AP05

การตรวจวิเคราะห์เอทิลกลูคูโรไนด์จากปัสสาวะของศพ โดยเทคนิค GC-MS
Analysis of Ethyl Glucuronide in Urine at Autopsy by GC-MS

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Abstract

In post-mortem cases, there are difficult to classify whether blood ethanol arises from ante mortem ingestion or post mortem formation of alcohol. Measurement of the stable ethanol metabolite, ethyl glucuronide (EtG) has been proposed as a marker of ante mortem ingestion of ethanol. The present study measured EtG in urine collected from autopsy cases by GC-MS to compare with blood ethanol level. The results showed that urine EtG was only detected in cases with positive for blood ethanol but not in those with negative for blood ethanol. Moreover, there was no correlation (n = 20, r^2 = 0.22) between urine EtG (0-0.70 mg/ml) and blood ethanol concentrations (157.59-312.52 mg%). This could be due to the difference in time interval between collection of blood and urine samples for each subject.

Keywords: *Ethyl Glucuronide / EtG / Ethanol / Urine*

บทคัดย่อ

ในผู้เสียชีวิต เป็นการยากที่จะจำแนกได้ว่าแอลกอฮอล์ที่ตรวจพบนั้นเกิดขึ้นก่อน หรือ ภายหลังการตาย ซึ่งการตรวจหาเมทาบอไลท์ของเอทานอลนั่นคือ เอทิลกลูคูโรไนด์ (EtG) สามารถใช้เป็นตัวบ่งชี้ว่าเป็นแอลกอฮอล์ที่ได้รับเข้าไปก่อนตาย การศึกษานี้ได้ทำการวิเคราะห์ EtG ในปัสสาวะของผู้เสียชีวิต โดยใช้เทคนิค GC-MS เพื่อเปรียบเทียบกับความเข้มข้นของเอทานอลในเลือด รวมทั้งวิเคราะห์หาความสัมพันธ์ของข้อมูล จากผลการศึกษาพบว่า EtG ในปัสสาวะนั้นจะตรวจพบเฉพาะในศพที่ตรวจพบเอทานอลในเลือด และความเข้มข้นของ EtG ในปัสสาวะ (0–0.70 mg/ml) และเอทานอลในเลือด (157.59–312.52 mg%) ไม่มีความสัมพันธ์กันทางสถิติ (n=20, r^2=0.22) อาจเป็นเพราะความแตกต่างในช่วงเวลาระหว่างการเก็บตัวอย่างเลือดและปัสสาวะของศพแต่ละราย

คำสำคัญ: *เอทิลกลูคูโรไนด์ EtG เอทานอล ปัสสาวะ*

Introduction

Alcohol is a serious public health problem worldwide. It causes health problem, family breakup, accident and crime, which affects society and economy. In Thailand, death caused by alcohol (ethanol) has been increasing steadily. This includes natural mortality from alcoholism and cirrhosis as well as unnatural deaths from accidents and physical abuse. In post-mortem cases, it is difficult to determine and interpret a blood ethanol concentration which reflects ante mortem ingestion or post mortem synthesis of alcohol, since microorganisms in the body can generate ethanol after death. Therefore, the metabolites of ethanol are alternative markers that can help in the interpretation. One such metabolite which has received much attention is ethyl glucuronide (EtG). Ethyl glucuronide (EtG) is a non-oxidative, minor metabolite of ethanol (alcohol) formed by glucuronidation of ethanol catalyzed by the enzyme UDP-glucuronosyl transferase

(Foti and Fisher, 2005). The determination of EtG in body fluid has become an important factor in forensic and other legal decisions (Droenner et al., 2002). EtG is a good candidate as it is a sensitive, specific and reliable marker of recent alcohol intake (Wurst et al., 2002). In post-mortem cases with negative ethanol intake, EtG is not produced endogenously (Hoiseth et al., 2007). In the literature, several methods for EtG analysis have been reported but there is no reference or analytical method reported in Thailand. Gas chromatography-mass spectrometry (GC-MS) is one of the most accurate techniques for analyzing complex samples. It is used to separate volatile compounds in a mixture. The separated compounds can be identified and quantified.

