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Note

Studies on steroïds

CL*. Separation of catechol estrogens by high-performance liquid chromatography with electrochemical detection

KAZUTAKE SHIMADA, TOUICHI TANAKA and TOSHIO NAMBARA**
Pharmaceutical Institute, Tohoku University, Sendai (Japan)
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Catechol estrogens are now recognized as the major metabolites of estradiol in human and other species[1]. Recent studies disclosed that these substances are not necessarily final biotransformation products but active metabolites having biological and endocrine potencies. The instability of catechol estrogens under aerobic conditions, as well as their relatively low concentrations in biological materials, has prevented previous attempts at determining these compounds. Recently, Paul and Axelrod[2] developed a sensitive radioenzymatic method for determining catechol estrogens. In addition, radioimmunoassay of 2-hydroxyestrone has also been reported by several groups[3–5]. These methods, however, have the disadvantage that radioactive compounds are prerequisite.

The present paper deals with the separation of catechol estrogens and related compounds by high-performance liquid chromatography (HPLC) with an electrochemical detector (ElCD).

EXPERIMENTAL

Instruments

The apparatus used was a Waters Model ALC/GPC 202 high-performance liquid chromatograph, equipped with an ultraviolet (UV) detector (Waters Assoc., Milford, Mass., U.S.A.) or a Yanagimoto Model VMD-101 ElCD (Yanagimoto Co., Kyoto, Japan). The UV detector was used for monitoring the absorbance at 280 nm. The potential of the ElCD was set at +0.7–1.1 V *vs.* the silver–silver chloride reference electrode. The samples were introduced by means of a Model U6K sample loop injector (Waters Assoc.) with an effective volume of 2 ml. The μPorasil (1 ft. × 1/4 in. I.D.) and μBondapak C_{18} (1 ft. × 1/4 in. I.D.) columns (Waters Assoc.) were used under ambient conditions.

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** To whom correspondence should be addressed.

Materials

2-Hydroxyestrone (2-OHE_1), 4-hydroxyestrone (4-OHE_1), 2-hydroxyestradiol (2-OHE_2), 4-hydroxyestradiol (4-OHE_2), 2-hydroxyestrone 1-glutathione thioether (2-OHE_1-1-GS), 2-hydroxyestrone 4-glutathione thioether (2-OHE_1-4-GS), 2-hydroxyestrone 3-methyl ether (3-$MeOE_1$), 2-methoxyestrone (2-$MeOE_1$) and 2-methoxyestradiol (2-$MeOE_2$) were prepared by the methods previously reported from this laboratory. Estrone (E_1), estradiol (E_2) and estriol (E_3) were kindly donated by Teikoku Hormone Mfg. Co. (Kawasaki, Japan). All reagents employed were of analytical grade. Solvents were purified by distillation prior to use.

RESULTS AND DISCUSSION

Initially, separation of a mixture of catechol estrogens on a normal-phase column was undertaken. The use of cyclohexane–ethyl acetate as a mobile phase gave broad peaks, but on addition of acetic acid to the solvent system the peak broadening was efficiently suppressed. Two pairs of positional isomers of catechol estrogens were resolved when chromatographed on a μPorasil column with cyclohexane–ethyl acetate–acetic acid, as shown in Fig. 1a. Separation of 2-OHE_1 and 4-OHE_2 was improved by increasing the ratio of cyclohexane in the mobile phase (see Table I). As illustrated in Fig. 1b, complete resolution of 2-$MeOE_1$, 3-$MeOE_1$ and E_1 was attained by using cyclohexane–ethyl acetate–acetic acid (100:5:1) as the mobile phase.

