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Separation and quantification of alkylphosphocholines by reversed phase high performance liquid chromatography

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Abstract

Alkylphosphocholines represent a new class of drugs with remarkable antineoplastic and antiprotozoal activity. For instance, hexadecylphosphocholine has been approved for the topical treatment of skin metastasis. In addition, it was successfully studied in India for the treatment of leishmaniasis. Different phase-I and phase-II-trials resulted in cure rates of more than 97%. To optimize antitumor or antiprotozoal activity, we have prepared alkylphosphocholines differing in chain length and unsaturation. For the qualitative and quantitative analysis of these longer chain analogues, we have used isocratic high performance liquid chromatography. The separation of the alkylphosphocholines with different chain lengths in this reversed phase HPLC system was achieved on a YMC-TMS column with a mobile phase consisting of methanol–water (85:15; v/v) at a flow rate of 1.0 ml/min. Furthermore the *cis*-/*trans*-isomers such as oleylphosphocholine and elaidylphosphocholine were clearly separated on a YMC-C8 column with a methanol–water mixture (80:20; v/v) as mobile phase. In the described reversed phase HPLC systems simple refractive index detection and UV detection allow the sensitive and quantitative determination of alkylphosphocholines. These methods are very important for reproducible identification and quantitative determination of saturated and mono-unsaturated alkylphosphocholines with alkyl residues containing up to 25 carbon atoms. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

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1. Introduction

Alkylphosphocholines have been identified as a new and exciting class of phospholipid-like analogues with remarkable biological and therapeutic activities (Eibl and Unger, 1989, 1990). The selective action against tumors (Muschiol et al., 1987; Hilgard et al., 1988; Unger et al., 1989; Vehmeyer et al., 1989; Fleer et al., 1990) and protozoan diseases (Kuhlencord et al., 1992; Kaufmann-Kolle et al., 1996) is the basis for extended studies in this area of membrane directed drug research since side effects at therapeutic effective concentrations are marginal.

Hexadecylphosphocholine as prototype is the first phospholipid like molecule that was approved as drug. It is used for the therapy of skin

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metastases of mammary carcinomas (Scherf et al., 1987; Unger et al., 1990) and cutaneous lymphomas (Dummer et al., 1992). Chemically, the alkylphosphocholines have a simple structure. They are phosphocholine esters of aliphatic long chain alcohols differing in chain length, unsaturation and position of the *cis*-double bond (Eibl and Engel, 1992; Hottkowitz, 1997).

The increasing number of laboratory experiments, which include clinical studies with saturated and unsaturated alkylphosphocholines and the search for a more quantitative understanding of the distribution and action of these pharmacologically active compounds in different tissues require fast and sensitive analytical methods for the detection and the determination of tissue concentrations. We have developed HPLC systems for the separation and quantitative determination of alkylphosphocholines differing in chain length (14–25 carbon atoms) and unsaturation (mainly *cis*-double bonds). The described methods allow the separation of alkylphosphocholines, which differ by only one methylene group in the alkyl chain. Furthermore it is possible to separate and to determine alkylphosphocholines containing *cis*- or *trans*-double bonds. These separations are not possible with high-performance thin layer chromatography (HPTLC) described in earlier publications (Rustenbeck and Lenzen, 1990; Kötting et al., 1992; Reitz et al., 1992).

2. Materials and methods

Methanol (gradient grade) and water (LiChrosolv) were purchased from Merck (Darmstadt, Germany). The alkylphosphocholines have been synthesized in our laboratory (Eibl and Engel, 1992; Hottkowitz, 1997). The purity of the lipids was checked by the methods described here. Fig. 1 shows the chemical structure of the investigated alkylphosphocholines.

2.1. High-performance liquid chromatography

The isocratic Gynkotek HPLC system consisted of a gradient pump Gynkotek M 480, an automatic injection system Gynkotek GINA 50, a solvent degasser DG-503, a column thermostat STH 585, an UV/VIS-detector 340 S and a refractive index (RI) detector Shodex RI-71. Alkylphosphocholines with different chain length were separated using a YMC-TMS column (250 × 4.6 mm; 5 μ) purchased from YMC Europe GmbH, Schermbeck (Germany) as the stationary phase. Samples were dissolved in methanol and elution was performed at 35°C with the solvent system methanol–water 85:15 (v/v) as mobile phase. The flow rate was 1 ml/min.

