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Determination of estradiol 2- and 4-hydroxylase activities by gas chromatography with electron-capture detection

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ABSTRACT

A highly sensitive assay has been developed for measuring the rate of formation of 2-hydroxyestradiol and 4-hydroxyestradiol from estradiol by microsomal preparations. Catechol estrogens were converted to heptafluorobutyryl esters, which were separated by capillary column gas chromatography and quantified using electron-capture detection. 2-Hydroxyestradiol 17-acetate was used as an internal standard. The identity of catechol estrogen derivatives was verified by gas chromatography–mass spectrometry using negative-ion chemical ionization. Estrogens were identified by negative molecular ions and/or by characteristic fragments. This procedure permits quantification of catechol estrogens at the subpicogram level. The assay was validated by comparing estrogen 2- and 4-hydroxylase activities in microsomes from hamster and rat liver with values reported previously.

INTRODUCTION

Catechol estrogens are metabolites of estrogenic hormones and have been postulated to play a role in physiological and pathophysiological processes [1,2]. The validity of these postulates can only be examined by accurate and reliable assays for catechol estrogen formation. The direct measurement of catechol estrogens requires assay conditions which minimize air oxidation, dimerization, binding to cellular macromolecules or other reactions characteristic of these catechols [3,4]. To circumvent problems caused by the high reactivity of catechol estrogens, two indirect assays have been developed. (1) The release of ^3H_2O generated from 2-[^{3}H]estradiol is utilized to monitor 2-hydroxylation of estradiol

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(E_2) [5]. (2) Reactive catechol estrogens are converted to more stable [methyl-^{14}C]- or [methyl-^{3}H]methoxyestrogens catalyzed by catechol-O-methyltransferase and radiolabeled S-adenosylmethionine [6–8]. Direct product isolation and analysis by gas chromatography–mass spectrometry (GC–MS) revealed that the release of tritium from [^{3}H]estradiol at neutral pH is consistent with values for 2-hydroxyestradiol (2-OH-E_2) formation but not 4-hydroxyestradiol (4-OH-E_2) formation [9]. At elevated pH, which is optimal for extra-hepatic NADPH-dependent catechol estrogen synthase activity [10], values for catechol estrogen formation by tritium release assay may exceed those by product isolation assay [11]. The catechol-O-methyltransferase-linked radioenzymatic assay [6–8] has been shown to both underestimate total catechol estrogen formation and to generate erroneous ratios of 2-OH-E_2/4-OH-E_2 [12]. In order to reliably and accurately measure catechol estrogens, several direct product isolation procedures have been developed making use of GC–MS [9], high-performance liquid chromatography (HPLC) [13,14], or column chromatography on neutral alumina to isolate the catechol estrogens coupled with thin-layer chromatographic (TLC) analysis [11,15].

In this report, we describe a new method for the measurement of catechol estrogen formation which has been developed because of several advantages over the already available methods such as simplicity, versatility, higher sensitivity, and the possibility for structure confirmation by GC–MS.

EXPERIMENTAL

Chemicals

E_2, 2-OH-E_2, 4-OH-E_2, and NADPH were purchased from Sigma (St. Louis, MO, U.S.A.). 2-Hydroxyestradiol 17-acetate was obtained from Steraloids (Wilton, NH, U.S.A.) and heptafluorobutyric anhydride and anhydrous triethylamine from Pierce (Rockford, IL, U.S.A.).

Microsomal preparation

Male Syrian hamsters and Sprague–Dawley male rats were purchased from Harlan Sprague Dawley (Houston, TX, U.S.A.). Liver microsomes were prepared by the method of Dignam and Strobel [16].

