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## Note

# Improved gas chromatographic method for measuring phenylethylene glycol

## Application to the determination of styrene monooxygenase and epoxide hydrase activities

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We have previously described a gas chromatographic (GC) method for the simultaneous determination of styrene monooxygenase and styrene oxide hydrase[1]. This method determined both activities by measuring only phenylethylene glycol (diol), which was quantitated after derivatization with *n*-butylboronic acid and detected with a flame-ionization detector. In this paper we present a more sensitive method which measures picomoles of phenylethylene glycol after its esterification with trifluoroacetic anhydride, using a gas chromatograph equipped with an electron-capture detector. We applied the method to the quantitative measurement of nuclear or microsomal styrene monooxygenase and styrene epoxide hydrase activities.

## EXPERIMENTAL

### *Chemicals*

Styrene (Carlo Erba, Milan, Italy) phenylethylene glycol (Merck, Darmstadt, G.F.R.), trifluoroacetic anhydride (Pierce, Rockford, Ill., U.S.A.), trimethylamine (Carlo Erba), 1-bromo-2-phenylethane (Merck), NADH (Boehringer, Mannheim, G.F.R.), NADPH (Boehringer) and styrene oxide (Merck) were used.

### *Apparatus*

A Carlo Erba gas chromatograph with a nickel-63 electron-capture detector was used. The column was a glass tube (2 m × 4 mm I.D.) packed with 3% OV-17 on 100–120-mesh Gas-chrom Q (Supelco, Bellefonte, Pa., U.S.A.). The column temperature was 140°, the injector port temperature 250° and the detector temperature 275°. The carrier gas was nitrogen at a flow-rate of 30 ml/min and the chart speed was 1 cm/min.

### *Animals*

Male CD-COBS rats (200 ± 20 g) were obtained from Charles River (Calco, Como, Italy). The rats were given a commercial laboratory feed and water *ad libitum*

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and kept in air-conditioned quarters with 12-h light–dark cycles. The rats were fasted for 12 h, then killed. The livers were removed immediately and used for the isolation of microsomes and nuclei.

*Isolation of microsomes and nuclei*

Rat liver microsomes were isolated as described previously[2], except for the buffer, to which sucrose was added to a final concentration of 0.25 *M*. Rat liver nuclear preparations were purified by means of a discontinuous sucrose gradient according to the method of Berezney *et al.*[3]. The protein concentration was determined by the method of Lowry *et al.*[4].

*Assay of styrene monooxygenase*

Intact nuclear or microsomal preparations were suspended in 50 mM phosphate buffer containing 150 m*M* potassium chloride, 5 m*M* magnesium chloride and 0.25 *M* sucrose to obtain a final protein concentration of about 1–2 mg/ml. To 1 ml of the suspension, 50 $\mu$l of a 20 mg/ml NADPH buffer solution were added. After 5 min of pre-incubation the reaction was started by adding 10 $\mu$l of a 250 m*M* methanolic solution of styrene. After 10 min of incubation at 37° in a Dubnoff incubator, the reaction was stopped with 0.4 ml of 0.6 *N* sulphuric acid and the preparation was left overnight. The samples were made alkaline with 0.8 ml of 0.6 *N* sodium hydroxide solution, then extracted twice with 3 ml of ethyl acetate. Acidification of the styrene resulted in more than 95% transformation into the diol. The combined extracts were dried under a gentle stream of nitrogen in a thermostatic bath at 37° and the phenylethylene glycol was determined as described below. The recovery of phenylethylene glycol under these conditions was 96%.

For all experiments a series of blanks was prepared, consisting either of boiled nuclei or microsomes or of the buffer solution alone, containing the amounts of NADPH and styrene used for fresh nuclei or microsomes.

