Confirmatory Analysis of Ethylglucuronide in Urine by Liquid-Chromatography/Electrospray Ionization/Tandem Mass Spectrometry According to Forensic Guidelines

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β-D-ethylglucuronide (EtG) is a stable Phase II metabolite of ethanol which can be detected in urine samples several days after elimination of ethanol. It is a useful diagnostic parameter for monitoring abstinence of alcoholics in alcohol withdrawal treatment. For this purpose, determination in urine is mainly performed by LC-MS, LC-MS/MS, or by GC-MS. For the mass spectrometric identification and detection of controlled substances in more sensitive fields such as forensic toxicology, workplace drug testing, doping analysis, and veterinary organic residue control, official guidelines have been released requiring a chromatographic separation and a minimum of two mass spectrometric transitions of the analyte. However, for detection of EtG none of the published LC-MS/MS methods could fulfill the minimum requirements of any of these guidelines. Therefore, an existing LC-MS/MS method has been modified by monitoring further MS/MS transitions instead of only one (deprotonated molecule $[M - H]^-$/product ions: *m/z* 75, 85, 113, and 159 optional) with the aim of withstanding administrative or court scrutiny in forensic or workplace drug testing cases. Full method validation has been performed in accordance to guidelines of the German Society of Toxicology and Forensic Chemistry (GTFCh) and requirements of ISO 17025. One application field in the United States is a workplace monitoring program to detect surreptitious alcohol use among recovering health professionals, who by contract had agreed on total abstinence after drug and alcohol withdrawal therapy. (J Am Soc Mass Spectrom 2004, 15, 188−193) © 2004 American Society for Mass Spectrometry

Current biological markers for monitoring recent alcohol consumption remain suboptimal with regard to sensitivity and specificity. Furthermore, these biomarkers can be influenced by age, gender, and a variety of substances and non-alcohol-associated diseases, and do not fully cover the time axis for alcohol intake. Conjugation of ethanol with activated glucuronic acid in the presence of membrane-bound mitochondrial UDP glucuronyl transferase represents a minor detoxification pathway for ethanol: About 0.02–0.06% (mean) of the dose of ethanol administered is recovered as β-D-ethylglucuronide (EtG) in urine in humans [1] and—dose dependent—0.5–1.5% in rabbits [2]. EtG is a non-volatile, water-soluble, stable, direct metabolite of ethanol that can be detected in various body fluids, tissues and hair. EtG ($C_8H_{14}O_7$) has a molecular weight of 222 g/mol, and the melting point (decomposition temperature) is about 150°C. Shortly after the initial consumption of even small amounts of ethanol, EtG is formed. It has been detected in urine up to 80 h after the complete elimination of alcohol from the body and was not detectable in teetotalers with a 0.1 mg/L cut-off [3, 4]. EtG is unique in covering this important time span of one to three days after alcohol uptake. In urine, it can be detected longer than ethanol. Therefore, EtG meets the need for a sensitive and specific marker to elucidate alcohol use not detected by

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Table 1. Methods which have been successfully used for the detection of EtG

Method	Derivatization Specimen Internal standard	Ionization *m/z*-values or transitions for detection	Chromatographic details	Ref.
GC-MS	Silylation (MSTFA) Serum/urine D_5-EtG[b]	EI *m/z* 160, 261, 405[a] (ISTD: 164, 266, 410[a])	(5%-phenyl)-methylpolysiloxane (DB 5)	[13, 31]
GC-MS	Silylation (MSTFA)[b] Hair Methylglucuronide	EI *m/z* 261, 292, 375, 405[a] (ISTD: 391[a])	(5%-phenyl)-methylpolysiloxane (DB 5)	[16]
GC-MS	Perfluorpropionylation Serum Propylglucuronide	EI *m/z* 405[a], 365, 289, 259 (ISTD: 405[a], 365, 289, 259)	100% dimethylpolysiloxane (DB 1)	[32]
LC-MS (SIM)	No derivatization Serum No internal standard	ESI neg. *m/z* 221[a]	(a) Column: TSK-Gel Amide-80, 250mm × 4.6mm, (acrylamide-type); Tosoh, Tokyo, Japan) Eluent: 30 mM ammonium acetate-acetonirile (30:760, v/v)	[11]
			(b) Column: Thermoquest Hypercarb (100mm × 2mm), Keystone/Thermo Sci.[c] Eluent: 25 mmol formic acid/acetronitrile (95:5 v/v)	[4]
LC-MS MS	No derivatization Serum, urine, hair D_5-EtG	ESI neg. 221–75[a] (ISTD: 226–75)	(a) Column: RP C18[b]; Eluent: 0.1% formic acid (isocratic) (b) Column: Synergy polar RP; polar-endcapped phenyl-propyl (Phenomenex)[b] [3,14], Eluent: 0.1% formic acid (isocratic) with post-column addition of acetonitrile	[12]