Incubation conditions

The incubation mixture consisted of 1 mg/ml microsomal protein, 5 m*M* NADPH, 50 m*M* Tris buffer, pH 7.5, containing 5 m*M* ascorbate and 100 μM E_2 in a final volume of 250 μl. Incubations were carried out at 37°C for 10 min and were stopped by placing culture tubes on ice. Ice cold 50 m*M* Tris buffer (1 ml) containing 5 m*M* ascorbate, pH 7.5, and 2.5 μg 2-OH-E_2 17-acetate as internal standard were added. The steroids were extracted twice with 5 ml of ice cold ethyl acetate saturated twice with water. The ethyl acetate layer was dried for 1 h using

anhydrous sodium sulfate (1 g) and then concentrated under a stream of nitrogen. Control incubations were carried out as described above but without substrate.

Derivatization

The dry extracts were redissolved in 100 μl of ethyl acetate and triethylamine (final concentration 100 m*M*) and acylated by addition of 100 μl of heptafluorobutyric anhydride. Derivatization was carried out for 1 h at 55°C. Samples were concentrated under a stream of nitrogen, and residues were redissolved in 100 μl of ethyl acetate. A 1-μl aliquot of this solution was injected into the gas chromatograph. It is important to note that heptafluorobutyryl (HFB) esters of estrogens are stable for several hours, but cannot be stored for longer time periods without appreciable decomposition.

Gas chromatography

The gas chromatographic analysis of E_2 and its metabolites was carried out using a Hewlett-Packard 5890 gas chromatograph equipped with a ^{63}Ni pulsed electron-capture detector and interfaced with a 3393A integrator. A bonded-phase DB-5 fused-silica capillary column (30 m × 0.25 mm) from J & W Scientific (Rancho Cordova, CA, U.S.A.) was used. The GC conditions were: 776 Torr helium carrier gas head pressure, splitless injection, 280°C injection temperature, 100°C initial temperature. A temperature gradient of 30°C/min from 100 to 245°C was followed by a 5-min isothermal period at 245°C and a second temperature gradient of 1°C/min from 245 to 265°C. The column was held at this final temperature for 5 min. Blank values were obtained form control incubations carried out without E_2 substrate. Any catechol estrogens possibly contained by the microsomal preparations were below the detection limits of the assay.

Gas chromatography–mass spectrometry

GC–MS was carried out using a Hewlett-Packard 5988A instrument equipped with an HP-5890 gas chromatograph (Hewlett-Packard, Palo Alto, CA, U.S.A.). The HFB derivatives of the steroids were separated on a 30 m × 0.32 mm DB-5 column (J & W Scientific). The column temperature was programmed from 200 to 330°C at 10°C/min and the flow-rate was 2.3 ml/min. Samples were chromatographed using a 30:1 split ratio and an injection temperature of 250°C. Methane negative-ion chemical ionization (NCI) analyses were performed using an ion source pressure of 0.7 Torr [17], a temperature of 200°C and an ionization energy of 120 eV. Mass spectra were scanned from *m/z* 100 to *m/z* 900 at approximately 1 scan/s.

