Journal of Chromatography, 272 (1983) 233–241
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1494

RAPID SIMULTANEOUS ASSAY OF SERUM ESTRONE, ESTRADIOL, AND ESTRIOL IN PREGNANT WOMEN USING METHYL ETHER ACETATE DERIVATIVES BY CAPILLARY GAS CHROMATOGRAPHY AND ELECTRON-IMPACT MASS SPECTROMETRY*

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(First received June 25th, 1982; revised manuscript received August 30th, 1982)

SUMMARY

A simple gas chromatographic—mass spectrometric method capable of measuring estrone, estradiol, and estriol simultaneously with a sensitivity close to that of radioimmunoassay has been developed. The estrogens in serum were extracted with diethyl ether, and internal standards (3-O-C^2H_3-estrone, 3-O-C^2H_3-estradiol, and 3-O-C^2H_3-estriol) were added, followed by converting to methyl ether compounds with an extractive alkylation procedure. The methyl ethers were then acetylated. Analyses were performed using a SP-2250 capillary column gas chromatograph coupled with an electron-impact mass spectrometer.

The estrogen methyl ether acetate derivatives were more stable chemically and gave less fragmentation upon electron impact than the conventional trimethylsilyl derivatives. The use of selected ion monitoring of molecular ions and that of the corresponding internal standards (M + 3) provides a sensitivity down to 10 pg for estrone and estradiol and to 200 pg for estriol. The time required for the preparation of multiple samples is within 4 hours.

INTRODUCTION

The measurement of estrone (E_1), estradiol (E_2), and estriol (E_3) serum levels is usually done by radioimmunoassay (RIA), but this requires three separate procedures [1]. Alternatively, measurement by gas chromatography—mass spectrometry (GC—MS) with selected ion monitoring permits simultaneous measurement of these three classical estrogens with sensitivity comparable to that of RIA [2]. Over the past several years, a number of GC—MS methods for the analysis of estrogens have been developed [3—7]. However, these methods require elaborate and lengthy procedures, which make them

*This paper was presented in part at the 28th Annual Conference on Mass Spectrometry and Allied Topics, New York, NY, May 25—30, 1980.

0378-4347/83/0000—0000/$03.00

impractical for multiple sample studies in clinical investigations. In evaluating dehydroepiandrosterone sulfate (DHAS) loading test for predicting fetus outcome [8] in our laboratory, the serum levels of E_1, E_2, and E_3 are to be monitored. We have developed a new GC—MS method using a methyl ether acetate derivative which simplified and shortened the preparation procedure and, in conjunction with the use of a capillary column, provided increased sensitivity. This method is simple enough to permit measurement of multiple samples. It also offers the opportunity to study estrogen metabolism safely with the use of stable isotopically labelled estrogens.

MATERIALS AND METHODS

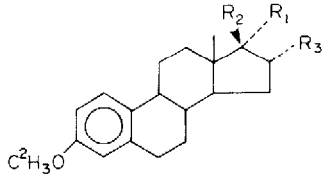
Reagents

Estrone, estradiol, and estriol were obtained from Steraloids (Wilton, NH, U.S.A.). Pyridine and acetic anhydride (commercial reagent grade) were distilled before use. Methylene chloride, diethyl ether, and methanol were of glass-distilled grade (Burdick and Jackson Labs., Muskegon, MI, U.S.A.).

0.1 M Tetrabutylammonium hydrogen sulfate solution. Tetrabutylammonium hydrogen sulfate (TBAHS) was obtained from Aldrich (Milwaukee, WI, U.S.A.). An amount of 1.963 g was dissolved in 4 ml of water, and after washing with methylene chloride (4 × 4 ml), the aqueous solution was diluted to a final volume of 50 ml with water.

