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High-performance liquid chromatographic method for the determination of polycyclic hydrocarbon metabolite distributions

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ABSTRACT

Analytical separations and quantification of eight metabolites of benzo[*a*]pyrene, nine metabolites of 7-methylbenz[*c*]acridine and nine metabolites of dibenz[*a,j*]acridine have been carried out on two reversed-phase high-performance liquid chromatographic systems using 3- and 10- μm C₈ packings. Quantitative results for the distributions of primary metabolites for the two systems closely agreed and showed that up to 70% savings in chromatographic time could be achieved with the 3 μm particle size columns.

INTRODUCTION

Investigations aimed towards the identification of the mechanisms of initiation of cancer by polycyclic aromatic hydrocarbons have been abundantly published over the last twenty years or so since the unequivocal identification of naphthalene oxide as an intermediate in the mixed function oxidase-catalysed oxidation of naphthalene [1] and the proposal that a diolepoxide was the ultimate carcinogen of benzo[*a*]pyrene (BP) [2] (see Fig. 1). A key feature of these researches has often been the identification of metabolites and their quantification by high-performance liquid chromatography (HPLC) [3,4] or thin-layer chromatography [5]. Of these two techniques, HPLC has been much more extensively used, and the quantitative work has usually employed a radioisotopically labelled carcinogen. Recently published reviews on polycyclic aromatic hydrocarbon carcinogenesis [6,7] refer to signal papers on metabolic investigations using HPLC. Other analytical methods measure the formation of fluorescent phenols of BP [8] or total metabolites using the very different polarities and partition coefficients of metabolites and substrate between hexane and aqueous alkaline dimethylsulphoxide [9,10].

A problem frequently encountered in the HPLC methods is the prolonged time

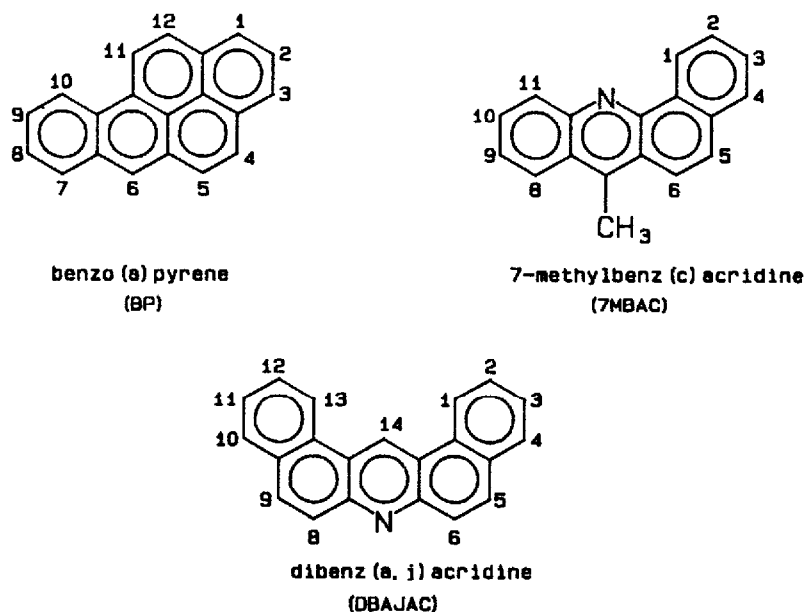


Fig. 1. Structures of benzo[*a*]pyrene, 7-methylbenz[*c*]acridine and dibenz[*a,j*]acridine.

taken to separate the dihydrodiols, phenols and other oxidized metabolites from unchanged substrate on the reversed-phase HPLC columns employed. The time taken for typical separations is 60–120 min, and this includes a wash-out procedure which allows satisfactory subsequent separations of metabolites. In the present work the use of 3- μ m short columns allowed comparable separations and quantitations of metabolite distributions in 50–70% shorter times. Data are presented for BP, 7-methylbenz[*c*]acridine (7MBAC) and dibenz[*a,j*]acridine (DBAJAC) (Fig. 1).

