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Studies on Immunological Assay of Urinary Estrogens. III.[1] A New Latex Agglutination Inhibition Reaction Method

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A new, simple and rapid method for the immunological determination of estrogens in pregnancy urine is described. The principle of this method is based on the latex agglutination inhibition reaction in a solid-solid system. An antibody latex reagent (Ab-Latex) was obtained by sensitizing latex particles with anti-estriol 16-glucuronide antibody which had been raised in a goat. Estriol 16-glucuronide-bound latex reagent (E_3-16-G-Latex) was prepared by the condensation of polyacrylic acid combined with estriol 16-glucuronide (E_3-16-G) and lysine-latex which had been prepared from carboxylate modified latex. Hexamethylenediamine was used for the binding of E_3-16-G to polyacrylic acid.

The latex agglutination inhibition tests were performed on opaque glass slides. One drop (30 μl) of Ab-Latex suspension followed by one drop of E_3-16-G-Latex suspension was added to one drop of diluted urine sample and mixed thoroughly. The slides were rotated gently for 2 minutes, and a macroscopically visible agglutination inhibition pattern was observed in the presence of an estrogen concentration of 0.1 μg/ml (as estriol) or more.

The estrogen values in urine can be obtained by multiplying the sensitivity by the highest dilution factor able to give a positive reaction. This test cross-reacted not only with free estriol but also with all free and conjugated estrogens having a free hydroxy group at position 3 in the steroid ring. The urinary estrogen values obtained by this method showed a good correlation with those measured by a radioimmunoassay method (correlation coefficient; 0.9845, regression line; $y=0.823x-0.614$). When this method was compared with a semi-quantitative determination method, the hemagglutination inhibition reaction (E_3 HAIR kit), the results of the two methods were in fairly good agreement.

Keywords——latex agglutination inhibition reaction; anti-estriol 16-glucuronide antibody-coated latex; estriol 16-glucuronide-bound latex; feto-placental functions; immunoassay; determination of urinary estrogens

The estriol or total estrogen value in the urine of pregnant women is one of the most reliable indices of fetal well-being in late pregnancy.

There are several assay methods for the determination of urinary estrogens,[2] but a long time is required to obtain the results, as well as special equipment. In the preceding paper,[1] we reported a simplified immunological determination method for urinary estrogens using the hemagglutination inhibition reaction (HAIR). This method is sensitive and useful as a screening test for feto-placental functions of pregnant women,[3] but it requires 2 hours to obtain the results. Bedside diagnostic measurements are required to provide rapid results with simple procedures. Therefore, we tried to develop a faster and simpler method for measuring urinary estrogens. This paper deals with a new latex agglutination inhibition reaction system consisting of anti-estriol 16-glucuronide antibody-coated latex particles and estriol 16-glucuronide-bound latex particles.

Experimental

Materials——Bovine serum albumin (BSA), goat serum albumin (GSA), human serum albumin (HSA), human gamma globulin (HGG), and bovine gamma globulin (BGG) were purchased from Miles Laboratories Inc., Ill., U.S.A. Polystyrene latex particles (diameter 0.81 μ) and carboxylate-modified latex particles were obtained from Dow Chemicals, Ind., U.S.A. Polyacrylic acid (molecular weight 250000) was purchased

from Aldrich Chemicals, Wis., U.S.A. Estriol 16-glucuronide was synthesized by the method reported by Nambara *et al.*[4] CN-Br-activated Sepharose 4B was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Urinary protein was prepared from pooled female urine by the method reported by Tsubota and Ozawa.[5]

Radioimmunoassay (RIA) and HAIR——RIA was performed by the method described in a previous paper[6] and HAIR was performed by using the E_3-HAIR kit (Teikoku Hormone Mfg. Co., Ltd., Tokyo, Japan).

Production of Anti-estriol 16-Glucuronide Antibody——Estriol 16-glucuronide-BSA conjugate was prepared by the method described in a previous paper.[6] The estriol 16-glucuronide-BSA (5 mg) was dissolved in 2.5 ml of physiological saline, and the solution was emulsified with an equal volume of complete Freund's adjuvant. The emulsion was injected subcutaneously into several sites of a mature goat at one month intervals. After confirming the increase of the antibody titer, the total blood was collected and antiserum was obtained. The complement contained in antiserum was inactivated at 56° for 30 min and anti-BSA antibody was removed by absorption with BSA-Sepharose as described previously.[6] Subsequent fractionation with ammonium sulfate afforded a partially purified preparation of anti-estriol 16-glucuronide antibody (gamma globulin).

