Research Article

Emre Karacaoğlu, Aysun Balseven Odabaşı, Ramazan Akçan, Ali Rıza Tümer, Aykut Lale*, Tolgahan Kocadağlı and Vural Gökmen

Time dependent change of ethanol consumption biomarkers, ethyl glucuronide and ethyl sulphate, after single dose ethanol intake

Etanol Tüketim Biyogöstergeleri Etil Glukuronid ve Etil Sülfatın Tek Doz Etanol Alımı Sonrasında Zamana Bağlı Değişimi

<https://doi.org/10.1515/tjb-2018-0173>

Received May 4, 2018; accepted September 11, 2018; previously published online December 11, 2018

Abstract

Background and objective: Ethyl glucuronide (EtG) and ethyl sulfate (EtS), minor metabolites of ethanol, aid to forensic scientist and clinicians to determine recent ethanol uptake when ethanol elimination is occurred. Present study aimed to show (a) kinetics of EtG and EtS in blood and urine after 0.5 g/kg ethanol intake (b) peak concentrations and time of disappearance in Turkish people.

Material and methods: Seventeen (10 male, 7 female) healthy volunteers participated in the study. Blood and urine samples were obtained during 48 h after consumption and analyzed in LC-MS/MS.

Results and conclusion: Blood peak concentrations of EtG and EtS were 0.13–0.389 mg/L and 0.211–0.5 mg/L, urine peak concentrations were 6.89–30.42 mg/L and 10.5–58.17 mg/L, respectively. There was no EtG and EtS in all samples 24 h later in blood and 48 h later in urine. Correlation was found between EtG and EtS concentrations

in blood. Most of obtained data are similar to literature, except EtS dominancy to EtG in urine and blood.

Keywords: Ethyl glucuronide; Ethyl sulfate; Ethanol; Kinetics; Liquid chromatography-mass spectrometry; Polymorphism.

Öz

Amaç: Etanolün minör metabolitleri olan etil glukuronid (EtG) ve etil sülfat (EtS), etanol eliminasyonu meydana geldiğinde son etanol alımını belirlemek için adli bilimci ve klinisyenlere yardım etmektedir. Bu çalışma a) EtG ve EtS kinetiğinin kan ve idrarda 0.5 g/kg etanol alımından sonra değişimini b) pik konsantrasyonlarını ve Türk popülasyonunda kaybolma zamanını göstermeyi amaçlamıştır.

Yöntem: Çalışmaya 17 hasta (10 erkek, 7 kadın) sağlıklı gönüllü katılmıştır. Kan ve idrar örnekleri, tüketimden 48 saat sonrasına kadar toplanmış ve LC-MS/MS ile analiz edilmiştir.

Bulgular ve Sonuç: EtG ve EtS'nin kan pik konsantrasyonları sırasıyla 0.13–0.389 mg/L ve 0.211–0.5 mg/L, idrar pik konsantrasyonları sırasıyla 6.89–30.42 mg/L ve 10.5–58.17 mg/L idi. EtG ve EtS 24 saat sonra tüm kan örneklerinde ve 48 saat sonra idrar örneklerinde tespit edilememiştir. Kandaki EtG ve EtS konsantrasyonları arasında korelasyon saptanmıştır. Elde edilen verilerin çoğu, idrar ve kandaki EtG'ye göre EtS dominantlığı hariç, literatüre benzer şekilde bulunmuştur.

Anahtar kelimeler: Etil Glukuronid; Etil Sülfat; Etanol; Kinetik; Sıvı Kromatografi Kütle Spektrometrisi; Polimorfizm

*Corresponding author: Aykut Lale, MD, Hacettepe University School of Medicine Department of Forensic Medicine, Ankara 06080, Turkey, Phone: +905422048554, e-mail: aykutlale@gmail.com. <https://orcid.org/0000-0002-2259-2209>

Emre Karacaoğlu: The Ministry of Justice, Kirikkale Council of Forensic Medicine, Kirikkale, Turkey

Aysun Balseven Odabaşı, Ramazan Akçan and Ali Rıza Tümer: Hacettepe University School of Medicine Department of Forensic Medicine, Ankara, Turkey

Tolgahan Kocadağlı and Vural Gökmen: Department of Food Engineering, Hacettepe University, Ankara, Turkey

Introduction

In recent ethanol intake monitoring, biological markers and metabolites have gained more importance because of rapid clearance of ethanol. Indirect markers like carbohydrate deficient transferrin, gamma glutamyl transferase, or mean corpuscular volume have low specificity and sensitivity due to the effects of non-alcoholic factors [1]. Ethyl glucuronide (EtG), ethyl sulfate (EtS), phosphatidyl ethanol, and fatty acid ethyl esters are direct markers of ethanol produced by nonoxidative metabolism. Small amounts of ethanol (>0.1%) are transformed EtG and EtS which are glucurono- and sulfo-conjugated forms of ethanol by phase II metabolism enzymes UDP-glucuronosyltransferase (UGT) and sulfotransferase (SULT), respectively [2].

