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Studies on Immunological Assay of Urinary Estrogens. IV.[1] Comparison of the Latex Agglutination Inhibition Reaction Method with Gas Chromatography-Mass Spectrometry

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Estrogen levels in the urine of women at various stages of pregnancy were determined semi-quantitatively and quantitatively by the latex agglutination inhibition reaction (LAIR) method. Quantitative processing according to the LAIR method was possible by strictly standardizing the volume of the sample (30 μl) and latex reagents (35 μl), and the rotating speed of the glass slide (30 rpm). The values obtained by the LAIR method were compared with those by gas chromatography–mass spectrometry–selected ion monitoring (GC–MS–SIM) in which deutrated estriol (estriol-2,4-d_2) was used as an internal standard. There was a significant correlation between these values. The correlation coefficient was 0.9865 and the regression line was expressed as $y=1.082x-2.10$. The results indicate that the estrogen values semi-quantitatively determined by the LAIR method are reliable ones and that this procedure can be used effectively to determine estrogen levels in four ranges, less than 5 μg/ml, 5—10 μg/ml, 10—20 μg/ml, and more than 20 μg/ml, as a clinical screening test for monitoring feto-placental functions. The LAIR method is suitable for routine measurement of urinary estrogens.

Keywords——gas chromatography-mass spectrometry; selected ion monitoring; stable isotope; urinary estrogens; latex agglutination inhibition reaction

The estrogen value in the urine of pregnant women is one of the most reliable indices of the fetal well-being in late pregnancy. Therefore, a conventional assay method is required for the routine screening measurement of estrogen in urine. In a series of immunological determination studies of urinary estrogens,[2] we have reported a simplified and sensitive semi-quantitative determination method using the latex agglutination inhibition reaction (LAIR) in the preceding paper.[1] In clinical diagnosis, it is widely accepted that the determination of urinary estrogens in four ranges, less than 5 μg/ml, 5—10 μg/ml, 10—20 μg/ml, and more than 20 μg/ml, is valuable for the screening of feto-placental functions.[3]

Recently, the usefulness of selected ion monitoring (SIM), in which gas chromatography–mass spectrometry with a multiple ion detector (GC–MS–MID) is used in conjunction with stable isotope labelling, has been of great interest for the measurement of trace amounts of a substance in biological materials because of its high sensitivity and high selectivity.[4] In this technique, stable isotope-labelled carriers serve as the ideal internal standard to correct for the loss of a substance under study during isolation.

In this work, we studied the application of the GC–MS–SIM technique in the determination of urinary estriol, and the values were compared with those for urinary estrogens obtained by quantitative processing according to the LAIR method.

Experimental

Materials——Estriol-2,4-d_2 (E_3-2,4-d_2) was synthesized by the method described by Kanno *et al.*[4f] We calculated the deuterium contents, using the ions around the molecular ion of the trifluoroacetate of the

labelled and unlabelled compounds. The isotopic composition was 98.3 atom % deuterium (d_2, 88.2%, d_1, 10.1%, d_0, 1.7%). Estriol-6,7-^{3}H 16-glucuronide (^{3}H-E_3-16-G) (40 Ci/mmol) was obtained from the Radiochemical Centre and was purified shortly before use by chromatography on Sephadex LH-20, using a benzene–methanol system, 85: 15. β-Glucuronidase from helix-pomatia was purchased from Sigma Chemical Co. (St. Louis, Mo.). All solvents and chemicals were of analytical-reagent grade.

Selected Ion Monitoring (SIM)——SIM measurements were made with a Shimadzu LKB-9000 gas chromatograph-mass spectrometer equipped with a Shimadzu high-speed multiple ion detector (MID)-peak matcher (PM). The ionizing voltage was set at 70 eV and the trap current at 60 μA. The MID was focussed on the ions at *m/z* 576 and *m/z* 578 and the peak height ratio was determined. GC was performed on a coiled glass column (1 m) packed with 1% OV-1 Chromosorb W (60—80 mesh). The column temperature was 215°C and the temperature of the flash heater and the separator was 270°C. The temperature of the ion source was 290°C. Helium carrier gas flow-rate was about 25 ml/min.

Preparation of Calibration Curve——To each of the four standards containing 10—100 ng of estriol in 50 μl of ethanol, 50 ng of E_3-2,4-d_2 dissolved in 10 μl of ethanol was added. After evaporation of the solvent, each standard was dried under a nitrogen stream. Each sample was dissolved in 50 μl of ethyl acetate and then 50 μl of trifluoroacetic anhydride was added. A 1—5 μl volume of the above reaction mixture was analyzed by GC–MS.

