(Bellefonte, PA, U.S.A.). Five deuterated PAHs (Nap-d_8, Ace-d_{10}, Phe-d_{10}, Pyr-d_{10} and BaP-d_{12}) were purchased from Wako Pure Chemical (Osaka, Japan) as internal standards. Both PAHs and deuterated PAHs were dissolved in acetonitrile (Kanto Chemical, Tokyo, Japan). All other chemicals used were of analytical reagent grade.

Collection and Pretreatment of Seawater Sample Seawater samples were collected at five sites (S1–5), whose latitudes and longitudes were respectively 32.8° and 129.5° (S1), 33.8° and 129.7° (S2), 34.8° and 130.5° (S3), 35.5° and 131.5° (S4), 36.0° and 132.5° (S5), in the Japan Sea in August 2008. Seawater samples were collected from the bow of Nagasaki-maru, expedition ship, moving forward, and were collected by immersing pre-cleaned borosilicate amber glass bottles (2 L) at *ca.* 0.5 m below the water surface (opening and closing it underwater). Each water sample was immediately filtered through a 0.45 μm micropore membrane (GC-50,diameter 90 mm, Advantec, Tokyo, Japan) and 10% (v/v) ethanol was added to each bottle to prevent the adsorption of PAHs on the wall. The seawater samples were stored in the refrigerator (at 4°C) no more than 1 week before analysis and no other preservatives were needed. Seawater samples collected at two beaches in Noto Peninsula, Japan damaged by the oil spill in 1997 were also used for comparison.

Artificial seawater was prepared by dissolving 32 g of NaCl, 14 g of $MgSO_4 \cdot 7H_2O$ and 0.2 g $NaHCO_3$ in 1 L of Milli-Q water.

On-line Concentration and Determination HPLC System for PAHs in Seawater The proposed HPLC system was shown in Fig. 1. The system consisted of three Hitachi L-2130 pumps (Tokyo, Japan), a Hitachi degasser, a Hitachi L-2485 fluorescence detector and a Hitachi organizer. An Asahipak ODP-50G column (4.6 mm i.d.×10 mm, Shodex, Tokyo, Japan) was used as the concentrator column. An Inertsil ODS-P column (4.6 mm i.d.×250 mm, GL-Science Company, Tokyo, Japan) and an Inertsil ODS-P column (4.6 mm i.d.×33 mm) were used as the separator and guard columns, respectively. PAHs were concentrated on the concentrator column by loading seawater sample through the solid-line in Fig. 1.

The on-line concentration and elution were operated as follows with the Teflon tubing. After seawater sample (with 10% ethanol) was added with internal standards of Nap-d_8, Ace-d_{10}, Phe-d_{10}, Pyr-d_{10} and BaP-d_{12}, an aliquot (1.0–100 mL) of the mixture was introduced into the HPLC system through the HPLC pump 1. The inlet tubing was rinsed with the sample

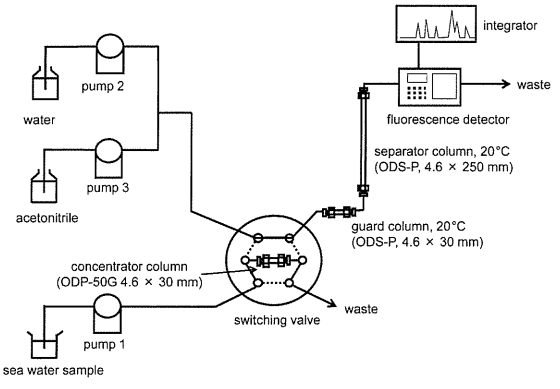

Fig. 1. Schematic Diagram of the Proposed System

for 10 min before loading. The loading volume depended on the concentrations of PAHs in the samples. PAHs in the sample were adsorbed and concentrated on the concentrator column (ODP-50G). The column was washed with 15 mL of distilled water to remove the interfering compounds and salts. The volume of samples and washing solutions was controlled by the loading time when the optimized flow rate of pump 1 was 1.0 mL min^{-1}. After the valve was switched (from the solid line to the dotted line in Fig. 1), PAHs were eluted from the concentrator column and separated on the separator column (ODS-P) with the HPLC mobile phase. Then, PAHs were determined fluorogenically under the following conditions according to our previous report[15] with minor modification as follows. The mobile phase consisted of acetonitrile and Milli-Q water and the flow rate of mobile phase was kept constant at 1.0 mL min^{-1}.

The gradient time program of pumps 2 and 3 was controlled by the Hatachi organizer. Initially, the content of acetonitrile in the mobile phase was 55% (v/v) for the first 20 min, and then was changed from 70 to 80% (20–35 min), after that, it was kept at 90% (35–45 min), then was increased to 100% (45–60 min) and kept for 20 min. The excitation and emission wavelengths were set at 280 and 340 nm (0–31.5 min) for Nap, Nap-d_8, Ace, Ace-d_{10}, Fle, Phe, Phe-d_{10}, 250 and 400 nm (31.5–34 min) for Ant, 286 and 433 nm (34–36 min) for Flu, 331 and 392 nm (36–41 min) for Pyr and Pyr-d_{10}, 264 and 407 nm (41–68.9 min) for BaA, Chr, BbF, BkF, BaP, BaP-d_{12}, DBA, BgPe and 294 and 482 nm (68.9–80 min) for IDP, respectively.

The whole system was checked routinely with blank samples for preventing from contamination.

Results and Discussion

Optimization of On-line Concentration As the solid-phase extraction sorbent for the on-line determination of PAHs in natural water, a kind of fluorocarbon polymer was used. In the system, only PAHs with high concentration, *e.g.* Nap, Ant, and Pyr, were determined.[14] In the present work, an octadecyl polyvinyl alcohol polymer (ODP) column, which was made of porous vinyl alcohol copolymer modified with octadecyl groups on the surface, was used as the concentrator column for enrichment of PAHs having 2 to 6 rings in seawater samples. The polymeric ODP column was more resistant to strong acid and basic solutions than commonly used silica-based octadecyl silyl (ODS) columns. Moreover, the polymeric ODP column showed selective retention power for PAHs compounds due to the existence of π-electron interaction between PAHs molecule and ODP polymer.[16] Therefore, we first compared the PAHs retention abilities on ODP and ODS columns. The recoveries of PAHs were higher on the polymeric ODP column than on the ODS column, indicating that the ODP column had stronger retention of PAHs and greater selectivity for PAHs. The elution profiles showed that PAHs were more stable on the ODP column than on the ODS column when the columns were loaded with PAHs and stored for several days before elution respectively. The higher stability on the ODP column might be because octadecyl polyvinyl alcohol polymers was more resistant to water than silica. For these reasons, an ODP column was selected for the concentrator column. The new ODP concentrator column was conditioned with 20 mL methanol and distilled water, respectively, at the flow rate of 0.5 mL min^{-1}.

The recoveries of PAHs depend on the flow rate of seawater, although a short time concentration is better. The peak heights were measured at flow rates from 0.5 to 2.0 mL min^{-1}, and the maximum peak heights were observed at flow rates of 0.5 and 1.0 mL min^{-1} for most PAHs. At flow rates over 1.0 mL min^{-1}, the peak heights decreased with the increase in the flow rate (Fig. 2). So, the flow rate was set at 1.0 mL min^{-1} for the following experiments.

The recoveries of PAHs were lower without the addition of ethanol. This might be attributed to the absorption of PAHs on the bottle walls due to the high hydrophobic property.[17,18] Here, methanol and ethanol were added to the water samples respectively. We found that 10% ethanol was just as effective as 10% methanol to decrease the adsorption of PAHs and that the recoveries of PAHs were constant at the concentrations over 10%. Considering that ethanol was nontoxic and safe to bring it on the ships, 10% ethanol was immediately added to the collected seawater samples after filtration. The seawater sample containing 10% ethanol was loaded on the column at 1.0 mL min^{-1}. The column was washed with 15 mL distilled water after trapping the PAHs in order to remove the adsorbed interfering compounds and prevent salts out from the column.

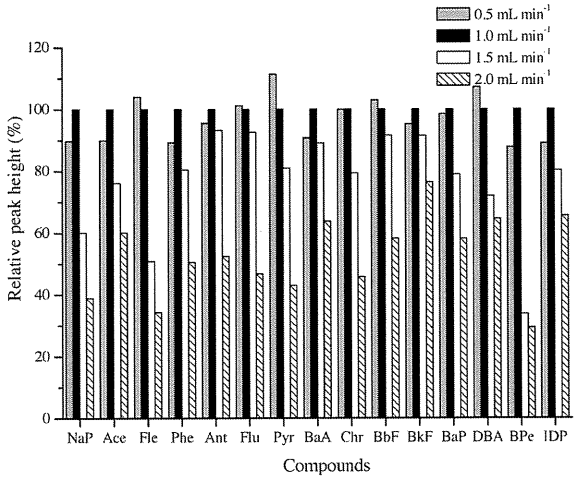

Fig. 2. Effect of Flow Rate of Pump 1 on the Relative Peak Height of PAHs

The samples were the spiked artificial seawater samples and the spiked concentration was shown in the text. The loading volume was 100 mL. The peak height at flow rate of 1.0 mL/min was defined as 100%.

The breakthrough volume was tested to evaluate the maximum sample volume which can be applied with a theoretical 100% recovery. The different volume of artificial seawater samples containing a constant concentration of analyte were treated as described in 'On-line Concentration and Determination HPLC System for PAHs in Seawater.' When the breakthrough volume of analyte began, this relationship started to deviate from linearity.[19] There was no deviation from linearity observed when the tested volume was changed from 100 to 1000 mL. The relationships between the peak height ratios of PAHs and their respective internal standards against the sample volume were linear. This result suggested that the concentrator column (ODP) had enough capacity for concentrating PAHs in seawater samples and the breakthrough volume of the column was larger than 1000 mL of the polluted seawater such as samples collected in harbors.

Performance of the Proposed Method In order to evaluate the practical applicability of the proposed system, performance parameters such as linearity, precision, limits of detection (LODs) and limits of quantization (LOQs) were measured under optimum analytical conditions using 100 mL artificial seawater samples spiked with PAHs. Results were given in Table 1. Concentrations of PAHs in the artificial seawater samples were as follows: Nap and Ace, 20 ng L^{-1}; Fle, Flu, BbF, DBA and BgPe, 4 ng L^{-1}; IDP, 3.2 ng L^{-1}; Phe, Ant, Pyr, BaA, Chr, BkF and BaP, 2.0 ng L^{-1}. All the PAHs showed good linearity with correlation coefficients (r) ranging from 0.951 to 0.998 (Table 1). Recoveries ranged from 74% (IDP) to 110% (Ant) with relative standard deviation (R.S.D.) of 1.5–9.4%. R.S.D.s were between 0.4–5.0% for repeatability (n=3) and 1.8–9.8% for reproducibility (n=5).

