Journal of Pharmaceutical and Biomedical Analysis 48 (2008) 702–707

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Quantification of pegylated phospholipids decorating polymeric microcapsules of perfluorooctyl bromide by reverse phase HPLC with a charged aerosol detector

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ARTICLE INFO

Article history:
Received 11 March 2008
Accepted 28 June 2008
Available online 23 July 2008

Keywords:
HPLC
Charged aerosol detection
Pegylated phospholipids
Polymeric microcapsules
Perfluorooctyl bromide

ABSTRACT

Polyethylene glycol (PEG) chains covalently linked to phospholipids are often used in the preparation of lipid or even polymer colloidal particles to avoid recognition and clearance by the reticuloendothelial system and to increase their plasmatic half-life. To the best of our knowledge, no direct method allows yet to quantify these pegylated phospholipids. The aim of this work was to develop a method for the quantification of a typical pegylated phospholipid, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-2000], DSPE-PEG2000, associated to polymeric microcapsules of perfluorooctyl bromide (PFOB). Reverse phase high-performance liquid chromatography (HPLC) was used, coupled with a corona charged aerosol detection (HPLC–CAD). Calibrations standards consisted of plain microcapsules and pegylated phospholipids (DSPE-PEG2000) in the concentration range of 2.23–21.36 μg/mL (0.22–2.14 μg injected). Calibration curve was evaluated with two different model, linear and power model. The power model describes experimental values better than the linear model, for pegylated phospholipids with the CAD detector. The correlation coefficient for the power model was 0.996, and limits of detection and quantification obtained were 33 and 100 ng, respectively. This method proved to be selective and sensitive; the accuracy of the method ranged from 90 to 115% and the relative standard deviation was ≤5.3%. Pegylated phospholipids associated to microcapsules, as well as the phospholipids and total phospholipids in the suspensions were successfully quantified in three different preparations of microcapsules.

1. Introduction

Recent ultrasound contrast agents (UCAs) generally consist of polymer microcapsules encapsulating gaseous perfluorocarbons. These agents are injected intravenously in a strategy to better visualize specific tissues [1,2]. Due to their rather hydrophobic surface, these microcapsules are quickly eliminated by the reticuloendothelial system and end up in the liver. To avoid a rapid clearance from the systemic circulation, it has been shown that covering particle's surface with polyethylene glycol (PEG) is very efficient [3,4]. Pegylation can be achieved by different methods such as physical adsorption, covalent grafting or using PEG copolymers [5–7]. By its hydrophilic nature PEG provides protection from blood proteins adsorption and uptake by the reticuloendothelial system is drastically reduced [8]. Furthermore, PEG is non-toxic, and chains shorter than 30 kDa are readily eliminated from the body by renal filtration [9]. PEG can therefore be considered biocompatible and safe [7,10].

In this work, microcapsule surface was modified using a pegylated phospholipid: 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-2000], noted DSPE-PEG2000 (Fig. 1), following a recent method developed for drug targeting [11]. Because of the micrometric size of the system developed, most of the qualitative techniques for the surface characterization of smaller pegylated nanoparticles and liposomes could not be used [3,12–15]. A method to directly quantify the amount of pegylated lipids associated to microcaspules was then developed based on HPLC and charged aerosol detection.

Until recently the evaporative light scattering detector (ELSD) was the most commonly used detector for lipid analysis. Dixon and Peterson [16] described a new detection principle using the charged aerosol detection (CAD). In the CAD, the eluent is nebulized and droplets are dried to remove the mobile phase, producing non-volatile analyte particles. A secondary stream of gas becomes positively charged as it passes a high-voltage platinum corona wire.

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doi:10.1016/j.jpba.2008.06.027

This charge transfers to the opposing stream of analyte particles and is then transferred to a collector where it is measured by a highly sensitive electrometer. The signal intensity generated by a CAD is said to be more sensitive than with ELSD in most cases [17]. High-performance liquid chromatography (HPLC) with a corona charged aerosol detection (HPLC–CAD) has been reported to be a sensitive technique for the direct identification and quantification of lipids at low concentrations [18,19].

The main objective of the present work was to develop a reverse phase HPLC method with CAD detection for the quantification of pegylated phospholipids associated to polymeric microcapsules of perfluorooctyl bromide (PFOB). To the best of our knowledge this is the first quantitative method for the analysis of pegylated phospholipids associated to polymeric microcapsules. We show that by using this method it is possible to obtain adequate quantitative data in a short run time (20 min only) using directly microcapsules. This method could easily be adapted to the quantification of pegylated phospholipids in any other colloidal carrier.

