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Determination of alkylphosphocholines by high-performance liquid chromatography with light-scattering mass detection

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Abstract

A rapid and sensitive method for the determination of five different alkylphosphocholines including the antineoplastic phospholipid analogues hexadecylphosphocholine and octadecylphosphocholine is presented. The method is based on the separation of the lipids by high-performance liquid chromatography and quantitation by light-scattering mass detection. The lower limit of detection is approximately 50 pmol for each alkylphosphocholine tested. Quantitation is linear over the range 0.05–75 nmol. Hexane–isopropanol extracts of cultured cells can be applied to the column without further cleanup. The high resolution of separation and the sensitivity of detection render this method useful for pharmacokinetic investigations dealing with the uptake of alkylphosphocholines into different types of cells.

1. Introduction

Alkylphosphocholines represent a group of phospholipid analogues which were shown to inhibit cell proliferation [1–4], phosphatidylcholine biosynthesis [5,6] and protein kinase C [2,7–9]. The prototype for the alkylphosphocholines, hexadecylphosphocholine (HePC; Miltefosine) was introduced for the therapy of skin metastases of mammary carcinomas [10–12] and cutaneous lymphomas [13]. Therefore, an analytical method became necessary to determine alkylphosphocholines in biological samples. Recently, a high-performance thin-layer chromatography (HPTLC) method was published to separate HePC from other physiologically occurring lipids like lysophosphatidylcholine and sphingomyelin [14]. However, this method is not able to separate the different alkylphosphocholines varying in their alkyl side chains (Fig. 1). Furthermore, an HPLC method using a reversed-phase system was reported to separate HePC from other alkyllysophospholipids [15]. In that study, elution profiles of phospholipids and phospholipid analogues were obtained by measuring inorganic phosphorus. Especially with regard to sensitivity and quickness, the procedure used does not seem to be sufficient for drug monitoring in clinical studies.

In the present paper, we report a rapid and sensitive method for the simultaneous determination of alkylphosphocholines with a side chain length between 12 and 20 carbon atoms using

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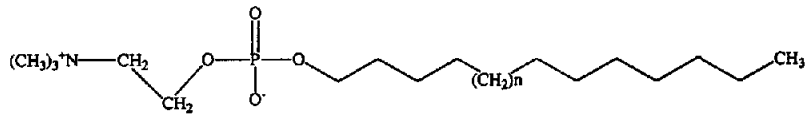

Alkylphosphocholines (n=1,3,5,7 and 9)

Fig. 1. Chemical structure of different alkylphosphocholines.

HPLC and light-scattering mass detection. In a light-scattering mass detector the effluent from the column enters a nebulizer and is converted to a fine mist by a stream of nitrogen [16]. The fine mist is then carried through a temperature-controlled tube in which the mobile phase evaporates. The non-volatile lipids pass through a light beam causing light scattering that is detected by a photodiode. The measured signal is proportional to the amount of sample.

The application of the developed method for the determination of alkylphosphocholines in cellular extracts and its potential use for other biological samples like serum and urine will be discussed.

2. Experimental

2.1. Materials

All solvents and chemicals were of analytical grade unless otherwise stated. HPLC-grade *n*-hexane and isopropanol were from Merck (Darmstadt, Germany). Hexadecylphosphocholine was synthesized as described [17]. Tetradecylphosphocholine, octadecylphosphocholine and eicosanylphosphocholine were a gift from D. Arndt (Max-Delbrück Zentrum, Berlin-Buch, Germany). Dodecylphosphocholine was synthesized by M. Mickeleit and J. Mulzer (Freie Universität Berlin, Berlin, Germany). Alkylphosphocholines were dissolved in isopropanol. The stock solutions (10 mmol/l of each compound) were diluted in isopropanol to reach the appropriate concentrations. Silica-gel 60 high-performance thin layer plates without fluorescent indicator were from Merck (Darmstadt, Germany).

