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Superoxide chemical transformation of diolepoxide polyaromatic hydrocarbon DNA adducts

Determination of benzo[*a*]pyrene-*r*-7,*t*-8,9,*c*-10-tetrahydrotetrol by gas chromatography☆

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ABSTRACT

Benzo[*a*]pyrene-*r*-7,*t*-8,9,*c*-10-tetrahydrotetrol (100 pg, 342 fmol) was measured using the following sequence of steps: (1) chemical transformation with potassium superoxide to 2,3-pyrenedicarboxylic acid; (2) electrophore derivatization with pentafluorobenzyl bromide; (3) sample clean-up by high-performance liquid chromatography and (4) measurement by gas chromatography with electron-capture detection and by gas chromatography with electron-capture negative-ion mass spectrometry. The overall, absolute yields obtained by the two procedures were 69% and 60%, respectively. This work completes the first stage towards the establishment of a general method for detecting diolepoxide polyaromatic hydrocarbon DNA adducts by gas chromatography.

INTRODUCTION

Many polyaromatic compounds have been found to be carcinogenic or mutagenic in animal exposure studies, including bacterial testing. It is generally considered, and there is much evidence, that many of these compounds exert their toxicity by chemically damaging DNA, producing so-called "DNA adducts" [1]. One important class of such adducts arises from the metabolic activation of polynuclear aromatic hydrocarbons (PAHs) to corresponding diolepoxide intermediates [2]. The latter electrophilic compounds can react with nucleophilic sites on the DNA bases, such as the N-2 position of guanine. Hence there is interest in the detection of diolepoxide PAH DNA adducts in biological samples.

Principally three kinds of methods have been employed to date for the measurement of PAH DNA adducts in biological samples: ^{32}P post-labeling, immunoassays and fluorescence. Examples of the effectiveness of these methods, and their shortcomings for the detection of PAH DNA adducts, have been summarized recently [3].

Gas chromatography (GC) is a useful analytical technique, especially when coupled with detection by mass spectrometry (MS). High sensitivity can be achieved by introducing strongly electrophoric analytes into a system comprising GC with detection by electron-capture negative-ion MS (GC–ECNI-MS). Attomole amounts of analytes can be measured in this way [3]. In order to subject diolepoxide PAH DNA to such detection, they first need to be

☆ *r* = Reference, *t* = trans and *c* = cis.

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chemically transformed and electrophore derivatized. Inherently they are not strong electrophores, nor can they become so by derivatization alone. In part, this is because of the large size of these adducts, including a diversity of functional, thermally labile groups.

In this paper, we present a novel chemical transformation technique for diolepoxide PAH DNA adducts. The technique was applied to femtomole amounts of the model analyte benzo[*a*]pyrene-*r*-7, *t*-8,9,*c*-10-tetrahydrotetrol. This compound is oxidized by potassium superoxide, the chemical transformation reagent, to a corresponding dicarboxylic acid, which in turn is electrophore derivatized and detected by GC. Both GC with electron-capture detection (GC–ECD) and GC–ECNI-MS were used, allowing them to be compared.

Recently we introduced methodology similar in concept to that here, in which an amino-PAH DNA adduct was chemically transformed by hydrazinolysis [3].