Preparation of Sample for SIM——To 0.1 ml of pregnancy urine samples diluted fifty-fold with 0.1 M acetate buffer (pH 4.8) was added 600 U of β-glucuronidase in 0.1 ml of acetate buffer (pH 4.8), and the solution was incubated at 48°C for 24 h. After hydrolysis of estriol glucuronide, 50 ng of E_3-2,4-d_2 dissolved in 0.1 ml of ethanol was added, then the urine sample was extracted with 3×2 ml of ethyl acetate. The layer of ethyl acetate was collected, washed with 2×0.5 ml of water, and dried over anhydrous sodium sulfate. The solvent was evaporated off, and the residue was dissolved in ethyl acetate. The trifluoroacetate (TFA) derivative was formed as described above and 1—5 μl of the sample was subjected to GC–MS. In order to determine the effect of purification, the extract was treated as follows. After removal of the solvent by evaporation, the residue was dissolved in ethyl acetate and subjected to thin-layer chromatography (TLC) on Kieselgel plates. The TLC plate was developed with chloroform: isopropyl alcohol: formic acid (15: 5: 4) and the UV-positive zone corresponding to 16-epi estriol with an *Rf* value of 0.69 was scraped off. Estriol was eluted with 10 ml of ethyl acetate and dried over anhydrous sodium sulfate. After removal of the solvent by evaporation, the TFA derivative was formed and subjected to GC–MS as described above.

Determination of Accuracy——Estriol 16-glucuronide in amonuts of 10.32, 20.64 and 30.96 ng equivalent to estriol was added to 0.1 ml aliquots of a fifty-fold diluted, pooled pregnancy urine sample. The estriol content of the diluted urine was 22.40±0.13 (S.D.) ng/0.1 ml in triplicate determinations. After preparation of the sample for SIM as described above, the observed peak height ratio of *m/z* 576 to *m/z* 578 was determined in triplicate.

Quantitative Processing by the Latex Agglutination Inhibition Reaction Method——Latex linked to polyacrylic acid combined with estriol 16-glucuronide (estriol 16-glucuronide bound latex) and anti-estriol 16-glucuronide antibody-coated latex were prepared by the method described in the preceding paper.[1]

On a clean opaque glass slide was placed 30 μl of urine sample diluted in glycine-NaOH-buffered saline (pH 9.6). Then, 35 μl each of the antibody-latex reagent and the estriol 16-glucuronide bound latex reagent were added in that order to each diluted sample, and the mixture was stirred thoroughly with an applicator stick. The slide was rotated at a speed of 30 rpm for 2 minutes, and the reaction patterns were observed. A macroscopically visible agglutination pattern was judged to be "negative," and an agglutination inhibition pattern(non-agglutination pattern), "positive." The estrogen value in the original urine was calculated by multiplying the sensitivity (0.1 μg/ml) by the highest dilution factor found to cause the positive reaction.

Results and Discussion

Factors in SIM Analysis

Hydrolysis of Urinary Estriol 16-Glucuronide——Since the main estrogen in the urine of pregnant women is estriol 16-glucuronide,[5] hydrolysis is essential for the application of the SIM technique. In order to determine the hydrolysis condition, 1 nCi of ^{3}H-E_3-16-G was added to fifty-fold diluted, pooled pregnancy urine (the concentration of estriol 16-glucuronide was *ca.* 1 μg/ml), and the mixture was incubated with 300 U, 600 U or 1200 U of β-glucuronidase in 1 ml of 0.1 M acetate buffer (pH 4.8) at 48°C. After a certain period of time, radioactivity which was extracted with 4×2 ml of ethyl acetate was counted and the extent of hydrolysis was obtained. As shown in Fig. 1, the extents of hydrolysis with 300 U, 600 U and 1200 U of β-glucuronidase at 4 h were 89, 92 and 94.5%, respectively, and those at 24 h were 95.0, 99.4 and 99.0%. Therefore, the hydrolysis of diluted, pregnancy urine samples was performed with 600 U of β-glucuronidase at 48°C for 24 h throughout this work.

Extraction and Purification——The assay was performed by adding 50 ng of the deuterated internal standard (E_3-2,4-d_2) to the hydrolyzed pregnancy urine sample. The results were compared to judge whether the purification step by TLC after ethyl acetate extraction is necessary or not. As shown in Table I, the results of the assays for five samples with and without TLC purification are in fairly good agreement. Therefore, it seems likely that there was no interference in the molecular ion peaks by contributions from other materials in the urine extract.

