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Assay of enzymic O-methylation of catechol oestrogens by high-performance liquid chromatography with coulometric detection

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ABSTRACT

A simple and sensitive method for the determination of guaiacol oestrogens enzymically formed from 2- or 4-hydroxyoestradiol, by menas of high-performance liquid chromatography with coulometric detection, has been developed. Catechol and guaiacol oestrogens were efficiently separated on a reversed-phase column, using 0.5% ammonium phosphate buffer (pH 3.0)–acetonitrile (59:41, v/v) as the mobile phase, and detected coulometrically in a screening-oxidation mode at +0.10 V and +0.35 V, respectively. The method was applied to the assay of *in vitro* enzymic O-methylation of catechol oestrogens. After 2- or 4-hydroxyoestradiol had been incubated with rat red blood cells in the presence of S-adenosylmethionine, the resulting guaiacols and unchanged substrate were percolated through an Extrelut-3 cartridge. The dried eluate was redissolved and directly injected. This simple procedure was as sensitive as the previously reported method using gas chromatography–mass spectrometry in a selected ion monitoring mode.

INTRODUCTION[a]

Catechol oestrogens, such as 2- or 4-hydroxyoestradiol (2- or 4-OHE_2), are currently recognized as active intermediates in oestrogen-induced carcinogenesis [1]. Enzymic O-methylation of catechol oestrogens yielding less active guaiacol oestrogens may have an important significance for the availability of active intermediates in the target tissues. In a previous paper, we reported the *in vitro* enzymic O-methylation of catechol oestrogens [2]. In the presence of S-adenosylmethionine (SAM), 2-OHE_2 or 4-OHE_2 was incubated with red blood cells obtained from rats and other animal species. The guaiacols formed and the unchanged substrate were determined by gas chromatography–mass spectrometry (GC–MS) in selected ion monitoring (SIM) mode, employing the corresponding $^2H_4$-labelled compounds as the internal standard (I.S.). For the reliable analysis of unstable compounds by GC–MS, the use of stable isotope-labelled substrates is essential, irrespective of the difficulty of their availability.

In recent years, high-performance liquid chro-

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[a] In this paper, the following trivial names and abbreviations are used: 2-OHE_2, 2-hydroxyoestradiol, 1,3,5(10)-oestratriene-2,3,17β-triol; 4-OHE_2, 4-hydroxyoestradiol, 1,3,5(10)-oestratriene-3,4,17β-triol; 2-OHE_2 2Me, 2-methoxyoestradiol, 2-methoxy-1,3,5(10)-oestratriene-3,17β-diol; 2-OHE_2 3Me, 2-hydroxyoestradiol 3-methyl ether, 3-methoxy-1,3,5(10)-oestratriene-2,17β-diol; 4-OHE_2 3Me, 4-hydroxyoestradiol 3-methyl ether, 3-methoxy-1,3,5(10)-oestratriene-4,17β-diol; 4-OHE_2 4Me, 4-methoxyoestradiol, 4-methoxy-1,3,5(10)-oestratriene-3,17β-diol; 2-OHE_1, 2-hydroxyoestrone, 2,3-dihydroxy-1,3,5(10)-oestratrien-17-one; 2-OHE_1 2Me, 3-hydroxy-2-methoxy-1,3,5(10)-oestratrien-17-one; 4-OHE_1, 4-hydroxyoestrone, 3,4-dihydroxy-1,3,5(10)-oestratrien-17-one; 4-OHE_1 4Me, 4-methoxyoestrone, 3-hydroxy-4-methoxy-1,3,5(10)-oestratrien-17-one.

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matography (HPLC) with electrochemical detection (ED) has been applied to the analysis of electrochemically active oestrogens, as well as catecholamines, in biological fluids [3,4]. Amperometric detection of catechol oestrogens was performed by oxidation on a working electrode at the applied potential of +0.9–1.0 V. Furthermore, the sensitivity has been markedly improved for the analysis of oestrogens by the use of a coulometric detection system [5–7]. However, there have been few papers dealing with the simultaneous determination of catechol and guaiacol oestrogens.

This paper describes a simple and sensitive assay method for *in vitro* enzymic O-methylation of 2-OHE_2 or 4-OHE_2 by HPLC with coulometric detection.

EXPERIMENTAL

Chemicals

Catechol and guaiacol oestrogens used in this study were synthesized in our laboratory according to the methods described in our previous paper [2]. SAM was purchased from Boehringer Mannheim (Mannheim, Germany). All other reagents and chemicals used were of analytical-reagent grade from commercial suppliers.

