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Determination of ethyl glucuronide in fingernails by LC/MS-MS

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Abstract: Introduction: Ethyl glucuronide (EtG) is a biomarker used for detection of alcohol consumption. Fingernail is a keratinous tissue capable of accumulating a number of biomarkers and metabolites of toxic agents. Therefore, nails are used especially for determination toxic exposure in forensic analyses. In this study, we aimed to demonstrate EtG in fingernail tissue and show its correlation with alcohol intake behavior.

Materials and Methods: Michigan Alcohol Screening Test (MAST) was performed to the individuals participated in the study. Sixteen fingernails were obtained from each individual. Afterwards, EtG analysis was carried out in fingernail tissue using LC/MS/MS.

Results: Michigan Alcohol Screening Test revealed that 4 participants showed non-hazardous drinking behavior and 8 cases were alcohol abusers, while 4 cases verbally stated they were abstainers. On the analyses, EtG was found between EtG level of <LOD and 90.52 ppb. EtG was observed as <LOD in fingernails of 4 participants who declared they were abstainers. A significant correlation was found between EtG levels measured in the fingernail samples and MAST outcomes on the statistical analyses ($p<0.001$; $r=0.801$).

Conclusion: In this study, we demonstrated that fingernail tissue is a useful sample for determination of ethyl glucuronide by LC/MS-MS and can be used in detection of alcohol intake.

Key Words: Fingernails, Alcohol intake, Ethyl glucuronide, LC/MS/MS.

Detection of alcohol intake remains crucial for forensic medicine. However, since the ethanol level decreases by 12-20 mg/dL per hour due to metabolizing following intake, alcohol might not be demonstrated in some cases despite the intake [1]. Thus, determination of minor metabolites of ethyl alcohol, which are ethyl glucuronide (EtG), fatty acid ethyl esters (FAEEs), ethyl sulphate (EtS) and phosphatidyl-ethanol (PEth), can be utilized [2,3,4,5,6]. These minor metabolites of ethanol can be used for detection of acute and chronic alcohol intake behavior.

EtG is a minor non-oxidative metabolite of ethanol. Formation of EtG is characterized by the net addition of glucuronic acid to ethanol. This clearance pathway is catalyzed by the UDP-glucuronosyltransferase (UGT) superfamily of enzymes, which utilize UDP-glucuronic acid as a cofactor. While normally serum and/or urine ethanol levels can only be detected for a few hours post-intake, urinary levels of EtG have been detected as long as 3–5 days following alcohol consumption [7,8].

EtG has previously been studied in blood, urine, sweat, and vitreous humor as well as in bone, hair,

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muscle, bone marrow and adipose tissue for detection of alcohol consumption [9,10,11,12,13]. However, there are only few studies dealing with the demonstration of EtG in fingernail tissue [14, 15].

Fingernail, a layered partially crystalline keratinized structure, originates at the fingernail matrix from specialized epidermal cells and grows along the nail bed at a rate of approximately 0.1 mm/day. Analytes are incorporated into the fingernail by blood flow in the nail matrix and blood flow from the nail bed underneath as the fingernail grows in length and thickness [16].

Together, these mechanisms allow for detection as recently as a few weeks ago and extending several months back [15]. Fingernail is a keratinous tissue capable of accumulating a number of biomarkers and metabolites of toxic agents. Therefore, nails are used especially for determination toxic exposure in forensic analyses [17,18, 19].

In this study, we aimed to demonstrate EtG in fingernail tissue and reveal its correlation with alcohol intake behavior.

MATERIALS AND METHODS

Ethics Statement and subject

This study was approved by the Ethical Committee of Mustafa Kemal University. Fingernail tissues used in the study were obtained from the individuals (n= 16) presented to Mustafa Kemal University School of Medicine Department of Forensic Medicine for forensic examination. Informed consents were received from the cases to be included in the study. Afterwards, Michigan Alcohol Screening Test (MAST) was carried out. Following the tests, cases were separated as non-hazardous (n=3) users and alcohol abusers (n=9). Fingernails were collected from these groups and those who declared to be abstainers (n=4), as 16 cases in total. The nails were collected with a clean scissor and leading any damage to hyponychium (the quick) zone was avoided. The fingers were put and kept in the clean Eppendorf tubes. Then, these samples were analyzed using LC/MS/MS method.