Synthesis of 3-O-C^2H_3-estrone, 3-O-C^2H_3-estradiol and 3-O-C^2H_3-estriol, internal standards

Estrone, estradiol, and estriol (1 mg of each) were dissolved in 1 ml of 0.5 *N* sodium hydroxide solution, followed by the addition of 0.5 ml of 0.1 *M* TBAHS, 2 ml of methylene chloride, and 0.2 ml of [$^2H_3$]iodomethane (99+ atom % deuterium, Aldrich). The mixture was shaken for 20 min. An additional 0.2 ml of [$^2H_3$]iodomethane was then added with repeat shaking for 20 min and then centrifuged to separate the layers. The methylene chloride layer was washed with 0.5 *N* sodium hydroxide (2 × 2 ml), water (3 × 2 ml), and evaporated to dryness. The residue was dissolved in 8 ml of methanol and stored at −20°C. The recovery of estrogens was quantitative.



Ia. $R_1 + R_2 = =O$, $R_3 = H$

b. $R_1 = H$, $R_2 = OH$, $R_3 = H$

c. $R_1 = H$, $R_2 = R_3 = OH$

Fig. 1. Structural formulae of internal standards, 3-O-C^2H_3-estrone (Ia), 3-O-C^2H_3-estradiol (Ib), and 3-O-C^2H_3-estriol (Ic).

Internal standard solution (E-d_3)

The above internal standard stock solution was diluted 50-fold with methanol to a final concentration of 2.5 μg/ml of each estrogen. It was stored at −20°C, and was stable for at least one year under these conditions.

E_1, E_2, and E_3 standards

Unlabelled E_1, E_2, and E_3 were dissolved in methanol to a final concentration of 10 ng per 20 μl, 2.5 ng per 20 μl, 1 ng per 20 μl, 500 pg per 20 μl, 50 pg per 20 μl, and 10 pg per 20 μl for each estrogen.

Standard curve

A 20-μl aliquot of internal standard solution (E-d_3), containing 50 ng of each estrogen, and 20 μl of a mixture of E_1, E_2, and E_3 standard solutions were combined and mixed with 0.25 ml of 0.5 *N* sodium hydroxide, 0.125 ml of 0.1 *M* TBAHS, 2 drops of iodomethane (freshly distilled), and 0.5 ml of methylene chloride. The mixture was mixed by a vortex mixer for 1 min, then centrifuged to separate the layers. The upper layer was discarded and the lower organic layer was washed with water (2 × 0.5 ml), and evaporated to dryness in a stream of air. To the dry residue, 0.15 ml of pyridine and 0.1 ml of acetic anhydride were added. The solution was kept at room temperature for 0.5 h. Water (0.5 ml) was then added. After mixing, the aqueous solution was extracted with methylene chloride (0.4 ml). The lower organic layer was transferred to a 1-ml serum vial (Wheaton Scientific, Millville, NJ, U.S.A.) with the aid of a syringe equipped with a flat-tipped needle and was evaporated to dryness with an air stream. The vial was then capped (PTFE-lined serum cap, Fisher Scientific, Fair Lawn, NJ, U.S.A.) and stored at −20°C. Prior to analysis by GC—MS, 7 μl of isooctane were introduced into the vial by a Hamilton microsyringe (Hamilton, Reno, NV, U.S.A.). After mixing well, 1 μl of this solution was used for GC—MS analysis.

Analysis of serum samples

Serum samples (200 μl) were diluted with equal volumes of water, then extracted with diethyl ether (freshly opened, 3 × 3 ml). The combined extract was dried and the residue was reconstituted with 200 μl of methanol. Aliquots (25—100 μl) of this solution were used for the GC—MS analysis of E_1, E_2, and E_3. The rest of the solution was used for RIA of E_2. The methanol solution (25—100 μl) was evaporated to dryness and 0.25 ml of 0.5 *N* sodium hydroxide was added. This solution was washed with cyclohexane (2 × 0.3 ml), followed by the addition of 20 μl of internal standard solution (E-d_3), 0.125 ml of 0.1 *M* TBAHS, 0.5 ml of methylene chloride, and 2 drops of iodomethane (freshly distilled). The procedure for the preparation of the standard curve was then followed.

