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Ultra-performance liquid chromatography–tandem mass spectrometry for rapid and highly sensitive analysis of stereoisomers of benzo[a]pyrene diol epoxide–DNA adducts

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ABSTRACT

An ultra-performance liquid chromatography tandem mass spectrometry with multiple reaction monitoring method (UPLC-MRM/MS) is developed for fast and sensitive analysis of four genotoxic stereoisomers of *anti*-benzo[a]pyrene diol epoxide (BPDE)–N^2dG adducts (*trans*-(+), *trans*-(−), *cis*-(+) and *cis*-(−)), which result from environmental exposure to ubiquitous pollutant benzo[a]pyrene (B[a]P). The developed method displays a low limit of detection of <0.7 fmol (S/N = 3) for the four stereoisomers of *anti*-BPDE–N^2dG, a dynamic range of 2 orders of magnitude (2.3–630 fmol, $R^2 \geq 0.997$), and one separation of 2–4 min. The developed method enables us to use the stereoisomers of *anti*-BPDE–N^2dG as a biomarker and to study the stereoselectivity of metabolic activation of B[a]P in human lung A549 cells. The UPLC-MRM/MS analysis of cellular DNA exposed to B[a]P show that activation of B[a]P in A549 cells predominantly induces *trans*-(+)-*anti*-BPDE–N^2dG with *cis*-(+)-*anti*-BPDE–N^2dG and one *syn*-BPDE–N^2dG as two minorities, while *trans*-(−)-*anti*-BPDE–N^2dG and *cis*-(−)-*anti*-BPDE–N^2dG are absent. The observed preferential formation of *trans*-(+)-*anti*-BPDE–N^2dG in B[a]P treated A549 cells may result from combined stereoselectivity of the metabolic activation of B[a]P and the reaction of *anti*-BPDE with dsDNA. The results also suggest that a number of key optical intermediates are formed during activation of B[a]P in A549 cells, including *trans*-(+)-B[a]P-7,8-dihydrodiol and *trans*-(−)-B[a]P-7,8-dihydrodiol and their corresponding downstream metabolites (+)-*anti*-BPDE and (+)-*syn*-BPDE.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental pollutants, found in automobile exhaust, forest fire, charbroiled food, tobacco smoke [1,2] and certain occupational settings [3,4], and may cause lung [5], skin [6] and bladder cancer [7]. Benzo[a]pyrene (B[a]P) is a representative of carcinogenic PAH. It is metabolized *in vivo* to an ultimate carcinogen, *anti*-benzo[*a*]pyrene diol epoxide (BPDE), which may bind to an exocylic amine group at N^2-deoxyguanosine (dG) or N^6-deoxyadenosine(dA) in DNA [8]. It is possible to form four stereoisomers of *anti*-BPDE–N^2dG and four stereoisomers of *anti*-BPDE-N^6dA, derived from the reaction of (±)-*anti*-BPDE with DNA [9]. However, the amount of BPDE-N^6dA adducts formed in lung cells are 2 orders of magnitude lower than that of the *anti*-BPDE–N^2dG adducts [10]. Therefore, it is more important to develop a fast and sensitive analytical method specifically for detection of the four stereoisomers of *anti*-BPDE–N^2dG. The *in vivo* activation, detoxification, and elimination of benzo(a)pyrene, formation of DNA adducts and followed enzyme processing all display stereoselectivity at certain degree [9,11]. The metabolic intermediates of benzo[a]pyrene, 7,8-epoxide and *trans*-7,8-diol, as well as the two stereoisomeric diol epoxides (*syn*- and *anti*-BPDE) are all optically active. Glutathione conjugates (for detoxification) also display optical activity [12]. The frequency and ratio of stereoisomeric BPDE–DNA adducts, the endpoint of metabolic intermediates, can be used as a stereochemical indicator of these optically active intermediates if the developed analytical method has adequate sensitivity. Therefore, the sensitive analysis of the stereoisomers of the *anti*-BPDE–N^2dG may provide subtle information on the stereoselectivity of each cellular process, and is of particular importance for understanding the roles of the stereochemistry played in mutagenesis, teratogenesis, and carcinogenesis of B[a]P in human and other mammalians.

A number of methods have been developed for detection of BPDE–DNA adducts, including capillary electrophoresis (CE)-laser-induced fluorescence (LIF) [13–16], accelerator mass spectrometry [17,18], liquid chromatography or capillary electrophoresis-mass spectrometry [19–26], and ^{32}P-postlabelling [27]. However, only few methods demonstrate the capability of resolving the stereoisomers of BPDE–DNA adducts. The method development for

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stereoisomeric analysis of BPDE–DNA adducts takes multiple steps and labor-intensive. It often involves with the preparation of single stereoisomers of adducted nucleosides or nucleotides (which are not commercially available), and the structural and stereochemical characterization, enzymatic digestion of genomic DNA, and highly efficient and stereoselective separation and coupled sensitive detection [10,21,28]. Among these steps, the stereoselective separation of the adduct stereoisomers is most critical and tunable factor for achieving high sensitivity of detection. The separation of the stereoisomers has been conducted mostly on HPLC because of the possible injection of relatively large sample (several microliters to hundred microliters), resulting in significant enhancement of concentration sensitivity. However, traditional HPLC separation takes too long time (25–100 min) because of its limited separation performance. Because of the long separation, the peaks of stereoisomers are broadened and the analytes are proportionally diluted. The peak broadening and analyte dilution could significantly cause poor detection sensitivity. The separation of the stereoisomers of *anti*-BPDE–N^2dG can be interfered with by the accompanying BPDE hydrolysis byproducts tetrols when UV or fluorescence detection is adopted, especially for the latter in which the tetrols have 20 times stronger response than the BPDE–N^2dG. In such case, six components (four *anti*-BPDE–N^2dG adducts and two BPDE tetrols) that have similar retention property are required to be well separated [29]. It often takes 60–100 min to achieve base-line separation [29]. The selective detection by mass spectrometry may partially solve the separation dilemma. In the mode of multiple-reaction monitoring (MRM) when a triple quadrupole mass spectrometry is adopted, the specific MS transition (e.g. m/z 570 → 454) can preclude the presence of BPDE tetrols and other interference substance, thus, only four stereoisomers of the adducted dG are needed to be resolved regardless of the other components. Because of the improved detection selectivity, it takes shorter time (20–50 min) for the stereoslective separation [21,22,30]. Due to large sample consumption (30–300 μg DNA), the LC–MS based methods are often used for identification of new DNA damage and analysis of DNA adducts from cultured cells or animal tissues rather than human biomonitoring [19,31,32].

