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GAS CHROMATOGRAPHIC AND MASS FRAGMENTOGRAPHIC ASSAYS OF CARCINOGENIC POLYCYCLIC HYDROCARBON EPOXIDE HYDRATASE ACTIVITY

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SUMMARY

A specific and very sensitive procedure for the determination of epoxide hydratase activity in hepatic microsomes is described. Any polycyclic hydrocarbon epoxide can be used as a substrate; in this study, benzo(*a*)anthracene-5,6-oxide, benzo(*a*)pyrene-4,5-oxide and 3-methylcholanthrene-11,12-oxide were utilized. The corresponding *trans*-diols formed during incubation are separated and evaluated using either an electron-capture gas chromatographic method for the determination of their chloromethyldimethylsilylated derivatives or gas chromatographic–mass fragmentographic measurement of their trimethylsilylated derivatives. Concentrations as low as 1 ng per millilitre of incubation mixture can be estimated.

INTRODUCTION

Considerable experimental evidence suggests that aromatic hydrocarbons are converted into biologically active epoxides by the microsomal mixed-function oxidases[1–3]. These highly reactive intermediates act as the ultimate carcinogens[4,5], mutagens[6–9] or cytotoxins[10] by reacting with DNA, RNA and protein *in vitro*[11] and *in vivo*[12]. They also rearrange non-enzymatically to phenols, are metabolized to glutathione conjugates by cytoplasmic glutathione-S-epoxide transferases[13] and to inactive *trans*-dihydrodiols by microsomal epoxide hydratases[14] and are back-converted to the parent hydrocarbon by a microsomal epoxide reductase[15].

It seems obvious that the carcinogenicity of a polycyclic hydrocarbon for a given tissue will depend on the relative rate of metabolic activation and deactivation of the hydrocarbon; in this respect, the currently used assay for mixed-function oxidase activity that utilizes benzo(*a*)pyrene as substrate must be supplemented by methods that allow the specific measurement of the respective activating and inactivating enzymatic activities.

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Several procedures have been described for the measurement of the epoxide hydratase activity. The most widely used method, which employs [7-^{3}H]styrene oxide as substrate, is very sensitive but suffers from the disadvantage of utilizing a non-carcinogenic and non-aromatic epoxide[16]. A gas chromatographic (GC) method[17,18] and, more recently, a liquid chromatographic method[19] that utilize 3-methylcholanthrene-11,12-oxide as substrate have been reported; these methods, while simple and rapid, are not sensitive enough to be applicable to the determination of the very low enzymatic activities present in some tissues, to the determination of their kinetic parameters or to the proper evaluation of their modifications under the influence of several inducers and inhibitors.

During the course of an investigation of the role of the microsomal system in chemical carcinogenesis, it became necessary to develop a method that could measure with both selectivity and high sensitivity the epoxide hydratase activity towards epoxides of different polycyclic aromatic hydrocarbons. The method described here allows the measurement of the dihydrodiols formed at the picogram level.

EXPERIMENTAL

Reagents and chemicals

All chemicals were of reagent grade and were used without further purification.

The polycyclic hydrocarbons benzo(*a*)anthracene-5,6-oxide (BA-5,6-oxide), benzo(*a*)pyrene-4,5-oxide (BP-4,5-oxide) and 3-methylcholanthrene-11,12-oxide (3-MC-11,12-oxide) were purchased from Fluka (Buchs, Switzerland). The silylating reagents were obtained from Supelco (Bellefonte, Pa., U.S.A.). The arene oxides and their corresponding *cis*- and *trans*-dihydrodiols were prepared according to the method described by Sims[20]. The deuterated dihydrodiols were prepared in a similar manner, but using $LiAlD_4$ instead of $LiAlH_4$ as the reducing agent.

1,2,3,4,10,10-Hexachloro-*trans*-6,7-dihydroxy-1,4,4a,5,6,7,8,8a-octahydro-1,4,-5,8-*endo,exo*-dimethanonaphthalene (aldrin-6,7-dihydrodiol) was a gift from Shell (The Hague, The Netherlands).

Apparatus and conditions

Gas chromatography. A Hewlett-Packard Model 5750G gas chromatograph equipped with a ^{63}Ni electron-capture detector (ECD) was employed. A spiral borosilicate-glass column (2.5 m × 2 mm I.D.) packed with 3% OV-1 on Chromosorb W (80–100 mesh) was used. The operating conditions were as follows: column temperature, 250°; injector and detector temperature, 300°; carrier gas, argon–methane (95:5) at a flow-rate of 15 ml/min.