EXPERIMENTAL

[¹⁴C]BP was obtained from Amersham International (Amersham, U.K.) while [³H]7MBAC [11] and [³H]DBAJAC [12] were available from previous studies. Synthetic marker compounds were either purchased from the NCI Chemical Carcinogen Repository (Bethesda, MD, U.S.A.) or synthesized previously [13,14]. The compounds available are shown in the captions to Figs. 2–4.

Rat liver microsomal suspensions from either untreated animals or 3-methylcholanthrene-pretreated animals were prepared as described [15] and used to convert radioactive BP [16], 7MBAC [17] and DBAJAC [15] to mixtures of metabolites in the presence of air and NADPH. The metabolites were extracted from 1.0-ml incubation mixtures which contained initially BP (40 μ M), 7MBAC (40 μ M) or DBAJAC (65 μ M), with ethyl acetate (3 \times 1.5 ml). Appropriate synthetic

marker compounds were added to the ethyl acetate extract which was then evaporated to dryness at $<30^{\circ}\text{C}$, and the residue was dissolved in dimethylformamide (20 μl) and analysed by reversed-phase HPLC.

Two Beckman Altex HPLC pumps, Model 110A, were controlled with an Altex 421 controller, and samples were introduced with an ICI Kortec automatic injector. A Joel Uvidec V ultraviolet detector was used at 254 nm for BP metabolites and at 275 nm for 7MBAC and DBAJAC metabolites.

Two separation systems were compared for the three substrates: system 1 separations were performed on a 10- μm Hibar RP-8 column (Merck, Darmstadt, F.R.G., 25 cm \times 0.46 cm I.D.) with a flow-rate of 1.0 ml/min while system 2 separations were performed on a 3- μm velosep RP-8 column (Brownlee Labs., Santa Clara, CA, U.S.A.; 10 cm \times 0.32 cm I.D.) fitted with a 7- μm RP-8 precolumn (Brownlee Labs.; 1.5 cm \times 0.32 cm I.D.), and a flow-rate of 0.5 ml/min was used. For both systems multi-step linear water-methanol gradients were necessary to separate metabolites from each other and the parent substrate. For BP ambient temperature was used for HPLC while for the other substrates an HPLC column oven temperature of 40°C was used.

For radiochemical quantitation of the metabolite distributions 1-min fractions of eluent were collected and assayed for HPLC system 1 separations, and ten-drop fractions were collected for system 2 separations.

System 1 HPLC gradients

BP. 40–55% methanol over 15 min, then isocratic for 25 min, 55–68% methanol over 2 min, isocratic for 20 min and 68–100% methanol over 2 min. Column eluent fractions were collected for a further 16 min giving a total of 80 fractions.

7MBAC. 20–35% methanol over 15 min, then 35–70% methanol over 70 min, 70–100% methanol over 5 min and a further 10 min at 100% methanol giving a total of 100 column eluent fractions.

DBAJAC. 28–52% methanol over 40 min, then isocratic for 15 min, 52–60% methanol over 10 min, isocratic for 20 min, 60–100% methanol over 5 min and a further 10 min a 100% methanol giving a total of 100 column eluent fractions.

System 2 HPLC gradients

BP. 40–52% methanol over 7 min, isocratic for 3 min, 52–68% methanol over 20 min, 68–100% methanol over 1 min and isocratic for a further 13 min giving a total of 44 min of column eluent collection.

7MBAC. 20–60% methanol over 12 min, isocratic for 3 min, 60–72% methanol over 2 min, isocratic for 8 min, 72–100% methanol over 1 min and isocratic for 10 min giving a total of 36 min of column eluent collection.

DBAJAC. 28–48% methanol over 7 min, 48–60% methanol over 9 min, isocratic for 7 min, 60–68% methanol over 5 min, 68–100% methanol over 10 min giving a total of 38 min of column eluent collection.

