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DETERMINATION OF SERUM UNCONJUGATED ESTRONE, ESTRADIOL-17β AND ESTRIOL DURING PREGNANCY BY SELECTED ION MONITORING

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SUMMARY

A simple, rapid and highly specific method by selected ion monitoring (SIM), using 9α,11α-[$^2H_2$]estrone, [2,4-$^2H_2$]estradiol-17β and 2,4-[$^2H_2$]estriol as internal standards, was developed for the determination of serum estrogens during pregnancy. Serum samples were submitted to a simple extraction procedure and were analysed after formation of the trifluoroacetic anhydride derivative. The inter-assay coefficients of variation for estrone, estradiol-17β and estriol were 3.73%, 3.42% and 3.49%, respectively. The results obtained by SIM were compared with analysis performed using radioimmunoassay.

INTRODUCTION

Quantification of estrogens has made considerable progress since the introduction of radioimmunoassays (RIA). These immunological methods are limited, however, by the nonspecificity of antibodies and are complicated by the need for using radioisotopes.

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The purpose of this investigation was to develop a simple, rapid and highly specific assay procedure for the three classical estrogens (estrone, estradiol-17β and estriol) using the technique of selected ion monitoring (SIM).

EXPERIMENTAL

Materials

Blood was collected between 9.00 a.m. and 12 noon from a group of 120 healthy pregnant women visiting Showa University Hospital. Serum was obtained by centrifugation and stored below −10°C until analyzed.

Reagents

Methanol, *n*-hexane, ethyl acetate and trifluoroacetic anhydride from freshly opened bottles were used without any additional purification. Ethanol was redistilled before use.

Synthesis of deuterium-labelled estrogens

[2,4-$^2H_2$]Estriol was prepared by the deuterium exchange reaction according to the method reported by Tökès and Throop [1]. A solution of estriol (100 mg) in deuterated methanol ($CH_3O^2H_2$) (10 ml) and 10% deuteriosulfuric acid in deuterium oxide (4 drops) was refluxed for 2 days. Most of the solvent was removed by distillation and water was added. The precipitates were collected by filtration and dried. Recrystallization from methanol gave the deuterated estriol (72 mg). Analysis by mass spectrum showed that the product consisted of 2,4-d_2-estriol (88.2%), d_1-estriol (10.1%) and d_0-estriol (1.7%).

[2,4-$^2H_2$]Estradiol-17β (50 mg) was prepared in the same manner as described above for estriol. 2,4-d_2-Estradiol-17β (32 mg, 80%) containing d_1-estradiol-17β (16%) and d_0-estradiol-17β (4%) was obtained.

[9α,11α-$^2H_2$]Estrone was prepared by the method of Tsuda et al. [2]. A solution of 9(11)-dehydroestrone (1.0 g) in dimethoxyethane (50 ml) was shaken vigorously in deuterium atmosphere with 5% palladium charcoal (0.4 g). The absorption was stopped after 10 min. The catalyst was removed by filtration and the filtrate was concentrated. 9α,11α-d_2-Estrone (0.9 g, 27%) containing d_0-estrone (2.9%), d_1-estrone (15.2%), d_3-estrone (38.0%), d_4-estrone (13.4%) and d_5-estrone (3.5%) was obtained.

Analysis by nuclear magnetic resonance (NMR) spectrometry showed that two deuteriums were induced into positions 2 and 4. Analysis of the synthesized deuterated estrone by NMR was not performed. However, under the procedure used in this experiment, deuterium should be induced into positions 9 and 11.

Gas chromatography—mass spectrometry

The instrument used in the present study was a Shimadzu LKB-9000 equipped with a multiple-ion detector and was operated under the following conditions. The column was a 1-m glass coil with 1% OV-1 coated on Chromosorb W 50—80 mesh, and was operated at 210°C, with a helium flow-rate of 25 ml/min. The temperature of the separator and ionization source were kept at 270°C and 290°C, respectively. The ionization energy and trap current were

70 eV and 50 μA, respectively. The retention times of estrone trifluoroacetate (TFA), estradiol-17β-TFA and estriol-TFA were 3 min 3 sec, 4 min 30 sec and 1 min 30 sec, respectively. The multiple-ion detector was focused on the molecular ions of TFA derivatives of unlabelled and labelled compounds. Monitor masses were as follows: m/e = 366, 368 for estrone; m/e = 464, 466 for estradiol-17β; and m/e = 576, 578 for estriol. Mass spectra of estrone-TFA, estradiol-17β-TFA, and estriol-TFA are demonstrated in Fig. 1.

