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DETERMINATION OF PHOSPHATIDYLGLYCEROL IN AMNIOTIC FLUID FOR PREDICTION OF FOETAL LUNG MATURITY BY MICROBORE-COLUMN LIQUID CHROMATOGRAPHY*

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SUMMARY

Phosphatidylglycerol (PG) has been determined in amniotic fluid from 55 patients by using a microbore-column liquid chromatographic system. The present analysis time is 1 h 40 min for pretreatment of amniotic fluid and 20 min for a chromatographic run. From 2 ml of amniotic fluid, the PG content has been determined between 1.0 and 0.05 mg/dl. The detection limit of PG is 10 ng. As the injection volume (0.5 μl) is small, repeated analyses are possible if necessary. It is concluded that, in the case of PG values over 0.10 mg/dl, a mother can deliver an infant without respiratory distress syndrome. This method is useful not only for the prenatal evaluation of lung maturity, but also for the assessment of any therapeutic effect.

INTRODUCTION

It is very important to predict foetal lung maturity by measuring the phospholipids of the amniotic fluid in order to decide on the delivery time in complicated cases. The most widely used method of analysing amniotic fluid

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in order to assess foetal lung maturity is the determination of the lecithin/sphingomyelin (L/S) ratio by thin-layer chromatography (TLC) [1–5]. The principal drawback encountered with the above method is a relative lack of quantitation. High-performance liquid chromatography (HPLC) is a powerful method for the separation of complex mixtures, and might be preferable to TLC. The determination of phospholipids in amniotic fluid by HPLC has been reported by Briand et al. [6], Hsieh et al. [7], Paton et al. [8] and Andrews [9].

Briand et al. [6] used a LiChrosorb Diol column with UV detection and Hsieh et al. [7] used silica-gel columns with a wire-transfer flame-ionization detector. In the above methods, detectable amounts of phospholipids are only 1 μg. Recently, Paton et al. [8] reported the application of HPLC to amniotic fluid analysis. They used a rather large amount of amniotic fluid (5—10 ml) and concluded that HPLC lacked sufficient reliability for the application to routine assessment of foetal lung maturity. This conclusion by Paton et al. [8] was derived partly from their liquid chromatographic (LC) system using a refractive index (RI) detector (relatively low sensitivity) and partly from chromatograms of amniotic fluid, which were incomplete. Andrews [9] demonstrated the determination of seven lipids in one chromatographic run, but he also encountered the problem of instability of the chromatographic baseline.

The enzymatic method for the specific determination of phosphatidylglycerol (PG) was reported recently by Muneshige and co-workers [10, 11]. They used 6 ml of amniotic fluid for one determination, and their procedure needed a relatively long analysis time (ca. 3 h).

In this paper, we have adopted an HPLC system with a microbore column for the determination of PG and have aimed to perform the analysis over a relatively short time and with enough high sensitivity for predicting foetal lung maturity or for the evaluation of therapeutic effect. In this study, we have been concerned particularly with the quantitation of PG, because it has been suggested recently that the quantity of PG is a better indicator of foetal lung maturity than the L/S ratio, especially in diabetic cases [3, 4, 10—12]. Since PG is not found in body fluids other than amniotic and bronchoalveolar fluids, it is possible to analyse it using a sample from the vagina.

EXPERIMENTAL

PG from egg yolk, bovine phosphatidyl ethanolamine (PE) and soy-bean phosphatidyl inositol (PI) (Sigma, St. Louis, MO, U.S.A.) were used as standard phospholipids.

An HPLC system consisting of pump (LC-5A), UV detector at 210 nm (SPD-1AM) and a microbore silica gel column (50 cm × 1 mm I.D., particle size 10 μm) (Shimadzu, Kyoto, Japan) was used. For recording and calculation of peak area, Shimadzu Chromatopak (C-R1B) was used. The flow-rate of the eluent was usually set at 90 μl/min. An injector with a 0.5-μl loop (No. 7410, Rheodyne, Cotati, CA, U.S.A.) was used. Short stainless-steel tubing of 0.25 mm I.D. (instead of 0.1 mm I.D. as recommended by the supplier) was used between the column and the injector, in order to avoid clogging up the inside

of the tubing by the precipitation of protein, which is sometimes included in the sample solution. When HPLC is stopped for more than 30 h, it is better to replace the effluent by a solvent whose composition is similar to that of the effluent but excluding phosphoric acid, because sometimes the system is clogged by crystallization of phosphoric acid from the effluent. If we do this, the present microbore LC system could analyse more than 1000 samples for three months without any trouble. Every three months, we replace the column.

