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Quantitative analysis of menthol in human urine using solid phase microextraction and stable isotope dilution gas chromatography–mass spectrometry

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ABSTRACT

To accurately measure menthol levels in human urine, we developed a method using gas chromatography/electron ionization mass spectrometry with menthol-d_4 stable isotope internal standardization. We used solid phase microextraction (SPME) headspace sampling for collection, preconcentration and automation. Conjugated forms of menthol were released using β-glucuronidase/sulfatase to allow for measuring total menthol. Additionally, we processed the specimens without using β-glucuronidase/sulfatase to quantify the levels of unconjugated (free) menthol in urine. This method was developed to verify mentholated cigarette smoking status to study the influence of menthol on smoking behaviour and exposure. This objective was accomplished with this method, which has no carryover or memory from the SPME fiber assembly, a method detection limit of 0.0017 μg/mL, a broad linear range of 0.002–0.5 μg/mL for free menthol and 0.01–10 μg/mL for total menthol, a 7.6% precision and 88.5% accuracy, and an analysis runtime of 17 min. We applied this method in analysis of urine specimens collected from cigarette smokers who smoke either mentholated or non-mentholated cigarettes. Among these smokers, the average total urinary menthol levels was three-fold higher ($p < 0.001$) among mentholated cigarette smokers compared with non-mentholated cigarette smokers.

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1. Introduction

Menthol is a naturally occurring compound with topical cooling and anesthetic properties used in a wide range of products including common cold medications, toothpastes, confectionery, pesticides, and cigarettes. Relevant to this study is its use as a flavor additive in milligram quantities (typically as L-menthol) in mentholated cigarettes. Although menthol is not considered a carcinogen, it may increase carcinogen uptake by numbing the respiratory tract so that smoke is inhaled deeper and held longer. Indeed, the high prevalence of mentholated cigarette use among African Americans has been hypothesized to explain this population's disproportionately higher lung cancer risk per cigarette smoked (menthol cigarettes smoked by >88% vs. 26% for whites) [1].

Results from studies evaluating the association between mentholated cigarette usage and increased lung cancer risk have been varied [2–6]. For example, a Kaiser Permanente study conducted between 1979 and 1986 found a statistically significant increase in risk for menthol cigarette smokers [2]. In males, the relative risk for menthol smokers was 1.45 (95% confidence interval 1.03–2.02). In females, the relative risk was 0.75 (95% confidence interval 0.52–1.11). On the contrary, the Southern Community Cohort Study, found a statistically significant reduced risk of lung cancer in non-mentholated smokers vs. mentholated cigarette smokers [7]. To better understand conflicting studies and discern the influences of menthol on smoking behaviour and associated health outcomes, a highly accurate and precise analytical method to verify biomarkers of menthol exposure is necessary.

Several researchers have investigated menthol analysis methods for biological specimens. Although high pressure liquid chromatography/mass spectrometry (HPLC–MS) has been demonstrated [8], most method development has involved gas chro-

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matography (GC) separation, because menthol is still within the realm of a volatile organic compound, with a boiling point of 212 °C and thermal stability [9,10]. As a result, GC methods have yielded better sensitivity than LC methods by at least two orders of magnitude, especially when combined with mass spectrometric detection. In 2004, Spichiger et al. incorporated solid phase microextraction (SPME) as a means to preconcentrate menthol collected in the headspace (HS) over urine and serum specimens before and after hydrolysis of menthol glucuronide adducts [11]. This SPME method experienced persistent background levels, especially for urine specimens, possibly from menthol penetration within the SPME fiber assembly caused by a high (80 °C) collection temperature and a relatively long (20 min) collection time. Schulz et al. used a similar method to measure menthol and three other compounds in serum specimens lowering the collection temperature to 50 °C over a 30 min collection time. They reported no carryover or memory, but a similar limit of detection (LOD) of 0.0046 μg/mL for the analysis of serum specimens [12]. However, analysis of menthol in urine is advangateous to analysis in serum because urine collection is non-invasive and menthol is readily partitioned from urine, which is a more polar matrix than serum. Moreover, menthol persists longer in urine than in blood [13] with an α-phase half-life of 56.2 min for blood vs. 74.9 min for urine.

Here we present an improved GC–MS method for analysis of free menthol and total menthol in urine specimens using a HS/SPME based method that eliminates carryover from the SPME fiber assembly. Our improved GC–MS method is validated with internal quality control, performance testing, and subsequent method verification involving analysis of menthol levels in urine from non-mentholated and mentholated cigarette smokers.