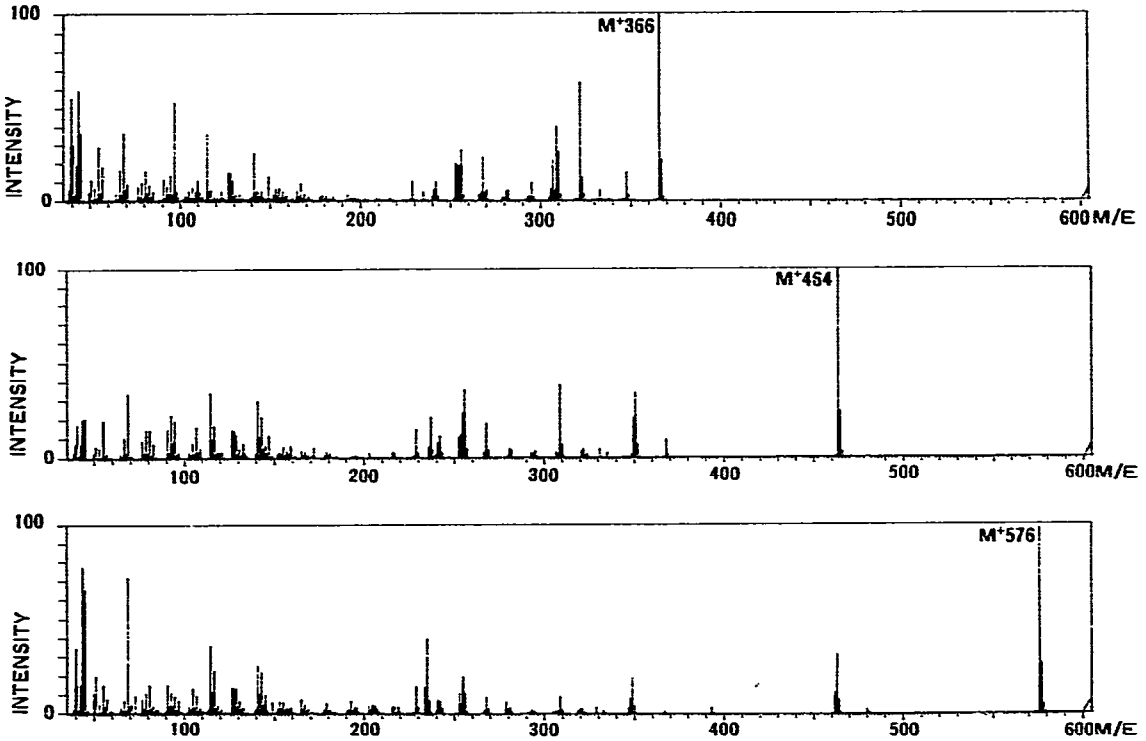


Fig. 1. Mass spectra in the analysis of trifluoroacetates of estrone, estradiol-17β and estriol. Upper spectrum: estrone, M^+=366; centre spectrum, estradiol-17β, M^+=464; lower spectrum, estriol, M^+=576. The mass spectra were measured on a Jeol JMS-D300.

Procedure

The extraction and derivative formation were carried out as summarized in Fig. 2. From a standard solution containing 10 ng/ml deuterium-labelled estrogens in ethanol, 2 ml (20 ng) were added to 0.5 ml of the serum and the serum was allowed to stand for 30 min at room temperature prior to the extraction procedure [3]. After the addition of 2 ml of methanol and stirring, the precipitates were removed by centrifugation. The extracts were washed twice with 2 ml of *n*-hexane to remove lipid and were evaporated to dryness. After the addition of 1 ml of water, estrogens were extracted with 2 ml of water-saturated ethyl acetate. The solvent was removed by evaporation under nitrogen gas. The residue was dissolved in 4 μl of ethyl acetate, and 4 μl of trifluoroacetic anhydride were added.

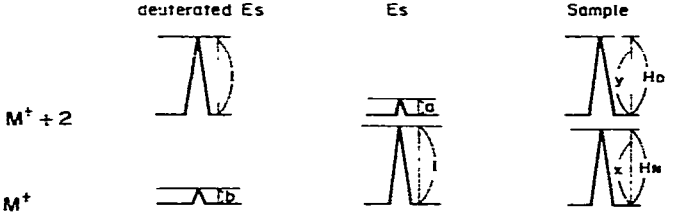
Serum 0.5ml
↓ add deuterium-labelled estrogens as internal standards
Extract with 2 ml methanol
↓
Centrifuge 3000rpm 5 min
↓
Methanol
↓ Removal of lipid with n-Hexane 2 ml×2:discard
Evaporate under N_2 gas stream
↓
H_2O 1 ml
↓
Ethyl acetate 2 ml
↓
Take supernatant
↓
Evaporate under N_2 gas stream
↓
TFA derivative formation
↓
GC-MS

Fig. 2. Extraction and derivatization of estrone, estradiol-17β and estriol to be determined by GC—MS.

