Validation of HPLC-ESI-MS/MS Protocol to Analyze EtG in Hair for Assessment of Chronic Excessive Alcohol Use in Thailand in Conjunction with AUDIT

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Background: *Hair analysis for chronic excessive alcohol (ethanol) use has focused on ethyl glucuronide (EtG), a minor metabolite of ethanol. Preferred methods have involved high-performance liquid chromatography (HPLC) combined with tandem mass spectrometry (MS/MS) in line with an electrospray ionization (ESI) source. EtG analysis in hair has not yet been introduced to Thailand.*

Objective: *To validate an in-house HPLC-ESI-MS/MS hair analysis protocol for EtG and to apply it to a field sample of alcohol drinkers to assess different risk levels of alcohol consumption as measured by the Alcohol Use Disorders Identification Test (AUDIT).*

Material and Method: *Validation procedures followed guidelines of the US Food and Drug Administration, the European Medicines Agency, and the Scientific Working Group for Forensic Toxicology. One hundred twenty subjects reported consuming alcohol during a 3-month period prior to enrollment. After taking the Thai-language version of AUDIT, subjects were divided on the basis of test scores into low, medium, and high-risk groups for chronic excessive alcohol use.*

Results: *The protocol satisfied the international standards for selectivity, specificity, accuracy, precision, and calibration curve. There was no significant matrix effect. Limits of detection and quantification (LOD/LOQ) were set at 15 pg of EtG per mg of hair. The protocol was not able to detect EtG in low-risk subjects (n = 38). Detection rates for medium-risk (n = 42) and high-risk subjects (n = 40) were 14.3% and 85%, respectively. The median of EtG concentration between these two groups were significantly different. Sensitivity and specificity were both more than 90% when EtG concentrations of high-risk subjects were compared with the 30 pg/mg cutoff recommended by the Society of Hair Testing (SoHT) for diagnosing chronic excessive alcohol consumption, based on an average ethanol daily intake greater than 60 g.*

Conclusion: *The in-house protocol for EtG analysis in hair was validated according to international standards. The protocol is a useful tool for evaluating risk for chronic excessive drinking as defined by AUDIT scores. It strongly predicted the highest level of risk, although it was inadequate for assessing lower levels of risk.*

Keywords: *HPLC-ESI-MS/MS, hair analysis, chronic excessive alcohol use, EtG, AUDIT, Thailand*

J Med Assoc Thai 2016; 99 (6): 711-22
Full text. e-Journal: *http://www.jmatonline.com*

Ethanol (ethyl alcohol) is widely consumed and often misused in Thailand. Medically and socially, the harmful consequences of chronic excessive alcohol use are numerous and well known[1]. Unlike an illicit substance such as heroin, ethanol is legal, readily obtainable, and socially acceptable in many segments of Thai society. Therefore, the diagnosis of alcohol abuse is not a matter of establishing that ethanol consumption has occurred. Rather, it depends on identifying the magnitude, frequency, and circumstance of ethanol consumption in order to evaluate different levels of risk that are commonly associated with different types of drinkers.

Outside the field of genetics, two basic screening techniques have been developed to assess an individual's level of risk for what is often called "alcohol use disorder". The first relies upon self-report by means of structured interview, self-administered test, or some combination of the two. A well-established and widely used screening technique of this type is the Alcohol Use Disorders Identification Test (AUDIT)[2].

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Translated into several languages including Thai[3], AUDIT was introduced to Thailand as a successful diagnostic tool in the late 1990s[4].

The second basic screening technique relies on laboratory analysis of biological samples to determine risk-correlated metrics for ethanol consumption. Ethanol itself can serve as a biochemical marker, but owing to its rapid metabolism its detection period is measured in hours for commonly assayed bodily fluids such as blood, urine, and saliva[5]. Although hair analysis is valuable for detecting long-term use of many drugs, it is inapplicable to ethanol because the compound is highly volatile and readily absorbed by hair from external sources, such as pubs and laboratories. In most countries with developed medical laboratory facilities, the diagnosis of alcohol use disorder has usually relied on blood assays that detect significant changes in liver biochemistry, but such analyses are most useful after chronic excessive use has already occurred. In addition, they often lack specificity because they detect pathogenic conditions that may or may not have resulted from ethanol consumption[6,7]. In Thailand, the diagnostics of chronic excessive alcohol use have not been the subject of systematic research. There are no previously published studies on any aspect of laboratory screening, so that the present investigation is the first of its kind. Based on our knowledge of Thai medical practice, the diagnosis of chronic excessive alcohol consumption is generally based on some form of psychiatric counseling. Blood assays to assess changes in liver biochemistry are used to corroborate clinical findings.

The challenge in laboratory ethanol screening has been to find a stable biochemical marker directly derived from the ethanol molecule that can be quantified with good specificity and sensitivity in an easily obtainable biological matrix that provides a wide enough window of detection to diagnose chronic long-term use. For over a decade, researchers specializing in hair analysis and alcohol use disorder have recognized the diagnostic potential of two types of ethanol metabolites: fatty acid ethyl esters (FAEE) and ethyl glucuronide (EtG)[7-11].

