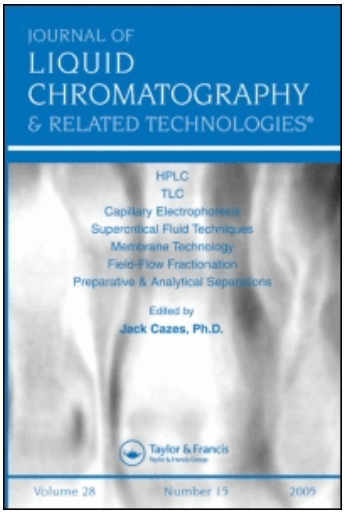

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PHOSPHATIDYLCHOLINE-GRAMICIDIN A INTERACTION STUDY IN NON-AQUEOUS SOLVENT WITH A NEW HPLC-SEC COLUMN

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ABSTRACT

A new HPLC-SEC column, Ultrastyragel 500 Å, has been assayed in order to check the separation between gramicidin A and phosphatidylcholine in tetrahydrofuran. The good resolution of both compounds has allowed the quantitation of lipid-polypeptide interaction in terms of the binding ratio parameter, BR. A BR value of 3.6 moles of phosphatidylcholine per mole of gramicidin A has been obtained for a 0.1 % (w/v) phosphatidylcholine solution as eluent. The suitability of this support for studies on lipid-polypeptide interactions in non-aqueous solvents is demonstrated.

INTRODUCTION

One of the major interests of investigators currently studying proteolipids concerns how these proteins interact with membrane lipids, since in many cases lipid-protein interactions are essential for protein functional activity (1). The solvation

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of a membrane hydrophobic protein by lipids may be compared to protein solvation in aqueous solution (2). Most of the present data on lipid solvation, in reconstituted and natural membranes, come from lipid activation studies (1), fluorescence-quenching spectroscopy (3,4), electron-spin resonance spectroscopy (5-7), nuclear magnetic resonance spectroscopic studies (5,8) and difference infrared spectroscopy (9).

Recently, a novel approach has been proposed to the study of lipid-lipid and lipid-polypeptide interactions in non-aqueous solvents by HPLC using μ-Styragel columns (10), based on column equilibration with binary lipid solutions according to the Hummel and Dreyer method (11). Gramicidin A (GA), a linear polypeptidic antibiotic, was selected for this purpose because it has been extensively used as a model of intrinsic membrane protein in reconstituted vesicles and liposomes (9,12,13). The preferential solvation parameters in ternary systems containing tetrahydrofuran (THF)/ phosphatidylcholine (PC)/ cholesterol (CH) were calculated and interpreted in terms of solute-solute interactions. The quantitation of GA preferential solvation by PC was not possible on this support due to partial peak overlapping, but it was inferred that PC solvates better GA than CH (10).

This paper deals with the use of a new SEC support, Ultrastyragel, for the analysis of PC-GA interactions in THF. The resolving power of Ultrastyragel 500 Å column has been checked. The resolution and reproducibility achieved, together with the linearity of calibration plots, have revealed that the application of this methodology to this support is extremely useful for the quantitative determination of binding parameters. Finally, the effect of polypeptide concentration has been also investigated.

EXPERIMENTAL

GA (mw ca. 1800) was obtained from Koch Light Lab. Egg yolk PC (mw ca. 770) was purchased from Merck, Darmstadt. Its purification was performed by preparative column chromatography,

according to Singleton et al. (14), and its purity controlled by thin layer chromatography. THF was a Merck spectroscopic reagent.

An M-45 solvent delivery system, a U6K universal injector and a differential refractometer, model R 401, from Waters Assoc., were used in all the experiments. Samples were also simultaneously monitored with a Varian Varichrom variable wavelength UV detector at 290 nm. The system was equipped with a 500 Å pore size Ultrastyragel column from Waters Assoc. The column was equilibrated at room temperature with a solution of PC in THF at a concentration of 0.1 % (w/v) (1.30×10^{-3} M), previously filtered through a 0.45 µm Micro Filtration Systems regenerated cellulose filter. The flow rate was 1.0 $mL.min^{-1}$.

Before injecting GA samples, several volumes of THF were injected corresponding to known defects of absolute amount of PC, $\Delta m°$, in mg. A calibration plot of $\Delta m°$ vs A° was obtained, A° being the area of defect peak appearing in the chromatogram. The subsequent injection of 50 µL GA samples at concentrations ranging from 1.0 to 10.0 $mg.mL^{-1}$, prepared by dissolving the polypeptide in the equilibrating solution, caused a PC defect "vacant" peak of area A (15). The Δm value corresponding to the area A was deduced from the calibration plot in a similar manner as described previously (10,15). Each measurement was repeated three times and the average values were calculated. The relative mean deviation was in all cases lower than 2 % for Δm values and lower than 1.5 % for elution volume measurements.