2. Materials and methods

2.1. Materials

Methylene chloride RPE-ACS 99.5% was purchased from Carlo Erba Reactifs (France). Chloroform and methanol were of HPLC-grade and purchased from VWR International (France). Acetic acid 99–100% RECTAPUR and ammonia solution at 32% RECTAPUR were purchased from VWR International (France). Water was purified using a RIOS system from Millipore (France). Poly(lactide-*co*-glycolide) acid 50:50, PLGA (Resomer RG502) was provided by Boehringer-Ingelheim (Germany). Sodium cholate (SC) and Nile Red were obtained from Sigma–Aldrich. PFOB was purchased from Fluorochem (UK). Pegylated phospholipids (1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[poly(ethylene glycol) 2000-*N*-carboxyfluorescein] (DSPE-PEG-CF), and DSPE-PEG2000 were provided as chloroform solutions by Avanti Polar Lipids Inc. (USA).

2.2. Instrumentation and chromatographic conditions

The chromatographic system consisted of a Hewlett Packard HP 1050 pump connected to a Rheodyne manual injection valve, equipped with a 100 μL sample loop. The analytical column was a Zorbax Eclipse XDB-C8, 150 mm × 4.6 mm and I.D., 5 μm (Agilent-Technologies, France). Detection was performed with the corona CAD, a charged aerosol detector (ESA Biosciences, USA). The sensitivity was 200 pA, the air pressure was of 35 psi with a "medium filter" setting. Data acquisition and peak integrations were performed on a Kroma System 2000 software (Kontron Instruments, France). The mobile phase consisted of acetonitrile, methanol, ammonia and acetic acid (84.63:15:0.26:0.11, v/v/v/v) (phase A) and methanol, ammonia and acetic acid (99.63:0.26:0.11, v/v/v) (phase B). The different components of the plain microcapsules and pegylated microcapsules were separated by running a gradient starting at 100% mobile phase A, decreasing to 40% A in 9 min and then back to 100% A in 1 min and finally isocratic condition 100% A for 10 min. Total run time for each sample was set to 20 min.

2.3. Samples preparation

2.3.1. Preparation of plain microcapsules added with DSPE-PEG2000 as standards

Plain microcapsules were prepared by modifying the classical solvent emulsion/evaporation method for microspheres to obtain microcapsules with a polymeric shell encapsulating PFOB [20]. Briefly, PLGA (0.1 g) was dissolved into 4 mL of methylene chloride along with 60 μL of PFOB and the desired amount of phospholipids dissolved into chloroform. The organic solution was placed in a thermostated bath maintained at 20 °C to ensure full miscibility of the PFOB. It was then emulsified into 20 mL of 1.5% (w/v) sodium cholate aqueous solution using an Ultra-turrax T25 (IKA, France) operating with a SN25-10G dispersing tool at a velocity of 8000 rpm. Emulsification was performed in a 50 mL beaker placed over ice. Methylene chloride and chloroform were then evaporated by magnetic stirring for about 3 h at 300 rpm in a thermostated bath (20 °C). For fluorescent or confocal microscopy, Nile Red was added to the organic solution prior to emulsification to label the polymer. Typically, about 100 μL of a concentrated Nile Red solution (0.057 mg/mL in methylene chloride) was added to the organic solution. After full evaporation of the solvents, the suspension volume was completed to 20 mL with Milli-Q water in a volumetric flask and fresh microcapsules were frozen at −20 °C. Samples were then freeze-dried for 48 h using a LYOVAC GT2. Then different solutions for the calibration curve were prepared by dissolving 200 mg of freeze dried plain microcapsules first into 6.5 mL chloroform before adding 3.3 mL of methanol. Then different volumes of DSPE-PEG2000 solution (440 μg/mL): 50, 100, 200, 300, 400 and 500 μL were added to obtain the following DSPE-PEG2000 concentrations: 2.23, 4.44, 8.80, 13.07, 17.25 and 21.36 μg/mL. Solutions were filtered on a 0.22 μm PTFE filter prior to injection and analysis.