In 2011, the Society of Hair Testing (SoHT) adopted the hair analysis of either EtG or FAEE as preferred methods of testing for drinking behavior that it defined as "chronic excessive alcohol consumption"[12]. A year later, SoHT selected EtG as "the first choice for abstinence assessment"[13]. Although Pragst et al argued shortly before the publication of the SoHT guidelines that the most accurate diagnosis of alcohol use disorder involves the combined analysis of both EtG and FAEE, the authors recognized that "in the last few years particularly EtG was increasingly introduced as a single parameter in forensic toxicological practice"[14]. A recently conducted review of the hair-analysis literature reached the same conclusion[15]. The present study therefore focused on the analysis of EtG in hair as the de facto biomarker of choice for the clinical and forensic diagnosis of alcohol use disorder.

EtG ($C_8H_{14}O_7$) is a stable, non-volatile, water-soluble compound that is a phase II-metabolite of ethanol. Its biochemical synthesis occurs mainly in the liver as an ethanol-glucuronic acid conjugate enzymatically catalyzed by UDP-glucuronosyltransferase. Because EtG contains the unchanged ethyl group of alcohol, the compound offers a highly specific biomarker for the detection of alcohol use disorder[16]. However, detection of EtG in hair presents many challenges, not only because the metabolite itself is synthesized in minute amounts (about 0.06 to 0.37% of ingested ethanol is eliminated as EtG) but also because EtG is both acidic and highly hydrophilic. Both these conditions tend to reduce a compound's incorporation into hair. Consequently, EtG hair assays require a high degree of sensitivity capable of accurate and reliable measurement in the picogram range[8,15-17]. Although prior researchers in several countries have validated EtG hair analysis protocols using a variety of instrumentation[15], EtG hair detection has not yet been introduced to Thailand.

The present study had two objectives. The first was to modify for in-house use a previously developed protocol for EtG analysis and to validate this modification according to international standards. The second was to apply the protocol to a sizeable field sample to determine if it could assess different risk levels of alcohol consumption as measured by AUDIT results.

Material and Method

In developing our methodology, we adapted a validated EtG hair analysis protocol published by Morini et al in 2006[18]. This technique combined high-performance liquid chromatography (HPLC) with electrospray-ionization (ESI) and tandem mass spectrometry (MS/MS). The Morini protocol had been successfully used in several investigations of EtG in hair[19-23]. As explained in detail below, our study involved the participation of several subjects. All received modest financial compensation for

furnishing hair samples, and all signed informed consent agreements regarding their participation. The objectives and procedures of the present study were reviewed and approved by the Research Ethics Committee of the Faculty of Medicine of Chiang Mai University, as recorded in Document Number 225/2555.

Chemicals and reagents

Except where otherwise noted, all compounds were HPLC grade. The EtG and the internal standard ethyl glucuronide-D5 (EtG-D5) were purchased from Cerilliant (Round Rock, Texas, USA). Both reagents came in the form of methanol dilutions, EtG at 1 mg/mL and EtG-D5 at 100 µg/mL. These stock solutions were stored at -20°C. Working solutions of EtG were prepared in methanol at concentrations of 25, 50, 75, 100, 150, 250, 500, 1,000, 1,500, and 2,500 ng/mL. The EtG-D5 working solution was prepared in methanol at 500 ng/mL. These working solutions were stored at 4°C. Formic acid was purchased from Fisher Scientific (Loughborough, Leicestershire, UK), which also supplied methanol. Acetonitrile was purchased from J.T.Baker (Center Valley, Pennsylvania, USA). Dichloromethane (AR grade) was obtained from Merck (Kenilworth, New Jersey, USA), while distilled water and ultrapure water were produced by the Elga system (Woodridge, Illinois, USA).

Numerous compounds were used for specificity/selectivity analysis as part of the general protocol validation. The following were purchased from Lipomed (Alesheim, Switzerland): methamphetamine (MA.HCl, 99.42% purity); amphetamine (AM.HCl, 99.84% purity); morphine (morphine.HCl, 98.9% purity); 3,4-methylenedioxyamphetamine (MDA.HCl, 99.74% purity); 3,4-methylenedioxymethamphetamine (MDMA.HCl, 99.62% purity); and 3,4-methylenedioxy-N-ethylamphetamine (MDE.HCl, 99.85% purity). Stock methanol solutions of these reagents were prepared in-house and stored at -20°C as follows: MA (1134.60 µg/mL), AM (1163.80 µg/mL), morphine (1350.23 µg/mL), MDA (1193.46 µg/mL), MDMA (1173.32 µg/mL), and MDE (1185.58 µg/mL). Working solutions of all of the above were prepared in methanol at 1 µg/mL. Specificity/selectivity analysis also utilized a stock acetonitrile solution of eight benzodiazepines (250 µg/mL of each component, greater than 99.8% purity). This mixture, purchased from Cerilliant, contained alprazolam, clonazepam, diazepam, flunitrazepam, lorazepam, nitrazepam, oxazepam, and temazepam. A working solution, stored at 4°C, was prepared in methanol, with each benzodiazepine at 1 µg/mL.

Hair sample preparation for EtG extraction and analysis

Hair was collected with clean scissors by cutting as closely to the scalp as possible from the posterior vertex region. The samples were stored in a clean plastic bag, with root ends marked and aligned. Hair sample preparation followed Morini et al with minor modifications[18]. The proximal 3 cm strands of each subject were washed by vortexing for 30 seconds with 5 mL of dichloromethane and then ultrasonicated for 10 minutes. This cleansing was followed by vortex washing with 5 mL of methanol for 30 seconds. The samples then were dried at room temperature and cut into small pieces. The analyte was prepared for aqueous extraction by combining 100 mg of each hair sample with 700 µL of distilled water, 20 µL of EtG-D5 working solution and 20 µL of methanol. This mixture was centrifuged at 5,000 rpm for 5 minutes, incubated overnight at room temperature, ultrasonicated for 2 hours, and then centrifuged at 13,000 rpm for 10 minutes. The supernatant was filtered by nylon syringe filter (0.2 µm) and evaporated under nitrogen flow at 40°C. The reconstitution solvent was 200 µL of 0.1% formic acid in ultrapure water and acetonitrile (95:5, v/v). Twenty µL of the reconstituted extract was injected into the HPLC-ESI-MS/MS system for analysis.

