Quantitative determination of thiolipids in organic solution, in membranes, and on HPTLC plates

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Abstract In the presence of *ortho*-phthalaldehyde and glucosamine, thiolipids form fluorescent isoindole derivatives. This reaction can be used to quantify single- and double-chain mercaptans in membranes (liposomes) and micellar solutions. The lower detection limit is 100 pmol. In addition, the assay allows the detection of 1.9 nmol thiolipids on HPTLC plates and the fluorescence signal is stable for days. A minor modification of the commonly used DTNB (Ellman's) assay allows the quantification of thiolipids in organic solutions at a concentration down to 3 nmol.—**Zellmer, S., J. Lasch, and U. Rothe.** Quantitative determination of thiolipids in organic solution, in membranes, and on HPTLC plates. *J. Lipid Res.* 1997. **38:** 2374–2379.

Supplementary key words *o*-phthalaldehyde • fluorescence

Chemical headgroup modifications of naturally occuring phospholipids increase the number of possible applications. Thiolation, for example, increases the chemical reactivity of lipids and allows the attachment of numerous substances by disulfide bridges. Subsequent incorporation of thiolipids into liposomal bilayers, for example, enables the binding of proteins to vesicle surfaces (1). In this way it is possible to link enzymes (2), antibodies and prodrugs, leading to numerous applications in biomedical research and pharmacology. In addition, thiolipids are used in biosensor technology to cover the surface of gold particles with a monomolecular lipid film (3, 4). Therefore it is important to quantify the efficiency of headgroup thiolation and the concentration of thiolipids.

Commonly, the photometric assay with DTNB (5,5′-dithiobis-(2-nitrobenzoic acid)) is used to quantify HS-groups of dissolved proteins and amphiphile molecules (5). However, this assay is limited to water-soluble substances. In consequence, long-chain thiolipids are frequently quantified after incorporation into liposomal membranes (2).

Different fluorimetric assays for the detection of HS-groups have been established, based on the use of maleimide (6) and dansyl derivatives (7). These assays, however, are generally applied in the field of protein research and are not useful for the quantification of thiolipids.

Here we present a novel fluorimetric assay of thiolipid determination in liposomal suspensions and on HPTLC plates with *o*-phthalaldehyde. OPA is commonly used to quantify amino acids (8), based on the fluorometric detection of an isoindole derivative formed by OPA, primary amines (amino acids or aminolipids (9)), and a mercaptan. In this study we show that the fluorescent isoindole derivative also forms with glucosamine (primary amine) and long-chain thiolipids, allowing the detection and quantification of different thiolipids in solution and on HPTLC plates. In addition, we introduce a modification of the commonly used DTNB reaction, which allows the quantification of long-chain thiolipids in organic solutions.

MATERIAL AND METHODS

Chemicals

The soybean phospholipid Phospholipon 90 is a product of Nattermann Phospholipid GmbH (Köln, Germany). OPA, $NaBH_4$, PDPH, and hexadecanoylmer-

Abbreviations: AU, absorbance units; DHM, 1,2-dihexadecyl-3-mercaptoglycerol; DHPPPA, 1,2-dihexadecyl-*sn*-glycero-3-phosphoethanol-3-(2-pyridyldithio)propionylhydrazide; DMPG, 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol; DMPM, 1,2-dimyristoyl-*sn*-glycero-3-phospho-ethanolmercaptan; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DSPC, 1,2-distearoyl-*sn*-glycero-3-phosphocholine; DTE, dithioerythritol; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); GluN, D-glucosamine hydrochloride; HD-SH, hexadecanoylmercaptan; HEPES, N-[2-hydroxyethyl]piperazine-N′[2-ethanesulfonic acid]; HPTLC, high-performance thin-layer chromatography; MOPS, 3-[morpholino]propanesulfonic acid; OPA, *o*-phthalaldehyde; PDPH, 3-(2-pyridyldithio)-propionylhydrazide; RU, relative units; Tris, 2-amino-2-(hydroxymethyl)aminomethane.

