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Highly Sensitive Fluorescent Method for the Detection of Cholesterol Aldehydes Formed by Ozone and Singlet Molecular Oxygen

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Cholesterol oxidation gives rise to a mixture of oxidized products. Different types of products are generated according to the reactive species being involved. Recently, attention has been focused on two cholesterol aldehydes, 3β-hydroxy-5β-hydroxy-B-norcholestane-6β-carboxyaldehyde (1a**) and 3β-hydroxy-5-oxo-5,6-secocholestan-6-al (**1b**). These aldehydes can be generated by ozone-, as well as by singlet molecular oxygen-mediated cholesterol oxidation. It has been suggested that** 1b **is preferentially formed by ozone and** 1a **is preferentially formed by singlet molecular oxygen. In this study we describe the use of 1-pyrenebutyric hydrazine (PBH) as a fluorescent probe for the detection of cholesterol aldehydes. The formation of the fluorescent adduct between** 1a **with PBH was confirmed by HPLC-MS/MS. The fluorescence spectra of PBH did not change upon binding to the aldehyde. Moreover, the derivatization was also effective in the absence of an acidified medium, which is critical to avoid the formation of cholesterol aldehydes through Hock cleavage of 5α-hydroperoxycholesterol. In conclusion, PBH can be used as an efficient fluorescent probe for the detection/quantification of cholesterol aldehydes in biological samples. Its analysis by HPLC coupled to a fluorescent detector provides a sensitive and specific way to quantify cholesterol aldehydes in the low femtomol range.**

Cholesterol (cholest-5-en-3β-ol) is a neutral lipid found in the cellular membranes of mammals.[1] Cholesterol is susceptible to oxidation mediated by enzymatic and nonenzymatic mechanisms.[2−5] The nonenzymatic oxidation can be mediated by reactive oxygen species.[2] Several oxidized products of cholesterol have been characterized including hydroperoxides, epoxides, and aldehydes.[2,6,7] These oxysterols have been detected in biological tissues and their formation has been associated to neurodegenerative and cardiovascular diseases.[2−5] Recently, attention has been focused on cholesterol aldehydes that can be formed by the oxidation of cholesterol by ozone[8] and singlet molecular oxygen.[9,10]

The ozonation of cholesterol produces several oxidized products, in special two aldehydes (Figure 1), the cholesterol 5,6-secosterols, 3β-hydroxy-5β-hydroxy-B-norcholestane-6β-carboxyaldehyde (**1a**) and 3β-hydroxy-5-oxo-5,6-secocholestan-6-al (**1b**).[11,12] Wentworth and co-workers showed the presence of **1a** and **1b** in atherosclerotic plaques[8] and LDL oxidized with several oxidants.[13] These aldehydes have been also detected in neurodegenerative diseases, like Lewy body dementia[14] and Alzheimer disease.[15] The role of **1a** and **1b** in the pathogenesis of cardiovascular and neurodegenerative diseases has been investigated. In vitro studies have shown that cholesterol aldehydes can covalently modify proteins, as well as, accelerate their aggregation, as in the case of amyloid β-peptide formation[15−17] and α-synuclein[14]. Further studies have shown that covalent modification of apo-B by **1b** causes this protein to misfold, rendering the LDL particle more susceptible to macrophage uptake.[18] Moreover, some reports have also shown that cholesterol aldehydes can induce apoptosis in macrophages and cardiomyoblasts.[19,20] The induction of apoptosis in cardi-

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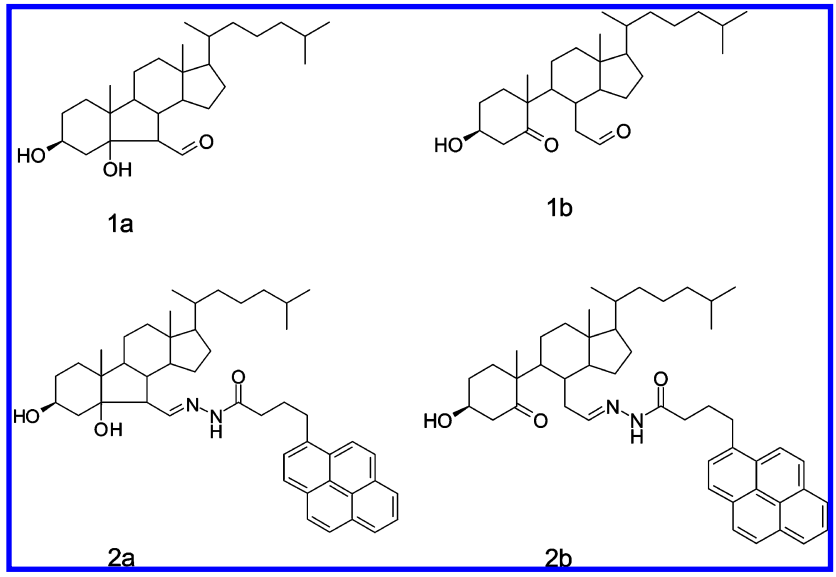

