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High-performance liquid chromatography post-column derivatization with fluorescence detection to study the influence of ambroxol on dipalmitoylphosphatidylcholine levels in rabbit eustachian tube washings

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ABSTRACT

In this work an appropriate high-performance liquid chromatography method was set up to guarantee specificity, sensitivity, precision and accuracy in analyzing dipalmitoylphosphatidylcholine (DPPC) in rabbit eustachian tube washings, as well as to determine its varying levels after administration of ambroxol chloride. The procedure is based on a post-column derivatization with fluorescence detection using 1,6-diphenyl-1,3,5-hexatriene which exhibits increased fluorescence in a lipid environment. DPPC was chromatographed on a Hypersil C_{18}. The mobile phase for the isocratic elution consisted of 40 mmol/l choline chloride in methanol–tetrahydrofuran (97:3). Ambroxol was given to a group of New Zealand white rabbits at a dose of 30 mg/kg. A second group receiving vehicle only acted as controls.

INTRODUCTION

The existence of a surface-active agent in the mammalian eustachian tube has been proved and it has also been demonstrated that this surfactant functions as a surface tension-lowering substance in a manner similar to lung [1–6]. Literature data, in particular Hills [5] thin-layer chromatography (TLC) studies on dog and rabbit eustachian tube washings, show that tubal surfactant, as well as pulmonary surfactant [7–9], mainly consist of phosphatidylcholines (PCs); phosphatidylethanolamines (PEs) and sphingomyelins are present in lesser quantities [10].

In a previous work [11], dipalmitoylphosphatidylethanolamine was identified and determined in rabbit eustachian tube washings; in order to extend the research, PCs are now investigated.

As reported by Wheeler *et al.* [12], the eustachian tube synthetizes an elevated quantity of disaturated PCs and dipalmitoylphosphatidylcholine (DPPC) is either the major factor responsible for the surface-active properties [13] or is the predominating

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compound in tubal and lung washing [10]; the high percentage of palmitic acid in tubal washings (60% in comparison to 70% in lung washings) [14] confirms the presence of disaturated PCs in eustachian tube.

In secretory otitis media a decrease in tubal surfactant levels has been reported [15]; thus the administration of a drug which promotes surfactant secretion should be successful in treating this type of condition. Ambroxol chloride [16], 4-{[(2-amino-3,5-dibromophenyl)methyl]amino}cyclohexanol, is a drug with fluidizing activity on bronchial secretion; as it stimulates the secretion of alveolar surfactant, it is also supposed to stimulate the secretion of tubal surfactant. The aim of this work is to verify its effect on DPPC levels in both bronchial and eustachian secretions, by means of comparative studies on treated and control animals. This approach requires a specific, sensitive, accurate and precise analytical method for the determination of DPPC in biological matrix (tubal and lung washings).

EXPERIMENTAL

Materials

Standard DPPC was obtained from Sigma (St. Louis, MO, U.S.A.). 1,6-Diphenyl-1,3,5-hexatriene (DPH) and choline chloride were from Aldrich-Chemie (Steinheim, Germany). Methanol and tetrahydrofuran were from J. T. Baker (Deventer, The Netherlands). Physiologic saline was from Abbott (Aprilia, Italy) and ambroxol was from De Angeli (Milan, Italy). All reagents were high-performance liquid chromatography (HPLC) grade.

Equipment

Qualitative and quantitative determinations were performed on an HP 1090 M liquid chromatograph (Hewlett-Packard, Palo Alto, CA, U.S.A.) equipped with a sample valve (Model 7410 Rheodyne) with a 100-μl loop.

The post-column derivatization system consisted of a Milton-Roy minipump with pulse damper connected via a T-junction to a mixing chamber (30 cm $\times$ 0.5 mm I.D. TFE, filled with 250-μm glass beads), 10 ft. $\times$ 0.5 mm I.D. TFE tubing (Supelco, Bellefonte, PA, U.S.A.) maintained at 50°C with a water jacket and then a 1046 A fluorescence detector (Hewlett-Packard). Data were elaborated by a HP 9000 Model 310 work-station (Hewlett-Packard).

Analytical conditions

The column was an ODS Hypersil 100 mm $\times$ 4.6 mm I.D., 5-μm spherical particles (Hewlett-Packard). Isocratic elution was carried out with mobile phase A (40 μM choline chloride in methanol) and mobile phase B (tetrahydrofuran, 97:3, v/v) at a flow-rate of 1 ml/min.

The detection reagent was distilled water containing, per litre, 150 μl of 3 μM DPH solution in tetrahydrofuran and 0.001% (v/v) Tween 20, at a flow-rate of 1.2 ml/min. The detergent was necessary to prevent the build-up of background fluorescence due to adsorption of PC vesicles in the flow cell.

The excitation and the emission wavelengths were 340 and 460 nm, respectively.

