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^{1}H-NMR assay of phosphatidylcholine and phosphatidylethanolamine in AL721*

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Abstract: A ^{1}H-NMR based method has been developed for the assay of phosphatidylcholine (PC), and phosphatidylethanolamine (PE) in AL721. The assay was based on the comparison of NMR signal intensities of the choline and the ethanolamine moiety of PC and PE, respectively, to the signal of imidazole, the internal standard. The assay was reliable and more accurate than the commonly used LC or TLC methods. The uncertainty, due primarily to signal intensities, of the ^{1}H-NMR method was estimated at <3%.

Keywords: *AL721; LC; ^{1}H-NMR; phosphatidylcholine; phosphatidylethanolamine.*

Introduction

AL721 is a 7:2:1 mixture of neutral glycerides (NG), phosphatidylcholine (PC), and phosphatidylethanolamine (PE). Through the action of PC (a membrane fluidizer) and PE (a bilayer disruptor), AL721 is able to extract cholesterol from cellular membranes [1, 2]. AL721 alters the HIV envelope composition and interferes with the HIV attachment and penetration to target cells [3–5]. The NG–PC–PE ratio is important in increasing the fluidity of cell membranes and a ratio of 7:2:1 is deemed most active [1]. Because of the current interest in AIDS chemotherapy and the purported benefit of AL721 to AIDS patients, an accurate determination of PC and PE in AL721 is desirable.

Quantitation of phospholipids has been carried out commonly by thin-layer chromatography (TLC) and liquid chromatography (LC). TLC, which suffers from inadequate separation, is inherently time-consuming and insensitive. In addition, the staining process during detection could lead to loss of polyunsaturated fatty acids [6]. LC is hampered by the lack of a universal detector for accurate measurement of phospholipids. Fluorescence, flame ionization, light scattering and infrared detections require either extensive sample preparation or are incompatible with many eluting solvents such as aqueous buffers [7–10]. The most widely used refractive index (RI) and ultraviolet (UV) detections are not without limitations. RI detection is insensitive. The possibility of both positive and negative peaks, as well as the detector's sensitivity to temperature and mobile phase changes limit RI detector to qualitative analysis of phospholipids [11]. UV detection is based on the absorption of the carbonyl and unsaturation of the fatty acid moiety; quantitation of phospholipids is accurate only when the analytes and the reference standards have identical fatty acid compositions. These requirements are difficult to meet, therefore, alternate methods that are specific and reliable for the assay of PC and PE in AL721 are needed.

Proton nuclear magnetic resonance (NMR) is a versatile structural determination method. Comparison of resonance intensities in a spectrum can give the ratio of different protons in a pure compound, or the molar composition of a mixture [12]. Because of the complexity of the spectrum and overlapping signals, accurate NMR quantitation of phospholipid mixtures is not easily achievable. This paper presents a ^{1}H-NMR based method for the simultaneous assay of PC and PE in AL721.

Experimental

Reagents and materials

PC (L-*a*-phosphatidylcholine, from egg yolk, 100 mg ml^{-1} chloroform) and PE (L-*a*-phosphatidylethanolamine, from egg yolk, 10 mg

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ml^{-1} chloroform), were purchased from Sigma Chemical. Another set of PC and PE (99+% pure) were obtained from Avanti Polar-Lipids Inc. 2-Aminothiadiazole (NSC4728, 99+% pure) was received from the National Cancer Institute (NCI). Imidazole (99+% pure) was purchased from Matheson, Coleman and Bell. These chemicals were used without further treatment. $CDCl_3$, D_2O, and tetramethysilane (TMS) were obtained from Norell Inc. Methanol and acetonitrile were HPLC grade (Burdick and Jackson Labs, Inc.). AL721 lots H1019871LA and H1019871LB (Matrix Research Labs, Inc.) were received from NCI, and lot P030689 (Ethigen Corporation) was purchased from Au Naturael (San Francisco, CA, USA).

Sample preparation

Each package of AL721 was weighed. A slit was cut in the package, the contents were quantitatively transferred to individual 50-ml volumetric flasks and diluted to the mark with chloroform to form the clear yellow stock solutions. Each empty package was dried and re-weighed. The difference between the two weighings was the net content weight (NCW).