Limits of detection (LODs) (concentrations giving a signal-to-noise ratio of 3) ranged from 0.002 (Ant) to 0.50 ng L^{-1} (IDP) as the loading sample volume was 100 mL. LODs by the proposed method were smaller than those by GC-MS/MS

Table 1. Linearity, Recovery, Repeatability and Reproducibility of the Proposed Method[a]

PAHs	Linearity (r)	LOD (ng L^{-1})	LOQ (ng L^{-1})	Recovery±R.S.D. (%, n=5)	Repeatability (R.S.D., %, n=3)	Reproducibility (R.S.D., %, n=5)
Nap	0.951	0.042	0.14	98±1.5	0.9	5.6
Ace	0.970	0.013	0.042	95±1.5	1.6	7.7
Fle	0.984	0.020	0.067	101±2.4	0.4	8.4
Phe	0.976	0.011	0.036	93±4.2	4.7	5.8
Ant	0.998	0.002	0.007	110±6.8	2.4	5.3
Flu	0.988	0.030	0.10	88±9.4	4.9	9.8
Pyr	0.994	0.012	0.040	95±9.3	2.5	7.3
B*a*A	0.990	0.012	0.040	92±6.5	1.1	3.8
Chr	0.990	0.018	0.061	88±5.3	1.2	1.8
B*b*F	0.996	0.060	0.20	84±5.2	4.1	9.6
B*k*F	0.988	0.013	0.044	89±5.6	2.6	5.1
B*a*P	0.988	0.012	0.039	80±7.4	1.3	8.9
DBA	0.990	0.20	0.67	75±8.2	5.0	7.1
BgPe	0.986	0.22	0.73	79±7.3	2.3	9.1
IDP	0.986	0.50	1.7	74±7.7	1.8	8.9

a) Loading sample volume, 100 mL.

method coupled with solid phase microextraction and HPLC-fluorescence detection method coupled with nanoextraction recently reported.[8–12,17,20] Especially LODs of Ace, Ant, Pyr, BaA, Chr, BkF and BaP by the proposed method were 1/10 to 1/100 of the reported values.

On the other hand, artificial seawater samples spiked with 100 times higher concentrations of PAHs were prepared and 1.0 mL of the solution was loaded to the system. The slopes of the calibration curves and LOQs of PAHs were almost the same as those obtained above, suggesting that the proposed method is useful for both highly polluted and clean seawater samples.

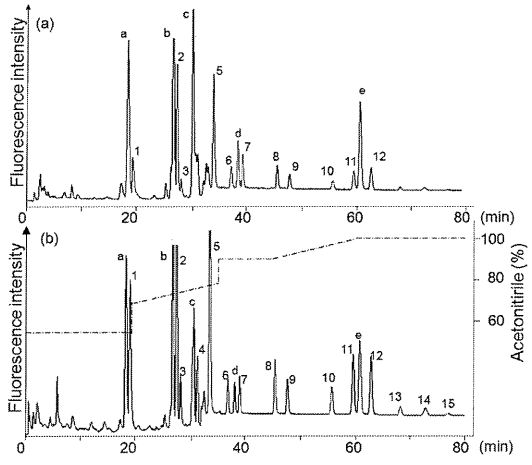

Fig. 3. Typical Chromatograms of (a) Real Japan Sea Water Sample and (b) Artificial Seawater Sample Spiked with PAHs

The sampling site of (a), S1. The spiked PAH concentrations, seen text; the dotted line in (b) indicated the acetonitrile concentration (%) in the mobile phase. Peaks: 1, Nap; 2, Ace; 3, Fle; 4, Phe; 5, Ant; 6, Flu; 7, Pyr; 8, BaA; 9, Chr; 10, BbF; 11, BkF; 12, BaP; 13, DBA; 14, BgPe; 15, IDP; a, Nap-d_8; b, Ace-d_{10}; c, Phe-d_{10}; d, Pyr-d_{10}; e, BaP-d_{12}.

PAHs Concentrations in Japan Sea Samples The proposed method was applied to seawater samples collected from the Japan Sea to investigate the stage. Figure 3 showed representative chromatograms of the Japan Sea water and a spiked artificial seawater sample obtained by the proposed method under the optimized conditions. The loading volume was 100 mL. Table 2 showed the concentrations of PAHs at the five sampling sites. Average concentrations ranged from 0.1 (Ant) to 22.9 (Nap) ng L^{-1} in the southwest part of the Japan Sea. Nap, a 2-ring PAH, was the predominant compound. BaA, BbF and BkF, whose concentrations were lower than LODs the reported in traditional methods, were quantified by the proposed method. DBA, BgPe and IDP showed trace peaks in Fig. 3, although they were lower than the LODs. The recoveries of PAHs spiked to the Japan Sea samples showed similar result as that of the spiked artifical seawater samples. These results suggested that the proposed method could determine PAHs at sub ppt (ng L^{-1}) in marine water.

Recovery of Beaches Damaged by Oil Spilled from Nakhodka from the View Point of BaP Finally the BaP concentration was determined in seawater samples collected at two beaches (Kaiso and Hagahashi) in Noto Peninsula, which were heavily damaged by the beached oil from the Nakhodka in January 1997, in Table 3. The BaP concentrations at Kaiso (8.1 ng L^{-1}) and Nagahashi (7.4 ng L^{-1}) were very high one month after the accident and decreased with time. Then the concentrations at 3 years after the accident (April, 2000) became to the normal level of the Japan Sea (0.2–0.3 ng L^{-1}).

Conclusion

An on-line HPLC system for concentrating and determining PAHs in small volume seawater samples was proposed with the advantages of automatic, simple and on-line sampling pretreatment. The polymeric ODP column concentrated PAHs in the samples with excellent matrix removal, and on-line elution and determination through switching valve guaranteed the

Table 2. Concentrations (ng L^{-1}) of PAHs Collected at Different Sampling Sites in the Japan Sea

Method	Proposed work						MASE[a]-GCMS[21]	LLE-GCMS[8]	SPE-HPLC[9]
Sampling site	Japan Sea						Bilbao, Spain	Alexandria Coast, Egypt	Saronikos Gulf, Greece
	S1[b]	S2	S3	S4	S5	Ave.±S.D.			
NaP	23.9	26.0	23.9	23.7	16.8	22.9±3.5	166	n.d.[c]	160
Ace	1.5	1.7	1.5	1.2	1.3	1.4±0.2	110	n.d.	41
Fle	1.6	1.6	1.6	1.2	1.3	1.5±0.2	37	2.1	42
Phe	i.p.[d]	i.p.	i.p.	i.p.	i.p.	—	118	14.8	54
Ant	0.1	0.1	0.1	0.1	0.1	0.1±0	76	3.8	30
Flu	1.5	1.6	1.5	1.6	1.4	1.5±0.1	21	6.7	34
Pyr	0.8	1.0	0.8	0.7	0.9	0.8±0.1	30	5.6	18
BaA	0.1	0.2	0.1	0.1	0.3	0.2±0.1	n.d.	n.d.	—
Chr	0.2	0.2	0.2	0.2	0.4	0.3±0.1	n.d.	5.1	—
BbF	0.6	0.6	0.6	0.5	0.5	0.6±0.1	9	n.d.	55
BkF	0.3	0.4	0.3	0.2	0.2	0.3±0.1	10.1	n.d.	—
BaP	0.2	0.3	0.2	0.2	0.2	0.2±0.1	9.7	9.0	25
DBA	n.q.[e]	n.q.	n.q.	n.q.	n.q.	—	n.d.	n.d.	—
BgPe	n.q.	n.q.	n.q.	n.q.	n.q.	—	n.d.	n.d.	—
IDP	n.q.	n.q.	n.q.	n.q.	n.q.	—	n.d.	n.d.	—
Total	30.8	33.7	30.8	29.7	23.4	29.7±3.8	679	47.0	459

a) MASE, membrane-associated solvent extraction; *b*) sampling site (locations of each sampling site seen text); *c*) not detected; *d*) not quantified because of the interfering peaks; *e*) detected but not quantified because lower than LOD.

Table 3. BaP Concentrations ($ng\,L^{-1}$) in Seawater Samples Collected at Beaches in Noto Peninsula Damaged by Nakhodka Spilled Oil in 1997 and South Part of the Japan Sea

Sampling date	Noto peninsula		Japan Sea (ave. of S1–5)
	Kaiso	Nagahashi	
Feb., 1997	8.1[a]	7.4[a]	—[b]
Jan., 1998	1.6[a]	—	—
April, 2000	0.3[a]	0.2[a]	—
April, 2008	0.2	0.2	0.2

a) Cited from ref. 3. *b*) Sample was not collected.

higher sensitivity than currently available systems. Thus the proposed method which required a small volume sample was successfully applied for the determination of PAHs in not only polluted seawater but also clean background seawater. This is the first report to determine the normal levels of PAHs. This method also found that the BaP concentration in seawater at beaches in Noto Peninsula which were damaged by the oil spill in 1997 had become to the normal level.

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—Review—

多環芳香族炭化水素類の生体影響に対し輸送及び生体内代謝がどのように関与しているのか

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滝上英孝,[c] 鈴木 剛,[c] 早川和一[b]

How Do Transport and Metabolism Affect on the Biological Effects of Polycyclic Aromatic Hydrocarbons?

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Polycyclic aromatic hydrocarbons (PAHs), some of which are carcinogenic/mutagenic, are generated by combustion of fossil fuels and also released through tanker or oilfield accident to cause a large scale environmental pollution. PAHs concentration in China is especially high in East Asia because of many kinds of generation sources such as coal heating systems, vehicles and factories without exhaust gas/particulate treatment systems. So, the atmospheric pollution caused by PAHs in China has been seriously concerned from the view point of health effects. Like yellow sand and sulfur oxide, PAHs exhausted in China are also transported to Japan. Additionally, strongly mutagenic nitrated PAHs (NPAHs), estrogenic/antiestrogenic PAH hydroxides (PAHOHs) and reactive oxygen species-producing PAH quinones (PAHQs) are formed from PAHs by the chemical reaction during the transport. Furthermore these PAHOHs and PAHQs are produced by the metabolism in animal body. In the biological activities caused by the above PAH derivatives, the structure-activity relationship was observed. In this review, our recent results on the generation of PAH derivatives by atmospheric transport and metabolism are reported. Also, the existing condition of PAHs as atmospheric pollutants is considered.

Key words——polycyclic aromatic hydrocarbon derivative; atmospheric transport; metabolism

1. はじめに

多環芳香族炭化水素（PAH）類は，石炭や石油などの化石燃料の燃焼に伴い生成され，タンカーや油田事故でも大規模な環境汚染を引き起こす環境汚染物質の1つである．PAH類はヒトや野生生物への影響が懸念されると同時に，偏西風等によって長距離輸送されることから越境汚染を引き起こす可能性も危惧されている．さらには，PAHは大気中を輸送される際には，様々な化学反応によって誘導体へと姿を変え，例えばPAHのニトロ化体（NPAH）が強い変異原性を示すように，多岐に渡る毒性を発現する可能性も示唆される．

本シンポジウムでは，PAHの大気輸送及び生体内代謝により生成されるPAH誘導体を中心に，これらが示す毒性と生体影響との係わりについて，筆者らの取り組みを紹介するとともに，PAH類の大気汚染物質としての現状について考えてみたい．

2. 環日本海域の主要都市におけるPAH/NPAH濃度の実態

筆者らは，環日本海域に位置する各都市の汚染状況を把握すべく，日本（札幌，金沢，東京，北九州），韓国（釜山），中国（瀋陽，撫順，鉄嶺，北京），ロシア（ウラジオストク）における，大気粉塵中のPAHとNPAHの濃度測定を実施した．分

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本総説は，日本薬学会第131年会シンポジウムGS03で発表したものを中心に記述したものである．

析の対象としたのは，US Environmental Protection Agency（EPA）に環境汚染物質として測定すべき項目としてリストアップされた16種類のPAHのうち，毒性の観点からヒトの健康に影響を及ぼし易いとされる4環以上の6種PAHを選択した．また，NPAHについては，特に変異原性が強いことが示されている11種を分析対象とした．その結果，大気中6種PAHの年平均濃度和は，撫順（1205 $pmolm^{-3}$）＞瀋陽（313 $pmolm^{-3}$）＞北京（270 $pmolm^{-3}$）＞鉄嶺（258 $pmolm^{-3}$）＞ウラジオストク（34 $pmolm^{-3}$）＞北九州（12 $pmolm^{-3}$）＞札幌（11 $pmolm^{-3}$）＞東京（5.9 $pmolm^{-3}$）＞釜山（5.6 $pmolm^{-3}$）＞金沢（3.6 $pmolm^{-3}$）の順となった．中国の都市のPAH, NPAHの年平均濃度は他国の都市に比して非常に高い値を計測した．近年，中国では発展する産業や冬季の暖房に多量の石炭を使用しているが，[1] 排煙脱粒子装置の普及は進んでいない．このため，PAHやNPAHが吸着した粒子状物質が多く排出されている．

