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QUANTIFICATION OF PENTAFLUOROBENZYL OXIME DERIVATIVES OF LONG CHAIN ALDEHYDES BY GC-MS ANALYSIS §

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Abstract

Negative ion mass spectrometric techniques for compounds having good ionization properties, such as pentafluorobenzyl derivatives, are believed to be more sensitive than positive ion methods. In this study we develop a technique of quantification of long-chain aldehydes by analysis of their pentafluorobenzyl oxime derivatives utilizing gas chromatography-mass spectrometry in the negative ion chemical ionization mode. We establish that separation of plasmalogens prior to pentafluorobenzyl derivatization is essential and achieve this separation over a silica column. We further demonstrate the sensitivity of this method and utilize this technique to identify increases in hexadecanal and octadecanal in 3-aminotriazole treated human neutrophils.

Keywords

gas chromatography; mass spectrometry; aldehydes; pentafluorobenzyl; hexadecanal; octadecanal; aminotriazole; negative ion chemical ionization

Introduction

Lipid oxidation through a variety of enzymatic and non-enzymatic mechanisms can lead to the formation of aldehydes [1,2]. The generation of aldehydes from polyunsaturated fatty acids through the attack of reactive oxidizing species is considered particularly facile and leads to the formation of aldehydes such as 4-hydroxynonenal (HNE) and malondialdehyde (MDA) [3–5]. Both, HNE and MDA have been extensively studied and are involved in numerous signaling pathways [1,2,6]. Besides short-chain aldehydes, the formation of long-chain aldehydes such as 2-chlorohexadecanal (2-CIHDA) and 2-hexadecenal is also known [7,8]. 2-CIHDA is a neutrophil chemoattractant and can also inhibit endothelial nitric oxide synthase [9,10]. Thus, knowledge of the overall load of aldehydes can provide an insight into the pathogenesis of diseases related to oxidative stress.

§ Abbreviations: 1,2-dihexadecanoyl-*sn*-glycero-3-phosphocholine, di-16:0 GPC; 1,2-diicosanoyl-*sn*-glycero-3-phosphocholine, di-20:0 GPC; 1-*O*-hexadec-1'-enyl-2-octadec-9'-enoyl-*sn*-glycero-3-phosphocholine, 16:0–18:1 pPC; 2-chlorohexadecanal, 2-CIHDA; 3-amino-1,2,4-triazole, ATZ; direct-infusion electrospray ionization tandem mass spectrometry, DI-ESI-MS/MS; gas chromatography-mass spectrometry, GC-MS; human coronary artery endothelial cells, HCAEC; 4-hydroxynonenal, HNE; malondialdehyde, MDA; negative ion chemical ionization, NICI; N-octadecanoyl sphingosylphosphorylcholine, 18:0 SM; pentafluorobenzyl, PFB; phosphatidylcholine, PC; selected ion monitoring, SIM; thin layer chromatography, TLC.

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Aldehydes have been measured by a variety of techniques including spectrophotometry [11] and mass spectrometry. Pentafluorobenzyl (PFB) oxime derivatization of aldehydes followed by gas chromatography-mass spectrometry (GC-MS) analysis has been a commonly employed technique for aldehyde quantification. Mass spectrometric determination of PFB oximes has been performed by electron impact [12] and negative ion chemical ionization (NICI) [13]. While most of these studies were performed for short-chain aldehydes, detection of long-chain aldehydes in biological tissue has not been studied in depth. We have previously quantified α -chlorinated fatty aldehydes and 2-hexadecenal as their PFB oximes utilizing GC-MS in NICI mode [7,8]. However, for measurement of long-chain saturated aldehydes in biological tissue, thin layer chromatography (TLC) separated aldehydes were oxidized to the corresponding acids, followed by their quantification as bromophenacyl esters [14].

It has been suggested that mass spectrometric determination in negative ion mode can be two orders of magnitude more sensitive than positive ion methodologies [15]. Accordingly, in this report, we develop a method for quantification of long-chain aldehydes utilizing PFB oxime formation and GC-MS analysis in the NICI mode. We establish a need for separation of plasmalogens from aldehydes prior to their derivatization and utilize a silica (Si) column to achieve this. We use the method to detect changes in 3-amino-1,2,4-triazole (ATZ) treated human neutrophils.

