Journal of Chromatography, 612 (1993) 21–26
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6632

Gas chromatographic determination of alkyl lysophospholipids after solid-phase extraction from cell culture media

J. Coene, E. Van den Eeckhout and P. Herdewijn

Laboratory for Drug Quality Control and Medicinal Chemistry, University of Ghent, Harelbekestraat 72, B-9000 Ghent (Belgium)

P. Sandra

Laboratory for Organic Chemistry, University of Ghent, Krijgslaan 281 (S4), B-9000 Ghent (Belgium)

(First received June 23rd, 1992; revised manuscript received October 19th, 1992)

ABSTRACT

The gas chromatographic determination of 1-O-octadecyl-2-O-methyl-DL-glycero-3-phosphorylcholine (Et-18-OMe), an anti-invasive alkyl lysophospholipid, in cell culture media is described. Sample clean-up was performed by solid-phase extraction on a weak cation-exchange column of the CBA type (carboxylic acid). For quantitation, the structural analogue Et-16-OMe as the internal standard was used after derivatization with trimethylsilyl bromide. The described method was free of interferences in cell culture media. The overall precision for twenty determinations was 14.99%.

INTRODUCTION

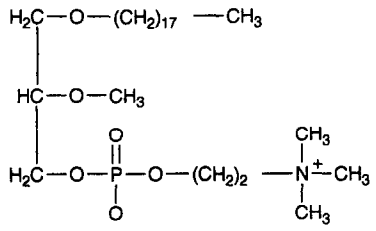
Alkyl lysophospholipids (ALPs) are synthetic analogues of the naturally occurring 2-lysophosphatidylcholine, which are selectively cytotoxic for tumour cells [1]. One of the most promising molecules of this group is 1-O-octadecyl-2-O-methyl-DL-glycero-3-phosphorylcholine (Et-18-OMe, Fig. 1).

In order to gain more insight into the mechanism of action of ALPs and their metabolism in cell cultures, precise analytical methods are necessary. The analysis of phospholipids in cow's milk, using high-temperature injection (350°C) gas chromatography (GC), has been described [2]. The disadvantage of this method is that pyrolysis of the phospholipids occurs. The class of the glycerophospholipids was analysed in erythrocytes and human plasma, after liquid extraction by GC of their silyl ethers after high-performance liquid chromatographic (HPLC) separation [3,4]. This methodology allowed a thorough classification of the molecular species of glycerophospholipids in erythrocytes and human plasma. However, for our purposes that method was too elaborate, so simpler analytical methods were investigated. High-performance thin-layer chromatography was evaluated for the quantitation of ether phospholipids, but had several drawbacks such as lack of selectivity on silica gel surfaces and detection problems on reversed-phase plates [5]. HPLC combined with light scattering detection was investigated for Et-18-OMe

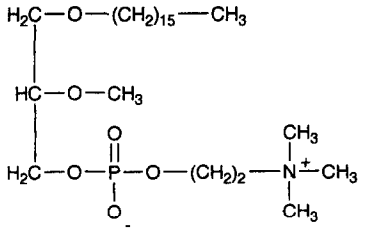
Correspondence to: E. Van den Eeckhout, Laboratory for Drug Quality Control and Medicinal Chemistry, University of Ghent, Harelbekestraat 72, B-9000 Ghent, Belgium.

0378-4347/93/$06.00

ET-18-OMe



ET-16-OMe



RADIOACTIVE ALP

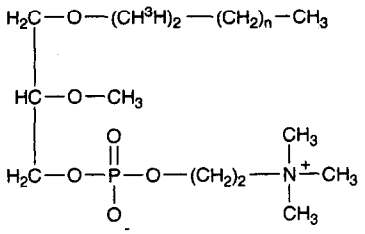


Fig. 1. Structures of Et-18-OMe, Et-16-OMe and radioactive ALP.

and its homologue Et-16-OMe, which was used as the internal standard (Fig. 1). The retention and elution characteristics of both compounds were studied on silica, poly(ethylene glycol)-coated silica, reversed-phase materials and polymeric resins. The problems of silanophilic interactions and lack of sensitivity were discussed [6].