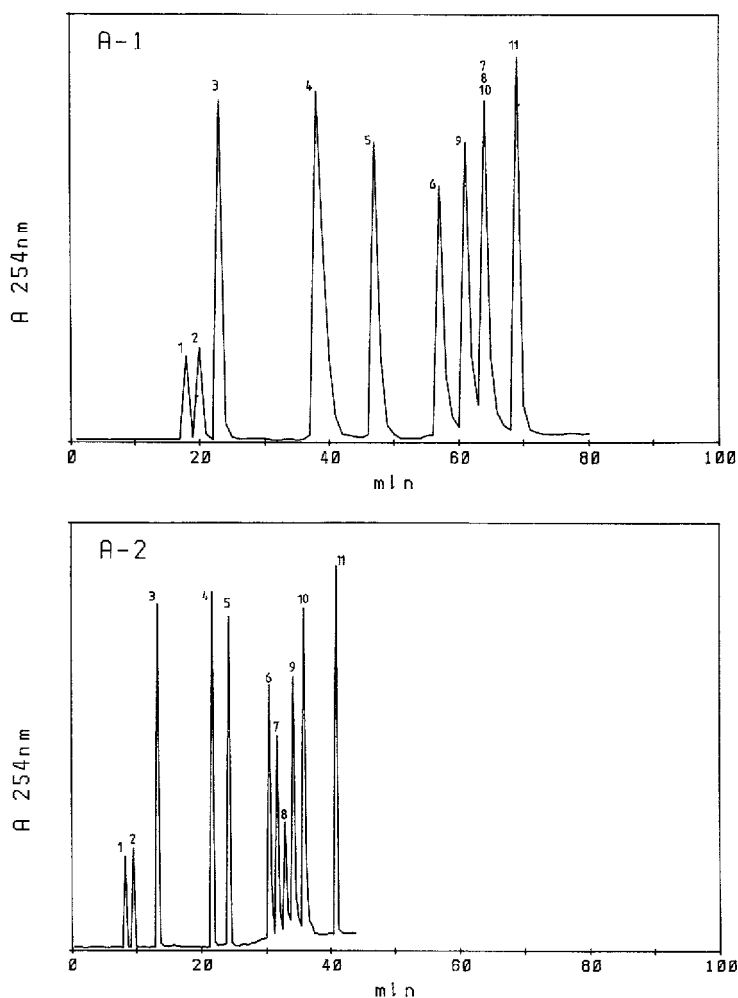


Fig. 2. Reversed-phase HPLC separations of mammalian liver metabolites of BP (A-1, A-2) on 10 μm particle size columns (A-1, system 1) and on 3 μm particle size columns (A-2, system 2). BP metabolites are: 1 = BP-r-7,t-8,9,c-10-tetraol; 2 = BP-r-7,t-8,9,10-tetraol; 3 = *trans*-9,10-dihydro-9,10-dihydroxy-BP (BP-9,10-DHD); 4 = *trans*-4,5-dihydro-4,5-dihydroxy-BP (BP-4,5-DHD); 5 = *trans*-7,8-dihydro-7,8-dihydroxy-BP (BP-7,8-DHD); 6 = BP-1,6-quinone (BP-1,6-Q); 7 = BP-3,6-quinone (BP-3,6-Q); 8 = BP-6,12-quinone (BP-6,12-Q); 9 = 9-hydroxy-BP (9-HOBP); 10 = 3-hydroxy-BP (3-HOBP); 11 = BP.

