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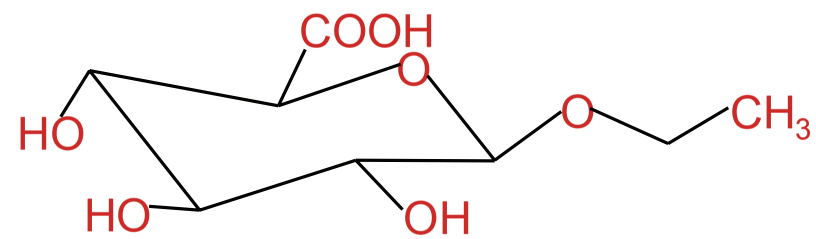
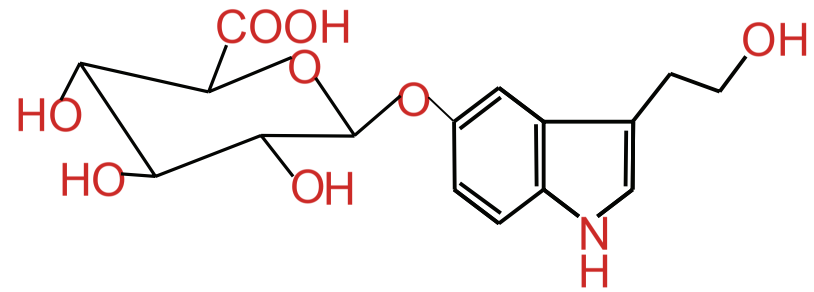
Liquid Chromatography-Mass Spectrometry Study of Two Biochemical Alcohol Markers





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Karolinska Institutet

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Liquid Chromatography
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LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY STUDY OF TWO BIOCHEMICAL ALCOHOL MARKERS

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“Although I have been through all that I have, I do not regret the many hardships I met”

Paulo Coelho

ABSTRACT

The interest in biochemical alcohol markers for detecting acute and chronic alcohol consumption has expanded greatly during recent years. The development and application of laboratory tests identifying early problematic drinking and monitoring abstinence have the potential of reducing the healthcare costs and suffering associated with alcohol misuse. Laboratory tests which are sensitive enough to detect single alcohol intake include ethanol, ethyl glucuronide (EtG) and 5-hydroxytryptophol glucuronide (GTOL). The overall aim of the present work was development of sensitive and specific liquid chromatography-mass spectrometry (LC-MS) methods for direct quantification of EtG and GTOL in urine, and to apply these for clinical studies.

EtG is a minor direct metabolite of ethanol, and is present for some time after ethanol is eliminated. A simple analytical procedure was developed based on direct injection of diluted urine into the LC-MS system. EtG was found to be stable in urine with no breakdown or artificial formation on storage in room temperature. Presence of ethanol in urine did not result in any artificial formation. EtG was not detectable in urine samples collected after abstinence from alcohol. EtG remains in the urine for many hours after ethanol itself has been eliminated. Thus, testing urine for the presence of EtG provides a mean for determination of recent alcohol consumption. Expressing urinary EtG as a ratio to creatinine should be recommended in routine clinical use to compensate for urine dilution. The method fulfils the need for a simple and reliable assay to be used as a routine test of recent alcohol intake.

GTOL is the major excretion form of 5-hydroxytryptophol (5-HTOL), a minor serotonin (5-HT) metabolite. Because the concentration of 5-HTOL is markedly increased following consumption of alcohol, measurement of 5-HTOL is used as a sensitive biomarker for detection of recent alcohol intake. An LC-MS procedure, including solid-phase extraction for direct quantification of GTOL was developed. The method was highly correlated with an established gas chromatography-MS method for urinary 5-HTOL (r^2 = 0.99, n = 70; mean 5-HTOL/GTOL = 1.10). This was the first direct assay for quantification of GTOL in urine, suitable for routine application.

In clinical use, GTOL is expressed as a ratio to the main 5-HT metabolite 5-hydroxyindoleacetic acid (5-HIAA), in order to compensate for variations in urine dilution and 5-HT turnover. A fully validated and robust LC-MS/MS method for measurement of urinary GTOL and 5-HIAA, based on direct injection was developed. The method was capable of measuring endogenous GTOL and 5-HIAA levels in urine that agreed with literature data. The method was applied and compared with a new developed enzyme-linked immunosorbent assay (ELISA) for GTOL in clinical material. Determination of GTOL by ELISA showed 82% sensitivity in detecting positive samples, compared to the LC-MS/MS method. When 10 alcoholic patients were followed during detoxification, the GTOL/5-HIAA ratio gave a median detection time of 39 hours, while EtG was detectable for a median of 65 hours. The lower sensitivity of the urinary GTOL/5-HIAA ratio compared with EtG for recent drinking may be clinically useful, in cases where the EtG test provides an unwanted high sensitivity for intake of only small amounts of alcohol or unintentional ethanol exposure. The present work demonstrated the potential of developing robust and selective methods for quantification of analytes in urine using electrospray ionization LC-MS and LC-MS/MS with minimal sample preparation.

LIST OF PUBLICATIONS

This thesis is based on the following original papers, which will be referred to in the text by their Roman numerals I-V.

I. Stephanson, N., Dahl, H., Helander, A. and Beck, O. (2002)
Direct quantification of ethyl glucuronide in clinical urine samples by liquid chromatography-mass spectrometry. *Ther Drug Monit*, **24**, 645-651.

II. Dahl, H., Stephanson, N., Beck, O. and Helander, A. (2002)
Comparison of urinary excretion characteristics of ethanol and ethyl glucuronide. *J Anal Toxicol*, **26**, 201-204.

III. Stephanson, N., Dahl, H., Helander, A. and Beck, O. (2005)
Determination of urinary 5-hydroxytryptophol glucuronide by liquid chromatography-mass spectrometry. *J chromatogr B Analyt Technol Biomed Life Sci*, **816**, 107-112.

IV. Stephanson, N., Helander, A. and Beck, O.
Determination of 5-hydroxytryptophol glucuronide and 5-hydroxyindoleacetic acid by direct injection of urine using ultra-performance liquid chromatography-tandem mass spectrometry. *Submitted to Journal of Mass Spectrometry.*

V. Beck, O., Stephanson, N., Böttcher, M., Dahmen, N., Fehr, C. and Helander, A.
Biomarkers to disclose recent intake of alcohol: potential of 5-hydroxytryptophol glucuronide testing using new direct UPLC-tandem MS and ELISA methods. *Submitted to Alcohol and Alcoholism.*

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LIST OF ABBREVIATIONS

ADH	alcohol dehydrogenase
ALDH	aldehyde dehydrogenase
APCI	atmospheric pressure chemical ionization
API	atmospheric pressure ionization
CID	collision-induced dissociation
ELISA	enzyme-linked immunosorbent assay
ESI	electrospray ionization
EtG	ethyl glucuronide
GC	gas chromatography
GC-MS	gas chromatography-mass spectrometry
GTOL	5-hydroxytryptophol glucuronide
5-HIAA	5-hydroxyindole-3-acetic acid
5-HIAL	5-hydroxyindole-3-acetaldehyde
HPLC	high performance liquid chromatography
5-HT	5-hydroxytryptamine
5-HTOL	5-hydroxytryptophol
LC	liquid chromatography
LC-MS	liquid chromatography-mass spectrometry
LC-MS/MS	liquid chromatography-mass spectrometry/mass spectrometry
LOD	limit of detection
LOQ	limit of quantification
m/z	mass-to-charge ratio
SPE	solid phase extraction
SRM	selected reaction monitoring
UPLC	ultra-performance liquid chromatography

1.0 INTRODUCTION

1.1 URINE DRUG TESTING

Drug and alcohol abuse poses major health and safety risks in society worldwide [1]. Laboratory investigations are important and have criminal, forensic and clinical applications. Drug intake can be detected by investigating blood, saliva, hair and urine. Urine is the commonly tested body fluid in clinical and forensic settings, because urine is easily obtainable and generally contains drugs and their metabolites, which can be detected for a reasonable time after ingestion [2]. In addition, to its use in clinical context, many workplaces mandate urine testing as a pre-employment and random screen [3]. The goal of drug testing is to achieve accurate results with no false positive or false negative results, and includes two steps. The first is screening, used to identify presumably positive specimens. The screening is commonly based on immunoassay using antibody binding to the drug, which has limited selectivity, so that chemically related substances can interfere and cause a false positive result. False negative results may occur when the concentration of the substance in the urine is below the accepted threshold or when the sample has been diluted or adulterated to obscure the presence of a drug. Furthermore, screening is often directed towards a class of drugs and not an individual substance [4], and preliminary positive results are confirmed by a second, more specific technique, such as mass spectrometry (MS) [2,5]. The common drugs of abuse being tested for include amphetamines, opiates, cannabinoids, benzodiazepines, buprenorphine, cocaine, phencyclidine, methadone, dextropropoxyphene, LSD and also ethanol.

1.2 METHODS FOR DETECTION OF ALCOHOL USE AND ABUSE

Following oral alcohol intake, alcohol is absorbed from the stomach and duodenum [6]. Approximately 92-95% of ethanol is metabolized in the liver by enzymes located in the cytosol fraction of the hepatocytes. Ethanol is metabolized first to acetaldehyde through oxidation by alcohol dehydrogenase (ADH) [7] in a reversible reaction that requires nicotinamide adenine dinucleotide (NAD^+) as the co-factor. The formed acetaldehyde is effectively further oxidized to acetic acid (acetate) by aldehyde dehydrogenase (ALDH) in a nonreversible reaction, again using NAD^+. Both ADH and ALDH have various isoforms, which are coded by different genes and seem to contribute to genetic differences in ethanol sensitivity and metabolic rate [8]. Acetate is then converted rapidly to energy, water and carbon dioxide in the Krebs cycle. During the hepatic oxidation of ethanol, the ratio of $NADH/NAD^+$ in the liver increases significantly, which accounts for many of metabolic disturbances associated with consumption of alcohol [7].

In addition to oxidation, ethanol and acetaldehyde can react with biomolecules to form various stable compounds. Esterase enzymes, present in brain, heart, pancreas and adipose tissue (fatty acid ethyl ester synthetases; FAEEs) can cause ethanol to react with fatty acids to form ethyl esters (FAEE) [6].

The need for objective methods revealing alcohol abuse has also been widely acknowledged [9]. The measurement of ethanol in body fluids or breath is the most common method to test for recent alcohol consumption [10]. Ethanol has a rapid

distribution in the body fluids and can be measured in blood, saliva, urine, sweat and exhaled air (breath). The determination of the ethanol is highly specific and therefore it can be used as a screening for patients with suspected ethanol intoxication or in follow-up studies to assess non-compliance for abstinence [9]. The methods for determination of ethanol in urine are based on enzymatic and gas chromatographic methods, the latter is generally considered the gold standard in forensic toxicology [11]. However, measurement of ethanol in urine is limited to detect only the very recent alcohol intake, because of the rapid elimination of ethanol from the body [12]. Biomarkers of alcohol consumption are needed to provide direct and indirect ways to estimate roughly the amounts of alcohol consumed and the duration of ingestion, and to obtain complementary information in assessment of problematic drinking and alcohol-related tissue damage resulting from long-term misuse [12]. A number of successful tests of acute or chronic alcohol consumption are already used clinically [12]. Use of biomarkers can help to improve compliance and treatment outcome [9,13,14].

Several laboratory tests or biological markers in blood and urine have become available, and are continuously being developed [9,12,15]. Common tests for identification of excessive alcohol intake include the liver enzymes, γ-glutamyltransferase (GGT) [9,16,17], aspartate aminotransferase (AST) [18] and alanine aminotransferase (ALT) [18]. GGT, AST and ALT are used as standard diagnostic tools to indicate non-specific liver dysfunction [12]. A more recently introduced and more specific indicator of excessive alcohol consumption is carbohydrate-deficient transferrin (CDT) [19]. CDT has proven useful for identifying excessive alcohol consumption and monitoring abstinence during outpatient treatment [13,20,21].

To fill the time gap between the markers of long-term drinking and ethanol testing to spot the recent use, other markers have been employed. The non-oxidative direct ethanol metabolite phosphatidyl ethanol (PEth) is a promising new biomarker of alcohol abuse [22]. Some studies have shown the superior sensitivity of PEth, which can remain in circulation for more than 2 weeks [9,22].

