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Original Article

Detection of BPDE-DNA adducts in human umbilical cord blood by LC-MS/MS analysis

Ling Guo ^{a,b,c,1}, Xiao Jiang ^{a,b}, Hao-Yuan Tian ^{a,b}, Shang-Jin Yao ^{a,b,c},
Bo-Ya Li ^{a,b}, Rong-Jie Zhang ^d, Shu-Sheng Zhang ^c, Xin Sun ^{a,b,*}

^a Key Laboratory of Chemical Safety and Health, Chinese Center for Disease Control and Prevention, Chinese Center for Disease Control and Prevention, 100050 Beijing, China

^b National Institute of Occupational Health and Poison Control, Chinese Center for Disease Control and Prevention, 100050 Beijing, China

^c Department of Chemistry, Zhengzhou University, Zhengzhou 450001, China

^d Henan Center for Disease Control and Prevention, Zhengzhou 450016, China

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ABSTRACT

Benzo [a]pyrene (BaP) is a model compound for the study of polycyclic aromatic hydrocarbon (PAH) carcinogenesis. Upon metabolism, BaP is metabolized to the ultimate metabolite, BaP *trans*-7,8-diol-*anti*-9,10-epoxide (BPDE), that reacts with cellular DNA to form BPDE-dG adducts responsible for BaP-induced mutagenicity, carcinogenicity, and teratogenicity. In this study, we employed our developed LC-MS/MS method to detect and quantify BPDE-dG adducts present in 42 normal human umbilical cord blood samples and 42 birth defect cases. We determined that there is no significant difference in the level of BPDE-dG formation between the normal and birth defect groups. This represents the first time to use an LC-MS/MS method to quantify BPDE-dG in human umbilical blood samples. The results indicated that under experimental conditions, BPDE-dG adducts were detected in all the human umbilical cord blood samples from the normal and birth defect groups.

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1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous carcinogenic environmental pollutants present in the environment, e.g. air, water, soil and sediment [1–5]. PAHs are present in tobacco smoke in high concentrations, in foods,

and occupational exposure [6,7]. PAHs induce malignant tumors in rodents, including mammary gland, lung, bladder, and skin [8–10]. International Agency for Research of Cancer (IARC) classified a group of PAHs as Group 1 human carcinogens, Group 2A probably carcinogens, or Group 2B possible human carcinogens [2,3]. It has also been reported that

* Corresponding author. Key Laboratory of Chemical Safety and Health, Chinese Center for Disease Control and Prevention, Chinese Center for Disease Control and Prevention, 100050 Beijing, China. Fax: +86 10 8313 2573.

E-mail address: sunxin@niohp.chinacdc.cn (X. Sun).

¹ Current Institute and address: Beijing Municipal Institute of Labour Protection, 100054 Beijing, China

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consumption of PAHs in foods increases risks of colorectal adenoma and pancreatic cancer [2,3,11–14].

Benzo [a]pyrene (BaP) is a model compound for the study of PAH carcinogenesis. Carcinogenic PAHs, including BaP, require metabolic activation to exert mutagenicity, teratogenicity, and carcinogenicity. BaP is metabolized to form the ultimate metabolite, *trans*-7,8-dihydroxy-*anti*-9,10-epoxy-7,8,9,10-tetrahydrobenzo [a]pyrene (BPDE). BPDE can bind with cellular DNA to form BPDE-DNA adducts, with 10-(deoxyguanosin-*N*²-yl)-7,8,9-trihydroxy-7,8,9,10-tetrahydrobenzo [a]pyrene (BPDE-dG) as a major DNA adduct [5]. Therefore, development of sensitive and reliable analytical methods to identify and quantitate BPDE-dG adducts in human samples is required for molecular epidemiological studies [15–18].

Molecular epidemiology studies have evidenced that the fetus is more susceptible than the adults to the toxic effects of PAHs and other toxicants [19–22]. The previous studies have suggested that the developing fetus may be 10 times more susceptible than the mother to PAH-induced DNA damage [20]. Perera et al. measured PAH-DNA and other bulky aromatic adducts in umbilical cord white blood cells using the ³²P-postlabeling assay [20]. They determined the association between this molecular dosimeter and behavioral attention problems in childhood and the results suggested that PAH exposure, measured by DNA adducts, may adversely affect child behavior, potentially affecting school performance [20].

