**Master thesis performed at:**


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**Department of Bioanalysis**

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# N-ACETYLTAURINE: A NOVEL URINARY ALCOHOL MARKER


**Sofie RUTJENS**

First Master of Pharmaceutical Care

Promoter

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**SUMMARY**

Alcohol, a legal drug, is an important cause of sickness and death. It is often abused, which leads to both health and socioeconomic consequences. In forensic sciences there are already a few routine methods to determine if there was ethanol ingestion and when it approximately took place.

The main objective of this thesis was to determine whether N-acetyltaurine in urine can serve as a forensic alcohol marker and to compare it to the markers already used in routine settings. A HILIC based LC method for the mass spectrometric analysis of N-acetyltaurine was developed and validated. After validation of the method, it was used to examine urine samples of a drinking study. The LLOQ was set at 50 ng/ml for N-acetyltaurine.

In this drinking study, eight subjects ingested an amount of alcohol which led to a blood alcohol concentration (BAC) of 0.8‰. For the first eight hours urine samples were taken every 1.5 - 2 hours, afterwards samples were taken every 24 hours. Both the levels of N-acetyltaurine and taurine were normalized to a creatinine concentration of 1 mg/ml to correct for the dilution of the urine. While evaluating the samples, it was seen that N-acetyltaurine is an endogenous compound, present at a range of 1.0 - 2.3 µg/ml in the urine of abstinent persons.

The N-acetyltaurine peak concentrations of 14 ± 2.6 µg/ml (range 9 - 17.5 µg/ml) appeared in every subject four to five hours after alcohol ingestion. It is seen that somewhere between 8 and 24 hours the concentration falls back to its endogenous level, this happens while low amounts of EtG are still present in the urine. This means that N-acetyltaurine can serve as a forensic alcohol marker, but that it is not a better marker than those routinely used.

**SAMENVATTING**

Alcohol, een legale drug, is een belangrijke oorzaak van ziekte en sterfte. Misbruik komt veelvuldig voor, wat aanleiding geeft tot consequenties op gezondheids- en socioeconomisch vlak. In de forensische wetenschappen zijn er al een aantal methodes om te bepalen of iemand alcohol gedronken heeft en wanneer dit plaatsvond.

Het hoofddoel van deze thesis is bepalen of N-acetyltaurine in urine dienst kan doen als een forensische alcohol merker en om deze te vergelijken met de merkers die reeds in routine settings worden toegepast. Een HILIC gebaseerde LC methode voor massa spectrometrische analyse van N-acetyltaurine werd ontwikkeld en gevalideerd. Na validatie werd de methode gebruikt om de urine stalen van een alcohol studie te testen. De LLOQ voor N-acetyltaurine werd vastegesteld als zijnde 50 ng/ml.

In deze alcohol studie dronken acht testpersonen een hoeveelheid alcohol die aanleiding gaf tot een promillage van 0.8‰. De eerste acht uur werden urine stalen genomen om de 1.5 - 2 uur, daarna werden stalen genomen om de 24 uur. Zowel het gehalte aan N-acetyltaurine als aan taurine werd genormaliseerd tot een 1 mg/ml creatinine concentratie, dit om te corrigeren voor de verdunning van de urine. Tijdens de metingen werd vastgesteld dat N-acetyltaurine een endogene component is. Het gehalte aan N-acetyltaurine in de urine van abstinente personen varieerde over een bereik van 1.0 - 2.3 µg/ml.

De N-acetyltaurine piek concentratie van 14 ± 2.6 µg/ml (bereik 9 - 17.5 µg/ml) verscheen in elke testpersoon vier tot vijf uur na alcohol ingestie. Endogene gehaltes worden terug bereikt tussen 8 en 24 uur na ingestie, op dit moment zijn er nog lage concentraties van EtG meetbaar. Dit betekent dat N-acetyltaurine dienst kan doen als een forensische alcohol merker, maar niet beter is in vergelijking met deze die reeds in routine settings worden gebruikt.

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| 5-HIAA | 5-hydroxyindole acid |
| 5-HIAL | 5-hydroxyindole acetaldehyde |
| 5-HTOL | 5-Hydroxytryptophol |
| ADH | Alcohol dehydrogenase |
| AUDIT | Alcohol Use Disorders Identification Test |
| BAC | Blood alcohol concentration |
| *C. Sordellii* | *Clostridium sordellii* |
| CDT | carbohydrate-deficient transferrin |
| CV | Coefficient of variation |
| CYP | Cytochrome P450 |
| *E. Coli* | *Escherichia coli* |
| ESI | Electrospray ionization |
| EtG | Ethylglucuronide |
| EtS | Ethylsulphate |
| FAEEs | Fatty acid ethyl esters |
| FDA | Food and drug administration |
| GGT | Gamma-glutamyl transpeptidase |
| H1-NMR | Proton-nuclear magnetic resonance |
| ISTD | Internal standard |
| LC-MS/MS | Liquid chromatography – tanden mass spectrometry |
| LLOQ | Lower limit of quantitation |
| LOD | Limit of detection |
| MCV | Mean corpuscular volume |
| MRM | Multiple reaction monitoring |
| NAD+ & NADH | Nicotinamide adenine dinucleotide |
| NAT | N-acetyltaurine |
| PEth | Phosphatidylethanol |
| PPM | Parts per million |
| RCF | Relative centrifugal force |
| $R_f$ | Retention factor |
| Rpm | Rotations per minute |
| RT | Retention time |
| TLC | Thin layer chromatography |

# 1. INTRODUCTION

## 1.1. ALCOHOL AND HEALTH PROBLEMS

Alcohol, a popular socially accepted drug, is consumed worldwide and is one of the top five risk factors causing illness, impairment and premature mortality.(1) As illustrated in figure 1.1, the correlation between alcohol and health is complex due to its J-shaped curve. It is seen that a small amount of alcohol is beneficial to the health of both men and women.(2) However, excessive consumption is worldwide the cause of 3.3 million deaths a year. This represents 5.9% of all deaths.(1)

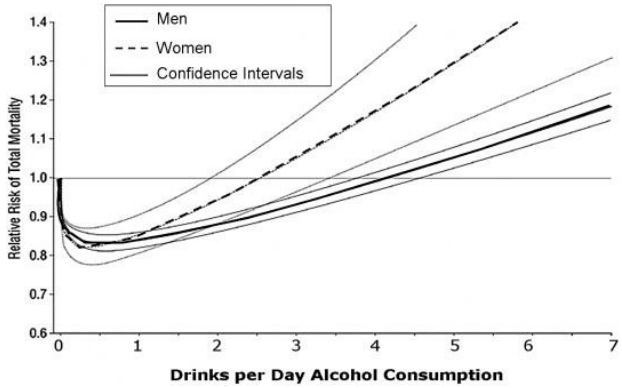



**Figure 1.1: The relationship between relative risk of total mortality and alcoholic drinks per day: a J-shaped curve.(2)**

Excessive alcohol consumption leads to health and socioeconomic consequences. People are more likely to develop different types of cancer like female breast cancer, mouth cancer, liver cancer, rectum cancer and other gastrointestinal cancers. The relationship between alcohol consumption and cancer is dose-dependent. The risk of injury is also significantly higher and people are at greater risk of getting infectious diseases like pneumonia and tuberculosis. Mental health gets compromised by alcohol abuse, leading to depression, anxiety and increased risk of suicide. Because of the effect of alcohol and alcohol withdrawal on the behavior, alcohol consumption has a negative influence on productivity, relationships, friendships and parenting. This means that not only the health cost burden for society is higher, but that it also has an impact on the economy and the social wellbeing of the person concerned and his surroundings. (1, 3, 4)

There are a few specific groups that are more sensitive to the harmful effects of alcohol including the elderly, those with underlying diseases or with concomitant medication-use, women and adolescents. Women are more sensitive due to their lower body mass, their higher body fat percentage and their smaller liver capacity in comparison with men. Because alcohol is hydrophilic, it will lead to a higher blood alcohol level when drinking the same amount as men, causing higher concentrations of alcohol in the brain. In adolescents alcohol has a harmful impact on the brain, because their brain is still maturating and more susceptible to the negative effects of alcohol in comparison with a completely developed adult brain. Furthermore, adolescents like to take risks and have less insight in the consequences of heavy drinking, thus they easily drink too much.(1, 4-6)

### 1.1.1. Trends in alcohol consumption

The recent trend in alcohol consumption is an increase in alcohol per capita. In Belgium 11 liters of pure alcohol per year was consumed per capita between 2008 and 2010. That is average compared to other European regions. Although it is expected that there will be a worldwide decrease in alcohol consumption the European region will still be the region where alcohol consumption is highest, due to cultural reasons.(1, 7)

In Belgium, the consumption peaks on Saturday and Sunday with the highest intake in the bigger cities like Antwerp and Brussels.(8) This leads to more alcohol-related traffic accidents. In 2012 about 63% of the drivers involved in a traffic accident underwent a breath alcohol test and 10.5% tested positive. This is a decrease of 0.1% compared to the amount of positive tests in 2011. Also the amount of traffic fatalities caused by excessive alcohol consumption decreased 6.3%, from 5829 fatalities in 2011 to 5461 in 2012. However, it has to be taken into account that this fatality rate might be underrated due to the fact that in most cases a breath alcohol test cannot be performed on seriously injured persons and no blood alcohol analysis might be requested after serious injury or death.(9)

Another trend seen in Belgium is that there are more and more reports about drunkenness in adolescents. Adolescents start drinking earlier, around the age of 12. The first contact with alcohol mostly happens in family environment.(5) Also the rate of binge

drinking is increasing among adolescents and young adults. Belgian first-year students at the faculties of engineering and psychology have approximately two to five episodes of binge drinking a week. This type of excessive alcohol consumption can lead to alcohol dependence in a later stage of life.(10)

Not only among young adults but also among adults the prevalence of binge drinking is increasing in several European countries, including Belgium. Binge drinking is defined as drinking 5 drinks or more during one consumption session or drinking more than 60 g of pure alcohol. Sometimes it is also called heavy episodic drinking.(11)

When comparing drinking habits in men and women, there are some remarkable differences. The percentage of abstainers is higher among women than men, and when women drink they drink less than men. Also the percentage of binge drinking is lower among women than among men. However, this difference between men and women is smaller in the European regions compared to worldwide drinking habits.(1) Furthermore, it is seen that women with higher education and higher socioeconomic standards drink more. The opposite behavior is seen among men. An explanation that has been put forward is that women experience more stress because of their bigger work-related responsibilities and thus drink more alcohol. The drinking habits of men do not seem to be affected by work-related stress.(12)

### 1.1.2. Alcohol metabolism in the human body

When consuming alcohol, the absorption takes place especially in the duodenum and the jejunum. The absorption in the stomach is rather limited. The rate of gastric emptying has a greater influence on absorption than the absorption in the stomach itself.(13)

Although there is not much absorption in the stomach, ethanol can get metabolized there. In the stomach ADH3 is present, an isoform of alcohol dehydrogenase, which can metabolize a small part of the ethanol in the stomach. Because this happens before absorption, it is a form of first pass metabolism. The same enzyme is also present in the liver. Thus the liver contributes to the first pass metabolism.(14)

When ethanol leaves the stomach and reaches the duodenum and jejunum it gets absorbed. Ethanol can cross biological membranes due to its hydrophilic character. Because of this, ethanol spreads rapidly in the blood and diffuses in different tissues according to the amount of water the tissue contains. Ethanol doesn't have affinity for plasma proteins.(13)

After being absorbed into blood, ethanol metabolism particularly takes place in the liver, but there are also non-liver tissues that can metabolize ethanol. There are two major elimination pathways: an oxidative pathway and a non-oxidative pathway.(15)

### 1.1.2.1. Oxidative metabolism

As illustrated in figure 1.2, there are three enzymatic systems responsible for the oxidative metabolism. The alcohol dehydrogenase (ADH) that is present in the cytosol of liver cells, the cytochrome P450 isozymes in the microsomes of the liver cells and other tissues like the brain, and catalase, that is present in peroxisomes. Approximately 90 - 95% of ethanol gets oxidized. Most of the remaining amount of ethanol is excreted unchanged in urine (0.5 - 2%), exhaled air (1.6 - 6%) and sweat (max. 0.5%). A rather small amount of the ethanol undergoes non-oxidative metabolism. (14, 16-18)