The ideal biochemical marker that is sensitive to small changes in ethanol use would be a direct ethanol metabolite or a product of ethanol catabolism [23]. The biochemical markers of recent ethanol consumption FAEE and 5-hydroxytryptophol (5-HTOL), have been proposed as useful in assessing ethanol intake, particularly in the setting of a clinical trial [23]. FAEE concentration rapidly increases after ingestion of ethanol and persists hours to days in the serum after ethanol can no longer be detected [24]. 5-HTOL is a minor metabolite of serotonin (5-hydroxytryptamine; 5-HT) that becomes formed at a higher rate during metabolism of ethanol and the urinary level of 5-HTOL remains increased for several hours after ethanol is no longer measurable in body fluids or breath [25-29]. In addition, direct derivates of ethanol metabolism, such as ethyl glucuronide (EtG) [30] and ethyl sulphate (EtS) [31], being formed by phase-II metabolic enzyme systems, can be used as new biochemical markers of recent alcohol intake [31].

However, the validation of the new biomarkers has been incomplete and the information on the sensitivities and specificities of the tests remain controversial [9]. Thus, further improvement of methodology for measurement of alcohol biomarkers suitable for routine use is needed.

1.3 ETHYL GLUCURONIDE

In 1952, EtG was isolated by Kamil *et al* as the triacetyl methyl ester from urine of rabbits and estimated to represent 0.5 – 1.6% of the total ethanol elimination [30]. EtG is also a minor detoxifying pathway for ethanol in man [32-34]. EtG (see Fig. 1) is a conjugate of ethanol and activated glucuronic acid (uridine-5'-diphospho-β-glucuronic acid; UDPGA) and is formed by action of membrane-bound mitochondrial uridine diphosphate glucuronyl transferase (UGT). EtG can be detected in various body fluids, tissues and hair [35-37]. EtG is a water soluble, direct metabolite of ethanol considered to be highly specific for alcohol intake [38] and the prolonged washout time compared to ethanol results in the high sensitivity [39,40] when used as a biomarker. EtG has the potential to detect alcohol intake up to several days [41] after the elimination of alcohol from the body and can be used as a marker for single alcohol intake.

Studies of EtG as an alcohol marker in urine and serum started in 1995. The determination of EtG has been performed by gas chromatography-mass spectrometry (GC-MS) after derivatisation (acetylation or silylation), either with or without previous solid-phase extraction (SPE) [37,40,42,43].

After these initial studies, enzyme-linked immunosorbent assay (ELISA) [44] and enzyme-linked immunoassay (EIA) [45] have been developed. However, the ELISA procedure, based on a polyclonal antibody, did not meet performance requirements (23% false positives and 24% false negatives in urine [44]) and has not come into practical use. EIA procedure has shown a high specificity [45], but has not been made commercially available due to a high measuring range.

Furthermore, the availability of liquid chromatography-mass spectrometry (LC-MS) [37,46] has resulted in increased analytical sensitivity and selectivity with great potential for clinical laboratories [47].

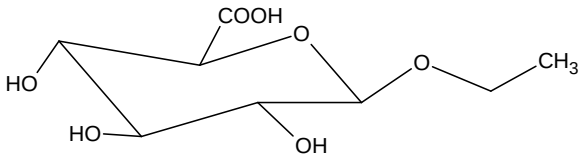

Figure 1 – The molecular formula of EtG (molecular weight is 222.3 g/mol).

1.4 5-HYDROXYTRYPTOPHOL GLUCURONIDE

The discovery of 5-HT in mammals in the early 1950's led to subsequent investigations of its metabolism. 5-HT is formed in the body from the amino acid tryptophan and found throughout the body, with appreciable concentrations in the gastrointestinal tract and blood platelets [48]. The metabolism of 5-HT initially involves oxidative deamination to form the intermediate aldehyde, 5-hydroxyindole-3-acetaldehyde (5-HIAL). This intermediate substance undergo either oxidation to form the main metabolite, 5-hydroxyindole-3-acetic acid (5-HIAA) or reduction to form a minor metabolite 5-HTOL [49,50]. Oxidation of the aldehyde is catalyzed by ALDH and the reduction is catalyzed mainly by ADH. Studies by Davis and coworkers found 5-HTOL to be formed at the expense of 5-HIAA [51] during ethanol metabolism. The shift in 5-HT metabolism is due to a metabolic interaction [52].

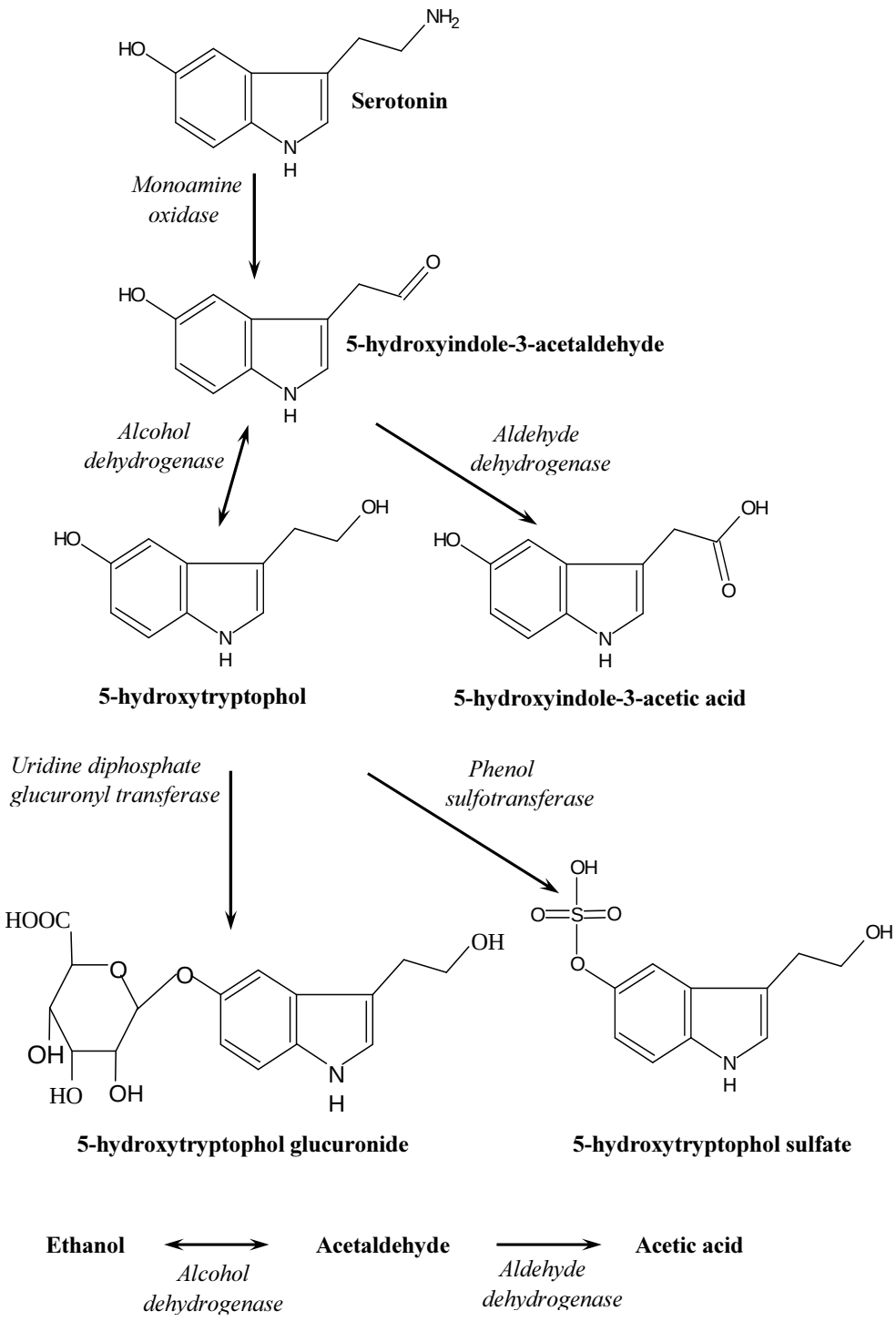

Figure 2 – Metabolic scheme showing the biochemical basis for the interaction between ethanol and serotonin.

Competitive inhibition of 5-HIAA formation by acetaldehyde was considered to be the main mechanism for the shift in metabolism (see Fig. 2). An alternative, possibly even more important, mechanism for the shift in metabolism of 5-HT is the increased NADH levels that results from ethanol metabolism, which increase 5-HTOL formation [53]. The increase in the urinary 5-HTOL excretion following alcohol intake is dose dependent, and the urinary output of 5-HTOL does not recover to baseline levels until several hours after ethanol itself is no longer measurable in body fluids or breath [25-29]. Unlike 5-HIAA, which is excreted from the body in free form, excretion of 5-HTOL is facilitated by formation of glucuronide and sulfate conjugates (Fig. 2) [54].

Free 5-HTOL accounts for less, than 5% of the total excretion in urine whereas the majority (about 80%) is excreted as 5-HTOL glucuronide (GTOL) [54], making GTOL an attractive target analyte [10]. To improve the precision of this acute alcohol marker in clinical use, 5-HTOL is expressed as a ratio to the main metabolite 5-HIAA, in order to compensate for variations in urine dilution, dietary intake of 5-HT (high amounts in banana) [55] and treatment with antidepressant drugs [56]. The urinary 5-HTOL/5-HIAA ratio has been successively applied as a sensitive and specific marker for recent alcohol consumption in a number of clinical and forensic settings [10,53,57-62].

Determination of 5-HIAA is usually performed by using high performance liquid chromatography (HPLC) with electrochemical detection [63]. A common analytical approach for 5-HTOL has been the use of hydrolysis followed by quantification of the free substance. Methods using GC-MS [10], HPLC [64,65] or LC-MS [62] have been employed. These methods involve extraction and derivatisation in addition to enzyme hydrolysis. Moreover, the employment of two different techniques makes the analysis of 5-HTOL and 5-HIAA more complicated and costly. One method exists for simultaneous determination of 5-HTOL and 5-HIAA [62], that showed the potential of liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS). However, involvement of enzymatic hydrolysis and derivatisation makes it less suitable in routine use. A recently developed ELISA procedure for determination of GTOL [66] should improve the utility of this marker in routine use.

2 BIOANALYSIS BY LC-MS

Drugs are xenobiotics and their uptake is followed by inactivation through biotransformation reactions catalyzed by enzymes in the liver [67]. Biotransformation of drugs proceeds in at least two distinct steps, divided into phase-I and phase-II reactions. In phase-I reactions, enzymes modify the parent compound via oxidation, hydrolysis or reduction [68], which is most often preparative stages for subsequent conjugation. Glucuronidation is a major phase-II reaction in mammals [69], resulting in increased hydrophilicity and thus enhanced excretion in urine [67]. Since the highly polar water soluble conjugates in urine are not amenable to traditional analysis by chromatography, the most common approach has been to cleave the conjugate by chemical or enzymatic hydrolysis prior to analysis.

A simpler approach is offered by analyzing the unhydrolysed conjugate in urine samples by LC-MS. The analysis procedure can be divided into several steps: sample preparation, chromatographic separation, followed by ionization and MS detection [70].

2.1 URINE AS A MATRIX

Urine has been used as a diagnostic fluid spanning from antiquity until the present time and urine analysis is considered the oldest clinical laboratory test [71]. Urine is produced by the kidneys at a volume of approximately 1-2 L/24 h. Urine contains large amounts of highly polar and low molecular weight urea, which is produced continuously by the liver as an end product of protein metabolism. Other common urine components include creatinine, various inorganic salts (electrolytes), and also traces of proteins. Creatinine is a metabolic waste product removed from the blood by the kidneys and excreted in the urine [72]. The 24 h creatinine content of urine remains roughly constant for an individual and is often used as a normalization factor when measuring other urinary components [73]. Although the 24-hour creatinine excretion is relatively constant within an individual, the concentration of creatinine in urine may vary widely in spot samples throughout the day [74].

2.2 SAMPLE PREPARATION

Sample preparation prior to analysis is often needed in order to enable quantification of compounds that are present together with large amounts of interfering substances in biological matrix [75]. A range of procedures is available, depending on the analyte and the method of determination [76]. Liquid-liquid extraction (LLE) [77] and SPE [78] are common sample preparation techniques, which can be applied as off-line and on-line methods [79]. The main advantage of off-line sample preparation is that it can be optimized independently from the other instrumentation. On-line methods are readily automated and eliminate the manual sample wash and transfer steps associated with off-line preparation [70]. LLE has been the common preparative procedure for gas-chromatographic (GC) and other chromatographic techniques [76]. However, it is time consuming, difficult to automate and it consumes large amounts of organic solvents. In addition, safe disposal of toxic solvents can be problematic and expensive.