To improve the sensitivity of LC–MS and reduce its sample consumption, here we report a stable isotope dilution ultra-performance liquid chromatography (UPLC)–tandem MS for fast and sensitive detection of the four stereoisomers of *anti*-BPDE–N^2dG. This is the first application of UPLC in DNA damage analysis. The developed UPLC-MRM–MS/MS method is the fastest (2–4 min) among all known methods, and applicable to quantitate four individual *anti*-BPDE–N^2dG stereoisomers in cellular DNA treated with *anti*-BPDE and B[a]P at low doses. By use of the developed method, we examine the distribution pattern of the stereoisomers of *anti*-BPDE–N^2dG in B[a]P or *anti*-BPDE treated lung cells.

2. Experimental

2.1. Chemicals and reagents

(±)-*anti*-BPDE was a kind gift from Dr. X. Chris Le at the University of Alberta. 2′-Deoxyguanosine, triethylamine and tetrahydrofuran were purchased from Sigma–Aldrich (St. Louis, MO, USA). Methanol and acetonitrile were of HPLC grade and purchased from Fisher Scientific (Pittsburgh, PA, USA). Ultrapure water was prepared by a Milli-Q water system (Millipore, Bedford, MA, USA). All other reagents and solvents were of analytical or HPLC grade.

2.2. Preparation of anti-BPDE–N^2dG and $^{15}N_5$-BPDE–N^2dG Stereoisomers

Four stereoisomers of *anti*-BPDE–N^2dG were first synthesized by a direct reaction of racemic *anti*-BPDE with excess 2′-deoxyguanosine, and the products were fractionated by SPE as described previously [29]. The fractions of *anti*-BPDE–N^2dGs were further purified by a modified HPLC method. The HPLC purification was conducted on a Hitachi L-2000 series liquid chromatography system (Hitachi, Tokyo, Japan). A Capcell Pak Ph UG column (250 mm × 4.6 mm, 5 μm particle size) from Shiseido (Tokyo, Japan) was employed, and acetonitrile/0.1% formic acid in water (18/82, v/v) was used as a mobile phase for isocratic elution. The flow-rate was kept at 1.2 mL/min. The column temperature was maintained at 25 °C. In this case, acidic mobile phase was used. Compared with neutral or basic mobile phase, acidic mobile phase may enhance the stability of *cis*-(+)-*anti*-BPDE–N^2dG and improve the separation and purification of the four stereoisomers (data not shown). The purified *anti*-BPDE–N^2dG stereoisomers were lyophilized and then individually reconstituted in 33% methanol/water (v/v). Their structure and stereochemistry were identified and characterized by a combination of HPLC-Diode Array Detector (DAD)–MS/MS (Waters, MA, USA), UV spectroscopy (Hitachi, Tokyo, Japan), and circular dichroism (CD) spectroscopy (Jasco, Tokyo, Japan). The concentration of each purified *anti*-BPDE–N^2dG stereoisomer was calculated by determining absorbance at 345 nm from triplicate measurements and reported maximum absorbance coefficient (ε 345 = 3.43 × 10^4 L/mol cm) [21].

The stereoisomers of $^{15}N_5$-*anti*-BPDE–N^2dG were synthesized, purified and characterized following the same protocol except that $^{15}N_5$-dG was used in the synthesis.

2.3. Reaction of (±)-anti-BPDE with naked dsDNA from A549 cells

0.1 mg (±)-*anti*-BPDE was dissolved in 1 mL freshly prepared tetrahydrofuran/triethylamine solution (19:1, v/v) at a concentration of 0.3 mM, then diluted to 3 μM by freshly prepared tetrahydrofuran/triethylamine solution. The 5 μL solution of 3 μM BPDE was finally mixed with 70 μL genomic DNA solution (3 mg/mL in 100 mM Tris–HCl buffer, pH 7.5) that was extracted from A549 cells with the Promega wizard® genomic DNA purification kit according to the manufacturer instructions. The reaction was conducted in dark at 37 °C for 16 h. After the reaction, DNA was precipitated by adding 3 μL of 3 M CH_3COONa buffer (pH 5.4) and 200 μL of ice-cold ethanol. The DNA pellet was then collected and washed by 75% ethanol three times. The collected DNA pellet was air-dried and reconstituted in a buffer of 40 mM Tris–HCl, 10 mM $MgCl_2$, pH 8.5. The samples were stored at −20 °C prior to enzymatic digestion and quantification.

2.4. Cell treatment by B[a]P and anti-BPDE

Human lung epithelial carcinoma A549 cells (1 × 10^6 cells/dish) were seeded in six 10-mm plastic dishes (Corning, NY, USA) and cultured in 10 mL per dish RPMI 1640 growth medium supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen, Carlsbad, CA), 100 U/mL penicillin G and 100 U/mL streptomycin sulfate. The dishes were kept for 48 h in a humidified 37 °C incubator supplied with 5% CO_2, which allowed cells to grow to the confluence in dishes. The confluent cells were incubated with 5.0 μM B[a]P for 16 h or 1.0 μM freshly prepared *anti*-BPDE for 2 h. A B[a]P stock solution of 10 mM was prepared in dimethyl sulfoxide (DMSO, Sigma). The BPDE was dissolved in freshly prepared tetrahydrofuran/triethylamine solution (19:1, v/v).The final concentration of DMSO or tetrahydrofuran/triethylamine mixture in each dish was about 0.05% (v/v). The genomic DNA was extracted from A549 cells with the Promega wizard® genomic DNA purification kit according to the manufacturer's instructions. The isolated DNA was air-dried and reconsitituted in the buffer of 40 mM Tris–HCl, 10 mM $MgCl_2$, pH 8.5. The concentration of each DNA sample was calculated by determining the mean absorbance

at 260 nm from triplicate measurements and assuming that one absorbance unit equals 50 μg/mL for dsDNA. The samples were stored at −20 °C prior to enzymatic digestion and quantification.

2.5. Enzymatic digestion of dsDNA

The DNA samples (about 120 μg) were digested with 10 U deoxyribonuclease I (DNase I, NEB), 20 U calf intestinal alkaline phosphatase (CIP, NEB) and 0.05 U snake venom phosphodiesterase I (Sigma, MO) at 37 °C for 24 h. The digested DNA sample was dissolved in 300 μL of methanol/HPLC grade water (5:95), and then fractionated by SPE on a Cleanert C18 cartridge (100 mg, Agela Technologies, Newark, DE). The cartridge was washed for removing unmodified nucleotides with 0.8 mL 30% methanol/water (v/v), and eluted with 0.8 mL 100% methanol for collecting the fraction of *anti*-BPDE-N^2dG. The collected fraction was dried under gentle nitrogen gas flow, and reconstituted to a final volume of 20 μL.