2.3.2. Preparation of pegylated microcapsules

For pegylated microcapsules, the preparation was similar to what was described for plain microcapsules, except that the DSPE-PEG2000 was introduced in the organic phase with PFOB and PLGA. Three independent preparations of pegylated microcapsules were prepared, each one with a different volume of pegylated lipids at 440 μg/mL in chloroform: 100, 300 and 500 μL. For each preparation of pegylated microcapsules the total, free and associated DSPE-PEG2000 were quantified. For the quantification of total pegylated phospholipids, microcapsules after their fabrication were frozen at −20 °C and then freeze-dried for 48 h using a LYOVAC GT2. In the case of free and associated pegylated lipids, pegylated microcapsules were separated from the aqueous solution containing the free lipids and the surfactant by centrifugation (2000 × *g*, 10 min, 4 °C) (MR 1812 centrifuge, Jouan, France). Microcapsules were resuspended with water (5 mL) by vortexing (30 s). Then microcapsules and supernatants were frozen separately and freeze-dried in the same conditions. Freeze-dried pellets of microcapsules were dissolved by adding first 2 mL of chloroform followed by 1 mL of methanol. Volumes of chloroform and methanol used to dissolve the freeze-dried pellets of supernatant were adjusted according to the initial concentration of DSPE-PEG in the sample. As

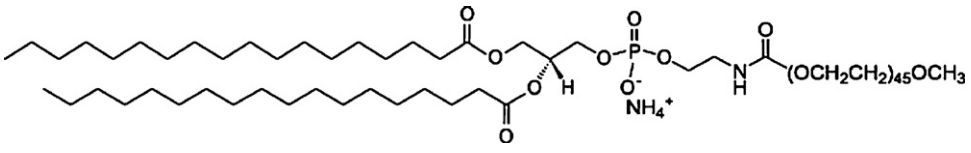

Fig. 1. Chemical structure of the pegylated phospholipid, DSPE-PEG2000.

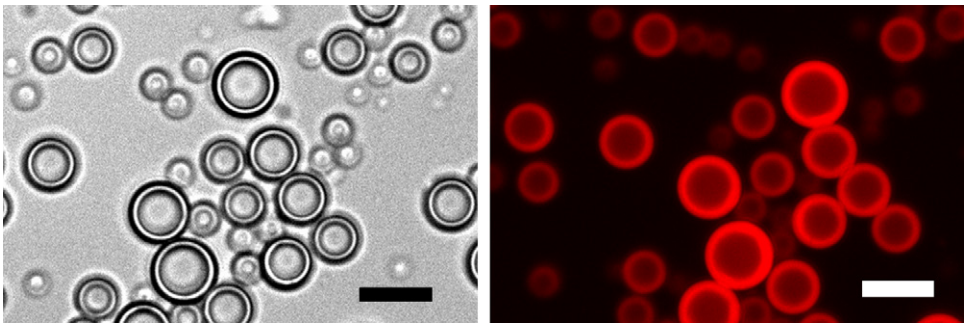

Fig. 2. Microscopy images of a suspension of microcapsules with DSPE-PEG, top with 57.2 μg (at 440 μg/mL). Bright field is presented on the left, whereas fluorescence is on the right (the polymer appears red, whereas PFOB is not fluorescent). Scale bars represent 10 μm in both images. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

an example, for the preparation obtained with 100 μL DSPE-PEG (at 440 μg/mL), volumes were 2 and 1 mL (chloroform and methanol), whereas for the one obtained with 300 μL the volumes were 7.8 and 3.9 mL. Solutions were filtered on a 0.22 μm PTFE filter prior to injection and analysis.

2.4. Characterization of pegylated microcapsules

2.4.1. Optical and fluorescence microscopy

Microcapsule suspensions were placed between glass slides and observed with a Leitz Diaplan microscope equipped with a Coolsnap ES camera (Roper Scientific, France). Fluorescent samples dyed with Nile Red were excited at 543 nm and observed at 560 nm (long-pass filter). Fluorescent samples dyed with fluorescein were excited at 488 nm and observed between 505 and 550 nm (band-pass filter).

2.4.2. Confocal microscopy

Glass slides were examined with a Zeiss LSM-510 confocal scanning laser microscope equipped with a 1 mW helium neon laser, using a Plan Apochromat 63× objective (NA 1.40, oil immersion). Red fluorescence was observed with a long-pass 560 nm emission filter and under a 543 nm laser illumination. Green fluorescence was observed with a band-pass emission filter between 505 and 550 nm, under a 488 nm laser illumination. The pinhole diameter was set at 71 μm. Stacks of images were collected every 0.42 μm along the *z*-axis.

