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Note

Separation of lipid classes by high-performance liquid chromatography with the "mass detector"

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A rapid method for the analysis of lipid classes from animal tissues by means of high-performance liquid chromatography (HPLC) on a column of silica gel with a ternary gradient elution scheme was recently described[1]. Lipids ranging in polarity from cholesterol esters to lysophosphatidylcholine were separately eluted in only 20 min. The detector employed was the "mass detector" manufactured by Applied Chromatography Systems (ACS, Macclesfield, U.K.), also known as an "evaporative analyser" or "light-scattering detector". With this system, the solvent emerging from the end of the HPLC column is evaporated in a stream of heated air; the lipid solute does not evaporate and passes in the form of minute droplets through a light beam, which is reflected and refracted. The degree of light scattering bears a relationship to the amount of material eluted. The analytical system has been in continuous use in this laboratory for lipid class analyses for over a year now, and some small but valuable modifications have been made to extend the working life of the column and to improve the separations, especially with respect to the more acidic lipids, such as free fatty acids, phosphatidylinositol and phosphatidylserine. In particular, it has been shown that it is possible to add small amounts of involatile salts to the eluent as ion suppressants without increasing the detector background noise levels.

EXPERIMENTAL

The HPLC equipment, including the column of silica gel, was as described previously[1], as in essence was the elution scheme. The three solvents in the ternary gradient were as follows: (A) hexane–tetrahydrofuran (99:1), (B) chloroform–isopropanol (1:4), (C) isopropanol–water (1:1). Briefly, solvent B was introduced into solvent A over a 5-min period to separate the simple lipids, then a gradient of solvent C into a mixture of A and B was generated to elute each of the phospholipids; finally, part of the gradient was run in reverse to remove bound water from the column and to re-equilibrate it before the next sample was injected.

Ionic materials were later added to the aqueous component to sharpen the resolution and to extend column life. In the optimum system, the water in solvent C contained 0.5 m*M* serine adjusted to pH 7.5 with ethylamine. No guard column was used, but an Upchurch filter with a stainless-steel frit (2-μm pores; Scotlab Instrument Sales, Bellshill, U.K.) was incorporated between the injector and the column

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to trap microparticulate impurities. All natural samples were filtered through Acrodisc™-CR disposable filters (0.45-μm pores; Gelman Sciences, MI, U.S.A.) prior to analysis.

RESULTS AND DISCUSSION

In order to separate lipid classes from animal tissues differing as markedly in polarity as cholesterol esters and lysophosphatidylcholine, it was necessary to employ a complex ternary gradient elution system in which a hydrocarbon solvent was used to elute the non-polar lipids and an aqueous solvent mixture to elute the phospholipids. To ensure miscibility between these extremes, there was a requirement for a solvent such as isopropanol to mediate the transfer from one to the other. Initially[1], isooctane was used as the hydrocarbon solvent as this has excellent cavitation properties at high pressures, but it has now been found preferable to use hexane, which has lower viscosity, as this permitted the system to be run at rather lower pressures; during elution, the pressure rose from approximately 900 p.s.i. initially to about 3400 p.s.i., when it fell as the column was re-equilibrated prior to the next analysis.

When the column was used continuously with this system in the analysis of real samples, excellent resolution was obtained for the first 300 to 400 chromatographic runs with no change in the elution times of individual components. Then the nature of the separation appeared to change rather abruptly and, in particular, peak broadening was observed and the response of the detector towards the acidic lipids, especially phosphatidylserine, appeared to diminish. Two factors were found to be involved, *i.e.* slow dissolution of silica gel from the column, leaving a slight void, and irreversible adsorption of lipids to the column. The former problem was resolved simply by unscrewing the ends of the column, topping up with fresh silica gel, reassembling, and eluting the column in the reverse direction.

Restoring the activity of the column by removing adsorbed materials proved somewhat more difficult initially, but was eventually solved also. A number of workers have obtained improved separations of phospholipids by adding acids or other ionic species to the eluent as ion suppressants (reviewed elsewhere[2,3]). Small amounts (less than 1% of the aqueous component) of formic, acetic, oxalic, trifluoroacetic and orthophosphoric acids did not appear to assist the separation and caused appreciable background noise. The last two components caused some breakdown of plasmalogens in natural samples, and the effect was still observed after the acid was removed from the eluent, presumably because some remained adsorbed onto the silica gel. Addition of dilute ammonia to the aqueous component removed the adverse effects of the acids but did not otherwise improve the separations. Similarly, addition of small amounts of detergents (Triton) or chelating agents (EDTA) to the aqueous component had no beneficial effects.

