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Note

High-performance liquid chromatographic separation of catechol estrogens: use as a screening procedure for evaluation of *in vitro* metabolism of [^{3}H]estradiol

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Recent studies suggest that catechol metabolites of estradiol may be important modulators of the biological effects of estradiol[1–3]. Exploration of the estradiol/catechol estrogen relationships would be greatly facilitated if the estradiol-metabolizing activity of a tissue could be easily assessed under a variety of conditions. The lability of catechol estrogens *in vitro* and the structural similarity of the various catechol molecules have made evaluation of their production by tissues difficult and time consuming[4–10]. In this study, a normal-phase isocratic high-performance liquid chromatographic (HPLC) technique is presented which separates estrone, estradiol and their 2- and 4-hydroxy metabolites in one chromatographic step. This HPLC technique, used in conjunction with an *in vitro* incubation system employing a radioactive precursor, provides a simple means of screening tissues for capacity to produce catechol estrogens.

A pilot study of estradiol metabolism by rat uterine paracervical ganglion is included to serve as an example of the information that can be gained with this technique. This tissue was selected for study, as the activities of nerves emanating from this ganglion are known to be altered by administration of estradiol[11,12]. One aspect of this estrogen responsiveness could be the metabolism to catechol forms capable of interacting with local neurotransmitter regulatory mechanisms[13–15]. The data provide evidence that local metabolism is possible and that the array of metabolites formed may vary with the stage of the estrous cycle.

EXPERIMENTAL

The tritiated compounds [6,7-^{3}H]estrone, (6,7-^{3}H)estradiol, [6,7-^{3}H)estriol and [2,4,6,7,16,17-^{3}H)estradiol were purchased from New England Nuclear (Boston, MA, U.S.A.). All non-radioactive estrogens were obtained from Steraloids (Wilton, NH, U.S.A.) and Fremy's salt (potassium nitrosodisulfonate) from ICN Pharmaceuticals (Plainview, NY, U.S.A.). Nicotinamide adenine dinucleotide, reduced form (NADPH), tetrasodium salt (Type X), isocitric dehydrogenase (Type IV), DL-isocitric acid (trisodium salt) and butylated hydroxytoluene (BHT) were obtained

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from Sigma (St. Louis, MO, U.S.A.). [6,7-^{3}H]-2- and -4-hydroxyestrone and [6,7-^{3}H]-2- and -4-hydroxyestradiol were prepared using the Fremy's salt technique of Gelbke *et al.*[16]. Prior to chromatographic separation of the hydroxyestrogens, the Fremy's salt reaction mixtures were treated with borate buffer to remove as much starting material as possible[17]. Extracts of the acidified borate buffer were taken to dryness under vacuum and applied to the chromatography system described below. Budget Solve Liquid Scintillation Cocktail (RPI, Elk Grove Village, IL, U.S.A.) was used for the determination of radioactivity.

High-performance liquid chromatography

The retention time of each estrogen was determined by chromatography on an Ultrasphere-Si (5 μm) 250 × 4.6 mm I.D. column (Rainin, Woburn, MA, U.S.A.) using as the mobile phase heptane–chloroform–acetic acid (75:25:5) containing 0.5% (w/v) of BHT. The flow-rate was maintained at 2.5 ml/min (1800 p.s.i.) and 250-drop (*ca* 4 ml) fractions were collected for a total of 100 fractions. Estrogens were prepared for application to the column by dissolving them in methanol or chloroform and adding appropriate solvents such that composition of the final solution was methanol–chloroform–heptane (1:1:2). Chromatographic fractions to be counted for radioactivity were evaporated to dryness under air, the residue was dissolved in 0.2 ml of methanol and 5 ml of liquid scintillation cocktail were added. Samples were counted to 5 min or 10,000 cpm in a Searle Delta 300 liquid scintillation counter with a 62% efficiency for tritium. BHT was omitted from the mobile phase in experiments defining retention times of non-radioactive estrogens because BHT absorbs UV light in the same wavelength range as the estrogens tested. In these experiments, each chromatographic fraction was read at 280 nm using a Beckman 35 spectrophotometer and peaks were identified by UV absorbance.

Pilot study of [^{3}H]estradiol metabolism by rat uterine paracervical ganglion

Animals. Sprague–Dawley rats (200 g) were housed six to a cage under a 12:12 light cycle. Estrous cycles were monitored via vaginal smears, and graded according to the criteria of Long and Evans[18].