EXPERIMENTAL

Chemicals and reagents

Potassium superoxide (KO_2), 18-crown-6 (a crown ether; CE), 9,10-dihydrobenzo[*a*]pyren-7 (8*H*)-one, pentafluorobenzyl bromide, triethylamine, succinic anhydride (99%), aluminum chloride (anhydrous, 99.99%), zinc (20 mesh), mercury (II) chloride (99.5%), phosphorus pentachloride, tin(IV) chloride, high-performance liquid chromatographic (HPLC)-grade dichloromethane, HPLC-grade benzene, nitrobenzene (99 + %), HPLC-grade *p*-xylene, HPLC-grade N,N-dimethylformamide (DMF) and HPLC-grade glacial acetic acid were purchased from Aldrich (Milwaukee, WI, USA). 2,3,5,6-Tetrafluorobenzyl bromide was obtained from Alfa Products (Danvers, MA, USA), benzo[*a*]pyrene-*r*-7,*t*-8,9,*c*-10-tetrahydrotetrol (Tetrol) from the NCI Chemical Carcinogen Repository (Kansas City, MO, USA), [$^2H_{10}$]pyrene from Icon Services (Summit, NJ, USA) and acetonitrile (UV) and hexane (GC^2) from American Burdick and Jackson (American Scientific Products, Boston, MA, USA). Distilled water was purified to HPLC grade with a Nanopure/Organicpure system (Barnstead, Boston, MA, USA). For the reactions, DMF and benzene were dried with type 4Å molecular sieves (Aldrich). The molecular sieves were washed with hexane three times followed by activation overnight at 250°C before use. USP-grade carbon dioxide (99.9%), ultra-high-purity helium (99.999%), high-purity helium, ultra-high-purity nitrogen (99.999%), high-purity nitrogen. C.P.-grade methane (99.998%) and Oxisorb-LP cartridges were purchased from Medical Technical Gases (Medford, MA, USA).

Equipment

High-performance liquid chromatography. The HPLC system consisted of a Series-4 liquid chromatograph from Perkin-Elmer (Norwalk, CT, USA), a Rheodyne Model 7125 injector from Rainin (Woburn, MA, USA), a Spectroflow 773 UV detector from Kratos (Ramsey, NJ, USA) and a SP-4270 integrator (Spectra-Physics, Piscataway, NJ, USA). The solvents were degassed and the solvent chamber pressurized to 7 p.s.i. with helium. The analytical HPLC clean-up was carried out on a Microsorb C_{18} column (150 × 4.6 mm I.D., 5-μm diameter particles; Rainin) fitted with a Microsorb C_{18} guard column (15 × 4.6 mm I.D., 5-μm diameter particles; Rainin). Detection was at 263 nm for 2,3-bis(pentafluorobenzyl)pyrenedicarboxylate.

Gas chromatography with electron-capture detection. A Model 3740 gas chromatograph from Varian (Walnut Creek, CA, USA), was fitted with a ^{63}Ni electron-capture detector, a Model 1095 on-column capillary injector and a Waters Model 840 data system (Millipore–Waters, Milford, MA, USA). The determination of 2,3-bis(pentafluorobenzyl)-pyrenedicarboxylate was carried out on an HP-1 (cross-linked methylsilicone gum) capillary column (10 m × 0.32 mm I.D., 0.17-μm film thickness; Hewlett-Packard, Palo Alto, CA, USA). The flow-rate of the carrier gas (helium) was 4 ml/min at 250°C and that of the make-up gas (nitrogen) was 26 ml/min at 250°C. The initial injector and column temperatures in the GC–ECD were 90 and 120°C, respectively. Immediately after injection, the injector temperature was programmed to 280°C at 180°C/min. The column, after a 3-min hold, was programmed to 290°C at 10°C/min. The detector temperature was kept constant at 340°C.

Gas chromatography–mass spectrometry. The equipment consisted of a Hewlett-Packard Model 5988A mass spectrometer equipped with an HP-5890 gas chromatograph. The mass spectrometer

was interfaced to an HP 59970C (Rev. 3.2) MS ChemStation computer and an HP-7957B disc drive (the formatted storage capacity of the disc is 81 Mbyte). The GC separations were carried out on an Ultra-1 (cross-linked methylsilicone) capillary column (12 m × 0.20 mm I.D., 0.11-μm film thickness) (Hewlett-Packard). The column was interfaced directly to the mass spectrometer, using an interface temperature of 290°C. Helium (UHP) at a column head pressure of 20 p.s.i. was used as the carrier gas. Methane (UHP) was used as the reagent gas with a source temperature of 250°C and a source pressure of 2.0 Torr. The instrument was manually tuned daily for maximum sensitivity. For the determination of 2,3-bis(pentafluorobenzyl)pyrenedicarboxylate, the oven was programmed from 160 to 290°C at 70°C/min.