Because of the disadvantages of the aforementioned methods, the possibilities of capillary GC, with its intrinsic high resolving power and good detection characteristics, were investigated. Derivatization of the ALPs with trimethylsilyl bromide allows quantitative analysis of ALPs by capillary GC [7].

The goal of this work is the determination of ALPs in cell culture media, so sample preparation is necessary. Owing to their tensioactive nature, liquid–liquid extraction seems not to be a good approach for these compounds, so solid-phase extraction was evaluated. Retention characteristics on normal-phase, reversed-phase and ion-exchange columns were investigated. The retention behaviour of a radioactive ALP was thoroughly studied on ion-exchange material. The developed methodology was applied to the determination of Et-18-OMe in cell culture media.

EXPERIMENTAL

Chemicals

Et-18-OMe (clinical grade) was obtained from Medmark Pharma (Munich, Germany). Et-16-OMe and Et-18-OMe (analytical grade) were supplied by Sigma (St. Louis, MO, USA). Trimethylsilyl bromide was obtained from Sigma. Bondelut columns containing a weak cation-exchange sorbent of the CBA type (carboxylic acid) were obtained from Analytichem International (Harbor City, CA, USA). DL-α-Phosphatidylcholine-β-O-methyl-γ-O-[^{3}H]alkyl was obtained from Amersham Belgium (Ghent, Belgium): the alkyl group was composed mainly of hexadecyl and octadecyl chains. RIA Luma was purchased from Lumac LSC (Olen, Belgium).

Instrumentation

Samples derivatized with trimethylsilyl bromide [7] were injected into a Hewlett-Packard Series II gas chromatograph, connected via an HP A/D convertor interface 35900 to an HP Chemstation. Cold on-column injections of 1 μl were done manually in the oven-track mode. The column was a polydimethylsiloxane phase HP-1 column (25 m × 0.32 mm I.D.; film thickness 0.17 μm). The oven temperature was held at 50°C for 1 min following injection, and programmed at 20°C/min to 200°C and then at 10°C/min to 300°C. Helium was used as carrier gas (3.0 ml/min) and nitrogen as make-up gas for flame ionization detection at 250°C.

A 1211 Rackbeta liquid scintillation counter, from LKB Wallac (Turku, Finland), was used.

Derivatization

Standards of ALPs in a concentration range of 5–60 μg/ml in chloroform were prepared. The internal standard, Et-16-OMe, was prepared at a concentration of 50 μg/ml in chloroform. In round-bottomed flasks of 25 ml, equipped with a condenser and a calcium chloride drying tube, 1.0 ml of standard and 1.0 ml of internal standard were added, and the solvent was evaporated under a gentle stream of nitrogen.

Derivatization was carried out with 50 μl of trimethylsilyl bromide, after dissolving the residues in 2 ml of dry acetonitrile. The mixtures were magnetically stirred and heated under reflux for 2 h, protected from light. The reagent and acetonitrile were evaporated under nitrogen after reaction, and the residue was redissolved in 1.0 ml of hexane. If necessary, the mixture was centrifuged before injection.

Solid-phase extraction

Standards. A CBA-type Bondelut column containing 500 mg of sorbent was conditioned with 2 ml of methanol, 2 ml of water and 2 ml of 0.1 *M* phosphate buffer (pH 5.50). Each ALP, *i.e.* Et-18-OMe and Et-16-OMe (50 μg), was dissolved in 6 ml of 0.1 *M* phosphate buffer (pH 5.50). This buffered sample solution was applied to the column. The column was washed consecutively with 2 ml of 0.01 *M* KCl–0.01 *M* HCl in water (pH 2) and 2 ml of 30% (v/v) methanol in water. Finally the compounds were eluted with 2 ml of methanol. The methanol was evaporated under reduced pressure, making the sample ready for derivatization with trimethylsilyl bromide as described above.

Cell culture media. Et-18-OMe and Et-16-OMe (50 μg), dissolved in 5 ml of 0.1 *M* phosphate buffer (pH 5.2), were added to 1 ml of cell culture media. This solution was applied to the CBA column, which was treated as described under solid-phase extraction for standards. After derivatization the samples were subjected to capillary GC.