2.6. UPLC separation of anti-BPDE–N^2dG stereoisomers

To optimize the UPLC separation of the four stereoisomers of *anti*-BPDE–N^2dG, two UPLC BEH C18 columns (Waters, Milford, MA) with same stationary phase but different dimension were tested. The column 1 was 100 mm × 2.1 mm i.d., and the column 2 was 50 mm × 1.0 mm i.d. Both the stationary phases are in same particle size (1.7 μm). The column temperature was controlled at 35 °C. For column 1, the four stereoisomers of *anti*-BPDE–N^2dG were separated by a mobile phase of acetonitrile/0.1% formic acid in water at a volume ratio of 28:72, 26:74 or 24:78. The flow-rate was of 0.45 mL/min. For BEH C18 column 2, the separation was also tested by the mobile phases of acetonitrile/0.1% formic acid in water at a volume ratio of 22:78, 21:79 or 20:80, (v/v). The corresponding flow-rate was of 0.25 mL/min. The sample was injected by 3 μL for each. The UPLC separation was conducted on an acquity ultra-performance liquid chromatography system (Waters, Milford, MA, USA) that equipped with a Waters Micromass Quattro Premier XE triple quadrupole mass spectrometer (Waters, Manchester, UK). Data acquisition was handled by MassLynx 4.1 software (Waters).

2.7. Mass spectrometry conditions

The eluate from the UPLC columns was directly introduced into an ESI-Quattro Premier XE triple quadrupole mass spectrometer without flow-splitting. The mass spectrometer was calibrated using pure dG (10 pmol/μL). The mass spectrometer was operated in the positive ion mode. A multiple reaction monitoring (MRM) mode (dwell time of 0.15 s and inter-channel delay of 0.05 s) for transitions of the protonated BPDE-dG molecule [(M+H)$^+$] (*m*/*z* 570) to [(M−dR+2H)$^+$](*m*/*z* 454) was adopted for selective detection of *anti*-BPDE–N^2dG adducts. The optimum collision energy is 12 V for the transitions. Nitrogen was used for the desolvation, cone and nebulizing gas. The flow-rate of the nebulization gas was set at 800 L/h for column 1 (100 mm × 2.1 mm) and 500 L/h for column 2 (50 mm × 1.0 mm), and the desolvation temperature was set at 350 °C. The cone gas was set at a flow-rate of 50 L/h and the source temperature was set at 110 °C. The capillary and cone voltages were set to 3500 V and 35 V, respectively. Inert argon was used as the collision gas at a collision cell pressure of 3.5×10^{-3} mbar.

2.8. Calibration and recovery

Quantitative analysis of *anti*-BPDE–N^2dG stereoisomers was conducted on UPLC BEH C18 column 1 (100 mm × 2.1 mm) with a mobile phase of 24% acetonitrile in 0.1% formic acid at a flow-rate of 0.45 mL/min. Calibration curves for the BPDE–N^2dGs were delineated by using synthesized stereoiosmers of *anti*-BPDE–N^2dG. The mixed stock solution of four stereoisomers (211 nM *trans*-(−), 172 nM *cis*-(+), 178 nM *trans*-(+) and 178 nM *cis*-(−)) were dissolved in 33% methanol/water, and diluted 3–5 times in series. The lowest one contained 0.94 nM *trans*-(−), 0.76 nM *cis*-(+), 0.79 nM *trans*-(+), and 0.79 nM *cis*-(−). Calibration curves were linearly plotted by peak area vs. concentration. The frequency of DNA adducts was calibrated by the internal standards of isotopic $^{15}N_5$-*anti*-BPDE–N^2dG. The triplicate measurements were conducted for each sample.

To determine the recovery of the method, 45 μL of control calf thymus (CT)–DNA solution (120 μg) was spiked with 5 μL mixture of four stereoisomers of *anti*-BPDE–N^2dG (211 nM *trans*-(−), 171 nM *cis*-(+), 177 nM *trans*-(+), 177 nM *cis*-(−)). The mixture was subjected to the same enzymatic digestion, SPE fractionation, and UPLC–tandem MS analysis as described above. The recovery of the four stereoisomers was estimated from the ratio of finally measured peak areas to that obtained by direct injection of standards for UPCL–tandem MS analysis.

3. Results and discussion

3.1. Preparation of the stereoisomers of BPDE–N^2dG adduct

The four stereoisomers of *anti*-BPDE–N^2dG (*trans*-(+), *trans*-(−), *cis*-(+), and *cis*-(−)) were synthesized by the direct reaction of *anti*-BPDE with 2′-deoxyguanosine (dG). The reaction products were analyzed by HPLC-DAD-Q-TOF–MS/MS (Fig. 1A). The total yield of *anti*-BPDE–N^2dG is about 40.4% by accounting the four stereoisomers of *anti*-BPDE–N^2dG in the sum of *anti*-BPDE hydrolysis byproducts and *anti*-BPDE–N^2dG, which were calculated from their peak areas measured from the absorbance at 345 nm by assuming that these *anti*-BPDE related products have the same absorbance coefficient at 345 nm. The stereoisomers of *trans*-(+), *trans*-(−), *cis*-(+), and *cis*-(−) account for 5.7%, 7.7%, 14.0%, and 13.0%, respectively. The four stereoisomers were purified by an optimized reversed-phase HPLC method (Fig. S1). The purity of the four purified stereoisomers evaluated by HPLC-DAD-FL is satisfactory (data not shown). The structure and stereochemistry of the four *anti*-BPDE–N^2dGs were identified and validated by a combination of liquid chromatography, mass spectrometry, UV spectrophotometry and CD spectroscopy. From the HPLC-Q-TOF–MS/MS analysis, all four stereoisomers of *anti*-BPDE–N^2dG display a protonated molecular ion [(M+H)$^+$] at *m*/*z* 570 in the positive mode, and product ions at *m*/*z* 454, 303, 285, 257, 152 and 117 from the selected precursor ion of *m*/*z* 570 (Fig. 1B). The fragment ion at *m*/*z* 454 is the protonated *anti*-BPDE-guanine, resulting from a neutral loss of deoxyribose (dR) from the precursor ion [(M+H)$^+$]. The fragment ion at *m*/*z* 303 results from a loss of dG from [(M+H)$^+$], and can be further dehydrated (−H_2O) into the fragment ion at *m*/*z* 285. Loss of the CO group from *m*/*z* 285 can give rise to *m*/*z* 257. These results from MS and MS/MS analysis are consistent with previous work [10], supporting the assigned chemical structure of *anti*-BPDE–N^2dG for the four purified stereoisomers.