2.5. Statistical analysis

The linear regression was calculated using Microsoft Excel v.5 and the statistical software Statgraphic plus v.5.0 whereas non-linear regression using Marquardt's algorithm was implemented using Matlab 6.0 R 12 to statistically assess the power model.

3. Results and discussion

The objective of this work was to develop a method for the quantification of pegylated phospholipids associated to polymeric microcapsules of perfluorooctyl bromide by reverse phase HPLC with CAD detection. Indeed, the exact quantity of pegylated phospholipids is of the utmost importance for surface properties of colloidal carriers. Usually it is only determined indirectly by measuring the zeta potential or the complement activation.

3.1. Localization of pegylated phospholipids in the microcapsules

The addition of pegylated phospholipids did not modify the core-shell morphology of microcapsules as observed by optical, fluorescent and confocal microscopy. When between 2.64 and 57.2 μg DSPE-PEG were added shells were clearly visible by optical microscopy (Fig. 2, left). To underline the structure of the capsules, the fluorescent marker Nile Red was added to the organic solution prior to emulsification. This marker colors the hydrophobic polymer in red but does not color PFOB. Fluorescent microscopy images present spherical particles with a nice red shell of homogeneous thickness and a darker core (Fig. 2, right) as already observed without phospholipids [20]. To ascertain that DSPE-PEG was indeed associated to microcapsule's shell, fluorescently labeled pegylated phospholipids, DSPE-PEG-CF, were used, along with Nile Red as a marker for PLGA. Since PFOB is denser than water (1.93 g/cm^3), as microcapsules were sedimenting, we noticed that the pellet was mostly red, whereas the supernatant was limpid and bright green. This indicates that some phospholipids are not associated to

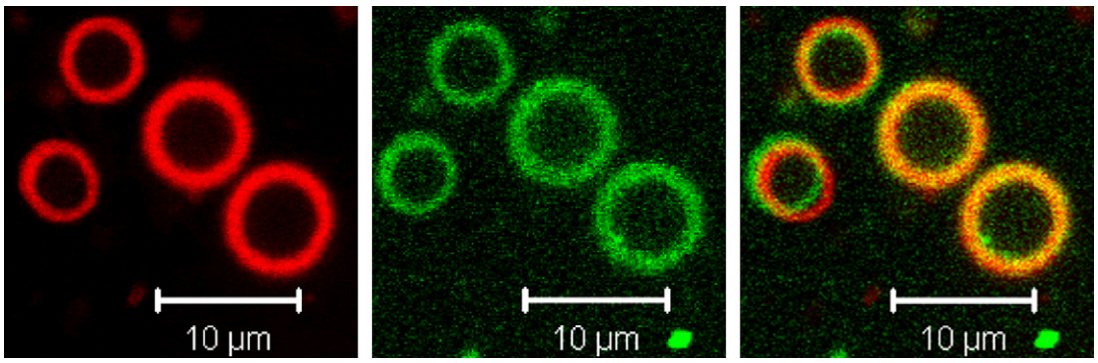

Fig. 3. Confocal microscopy images (scale bar = 10 μm) of a suspension of microcapsules with DSPE-PEG-CF (0.05 mg at 1 mg/mL, 1.58×10^{-5} mmoles). PLGA is dyed in red (left), pegylated lipids appear in green (center) and superposition of both images (right).

