DETERMINATION OF EPOXIDE HYDROLASE ACTIVITY BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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Expoxide hydrolase is a key enzyme in the metabolism of highly reactive epoxides both of exogenous origin and formed in the body during metabolic oxidation of various foreign compounds in the microsomal mono-oxygenase system [9]. Epoxide hydrolase converts epoxides into less dangerous dihydrodiols, and thus protects the cell against the toxic action of epoxide-containing compounds. In some cases during biotransformation of polycyclic aromatic hydrocarbons, epoxide hydrolase converts monofunctional epoxides into more reactive dihydrodiol epoxides, which can interact with cellular macromolecules and which are initiators of carcinogenesis [9, 12]. In these cases epoxide hydrolase behaves as an activating carcinogenic factor.

Various methods of determining epoxide hydrolase activity are known: radiometric, the method most frequently used [8, 10], fluorometric [1, 4], and chromatographic [3, 7, 11], each of which has its limitations for widespread use: involving working with radioactive substances, insufficient sensitivity, the need to form derivatives of products of the enzyme reaction before chromatographic analysis. Moreover, in the overwhelming majority of cases the substrates used to determine activity of the enzyme are not commercial preparations, and their synthesis is a laborious process [3, 4, 7, 10].

In this paper we suggest a method of determining microsomal epoxide hydrolase by the use of high performance liquid chromatography (HPLC), which differs from methods described previously in its higher sensitivity, accuracy, and good reproducibility.

EXPERIMENTAL METHOD

Sexually mature male Wistar rats were used. Styrene oxide ("Fluka," Switzerland) was used as the substrate and phenylethylene glycol (PEG, from "Aldrich," USA) as the external standard. Microsomes were isolated from the rat liver in 0.15 M KCl, containing 50 mM Tris-HCl, pH 7.4, by the method in [6].

The incubation medium, containing 340 μl of 0.15 M Tris-HCl, pH 8.7, and 50 μl of a suspension of microsomes (0.1-0.2 mg protein), was heated for 1 min at 37°C, after which 10 μl of a 40 mM solution of styrene oxide in acetonitrile was added. To estimate the level of nonenzymic hydrolysis of styrene oxide, in the control samples the microsomal suspension was heated to 100°C for 5 min before addition to the incubation medium. Experimental and control samples were incubated at 37°C for 15 min, after which they were transferred to an ice bath, and 1 ml each of n-butanol and 0.5 ml of a saturated solution of ammonium sulfate was added, the mixture was shaken for 10 sec, and 5-μl samples of the n-butanol extract were withdrawn for subsequent chromatographic analysis.

An "Altex" model 332 chromatograph (USA), a column (25 cm × 4.6 mm) and precolumn (4.5 cm × 4.6 mm) with "Ultrasphere-Si" silica-gel (particle size 5 μ) were used for analysis; the mobile phase was a system of hexane—isopropanol—water (80:28:2) and the rate of flow 1.5 ml/min. The hydrolysis product of styrene oxide (PEG) was determined by means of a "Kratos-SF-757" UV detector (Great Britian) at a wavelength of 210 nm. The retention time of PEG was 4-4.5 min (Fig. 1). The PEG concentration was determined by means of a "Shimadzu C-R3A" integrator (Japan), using an external standard of PEG. Enzyme activity (A) was calculated by the formula:

$$A = \frac{P_0 - P_c}{t \cdot m}$$

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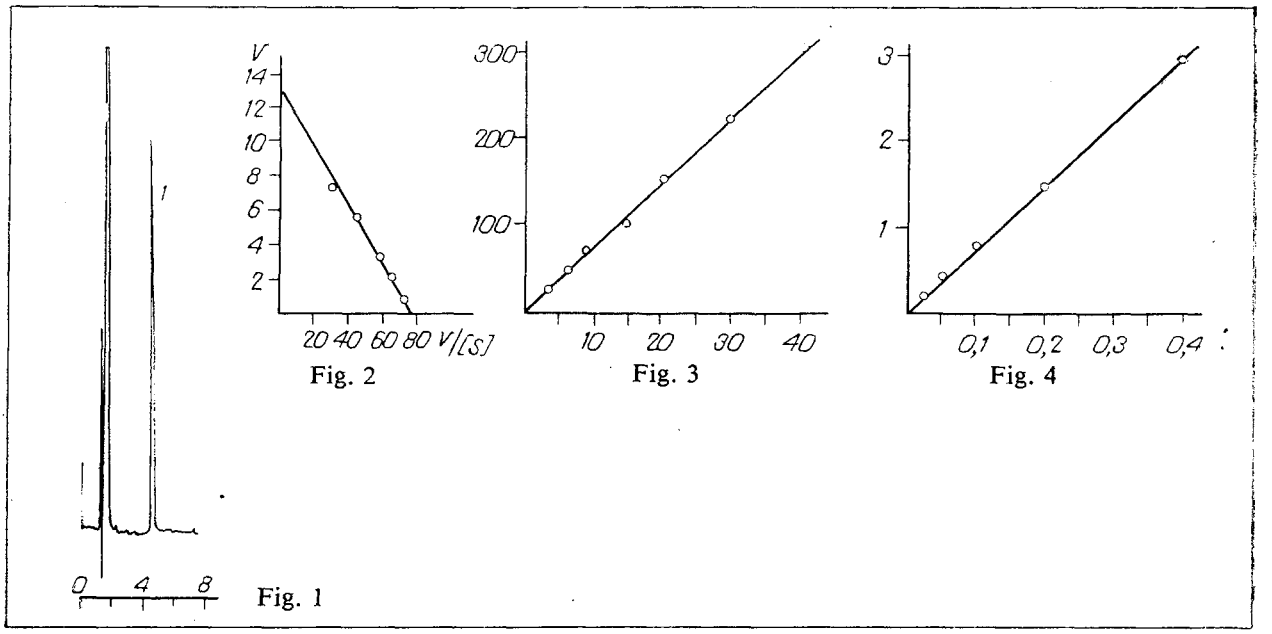