In SPE, the analytes are extracted from solution by adsorption onto a solid-phase surface due to greater affinity for the solid phase than to the liquid phase. Unwanted

matrix components are rinsed off and thereafter the analytes are eluted with an appropriate solvent. A wide range of high-quality materials for SPE is available, including “designer phases” developed for target extractions of drugs of abuse, offering considerable versatility [76]. The range of sampling formats for SPE has also expanded from simple packed syringes to cartridges, disks and 96-well plates. This technological development has facilitated automated off-line and on-line sample processing, widely used in pharmaceutical and environmental analysis [76]

2.3 LIQUID CHROMATOGRAPHY

Chromatography is used as an efficient and rapid procedure for separation and determination of a wide array of substances [80]. The principle of the separation is the partition of analytes between the mobile and stationary phases, where the stronger binding of a molecule to the stationary phase results in longer retention time. The development of chromatographic methods is usually a compromise between desired resolution and analysis time. The reduction in the stationary phase particle size can result in the benefit to the chromatographic process [81].

Recent technological advances have made available reversed-phase chromatographic media with a 1.7 µm particle size with a liquid chromatograph system (Ultra-performance liquid chromatography; UPLC™) that can operate at much higher pressures compared to conventional liquid chromatography (LC). The great advantage of small particle size is that it allows analysis at flow rates much higher than the optimum with minimal loss in column efficiency. This is due to the flatter nature of the van Deemter plot of linear velocity versus height equivalent to a theoretical plate (HETP). The use of small-particle columns should provide improved resolution to the chromatographic analysis [82] and the use of higher flow rate together with a shorter column can result in reduced analysis time. LC can be used in the separation of glucuronide conjugates. An advantage of LC is the direct chromatographic analysis of glucuronide conjugates without prior hydrolysis or chemical derivatisation, resulting in a simpler and more robust sample preparation procedure.

Previous LC systems had low detector selectivity. During recent years coupling of LC-systems with mass spectrometers with high selectivity and sensitivity have been developed. This technique in combination with simple sample preparation for LC may be the “gold standard” for bioanalytical application.

2.4 LC-MS ELECTROSPRAY IONIZATION INTERFACE

In the early 1990s atmospheric pressure ionization (API) interface was introduced for applications in toxicology [83]. The combination of LC and MS (LC-API-MS) is considered as a major breakthrough in analytical technology and is accepted as alternative and complement to GC-MS [84]. The challenge of coupling two systems like LC and MS, one operating in liquid phase and the other in high vacuum, has been overcome by creating dedicated interfaces. API interfaces have several available modes for operation, such as electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI) and atmospheric pressure photoionization (APPI) [82]. APCI is generally regarded as the more robust ionization method being less susceptible to signal suppression from co-eluting matrix components [70,85]. However, the more used ESI

is softer and more versatile of the two methods enabling to ionize very polar/non-volatile molecules [70].

The operational principle of an API interface/ESI ion source is the following: The eluent from the LC system is sprayed from the tip of the capillary into an API source region. The eluent is nebulized by use of a pneumatically assisted gas stream (nebulizer) and an applied high temperature. The applied voltage to the capillary provides the electric-field gradient required to produce charge separation at the surface of the liquid. As a result, the liquid pushes out from the capillary tip as a "Taylor cone" [86] (see Fig. 3). When the solution that compromises the Taylor cone reaches the Rayleigh limit (the point at which Coulombic repulsion of the surface charge is equal to the surface tension of the solution) [86], droplets with an excess of charges detach from the capillary tip. These droplets move towards the entrance of the mass spectrometer by an electrical field and generate charged analyte molecules (ions) by several proposed mechanisms [87]. Independent mechanisms by which the ions are produced, the ESI process generates gas phase ions that can be analyzed for mass-to-charge (m/z) ratio within the mass spectrometer. The residual clusters originating from the solvent are disintegrated and accelerated in the electrical field. Increasing electrical potential leads to increased dissociation of ions by collision with the residual solvent and gas molecules. The fragments produced by this collision-induced dissociation (CID), called "in-source CID", can be used as confirmation ions for identification and quantification in the selected ion monitoring (SIM) mode or for structure elucidation. Knowledge of the factors that effect ESI responsiveness is helpful in predicting the suitability of a given analyte for analysis with ESI-MS. An analyte, suitable for analysis with ESI-MS can either exist as a preformed ion in solution or to be chargeable through protonation, deprotonation and adduct formation [86]. The structure of the analyte can influence its ESI response. Analytes with significant nonpolar portions generally have a higher electrospray response than polar analytes, because hydrophobic analytes have higher affinities for electrospray droplet surfaces and thus, tend to carry a greater fraction of the excess charge produced in the electrospray process.

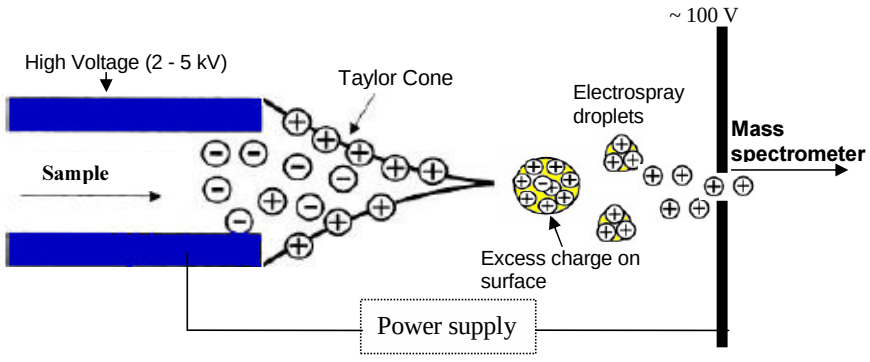

Figure 3 – Schematic of the electrospray ionization process (Reproduced after [86]).

ESI-MS is the method of choice to accomplish mass determination of biomolecules [86]. It is ideally suited to biochemical analysis allowing for substances to be analyzed directly from the liquid phase, and thus can be coupled to separation techniques such as LC [86].

2.5 LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY

The first system that combined HPLC with MS detection involved a single quadrupole analyzer (LC-MS) (see Fig. 4) [47]. The quadrupole consists of four precisely parallel rods (see Fig. 5). Between each pair of opposite and electrically connected rods, separated by a distance $2r_0$, a DC voltage (U) and superimposed radiofrequency (RF) potential is applied. Ions are propelled from the source into the quadrupole analyzer by a small accelerating voltage and under the influence of the combination of electric fields, the ions follow complex trajectories. The oscillations of ions in the quadrupole analyzer may have finite amplitudes, in this case the ion trajectory is "stable" and the ions are transmitted to the detector. If oscillations are unstable, the amplitude becomes infinite and the ions oscillate wildly. In this case, the ions collide with the rods and do not reach the detector.
The choice of solvent is of great importance for the functioning of LC-MS system. Volatile buffers are used to modify mobile phase. Manipulation of pH can enhance performance for analytes that are not normally ionized in solution. The pH may favour the formation of positive and negative ions, respectively. Non-volatile buffers are usually not used, due to the risk of contamination of the MS with salt crystals. Thus, chromatographic separation may not always be optimized by the restriction in choice of the buffer in the mobile phase. Traditionally, LC in combination with ultraviolet (UV), fluorescence, or electrochemical detection was employed in routine clinical analysis of biomolecules. The successful combination of LC and MS detection provides better sensitivity and selectivity than UV techniques and is applicable to a significantly larger group of compounds than fluorescence and electrochemical detection [88]. The selectivity of a MS-detector is superior to conventional HPLC detectors [47]. However, the complexity of biological samples with different compounds of identical molecular mass and multiple charging of analytes in ESI still may require the sample preparation and chromatographic separation of relevant compounds.

2.6 LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY/MASS SPECTROMETRY

One essential step in the development of LC-MS was the use of CID [47], which remains the most common ion activation method used in present day instruments [89-91]. The low-energy collisions are used in the tandem mass spectrometer (tandem MS; TMS), where a collision cell is introduced with a second quadrupole for generation of fragments ions (see Fig. 6). TMS implies that the generation of secondary ions is distinct from the ionization step, and that the precursor and product ions are both characterized independently by their m/z ratios [91]. In CID, generation of the product ions occurs by single or multiple collisions with neutral gas molecules in the collision cell (see Fig. 6). Collisions between precursor ion and a neutral target gas (N_2, Ar) are followed by an increase in internal energy of the ion, which induces decomposition,

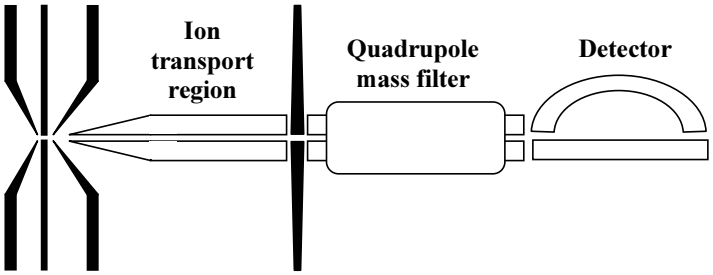

Figure 4 – Scheme of a single quadrupole mass spectrometer (Reproduced after Sciex instruction manual).

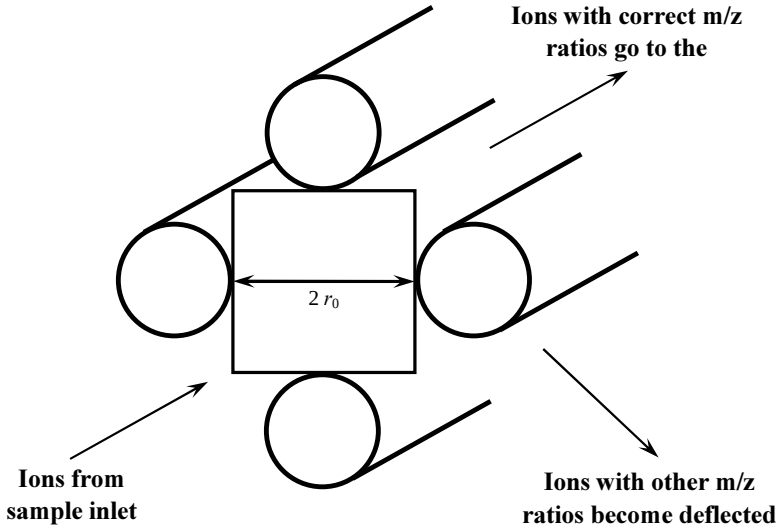

Figure 5 – Schematic figure of a quadrupole mass filter.

providing structurally informative fragments (product ions). The nature of the collision gas and the pressure of the collision cell are important. At higher gas pressures, both the number of ions undergoing collisions and the probability for an individual ion to collide several times with the target gas molecules increases. Furthermore, at higher gas pressures the product ions formed by dissociation of the precursor ion can be further activated by subsequent collisions and then also dissociate. In addition, several scan modes can be implemented using this technique [91]. The most common mode is the product ion scan, where a specific precursor is selected in Q1, fragmented in the collision cell and the products subsequently monitored in Q2. A second mode is the precursor ion scan, which is the reverse of the product ion scan. The Q2 is set to select a specific product ion formed in collision cell and the Q1 is scanned for all precursor ions forming the chosen product ion. An important use of those scans is in identifying drug metabolites having similar fragmentation behaviour and producing common fragment ions [91]. A third mode is the neutral loss scan, which is routinely used for rapid diagnosis in neonatal screening of metabolic diseases [91], and also is useful for identifying phase II metabolites, e.g. glucuronide and sulfate conjugates.