RESULTS

Characteristics of the assay

E_2, 2-OH-E_2, 4-OH-E_2 and the internal standard were readily derivatized and

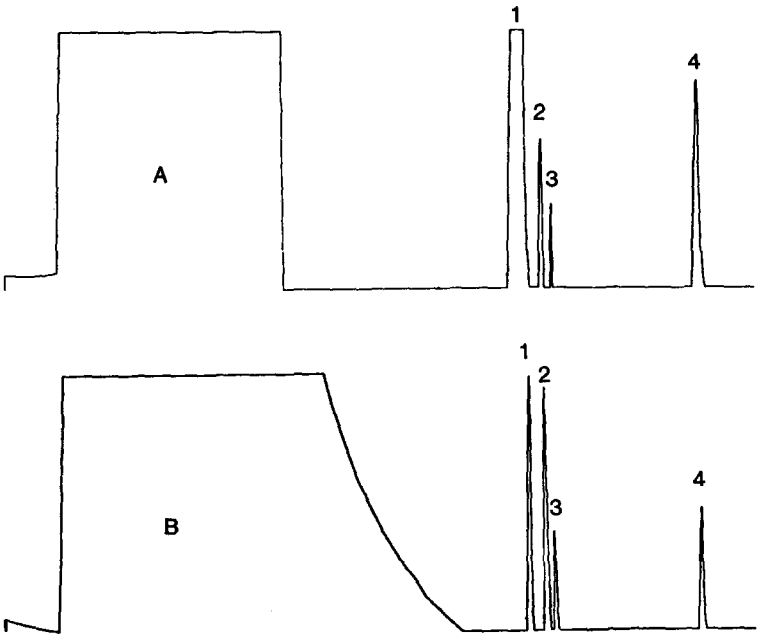

Fig. 1. Capillary gas chromatograms of catechol estrogens detected by electron capture. After incubation with microsomes and NADPH, steroids were extracted, derivatized and analyzed (A). Peaks were identified as E_2 (1), 2-OH-E_2 (2), 4-OH-E_2 (3) and 2-hydroxyestradiol 17-acetate (4), the internal standard. (B) A mixture of authentic reference substances.

well separated by capillary GC as shown in Fig. 1. Under the chromatographic conditions used, the derivatives of E_2, 2-OH-E_2 and 4-OH-E_2 had retention times of 14.9, 15.8, and 16.1 min, respectively. 2-Hydroxyestradiol 17-acetate was chosen as internal standard, because its derivative was demonstrated to be stable under the assay conditions and eluted well after the estrogens to be analyzed with a retention time of 20.2 min. The estrogen derivatives eluted in an area of the chromatogram which was free of any interfering peaks from the microsomal preparation (Fig. 1). The dose–response curves are linear for the three estrogen derivatives over wide ranges (Table I). The intercepts are very close to the origin (Table I).

Extraction efficiency, accuracy, and sensitivity

Extraction efficiencies were determined for the three estrogens and the internal standard and were routinely above 90% (Table II). Intra-assay variations were low.

Accuracies of determination, defined as the mean percentage deviation of concentrations from theoretical values, were determined from quadruplicate measurements and were found to be 10–11% or better. The assay has been designed

TABLE I

LINEAR REGRESSION PARAMETERS CALCULATED FROM STANDARD CURVES OF E_2, 2-OH-E_2, AND 4-OH-E_2

Compound	γ-Intercept	Slope	r^2
E_2	0.053	0.19	0.99
2-OH-E_2	0.016	0.22	0.99
4-OH-E_2	0.057	0.27	0.98

to utilize the high sensitivity achieved with electron-capture detection of halogenated compounds [18,19]. Catechol estrogens were detected when 500 fg of catechol estrogen were injected into the instrument (data not shown).

Gas chromatography-mass spectrometry

Characteristic mass spectra of the estrogens were obtained by NCI (Table III and Fig. 2 and 3). The estrogen spectra were characterized by negative molecular ions and by fragments $[M-H]^-$ (except in the case of E_2) and $[M-HF]^-$. With the exception of the spectrum of the internal standard, fragments $[M-HF-HF]^-$ were also observed. Base peaks or intense fragment peaks were

TABLE II

EXTRACTION EFFICIENCY AND ACCURACY OF MEASUREMENT OF E_2, 2-OH-E_2, 4-OH-E_2, AND 2-HYDROXYESTRADIOL 17-ACETATE IN MICROSOMES

Amount added (ng/ml)	Amount recovered (mean, n = 2) (ng/ml)	Coefficient of variation (%)	Accuracy[a] (%)
E_2			
1000	1025	8	10
2500	2478	6	8.5
2-OH-E_2			
1000	1075	4	7.5
2500	2750	8	10.0
4-OH-E_2			
1000	950	2.5	5.0
2500	2320	5.0	11.2
2-Hydroxyestradiol 17-acetate			
250	260	3.5	4.0
1000	1080	6.2	8.0

[a] Mean percentage deviation of concentrations from the theoretical value.

TABLE III

NEGATIVE-ION CHEMICAL IONIZATION MASS SPECTRA OF ESTROGENS

HFB derivatives of estrogens were prepared and analyzed by GC–MS. Each estrogen gave a single, sharp GC peak without separation of fully or partially derivatized species. Values in parentheses represent relative intensity (%).