In this study, we measured the BPDE-dG adducts in the DNA of umbilical cord white blood cell samples from a subset of birth cohort. A total of 84 umbilical cord white blood cell samples, with 42 birth defect cases and 42 normal cases, were studied.

2. Materials and methods

2.1. Chemicals

[¹⁵N₅]-2'-Deoxyguanosine was purchased from Cambridge Isotope Laboratories (Andover, MA, USA). Calf thymus DNA, deoxyribonuclease I from bovine pancreas (DNase I), phosphodiesterase I from *Crotalus adamanteus* venom (phosphodiesterase I), phosphatase, alkaline from *Escherichia coli* (alkaline phosphatase) were obtained from Sigma-Aldrich Corp. (St. Louis, MO, USA). BPDE, synthesized by reaction of BaP *trans*-7,8-dihydrodiol with DDQ [23,24], was kindly provided by Dr. Peter P. Fu of the National Center for Toxicological Research (NCTR). All solvents were HPLC grade.

2.2. Umbilical cord white blood samples

Umbilical cord white blood samples were collected between November 2013 and June 2014 from pregnant women enrolled from the local hospital of the Shenqiu county, Henan province (Huaihe River Basin). A total of 84 human umbilical cord blood samples, including 42 birth defect cases and 42 normal cases, were randomly selected from a subset of birth cohort. Before

umbilical cord blood was obtained, the pregnant women were required to sign a consent form after receiving a detailed explanation of the study. Approximately 25–30 mL of umbilical cord blood was collected in SST coagulation tubes at the time of delivery, and the centrifuged plasma samples were stored at –80 °C before use. This study was reviewed and approved by the Ethics Committee of the National Institute for Occupational Health and Poison Control, Chinese Center for Disease Control and Prevention.

2.3. DNA isolation from umbilical cord blood

Umbilical cord white blood cell DNA preparation were collected in vacuum blood collection tube with EDTA-K₂ (Becton, Dickinson and Company, USA) (0.05 M sodium citrate, pH 7.0), cooled to 4–8 °C and followed by centrifugation (2900 rpm, 20 min). Buffy coat (PBMC, mainly leukocytes) and plasma fractions were separated and stored at –80 °C for further analyses. The isolation of genomic DNA from buffy coats was achieved using the DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol except for the modification of the supplied pH to 7.4 and adjusting the NaCl concentration in the elution buffer to 1.4 M.

2.4. Synthesis of BPDE-dG and [¹⁵N₅]BPDE-dG standards

BPDE-dG was synthesized followed the protocol reported earlier by Beland et al. with minor modifications [25]. Briefly, BPDE (3.0 mg) was dissolved in 5.0 mL THF and added 18 mg of 2'-deoxyguanosine (dG) dissolved in 1.0 mM Tris-HCl buffer (pH 6.8). The mixture was incubated 37 °C for 4 h and extracted sequentially with 10 mL of water saturated *n*-butanol and washed with 10 mL of *n*-butanol saturated water three times respectively. The *n*-butanol extracts were evaporated and the residue was dissolved in methanol and purified by reverse phase HPLC using linear gradient program with acetonitrile (A) and 0.1% formic acid (B) as follows: 0–5 min, 5% A isocratic; 5–30 min, 5%–35% A in B; 30–35 min, 35%–90% A in B; 35–40 min, 90% A isocratic; 40–41 min, 90%–5% A in B; 41–50 min, 5% A isocratic. The flow rate was 0.3 mL/min. The purified BPDE-dG was quantitated by UV spectrometry using the absorption coefficients as an external standard [26]. [¹⁵N₅]BPDE-dG was synthesized by the reaction of BPDE with [¹⁵N₅]2'-deoxyguanosine ([¹⁵N₅]dG) followed by same procedures described above as an internal standard (I.S.). The purified [¹⁵N₅]BPDE-dG was quantitated by UV spectrometry using the absorption coefficients [26].