Production of Latex Sensitized with Anti-estriol 16-Glucuronide Antibody (Ab-Latex)——The antibody-sensitized latex was prepared by the modified method of Fritz *et al.*[7] The anti-estriol 16-glucuronide antibody (4 mg) was dissolved in 15 ml of glycine–NaOH buffered saline (pH 8.2), and 1 ml of a 10% polystyrene latex particle suspension was added. The mixture was stirred at 56° for 30 min, and the latex particles were precipitated by centrifugation then washed with glycine–NaOH buffered saline. The latex particles were suspended in 15 ml of glycine–NaOH buffered saline containing 0.05% GSA to obtain the Ab-Latex reagent.

Production of Lysine-latex (III)——A solution of 260 mg of *ε-tert*-butoxycarbonyl lysine methyl ester[8] in 3 ml of dimethylformamide was added to 5 ml of a 10% suspension of carboxylate–modified latex particles (I). The mixture was cooled to 0°, and 234 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (water-soluble carbodiimide) was added with stirring. The mixture was stirred at 0° for 1 hr and at room temperature for 3 hr, and was then allowed to stand overnight at room temperature. After centrifugation, the supernatant was discarded, and the precipitates were washed with a 50% aqueous solution of dimethylformamide and then with water. After 5 ml of ice-cold concentrated hydrochloric acid had been added, the mixture was allowed to stand at 0° for 15 min with occasional shaking. It was then diluted with an equal volume of ice-water, and centrifuged. The precipitates were washed with water until the supernatant became neutral, then 10 ml of a 10% aqueous solution of trimethylamine was added. The mixture was stirred at room temperature for 15 min, centrifuged, and repeatedly washed with water until the supernatant became neutral. Finally, the concentration of the resulting suspension was adjusted to 10% to form the desired lysine–latex (III). This product showed a positive ninhydrin reaction.

Production of Polyacrylic Acid combined with Estriol 16-Glucuronide (VII)——1) N-(6-Benzyloxycarbonylaminohexyl)-(3,17β-dihydroxy-1,3,5(10)-estratrien-16α-yl-β-D-glucopyranosid)uronamide (V): Estriol 16-glucuronide (IV) (93 mg) and N-hydroxysuccinimide (25 mg) were dissolved in 1.5 ml of dimethylformamide, then 41 mg of dicyclohexylcarbodiimide (DCC) was added with stirring under ice-cooling. After 30 min, a solution of 55 mg of monobenzyloxycarbonyl hexamethylenediamine hydrochloride[9] and 0.03 ml of triethylamine in 1 ml of dimethylformamide was added. The mixture was stirred for 2 hr with ice-cooling, and for 12 hr at room temperature. It was then evaporated to dryness under reduced pressure. The residue was subjected to preparative thin-layer chromatography to afford 82 mg (55% of the theoretical yield) of the desired product (V). This product showed a single spot at $Rf=0.42$ (chloroform-methanol 5:1) on silica gel thin-layer chromatography.

2) N-(6-Aminohexyl)-(3,17β-dihydroxy-1,3,5(10)-estratrien-16α-yl-β-D-glucopyranosid)uronamide (VI): Compound V (50 mg) was dissolved in 3 ml of methanol, and 10 mg of palladium-charcoal was added. The mixture was stirred at room temperature under an H_2 atmosphere. The reaction was followed by thin-layer chromatography; the starting material disappeared within 1.5 hr. The catalyst was separated by filtration, and washed with methanol. The filtrate was evaporated to dryness under reduced pressure. Addition of ether to the residue gave 35 mg of the desired product (VI) as a white solid.

3) Polyacrylic Acid combined with Estriol 16-Glucuronide (VII): Compound VI (80 mg) and polyacrylic acid (5 g) were dissolved in 100 ml of dimethylformamide and 40 mg of dicyclohexylcarbodiimide was added. The mixture was stirred for 48 hr. The reaction mixture was transferred to a cellophane tube and dialyzed against water for 80 hr. The nondialysable fraction was filtered and made up to 500 ml, and the amount of estriol 16-glucuronide introduced was determined spectrometrically (280 nm). Lyophilization afforded 5 g of the desired product (VII) as a white solid. It contained 0.85—1.10 mg of estriol 16-glucuronide per 100 mg of solid.