3. 日本海域及び関連河川におけるPAH濃度

日本海域は，地震や火山活動の盛んな地帯であるとともに，タンカーやパイプライン等の事故の危険性も増している．[2,3] また中国等からの大気汚染の影響も受け易く，両端が狭い海峡で閉鎖性が高く環境変化に敏感である．したがって日本海の汚染調査は国際的にも要望されているものの，これまでその実態に関する報告はほとんどなかった．そこで筆者らは，日本海におけるPAH汚染の実態を把握すべく，海水中のPAH濃度測定を実施することとした．測定の対象は，人為起源汚染物質である3環から6環の合計13種のPAHとした．

総PAH濃度は，日本列島側，大陸側外洋域でそれぞれ，9.63 ng/L（3.44–24.0 ng/L），7.67 ng/L（4.10–30.6 ng/L）であり，大陸側がやや低かった．さらに，瀬戸内海や東北沿岸太平洋域の総PAH濃度と比較すると，日本海は瀬戸内海より低く，東北沿岸太平洋側外と同じレベルであったが，大西洋より数桁高かった．

今後，さらにデータを蓄積させることにより，汚染の現状はより明らかになっていくと考える．

4. PAH誘導体の生成

上で述べたように，発生したPAH類は発生源周辺に留まるに限らず，様々な要因により長距離を輸送され，輸送中に，PAHは紫外線やニトロラジカ

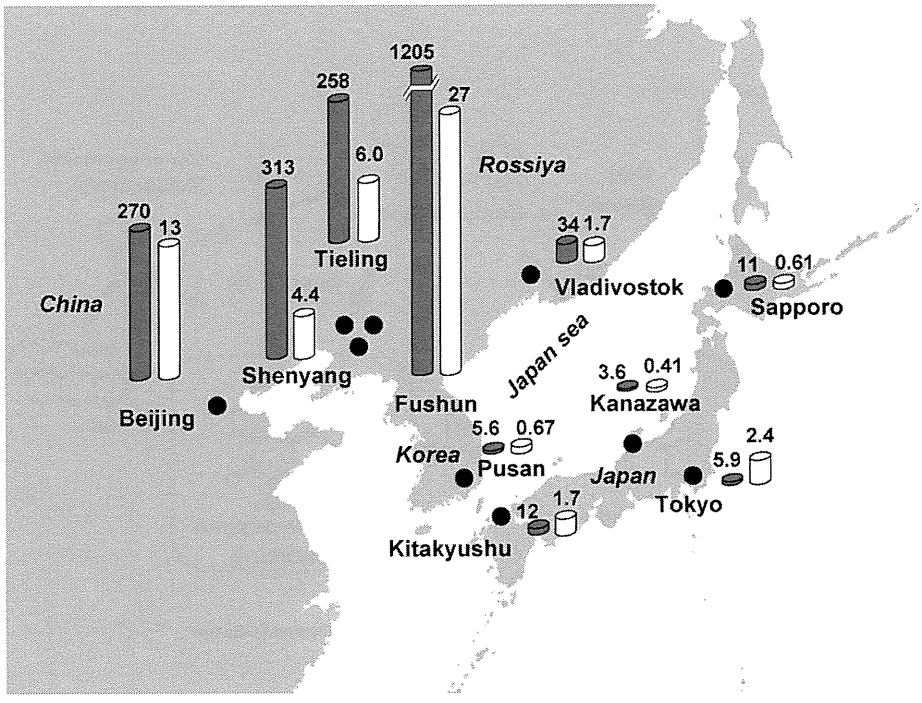

Fig. 1. Annual Average Atmospheric Concentrations of PAHs and NPAHs

Unit: pmol/m³ ▮ [PAHs] = [Pyr] + [BaA] + [Chr] + [BbF] + [BkF] + [BaP], ▯ [NPAHs] = [1,3-Dnp] + [1,6-Dnp] + [1,8-Dnp] + [9-NA] + [1-NP] + [2-NFR1 + 2-NP] + [6-Nc] + [7-NBaA] + [6-NBaP] + [3-Nper], Year: 2001–2002 (Shenyang), 2002–2003 (Pusan), 2003–2004 (Tieling), 2004–2005 (Beijing, Fushun, Vladivostok, Kanazawa, Sapporo, Tokyo, Kitakyushu).

ル（$\cdot NO_3$），ヒドロキシラジカル（$\cdot OH$）などと化学反応すると，PAH ニトロ体（NPAH），PAH 水酸化体（PAHOH），PAH キノン体（PAHQ）等の誘導体となる.[4-6]

一方，これら PAH 誘導体は，生体内に取り込まれた後，代謝によっても生成する．例えば，PAH 類は，主に肝臓において，アリール炭化水素受容体（AhR）を活性化することにより薬物代謝酵素，CYP1A1 等のシトクロム P450 酵素などを誘導し，ついで P450 の存在下で PAH 類は PAHOH や PAHQ といった PAH 誘導体へと代謝される（Fig. 2）.[7]

5. PAH 誘導体による毒性影響

上で述べたように環境中に放出された PAH は PAH 誘導体となって存在しているため，PAH 誘導体が生体に対してどのような作用を示すかが重要となってくる．PAH 誘導体の毒性としては，NPAH による変異原性が古くからよく知られている．NPAH は，PAH がニトロ還元酵素（Nitroreductase: NTR）により代謝を受けることにより生成し，それらは最終的に DNA へ損傷を及ぼす付加体となることで，強い変異原性を示すことが知られている.[8] また，PAHQ についても活性酸素種（ROS）産生能を有すことから,[9] ラジカルの生成を経て物理的に DNA を損傷させ，細胞機能障害や細胞死を引き起こすことも知られている.[10-13] このような酸化ストレスは，多くの経路で，がんを始めとし，循環器系疾患，感染症，老化など様々な疾患に関与するとされる.[14]

一方で，近年，着目されているのが，PAH 誘導体による内分泌撹乱作用である．酵母 two-hybrid assay を用いた PAH 代謝産物によるエストロゲン受容体への作用を調べたわれわれの実験結果から，PAH 代謝産物である PAHOH 及び PAHQ がエストロゲン様/抗エストロゲン様作用を示すことが明らかとなっている.[15,16]

このように，大気中での様々な化学反応によって PAH 誘導体が生成するのと同時に，体内に取り込まれた PAH は，生体内の様々な代謝を介して無数の PAH 誘導体を生成することから，われわれは様々な生理活性作用を示す PAH 誘導体に高濃度曝露されている可能性が高い.

6. PAH 誘導体が示す構造活性相関

PAH 等の環境化学物質が示すエストロゲン受容体（ER）を中心としたホルモン受容体への結合及び活性化といった作用は，その立体構造によって，作用の有無や強弱が異なるものと推測される．そこで，PAHOH 及び PAHQ が示すエストロゲン/抗

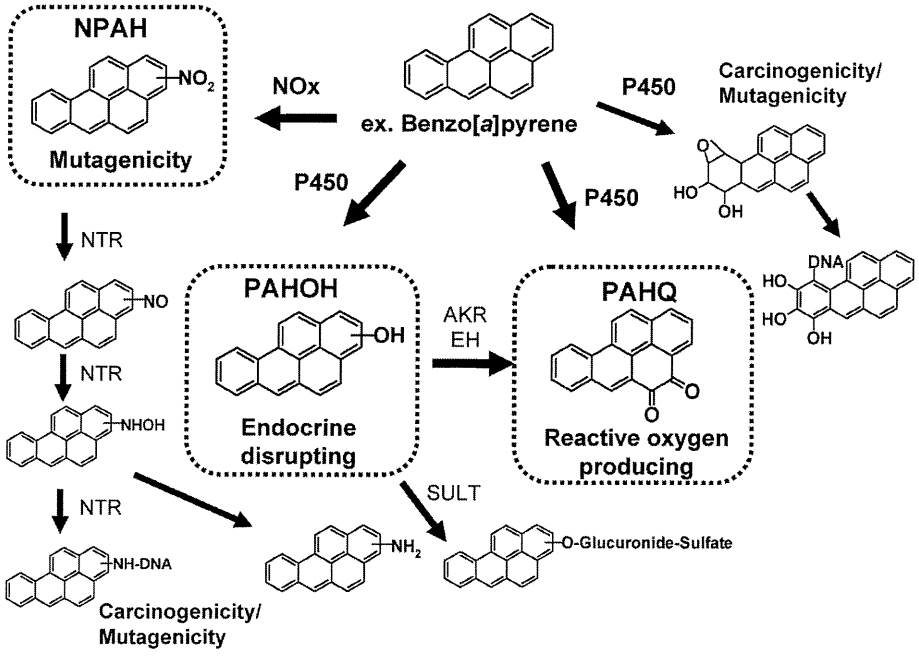

Fig. 2. Metabolic Activation of PAH/NPAH
NTR: Nitroreductase, AKR: Aldo-keto reductase, EH: Epoxide hydrase, SULT: Sulfotransferase.

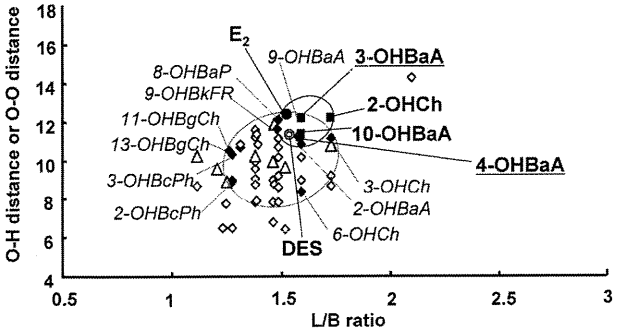

Fig. 3. Relatioship between L/B Ratios and O-H Distances of Strongly Estrogenic and Antiestrogenic PAHOHs

Estrogenic activity of each test compound was assayed in the concentration range from 1×10^{-6} M to 1×10^{-9} M. Reactive effective potency of estrogenic activity (REP_E) was calculated from the value of E_2 as a positive control. Antiestrogenic activity of each test compound was assayed in the concentration range from 1×10^{-6} M to 1×10^{-9} M. Reactive effective potency of antiestrogenic activity (REP_{AE}) was calculated from the value of 4-hydroxytamoxifen (4-OHT) as a positive control. Symbols: ■; Strong estrogenic activity ($REP_E\geq0.001$), ◆; Strong antiestrogenic activity ($REP_{AE}>0.1$), ◎; DES, ●; E2. In the case of E_2 and DES, O-O distance was used instead of O-H distance.[15]

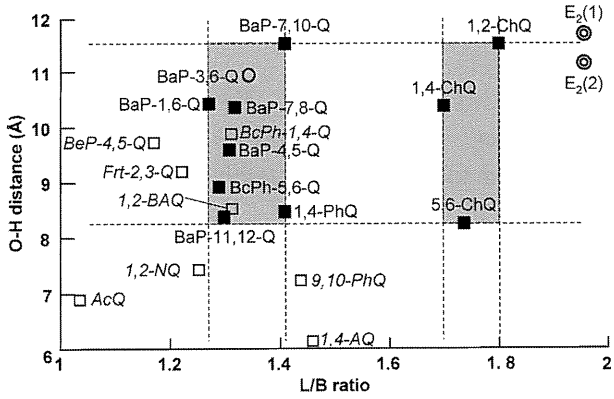

Fig. 4. Relatioship between L/B Ratios and O-H Distances of Strongly Estrogenic and Antiestrogenic PAHQs

