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THIN-LAYER CHROMATOGRAPHIC AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF METABOLITES OF THE WEAK CARCINOGEN, 7-METHYLBENZ[*c*]ACRIDINE

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SUMMARY

The metabolism of [^{14}C]7-methylbenz[*c*]acridine, a weakly carcinogenic polycyclic aza aromatic hydrocarbon, was studied in rats in vivo, and in rat hepatocytes and hepatic microsomes. Evidence for oxidation of the methyl group and excretion of 7-hydroxymethylbenz[*c*]acridine and benz[*c*]acridine-7-carboxylic acid in rat bile was obtained by thin-layer chromatography. A reversed-phase high-performance liquid chromatographic separation of metabolites was developed and chromatographic profiles of metabolites found in vivo and formed in vitro are presented. Several synthetic potential oxidation products were used to characterise the chromatographic profiles and evidence for the in vitro formation of 7-hydroxymethylbenz[*c*]acridine and 5,6-dihydro-5,6-dihydroxy-7-methylbenz[*c*]acridine was obtained.

INTRODUCTION

Studies with polycyclic aromatic hydrocarbons (PAH) such as benzo[*a*]-pyrene (BP) and 7-methylbenz[*a*]anthracene have indicated that metabolites of these substances, rather than the parent compound are responsible for their observed mutagenic and carcinogenic effect [1—3]. Many aza aromatic compounds are found as tobacco smoke and tar constituents [4] and occur as environmental air pollutants [5—7], and some of these have been shown to be tumorigenic [8] and mutagenic [9,10]. We are engaged in the study of the metabolism and mode of action of these compounds and have subjected 7-methylbenz[*c*]acridine to intensive examination. For metabolic studies on the PAH thin-layer chromatography (TLC) [11—13], gas chromatography (GC) [14—16], and high-performance liquid chromatography (HPLC) [17,18] have all been used. TLC techniques have the disadvantage that separation of isomeric dihydrodiols and phenols of PAH is usually poor, and GC techniques used with dihydrodiol and arene oxides may result in chemical alteration despite initial

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derivatization. HPLC systems used for PAH metabolites comprise reversed-phase octadecylsilane stationary phases with methanol—water or acetonitrile—water gradients as eluents. Amongst PAH metabolites examined are those of 3-methylcholanthrene [19], 7,12-dimethylbenz[*a*]anthracene [20], benz[*a*]-anthracene [21], dibenz[*a*,*h*]anthracene [22], 5-methylchrysene [23] and BP [17,18]. We report herein an examination of the metabolites of 7-methylbenz[*c*]acridine (7MBAC) formed by the rat in vivo or in vitro by HPLC using a reversed-phase gradient.

EXPERIMENTAL

Chemicals

[^{14}C]7-Methylbenz[*c*]acridine [24], *trans*-5,6-dihydroxy-5,6-dihydro-7-methylbenz[*c*]acridine, 5-hydroxy-7-methylbenz[*c*]acridine, 7-methylbenz[*c*]-acridine-5,6-oxide [25], 7-hydroxymethylbenz[*c*]acridine [26] and benz[*c*]-acridine-7-carboxylic acid [27] were synthesised according to literature methods. 9-Hydroxy-7-methylbenz[*c*]acridine and 11-hydroxy-7-methylbenz-[*c*]acridine were prepared in this laboratory [28].

Glucose-6-phosphate, glucose-6-phosphate dehydrogenase, NADP and β-glucuronidase containing arylsulphatase (from *Helix pomatia*) were purchased from Sigma (St. Louis, MO, U.S.A.). Penicillin, streptomycin, foetal calf serum, tryptose phosphate broth and Leibovitz L-15 medium were purchased from Flow Laboratories (Sydney, Australia).

Chromatography

Reversed-phase HPLC. An Altex Model 334 gradient liquid chromatograph fitted with 10-μm RP-8 column (25 cm × 4.6 mm I.D.; Browlee Labs., Santa Clara, CA, U.S.A.) was used with a 3.5 cm × 4.6 mm precolumn of Corasil C_{18} Bondapak (37 μm, Waters Assoc., Milford, MA, U.S.A.). The column system was enclosed within a water jacket at 40.0 ± 0.1°C, and was eluted with a methanol—water gradient comprised of three sequential linear ramps. They were (1) 20—30% methanol over 15 min, (2) 35—70% methanol over 70 min and (3) 70—100% methanol over 5 min. A flow-rate of 1.2 ml min^{-1} and UV detection at 254 nm were used. One-minute fractions were collected and assayed for radioactivity by liquid scintillation counting in ACS cocktail (10 ml, Amersham Australia, Sydney, Australia).