2.5. LC-MS/MS analysis for BPDE-dG adduct in umbilical cord white blood cell samples

LC-MS/MS analysis of BPDE-dG adduct was conducted following the protocol reported earlier with minor modifications [25]. Briefly, umbilical cord white blood cell DNA (20 µg) was added 10 µL DNase I (0.5 unit/µL) and 10 pg of [¹⁵N₅]BPDE-dG as I.S. for correcting recovery of BPDE-dG adduct. The

mixture was incubated at 37 °C for 3 h. After incubation, the mixture was added 10 μ L phosphodiesterase I (0.0002 unit/ μ L) and 10 μ L alkaline phosphatase (0.004 unit/ μ L). After incubation at 37 °C for 4 h, the mixture was extracted by adding 500 μ L of water-saturated *n*-butanol three times. The *n*-butanol extracts were evaporated and dissolved in 50 μ L of methanol and injected into LC-MS/MS for BPDE-dG adduct analysis. The liquid chromatography handling system consisted of a Thermo TSQ Quantum Access Max module (Thermo Fisher, Milford, MA, USA) connecting with a Hypersil Gold 1.9 μ m C18 column (100 \times 2.10 mm, Thermo Fisher, Milford, MA, USA). Each sample was eluted at 0.35 mL/min using a linear program with acetonitrile (A) and 0.1% formic acid (B) gradient as follows: 0–1 min, 5% A isocratic; 1–1.5 min, 5–20% A in B; 1.5–20 min, 20–28% A in B; 20–21 min, 28–90% A in B; 21–22 min, 90% A isocratic; 22–22.5 min, 90–5% A in B; 22.5–24 min, 5% A isocratic. A Thermo TSQ Access Max mass spectrometer (Thermo Fisher, Milford, MA, USA) equipped with an HESI interface, was used with a spray voltage of 3500 v, a vaporizer temperature of 300 °C and a capillary temperature of 270 °C. Nitrogen was the sheath gas (45 arb), aux gas (10 arb), and collision gas (0.12 mL/min). Positive ions were acquired in the multiple reaction monitoring (MRM) mode (collision energy, 10 eV).

2.6. Standard calibration curve

Standard curve was generated by plotting the amounts of standard compounds against peak area. Each sample was tested at 10 μ L injection volume containing 10 pg [$^{15}\text{N}_5$]BPDE-dG internal standard. The calibration curve was linear over the concentration range of 1–500 pg BPDE-dG. The best linear fit and least variability for the calibration curve was achieved with a weighting factor of $1/X$, with the correlation coefficients (r^2) above 0.99 (Fig. 1).

3. Results

3.1. Analysis of BPDE-dG and [$^{15}\text{N}_5$]BPDE-dG adducts

Following the method of Beland et al. with modification [25], reaction of BPDE in THF with 2'-deoxyguanosine dissolved in

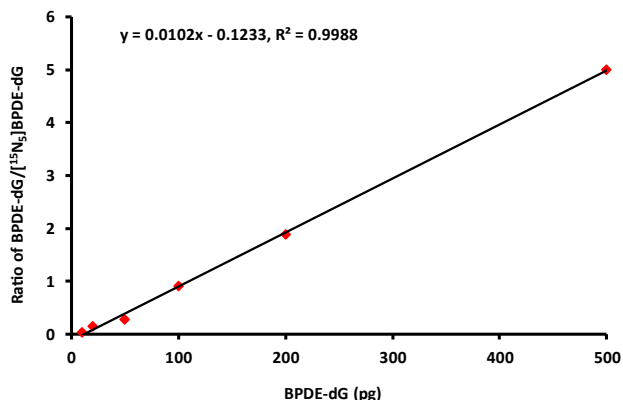


Fig. 1 – Standard calibration curve of BPDE-dG.

Tris–HCl buffer (pH 6.8) at 37 °C for 4 h resulted in the formation of four enantiomeric BPDE-dG adducts, with the retention times at 10.56, 11.20, 11.52, and 11.84 min, respectively (Fig. 2). The structures of these enantiomeric BPDE-dG adducts are *cis*-(+)-anti-BPDE-dG; *trans*-(-)-anti-BPDE-dG; *cis*-(-)-anti-BPDE-dG, and *trans*-(+)-anti-BPDE-dG, respectively (Fig. 3). These BPDE-dG adducts had a protonated molecule $[(M + H)^+]$ at m/z 570 (data not shown). The full scan LC-MS/MS spectrometric analysis indicated that these adducts all had identical fragment ions pattern, with fragment ions at m/z 454, m/z 303, m/z 285, m/z 257 and m/z 152 (Fig. 4A). The mass spectrum of [$^{15}\text{N}_5$]BPDE-dG shown in Fig. 2B also exhibited four chromatographic peaks, with the retention times at 10.58, 11.21, 11.53, and 11.84 min, respectively.