Rapid clearance of ethanol limits detection time to <12 h in blood and breath, EtG and EtS gives longer detection time window in body fluids [3]. Previous controlled drinking studies showed that terminal half-life of EtG is 2–4 h in blood and 3.5–5.5 h in urine following ingestion of low dose of ethanol, EtG and EtS are detectable up to 12 h at low amounts of consumption, up to 24 h at repeated or large doses [3–7]. On the other hand studies indicated that EtG and EtS levels show inter individual variations independent from sexes [4, 6]. Genetic polymorphisms and ethanol related isotypes of UGT and SULT enzymes were demonstrated previously [8–10]. Although UGT and SULT exist as a super family of about 22 and 13 enzymes in human, respectively, UGT1A1, UGT2B7 isotypes for EtG and SULT1A1, SULT2A1 for EtS are predominant in phase II metabolism of ethanol [8, 11]. UGT1A1 is, the only proven isotype, responsible for dysfunctional glucuronidation in humans due to genetic variations, additionally polymorphism of SULT isotypes particularly SULT1A1 were discussed by Glatt et al. [9, 12]. The authors also indicated that altered enzyme function or expression caused by polymorphic variations may be seen for all UGT isotypes [12]. Besides population differences affect polymorphism variation frequency for both enzyme isotypes [9, 13].

The aim of this study is to (a) investigate time depended change of EtG and EtS in Turkish population with controlled drinking experiment and (b) to evaluate the effects of ethnic differences on EtG and EtS levels.

Materials and methods

Study protocol

Seventeen healthy volunteers (10 men and 7 women) with a median age of 34 years (range 19–52 years) and a median

body mass index of 23.1 kg/m² (range 18.9–32.5 kg/m²) participated in a controlled drinking experiment.

They were all social drinkers with a range use of 45–230 g pure ethanol/month and had abstained from alcohol during the week preceding the study, according to self-reports.

Exclusion criteria were somatic or psychiatric illness, use of regular medication, and pregnancy. Hemograms, liver and kidney function tests of all subjects were examined before the study.

From 1 to 4 h after the start of drinking, venous blood samples were taken at intervals of 30 min, from 4 to 8 h at intervals of 60 min, and at 10 h and 24 h. Urine samples were obtained approximately every 2 h until 10 h after the start of drinking, and at 24 and 48 h. Approximately 2 and 7 h after the start of drinking, the volunteers were given standardized meals (after 2 h sandwich and mineral water, after 7 h sandwich and mineral water). After taking blood samples at 10 h, the volunteers were sent home with instructions to abstain from ethanol contained in drinks, food, or medications until the end of urine sampling.

All blood and urine samples were stored at +4°C during collecting, and at –80°C until the time of analysis.

Analytical method

Chemicals

EtG, EtS and EtG-d5, EtS-d5 [as internal standard (IS)] standard solutions were purchased from Lipomed (Weil am Rhein, Germany). Acetonitrile and methanol (HPLC grade) were purchased from Merck (Darmstadt, Germany). Formic acid (98%) was purchased from J.T. Baker (Deventer, Holland). Ultra-pure water was used throughout the experiments (Milli Q-System, Millipore, Milford, MA, USA).

Sample preparation

Urine sample of 1 mL was centrifuged at 12,000 rpm for 5 min. Fifty microliter of the sample was mixed with 10 µL of IS and 940 µL of 0.1% formic acid in water. After vortexing, the sample was filtered through 0.45 µm syringe filter into an autosampler vial prior to analysis.

Blood sample of 200 µL was mixed with 10 µL of IS and 790 µL of acetonitrile and then it was vortexed for 1 min. The mixture was centrifuged at 12,000 rpm for 5 min and the clear supernatant was filtered through 0.45 µm syringe filter into an autosampler vial prior to analysis.

