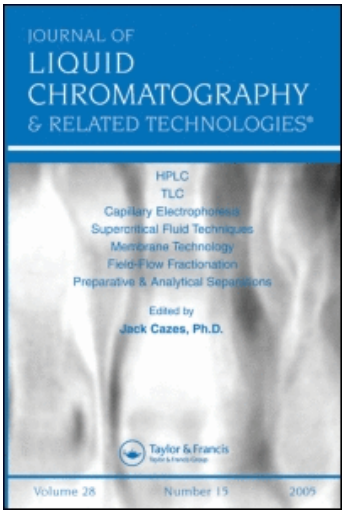

Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Simultaneous Quantification of Complex Phospholipid Compositions Containing Monophosphoryl Lipid-A by RP-HPLC

Karola Vorauer-Uhl[a]; Dominik Jeschek[a]; Gabriele Lhota[a]; Andreas Wagner[b]; Stefanie Strobach[a]; Hermann Katinger[ab]

[a] Department of Biotechnology, University of Natural Resources and Applied Life Sciences, Vienna, Austria [b] Polymun Scientific, Immunbiologische Forschung GmbH, Vienna, Austria

To cite this Article Vorauer-Uhl, Karola , Jeschek, Dominik , Lhota, Gabriele , Wagner, Andreas , Strobach, Stefanie and Katinger, Hermann(2009) 'Simultaneous Quantification of Complex Phospholipid Compositions Containing Monophosphoryl Lipid-A by RP-HPLC', Journal of Liquid Chromatography & Related Technologies, 32: 15, 2203 — 2215

To link to this Article: DOI: 10.1080/10826070903163180

URL: http://dx.doi.org/10.1080/10826070903163180

Journal of Liquid Chromatography & Related Technologies®, 32: 2203–2215, 2009

ISSN: 1082-6076 print/1520-572X online
DOI: 10.1080/10826070903163180

Simultaneous Quantification of Complex Phospholipid Compositions Containing Monophosphoryl Lipid-A by RP-HPLC

Karola Vorauer-Uhl,[1] Dominik Jeschek,[1] Gabriele Lhota,[1] Andreas Wagner,[2] Stefanie Strobach,[1] and Hermann Katinger[1,2]

[1]Department of Biotechnology, University of Natural Resources and Applied Life Sciences, Vienna, Austria
[2]Polymun Scientific, Immunbiologische Forschung GmbH, Vienna, Austria

Abstract: High performance liquid chromatography (HPLC) is a powerful technique for lipid quantification. However, for the characterization of lipid based vaccines more sensitive, high resolution methods for different lipid compositions are still evident. For this purpose, we have established a RP-HPLC method for the simultaneous quantification of saturated and unsaturated phospholipids with different acyl-chains and head groups, cholesterol and monophosphoryl Lipid-A without labeling. A novel ELSD feature enables adjustment of sensitivity within one run. By this method, we could demonstrate that complex lipid formulations can be quantified in concentrations of few micrograms but also in milligram amounts with the same performance characteristics.

Keywords: ELSD, Liposomes, Monophosphoryl Lipid-A, MPLA, Phospholipids, RP-HPLC

INTRODUCTION

High performance liquid chromatography (HPLC) is a commonly used technique for lipid quantification. For complex lipid compositions such

Correspondence: Karola Vorauer-Uhl, Department of Biotechnology, University of Natural Resources and Applied Life Sciences, Muthgasse 18, A-1190 Vienna, Austria. E-mail: karola.vorauer-uhl@boku.ac.at

as liposomal formulations, there is still a need to develop more sensitive and high resolution techniques. Liposomes, which consist of different phospholipids but also other bilayer forming molecules, are investigated for drug delivery and vaccination strategies. For vaccines, primarily mixtures of phosphatidylcholins, phosphatidylglycerols, and cholesterol combined with monophosphoryl Lipid-A (MPLA) as adjuvant are applied.[1–3] For their characterization, analytical methods must be selected carefully. Presently, different methods for the simultaneous quantification of phospholipids and cholesterol are available but none that include monophosphoryl Lipid-A.