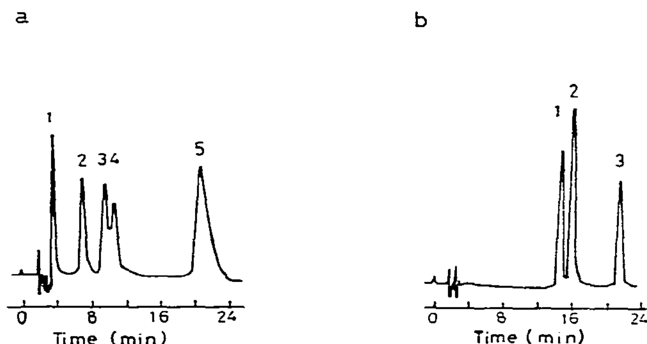

Fig. 1. (a) Separation of catechol estrogens on a normal-phase column. Peaks: 1 = E_1; 2 = 4-OHE_1; 3 = 2-OHE_1; 4 = 4-OHE_2; 5 = 2-OHE_2. Conditions: μPorasil column; mobile phase, cyclohexane–ethyl acetate–acetic acid (500:100:9), 2 ml/min; detection, Waters UV detector (280 nm). (b) Separation of isomeric 2-hydroxyestrone monomethyl ethers. Peaks: 1 = 2-$MeOE_1$; 2 = 3-$MeOE_1$; 3 = E_1. Mobile phase: cyclohexane–ethyl acetate–acetic acid (100:5:1), 2 ml/min; other conditions as in (a).

It is reasonably well substantiated that 2-OHE_1 undergoes both *in vitro* and *in vivo* O-methylation, producing the isomeric monomethyl ethers, 2-$MeOE_1$ and 3-$MeOE_1$. Separation of the two positional isomers has been attempted by paper chromatography and thin-layer chromatography[2]. These chromatographic procedures, however, are somewhat tedious and unsatisfactory for complete resolution. HPLC under the conditions established in this study is much more effective for resolution of these two compounds than the known methods. For normal-phase chromatography,

TABLE I

RELATIVE RETENTION TIMES OF CATECHOL ESTROGENS

Mobile phase: A = cyclohexane–ethyl acetate–acetic acid (175:25:3); B = acetonitrile–0.5% $NH_4H_2PO_4$ (pH 3.0) (1:2); C = methanol–0.5% $NH_4H_2PO_4$ (pH 3.0) (7:6). Detection, Waters UV detector (280 nm).

Compound	*μPorasil*	*μBondapak C_{18}*	
	A (*2 ml/min*)	*B* (*2 ml/min*)	*C* (*2 ml/min*)
2-OHE_1	2.67	0.54	0.59
2-OHE_2	8.27	0.46	0.76
4-OHE_1	1.75	0.62	0.62
4-OHE_2	3.08	0.49	0.68
2-$MeOE_1$	1.00	1.13	1.82
2-$MeOE_2$	2.25	0.87	2.27
3-$MeOE_1$	1.00	1.18	1.65
E_2	1.83	0.77	1.21
E_3	>10	0.23	0.38
E_1	1.00 (4.8 min)	1.00 (15.0 min)	1.00 (11.2 min)

the highly sensitive ElCD is not applicable, but the sensitivity obtained with the UV detector (500 ng per injection: signal-to-noise ratio 10 at 0.04 a.u.f.s.) seems adequate for determination of catechol estrogens in human late-pregnancy urine.

Next, we investigated the separation of catechol estrogens on a reversed-phase column combined with ElCD. Preliminary experiments indicated that basic solvent systems did not give satisfactory chromatograms. The use of a mobile phase containing ammonium dihydrogen phosphate, adjusted to pH 3.0, was found to be effective for separation. As illustrated in Fig. 2, almost all compounds were clearly resolved under isocratic conditions. Satisfactory separation of 2-OHE_2 and 4-OHE_2 was not obtained under these conditions. However, these isomeric catechols were evidently distinguishable on the normal-phase column (see Table I).