The resolution of the column was rapidly restored, however, by adding small amounts of salts (1 to 5 m*M*) to the water in solvent C. Ammonium chloride was tried initially and brought about an immediate improvement, although the detector baseline was noisy. Ammonium acetate was almost as effective and did not disturb the detector background level to the same extent. Many different salt combinations were then tried at various pH values, including ammonium, ethylamine, piperazine and serine salts of acetic and phosphoric acids. Different salts and pH values had

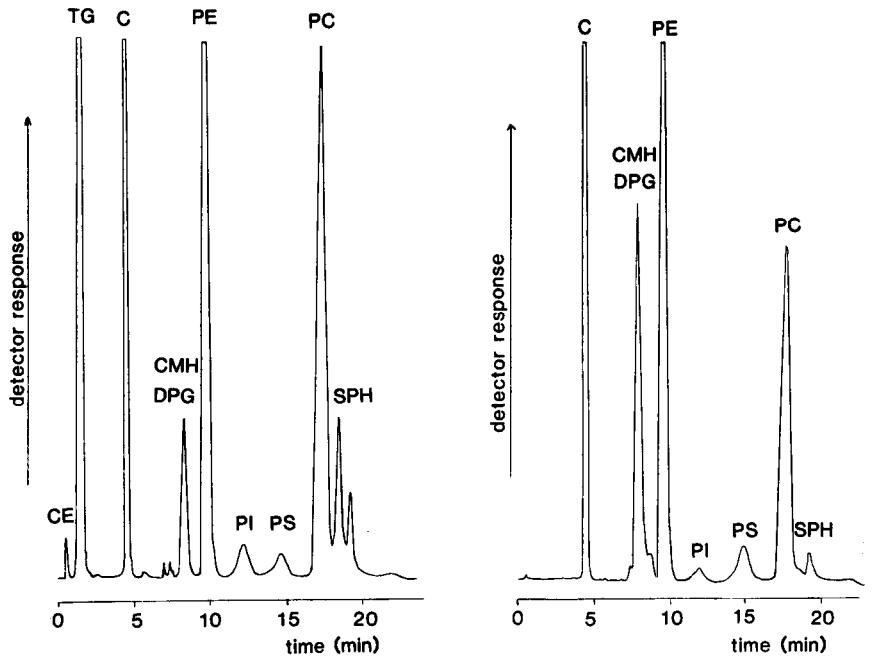

Fig. 1. Separation of a lipid extract from rat kidney by HPLC on a silica gel column with mass detection. See text for conditions. Abbreviations: CE = cholesterol esters; TG = triacylglycerols; C = cholesterol; DPG = diphosphatidylglycerol (cardiolipin); CMH = ceramide monohexoside; PE = phosphatidylethanolamine; PI = phosphatidylinositol; PS = phosphatidylserine; PC = phosphatidylcholine; SPH = sphingomyelin (usually seen as a double peak).

Fig. 2. Separation of a lipid extract from rat brain tissue by HPLC on a silica gel column with mass detection. See text for conditions. For abbreviations see Fig. 1.

small effects also on the order of elution of some of the minor components. The sharpest peaks for unesterified fatty acids and phosphatidylserine, especially, were obtained with serine buffered to pH 7.5 with ethylamine. At a concentration of 0.5 m*M* serine, the background noise from the detector was negligible at the highest concentration of solvent C in the elution scheme (16%). An old column that had previously been abandoned was completely restored in this way, and the retention times for individual lipids were the same as when the column was new.

The nature of the separation obtained with a lipid extract from rat kidney, with the system as described previously[1] but with hexane in solvent A and with 1 m*M* serine (pH 7.5) in the water of solvent C, is shown in Fig. 1. All the main simple and complex lipid classes, including cholesterol esters, triacylglycerols, and each of the more abundant phospholipid classes, were clearly resolved. In particular, phosphatidylinositol and phosphatidylserine both gave acceptable peaks, in spite of the fact that this particular column had been used for at least 1000 analyses over an 8-month period, during which the level of silica gel had been topped up on three occasions. A sample of 0.4 mg of lipid in 5 μl of hexane–chloroform (1:1) was applied to the column.

Similar results were obtained with a lipid extract from rat brain tissue as shown in Fig. 2. In this instance, free cholesterol was the only simple lipid component, but each of the main phospholipid classes, including the acidic lipids, was separated.

The relationships between the nature of the solvents, sample size and detector response with the ACS mass detector has been studied from a theoretical stand point in some detail[4,5]. It was shown earlier[1] that for most lipid classes the detector response was approximately linear in the range 50 to 200 μg, but tended to fall off rapidly below 10 μg. A different calibration curve was necessary for each compound in the sample. After changing the elution conditions in the manner described, it was necessary to re-calibrate the system. The calibration lines were very similar to those obtained before, although they had moved a little from their original positions, but not in any consistent direction. The addition of ions appeared to accentuate minor differences in the responses to each of the phospholipids. Experience has shown that, in order to obtain reproducible results in quantitative analyses with the mass detector, it is essential to set up the detector parameters, *i.e.* evaporation temperature, air flow-rate and sensitivity, as well as the elution system in exactly the same way each day both for calibration and then for routine analytical purposes. The manner of application of the sample also appeared to be important, and a 10-μl loop was utilized in the injection valve, and the sample was always injected onto the column in 5 μl of hexane–chloroform (1:1). If these precautions are followed, the system is capable of giving results that are at least as good as those attainable by any other technique, and in much less time than most.

The finding that the addition of small amounts of involatile ionic materials to eluents need not affect the baseline of the mass detector may be of value in many other types of analysis.

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