Amniotic fluid obtained from 55 patients were studied consecutively between September, 1983 and April, 1984. Amniotic fluid is collected through the vagina, by amniocentesis or at the time of Caesarean operation.

The pretreatment procedure proposed by Gruck and co-workers [1, 2] was modified as follows. After 2 ml of amniotic fluid were centrifuged for 15 min at 3000 rpm (2700 *g*), the insoluble material was filtered off. Then 6 ml of a solution of chloroform and methanol (2:1) were added and the mixture was centrifuged again for 10 min. After centrifugation, the chloroform layer was taken up, evaporated to dryness under vacuum using a rotary evaporator, then 50 μl of ethanol were added. This ethanol solution was injected into the liquid chromatograph in aliquots of 0.5 μl.

The L/S ratio has been measured by TLC (Fetal-Tek 200, Helena Labs., Beaumont, TX, U.S.A.) with the procedure of ref. 5.

In this study we use mg/dl units, because these units are commonly used in clinical laboratories (1 mg/dl PG is equivalent to 10 μg/ml, or 12.5 nmol/ml; the average molecular mass of PG is assumed to be 800).

RESULTS AND DISCUSSION

Phospholipids are a difficult class of compounds to separate by HPLC, because they include very different characteristic compounds; for example, some of them have a negative or positive charge and some have very long saturated or unsaturated fatty acid chains. Therefore, it is very difficult to select a suitable eluent for the complete separation of several phospholipids. There are two standard methods for the separation of phospholipids: class [13—18] or individual molecular species [14, 19]. The latter is the separation of each phospholipid based on differences in the fatty acid components. For medical use, we obtain more information from class analysis. For this purpose, it is common to use a silica gel column and a mixture of hexane (as major component), alcohol, water, acid and/or base (as minor components) as the mobile phase [14—18]. The analytical system of a silica gel column and acetonitrile—methanol—phosphoric acid [13] was not suitable for amniotic fluid, so we adopted the former composition: a mixture of hexane, 2-propanol, 25 m*M* phosphate buffer (pH 7), ethanol and acetic acid was used as the mobile phase [14]. After mixing the above components, it was necessary to keep it for 24 h before use in order to obtain a homogeneous solution. When the concentration of 2-propanol in the mobile phase is increased, the retention times of PG, PE and PI increase. When the concentration of ethanol is increased, the retention times of PE and PI increase but the retention time of PG remains virtually constant. If the retention time of PG is decreased by adjusting the component ratio of the mobile phase, the peak of PG approaches almost the

same retention time as the neutral lipids. If the retention time of PG is lengthened, the peak is broadened, thus making it unsuitable for quantitation. The best composition of mobile phase with a microbore silica gel column for the separation of PG, PE and PI in amniotic fluid is as follows: *n*-hexane–2-propanol–15 m*M* phosphate buffer (Na_2HPO_4–KH_2PO_4, pH 7)–ethanol–acetic acid (795:367:216:62:0.12). The chromatographic separation of amniotic fluid takes ca. 20 min per sample. A typical chromatogram of amniotic fluid is shown in Fig. 1. Retention times of PG, PE, phosphatidyl serine and PI are 5.5, 8.8, 10.6 and 11.8 min, respectively. In our present conditions, lecithin and sphingomyelin do not appear as peaks.

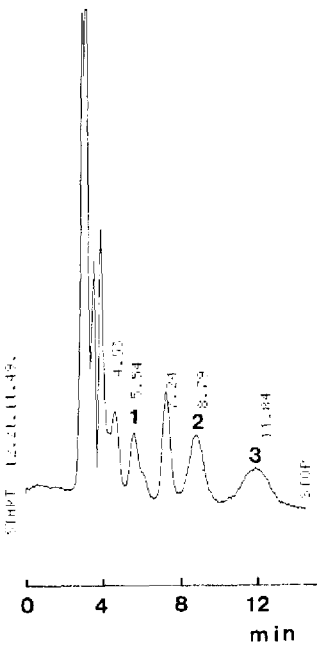

Fig. 1. Liquid chromatogram of amniotic fluid. Amniotic fluid with normal case was obtained at gestational week 38. Peaks: 1 = PG; 2 = PE; 3 = PI.