RESULTS AND DISCUSSION

A comparison of the two chromatographic separation systems for the three polycyclic aromatic hydrocarbon substrates is shown in Figs. 2–4. Two of the substrates, 7MBAC and DBAJAC, are aza-aromatic compounds. In each case synthetic marker compounds have been added to extracts of incubation mixtures to aid quantitation by the radiochemical assay, and the comparative separations

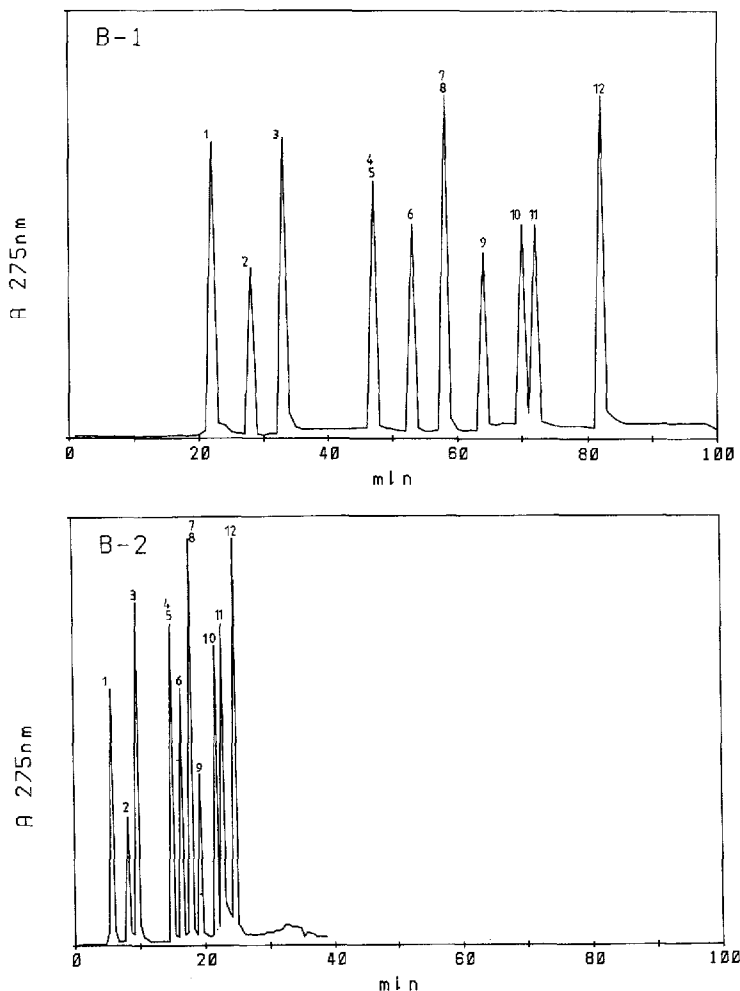


Fig. 3. Reversed-phase HPLC separations of mammalian liver metabolites of 7MBAC (B-1, B-2) on 10 μm particle size columns (B-1, system 1) and on 3 μm particle size columns (B-2, system 2). 7MBAC metabolites are: 1 = *trans*-5,6-dihydro-5,6-dihydroxy-7MBAC (7MBAC-5,6-DHD); 2 = 7MBAC-r-1,t,2,3,c-4-tetraol; 3 = *trans*-8,9-dihydro-8,9-dihydroxy-7MBAC (7MBAC-8,9-DHD); 4 = *trans*-3,4-dihydro-3,4-dihydroxy-7MBAC (7MBAC-3,4-DHD); 5 = 7-hydroxymethyl-BAC-5,6-oxide (7-HOMBAC-5,6-oxide); 6 = *trans*-1,2-dihydro-1,2-dihydroxy-7MBAC; 7 = *trans*-10,11-dihydro-10,11-dihydroxy-7MBAC (7MBAC-10,11-DHD); 8 = 7-hydroxymethyl-BAC (7-HOMBAC); 9 = 7MBAC-5,6-oxide; 10 = 5-hydroxy-7MBAC; 11 = 9-hydroxy-7MBAC; 12 = 7MBAC.

and time required for adequate separation to allow quantitations can be compared. It is clear that for these carcinogens comparable or superior separations of closely related metabolites were achieved with the 3- μm column of Velosep RP-8 to those that were obtained with the 10- μm reversed-phase columns. In system 2 separations, unchanged BP, 7MBAC and DBAJAC eluted from the column at