The assigned stereochemistry of each stereoisomer was characterized and validated by UV and CD spectra. All four stereoisomers display characteristic absorbance of *anti*-BPDE at 320–350 nm (Fig. 1C). The stereoisomers of assigned *cis*-(+) and *cis*-(−) exhibit a characteristic absorbance maximum at 346 nm, and the stereoismers of *trans*-(+) and *trans*-(−) exhibit a maximum absorbance at shorter wavelength (345/344 nm). Since *anti*-BPDE–N^2dGs in the *cis* configuration show a stronger red shift than those in *trans* configuration [10], these recorded UV spectra are in agreement with their assigned *cis* or *trans* configurations. Stereoisomeric adducts (*trans*-(+) vs. *trans*-(−); *cis*-(+) vs. *cis*-(−)) exhibit similar CD spectra that are opposite in sign (Supporting information, Fig. S2). Both *trans*-(+)- and *cis*-(−)-*anti*-BPDE–N^2dGs have a 10S absolute con-

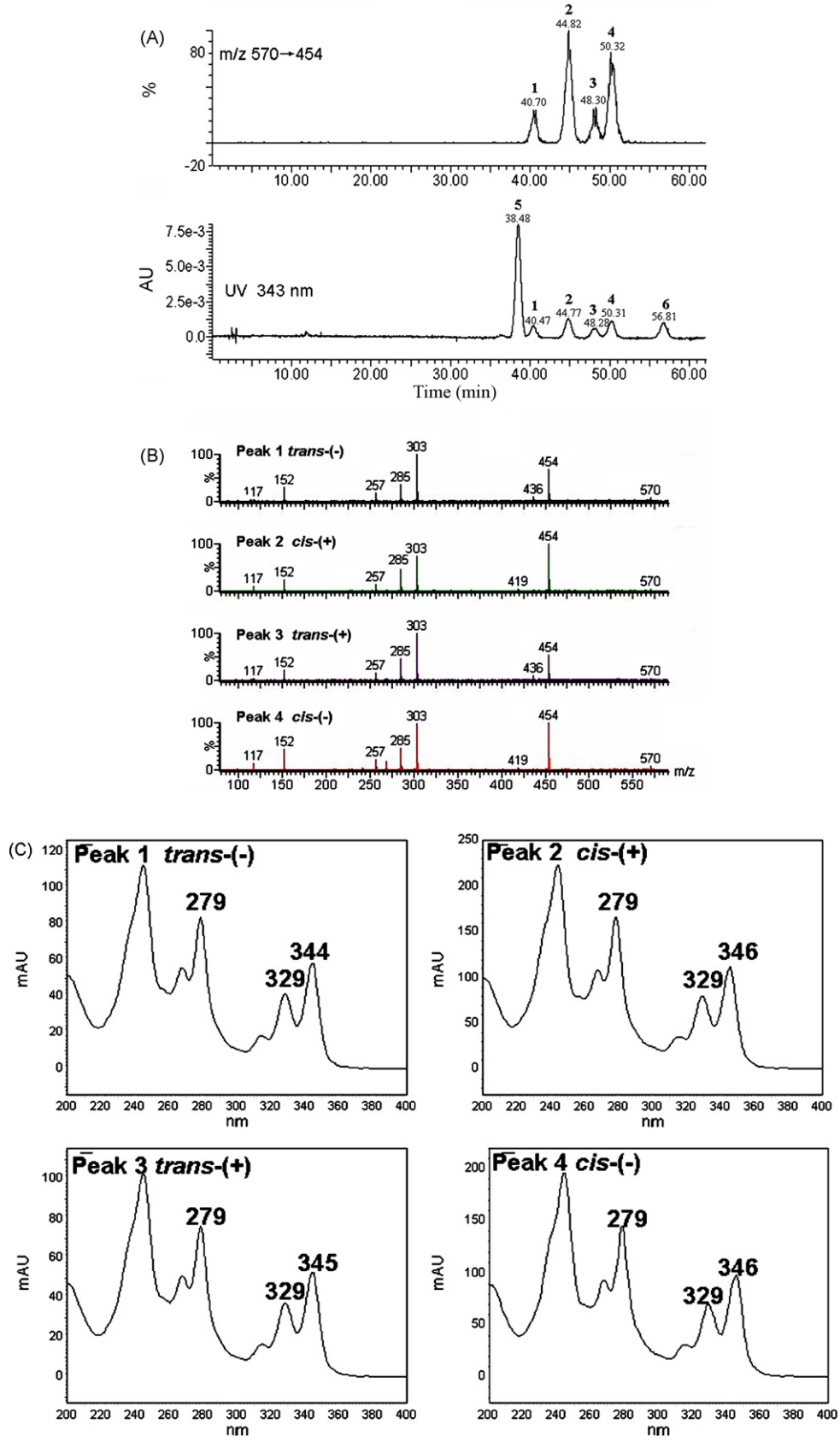

Fig. 1. HPLC-DAD-Q-TOF analysis of four stereoisomers of *anti*-BPDE–N^2dG from the reaction mixture of (±)-*anti*-BPDE and 2′-deoxyguanosine. The separation was conducted on a Capcell Pak Ph UG column (250 mm × 4.6 mm i.d., 5 μm, Shiseido, Tokyo, Japan) under an isocratic elution of 18% acetonitrile/0.1% formic acid in water at a flow-rate of 1.2 mL/min, and the column temperature was maintained at 25 °C. (A) Chromatograms for separation of the four stereoisomers recorded by ESI–MS/MS of (*m*/*z* 570→454) (upper trace), and by UV absorbance at 343 nm (lower trace). The peaks of 5 and 6 represent two BPDE tetrols without identified stereochemistry, and peaks of 1–4 represent *trans*-(−)-, *cis*-(+)-, *trans*-(+)-, and *cis*-(−)-*anti*-BPDE–N^2dG, respectively. (B) Product ion spectrum of the four stereoisomers of *anti*-BPDE–N^2dG obtained from LC/ESI–MS/MS analysis. (C) Ultraviolet spectra of the four stereoiosmers of *anti*-BPDE–N^2dG.

