Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography B, 824 (2005) 166–174
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

A novel, sensitive method for determining benzo[*a*]pyrene-diones using high-performance liquid chromatography with post-column zinc reduction

Kai H. Liao [a,b,1], Arthur N. Mayeno [a,c], Kenneth F. Reardon [a,b], Raymond S.H. Yang [a,c,*]

[a] *Quantitative and Computational Toxicology Group, Center for Environmental Toxicology and Technology, Colorado State University, Fort Collins, CO 80523, USA*
[b] *Department of Chemical and Biological Engineering, Colorado State University, Fort Collins, CO 80523, USA*
[c] *Department of Environmental and Radiological Health Sciences, Colorado State University, Campus Delivery 1680, Physiology Building, Fort Collins, CO 80523-1680, USA*

Received 17 April 2005; accepted 12 July 2005
Available online 2 August 2005

Abstract

A novel and sensitive high-performance liquid chromatography (HPLC) method was developed to analyze dione metabolites of benzo[*a*]pyrene (BaP). Because BaP-diones do not fluoresce, detection of low concentrations is difficult to achieve when analyzing these chemicals with a simple HPLC system. We developed a method to increase the detection sensitivities for BaP-diones using reduction by zinc after the chromatographic separation. A post-column zinc reducer was used to convert BaP-diones, in-line, to their corresponding fluorescent BaP-hydroquinones, which can be measured by fluorescence detection with high sensitivity. With 20-μL injections, the limits of detection for the BaP-diones tested (BaP-1,6-dione, BaP-3,6-dione, and BaP-6,12-dione) were all below 1.0 nM. In addition to the high detection sensitivity, this HPLC method provides a wide linear dynamic range for BaP-dione detection (1.0–220 nM). We also studied the extraction recovery of BaP-diones from recombinant human cytochrome P450 and epoxide hydrolase. To demonstrate the application of this method, the kinetics of BaP-dione formation was studied by incubating BaP with these recombinant enzymes. The present method enhances the detection sensitivity for BaP-diones by more than two orders of magnitude compared with traditional ultraviolet detection. Moreover, the method avoids the time-consuming derivatization or reduction steps required by other methods.

Keywords: Benzo[*a*]pyrene; Benzo[*a*]pyrene-dione; Cytochrome P450; Post-column; Zinc reduction; High-performance liquid chromatography (HPLC); Metabolites

1. Introduction

Benzo[*a*]pyrene (BaP) is a probable human carcinogen [1] that is formed by incomplete combustion of organic matter and is widespread in the environment. Like many other xenobiotics that require activation, BaP undergoes biotransformation by cytochrome P450 and epoxide hydrolase to its mutagenic forms, e.g. 7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene and BaP-4,5-dihydroepoxide [2–5]. Biotransformation of BaP was studied in our laboratory using recombinant human cytochrome P450 1A1 (CYP1A1) and microsomal epoxide hydrolase (EH), as part of our research to study and simulate, by computer, the metabolic reaction networks of xenobiotics [6–8]. Due to the high cost of the enzymes, these experiments were carried out in small volumes (1000 μL), resulting in limited amounts of metabolites. Therefore, analytical methods with high detection sensitivities were required to determine BaP metabolites.

* Corresponding author. Tel.: +1 970 491 5652.
E-mail address: raymond.yang@colostate.edu (R.S.H. Yang).
[1] Present address: CIIT Centers for Health Research, 6 Davis Drive, Research Triangle Park, NC 27709, USA.

1570-0232/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2005.07.021

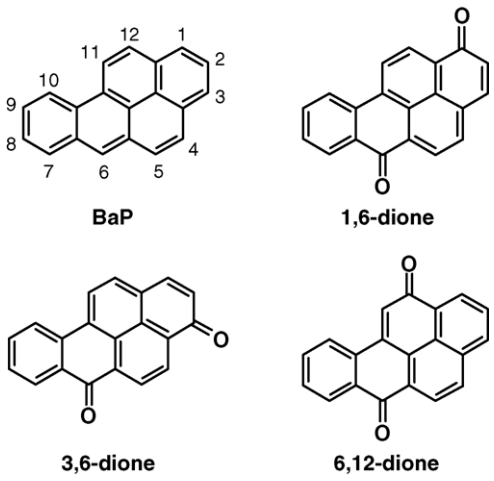

Fig. 1. Chemical structures of benzo[*a*]pyrene (BaP), BaP-1,6-dione (1,6-dione), BaP-3,6-dione (3,6-dione), and BaP-6,12-dione (6,12-dione).

Previously published studies utilized high-performance liquid chromatography (HPLC) methods, with various detectors, e.g. ultraviolet (UV), fluorescence, radioactivity, and mass spectrometry (MS), to analyze BaP metabolites [9–12]. Similarly, we used HPLC to monitor the formation of 11 BaP metabolites, namely BaP-1,6-dione (1,6-dione), BaP-3,6-dione (3,6-dione), BaP-6,12-dione (6,12-dione), 3-hydroxy-BaP (3-OH), 7-hydroxy-BaP (7-OH), 9-hydroxy-BaP (9-OH), BaP-*trans*-4,5-dihydrodiol(±) (4,5-diol), BaP-*trans*-7,8-dihydrodiol(±) (7,8-diol), BaP-*trans*-9,10-dihydrodiol(±) (9,10-diol), BaP-7,8,9,10-tetrol-7,8,9,10-tetrahydro-(7α,8β,9β,10α)-(±) (7,10/8,9-tetrol), and BaP-7,8,9,10-tetrol-7,8,9,10-tetrahydro-(7α,8β,9α,10β)-(±) (7,9/8,10-tetrol). Except for the BaP-diones (1,6-, 3,6-, and 6,12-diones; structures shown in Fig. 1), all of these BaP metabolites fluoresce. Thus, fluorescence detection was used to analyze the metabolite mixtures. Due to the non-fluorescent nature of BaP-diones, alternative detectors are required to achieve the same detection sensitivities [12]. Using UV absorbance, the detection limits of BaP-diones are at least two orders of magnitudes higher than those of the other fluorescent BaP metabolites using a fluorescence detector. An interesting and useful characteristic of BaP-diones is that they yield the corresponding BaP-hydroquinones upon chemical reduction. The BaP-hydroquinones are highly fluorescent [13], and thus can be monitored by a fluorescence detector at very low levels. However, BaP-hydroquinones are unstable in the presence of air and readily autoxidize to their BaP-diones [14]. To develop a simple technique to reduce BaP-diones to their respective hydroquinones, we adapted a method that had been used to analyze Vitamin K compounds by HPLC, in which Vitamins K_1 and K_3 were reduced in-line to their fluorescent analogues using reduction by zinc metal following the chromatographic separation [15,16]. Here we report the development and validation of a simple HPLC method based on this post-column zinc reducer strategy. Reduction of the BaP-diones increased the detection sensitivity by more than two orders of magnitude. This new method is simple, uses readily available equipment, and avoids labor-intensive and time-consuming sample preparation involving reducing or derivatizing reagents.

