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A FLUOROMETRIC-HPLC ASSAY FOR QUANTITATING THE BINDING OF BENZO[a]PYRENE METABOLITES TO DNA*

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Summary. A nonradiometric method is presented for quantitating low levels of benzo[a]pyrene (BP) derivatives that are covalently bound to the DNA of BP-treated mice. This method consists of hydrolyzing the DNA with acid to liberate the BP-adducts in the form of the isomeric tetrols of BP. These tetrols have fluorescence quantum yields of ~0.7 in deoxygenated solution at 298 K. Hence they are easily quantitated, following HPLC separation, by means of fluorescence detection. The sensitivity of the method is such that one bound BP residue per 10^7 bases can be detected in 100 μg of DNA.

The cellular metabolism of benzo[a]pyrene (BP)† to the chemically reactive isomers BPDE I and II results in the formation of covalent BPDE-adducts with DNA (1) which may be the initiating events responsible for the observed carcinogenic properties of BP (2). The common method for quantitating the binding of BPDE to DNA makes use of radioactively labeled BP (3). However, in those instances in which the sources of BP are substances found in the environment, it is not possible to use radiometric detection because the BP is unlabeled. Hence, only meager estimates exist of the burden in human tissue of BP derivatives (4).

Fluorimetry, with its intrinsically high sensitivity, is an alternative to radiometric analysis for detecting carcinogens bound to DNA (5). Previously, fluorescence studies of the intact BPDE-DNA complex have utilized either photon counting (6) or low temperature fluorimetry (7) or a combination of both of these methods (8) to detect as little as one molecule of BPDE bound per 10^5

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†Abbreviations used are: BP, benzo[a]pyrene; BPDE I or II = (±)-7β,8α-dihydroxy(9α,10α or 9β,10β)-epoxy-7,8,9,10-tetrahydro-BP; tetrol I-1 or I-2 or II-1 or II-2 = (±)-7β,8α(9α,10β, or 9α,10α, or 9β,10α, or 9β,10β)-tetrahydroxy-7,8,9,10-tetrahydro-BP; HPLC, high performance liquid chromatography; PB, phosphate buffer (0.01 M, pH 7); BPDE-DNA, DNA containing covalently bound adducts derived from BPDE; C.T. DNA, calf thymus DNA.

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DNA nucleotides. However, in order to analyze the minute amounts of BP derivatives bound to the DNA of animals exposed to polyaromatic hydrocarbons in the environment, more sensitive detection methods are necessary.

Other workers (9-11) have pointed out that the interaction of the bases in DNA with covalently bound BPDE strongly quenches the fluorescence of the pyrene moiety. On the other hand, when the pyrene moiety is in the form of the free tetrol, the fluorescence quantum yield in deoxygenated solution is similar to that of pyrene itself (ϕ_f ~0.7) (9). These results suggest that acid-induced removal of the pyrenyl moieties from the DNA as tetrols, as has been demonstrated previously (12-14), could be used to increase the sensitivity of the fluorescence assay.

EXPERIMENTAL

Calf thymus DNA (Sigma) in PB was treated with tritiated BPDE I or II (Midwest Research Institute) for one hour at 37°C. Following phenol extraction (3×) the samples were dialyzed against PB (3×). This treatment removes all noncovalently associated BP derivatives from the DNA (8). The relative amount of BPDE reacted with the DNA was determined from the absorbance at 350 nm as described previously (15). Purified tetrols I-1, I-2, II-1 and II-2, were obtained by hydrolysis of the corresponding BPDE isomers followed by HPLC separation. Their concentration was determined from the absorbance at 343 nm using ε° = 49,000 $cm^{-1}M^{-1}$ for BPDE (16) and assuming that ε° for BPDE and the tetrol are the same. DNA containing BPDE adducts was also obtained from Sencar mice exposed to BP (100 μg) over an area on the back of 20 cm^2. After 24 hr the skin was removed and the DNA extracted as described elsewhere (17). For a control, DNA was extracted from untreated C_3H mice.