Other techniques. Fluorescent indicator plates for analytical and preparative separations by thin-layer chromatography (TLC) were obtained from Analtech. ^{1}H and ^{13}C NMR spectra were recorded on a Varian XL-300 spectrometer. All the glassware was cleaned according to a previously described procedure [4].

Syntheses

2,3-Pyrenedicarboxylic acid. Finely powdered potassium superoxide (657 mg, 9.2 mmol), 18-crown-6 (611 mg, 2.3 mmol) and 9,10-dihydrobenzo[*a*]pyrene-7(8*H*)-one (50 mg, 0.18 mmol) were suspended in dry benzene (20 ml). The mixture was stirred vigorously for 20 h at room temperature, in the dark, then 40 ml of water were added. The benzene layer was separated and the water layer was filtered through a filter-paper (Whatman). The filtrate was acidified to *ca.* pH 2 (pH paper) with concentrated hydrochloric acid under stirring, and extracted with ethyl acetate. After the ethyl acetate had been evaporated on a rotary evaporator, the product was purified on a Dynamax C_{18} semi-preparative HPLC column (Rainin Microsorb, 250 mm × 10 mm I.D., 5-μm particles) with a Rainin C_{18} guard column (50 mm × 10 mm I.D., 5-μm particles). Detection was effected at 264 nm, and the mobile phase program was (1) a 15-min equilibrium period with acetonitrile–0.1 *M* acetic acid (50:50) at a flow-rate of 5 ml/min; (2) a linear increase in the proportion of acetonitrile to 58:42 over a 8-min period; (3) a linear increase over 1 min to 100% acetonitrile, which was maintained for 10 min; and (4) a linear change in 10 min back to the initial composition (50:50). The time window for peak collection was 5.7–6.7 min. After the evaporation, the product gave ^{1}H NMR ([$^2H_6$]acetone), δ 8.21–8.45 (m, 7H, Ar), 8.90 (s, 1H, H_1), 11.94 (br s, 2H, COOH); MS[electron impact (EI)], *m/z* 290 (M, 4%), 272 (M − H_2O, 50%), 200 (M − $2CO_2H$, 100%). Analysis: calculated for $C_{18}H_{10}O_4$, C 74.47, H 3.47; found, C 74.37, H 3.41%.

2,3-Bis(pentafluorobenzyl)pyrenedicarboxylate. Finely powdered potassium carbonate (210 mg, 1.52 mmol, dried at 250°C) in 20 ml of acetonitrile was stirred at room temperature overnight. 2,3-Pyrenedicarboxylic acid (32 mg, 0.11 mmol) and pentafluorobenzyl bromide (1.4 ml, 9.27 mmol) were added. Derivatization was performed at 60°C (water condenser) with stirring until no more starting material could be seen on silica TLC [hexane–ethyl acetate (9:1)]. The reaction mixture was filtered and the acetonitrile was evaporated with a rotary evaporator. The product was purified twice by preparative silica TLC (20 × 20 cm plate, 1000-μm layer) with hexane–ethyl acetate (9:1). This led to a light yellow powder which gave a single peak on analytical HPLC. ^{1}H NMR (C^2HCl_3), δ 5.53, 5.64 (s, 2H each, CH_2), 8.05–8.26 (m, 7H, Ar), 8.67 (s, 1H, H_1); MS (EI), *m/z* 650 (M, 11%), 181 ($C_6F_5CH_2$, 100%).

2,3-Bis(tetrafluorobenzyl)pyrenedicarboxylate. This compound was synthesized and purified using the same procedure as for 2,3-bis(pentafluorobenzyl)pyrenedicarboxylate except that 2,3,5,6-tetrafluorobenzyl bromide was used instead of pentafluorobenzyl bromide. ^{1}H NMR (C^2HCl_3), δ 5.58, 5.67 (s, 2H each, CH_2), 7.08–7.14 (m, 2H, C_6F_4H), 8.03–8.23 (m, 7H, Ar), 8.67 (s, 1H, H_1); MS (EI), *m/z* 614 (M, 13%), 163 ($C_6F_4HCH_2$, 100%).

