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^{31}P-NMR assay of phosphatidylcholine and phosphatidylethanolamine in AL721*

LORI L. OLSON and ANDREW P. CHEUNG†

Life Sciences Division, SRI International, 333 Ravenswood Ave, Menlo Park, CA 94025, USA

Abstract: A ^{31}P-NMR based method has been developed for the assay of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) in AL721. The assay is based on the comparison of NMR signal intensities of the phosphatidyl moiety of PC and PE to the signal of triphenylphosphate (TPP), the internal standard. The assay is specific and reliable for the quantitation of phospholipid mixtures. Its uncertainty, due primarily to low intensity of ^{31}P signals, was estimated at <6%.

Keywords: *AL721; ^{31}P-NMR; phosphatidylcholine; phosphatidylethanolamine.*

Introduction

AL721 is a 7:2:1 mixture of neutral glyceride (NG), phosphatidylcholine (PC) and phosphatidylethanolamine (PE). It has been shown to extract cholesterol from cell membranes, alter HIV envelope composition, and interfere with virus attachment to T-cells [1–5]. Shinitsky [1] indicated that the NG–PC–PE ratio of 7:2:1 was most active. Because of the purported benefits of AL721 to HIV-infected patients, a reliable and accurate assay of PC and PE in AL721 is needed. Thin-layer chromatography (TLC) and liquid chromatography (LC) have been the common methods to assay phospholipid mixtures. TLC suffers from inadequate separation, insensitivity, and is time consuming [6]. LC offers good separation but lacks a reliable and accurate detection for quantitative measurements of PC and PE [7–11]. Cheung and Olson [12] developed an ^{1}H-NMR (proton nuclear magnetic resonance) based assay for PC and PE in AL721. Their method required an advance knowledge of the PC–PE molar ratio in the samples.

Because PC and PE each contain a single phosphatidyl moiety which is absent in NG, ^{31}P-NMR spectroscopy appears to be a logical technique for their quantitation in AL721. Due to problems associated with poor signal intensity, relaxation time (T_1) and Nuclear Overhauser Enhancement (NOE) differences between dissimilar molecules, ^{31}P-NMR has been used primarily as a qualitative tool. This paper presents a successful application of ^{31}P-NMR for the simultaneous quantitation of PC and PE in AL721.

Experimental

Reagents and materials

AL721 lots H1019871LA and H1019871LB (Matrix Research Labs, Inc.) were received from the National Cancer Institute, lot P030689 (Ethigen Corporation) was purchased from Au Naturael (San Francisco, CA, USA). L-*a*-Dipalmitoylphosphatidylcholine (PCdp, synthetic, 99+% pure), L-*a*-phosphatidylcholine (PC from egg yolk, 100 mg ml^{-1} chloroform) and L-*a*-phosphatidylethanolamine (PE from egg yolk, 10 mg ml^{-1} chloroform) were purchased from Sigma Chemical. Triphenylphosphate (TPP, Aldrich Chemical Co.) was characterized by UV, NMR and elemental analyses as 99+% pure. NMR reagents, $CDCl_3$, D_2O, and tetramethysilane (TMS) were obtained from Norell, Inc.

Sample preparation

Each package of AL721 was weighed. A slit was cut in the package, and the contents were quantitatively transferred to a 50-ml volumetric flask and diluted to the mark with chloroform to form the clear yellow stock

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† Author to whom correspondence should be addressed.

solution. The empty package was dried and reweighed. The difference between the two weighings was the net content weight (NCW).

Phosphorus nuclear magnetic resonance (^{31}P-NMR)

A 2.00-ml aliquot from each stock solution was transferred to individual round bottom flasks containing 26.0 mg of TPP and was evaporated to near dryness. The residue was redissolved in 0.5 ml $CDCl_3$ and transferred to an NMR tube. The ^{31}P-NMR spectrum of each final solution was acquired with a JEOL FX90Q NMR spectrometer set with a single pulse sequence with gated proton decoupling without NOE, operating at 36.19 MHz, observation frequency of 8000 Hz, 8K data points for acquisition, 60 s pulse delay, a 45° pulse angle (approximately 5.8 μs), 0.5 s acquire time and 30–60 accumulations. The peak intensities for the PC, PE and TPP signals were carefully integrated.

