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High-performance liquid chromatographic analysis of β-carbolines in human scalp hair

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Abstract

A chromatographic method was studied for the quantitation of β-carbolines in hair as potent biomarkers. Under optimal conditions, human scalp hair was enzymatically digested to release analytes effectively. The hair digests were treated with fluorescamine before serial extractions to inhibit the artifactual production of β-carbolines during analysis and purify them selectively, followed by reversed-phase high-performance liquid chromatography with fluorometric detection. Hair samples were found to contain β-carboline and 1-methyl-β-carboline, which were identified by tandem mass spectrometry, but not their reduced form 1,2,3,4-tetrahydro-β-carboline and 1-methyl-1,2,3,4-tetrahydro-β-carboline. Both β-carboline and 1-methyl-β-carboline were quantified in the concentration range of 0.1–10.0 ng/ml. Their mean recoveries from hair digests were 70–72%, and the intra- and inter-assay RSD ranged between 6.0 and 10.3% in spiking experiments with standards (1.0 ng/ml). When quantitatively analyzing scalp hair collected from alcoholics, smokers, non-smokers and autistics, β-carboline and 1-methyl-β-carboline showed the concentrations of ng/mg levels or less which characterized different hair samples. The proposed method will be useful for detecting the in vivo concentration changes of β-carbolines associated with alcohol abuse, smoking behavior and neuropsychiatric disorder.
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Keywords: Hair; Carbolines

1. Introduction

A series of bioactive pyridoindole compounds are produced from indoleamines through the Pictet–Spengler condensation with aldehydes [1]. The representative products are formaldehyde-derived 1,2,3,4-tetrahydro-β-carboline (TBC) and acetaldehyde-derived 1-methyl-1,2,3,4-tetrahydro-β-carboline (MTBC) which are oxidized to β-carboline (BC) and 1-methyl-β-carboline (MBC), respectively (Fig. 1). Since the production reaction readily occurs during fermentation and pyrolysis, and also under physiological conditions, significant amounts of β-carbolines are contained in alcoholic beverages and tobacco smoke [2,3], and found in the mammalian body fluids and tissues when availability of the precursor aldehydes is enhanced [4,5]. TBC, MTBC, BC and MBC levels increase in human plasma, urine and platelet by alcohol drinking and tobacco smoking because of their increased exogenous supply and/or their promoted endogenous production [2,6–8]. These β-carbolines and related condensation products show a variety of pharmacological and toxicological effects including neuropsychoactivity, and variations of their in vivo concentrations are associated with pathological states [5,9,10]. They have been considered to play an etiological role in alcoholism and neuropsychiatric disorders [6,11–13].

Compared with other biological matrices, hair sampling is easy, less invasive and suitable for multisample analysis. Hair analysis is useful for detecting the in vivo changes of biochemicals as well as for drug monitoring and forensic toxicology [14–16]. However, no data on the detection of β-carbolines in hair samples are found in the literature, although they have the property amenable to incorporation into hair and the possibility to be biomarkers of alcohol abuse, chemical exposure, and psychiatric and neurological diseases [17].

In the present study, β-carbolines in human scalp hair were analyzed by high-performance liquid chromatography (HPLC) using a hair digestion-extraction procedure designed to avoid their artifactual production. After identification of β-carbolines in hair by HPLC tandem mass spectrometry (HPLC MS MS), the proposed HPLC method was applied to the quantitative analysis of hair samples collected from individuals with alcoholism, autism and smoking habits.