Calculation of the content of estrogens

SIM responses were measured as peak heights, and the concentrations of the three classical estrogens were calculated with the aid of the formula given in Fig. 3.

Formula



a : ratio of deuterated Es containing Es
= 0.035, 0.040 and 0.053 for E_1, E_2 and E_3
b : ratio of Es containing deuterated Es
= 0.092, 0.016 and 0.061 for E_1, E_2 and E_3
X : amount of Es in Sample

A : ratio of deuterization
= 0.270, 0.800 and 0.882 for E_1, E_2 and E_3
B : amount of added d_2—Es

$$H_O = y + aH_N$$

$$H_N = x + by$$

$$X = AB\frac{x}{y} \fallingdotseq AB\frac{\frac{H_N}{H_O} - b}{1 - a\frac{H_N}{H_O}}$$

Fig. 3. Formula for calculation of the content of estrogens (Es) [estrone (E_1), estradiol-17β (E_2) and estriol (E_3)]. Hormone concentrations were calculated by multiple-ion detection responses as peak heights in mm.

RESULTS

Evaluation of the method

Known amounts of estrone, estradiol-17β and estriol were added to 0.5 ml of healthy male serum. The extraction and derivative formation were carried out as shown in Fig. 2. As seen from Table I, the values for percentage recovery and coefficient of variation (C.V.) were satisfactory.

TABLE I

RESULTS OF THE EVALUATION OF THE METHOD

Estrogen added to male serum (ng)		*n*	Recovered ng/ml (mean ± S.D.)	Percentage recovery (mean ± S.D.)	C.V. (%)
Estrone	10	6	9.65 ± 0.36	96.47 ± 3.57	3.73
Estradiol-17β	20	6	19.32 ± 0.66	96.58 ± 3.29	3.42
Estriol	20	6	19.58 ± 0.68	97.88 ± 3.41	3.49

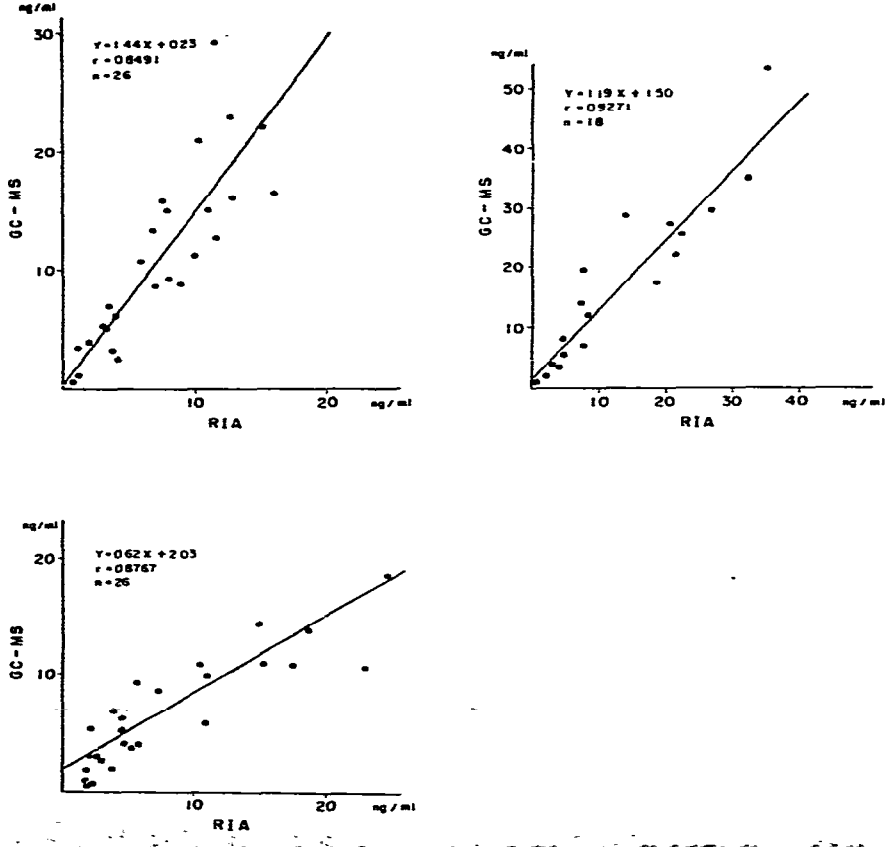


Fig. 4. Correlation between estrone (top left), estradiol-17β (top right) and estriol (bottom left) concentrations obtained by GC—MS and RIA.

