QUANTITATIVE DETERMINATION OF PHOSPHOCHOLINE AND THE ACTIVITY OF PHOSPHOLIPASE C

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At the present time, thin-layer chromatography on silica gel is widely used for the quantitative study of phospholipids [1-9]. However, in only one of the papers mentioned [8] is a method described for the analysis of the products of the hydrolytic cleavage of phospholipids, in particular phosphocholine. It has been reported recently [7] that this method does not give accurate results, since the silica gel interferes with the establishment of the presence of phosphorus.

We have developed a method for determining phosphocholine based on the thin-layer chromatography of a hydrolyzate of phospholipids on silica gel, elution of the phosphocholine with 1 N hydrochloric acid, and spectrophotometric measurement of the amount of phosphorus in the eluate. When less polar eluants are used, the phosphocholine is eluted incompletely. The results obtained with standard solutions of the Ca salt of phosphocholine (Table 1) show that the mean error of the method does not exceed ± 3%.

Table 1

Quantitative Determination of the Ca Salt of Phosphocholine

Ca salt of phosphocholine, ml (with 162 γ of P/ml)	Phosphorus content, γ		Δ, %
	calculated	found*	
0.025	4.0	4.1	+2.4
0.05	8.1	8.2	+1.8
0.1	16.2	16.7	+3.1
0.15	24.3	24.0	−1.3
0.2	32.4	32.0	−1.2

* The figures given are the means of three measurements.

Table 2

Determination of the Activity of Phospholipase C

Time of incubation, min	Amount of substances formed	
	phosphocholine (in % P)	diglycerides (in % COOH groups)
1	48.4	47.0
15	66.0	68.1
30	71.8	73.6
60	93.5	94.7

Note: The amount of phosphorus and COOH groups in 0.05 ml of lecithin solution was taken as 100%. The figures given are the means of three experiments.

We have used this method to determine the phosphocholine formed in the enzymatic cleavage of lecithin with phospholipase C isolated from Clostridium perfringens. Preparative chromatography of the substrate in a thin layer of silica gel [5] led to the formation of three zones: phosphocholine (R_f 0.01), unchanged lecithin (R_f 0.35), and diglycerides (R_f 0.95). The results obtained by the elution of the phosphocholine and subsequent determination of the phosphorus are given in Table 2.

A comparison of the amounts of phosphocholine split off after predetermined intervals of time (for example, after 15 min [10]) with different preparations of phospholipase C can be used to evaluate their activities.

The advantage of the proposed method consists of the fact that, using the same chromatogram, it is possible simultaneously to determine the activity of the enzyme not only from the amount of phosphocholine but also from the amount of diglycerides formed, these being determined by the hydroxamate method [11] (cf. Table 2). The agreement of the results of the two independent determinations is a reliable criterion of the activity of the phospholipase C.

Experimental

The phosphorus was determined by a modification [12] of Bartlett's method [13]. The lecithin was isolated from egg yolk [14]. The enzymatic hydrolysis of the lecithin was carried out by the method described by Matsumoto [15].

Determination of the activity of phospholipase C. To 33 mg of lecithin suspended in 2.2 ml of a 0.001 M solution of $CaCl_2$ (pH 7) was added 2.2 ml of the same solution containing 1.2 mg of phospholipase C in 1 ml. After careful stirring, 0.4 ml of the mixture was removed, and the remainder was placed in a thermostat at 37° C. The sample taken was treated with 0.4 ml of ethanol to denature the enzyme and stirred, and 0.2 ml of the resulting mixture was trans-

ferred to a plate coated with a thin layer of silica gel [16]. Other samples of the same size were taken after 15, 30, and 60 min and were deposited on the same plate. To determine the zones on the plate, one additional spot was deposited on it (from any sample), and after the chromatogram had been developed it was sprayed (see below) with Dragendorff's reagent and also with a solution of phosphomolybdic acid [17], the other part of the plate being protected from the reagents.

The chromatograms were developed in the chloroform–methyl alcohol–acetic acid–water (65 : 25 : 8 : 4) system. In this system, the diglycerides migrate almost with the front (R_f 0.95), the unchanged lecithin has R_f 0.35, and the R_f value of the phosphocholine formed is 0.01. After the chromatogram had been dried, the whole of the plate was covered with a sheet of glass and its edge was carefully sprayed with the reagents to reveal the zones.

Determination of the phosphocholine. The zone containing the phosphocholine was removed and placed in a centrifuged tube. For a blank experiment, a strip of pure silica gel of the same size was taken from the same place. Each tube containing silica gel was treated with 4 ml of 1 N hydrochloric acid and it was heated with shaking for 15 min in a water bath (50°–55° C). After cooling, the mixture was centrifuged for 15 min (4000 rpm). The liquid was decanted and the residue was extracted by the same method twice more. The combined extract was evaporated to dryness in a graduated tube. Then 0.7 ml of a mixture of concentrated sulfuric acid and 72% perchloric acid (1 : 1) was added to each tube and the contents were ignited for 10 min at 300° C until the yellow-green color of chlorine appeared. After cooling, 4.4 ml of a freshly-prepared 1% aqueous solution of ammonium molybdate and 0.2 ml of the Fiske-Subbarow reagent [12] were added to each tube and, if necessary, the volume was made up to 5 ml with water. After the contents had been mixed, the tubes were placed in a boiling water bath for 10 min, and then they were cooled for 20 min at room temperature and the optical density of the solution was measured in the 830 mμ region with a SF-4A spectrophotometer (cell thickness 1 cm).

Diglycerides. These were determined without elution from the silica gel by the hydroxamate method [11].

Summary

1. A quantitative method for determining phosphocholine by means of thin-layer chromatography has been proposed.

2. In the enzymatic cleavage of lecithins with phospholipase C, this method permits the amount of diglycerides formed to be determined as well as the phosphocholine.

3. The agreement of the results of the analysis of phosphocholine and the diglycerides is a reliable criterion of the activity of the phospholipase C.

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