Clinical Experimental Obst. & Gynecology
Vol. IV, nn. 3-4, 1977 pp. 106-110

vention is not always possible due to the rapid spread of the neoplasms which tends to involve various endoabdominal structures, thus rendering complete exeresis impossible. The average survival time after tumor appearance is approximately one year.

SUMMARY

A case of pure endodermal sinus tumor of the ovary is described in a 5 year old girl. Histological features of this rare neoplasm are described. Despite various therapeutic attempts, the child died within one year.

BIBLIOGRAPHY

1. Abell N. R., Johnson S. V., Holtz F.: *Am. J. Obst. Gynec., 92,* 1059, 1956. - 2. Blythe J. G., Buchsbaum H. J.: *Hum. Path., 4,* 595, 1973. - 3. Huntington R. N., Bullock W. K.: *Acta Path. Microbiol Scand. Sect. A, Suppl., 233,* 26, 1972. - 4. Norris J. H., Jensen R. D.: *Cancer, 30,* 713, 1972. - 5. Novak E. R., Woodruff J. D.: *Novak's gynecologic and obstetric pathology.* 6th Ed. Philadelphia, Saunders 1967. - 6. Santesson L., Barrubini G.: *Acta Obst. Gynec. Scand., 36,* 399, 1957. - 7. Schiller W.: *Am. J. Cancer, 35,* 1, 1939. - 8. Teilum G.: *Acta Path. Microbiol. Scand., 64,* 407, 1965.

Description of a simple method for the dosage of glycerophospholipids in human amniotic fluid

by

A. Casu *, R. Monacelli * and D. Pecorari **

Amniotic fluid phospholipids have been the object of increasing research activity in recent years owing to their relationship with the respiratory distress syndrome of the newborn. In most mammals toward the term of gestation there is a marked rise of concentration of dipalmitoil-phosphatidyl-choline in amniotic fluid, which is coincident with the acquisition of surface tension lowering properties by fetal lung extracts ([10]). Among the many papers on this argument, however, only a minority report comparable and reproducible data; this is mainly due to the fact that:

a) amniotic fluid often is not centrifuged, so that the phospholipids measured are the sum of phospholipids actually present in amniotic fluid and of those present in much greater aumont in cells and subcellular membranes suspended in amniotic fluid.
b) Amniotic fluid is centrifuged, but not immediately after it is obtained; hence, there is a diffusion of phospholipids into the liquid phase, owing to decomposition of subcellular particles. An important review of the main errors that

* From the Institute of General Pathology and Chair of Molecular Pathology University of Genova.

** From the Department of Obstetrics and Gynaecology I., University of Genova.

can be done in the evaluation of amniotic fluid phospholipids can be found in the recent paper by Wagstaff and co-workers ([12]).

Most data available today have been obtained by the use of classical techniques for lipid analysis; they are rather complex procedures, requiring extraction, thin layer chromatographic separation and chemical dosage of phospholipidic phosphorus or densitometric measurements; a similar technique has been used also in our laboratory ([2,11]).

In the search for easier procedures, but without giving priority to simplicity over reliability and precision, we have developed the method described in this paper, based on enzymatic analysis of glycerophospholipidis.

In summary, pre-treatment with phospholipase C removes phosphorilated bases; secondly, the glycerides thus formed are hydrolyzed; finally, glycerol is measured with the standard methods of clinical chemistry. A similar method has been already described for glycerophospholipids of blood serum ([3]).

MATERIAL AND METHODS

Most samples of amniotic fluid have been obtained by means of high puncture of the membranes with a Drew Smythe catheter; a few were obtained by means of uterine puncture at caesarean section.

All patients were healthy gravidas at term. Samples contaminated with blood or meconium were discarded.

All samples were immediately centrifuged in a cold chamber for 40 min at 20.000 x g in a Lourdes A x 9RA centrifuge in order to remove all cells and particulate materials. After centrifugation the liquid was filtered on Whatman paper in order to remove all the floating material; the clear fluid was processed immediately or stored at −20°C.

Treatment with phospholiphase C:

to 5 ml of clear fluid were added 0.15 ml of 0.1 M $CaCl_2$ and 0.15 ml of phospholiphase C (Bacillus cereus, Sigma, lot N° P-6135, type III; phosphatidyl-choline-choline phosphodiesterase, E.C. N° 3.1.4.3.). The phospholiphase C was dissolved in triethanolamine buffer 0.1M, pH 7.6 with $MgSO_4$ 4mM; 0.5 mg of enzyme protein were contained in 1.0 ml of buffered solution.

A blank was prepared in parallel with the same amount of buffer but omitting the phospholiphase C.

Mixtures were incubated at 37°C for 40 minutes, thereafter extraction was performed.

Preliminar experiments by means of thin layer chromatography and dosage of phospholipidic phosphorus on the washed extract with the method already described ([4]) had shown that hydrolysis of glycerophospholipids was complete.