The aims of the study were 1) to determine ethyl glucuronide, a metabolite of ethanol, in urine samples collected from autopsy cases by GC-MS, 2) to compare the concentration of EtG in urine with blood ethanol level (positive and negative) to determine whether EtG is a reliable post-mortem indicator of ante-mortem alcohol consumption, and 3) to find the correlation between EtG concentration in urine and blood alcohol level.

Research methodology

Chemical Reagents

Ethyl glucuronide (EtG) and d_5-Ethyl glucuronide (d_5-EtG) standards were obtained from Cerilliant (USA). N-Methyl-N-(trimethylsilyl) trifluoroacetamine (MSTFA) was from MACHERY-NAGEL (Germany). Pyridine was from LAB-SCAN (Poland) and HPLC grade methanol was from Fisher Scientific (UK).

Samples collection

Urine samples of 20 autopsy cases (male, 14-69 yr, collection time was less than 2 days after death) were collected at Central Institute of Forensic Science (CIFS), Department of Justice, Thailand. The samples were kept in tube containing sodium fluoride as preservative, storage at 4°C. Ten samples had blood ethanol level above the legal limit of 50 mg% and 10 samples with negative blood ethanol level. EtG was analyzed by GC-MS at the National Doping Control Centre, Mahidol University.

Preparation of urine samples

Internal standard d_5-EtG (60 µl, 0.01 mg/ml) was added to 60 µl urine in a glass test tube and the solution was dried under nitrogen stream at 40°C and 5 psi gas pressure in the fume hood. The dried sample was then placed in a vacuum oven at 40°C for 30 min. The sample was then dissolved in 25 µl pyridine and 75 µl MSTFA (the silylating reagent) and heated in an oven at 60°C for 30 minute. After cooling to room temperature the supernatant was then transferred to a GC glass insert for GC-MS analysis.

GC-MS condition

An Agilent 6890/5973 GC/MSD (Agilent, Germany) instrument was used for analysis of the sample. The capillary column was Zebron ZB-1 (20 m×250 µm id, 0.10 µm film thickness, Phenomenex, USA). The oven temperature program was as follows: initial 60°C for 2 min, then increasing at 10°C/min to 200°C, then at 15°C/min to 250°C, and hold for 1 min. The carrier gas was helium at the flow rate of 1 ml/min. Injections were made in the split mode (split ratio 5:1). The injector and transfer line were maintained at 250°C and 280 °C, respectively. The quadrupole temperature was 150°C and ion source set at 230°C. The mass spectrometer was operated in the full scan mode (m/z 50-550). The extracted ions for quantitation and identification of EtG and d_5-EtG were m/z 261, 160, 405 and m/z 266, 165, 410, respectively.

Data analysis

Calibration curve was constructed for each batch of analysis. The concentrations of the standard EtG solutions used in the calibration were 0.01, 0.02, 0.1 and 0.2 mg/ml, respectively. Limit of quantitation (LOQ) is the lowest amount that can be quantitatively measured in a sample with suitable precision and accuracy. The LOQ analysis was carried out using EtG concentrations of 0.05 and 0.01 mg/ml in blank urine. The standard deviation (SD) of the analysis was calculated. Inter-day and intra-day precisions were measured using EtG concentration of 0.02 and 0.1 mg/ml in blank urine. The percent relative standard deviation (%RSD) was calculated. Correlation between EtG concentrations in urine samples and blood alcohol levels was determined.

Results and Discussion

In this study, the characteristic ions for the derivatized ethyl glucuronide were selected using the published data (Janda and Alt, 2001). For EtG the ions are m/z 261 (the quantifying ion), m/z 160, and m/z 405 (the qualifying ions). For d_5-EtG, the internal standard, the ions are m/z 266 (the quantifying ion) with m/z 165 and 410 as qualifier ions. The retention times of EtG and d_5-EtG are 15.71 min and 15.68 min, respectively (see Figs. 1– 4).