Liquid chromatography (LC)

The LC assays were performed isocratically with conc. sulphuric acid–methanol–acetonitrile (0.05:3:97, v/v/v) at 0.8 ml min^{-1} on a 4.6 × 250 mm stainless steel, silica column (IBM Instruments) using a Model 600 Solvent Delivery System, a WISP 712 injector and a Model 401 LC Spectrophotometer (Waters Assoc.). A Maxima 820 Integration System (Dynamic Solution) was used to collect and process the chromatographic data. Aliquots of 1.00 ml of stock solutions were each mixed with 1.00 ml of an internal standard solution (ISS, 10 mg 2-aminothiadiazole in 25 ml methanol) before chromatography. Analytical reference solutions were analogously prepared with authentic PC and PE (both Sigma and Avanti lots). The PC and PE contents in each AL721 package were calculated by the internal standard method.

Proton nuclear magnetic resonance (NMR)

A 2.00-ml aliquot of each AL721 stock solution was mixed with 20.0 mg of imidazole. Each resulting solution was evaporated to dryness, the oily residue was re-dissolved in 0.5 ml $CDCl_3$ and transferred to an NMR tube containing TMS and external D_2O. The NMR spectrum was recorded using a JEOL FX-90Q NMR spectrometer operating at 89.55 MHz, observation frequency of 1800 Hz, 8K data points, 100 μs pulse delay, 2.3 s acquire time and 100 accumulations. The 2.8–3.8 and 6.8–7.8 ppm regions were carefully and accurately integrated.

Results and Discussion

Determination of the PC and PE contents in two lots of AL721 samples were attempted with the LC assay which was adapted from Kaduce *et al.* [12]. The results, presented in Table 1, varied with the analytical standards. Results based on Avanti standards were higher than those based on Sigma standards. Because the fatty acid compositions in the phospholipid standards were different, these two sets of standards yielded non-equivalent LC detection responses which resulted in precise but unreliable PC and PE contents in AL721. NMR has been employed as a reliable quantitation for simple mixtures [13]. From the spectrum of

Table 1
LC assay results of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) in AL721 samples

Sample	NCW (g)	Based on Sigma Ref. PC (g)	PE (g)	Based on Avanti Ref. PC (g)	PE (g)
Lot H101987ILA	11.23	3.03	0.30	3.48	0.98
SD	0.53	0.26	0.02	0.30	0.10
n	3	3	3	3	3
Lot H101987ILB	16.77	3.57	0.36	3.91	1.18
SD	0.18	0.06	0.01	0.06	0.01
n	6	6	6	6	6

NCW = net content weight. The results were expressed as grams (g) per package of AL721 sample. The concentrations and purities of analytical references were determined by ^{1}H-NMR assay. They were: 104.6 mg ml^{-1} for Sigma PC, 8.3 mg ml^{-1} for Sigma PE, 100% pure for Avanti PC and 89% pure for Avanti PE.