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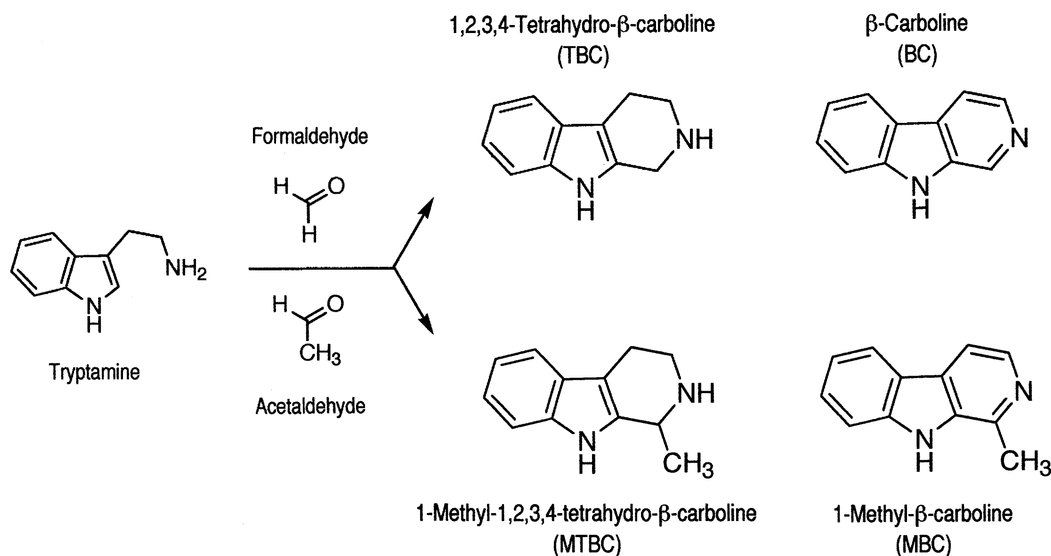

Fig. 1. Condensation schema and structures of β-carbolines.

2. Experimental

2.1. Chemicals and materials

BC, MBC and 3-hydroxymethyl-β-carboline (HMBC) were obtained from Funakoshi (Tokyo, Japan). TBC, MTBC and 2-ethyl-1,2,3,4-tetrahydro-β-carboline (ETBC) were synthesized as reported previously [18,19]. HMBC was used as an internal standard for BC and MBC, and ETBC for TBC and MTBC. All of them were dissolved in acetonitrile and diluted with water as required. A hair digestion kit, Isohair®, was purchased from Nippon Gene (Tokyo, Japan), and fluorescamine from Fluka (Buchs, Switzerland). Acetonitrile and water of HPLC grade from Kishida (Osaka, Japan) were used for preparing the reagent and mobile phase solutions. All other reagents were of the highest analytical grade available.

2.2. Sample preparation

Scalp hair from individuals either with alcoholism or autism was kindly supplied by Dr. Toshihiko Uematsu, the former professor of Gifu University School of Medicine (Gifu, Japan). Scalp hair was also collected from smokers and non-smokers of Asahi University School of Dentistry (Mizuho, Japan). The non-smokers were not habitual alcohol consumers. Informed consent was obtained from all subjects after the nature and consequences of their participation were explained.

Hair samples were washed successively with 1.0% (w/v) sodium dodecyl sulfate solution and water for 30 min to remove contamination due to absorption from environmental tobacco smoke containing β-carbolines [5]. This washing procedure was repeated twice. After drying naturally at room temperature, one or two pieces of hair were weighed and sectioned into ≤2 mm lengths from the scalp end. The hair sections were incubated at 55 °C for 30 min in a mixture of 10 mM semicarbazide (10 μl) and Isohair® reagent solutions consisting of an extraction buffer (200 μl), an enzyme solution (20 μl) and a lysis solution (10 μl). If the digestion was not complete, more enzyme solution (10–30 μl) was added as required and the incubation was continued. To the resulting digest solution, 50 μl of an HMBC (25 ng/ml) and ETBC (250 ng/ml) solution, 0.5 ml of 2 M potassium phosphate buffer (pH 8.5) and 0.5 ml of a fluorescamine solution in acetonitrile (5 mg/ml) were added under vortex-mixing for 30 s. Immediately after that, 0.5 ml of an L-glycine solution (10 mg/ml) in 2 M potassium phosphate buffer (pH 8.5) was added under vortex-mixing for 30 s to consume an excess of fluorescamine. The mixture was extracted with 7.0 ml of ethyl acetate after adding 1.0 ml of 0.5 M NaOH. The organic phase was extracted with 1.0 ml of 0.1 M HCl, and then the aqueous phase was re-extracted with 7.0 ml of ethyl acetate after adding 1.0 ml of 0.2 M K_3PO_4. The finally obtained extract was dried over anhydrous Na_2SO_4 and evaporated to dryness. The residue was dissolved in 200 μl of 0.2% (v/v) trifluoroacetic acid solution. An aliquot (50–100 μl) of the resulting solution was subjected to HPLC analysis as described in Section 2.3.