HPLC. Samples (50 or 200 λ) in 50% methanol were injected into a Zorbax ODS column (250 × 4 mm) connected to a Dupont 850 liquid chromatographic system. Elution of the sample was done with 50% methanol in water (v/v) using a flow rate of 1 ml/min and a column temperature of 50°C. The 50% methanol solution had helium bubbled through it prior to elution to remove oxygen. The fluorescence of the elutant was continuously monitored using a Schoeffel FS-970 fluorometer with a deuterium lamp as an excitation source. The excitation monochromator was set at 246 nm and the fluorescence emission was selected using a 389 nm cut-off filter. The spectral properties of the tetrols were found to be virtually identical to those of pyrene (9).

In order to remove fluorescent impurities eluting from the column near the void volume, the DNA solution, following acid hydrolysis, was passed through a C_{18} Sep-Pak cartridge (Waters Associates, Inc.). Non-tetrol material, such as DNA bases, was removed with water and the tetrols eluted with 50% methanol. The solution containing the tetrols was then injected into the HPLC column. Recovery of tetrols from the Sep-Pak treatment was ~100%.

RESULTS AND DISCUSSION

A. Relative Fluorescence of BPDE-DNA at 298 K and 77 K Following Various Treatments. The relative fluorescence yields at 298 K and 77 K of the pyrenyl moiety either bound to DNA or free as either the BPDE-deoxynucleoside adduct or the tetrol are given in Table I. These results demonstrate that decreasing the interaction between the pyrenyl moiety and the nucleotides reduces quenching of the singlet state, i.e., the free tetrol has an order of magnitude higher fluorescence yield that the intact BPDE-DNA adducts and at least a 2-fold higher fluorescence yield than the BPDE-deoxynucleoside adducts.

TABLE I

Relative Fluorescence Yields at 298 K and 77 K of the Pyrenyl Moiety Following Various Treatments of BPDE-DNA

	Treatment of BPDE-DNA	State of Pyrenyl Moiety	Medium	Relative Fluorescence Yield	
				298 K	77 K
1.	None	BPDE-double stranded DNA	PB	0.01	0.39
2.	Heat denaturation	BPDE-single stranded DNA	PB	0.02	0.39
3.	Enzyme digestion plus LH20 separation	BPDE-deoxynucleoside	50% methanol	0.05	0.48
4.	Acid hydrolysis plus LH20 separation	Tetrol	50% methanol	0.11	1.0
		Tetrol ($-O_2$)	50% methanol	0.50	1.0

Calf thymus DNA treated with (^{3}H)BPDE was phenol extracted and dialyzed against PB; the resulting BPDE-DNA had a BPDE-adduct per base ratio of 0.001. Heat denaturation was done by heating for 15 min at 100°C. Enzyme hydrolysis to the deoxynucleoside level was done as described previously (18). Removal of the tetrols with acid was done using 0.12 NHCl at 80°C for 6 hr (12-14). Following either enzyme or acid hydrolysis, the hydrolysate was run through a Sephadex LH20 column and the fraction containing the pyrenyl moiety, either in the form of BPDE-deoxynucleoside or as the free tetrol, was eluted using 50% methanol. Samples (0.15 ml) were placed in cylindrical quartz sample tubes (3 mm ID) and held in a quartz "fingertip dewar" filled with either ethanol for measurements at 298 K or liquid nitrogen for measurements at 77 K. The fluorescence was measured using a Spex Fluorolog exciting at the excitation maximum (345-351 nm) and observing at the emission maximum (398-406 nm). All measurements were made in the presence of oxygen except for the tetrol($-O_2$) which was deoxygenated by nitrogen bubbling. Samples in 50% methanol formed optically clear glasses at 77 K. On the other hand, samples in PB formed optically opaque matrices at 77 K in which the DNA remains homogeneously dispersed, a condition suitable for quantitative fluorescence measurements as determined by comparison with results obtained using 50% ethylene glycol which forms a glass at 77 K. The signal levels at 77 K were the same regardless of whether ethylene glycol was present or not. Yields are expressed relative to that of the tetrol at 77 K being assigned a value of 1.0 and have been normalized to one another on the basis of the radioactivity of the BP-moiety.