Other instruments using also low-energy collisions, are trapping devices, such as quadrupole ion traps (QIT) and Fourier-transform ion cyclotron resonance (FTICR) [91]. In QIT, the precursor ions are isolated and accelerated by 'on-resonance' excitation causing collisions to occur and product ions are detected by subsequent ejection from the trap. As is the case for QIT, in FTICR instruments isolation and excitation take place in the same confined space, where the ions are trapped for a specific time in combined magnetic and electrostatic field [91]. It is important to increase the specificity in analytical methods. This task is relatively difficult to achieve through enhancing the chromatographic resolution [92]. Better selectivity can be obtained with time-of-flight mass analyzers (TOF) that provide a high resolution (> 5000). One of these techniques is the quadrupole TOF (qTOF or QqTOF) mass spectrometer [93], which incorporates a series of multipoles (quadrupoles, hexapoles and octapoles) through which ions are focused, stored and, during MS/MS selected prior to activation with inert gas. The observed fragmentation shows low energy decomposition, similar to those recorded on TMS, due to the energy used to accelerate ions into the collision cell [93]. In MS/MS regime, the ions are pulsed orthogonally into the TOF by an accelerating voltage. The operational principle of TOF detection is based on the fact that the precursor and product ions acquire the same kinetic energy and thus can be differentiated by their velocities and flight times, which are related to their m/z ratios. This technique is broadly used in proteomic applications [93,94], where the identification of compounds (but not quantification) is of the primary importance.

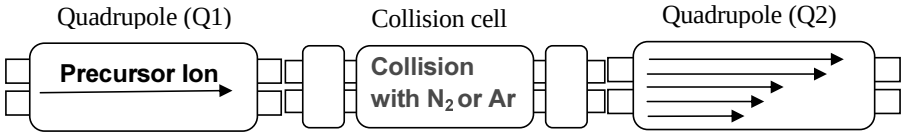

Figure 6 – Scheme of a tandem mass spectrometer (Reproduced after Sciex instruction).

2.6.1 Selected reaction monitoring

A great part of present day bioanalysis occurs using tandem MS [70]. Complex matrices often afford interferences over the chromatographic separations, and require a two-stage filtering process for accurate and precise quantification [92]. This process, termed selected reaction monitoring (SRM) is performed using the arrangement of TMS (see Fig. 6). The precursor of an analyte is selected by Q1 and the predominant fragment ions produced are monitored by the final quadrupole Q2. The unique selectivity offered by SRM is essential for bioanalysis where detection limits are frequently determined by the extent of chemical background originating from the sample matrix [70]. SRM is most commonly used in bioanalysis for quantification of the target analytes [92]. TMS detection provides a possibility to simplify sample preparation, which reduces sample analysis time in routine application and may allow direct analysis of diluted urine matrix [95]. However, one limitation associated with LC-MS analysis is its susceptibility to matrix effect [85,96-99].

2.6.2 Matrix effect

A commonly observed property of LC-API-MS is that analyte signal is affected by the sample matrix [70]. Matrix effect during the ionization process results in either signal suppression or enhancement. These effects are strongly compound-dependent, and are also more pronounced with ESI than with APCI [85]. While the analyte is in the gas phase, the charge might be lost through neutralization reactions, charge stripping, or charge transfer to another gas phase species. Investigation of the mechanism of ion suppression in ESI has shown that the gas phase reactions leading to the loss of net charge of the analyte is not the most important process causing ion suppression [85]. However, the presence of non-volatile solutes is much more important since it changes the droplet solution properties [85]. The pH, electrolyte concentration, and properties of the droplets are depending on the exact composition of the droplet, which may vary between biological samples. One of the consequences of unexpected changes in these factors can be the variability in analyte response, commonly referred to as ion suppression [85]. Molecules with higher mass may suppress the signal of smaller molecules [99] and more polar analytes are more susceptible to ion suppression [100]. Furthermore, for ESI, a predicative model based on competition among the ions in the solution for the limited number of excess charge sites has been proposed [101]. According to this model, at low concentrations of the analyte, relation between response and concentration is linear. However, at higher concentrations, the response becomes independent of the analyte concentration and is highly affected by the presence of other analytes [101]. Signal suppression of a substance, by increasing the concentration of the co-eluting analytes in a calibration series, is also quite common when using isotope dilution. However, the calibration curve is not affected because of this decrease, if a stable isotope analogue is used as internal standard. It may become more critical if the selected internal standard for an analyte does not co-elute [102].
Earlier, it was generally assumed that the highly selective LC-MS/MS technique permits the use of short chromatographic retention times and minimal or eliminated sample clean-up procedures [103]. Contrary to this common belief, those conditions can easily cause serious matrix effect problems effecting analyte response and sensitivity [98]. A number of approaches to assess matrix effect, recommended for LC-MS/MS assay development and validation have been proposed [98]:

1. Post-column infusion of analyte of interest, while injecting the blank matrix [100] allows to determine the extent of the effect of endogenous components present in the matrix on the analyte response as a function of chromatographic retention time. While increasing extraction selectivity is the most safe way to reduce ion suppression, it is often possible to reduce the impact by increasing the capacity factor (k') of the analyte [70].
2. The matrix effect may be examined by comparing the MS/MS response of an analyte at any given concentration spiked post-extraction **(B)** into biological matrix extract to the MS/MS response of the same analyte present in the interference free solution (mobile phase) **(A)**. The obtained value **(ME% = B/A x 100)**, showing the difference in response is assumed to be caused by components of the extract not present in the mobile phase [100] and may be considered as an absolute matrix effect [98]. The experiment may also be done by comparing the MS/MS response of an analyte at any given concentration spiked post-extraction **(B)** into biological matrix of different sources and relative matrix effect between different lots may be ascertained [98]. The presence of an absolute or a relative matrix effect for a given analyte does not necessarily indicate that the bioanalytical method may not be useful [98], because the analyte-to-internal standard (which is isotope labeled) ratio should not be affected.
3. The influence of late eluting components from the biological matrix, interfered with response of an analyte in subsequent run may be studied by comparing the MS/MS response of an analyte spiked into mobile phase and injected every second injection in the sequence to the response of the same analyte spiked into mobile phase before the first biological matrix injection.
Since polar matrix components causing matrix effects are eluted early in reversed-phase chromatography system, they can easily be separated from the analytes of interest. The most difficult matrix effect influence can be caused by hydrophobic components with retention times that overlap the analytes [96], resulting in decreased precision and accuracy of subsequent measurements [96,103].

2.6.3 Identification criteria

As LC-MS/MS methods become more frequent for quantitative confirmatory analysis in forensic toxicology, and guidelines for necessary requirements have been published by several organizations [104]. However, these guidelines has not yet been fully included in procedures for drugs analysis by LC-MS/MS [104].
The criteria for identification that the particular method has to fulfil to ensure reliable and objective identification of the analytes [104] is based on identification points (IP) [105] (Table 1). For MS/MS and related techniques, two SRM transitions are generally required [104], which is considered equivalent to three diagnostic ions [105]. The first SRM transition (usually the most intensive) is used for quantification (quantifier), and the second transition (qualifier) for confirmation of identity of substance. In addition, the relative intensities of the detected ions using various LC-MS techniques are expressed as a percentage of the intensity of the most intense ion or SRM transition and should fulfil criteria for permitted tolerance [104] (Table 2). Finally, the signal-to-noise (S/N) for all diagnostic ions should be greater than 3:1 [105].

Table 1. Selected examples of the number of IP earned for a range of MS techniques and combinations thereof (adapted from [104])

Technique	Number of ions	IP
LC-MS	n	n
GC-MS/MS or LC-MS/MS	1 precursor and 2 products	4
GC-MS/MS or LC-MS/MS	2 precursors, each with 1 product	5
$LC\text{-}MS^3$	1 precursor , 1 product and 2 of its fragments	5.5
LC-TOF	n	n
GC-MS and LC-MS	2+2	4
GC-MS and LC-TOF	1+1	4

Table 2. Maximum permitted tolerance for relative ion intensities using LC-MS/(MS) techniques (adapted from [104])

Relative intensity (% of base peak)	$LC\text{-}MS/(MS)^n$ (relative, %)
>50	±20
20-50	±25
10-20	±30
≤10	±50

2.7 METHOD VALIDATION

New analytical methods, to be used in forensic and/or clinical toxicology require careful method development followed by a thorough validation of the final method in order to generate reproducible and reliable data [106]. It is essential to employ well-characterized and fully validated analytical methods to yield reliable analytical results which can be correct interpreted [107] in the evaluation of scientific studies, as well as in daily routine work [106]. For quantitative bioanalytical procedures the validation parameters should include the following:

2.7.1 Selectivity

Selectivity is defined as “the ability of the bioanalytical method to measure unequivocally and to differentiate the analyte(s) in the presence of components, which may be expected to be present. Typically, these might include metabolites, impurities, degradants, matrix components, etc“ [107]. To establish method selectivity is to prove the lack of response by analyzing blank matrix from different lots [106-108], i.e., that there are no signals interfering with the signal of the analyte(s) or the internal standard (IS).

2.7.2 Calibration model (linearity)

The relationship between the concentration ratio analyte versus IS and the corresponding response. Calibrators should be matrix-based, cover the whole calibration range [106-108] and be evenly spaced across it [109]. Most guidelines require a minimum of five to eight concentration levels [107,108].

2.7.3 Accuracy and precision

The accuracy of the method is the combination of systematic and random error components, whereas the estimate of the pure systematic error should be indicated with bias [110]. Bias is "the difference between the expectation of the results and an accepted reference value" [110]. Bias can be reported as percentage and is calculated from expression [111]:

% Bias = [(measured value – true value)/true value)] ×100.

Precision is the closeness of agreement (degree of scatter) between a series of measurements obtained under the prescribed conditions and may be considered at three levels: repeatability, intermediate precision and reproducibility [112]. Precision is commonly measured in terms of imprecision, which expressed as an absolute or relative standard deviation (R.S.D.) and does not relate to reference interval. Repeatability expresses the precision under the same operating conditions over a short interval of time and termed as intra-assay or within-assay precision [112]. Intermediate precision is a measure of the within-laboratory variation due to different days, analysts, equipments, etc. [112]. Intermediate precision can also be expressed as the total precision under varied conditions [106]. Reproducibility expresses the precision between laboratories (collaborative studies, usually applied to standardization of methodology) [112].

2.7.4 Range of measurement

The lower limit of quantification (LOQ) is the lowest amount of an analyte in a sample that can be quantitatively determined with suitable precision and accuracy (bias) [107,112]. The acceptance criteria for both parameters at LOQ are 20% R.S.D. for precision and ± 20% for bias [107]. The LOQ can also be estimated by signal-to-noise ratio [108,112] and is usually required to be equal to or greater than 10.

The upper limit of quantification (ULOQ) is the highest calibration standard that can be quantified with fulfilled acceptable precision and accuracy (bias).

The limit of detection (LOD) is the lowest amount of analyte that can be detected. The most common way to define the LOD is by using the signal to noise ratio of 3:1.

In the field of forensic toxicology or doping control, the LOD as the lowest concentration of analyte in a sample should be defined in agreement with fulfilment of specific identification criteria [105].

2.7.5 Recovery

The recovery can be calculated as the percentage of the analyte response after sample workup compared to that of a solution containing the analyte at a concentration corresponding to 100% recovery [106]. In LC-MS/(MS) analysis, the absolute recovery and matrix effect should be determined by different experimental design [98], because

part of the change of the response in prepared samples in comparison to respective standard solutions might be attributable to matrix effect [98].

2.7.6 Robustness

The robustness/ruggedness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage [112]. Full validation must not necessarily include robustness testing, but it should be performed if a method is to be transferred to another laboratory [112,113].

2.7.7 Stability

Stability can be defined as follows: “The chemical stability of an analyte in a given matrix under specific conditions for given time intervals” [107].
It may be subjected to change prior to analysis, under sample preparation and under conditions of analysis [106] . Unless data on analyte stability are available in the literature, full validation of method must include stability experiments for the various stages of analysis including storage prior to analysis [106].

2.7.8 Matrix effect

Studies of ion suppression/enhancement should be an integral part of any LC-MS/(MS) method [106]. The approaches to study ion suppression/enhancement are described above (see “2.6.2”).

2.7.9 Carry-over

Carry-over is a commonly encountered analytical problem that can compromise the accuracy of assays. The carry-over may be determined after injection of a sample spiked with a very high concentration of an analyte.

3 AIMS OF THE STUDY

The aims of the present study were:

1. To develop an LC-MS method for determination of EtG in urine and to apply the method to clinical samples.

2. To compare the urinary excretion of EtG with that of ethanol with focus on the effect of water-induced diuresis.

3. To develop an LC-MS method for GTOL in urine suitable for clinical application.

4. To develop a direct injection LC-MS/MS method for simultaneous determination of GTOL and 5-HIAA in urine.

5. To apply the LC-MS/MS for GTOL/5-HIAA and compare it with ELISA assay for GTOL in clinical material and to study the detection time of urinary GTOL/5-HIAA with ethanol and EtG.