Figure 1. Structures of cholesterol carboxyaldehyde (**1a**), cholesterol secocholestanal (**1b**) and their corresponding fluorescent adducts formed upon derivatization with 1-pyrenebutyric hydrazide (**2a** and **2b**).

omyoblasts was associated with the induction of ROS generation and the activation of extrinsic and intrinsic pathway.[19]

Besides ozone, recent studies have evidenced the generation of cholesterol aldehydes in the reaction of cholesterol with singlet molecular oxygen. The proposed mechanisms involve Hock-cleavage of cholesterol 5α-hydroperoxide (5α-OOH) in the presence of acids[9] or cholesterol dioxetane decomposition.[10] In both cases, **1a** was detected as the major product. Supporting these studies, Tomono et al.[21] have also detected **1a** as the major product in the incubation of cholesterol with human myeloperoxidase in the presence H_2O_2 and chloride ions, a system that is suggested to generate singlet molecular oxygen in activated neutrophils.[22]

Several methods have been used for the determination of cholesterol aldehydes, including mass spectrometry,[10] UV−visible,[8,13,23] and fluorescence detection,[14,21] most of them coupled to liquid chromatography. Although mass spectrometry based methods have shown a good sensitivity, this technique requires specialized people and expensive equipment. UV−visible detection methods using 2,4-dinitrophenylhydrazine (DNPH) have been widely used to detect and quantify aldehydes derived from lipid peroxidation. However, this method is usually carried out in a strong acidic media, which is able to induce the cleavage of cholesterol 5α-OOH to form **1a** and **1b**,[9] therefore leading to an overestimation of the real aldehyde concentration in biological samples. An alternative method used to detect aldehydes involves their reaction with fluorescent probes and the detection of the fluorescent adducts formed. The fluorescent methods have been used to increase detection limits and exclude interferences. The fluorescent probe described in the literature for cholesterol 5,6-secosterols detection is dansyl hydrazine. Bosco and co-workers used this probe in acidic media to detect cholesterol aldehydes in brain.[14]

This manuscript describes a new fluorescence-based method to detect and quantify cholesterol aldehydes using the probe 1-pyrenebytiric hydrazine (PBH) (Figure 1). This method proved to be highly sensitive and has the advantage of minimizing the effect of interfering compounds and not requiring acidic media. The use of acid conditions showed to cause an overestimation of **1a** in a sample containing cholesterol 5α-OOH. Using PBH as the fluorescent probe we could detect **1a** in the low femtomolar range by HPLC coupled to fluorescence detector. Moreover, this methodology allowed detecting and calculating the ratio of **1a** and **1b** formed by the oxidation of cholesterol exposed to singlet molecular oxygen and ozone, respectively.

EXPERIMENTAL SECTION

Reagents. Cholesterol, silica gel (200−400 mesh, 60 Å), 1-pyrenebutyric hydrazide (PBH), deuterated chloroform ($CDCl_3$), sodium phosphate monobasic and dibasic were purchased from Sigma (St. Louis, MO). Methylene blue was purchased from Merck (Rio de Janeiro, Brazil). All the other solvents were of HPLC grade and were acquired from Mallinckrodt Baker (Phillipsburg, NJ). The water used in the experiments was treated with the Nanopure Water System (Barnstead, Dubuque, IA).

Synthesis of 3*β*-Hydroxy-5*β*-hydroxy-B-norcholestane-6*β*-carboxyaldehyde (1a). 1a was synthesized by photooxidation of cholesterol in the presence of methylene blue as described previously.[10] Briefly, 200 mg of cholesterol dissolved in 20 mL of chloroform was mixed with 250 μL of methylene blue (10 mM in methanol). This solution was cooled at 4 °C and irradiated under continuous agitation using two tungsten lamps (500 W) during 2.5 h. The formation of cholesterol oxidation products were checked by thin-layer chromatography using ethyl acetate and isooctane (1:1, v/v) as the eluent. **1a** was purified from the reaction mixture by flash column chromatography, using silica gel and a gradient of hexane and ethyl eter. The purified **1a** was analyzed and quantified in $CDCl_3$ by NMR spectroscopy using DRX500 instrument, AVANCE series (Bruker-Biospin, Rheinstetten, Germany) as described previously.[10] For the quantifica-

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tion, 2 μL of proprionaldehyde were added as the internal standard into 750 μL of the **1a** solution in $CDCl_3$. The ^1H signal corresponding to the aldehyde group of the internal standard and **1a** appeared at 9.73 and 9.62 ppm, respectively. The relative signals of ^1H of aldehydes were integrated by Mestre C software (Figure S1 in the Supporting Information (SI)).

