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Degradation of the ethyl glucuronide content in hair by hydrogen peroxide and a non-destructive assay for oxidative hair treatment using infra-red spectroscopy

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ABSTRACT

The assessment of quantification results of the alcohol abuse marker ethyl glucuronide (EtG) in hair in comparison to the cut-off values for the drinking behavior may be complicated by cosmetic hair bleaching. Thus, the impact of increasing exposure to hydrogen peroxide on the EtG content of hair was investigated. Simultaneously, the change of absorbance in the range of 1000–1100 cm^{-1} indicative for the oxidation of cystine was investigated non-destructively by attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR) using pulverized portions of the respective hair samples. Hair samples treated with hydrogen peroxide consistently displayed a significantly increased absorbance at 1040 cm^{-1} associated with the formation of cysteic acid. The EtG content decreased significantly if the hair was treated with alkaline hydrogen peroxide as during cosmetic bleaching. It could be shown that ATR-FTIR is capable of detecting an exposure to hydrogen peroxide when still no brightening was visible and already before the EtG content deteriorated significantly. Thus, hair samples suspected of having been exposed to oxidative treatment may be checked non-destructively by a readily available technique. This assay is also possible retrospectively after EtG extraction and using archived samples.

1. Introduction

In recent years, the determination of ethyl glucuronide (EtG), a minor metabolite of ethanol, in human hair gained increasing importance for the assessment of the overall drinking behavior [1–5]. Correct quantification of the actual EtG content in a person's hair sample is prerequisite for its comparison with generally accepted cut-off values [6] especially in legal cases and further scenarios with serious consequences for the involved parties. Hair bleaching is a possible way to dissemble lower EtG assays or generate false negative EtG results. It has been reported that treatment of hair involving cosmetic bleaching leads to complete loss of EtG [7]. However, it could not be decided, to which extent the EtG losses were induced by detergent mediated washing-off or by oxidative degradation. Meanwhile, there is evidence that EtG is chemically degraded by hydrogen peroxide in aqueous solution [8]. However, oxidative treatment of hair and potential EtG loss may not be obvious to the analyst and an assay to detect manipulated samples would increase the reliability of EtG results. This investigation aimed at clarifying two questions: Under which conditions does oxidative treatment of hair comparable to cosmetic bleaching lead to a significant decrease of the EtG content pretending an underestimation of the drinking behavior? Is the exposure of a given hair sample to oxidative agents detectable by technical means with practical relevance? Therefore, no commercial bleach was applied but the effects of detergent-free hydrogen peroxide alone and in combination with a buffer and ammonia were investigated in detail. Concentrations and exposure periods matched the conditions of cosmetic bleaching. Fourier transform infrared spectroscopy (FTIR) was used to investigate significant changes in the hair matrix induced by oxidative treatment because of its availability in forensic laboratories, its sensitivity and its non-destructive operation principle. Furthermore, FTIR has successfully been employed to detect the oxidation induced alteration of hair [9–11] and wool [12–14]. The ATR technique allowed recording of IR spectra directly from powdered hair samples without further sample preparation.

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2. Materials and methods

2.1. Chemicals and reagents

Native EtG and labeled EtG-d_5 were obtained from Medichem (Steinenbronn, Germany). Hydrogen peroxide (trace select, ≥30%), Trizma® base (≥99.9%), hydrochloric acid (37%), ammonium hydroxide (trace select, ≥25%), and formic acid (for mass spectrometry, 98%) were purchased from Sigma–Aldrich (Steinheim, Germany). Dichloromethane and methanol were obtained in picograde quality from LGC Standards GmbH (Wesel, Germany). Acetonitrile (HPLC grade) was from VWR International (Leuven, Belgium). Deionized water was prepared with a Milli-Q system (Millipore, Billerica, MA, USA).

2.2. Origin of hair samples and sample pre-treatment

Hair samples were anonymously received from male volunteers with known age and abstinence from hair dying or bleaching but suspected or known alcohol consumption. Each hair strand as received from an individual was humidified with a small amount of water and gently pressed between paper filters to remove excess water. Then the strand was aligned on a plastic board and manually cut with a ceramic household knife to pieces of 1–2 mm of length. The moist cuttings were cooled to −28 °C and lyophilized for 24 h using a LYOVAC GT 2/GT 2-E lyophilizer (FINN-AQUA, Hürth, Germany). After equilibration with the laboratory atmosphere the cut hair material was homogenized by overhead shaking for 48 h using an REAX 20 end over end mixer (Heidolph, Schwabach, Germany). The hair pool was obtained from a local barber and contained hair without oxidative treatment from a number of male donors and was cut and homogenized as reported elsewhere [15].

