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QUANTITATION OF HEXADECYLPHOSPHOCHOLINE BY HIGH-PERFORMANCE THIN-LAYER CHROMATOGRAPHY WITH DENSITOMETRY

INGO RUSTENBECK* and SIGURD LENZEN

Institute of Pharmacology and Toxicology, University of Göttingen, Robert-Koch-Strasse 40, D-3400 Göttingen (F.R.G.)

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SUMMARY

A method for the determination of the antineoplastic ether phospholipid hexadecylphosphocholine (HePC) is presented, based on the separation of the lipids by high-performance thin-layer chromatography charring with a cupric sulphate reagent and quantitation by in situ densitometry. The lower limit of determination is ca. 25 pmol. Concentrated hexane–isopropanol extracts of plasma samples can be applied to the plate without further clean-up, making this method useful for clinical drug monitoring. Additional ion-exchange chromatography and removal of the salt contaminants by gel filtration permits the study of endogenous phospholipids together with HePC from the same sample.

INTRODUCTION

Some synthetic phospholipids have been shown to possess antineoplastic activity [1]. Two examples of this substance class are currently in clinical trials: octadecylmethylglycerophosphocholine and hexadecylphosphocholine (HePC, proposed INN: Miltefosine). The latter belongs to a recently synthesized class of phospholipid analogues, the alkylphosphocholines [2]. HePC has been used successfully for the treatment of cutaneous metastases of mammary carcinoma [3], and further indications for its use are under investigation. Studies on its biodistribution in the whole animal and on its metabolism in cultured cells have been performed with the radioactively labelled substance [4, 5]. The absolute amount of the alkylphosphocholine taken up by the cell

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cannot be easily determined in this manner, and the data on the long-term metabolism are difficult to interpret owing to migration of the radioactive label. Here we present a method for the determination of HePC in plasma, which is useful for drug monitoring. This method can also be applied to the determination of absolute amounts of HePC in tissues. An additional step in sample preparation allows the determination of up to seventeen endogenous lipids or groups of lipids from small amounts of tissue. This may prove advantageous in future research on the mechanism of action of alkylphosphocholines.

EXPERIMENTAL

Chemicals

Phospholipids and neutral lipids were purchased from Sigma (St. Louis, MO, U.S.A.), Calbiochem (San Diego, CA, U.S.A.) or Serdary (London, Canada). Solvents were of HPLC grade from Merck (Darmstadt, F.R.G.) or purissimum grade from Fluka (Buchs, Switzerland). HPTLC 60 F_{254} plates were from Merck.

Apparatus

The lipid solutions were concentrated with a vacuum concentrator (α-RVC, Christ, Osterode, F.R.G.). Samples and solutions of lipid standards were streaked on the plate by use of a modified microdoser (Desaga, Heidelberg, F.R.G.). The plates were developed in a double-trough chamber (Camag, Muttenz, Switzerland). The post-chromatographic colouring reaction was performed on a Desaga thermoplate. The coloured plates were scanned in a Desaga CD60 densitometer interfaced with an IBM AT 03 microcomputer.

Sample preparation

Lipids were extracted by vortex-mixing a sonicated sample (homogenates from liver cells, plasma, whole blood or resuspended erythrocytes) with the twenty-fold volume of hexane–2-propanol (3:2, v/v) [6, 7]. Centrifugation at 2500 *g* for min 5 pelleted the lipid-insoluble compounds, and the supernatant was concentrated to dryness. This total lipid extract was redissolved in 300 μl of solvent A (chloroform–methanol–water, 30:60:8, v/v) [8] and desalted by application on a Sephadex G15 minicolumn (1.6 ml gel volume) and elution with 1.3 ml of solvent A. The effluent was redried, redissolved in solvent A, and a fraction (depending on the estimated lipid content) was applied on the chromatoplate. If an analysis of the endogenous lipids of the tissue exposed to HePC was required, the total lipid extract was divided into a neutral and an acidic fraction by passage through a DEAE-Sephadex minicolumn (1 ml gel volume, acetate as counter-ion). The neutral lipids and zwitterionic phospholipids were eluted with 1.5 ml of solvent A, and the acidic phospholipids were eluted with 5 ml of solvent B (chloroform–methanol–0.8 *M* sodium acetate,

30:60:8, v/v) [8, 9]. Both fractions were then desalted as described above. In some cases the redissolved total lipid extract was chromatographed without prior desalting.