Thin-layer chromatography. This was conducted using 0.25-mm Merck Kieselgel GF_{254} plates with visualisation by fluorescence or fluorescence quenching. The silica gel was scraped at 1-cm intervals and radioactivity was determined to confirm the presence of metabolites. Alternatively a Nuclear Chicago Actigraph III was used for radiochromatogram scans.

Gas chromatography—mass spectrometry. A Finnigan 9500 gas chromatograph interfaced with a Finnigan 3200 mass spectrometer and Finnigan 6100 data system was used. Extracts were derivatized with Trisil (Pierce, Rockford, IL, U.S.A.) for 1 h at 60°C before GC. For acidic fractions the temperature program after 2 min at 200°C was 10°C min^{-1} to 280°C while for neutral fractions a gradient of 4°C min^{-1} was used. Gas-Chrom Q (100—120 mesh) with 3%

OV-101 and a methane [for chemical ionization mass spectrometry (CIMS)] flow-rate of 20 ml min^{-1} were used.

Microsomal metabolism

Hepatic microsomes were prepared from male Wistar rats (100—150 g) according to a published method [29]. Incubation mixtures (3 ml) contained magnesium chloride (9 μmol), NADP (1.5 μmol), glucose-6-phosphate (12.5 μmol), glucose-6-phosphate dehydrogenase (1 unit), microsomal protein (0.2 mg/ml), potassium phosphate buffer, pH 7.4 (300 μmol) and [^{14}C]-7MBAC (200 nmol). Incubations were carried out in diffuse light at 37°C under air with constant shaking for 10 min. The reaction was stopped by cooling the incubation flasks in ice. The pooled contents of four flasks were extracted with ethyl acetate (3 volumes). The extract was treated with anhydrous sodium sulphate and the solvent evaporated under reduced pressure. The residue was dissolved in a methanolic solution of the available synthetic standards and analysed by HPLC.

Isolated hepatocyte metabolism

Isolated hepatocytes were prepared by slight modifications of the method of Jones et al. [30]. Washed liver slices from three male 50-day-old Wistar rats were digested with collagenase—hyaluronidase (0.05%:0.06%) in Hank's buffered salt solution (lacking calcium and magnesium salts) containing 20 m*M* HEPES buffer (pH 7.4) for 90 min at 37°C. The isolated hepatocytes were washed twice in Hank's solution before resuspending in culture medium.

Incubation mixtures (10 ml) contained $1.2 \cdot 10^7$ hepatocytes, 100 μ*M* [^{14}C]-7MBAC and 0.2% v/v dimethylformamide. Mixtures were incubated for 20 min at 37°C in diffuse light, under air, then centrifuged (15 sec, 500 *g*) to separate the cells from the incubation medium. The cells were homogenised in a Potter Elvejhem mortar and portions of both the incubation medium and the cell homogenate were extracted three times with ethyl acetate. Pooled extracts were treated as above for microsomes.

In vivo metabolism

Male Wistar rats (250—300 g), anaesthetised with urethane (1.25 g/kg), were cannulated at the bile duct and injected via the femoral vein with [^{14}C]-7MBAC (2 mg/kg) dissolved in 20% dimethylsulphoxide (DMSO)—water. Bile was collected for 6 h and then hydrolysed with β-glucuronidase at 37°C under nitrogen for 18 h. The bile was adjusted to pH 2.75 with concentrated hydrochloric acid and the solution was extracted with ethyl acetate (2 × 3 volumes). The ethyl acetate extracts were treated with anhydrous sodium sulphate and the solvent evaporated under reduced pressure after marker amounts of synthetic standards were added where appropriate. The residue was redissolved in ethyl acetate or DMSO and aliquots were analysed by TLC or HPLC respectively.