Estrogenic activity of each test compound was assayed in the concentration range from 1×10^{-6} M to 1×10^{-9} M. Reactive effective potency of estrogenic activity (REP_E) was calculated from the value of E_2 as a positive control. Antiestrogenic activity of each test compound was assayed in the concentration range from 1×10^{-6} M to 1×10^{-9} M. Reactive effective potency of antiestrogenic activity (REP_{AE}) was calculated from the value of 4-hydroxytamoxifen (4-OHT) as a positive control. Symbols: ○; Estrogenic activity ($REP_E=2.3$), ■; Antiestrogenic activity ($REP_{AE}>0.42$), □; Inactive, ◎; E2.[16]

エストロゲン様作用について，分子構造又は物理化学的特性パラメーターによる予測が可能かどうか考察を行った．

われわれは，PAH の分子構造を考える上で 2 つの因子，すなわち，母核構造と水酸基の位置が活性に関与していることを予測し，母核構造の違いを表すパラメーターである Length-to-breadth ratio（L/B 比）[17,18]及び水酸基の位置を表すパラメーターとして O-H distance[19] を使用した構造活性相関を調べた．

まず，PAHOH における活性と L/B 比及び O-H-distance との関係を Fig. 3 に示した．図に示したように，ER のアゴニスト活性を示した化合物は，L/B 比が 1.5–1.7, O-H distance が 10.8–11.7 Å の範囲に，また抗エストロゲン活性を持つ PAHOH は L/B 比が 1.2–1.7, O-H distance が 8.4–11.6 Å の範囲に集中することが明らかとなった．また，PAHQ についても同様に調べた結果を Fig. 4 に示した．エストロゲン様活性を示した PAHQ は，L/B 比が 1.2–1.4 及び 1.7–1.8, O-H distance が 8.2–11.5 Å の狭い範囲に集中して存在していた．これらは，ER の既知アゴニスト化合物である 17β-Estradiol（E_2）及び diethylstilbestrol（DES）と類似しており，これらは活性を強く示す要因の 1 つであると考えられた．

以上の結果により，バイオアッセイにより示された PAHOH 及び PAHQ が有するエストロゲン/抗エストロゲン作用を *in silico* での構造活性相関により予測できたことは，環境中や生体内に無数に存在する PAH 誘導体のリスクを知る上での有効な手法となることが期待できる．

7. おわりに

以上，PAH 誘導体を中心にわれわれ東アジアを取り巻く環境について述べたが，主要な大気汚染物質の 1 つである PAH 類は，PAH 誘導体へと姿を変えてわれわれ人類の健康を脅かしている危険性があり，今後も PAH 誘導体の毒性や環境中濃度等，動態を突きとめていかなくてはならない大きな環境問題であると思われる．

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analytical chemistry

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Determination of Benzo[*a*]pyrene-7,10-quinone in Airborne Particulates by Using a Chemiluminescence Reaction of Hydrogen Peroxide and Hydrosulfite

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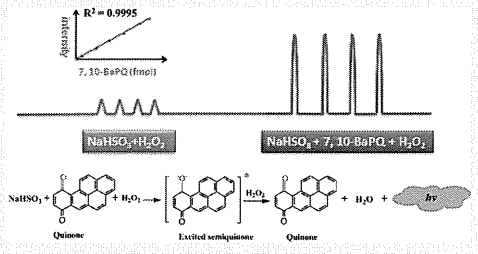
ABSTRACT: An ultraweak chemiluminescence (CL) was observed when sodium hydrosulfite ($NaHSO_3$) reacts with hydrogen peroxide (H_2O_2) and was enhanced 70 times by adding 10 pmol benzo[*a*]pyrene-7,10-quinone (7,10-BaPQ). The CL reaction is fast, and it reached maximum intensity in 0.1 s, and then decayed to baseline in 3 s. Mechanism of $NaHSO_3$-7,10-BaPQ-H_2O_2 system were investigated by CL spectrum, radical scavengers and electron spin resonance (ESR). Hydroxyl radical ($^{\bullet}OH$), super oxide anion radical ($^{\bullet}O_2^-$), and sulfite radical ($^{\bullet}SO_3^-$) were generated in the $NaHSO_3$-7, 10-BaPQ-H_2O_2 system. Reduction of 7,10-BaPQ by $^{\bullet}O_2^-$ radical gave excited semiquinone, which showed strong CL emission when decayed to its ground state. Maximum CL emission wavelength was located at 440 nm, which may belong to the excited semiquinone. This CL system was developed as post column detection of high performance liquid chromatography for the determination of 7,10-BaPQ. Linearity ranged from 50 fmol to 20 pmol (R^2 = 0.9995) with limit of detection of 30 fmol (S/N = 3). The proposed method was used to determine 7,10-BaPQ in airborne particulates. Average atmospheric concentrations of 7,10-BaPQ in Kanazawa in December 2010 and Wajima in October 2007 were 2.0 and 1.6 pg/m^3, respectively.



Polycyclic aromatic hydrocarbon (PAH) is an important class of environmental toxic organic compounds, which are formed by the burning of fossil fuels, industry waste, automobile exhausts, and tobacco smoke.[1−4] Degradation of PAHs leads to the generations of toxic derivatives, such as hydroxylated PAHs, nitro-PAHs, and PAH quinones.[5−8] PAH quinones are harmful environmental pollutants that induce the generation of reactive oxygen species (ROS) though their redox cycle in human body.[9−11] Many adverse health effects, such as aging, carcinogenesis, and inflammation, were related to the generations of ROS.[12,13] In addition, PAH quinones can combine with functional groups of enzymes to give covalent enzyme adducts, which can damage the activity of enzymes.[14,15] Furthermore, Hayakawa and co-workers have reported the estrogenic activity or antiestrogenic activity of PAH quinones.[16] Thus the health effects of PAHs and their derivatives became an important social concern. Therefore, it is necessary to establish a sensitive and reliable method to determine PAH quinones in the environment.

Gas chromatography mass spectrometry (GC-MS) has been reported as an efficient method to detect PAH quinones in airborne particulates. However, derivatization is necessary for GC-MS determination because of the high polarity and low vapor pressure of PAH quinones.[17] High performance liquid chromatography (HPLC) combined with atmospheric pressure ionization mass spectrometry (APCI-MS) is selective and sensitive for determination of PAH quinones.[18−21] In particular, tandem mass spectrometry (MS/MS) has been successfully applied to determine PAH quinones in airborne particulates. But the instrument is expensive for common use. Additionally, LC-MS/MS is not sensitive for four- and five-ring PAH quinones. HPLC with fluorescence detection was frequently used to determine PAHs and their derivatives. Unfortunately, most PAH quinones have no fluorescence emission. Thus, derivatization is needed for HPLC-fluorescence determination. Kishikawa and co-workers established several novel HPLC-fluorescence methods for determination of PAH quinones after derivatization.[22−24] These methods have high sensitivity and selectivity, but loss or contamination of analyte in the process of derivatization may affect accuracy of determination. The problem from derivatization was solved by Hayakawa and co-workers,[25] who combined a Pt−Rh catalytic column after separation column for reduction of PAH quinones. A strong fluorescence emission was observed after PAH quinone reduction. In the reported method,[25] PAH quinones can be detected online without derivatization. However, high cost and short lifetime of Pt−Rh catalytic column may limit the widespread use of the method. Chemiluminescence (CL) detection is a powerful method because excitation light is not needed compared with fluorescence detection. Low baseline signal of CL

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leads to higher sensitivity. HPLC with peroxyoxalate or luminol CL system with online post column ultraviolet irradiation was employed to determine PAH quinones with high selectivity and sensitivity.[26,27] But strict conditions make the methods not applicable for environmental samples.

In the present work, an ultrasensitive CL method for determination of 7,10-BaPQ based on $NaHSO_3$–H_2O_2 CL system was proposed. Although 7,10-BaPQ exhibits no fluorescence emission, a strong CL emission was observed when H_2O_2 was injected into $NaHSO_3$-7,10-BaPQ mixture solutions. 7,10-BaPQ can be detected without any derivatization or reduction in this method. Fmol level of 7,10-BaPQ can be determined easily. The proposed method was successfully applied to detect the concentrations of 7,10-BaPQ in airborne particulates for the first time.

EXPERIMENTAL SECTION

Chemicals and Reagents. Sodium hydrosulfite ($NaHSO_3$) and hydrogen peroxide (H_2O_2) were purchased from Kanto Chemical Co. Inc. (Tokyo, Japan). $NaHSO_3$ solution was freshly prepared by dissolving appropriate amount of $NaHSO_3$ powder in ultrapure water. H_2O_2 solution was freshly prepared by volumetric dilution of commercial 30% H_2O_2 solution. 7,10-BaPQ was obtained from NCI Chemical Carcinogen Repository (Kansas City, MO, U.S.A.). Nitro blue tetrazolium chloride (NBT) was purchased from Nacalai Tesque Inc. (Tokyo, Japan). 5, 5-Dimethyl-1-pyrroline-*N*-oxide (DMPO) was bought from Tokyo Kasei Kogyo Co. Ltd. (Tokyo, Japan). Thiourea and ascorbic acid were from Beijing Chemical Reagent, Co. (Beijing, China).

Solution of 7,10-BaPQ was prepared by dissolving powder with methanol. Standard solution of 7,10-BaPQ was diluted with methanol. All the organic solvents (methanol, hexane, dichloromethane, benzene, and ethanol) were HPLC grade from Wako Pure Chemical Industries, Ltd. Ultrapure water was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, U.S.A.).

CL Profile and Emission Spectra. CL intensity versus time profile was measured by an ultraweak chemiluminescence analyzer (Institute of Biophysics, Chinese Academy of Science, Beijing, China). The signal was recorded by a computer with a data acquisition interface. Data acquisition and treatment were performed with BPCL software.

CL emission spectra were obtained in the same instrument as CL profile. Cutoff filters (400–640 nm), which were inserted between the cuvette and the PMT, were applied to measure the CL emission spectra.

HPLC. The separation of 7,10-BaPQ from samples was carried out on HPLC system as previously reported with some minor changes.[28] First, the separation column was changed to Waters AR-II 5C18 column (250 mm × 4.6 mm). CL detector was from Shimadzu Company (CLD-10A, Shimadzu, Kyoto, Japan). The flow rates of $NaHSO_3$, H_2O_2, and mobile phase were 2.0, 3.0, and 1.5 mL/min, respectively. Mobile phase was 80% (v/v) methanol in water.

LC-MS/MS. The Agilent 1100 series LC system consisted of a G1379A degasser, a G1312A binary pump, a G1367A auto sampler, and a G1316A column oven (all from Agilent Technologies, Palo Alto, CA). Separation of 7,10-BaPQ was performed on an Inertsil ODS-P column (250 mm ×4.6 mm, i.d.; 5.0 μm). The column temperature was kept at 30 °C. Mobile phase was 90% methanol at 0.5 mL/min in isocratic mode.

The mass spectrometric analyses were performed using an API 4000 Q-Trap tandem mass spectrometer (Applied Biosystems, Foster City, CA) equipped with an atmospheric pressure chemical ionization (APCI) source and operated in a positive ion mode. The ion-spay voltage was set to 5500 V. Nitrogen gas was used in all cases. Instrument setting were: curtain gas (10.0), nebulizer gas (10.0), collision gas (5.0). The collision energy was 35 eV. The source and probe temperatures were 30 and 400 °C, respectively. The mass spectrometer was operated under multiple reaction monitoring (MRM) mode, and monitoring ions were m/z 283 → 255 and 283 → 226.[21]

ESR Spectra. Electron spin resonance (ESR) spectra were measured on a JEOL spectrometer (JES-FA200, Japan). Instrument conditions: microwave frequency, 9.75 GHz, power, 12.72 mW, modulation amplitude, 2.01 G, modulation frequency, 100 kHz.