Mass fragmentography. Mass fragmentographic analysis was carried out with an LKB 9000S instrument. All derivatives were injected into the gas chromatograph with a flash heater temperature of 250°, a carrier gas (helium) flow-rate of 30 ml/min and an oven temperature of 220°. The coiled glass GC column (2.2 m × 3 mm I.D.) was packed with 1% OV-1 on 60–80-mesh Chromosorb W. The Ryhage type of molecular separator was maintained at 270°; mass spectra were recorded at electron energy 70 eV, trap current 60 μA and ion-source temperature 270°.

Microsomal preparation

Hepatic microsomes from male Wistar rats, R strain, weighing between 200 and 250 g were prepared according to the method of De Duve as described by Amar-Costesec *et al.*[21]. Protein concentration was determined by the method of Lowry *et al.*[22]. All animals were fed normally and fasted for 24 h before sacrifice.

Methods

Assay of epoxide hydrase. Incubations were carried out as follows. Rat liver microsomes (0.033 mg of protein) were pre-incubated at 37° for 2 min in 0.07 *M* phosphate buffer (pH 8) in a total volume of 0.4 ml. The arene oxide (62.5–1250 ng in 25 μl of acetone) was added and the mixture vortexed for 2 sec and incubated for different periods. This amount of acetone had no effect on the enzyme activity. Boiled microsomes served as controls.

Gas chromatography. After the incubation period, the reaction was halted by the addition of 2 ml of cold ethyl acetate containing 50 ng of aldrin-6,7-dihydrodiol, used as internal standard. The epoxide and the dihydrodiols were extracted by shaking for 2 min. The organic layer was separated by centrifugation at 600 *g* for 5 min and removed; the aqueous layer was re-extracted with a further 1 ml of ethyl acetate. The organic layers were combined and dried over anhydrous magnesium sulphate.

The organic solvent was evaporated at 40° under a stream of nitrogen and the residue derivatized by addition of 20 μl of the silylating reagent mixture bischloromethyltetramethyldisilazane–chloromethyltrimethylchlorosilane–pyridine (1:0.5:2).

The stoppered mixture was heated at 60° for 30 min and then evaporated to dryness. The residue was dissolved in 50 μl of *n*-hexane and aliquots (1–2 μl) were injected on to the gas chromatograph.

Mass fragmentography. The procedure was as described above, except that the tetradeuterated dihydrodiol was used as the internal standard and the silylated derivatives were prepared by using a mixture of 20 μl of TRI-SIL and 10 μl of BSTFA.

RESULTS AND DISCUSSION

Gas chromatography

Typical GC results are shown in Fig. 1. No diol peak was observed in gas chromatograms with samples similarly obtained from extracts of control reaction mixtures using boiled microsomes, indicating that non-enzymatic hydration of the epoxides was negligible. The identities of the disilylated derivatives of the *trans*-diols were established by mass spectrometry.

The concentrations of the dihydrodiol formed after incubation of the corresponding epoxide with the microsomal preparations were calculated from a standard graph (Fig. 2) constructed from chromatograms for biological samples containing known amounts (10–100 ng) of the dihydrodiol and a fixed amount (50 ng) of the internal standard. The ratio of the peak height of derivatized dihydrodiol to that of derivatized internal standard was plotted against concentration.

Mass fragmentography

In order to check the specificity of the GC–ECD method, a mass fragmento-

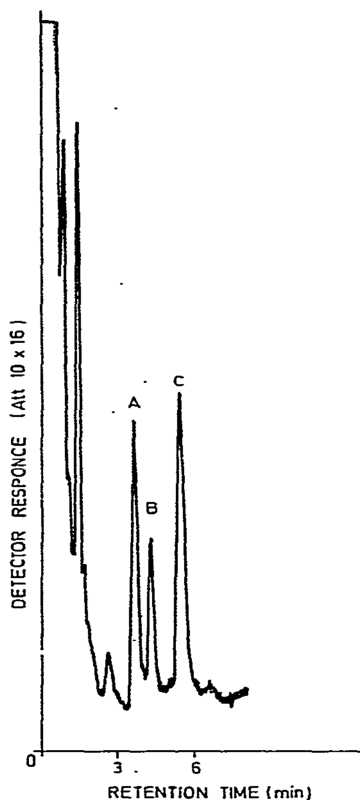

Fig. 1. Typical gas chromatogram of the derivatives of participants in the epoxide hydratase assay, as extracted from the incubation mixture. The figure shows the separation of the various diols: A, aldrin; B, BA; C, BP and 3MC. The other small peaks were also present in control extracts obtained from microsomes alone; no *cis*-diol could be detected in the gas chromatogram. Under the normal incubation and gas chromatographic conditions, the silylated phenols arising from the corresponding epoxides and which have longer retention times were never observed.