Determination of EtG and EtS

EtG and EtS were determined by a Waters Acquity UPLC system coupled to an Acquity TQD tandem mass spectrometer operated in ES negative mode. Chromatographic separations were performed on Acquity UPLC HSS C18 column (2.1×50 mm, 1.8 µm) using an isocratic mixture of 0.05% formic acid in water and methanol (90:10, v/v) as mobile phase at the flow rate of 0.3 mL/min. The column equilibrated at 50°C and Waters ACQUITY FTN autosampler was held at 10°C during the analysis. The electrospray source had the following settings: capillary voltage of

Table 1: Precursor ions and the product ions of EtG, EtS and internal standards.

Compound	Precursor ion (m/z)	Product ion (m/z)
EtG	221	85
	221	75
EtS	125	97
	125	80
EtG-D5	226	85
EtS-D5	130	98

Figure 1: MS chromatogram of EtG (m/z 221>75), EtS (m/z 125>97), EtG-d5 (226>85), EtS-d5 (130>98) and retention times.

Table 2: LOD and LOQ values for EtG and EtS in blood and urine.

	Blood (mg/L)	Urine (mg/L)
LOD		
EtG	0.002	0.01
EtS	0.002	0.012
LOQ		
EtG	0.007	0.032
EtS	0.008	0.04

3 kV; cone voltage of 28 V; extractor voltage of 3 V; source temperature of 130°C; desolvation temperature of 400°C; and desolvation gas (N₂) flow of 900 L/h. Collision gas (Ar) had the flow of 0.2 mL/min. Both compounds were identified by multiple reactions monitoring (MRM) of two channels. Precursor ions and the product ions of EtG, EtS and ISs were given in Table 1.

The 221>75 and 125>97 MRM transitions were selected to quantify EtG and EtS, respectively. IS was prepared to be containing 10 mg/L EtG-d5 and 5 mg/L EtS-d5 in water (Figure 1). Concentrations of both EtG and EtS were calculated by means of a calibration curve built in the range between 0.01 and 1 mg/L. The linearity was evaluated by plotting the peak area against the concentrations of EtG and EtS standards. Limit of detection (LOD) and limit of quantification (LOQ) were determined at a signal to noise ratio of 3 and 10, respectively. LOD and LOQ values for both matrixes are given in Table 2.

Statistics

Maximum concentration of EtG and EtS values were presented as range (mean – standard deviation). Statistic parameters were analyzed by using SPSS (Version 24.0). Areas under curve (AUC) were calculated for every volunteers' EtG and EtS levels. Spearman correlation test and general linear model with repeated measures were applied. Statistical significance was expressed as $p < 0.05$.

Results

All initial blood and urine samples were free for EtG, EtS and ethanol.

Blood

Mean peak concentrations for EtG and EtS in blood, after taking 0.5 g/kg ethanol, were 0.13–0.389 mg/L

Figure 2: Blood EtG and EtS data.

(A) Blood EtG concentrations box-plot graphics (B) blood EtS concentrations box-plot graphics (C) mean/median blood EtG and EtS concentrations (*median data were given for 4, 5, 7, 8th h blood EtG and 1, 2.5, 8th h blood EtS).

(mean 0.240 ± 0.075 mg/L SD) and $0.211\text{--}0.5$ mg/L (mean 0.334 ± 0.091 mg/L SD), respectively (Figure 2). Tmax levels were 2–3 h (mean 2.55 ± 0.3 h) for EtG and 2.5–4 h (mean 3.14 ± 0.3 h) for EtS, in blood.

Blood EtS values were significantly higher than blood EtG values ($p < 0.001$). There were no correlation blood EtG/EtS ratios with time, ratios were >1 for 18% of all analyzed sample which indicates that EtS was produced higher than EtG independent from time. Ten hours later following ingestion of ethanol, EtG was still positive in two cases ($>\text{LOD}$); whereas, EtS was positive in nine cases. EtS was positive for one of EtG positive case at 10 h, EtS was negative for another one. In the last collected blood samples (24 h after the start of drinking) all subjects were negative for both EtG and EtS. There was no statistically significant difference between genders for blood EtG and EtS concentration-time profiles. When comparing AUC (areas under curve) for EtG and EtS in blood, a correlation was found between them ($\rho = 0.588$). Additionally EtG and EtS values of same individuals significantly correlated ($\rho = 0.878$), in each time series.

Two successive measurement ratios ($t_{\text{previous}}/t_{\text{next}}$) in first 2 h were found <1 , and >1 after three and half hours for EtG in all cases. On the other hand this ratio was <1 in first 3 h except one case, and >1 after three and one half

hours for EtS except one case. Depending on the boxplot graphics especially for EtG in excretion phase outlier (extremely high or low) values were detected.