2.3. HPLC analysis

Chromatography was performed using a Shimadzu (Kyoto, Japan) HPLC system consisting of an LC-10ADVP liquid chromatograph connected to an SCL-10A system controller, a DGU-4A degasser, a 7125 sample injector (Rheodyne, Cotaci, CA, USA), and a Shim-pack CLC-C_8 column (250 mm × 4.6 mm i.d., particle size 5 μm) placed in a CTO-6A column oven. The mobile phase, a mixture of trifluoroacetic acid–acetonitrile–water (0.2:20.0:79.8,

v/v/v), was delivered at a flow-rate of 1.0 ml/min and at a column temperature of 50 °C. BC, MBC and HMBC were detected at excitation and emission wavelengths of 300 and 430 nm, and TBC, MTBC and ETBC at 275 and 350 nm, respectively, by RF-550 and RF-535 fluorometric detectors (Shimadzu) connected in series. A CR-6A Chromatopac data processor (Shimadzu) connected to each detector was used for data analysis. BC and MBC were quantified based on the calibration graphs prepared as described in Section 2.4. Their concentrations in hair were corrected by the recoveries obtained as described in Section 2.4.

2.4. Analytical evaluation

To evaluate the quantitative range, calibration graphs were prepared by plotting the peak area ratios of BC and MBC to HMBC against the known concentrations. Standard BC and MBC dissolved in 10 mM semicarbazide were added to the incubation mixture so that the final concentrations were 0.05–10.0 ng/ml for each, followed by digestion, extraction and HPLC analysis as described in Sections 2.2 and 2.3. The mean ratios of duplicated experiments were plotted at 0.1, 0.25, 0.5, 1.0, 2.0, 3.0, 5.0 and 10.0 ng/ml.

To evaluate the recovery and analytical precision, hair digest mixtures were spiked with standard BC and MBC (1.0 ng/ml for each), and replicate samples ($n = 5$) were subjected to incubation, extraction and HPLC analysis as described in Sections 2.2 and 2.3. The mean recovery, and intra- (analyzed on the same day) and inter-assay RSD (analyzed on different days) were determined.

2.5. HPLC MS MS analysis

Chromatographic separation was performed by an HP 1100 system (Agilent Technologies, Tokyo, Japan) with a Deverosil ODS-UG-5 column (150 mm × 2 mm i.d., particle size 5 μm, Nomura Chemical, Seto, Japan). The mobile phase, a mixture of formic acid–methanol–water (0.1:22.0:78.0, v/v/v), was delivered at a flow-rate of 0.2 ml/min and at a column temperature of 40 °C. The injection volume was set to 20 μl for the hair samples prepared as described in Section 2.2. MS MS analysis was performed by a triple stage quadrupole TSQ 7000 mass spectrometer equipped with an electrospray ionization interface (Thermoquest, Tokyo, Japan). In MS MS experiments, a collision gas pressure was 3.0 mTorr, Ar and the collision energy was 40 eV (1 Torr = 133.322 Pa). For selected reaction monitoring chromatography, product ions were detected by scanning from precursor ion m/z = 169–115 for BC and m/z = 183–115 for MBC.

3. Results and discussion

In the precise quantitation of condensation products like β-carbolines, there are methodological subjects such as