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captan were purchased from Sigma (München, Germany). A methanolic OPA stock solution (10 mg in 1 ml) was prepared and diluted to 0.1% (v/v) with phosphate buffer prior to the experiments. Octyl-D-glucopyranoside was obtained from Fluka (Neu-Ulm, Germany). The HPTLC chromatoplates (HPTLC 60 F_{254}), CH_3OH, and $CHCl_3$, all HPLC grade, and Tris were products of Merck (Darmstadt, Germany). NaCl, Na_2HPO_4, NaH_2PO_4, all p.a., were purchased from VEB Laborchemie (Apolda, Germany). The latter were used to prepare 100 mM phosphate buffer, pH 8. GluN and DTE were obtained from Serva (Heidelberg, Germany). DTNB is a product of Ferak (Berlin, Germany).

Synthesis

DHM was synthesized using 1,2-dihexadecylglycerol, according to Mitsunobu (10). DMPM was synthesized according to a modified procedure of Hirth and Berchthold (11). DHPPPA was synthesized using 1,2-diacylglycero-3-phosphoglycerol, which was oxidatively cleaved with HJO_4, thereby forming the corresponding aldehyde. The latter was coupled with PDPH, dissolved in $CHCl_3$. The obtained dithio structure of DHPPPA was reductively cleaved with $Na[BH_4]$, dissolved in MeOH for 20 min, according to Means and Feeney (12).

Liposome preparation

Phospholipids and HD-SH were weighed in ratios between 10:0 and 4:6 (w/w) into vials and dissolved in methanol–chloroform 1:1(v/v). The organic solvents were removed under a gentle stream of warm nitrogen and the lipids were dried overnight under reduced pressure. The dry lipid film was rehydrated with 0.1 M phosphate buffer (pH 8) and small oligolamellar vesicles (transparent suspension) were obtained by tip-probe sonication with a Branson Sonifier W250 (Danbury, CT). The final lipid concentration was 1% (w/v).

Fluorescence measurements of the isoindole derivative

For each measurement, 10 µl of the liposome suspension was diluted with 40 µl phosphate buffer and the vesicles were solubilized by addition of 100 µl octyl-glucopyranoside (10%, w/v). Finally, 400 µl *o*-phthalaldehyde solution (0.1%) followed by 400 µl glucosamine solution (0.1%) were added, and the fluorescence intensity of all samples was determined after an incubation of 16 min at room temperature with an Hitachi F-4500 fluorescence spectrophotometer (Hitachi Scientific Instr. Ltd., Wokingham, UK). The excitation and emission wavelengths were 360 nm and 455 nm, respectively. The nonspecific fluorescence intensity of the control sample, free of thiolipids, was substracted from the data of the samples.

Absorbance measurements of the nitrophenolate ion

A stock solution of DTNB (2.5 mM) and Tris (5.26 mM) in methanol was prepared. Of this solution 150 µl was added to 150 µl DTE (in methanol) or HD-SH (in chloroform). After stabilization of the signal intensity, the absorbance was determined at 400 nm.

High-performance thin-layer chromatography

Lipids were dissolved in chloroform–methanol 1:1 (v/v) and applied on HPTLC plates with the aid of a Linomat 4 (CAMAG, Muttenz, Switzerland). Subsequently, the chromatoplate was developed with two different solvent systems. To separate the apolar monoalkylthiols and the polar thiophospholipids, the chromatoplate was developed first with $CHCl_3$–MeOH–H_2O 65:35:0.4 (v/v/v) to a distance of 4 cm and then with $CHCl_3$–MeOH 95:5 (v/v) to a distance of 8 cm from the origin. After drying, the plate was dipped first in OPA (0.1%) and then in GluN solution (0.1%). A bright green fluorescence developed immediately and could be visualized with an ultraviolet lamp (neoLab, Heidelberg, Germany) at an excitation wavelength of 366 nm. In order to visualize all lipids, the plate was stained with iodine vapor.

Quantification of the fluorescent HD-SH derivatives on the HPTLC plates was performed with a CAMAG TLC SCANNER II at an excitation wavelength of 365 nm. A cut-off filter ($\tau_{440\ nm}$ = 0.827, $\tau_{410\ nm}$ = 0.04) was inserted into the emission light path to reduce the amount of reflected light. The beam dimensions were 4 mm × 2 mm and the plate was scanned at a speed of 4 mm/s. Peak areas of the fluorescence signal were used for further quantification, using the CAMAG standard software CATS.

