Studies on the Analysis of Benzo(a)pyrene and Its Metabolites in Biological Samples by Using High Performance Liquid Chromatograpy/Fluorescence Detection and Gas Chromatography/Mass Spectrometry

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An analytical method the determination of benzo(a)pyrene (BaP) and its hydroxylated metabolites, 1-hydroxybenzo(a)pyrene (1-OHBaP), 3-hydroxybenzo(a)pyrene (3-OHBaP), benzo(a)pyrene-4,5-dihydrodiol (4,5-diolBaP) and benzo(a)pyrene-7,8-dihydrodiol (7,8-diolBaP), in rat urine and plasma has been developed by HPLC/FLD and GC/MS. The derivatization with alkyl iodide was employed to improve the resolution and the detection of two mono hydroxylated metabolites, 1-OHBaP and 3-OHBaP, in LC and GC. BaP and its four metabolites in spiked urine were successfully separated by gradient elution on reverse phase ODS C_{18} column (4.6 mm I.D., 100 mm length, particle size 5 μm) using a binary mixture of MeOH/H_2O (85/15, v/v) as mobile phase after ethylation at 90 ^oC for 10 min. The extraction recoveries of BaP and its metabolites in spiked samples with liquid-liquid extraction, which was better than solid phase extraction, were in the range of 90.3-101.6% in *n*-hexane for urine and 95.7-106.3% in acetone for plasma, respectively. The calibration curves has shown good linearity with the correlation coefficients (R^2) varying from 0.992 to 1.000 for urine and from 0.996 to 1.000 for plasma, respectively. The detection limits of all analytes were obtained in the range of 0.01-0.1 ng/mL for urine and 0.1-0.4 ng/mL for plasma, respectively. The metabolites of BaP were excreted as mono hydroxy and dihydrodiol forms after intraperitoneal injection of 20 mg/kg of BaP to rats. The total amounts of BaP and four metabolites excreted in dosed rat urine were 3.79 ng over the 0-96 hr period from adminstration and the excretional recovery was less than 0.065% of the injection amounts of BaP. The proposed method was successfully applied to the determination of BaP and its hydroxylated metabolites in rat urine and plasma for the pharmacokinetic studies.

Key Words : Benzo(a)pyrene, Hydroxylated metabolites, Rat urine, Blood plasma, HPLC/FLD

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are commonly occurring environmental contaminants generated by various processes that involve incomplete combustion or pyrolysis of organic materials including fossil fuels.[1-3] PAHs are widely distributed in the environment as pollutants of air, water and soil.[4] Because a large number of these compounds are mutagenic and carcinogenic, the International Agency for Research on Cancer (IARC) has characterized different PAHs as carcinogens in animal experiments.[5-8] Exposure in occupational environments occurs mainly through the lung and skin. To carefully control exposure to these compounds, urinary excretion metabolites have been employed as biomakers.[9-11] PAHs were metabolized to a complex mixture of quinones, phenols, dihydrodiols, triol and tetrols in the biological system.[12-14] Measurement of their metabolites in urine or plasma can be used as a means of assessing the exposure to these compounds.

It is well known that 1-hydroxypyrene (1-OHP) is commonly used as a biological indicator of the overall exposure to PAH because it is not only a major metabolites of pyrene but also convenient for detection by a various analytical methods.[15] Jongeneelen *et al.*[16] developed a high performance chromatographic method for the determination of 1-OHP, the main metabolite of pyrene. However, pyrene shows less carcinogenic properties than benzo(a)pyrene (BaP) which is the most toxic compounds of the parent PAHs. Among the various PAHs, BaP is one of the most studied PAHs, a class well known environmental pollutants.[17] The simultaneous determination of the urinary metabolites thus enables the assessment of the internal dose of PAHs and provides an additional important information source for biological monitoring of occupational and environmental human exposure.[18] However, few reports were available for the analysis of the metabolites from human exposed to the mixture of PAHs. Therefore, the need exists for sensitive and practical methods for the routine analysis of PAH metabolites in urine or plasma, which result from carcinogenic PAHs such as BaP.

It has been known that BaP is metabolized by the mixed-functional oxidases of cytochrome P450 mono oxygenases to arene epoxides followed by hydrolysis to the *trans*-diols and further oxygenation or by the hydroxylation to hydroxylated metabolites.[12,13] Many researchers have reported

