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Short Communication

Liquid chromatographic analysis of phospholipids in washings from rabbit eustachian tube: dipalmitoyl phosphatidylethanolamine content*

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Introduction

This note is based on a larger body of work undertaken by the Otorhinolaryngology Clinic of the University of Pavia to demonstrate the phospholipidic nature of the Eustachian tube surfactant. Literature data and in particular Hill's [1] thin layer chromatography (TLC) studies on dog and rabbit Eustachian tube washings show that the tubal surfactant mainly consists of phosphatidylcholines (PC), phosphatidylethanolamines (PE) and sphingomyelins (S).

On this basis an appropriate liquid chromatographic (LC) method has been developed to guarantee specificity, sensitivity, precision and accuracy in analysing these particular phospholipids, as well as for monitoring them after administration of tubal surfactant regulatory drugs.

Quantitative analysis of phospholipid composition of biological fluids is frequently performed in biomedical research; TLC methods are widely used [2–6] but they are tedious and require additional assays for quantitation of fractions. For this purpose, alternative techniques such as high-performance liquid chromatography (HPLC) would be useful. Up to now, several LC methods for phospholipid separation and determination have been described [7–18].

When phospholipids are analysed directly by HPLC, one difficulty arises in the detection. The use of UV-detection does not allow direct quantitation of fractions,

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because the phospholipid absorbance in the 200-nm region reflects the number of double bonds rather than the number of molecules [12]. LC with flame ionization detection has been used to quantitate phospholipid fractions, but it involves specialized equipment and is relatively insensitive. In order to improve the sensitivity for the molecules containing the primary amino group (e.g. PE), fluorescent derivatives such as biphenylcarbonyl [19], naphthyl [20] or dansyl [21] derivatives can be used. In this report an analytical method is described for identifying and determining dipalmitoyl phosphatidylethanolamine (the predominant species present naturally in Eustachian tube washing) as the dansyl derivative.

Experimental

Reagents and chemicals

Standard L-α-phosphatidylethanolamine dipalmitoyl (DPEA) and *N*-dansyl dipalmitoyl L-α-phosphatidylethanolamine (2 mg ml^{-1}; $CHCl_3$–MeOH, 4:1) (*N*-Dns-DPEA) were obtained from Sigma Chemical Co. (St. Louis, MO, USA); dansyl chloride (Dns) was from BDH Chemicals (Poole, Dorset, UK); methanol and chloroform were HPLC grade from Merck (Darmstadt, FRG); reagent grade potassium dihydrogen phosphate was obtained from Merck (Darmstadt, FRG).

Apparatus

The LC system used consisted of a Varian 5060 liquid chromatograph (Palo Alto, CA, USA) equipped with a Valco semiautomatic sampling valve with a 10-μl loop, an Alltex 250 × 4.5 mm i.d. Econosphere C_8 (10-μm spherical particles) column, a Varian Fluorichrom fluorescence detector and a Varian Vista CDS-401 printer–plotter data system.

Analytical conditions

The operating conditions were: ambient temperature; flow rate, 1 ml min^{-1}; detector waelength, 360 nm (excitation) and 420 nm (emission); chart speed, 0.3 cm min^{-1}; sensitivity, 0.032 AUFS.

The analyses were performed with gradient elution: mobile phase A = 0.005 M aqueous potassium phosphate buffer (pH = 7); mobile phase B = methanol. The gradient profile adopted was: 80% B for 5 min, then a linear gradient to 95% B in 10 min, 95% B for 5 min, then a linear gradient back to 80% B in 5 min.

Standard solution. The standard solution was prepared by dissolving 50 mg of DPEA standard in 100 ml of chloroform.

Dansylation. Dns derivatization of DPEA standard was carried out according to the Chen method [21]: 1 mg of DPEA was dissolved in 2.5 ml of Dns-Cl solution (1 mg ml^{-1} in chloroform) and 0.8 ml of triethylamine was added. Vials were tightly capped and vortexed vigorously with Vortex for 10 s. They were incubated in the dark in a 50°C water bath for 3 h or kept at ambient temperature for 1 night.

Extraction of DPEA from pulmonary and Eustachian tube washings. The biological samples were taken from two New Zealand white rabbits (A and B, mean weight 3.5 kg) as described in the literature [1]. The extraction of phospholipids was carried out by a

method modified from that of Folch *et al.* [22]. To 2 ml of sample was added 2 ml of chloroform–methanol (2:1, v/v) in a dark centrifuge tube; the mixture was shaken on a Vortex shaker for 10 s and then centrifuged at 3000 rpm for 10 min. The chloroform layer was dried under a stream of nitrogen and the dry residue was subjected to derivatization as described for the DPEA standard.