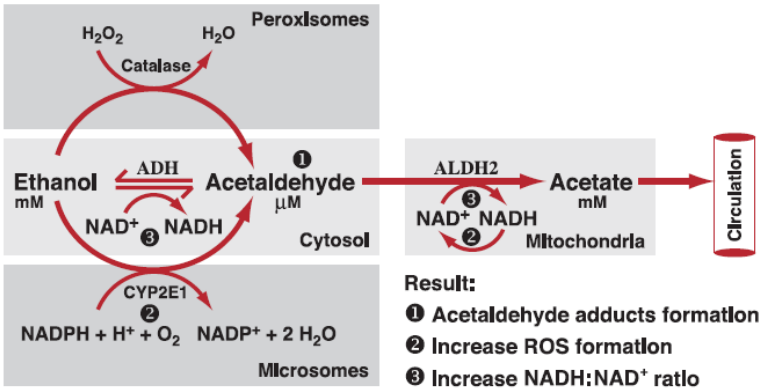



**Figure 1.2: The three enzymatic systems contributing to the oxidative metabolism of alcohol: alcohol dehydrogenase (ADH), cytochrome P450 2E1, catalase.(14)**

The most important of the three oxidative enzymatic systems is ADH. This enzyme oxidizes ethanol to acetaldehyde. Acetaldehyde can bind proteins and is seen as the product

that causes main tissue damage. Further oxidation of acetaldehyde by aldehyde dehydrogenase in the mitochondria leads to acetate. Both enzymes use $NAD^+$ as a cofactor in the oxidation process. This cofactor accepts the reducing equivalents from the ethanol and gets reduced to NADH + $H^+$. There are several isoforms of this enzyme, which explains the inter-individual differences in metabolism rate of ethanol. ADH is also present in other tissues like the kidneys and the uterus, but in smaller amounts compared to the amounts in the liver. (13, 15, 16)

The cytochrome P450 isoenzymes (CYPs) are a second group of enzymes that contribute to the oxidative metabolism of ethanol. CYP2E1 is the most important of the different isoenzymes considering the alcohol metabolism. It oxidizes ethanol to acetaldehyde. When drinking a moderate amount of alcohol, about 10% gets oxidized by CYP2E1. However, when drinking excessively the enzymes get induced and the amount of ethanol oxidized by CYP2E1 increases. CYP2E1 is also important in oxidizing ethanol in other tissues than the liver like the brain where the amount of ADH is limited.(13, 14)

The third pathway, catalase, is only an important pathway in fasted state. The enzyme uses $H_2O_2$ to oxidize ethanol, but due to the rather small amounts of $H_2O_2$ in the human body, this pathway is considered as being insignificant.(13, 14)

The elimination of ethanol occurs with a zero order kinetics (linear) at a rate of 0.1 to 0.2 per mille alcohol/hour, that is why after several hours – depending on the amount of consumed alcohol - ethanol cannot be detected in blood anymore.

#### 1.1.2.2. Non-oxidative metabolism

Although the amount of ethanol that undergoes non-oxidative metabolism is small, this process forms a lot of important diagnostic products. Non-oxidative metabolism consists of the formation of different types of products. These include ethyl esters of fatty acids, phosphatidyl ethanol, ethyl glucuronide and ethyl sulfate.(19)

Ethyl esters of fatty acids also known as fatty acid ethyl esters (FAEEs) originate from the reaction between ethanol and a fatty acid catalyzed by fatty acid ethyl ester synthases. This reaction occurs mainly in the endoplasmic reticulum. The catalyzing enzymes are present in

great numbers in the liver and the pancreas but are also present in brain, hearth and blood.(13, 14, 19)

Phospholipase D is the enzyme responsible for the formation of phosphatidyl ethanol (PEth). The enzyme converts phosphatidylcholine into phosphatidyl ethanol by transphosphatidylating it, which only happens in the presence of ethanol. When ethanol is not present, phosphatidic acid will be formed instead. This reaction occurs in brain, renal tissue and blood.(14, 19)

In the liver the enzyme uridine 5'-diphospho-glucuronosyltransferase (UDP-glucuronyltransferase) catalyzes the reaction between ethanol and glucuronic acid. This results in the formation of ethyl glucuronide (EtG). UDP-glucuronyltransferase is also present in other tissues like, the kidneys, the suprarenal gland, the spleen and others. This marker is not detectable when there is no ethanol intake.(13, 19)

Aside from glucuronidation, ethanol is also conjugated in the liver with sulfate to form ethyl sulfate (EtS). This reaction is catalyzed by cytosolic sulfotransferases. Less than 0.1% of the amount of ethanol that is ingested gets eliminated as EtS. (20-22)

## 1.2. ALCOHOL BIOMARKERS AND THEIR INTERPRETATION

### 1.2.1. General

Because excessive alcohol intake leads to several health and socioeconomic consequences, there is a growing demand for measures to control the alcohol consumption. There are a few alcohol consuming questionnaires like the Alcohol Use Disorders Identification Test (AUDIT) and CAGE (acronym for four questions), but these questionnaires lack reliability, because people tend to under-report their alcohol intake both deliberately as undeliberately. Due to this fact there is a need for an objective method of measuring the alcohol intake both on short-term and on long term.(1, 20, 23-25)

During the past ten years the focus has shifted from questionnaires to alcohol biomarkers, to assess the amount of alcohol consumed. A biomarker is defined as a substance that provides information about biological processes and gives an insight into the

current condition of the person and the possible future risks this condition may lead to. Ideally, an alcohol biomarker is sensitive and specific and allows differentiation between normal drinking, excessive drinking and alcohol abstinence. (16, 26, 27)

There are two types of alcohol biomarkers: indirect and direct markers. The difference between the two is that direct markers contain the ethanol structure and the indirect markers do not. The indirect markers consist of all types of liver enzymes like gamma-glutamyl transpeptidase (GGT) and carbohydrate-deficient transferrin (CDT). Also the mean corpuscular volume (MCV) is an indirect alcohol marker. The problem concerning indirect markers is that they are influenced by other factors than alcohol intake like pathological conditions, age, gender, body mass index, smoking status, anorexia and drugs. The sensitivity and the specificity of those markers are low and it takes a large amount of alcohol intake for a long time before there are any changes visible.(20, 24, 27-30) The direct markers are more suitable in the forensic and clinical assessment of alcohol intake. Ethanol, ethyl glucuronide, ethyl sulfate, phosphatidyl ethanol and fatty acid ethyl esters are the most important direct markers of alcohol intake. These markers are upregulated in the body after exposure to ethanol.(20, 24, 30) It is important to notice that exposure to ethanol is not always due to the intake of alcoholic beverages, ethanol is also present in maturated bananas, non-alcoholic beers, fruit juices and sauerkraut.(31) And even the use of alcohol containing hand sanitizers can lead to a positive alcohol marker result.(32)

Each marker can cover a certain period after the alcohol consumption. While the blood ethanol concentration only covers a short period after the alcohol intake, substances like EtG, EtS, PEth and FAEEs cover a longer period in which alcohol consumption can be detected. EtG and FAEEs can even be detected for several months in hair, but because hair has different growth phases a waiting period is necessary before these components can be found. Hair analysis is useful, but is prone to errors and contaminations.(20, 27)

The main disadvantage of these markers is that they still have a quite short detection window. Therefore the search for a better marker that can detect recent alcohol consumption over a longer period and can differentiate between social drinking, excessive drinking and alcohol abstinence is still ongoing. The purpose of this study is to detect an even better alcohol marker in urine. The emphasis is on N-acetyltaurine, a component already detected in mouse urine when they ingested alcohol.(33)

### 1.2.2. Ethanol

Ethanol as marker of alcohol consumption can only be used after recent alcohol intake. The peak concentration in blood is reached 20 to 60 minutes after alcohol ingestion. Depending on the amount ingested, ethanol cannot be detected in blood anymore approximately after six to eight hours.(16, 20) Only a small amount of the ingested ethanol reaches the urine unchanged with peak concentration two hours after ingestion. Ethanol can be detected in urine up to approximately seven hours after alcohol consumption, depending on the amount ingested.(17, 34) The combination of the amount of alcohol in the blood and the current state of the person concerned makes it possible to determine whether the person is a heavy drinker and thus tolerant to alcohol. A blood alcohol concentration higher than 1.5‰ without signs of alcohol intoxication indicates long-term heavy drinking.(28, 30) Due to its short half-life, ethanol is not a good marker for the detection of alcohol consumption for more than one day after the end of consumption. Therefore it is necessary to use other markers to cover longer time periods.

### 1.2.3. Ethyl glucuronide

Ethyl glucuronide is formed in the body only after consumption of alcohol. It can be detected in blood, hair and in urine. (35, 36) 0.5 - 1.6% of the total amount of alcohol ingested will be conjugated to EtG and 0.02 - 0.06% of the amount ingested will be excreted in the urine as EtG.(19, 20)

In blood, EtG reaches its peak concentration three to six hours after alcohol intake and is still detectable for six to eighteen hours after consumption, depending on the amount of alcohol that was ingested.(19) In urine EtG can still be measured thirteen to twenty hours after intake and even eighty-four hours if the ingested amount was high.(34) It is important when analyzing the urinary EtG that the amount of creatinine is also analyzed so that a correction for the dilution of the urine can be made.(31) In hair, EtG can be detected for several months. Really low amounts of alcohol intake are not detectable due to wash-out

effects. It must be taken into account that aggressive hair treatments can result in false negative results.(25, 37)

The disadvantage of using EtG as a marker is that it is so sensitive that even incidental intake of alcohol, like maturated bananas and the use of alcohol containing hand sanitizers can lead to a positive urinary result. That is why it is important that a good cutoff concentration is selected in order to prevent false positive results.(31, 38) Another disadvantage is that there can be a post-collection degradation of EtG when the urine sample is not stored properly. *E. coli* and *C. sordellii* are able to degrade EtG due to their β-glucuronidase activity, resulting in a false negative result. Because *E. coli* is the most common cause of a urinary tract infection it is important to keep this in mind. By using dried urine spots this phenomenon can be prevented.(15, 39, 40) Sex, age and renal function also have some influence on the amount of EtG in the urine.(35)

### 1.2.4. Ethyl sulfate

Like EtG, ethyl sulfate is only formed in the presence of ethanol. Approximately 0.1% of the alcohol amount ingested is conjugated with sulfate which results in the formation of EtS and 0.010 - 0.016% of the total amount of ethanol is excreted in the urine as EtS.(20) EtS reaches its peak concentration in urine after maximum two hours and can be measured for one-and-a-half days when 49 g ethanol was ingested.(27) In contrast to EtG the amount of EtS in the urine is not affected by the presence of bacteria.(40)

### 1.2.5. Phosphatidylethanol

In the past PEth was considered a good biomarker in blood due to its linear relationship with the amount of alcohol consumed. The half-life of PEth in blood is four days and it is possible to detect alcohol consumption several weeks longer than based on ethanol detection in blood.(19, 35, 41)

Because PEth is not influenced by age, sex, liver disease or hypertension, it is a good biomarker for alcohol intake.(35, 42) The only disadvantage is that when stored at a temperature of -20 ˚C or at room temperature additional PEth is formed from ethanol, present in the sample, which can cause false positive results. This can be prevented by storing the samples at -80˚C or by using dried blood spots.(35, 43)

In the past only prolonged and more than moderate intake of alcohol could increase PEth in a detectable amount, but thanks to improvements to the sensitivity of the analytical method a single dose of alcohol can result in a detectable amount of PEth.(41, 44)

### 1.2.6. 5-HTOL/5-HIAA ratio

5-Hydroxytryptophol (5-HTOL) is an indirect marker of alcohol consumption and is a component derived from serotonin that is always present in the human body. The normal ratio in blood between 5-HTOL and 5-hydroxyindole acid (5-HIAA), both degradation products of serotonin, is 0.007 - 0.01, but after the intake of ethanol the ratio increases to 0.4 - 0.5. Ethanol is responsible for this increase in ratio because acetaldehyde, a degradation product of ethanol, gets metabolized by aldehyde dehydrogenase and thus competes with 5-hydroxyindole acetaldehyde (5-HIAL), the precursor of both 5-HTOL and 5-HIAA, for this enzyme. This leads to a decrease in 5-HIAA formation and an increase in 5-HTOL.(19, 45, 46)

5-HTOL/5-HIAA is elevated in blood four to seven hours after the end of the alcohol intake and up to eighty hours this is still detectable. Although 5-HTOL and 5-HIAA are degradation products of serotonin, the intake of serotonin rich foods and selective serotonin reuptake inhibitors does not influence this ratio. The disadvantage of using the 5-HTOL/5-HIAA ratio as an alcohol marker is that the ratio is difficult to interpret and thus is not very useful in distinguishing between social and excessive alcohol consumption.(16, 30, 45)

### 1.2.7. Fatty acid ethyl esters

FAEEs are a group of ethyl esters that consists among others of ethyl myristate, ethyl palmitate, ethyl oleate and ethyl stearate. They are detectable both in blood and hair. The half-life of FAEEs in blood is sixteen hours and FAEEs are still measurable twenty-four hours after alcohol consumption and even up to ninety-nine hours in heavy drinkers. In hair, the detection of FAEEs serves as a marker for long term heavy drinking. The disadvantage of FAEEs is that even teetotalers have small amounts of FAEEs in blood and hair, as FAEEs seem to be endogenous substances.(19, 20, 30, 47, 48)

