Quantitation of ethyl glucuronide in serum & urine by gas chromatography - mass spectrometry

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Background & objectives: Alcohol misuse has now become a serious public health problem and early intervention is important in minimizing the harm. Biochemical markers of recent and high levels of alcohol consumption can play an important role in providing feedback regarding the health consequences of alcohol misuse. Existing markers are not sensitive to recent consumption and in detecting early relapse. Ethyl glucuronide (EtG), a phase-II metabolite of ethanol is a promising marker of recent alcohol use and can be detected in body fluids. In this study an analytical technique for quantitation of EtG in body fluids using solid-phase extraction (SPE) and gas chromatography (GC) with mass spectrometric detection (MS) was developed and validated.

Methods: De-proteinization of serum and urine samples was done with perchloric acid and hydrochloric acid, respectively. Serum samples were passed through phospholipids removal cartridges for further clean up. EtG was isolated using amino propyl solid phase extraction columns. Chromatographic separation was achieved by gas chromatography with mass spectrometry.

Results: Limit of detection and limit of quantitation were 50 and 150 ng/ml for urine and 80 and 210 ng/ml for serum, respectively. Signal to noise ratio was 3:1, mean absolute recovery was 80-85 per cent. Significant correlation was obtained between breath alcohol and serum EtG levels (r=0.853) and urine EtG and time since last abuse (r = -0.903) in clinical samples.

Interpretation & conclusions: In the absence of other standardized techniques to quantitate EtG in biological samples, this GC-MS method was found to have high throughput and was sensitive and specific.

Key words Ethanol - ethyl glucuronide - gas chromatograph - mass spectrometer - solid phase extraction

Alcohol is one of the most frequent addictions worldwide that results in many severe health problems and pathologies\(^1,2\). The World Health Organization has estimated that two billion people consume alcoholic beverages globally, with 76.3 million of those suffering from a diagnosable alcohol-use disorder\(^3\). The global burden of diseases from alcohol exceeds that of tobacco and is on par with the burden attributable to unsafe sex practices world-wide\(^4,5\). Alcohol has detrimental effects, contributing to liver disorders, gastrointestinal...
problems, cardiovascular and diabetic complications, sexual function disorders, bone loss, neurological complications and increased risk for cancer\textsuperscript{2,5}. Objective screening for alcohol use can provide an opportunity to intervene for improving patient health and quality of life. However, in the absence of specific and sensitive biomarkers for alcohol abuse, physicians often fail to recognize such patterns\textsuperscript{1,7,8}. Questionnaires sometimes provide unreliable information or may not be applicable to all patients. The value of breath ethanol testing is restricted by the rapid elimination rate of ethanol from the body resulting in short detection time (typically <12 h)\textsuperscript{9}. Although ethanol is a good marker of alcohol consumption, its detection in body fluids is possible for a relatively short time after alcohol intake and, therefore, its use is limited\textsuperscript{10}. Alcohol biomarkers such as carbohydrate deficient transferrin (CDT) and gamma glutamyl transferase (GGT) mainly identify persons engaged in long-term heavy drinking\textsuperscript{7} whereas their sensitivity to detect current infrequent use is low\textsuperscript{11}.

For this reason, there is a need to find a useful and reliable marker of alcohol consumption. A sensitive short-term alcohol biomarker with longer detection windows than ethanol may add important information about individual drinking patterns. Ethyl glucuronide [(ethyl β-D-6 glucuronic acid, (EtG)] is a direct metabolite of ethanol and can be detected in body fluids up to 3 days. It is formed by the conjugation of glucuronic acid with ethanol\textsuperscript{12,13}. EtG is a non volatile, water soluble, acidic and stable metabolite of alcohol. It can be detected in various body fluids, tissues and hair. Compared to other short term and long term biomarkers, it is a more specific and sensitive marker of alcohol abuse\textsuperscript{10,11}, which can be applied for both clinical and forensic analysis\textsuperscript{14,15}. Many analytical methods have been developed and validated for detection and quantitation of EtG in the biological matrices, such as nuclear magnetic resonance (NMR)\textsuperscript{16}, capillary zone electrophoresis (CZE)\textsuperscript{3}, liquid chromatography–mass spectrometry (LC-MS)\textsuperscript{14} and gas chromatography–mass spectrometry (GC-MS)\textsuperscript{18,15}. But because of the complex nature of urine and serum, these methods are frequently hindered by the matrix compounds co-eluting with EtG in urine and serum. Therefore, the objective of the present work was to develop and validate an accurate and precise analytical method for quantitation of alcohol metabolite EtG in biological fluids using gas chromatography with mass spectrometry (GC-MS).

Material & Methods

All chemicals used were of analytical grade. Bulk solvents and routine chemicals were procured from SISCO research laboratory (Mumbai, India). Ethyl glucuronide was procured from Enzo-life, (Switzerland) and methyl β-D-glucuronide (internal standard) and derivatizing agent MSTFA (N-methyl–N-trimethylsilyl tri fluoracetamide with 1% trimethylchlorosilane) were procured from Sigma Aldrich, USA.