Correlation with radioimmunoassay

The radioimmunoassay of the three classical estrogens was carried out according to methods currently used in this laboratory [4]. Anti-estriol-16,17-dihemisuccinate—bovine serum albumin (BSA) was used as an antibody. The cross-reactivity of this anti-serum with various steroids was studied [5]. Specimens were extracted with dichloromethane and separation of all three estrogens was performed by column chromatography using Sephadex LH-20.

Serum samples were quantitated by two different methods — RIA and gas chromatography—mass spectrometry (GC—MS). The correlation between these methods is shown in Fig. 4. The values for the three classical estrogens obtained by GC—MS correlated well with those obtained using RIA.

Clinical results

Normal pregnant women (n = 120) were studied. The levels of the three estrogens increased with progressing gestation (Table II). The mean ± S.D. levels of three estrogens from the 36th to the 40th week were as follows: estrone, 19.8 ± 8.5 ng/ml; estradiol-17β, 31.7 ± 13.4 ng/ml; estriol, 16.5 ± 3.1 ng/ml.

TABLE II

SERUM LEVELS OF UNCONJUGATED ESTROGENS IN NORMAL PREGNANT WOMEN DETERMINED BY GC—MS

Values are expressed as mean ± S.D. Number of samples in parentheses.

Weeks of gestation	Estrone (ng/ml)	Estradiol-17β (ng/ml)	Estriol (ng/ml)
4—7	2.0 ± 1.2 (6)	2.1 ± 1.5 (7)	1.8 ± 0.9 (8)
8—11	2.7 ± 1.0 (11)	3.7 ± 1.6 (6)	1.9 ± 0.9 (12)
12—15	4.5 ± 2.3 (7)	9.8 ± 4.5 (10)	3.9 ± 1.1 (6)
16—19	6.0 ± 1.9 (12)	9.9 ± 4.5 (11)	5.0 ± 2.3 (11)
20—23	7.5 ± 2.9 (16)	12.7 ± 5.6 (14)	5.7 ± 2.1 (11)
24—27	10.4 ± 4.7 (16)	16.7 ± 4.2 (12)	7.7 ± 1.5 (8)
28—31	12.2 ± 2.8 (14)	17.8 ± 6.7 (18)	10.1 ± 4.0 (21)
32—35	13.3 ± 6.7 (20)	22.3 ± 8.5 (21)	10.5 ± 5.3 (21)
36—40	19.8 ± 8.5 (18)	31.7 ± 13.4 (21)	16.5 ± 3.1 (22)

DISCUSSION

The labelled compounds used in the present study were found to be stable in all procedures including extraction, derivative formation and GC—MS measurement. Although the isotopic purity of 9,11-deuterated estrone was

only 27%, the ratio of d_2-estrone to d_0-estrone in the standard labelled estrone was high. Thus this compound could be used as a standard with satisfactory results. 2,4-Di-deuterated estrogens used for estradiol-17β and estriol were of high isotopic purity and could be used as good standard compounds.

Recently, there have been several reports concerning determination of estrogen by GC—MS. Björkhem et al. [6] and Breuer and Siekmann [7] prepared di-deuterated estrogens ([2,4-$^2H_2$]estriol, and [6,7-$^2H_2$]estradiol, respectively), as used in this study. Zamecnik et al. [8] determined estradiol-17β with tetra-deuterated estradiol-17β. These investigators prepared trimethylsilyl ether derivatives. Knuppen et al. [9] determined estradiol as its heptafluorobutylate derivative with [$^2H_2$]estradiol. Derivative formation of estrogens by polyfluorocarboxylic acid seems to be suitable for steroid measurement by GC or GC—MS, since derivatives could be prepared easily with high volatility, and molecular ions were intensive at high masses. In our present study, TFA derivatives were used because of the reason mentioned above.

We believe that the present study is the first to demonstrate the determination of serum unconjugated estrone, estradiol-17β and estriol during pregnancy by the technique of SIM from the same samples.