RESULTS AND DISCUSSION

Figure 1 shows the calibration curve of defect peak areas, A°, in arbitrary units, versus PC defect, $\Delta m°$, in mg, for a 1.30×10^{-3} M PC solution as eluent for Ultrastyragel 500 Å column.

The elution volumes, V_e, of defect peaks increase from 7.5 to 7.9 mL when increasing the amount of injected THF (PC defect

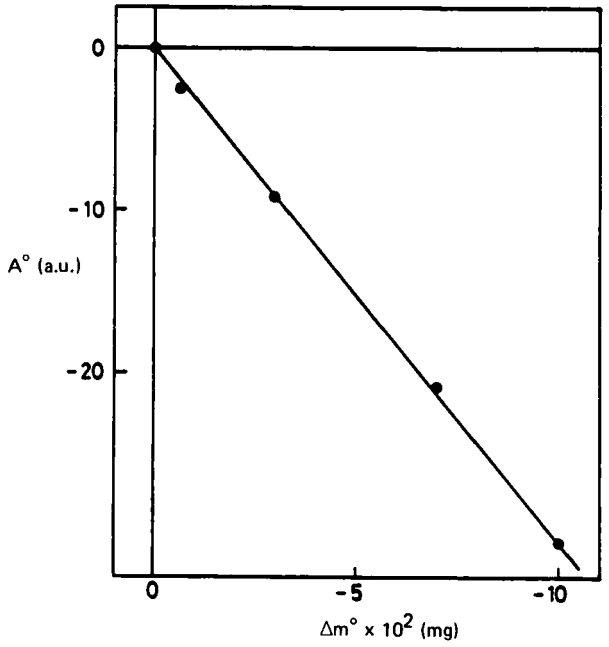

FIGURE 1. Calibration curve of defect peak areas, A°, in arbitrary units vs. PC defect, Δm°, for a 0.1 % (w/v) PC in THF solution as eluent.

with respect to eluent composition). The shift observed in V_e can be attributed to adsorption mechanism of PC on the gel rather than to a pure size exclusion effect. In fact, this behavior is quite common in gel permeation chromatography when binary equilibrating solutions are used (15,16). In our particular case, this phenomenon can be easily evidenced by the pronounced peak tailing obtained after injection of PC on the column equilibrated with pure THF.

Figure 2 shows the chromatograms of several injections of GA at concentrations ranging from 1.0 to 10.0 $mg.mL^{-1}$, with a 1.30×10^{-3} M solution of PC in THF as eluent. The first eluting peak in the chromatogram corresponds to the solvated GA and the second one to the vacant peak, involving a PC defect with respect to eluent composition, which represents the amount of lipid interacting with GA. As it can be observed, peak resolution on this

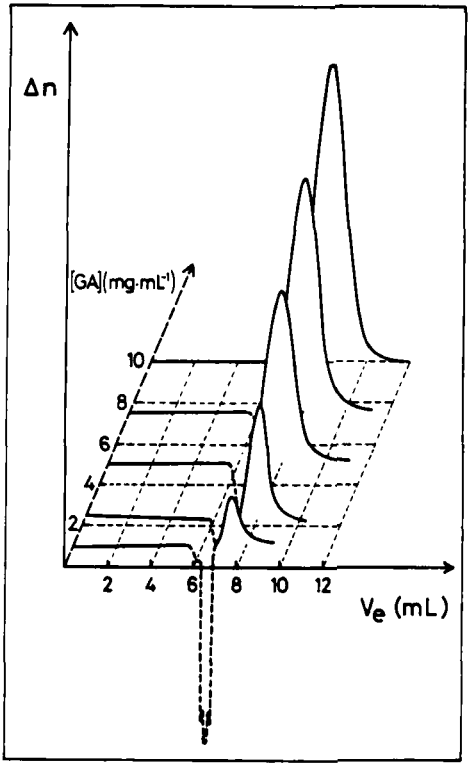

FIGURE 2. Chromatograms corresponding to GA injections at concentrations 1.0; 2.5; 5.0; 7.5; and 10.0 $mg.mL^{-1}$ for a 0.1 % (w/v) PC in THF solution as eluent. In order not to overcrowd the figure only the peak of solvated GA corresponding to the concentration of 1.0 $mg.mL^{-1}$ is included.

column is satisfactory for this rather difficult separation, so far not achieved with any other column. Resolution was much better than that obtained on μ-Styragel as previously described (10). In a similar manner to the behavior observed in the calibration for PC defect peaks, the elution volumes of vacant peak increase from 7.5 to 8.2 mL with increasing injected sample concentration (figure 2).

The elution volume of GA with lipid-containing eluent is always decreased when compared with the chromatograms obtained with pure THF as eluent. The shift in V_e values of GA can be explained by the strong interactions between GA molecules and the lipid. The same effect has been observed as a result of the formation of stable polymer-surfactant complexes (17). Moreover, lower elution volumes for solvated GA were found at higher concentrations of the injected sample, in contrast with pure THF as eluent where GA did not appreciably vary its V_e in the range of assayed concentrations. The dependence of V_e on GA concentration was linear, as shown in figure 3.

