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Chemometric investigation of some analytical methods used for the chemical test of foetal lung maturity*

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Abstract: The lecithin/sphingomyelin (L/S) ratio is particularly important for the prediction of foetal lung maturity. A package for pattern recognition of analytical chemical data, "Parvus", was used to handle the different values of the L/S ratio obtained experimentally by common amperometric, spectrometric and chromatographic methods for the determination of lecithin. Eight subjects were considered and some interesting conclusions drawn on the equivalence of different analytical methods of determining lecithin in amniotic fluid.

Keywords: *Foetal lung maturity; L/S ratio; amniotic fluid; pattern recognition analysis; chemometrics.*

Introduction

It is well known that the most important problem in assessing the foetus, is to obtain reliable information about the degree of pulmonary maturity [1], for which a simple procedure is required. Some researchers have suggested that phosphatidylglycerol is an important indicator of foetal lung maturity [2]; others [3] have found that the lecithin concentration in the amniotic fluid is a better indicator. However, the ratio of the concentrations of lecithin and sphingomyelin (L/S ratio) is the most currently used value for the determination of foetal lung maturity [1, 4, 5]. Phospholipids in amniotic fluid have been measured by several techniques; most methods require time-consuming extraction of the phospholipids by a mixture of organic solvents [6], from the amniotic fluid. Although the concentrations of lecithin and sphingomyelin have been determined by thin layer chromatography (TLC) techniques, the reproducibility of these methods is unsatisfactory [7]; various enzymic assays have been developed to measure lecithin [8] and sphingomyelin [9] in order to circumvent some of the problems associated with the chromatographic methods.

Several methods (enzymic-spectrometric, amperometric or chromatographic [10]), have been used to determine lecithin concentration, needed to calculate the value of the L/S ratio, but the real equivalence for this purpose of the analytical methods studied was not clear. On the other hand, some interesting conclusions [11] were drawn on the real equivalence of some analytical methods for the determination of phospholipid concentration; this was done to calculate another important clinical index (IL) for human bile [12] in which elaboration of the row data, with the package for pattern recognition analysis, "Parvus" [13], is performed.

In the same way, 32-values of the L/S ratio, obtained by four different techniques for the determination of lecithin have been calculated and elaborated by pattern recognition analysis, using the same package of programs; results are outlined in this note.

Experimental

Samples

The samples of amniotic fluid, provided by the First Obstetrics and Gynaecology Clinic of Rome University, were obtained from eight women, aged 25–35 years, undergoing caesarian section. All samples were stored at −20°C before analysis.

Methods, apparatus and chemical reagents

Spectrophotometric measurements were

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performed with a Perkin–Elmer spectrophotometer model 320, and 1-cm silica cell, by the procedures described previously [14–16].

The enzymic colour test for lecithin [14, 15] was obtained from Poli SPA (Milano, Italy).

Two sets of values were obtained spectrophotometrically, one using Takayama's procedure [16] with subtraction of the reagent blank only, and the other by a specially developed procedure [14], with subtraction of the reagent and sample blanks. This second technique seemed generally to provide better results [14, 15].

Enzymic-amperometric measurements for lecithin were carried out with the enzyme sensor developed previously [14] and by the procedure and the flow apparatus previously described [14, 16]. The reagents and the enzymic immobilization method were those previously described [14, 17]; a commercial oxygen probe and an amperometric apparatus (IL 213), were supplied by Instrumentation Laboratory (Milan, Italy).

Choline oxidase was supplied by Sigma Chemical Co. (St Louis, MO, USA) and phospholipase D by Boehringer Biochemia (Mannheim, FRG).

Chromatographic procedures for determination of lecithin and sphingomyelin were those described in detail previously [18]. In brief, the samples, after centrifugation, were extracted by Bligh and Dyer's method [19]; phospholipids were then separated by TLC on silica gel plates (Merck Darmstadt, FRG), by Yavin and Zutra's method [20]. The separated spots were sprayed and removed by scraping the plate. For each spot, organic phosphorus was determined by Ames and Dubin's procedure [21], based spectrometry at 700 nm. The concentration of phospholipid was obtained from that of phosphorus by means of a calibration graph.