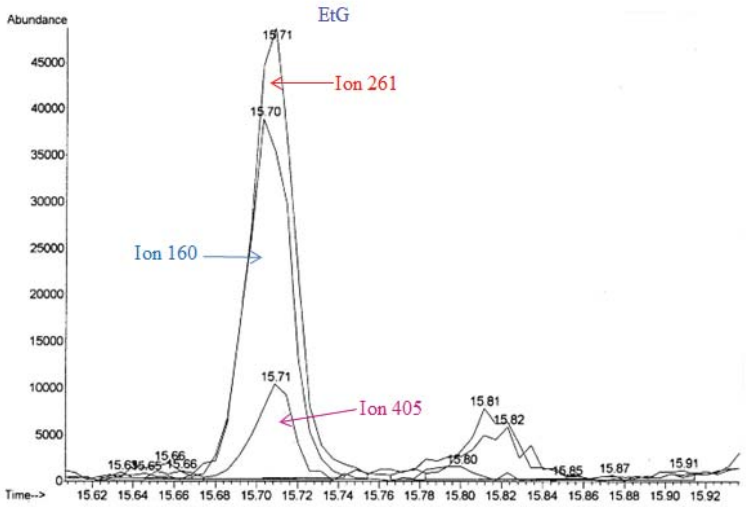

Figure 1 Extracted ion chromatograms from the MS scan mode of EtG spiked in blank urine at 0.02 mg/ml. The ions are m/z 261, 160, and 405.

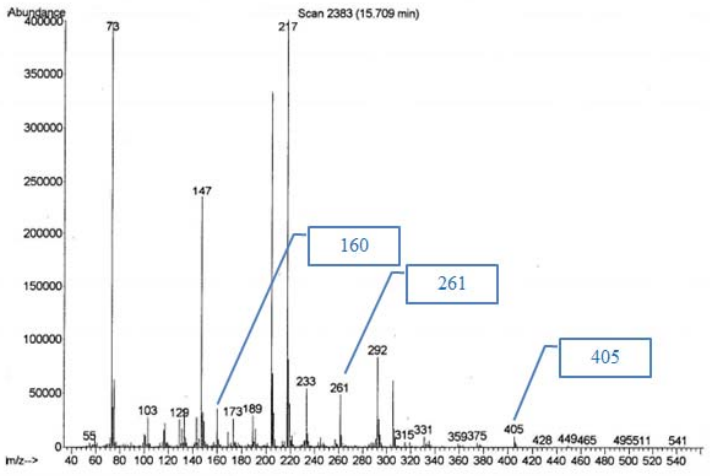

Figure 2 Mass spectrum at RT 15.71 min of EtG spiked in blank urine at 0.02 mg/ml.

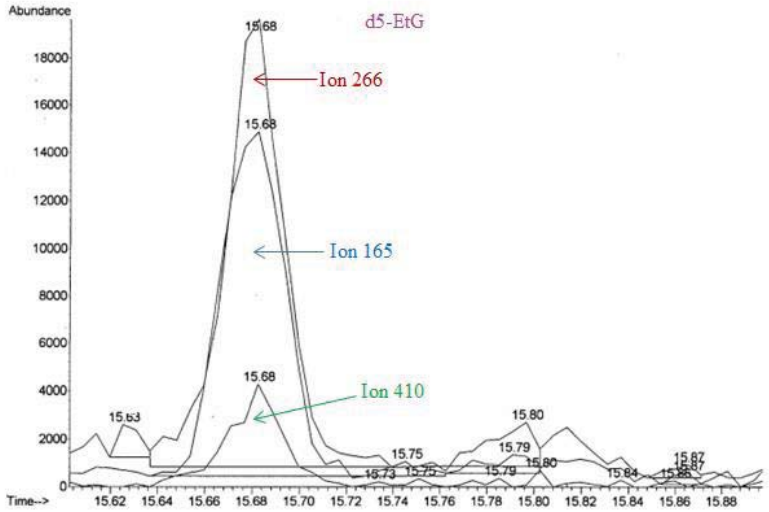

Figure 3 Extracted ion chromatograms from the MS scan mode of d_5-EtG spiked in blank urine at 0.01 mg/ml. The ions are m/z 266, 165, and 410.

