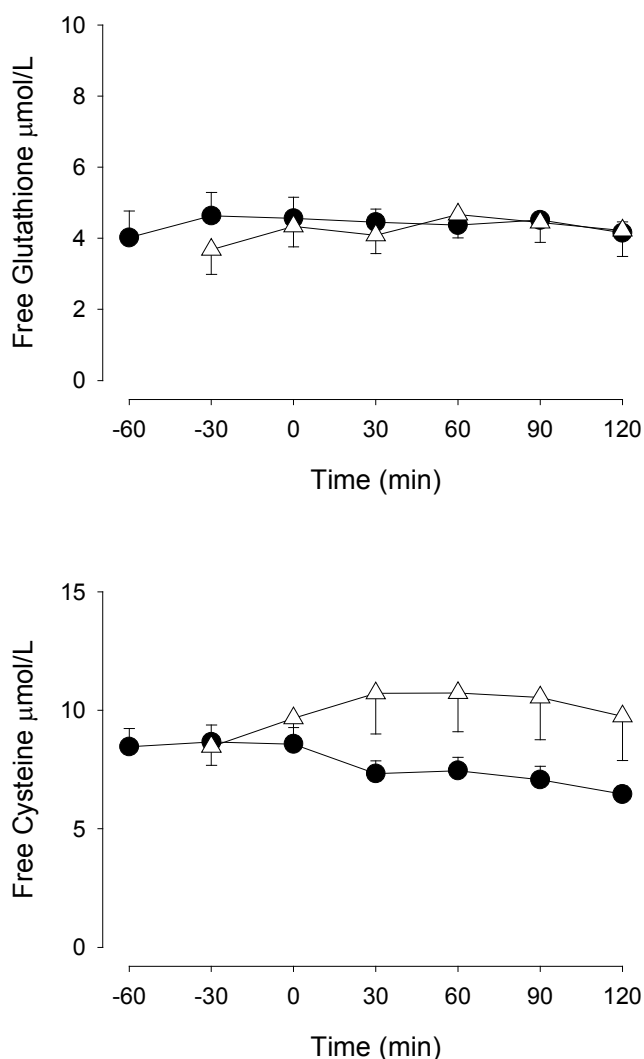
Fig. 70: free thiols as mean ± SE with (triangles) and without (circles) paracetamol

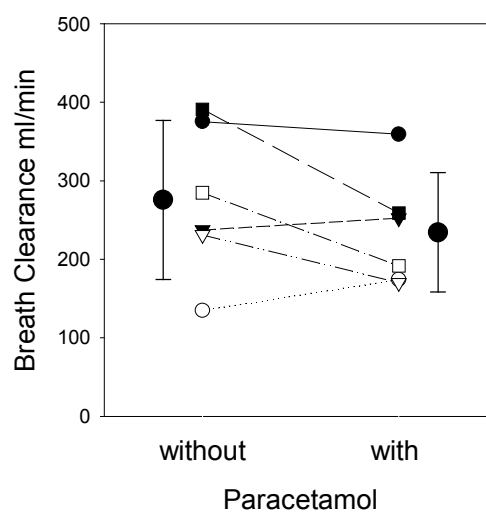The plasma concentration of paracetamol amounted to 0.13 +/- 0.013 µmol/L at 30 minutes after ingestion (just before the ingestion of the labelled Met).

Discussion

Following the oral ingestion of ^{13}C-Met approximately 12% of the administered dose appear in breath in form of $^{13}CO_2$ within 2 h of ingestion. This provides a minimal estimate of the metabolism of Met since only a fraction of administered labelled bicarbonate appears in breath as CO_2[123], indicating that a substantial fraction of the one carbon pool is not eliminated via breath. The amount of CO_2 appearing in breath after administration of S-methyl-labelled Met was similar to the amount exhaled after administration of Met

labelled in the carboxyl group ([1-^{13}C]-Met) where approximately 11% of the administered dose appeared in breath within 2h[99].

When the administered Met is labelled in the S-methyl group the $^{13}CO_2$ appearing in breath reflects mainly the fraction of Met that has been incorporated into SAM and has been demethylated to SAH. The subsequent fate of the homocysteine originating from SAH, either transsulfuration, remethylation, transamination etc. cannot be deduced. When the administered Met is labelled in the carboxyl group the $^{13}CO_2$ appearing in breath mainly arises either from transsulfuration of homocysteine to Cys, decarboxylation of SAM or transamination and decarboxylation of Met. Decarboxylation of SAM is a minor pathway in healthy subjects[75]. Only negligible amounts of $^{13}CO_2$ arise from transamination in humans[14,15]. It has been estimated that the transsulfuration pathway accounts for approximately 13% to 17% of Met turnover[32,74,123,133]. The fraction of the administered dose exhaled as $^{13}CO_2$ following [S-^{13}C]-Met would thus be expected to exceed the fraction in the case of [1-^{13}C]-Met. That the exhalation of $^{13}CO_2$ following [S-^{13}C]-Met is not considerably higher than following [1-^{13}C]-Met is probably due to the utilization of the methyl group of Met for methylation reactions so that only a fraction of the transferred methyl groups rapidly enters the one carbon pool to yield $^{13}CO_2$. Nevertheless, an increased utilisation of Met for the synthesis of Cys should result in an increased turnover of Met and an increased exhalation of $^{13}CO_2$.