High-performance liquid chromatography

The apparatus used was a JASCO 880-pu Intelligent HPLC pump from Japan Spectroscopic (Tokyo, Japan) equipped with a Coulochem Model 5100A electrochemical detector (Environmental Sciences Assoc., Bedford, MA, USA). The applied potential was set *versus* a palladium reference electrode. The potentials of a Model 5010 analytical cell consisting of a dual-detector system were set at +0.10 V (first electrode for catechol oestrogens) and +0.35 V (second electrode for guaiacol oestrogens). A guard cell Model 5020 was set at +0.45 V. HPLC was carried out at 25°C on an Inertsil ODS-2 column (particle size 5 μm; 25 cm × 0.46 cm I.D.) (GL Sciences, Tokyo, Japan). The mobile phase was 0.5% $NH_4H_2PO_4$ (pH 3.0)–acetonitrile (59:41, v/v); the flow-rate was 0.8 ml/min.

Preparation of blood lysate

Male spontaneously hypertensive rats [8], nine weeks old, weighing 216–230 g, were supplied by Charles River Japan (Kanagawa, Japan). Before collection of blood, the animals were starved for one night. Heparin-treated venous blood was diluted with a nearly equal volume of ice-cooled 0.15 *M* KCl, which in turn was centrifuged at 800 *g* for 10 min at 4°C. The red blood cells (0.5 ml) were transferred to a tube, frozen rapidly on dry ice–methanol, and stored at −80°C until use. Prior to the assay, the red blood cells were diluted with ice-cooled 0.15 *M* KCl (2.0 ml) and lysed by thawing and sonicating for 2 min at 0°C. The whole was centrifuged at 1500 *g* for 15 min at 4°C, and the blood lysate obtained was stored in an ice-bath.

Assay of enzymic O-methylation

An incubation mixture was made up with 2-OHE_2 or 4-OHE_2 (1 μg), ascorbic acid (60 μg), ethanol (5 μl), 1 m*M* $MgCl_2$, 1 m*M* SAM · HSO_4, blood lysate (100 μl) and 0.1 *M* phosphate buffer (pH 7.4) to bring the volume to 300 μl. Incubation was carried out in a 10-ml glass-stoppered brown tube for 30 min at 37°C in a shaking water-bath.

The reaction was terminated by the addition of 300 μl of 0.2 *M* HCl containing the I.S. mixture and ascorbic acid (300 μg) to the incubation mixture on ice. The I.S. mixture consisted of 4-OHE_1 (500 ng) and 4-OHE_1 4Me (125 ng) for the determination of 2-OHE_2 and its monomethyl ethers, and 2-OHE_1 (500 ng) and 2-OHE_2 2Me (50 ng) for 4-OHE_2 and its monomethyl ethers.

After the addition of 2.5 ml of 0.1 *M* acetate buffer (pH 5.0), the mixture was percolated through an Extrelut-3 cartridge (E. Merck, Darmstadt, Germany). The desired fraction was eluted with dichloromethane (12 ml), and the eluate was dried at 40°C under a stream of nitrogen. To the residue was added 0.01% ascorbic acid–methanol (100 μl), and a 10–20 μl aliquot of the solution was injected into the analytical column without further purification.

The amount of each product or substrate was determined from the peak-area ratio of guaiacol

or catechol to the corresponding I.S. For constructing the calibration graph, three to four different amounts of guaiacols and catechols were added to the incubation mixture without blood, and processed as described above except for the incubation step.

Recovery test for catechol oestrogens

The recovery test was performed without incubation for the standard assay mixture fortified with known amounts of catechol and guaiacol oestrogens.

RESULTS AND DISCUSSION

Initially, a hydrodynamic voltammogram of each compound was obtained to select the suitable applied potential (Fig. 1). On the basis of these data, oxidative potentials of +0.10 V and + 0.35 V were chosen as the working potentials for catechol oestrogens and guaiacol oestrogens, respectively. A mixture of 2-OHE_2 or 4-OHE_2 and its monomethyl ethers was efficiently separated by HPLC on a reversed-phase column, using 0.5% ammonium phosphate buffer (pH 3.0)–acetonitrile (59:41, v/v) as the mobile phase.