Experimental Procedures

Materials

Hexadecanal and octadecanal were synthesized using Dess-Martin oxidation as described [16]. [*d*₄]-hexadecanal was synthesized utilizing Mancuso oxidation of [*d*₄]-hexadecanol, which was prepared by reduction of the corresponding acid. A similar procedure has been described earlier [9]. Bovine heart phosphatidylcholine (PC), 1,2-dihexadecanoyl-*sn*-glycero-3-phosphocholine (di-16:0 GPC) and 1,2-diicosanoyl-*sn*-glycero-3-phosphocholine (di-20:0 GPC) were purchased from Avanti Polar Lipids. 1-*O*-hexadec-1'-enyl-2-octadec-9'-enyl-*sn*-glycero-3-phosphocholine (16:0–18:1 pPC) was synthesized as described previously [17] and N-octadecanoyl sphingosylphosphorylcholine (18:0 SM) was purchased from Matreya, Inc. Human coronary artery endothelial cells (HCAEC) were purchased from Cambrex and maintained according to manufacturer's protocol. Silica (Si) columns (1 ml) were purchased from Sigma. All other reagents were obtained either from Sigma or Fisher.

Si column separation

Organic extracts of HCAEC and neutrophils were obtained after a modified Bligh and Dyer extraction [18]. [*d*₄]-hexadecanal was added in methanol as the internal standard. Organic extracts or 150 μ g bovine heart PC were dried and resuspended in 500 μ l chloroform. Each extract (500 μ l $\times$ 2) was loaded onto a pre-equilibrated Si column (pre-equilibration was performed with 10 ml chloroform) and eluted with 5 ml of chloroform. The void and elute volumes were collected in 15 ml screw-top tubes. This was dried and either derivatized to their PFB oximes for GC-MS analysis or resuspended in a 4:1 mixture of methanol/chloroform for direct-infusion electrospray ionization tandem mass spectrometry (DI-ESI-MS/MS).

PFB oxime derivatization and GC-MS analysis

Si-purified extracts or known amounts of aldehydes or lipids (100 μ g) were taken, dried and derivatized to their respective PFB oximes utilizing PFB hydroxylamine as the reagent. The PFB oximes were resuspended in 50–100 μ l of petroleum ether and analyzed by GC-MS as described earlier [8]. The analyses were performed either by mass scan analysis from *m/z* 100 to 800 or by selected ion monitoring (SIM) of the [*M*-HF][−] ion of the PFB oximes of respective

aldehydes. The ions monitored for the PFB oximes of hexadecanal, octadecanal and [*d*₄]-hexadecanal were *m/z* 415, *m/z* 443 and *m/z* 419, respectively.

DI-ESI-MS/MS

Following Si column separation, organic extracts were dried and resuspended in a 4:1 mixture of methanol/chloroform. These were injected onto the electrospray ionization interface (TSQ Quantum Ultra, Thermo-Fisher Corporation) at a flow rate of 3 μ l/min (after addition of the internal standard di-20:0 PC at 1.3 pmol/ μ l). Spectra were averaged over 3–5 min and processed utilizing Xcalibur software. Ions were monitored in the positive ion mass scan mode.

Limit of detection

HCAEC were incubated on 60 mm plates to confluency. The media was discarded and the cells were scraped with 1:1 mixture of methanol/saline (1 ml $\times$ 2). Varying amounts of [*d*₄]-hexadecanal were added in methanol (0 to 50 pmol). Organic extracts collected using a Bligh and Dyer extraction were Si column separated. The chloroform extracts were derivatized by PFB hydroxylamine and analyzed by GC-MS for [*d*₄]-hexadecanal. The area under the curve was integrated using the Chemstation software and plotted against the amount of [*d*₄]-hexadecanal added.

Neutrophil ATZ treatment

Human neutrophils were isolated from whole blood as described earlier [9]. 0.5×10^6 neutrophils/ml were incubated in the presence or absence of 10 mM ATZ at 37°C for 10 minutes. The reactions were terminated with the addition of methanol containing 20 pmol of [*d*₄]-hexadecanal as the internal standard. Following an extraction, the samples were separated over a Si column, derivatized and analyzed by GC-MS. The calculations were based on linear response curves generated for hexadecanal and octadecanal against [*d*₄]-hexadecanal.

Results

Plasmalogens are a subclass of lipids with a vinyl ether bond at the *sn*-1 position. The vinyl ether bond is readily susceptible to breakdown under acidic [19,20] and oxidative conditions [7,14,21]. Since PFB hydroxylamine derivatization requires acidic conditions we tested the stability of the vinyl ether bond, the acyl ester and the amide bond under derivatization conditions. Derivatization was performed in the presence of 16:0–18:1 pPC, di-16:0 GPC and 18:0 SM and the derivatives were analyzed by GC-MS. As shown in Fig. 1, the vinyl ether bond in plasmalogens degrades to generate the PFB oxime of hexadecanal (Fig. 1, trace a). For reference, the chromatogram, mass spectrum and fragment ions of the PFB oxime of hexadecanal are provided as supplementary data (supplementary data Fig. 1). However, the acyl ester bonds and the amide bond are stable (Fig. 1, trace b,c). Thus to quantify aldehydes, which would be of similar structure to the alkenyl chains of plasmalogens, prior separation is necessary.

