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CHAPTER 4

REVERSED-PHASE LIQUID CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF 7-NITROBENZ-2-OXA-1,3-DIAZOL-4-YL-LABELED LIPID ANALOGUES


Reversed-phase liquid chromatographic method for the
determination of 7-nitrobenz-2-oxa-1,3-diazol-4-yl-labelled lipid
analogues

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Abstract

This paper reports the development of a dual column system for the simultaneous separation of fluorescent short-chain
ceramide, 6-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)[NBD]amino]hexanoyl-sphingosine and its metabolites, C6-NBD-sphingomyelin and C6-NBD-glucosylceramide, as well as the fluorescent derivatives of choline and serine phosphatides. The method enables the separation of these lipids in a single run on the basis of the polarity of their headgroups and hydrophobicity of their acyl backbone. The fluorescent properties of the NBD-label make it possible to quantify small amounts of NBD-lipid analogues. The sensitivity of the presented method thus permits the use of small sample volumes and the determination of NBD-lipid analogues secreted into mouse bile directly, without prior extraction or concentration steps.

Keywords: Derivatization, LC; Lipids

1. Introduction

Cholesterol and phospholipids (PLs) are major constituents of the organic fraction of bile. The "classical" model for biliary lipid secretion supposed the passive extraction of canalicular membrane lipids by micellar bile salts in the canalicular lumen (reviewed in Ref. [1]). Recently, it was demonstrated that a protein present in the canalicular membrane of the hepatocyte also has a major regulatory function in lipid secretion from the liver to the bile [2]. This mdr2 P-glycoprotein translocates phosphatidylcholine (PC) from the inner leaflet to the outer leaflet of the hepatocyte canalicular membrane ensuring a steady supply of PC available to be extracted by bile salts present in the bile canalculus [2-5]. The exact mechanism of biliary PL secretion is not yet known but the appreciation of an important role for membrane bound ATP-dependent transport proteins in this process provided a new impulse and tools to study this mechanism.
The transbilayer movement of lipids by a translocator (flippase) in biological membranes can be studied by monitoring the distribution of fluorescent lipid analogues over both leaflets of the bilayer [6,7]. The most commonly used PL analogues in this respect are lipids with a fluorescent short acyl chain (C6 or C12) with a 7-nitrobenz-2-oxa-1,3-diazol-4-yl group (NBD) as the fluorophore. By replacing one of the naturally occurring fatty acids by the short-chain labelled fluorescent fatty acid, the resulting lipid analogues can be readily integrated into biological membranes by spontaneous lipid transfer from exogenous sources like bovine serum albumin (BSA) or liposomes.

In most studies a thin layer chromatographic method is used to characterise and quantify NBD-lipid metabolites [8,9]. This method is laborious, time consuming and insensitive.

A HPLC method circumvents the problems with the aqueous matrix of the sample. Martin and Pagano used a C18 column in order to separate NBD-labelled lipids [10]. However, this separation is mainly based on the length of the acyl chains and not the polarity of the different headgroups (extensively reviewed by Olsson and Salem [11]). As studied by Andrews [12] and Samet et al. [13] use of a column material modified with more polar groups like cyanopropyl or diol permits separation of several classes of underivatized PLs.

The polarity and chain length differences of the biliary NBD-lipids of interest prohibited the use of the methods mentioned above. In this paper, we describe a new method, which allows separation and quantification of several NBD-lipid analogues.

