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A high-performance liquid chromatography method for determination 2-(*n*-(*N*,*N*,*N*-trimethyl)-*n*-alkyl)-5-alkylfuryl halides in dipalmitoylphosphatidilcholine liposome solutions

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Abstract

A high-performance liquid chromatography (HPLC) method for the determination of 2-(4-(*N*,*N*,*N*-trimethyl)-butyl)-5-dodecylfuryl bromide (DFTA) in dipalmitoylphophatidil-choline (DPPC) liposome solutions has been developed. Lipid-soluble furan derivatives, 2,5-disubstituted with different *n*-alkyl chains and a terminal trimethylammonium group are useful probes for studying singlet oxygen dynamics and equilibria in microcompartmentalized systems. The actual HPLC method uses a gradient elution and DAD detection. The chromatographic separation of these components is achieved using a C18 analytical column with a 10 mM solution of 1-heptanesulfonic acid (PIC-7)–methanol (10:90, v/v) as initial mobile phase. Both DFTA peaks are well resolved and free of interference from matrix components and reaction products. The method has been found to be linear ($r > 0.999$) over a wide concentration range and reliable to perform kinetic experiments in which the time dependent consumption of a tetraalkylammonium surfactant in a microorganized systems composed by lipidic surfactants is followed.

Keywords: HPLC; Tetraalkylammonium-derivatives; Liposomes; Singlet oxygen; Furane-derivatives; Phospholipids

1. Introduction

Surfactants are employed in a vast number of uses including domestic and industrial detergents, solubilization of membranes, and pharmaceutical and cosmetic formulations. With these extensive uses, a number of sensitive and high resolution techniques have been developed for the determination of low concentrations of these analytes in different matrices. Many of these techniques and some applications have been reviewed by Barco et al. [1], Morelli and Szajer [2,3] and Vogt and Heinig [4]. A substantial number of these assays were directed to evaluate trace detergents and their degradation products in environmental analyses. In process development and basic research, the quantitation of detergents is more complex than in trace analyses from aqueous matrices. In some cases, a fast and simple assay using routine laboratory equipment can be of significant value. In many circumstances, more than one type of surfactant is present in a given system. For example, our studies of singlet molecular oxygen reactions in biological systems, where singlet oxygen have important deleterious and/or beneficial roles [5–7], involves the use of fast response detectors with near-IR sensitivity allows direct detection of $O_2(^1\Delta_g)$ by analyzing its weak emission at 1270 nm in both laser pulsed flash photolysis and steady-state experiments [8–10]. However, employment of this technique in biological systems has serious limitations [11–13]. In a previous work [14], we reported on the synthesis of several lipid-soluble singlet oxygen quenchers, including a furan moiety in their structure and anchored to the water–lipidic interphase by means of a charged head group. These compounds are useful probes for studying singlet oxygen dynamics and equilibria in microcompartmentalized systems because they react very rapidly with singlet oxygen, physical quenching can be neglected and medium effects on reactivity are small. Furthermore, to measure critical kinetic parameters accounting for singlet oxygen mobility and concentration in organized systems, it is necessary to monitor concentration changes of the probe in these complex systems. Absorption spectroscopic analysis of these furan-derivatives in microheterogeneous systems such as dipalmitoylphosphatidyl-

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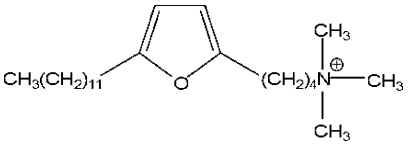

Fig. 1. Structure of 2-(4-(*N*,*N*,*N*-trimethyl)-butyl)-5-dodecylfuryl bromide (DFTA).

choline liposomes is unviable because they present short wavelength transitions, non-distinguishable from absorptions of the bulk system components. Therefore, only liquid chromatography and/or capillary electrophoresis methods can lead to a robust protocol allowing separation of the probe, which behaves as surfactant, from the surfactant employed in preparing the organized aggregate and subsequent probe quantitation. Although a general approach for the development of HPLC protocols for surfactant analysis and purification has been proposed [15], and the simultaneous quantitative trace analysis of ionic and non-ionic surfactant mixtures by reversed-phase liquid chromatography has been described [16,17], studies regarding the separation and quantitation of trimethylammonium-derivative cationic surfactant in a phospholipid containing suspension has not been published.