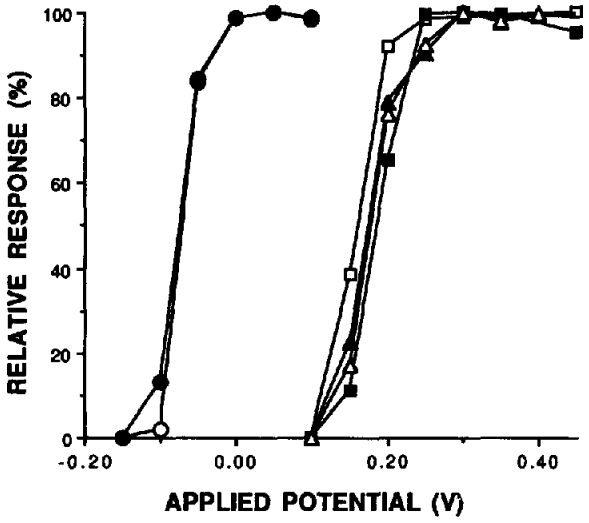

Fig. 1. Hydrodynamic voltammograms of 2-OHE_2, 4-OHE_2 and their isomeric monomethyl ethers: (○) 2-OHE_2; (△) 2-OHE_2 2Me; (□) 4-OHE_2 3Me; (●) 4-OHE_2; (▲) 2-OHE_2 3Me; (■) 4-OHE_2 4Me.

TABLE I

RECOVERY OF CATECHOL OESTROGENS ADDED TO THE INCUBATION MEDIUM

Added (ng)	Recovered (mean ± S.D., n = 5) (ng)	Recovery (%)
2-OHE_2		
750	759 ± 4.5	101
75	74.7 ± 3.2	99.6
7.5	7.47 ± 0.12	99.6
4-OHE_2		
750	762 ± 9.1	102
75	73.7 ± 1.4	98.3
7.5	7.43 ± 0.13	99.1

The calibration graph was constructed by plotting the peak-area ratio of each catechol or guaiacol to the corresponding I.S. against the amount of the former, respectively, where good linearity was observed for all oestrogens over the practical working range 1–1000 ng per tube. Electrochemical responses obtainable with catechols and guaiacols were substantially the same among isomeric compounds. The recovery test was performed with blood lysate samples spiked at three levels. Each oestrogen was recovered at 98–104% (Tables I and II), indicating the ability of 0.01% ascorbic acid–methanol to prevent oxidative decomposition of labile catechol oestrogens. Their stability during incubation with blood lysate and extraction procedure has previously been confirmed [2]. It is evident from the data in Tables I and II that the proposed method is also satisfactory with respect to accuracy and precision (C.V. < 4.7%).

The substrate, 2-OHE_2 or 4-OHE_2, was incubated with blood lysate under the assay conditions previously reported. Typical chromatograms obtained with 2-OHE_2 and 4-OHE_2 are illustrated in Figs. 2 and 3, respectively. No interfering peaks were observed on either chromatogram. The detection limit (signal-to-noise ratio = 2) obtainable under the standard assay conditions was 0.3 ng per tube (50 pg per injection). Besides the main peaks of 2-OHE_2, 4-

TABLE II

RECOVERY OF GUAIACOL OESTROGENS ADDED TO THE INCUBATION MEDIUM

Added (ng)	Recovered (mean ± S.D., n = 5) (ng)	Recovery (%)
2-OHE$_2$ 2Me		
300	302 ± 7.0	101
30	29.5 ± 0.45	98.3
3	3.00 ± 0.28	100
2-OHE$_2$ 3Me		
300	299 ± 4.2	99.7
30	29.7 ± 0.67	99.0
3	2.95 ± 0.14	98.3
4-OHE$_2$ 4Me		
300	300 ± 1.9	100
30	29.9 ± 0.18	99.7
3	3.12 ± 0.06	104
4-OHE$_2$ 3Me		
300	299 ± 4.0	99.7
30	29.9 ± 0.18	99.7
3	3.00 ± 0.08	100

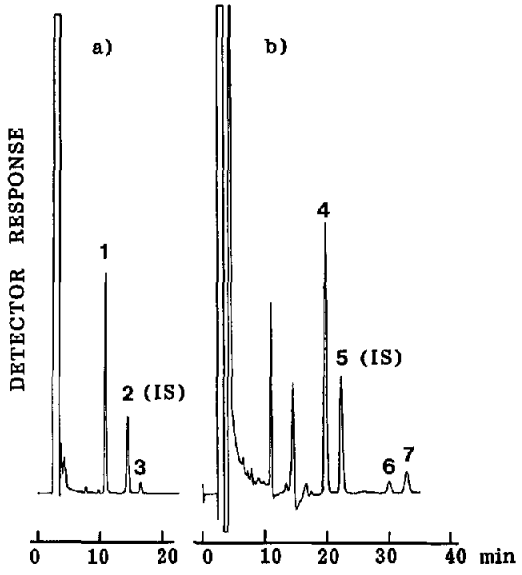

Fig. 3. Chromatograms of the products formed from 4-OHE$_2$ at different applied potentials: (a) + 0.10 V; (b) + 0.35 V. Other conditions as in Fig. 2. Peaks: 1 = 4-OHE$_2$; 2 = 2-OHE$_1$ (I.S.); 3 = 4-OHE$_1$; 4 = 4-OHE$_2$ 4Me; 5 = 2-OHE$_2$ 2Me (I.S.); 6 = 4-OHE$_1$ 4Me; 7 = 4-OHE$_2$ 3Me.

