^{1}H NMR Quantification of poly(Ethylene Glycol)-Phosphatidylethanolamine in Phospholipid Mixtures

E. A. A. M. Vernooij,[1,4] C. A. Gentry,[2] J. N. Herron,[2] D. J. A. Crommelin,[1] and J. J. Kettenes-van den Bosch[3]

Received June 10, 1999; accepted July 9, 1999

KEY WORDS: ^{1}H NMR; poly(ethylene glycol)-phosphatidylethanolamine; PEG; phospholipids; quantification.

INTRODUCTION

In recent years, *N*-poly(ethylene glycol)-derivatized phosphatidylethanolamines (PEG-PE, Fig. 1) have been used extensively as components of liposomes to avoid uptake by the phagocytic cells of the mononuclear phagocyte system (MPS) (1–3). A prolonged lifetime of the liposomes within the circulation results in an increased probability of liposomes to extravasate in areas of enhanced vascular permeability, e.g., at tumors and at sites of infection and inflammation (4–6). In addition, long-circulating liposomes can be used as a vascular imaging agent for both nuclear medicine and magnetic resonance imaging (MRI).

The properties of these long-circulating liposomes are directly related to the presence of PEG chains. Therefore, there is a need for validated methods for the quantification of PEG(-phospholipids) present in the liposomal membrane. No simple and reliable method has been published as yet. Quantification is hampered by both the molecular mass distribution of the PEG polymer and the lack of a chromophore. We now report a simple proton nuclear magnetic resonance (^{1}H NMR) method to determine the relative amounts of PEG-PE, phosphatidylcholine (PC) and phosphatidylglycerol (PG) in regularly used mixtures of these three types of phospholipids.

MATERIALS AND METHODS

Egg phosphatidylcholine (EPC), egg phosphatidylglycerol (EPG), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and 1,2-dipalmitoyl-*sn*-glycero-3-phospho-*rac*-glycerol (DPPG) were a gift from Lipoid GmbH (Ludwigshafen, Germany). The purity of these lipids was ≥99.1% on an anhydrous weight basis, with only trace amounts of lysophospholipid present and a residual water content of ≤0.9%, as stated by the manufacturer. PEG2000-DSPE (poly(ethylene glycol) with an average molecular weight of 2000 Da, *N*-linked to distearoylphosphatidylethanolamine, >99%) was purchased from Avanti Polar Lipids (Alabaster, Alabama, USA). Lipids were dissolved in deuterated chloroform (Chloroform-D, 99.6+ atom % D, Acros Organics, Geel, Belgium), in a concentration of 25 mg/mL.

^{1}H NMR

For 34 phospholipid mixtures of different composition a ^{1}H NMR spectrum was recorded with a Gemini 300 MHz spectrometer (Varian Associates Inc. NMR Instruments, Palo Alto, CA, USA). The number of accumulated transients was 16. For quantitative measurements the relaxation delay D1 was set at 13 seconds, corresponding to $5 \cdot T_1$ (longitudinal relaxation time).

The signals in the spectra of the individual phospholipids and phospholipid mixtures were assigned on the basis of chemical shift and intensity data and confirmed by homo- and heteronuclear double resonance experiments. The spectra were in good agreement with literature data (7–11). A spectrum of a mixture of DPPC, DPPG and PEG-DSPE is shown in Fig. 2. The assignment of the partially overlapping signals is given in Table I.

Calculations

The integral value I_1 for one proton was calculated from the integral of the fatty acid methyl signal ω1 at δ 0.9. Since PC, PG and PEG-PE all contain two fatty acid residues, this signal integrates for 6 protons per lipid molecule. Therefore, $I_1 = I(\omega 1)/6$. The integral value for one proton could also be calculated as $I_1 = (I(\omega 1) + I(C2) + I(C3))/14$, with similar results. The relative amount of the individual components was then calculated as follows.

Amount of PEG-PE

The number of protons in the PEG signal at δ 3.6 (A) per lipid molecule is calculated with equation 1, correcting the integral of the δ 3.6 signal for the contribution of the ^{13}C satellites of the δ 3.3 signal.

$$A = \{I(\delta\ 3.6) - 0.0055 \cdot I(\delta\ 3.3)\}/I_1 \qquad (1)$$

The ^{13}C satellite signals arise from proton-^{13}C coupling and are found at equal distances from the central signal (here at δ 3.3), the distance depending on the value of the coupling constant; ^{12}C is not NMR-active and no coupling is observed between a proton and the ^{12}C atom to which it is attached. Since the natural abundance of ^{13}C is 1.1%, each ^{13}C satellite signal has an intensity of 0.55% of the main signal; therefore, the correction factor is $0.0055 \cdot I(\delta\ 3.3)$. Because of the high intensity of the phosphatidylcholine H3 signal, this contribution can not be neglected.