RESULTS AND DISCUSSION

In order to obtain information on the elution characteristics of ALPs on solid-phase columns the radioactive tracer, DL-α-phosphatidylcholine-β-O-methyl-γ-O-[^{3}H]alkyl (Fig. 1) was used.

Experiments on normal-phase silica gel columns showed that the recovery of ALPs on this kind of material is good, but that no really selective clean-up of the sample components is achieved [8]. A second limitation of normal-phase silica gel columns is samples must be dissolved in organic solvents, which means that the aqueous cell cultures have to be lyophilized and redissolved in an organic solvent. This is a time-consuming process.

Reversed-phase material, on the other hand, was not suitable for the clean-up of cell culture samples containing ALPs. In spite of the fact that ALPs contain hydrophobic chains, the retention characteristics in reversed-phase material were bad, probably owing to the formation of micelles, which are not disrupted by the addition of an organic solvent. The ALPs were not sufficiently retained on the column, so that no real clean-up took place. Finally, the use of ionic columns was considered. Three cationic columns are commercially available, namely CBA, propylsulphonic acid (PRS) and benzenesulphonic acid (SCX) columns. PRS and SCX columns are strong cation-exchange columns. CBA columns consist of a silica matrix derivatized with a carboxylic acid group. As ether phospholipids contain a quaternary nitrogen, the CBA columns were preferred since the ALPs will be easily retained and eluted from this column.

Retention characteristics of radioactive ALP on CBA columns

The radioactive tracer DL-α-phosphatidylcholine-β-O-methyl-γ-O-[^{3}H]alkyl was diluted in methanol to 50 000 cpm (stock solution). In order to check the elution characteristics of this ALP at different pH values, buffers in the pH range 4.80–8.50 were prepared.

A CBA column was treated consecutively with 2 ml of methanol, 2 ml of distilled water and 2 ml of each buffer solution. From the radioactive stock solution, 50 μl were diluted with 1 ml of buffer in a small test-tube. The contents of the

test-tube were applied to the column, collected in a liquid scintillation vial and counted. The column was then eluted with 1 ml of buffer of each pH value. Each buffer fraction was counted. Fig. 2 shows the influence of the buffer on the retention. The retention of ALP is optimal between pH 4.80 and pH 5.60.

Elution characteristics of radioactive ALP in cell culture media on CBA columns

When ALPs are dissolved in a cell culture medium two factors have to be considered: ionic strength and pH value. A cell culture medium consists mainly of amino acids, vitamins, inorganic salts, antibiotics and a plasma fraction. This medium has a high intrinsic ionic strength and a pH value of 7.68. To 1 ml of culture, 5 ml of 0.1 *M* phosphate buffer (pH 5.20) were added, to adjust the pH to 5.60. The mixture of ALP in buffered cell culture medium was applied to the column, which had been previously washed as described above. Elution of ALP was tried with the following solvent systems: 18 *M* HCOOH in methanol; 18 *M* HCOOH in acetonitrile (0.01 *M*); KCl–0.01 *M* HCl in water (pH 2); 2.5 *M* K_2HPO_4 (pH 12); and methanol. The results are summarized in Table I.

From this experiment it can be concluded that elution solvents with organic solvents at acidic pH give satisfactory results, in contrast to the aqueous acidic solvent systems, which are unable to elute ALP.

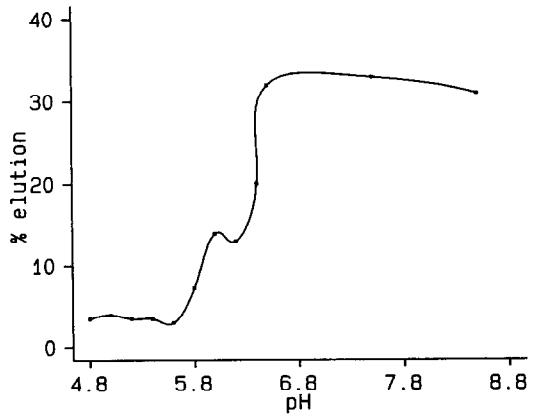


Fig. 2. Influence of pH on the retention of DL-α-phophatidylcholine-β-O-methyl-γ-O-[^{3}H]alkyl on a CBA column.

