Journal of Chromatography, 202 (1980) 447–452
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 13,269

SIMPLIFIED LIQUID CHROMATOGRAPHIC ASSAY FOR EPOXIDE HYDROLASE

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(Received August 1st, 1980)

SUMMARY

A simplified liquid chromatographic assay for epoxide hydrolase (E.C. 3.3.2.3) is described. The enzyme substrate employed, 3-(*p*-nitrophenoxy)-1,2-propene oxide, is commercially available and is readily resolved by reversed-phase liquid chromatography from the enzymatic hydration product, 3-(*p*-nitrophenoxy)-1,2-propane diol. As little as 100 pmol of the diol can be detected by absorbance monitoring at 315 nm. This assay has been employed to measure epoxide hydrolase in rat liver microsomes from animals treated with the dietary antioxidant, 2(3)-*tert.*-butyl-4-hydroxyanisole, a known inducer of the enzyme. The assay can also be used to measure rat liver cytosolic epoxide hydrolase.

INTRODUCTION

There is great interest in the properties of the microsomal enzyme, epoxide hydrolase (E.C. 3.3.2.3) especially in relation to its role in the detoxification of mutagenic epoxides[1–3]. A wide range of assays for epoxide hydrolase have been described, including radiometric[4–6], photometric[7] and fluorimetric[8] assays, as well as methods based on gas–liquid or liquid chromatographic separation of the diol product from the epoxide substrate[9–15]. Each of these methods has one or more drawbacks, such as non-linearity, the need to extract the diol product, derivatization of the diol product, synthesis of the epoxide substrate, high blank values, etc.; some of these problems have been discussed[5,8,15].

Recently, Westkaemper and Hanzlik[15] have described a convenient method using *p*-nitrostyrene oxide as the epoxide hydrolase substrate. The separation of the epoxide and diol product was readily achieved by liquid chromatography on a reversed-phase column. We have been developing a similar approach but using 3-(*p*-nitrophenoxy)-1,2-propene oxide (NP-oxide)* as the substrate. The advantages of our approach are that (a) this epoxide substrate is commercially available, and (b) its diol derivative, 3-(*p*-nitrophenoxy)-1,2-propane diol (NP-diol) has an absorption

* Abbreviations used: BHA = 2(3)-*tert.*-butyl-4-hydroxyanisole; NP-diol = 3-(*p*-nitrophenoxy)-1,2-propane diol; NP-oxide = 3-(*p*-nitrophenoxy)-1,2-propene oxide.

0021-9673/80/0000–0000/$02.25

maximum at 315 nm[16], a wavelength shifted away from ultraviolet-absorbing materials present in cell extracts that interfere with the detection of aromatic diols such as styrene glycol or *p*-nitrostyrene glycol. The epoxide hydrolase reaction with NP-oxide as substrate is diagrammed in Fig. 1.

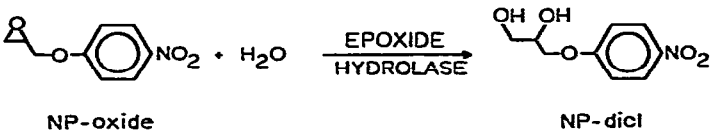

Fig. 1. Reaction of NP-oxide with epoxide hydrolase.

MATERIALS AND METHODS

Materials

Styrene oxide and styrene glycol were obtained from Aldrich (Milwaukee, WI, U.S.A.). NP-oxide was obtained from Eastman (Rochester, NY, U.S.A.). NP-diol was synthesized from NP-oxide according to Tang[16] and its concentration was measured using an extinction coefficient of 11,000 M^{-1} cm^{-1} at 315 nm[16]. Acetonitrile (HPLC grade) was obtained from Fisher Chemicals (Denver, CO, U.S.A.).

Liquid chromatography

The chromatographic separation was performed on an Altex Model 323 gradient liquid chromatograph equipped with an Hitachi Model 100-10 variable-wavelength spectrophotometer operating at 315 nm for the detection of NP-diol and at 260 nm for the detection of styrene glycol. A C_{18} reversed-phase column (15 cm × 4.6 mm I.D., Ultrasphere-ODS; Altex, Berkeley, CA, U.S.A.) was employed for separations. A solvent system of acetonitrile–water (50:50) at a flow-rate of 2.0 ml/min was used when assaying for NP-diol. When chromatographing styrene glycol, a solvent system of acetonitrile–water (15:85) flowing at 2.0 ml/min was used.