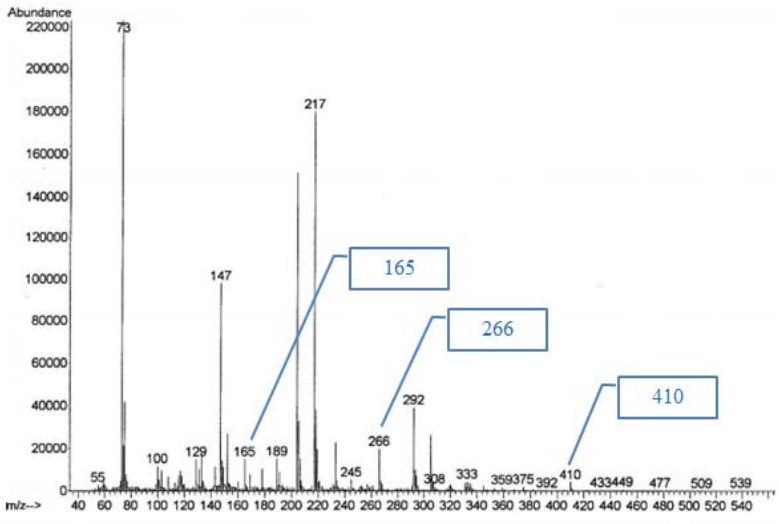

Figure 4 Mass spectrum at RT 15.68 min of d_5-EtG spiked in blank urine at 0.01 mg/ml.

Validation study

Calibration curve was constructed using the area ratio of the quantifying ions of EtG (m/z 261) and d_5-EtG (m/z 266). The calibration was linear for EtG concentration in the range 0.01 – 0.2 mg/ml, with correlation coefficient r^2 = 0.999

In this study LOQ is defined as the lowest concentration for which the percent relative standard deviation (%RSD) is not greater than 20% but the chromatographic peak can still be identified as the target compound. The LOQ of EtG was 0.01 mg/ml (see Table 1) because %RSD of EtG concentration 0.005 mg/ml was more than 20%. The precisions for intra-day and inter-day analyses of EtG spiked in blank urine at concentrations of 0.02 and 0.1 mg/ml are given in Table 2.

Janda and Alt (2001) analyzed EtG in urine in the range of 70 to 700 ng/ml. They employed SPE extraction to achieve this concentration range of EtG. The LOQ was 560 ng/ml and inter-day precision was 3.8%. Freire *et al*. (2008) analyzed EtG in urine samples by using microwave assisted extraction and obtained linear calibration curve for 0.1 to 100 µg/ml EtG. LOQ was 0.1 µg/ml and %RSD for intra-day and inter-day precisions were 1.49% and 4.51%, respectively, at 100 µg/ml.

Table 1 %RSD of EtG at concentration of 0.01 mg/ml in spiked urine.

Mean of area ratio	Standard deviation	% Relative standard deviation
1.08	0.05	5.00

Table 2 Inter-day and intra-day precisions for EtG in spiked urine

Concentration of EtG (mg/ml)	Intra-day %RSD	Inter-day %RSD
0.02	11.20	10.73
0.1	13.69	14.26

Analysis of EtG in urine samples

Results of the analysis of 20 urine samples collected from postmortem subjects are shown in Tables 3 and 4. EtG was not detected (LOQ 0.01 mg/ml) in all 10 urine samples from which blood ethanol was not found. However, in the other 10 urine samples, which had positive for blood ethanol, EtG was detected. The extracted ion chromatograms and full scan mass spectra of EtG in urine samples with negative and positive blood ethanol are shown in Figs. 5 and 6.