2. Experimental

2.1. Materials

Krebs–bicarbonate buffer containing, 120 mM NaCl, 24 mM NaHCO3, 1.2 mM KH2PO4, 4.8 mM KCl, 1.2 mM MgSO4 and 1.3 mM CaCl2; pH 7.4, N-2-hydroxyethylpiperazine-N’-2-ethane sulphonic acid (HEPES) and BSA (essentially fatty acid free) were obtained from Sigma (St. Louis, MO, USA). Tauroursodeoxycholic acid (TUDC, 99%) was obtained from Calbiochem–Novabiochem (La Jolla, CA, USA). Methanol, HPLC-grade, was obtained from Baker (Deventer, The Netherlands). Triethyl-amine, 99%, was obtained from Merck–Schuchardt (Hohenbrunn, Germany). O-phosphoric acid, 85%, was obtained from Merck (Darmstadt, Germany). C6-NBD-ceramide (6-[(N-[7-nitrobenz-2-oxa-1,3-diazol-4-yl]amino)hexanoyl]phosphoglycerol) and C6-NBD-sphingomyelin (6-[(N-[7-nitrobenz-2-oxa-1,3-diazol-4-yl]amino)hexanoyl]phosphoglycerol-phosphocholine) and C6-NBD-C16-PC (2-[(N-[7-nitrobenz-2-oxa-1,3-diazol-4-yl]amino)hexanoyl]1-hexadecanoyl-sn-glycero-3-phosphocholine) and C6-NBD-C12-PC (2-[(N-[7-nitrobenz-2-oxa-1,3-diazol-4-yl]amino)hexanoyl]1-dodecanoyl-sn-glycero-3-phosphocholine) were obtained from Molecular Probes (Eugene, OR, USA), C6-NBD-C16-PE (2-[(N-[7-nitrobenz-2-oxa-1,3-diazol-4-yl]amino)hexanoyl]1-hexadecanoyl-sn-glycero-3-phosphoethanolamine), C6-NBD-C16-PS (2-[(N-[7-nitrobenz-2-oxa-1,3-diazol-4-yl]amino)hexanoyl]1-hexadecanoyl-sn-glycero-3-phosphoserine), C6-NBD-C14-PC (2-[(N-[7-nitrobenz-2-oxa-1,3-diazol-4-yl]amino)hexanoyl]1-tetradecanoyl-sn-glycero-3-phosphocholine) were obtained from Avanti Polar Lipids (Alabaster, AL, USA), C6-NBD-glucosylceramide was a kind gift of Dr. G. van Meer.

2.2. Methods

2.2.1. Fluorescent probe preparation

C6-NBD-ceramide was bound to BSA adapting the method described by Crawford et al. [8]. C6-NBD-ceramide (109 nmol dissolved in 25 µl ethanol) was added to 1.25 ml Krebs–bicarbonate, pH 7.45, containing 10 mM HEPES and 30 mg/ml BSA (essentially fatty acid free). After dialysing the solution fourtimes against this medium at 4°C, aliquots were prepared finally containing 6.6 nmol C6-NBD-ceramide bound to BSA in a 1:5 molar ratio.

2.2.2. In situ mouse liver perfusion system

Mouse surgery and perfusion technique and solution preparation were performed as described previously [14]. Briefly, under anaesthesia (Hypnorm (fentanyl/luanisone), 1 ml/kg, Janssen, Beerse, Belgium) the vena cava superior, the gallbladder and
portal vein were cannulated. Perfusion was performed in orthograde direction with Krebs--bicarbonate buffer in a perfusion cabinet thermostated at 37°C. The perfusion medium was gassed with carbogen (5% CO₂, 95% O₂, Hoek Loos, Schiedam, The Netherlands) in an oxygenator. Directly after start of the perfusion with Krebs--bicarbonate buffer, TUDC was infused through a three-way connector attached to the portal vein cannula, at a steady rate of 500 nmol/min/100 g body weight during the whole experimental period to maintain constant biliary excretion. After a stabilisation period of 20 min, a bolus NBD-ceramide was given into the perfusion medium by infusion through to the portal vein cannula.

Bile samples were collected at 5 min intervals, ten times diluted with distilled water and immediately frozen at −20°C. C6-NBD-C16-PC was dissolved in eluent (85% 0.45 mM triethylammoniumphosphate in methanol+15% 0.45 mM triethylammoniumphosphate at pH 3) and was used as internal standard. Before injection the samples were diluted four times with internal standard solution that resulted in a final amount of 1.2 pmol per injection.

2.3. Instrumentation

The HPLC system used consisted of a Gynkotek 480 HPLC gradient system (Germering, Germany) connected to a Rheodyne 7125 injection valve (Cotati, CA, USA) with an 20 µl injection loop and a Jasco FP920 Fluorimeter (Tokyo, Japan); excitation wavelength set to 470 nm and emission wavelength on 530 nm. The LC columns (Inertsil ODS-2, 5 µm, 100 mm×3 mm I.D. and Spherisorb CN, 3 µm, 100 mm×4.6 mm I.D.) used were obtained from Chrompack (Bergen op Zoom, The Netherlands). Data acquisition was performed on a computer with Gynkosoft integration software. The eluent flow-rate was 0.6 ml/min. Gradient runs were performed using (A) 0.45 mM triethylammoniumphosphate at pH 3.0 (unless otherwise mentioned) and (B) 0.45 mM triethylammoniumphosphate in methanol. The initial eluent composition was 15% A and 85% B, after 0.5 min this was linearly changed to 100% B in 9 min and kept for 6 min at 100%. Finally the system was reset to its initial composition in 5.5 min and stabilised for 8 min before the next analysis was started.