Synthesis of 3β-Hydroxy-5-oxo-5,6-secocholestan-6-al (1b). **1b** was prepared by ozonation of cholesterol as described by Wentworth et al.[8] and Wang et al.[24] Ozone was produced at a rate of 100 mg/h by passing pure oxygen through AquaZone PLUS 200 instrument (Red Sea Fish Pharm. Ltd., Houston, TX). Oxygen flow rate was set to 10 mL/min. A solution of 10 mg/mL of cholesterol in chloroform was cooled at dry ice temperature and oxidized by bubbling ozone for 5 min. After oxidation, chloroform was evaporated with nitrogen gas and the residue was stirred for 2 h at room temperature with Zn powder (19.4 mg) in water-acetic acid (1:19 v/v; 3.1 mL). Dichloromethane (15 mL) was added and the mixture was washed five times with deionized water (5 × 15 mL). The organic phase was evaporated to dryness in vacuo. The residue was dissolved in isopropyl alcohol and kept at −80 °C for further analysis. The characterization of **1b** was performed by NMR spectroscopy using a DRX500 instrument, AVANCE series (Bruker-Biospin, Rheinstetten, Germany) operating at 11.7 T. Cholesterol ozonation followed by Zn reduction in acetic acid gave **1b** as a major aldehyde, as confirmed by ^1H NMR analysis (δ, ppm, $CDCl_3$): δ 9.607 (s, J = 0.5 Hz, 1H, CHO), 4.466 (s, 1H, H-3), 3.097 (dd, J = 13.5, 4.0 Hz, 1H, H-4e), 1.010 (s, 3H, CH_3-19), 0.671 (s, 3H, CH_3-18) (Figure S2 in the SI).

Derivatization of Cholesterol Aldehydes with PBH. The optimal conditions for the derivatization of cholesterol aldehydes were established using the purified **1a**. Time-course of PBH adduct formation with **1a** was monitored by incubating 100 μM **1a** with 2 mM PBH in isopropanol at 37 °C under continuous agitation for up to 8 h. Aliquots of 1 μL of the reaction mixture were taken at specified times and analyzed by HPLC coupled to fluorescence detector. The ideal concentration of the probe was determined by incubating 1 μM of **1a** in the presence of 1−1000 μM of PBH in isopropanol at 37 °C under continuous agitation for 6 h. The effect of the pH in the formation of **2a** was analyzed by incubating 1 μM of **1a** with 50 μM PBH in a reaction system consisted of isopropanol:10 mM phosphate buffer at pH 5.7, 7.4, and 8.0 (90:10, vol/vol). Quantitative analysis of **1a** was done by incubating an aliquot of sample with 600 μM of the probe in isopropanol containing 1 mM phosphate buffer at pH 7.4 (neutral condition) or 0.1 mM HCl (acid condition) at 37 °C for 6 h.

Analysis of the Fluorescent Adduct by HPLC Coupled with Fluorescence Detector. HPLC analysis was carried out on a Shimadzu Prominence system (Tokyo, Japan), consisted of LC-20AT pumps, SIL-20AV autosampler, RF-10Axl fluorescence detector, and a CBM-20A controller. One microliter of the sample was injected into a reversed-phase column Synergi C18 (50 × 4.6 mm, 2.5 μm particle size, Phenomenex, Torrance, CA). The HPLC mobile phase consisted of water (A) and methanol (B), and the flow rate was 1 mL/min. The separation of the fluorescent adducts was done using the following condition: 86% B for 5 min, 86−92% in 1 min, 92% B for 15 min, and 92−86% B in 1 min. The excitation and emission wavelengths were fixed at 339 and 380 nm, respectively.[25] For the analysis 1 μL was injected through the autosampler. Data were acquired at high sensitivity and gain of 1× in the fluorescence detector. Fluorescence spectra were acquired by the fluorescence detector RF-551 (Shimadzu, Japan). HPLC data were processed using the LC solution software (Shimadzu, Japan).