Figure 7 shows the plot of the concentration of EtG for all 20 urine samples against the blood ethanol level. There was no correlation (r^2 = 0.22) between the level of urine EtG and blood ethanol. These results were similar to those reported by Wurst et al. (1999) in which; the average urine EtG (344±242 mg/l; range 3.6-710 mg/l) and average blood ethanol concentration (183±86 mg/dl; range 39-360 mg/dl) was not found to be correlated (r=0.17). Later, Bergstrom et al. (2003) reported that urine alcohol concentration was not correlated with urine EtG (r = -0.03), but the urine EtG was positively correlated with creatinine, indicating that the excretion of EtG in urine is influenced by diuresis. There are studies that found patients with renal disease decreased and delayed excretion of EtG. Therefore it has been proposed that the measured EtG concentration should be corrected for urine dilution using either creatinine, osmolality, or specific gravity (Wurst et al., 2004, Hoiseth et al., 2009). However, ethanol concentration, time of consumption, and incidental exposure before death should also be considered.

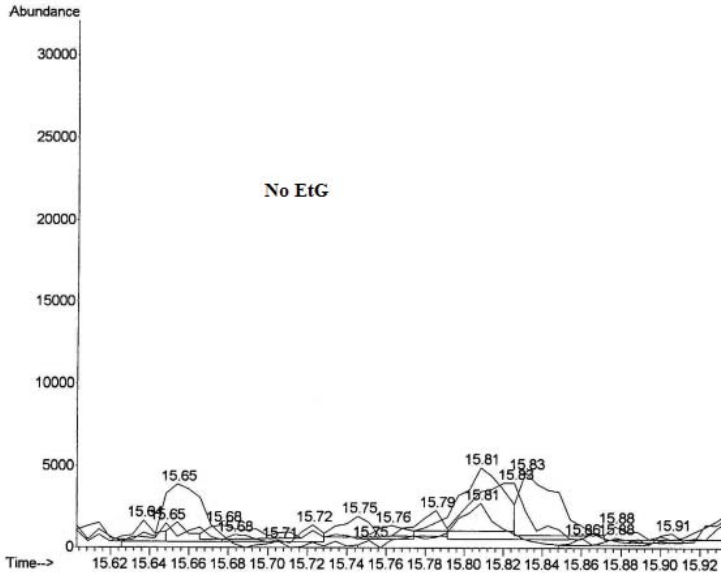

Figure 5 Extracted ion chromatograms from the MS scan mode of urine sample (case No. 4) with negative blood ethanol. The ions are m/z 261, 160, and 405.

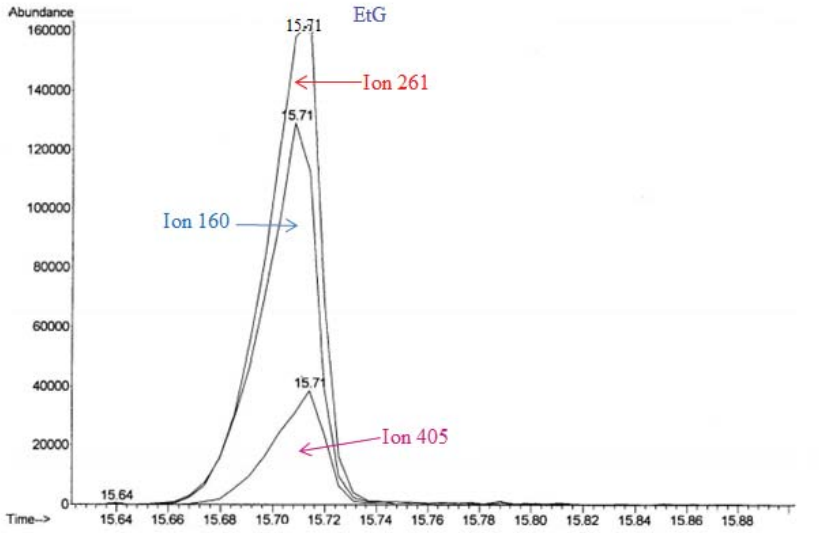

Figure 6 Extracted ion chromatograms from the MS scan mode of urine sample (case No.8) with positive blood ethanol. The ions are m/z 261, 160, and 405.