Gas chromatography—mass spectrometry

A Hewlett-Packard (Palo Alto, CA, U.S.A.) 5985A GC—MS system was used, equipped with a Hewlett-Packard 18835A capillary inlet system. The

original interface between gas chromatograph and mass spectrometer ion source was removed (the isolation valve section was kept), and replaced with a glass-lined stainless-steel tube (GLT) from Scientific Glass Engineering (Austin, TX, U.S.A.). The ends of the glass capillary (WCOT SP-2250, 10 m × 0.25 mm, J & W grade AA, supplied by Supelco, Bellefonte, PA, U.S.A.) were straightened with a glass capillary end straightener (Supelco), then coated with Carbowax 20M (Supelco) according to the recommended procedure. The outlet of this glass capillary column was connected to the GLT interface with a low-dead-volume valve (Scientific Glass Engineering). A splitless mode of analysis was used. Initially the column temperature was held at 90°C, programmed to rise at 30°C/min for 5 min, then changed to 2°C/min for the rest of the 13-min run. The helium flow-rate was 1 ml/min and the solvent purging valve was activated at 0.7 min after injection. The various zone temperatures of this system were as follows: injection port, 250°C; GC—MS interface, 250°C; ion source, 200°C; and analyzer manifold, 200°C.

The sample was ionized with an electron beam at 70 eV, then analyzed with selected ion monitoring (software provided by the manufacturer). From 5 to 8 min it was focused on *m/z* 284 vs. *m/z* 287 with dwelling times 50 msec and 200 msec, respectively. It was then switched to *m/z* 328 vs. *m/z* 331 from 8 to 10 min; then to *m/z* 386 and *m/z* 389 from 10 to 13 min. In a typical analysis, E_1 has a retention time of 7.5 min; E_2, 8.3 min; and E_3, 12.3 min. The total analysis time was 13 min.

Radioimmunoassay

Estradiol in serum was analyzed by the following procedure. An aliquot (25 μl) of methanol solution of E_2 extracted from plasma was diluted to a final volume of 500 μl. Three different sample volumes (10 μl, 30 μl, 50 μl) of the methanol solution were pipetted into separate tubes and evaporated to dryness. The residue was mixed with 200 μl of first antibody tracer solution ([2,4,6,7-^{3}H]estradiol, phosphate buffer (pH 7.8), rabbit gamma-globulins, and anti-E_2 antibody), and kept at 38°C for 2 h, then chilled at 0°C for 0.5 h. The bound E_2 was precipitated by the addition of a second antibody (goat anti-gamma-globulins precipitating antibody). After incubating at 0°C for 1 h, the supernatant (100 μl) was pipetted into a scintillation vial and scintillation mixture was added. The radioactivity was determined with a Packard (Downers Grove, IL, U.S.A.) 3320 liquid scintillation counter. The data were treated by the standard logit—log procedure.

RESULTS AND DISCUSSION

Internal standards

In a GC—MS assay with stable isotope labelled internal standard, the isotopic purity of this labelled compound should be high in order to increase the sensitivity. Besides, this internal standard should be added in the early stage of the procedure. A number of stable isotope labelled estrogens were used for GC-MS procedures by various investigators [4—7]. However, they were either being added late in the procedure [4, 5] or were of insufficient isotopic

purity for a sensitive assay [6, 7]. In addition, the procedures for synthesizing some of these standards are lengthy and time consuming.

The internal standards used in the present procedure, 3-O-C^2H_3-E_1, 3-O-C^2H_3-E_2, and 3-O-C^2H_3-E_3, are easy to prepare. They can be synthesized conveniently from [$^2H_3$]iodomethane. The high isotopic purity of [$^2H_3$]iodomethane (99+ %) made the labelled end-products devoid of residual unlabelled species, therefore the sensitivity of this method was increased. The disadvantage of these internal standards is that losses in the initial extraction procedure were not corrected. However, when recovery was checked by the addition of trace amounts of radioactive labelled E_2, the yields were consistently greater than 90%. The extractive methylation was quantitative and the washing with cyclohexane in the analysis of serum samples did not affect the recovery (see below).