Lipids like phosphatidylcholins and phosphatidylglycerols are predominantly separated on normal phase HPLC columns using bi-, respectively, quaternary gradients[4] with frequently applied mobile phases such as hexane, chloroform, and different alcohols. Silica, alumina, aminopropyl, cyanopropyl, and diol, as stationary phases, interact with the more polar part of the molecules. Alternatively, on reversed phase columns lipophilic molecules are typically bound to an "inert" substrate. Most common forms are C_8 and C_{18} phases. By definition, the mobile phase of RP-HPLC systems is more polar than the stationary phase. Acetonitrile, different alcohols, and water are some of the commonly used mobile phases. However, for the separation of phosphatidylcholins, and phosphatidylglycerols the RP-HPLC separation is less frequently used due to its insufficient resolution with conventional gradients.[5]

Cholesterol, which is a common membrane stabilizing molecule, is frequently present in liposomal formulations in varying amounts between 10 and 50 mole percent. Its quantification is uncritical and can be performed with both HPLC techniques.[6]

MPLA is currently analyzed separately by more complex methods based on semi-quantitative detection by mass spectroscopy or UV detection at 254 nm combined with RP-HPLC separation, after labelling with 3,5-dinitrobenzyloxyyamine in pyridine.[7]

Since complex liposomal formulations are gaining more and more interest for therapeutic application, therefore, simultaneous quantification methods of singular components are favorable for product release.

Especially, the insufficient separation of the lipids by certain HPLC methods, unbalanced lipid ratios and the varying detection limits of these membrane integrated molecules are critical parameters. Furthermore, the solubility of these compounds differs significantly in individual solvents. Hence, the selected solvent must be suitable to dissolve all molecules quantitatively, facilitating adsorption onto the stationary HPLC phase but avoiding precipitation onto the column.

Sophisticated separation methods must be combined with adequate detection systems.[8] For the simultaneous detection, low temperature

evaporative light scattering detectors (ELSD) are gaining more and more interest due to their high sensitivity and their applicability for different classes of lipophilic molecules. However, their appropriateness for this intended approach needs to be demonstrated.[9]

In the present work, we introduce a reversed phase HPLC method applicable for complex lipid mixtures, which demonstrates high resolution properties combined with variable adjustable detection. The challenge was based on the completely different solubility properties of the lipophilic molecules, rather similar retention behaviour of phospholipids, and different sensitivities.

EXPERIMENTAL

Materials

1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), 1,2-dimyristoyl-sn-glycero-3-[phospho-rac-(1-glycerol)] (DMPG) were purchased from Lipoid (Ludwigshafen, Germany). Cholesterol was obtained from Solvay, (Weesp, The Netherlands) and monophosphoryl Lipid-A from Avanti Polar Lipids (Alabaster, AL, USA). The purity of all materials was higher than 99%.

HPLC analysis was performed with gradient grade isopropanol (IP), methanol (MeOH) and trifluoracetic acid (TFA). All solvents including chloroform were purchased from Merck (Darmstadt, Germany). The purity of chloroform and trifluoracetic acid was >98%.

Preparation of Standard Stock-Solutions

About 12.5 mg of each substance, except MPLA, were dissolved separately in a mixture of chloroform/isopropanol (6/94%, v/v) and diluted to a final concentration of 250 μg/mL. MPLA was dissolved in chloroform to a final concentration of 1 mg/mL. Stock solutions were mixed to obtain final concentrations of 90, 70, 50, 30, and 10 μg/mL in chloroform/isopropanol (10/90%, v/v), respectively.

HPLC-Equipment

All analyses were performed on a HPLC 1200 system (Agilent Technologies, Vienna, Austria). The system was equipped with a binary pump, an online membrane degasser, a temperature controlled autosampler, and

column oven. Detection was performed with a low temperature evaporative light scattering detector, Sedex-85 (Sedere, Alfortville, France). For separation a Luna C_{18} column, 5 μm, 100 A (OOF-4041-EO, Phenomenex LTP, Aschaffenburg, Germany) was used.

Chromatographic Conditions

The separation was performed at a constant flow rate of 1 mL/min at 30°C. The gradient is shown in Table 1. Samples were tempered at 10°C in the autosampler and 20 μL aliquots were injected. Detection was performed with varying gains of 7, 10, 11, and 12. Filter settings were adjusted to 10 s. For evaporation, inert gas flow was adjusted to 3.5 bar at 40°C.