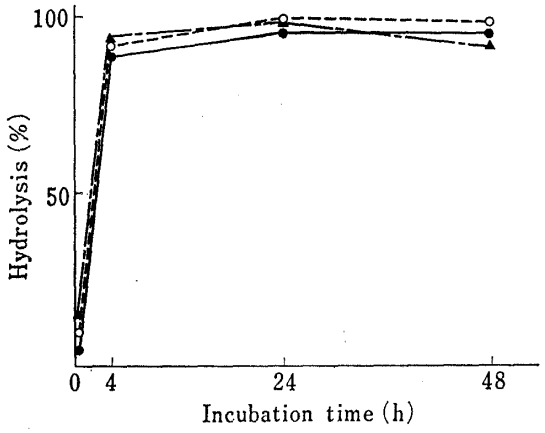

Fig. 1. The Extents of Hydrolysis with 300 U, 600 U and 1200 U of β-Glucuronidase

One nCi of ^{3}H-E_3-16-G was added to fifty-fold diluted, pooled pregnancy urine (the concentration of E_3-16-G was *ca.* 1 μg/ml as E_3), and the mixture was incubated with 300 U (●), 600 U (○) and 1200 U (▲) of β-glucuronidase in 1 ml of 0.1 M acetate buffer (pH 4.8) at 48°C. After a certain period of time, radioactivity which was extracted with 4×2 ml of ethyl acetate was counted and the extent of hydrolysis was obtained. Each value represents the mean of triplicate measurements.

Calibration Curve——Known mixtures of estriol and E_3-2,4-d_2 were prepared so that the sample size (1—5 μl) injected into the gas chromatograph-mass spectrometer covered the estriol range of 1—10 ng with a fixed amount (5 ng) of E_3-2,4-d_2. Each mixture was then analyzed as the TFA derivative, focusing on the molecular ions at *m/z* 576 for estriol and *m/z* 578 for E_3-2,4-d_2. There was a good correlation between the mixed molar ratio and the observed peak height ratio, as shown in Table II. The peak height ratio was plotted against the molar ratio of estriol to E_3-2,4-d_2 and a calibration curve was obtained.

Accuracy——Estriol 16-glucuronide in amounts of 10.32, 20.64 and 30.96 ng equivalent to estriol was added to 0.1 ml aliquots of a fifty-fold diluted, pooled pregnancy urine sample. The amounts of estriol in these samples were then measured by the present method. The

Table I. Comparison of the Values determined by SIM for Five Pregnancy Urine Samples with and without TLC Purification

Sample	Without TLC			With TLC		
	Individual values		Mean (μg/ml)	Individual values		Mean (μg/ml)
1	22.58	22.55	22.57	22.15	23.30	22.23
2	6.35	6.42	6.39	6.34	6.44	6.39
3	56.37	56.43	56.40	57.03	55.98	56.51
4	11.62	12.96	12.26	11.64	12.18	11.91
5	45.94	47.57	46.76	44.42	46.24	45.33

Each individual value represents the mean of duplicate measurements.

Table II. Relationship between Mixed Molar Ratio and Observed Peak Height Ratio determined by SIM

Mixed ratio[a]	Observed ratio[b]
0.2256	0.2718±0.0015
0.5390	0.5865±0.0087
1.0461	1.1448±0.0138
1.9900	1.9660±0.0187

a) Molar ratio of estriol to estriol-2,4-d_2.
b) Mean±S.D. of triplicate measurements.

Table III. Accuracy of SIM Analysis of Estriol in Urine

Added (ng)	Expected (ng)	Found (ng) Individual values[a]			Mean±S.D.	Relative error (%)
		22.50	22.31	22.40	22.40±0.13	
10.32	32.72	32.49	32.27	32.90	32.55±0.32	−0.52
20.64	43.04	42.56	42.30	41.60	42.15±0.49	−2.07
30.96	53.36	52.13	52.06	52.92	52.37±0.48	−1.86

a) Each individual value represents the mean of triplicate measurements.

estriol content in the diluted urine measured in triplicate was 22.40±0.13 ng/0.1 ml. The results presented in Table III show that the amounts of estriol added were in good agreement with those of estriol measured, the relative error being less than 2.1%.