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several kinds of BaP metabolites monohydroxy and diol form such as 3-hydroxyBaP (3-OHBaP) and BaP-trans-9,10-dihydrodiol (9,10-diol BaP), etc from rat urine.[19-21] They also reported significant individual and species variations in the excretion of these metabolites. It is necessary to develop a method for the simultaneous quantification of a variety of PAH metabolites in urine such as monohydroxy and dihydrodiols of BaP. Currently, analytical methods for the determination of BaP metabolites in urine are based on chromatographic techniques, both GC and HPLC. Grimer *et al*.[22] have developed a procedure for the determination of urinary BaP metabolites level using GC/MS. Also the use of HPLC with UV detection has been reported by several authors.[23] Because of the low molar absorption coefficient of metabolites, direct UV detection without any analyte enrichment lacks the sensitivity to determine the relatively low urinary amount of these metabolites. A sensitive HPLC method for the determination of 3-OHBaP as the major metabolite of BaP in urine has been developed using a coupled column HPLC system with a special enrichment precolumn and fluorescence detection.[24-27] However, some isomers of metabolites, especially 3-OHBaP and 1-hydroxybenzo(a)pyrene (1-OHBaP), could not be completely separate from each other without any treatment due to a similar chemical and physical properties of these isomers. Because of the difficulty in separating these isomers, the analytical results of BaP metabolites sometimes became incorrect one. On the other hand, D`Silvia *et al*.[27] have reported a method of detecting selectively isomer of BaP metabolites in urine and blood sera samples by Laser Excited Shpol`s kii Spectrometry (LESS). BaP is a relatively intense fluorescent compound. However, the absorption and luminescence profiles of the BaP and their isomers overlap, and therefore would require isolation of the individual compounds prior to analysis.

In this paper, a sensitive and selective method for the detection and quantitative determination of BaP and its hydroxylated metabolites, 1-OHBaP, 3-OHBaP, 4,5-diolBaP, and 7,8-diolBaP by high performance liquid chromatography with fluorescence detection and gas chromatography/mass spectrometry. The derivatization was carried out by alkylation to improve the chromatographic resolution and detection of BaP metabalites. An extraction for BaP and its metabolites from spiked rat urine was performed with various solvents and adjusting pH based on liquid-liquid extraction technique. The proposed method can be applied to the study on the metabolism of BaP in biological samples and be provided the basic informations for human risk assessment.

Experimental Section

Materials. Bezo(a)pyrene (BaP) was obtained from Aldrich Co (St. Louis, MO, USA). Four metabolites of BaP, 1-hydroxybenzo(a)pyrene (1-OHBaP), 3-hydroxybenzo(a)-pyrene (3-OHBaP), benzo(a)pyrene-4,5-dihydrodiol (4,5-diolBaP) and benzo(a) pyrene-7,8-dihydrodiol (7,8-diolBaP) were supplied by National Cancer Institute (Bethesda, MD, USA), and they are shown in Figure 1. The derivatization reagent, iodomethane, iodoethane and iodopropane, were purchased from Merk (Danstadt, Germany). Phenanthrene-d_{10} as internal standard was obtained from Supelco Co. (St. Louis, MO, USA). All other chemicals and solvents were the highest purity grade available and were used without further purifications and deionized water used was produced in a Millipore Super-Q system (Millipore, Milford, MA, USA). Standard stock solutions of benzo(a)pyrene and its metabolites were prepared in *n*-hexane at concentration of 10 μg/mL and stored at 4 °C in the dark. Sep-Pak C_{18} cartridge purchased from Waters (Miliford, MA). Adult male Sprague-Dawley rats, 200 g in body weight, were obtained from DaeHan Laboratory for Animal Research Center (Umsung, Chungbuk, Korea)

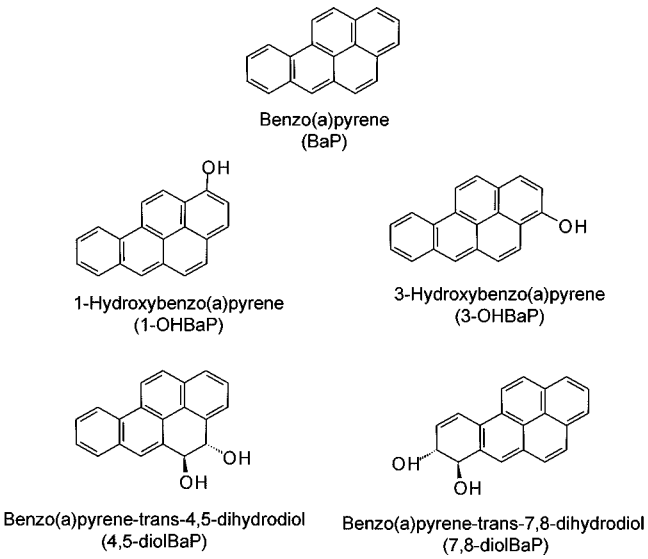

Figure 1. Chemical structures of BaP and its metabolites.

Instruments. A Hewlett-Packard (Palo Alto, CA, USA) HP 1100 series high performance liquid chromatograph with HP 1046A programmable fluorescence detector was used with an ODS C_{18} column, particle size 5 μm, 4.6 mm i.d., 100 mm length (Hewlett-Packard). The mobile phase was eluted using a gradient program. A Hewlett-Pakard (Palo Alto, CA, USA) HP 5890 series II plus gas chromatograph coupled with a 5972 mass selective detector was used. The column used for GC was Ultra-2, 5% phenylmethylsilicone capillary column with a length of 25 m, 0.2 mm i.d., 0.33 μm film thickness (Hewlett Packard, Palo Alto, CA, USA).

