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Short Communication

# Improved separation of radioactively labelled cellular phospholipids by high-performance liquid chromatography

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**Abstract**

An improved high-performance liquid chromatographic method for the separation and determination of radioactively labelled cellular phospholipids is described. The method is based on separation of phospholipids on a 250 × 4 mm I.D. LiChrospher DIOL 100 (5 μm) column, fitted with a 50 × 4 mm I.D. LiChrospher Si 60 (5 μm) precolumn and a gradient of 5% $H_3PO_4$ and acetonitrile. It allows the determination of small amounts of labelled phosphatidylcholine and sphingomyelin due to the sharp elution profile in spite of long retention times.

## 1. Introduction

$^3$H-labelled phosphatidylcholine (PC), lysophosphatidylcholine (LPC), sphingomyelin (SPM) and [$^{14}$C]-sphingomyelin had to be separated and quantitated from cell homogenates and culture media from mononuclear phagocytes and fibroblasts of controls and patients with Tangier disease, which is characterized by a disturbance of cellular lipid and lipoprotein metabolism [1]. Under the culture conditions required for our experiments the amount of SPM in lipid extracts from cells and culture medium is very low.

Therefore, the major analytical problem encountered is the reliable quantitation of small amounts of SPM. This requires a good separation of the SPM peak from the PC area. Tailing of the PC peak will prevent the accurate determination of SPM in human cells. Since radio detection causes additional peak broadening, it was essential to design an HPLC protocol which provides maximal separation of these two phospholipids.

Earlier protocols applying a combination of a DIOL column with a silica precolumn [2,3], and also their improvement by Kuhnz *et al.* [4] or Kynast and Schmitz [5], showed sufficient separation of the major phospholipid classes but with tailing of the PC peak and only small differences in retention times for PC and SPM. Thus these methods do not allow the determination of small amounts of SPM found in our cell culture experiments. Therefore, we attempted to overcome

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these problems by optimizing the separation conditions.

## 2. Experimental

### 2.1. Materials

Dulbeccos Modified Eagles Medium (DMEM) was obtained from Gibco-BRL, (Gaithersburg, Maryland, USA). [$^3$H]-choline (80 Ci/mmol), [$^3$H]-phosphatidyl-choline (50 Ci/mmol) and [$^{14}$C]-sphingomyelin (54.5 mCi/mmol) were purchased from NEN (Boston, MA, USA), phospholipids were purchased from Sigma Chemicals, (St. Louis, MO, USA), acetonitrile (HPLC grade) was obtained from Baker (Phillipsburg, NJ, USA). All other chemicals and solvents were from Merck (Darmstadt, Germany). Water was purified by means of a Millipore Milli-Q-UF-Plus Water System. All solvents were degassed by sonication prior to use.

### 2.2. Cell culture

Cutaneous fibroblasts were obtained from three patients homozygous for Tangier disease by skin biopsy. Normal control fibroblasts were cultured from the cutis of normolipemic individuals who underwent abdominal surgery. Fibroblasts were cultured according to standard conditions in DMEM supplemented with 1% L-glutamine, 1% non-essential amino acids and 10% fetal calf serum (FCS) (Gibco) in a humidified 5% $CO_2$ atmosphere at 37°C. Human mononuclear phagocytes were isolated from patients and normolipemic donors by leukapheresis in a Cobe cell separator system as described earlier [6]. To obtain peaks of satisfactory height for exact analysis it was necessary to use *ca.* $5 \cdot 10^5$ fibroblasts or *ca.* $3.5 \cdot 10^6$ macrophages (*ca.* 0.5 mg cell protein each). Cells were pulsed by incubation in a medium containing 3 $\mu$Ci/ml [$^3$H]-choline for 48 h at 37°C. The chase medium containing varying amounts of high density lipoprotein$_3$ ($HDL_3$) as lipid acceptor was collected after a period of 24 h and lipids were extracted according to the method of Bligh and Dyer [7].

### 2.3. Equipment

Chromatography was performed with a Beckman System Gold HPLC consisting of an analog interface module, a binary pump module with high-pressure mixing, a variable-UV detector, an autosampler and an IBM AT. Additionally, the system was equipped with a column oven (W.O. Industrie Elektronik, Langenzersdorf, Austria), an HPLC radioactivity monitor LB 507 A (Berthold, Wildbad, Germany) and a Merck fraction collector L-5200.

### 2.4. Columns

The chromatographic column system was composed of a 250 × 4 mm I.D. LiChrospher DIOL 100, 5 $\mu$m column, fitted with a 50 × 4 mm I.D. LiChrospher Si 60, 5 $\mu$m precolumn (both Merck). Columns were maintained at 60°C while solvents were kept at room temperature. A gradient of 5% $H_3PO_4$ (solvent A) and acetonitrile (solvent B) was used at variable flow-rates according to Table 1 and Fig. 1.