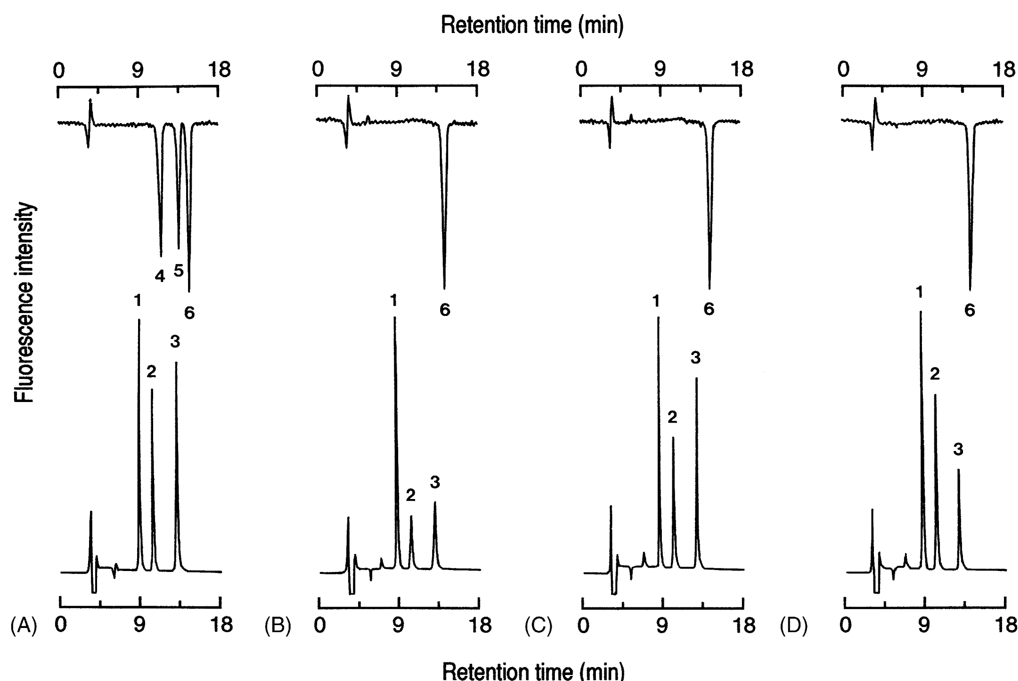

Fig. 2. High-performance liquid chromatograms obtained from standard and hair samples. Fluorometric detection: (lower) excitation at 300 nm and emission at 430 nm for β-carbolines; and (upper) excitation at 275 nm and emission at 350 nm for tetrahydro-β-carbolines. (A) Standard BC (1.5 ng/ml), MBC (1.5 ng/ml), TBC (30.0 ng/ml) and MTBC (30.0 ng/ml); (B) control hair; (C) alcoholic hair; and (D) autistic hair. Peaks: (1) HMBC used as an internal standard for BC and MBC; (2) BC; (3) MBC; (4) TBC; (5) MTBC; and (6) ETBC used as an internal standard for TBC and MTBC.

artifactual formation, specificity and sensitivity [19,20]. Since the condensation between indoleamines and aldehydes readily proceeds even under laboratorial conditions, β-carbolines are artifactually produced during sample work-up and the resulting artifacts are analyzed concomitantly with the original ones [21,22]. Beta-carbolines are contained in biological samples together with structurally related compounds and at low concentrations (pg/ml to ng/ml levels), requiring a very specific and sensitive method for their quantitation [20,23,24]. Hair samples have been routinely digested by heating in 0.1–1.0 M HCl or NaOH at 56–80 °C for 0.5–12 h [14,15,25]. However, such digestive conditions are not necessarily favorable for the stability of β-carbolines and possibly promote their artifactual production [19,20]. In order to solve these problems of chemical reactions, enzymatic digestion was employed, resulting in effective release of β-carbolines from hair under relatively mild conditions and in a short time (completed within 30–60 min). When standard tryptamine solutions (50 ng/ml) were incubated under enzymatic reaction conditions for 30 min and extracted by the solid-phase and liquid-phase methods widely used for purifying biological samples, β-carbolines of pg levels were produced from contaminant aldehydes [23]. In order to inhibit the artifactual production of β-carbolines during hair digestion, sample solutions were treated with semicarbazide that trapped the precursor aldehydes contaminated in samples [24,26]. The hair digests were subsequently reacted with fluorescamine to convert precursor amines to carboxylic derivatives which were removed from the analytical system by solvent extractions, resulting in not only effective inhibition of the artifactual formation but also selective purification of β-carbolines as reported previously [3,20,23]. When standard TBC, MTBC, BC and MBC (1.0–100 ng/ml for each) were subjected to