BaP administration and sample collection. Rats were intraperitoneally dosed the 20 mg BaP/kg of body weight which was dissolved in corn oil (10 mg/mL). Rat urine samples, which were collected at 4-12 hr intervals over the 0-96 hr period following injection, were stored in polyethylene bottles after sampling, and then these samples were kept frozen at −20 °C until the analysis. Control samples were also collected during 24 hr period before dose in the rat.

Sample preparation. Five milliliters of rat urine sample was placed in a test tube, then pH of solution was adjusted to

7.0 with a phosphate buffer, and 0.5 g of anhydrous sodium sulfate and 10 mL of *n*-hexane were added. After shaking for 10 min and centrifuging for 5 min, the organic phase was transfered into test tube with 20 μL of internal standard (1 μg/mL, phenanthrene-d_{10}). Organic layer was evaporated in vacuum rotary evaporator and dried finally with a nitrogen stream. In the residue, 150 μL of acetone, 50 μL of iodoethane as derivatizing agent and 0.1 g of potassium carbonate were added, and heated for 10 min at 90 ^{o}C. After cooling, the solution collected by filtration with membrane filter (0.45 μm).

A 1 mL of blood plasma sample was placed in a test tube added 20 μL of internal standard (1 μg/mL of phenanthrene d-$_{10}$). And 2 mL of acetone were added. After vortexing for 5 min and centrifuging for 10 min, the organic phase was transfered into test tube, evaporated in vacuum rotary evaporator and dried finally with a nitrogen stream. In the residue, 150 μL of acetone, 50 μL of iodoethane as derivatizing agent and 0.1 g of potassium carbonate were added, and heated for 10 min at 90 ^{o}C. Approximately 1-3 μL of each sample solutions were injected for analysis by HPLC/FLD and GC/MS. The operating conditions of each instrument are listed in Table 1.

Calibration and quantification. Calibration samples were prepared by adding the appropriate amounts of BaP and its metabolites to 5 mL of blank urine or 1 mL of blood sample. The concentration range of standards was 0.1-8.0 ng/mL for urine and 0.1-40.0 ng/mL for plasma. Calibration curves were established by extraction and derivatization after adding above concentration range of standards and 20 mL of internal standard (1 μg/mL). The ratio of peak area of standard to that of the internal standard was used in the quantification of the each analytes.

Results and Discussion

Chromatogram in HPLC and derivatization. First, in order to separate simultaneously and its four in spiked urine by HPLC, chromatogram was obtained by gradient elution on reverse phase ODS C_{18} column (4.6 mm i.d., 100 mm length, particle size 5 μm) using a binary mixture of MeOH/H_2O as an eluent. As shown in Figure 2(b), the peaks of BaP, 3,4-diol BaP and 7,8-diol BaP including internal standard are symmetrical and the separation above three analytes from spiked urine matrices was very good. The retention times were 5.3 min (4,5-diolBaP), 6.7 min (7,8-diolBaP), 13.5 min (ISTD, phenanthrene d_{10}) and 22.4 min (BaP), respectively. However, only one peak probably corresponding to 1-OHBaP and 3-OHBaP overlapped was appeared at 13.5 min with no separation. This result was assumed that two mono hydroxylated metabolites, 1-OHBaP and 3-OHBaP, could not be separated from each other under the above chromatographic conditions without any sample treatment due to a similar chemical structure and some physical properties. Therefore, we tried to separate these metabolites by alkylation in HPLC work. Usually, the derivatization has been widely used to increase the volatility and detection signal of the analyte in GC. However, in this work, to enhance the chromatographic resolution of two mono hydroxylated metabolites in HPLC, the derivatization of the analytes was carried out by alkylation with iodomethane, iodoethane and iodopropane. The results are summarized in Table 2. In comparison to these alkylations, the ethylated derivatives showed better separation and response than

Table 1. Instrumental operation conditions for BaP and its metabolites

HPLC/Fluorescence detection

Column : ODS Hypersil, 100 mm length × 4.6 mm i.d., particle size 5 μm

Mobile phase :

MeOH/H_2O = 70/30 $\xrightarrow{1\%/\text{min}}$ 85/15 (15 min hold)

Injection volume : 5 μL

Oven temperature : 40 ^{o}C

Run time : 30 min

Fluorescence detection time program

time	excitation (nm)	emission (nm)
1.0	250	400
20.0	230	450

GC/MS

GC/MS : Agilent 5890 Gas Chromatograph II plus/5972 Mass Selective Detector

Column : Ultra-2 (cross-linked 5% phenylmethylsilicon, 25 m length × 0.2 mm i.d., 0.33 μm film thickness)

Carrier gas : He at 0.8 mL/min

Splitless (purge on at 0.75 min)

Injection port temp. : 280 ^{o}C

Transfer line temp. : 280 ^{o}C

Oven temp. program :

initial temp.	initial time	rate	final temp.	final time
100 ^{o}C	0 min	10.0 ^{o}C/min	300 ^{o}C	5 min

