Ethanol Contamination Leads to Fatty Acid Ethyl Esters in Hair Samples

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Abstract

The diagnosis of alcoholism is a topical subject of discussion; in fact, many studies have been published on the determination of biochemical markers useful to this target. Fatty acid ethyl esters (FAEE) are minor metabolites of ethanol, and their usefulness has been demonstrated by their detection in hair using a headspace solid-phase microextraction-gas chromatographic–mass spectrometric technique. Environmental contamination in the analysis of drugs of abuse is a well-known focus of discussion between scientists. In the same way, interference from the surroundings could be hypothesized in FAEE detection. To assess the influence of ethanol contamination, an in vitro experiment was performed, leaving hair in an atmosphere saturated with ethanol vapors for 15 days. The spontaneous production of FAEE was demonstrated by analyzing hair day by day. In fact, we observed a constant increase of ethyl myristate, palmitate, and stearate that reached very high concentrations at the end of the investigation. Although the experiment was managed in a stressed way and could not represent real life, its purpose was to focus the attention of researchers on the problem of hair contamination that can occur, for example, with ethanol-containing cosmetics. Therefore, care in interpretation must be taken into account, especially with such a volatile molecule.

Introduction

The detection of biological markers for the diagnosis of alcoholism is a serious aim with important implications, both clinical and forensic (1–3).

Alcoholism is a social problem that involves a large part of the world population, and finding evidence of alcohol abuse can be problematic because people can abstain before a medical examination. Therefore, it is to be hoped to have a diagnostic medium that allows the objective detection of excessive alcohol use/abuse (4,5).

In previous years, various markers were investigated for the objective diagnosis of alcohol abuse, and some of them are sometimes employed in current clinical practices (5,6). Nowadays, alcoholism diagnosis, using mainly some enzymatic and hematological parameters (5), is usually left to doctor competence. Unfortunately, their sensitivity and specificity are not always satisfactory because of the influence of metabolic disorders, nutritional problems, and other pathologies unrelated to the alcohol abuse.

Other biochemical markers that may offer some chances to determine alcohol abuse are under investigation. To search for specific markers, some experts focused their attention on some minor ethanol metabolites originating from its secondary metabolism, such as ethylglucuronide (7–9) and fatty acid ethyl esters (FAEE) (10–12). The possibility of entrapping of these compounds in hair matrix, like drugs of abuse, has been considered (13–15). The scientific community has already expressed a favorable response to the role of “drug testing” in hair and published opinion consensus (16); in the same manner, hair analysis could probably be used for the diagnosis of heavy alcohol consumption.

The acronym FAEE includes a group of compounds synthesized from ethanol and fatty acids, lipoprotein and phospholipids, catalyzed by the specific “FAEE synthase.”

Since 2000, the detection of FAEE in hair for the diagnosis of heavy alcohol consumption has been proposed (13). Auwärter et al. (17) found that FAEE are trapped in hair matrix mainly via sebum, after their synthesis from ethanol and fatty acids in sebaceous glands. They documented their identification and quantification with headspace solid-phase microextraction–gas chromatography–mass spectrometry (HS-SPME–GC–MS) analysis, and suggested a slight influence on ethanol-containing cosmetics (18). In particular, ethyl myristate, palmitate, stearate, and oleate have been found in the hair of alcoholics, and it was assumed that the sum of FAEE concentrations higher than 1 ng/mg could be used as a biochemical marker of high consumption of alcoholic beverages (15,17).

No exhaustive studies on possible environmental contamination have been carried out yet, but a brief annotation has been reported in the literature (18).

Environmental contamination in hair analysis is a very serious problem because of the high surface/volume ratio, hence it is important to distinguish active intake from passive consumption and environmental contamination; furthermore, it...
could probably be more evident with ethanol, which is a volatile molecule easily accessible to the majority of the population. Hence, we believe that the evaluation of external contamination could be interesting and important.

In a previous paper, authors tested the potentiality of FAEE determination in hair with satisfactory results (19). In the present paper, the possible interference from the surroundings on the determination of FAEE in hair is evaluated. For this purpose, an in vitro test was performed, preserving hair collected from teetotalers in an atmosphere saturated with ethanol vapors.

**Materials and Methods**

To isolate ethyl myristate, palmitate, and stearate from hair samples, a liquid–liquid extraction procedure followed by HS-SPME was applied to hair specimens, as previously reported (19).

**Reagents and standards**

Ethyl myristate, ethyl palmitate, ethyl stearate, and the corresponding free carboxylic acids were obtained from Sigma-Aldrich (St. Louis, MO). Ethanol-d₆ and thionyl chloride were also obtained from Sigma-Aldrich. All other reagents were analytical grade.

**Internal standard synthesis**

Pentadeuterated internal standard analogues of each FAEE were made in-house. Their synthesis was performed at −78°C by adding 50 μL of ethanol-d₆ to 10 mg of the free acids and using 10 μL thionyl chloride as the catalyst (14). The temperature was then slowly increased to room temperature, then to 40°C, and maintained at 40°C for 2 h. The solution was finally dried and the residue dissolved in chloroform to obtain a 2 mg/mL stock solution.

**In vitro experiment**

A lock of hair of about 1 g was collected from a teetotaler and tested negative for FAEE. After cutting hair in segments of 3 cm length, the whole specimen was placed in a confined space. They were stored at room temperature for 15 days in a small glass vessel (250-mL volume) in an ethanol-containing atmosphere (Figure 1). For this purpose, a small layer of 96% ethanol (approximately 5 mL) was poured into a bigger beaker (500 mL) in which the smaller container was inserted, and the system was then sealed. Hair was tested in duplicate at different times during the storage; in particular, two 30-mg samples of hair were submitted to the analysis of FAEE (as depicted later) after 1, 2, 3, 4, 7, 10, and 15 days from the first exposure. The mean concentration between the two samples for each ester is reported in the present paper.

