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Deposition of ethyl glucuronide in WHP rat hair after chronic ethanol intake

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Abstract:
Background: This study investigated the relationship between ethanol intake in rats and the resulting level of ethyl glucuronide (EtG) in rat hair.
Methods: Rats (n = 50) consumed a 10% ethanol solution for 4 weeks, then EtG was extracted from samples of their hair using a novel extraction procedure involving freezing and thawing. The EtG concentration was measured using gas chromatography and mass spectrometry. The animals voluntarily drank ethanol, with daily consumption in most rats exceeding 5 g/kg b.w. The silylated EtG was stable for at least 28 h. The limit of detection was 0.03 ng/mg, and the limit of quantification was 0.1 ng/mg.
Results: Hair samples from rats that consumed ethanol had EtG levels ranging from 0.17–20.72 ng/mg in female rats and 0.15–13.72 ng/mg in males. There was a correlation between the amount of alcohol consumed and the EtG levels in hair from female ($p < 0.01$), but not male, rats.
Conclusion: The method presented allows detection and quantification of EtG in rat hair. We also observed differences in EtG deposition in male and female rats.

Key words:
alcohol abuse, ethyl glucuronide, rat hair, WHP rats, gas chromatography-mass spectrometry

Abbreviations: AUC – area under the curve, EI – electron impact, EtG – ethyl glucuronide, ICR – index of the incorporation rate, MG – methyl glucuronide, TMS – trimethylsilyl, WHP – Warsaw high-preferring

Introduction

Alcohol abuse is a serious problem worldwide, and suitable markers are needed that allow physicians and researchers to identify individuals with elevated alcohol consumption. Many such markers have been investigated in blood, urine and hair. Ethyl glucuronide (EtG) is a direct metabolite of ethanol that can be detected in hair for an extended period of time after complete elimination of ethanol from the body [9, 29, 34].

EtG is deposited in human hair and is detectable following heavy alcohol consumption. It has thus been proposed as a marker of alcohol abuse [27, 35]. There has been growing interest in this marker in the last few years, and several studies have been conducted to determine the correlation between ethanol intake and the EtG levels in hair. Specifically, EtG concentrations in human hair have been correlated

with self-reported alcohol (ethanol) consumption information [3, 13, 22]. However, precise data regarding reproducible dosing of ethanol and the corresponding EtG levels has not been obtained in experimental animals.

Rat hair is used to study the deposition of xenobiotics, particularly drugs of abuse such as methamphetamine [20], ephedrine [19] and methylenedioxyamphetamines [16]. To clarify the mechanisms underlying drug incorporation into hair, deposition of 3,4-methylenedioxyamphetamines and their metabolites has been investigated in rats. The results confirmed that hair samples can be used to confirm previous methylenedioxyamphetamine use. The disposition of these drugs into rat hair from rat plasma was investigated by comparison between concentration in hair and areas under the concentration *vs.* time curves (AUCs) in plasma that represented an index of the incorporation rate (ICR) of the drug into hair [16].

To study the disposition of dimethylamphetamine (DMAP) and its metabolites, from plasma to rat hair, a method for simultaneous determination of these compounds in biological samples using GC/MS with selected ion monitoring was developed. After intraperitoneal administration of DMAP HCl to pigmented hairy rats, the concentrations of DMAP and its metabolites were measured in rat urine, plasma and hair. The tendency of DMAP N-oxide to be incorporated into hair was low compared to the other compounds [15].

Determination of three benzodiazepines (alprazolam, estazolam and midazolam) and their metabolites in rat hair roots and plasma was also carried out. The concentrations of the parent drug and the metabolites in hair were mainly dependent on their plasma levels [31]. Incorporation of the main metabolites of toluene and xylenes from thinners (o-, m-, p-methylhippuric acid and hippuric acid) into rat hair has also been studied. The metabolite concentration in the hair of exposed rats changed according to exposure concentration. The mean concentration was 7.6 ng/mg for hippuric acid and 13.8, 10.1 and 9.2 ng/mg hair for o-, m-, and p-methylhippuric acid, respectively. These results indicated that the metabolite concentration in hair reflects exposure to thinner [25].

