Harmful effects of alcohol consumption have caused a recent interest in the search for biomarkers of alcohol abuse. These biomarkers play an important role in identifying the nature of alcohol consumption from an occasional alcohol intake to abuse or dependency status (1-3). Ethyl glucuronide (EtG) is a direct ethanol metabolite formed by ethanol conjugation with glucuronic acid mediated by UDP-glucuronyltransferase (4). EtG is non volatile, water soluble and stable upon storage; it can be detected in urine for extended time periods of up to several days after complete elimination of alcohol from the body (5). It has been estimated that only about 0.02% of total ethanol dose was recovered as EtG in the urine in humans and about 0.13% in rats (6, 7). As a direct ethanol metabolite, EtG can be regarded as a highly specific marker of alcohol intake, which may be useful in monitoring drinking during withdrawal treatments or in situations where it is important to exclude alcohol use for example, in workplace, during pregnancy and in transplantation patients (8). The presence of EtG in urine provides a strong indication of recent drinking, even if ethanol is no longer detectable. Thus, urine EtG levels can be used as a laboratory test to detect recent alcohol consumption.

Determination of EtG in urine samples helps to fill the gap in evaluations between short-term biomarkers (ethanol) and long-term ones [carbohydrate deficient transferrin (CDT), γ glutamyltransferase (GGT), mean corpuscular volume (MCV)], used as traditional markers of alcohol consumption (9).

In recent years, EtG has been receiving more and more attention as a biomarker of acute alcohol intake and has been recommended for use in clinical and forensic investigation of alcohol intake (10, 11). The usefulness of its detection has been repeatedly demonstrated in situations where alcohol consumption was tentatively assumed, but not confirmed.
One study conducted in alcohol-dependent patients during an alcohol detoxification period after admission to the hospital demonstrated high inter-individual variability in EtG detectability, ranging from 40 to 130 h (5).

Several earlier studies in humans revealed that the same amount of ethanol ingested at different concentrations results in different blood ethanol concentrations (blood alcohol content, BAC) depending on the prandial state (12-14).

These differences in BAC were also observed in rats. The studies in rats have shown that first-pass metabolism of ethanol is also dependent on the concentration of administered ethanol (13).

The aim of this study was to establish whether the amount of ethyl glucuronide formed and excreted with the urine of WHP rats could be affected by the concentration of alcohol given orally. In addition, the EtG concentration in rat hair was measured and evaluated.

MATERIALS AND METHODS

Animal experiments
This study was conducted using adult male Warsaw high preferring (WHP) rats, weighting 320–390 g at the beginning of the experiment. The WHP rat line is selectively bred from Wistar rats for voluntary alcohol consumption. WHP rats were obtained from Department of Pharmacology and Physiology of the Nervous System, Institute of Psychiatry and Neurology, Warszawa, Poland. Rats were kept in individual cages in a temperature-controlled room with a 12-h light/12-h dark cycle. Animals were quarantined for 1 week before ethanol dosing. Rats received the ethanol solution orally according to their body weight on 5 consecutive days per week for five weeks. During weeks 1, 2 and 3, the rats were administered 1.5 g/kg of ethanol at concentrations 15, 30 and 50%, respectively. During weeks 4 and 5, the same rats were administered 3 g/kg of ethanol at concentrations 30 and 50%, respectively.

Urine was collected in metabolic cages at the following time periods: 0–3, 3–6, 6–9, 9–12, 12–15, 15–21, 21–27, 27–33 h after ethanol administration. The rats received continuously water ad libitum and food was withdrawn two hours before ethanol intake.

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

Urine samples were collected weekly from each animal by housing each animal separately in metabolic cages, with water ad libitum. Samples were stored frozen (-20°C) in Eppendorf vials until their analysis. 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 ethanol administration and on the seventh day after the last ethanol intake.

Materials
The following chemical reagents were used in this study: ethyl glucuronide (EtG) and methyl glucuronide (MG) standard from Medichem (Germany), GC/MS-grade ethyl acetate (Sigma), acetone (POCH S.A.), methanol (LAB-SCAN) and N-methyl-N-[(trimethylsilyl)trifluoroacetamide (MSTFA; Sigma-Aldrich).

Stock standard solutions of EtG and MG were prepared by dissolving each compound in methanol to obtain a concentration of 1 mg/mL.

Analytical procedures
EtG levels were determined using a gas chromatograph / mass spectrometer (GC/MS-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 maintained 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 the electron impact (EI) mode with 70 eV ionization energy. Under these conditions the retention time was 10.82 min for silylated EtG 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, biological background interference was negligible. Methyl glucuronide was chosen as the internal standard according to Skopp et al. (15).

Sample preparation and EtG extraction procedure
Two hundred microliters (10 µg/mL) of internal standard (IS) was added to the 50-µL urine sample and the solution was made up to 1 mL in volume with methanol.