Chemicals, Reagents and Materials

EtG and deuterium-labeled EtG-d5 standards (internal standard) were obtained from Medichem (Stuttgart, Germany). All solvents were hyper graded for LC-MS LiChrosolv and purchased from Merck KGaA (Darmstadt, Germany). Deionized water was obtained from the Mili-Q (Millipore, Bedford, USA) water purification system.

Calibrator, Control And Internal Standard Spiking Solutions

10 ppm of EtG Standard; 1 mg of EtG was dissolved in 1 mg methanol and, 250 µl from this solution was diluted to a volume of 25 mL with methanol in a 25-mL volumetric flask. 2500 ppb of EtG-d5 Standard; 0.25mg of EtG-d5 was dissolved in 1ml methanol and, 250 µl from this solution was diluted to a volume of 25 mL with methanol in a 25-mL volumetric flask. Then, standard solutions of 2, 5, 10, 20, 50, 100, 200, 1000 and 2000 were prepared using the above-mentioned standards.

Specimen Preparation

The powderized fingernail specimen of 50 mg was weighed with a sensitive scale and put in a tube. A mixture of 50% acetonitrile/50% water was added on it, and this mixture was kept in an ultrasonic bath at 25°C for 2 hours. Then, internal standard of 50 µl was added on it and mixed with vortex. Then it centrifuged at 4000 rpm for 10 minutes. Two ml was extracted from the upper part and put in the autosampler vials. The specimens were separately subjected to extraction with mixtures of water/acetonitrile/methanol, acetonitrile/water of 80% and acetonitrile/water of 50% and the best result was obtained with the mixture of 50% acetonitrile/50% water.

LC-MS/MS Conditions

The specimens were analyzed using an Agilent Technologies 1200 system that consisted of a G1367C autosampler, a G1379B degasser, G1312B binary pump. Separation was achieved using two Zorbax Hilic Plus (4.6x100 mm, 3.5 micron particle size) serial connected columns. Reverse-reverse chromatographic technique was used. The column was held at 25°C in a G1316B Thermostatted Column Compartment (Wilmington, DE, USA).

The solvent system was a gradient that consisted of A (1mM NH4Ac) and B (acetonitrile), using a flow rate of 0.8 mL/min. The solvent program held B at 65 % from 0.0 min to 2.2 min. Solvent B was decreased to 20 % between 2.3 min and 9.5 min. Solvent B was increased to 20% at 5.1 min and held at 65 % until 10.0 min.

The detector was Agilent Technologies 6460 Triple Quad LC/MS System using electro–spray ionization (ESI) in the negative mode (Wilmington, DE, USA). The capillary voltage was set at 4000 V, the nozzle voltage set at 0 V and the desolvation gas (nitrogen) was heated to 350 °C with a flow of 11 l/min. Nebulazator pressure: 50 psi. The sheath gas (nitrogen) was heated to 350 °C and delivered at 11 l/min.

The internal standard (ETG -d5) was monitored using the m/z 226.0 > 75.0 (quantification ion) transition and the m/z 226.0 > 85.0 (qualifying ion) transition. The m/z 221.0 > 75.0 (quantification ion) and m/z 221.0 > 85.0 (qualifying ion) transitions were used to monitor ETG. All three transitions used a fragmentor voltage of 100V and collision energy of 12V. All data were processed using MassHunter B.04.01 (Wilmington, DE, USA).

Identification Criteria

The identification criteria used for this procedure included four components: retention time, relative ion intensity signal to noise and baseline resolution.

Validation

Analytical quality assurance study of the method was performed with definitions of the parameters, including selectivity, linearity, recovery, limit of detection (LOD) and limit of quantification (LOQ).

Selectivity

Selectivity (specificity) is an indicator of the extent of interference for a particular analyte with the other components in a mixture. Within the selectivity studies, 5 blind specimens were analyzed. These specimens were subjected to the Specimen preparation process and analyzed. No any peak was found to lead to interference.