TABLE I

ELUTION OF ALP IN CELL CULTURE MEDIA ON CBA COLUMNS (n = 5) WITH DIFFERENT ELUENTS

Eluent	Percentage elution
18 *M* HCOOH–methanol[a]	90
18 *M* HCOOH–acetonitrile[b]	95
KCl–HCl in water (pH 2)[c]	0.63
KCl–HCl in methanol[d]	96
2.5 *M* K_2HPO_4 (pH 12)[e]	1.4
Methanol	83

[a] 678 ml of HCOOH and 322 ml of methanol.
[b] 678 ml of HCOOH and 322 ml of acetonitrile.
[c] 0.01 *M* KCl–0.01 *M* HCl (1:1) in water.
[d] 0.01 *M* KCl–0.01 *M* HCl (1:1) in methanol.
[e] 435 g of K_2HPO_4, adjusted with 2.5 *M* KOH in water to 1 l.

Clean-up of cell culture media samples containing radioactive ALP

It was our purpose to elute cell culture material from the column, before eluting radioactive ALP. Theoretically we could use the "dual-retention" mechanism. This means eliminating the hydrophobic interactions and washing out these impurities, while retaining ALP on the column by a cation-exchange effect. Afterwards this effect is neutralized, ALP is retained through hydrophobic interactions and the other charged impurities are eliminated.

We tested this approach as follows. The radioactive tracer was diluted in 0.1 *M* phosphate buffer (pH 5.20) and added to the cell culture as described above. This sample was applied to a 500-mg CBA column, which was then washed consecutively with 2 ml of water, 2 ml of 0.01 *M* KCl–0.01 *M* HCl in water (pH 2), 2 ml of 2.5 *M* K_2HPO_4 (pH 12), and 30% (v/v) methanol. ALP was finally eluted with methanol. For each washing with aqueous solvent and with 30% methanol the percentage loss of ALP was calculated. This amounted to a total of 17%, so that 83% of Et-18-OMe was eluted from the column with methanol.

Polar impurities are eluted with 30% methanol, but a higher percentage of methanol eliminates the hydrophobicity interaction between the column and ALP and gives rise to elution.