Enzyme preparations

Female Sprague-Dawley rats (Charles River) were divided into two groups and housed four per cage. One group had free access to water and powdered fat-free, high-protein special diet (Nutritional Biochemicals, Cleveland, OH, U.S.A.). The other group was allowed the same diet but containing 1% (w/w) BHA (Sigma, St. Louis, MO, U.S.A.). Both groups were left on the diet for 6 days then starved 24 h before sacrifice. Liver microsomes were prepared separately from each liver according to Jerina *et al.*[5]. Microsomes were resuspended in 0.25 *M* sucrose and stored at −80°C for up to 3 weeks with no significant loss in activity. Protein was measured using a microbiuret method[17] with bovine serum albumin as standard.

Epoxide hydrolase assay

The assay mixture contained 20 μl of 0.5 *M* Tris–HCl buffer (pH 7.5), 10 μl 0.8% (v/v) Tween-80 and resuspended microsomes in a total volume of 70 μl. The assay mixture minus substrate was incubated at 37°C for 2 min at which time the reaction was initiated by the addition of 10 μl of a 25 m*M* solution of substrate (in acetonitrile) giving an initial substrate concentration of 3.1 m*M* in a final volume of

80 μl. The reaction was terminated by the addition of 100 μl of ice-cold spectral grade carbon tetrachloride, vortex mixing for 10 sec and placing the tubes on ice.

At the end of an experiment, tubes were revortexed for 30 sec. and centrifuged in a swinging bucket rotor at 4000 *g* for 2 min at 4°C to separate phases and sediment the microsomal protein to the water–carbon tetrachloride interface. An aliquot of the supernatant was removed (usually 60 μl) and diluted with an equal volume of acetonitrile. This solution was vortexed and centrifuged again to remove any precipitated protein. A 20-μl sample of the clear supernatant was injected on stream into the liquid chromatograph. The retention times (min) for the following compounds were: NP-diol, 1.1; NP-oxide, 2.5 (see Fig. 2, left).

To determine if any NP-diol was being extracted into the carbon tetrachloride layer, known amounts of NP-diol and water were mixed and extracted with carbon tetrachloride in the same proportions used in the assay. The extracted solution showed no significant decrease in absorbance at 315 nm over the unextracted mixture excluding the need for a correction for product recovery.

In some experiments we assayed for epoxide hydrolase activity with styrene oxide as substrate. The assay was identical to that with NP-oxide except for the chromatography conditions (see above). Quantitation of styrene glycol production (retention time 2.9 min) was difficult due to the inability to completely separate the product, styrene glycol, from a solvent front peak containing metabolites that absorb strongly at 260 nm, and the low extinction coefficient of styrene glycol at 260 nm. To assay for activity using styrene oxide as a substrate the assay mixtures contained from 1.5 to 2 mg microsomal protein and an incubation time of at least 15 min to allow for significant product formation. Despite these limitations it was possible to obtain values for epoxide hydrolase activity with styrene oxide as substrate that were close to published values using the radiometric assay[3].

RESULTS AND DISCUSSION

Our basic motivation in these studies was to find an epoxide hydrolase substrate that would yield a diol product that could be easily detected by absorbance monitoring. There are many epoxide hydrolase substrates containing chromophores (see ref. 1), but the absorption maxima of the derivative diols is generally in the ultraviolet region where many cellular metabolites also absorb. Hanzlik and Hilbert[18] reported their attempts to synthesize a photometric substrate for epoxide hydrolase, but the most promising, α-acetoxy-*p*-nitrostyrene, exhibited a relatively rapid rate of non-enzymatic hydrolysis.

We were attracted to the use of NP-oxide because (a) it was shown to be hydrolyzed to NP-diol [as monitored by thin-layer chromatographic (TLC) analysis] by a guinea pig liver microsomal preparation[19], and (b) its diol product, NP-diol, was shown to have an absorption maximum at 315 nm. The highly absorbing NP-diol ($\varepsilon = 11{,}000\ M^{-1}\ cm^{-1}$) was found as a product of the inactivation of pepsin by NP-oxide[16]. NP-oxide and NP-diol are readily resolved by reversed-phase liquid chromatography (Fig. 2, left) using absorbance detection at 315 nm.

To obtain a calibration curve for NP-diol, known quantities in acetonitrile–water (50:50) were chromatographed directly in 20-μl aliquots using the same chromatographic conditions described for the assay. Peak heights were used to quantitate