[a]quantifier ion;
[b]validation was performed using linear regression;
[c]confirmation was performed with LC-MSMS with selected samples (one transition: *m/z* 221–*m/z* 75)

standard testing [5–13]. Furthermore, EtG has been detected in hair samples [14–16] and post-mortem tissues (liver, brain, fat tissue [17]) of alcohol addicts. Recently, the determination of ethylglucuronide in urine was shown to be superior to ethanol as a proof of recent alcohol consumption in a workplace drug testing program of recovering health professionals [18].

For the detection of EtG in urine and serum, immunoassays are under current development [19–21]. However, to date the development of an immunoassay with sufficient sensitivity and specificity has been unsuccessful. Therefore, mass spectrometric methods, such as GC-EI-MS after derivatization, and LC-MS or LC-MS/MS with negative electrospray ionization, using selected ion monitoring (of the deprotonated molecule, *m/z* 221) and one MS/MS-transition (deprotonated molecule to fragment ion; *m/z* 221–75), respectively, have been applied to the analysis of urine (Table 1). Quantitative analysis has been performed by use of penta deuterium-labeled internal standard (D_5-EtG) or the glucuronides of ethanol homologues (methylglucuronide, propylglucuronide). Since EtG is a very polar substance, precipitation or dilution prior to analysis by GC-MS and LC-MS have been used for sample clean-up in most published papers. LC-MS or LC-MS/MS methods are advantageous in comparison with GC-MS methods because of the reduced need for derivatization and shorter analysis times (10 min per chromatographic run) [4, 12]. In our experience, GC-MS with derivatization requires much more system maintenance than does LC-MS, because of the high load of polar matrix which is present in urine samples. This is important for the analysis of large numbers of samples.

Guidelines for urine drug testing in the clinical laboratory have been approved by the National Committee for Clinical Laboratory Standards (NCCLS) [22]. When the results of urine drug testing can affect an individual's reputation, job status, or freedom, forensically-acceptable analytical procedures must be utilized, and the findings must be legally defensible. For a confirmatory analysis in forensic toxicology, guidelines require chromatographic separation and at least three ions for compound detection or identification with SIM when a single quadrupole GC-MS is used [23]. For the identification of residues by LC-MS/MS analysis, European guidelines require chromatographic separation and a minimum of two transitions for substance detection; when LC-MS (single MS) is used, three characteristic ions are required [24, 25]. None of the published LC-MS or LC-MS/MS methods for EtG listed in Table 1 fulfilled these requirements, since either only one ion with optimized "in-source" CID conditions was used with a single-quadrupole instrument [4, 11], or only one

characteristic transition was used for EtG by LC-MS/MS-analysis [12, 14].

Our aim was to develop and to validate an LC-MS/MS method with at least two transitions for EtG which would fulfill the requirements for forensic confirmatory analysis, thus being suitable for workplace drug testing programs and forensic toxicological analysis.

