Journal of Chromatography B, 953–954 (2014) 126–131

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Effect of cigarette smoking on urinary 2-hydroxypropylmercapturic acid, a metabolite of propylene oxide

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ARTICLE INFO

Article history:
Received 25 October 2013
Accepted 3 February 2014
Available online 14 February 2014

Keywords:
Propylene oxide
Mercapturic acid
Cigarette smoke
Smoking cessation

ABSTRACT

2-Hydroxypropylmercapturic acid (2-HPMA) is a urinary biomarker of exposure to propylene oxide, a mutagen and carcinogen to which humans are exposed through inhalation of cigarette smoke as well as in certain environmental and occupational settings. 2-HPMA is the final product of a detoxification pathway in which propylene oxide is conjugated with glutathione, and the resulting conjugate is further metabolized and excreted. We have developed and validated a liquid chromatography-atmospheric pressure chemical ionization–tandem mass spectrometric (LC-APCI–MS/MS) method for the rapid quantitation of 2-HPMA in human urine. The method was applied to an analysis of urine samples from 40 smokers and 40 nonsmokers as well as from a group of 15 subjects who quit smoking. The results demonstrate that smokers have significantly ($P < 0.001$) higher levels of urinary 2-HPMA (median = 480 pmol/mg creatinine) than do nonsmokers (208 pmol/mg). Similarly, subjects who quit smoking for four weeks exhibited a significant ($P < 0.001$) 52% median decrease in urinary 2-HPMA upon cessation. Approximately 5% of all urine samples had unusually high levels of 2-HPMA (>10 times higher than the median), apparently unrelated to tobacco smoke exposure or available demographic data. The method presented here can be used to rapidly quantify an individual's exposure to propylene oxide via tobacco smoke or other sources.

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1. Introduction

Propylene oxide (PO; 2-methyloxirane) is a colorless, volatile liquid and strong irritant. PO has shown clear evidence of carcinogenicity in rats, some evidence of carcinogenicity in mice [1], and is "reasonably anticipated to be a human carcinogen" by the National Toxicology Program [2]. The International Agency for Research on Cancer evaluated PO as "possibly carcinogenic to humans" (Group 2B) [3].

Cigarette mainstream smoke contains PO at levels reported as 0.65–0.93 μg/cigarette [4]. It has been listed by the US Food and Drug Administration as one of the "harmful and potentially harmful constituents" of cigarette smoke [5]. Nonsmokers can also be exposed to PO. It is used as a soil fumigant, herbicide, insecticide, and fungicide; and also as a means of sterilization for packaged foods [2,6]. Certain household products, such as carpet cleaners and automobile lubricants, contain PO [7]. PO is used industrially as an intermediate in making polyurethane foams and propylene glycol resins, and workers in these industries can be exposed via inhalation or dermal contact. Another potential source of PO exposure is metabolism of propylene, but this seems to play a relatively minor role [3,8].

PO may contribute to the toxic and carcinogenic effects of cigarette smoking, but there is relatively little information available. One way of monitoring PO uptake in smokers and nonsmokers is by quantifying its metabolites. PO is conjugated with glutathione, leading ultimately to excretion of 2-hydroxypropylmercapturic acid (2-HPMA) in urine (Scheme 1). There are only 3 previous reports of 2-HPMA levels in the urine of smokers and nonsmokers [9–11]. They all indicated high levels in smokers. We are not aware of any reports on the effect of smoking cessation on urinary 2-HPMA levels. In the study reported here, we have developed an accurate and precise liquid chromatography-atmospheric pressure chemical ionization–tandem mass spectrometry (LC-APCI–MS/MS) method for analysis of 2-HPMA in human urine and have applied

Abbreviations: PO, propylene oxide; 2-HPMA, 2-hydroxypropylmercapturic acid; 3-HPMA, 3-hydroxypropylmercapturic acid; LOD, limit of detection; LOQ, limit of quantitation; CV, coefficient of variation; LC-APCI–MS/MS, liquid chromatography-atmospheric pressure chemical ionization–tandem mass spectrometry; SRM, selected reaction monitoring; Q_{high}, high-concentration quality control urine sample; Q_{low}, low-concentration quality control urine sample.

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http://dx.doi.org/10.1016/j.jchromb.2014.02.001
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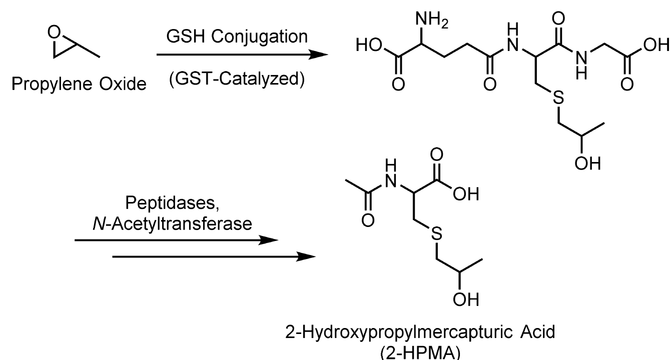

Scheme 1. Formation of 2-HPMA from propylene oxide. Propylene oxide reacts with glutathione, which can be catalyzed by GSTs. The initial glutathione conjugate is further metabolized to 2-HPMA and excreted in the urine.

it to urine samples from smokers, nonsmokers, and smokers who quit smoking.