Comparison of Results obtained by SIM and by the Latex Agglutination Inhibition Reaction (LAIR) Method

Since the transitional pattern from inhibition to agglutination in the LAIR method is clear, as described in the preceding paper,[1] quantitative processing by the LAIR method could be achieved by strictly standardizing the volumes of the sample and latex reagents, and the rotating speed of the glass slide.

Table IV shows the sensitivity of this method. When the volumes of the standard estriol 16-glucuronide (as estriol) and latex reagents were 30 μl and 35 μl, respectively, and the rotating speed was 30 rpm, it was possible to distinguish the inhibited pattern at 0.1 μg/ml and the agglutination pattern at 0.09 μg/ml. Therefore, first, urine samples of healthy pregnant women in various stages of gestation were semi-quantitatively analyzed at four ranges of estrogen level, and then quantitatively analyzed by the above method using accurately diluted urine. The results obtained by this method were compared with those obtained by the GC–MS–SIM method described above. As shown in Table V, a satisfactory correlation was found between the results obtained by the two methods. The inter-assay coefficients of variation (C.V.) obtained with the 20 samples were 4.86% for the SIM method and 12.9% for the LAIR method. The correlation coefficient was 0.9865 and the regression line was expressed as $y=1.082x-2.10$. The inter-assay C.V. for this SIM method was slightly higher than that for the plasma testosterone analysis reported by Baba *et al.*[4e] and for the serum estriol analysis reported by Sugano *et al.*[4f] It is considered that this difference is due to the hydrolysis step, since the addition of the isotope-labelled carriers could not cancel out the error caused by the hydrolysis in this experiment.

The LAIR method is fundamentally semi-quantitative and quantitative processing is of little practical utility for clinical purposes because of the complicated procedure of accurate dilution of sample urine and the requirement, to some degree, for skillful judgement of reaction

Table IV. Sensitivity of the Latex Agglutination Inhibition Test

Reaction time (min)	E_3-16-G (μg/ml as E_3)							
	0.12	0.10	0.09	0.08	0.07	0.06	0.05	0
0.5	+	+	+	+	−−	−−−	−−−	−−−
1.0	+	+	+	−	−−−	−−−	−−−	−−−
1.5	+	+	−	−−	−−−	−−−	−−−	−−−
2.0	+	+	−	−−	−−−	−−−	−−−	−−−

+: A complete agglutination-inhibited pattern.
−: Small agglutinates observed visually to a slight extent.
−−: Comparative large agglutinates with a turbid background.
−−−: Large agglutinates with a clean background.

TABLE V. Measurement of Estriol in Pregnancy Urine Samples by SIM and LAIR

Urine	SIM[a] (μg/ml)	LAIR (μg/ml)	
		Quantitative[a]	Semi-quantitative
1	22.57±0.02	21.3±1.2	≧20
2	15.61±0.04	16.3±1.2	10—20
3	25.79±0.52	27.0±1.0	≧20
4	6.39±0.05	5.1±0.4	5—10
5	56.40±0.04	64.0±4.0	≧20
6	15.58±0.17	14.7±0.6	10—20
7	5.89±0.98	6.0±1.0	5—10
8	42.69±1.00	40.7±3.1	≧20
9	12.29±0.95	10.7±1.2	10—20
10	35.73±0.57	35.3±2.3	≧20
11	49.68±0.21	52.0±4.0	≧20
12	15.42±0.38	14.0±1.0	10—20
13	44.43±1.46	49.3±2.3	≧20
14	6.40±0.10	5.8±0.6	5—10
15	21.63±0.05	19.0±1.0	≧20
16	11.12±0.05	10.2±0.8	10—20
17	13.53±0.00	12.3±1.5	10—20
18	9.14±0.10	8.3±0.8	5—10
19	28.54±0.56	27.3±3.1	≧20
20	46.76±1.15	44.0±4.0	≧20
Inter-C.V. (%)	4.86	12.90	

a) Each value represents the mean±S.D. of triplicate measurements.
The correlation coefficient was 0.9865 and the regression line was expressed as $y=1.082x-2.10$. (y; quantitative LAIR values, x; SIM values).

patterns. However, the estrogen values obtained by quantitative processing according to the LAIR method agreed fairly well with the estriol values obtained by the GC–MS–SIM method, and a satisfactory correlation was found between the values obtained by the two methods. From these results, we believe that the determination of estrogen values in four ranges, less than 5 μg/ml, 5—10 μg/ml, 10—20 μg/ml and more than 20 μg/ml, can be carried out reliably by the semi-quantitative LAIR method as a clinical screening test for feto-placental functions, and that this method is suitable for the routine measurement of urinary estrogens.

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