All reagents were of analytical grade and supplied by Carlo Erba (Milano, Italy).

Software and hardware

To calculate the 32 values of the L/S ratio from the 32 experimental values for lecithin and from the eight values for sphingomyelin (Table 1), to obtain row normalization and to produce histograms, a personal IBM XT computer with 640 KBytes, a CGA card and graphic printer Epson FX85, was used, with the "Lotus 2.01" program of the Lotus Corporation [22]. Graphic representation of the "scores" (Fig. 1) and all the relative calculations in respect of the methods used for pattern recognition, were performed by the "Parvus 1.0" program [13, 23], on the same IBM computer and Epson printer.

Table 1
Experimental values for lecithin (L) and sphingomyelin (S)

Subject No.	L (a)	L (b)	L (c)	L (d)	(S)
1	10.0	9.0	12.0	8.1	2.1
2	8.6	9.0	11.5	14.9	2.8
3	14.8	15.2	25.5	54.5	2.0
4	14.8	11.9	20.0	41.2	2.0
5	6.7	6.2	8.0	6.0	1.4
6	6.2	6.7	7.0	7.3	1.1
7	2.9	2.9	3.5	1.9	1.6
8	2.9	3.1	4.1	2.3	2.7

(a) Lecithin by enzymic–amperometric method.
(b) Lecithin by enzymic–spectrometric method with the absorbance of the sample corrected for the reagent blank and for the sample blank.
(c) Lecithin by enzymic–spectrometric method with the absorbance corrected for the reagent blank only.
(d) Lecithin by chromatographic method.
(S) Sphingomyelin by chromatographic method.
All concentrations are expressed as nmol ml^{-1} of lecithin, or sphingomyelin, phosphorus.

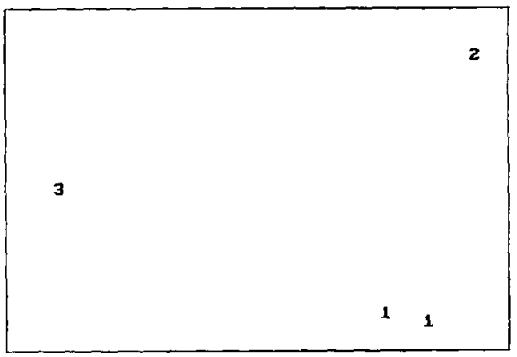

Figure 1
Graphical representation of the "scores" of "eigenvectors" with 98.3% of the total information. Four objects (methods) and seven variables (subjects); after autoscaling, normalization and generalized covariance matrix. Points marked by the index "1" are those relative to methods (a) and (b), the point marked "2" is relative to method (c) and the point marked as "3" is relative to the method (d) of Table 1.

Results and Discussion

All the values obtained for lecithin and sphingomyelin concentrations in the biological samples are reported in Table 1. The lecithin

concentrations were determined, by enzymic-spectrometry, with or without sample blank subtraction [14, 15], by enzymic-amperometry [14], or by an extraction-chromatographic method [18]. The extraction-chromatographic method was also used to determine sphingomyelin in amniotic fluid.

The lecithin concentration found by the four methods and the sphingomyelin concentration by TLC were used to obtain four values of the L/S ratio for each of the eight subjects examined. In Table 2 the values of the L/S ratio are summarized.

A particularly useful normalization of row, between zero and one, of these values was carried out by the "Lotus" program [22] (Table 3). It can be observed that method (d) for lecithin yields the highest values of L/S for four subjects and the lowest values of L/S for the other four subjects; in contrast method (a) yields the lowest L/S values for three of the eight subjects and method (c) yields the highest values for four subjects. In general the method (b) yields intermediate L/S values. Moreover the mean values, for each column of this table, are the same for the methods (a) and (b) but higher for methods (c) and (d).

