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Quantitative determination of hydroxy polycylic aromatic hydrocarbons as a biomarker of exposure to carcinogenic polycyclic aromatic hydrocarbons

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ABSTRACT

A high-resolution gas chromatography/high-resolution mass spectrometry (HRGC/HRMS) method was developed for quantitative analysis of hydroxy polycyclic aromatic hydrocarbons (OH-PAHs). Four hydroxy metabolites of known and suspected carcinogenic PAHs (benzo[*a*]pyrene (B[*a*]P), benz[*a*]anthracene (B[*a*]A), and chrysene (CRY)) were selected as suitable biomarkers of PAH exposure and associated risks to human health. The analytical method included enzymatic deconjugation, liquid – liquid extraction, followed by derivatization with methyl-*N*-(trimethylsilyl) trifluoroacetamide and instrumental analysis. Photo-induced oxidation of target analytes – which has plagued previously published methods – was controlled by a combination of minimizing exposure to light, employing an antioxidant (2-mercaptoethanol) and utilizing a nitrogen atmosphere. Stability investigations also indicated that conjugated forms of the analytes are more stable than the non-conjugated forms. Accuracy and precision of the method were 77.4–101% (<4.9% RSD) in synthetic urine and 92.3–117% (<15% RSD) in human urine, respectively. Method detection limits, determined using eight replicates of low-level spiked human urine, ranged from 13 to 24 pg/mL. The method was successfully applied for analysis of a pooled human urine sample and 78 mouse urine samples collected from mice fed with PAH-contaminated diets. In mouse urine, greater than 94% of each analyte was present in its conjugated form.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are formed from incomplete combustion of organic matter, and are widely distributed in the ambient environment. Sources of human exposure to PAHs include industrial processes (e.g. petroleum refining), domestic heating, waste incineration, motor vehicle emissions as well as from smoking and dietary sources [1–3]. Approximately 500 individual PAHs have been detected in air, of which 16 are considered a priority by the United States Environmental Protection Agency, owing to their potential adverse health effects in humans [4].

Following exposure, PAHs undergo oxidation by cytochrome P450 enzymes to form hydroxylated PAHs (OH-PAHs). These metabolites may be further biotransformed to reactive electrophiles, which can bind covalently to DNA, leading to carcinogenicity and mutagenicity [5–10]. Deactivation and excretion is facilitated through phase 2 enzymes which produce glucuronide or sulfate conjugates. As the metabolism of PAHs occurs rapidly *in vivo*, exposure characterization relies upon determination of both hydroxylated and conjugated forms of PAH metabolites [11].

OH-PAHs can be analyzed by derivatization gas chromatography high resolution mass spectrometry (GC-HRMS) [12,13], liquid chromatography tandem mass spectrometry (LC–MS/MS) [14–16], or derivatization LC–MS/MS [17–19] but analyte instability remains a frequent and unresolved problem [20–23]. For example, both Whiton et al. [20] and Olmos-Espejel et al. [22] observed significant instability of 3-OH-B[a]P, which prevented determination of this compound with adequate accuracy and precision. During the 2001–2002 and 2003–2004 national health and nutrition exam-

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ination survey (NHANES), less than 50% of hydroxylated PAHs monitored were reported due to unreliable test results for these compounds [21]. Recent NHANES reports continued to include similar analytes [24]. Further investigations into the instability of various OH-PAHs were conducted by Schantz et al. [25] but the problem of analyte losses remains unresolved and low analyte recoveries continue to be reported in recent publications [26]. Some studies have speculated that OH-PAH instability is due to oxidation and have employed antioxidants such as 2-mercaptoethanol (2-ME), for example in measurement of OH-PAHs in fish bile [27]. However, the effectiveness of this antioxidant remains unclear. Tert-butyl hydroquinone has been employed in a similar manner for analysis of OH-PAHs in milk and manure matrices [28,29], resulting in improved recoveries. However, the exact cause of analyte loss remains unclear, as does the relative stability of conjugated versus non-conjugated species, which is particularly relevant for urine matrices.

In this study, hydroxy metabolites of benzo[*a*]pyrene (B[*a*]P), benz[*a*]anthracene (B[*a*]A), and chrysene (CRY), which all contain 'bay regions' that favor production of reactive and potentially carcinogenic metabolites [30–32] were selected as suitable biomarkers of PAH exposure. The objectives of this work were to: 1) develop a method for quantitative analysis of OH-PAHs in urine; 2) investigate the cause of rapid degradation of OH-PAHs during sample handling; including relative stability of conjugated versus non-conjugated OH-PAHs and implications for sample analysis, storage and experimental designs; and 3) implement measures to control analyte instability during analysis and storage in order to produce a reproducible and defensible analytical method suitable for measurements of OH-PAHs in occupational exposure and pharmacokinetic studies.