Instrumentation

HPLC-ESI-MS/MS was performed with Agilent (Palo Alto, California, USA) equipment (LC 1290 Infinity Series coupled with Triple Quadrupole 6460 Series and an electrospray ionization source). Chromatography separation used Phenomenex (Torrance, California, USA) apparatus in the form of a Gemini C6 phenyl column (150x4.6 mm, 5-µm particle size) operated at 40°C. The mobile phase consisted of (A) 0.1% formic acid in ultrapure water and (B) acetonitrile. Gradient elution was 95% A:5% B at 0 to 7 minutes. This condition was then adjusted to 0% A: 100% B within 2 minutes and retained up to 15 minutes at 0.5 mL/minute flow rate. Electrospray ionization (ESI) was in negative mode. Temperature and flow rate of nitrogen as drying gas were 300°C and 12 L/minute, respectively. Nitrogen sheath gas temperature was 400°C at 12 L/minute flow rate. Nebulizer pressure was 60 psi. Capillary and nozzle voltages were 4,000 and 1,000 V, respectively. The deprotonated ion $(M-H)^-$ was assayed in multiple

reaction monitoring (MRM) mode. The transitions m/z 221/75 and 226/75 were used as quantifiers for EtG and EtG-D5, respectively. The transitions m/z 221/85 and 226/85 were used for confirmation of EtG and EtG-D5, respectively. Fragmentor voltage of EtG and EtG-D5 was 80 V, while collision energy was 6 and 10 V, respectively. Relative abundance of ion transition was considered to be analytically satisfactory at 60 to 80% of the quantifier transition. All data were processed using Agilent MassHunter Workstation Analysis B.04.00.

Method validation

Validation procedures followed guidelines of the US Food and Drug Administration (USFDA)[24], European Medicines Agency (EMA)[25], and Scientific Working Group for Forensic Toxicology (SWGTOX)[26]. The protocol was validated for selectivity/specificity, linearity and range, accuracy, precision, limit of detection (LOD), limit of quantitation (LOQ), matrix effect, recovery, process efficiency, and application.

Blank matrix and fortified matrix preparations

Several of the validation procedures utilized hair from volunteers who had never consumed ethanol. This material was used to create two types of hair samples. According to SWGTOX terminology, the first type was a "blank matrix" preparation, defined as lacking both a target analyte and an internal standard. The second type was a "fortified matrix" preparation, defined as a blank matrix preparation spiked with a target analyte and/or an internal standard[26]. Hair cleaning and extraction procedures were the same as those previously described.

Selectivity/specificity

Selectivity/specificity analysis used three different sets of solutions. Each set comprised nine samples containing 100 mg of blank hair. The nine samples differed by containing blank hair from a different individual. The first set was a blank matrix preparation that contained 700 µL of distilled water and 40 µL of methanol. The second set was a fortified matrix preparation that contained 700 µL of distilled water, 20 µL of the working internal standard solution and 20 µL of methanol. The third set also was a fortified matrix preparation; it consisted of 700 µL of distilled water, 20 µL of the working internal standard solution, and 20 µL of a methanol solution containing 1µg/mL concentrations of each of the 14 compounds under investigation for interference.

Linearity and range

For calculating the calibration curve, the blank hair cuttings of nine individuals were thoroughly mixed to create a composite sample. Linearity was evaluated by intraday analysis of three replicates of three fortified matrix preparations containing 100 mg of the composite blank hair mixture, 700 µL of distilled water, 20 µL of the working internal standard solution, and 20 µL of seven different EtG working solutions at concentrations of 100, 150, 250, 500, 1,000, 1,500, and 2,500 ng/mL. The final EtG concentrations were 20, 30, 50, 100, 200, 300, and 500 pg/mg. Each fortified matrix preparation also was analyzed on five different days. Abundance ratios of EtG and EtG-D5 were plotted against the various concentrations. The correlation coefficient (r) was computed by least-squares regression analysis with required acceptance criterion higher than 0.995. If HPLC-ESI-MS/MS findings yielded EtG concentrations higher than calibration curve values, the sample under investigation was appropriately diluted for reanalysis.

Accuracy and precision

Accuracy and precision were determined by analyzing three sets of fortified matrix preparations. Each set contained 700 µL of distilled water, 20 µL of the working internal standard solution, and 100 mg of the same composite blank hair sample used in calculating the calibration curve. The three sets differed in their amount of EtG, which was added by means of a 20 µL aliquot of an EtG working solution containing 150, 500, or 1,500 ng/mL. Final EtG concentrations were 30, 100, and 300 pg/mg. Intraday analysis used five replicates of each set, while interday analysis used triplicates on five different days. Accuracy expressed as the relative recovery (%RR) was considered acceptable within the range of 85 to 115%. Precision expressed as coefficient of variation (%CV) was considered acceptable within ±15%[24,25].