3. Results and discussion

A bolus of NBD-labelled ceramide, added to the perfusate of a mouse liver [8,15] was taken up by the hepatocyte and partially metabolised to NBD-sphingomyelin and NBD-glucosylceramide. Secretion of the precursor molecule and its metabolites into the bile was measured in a fluorometer. In order to separate and quantify these PLs of interest, the RPLC method described by Martin and Pagano [10] was used and C6-NBD-C16-PC was included for use as internal standard. Following the conditions used in this method, it was not possible to separate NBD-sphingomyelin from NBD-glucosylceramide (Fig. 1A). In an attempt to improve the separation, the effect of varying the amount of methanol was studied. From Fig. 1B, it can be seen that the amount of methanol has dramatic effects on the retention of the lipids. A change from 95 to 75% methanol in the mobile phase led to an increase of 30 to 40 min for ceramide and sphingomyelin and glucosylceramide while for the fluorescent lipid analogue of phosphatidylcholine an increase of about 75 min was observed. However, with the variation of the methanol concentration no separation between sphingomyelin and glucosylceramide was achieved. Variation of the pH between 2.5 and 6.0 had little influence on the separation (results not shown) as could be expected from the fact that the separation on a C₁₈ column is based on apolar interactions of the lipids with the stationary phase. Thus, compounds with the same polar headgroup could be separated (i.e., sphingomyelin and phosphatidylcholine) due to their difference in acyl backbone. In contrast, sphingomyelin and glucosylceramide have identical sphingosine backbones but different headgroups and these could not be separated on a C₁₈ column. To minimise the retention time and to obtain better peak shapes, we chose for gradient elution (85–100% methanol). With this procedure, the peaks elute within 17 min.

When a cyano column was used, it was possible to separate the PLs on the basis of their polar behaviour. With this column, it was possible to separate
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Fig. 1. Isocratic separation of the NBD-lipids on C18 column. (A) Separation of NBD-sphingomyelin (0.3 pmol) and NBD-glucosylceramide (0.3 pmol) (peak 1), NBD-ceramide (0.4 pmol; peak 2) and C6-NBD-C16-phosphatidylcholine (0.5 pmol; peak 3); mobile phase flow-rate 0.6 ml/min; 15% 45 mM triethylammoniumphosphate (TEAP) (pH 3) and 85% 45 mM TEAP in methanol. (B) Influence of the amount of methanol on the retention time of the NBD-lipids: C6-NBD-C16-phosphatidylcholine (Δ), NBD-ceramide (■) and NBD-sphingomyelin + NBD-glucosylceramide (♦).

NBD-sphingomyelin from NBD-glucosylceramide, but then the peaks of NBD-ceramide and NBD-glucosylceramide overlapped. As the separation of the compounds with this stationary phase is based on polarity, the effect of varying the pH on the selectivity was studied. In the pH range of 2.5–6, no effect on the retention nor on the resolution of the compounds was observed. This could be due to the amount of methanol (85%) used. However, the difference between ceramide and glucosylceramide is only a sugar moiety and influence of the pH on the separation was therefore not expected. Variation of the amount of methanol had drastic effects on the peak shape of the PLs. With relatively low amounts of methanol (<80%) the plate number fell off to about 1000 while it is about 4000 with methanol percentages above 80%. Due to this reduction in plate number there was no separation between sphingomyelin and phosphatidylcholine possible even at 60% methanol although there was a difference in retention time (Fig. 2). The sharp decrease in plate number is probably caused by the change in
retention mechanism: above 80% methanol the retention mechanism is based upon the polar interactions of the stationary phase with the polar moieties of the lipids giving more retention at higher amounts of methanol. Under these conditions, separation between the choline moiety and hydroxyl groups is possible. At lower amounts of methanol, the apolar interactions of the acyl backbone with the propylchain of the stationary phase come into account.

The individual retention times of the PLs on both columns described above, suggests that with a combination of both columns separation of all compounds is possible. It can be calculated with the data shown (Figs. 1 and 2) that the retention times for both columns in series would become 7.5, 7.9, 8.7 and 11.1 min, respectively for NBD-glucosylceramide, NBD-sphingomyelin, NBD-ceramide and C6-NBD-C16-phosphatidylcholine, suggesting separation of all compounds mentioned. However, these calculations do not take into account the effect of a gradient program used. As a consequence of the use of a gradient program with coupled columns the conditions for separation on both columns are not identical. Consequently, it is hard to predict which column should be placed in front of the other. It can be suggested that the effect of the gradient leads to a better separation for the first three peaks as the resolution between sphingomyelin and glucosylceramide and ceramide on the cyanopropyl column is increased at higher methanol percentages.

