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Total phospholipid choline concentration of amniotic fluid in the assessment of foetal gestational and lung maturity. I. Establishment of a direct enzymic method

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Summary

An enzymic assay of the choline-containing phospholipids of amniotic fluid which does not require lipid solvent extraction has been developed. Lecithin and sphingomyelin are the dominant phospholipids in mature lung surfactant. The total phospholipid choline content of amniotic fluid, which represents the molar sum of lecithin and sphingomyelin, may be expected to correlate with foetal lung maturity. The merits of our new analytical method of determination of total phospholipid choline concentration in amniotic fluid are discussed.

Résumé

On a développé un essai d'enzyme de choline renfermant les phospholipids de l'échantillon de liquor amnii qui ne demande point l'extraction du solvant de lipide. Lecithin et sphingomyelin sont les phospholipids principaux dans le surfactant de poumon mûr. Le contenu du concentré total de phospholipid choline de l'échantillon de liquor amnii qui représente la somme totale de lecithin et sphingomyelin, pourrait correspondre à la maturité de poumon du foetus. On discute ici le grand mérit de notre nouvelle méthode pour déterminer le concentré total de phospholipid choline.

Introduction

Lecithin and sphingomyelin — the major choline-containing phospholipids — constitute the largest percentage of the phospholipids that are thought to correlate with foetal lung maturity [1]. Since the establishment of the determination of the lecithin/sphingomyelin (L/S) ratio as an index of foetal lung maturity by Gluck *et al.* [2], various methods for the estimation of the phospholipid content of amniotic fluid to determine lung maturity have been described [1,3,4]. As far as we know, an estimate of the total choline-containing phospholipids of amniotic fluid has not been used as an index of foetal lung maturity. Enzymic colorimetric assay of lecithin and sphingomyelin in aqueous solution was described by McGowan *et al.* [5]. Muneshige *et al.* [6] also described an enzymic colorimetric assay of phospholipids obtained from lipid solvent extracts of amniotic fluid. We are not aware that a direct enzymic assay of the choline-containing phospholipids of amniotic fluid, without the need for a solvent extraction, has yet been described. We describe a direct and specific enzymic colorimetric assay of the total choline-containing phospholipids of amniotic fluid. We suggest that the phospholipid choline content of amniotic fluid, which represents the molar sum of lecithin and sphingomyelin, measures the major surface-active component of surfactant. Total phospholipid choline may therefore be expected to correlate with lung maturity.

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Materials and methods

The assay is based on the hydrolysis of lecithin and sphingomyelin by phospholipase D, followed by the oxidation of choline released from this reaction [5].

$$\text{Lecithin} \xrightarrow{\text{PLD}} \text{phosphatidic acid} + \text{choline};$$

$$\text{sphingomyelin} \xrightarrow{\text{PLD}} \text{ceramide} + \text{choline};$$

$$\text{choline} + 2O_2 \xrightarrow{\text{COD}} \text{betaine} + 2H_2O_2;$$

$$2H_2O_2 + \text{DCP} + \text{AP} \xrightarrow{\text{PEROX}} \text{red chromogen}$$

where PLD is phospholipase D; COD, choline oxidase; PEROX, peroxidase; AP, 4-aminophenazone; and DCP, sulphonated 2,4-dichlorophenol.

All reagents were of analytical or reagent grade. The following reagents were obtained from Sigma Chemical Co. (Poole, U.K.): Tris-(hydroxymethyl)aminomethane, lecithin (synthetic dipalmitoyl phosphatidyl choline), sphingomyelin (bovine brain), choline chloride, choline oxidase (E.C. 1.1.3.17 from Alcaligenes species), peroxidase (E.C 1.11.1.7 from horseradish), phospholipase D (E.C. 3.1.4.4 from *Streptomyces chromofuscus*), triton-X-100, calcium chloride, and 4-aminophenazone. Sulphonated 2,4-dichlorophenol was obtained from BDH Chemicals Ltd. (Poole, U.K.)

Buffer. All enzymes used in this procedure were made up in a buffer consisting of 0.5% (w/v) Triton-X-100 and 50 mM Tris hydrochloride pH 7.5.

Enzyme reagents. All enzyme reagents were made up in the buffer above in the strengths indicated: phospholipase D (20 U/ml), choline oxidase (40 U/ml), peroxidase (4 U/ml). Deep frozen at −20°C the reagents are stable for at least 6 months.