Run time : 25.0 min

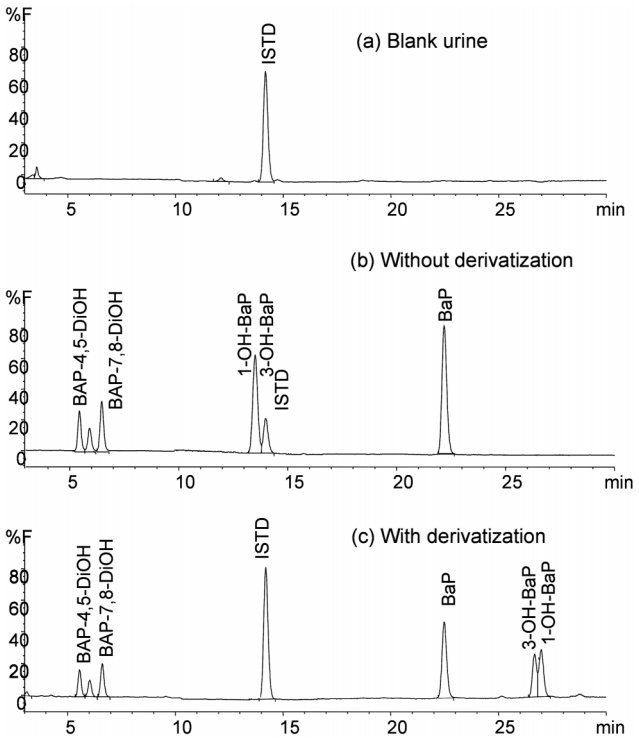

Figure 2. Chromatograms of blank urine (a), spiked of BaP and its hydroxy metabolites without derivatization (b) and with derivatization (c). *ISTD: internal standard (phenathrene-d_{10})

Table 2. Comparison of peak area ratio for derivatives of monohydroxylated BaP with alkylation

BaP metabolites	Peak area ratio*		
	Methylation	Ethylation	Propylation
1-OHBaP	0.0541	0.1343	0.1219
3-OHBaP	0.1524	0.2974	0.2755

*ratio = peak area of derivative of hydroxy BaP metabolites (10 μg/mL)/ peak area of ISTD (phenanthrene d_{-10}, 10 μg/mL). Reaction temperature and time: at 90 °C for 30 min.

another alkylations. In general, the yields of derivatization depend on mainly reaction time and temperature. The effect of the reaction time and temperature on the peak area of 1-OHBaP and 3-OHBaP examind by ethylation. The highest peak area of two ethylated mono hydroxy metaboltes was obtained by derivatization at 90 °C for 30 min.

BaP and four metabolites were successfully separated by gradient elution using a binary mixture of MeOH/H_2O (85/15, v/v) as mobile phase after the derivatization of 1-OHBaP and 3-OHBaP under the optimum condition as shown in Figure 2(c). The retention times of BaP, 1-OHBaP, 3-OHBaP, 4,5-diol BaP, and 7,8-diol BaP were 22.4 min, 26.1 min, 25.6 min, 5.3 min, and 6.7 min, respectively. The elution order of five analytes was as follow; 4,5-diolBaP, 7,8-diolBaP, BaP ethylated 3-OHBaP, ethylated 1-OHBaP. This order was in good agreement with that of polarities of analytes. In HPLC system, it is well known that the retention time of the solutes decreases with the increasing the polarity of the solutes to increasing interaction of solute with mobile phase.

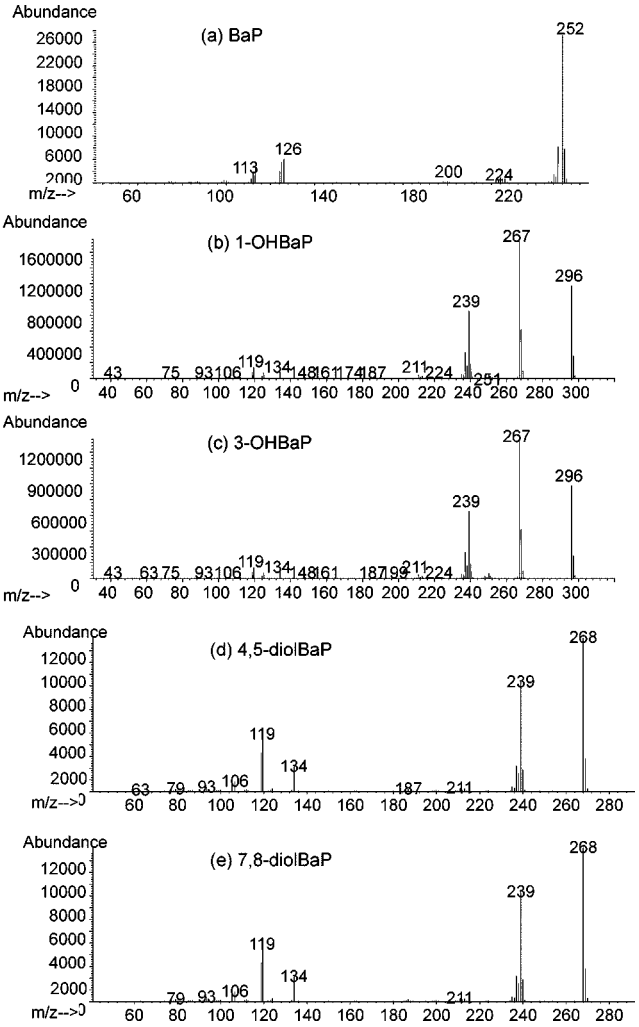

Figure 3. Mass spectra of BaP and its metabolites with ethylation.