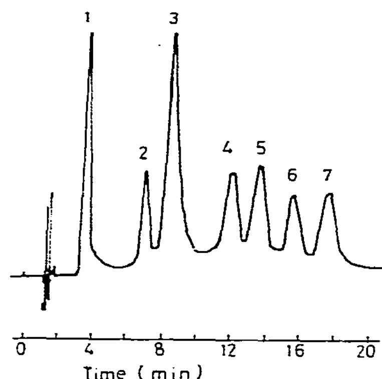

Fig. 2. Separation of a mixture of principal estrogens. Peaks: 1 = E_3; 2 = 2-OHE_2; 3 = 2-OHE_1; 4 = E_2; 5 = 2-$MeOE_2$; 6 = E_1; 7 = 2-$MeOE_1$. Conditions: μBondapak C_{18} column; mobile phase, acetonitrile–0.5% $NH_4H_2PO_4$ (pH 3.0) (1:2), 2 ml/min; detection, Waters UV detector (280 nm).

The elution order of the corresponding 17-keto and 17-hydroxyl compounds (relative retention time: $E_1 > E_2$, 2-OHE$_1$ > 2-OHE$_2$ and 2-MeOE$_1$ > 2-MeOE$_2$) in acetonitrile–phosphate buffer was reversed when methanol–phosphate buffer was employed as the mobile phase. The combined use of the two mobile phases may serve for the qualitative and quantitative analysis of these compounds with high reliability. The retention times of catechol estrogens relative to estrone, obtained with μPorasil and μBondapak C_{18} columns under identical conditions, are listed in Table I.

Separation of the isomeric glutathione conjugates of 2-hydroxyestrone, 2-OHE$_1$-1-GS and 2-OHE$_1$-4-GS, has been carried out by thin-layer chromatography on Avicel microcrystalline cellulose and by gel chromatography on Sephadex G-25[6,7]. However, these procedures are not satisfactory, because separation is incomplete and time-consuming. As shown in Fig. 3, the two positional isomers were readily resolved when chromatographed on a μBondapak C_{18} column by use of acetonitrile–0.5% ammonium dihydrogen phosphate, adjusted to pH 3.0 with phosphoric acid, as a mobile phase.

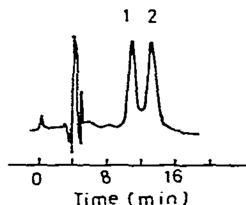

Fig. 3. Separation of isomeric 2-hydroxyestrone glutathione thioethers. Peaks: 1 = 2-OHE$_1$-1-GS; 2 = 2-OHE$_1$-4-GS. Conditions: μBondapak C_{18} column; mobile phase, acetonitrile–0.5% $NH_4H_2PO_4$ (pH 3.0) (1:3), 1 ml/min; detection, Waters UV detector (280 nm).

A sensitive and selective ElCD is widely used for HPLC of phenolic compounds, in particular catecholamines, in biological materials[8,9]. The utilization of ElCD for HPLC of phenolic steroids has not previously been attempted. The effect of the applied potential on the sensitivity in electrochemical detection was tested for typical estrogens in the range of +0.7–1.1 V *vs.* the silver–silver chloride electrode. The results obtained with 30 ng each of 2-OHE$_1$, 2-MeOE$_1$ and E_3 per injection are shown in Fig. 4. The detector gave a linear response up to +1.0 V and then showed a plateau. As a compromise between sensitivity and stability, the applied potential was set at +0.8–1.0 V *vs.* the reference electrode. Typical chromatograms obtained with catechol estrogens and classical estrogens are illustrated in Fig. 5a and b, respectively. It is to be noted that the responses of ElCD for catechol estrogens and their methyl ethers were almost identical. The detection limits of 2-OHE$_2$ and E_3 were determined to be 1 ng and 5 ng per injection (signal-to-noise ratio 10), respectively, when acetonitrile was used as a mobile phase. These results imply that the HPLC–ElCD method should be capable of determining catechol estrogens in rat brain[2].

To the best of our knowledge, this is the first reported separation of catechol estrogens and related compounds by HPLC–ElCD. The application of the present method to biological specimens is being conducted in this laboratory; the details will be reported elsewhere.

