Cholesterol in Amniotic Fluid, Determined by Gas Chromatography

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Cholesterol was extracted from amniotic fluid, saponified, converted to its trimethylsilyl derivative, and gas chromatographed, with cholesteryl acetate as the internal standard. The method is sufficiently accurate and precise for use with the range of concentrations of cholesterol found in amniotic fluid (5 to 46 mg/litre). Total cholesterol was measured in amniotic fluids collected at different stages of gestation. No significant trend or change was observed nor was cholesterol in the amniotic fluid and the mother's serum correlated at any stage of gestation. Thus we conclude that cholesterol is not a useful indicator of fetal age or maturity. Cholesterol concentrations in amniotic fluid from complicated pregnancies were within the range found for normal pregnancies.

Additional Keyphrases: indexes to fetal maturity • cholesterol in serum • gas chromatography and continuous-flow analysis compared

The technique of amniocentesis has greatly increased the feasibility of sampling amniotic fluid at different stages of gestation, for use in assessing fetal maturity and status. Of the many chemical constituents investigated, creatinine and lecithin in amniotic fluid have been shown to be useful indicators of fetal maturity (1, 2). We decided to measure cholesterol in amniotic fluid and see if this measurement has any clinical significance and application.

Wolf et al. (3) and Fennefrohn (4) reported that the cholesterol concentration did not change significantly throughout gestation, but it is difficult to see how they measured the cholesterol with precision, because they both used AutoAnalyzer SMA (Technicon) systems but neither reported any modification of the method to allow for the fact that the cholesterol concentration in amniotic fluid is only about one hundredth that in normal serum. Because the usual methods for estimation of serum cholesterol are not appropriate for use with small volumes of amniotic fluid, we developed a gas-chromatographic method for the determination of total cholesterol, and assessed its precision and accuracy.

We then measured cholesterol in amniotic fluid, to see if there is any correlation with other variables such as fetal age or the mother's serum cholesterol concentration.

Materials and Methods

Samples

Amniotic fluid was obtained by amniocentesis, at cesarian section, by hysterotomy or amniotomy. After centrifugation, those samples that could not be analyzed immediately were frozen. The mother's blood was sampled at the same time as the amniotic fluid, and the serum was analyzed.

Reagents

*Cholesterol stock standard.* About 25 mg of cholesterol (Fisher Scientific Co., Certified Reagent) was weighed out accurately and diluted to 200 ml with chloroform. Stored refrigerated, this solution can be used for as long as a month.

*Cholesteryl acetate standard.* This compound was prepared by dissolving cholesterol in anhydrous pyridine and adding anhydrous acetic anhydride.

The solution was refluxed for 30 min, cooled, and water added until white crystals precipitated. The crystals were collected by filtration, recrystallized from ethyl acetate, and dried. Cholesteryl acetate standard was prepared to contain 10 mg of cholesteryl acetate per 100 mg of dry pyridine. The solution can be stored in a fume hood for as long as one month. We also used cholesteryl acetate “Standard for Chromatography” obtained from Sigma Chemical Co., St. Louis, Mo. 63178.

*Cholesterol palmitate standard.* About 15 mg of cholesterol palmitate (Sigma Chemical Co., “Standard for Chromatography”) was weighed out accurately and diluted to 100 ml with chloroform.

*Hexamethyldisilane and trimethylchlorosilane* were obtained from Sigma Chemical Co.

*Pyridine “Spectro grade”* was obtained from Eastman Kodak Co., Rochester, N. Y. 14650.

*n-Hexane* was laboratory-reagent grade, from BDH Chemicals, Toronto, Canada.

*KOH in ethanol.* Ten grams of “Certified Re-
agent” (Fisher Scientific Co., Montreal, Canada) was dissolved in 20 ml of distilled water and 6 ml of this was diluted to 100 ml with ethanol immediately before use.

Equipment

We used an F and M Scientific 402 High Efficiency Gas Chromatograph with a hydrogen flame-ionization detector (Hewlett-Packard, Avondale, Pa. 19311). The 150-cm column was packed with 3% OV-17 on Gas Chrom Q, 100–120 mesh (Chromatographic Supply, Brockville, Ontario, Canada). The oven temperature was 280 °C, and the carrier gas was helium.

Methods

Cholesterol standards were prepared by pipetting 0.1-, 0.2-, and 0.3-ml aliquots of cholesterol stock standard into test tubes and evaporating under nitrogen. Then 1 ml of a saline solution (NaCl, 8.5 g/litre) was added to each tube.

To 1 ml of either amniotic fluid or the cholesterol standard in saline was added 5 ml of ethanolic KOH and the tube contents were vortex-mixed for 15 s and incubated at 37 °C for 90 min. After the mixture had cooled to room temperature, 10 ml of n-hexane was added.

The tubes were vortex-mixed for 1 min and the layers allowed to separate. Seven milliliters of the hexane (upper) layer was removed and evaporated in a stream of air at room temperature. To the residue were added 0.5 ml of cholesteryl acetate in pyridine, 0.2 ml of hexamethyldisilane, and 0.1 ml of trimethylchlorosilane; the tubes were vortex-mixed for 1 min, and the mixture was allowed to stand for 15 min at room temperature. Then we injected 2 μl into the gas chromatograph and measured the heights of the peaks corresponding to the internal standard, cholesteryl acetate, and the trimethylsilyl derivative of cholesterol. A calibration curve was prepared by plotting the cholesterol concentration in the standard vs. the height of the trimethylsilyl-cholesterol peak, divided by the height of the cholesteryl acetate peak. The use of the internal standard corrected for mechanical loss of the sample during preparation and variation in the amount injected into the gas chromatograph.