RESULTS

Figure 1 shows excitation and emission spectra of the fluorescent derivative, formed 15 min after addition of GluN and OPA to solubilized DPPC–HD-SH liposomes 7:3 (w/w). The two peaks of the excitation spectrum are at 390 and 411 nm. The maximum of the emission spectrum is at 456 nm with a shoulder at 435 nm. The shape of the spectra depends on the kind of primary amine. Substitution of phosphate buffer by HEPES and of GluN by glycine, for example, results in a single peak emission spectrum with a maximum at 458 nm.

The isoindole derivative is formed immediately after addition of OPA to the reaction mixture. However, in the absence of mercaptans, fluorescence intensity also increases, due to an unspecific reaction. The intensity of this unspecific fluorescence and the specific mercap-

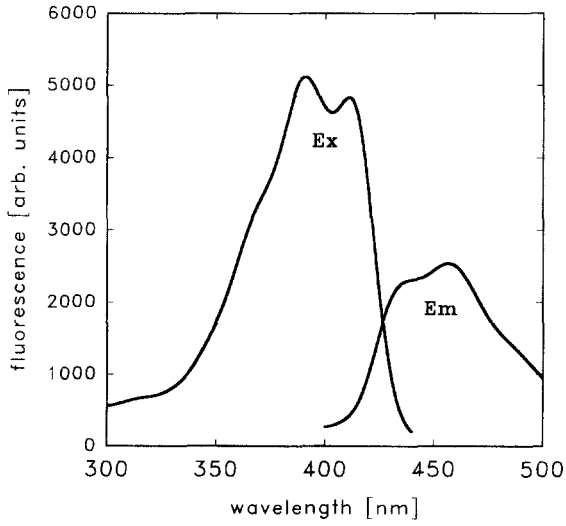

Fig. 1. Excitation and emission spectrum of the fluorescent isoindole derivative, formed 15 min after the incubation of 400 µl *o*-phthalaldehyde (0.1%), 6 µg hexadecanoylmercaptan, and 400 µl glucosamine solution (0.1%) in 0.1 M phosphate buffer (pH 8) at room temperature. Hexadecanoylmercaptan was incorporated into DPPC liposomes at a weight ratio of 3:7 and 2 µl of this liposomal suspension (10 mg/ml) was used in the experiment.

