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A SPECIFIC GAS CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF MICROSOMAL STYRENE MONOOXYGENASE AND STYRENE EPOXIDE HYDRATASE ACTIVITIES

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SUMMARY

A gas chromatographic (GC) method for the determination of the metabolite resulting from the activities of microsomal styrene monooxygenase (epoxide synthetase) and epoxide hydratase using styrene or styrene epoxide as substrates has been developed. The determination of the activities of both enzymes is based on the GC determination of phenylethylene glycol after its esterification with *n*-butylboronic acid. Kinetic parameters for both enzymes are given.

INTRODUCTION

The biotransformation of a variety of chemicals through the epoxide–diol pathway[1,2] seems to be important in the formation of active intermediates that may cause damage to cellular components[3,4]. The enzymes involved in the formation (monooxygenase, E.C. 1.14.1.1) and hydration of epoxides (epoxide hydratase, E.C. 4.2.1.63) play a crucial role in this pathway. The comprehension of their behaviour to different stimuli *in vivo* and *in vitro* and the study of their distribution in various tissues could be helpful in establishing if there is a correlation between the accumulation of epoxide and the onset of toxic effects.

While the method for determination of the activities of monooxygenase and hydratase separately (with different substrates) has been reported by several workers[5–7], no methods that allow the simultaneous determination of activities of these two enzymes utilizing homogeneous substrates seem to be available.

This paper presents a simple gas chromatographic (GC) method for the assay of the metabolite resulting from the activities of microsomal monooxygenase and epoxide hydratase when styrene and styrene oxide are utilized as substrates.

EXPERIMENTAL

Chemicals

Styrene, styrene oxide and phenylethylene glycol were purchased from Merck

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(Darmstadt, G.F.R.); *n*-butylboronic acid from Pierce (Rockford, Ill., U.S.A.); nicotinamide-adenine dinucleotide phosphate ($NADP^+$), glucose-6-phosphate and glucose-6-phosphate dehydrogenase from Boehringer (Mannheim, G.F.R.); and *m*-dinitrobenzene from EGA-Chemie (Steinheim/Albuch, G.F.R.). All other reagents were obtained from Carlo Erba (Milan, Italy).

Microsomal preparation

The microsomes were prepared using Charles River CD male (150–180 g) rats according to the method of Kato and Takayanaghi[8]. Only a slight modification to the composition of the solution used for the homogenization was made. This solution consisted of Tris–hydrochloric acid buffer (0.05 *M*, pH 7.4) that was 0.15 *M* in potassium chloride and 5 m*M* in magnesium chloride.

The microsomal pellets were re-suspended in the same buffer to a final protein concentration of 10–20 mg/ml. The protein concentration was determinated by the method of Lowry *et al.*[9] using bovine serum albumin as a standard.

Assay of styrene monooxygenase

The incubation mixture in a final volume of 5 ml contained the NADPH-generating system (2.5 μmoles of $NADP^+$, 50 μmoles of glucose-6-phosphate and 2 units of glucose-6-phosphate dehydrogenase), the Tris–hydrochloric acid buffer and about 10 mg of microsomal protein. After pre-incubation at 37° for 10 min, the reaction was started by the addition of 100 μl of an acetone solution of styrene to obtain a final substrate concentration of 5 m*M*. The reaction was carried out with shaking for 5 min and stopped by the addition of 1 ml of 0.6 *N* sulphuric acid. The samples were then agitated for 12 h at room temperature and subsequently extracted twice with 5 ml of ethyl acetate after being made alkaline with 2 ml of 0.6 *N* sodium hydroxide solution.

The acidification of the styrene epoxide induces more than 95% transformation into the diol. The combined extracts were dried under vacuum at room temperature and the phenylethylene glycol content in the residue was determined as described below. The recovery of phenylethylene glycol under these conditions was about 90%.

The enzymatic activity was expressed in nanomoles of glycol formed per minute of incubation per milligram of microsomal protein.

Assay of epoxide hydratase

The activity of this enzyme was determined as described previously for styrene monooxygenase with the following exceptions: (a) 5 m*M* styrene oxide was used instead of styrene as substrate, (b) the NADPH-generating system was omitted and (c) the reaction was stopped by adding 1 ml of 0.6 *N* sodium hydroxide solution and samples were immediately extracted twice with ethyl acetate.

The spontaneous hydration of styrene oxide that occurred under the conditions used was less than 5% with respect to the enzymatic hydration and it was subtracted in the calculation of the enzymatic activity.

