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Derivatization method for sensitive determination of 3-hydroxybenzo[a]pyrene in human urine by liquid chromatography–electrospray tandem mass spectrometry

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ABSTRACT

While urinary 3-hydroxybenzo[a]pyrene (3-OHBaP) can indicate exposure to carcinogenic benzo[a]pyrene (BaP) in humans, most previous analytical methods could not detect it in the general population due to a lack of sufficient sensitivity. In this study, a new liquid chromatography–electrospray mass–mass spectrometry (LC–ESI-MS/MS) method was developed to analyze 3-OHBaP by employing an optimized derivatization reaction with dansyl chloride (DNS) in the aqueous phase. The instrument detection limit (IDL) of the newly developed method was 10 ng/L, which is 60-fold lower than without derivatization. The method was successfully applied to analyze 3-OHBaP and 1-hydroxypyrene (1-OHPy) in human urine combined with enzymatic hydrolysis, C18 enrichment, liquid–liquid extraction and silica cartridge cleanup. The overall method recoveries were 68.7 ± 5.47% for 3-OHBaP and 72.3 ± 4.96% for 1-OHPy, with matrix effects of <10%. The limits of quantification (LOQs) in urine were 0.3 ± 0.02 ng/L and 0.5 ± 0.02 ng/L (n = 3), respectively. The method was applied to analyze seven urine samples of volunteers, and 3-OHBaP was detected in five samples. The concentration of urinary 3-OHBaP ranged from <LOD to 2.30 ng/L, much lower than those (42.0–216 ng/L) of 1-OHPy. The developed method provides a new way to sensitively determine 3-OHBaP in urine of the general population.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a group of organic compounds ubiquitously distributed in the environment mainly due to incomplete combustion of organic matter, and exposure has been associated with lung, skin and bladder cancer [1–4]. PAHs are indirect carcinogens that acquire carcinogenicity only after they have been activated by xenobiotic-metabolizing enzymes to form highly reactive metabolites. Cytochrome P450 (CYP) enzymes play a dominant role in the metabolic activation of PAHs to epoxide intermediates, which are converted with the aid of epoxide hydrolase to diol-epoxides, the ultimate carcinogens. The epoxide intermediates can also spontaneously rearrange to form phenols or be catalytically hydrolyzed to form dihydrodiols, and are excreted in feces or urine in the form of glucuronide or sulfate conjugates [5–8]. Thus, analysis of urinary metabolites of PAHs has been used to assess internal exposure to PAHs via various pathways.

The monohydroxylated metabolite of pyrene, 1-hydroxypyrene (1-OHPy), has been accepted as a biomarker in human urine to indicate internal exposure to PAHs, and has been used to estimate PAHs exposure in the occupational and general population due to its relatively high concentration, even though pyrene is not carcinogenic. For example, the atmospheric concentration of BaP or carcinogenic PAHs is usually estimated to assess the risk of PAHs in air on the basis of the pre-established ratio of urinary 1-OHPy concentration and atmospheric concentration of BaP [9–13]. However, the profile of PAHs is dependent on the emission sources, and therefore such extrapolation would introduce uncertainty, and direct determination of BaP metabolites such as 3-hydroxybenzo[a]pyrene (3-OHBaP) should more accurately assess the internal exposure to carcinogenic BaP. Urinary 3-OHBaP has been used to assess BaP exposure based on workers exposed to coke-ovens and dermatological patients treated with a coal tar ointment [14,15]. Liquid chromatography–tandem mass spectrometry (LC–MS/MS) and derivatization gas chromatography–mass spectrometry (GC–MS) have been applied in the determination of urinary OHPAHs in coke-oven workers [16,17], but failed to detect 3-OHBaP in the general human population due to low sensitivity (instrument detection limit of 105 ng/L for LC–MS–MS

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Table 1
Analytes, surrogate standard, and selected reaction monitoring conditions for UHPLC–MS/MS.