The mol percentage PEG-PE in the mixture was calculated with equation 2.

[1] Department of Pharmaceutics, Faculty of Pharmacy, Utrecht Institute for Pharmaceutical Sciences (UIPS), Utrecht University, PO Box 80 082, 3508 TB Utrecht, The Netherlands.

[2] Department of Pharmaceutics and Pharmaceutical Chemistry, University of Utah, Center for Biopolymers at Interfaces, Biomedical Polymers Research Building, RM 108, Salt Lake City, Utah 84112-9450.

[3] Department of Pharmaceutical Analysis, Faculty of Pharmacy, Utrecht Institute for Pharmaceutical Sciences (UIPS), Utrecht University, PO Box 80 082, 3508 TB Utrecht, The Netherlands.

[4] To whom correspondence should be addressed. (e-mail: e.a.a.m.vernooij@pharm.uu.nl)

0724-8741/99/1000-1658$16.00/0

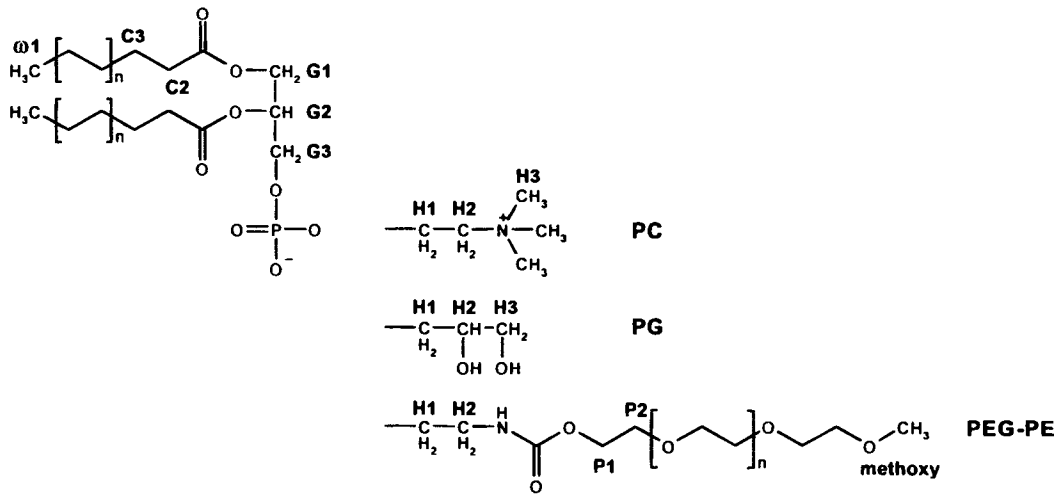

Fig. 1. Molecular structure of phosphatidylcholine, phosphatidylglycerol and poly(ethylene glycol)-phosphatidylethanolamine.

$$\%\ \text{PEG-PE} = [A \cdot \{((2000 - 59)/44)\cdot 4 + 2\}^{-1}] \cdot 100\% \quad (2)$$

in which 2000 is the average PEG molecular weight, 59 the correction for the PEG-PE P1 and P2 CH_2's, and for the PEG-PE methoxy CH_3O: these groups show a chemical shift different from the bulk PEG CH_2's. In the equation, 2 is the correction for H2 of PEG-PE, which overlaps with the PEG signal. The molecular mass per ethylene glycol moiety is 44 Da and 4 is the number of protons per ethylene glycol group. In this calculation, the contribution of the PG H3 protons to the δ 3.6 signal is not taken into account, as explained in the discussion.

Amount of PC

The amount of PC was calculated from the integral B of the signal at δ 3.3. The integral of this signal was corrected for the overlapping signals of the δ 3.6 ^{13}C satellites, and for the PEG-PE P1 (2 protons) and methoxy protons (3 protons).