RESULTS AND DISCUSSION

Preliminary experiments with TLC of the rat bile extracts indicated that

TABLE I

BILIARY 7MBAC METABOLITES IDENTIFIED BY TLC

Solvent system	R_F standard	R_F metabolite	Percentage of dose*
7-Hydroxymethylbenz[c]acridine			
Methanol—toluene (1:9)	0.42	0.42	4.6
Ethyl acetate—hexane (1:3)	0.23	0.23	
Acetone—hexane (7:13)	0.42	0.42	
Benz[c]acridine-7-carboxylic acid			
Methanol—chloroform (3:7)	0.31	0.31	6.5
Acetic acid—ethanol—ethyl acetate (1:12:19)	0.50	0.50	
Ethanol—chloroform (2:3)	0.40	0.40	

*Excreted in 5-h bile after two 1-mg doses of [^{14}C]-7MBAC and determined by integration of radiochemical TLC scans.

both the alcohol, 7-hydroxymethylbenz[c]acridine (7-hydroxy MBAC), and benz[c]acridine-7-carboxylic acid were present in amounts of about 4—6% of an intravenous dose of 7MBAC in a neutral and acidic fraction respectively of the ethyl acetate extract. This conclusion was supported by cochromatography of marker compounds with radioactivity in three systems (Table I) and the proportions were determined by integration of radiochemical scans. Regions of the thin-layer chromatograms corresponding to the alcohol and the carboxylic acid were examined by gas chromatography—mass spectrometry (GC—MS) after derivatization as the trimethylsilyl ether and ester respectively. For the acidic metabolite a small peak emerged from the column with a retention time of 8.90 min, corresponding to that of the trimethylsilyl ester of the authentic carboxylic acid. The methane CI mass spectra of the derivatized metabolite and acid were in close agreement. Ions present were found at *m/e* 346 (100%, the base peak, quasimolecular ion, MH^+); 374 (20%, $M + C_2H_5^+$); 330 (11%, $MH^+ - CH_4$); 256 (12%, MH^+ — trimethylsilanol); and 230 (18%, 256 $- C_2H_2$). These fragmentation pathways from the protonated molecular ion or quasimolecular ion, MH^+, have not been proven by accurate molecular weight determinations since the spectrometer available was a low-resolution instrument. They are, however, reasonable postulates based on the expected loss of a neutral molecule from an even electron ion [31]. For the neutral metabolite a peak was not clearly visible in the gas chromatogram (Fig. 1) but selected ion monitoring at *m/e* 332, 360 and 316 showed pronounced peaks at a retention time of 7.30 min. The ions monitored were chosen to correspond to the quasimolecular ion MH^+, $M + C_2H_5^+$ and $MH^+ - CH_4$, respectively, of the trimethylsilyl derivative of 7-hydroxy MBAC, and the identity of the 7.30-min peak as the alcohol derivative was confirmed by its mass spectrum. Close correspondence of the metabolite derivative spectrum with that of authentic 7-hydroxy MBAC derivative was seen. Ions were present at *m/e* 360 (26%); 332 (100%); 316 (22%); 270 (22%, $M + C_2H_5^+$ — trimethylsilanol); and 242 (89%, MH^+ — trimethylsilanol). Other peaks seen in the limited mass scans probably correspond

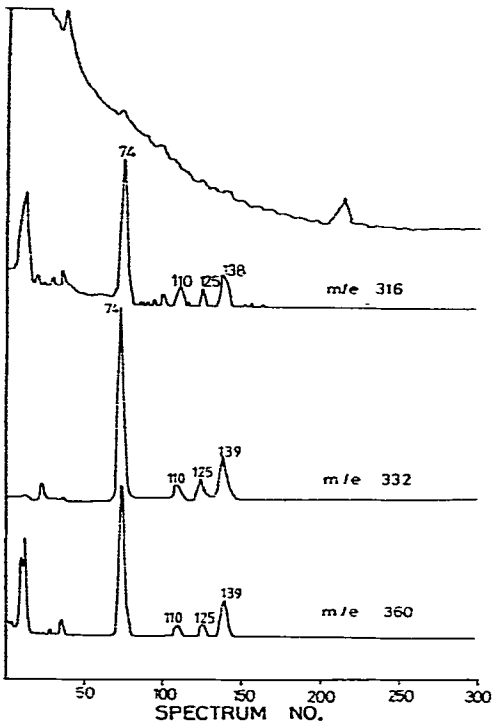
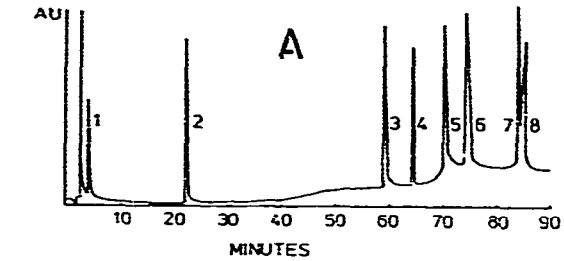