Calculation

Calibration curves were calculated for a quantification range of 10–90 μg/mL using 5 standard concentration levels. For DMPC an additional calibration curve in the working range between 50–1000 μg/mL was calculated using 6 levels. For both calibrations, the individual levels were analyzed in triplicates. Calibration functions, their correlation coefficient, quantification limits, selectivity factors, and resolution were

Table 1. The timetable of the finalized gradient with solvent A (95% methanol/5% water) and solvent B (100% isopropanol/0.1% TFA)

Gradient (min)	Solvent-A (%)	Solvent-B (%)
0.00	95	5
32.00	95	5
34.00	80	20
42.00	80	20
43.00	60	40
51.00	60	40
52.00	40	60
60.00	40	60
61.00	20	80
69.00	20	80
70.00	0	100
78.00	0	100
82.00	95	5
90.00	95	5

calculated using Microsoft Excel. Raw data analyses were calculated online with Agilent software, version 3.8. Quantification limits for all substances were calculated by the signal/noise ratio. Peak heights were taken by the chromatograms and the corresponding noise was calculated by the peak to peak method in a separate run. Selectivity factor and resolution of subsequent peaks were calculated as follows. The selectivity factor $\alpha = k'_B/k'_A$, in which k' is the retention factor calculated as $k' = (t_R - t_M)/t_M$, whereas t_R is the retention time of an individual peak and t_M the time taken for the mobile phase to pass through the column. The resolution (R) was calculated as $R = 2[(t_R)_B - (t_R)_A]/(W_A + W_B)$. W is the peak width of the corresponding peak, A and B are adjacent peaks.

RESULTS AND DISCUSSION

Solubility and Mode of Separation

The development of a HPLC method for simultaneous quantification of lipophilic molecules includes selection of adequate solvents to dissolve all components, optimization of an appropriate separation mode, fine tuning of the detection performance, and statistical analysis of the performance characteristics.

Conventional polar solvents like water and buffer solutions are inconvenient for phospholipids. However, alcohols, which are also defined as polar, respectively slightly non-polar solvents such as methanol and isopropanol, are suitable to dissolve cholesterol, phosphocholines (PC), and phosphoglycerols (PG) but not MPLA. For monophosphoryl Lipid-A, pure chloroform or a mixture of chloroform/methanol (2:1) are recommended. In order to prepare calibration standards containing all analytes, we dissolved MPLA in pure chloroform and all other molecules in chloroform/isopropanol. By mixing them, a final isopropanol/chloroform ratio of 90% IP (v/v) was demonstrated to obtain stable standard solutions in defined concentrations. This solvent ratio was sufficient to prepare stable standard stock solutions and also prevented precipitation onto the column.

With this matrix the RP-HPLC method was developed. The advantage of reversed phase HPLC systems is the availability of stable and reproducible stationary phases, which is at least important for method validation. In particular, column bleeding, which is a minor concern in UV detection but is of major relevance in ELS detection, leads to inadequate baselines, respectively ghost peaks, and must be prevented.[10] Furthermore, RP-columns can be easily equilibrated between the runs and are applicable for many analytes and solvents. Another argument

is that the stationary phases are not plagued by an abundance of secondary interactions with the analytes.

To define the mobile phase for the separation of phospholipids, cholesterol, and MPLA, various methanol/isopropanol gradients were tested by linear and step elution profiles. Methanol, applied as mobile phase-A is definitely polar because the –OH group dominates the molecule. The –OH group of isopropanol, used as mobile phase-B still gives a polar effect but the total of free carbons and multiple hydrogens induces slightly non-polar properties. Trifluoracetic-acid in solvent-B reduces the binding strength by ionization of DMPG, which improves its elution prior to MPLA. Finally, an isocratic-step methanol-isopropanol-TFA gradient as demonstrated in Table 1 proved to be the most suitable.