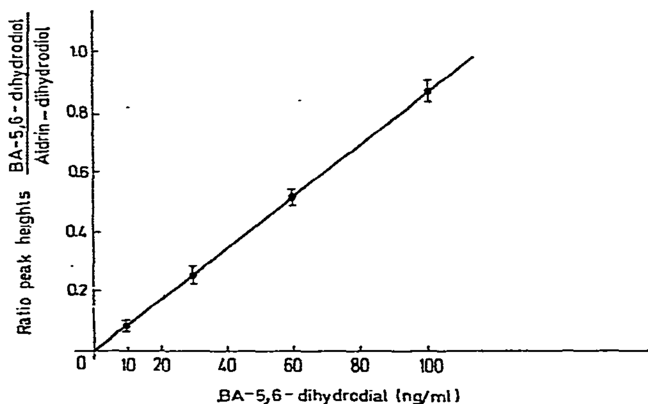

Fig. 2. Calibration graph for the determination of BA-5,6-dihydrodiol in microsomal suspension (50 ng/ml of internal standard added).

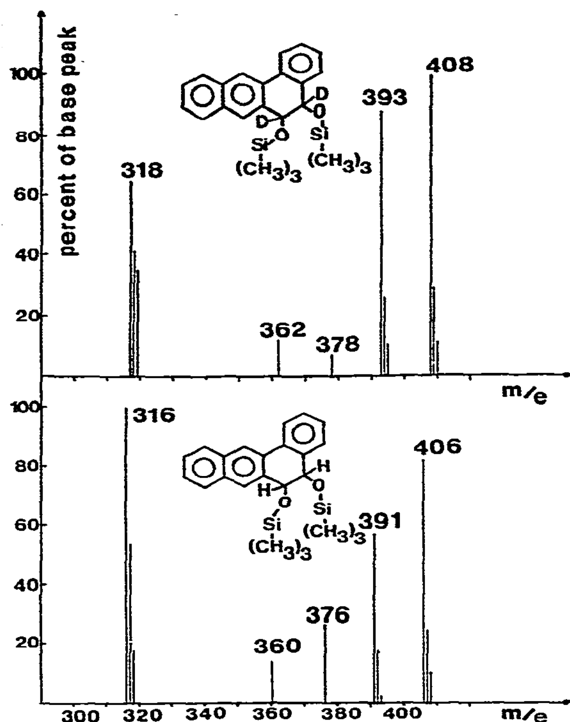

Fig. 3. Mass spectra of the trimethylsilylated derivatives of BA-5,6-dihydrodiol (A) and its deuterated analogue (B).

graphic method was developed for the measurement of the epoxide hydratase activity; this procedure has been shown to be very sensitive and can be used as an alternative assay. Fig. 3 presents the mass spectra of the trimethylsilylated derivatives of BA-5,6-dihydrodiol and its deuterated analogue between 300 and 400 mass units. The results of the mass fragmentographic analysis are shown in Fig. 4.

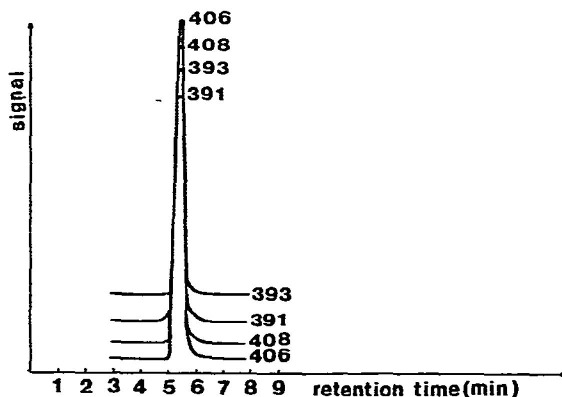

Fig. 4. Fragmentogram of TMS derivatives of 1 ng of BA-5,6-dihydrodiol (*m/e* = 391 and 405) and 5 ng of the internal standard (*m/e* = 393 and 408).