Target compounds	Molecular weight	Transition monitored (*m*/*z*)	Cone voltage (V)	Collision energy (V)
Dansylated 3-OHBaP	501.61	502.48 > 268.20[a]	25	46
		502.48 > 171.13	25	22
		502.48 > 156.08	25	56
Dansylated 1-OHPy	451.55	452.49 > 218.17[a]	23	49
		452.49 > 171.13	23	26
		452.49 > 155.89	23	58
Dansylated ^{13}C6-1-OHBaA	483.54	484.42 > 250.21[a]	25	40
		484.42 > 170.94	25	30
		484.42 > 156.02	25	55

[a] MRM transition used for quantitation.

and limit of quantification of 1000 ng/L for derivatization GC–MS) [18,19]. While derivatization GC/isotope dilution high-resolution MS (GC–IDHRMS) [7,20] improved the sensitivity for determination of urinary 3-OHBaP with limit of detection (LOD) of 10 ng/L, it still could not detect 3-OHBaP in urine of the general human population; therefore, a more sensitive analytical method is necessary.

In this paper, we newly developed a derivatization method with dansyl chloride (DNS) for sensitive determination of 3-OHBaP in human urine by ultra-high performance-liquid chromatography–electrospray tandem mass spectrometry (UHPLC–ESI-MS/MS), and then applied it to the quantification of urinary 3-OHBaP concentrations in volunteers who were not occupationally exposed to PAHs. For comparison, the method variation for 1-OHPy was also investigated in this study.

2. Experimental

2.1. Chemicals and reagents

3-OHBaP, 1-OHPy and stable isotope-labeled standard 1-hydroxy[^{13}C6]benzo[a]anthracene (^{13}C6-1-OHBaA) were purchased from the NCI Chemical Carcinogen Reference Standard Repository (Kansas, MI). Triethylamine, sodium carbonate, 4-(dimethylamino)-pyridine (DMAP), DNS and β-glucuronidase/arylsulfatase (glucuronidase activity ≥100,000 units/mL, sulfatase activity ≤7500 units/mL) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Sep-Pak C18 (3 cm^3, 200 mg) and silica (6 cm^3, 1 g) solid phase extraction (SPE) cartridges were purchased from Waters (Milford, MA, USA). All solvents including hexane, dichloromethane (DCM), tetrahydrofuran (THF), acetone, acetonitrile (ACN), and methanol were HPLC grade and purchased from Fisher Chemicals (New Jersey, USA). Water obtained by a Milli-Q Synthesis water purification system (Millipore, Bedford, MA, USA) was used throughout the study.

2.2. Optimization of dansylation conditions

Dansylation was carried out in a sealed 1.5 mL glass sample vial (Waters, Milford, MA, USA). The dansylation conditions of 3-OHBaP and 1-OHPy were optimized by changing the reaction system (water or DCM) with different catalysts (sodium bicarbonate, DMAP and triethylamine), different pH (9.4, 10, 11 and 12) and different DNS concentrations (0.1–5 mg/mL). Aliquots of a mixture of standards were dissolved in 200 μL of solvent, and then a mixture (200 μL) of DNS and catalysts was added and shaken vigorously for 1 min. The resulting mixture was kept at 65 °C for 60 min followed by mixing with a vortex device for 30 s. The reaction temperature and time were optimized by varying the reaction temperature (45, 65, and 85 °C) and the derivatization time (10, 30, 60, and 90 min).