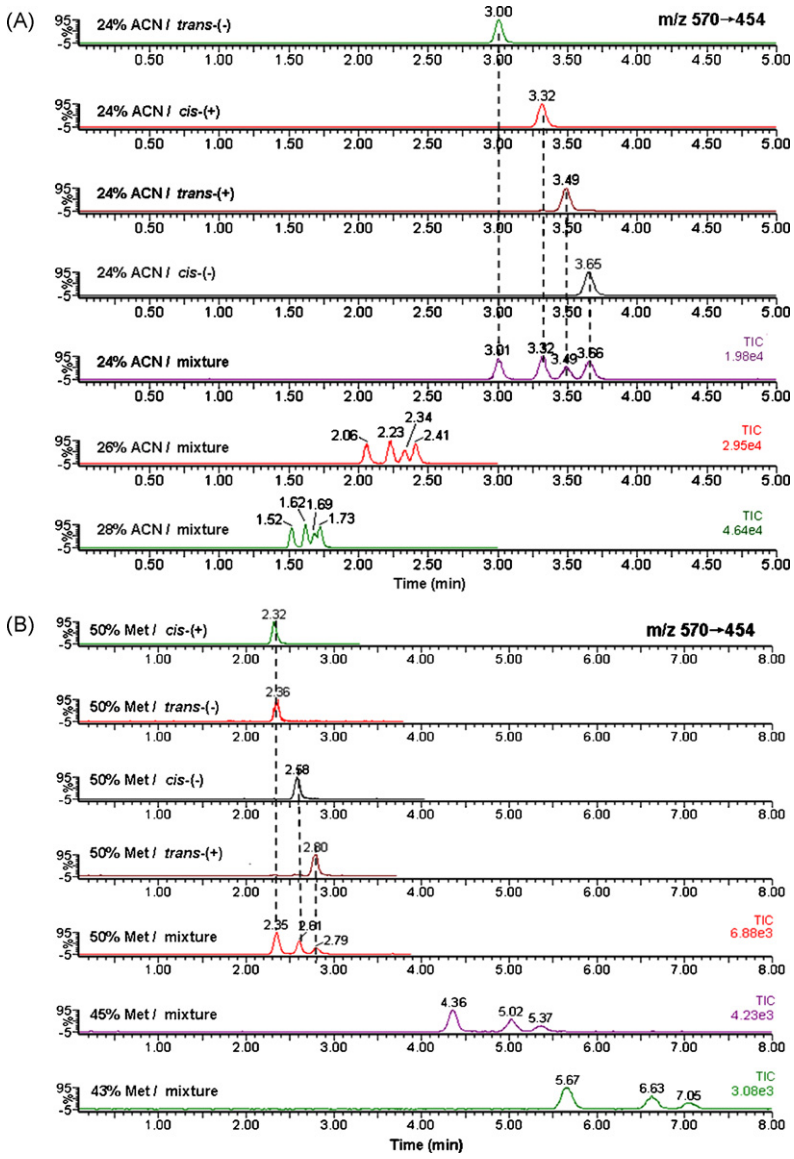

Fig. 2. UPLC-MRM/MS analysis of the four stereoisomers of *anti*-BPDE–N^2dG. The separation was conducted on a UPLC BEH C18 column (100 mm × 2.1 mm i.d, 1.7 μm) under an isocratic elution of acetonitrile(ACN)/0.1% formic acid in water (A) or of methanol (Met)/0.1% formic acid (B) at a flow-rate of 0.45 mL/min, and column temperature was maintained at 35 °C.

figuration and exhibit a positive signal for their most intense CD band (250 nm). In contrast, *trans*-(−)- and *cis*-(+)-*anti*-BPDE–N^2dG have a 10R absolute configuration and exhibit a negative signal for the same CD band (250 nm). The measured CD spectra of the purified *trans*-(−)-, *trans*-(+)-, *cis*-(−)-, and *cis*-(+)-*anti*-BPDE–N^2dGs are consistent with previous work [22,23], further validating the assigned chirality and stereochemistry of the stereoisomers of *anti*-BPDE–N^2dG.

3.2. UPLC separation of four stereoisomers of anti-BPDE–N^2dG

Fig. 2 shows the separation of the four stereoisomers of *anti*-BPDE–N^2dG by use of an optimized stationary phase (UPLC BEH C18 column, 100 mm × 2.1 mm i.d., column 1). The four stereoisomers were rapidly and well separated under the isocratic elution by the use of the mobile phase of acetonitrile/0.1% formic acid in water of 24:76 (Fig. 2A). The resultant separation resolutions are $R_{1,2}$ = 2.70, $R_{2,3}$ = 1.36, and $R_{3,4}$ = 1.30, indicating baseline separation of the four stereoisomers ($R \geq 1.14$, baseline separation). One separation of the four stereoisomers can be achieved within 3.8 min at a flow-rate of 0.45 mL/min. The peaks of the four stereoisomers were identified by injection of purified single stereoismers as shown in Fig. 2A. The four stereoisomers were eluted in the order of *trans*-(−) (3.01 min), *cis*-(+) (3.32 min), *trans*-(+) (3.49 min), and *cis*-(−)-*anti*-BPDE–N^2dG (3.66 min). Even with such short separation (<3.8 min), the separation efficiency of the stereoisoemrs by UPLC was over (5.6–9.6) × 10^4 theoretic plates per meter. The retention of the four stereoisomers decreases with increasing concentration of acetonitrile in the mobile phase from 24% to 28%, and the separation of the four stereoisomers becomes worse (Fig. 2A). It is evident that the separation of the four stereoisomers of *anti*-BPDE–N^2dG is very sensitive to the change in the concentration of acetonitrile in the mobile phase. When the concentration of acetonitrile in the mobile phase slightly increased (from 24% to 26%), the resolutions of four stereoisomers reduced to $R_{1,2}$ = 1.89, $R_{2,3}$ = 1.15, and $R_{3,4}$ = 0.67. However, the elution order is kept as the same (data not shown).

Methanol was further tested whether it could be used as strong elution solvent instead of acetonitrile. If methanol could be used, much higher concentration of methanol should be involved in the mobile phase and increase the evaporation of the mobile phase,

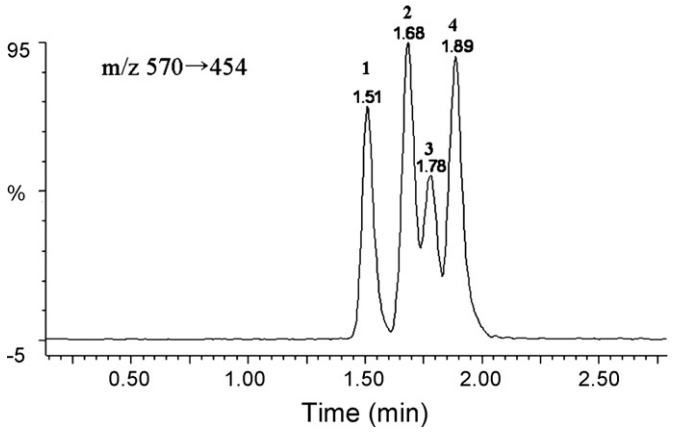

Fig. 3. The fastest analysis (<2 min) of the four stereoisomers of *anti*-BPDE–N^2dG by UPLC-MRM/MS. The separation was conducted on a narrower UPLC BEH C18 column (50 mm × 1.0 mm i.d, 1.7 μm) under an isocratic of 20% acetonitrile in 0.1% formic acid aqueous solution at a flow-rate of 0.25 mL/min, and the column temperature was kept at 35 °C. Peaks 1–4 are corresponding to *trans*-(−), *cis*-(+), *trans*-(+), and *cis*-(−)-*anti*-BPDE–N^2dG.