The observation that ethanol is excreted by rats as ethyl β-D-glucosiduronic acid was made by Jaakonmaki et al. [10]. In that study, young adult male and female rats were given 6 g/kg of ethanol orally as a 50% aqueous solution. EtG was isolated from the rat urine and identified by gas-liquid chromatography (GLC) and the associated technique, GLC/MS.

The practical use of EtG in rat hair has not been fully explored, although it is known that alcohol administration results in EtG incorporation into hair. Rats provide a useful animal model as they can be given known amounts of alcohol over a relatively long time period. In this study, we developed a new method to extract and evaluate the EtG concentration in rat hair.

Materials and Methods

Animal experiments

Hair samples were collected from Warsaw high-preferring (WHP) rats weighing 340–380 g. This line is selectively bred from Wistar rats for voluntary alcohol consumption. The WHP rats were obtained from Department of Pharmacology and Physiology of the Nervous System, Institute of Psychiatry and Neurology, Warszawa, Poland. A total of 25 female and 25 male adult rats were used in this study.

Rats were maintained in individual cages in a temperature-controlled room with a 12 h light/12 h dark cycle. They were first given single-bottle access to 10% ethanol for 1 week (water was not available, but food was available *ad libitum*). Then, a two-bottle preference choice (10% ethanol *vs.* water) was given over 3 weeks; food remained *ad libitum* [4, 5]. The estimated ethanol daily intakes ranged from 0.07 g/kg/day EtOH to 13.8 g/kg/day EtOH for females and from 0.24 g/kg/day EtOH to 12.1 g/kg/day EtOH for males.

Hair samples were collected as close as possible to the skin from the back right side (the same place on each animal) using an electric shaver before the experiment and on the seventh day after the last intake. The hair samples were stored at room temperature until analysis.

EtG-free rat hairs were obtained from rats which were not given ethanol.

The study protocol was approved by the II Local Ethics Commission at the Medical University of Warsaw, Poland (37/2009).

Materials

The following chemical reagents were used in this study: ethyl glucuronide (EtG) and methyl glucuronide (MG) standards [both from Medichem (Germany)], GC/MS-grade ethyl acetate (Sigma), acetone (POCH SA), methanol (LA B-SCAN) and N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA; Sigma-Aldrich).

Analytical procedures

EtG was determined using a GC/MS apparatus (GCMS-QP2010 Plus, Shimadzu), equipped with a ZB-5MSi column (length, 30 m; inner diameter, 0.25 mm; film thickness, 0.25 µm; Zebron, Phenomenex). The injector and GC/MS interface temperatures were 270°C, and the ion source temperature was 250°C. The oven temperature was held at 100°C for 10 min, then increased to 270°C at a rate of 10°C/min, then maintained at 270°C for 5 min. Helium was used as a carrier gas with a flow rate of 1 ml/min. The mass selective detector was operated in electron impact (EI) mode with electron energy of 70 eV. In these conditions the retention time of silylated EtG was 10.82 min and 10.44 min for silylated MG. The following ions were monitored: 261, 292 and 405 for EtG and 261, 292 and 391 for MG, the underlined ones being used for quantification. With 261 and 391 ions, the biological background interference was negligible. Methyl glucuronide was chosen as the internal standard according to Skopp et al. [28]. This compound was not found in any investigated hair sample.

Optimization of the analytical method

Several procedures were tested to optimize extraction of EtG from samples of rat hair. To make comparison possible, all samples consisted of rat hair from a single animal. Hairs were washed and cut into small pieces. Then, 50 µl of a 10 µg/ml aqueous solution of internal standard (MG) and distilled water (350 µl) were added to 30 mg of the hair. For method A, the sample was incubated for 24 h at room temperature; for method B, the sample was sonicated for 1 h at room temperature; for method C, the sample was frozen and thawed twice; for method D, the sample was frozen and thawed twice, then incubated at room temperature for 24 h; for method E, the sample was frozen and thawed twice, then sonicated for 1 h at room temperature; for method F, the sample was sonicated for 1 h at room temperature, then incubated for 24 h at room temperature (Tab. 1).