Limit of detection and limit of quantitation

Limit of detection (LOD) and limit of quantitation (LOQ) were determined by analyzing five replicates of fortified matrix preparations containing 700 µL of distilled water, 20 µL of the working internal standard solution, 100 mg of the composite blank hair sample, and 20 µL of an EtG working solution. Samples were prepared for EtG working solutions containing 25, 50, 75, 100, 125, and 150 ng/mL. Final EtG concentrations were 5, 10, 15,

20, 25, and 30 pg/mg. LOD was defined by the lowest concentration of mass/charge ratio within acceptable limits. LOQ, defined as the lowest analyte concentration that can be reliably calculated with accuracy and precision, was derived from data pertaining to relative recovery and the coefficient of variation. Acceptable deviation was ±20%[24,25].

Matrix effect, recovery, and process efficiency

The determination of matrix effect, recovery, and process efficiency followed procedures described by Matuszewski et al[27]. Analysis used three types of samples (A, B, and C). Samples A and B both contained 100 mg of blank hair collected from the same six individuals and a given amount of EtG. Both samples also were extracted by the addition of 700 µL of distilled water. However, sample A was spiked after extraction by the addition of EtG and the internal standard (EtG-D5), while sample B was spiked before extraction by the addition of EtG and the internal standard. In both cases, the analytes were added by means of a 20 µL aliquot of the internal standard working solution and a 20 µL aliquot of an EtG working solution. Samples were prepared for EtG working solutions containing 150, 500, and 1,500 ng/mL. Sample C was prepared in 700 µL of distilled water with the same analyte concentrations as samples A and B, but it did not contain any hair. Matrix effect (ME), recovery (R), and process efficiency (PE) were calculated by abundance ratio of EtG/EtG-D5 according to the following equations: ME = A/C x100; R = B/A x100; and PE = B/C x100.

Application

Although USFDA, EMA, and SWGTOX guidelines do not specifically discuss the matter, the validation of an analytical protocol often includes a study that applies the procedure to a field sample in order to test the method under "real life" conditions. Our field sample comprised 120 subjects who reported consuming alcohol during a 3-month period prior to enrollment. Forty subjects were patients in alcohol withdrawal programs at Chiang Mai Thanyarak Hospital, an addiction treatment center, or Maharaj Nakorn Chiang Mai Hospital. Eighty subjects were volunteers recruited by one of the authors through collegial and kinship networks. Ranging in age from 19 to 63 years, the sample consisted of 86 males and 34 females. Participation required all subjects to provide an appropriate hair sample and to answer the Thai-language version of AUDIT.

AUDIT was administered to all subjects by the same researcher, who explained the meaning of test questions to those subjects who requested clarification. Briefly, AUDIT consists of 10 questions concerning the nature and consequences of an individual's alcohol consumption[2]. Although two questions deal with consequences that may have occurred "during the last year", the other eight questions focus on the recent past by requesting subjects to describe "daily", "weekly", or "monthly" behavior. Depending on the question, the subject is offered three to five possible responses that are graded on a scale of 0 to 4, with a total possible score of 40. Although AUDIT responses may be interpreted in a variety of ways, the AUDIT manual identifies various "risk levels" for chronic excessive alcohol consumption that may warrant professional intervention, ranging from "alcohol education" to "brief counseling" to "referral to specialist for diagnostic evaluation and treatment". For the purposes of our application study, we adopted AUDIT terminology and scoring to identify three types of drinking behavior: "low risk" (0 to 7), "harmful/hazardous" (8 to 19), and "alcohol-dependent" (20 to 40). We then correlated EtG concentrations with AUDIT test scores to determine whether our protocol could discriminate between AUDIT-defined risks levels of drinking behavior.

Statistical analysis

All data were analyzed by using SPSS software v.17 (IBM, Armonk, NY, USA). EtG concentrations in hair between groups were compared using non-parametric one-way analysis of variance; the Kruskal-Wallis test was considered statistically significant at p-value <0.05.

Results

Under the protocol's HPLC-ESI-MS/MS conditions, a retention time of approximately 6.1 minutes (Fig. 1) was recorded for quantifier and qualifier transitions of EtG (m/z 221/75 and 221/85) and EtG-D5 (m/z 226/75 and 226/85). There was no peak interferences from any of the 14 commonly abused drugs that were investigated as part of the selectivity/specificity analysis. Nor were there interferences from the blank matrix or from the internal standard. The calibration curve of EtG was linear between 20 and 500 pg/mg of hair (Fig. 2), with the correlation coefficient (r) for both intraday and interday analysis at 0.999±0.001. Intraday and interday analysis of the protocol's accuracy (% of relative

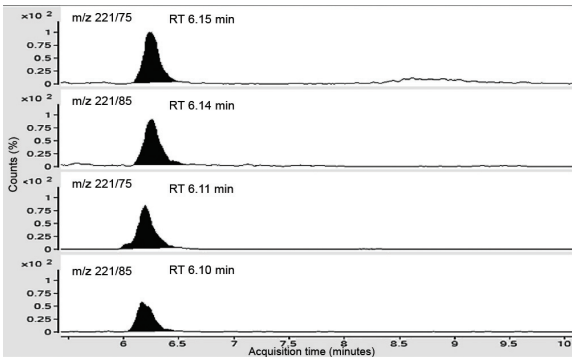

Fig. 1 Chromatograms of 200 pg/mg of EtG (m/z 221/75, 221/85) and 100 pg/mg EtG-D5 (m/z 226/75, 226/85) spiked in blank hair. Retention time of EtG was 6.1 minutes and EtG-D5 was 6.1 minutes. The relative abundance of m/z of EtG was 80% and EtG-D5 was 76%).