*Assay of styrene epoxide hydrase*

The activity of this enzyme was determined as described above for styrene monooxygenase with the following exceptions: (a) 42 m*M* methanol solution of styrene oxide instead of styrene was used as substrate; (b) the NADPH was omitted; and (c) the reaction was stopped by adding 0.4 ml of 0.6 *N* sodium hydroxide solution.

In the experiments to determine nuclear hydrase activity, as spontaneous hydrolysis may be quantitatively important, a series of blanks was always prepared, consisting of boiled nuclei or buffer solution alone. In experiments to determine microsomal hydrase activity, the same kind of blanks were prepared but, as microsomal is much greater than nuclear activity, they are less important. The enzymatic activity was calculated by subtracting the blank value from that for the fresh nuclei; for the nuclear preparations we took into account only activity values that were at least twice as high as the blank values.

*Derivative formation*

The extraction residue was dissolved in 1 ml of toluene and 200 $\mu$l of 0.05 *M* trimethylamine in toluene and 100 $\mu$l of pure trifluoroacetic anhydride were added. Derivatization was carried out by holding the samples at 60° for 30 min. They were

then left to cool at room temperature; 1 ml of distilled water was added to the organic phase and, after 1 min, 1 ml of 5% ammonia solution was added. The samples were shaken for 5 min and centrifuged at 3500 *g*. To 50 $\mu$l of the organic phase, 50 $\mu$l of a 28 $\mu$g/ml toluene solution of 1-bromo-1-phenylethane used as internal standard were added. A 1-$\mu$l volume of this solution was injected into the gas chromatograph.

## RESULTS AND DISCUSSION

Fig. 1 shows a typical chromatogram, where peak 1 corresponds to the trifluoroacetyl derivative of phenylethylene glycol and peak 2 to the internal standard, 1-bromo-2-phenylethane. The overall sensitivity of the method is 5 ng/ml, but more reliable values were above 100 ng/ml and all the experiments were carried out in a linearity range from 125 to 2000 ng/ml. The calibration graph for phenylethylene glycol is shown in Fig. 2.

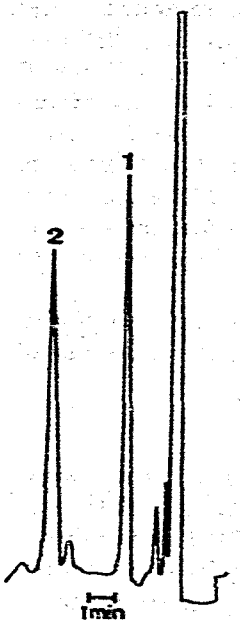


Fig. 1. Gas chromatogram of phenylethylene glycol trifluoroacetyl derivative. Peak 1 corresponds to the derivatized diol and peak 2 to 1-bromo-2-phenylethane (internal standard).

The trifluoroacetylderivative of phenylethylene glycol was identified by mass spectrometry (MS) and GC–MS. The mass spectrum of the glycol ester with trifluoroacetic anhydride is shown in Fig. 3. The amounts of glycol formed during the enzymatic reaction catalysed by monooxygenase or hydratase are linear up to incubation times of 15 and 30 min, respectively, and in the ranges of nuclear protein from 0.5 to 2.5 mg/ml and 1 to 2.5 mg/ml, respectively. The coefficient of variation of the method, calculated for two concentrations, is about 7% for the lower concentration (125 ng/ml) and about 11% for the higher concentration (1500 ng/ml).

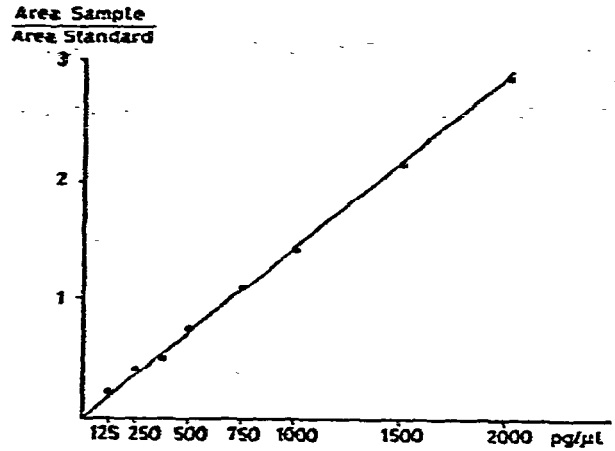


Fig. 2. Calibration graph for derivatized phenylethylene glycol.