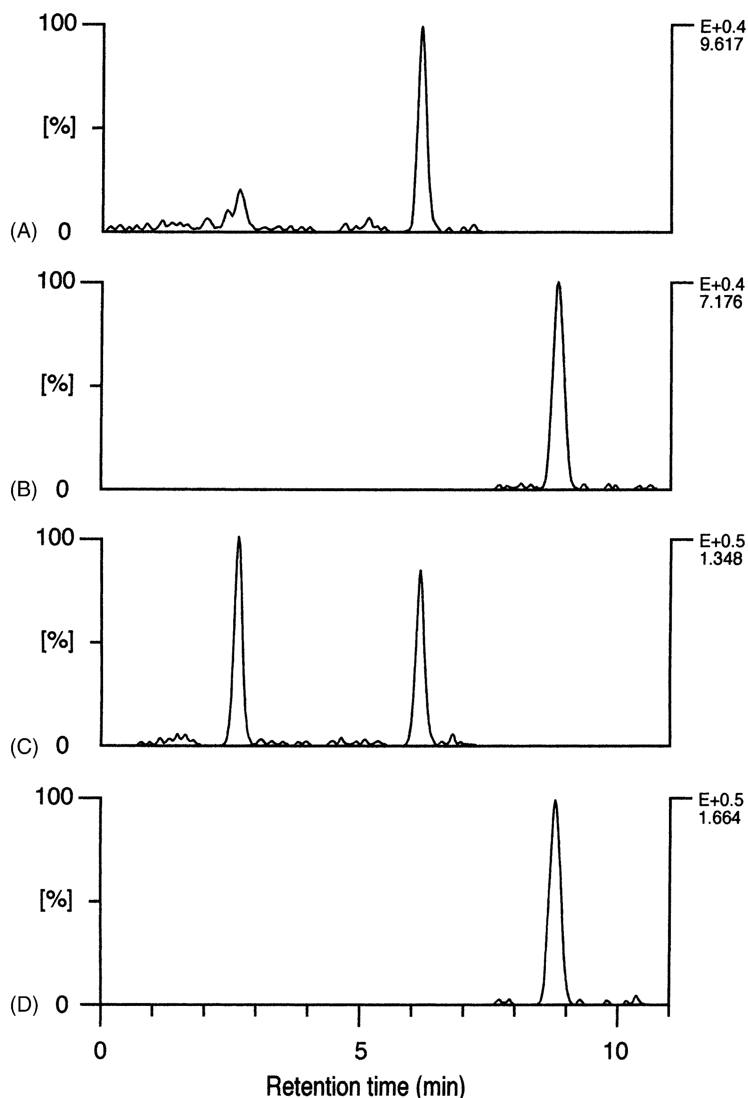

Fig. 3. Mass chromatograms obtained from standard and hair samples. Monitoring ion: *m/z* 169 for (A) and (C); and *m/z* 183 for (B) and (D). (A) Standard BC at 6.1 min and unknown contaminant at 2.6 min; (B) standard MBC at 8.8 min; (C) BC in hair at 6.1 min and unknown contaminant at 2.6 min; and (D) MBC in hair at 8.8 min.

digestion and extraction, they showed neither oxidation nor decomposition, indicating that all analytes are stable during hair sample preparation and the proposed procedure does not cause the detectable oxidation of TBC and MTBC.

Many compounds with the β-carboline structure naturally fluoresce, which allows for their sensitive detection. However, maximum excitation and emission wavelengths were different between β-carbolines (BC and MBC) and tetrahydro-β-carbolines (TBC and MTBC) [27]. Two fluorometric detectors were connected in series to quantify both types of analytes simultaneously (Fig. 2). When β-carbolines (1.5 ng/ml for each) and tetrahydro-β-carbolines (30.0 ng/ml for each) were chromatographed under detection conditions for TBC and MTBC (excitation at 275 nm and emission at 350 nm) and for BC and MBC (excitation at 300 nm and emission at 430 nm), respectively, they produced no peaks on each individual chromatogram. Since no peaks were detected at the retention times of HMBC and ETBC in all the tested scalp hair, they were used as the internal standards for extraction procedure and peak identification. The digested and extracted hair samples provided two peaks in the detection system for β-carbolines, but not in that for tetrahydro-β-carbolines.

The two peaks obtained from HPLC separation were identified as BC and MBC in the elution order by comparing selected reaction monitoring chromatograms between standard and hair samples (Fig. 3). Mass spectra showed *m/z* 169 (MH^+) for BC and *m/z* 183 (MH^+) for MBC. Hair samples provided two peaks at *m/z* 169 and 183 which were eluted at the retention times (6.1 and 8.8 min) identical to those of standard BC and MBC. Although BC and MBC were found in all pieces of hair, their precursors in a reduced form, TBC and MTBC, were not detected in any hair samples. The oxidation of TBC and MTBC is negligible during digestion and extraction as described in determination of sample preparation conditions. It is unknown whether BC and MBC are exclusively incorporated into scalp hair or all of TBC and MTBC are oxidized in hair after incorporation. In the following studies, the quantitative analysis was focused on BC and MBC which were present in hair as the potent biomarkers.