Sample preparation and EtG extraction procedure

The rat hair samples were washed with water and acetone. After drying, the hairs were cut into small pieces. Then, 50 µl of a 10 µg/ml aqueous solution of internal standard (MG) and distilled water (350 µl) were added to 30 mg of the hair and the samples were frozen and thawed twice. After incubation for 24 h at room temperature, the samples were centrifuged at 10,000 rpm for 10 min. The supernatant was evaporated to dryness under a stream of nitrogen at 70°C using a heated metal block. Anhydrous ethanol (100 µl) was added and evaporated to dryness. The residue was derivatized with 50 µl MSTFA and 50 µl ethyl acetate (60 min, 80°C) and 1 µl was injected into a GC/MS system for analysis.

Statistical analysis

For statistical analysis, Levene's test for homogeneity of variance, analysis of variance (ANOVA), and Newman-Keuls *post-hoc* test were used to determine the best extraction procedure. Spearman's correlation coefficient was used to correlate the amount of alcohol consumed with the EtG concentration. Analyses were performed using Statistica 9 software (StatSoft, Poland).

Results

The efficiency of extraction procedures is presented in Table 1. The best results were achieved with procedure D. ANOVA revealed significant differences between the procedures in terms of how much EtG was extracted [$F(5, 48) = 20.96$; $p < 0.0001$]. The Newman-Keuls *post-hoc* test showed that procedure D had a significantly higher effect ($p < 0.001$) and procedure A had a significantly less effect ($p < 0.001$). Extraction using 1, 2, and 3 freeze/thaw cycles was also tested. The most EtG was extracted using 2 freeze/thaw cycles, so 2 cycles were routinely used for extraction [$F(2, 15) = 4.73$, $p = 0.0256$].

Tab. 1. Efficiency of EtG extraction from rat hair samples. Analysis of variance (ANOVA) revealed significant differences between the procedures [F (5, 48) = 20.96; p < 0.0001]. The Newman-Keuls *post-hoc* test showed that procedure D had a significantly higher effect on EtG extraction from samples of rat hair (p < 0.001) and procedure A had a significantly less effect (p < 0.001). SD – standard deviation, RSD – relative standard deviation

Extraction method	EtG ng/mg Mean ± SD (n = 9)	RSD (%)
A. 24 h incubation	9.03 ± 0.66	7.30
B. 1 h sonication	10.00 ± 0.28	2.81
C. Two freezing/thawing cycles	10.48 ± 0.55	5.29
D. Two freezing/thawing cycles and 24 h incubation	11.61 ± 0.69	5.94
E. Two freezing/thawing cycles and 1 h sonication	10.54 ± 0.41	3.92
F. 1 h sonication and 24 h incubation	9.92 ± 0.64	6.47

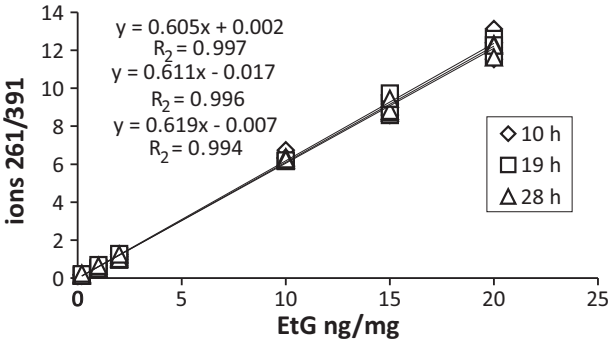

Fig. 1. Stability of the TMS derivative of ethyl glucuronide after 10, 19 and 28 h. The differences between three regression lines were insignificant: F (2, 48) = 0.544, p = 0.583 for the slopes and F (2, 50) = 0.541, p = 0.584 for the elevations [36]

Method validation results are presented in Table 2. The calibration curve consisted of six points, ranging from 0.1 to 20 ng of EtG/mg of rat hair. The curve was established by adding EtG standards to methanol solutions and 0.5 μg of MG (as an internal standard) to samples of EtG-free rat hairs, and then using procedure D. The linear relationship obtained, $y = 0.605x + 0.0024$, $R^2 = 0.998$, showed good proportionality between the GC/MS data and the studied range of EtG concentrations. The limits of detection and quantification were calculated to be 0.03 ng/mg and 0.10 ng/mg, respectively.