4 MATERIAL AND METHODS

4.1 ETHICAL APPROVAL

The projects included in paper I-IV were approved by the ethics committee at the Karolinska Institutet (Dnr KI 99-338). The project of paper V was approved by the ethics committee of Landesärztekammer Rheinland Pfalz, Mainz, Germany Dnr 837.095.05(4753) 20.

4.2 URINE SAMPLES

Urine samples were obtained from patients treated for alcohol and drug abuse in an outpatient treatment program in Stockholm (Sweden) or patients being hospitalized for recovery from acute alcohol intoxication in Rheinland Pfalz (Germany). In addition, samples were also obtained from healthy individuals (social drinkers).
The samples were stored at –20°C until analysis. The clinical urine samples used for assessment of the stability of EtG on storage as well as the blank urine samples used for the study of potential artificial formation of EtG were left at 4°C or room temperature. The clinical urine samples used for the study of stability of GTOL and 5-HIAA on storage were left at room temperature, 4º C or -20º C.

4.3 SAMPLE PREPARATION

4.3.1 EtG analysis by LC-MS (study I, II and V)

Prior to analysis, the samples were diluted to 1:10 of the initial concentration by adding deionised water containing the internal standard.

4.3.2 GTOL analysis by LC-MS (study III)

The samples were extracted manually by SPE. The SPE procedure used octadecyl (C_{18}) cartridges. First, the cartridges were conditioned with methanol and deionised water. The urine sample, internal standard and trifluoroacetic acid were mixed, applied and allowed to flow through the cartridge. The cartridges were then washed with deionised water and dried applying light pressure. The analytes in the cartridges were eluted using freshly prepared methanol-water (50:50, v/v) and the extracts were evaporated to dryness under nitrogen.

4.3.3 GTOL and 5-HIAA analysis by LC-MS/MS (study IV and V)

Prior to analysis, the samples were diluted to 1:1 (25 µL:25 µL) of the initial concentration by adding deionised water containing the internal standard. To the diluted sample, 10 µL of 1% ammonia was added.

4.4 LC-MS ANALYSIS OF ETG (STUDY I, II AND V)

The mass spectrometer equipped with an electrospray interface set to negative ionization mode and selected ion monitoring (SIM) mode. A Hypercarb column combined with a Hypercarb guard cartridge was used. A mobile phase consisting of 5% acetonitrile and 25 mmol/L formic acid. The flow was 200 µL/min.

4.5 LC-MS ANALYSIS OF GTOL (STUDY III)

The electrospray interface used with the mass spectrometer was operating in the negative mode and the SIM method was applied. The urine extracts were reconstituted in 100 µL deionised water (sample). 15 µL of the sample was injected on a Hypurity C_{18} separation column. The system was run isocratically at 2% acetonitrile in 50 mmol/L formic acid following injection of the sample. After 2 minutes, the concentration of acetonitrile was gradually increased to 50% within 5 minutes and kept there for 8 minutes. The column was re-equilibrated for 10 minutes. The mobile phase flow was 200 µL/min.

4.6 LC-MS/MS ANALYSIS OF GTOL AND 5-HIAA (STUDY IV AND V)

The electrospray interface used with the mass spectrometer was operating in the negative mode and the SRM method was applied. A 1.8 µm 100 x 2.1 mm (inner diameter) high strength silica (HSS) trifunctional C_{18} column, preceded by 0.2 µm column filter was used. The system was operated at 97% of 0.1 % formic acid and 3% of acetonitrile following injection of the sample and the concentration of acetonitrile gradually increased to 20% at 2 min. After 2 min, the concentration of acetonititrile gradually increased to 100%, which was reached at 2.5 min. 100 % acetonitril was maintained for 0.5 min. The column was re-equilibrated for 0.6 minutes. The mobile phase flow rate was 400 µL/min.

4.7 ELISA ANALYSIS OF GTOL (STUDY V)

GTOL was determined by ELISA, using a monoclonal antibody (AlcoDia Co, Stockholm, Sweden). The optical density reading was performed at 450 nm.

4.8 LC-ELECTROCHEMICAL DETECTION ANALYSIS OF 5-HIAA (STUDY IV AND V)

5-HIAA in study IV was analysed by LC with electrochemical detection, as previously described [63].
5-HIAA in study V was analysed on an isocratic HPLC system with electrochemical detection (CLC 100 electrochemical detector). The analytical column and chromatography was used according to the manufacturers instructions (Chromsystems, Munich, Germany).

4.9 ANALYSIS OF ETHANOL AND CREATININE (STUDY II)

The ethanol concentration was determined enzymatically using yeast ADH. Urinary creatinine was determined by the Jaffe reaction on a Hitachi 917 analyzer.

5 RESULTS AND DISCUSSION

5.1 STUDY I: DIRECT QUANTIFICATION OF ETHYL GLUCURONIDE IN CLINICAL URINE SAMPLES BY LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY

Chromatographic conditions

EtG had a low capacity factor and separation from the void was not obtained by reversed-phase chromatography. Instead a Hypercarb column containing porous graphite material yielded a suitable retention time with a capacity factor of about 4 (see Fig. 7). A novel feature of this work was development of a chromatographic system suitable for the direct analysis of EtG in urine. The porous graphite material shows higher selectivity for polar substances as compared to conventional reversed-phase chromatography on silica-based systems [114-116]. This porous graphite chromatographic system can be used with isocratic elution instead of gradient elution, which is often used in reversed-phase chromatography [35,37]. However, the mobile phase was not strong enough to elute all components of the urine matrix from the column. Therefore, following approximately 1000 injections, recovery of the column with solvent in order to remove adsorbed components was required. The use of diluted samples (1:10) resulted in improved system robustness and decreased risk for the contamination of the chromatographic column. Routinely LC-MS analysis of EtG was performed in the negative ion mode, using SIM of the deprotonated molecules for EtG (m/z 221) and internal standard EtG-$^{2}H_5$ (m/z 226).

Method validation

The validation showed that linear response curves were obtained within the range 0.1 to 1500 mg/L. Repeated injection and intra- and inter-assay coefficients of variation never exceeded 12.2%.

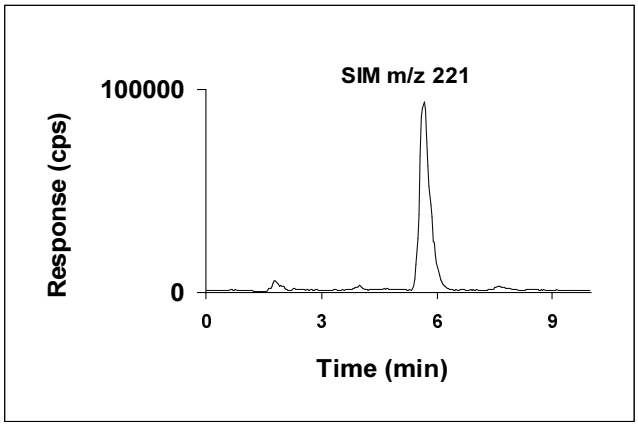

Figure 7 – Chromatogram showing the peak for EtG injected on the Hypercarb column in a clinical urine sample with a concentration of 25 mg/L by the LC-MS method.

The limit of detection (LOD) (s/n ~ 3:1) was estimated to 0.05 mg/L and carry-over was below 0.005%. In the SRM mode, the LOD was about 0.3 mg/L (s/n ~ 3:1).

Stability of EtG in urine

The stability of EtG in urine was confirmed by the following results:

1. The concentration of EtG in urine positive for EtG did not change during storage for 4 days at room temperature.
2. No EtG can be found in urine of volunteers, who abstained from alcohol for at least 10 days.
3. Urines negative for EtG remain negative following 14 days at room temperature.
4. Addition of alcohol (1%) to negative urine samples and storage for 10 days at 4°C does not result in any measurable EtG formation.

Application of the method

Analysis of 252 clinical urine samples from outpatients treated for alcohol abuse. The EtG concentration ranged from the LOQ to 1240 mg/L, with 63% samples being positive (see Fig. 8). Approximately 32% of the positive samples showed values above 100 mg/L and 37% were below 10 mg/L. The urinary excretion pattern of EtG after ingestion of a very low ethanol dose (7 g as a low-alcohol beer) with or without food by healthy volunteers is shown in Fig. 9. The highest levels of EtG (range 0.6-8.4 mg/L) were observed when ethanol was ingested without food. Alcohol intake in combination with food produced much lower EtG levels (0.2-0.6 mg/L). This experiment demonstrated a high sensitivity of LC-MS for EtG in detecting a single alcohol intake. A concentration of EtG less than 10 mg/L, which was observed in 37% of the EtG-positive clinical urine samples, could result either from heavy drinking the days before or the intake of a low-alcohol drink a few hours before voiding.

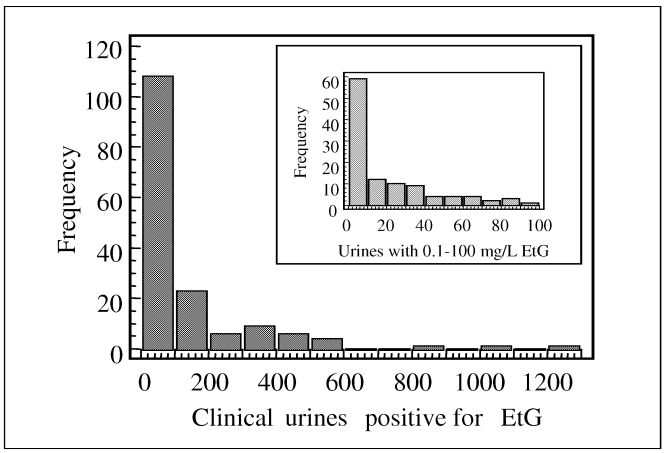

Figure 8 – Distribution of EtG concentrations in the 159 clinical urine samples. Inset: Distribution of the 108 urine samples with an EtG value of < 100 mg/L.

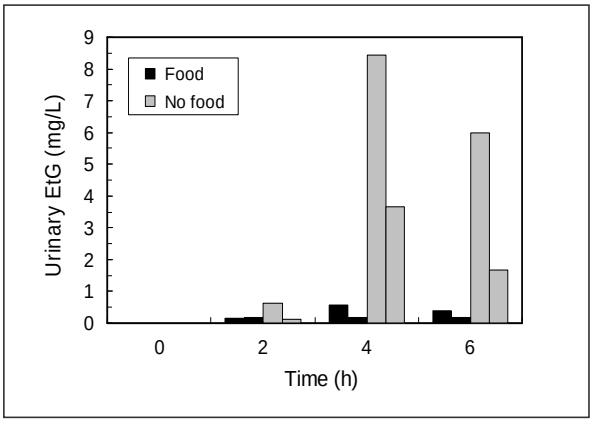

Figure 9 – Urinary excretion profile of EtG after oral intake of a low-ethanol dose (7 g). Two subjects injected the ethanol together with food, and two did so in a fasted state. Data presented are the individual values.

This experiment demonstrated a high sensitivity of LC-MS for EtG in detecting a single alcohol intake. A concentration of EtG less than 10 mg/L, which was observed in 37% of the EtG-positive clinical urine samples, could result either from heavy drinking the days before or the intake of a low-alcohol drink a few hours before voiding. Lower reference levels than 10 mg/L may detect recent intake of small amounts of low-alcohol beverages. Thus, EtG levels higher than 10 mg/L could suggest a positive cut-off for clinically relevant alcohol intake.

5.2 STUDY II: COMPARISON OF URINARY EXCRETION CHARACTERISTICS OF ETHANOL AND ETHYL GLUCURONIDE

Intake of an ethanol dose of 0.5 g/kg (beer) resulted in an increase in urine production and a concomitant drop in the creatinine concentration in urine. The concentration of ethanol increased rapidly and reached a mean peak value of 17.0 ± 2.5 (SD) mmol/L in the 1.5-h urine sample. EtG was already detectable in the first urine collection 1.0 h after alcohol intake. At 3 h after starting drinking the beer, intake of the same volume of the water resulted in another increase in urine production and a drop in the creatinine concentration. The urine ethanol concentration decreased independent of water intake, whereas the EtG concentration decreased from a mean value of 44.6 mg/L three hours after drinking alcohol to 13.8 mg/L 1 h after water intake. The urine ethanol concentration reached zero after 6-h after alcohol intake. In contrast, the EtG concentration reached a peak 6 h after alcohol intake and started to decline with a half-life of ~ 2.5 h. The EtG/creatinine ratio in urine was not markedly affected by water intake (see Fig.10). The urinary ethanol concentration returned to below the LOQ at 6.5 h, whereas EtG was detectable at low levels (< 1 mg/L) for up to 22.5 – 31.5 h after starting the experiment. Applying 10 mg/L as a positive cut-off, EtG detection time was in the range of 8.5 – 12.5 h. The amount of ethanol converted to EtG and detected in urine as EtG was only 0.02% of the orally administered ethanol on molar basis.

