Journal of Chromatography, 299 (1984) 445–449
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 16,946

Note

High-performance liquid chromatographic methods designed for metabolic studies of the proallatocidin precocene II

SCOTT J. GROSSMAN* and M. T. STEPHEN HSIA*

Department of Entomology and Environmental Toxicology Center, University of Wisconsin, Madison, WI 53706 (U.S.A.)

(First received April 19th, 1984; revised manuscript received May 17th, 1984)

The insect growth regulators, precocene I (7-methoxy-2,2-dimethylchromene) and precocene II (6,7-dimethoxy-2,2-dimethylchromene), have been shown to induce precocious metamorphosis and other symptoms of juvenile hormone deficiency in certain insects[1]. It has been proposed that precocenes act directly on the corpora allata, but require metabolic activation within the glands in order to exert their effect[2,3]. In addition, Hsia and coworkers[4,5] indicated that the generation of a reactive precocene 3,4-epoxide may be an important event in the hepatotoxic action of precocene II seen in rats. Because precocene II is now widely used as a molecular probe in experimental arthropod endocrinology, there is an increasing need for developing efficient separation methods to be used in various metabolic studies. This paper describes several high-performance liquid chromatographic (HPLC) methods which are suitable for the metabolic studies of either non-radioactive or radiolabeled precocene II.

EXPERIMENTAL

All solvents used in this study were HPLC grade (Fisher Scientific) and water was twice glass-distilled. Both radiolabeled and non-radioactive precocene II were synthesized as previously reported[4]. Analytical standards of *cis*- and *trans*-precocene-3,4-dihydrodiols (6,7-dimethoxy-2,2-dimethylchromane-3,4-diol), 3-hydroxyprecocene (3-hydroxy-6,7-dimethoxy-2,2-dimethylchromane), and 6-hydroxyprecocene (6-hydroxy-7-methoxy-2,2-dimethylchromene) were prepared in this laboratory[4]. All other reagents were commercially available.

For the kinetic studies, precocene II was incubated with rat liver microsomes in an NADPH-generating system[6]. Assays for precocene levels were done by withdrawing 1-ml aliquots of the incubation mixture and quenching in 3 ml of ice-cold water. The solutions were immediately extracted twice with 5 ml of ethyl acetate, and the organic extracts concentrated and redissolved in acetonitrile–ethanol (1:1) for HPLC analysis. This simple extraction procedure was found to yield a recovery ef-

* Submitted in partial fulfillment of the requirements for the degree of Master of Science in Environmental Toxicology.

0021-9673/84/$03.00

ficiency of 92.3 ± 3.6% when boiled microsomes and non-radiolabeled precocene II were used under the same experimental conditions.

Analysis of all samples was carried out with an Altex Model 312 MP liquid chromatograph equipped with a Model 152 dual-wavelength detector. Two basic reversed-phase HPLC systems were used. For isocratic HPLC analysis, a C_{18} Ultrasphere ODS column (5 μm, 250 × 4.6 mm I.D.) connected with an ODS precolumn (10 μm, 40 × 3.2 mm I.D.) was used. For the reversed-phase gradient HPLC analysis, the same column was used in combination with a low-cost linear-gradient generator. This system was composed of the Altex Model 100A solvent pump and an ISCO Model D density gradient fractionator. For normal-phase HPLC analysis of the stereoisomeric mixture of *cis*- and *trans*-precocene-3,4-dihydrodiols, a LiChrosorb DIOL column (10 μm, 250 × 4.6 mm I.D.; Laboratory Data Control) was used.

RESULTS AND DISCUSSION

Several HPLC methods have been employed in the structural elucidation of various metabolites derived from radiolabeled precocene II[7,8]. However, the need for a rapid and simple HPLC analytical method suitable for kinetic studies using non-radioactive substrate was not fulfilled by any of these published procedures. We now have developed an isocratic reversed-phase HPLC method which is capable of separating precocene II from all of its metabolites in less than 8 min (Fig. 1). Minimal sample clean-up was required. With the aid of a standard curve obtained by plotting