β-3-[$^2H_9$]Pyrenoylpropanoic acid [5]. Anhydrous aluminum chloride (1.20 g, 9 mmol) was added slowly, with cooling (ice-bath) and stirring, to a solution of succinic anhydride (0.48 g, 4.8 mmol) in nitrobenzene (20 ml). [$^2H_{10}$]Pyrene (1 g, 4.7 mmol) was then gradually introduced, and the color of the solution (yellow) immediately turned deep red. The mixture was stirred at 0°C for 3 h, then hydrolyzed by careful addition of dilute hydrochloric acid (3 ml of concentrated hydrochloric acid in 9 ml of water). The yellow precipitate was filtered and washed with water. The filter cake was then dis-

solved in hot aqueous sodium carbonate (12 g of sodium carbonate in 100 ml of water) and filtered immediately in order to remove aluminum hydroxide. The filtrate, on cooling to room temperature, gave a solid, the sodium salt of β-3-[$^2H_9$]pyrenoylpropanoic acid, which was filtered and recrystallized from acetic acid to yield yellow needles (1.12 g, 82%). ^1H NMR [dimethyl sulfoxide (DMSO)], δ 2.77, 3.49 (t, 2H each, CH_2), 12.26 (br s, 1H, CO_2H).

γ-3-[$^2H_9$]Pyrenylbutanoic acid [5]. Zinc (10 g, 20 mesh) was cleaned by stirring in 10% hydrochloric acid (2.6 ml of concentrated hydrochloric acid in 7.4 ml of water) for 2 min followed by washing with water (3 × 10 ml). It was then amalgamated with mercury(II)chloride (1 g). The amalgamated zinc was placed in a 100-ml round-bottom flask, followed by the addition of 7.5 ml of water, 10 ml of concentrated hydrochloric acid, 10 ml of *p*-xylene and 1 g of β-3-[$^2H_9$]pyrenoylpropanoic acid. The mixture was refluxed for 6 h, during which period concentrated hydrochloric acid (2 × 5 ml) was added. The reaction mixture was allowed to cool until crystallization was completed. The crystals, together with the zinc, were filtered, washed with water and dried under vacuum. The product, with the zinc, was digested with hot sodium hydroxide solution (3 g of sodium hydroxide in 150 ml of water) to separate the reduced acid from the insoluble colorless by-product and zinc. On acidifying the alkaline solution with concentrated hydrochloric acid, the product was obtained as a chalk-white solid (0.87 g, 91%) which was washed (water, then a small amount of benzene) and dried under vacuum. ^1H NMR ([$^2H_6$]DMSO), δ 2.02 (m, 2H, CH_2), 2.40, 3.34 (t, 2H each, CH_2), 12.10 (br s, 1H, CO_2H).

[1,2,3,4,5,6,11,12-$^2H_8$]9,10-Dihydrobenzo[a]-pyren-7(8H)-one [5]. Phosphorus pentachloride (460 mg, 2.20 mmol) was added in portions to a stirred solution of γ-3-[$^2H_9$]pyrenylbutanoic acid (600 mg, 2.02 mmol) in 50 ml of dry benzene. The mixture was stirred at room temperature under nitrogen for 1.5 h. To the clear, dark-yellow solution, 0.3 ml (2.55 mmol) of tin(II)chloride was added and the mixture was stirred at room temperature under nitrogen for 8 h. The purple complex was decomposed by adding ice and concentrated hydrochloric acid with vigorous stirring until a clear, yellow, two-phase solution was formed. The benzene layer was separated, washed twice with water, dried with anhydrous sodium sulfate and the solvent was removed under vacuum. The residual solid was recrystallized from dichloromethane–methanol to give a yellow product (260 mg, 46%). ^1H NMR (C^2HCl_3), δ 2.24 (m, 2H, CH_2), 2.74, 3.30 ppm (t, 2H each, CH_2); ^{13}C NMR (C^2HCl_3), δ 22.9, 25.8, 38.7, 122.8, 124.0, 124.8, 125.0, 126.7, 128.0, 128.9, 129.3, 131.1, 137.3, 199.0 (CO).