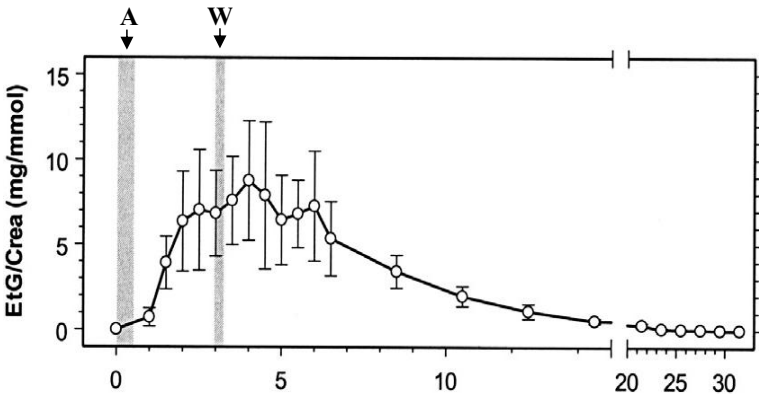

Figure 10 – Time course of the urinary EtG/creatinine ratio after six healthy volunteers drank 594–986 ml export beer (range 25.0–41.5 g ethanol, corresponding to 0.5 g/kg) in 30 min (A, first shaded area) and 3 h later the same volume of tap water in 10 min (W, second shaded area). Data represent mean values ± SD.

This study demonstrates that ingestion of a water load prior to urine sampling leads to a reduction in the EtG urine concentration, while the concentration of ethanol in urine was not affected. EtG could play an important role as a test for recent alcohol consumption during the descending part of the concentration-time curve of EtG excretion, but the water-induced diuresis may then cause the already low EtG concentration to fall below the limit of detection.

The normalization of values to creatinine is common practice to compensate for dilution of urine samples and has been used to improve interpretation of the pharmacokinetics of conjugated metabolites of for example cannabis [117].

In urine drug testing, intentional dilution of urine by excessive water intake is often common practice to decrease the chances of positive drug concentration in urine. It is highly likely that this also will be used by alcohol patients. Thus, it is important to verify EtG in urine independent of urine dilution. This study may suggest that calculation of the EtG/creatinine ratio can be applied either routinely or only in patients with EtG levels lower than 10 mg/L.

5.3 STUDY III: DETERMINATION OF URINARY 5-HYDROXYTRYPTOPHOL GLUCURONIDE BY LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY

Initial experiments revealed that direct analysis of urine, diluted with water containing internal standard, was not feasible because the target LOD (<25 nmol/L) [64] was not achieved and chromatographic interference occurred frequently. Furthermore, because of polar interferences in urine, simple filtration of urine using SPE columns with reversed-phase (C_{18}, C_8) and strong anion exchanger (SAX) phase did not show sufficient recovery and good linearity. Thus, there was a need for SPE purification of the sample and analyte concentration for detection of the target LOQ for endogenous concentrations of GTOL. By sample purification on C_{18} or SAX SPE columns this problem was solved. The C_{18} phase was selected due to a higher sensitivity and

reliability as compared to SAX phase. Moreover, elution of GTOL with 50% methanol in water was more selective with less interference than 100% methanol. Finally, the analytes in the eluate were concentrated to further increase the sensitivity.
A Hypercarb column with porous graphite was tested but resulted in long retention times and poor chromatography due to tailing. In contrast, a Hypurity C_{18} analytical column showed a suitable retention time about 7 minutes with a capacity factor about 4.8 (see Fig. 11). Gradient elution was chosen because of shorter retention time and improved peak shape, resulting in better separation and less interference. However, an analysis time of 25 minutes was needed due to interference of late eluting compounds and column equilibration. Linear response curves were obtained between 6.4 and 8500 nmol/L. The LOD (s/n ~ 3:1) was estimated to be 2 nmol/L, and the LOQ was 6.4 nmol/L (s/n ~ 10:1). The intra- and inter-assay CV were <3.5% and <6% respectively. The carry-over in the LC-MS system was less than 0.003%. The endogenous GTOL concentration in urine samples obtained from 13 volunteers who had abstained from ethanol for at least 10 days prior to sampling (38-327 nmol/L) was in the same range as the total 5-HTOL levels previously reported [54] (range 98-301 nmol/L) for controls individuals. When urinary GTOL is used as an alcohol biomarker [10], it is reported as ratio to 5-HIAA, which compensates for variations in 5-HT turnover and urine dilution. This results in an overall improved specificity for alcohol consumption. To compensate for urine dilution, the 5-HTOL(GTOL)/creatinine ratio may also be used, but this will not compensate for fluctuations in 5-HT metabolism due to dietary intake of 5-HT [118]. The GTOL/5-HIAA mean ratio (6.24) was similar to previously reported 5-HTOL/5-HIAA ratios (7.0 [54] and 7.6 [58]). Also the GTOL/creatinine ratio (12.1) was in the same range as the reported 5-HTOL/creatinine ratio [119] for non consumers of alcohol (9.8) and in social drinkers (14.6).

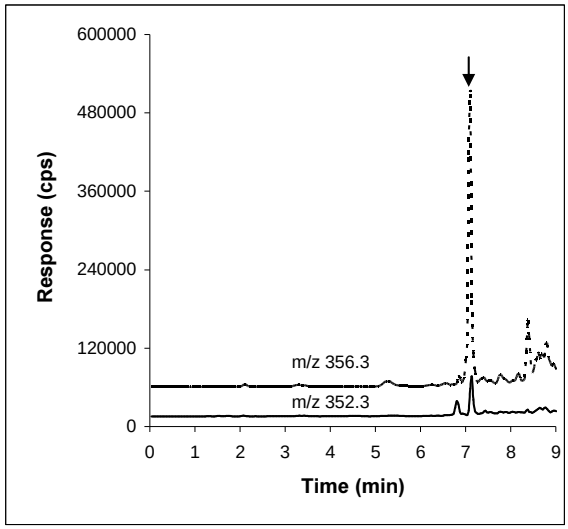

Figure 11 – LC-MS chromatograms of a human urine sample containing 1000 nmol/L GTOL injected on the Hypurity C_{18} column, showing the peaks for GTOL (m/z 352.3) and internal standard GTOL-D4 (m/z 356.3).

The comparison of the present LC-MS method for GTOL and the GC-MS method for 5-HTOL (free plus liberated from GTOL) included a total of 70 urine samples, obtained from healthy volunteers who abstained from alcohol and from patients undergoing treatment for alcohol and drug abuse. The methods showed a high correlation ($R^2 = 0.99$) with no outliers. The mean ratio for GC-MS (5-HTOL)/LC-MS (GTOL) for all samples, covering the range 10-6000 nmol/L, was 1.10 (SD, 0.16).
In previous studies [54] the free level of 5-HTOL was determined by excluding the enzymatic treatment by the GC-MS method. The sum of free and glucuronide conjugated 5-HTOL was determined after hydrolysis with β-glucuronidase. Free 5-HTOL accounted for less than 5 percent of total 5-HTOL [54] in 27 urine samples, which is slightly lower than the GTOL concentrations by LC-MS (10%). This was expected as the GC-MS method measures the sum of free and glucuronide conjugated 5-HTOL.
The present study (III) demonstrates for the first time that direct quantification in urine of GTOL can be performed by LC-MS with high accuracy and sensitivity, which represents an analytical improvement over previous indirect methods, including HPLC [64,65], GC-MS [10], LC-MS [62] with either enzymatic hydrolysis or derivatisation. This method may be very helpful as a reference method for the development of an ELISA assay for direct measurement of GTOL as a biomarker attractive for clinical use.

5.4 STUDY IV: DETERMINATION OF 5-HYDROXYTRYPTOPHOL GLUCURONIDE AND 5-HYDROXYINDOLEACETIC ACID BY DIRECT INJECTION OF URINE USING ULTRA-PERFORMANCE LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY

Chromatographic conditions
A suitable chromatographic solution was achieved on a UPLC Acquity C_{18} analytical column with a capacity factor (k') of about 2.5 for GTOL and 3.6 for 5-HIAA (see Fig. 12). Gradient elution was chosen over isocratic because of shorter retention times and sharper peaks, which resulted in higher sensitivity and less interferences from neighbouring peaks. A novel feature of development of a chromatographic system was use of a reversed-phase chromatographic media with a small particle size (<2 μm), which provided improved resolution to the chromatographic analysis [81,82]. The UPLC analysis produced higher sensitivity for both GTOL and 5-HIAA analytes through improvements in the column efficiency. The use of the flow rate of 400 μL/min in combination with a column temperature of 60º C and a column length of 100 mm resulted in a total analysis time of 3.6 min. The capacity of the method was 16 samples per hour. In comparison, the previous LC-MS method for measurement of urinary GTOL [120] required an initial sample preparation and 25 min run time in order to separate interfering matrix components. The use of the UPLC technology was crucial in this study, particularly for determination of the low endogenous levels of GTOL. Coupling of the UPLC system to the high speed MS/MS provided with the possibility to simplify sample preparation, which resulted in a simple urine dilution with internal standard.

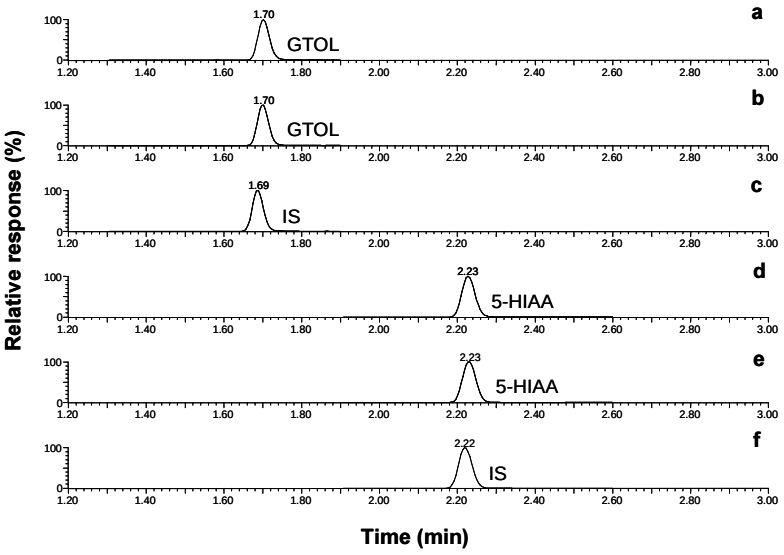

Figure 12 – UPLC-MS/MS chromatograms showing the peaks for GTOL (**a**: *m/z* 352.1→131.0; **b**: *m/z* 352.2→175.9), GTOL-$^2H_4$ (**c**: *m/z* 356.2→131.0), 5-HIAA (**d**: *m/z* 189.9→146.2; **e**: *m/z* 189.9→116.2), and 5HIAA-$^2H_2$ (**f**: *m/z* 191.9→148.2) on the UPLC Acquity C_{18} analytical column for a water calibrator containing 2500 nmol/L GTOL and 25 µmol/L 5-HIAA.

MS/MS conditions

The electrospray negative ionisation mode was chosen, because of a stronger response for deprotonated molecules $[M–H]^-$ of each compound, as compared to the signal of either electrospray positive or APCI modes.Addition of ammonia to the injected urine extract reduced the background signal up to 35% and as a result, an increased signal-to-noise ratio was obtained. In order to optimize further the sensitivity for GTOL, detection and quantification was divided into separate time periods, one for GTOL and another for 5-HIAA. Routinely LC-MS/MS analysis was performed with selected-reaction monitoring (SRM) using two most intense transitions for each compound and one transition for each internal standard. The presence of interferences for the available ions to monitor was evaluated by injecting different urine samples. This resulted in a choice of the transition 352→131 as the target for GTOL, and the transition 356→131 for GTOL-D4.