The administration of two grams of paracetamol did not have a statistically significant measurable effect on the kinetics and metabolism of Met. One possible explanation for this negative finding is that in man Met is not an important source of Cys for the synthesis of GSH. The Cys consumed in the form of GSH for the detoxification of paracetamol would then have to be replaced by Cys equivalents in the circulation since the intracellular concentration of Cys is low. Neither free Cys nor small molecular cysteine disulfides, mostly cystine, however, exhibited a measurable decrease during the study, thus arguing against this hypothesis. An alternative explanation may be that the pool of hepatic GSH is not stressed to the extent where a measurable effect on Met kinetics can be expected.

Approximately 5% of a dose of paracetamol are metabolised to GSH adducts and their metabolites[90,93]. Thus, 2 g, corresponding to 13.2 mmol of paracetamol will consume approximately 0.7 mmol of GSH. A normal liver of 1.5 kg contains approximately 6 mmol of GSH. In a static system the consumption of approximately 10 % of the pool of GSH would be expected to have a measurable effect. However, the turnover of GSH is quite high even in the absence of paracetamol and has been estimated at 20–30 μmol/min, corresponding to approximately 43 mmol or 14 g per day[13,19]. This represents a minimal

estimate of the total synthesis of GSH, since it does not account for the intracellular consumption of GSH and its secretion into compartments other than the circulation, such as bile and epithelial lining fluid of the lungs. An additional 0.7 mmol during the hour when most of the metabolism of the administered paracetamol will take place may not have a measurable effect on the turnover.

In previous studies the ingestion of the same dose of paracetamol had a measurable effect on the concentration of circulating GSH[19,62]. GSH decreased moderately and recovered within 2-3 h consistent with a paracetamol-induced consumption of GSH and its resynthesis. No such change in either free or total GSH in plasma was seen in the six subjects participating in the present study. Except for the use of a different formulation of paracetamol the experimental design and the analytical methods were similar. The concentrations of paracetamol in plasma were not measured in the previous studies and might have been higher, although the formulation used in the present study is thought to release paracetamol rapidly and completely. Alternatively, the stress on GSH pool produced by the 2 g of paracetamol may not have been sufficient in these mostly young and healthy volunteers to measurably decrease circulating GSH and Cys.

In summary, the administration of 2 g of paracetamol to healthy subject does not measurably stimulate the oral clearance of Met and does not increase the utilisation of Met for GSH synthesis via the transsulfuration pathway.

Reference List

1. P450 Drug-Interactions Table. Internet (2003). 2003.
    Ref Type: Electronic Citation

2. Aleynik S,.Lieber CS. Role of S-adenosylmethionine in hyperhomocysteinemia and in the treatment of alcoholic liver disease. *Nutrition* 2000;16:1104-8.

3. Allemann PI. [Markers for excessive alcohol use (screening)]. *Ther.Umsch.* 2000;57:185-90.

4. Allen JP, Litten RZ, Fertig JB, Babor T. A review of research on the Alcohol Use Disorders Identification Test (AUDIT). *Alcohol Clin Exp Res* 1997;21:613-9.

5. Alt A, Wurst F, Seidl S. Evaluation of the ethylglucuronide in urine samples with the internal standard d5-ethylglucuronide. *Blutalkohol* 1997;34:360-5.

6. Alterman AI, Gelfand LA, Sweeney KK. The Alcohol Clinical Index in lower socioeconomic alcohol-dependent men. *Alcohol Clin Exp Res* 1992;16:960-3.

7. Altomare E, Grattagliano I, Vendemiale G, Palmieri V, Palasciano G. Acute ethanol administration induces oxidative changes in rat pancreatic tissue. *Gut* 1996;38:742-6.

8. Arndt T. Möglichkeiten und Grenzen des Kohlehydrat-defizienten Transferrins (CDT) als Kenngrösse missbräuchlichen Alkoholkonsums. In Aderjan R, ed. *Marker missbräuchlichen Alkoholkonsums: klinische und rechtliche Bedeutung,* pp 82-96. Stuttgart: Wissenschaftliche Verlagsgesellschaft mbH, 2000.

9. Arndt T. Carbohydrate-deficient transferrin as a marker of chronic alcohol abuse: a critical review of preanalysis, analysis, and interpretation. *Clin Chem.* 2001;47:13-27.

10. Auwarter V, Sporkert F, Hartwig S, Pragst F, Vater H, Diefenbacher A. Fatty acid ethyl esters in hair as markers of alcohol consumption. Segmental hair analysis of alcoholics, social drinkers, and teetotalers. *Clin Chem.* 2001;47:2114-23.

11. BAG. Alkoholmissbrauch kostet Milliarden. Bundesamt für Gesundheit. 24-10-2003. Bern.
    Ref Type: Report

12. Behrens UJ, Hoerner M, Lasker JM, Lieber CS. Formation of acetaldehyde adducts with ethanol-inducible P450IIE1 in vivo. *Biochem Biophys.Res Commun.* 1988;154:584-90.

13. Bianchi G, Bugianesi E, Ronchi M, Fabbri A, Zoli M, Marchesini G. Glutathione kinetics in normal man and in patients with liver cirrhosis. *J Hepatol.* 1997;26:606-13.

14. Blom HJ, Boers GH, Trijbels JM, van Roessel JJ, Tangerman A. Cystathionine-synthase-deficient patients do not use the transamination pathway of methionine to reduce hypermethioninemia and homocystinemia. *Metabolism* 1989;38:577-82.