2,3-[$^2H_8$]Bis(pentafluorobenzyl)pyrenedicarboxylate. A solution of [1,2,3,4,5,6,11,12-$^2H_8$]9,10-dihydrobenzo[*a*]pyren-7(8*H*)-one (100 mg, 0.36 mmol) in 10 ml of dimethylformamide was added to a suspension of potassium superoxide (767 mg, 10.80 mmol) and 18-crown-6 (1.14 g, 4.30 mmol) in 10 ml of dimethylformamide in a 100-ml round-bottomed flask. The oxidation was conducted at room temperature, in the dark, with stirring. The reaction was stopped after 24 h by adding water (5 ml) and carefully acidified with concentrated hydrochloric acid until a precipitate formed. The reaction mixture was then extracted with 3 × 20 ml of ethyl acetate, dried over sodium sulfate and evaporated to dryness. The crude product was esterified directly with pentafluorobenzyl bromide (1 ml) and triethylamine (0.5 ml) in acetonitrile (20 ml) at 60°C for 4 h. The product was purified by preparative TLC using hexane–dichloromethane (1:1) and recrystallized from dichloromethane–methanol to give white crystals {144 mg, overall yield of 61% from [1,2,3,4,5,6,11,12-$^2H_8$]9,10-dihydrobenzo[*a*]pyren-7(8*H*)-one}. ^1H NMR (C^2HCl_3), δ 5.53 (s, 2H each, CH_2).

Analytical procedure

Oxidation. In triplicate, 70 μl of a Tetrol stock solution (1.56 pg/μl in methanol) were added to a 1-ml clear silanized Micro-V vial (American Scientific Products). The sample was dried at 40°C under nitrogen, followed by addition of 25 μl of DMF containing 200 μg of potassium superoxide and 190 μg of 18-crown-6. The vial was capped with a PTFE-lined screw-cap and kept at room temperature, in the dark, for 20 h, with vortex mixing for 30 s every 30 min for the first 6 h. Water (25 μl) was added, followed by two drops of acetic acid and removal of solvent at 70°C under nitrogen.

Derivatization. The 2,3-pyrenedicarboxylic acid formed was derivatized in the same vial by adding 30 μl of acetonitrile containing 0.8 μl of pentafluoro-

benzyl bromide and 0.15 μl of triethylamine. The vial was capped and kept at 60°C for 6 h with vortex mixing for 30 s every 30 min. After cooling and addition of 70 μl of acetonitrile, the sample was kept at 4°C overnight prior to post-derivatization clean-up by HPLC.

HPLC clean-up. The mobile phase program for the determination of 2,3-bis(pentafluorobenzyl)pyrenedicarboxylate was (1) a 10-min equilibrium period with water–acetonitrile (15:85) at 1 ml/min and (2) on injection, a linear change of water–acetonitrile to 5:95 in 10 min, followed by a 5-min hold at this composition. A 100-μl volume of 2,3-bis(pentafluorobenzyl)pyrenedicarboxylate standard (36.05 pg/μl in acetonitrile) was injected to establish the retention time. The injector was washed with 2 × 1 ml of hot acetonitrile. Three 100-μl volumes of acetonitrile were then injected. The fraction from 10.4 to 11.6 min was collected from the third acetonitrile injection, evaporated and analyzed by GC–ECD to assure that the HPLC system was clean.

The sample was removed from the refrigerator, left at room temperature for 30 min, vortex mixed well and allowed to stand for 5–10 min. The entire volume was then injected into the HPLC column. Three blanks were injected first, followed by three samples. The fraction from 10.4 to 11.6 min for each was collected in a 2-ml silanized Reacti-Vial. The injector was washed with 2 × 1 ml of hot acetonitrile after each injection. After evaporation at 60°C under nitrogen, the residue was dissolved in 100 μl of acetonitrile, and the entire volume was again purified as before on the same HPLC column. A 30-μl volume of GC internal standard, 2,3-bis (tetrafluorobenzyl)pyrenedicarboxylate, was added to each of the second collection vials prior to evaporation.