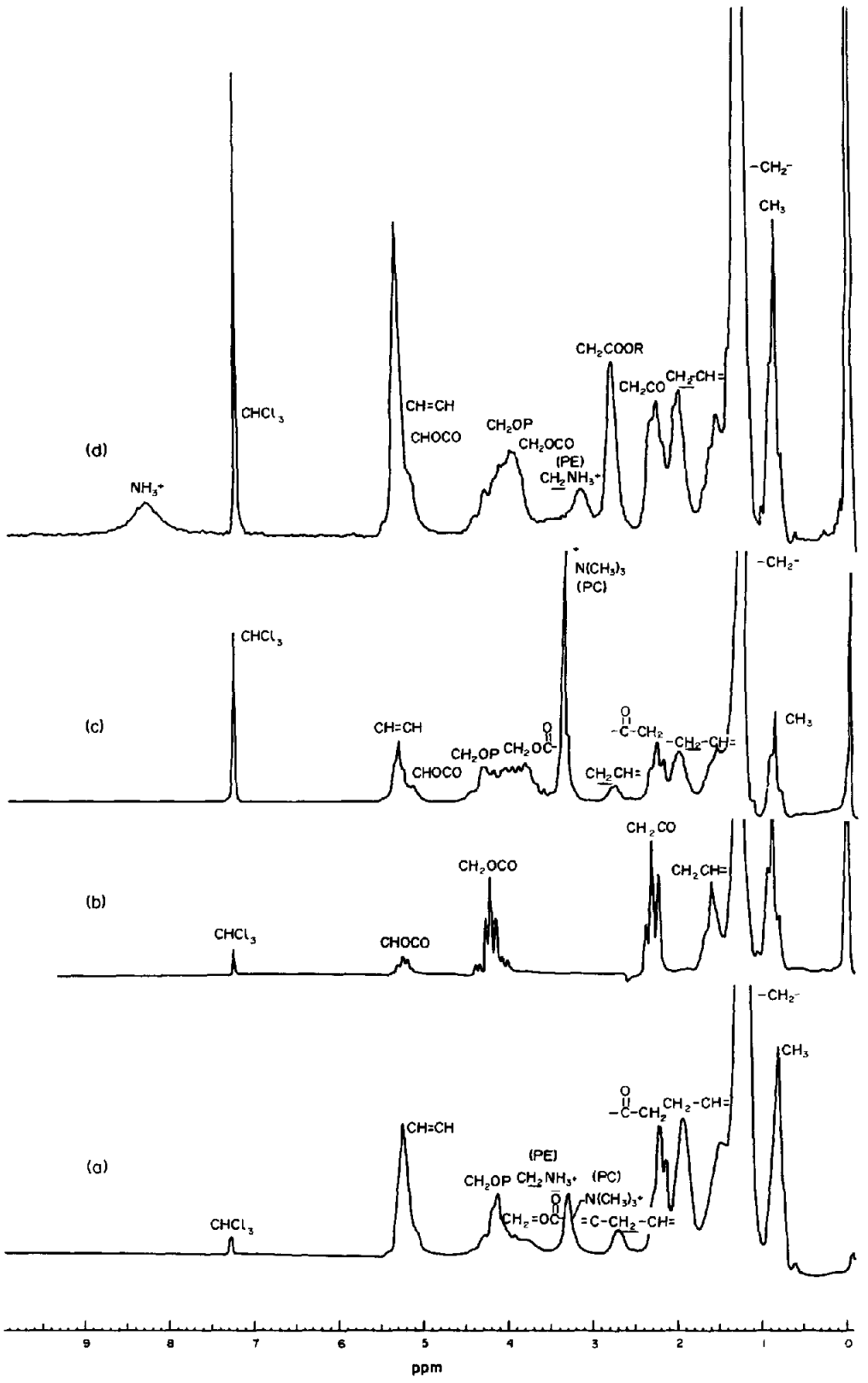

Figure 1
^{1}H-NMR spectra of (a) AL721, (b) trimyristin (NG), (c) egg yolk phosphatidylcholine (PC), and (d) egg yolk phosphatidylethanolamine (PE) in $CDCl_3$ solutions.

a mixture of the unknown analyte (U) and the reference standard (R), the molar concentration of the analyte (C_U) can be calculated according to equation (1), where C_R is the molar concentration of the reference, A_U and A_R are the signal intensities associated with N unique nuclei of U and R, respectively:

$$N_R \times A_U/C_U = N_U \times A_R/C_R. \quad (1)$$

Equation (1) requires that N_U and N_R be known and that A_U and A_R are free of interference. NMR spectra of phospholipid mixtures are complex, and signals of one phospholipid overlapped with those of other phospholipids in the mixture. Therefore, NMR quantitations of phospholipid mixtures have been unsuccessful. However, for AL721 which is a binary mixture of phospholipids PC and PE, this difficulty can be mathematically overcome.