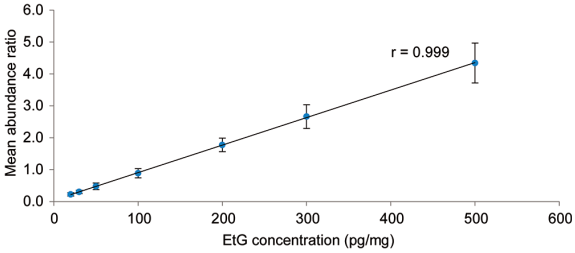

Fig. 2 The calibration curve of EtG concentration in hair plotted against mean abundance ratio of EtG and EtG-D5 were linearity (r = 0.999) over a wide range (20-500 pg/mg) (n = 9).

recovery) and precision (% of coefficient of variation) were shown in Table 1. Five concentrations of EtG (5, 10, 15, 20, and 30 pg/mg of hair) were used to determine the LOD and LOQ. EtG at 15 pg/mg was the lowest concentration to yield quantifier and qualifier transitions (m/z 221/75 and 221/85) with acceptable abundance ratio. Accuracy and precision were 90.19% and 15.65%, respectively. Therefore, the protocol's LOD and LOQ were set at 15 pg/mg. The investigation of co-eluting matrix components permitted the calculation of mean values of matrix effect. These values, along with data on recovery and process efficiency, are presented in Table 2.

At the protocol's cutoff of 15 pg/mg, the application study was not able to detect EtG in the hair of any of the AUDIT-defined low-risk group of drinkers (n = 38). The EtG detection rates for the hazardous/harmful group (n = 42) and alcohol-dependent group (n = 40) were 14.3% and 85%, respectively. These data, as well as median of EtG concentrations for the three groups, are presented in Table 3. Using a non-parametric Kruskal-Wallis test, the median (IQR) of the three groups were significantly different. With 95% CI, there was no overlapping of mean EtG concentrations between the hazardous/harmful group and the alcohol-dependent group (Fig. 3).

Discussion

Validation results

Chromatograms of EtG and EtG-D5 presented clearly delineated peaks with consistent retention

Table 1. Accuracy and precision of analysis of EtG in hair

Nominal EtG concentration (pg/mg)	Intraday (n = 5)			Interday (n = 15)		
	Detected concentration (mean ± SD)	Accuracy (%RR)	Precision (%CV)	Detected concentration (mean ± SD)	Accuracy (%RR)	Precision (%CV)
30	32.7±1.1	109.1	3.5	31.7±1.9	105.6	5.9
100	102.8±1.9	102.8	1.9	100.4±4.4	100.4	4.4
300	310.7±4.4	103.6	1.4	303.6±2.4	101.2	0.8

EtG = ethyl glucuronide; %RR = % relative recovery; %CV = % coefficient of variation; SD = standard deviation

Table 2. Matrix effect, recovery, and process efficiency of EtG analysis in hair (n = 6)

Nominal EtG concentration (pg/mg)	Matrix effect (%)		Recovery (%)		Process efficiency (%)	
	Mean ± SD	%CV	Mean ± SD	%CV	Mean ± SD	%CV
30	92.9±14.0	15.0	92.5±5.6	6.1	85.4±9.5	11.2
100	88.5±6.7	7.6	102.1±12.4	12.1	90.0±8.8	9.8
300	93.2±12.5	13.5	93.9±12.7	13.5	86.5±8.4	9.7

%CV = % coefficient of variation; SD = standard deviation

Table 3. EtG concentration in hair samples of 120 alcohol-consuming subjects

Groups	AUDIT score	n	Rate of detection (%)	EtG concentrations (pg/mg), median* (IQR)
Low-risk drinkers	1-7	38	0	0
Hazardous/harmful drinkers	8-19	42	14.3	37.1 (17.1-378.1)
Alcohol-dependent drinkers	20-40	40	85.0	106.6 (62.9-211.4)

AUDIT = Alcohol Use Disorders Identification Test; IQR = interquartile range
* Kruskal-Wallis test, $p<0.001$

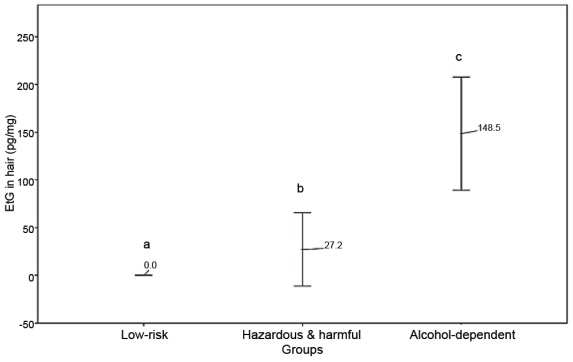

Fig. 3 Comparison of 95% CI of mean EtG concentrations in hair for low-risk, hazardous/harmful and alcohol-dependent groups. There was no overlapping between groups.

times and appropriate quantifier/qualifier transitions. There were no interferences. Therefore, the protocol demonstrated good selectivity and specificity. Data for accuracy, precision, and LOD/LOQ satisfied international standards in terms of acceptable variation, while the calibration curve for EtG quantitation was linear (r = 0.999) over a wide range (20 to 500 pg/mg). In LC-MS/MS analysis, unsuspected and unidentified substances in the sample sometimes co-elute with target analytes so as to increase or decrease ionization resulting in distortion of analytical results. The calculation of this "matrix effect" is commonly expressed in percentages with ionization enhancement or suppression measured, respectively, by values above or below 100%. In our protocol, the matrix effect was 88.5 to 93.2%, which indicated some degree of ionization suppression. In addition, the presence of an internal standard in combination with the analyte may affect analyte extraction and subsequent measurement or "recovery" of EtG. Recovery was 92.5 to 102%, which indicated that the protocol's extraction procedure did not affect analysis. Finally, the study's "process efficiency" rating of 85.4 to 90% showed that matrix effect did not significantly influence overall performance of the protocol. These various findings demonstrated that our method for analyzing EtG in hair had been successfully validated according to international standards[24-26].