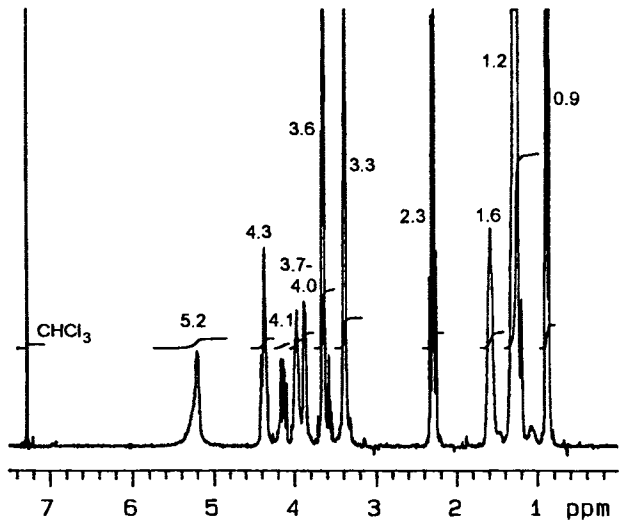

Fig. 2. ^{1}H NMR spectrum of a mixture of DPPC/DPPG/PEG-PE 83.4/9.3/7.2. Assignment of the signals is described in Table I.

The correction factor for these 5 protons is %PEG-PE(100)$^{-1}\cdot 5I_1$. The integral I(PC H3) of the phosphatidylcholine H3 groups is then:

$$I(\text{PC H3}) = B - 0.0055 \cdot I\ (\delta\ 3.6) - \%\ \text{PEG-PE} \cdot (100)^{-1} \cdot 5I_1 \quad (3)$$

and the relative amount of PC:

$$\%\ \text{PC} = [\{I(\text{PC H3})/9\}/I_1] \cdot 100\% \quad (4)$$

9 being the number of protons in the phosphatidylcholine H3 groups.

Amount of PG

The amount of PG was calculated from:

$$\%\ \text{PG} = 100 - \%\ \text{PEG-PE} - \%\ \text{PC} \quad (5)$$

Additional Corrections

Finally, A was corrected for the contribution of the two PG H3 protons with equation 6, and relative amounts of PEG-PE, PC and PG were recalculated with Eqs. 2–5.

$$A_{corr} = A - 2 \cdot I_1 \cdot \%\ \text{PG} \cdot (100)^{-1} \quad (6)$$

Accuracy and Precision

Since in the studied phospholipid mixtures, as well as in pharmaceutically relevant mixtures, the amount of PC is usually far higher than that of PG or PEG-PE, absolute values of accuracy and precision for each individual lipid are more illustrative than relative values. Moreover, for each phospholipid in the examined mixtures the absolute accuracy was constant over the entire concentration range (0–100%). Therefore, the accuracy for each lipid was calculated from

$$\text{Accuracy} = \sum_{i=1}^{n} (\%\ \text{lipid}_{\text{calculated, i}} - \%\ \text{lipid}_{\text{theoretical, i}})/n \quad (7)$$

in which n is the total number of phospholipid mixtures measured. The standard deviation was calculated as

Table I. Assignment of the Signals in the ^1H NMR Spectrum of a Mixture of DPPC/DPPG/PEG-DSPE 83.4/9.3/7.2, Shown in Fig. 2

Chemical shift	Assignment
0.9	ω1 PC/PG/PEG-PE
1.2	methylene bulk acyl chain PC/PG/PEG-PE
1.6	C3 PC/PG/PEG-PE
2.3	C2 PC/PG/PEG-PE
3.3	methoxy + H3 PC + P1 PEG-PE + ^{13}C satell. δ 3.6
3.6	methylene bulk PEG + H2 PEG-PE + H3 PG + ^{13}C satell. δ 3.3
3.7–4.0	G3 PC/PG/PEG-PE + H2 PC + H1 PG + H2 PG + P2 PEG-PE + ^{13}C satell. δ 3.6
4.1	G1 PC/PG/PEG-PE + H1 PEG-PE
4.3	G1 PC/PG/PEG-PE + H1 PC
5.2	G2 PC/PG/PEG-PE + H_2O

Note: The functional group labels are the same as in Fig. 1.

$$\text{Standard deviation}_{\text{accuracy}} = (n-1)^{-1} \cdot \sum_{i=1}^{n} (\%\ \text{lipid}_{\text{calculated, i}} - \%\ \text{lipid}_{\text{theoretical, i}})^2 \quad (8)$$