**Blood and urine samples:** Laboratory tests including blood sugar, urea, liver function test, breath alcohol and EtG are offered as part of the clinical evaluation at the Centre for Addiction Medicine at the National Institute of Mental Health and Neuro Sciences (NIMHANS), Bengaluru, Karnataka, India. The standardization of ethyl glucuronide was carried out in 2011-2012, for a planned prospective study on evaluating EtG as a biomarker of recent alcohol consumption. The study protocol was approved by the institutional ethics committee. For the purpose of EtG estimation, blood (2-3 ml) and urine (2-5 ml) were collected from both outpatients and inpatients at Centre for Addiction Medicine, NIMHANS. All consecutive patients with diagnosis of alcohol dependence during the study period were included in the study. Those with severe morbidities were excluded. Urine samples were collected from all 76 subjects [outpatients (n=63) and inpatients (n=13)] among these, urine and serum was available for 35 subjects while urine, serum and breath alcohol were available for 20 subjects. Method validation was done with 10 anonymous samples for which urine, serum and breath alcohol samples as well as history of last use were available. Each sample was accompanied by a requisition form containing details including clinical details and self reported alcohol use\textsuperscript{12,17}. Serum was separated from blood samples and stored at -20°C. Urine samples were centrifuged to settle endogenous impurities and were stored in -20°C until analysis.

**Sample preparation:** For serum deproteinization, acetonitrile with 1 per cent formic acid serum was added to an aliquot containing serum in a ratio of 1: 3. Aliquot was vortexed for two minutes at 2000 g. Sample was passed through phospholipids removal cartridges (Phenomenex), filtrate was collected and was subjected to solid phase extraction. Methyl glucuronide (2500mg/ml) was added as an internal standard (IS). To 1.5 ml of urine sample, 100µl of 3M hydrochloric acid was added. Sample was centrifuged at 3000 g for 5 min.
Clear supernatant was added to 3.5 ml of acetonitrile containing 2500 ng/ml of internal standard.

**Solid phase extraction (SPE):** Mega BE amino cartridge (Agilent, USA) used for SPE was conditioned with 3.0 ml of methanol, 3.0 ml of de-ionized water and 3.0 ml of acetonitrile. It was ensured that the column did not get dry in between the conditioning steps. Pretreated sample was loaded at the rate of 0.5 ml/min to the amino cartridge. Cartridge was washed with 3.0 ml of n-hexane at the rate of 0.5 ml/min. Vacuum was applied for 15 min to remove all other impurities. EtG was eluted with 1.0 ml of water containing 2 per cent ammonia. Elute was evaporated to dryness under a gentle stream of nitrogen using evaporator at 35°C.

**Derivatization:** Vacuum dried sample was reconstituted in 100 µl of N-methyl -N-tri-methylsilyl trifluoroacetamide with 1 per cent tri-methylchlorosilane (MSTFA). The vial was capped and heated in an oven at 70°C for 20 min. After cooling, the sample (1-2 µl) was injected into the GC-MS system.

**Preparation of EtG standard solution:** EtG stock solution was prepared in methanol to obtain a concentration of 1 mg/ml. Different concentrations of EtG (30 to 7000 ng/ml) were used for preparing calibration curve for both urine and serum analysis. Methyl glucuronide was used as internal standard and its stock of 1mg/ml was prepared in double distilled water. These solutions were stored at -20°C and were stable for several months.

**GC-MS conditions:** The analysis was performed using 5975C MS equipped with 7890 GC and 7693 auto sampler (Agilent Technologies, USA). Chromatographic separation was achieved on DB-5 fused-silica capillary column, 30m x 0.25 mm internal diameter. Oven temperature was programmed as 60°C for 2 min, temperature was increased at the rate of 10°C/min to 200°C and finally at the rate of 15°C/min to 250°C. Helium was used as the carrier gas. Injection was made in split-less mode. The injector and transfer line were maintained at 250°C and 280°C, respectively.

The mass selective detector was operated in electron impact mode at 70 eV with an ion source temperature of 150°C. Data were acquired in the selected-ion monitoring mode (SIM mode). The trimethylsilyl derivatives were identified with masses m/z 217 (target ion), 204, and 147 were the qualifiers for TMS EtG (Figs 1, 2).

**Results**

This exploratory work was planned to generate a standardized protocol for detection and quantization of EtG in urine and blood samples of patients with a history of alcohol abuse. A sensitive, simple and reliable solid phase extraction procedure combined with gas chromatograph and mass spectrometer (GC-MS) method was developed and validated. Since EtG testing needs to be done frequently for patients admitted to wards or attending OPD, small volume of samples is preferable. Pretreated biological sample was passed through a conditioned SPE cartridge. It combined by both anion exchange and reverses phase interaction. EtG stayed on the SPE column by ionic interactions. This characteristic permitted washing with hexane to remove interfering compounds. Finally, sample was eluted with 1 ml of 2 per cent ammonical water, evaporated and derivatized before injecting into the GC-MS. Derivatization of polar N-H group, resulting in less polar and more volatile derivatives, allows analysis by GC-MS. This method was found to be time-effective with total turn-around duration of less than an hour.