2.3. Urine sample collection and preparation

Urine samples were collected from volunteers in our laboratory in April 2014, and then were analyzed immediately. Since significant differences in the averaged concentration values of 1-OHPy between supernatant and whole urine have been observed [15], we analyzed well-mixed thawed whole urine sample for quantitating 3-OHBaP and 1-OHPy. A human urine sample of 15 mL was diluted with acetate buffer (15 mL) to adjust the pH to 5.0, and ^{13}C6-1-OHBaA was added to obtain a final concentration of 10 ng/L. β-Glucuronidase/arylsulfatase (glucuronidase activity ≥100,000 units/mL, sulfatase activity ≤7500 units/mL) of 20 μL was added to the sample solution, which was then incubated for 20 h at 37 °C. After hydrolysis, the mixture was loaded on the Sep-Pak C18 (3 cm^3, 200 mg) cartridge that had been preconditioned successively with 6 mL methanol, 6 mL THF, and 6 mL Milli-Q water. After washing with 6 mL methanol/water 2/8 (v/v), the cartridge was dried with nitrogen flow, and the analytes were eluted with 3 mL methanol. After methanol was removed by a gentle stream of nitrogen, 200 μL sodium bicarbonate buffer (pH 11) and 200 μL 0.3 mg/mL DNS in acetone were added to the residue and shaken vigorously for 1 min. The resulting mixture was kept at 65 °C for 60 min, transferred to a 15 mL centrifuge tube, and then 1 mL of ultrapure water and 3 mL of hexane were added. After being shaken vigorously for 10 min, the organic layer was separated by centrifugation at 3000 rpm for 5 min. The extraction was repeated and the combined extracts were loaded onto silica cartridges preconditioned with 8 mL DCM and 8 mL hexane. The dansylated 3-OHBaP and 1-OHPy were eluted with 8 mL hexane:DCM (1:1) and then blown to dryness and dissolved in 0.1 mL of ACN for UHPLC–ESI-MS/MS analysis.

2.4. UHPLC–ESI-MS/MS analysis

The LC apparatus was an ACQUITY™ Ultra Performance LC system (Waters, Milford, MA, USA). Separation of dansylated 3-OHBaP and 1-OHPy was conducted using a Waters ACQUITY UPLC® BEH phenyl column (1.7 μm; 2.1 mm × 100 mm). The column was maintained at 40 °C, and the flow rate and injection volume were 0.3 mL/min and 5 μL, respectively. Methanol (A) and ultrapure water containing 0.1% formic acid (B) were used as the mobile phases. The initial composition of 20% A was increased to 80% in 1 min, then increased to 100% at 5 min and kept for 2 min, followed by a decrease to 10% A, and held for 2 min to allow for equilibration. Mass spectrometry was performed using a Waters Micromass Quattro Premier XE triple quadrupole instrument detector equipped with an electrospray ionization source (Micromass, Manchester, UK) in positive ion mode. The optimized parameters were as follows: source temperature, 110 °C; desolvation temperature, 350 °C; capillary voltage, 3.50 kV; desolvation gas flow, 800 L/h; cone gas flow, 50 L/h; and multiplier, 650 V. MS/MS

Table 2
Limits of detection (LODs), limits of quantification (LOQs), and recoveries ($n = 3$) spiked with standards at low levels.

	LODs (ng/L)	LOQs (ng/L)	Recoveries[a]
1-OHPy	0.2 ± 0.01	0.5 ± 0.02	72.3 ± 4.96%
3-OHBaP	0.1 ± 0.01	0.3 ± 0.02	68.7 ± 5.47%
^{13}C6-1-OHBaA	–	–	84.6 ± 6.92%

[a] The spiked levels were 1 ng/L for 3-OHBaP, 10 ng/L for 1-OHPy and 10 ng/L for ^{13}C6-1-OHBaA.

parameters for the analytes including their precursor and product ions, cone voltage, and collision energy are summarized in Table 1.

2.5. Quantitation

Identification of 3-OHBaP and 1-OHPy was accomplished by comparing the retention time (within 2%) and the ratio (within 20%) of the two selected precursor ion-product ion transitions with those of standards. To automatically correct for the losses of analytes during sample preparation and the matrix-induced change in ionization, and to compensate for variations in instrument response from injection to injection, ^{13}C6-1-OHBaA was used as surrogate standard in this study.