The peak of PG generally shows a shoulder, as in Fig. 1. This is probably due to the individual fatty acid chains of PG. The calculation of the peak area of PG includes the shoulder. UV adsorption of lipids at 210 nm is believed to be due largely to the unsaturated fatty acid chains. UV adsorption of PG might be dependent on its fatty acid chain composition. Although the fatty acid chain composition of PG in amniotic fluid is not clear, it is possible to estimate the PG value by using UV detection at 210 nm for clinical use.

Characteristic points using microbore-column liquid chromatography

The most remarkable advantage owing to the use of a microbore column is that the consumption of solvent is very small, i.e. 250–500 ml for one week of operation. This is important for reducing the cost of solvents and labour and is convenient for routine analytical systems. The second advantage is that the sample volume per injection is only 0.5 μl of ethanol solution, so it is possible

to repeat the analysis several times in complicated cases. Although, owing to the extra space in the injector a few μl are necessary to fill a loop of 0.5 μl, it is still smaller than that using a conventional-column system; this could be important in the future, if we have a small injector which really has no extra space inside it. The third advantage is that the sample collected from a patient can be kept small.

It is not clear at present whether there is any advance in the sensitivity of the microbore-column system compared to the conventional-column system. We suppose, for comparing the two systems, that a solute passes through the detector per unit time. Injection amounts for microbore- and conventional-column systems are 0.5 and 5 μl, flow-rates are 90 μl/min and 1 ml/min, and cell volumes are 0.5 μl (pass length 3 mm) and 6.4 μl (pass length 8 mm), respectively. With the above conditions, the concentration of sample in the cell is the same for both systems. At the detector, if the light beam is focused to the cell window and the quantity of light is the same for both cells, the sensitivity of the detector for the microbore-column system is three eights that of the detector for the conventional-column system. Therefore, it is difficult to say that there is any advance in sensitivity for the microbore-column system. In other words, there is little sensitivity decrease by using a microbore-column system.

Recovery and reproducibility

Recovery of PG in the present system was checked by using isotonic sodium chloride solution or amniotic fluid at gestational week 20, in which PG is not generally present. After 50 μg of PG were added to 2 ml of the above solutions, the pretreatment procedure was followed. The recovery of PG, estimated by the present method, was 91%. The pretreatment procedure takes ca. 40 min.

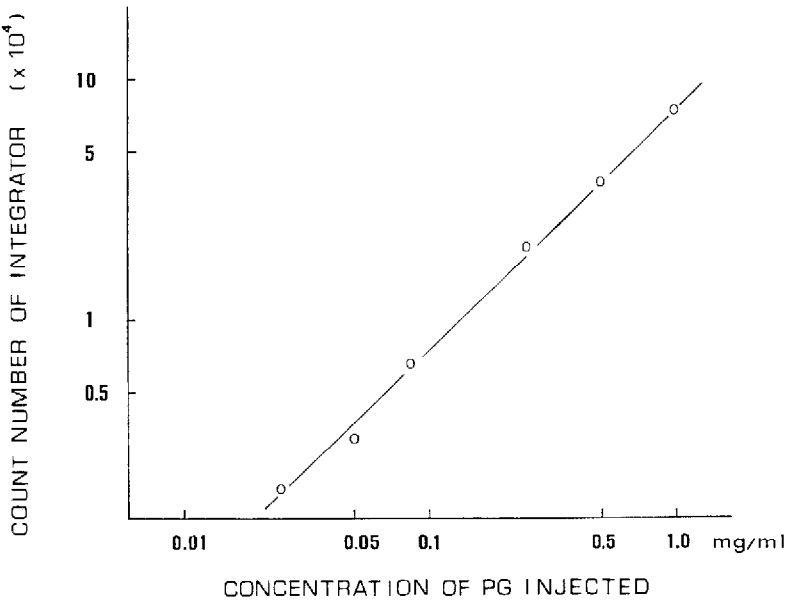

Fig. 2. Calibration curve for PG. The injection amount was 0.5 μl. The concentration of PG in the sample solution was varied from 0.02 to 1.00 mg/ml.