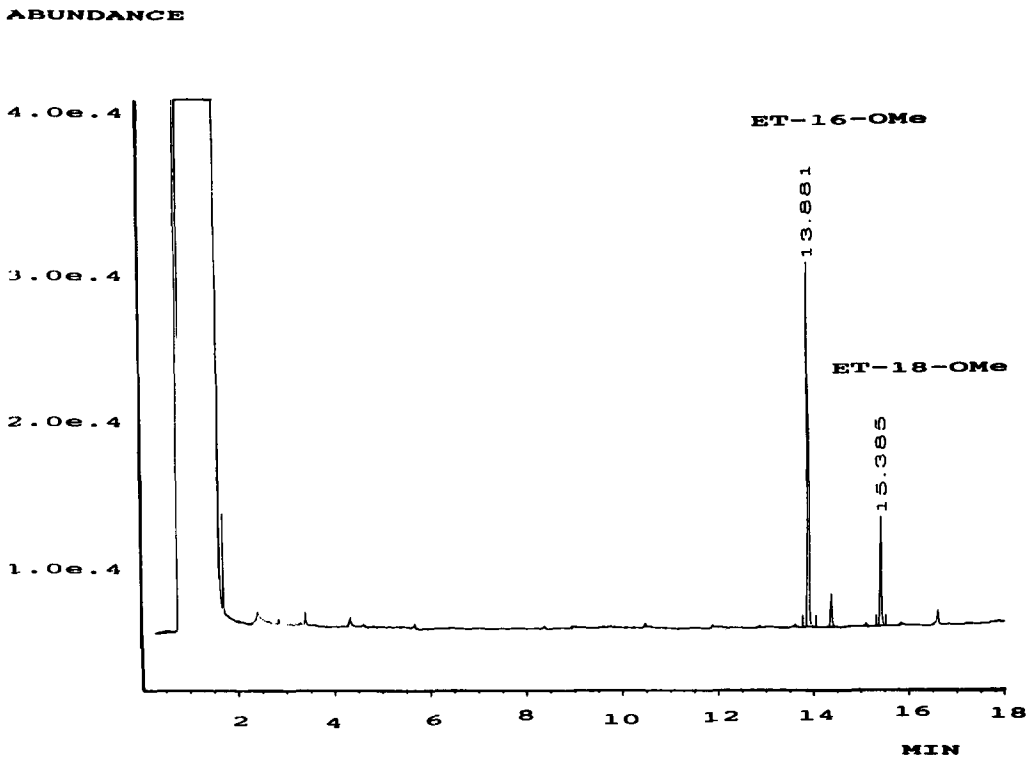


Fig. 3. Chromatogram of Et-18-OMe (5 μg/ml) and Et-16-OMe (50 μg/ml) in cell culture medium after derivatization with trimethylsilyl bromide.

Having established the retention and elution behavior of radioactive ALP, we applied the procedure to the compounds of interest, namely Et-18-OMe and Et-16-OMe.

Quantitation of the solid-phase extraction of Et-18-OMe from cell culture media

The recovery of the solid-phase extraction, using labeled ALP, was 81.0 ± 1.4% (n = 5). The loss during extraction was compensated by the use of the structurally related internal standard Et-16-OMe.

Et-18-OMe and Et-16-OMe, 5 and 50 μg, respectively, were dissolved in 5 ml of 0.1 M phosphate buffer (pH 5.5) and added to 1.0 ml of cell culture medium and subjected to solid-phase extraction and capillary GC as described above. The overall reproducibility for twenty determinations, including solid-phase extraction, was 14.99%.

The linearity of the derivatization reaction was checked for Et-18-OMe using Et-16-OMe as the internal standard in the concentration range 5–50 μg/ml. A linear correlation between the concentration and the area of the GC peaks was found. The reproducibility was 4.65% for ten determinations.

A typical chromatogram is shown in Fig. 3. Et-18-OMe was present in a concentration of 5 μg/ml of cell culture medium, to which 50 μg/ml Et-16-OMe was added. No interfering peaks were observed.

CONCLUSION

The retention, washing and elution steps on CBA columns were quantitatively evaluated for cell culture media samples spiked with Et-18-OMe. Et-18-OMe is sufficiently separated from the cell culture compounds, and the capillary GC chromatograms are free from interfering peaks. The method can be used for the determination of

Et-18-OMe in cell culture media, and will be applied to investigate the mechanism of action of Et-18-OMe in the cell invasion process. This investigation is now in progress, and the results will be published elsewhere.

REFERENCES

1 W. E. Berdel, R. Andreesen and P. G. Munder, in J. F. Kuo (Editor), *Phospholipids and Cellular Regulation*, Vol. 2, CRC Press, Boca Raton, FL, 1985, pp. 41–73.
2 M. F. Caboni, G. Lereker and A. M. Ghe, *J. Chromatogr.*, 315 (1984) 223.
3 J. J. Myher, A. Kuksis and S. Pind, *Lipids*, 24 (1989) 396.
4 J. J. Myher, A. Kuksis and S. Pind, *Lipids*, 24 (1989) 408.
5 J. Coene, M. E. Ramos, E. Van den Eeckhout, P. Sandra and W. Van den Bossche, *Chromatographia*, 30 (1990) 414.
6 J. Coene, M. Ghys, E. Van den Eeckhout, W. Van den Bossche and P. Sandra, *J. Chromatogr.*, 553 (1990) 285.
7 J. Coene, P. Herdewijn, E. Van den Eeckhout, W. Van den Bossche and P. Sandra, *J. High Resolut. Chromatogr.*, 14 (1991) 699.
8 J. Coene, M. E. Ramos, E. Van den Eeckhout, W. Van den Bossche and P. Sandra, *Pharm. Weekbl.*, 13 (1991) 1.