The stability of the trimethylsilyl (TMS) derivative of EtG was assessed by comparing the values of six concentrations 10, 19, and 28 h after the derivatization process. The three regression functions were compared statistically (Fig. 1) and no significant differences were found [36]. These results show that the TMS derivatized product was stable for at least 28 h at room temperature.

EtG concentration in rat hair

The mean EtG concentration in rat hair was calculated to be 3.89 ng/mg (range: 0.17–20.72 ng/mg) for females and 2.18 ng/mg (range: 0.15–13.72 ng/mg) for males. For female rats, plotting the EtG hair concentration *vs.* daily ethanol intake showed a correlation (Spearman's $r_s = 0.7049$, $p = 0.000083$; Fig. 2), but there was no correlation between the amount of ethanol consumed and the EtG concentration in hair from male rats (Spearman's $r_s = 0.2251$, $p = 0.2792$, Fig. 3).

Tab. 2. Determination of ethyl glucuronide (EtG) in rat hair samples. The intra-day precision was determined by analyzing, on the same day and in the same conditions, three aliquots of each type of sample. Inter-day precision data were obtained from analyses of nine replicates of the samples performed on three different days

	Intra-day precision RSD (%) (n = 3)	Inter-assay precision RSD (%) (n = 9)	Accuracy (%)	Recovery (%)
EtG in soaked rat hair samples				
2 ng/mg	6.28	8.52	92.84	98.04
20 ng/mg	7.48	3.08	98.68	97.27
EtG in rat hair samples				
8.81 ng/mg	4.83	5.28		

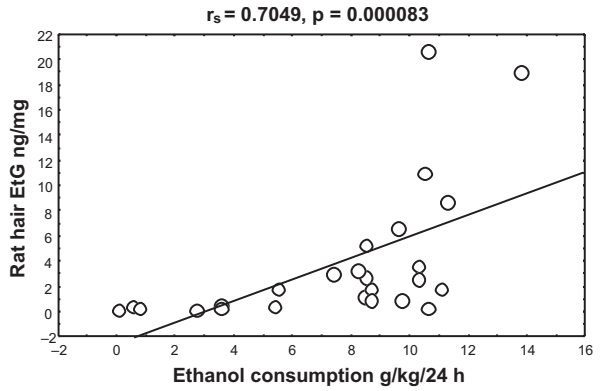

Fig. 2. Ethyl glucuronide (EtG) concentration in hair from female rats *vs.* daily ethanol consumption (n = 25)

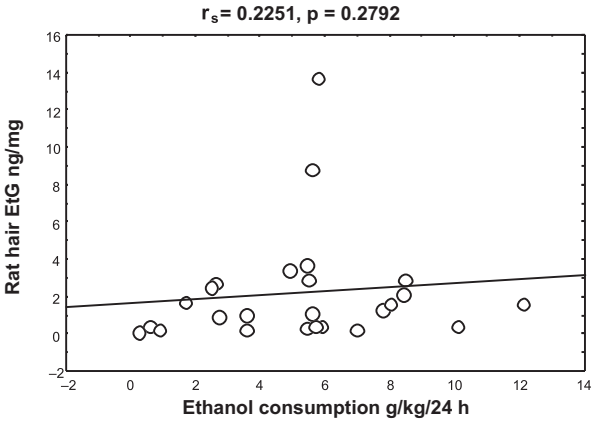

Fig. 3. Ethyl glucuronide (EtG) concentration in hair from male rats *vs.* daily ethanol consumption (n = 25)

Discussion

Extraction procedure

Quantitation of compounds in human and animal hair requires extraction of the chemicals from the hair matrix. It is possible to achieve this by dissolving the hair; however, dissolving the hair releases a large number of compounds that interfere with the analytical process. The extraction method most frequently reported in published studies is sonication for as long as 2 h. Sometimes samples are also subjected to maceration and incubation periods of up to 24 h [1, 12, 18]. Our method of extracting EtG from hair *via* 2 freezing/thawing cycles rather than sonication, has not been used previously.