The whole procedure of pretreatment and chromatography takes 1 h. The present method is very rapid when compared to other [8, 10, 11, 14].

The calibration curve of PG is shown in Fig. 2. The relation between the concentration of phospholipid and peak area has a good linear relationship from 0.02 to 1.00 mg/ml ethanolic solution of PG. The correlation coefficient is 0.996. Repeat injections of an ethanolic solution of PG at concentrations of 0.02—1.00 mg/ml give peak areas in which deviation is within 10%.

The absolute limit of detection in this system is 10 ng of PG. The detection limit of PG in amniotic fluid is down to 0.05 mg/dl, because in pretreatment the sample of amniotic fluid is concentrated 40 times, i.e. from 2 ml of amniotic fluid to 50 μl of ethanol solution.

For checking the validation of this method, the PG content in one amniotic fluid sample was measured three times. Variances of observed values of PG in five different amniotic fluid samples are 15%. It is also possible to determine PE from 0.02 to 1.00 mg/ml (the correlation coefficient of the calibration curve is 0.991) and its recovery is the same as that of PG.

PG content of amniotic fluid in normal cases

For evaluation of the PG content in normal cases, 30 amniotic fluid samples were collected from the vagina or at the time of Caesarean operation. The numbers of amniotic fluid samples from the vagina and at the time of Caesarean operation were 24 and 6, respectively. The 30 amniotic fluid samples were taken as follows: four, ten, eight, six and two samples were collected at gestational weeks 37, 38, 39, 40 and 41, respectively. The PG contents in the 30 amniotic fluid samples are distributed from 0.08 to 1.37 mg/dl, and give an average value of 0.51 ± 0.31 mg/dl. The average value at gestational weeks 37, 38, 39, 40 and 41 are 0.46 ± 0.25, 0.49 ± 0.39, 0.50 ± 0.37, 0.59 ± 0.10 and 0.52 ± 0.42 mg/dl, respectively. Namely, the PG content slightly increases with the gestational week number.

PG content of amniotic fluid in complicated cases

In complicated cases, the continuation of pregnancy is sometimes not favourable for treating a mother. Thus, it is very important to decide the right period for delivery of an infant who has mature enough lungs, i.e. one who will have an outside life without respiratory distress syndrome (RDS). In the case of early rupture of the membrane, if the baby has a mature lung even though the gestational week is less than 36 (its amniotic fluid has a high PG content), it is not necessary to treat the mother with tocolytic drugs for the suppression of early delivery. Furthermore, an infant with early rupture of the membrane could avoid infection. Therefore, under the clinical treatment of a complicated or abnormal case, it is very important to know the lung maturity, i.e. to know the PG content in the amniotic fluid of a patient.

We analysed 33 amniotic fluid samples from 25 cases, which were complicated or abnormal cases, as follows: diabetic case (six cases), severe pre-eclampsia (two cases), premature rupture of membrane (five cases), threatened premature delivery (two cases), placenta previa (three cases), polyhydramnios (four cases) and chromosome analysis (one case). We discuss

here 19 of the 25 cases, whose amniotic fluid samples were collected within 72 h before delivery. These clinical data are summarized in Table I.

The number of cases where PG content was lower than the present detection limit (0.05 mg/dl) was four. Two cases out of four had developed RDS. The PG contents of the other fifteen cases were as follows: one, three and eleven cases were between 0.05 and 0.1 mg/dl, between 0.1 and 0.2 mg/dl and more than 0.2 mg/dl, respectively. All of the above fifteen cases were without RDS.

From our present data, an infant whose PG content is higher than 0.05 mg/dl does not suffer with RDS. Therefore, we assume that the "safety boundary value of PG" is 0.10 mg/dl. Muneshige et al. [11] have proposed the value of 0.29 mg/dl as the critical concentration for foetal lung maturity. The value of 0.29 mg/dl is nearly three times our proposed value. Our safety boundary value is set at one fifth of the average PG content of normal cases, i.e. 0.51 mg/dl. Muneshige's estimation for the critical value is also set at one quarter to one fifth for normal cases. Therefore, the difference in the safety boundary value might come from the difference between the present analytical method and that of Muneshige et al. [11].