Experimental

EtG and D_5-EtG standards were obtained from Medichem (Stuttgart, Germany). HPLC-grade acetonitrile and formic acid (analytical grade) were purchased from Merck (Darmstadt, Germany). Deionized water was prepared with a cartridge-deionizer from Memtech (Moorenweis, Germany). Sample preparation: To 0.1 mL blank urine or an EtG spiked urine 50 ng D_5-EtG (10 μL of an aqueous solution, 5 μg/mL) was added and the sample diluted with 0.25 mL methanol. The sample was centrifuged (14,000 rpms) for ten min at 4°C. Next, 0.25 mL of the clear supernatant were transferred to a glass autosampler vial (1.5 mL) and evaporated to dryness by use of a vacuum concentrator (60 min, 45°C, 1 mbar, Alpha RVC, Martin Christ, Osterode, Germany). The dried residue was then redissolved with 0.14 mL of 0.1% formic acid, transferred to a glass micro insert (0.2 mL volume), and 10 μL were injected into the LC-MS/MS system.

The LC-MS/MS system consisted of an API 365 triple-quadrupole mass-spectrometer fitted with a turbo ionspray interface (Applied Biosystems/Sciex, Darmstadt, Germany) and a Shimadzu HPLC system (two pumps LC10AD Shimadzu, Duisburg, Germany). Analyses were performed with electrospray ionization using a turbo ionspray source in negative mode. EtG was separated at 40°C on a polar-endcapped phenylpropyl reversed phase column (Synergy Polar-RP 250 × 2 mm, 4 μm) with a guard column (4 mm × 2 mm, same packing material) (Phenomenex, Aschaffenburg, Germany). The mobile phase consisted of aqueous 0.1% formic acid (vol/vol) at a flow-rate of 0.2 mL/min. To enhance signal intensity, acetonitrile was added with a post-column "tee" before the effluent enters the turbo ionspray interface. With a six-port/two-way switching valve (Labpro, Rheodyne, Rohnert Park, CA) the LC-effluent was admitted to the MS interface only between 4 and 6 mins of the chromatographic retention time. The turbo ionspray source was operated at 400°C with an ionization voltage of −4 kV, and nitrogen as curtain gas and nebulizer gas. Optimization of the ion source and MS/MS parameters for data acquisition were done by infusion of a 1 μg/mL solution of EtG (20 μl/min) with a syringe-pump coupled with a tee-union to the outlet of the HPLC system using the autotune function of the Analyst software (Applied Biosystems/Sciex, Darmstadt, Germany). Analysis was performed by multi-reaction monitoring, using the precursor ion at *m/z* 221 and the fragment ions at *m/z* 75, 85, 113, and 159 for EtG, and precursor ion at *m/z* 226 and fragment ion at *m/z* 75 for the internal standard D_5-EtG (dwell-times: 200 msec for each transition, defragmentation potential: −16 V, focusing potential: −140 V, entrance potential: 3 V, collision energy: −16 eV, collision cell exit potential: −10 V, collision gas pressure: 2.4–0.7 × 10^{-5} torr nitrogen; unit resolution for Q1 and Q3). For method validation VALISTAT program was used [26].

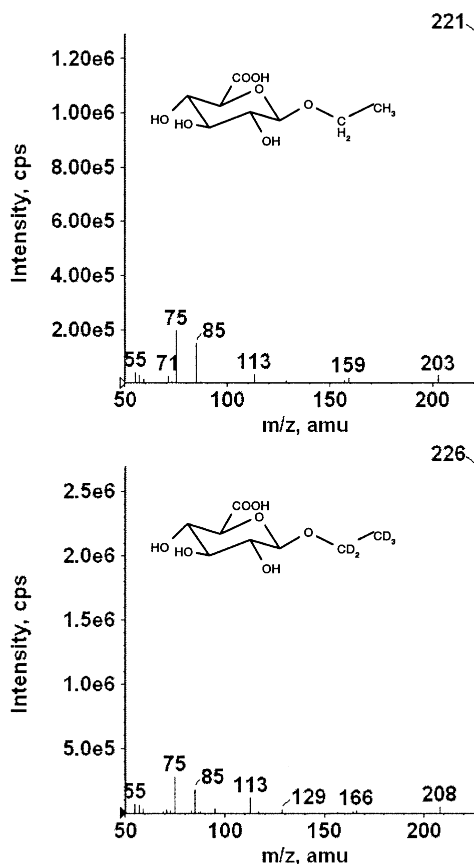

Figure 1. Product-ion spectra of EtG and D_5-EtG (precursor ions *m/z* 221 and 226).

