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REVERSED-PHASE SEPARATION OF BENZO[*a*]PYRENE METABOLITES BY THIN-LAYER CHROMATOGRAPHY

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SUMMARY

The use of reversed-phase thin-layer chromatography for the separation of benzo[*a*]pyrene metabolites has been investigated. Two systems are described for the separation of the major metabolites of benzo[*a*]pyrene, including sulfate and glucuronide conjugates.

INTRODUCTION**

Adequate resolution of the major metabolite of B(*a*)P has not been obtained by normal-phase TLC using solvents such as benzene–ethanol[1–4]. The advent of re-

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** Abbreviations: B(*a*)P = Benzo[*a*]pyrene; NADH = nicotinamide–adenine dinucleotide, reduced; NADPH = nicotinamide–adenine dinucleotide phosphate, reduced; THF = tetrahydrofuran; TBAH = tetrabutylammonium hydrogen sulfate; UDPGA = uridine-3′-phosphoglucuronic acid; S.D. = standard deviation; TLC = thin-layer chromatography; HPLC = high-performance liquid chromatography; S-9 = 9000 *g* supernatant.

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versed-phase HPLC offered rapid separation of the metabolites with increased resolution of the phenols and quinones[5–7]. Use of microparticulate columns has increased resolution and decreased analysis time. In addition, greater resolution of the more polar compounds, including triols and tetrols, has been observed[8,9].

Most studies involving the metabolism of B(*a*)P use HPLC for quantitation of the metabolites. In this report, an inexpensive system utilizing reversed-phase TLC and an ion-pairing reagent is described for the analysis of B(*a*)P metabolites. This procedure offers rapid separation with good resolution of the more polar metabolites, including conjugates.

EXPERIMENTAL

Materials

B(*a*)P metabolite standards were obtained from Dr. David Longfellow, Division of Cancer Cause and Prevention, National Cancer Institute (Bethesda, MD, U.S.A.) Whatman $KC_{18}F$ reversed-phase TLC plates were obtained from Whatman (Clifton, NY, U.S.A.). Solvents were HPLC grade from Fisher Scientific (Fairlawn, NJ, U.S.A.). Aroclor 1254 was a generous gift of Monsanto (St. Louis, MO, U.S.A.). B(*a*)P, *α*-tocopherol, *E. coli β*-glucuronidase, UDPGA, NADPH, NADH and TBAH were products of Sigma (St. Louis, MO, U.S.A.). [G-^{3}H]Benzo[*a*]pyrene, specific activity 18,000 mCi/mmol, was obtained from Amersham (Arlington Heights, IL, U.S.A.). Silica gel Sep-Paks were purchased from Waters Assoc. (Milford, MA, U.S.A.).

TLC sheets

Whatman $KC_{18}F$ TLC plates were unwrapped just prior to spotting samples to minimize contact with moisture in the air. The tanks were allowed to equilibrate in the solvent for 30–60 min prior to development of the chromatogram. The use of filter paper at the top of the tank was not found to be necessary but does afford better reproducibility. For development of the solvent system, 20 × 5 cm TLC plates were used; for radioactive samples, 20 × 20 cm sheets were used. Generally, 10–20 *μ*l were spotted for each sample.

Solvent system

For separation of the major metabolites of B(*a*)P, a solvent system consisting of acetonitrile–THF–0.071 *M* TBAH (85:1:14) (solvent A) was used. To separate sulfate and glucuronide conjugates from the major metabolites, acetonitrile–methanol–THF–0.042 *M* TBAH (65:10:1:24) (solvent B) was used.

Metabolite formation

Male Sprague-Dawley rats were induced with 500 mg/kg Aroclor 1254 for 3 days according to Fang and Strobel[10]. [^{3}H]B(*a*)P was diluted to a specific activity of 1000 mCi/mmole and purified by silica gel chromatography just prior to incubation[11]. The incubation mixture has been previously described[6]. Briefly, 200 *μ*g rat liver S-9 was added to 100 nmoles [^{3}H]B(*a*)P in 0.05 *M* Tris (pH 7.5), 0.003 *M* $MgCl_2$ with 0.75 mg NADH and 0.75 mg NADPH in 1 ml. Samples were incubated in 5 tubes for 0, 15, 30 or 60 min.