The detection of FAEEs in hair is often used in postmortem analysis to determine whether there was an excessive alcohol intake two weeks prior to death. The incorporation of FAEEs in hair can be caused by three possible pathways. The first pathway is assumed to be incorporation of FAEEs from the blood in the cells of the hair root. The second pathway is that ethanol from the blood diffuses in the hair roots and there it gets metabolized to FAEEs. The third possible pathway is that ethanol gets metabolized to FAEEs in the sebaceous glands and then the FAEEs diffuse in the hair when excreted with the sebum. It has been reported that the second and third pathway are the most probable routes.(47)

### 1.2.8. N-acetyltaurine

The aim of this study is to determine whether N-acetyltaurine (NAT) can be used as a biomarker of alcohol consumption, to develop and validate an LC-MS/MS method, and finally to investigate if NAT has any added value in comparison to the other known biomarkers mentioned before.(49)

Recently, it has been reported that in mice the intake of ethanol leads to an increase in NAT excretion. After the metabolization of ethanol, a large amount of acetyl-CoA and acetate are present in the body. These compounds deliver the acetyl group necessary for the synthesis of NAT from taurine. The biosynthesis has not completely been figured out yet, but there are two possible routes. The first route, probably the main route, is a direct interaction

between the acetate and the taurine, whilst the second route is a reaction between acetyl-CoA and taurine.(33)

Taurine is a component that is already present in the body and 0.1% of the total body weight consists of taurine. Although it is chemically not an amino acid due to the lack of the carboxyl group, it is seen as the most abundant amino acid in the human body. It is a zwitterion at every physiological pH value. Because of this, it is highly water soluble and cannot cross biological membranes. Taurine has different important biochemical roles in the human body. Taurine plays an important role in protecting cell membranes, either because it can eliminate toxic compounds or acts as an osmoregulator. Also during the development taurine is quite important. It is even considered as an essential amino acid in newborns. Besides this, there are several other important functions of taurine in the human body.(50)

Because newborns are not yet able to synthesize taurine by themselves they have to ingest it. Breast milk is a great source of taurine for newborns but also other food products can provide taurine. The highest concentrations of taurine are found in animal products like meat, seafood, and milk. There is no taurine in vegetables, which means that vegetarians have rather low taurine plasma concentrations. However, in the liver and in the brain synthesis of taurine can take place. The synthesis consists of different steps, with involvement of methionine and cysteine. Furthermore, vitamin $B_6$ is necessary, as it acts as a co-factor for the different enzymes involved in the process.(50, 51)

The main disadvantage of N-acetyltaurine as a biomarker for alcohol intake is that this compound is also formed after an endurance exercise like a marathon. In order to determine whether N-acetyltaurine can be used as an alcohol marker, further investigation is needed to compare the amount that is detectable after an endurance exercise and the amount that is detectable after alcohol intake. If the NAT-level after alcohol consumption is much higher than after an endurance exercise, NAT can be used as an alcohol marker. Another option is determining more than one alcohol marker in order to detect alcohol consumption.(52)

## 1.3. INSTRUMENTATION

### 1.3.1. LC-MS/MS

LC-MS/MS is a technique that is widely used in forensic toxicology. It is the combination of liquid chromatography and tandem-mass-spectrometry. In this study LC-MS/MS is applied to detect NAT and other compounds such as creatinine and taurine in urine.

In order to detect the different compounds, these need to be separated. This happens during LC by using an appropriate column, stationary phase and liquid (mobile) phase. Because of the different interactions between the stationary phase and the mobile phase with the compounds in the sample, a good separation can be achieved. After the sample is injected, the mobile phase drags the sample along through the column. Depending on the affinity of the different compounds in the sample, they will either stay in the column or move with the mobile phase. By slowly changing the composition of the mobile phase the affinity of the compounds to the stationary phase changes and they elute at different retention times (RT). By searching the ideal combination of mobile phase and stationary phase and by adjusting the parameters, the different compounds can get released of the column one at a time.(53, 54)

After being separated, the different compounds are detected by a tandem mass-spectrometer. First the compounds are charged by using electrospray ionization (ESI). This technique makes use of charged droplets that shrink due to a drying gas until electrostatic forces make them explode and charge the analytes in the sample. After this the desired analytes are selected in the quadrupoles. As illustrated in figure 1.3, there are three quadrupoles. The first quadrupole ($Q_1$) of the mass spectrometer acts as a filter and selects the desired analytes based on the mass-to-charge ratio. In the second quadrupole ($Q_2$) the desired analytes get fragmented by collision of the compound with a neutral collision gas. In the third quadrupole ($Q_3$) there is another selection step based on the mass-to-charge ratio of the fragments. This process is called multiple reaction monitoring (MRM).(55-59)

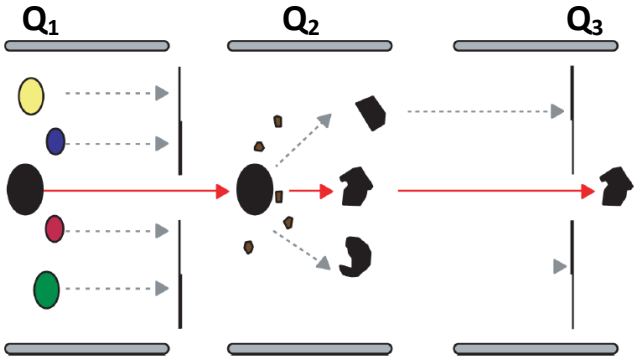



**Figure 1.3: The three quadrupoles. $Q_1$ acts as a filter: only the analyte with the desired mass-to-charge ratio can get passed. Then fragmentation happens in $Q_2$ due to a neutral collision gas. In $Q_3$ a specific fragment is selected based on the mass-to-charge ratio.(59)**

## 2. OBJECTIVES

Nowadays, several different alcohol markers are used to determine whether a person abstained from alcohol or not. Among others, these include ethylglucuronide, ethylsulfate, phosphatidylethanol and fatty acid ethyl esters. However, examining the amounts still left in urine is only possible up to approximately four days after drinking, depending on which marker is observed and which amount of ethanol was ingested. Furthermore, all these markers have their own disadvantages, for example post-collection degradation or formation of EtG, which have to be taken into account. That is why the search for a marker that is longer detectable both in urine and in blood, and has no disadvantages is still ongoing.

The aim of this study was to validate an LC-MS/MS method to measure N-acetyltaurine in urine, in order to investigate this newly discovered alcohol marker and its potential for abstinence monitoring or other purposes. The sample preparation was based on protein precipitation of the urine samples and dilution. After work up, the samples underwent chromatographic separation with a SeQuant® ZIC®-HILIC column and were measured in MRM mode with a QTRAP 5500 triple-quadrupole mass spectrometer.

To validate the method, internal standards for every compound were added to the standard solutions made in water. The criteria for validation were defined by the Guidance for Industry with focus on Bioanalytical Method Validation from the U.S. Department of Health and Human Services (FDA guidelines) and by the guideline on bioanalytical method validation from the European Medicines Agency. The mean accuracy had to be within 15% of the predetermined concentration, only at the LLOQ the mean accuracy was allowed to be within 20% of the actual value. The precision, expressed as CV%, should be kept below 15%, except at the LLOQ where a maximum of 20% was accepted. The different experiments for validation should include: selectivity, matrix effect, extraction efficiency, process efficiency, carry over, autosampler stability and freeze-thaw stability. Finally, after validation this procedure should be used to measure the amount of N-acetyltaurine in urine sampled during a drinking study with eight volunteers, which was previously performed at the institute.

# 3. MATERIALS AND METHODS

## 3.1. MATERIALS

### 3.1.1. Chemical and reagents

Taurine, acetic anhydride, acetyl chloride, creatinine and $D_3$-creatinine were obtained from Sigma Aldrich (Buchs, Switzerland). Dichloromethane, ethanol, pyridine, acetic acid and ammonium acetate were purchased from Merck (Darmstadt, Germany). Water was deionized with a Milli-Q water system from Millipore (Billerica, USA). $D_4$-taurine was ordered from Toronto Research Chemicals (Toronto, Canada). Acetonitrile, HPLC for gradient analysis, was obtained from Acros Organics (Geel, Belgium). Triethylamine, formic acid and ninhydrin were purchased from Fluka Chemie (Buchs, Switzerland). N-Acetyltaurine was obtained from Carbosynth (Berkshire, UK). Deuterated DMSO (99.9%) was obtained from Cambridge Isotope Laboratories (Tewksbury, MA, USA)

### 3.1.2. Material and laboratory instrumentation

For handling liquids different types of pipettes were used, such as the electronic Gilson Pipetman® 200M (systematic error ±1.00 µL, random error ≤0.26 µL) and 1000M (systematic error ±5.00µL, random error ≤1.1 µL). Urine and internal standard stock solution were inserted using a Gilson REPETMAN® positive-displacement pipette. Smaller amounts were added by using positive-displacement pipettes from Gilson MICROMAN®, the M10 (systematic error ±0.15 µL, random error ≤0.06 µL), M25 (systematic error ±0.30µL, random error ≤0.10 µL) and M50 (systematic error ±0.70 µL, random error ≤0.30 µL) were used.

To homogenize the different samples a Vortex-Genie® 2 from Scientific Industries, Inc. (New York, USA) and a VIBRAX® VXR basic from IKA (Staufen, Germany) was used. The MIKRO 22 R centrifuge form Hettich (Tuttlingen, Germany) was used for centrifugation. The samples were dried under reduced pressure (approximately 0.18 bar) by using a CentriVap concentrator from LABCONCO® (Missouri, USA) at 60°C. A Mettler Toledo analytical balance type XS603S and AT200 was used for weighing substances (Greifensee, Switzerland). During

the different synthesis attempts both the lab disc from IKA (Staufen, Germany) and the magnetic stirrer hot plate from R. C. Kuhn (Bern, Switzerland) were used.

## 3.2. METHODS

### 3.2.1. Liquid chromatography gradient

For the liquid chromatography an UltiMate® 3000 UHPLC+ focused system with an UltiMate® 3000 RS auto sampler and a heated column compartment from Dionex (Olten, Switzerland) was used. 10 µl of the samples were injected onto a SeQuant® ZIC®-HILIC 3.5 µm, 100Å, 150*2.1mm PEEK coated HPLC column from Merck (Darmstadt, Germany) with a 100 µl auto sampler. The flow-rate during the chromatography was set at 0.30 ml/min. The LC-gradient is illustrated in table 3.1. Mobile phase A consisted of water with a 5 mM ammonium acetate buffer at pH 5.72, while mobile phase B consisted of acetonitrile with 0.1% formic acid. During the run the column compartment was kept at 30°C.

**Table 3.1: LC-gradient for the separation of the different compounds.**

| Retention (min) | %B |
|---|---|
| **0** | 95% |
| **1** | 95% |
| **4.5** | 20% |
| **9** | 20% |
| **10** | 95% |
| **14p** | 95% |

### 3.2.2. Tandem mass spectrometry

For the mass spectrometric detection a QTRAP 5500 triple-quadrupole mass spectrometer from AB Sciex (Rotkreuz, Switzerland) with Analyst software (version 1.6.2) was used in MRM mode. The analytes were ionized by electrospray ionization in negative mode. The instrument parameters were: -4250V ion source voltage, 550°C temperature, medium collision gas, curtain gas: 35, gas1: 40 and gas2: 40. The MRM transitions for the

different compounds are illustrated in table 3.2. The first MRM transition is the quantifier and the second is the qualifier, the latter to confirm the presence of the compounds. Each measurement took approximately 14 minutes, which consisted of 3731 cycles of 0.2751 seconds per cycle.