This observation seems to indicate that the different methods for the quantitative determination of lecithin are not always equivalent when used for calculation of the L/S value; however, the extent of the difference is not known. On the other hand, application of the Mann–Whitney [24] and Wilcoxon [25] tests to the L/S values of Table 2 generally supports the H_0 (null hypothesis) [25]. These considerations suggested that further investigations were necessary since the question is of great importance from the analytical and the diagnostic points of view.

To this aim, all the L/S values of Table 2 were submitted to the "Parvus" software package for pattern recognition analysis; the values were first normalized by the "Normal" program and the series of four L/S values, corresponding to the four methods of analysis for lecithin, were considered as objects while the eight subjects were considered as variables. The convergence of the four methods, was estimated by the "KNN" program, which performs a piecewise-linear classification of the objects and, for each object, computes the Euclidian distance [25] from the others and recognizes the K nearest [13, 23]. Table 4 and the histograms of Fig. 2 summarize these findings. The L/S values, in the last column of Table 2, in respect of method (d) (see Table 1), have a greater Euclidian distance from the remaining L/S values than those of the other three methods. Moreover, the L/S values in columns (a) and (b) are very near and almost equidistant from those of column (c); values in column (c) are sufficiently distant both from those of columns (a) and (b) and from the values of column (d) but are nearer to the values of the first two.

These conclusions are better shown by the relative projection of the "scores" of the "eigenvectors" with 96.5% of the total information (Fig. 1), obtained by the "Varvar" program [23] of the "Parvus" package. To obtain these graphical representations, the original values were normalized by "Autoscaling"; then, the generalized covariance matrix was carried out by the "Matcal" program [23]; finally the "loadings" and the "scores" were calculated by the "Eigen" program [23]. In Fig. 1 it can be easily observed that a group of two points (correlated by methods (a) and (b)) is

Table 2
The thirty-two values of the lecithin/sphingomyelin (L/S) ratio, from the experimental data reported in Table 1, using lecithin values reported in the corresponding (a), (b), (c) or (d) column of Table 1. Sphingomyelin values are those reported on the last column (S) of Table 1

Subject No.	L/S (a)	L/S (b)	L/S (c)	L/S (d)
1	4.76	4.29	5.7	3.86
2	3.07	3.21	4.11	5.32
3	7.40	7.60	12.75	27.25
4	7.40	5.95	10.00	20.60
5	4.79	4.43	5.71	4.29
6	5.64	6.09	6.36	6.64
7	1.81	1.81	2.19	1.19
8	1.04	1.13	1.49	0.82

Table 3
L/S ratio, of values in Table 2, normalized from 0 to 1 by "row normalization"

Subject No.	(a)	(b)	(c)	(d)
1	0.49	0.23	1.00	0.00
2	0.00	0.06	0.46	1.00
3	0.00	0.01	0.27	1.00
4	0.10	0.00	0.28	1.00
5	0.35	0.10	1.00	0.00
6	0.00	0.45	0.73	1.00
7	0.62	0.62	1.00	0.00
8	0.33	0.46	1.00	0.00
mean	0.24	0.24	0.72	0.50
Δ	0.62	0.62	0.73	1.00

Table 4
"Euclidian distances" from different "objects", corresponding to the four series of L/S ratio values, relative to the four methods for lecithin determination, obtained by the "KNN" program

Methods	(a)	(b)	(c)	(d)
(a)	0.00	1.36	2.95	4.78
(b)	1.36	0.00	3.15	4.21
(c)	2.95	3.15	0.00	4.79
(d)	4.78	4.21	4.79	0.00

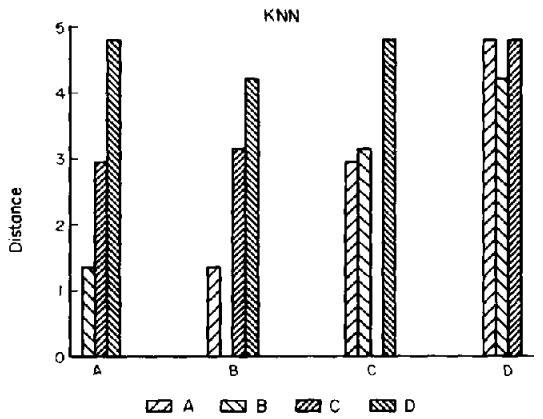

Figure 2
Histogram of "Euclidian distances" obtained by the values reported in Table 4.

compact whereas the other two points (correlated by methods (c) and (d)) are separated from each other and from the group of the other two points; however point (c) is nearer to the last one than point (d). These findings are in good agreement with results by the "KNN" method and improves and explains the previous observations, derived from "row normalizations" of Table 3.