In this report, we present a reversed phase assay for the separation and determination of 2-(4-(*N*,*N*,*N*-trimethyl)-butyl)-5-dodecylfuryl bromide (DFTA) (Fig. 1), a surfactant employed as singlet oxygen probe, in a dipalmitoylphosphatidilcholine (DPPC) liposome matrix. The assay is robust and accurate requiring only routine laboratory equipment such as an HPLC with a diode array detector.

2. Experimental details

2.1. Chemicals

All solvents and reagents used were of reagent grade, spectroscopic or HPLC quality. Water was purified and deionized using a Waters Milli-Q system. 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) (Sigma) was used as received. 2-(4-(*N*,*N*,*N*-Trimethyl)-butyl)-5-dodecylfuryl bromide was synthesized as previously described [14] and purified by successive recrystallizations from acetone before use.

2.2. Liposome preparation [18,19]

Blank multilamellar large liposomes, MLVs, were prepared by the thin layer evaporation method. In a typical experiment, 14.7 mg of dipalmitoylphosphatidylcholine were dissolved in a small amount of chloroform. The solution was put in a small round-bottomed flask, the organic solvent was evaporated under nitrogen stream and the dry lipid films were maintained 2 h under reduced pressure to remove solvent traces. Films were hydrated by adding an appropriate amount of 100 mM phosphate buffer pH 7.4, heated at a temperature 10 °C above the phospholipid gel-liquid crystalline phase transition temperature, to yield 10 mM phospholipid concentration, while shaking in vortex mixer. The phospholipid–buffer mixture was heated and shaked by short periods (four to six intervals of 1 min), until homogeneous milky suspensions were obtained. Then, the homogeneous suspension was carefully frozen using a liquid nitrogen bath for 5 min and thawed in a water bath held at 60 °C for the same period of time. This cycle was repeated five times. The MLVs suspensions were repeatedly extruded (10 times) through a polycarbonate filter (pore size 200 nm) using an 10 mL Lipex extruder (Northern Lipids Inc.). During extrusion, the temperature of the extruder was maintained at 60 °C. The LUVs obtained were stored at 5 °C. DFTA containing liposomes were prepared by adding aliquots of a stock solution of DFTA in chloroform, to yield the desired concentration, to the chloroform DPPC solution before preparing the MLVs. The DFTA loaded MLVs were frozen and stored at −22 °C and just thawed before the extrusion procedure.

2.3. Preparation of reagents

Phosphate buffer (100 mM, pH 7.4) was typically prepared by mixing 100 mL of 0.1 M KH_2PO_4 and 78.2 mL of 0.1 M NaOH and adjusting pH to 7.4. The clear solution was filtered through a 0.45 μm nylon membrane filter and stored at 5 °C and used for 1–2 weeks. Standard stock solution of DFTA in ethanol was prepared by weighing on a microbalance 2.15 mg of DFTA, dissolving in 3 mL of solvent in a ultrasonic bath at room temperature to get a clear solution and adjusting to 10 mL with solvent. Calibration standards were prepared in ethanol-phosphate buffer pH 7.4 (50:50, v/v) by further diluting the standard stock solution. DFTA solutions were light protected and daily prepared. PIC-7 solutions were prepared by weighting 202.15 mg of PIC-7, dissolving in 50 mL of deionized water, sonicating in a ultrasonic bath at room temperature to get a clear solution and adjusting to 100 mL with deionized water. Initial mobile phase was prepared by mixing the PIC-7 solution and methanol in 10:90 (v/v) ratio.