Chromatographic conditions

Six to eight samples and five standards were streaked in 8×1 mm bands 8 mm above the lower edge of the plate. Development was performed in a paper-lined chamber (30-min chamber saturation) with chloroform–methanol–triethylamine–water (30:35:34:8, v/v) [9, 10]. Sorbent saturation [11] was reached by suspending the plate for 10 min above the elution trough before development. This made the chromatographic separations very reproducible. When the solvent front had reached 90 mm, the plates were removed and dried at 180°C for 10 min. The cooled plate was dipped into a solution of 10% copper sulphate in 8% aqueous phosphoric acid (w/v) for 15 s [12]. The lipids were charred by heating the plate for 2 min at 110°C and for 10 min at 175°C. Densitometry of the charred spots was performed with a Desaga CD60 densitometer using the following conditions: 530 nm, transmission mode, 0.1×6 mm light spot, linear scan. The calibration curves were set up from five concentrations of standards run on the same plate using the peak area.

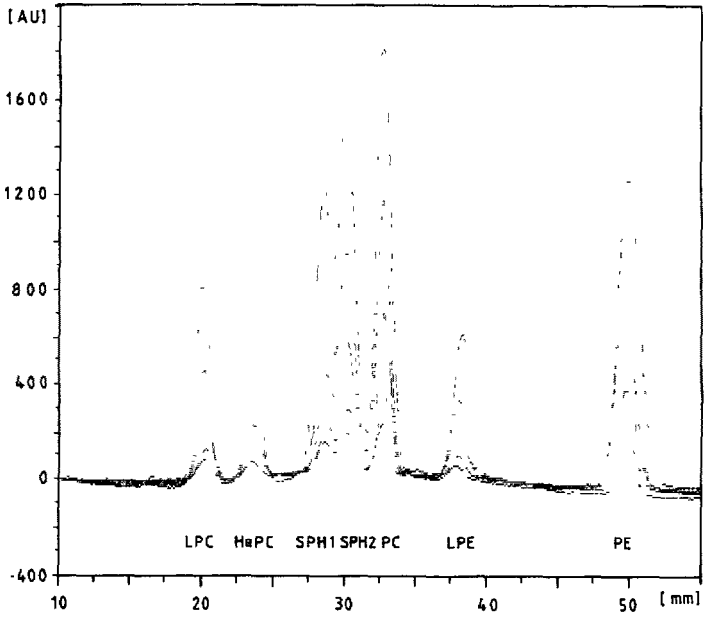

Fig. 1. HPTLC of zwitterionic phospholipid standards. The amounts of the substances are from 55 pmol to 1.1 nmol, except for those of HePC, which range from 27 pmol to 0.55 nmol.

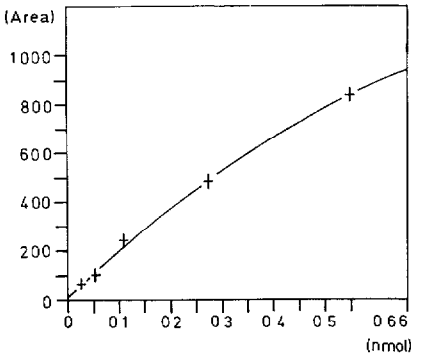

Fig. 2. Calibration curve of HePC from 27 pmol to 0.55 nmol. The curve is close to linearity in the range 0–200 pmol, and its overall shape was calculated as a second-order polynomial. The S.D. is 18.2 area units. Depending on the S.D. of the curve, which varies from plate to plate, peak areas between 50 and 70 area units (25–30 pmol) are sufficient for accurate determinations.

RESULTS

HePC as a lysophosphatidylcholine analogue was baseline-separated in this development system from lysophosphatidylcholine and the lower spot of sphingomyelin (Fig. 1). The only natural substance of possible interference is PAF (platelet-activating factor) which occurs on cell stimulation in minute amounts, but migrates closer to sphingomyelin. The other therapeutic phospholipid, octadecylmethylglycerophosphocholine, nearly comigrates with PAF.

The calibration curve (peak area versus concentration) of HePC can be described by a second-order polynomial, which is close to linearity in the lower concentration range (Fig. 2).