Compound	*m/z* (a.m.u.)				
	M^-	$[M-H]^-$	$[M-HF]^-$	$[M-HFB]^-$	$[M-H-HFB]^-$
$E_2(HFB)_2$[a]	664(0.7)		644(100)	467(32)	
2-OH-$E_2(HFB)_3$	876(0.03)	875(0.1)	856(2)	679(46)	678(100)
4-OH-$E_2(HFB)_3$	876(0.1)	875(0.1)	856(7)	679(100)	678(86)
2-Hydroxyestradiol 17-acetate $(HFB)_2$[b]		721(0.1)	702(0.1)	525(57)	524(100)

[a] Other fragment ions were: 624(9) for $[M-HF-HF]^-$ and 427(59) for $[m/z\ 467-HF-HF]^-$.

[b] Other fragment ions were: 684(2) for $[M-F_2]^-$.

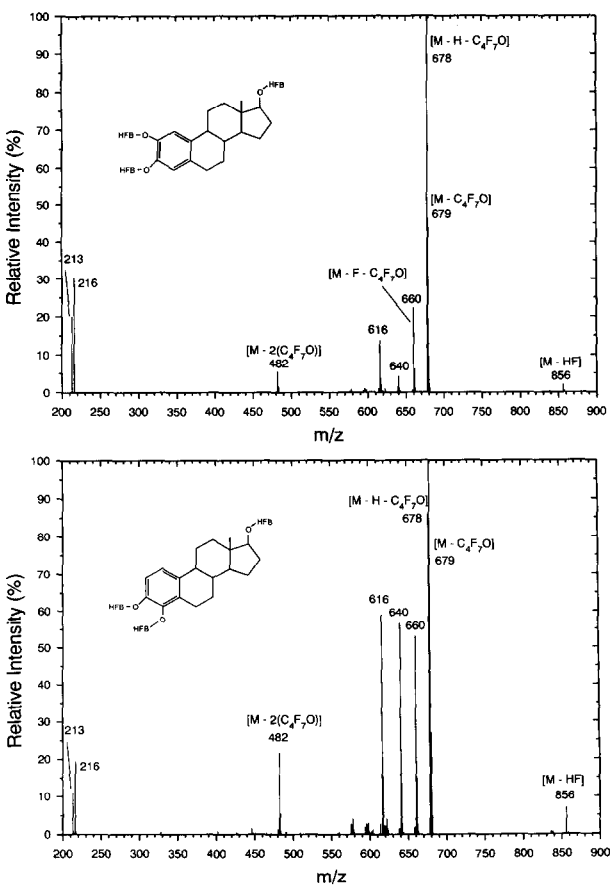

Fig. 2. Negative-ion chemical ionization mass spectra of HFB derivatives of 2-OH-E_2 (upper panel) or 4-OH-E_2 (lower panel). The catechol estrogens were derivatized using heptafluorobutyric anhydride. The esters were separated by capillary gas chromatography and analyzed by mass spectrometry. The most important ions are identified by a.m.u. and/or most likely fragmentation.

