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Note

High-performance liquid chromatographic separation of the two estrogen isomers of estradiol with electrochemical detection

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Catechol estrogens, the 2- or 4-hydroxylated metabolites of phenolic estrogens, have been the focus of increasing attention in recent years. They are major metabolites of estrogens present in the circulation and are formed within certain estrogen target organs[1]. Investigation of the labile catechol estrogens is complicated by rapid oxidative degradation that occurs during isolation and purification. This degradation can be decreased, but not eliminated when separations are carried out under reducing conditions, for example, by using ascorbic acid-saturated plates for thin-layer chromatography[2]. In the course of developing an assay for estrogen-2/4-hydroxylase[3], we explored the use of high-performance liquid chromatography (HPLC), coupled with electrochemical detection, for separating the labelled products, 2- and 4-hydroxyestradiol, formed during an *in vitro* incubation of brain tissue with a radioactive estradiol substrate.

The polarographic (electrochemical) detection method, coupled with HPLC is considered to be one of the most sensitive methods for detection of catechol compounds[4–6]. Greater sensitivity and reproducibility and lower interference from impurities make this an attractive system for separation of the catechol metabolites of estrogen. We report here on the HPLC separation of the two isomers of catechol estrogens, 2-OHE_2 from 4-OHE_2, with electrochemical detection. This technique was shown to be applicable to isolation of small amounts of radioactive product formed by estrogen-2/4-hydroxylase from brain *in vitro*[3], and for verifying the purity of radioactive enzymatically synthesized 2-OHE_2 (ref. 7).

MATERIALS AND METHODS

A Hewlett-Packard Model 1081 B liquid chromatograph (Palo Alto, CA, U.S.A.) was used, equipped with an automatic injector, and with a 10-μm HP C_{18} reversed-phase column (250 × 4.6 mm I.D.). A digital multimeter (Hewlett-Packard 3465 A) was used for zeroing the detector and for on-line monitoring of the output. The control of a Rheodyne 5001 (Cotati, CA, U.S.A.) three-way pneumatic valve and a LKB Ultrarack (Rockville, MD, U.S.A.) fraction collector was integrated into the events board of the instrument. These additions, along with the auto-injector, allowed fully automated separation and collection of samples. For detection, a glass-

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TABLE I

RELATIVE RETENTION TIMES OF ESTRADIOL AND METABOLITES

Mobile phase: varied as indicated and made 0.2 *N* with respect to acetic acid.

Solvent system		*Compounds*			
Water (%)	*Methanol (%)*	*4-OHE₂*	*2-OHE₂*	*E₂*	*2-Methoxy-E₂*
65	35	0.40	0.50	1.00 (24.25 min)	–
62	38	0.39	0.50	1.00 (21.36)	–
60	40	0.41	0.51	1.00 (19.31)	1.32
55	45	0.44	0.53	1.00 (12.11)	–
35	65	–	0.69	1.00 (8.65)	1.15

carbon electrochemical device (Bio-Analytical Systems, West Lafayette, IN, U.S.A.), was used at an applied potential of +0.86 V *vs.* the reference electrode.

Glass-distilled methanol (Burdick & Jackson, Muskegon, MI, U.S.A.), acetic acid (Baker, Phillipsburg, NJ, U.S.A.) and deionized water were filtered through a Millipore vacuum filtration device (Millipore, Bedford, MA, U.S.A.). The solvent systems consisted of methanol and water in various proportions, made 0.2 *N* with respect to glacial acetic acid (pH 2.9). Solvents were degassed immediately before use. Separations were carried out at ambient temperature at an attenuation level of 10. The flow-rate of the mobile phase was 3 ml/min.

The steroids and their sources were: estradiol [1,3,5(10)-estratriene-3,17β-diol], Steraloids; 2-methoxy-estradiol [1,3,5(10)-estratriene-3,17β-diol-2-methylether], Research Plus; 2-OHE_2 [1,3,5(10)-estratriene-2,3,17β-triol] and 4-OHE_2 [1,3,5(10)-estratriene-3,4,17β-triol] were supplied by Dr. K. I. H. Williams (Worcester Foundation, Shrewsbury, MA, U.S.A.). All steroids were injected in 10 μl methanol containing ascorbic acid (1 m*M*) as an anti-oxidant.