Application

Quantitative biomarker analysis of alcohol consumption faces a linguistic obstacle resulting from imprecise and ambiguous terminology used to describe types of drinking behavior. There is agreement that adjectives such as "light," "social," "low-risk," "occasional," "moderate," "hazardous," "harmful," "non-moderate," "heavy," "alcohol-dependent," and "chronic excessive" describe a generally ascending scale of alcohol consumption and injurious health consequences, but many of these terms are used synonymously and often with considerable overlapping. In terms of EtG analysis, SoHT attempted to introduce greater linguistic precision by proposing two guidelines. First, it adopted the World Health Organization's definition of "chronic excessive" alcohol drinking as "an average consumption of 60 g of pure ethanol per day over several months." Second, it demarcated this highly injurious level of drinking by an EtG concentration level greater than 30 pg/mg in proximal scalp hair samples of at least 3 cm in length[12]. A subsequent meta-analysis of EtG hair-detection studies supported SoHT's 30 pg/mg cutoff as "a promising threshold value for limiting the rate of false negatives when differentiating heavy from social drinkers"[15]. Nevertheless, there is continuing debate concerning the appropriate EtG cutoff value for chronic excessive drinking as defined by ethanol daily intake (EDI) greater than 60 g. Recently, Kharbouche et al[28] recommended a cutoff of 25 pg/mg, while Shi et al[17] proposed a cutoff as low as 20 pg/mg. If one of these lower values were to be adopted, our protocol's LOQ of 15 pg/mg would have ample sensitivity to identify chronic excessive alcohol consumption. However, our protocol cannot be used for the monitoring of alcohol abstinence. For such assessment, SoHT recommends considerably lower LOQ values in the range of less than 3 pg/mg[13].

Scalp hair typically grows at approximately 1 cm per month[16]. In recommending that EtG analysis use a proximal hair sample at least 3 cm in length, SoHT indicated that detection studies based upon self-report should use a procedure that elicited information concerning drinking behavior for at least the prior three months. AUDIT satisfies this requirement. However, our use of AUDIT-defined drinking behavior in conjunction with LC-MS/MS hair detection of EtG departed from general practice. Most prior research correlated EtG concentrations in hair with retrospective self-reported EDI. AUDIT scores cannot be directly translated into EDI. However, the 30 pg/mg cutoff appears to be a serviceable value for distinguishing the two groups in our study for which EtG concentrations were detected. The median (IQR) of EtG concentrations for the medium-risk group (AUDIT scores 8 to 19) and the high-risk group (AUDIT scores 20 to 40) were, respectively, 37.1 (17.1-378.1) pg/mg and 106.6 (62.9-211.4) pg/mg (Table 3). As correlated with our findings of EtG concentration, AUDIT scores greater than 20 are useful predictors of "chronic excessive" drinking. Additional substantiation is provided by data in Table 4, which compares the sensitivity and specificity of AUDIT scores greater than 20 to the SoHT EtG cutoff for chronic excessive drinking. Both sensitivity and specificity were above 90%. All subjects in our study (n = 40) who had AUDIT scores greater than 20 were already in alcohol withdrawal programs.

As Boscolo-Berto et al had noted, prior studies of EtG hair detection using methods with sufficiently sensitive LOQ values offered numerous instances of downward and upward trespass for reported EDI-defined levels of drinking[15]. In our study, 15% (6/40) of the alcohol-dependent subjects (AUDIT scores 20 to 40) tested negative for EtG, while 50% (3/6) of the medium-risk subjects (AUDIT scores 8 to 19) had EtG concentrations exceeding 30 pg/mg, which would place them in the SoHT category of chronic excessive consumption. Indeed, one of these medium-risk subjects had the highest EtG concentration (796.7 pg/mg) in the present study, despite a relatively low AUDIT score of 8. The customary explanations for such overlapping results are individual metabolic variability, unreliable self-reporting, and cosmetic hair treatment, such as shampooing, dyeing, and bleaching[15,19]. Because of these variables, it is generally acknowledged that EtG hair test results should not be evaluated in isolation. They should be interpreted in combination with clinical diagnosis and, when possible, with test results of other suitable biomarkers[7,12,14,15,29].