### 2.5. Detection

After passing the UV-detector (detection of non-labeled compounds at 201 nm) the effluent was mixed with Flow Scint A (Packard, Frankfurt, Germany) and directed to the radiodetector. For pure $^3$H-measurements scint was mixed 1:1 with effluent and the flow cell volume was 500 $\mu$l. If $^3$H and $^{14}$C were detected simultanously scintillator was mixed 3:1 with effluent and a 1-ml flow cell was used.

The fractionated effluents of four different chromatograms were collected and liquid scintillation counting was used to determine the dpm per fraction. The results were correlated to the corresponding peak areas and used for calculation of the ratio (mol phospholipid)/(peak area from radio chromatogram) of each phospholipid. The resulting ratio was used for further chromatograms to calculate the molar amount of detected radioactive phospholipid.

Table 1
Mobile phase composition and flow-rates for the separation of phospholipids as a function of time

| Time (min) | Flow-rate (ml/min) | %B | | Duration of gradient (min) |
|---|---|---|---|---|
| | | from | to | |
| 0.00 | 2.2 | 92.0 | | – |
| 4.55 | 0.7 | 92.0 | 80.0 | 0.50 |
| 5.15 | 0.7 | 80.0 | 92.0 | 0.50 |
| 7.50 | 0.7 | 92.0 | 80.0 | 0.50 |
| 8.50 | 0.7 | 80.0 | 92.0 | 0.50 |
| 20.00 | 2.2 | 92.0 | | – |
| 24.00 | 0.0 | 92.0 | | – |

Solvent A: 5% $H_3PO_4$; solvent B: acetonitrile.

## 3. Results and discussion

We developed a separation protocol which is characterized by very short retention times for phosphatidylinositol (2.1 min), phosphatidylserine (2.8 min) and phosphatidylethanolamine (3.5 min). PC elutes in 6.4 min. SPM elutes more than 5 min later as a double peak at 11.7 min and 11.8 min. It is followed by LPC after 15.7 min. The SPM peak is extremly sharp (peak width 6 s) in relation to its late appearance (Fig. 2). The PC peak shows only little tailing as demonstrated in Figs. 2–4. This was achieved by a very low pH of eluent A (5% $H_3PO_4$, pH 0.9). These properties permit accurate separation and quantitation of the phospholipids, *e.g.* in medium extracts of fibroblasts (Fig. 3) and macrophage cell extracts (Fig. 4) despite the low concentrations of the labelled phospholipids.

The improvement of phospholipid separation is obtained using a complex gradient profile (Table 1, Fig. 1). The flow-rate as well as the solvent composition was changed compared to the basic protocols [2,4]. In order to obtain a maximal separation of PC and SPM without prolonging the retention time of both phospholipids unnecessarily the initial flow-rate is set at 2.2 ml/min. Before the PC peak starts it is reduced to 0.7 ml/min (Fig. 1).

The solvent gradient is divided into two parts both comprising relatively sharp steps in eluent composition from 92% eluent B (acetonitrile) to 80% B in 0.5 min and returning to the preceding composition. Returning to an eluent mixture

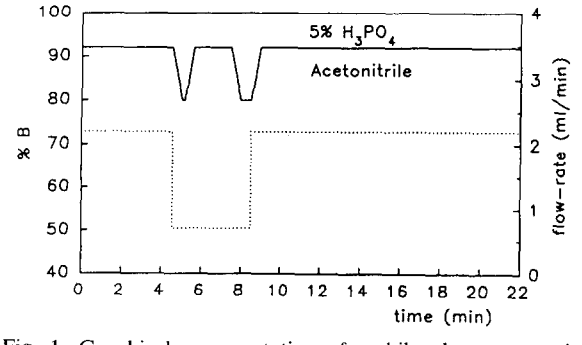


Fig. 1. Graphical representation of mobile phase composition (solid line) and changes in flow-rate (doted line) for the separation of phospholipids as a function of time.