15. Blom HJ, Boers GH, van den Elzen JP, Gahl WA, Tangerman A. Transamination of methionine in humans. *Clin Sci.(Lond)* 1989;76:43-9.

16. Bode JC,.Bode C. [Alcohol, the gastrointestinal tract and pancreas]. *Ther.Umsch.* 2000;57:212-9.

17. Braun KP, Cody RB, Jr., Jones DR, Peterson CM. A structural assignment for a stable acetaldehyde-lysine adduct. *J Biol.Chem.* 1995;270:11263-6.

18. Brenner H, Rothenbacher D, Bode G, Marz W, Hoffmeister A, Koenig W. Coronary heart disease risk reduction in a predominantly beer-drinking population. *Epidemiology* 2001;12:390-5.

19. Burgunder JM,.Lauterburg BH. Decreased production of glutathione in patients with cirrhosis. *Eur.J Clin Invest* 1987;17:408-14.

20. Cap L,.Lauterburg BH. [Biological changes caused by ethanol: their sequelae and importance in the diagnosis of alcoholism]. *Ther.Umsch.* 1990;47:350-7.

21. Chick J, Kreitman N, Plant M. Mean cell volume and gamma-glutamyl-transpeptidase as markers of drinking in working men. *Lancet* 1981;1:1249-51.

22. Cho ES, Johnson N, Snider BC. Tissue glutathione as a cyst(e)ine reservoir during cystine depletion in growing rats. *J Nutr.* 1984;114:1853-62.

23. Corrao G, Rubbiati L, Bagnardi V, Zambon A, Poikolainen K. Alcohol and coronary heart disease: a meta-analysis. *Addiction* 2000;95:1505-23.

24. Dahl H, Stephanson N, Beck O, Helander A. Comparison of urinary excretion characteristics of ethanol and ethyl glucuronide. *J Anal.Toxicol.* 2002;26:201-4.

25. Davis M, Labadarios D, Williams RS. Metabolism of paracetamol after therapeutic and hepatotoxic doses in man. *J Int.Med.Res* 1976;4:40-5.

26. DeLeve LD,.Kaplowitz N. Glutathione metabolism and its role in hepatotoxicity. *Pharmacol Ther.* 1991;52:287-305.

27. Donohue TM, Jr., Tuma DJ, Sorrell MF. Acetaldehyde adducts with proteins: binding of [14C]acetaldehyde to serum albumin. *Arch.Biochem Biophys.* 1983;220:239-46.

28. Doyle KM, Cluette-Brown JE, Dube DM, Bernhardt TG, Morse CR, Laposata M. Fatty acid ethyl esters in the blood as markers for ethanol intake. *JAMA* 1996;276:1152-6.

29. Droenner P, Schmitt G, Aderjan R, Zimmer H. A kinetic model describing the pharmacokinetics of ethyl glucuronide in humans. *Forensic Sci.Int.* 2002;126:24-9.

30. Escobar F, Espi F, Canteras M. Diagnostic tests for alcoholism in primary health care: compared efficacy of different instruments. *Drug Alcohol Depend.* 1995;40:151-8.

31. Frei A. Die sozialen Kosten des Alkoholkonsums in der Schweiz 1998 - Direkte Kosten. *Suchtforschung des BAG / Recherches de l'OFSP en matière de dépendances 1999 - 2001*, pp 169-74. Bern: Bundesamt für Gesundheit, 2003.

32. Fukagawa NK, Ajami AM, Young VR. Plasma methionine and cysteine kinetics in response to an intravenous glutathione infusion in adult humans. *Am.J Physiol* 1996;270:E209-E214.

33. Gaizutis M, Pesce AJ, Pollak VE. Renal clearance of human and rat albumins in the rat. *Proc.Soc.Exp Biol.Med.* 1975;148:947-52.

34. Gmel G, Rehm J. Durchschnittskonsum, Trinkmuster, soziale und gesundheitliche Konsequenzen. *Suchtforschung des BAG / Recherches de l'OFSP en matière de dépendances 1999 - 2001,* pp 101-8. Bern: Bundesamt für Gesundheit, 2003.

35. Goll M, Schmitt G, Ganssmann B, Aderjan RE. Excretion profiles of ethyl glucuronide in human urine after internal dilution. *J Anal.Toxicol.* 2002;26:262-6.

36. Goya RG, Sosa YE, Chang J, Meites J. Degradation of immunoreactive albumin in young and old conscious female rats. *Mech.Ageing Dev.* 1986;37:69-78.

37. Grattagliano I, Lauterburg BH, Portincasa P, Caruso ML, Vendemiale G, Valentini AM *et al.* Mitochondrial glutathione content determines the rate of liver regeneration after partial hepatectomy in eu- and hypothyroid rats. *J Hepatol.* 2003;39:571-9.

38. Gross MD, Gapstur SM, Belcher JD, Scanlan G, Potter JD. The identification and partial characterization of acetaldehyde adducts of hemoglobin occurring in vivo: a possible marker of alcohol consumption. *Alcohol Clin Exp Res* 1992;16:1093-103.