It is tedious and time-consuming to perform and draw a standard curve, especially when a limited number of samples are determined as applied for RIA. The hormone concentrations in this paper were calculated with the aid of the described formula without using standard curves. This approach has been used to measure testosterone and progesterone by Dehennin et al. [10]. The estimation of peak heights from SIM enables immediate determination of hormone concentration. The amount of estrogens introduced into the GC—MS instrument ranged between 100 and 500 pg in this study. Such amounts were sufficient for accurate determination. This is consistent with the sensitivity of GC—MS.

The mean levels of plasma unconjugated estrone estimated from the 36th week of pregnancy until term by different investigators using chemical methods ranged from approximately 8 to 15 ng/ml [11,12], while values from the radioimmunological method ranged from 4.9 to 25.0 ng/ml [13,14]. The mean serum levels published for unconjugated estradiol-17β determined by chemical methods ranged from approximately 8 to 25 ng/ml [11,12], and for radioimmunological methods ranged from 18 to 25 ng/ml [15,16]. The mean levels of estrone and estradiol-17β measured by the present method seem to be higher than those reported by the others. The mean levels of estriol were also higher than those noted with the chemical method [11]. However, the mean levels of estriol in our study seem to be lower than concentrations reported using radioimmunological methods [17,18].

In this study, conjugated estrogens were not determined, but values of steroid sulfates [19] have to be considered when levels of unconjugated steroids are evaluated.

The possibility of hydrolysis of estrone sulfate in the preparation of the sample for GC—MS can not be completely ruled out, since estrone sulfate is known to be labile and its concentration in blood is relatively high. However, addition of 100 ng of estrone sulfate to male serum resulted in a 0.13% higher value than that in a sample without addition (our recent observation).

With this method, it is possible to complete approximately 30 samples during one working day.

In conclusion, the assay described here is a simple, rapid and specific method for measuring the three classical estrogens.

REFERENCES

1 L. Tōkēs and L.J. Throop, in J. Fried and J.A. Edwards (Editors), Organic Reactions in Steroid Chemistry, Vol. 1, Van Nostrand Reinhold, New York, 1972, p. 156.
2 K. Tsuda, E. Ohki and S. Nozoe, J. Org. Chem., 28 (1963) 781.
3 S. Baba, Y. Shinohara and Y. Kasuya, J. Chromatogr., 162 (1979) 529.
4 F. Seo, H. Suzuki, K. Uehara, T. Yanaihara and T. Nakayama, Acta Obstet. Gynaecol. Jap., 32 (1980) 1089 (in Japanese).
5 K. Arai, T. Yanaihara and S. Okinaga, Amer. J. Obstet. Gynecol., 123 (1975) 804.
6 I. Bjōrkhem, R. Blomstrand, L. Svensson, F. Tietz and K. Carlstrōm, Clin. Chim. Acta, 62 (1975) 385.
7 H. Breuer and L. Siekmann, J. Steroid Biochem., 6 (1975) 685.
8 J. Zamecnik, D.T. Armstrong and K. Green, Clin. Chem., 24 (1978) 627.
9 R. Knuppen, O. Haupt, W. Schramm and H.O. Hoppen, J. Steroid Biochem., 11 (1979) 153.
10 L. Dehennin, A. Reiffsteck and R. Scholler, J. Steroid Biochem., 5 (1974) 81.
11 O.W. Smith, Acta Endocrinol. Copenhagen, Suppl., 104 (1966) 9.
12 J.C. Touchstone and T. Murawec, Biochemistry, 4 (1965) 1612.
13 H.H. Simmer, D. Tulchinsky, E.M. Gold, M. Frankland, M. Greipel and A.S. Gold, Amer. J. Obstet. Gynecol., 119 (1974) 283.
14 J.D. Townsley, N.H. Dubin, G.F. Grannis, L.J. Gartman and C.D. Crystle, J. Clin. Endocrinol. Metab., 36 (1973) 289.
15 D. Tulchinsky, C.J. Hobel and S.G. Korenman, Amer. J. Obstet. Gynecol., 111 (1973) 311.
16 D.M. Okada, D. Tulchinsky, J.W. Ross and C.J. Hobel, Amer. J. Obstet. Gynecol., 119 (1974) 502.
17 A. Klopper, G. Masson, D. Campbell and G. Wilson, Amer. J. Obstet. Gynecol., 117 (1973) 21.
18 S. Ichimaru, M. Ito, I. Miyakawa and M. Maeyama, Acta Obstet. Gynaecol. Jap., 30 (1978) 439 (in Japanese).
19 O. Jänne, R. Vihko, J. Sjövall and K. Sjövall, Clin. Chim. Acta, 23 (1969) 405.