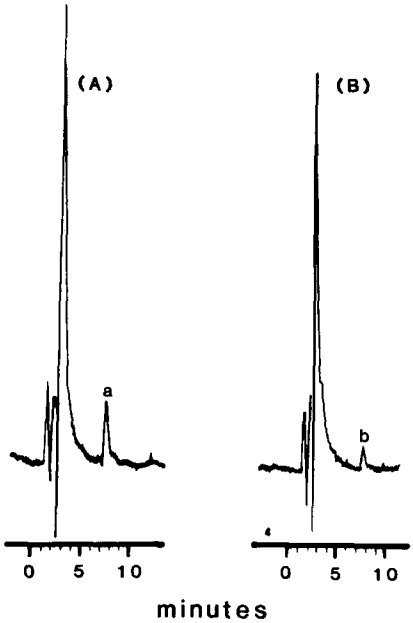

Fig. 1. Representative chromatograms of non-radioactive precocene II in the organic extracts of rat liver microsomes. (A) Peak a corresponds to 100 ng of unmetabolized precocene II. (B) Peak b corresponds to 20 ng of unmetabolized precocene II. In both chromatograms, the various polar metabolites are unresolved and eluted immediately following the solvent front.

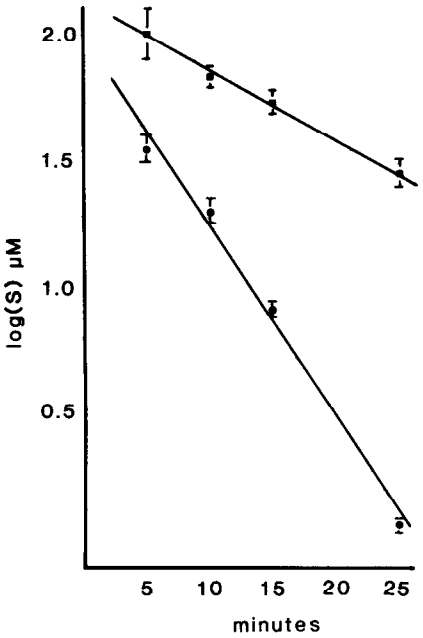

Fig. 2. First-order disappearance of precocene II in a microsome-mediated, NADPH-O_2 dependent system. Each data point represents concentration of precocene II remaining in the incubation media at specified time from initial concentration of 200 μM (■) or 80 μM (●). Expressed as mean ± S.E.M. of triplicate samples.

known amounts of precocene II *versus* the peak height, non-radioactive material may be used in these metabolic studies. When monitoring at the wavelength of 280 nm and the absorbance range of 0.005 a.u.f.s., a 10-ng detection limit of precocene II was attained. The sensitivity of our technique could almost double if a variable-wavelength detector set at 323 nm is used. By using this HPLC method, the *in vitro* metabolism of precocene II by rat liver mitrosomes can be easily monitored. The

TABLE I

RATES OF PRECOCENE II DISAPPEARANCE IN A MICROSOME-MEDIATED, NADPH-O_2 DEPENDENT SYSTEM

Data obtained from plots of log (precocene II)$_{remaining}$ *versus* time at specified precocene II concentration. Assay samples were taken and analyzed at four time points (5, 10, 15 and 25 min after incubation started) for each concentration.

*Parameter**	*Substrate concentration (mM)*			
	0.08	*0.12*	*0.16*	*0.20*
Slope	−0.078	−0.047	−0.028	−0.027
k	0.034	0.020	0.012	0.012
r	0.994	0.961	0.989	0.997

* k = First-order rate constant (min^{-1}); r = correlation coefficient obtained by linear regression analysis.

TABLE II

RETENTION TIMES OF PRECOCENE II AND ITS POTENTIAL METABOLITES DETERMINED BY FOUR HPLC ANALYTICAL SYSTEMS

Column	*Solvent system*	*Retention time (min)**					
		PII	*3-OH PII*	*4-OH PII*	*6-OH PII*	*cis-PII diol*	*trans-PII diol*
C_{18} ODS	Gradient, 15% acetonitrile in 1 m*M* citrate buffer (pH 4.7) to 70% acetonitrile in 1 m*M* citrate buffer (pH 4.7); flow-rate, 2 ml/min	18.37	11.95	10.57	15.60	8.00	8.00
C_{18} ODS	Gradient, 15% acetonitrile in 1 m*M* citrate buffer (pH 3.8) to 60% acetonitrile in 1 m*M* citrate buffer (pH 3.8); flow-rate, 2 ml/min	19.35	10.89	9.42	14.20	6.24	6.24
C_{18} ODS**	Isocratic, 55% methanol in 1 m*M* KH_2PO_4 (pH 5.75); flow-rate, 1 ml/min	36.17	13.48	11.36	18.55	5.45	5.45
LiChrosorb DIOL	Isocratic, dichloromethane; flow-rate, 1 ml/min					6.48	14.01

* PII = precocene II; 3-OH PII = 3-hydroxyprecocene; 4-OH PII = 4-hydroxyprecocene; 6-OH PII = 6-hydroxyprecocene; PII diol = precocene-3,4-dihydrodiol.