39. Gutjahr E, Gmel G. Die epidemiologischen Grundlagen zur Schätzung der sozialen Kosten des Alkoholkonsums in der Schweiz, 1995-1998. *Suchtforschung des BAG / Recherches de l'OFSP en matière de dépendances 1999 - 2001,* pp 161-8. Bern: Bundesamt für Gesundheit, 2003.

40. Hall AG. Glutathione and the regulation of cell death. *Adv.Exp Med.Biol.* 1999;457:199-203.

41. Hammond CL, Lee TK, Ballatori N. Novel roles for glutathione in gene expression, cell death, and membrane transport of organic solutes. *J Hepatol.* 2001;34:946-54.

42. Hansson P, Caron M, Johnson G, Gustavsson L, Alling C. Blood phosphatidylethanol as a marker of alcohol abuse: levels in alcoholic males during withdrawal. *Alcohol Clin Exp Res* 1997;21:108-10.

43. He ZJ, Ericksson P, Alho H, Harmoinen A, Nordback I. Effects on endogenous acetaldehyde production by disulfiram and ethanol feeding on rat pancreas. *J Gastrointest.Surg.* 2001; 5:531-9.

44. Helander A,.Eriksson CJ. Laboratory tests for acute alcohol consumption: results of the WHO/ISBRA Study on State and Trait Markers of Alcohol Use and Dependence. *Alcohol Clin Exp Res* 2002;26:1070-7.

45. Helander, A. and Jones, A. W. Application of Alcohol Markers in Traffic Medicine. 2000. International Conference on Alcohol, Drugs and Traffic Safety, May 22 - 26, 2000 in Stockholm.
   Ref Type: Conference Proceeding

46. Hoffmann T, Meyer RJ, Sorrell MF, Tuma DJ. Reaction of acetaldehyde with proteins: formation of stable fluorescent adducts. *Alcohol Clin Exp Res* 1993;17:69-74.

47. Honda S, Fujioka T, Shiota K, Fujiyama K, Kubota T, Murakami K *et al.* Effects of ethanol on the pancreas of disulfiram-treated rats. *J Gastroenterol.* 1995;30:231-6.

48. Horowitz JH, Rypins EB, Henderson JM, Heymsfield SB, Moffitt SD, Bain RP *et al.* Evidence for impairment of transsulfuration pathway in cirrhosis. *Gastroenterology* 1981;81:668-75.

49. Iffland R. Zur Analyse des Alkoholmissbrauchs in der Forensik. In Aderjan R, ed. *Marker missbräuchlichen Alkoholkonsums: klinische und rechtliche Bedeutung*, pp 44-56. Stuttgart: Wissenschaftliche Verlagsgesellschaft mbH, 2000.

50. Itala L, Seppa K, Turpeinen U, Sillanaukee P. Separation of hemoglobin acetaldehyde adducts by high-performance liquid chromatography-cation-exchange chromatography. *Anal.Biochem* 1995;224:323-9.

51. Jaakonmaki PI, Knox KL, Horning EC, Horning MG. The characterization by gas-liquid chromatography of ethyl-beta-D-glucosiduronic acid as a metabolite of ethanol in rat and man. *Eur.J.Pharmacol.* 1967;1:63-70.

52. Janda I,.Alt A. Improvement of ethyl glucuronide determination in human urine and serum samples by solid-phase extraction. *J Chromatogr.B Biomed.Sci.Appl.* 2001;758 :229-34.

53. Janda I, Weinmann W, Kuehnle T, Lahode M, Alt A. Determination of ethyl glucuronide in human hair by SPE and LC-MS/MS. *Forensic Sci.Int.* 2002;128:59-65.

54. Jeppson J-O. Carbohydrate-deficient Transferrin (CDT). In Thomas L, ed. *Labor und Diagnose: Indikation und Bewertung von Laborbefunden für die medizinische Diagnostik*, pp 672-5. Frankfurt/Main: TH-Books Verlagsgesellschaft mbH, 1998.

55. Kamill I, Smith J, Williams R. A New Aspect of ethanol metabolism: Isolation of Ethyl-Glucuronide. *Biochem J* 1952;51:32.

56. Karkkainen P, Poikolainen K, Salaspuro M. Serum beta-hexosaminidase as a marker of heavy drinking. *Alcohol Clin Exp Res* 1990;14:187-90.

57. Karlberg HI, Kern KA, Fischer JE. Albumin turnover in sarcoma-bearing rats in relation to cancer anorexia. *Am.J Surg.* 1983;145:95-101.

58. Klatsky AL, Friedman GD, Armstrong MA, Kipp H. Wine, liquor, beer, and mortality. *Am.J Epidemiol.* 2003;158:585-95.

59. Landolt HP,.Borbely AA. [Alcohol and sleep disorders]. *Ther.Umsch.* 2000;57:241-5.

60. Laposata EA,.Lange LG. Presence of nonoxidative ethanol metabolism in human organs commonly damaged by ethanol abuse. *Science* 1986;231:497-9.

61. Laposata M. Fatty acid ethyl esters: short-term and long-term serum markers of ethanol intake. *Clin Chem.* 1997;43:1527-34.

62. Lauterburg BH,.Velez ME. Glutathione deficiency in alcoholics: risk factor for paracetamol hepatotoxicity. *Gut* 1988;29:1153-7.