The identification of GTOL and 5-HIAA compounds was done by the following criteria: (i) the relative intensities of the transitions (qualifier-to-target) were within the accepted range (± 20%) as compared to the relative intensities of transitions of the reference standard. However, in the samples with low GTOL concentration (about 15%), the interference was detected and the automatic integration of the transitions area resulted in the relative intensities outside ± 20% range. The manual integration was performed to correct the integrated area (see Fig. 13). The target ions were used for all validation parameters and for calculation of LOD, and both transitions were estimated for a signal-to-noice ratio of >3; (ii) for chromatography, the retention time (RT) and the relative retention time (RRT) for both transitions of the analyte did not differ by more than 1% from the reference standard in the same batch.

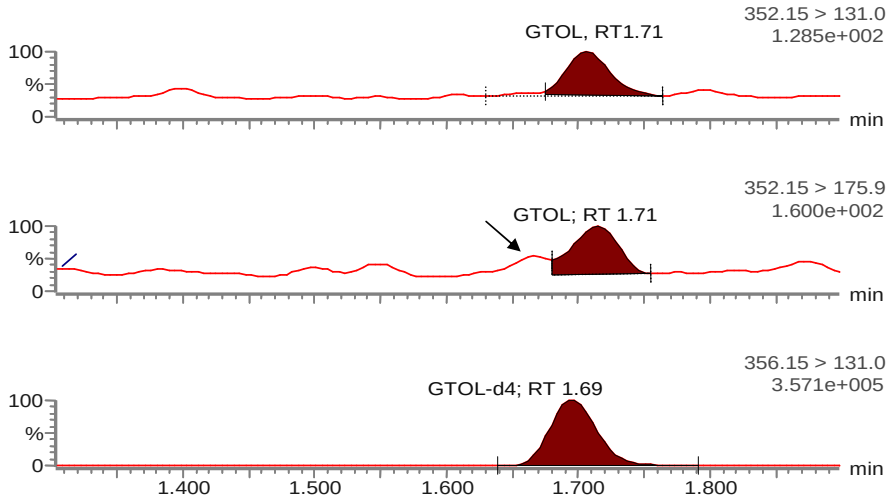

Figure 13 – UPLC-MS/MS spectrometry chromatograms of patient urine sample (about 25 nmol/L of GTOL) showing the presence of interference for *m/z* 352.2→175.9.

Matrix effect

The presence of interferences in the LC-MS/MS analysis may not always be observed, due to the use of SRM detection. As a result, the response of electrospray for a given concentration of an analyte can change in the presence of other sample components. This inability to direct detect the interference with MS/MS makes identification and elimination of the interference more difficult [100]. The study of matrix effect was done in several experiments:

1. In order to determine the extent of change in the analytes response in the presence of the interference under LC-MS/MS assay conditions, post-column infusion of water solutions of GTOL or 5-HIAA into the MS was used. A simultaneous injection of a urine matrix with either gradient or isocratic elution showed that the most intense response loss (99%) occurred after the column void had eluted (see Fig. 14).

The recovery time was longer for 5-HIAA (>3 min) than for GTOL (~ 1.7 min). Use of gradient elution gave a greater loss of MS/MS response compared to isocratic elution, which might be due to the fact that interfering components elutes faster with gradient elution. A greater ion suppression was present at the time of elution for 5-HIAA, compared to GTOL (see Fig. 15).

In conclusion, this experiment demonstrated that urine sample components affect both analytes. In addition, detailed information about the time profile of the interference as it elutes from the column under the chromatographic conditions of the method was obtained.

2. The matrix effect was examined by comparing the response of internal standards (GTOL-$^2H_4$, 5-HIAA-$^2H_2$) in 20 randomly selected urine samples to the response of the same amount of internal standards present in 5 calibrators (water matrix). The obtained values were on average 83% for GTOL-$^2H_4$ and 48% 5-HIAA-$^2H_2$, showing the difference in response (on average 17% and 52% lower for GTOL and 5-HIAA, respectively), assumed to be caused by components of the urine samples not present in

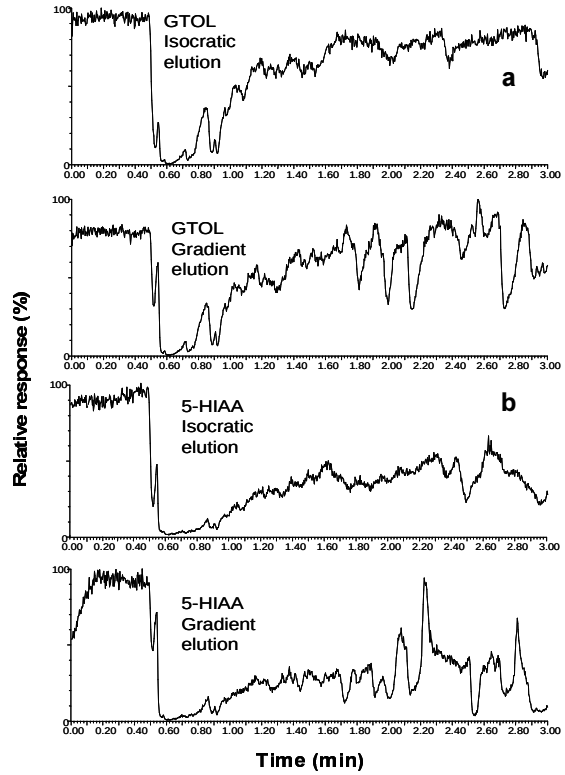

Figure 14 – (a) Effect of injecting urine sample with isocratic and gradient elution on the mass spectrometric response of an infused GTOL solution. (b) Effect of injecting urine sample with isocratic and gradient elution on the mass spectrometric response of an infused 5-HIAA solution.

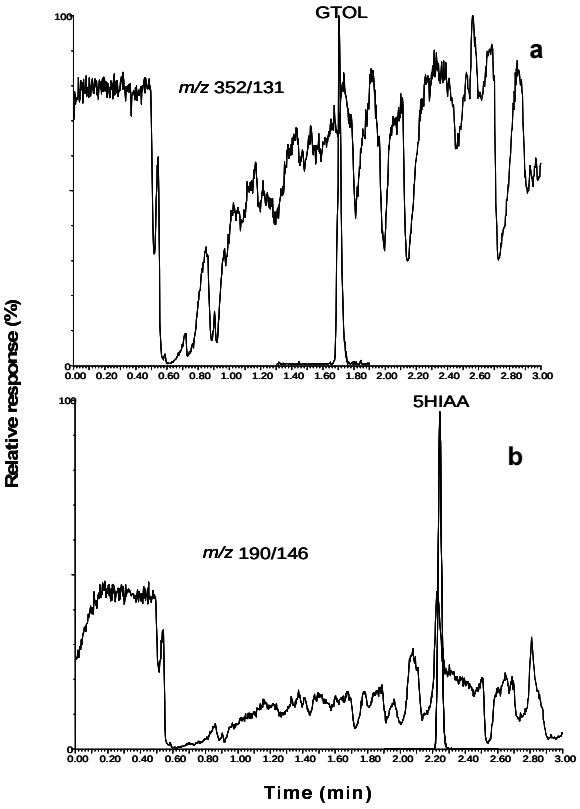

Figure 15 – (a) Effect of injecting urine sample on column with post-column infusion of GTOL. The infusion chromatogram from GTOL is overlaid with the GTOL calibrator chromatogram. (b) Effect of injecting urine sample on column with post-column infusion of 5-HIAA. The infusion chromatogram from 5-HIAA is overlaid with the 5-HIAA calibrator chromatogram.

water matrix [98]. This experiment demonstrated the influence of different matrix contents on ion suppression of the analytes.
3. The influence of late eluting components from the urine samples, interfered with the response of the internal standards in subsequent injections was studied by comparing the MS/MS response of the internal standards in water solution injected every second injection in the sequence of ten urine samples to the response of the same internal standards before the first urine injection. No significant long-lasting influence on response of internal standards during the sequence was observed. Ion suppression or ion enhancement may affect the method validation parameters, such as LOD, LOQ, linearity, precision and/or bias; especially the latter three in the absence of an isotopically labeled internal standard [106]. In this study, stable isotope labeled analogues were used as internal standards, since matrix effect should not affect the relative efficiency of ionization of the analyte and internal standard [98].

Method validation
Linear response curves were obtained for GTOL up to 10000 nmol/L and for 5-HIAA up to 100 µmol/L. The LOD (s/n ~ 3:1) and the LOQ (s/n ~ 10:1) for GTOL were 2 nmol/L and 6.7 nmol/L, respectively, and for 5-HIAA 0.02 µmol/L and 0.07 µmol/L, respectively. The intra- and inter-assay CV for GTOL were <5.8% and 6.2%, respectively, and for 5-HIAA <3.3% and 3.5% respectively. The values of quality control (QC) samples observed over 2 weeks (N = 23) for the method in routine use for GTOL were <6.5% (mean, 111 nmol/L) and <5.2% (mean, 1995 nmol/L), respectively, and for 5-HIAA <4.8% (mean, 14 µmo/L) and <2.7% (mean, 31 µmo/L), respectively. The carry-over in the LC-MS/MS system was <0.01% for GTOL and <0.001% for 5-HIAA. The analytical recovery was closed to 100%.

Stability of GTOL and 5-HIAA on storage in urine
The concentration of GTOL and 5-HIAA did not change during storage for 4 days at 22°C, 4°C, or –20°C.

Method comparison
The GTOL and 5-HIAA results of the UPLC-MS/MS method for 25 patient urine samples were highly correlated with the 5-HTOL (r^2 = 0.975) (see Fig. 16) and 5-HIAA (r^2 = 0.969) (see Fig. 17) results from the reference methods [10,63]. The median ratio for total 5-HTOL (GTOL plus free 5-HTOL; by GC-MS) over GTOL (UPLC-MS/MS), covering the range 50–5000 nmol/L, was 1.14 (95% CI, 1.10–1.26), which is in the same range as previously reported (mean, 1.10; SD, 0.16; N=70) [120]. The free level, determined by excluding the enzymatic treatment in the GC-MS method, accounted for less than 5% of the total 5-HTOL [54] in 27 urine samples, which is slightly lower than the direct measurements of GTOL concentrations by LC-MS techniques. This was expected as the GC-MS method measures the sum of free and glucuronide conjugated 5-HTOL. For 5-HIAA, the median ratio between HPLC and UPLC-MS/MS in the range 1.6 – 60 µmol/L was 1.05 (95% CI, 0.78-2.04). The 5-HIAA concentrations by UPLC-MS/MS were in the same range as those determined by HPLC, except for two samples that showed much lower values by UPLC-MS/MS, which resulted in a ratio between methods outside the 95% CI. Exclusion of those two samples identified as outliers by Box-and-Whisker plot did not change the median ratio but the 95% CI was reduced to 0.97–1.13. This difference in the measurements might be due to a lack of

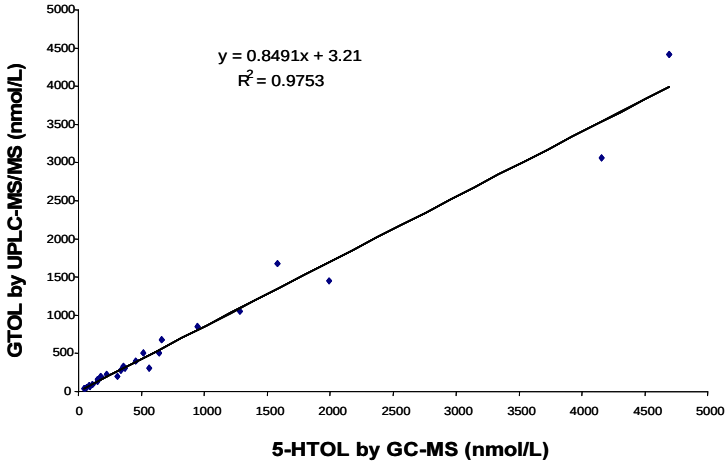

Figure 16 – Correlation between GTOL values obtained with the UPLC-MS/MS method and the corresponding 5-HTOL values (sum of free and GTOL) obtained by the GC-MS method for 25 human urine samples.