2.4. High-performance liquid chromatography (HPLC)

The HPLC system was equipped with Waters system consisting of a Waters 600 controller, helium degasser, column thermostat, quaternary pump and a Waters 996 photodiode array detector. Chromatographic analysis was performed using a Chromolith RP-18e (2 μm macropores, 10 cm × 4.6 mm i.d.) column from Merck. Also, a ODS Hypersil (5 μm, 20 cm × 4.6 mm i.d.) column from Hewlett Packard and a LiChrospher RP-select B (5 μm, 25 cm × 4.6 mm i.d.) from Merck were tested. All experiments were carried out at column temperature of 25 °C. A gradient elution was the most convenient method to achieve optimal separation between DFTA and DPPC. The initial mobile phase consisted of a 10 mM solution of 1-heptanesulfonic acid (PIC-7)–methanol (10:90, v/v) at a flow-rate of 2 mL/min. The initial conditions were held for 15 min, time in which DFTA elutes. After this time, isopropyl alcohol was added to the phase mobile by 2 min until reach a 60% (PIC-7)-methanol (10:90, v/v) and 40% isopropyl alcohol mixture. These conditions were held for 18 min in which elutes the DPPC. Initial conditions then were restored in 2 min and maintained during 13 min before a

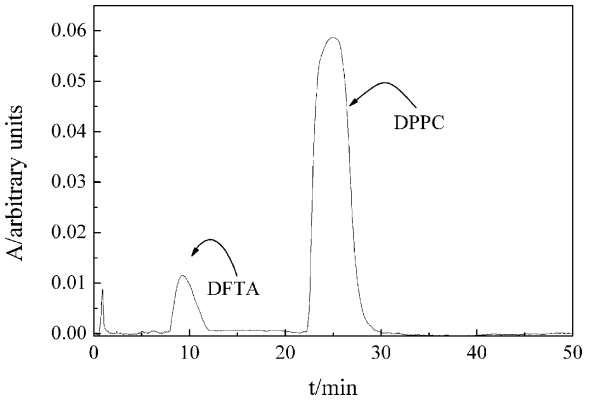

Fig. 2. Chromatogram of a sample containing 100 μg/mL of DFTA in 10 mM DPPC large unilamelar vesicles.

new injection giving a total run time of 50 min. The diode array detector was operated at 222 nm with 4 nm of bandwidth. Injection volume was set at 20 μL.

2.5. Preparation of DFTA loaded liposome samples for HPLC analysis

Samples of DFTA loaded liposomes for HPLC injections were prepared by diluting 300 μL of the the liposome solution with 300 μL of ethanol in a conical plastic tube. The mixture was shaked in a vortex mixer for 2–3 min and then centrifuged at 3600 × *g* during 30 min. Supernatant was employed for HPLC injection.

3. Results and discussion

The HPLC method described here was developed for DFTA quantitation following the FDA guidelines [20].

Linearity, accuracy, precision, and method quantitation limit were tested to ensure method suitability for identification and quantitation of DFTA included in DPPC LUVs. The UV detection wavelength set at 222 nm was considered as a compromise between the sensitivity of the compound of interest and eventual interferences: photooxidation products generated in sensitized reactions, the sensitizer present in the same experiments, and the stability of the baseline. After several trials with mixtures of (PIC-7)-methanol and (PIC-7)-acetonitrile, an appropriate initial mobile phase composed of 10 mM solution of 1-heptanesulfonic acid (PIC-7)–methanol (10:90, v/v) was preferred. Fig. 2 shows that the gradient elution described in Section 2.4 provides a good separation of the DFTA from DPPC with retention times of 9.28 and 24.93 min, respectively, without interference of 5,10,16,20-tetraphenyl-21H,23H-porphine-*p*,*p*′,*p*″,*p*‴-tetrasulfonic acid tetrasodium hydrate (TPPS) or methylene blue (MB) employed as sensitizers neither of the photo-oxidation products. Chromatographic performance data for a typical run are presented in Table 1.