63. Lieber CS. Hepatic, metabolic and toxic effects of ethanol: 1991 update. *Alcohol Clin Exp Res* 1991;15:573-92.

64. Lieber CS. Ethanol metabolism, cirrhosis and alcoholism. *Clin Chim.Acta* 1997;257:59-84.

65. Lieber CS. Microsomal ethanol-oxidizing system (MEOS): the first 30 years (1968-1998)--a review. *Alcohol Clin Exp Res* 1999;23:991-1007.

66. Lieber CS. Alcohol and the liver: metabolism of alcohol and its role in hepatic and extrahepatic diseases. *Mt.Sinai J.Med.* 2000;67:84-94.

67. Lieber CS,.Packer L. S-Adenosylmethionine: molecular, biological, and clinical aspects--an introduction. *Am.J Clin Nutr.* 2002;76:1148S-50S.

68. Lin RC, Lumeng L, Shahidi S, Kelly T, Pound DC. Protein-acetaldehyde adducts in serum of alcoholic patients. *Alcohol Clin Exp Res* 1990;14:438-43.

69. Lin RC, Shahidi S, Kelly TJ, Lumeng C, Lumeng L. Measurement of hemoglobin-acetaldehyde adduct in alcoholic patients. *Alcohol Clin Exp Res* 1993;17:669-74.

70. Lin RC, Shahidi S, Lumeng L. Production of antibodies that recognize the heterogeneity of immunoreactive sites in human hemoglobin chemically modified by acetaldehyde. *Alcohol Clin Exp Res* 1993;17:882-6.

71. Lin RC, Smith JB, Radtke DB, Lumeng L. Structural analysis of peptide-acetaldehyde adducts by mass spectrometry and production of antibodies directed against nonreduced protein-acetaldehyde adducts. *Alcohol Clin Exp Res* 1995;19:314-9.

72. Loe DW, Almquist KC, Deeley RG, Cole SP. Multidrug resistance protein (MRP)-mediated transport of leukotriene C4 and chemotherapeutic agents in membrane vesicles. Demonstration of glutathione-dependent vincristine transport. *J Biol.Chem.* 1996;271:9675-82.

73. Lumeng L. New diagnostic markers of alcohol abuse. *Hepatology* 1986;6:742-5.

74. MacCoss MJ, Fukagawa NK, Matthews DE. Measurement of intracellular sulfur amino acid metabolism in humans. *Am.J Physiol Endocrinol.Metab* 2001;280:E947-E955.

75. Mato JM, Corrales FJ, Lu SC, Avila MA. S-Adenosylmethionine: a control switch that regulates liver function. *FASEB J* 2002;16:15-26.

76. Mehlen P, Kretz-Remy C, Preville X, Arrigo AP. Human hsp27, Drosophila hsp27 and human alphaB-crystallin expression-mediated increase in glutathione is essential for the protective activity of these proteins against TNFalpha-induced cell death. *EMBO J* 1996;15:2695-706.

77. Mitchell JR, Thorgeirsson SS, Potter WZ, Jollow DJ, Keiser H. Acetaminophen-induced hepatic injury: protective role of glutathione in man and rationale for therapy. *Clin Pharmacol Ther.* 1974;16:676-84.

78. Moncada C,.Israel Y. Generation of acetate and production of ethyl-lysine in the reaction of acetaldehyde plus serum albumin. *Alcohol* 1999;17:87-91.

79. Morse, RM. and Flavin, DK. Definition of Alcoholism. Internet (http://www.ncadd.org/facts/defalc.html). 3-2-1990. NCADD.
     Ref Type: Electronic Citation

80. Musshoff F. Chromatographic methods for the determination of markers of chronic and acute alcohol consumption. *J.Chromatogr.B Analyt.Technol.Biomed.Life Sci.* 2002;781:457-80.

81. Musshoff F,.Daldrup T. Determination of biological markers for alcohol abuse. *J.Chromatogr.B Biomed.Sci.Appl.* 1998;713:245-64.

82. Mutschler E, Schäfer-Korting M. Ethanol. In Mutschler E, Schäfer-Korting M, eds. *Arzneimittelwirkungen*, pp 816-8. Stuttgart: Wissenschaftliche Verlagsgesellschaft mbH, 1997.

83. Neubauer O. Über Glykuronsäurepaarung bei Stoffen der Fettreihe. A Exp Pathol Pharmacol 46, 133-154. 1901.

    Ref Type: Journal (Full)

84. Niemela O, Israel Y, Mizoi Y, Fukunaga T, Eriksson CJ. Hemoglobin-acetaldehyde adducts in human volunteers following acute ethanol ingestion. *Alcohol Clin Exp Res* 1990;14:838-41.

85. Niemela O, Juvonen T, Parkkila S. Immunohistochemical demonstration of acetaldehyde-modified epitopes in human liver after alcohol consumption. *J Clin Invest* 1991;87:1367-74.

86. Niemela O, Klajner F, Orrego H, Vidins E, Blendis L, Israel Y. Antibodies against acetaldehyde-modified protein epitopes in human alcoholics. *Hepatology* 1987;7:1210-4.

87. Niemela O, Parkkila S, Pasanen M, Iimuro Y, Bradford B, Thurman RG. Early alcoholic liver injury: formation of protein adducts with acetaldehyde and lipid peroxidation products, and expression of CYP2E1 and CYP3A. *Alcohol Clin Exp Res* 1998;22:2118-24.