Analysis of the Fluorescent Adducts by HPLC-MS/MS. The PBH fluorescent adducts formed with **1a** and **1b** were analyzed by a HPLC system connected to a UV−visible detector (SPD 10 AVVP, Shimadzu, Kyoto, Japan) and a Quattro II triple quadrupole tandem mass spectrometer (Micromass, Manchester, UK) (HPLC-MS/MS). HPLC separation was carried out as described above. The eluent from the column was monitored at 342 nm and 10% of the HPLC flow rate was directed into the mass spectrometer. The fluorescent adducts were detected using electrospray ionization (ESI) in the positive ion mode. The source and desolvation temperature of the mass spectrometer were set at 100 and 200 °C, respectively. The cone voltage was set to 50 V, the extractor cone voltage was set to 5 V and the capillary and the electrode potentials were set at 4.5 and 0.5 kV, respectively. Collision energy was set at 20 eV for **2a** and 30 eV for **2b**. Full-scan data was acquired over a mass range of 100−900 m/z. Data was processed by means of the MassLynx NT software.

Cholesterol Oxidation Induced by Photooxidation, Ozone and HOCl/H_2O_2. Cholesterol (10 mg/mL in chloroform) was photooxidized in the presence of methylene blue for 2.5 h. An aliquot of this sample was taken for cholesterol aldehyde determination. The ozonation of cholesterol (10 mg/mL in chloroform) was conducted essentially as described for the synthesis of **1b**. After 5 min ozonation, chloroform was evaporated by nitrogen gas and the residue was dissolved in isopropanol. An aliquot of this sample was diluted and used for cholesterol aldehyde determination. The oxidation of cholesterol by HOCl/H_2O_2 system was conducted by incubating a 10 mM cholesterol solution containing 1% ethanol in 10 mM phosphate buffer at pH 7.4 with 1 mM HOCl and H_2O_2, under continuous agitation at 37 °C for 5 min.

Method Validation. The limit of quantification (LOQ) was established as the amount of adduct formed that generated a signal 6-fold higher than baseline. The limit of detection (LOD) was established as the amount of adduct formed that generated a signal 3-fold higher than baseline. The reproducibility was checked by intra- and interday analysis. Interday analyses were conducted on three consecutive days.

RESULTS

Synthesis and Characterization of 1a. Cholesterol carboxyaldehyde (**1a**) was synthesized by photooxidation of cholesterol.[10] After the reaction, the oxidized products were purified by flash column chromatography and **1a** was isolated. The identity of **1a** was confirmed by NMR spectroscopy as described by Uemi et al.[10] The ^1H NMR analysis showed a doublet peak at 9.62 ppm consistent with the presence of the aldehyde group in **1a** structure (Figure S1 in the SI). **1a** was quantified by ^1H NMR analysis using proprionaldehyde as the internal standard as described in the experimental section.

Characterization of 2a Fluorescent Adduct. Using the purified **1a** sample, several experiments were conducted to

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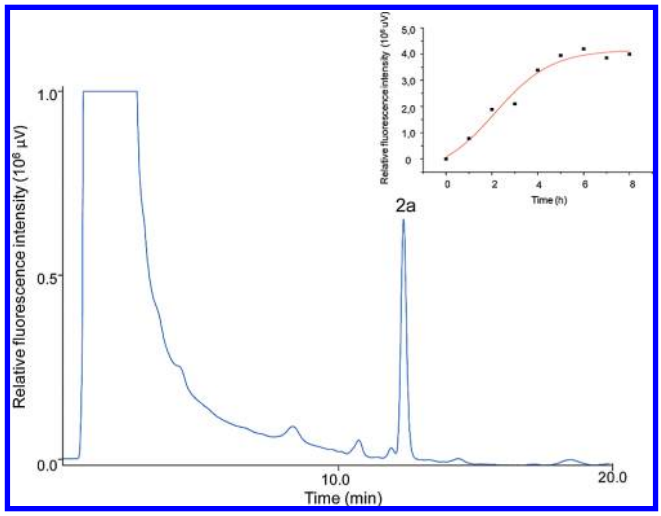

Figure 2. Analysis of **2a** fluorescent adduct, formed in the reaction of **1a** with PBH by HPLC coupled to fluorescence detector. HPLC/fluorescence chromatogram obtained for the analysis of **2a** using excitation at 339 nm and emission at 380 nm. For the derivatization, 5 pmol of **1a** was incubated with PBH 100 μM in a final volume of 100 μL isopropanol at 37 °C for 6 h under agitation. Analysis was done by injecting 1 μL of the reaction mixture into the HPLC. The inset shows the time dependent formation of **2a** during incubation of 100 μM of **1a** in the presence of 2 mM of PBH for up to 8 h.