Sample Collection and Preparation. Airborne particulates were collected on the roof of a seven-storey building of Kanazawa University (Kakuma-machi) in Kanazawa, Japan. Wajima samples were collected in Wajima City in October, 2007. Sampling was conducted from November 28, 2010 to December 5, 2010 using a high-volume air sampler (Model 120, Kimoto Electrics, Osaka, Japan) with a 2500 QAT-UP quartz fiber filter (8 in. × 10 in., Pallflex Products, Putnam, CT, U.S.A.) at a flow rate of 1000 L/min (total volume 1434.9 m^3). Filter was changed every 24 h. The filters with airborne particulates were stored in a refrigerator at −20 °C until analysis.

Airborne particulates were extracted with the same method as previously reported with some small change.[28] First, in the previous work, whole filter was used. However, in this study, only half filter was needed. Second, the target compound 1-hydroxypyrene was eluted by 10 mL hexane/ethyl acetate in solid phase extraction step of the previous study, while

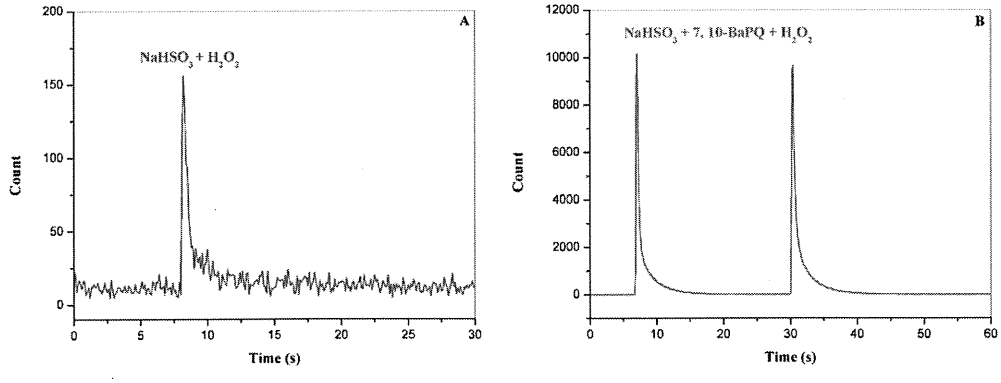

Figure 1. Observation of chemiluminescence emission. (A) $NaHSO_3$ solution was injected into H_2O_2 solution; and (B) $NaHSO_3$ solution was injected into H_2O_2–7,10-BaPQ mixture solutions. $NaHSO_3$, 0.1 mol/L; H_2O_2, 0.15 mol/L; injection volume, 100 μL.

dichloromethane was used to elute 7,10-BaPQ in the present work.

RESULTS AND DISCUSSION

CL Phenomenon. A batch CL system was used for the kinetic study of CL reaction. Figure 1A showed the kinetic curve of $NaHSO_3$–H_2O_2 CL reaction. $NaHSO_3$ reacted with H_2O_2 rapidly. An ultraweak CL emission which lasted less than 2s was observed when H_2O_2 was injected into $NaHSO_3$ (Figure 1A). CL intensity of $NaHSO_3$–H_2O_2 was increased 70 times after addition of 10 pmol 7,10-BaPQ (Figure 1B). However, the shape of kinetics curve of $NaHSO_3$–H_2O_2 was not changed by the addition of 7,10-BaPQ. No CL emission was observed when 7,10-BaQ was injected into with only $NaHSO_3$ or H_2O_2, which means 7,10-BaPQ can not react with only $NaHSO_3$ or H_2O_2. In addition, no signal was recorded when 7,10-BaPQ was injected into the mixture of $NaHSO_3$–H_2O_2 solution. One reason can explain this phenomenon. $NaHSO_3$ reacted with H_2O_2 in less than one second, when 7, 10-BaPQ was adding into the mixture, the reaction between $NaHSO_3$–H_2O_2 has finished.

Identification of the Radicals, Products, and Emitting Species in the CL System. To explain the CL phenomenon, radicals, emitting species, and products which were generated in the $NaHSO_3$–7,10-BaPQ–H_2O_2 CL system were investigated by radical scavengers, ESR spectrum, HPLC, and CL spectrum. The results were showed in Table 1 and Figures 2–4.

Table 1. Effect of Radical Scavengers on the $NaHSO_3$–7,10-BaPQ–H_2O_2 System[a]

radical scavengers	radical	concentration (mol/L)	CL intensity[b] of $NaHSO_3$–H_2O_2–7,10-BaPQ
without scavengers			1.00×10^4
ascorbic acid	$^{\bullet}OH$, $^{\bullet}O_2^-$	0.01	30.0
thiourea	$^{\bullet}OH$	0.01	800
NBT	$^{\bullet}O_2^-$	0.01	15.0
DMPO	$^{\bullet}OH$, $^{\bullet}SO_3^-$	0.05% (v/v)	6.50×10^3

[a]Concentrations of $NaHSO_3$ and H_2O_2 were 0.2 and 0.5 mol/L, injection volume was 100 μL, radical scavengers were 50 μL; 7,10-BaPQ was 10 pmol. [b]Average value of three times.

In Table 1, we can find the CL intensity decreased to 30 with the addition of ascorbic acid. As we known, ascorbic acid is an effective active oxygen free radical ($^{\bullet}OH$, $^{\bullet}O_2^-$) scavenger.[32] Therefore, it can be considered that $NaHSO_3$ reacted with H_2O_2 via radical way. $^{\bullet}OH$ radical has been reported in the oxidation of HSO_3^- ion by H_2O_2.[31,33] Thiourea was commonly used as $^{\bullet}OH$ radical scavenger.[33] When thiourea was added into the CL system, strong inhibition of CL intensity by thiourea was observed, which indicated the generations of $^{\bullet}OH$ radical. In addition, the existence of $^{\bullet}OH$ radical can be confirmed by the electron ESR spectrum (Figure 2).

Production of $^{\bullet}SO_3^-$ radical can be identified by inhibition effect of DMPO, which can trap $^{\bullet}SO_3^-$ radical and $^{\bullet}OH$ (Reactions 1 and 2).[34] The CL reaction stopped when the $^{\bullet}SO_3^-$ radical was trapped by DMPO, which lead to the decreasing of CL intensity.

$$DMPO + {}^{\bullet}SO_3^- \rightarrow DMPO/{}^{\bullet}SO_3 \quad (1)$$

$$DMPO + {}^{\bullet}OH \rightarrow DMPO/{}^{\bullet}OH \quad (2)$$

$^{\bullet}SO_3^-$ radical reacted with excess H_2O_2 to give HO_2^- and finally forming $^{\bullet}O_2^-$ radical.[33] NBT is an specific $^{\bullet}O_2^-$ radical scavenger (reaction 3).[35,36] When NBT was added into the $NaHSO_3$-7, 10-BaPQ-H_2O_2 system, CL intensity decreased to 15 (Table 1). Therefore, we can speculate $^{\bullet}O_2^-$ radical was generated in the $NaHSO_3$–7,10-BaPQ–H_2O_2 system, which played a key role in CL emission.

$$NBT^{2+} + {}^{\bullet}O_2^- \rightarrow {}^{\bullet}NBT^+ + O_2 \quad (3)$$

In addition, ESR method was also employed to confirm the existence of $^{\bullet}OH$ and $^{\bullet}SO_3^-$ radicals, which were generated in the $NaHSO_3$–H_2O_2 system. The life times of $^{\bullet}OH$ and $^{\bullet}SO_3^-$ radicals are short, therefore, DMPO was used to trap them.[31,33,34,37] The ESR spectrum of $^{\bullet}OH$ and $^{\bullet}SO_3^-$ radicals is shown in Figure 2A. In this spectrum, $^{\bullet}OH$ and $^{\bullet}SO_3^-$ radicals overlapped.[31,37] Thus, the spectra in 0, 2, and 4 min were compared (Figure 2B). In the $NaHSO_3$–H_2O_2 system, the lifetime of DMPO/$^{\bullet}OH$ is longer than DMPO/$^{\bullet}SO_3^-$, after 4 min, DMPO/$^{\bullet}SO_3^-$ decomposed or reacted with other ions, while DMPO/$^{\bullet}OH$ still existed in the system (Figure 2B).

With the addition of 7,10-BaPQ, a strong distinct approximate 1:3:3:1 four-line spectrum (g = 2.0042) appeared (Figure 2C), which may belong to the semiquinone of 7,10-BaPQ.[38] The irregular peak was unstable, disproportionate in five minutes (Figure 2D).

The construction of 7,10-BaPQ was confirmed by HPLC before and after it reacting with $NaHSO_3$–H_2O_2. The chromatograms showed that 7,10-BaPQ appeared in the same retention time before and after reacting with $NaHSO_3$–H_2O_2 CL system, which means that 7,10-BaPQ returned back to its original structure after CL emission occurred (Figure 3). However, we also found two small peaks appeared in the chromatogram after the reaction, which may be generated by the reaction of semiquinone with other radicals or ions in the $NaHSO_3$–H_2O_2 system.

The cut off filters (400–640 nm) were used to determine the CL emission spectrum of $NaHSO_3$–H_2O_2. The CL emission from $NaHSO_3$–H_2O_2 was too weak to measure the CL wavelength. Sodium dodecyl sulfate, which is a common surfactant, was applied to enhance the CL intensity. As shown in Figure 4a, a wide peak with a maximum appeared in the spectrum of $NaHSO_3$–H_2O_2 system, which may be ascribed to excited sulfur dioxide. The maximum of the peak moved to 440 nm after addition of 7,10-BaPQ (Figure 4b).

CL Mechanism. On the basis of the study of radicals, products, and CL emission spectrum in the $NaHSO_3$–7,10-BaPQ–H_2O_2 system, mechanism of this CL system can be speculated as the following reactions and graphically showed in Scheme 1.

First, HSO_3^- reacted with H_2O_2 via nucleophilic dispalcement to form a peroxomonosulfurous acid intermediate,[29,30] which decomposed to generate $^{\bullet}OH$ and $^{\bullet}SO_3^-$ radicals (reaction 4).[31,33,37]The $^{\bullet}OH$ radical as a strong oxidant reacted with HSO_3^- to give $^{\bullet}SO_3^-$ radical in a rapid rate.[39,40]

$$H_2O_2 + HSO_3^- \rightleftharpoons \begin{matrix} {}^{-}O \\ S \\ O \end{matrix} \!\!-\!O\!-\!OH \longrightarrow {}^{\bullet}OH + {}^{\bullet}SO_3^- \quad (4)$$