All equipment rinses were done with methanol to avoid sample contamination, and laboratory blanks were analyzed to assess potential sample contamination. Since it is impossible to obtain samples free of analytes, the recoveries ($n = 3$) were calculated by subtracting the background concentrations in non-spiked samples from spiked samples at three spiking levels (1, 5, and 10 ng/L for 3-OHBaP and 10, 100, and 500 ng/L for 1-OHPy). Since 3-OHBaP and 1-OHPy can be detected in human urine, the method limits of detection (LODs) and limits of quantification (LOQs) were based on the peak-to-peak noise of the baseline near the analyte peak obtained by analyzing urine samples and on a minimal value of signal-to-noise of 3 and 10, respectively [21,22]. Since the matrix effect is a general problem in LC–MS/MS analysis, we evaluated the extent of signal suppression or enhancement by spiking standards of dansylated 3-OHBaP (100 ng/L) and 1-OHP (1000 ng/L) into the extracts of urine samples. The matrix effect observed for each analyte was calculated using the percentage of signal intensity in the sample matrix versus the signal of the same concentration in acetonitrile (Table 2).

3. Results and discussion

3.1. Optimization of dansylation conditions

To optimize the dansylation conditions of 3-OHBaP and 1-OHPy, solvents (water and DCM), catalysts (sodium bicarbonate, DMAP, or triethylamine), reaction temperature (45, 65, or 85 °C), incubation time (10, 30, 60, or 90 min), pH (9.4, 10, 11 or 12), and DNS concentrations (0.1, 0.25, 0.5, 1, 2 or 5.0 mg/mL) were tested. Dansylation yielded the highest signal level for 3-OHBaP at 1 mg/mL of DNS in water at 65 °C for 60 min with catalysis by sodium bicarbonate (pH 11) (Fig. 1), and therefore these conditions were selected for the dansylation of 3-OHBaP and 1-OHPy in the following experiments. Although dansylation in DCM also yielded relatively high signal compared to water, the solvent was not used since DCM causes significantly greater chromatogram baseline noise and is volatile during reaction at 65 °C.

3.2. Optimization of analytical conditions

Full-scan ESI-MS analysis in the positive ion mode was performed for the determination of the dansylated 3-OHBaP. The protonated molecules of dansylated 3-OHBaP and 1-OHPy were

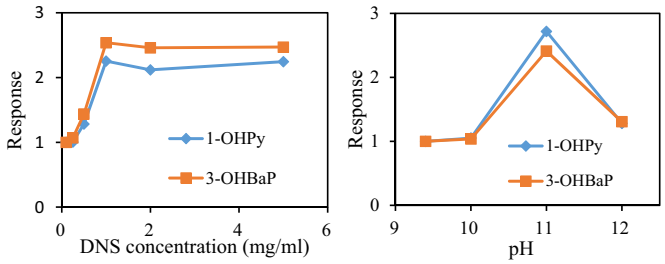

Fig. 1. Effects of dansyl chloride (DNS) concentrations and pH on dansylation efficiencies of 3-hydroxybenzo[a]pyrene (3-OHBaP) and 1-hydroxypyrene (1-OHPy) ($n = 3$). Response indicates the fold increase of signal intensity of dansylated 3-OHBaP and 1-OHPy at each point relative to the first point (mean value with standard deviation).

detected in the reaction solution at the optimized cone voltage from 23 to 25 V and collision energy from 22 to 58 V as shown in Table 1. The CID spectrum of the protonated molecule obtained from dansylated 3-OHBaP was governed by cleavage of the S—O bond and yielded the most predominant product ion at m/z 268. The product ions at m/z 171 ([dimethylaminonaphthalene+H]$^+$) from the cleavage of a C—S bond in the dansyl portion of the molecule and m/z 156 ([dimethylaminonaphthalene−CH_3+H]$^+$) from the loss of a methyl group from the ion at m/z 171 were also observed in the spectrum. Such fragmentation was different from the dansylated derivatives of phenolic chemicals such as bisphenol A and hydroxylated polybrominated diphenyl ethers where only two ions at m/z 156 and m/z 171 were observed in their mass spectra [23,24]. In the spectrum of dansylated 1-OHPy, while m/z 171 was the most predominant product ion (Fig. 2), the product ion at m/z 218 can be observed as the most abundant when deceasing collision energy to 15 V.