88. Niessen WMA. Liquid Chromatography - Mass Spectrometry. New York, Basel, Hong Kong: Marcel Dekker, Inc., 1999.

89. Nishikawa M, Tsuchihashi H, Miki A, Katagi M, Schmitt G, Zimmer H *et al.* Determination of ethyl glucuronide, a minor metabolite of ethanol, in human serum by liquid chromatography-electrospray ionization mass spectrometry. *J.Chromatogr.B Biomed.Sci.Appl.* 1999;726:105-10.

90. Nuttall SL, Khan JN, Thorpe GH, Langford N, Kendall MJ. The impact of therapeutic doses of paracetamol on serum total antioxidant capacity. *J Clin Pharm.Ther.* 2003;28:289-94.

91. O'Donovan DJ,.Fernandes CJ. Mitochondrial glutathione and oxidative stress: implications for pulmonary oxygen toxicity in premature infants. *Mol.Genet.Metab* 2000;71:352-8.

92. Oneta CM. [Alcohol and drug interactions]. *Ther.Umsch.* 2000;57:220-6.

93. Prescott LF. Paracetamol overdosage. Pharmacological considerations and clinical management. *Drugs* 1983;25:290-314.

94. Reed RG, Davidson LK, Burrington CM, Peters T, Jr. Non-resolving jaundice: bilirubin covalently attached to serum albumin circulates with the same metabolic half-life as albumin. *Clin Chem.* 1988;34:1992-4.

95. Renner EL. [Biological markers of alcoholism]. *Schweiz.Med.Wochenschr.* 1992;122:614-8.

96. Renner EL,.Dallenbach A. [Increased liver enzymes: what should be done?]. *Ther.Umsch.* 1992;49:281-6.

97. Rimm EB, Williams P, Fosher K, Criqui M, Stampfer MJ. Moderate alcohol intake and lower risk of coronary heart disease: meta-analysis of effects on lipids and haemostatic factors. *BMJ* 1999;319:1523-8.

98. Roth H, Eger K, Troschütz R. Pharmazeutische Chemie II; Arzneistoffanalyse; Reaktivität, Stabilität, Analytik. Stuttgart, Jena, Leipzig, Ulm: Gustav Fischer, 1997.

99. Russmann S, Junker E, Lauterburg BH. Remethylation and transsulfuration of methionine in cirrhosis: studies with L-[H3-methyl-1-C]methionine. *Hepatology* 2002;36:1190-6.

100. Sachs H. Drogennachweis in Haaren. Lübeck: Schmidt-Römhild, 1997.

101. Salaspuro M. Conventional and coming laboratory markers of alcoholism and heavy drinking. *Alcohol Clin Exp Res* 1986;10:5S-12S.

102. Salaspuro M. Recent Developments in the Research on Markers of Alcoholism. In Aderjan R, ed. *Marker missbräuchlichen Alkoholkonsums: klinische und rechtliche Bedeutung,* pp 69-81. Stuttgart: Wissenschaftliche Verlagsgesellschaft mbH, 2000.

103. San George RC,.Hoberman HD. Reaction of acetaldehyde with hemoglobin. *J Biol.Chem.* 1986;261:6811-21.

104. Schlapfer TE. [Alcohol and the brain--morphological and functional brain changes]. *Ther.Umsch.* 2000;57:191-5.

105. Schmitt G, Aderjan R. Abstinenzkontrolle durch Überprüfung des Ethanolstoffwechsels (Ethylglucuronid). In Aderjan R, ed. *Marker missbräuchlichen Alkoholkonsums: klinische und rechtliche Bedeutung,* pp 97-102. Stuttgart: Wissenschaftliche Verlagsgesellschaft mbH, 2000.

106. Schmitt G, Aderjan R, Keller T, Wu M. Ethyl glucuronide: an unusual ethanol metabolite in humans. Synthesis, analytical data, and determination in serum and urine. *J Anal.Toxicol.* 1995;19:91-4.

107. Schmitt G, Droenner P, Skopp G, Aderjan R. Ethyl glucuronide concentration in serum of human volunteers, teetotalers, and suspected drinking drivers. *J.Forensic Sci.* 1997;42:1099-102.

108. Seitz HK,.Poschl G. [Alcohol and the liver]. *Ther.Umsch.* 2000;57:227-31.

109. Seitz HK, Werle E. Klinische Diagnostik für Alkoholmissbrauch und Alkoholassoziierte Organschäden. In Aderjan R, ed. *Marker missbräuchlichen Alkoholkonsums: klinische und rechtliche Bedeutung,* pp 57-62. Stuttgart: Wissenschaftliche Verlagsgesellschaft mbH, 2000.

110. Seitz S,.Boelsterli UA. Diclofenac acyl glucuronide, a major biliary metabolite, is directly involved in small intestinal injury in rats. *Gastroenterology* 1998;115 :1476-82.

111. Sharpe PC. Biochemical detection and monitoring of alcohol abuse and abstinence. *Ann.Clin Biochem.* 2001;38:652-64.

112. Shirota FN, DeMaster EG, Shoeman DW, Nagasawa HT. Acetaminophen-induced suppression of hepatic AdoMet synthetase activity is attenuated by prodrugs of L-cysteine. *Toxicol.Lett.* 2002;132:1-8.

113. Sillanaukee P,.Koivula T. Detection of a new acetaldehyde-induced hemoglobin fraction HbA1ach by cation exchange liquid chromatography. *Alcohol Clin Exp Res* 1990;14:842-6.