Extraction of metabolites

Following incubation, 0.03 ml of 0.1 *M* α-tocopherol in ethanol was added to each tube prior to extraction with an equal volume of acetone and an equal volume of ethyl acetate[9]. The aqueous layer was reextracted with an equal volume of ethyl acetate. The organic layers were pooled from each of 5 tubes, dried over anhydrous $MgSO_4$, and evaporated under nitrogen. Samples were stored in the dark at 4°C prior to chromatography.

Removal of [^{3}H]Benzo[a]pyrene

[^{3}H]B(*a*)P was removed from the metabolites by dissolving the evaporated organic extract in benzene then applying the sample to a Waters silica gel Sep-Pak. [^{3}H]B(*a*)P was eluted with 5 ml benzene, and the metabolites were eluted with 5 ml methanol. 30 μl of 0.1 *M* α-tocopherol was added to the metabolite fraction prior to evaporation under nitrogen.

TLC

Dried metabolites were dissolved in 100 μl and 15 μl were spotted in two different lanes, along with 5 μl of a mixture of unlabeled metabolite standards. Development times were 55 min for solvent A and 80 min for solvent B.

Autoradiography

After development, the TLC sheets were dried, marked with ^{14}C-labeled ink and a sheet of LKB [^{3}H]Ultrofilm was placed on top. The sheets were developed for 7–14 days.

RESULTS AND DISCUSSION

Polar solvent selection

In attempting to develop a solvent system to separate the B(*a*)P metabolites, efforts were centered around use of a methanol–water system that was analogous to solvent systems used with by HPLC and octadecyl silane column packing. However, this solvent system did not yield adequate separation of the B(*a*)P phenols and quinones (Table I). Thus, the ion-pairing agent TBAH was added (Table I). Increased

TABLE I

R_F VALUES OF BENZO[*a*]PYRENE METABOLITES

Metabolite	*Solvent system*		
	Methanol–water (95:5)	*Methanol–0.04 M TBAH (95:5)*	*Methanol–0.10 M TBAH (95:5)*
B(*a*)P	0.27	0.24	0.27
Quinones*	0.37	0.35	0.37
3-OH	0.43	0.39	0.43
9-OH	0.43	0.40	0.44
4,5-epoxide	0.43	0.40	0.45
7,8-dihydrodiol	0.57	0.55	0.59
4,5-dihydrodiol	0.61	0.60	0.64
9,10-dihydrodiol	0.73	0.72	0.75

* Mixture of 1,6-, 3,6- and 6,12-quinones.

resolution of the quinones and phenols was observed at higher concentrations of TBAH, but adequate resolution of 3-OH- and 9-OH-B(*a*)P was not achieved. Thus, a system using acetonitrile–water was tried (Fig. 1). Increasing the acetonitrile concentration from 80% to 85% or the addition of 1% THF still did not adequately resolve the phenols, but better separation was obtained than with methanol–water (Fig. 1A). Very poor separation was observed for the 4,5-epoxide from the phenols and quinones in the first five solvents. The best separation of all the metabolites was obtained with acetronitrile–THF–0.071 *M* TBAH (85:1:14) as shown in Fig. 1 (solvent 6). Adequate separation of 3-OH- and 9-OH-B(*a*)P was obtained but not between 7-OH- and 3-OH- or 9-OH-B(*a*)P. The 4,5-epoxide was separated from the quinones and 3-OH-B(*a*)P in this system, with an R_F value of 0.45 (Table II). The quinones were not well separated from each other and tended to migrate as a large, diffuse spot.