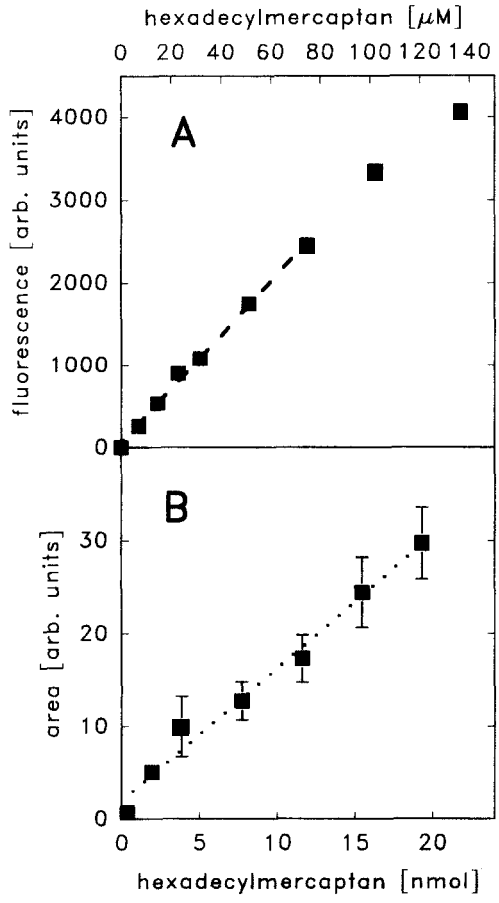

Fig. 2. A: Measurement in aqueous micellar solutions. The fluorescence intensity (Ex. 360 nm, Em. 455 nm) increases linearly with the amount of mercaptan (HD-SH) added, using OPA and GluN as derivatization reagents at room temperature. At concentrations above 100 µM linearity is lost. HD-SH was incorporated in liposomes (10 mg/ml) at different concentrations. Each point represents the mean of three separate measurements and error bars are smaller than dot size. B: Measurement on HPTLC plates. The fluorescence intensity (Ex. 366 nm, Em. ≥ 440 nm) of the HD-SH, OPA, and GluN adduct increases linearly with the amount of HD-SH (n = 4, error bars represent the SD).

tan-GluN-OPA derivative depends on the concentration and the kind of primary amine. The incubation temperature has a high impact on the intensity of the blank value. At 45°C the blank value increases 10-fold and therefore the signal to noise ratio is reduced. In all experiments, however, the fluorescence intensity of the unspecific reaction product(s) was below that of the specific GluN-OPA-mercaptan reaction product.

In a series of experiments, the amount of HD-SH incorporated into liposomal membranes was kept constant (10 µl DPPC-HD-SH, 7:3) while the relative amounts of GluN (0.1%) and OPA (0.1%) were varied between 5 and 600 µl. The total volume in the cuvette was 1270 µl. Maximal fluorescence intensity was obtained after the addition of equal amounts of GluN and OPA. Therefore we used equal amounts of GluN and OPA in all further experiments.

Furthermore, the relationship between fluorescence intensity and amount of mercaptanes added was investigated using the following technique. Vesicles of soybean phospholipid and HD-SH were prepared and lipid ratio was varied between 10:0 (w/w) and 4:6 (w/w), respectively. **Figure 2A** shows that the fluorescence intensity increases in a linear manner with increasing amounts of HD-SH. The linear regression line has a slope of 32.8 RU · µM^{-1} and intersects the y-axis at 52 RU ($r = 0.998$). At concentrations above 80 µM HD-SH linearity is lost under our assay conditions. At the lowest mercaptan concentration (1.8 µg, 7 nmol in 1 ml), the fluorescence intensity is still 254.8 RU. Therefore we conclude that OPA and GluN can be used to quantify thiolipids dissolved in aqueous micellar solutions. As all liposomes were solubilized into micelles, the assay allows no discrimination between inner and outer monolayer thiolipids.

In the next experiments we compared the novel OPA-GluN assay with the commonly used DTNB assay. The latter was modified for the use in organic solvents by dissoving TRIS in methanol. During the assay reaction a nitrothiophenolate ion is produced, which can be detected at 400 nm. **Figure 3** shows that nitrothiophenolate is formed in methanolic solution after addition of dithioerythritol. The absorbance increases with

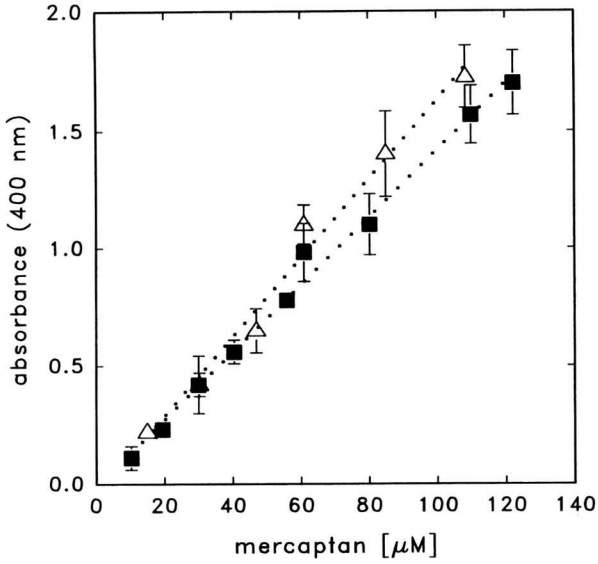

Fig. 3. Thiolipids can be quantified in methanolic solution with DTNB. The concentration of the nitrophenolate ions increases proportional with the amount of HD-SH (■) and DTE (△) in solution (n = 10, error bars represent the SD).

