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Note

Sensitive gas chromatographic determination of certain glycerophosphates by use of the flame photometric detector

Application to glycerophospholipids

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One of the most important recent developments in the field of gas chromatography (GC) has been the introduction of a flame photometric detector (FPD) for the sensitive and selective detection of compounds containing sulphur or phosphorus. This FPD has been applied extensively in the detection of residual pesticides[1], atmospheric pollutants[2] and trace impurities in chemicals[3]. Phospholipids attract our interest because of their biological functions and specific polar structures. Gas-phase determination of these polar lipids, either directly or after conversion into trimethylsilyl (TMS) derivatives, has been reported by different groups[4,5]. However, detection of phosphorus-containing compounds on the chromatograms were unsuccessful. Previously Duncan *et al.*[6] reported a combined GC–mass spectrometric (MS) study of glycerophospholipids by removal of fatty acid residues with sodium hydroxide and subsequent silylation.

Cicero and Sherman have documented the GC–MS of phosphatidylinositides[7] and cardiolipin[8]. But hitherto no report on the use of FPD for GC analysis of glycerophosphates or glycerophospholipids has been published, possibly because of practical difficulties. The method described by Duncan *et al.*[6] was excellent but involved certain problems in its application to GC–FPD analysis. For instance, undesired side-reactions took place during alkaline hydrolysis, such as breakdown of phosphate ester linkages to the extent that the corresponding glycerophosphate esters are scarcely detectable. Moreover, the conditions used for silylation appear to be not always suitable for complete reactions of the respective glycerophosphate esters, deacylated products of glycerophospholipids. Therefore, our primary aim was to establish whether GC–FPD determination of glycerophosphates and their esters, parent substances of glycerophospholipids, is possible or not after silylation. We also attempted to reduce the side-reactions occurring during alkaline hydrolysis and to establish the necessary conditions for silylation for developing a sensitive method of GC–FPD determination of glycerophospholipids.

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This paper reports preliminary studies on convenient and sensitive GC–FPD analysis of certain glycerophosphates, and also further trial applications to assays of certain glycerophospholipids, such as phosphatidic acid, phosphatidylglycerol, phosphatidylinositol and phosphatidylinositol-4-monophosphate. In the application to glycerophospholipids, the method consists of two steps: first, selective hydrolysis of phospholipids with lithium hydroxide and, second, silylation of the resulting glycerophosphate esters.

EXPERIMENTAL

Chemicals were obtained commercially as follows: disodium DL-α-glycerophosphate (Sigma, St. Louis, MO, U.S.A.), disodium β-glycerophosphate (Sigma), L-α-glycerophosphorylinositol (Supelco, Bellefonte, PA, U.S.A.), disodium L-α-phosphatidate (dipalmitoyl, Sigma), L-α-phosphatidylinositol (from pig liver, Serdary London, Canada), L-α-phosphatidylinositol-4-monophosphate (from bovine brain, Sigma) and L-α-phosphatidylglycerol (from egg-yolk lecithin, Serdary). L-α-Glycerophosphorylglycerol and L-α-glycerophosphorylinositol-4-monophosphate were prepared from L-α-phosphatidylglycerol (dipalmitoyl, Serdary) and L-α-phosphatidylinositol-4-monophosphate, respectively.

For silylation, N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS) (Pierce, Rockford, IL, U.S.A.) was used. Test samples were prepared by the following three methods.

(1) Sodium DL-α-glycerophosphate or sodium β-glycerophosphate (2–5 μg, containing *ca.* 100–250 ng of phosphorus) dissolved in a small volume of methanol–water (50:50) was passed through a Dowex 50-X8 column 3.0 × 0.6 cm I.D. and eluted with methanol–water (80:20); 5 ml of the fraction was collected. An aliquot of the eluate was evaporated *in vacuo* in a 2–3 ml test-tube, 25 μl of BSTFA containing 1% TMCS and 25 μl of pyridine was added, and the solution was allowed to stand at 30°C for 1 h with occasional shaking, before GC–FPD analysis.

(2) For silylation of authentic glycerophosphorylglycerol, the sample was mixed with BSTFA containing 1% TMCS and pyridine, and kept for 1 h at 30°C. Standing with the silylating agent at 30°C for 24 h was necessary for authentic glycerophosphorylinositol or glycerophosphorylinositol-4-phosphate.