2.2. Sample preparation

Madin-Darby canine kidney (MDCK) cells were grown to confluence in Dulbecco's minimal essential medium supplemented with 10% heat-inactivated fetal-calf serum, 0.56 g/l glutamine, 100 000 I.U./l penicillin and 0.1 g/l streptomycin in plastic culture dishes (Nunc, Wiesbaden, Germany). Media and culture reagents were from Gibco (Karlsruhe, Germany). Penicillin and streptomycin were from Boehringer (Mannheim, Germany).

Prior to extraction, $7 \cdot 10^6$ cells were incubated in medium containing 50 μmol/l HePC for 5 h. Control medium did not contain any additional supplements. Cells were harvested in 1 ml of distilled water and pelleted at 13 000 *g* and 4°C for 5 min. After washing the cells once with distilled water, HePC was extracted with 1 ml of *n*-hexane–isopropanol (3:2, v/v) by sonication for 5 min. Then 25 μl of the organic phase were applied to a silica-gel column as described below.

2.3. HPLC analysis

A Bischof 2200 HPLC gradient system equipped with a Rheodyne sample injection valve was used (Bischoff, Leonberg, Germany). The HPLC system was connected with a light-scattering detector Sedex 55 (ERCs, Regensburg, Germany). The signals were recorded with a Nelson Analytical 900 series data module (Nelson Analytical, Bub Road Cupertino, CA, USA) connected with a personal computer. Data were analysed using the appropriate software.

A silica-gel column (125 mm × 4 mm I.D., 3 μm particle size) was used (Säulentechnik, Berlin, Germany) in this study. The column was equilibrated with *n*-hexane–isopropanol–water

(300:200:29, v/v/v) at a constant flow-rate of 1.2 ml/min. After sample application (25–50 μl), separation of alkylphosphocholines was achieved by a 20-min wash with the eluent indicated above. After completion of the run, the column was washed for another 10 min. The signals of the light-scattering detector were collected during the first 18 min of the run and the data were analysed.

2.4. High-performance thin-layer chromatography

Samples were applied to silica-gel high-performance thin layer plates using an applicator (Linomat III; Camag, Berlin, Germany). Development was performed with chloroform–methanol–triethylamine–water (30:35:34:8, v/v) as described by Rustenbeck and Lenzen [14]. Alkylphosphocholines were stained with a $CuSO_4$ solution (156 g/l in 8.5% H_3PO_4) and quantified by use of a videodensitometer (Fischer Biotec, Reiskirchen, Germany).

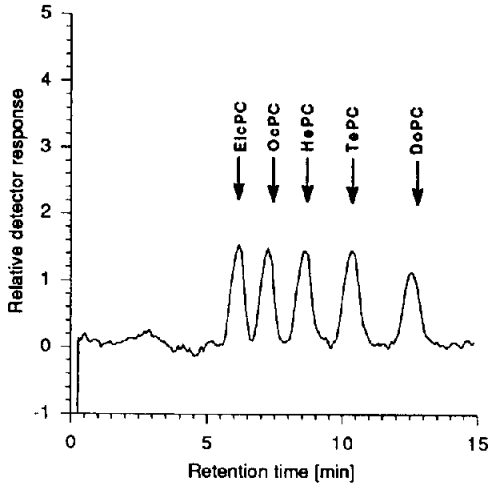

Fig. 2. Separation of alkylphosphocholines with different chain lengths. Alkylphosphocholines dissolved in isopropanol were applied to a silica-gel column and separated as described in Experimental. Detection of alkylphosphocholines was performed by use of a light-scattering detector. EicPC = eicosanylphosphocholine, OcPC = octadecylphosphocholine, HePC = hexadecylphosphocholine, TePC = Tetradecylphosphocholine, DoPC = Dodecylphosphocholine.

3. Results and discussion

High-performance liquid chromatography (HPLC) has been widely used in lipid analysis, but application of direct HPLC analysis of underivatized probes using UV-detection has been limited by the low sensitivity of the detectors and the baseline shift during gradient elution. Especially, UV-detection of phospholipids at 200–215 nm is limited by the poor absorptivity of phospholipids at that wavelength. Impurities of the samples and the use of aqueous gradient systems greatly affect the identification.