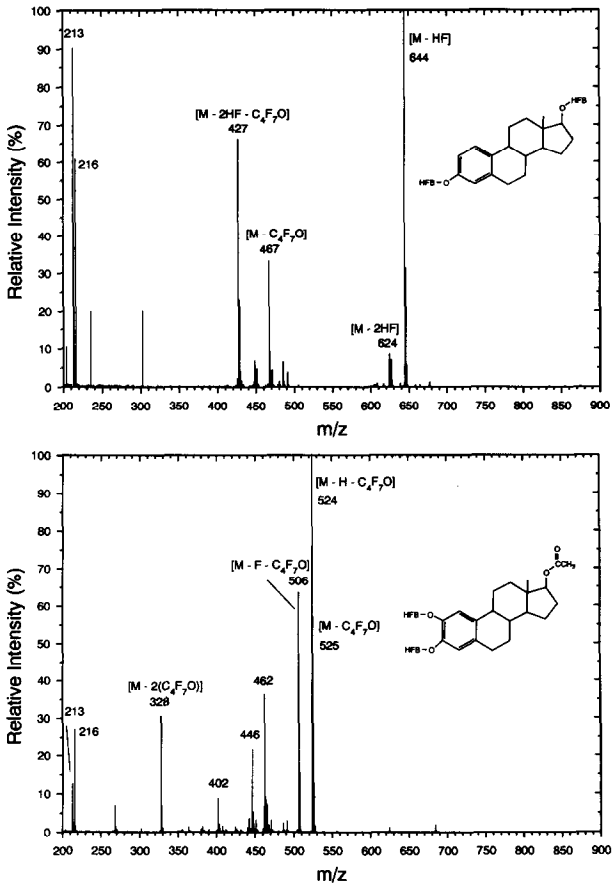

Fig. 3. Negative-ion chemical ionization mass spectra of HFB derivatives of E_2 (upper panel) and 2-hydroxyestradiol 17-acetate, the internal standard (lower panel). The estrogens were derivatized using heptafluorobutyric anhydride. The esters were separated by capillary gas chromatography and analyzed by mass spectrometry. The most important ions are identified by a.m.u. and/or most likely fragmentation.

formed by scission of the O–CO bond of the HFB ester derivatives. These fragmentations occurred in molecular anions of E_2 or $[M-H]^-$ ions of the other estrogens analyzed to form phenoxylate anions. There were only minor differences in intensity of ions in the spectra of 2-OH-E_2 and 4-OH-E_2. The molecular anions of the catechol estrogens including the internal standard also fragmented by elimination of two acyl moieties to form quinone anions. Fragment ions formed by minor fragmentation pathways are outlined in the spectra (Figs. 2 and 3).

Validation of the assay

The assay described in this report was validated by comparing the values for catechol estrogen formation from rodent liver microsomes with values published

TABLE IV

ESTROGEN 2- AND 4-HYDROXYLASE ACTIVITIES IN LIVER MICROSOMES OF RATS AND HAMSTERS

Microsomes were prepared from male rodents by the method of Dignam and Strobel [16]. Each value is a mean (± S.D.) of six experiments. Each experiment was done in duplicate.

Species	Estrogen 2- and 4-hydroxylase activity (pmol/mg protein per min)	
	2-OH-E_2	4-OH-E_2
Rat	2043 ± 191 (2329)[a]	264 ± 35 (211)[a]
Hamster	1580 ± 196 (1333)[b]	469 ± 98 (232)[b]

[a] Porubek and Nelson [9].
[b] Roy *et al.* [12].

previously [9,12]. As shown in Table IV, estrogen 2-hydroxylase activities in liver microsomes of rats and hamsters were within 20% of values published previously. These values reported previously have been obtained by product isolation procedures using GC–MS [9] or a TLC procedure using radiolabeled standards [12].

DISCUSSION

The assay for catechol estrogen formation described in this report is attractive for several reasons. The sensitivity in the femtogram range is better than that reported with any of the other assay procedures. High sensitivity is a characteristic of electron-capture detection of halogenated and aromatic compounds [18–22]. Because of its sensitivity in the subpicogram range, this method can be utilized for the detection of estrogen hydroxylation in microsomes of tissues with very low metabolic potential as compared to liver. Moreover, this procedure can be used to detect the catechol estrogen concentrations in tissues and body fluids.

Although highly sensitive, the assay is relatively simple and uncomplicated and utilizes commercially available instrumentation and standards. The method is also versatile and may easily be adapted to measure catechol formation from various estrogens of differing structure [23]. For structural characterization, the procedure is applicable to GC–MS analysis.

The NCI spectra of HFB esters of estrogens are useful for the identification of the estrogens. The fragmentation of molecular anions, elimination of hydrogen, HF or HFB moieties, is in agreement with fragmentation patterns described previously for NCI spectra [20–22]. Although the intensities of molecular anions were low, the substances were easily identified by their fragmentations. The differences between the spectra of derivatives of 2-OH-E_2 and 4-OH-E_2 were insufficient for structural identification of a specific isomer. Only the GC retention times

provide sufficient discrimination for the identification of a specific catechol estrogen. There was no indication of a partial derivatization, when the corresponding negative molecular ions of mono- and bis-HFB esters of E_2 were analyzed by single-ion monitoring.