After 10 min shaking and centrifugation at 10,600 x g, 50 µL of the supernatant was transferred to vials and evaporated to dryness under a stream of nitrogen at 70°C using a heated metal block. Anhydrous ethanol (100 µL) was added and evapo-
rated to dryness. The residue was derivatized with 50 µL MSTFA and 50 µL ethyl acetate (60 min, 80°C) and the volume of 1 µL of the processed samples was injected into the GC/MS system.

The calibration curve covered a concentration range of 10–2,000 µL/mL for the analyte in urine. The limit of detection (LOD) and limit of quantitation (LOQ) were calculated to be 3.3 µL/mL and 10 µL/mL, respectively.

The rat hair EtG concentrations were measured according to a previously published validated method (16). The limit of detection was 0.03 ng/mg, and the limit of quantification was 0.1 ng/mg.

Statistical analysis
The Shapiro-Wilk test was used to test for normal distribution of data and the Levene’s test for homogeneity of variance. Analysis of variance (ANOVA) was used to test for significance between the different ethanol concentrations and total EtG excreted with urine.

The nonparametric test of Wilcoxon was used when the data did not meet the requirements for a parametric test (such as normal distribution). Values of p < 0.05 were considered statistically significant.

The analyses were performed using Statistica 10 software (StatSoft, Poland).

RESULTS
Urine EtG cumulative profiles after ethanol administration at 1.5 g/kg and 3 g/kg in different concentrations are presented in Figure 1. All urine samples collected immediately before alcohol.
administration were negative for EtG. The synthesis of EtG was dependent on the amount of ethanol ingested and increased with the dilution of ethanol. EtG was detectable for up to 33 h but the EtG concentration in the last collected urine samples (between 27–33 h after ethanol administration) was 6 times higher after the dose of 3 g of ethanol/kg (93.4 ± 12.41 µg/mL) than after the smaller one (15.5 ± 10.46 µg/mL).

This indicates that higher doses of ethanol resulted in a greater urine EtG concentration.

Individual profiles of urinary excretion of EtG revealed varied EtG formation between rats. We observed a tendency for reduced total EtG excreted with urine depending on increasing the ethanol concentration.

Total amounts of EtG excreted with urine in individual rats are depicted in Figure 2.

This study showed an ethanol dose- and concentration-dependent correlation with EtG excretion in rat urine. The higher ethanol dose of 3 g/kg b.w. resulted in higher EtG levels and the total amount excreted with urine were significantly higher (p < 0.01) (Table 1).

When the administered ethanol was more concentrated, the total excretion of EtG was decreased. The difference was statistically significant at the higher dose of 3 g/kg (p < 0.01). Furthermore, we observed a tendency toward a reduction in total amount of EtG excreted with urine depending on the ethanol concentration after the lower ethanol dose of 1.5 g/kg as well, however, the differences between the three concentrations 15, 30 and 50% were not statistically significant (p > 0.05) (Table 1).

It was interesting to find out if the inter-individual differences in rats alter the EtG deposition in rat hair. Table 2 presents the EtG content in rat hair after a completed set of experiments. The rats received the same amounts of ethanol at the same time and at the same concentration. A mean of 1.5 ng EtG/mg hair was found. This result reflects a 43% coefficient of variance that is comparable to the mean urine AUC coefficient of variance (49%) or the coefficient of variance (44%) of the total amount of EtG excreted.

**DISCUSSION**

Biotransformation of ethanol to EtG was observed in various rat strains and lines (17, 18). We chose to study EtG production in WHP rats because this line has been used in experimental models in alcohol studies (19-21).

Ethyl glucuronide (EtG) is formed by the net addition of activated glucuronic acid to ethanol. This reaction is catalyzed by the UDP-glucuronosyltransferase (UGT) enzymes, which are characterized by a high genetic polymorphism. Foti and Fisher (22) observed the highest rate of EtG formation in humans by isoform UGT 1A1, whereas Schwab and Skopp found high activity of UGT 1A9 and UGT 2B7 (23). The increase of UGT 1A1 expression was also reported in the liver of ethanol consuming Wistar rats (24). While comparing to humans, more efficient glucuronidation of ethanol has been observed in tree shrews and Sprague-Dawley rats (7). Ethanol biotransformation to EtG after a single dose has been described in Sprague-Dawley (SD) rats.