Phospholipase C of Bacillus cereus has been selected because it attacks specifically the lecithins and not the sphingomyelins; phospholipase C of Cl. perfringens, on the contrary, does not completely hydrolyze the lecithins, so that its use did not seem indicated in the case of this body fluid.

Saponification and dosage of free glicerol:

after incubation the mixture is extracted with 4 volumes of chloroform/methanol 1/1 (v/v) by means of agitation in Vortex for two minutes.

Clinical Experimental Obst. & Gynecology
Vol. IV, nn. 3-4, 1977 pp. 106-110

Separation of the two phases is accelerated by means of centrifugation, the superior and the intermediate phases are discarded and the inferior phase is dried-evaporated in nitrogen atmosphere.

Control samples for a preliminary dosage of phosphorus ([4]) were washed according to Folch ([7]).

Saponification of glycerides is finally performed by incubation in glass tubes with well fitted glass stoppers with 0.5 ml of KOH 2% in 96% ethanol, for 30 minutes at 60°C.

Dosage of glycerol was done using the method of Eggstein and Kreutz (1966), for which special kits are commercially available. To the hydrolysate 1 ml of 0.30 N $MgSO_4$ is added.

The mixture is centrifuged and on 0.5 ml of the supernatant free-glycerol is measured in a system containing in a final volume of 3.10 ml at pH 7.6: 250 μM of triethanolamine; 10 μM of $MgSO_4$; 0,6 μM of NADH; 3,3 μM of ATP; 1,1 μM of phosphoenolpiruvate; lactic dehydrogenase corresponding approximately to 3 units of activity.

Aspecific variations of extinction were measured for a few minutes in the spectrophotometer at 340 or 366 nm; subsequently the reaction was started by adding 0.02 ml of glycero-kinase containing approximately 3 units of activity.

The reading is done against the blank and the end of the reaction is indicated by the shape of the curve.

In order to evaluate the glycerol, the reading is multiplied by the extinction coefficient of NADH, which at 340 and 360 nm is respectively 0.161×10^{-3} and 0.303×10^{-3}, taking into account the dilution.

All reagents used were BDH Analar, unless otherwise specified.

RESULTS AND DISCUSSION

The results of glycerophospholipid determinations both by the classical method of chloroform/methanol extraction and dosage of phosphorus (as already described in our preceding papers) and with the present method are summarized in table 1.

There is a good correspondence between the two methods as the results with the dosage of phosphorus is 9,88 μmoles of glycerophospholipids/liter, while the results with the present method (glycerol determination) is 9,4 μmoles/liter.

Total glycerol has been measured by means of the enzymatic method: it corresponds to the sum of phospholipidic glycerol and of glycerol from other glyce-

Tab. 1. *Results of evaluation of glycerophospholipids in human amniotic fluid. Pool of normal amniotic fluid at term.*

Total glycerol μmoles/liter	Phospholipidic glycerol μmoles/liter	
(I)	(II)	(III)
48,2	9,40	9,88

(I) Enzymatic dosage of glycerol after hydrolysis of phosphorylated bases by means of phospholipase C and KOH.

(II) Difference between total glycerol and glycerol values obtained after hydrolysis using only KOH.

(III) Dosage of phospholipidic phosphorus after hydrolysis with phospholipase C, extraction with chloroform/methanol and washing of the extract. Glycerophospholipids are calculated from the difference between total phospholipidic phosphorus and residual phosphorus.

rides present in the amniotic fluid. The amount of glycerophospholipids is calculated from the difference between total glycerol, measured after using phospholipase C combined with KOH and glycerol measured after using only KOH.

The results shown have been all obtained from the same pool of 6 normal amniotic fluids at term of gestation. The basic principles of the method are outlined in table 2. In order to ascertain that hydrolysis of phospholipids by

Tab. 2. *Schematic representation of evaluation of glycerophospholipids in amniotic fluid.*

■ Amniotic fluid sample, centrifuged immediately
↓ 5,00 ml
■ Removal of phosphorylated bases by means of phospholipase C
↓ (75 μg phospholipase C+0,15 ml of $CaCl_2$ 0,1M)
■ Incubation at 37° C for 40 minutes
↓
■ [4 volumes chloroform/methanol 1:1 (v/v); evaporation of the chloroform phase]
■ Saponification of neutral fats
↓ (0,5 ml KOH 2% in ethanol 96%; 30 minutes at 65° C)
■ Enzymatic evaluation of glycerol with the method of Eggstein and Kreutz.

phospholipase C be complete, an internal standard of a mixture of lecithins was added; the mixture was prepared as already described ([3]).

As in the case of serum, the internal standard is not completely hydrolyzed by phospholipase C, because the enzyme requires peculiar physico-chemical properties of the substrate ([1,5]). Consequently, the control of hydrolysis was performed by means of phospholipid analysis using thin layer chromatography.