Results

The most abundant product ions of EtG and D_5-EtG (*m/z* 75, 85, and 113) (see Figure 1) result from the

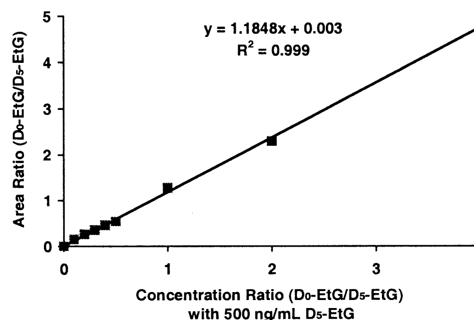

Figure 2. Calibration with spiked urine samples.

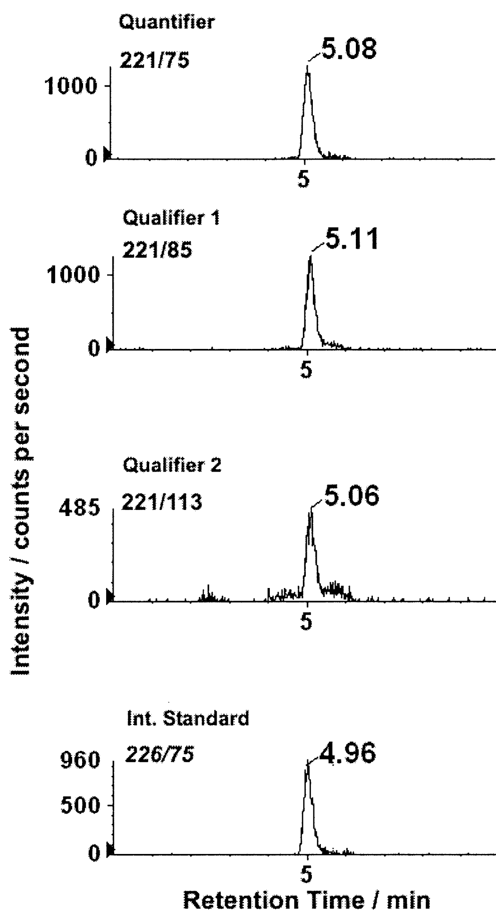

Figure 3. Ion chromatograms of urine sample of a female volunteer 8 h after drinking sparkling wine with 9 g ethanol content (495 ng/mL EtG was determined).

glucuronic acid not including the aglycon ethanol. The ions with low abundance result from lactonization or loss of water (*m/z* 203), additional decarboxylation (*m/z* 159), loss of ethanol (*m/z* 113), and water (95) or CO (85). In positive ionization mode, β-D-glucuronides, e.g., of morphine, codeine and nor-Δ^9-carboxy-tetrahydrocannabinol (cannabis metabolite), show a totally different fragmentation pattern (neutral loss of the glucuronide at C_1, mass difference 176 u and further fragmentation of the aglycon) [27, 28]. However, in negative mode the ions at *m/z* 75, 85, and 113 could also be generated either by CID or by in-source CID of other glucuronides of xenobiotics or endogenous substances and could give rise to mass spectrometric interferences if not separated by liquid chromatography and by precursor ion filtering using MS/MS.

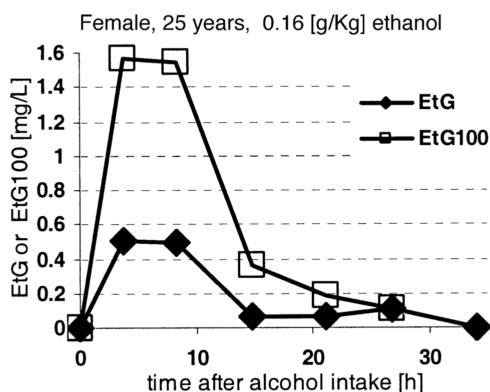

Figure 4. Time course of urine EtG and EtG100 (normalized to creatinine concentration of 100 mg/dL) of a female volunteer after drinking sparkling wine (0.1 L) with a total of 9 g ethanol.