In Figure 2(b) and (c), comparing two chromatograms of spiked urine with and without derivatization, the retention times of BaP and two dihydrodiol metabolites as well as internal standard are the same as that of their underivatized species. The results mean that three analytes were not derivatized by ethylation. Therefore, simultanous separation of BaP and its four metabolites in HPLC can be achieved by applying the difference in the reactivity of analytes for derivatizing reagents.

Characterization of BaP and its metabolites by GC/MSD. As can be seen chromatograms in Figure 2, it was assumed that only two 1-OHBaP and 3-OHBaP are formed ethylated derivatives except BaP, 3,4-diolBaP and 7,8-diolBaP among five analytes under the derivatization condition mentioned above. In order to identify the result, the mass spectra of analytes with ethylation are obtained by GC/MS as presented in Figure 3. As expected, the mass spectra of two ethylated mono hydroxylated metabolites are almost the same and the molecular ions are detected at m/z 296 corresponding to 1-ethoxyBaP and 3-ethoxyBaP. Also, the base peaks of two derivatives are presented m/z 267 [M-

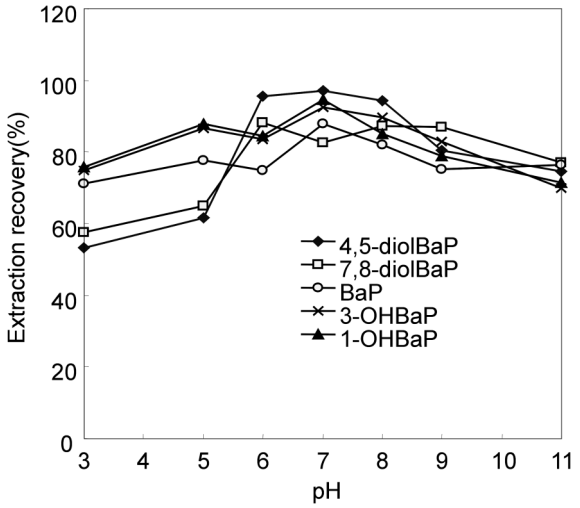

Figure 4. Effect of pH on the extraction recoveries of BaP and its metabolites using *n*-hexane in urine.

$CH_2CH_3]^+$ which are to the loss of the ethyl group from the ethylated molecular ions. But, BaP shows molecular ion at m/z 252 $[M]^+$ and the base peaks of two dihydrodiol metabolites are shown at m/z 268 $[M\text{-}H_2O]^+$ owing to the loss of water molecule from the molecular ion. The results were assumed that they were probably not derivatized by alkylation with iodoethane.

Extraction recoveries. The optimum conditions for the extraction of analytes were examined with respect to the pH, species of solvent and solvent volume in the medium.

The influence of pH. The pH of the medium is one of the important parameter for solvent extraction because the analytes should not be ionized to anion species, which can be form in the higher pH range to reduce extraction efficiency. The influence of the pH on the extraction recoveries for analytes was investigated in the range of pH 3-11. In Figure 4, the results are indicated that relatively high extraction recoveries were obtained at the pH range from 6 to 8. Therefore, phosphate buffer of pH 7.0 solution was recommended for the further examination.

Extraction solvent. It is well known that the distribution of analytes into solvent depends upon the affinity of each analyte as solute to solvent. To examine simultaneous extraction of BaP and its metabolites from matrices, various solvents such as n-hexane, diethyl ether, ethyl acetate, and methyl-*t*-butyl ether were tested. As given in Table 3, the higher efficiency of extraction was obtained using n-hexane and methyl-*t*-butyl ether. The extraction recoveries of analytes from spiked urine sample were in the range of 90.3-101.6% for *n*-hexane and 90.2-105.3% for methyl-*t*-butyl ether with relative standard deviation between 1.7-8.6% and 4.7-8.0% for all analytes ($n = 5$), respectively. In this study, *n*-hexane was used as an extracting solvent because baseline of chromatogram for all analytes in n-hexane shows better than that in methyl-*t*-butyl ether. In the case of blood plasma sample, acetone as an extracting solvent was chosen to improve the analytical sensitivity of analytes by removing proteins and fatty acids from biological matrix. As listed in Table 4, the highest recovery of analytes from spiked blood was obtained in the range of 95.7-106.3% using acetone. BaP and its metabolites in urine and blood plasma were efficiently extracted by up to 2.5 times volume of solvent to that of sample. This is because the amount of analytes extracted is proportional to the volume of solvent at 2.5 times. However, if solvent volume is larger than 10 mL, all analytes would be lost from evaporation step for concentration.