To our knowledge, only one other study has attempted to correlate AUDIT scores with LC-MS/MS hair testing of EtG[30]. In a study of 103 pregnant Swedish women, Wurst et al found that none of the subjects had an AUDIT score greater than 5, which would place them in our "low-risk" (1 to 7) category. However, 11.6% (12/103) had EtG concentrations ranging from 7 to 25 pg/mg. Since the LOD/LOQ of our protocol was not sensitive enough to detect/measure EtG in our low-risk sample, it is not possible to compare results. However, the Swedish participants who approximated or equaled an EtG value of 25 pg/mg approached the 27.2 pg/mg mean concentration that separated our hazardous/harmful (AUDIT scores 8 to 19) drinkers from alcohol-dependent drinkers (AUDIT scores 20 to 40). The Swedish data suggest upward trespass between AUDIT-defined risk levels, possibly because the study's pregnant women under-reported an activity that is well known in European countries to be harmful to a fetus. In a wider sense, the possibility of trespass of AUDIT-defined risk levels as correlated with EtG detection is built into the AUDIT questionnaire. AUDIT scores measure more than EDI in assessing risk levels. For example, it is possible for

Table 4. Sensitivity and specificity of AUDIT scores compared to EtG in hair

AUDIT Scores	EtG in hair (pg/mg)			
	≥30 pg/mg	<30 pg/mg	Total (n)	
20-40 (alcohol dependent)	32	8	40	PPV = 80.0% (95% CI = 64.3-91.0%)
1-19 (non-alcohol dependent)	3	77	80	NPV = 96.3% (95% CI = 89.4-99.2%)
Total	35	85	120	
	Sensitivity = 91.4% (95% CI = 76.9-98.2%)	Specificity = 90.6% (95% CI = 82.3-95.9%)		

PPV = positive predictive value; NPV = negative predictive value; CI = confidence interval

subjects who drink only twice a month to have an AUDIT score greater than 20, if on those occasions they consumed a lot of alcohol resulting in various harmful personal and social consequences. In AUDIT terms, they would qualify as "alcohol-dependent" drinkers, although their EtG concentrations in a 3 cm proximal hair segment might fall below the SoHT cutoff for chronic excessive alcohol consumption. In our study, none of the six alcohol-dependent subjects who tested negative for EtG fell into this category, but it is possible that frequent shampooing of hair affected their analytical results. As is true for EtG detection itself, AUDIT scores often are best interpreted in combination with clinical counseling that examines a variety of life-style issues[2,30].

Conclusion

The method for determining EtG level in hair by HPLC-ESI-MS/MS was validated according to international standards, with LOD/LOQ values of 15 pg/mg of hair. The calibration curve was linear from 20 to 500 pg/mg (r = 0.999). Accuracy and precision of analysis were within 15%, and there was no significant matrix effect. This protocol was used to measure EtG in the hair of 120 subjects who reported consuming alcohol within the prior three months. After taking the AUDIT, subjects were categorized by test scores into three ascending risk levels for chronic excessive alcohol use. As defined by AUDIT guidelines and terminology, these levels and corresponding scores were "low-risk" (1 to 7) "hazardous/harmful" (8 to 19) and "alcohol-dependent" (20 to 40). No EtG was detected in the hair of the low-risk group. EtG detection rates for the hazardous/harmful and alcohol-dependent groups were 14.3% and 85%, respectively. The mean (95% CI) of the hazardous/harmful group was 27.2 (-12 to 66.8) pg/mg, while the mean (95% CI) of the alcohol-dependent group was 148.5 (87 to 209.6) pg/mg. With 95% CI, there was no overlapping of mean EtG concentrations between these two groups. These results indicated that our protocol is a useful tool for evaluating risk for chronic excessive drinking as defined by AUDIT scores. It strongly predicted the highest level of risk (alcohol-dependent drinking), although it was inadequate for assessing lower levels of risk. In our study, the sensitivity and specificity of AUDIT scores greater than 20 were above 90% compared to 30 pg of EtG per mg of hair. These data supported AUDIT's diagnostic value in predicting chronic excessive alcohol consumption, as defined by the SoHT.

What is already known on this topic?

EtG is a minor metabolite of ethanol. Because the EtG molecule contains an unchanged ethyl group, it is a highly specific biomarker of alcohol consumption. For purposes of evaluating alcohol abuse, protocols for the detection and quantification of EtG in hair have been validated in many countries using a variety of instrumentation. HPLC-ESI-MS/MS is a favored technique.

What this study adds?

This study is the first to develop and validate an HPLC-ESI-MS/MS protocol for EtG hair analysis for use in Thailand. It is also the first study to attempt to assess levels of risk for alcohol abuse among a sizeable population of self-reported alcohol drinkers by correlating EtG hair concentrations with the results of AUDIT, a widely used clinical screening procedure.

Acknowledgement

The present project received financial support from the Faculty of Medicine of Chiang Mai University and the Center for Alcohol Studies. The authors would like to express their sincere thanks to the directors and staff of the Chiang Mai Thanyarak Hospital and Maharaj Nakorn Chiang Mai Hospital. We also would like to thank Mrs. Rochana Phuackchantuck from the Research Department of the Faculty of Medicine, Chiang Mai University, and Professor Timothy O'Brien from the Department of Mathematics and Statistics, Loyola University, Chicago, USA, for their advice concerning statistics. We also appreciate the assistance of Jeffrey Hess, who served as English language writer/editor. Our greatest debt, however, is to the subjects who participated in the present study.

Potential conflicts of interest

None.