Production of Latex linked to Polyacrylic Acid combined with Estriol 16-Glucuronide (VIII)——Compound III (0.1 g) was suspended in 1 ml of distilled water, and 4 mg of VII was added to the suspension. Next, 10 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride was added, and the reaction mixture was stirred overnight. The mixture was centrifuged, and the resulting precipitates were washed three times

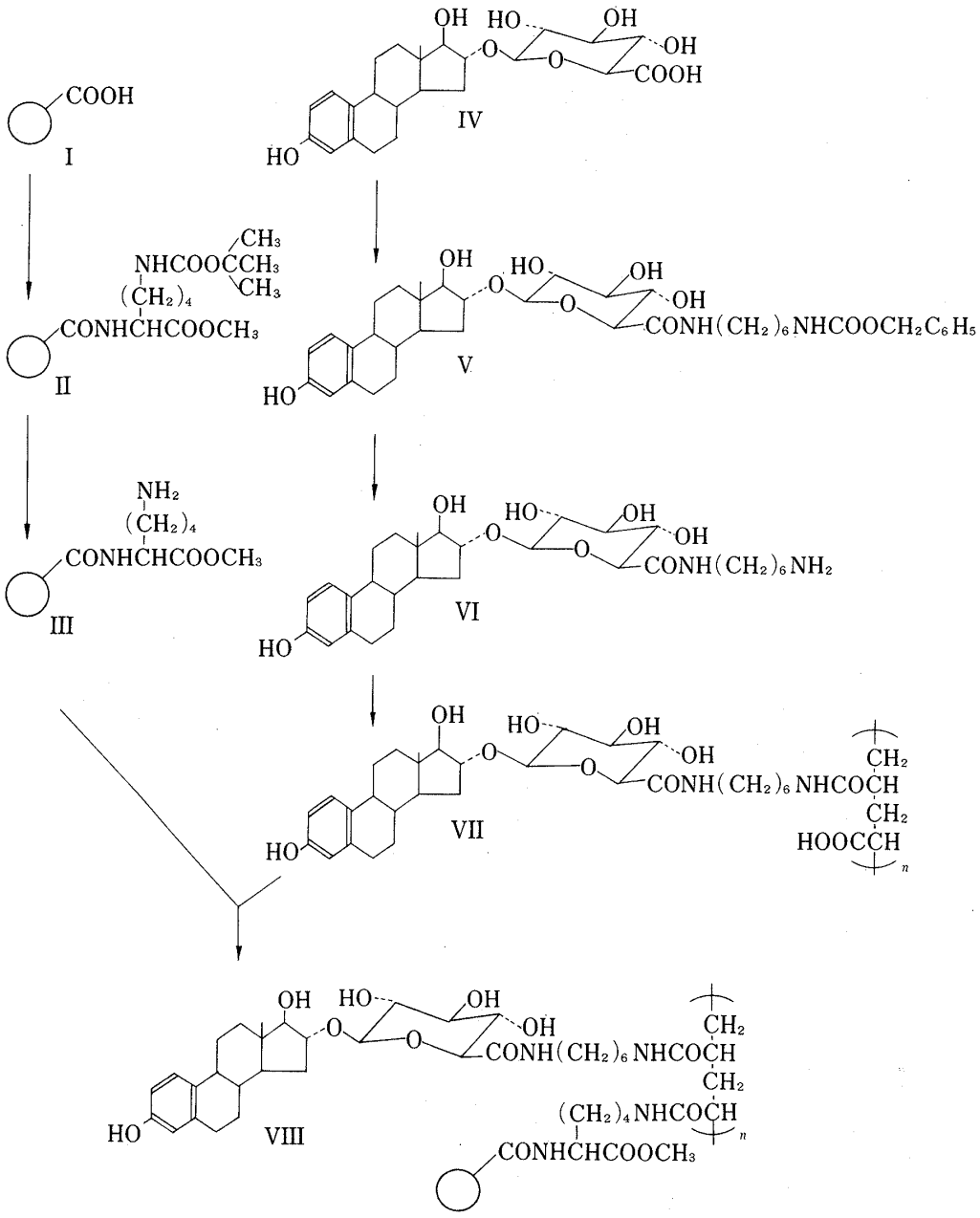

Chart 1

with 10 ml of glycine–NaOH buffered saline (pH 9.6). The precipitates were suspended in glycine–NaOH buffered saline containing 0.05% GSA to produce the suspension of VIII.