Product ion analysis of BPDE-dG gave fragments consistent with sequential loss of deoxyguanosine (dG) (m/z 303), loss of H_2O (m/z 285), and loss of CO (m/z 257) as well as the fragment indicative of guanine (m/z 152) (Fig. 4A). The structures of the fragment ions from BPDE-dG are shown in Fig. 5. Similarly, in the full scan mass spectrum of [$^{15}\text{N}_5$]BPDE-dG, four chromatographic peaks were also observed (Fig. 4B). The fragment ion at m/z 459 is the loss of a ribose $[(M\text{-ribose})^+]$.

3.2. Response curve of LC-MS/MS analysis

The substrate concentration-dependent response curve of LC-MS/MS analysis was obtained by adding 2.5, 5, 10, 15, 20 and 25 pg of BPDE-dG standards in calf thymus DNA samples and each added 10 pg of [$^{15}\text{N}_5$]BPDE-dG as internal standards. After incubation and enrichment, these single calf thymus DNA samples were then subjected into LC-MS/MS (data not shown). There is a linear correlation between the quantity of BPDE-dG and area ratios of BPDE-dG/[$^{15}\text{N}_5$]BPDE-dG, within the range studied. It was determined that the limit of detection ($s/n = 3$) of BPDE-dG is 2.7 BPDE-dG/ 10^9 dG. BPDE-dG in an aliquot equivalent about 10 μ g of DNA could be detected.

3.3. Reproducibility of LC-MS/MS method

The LC-MS/MS method was validated with respect to intra-assay and inter-assay by adding 5 pg and 10 pg of [$^{15}\text{N}_5$]BPDE-dG as internal standards in calf thymus DNA. The calf thymus DNA samples were injected into LC-MS/MS at the same time for the intra-assay. The relative standard deviation (RSD) was 13.05%. For the inter-assay, the calf thymus DNA samples were injected into LC-MS/MS with a 24 h interval, RSD was 23.1%.

3.4. Recovery of LC-MS/MS method

The recovery of LC-MS/MS method was achieved by adding 5 pg and 10 pg of [$^{15}\text{N}_5$]BPDE-dG in calf thymus DNA, respectively. These single DNA samples were injected into LC-MS/MS for the calculation of the recovery. The recovery of [$^{15}\text{N}_5$]BPDE-dG with adding 5 pg ranged from 74.6% to 89.6% and the mean recovery was $83.7\% \pm 6.1$, RSD was 9.54%. The recovery of [$^{15}\text{N}_5$]BPDE-dG with adding 10 pg ranged from 80.6% to 96.4%, mean recovery was $88.3\% \pm 5.4$, RSD was 8.96%.