Phospholipid standards. Standard solutions of lecithin and sphingomyelin were made in the same buffer as for the enzymes. Choline chloride standards were made in distilled water.

Calcium chloride. Made up to 2 mM and 8 mM in Tris hydrochloride buffer, pH 7.5.

Choline colour reagent. This reagent, freshly made before each assay, consisted of choline oxidase (2 U/ml), peroxidase (0.2 U/ml), 5 mM sulphonated 2,4-dichlorophenol, and 0.1 mM 4-aminophenazone in 50 mM Tris hydrochloride buffer, pH 7.5.

Amniotic fluid. Amniotic fluid samples were obtained from term pregnancies at Caesarean section or *per vaginam* following artificial rupture of membranes. Only amniotic fluids free of blood and meconium contamination were used. The samples of amniotic fluid were centrifuged at 1000 *g* for 10 min to remove cellular debris. Amniotic fluid assays were performed within 2 h of collection of samples.

Method

Standard solutions (50 μM, 100 μM, 150 μM, and 200 μM) of lecithin and sphingomyelin were each made up in the reagent buffer. Choline chloride solutions were made up in distilled water.

Choline chloride assay

To 100 μl choline chloride standard and 100 μl of reagent buffer was added 1 ml of choline colour reagent, pre-warmed to 37°C. The mixture was transferred into a 1 cm light-path cuvette, and the colour development was monitored in a Pye Unicam SP 800 spectrophotometer (Pye Unicam Ltd., Cambridge, U.K.) with a thermostated cuvette chamber at 37°C. A reddish colour, measured at 510 nm, was produced, reaching maximum intensity at 6 min. The reagent blank, treated exactly as the standards, was 1 ml of colour reagent mixed with 200 μl of reagent buffer. A calibration curve was produced for choline chloride standards within the range of 50–200 μM.

Phospholipid assays

To 100 μl of each of the aqueous lecithin and sphingomyelin standards was added 50 μl of 2 mM calcium chloride solution and 50 μl phospholipase D (1 U). The mixture was incubated at 37°C in a water bath for 5 min. One millilitre of colour reagent was added and the mixture transferred into a 1 cm light-path cuvette. The colour development was monitored as for the choline chloride standards. A blank was prepared for each phospholipid by substituting 50 μl of reagent buffer for 50 μl of phospholipid D solution. Calibration curves were drawn for each of the phospholipid stan-

dards within the range of 50–200 μM. Mixtures of lecithin and sphingomyelin, in various concentrations, were also assayed to simulate concentrations of the phospholipids in amniotic fluid.

Amniotic fluid assay

One hundred microlitres of freshly centrifuged amniotic fluid was treated exactly like the aqueous phospholipid standards except that 8 mM calcium chloride solution was substituted for the 2 mM calcium chloride used for aqueous standards. One blank was prepared for each amniotic fluid assayed in the same way as for phospholipid standards.

Verification of completion of hydrolysis of amniotic fluid phospholipids was carried out by comparing the values of total phospholipid choline concentrations of 10 amniotic fluid samples from term pregnancies with the corresponding values of lipid solvent extracts of each amniotic fluid sample. The lipid solvent extracts were prepared by the method of Gluck *et al.* [2] Three millilitres of freshly centrifuged amniotic fluid was mixed with 9 ml of a mixture of chloroform and methanol (2:1, v/v) on a vortex mixer. The mixture was centrifuged at 1500 *g* for 10 min. The upper aqueous layer was removed by aspiration. Five millilitres of the lower chloroform layer was carefully removed, avoiding the insoluble proteinaceous material at the interphase. The 5 ml chloroform extract was evaporated to dryness under a stream of nitrogen. The residue was redissolved in 2.5 ml of the same buffer used to prepare the aqueous phospholipid standards. This solution was assayed for its total choline content as was the unextracted amniotic fluid. If there is complete extraction of amniotic fluid phospholipids into the lipid solvent, and complete hydrolysis of the phospholipids in amniotic fluid and in the reconstituted lipid solvent extract, then the values of total phospholipid choline concentration in each amniotic fluid and its matched extract can be expected to be the same.