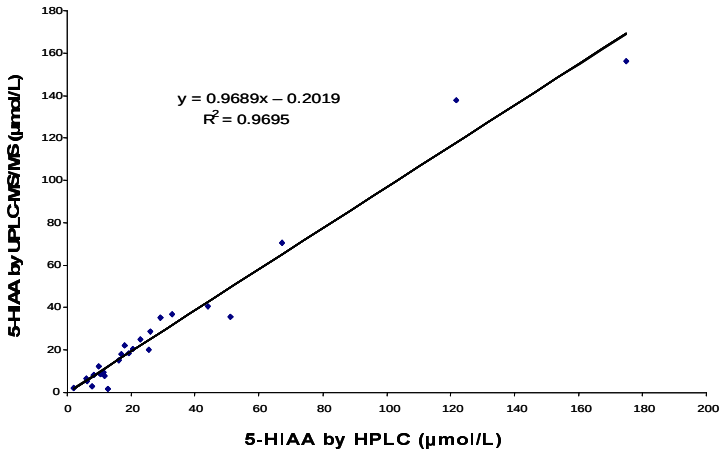

Figure 17 – Correlation between the 5-HIAA values obtained with the UPLC-MS/MS method and the HPLC method for 25 human urine samples.

selectivity of the HPLC method. The median ratio for GTOL/5-HIAA over 5-HTOL/5-HIAA for all samples was 0.92 (95% CI, 0.74–1.56).

Exclusion of two outliers with high 5-HIAA levels by HPLC and low levels by UPLC-MS/MS did not affect the median ratio but the 95% CI was 0.85–0.96. The high correlation obtained between GTOL/5-HIAA and 5-HTOL/5-HIAA, and the agreement between the median ratios for GTOL/5-HIAA and 5-HTOL/5-HIAA, indicates that the

ratio of GTOL/5-HIAA is equivalent to the ratio of 5-HTOL/5-HIAA, which is used as an alcohol biomarker.

Endogenous levels of GTOL and 5-HIAA
The endogenous GTOL concentrations in urine samples, obtained from 10 volunteers who had abstained from ethanol for several days prior to sampling (14 – 197 nmol/L) were in the same range, as the GTOL levels (range 38 – 327 nmol/L) [120] and the total 5-HTOL levels (range 98 – 301 nmol/L) [54] previously reported for control individuals. When urinary GTOL is used as alcohol biomarker [10], it is reported as ratio to 5-HIAA to compensate for variations in 5-HT turnover and urine dilution [55]. This results in an overall improved specificity for alcohol consumption. The GTOL/5-HIAA mean ratio (5.1) was similar to previously reported GTOL/5-HIAA ratio 6.24 [120] and 5-HTOL/5-HIAA ratios (7.0 [54] and 7.6 [58]). To compensate for urine dilution, the 5-HTOL(GTOL)/creatinine ratio may also be used, but this will not compensate for fluctuations in 5-HT metabolism due to dietary intake of 5-HT [118]. Also the GTOL/creatinine ratio (mean 24.7; median 14.5) was in the same range as the reported the GTOL/creatinine ratio (12.1 [120]) and the total 5-HTOL/creatinine ratio [119] for non consumers of alcohol (9.8) and in social drinkers (14.6).

In conclusion, the present study demonstrated for the first time that direct measurement of the GTOL/5-HIAA ratio in diluted urine, being used as an alcohol biomarker, can be performed by the validated UPLC-MS/MS method. The simultaneous quantification of urinary GTOL and 5-HIAA represents an important improvement over previous methods that have involved two separate analytical techniques; usually HPLC for 5-HIAA [63] and GC-MS for 5-HTOL [10]. The simplicity of this method, achieved by direct injection of urine, should have advantages over earlier methods using solid phase [120] or solvent extraction procedures in combination with derivatisation [62].This method can be used for direct measurement of GTOL/5-HIAA ratio as a biomarker in clinical context.

5.5 STUDY V: BIOMARKERS TO DISCLOSE RECENT INTAKE OF ALCOHOL: POTENTIAL OF 5-HYDROXYTRYPTOPHOL GLUCURONIDE TESTING USING NEW DIRECT UPLC-TANDEM MS AND ELISA METHODS

In ten alcoholic patients studied during detoxification, the median detection time for ethanol in urine was ~ 17 h. However, as sampling occurred infrequently, ethanol elimination curve may overestimate the elimination time for ethanol in the body, because the last point for several individuals were at times when ethanol was no longer detected in urine. Accordingly, the blood ethanol concentration may have reached zero many hours before [11,57].
The detection time for EtG was between 10 to >90 h (median, ~ 65 h), which is considerably longer than observed in experiments with healthy volunteers [39,121]. However, a marked inter-individual variability in the EtG concentrations was observed, even after correcting the values for urine dilution [39,122]. Because prolonged drinking does not cause accumulation of EtG [123], the longer detection times may be related to the higher doses of ethanol ingested.

An increased urinary GTOL/5-HIAA ratio was observed between 9 and >100 h (median, ~ 39 h). The shorter detection time of GTOL/5-HIAA compared with EtG is in accordance with observations on alcohol patients and from experiments in healthy controls [39,121,123]. The lower sensitivity of GTOL/5-HIAA compared with EtG may be useful to differentiate between intentional intake of a more substantial (moderate-to-heavy) dose of alcohol but not very small amounts and unintentional intake.

The comparison of the UPLC-MS/MS and ELISA methods for GTOL included a total of 88 urine samples from randomly selected patients being hospitalized for recovery from acute alcohol intoxication. The ELISA method correlated (r=0.935) with the UPLC-MS/MS (see Fig. 18), albeit with a marked scatter in the low concentration range. The result agreement between the UPLC-MS/MS method for GTOL/5-HIAA and the ELISA method for GTOL with a commercial HPLC method for 5-HIAA (applying a molar ratio of 0.015 as cut-off limit) was 61%, with an 82% sensitivity for finding samples for elevated levels. However, in terms of sensitivity and specificity, the results of the present study rather favour the UPLC-MS/MS method.

The present study (V) demonstrated that two new methods are available for measurement of urinary GTOL and confirmed the usefulness of the LC-MS/MS method. This might facilitate the use of GTOL as an alcohol biomarker of recent drinking in routine laboratories.

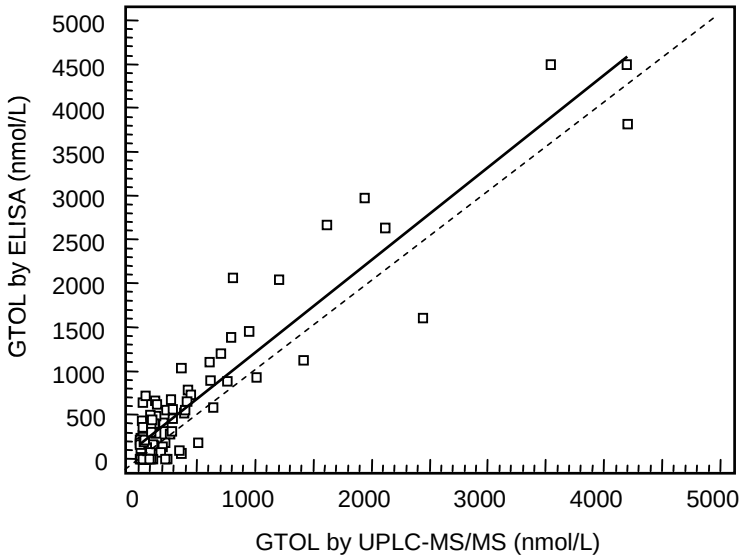

Figure 18 – Agreement between the ELISA and the UPLC-MS/MS methods for determination of urinary GTOL in the measuring range of the ELISA assay: $r = 0.9351$, $P < 0.0001$, $N = 88$ (Equation: Y = 161.781 + 1.054X). Dotted line: $x = y$.

6 GENERAL DISCUSSION

The development and application of laboratory tests that can identify early problematic drinking and monitor abstinence may have the potential of reducing the healthcare costs and suffering associated with alcohol misuse. The field of biochemical alcohol markers, both for clinical and forensic applications has expanded greatly over the years. This has provided an improved knowledge of drinking patterns in both individuals and populations, and the use of biomarkers is of a great value for the objective evaluation of treatment efforts [12]. Determination of the urinary GTOL/5-HIAA ratio and EtG in urine is much more sensitive than conventional ethanol testing, and thus provides a means of monitoring recent alcohol consumption. The GTOL/5-HIAA ratio in urine has proven valuable for the purpose of monitoring adherence of alcohol-dependent subjects [28], and also used in hospital settings to improve the possibility of identifying high-risk patients at an early stage [124]. Since introduction of testing capability for EtG in US (2003) [22], it has become a valuable tool in physician monitoring programs [125]. Use of EtG testing randomly or routinely in high-risk individuals with multiple relapses may be very effective in detecting covert alcohol use [22]. Testing for acute alcohol consumption may also be applied in certain workplaces upon return to safety-sensitive duties [126], because it is known that the acute and post-intoxication effects (hang-over, fatigue) of heavy drinking will impair performance, resulting in an increased risk for accidents and injury [127]. In forensic toxicology, testing for the GTOL/5-HIAA ratio and EtG can be used to estimate whether the ethanol originates from alcohol ingestion prior to death or has been generated artificially (i.e. post-mortem) [29] or if ethanol formation occurred due to incorrect storage of unpreserved biological specimens prior to analysis [128].

Urine testing for drugs of abuse, including screening and confirmation methods, has increased dramatically and become well established in clinical and forensic applications [2]. Alcohol markers may be considered to be a compliment to urine testing for drugs of abuse. Thus, further improvement of methodology for measuring these alcohol biomarkers suitable for routine use is needed. In this study, the developed LC-MS assay for the measurement of EtG in urine fulfils the requirement of a routine clinical method. In addition, it has been used in the clinical laboratory for several years. The measurement of urinary EtG is superior to GTOL/5-HIAA ratio regarding detection time [121,123], but provides sensitivity for intake of very low amounts of ethanol or even unintentional ethanol exposure [129]. The use of GTOL/5-HIAA ratio may offer a useful alternative, as it focuses on moderate-to-heavy drinking. The aim of the present study was to develop a method for direct measurement of GTOL, including simultaneous quantification of GTOL and 5-HIAA, for direct determination of the GTOL/5-HIAA ratio in urine. Since urine is a complex and variable matrix, the performance of LC-MS/MS can be affected, resulting in invalidation of qualitative and quantitative results. As a consequence, the method development included careful validation in order to generate reproducible and reliable data.

The requirement on chromatographic technique was to separate the analytes from matrix endogenous components and also from co-eluting interferences. Gradient elution was chosen over isocratic because of shorter retention times and sharper peaks, which resulted in higher sensitivity and less interferences from neighbouring peaks. A novel feature of development of a chromatographic system was use of a reversed-phase

chromatographic media with a small particle size (<2 µm), which provided improved resolution to the chromatographic analysis [81,82]. The UPLC analysis produced higher sensitivity for both GTOL and 5-HIAA analytes through improvements in the column efficiency. Furthermore, the co-eluting interferences, which were not detectable by ion chromatograms due to the high selectivity of MS, were evaluated by several experiments for matrix effect. The use of isotope labeled internal standards was very important, because it corrected for variations in ion response and minimized the influence from matrix effect [98]. When using the SRM method, the identification of the analytes was performed by suggested identity confirmation criteria in accordance with guidelines [104,105].

The present study demonstrated the potential of developing robust and selective methods for quantification of analytes in urine using ESI LC-MS and LC-MS/MS with minimal sample preparation.

7 CONCLUSIONS

- Analysis of EtG in urine by ESI-LC-MS or ESI-LC-MS/MS is a technique with advantages over GC-MS analysis because of higher sample throughput and shorter analysis time (10 min per run instead of approximately 20 min), no need for extensive sample preparation or derivatisation as in GC-MS, and less contamination in the chromatographic system.

- EtG is a robust and stable analyte, which is a great advantage for sampling, handling and transportation to the laboratory for analysis. Ethanol in urine does not result in any artificial EtG formation.

- EtG remains in the urine for many hours after ethanol itself has been eliminated. Thus, testing urine for the presence of EtG provides a mean for determination of recent alcohol consumption. Expressing urinary EtG as a ratio to creatinine is recommended in routine clinical use to compensate for urine dilution.

- To overcome the potential risk of “false positive” results due to the high sensitivity of the LC-MS method, further studies must include the definition of cut-off values for EtG determination for diagnostic purposes.

- The LC-MS method for GTOL developed for this study correlates well with previous GC-MS methods and provides the possibility to use the main metabolite GTOL as an alcohol marker in clinical and forensic purposes.

- The LC-MS for EtG and GTOL method may represent a major improvement for routine clinical application by its simplicity.

- The UPLC-MS/MS method for simultaneous quantification of GTOL and 5-HIAA developed in this study represents an important improvement needed for clinical routine application.

- Use of the same analytical technique for urinary GTOL/5-HIAA as for EtG provides a good option, for use of these alcohol markers in combination or to identify the most appropriate single marker in clinical cases.

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