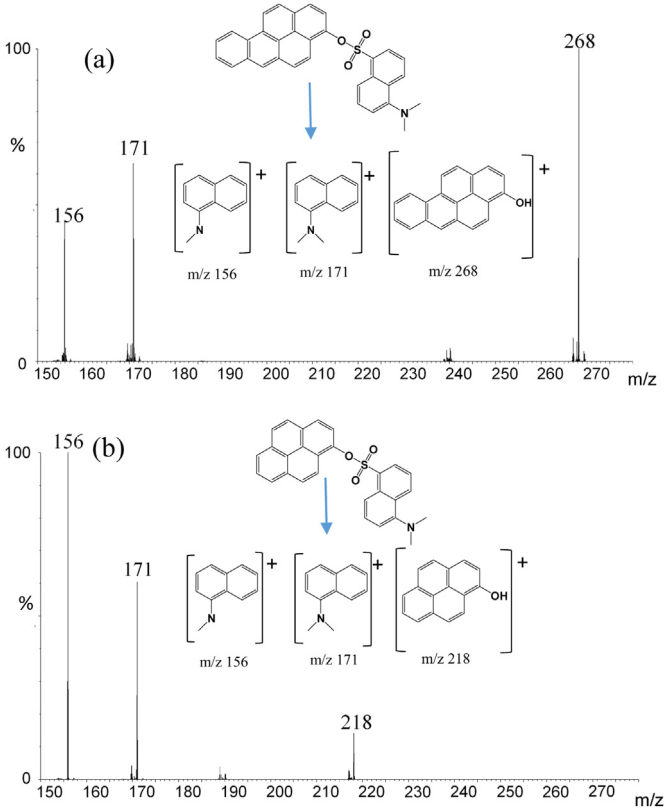

Fig. 2. Typical MS/MS spectra of dansylated 3-OHBaP (a) and dansylated 1-OHPy (b).