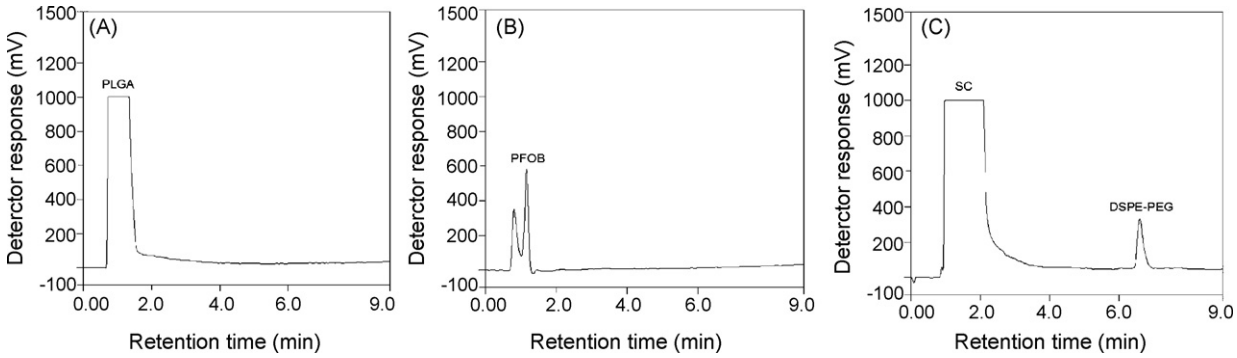

Fig. 4. (A) Chromatogram of PLGA in chloroform corresponding to the 100 mg normally used for the microcapsules preparation (5 mg/mL). (B) Chromatogram of PFOB in chloroform corresponding at 60 μL used for the microcapsules (0.003 mg/mL). (C) Chromatogram of DSPE-PEG in sodium cholate solution at 1.5% (21.95 μg/mL). Conditions are described in the text.

microcapsules but probably solubilized by sodium cholate, a bile salt known to be a good solubilizing agent [21–23]. However, as microcapsule suspensions were examined by confocal microscopy, after centrifugation to eliminate free fluorescent phospholipids, green fluorescence could be observed within the shell. The green fluorescence superimposes perfectly with the red one arising from Nile Red unless capsules have moved (Fig. 3). Despite solubilization of some lipids by sodium cholate, a fraction of pegylated lipids remains associated to microcapsules either at their surface or mixed with the polymer shell. Quantification of lipids associated to microcapsules or solubilized along with the surfactant is of major importance to estimate if our method allows to efficiently cover microcapsule surface.

3.2. Validation of the HPLC–CAD method for the quantification of DSPE-PEG

Generally, for lipid analysis, two chromatographic modes are available. The first one consists of a normal phase method used to separate different class of lipids containing different molecular species. The second one is a reverse phase method used to separate different molecular species from a same class of lipids. Since DSPE-PEG is a single molecular species, it appeared more logical to use a reverse phase separation based on a non-aqueous reverse phase (NARP). Acetonitrile and methanol were used as weak and strong solvent, respectively [24]. In addition, both phases were supplemented with ammonia and acetic acid to fix the state of ionization of the DSPE-PEG polar head and improve the peak symmetry [25,26]. Moreover, our solvent conditions were compatible with the use of chloroform to solubilize our sample.

Plain microcapsules added with DSPE-PEG were chosen as standards for the calibration curve, because standard solutions should have the same composition as samples, particularly for the quantification of total phospholipids in the suspension. This choice also allows controlling precisely pegylated phospholipids concentration and decreasing possible preparation losses.

3.2.1. Selectivity

Possible interference of the different chemicals in the microcapsule suspension was evaluated. Solutions of each chemical were injected using the chromatographic conditions described above. Neither the polymer (PLGA), nor the PFOB dissolved into chloroform, nor the sodium cholate interfered with the peak obtained for DSPE-PEG (retention time = 6.6 min) (Fig. 4). Only the pegylated lipid (DSPE-PEG) was retained while the other solutes (PLGA, PFOB and SC) were eluted in the solvent front, which proves the selectivity of the method. Since chloroform/methanol mixture (2:1, v/v) is a mixture of polar and non-polar solvents [27,28] it has allowed us to solubilize all the analytes of the suspensions. For plain microcapsules added with DSPE-PEG, the addition of chloroform and methanol led to the obtention of a transparent and limpid solution. Furthermore, this solvent mixture is compatible with the non-aqueous mobile phase used in our gradient program without the need on an extraction.

3.2.2. Detector response assessment

The method we propose is aimed at quantifying DSPE-PEG either associated to microcapsules, free in the surfactant solution or to the whole suspension. For this reason a wide calibration range is needed and the detector response is assessed over a decade. Since the expected maximum lipid concentration could reach 21.36 μg/mL, the range of the calibration curve was evaluated with six solutions of plain microcapsules added with DSPE-PEG, where the concentrations varied from 2.23 to 21.36 μg/mL (0.22–2.14 μg injected). The resulting peak areas were plotted as a function of lipid concentration and fitted using two different models. The linear model:

$$y = a_0 + a_1 x \quad (1)$$

and the power model:

$$y = Ax^b \quad (2)$$

were compared, since for the corona detector, a non-linear power response has been proposed [16] (where y is the peak area and x the analyte concentration). Results for both models (Table 1) showed