2. Materials and methods

2.1. Chemicals and reagents

BaP, benzo[ghi]perylene (BghiP), acetic acid (99.7%, A.C.S. reagent), and Trizma Pre-set crystal (pH 7.4 at 37 °C) were purchased from Sigma–Aldrich (St. Louis, MO). 1,6-Dione, 3,6-dione, 6,12-dione, 3-OH, 7-OH, 9-OH, 4,5-diol, 7,8-diol, 9,10-diol, 7,10/8,9-tetrol, and 7,9/8,10-tetrol were purchased from National Cancer Institute Chemical Carcinogen Reference Standard Repository (Kansas City, MO) and stored at −20 °C. HPLC grade methanol, acetone, ethyl acetate, and dimethyl sulfoxide (DMSO) were purchased from Fisher Scientific (Hampton, NH). Zinc powder (−140 + 325 mesh; 99.9% on metals basis) was purchased from Alfa Aesar (Ward Hill, MA).

2.2. Enzymes

Microsomal preparations of recombinant human cytochrome P450 1A1 expressed in *Escherichia coli* (106 pmol CYP1A1/mg protein) were purchased from XenoTech, LLC (Lenexa, KS). Human lymphoblast microsomes containing human microsomal epoxide hydrolase (styrene oxide hydrolase activity of 25 nmol/min mg protein) were purchased from BD Gentest (Bedford, MA). NADPH regenerating system, containing glucose-6-phosphate dehydrogenase, NADP+, glucose-6-phosphate, and $MgCl_2$, was also purchased from BD Gentest.

2.3. HPLC instruments

The HPLC system consisted of a Beckman Coulter System Gold (Fullerton, CA), composed of Model 126 pump, Model 507 autosampler, and Model 166 UV detector, coupled, in line, to a Jasco (Easton, MD) FP-920 fluorescence detector. BaP-diones were separated on a Discovery C18 Column (250 mm × 4.6 mm I.D., 5 μm; Supelco, Bellefonte, PA). A 0.5-μm precolumn filter (Upchurch Scientific, Oak Harbor, WA) and a C18 SecurityGuard cartridge (4.0 mm × 3.0 mm; Phenomenex, Torrance, CA) were used to protect the analytical column. An eight-channel Degasys Populaire vacuum degasser (Chrom Tech, Inc., Apple Valley, MN) was used to de-gas the mobile phases.

2.4. Chromatographic conditions

Twenty microliters of sample, dissolved in methanol, was injected for HPLC analysis. The flow rate was 0.7 mL/min during separation (0–57 min) and increased to 1.0 mL/min at

Table 1
Time steps for the mobile phase gradient for analyzing BaP-diones

Time (min)	Composition of mobile phase (%)	
	A[a]	B[a]
0	40	60
1	40	60
26	20	80
37	20	80
39	4	96
56	4	96
57	40	60
66	40	60

[a] Mobile Phase A: H_2O with 0.003% (v/v) acetic acid; Mobile Phase B: methanol.

the end of the run to re-equilibrate the column (57–66 min). Mobile Phase A contained water with 0.003% (v/v) acetic acid (pH 4.1). Mobile Phase B was methanol. The time program for the multi-step gradient is described in Table 1. BaP-diones were reduced to BaP-hydroquinones by a post-column zinc reducer (described below) and monitored using the fluorescence detector at 400 nm for excitation and 500 nm for emission. The wavelengths used for monitoring other BaP metabolites were listed in Table 2. UV absorbance at 280 nm was also monitored. HPLC analyses were performed at ambient temperature.

2.5. Post-column zinc reducer

An apparatus was designed to reduce BaP-diones to their corresponding fluorescent BaP-hydroquinones in-line (Fig. 2). The key component of this apparatus is the post-column zinc reducer, which consisted of a 20 mm × 2 mm I.D. guard column (Upchurch Scientific) with 0.5-μm frits packed with zinc powder (−140 + 325 mesh; equivalent to 44–105 μm). To pack the zinc reducer, the guard column was

Table 2
Time program for the fluorescence detector for measuring BaP-diones and other BaP metabolites

Time (min)	Wavelength (nm)		Compounds[a]
	Excitation	Emission	
0.0–14.0	278	407	9,10-Diol, 7,10/8,9-tetrol, 7,9/8,10-tetrol
14.0–22.5	263	388	4,5-Diol
22.5–25.5	348	402	7,8-Diol
25.5–35.0	400	500	1,6-Dione, 3,6-dione, 6,12-dione
35.0–66.0	375	441	BaP, 3-OH, 7-OH, 9-OH, BghiP

[a] *Abbreviations*: BaP: benzo[*a*]pyrene; 1,6-dione: BaP-1,6-dione; 3,6-dione: BaP-3,6-dione; 6,12-dione: BaP-6,12-dione; 3-OH: 3-hydroxy-BaP; 7-OH: 7-hydroxy-BaP; 9-OH: 9-hydroxy-BaP; 4,5-diol: BaP-*trans*-4,5-dihydrodiol(±); 7,8-diol: BaP-*trans*-7,8-dihydrodiol(±); 9,10-diol: BaP-*trans*-9,10-dihydrodiol(±); 7,10/8,9-tetrol: BaP-7,8,9,10-tetrol-7,8,9,10-tetrahydro-(7α,8β,9β,10α)-(±); 7,9/8,10-tetrol: BaP-7,8,9,10-tetrol-7,8,9,10-tetrahydro-(7α,8β,9α,10β)-(±); BghiP: benzo[ghi]perylene.