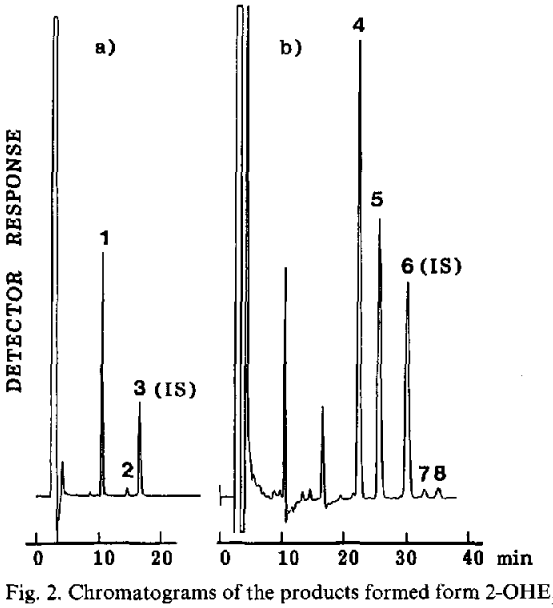

Fig. 2. Chromatograms of the products formed form 2-OHE$_2$ at different applied potentials: (a) +0.10 V; (b) +0.35 V. Column, Inertsil ODS-2, particle size 5 μm; mobile phase, 0.5% $NH_4H_2PO_4$ (pH 3.0)–acetonitrile (59:41, v/v). Peaks: 1 = 2-OHE$_2$; 2 = 2-OHE$_1$; 3 = 4-OHE$_1$ (I.S.); 4 = 2-OHE$_2$ 2Me; 5 = 2-OHE$_2$ 3Me; 6 = 4-OHE$_1$ 4Me (I.S.); 7 = 2-OHE$_1$ 2Me; 8 = 2-OHE$_1$ 3Me.

OHE$_2$ and their monomethyl ethers, minor peaks due to 2-OHE$_1$, 2-OHE$_1$ 2Me, 2-OHE$_1$ 3Me, 4-OHE$_1$ and 4-OHE$_1$ 4Me were also detected, the amounts of the monomethyl ethers being less than 1 ng per tube (Figs. 2 and 3).

The results of the formation of isomeric monomethyl ethers, together with the unchanged substrate, in comparison with those obtained by GC–MS–SIM [2] are listed in Tables III and IV. A good correlation was observed between the two methods. The use of dual-electrode coulometric detection is of great advantage in the present method, because labile catechol and guaiacol oestrogens can be determined simultaneously with only solid-phase extraction and without tedious derivatization. With respect to versatility and sensitivity, the newly developed method has proved to be better than the GC–MS–SIM method. Moreover, the proposed method appears to be widely applicable to the assay of enzymic O-methylation of catechols in biological materials such as brain, kidney and liver cytosolic fraction. The details will be reported elsewhere.

TABLE III

O-METHYLATION OF 2-OHE_2 WITH RAT RED BLOOD CELLS

HPLC conditions as in Fig. 2.

Method	Products formed[a] (ng/tube)		Ratio (2Me/3Me)	2-OHE_2 unchanged[a] (ng/tube)
	2-OHE_2 2Me	2-OHE_2 3Me		
HPLC–ED	170 ± 4.2	111 ± 2.5	1.5	615 ± 8
GC–MS–SIM	189 ± 2.6	131 ± 13	1.4	573 ± 43

[a] Mean ± S.D. (n = 3).

TABLE IV

O-METHYLATION OF 4-OHE_2 WITH RAT RED BLOOD CELLS

HPLC conditions as in Fig. 3.

Method	Products formed[a] (ng/tube)		Ratio (4Me/3Me)	4-OHE_2 unchanged[a] (ng/tube)
	4-OHE_2 4Me	4-OHE_2 3Me		
HPLC–ED	101 ± 4.7	8.41 ± 0.36	12	805 ± 10
GC–MS–SIM	102 ± 3.0	9.61 ± 0.36	11	739 ± 30

[a] Mean ± S.D. (n = 3).

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