It was not possible to evaluate the extraction methods by simply adding an external standard to the hair sample. Instead, we tested the extraction procedures by analyzing a homogenous pool of hair containing an unknown amount of EtG (Tab. 1). Incubating the sample after 2 freezing/thawing cycles allowed for a relatively selective isolation of the investigated substances.

Derivatization

The conditions used for derivatizing EtG are very important in order to obtain a stable derivative that will facilitate automation of the sample dosing process. Silylation is the most widely used derivatization procedure for sample analysis by GC/MS. EtG derivatized with BSTFA was evaluated for stability and found to be stable during a period of 4 h [2], but in another study EtG-TMS was stable for a very short period of time, less than 2 h [12].

Of note is that even a negligible amount of water contributes to the dissolution of the derivative. To avoid this, we treated the sample residues with anhydrous ethanol that was evaporated before derivatization. The resulting TMS derivative of EtG in our study was stable for 28 h (Fig. 1).

Determination of EtG in rat hair

The WHP rats voluntarily consumed 10% ethanol for one month and each rat's average daily consumption was calculated. This daily intake was plotted against the level of EtG in the rat hair samples (Figs. 2 and 3). The distribution pattern was different in female and male rats. Specifically, we did not find any statistically significant correlation between daily consumption of ethanol and EtG level in the hair of male rats, although 2 animals showed remarkably high levels of EtG (8.8 and 13.7 ng/mg of hair). Excluding these results did not change the statistical conclusion of a lack of correlation. Interestingly, there were also 2 female rats with high EtG levels (20.7 and 19.1 ng/mg of hair). The distribution pattern revealed a statistically significant correlation that was present even after excluding the highest values.

Politi et al. [22] found a proportional relationship between the EtG concentration in hair and the amount of alcohol consumed (R^2 = 0.8591) in a group of 21

volunteers who had been asked to complete a written questionnaire about their drinking habits. This correlation was determined by plotting the concentration of EtG in samples of the volunteers' hair *vs.* ethanol daily intake (EDI), which was normalized by body weight. Another study showed a significant correlation ($R^2 = 0.5357$) between EtG concentration in segmental hair analysis and the amount of alcohol consumed [3]. Some researchers found no correlation between the level of EtG in hair and drinking behavior. In some hair samples of alcoholics with known excessive alcohol consumption no EtG was detected [1, 11]. The lack of correlation between the detection of EtG in hair and drinking behavior was also observed by Skopp et al. [28]. Several reasons may contribute to this lack of correlation, one of them is that information on alcohol consumption is based, at least in part, on personal statement and therefore, may not be sufficiently reliable. This problem does not exist in experiments with animals which consume precisely dosed amounts of ethanol.

There are several sex-related differences in ethanol consumption, metabolism, kinetics and pharmacodynamic effects in laboratory animals. Starting as early as a few hours after birth, gender differences in responsiveness and sensitivity to ethanol could be detected in rat pups. Female neonates were more responsive to ethanol than males [32]. Ethanol absorption from the stomach seemed to occur faster in females than males, with the females reaching maximum blood concentrations more rapidly. Distribution of ethanol to the brain extracellular fluid was faster in females than males. Female rats exhibited faster ethanol elimination than male rats, both from brain and blood, and therefore, the differences in the pharmacological response to ethanol can be expected [24]. The elimination rate of ethanol decreases with age in male rats, as it was shown in F344 rats [26] and Sprague-Dawley rats [21]. The elimination rates for Wistar female rats and for Wistar male rats were calculated to be 428.3 ± 30.2 mg/kg/h and 356.6 ± 16.3 mg/kg/h, respectively. There was no correlation between the rate of elimination and the preference for alcohol [7]. In male spontaneously hypertensive (SH) rats, ethanol metabolism rate fell markedly between 4 and 10 weeks of age, which coincided with the time when the rats reach sexual maturity [23].