Linearity and detection limits. The linearity of a typical calibration curves for the each analytes in rat urine and blood plasma was studied by computing a regression line of peak area ratio of standard to internal standard on their concentration ratio using least-squares fit. As given in the Table 5, the results are demonstrated a linear relationship with the correlation coefficients (R^2) range from 0.992 to 1.000 for urine and from 0.996 to 1.000 for plasma, respectively. The method detection limits were in the range of 0.01-0.10 ng/mL in urine and 0.1-0.4 ng/mL in plasma sample. The detection limits were evaluated by minimum signal to noise

Table 4. Extraction recoveries of BaP and its metabolites from plasma with LLE

Compounds	Extraction recoveries (%)					
	n-Hexane		Acetone		Acetonitrile	
	Mean	RSD	Mean	RSD	Mean	RSD
BaP	90.3	7.5	106.3	6.1	106.3	3.0
1-OHBaP	86.7	11.8	104.7	5.3	94.0	7.4
3-OHBaP	99.3	10.3	102.0	8.0	95.3	10.9
4,5-diolBaP	23.7	12.7	95.7	4.7	97.7	2.1
7,8-diolBaP	15.7	13.3	105.7	3.9	115.3	3.3

Table 3. Extraction recoveries of BaP and its metabolites from spiked urine with LLE

Compounds	Extraction recoveries (%)							
	n-Hexane		Methyl t-butyl ether		Ethyl acetate		Diethyl ether	
	Mean	RSD	Mean	RSD	Mean	RSD	Mean	RSD
BaP	100.9	3.5	105.3	1.4	92.8	1.3	86.3	22.0
1-OHBaP	101.6	1.7	96.2	5.2	50.0	16.0	0.0	0.0
3-OHBaP	99.1	8.6	90.8	3.7	56.7	16.1	0.0	0.0
4,5-diolBaP	98.7	4.5	90.2	7.0	60.6	6.2	59.7	9.2
7,8-diolBaP	90.3	7.6	103.3	9.2	52.0	2.2	85.1	6.7

RSD: relative standard derivation.

Table 5. Calibration table and detection limits of BaP and its hydroxy metabolites from urine and plasma

Sample	Compounds	RRT[a]	Conc. range (ng/mL)	Y = aX + b			DL[c] (ng/mL)
				a	b	R^{2b}	
Urine	BaP	1.6428	0.01-8.0	0.857	−0.0052	0.998	0.01
	1-OHBaP	1.9946	0.02-8.0	0.825	−0.010	1.000	0.02
	3-OHBaP	1.9728	0.02-8.0	0.624	0.010	0.999	0.02
	4,5-diolBaP	0.3859	0.1-8.0	0.139	0.018	0.996	0.1
	7,8-diolBaP	0.4853	0.04-8.0	0.175	0.036	0.992	0.04
Plasma	BaP	1.6428	0.1-40.0	0.746	0.0057	1.000	0.1
	1-OHBaP	1.9946	0.2-40.0	0.619	−0.0057	0.999	0.2
	3-OHBaP	1.9728	0.2-40.0	0.812	−0.0057	0.999	0.2
	4,5-diolBaP	0.3859	0.4-40.0	0.138	0.00023	0.998	0.4
	7,8-diolBaP	0.4853	0.2-40.0	0.209	−0.0019	0.996	0.2

[a]RRT: relative retention time to phenanthrene d-$_{10}$. [b]R^2: regression coefficient. [c]DL: method of detection limit.

Table 6. Precision and accuracy of BaP and its metabolites in urine and plasma (n = 5)

Sample	Compound	Spiked Concentration (ng/mL)	Mean (ng/mL)	RSD (%)	Recovery (%)
Urine	BaP	1.0	1.02	6.51	102
	1-OHBaP	1.0	1.07	9.46	107
	3-OHBaP	1.0	1.05	10.02	105
	4,5-diolBaP	1.0	1.05	6.49	105
	7,8-diolBaP	1.0	1.03	7.96	103
Plasma	BaP	5.0	4.90	9.48	98.0
	1-OHBaP	5.0	4.75	8.95	95.0
	3-OHBaP	5.0	5.18	7.91	103
	4,5-diolBaP	5.0	5.05	7.34	101
	7,8-diolBaP	5.0	4.98	9.05	99.6

ratio of 3.

Precision and accuracy. The reproducibility and accuracy of BaP and its four metabolites in rat urine and plasma were very good as shown in Table 6. For five independent determination for each metabolites, the coefficient of variation (RSD %) was less than 10.02% and the mean recovery was in the range of 95.0-107% for both urine and plasma samples.