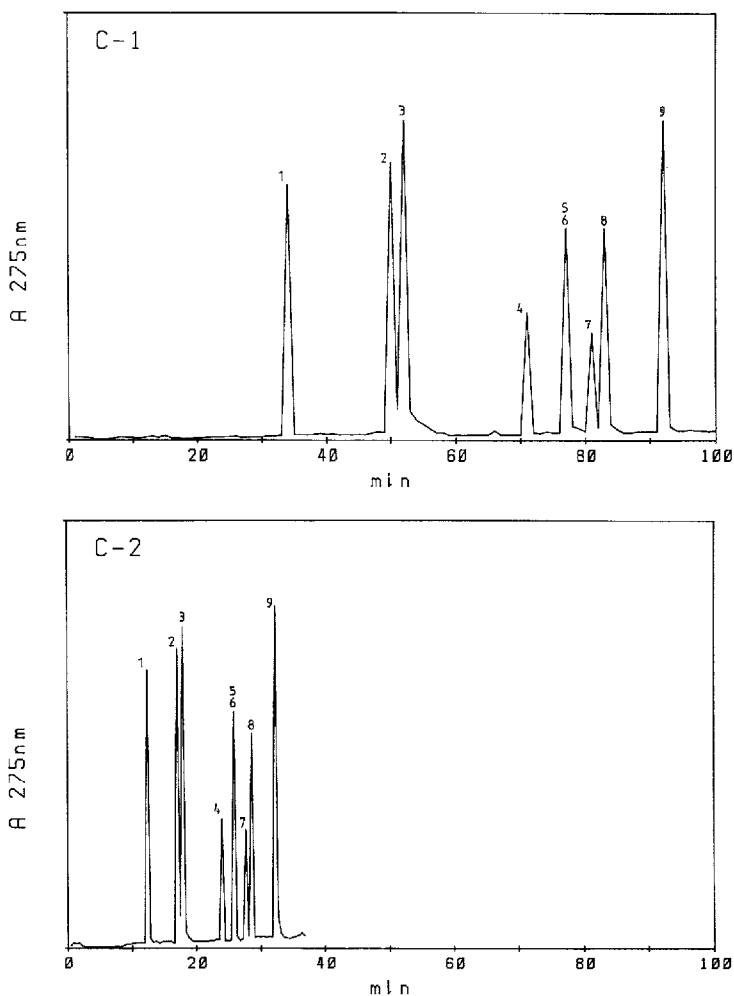


Fig. 4. Reversed-phase HPLC separations of mammalian liver metabolites of DBAJAC (C-1, C-2) on 10 μm particle size columns (C-1, system 1) and on 3 μm particle size columns (C-2, system 2). DBAJAC metabolites are: 1 = *trans*-1,2-dihydro-1,2-dihydroxy-DBAJAC; 2 = *trans*-5,6-dihydro-5,6-dihydroxy-DBAJAC (DBAJAC-5,6-DHD); 3 = *trans*-3,4-dihydro-3,4-dihydroxy-DBAJAC (DBAJAC-3,4-DHD); 4 = DBAJAC-14-one; 5 = DBAJAC-5,6-oxide; 6 = DBAJAC N-oxide; 7 = 3-hydroxy-DBAJAC (3-HODBAJAC); 8 = 4-hydroxy-DBAJAC (4-HODBAJAC); 9 = DBAJAC.

42, 26 and 33 min, respectively. For BP, the lower temperature of separation was employed although peak symmetries were poorer, and some separations were inferior or absent on system 1 compared to those obtained at 40°C. This was chosen because the quantitative data for BP metabolite profiles obtained at higher temperatures resulted in a slightly increased proportion of quinones compared to phenols. Such an artifact of the chromatography probably occurs through

oxidation of labile metabolites by solute oxygen during chromatography at the higher temperatures. For BP system 2 was superior; the three quinones and two phenols were separated from each other sufficiently to allow estimation of every metabolite.