Maternal serum. The cholesterol concentration in the mother’s serum was estimated with the AutoAnalyzer (5).

Analytical Variables

Recovery studies. The analytical recovery of cholesterol esters was checked by adding known amounts of cholesterol palmitate to amniotic fluid and estimating the amount of cholesterol in the sample before and after the addition.

Precision. Precision was investigated by analyzing one pool of amniotic fluid 20 times during four days. Also duplicate samples of amniotic fluid were analyzed during several weeks. The duplicates were sometimes run on the same day, sometimes on separate occasions.

Correlation studies. Serum samples were analyzed by using an AutoAnalyzer (5). The same serum samples were diluted 100-fold with a saline solution (NaCl, 8.5 g/litre), and analyzed by the gas-chromatographic method. The results obtained by gas chromatography were multiplied by 100 and compared with those obtained on the AutoAnalyzer.

Results

The calibration curve was linear up to at least 500 mg of cholesterol per litre.

Table 1 shows that, on average, 97% of the cholesterol added to amniotic fluid in the form of cholesterol palmitate could be analytically accounted for. These results were obtained for cholesterol at the concentrations found in amniotic fluid.

For the pool of amniotic fluid analyzed 20 times, a mean value of 17.3 mg/litre was obtained (SD, 1.3; CV, 7.11%). The SD for the results obtained on the duplicate estimation of 68 samples was 1.6 mg/litre. Comparison of the results of the AutoAnalyzer method (x) with the gas-chromatographic method (y) showed the following results: y = 1.14x - 191.2, correlation coefficient = 0.99 (n = 22). The serum samples were in the range 1000 to 4000 mg/litre.

Table 2 gives the results of the analysis of cholesterol in amniotic fluid collected at different times during gestation. The results for any given time were not normally distributed, being skewed toward the low side. The number of samples collected before 30 weeks of gestation was small and therefore not appropriate for meaningful statistical comparison. The number of samples collected after 30 weeks was greater and the results were compared statistically. The mean value increased after the 31st week of gestation, but not statistically significantly, except for the mean at 31 weeks, which was significantly (P = 0.05) lower than the means obtained later in gestation.

Table 2 shows the distribution of the results for cholesterol in the maternal serum throughout gestation; values are about 100-fold those for amniotic

| Table 1. Analytical Recovery of Cholesterol Added to Amniotic Fluid (as Cholesterol Palmitate) |
|-------------------------------------------------|-----------------------------------------------|
| Cholesterol | Added mg/litre | Found mg/litre | Recovery, % |
| 0.0         | 6.8            |                 | 100.0       |
| 8.6         | 15.4           |                 | 96.3        |
| 17.2        | 23.2           |                 | 94.2        |
| 25.8        | 31.1           |                 | 96.3        |
| 34.4        | 39.9           |                 | 97          |

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fluid. We examined the ratio for cholesterol in serum and amniotic fluid for each case in which we had both of these data, but found no correlation at any time of gestation.

We measured cholesterol in amniotic fluid from 28 pregnancies complicated by abnormalities such as hypertension, diabetes, rhesus sensitization, hydramnios, or interuterine growth retardation. Our results were within the range found in normal pregnancies.

In one case, which was excluded from the statistical analysis, the amniotic fluid cholesterol concentration was 139 mg/litre. The corresponding value for serum was 2720 mg/litre, which was within the usual range during pregnancy. No abnormalities were noted in this pregnancy, which was in the 42nd week of gestation when the amniotic fluid was collected. In eight other similar post-mature pregnancies, the mean amniotic fluid cholesterol was 20.5 mg/litre, ranging up to 38 mg/litre.

Discussion

This gas-chromatographic method for estimating cholesterol has been shown to be appropriate for use with amniotic fluid. Our analytical recoveries of a cholesterol ester, cholesterol palmitate, were good at the concentrations of total cholesterol found in amniotic fluid. Serum and serum dilutions were used to show that results of this method are similar to those obtained with a continuous-flow system. Some of the variation in the results obtained on duplicate analysis may be due to the presence of vernix in samples taken late in gestation. The vernix is difficult to separate by centrifugation, and small particles may be inadvertently included in the sample for analysis. Vernix is rich in cholesterol.

We agree with Wolf et al. (3) and Fennerfrohn (4) that the cholesterol concentration in amniotic fluid is not significantly correlated with gestational age. For some stages of gestation the number of cases was small; differences in the means may well have been of greater statistical significance if more cases had been studied, but it is apparent from this limited study that there is a wide overlap in the values for cholesterol in amniotic fluid collected at different stages of gestation. We conclude that cholesterol is not a useful indicator of fetal age or maturity.

The concentration of cholesterol in the maternal serum was 100-fold that in amniotic fluid, an extreme difference that has not been seen in other components of amniotic fluid, such as protein, that we have studied (6). The lack of correlation between values for maternal serum cholesterol and amniotic fluid cholesterol suggests that there is no equilibrium or free exchange between the maternal serum and the amniotic fluid cholesterol. Robertson and Sprecher (7) report that in guinea pigs the proportion of the fetal serum cholesterol of maternal origin can vary with the mother’s diet, but that concentrations of circulating cholesterol in the fetus remain the same whether the mother’s diet is cholesterol free or rich in cholesterol. In rats, during early gestation fetal cholesterol is of maternal origin; later, the fetus synthesizes its own cholesterol (8).

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References

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