Procedure of the Latex Agglutination Inhibition Test——On a clean opaque glass slide was placed 0.03 ml of urine sample, which was serially diluted in glycine–NaOH buffered saline (pH 9.6). One drop of Ab-Latex reagent followed by one drop of the reagent (VIII) was added to each diluted sample, and the mixture was stirred thoroughly with an applicator stick. The slide was rotated gently for 2 min, then the reaction patterns were observed. A macroscopically visible agglutination pattern is judged to be "negative," and an agglutination inhibition pattern (non-agglutination) "positive."

The estrogen value in the original urine was calculated by multiplying the sensitivity (0.1 μg/ml) by the highest dilution factor able to cause a positive reaction. For example, if the result is positive with the sample diluted with 100 volumes of buffer, urinary estrogens should be present at a concentration equal to or higher than 10 μg/ml.

Production of Latex linked to Polyacrylic Acid combined with 17β-Amino-1,3,5(10)-estratrien-3-ol[10]—— The latex reagent was prepared as described above from 17β-amino-1,3,5(10)-estratrien-3-ol by two steps; the reaction of the aminosteroid and polyacrylic acid and the condensation of the product with lysine–latex.

Results

Reaction Pattern and Sensitivity of the Latex Agglutination Inhibition Test

Latex agglutination inhibition test were performed with standard solutions of estriol 16-glucuronide (0—0.2 μg/ml as estriol) prepared in glycine-NaOH buffered saline (pH 9.6). The reaction patterns were checked at 0.5 min intervals up to 3 min after mixing the latex reagents. As shown in Table I, the transition from an inhibition pattern (at 0.1 μg/ml) to an agglutination pattern (at 0.08 μg/ml) was clearly apparent between 1 and 3 min. Agglutination inhibition was seen in a 0.08 μg/ml solution at 0.5 min, but slight agglutination occurred after 1 min, and then a clear agglutination pattern emerged. It was concluded from the above tests that the results should be evaluated at 2 minutes after mixing of the latex reagents, and the sensitivity of this test was found to be 0.1 μg/ml as estriol.

Table I. Sensitivity of the Latex Agglutination Inhibition Test

Reaction time (min)	E_3-16-G (μg/ml as E_3)						
	0.20	0.12	0.10	0.08	0.06	0.05	0
0.5	+	+	+	+	− − −	− − −	− − −
1.0	+	+	+	−	− − −	− − −	− − −
1.5	+	+	+	− −	− − −	− − −	− − −
2.0	+	+	+	− −	− − −	− − −	− − −
2.5	+	+	+	− −	− − −	− − −	− − −
3.0	+	+	+	− −	− − −	− − −	− − −

+ : a completely agglutination-inhibited pattern.
− : some small agglutinates observed visually.
− − : comparatively large agglutinates with a turbid background.
− − − : large agglutinates with a clean background.

Sensitivity and Accuracy

Estriol 16-glucuronide was dissolved in male urine at concentrations from 1 μg/ml to 24 μg/ml (as estriol). The sample was diluted 50, 100, and 200 times with the buffer described above, and the tests were performed (Table II).

Positive results were obtained in all three runs with 50-fold dilution of urine containing not less than 5 μg/ml estriol, with 100-fold dilution of urine with $\geqq$10 μg/ml estriol, and with 200-fold dilution of urine with $\geqq$20 μg/ml estriol. When the samples were diluted nearly to the limit of sensitivity as shown in Table II, it was possible to distinguish an agglutination or agglutination inhibition pattern with less than 10% of the concentration of estriol contained in each sample before dilution. Since the same results were obtained in three consecutive tests, this method is reliable in terms of both sensitivity and accuracy.