The peak area ratios of standards of known concentrations to HMBC were plotted after all procedures including digestion, extraction and HPLC separation. The prepared calibration graphs showed good linearity in the concentration range of 0.1–10.0 ng/ml for both BC and MBC. The regression equations were found to be $y = 0.1283x$ ($r^2 = 0.966$) for BC and $y = 0.2073x$ ($r^2 = 0.9717$) for MBC. Analytical recovery and reproducibility of the proposed method were evaluated by analyzing replicate hair digest samples spiked with standard BC and MBC (1.0 ng/ml, corresponding to the concentration in hair digests). The recoveries (mean ± S.D.) of BC and MBC were 72.2 ± 3.6 and 70.7 ± 3.3%, respectively. The intra- and inter-assay RSD were 6.0 and 10.3% for BC and 6.3 and 7.5% for MBC.

The proposed method was applied to human scalp hair collected from different subjects. The quantitative results are shown in Table 1. While the number of subjects was limited, β-carbolines were likely to increase in hair samples of alcoholics (BC and MBC, $P < 0.01$), autistics (BC, $P < 0.01$ and MBC, $P < 0.05$), and smokers (BC, $P < 0.05$) compared with non-smokers who had no alcohol drinking habits. Difference in hair concentrations suggests the potential of β-carbolines as biomarkers in hair analysis.

Table 1
Beta-carbolines in human scalp hair

Hair sample	Concentration (ng/mg)	
	BC	MBC
Alcoholic 1	2.29	2.94
Alcoholic 2	1.57	1.53
Alcoholic 3	1.91	1.41
Alcoholic 4	1.98	2.33
Alcoholic 5	0.94	1.07
Mean ± S.E. (n = 5)	1.74 ± 0.23	1.86 ± 0.34
Autistic 1	1.43	0.98
Autistic 2	2.06	0.69
Autistic 3	1.25	0.89
Autistic 4	1.84	0.57
Autistic 5	2.53	1.33
Mean ± S.E. ($n = 5$)	1.82 ± 0.23	0.89 ± 0.13
Non-smoker 1	1.15	0.71
Non-smoker 2	1.05	0.68
Non-smoker 3	1.22	0.71
Non-smoker 4	0.87	0.52
Non-smoker 5	0.72	0.40
Mean ± S.E. ($n = 5$)	1.00 ± 0.09	0.60 ± 0.06
Smoker 1	1.15	0.99
Smoker 2	1.58	0.34
Smoker 3	2.98	0.66
Smoker 4	1.57	0.55
Smoker 5	1.44	0.21
Mean ± S.E. ($n = 5$)	1.74 ± 0.32	0.55 ± 0.14

Hair samples were collected from individuals with alcoholism or autism, smokers, and non-smokers without alcohol drinking habits. They were subjected to digestion, extraction and HPLC analysis for determining BC and MBC concentrat ions in hair.

Hair analysis is able to provide information reflecting the in vivo concentration changes of endogenous and/or exogenous substances, chemicals, drugs, toxicants, etc. [14–17,25]. Since the compounds with relatively short lifetime and low concentration in blood are time-dependently entrapped and concentrated in hair, they are detectable for a long time and hair-segmental analysis indicates the personal history concerning drug addiction, chemical exposure and pathological state. A range of compounds have been subjected to screening for the biomarkers in hair analysis: ethylglucuronide, fatty acid ethyl esters and acetaldehyde–protein adducts for alcohol abuse and chronically elevated alcohol consumption [17,28,29]; nicotine and cotinine for smoking behavior and exposure to tobacco smoke [30,31]; and minerals including calcium, magnesium, copper, chromium,

etc. for autism [32,33]. However, there are still unresolved issues such as hydrophilic ethylglucuronide unsuitable for incorporation into hair, acetaldehyde–protein adducts not tested in human hair, nicotine and cotinine influenced by hair treatments, and fatty acid ethyl esters and minerals not closely linked to pathogenesis (alcoholism or autism). In contrast, β-carbolines have the basicity and lipophilicity required for deposition into hair [17], the character to increase in the human body fluids and tissues in association with alcohol ingestion and tobacco smoking [2,6–9], and the etiological relevance for neurospychiatric disorders [5,12,13]. BC and MBC would be useful biomarkers for hair analysis.

This study is the first to reveal the incorporation of BC and MBC into human scalp hair and determine their concentrations therein. The proposed HPLC method may be a diagnostic tool for chronic alcohol drinking, tobacco smoking and neuropsychiatric disorders which are related to the in vivo concentration changes of β-carbolines.

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