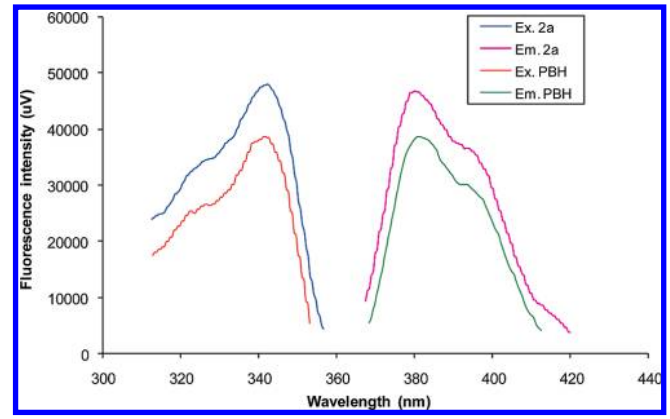

Figure 3. Fluorescence excitation and emission spectra of PBH and **2a**. Both compounds showed the same excitation and emission maximum wavelengths at 339 and 380 nm, respectively.

establish the optimal conditions for the reaction with PBH. Incubations of **1a** with PBH were conducted in isopropanol at 37 °C under continuous agitation. HPLC analysis using fluorescence detection showed the appearance of an intense peak at 12.5 min, corresponding to the fluorescent adduct **2a** (Figure 2). The pH effect on **2a** adduct formation was analyzed. Incubations conducted at pH 5.7, 7.4, and 8.5 showed that this adduct was preferentially formed at pH 5.7 (Figure S3 in the SI), which is in accordance with favorable formation of Schiff base adduct at slightly acidic conditions.

A time-course analysis of **2a** showed a time-dependent increase of the peak area up to 4 h, reaching a *plateau* after this time (inset, Figure 2). Based on this, the incubation time for the derivatization was fixed to 6 h. The ideal concentration of PBH for the reaction was established by incubating **1a** (1 μM) in the presence of 1−1000 μM of PBH. The fluorescent adduct formation reached its maximum with PBH concentration higher than 50 μM.

The fluorescence of **2a** was characterized to establish the optimal excitation and emission wavelengths. Fluorescence analysis of **2a** showed a spectrum similar to the original probe with excitation and emission wavelengths maximum at 339 and 380 nm, respectively (Figure 3). Thus, indicating that adduct formation does not alter the fluorescence spectra of the probe.

The formation of **2a** was also confirmed by HPLC-MS/MS. The mass spectrum of **2a** acquired by ESI in the positive ion mode showed peaks corresponding to **2a** molecular ion ($[M+H]^+$) and its sodium adducts ($[M+Na]^+$) at m/z 703 and 725, respectively (Figure 4A and 4B). Two other peaks at m/z 685 and 667 were also detected, corresponding to the loss of one ($[M+H-H_2O]^+$) and two water molecules ($[M+H-2H_2O]^+$), respectively. These ions were also observed in the MS/MS spectrum of m/z 703 (Figure 4C). The MS/MS spectrum also showed other fragment ions at m/z 431, 398, 365, and 303. The first three, correspond to the ions formed by the loss of pyrene butyric (PB) group ($[M+H-PB]^+$, m/z 431), PBH and two protons ($[M+H-PBH-2H]^+$, m/z 398) and PBH and two water molecules ($[M+H-PBH-2H_2O]^+$, m/z 365), respectively. The fragment ion at m/z 303 corresponds to the positively charged PBH ion.

Reproducibility and Stability. The intra- and interday reproducibility of the assay procedure was determined by evaluating three individual reactions containing **1a** (1 pmol; 1 μM final concentration) and PBH (50 μM). The relative standard deviation for the intra- and interday reproducibility at this concentration was 1.7% and 7.6%, respectively.

The stability of **2a** adduct was evaluated in triplicate reactions of **1a** (1 pmol; 1 μM final concentration) with PBH (50 μM). After derivatization, these samples were kept in vials inside the autosampler at 4 °C and the **2a** peak was monitored up to 6 h. There was no significant change in the peak area of the fluorescence signal during this period.

Standard Curve, Limit of Detection and Limit of Quantification. A calibration curve was constructed using 5−100 fmol (50−1000 nM) of **1a** in triplicate reaction in a final volume of 100 μL. Peak area of **2a** increased linearly over this concentration range with R^2 value of 0.9962 and $Y = 6.64 \times 10^3 X + 1.65 \times 10^4$. The limit of detection (LOD) and quantification (LOQ) of **1a** was 10 fmol (10 nM) and 20 fmol (20 nM), respectively.