Oleylphosphocholine and elaidylphosphocholine (OlPC and ElPC) were separated on a YMC-C8 column (250 × 4.6 mm; 5 μ) with a mixture of methanol–water 80:20 (v/v) at a flow rate of 1 ml/min. The same conditions were applied for the separation of ErPC and (Z)-10-docosenyl-1-phosphocholine ($C_{22:1}$-PC). The temperature of the column thermostat was 35°C.

High-sensitivity refractive index detection (saturated and unsaturated alkylphosphocholines) or UV detection at $\lambda = 206$ nm (unsaturated alkylphosphocholines) were used for quantitation based on calibration standards, whereas the detectors were set up in series with the column effluent first passing through the UV-detector. Linear regression constants of the calibration curves were calculated by SQS-Software (Perkin-Elmer, version 2.01). Each point of all of the calibration curves was the mean of the two measurements, each with a variation coefficient of less than 1%.

3. Results and discussion

We have already described a HPLC method for the separation and quantitative determination of different even-numbered saturated and mono-unsaturated alkylphosphocholines (Thaler et al., 1998). Since small structural alterations, for instance *cis*/*trans* in OlPC versus ElPC, may have a strong impact on the side effects of these drugs by in vivo applications, we describe new and sensitive methods for the separation of alkylphosphocholines differing only in the *cis*/*trans* configuration. The methods include the analysis of extremely long chain alkylphosphocholines, which can be separated according to the number of methylene