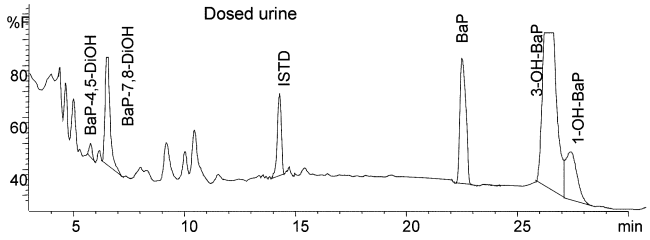

Figure 5. Chromatogram of BaP and its metabolites in BaP dosed rat urine after 34 hr from adminsration.

Excretion in rat urine. First, in order to estimate the urinary excretion metabolites, reverse phase liquid chromatogram of BaP dosed urine after extraction and derivatization are obtained over 0-96 hr following a single intraperitoneal injection of 20 mg Bap per kg rat as shown Figure 5. By comparing the retention of analytes in dosed rat urine and spiked rat urine (Figure 5 and 2c) by HPLC. It was identified

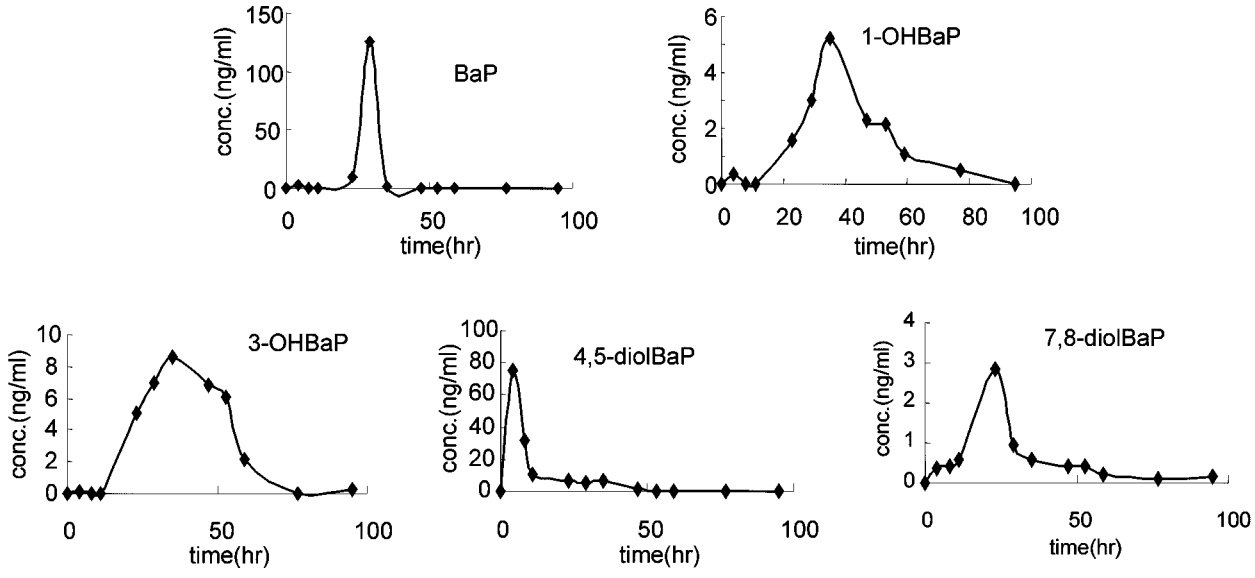

Figure 6. Excretion profile of BaP and its metabolites from BaP dosed rat urine.

that BaP and four hydroxylated metabolites, 1-OHBaP, 3-OHBaP, 4,5-diolBaP and 7,8-diolBaP, are excreted from dosed rat urine. For given dose, relative excretion amounts of BaP and four metabolites were as follows; BaP (1.33 μg) > 4,5-diolBaP (1.17 μg) > 3-OHBaP (0.82 μg) > 1-OHBaP (0.33 μg) > 7,8-diolBaP (0.15 μg) and the total excretional recoveries were less than 0.065% of the injection amounts of BaP as shown in Figure 6. Especially, we have observed 1-OHBaP as a new in this urinary excretion study. From previous studies by Bouchard *et al.*,[19,27] the results were assumed that the excretion amounts of hydroxylated metabolites and its excretion profile was depended on the administration route, dose, and/or time of sampling, etc.

Conclusion

In the present work, an analytical method for the determination and confirmation of benzo(a)pyrene and its four metabolites in rat urine and plasma sample has been developed by using HPLC/FLD and GC/MS. All analytes could be separated simultaneously by gradient elution on reverse phase ODS C_{18} column using a binary mixture of MeOH/H_2O (85/15, v/v) as mobile phase after ethylation at 90 ^oC for 10 min. Optimum conditions for extraction of analytes were obtained by < 10 mL of *n*-hexane in urine and by < 10 mL of acetone in plasma at pH 7.0. The extraction recoveries of all analytes for spiked urine and plasma samples are in the range of 90.3-101.6% and 95.7-106.3%, respectively. The calibration curves are linear relationship with the correlation coefficients (R^2) varying from 0.992 to 1.000 for urine and from 0.996 to 1.000 for plasma. The method detection limits of all derivatized analytes are obtained in the range of 0.01-0.1 ng/mL in 5 mL of urine and 0.1-4.0 ng/mL in 1 mL of plasma, respectively. The total amounts BaP and four hydroxylated metabolites in dosed rat urine were 3.79 g over the 0-96 hr period from administration and the excretional recovery was less than 0.065% of injection amounts of BaP. The proposed method can be applied to the study on the metabolism of BaP in biological samples for the pharmacokinetic studies.