(3) Samples of authentic glycerophospholipids, such as phosphatidic acid, phosphatidylglycerol, phosphatidylinositol and phosphatidylinositol-4-phosphate (2–5 μg, containing *ca.* 80–200 ng of phosphorus) dissolved in a small volume of solvent were mixed with 10 μl of 1 *N* LiOH and 100 μl of methanol, and kept at 37°C for 15 min. The reaction mixture was processed through the cation-exchange resin as describe above.

Lipids from biological sources can also be determined after appropriate purification. For instance, the lipid to be analysed and authentic samples were spotted on a silica gel plate, and developed with an appropriate solvent system. The part of the plate spotted with the authentic samples was cut off and visualized by exposure in iodine vapour or charring with ethanolic sulphuric acid. The gel at the required area of the residual part of the plate was scraped, extracted three times with chloroform–methanol (1:1) and an aliquot of the combined extracts was dried *in vacuo* for assay.

For GC assay, a Hitachi Model 163 gas chromatograph equipped with an FPD

and a flame ionization detector (FID) was used. In FPD, the emission was measured with a photomultiplier tube through an interference filter with a transmission maximum at 526 nm. The inlet system was equipped with a glass column (2 m × 3 mm) packed with 2% OV-17 on 80–100 mesh Chromosorb W AW DMCS. The column temperature was programmed from 150°C to 290°C at 5°/min, the injection temperature was 290°C, the carrier gas was nitrogen at a flow-rate of 40 ml/min, the fuel gas was hydrogen (100 ml/min) and oxygen (20 ml/min), and the detector gas was nitrogen (40 ml/min). The photomultiplier voltage was 700 V and the chart speed 5 mm/min. Additionally, GC–MS of TMS derivatives of respective glycerophosphates and esters was carried out, in a Shimadzu LKB 9000 instrument, in the manner reported by Duncan *et al.*[6] and Cicero and Sherman[7].

RESULTS

On GC–FPD analysis, α- and β-glycerophosphates are detected with a lower limit of 200 pg–1 ng of phosphorus, and the isomers are separated (Fig. 1).

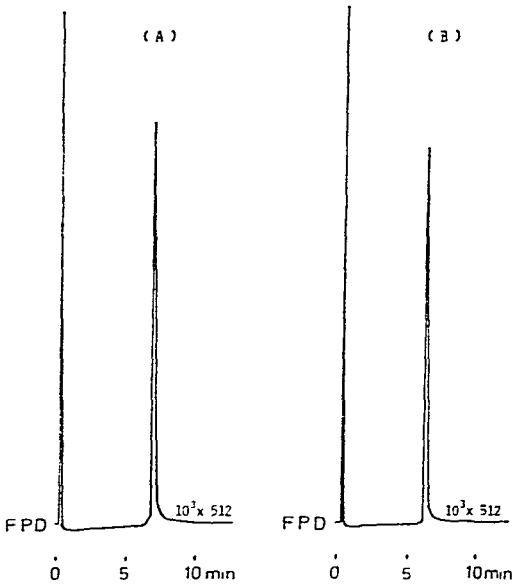

Fig. 1. Gas chromatograms of tetrakis(trimethylsilyl)-α-glycerophosphate (A) and its β-isomer (B) with monitoring by FPD. They correspond to 20 ng of α- and β-glycerophosphate, respectively.

In the examination of glycerophosphorylglycerol, glycerophosphorylinositol and glycerophosphorylinositol-4-phosphate, peaks of the respective TMS derivatives were also detected sensitively for *ca.* 1 ng of phosphorus. Thus the superiority of GC–FPD method for determination of glycerophosphates and their esters seems to be established. The retention times of glycerophosphates and esters on GC–FPD were as follows: β-glycerophosphate (6.3 min), α-glycerophosphate (7.3 min), α-

glycerophosphorylglycerol (15.1 min), α-glycerophosphorylinositol (24.0 min) and α-glycerophosphorylinositol-4-phosphate (29.3 min).