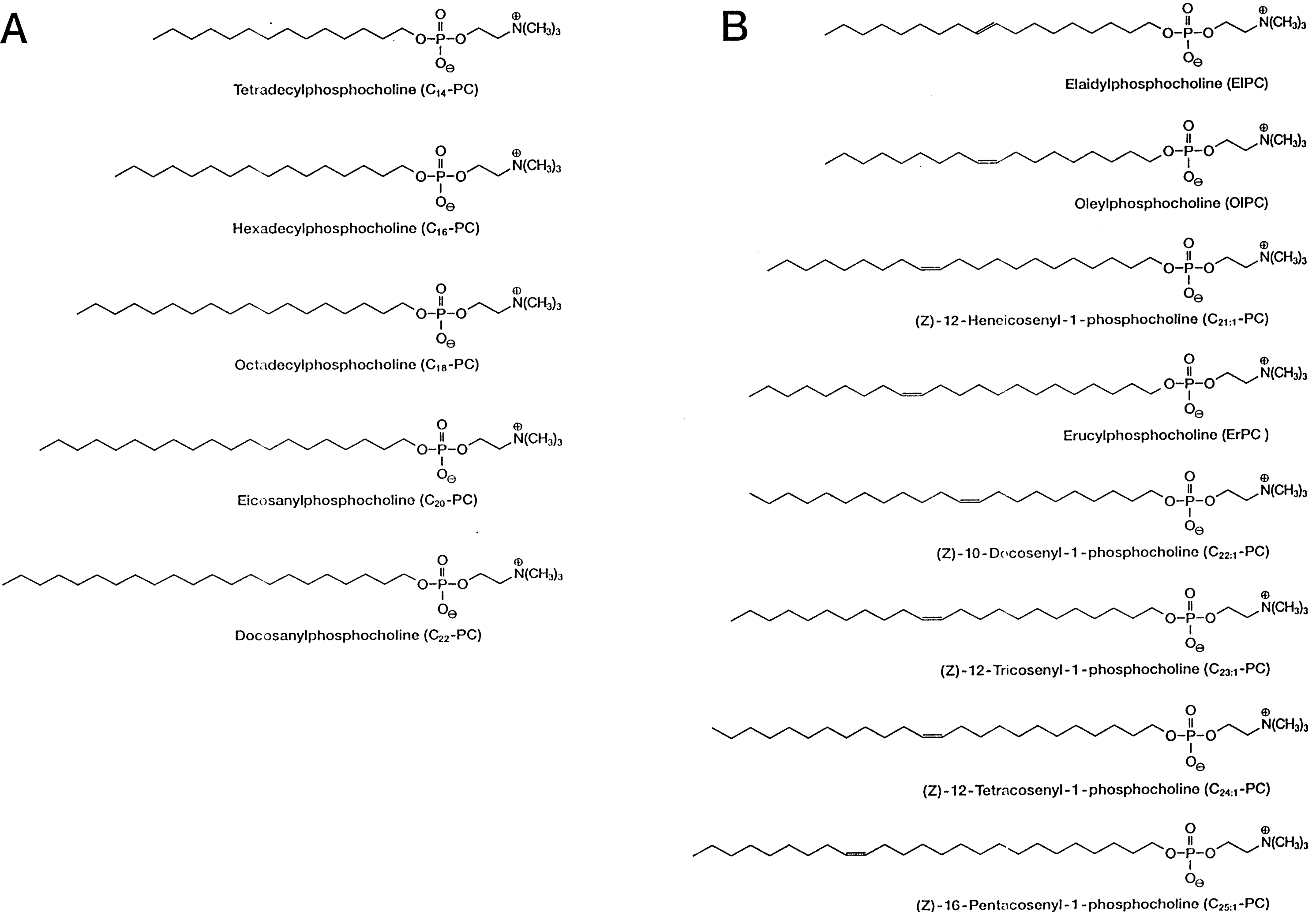

Fig. 1. Chemical structures of five saturated alkylphosphocholines (A) and eigth mono-unsaturated alkylphosphocholines (B)

groups in their alkyl chain, the configuration of the double bonds of the unsaturated species and the position of the double bond in their alkyl residue.

3.1. Separation of saturated alkylphosphocholines

A rapid and sensitive HPLC method with high-sensitive RI-detection in the case of saturated alkylphosphocholines is described for the separation and quantitative determination. Since RI-detection is very sensitive to changes of the mobile phase composition, isocratic conditions with a constant flow of the mobile phase are required. Purities of $>98\%$ were determined by this method in the case of saturated alkylphosphocholines.

Fig. 2 shows the simultaneous separation of mixtures containing five saturated alkylphosphocholines from tetradecylphosphocholine to docosanylphosphocholine (chemical structures see Fig. 1a). This separation was achieved on a reversed-phase system consisting of a YMC-TMS column with the solvent system methanol–water 85:15 (v/v). The structural analogs are separated clearly from each other with retention times of 5.3, 6.0, 6.9, 8.1, and 9.8 min for C_{14}-PC, C_{16}-PC, C_{18}-PC, C_{20}-PC and C_{22}-PC, respectively. The saturated alkylphosphocholines elute in the order of increasing number of methylene groups. An increase in chain length prolongs the retention time as a result of the lower polarity.

The calibration curves for each of the five saturated alkylphosphocholines show a linear increase of area unit versus amount of alkylphosphocholine. The linearity is well maintained between 5 and 200 nmol. The linear regression coefficients (r) for each of the phospholipid calibration curves was better than 0.9999 and the slopes of the five calibration curves depend on the molecular mass