Table 3 Results of urine EtG level for samples with negative blood ethanol

Case No.	Gender	Age	Cause of Death	Blood Alcohol Concentration (mg%)	EtG Concentration (mg/ml)
1	male	16	unknown	ND	ND
2	male	50	unknown	ND	ND
3	male	14	accident	ND	ND
4	male	53	unknown	ND	ND
5	male	47	unknown	ND	ND
6	male	20	unknown	ND	ND
7	male	50	unknown	ND	ND
8	male	17	unknown	ND	ND
9	male	54	unknown	ND	ND
10	male	60	unknown	ND	ND

ND= Not detect, unknown= unexpected cause of death from accident, suicide and homicide such as sudden unexpected death syndrome, drowning.

Table 4 Results of urine EtG level in samples with positive blood ethanol

Case No.	Gender	Age	Cause of Death	Blood Alcohol Concentration (mg%)	EtG Concentration (mg/ml)
1	male	20	accident	157.59	0.24
2	male	57	unknown	181.10	0.70
3	male	24	accident	189.48	0.01
4	male	27	homicide	202.62	0.01
5	male	25	accident	205.24	0.08
6	male	49	suicide	216.42	0.08
7	male	51	accident	219.46	0.03
8	male	69	accident	239.34	0.09
9	male	48	accident	269.62	0.28
10	male	29	homicide	312.52	0.18

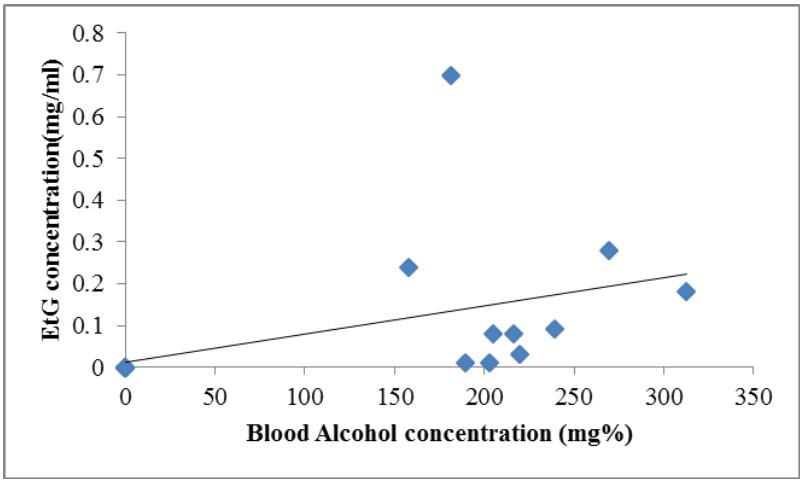

Figure 7 Plot of urine EtG concentration against blood alcohol concentration for all 20 samples (r^2 = 0.22).

Conclusion

Ethylglucuronide, a metabolite of ethanol, is a suitable marker to confirm previous ethanol intake from postmortem urine samples. Unlike ethanol, EtG is stable and not a product of bacterial growth, which can produce after death in blood and urine. In this work, stable isotopic labeled EtG (d_5-EtG) is available for use as the internal standard, providing high precision in quantitation. EtG was not detected in the samples with negative blood ethanol. Urine EtG (0.01–0.70 mg/ml) were found in 10 samples with positive blood ethanol (158–312 mg%). There was no correlation (r^2 = 0.22) between the level of urine EtG and blood ethanol. The results of the study would help to confirm that, ethanol could only come from ante-mortem ingestion in autopsy cases with blood ethanol over the legal limit (≥50 mg%).

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