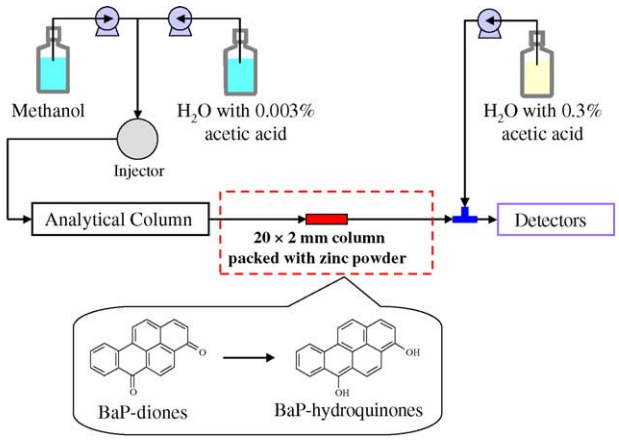

Fig. 2. Apparatus for reducing BaP-diones in-line to their corresponding fluorescent BaP-hydroquinones.

first manually filled with dry zinc powder. The packed zinc column was then placed in a vertical position with mobile phase entering from the top, and conditioned using the following steps: (1) pumping 100% Mobile Phase B at a flow rate going from 0 to 1.5 mL/min over 5 min; (2) changing from 100% Mobile Phase B to 100% Mobile Phase A over 5 min; (3) switching back to 100% Mobile Phase B over 5 min; and (4) decreasing the flow rate to 0 over 2 min. The inlet of the zinc column was disassembled after Step 4 to visually examine the filling. If any head space was observed, more zinc powder was added to fill the void. Acetic acid was added to Mobile Phase A to facilitate the reduction of BaP-diones to BaP-hydroquinones. In order to prevent the coating of the flow cells by a reducer-derived material (as yet unidentified), an additional pump (L-6200A Smart Pump; Hitachi, San Jose, CA) was used to deliver 0.3% (v/v) acetic acid in H_2O (de-gassed by a vacuum degasser) at 0.3 mL/min directly to the detectors via a T-connector between the zinc column and the first detector (Fig. 2).

2.6. Calibration procedures

Seven calibration standards, each containing a mixture of 1,6-dione, 3,6-dione, 6,12-dione, BaP, BghiP (internal standard), 3-OH, 7-OH, 9-OH, 7,8-diol, 4,5-diol, 9,10-diol, 7,10/8,9-tetrol, and 7,9/8,10-tetrol, were prepared in methanol. The concentrations of the metabolite standards ranged from 1.0 to 220 nM, while the concentrations of BaP and BghiP were fixed at 10 and 1.6 μM, respectively. Standard calibration curves were obtained by plotting the peak area ratio of individual BaP-hydroquinones to internal standard (A_D/A_{IS}) as a function of the concentrations of their corresponding BaP-diones (C_D). Linear regression of the standard curves and the determination of whether the *y*-intercept significantly differed from zero were performed using the Regression Analysis Tool in Microsoft Excel (Microsoft Corporation, Redmond, WA). Standard curves were then used to determine the recovery of BaP-diones from solutions con-

taining recombinant human enzymes. The equations used to calculate the recovery are listed in Appendix A.

2.7. Limits of detection, limits of quantitation, and instrument precision

The limit of detection (LOD) was determined as the concentration of the metabolite corresponding to a peak height that is three times the baseline noise of the fluorescence detector [17]. A 10:1 ratio of peak height to baseline noise was used to determine the limit of quantitation (LOQ) [17]. Instrument precision was measured as the relative standard deviation (RSD) of the peak area ratio of BaP-hydroquinones to internal standard (A_D/A_{IS}) from six consecutive injections of a mixture of authentic standards, each at 170 nM.

2.8. Samples for extraction recovery study

One milliliter of a solution containing CYP1A1 (0.085 mg microsomal protein) and EH (0.05 mg microsomal protein) in 50 mM Tris–HCl buffer (pH 7.4 at 37 °C) was spiked with a mixture of 1,6-dione, 3,6-dione, and 6,12-dione in 10 μL of DMSO to give final BaP-dione concentrations of 10–226 nM. After incubation at 37 °C for 50 min, 1.0 mL of acetone containing 1.6 nmol of BghiP as an internal standard was added to mimic the reaction termination step. The extraction procedures were adapted from a previously published method [10]. BaP and its metabolites were extracted with ethyl acetate (1 × 4.0 mL). After thorough mixing, the solution containing the reaction mixture, acetone, and ethyl acetate was centrifuged at 3000 rpm (1841 × *g*) for 10 min to separate the organic and aqueous layers. The organic layer (4.0 mL) was collected and evaporated to dryness using a SPD121P SpeedVac concentrator (Thermo Electron, Woburn, MA) at 35 °C. The residues were dissolved in 1.0 mL methanol and analyzed by HPLC. The equations used to calculate the percentage recovery of BaP-diones are listed in Appendix A.

2.9. Time-course study of BaP metabolism using recombinant human enzymes

Incubation mixtures contained 0.085 mg microsomal CYP1A1 protein, 0.05 mg microsomal EH protein, 1.3 mM $NADP^+$, 3.3 mM glucose-6-phosphate, 0.4 U/mL glucose-6-phosphate dehydrogenase, 3.3 mM magnesium chloride in 50 mM Tris–HCl buffer (pH 7.4 at 37 °C), in a final volume of 1.0 mL. After pre-incubation at 37 °C for 2 min, reactions were initiated by adding BaP in DMSO to achieve a final concentration of 10 μM of BaP and 1% (v/v) DMSO. Reactions were stopped at various time points by adding 1.0 mL acetone containing 1.6 nmol of internal standard (BghiP). The same extraction procedures described in the previous section were used to recover BaP and its metabolites from the reaction mixture.