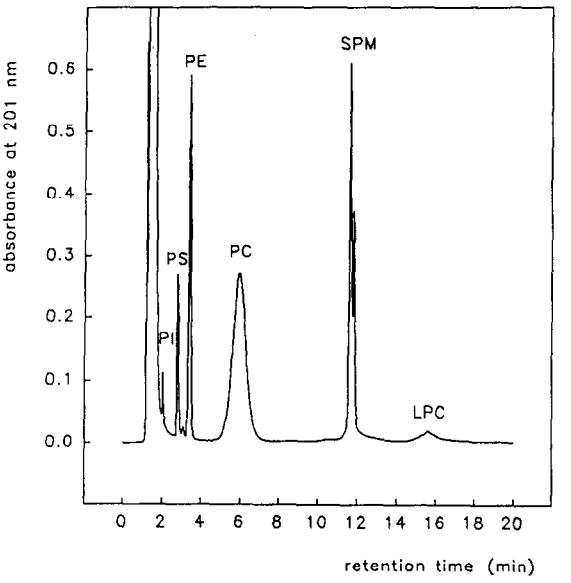


Fig. 2. Typical elution profile for a standard mixture of 6 phospholipids as detected by UV-monitor. The amount injected was 8 μg for each phospholipid. PI = phosphatidylinositol, PS = phosphatidylserine, PE = phosphatidylethanolamine, PC = phosphatidylcholine, SPM = sphingomyelin, LPC = lysophosphatidylcholine.

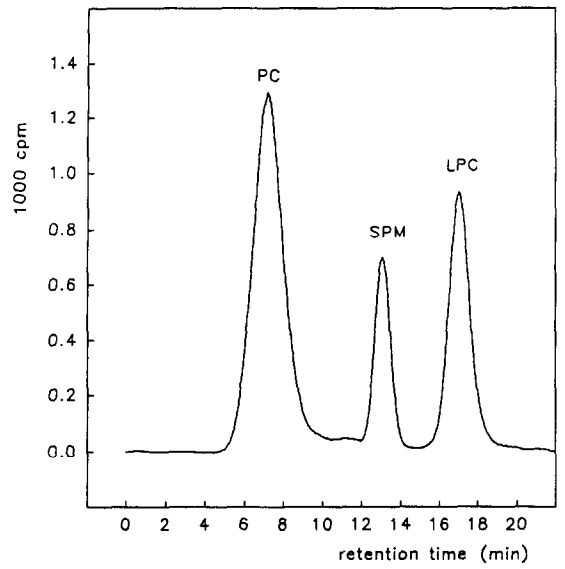


Fig. 3. Representative elution profile for [$^3$H]-choline labelled phospholipids in incubation medium extracts of fibroblasts as measured by the radio detector. The increase in retention times compared to the UV detection (Fig. 2) result from the arrangement of the system in which the effluent passes first the UV detector and than the radio-monitor. The determined amounts are 0.409 pmol [$^3$H]-PC, 0.077 pmol [$^3$H]-SPM and 0.016 pmol [$^3$H]-LPC.

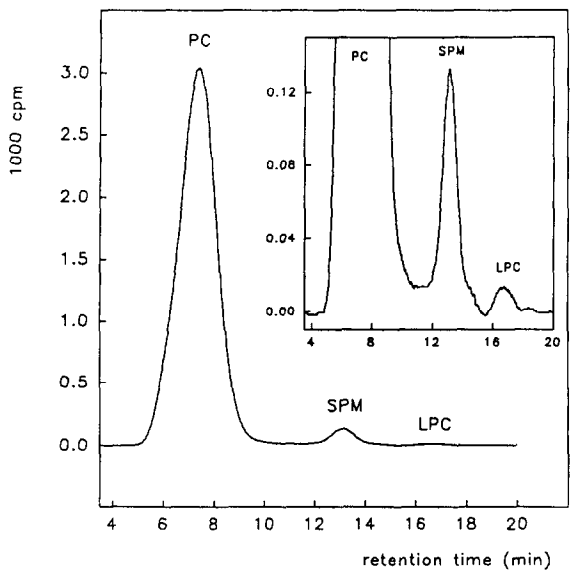


Fig. 4. Typical elution profile for [$^3$H]-choline labelled phospholipids in cell extracts of human monocytes/macrophages which were pulsed for 1 h with 5 $\mu$Ci $^3$H-choline/ml medium. The inset shows a 25-fold amplification of the $y$-axis showing that good peak symmetry and a relatively big difference in retention time for PC and SPM is critical for accurate quantitation at low SPM concentrations. The determined amounts are 0.840 pmol [$^3$H]-PC, 0.014 pmol [$^3$H]-SPM and 0.003 pmol [$^3$H]-LPC.

with lower elution capacity was essential in order to separate LPC from SPM. The second part of the gradient (at 7.5 min) is necessary to obtain a relatively sharp LPC peak (Fig. 3).

The method described was used to study high-density-lipoprotein mediated efflux of newly synthesized PL in control and Tangier fibroblasts. We detected an 80% reduction in the efflux of newly synthesized PC and an 30% reduction in the efflux of newly synthesized SPM from Tangier fibroblasts compared to control.

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