Linearity

The linearity is the ability of analytical procedure to produce test results, which are proportional to the concentration (amount) of analyte in samples within a given concentration range. In order to define the linearity, EtG standard solutions were prepared in a concentration of 10 ppm, and the linear range was defined as 2 to 2000 pg/mg.

Table 1. EtG spikes of the same sample of 5,20 and 50 pg/mg

50 ACN ext.d	2,1450*
50 ACN ext+5 pg/mg EtG spiked.d	7,4771
50 ACN ext+20 pg/mg EtG spiked.d	22,1641
50 ACN ext+50 pg/mg EtG spiked.d	51,1913

*pg/mg

Table 2. Integration Peak List

Start	RT	End	Height	Area	Signal To Noise
1.986	2.045	2.13	14	62	12.4

Accuracy

The accuracy can be determined by extracting a relevant certified reference material (CRM) and calculating the percentage recoveries relative to the certified values. Because we had not certified reference materials of fingernail samples, accuracy examinations were made with calculating the percentage recoveries. Recovery is a measure of the retaking rate of the sought analyte added into the sample. For this purpose, standard EtG levels of 5.20 and 50 pg/mg were added to the samples and re-analyzed (Table1).

Sensitivity

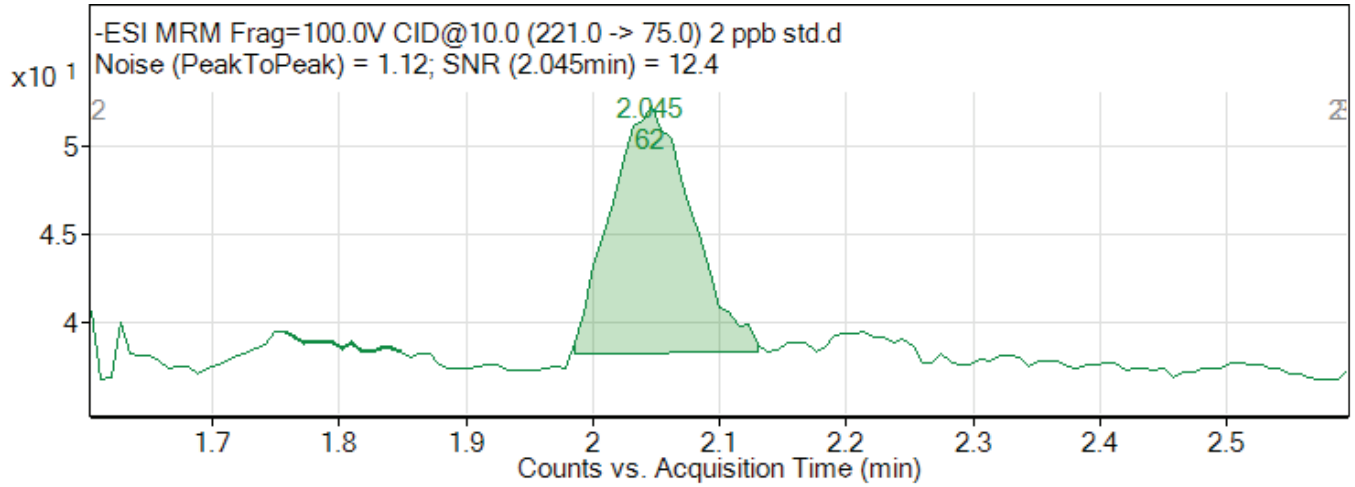
The sensitivity result for EtG is given by table and graphical data, respectively. The result for signal to noise ratio is determined to be 12.4. However, it can be seen that the peak is clearly separated according to 2 pg/mg noise background (Table 2).

Limit of detection (LOD) and Limit of Quantitation (LOQ)

The detection limit of an analytical procedure is the lowest amount of an analyte in a sample that can be detected, but not necessarily quantitated as an exact value. It is expressed as a concentration at a specified signal:noise ratio (SNR), usually as 3:1. The limit of quantitation is the lowest amount of the analyte in the sample that can be quantitatively determined with defined precision under the stated experimental conditions. It is expressed as a concentration at a specified signal:noise ratio (SNR), usually as 10. For the method used in this study, limit of detection was found as 0.48 ppb and limit of quantitation as 1.61 ppb. The values obtained were confirmed by analyzing of the samples with addition in the specified concentrations.