Resolution of 2.0 or greater is desired for critical band pair. Critical resolution of 3.16 was observed between DFTA and DPPC peaks. Tailing factors for both DFTA and DPPC are near

Table 1
Chromatographic performance data of the method

Analite	Retention time (min)	Tailing[a]	Retention factor[b]	Resolution between critical band pair
DFTA	9.28	1.50	9.34	3.16
DPPC	24.93	1.46	26.79	

[a] Tailing is defined as $W_{0.05}/2t_w$, where $W_{0.05}$ is peak width at 5% of peak height (min) and t_w is distance between peak front and peak retention measured at 5% of the peak height (min).

[b] Retention factor is defined as $(t_R - t_0)/t_0$, where t_R is retention time of peak (min) and t_0 is void time (min). Void time = 0.891 min for the method.

to 1.5. Retention factor in the range of $0.5 < k' < 20.0$ is desired to clearly separate the first peak from void time and to avoid higher retention time for the last band. Retention factors of 9.34 and 26.79 (with solvent front as unretained compound) were found for DFTA and DPPC, respectively, indicating a very good separation of the DFTA peak from void time and the second peak corresponding to DPPC, however, retention factor for DPPC is larger than 20.0 increasing the analysis time. We attempt to improve retention factors of the DFTA and DPPC peaks by varying mobile phase composition and changing the chromatographic column. However, not good results were found employing mixtures 10:90 (v/v) of PIC-7 (5, 10, 20 and 40 mM)-methanol and PIC-7 (5, 10, 20 and 40 mM)-acetonitrile, neither using both a ODS Hypersil (5 μm, 20 cm × 4.6 mm i.d.) column from Hewlett Packard or a LiChrospher RP-select B (5 μm, 25 cm × 4.6 mm i.d.) from Merck. With these columns and the gradient elution described in Section 2.4, enlarges retention time (15 and 18 min for DFTA in Hypersil and LiChrospher RP-select B columns, respectively), excessive tailing (>2.0) and back pressures were observed.

3.1. Linearity and range

Linearity of the DFTA calibration standards was tested in the concentration rage of 20.4–306.5 μg/mL. Calibration standards were prepared at various concentration levels.

It is clear from Fig. 3 that the curve is linear in this range of concentration and the correlation is suitable for quantitation.

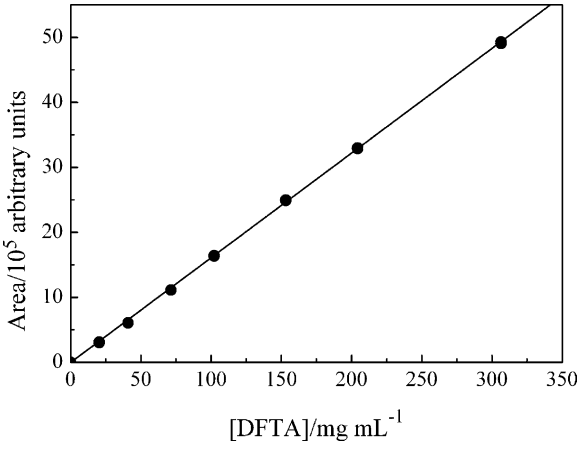

Fig. 3. Response curve of DFTA in ethanol-phosphate buffer pH 7.4 (50:50, v/v).

Table 2
Summary of method accuracy results

Recovery solution at target level (%)	Theoretical concentration (μg/mL)	Recovered average concentration[a] (μg/mL)	RSD[a] (%)	Analytical recovery[a] (%)
16.6	21.49	12.70 (0.057)	0.45	59.01
33.3	42.99	25.51 (0.219)	0.86	59.33
100.0	128.96	75.74 (0.234)	0.31	58.73
133.3	172.16	100.59 (0.151)	0.15	58.42
166.6	214.64	127.98 (2.329)	1.82	59.62

[a] Based on six data points. Standard deviation is given in parentheses.

