Decrease of ethyl glucuronide concentrations in hair after exposure to chlorinated swimming pool water.

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**Abstract**

The direct alcohol marker ethyl glucuronide (EtG) is widely used for the assessment of alcohol consumption behavior and abstinence monitoring by hair analysis. We investigated the influence of chlorinated swimming pool water on EtG concentrations in hair in comparison to deionized water (Milli-Q) containing no chlorine. EtG concentrations were measured with a validated online-SPE-LC-MS/MS method. EtG positive hair samples were obtained from three regular drinkers and incubated for 0, 2, 4, 6, 8, and 10 hours at room temperature. EtG concentrations in hair were reduced after two hours of incubation in chlorinated water by 20±12% (range:4-33%), in deionized water by 24±5% (range:18-29%). Incubation for 10 hours resulted in a decrease in EtG concentrations of 57±6% (range:52-65%) for chlorinated water and 47±11% (range:32-60%) for deionized water. To demonstrate washout in forensic hair samples, 20 samples from subjects with known alcohol consumption behavior were investigated additionally. The samples were divided into two strands and analyzed with incubation in chlorinated water for 10 hours and for comparison without any incubation. A mean decrease of 53±18% (range:26-88%) was observed. These results clearly demonstrate that washout effects are caused by water and have a significant impact on EtG concentrations in hair. For people with hair that are regularly exposed to water for a longer period of time (e.g. swimmers), washout effects may lead to a significant decrease of EtG concentrations in hair. Concentrations may fall below threshold concentrations used for the interpretation of consumption habits (7 pg/mg for social consumption, 30 pg/mg for excessive consumption).

**Keywords**

**ethyl glucuronide, EtG, hair analysis, alcohol marker, drug test adulteration**

**Introduction**

Alcohol abuse and the associated medical and social problems have a major impact on today’s society. To investigate drinking habits, a self-report questionnaire such as AUDIT (subjective) or the analysis of alcohol markers (objective) may be considered ([1](#_ENREF_1), [2](#_ENREF_2)). Ethyl glucuronide (EtG), a direct alcohol marker generated during phase II metabolism, detectable in blood, urine, and hair, is one of the routine markers to monitor alcohol abuse or abstinence ([3](#_ENREF_3), [4](#_ENREF_4)). The assessment of the consumption behavior and abstinence monitoring is frequently performed by determination of EtG concentrations in hair. The detection window of EtG can be estimated from the analyzed hair length, assuming a growth rate of scalp hair of about 1 cm/month ([5](#_ENREF_5)). Segmentation of hair samples permits a time-resolved investigation of exposition to ethanol and drugs, dependent on the route of incorporation. Since the outcome of such an assessment is often linked to significant personal or social consequences (e.g. loss of driving license, loss of custody for children), the integrity of the test’s outcome is very important. Various methods potentially altering the outcome of EtG determination in hair, such as bleaching, dyeing, perming, thermal hair straightening, cosmetic treatment and a single application of a cleansing shampoo have been investigated ([6-11](#_ENREF_6)). Chemical treatment or the use of hairstyling tools can lead to changes in the hair structure, causing drugs to leak out. With respect to EtG, this may change the concentration over time, due to its high water solubility. It was found that normal hair hygiene might wash out EtG from the hair, however, no specific data is currently available about the effects of water, especially chlorinated water, on EtG concentrations in hair ([12](#_ENREF_12)). Considering water as a potential reason for a reduction of EtG concentrations in hair, extended washout effects due to chlorinated water could have an influence on EtG concentrations in hair samples from regular swimmers, wellness/spa facility visitors or subjects living in an area with drinking water chlorination as standard procedure. The process of water chlorination is frequently used, leading to a primary sanitizer for swimming pools or a disinfectant for drinking water, as chlorine oxidizes undesired contaminants (11). Recently, a reduction of benzodiazepine concentrations in hair after prolonged exposure to chlorinated water (0.1 % sodium dichloroisocyanurate and 0.1 M sulfuric acid at pH 5.5) was investigated by Morini et al. ([13](#_ENREF_13)).