Phosphocholins and cholesterol were separated under isocratic conditions with 95% methanol. DMPG and MPLA eluted with a step gradient with increasing concentrations of isopropanol and TFA up to 80% isopropanol. With this optimized gradient, the substances elute in the order DMPC, cholesterol, DPPC, POPC, DMPG, and MPLA as shown in Figure 1. The combination of an isocratic and a step gradient was developed empirically, since the separation of phospholipids (PLs) is especially difficult to predict as they consist of different polar and non-polar moieties. Nevertheless, the ionic charges of the phosphate

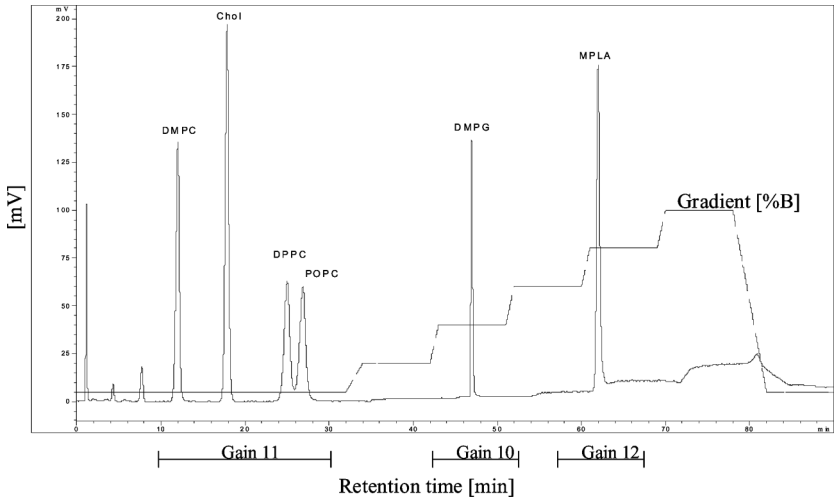

Figure 1. Separation of individual standard molecules in a concentration of 90 µg/mL. Standard mixture was prepared in 10% chloroform/90% isopropanol (v/v). The isocratic/stepwise gradient is illustrated in Table 1. Detection was performed at different gains.

and the polar head groups vary depending on the applied mobile phase and, therefore, the elution order in our case is different than the literature.[11]

ELSD

Besides the mode of separation, the mode of detection needs to be adjusted carefully. In principle, most of the PLs are not sufficiently measured with commonly used UV detectors. Alternatively, mass spectroscopy, refractive index, or evaporative light scattering detectors are used. In particular, ELSDs gain more and more interest due to their high sensitivity, compatibility with most solvents used in HPLC application, and suitability for step and linear gradients. The applied ELSD enables individual gain adjustment within the gradient, as shown in Figure 1, by an integrated supplementary function. This option is necessary for analytes with low signal intensities but even more for samples with significantly different amounts of lipids. Adjusting a higher gain that induces increased voltage on the photomultiplier facilitates the improved sensitivity. Thereby the individual detection limit of a substance is reached at lower volumetric concentrations. On the other hand, by decreasing the gain sensitivity, molecules with high intensity signals or higher volumetric amounts can also be quantified in higher concentrations. These adjustments enable optimal working ranges individually adapted to the lipid ratios in liposomal samples. Figure 2 demonstrates the increase of the peak intensities measured with different gains. Changing the gain from

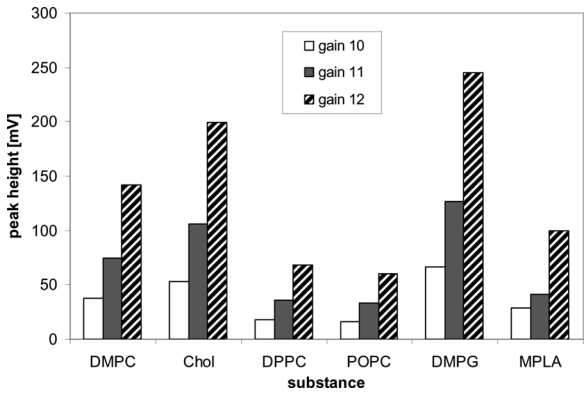

Figure 2. Peak heights of the subsequent molecules at different gains are illustrated. Analyses were performed with standard solutions containing each substance in a concentration of 50 μg/mL.

10 to 11 and 12 enhances the peak height approximately twofold. Nevertheless, the effect of gain adjustment is not predictable and must be optimized empirically. Additionally, corresponding blank runs are required to adjust sensitivity properly, since the increase of the gain may also increase baseline noise.