Validation has been performed using a common validation procedure [29] with a Microsoft Excel based validation program (VALISTAT [26]). A linear calibration model was used for transition 221/75 (base peak) as quantifier and transition 221/85 (96% base peak intensity) as qualifier. The less abundant transitions 221/113 (46% base peak intensity) and 221/159 (15% base peak intensity) were not used for method validation, but can be used as additional qualifiers for analyte identification. The calibration curve was linear over the range from 50 to 2000 ng/mL (see Figure 2). Linear regression [23] with equidistant calibration levels (0, 50, 100, 150, 200, and 250 ng/mL) was used for the calculation of LOD and LOQ with a significance level of 33% (k = 3): LOD: 52 ng/mL (with α-error 10% for the qualifier) LOQ: 152 ng/mL (α-error 1% for quantifier). Intra-assay repeatability and accuracy (bias) were tested by replicate analysis of two quality control samples (200 ng/mL and 2000 ng/mL, respectively) in eight assays, and produced results as follows. Intra-assay repeatabilty: for 200 ng/mL, 4.19% relative standard deviation (RSD) and 0.03% bias; for 2000 ng/mL, 3.78% RSD and 1.94% bias.

Figure 3 shows the typical ion chromatograms of the quantifier and two additional qualifiers for EtG in a urine sample (495 ng/mL EtG) obtained from a female volunteer 8 h after drinking 0.1 L of sparkling wine (9 g ethanol) in a controlled study. Figure 4 shows the time course of EtG elimination in this study; due to the correlation of EtG concentration and creatinine concentration [30], EtG100 was calculated by normalizing the measured EtG- and creatinine concentrations to a creatinine concentration of 100 mg/dL. In this study with nine volunteers, no EtG was detected in the baseline urine samples collected after one week of abstinence, and no interferences with other compounds were found in the retention time window of EtG (5.1 ± 0.3 min) for the transitions of the quantifier and qualifiers.

Conclusions

A validated LC-MS/MS method has been developed which fulfills the requirements for forensic confirmatory analysis for EtG in urine through the use of at least two MS/MS-transitions for compound identification, instead of only one transition as published recently by others. Although this could easily be achieved, none of the previous LC-MS(MS) methods ever took this possibility into consideration, mainly because the discussion

of validity of results produced by LC-MS/MS methods by selected reaction monitoring has just started in the workplace and forensic drug testing environments.

Method validation has been performed according to guidelines of the GTFCh, ISO 17025, and general rules for bioanalytical method validation [29], which are rather detailed for testing specificity, regression models, intra-assay repeatability, and accuracy. In analyzing more than 1000 urine samples from clinical and forensic cases, we have found that LC-MS/MS has advantages over GC-MS analysis [16, 31, 32] because of higher sample throughput by shorter analysis time (10 min per run instead of approximately 20 min), no need for derivatization as in GC-MS, and less contamination of the chromatographic system.

Until now, no EtG has been detected in baseline urine samples of volunteers of our study or those studied by others. These volunteers were social drinkers; baseline urine was analyzed after total abstinence for several days. However even low amounts of ethanol in the range of 1 to 2 g, which could be ingested with "alcohol-free" beverages, pharmaceutical preparations containing ethanol, or by food containing low amounts of ethanol (e.g., cakes, fruit juices) could give rise to low urine EtG concentrations. To overcome the potential of "false positive" results due to the high sensitivity of the LC-MS/MS method, further studies must include the definition of cut-off values for EtG determination for diagnostic purposes. Furthermore, inter-individual differences of glucuronidation rates have to be investigated, and the proposal of a cut-off should include normalization to the creatinine concentration.

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