The apparent singlet at 3.1 ppm in the NMR spectrum of AL721 (Fig. 1) is attributed to the nine $-N(CH_3)_3$ and the two $-CH_2N-$ protons of PC and PE, respectively. Therefore, this singlet represents $9a + 2$ equivalent PE protons, where a is the molar ratio of PC–PE. Substitution and rearrangement of equation (1) resulted in equations (2) and (3), where C_U is the molar concentration of PE:

$$A_R/(N_R \times C_R) = A_U/(9a + 2) \times C_U, \quad (2)$$

$$[A_7 \times M_R]/[2 \times W_R \times P_R] = [A_3 \times M_{PE}]/[(9a + 2) \times W_{PE} \times P_{PE}]. \quad (3)$$

Thus, from the NMR spectrum of an AL721 and imidazole mixture (Fig. 2), the amount (W) of PE in the mixture can be calculated from equation (3), where M is molecular weight and P is purity. Imidazole was chosen as the reference because it was commercially available of suitable purity and compatible with the NMR experiment. Its NMR spectrum was simple and well defined with singlets at 7.0 and 7.5 ppm for the $H_{4\&5}$ and H_2 protons, respectively. Rearranging equation (3) and substituting W_{PE} with $W_{PC} \times M_{PE}/(a \times M_{PC})$ resulted in equations (4) and (5) which were used to assay the amounts of PC and PE in AL721:

$$W_{PE} = [2 \times A_3 \times M_{PE} \times W_R \times P_R]/[(9a + 2) \times A_7 \times M_R], \quad (4)$$

$$W_{PC} = [2 \times a \times A_3 \times M_{PC} \times W_R \times P_R]/[(9a + 2) \times A_7 \times M_R]. \quad (5)$$

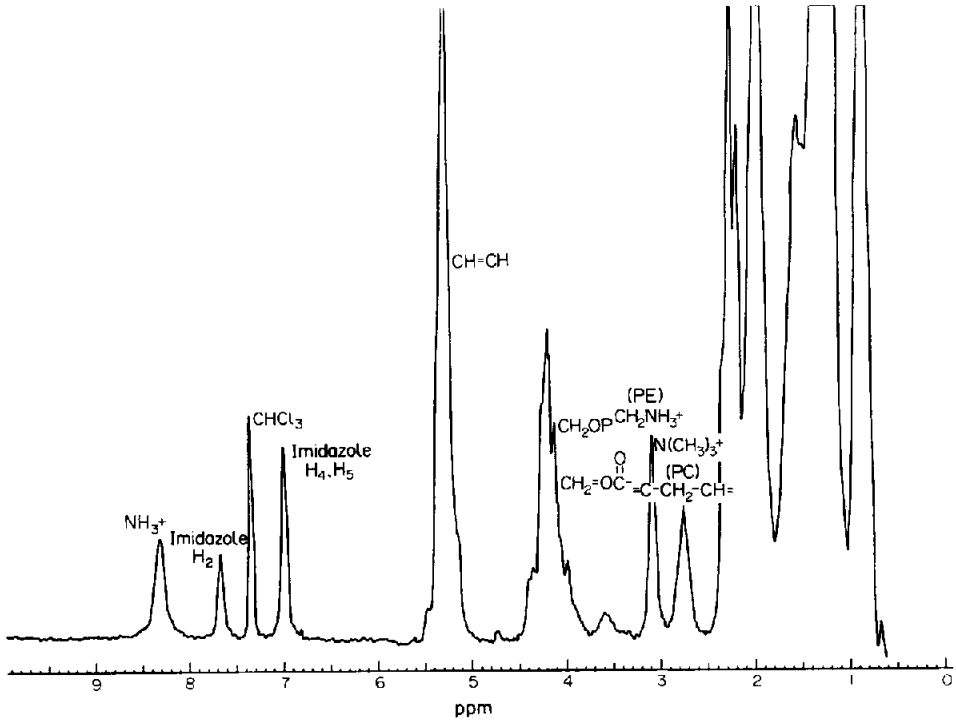

Figure 2
^{1}H-NMR spectrum of a mixture of AL721 and imidazole in $CDCl_3$ solution.

To test the validity of equations (4) and (5), individual and combinations of authentic PC and PE references were mixed with imidazole and their NMR spectra were recorded. Results are presented in Table 2. Samples 1–12 determined the purity or the concentration of individual references, which are 83 to 105% of the labelled values (last column in Table 2). The nearly 100% found/actual values for Samples 13 and 14 validate equations (4) and (5). Thus, the PC and PE contents in AL721 can be determined by this NMR technique if a, the molar ratio of PC–PE can be independently determined.