Statistical Analysis of Performance Characteristics

The calculation of calibration functions is commonly solved with linear equations. Using conventional systems, a linear correlation between the amount and the signal is expected following the Lambert-Beer-Law. In ELSD the concentration related peak area is defined by light scattering intensities following a non-linear correlation. The intensity of the scattered light is a function of the mass scattered particles and is calculated by the exponential equation: $I = k \times m^b$. The signal intensity (I) is determined by the mass of the analyte (m) and two nebulization factors, (k and b), which are affected by the solvents and the evaporation temperature. Therefore, the calibration function needs to be estimated carefully, using more standard levels within the working range compared to UV methods. Several authors suggest that linear working ranges are obtained by selecting a narrow concentration range.[4] This approach cannot be generalized and must be determined individually. For our application, we have found that the calibration is non-linear and the linearization would limit the working range in a too narrow range.

The quantification of all analytes in similar concentration ranges between 10 and 90 μg/mL was performed at gain 11 for DMPC, Chol, DPPC, and POPC, which elute isocratically. DMPG was measured at gain 10 and MPLA at gain 12. Figure 3 shows second ordered polynomial curve fittings of all lipids. The correlation coefficients of all substances are in the range of 0.999.

This calibration range is practicable for liposomal compositions with lower cholesterol and MPLA concentrations and balanced ratios of phosphocholins (PC) and phosphoglycerols (PG).[12] Nevertheless, in certain lipsomal formulations the PC content is ten times higher than PG.[2,13] In this case, PCs need to be calibrated in a different working range. As exemplified for DMPC, we have adjusted the working range to 50–1000 μg/mL by reducing the gain at level 7. Transformation of the calibration ranges can be properly performed as demonstrated in Figure 4. Just these individual gain adjustments within one run enable the simultaneous quantification of unbalanced liposomal components more efficiently than with other detectors used for lipid analysis.

In the next step, we evaluated performance parameters by calculating the signal to noise ratios, selectivity factors, and resolution for each

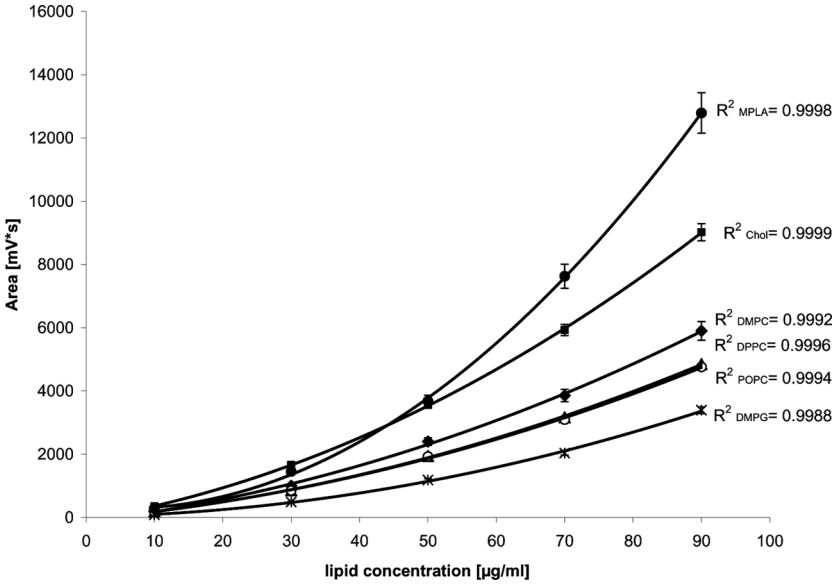

Figure 3. All substances were measured in a concentration range between 10–90 µg/mL. Each concentration level was measured in triplicates. The calibration curves fit to second order polynomial functions with correlation coefficients in the range of 0.999.