$$^{\bullet}OH + HSO_3^- \rightarrow {}^{\bullet}SO_3^- + H_2O \quad (5)$$

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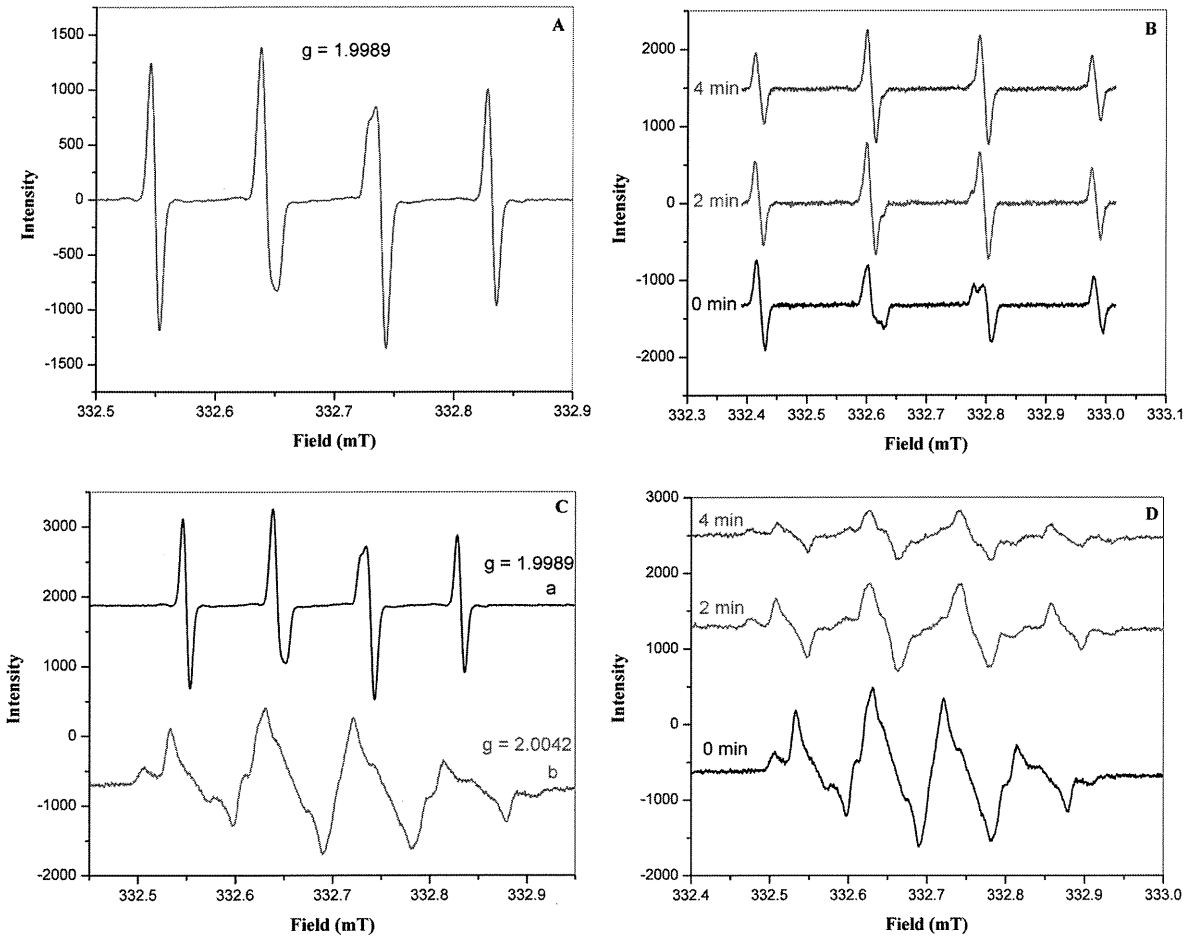


Figure 2. ESR spectra (A) $^{\bullet}OH$ and $^{\bullet}SO_3^-$ radical in $NaHSO_3$–H_2O_2 CL system. (B) DMPO/$^{\bullet}OH$ and DMPO/$^{\bullet}SO_3^-$ in 0, 2, 4 min. (C) ESR spectra of $NaHSO_3$–H_2O_2 CL system before (a) and after addition of 7,10-BaPQ (b). (D) ESR spectra of semiquinone radicals from 7,10-BaPQ. Conditions: DMPO 0.2 mol/L prepared in water; $NaHSO_3$ 0.1 mol/L, H_2O_2 0.5 mol/L. Each volume of DMPO, $NaHSO_3$, and H_2O_2 was 30 μL.

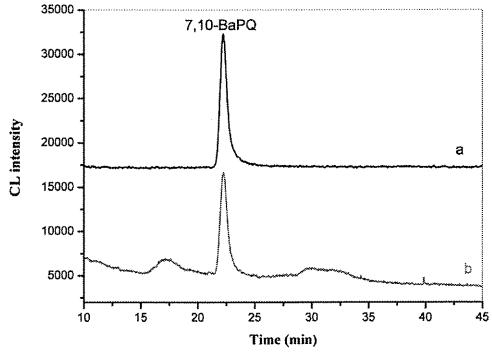


Figure 3. Chromatograms of 7,10-BaPQ (1.5 pmol/injection) before (a) and after (b) reacting with $NaHSO_3$–H_2O_2 system.

$^{\bullet}SO_3^-$ radical reacted with H_2O_2 to give $^{\bullet}O_2^-$ radical (reactions 6 and 7),[33] which is an important radical in the CL system.

$$^{\bullet}SO_3^- + H_2O_2 \rightarrow HSO_3^- + HO_2^{\bullet} \quad (6)$$

$$HO_2^{\bullet} \rightarrow H^+ + {}^{\bullet}O_2^- \quad (7)$$

Several groups have reported the reduction of quinone to semiquinone by $^{\bullet}O_2^-$ radical.[41−44] CL emission was observed with the decay of excited semiquinone,[45−47] which was then oxidized to 7,10-BaPQ (reaction 8).

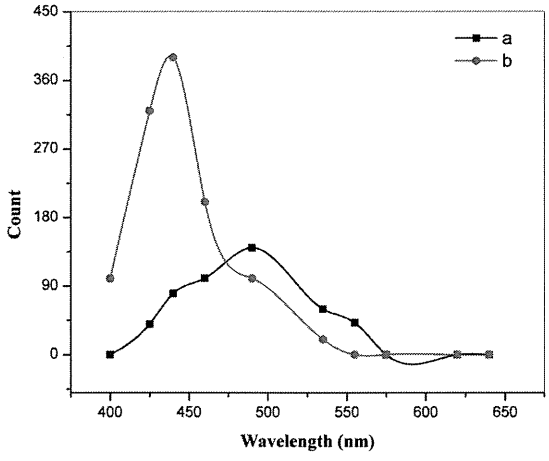
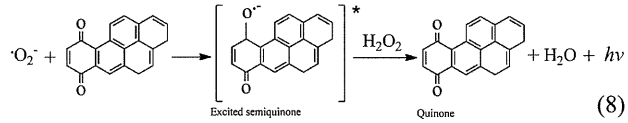


Figure 4. CL emission spectrum of $NaHSO_3$-7, 10-BaPQ-H_2O_2 CL system. (a) Emission spectrum of $NaHSO_3$–H_2O_2 system. (b) Emission spectrum of $NaHSO_3$–7,10-BaPQ–H_2O_2 system.



(8)

Other PAH quinones (1,4-benzoquinone, 1,4-naphthoquinone, 1,4-anthraquinone, and 1,4-chrysenequinone), whose

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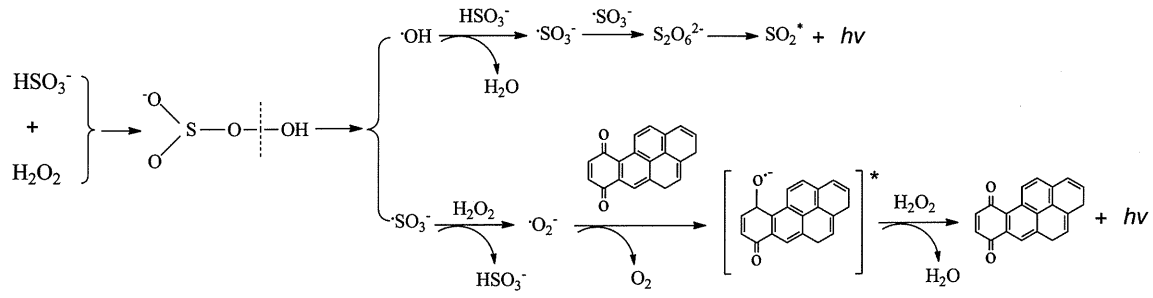
Scheme 1. Mechanism of $NaHSO_3$–7,10-BaPQ–H_2O_2 CL System



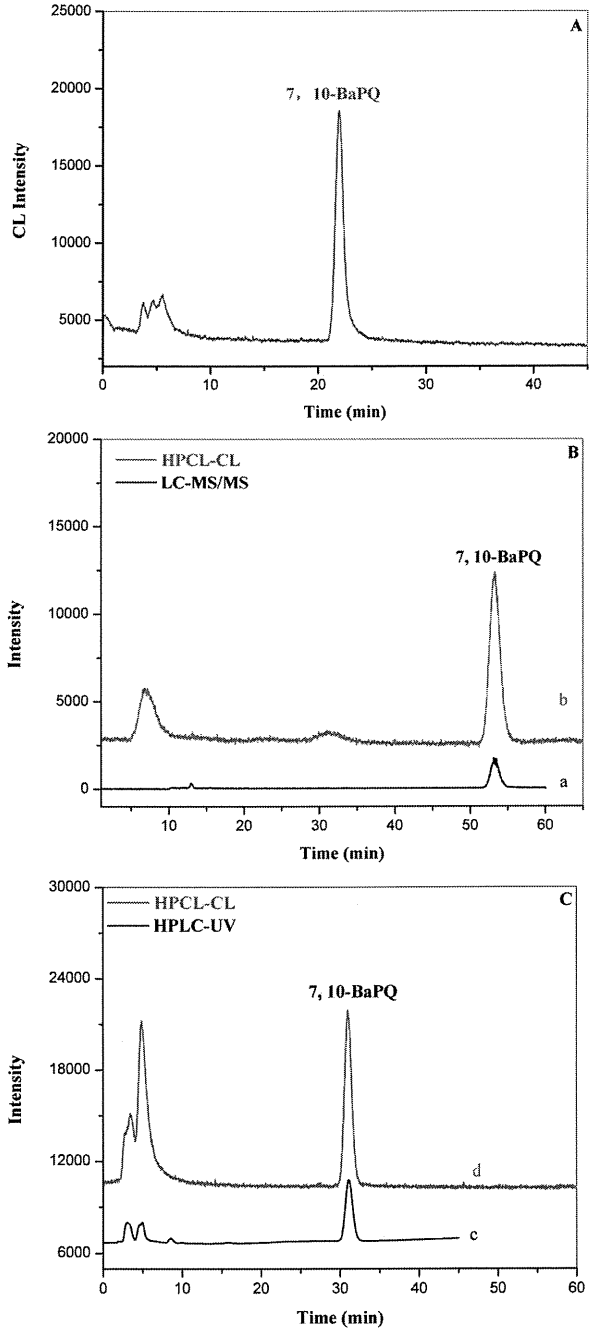


Figure 5. (A) Typical chromatogram of standard 7,10-BaPQ (1.5 pmol per injection) in HPLC-$NaHSO_3$–H_2O_2 system. (B) Chromatograms of 7,10-BaPQ in HPLC-CL (7,10-BaPQ, 1.0×10^{-8} mol/L) and in HPLC-UV (7,10-BaPQ, 1.0×10^{-6} mol/L). (C) Chromatograms of 7,10-BaPQ in HPLC-CL and in LC-MS/MS (7,10-BaPQ, 1.0×10^{-7} mol/L).

structures are similar to 7,10-BaPQ, were also added into the $NaHSO_3$–H_2O_2 system. It was found that with the increase of benzene ring, the CL intensity increased. However, the CL intensities of the PAH quinones were still much lower than 7,10-BaPQ.

Furthermore, generation of excited sulfur dioxide (SO_2*) from $NaHSO_3$–H_2O_2 reaction has been reported.[33] $^{\bullet}SO_3^-$ radical dimerized to give $S_2O_6^{2-}$ ion (reaction 9). SO_2* was produced by the decomposition of $S_2O_6^{2-}$ ion (reaction 10).[48,49]

$$^{\bullet}SO_3^- + {}^{\bullet}SO_3^- \rightarrow S_2O_6^{2-} \quad (9)$$

$$S_2O_6^{2-} \rightarrow SO_4^{2-} + SO_2^* \quad (10)$$

Analytical Figures of Merit. The CL system was developed as an ultrahighly sensitive postcolumn detection for the determination of 7,10-BaPQ in airborne particulates. Organic solvents which were used for HPLC mobile phase have critical effect on $NaHSO_3$–7,10-BaPQ–H_2O_2 CL system. Methanol and acetonitrile were compared. It was found 80% (v/v) methanol/water gave baseline separation of 7,10-BaPQ from interference compounds and highest peak height. The typical chromatogram of standard 7,10-BaPQ in the optimized conditions is shown in Figure 5A.