Absolute ethanol consumption generally is higher in males but their body weight is generally higher. The females consume relatively much more ethanol per unit of body weight than the males. The total ethanol metabolism in females per unit of body weight is considerably faster, as their total calorie consumption was significantly greater than that in the males of Wistar strain [7]. Similar observations were made in Sprague-Dawley rats regarding weight gain and ethanol intake, however, blood ethanol levels in males and females consuming liquid ethanol diet were found to be similar in both groups [21].

Liver alcohol dehydrogenase (LADH) plays a crucial role in ethanol oxidation *in vivo*. Specific LADH activity did not significantly change with age in F344 rats of either sex, while it was significantly reduced with age in males but not in females when related to body weight [26]. Female rats of both Alko strains (either preferring or not preferring ethanol) had a more rapid ethanol oxidation and oxygen consumption than the respective males [6].

When ethanol-fed female Sprague-Dawley rats were compared with ethanol-fed male rats, there was a significant increase in secretion of trypsinogen and amylase, and statistically nonsignificant but proportional increase in lipase in female rats. An increased ratio of trypsinogen to trypsin inhibitor can facilitate premature activation of trypsinogen, as postulated in acute pancreatitis and may explain earlier occurrence of alcoholic pancreatitis in females when compared with males [17].

Some sex differences in SH rats were shown to be modulated by testosterone. In male SH rats testosterone has an inhibitory effect and estradiol has a testosterone-dependent stimulatory effect on the ethanol metabolism rate and alcohol dehydrogenase activity in these animals. In female SH rats, alcohol dehydrogenase does not appear to limit the ethanol metabolism rate. Testosterone decreases the ethanol metabolism rate and alcohol dehydrogenase activity in female SH rats to the level found in mature male SH rats [23].

EtG is formed by a phase-II conjugation reaction with glucuronic acid catalyzed by multiple UDP-glucuronosyltransferase (UGT) isoforms. In humans, the most important UGT isoforms are UGT1A1 and UGT2B7 [8]. In alcohol-fed female Wistar rats UDP-glucuronosyltransferase is down-regulated, and in males an increased expression was observed, which suggests a sex-related difference and a potentially greater susceptibility of female rats to the effects of ethanol [30].

The results presented here demonstrate a newly discovered sex-related difference in the deposition of

EtG in rat hair. The absence of a correlation between daily ethanol consumption and EtG levels in hair samples from male rats and the presence of such correlation in females suggest that multiple factors may affect EtG incorporation, such as the activity of UGT, blood ethanol concentration, EtG persistence in the bloodstream, and hair follicle cell permeability.

Adult female rats consumed more ethanol per kg body weight than did adult males (an average of 7.5 g/kg/day *vs.* 5.2 g/kg/day [146% more ethanol]), and more EtG was deposited in the hair of adult female rats (an average of 3.89 ng/mg hair *vs.* 2.18 ng/mg hair [172% more EtG]). These observations support earlier findings that adult female rats consume relatively more ethanol than adult male rats [33].

Recently, Kharbouche et al. [14] has reported a median EtG concentration in Long-Evans rat hair of 0.021, 0.104, and 0.189 ng/mg following administration of ethanol at doses of 1, 2 and 3 g/kg b.w., respectively. Ethanol was administered on 4 consecutive days per week for 3 weeks. These values are significantly lower than our results for WHP rats. WHP rats consumed voluntarily nearly twice as much ethanol per day and for the whole week. The drinking behavior may be another important factor for EtG incorporation in rat hair. The time of exposure to blood EtG is much more prolonged during voluntarily consumption of ethanol by rats than after a single daily dose, which may be crucial for EtG incorporation.

In summary, the method presented here allows detection and quantification of EtG in rat hair. We also observed differences in EtG deposition in male and female rats, and these observations merit further investigation.

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