Preparation of samples for GC analysis

The residue, after evaporation of ethyl acetate, was dissolved in 100 μl (monooxygenase assay) or 400 μl (hydratase assay) of an acetone solution of *m*-dinitro-

benzene (0.5 μg/μl) used as an internal standard. To each 100 μl of above solution, 2.5 μl of *n*-butylboronic acid solution (100 mg/ml in dimethylformamide) were added and 1 μl of the sample was injected into the gas chromatograph.

Apparatus and conditions

A Carlo Erba Model GI gas chromatograph equipped with a flame ionization detector was used. The GC column was a 2.5-m glass tube (I.D. 2 mm; O.D. 4 mm) packed with 3% OV-17 and Gas-Chrom Q (100–120 mesh). The operating conditions were: column temperature, 170°; oven temperature, 240°; nitrogen (carrier gas) flow-rate, 30 ml/min; and chart speed, 1 cm/min.

Mass spectrometry

In order to confirm the formation of the *n*-butylboronate of phenylethylene glycol[10], a mass spectrometric analysis was carried out on an LKB 9000 instrument under the following conditions: ion source temperature, 290°; ionization energy, 70 eV; and trap current, 60 μA. The sample was introduced either by a direct inlet system (DIS) or by GC under the conditions described above but with helium as the carrier gas at a flow-rate of 30 ml/min.

RESULTS

The gas chromatograms of the *n*-butylboronic ester of phenylethylene glycol and the internal standard *m*-dinitrobenzene are shown in Fig. 1.

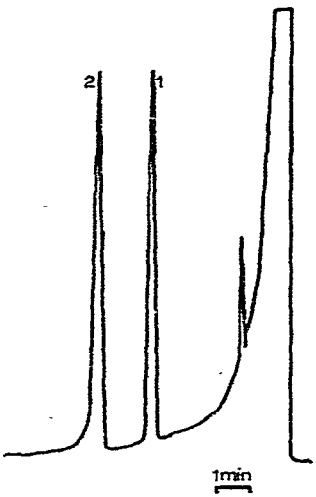

Fig. 1. Gas chromatographic separation of the ester of phenylethylene glycol with *n*-butylboronic acid (1) and *m*-dinitrobenzene (2) under the conditions described in the text.

The identification of the *n*-butylboronate of phenylethylene glycol was checked by means of mass spectrometry (MS) and GC–MS. The mass spectra of the glycol and of its ester with butylboronic acid are shown in Fig. 2.