GC–ECD. A calibration graph was established by injecting 1 μl of three GC standards containing 1.2–12.1 fmol/μl of 2,3-bis(pentafluorobenzyl)pyrenedicarboxylate (A) and 6.6 fmol/μl each of 2,3-bis(tetrafluorobenzyl)pyrenedicarboxylate (B) in isooctane. The ratio of the concentration of A to that of B was plotted *versus* the ratio of the peak areas, using the least-squares quantitation equation (Waters 840 data system), along with the correlation coefficient. Isooctane was injected before and after each standard injection.

After evaporation of the HPLC solvent at 60°C under nitrogen, the residue was dissolved in 30 μl of isooctane, followed by vortex mixing, and 1 μl of each sample was injected into the GC–ECD system. The peak area was obtained by manual integration using the Model 840 data system.

GC–ECNI-MS. After GC–ECD analysis, 100 μl of a 524.6 fg/μl solution of deuterated internal standard in acetonitrile were added to both blanks and samples, followed by evaporation at 60°C under nitrogen. The residue was dissolved in 100 μl of isooctane and 1 μl was injected into the GC–ECNI-MS system.

RESULTS AND DISCUSSION

Our long-term aim is to establish a general assay, based on MS, for diolepoxide PAH DNA adducts. One such adduct, as a prototype, has been studied previously by several investigators [2]. This adduct arises from the reaction of *r*-7,*t*-8-dihydroxy-*t*-9,10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene with the N-2 position of guanine residues in DNA. The resulting adduct, no doubt like related adducts at this site, can be hydrolyzed by mild acid hydrolysis of the DNA [6]. For the prototype, this releases a corresponding benzo[*a*]pyrenetetrahydrotetrol (Tetrol). Thus, as a first step towards our long-term aim, we set up an assay for the latter compound, as reported here.

It is known that carboxylic acids can be converted by reaction with pentafluorobenzyl bromide into corresponding esters that are sensitive for detection by GC–ECD [7] and GC–ECNI-MS [8]. Importantly, these esters undergo dissociative electron capture to form a structurally characteristic carboxylate anion in high yield; hence the detection of such esters by GC–ECNI-MS can be specific. This makes it potentially attractive to oxidize the Tetrol to 2,3-pyrenedicarboxylic acid. We demonstrated previously that this can be achieved in high yield not only for the Tetrol, but also for a variety of related compounds by reaction with potassium superoxide [9]. This prior work was performed on milligram amounts of the PAHs. Here we extended this approach for analytical purposes by applying it to a trace amount of Tetrol, and conducted subsequent steps leading to the detection of the Tetrol, as a corresponding pentafluorobenzyl diester (Diester), by GC–ECD and GC–ECNI-MS.

In this procedure, Tetrol is first oxidized by potassium superoxide to 2,3-pyrenedicarboxylic acid (Diacid) which, in turn, is converted by pentafluorobenzyl bromide into Diester, followed by purificatio n by HPLC and determination by GC. This procedu re is a consequence of previous work in which we first determined microgram and then nanogram amounts of the Tetrol. The Diacid and the Diester could be determined by HPLC down to the low nanogram level (data not shown). This prior work helped us to optimize the conditions for the overall procedure.

Superoxide oxidation

The Tetrol is oxidized with potassium superoxide in the presence of 18-crown-6 in dimethylformamide at room temperature in the dark for 20 h (Fig. 1, step 1). On acidic aqueous work-up, 2,3-pyrenedicarboxylic acid is formed. It is attractive that one obtains the product dissolved in a clear, aqueous solution, which is simply evaporated prior to the next step. This is an advantage of using potassium superoxide for the oxidation.