The PC and PE molecules each contain a single phosphate group. Relative measurement of phosphate content in PC and PE reveals their molar ratio. This measurement could be accomplished with a phosphate specific detection such as ammonium molybdate after TLC separation or by ^{31}P-NMR. In a further investigation, ^{31}P-NMR was used because it gave a reliable molar ratio of PC–PE [14]. The ratio, a, obtained by ^{31}P-NMR for AL721 lots

Table 2
Validation and quantitation of PC and PE by ^{1}H-NMR

Sample*	Actual weight (mg) PC	PE	IS	Found weight (mg) PC	PE	Found (%) Actual
1. PCdp	25.51		21.51	24.81		97.3
2. PEdp		18.56	25.27		18.17	97.9
3. PEdp		13.56	9.57		13.37	98.6
4. PC_S	50.00†		10.73	52.31		104.6
5. PC_S	50.00†		12.62	51.54		103.1
6. PC_S	100.00†		19.97	105.98		106.0
7. PC_S	100.00†		19.42	105.20		105.2
8. PC_A	47.95		20.23	48.10		100.0
9. PE_S		30.00†	12.07		23.91	79.7
10. PE_S		30.00†	6.28		25.57	85.2
11. PE_S		60.00†	9.98		49.90	83.2
12. PE_A		56.21	22.22		50.19	89.3
13. PC_S + PE_S	52.35‡	8.27‡	10.02	52.76	8.33	100.8
14. PC_S + PE_S	52.35‡	33.08‡	17.23	54.31	34.37	103.7

Please see text under NMR for experimental details.

*Weighed or pipetted amount of PCdp (dipalmitoylphosphatidylcholine), PEdp (dipalmitoylphosphatidylethanolamine), PC_S (Sigma PC reference), PE_S (Sigma PE reference), PC_A (Avanti PC reference) or PE_A (Avanti PE reference) was mixed and dissolved with weighed amount of imidazole (IS) in $CDCl_3$.

†Volume of aliquot pipetted × labelled concentration.

‡Volume of aliquot pipetted × concentration determined by NMR as indicated by averages of samples 4–7 or samples 9–11.

Table 3
NMR assay results of PC and PE in AL721 samples

Sample	NCW (g)	Found per package PC (g)	PE (g)	PC–PE by weight
Lot H101987ILA	11.08	2.65	0.35	7.6
SD	0.46	0.10	0.02	0.1
n	2	2	2	2
Lot H101987ILB	16.83	2.76	0.39	7.3
SD	0.20	0.27	0.04	0.2
n	4	4	4	4
Lot P030689	15.37	1.87	0.29	6.6
SD	0.11	0.09	0.02	0.2
n	3	3	3	3

The NCW (net content weight), and PC and PE were expressed as grams per package of AL721. The PC and PE results were calculated with equations (4) and (5), respectively. Molecular weights for PC and PE were assumed 785 and 743, respectively, as the dioleates. PC–PE molar ratios, a, for equations (4) and (5) were obtained with ^{31}P-NMR. They were 7.25, 6.77 and 6.08 for Lots H101987ILA, H101987ILB and P030689, respectively.

H101987ILA, H101987ILB and P030689 were 7.3, 6.8 and 6.1, respectively. Using these a values and the proton NMR spectra, the PC and PE contents in each AL721 sample were calculated using equations (4) and (5). The results, listed in Table 3, were lower than those determined by LC (Table 1). The PC–PE weight ratio was 7, which differs greatly from 2, a value claimed by the manufacturers.

Conclusion

This paper presents a ^{1}H-NMR based method to assay the PC, PE content and the PC–PE ratio in AL721 samples. The results thus obtained were different from those obtained by an LC method. The NMR method, an absolute, was more accurate than the LC method when the fatty acid compositions of the analytes and the analytical references were different. The precision of the NMR method, however, was inferior to that of LC. The precision of the NMR method depended on the signal integral measurements and was estimated at <3%. However, it could be improved with increased number of spectral accumulation.

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