Fig. 1. Selected ion monitoring of neutral bile metabolites extracted from TLC plates in region corresponding to 7-hydroxymethylbenz[*c*]acridine standard.

to phenol derivatives which cochromatographed with 7-hydroxy MBAC on TLC. Extracts of the earlier TLC fractions (lower R_F values) only gave peaks on GC—MS selection ion scans at *m/e* values of 332 and 316 suggesting that possible dihydrodihydroxytrimethylsilyl derivatives of 7MBAC had decomposed by loss of trimethylsilanol during GC [16] or fragmented normally during MS examination. The available K region dihydrodiol, however, behaved normally on GC after trimethylsilylation and gave a single GC peak with ions at values expected for a bis-trimethylsilyl ether derivative at *m/e* 450, (20%, $M + C_2H_5^+$); 422 (100%, MH^+); 406 (80%, $MH^+ - CH_4$); 360 (15%, $M + C_2H_5^+$ — trimethylsilanol); and 232 (90%, MH^+ — trimethylsilanol). Efforts to analyse liver microsomal metabolites by TLC and GC—MS similarly gave evidence for the formation of several dihydrodiols by the appearance of *m/e* peaks at 332 presumably by loss of trimethylsilanol from bis-trimethylsilyl derivatives.

The limited ability of TLC to separate the metabolites from 7MBAC led us to examine HPLC as an analytical method. Using the standards synthetically available a gradient was developed which offered the possibility of analysing for the carboxylic acid in the presence of the essentially neutral dihydrodiols and phenols (Fig. 2A). The separation of the 5- and 9-phenols (peaks 5 and 6) was increased very considerably by the use of a higher temperature (40°C) for the analytical column. At ambient temperatures (22—28°C) the two peaks were incompletely separated, and the retention times of all the standards were more variable. The gradient separation has the disadvantage that the 11-phenol emerges from the column after the unchanged hydrocarbon and is incompletely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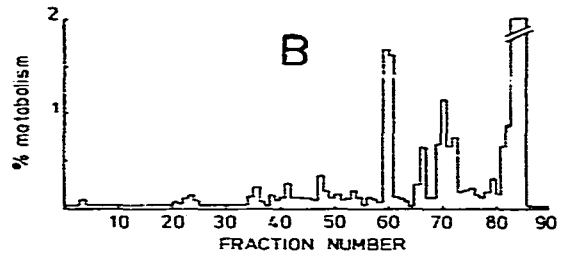
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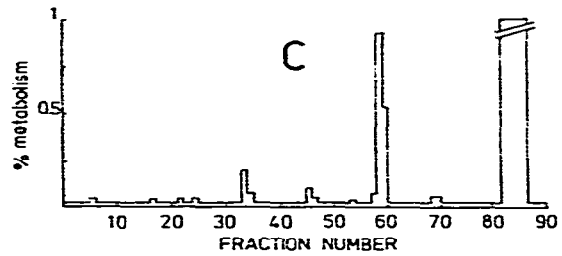
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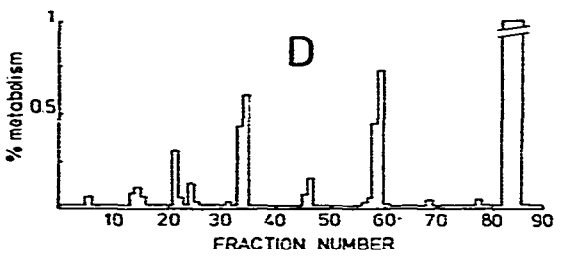
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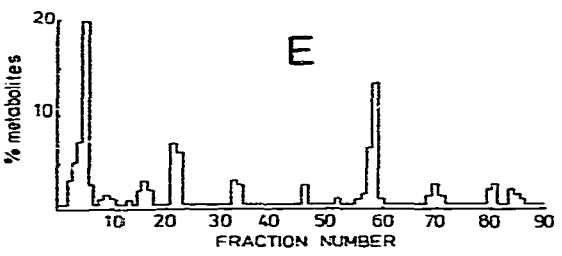
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separated from it. Consequently this method cannot be used to identify and determine 11-hydroxy-7-methylbenz[*c*]acridine as a 7MBAC metabolite where unchanged parent hydrocarbon is present (Fig. 2B, C and D).