Results and Discussion

Quantitative NMR is based on the comparison of signal intensities (A) associated with N unique nuclei of the unknown analyte (U) and an internal standard reference (R) according to equation (1), where C is molar concentration:

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

Though ^{31}P-NMR has been used quantitatively for simple compounds, its application to phospholipids has been primarily qualitative. Although signals of phospholipids are distinct and resolved [13–15], their use in quantitation has been inhibited by T_1 and NOE differences between dissimilar molecules [16, 17]. In addition, the high molecular weights of phospholipids leads to low molar concentrations for NMR solutions and results in poor signal intensities. Because of these difficulties, only a few reports on the ^{31}P-NMR assay of phospholipids appeared in the literature. These assays either used expensive high field (400 MHz) instruments [17], or required sample pretreatment and/or detergent addition to the sample solutions [14, 15]. The simplicity of the ^{31}P-NMR of AL721 (Fig. 1) and the need for a reliable assay prompted further investigation on the quantitative use of ^{31}P-NMR for AL721.

In NMR quantitation, the choice of the internal standard reference is most important. Triphenylphosphate (TPP) was chosen because

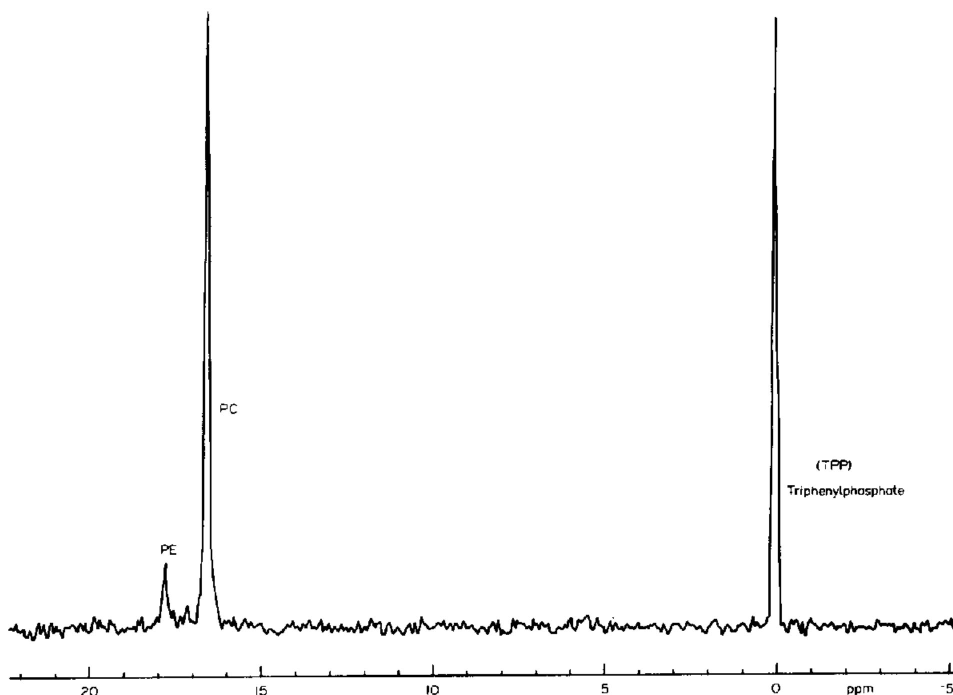

Figure 1
^{31}P-NMR spectrum of a mixture of AL721 and triphenylphosphate (TPP) in $CDCl_3$ solution.

its signal was well resolved from phospholipids (Fig. 1) and it was commercially available in high purity. Attempts to overcome quantitative problems caused by non-equivalent NOE between TPP, PC and PE by gated proton decoupling [18] resulted in inconsistent results. Canet [19] pointed out that gated decoupling could lead to systematic error in peak intensities due to T_1 differences. He suggested that proper choice of instrument settings such as pulse delay time could overcome the difficulty. In light of this, the effect of pulse delay time and pulse angle on the accuracy of relative peak intensities for mixtures of TPP and phospholipid (PCdp) was investigated. The data in Table 1 indicate that a pulse angle of 45° and 60 s pulse delay time resulted in a peak area ratio equivalent to the actual molar ratio. This finding is consistent with the T_1s of TPP (13.5 s [20]) and phospholipids (2–4 s [14, 17]). At a pulse angle of 90°, 10 times T_1 or 135 s pulse delay time is needed for complete recovery of the nuclear magnetization of both TPP and PCdp [19]. Only in this way can one expect to have quantitative results between nuclei with dissimilar T_1s. A shorter delay time would attenuate the TPP signal due to saturation. With a 45° pulse angle, only four to five times T_1 or 55–65 s is required [17].