2.3. Bleaching procedure

Three different bleaching solutions were prepared: Solution A: 10% neutral H_2O_2 in water; solution B: 10% H_2O_2 in 50 mM Trizma®-HCl buffer at pH 8.0; solution C: 10% H_2O_2 in 50 mM Trizma®-HCl buffer adjusted to pH 10.0 with ammonium hydroxide. 50 mg of the hair sample under investigation (snippet size: 1– 2 mm) were weighed into a 2 mL conical plastic self-lock tube with attached lid (Eppendorf AG, Hamburg, Germany) and submerged in 1 mL of dichloromethane for 15 min. After pipetting the dichloromethane off, 1 mL of methanol was added and allowed just to perfuse to hair and then removed immediately. After air-drying over night the sample was submerge in the respective solution A, B or C for 10, 30, 45 or 60 min. Thereafter, the solution was removed and the hair sample was washed three times with 1 mL of water. The moist sample was cooled to −28 °C and then lyophilized for 24 h.

2.4. Infrared spectroscopy

50 mg of the designated sample were filled in 5 mL stainless steel milling flask together with two stainless steel milling balls of (Ø = 5 mm) and pulverized in a Retsch Mixer mill MM400 (Retsch, Haan, Germany) for 10 min at frequency of 30 s^{-1}. 10 mg of the powdered hair was applied to an ATR Diamond Golden Gate accessory mounted on an IFS66v FTIR vacuum spectrometer (Bruker, Ettlingen, Germany). Every measurement included 2000 background and samples scans covering the range between 4000 and 600 cm^{-1} with a resolution of 2 cm^{-1}. IR spectra were processed using the Origin 9.0 software package (OriginLab Corp., Northampton, MA, USA).

2.5. Quantification of ethyl glucuronide with HPLC–MS/MS

50 mg of the hair (snippet size: 1– 2 mm) were washed with dichloromethane and methanol as described above. After air-drying, the sample was submitted to a combined grinding and extraction procedure known as micropulverization and described elsewhere in detail [16]. In brief, two stainless steel balls (Ø = 5 mm) were added to the Eppendorf tube followed by 550 μL of water and 50 μL of an aqueous EtG-d_5 solution (100 ng/mL) as internal standard. Up to ten closed tubes were placed equally into each of the two PTFE-adaptors with a capacity of ten conical plastic tubes (Retsch, Haan, Germany). The samples were milled for 30 min with a frequency of 30 s^{-1} using the mixer mill MM400. Thereafter, the extract was filtered through a 0.2 μm regenerated cellulose Phenex® filter (Phenomenex, Aschaffenburg, Germany) and 150 μL of the filtrate allowing three injections were submitted to HPLC analysis.

Quantification of EtG was performed on an Agilent 1200 series HPLC binary pump system (Agilent Technologies, Waldbronn, Germany) equipped with an autosampler and coupled to an API 4000 Q-Trap® high performance hybrid triple quadrupole/linear ion trap mass spectrometer (Applied Biosystems/MDS SCIEX, Foster City, California/Concord, Ontario, Canada). Chromatographic separation was carried out using a combination of a 10 mm × 2.1 mm Hypercarb guard column (3 μm particle size) with a 100 mm × 2.1 mm Hypercarb column (Thermo Scientific, Waltham, USA) with 3 μm particle size. A mixture of 93% water, 7% acetonitrile and 0.1% formic acid was used as mobile phase at a flow rate of 0.3 mL/min. The injection volume was 50 μL. The separation was carried out isocratically over a total run time of 10 min and the analytes displayed retention times of 3.5 min (native EtG) and 3.4 min (EtG-d_5). The oven temperature was held at 30 °C.

The mass spectrometer was run in the multiple reaction mode (MRM, dwell time: 100 msec) with negative ionization. Transitions monitored were m/z 220.8 → 74.9 (quantifier) for native EtG and m/z 226.0 → 74.9 for EtG-d_5 as well as m/z 220.8 → 84.9 (qualifier) for native EtG and m/z 226.0 → 84.9 for EtG-d_5. The first and third quadrupole were set to unit resolution. Source parameters were: ion spray voltage, −4000 V; desolvation temperature, 500 °C; ion source gas 1, 80 (arbitrary units) a.u.; ion source gas 2, 90 a.u.; curtain gas, 25 a.u.; collision gas 12 a.u.; declustering potential, −28 V for both transitions of native EtG and −27 V for both transitions of EtG-d_5; collision energy, −27 eV for the transition m/z 220.8 → 74.9 and −26 eV for the other ones; entrance potential, −9 V for the quantifier transitions and −10 V for the qualifier transitions; and collision cell exit potential, −7 V for all four transitions. Data were collected with the Analyst 1.5.2 and processed with the Analyst 1.6.2 software packages (Applied Biosystems/MDS SCIEX).

2.6. Calibration and quality control

Fourteen aqueous calibration solutions in the concentration range between 100 pg/mL and 80,000 pg/mL, corresponding to a concentration range between 1 and 800 pg EtG per mg hair were prepared gravimetrically. At least six calibration points were used for each measurement sequence depending on the concentration of the samples. Coefficients of determination R^2 were in all cases better than 0.998. Procedural blanks were analyzed with each sequence of 30 injections in duplicate. Procedural background levels of EtG were carefully controlled and throughout insignificant. Additionally, duplicate injections of two quality control materials (QM) were done after each sequence of 20 injections to monitor the quantitative reproducibility of the analytical procedure. No significant drift was observed. These QM materials