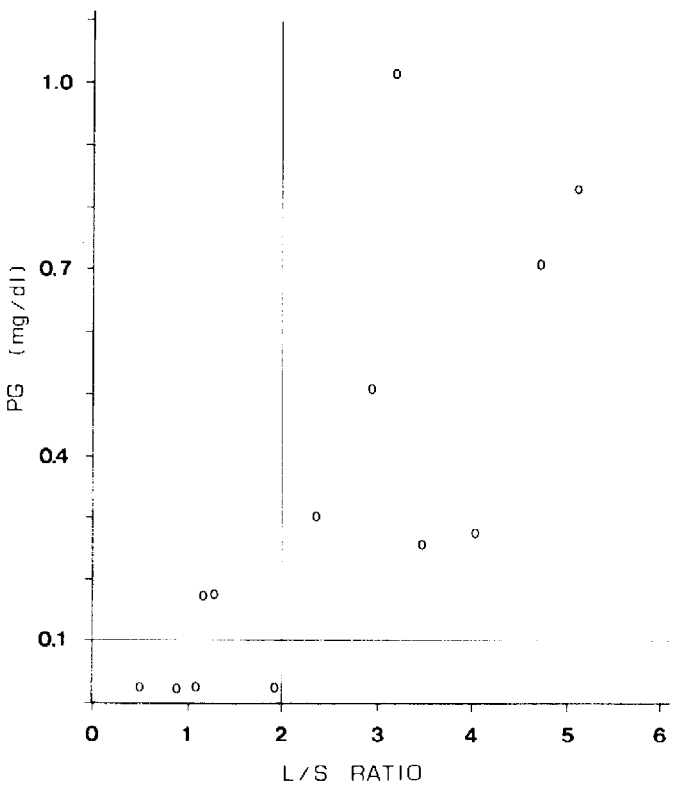

Fig. 3. The relationship between concentration of PG in amniotic fluid and L/S ratio. Concentrations of PG and L/S ratios in amniotic fluid were determined by HPLC and TLC, respectively. The straight line at 0.1 mg/dl PG means "safety boundary value line". The straight line at L/S ratio 2 also means the boundary value line for foetal lung maturity. PG content and L/S ratio were obtained by HPLC and TLC, respectively.

TABLE I

CLINICAL DATA OF COMPLICATED CASES

PROM = Premature rupture of membrane; D.M. = diabetes mellitus; T.A. = trans-abdominal; T.V. = trans-vaginal; C.S. = caesarean section; M = male; F = female.

Case	Gestational week	Complication	Collecting method	Duration* (h)	Birth weight (g)	Sex	PG content (mg/dl)	L/S ratio	RDS
1. Y.A.	34	PROM	T.A.	9	1955	M	0.11		—
2. M.F.	30	PROM	T.A.	60	1615	M	0.23		—
3. M.M.	35	PROM	T.V.	5	2950	M	0.44		—
4. Y.O.	36	PROM	T.V.	3	2930	M	0.70		—
5. M.T.	33	PROM	T.V.	48	1695	F	0.27		—
		Twin			2140	F			—
6. K.T.	29	Pre-eclampsia	C.S.	0	660	F	0.18	1.3	—
7. S.T.	36	Pre-eclampsia	C.S.	0	1490	F	0.21		—
8. H.B.	37	D.M. class A	T.A.	30	3770	F	0.25	3.5	—
9. M.S.	37	D.M. class D	T.V.	7	3125	M	1.01	3.3	—
10. C.A.	32	D.M. class B	T.A.	62	2645	F	<0.05	1.1	—
11. S.O.	37	D.M. class B	T.A.	4	3540	F	0.30	2.4	—
12. K.O.	37	D.M. class A	T.V.	6	2945	M	0.44		—
13. K.K.	42	D.M. class A	T.A.	70	2590	M	0.33		—
14. H.S.	36	Placenta Previa	C.S.	0	2660	F	<0.05	1.9	—
15. Y.F.	33	Placenta Previa	C.S.	0	2225	M	0.17	1.2	—
16. M.S.	29	Placenta Previa	C.S.	0	1005	F	<0.05	0.9	+
		Twin			1050	M			+
17. K.K.	33	Polyhydramnios	T.A.	18	2260	M	0.51	3.0	—
		Twin			1580	M			—
18. H.N.	31	Polyhydramnios	T.A.	48	1855	M	<0.05	1.1	+
19. Y.Y.	35	Heart disease Threatened premature labour	T.V.	5	2675	F	0.07		—

*From collection of amniotic fluid to delivery.