The aim of this study was to systematically investigate the effect of swimming pool water (chlorinated water) on EtG concentrations in hair. Therefore hair samples from different subjects with a regular alcohol consumption behavior were incubated with chlorinated water from a local swimming pool and for comparison with Milli-Q water or without any treatment. After extraction in an ultrasonic bath, samples were analyzed by a validated liquid chromatography tandem mass spectrometry method (LC-MS/MS).

**Materials and methods**

**Chemicals and Reagents**

Acetone (LiChrosol), dichloromethane (Reag. Ph Eur), aqueous ammonia solution (25%, EMSURE), and methanol (Reag. Ph Eur) were obtained from Merck (Darmstadt, Germany). Formic acid solution (puriss. p.a., 50% in water) was from Fluka/Honeywell (New Jersey, USA). Ampoules containing ethyl-β-D-glucuronide in methanol (1 mg/mL) and ethyl β-D-glucuronide-*d5* in methanol (1 mg/mL) were ordered from Lipomed (Arlesheim, Switzerland) and Cerilliant (Texas, USA). External quality control samples were purchased form ACQ Science GmbH (Rottenburg-Hailfingen, Germany). Deionized water was produced with a Milli-Q water system from Millipore (Billerica, USA).

**Chlorinated water**

Authentic chlorinated water was obtained from a local swimming hall (Bern-Brünnen, Switzerland). Sampling of the water was performed in the morning, directly after the opening of the swimming hall, by using an empty 1.5 L PET bottle (previously containing drinking water) to obtain water directly from the pool. Sampling in the morning was favored to ensure proper cleaning of the water by the filtration system during the previous night, when customers were absent. After sampling, the water was transported to the laboratory and the incubation experiments were started. With respect to the guidelines of the Swiss Society of Engineers and Architects (SIA), the target values for chlorinated water at the investigated facility were the following: chlorine content: 0.30-0.40 mg/L, pH 7.15-7.35, target redox potential 750-830 mV. Chlorination was achieved by using sodium hypochlorite, created by electrolysis from sodium chloride tablets. Additional sterilization of the water was achieved by ozone, generated by a high voltage ozone generator (Rheno, Schlieren, Switzerland).The regulation of the pH was achieved by the addition of sulfuric acid (38%).

**Preparation of standard solutions, calibrators, and quality controls**

Two individual EtG stock solutions, for calibrators and quality control samples, respectively, were prepared in methanol at concentrations of 2.5 µg/mL. To verify the reference standard concentration of 1 mg/mL, different sources of reference standards were used. Based on the individual stock solutions, six calibrator working solutions with concentrations from 1.5-150 ng/mL and two quality control working solution at 9 and 45 ng/mL were prepared. All solutions were stored at -18 °C. Calibration and quality control samples were prepared by adding 20 µL of working solution to 30 mg of pulverized, blank hair. The final concentration of calibration samples was from 1 – 100 pg/mg in hair. Commercially available EtG positive quality control hair samples with concentrations of 23 and 44 pg/mg were included within each measurement series.

**Hair samples**

 **Pretest**

For the systematic investigation of EtG concentrations during 0-10 hours of incubation, hair samples were obtained from three male subjects with short hair (<5 cm total length), with a self-reported alcohol consumption of: 1 L of beer per day (subject 1, about 2 cm hair segment), 1 L of beer per day and larger amounts at the weekend (subject 2, about 3.5 cm hair segment), and about half a liter of beer per day (subject 3, about 2.5 cm hair segment). The hair samples were cut from the distal end during a standard hair cut and collected in envelopes. Afterwards, the samples were mixed individually, added into individual glass vials and incubated for 0, 2, 4, 6, 8 and 10 hours in chlorinated water, and for control in Milli-Q water for the same duration, by shaking on a Multi Reax mixer (Heidolph Instruments GmbH, Schwabach, Germany) at 2’000 U/min at room temperature. In order to simulate a visit to the swimming hall or frequent hair washing, the water (4 mL per tube) was changed every 30 min.

