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Note

Rapid technique for the radiometric assay of phospholipase A_2

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Phospholipase A_2 (E.C. 3.1.1.4) catalyses the cleavage of a fatty acid from the β-position of phosphatidylcholine (PC) to yield lysolecithin and the free fatty acid (FA). This enzyme has recently received much attention because arachadonic acid released by the reaction is the rate-limiting precursor of inflammatory mediators such as prostaglandins and leukotrienes[1].

Radiometric assays provide the most sensitive and widely used means of determining phospholipase activity. After incubation of labelled PC with the sample, the FA is separated from excess of PC prior to measurement of radioactivity[2]. Most published techniques employ thin-layer chromatography (TLC) for this purpose, despite its obvious practical limitations. The recent advent of commercially available disposable micro-columns prompted us to assess their value as an alternative means of separating FA from PC.

EXPERIMENTAL

Materials

The TLC plates (20 × 20 cm) coated with a 0.25 mm layer of silica gel without fluorescent indicator were obtained from Merck (Darmstadt, G.F.R.). The columns were pre-packed disposable units (1-ml capacity, Type Octadecyl, Cat. No. 7020-1) designed for use with the Baker-10 extraction system (J. T. Baker, Deventer, The Netherlands). Radiochemicals were obtained from New England Nuclear (Dreieich, G.F.R.). Radioactivity was measured by scintillation counting in an Isocap-300 (Searle Nederland, Uithoorn, The Netherlands) using Insta-gel scintillation fluid (Packard, Brussels, Belgium). All counts were corrected for quenching and expressed as dpm prior to calculation of recoveries and enzyme activities.

Enzyme assay

A standard procedure[3] with minor modifications was followed. The reaction mixture (total volume 50 μl) contained 0.5 μmol of glycylglycine, 0.1 μmol of calcium chloride, 0.5 mg of bovine serum albumin, 4 nmol of "cold" dipalmitoyl PC, approximately 20,000 dpm of "hot" PC (phosphatidylcholine, *L*-α-dipalmitoyl, [2-

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palmitoyl-1-^{14}C]) and the enzyme source at a pH of 7.4. After incubation for 1 h at 37°C the reaction was stopped by the addition of 1 ml of chloroform–methanol (2:1) or 0.5 ml of ethanol and the mixture was processed further as described below.

TLC separation

Following the addition of chloroform–methanol, 200 μl of 0.1 *N* potassium chloride solution were added and the tubes were shaken and centrifuged. The upper phase was removed and the remaining lower phase dried under nitrogen. The residue was applied to a TLC plate in a minimal volume of chloroform–methanol and the plate developed in chloroform–methanol–acetic acid–water (100:60:16:8). After drying, reference spots were identified with iodine vapour and the radioactive zones corresponding to FA and PC were scraped off. The scrapings were suspended in 1 ml of water, 10 ml of scintillation fluid were added and the radioactivity was measured.

The recovery was determined by the addition of [9, 10-^{3}H]palmitic acid (about 50,000 dpm) to the chloroform–methanol extract prior to TLC analysis.

Column separation

After stopping the reaction with 0.5 ml of ethanol, the entire reaction mixture was placed on a column which had been pre-wetted with ethanol. The reaction tube was rinsed with a second 0.5-ml volume of ethanol and the rinsings were added to the column. A brief reduction in pressure eluted the FA directly from the bottom of column into a scintillation vial. Scintillation fluid was added and radioactivity measured as before.

The recovery was determined by addition of [9, 10-^{3}H]palmitic acid to the reaction mixture immediately prior to chromatography.

RESULTS

The recovery of tritiated palmitc acid after fractionation by TLC was 79.9 ± 3.7% (n = 3), compared with a recovery of 95.7 ± 1.6% (n = 5) after column chromatography.

The results of three sets of experiments to compare the alternative techniques are summarized in Table I. In the first experiment, the enzyme was omitted from the reaction mixture (blank); the second and third experiments employed different prep-

TABLE I

COMPARISON OF TLC AND COLUMN TECHNIQUES

Values for percentage cleavage of PC (dpm FA/dpm total) have been corrected for recovery and represent the mean ± standard deviation of six experiments.

Sample	*dpm FA/dpm total*	
	TLC	*Column*
Blank	1.06 ± 0.08	0.98 ± 0.04
Snake venom A	3.24 ± 0.25	2.93 ± 0.20
Snake venom B	7.35 ± 0.49	7.05 ± 0.47

arations of snake venom phospholipase A_2. It can be seen that the absolute values using the two techniques are very similar, and that the reproducibility of the column separation is at least as good as that obtained using TLC.

DISCUSSION

It is clear that the use of disposable micro-columns offers a far quicker and less laborious method of separating FA from PC, the overall time being reduced from several hours to a few minutes. It is particularly suited to batch analysis when using the Baker-10 system, a point of importance in routine assay of enzymes. The recovery (95.7%) is sufficiently high that in practice no correction is necessary to calculate absolute values.

At present prices the cost per assay using commercial disposable columns is about the same as TLC employing six channels per plate.

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