The proposed method was evaluated in terms of linearity, sensitivity, and precision. Calibration curve was obtained by the standard 7,10-BaPQ solution over the range from 50 fmol to 20 pmol per injection. A highly linear relationship between peak height and concentrations was obtained (12 calibration points; $R^2 = 0.9995$). The slope and intercept of the regression equation of calibration curve were 1.22×10^{12} and −463. The detection limit ($S/N = 3$) of 7,10-BaPQ was 30 fmol per injection. Till now, LC-MS/MS is the most sensitive method for the determination of 7,10-BaPQ.[50] However, the sensitivity of the proposed HPLC-CL method for detecting 7,10-BaPQ was 2 orders of magnitude higher than LC-MS-MS method and 3 orders of magnitude higher than HPLC-UV (Figure 5B and Figure 5 C). The precision and accuracy of the proposed method for determination of 7,10-BaPQ were investigated with

Table 2. Precision of the Method for Determining 7,10-BaPQ in Airborne Particulates and Recovery of 7,10-BaPQ in Airborne Particulates

	intraday ($n = 3$)			interday ($n = 3$)		
added (pmol)	0	15.0	50.0	0	15.0	50.0
found (pmol)	3.90	19.3	53.7	3.90	18.5	54.1
recovery (%)[a]		102.7	99.6		97.3	100.4
RSD (%)	7.80	4.80	4.80	5.70	1.60	1.00

[a]Expressed as (found amount − nonspiked amount)/added amount.

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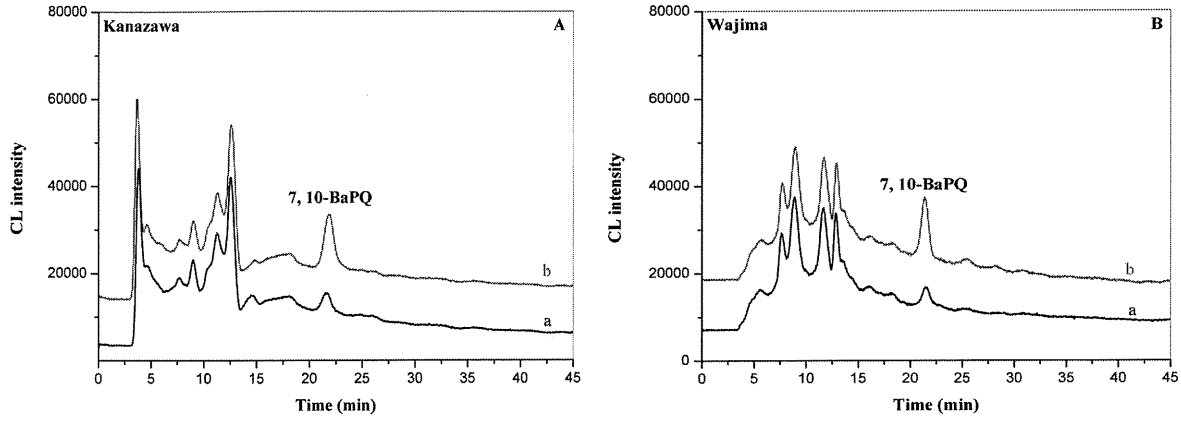

Figure 6. Chromatograms of 7,10-BaPQ (a) in airborne particulates and (b) airborne particulates spiked with 1.5 pmol 7,10-BaPQ.

spiking the airborne particulates at two different level of 7,10-BaPQ standard solutions. The results are shown in Table 2.

Determination of 7,10-BaPQ in Airborne Particulates. Benzene[*a*]pyrene (BaP), which is one of most important PAH pollutants in environment, has high carcinogenicity, mutagenicity. In airborne particulates, BaP reacted with some reactive oxygen species to generate BaP quinones. The toxicity of these BaPQs is higher than BaP. Therefore, it is urgency to monitoring the concentration of these BaPQs. However, common methods, such as LC-MS/MS, GC-MS/MS, HPLC-UV, and HPLC-fluorescence, are difficult to detect the BaPQs because their sensitivities are not high enough for the really application, especially for 7,10-BaPQ.[50] The proposed HPLC-CL method was employed to determine 7,10-BaPQ in airborne particulates successfully. Average concentrations of 7,10-BaPQ in Kanazawa, Japan in December 2010 and Wajima, Japan in October 2007 were 2.0 and 1.6 pg/m^3, respectively. Chromatograms of airborne particulates were show in Figure 6A and B.

CONCLUSIONS

7,10-BaPQ (10 pmol) is found to greatly enhance the ultra-weak CL emission, which emits from $NaHSO_3$–H_2O_2 system. The radicals of $NaHSO_3$–7,10-BaPQ–H_2O_2 system are confirmed by CL spectrum, radical scavengers and ESR spectrum. Superoxide ($^{\bullet}O_2^-$) radical reacted with 7,10-BaPQ to give semiquinone which give out a strong CL signal at 440 nm. An ultrasensitive CL detection method was established based on this mechanism.

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Notes

The authors declare no competing financial interest.

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Short communication

Analysis of 8-hydroxy-2′-deoxyguanosine in human urine using hydrophilic interaction chromatography with tandem mass spectrometry

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ABSTRACT

Urinary 8-hydroxy-2′-deoxyguanosine (8-OHdG) is a widely used noninvasive biomarker of oxidative stress. A selective, sensitive and rapid method for determining 8-OHdG in human urine was developed using hydrophilic interaction chromatography–tandem mass spectrometry (HILIC–MS/MS) with electrospray ionization. 8-OHdG and isotopically labeled 8-OHdG (internal standard) were separated on a HILIC column with a mobile phase of 10 mM ammonium acetate: acetonitrile (1:9, v/v) within 10 min and detected by using a positive electrospray ionization interface under the selected reaction monitoring mode. The detection limits of 8-OHdG (corresponding to a signal-to-noise ratio of 3) for the HILIC-MS/MS system and the conventional method using a reversed-phase column with MS/MS were 1.0 and 26.0 fmol/injection, respectively. The proposed method makes it possible to monitor the basal level of urinary 8-OHdG from non-exposed healthy subjects and can be used for large-scale human studies.

1. Introduction

Oxidative stress in an organism arises from excessive generation of reactive oxygen species (ROS) such as superoxide radicals, hydrogen peroxide and hydroxyl radicals or from depletion of antioxidants [1]. The production of ROS can be induced by both endogenous and exogenous factors [2]. While endogenous factors include physiological processes, exogenous factors include environmental sources such as smoking, diet and pollution [3]. ROS may cause oxidative damage to nucleic acids, proteins, and lipids [2]. In particular, oxidative damage to DNA has been associated with numerous pathological conditions, having both genetic and epigenetic consequences [4–6]. To understand how ROS affect normal and pathological processes, an indicator to assess oxidative stress *in vivo* is required.

The oxidized product of DNA, 8-hydroxy-2′-deoxyguanosine (8-OHdG), is the most frequently measured biomarker of the oxidative stress [7,8]. The 8-OHdG has been analyzed in various kinds of samples, such as urine, serum, peripheral blood leukocyte, and organ tissue [9]. Measurements of 8-OHdG in urine samples are especially well-suited to large-scale human studies and clinical applications because they are noninvasive [10,11]. Urinary 8-OHdG has been analyzed by several methods, such as enzyme-linked immunosorbent assay (ELISA) [12,13], high-performance liquid chromatography with electrochemical detection (HPLC-ECD) [14], gas chromatography with mass spectrometry (GC/MS) [15,16], and liquid chromatography with tandem mass spectrometry (LC/MS/MS) [17–26]. The ELISA method suffers from the problem of non-selectivity because the antibody may cross-react with other substances present in urine [12,27,28]. HPLC-ECD has often been used [29], but it suffers from possible interference from the biological matrix, incompatibility of a stable isotope labeled internal standard [16,21]. For GC/MS analysis, 8-OHdG must be purified by HPLC and derivatized before analysis [15,16,28].

LC–MS/MS has been increasingly applied to detect urinary 8-OHdG. LC–MS/MS, when combined with the isotope dilution technique, is highly selective, sensitive, and accurate, and does not require derivatization [28]. In most of previous LC–MS/MS studies, reversed-phase columns have been used to separate 8-OHdG [17–26]. As a polar compound, 8-OHdG is hardly retained on a reversed-phase column, even though aqueous mobile phases are used. Such poor retention of 8-OHdG and its insufficient separation from polar components in the matrix may lead to matrix effects, which can increase or decrease the 8-OHdG MS signal [30]. In addition, aqueous mobile phases that are used to retain polar compounds on reversed-phase columns are not suited for electrospray ionization (ESI) conditions [30]. Indeed, urinary 8-OHdG levels have even been below the detection limit of LC–MS/MS

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method (7.5 fmol/injection, $S/N = 3$) [27,31]. Therefore, a more sensitive LC–MS/MS method is required to measure basal levels of urinary 8-OHdG in non-exposed healthy subjects.

In recent years, hydrophilic interaction chromatography (HILIC) has been increasingly used in LC–MS/MS for analyzing polar compounds such as metabolites in biological samples. Under HILIC conditions, the analyte interacts with a hydrophilic stationary phase and is eluted with a high concentration of organic solvent (typically acetonitrile with a small percentage of water/buffer). The highly organic mobile phase can result in increased sensitivity with ESI-MS detection [30,32].

In this study, we developed an improved LC–MS/MS method for measuring urinary 8-OHdG using a HILIC column. We found that the HILIC column provided much greater sensitivity than a conventional reversed-phase column with the same MS/MS detection conditions.

2. Experimental

2.1. Materials

The 8-OHdG and [$^{15}N_5$]8-OHdG were purchased from Sigma (MO, USA) and Cambridge Isotope Laboratories (MA, USA), respectively. HPLC grade acetonitrile was obtained from Kanto Chemical (Tokyo, Japan), and water from a Milli-Q water purification system (Millipore, Bedford, MA, USA). All other chemicals and solvents used were of an analytical grade.

2.2. Sample preparation

Urinary samples were pretreated as described previously with a slight modification [22,28]. After centrifugation at 2150 × *g* for 10 min, a 100 μL aliquot of each supernatant was diluted with 900 μL of water and spiked with 10 pmol of the stable isotope labeled internal standard (IS), [$^{15}N_5$]8-OHdG. The diluted sample was subjected to solid-phase extraction using Oasis HLB cartridge (3 cc, 60 mg; Waters, Milford, MA, USA) that had been primed with 1 mL of methanol and 1 mL of water. After sample loading, the cartridge was sequentially washed with 1 mL of water. The 8-OHdG was eluted from the cartridge with 500 μL of water: acetonitrile (1:1, v/v), and evaporated to dryness using a centrifugal vacuum evaporator. The residue was redissolved in 100 μL of water: acetonitrile (1:9, v/v), and an aliquot of 20 μL was injected into the LC–MS/MS system.

2.3. 8-OHdG analysis by HILIC-MS/MS

2.3.1. Apparatus and chromatographic conditions

The Agilent 1100 series LC system consists of a G1379A degasser, a G1312A binary pump, a G1367A autosampler, and a G1316A column oven (all from Agilent Technologies, Palo Alto, CA, USA). The chromatographic separation of 8-OHdG in the pretreated urine samples was performed on a COSMOSIL HILIC (150 mm × 2.0 mm i.d., 5 μm, Nacalai Tesque, Kyoto, Japan), kept at the temperature of 40 °C. The column was eluted isocratically with 10 mM ammonium acetate: acetonitrile (1:9, v/v) at a flow rate of 0.4 mL/min. The retention time of the analyte was optimized by varying the mobile phase acetonitrile content between 70% and 95% with 10 mM ammonium acetate and by varying the aqueous buffer concentration between 10 and 50 mM with 90% acetonitrile. The retention factor (k) of 8-OHdG was defined as $k = (t_R - t_0)/t_0$, where t_R and t_0 are the retention times of the analyte and the hold-up time, respectively. Sample volumes of 20 μL were injected for each analysis. The mass spectrometric analyses were performed using an API 4000 Q-Trap tandem mass spectrometer (Applied Biosystems, CA, USA) equipped with an electrospray ionization (ESI) and operated in a positive ion mode. Sensitivity of the selective reaction monitoring (SRM) was optimized by testing with an infusion of 8-OHdG under the mobile phase condition. The mass spectrometer was operated under SRM mode of the transitions at m/z 284.3 → 167.9 for 8-OHdG and at m/z 289.1 → 173.0 for [$^{15}N_5$]8-OHdG (IS) with dwell times of 1000 ms. The spray voltage was maintained at 5.5 kV. Nitrogen gas was used as the collision gas (4 psi) and curtain gas (20 psi), whereas zero grade air was used as the nebulizer gas (40 psi) and heated gas (60 psi). Source temperature was set at 600 °C. The collision energy and declustering potential were set at 19 V and 71 V, respectively. Analyst software (version 1.4, Applied Biosystems) was used to control the LC–MS/MS system, and to acquire and process the data.