The precision of the method was determined with equation 9 for n spectra of the same lipid mixture.

$$\text{Precision} = (n-1)^{-1} \cdot \sum_{i=1}^{n} (\%\ \text{lipid}_{\text{calculated, i}} - \%\ \text{lipid}_{\text{calculated, mean}})^2 \quad (9)$$

Table II. Quantification of Some Representative Phospholipid Mixtures with ^1H NMR

Sample	Lipids	Theoretical amount (%)[a]	Calculated amount (%)[b]
A	EPC	90.4	89.4
	EPG	9.6	10.5
	PEG-DSPE	0.0	0.0
B	EPC	97.3	95.4
	EPG	0.0	1.7
	PEG-DSPE	2.6	2.9
C	EPC	73.8	72.3
	EPG	14.4	16.4
	PEG-DSPE	11.8	11.3
D	DPPC	88.2	85.1
	DPPG	0.0	3.7
	PEG-DSPE	11.8	11.2
E	DPPC	83.4	82.0
	DPPG	9.3	9.7
	PEG-DSPE	7.2	8.3
F	DPPC	0.0	0.4
	DPPG	55.3	56.2
	PEG-DSPE	44.7	43.4

[a] Based on the weight of phospholipids in the sample.
[b] Calculated from spectra.

RESULTS

The results of the calculations described above, carried out on a few representative mixtures of phospholipids, are shown in Table II. The calculated results are in good agreement with the actual composition of the lipid mixture, and also for solutions of pure phospholipids. The accuracy was −0.3% for PEG-PE, −1.5% for PC and +1.8% for PG, determined from the spectra of 34 mixtures of different phospholipid composition (n = 34). The standard deviations were 1.1, 1.2 and 1.1%, respectively. The precision was 0.03% for PEG-PE, 0.14% for PC and 0.14% for PG, determined from 10 spectra of the same phospholipid mixture (n = 10).

DISCUSSION

Table II shows that our ^1H NMR method for quantification of PEG-PE in mixtures of phosphatidylcholine, phosphatidylglycerol and PEG phosphatidylethanolamine gives values which are in excellent agreement with the known composition of a wide range of phospholipid mixtures. Analysis of the spectra of pure (PEG-)phospholipids was carried out to validate the calculations and corrections. For all spectra of pure phospholipids, the calculated relative amount was 100%, indicating and supporting the accuracy of the method. For determining the amount of PEG-PE in liposomes, lipids are extracted into chloroform (12). After solvent evaporation, the lipids are redissolved in deuterated chloroform and analyzed. The method is applicable for mixtures of both saturated and unsaturated phospholipids, irrespective of the acyl chain length, and for all PEG-PE's in which PEG is anchored to the ethanolamine NH_2 group via a carbamide bond. Since the chloroform signal is well away from all phospholipid signals, trace amounts of chloroform do not influence the outcome of the calculations. Other solvents do interfere, in particular hydrocarbons such as hexane. The CH_3 signal overlaps with the ω1 signal of the phospholipids. Since even traces will lead to erroneous results, such solvents should be avoided.

In the calculations, the average weight of the poly(ethylene glycol) chains is assumed to be 2000 Da, as stated by the manufacturer. Information about the exact average mass of the PEG chains is not essential. Even if the exact average mass of the poly(ethylene glycol) chains deviates from the estimated average mass by 10%, this leads to an error in relative amount of PEG-PE in relevant phospholipid mixtures of only 0.1%. The exact average molecular weight of the PEG chains can be calculated from the ^1H NMR spectrum of pure PEG-PE: the amount of PEG methylene protons per molecule is determined from the integral of the PEG signal at δ 3.6 and the fatty acid methyl signal ω1 at δ 0.9. Corrections are required for the contribution of the PEG-PE H2 protons to the PEG CH_2 signal at δ 3.6, and the PEG-PE P1, P2 and methoxy protons, which show a chemical shift different from the bulk PEG methylene protons.

In the initial calculations for determining the amount of PEG-PE (Eq. 2), the contribution of the H3 protons of phosphatidylglycerol to the signal at δ 3.6 is not taken into account. The following example demonstrates that this simplification is justified.