The tetrol, because of its long lived excited singlet state, is also sensitive to quenching by oxygen so that removal of oxygen from the solution also enhances the fluorescence yield. Hence, the fluorescence yield of the tetrol in deoxygenated solution is 50 times greater than that of an equivalent amount of pyrenyl moiety bound to native DNA in the presence of oxygen.

At 77 K a rigid matrix is formed which imposes restrictions on the ability of both nucleotides and oxygen to quench the fluorescence of the pyrenyl moieties; consequently, as shown in Table I, the yields obtained for BPDE-DNA are ~40 times greater at 77 K than they are at 298 K. Hence, the fluoresence

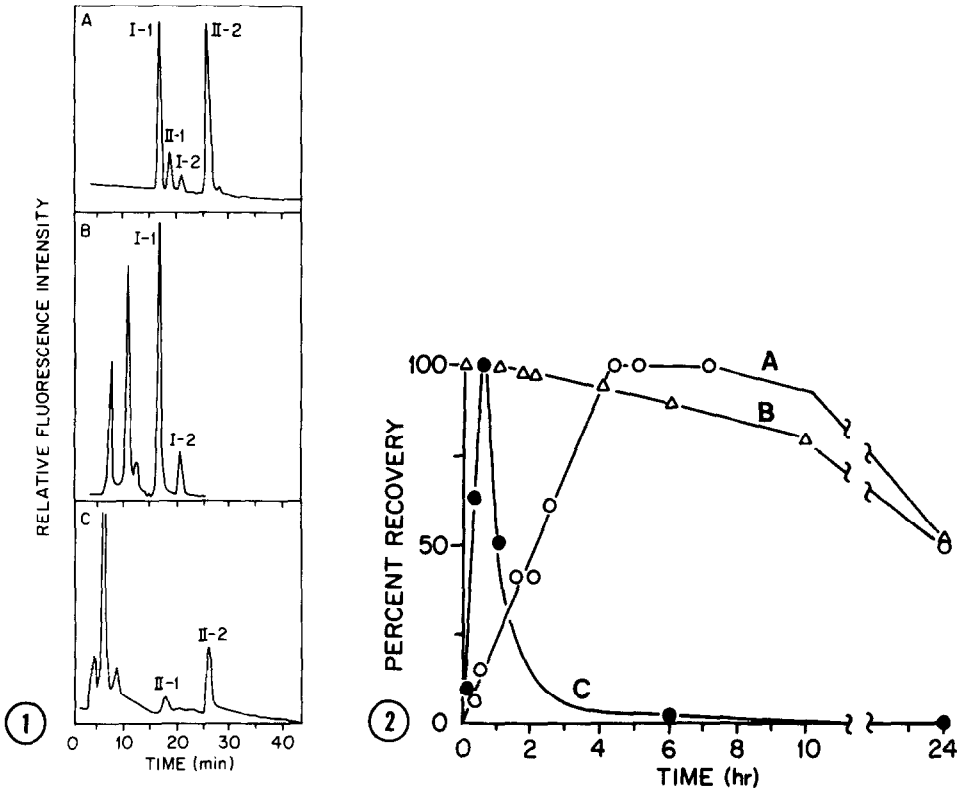


Fig. 1. HPLC analysis of tetrols obtained from various sources: (A) Derived from hydrolysis in water of 1 ng of BPDE-I (peaks I-1 and I-2) and 3.5 ng of BPDE-II (peaks II-1 and II-2). (B) Liberated by acid hydrolysis of 0.47 μg C.T. DNA previously treated with BPDE-I to give a final binding level of 0.004 BPDE/DNA (w/w). (C) Liberated by acid hydrolysis of 12 μg DNA previously treated with BPDE-II to give a final binding level of 0.0001 BPDE/DNA (w/w).