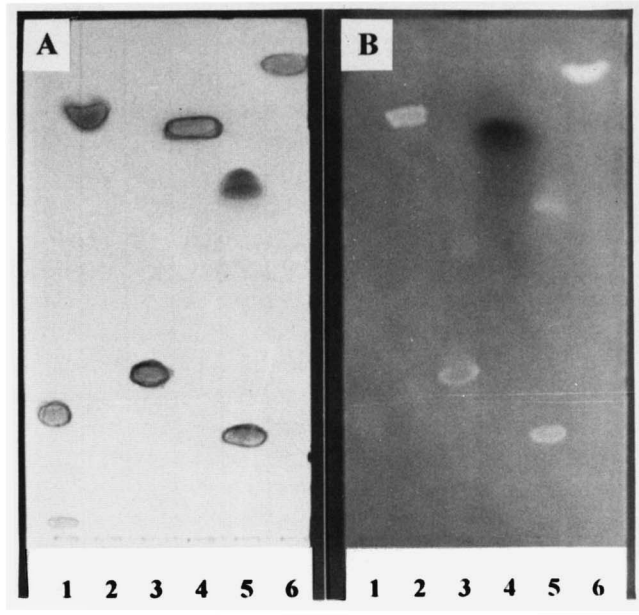

Fig. 4. Visualization of different single- and double-chain thiolipids on HPTLC plates after separation with two different solvent systems and postchromatographic derivatization with iodine vapor (A) and with OPA and GluN (B). The following lipids were applied: lane 1: 5 μg DSPC-DMPG (1:1), lane 2: 5 μg DHM, lane 3: 5 μg DMPM, lane 4: 5 μg DHPPPA, lane 5: same as lane 4 after the reduction with sodium borohydride, lane 6: 1 μg HD-SH.

the amount of DTE added. The linear regression line has a slope of 0.0136 AU · μM^{-1} and intersects the y-axis at 0.0319 AU ($r = 0.996$). At the lowest DTE concentration used (10.34 μM), we obtained 0.11 AU. This corresponds to an absolute amount of 3 nmol DTE, the lower limit of this assay. The concentration of nitrothiophenolate ions increased also with increasing amounts of HD-SH. The parameters of the calculated regression line were 0.0168 AU · μM^{-1} and −0.0514 AU for slope and y-intercept, respectively ($r = 0.991$). Therefore we conclude that long chain thiolipids can be quantified after the substitution of water by methanol.

In order to see whether OPA and GluN can be used to quantify thiolipids on HPTLC plates, we applied different amounts of HD-SH on a silica plate. The peak areas of the fluorescent OPA-GluN-HD-SH derivative increased linearly with the amount of HD-SH applied (Fig. 2B). The linear regression line has a slope of 5.53 RU · $nmol^{-1}$ and a y-intercept at 1.94 RU ($r = 0.996$). The lowest detectable HD-SH amount was 1.9 nmol, applied on a 6-mm-long band.

In a subsequent set of experiments the assay's use and specifity for the detection of other thiolipids were studied. Double-chain thiolipids with different head-group structure were separated by HPTLC, and post-chromatographic derivatization was performed with OPA and GluN solution as well as with iodine vapor (**Fig. 4**). The fluorescent lipid derivatives were visualized at an excitation wavelength of 366 nm (Fig. 4B). DHM, DMPM, and HD-SH formed fluorescent isoindole derivatives, which were stable for days. DHPPPA reacted only after the reduction with methanolic $Na[BH_4]$ before the chromatographic separation. Two spots with different R_f values appeared, being most likely the two thiols obtained after the cleavage of the dithio-structure of DHPPPA. DHPPPA in the non-reduced form (Fig. 4B, lane 4) did not form any fluorescent chromophore, indicating that the reaction is specific for thiol-groups. In addition, DSPC and DMPG did not form any chromophores after the OPA-GluN derivatization. A subsequent staining with iodine vapor, however, made all substances visible (Fig. 4A).

Finally, we tried to stain different thiolipids by a modification of the DTNB assay. However, selective staining was not possible due to rapid bleaching of the nitrophenolate ion.