Tissue preparation. Ganglia for metabolic studies were excised between 0800 and 1200 hours each day. Rats were anesthetized with pentobarbital (22.8 mg/kg, i.p.) and the paracervical ganglia were excised, trimmed of all fat and placed in ice-cold 0.15 *M* sodium chloride solution. Within 1 h of excision, ganglia from rats of the same estrous cycle stage were transferred to 200-μl glass homogenizers and homogenized in 0.1 *M* Tris–HCl buffer, pH 7.4 (10 μl per ganglion). Homogenates were transferred to conical polypropylene vials and the homogenizers rinsed twice with the volume of buffer used for the homogenization. The homogenates and rinse solutions were combined and stored at −20°C until analysis.

Metabolism studies and HPLC analysis. Ganglion homgenates were subjected to [2,4,6,7,16,17-^{3}H]estradiol metabolism studies within 1 week of excision. The incubation system chosen was based upon previous reports[6,19,20] and upon prior chromatographic evaluation of catechol estrogen formation by ganglia homogenates with the NADPH generating system and with varying concentrations of ascorbic acid. On the day of the incubation study, the homogenates were thawed and the homogenates of six ganglia of like cycle stage combined. Each ganglion homogenate pool was

incubated with 15 μCi (30 ng) of [^{3}H]estradiol in a total volume of 200 μl of 0.1 *M* Tris–HCl buffer containing $5 \cdot 10^{-7}$ *M* of ascorbic acid, 7 μM of NADPH, 2 μM of isocitric acid and 2 units of isocitric dehydrogenase. The control sample consisted of [^{3}H]estradiol incubated with the buffer and cofactors, but no tissue. Samples were incubated at 37°C in 1-ml stoppered glass containers with two pieces of PE50 tubing piercing the stopper. Oxygen–carbon dioxide (95:5) was delivered to the mixture via one piece of tubing. The outflow tubing was connected to a desiccant trap to prevent loss to the atmosphere of any [^{3}H]water formed from the hydroxylation of estradiol[21]. After 1 h of incubation, the metabolism of [^{3}H]estradiol was stopped by the addition of 100 μl of 0.014 *M* ice-cold ascorbic acid solution. [^{3}H]Estradiol and its metabolic products were extracted from the incubate with two 500-μl portions of ice-cold ethyl acetate. The extracts were evaporated to dryness in a vacuum centrifuge before adding 25 μl of ascorbic acid solution (3.75 g of ascorbic acid, 100 ml of methanol, 1 ml of acetic acid) to prevent oxidation of the products[4] and stored at −20°C. Just prior to chromatography, 25 μl of chloroform and 50 μl of heptane were added to each stored extract. Volumes of 20 μl were chromatographed as described above. Peak areas above the background were determined by triangulation. Analysis of water-soluble and protein-bound materials was not attempted in this study.

RESULTS

Fig. 1 shows the following elution patterns: (A) products of Fremy's salt reaction with [^{3}H]estrone, (B) products of Fremy's salt reaction with [^{3}H]estradiol and (C) resolution of a mixture of (A) and (B). Fig. 1 verifies that the products of the Fremy's salt reactions with estrone and estradiol are each well resolved and that the system is capable of resolving all of these materials should they occur in the same sample.

Fig. 2 gives data obtained from the chromatography of individual estrogen standards. Elution of estrone and estradiol in both the radioactive and non-radioactive forms are included and reveal no effects of sample concentration on retention time in this system. Elution patterns for 2-hydroxyestriol and estetrol are not presented