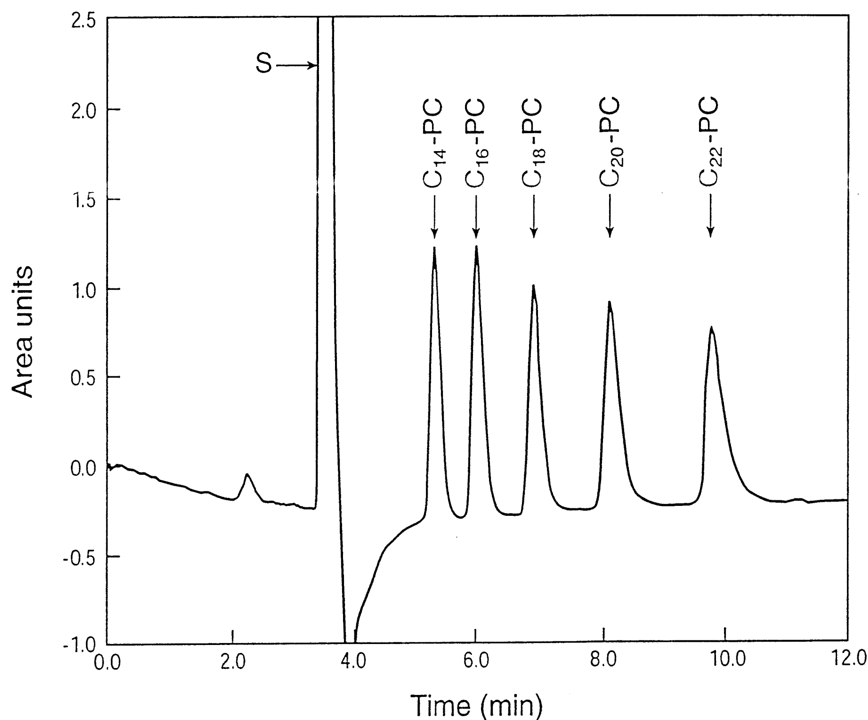

Fig. 2. Separation of saturated alkylphosphocholines (C_{14}-PC, retention time 5.3 min; C_{16}-PC, retention time 6.0 min; C_{18}-PC, retention time 6.9 min; C_{20}-PC, retention time 8.1 min; C_{22}-PC, retention time 9.8 min). A total amount of 6 nmol per saturated alkylphosphocholine was injected (S, solvent front). Detection: refractive index. Chromatographic conditions: YMC-TMS column; methanol–water 85:15 (v/v); 1.0 ml/min.

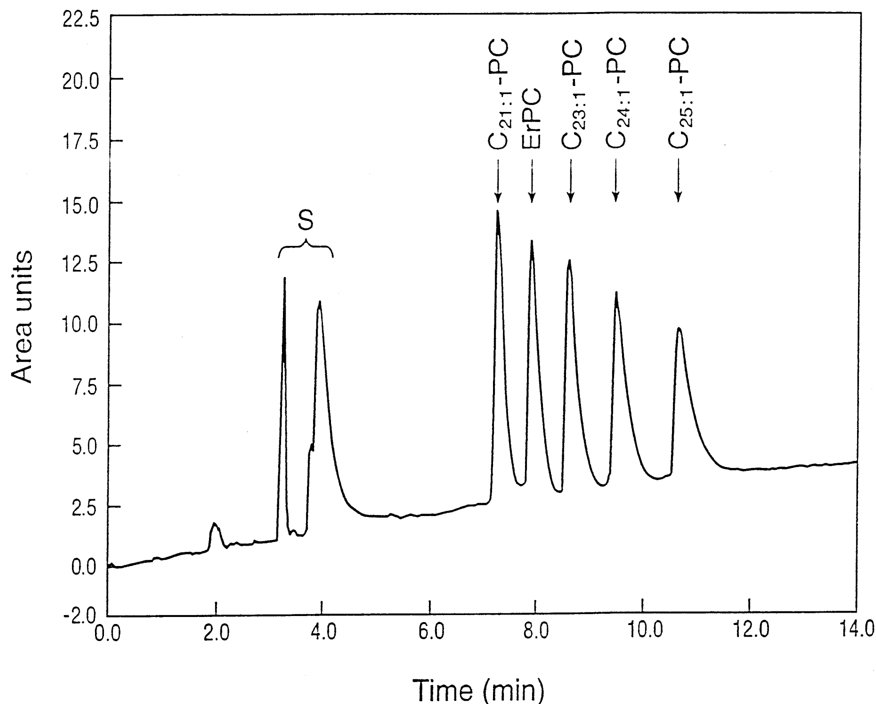

Fig. 3. Separation of unsaturated alkylphosphocholines ($C_{21:1}$-PC, retention time 7.2 min; ErPC, retention time 7.9 min; $C_{23:1}$-PC, retention time 8.6 min; $C_{24:1}$-PC, retention time 9.5 min; $C_{25:1}$-PC, retention time 10.6 min). A total amount of 4 nmol per unsaturated alkylphosphocholine was injected (S, solvent front). Detection: UV ($\lambda = 206$ nm). Chromatographic conditions: YMC-TMS column; methanol–water 85:15 (v/v); 1.0 ml/min.

of the saturated alkylphosphocholines. They vary between C_{14}-PC to C_{22}-PC from 5.38 to 6.75 units, respectively.