114. Skinner HA, Holt S, Sheu WJ, Israel Y. Clinical versus laboratory detection of alcohol abuse: the alcohol clinical index. *Br.Med.J.(Clin Res Ed)* 1986;292:1703-8.

115. Slanger BH, Kusubov N, Winchell HS. Effect of exercise on human CO2-HCO3 minus kinetics. *J Nucl.Med.* 1970;11:716-8.

116. Smith PC, McDonagh AF, Benet LZ. Irreversible binding of zomepirac to plasma protein in vitro and in vivo. *J Clin Invest* 1986;77:934-9.

117. Soyka M. Alkoholismusdiagnose in psychiatrischer und laborchemischer Sicht. In Aderjan R, ed. *Marker missbräuchlichen Alkoholkonsums: klinische und rechtliche Bedeutung,* pp 24-43. Stuttgart: Wissenschaftliche Verlagsgesellschaft mbH, 2000.

118. Stein AF, Bracken WM, Klaassen CD. Utilization of methionine as a sulfhydryl source for metallothionein synthesis in rat primary hepatocyte cultures. *Toxicol.Appl.Pharmacol* 1987;87:276-83.

119. Stephanson N, Dahl H, Helander A, Beck O. Direct quantification of ethyl glucuronide in clinical urine samples by liquid chromatography-mass spectrometry. *Ther.Drug Monit.* 2002;24:645-51.

120. Stevens VJ, Fantl WJ, Newman CB, Sims RV, Cerami A, Peterson CM. Acetaldehyde adducts with hemoglobin. *J Clin Invest* 1981;67:361-9.

121. Sticht G, Käferstein H, Staak M. GC-MS-Bestimmung von Ethylglucuronid im Serum mit Propylglucuronid als internem Standard. *Rechtsmedizin* 1997;7:184-8.

122. Stockham TL,.Blanke RV. Investigation of an acetaldehyde-hemoglobin adduct in alcoholics. *Alcohol Clin Exp Res* 1988;12:748-54.

123. Storch KJ, Wagner DA, Burke JF, Young VR. Quantitative study in vivo of methionine cycle in humans using [methyl-2H3]- and [1-13C]methionine. *Am.J Physiol* 1988;255 :E322-E331.

124. Suter PM. [Alcohol, lipid metabolism and body weight]. *Ther.Umsch.* 2000;57:205-11.

125. Swift R. Direct measurement of alcohol and its metabolites. *Addiction* 2003;98 Suppl 2:73-80.

126. Tateishi N, Higashi T, Naruse A, Hikita K, Sakamoto Y. Relative contributions of sulfur atoms of dietary cysteine and methionine to rat liver glutathione and proteins. *J Biochem (Tokyo)* 1981;90:1603-10.

127. Thiele GM, Tuma DJ, Miller JA, Wegter KM, McDonald TL, Klassen LW. Monoclonal and polyclonal antibodies recognizing acetaldehyde-protein adducts. *Biochem Pharmacol* 1998;56:1515-23.

128. Thomas L. Alanin-Aminotransferase (ALT), Aspartat-Aminotransferase (AST). In Thomas L, ed. *Labor und Diagnose: Indikation und Bewertung von Laborbefunden für die medizinische Diagnostik,* pp 56-67. Frankfurt/Main: TH-Books Verlagsgesellschaft mbH, 1998.

129. Thomas L. Gamma-Glutamyl-Transferase (GGT). In Thomas L, ed. *Labor und Diagnose: Indikation und Bewertung von Laborbefunden für die medizinische Diagnostik,* pp 83-8. Frankfurt/Main: TH-Books Verlagsgesellschaft mbH, 1998.

130. Tossmann P, Boldt S, Tensil M. The Use of Drugs within the Techno Party Scene in European Metropolitan Cities. *Suchtforschung des BAG / Recherches de l'OFSP en matière de dépendances 1999 - 2001,* pp 132-49. Bern: Bundesamt für Gesundheit, 2003.

131. Tuma DJ, Donohue TM, Jr., Medina VA, Sorrell MF. Enhancement of acetaldehyde-protein adduct formation by L-ascorbate. *Arch.Biochem Biophys.* 1984;234:377-81.

132. Tuma DJ, Newman MR, Donohue TM, Jr., Sorrell MF. Covalent binding of acetaldehyde to proteins: participation of lysine residues. *Alcohol Clin Exp Res* 1987;11:579-84.

133. van Guldener C, Kulik W, Berger R, Dijkstra DA, Jakobs C, Reijngoud DJ *et al.* Homocysteine and methionine metabolism in ESRD: A stable isotope study. *Kidney Int.* 1999;56:1064-71.

134. Varga A, Hansson P, Johnson G, Alling C. Normalization rate and cellular localization of phosphatidylethanol in whole blood from chronic alcoholics. *Clin Chim.Acta* 2000;299:141-50.

135. von Wartburg JP. [Alcohol metabolism]. *Ther.Umsch.* 1981;38:414-9.

136. Weinmann W, Schaefer P, Thierauf A, Schreiber A, Wurst FM. Confirmatory analysis of ethylglucuronide in urine by liquid-chromatography/electrospray ionization/tandem mass spectrometry according to forensic guidelines. *J Am.Soc.Mass Spectrom.* 2004;15:188-93.