Specificity

Since the antibody used for the production of Ab-Latex reagent was obtained with estriol 16-glucuronide-BSA as an antigen, it is capable of binding not only with estriol but also with all estrogens having a free hydroxy group at position 3 in their steroid ring.[6] Table III shows the results of the agglutination inhibition test on various steroids. Cross reaction was observed and the agglutination was almost equally inhibited with various estrogens having a hydroxy group at position 3. On the other hand, negative results were obtained with estrogens conjugated at position 3, as well as with steroids having no phenolic hydroxyl group at position 3.

TABLE II. Accuracy of the Latex Agglutination Inhibition Test

Added E_3-16-G (μg/ml as E_3)	1st Dilution 50	1st Dilution 100	1st Dilution 200	2nd Dilution 50	2nd Dilution 100	2nd Dilution 200	3rd Dilution 50	3rd Dilution 100	3rd Dilution 200
1	− − −	− − −	− − −	− − −	− − −	− − −	− − −	− − −	− − −
2	− − −	− − −	− − −	− − −	− − −	− − −	− − −	− − −	− − −
3	− − −	− − −	− − −	− − −	− − −	− − −	− − −	− − −	− − −
4	− −	− − −	− − −	− −	− − −	− − −	− −	− − −	− − −
5	+	− − −	− − −	+	− − −	− − −	+	− − −	− − −
6	+	− − −	− − −	+	− − −	− − −	+	− − −	− − −
7	+	− − −	− − −	+	− − −	− − −	+	− − −	− − −
8	+	− −	− − −	+	− −	− − −	+	− −	− − −
9	+	−	− − −	+	− −	− − −	+	−	− − −
10	+	+	− − −	+	+	− − −	+	+	− − −
12	+	+	− − −	+	+	− − −	+	+	− − −
14	+	+	− − −	+	+	− − −	+	+	− − −
16	+	+	− −	+	+	− −	+	+	− −
18	+	+	−	+	+	−	+	+	−
20	+	+	+	+	+	+	+	+	+
24	+	+	+	+	+	+	+	+	+

+: a completely agglutination-inhibited pattern.
−: some small agglutinates observed visually.
− −: comparatively large agglutinates with a turbid background.
− − −: large agglutinates with a clear background.

TABLE III. Cross-reaction in the Latex Agglutination Inhibition Test with Various Steroids

Compound	Concentration (μg/ml)[a] 0.40	0.20	0.10	0.05	0.025
Estrone	+	+	+	−	−
Estrone 3-sulfate	−	−	−	−	−
Estrone 3-glucuronide	−	−	−	−	−
Estradiol	+	+	+	−	−
Estradiol 3-sulfate	−	−	−	−	−
Estradiol 3-glucuronide	−	−	−	−	−
Estradiol 17-sulfate	+	+	+	−	−
Estradiol 17-glucuronide	+	+	+	−	−
Estriol	+	+	+	−	−
Estriol 3-sulfate	−	−	−	−	−
Estriol 3-glucuronide	−	−	−	−	−
Estriol 16-glucuronide	+	+	+	−	−
Estriol 17-glucuronide	+	+	+	−	−
16-epiestriol	+	+	−	−	−
Dehydroepiandrosterone	−	−	−	−	−
16α-hydroxy dehydroepiandrosterone	−	−	−	−	−
Pregnanediol	−	−	−	−	−
Pregnanediol 3-glucuronide	−	−	−	−	−
Testosterone	−	−	−	−	−
Testosterone 17-glucuronide	−	−	−	−	−
Cortisol	−	−	−	−	−

a) Concentrations of conjugated steroids are shown as equivalents in free steroids.
+: agglutination-inhibited pattern.
−: agglutinated pattern.

Table IV. The Influence of Glucose, NaCl, HSA, HGG and Urinary Protein on the Latex Agglutination Inhibition Test

Compound	Amount of added (mg/ml)	Pregnancy urine A				Pregnancy urine B				Pregnancy urine C			
		Dilution			Estimated estrogen (μg/ml)	Dilution			Estimated estrogen (μg/ml)	Dilution			Estimated estrogen (μg/ml)
		50	100	200		50	100	200		200	300	400	
Glucose	125	+	−	−	5	+	+	−	10	+	−	−	20
NaCl	125	+	−	−	5	+	+	−	10	+	−	−	20
HSA	12.5	+	−	−	5	+	+	−	10	+	−	−	20
HGG	12.5	+	−	−	5	+	+	−	10	+	−	−	20
Urinary protein	12.5	+	−	−	5	+	+	−	10	+	−	−	20
No addition		+	−	−	5	+	+	−	10	+	−	−	20

+: agglutination-inhibited pattern.
−: agglutinated pattern.