** Two C_{18} Spherisorb ODS columns (10 μm, 250 × 4.6 mm I.D.) connected in series were used.

enzyme-mediated biotransformation of precocene II was rapid and displayed first-order kinetics with respect to substrate concentrations in the range of 80–200 μM (Fig. 2). The calculated first-order rate constants are shown in Table I. The data indicated an apparent V_{max} of 9.5 nmole/min/mg protein as calculated from the Lineweaver–Burk reciprocal plot.

Although the preceding HPLC method has proven to offer a simple and efficient means to study the kinetics of precocene II metabolism, it was not designed for the separation of the various metabolites. Thus, we proceeded to investigate other HPLC solvent systems and columns which may be used for the analysis and isolation of closely related precocene II metabolites. The primary objective was to develop an analytical system capable of separating the following components: precocene II, 3-hydroxyprecocene, 4-hydroxyprecocene, 6-hydroxyprecocene, and the *cis*- and *trans*-dihydrodiol derivatives of precocene II. Using the C_{18} ODS reversed-phase column, the performance of the following isocratic solvent systems was evaluated: acetonitrile–water (50:50); methanol–water (65:35); tetrahydrofuran–water (30:70); and acetonitrile–methanol–water (25:32.5:42.5). Due to peak broadening and/or lack of resolution, none of these solvent systems provided satisfactory results. However, further investigation has demonstrated the successful performance of several new HPLC procedures as shown in Table II. Although both gradient elution methods produced satisfactory results, the isocratic elution procedure using 55% methanol in 1 mM potassium dihydrogen phosphate (KH_2PO_4) was slightly favored as the method of our choice because of its convenience. Nonetheless, all three procedures failed to resolve the stereoisomeric *cis*- and *trans*-dihydrodiol derivatives of precocene II, which were separated by the normal-phase HPLC system using a DIOL column and dichloromethane. It is noteworthy that Sephadex LH-20 column chromatography, though less convenient, was also capable of separating these two stereoisomers[4]. The utility of the chromatographic methods as described in this report was demonstrated by our recent success to isolate and identify a 30:70 *cis:trans* precocene-3,4-dihydrodiol metabolite generated by freshly isolated rat hepatocytes[9]. The availability of these HPLC methods may provide impetus for further metabolic and pharmacokinetic studies of precocene II in other biological systems.

ACKNOWLEDGEMENTS

This work was supported in part by the College of Agricultural and Life Sciences, University of Wisconsin-Madison, and the NIH Biomedical Research Support Program.

REFERENCES

1 W. S. Bowers, T. Ohta, J. S. Clure and P. A. Morsella, *Science*, 193 (1976) 542.
2 G. E. Pratt, R. C. Jennings, A. F. Hamnett and G. T. Brooks, *Nature (London)*, 284 (1980) 320.
3 G. E. Pratt, R. C. Jennings and A. F. Hamnett, in F. Sehnal, A. Zabza, J. J. Menn and B. Cymborowski (Editors), *Regulation of Insect Development and Behavior*, Technical University of Wroclaw, 1981, p. 347.
4 M. T. S. Hsia, S. J. Grossman and K. R. Schrankel, *Chem. Biol. Interact.*, 37 (1981) 265.
5 S. J. Grossman and M. T. S. Hsia, *Toxicology*, 25 (1982) 293.
6 M. T. S. Hsia and B. L. Kreamer, *Chem. Biol. Interact.*, 34 (1981) 19.
7 D. M. Soderlund, A. Messeguer and W. S. Bowers, *J. Agric. Food Chem.*, 28 (1980) 724.
8 B. J. Bergot, K. J. Judy, D. A. Schooley and L. W. Tsai, *Pestic. Biochem. Physiol.*, 13 (1980) 95.
9 M. T. S. Hsia and B. L. Kreamer, unpublished results.