A standard graph was prepared by adding known amounts (500, 250, 125 and 25 ng/ml) of BA-5,6-dihydrodiol and a fixed amount of BA-5,6-dihydrodiol-d_4 (500 ng/ml) to microsomal suspensions and carrying out the described procedure. The standard graph was constructed by plotting the ratio of the peak height of the TMS derivative of BA-5,6-dihydrodiol (*m/e* 406) to that of the TMS derivative of BA-5,6-dihydrodiol-d_2 (*m/e* 408) against known amounts of added BA-5,6-dihydrodiol in microsomal suspension (Fig. 5).

With the various control samples that we have analyzed, we have so far en-

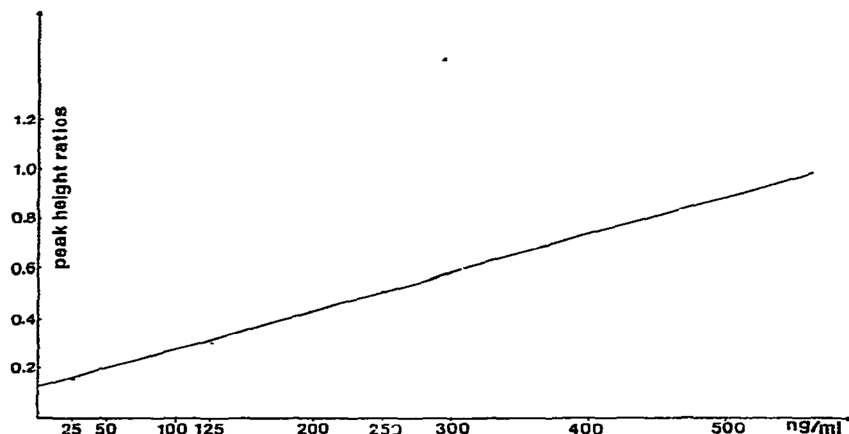

Fig. 5. Calibration graph for the determination of BA-5,6-dihydrodiol in microsomal suspension (500 ng/ml of internal standard added).

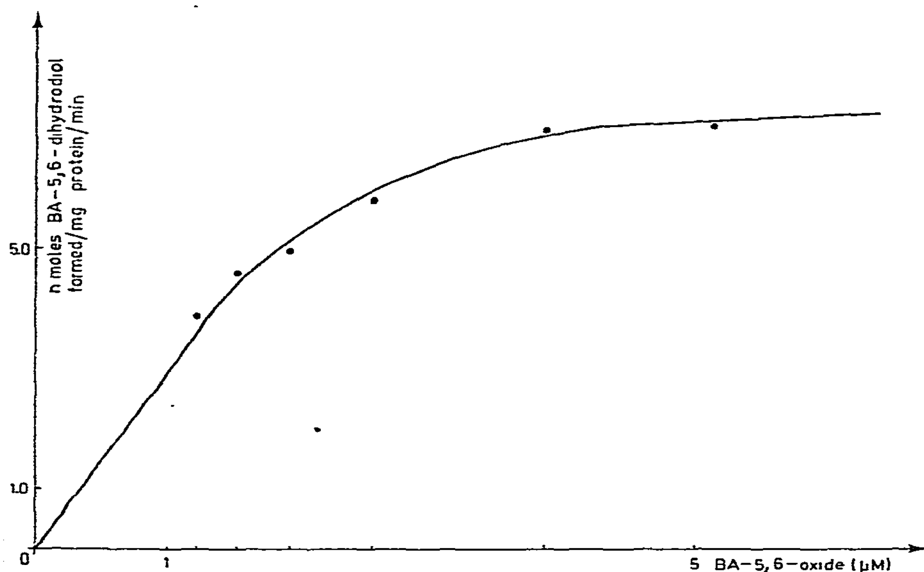

Fig. 6. Relationship between the BA-5,6-dihydrodiol formed per milligram of protein per minute and the substrate concentration.

countered no background interference at the retention time of the BA-5,6-dihydrodiol derivative when recording the selected four mass numbers.

A concentration of 4 μM BA-5,6-oxide was sufficient to saturate the enzyme (Fig. 6).