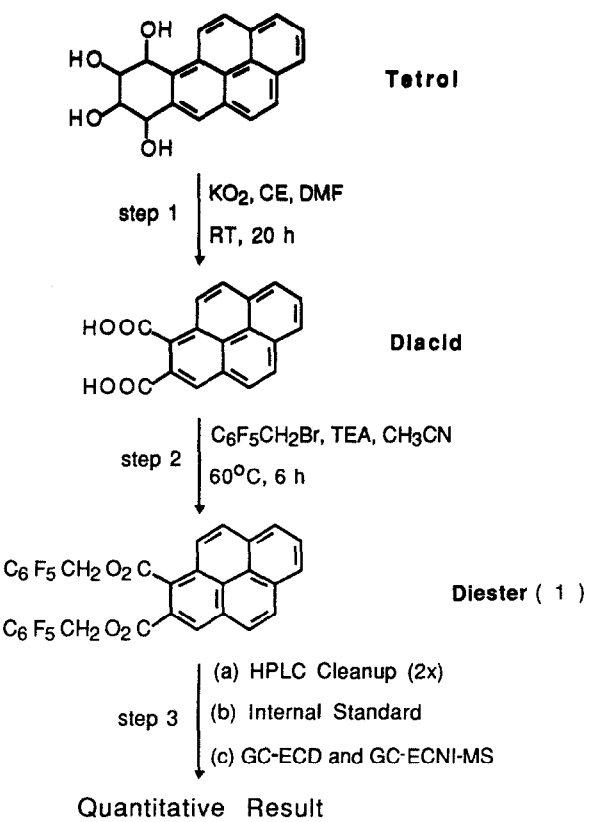


Fig. 1. Scheme for the measurement of benzo[*a*]pyrene-*r*-7,*t*-8,9,*c*-10 tetrahydrotetrol (Tetrol) by KO_2 chemical transformation, electrophore derivatization, GC–ECD and GC–ECNI-MS. CE = 18-Crown-6 (a crown ether); TEA = triethylamine.

Derivatization

The 2,3-pyrenedicarboxylic acid is derivatized with pentafluorobenzyl bromide in the presence of triethylamine in acetonitrile at 60°C for 6 h (Figure 1, step 2). The reaction volume is only 30 μl in order to allow the entire sample to be simply diluted with acetonitrile (70 μl) and injected into an HPLC column for post-derivatization clean-up. Vortex mixing instead of magnetic stirring is done as a PTFE-coated stirring bar causes an adsorption loss of the analyte and a glass-coated stirring bar is too fragile.

HPLC clean-up

Post-derivatization sample clean-up by HPLC is attractive because it can be reproducible and convenient and give a high recovery. For example, a quantitative recovery was obtained when 602 fg (in duplicate) of standard Diester was injected onto the

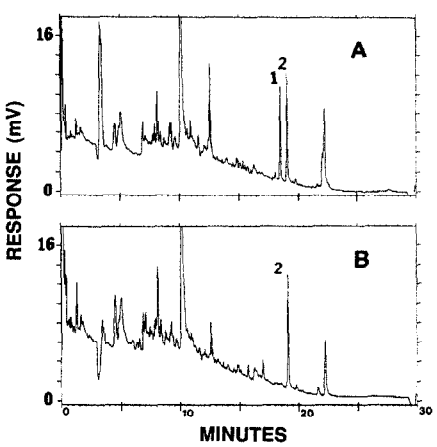
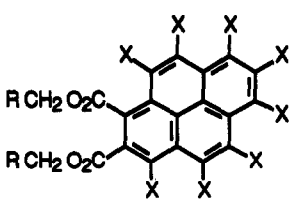


Fig. 2. GC–ECD for the oxidation and derivatization of 100 pg of Tetrol. After the second HPLC clean-up, 30 μl of internal standard solution of **2** (6.6 fmol/μl in acetonitrile) were added to blanks and samples, followed by evaporation at 60°C under nitrogen. The residue was dissolved in 30 μl of isooctane and 1 μl was injected. (A) Reaction mixture; (B) reaction blank. Peaks: 1 = 2,3-bis(pentafluorobenzyl)pyrenedicarboxylate; 2 = 2,3-bis(tetrafluorobenzyl)pyrenedicarboxylate.

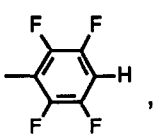
HPLC column, followed by collection, evaporation, dissolution and GC–ECNI-MS (data not shown). The potential to automate the HPLC separation in the future for this method is also important. As interferences are encountered in the subsequent GC–ECD step after a single passage of the sample through the HPLC column, the HPLC purification is done twice (step 3a in Fig. 1). The same column is used twice for each sample, and the column is carefully washed between the injections to prevent analyte carryover.