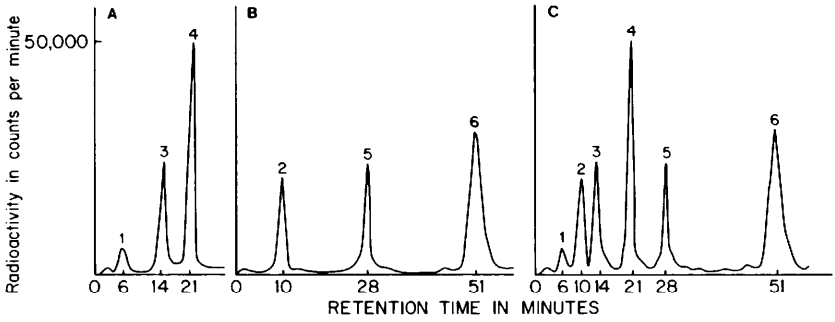

Fig. 1. Retention time in minutes of the products of Fremy's salt reaction with [^{3}H]estrone or [^{3}H]estradiol: (A) products from reaction with [^{3}H]estrone; (B) products from reaction with [^{3}H]estradiol; (C) a mixture of (A) and (B). Conditions: column, 250 × 4.6 mm I.D. Ultrasphere-Si (5 μm); mobile phase, heptane–chloroform–acetic acid (75:25:5) containing 0.5% (w/v) of BHT; flow-rate, 2.5 ml/min. Peaks: 1 = estrone; 2 = estradiol; 3 = 4-hydroxyestrone; 4 = 2-hydroxyestrone; 5 = 4-hydroxyestradiol; 6 = 2-hydroxyestradiol.

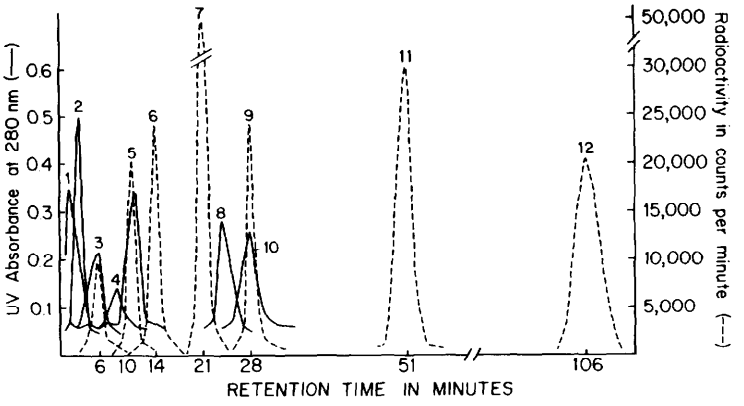

Fig. 2. Retention time in minutes of various estrogens detected either by UV absorbance or by amount of radioactivity. Conditions as in Fig. 1. Peaks: 1 = 3-methoxyestrone; 2 = 3-methoxyestradiol; 3 = estrone; 4 = 3-methoxyestriol; 5 = estradiol; 6 = 4-hydroxyestrone; 7 = 2-hydroxyestrone; 8 = 16-ketoestradiol; 9 = 4-hydroxyestradiol; 10 = 16-hydroxyestrone; 11 = 2-hydroxyestradiol; 12 = estriol.

because these unlabelled materials could not be dissolved in the application solvents in high enough concentration to be detected by UV absorbance. 16-Hydroxyestrone appears to elute only slightly ahead of 4-hydroxyestradiol. Therefore, a peak of either of these materials in a tissue incubation extract would be identified as 4-hydroxyestradiol/16-hydroxyestrone.

Table I shows the distribution of radioactivity in the control and paracervical ganglion incubates among the metabolite peaks and summarizes the data generated by the HPLC screening technique. The control incubation of [^{3}H]estradiol without

TABLE I

DISTRIBUTION OF RECOVERED RADIOACTIVITY AMONG ESTRADIOL METABOLITE PEAKS AS DETERMINED BY HPLC SCREENING

Uterine paracervical ganglia were homogenized and incubated with [^{3}H]estradiol (30 ng) in Tris-HCl buffer (pH 7.4) containing $5 \cdot 10^{-7}$ *M* of ascorbic acid and an NADPH generating system. The control incubate contained precursor and cofactors but no tissue. Extracts of the incubation mixtures were subjected to normal-phase HPLC as described under Experimental. Peak areas are expressed as a percentage of the radioactivity recovered from the control incubate. The values in each column represent one determination of products of metabolism of [^{3}H]estradiol by the combined ganglia of three animals (approximately 15 mg of tissue) at a particular stage of the estrous cycle.