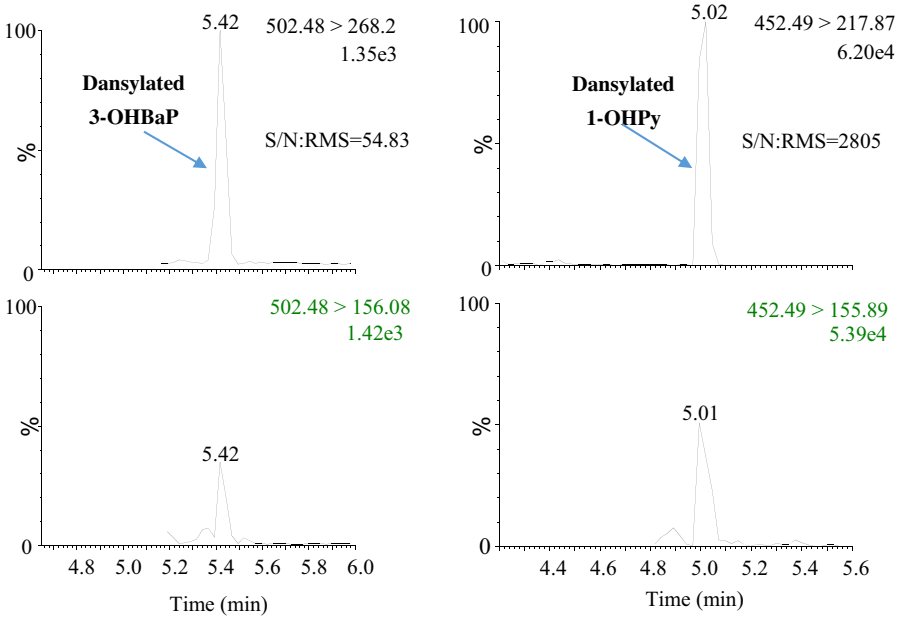

Fig. 3. Typical UHPLC–MS/MS chromatograms of dansylated 3-OHBaP and dansylated 1-OHPy detected in a urine sample (Sample 1). The ratios of the two selected precursor ion-product ion transitions were within the scope of the 20% deviation compared with the standards.

C18 column has been well used in the chromatographic separation of 3-OHBaP and 1-OHPy [18,25]. In this study, when BEH C18 column was used to analyze dansylated 3-OHBaP and 1-OHPy, the serious system residue of dansylated 3-OHBaP was observed due to its strong retention on BEH C18 column. Therefore, we made an attempt to use BEH phenyl column. Since high separation efficiency with high signal-to-noise ratios at the same concentration was achieved, BEH phenyl column was finally selected as the analytical column for separating dansylated 3-OHBaP and 1-OHPy.

3.3. Calibration, precision, and recovery

A series of calibration standard solutions of 3-OHBaP and 1-OHPy were prepared for dansylation to evaluate the dynamic linear response of the analysis. Dansylation showed good linearity in the range from 50 to 4000 ng/L, and the values of r^2 were 0.9969 and 0.9941 for 3-OHBaP and 1-OHPy, respectively. The IDLs for dansylated 3-OHBaP and 1-OHPy using UHPLC–MS/MS were defined as the concentration of analyte producing a peak with a signal-to-noise (S/N) ratio of 3. Thus, IDLs for dansylated 3-OHBaP and 1-OHBaP were estimated to be 8 ng/L and 13 ng/L, respectively. The IDL for 3-OHBaP was 60-fold lower than that using the same instrument without dansylation.

The repeatability of dansylation (in terms of intravial and intervial) was investigated to evaluate the precision of the method. The intravial repeatability was determined by repeated analysis ($n = 6$) of the same dansylated 3-OHBaP (100 ng/L) and 1-OHPy (500 ng/L). The repeatability of dansylation for intervial comparisons was determined by measuring samples of dansylated 3-OHBaP (100 ng/L) and 1-OHPy (500 ng/L) incubated in different vials ($n = 6$). The intravial relative standard deviations (RSD) of the peak intensity for dansylated 3-OHBaP and 1-OHPy were 5.43% and 4.11%, and the intervial RSDs were 6.22% and 4.90%, respectively, indicating an acceptable precision. To assess the stability of each dansylated derivative, the peak area of each derivative was measured each week during 4-week storage at −20 °C. All dansylated compounds were stable, and the relative variations of signal intensities of dansylated 3-OHBaP, 1-OHPy and ^{13}C6-1-OHBaA after 4-week storage were within 10%.

Prior to the analysis of samples, a recovery test was conducted through spiking of each target compound at different concentrations, with subsequent passage through the entire analytical procedure ($n \geq 3$). In the urine samples, the absolute recoveries (mean ± SD) were 68.7 ± 5.47%, 79.9 ± 6.21%, and 93.5 ± 3.44% for 3-OHBaP at 1, 5 and 10 ng/L, 72.3 ± 4.96%, 85.6 ± 7.73% and 94.3 ± 5.94% for 1-OHPy at 10, 100, and 500 ng/L, and 84.6 ± 6.92%, 90.3 ± 5.70% and 92.2 ± 6.11% for ^{13}C6-1-OHBaA at 10, 50 and 100 ng/L, respectively. These results indicated that no significant loss of target compounds occurred during the analytical procedure.

When analyzing samples, procedural blanks containing milli-Q water in place of urine were analyzed as a check for interferences or laboratory contamination. No target compounds were found in procedural blanks.