2.10. Other aspects evaluated during method development

During method development, several method parameters were adjusted or evaluated, as described here in part. None of the methods described in this section involved the use of the third pump delivering 0.3% acetic acid to the detectors. Zinc powder with particle size (median 6–9 μm; Alfa Aesar) smaller than the one described above was also used to pack the zinc reducer. However, use of the smaller particles resulted in back pressure as high as 3800 PSI, which can potentially damage the analytical column. Various acid concentrations in the mobile phases were tested before reaching the optimized method. With Mobile Phases A (water) and B (methanol) both containing 0.1% (v/v) acetic acid, no separation of the three BaP-diones was found. Instead, only a large, broad peak was observed, possibly a result of the mixing of the three BaP-diones within the space in the zinc reducer produced by channeling, which was observed after the column was disassembled. When the concentration of acetic acid in water and methanol was reduced to 0.01%, no channeling was observed. However, at this acid concentration, the baseline from the fluorescence and UV detectors became steeper and the offset value on the UV detector increased progressively from run to run. This baseline rise was a result of contamination of the flow cells by unknown compounds from the zinc reducer, observed as a white coating inside the flow cell. The coating in the flow cells could be eliminated by washing the flow cells with 0.3% (v/v) acetic acid in water.

Other modifications of the mobile phases included the use of zinc chloride (10 mM), sodium acetate (5 mM), and acetic acid (4.5 mM) in both water and methanol, as adapted from a previous report [18]. However, severe channeling was observed under these conditions. On the other hand, when the mobile phases contained methanol and HCl in water (pH 4.05), high back pressure was observed.

3. Results

3.1. Chromatograms

A representative HPLC profile for the authentic standards of BaP-diones and other BaP metabolites is shown in Fig. 3. As shown in the chromatogram, the method can also be used to separate eight other BaP metabolites. All peaks in the chromatogram were well separated, except for those corresponding to 7-OH and 3-OH. A different method was developed to separate 7-OH and 3-OH (manuscript in preparation). A representative HPLC chromatogram showing the metabolites produced from BaP by recombinant human CYP1A1 is shown in Fig. 4. The metabolites observed were 1,6-dione, 3,6-dione, 6,12-dione, 9-OH, 7-OH, and 3-OH. No unknown peaks interfered with the separation of these metabolites. The sharp shifts of the baseline observed at 22.5 and 35 min (Fig. 4) were caused by the programmed wave-

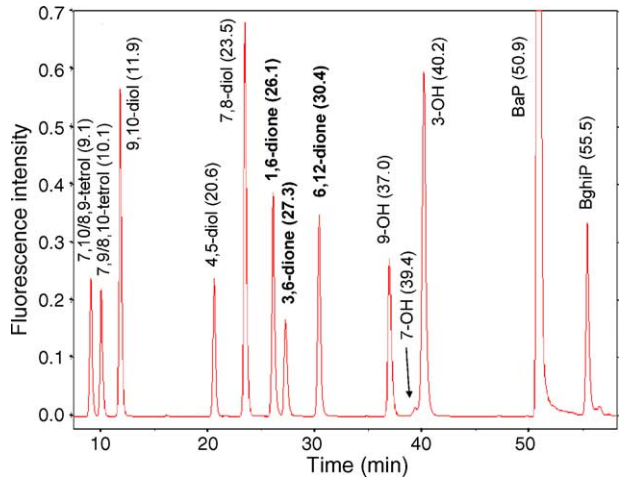

Fig. 3. HPLC profile of the authentic standards of BaP and its metabolites. The BaP-hydroquinones (labeled in boldface) are formed from their corresponding BaP-diones by the post-column zinc reducer. The numbers enclosed in parentheses represent the retention time of the compounds in minutes. The metabolite peaks and BghiP peak represent 3.4 and 32 pmol of the compounds, respectively. The BaP peak was off-scale and represented 200 pmol of the compound. See Table 2 for detection methods and list of abbreviations.

length changes of the fluorescence detector. In addition, it was observed that the retention time of all peaks was dependent on the ambient temperature (data not shown), with increasing temperature leading to slightly shorter elution times.

3.2. Standard calibration curves and detection limits

The slopes and coefficients of determination (R^2) for the standard calibration curves, i.e. peak area ratio (A_D/A_{IS}) versus dione concentration (C_D), are shown in Table 3. The data were gathered from four different HPLC sequences

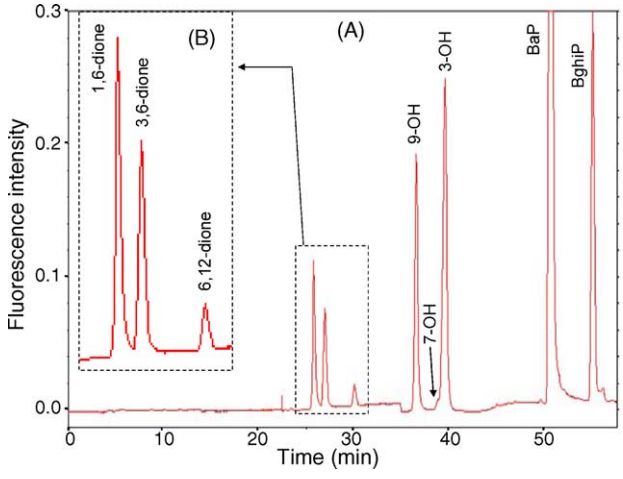

Fig. 4. HPLC profile of BaP metabolism using recombinant human CYP1A1 (A). The peaks shown in the inset (B) are from BaP-hydroquinones, which were formed by reduction of their corresponding BaP-diones by the post-column zinc reducer. BaP (10 μM) was incubated with 0.085 mg microsomal CYP1A1 in 1.0 mL solution (pH 7.4) at 37 °C for 24 min.