Urine

Following the ingestion of 0.5 g/kg ethanol, Cmax for EtG and EtS in urine samples were 6.89–30.42 mg/L (mean 19.361 ± 5.98 mg/L) and 10.5–58.17 mg/L (mean 30.0 ± 14.06 mg/L), respectively (Figure 3). Tmax levels were found 4–6 h (4.4 ± 0.8 h) for EtG and 4–6 h (4.5 ± 0.9 h) for EtS.

EtS levels were higher than EtG levels in urine ($p < 0.01$). No correlation were detected between urine EtG/EtS ratios and time. On the other hand EtG/EtS ratio were >1 for 26% of samples analyzed in first 3 h. The augmentation trend was observed for urine EtG/EtS ratio time dependent manner. All urine samples collected 24 h later from starting, were positive for EtG and EtS ($>\text{LOD}$); whereas, last urine samples (collected 48 h later) were all negative for both.

No correlation was found between AUC levels of EtG and EtS in urine. When comparing AUC levels of metabolites, there was also no correlation between blood and

Figure 3: Urine EtG and EtS data.

(A) Urine EtG concentrations box-plot graphics (B) urine EtS concentrations box-plot graphics (C) mean urine EtG and EtS concentrations (*median data were given for 2, 24, 48th h urine EtG EtS).

urine. Comparing urine EtG and EtS values as paired samples for same individuals at each time series, EtG and EtS values were correlated ($\rho = 0.945$).

Two successive measurement ($t_{\text{previous}}/t_{\text{next}}$) ratios in first 4 h were found <1 , and >1 after 6 h for EtG and EtS for all cases. Depending on the boxplot graphics outliers were lower than blood, detected outliers belonged to same individuals for both metabolite in urine.

Discussion

Determination of recent alcohol use is critical for forensic purposes to evaluate cases such drunk drivers, sexual assault victims, also postmortem suspected poisoning cases [14]. Besides, for management of clinical cases diagnosed substance use disorder, measurement of recent usage is important in order to treat successively [15]. Estimation the time of ethanol ingestion is useful information in this cases. To understand, estimate and evaluate better the EtG and EtS values, pharmacokinetic studies of EtG and EtS in blood and urine with controlled drinking experimental design were performed by various authors in various population [3–7]. In the present study

we tested a different population included all Turkish individuals, whose genetic properties, culture of life, diet profiles, life conditions were different, given us opportunity to see EtG and EtS pharmacokinetics from a different perspective.

Depending on our findings EtG and EtS were detectable up to 10 h in blood and up to 24 h in urine after ingestion. In all four controlled experiment Hoiseith et al. [7] detected EtG in blood up to 14 h as the longest detection time. Other experiments limitation to detect longer time period were lower sampling time after 10 h. Lostia et al. [3] collected blood samples every hour for the first 6 h and then after 24 h and 48 h. In the other study by Hoiseith et al. [6] samples were collected at 1.5, 3.5, 5.5, 8.5, 11.5, and 24 h, for Halter et al. [4] every 30 min from 1 to 5 h and every 60 min from 5 to 10 h after ingestion. The studies either did not perform any sample collection after 10 h or a large time gap existed between the previous one and 24 h. On the other hand all experiments include us indicate EtG and EtS are detectable up to 10 h in blood (Lostia et al. [3] emphasized in their results that EtG and EtS were detectable for 10 h in four females blood samples). For urine samples both metabolites were detected at 24 h in all studies.

Comparison of C_{max} and T_{max} data of studies were shared in Table 3 and Figures 4, 5. Maximum and minimum C_{max} values were measured the study by Halter et al. 1.09 mg/L and by us 0.13 mg/L for EtG, by Lostia et al. 0.1 mg/L and by Halter et al. 0.8 mg/L for EtS in blood at the similar consumption level, respectively [3, 4]. The highest urine EtG and EtS C_{max} were 189.5 mg/L and 74.5 mg/L by Lostia et al. [3] in all controlled experiments, the least values were 6.9 mg/L by us and 5 mg/L by Lostia et al. [3], respectively. The lowest C_{max} mean value for EtG were detected in our study for both biological samples. The analytical methods of all experiments were almost similar except minor differences that could cause minor deviation for results. The analytical instruments were same (LC-MS/MS) in all experimental methods and the authors declared their methods were fully validated. Calculated validation parameters were within expected range according to international guidelines in the studies. Comparison of LOD and LOQ data of methods were given in Table 4. On the other hand when the results are taken consideration separately for each studies, great variation are seen between the lowest and highest values for C_{max} which is not explained by methodological differences. The dispersion patterns of data are not well understandable from studies and lack of some statistical outputs such as standard deviations, interquartile range bring on insufficient interpretation for valuable informations. The studies of Høiseth et al. [6, 7] had lower dispersion range than other studies with their smaller number of volunteers. Otherwise in the study by Lostia et al. [3] which has the largest number of volunteers, dispersion range of C_{max} values was the broadest for both metabolites in urine and blood EtG, also blood