leading to an improvement of the MS sensitivity. In this case, same UPLC column (column 1) was used. However, our results demonstrate that methanol can improve the separation of *cis*-(−)- and *trans*-(+)- isomers but not the *cis*-(+)- and *trans*-(−) isomers, which are overlapped seriously (Fig. 2B). As shown in Fig. 2B, it is hard to resolve the four stereoisomers within 8 min by the use of methanol/0.1% formic acid in water as the mobile phase. The stereoisomers were eluted in the order of *cis*-(+)-/*trans*-(−)-, *cis*-(−)- and *trans*-(+)-*anti*-BPDE–N^2dG by the mobile phase of methanol/0.1% formic acid in water (Fig. 2B), which is different from the elution order (*trans*-(−)-, *cis*-(+)-, *trans*-(+)-, and *cis*-(−)-isomers) by the use of acetonitrile/0.1% formic acid in water as the mobile phase (Fig. 2A). It is possible to use the mixture of acetonitrile/methanol in the mobile phase. However, the four stereoiomers cannot be well separated by the mobile phase consisted of acetonitrile/methanol/0.1% formic acid in water (data not shown).

Taken together, it is concluded that the acetonitrile/0.1% formic acid in water is the optimum mobile phase for the fast separation of the four stereoisomers of *anti*-BPDE–N^2dG on the UPLC BEH C18 column.

A faster separation of the four stereoisomers (<2 min) is achieved by use of the same stationary phase but with a narrower and shorter dimension (UPLC BEH C18 column, 50 mm × 1.0 mm i.d., column 2). In this case, the column was isocratically eluted by a mobile phase consisted of acetonitrile/0.1% formic acid in water of 20:80. The four stereoisomers of *anti*-BPDE–N^2dG are well separated in 2 min at a flow-rate of 0.25 mL/min, and eluted in the order of *trans*-(−)- (1.51 min), *cis*-(+)- (1.68 min), *trans*-(+)- (1.78 min) and *cis*-(−)- (1.89 min) (Fig. 3). The elution order is same as that on the column 1(BEH C18, 100 mm × 2.1 mm i.d.). The four stereoisomers show a resolution of $R_{1,2}$ = 1.79, $R_{2,3}$ = 0.83 and $R_{3,4}$ = 0.85. The widths of half peak height for these stereoisomers ($t_{1/2}$) in the optimized UPLC separation are about 0.06–0.08 min, indicating the distribution of about 75% analytes in 15–20 μL by accounting the flow-rate of 0.25 mL/min and assuming that the peaks are Gaussian distribution and the distribution volume equals the product of $t_{1/2}$ and the flow-rate. In contrast, the widths of half peak height of the four stereoisomers under well-resolved conditions in conventional HPLC separation are about 0.85–1.06 min (Fig. 1A), similarly indicating the distribution of about 75% analytes in 1.02–1.27 mL by accounting the flow-rate of 1.2 mL/min. The analytes were concentrated in 60 times smaller volume in UPLC separation than that in conventional HPLC separation, indicating 60 times improvement in detection sensitivity, provided that the response of the applied detector is concentration-dependent.

Table 1
Test range, LOD, and LOQ of *anti*-BPDE–N^2dG standards by UPLC-MRM/MS.

Analyte	Test range (nM)	Linearity	R^2	LOD (nM)	LOQ (nM)
trans-(−)	0.94–211	$Y = 1.70x + 5.51$	0.999	0.12	0.40
cis-(+)	0.76–172	$Y = 2.23x + 2.79$	0.997	0.16	0.56
trans-(+)	0.79–178	$Y = 1.56x + 0.22$	0.998	0.23	0.74
cis-(−)	0.79–178	$Y = 1.89x + 3.74$	0.999	0.19	0.60

Because of the high speed and high efficiency of the developed UPLC method, the throughput is significantly improved over 25–30 times. Since one analysis can be completed in 2 min and no recondition is required for the UPLC analysis under isocratic elution, the developed UPLC–MS/MS method provides the capability of 25–30 runs/h and 200–240 runs/day (8 working hours per day) for detection and quantitiation of the four stereoisomers of *anti*-BPDE–N^2dGs. In contrast, the throughput for conventional HPLC analysis is about <1 run per hour and <8 run per working day (8 working hours per day).

3.3. Calibration curves, limits of detection, and precision

Calibration curves of the four stereoisomers (ranging from 0.76 to 211 nM) were established by plotting the peak area vs. concentration. The stereoisomers were analyzed using column 1 (100 mm × 2.1 mm), and the mobile phase was 24% acetonitrile in 0.1% formic acid at a flow-rate of 0.45 mL/min. The analytes eluted from the UPLC column was detected in MRM mode of electrospray ionization-triple-quadrupole mass spectrometry. The MRM mode of detection provides the high sensitivity due to decreased solvent and matrix interferences, and increased specificity due to the monitoring of a characteristic dissociation in the collision cell of the compound under investigation [33]. The selected ion is the parent ion of m/z 570 → 454, which corresponds to the *anti*-BPDE adducted 2′-deoxynucleoside $[M+H]^+$ → adducted base $[M-dR+2H]^+$. This transition was used due to the observation that the major product ion of the adducted base, *anti*-BPDE-N^2guanine, could be formed following ESI-MS/MS CID analysis. The observation is consistent with previous works [21,22]. Excellent linearity for each stereoisomer was achieved with a linear regression coefficient of $R^2 \geq 0.99$ (Table 1). The limits of detection (LOD) and quantification (LOQ) were calculated at S/N of 3 and 10, respectively. The estimated LODs and LOQs for the four stereoisomers are less than 0.23 nM and 0.74 nM (Table 1), respectively. In this case, only 3 μL of each sample was injected, the estimated LODs and LOQs for four stereoisomers were less than 0.7 fmol and 2 fmol. Intraday precision data are shown in Table 2. The precision of the UPLC-MS/MS method was defined by examining the variance of peak areas of four stereoisomers standards in repeated injection analysis (n = 4). The intraday RSD values for the four stereoisomers are less than 5%.