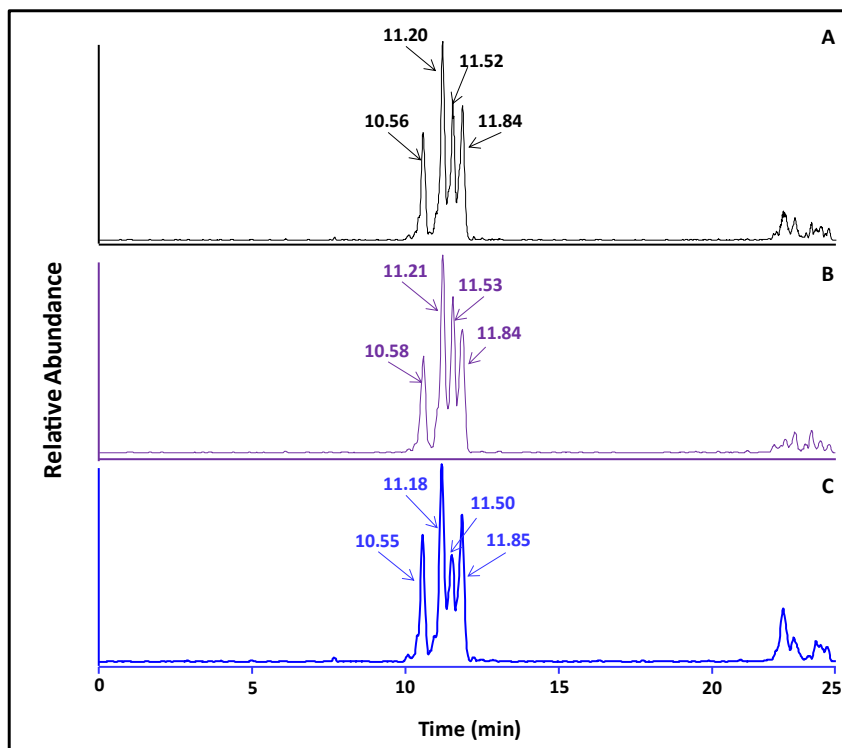


Fig. 2 – LC-MS/MS chromatographic profiles of BPDE-dG adducts: (A) BPDE-dG standard; (B) [¹⁵N₅]BPDE-dG internal standard; and (C) BPDE-dG detected in cord blood cell DNA. 10 pg of [¹⁵N₅]BPDE-dG was added to each sample as an internal standard for correcting recovery of each sample.

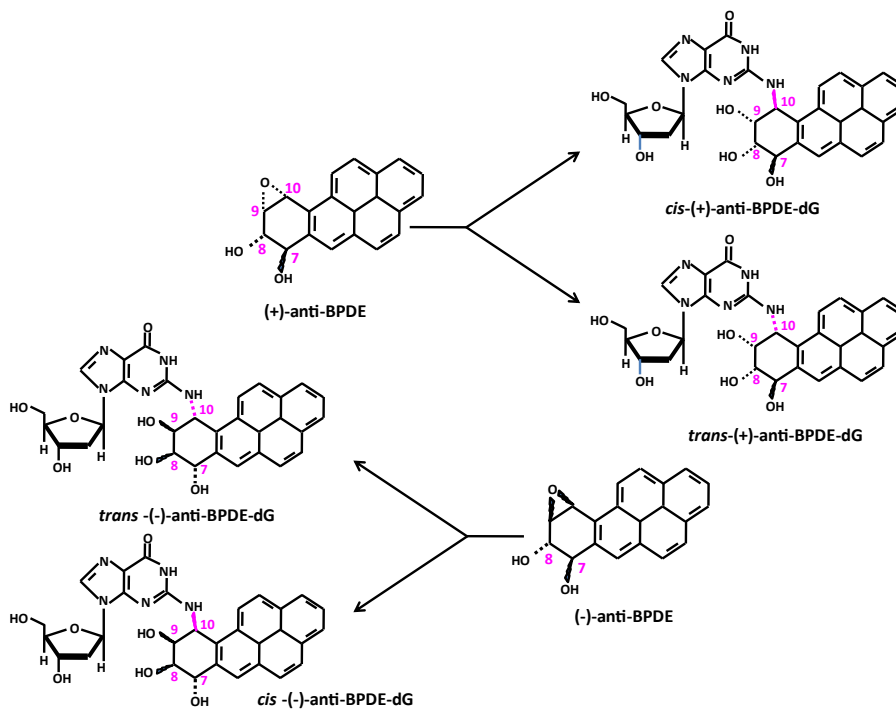


Fig. 3 – Structures of four enantiomeric of BPDE-dG adducts formed from organic reactions.

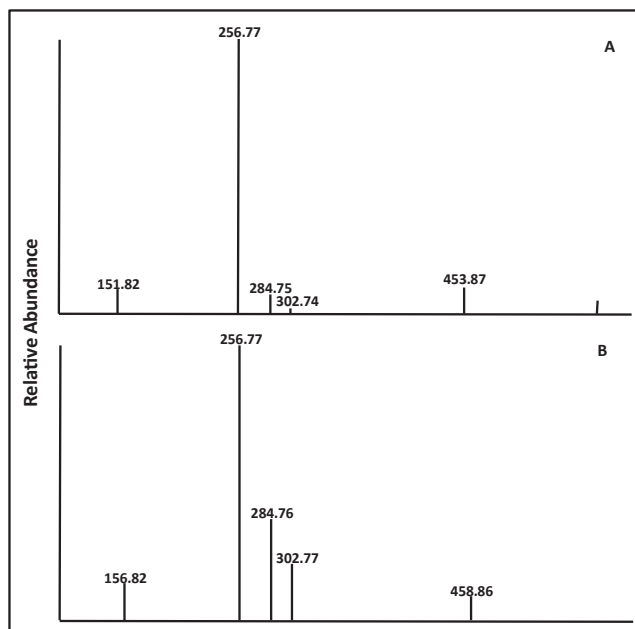


Fig. 4 – Mass spectra of synthesized standards: (A) BPDE-dG; and (B) [$^{15}\text{N}_5$]-BPDE-dG.