Application of PBH for Cholesterol Aldehyde Detection in Oxidized Cholesterol Samples. The newly developed fluorescence-based method was applied for the detection and quantification of **1a** in cholesterol samples oxidized by photooxidation or ozone. Cholesterol photooxidation gave rise to a major peak at 12.5 min (Figure 5A). On the other hand, cholesterol ozonation yielded an intense peak at 10.3 min as well as some other smaller peaks, including the same peak at 12.5 min observed for the photooxidation (Figure 5B). Peak assignment was done by comparison of the retention times of the peaks with those obtained for the standard samples of **1a** (Figure 5C) and **1b** (Figure 5D). In this way, the peaks observed at 10.3 and 12.5 min were assigned to the fluorescent adducts **2b** and **2a**, respectively. Additionally, the identity of the peaks was confirmed by HPLC-MS/MS analysis (Figure S4 in the SI). It should be noted that the overall retention times for the analysis by HPLC-MS/MS were increased by almost 2 min compared to the analysis by HPLC/fluorescence detection. This retention time delay was due to the differences in room

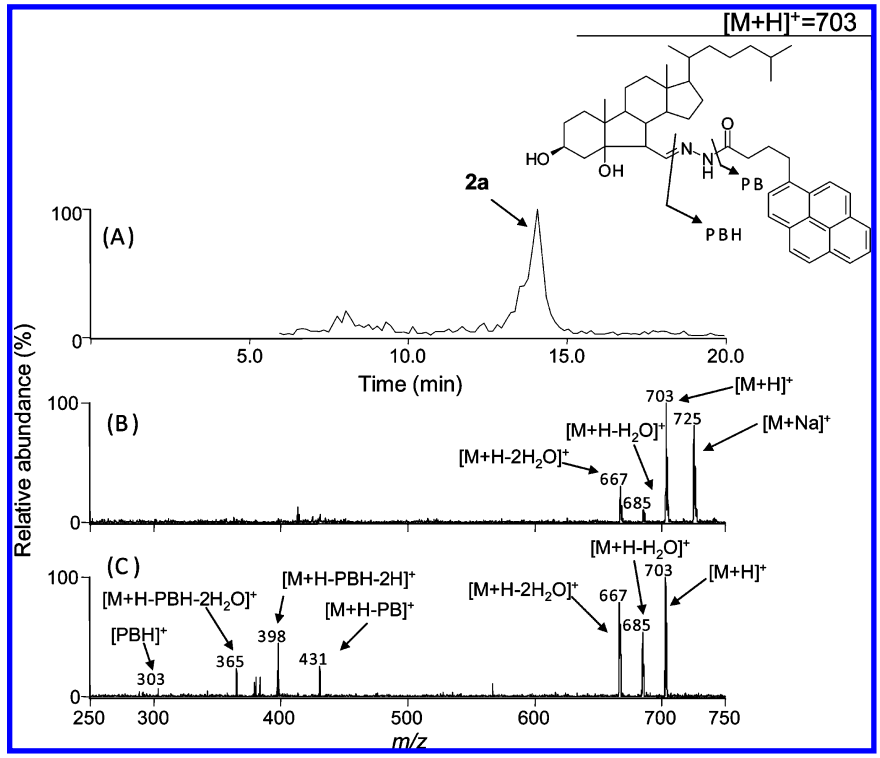

Figure 4. Analysis of **2a** by HPLC-MS/MS using ESI in the positive ion mode. The cone voltage and the collision energy were set to 50 V and 20 eV, respectively. Selected ion chromatogram of the ion at *m/z* 703 (A). Spectrum of the peak corresponding to **2a** at 14 min (B). Fragment ion spectrum of *m/z* 703 (C).

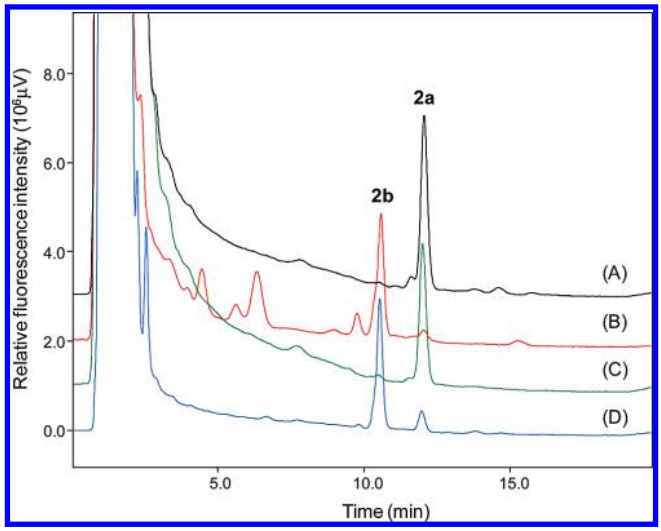

Figure 5. Analysis of cholesterol aldehydes formed in the photooxidation (A) and ozonation (B) of cholesterol using PBH. An aliquot of the sample was reacted with PBH (600 μM) for 6 h and analyzed by HPLC coupled to fluorescence detector. For peak assignment standard samples containing **1a** (C) or **1b** (D) were also reacted with PBH and analyzed.

temperatures, which was about 5 °C lower in the case of HPLC-MS/MS analysis.