**Table 3.2: MRM transitions for the different compounds.**

| Compound | Q1 (m/z) | Q3 (m/z) | Time (msec) | DP (volts) | CE (volts) | CXP (volts) |
|---|---|---|---|---|---|---|
| **AcTaur MRM1** | 166 | 107 | 20 | -120 | -30 | -16 |
| **AcTaur MRM2** | 166 | 124 | 20 | -120 | -30 | -16 |
| **D4-AcTaur** | 170 | 128 | 20 | -120 | -30 | -16 |
| **Creatinine MRM1** | 112 | 68 | 20 | -55 | -27 | -8 |
| **Creatinine MRM2** | 112 | 41 | 20 | -55 | -27 | -8 |
| **D3-Creatinine** | 115 | 42 | 20 | -55 | -27 | -8 |
| **Taurine MRM1** | 124 | 80 | 20 | -5 | -26 | -21 |
| **Taurine MRM2** | 124 | 124 | 20 | -5 | -10 | -16 |
| **D4-Taurine** | 128 | 79 | 20 | -105 | -30 | -19 |

### 3.2.3. Synthesis of N-acetyltaurine

#### 3.2.3.1. Synthesis attempt 1

The first synthesis attempt was based on the paper from Jokiel *et al*..(49) First 1 g of taurine was dissolved in 1 ml of water. Then 5 ml pyridine and 4 ml acetic anhydride were added separately. The reaction is illustrated in figure 3.1. The mixture was stirred for eight hours at room temperature and then left at -20°C for 16 hours. To obtain pure N-acetyltaurine, the mixture was dried under reduced pressure (1*10^-2 mbar). The dry load method with 2 g of SiliaFlash® P60 from Silicycle Inc. (Québec, Canada) with size 230-400mesh was used to get the mixture completely dried. After this, the powder was resolubilized in just enough water to dissolve. Then the reaction was checked by spotting the mixture with glass capillaries onto a thin layer chromatography (TLC) silica gel 60 $F_{254}$ plate from Merck (Darmstadt, Germany). After six minutes elution with a solution of ethanol/dichloromethane (1:1; v/v), the plates were dried and sprayed with a ninhydrin-solution consisting of 100 ml 1-butanol mixed with 3 ml acetic acid and 0.6 g of ninhydrin. The plates were then dried with a heat-gun until colored spots appeared. As illustrated in

figure 3.2, coloring resulted in reddish spots for the taurine (primary amine) and yellow spots for the N-acetyltaurine (secondary amine). The $R_f$ value (Retention factor) was 0.25 for taurine and was 0.52 for N-acetyltaurine.

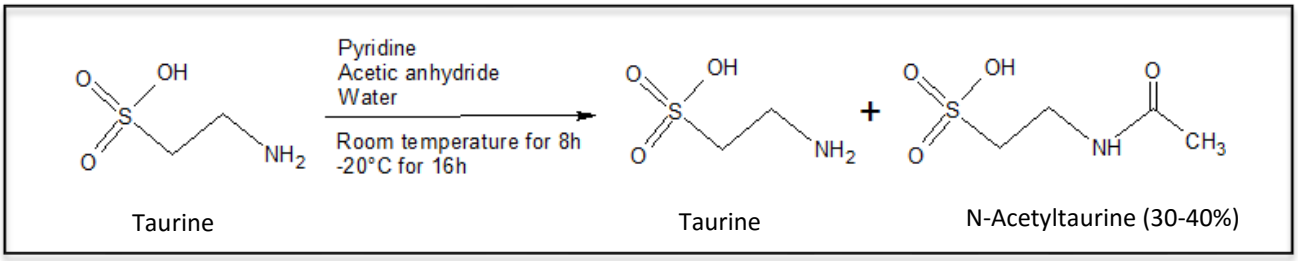



**Figure 3.1: First method of synthesis of N-acetyltaurine out of taurine in aqueous conditions.**

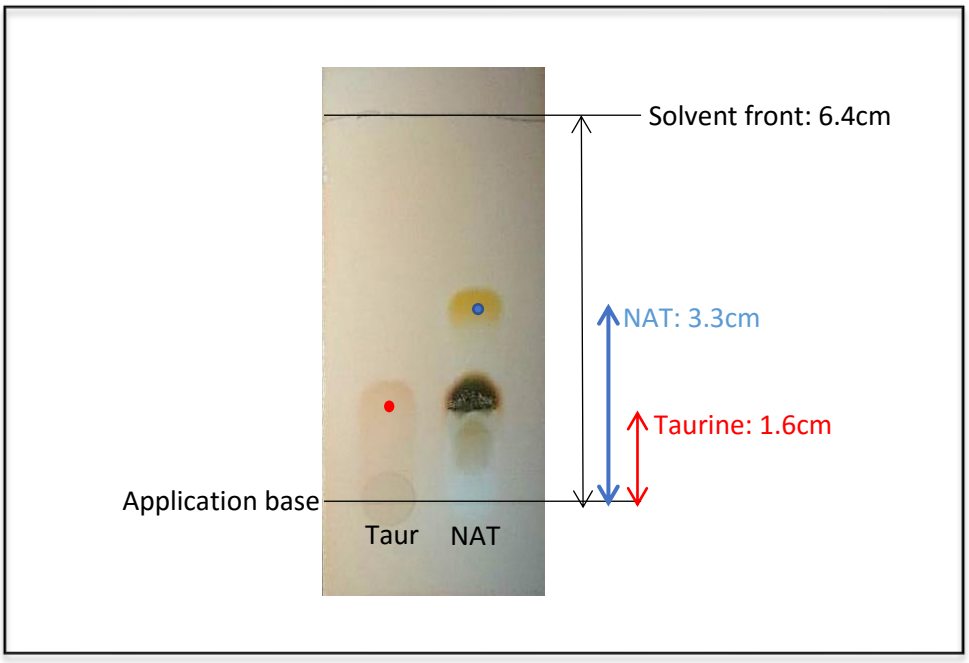



**Figure 3.2: TLC plate from the mixture after the reaction compared to a taurine reference solution. $R_f$ value taurine: 0.25. $R_f$ value NAT: 0.52.**

After the TLC confirmed that N-acetyltaurine was formed, an excess of ethanol was added to the mixture to lower the boiling point of the water so that evaporation of water was easier. Then another drying step was executed by using the Rotavapor®-RE from Büchi (Flawil, Switzerland) at a pressure of 175 mbar and with the Waterbath B-480 from Büchi

(Flawil, Switzerland) at 60°C. Afterwards, to get the mixture completely dry, a high vacuum line was applied.

The dried powder was then transferred onto a 40 g RediSep Rf Gold® Normal-Phase Silica column from Teledyne Isco (Lincoln, USA) for clean-up. For this clean-up ethanol/dichloromethane (1:1; v/v) was used. This procedure was carried out by the CombiFlash® Rf 200 from Teledyne Isco (Lincoln, USA). The flow rate was set at 30 ml/min and the column was first wetted with five column volumes. At the end 52 fractions of approximately 16 ml were collected and tested by using the same TLC conditions as before. Despite the fact that this method was mentioned in literature, it was seen on the TLC plates that there were no clean fractions. A mixture of both taurine and N-acetyltaurine was present in the different fractions.

#### 3.2.3.2. Synthesis attempt 2

A second method for the synthesis of N-acetyltaurine, based on a Chinese patent, was executed as illustrated in figure 3.3. The main difference with this method was the fact that they used high-taurine to synthesize high-acetyltaurine, which both have one carbon more than the normal taurine and N-acetyltaurine.(60) For this method 8.5 ml of absolute ethanol was mixed with 1 g of taurine whilst stirring. Then 1.95 ml of triethylamine and 0.85 ml of acetic anhydride were added dropwise to the mixture at room temperature. For 20 - 24 hours the mixture was heated under reflux at a temperature of approximately 40°C by using the magnetic stirrer hot plate. After this, the mixture was cooled on an ice bath and then 5 ml of acetyl chloride was added.

The mixture was then stirred at room temperature for about 20 hours. Around 30 ml of ethanol was then used to filter the mixture over a Faltenfilter Ø185 mm from Schleicher & Schüll AG (Feldbach, Switzerland). The filtrate was dried under reduced pressure with the Rotavapor®-RE 111 from Büchi (Flawil, Switzerland) and with the Waterbath 461 from Büchi (Flawil, Switzerland) at 50°C. When completely dry, approximately 15 ml of dichloromethane was added and the mixture was dissolved under reflux. Crystallization happened by leaving the mixture at room temperature for approximately 20 hours. Afterwards the last residue of

solvent was removed under reduced pressure. The crystals were cleaned by filtrating them with ice cold ethanol over a P4 glass frit Buchner filter. Eventually the crystals were dried under reduced pressure. The method resulted in 1.030 g of white powder.

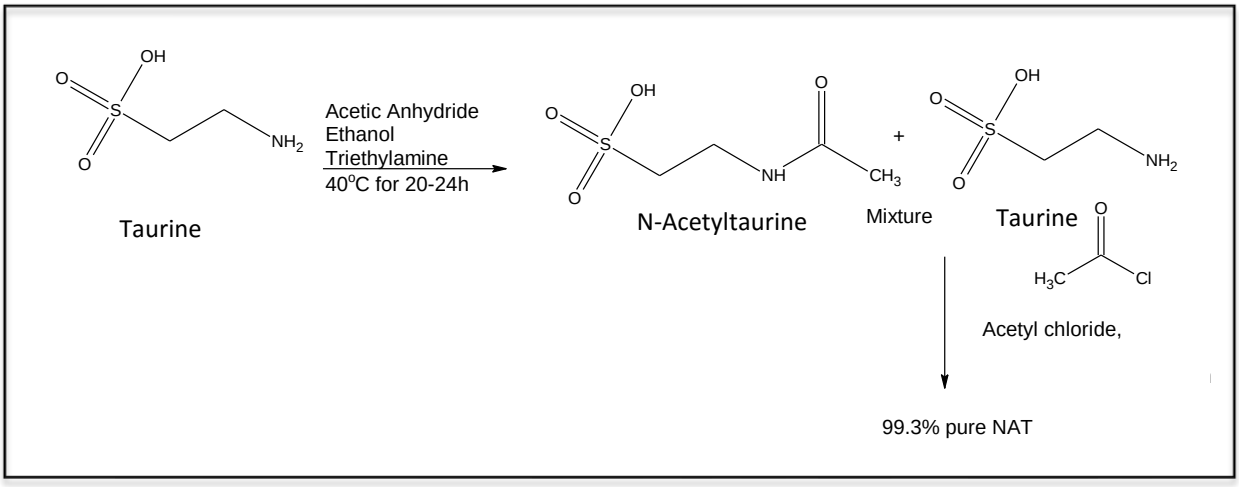



**Figure 3.3: Second method of synthesis of N-acetyltaurine out of taurine in organic conditions.**

To determine whether the expected yield of 99.3% of N-acetyltaurine in the second method was achieved, LC-MS/MS and $H_1$-NMR were performed. The residue of taurine in the sample was determined by measuring the concentration of taurine with LC-MS/MS in 4000 ng synthesized powder dissolved in 0.1 ml water. The calibrators of taurine were made by diluting taurine in water. Seven calibrators were made with concentrations of 10 ng/ml, 20 ng/ml, 100 ng/ml, 500 ng/ml, 1000 ng/ml, 1500 ng/ml and 4000 ng/ml. At each calibrator a fixed amount of internal standard for taurine was added. N-acetyltaurine internal standard was added to the sample in order to compare the signal intensity for 40 ng/ml D4-acetyltaurine to 400 ng/ml N-acetyltaurine.

With the AVANCE II 500 IOCSP2 $H_1$-NMR from Bruker (Fällanden, Switzerland) the amount of N-acetyltaurine in the powder was measured and the presence of other compounds was checked. The $H_1$-NMR was executed by dissolving approximately 0.5 mg of the synthesized powder in 45 µl of DMSO-*d6* and transferred into 1.7 mm NMR tubes.

### 3.2.4. Synthesis of deuterated-acetyltaurine

Deuterated-acetyltaurine was synthesized by dissolving 2 mg of D4-taurine in 400 µl of water. Then 143 µl pyridine and 114.5 µl acetic anhydride were added. The reaction mixture was stirred for eight hours at room temperature and then left at -20°C for sixteen hours. The solvents were evaporated to dryness at 60°C under vacuum (approximately 0.18 bar) with a CentriVap concentrator from LABCONCO® (Biolabo Scientific Instruments, Switzerland). The residue was reconstituted in 0.8 ml water. According to literature, the yield of the reaction is approximately 40%, which means that the concentration of the solution is around 1 mg D4-acetyltaurine per milliliter. In contrast to the synthesis of N-acetyltaurine, a clean-up step is not necessary due to the fact that there is no D4-taurine in the urine. This means that although there will be D4-taurine still present in the mixture, this will not interfere with the measurements.

### 3.2.5. Preparation of the internal standard solution

The internal standard solution was made by adding 0.1 ml of a 1 mg/ml D3-creatinine solution to 0.12 ml of a 0.025 mg/ml D4-taurine solution. Then 0.12 ml of a 0.025 mg/ml D4-acetyltaurine solution in 95/5 acetonitrile/water with 0.1% formic acid was added and the mixture was filled with acetonitrile in a volumetric flask to a total of 20 ml. The concentration of the internal standard solution is illustrated in table 3.3.