Table 1
Regression analysis with the two models used

Parameter	Linear model $y = a_0 + a_1 x$
Range (μg/mL)	2.23–21.36 μg/mL (0.22–2.14 μg injected)
Calibration curve equation	$y = 1.10 + 1.87x$
Correlation coefficient, R^2	0.9942
Intercept, $a_0 \pm$ S.D.	1.10 ± 0.47
Slope, $a_1 \pm$ S.D.	1.87 ± 0.04
F-value for the regression	2749
F-value for lack of fit	7.25
p-value of the lack of fit	0.003
Parameter	**Power model $y = Ax^b$**
Range (μg/mL)	2.23–21.36 μg/mL (0.22–2.14 μg injected)
Calibration curve equation	$y = 2.47x^{0.91}$
Correlation coefficient, R^2	0.9961
Coefficient $A \pm$ S.D.	2.47 ± 0.14
Coefficient $b \pm$ S.D.	0.91 ± 0.02
F-value for the regression	4374
F-value for lack of fit	3.2
p-value of the lack of fit	0.052

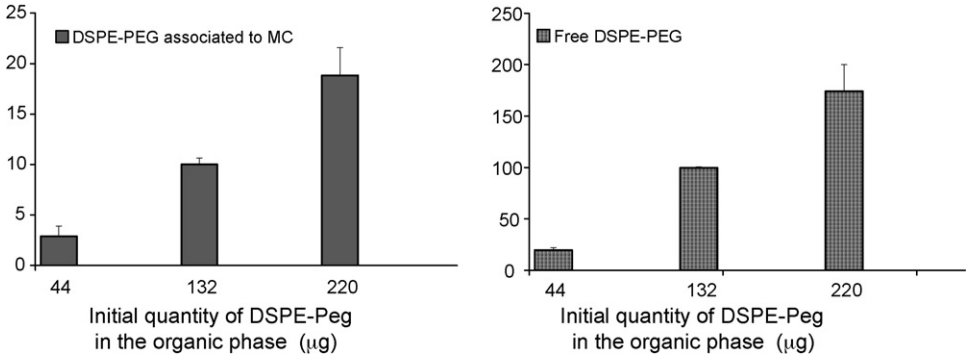

Fig. 5. Influence of the initial pegylated phospholipid in the organic phase on the free and associated DSPE-PEG in the different batches of pegylated microcapsules samples ($n = 3$ for each batch).

high determination coefficient, $R^2 = 0.9942$ and 0.9961 for linear and power models, respectively. The F for regression (Freg) was also calculated to compare linear and power model. The bigger the Freg value, the better the regression and the lower the residual. The Freg value is higher for the power model and, therefore, the power model describes experimental values better than the linear model. This is further confirmed by testing the lack of fit that is acceptable for the power model ($p = 0.052$), whereas the linear model exhibits a low p-value ($p = 0.003$). Hence, from a statistical point of view the data are better described by the power function than the linear model. In addition, the value of b from Eq. (2), is statistically different from 1.0 ($p = 0.00$). Taking this into account, we can conclude that the response of the detector is significantly different from linearity when calculated over the whole concentration range (2.23–21.36 μg/mL). This is further confirmed by the value of the intercept of the linear model (1.10 ± 0.47), statistically different from zero. Although the linear model could be used for a restricted calibration range, we preferred to use the power model over the whole calibration range.

3.2.3. Sensitivity and limits of detection and quantification

Using a linear detector, the limit of detection (LOD) can be calculated based on the standard deviation (S.D.) of the response and on the slope (S) of the calibration curve according to the following formula: LOD = 3.3(S.D./S), while the limit of quantification (LOQ) can be calculated according to: LOQ = 10(S.D./S). The S.D. of the response is usually calculated as the S.D. of y-intercept of regression line [29]. In the previous expression of LOD and LOQ, S, the slope of the linear model is in fact the sensitivity. When the response function of the detector is non-linear, the sensitivity changes with concentration. In the case of the CAD detector, the sensitivity can be expressed as the change of response per unit change of concentration: $S = dy/dx = Abx^{(b-1)}$. Using the lower value of x in the calibration curve gives an estimate of the sensitivity of the detector at this concentration levels. Using values A and b given in Table 1, and the standard deviation of the response at the lower concentration of the calibration curve, the detector sensitivity established at 2.23 μg/mL is 2.11 (AU mL μg^{-1}). As a consequence, the LOD and LOQ, are 33 and 100 ng, respectively.