The major fluorescent adduct detected in cholesterol ozonation was further characterized by HPLC-MS/MS analysis (Figure 6). Selected ion mass chromatogram of the ion at *m/z* 703 showed a single peak at 12 min. (Figure 6A). Mass spectrum for this peak showed two major ions, one at *m/z* 703 and other at *m/z* 725 (Figure 6B), which corresponds to the molecular ion ($[M+H]^+$) and the sodium adduct ($[M+Na]^+$) of **2b** (Figure 6B). Collision induced dissociation of the ion at *m/z* 703, showed the same fragment ions observed for **2a**, suggesting that the peak corresponds to its isomer, **2b** (Figure 6C). Fragments ions observed at *m/z* 685 and 667 are formed by the loss of one and two water molecules from **2b**, respectively. The ions at *m/z* 431, 398, 383, and 365 are formed by the loss of PB, PBH and two protons, PBH and one water, and PBH and two water molecules from **2b**, respectively (Figure 6C).

Aiming to get a quantitative data on cholesterol aldehyde formation, a calibration curve constructed for **1a** was used to determine its concentration. For the quantification, oxidized cholesterol samples were diluted to get a final cholesterol concentration of 219 μM. The amounts of **1a** detected in the samples by PBH method at neutral pH were 19.1 ± 4.0 μM and 0.7 ± 0.3 μM for the photooxidation and ozonation, respectively. This corresponds to a **1a** yield of 8.7% for the photooxidation and 0.3% for the ozonation. For comparison, **1a** quantification was also done using dansyl hydrazine method in the presence of acid (0.1 mM HCl) (method in the SI). The concentrations of **1a** determined by this method were 35.2 ± 0.7 μM and 1.0 ± 0.1 μM in the photooxidation and ozonation, respectively. This provides **1a** yields of 16% for the photooxidation and 0.4% for the ozonation. As can be clearly noticed, the **1a** yield determined by the dansyl hydrazine method was overestimated by almost 2-folds for the photooxidation. The same result was obtained when PBH derivatization was conducted in the presence of acid. Under this condition, the **1a** concentration was 32.6 ± 0.7 μM, which corresponds to a **1a** yield of 15% in the photooxidation. These results are consistent with the fact that photooxidized cholesterol

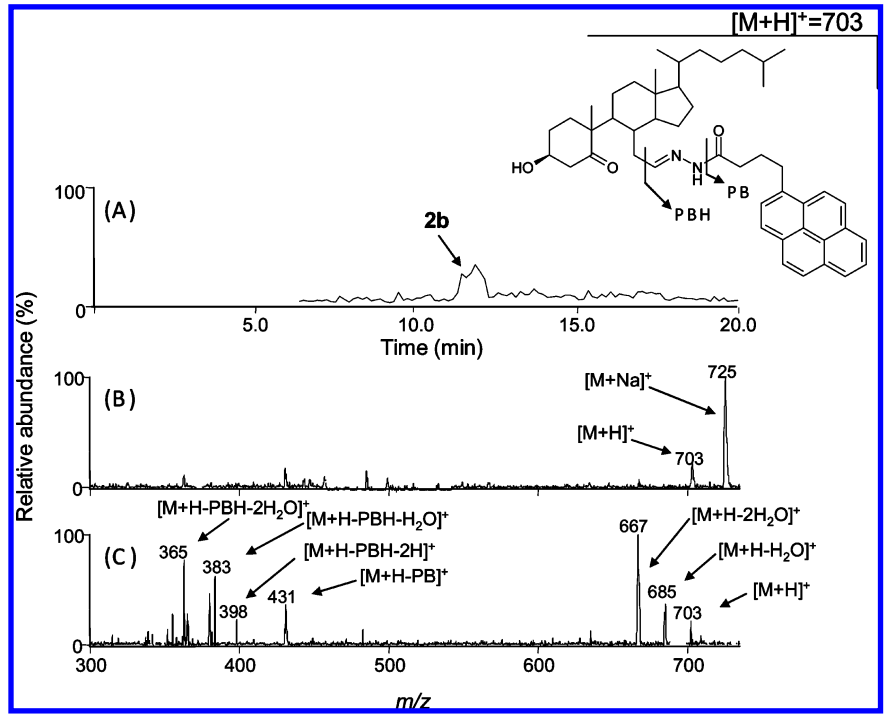

Figure 6. Analysis of **2b** by HPLC-MS/MS using ESI in the positive ion mode. The cone voltage was set to 50 V and the collision energy was set to 30 eV. Selected ion chromatogram for the ion at *m*/*z* 703 (A). Mass spectrum of the peak corresponding to **2b** at 12 min (B). Fragment ion spectrum of *m*/*z* 703 (C).

samples contain cholesterol 5α-OOH, which in the presence of acids is easily converted to **1a**.[9]

Moreover, the sensitivity of PBH and dansyl hydrazine methods were also compared (Figure S5 in the SI). PBH method conducted at neutral pH was almost 10 times more sensitive than dansyl hydrazine method. This difference was even larger when PBH reaction was carried out with acid, reaching a 90 times higher sensitivity.