Standard solution

The optimal analytical conditions were assessed in rabbit lung washings. Since the DPPC concentrations in lung and tubal washings differed considerably, it became necessary to prepare two calibration curves. Standard solution was prepared by dissolving DPPC in methanol and diluting this stock solution to obtain concentrations ranging from 500 to 3000 μg/ml (first curve) and from 5 to 300 μg/ml (second curve).

The detector response was linear within the ranges above described and the two regression equations were: $y = 0.0253x + 0.379$, $r = 0.991$ and $y = 0.228x + 7.76$, $r = 0.968$, respectively.

The precision of the chromatographic procedure was indicated by five replicate injections of standard solutions (250 and 1500 μg/ml DPPC). The standard deviations were ± 0.7 and ± 0.5, and the accuracies were 0.19 and 0.17%, respectively. The limit of detection was 1.5 μg/ml with a signal-to-noise ratio 2:1.

Biological samples

The biological samples were taken from New Zealand white rabbits (average weight: 3.5 kg) and the washing was carried out with physiologic saline as described in the literature [5,10,15].

Ambroxol (30 mg/kg) was given by means of intravenous administration to a group of twelve white New Zealand rabbits in order to verify the effect of the drug on DPPC levels. A second group of twelve rabbits acted as controls.

Sample preparation

A 10-ml volume of Eustachian tube washings (or lung washings) was centrifuged at 1000 *g* for 5 min in order to exclude the risk of washing contamination by erythrocytes and cell debris. The supernatant was transferred to a Petri dish and lyophilized. To the lyophilized sample were added 5 ml of chloroform. After filtration on Millipore HF filters (0.45 μm), the solution was dried under a stream of nitrogen. To the dry residue were added 500 μl of methanol. Triplicate 100-μl aliquots were directly chromatographed.

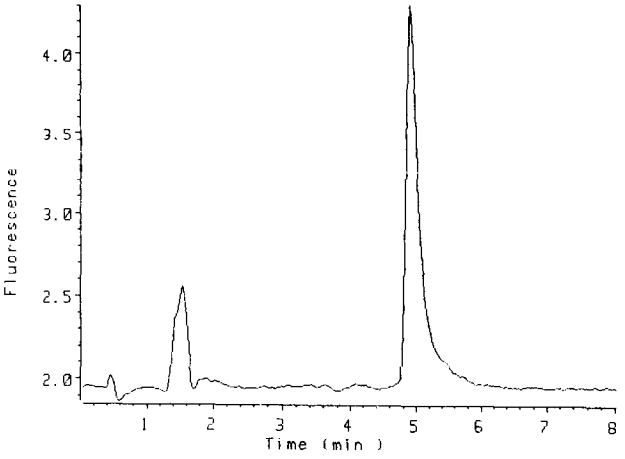

Fig. 1. Chromatogram of standard solution of DPPC (250 μg/ml).

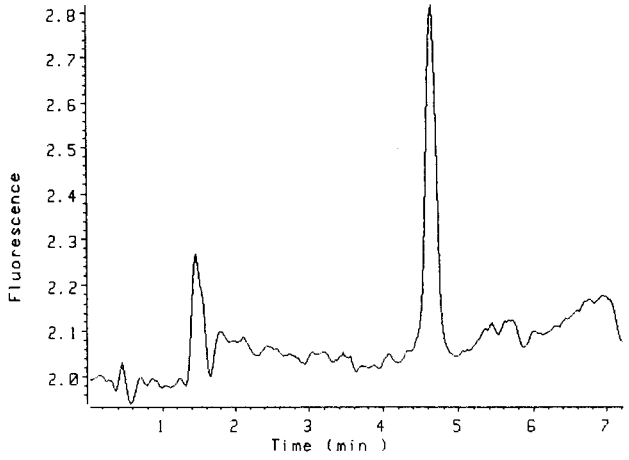

Fig. 2. Chromatogram of DPPC in eustachian tube washings.