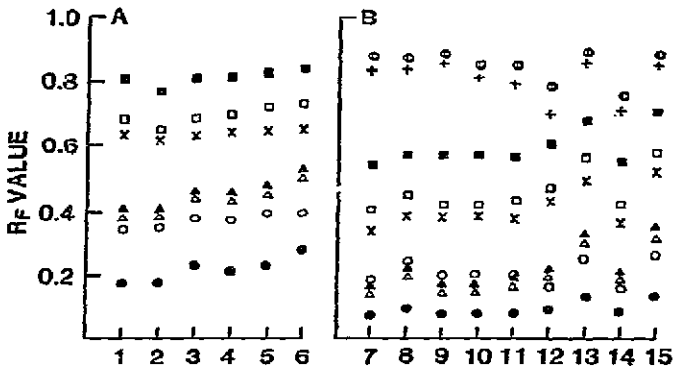

Fig. 1. A, Reversed-phase TLC of major B(*a*)P metabolites in various solvents. Solvent systems: 1 = acetonitrile–water (80:20); 2 = acetonitrile–THF–water (80:1:19); 3 = acetonitrile–water (85:15); 4 = acetonitrile–0.007 *M* TBAH (85:15); 5 = acetonitrile–0.07 *M* TBAH (85:15); 6 = acetonitrile–THF–0.071 *M* TBAH (85:1:14) (solvent system A). B, Reversed-phase TLC of primary B(*a*)P metabolites with sulfate and glucuronide conjugates in various solvents. Solvent systems: 7 = acetonitrile–methanol–water (50:25:25); 8 = acetonitrile–methanol–THF–water (50:25:1:24); 9 = acetonitrile–methanol–THF–water (50:25:2:23); 10 = acetonitrile–methanol–THF–0.004 *M* TBAH (50:25:2:23); 11 = acetonitrile–methanol–0.004 *M* TBAH (50:25:25); 12 = acetonitrile–methanol–0.04 *M* TBAH (50:25:25); 13 = acetonitrile–methanol–THF–0.004 *M* TBAH (50:25:1:24); 14 = acetonitrile–methanol–THF–0.04 *M* TBAH (50:25:1:24); 15 = acetonitrile–methanol–THF–0.04 *M* TBAH (65:10:1:24) (solvent system B). Metabolites: ● B(*a*)P; ○, quinones; △, 3-OH; ▲, 9-OH; ×, 7,8-dihydrodiol; □, 4,5-dihydrodiol; ■, 9,10-dihydrodiol; +, 3-SO_4; ○, 3-glucuronide.

Separation of conjugates

A great deal of attention has focused on the detoxification of B(*a*)P through conjugation. Thus, a system was developed to simultaneously identify the presence of sulfate and glucuronide conjugates. The use of acetonitrile–water solvent systems with a concentration of water of greater than 40% led to the TLC sorbent detaching from the glass; therefore, a mixture of acetonitrile–methanol–water was used. The solvent systems of acetonitrile–methanol or acetonitrile–methanol–THF did not yield

TABLE II

R_F VALUES OF MAJOR BENZO[a]PYRENE METABOLITES IN SOLVENT SYSTEM A

Metabolite	$\bar{X}^* \pm S.D.$
B(a)P	0.27 ± 0.03
1,6-quinone	0.37 ± 0.03
3,6-quinone	0.38 ± 0.03
6,12-quinone	0.38 ± 0.03
4,5-epoxide	0.45 ± 0.03
3-OH	0.50 ± 0.03
7-OH	0.52 ± 0.02
9-OH	0.53 ± 0.02
7,8-dihydrodiol	0.65 ± 0.04
4,5-dihydrodiol	0.73 ± 0.02
9,10-dihydrodiol	0.84 ± 0.03

* Mean value from eight different samples on separate TLC sheets.

adequate separation of the B(a)P metabolites in the quinone–phenol region, as shown in Fig. 1B. Addition of TBAH at 0.001 *M* was not much different from acetonitrile–methanol alone. Increasing the concentration of TBAH to 0.01 *M* led to a retardation of the quinones; however, the separation of 4,5-epoxide and 3-OH-B(a)P was not accomplished. A combination of acetonitrile–methanol–THF–TBAH was also used, as shown in Fig. 1B, solvents 13, 14, and 15. It appears that 0.001 *M* TBAH gives the best separation at lower organic solvent concentrations (50% acetonitrile and 25% methanol, compared to 85% acetonitrile). The R_F values obtained in solvent system B are shown in Table III.

TABLE III

R_F VALUES OF MAJOR BENZO[a]PYRENE METABOLITES IN SOLVENT SYSTEM B

Metabolite	$\bar{X}^* \pm S.D.$
B(a)P	0.13 ± 0.02
Quinones	0.22 ± 0.03
4,5-epoxide	0.25 ± 0.03
3-OH	0.27 ± 0.04
7-OH	0.28 ± 0.03
9-OH	0.30 ± 0.04
7,8-dihydrodiol	0.47 ± 0.04
4,5-dihydrodiol	0.53 ± 0.04
9,10-dihydrodiol	0.66 ± 0.04
3-SO_4	0.82 ± 0.04
3-glucuronide	0.86 ± 0.03

* Mean value from thirteen different samples.