2. Methods

2.1. Materials

2-HPMA dicyclohexylammonium salt and [D_3]2-HPMA dicyclohexylammonium salt were purchased from Toronto Research Chemicals. Oasis MAX solid-phase extraction 96-well plates (60 mg, 60 μm, 2 mL reservoir) were acquired from Waters Corporation. Square 2 mL 96-well plates with TrueTaper^{TM} 100 μL tapered reservoirs were purchased from Analytical Sales and Services. Sealing mats for square 96-well plates and a 50 mm × 3.0 mm Synergi C12, 2.5 μm, Max-RP, 100 Å HPLC column were obtained from Phenomenex. All other materials and chemicals were purchased from U-Stores, University of Minnesota. All solutions and buffers were prepared freshly on the same day as the assay.

2.2. Urine samples

These studies were approved by the University of Minnesota Institutional Review Board. The levels of 2-HPMA were measured in a set of 40 smokers' and 40 nonsmokers' urine, obtained through the University of Minnesota Tobacco Programs Biorepository, which was established to collect biological samples for tobacco-related biomarker analysis and development. Demographic information (gender, age, and cigarettes smoked per day) was collected from each of the subjects. Pooled smokers' and nonsmokers' urine samples used for method validation were also obtained from this biorepository and from voluntary subjects, respectively.

2-HPMA levels were also analyzed in a smoking cessation study, the details of which have been previously reported [12]. Briefly, cigarette smokers who wanted to quit smoking were recruited from the local Twin Cities area via advertisements on the radio, cable TV, the internet, and in brochures in doctors' offices. Inclusion criteria were 18–70 years of age, smoked ≥10 cigarettes per day for ≥1 year, generally in good physical and mental health, and possible candidates for nicotine replacement therapy. To aid in cessation, the subjects were offered a nicotine patch, gum, lozenge, or combination thereof, as needed. Subjects were paid in increasing amounts as they abstained for the duration of the study. Twenty-four-hour urine samples were collected seven days before cessation to establish baseline biomarker levels and collected again four weeks after cessation.

2.3. Analysis of 2-HPMA in urine

Urine samples (0.4 mL) were transferred into a 96-well plate and 0.1 mL internal standard solution [100 ng (0.45 nmol) of [D_3]2-HPMA dicyclohexylammonium salt] was added. Mixed-mode anion exchange solid phase extraction cartridges (Oasis MAX) were preconditioned with CH_3OH (0.7 mL) and 2% aq NH_4OH (0.7 mL, pH = 11). The urine samples were applied, and the cartridges were washed with 2% aq NH_4OH (0.7 mL) and CH_3OH (0.7 mL) and then dried for 5 min with a stream of N_2. The cartridges were washed with 2% aq HCOOH (0.7 mL, pH = 1), and then the analyte was eluted with 30% CH_3OH in 2% aq HCOOH (0.7 mL). This fraction was concentrated to dryness *in vacuo* and reconstituted in 20% CH_3OH (100 μL) for LC-APCI–MS/MS analysis.

For the chromatographic conditions, the aqueous phase consisted of 15 mM NH_4OAc (pH 6.8, unadjusted) and the organic phase was CH_3OH. The gradient program (% aqueous:% organic) was as follows: 98:2 from 0 to 4 min, ramp for 0.5 min and held at 30:70 from 4.5 to 6.5 min, ramp for 0.5 min and held at 98:2 from 7 to 12 min. The flow rate was 400 μL/min, the column temperature was 40 °C and the injection volume was 3 μL. LC-APCI–MS/MS analysis was conducted on a TSQ Quantum Discovery Max instrument (Thermo Scientific) with conditions as follows: ionization source, negative mode APCI; collision energy, 13 V; peak width parameters, Q1 = 0.7, Q3 = 0.7; scan width, 0.4 *m/z*; scan time, 0.1 s; and selected reaction monitoring (SRM), *m/z* 220.07 → 91.0 ± 0.2 for 2-HPMA and *m/z* 223.09 → 91.0 ± 0.2 for [D_3]2-HPMA.

Quantitation of 2-HPMA was based on a linear calibration curve, constructed in water from five standard solutions of 2-HPMA (0.4, 2, 10, 50, and 100 ng/μL), each with the same concentration of [D_3]2-HPMA (1.1 ng/μL). The measured area ratio of 2-HPMA:[D_3]2-HPMA was plotted against the known concentration ratio. The slope of the calibration curve was 1.01 (range 0.98–1.03), and the calibration covered the range of observed 2-HPMA values in urine samples. The same stock solution of [D_3]2-HPMA was used to construct the calibration curve as was added to each sample as the internal standard. 2-HPMA in the samples was calculated by relating the measured area ratio of 2-HPMA:[D_3]2-HPMA to the unknown 2-HPMA concentration via the slope of the calibration curve and the known concentration of [D_3]2-HPMA. Four samples of pooled smokers' urine were included with each set of urine to monitor assay performance.

2.4. Analysis of nicotine, cotinine, and creatinine

Urinary nicotine and cotinine levels were analyzed as described [13]. Briefly, 0.1 mL urine was added to 0.9 mL 50% aq K_2CO_3 (pH 12), and to this was added 10 μL of [CD_3]nicotine and [CD_3]cotinine internal standard mixture (47.6 ng and 17 ng, respectively). The mixture was extracted into 1 mL CH_2Cl_2, and the organic layer was concentrated and analyzed by gas chromatography–mass spectrometry. Urinary creatinine was analyzed by an established colorimetric assay using a Creatinine Microplate Assay from Eagle Biosciences (Boston, MA) [14].

2.5. Statistical analysis

There were 78 urine samples from 39 smokers and 39 nonsmokers with complete 2-HPMA data and two samples below the limit of detection. Our goal was to compare smokers versus nonsmokers, but any difference between these two groups might be confounded by a number of factors (age, gender, and creatinine level). We applied multiple regression analysis, with 2-HPMA as the dependent variable in order to adjust for the potential confounders. Data for 2-HPMA were highly skewed to the right, so we performed the analysis on the regular scale and the log scale. The

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