3.2. Separation of mono-unsaturated alkylphosphocholines

Mono-unsaturated alkylphosphocholines ($C_{21:1}$-PC, ErPC, $C_{23:1}$-PC, $C_{24:1}$-PC and $C_{25:1}$-PC; see also Fig. 1b) offer the possibility of UV-detection additionally to RI-detection for their qualitative and quantitative determination. The molar response of the short wavelength UV-detector is highly dependent on the nature of the fatty alcohol residue and varies with the degree of unsaturation. Unsaturated alkylphosphocholines yield a good response at $\lambda = 206$ nm. Fig. 3 illustrates the chromatographic separation for mixtures of all five mono-unsaturated alkylphosphocholines from $C_{21:1}$-PC to $C_{25:1}$-PC using a YMC-TMS column and isocratic elution with methanol–water 85:15 (v/v). The detection was also carried out with a RI-detector. The separation system showed a very high selectivity (Fig. 3). It is emphasized that neighbouring peaks differ only by one methylene group (-CH_2-) in chain length of the alkyl residue and are still quantitatively separated. In our reversed-phase system the methylene groups have exactly the same effect as in the case of the saturated alkylphosphocholines, i.e. they prolong the retention time as a result of higher apolarity. The plots of RI-detector responses against the amounts of the mono-unsaturated alkylphosphocholines were linear in the range between 5 and 160 nmol (Figure not shown) and the linear regression coefficients (r) were higher than 0.9999.

Compared with the data of the saturated alkylphosphocholines the RI-detector responses were also affected by the chain length of the alkyl residue with values of 6.60–7.59 for $C_{21:1}$-PC to $C_{25:1}$-PC, respectively.

The UV-detector-response at $\lambda = 206$ nm of the five mono-unsaturated compounds was also investigated. The calibration curves for each of the unsaturated alkylphosphocholines were determined with amounts of components (x-values) ranging from 5 to 160 nmol and second-order polynomial curves were obtained according to the equation $y = a_2x^2 + a_1x + a_0$ (y-values: area units), whereas linearity was observed between 5 and 80 nmol unsaturated alkylphosphocholine and the linear regression coefficients (r) for each unsaturated alkylphosphocholine calibration curve were higher than 0.9999. The results showed that the quadratic regression constants (a_2, a_1, a_0) are not significantly influenced by the length of alkyl chain like in case of RI-detection.

For the RI- and UV-detectors the limit of detection is defined as the concentration that resulted in a signal-to-noise ratio of two. Detection limits obtained were 380 pmol by RI-detection and 20 pmol by UV-detection at $\lambda = 206$ nm for ErPC.

A comparison of short wavelength UV-detection and RI-detection shows, that UV-detection is nearly twenty times more sensitive than RI-detection for unsaturated alkylphosphocholines. Beside sensitivity, an obvious disadvantage of RI-detection is, that gradient elution is almost impossible due to strong drifts in the base line during elution. Therefore an isocratic HPLC method is essential for the analysis of saturated alkylphosphocholines. An advantage of RI-detection over UV-detection is the qualitative and quantitative determination of both saturated and unsaturated compounds with a linearity range between 5 and 160 nmol, whereas for UV-detection the linear range was only 5–80 nmol.

3.3. Separation of cis-/trans-isomers of mono-unsaturated alkylphosphocholines

As discussed in the earlier publication (Thaler et al., 1998) the HPLC method described of either saturated or mono-unsaturated alkylphosphocholines with different chain lengths cannot separate *cis*-/*trans*-isomers of mono-unsaturated alkylphosphocholines. This fact is extremely important in the case of synthetic compounds with introduction of the *cis*-double bond via the Wittig reaction. This synthetic procedure can result in the formation of *trans*-isomers, which may amount to about 3–6% in comparison to the *cis*-isomer.

We describe a HPLC method for separation of the isomers OlPC and ElPC by using an YMC-C8 column as stationary phase and a methanol–water mixture (80:20; v/v) as mobile phase, whereas the purity of the unsaturated alkylphosphocholines checked by this method is higher than 97%. Fig. 4a shows the quantitative separation of the two isomers in the presence of the saturated analog C_{18}-PC. Of the three compounds studied, OlPC eluted first (23.4 min), followed by ElPC (24.9 min) and C_{18}-PC (34.1 min). The elution order of the three compounds was dependent on the polarity of the fatty alcohol residue.