The lower limit of determination is ca. 25 pmol, when the S.D. of the calibration curve usually approaches 25% of the peak area value; the limit of detection is at 5–10 pmol. The determination of four samples (desalted extract from plasma of HePC-incubated blood) each containing the same amount of HePC ($x = 117$ pmol) gave a coefficient of variation of 8.5%.

The overall recovery was determined by assaying four samples (liver homogenates) spiked with 4 nmol of HePC each. Chromatography of 3.3 or 6.6% of the samples yielded recovery rates of $89 \pm 7.5\%$ for both types of sample preparation, i.e. with or without prior desalting of the total lipid extract.

In blood nearly all HePC is bound to plasma proteins. When 2 ml of whole blood were incubated with 15 μM HePC (plus 5 mM glucose and 5 mM EDTA), the amount of HePC in plasma was two to three times that of the same volume of whole blood, whereas virtually no HePC could be detected in washed erythrocytes (Figs. 3a–c and 4). The high amount of lysophosphatidylcholine is not an artifact but typical for rat plasma, where it comprises 15% of the phosphatidylcholine content [13].

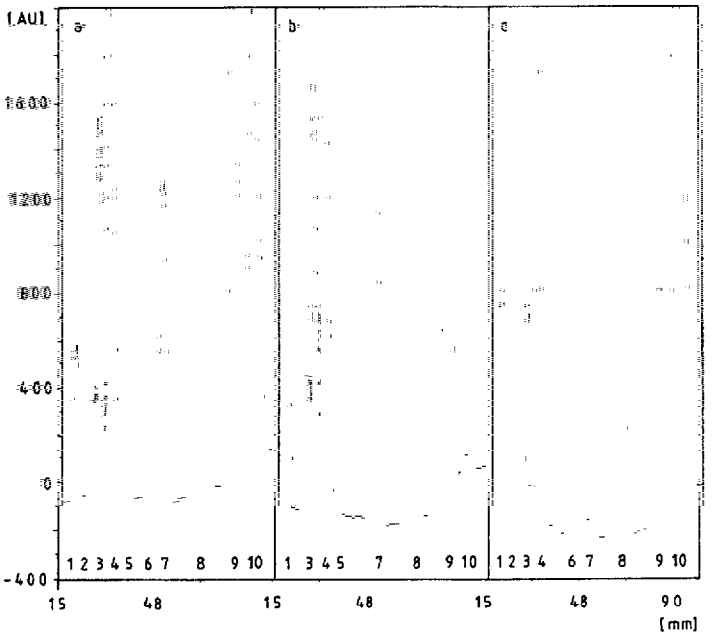

Fig. 3. HPTLC of zwitterionic phospholipids and neutral lipids from (a) whole blood, (b) erythrocytes and (c) plasma. Peaks: 1 = lysophosphatidylcholine (LPC); 2 = HePC; 3 = hydroxyl- and acylsphingomyelin (SPH1 and SPH2); 4 = phosphatidylcholine (PC); 5 = lysophosphatidylethanolamine (LPE); 6 = unidentified (glycolipid?); 7 = phosphatidylethanolamine (PE); 8 = monoacylglycerol (MG); 9 = cholesterol (CHOL); 10 = triacylglycerol (TG) and cholesteryl ester (CE). Note the absence of HePC in the erythrocytes. The amount of HePC in (c) is 45 pmol.

For research on the molecular mechanism of action of HePC, analysis of the possible changes in the phospholipid pattern of the target organ can be of interest. As shown in Fig. 5a and b, separation of the total lipid extract into a neutral and an acidic fraction allows the simultaneous determination of HePC plus all major and most minor phospholipids, as well as cholesterol and some neutral lipids.

DISCUSSION

The flexible chromatographic system presented here allows the determination of HePC with only modest requirements for sample clean-up. Hydrolysis of interfering phospholipids is not necessary. Desalting can be omitted when the salt load of the sample is low. The determination of plasma levels of HePC from patients thus becomes possible with relative ease. Even if the concentrations in the blood prove to be lower than 15 μM (an effective concentration in cell culture [14]), this method is still sufficiently sensitive, since only 10% of