**Table 3.3: Concentration of the different compounds in the internal standard stock solution**

| Compound | Concentration in ISTD stock solution (µg/ml) |
|---|---|
| **D3-Creatinine** | 5.00 |
| **D4-taurine** | 0.15 |
| **D4-Acetyltaurine** | 0.15 |

### 3.2.6. Quality control and calibration standards

For calibration, a calibration series with N-acetyltaurine, taurine and creatinine was made in water. For NAT and taurine quality control and calibration samples, two individual stock solutions were made. One of 2.5 mg/ml was made by dissolving 13.2 mg of 95% pure N-acetyltaurine and 12.5 mg of >99.5% pure taurine in 5 ml of water (StockA) and one of 1.625 mg/ml was made by dissolving 8.5 mg of NAT and 8.1 mg of taurine in 5 ml of water (StockB). Two stock solutions were also made for creatinine. The first was made by dissolving 5 mg of creatinine in 1 ml of water (StockC). The second was made by dissolving 2.6 mg of creatinine in 1 ml of water (StockD). Seven calibration solutions ($K_1$-$K_7$) and five quality control solutions (LLOQ-$QC_4$) were made according to the dilution series in table 3.4 and 3.5.

**Table 3.4: Dilution series for the calibration solutions.**

| Calibration working solutions | K7 | K6 | K5 | K4 | K3 | K2 | K1 |
|---|---|---|---|---|---|---|---|
| H2O (µl) | 480 | 760 | 500 | 600 | 800 | 750 | 500 |
| From solution | StockA[a] | C7 | C6 | C5 | C4 | C3 | C2 |
| Addition of (µl) | 20 | 240 | 500 | 300 | 200 | 250 | 500 |
| From solution | StockC[b] | / | / | / | / | / | / |
| Addition of (µl) | 500 | / | / | / | / | / | / |
| Conc. Crea (µg/ml) | 2500 | 600 | 300 | 100 | 20 | 5 | 2.5 |
| Conc. NAT (µg/ml) | 50 | 12 | 6 | 2 | 0.4 | 0.1 | 0.05 |
| Conc. Taurine (µg/ml) | 50 | 12 | 6 | 2 | 0.4 | 0.1 | 0.05 |

[a] 2.5mg/ml solution of taurine and NAT
[b] 5mg/ml solution of creatinine

**Table 3.5: Dilution series for the quality control solutions.**

| Quality control working solution | QC4 | QC3 | QC2 | QC1 | LLOQ |
|---|---|---|---|---|---|
| **H2O (µl)** | 480 | 846 | 500 | 800 | 900 |
| **From solution** | **StockB**[a] | QC4 | QC3 | QC2 | QC1 |
| **Addition of (µl)** | 20 | 154 | 500 | 200 | 100 |
| **From solution** | **StockD**[b] | / | / | / | / |
| **Addition of (µl)** | 500 | / | / | / | / |
| **Conc Crea (µg/ml)** | 1300 | 200 | 100 | 20 | 2 |
| **Conc. NAT (µg/ml)** | 32.5 | 5 | 2.5 | 0.5 | 0.05 |
| **Conc. Taurine (µg/ml)** | 32.5 | 5 | 2.5 | 0.5 | 0.05 |

### 3.2.7. Method validation criteria for LC-MS/MS

For validation, the criteria from the Guidance for Industry with focus on Bioanalytical Method Validation from the U.S. Department of Health and Human Services (FDA guidelines) and the criteria of the guideline on bioanalytical method validation from the European Medicines Agency were used.(61, 62)

#### 3.2.7.1. Selectivity

Selectivity was investigated by spiking different analyte concentrations and internal standards to a urine sample resulting in a calibration curve. It was compared to water spiked with the three analytes at calibration concentrations. This was measured together with six blank urine samples from abstinent people, to which internal standard was added as well.

[a] 1.625mg/ml solution of taurine and NAT
[b] 2.6mg/ml solution of creatinine

#### 3.2.7.2. Calibration in double

To validate the whole procedure, a calibration in double was used. This means, that the calibration samples were measured first, followed by the quality controls and blanks. Afterwards another calibration series was measured. The calibration curve consisted out of the mean of the two measurements during each of the three series.

#### 3.2.7.3. Accuracy and precision

To determine accuracy and precision, two seven point calibrations, four quality controls for creatinine and five for both taurine and N-acetyltaurine were measured. The mean accuracy had to be within 15% of the predetermined concentration, only at the LLOQ the mean accuracy was allowed to be within 20% of the actual value.

The coefficient of variation (CV) was used to determine the precision. The precision, expressed as CV%, could not be more than 15% at each quality control concentration level, except at the LLOQ, there the precision could not be more than 20%. To determine the inter-assay precision and accuracy the quality controls were each measured six times during one run and three runs were performed.

#### 3.2.7.4. Autosampler stability

The quality control samples, in the range of the expected concentrations, were reinjected after being stored in the autosampler at 8°C for three and seven days to check autosampler stability. The LLOQ was not reinjected, due to the fact that its amount is lower than normally seen in real samples. The results were calculated by using the calibrator curve from the initial measurement, followed by comparing the mean results of the seven samples per concentration to the mean results of the calculated concentrations that were obtained

from the first measurement right after extraction. The mean concentration should be within ±15% of the concentration from the first measurement.

#### 3.2.7.5. Freeze-thaw stability

Six blank urine samples were frozen over night at -20°C and afterwards thawed for at least eight hours at room temperature. This was repeated three times in order to check the freeze-thaw stability of the different compounds in urine. After the third thawing step, the endogenous concentrations were measured by using the initial calibrator curve. This was then compared to the concentration of the different compounds in urine samples of the same stock solution that did not undergo freeze-thaw-cycles. The difference should not be higher than ±15%.

#### 3.2.7.6. Limit of detection and lower limit of quantitation

To determine the limit of detection (LOD), $K_1$ was diluted with water into four samples with concentrations of 25 ng/ml, 12.5 ng/ml, 6.25 ng/ml and 3.125 ng/ml. These samples were then measured and the LOD was the concentration of the sample which had a 3-to-1 signal to noise ratio.

The lower limit of quantitation (LLOQ) was set at the concentration which had a 10-to-1 signal to noise ratio and that had a precision of 20% and accuracy of 80-120%.

#### 3.2.7.7. Matrix effect, extraction efficiency and process efficiency

To determine matrix effect, extraction efficiency and process efficiency, three sample sets were made as illustrated in table 3.6. Each sample set consisted out of three times three samples with different concentrations.(63) The sample with the highest concentration

contained 32.5 µg/ml taurine and NAT, and 1250 µg/ml creatinine. The second highest sample contained 16.25 µg/ml taurine and NAT, and 625 µg/ml creatinine. The third sample contained the lowest concentration of 8.125 µg/ml taurine and NAT, and 312.5 µg/ml creatinine.

**Table 3.6: Sample preparation and concentration of the sample sets used to determine matrix effect, extraction efficiency and process efficiency.**

| | **Sample preparation** |
|---|---|
| **Set 1** | Spiked urine was extracted, afterwards ISTD was added. Then the mixture was dried and reconstituted. |
| **Set 2** | Urine was extracted and dried. Then it was spiked with analyte in water and ISTD was added. Then it was dried again and reconstituted. |
| **Set3** | Spiked water with ISTD, total volume dried and reconstituted. |

The extraction efficiency was determined for each compound by dividing the area of set 1 by set 2. To calculate the process efficiency the area of set 1 was divided by the area of set 3. For the matrix effect the area of set 2 was divided by the area of set 3. Due to the fact that in blank urine the three compounds are always present, the signals of set 1 and set 2 had to be corrected. The corrected signal was calculated as following:

$$corrected\ signal = measured\ signal * \frac{spiked\ concentration}{spiked\ concentration + (0.96) * endogenous\ concentration}$$

The 0.96 correction factor was only necessary for set 1 due to the fact that in set 1 a dilution happened of the endogenous amount by adding the spiked amount of the three analytes to the sample.

The same calculations were made based upon the calculated concentrations. By comparing the ratio of the areas to the ratio of the concentrations it can be determined if

the internal standard corrects for ionization and for matrix effect. Extraction efficiency is not corrected by adding ISTD, due to the fact that ISTD is added after extraction.

#### 3.2.7.8. Carry over

Carry over was checked by measuring $K_7$ three times, followed by two times pure water. The signal for the compounds measured in pure water was compared to the signal of the compounds in $K_7$. Due to the fact that the amount in the LLOQ is lower than the amount in real samples, the signals are compared to the signals in $K_7$. The ratio should be preferably lower than 1%.

### 3.2.8. Sample preparation

To prepare the urine samples, to 50 µl of urine 150 µl internal standard stock solution was added (in a microtube) and then vortexed for 10 minutes. Then the samples were centrifuged at a speed of 13000 rpm (16000 RCF) at a temperature of 8°C. After centrifuging, 100 µl of the supernatant was transferred into a glass vial. The samples were then dried under reduced pressure (approximately 180 mbar) at 60°C and reconstituted with a 400 µl solution of acetonitrile/water (95/5) with 5 mM ammonium acetate buffer of pH 5.72 and 0.1% of formic acid. The same procedure was used to prepare the calibrators and the quality control samples for injection.

### 3.2.9. Drinking study

To determine the changes of N-acetyltaurine in urine after drinking alcohol, a drinking study was performed. It consisted out of eight volunteers, two female and six male. Before the drinking study, the volunteers had to be abstinent for at least two weeks. Based on the

Widmark equation, the amount of vodka mixed with soft drink which led to a BAC of 0.8‰, was calculated.(64)

At the start of the drinking study, blank urine samples were obtained (t=0). Then alcohol was ingested in the hour after the blank samples were taken. The second urine sample was obtained one hour after the ingestion, thus two hours after the start of the drinking study. Other urine samples were obtained approximately at 4, 5.5, 7, 24, 48, 72, 96, 168 and 216 hours after the start of the drinking study. The amount of EtG in the urine of the subjects was measured beforehand by A. Shröck based on an in-house LC-MS/MS method on a 3200 QTrap from AB Sciex (Rotkreuz, Switzerland) in combination with a Phenomenex Synergi Polar RP 4µm (Schlieren, Switzerland).(65) This was then compared to the amounts of N-acetyltaurine. It is important to keep into account that the conentrations of EtG and EtS in the urine were not normalized to the concentrations of creatinine.

## 4. RESULTS

### 4.1. SYNTHESIS OF N-ACETYLTAURINE

The accuracy of the calibrators of the LC-MS/MS method is illustrated in table 4.1. The powder contains approximately 7.2 ng/ml taurine. This means that only 0.18% of the 4000 ng/ml sample consists out of taurine.

**Table 4.1: Calibrators for the determination of residual taurine. $R^2$ was 0.993.**

| Expected Concentration (ng/ml) | Sample Name | Accuracy |
|---|---|---|
| **10** | K1 taurine | 98.9 |
| **20** | K2 taurine | 92.9 |
| **100** | K3 taurine | 100.2 |
| **500** | K4 taurine | 101.9 |
| **1000** | K5 taurine | 104.9 |
| **1500** | K6 taurine | 104.3 |
| **4000** | k7 taurine | 96.9 |

The peak heights for N-acetyltaurine and D4-acetyltaurine in the sample are illustrated in figure 4.1. The peak height for the 400 ng/ml N-acetyltaurine is $2.85e^4$ and for the 40 ng/ml D4-acetyltaurine it is $1.00e^5$.

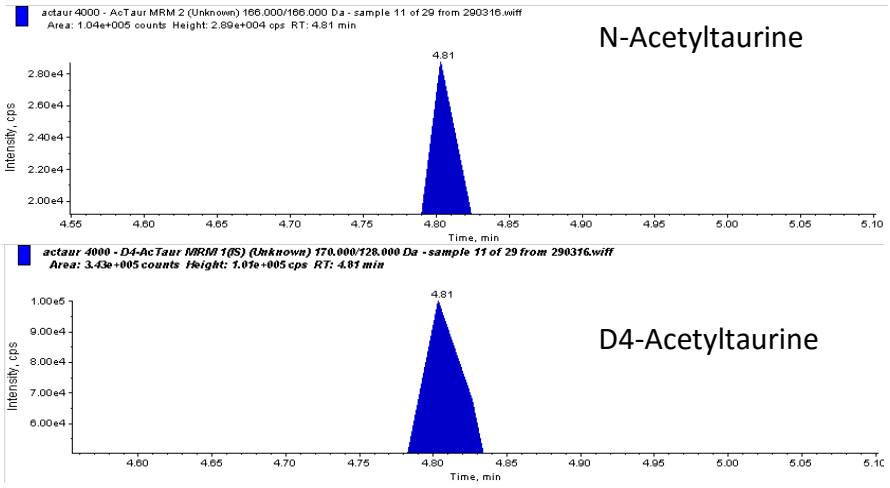



**Figure 4.1: Peak for 400ng/ml N-acetyltaurine in sample and for 40ng/ml D4-acetyltaurine in internal standard.**

The results of the $H_1$-NMR are illustrated in figure 4.2. N-acetyltaurine has peaks at 1.98, 3.08 and 3.55 ppm. The amount of powder that consists out of N-acetyltaurine is determined as approximately 1%. The main compound appears to be $CH_3CH_2SO_3^-$ or something alike, with peaks at 1.27 and 3.19 ppm.