A careful analysis of the *trans*-content in alkylphosphocholines with *cis*-double bonds is very important. Side effects due to *trans*-impurities are remarkable and can negatively effect the treatment with OlPC (Sobottka et al., 1993). The preparation of *cis*-alkenols by Wittig synthesis leads to *trans*-isomers in amounts up to 3–6% (Hottkowitz, 1997). A representative chromatogram is shown in Fig. 4b, where $C_{21:1}$-PC is separated clearly from its *trans*-isomer. In this case the percentage in amount of the *trans*-isomer is about 1%. Furthermore it is possible to separate by this HPLC-method the two compounds ErPC and $C_{22:1}$-PC with retention times of 66.0 and 69.2 min. Both mono-unsaturated alkylphosphocholines differ only in the position of the *cis*-double bond in the alkyl chain (Fig. 5). The *cis*-double bond in $C_{22:1}$-PC (Z-10) is shifted by three methylene groups in comparison to ErPC (Z-13).

4. Conclusions

In summary, sensitive reversed-phase HPLC systems have been developed for the separation and quantitative analysis of different saturated and mono-unsaturated alkylphosphocholines. All investigated alkylphosphocholines elute in the order of increasing number of carbon atoms in their alkyl chains. The methods can also distinguish

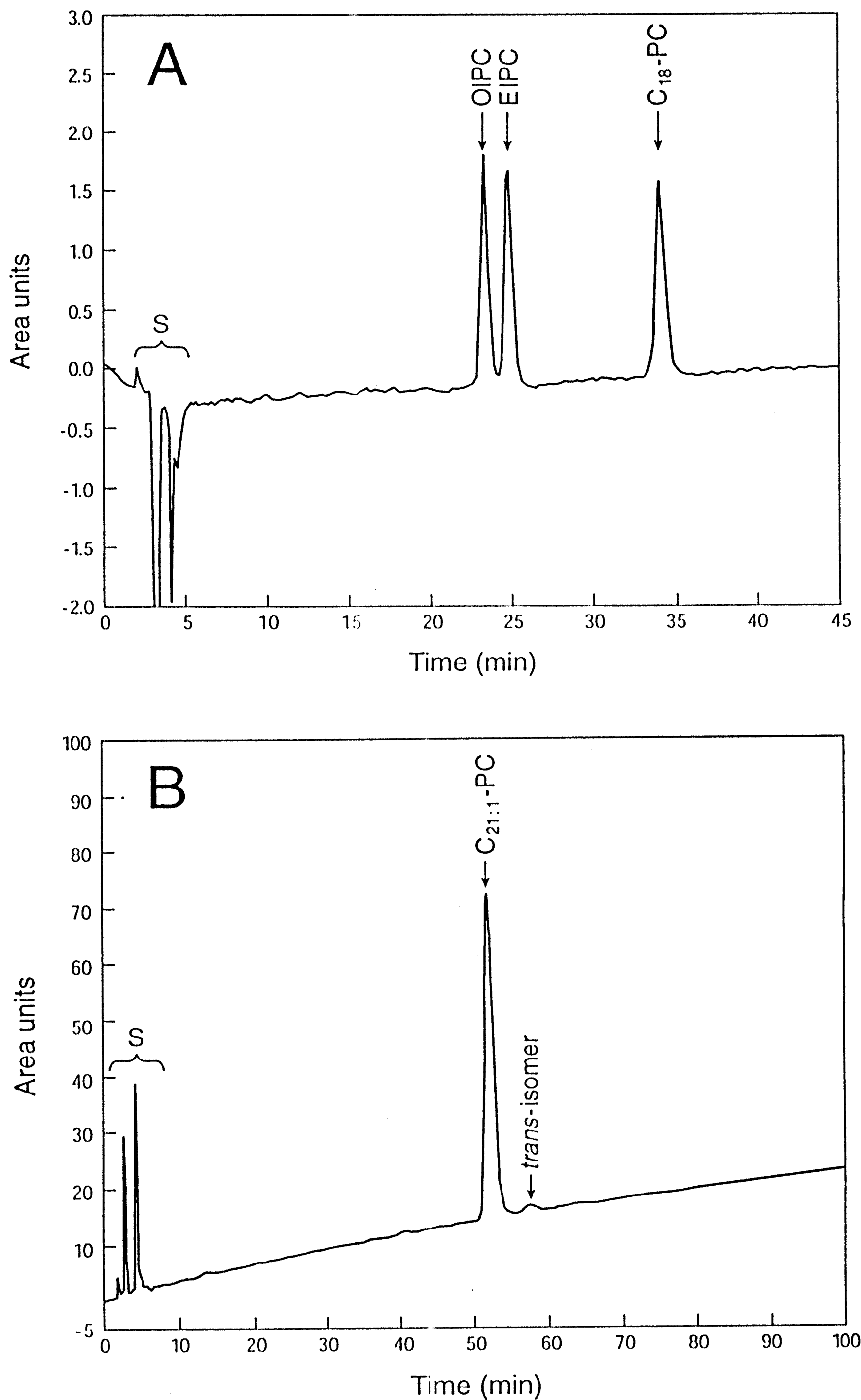