Table V. The Influence of Urine pH on the Latex Agglutination Inhibition Test

pH of urine	Pregnancy urine A				Pregnancy urine B				Pregnancy urine C			
	Dilution			Estimated estrogen (μg/ml)	Dilution			Estimated estrogen (μg/ml)	Dilution			Estimated estrogen (μg/ml)
	50	100	150		100	200	300		50	75	100	
2	+	+	−	10	+	+	−	20	+	−	−	5
4	+	+	−	10	+	+	−	20	+	−	−	5
6	+	+	−	10	+	+	−	20	+	−	−	5
8	+	+	−	10	+	+	−	20	+	−	−	5
10	+	+	−	10	+	+	−	20	+	−	−	5

+: agglutination-inhibited pattern.
−: agglutinated pattern.

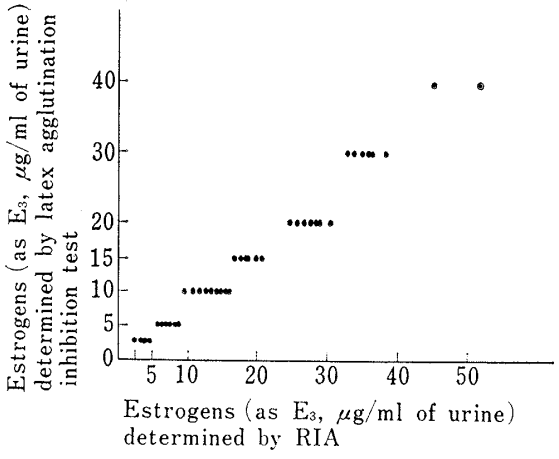

Fig. 1. Correlation of Urinary Estrogen Values obtained by the Latex Agglutination Inhibition Test and by RIA

$n=40$
$r=0.9845$
$y=0.823x-0.614$

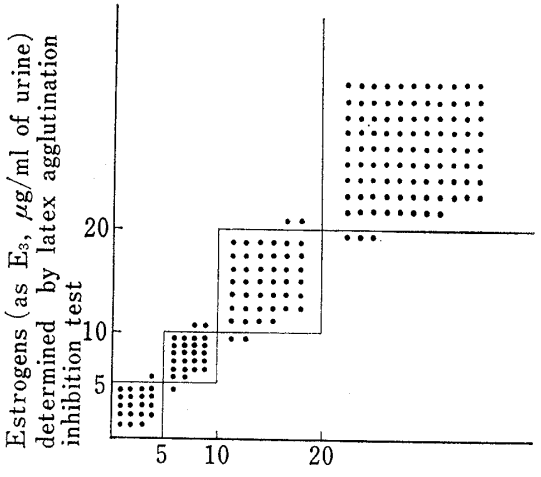

Fig. 2. Coincidence of Urinary Estrogen Values obtained by the Latex Agglutination Inhibition Test and by HAIR

The coincidence rate was 94.1% ($n=188$).

The Influence of Glucose, Sodium Chloride, Serum Protein, Urinary Protein, and Urine pH on This Method

Glucose, sodium chloride, HSA, HGG and urinary protein were separately dissolved in samples of pregnancy urine, and the urine was diluted. As shown in Table IV, these materials did not affect the results.

Urine pH was adjusted with 1 N sodium hydroxide or 1 N hydrochloric acid from 2 to 10, and the samples were allowed to stand for 1 hour before the test. It was found that urine pH did not affect the results. These data are shown in Table V.

Correlation with Other Methods

Urine of healthy pregnant women in various periods of gestation was used as test samples to investigate the correlation between the results obtained by this method and those obtained by RIA. As Fig. 1 shows, there was a good correlation between the values determined by these two methods. The correlation coefficient was 0.9845 and the regression line can be expressed as $y=0.823x$-0.614. Good agreement of the results obtained by this method with those obtained by the HAIR method (a semi-quantitative method for the determination of estrogens) was obtained using pregnancy urine samples at four levels of estrogens: less than 5 μg/ml, 5—10 μg/ml, 10—20 μg/ml, and more than 20 μg/ml. As shown in Fig. 2, the coincidence rate was about 94%, which is satisfactory.