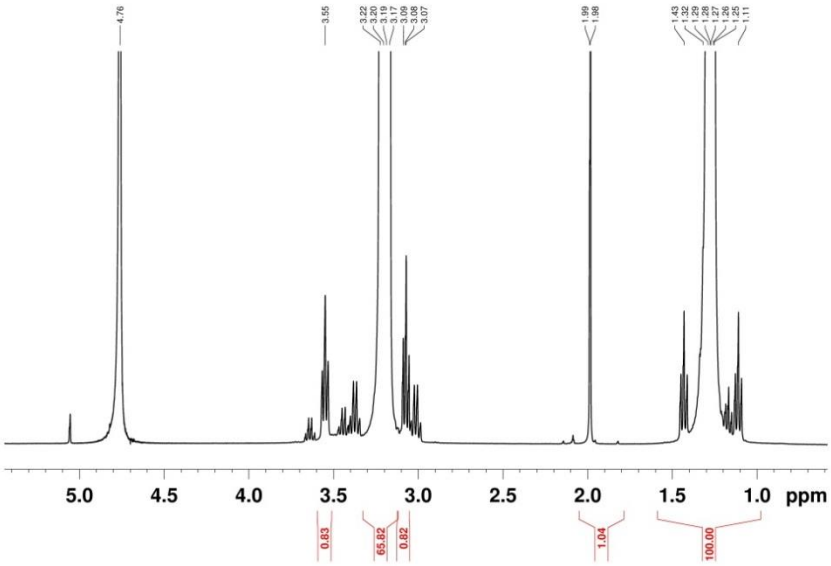



**Figure 4.2: $H_1$-NMR of the synthesized sample. Peaks for N-acetyltaurine at 1.98, 3.08 and 3.55 ppm.**

## 4.2. METHOD VALIDATION LC-MS/MS

### 4.2.1. Selectivity

The signal of the analytes showed the same retention times on the column as their internal standards. An increase in analyte peak area with increasing concentration was visible for both calibrations, in water and urine. Linear regression of the spiked urine samples and the blank urine from abstinent people revealed the presence of endogenous concentrations for all the three substances. The mean endogenous concentrations, which were investigated in the six blank urine samples, were 14 µg/ml for taurine, 0.57 µg/ml for N-acetyltaurine and 453 µg/ml for creatinine. Corrected to 1 mg creatinine/1 ml urine the mean amounts are 34 µg/ml for taurine and 1.4 µg/ml for N-acetyltaurine. Due to the

endogenous presence of all three compounds in blank urine, water was chosen as a matrix for calibration.

As illustrated in figure 4.3, the retention times for the different compounds were: 4.87 min for taurine, 4.17 min for N-acetyltaurine, 4.96 min for creatinine.

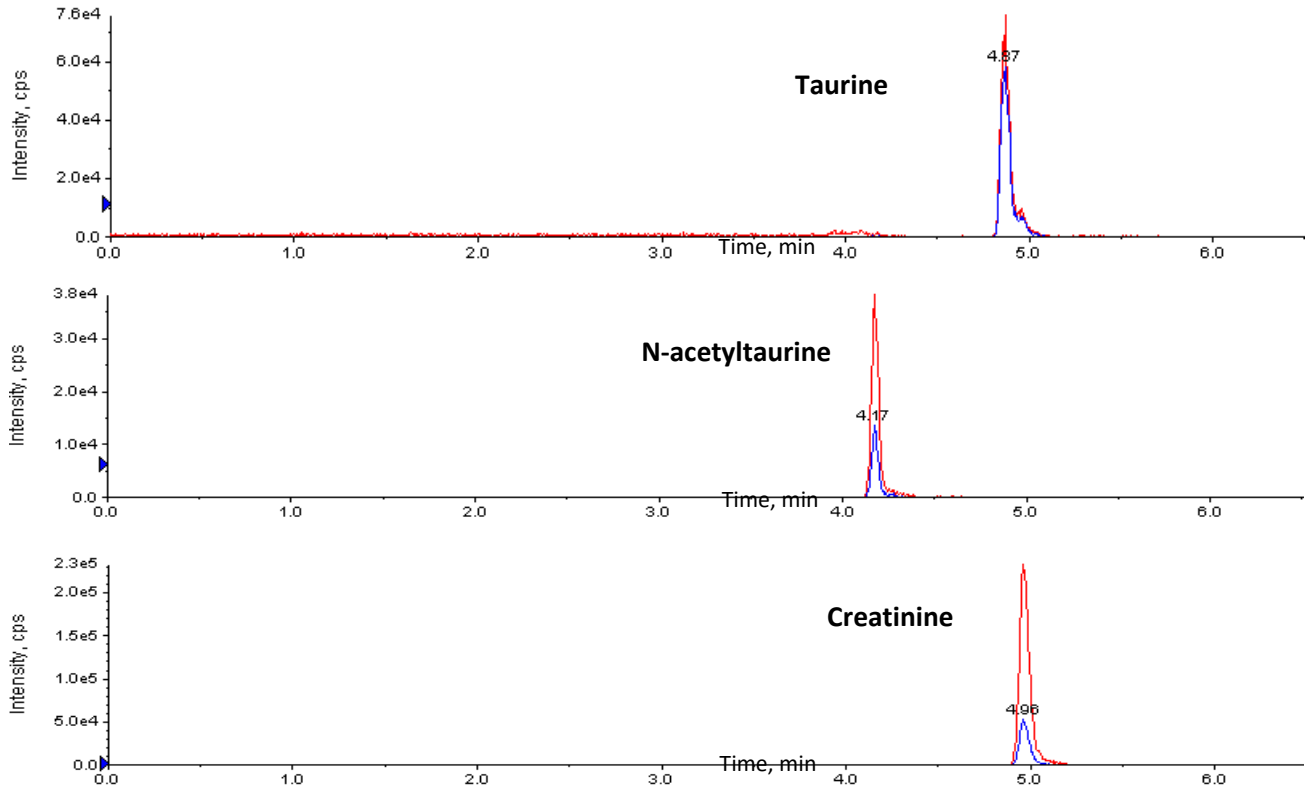
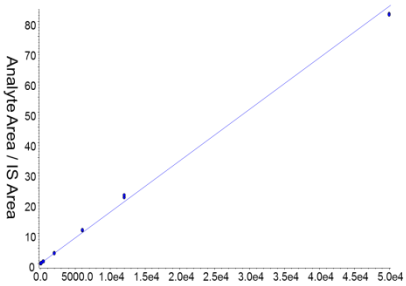



**Figure 4.3: Retention times for the different compounds in a spiked water sample: 4.87 min for taurine, 4.17 min for N-acetyltaurine, 4.96 min for creatinine.**

### 4.2.2. Calibration in double

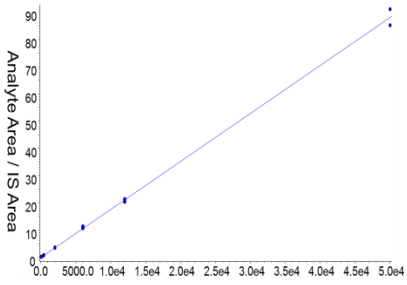
The calibration curves for the three compounds and the three series are illustrated in figure 4.4. The calibration curve is the mean of two independently created and extracted water calibrations out of one stock solution. The x-axis represents the analyte concentration in ng/ml, while the y-axis represents the analyte area over the ISTD area. N-acetyltaurine underwent linear regression with a 1/x weighting, while both taurine and creatinine underwent linear regression with a $1/x^{\wedge 2}$ weighting.

## N-acetyltaurine



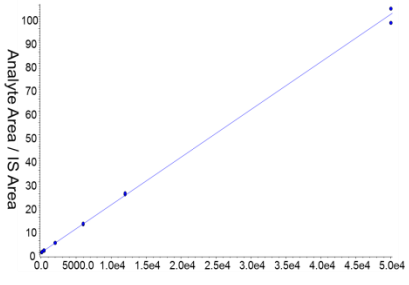

Serie 1

y = 0.0017x + 0.0165 (r=0.9986)




Serie 2

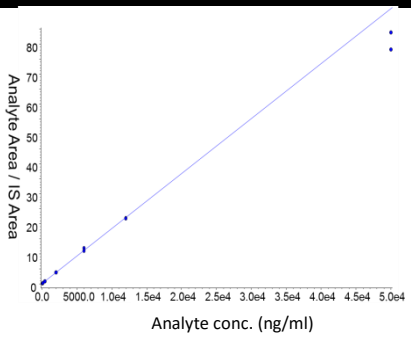
y = 0.00176x - 0.0012 (r=0.9994)




Serie 3

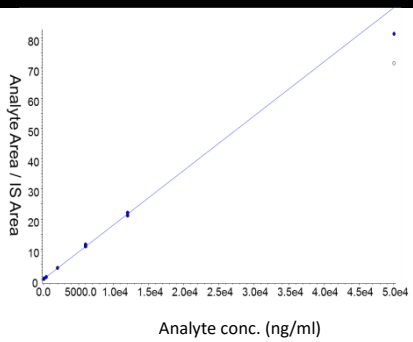
y = 0.00202x - 0.00104 (r=0.9996)

## Taurine



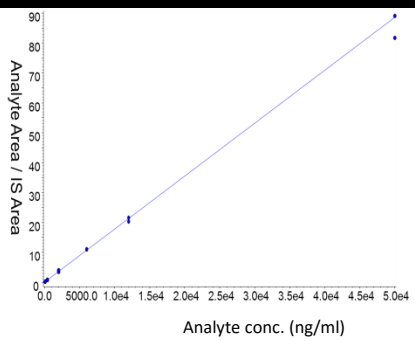

Serie 1

y = 0.00183x + 0.0642 (r=0.9972)




Serie 2

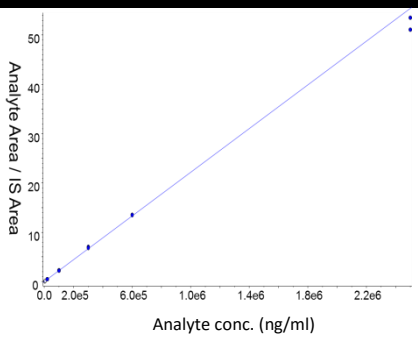
y = 0.0018x + 0.0654 (r=0.9973)




Serie 3

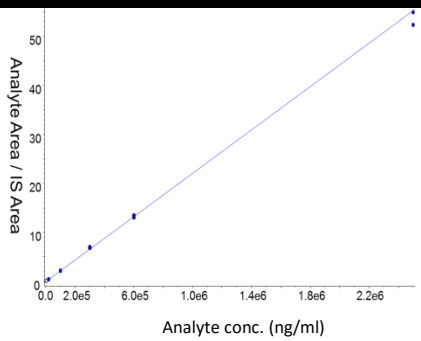
y = 0.00177x + 0.0993 (r=0.9978)

## Creatinine



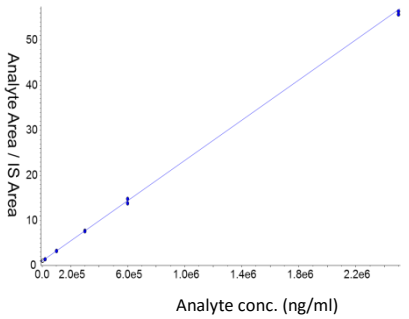

Serie 1

y = 2.22e^-5x + 0.0207 (r=0.9990)




Serie 2

y = 2.21e^-5x + 0.00734 (r=0.9994)




Serie 3

y = 2.23e^-5x - 0.00691 (r=0.9997)

**Figure 4.4: Calibration curves from validation series 1-3 for the three different compounds. X-axis represents analyte concentration in ng/ml. Y-axis represents analyte area over the ISTD area.**

### 4.2.3. Accuracy and precision

Accuracy and precision are illustrated for every compound separately in table 4.2, 4.3 and 4.4. For creatinine the lowest concentration is set at 20 µg/ml due to the fact that lower amounts of creatinine are only seen in diluted urine. Accuracy and precision were in an acceptable range (<15%).