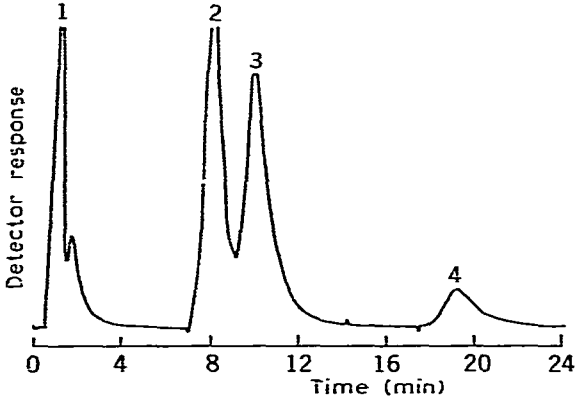

Fig. 1. Separation of isomeric catechol estrogens. Peaks: 1 = ascorbic acid; 2 = 4-OHE_2; 3 = 2-OHE_2; 4 = E_2. Conditions: reversed-phase C_{18} column (250 × 4.6 mm I.D.); mobile phase water–methanol (60:40) 0.2 *N* with respect to acetic acid; electrochemical detection at +0.86 V; flow-rate 3 ml/min; quantity of steroids 1 μg each.

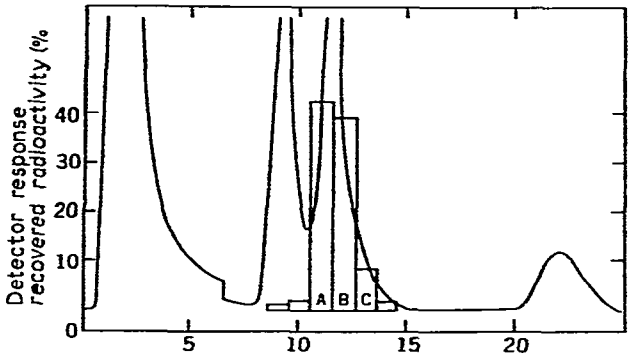

Fig. 2. Correspondence of radioactivity from bio-synthesized 2-OHE_2 with authentic 2-OHE_2. Conditions as in Fig. 1. Bars, radioactivity in percent of total recovered per minute. Bars A, B and C account for approximately 91% of recovered radioactivity. Fractions not represented by bars contained less than 1% of the recovered radioactivity.

RESULTS AND DISCUSSION

Different absolute retention times for estradiol and its metabolites were obtained by varying the proportions of methanol and water in the mobile phase. The relative retention times remained constant over the range of concentrations shown in Table I until the ratio of methanol to water exceeded 1. To separate 2- and 4-OHE_2 from each other and from estradiol, we chose water–methanol (60:40). This solvent system offered the shortest absolute retention times while maintaining adequate separation (> 1.5 min) of the catechol isomers of E_2. The ascorbic acid used as an antioxidant was eluted from the column near the solvent peak and did not interfere with the detection of the estrogens (Fig. 1).

The separations afforded by this system allowed us to examine the chemical purity of our enzymatically synthesized radiolabelled 2-OHE_2. It was injected with non-labelled carrier, and fractions were collected directly in scintillation vials. Purity was established by correspondence of the radioactivity eluted with the authentic 2-OHE_2 standard (Fig. 2).

This separatory technique was also applied to a quantitative assay for brain 2/4-hydroxylase activity[3]. The tissue was incubated with 6,7-tritiated estradiol as the substrate. The quantities of radiolabelled catechol estrogens formed during the incubations were in the picogram (fmole) range. Therefore, they were diluted with authentic catechol estrogens to permit visualizing the compounds in the chromatogram and to protect them from spontaneous oxidative degradation. By injecting the radioactive products with microgram amounts of carrier and using a low attenuation level, recordings with minimal noise were obtained. Degradative losses were also minimized and corrected for by using an internal ^{14}C-labelled standard.

Separation of estrogen metabolites by HPLC coupled with electrochemical detection has been recently reported by Shimada *et al.*[8,9]. The present paper is the first report of separation of the hydroxylated metabolites of estradiol from the parent molecule by HPLC with the benefits of electrochemical detection. We have taken advantage of this resolution, and here report on the efficacy of this fully automated separatory system to measure the enzymatic hydroxylation of estradiol.

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