Figure 1. shows a chromatograph of phospholipids of the control fluid before

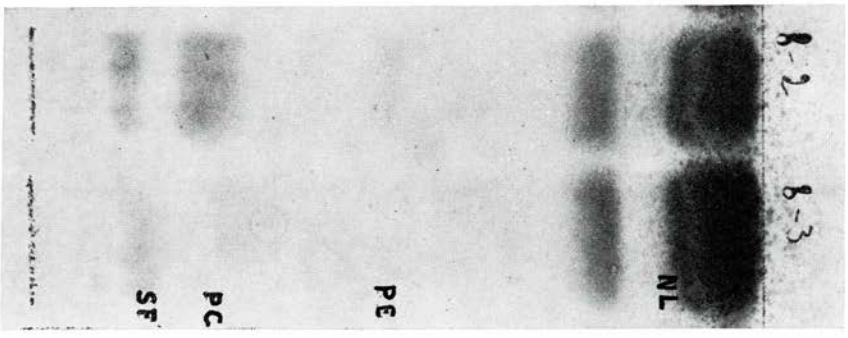

FIG. 1 - Thin layer chromatography on silica gel G of amniotic fluid phospholipids. Elution by means of chloroform, methanol and water (65:25:4; v/v/v). Evidentiation by means of H_2SO_4 and charring at 180° C. - A. Control phospholipids; B. After hydrolysis with phospholipase C: the lecithin (PC) and phosphatidyl-ethanolamine (PE) fractions have disappeared. SF=sphyngomyelins. NL=Neutral lipids.

and after treatment with the enzyme. After treatment with the enzyme lecithins and phosphatidylethanolamines disappear while sphyngomyelins are not hydrolized.

The interest of the dosage of glycerophospholipids in amniotic fluid depends from the fact that lecithins represent approximately 80% of glycerophospholipids and from the fact that lecithins are the main component of lung surfactant ([10]).

Clinical Experimental Obst. & Gynecology
Vol. IV, nn. 3-4, 1977 pp. 110-114

The main purpose of our study was to develop a method having a satisfactory accuracy and precision and, at the same time, beeing sufficiently easy to be used in routine clinical work.

SUMMARY

A simple method for the dosage of glycerophospholipids in amniotic fluid is described. The method is based on liberation of glycerol from the phospholipidic molecule by means of the combined action of phospholipase C and KOH; this is followed by enzymatic determination of glycerol.

The new method is compared with phospholipid dosage by means of the traditional method using extraction and thin layer chromatography.

BIBLIOGRAPHY

1. Bangham A. P.: *Progr. biophys. molec. Biol., 18*, 29, 1968. - 2. Casu A., Grimaldi M., Monacelli R., Pantarotto M. F., Vicino P., Pecorari D.: *Pathologica, 67*, 395, 1975. - 3. Casu A., Monacelli R.: *Ital. J. Biochem., 25*, 390, 1976. - 4. Casu A., Pala V., Pantarotto M. F., Pecorari D.: *Ital. J. Biochem., 23*, 38, 1974. - 5. De Haas G. H., Bonsen P. P. M., Pieterson W. A., Van Deenen L. L. M.: *Biochim. Biophys. Acta, 239*, 252, 1971. - 6. Eggstein M., Kreutz F. H.: *Klin. Wschr., 44*, 262, 1966. - 7. Folch E., Lees M., Sloane Stanley G. M.: *J. biol. Chem., 226*, 497, 1957. - 8. Gluck L., Kulovich M. V., Borer R. C.: *Clinics in Perinatology, 1*, 125, 1974. - 9. Gluck L., Kulovich M. V., Borer R. C., Brenner P. H., Anderson G. G., Spellacy W. N.: *Am. J. Obst. Gyn., 109*, 440, 1971. - 10. Goerke J.: *Biochim. Biophys. Acta, 344*, 241, 1974. 11. Pantarotto M. F., Casu A., Monacelli R., Pala V., Pecorari D.: *Pathologica, 65*, 217, 1973. - 12. Wagstaff T. I., Whyley G. A., Freedman G.: *J. Obst. Gyn. Brit. Cwlth., 81*, 264, 1974.

Chemoprophylaxis in trophoblastic disease

by

M. MARCHETTI, T. MAGGINO and B. DE BASTIANI *

It is known that recurrences occur, also in the form of metastases, in about 20% of cases of hydatidiform mole ([3,6,11]). Nevertheless, prophylaxis by means of antiblastic chemotherapy in the pre- and post-evacuation treatment of hydatidiform mole is still controversial, both as regards its real efficacy ([19]) and as regards the advisability of administering potentially toxic agents (methotrexate, actinomycin D and others) to patients who, in the majority of cases, should run a benign post-molar course ([3, 6]). On the other hand, the view expressed by various authors ([14,15,16,18]) is that chemoprophylaxis in trophoblastic disease is really effective on various technical grounds and according to incontestable clinical data, even when administered in low doses, thus avoiding all the toxicity risks of these substances. In fact, it is not necessary to make use of high (and thus toxic) doses of antiblastic agents in order to achieve effective chemoprophylaxis, but the doses

From the Obstetric and Gynaecological Clinic, University of Padova.