**Table 4.2: Accuracy and precision for creatinine. Three validation series in water were executed to determine inter-assay accuracy and precision.**

| Expected Concentration (µg/ml) | Results Name | Number of values used | Mean (µg/ml) | %CV | Accuracy (%) |
|---|---|---|---|---|---|
| 20 | Series 1 | 6 | 19.6 | 1.8 | 98 |
| | Series 2 | 6 | 20.3 | 2.4 | 101 |
| | Series 3 | 6 | 20.5 | 1.5 | 103 |
| | Inter assay | 3 | 20.1 | 2.3 | 101 |
| 100 | Series 1 | 6 | 101 | 2.6 | 102 |
| | Series 2 | 6 | 101 | 0.7 | 101 |
| | Series 3 | 6 | 100 | 1.6 | 100 |
| | Inter assay | 3 | 101 | 0.7 | 101 |
| 200 | Series 1 | 6 | 203 | 1.5 | 102 |
| | Series 2 | 6 | 204 | 2.0 | 102 |
| | Series 3 | 6 | 201 | 1.5 | 101 |
| | Inter assay | 3 | 203 | 0.6 | 101 |
| 1300 | Series 1 | 6 | 1318 | 3.0 | 101 |
| | Series 2 | 6 | 1284 | 2.3 | 99 |
| | Series 3 | 6 | 1255 | 1.8 | 97 |
| | Inter assay | 3 | 1286 | 2.5 | 99 |

**Table 4.3: Accuracy and precision for N-acetyltaurine. Three validation series in water were executed to determine inter-assay accuracy and precision.**

| Expected Concentration (ng/ml) | Results Name | Number of values used | Mean (ng/ml) | %CV | Accuracy (%) |
|---|---|---|---|---|---|
| 50 | Series 1 | 6 | 47.2 | 6.8 | 94 |
| | Series 2 | 6 | 50.4 | 5.3 | 101 |
| | Series 3 | 6 | 50.8 | 3.1 | 102 |
| | Inter assay | 3 | 49.5 | 3.9 | 99 |
| 500 | Series 1 | 6 | 524 | 3.0 | 105 |
| | Series 2 | 6 | 508 | 3.7 | 102 |
| | Series 3 | 6 | 516 | 1.5 | 103 |
| | Inter assay | 3 | 516 | 1.6 | 103 |
| 2500 | Series 1 | 6 | 2709 | 3.1 | 108 |
| | Series 2 | 6 | 2530 | 2.8 | 101 |
| | Series 3 | 6 | 2631 | 2.7 | 105 |
| | Inter assay | 3 | 2624 | 3.4 | 105 |
| 5010 | Series 1 | 6 | 5310 | 2.5 | 106 |
| | Series 2 | 6 | 5148 | 1.7 | 103 |
| | Series 3 | 6 | 5137 | 1.6 | 103 |
| | Inter assay | 3 | 5199 | 1.9 | 104 |
| 32500 | Series 1 | 6 | 34080 | 2.3 | 105 |
| | Series 2 | 6 | 32114 | 1.9 | 99 |
| | Series 3 | 6 | 32091 | 5.0 | 99 |
| | Inter assay | 3 | 32762 | 3.5 | 101 |

**Table 4.4: Accuracy and precision for taurine. Three validation series in water were executed to determine inter-assay accuracy and precision.**

| Expected Concentration (ng/ml) | Results Name | Number of values used | Mean (ng/ml) | %CV | Accuracy (%) |
|---|---|---|---|---|---|
| 50 | Series 1 | 6 | 46.6 | 14.1 | 93 |
| | Series 2 | 6 | 47.6 | 5.7 | 95 |
| | Series 3 | 6 | 46.3 | 5.1 | 93 |
| | Inter assay | 3 | 46.8 | 1.4 | 94 |
| 500 | Series 1 | 6 | 513 | 2.3 | 103 |
| | Series 2 | 6 | 508 | 2.0 | 102 |
| | Series 3 | 6 | 503 | 2.6 | 101 |
| | Inter assay | 3 | 508 | 2.3 | 102 |
| 2500 | Series 1 | 6 | 2559 | 1.9 | 102 |
| | Series 2 | 6 | 2505 | 1.5 | 100 |
| | Series 3 | 6 | 2570 | 1.5 | 103 |
| | Inter assay | 3 | 2544 | 1.4 | 102 |
| 5010 | Series 1 | 6 | 5097 | 2.5 | 102 |
| | Series 2 | 6 | 4979 | 1.1 | 99 |
| | Series 3 | 6 | 5127 | 1.7 | 102 |
| | Inter assay | 3 | 5067 | 1.6 | 101 |
| 32500 | Series 1 | 6 | 30996 | 1.5 | 95 |
| | Series 2 | 6 | 30491 | 1.3 | 94 |
| | Series 3 | 6 | 31444 | 4.0 | 97 |
| | Inter assay | 3 | 30977 | 1.5 | 95 |

### 4.2.4. Autosampler stability

Table 4.5 illustrates the mean differences and percentage deviation for the different compounds in the reinjected quality controls between first measurement right after extraction and measurement after respectively three and seven days storage in the auto sampler. The LLOQ was left out, due to the fact that these amounts are rarely seen in real samples.

**Table 4.5: Changes in compound concentration in the quality control samples after storage of respectively three and seven days in the autosampler at 8°C.**

| Analyte | Expected concentration (µg/ml) | Mean difference after 3 days (µg/ml) | Mean difference/Expected concentration (%) after 3 days | Mean difference after 7 days (µg/ml) | Mean difference/Expected concentration (%) after 7 days |
|---|---|---|---|---|---|
| NAT | 0.50 | 0.01 | 2.0 | 0.01 | 2.0 |
| | 2.50 | -0.05 | -2.0 | -0.03 | -1.2 |
| | 5.00 | -0.09 | -1.8 | -0.08 | -1.6 |
| | 32.5 | -0.07 | -0.2 | -0.96 | -3.0 |
| Taurine | 0.50 | -0.01 | -2.0 | 0.02 | 4.0 |
| | 2.50 | -0.04 | -1.6 | 0.07 | 2.8 |
| | 5.00 | -0.05 | -1.0 | 0.37 | 7.4 |
| | 32.5 | -0.11 | -0.3 | 2.90 | 8.9 |
| Crea | 20.0 | 0.43 | 2.2 | 1.27 | 6.4 |
| | 100 | -5.63 | -5.6 | 0.73 | 0.7 |
| | 200 | -12.1 | -6.1 | 2.00 | 1.0 |
| | 1300 | -95.7 | -7.4 | -1.43 | -0.1 |

### 4.2.5. Freeze-thaw stability

The difference in concentration of the different compounds measured in the urine samples before and after three freeze-thaw-cycles is illustrated in table 4.6. All analytes were stable (less than 20% difference).

**Table 4.6: Changes in concentration of the three analytes in urine samples after three freeze-thaw-cycles compared to the concentration before the freeze-thaw-cycles**

| Analyte | Sample | Concentration before (µg/ml) | Concentration after (µg/ml) | Difference (µg/ml) | Difference (%) |
|---|---|---|---|---|---|
| NAT | U1 | 0.52 | 0.48 | -0.04 | -6.95 |
| | U2 | 1.36 | 1.22 | -0.14 | -10.3 |
| | U3 | 0.30 | 0.34 | 0.38 | 12.8 |
| | U4 | 0.25 | 0.27 | 0.02 | 7.09 |
| | U5 | 0.62 | 0.61 | -0.01 | -1.77 |
| | U6 | 0.46 | 0.47 | 0.01 | 0.65 |
| Taurine | U1 | 14.2 | 13.4 | -0.80 | -5.63 |
| | U2 | 24.4 | 23.0 | -1.40 | -5.74 |
| | U3 | 27.4 | 24.7 | -2.70 | -9.85 |
| | U4 | 6.27 | 6.05 | -0.22 | -3.51 |
| | U5 | 8.36 | 8.51 | 0.15 | 1.79 |
| | U6 | 3.03 | 3.01 | -0.02 | -0.66 |
| Creatinine | U1 | 228 | 197 | -31.0 | -13.6 |
| | U2 | 1030 | 880 | -150 | -14.6 |
| | U3 | 574 | 550 | -24.0 | -4.18 |
| | U4 | 173 | 155 | -18.0 | -10.4 |
| | U5 | 386 | 354 | -32.0 | -8.29 |
| | U6 | 329 | 288 | -41.0 | -12.5 |

### 4.2.6. Limit of detection and lower limit of quantitation

For the limit of detection the peak heights of the signal were compared to those of the noise. As illustrated in figure 4.5, a concentration of 25 ng/ml for N-acetyltaurine gives a signal to noise ratio of 3.

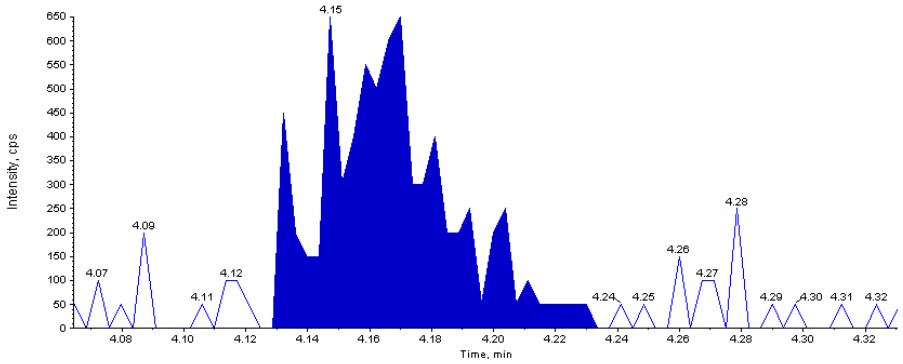



**Figure 4.5: Determination of LOD of N-acetyltaurine based on the signal to noise ratio of MRM1. Signal to noise ratio has to be 3.**

To determine the LLOQ the same procedure was followed. A 50 ng/ml concentration of N-acetyltaurine gives a signal to noise ratio of 10 as illustrated in figure 4.6. For this concentration the accuracy and precision fulfilled the predefined criteria, which is also necessary to determine the LLOQ.

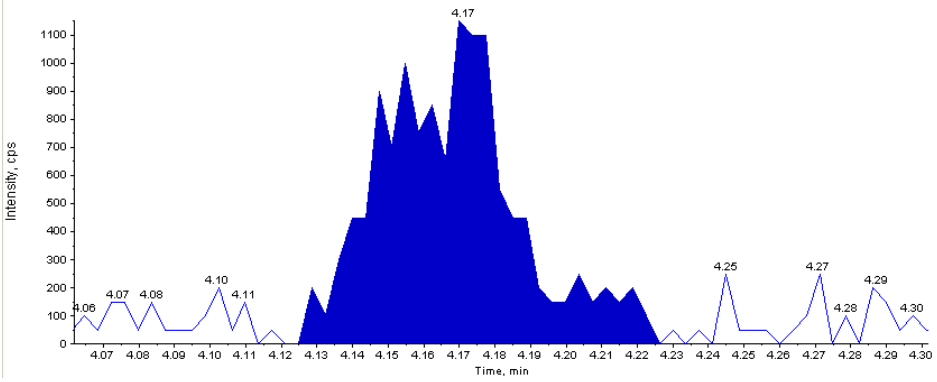



**Figure 4.6: Determination of LLOQ of N-acetyltaurine based on the signal to noise ratio of MRM1. Signal to noise ratio has to be 10.**

### 4.2.7. Matrix effect, extraction efficiency and process efficiency

As illustrated in table 4.7, extraction efficiency and process efficiency were around 50%, except for creatinine which showed an extraction efficiency around 60%. Ion enhancement was observed for N-acetyltaurine when comparing the peak areas of set 2 to set 3. By using internal standards this matrix effect was partially corrected.

**Table 4.7: Summary of extraction efficiency, process efficiency and matrix effect for the MRM1 of the three compounds. The mean values of three samples were used.**

| Analyte | Experiment | Spiked concentration (µg/ml) | Peak Area/Peak Area (%)[a] | Calc. Con./ Calc. Con. (ISTD corrected) (%)[b] |
|---|---|---|---|---|
| NAT | Extraction efficiency | 8.13 | 65 | 49 |
| | | 16.3 | 69 | 51 |
| | | 32.5 | 54 | 51 |
| | Process efficiency | 8.13 | 526 | 47 |
| | | 16.3 | 319 | 48 |
| | | 32.5 | 277 | 46 |
| | Matrix effect | 8.13 | 807 | 95 |
| | | 16.3 | 488 | 94 |
| | | 32.5 | 433 | 90 |
| Taurine | Extraction efficiency | 8.13 | 86 | 53 |
| | | 16.3 | 71 | 53 |
| | | 32.5 | 43 | 51 |
| | Process efficiency | 8.13 | 67 | 45 |
| | | 16.3 | 31 | 48 |
| | | 32.5 | 29 | 50 |
| | Matrix effect | 8.13 | 78 | 85 |
| | | 16.3 | 47 | 91 |
| | | 32.5 | 67 | 99 |
| Creatinine | Extraction efficiency | 313 | 70 | 62 |
| | | 625 | 66 | 58 |
| | | 1250 | 61 | 57 |
| | Process efficiency | 313 | 69 | 53 |
| | | 625 | 64 | 52 |
| | | 1250 | 69 | 50 |
| | Matrix effect | 313 | 98 | 86 |
| | | 625 | 97 | 88 |
| | | 1250 | 114 | 87 |

### 4.2.8. Carry over

Carry over was negligible. For creatinine the ratio of the signal in water to the signal in $K_7$ was 0.22%. For N-acetyltaurine this was 0.06% and for taurine 0.03%.