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References

1. Konig, J.; Balfanz, E.; Funcke, W.; Romanowski, T. *Anal. Chem*. **1983**, *55*, 599.
2. Grimmer, G.; Jacob, J.; Naujack, K.-W.; Dettbarn, G. *Anal. Chem*. **1983**, *55*, 892.
3. Schuetzle, D.; Rilley, T. L.; Prater, T. J. *Anal. Chem*. **1982**, *54*, 265.
4. International Agency for Research on Cancer, IARC; *Polynuclear Aromatic Compounds*, Part 1. Chemicals, Environmental and Experimental Data; 1983; Vol. 32, p 211.
5. International Agency for Research on Cancer, IARC; *Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Man*; 1972-PRESENT (Multivolume work); World Health Organization: Geneva, 1987; p S7 58.
6. International Agency for Research on Cancer, IARC; *Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Man*; 1972-PRESENT (Multivolume work); World Health Organization: Geneva, 1973; p V3 107.
7. U.S. EPA., *Drinking Water Criteria Document for PAH. Prepared by the Office of Health and Environmental Assessment*; Environmental Criteria and Assessment Office: Cincinnati, OH for the Office of Water Regulations and Standards, Washington, DC., 1991.
8. International Agency for Research on Cancer, IARC; *Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Man*; 1972-PRESENT (Multivolume work); World Health Organization: Geneva, 1983; p V32 216.
9. Strickland, P.; Kang, D. *Toxicology Letters* **1999**, *108*(2-3), 191.
10. Lindstedt, G.; Sollenberg, J. *Scand. J. Work Environ. Health* **1982**, *8*, 1.
11. Van de Wiel, J. A. G.; Fijneman, P. H. S.; Duijf, C. M. F.; Anzion, R. B. M.; Theuws, J. L. G.; Bos, R. P. *Toxicology* **1993**, *80*, 103.
12. Lee, M. L.; Novotny, M. V.; Bartle, K. D. *Analytical Chemistry of Polycyclic Aromatic Compounds* **1981**, 70.
13. Weeks, S. J.; Gilles, S. M.; D'Silva, A. P. *Appl. Spectroscopy* **1991**, *45*(7), 1093.
14. Bouchard, M.; Viau, C. *Arch. Toxicol*. **1995**, *69*, 540.
15. VanOrden, S. L.; Malcomson, M. E.; Buckner, S. W. *Anal. Chim. Acta* **1991**, *246*, 199.
16. Barcelo, D. *Anal. Chim. Acta* **1992**, *263*, 1.
17. Elchelberger, J. W.; Kerns, E. H.; Olynyk, P.; Buddle, W. L. *Anal. Chem*. **1983**, *55*, 1471.
18. Areise, F.; Verkeik, M.; Hoornweg, G. P.; Vandenesse, R. J.; Ukemaleenstra, S. R.; Hofstraat, J. W.; Gooijer, C.; Velthorst, N. H. *J. Anal. Toxicol*. **1994**, *18*, 195.
19. Bouchard, M.; Viau, C. *Can. J. Physiol. Pharmacol*. **1997**, *75*(3), 185.
20. Jongeneelen, F. J.; Anzion, R. B.; Henderson, P. T. *J. Chromatogr.* **1987**, *413*, 227.
21. Ariese, F. *J. Anal. Toxicol*. **1994**, *18*(4), 195.
22. Vock, E. H.; Wolfe, A. R.; Meehan, T. *Mutation Research/ Fundamental and Molecilar Mechanisms of Mutapenesis* **2001**, *478*(1-2), 199.
23. Quilliam, M. A.; Sim, P. G. *J. Chromatogr. Sci*. **1988**, *26*, 160.
24. Chen, S. H.; Evans, C. E.; McGuffin, V. L. *Anal. Chim. Acta* **1991**, *246*, 65.
25. Gundel, J.; Angerer, J. *J. Chromatogr. B* **2000**, 47.
26. Ramesh, A. *Experimental and Toxicologic Pathology* **2001**, *53*(4), 275.
27. Strickland, P. T.; Kang, D.; Bowman, E. D.; Fitzwillam, A.; Downing, T. E.; Rothman, N.; Groopman, J.; Weston, A. *Carcinogenesis* **1994**, *15*(3), 483.
28. Weeks, S. J.; Gilles, S. M.; D'Silvia, A. P. *Appl. Spectrosc*. **1991**, *45*(7), 1093.