DISCUSSION

Ortho-phthalaldehyde, primary amines, and mercaptans can form fluorescent isoindole derivatives at alkaline pH. However, several substances may interfere with the reaction. Tryptophan and OPA form a fluorophore in the absence of mercaptans (13). In addition, experi-

ments confirmed that glycine (0.1%, w/v) and OPA form a yet unidentified fluorophore. Furthermore, glycine or histamine (both 0.1%, w/v) precipitate within a few minutes in 0.1 M sodium borate, 0.1 M MOPS, 0.1 M Tris, and 0.1 M HEPES buffer (all pH 8) in the presence of OPA, making these substances unsuitable for an analytical assay. Therefore, 0.1 M phosphate buffer (pH 8) and a glucosamine solution, which did not form precipitates in the presence of OPA, were used in all experiments. However, even in the absence of thiolipids, a nonspecific fluorescence appeared within a few minutes. We propose that this is due to an OPA-GluN adduct, similar to the histamine-OPA adduct described by Rönnberg and coworkers (13).

In the presence of mercaptans, however, the fluorescence is always higher than in their absence, probably due to a specific mercaptan-GluN-OPA isoindole derivative. It is very likely that the structure is similar to the well-known isoindole derivatives formed by OPA, 2-mercaptoethanol, and primary amines (8). This is supported by the observation that fluorescence intensity was highest when GluN and OPA were present in equal concentrations. The fluorescent isoindole derivative is formed rapidly and the appearance of the unspecific reaction product is always delayed. The unspecific reaction could not be suppressed by changing concentration, pH value, or incubation temperature. Acidification stops the formation of the unspecific reaction products and the isoindole derivative resulting in a decreased fluorescence intensity and low reproducibility. Therefore we did not change the reaction conditions, but subtracted the fluorescence intensity value of the unspecific reaction from samples containing thiolipids.

The reactivity of thiolipids with GluN and OPA differ and the resulting isoindole derivatives differ in quantum yield as well as in the excitation and emission spectra. This has important consequences for analytical procedures: reference lipids and the lipids to be quantified should be the same. If reference thiolipids are not available, lipids with similar conformation at the HS-group should be used.

We did not attempt to determine the lower detection limit of the OPA-GluN assay as the mercaptans differed in their reactivity and quantum yield of the isoindole derivative. The lowest HD-SH concentration in our study was 7 μM, which resulted in a fluorescence intensity of 254 units. Using the regression function obtained from Fig. 2A, a HD-SH concentration of 100 nM is detectable with conventional instruments. This corresponds to 100 pmol lipid in 1 ml buffer and is in good agreement with previously described detection limits of aminolipids using OPA (9) and of amino acids and peptides (50–200 pmol) (8).

The modified DTNB assay allows the quantification of thiolipids dissolved in methanol or chloroform (Fig. 3). Similar to the OPA-GluN assay, the mercaptans differ in their reactivity. DTE reacts within seconds with DTNB while HD-SH achieves stable absorbance values after 15 min. The advantage of the modified DTNB assay, compared to the non-modified DTNB assay and the OPA-GluN assay, is the applicability in organic solutions. The detection limit is 3 nmol for DTE and 10 μM for HD-SH (Fig. 3). This is less than the for OPA-GluN assay.

The OPA-GluN assay can be used for the detection (Fig. 4) and quantification (Fig. 2B) of thiolipids on HPTLC plates. It is specific for free HS-groups as dithio-structures DHPPPA, choline- or glycerol-headgroups do not form fluorescent derivatives. The sensitivity and fluorescence maxima of the fluorophores formed (observed with the naked eye) depend on the chemical nature of the reacting HS-head group, and the detection limit on HPTLC plates is 1.9 nmol (HD-SH).

We thank U. Bakowsky for the synthesis of the thiolipids, S. Sommer for her technical assistance, and G. Sänger for the photographs. The financial support of the Deutsche Forschungsgemeinschaft through grants SFB 197, Ze 376/1-1, and of the Fonds der Chemischen Industrie is gratefully acknowledged.

Manuscript received 6 May 1997 and in revised form 11 July 1997.

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