EtS was relatively higher (highest/lowest ratio: 4.17, 4, 15, 10 for blood EtG, EtS and urine EtG, EtS, respectively). The broadest dispersion range were detected by Halter et al. [4] for blood EtS (highest/lowest ratio: 6.15). In the present study our dispersion ranges were the narrowest for EtS in blood and urine, the midmost of the studies for EtG. Urine dispersion ranges both metabolites are broader than blood in all studies. They concentrate in the urine due to renal functions, hydration status of body, acid trap, and their hydrophilic chemical properties, also these factors are additional variables affecting the urine concentrations [6, 7, 16].

Beside, EtG and EtS in blood are affected by various factors that could explain dispersion ranges. Inter individual variations are the common cause of this broad dispersion range beginning from ethanol absorption to metabolic pathways. Bioavailability and first pass effect by liver on ethanol depend on the beverage type, the consumption speed and amount, the fed or fasted state of person [17]. Alcohol and aldehyde dehydrogenase enzymes saturation which is the major metabolic pathway for biotransformation of ethanol and free substrate amount for other phase II enzymes alter the production of EtG and EtS [18, 19]. Doubling the ethanol dose resulting triplicated blood EtG and EtS is demonstrated by Høiseth et al. [6] and Lostia et al. [3]. Induction of alcohol dehydrogenase (ADH) enzymes by higher consumption amount per month and enzyme polymorphisms as ADH2*2 and ADH2*3 which metabolize ethanol more rapidly, CYP2E1 which is the major enzyme of nonoxidative pathway could change free ethanol amount targeted by UGT and SULT, albeit there is no published data [20, 21]. Additionally genetic

Table 3: Kinetics of EtG and EtS in blood and urine in previous studies and our study.

Studies	Ethanol intake (g/kg)	C _{max} (mg/L) and T _{max} (h) for blood EtG	C _{max} (mg/L) and T _{max} (h) for blood EtS	C _{max} (mg/L) and T _{max} (h) for urine EtG	C _{max} (mg/L) and T _{max} (h) for urine EtS
Høiseth et al. [7]	0.5	0.27–0.5 4.0	– –	41–73 4.75	– –
Halter et al. [4] ^a	0.5–0.78	0.27–1.09 4.0±0.9	0.13–0.8 3±0.5	23.1–178.9 6.2±0.9	5.8–67.2 5.3±1.2
Høiseth et al. [6]	0.5	0.28–0.41 3.5	– –	47.1–96.5 5.5	– –
Høiseth et al. [6]	1.0	0.8–1.22 5.5	– –	97.2–225.5 5.5	– –
Lostia et al. [3]	4 Units (0.76 g/kg, mean)	0.24–1.0 2.3±0.6	0.1–0.4 1.3±0.5	19–189.5 3.5±1.5	5–74.5 3.4±1.7
Lostia et al. [3]	8 Units (1.53 g/kg, mean)	0.5–2.4 3.8±1.4	0.3–0.9 2.8±1.2	38–467 5.7±1.5	12–85.5 5.1±1.2
Our study	0.5	0.13–0.389 2.55±0.3	0.211–0.5 3.14±0.3	6.9–30.4 4.4±0.8	10.5–58.2 4.5±0.9

^aThe data were given as µmol/L in paper and concentrations were converted to mg/L getting EtG: 222.193 g/mol and EtS: 126.126 g/mol.

Figure 4: Literature comparison of blood and urine EtG concentrations. Graphical comparison of Cmax concentrations (A) blood EtG concentrations (B) urine EtG concentrations (the highest and lowest Cmax concentrations are given tip of lines, spot on the lines indicate mean Cmax concentrations).