In patients’ samples, serum ethyl glucuronide levels correlated significantly with breath alcohol levels (r=0.853, P=0.001). Urinary EtG levels correlated inversely with the time since last use of alcohol (r= -0.903, P<0.01).

**Method validation:**

Concentration range, linearity and selectivity: Linearity of a method is its ability to obtain results (within a given range) which is directly proportional to the analyte concentration in the sample. Seven replicate measurements at seven different spiked concentrations were analyzed (50, 218, 437, 870, 1700, 3500 & 7000 ng/ml) for urine and (30, 218, 437, 875, 1750, 3500 & 7000 ng/ml) for serum. In this concentration range, positive correlation of nominal concentration and detector response was observed. The correlation coefficient of the calibration curve was >0.998. Signal to noise ratio was 3:1 at a concentration of 20 ng/ml.

Recovery: Recovery was evaluated by comparing peak area of EtG standard with peak resulted from spiked samples. Good recoveries were reported at all the concentration levels. For serum sample of 80 ng/ml concentration recovery was 78 per cent while for 1000 ng/ml it was 86 per cent. For urine sample containing 200 ng/ml concentrations recovery was 78 per cent while for 5000 ng/ml recovery was 84 per cent.
SPE allows greater recovery due to selective extraction of the analyte before analysis irrespective of the sample volume and matrix.

Sensitivity, precision and accuracy - Limit of detection was 50 ng/ml for urine and 80 ng/ml for serum, limit of quantitation was 150 ng/ml for urine and 210 ng/ml for serum. Methyl glucuronide was used as internal standard to eliminate injection errors. Ten blank samples (urine and serum) from abstainers were extracted and injected into GC-MS to detect the selectivity of method. In these samples, neither any interference nor EtG itself could be detected suggesting that endogenous EtG can be ignored.

For inter- and intraday precision and accuracy of the method, six replicates of each of three quality control samples (50, 300 and 1000 ng/ml for both urine and serum) were assayed on five different days. The results were expressed as the relative standard deviation (RSD %). The variability was less than 20 per cent across the concentration range. Absolute recoveries of EtG from serum and urine samples were calculated by comparing the peak area of extracted samples spiked with EtG at each level. To exclude potential interfering peaks, blank samples were extracted and analyzed. Fig. 1 shows the representative chromatogram of positive serum sample and standard.

Discussion

Ethanol has high specificity for excessive alcohol intake but time window of positivity is quite small.
(4-6 h for breath, 10-12 h for blood, 18-24 h for urine). Gamma-glutamyl transeptidase (GGT) and erythrocyte mean corpuscular volume (MCV) are elevated after significant alcohol use over a period of time but have poor specificity as both can be elevated in other chronic diseases and nutritional deficiencies. CDT has good specificity for alcohol but gets elevated after 10-15 days of abuse. EtG, a direct metabolite of ethanol formed by the enzymatic conjugation of ethanol with glucuronic acid in liver is the most effective method of detecting surreptitious alcohol use. It is non-volatile, water soluble and a stable metabolite of ethanol that can be detected in urine, blood/serum, hair and nail. Studies have shown that this minor metabolite is present in higher concentration in serum than whole blood. Urine EtG remains positive up to 48-72 h following heavy alcohol consumption. EtG is a marker with an intermediate time frame between ethanol measurement and GGT/CDT ratio.

On comparing breath alcohol levels with serum EtG levels of users and abstainers a positive correlation was obtained. EtG levels varied significantly with self reported duration of last use. Similar findings have been reported earlier. In cases where alcohol use was 1-2 days earlier and breath alcohol could not be detected, raised urinary EtG values were still observed.

At present, there is no universal consensus on cut-off value for EtG. Studies in healthy volunteers given alcohol at a dose of 0.1 and 0.8 g/kg body weight have shown a cut-off values between 100-110 ng/ml in urine. In routine clinical use, cut-off values as high as 500 ng/ml can be used to reduce the risk of false positive results. To date, there have been insufficient clinical studies on serum EtG to allow the reliable determination of most appropriate cut-off values. Since EtG lacks a universally established cut-off, further research needs to be done to develop cut off for our population. This is necessary for other biomarkers of alcohol abuse as well. EtS (ethyl sulphate) is also a good marker of alcohol abuse. Presence of EtS in samples adds value to EtG confirmation. Requirement of liquid LC-MS methods for EtS determination is the main constraint. A better correlation can be obtained by comparing EtG: EtS along with GGT and CDT. As EtS estimation techniques are not available in our setting, the current method was developed with required modification.

In conclusion, a simple, sensitive and specific method of EtG quantitation in biological fluids was developed and standardized. This has important implications for clinical as well as medico-legal evaluation for recent alcohol use.

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