GC–ECD

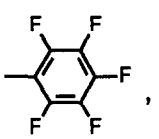
After the second HPLC separation, the first internal standard, compound **2** (for GC–ECD purposes; see below) is added to each sample (step 3b in Figure 1), followed by evaporation. The sample is dissolved in 30 μl of isooctane and 1 μl is injected into the GC–ECD system (step 3c in Fig. 1). This results in the chromatograms shown in Fig. 2, where A represents the reaction mixture (starting from 100 pg of Tetrol) and B is the blank (entire procedure starting from 0 pg of Tetrol). As shown in Figure 2, the chromatogram for the reaction blank is reasonably clean at this level of sensitivity in the elution region of interest. The overall, absolute yield for the triplicate sample is 69% (77, 68 and 62%), based on a calibration graph constructed by measuring samples containing increasing amounts of the analyte and a constant amount of **2**.



Internal Standards



2: R = X = H (for GC-ECD)



3: R = X = D (for GC-ECNI-MS)

GC–ECNI-MS

After GC–ECD, the second internal standard, compound **3**, is added to all of the same samples followed by GC–ECNI-MS, giving the chromatograms shown in Fig. 3. Chromatogram A represents the reaction mixture and B the blank. In this instance the yield is 60% (80, 50 and 49%), which agrees with the GC–ECD value.

In future work, we shall extend the method to lower Tetrol levels and to related tetrols, and apply it to biological samples. As is apparent from comparing Figs. 2 (GC–ECD) and 3 (GC–ECNI-MS), only the latter method can be trusted at lower analyte levels. This reaffirms our earlier observation that GC–ECD tends to be useful in procedures such as this only down to about the picogram level [4]. We plan to use the [1,2,3,4,5,6,11,12-$^2H_8$]9,10-dihydrobenzo[*a*]pyren-7(8*H*)-one that we synthesized as the internal standard for future work. This compound can be added early in the procedure, prior to the superoxide oxidation step. Like the Tetrol, this compound is oxidized by potassium superoxide into the corresponding dicarboxylic acid [9]. As GC–

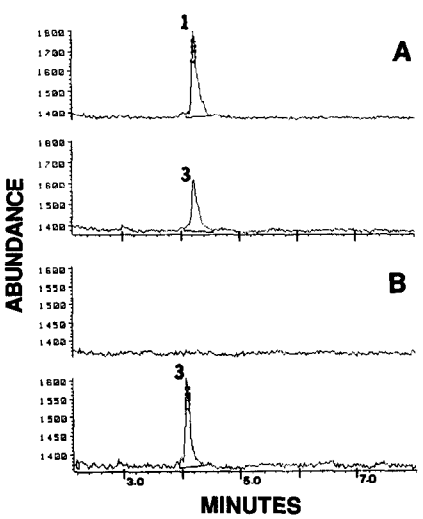


Fig. 3. GC–ECNI-MS for the oxidation and derivatization of 100 pg of Tetrol. After GC–ECD, 100 μl of an internal standard solution of **3** (524.6 fg/μl in isooctane) were added to both blanks and samples, followed by evaporation at 60°C under nitrogen. The residue was dissolved in 100 μl of isooctane and 1 μl was injected. (A) Reaction mixture; (B) reaction blank. Peaks: 1 = 2,3-bis(pentafluorobenzyl)pyrenedicarboxylate; 3 = [$^2H_8$]2,3-bis(pentafluorobenzyl)pyrene-dicarboxylate.

ECD was compared with GC–ECNI-MS in this study, internal standards **2** and **3** were added late in the current procedure just to monitor the GC step.

CONCLUSION

A novel procedure relying on superoxide oxidation shows promise for the general measurement of diolepoxide PAH DNA adducts. While both GC–ECD and GC–ECNI-MS can be used to detect as little as 100 pg of the model analyte, benzo[*a*]pyrene-*r*-7,*t*-8,9,*c*-10-tetrahydrotetrol, only the latter technique gives a clean baseline that encourages its further application to lower analyte levels including, eventually, a diversity of such analytes present in biological samples.

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