To separate plasmalogens from aldehydes, we tested a Si column method. Pre-equilibrated Si columns were loaded with bovine heart PC in chloroform. Bovine heart PC was chosen because it contains ~33% plasmalogens. The void and the chloroform elute were collected and analyzed by DI-ESI-MS/MS. As seen in Fig. 2, the sodiated adduct of plasmalogens present at *m/z* 764.7 and 790.7 in bovine heart PC (Fig. 2a) are absent in the chloroform elute after Si column separation (Fig. 2b). The major PC species are outlined in supplementary data Table 1. The internal standard seen at *m/z* 868.8 (sodiated adduct of di-20:0 GPC) was added for comparison after Si column separation. Thus, it was concluded that Si column separation would successfully be able to separate the plasmalogens and since aldehydes are neutral lipids they

should readily elute in chloroform. Following this, the aldehydes could be derivatized to their PFB oximes and analyzed by GC-MS.

To test limit of detection of our method we synthesized [*d*₄]-hexadecanal and added it to HCAEC prior to Bligh and Dyer extraction. The cellular extracts were separated on a Si column, derivatized and analyzed for [*d*₄]-hexadecanal. Fig. 3 shows the plot of the amount of [*d*₄]-hexadecanal added against the area under the chromatographic peak for [M-HF][−] ion of its PFB oxime at *m/z* 419. The lowest amount we were able to easily detect was 0.5 pmol (inset). The dashed line represents 95% confidence limits of the linear detection curve.

Further, we treated human neutrophils with ATZ, an inhibitor of hydrogen peroxide scavenging enzymes such as catalase (EC 1.11.1.6) [22] and myeloperoxidase (EC 1.11.1.7) [23]. It is expected that ATZ will create an oxidative state and hence an increase in aldehyde load. Indeed, as shown in Fig. 4, ATZ causes an increase in hexadecanal and octadecanal. The calculations were based on linear response curves generated for hexadecanal and octadecanal against the internal standard, [*d*₄]-hexadecanal (supplementary data, Fig. 2). Collectively, these data suggest the sensitivity and utility of the method for quantification of long-chain aldehydes under biologically relevant conditions.

Discussion

Quantification of long-chain aldehydes as PFB derivatives from biological tissue poses a special problem due to the presence of masked aldehydes in plasmalogens. Earlier, TLC separation has been used to separate polar lipids such as plasmalogens from long-chain aldehydes prior to their measurement after oxidation to the acid followed by bromophenacyl esterification and liquid chromatography [14]. However, the drawbacks of that method include the acidic conditions used for TLC separation, a tedious procedure and low sensitivity. Separation of plasmalogens prior to the acidic derivatization conditions decreases the possibilities of degradation of the vinyl ether bond. Furthermore, the advantage of attaining a sensitive quantification, by utilization of PFB oxime derivatives, is retained.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Vinyl ether susceptibility of PFB hydroxylamine derivatization. 16:0–18:1 pPC (trace a), di-16:0 GPC (trace b) and 18:0 SM (trace c), were derivatized by PFB hydroxylamine and analyzed by GC-MS in the NCI utilizing the mass scan mode. Representative chromatograms obtained are shown. Each condition was repeated three times.

Figure 2. Si column separation. 150 μg of bovine heart PC was analyzed either directly (a) or after Si column separation (b) by DI-ESI-MS/MS. The internal standard di-20:0 GPC was added just prior to analysis.

Figure 3.

Limit of detection. Varying amounts of [*d*₄]-hexadecanal (0–50 pmol) was added to HCAEC prior to extraction. The cell extracts were then separated over a Si column and analyzed by GC-MS after derivatization. The figure shows the linear plot of the integrated area under the curve for chromatographic peak of *m/z* 419 against the known initial amount of [*d*₄]-hexadecanal. The individual points represent the mean with standard deviation of the measured area for that amount. The dashed lines represent the 95% confidence limits of the linear curve. The inset shows magnification of the lower end of the plot. GraphPad Prism (GraphPad Software, Inc, CA) was used for curve plotting and statistical analyses.

Figure 4.

Long-chain aldehydes increase in ATZ treated neutrophils. Isolated human neutrophils were treated with 10 mM ATZ for 10 min. The amount of hexadecanal and octadecanal in untreated and treated neutrophils was measured utilizing [*d*₄]-hexadecanal as the internal standard as outlined in 'Experimental Procedures'.