Derivative

Conventionally, the trimethylsilyl (TMS) derivative of estrogen is used for GC—MS procedures [4—7]. The methyl ether acetate derivative used in the present method offers several advantages over the TMS derivative. First, it is more stable and results in less fragmentation. Secondly, extractive alkylation, used to prepare the methyl ether derivative, also serves a purification purpose. The mass spectra of the methyl ether acetate derivative of E_2 and 3-O-C^2H_3-E_2 are shown in Fig. 2.

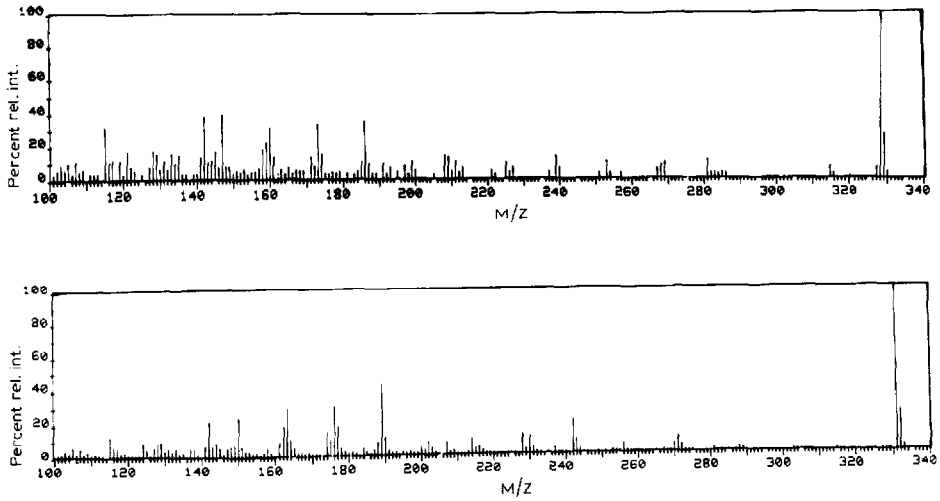


Fig. 2. Electron-impact (70 eV) mass spectra of estradiol methyl ether acetate (top), and 3-O-C^2H_3-estradiol acetate (bottom).

Analytical procedure

A chromatographic (column or thin-layer) purification procedure usually precedes the GC—MS analysis as a preliminary clean-up in estrogen analysis [3—7]. However, column or thin-layer chromatographic pre-cleaning is usually the most time-consuming step, and results in both sample loss and contamination. It prolongs the total time required for a complete analysis. The present

procedure eliminated all the chromatographic pre-cleaning procedure. The cleaning of the sample resulted from solvent washing and differential extractive methylation. The final removal of interfering peaks was achieved with a high-resolution capillary column.

The estrogens were dissolved in 0.5 *N* sodium hydroxide solution. For plasma samples, the alkaline solution was then washed twice with cyclohexane. This step removed cholesterol, triglycerides, neutral steroids, and other non-polar neutral compounds. The solvent washing was necessary when E_3 was analyzed. It removed cholesterol, which was eluted immediately before E_3 in GC and elevated the baseline to mask the E_3 peak. However, cyclohexane washing was not necessary when only E_1 and E_2 were to be analyzed. None of the neutral, non-polar components in the plasma interfered with the assay of E_1 and E_2. The difference between the standard curve and plasma sample is the cyclohexane step. Even though the possible sample loss during this step was not compensated by internal standards, the data in Table I show that

TABLE I

EFFECT OF CYCLOHEXANE WASHING ON THE ASSAY RESULTS OF ESTRONE AND ESTRADIOL

Method 1 is without cyclohexane washing and method 2 is with cyclohexane washing.