Fig. 2. Time course for changes in the concentration of tetrol I-1 as monitored by HPLC-fluorescence: effect of HCl concentration and temperature on (A) 0.47 μg of C.T. DNA [previously treated with BPDE-I to give 0.004 BPDE/DNA (w/w)] incubated at 80°C in 0.12 N HCl. (B) 2.9 ng of tetrol I-1 previously purified by HPLC and incubated at 80°C in 0.12 N HCl. (C) Same as A except incubation at 85°C in 1.0 HCl.

yield of the free tetrol at 298 K and the BPDE-DNA complex at 77 K are about the same. However, the intrinsic background fluorescence due to solvent impurities, etc., is about an order of magnitude greater at 77 K than at 298 K. Therefore, the maximum signal to background ratio for a fixed amount of pyrenyl moiety is an order of magnitude greater for the free tetrol at 298 K than it is for the BPDE-DNA complex at 77 K. This result suggested the use of HPLC coupled with fluorescence detection to analyze the pyrenyl content of BPDE-DNA following acid hydrolysis to release the tetrols.

B. Fluorescence - HPLC Detection of Tetrols Obtained from Acid Hydrolyzed BPDE-DNA. The HPLC-fluorescence profile obtained for a mixture of tetrols derived from hydrolysis of BPDE I and II (Fig. 1A) is essentially identical to that reported previously (19) in which absorbance was used to

detect the eluted tetrols. According to Yang et al. (19) nucleophilic addition of water to BPDE proceeds trans-sterioselectively for I and cis-sterioselectively for II both at the C(10) position.

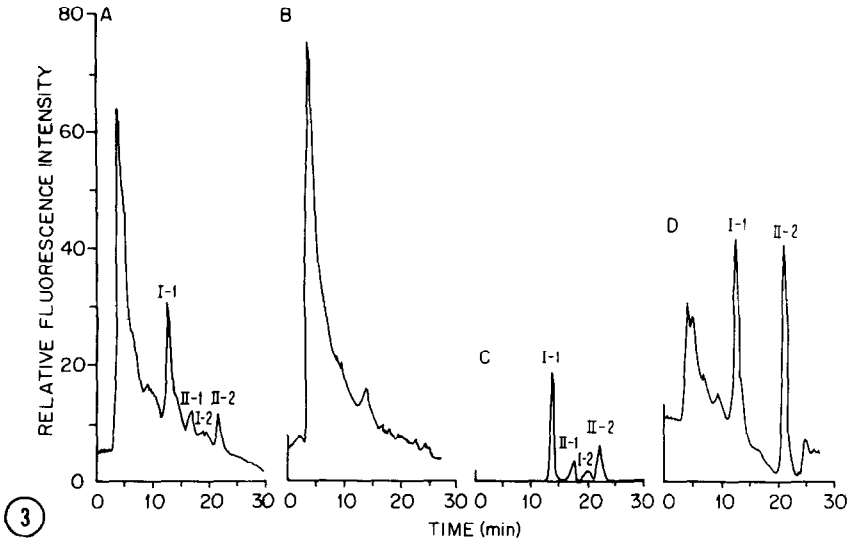
C.T. DNA treated with either BPDE I or II, acid hydrolyzed, and subjected to HPLC, gives rise to the chromatographic profiles shown in Fig. 1B and 1C, respectively. For each isomer there is a major and minor peak corresponding to the same pair of tetrols obtained by hydrolysis of the BPDE isomer itself. Also shown in Figs. 1B and 1C are several peaks eluting near the void volume which appear regardless of whether BP derivatives are present or not in the DNA. Passage of the hydrolyzed DNA solution through a C_{18} Sep-Pak prior to HPLC removes the majority of this non-tetrol fluorescent material, however.

C. Optimization of Hydrolysis Conditions. In order to verify that the conditions used for acid hydrolysis provide the optimal fluorescence signal, the conditions of hydrolysis were varied and the results shown in Fig. 2 were obtained. Curve A shows the time dependent variation of the fluorescence yield for hydrolysis of BPDE-DNA using 0.12 N HCl at 80°C. The signal reaches a maximum in 5 hr after which it begins to decrease slightly. As shown by curve B, which is for hydrolysis of the tetrol alone, this decrease in fluorescence amounts to about a 50% loss in 24 hr. Acid hydrolysis in 1 N HCl at 85°C (curve C) results in a rapid conversion of BPDE-DNA into the tetrol form followed by an equally rapid degradation of the tetrol. Based on these results, 6 hr at 80°C in 0.12 N HCl are judged to be the optimal conditions for maximizing the yield of tetrol from BPDE-DNA.