Statistical Analysis

Statistical analysis was performed using SPSS Statistics version 15.0. Pearson's correlations were used to evaluate the associations of MAST point and nail EtG concentrations. Independent student t-test (t) was used to compare the means of EtG concentrations in mast point



and fingernails. A probability of $p < 0.001$ was considered to be significant.

RESULTS AND DISCUSSION

All the cases included in the study were males aged between 31 and 68 with a mean age of 45.5 years.

Table 2. Demographic features and EtG outcomes of the cases

Case	Gender	Age	MAST Point *	EtG (pg/mg)
1	M	40	4	5,9694
2	M	31	3	6,1523
3	M	37	4	9,5448
4	M	35	5	12,2396
5	M	42	6	40,6497
6	M	68	9	41,9403
7	M	60	7	48,5382
8	M	54	7	57,0498
9	M	31	7	66,7349
10	M	51	9	77,8820
11	M	46	9	83,1661
12	M	41	9	90,5202
13	M	36	No drink alcohol; declaration	<LOD**
14	M	37	No drink alcohol; declaration	<LOD**
15	M	39	No drink alcohol; declaration	<LOD**
16	M	40	No drink alcohol; declaration	<LOD**

*) MAST point 0-4: Non-hazardous drinking , 5-9: alcohol abuser, >10: alcohol addiction
**) LOD: Limit of Detection

According MAST, 4 of the cases were found to have a non-hazardous drinking behavior and 8 cases were alcohol abusers, while 4 cases verbally stated they were abstainers.

On the analyses, EtG levels were found to be between EtG <LOD and 90.52 pg/mg in fingernail tissues. EtG was observed as < LOD in fingernails of 4 cases who declared they were abstainers (Table 3).

A significant correlation was found between EtG values measured in the fingernail tissues and MAST outcomes on the statistical analyses ($p<0.001$; $r=0.801$) (Figure 1).

In this study, we demonstrated that fingernail is a useful sample for determination of ethyl glucoronide by LC/MS-MS and measurement of EtG in fingernail tissue can be used in detection of alcohol intake behavior. In the studies about using EtG in the demonstration of alcohol intake behavior; Neumann *et al.* showed high EtG values in the chronic alcohol users, while Wurst *et al.* (2003) demonstrated that EtG could be used in monitoring the alcohol regime in the treatment plans of chronic alcoholics [20,21]. There are also studies indicating usefulness of measurements of EtG levels in revealing the alcohol intake behavior, in postmortem cases [13].

It is stated, in the literature, that demonstration of EtG in blood and urine is possible after a certain period of time. EtG is considered to be superior to ethanol, although demonstration of it in the body fluids such as blood and urine is possible only within certain time limits [12].

Thereby, keratinous tissues such as hair and nails might be needed to allow assessment in a longer time periods. In previously conducted studies, among the keratinous tissues hair samples have been demonstrated to show a higher specificity and sensitivity for EtG analysis [22]. As in hair, in fingernails tissue, measurement EtG levels have been shown to have higher specificity and sensitivity than the other classical alcohol indicators (CDT, GGT) [23, 24, 25, 26, 27]. Furthermore, Gareri *et al.* [28] reported that EtG was not detected in hair following external ethanol contamination and a recent report demonstrated that hair bleaching adversely affects the detection of EtG [29].

Therefore, analysis using fingernail tissue is thought to be more reliable compared hair. In a study, Jones *et al.* analyzed EtG in the hair and fingernail samples and emphasized that fingernail samples might be used in revealing of the alcohol intake behavior [15].