3.4. Method performance for human urine samples

To apply the dansylation method for the detection of 3-OHBaP in complex urine samples, sample preparation and cleanup before dansylation were necessary to reduce potential interferences, which would react with DNS and then decrease the dansylation efficiency. The C18-SPE-based method was used in this study for urine samples cleanup prior to dansylation. For assessment of potential matrix effects on dansylation efficiency, extracts from urine after C18 cartridge cleanup were spiked with standards of 3-OHBaP (100 ng/L) and 1-OHPy (500 ng/L) prior to dansylation. The results showed that the matrix effects on dansylation efficiencies after cleanup were less than 10% for 3-OHBaP and 1-OHPy. It was found that small volumes of methanol (50 μL) greatly decreased the sensitivities by 90% for 3-OHBaP and 1-OHPy, possibly due to the reaction of methanol with DNS, and therefore the methanol fraction after C18 should be blown to dryness before dansylation. In addition, a relatively high concentration of DNS was found to accumulate in the mass spectrometer, causing a significant loss of sensitivity if dansylation solutions were directly injected to instrument. Therefore, dansylated 3-OHBaP was extracted with hexane from water-diluted derivatization solutions and then passed through a silica cartridge for further cleanup.

Table 3
Concentrations of target chemicals (ng/L) in human urine samples.

Analytes	Sample						
	1	2	3	4	5	6	7
1-OHPy	137.48	172.63	138.85	216.12	42.03	168.06	49.68
3-OHBaP	1.75	2.23	1.58	2.30	ND[a]	1.50	ND

[a] Not detected.

LODs, LOQs and inter-day precision of dansylated 3-OHBaP and 1-OHPy were investigated for method validation using human urine samples. The LODs of dansylated 3-OHBaP and 1-OHPy in urine samples ($n = 3$) were determined to be 0.1 ± 0.01 ng/L and 0.2 ± 0.01 ng/L, respectively, and their LOQs were 0.3 ± 0.02 ng/L and 0.5 ± 0.02 ng/L, respectively. The LOQ of 3-OHBaP was 30-fold lower than that (10 ng/L) using HPLC–MS/MS without dansylation and 3000-fold lower than that (1000 ng/L) using GC/MS reported in previous studies [18,19,25]. Matrix spiked samples (1 ng/L for 3-OHBaP and 10 ng/L for 1-OHPy) were analyzed, and the recoveries of 3-OHBaP and 1-OHPy spiked in urine matrices were 63.9% and 71.4%, respectively. The inter-day precision, based on the means for three replicate spiked samples assayed in each of 3 days, was 5.44% and 8.50% for 3-OHBaP and 1-OHPy, respectively. Less than 10% signal suppression or enhancement was observed for dansylated 3-OHBaP and 1-OHPy.

3.5. Human urine samples

The method was successfully applied to the analysis of 3-OHBaP and 1-OHPy in seven human urine samples collected from volunteers in our laboratory. 3-OHBaP was detected in five samples, and 1-OHPy was detected in all seven samples. Fig. 3 shows typical chromatograms of 3-OHBaP and 1-OHPy detected in a urine sample (Sample 1). The concentrations of urinary 3-OHBaP ranged from <LOD to 2.30 ng/L, much lower than those (42.0–216 ng/L) of 1-OHPy (Table 3). Urinary 3-OHBaP showed a relatively weak correlation ($r^2 = 0.53$) with 1-OHPy, and further study on such a correlation in the general population is warranted on the basis of large sample numbers.

4. Conclusions

UHPLC–MS/MS combined with dansylation allowed the quantitative analysis of 3-OHBaP with good sensitivity and reproducibility. A 60-fold enhancement of sensitivity was achieved compared with the method without dansylation. The performances of the method newly developed in this paper make it possible to determine the urinary 3-OHBaP concentration in the general populations.

Acknowledgments

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