The absolute amounts of PC defects, in mg, were calculated for the different concentrations of injected GA from the vacant peak areas in chromatograms (figure 2) and by using the calibration curve (figure 1). An apparent linear relationship between the amount of lipid interacting with the polypeptide and GA concentration was observed (figure 3), though chromatograms for the more concentrated samples showed a very slight peak overlapping, what could imply actual little deviations from linearity for higher GA concentrations. The results in figure 3 indicate that a correlation apparently exists between both the increase in PC defect amounts and the decrease in V_e with increasing GA concentration. This may be interpreted in terms of larger hydrodynamic volumes (for solvated GA) as the amount of solvating lipid becomes higher. In fact, the lowest V_e value of GA, 6.1 mL, corresponding to an injected GA concentration of 10.0 $mg.mL^{-1}$, gives an approximate molecular weight of 3000 by interpolation in a column calibration plot in THF (not shown).

It is possible to determine the "binding ratio", BR, from the slope of the curve using the data in figure 3, as described by Szmerekova et al. (18). We find more appropriate to use here the molar binding ratio instead of the preferential solvation parameter λ as a measure of binding, since in biomembrane studies it is more usual to express lipid-protein interaction as a molar

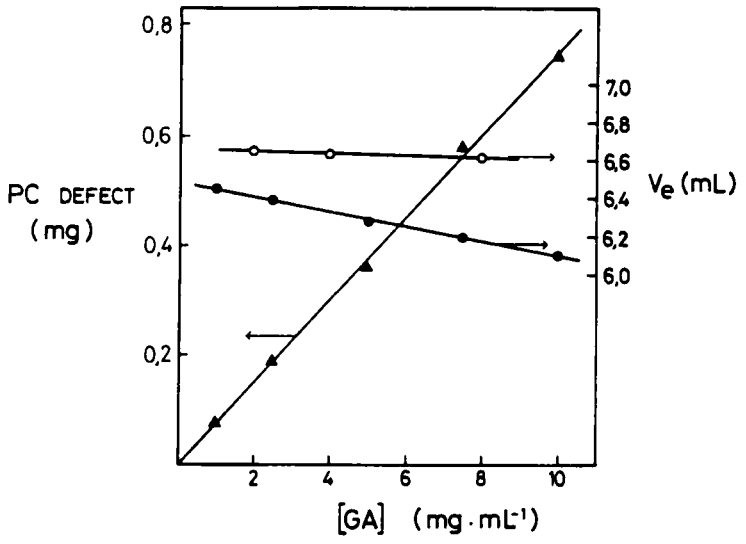

FIGURE 3. Dependence of GA elution volume and absolute amount of PC defect vs. injected GA concentration. (o) THF as eluent; (●,▲) a 0.1 % (w/v) PC in THF solution as eluent.

ratio. The BR (moles of PC bound to one mole of GA) is 3.6 for a 0.1 % (w/v) PC solution in THF. According to this, it is very significant that GA-PC interactions are at least several thousand-fold stronger than CH-PC ones (BR = 5×10^{-4} moles of PC/ mole of CH), as deduced for the same PC eluent composition in THF/PC/CH system from our previous results (10). On the other hand, the observation that GA is preferentially solvated by THF in THF/CH/GA systems (10) is of particular importance for further considerations.

An interesting conclusion emerges from the quantitative studies of these systems: the polypeptide shows a lipid selectivity with a clear preference for the phospholipid. This is in good agreement with experimental evidences that CH is excluded from the protein surface, deduced from studies with reconstituted proteoliposomes where interactions are mainly directed by the presence of

aqueous solvent (7,18,19). So, the fact that the strong PC-GA interactions occur in solution in a relatively low polarity solvent seems to indicate a pronounced specificity probably due to some structural features of both GA and PC molecules. The lack of an appreciable interaction of CH with GA suggests that the planar rigid steroid molecule is not easily accommodated at the polypeptide surface, but it is better solubilized in a lipid environment (CH is preferentially solvated by PC in THF/PC/CH systems (10)). A similar selectivity has been found in lipid-myelin proteolipid apoprotein complexes (7).

A more detailed study is now being carried out in our laboratory in order to better characterize PC-GA interactions by determining the influence that factors such as temperature, type and length of lipid hydrocarbon chains, solvent polarity, etc., have on this interaction.

In summary, the proposed experimental method has proved to be very suitable for the determination by HPLC-SEC of interaction parameters among biomembrane components in non-aqueous solutions, and it may be envisaged as a powerful tool in the study not only of model compounds but also of membrane proteolipids, whose lipid-protein interactions are a current matter of research.

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