Results

Phospholipase D is a calcium-dependent enzyme [7]. We found that a calcium concentration of 0.5 mM in the reaction medium was adequate to ensure complete hydrolysis of aqueous phospholipid standards, while amniotic fluid samples required 2 mM of calcium. Figure 1 shows the standard calibration curve for choline chloride. Equimolar concentrations of aqueous lecithin and sphingomyelin standards gave identical colour responses to comparable molar concentrations of choline chloride

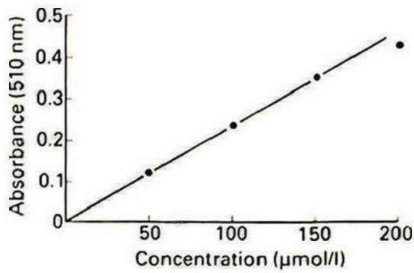

Fig. 1. Standard calibration of choline chloride.

(Fig. 2). Standard calibration with choline chloride and the aqueous phospholipids gave linear colour response up to 150 μM. Mixtures of aqueous phospholipid standards of various concentrations gave absorbance values consistent with the total molar concentrations of the phospholipids. Within-batch analytical imprecision of the assay using aqueous phospholipid solutions was 3%. Table 1 shows concentrations of total phospholipid choline in amniotic fluid samples and their matched lipid extracts. Amniotic fluid samples assayed by the direct enzymic method yielded between 96% and 106% of total choline obtained from matched amniotic fluid extracts. There was no detectable

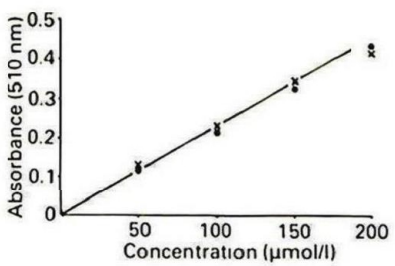

Fig. 2. Standard calibration of phospholipids, (x) lecithin and (●) sphingomyelin.

Table 1. Total phospholipid choline concentration of amniotic fluid by direct and lipid extract assays

	Amniotic fluid number									
	1	2	3	4	5	6	7	8	9	10
Direct assay (μmol/l)	95	52	80	88	52	126	104	38	117	142
Extract assay (μmol/l)	93	54	76	92	50	119	107	38	116	137
Recovery (%) (direct/extract)	102	96	105	97	104	106	97	100	101	104

free choline in all the fresh amniotic fluid samples we assayed. However, free choline was detected in amniotic fluid stored for 7 days at −20°C immediately after centrifugation. This finding suggested that either a spontaneous hydrolysis of phospholipids was taking place even in frozen amniotic fluid samples or the freezing and thawing caused phospholipid hydrolysis. The use of deep frozen amniotic fluid samples stored over a long time will therefore lead to erroneous detection of free choline, as reported by McGowan [5].

Discussion

Lung surfactant consisting of various phospholipids and proteins is essential for the ventilatory activity of the healthy lung, especially of the newborn [8]. Secretion of phospholipids of surfactant by lung alveolar cells starts early during foetal life but the phospholipid composition of surfactant of foetal lung changes until maturity [9,10]. The free exchange of fluid between the foetal lung and the amniotic fluid in which the foetus lies [11], has enabled the estimation of amniotic fluid phospholipids to be used as an estimate of phospholipid secretion from the foetal lung. Most of the methods for estimating amniotic fluid phospholipids are chromatographic techniques involving the extraction of phospholipids into lipid solvents [2,4,6,10]. The analytical imprecision inherent in chromatographic techniques may be an important factor in the lack of agreement on the concentrations or the ratios of concentration of the various phospholipids in amniotic fluid that correlate with foetal lung maturity. Our assay of total choline-containing phospholipids in unextracted amniotic fluid is specific and precise. The values of phospholipid choline concentration in each amniotic fluid and its matched lipid extract suggest complete hydrolysis of choline-containing phospholipids in the amniotic fluid samples. We believe that the specificity and precision of the assay will enable accurate data of the total concentrations of amniotic fluid lecithin and sphingomyelin at different gestational periods to be established. The analysis of phospholipid choline by our method can be completed within 30 min by an averagely skilled technologist. This compares with the 3 h or longer needed by a skilled technologist to carry out a L/S ratio determination. We suggest that our assay is a good substitute for determination of L/S ratio in the assessment of foetal lung maturity.

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