137. White IR. The level of alcohol consumption at which all-cause mortality is least. *J.Clin Epidemiol.* 1999;52:967-75.

138. White IR, Altmann DR, Nanchahal K. Alcohol consumption and mortality: modelling risks for men and women at different ages. *BMJ* 2002;325:191.

139. WHO. Adult per capita alcohol consumption. 18-1-2004. WHO. Ref Type: Data File

140. Wickramasinghe SN, Hasan R, Khalpey Z. Differences in the serum levels of acetaldehyde and cytotoxic acetaldehyde-albumin complexes after the consumption of red and white wine: in vitro effects of flavonoids, vitamin E, and other dietary antioxidants on cytotoxic complexes. *Alcohol Clin Exp Res* 1996;20:799-803.

141. Wilson JS, Apte MV, Thomas MC, Haber PS, Pirola RC. Effects of ethanol, acetaldehyde and cholesteryl esters on pancreatic lysosomes. *Gut* 1992;33:1099-104.

142. Worrall S, de Jersey J, Shanley BC, Wilce PA. Acetaldehyde-modified proteins and associated antibodies in ethanol-fed rats. *Alcohol Alcohol Suppl* 1991;1:255-60.

143. Worrall S, de Jersey J, Wilce PA. Comparison of the formation of proteins modified by direct and indirect ethanol metabolites in the liver and blood of rats fed the Lieber-DeCarli liquid diet. *Alcohol Alcohol* 2000;35:164-70.

144. Worrall S, de Jersey J, Wilce PA, Seppa K, Hurme L, Sillanaukee P. Relationship between alcohol intake and immunoglobulin a immunoreactivity with acetaldehyde-modified bovine serum albumin. *Alcohol Clin Exp Res* 1996;20:836-40.

145. Wurst FM, Kempter C, Metzger J, Seidl S, Alt A. Ethyl glucuronide: a marker of recent alcohol consumption with clinical and forensic implications. *Alcohol* 2000;20:111-6.

146. **Wurst FM, Kempter C, Seidl S, Alt A. Ethyl glucuronide--a marker of alcohol consumption and a relapse marker with clinical and forensic implications.** *Alcohol Alcohol* 1999;34:71-7.

147. **Wurst FM,.Metzger J. The ethanol conjugate ethyl glucuronide is a useful marker of recent alcohol consumption.** *Alcohol Clin Exp Res* 2002;26:1114-9.

148. **Wurst FM, Schuttler R, Kempter C, Seidl S, Gilg T, Jachau K** *et al.* **Can ethyl glucuronide be determined in post-mortem body fluids and tissues?** *Alcohol Alcohol* 1999;34:262-3.

149. **Wurst FM, Seidl S, Alt A, Metzger J. [Direct ethanol metabolite ethyl glucuronide. Its value as alcohol intake and recurrence marker, methods of detection and prospects].** *Psychiatr.Prax.* 2000;27:367-71.

150. **Zybko WC, Cluette-Brown JE, Laposata M. Improved sensitivity and reduced sample size in serum fatty acid ethyl ester analysis.** *Clin Chem.* 2001;47:1120-1.

Publications

Schmid A, Reich E; HPTLC and Video-technology for stability tests of plant extracts. CAMAG Bibliography Service (CBS) 86, March 2001, 10-12

Schmid A, Reich E; HPTLC and Video-Technologie für Stabilitätstests an Pflanzenextrakten. Chemie plus 2001, 10 (10) 36-38

Schmid A, Lauterburg BH; Alkohol in Arzneimitteln: Wieviel ist bei Kindern vertretbar? Phytotherapie 2002, 2 (1) 13-14

Schmid A, von Heeren F, Widmer HR; Hygiene im Spital: Rolle der Antibiotika, Antiseptika und Desinfektionsmittel. Hospitalis 2002, 72 (3) 115-125

Presentation

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Workshop-Presentation: Fingerprint Analysis of Valerian by HPTLC

Curriculum vitae

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Gehring,W; Grünig,HM; Guenthert,T; Haag,D; Hädener,A; Haefeli,W; Hauri,HP; Hersberger,K; Heusser,P; Huber,H; Iberg,S; Imhof,H; Inderbitzin,S; Jungen,M; Kaden,P; Kenzelmann,R; Kress,A; Kupferschmidt,H; Lampert,M; Lauterburg,BH; Leu Marseiler,R; Leuenberger,H; Luef,W; Martinelli,E; Martin-Facklam,M; Marty,S; Mathys,U; Meier,B; Meier,C; Meister,T; Moor,M; Muff,P; Mühlebach,S; Müller,HJ; Müller,L; Müller-Morgenthaler,M; Oelhafen,P; Plattner,GR; Pretzel,K; Rätz,A; Reich,E; Renggli,V; Riewe,B; Rösch,B; Rothen,C; Rowell,CH; Rudin,H; Russmann,S; Saller,R ; Saxer,A ; Schacher,J ; Schaffner,W; Schatz,G; Scheffold,R; Schlienger,R; Schmitt,E; Schmutz,C; Scholer,A ; Séquin,U ; Sick,J; Sigel,H; Spornitz,UM; Strazewski,P; Surber,C; Szucs,T; Tannert,I; Tauscher,L; Thormann,W; Walder,L; Widmer,HR; Wienten,A; Wolf,P; Zelger,G; Zgraggen,B; Ziegler,H; Ziemiecki,A; Zuberbühler,A.