| Table 1. Maximum concentrations (C_{max}) time to-maximum concentrations (T_{max}) and area under the curve (AUC) for EtG and the total amount of EtG excreted in rat urine after alcohol administered in two doses at different concentrations. |
|---|---|---|---|---|---|
| Doses of ethanol | 1.5 g/kg b.w. | 3 g/kg b.w. | Mean RSD% |
| Ethanol concentrations (v/v) | | | |
| C_{max} (µL/mL) | 15% | 30% | 50% | 30% | 50% |
| T_{max} (h) | 3-6 | 3-6 | 3-6 | 6-9 | 6-9 |
| AUC µg/mL-h | 12713 ± 5244 | 9051 ± 5574* | 6036 ± 3262* | 26548 ± 9709* | 18524 ± 9401* |
| RSD% | 41.2 | 61.6 | 54.0 | 36.6 | 50.8 |
| Total urine EtG | 4600 ± 2110 | 3746 ± 1955 | 3162 ± 2219 | 12462 ± 3124* | 8428 ± 2068* |
| RSD% | 45.9 | 52.2 | 70.2 | 25.1 | 24.5 |
| EtG in hair ng/mg | 1.49 ± 0.64 | 1.49 ± 0.64 | 1.49 ± 0.64 | 1.49 ± 0.64 | 1.49 ± 0.64 |

*Denotes a statistically significant difference between 30 and 50% ethanol at dose 3 g/kg b.w. (p < 0.01) (Wilcoxon test). *The value differed significantly for ethanol administered in two doses of 1.5 g/kg b.w. and 3 g/kg b.w. (p < 0.01) (two-way ANOVA). RSD% - relative standard deviation.
rats and its similarity to that in humans was indicated (18).

The variations in urine EtG with different concentrations of the same ethanol dose observed in our study correspond to findings reported by other authors, who studied the effect of ingested ethanol concentration on blood alcohol levels (13, 25). As was demonstrated, the consumption of more concentrated ethanol resulted in lower blood alcohol levels than consumption of a dilute solution did. When rats were tested in a fed state, they exhibited decreasing mean AUC with increasing ethanol concentrations (4, 16 and 40%) after intragastrically administered ethanol (1.0 g/kg). In fasted rats, only the highest ethanol concentration (40%) produced a lower mean AUC (13).

Likewise in humans, when ethanol was ingested postprandially, the mean AUC and the mean peak blood alcohol concentrations were significantly lower with a concentrated (40% w/v) than with a dilute (4%) solution (13). Differences were also observed after ingestion of beer and whiskey and showed higher peak blood alcohol levels with beer than with whiskey in the postprandial condition, with the opposite findings in the preprandial state (25). Our assumption is that the changes in blood ethanol concentration in the postprandial state are followed by similar changes in the excretion of EtG with urine. In this study, rats were given ethanol two hours after their feed had been withdrawn in the morning. Therefore, it can be assumed that the rats were in postprandial state, considering the fact that rats feed mainly at night.

The $T_{\text{max}}$ of EtG in urine after 1.5 g/kg of alcohol was shorter than after 3 g/kg of alcohol (Table 1). Similar trends have been observed in humans. In a study conducted by Lostia et al. (26), the authors found a statistically significant increase in urine EtG $T_{\text{max}}$ after higher doses of alcohol. An increase in EtG $T_{\text{max}}$ after three doses of alcohol was also observed in rat serum. In rats receiving ethanol at 1, 2 and 3 g/kg body weight, the $T_{\text{max}}$ for blood EtG were 1, 2 and 4 h, respectively (17).

EtG concentrations in blood and hair of Long-Evans rats were compared after ingestion of different ethanol doses. Higher blood EtG AUC values were observed after higher ethanol doses and a correlation with hair EtG concentrations was found. Median hair EtG concentrations were 21, 104 and 189 pg/mg in groups receiving ethanol in doses of 1, 2 and 3 g/kg, respectively, on four consecutive days per week for three weeks (17). All WHP rats having received ethanol in our study showed a median EtG concentration in hair of 1490 pg/mg (range: 700–2300). This concentration was higher than that observed by Kharbouche et al. (17) but we administered ethanol for longer time, i.e. on 5 days a week for 5 weeks.

It is worth emphasizing that EtG that accumulated in hair was a result of five different ethanol dosing procedures, which is closer to the pattern of alcohol consumption by humans (in different forms and in different amounts). The rats in the experimental study group also varied in ethanol glucuronidation levels. Despite these differences, relative standard deviation (RSD) values for total urine EtG excretion (44%), urine EtG AUC values (49%) and EtG content in hair (43%) were very similar, which indicates a close relationship between EtG contents in urine and in hair, as both parameters are derived from the blood EtG concentration. Thus, the increased ethanol concentration in consumed beverages would decrease ethanol blood concentration and, in turn, decrease the EtG synthesis that results in lower urine EtG levels and likely EtG hair content.

In summary, the present study showed that EtG production in WHP rats was depended on the concentration of orally administered alcohol, with differences more pronounced at higher ethanol doses. These findings may have implications for individuals who consume alcoholic beverages with food. Drinking less concentrated ethanol is very likely to result in greater EtG production than ingesting the same amount of ethanol in a more concentrated form, particularly in the postprandial state. EtG levels in hair closely reflect the fate of EtG in the rat.

**Conflict of interest**

There are no conflicts of interest relevant to the presented study. The authors alone are responsible for the content and writing of the paper.
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