3.4. Recovery of analyte

To test the recovery, 5 μL of four mixed stereoisomers of *anti*-BPDE–N^2dG (211 nM *trans*-(−), 171 nM *cis*-(+), 177 nM *trans*-(+), 177 nM *cis*-(−)) was mixed with 45 μL of control CT–DNA solu-

Table 2
Intraday assay variations of UPLC-MRM/MS analysis of *anti*-BPDE–N^2dG in matrix (n = 4).

Analyte	Measured peak area	RSD (%)
trans-(−)	492 ± 20	4.2
cis-(+)	586 ± 19	3.3
trans-(+)	339 ± 15	4.5
cis-(−)	580 ± 24	4.1

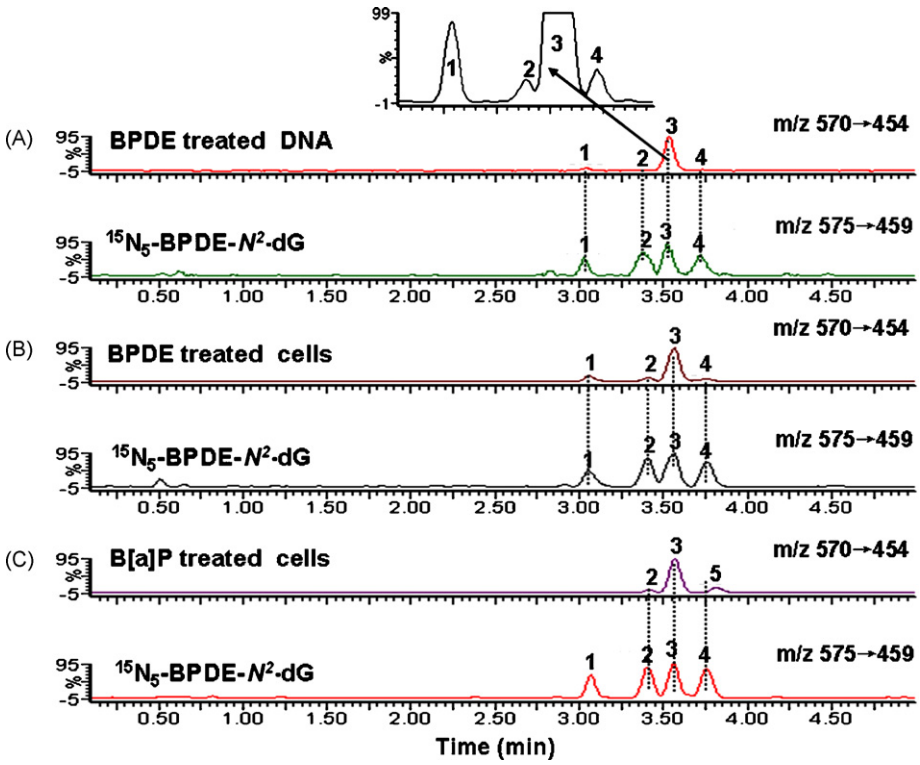

Fig. 4. Representative chromatograms of the four stereoisomeric *anti*-BPDE–N^2dG adducts obtained from stable isotope dilution UPLC-MRM/MS analysis of naked A549 DNA treated by 0.2 μM (±)-*anti*-BPDE and A549 cellular DNA treated by 1.0 μM (±)-*anti*-BPDE or 5.0 μM B[a]P. The mobile phase consists of 24% acetonitrile in 0.1% formic acid aqueous solution. The other chromatographic conditions are same as described in Fig 2.

tion (120 μg), followed by enzymatic digestion, SPE fraction and UPLC-MS/MS MRM analysis. The obtained recovery is 96.4(±6.6)% for *trans*-(−), 93.0(±6.1)% for *cis*-(+), 95.1(±5.4)% for *trans*-(+) and 92.4(±5.3)% for *cis*-(−) (n = 3). The excellent recovery also suggests the stability of the four stereoisomers of *anti*-BPDE–N^2dG throughout the enzymatic digestion and SPE fraction.

3.5. Reaction stereoslectivity of anti-BPDE and dsDNA

We first examined the direct reaction of racemic *anti*-BPDE with naked A549 DNA using the developed UPLC–MS/MS MRM method (Fig. 4). The frequency of BPDE–DNA adducts occurred in reacted A549 dsDNA were calibrated by the internal isotopic standards (four isomers of $^{15}N_5$-*anti*-BPDE–N^2dG). By incubating naked A549-dsDNA with 0.20 μM racemic *anti*-BPDE for 16 h, the estimated adduct frequency for the *trans*-(−)-, *cis*-(+)-, *trans*-(+)-, and *cis*-(−)-steroisomer are 3.5 ± 0.2, 0.30 ± 0.07, 58 ± 1.2, and 0.25 ± 0.05 adducts per 10^6 normal nucleotides, and accounting in 5.6%, 0.48%, 93.5% and 0.40% (Table 3), respectively. Benefiting from the high sensitivity and high efficient baseline-separation of the developed UPLC-MRM/MS method, the trace adduct of *cis*-(−)-steroisomer that is 270 times lower than the predominated *trans*-(+)- steroisomers can be accurately quantified. It is known that (+)-*anti*-BPDE only generates *trans*-(+)- and *cis*-(+)-*anti*-BPDE–N^2dGs, and (−)-*anti*-BPDE only generates *trans*-(−)- and *cis*-(−)-*anti*-BPDE–N^2dGs. Since the racemic *anti*-BPDE contained equaled amount of (+)-*anti*-BPDE and (−)-*anti*-BPDE, the measured adduct frequencies indicate that (+)-*anti*-BPDE prefers to form *trans*-(+)-*anti*-BPDE–N^2dG and (−)-*anti*-BPDE prefers to form *trans*-(−)-*anti*-BPDE–N^2dG. The predominant formation of *trans*-(+)-*anti*-BPDE–N^2dG is consistent with previous works [7,10]. In 1.0 μM racemic *anti*-BPDE treated A549 cells, the other three stereoisomers of *anti*-BPDE–N^2dG can also be detected (1.5%-11%) besides the predominated *trans*-(+)-*anti*-BPDE–N^2dG (83.5%). However, comparing with the direct reaction of racemic *anti*-BPDE with naked DNA, the percentage of the minor three stereoisomers (*trans*-(−)-, *cis*-(+)- and *cis*-(−)-*anti*-BPDE–N^2dG) in A549 cells treated by same racemic *anti*-BPDE increases over 1–7 times (Table 3).

3.6. Stereoselectivity of metabolic activation of B[a]P in A549 cells

In 5.0 μM B[a]P treated A549 cells (16 h), the amounts of (+)-*trans*-, (+)-*cis*-BPDE–N^2dG are 7.6 ± 0.21 and 0.11 ± 0.01 adducts/10^6 normal bases, respectively(n = 3) (Table 3). The adducts of *trans*-

Table 3
Quantitation of *anti*-BPDE–N^2dG stereoisomers in cellular and naked DNA from A549 cells (adducts per 10^6 bases).