Discussion

Methods employing the inhibition of latex agglutination, in which latex sensitized with an antigen (L–Ag) and the corresponding antiserum (Ab) solution are employed, have been widely used in the determination of so-called "complete antigens" such as protein and polysaccharide.[11] On the other hand, there have been few reports on the application of this method to determine a hapten.[12] In a recent Japanese patent,[12b] the determination of estriol in pregnancy urine has been described, where L–Pr–H (L, Pr, and H indicate latex, protein and hapten, respectively) is used instead of L–Ag. The determination of haptens should be possible by the combined use of other reagents such as latex sensitized with an antibody (L–Ab) and the corresponding Pr–H solution. In fact, such a method has been reported in a Japanese patent by the Mochida group.[13] The two methods described above can be considered to be solid-liquid systems. These observations prompted us to study a solid-solid system for the determination of a hapten. An attempt was made to use such a system for the determination of urinary estrogens, and many advantages, such as clearness of agglutination pattern, increase of sensitivity, and shortening of determination time, were found.

Anti-estriol 16-glucuronide antibody was used as the antiserum, since the main estrogen in the urine of pregnant women is estriol 16-glucuronide,[14] and, accordingly, the use of this antibody seemed to be the most reasonable for the determination of urinary estrogens. The antibody was sensitized to latex in a usual manner, and this L–Ab was used throughout this work.

Much effort was directed towards determining what kind of latex reagent bound with estrogen should be used. At first, 17β-amino-1,3,5(10)-estratrien-3-ol was selected as a model compound. Although this compound can be directly condensed with carboxylate-modified latex, a sufficiently long distance between the steroid and the latex surface seemed to be necessary to provide a clear agglutination pattern. Polyacrylic acid, a widely used polymer, seemed very attractive as a spacer. The reaction of the aminosteroid with polyacrylic acid in the presence of dicyclohexylcarbodiimide, followed by condensation with lysine latex, afforded a latex reagent. Mixing of the latex reagent and the Ab–Latex on a glass slide gave a clear agglutination pattern within a short period, usually 1—2 minutes. After succeeding in the preparation of aminosteroid-latex, we studied the preparation of latex bound with estriol

16-glucuronide. The synthesis was carried out as summarized in Chart 1. A detailed comparison of this solid-solid system with two solid-liquid systems reported in Japanese patents[13b,14] revealed the advantages mentioned above. These differences can be explained by the fact that agglutination takes place rapidly in the solid-solid system because the two reagents, latex particles bound with hapten and those with antibody, have binding sites on the particles themselves, and directly participate in the reaction. In the solid-liquid system, however, agglutination occurs gradually and weakly because the latex particles are indirectly bound through antibody (or Pr–H) in the solution.

It was found that hexamethylenediamine used for the binding of estriol 16-glucuronide to polyacrylic acid and lysine used for the binding of latex to polyacrylic acid can be replaced by lysine and hexamethylenediamine, respectively. The replacement of polyacrylic acid with vinyl methyl ether/maleic anhydride copolymer gave almost the same results.

It was found that polyacrylic acid, polyacrylic acid combined with estriol 16-glucuronide (VII), and moreover the latex reagent (VIII) itself have no antigenicity. The latex reagent (VIII), however, has sufficiently strong affinity for the antibody to cause agglutination. It should be pointed out that the synthetic latex reagent (VIII) is covalently bonded, and is very stable.

In this experiment, the latex reagents had sufficient sensitivity to detect 0.1 μg/ml of estriol in the agglutination inhibition test, which seems to be very suitable for the diagnostic determination of urinary estrogens.

Although this method is semi-quantitative and the change of pattern from inhibition to non-inhibition is continuous, as in the case of HAIR method, it was possible to detect the change of pattern in the region of the limit of sensitivity to within a concentration range of 10% as shown in Table II. This means that the accuracy of this method is more than 90%, which is sufficient for the clinical determination of urinary estrogens. The correlation between the results of this method and those of RIA was fully established, as shown in Fig. 1. It was also found that the addition of urinary protein, serum protein, glucose and sodium chloride to the urine, and change of pH of the urine (2—10) did not affect the results. We believe that this system represents a valuable clinical screening test for feto-placental functions.

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