Estradiol metabolite	*Control*	*Diestrus*	*Proestrus*	*Estrus*	*Metestrus*
Methoxyestrogens	0.6	8.7	7.1	2.9	2.1
Estrone	0.8	1.4	–	–	0.9
Estradiol	98.2	6.3	3.6	18.4	50.2
4-Hydroxyestrone	–	8.3	11.0	9.3	6.0
2-Hydroxyestrone	–	1.1	–	–	0.6
4-Hydroxyestradiol/16-hydroxyestrone	0.2	–	–	3.2	0.5
Peak eluting at 34 min	0.2	2.0	–	0.4	3.0
2-Hydroxyestradiol	–	–	4.8	0.4	0.1
Peak eluting at 66 min	–	–	–	0.3	–
Estriol	–	–	0.5	0.2	–

tissue showed 98.2% of the radioactivity eluting as a symmetrical estradiol peak. In contrast, incubates of [^{3}H]estradiol with paracervical ganglion homogenates showed, in addition to the [^{3}H]estradiol peak, four to seven metabolite peaks. The metabolites formed included estrone, 2- and 4-hydroxyestrone, 2-hydroxyestradiol, the 4-hydroxyestradiol/16-hydroxyestrone peak, estriol, a peak composed of methoxyestrogens, a peak of unknown identity eluting at 34 min and one eluting at 66 min.

DISCUSSION

The primary goal of this study was the development of an isocratic HPLC technique suitable for preliminary evaluation of *in vitro* metabolism of [^{3}H]estradiol. The normal-phase isocratic system described here accomplishes the rapid resolution of 2-hydroxyestrone, 4-hydroxyestrone, 2-hydroxyestradiol and 4-hydroxyestradiol and separates them from estrone and estradiol as well as from estriol, 16-ketoestradiol and the 3-methoxy metabolites of estrone, estradiol and estriol. Only 16-hydroxyestrone appears to interfere with detection of catechol estrogens. As this material is not resolved from 4-hydroxyestradiol, a peak eluting at 28 min of chromatography is most correctly referred to as a 4-hydroxyestradiol/16-hydroxyestrone peak. Non-catechol materials such as 16-hydroxyestrone may be removed prior to HPLC by a borate buffer extraction as reported by Shimada *et al.*[22]. Although this approach simplifies the identification of catechol estrogens, it sacrifies the ability to detect other estradiol metabolites which are not recovered from the borate buffer extraction procedure. Evaluation of the formation of catechol estrogens relative to other metabolites of estradiol may provide more information than observation of catechol estrogens alone. For this reason, the isocratic HPLC method presented here or the gradient HPLC technique recently described by Aten *et al.*[23] is recommended for the initial screening of estradiol metabolism *in vitro*.

The pilot study involving rat uterine paracervical ganglion was conducted to screen this tissue for capacity to metabolize estradiol *in vitro*. The data suggest not only metabolic activity, but a variation in the specific metabolites produced with stage of the estrous cycle. Recoverable metabolites consistently included large amounts of methoxyestrogens and 4-hydroxyestrone. In contrast, several other metabolites appeared in smaller amounts on specific days of the cycle: 2-hydroxyestradiol was produced primarily on proestrus and 4-hydroxyestradiol/16-hydroxyestrone on estrus; estrone, 2-hydroxyestrone and a peak of unknown identity at 34 min were produced on metestrus and diestrus. High specific activity estradiol (136 Ci/mM) was chosen as a precursor to enable picogram amounts of these metabolites to be detected. As loss of tritium could occur at least during the 2-hydroxylation of estradiol[21], the estimates of conversion of estradiol to metabolites listed in Table I may be conservative. It should be emphasized that the evaluation of the metabolic activity at each stage of the cycle is based on data from single incubations of pooled ganglia from three animals. Random biological variation, normally assessed by statistical tests, cannot be determined from these preliminary data. As random variation is probably present in each incubation mixture, small (2-fold) differences seen in 4-hydroxyestrone, for example, should be interpreted with caution. However, striking differences in estradiol metabolism, such as a 10-fold increase in 2-hydroxyestradiol at proestrus, are probably attributable to specific cyclic changes in the paracervical

ganglion rather than to random biological variation. This observation is not without precedent, as Fishman *et al.*[7] demonstrated fluctuations in metabolism of estradiol by rat brain with stage of the estrous cycle. Based on information obtained in this study, further investigation of the role of local metabolism of estradiol by paracervical ganglion as it contributes to the steroid responsiveness of this tissue would be justified.

The HPLC technique presented here emphasizes the detection of 2- and 4-hydroxy metabolites of estrone and estradiol formed from radioactive precursor *in vitro*. Use of this screening technique prior to pursuing more detailed studies can provide assurance that metabolism to catechol estrogens is an appropriate subject for investigation. If so, HPLC screening can help to define experimental parameters important to catechol estrogen production by a tissue. The relative ease with which these studies can be conducted should facilitate the definition of the role catechol estrogens play as modulators of the biological effects of estradiol.

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