Analytical application

Both solvent systems A and B were used to separate the metabolites of [^{3}H]-B(a)P. The results are shown in Figs. 2 and 3. Fig. 2 illustrates the separation of the B(a)P metabolites in solvent system A. The 3-OH- and 9-OH-B(a)P spots are not as well resolved due to the presence of other phenols (*i.e.* 7-OH-B(a)P). Fig. 3 illustrates

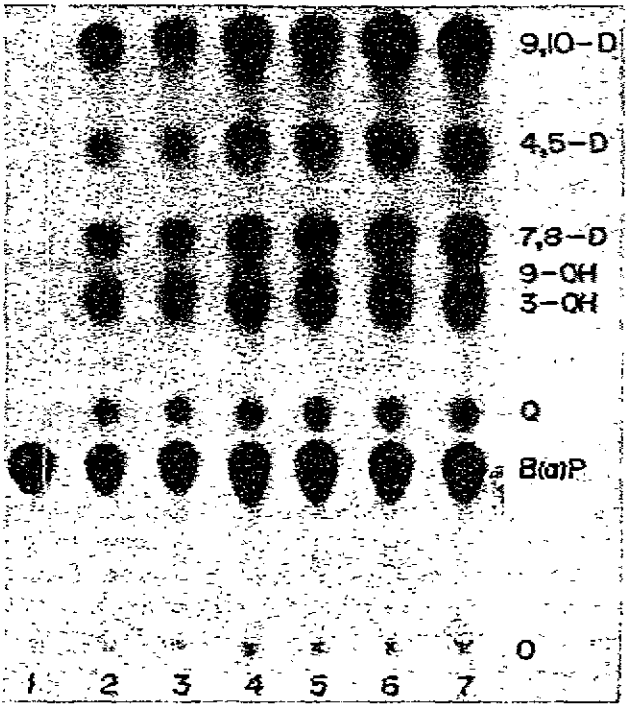

Fig. 2. Chromatography of [^{3}H]B(*a*)P metabolites in solvent system A. Autoradiogram of [^{3}H]B(*a*)P metabolites produced by rat liver S-9 in the presence of 2.5 m*M* UDPGA. Incubation times are 0 min (lane 1), 15 min (lanes 2, 3), 30 min (lanes 4, 5) and 60 min (lanes 6, 7). Autoradiography development was for 14 days. 9,10-D = 9,10-Dihydrodiol; 4,5-D = 4,5-dihydrodiol; 7,8-D = 7,8-dihydrodiol; 9-OH = 9-OH-B(*a*)P; 3-OH = 3-OH-B(*a*)P; Q = mixture of 1,6-, 3,6- and 6,12-quinones; B(*a*)P = benzo[*a*]pyrene; O = origin.

the separation of the B(*a*)P metabolites obtained with solvent B. Lanes 1, 3, 5, and 7 are from the incubation without UDPGA; lanes 2, 4, 6, and 8 were from the incubation with 2.5 m*M* UDPGA. Exposure time was 7 days for lanes 1, 3, 5, and 7, and 14 days for lanes 2, 4, 6, and 8. The glucuronide and sulfate conjugate spots were too faint to be observed, but they are visible following longer autoradiography times. Better resolution is obtained for the 3-OH- and 9-OH-B(*a*)P compared to solvent system A (Fig. 2).

Hydrolysis of the water soluble (glucuronide) conjugates was obtained with *E. coli* β-glucuronidase. Separation of these metabolites in solvent system B is shown in Fig. 4. As can be observed, the major conjugate is 3-OH-B(*a*)P, as previously observed by Nemoto and co-workers.[12,13] The large amount of quinones probably arise from the oxidation of 6-OH-B(*a*)P after hydrolysis. Other hydrolyzed conjugates are observed which migrate between the 7,8-dihydrodiol and 9-OH-B(*a*)P. Another spot is observed which migrates between the quinones and B(*a*)P.

Several chromatography systems have been developed for the quantitation of B(*a*)P and its metabolites. Early techniques include paper partition chromatography utilizing aqueous or organic solvents[14–16]. These techniques were unsatisfactory for resolving the large number of metabolites of B(*a*)P which have subsequently been