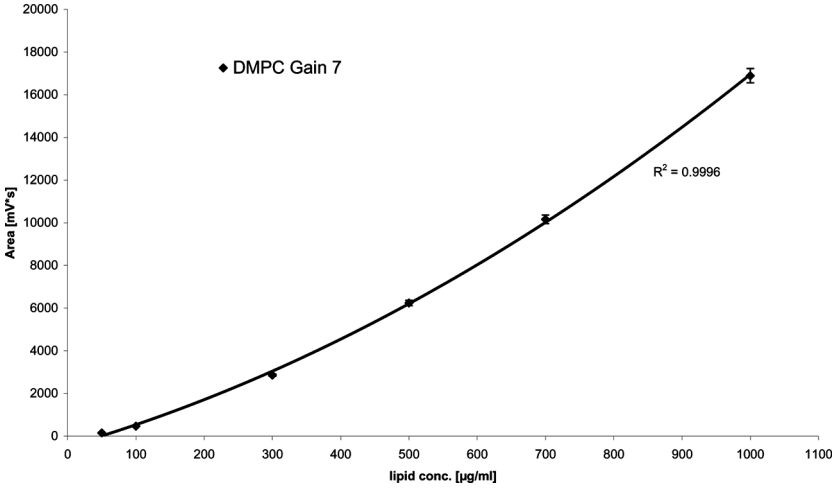

Figure 4. DMPC was measured in a concentration range between 50–1000 µg/mL. Each concentration level was measured in triplicates. The calibration curve fits to second order polynomial functions with a correlation coefficient of 0.9996.

Table 2. Signal to noise ratios were estimated at the lowest working concentration of 10 μg/mL each. The corresponding noise was estimated in a blank run by the peak to peak method

Lipids	Noise–P to P (mV)	Peak height (mV)	Signal/noise ratio
DMPC	0.37	6.13	16.69
Cholesterol	0.24	10.42	43.54
DPPC	0.29	3.39	11.88
POPC	0.25	3.31	13.49
DMPG	0.14	5.63	41.43
MPLA	1.01	10.12	10.05

substance. Therefore, we considered the appropriateness of the working range at the lowest standard concentration (Table 2). The baseline noise was measured in an independent blank run with the peak to peak method (noise-P to P).

The signal to noise ratios (column 4 in Table 2) comply with the international requirements for the limit of quantification, which is defined by a factor ten between the peak height (column 3 in Table 2) and the corresponding noise (column 2 in Table 2). The individual ratios considerably exceed the required factor of ten, proving that 10 μg/mL are well accepted as quantification limits. Similar results were obtained for the 50 μg level of DMPC calibrated at gain 7. In the case of cholesterol and DMPG, the Signal to Noise ratios were significantly higher compared to all other lipids. This offers the possibility to adjust the limit of quantification lower than 10 μg/mL.

Selectivity factors and resolution are routinely estimated in method development. The selectivity factor is a parameter that calculates the separation of two substances just by the migration rate at the peak maximum.

Column 4 in Table 3 describes the selectivity factor (alpha) of two adjacent peaks. The resolution considers the peak width (column 5 and 6 in Table 3 for 10 and 90 μg/mL), which is often increased at lower concentrations. Especially, the resolution between DPPC and POPC seemed to be critical due to their close retention times and their peak forms, which were broader than those of the other analytes. However, the calculated resolution (column 7 and 8 in Table 3) at both concentrations was significantly higher than 1.5 with values of 2.1 and 3.2 and, therefore, per definition sufficient for quantification.

The retention time (column 9 in Table 3) that defines the migration of each substance was additionally evaluated. We have observed very

Table 3. Selectivity factor and resolution of subsequent peaks were calculated. The selectivity factor $\alpha = k'_B/k'_A$, in which k' is the retention factor calculated as $k' = (t_R - t_M)/t_M$ that describes the separation of two species on the column. The resolution (R) was calculated as $R = 2[(t_R)_B - (t_R)_A]/(W_A + W_B)$. W is the peak width of corresponding peak. Resolution is acceptable if $R \geq 1.5$

	t_M (min)	t_R (min)	Selectivity factor (α)	Peak width (10 μg/mL)	Peak width (90 μg/mL)	Resolution 10 μg (R)	Resolution 90 μg (R)	Retention time (min)
DMPC	2.5	11.92		0.45	0.35			11.92 ± 0.08
Cholesterol	2.5	17.75	1.62	0.52	0.36	12.0	16.4	17.75 ± 0.09
DPPC	2.5	24.78	1.46	0.83	0.55	10.4	15.4	24.78 ± 0.20
POPC	2.5	26.63	1.08	0.93	0.61	2.1	3.2	26.63 ± 0.21
DMPC	2.5	46.82	1.84	0.23	0.22	35.1	48.9	46.82 ± 0.16
MPLA	2.5	62.50	1.35	0.48	0.37	44.8	53.6	62.50 ± 0.02

constant retention times within all repeats at one concentration and between different concentrations. This result demonstrates on the one hand, the stability and reproducibility of the separation and on the other hand, the reproducibility of the evaporation, which is caused by the low temperature nebulization and the constant gas flow of the detector.