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การพัฒนาการตรวจ EtG ในเส้นผม ด้วยวิธี HPLC-ESI-MS/MS ในประเทศไทย เพื่อประเมินการบริโภคแอลกอฮอล์แบบเรื้อรังร่วมกับการประเมินโดย AUDIT

ทิวาพร ทนันชัย, อนงพันธ์ จันทร์กุย, พันธุ์นภา กิตติรัตนไพบูลย์, พงษ์รักษ์ ศรีบัณฑิตมงคล

ภูมิหลัง: *Ethyl glucuronide (EtG) เป็นเมตาบอไลท์ของเอทานอล ที่ได้รับความสนใจเพื่อการตรวจวิเคราะห์ในเส้นผมของผู้ที่บริโภคแอลกอฮอล์ (เอทานอล) แบบเรื้อรังโดยวิธีการวิเคราะห์ใช้ลิควิดโครมาโตกราฟีแทนแดมแมสสเปคโตรเมตรีและแหล่งกำเนิดไอออนแบบอิเลคโตรสเปรย์ (HPLC-ESI-MS/MS) ทั้งนี้การวิเคราะห์ EtG ในเส้นผมยังไม่มีการศึกษาและนำมาใช้ในประเทศไทย*

วัตถุประสงค์: *เพื่อทดสอบความถูกต้องของวิธีวิเคราะห์ EtG ในเส้นผม โดยใช้ HPLC-ESI-MS/MS และนำไปตรวจวิเคราะห์ในกลุ่มตัวอย่างที่มีการบริโภคเครื่องดื่มแอลกอฮอล์ในระดับความเสี่ยงจากการบริโภคแอลกอฮอล์ที่ประเมินโดยแบบประเมินปัญหาการดื่มสุรา (AUDIT)*

วัสดุและวิธีการ: *การทดสอบความถูกต้องของวิธีวิเคราะห์ยึดตามแนวปฏิบัติของ US Food and Drug Administration, European Medicines Agency และ Scientific Working Group for Forensic Toxicology การศึกษาครั้งนี้ใช้กลุ่มตัวอย่างจำนวน 120 คน ซึ่งเป็นบุคคลที่มีประวัติการบริโภคเครื่องดื่มแอลกอฮอล์ในระยะเวลา 3 เดือนย้อนหลัง ก่อนที่จะเข้าสู่โครงการศึกษา โดยกลุ่มตัวอย่างทั้งหมดได้ตอบแบบประเมินปัญหาการดื่มสุรา (AUDIT) เพื่อทำการคัดแยกระดับความเสี่ยงจากการบริโภคเครื่องดื่มแอลกอฮอล์ตามความเสี่ยงระดับต่ำ ความเสี่ยงระดับปานกลาง ไปจนถึงความเสี่ยงระดับสูงสำหรับการบริโภคแอลกอฮอล์แบบเรื้อรัง*

ผลการศึกษา: *การทดสอบความถูกต้องของวิธีวิเคราะห์พบว่าความจำเพาะ ความแม่น ความเที่ยง และความเป็นเส้นตรงได้ผลตามมาตรฐานสากล ส่วนประกอบเส้นผมไม่ส่งผลกระทบต่อกระบวนการวิเคราะห์อย่างมีนัยสำคัญ ความเข้มข้นต่ำสุดที่สามารถวิเคราะห์ได้และความเข้มข้นต่ำสุดที่สามารถบอกปริมาณได้ มีความเข้มข้นที่ 15 พิโคกรัมต่อมิลลิกรัมเส้นผม ในการศึกษานี้ตรวจไม่พบ EtG ในเส้นผมของกลุ่มตัวอย่างความเสี่ยงต่ำ (จำนวน 38 ตัวอย่าง) แต่กลุ่มความเสี่ยงปานกลาง (จำนวน 42 ตัวอย่าง) และกลุ่มความเสี่ยงสูง (จำนวน 40 ตัวอย่าง) สามารถตรวจพบ EtG ในเส้นผม คิดเป็นร้อยละ 14.3 และร้อยละ 85 ตามลำดับ โดยระดับความเข้มข้น EtG ในเส้นผมของทั้งสองกลุ่มมีความแตกต่างกันอย่างมีนัยสำคัญ ค่าความไวและความจำเพาะมีค่ามากกว่าร้อยละ 90 เมื่อเปรียบเทียบระหว่างกลุ่มตัวอย่างความเสี่ยงสูงจากการบริโภคแอลกอฮอล์กับความเข้มข้น EtG ในเส้นผมตั้งแต่ 30 พิโคกรัมต่อมิลลิกรัมเส้นผมขึ้นไป ซึ่ง SoHT กำหนดเพื่อวินิจฉัยบุคคลที่มีการบริโภคเครื่องดื่มแอลกอฮอล์แบบเรื้อรังโดยพิจารณาจากการบริโภคเครื่องดื่มแอลกอฮอล์เฉลี่ยมากกว่า 60 กรัมต่อวัน*

สรุป: *วิธีการวิเคราะห์ EtG ในเส้นผมที่ได้รับการทดสอบความถูกต้องของวิธีนี้ สามารถใช้ประเมินบุคคลที่บริโภคเครื่องดื่มแอลกอฮอล์ที่มีความเสี่ยงสูงที่ได้รับการแบ่งกลุ่มจากคะแนนของแบบประเมินปัญหาการดื่มสุรา (AUDIT) แต่วิธีการวิเคราะห์นี้ยังไม่เหมาะสมสำหรับการประเมินบุคคลที่อยู่ในกลุ่มความเสี่ยงต่ำ ทั้งนี้การศึกษาครั้งนี้ให้ผลสนับสนุนการวินิจฉัยผู้ที่บริโภคเครื่องดื่มแอลกอฮอล์แบบเรื้อรังที่กำหนดโดยแบบประเมินปัญหาการดื่มสุรา (AUDIT) และ SoHT*