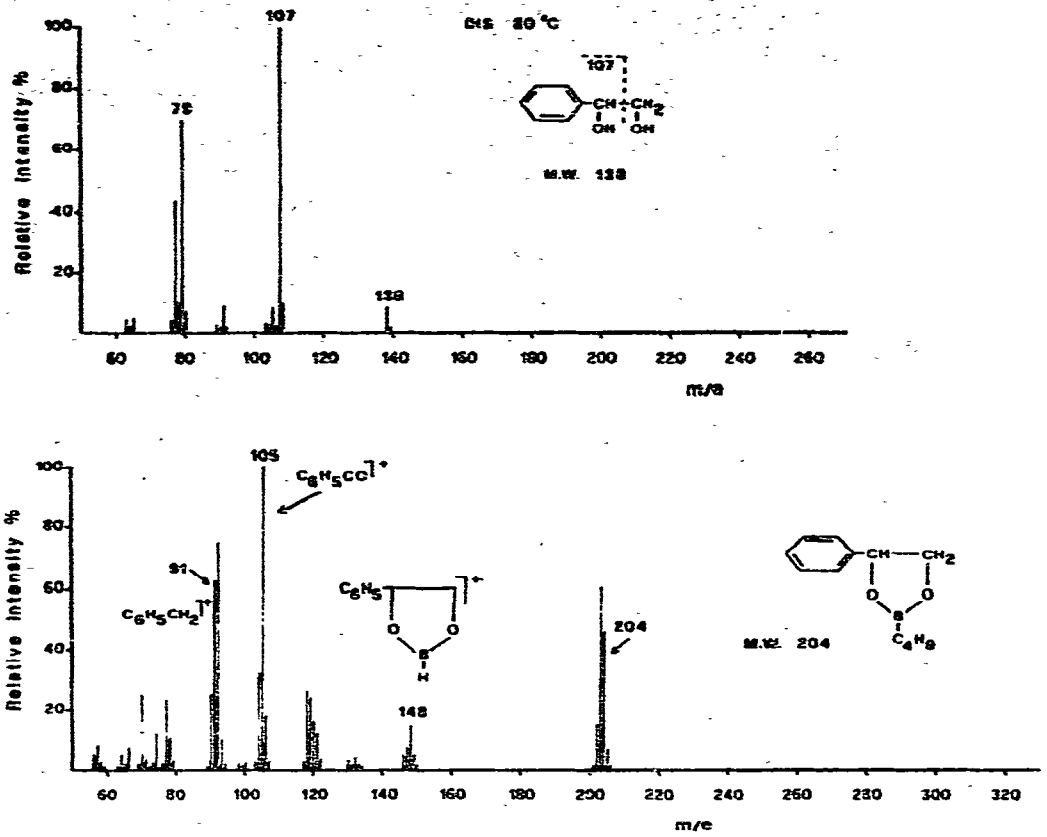

Fig. 2. Mass spectra of phenylethylene glycol (above) and butylboronic derivative of phenylethylene glycol (below).

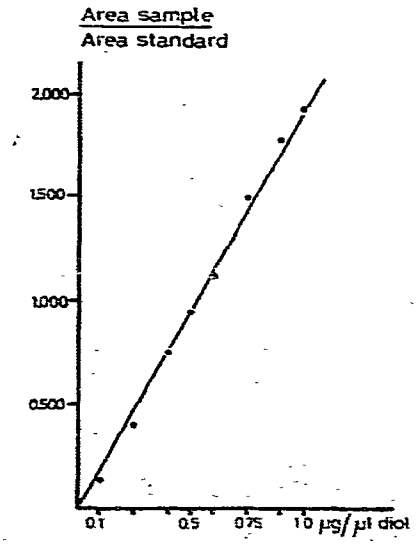

Fig. 3. Calibration graph for phenylethylene glycol after esterification with *n*-butylboronic acid. Internal standard: *m*-dinitrobenzene (0.125 μg/μl).

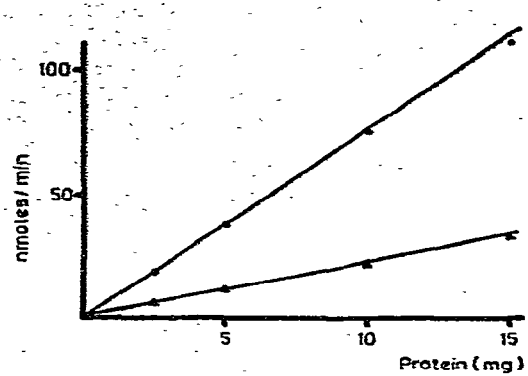

Fig. 4. Effect of microsomal protein concentration on the activity of hydratase (●) and monooxygenase (▲).

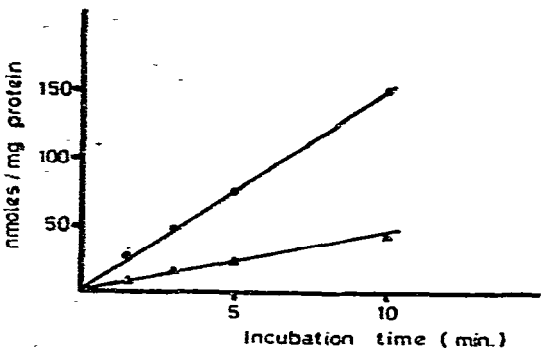

Fig. 5. Effect of incubation time on the amount of product formed from hydratase (●) and monooxygenase (▲).

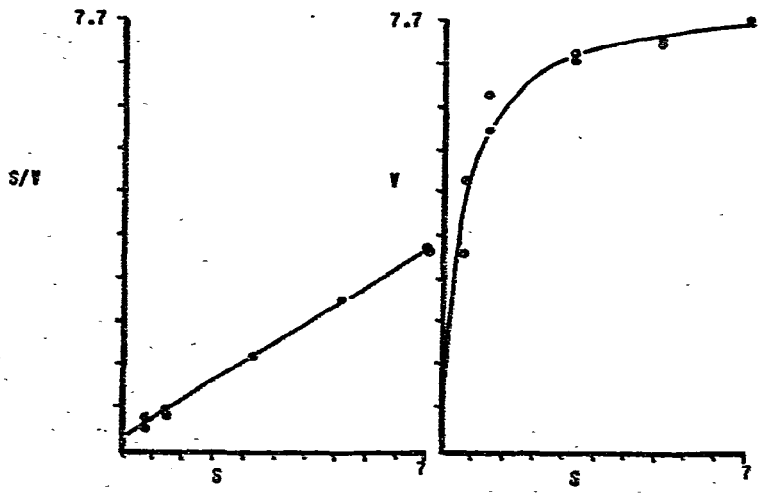

Fig. 6. Activity of monooxygenase in the presence of different concentrations of styrene. Left, Woolf plot; right, Michaelis–Menten plot.