2. Experimental

2.1. Chemicals

Native L(−)-menthol (5-methyl-2-[1-methylethyl] cyclohexanol, 99.7%), mentholglucuronic acid ammonium salt, Type H-1 β–D-glucuronidase/sulfatase (Type H-1, from *Helix pomatia*), trisodium citrate dihydrate (SigmaUltra grade), and citric acid monohydrate (ACS reagent grade) were purchased from Sigma Chemical Company (St. Louis, MO, USA). (−)-Menthol-1,2,6,6-d_4 (98%) was purchased from Cambridge Isotope Lab (Andover, MA, USA). Water (HPLC grade) was purchased from J.T. Baker (Phillipsburg, NJ, USA), and methanol (GC^2 Capillary GC/GC–MS grade) was purchased from Burdick and Jackson (Muskegon, MI, USA). Helium gas for GC–MS (ultra high-purity grade) was purchased from Airgas Inc. (Jacksonville, FL, USA).

2.2. Urine specimen collection

The method was applied to urine collected from established smokers (i.e., individuals who smoked at least 6 cigarettes per day for at least the past three years). Pregnant participants, participants arriving intoxicated to any visit, and participants with self-reported smoking-related diseases were excluded. Participants signed informed consent documents and subsequently provided urine specimens. The study protocol was approved by CDC's institutional review board. Urine from 95 smokers was used to verify this method. Smokers consisted of 27 non-mentholated and 68 mentholated cigarettes smokers.

This study was approved by the Battelle Centers for Public Health and Research Evaluation (CPHRE) Institutional Review Board (IRB# FG465925-04) to ensure the protection of participants' safety, rights, and welfare. The Center for Disease Control and Prevention's (CDC) role was limited to analysis of coded specimens and was determined to not constitute engagement in human subjects research.

2.3. Urine specimen preparation

Unknown specimens were prepared in 10 mL headspace SPME vials obtained from MicroLiter Analytical Supplies Inc. (Suwanee, GA, USA). For free menthol measurement, 100 μL of urine, 100 μL of 0.1 M trisodium citrate dihydrate buffer (pH 5.0), and 50 μL of 5 μg/mL menthol-d_4 internal standard solution were added to the vial. The total liquid volume was 0.25 mL. For total menthol measurements, the buffer was replaced with the same volume of an enzyme solution that was made by adding β-D-glucuronidase into the buffer at a concentration of 3 mg/mL. The SPME vial was then sealed with a 1-mm thick, 20-mm PTFE/silicone septum (Supelco, St. Louis, MO, USA) and capped using a M-10 flat washer spacer (Hillman, Cincinnati, OH, USA) and a 20-mm open-center steel seal (Supelco, St. Louis, MO, USA). Specimens prepared for free menthol measurement were ready to analyze immediately. Specimens prepared for total menthol measurement were put into an oven and incubated at 37 °C for 24 h to ensure complete deconjugation of the menthol glucuronide. Once samples are ready for analysis they equilibrate on the PAL autosampler in queue for approximately 5 h as instrument and fiber blanks, standards and QCs are run.

2.4. Standards preparation

All standards were prepared identically to unknown specimens with the exception of replacing 100 μL urine with 100 μL synthetic urine (CTSI, Great Neck, NY, USA). For total menthol measurement, the calibration curve consisted of 6 points (0.01, 0.1, 0.5, 2, 5, and 10 μg/mL). For free menthol measurement, the calibration curve consisted of 8 points (0.001, 0.002, 0.005, 0.01, 0.02, 0.05, 0.1, and 0.5 μg/mL).

2.5. Characterization of quality control (QC) samples

Total and free quality control pool samples were characterized prior to any urine specimen analysis by spiking menthol or menthol glucuronide into an anonymously donated and homogenized urine pool. Total menthol QC pools were prepared at two different levels by adding an aqueous solution of mentholglucuronic acid ammonium salt into the urine pools to achieve the desired levels. These total menthol QC samples were characterized from 20 independent batches within a 5-month period, using the same deconjugation method as for the specimens. This determination yielded mean concentrations of 1.43 ± 0.08 μg/mL (N = 20) for the total QC low (TQCL) pool and 8.77 ± 0.50 μg/mL (N = 20) for the total QC high (TQCH) pool.

Free menthol QC pools were prepared at two different levels by adding menthol solution to urine pools to achieve characterized levels of 0.039 ± 0.004 μg/mL (N = 13) for the free QC low (FQCL) pool and 0.267 ± 0.033 μg/mL (N = 14) for the free QC high (FQCH) pool. The concentrations for both the total menthol and free menthol QC pools reflect the sum of the background levels in the anonymous urine and the spiked amount.

A typical run included at least four QC samples where a QC low and QC high bracketed the unknown specimens. The two samples from the same QC pool were averaged. All measurements within a run batch were considered invalid if: (1) the difference between the two averaged QCs was within a factor of 4 of the SD of the independent characterization QCs, (2) the level of QC low or QC high was more than 3 SDs of the characterized mean, (3) both QC low and QC high concentrations were outside 2 SD limits, and (4)