Figure 5: Literature comparison of blood and urine EtS concentrations. Graphical comparison of Cmax concentrations (A) blood EtS concentrations (B) urine EtS concentrations (the highest and lowest Cmax concentrations are given tip of lines, spot on the lines indicate mean Cmax concentrations).

polymorphisms of UGT and SULT, and nutritional factors affect the formation of EtG and EtS. Endogenous and exogenous multiple substrates are targeted by these enzymes,

whereas all isoforms contribute the formation, especially UGT1A1 (at the study by Foti et al. [10]), UGT1A9 (at the study by Schwab and Skopp [11]), UGT2B7 and SULT1A1,

Table 4: Comparison of LOD and LOQ data of studies (all data were given as mg/L).

	Blood EtG		Urine EtG		Blood EtS		Urine EtS	
	LOD	LOQ	LOD	LOQ	LOD	LOQ	LOD	LOQ
Høiseth et al. [7]	0.02	0.09	0.09	0.2				
Halter et al. [4] ^a		0.1		0.1		0.1		0.1
Høiseth et al. [6]	0.03	0.06	0.17	0.37	0.007	0.02	0.06	0.16
Lostia et al. [3]	0.03	0.08	0.05	0.15	0.01	0.03	0.03	0.1
Our study	0.002	0.007	0.01	0.032	0.002	0.008	0.012	0.04

^aThe data were given as $\mu\text{mol/L}$ in paper and concentrations were converted to mg/L getting EtG: 222.193 g/mol and EtS: 126.126 g/mol.

SULT2A1 subtypes are predominant [9, 15]. Functional genetic polymorphisms of UGT1A1, UGT1A9, UGT2B7, SULT1A1, and SULT2A1 were demonstrated previously with ethnicity differences, furthermore inhibitors in diet component like flavonoids were observed to explain high inter individual variability [8, 9, 11–13, 22]. Contrary to other controlled drinking experiment our data presented higher EtS concentrations than EtG, as to be possible reflection of enzymatic activity discrepancy due to any polymorphism encountered specifically in our population. We need to perform further studies to prove this.

To reveal recent ethanol use Lostia et al. [3] demonstrated increased EtG/EtS ratio over time. At the present study EtG/EtS ratio was irrelevant to time and only limited samples was >1 independent from time. For indication of recent use, ratio of two subsequent EtG and EtS could be guide deducing from our data. If previous/subsequent ratio is <1 , it is indicated that at most 3.5 h in blood, 6 h in urine for both metabolites past last consumption, If it is >1 , at least 2 h for EtG and 3 h for EtS in blood, 4 h for both in urine past. On the other hand these deductions are validity for healthy individuals. Høiseth et al. [7] emphasized similarly with our results that two decreasing concentrations for EtG in blood abolish the possibility of consumption within 3.5–5 h.

The major limitation of present study was the limited number of volunteers, as clearly specified by the previous study too. There is need for researches containing larger number of volunteers and more controlled factors like disease, diet, recent and general drinking pattern, even ethnicity to explain inter individual variations. The second limitation of our study is lack of ethanol concentration of volunteers after drinking that might be important contribution of our data.

Pharmacokinetic studies of EtG and EtS in blood and urine have been performed in various populations. It is thought that the metabolites are influenced by many factors which can not be explained by previous studies due to their limitations. Determination of recent alcohol use has been investigating for a long time in forensic and

clinical sciences and recent studies focused on influences of genetic and environmental factors [8, 11]. Our study showed that detection of EtS has more determinant than EtG in Turkish population; since, EtS remained much more time and dominant than EtG in both blood and urine. In addition, further studies should be conducted to determine all factors in kinetics of the minor metabolites of ethanol in different populations with genetic confirmation to take advantage of these metabolites.

Acknowledgements: The project was funded by Hacettepe University BAP Department, Funder Id: 10.13039/501100005378, project number 013 D03 101 001. The study protocol was approved by the Hacettepe Committee for Research Ethics, decision date 20/12/2012 and number 07/07, in Turkey. The subjects gave informed consent. Dr. Emre Karacaoğlu, Dr. Ramazan Akçan and Prof. Dr. Aysun Balseven Odabaşı designed the study. Dr. Emre Karacaoğlu and Dr. Ramazan Akçan evaluated the objects, organized the study and collected the specimens. Dr. Tolgahan Kocadağlı and Prof. Vural Gökmen managed the analytical process of the specimens. Dr. Emre Karacaoğlu and Dr. Aykut Lale managed the literature searches and wrote the first draft of the manuscript. Dr. Ali Rıza Tümer provided critical input to final paper. All authors contributed to and approved the final manuscript.

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