In our analytical conditions, the calibration curve shows a linear regression equation of $y = 16{,}103x - 8426$, where y is the peak area in arbitrary units and x is the DFTA concentration in mg mL^{-1}. Correlation coefficient (r) and coefficient of determination (r^2) for DFTA were equal to 0.99991 and 0.9998, respectively. No calibration curve was obtained for DPPC because is not of current interest to quantify this phopholipid at high concentration levels. Typical chromatograms of DFTA standard solutions are shown in Fig. 4.

3.2. Accuracy

Accuracy studies were performed to determine the closeness between the true concentration value and the experimental results. Samples of DFTA loaded liposomes were prepared and treated as described in Section 2.5. Preliminary experiments show a recovery in the order of 60%, therefore, was crucial to determine if this value remains constant in a wide range of concentrations. Considering that in our kinetic experiments we employ an initial DFTA concentration near to 130 μg/mL, we define this value as the target concentration. In addition typically we follow the DFTA consumption not further than two lifetimes. Consequently, the recovery study was performed at five different concentration levels (16.6, 33.3, 100.0, 133.3 and 166.6%) of the target concentration, a sufficiently wide range to guarantee that all DFTA concentrations measured in our experiments, are covered. For each level, six preparations were tested. Table 2 summarizes the results from accuracy experiments. Although from the analytical point of view a recovery of 60% is not a good value, an average recovery of 59.02 was observed for DFTA, which is within ±2.0% of normally accepted value. For kinetic studies, a constant recovery value in the whole concentration range is a necessary condition, given that under pseudo order conditions, the ratio between the concentration at time zero and concentration at time t is currently employed.

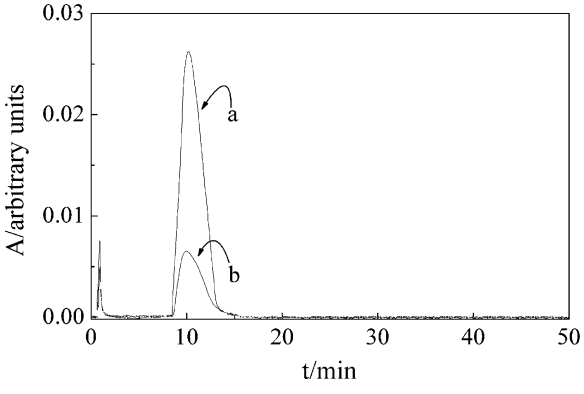

Fig. 4. Chromatogram of DFTA standards in ethanol-phosphate buffer pH 7.4 (50:50, v/v). (a) 306.6 μg/mL, (b) 71.5 μg/mL.

3.3. Precision

Instrument precision was performed as part of each sequence run at the beginning of the sequence. Four injections of the target level of calibration standard were performed and the data were evaluated. Table 3 shows typically determined values obtained for a liposome preparation loaded with 107.48 μg/mL of DFTA, the mean retention time, area response and the corresponding relative standard deviations.

Relative standard deviations for both retention times and area response are <1.50%, indicating sufficient instrument reproducibility for this method. For sample precision measurements, six replicate samples were prepared and analyzed on the first day. For each sample preparation two injections were performed. On the second day, the same analyst prepared a second set of six samples from the same sample vial and independently analyzed on the same system. Results are summarized in Table 4. The RSD values are found to be <1.4% on each day and between day 1 and 2. These results clearly indicate sufficient sample repeatability with this method.