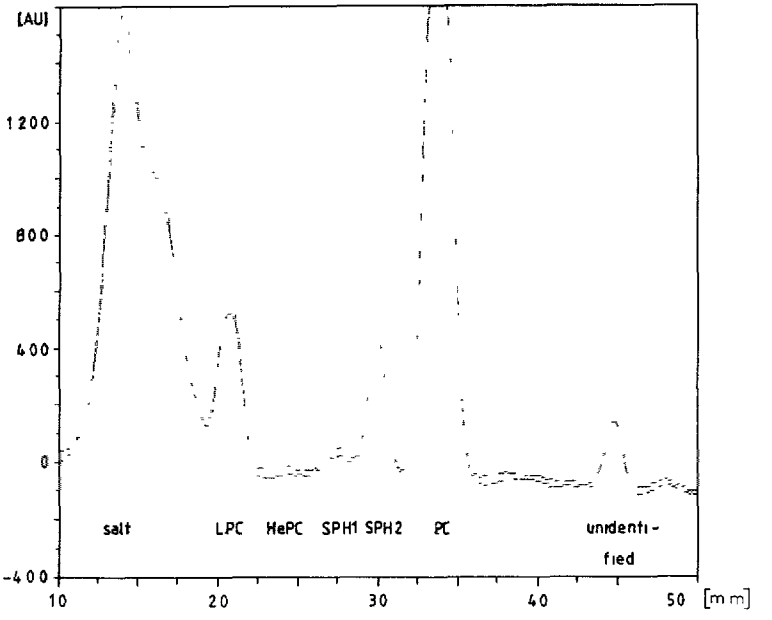

Fig. 4. HPTLC of undesalted hexane–2-propanol extracts from plasma of HePC-incubated whole blood and control. The contaminants are spontaneously coloured during the 180 °C step and further darkened by cupric sulphate. The quantification of HePC remains possible, as can be seen from comparison of the otherwise identical chromatograms. For peak identification, see Fig. 3.

a sample of 100 μl plasma were required for one determination, as in Fig. 3c. In routine diagnostics, use of a more sophisticated, commercially available thin-layer application apparatus can further increase the precision of the method. Separation of the extract makes possible detailed studies on the composition of membranes from HePC-treated cells. The absolute amount of HePC can be determined, as can the molecular percentage in relation to the other membrane phospholipids.

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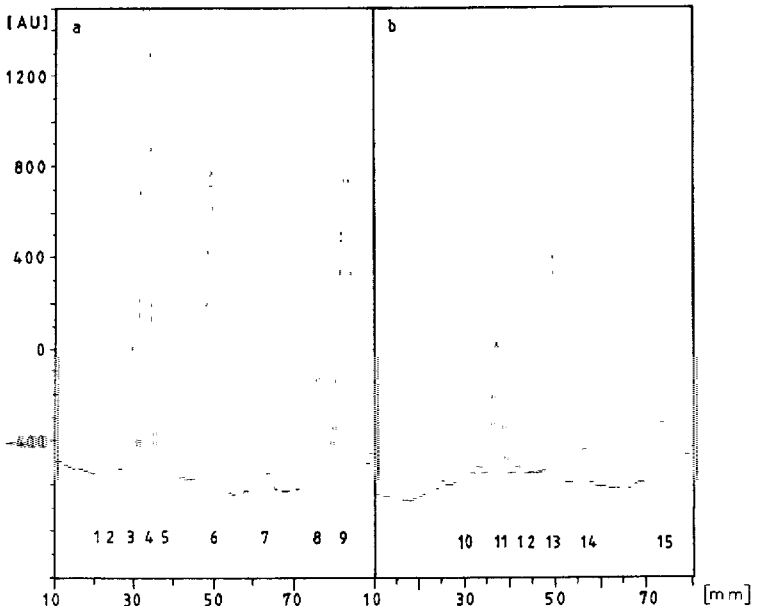

Fig. 5. HPTLC of the (a) neutral and (b) acidic fractions from the homogenate of HePC-incubated hepatocytes. Peaks: 1=LPC; 2=HePC; 3=SPH1 and SPH2; 4=PC; 5=LPE; 6=PE; 7=MG; 8=CHOL; 9=TG and CE; 10=lysophosphatidylserine (LPS); 11=phosphatidylserine (PS); 12=lysophosphatidylinositol (LPI); 13=phosphatidylinositol (PI); 14=phosphatidylglycerol (PG); 15=cardiolipin (CL). Fatty acids (FA) and phosphatidic acid (PA) are not visible in this sample.

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