CONCLUSION

The reversed phase HPLC method that has been developed for complex lipid compositions is suitable for the simultaneous quantification of the most commonly used components in liposomal vaccines. In particular, we could demonstrate for the first time that a methanol/isopropanol gradient enables the selective separation of phospholipids, cholesterol, and MPLA directly and without labeling of MPLA. Additionally, our data revealed that the individual mode of detection for each substance is a powerful opportunity to adjust the calibration ranges according to individually composed formulations. Therefore, we have documented that high resolution RP-HPLC combined with individual fine tuning of selectivity offers a promising technique for research and development as well as product release applications.

REFERENCES

1. Babai, I.; Barenholz, Y.; Zakay-Rones, Z.; Greenbaum, E.; Samira, S.; Hayon, I.; Rochman, M.; Kedar, E. A novel liposomal influenza vaccine (INFLUSOME-VAC) containing hemagglutinin-neuraminidase and IL-2 or GM-CSF induces protective anti-neuraminidase antibodies cross-reacting with a wide spectrum of influenza A viral strains. Vaccine **2001**, *20* (3–4), 505–515.
2. Fries, L.; Gordon, D.; Richards, R.; Egan, J.; Hollingdale, M.; Gross, M.; Silverman, C.; Alving, C. Liposomal malaria vaccine in humans: A safe and potent adjuvant strategy. Proc. Natl. Acad. Sci. USA **1992**, *89* (1), 358–362.
3. Alving, C.; Rao, M. Lipid A and liposomes containing lipid A as antigens and adjuvants. Vaccine **2008**, *26* (24), 3036–3045.
4. Descalzo, A.; Insani, E.; Pensel, N. Light-scattering detection of phospholipids resolved by HPLC. Lipids **2003**, *38* (9), 999–1003.
5. Mounts, T.; Abidi, S.; Rennick, K. HPLC analysis of phospholipids by evaporative laser light-scattering detection. J. Am. Oil Chem. Soc. **1992**, *69* (5), 438–442.
6. Lang, J. Quantitative determination of cholesterol in liposome drug products and raw materials by high-performance liquid chromatography. J. Chromatogr. **1990**, *507*, 157–163.
7. Hagen, S.; Thompson, J.; Snyder, D.; Myers, K. Analysis of a monophosphoryl lipid A immunostimulant preparation from Salmonella minnesota

R595 by high-performance liquid chromatography. J. Chromatogr. A **1997**, *767* (1–2), 53–61.
8. Peterson, B.; Cummings, B. A review of chromatographic methods for the assessment of phospholipids in biological samples. Biomed. Chromatogr. **2006**, *20* (3), 227–243.
9. Shimizu, Y.; Nakata, M.; Matsunuma, J.; Mizuochi, T. Simultaneous quantification of components of neoglycolipid-coated liposomes using high-performance liquid chromatography with evaporative light scattering detection. J. Chromatogr. B Biomed. Sci. Appl. **2001**, *754* (1), 127–133.
10. Megoulas, N.; Koupparis, M. Twenty years of evaporative light scattering detection. Crit. Rev. Anal. Chem. **2005**, *35* (4), 301–316.
11. Zhai, D.; Reilly, P. Effect of FA chain length on normal- and reversed-phase HPLC of phospholipids. J. Am. Oil Chem. Soc. **2002**, *79* (12), 1187–1190.
12. Arigita, C.; Luijkx, T.; Jiskoot, W.; Poelen, M.; Hennink, W.; Crommelin, D.; van der Ley, P.; van Els, C.; Kersten, G. Well-defined and potent liposomal meningococcal B vaccines adjuvated with LPS derivatives. VACCINE **2005**, *23* (43), 5091–5098.
13. Childers, N.; Miller, K.; Tong, G.; Llarena, J.; Greenway, T.; Ulrich, J.; Michalek, S. Adjuvant activity of monophosphoryl lipid A for nasal and oral immunization with soluble or liposome-associated antigen. Infect. Immun. **2000**, *68* (10), 5509–5516.

Received February 4, 2009
Accepted March 9, 2009
Manuscript 6477