L/S ratio and PG content

The relation between PG content and L/S ratio is shown in Fig. 3. Both PG content and L/S ratio were measured in thirteen amniotic fluid samples (three normal cases and ten complicated cases), which were collected within 72 h before delivery. The relation between PG (y, mg/dl) and L/S ratio (x) is expressed as follows: $y = 0.17x - 0.11$, and the correlation coefficient is 0.78. The PG content obtained by the present method shows a fair correlation with the L/S ratio obtained by TLC.

The ratios of six cases out of thirteen were < 2 (immature L/S ratio). Two cases out of six were with RDS, and the other cases were without RDS. There were two cases in which the L/S ratio was < 2 and the PG content was > 0.10 mg/dl. In these cases, mothers had delivered infants without RDS. These two cases were No. 6 (K.T.) and No. 15 (Y.F.) in Table I.

Case No. 6 (K.T.). A pregnant woman developed severe pre-eclampsia, following an emergent Caesarean operation owing to threatened eclamptic sign. She had an infant (girl), 660 g weight, at gestation week 29. The PG content of her amniotic fluid at the time of the operation was 0.17 mg/dl and the L/S ratio was 1.3.

Case No. 15 (Y.F.). A pregnant woman with placenta praevia had genital bleeding, following clinical treatment by tocolytic drugs for suppression of early delivery. She developed dysfunction of the liver due to a side-effect of the medicine, therefore we took a sample of her amniotic fluid by amniocentesis. Its PG content and L/S ratio were < 0.05 mg/dl and 1.1, respectively. Then, we treated her with betamethasone (6 mg, twice) for accelerating the foetal

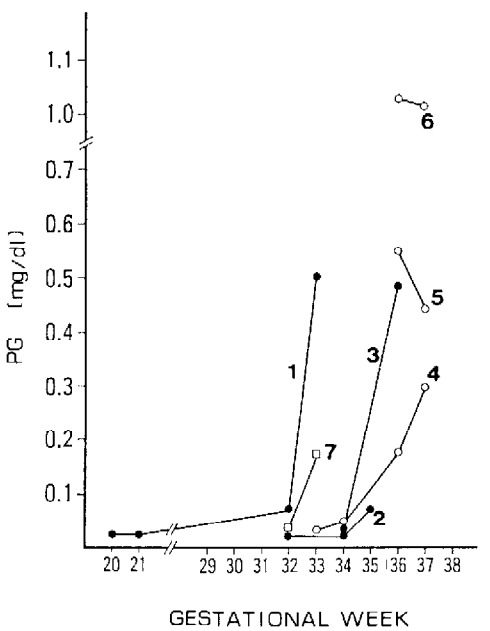

Fig. 4. Variation of PG value in amniotic fluid with the gestational week number for complicated cases. (1, 2, 3) Cases of threatened premature delivery; (4, 5, 6) diabetic cases; (7) the case of steroid therapy.

lung maturity. After that, she had an infant (boy), 2225 g weight, by Caesarean operation. The PG content and L/S ratio of amniotic fluid obtained at the operation were 0.17 mg/dl and 1.3, respectively.

From the above two cases, the patient whose PG content was > 0.10 mg/dl and L/S ratio < 2 could have an infant without RDS. From case No. 15 (Y.F.), it is suggested that we are able to estimate the therapeutic effect of a steroid drug from the increase in the PG content from one time to another.

Typical examples of individual variations of PG content in complicated cases are shown in Fig. 4, along with the gestational week. From Fig. 4, the PG content of an individual patient increases gradually at the beginning, and then starts to increase very rapidly at a certain gestational week. Therefore, it is very important to know the right period in each case, i.e. the time that the observed PG content becomes 0.10 mg/dl (more than the safety boundary value of PG).

Compared to the TLC method, the present method is more accurate and rapid. Comparing it with the recent enzymatic method proposed by Muneshige and co-workers [10, 11], the present method is simpler and more sensitive. This method is useful not only for prenatal evaluation of lung maturity, but also for assessment of any therapeutic effect. In our system, it is possible to estimate absolute amounts of PG, PE and PI in amniotic fluid simultaneously.

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