3.2.4. Accuracy and precision

To test the precision of the injections, six injections of the same solution of plain microcapsules added with DSPE-PEG (21.36 μg/mL) were performed in the conditions described above. The repetitive injections yielded a mean area of 43.19 AU, a standard deviation of 0.53 AU and a coefficient of variation of 1.2%, when monitoring the area under the DSPE-PEG peak. The accuracy of the method was also tested. The accuracy is defined as the closeness of the test results obtained by the analytical method to the true value. It was assessed by analyzing a sample of known concentration: different solutions of plain microcapsules added with DSPE-PEG, and the measured and the true values were compared (Table 2). The accuracy reported in Table 2 is the percent of recovery calculated. The recovery ranges from 90 to 115%, for three batches corresponding to three different concentrations. The measure of the degree of repeatability of the analytical method under normal operation, named intra-day precision was also assessed, using different amounts of DSPE-PEG (44, 132 and 220 μg). It was expressed as the relative standard deviation (R.S.D.) of the determination at three levels of amount, with three repetitions for each. R.S.D. values obtained are below or equal to 5.3% (Table 2).

3.3. Application of the HPLC–CAD method for the quantification of DSPE-PEG associated to microcapsules

Pegylated microcapsules were prepared using the same amount of DSPE-PEG than for plain microcapsules added with the phospholipid of the reproducibility test. The total DSPE-PEG in the whole suspension, the free DSPE-PEG in the supernatant and the pegylated phospholipids associated to microcapsules were quantified in three batches with different amount of pegylated phospholipids. Keeping constant the polymer (PLGA), the amount of DSPE-PEG associated to the microcapsules increases with increasing amounts of DSPE-PEG (Table 3). This is also the case for free phospholipids in the supernatant. Independently of the initial DSPE-PEG concentration, approximately 10% of the initial phospholipids are associated to microcapsules, whereas the remaining 90% are free in the supernatant (Fig. 5). So despite their solubilization by

Table 2
Analysis results of the accuracy and precision for DSPE-PEG with plain microcapsules added with pegylated lipids for the power model

Expected amount (μg)[a]	Measured amount[b] ± S.D. (μg)	Accuracy[b] (%)	R.S.D. (%)
44	40 ± 1.3	90	3.4
44	43 ± 1.6	97	3.8
44	41 ± 0.9	94	2.2
132	152 ± 8.1	115	5.3
132	141 ± 3.0	107	2.2
132	143 ± 0.9	108	0.6
220	225 ± 2.3	102	1.0
220	216 ± 2.9	98	1.3
220	233 ± 3.1	106	1.3

[a] DSPE-PEG corresponding at the volumes of 100, 300 and 500 μL at 440 μg/mL.
[b] Mean of three determination; S.D., standard deviation R.S.D., relative standard deviation.

Table 3
Quantification of DSPE-PEG associated to microcapsules and at the polymer

DSPE-PEG in the organic phase (μg)[a]	DSPE-PEG associated (μg/100 mg of PLGA)[b]
44	1.83 ± 0.8
132	5.65 ± 0.2
220	10.64 ± 1.9

[a] DSPE-PEG corresponding at the volumes of 100, 300 and 500 μL at 440 μg/mL.
[b] Mean of three samples per batch.

the surfactant, a minimum of 1.83 ± 0.8 μg of DSPE-PEG is present for 100 mg of polymer used. This corresponds to 4.5×10^6 pegylated phospholipids per microcapsules, which is probably enough to ensure sufficient surface modification of the microcapsules.

4. Conclusion

The HPLC–CAD method developed in this study proved to be simple, sensitive, accurate and repeatable for the analytical quantification of DSPE-PEG. This method was applicable to the quantification of DSPE-PEG associated to microcapsules, free in the surfactant solution or to the whole suspension. No interferences were observed from the other chemicals present in the formulation. Adequate quantitative data were obtained in a short run time of 20 min only using directly microcapsules. Experimental results were better fitted by a power model than a linear one for pegylated phospholipids with the CAD detector. The method is sensitive and rather simple because sample preparation for quantification does not require any complicated extraction of the phospholipid. This method could easily be applied for the quantification of pegylated phospholipids associated to liposomes.

Acknowledgments

Authors would like to thank V. Nicolas (IFR 141 ITFM) for confocal microscopy experiments, E. Pisani for help with microcapsule preparation, R. Lopez Arellano and R. Godoy Ramos for advice with data treatment, CONACYT and ANR ACUVA (NT05-3_42548) for funding.

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