The validity of the ^{31}P-NMR quantitation, using a 45° pulse angle and 60 s pulse delay time, was confirmed with mixtures of TPP and authentic PC and PE references. When this ^{31}P-NMR method was applied to AL721 samples, the PC and PE assay results (Table 2) were in close agreement with those determined by a ^{1}H-NMR assay [12]. The slightly higher ^{1}H-NMR results for lot P030689 was probably due to other phospholipids, such as sphingomyelin (a minor phospholipid in egg yolk) which interfered with the ^{1}H- but not the ^{31}P-NMR assay.

Conclusion

The ^{31}P-NMR assay presented in this paper for phospholipids circumvents quantitation problems associated with commonly used TLC and LC methods. Because ^{31}P-NMR signals for the phospholipids are resolved, the method is

Table 1
Effect of instrument settings on peak integrations of ^{31}P-NMR

Run	Pulse angle (degree)	Delay time (s)	Peak area A = PCdp	B = TPP	C = A/B	Molar concentration D = PCdp	E = TPP	F = D/E	Ratio G = C/F
1	45	30	44	40	1.10	0.0336	0.0336	1.00	1.10
2	45	45	64.8	67.5	0.96	0.0336	0.0336	1.00	0.96
3			20.5	33	0.62	0.0339	0.0539	0.63	0.99
4			49	98	0.50	0.0343	0.0704	0.49	1.02
5			35	23	1.52	0.0349	0.0211	1.65	0.92
6			46	32	1.44	0.0349	0.0211	1.65	0.87
								Average	0.95
								SD	0.06
								n	5
7	45	60	40.5	21.4	1.89	0.0340	0.0168	2.02	0.94
8			37.4	39.4	0.95	0.0339	0.0349	0.97	0.98
9			57	38	1.50	0.0503	0.0353	1.42	1.06
10			31.4	38.5	0.82	0.0262	0.0325	0.81	1.01
11			32	63	0.51	0.0171	0.0334	0.51	1.00
12			29	32	0.91	0.0426	0.0467	0.91	1.00
13			19.5	19.2	1.02	0.0337	0.0326	1.03	0.99
								Average	1.00
								SD	0.03
								n	7
14	90	60	38	36	1.06	0.0339	0.0349	0.97	1.09
15			59	73	0.81	0.0262	0.0325	0.81	1.00
16			42	69	0.61	0.0171	0.0334	0.51	1.20
								Average	1.10
								SD	0.10
								n	3

See text under ^{31}P-NMR for experimental details. Solutions of PCdp (dipalmitoylphosphatidylcholine, Sigma Chemical Co., mol. wt = 734) and TPP (triphenylphosphate, Aldrich Chemical Co., mol. wt = 326) were prepared in $CDCl_3$ and their ^{31}P-NMR spectra recorded under different instrument settings.

Table 2
Comparison of ^{31}P- and ^{1}H-NMR assay of PC and PE in AL721 samples

Sample	NCW (g)	PC (g) per package		PE (g) per package	
		^{1}H-NMR	^{31}P-NMR	^{1}H-NMR	^{31}P-NMR
Lot H101987ILA	11.08	2.65	2.69	0.35	0.37
SD	0.46	0.10	0.16	0.02	0.02
n	2	2	2	2	2
Lot H101987ILB	16.83	2.76	2.77	0.39	0.37
SD	0.20	0.27	0.06	0.04	0.01
n	4	4	4	4	4
Lot P030689	15.37	1.87	1.68	0.29	0.25
SD	0.11	0.09	0.06	0.02	0.01
n	3	3	3	3	3

The NCW (net content weight), and PC (phosphatidylcholine) and PE (phosphatidylethanolamine) were expressed as grams per package of AL721. ^{1}H-NMR results were obtained from ref. 12. ^{31}P-NMR results were calculated with equation (1). Molecular weights for PC and PE were assumed 785 and 743, respectively, calculated as the dioleates.

specific for individual phospholipids. The accuracy or precision of the method depends on the signal intensities. For this study, with 30–60 accumulations, the precision was 6%. Better precision can be obtained by an increase in the number of accumulations.

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