3.5. Analysis of BPDE-dG adduct in umbilical cord white blood cell DNA samples

Analysis of umbilical cord white blood cell DNA samples was conducted by LC-MS/MS method, with 84 human umbilical

cord blood samples, including 42 birth defect cases and 42 normal cases, randomly selected from a subset of birth cohort. The results are summarized in Table 1. A representative autoradiogram of BPDE-dG detected in human umbilical cord blood samples is shown in Fig. 2C. The results indicate that there is no significant difference in the level of BPDE-dG adducts formed from the normal and the birth defect groups.

4. Discussion

PAHs are mutagenic, teratogenic, and carcinogenic widespread environmental pollutants [1–7]. BPDE, the ultimate metabolite of BaP, covalently bound with DNA to form four enantiomeric BPDE-dG adducts *in vivo* (Fig. 2). The biological activities of *trans*-BPDE-dG enantiomers are extensively studied than the *cis* enantiomers [27,28]. Several analytical methods, including ^{32}P -postlabeling, immunoassay, and LC-MS/MS-based methods, have been used for the analysis of BPDE-DNA adducts [29–32]. Among which, the best analytical method is the LC-MS/MS. The recent improvements in LC-MS/MS method have been utilized in high-throughput applications for BPDE-DNA adducts analysis [33–35]. To analyze BPDE-dG adducts in small amounts in the umbilical cord white blood cell DNA, we developed an ultrasensitive LC-MS/MS method that is highly sensitive. The improved method is based on the effective purification and enrichment of BPDE-dG adducts in human umbilical cord blood samples followed by solvent extraction to efficiently enrich BPDE-dG

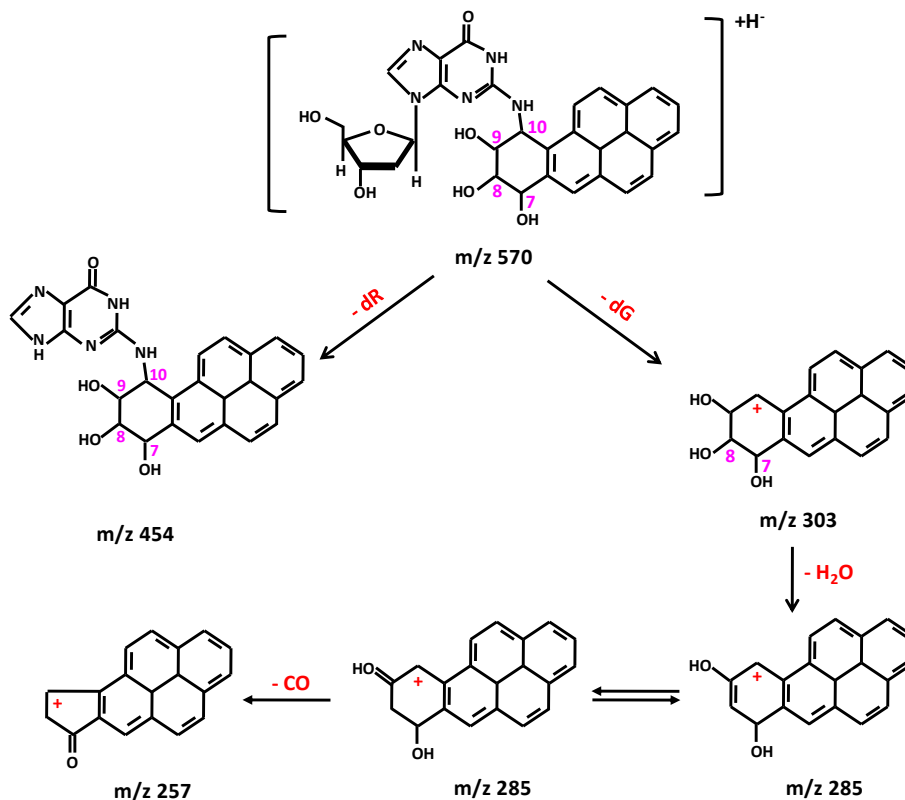


Fig. 5 – Structures of protonated molecular ions of BPDE-dG and its fragment ions formed from the LC-MS/MS full scan of BPDE-dG.