In the present study, we developed a method to analyse a new class of antitumoral phospholipid analogues, the alkylphosphocholines, by HPLC and light-scattering mass detection.

Fig. 2 shows the baseline-separation of five different alkylphosphocholines using a silica-gel column and isocratic elution with *n*-hexane–isopropanol–water (300:200:29, v/v/v). The separation was reproducible. However, since the retention times of the different alkylphosphocholines varied slightly it was necessary to calibrate the column with a known standard before each run. The alkylphosphocholines elute in order of the decreasing number of carbon atoms in their alkyl side chain. In contrast to the method using high-performance thin-layer chromatography [14], where baseline-separation of alkylphosphocholines is incomplete (data not shown), this method may be used for simultaneous determination of different alkylphosphocholines in the same sample.

In additional experiments, we determined the sensitivity and linearity of quantitation using light-scattering mass detection and compared the results with other procedures. The lower limit of detection of the light scattering detector is approximately 50 pmol which is in the same range as for the quantitation by video densitometry [14]. In our hands, video densitometry has a detection limit of 100 pmol. However, both methods are more sensitive than the determination of inorganic phosphorus as described by Unger *et al.* [15] where the detection limit of

hexadecylphosphocholine was found to be 2 nmol.

As shown in Fig. 3, the calibration curves of all analogues investigated are linear up to 75 nmol of each compound when quantitation is performed by light scattering mass detection. Samples with high concentrations of alkylphosphocholines may therefore be analysed without further dilution. This is of interest in cell culture experiments since some cell types seem to accumulate the analogues. In contrast, the calibration curves of the video densitometer are second-order polynomials and close to linearity in the range between 0.1–1.0 nmol (Fig. 4). The intensity of staining is chain length-dependent. For example, the peak area of eicosanylphosphocholine is tenfold higher than the peak area of dodecylphosphocholine.

Fig. 5 shows the HPLC profile of extracts of hexadecylphosphocholine (HePC) treated (50 μmol/l) and untreated MDCK cells. HePC is clearly separated from the other lipids of the extract. Furthermore, no lysophosphatidylcholine is detectable in control and HePC-treated cells. The lysophosphatidylcholine standard elutes at a higher retention time as compared with dodecylphosphocholine.

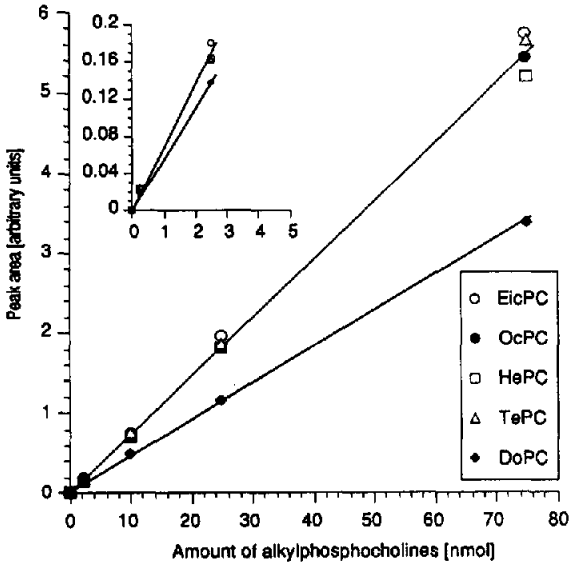

Fig. 3. Calibration curves of different alkylphosphocholines using light-scattering mass detection. Different amounts of alkylphosphocholines were separated on a silica-gel column and detected by use of a light-scattering detector. The data were recorded by a Nelson Analytical data module and analysed as described in Experimental. Each point represents the mean of two determinations. The variation of the two determinations was in all cases <5%. The experiment was repeated with similar results. For abbreviations of compounds see legend to Fig. 2.