The L/S ratio, as a numerical index to predict the foetal lung maturity of infants, has clinical significance [1, 2, 18, 26]: subjects for whom the L/S ratio is ≥ 2 are considered to be "mature"; those for whom the L/S ratio is <1.5 are considered to be "immature"; and those with L/S ratios of 1.5–1.9 are "intermediate". However, these values are not always indicative of pulmonary immaturity. If the values for the L/S ratio (Table 2) are considered from a clinical point of view, it is evident that, for the first six subjects, the L/S values are in every instance >2, apart from the analytical method adopted for lecithin measurement, whereas for subject No. 8, the L/S value is in all instances <1.5.

For subject No. 7, however, the calculated L/S values, are >2, or <1.5, or intermediate, depending on the analytical method adopted for the determination of lecithin. With the aim of investigating how with a package for pattern recognition analysis, "Parvus", a decisional procedure for subject No. 7 could be made available, seven of the eight subjects in Table 2, were divided into two classes: "mature" with L/S ≥ 2; and "immature" with L/S <1.5. The seven subjects were considered as objects; the L/S values, from the four methods of lecithin determination, were considered as variables. The values for L/S, calculated for subject No. 7, were placed in a "test set"; it was observed whether the corresponding values of the scores in the eigenvalues projection of the two eigenvectors with greater information (99.8%) fell in the zone of the "mature" subjects or in that of the "immature" subjects or in that of "intermediate" subjects.

Unfortunately "separation" of the scores of the two eigenvectors was unacceptable when the programs "Normal", "Matcal" and "Eigen" [23] were used; in previous research [11], those programs were able to achieve good separation of examined subjects in two different classes. All the other chemometric methods tried gave no better results. Thus subject No. 7, cannot be classified with satisfactory accuracy by these methods.

Conclusions

The analytical methods examined for the determination of lecithin in amniotic fluid are not equivalent from the analytical point of view and are not exchangeable for the determination of the L/S ratio. The greatest differences of results are observed when method (c) or (d) is adopted. An explanation is based on what each method actually measures. Method (d) measures only lecithin [18], whereas method (c) generally yields the sum of free choline and choline-containing phospholipids (in practice, lecithin in amniotic fluid [11, 14]). Nevertheless it is interesting that method (a) (amperometric), free of any interference, measuring only the lecithin concentration, is in good agreement with method (b) (spectrometric as the method (c), but providing that the correction for the sample blank is performed [11, 14]). In contrast, method (a) shows less agreement both with method (c) (spectrometric, but without the correction for the sample blank) and with method (d) (chromatographic). This leads to the conclusion that the observed differences cannot be completely

ascribed to the influence of free choline, but are probably caused by casual and systematic errors, as previously shown [11, 14]; these errors were due to interference from sample turbidity for method (c) and to the difficulty of quantitatively determining the TLC spots for method (d) [18]. On the other hand, in the samples of amniotic fluid previously examined, the content of free choline was of little significance [10, 18].

Elaboration of the data by the "Parvus" program for pattern recognition, represented in Fig. 1, was extremely useful and clearly confirmed the conclusions. From the clinical point of view, it is important to observe that independently of the method used to determine lecithin concentration, the L/S ratio was able to unequivocally indicate for seven of the eight cases considered, the maturity or immaturity of the subject. For subject No. 7, uncertainty about assignment to the right class has not been solved, probably not because of the inability of the chemometric method but because of the limited number of data. This limitation was known by the authors who preferred to give up several sets of data in favour of homogeneous experiments from the point of view of the provision of samples and the analytical characteristics of the methods.

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