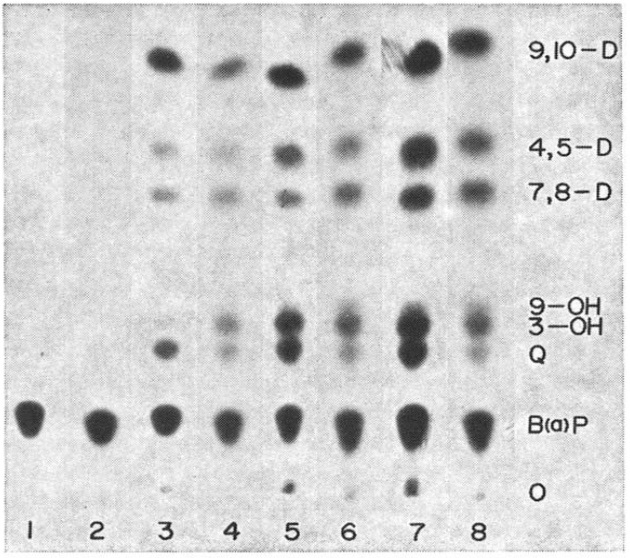

Fig. 3. Chromatography of [^{3}H]B(a)P metabolites in solvent system B. Autoradiogram of [^{3}H]B(a)P metabolites produced by rat liver S-9 in the absence of UDPGA (lanes 1, 3, 5, 7) or with 2.5 mM UDPGA (lanes 2, 4, 6, 8). Incubation times are 0 min (lanes 1, 2), 15 min (lanes 3, 4), 30 min (lanes 5, 6) and 60 min (lanes 7, 8). Autoradiographic exposure was for 7 days for the samples without UDPGA and 14 days for the samples with UDPGA.

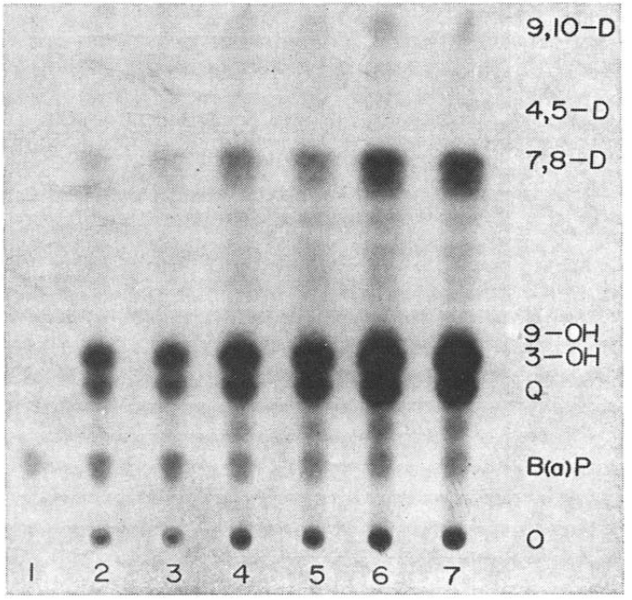

Fig. 4. Hydrolyzed glucuronide conjugates of [^{3}H]B(a)P chromatographed in solvent system B. Autoradiogram of hydrolyzed [^{3}H]B(a)P conjugates produced by rat liver S-9 in the presence of 2.5 mM UDPGA. Following incubation and extraction with ethyl acetate, the aqueous layer was digested with 500 μ/ml *E. coli* β-glucuronidase for 4 h then re-extracted with ethyl acetate as previously described. The evaporated samples were dissolved in 50 μl methanol and 15 μl were spotted in two different lanes, along with 5 μl of a mixture of unlabeled metabolite standards. Incubation times for the various lanes are identical to those in Fig. 2. Autoradiography development was for 14 days.

detected by HPLC[17]. In addition the paper chromatography takes several hours to complete and the R_F values are sensitive to moisture[18]. More recently, silica gel TLC has been used to separate the metabolites of B(*a*)P (including conjugates) based on their relative polarities[19,23]. Unfortunately, this technique does not adequately resolve the phenolic metabolites. The stability of B(*a*)P during silica gel chromatography is also doubtful[24,25]. Gas chromatography has been successfully used to separate various polycyclic aromatic hydrocarbons but this technique is inadequate for hydroxylated metabolites which are destroyed by pyrolysis during the vaporization stage. HPLC was thus the method of choice due to the rapid separation of the B(*a*)P metabolites, particularly phenols and quinones, the major metabolites in rat liver systems[26]. Reversed-phase TLC allows a more rapid analysis of samples, but the resolution is not as great as that obtained by HPLC. However, adequate resolution of sulfate and glucuronide conjugates of B(*a*)P metabolites has not been previously reported in a single chromatographic step. Thus reversed-phase TLC allows a more complete analysis of B(*a*)P metabolism with greater resolution of detoxification metabolites as well as activation metabolites of B(*a*)P.

In summary, a TLC procedure is described which can be utilized to separate both primary and secondary metabolites, including conjugates, of B(*a*)P. The separation of both sulfate and glucuronide conjugates is useful in that these are the primary means of detoxification of B(*a*)P[11–13,19–23,26].

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