**Forensic Samples**

20 hair samples from different individuals with known positive EtG concentrations (>6 pg/mg) were divided lengthwise into two equivalent strands. One strand was incubated for 10 hours in chlorinated water, analogous to the procedure described above, while the second one served as a reference without any pretreatment. Information about the origin, color, and processed segments can be found in table 1.

**Sample preparation for LC-MS/MS measurements**

EtG in hair by LC-MS/MS was performed with a fully validated method according to the guidelines of the German Society of Toxicological and Forensic Chemistry (GTFCh) with a limit of quantification (LOQ) of 3 pg/mg. ([14](#_ENREF_14), [15](#_ENREF_15)). The linearity was obtained from 1 – 300 pg/mg (r2=0.9989). The method is used for routine analysis of driving under the influence of alcohol (DAA) cases in an accredited laboratory (ISO 17025) of the Institute of Forensic Medicine in Bern, based on the guidelines of the “Swiss Society of Legal Medicine” (Schweizerische Gesellschaft für Rechtsmedizin (SGRM)).

In order to remove potential contaminants, the hair samples were washed in a glass tube by shaking them for 4 min with 4 mL of water, removal of the solvent by use of a pasteur pipette, followed by acetone and dichloromethane washes in the same way. Subsequently, the samples were dried at room temperature and manually cut into small pieces to ensure homogeneity. About 30 mg of cut hair were weighed in a 2 mL snap-cap Eppendorf tube together with two stainless steel balls and pulverized at 30 Hz during 5 min with a ball mill (MM 400, Retsch Technology GmbH, Haan, Germany). After pulverization, 1 mL of Milli-Q water and 10 µL of internal standard solution (100 ng/mL EtG*-d5*) were added. Samples were then placed in an overhead shaker (Heidolph Instruments GmbH, Schwabach, Germany) for 10 min and subsequently centrifuged for 1 min at 16’000 *g*, before they were extracted for 2 hours in a supersonic bath (Sonorex RK 100, Bandelin Electronic GmbH, Berlin, Germany). The samples were subsequently mixed in the overhead shaker for about one min and centrifuged for 10 min at 16’000 *g*, beforeperforming a solid-phase extraction (SPE) with OASIS Max cartridges (60 mg/3 mL, Waters, Baden, Switzerland). The cartridges were equilibrated by adding 2 mL of methanol, followed by 2 mL of water. The samples were subsequently added to the cartridges, followed by a washing step with 1 mL of a water/ammonia solution (1%). Afterwards, the cartridges were dried during 15 min by applying a vacuum. Finally, EtG was eluted with 1.6 mL of methanol, containing 2% formic acid. The eluate was subsequently evaporated to dryness in a heating block set at 45 °C, with a gentle stream of nitrogen. Prior to LC-MS/MS analysis, the residue was reconstituted in 100 µL (H2O/MeCN, 99:1 + 0.1% formic acid). Liquid chromatography was performed by using an UltiMate® 3000 UHPLC system (Dionex, Thermo Scientific Instruments, Reinach, Switzerland) in online-SPE mode using a Hypercarb column to trap the analyte (30 × 2.1 mm, 5 µm) (Thermo Fisher Scientific, Reinach, Switzerland) and a Zorbax Eclipse plus C18 (50 mm × 4.6 mm, 1.8 μm) (Agilent Technologies, Basel, Switzerland) for the analytical separation. Mass spectrometric analysis was performed on a 5500 QTrap system (Sciex, Toronto, Canada) in selected reaction monitoring mode (SRM), by measuring the following transitions: EtG: m/z 221/75 as quantifier and m/z 221/85 as qualifier, EtG-*d5* m/z 226/75 as quantifier and m/z 226/85 as qualifier. Analysis of the collected data was performed with Analyst software 1.6.2 (Sciex, Toronto, Canada).