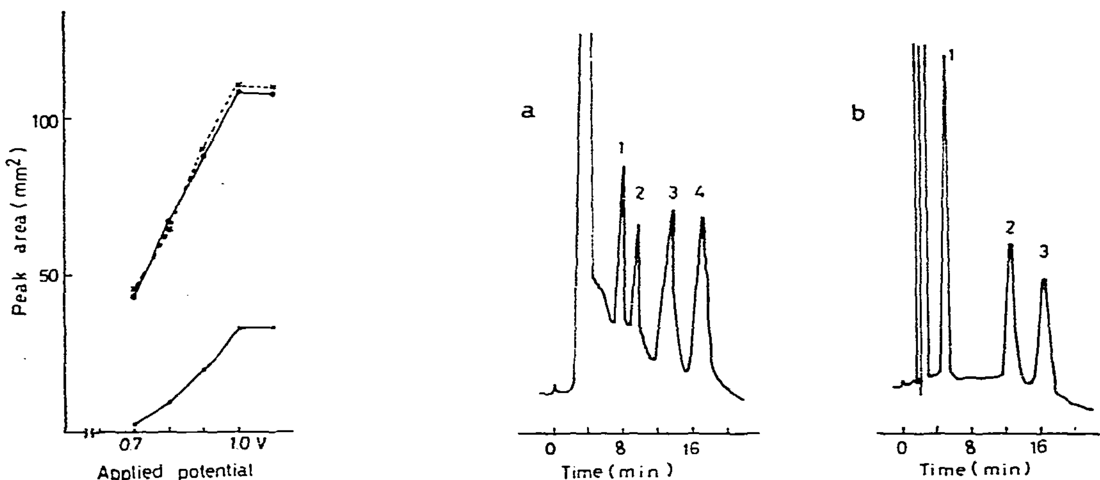

Fig. 4. Relationship between applied potential and sensitivity in electrochemical detection. ●, E_3 (30.5 ng); ×, 2-OHE$_1$ (31 ng); ○, 2-MeOE$_1$ (30 ng). Conditions: μBondapak C_{18} column; mobile phase, methanol–0.5% $NH_4H_2PO_4$ (pH 3.0) (6:5), 1 ml/min; detection, Yanagimoto Model VMD-101 ElCD, 16 nA full scale.

Fig. 5. (a) Separation of catechol estrogens on a reversed-phase column. Peaks: 1 = 2-OHE$_2$ (4.5 ng); 2 = 2-OHE$_1$ (3.1 ng); 3 = 2-MeOE$_2$ (5.3 ng); 4 = 2-MeOE$_1$ (6.0 ng). Conditions: μBondapak C_{18} column; mobile phase, acetonitrile–0.5% $NH_4H_2PO_4$ (pH 3.0) (2:3), 1 ml/min; detection, Yanagimoto Model VMD-101 ElCD, potential 0.8 V, 4 nA full scale. (b) Separation of classical estrogens. Peaks: 1 = E_3 (25 ng); 2 = E_2 (25 ng); 3 = E_1 (25 ng). Conditions as in (a).

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REFERENCES

1 H. P. Gelbke, P. Ball and R. Knuppen, *Advan. Steroid Biochem. Pharmacol.*, 6 (1977) 81.
2 S. M. Paul and J. Axelrod, *Science*, 197 (1977) 657.
3 I. Yoshizawa and J. Fishman, *J. Clin. Endocrinol. Metab.*, 32 (1971) 3.
4 P. Ball, G. Emons, O. Haupt, H.-O. Hoppen and R. Knuppen, *Steroids*, 31 (1978) 249.
5 S. C. Chattoraj, A. S. Fanous, D. Cecchini and E. W. Lowe, *Steroids*, 31 (1978) 375.
6 E. Kuss, *Hoppe-Seyler's Z. Physiol. Chem.*, 350 (1969) 95.
7 M. Numazawa and T. Nambara, *J. Steroid Biochem.*, 8 (1977) 835.
8 P. T. Kissinger, C. Refshauge, R. Dreiling and R. N. Adams, *Anal. Lett.*, 6 (1973) 465.
9 H. Hashimoto and Y. Maruyama, *J. Chromatogr.*, 152 (1978) 387.