Tables I–III show quantitative comparisons of data collected for incubations of rat liver microsomes from 3-methylcholanthrene-pretreated rats with the three carcinogenic substrates. The data are presented as metabolite profiles and total catalytic activities of the liver microsomal fractions determined using radioactivity emerging from the column before the unchanged substrate as the measure of total metabolites. Because the BP metabolites behaved differently to each other on the two chromatographic systems, comparisons of quantitative data can only be done with the three dihydrodiols, 9-hydroxybenzo[*a*]pyrene, benzo[*a*]pyrene-1,6-quinone, and the total catalytic activity. The close agreement of these data (Table I) indicates that the 3 μ m particle size column offers adequate distribution data. Similarly, the data for 7MBAC and DBAJAC, shown in Tables II and III, respectively, indicate that for quantitative purposes the two systems are virtually identical. 7MBAC and DBAJAC were also metabolized in incubations with rat liver microsomes from untreated animals, and the metabolite profiles were obtained on both analytical systems for two subsets of replicate incubations.

TABLE I

COMPARISON OF DISTRIBUTIONS OF [¹⁴C]BENZO[*a*]PYRENE METABOLITES FORMED BY 3-METHYLCHOLANTHRENE INDUCED RAT LIVER MICROSOMES DETERMINED BY HPLC AT 23°C

Metabolite ^a	Distribution ^b (%)	
	System 1 ^c	System 2 ^c
Tetraol(s)	1.4	1.0
BP-9,10-DHD	17.8	15.8
BP-4,5-DHD	8.0	7.9
BP-7,8-DHD	12.1	9.9
BP-1,6-Q	11.8	12.2
BP-3,6-Q	— ^d	12.5
BP-6,12-Q	— ^d	10.8
9-HOBP	14.1	10.8
3-HOBP	26.9 ^d	8.8
Total catalytic activity ^e	5.48	5.94

^a Metabolite abbreviations are shown in the caption to Fig. 2.

^b The distributions were expressed as percentages of total radioactivity eluted before unchanged substrate.

^c Means of two analyses for four replicate incubations.

^d BP-3,6-Q and BP-6,12-Q cochromatography with 3-HOBP in system 1.

^e Expressed as nmol BP metabolised per mg protein per min.

TABLE II

COMPARISON OF DISTRIBUTIONS OF [³H]7MBAC METABOLITES FORMED BY 3-METHYLCHOLANTHRENE INDUCED RAT LIVER MICROSOMES DETERMINED BY HPLC AT 40°C

Metabolite ^a	Distribution ^b (%)	
	System 1 ^c	System 2 ^c
7MBAC-5,6-DHD	7.8 ± 0.3	7.1 ± 0.3
7MBAC-8,9-DHD	48.0 ± 0.6	48.3 ± 1.4
7MBAC-DHD-N-oxide ^d	4.0 ± 0.2	3.9 ± 0.2
7MBAC-3,4-DHD and 7-HOMBAC-5,6-oxide ^e	2.5 ± 0.8	2.2 ± 0.2
7MBAC-10,11-DHD and 7-HOMBAC ^f	10.8 ± 0.1	11.4 ± 0.1
7MBAC-5,6-oxide	6.9 ± 0.2	7.9 ± 2.5
7MBAC phenols	15.3 ± 0.7	12.5 ± 3.3
Total catalytic activity ^g	3.73 ± 0.05	3.54 ± 0.04

^a Metabolite abbreviations are shown in the caption to Fig. 3.

^b See footnote *b* in Table I.

^c Six replicate incubations were analysed, three on each system, and the results shown are means ± S.D.

^d Not available as a synthetic standard.

^e Cochromatographing metabolites; previous analyses have shown that about equal amounts of each are formed [23].

^f Cochromatographing metabolites; by synchronous luminescence it was previously shown that 7-HOMBAC is the predominant metabolite [24].

^g Expressed as nmol 7-MBAC metabolised per mg protein per min.

Again, the results (data not shown) were in very close agreement for both systems. This again validated the use of the shorter 3- μ m column.