The radiochemical histograms (Fig. 2B—E) illustrate the value of this method to separate 7MBAC metabolites. Liver microsomes (Fig. 2B) convert the aza hydrocarbon principally to metabolites cochromatographing with the 7-hydroxy MBAC, and other radioactivity was present in regions corresponding to the 5,6-dihydrodiol and phenols. When hepatocytes were used to metabolise 7MBAC, the profiles of the metabolites inside and outside the cell (Fig. 2C and D, respectively) were different. 7-Hydroxy MBAC appeared to be the major metabolite inside, while outside considerable amounts of more polar metabolites were found. Amongst these, the 5,6-dihydrodiol and possibly benz[*c*]-acridine-7-carboxylic acid were present. Phenolic type metabolites were almost absent. When bile was collected from rats which had received intravenous [^{14}C]7MBAC, and treated with β-glucuronidase and arylsulphatase to cleave conjugates, the metabolites obtained gave substantially different HPLC profiles (Fig. 2E). The parent compound at a retention time of 84 min was absent as would be expected, and there was some resemblance to the profile of hepatocyte 7MBAC metabolites found outside the cells (Fig. 2D), especially in the 10—60-min region of the chromatogram. The presence of the 7-hydroxy MBAC and benz[*c*]acridine-7-carboxylic acid as major metabolites is clear and the 5,6-dihydrodiol at a retention time of 22 min was also found. Only small amounts of phenols were present. In all profiles there were unidentified peaks of radioactivity which emerged from the column with retention times less than 60 min (the retention time of the alcohol).

The metabolite peaks corresponding to the alcohol and K-diol obtained from microsomes of rats have mass spectra in agreement with these assignments. Others, for which no synthetic standards are available, await structure confirmation. Based on the HPLC cochromatography alone there is no certainty that peaks of radioactivity are chemically homogeneous and further work on these metabolites is in progress to establish their homogeneity, spectral characteristics and identity. It is probable, based on results obtained with 7,12-dimethylbenz[*a*]anthracene [32], that multiply oxidised metabolites arising by further metabolism of the alcohol are produced. The appearance of liver preparation profiles containing several metabolites is similar to observations with other polycyclic aromatic compounds [17,18], and is consistent with observations that 7MBAC oxidation by liver microsomes of variously treated rats is closely paralleled by BP oxidation [33].

The metabolic fractionation described is expected to provide information on the comparative profiles of metabolites of 7MBAC produced by variously

Fig. 2. HPLC of (A) [^{14}C]7-methylbenz[*c*]acridine metabolism standards, (B) metabolites produced by hepatic microsomes from Wistar rats, (C) metabolites produced from hepatocytes found inside and (D) outside cells, and (E) metabolites extracted from rat bile after β-glucuronidase—arylsulphatase hydrolysis. Standards are (1) benz[*c*]acridine-7-carboxylic acid, (2) *trans*-5,6-dihydro-5,6-dihydroxy-7-methylbenz[*c*]acridine, (3) 7-hydroxymethylbenz[*c*]acridine, (4) 7-methylbenz[*c*]acridine-5,6-oxide, (5) 5-hydroxy-7-methylbenz[*c*]-acridine, (6) 9-hydroxy-7-methylbenz[*c*]acridine, (7) 7-methylbenz[*c*]acridine, (8) 11-hydroxy-7-methylbenz[*c*]acridine.

treated animals and will allow testing of these metabolites in the test of Ames et al. [34] to determine which metabolites are responsible for the S9 fraction mediated mutagenicity of 7MBAC [35]. This work is in progress.

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