**Results and Discussion**

**Pretest in chlorinated water**

The systematic investigation of hair samples incubated in chlorinated water for 0 to 10 hours resulted in a steady decrease in EtG concentrations, see figure 1. The occurring decrease was observed in hair samples from all the observed subjects, which were incubated in independent tubes for the stated duration. Already after two hours of incubation, a mean decrease in concentration of 20±12% (range: 4-33%) was measurable. This indicates that an average stay at a spa facility of about 2 hours could decrease EtG concentrations in hair by about a fifth. The mean decrease after ten hours of incubation was 57±6% (range: 52-65%). These results show that prolongated incubation of hair in chlorinated water reduces the EtG concentration by more than half. These samples were additionally measured by GC-MS/MS to investigate if matrix effects potentially alter the outcome of the LC-MS/MS determination ([16](#_ENREF_16)). A decrease of 26±8% (range: 16-34%) after two hours and a decrease of 60±7% (range: 54-69%) after ten hours was observed with GC-MS/MS confirming the LC-MS/MS results.



Figure EtG concentrations from three different subjects after incubation in chlorinated water and Milli-Q water, respectively, from 0 to 10 hours. EtG concentrations prior to the incubation: subject 1: 314 pg/mg, subject 2: 50 pg/mg, subject 3: 37 pg/mg.

**Pretest in Milli-Q water**

The systematic investigation of hair samples incubated in Milli-Q water from 0 to 10 hours resulted in a decrease in EtG concentrations, occurring in hair samples from all the observed subjects, see figure 1. After two hours of incubation, a mean decrease in concentration of 24±5% (range: 18-29%) was measurable. The mean decrease over 10 hours of incubation was 47±11% (range: 32-60 %). When comparing the incubation of hair in swimming pool water to Milli-Q water, no significant difference was observable (10% mean difference), as the observed difference remains within the measurements imprecision of ±15%.

**Forensic samples**

The twenty forensic samples from different subjects represented a broad range of EtG concentrations (median: 50 pg/mg; range: 6-328 pg/mg), see table 1. Incubation for 10 hours in chlorinated water resulted in a mean decrease in EtG concentrations of 53±18% (range: 26-88%). In all the investigated samples, a decrease in EtG concentrations after incubation in chlorinated water was observed. With respect to the threshold concentration of 7 pg/mg (indicating moderate alcohol consumption from 7-30 pg/mg), two samples which were above the threshold concentration prior to the incubation showed concentrations below the cut-off concentration afterwards ([17](#_ENREF_17)). Considering the threshold concentration of 30 pg/mg (indicating excessive alcohol consumption), four samples which were above this limit prior to any incubation, fall below the threshold concentration after the incubation. These results indicate that prolonged exposure to chlorinated water may lead to a misinterpretation of drinking behavior.

Table Comparison between reference samples and samples washed for ten hours in chlorinated water, rounded figures.