3.1. Final procedure

The best option was to place the C18 column in front of the cyanopropyl column in order to elute the compounds with a relative high amount of methanol of this column. In Fig. 3, the chromatograms are shown for the separation of the PLs on the combined columns. Fig. 3A shows the chromatogram for the cyanopropyl column placed after the C18 column, while in Fig. 3B the cyanopropyl column is placed before the C18 column; the separation is carried out under exactly the same conditions. As can be seen the separation was optimal in the proposed sequence (Fig. 3A). The real and calculated retention times slightly differ but, as suggested by the simple calculations above, separation could be achieved. The resolution between NBD-glucosyleramide and NBD-sphingomyelin was about 1.1 causing a slight overlap of these peaks. Improvement of this separation by changing the pH or the amount of methanol was not
In order to show the feasibility of the system, other NBD-labelled PLs were also injected into the HPLC system. In Fig. 4, the separation of phosphatidylcholines with different acyl backbone length is shown. Retention time depended on chain length with the shortest lipid eluting first. The position of the acyl chain and the NBD-label on the glycerol backbone led to a slight difference in retention [10,11]; the acyl group at the end of the glycerol (sn1) has a stronger interaction with the C_{18} chains of the stationary phase and therefore the retention is increased. Some of the NBD-lipid standards showed a sn1−sn2 position-isomerism of the acyl chains. Other lipids with different headgroups can also be separated as shown in Fig. 5. With these lipids the effect of the polar headgroup can be seen, the rather polar serine group eluted earlier at the pH used compared to the less polar ethanolamine and choline groups.

Under the conditions mentioned above a calibration curve, $n=3$; range 0.06–17.28 pmol (nine data points), for the internal standard C6-NBD-C16-phosphatidylcholine was measured. Good linearity was obtained with the system (slope $= 12.65 \pm 0.015$, intercept $= 0.104 \pm 0.091$, $r^2 = 0.99999$); the detection limit was 2 fmol (signal-to-noise ratio $= 3$). As mouse bile samples are very small (<5 µL/5 min) the samples were diluted with an internal standard.
solution and subsequently injected. Addition of known amounts of C6-NBD-ceramide, C6-NBD-sphingomyelin or C6-NBD-C16-phosphatidylcholine to bile samples showed a similar relative fluorescent response (95–105%; n=2) at 1.2 pmol. This allows the use of a single calibration curve in order to quantify the amounts different NBD-labelled lipids.

Fig. 6 shows the chromatograms of bile of a NBD-ceramide infused mouse. At the start of the chromatogram, several peaks are present originating from fluorescent compounds normally present in bile (Fig. 6A). During the whole experimental phase these peaks remained, only towards the end of the perfusion did the amount decrease. When a liver perfusion was carried out without administration of NBD-ceramide, these early eluting peaks were still present indicating that these peaks are not metabolites or breakdown products of the applied fluorescent label. In Fig. 6B, details of chromatograms of the subsequent bile samples of a mouse undergoing a liver perfusion are shown after normalisation to internal standard. As can be seen the NBD-ceramide peak rose immediately after administration of the NBD-ceramide and subsequently the peaks of both metabolites appeared. The amount of C6-NBD-ceramide (peak 3) ranged from 23.1 pmol at 40 min down to 1.1 pmol at 110 min. The metabolites C6-NBD-glucosylceramide (peak 1) and C6-NBD-sphingomyelin (peak 2) had their secretion maximum at 3.4 pmol and 1.3 pmol at 50 min respectively. During the whole analysis of bile samples belonging to one liver perfusion the system remained stable with no changes in retention time and no increase in pressure. The C_{18} precolumn was changed only once a
week (after about 80 injections) mainly as a precaution.

4. Conclusion

The method discussed above is capable of separating and quantifying the fluorescent lipids analogues NBD-sphingomyelin and NBD-glucosylceramide appearing in bile after injection with the precursor molecule NBD-ceramide. The previously described HPLC methods [10,11] lack the ability to discriminate between the biliary secreted metabolites of NBD-ceramide. The two-column approach enables separation of PL analogues on the basis of their headgroup as well as backbone. The advantages of this method are its direct compatibility with the aqueous bile samples avoiding an extraction step and its sufficiently high sensitivity to monitor the secretion of the metabolites in extremely small bile volumes. Furthermore, this method can also be used to monitor the secretion of NBD-PL analogues with small changes in acyl chain length or changes in headgroup. In future studies we want to use these properties to study the mechanisms responsible for the selectivity of the transport processes involved in biliary PL secretion.

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