Fig. 1 Fig. 2 Fig. 3 Fig. 4

Fig. 1. Chromatogram of n-butanol extract of enzymic hydrolysis products of styrene oxide. Peak 1 corresponds to 0.2 nmole PEG with 0.005 AUFS detector and time constant of 0.5 sec. Abscissa, retention time (in min).

Fig. 2. Dependence of reaction velocity on substrate concentration (in Adey—Hofstee coordinates). Abscissa, V/[S], ordinate, V (in nmoles/min/mg protein).

Fig. 3. Dependence of reaction velocity on incubation time. Mean results of three determinations shown. Abscissa, time (in min); ordinate, reaction velocity (in nmoles/mg protein).

Fig. 4. Dependence of reaction velocity on protein content in sample. Mean results of three determinations given. Abscissa, protein content (in mg); ordinate, reaction velocity (in nmoles/min).

where P_0 is the quantity of PEG formed in the experimental samples as a result of enzymic hydrolysis (in nmoles); P_c the quantity of PEG formed in the control samples as a result of nonenzymic hydrolysis (in nmoles); t the incubation time (in min); and m the quantity of protein added to the incubation mixture (in mg).

EXPERIMENTAL RESULTS

The limit of detection of PEG by the suggested method is 5 pmoles per injection, corresponding to a sensitivity of the method of 0.6 nmole/min/mg protein, the volume of an aliquot of n-butanol extract being 5 μl. The coefficient of variation of the method is 3.7%. The sensitivity of the method can be increased tenfold by increasing the size of the aliquot of n-butanol extract for chromatographic analysis (if necessary, after preliminary concentration).

Epoxide hydrolase activity in rat liver microsomes, determined by the suggested method, was 9.54 ± 0.16 nmoles/min/mg protein, the apparent K_m was 0.18 mM, and V_{max} was 13.2 nmoles/min/mg protein (Fig. 2), in agreement with results obtained for the same substrate by other method [5]. The reaction was linear in character during incubation for 40 min (Fig. 3) and remained linear with a change in the protein content in the sample from 0.025 to 0.4 mg (Fig. 4).

The suggested method of determination of epoxide hydrolase activity has a number of advantages over existing methods using styrene oxide as the substrate, including methods based on the use of HPLC. To begin with, the method is highly sensitive, due to the use of a mobile phase (hexane—isopropanol—water) that is transparent at the wavelength of maximal absorption of PEG (210 nm). Thus when the mobile phase ethyl acetate—chloroform described in method [11] was used, the limit of detection of PEG was 250 pmoles per injection. Moreover, detection of PEG under optimal conditions makes it possible to eliminate the stage of concentration and redissolving, which greatly simplifies the method and shortens the time required to do the analysis.

High accuracy and reproducibility of the method are due to the use of n-butanol in the presence of ammonium sulfate solution to extract the PEG from the incubation mixture. It enables more complete extraction of PEG than the

ethyl acetate widely used for this same purpose, and it is transparent at the wavelength of PEG determination. On comparison of the completeness of extraction of PEG from the incubation mixture with chloroform, ethyl acetate, and n-butanol, the values obtained were 63.1 ± 1.8, 86 ± 3, and 93.2 ± 0.8% respectively.

Finally, it must be pointed out that the use of HPLC enables quantitative estimation of an individual compound PEG, formed as a result of the enzymic reaction, to be carried out, thus ensuring the high reliability of the method by comparison with fluorometric and radiometric methods.

The suggested method can be widely used to study the activity and properties of epoxide hydrolase in various organs and tissues, including those distinguished by low activity of the enzyme, for example, in the skin and intestinal mucosa, which are the "portals of entry" of various foreign substances into the body, and whose enzyme systems can play a definite role in the metabolism of these substances and in the transport of them and their metabolites into other organs and tissues.

The method can also be used to assess the functional state of the system for metabolism and detoxication of foreign substances, which comes high on the scale of priorities in the description of pathological states caused by drugs, by environmental pollutants, by alimentary disturbances, and so on [2].

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