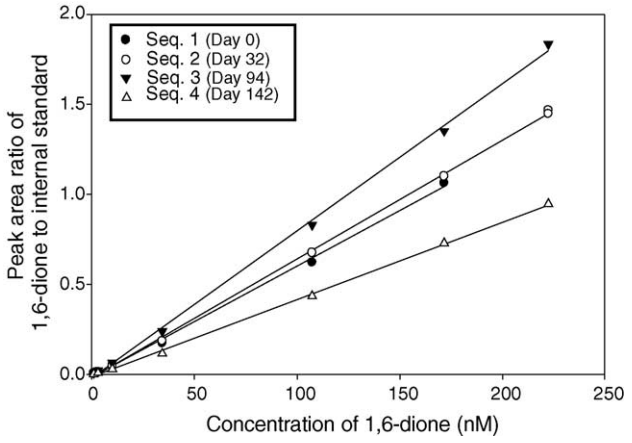

Fig. 5. Standard calibration curves for 1,6-dione. Symbols represent experimental data and lines represent linear regression results. The data were collected from four sequences, using the same zinc post column.

(batches), each performed on a separate day. Fig. 5 shows the standard calibration curves for the 1,6-dione from each of the separate sequences, and Fig. 6 shows the standard curves for each of the three BaP-diones, based on data from Sequence 4. The slope of the standard calibration curve for each BaP-dione varied from sequence to sequence (Fig. 5). However, excellent linear correlation was observed between A_D/A_{IS} and C_D within each sequence ($R^2 \geq 0.9976$) at BaP-dione concentrations ranging from 1.0 to 220 nM (Table 3; Figs. 5 and 6). The *y*-intercept for each standard curve was not significantly different from zero. The variations in the slopes of standard curves among the sequences were presumably the result of changes in the catalytic activity of the zinc toward the conversion of BaP-diones to BaP-hydroquinones. This hypothesis was supported by the observation that the peak area of the internal standard was constant for every sequence. The same zinc column was used to generate the data in Table 3 and Fig. 5, revealing that the catalytic activity remained effective even after 250 injections, over a span

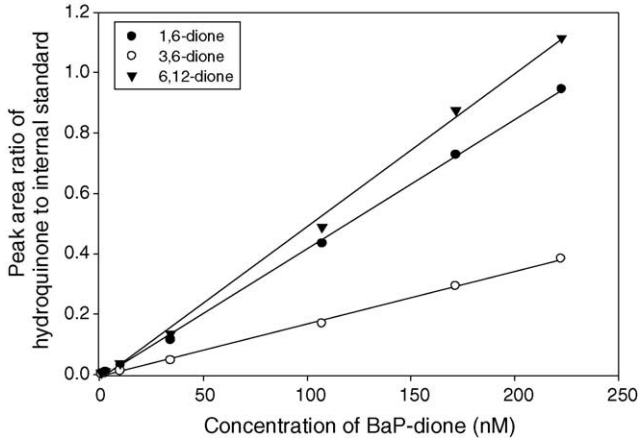

Fig. 6. Standard calibration curves for 1,6-dione, 3,6-dione, and 6,12-dione based on data from Sequence 4 (Table 3 and Fig. 5). Symbols represent experimental data and lines represent linear regression results.

Table 3
Standard calibration curves and detection limits of BaP-diones[a]

	Sequence #	Standard curve (A_D/A_{IS} vs. C_D)		LOD (nM)	LOQ (nM)
		Slope	R^2		
BaP-1.6-dione	1	6.16×10^{-3}	0.9981	0.18	0.61
	2	6.58×10^{-3}	0.9994	0.19	0.62
	3	8.16×10^{-3}	0.9988	0.12	0.39
	4	4.29×10^{-3}	0.9991	0.41	1.35
BaP-3.6-dione	1	2.82×10^{-3}	0.9978	0.40	1.32
	2	3.16×10^{-3}	0.9994	0.36	1.19
	3	3.91×10^{-3}	0.9986	0.21	0.69
	4	1.74×10^{-3}	0.9981	0.90	2.99
BaP-6,12-dione	1	5.73×10^{-3}	0.9983	0.17	0.55
	2	5.79×10^{-3}	0.9994	0.20	0.67
	3	7.71×10^{-3}	0.9985	0.11	0.37
	4	5.07×10^{-3}	0.9976	0.33	1.09

[a] Sequences 1, 2, 3, and 4 were performed on Days 0, 32, 94, and 142, respectively. The same zinc post column was used for all four sequences.

of 142 days. As expected from the changes in the slopes of the standard curves, LOD and LOQ values also varied from sequence to sequence (Table 3). Generally, detection sensitivities for 1,6-dione and 6,12-dione were similar, with LOD values approximately equal to one-half of the value for 3,6-dione.

3.3. Instrument precision

Instrument precision was measured as RSD of the peak area ratio (A_D/A_{IS}) from six consecutive injections of a mixture of authentic standards at 170 nM. The RSD for 1,6-dione, 3,6-dione, and 6,12-dione were 1.24%, 1.86%, and 0.81%. The differences in the RSD between 3,6-dione and 6,12-dione was due to the large difference in their respective peak areas.