D. Detection of Pyrenyl Moieties Isolated from the DNA of Treated Mice. DNA from BP-treated Sencar mice was isolated, acid hydrolyzed, and the liberated tetrols analyzed by fluorescence-HPLC. The results are shown in Fig. 3A, in which 4 peaks corresponding to the 4 tetrol isomers can be observed. If one subtracts the background profile observed for unmodified DNA (Fig. 3B) from the profile shown in Fig. 3A, then one obtains the profile shown in Fig. 3C in which the 4 tetrol peaks are more easily discerned. Of the 4 tetrol peaks shown in Fig. 3C, that corresponding to isomer I-1 is the largest with II-2 being the next largest.

In order to estimate the amount of tetrol represented by the peak heights shown in Fig. 3C, a mixture of known amounts of tetrol I-1 and II-2 was prepared and applied to the column. The peak heights shown in Fig. 3D were obtained and used as a means of calibration. In this way one estimates that 65 pg of tetrol I-1 and 31 pg of tetrol II-2 are present in the sample shown in Fig. 3C. From these amounts one calculates that approximately 100 pg of BPDE are bound to 100 μg of the mouse DNA (or 1 BP residue per 10^6 bases).

An estimate of the lower limit of detection was obtained by mixing the BPDE-DNA from BP-treated Sencar mice (Fig. 3A) and unmodified DNA isolated



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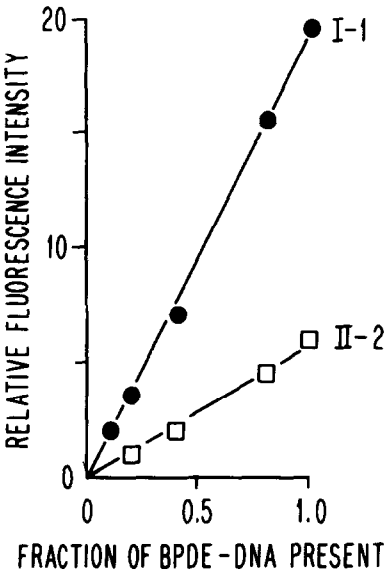

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Fig. 3. Tetrol analysis by fluorescence-HPLC on mouse skin DNA: (A) 100 μg of DNA from BP-treated Sencar mice acid hydrolyzed, put through a Sep-Pak C_{18} cartridge and resuspended in 200 μl of 50% methanol. (B) Same as A except DNA was isolated from untreated C_3H mice. (C) Difference between A and B. (D) Mixture of tetrol I-1 (100 pg) and tetrol II-2 (200 pg) passed through Sep-Pak C_{18} cartridge and resuspended in 200 μl of 50% methanol.

Fig. 4. Variation of the relative intensity of tetrol peaks I-1 and I-2 as a function of the fraction of modified DNA present in a mixture of BPDE-DNA (from BP-treated Sencar mice) and unmodified DNA (from untreated C_3H mice). The total amount of DNA was kept constant at 100 μg. The DNA was subjected to the same hydrolysis and chromatography conditions as presented in Fig. 3.

from C_3H mice (Fig. 3B). The two types of DNAs were mixed in varying amounts such that the total amount of DNA was kept fixed at 100 μg but the total amount of bound BPDE varied from 0 to 100 pg. Following acid hydrolysis, the samples were analyzed by HPLC and the fluorescence intensity of peaks I-1 and II-2

plotted as a function of the fraction of the modified DNA present. As shown in Fig. 4, the peak heights vary linearly with the amount of modified DNA present and a lower limit of 10 pg of tetrol appears to be detectible per 100 μg of BP-modified DNA. In other words, approximately 1 BP residue/10^7 bases/100 μg DNA represents the current limit of detection.

Other workers have used HPLC with absorbance detection to monitor the appearance of tetrols following acid hydrolysis of BPDE-DNA (13). HPLC with fluorescence detection has also been used to study the BPDE-deoxynucleoside adducts released following enzyme hydrolysis of BPDE-DNA (20). However, the method described here combines the simplicity of acid hydrolysis and the sensitivity of fluorescence detection to arrive at a reproducible method of analysis which is simple, sensitive and straightforward.

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