The assay has been validated by comparing values for estrogen 2- and 4-hydroxylase activity of rodent liver microsomes with product isolation data published previously [9,12]. These previously published data have been obtained with other validated product isolation assays and have thus been demonstrated to be reliable. Values obtained with the assay reported here are within 20% of values reported previously and are thus within a range attributable to biological variation.

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REFERENCES

1 M. Metzler and J. A. McLachlan, in D. Neubert, H. J. Merker, H. Nau and J. Langman (Editors), *Role of Pharmacokinetics in Prenatal and Perinatal Toxicology*, George Thieme, Stuttgart, 1978, p. 157.
2 P. Ball and R. Knuppen, *Acta Endocrinol.*, 232 (1980) 1.
3 H. P. Gelbke and R. Knuppen, *J. Chromatogr.*, 71 (1972) 465.
4 J. Fishman, *J. Clin. Endocrinol. Metab.*, 23 (1963) 207.
5 J. Fishman, H. Guzik and L. Hellman, *Biochemistry*, 9 (1970) 1593.
6 S. M. Paul, J. Axelrod and E. Diliberto, *Endocrinology*, 101 (1977) 1604.
7 R. L. Barberi, L. M. Canick and K. H. Ryan, *Steroids*, 32 (1978) 529.
8 R. H. Purdy, P. H. Moore Jr., M. C. Williams, J. W. Goldzieher and S. M. Paul, *FEBS Lett.*, 138 (1982) 40.
9 D. J. Porubek and S. D. Nelson, *Biomed. Environ. Mass Spectrom.*, 15 (1988) 157.
10 Q. D. Bui and J. Weisz, *Endocrinology*, 124 (1989) 1085.
11 R. M. Hersey, P. Gunsalus, T. Lloyd and J. Weisz, *Endocrinology*, 109 (1981) 1902.
12 D. Roy, Q. D. Bui, J. Weisz and J. G. Liehr, *J. Steroid Biochem.*, 33 (1989) 243.
13 K. Shimada, M. Kumai, H. Shinkai and T. Nambara, *Anal. Biochem.*, 116 (1981) 287.
14 C. Bunyagidj and J. A. McLachlan, *J. Chromatogr. Sci.*, 26 (1988) 24.
15 R. M. Hersey, K. I. H. Williams and J. Weisz, *Endocrinology*, 109 (1989) 1912.
16 J. D. Dignam and H. W. Strobel, *Biochemistry*, 16 (1977) 1116.
17 E. A. Stemmler, R. A. Hites, B. Arbogast, W. L. Budde, M. L. Deinzer, R. C. Dougherty, I. W. Eichelberger, R. L. Foltz, C. Grimm, E. P. Grimsrud, C. Sakashita and L. J. Sears, *Anal. Chem.*, 60 (1988) 781.

18 D. K. Knapp, in *Handbook of Analytical Derivatization Reactions*, Wiley, New York, 1979, p. 3.
19 E. D. Morgan and C. F. Poole, *J. Chromatogr.*, 89 (1974) 225.
20 C. S. Hsu, G. J. Dechert and K. D. Rose, *Proceedings of the 38th ASMS Conference on Mass Spectrometry and Allied Topics, Tuczon, AZ, June 3–8, 1990*, p. 595.
21 W. A. Korfmacher, T. Kinouchi, A. T. Lopez Miranda and F. A. Beland, *Proceedings of the 38th ASMS Conference on Mass Spectrometry and Allied Topics, Tuczon, AZ, June 3–8, 1990*, p. 686.
22 M. Saha, G. M. Kresbach, R. W. Giese, R. S. Annan and P. Vouros, *Biomed. Environ. Mass Spectrom.*, 18 (1989) 958.
23 D. Roy and J. G. Liehr, unpublished results.