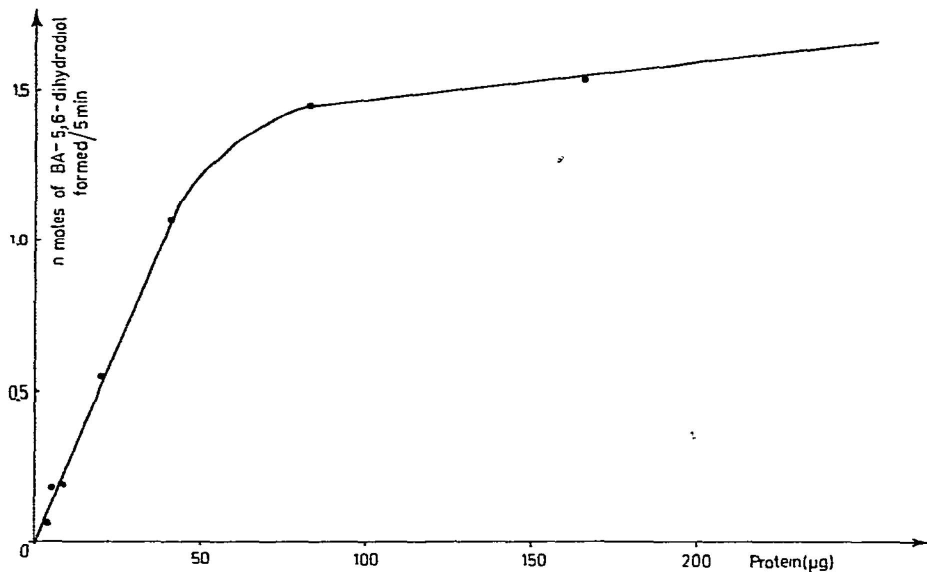

Fig. 7. Enzymatic hydration of BA-5.6-oxide as a function of protein concentration.

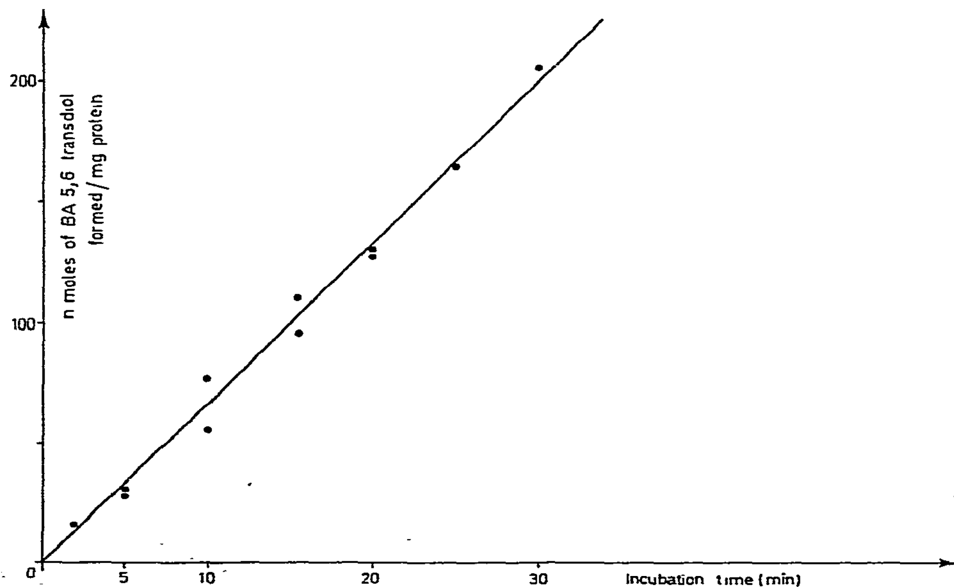

Fig. 8. Effect of incubation time on the amount of BA-5,6-dihydrodiol formed.

The relationship between BA-5,6-dihydrodiol formed and protein concentration was found to be linear up to 40 μg of protein (Fig. 7). The time course of the formation of BA-5,6-dihydrodiol was linear up to an incubation time of 30 min (Fig. 8).

Both analytical procedures are more specific than the widely used radiometric method[16] and more sensitive than the previously described chromatographic assays[17,18]. The method can be applied to the evaluation of the microsomal epoxide hydratase activity towards various polycyclic hydrocarbon epoxides.

We will subsequently report the evaluation of the very low levels of epoxide hydratase activity present in some extrahepatic tissues, the accurate determination of their kinetic parameters and the evaluation of their modifications under the influence of several inducers and inhibitors.

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