Before the analysis, a washing step was performed to eliminate ethanol deposited on the hair surface. Obviously, the chance is very small that hair may be contaminated with such a large amount of ethanol in real life, but the aim of the experiment was to demonstrate the production of FAEE, even when unlikely circumstances were simulated.

**FAEE extraction**

Hair samples (of approximately 30 mg) subjected to the previously specified protocol underwent the extraction procedure. For this purpose, n-heptane was added twice to hair samples that were left in contact with the solvent for 10 min; the supernatant was removed each time. After this step, hair samples were dried and cut in small pieces (2–3 mm length) and then added to a mixture of pentadeuterated internal standards corresponding to the three metabolites ethyl myristate, ethyl palmitate, and ethyl stearate (25 μL of a mixture 1 μg/mL each). The samples were extracted with 0.5 mL dimethylsulfoxide and 3 mL n-heptane shaking for about 20 h at room temperature. The organic layer was evaporated to dryness in a vial, reconstituted with 1 mL phosphate buffer (0.1M, pH 7.4) and 500 mg of natrium chloride (salting-out effect), and then submitted to HS-SPME at 90°C for 30 min after 5 min equilibration time. A 65-μm PDMS/DVB phase (Supelco, Bellefonte, PA) was used for the microextraction. The desorption was performed at 260°C for 5 min.

**GC–MS conditions**

A Focus GC (Thermo Electron, Waltham, MA) equipped with an Equity-5 capillary column (30 m × 0.25 mm × 0.25 μm, Hewlett-Packard, Palo Alto, CA) combined with a DSQ electron impact mass analyzer (Thermo Electron) set at 70 eV in SIM mode was used. The following temperature program was applied: 1 min at 70°C and then up to 290°C at 25°C/min with 5 min final isotherm.

The following ions were chosen for the analysis: 88, 101, 256 for ethyl myristate; 93, 106, 261 for ethyl myristate-d₆; 88, 101, 284 for ethyl palmitate; 93, 106, 289 for ethyl palmitate-d₆; 88, 101, 312 for ethyl stearate; and 93, 106, 317 for ethyl stearate-d₆.

**Calibration**

Calibration and validation parameters were referred to in...
detail in a previous paper (19). Linear curves for ethyl myristate, palmitate, and stearate were obtained in the range of 0.05–2 ng/mg by adding known amounts of each FAEE standard to 30 mg of hair and 25 μL of the internal standard solution (1 μg/mL).

Results and Discussion

In Figure 2, chromatograms are shown concerning hair analyses performed after 2 days' (A) and 15 days' saturation (B). The increase of the three compounds with the time is clear. In fact, in Figure 2B the high amount of FAEE with respect to their corresponding internal standards is evident.

Figure 3 shows the production of ethyl myristate, palmitate, and stearate during the 15 days' storage in the atmosphere saturated with ethanol. On the last day of the experiment, the concentration of ethyl myristate, palmitate, and stearate reached the values 3.39, 6.85, and 1.19 ng/mg, respectively. These concentrations are very high, particularly for ethyl myristate and palmitate. In fact, in a previous study performed with real samples (19), FAEE concentrations in alcoholics never reached these levels.

This experiment showed a surprising phenomenon of the in vitro synthesis of FAEE. We observed a constant and progressive increase of FAEE, especially ethyl myristate and palmitate, with the exposure of hair to an ethanol-saturated environment. Moreover, washing procedures performed on the samples were not effective at lower concentrations, unappreciable until seven days exposure, and had only a slight influence at higher levels.

The production of FAEE seemingly occurs without the catalyst's assistance, and high concentrations of these products were found even when a strong decontamination was performed before the analysis. It follows that besides the physical adsorption, ethanol is chemically bound with free fatty acids or lipids of the hair matrix or the sebum layer. From here, we can suppose the esterification of fatty acids layered on the external surface of hair (possibly under the action of bacterial enzymes), and then the incorporation of esters into the hair matrix. Alternatively, we can imagine ethanol entrance into the hair shaft and its reaction with free fatty acids.

External decontamination performed to remove ethanol and FAEE layered on the hair surface showed that the major part of FAEE was incorporated in hair matrix. In fact, a slight removal of FAEE was appreciable only at higher concentrations; heptane washings were analyzed by GC–MS, but traces of FAEE were identified only after day 7.

These results enable us to make important considerations...
about the environmental contamination, a problem often highlighted by forensic toxicologists when performing hair analysis for drugs of abuse.

Although the experiments were carried out in a stressful way which could not represent real life, the purpose of the present study was to focus the attention of researchers on the risk of hair contamination.

In light of the results obtained, we could conjecture the dangerous possibility of an external contamination (due, for example, to the use of ethanol-containing cosmetics) that may lead to false-positive results. We can also suppose a contamination may occur in a working environment, such as wine bar or wine cellar, which compels somebody to work in an ethanol-polluted surrounding.

All of these considerations testify that much care must be taken in the interpretation of data. Drawing up an accurate anamnesis of the patient, including notices about the use of hair cosmetics, working environment, and hobbies could help the toxicologist to explain the results.

After consideration about the absolute reliability of FAEE determination, we can therefore conclude that, although hair analysis could be an important source of information for the diagnosis of alcoholism, FAEE detection probably cannot be the absolute marker of alcohol abuse, and we suggest a very careful anamnesis to exclude any possibility of contamination.

Because an absolute biochemical marker for the diagnosis of alcoholism does not yet exist, it is necessary to detect various parameters (e.g., the combination of the seric CDT and FAEE in hair) to obtain reliable information.

It is therefore important to use these markers carefully to avoid interference due to external contamination.

References


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