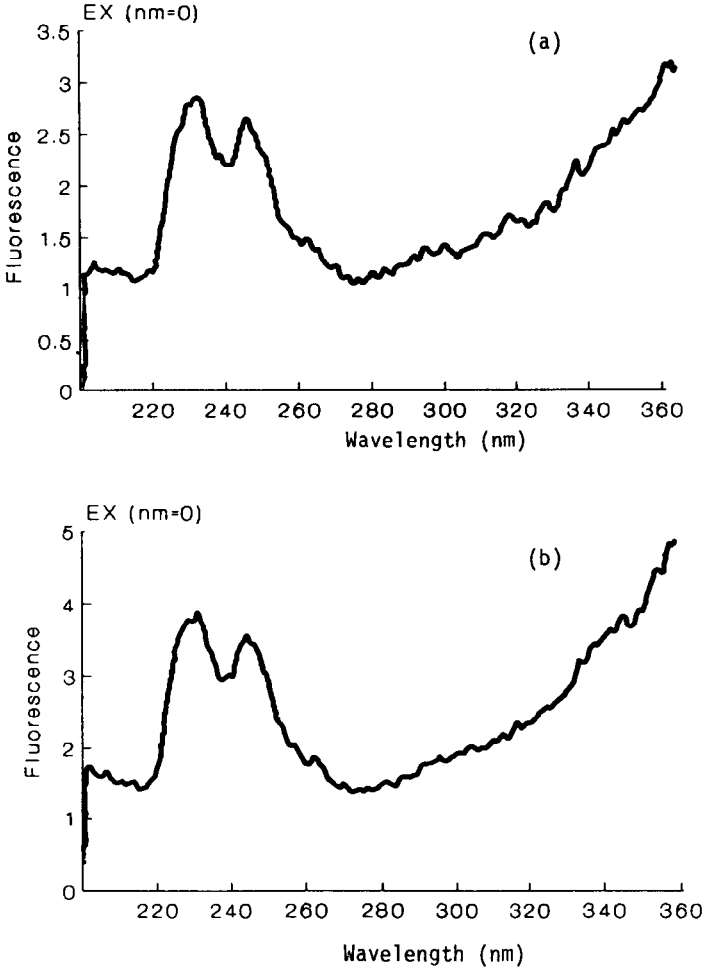

Fig. 3. Excitation spectra of DPPC standard solution (a) and biological sample (b).

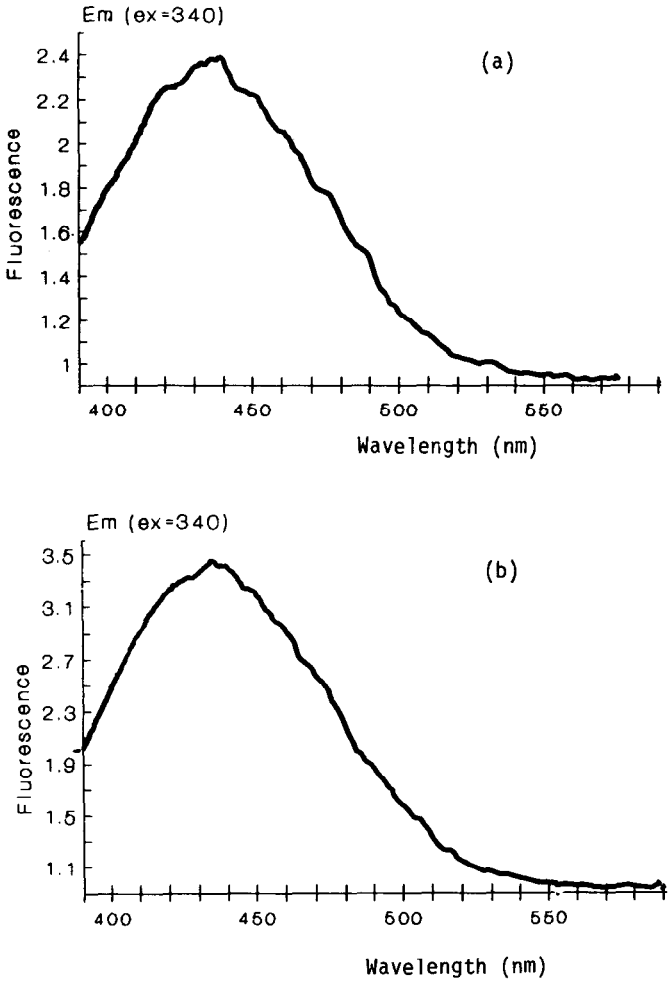

Fig. 4. Emission spectra of DPPC standard solution (a) and biological sample (b).

TABLE I

MEAN VALUES OF DPPC IN TWELVE RABBITS

Sample	Concentration (mean ± S.D.) (μg/ml)	t^a	P
Lung washings			
Control	787.78 ± 65.65	5.60	<0.001
Treated	1024.76 ± 131.11		
Eustachian tube washings			
Control	15.15 ± 1.07	10.63	<0.001
Treated	35.47 ± 6.53		

[a] t = Student's t-test.

RESULTS AND CONCLUSION

The starting point of the study was the method proposed by Postle [17] but it was found to be unsuitable for our purposes. Under the modified chromatographic conditions described above, DPPC gave a retention time of 5 min (Fig. 1); standard solutions of distearoyl- and palmitoyllinoleylphosphatidylcholine were chromatographed and the retention times (8.7 and 4.4 min, respectively) do not interfere with DPPC. Fig. 2 shows the chromatogram of DPPC in eustachian tube washings.

During the chromatographic analysis, excitation and emission spectra were recorded both for DPPC standard and biological sample (tubal washings). Spectra are shown in Figs. 3 and 4. The method was found to be rapid, sensitive, specific and accurate for the analysis of DPPC in the biological samples considered.

The mean concentrations (μg/ml) of DPPC in biological samples are reported in Table I together with statistical significance.

DPPC levels of treated rabbits were enhanced in comparison with the control values, both in lung washings and in eustachian tube washings. The extent of the enhancement was much higher in the second group.

These results confirm the phospholipid nature of tubal surfactant and suggest new therapeutic applications in the otorhinolaryngologic pathologies for drugs which stimulate the production of surfactant in the lung.

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