Recovery test. 0.2 ml of standard solutions of DPEA, ranging from 1 to 100 μg, were dried under a stream of nitrogen in a dark centrifuge tube. The dry residue was dissolved in 2 ml of physiological solution and subjected to extraction and derivatization, as described above.

The quantitative recovery of Dns derivatization was calculated by comparing the response of *N*-Dns-DPEA obtained by this reaction with that of *N*-Dns-DPEA standard solutions.

Results

The optimal analytical conditions were assessed in rabbit pulmonary washing fluid. Several solvent systems, in various proportions were tested for their ability to separate the Dns derivative of DPEA from the reaction products. The isocratic elution at the outset followed by the linear concentration gradient gave a very good separation of the Dns-DPEA peak from the other dansylation products, which were eluted in the initial phase of analysis. Under the chromatographic conditions described Dns-DPEA gave a peak with a retention time of 16 min (Fig. 1). The identity of the DPEA peak in the chromatogram was established by injecting the standard Dns-DPEA into the chromatograph.

Quantitative assays were performed by means of the external standard procedure. Different quantities of standard DPEA in the range 1–100 μg ml^{-1} were derivatized. In this range the detector response was linear, the regression data being: $y = 1.87x + 7.04$ ($n = 5$), with a correlation coefficient of 0.999. The recovery over the concentration range of interest was found to be 95% (RSD 0.35%).

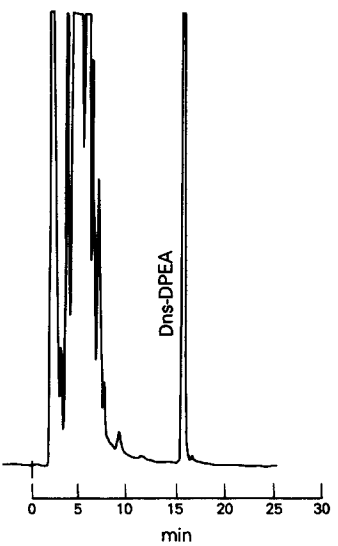

Figure 1
Chromatogram of standard N-dansyl dipalmitoyl phosphatidylethanolamine derivative.

The dansyl derivative is specific, sensitive and is suitable for the determination of DPEA. The detection limit of DPEA was found to be 1 $\mu g\ ml^{-1}$ at 0.032 AUFS, and a signal-to-noise ratio of 2:1. For DPEA in total Eustachian tube washing (10 ml) the precision of the method at 4.74 $\mu g\ ml^{-1}$ (rabbit A) and 5.11 $\mu g\ ml^{-1}$ (rabbit B) was RSD 10.2 and 4.5% ($n = 5$), respectively. The analytical conditions described enable the fast and reliable identification and determination of DPEA.

In Figs 2 and 3 are shown representative chromatograms for the Eustachian tube and lung rabbit washings. The quantitative results of DPEA determination in pulmonary washings from two rabbits were 9.78 (RSD 4.2%) and 9.60 (RSD 4.4%), respectively ($n = 5$). These results for DPEA determination were in good agreement with those obtained by other investigators by different methods [1].

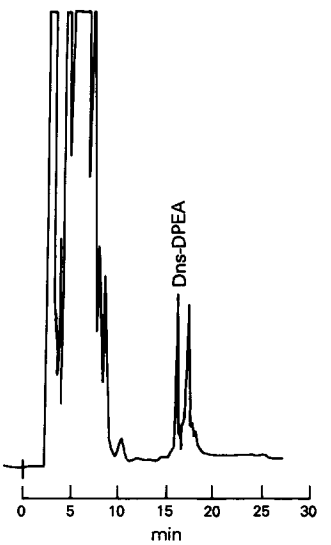

Figure 2
Chromatogram of rabbit Eustachian tube washing. The peak overlapping Dns-DPEA was not investigated, but is probably due to another molecular species of PE.

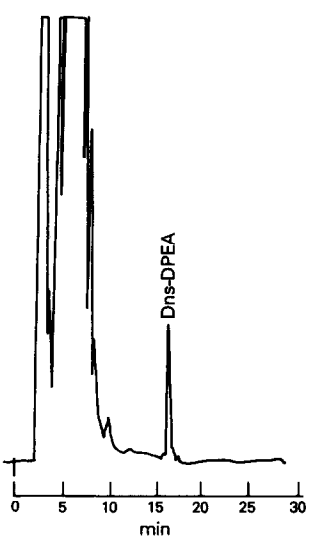

Figure 3
Chromatogram of rabbit pulmonary washing.

Conclusion

The present LC method was found to be rapid, sensitive, specific and accurate for the analysis of DPEA in Eustachian tube washing. It should be suitable for monitoring DPEA after administration of tubal surfactant regulatory drugs.

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