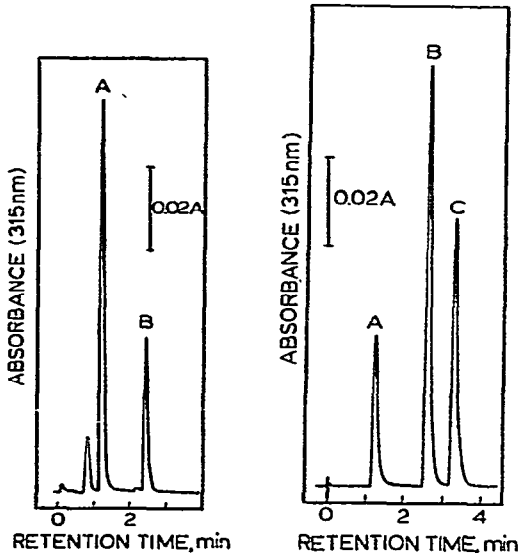

Fig. 2. Left: liquid chromatogram of an aliquot of a typical assay mixture. The mixture contained 0.36 mg microsomal protein with an incubation time of 7 min. After the standard extraction 20 μl of the 50% acetonitrile phase was injected. Peaks: A = NP-diol (3.7 nmol); B = unextracted NP-oxide remaining in the aqueous phase. Right: liquid chromatogram of NP-diol and NP-oxide with the addition of internal standard. Peaks: A = NP-diol (1.4 nmol); B = NP-oxide (3.7 nmol); C = *p*-nitroanisole (*ca.* 1.0 nmol).

the diol concentrations, and were found to be linear for the range of 0–9 nmol (injected). As little as 100 pmol could be reproducibly detected. If higher precision is required for the quantitation of NP-diol, an internal standard can be used. For example, *p*-nitroanisole, can be added to the acetonitrile used to dilute the aqueous extract. The same chromatographic conditions used in the assay will separate *p*-nitroanisole from NP-diol and NP-oxide, and still allow a total elution time of under 4 min (Fig. 2, right). After chromatography, the peak height ratio of NP-diol/*p*-nitroanisole can be calculated and plotted against nmol NP-diol injected to obtain a calibration curve.

Correction for non-enzymatic hydration of NP-oxide was not necessary since no detectable NP-diol was seen in boiled enzyme control incubations. The time course of the enzymatic hydration was linear to 10 min, and 7 min was routinely used as the standard assay time. The relationship of product formed *versus* microsomal protein concentration was also linear up to 0.8 mg protein in the assay.

Using this epoxide hydrolase assay we have determined the specific activity of the enzyme in liver microsomes from rats fed standard and BHA-supplemented diets. BHA has been shown to induce epoxide hydrolase in rodent tissues[3]. These data as well as assays using styrene oxide as substrate are shown in Table I. The specific activity of epoxide hydrolase in liver microsomes was 3.2 nmol NP-diol per min per mg protein, a value similar to that found with styrene oxide as substrate (Table I and ref. 3). The enzyme activity in liver microsomes from BHA-fed rats was 13 nmol NP-diol per min per mg protein, or an increase of approximately four-fold over the controls (Table I). A similar increase in epoxide hydrolase activity in BHA-fed animals was

seen with styrene oxide as substrate (Table I). These increases in enzyme activity are comparable to those previously noted in BHA-fed rats[3], where a radiometric assay was employed.

TABLE I

EFFECT OF DIETARY BHA ADMINISTRATION ON MICROSOMAL EPOXIDE HYDROLASE ASSAYED WITH NP-OXIDE AND STYRENE OXIDE

K_m values and error ranges were determined by the statistical method of Cleland[22].

Enzyme source	*Epoxide hydrolase activity (nmol product per min per mg protein)*		*K_m values (μM) with NP-oxide*
	*NP-oxide**	*Styrene oxide***	
Control microsomes	3.2±0.5	2.6	23±4
BHA-induced microsomes****	13 ±2	15	150±30

* Mean of activities from four separate liver microsome preparations ± standard deviation.

** Activity measured in a pool of liver microsome preparations from either control or BHA-fed animals.

*** Epoxide hydrolase was induced by adding BHA to the diet[3], as described in Materials and methods.

Using NP-oxide as substrate, K_m values for epoxide hydrolase from control and BHA-treated liver microsome preparations were determined (Table I). K_m values were 23 μM and 150 μM in control and BHA-treated microsomes, respectively. The higher K_m value for the induced microsomes could imply that a second form of the enzyme with lower affinity for NP-oxide was induced (see ref. 6), but a more detailed study is required to arrive at any definite conclusions.

When this work was nearly completed a report appeared describing the presence of a distinct epoxide hydrolase in mammalian liver cytosol[20]. The cytosolic enzyme was active with *trans*-β-methylstyrene oxide but not styrene oxide, in contrast to the microsomal form of the enzyme that is active with only the latter substrate. Both forms of the enzyme were active with allylbenzene oxide. Using rat liver cytosol we have detected significant epoxide hydrolase activity using NP-oxide[21]. Possibly NP-oxide will prove to be a useful substrate for assaying the cytosolic as well as microsomal forms of epoxide hydrolase.

As shown here NP-oxide is an excellent substrate for epoxide hydrolase. The assay method is sensitive and rapid, requiring only simple solvent extraction, and then liquid chromatography under isocratic conditions.

ACKNOWLEDGEMENT

This work was supported by grants from the University of Colorado Cancer Advisory Committee and Biomedical Science Support Grant.

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