The determination of phosphatidic acid, phosphatidylglycerol, phosphatidylinositol and phosphatidylinositol-4-phosphate was attempted. Phosphatidic acid or esters was treated with LiOH instead of NaOH to suppress the side-reactions, and silylated under the conditions described above for the respective glycerophosphoric acid or esters. The desired peaks of TMS derivatives of glycerophosphate and esters were detected, and were analysed by comparison with calibration curves of respective authentic samples. Fig. 2 shows the simultaneous recordings with FID and FPD monitors on GC of pentakis(trimethylsilyl)glycerophosphorylglycerol, which is produced by selective alkaline hydrolysis of phosphatidylglycerol and subsequent silylation. Even in the presence of TMS derivatives of fatty acids liberated by alkaline hydrolysis, a TMS derivative of α-glycerophosphorylglycerol was apparently detected as a main peak by the FPD monitor. With FID monitor, the peak of α-glycerophosphorylglycerol was difficult to detect, because peaks of TMS derivatives of fatty acids and others were also detectable. With tetrakis(trimethylsilyl)-α-glycerophosphate, octakis(trimethylsilyl)-α-glycerophosphorylinositol and nonakis(trimethylsilyl)-α-glycerophosphorylinositol-4-phosphate derived from phosphatidic acid or respective esters, GC–FPD analysis was performed in a similar manner. However, phosphate bond cleavage occurred to some extent even with the use of LiOH.

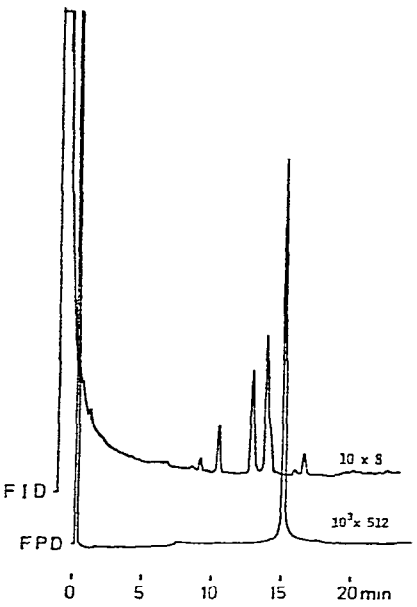

Fig. 2. Simultaneous recording with monitoring by FID and FPD of pentakis(trimethylsilyl)-glycerophosphorylglycerol and trimethylsilyl derivatives of fatty acids. L-α-Phosphatidylglycerol was selectively hydrolysed with methanolic lithium hydroxide, and silylated after passage through a cation-exchange resin.

CONCLUSION

The salient features of the present method are as follows.

(1) For selective alkaline hydrolysis of glycerophospholipids to liberate fatty acid residues, *ca.* 0.1 *N* LiOH in aqueous methanol (final concentration) is used, instead of *ca.* 0.1 *N* NaOH in chloroform–methanol mixture[6], to obtain more selective hydrolysis[9]. Under these conditions the side-reactions involving cleavage of phosphate ester linkages are minimized.

(2) The procedure is very simple, because the preliminary procedure of previous investigators[6] for partial purification of glycerophosphate esters involving neutralization with acetic acid, and partition procedure can be omitted. However, removal of lithium ion by passage through a cation exchange resin was necessary, to prevent accumulation of lithium salts at the injection port of the apparatus which may cause destruction of the samples. Such simplification is possible because of the high selectivity and sensitivity of the FPD, which allows analysis of the glycerophosphates. Furthermore, the GC–FPD method offers the possibility of sensitive and convenient determination of phosphatidic acid or esters, when the calibration curves of glycerophosphates or esters derived from corresponding phosphatidates or esters are available, even in the presence of considerable amounts of fatty acids liberated simultaneously with alkali.

(3) The sensitivity of the FPD is so high that assays of samples containing *ca.* 1 ng of phosphorus may be possible. Thus the method is convenient for measurements of numerous samples in routine work.

Other glycerophospholipids, such as glycerophosphorylethanolamine, glycerophosphorylserine and others, appear to be in a separate category from the above glycerophosphates because of fundamental differences in the conditions for silylation, and they are under investigation in our laboratories. The GC–FPD method may be useful for determination of other biologically important substances containing phosphorus, such as phosphorus derivatives of sugars and nucleic acids.

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