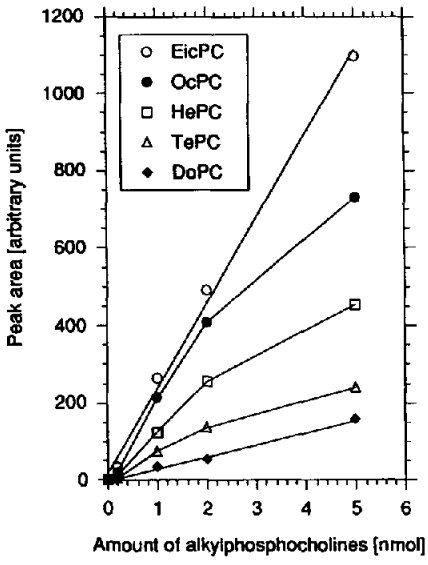

Fig. 4. Calibration curves of different alkylphosphocholines using video densitometry. Different amounts of alkylphosphocholines were stained on a silica-gel high-performance thin-layer plate as described in Experimental. Alkylphosphocholines were quantitated by video densitometry. Each point represents the mean of two determinations. The variation of the two determinations was in all cases <5%. The experiment was repeated with similar results. For abbreviations of compounds see legend to Fig. 2.

In summary, a fast and sensitive method for the quantitation of different alkylphosphocholines is described which is linear over a range of *ca.* three magnitudes. This method may be used for pharmacokinetic investigations with different alkylphosphocholines in cell culture systems as well as for drug monitoring of blood samples of patients treated with these antitumoral agents.

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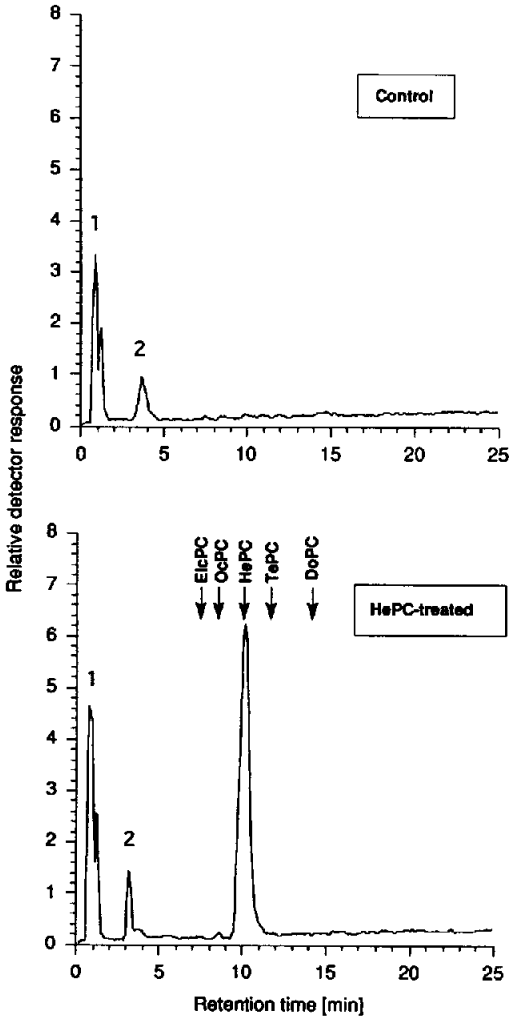

Fig. 5. HPLC profile of cell extracts from MDCK cells treated with hexadecylphosphocholine. Confluent MDCK cells were incubated with medium containing 50 μmol/l HePC or no supplements as a control. The cells were extracted and the lipids were separated on silica-gel column as described in Experimental. The experiment was repeated with similar results. 1 = cellular phospholipids, 2 = sphingomyelin. For abbreviations of compounds see legend to Fig. 2.

(NaFöG) of the Freie Universität Berlin. The light-scattering detector was made available by ERC GmbH (Alteglofsheim, Germany).

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