Similarly, in a study investigating demonstration of EtG in fingernail

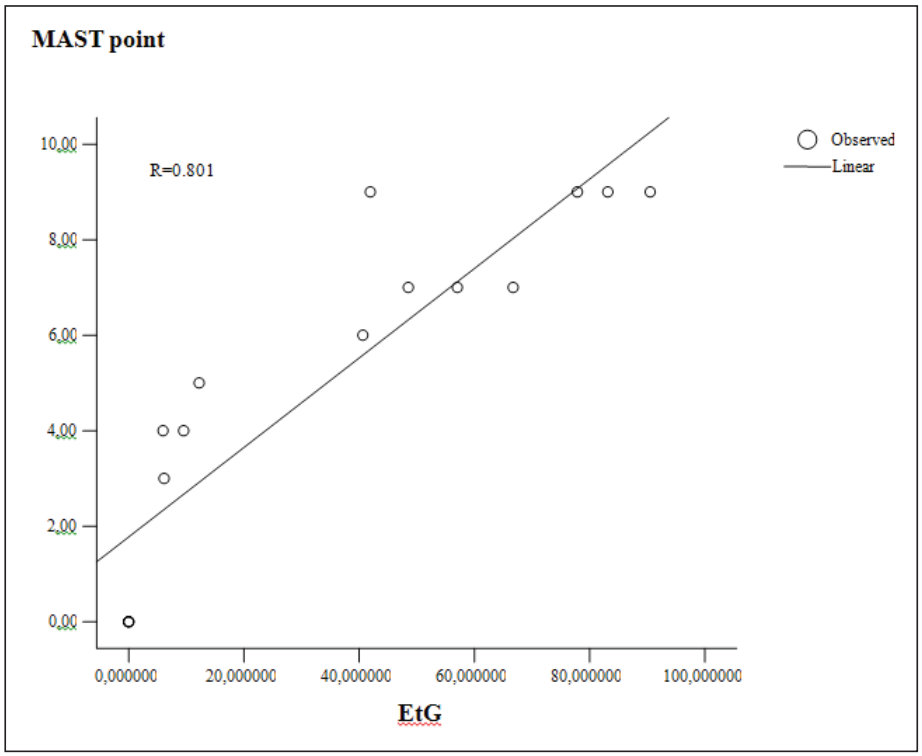


Figure 3. Correlation between alcohol intake behavior (MAST point) and EtG levels.

samples of 15 cases, Morini *et al.* reported that EtG levels were consistent with alcohol intake behavior [14]. Beside, these two studies dealing with EtG detection in fingernail, the present study positive correlation was observed between EtG levels in fingernail and alcohol intake behavior ($p<0.001$; $r=0.801$)

In a study evaluating alcohol intake in 103 pregnant women, Wurst *et al.* (2008) demonstrated that fatty acid ethyl esters (FAEE) might be used together with EtG in long term follow-up of the alcohol intake [30]. EtG level in fingernail is crucial in definition of the alcohol intake behavior of pregnant women. In addition, EtG analyses in fingernail samples may be useful in professionals (surgeons, pilots, drivers, etc.) in which the alcohol intake is of high importance, and before organ transplantation operations.

False positive and false-negative EtG values were observed in certain studies [29,30]. In a study conducted to reveal false positive and negative results, B-glucuronidase enzyme activity of bacteria, including Bacteroides fragilis, Bacteroides vulgatus, Clostridium perfringens, Clostridium sordellii and Escherichia coli were shown to cause false-negative results due to degradation of EtG [31]. In addition, there are drug interactions in which false-positive values were detected. In a study by Arndt *et al.*, using of Chloral hydrate was found to cause false positive results [32]. Given these conditions, we believe fingernail tissues may give levels of EtG more accurately.

CONCLUSION

In this study, we demonstrated that fingernail tissue is a useful sample for determination of ethyl glucoronide by LC/MS-MS and can be used in detection of alcohol intake. A correlation between EtG levels detected in the fingernail tissue and alcohol intake behavior was observed, which indicates that fingernail tissue can be used for alcohol intake behavior in longer time periods. Collecting of the fingernail tissue is a non-invasive method and easier to obtain than blood, urine and vitreous humor both in postmortem and antemortem investigations. Therefore, fingernail tissue can be used in cases requiring the evaluation of alcohol intake behavior. However, further studies with larger case numbers are needed to investigate the effects of various nail diseases, vascular disorders and metabolic diseases on fingernail tissue and consecutively EtG levels.

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