[a] Non ISTD corrected values
[b] ISTD corrected values (extraction efficiency is not corrected by adding ISTD, due to the fact that ISTD is added after extraction)

## 4.3. DRINKING STUDY

The changes of the amount of N-acetyltaurine in urine (normalized to 1mg creatinine/ml urine) over 25 hours for the eight subjects of the first drinking study are illustrated in figure 4.7.

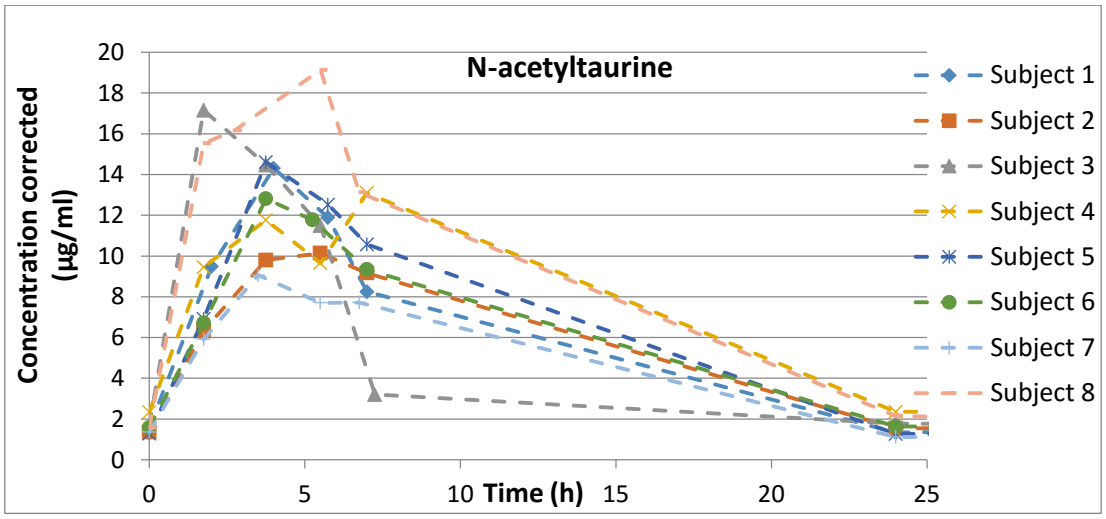



**Figure 4.7: Change of NAT concentration (normalized) over time for the eight subjects.**

The NAT concentration of subject two was compared to the EtG concentration in urine (Determined by A. Schröck). Samples were stored frozen (-20°C) since the drinking study (which was conducted in September 2015). Results are shown in figure 4.8. No samples were taken between 8 and 24 hours after start of drinking (over-night).

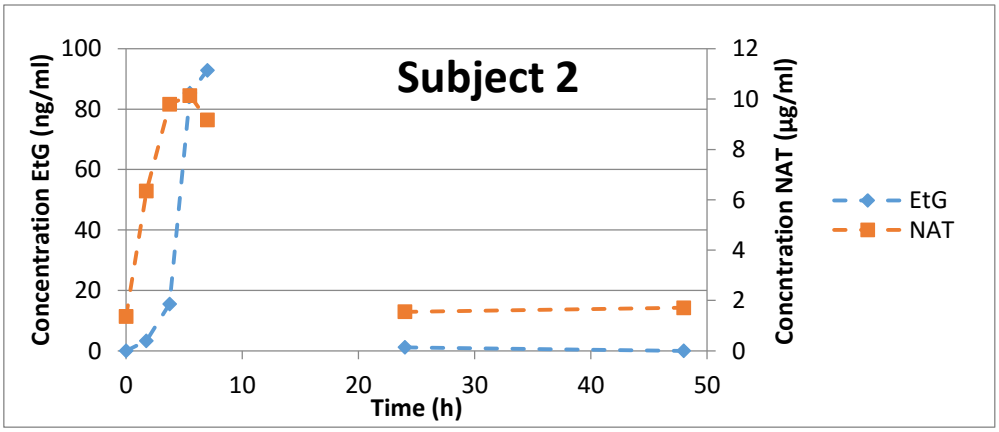



**Figure 4.8: Change of NAT in urine over time in subject two compared to the change of EtG. No trend line between 8 and 24 hours after drinking, because no samples were taken over-night.**

## 5. DISCUSSION

At first, two syntheses of N-acetyltaurine were performed. Both synthesis methods, the first according to the paper of Jokiel *et al*. and the second based on a Chinese patent, were not successful. In the first method, separation of N-acetyltaurine and taurine was not possible. While in the second method the LC-MS/MS showed that the amount of taurine was low, but $H_1$-NMR revealed that the amount of N-acetyltaurine was low as well. So there is a bad recovery. This is also seen in the peak height of N-acetyltaurine compared to the D4-NAT peak height. The peak height of the N-acetyltaurine is lower than that of the deuterated ISTD, although it was expected to be the other way around due to the higher concentration of the deuterated ISTD. Possibly there is a critical pH component in both synthesis methods, which was not thoroughly checked. The syntheses were not repeated, due to the fact that pure N-acetyltaurine could be obtained from a company. The first method, according to Jokiel *et al*., was used for synthesis of D4-acetyltaurine. There it did not matter that there was still D4-taurine left in the mixture, due to the fact that there is no endogenous D4-taurine present in urine.

With the commercial N-acetyltaurine, an LC-MS/MS method was developed for the measurement of the new alcohol marker N-acetyltaurine, and for taurine and creatinine in urine samples. The three analytes were measured by using negative ESI MS/MS coupled to a HILIC-HPLC separation. For the chromatographic separation, a SeQuant® ZIC®-HILIC column was used. This column was chosen because of its ability to retain zwitterionic compounds, such as N-acetyltaurine and taurine. In comparison to the method of Jokiel *et al*.(49) it is a different column, these authors used a C-18 column. This change was made due to the fact that in the method of Jokiel *et al*. only N-acetyltaurine was measured, while in the method of this thesis both N-acetyltaurine and taurine were measured and a C18-column is not capable of separating these two.

The next step in the thesis was validating the LC-MS/MS method. The validation occurred according to the Guidance for Industry with focus on Bioanalytical Method Validation from the U.S. Department of Health and Human Services (FDA guidelines) and the guideline on bioanalytical method validation from the European Medicines Agency.

This validation consisted out of a calibration in double. When comparing the resulting Pearson Coefficient of Determination ($R^2$) of the three measuring days for every compound, it is seen that the results are consistent and meet the criteria.

Another part of the validation is measuring accuracy and precision. The predefined goals for precision and accuracy are set at a percentage of less than 15% or 20% in case of the LLOQ for both precision and accuracy. The results demonstrate that these predefined goals are reached.

Stability is also a part of the validation procedure. When observing the changes in concentration after three and seven days of storage in the autosampler, it is seen that the measured concentrations change both in positive as in negative direction. This shows that the changes are probably caused by coincidence. Same changes can be seen when checking the freeze-thaw stability. Here the changes are a bit higher, but still in both directions.

The LOD and LLOQ were visually determined. The peaks are not really sharp, thus it is difficult to check the signal-to-noise ratios. A solution for this problem could be to change the mobile phase, or to change the liquid chromatography gradient. Both solutions were tested, without significant changes. This could be due to the fact that the HILIC column is very pH sensitive, slight changes of pH during the run change the charge of the zwitterionic compounds, N-acetyltaurine and taurine, which could lead to these kind of peaks. It is important to mention that although this happens, integration leads to reliable results.

The next part of the validation was checking for matrix effect, extraction efficiency and process efficiency. The main conclusion that can be made out of these results is that the internal standard does correct the ion enhancement of N-acetyltaurine. When checking the peak area ratios, the results of the process efficiency for N-acetyltaurine and taurine are not consistent. This is because of matrix effect, and is corrected by adding ISTD.

After validation, the method was used to test the changes over time in N-acetyltaurine concentration in urine after ingestion of alcohol and to determine the endogenous amounts of N-acetyltaurine in abstinent persons. The mean endogenous amount measured was 1.4 µg/ml (normalized to 1mg creatinine/ml urine) which is higher than the amounts that Jokiel *et al*. reported. The reason for this difference could be that in the report of Jokiel *et al*. a calibration range from 100 – 1000 ng/ml was used. In order to fit this small calibration curve

these authors diluted their samples. Another reason could be that because of making their own N-acetyltaurine, impurities were present and that these had an influence on the results. More blank samples should be tested to really pinpoint the mean endogenous concentration of N-acetyltaurine. There is also a difference between this thesis and the research of Jokiel *et al*. in the concentrations of N-acetyltaurine when alcohol is ingested. They reported a minimum of 0.7 µg/ml and a maximum of 110 µg/ml (both normalized) while our results showed a minimum of 8 µg/ml and a maximum of 19 µg/ml (both normalized). This could be due to the fact that another amount of ethanol was ingested, the amount in the study of Jokiel *et al*. is not mentioned. It was also not mentioned what they define as a drinker, and if the BAC of the subjects was elevated or not. Another reason for the deviations could be that the samples were taken at a different moment. In the paper of Jokiel *et al*. it is also not mentioned how creatinine is measured, so the correction to the creatinine could be different. In the future it would be interesting to examine more samples at different BAC's to determine the impact of the ingested amount on the presence of N-acetyltaurine in the urine. A relationship between N-acetyltaurine and taurine concentrations could not be found.

When measuring N-acetyltaurine in real samples, it has to be taken into account that endurance exercises also lead to a rise of concentration in urine. The amount mentioned in the study of Miyazaki *et al*. is 2.8 µg/ml, which is lower than our lowest peak concentration. This means that at the peak moment of N-acetyltaurine a distinction can be made between alcohol ingestion and endurance exercise, but that when N-acetyltaurine is partially metabolized this will become harder. Low amounts of ethanol will probably lead to comparable concentrations, so a differentiation between the two might be hard or even impossible. This pathway has to be examined further, maybe there are other additional markers that can help to make the distinction between the two.

Another pathway that needs further investigation is what happens with the amounts of N-acetyltaurine after drinking an amount of alcohol in vegans. It is seen that vegans have a lower amount of taurine in their body, because the major amount of taurine is ingested by eating meat, seafood and milk. This could influence the amount of N-acetyltaurine present in urine after drinking, so it is important to investigate this further.

More experiments are needed to determine how the concentration of N-acetyltaurine changes between 8 and 24 hours after drinking. Now it is seen that somewhere between 8 and 24 hours the concentration falls back to its endogenous level. Although there is some uncertainty when the endogenous level is reached, it can be concluded that this happens while low amounts of EtG are still present in the urine. This means that N-acetyltaurine is not a better marker than the ones already used in routine examinations.

Although the key outcome of this thesis was negative, the method is developed and validated and thus can be used for other studies. Maybe it is possible to see a correlation between the amount of N-acetyltaurine after an endurance exercise and the severity of overuse injuries – tissue damage, caused by repetitive action.(66) It would also be interesting to examine the difference in N-acetyltaurine between men and women, because it was seen that there probably is a difference in the endogenous amounts.

## 6. CONCLUSIONS

The method validation showed that the developed LC-MS/MS method was overall good. Accuracy and precision met the predefined criteria of 15% (20% for the LLOQ) for every examined compound. The stability of every compound during storage at 8°C for three and seven days, and after three freeze-thaw cycles at -20°C also showed good results. Both the matrix effect and the process efficiency were corrected by adding internal standard.

This method was then applied to real samples of a drinking study. It was seen that there is a relationship between ethanol intake and N-acetyltaurine levels in urine. The main conclusion of this thesis is that N-acetyltaurine cannot be detected over a longer period than the already routinely used alcohol markers. After ethanol ingestion up to a BAC of 0.8‰, the amount of N-acetyltaurine reaches its peak four to five hours after ingestion. More experiments are necessary to determine at which exact moment the concentration falls back to its endogenous level, but it is seen that this happens while low amounts of EtG are still present in the urine.

Other future studies are necessary to determine what the effect of higher amounts of alcohol ingestion is on the amounts of N-acetyltaurine, in blood as well as in urine. It should also be investigated if N-acetyltaurine can be found in hair and what the influence of a vegan diet is on N-acetyltaurine.

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