Plasma sample No.	E_1 (ng/ml)		E_2 (ng/ml)	
	Method 1	Method 2	Method 1	Method 2
1	4.8	4.3	24.0	26.5
2	4.5	4.2	23.0	24.8
3	5.0	5.0	23.0	21.0
4	5.1	5.5	23.5	25.5
5	9.7	9.0	83.0	85.0
6	10.2	9.9	63.0	67.5
7	11.3	10.7	60.0	62.4

essentially no estrogen loss occurred when a series of plasma samples were analyzed with and without cyclohexane washing for E_1 and E_2.

Daley et al. [9] used tetrahexylammonium as counter ion to synthesize estrogen methyl ethers in high yield. Rosenfeld and Taguchi [10] applied the same principle for the analysis of 2-hydroxyestradiol. It is known that by varying the type and concentration of the counter ions, anions of different ionization constants can be differentially extracted into an organic solvent, thereby achieving a degree of purification [11]. Experimentally, it was found that tetrabutylammonium ion in a concentration of 0.024 *M* served this purpose. Higher concentration or counter ions with higher hydrophobicity increased the background of GC—MS analysis. Lower concentration or counter ions with higher hydrophilicity ran the risk of incomplete recovery.

Gas chromatography—mass spectrometry

Estrogen methyl ether acetates have good GC properties. They showed

symmetric peaks when the glass capillary column was used under the conditions described above. Their 70-eV mass spectra (Fig. 2) show prominent molecular ions and were used for selected ion monitoring (Fig. 3). The area ratio of the molecular ion of estrogen versus the corresponding deuterated standards was plotted against the amount of unlabelled estrogen in the sample. The standard curves (Fig. 4) were linear over a range of at least 1000-fold, from 10 pg to 10 ng. Estrone and estradiol had similar standard curves, and the detection limits were 10 pg. The standard curve for E_3 shows a different slope from E_1 and E_2 and had a lower sensitivity (200 pg). The deviation of the E_3 standard curve is unclear at this moment. Since it was reproducible and was linear over the range studied, the possibility that it was due to incomplete recovery or decomposition of E_3 was highly unlikely. The reproducibility of this procedure was checked by analyzing a set of plasma samples in triplicate. The average coefficient of variation was 3.5% for sample concentrations ranging from 5—70 ng/ml of estrogen (n = 18). Theoretically,

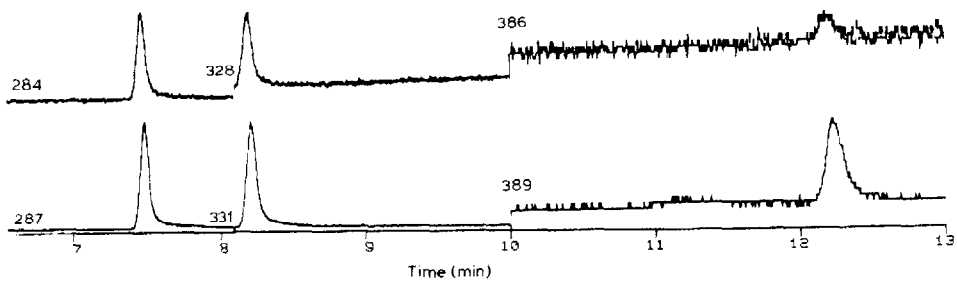


Fig. 3. Selected ion monitoring chromatogram of a serum sample for estrone (m/z 284 vs. 287), estradiol (m/z 328 vs. 331), and estriol (m/z 386 vs. 389). The displays are normalized to the maximum of each individual peak.