3.4. Impact of the post-column zinc reducer

The increase in the detection sensitivity for BaP-diones is shown in Fig. 7 by comparing the peak area of each BaP-dione measured under the following conditions: (A) no zinc reducer; (B) with zinc reducer and no acid in the mobile phase; and (C) with zinc reducer using acidic mobile phase. With no zinc reducer, the peak areas were determined using UV absorbance, while fluorescence detection was used for the latter two conditions. To compare the relative sensitivity of these three conditions, the measured peak areas were normalized, based on the baseline noise level observed under each condition. In addition, for Condition C, the normalized peak areas were corrected for the dilution caused by the use of the third pump. Specifically, acetic acid (0.3% in water) from the third pump (at 0.3 mL/min) was only delivered under Condition C, but not under Conditions A and B. As a result, BaP-diones passed through the detectors at a flow rate of 1.0 mL/min (0.7 mL/min for the mobile phases and 0.3 mL/min for the 0.3% acetic acid) for Condition C, while at a flow rate of 0.7 mL/min for Conditions A and B. The corrected peak areas for Conditions A–C are shown in Fig. 7. Even with no acid added to the mobile phase, the use of the zinc reducer resulted in increases of peak areas of 88-, 23-, and 67-fold for 1,6-dione, 3,6-dione, and 6,12-dione, respectively. With the combination of the zinc reducer and 0.003% (v/v) acetic acid in Mobile Phase A, the peak areas were 410, 126, and 183 times those measured without the zinc reducer.

3.5. Extraction recovery study

The results of the extraction recovery study are shown in Table 4. The percentage recovery of BghiP (RCV_{IS}) was 102.6% ± 2.8%. The percentage recoveries for 1,6-dione and

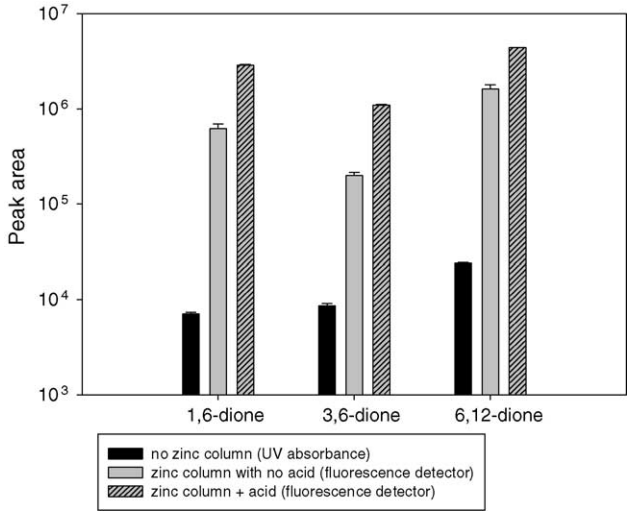

Fig. 7. Comparison of the peak area of BaP-diones measured in the presence and absence of the zinc post-column reducer. Error bars represent one standard deviation from six measurements, except for the zinc column with no acid samples (which were from three measurements). For zinc column with acid, 0.003% acetic acid in water was added to Mobile Phase A and 0.3% acetic acid in water was delivered from the third pump. UV absorbance was monitored at 280 nm.

Table 4
Percentage recovery of BaP-diones from 0.085 mg microsomal CYP1A1 protein and 0.05 mg microsomal EH protein at pH 7.4 and 37 °C

Concentration (nM)	% Recovery[a]		
	1,6-Dione	3,6-Dione	6,12-Dione
10.2	14.4 ± 1.6	19.7 ± 2.2	7.5 ± 1.8
34.0	19.3 ± 1.7	24.6 ± 4.6	7.2 ± 1.2
113	26.6 ± 4.6	32.8 ± 4.4	6.6 ± 2.7
227	33.1 ± 1.6	40.5 ± 3.0	7.9 ± 2.6

[a] Results are the mean of % recovery ± standard deviation from four data points (two injections from each duplicated sample).

3,6-dione increase with increasing spiked concentrations. The recovery for 1,6-dione ranged from 14.4% to 33.1%; the recovery for 3,6-dione ranged from 19.7% to 40.5%. On the other hand, less than 8% of 6,12-dione was recovered, and the percentage recovery was independent of the concentration of the dione from 10.2 to 227 nM.

3.6. Application of the method to study the time-course of BaP metabolism

This new analytical method was tested on samples from an experiment on the dynamics of BaP transformation by recombinant human CYP1A1 and EH (Fig. 8). Although the concentrations shown are not corrected for recovery or non-enzymatic transformations (e.g. autoxidation), it is evident that the levels of the 1,6- and 3,6-diones increased over time, while that of 6,12-dione remained relatively constant. Low concentrations of all three diones were detected with relatively low variability in this complex reaction system.

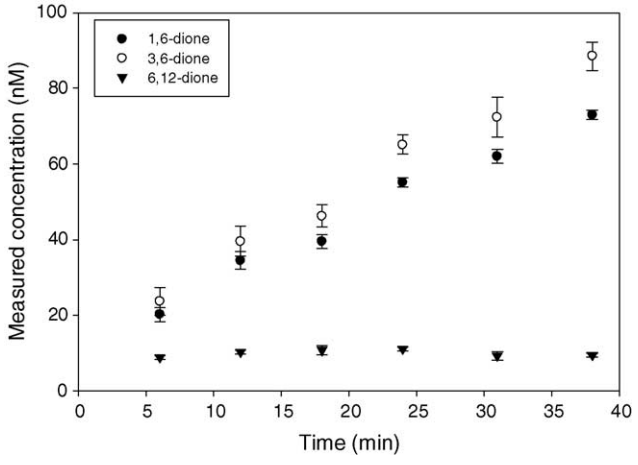

Fig. 8. Time course profiles of BaP-diones after incubating BaP (10 μM) with recombinant human CYP1A1 (0.085 mg microsomal protein/mL) and EH (0.05 mg microsomal protein/mL) at 37 °C. Results are the mean of the measured concentrations ± one standard deviation from four data points (two injections from each duplicated sample). The data shown are not corrected for recovery or for the amount of diones detected in controls with no added NADPH.

4. Discussion

This study reports the development and validation of a novel and relatively simple HPLC method for qualitative and quantitative analysis of BaP-diones with high detection sensitivity. A post-column zinc reducer was used to convert BaP-diones to their corresponding fluorescent BaP-hydroquinones. Since the BaP-hydroquinones can be analyzed using a fluorescence detector, the detection sensitivities are at least two orders of magnitude higher than those for non-converted BaP-diones using UV absorbance. The high detection sensitivities of this method make it suitable for studies where limited amounts of BaP-diones are present, such as metabolic studies using recombinant enzymes. In previous studies involving BaP metabolism using recombinant enzymes, the formation of BaP-diones was either not measured [19] or measured by radioactivity detectors using radiolabeled BaP [10,20,21]. Another method that can detect BaP-diones in airborne particulate matter with good sensitivity utilized liquid chromatography/mass spectrometry with atmospheric pressure chemical ionization [12].