2. Experimental

2.1. Standards and reagents

All solvents were HPLC grade. The native analytes 9-OH-benzo[*a*]pyrene (>99%; 9-OH-B[a]P), 3-OH-benz[*a*]anthracene (97%; 3-OH-B[a]A), 3-OH-chrysene (>99%; 3-OH-CRY), pyrene-1-sulfate, potassium salt, $^{13}C_6$-3-OH-chrysene >95%; $^{13}C_6$-3-OH-CRY,- and $^{13}C_6$-1-OH-benz[*a*]anthracene (>99%), ($^{13}C_6$-1-OH-B[*a*]A) were obtained from MRI Global Chemical Carcinogen Repository (Kansas City, Missouri, USA). 3-OH-benzo[*a*]pyrene (95%; (3-OH-B[*a*]P) was obtained from Toronto Research Chemicals Inc. (TRC; Ontario, Canada). 1-OH-naphthalene (>99%; 1-OH-NAP) and 1-OH-pyrene (98%; 1-OH-PYR) were obtained from Sigma-Aldrich, Saint Louis, MO, USA. $^2H_{11}$-3-OH-Benzo[*a*]pyrene (95%; $^2H_{11}$-3-OH-B[*aa*]P) was obtained from Toronto Research Chemicals Inc. (TRC), Ontario, Canada. $^{13}C_{12}$-4′-OH-3,3′,4,5′-TetraCB(>98%), $^{13}C_6$-1-OH−naphthalene (>98%; $^{13}C_6$-1-OH−NAP), and $^{13}C_6$-1-OH-pyrene- (>98%) $^{13}C_6$-1-OH-PYR, were obtained from Cambridge Isotope laboratories Inc., And over, MA, USA. $^{13}C_{12}$-PCB-81 (>98%) was obtained from Wellington Laboratories, Guelph, ON, Canada. 1-naphthyl-β-D-glucuronide sodium salt was obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). ß-Glucuronidase from *Helix pomatia* type H-1, partially purified powder, ≥300,000 units/g solid was obtained from Sigma Aldrich (St. Louis, MO, USA). *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) and the antioxidants 2-mercaptoethanol and butylated hydroxytoluene (BHT) were obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Preparation of standard solutions

All glassware including gas chromatography (GC) microvials were silanized by soaking in 5% dimethyldichlorosilane in heptane for 15 min. All native and isotopically-labeled standard solutions were prepared in the dark to minimize photo-induced degradation. In addition, 30 μL of 2-ME was added to solutions (not more than 10 mL volumes) to minimize oxidation of OH-PAHs. Native and surrogate standard solutions were prepared in acetonitrile to ensure quick mixing with urine or aqueous matrices. Prepared solutions were kept in amber containers and wrapped with foil to minimize exposure to light. The derivatization control and recovery standard solution were prepared in toluene. All standard solutions were stored at −80 °C.

A series of eight calibration solutions containing all native and labeled standards (Table S1) were derivatized and used to establish the initial calibration of the instrument. The concentration of native analytes varied from 0.25 to 1000 ng/mL, while the concentrations of surrogates, derivatization standards and recovery standards remained constant at 200, 50, and 50 ng/mL, respectively. Calibration solutions were prepared in toluene and were stored at −80 °C for a maximum of three weeks prior to use.

2.3. Sample collection

Synthetic urine was prepared as described in Gustafsson and Uzqueda [33]. A brief description of preparation of synthetic urine is provided in Table S3. Pooled human urine samples were collected from volunteers. Mouse urine samples were collected as part of a larger PAH exposure experiment conducted at Ricerca Biosciences (Concord, Ohio, USA). Briefly, mice were divided into 5 groups: Group 1 (controls) were administered a diet consisting of uncontaminated soil (i.e. <0.17 μg/g CRY, B[*a*]A, and B[*a*]P) mixed with feed. Groups 2, 3, and 4 were administered diets consisting of feed mixed with 5, 10, or 20% contaminated soil, respectively, which was obtained from a clay pigeon target military range site. The soil contained 253 μg/g CRY, 230 μg/g B[*a*]A, and 253 μg/g B[*a*]P. The mice in Group 5 were fed a diet consisting of feed mixed with site soil extract to match the 10% soil group. Urine samples were collected following 7 days of exposure for all groups.

2.4. Sample preparation

Sample processing was conducted in a dark room with limited yellow light. Urine samples (up to 4 mL) were placed in centrifuge tubes and spiked with 30 μL of 2-ME. The samples were then spiked with surrogate standard solution. The laboratory blank was spiked with deconjugation control standards containing 100 ng 1-naphthyl-β-D-glucuronide and 70 ng pyrene-1-sulfate potassium salt. All unknowns and Quality Control (QC) samples were spiked with 1 mL of enzyme solution (1 mg/mL β-glucuronidase enzyme prepared in a 1 M sodium acetate buffer at pH 5.5) [12]. The headspace over each sample was purged with nitrogen, sealed with Teflon tape, and incubated in the dark at 37 ± 2 °C. After 17 h, the samples were extracted in the dark with 4 mL of pentane using a Thermolyne Speci-mix test tube rocker, and then centrifuged at 2800 rpm for 20 min. The supernatant was removed and the extraction was repeated twice, after which the pentane layers were combined and dried by passing the extract through a Pasture pipette filled with anhydrous sodium sulfate. A 20 μL dodecane keeper solvent was added and the extracts were reduced to 20 μL under a gentle stream of nitrogen in a Turbovap evaporator (Caliper Life Sciences MA, USA) that was maintained at 6 psi and 40 °C. Extracts were subsequently reconstituted with 140 μL of a 10:65:65 of 2-mercaptoethanol: acetonitrile: toluene solution and transferred to GC microvial. The resulting extract was spiked with derivatization control standard and recovery standard solutions. The mixture was finally spiked with 20 μL of MSTFA derivatization