Table 1 – Detection of BPDE-DNA adduct in human umbilical cord blood by LC/MS analysis.

Case ID (healthy)	BPDE-dG/10 ⁸ nucleotides	Case ID (birth defect)	Type of defect	BPDE-dG/10 ⁸ nucleotides
0737002	0.27	0409019	pigmented nevus	0.27
1118001	0.27	0712004	cleft lip	0.27
1701001	0.27	1020001	melanoma	0.27
2004001	0.27	1908004	umbilical hernia	0.27
2023003	0.27	0523005	hemangioma	0.84
0408006	1.04	0524005	fissura auris congenita	1.04
0409002	0.98	0529007	locus coeruleus buttock	0.96
0409003	1.01	0536002	pigmented nevus	0.84
0523001	0.84	0702003	pigmented nevus	0.96
0524002	1.04	0718011	fissura auris congenita	0.96
0529001	0.82	0724004	polydactyly	0.93
0529002	0.87	0913005	strephepoxodia	0.96
0536003	0.93	1009001	fissura auris congenita	1.01
0706002	0.96	1013001	pigmented nevus	0.98
0706004	0.96	1122001	accessory auricles, hydrocele of tunica vaginalis	0.96
0712002	0.82	1210001	fissura auris congenita	0.98
0718003	0.82	1318002	pigmented nevus	0.87
0724002	0.96	1605004	fissura auris congenita	0.96
0913001	0.82	1610001	pigmented nevus	1.01
0913002	0.82	1702001	pigmented nevus	0.96
1014001	0.82	1702003	hypospadias	0.98
1030005	1.01	1712003	fissura auris congenita	0.96
2406002	0.98	2003004	locus coeruleus buttock	1.01
1111001	1.01	2018001	locus coeruleus buttock	1.04
1210002	0.98	2222006	aural deformity, accessory auricles	1.01
1605001	0.79	2406003	accessory auricles	1.01
1605002	0.93	2229003	fissura auris congenita	0.96
1610002	0.93	2304001	ectrodactyly (left)	0.98
1703001	1.01	2304003	chin deformity (right)	0.98
1908001	0.87	2318001	fissura auris congenita	0.98
2017001	0.93	2321002	hemangioma	1.01
2022001	0.98	2401002	hydrocele of tunica vaginalis	0.98
2027001	0.96	2228002	hydrocele of tunica vaginalis	1.10
2027002	0.84	0724001	down's syndrome	1.12
2303002	1.01	0706005	pigmented nevus	1.21
2315003	0.87	1041005	fissura auris congenita	1.29
2321001	0.82	1107002	pigmented nevus	1.35
2406001	0.82	0902005	pigmented nevus	1.41
1041001	1.18	2222005	retained testicle	1.43
2402001	1.15	0408004	pigmented nevus (black)	2.53
1713001	1.27	1032001	pigmented nevus (black)	6.38
1023001	1.55	0736002	cleft lip	11.84

adducts in cord blood samples. The sensitivity of this developed method can be applied to use human cord blood cell DNA in a quantity as low as ~10 µg of DNA. For improving the reliability of the method, [¹⁵N₅]BPDE-dG was added to each human umbilical cord blood sample as internal standards allowing the good correction of the recovery.

Our study represents the first time to use the LC-MS/MS method to quantify BPDE-dG adducts in human umbilical cord blood samples. As the data shown in Table 1, the levels of BPDE-DNA adducts present in 42 normal human umbilical cord blood samples and the 42 birth defect cases are generally close, with the adduct level in only three birth defect cases a little higher than all the others. Since only one measurement was conducted for each sample, it is

reasonably to conclude that there is no significant difference in the BPDE-dG formation between the normal and the birth defect groups.

It is important to point out that all the 82 human umbilical cord blood samples, from both normal and birth defect groups, contained BPDE-dG adducts. Thus, it is important to determine whether this is a general phenomenon, or a specific case. This warrants further investigation.

Conflicts of interest

The authors declare no conflict of interest.

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