Fig. 4. (A) Separation of OlPC (retention time: 23.4 min) from ElPC (retention time: 24.9 min) and C_{18}-PC (retention time: 34.1 min). A total amount of 18 nmol per alkylphosphocholine was injected (detection: refractive index). (B) Separation of $C_{21:1}$-PC (retention time: 51.6 min) from its *trans*-isomer (retention time: 57.5 min).A total amount of 125 nmol unsaturated alkylphosphocholine was injected (detection: UV; $\lambda = 206$ nm). Chromatographic conditions for both separations (A and B): YMC-C8 column; methanol–water 80:20 (v/v); 1.0 ml/min (S, solvent front).

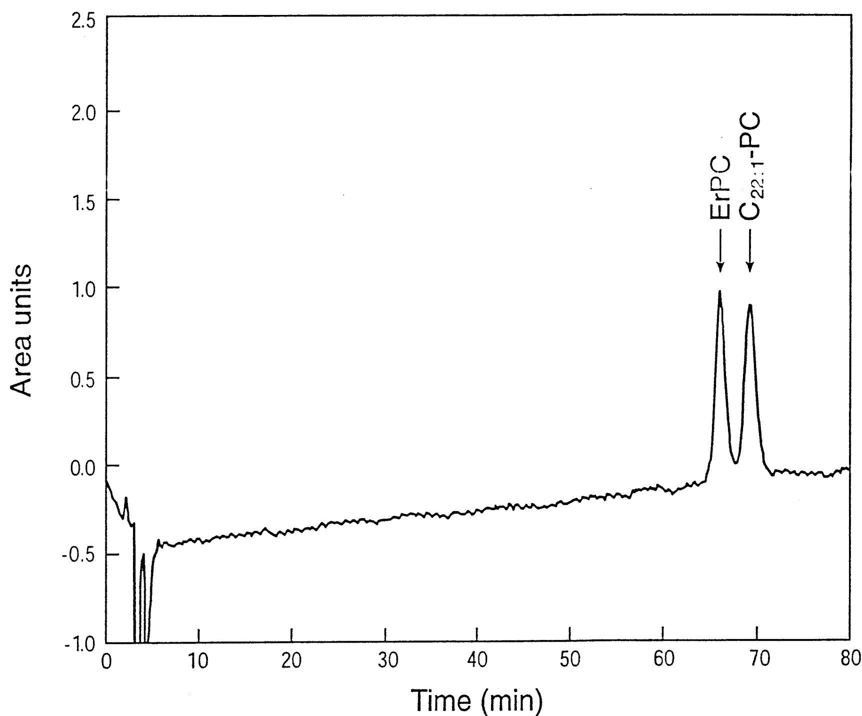

Fig. 5. Separation of ErPC (retention time: 66.0 min) from $C_{22:1}$-PC (retention time: 69.2 min) as described in Section 2. A total amount of 20 nmol per unsaturated alkylphosphocholine was injected. Detection: refractive index. Chromatographic conditions: YMC-C8 column; methanol–water 80:20 (v/v); 1.0 ml/min.

between *cis*-/*trans*-isomers of mono-unsaturated alkylphosphocholines and surprisingly, even the position of the *cis*-double bond in the alkyl chain.

UV-detection was about twenty times more sensitive than RI-detection with 20 versus 380 pmol, respectively. The linear range for detection was 5–200 nmol for RI-detection and 5–80 nmol for UV-detection.

Reversed phase HPLC with RI- and UV-detection is an excellent tool for the analysis of alkylphosphocholines with antineoplastic or antiprotozoal activity. Finally, this method is very useful to determine the purity of synthetic alkylphosphocholines containing *cis*-double bonds, which have been obtained by Wittig synthesis.

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