2.3.2. Calibration curve and validation

Calibration curves for quality control (QC) samples were obtained from the ratio of peak areas of 8-OHdG and [$^{15}N_5$]8-OHdG (IS) using 0.1 mL human urine samples from six humans spiked with 8-OHdG at final concentrations of 0.2, 5, 10, 30, 50, 100 nmol/L. Standard curves were also obtained from plotting the peak area ratio against the same six concentrations of the analyte as the spiked urine samples ($n = 6$ for each). The lower limit of quantification (LLOQ) was determined as the lowest standard on the calibration curve that gave a signal-to-noise ratio of more than ten and reached a precision of 20% and an accuracy of 80–120%. The limit of detection (LOD) was determined as the lowest concentration that gave a signal-to-noise ratio of more than three. To evaluate the intra and inter-day accuracy and precision, the stock solution of 8-OHdG was added to urine at concentrations of 10 and 50 nmol/L in 0.1 mL urine. The spiked samples, together with non-spiked samples, were analyzed using HILIC–MS/MS and 8-OHdG concentrations were calculated using a standard curve. Accuracy was expressed as the ratio of the quantified concentration to the known concentration of 8-OHdG. To evaluate the intra-day precision, the non-spiked samples and the samples spiked at the two levels were prepared five times per day. The inter-day precision was determined using five independent experiments. The precision was calculated as the relative standard deviation (RSD) (%) of the replicates.

2.4. 8-OHdG analysis by LC–MS/MS using reversed-phase column

On the basis of previously reported LC–MS/MS method [25], chromatographic separation of 8-OHdG and [$^{15}N_5$]8-OHdG (IS) in urine samples was performed on a XBridge C_{18} column (150 mm × 2.1 mm i.d., 3.5 μm, Waters) with a guard column (XBridge C_{18} column, 10 mm × 10 mm i.d., 5 μm, Waters). The elution was run isocratically with a mobile phase consisting of 10 mM ammonium acetate: methanol (19:1, v/v) at a flow rate of 0.4 mL/min. The column temperature was set at 40 °C. The mass spectrometric detection was carried out by the same SRM transitions as the HILIC mode.

3. Results and discussion

3.1. Mass spectrometry and chromatography

The full scan mass spectrum of 8-OHdG in the positive ESI mode and the fragmentation pattern of protonated molecular ion $[M+H]^+$ observed in this study were consistent with those of previous studies [17,18,24,26]. The transition from the molecular ion $[M+H]^+$ to the most intense fragment was recorded in the selective reaction monitoring (SRM) acquisition mode. The main product ions of 8-OHdG and [$^{15}N_5$]8-OHdG (IS) were m/z 167.9 and 173.0 $[M+H-116]^+$, respectively. Therefore, the $[M+H]^+ \rightarrow [M+H-116]^+$ transition was used in the SRM mode.

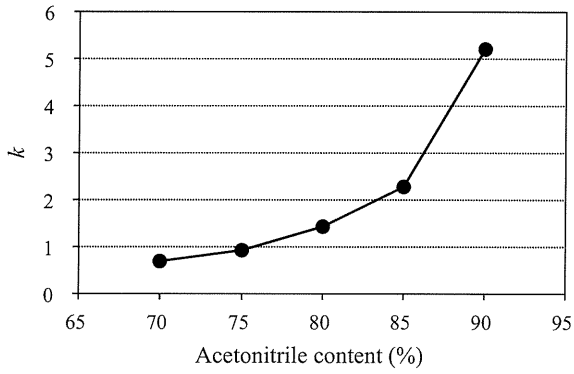

Fig. 1. Effect of acetonitrile content in the mobile phase on the retention of 8-OHdG.

We examined the effect of the acetonitrile concentration in the mobile phase of the HILIC column on the retention time of the analytes. The column was operated under isocratic elution conditions using acetonitrile concentration between 70 and 95%, at the interval of 5%. The retention factor (k) increased with the increasing acetonitrile concentration (Fig. 1). The high content of acetonitrile increases the hydrophilic interactions between the analytes and the stationary phase [33]. At 95% acetonitrile, 8-OHdG was retained for over 15 min on the column and its peak shape was broad. The retention time of charged analytes can be affected by adding salt to the mobile phase due to the electrostatic interactions between the column and analytes [33,34]. For a salt, we selected ammonium acetate because of its solubility in concentrated acetonitrile solutions, and its volatility at the ion source. The effect of the buffer concentration in the mobile phase on the retention of the analyte was investigated in the range 10–50 mM. Increasing the buffer concentration only slightly decreased the retention and did not influence the peak shape (data not shown). Finally, the analytes were separated by isocratic elution with 10 mM ammonium acetate: acetonitrile (1:9, v/v). The analyte and the internal standard eluted within 10 min using the optimized mobile phase (Fig. 2, A-1 and A-2).

3.2. Calibration curve and validation

The calibration curve for the standard compound was linear ($r^2 > 0.999$) for concentrations in the range of 0.2–100 nmol/L (LLOQ: 0.2 nmol/L), which covers the lower range of the reported levels of 8-OHdG in human urine [29], and the slope was 0.00630 ± 0.00026 (mean ± S.D. RSD, 4.1%).

Representative SRM chromatograms for the analyte and the internal standard of a urine sample from a non-smoker showed that the physiological components of the urine did not interfere with the identification and quantification of the analytes in the chromatograms (Fig. 2, B-1 and B-2). The matrix effect on the mass spectrometric response was evaluated by comparing the slope of the calibration curve with the slope obtained in the presence of urine matrix. Six curves were obtained using six different urine samples, each spiked with six different amounts of 8-OHdG. The mean slope was 0.00655 ± 0.00043 (mean ± S.D., RSD, 6.6%), which was almost identical to the mean slope obtained with the standard solutions. This clearly showed that the matrix did not affect the calibration curve. Therefore, 8-OHdG was quantified by using the calibration curve obtained from the standard solution.

The precision and accuracy of 8-OHdG determination in human urine with the HILIC–MS/MS system were examined by adding two different known amounts of 8-OHdG to a urine sample. The RSDs of the intra-day precision assay ($n = 5$) were in the range 2.3–2.6%, and those of the inter-day assay ($n = 5$) were in the range 2.1–4.0% (Table 1). The accuracy values (%) of the intra-day study and the inter-day assay were in the range 96–102%. Both intra and inter-day precision and accuracy values were satisfactory for determining 8-OHdG in human urine.

3.3. Comparison of the HILIC column with a reversed-phase column

The instrumental detection limit of 8-OHdG measured by the HILIC–MS/MS was 1.0 fmol/injection (signal-to-noise ratio, $S/N > 3$). In contrast, the detection limit achieved with a reversed-phase column and the same detection system was 26.0 fmol/injection under optimal instrumental conditions. Our detection limit of 1.0 fmol

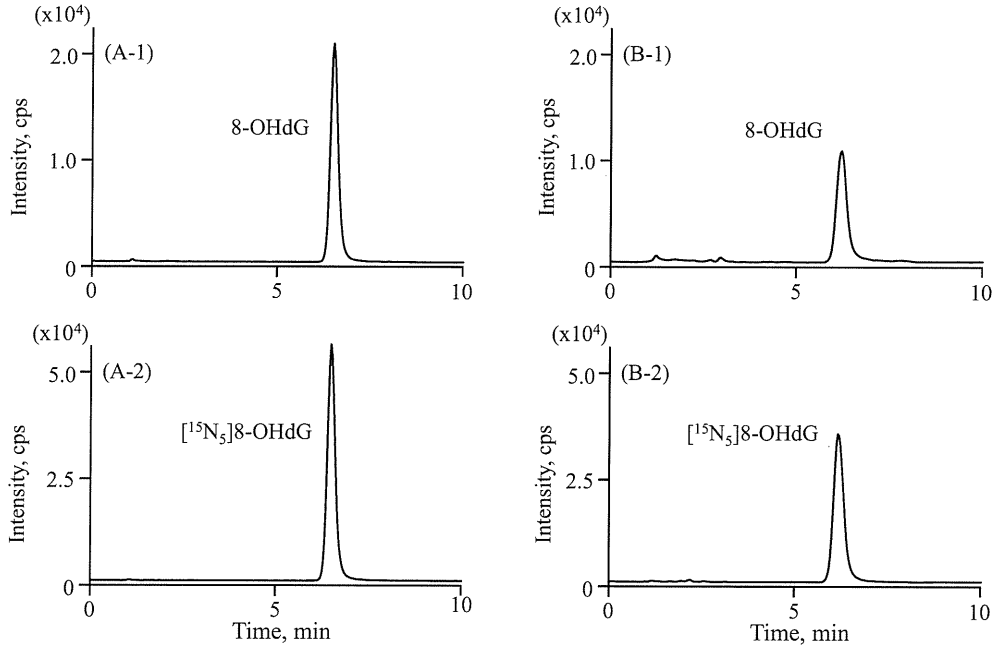

Fig. 2. Representative SRM chromatograms (transition: m/z 284.3 → 167.9 for 8-OHdG and m/z 289.1 → 173.0 for the internal standard ([$^{15}N_5$]8-OHdG)) of a standard solution corresponding to 200 fmol 8-OHdG/injection (A) and a non-smoker urine sample (B) (A-1 and B-1 panels for 8-OHdG; A-2 and B-2 panels for the internal standard).

Table 1
Precision and accuracy in the determination of 8-OHdG in urine samples.

	Intra-day assay ($n=5$)			Inter-day assay ($n=5$)		
Added amounts (pmol/mL urine)	0	10	50	0	10	50
Found ± SD (pmol/mL urine)	17.5 ± 0.4	27.0 ± 0.7	68.5 ± 1.6	16.8 ± 0.4	26.9 ± 0.7	67.5 ± 2.7
RSD (%)	2.3	2.6	2.4	2.1	2.8	4.0
Accuracy (%)	–	96	102	–	101	101

was considerably lower than the detection limits reported for other previous LC–MS/MS methods: 20 fmol [17], 7 fmol ($S/N=3$) [18], 5 fmol ($S/N=4$) [19] and 7.5 fmol/injection ($S/N=3$) [27,31]. The low detection limit of method enables the measurement of basal levels of urinary 8-OHdG in non-exposed healthy subjects that were not quantified in the previous report [27,31]. Furthermore, the analysis can be completed in 10 min and does not require washing and re-equilibrating the column, which makes it well suited for continuous analyses.

4. Conclusions

HILIC-MS/MS provides a selective, sensitive and rapid method for determining 8-OHdG in human urine. The method has acceptable linearity, accuracy, and precision, and is more sensitive than previously described LC–MS/MS methods that have been used in reversed-phase columns. The proposed HILIC–MS/MS method is well suited for large-scale human studies and clinical studies, and would also be applicable to analysis of 8-OHdG in not only urine but also other biological fluids such as plasma, serum and saliva, and in tissue.

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