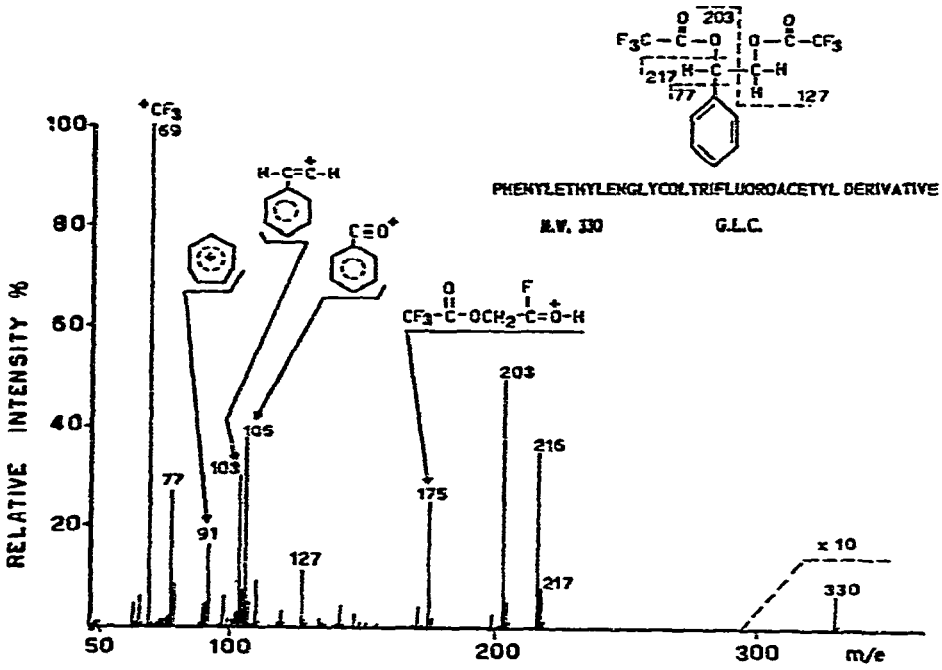


Fig. 3. Mass spectrum of phenylethylene glycol trifluoroacetyl derivative. Analysis was carried out by using an LKB 2091-051 instrument operated in the electron-impact mode at 70 eV.

Table I shows monooxygenase and hydrase activity values in nuclei and microsomal preparations of liver.

The GC method described here measures concentrations of phenylethylene glycol at the picomole level and enabled us to determine for the first time the presence on the nuclear envelope of an enzymatic activity capable of oxidizing the aliphatic

TABLE I

STYRENE MONOOXYGENASE AND EPOXIDE HYDRASE ACTIVITIES IN MICROSOMAL AND NUCLEAR PREPARATIONS

Both activities are expressed as pmole/min/mg protein. Each figure is the mean value ± standard error of at least 10 determinations.

| *Preparation* | *Styrene monooxygenase* | *Styrene epoxide hydrase* |
|---|---|---|
| Microsomes | 1950 ± 270 | 8660 ± 1640 |
| Nuclei | 128 ± 3 | 303 ± 36 |

double bond of styrene. We also found epoxide hydrase values in good agreement with the data available in literature, mainly based on radioactive studies[5].

The method described may be useful for investigations involving the determination of styrene monooxygenase and epoxide hydrase in small samples of tissues or in nuclei, monocytes and platelets which contain only limited amounts of such enzymatic activities[6–9].

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