It is clear that the 3- μ m reversed-phase columns used in the present study offer a considerable advantage in terms of time, and for BP separation and resolution. Three times the number of samples can be analysed each day and the quantitative data are not compromised. Operating pressures are of course considerably higher, but with the reduced flow-rate and the use of the 7- μ m short precolumn the operating life of the 3- μ m columns is greater than nine months. These quite considerable time and resolution advantages indicate that these columns deserve to be the analytical standard in this type of work.

A previous study of the reversed-phase separation of DNA adducts of the two racemic diol epoxides of BP similarly showed that separations achievable were dependent not only on the column type but also on the column manufacturer, the best results being obtained with an Ultrasphere ODS 5- μ m column and a methanol-water gradient over 60 min [18]. Typical recent studies have employed 5- and 10- μ m reversed-phase columns for the separation of metabolites of benzo[*k*]fluoranthene and its fluorinated derivatives [19], 7-ethyl- and 7-methylbenzo[*a*]anthracene [20], 6-fluorobenzo[*c*]phenanthrene [21] and 6-halobenzo[*a*]pyrenes [22], and

TABLE III

COMPARISON OF DISTRIBUTIONS OF [³H]DBAJAC METABOLITES FORMED BY 3-METHYLCHOLANTHRENE INDUCED RAT LIVER MICROSOMES DETERMINED BY HPLC AT 40°C

Metabolite ^a	Distribution ^b (%)	
	System 1 ^c	System 2 ^c
Tetraol (s)	1.2 ± 0.1	1.7 ± 0.4
Secondary metabo ^s of DBAJAC-5,6-DHD and DBAJAC-3,4-DHD ^d	7.6 ± 0.3	7.2 ± 0.3
DBAJAC-5,6-DHD	15.3 ± 2.1	15.9 ± 3.2
DBAJAC-3,4-DHD	35.5 ± 1.2	34.0 ± 1.9
Phenol of DBAJAC-5,6-oxide ^d	1.9 ± 0.2	1.5 ± 0.4
Unknown	1.0 ± 0.1	0.7 ± 0.2
DBAJAC-5,6-oxide and DBAJAC-N-oxide ^e	23.4 ± 0.6	20.3 ± 1.0
3-HODBAJAC	1.1 ± 0.1	1.6 ± 0.2
4-HODBAJAC	4.9 ± 0.3	6.2 ± 0.9
Total catalytic activity ^f	5.34 ± 0.15	5.91 ± 0.17

^a Metabolite abbreviations are shown in the caption to Fig. 4.

^b See footnote *b* in Table I.

^c Six replicate incubations were analysed, three on each system, and the results shown are means ± S.D.

^d These metabolite standards were not available; identifications are based on relative retention times compared to standards available [15].

^e The N-oxide has been identified as a metabolite only in liver microsomal incubations with phenobarbital-induced preparations [15]; this fraction is comprised predominantly of the epoxide.

^f Expressed as nmol DBAJAC metabolised per mg protein per min.

either prolonged retention times or poor resolution of analytes was observed. The use of the 3- μ m columns described herein would allow considerable improvements.

The distributions of liver microsomal metabolites reported herein were broadly consistent with previously obtained data which have been published for BP [4,16], 7MBAC [23] and DBAJAC [15]. Minor differences were the increased proportion of *trans*-5,6-dihydro-5,6-dihydroxy-DBAJAC in the present study of DBAJAC oxidation. For 7MBAC the proportion of the 8,9-dihydrodiol reached 48% in the present work compared with 36% previously measured, and the *trans*-7-methylbenz[*c*]acridine-3,4-dihydrodiol and 7-hydroxymethylbenz[*c*]acridine-5,6-oxide fraction was decreased. For BP the results obtained with system 2 contrast with the literature insofar as the proportion of phenols is reduced while that of the quinones is increased. The proportion of dihydrodiols agrees with literature values for liver microsomal metabolism [4,16].

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