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| sample nr. | reference hair sample [pg/mg] | incubated hair sample [pg/mg] | decrease in EtG | natural color | coloration | region of sampling | segment used |
| 1 | 59 | 32 | 46% | brown | none | posterior vertex  | 0.2-5.5 cm |
| 2 | 18 | 11 | 39% | blonde | none | posterior vertex | 0.2-6 cm |
| 3 | 14 | 2 | 86% | brown | none | leg | 0-2 cm |
| 4 | 66 | 36 | 45% | grey | none | posterior vertex | 0.2-6 cm |
| 5 | 6 | 4 | 33% | brown | none | posterior vertex | 0.2-44 cm |
| 6 | 253 | 95 | 62% | brown | none | leg | 0-2 cm |
| 7 | 166 | 93 | 44% | unknown | brown | posterior vertex | 0.2-30 cm |
| 8 | 20 | 15 | 25% | blonde | none | posterior vertex | 0.2-6 cm |
| 9 | 114 | 26 | 77% | brown | none | leg | 0-2.5 cm |
| 10 | 328 | 231 | 30% | blonde | none | posterior vertex | 0.2-7 cm |
| 11 | 175 | 53 | 70% | grey | none | posterior vertex | 0-8 cm |
| 12 | 20 | 9 | 55% | grey | none | posterior vertex | 0.2-4.5 cm |
| 13 | 64 | 43 | 33% | brown | none | posterior vertex | 0.2-8 cm |
| 14 | 38 | 12 | 68% | brown | none | posterior vertex | 0.2-8 cm |
| 15 | 80 | 43 | 46% | blonde | none | posterior vertex | 0.2-7 cm |
| 16 | 41 | 15 | 63% | black | none | posterior vertex | 0.1-6 cm |
| 17 | 100 | 19 | 81% | unknown | black | posterior vertex | 0.2-6 cm |
| 18 | 11 | 3 | 73% | brown | none | posterior vertex | 0.1-5.5 cm |
| 19 | 26 | 14 | 46% | grey | none | posterior vertex | 0.2-4.5 cm |
| 20 | 15 | 10 | 33% | brown | none | posterior vertex | 0.2-43 cm |

**General**

EtG concentrations decrease in a time-dependent manner when hair samples are incubated in water over a prolonged time. Washout effects of EtG are therefore not solely linked to the application of hygiene product such as shampoos or conditioners, but rather to the polarity of EtG itself. Contrary to basic substances such as cocaine, amphetamine, and codeine, we assume that EtG does not show a strong tendency to bind to melanins ([18](#_ENREF_18)). The application of chemical agents might increase washout effects due to adulterations in hair structure. However, the observed increase in washout by chlorinated water, in comparison to deionized water, remained within the range of the measurements imprecision. Variations observed in washout effects within the same group of samples might be linked to differences in the individual hair structure: Dependent on the thickness of the hair, the total surface exposed to the washing agent and the swelling rate may be different from subject to subject ([19](#_ENREF_19)).

Our observation raises the question, if EtG concentrations, measurable in hair, cover a shorter detection window than presumed. Over time, EtG concentrations, potentially incorporated during the growth of the hair, might be washed out and become replaced by EtG from sweat or sebum, if alcohol is consumed regularly ([20](#_ENREF_20), [21](#_ENREF_21)). Such an incorporation of substances from sweat or sebum has been observed for cannabinoids ([22](#_ENREF_22)). As a consequence of the vast reduction of EtG concentrations in hair after prolonged exposure to water, we recommend that the assessment of abstinence should be backed by more than one diagnostic parameter in more than one matrix.

**Conclusion**

Based on our results, EtG concentrations below the threshold value for abstinence (7 pg/mg) could be caused by extensive washout effects related to lifestyle habits, instead of an actual abstinence from alcohol. Furthermore, by extensive hair washing or bathing in water, the original EtG concentration from above 30 pg/mg may decrease to concentrations below this threshold used for the differentiation between social and extensive alcohol consumption habits. This assumption is accompanied by an unknown rate of false negative results for EtG determinations in hair, which could be caused by intentional or unintentional, prolonged exposure of hair to water prior to the sampling procedure. In order to minimize washout effect, EtG concentrations from the most recent hair sections (proximal), being affected by the lowest amount of washout effects due to hair or body care, should be analyzed. Although the in vitro experiments presented in this study clearly showed that exposure to water seems to be the main cause of the decrease of EtG in hair, as a further perspective, additional confirmation by the investigation of real cases (incubation of hair prior to a haircut) is recommended.

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**Conflict of Interest**

The authors declare that they have no conflict of interest.

**Compliance with Ethical Standards**

All subjects provided informed consent for the analysis of EtG in hair. Hair samples from regular drinkers for systematic washout investigations were obtained during a standard haircut. All samples were anonymized prior to the analysis by a specific sample identity code.

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