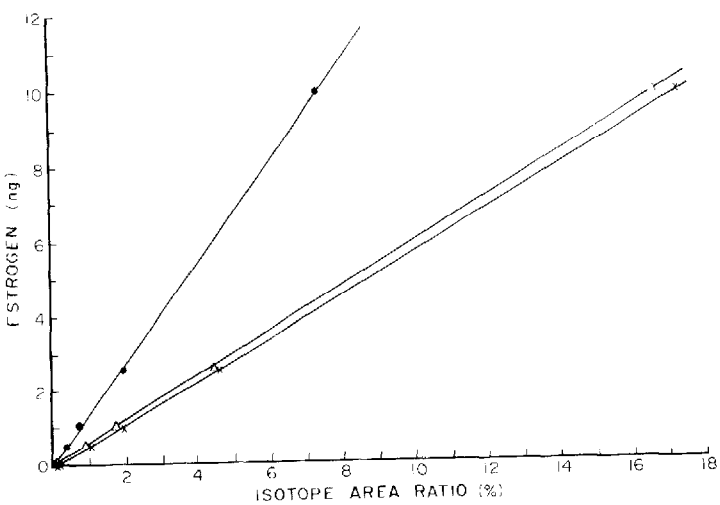


Fig. 4. Standard curves of estrogens: (△——△) estrone; (×——×) estradiol; (●——●) estriol. Isotope area ratio (%) was obtained by dividing the peak area of unlabelled estrogen by that of labelled estrogen.

chemical ionization results in less fragmentation which would tend to increase sensitivity. The methane chemical ionization of these estrogens did show the protonated molecular ion (MH^+) as the only fragment. Unfortunately, the sensitivity was not increased.

Clinical study

A typical result of a dehydroepiandrosterone sulfate loading test is shown in Fig. 5. The E_1 serum level rose slowly and peaked at the end of study (2 h), whereas the E_2 level rose rapidly after the loading dose, reaching a peak level at about 0.5 h after dose. The E_3 level was unchanged throughout the study. The average of four basal serum levels of unconjugated E_1, E_2, and E_3 in this subject were 8.2 ng/ml, 29.7 ng/ml, and 12.6 ng/ml, respectively. These are consistent with RIA assayed values reported in the literature for women in this stage of pregnancy [12]. The pattern of estrogen after DHAS loading is also consistent with the literature results measured by RIA [12—14].

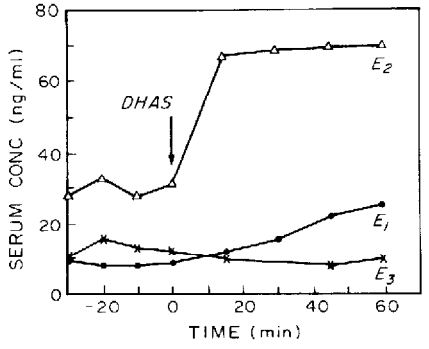


Fig. 5. Estrone, estradiol, and estriol levels before and after intravenous administration of a 50-mg bolus of dehydroepiandrosterone sulfate (DHAS). The patient was a woman in her 39th week of pregnancy, which resulted in delivery of a healthy, normal baby a week later.

TABLE II

CORRELATION OF E_2 PLASMA LEVELS AS MEASURED BY THE GC—MS PROCEDURE AND BY RIA

Sample No.	E_2 (ng/ml)	
	GC—MS	RIA
1	30.0	28.3
2	35.0	39.5
3	29.0	28.5
4	32.6	37.5
5	73.2	64.0
6	74.5	79.0
7	75.8	82.3
8	75.8	83.7

Correlation of E_2 plasma levels as determined by GC—MS and by RIA

The correlation of E_2 serum levels as determined by the GC—MS procedure and by RIA is shown in Table II. The samples were obtained from a DHAS loading study of a pregnant woman. Samples 1—4 were basal values before the administration of DHAS. Samples 5—8 were E_2 values after DHAS loading. Regression analysis of data obtained from RIA (Y) and GC—MS (X) gave the equation $Y = 1.0281\ X + 0.5187$; the correlation coefficient (r^2) was 0.9479.

ACKNOWLEDGEMENT

This work was supported by a grant from National Institute of Child Health and Human Development (HD-11089).

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