Acetic acid was used in Mobile Phase A to facilitate reduction and prevent the BaP-hydroquinones from autoxidizing to BaP-diones. However, the acidic mobile phase resulted in problems that were not reported in previous publications using a post-column zinc reducer [15,16]. Specifically, an unknown substance coated the flow cells of the detectors and resulted, eventually, in the inability to use the detectors. At higher concentrations of acetic acid in Mobile Phase A, channeling within the zinc column was observed although the flow cells were not contaminated. An extra pump that delivered a diluted acetic acid solution to the flow cells without passing through the zinc column successfully prevented fouling of the flow cells without damaging the zinc powder packing material.

While this method was designed to separate three BaP-diones and other fluorescent BaP metabolites, the run time may be shortened if BaP-diones are the only compounds to be analyzed. This may be achieved by using acetonitrile or other solvents stronger than methanol as the organic mobile phase.

Due to variations in the catalytic activity of the zinc in converting BaP-diones to BaP-hydroquinones, sequence-to-sequence variation was observed in the slopes of standard curves. However, good linear correlation was observed between A_D/A_{IS} and C_D within each sequence ($R^2 \geq 0.9976$) at BaP-diones concentrations ranging from 1.0 to 220 nM. This sequence-to-sequence variation can be addressed by injecting authentic standards every four to six runs.

The recoveries of 1,6-dione and 3,6-dione from the enzymatic reaction mixture increased with increasing spiked concentrations, with lower concentrations showing poor recoveries. These results suggest saturable binding of these diones to biomolecules such as proteins or DNA. This interpretation is supported by the identification of 3,6-dione DNA adducts after incubating 3,6-dione with mouse aortic smooth mus-

cle cells [22] and after incubating BaP with monkey kidney COS1 cells co-transfected with CYP1A1 and P450 reductase [23]. Reindl et al. [24] reported 62% recovery of 3,6-dione (2 μM) from rat liver microsomes (0.087 mg protein/mL). Although this level of 3,6-dione recovery is higher than that achieved in this study, the only concentration of 3,6-dione (2 μM) used by Reindl et al. [24] was 8.8 times the highest concentration used here with the protein concentration at a comparable level. In addition to possible binding to protein and/or DNA, another factor that can contribute to the low recovery of 6,12-dione from the reaction mixture may be dione degradation during the extraction processes.

Recovery efficiencies are likely to depend on the nature of the sample. Prior to the recovery study described above, we performed a pilot recovery study (data not shown). This study revealed that, in the presence of BaP and other metabolites, the recoveries of all three BaP-diones from the enzyme incubation were higher than those shown in Table 4 (performed in the absence of BaP and other metabolites). In this pilot study, the enzymatic reaction solution (containing 0.085 mg microsomal CYP1A1 protein, 0.05 mg microsomal EH protein and no added NADPH) was spiked with mixtures of 10 μM BaP and various concentrations of the eleven BaP metabolites. At a spiked concentration of 34 nM for each metabolite, the apparent recoveries of 1,6-dione, 3,6-dione and 6,12-dione were 39%, 116%, and 28%, respectively (cf. Table 4). The higher apparent recoveries may be due, in part, to the spontaneous conversion of BaP to BaP-diones, as low levels of BaP-diones (3–7 nM) were observed in control samples where 10 μM of BaP alone was incubated with CYP1A1 and EH, with no added NADPH. In addition, the presence of other metabolites may also contribute to the higher apparent recoveries of BaP-diones. It is known that 3-OH can be non-enzymatically oxidized to 3,6-dione [25]; thus, this reaction may occur during incubation and/or extraction, contributing to the apparent higher recovery of 3,6-dione in the presence of BaP metabolites other than BaP-diones in the spiking solution.

Further evidence of non-enzymatic oxidation of BaP to diones was observed in controls that were performed during the time-course experiment. In addition to the results shown in Fig. 8, a control experiment was performed in which the reaction mixture was incubated for 45 min with 10 μM BaP but no NADPH. In those samples, the measured concentrations of 1,6-dione, 3,6-dione, and 6,12-dione were 6.60 ± 0.73, 6.72 ± 0.88, and 7.19 ± 0.56 nM, respectively. Thus, it appears that most, if not all, of the 6,12-dione detected in the time-course samples (Fig. 8) was produced non-enzymatically. This, combined with the recovery issues discussed above, underscores the complexity of this reaction system and the need to perform careful control experiments to assess non-enzymatic formation or transformation of the analytes during the reaction and extraction steps, as well as any false positive peaks that may be generated should different sample matrices be studied. Nonetheless, it is clear that the analytical method developed here performed well, detecting low concentrations of the three diones in small sample volumes with minimal sample preparation.

5. Conclusions

A novel, sensitive HPLC method for measuring BaP-dione concentrations was developed and validated. With the combination of a zinc reducer and acidic mobile phase, the detection sensitivity for BaP-diones was enhanced by greater than two orders of magnitude. The LODs for all three BaP-diones tested were below 1 nM based on 20-μL injections. Wide linear ranges for these metabolites were also provided by this method. This method is ideal for studies that require the detection of low levels of BaP-diones when radiolabeled BaP or radioactivity detectors are not available. Moreover, the method avoids time-consuming derivatization or reduction steps required by other methods.

Acknowledgement

This work was supported by National Institute for Environmental Health Sciences Grant # R01 ES09655.