Analyte	Cellular DNA treated by 5 μM B[a]P (16 h)		Cellular DNA treated by 1 μM (±) *anti*-BPDE (2 h)		Naked DNA treated by 0.2 μM (±) *anti*-BPDE (16 h)	
	Number	Percent	Number	Percent	Number	Percent
trans-(−)	ND		12 ± 0.4	11	3.5 ± 0.20	5.6
cis-(+)	0.11 ± 0.01	1.4	4.4 ± 0.2	4.0	0.30 ± 0.07	0.48
trans-(+)	7.6 ± 0.21	93.7	91 ± 2.1	83.5	58 ± 1.2	93.5
cis-(−)	ND		1.6 ± 0.2	1.5	0.25 ± 0.05	0.40
syn-	0.40 ± 0.02[a]	4.9[a]	ND		ND	

Samples were analyzed in triplicate; ND: not detectable.
[a] Quantitation by assuming same response as *cis*-(−).

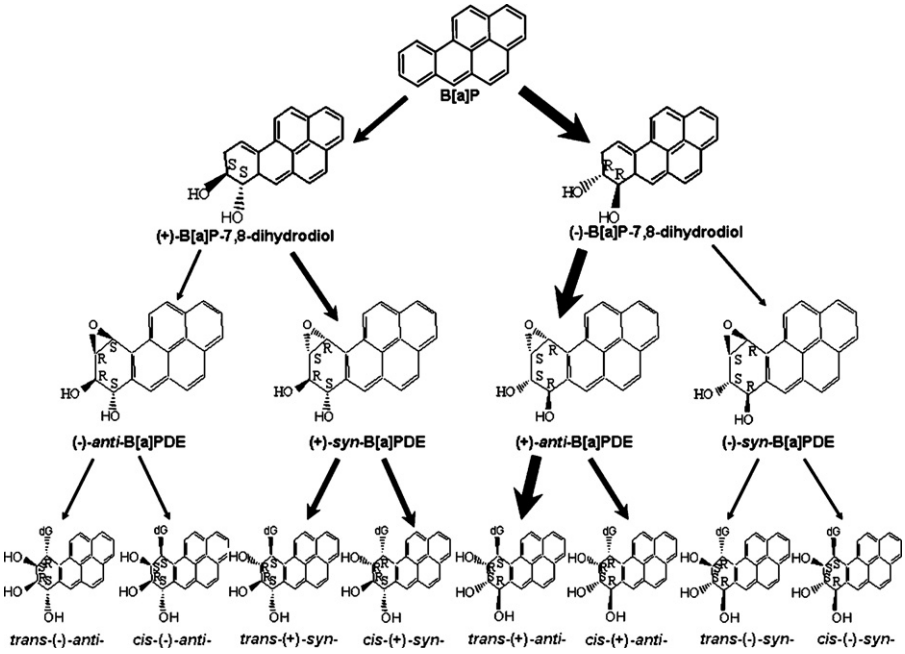

Fig. 5. Stereochemical activation of B[a]P for formation of optically active BPDE–N^2dG adducts.

(−)- and *cis*-(−)- are not detectable (Fig. 4). An unknown peak of BPDE-dG adduct (peak 5) that has same MS/MS MRM response of 570 → 454 was observed, presumably as a *syn*-BPDE–N^2dG [34]. The presumed *syn*-BPDE–N^2dG has slightly longer retention time (3.81 min) than *cis*-(−)-*anti*-BPDE–N^2dG (3.75 min). By use of the internal isotope standard, it was observed that the peak of presumed *syn*-BPDE–N^2dG was not completely overlapped with that of the internal $^{15}N_5$-*cis*-(−)-*anti*-BPDE–N^2dG. The result further excludes the possibility of peak 5 being *cis*-(−)-*anti*-BPDE–N^2dG (Fig. 4). Among the detected isomers, *trans*-(+)-*anti*-BPDE–N^2dG is predominated (93.7%) in A459 cells treated by B[a]P. In contrast, the percentage of *trans*-(+)-*anti*-BPDE–N^2dG (83.5%) in A549 cells treated by direct damage agent *anti*-BPDE is slight lower. The preferential formation of *trans*- (+)-*anti*-BPDE–N^2dG in B[a]P treated A549cells results from combined stereoselectivity of the metabolic activation of B[a]P and the reactivity of *anti*-BPDE with dsDNA. Following the general metabolic pathway of B[a]P (Fig. 5), the predomination of *trans*-(+)-*anti*-BPDE–N^2dG accompanying with the presence of *cis*-(+)-*anti*-BPDE–N^2dG, and the absence of *trans*-(−)- and *cis*-(−)-*anti*-BPDE–N^2dG together indicate the preferential formation of (+)-*anti*-BPDE and (−)-B[a]P-7,8-dihydrodiol.

In H358 cells, (+)-B[a]P-7,8-dihydrodiol is mainly activated into (+)-*syn*-BPDE, and preferentially forming (+)-*syn*-BPDE–N^2dG adduct [34,35]. In rat liver microsome, only (+)-B[a]P-diol can form predominant (+)-*syn*-BPDE that forms detectable *syn*-BPDE-dG adduct and minor (−)-*anti*-BPDE (3:1) and (−)-B[a]P-diol predominantly form (+)-*anti*-BPDE[35]. In contrast, exposure to (−)-B[a]P-7,8-dihydrodiol only induce *anti*-BPDE–N^2dG adduct [34,35]. The presence of un-negligible *syn*-BPDE–N^2dG adduct in B[a]P treated A549 cells should be the (+)-*syn* isomers, and indicates the formation of significant amount of (+)-*syn*-BPDE and its upstream metabolite (+)-B[a]P-7,8-dihydrodiol in B[a]P treated A549 cells. However, the exact stereochemistry of the *syn*-BPDE–N^2dG is unidentified.

4. Conclusion

In summary, we demonstrate a fastest and sensitive method for quantification of the four stereoisomers of *anti*-BPDE–N^2dG by use of UPLC-MRM/MS method. Compared with conventional HPLC based methods, the separation is speed up over 10–30 times. By such fast separation, the through-put of analysis is greatly increased (over 20–30 times). Benefiting from the high separation efficiency and concentration effect of UPLC, the detection sensitivity of the four stereoisomers of *anti*-BPDE–N^2dG is improved least 10–20 times. The developed method will be useful to evaluate the metabolic activation, detoxification of benzo[a]pyrene and DNA adduct formation and repair at low doses.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2009.05.054.

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