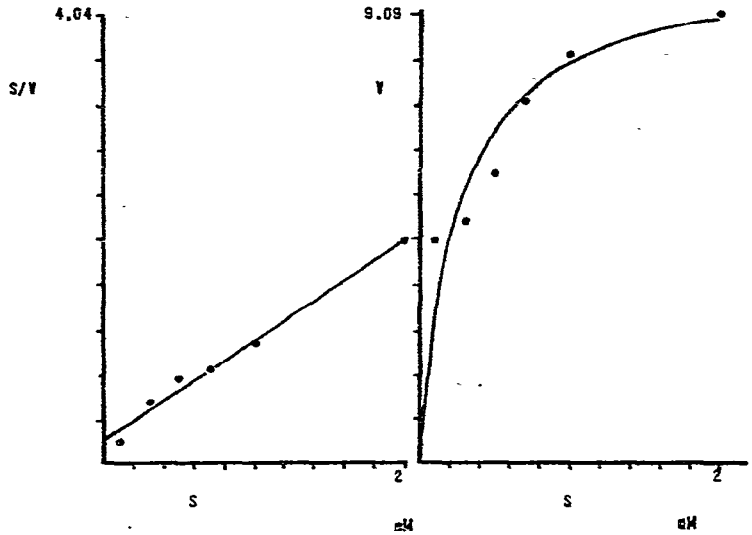

Fig. 7. Activity of epoxide hydratase in the presence of different concentrations of styrene epoxide. Left, Woolf plot; right, Michaelis–Menten plot.

The calibration graph for phenylethylene glycol is shown in Fig. 3. The graph is linear from 0.1 to 1 μg/μl.

The results in Figs. 4 and 5 indicate that the amounts of glycol formed during the enzymatic reaction catalyzed by monooxygenase or hydratase are linear up to an incubation time of 10 min and in the range of microsomal protein from 2.5 to 15 mg. In all of the subsequent experiments, therefore, an incubation time of 5 min and an amount of microsomal protein of 10 mg were used.

Fig. 6 shows the Woolf and Michaelis–Menten plots for the monooxygenase activity.

The saturation of the enzyme by the substrate (styrene) is observed at a concentration of 3 mM. The apparent K_m and $V_{max.}$ values are 0.43 mM and 4.77 nmoles/min per milligram of protein, respectively.

Fig. 7 shows the Woolf and Michaelis–Menten plots for epoxide hydratase using styrene epoxide as substrate. The apparent K_m and $V_{max.}$ values are 0.25 mM and 11.17 nmoles/min per milligram of protein, respectively.

DISCUSSION

The determination of epoxide hydratase involves the use of radioactive styrene epoxide[7], while the monooxygenase (epoxide-forming enzyme) activity is determined by using a variety of substrates[5–7]. This paper describes a simple GC method for the determination of both enzymatic activities by employing the homogeneous non-radioactive substrates styrene epoxide and styrene.

In the presence of monooxygenase obtained from rat liver, styrene is transformed into an epoxide but a consistent fraction of this epoxide is metabolized to form the diol, phenylethylene glycol. As the diol can be formed only by the epoxide

pathway[11,12], it is possible to determine the total epoxide formed by measuring the diol formed both enzymatically and chemically after acidification of the epoxide that is not hydrated during the incubation. The chemical hydration of the epoxide accomplished by addition of sulphuric acid is necessary in order to avoid under-estimation of the amount of epoxide formed.

In the presence of epoxide hydratase, the diol is derived quantitatively only from the disappearance of the epoxide. Therefore, by using the two substrates, but by measuring only one final metabolite, namely phenylethylene glycol, it is possible to determine the activities of two enzymatic systems separately. The determination of phenylethylene glycol is simple and quantitative because its butylboronic derivative is suitable for GC analysis.

The kinetic data obtained in these studies indicate that the affinity of styrene epoxide for the hydratase is about twice as great as that of styrene for the epoxide-forming enzyme. Not only the affinity constant is different but also the amount of the hydratase is greater than the epoxide-forming system, as shown by the $V_{max.}$ values. Therefore, the formation of the epoxide seems to be the rate-limiting step in the epoxide–diol pathway.

The method described may be of use in studies on the inhibition or the activation of these two enzymatic activities, which are of significance for the accumulation of toxic metabolites.

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