3.4. Quantitation limit

There are at least four different ways to determine quantitation limits of analytes [21] and signal-to-noise ratio (S/N)

Table 3
Reproducibility of retention times and peak areas of the standard compound

Sample	Retention time (min)	Area response (a.u.)
1	9.203	1000150
2	9.291	1019020
3	9.376	1045886
4	9.175	1023095
5	9.272	1025890
6	9.325	1019576
Mean value	9.274	1022270
RSD (%)	0.81	1.43

Table 4
Summary of method precision results

Sample	[DFTA] ($\mu g\,mL^{-1}$)	
	Day 1	Day 2
1	64.48	64.24
2	64.89	63.30
3	64.03	63.17
4	64.21	63.12
5	63.19	62.33
6	63.17	62.19
7	62.66	61.92
8	62.30	61.86
9	64.20	62.75
10	64.76	63.29
11	64.41	63.47
12	64.52	63.94
Mean value	63.91	62.97
RSD (%)	1.34	1.22

is one of the most commonly used procedures. We have used this procedure for determine quantitation limits of DFTA. Stock solutions of analyte were progressively diluted with the supernatant obtained after process blank liposomes according to the protocol described in Section 2.5. Signal-to-noise ratios were determined until a minimum S/N ratio of 10 was achieved. Using this method, a quantitation limit of 10 μg/mL was observed for DFTA. A detection limit (defined at signal to noise ratio of 3) of 4 μg/mL was also determined for DFTA. Values of 9.3 and 4.6 μg/mL for both quantitation limit and detection limit, respectively, were obtained with real samples if a constant recovery of 59% is assumed for liposomes loaded with low DFTA concentrations.

3.5. A kinetic experiment

Several steady-state experiments allow us to evaluate the method applicability to study the kinetics of reaction between singlet molecular oxygen and DFTA in liposomes. Typically, experimental chemical reaction rate constants were determined in 10 mM DPPC liposome solutions loaded with DFTA using a 10 mL double wall cell, light-protected by black paint. A centered window allows irradiation with light of a given wavelength using Schott cut-off filters. Circulating water maintained the cell temperature at $20 \pm 0.5\,^{\circ}C$. The irradiation of the sensitizer, MB or TPPS, was performed with a visible, 35 W, halogen lamp. Time dependent DFTA consumption was followed taking 300 μL samples of the reaction cell at several times, treating as described in Section 2.5 and analyzing by HPLC. Fig. 5 shows the results obtained in a duplicated analysis. Plot of Fig. 5 indicates that the decrease in DFTA concentration with the reaction time, follows a first-order kinetics as expected. Therefore, the experimental rate constant can be calculated from the slope of the linear fit. Results obtained with real samples account for the robustness and accuracy of the analytical method to quantify DFTA in DPPC liposome solutions.

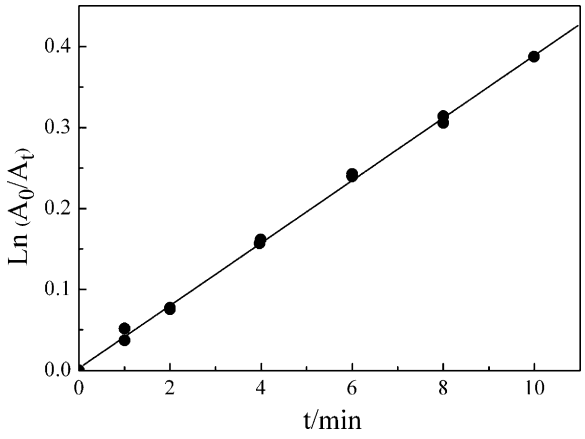

Fig. 5. First-order plot (correlation coefficient >0.999) for the reaction between DFTA and singlet molecular oxygen in 10 mM DPPC liposomes employing MB as sensitizer. $T = 22\,^{\circ}C$.

4. Conclusions

A rapid HPLC method for quantitation of DFTA in DPPC liposome solutions was developed. The method is simple, linear, precise, accurate, sensitive and corresponds to the first report for quantify an amphipatic tetraalkylammonium-derivative in the presence of DPPC liposomes. In addition, this is a reliable method to perform kinetic experiments where the time dependent consumption of a tetraalkylammonium surfactant is followed. These compounds are employed as probe to monitor the dynamics and equilibrium of singlet molecular oxygen in microorganized systems composed by lipidic surfactants.

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