Should a mixture contain 1% PEG-PE and 20% PG, an extreme situation, the δ 3.6 signal represents (for 100 molecules): 176 PEG CH_2 protons, 2 PEG-PE H2 protons and 40 PG H3 protons (218 protons in total). If no correction is made

for the contribution of the PG H3 protons to the signal, a PEG-PE percentage will be found that is 15% too high (40 PG headgroup protons in 218 protons in total): 1.15% will be found instead of 1.00%. This difference is small and hardly affects the other calculations. Therefore, in the first approximation, the area of the δ 3.6 signal is not corrected for the contribution of the H3 protons of phosphatidylglycerol.

In our experiments, the calculated composition of the mixture changed little after the corrections (equation 6). A second recalculation resulted in identical values and is, therefore, unnecessary.

The chemical shift of residual water present in the phospholipids appeared to depend on the composition of the lipid mixture. In a solution of pure PEG-phosphatidylethanolamine, the H_2O signal overlapped with the δ 3.3 signal, resulting in an incorrect relative composition. This problem could be overcome by adding a trace of D_2O, which shifted the H_2O out of the area of the δ 3.3 signal.

The presented ^{1}H NMR method for determining the amount of PEG-PE is also applicable for other types of PEG-PE or mixtures containing other lipid compounds with only minor modifications of the calculation procedure. This also holds for PEG5000-PE. Preliminary results indicate that the method can also be used for lipid mixtures containing cholesterol.

ACKNOWLEDGMENTS

The authors thank Lipoid GmbH for their generous gift of phospholipids and for financial support of this research project.

REFERENCES

1. M. C. Woodle and D. D. Lasic. Sterically stabilized liposomes. *Biochim. Biophys. Acta* **1113**:171–199 (1992).
2. G. Storm, S. O. Belliot, T. Daemen, and D. D. Lasic. Surface modification of nanoparticles to oppose uptake by the mononuclear phagocytic system. *Adv. Drug Del. Rev.* **17**:31–48 (1995).
3. M. C. Woodle and G. Storm (Eds.) *Long Circulating Liposomes: Old Drugs, New Therapeutics*, Springer Verlag, 1998.
4. G. Storm, M. T. ten Kate, P. K. Working, and I. A. J. M. Bakker-Woudenberg. Doxorubicin entrapped in sterically stabilized liposomes: effects on bacterial blood clearance capacity of the mononuclear phagocyte system. *Clin. Cancer Res.* **3**:111–115 (1998).
5. I. A. J. M. Bakker-Woudenberg, A. F. Lokerse, M. T. ten Kate, J. W. Mouton, M. C. Woodle, and G. Storm. Liposomes with prolonged blood circulation and selective localization in Klebsiella pneumonia-infected lung tissue. *J. Infect. Dis.* **168**:164–171 (1993).
6. O. C. Boerman, W. J. G. Oyen, L. van Bloois, E. B. Koenders, J. W. M. van der Meer, F. H. M. Corstens, and G. Storm. Optimization of technetium-99m-labeled PEG liposomes to image focal infection: effects of particle size and circulation time. *J. Nucl. Med.* **38**:489–493 (1997).
7. N. J. M. Birdsall, J. Feeney, A. G. Lee, Y. K. Levine, and J. C. Metcalfe. Dipalmitoyl-lecithin: assignment of the ^{1}H and ^{13}C nuclear magnetic resonance spectra, and conformational studies. *J. Chem. Soc., Perkin Trans.* **2**:1441–1445 (1972).
8. R. D. Hershberg, G. H. Reed, A. J. Slotboom, and G. H. de Haas. Nuclear magnetic resonance studies of the aggregation of dihexanoyllecithin and of diheptanoyllecithin in aqueous solutions. *Biochim. Biophys. Acta* **424**:73–81 (1976).
9. D. Marsh and A. Watts. NMR spin-spin splittings in lipid membranes. *FEBS Lett.* **85**:124–126 (1978).
10. H. Hauser, W. Guyer, I. Pascher, P. Skrabal, and S. Sundell. Polar group conformation of phosphatidylcholine. Effect of solvent and aggregation. *Biochemistry* **19**:366–373 (1980).
11. X. Han, X. Chen, and R. W. Gross. Chemical and magnetic inequivalence of glycerol protons in individual subclasses of choline glycerophospholipids: implications for subclass-specific changes in membrane conformational states. *J. Am. Chem. Soc.* **113**:7104–7109 (1991).
12. E. G. Bligh and W. J. Dyer. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37**:911–917 (1959).