Appendix A

All equations associated with the calculation of the response factors and percentage recovery for BaP-diones are listed in this section. The concentration of BaP-diones was correlated with the HPLC peak area using:

$$C_D = \frac{A_D}{A_{IS}} \frac{RF_{IS}\, C_{IS}}{RF_D} \quad (1)$$

where A_i represents the peak area measured by HPLC for compound i, and RF_i and C_i represent the response factor and concentration, respectively, for compound i. The subscripts D and IS were used to denote BaP-diones and internal standard (BghiP), respectively. Standard calibration curves were obtained by plotting the peak area ratio of individual BaP-hydroquinones to internal standard (A_D/A_{IS}) as a function of the concentrations of corresponding BaP-diones (C_D). The slope of the standard curve for metabolite D is equal to the value of $RF_D/(RF_{IS}\, C_{IS})$.

The percentage recovery of BaP-diones (RCV_D) from solutions containing recombinant human enzymes was then calculated as

$$RCV_D = \frac{A_D}{A_{IS}} \frac{1}{C_D} \frac{RF_{IS}\, C_{IS}}{RF_D} RCV_{IS} \quad (2)$$

where A_D/A_{IS} values were the peak area ratios analyzed by HPLC and C_D is the known spiked concentration of BaP-dione D. The values of $RF_D/(RF_{IS}\, C_{IS})$ were obtained from standard calibration curves. The percentage recovery of inter-

nal standard (RCV_{IS}) in Eq. (2) was calculated by

$$RCV_{IS} = \frac{A_{IS}}{RF_{IS}\, C_{IS}} \quad (3)$$

where C_{IS} is equal to 1.6 μM. A_{IS} is the mean of the peak area of BghiP from the entire recovery study. RF_{IS} is the mean of the values of (A_{IS}/C_{IS}) from the entire standard calibration study. Eq. (1) is the simplified form of Eq. (2), where RCV_{IS} and RCV_{IS} were assumed to be 100%.

References

[1] IARC, Polynuclear Aromatic Compounds, Part 1: Chemical, Environmental and Experimental Data, International Agency for Research on Cancer, Lyon, 1983.
[2] W. Levin, A. Wood, R. Chang, D. Ryan, P. Thomas, H. Yagi, D. Thakker, K. Vyas, C. Boyd, S.Y. Chu, A. Conney, D. Jerina, Drug Metab. Rev. 13 (1982) 555.
[3] ATSDR, Toxicological Profile for Polycyclic Aromatic Hydrocarbons (PAH) (Update), U.S. Department of Health and Human Services, Public Health Service, Agency for Toxic Substances and Disease Registry, Atlanta, 1995.
[4] R.G. Harvey, Polycyclic Aromatic Hydrocarbons: Chemistry and Carcinogenicity, Cambridge University Press, Cambridge, 1991.
[5] K.P. Miller, K.S. Ramos, Drug Metab. Rev. 33 (2001) 1.
[6] K.H. Liao, I.D. Dobrev, J.E. Dennison Jr., M.E. Andersen, B. Reisfeld, K.F. Reardon, J.A. Campain, W. Wei, M.T. Klein, R.J. Quann, R.S. Yang, Environ. Health Perspect. 10 (Suppl. 6) (2002) 957.
[7] M.T. Klein, G. Hou, R.J. Quann, W. Wei, K.H. Liao, R.S. Yang, J.A. Campain, M.A. Mazurek, L.J. Broadbelt, Environ. Health Perspect. 110 (Suppl. 6) (2002) 1025.
[8] K.H. Liao, Ph.D. dissertation in Chemical Engineering, Colorado State University, Fort Collins, CO, 2004.
[9] J.K. Selkirk, in: H.F. Kraybill, M.A. Mehlman (Eds.), Environmental Cancer, Hemisphere Pub. Corp., Washington, 1977, p. 1.
[10] M. Shou, K.R. Korzekwa, C.L. Crespi, F.J. Gonzalez, H.V. Gelboin, Mol. Carcinog. 10 (1994) 159.
[11] A. Toriba, H. Nakamura, T. Chetiyanukornkul, R. Kizu, T. Makino, H. Nakazawa, T. Yokoi, K. Hayakawa, Anal. Biochem. 312 (2003) 14.
[12] R. Koeber, J.M. Bayona, R. Niessner, Environ. Sci. Technol. 33 (1999) 1552.
[13] M.R. Osborne, N.T. Crosby, Benzopyrenes, Cambridge University Press, Cambridge, 1987.
[14] R.J. Lorentzen, P.O. Ts'o, Biochemistry 16 (1977) 1467.
[15] S.M. Billedeau, J. Chromatogr. 472 (1989) 371.
[16] Y. Haroon, D.S. Bacon, J.A. Sadowski, Biomed. Chromatogr. 2 (1987) 4.
[17] ICH, Validation of Analytical Procedures: Methodology (Q2B), The International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use, Geneva, 1996.
[18] T.J. Koivu-Tikkanen, V. Ollilainen, V.I. Piironen, J. Agric. Food Chem. 48 (2000) 6325.
[19] J.C. Gautier, P. Urban, P. Beaune, D. Pompon, Chem. Res. Toxicol. 9 (1996) 418.
[20] D. Schwarz, P. Kisselev, I. Cascorbi, W.H. Schunck, I. Roots, Carcinogenesis 22 (2001) 453.
[21] J.H. Kim, K.H. Stansbury, N.J. Walker, M.A. Trush, P.T. Strickland, T.R. Sutter, Carcinogenesis 19 (1998) 1847.
[22] B. Moorthy, K.P. Miller, W. Jiang, E.S. Williams, S.R. Kondraganti, K.S. Ramos, J. Pharmacol. Exp. Ther. 305 (2003) 394.
[23] P. Joseph, A.K. Jaiswal, Proc. Natl. Acad. Sci. U.S.A. 91 (1994) 8413.
[24] M. Reindl, A. Seidel, H. Frank, K.L. Platt, Polycyc. Aromat. Comp. 10 (1996) 77.
[25] J.K. Selkirk, R.G. Croy, P.P. Roller, H.V. Gelboin, Cancer Res. 34 (1974) 3474.