PBH was also used for the quantification of ChAld formed during cholesterol oxidation promoted by the $HOCl/H_2O_2$ reaction system. This is a biologically relevant reaction system that is known to generate stoichiometric amounts of singlet molecular oxygen. The formation of **2a** was analyzed by HPLC coupled with fluorescence detector (Figure 7). The complete reaction system containing cholesterol and $HOCl/H_2O_2$ showed the appearance of an intense fluorescent peak corresponding to **2a**. The fluorescent adduct was quantified using the calibration curve and a value of 0.2 μM of **1a** was found. This corresponds to a yield of 0.2%.

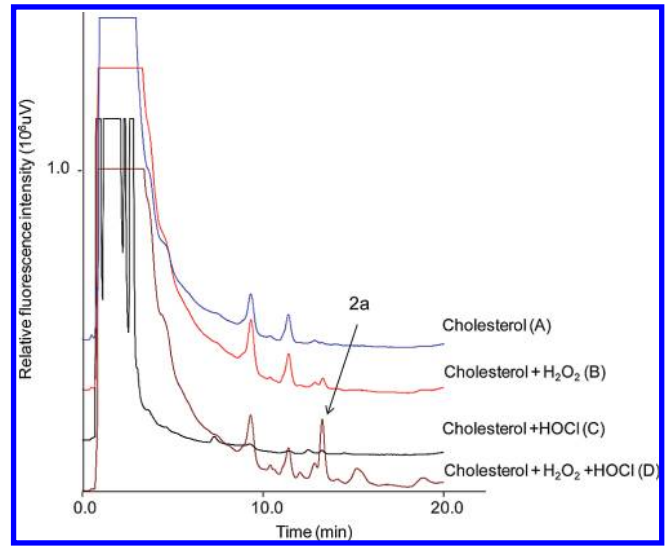

Figure 7. Analysis of cholesterol aldehydes generated by the reaction of cholesterol with H_2O_2 and HOCl using PBH. HPLC/fluorescence chromatograms obtained for cholesterol (A), and cholesterol incubated with H_2O_2 (B), HOCl (C), and H_2O_2 + HOCl (D). Reactions were carried out with 100 μM cholesterol in the presence of 1 mM HOCl, 1 mM H_2O_2 and 100 μM PBH for 6 h at 37 °C under continuous agitation.

DISCUSSION

Cholesterol is oxidized in the presence of reactive oxygen species generating aldehydes, hydroperoxides and epoxides.[6,7] Recently, two cholesterol aldehydes have attracted attention, the cholesterol carboxyaldehyde (**1a**) and cholesterol secoaldehyde (**1b**). The formation of **1a** and **1b** was first described in the oxidation of cholesterol with ozone.[11,12] Wentworth and co-workers reported the presence of **1a** and **1b** in atherosclerotic plaques and related the detection of these aldehydes as an evidence for ozone generation in human tissues.[8] On the other hand, two recent studies identified the generation of **1a** in the reaction of cholesterol with singlet molecular oxygen.[9,10] Indeed, Brinkhorst and co-workers reported the formation of **1a** from cholesterol 5α-hydroperoxide by Hock cleavage in acid media[9] and Uemi and co-workers reported the formation of **1a** in the reaction of cholesterol with singlet molecular oxygen generated either by photooxidation or by the thermodecomposition of endoperoxides.[10]

In this study, we have developed a new detection method for cholesterol carboxyaldehyde, **1a**, using the fluorescent probe PBH, which contains the highly fluorescent pyrene group. The

reaction of **1a** with PBH can be conducted in the absence of acids, which is important to avoid the formation of **1a** from acid catalyzed cholesterol 5α-OOH decomposition.[9] Bosco and co-workers[14] used dansyl hydrazine as a fluorescent label to detected **1a** and **1b** in a brain tissue sample from Lewy body disease. However, the derivatization of cholesterol aldehydes with dansyl hydrazine is conducted in the presence of strong acids, such as, 5 mM sulfuric acid.[14] In fact, we have done a comparative analysis of the two fluorescent probes (PBH and dansyl hydrazine) to detect **1a** in vitro and observed that the method using dansyl hydrazine can overestimate the basal level of **1a**, especially in samples containing cholesterol 5α-OOH. The **1a** yields in photooxidized cholesterol samples estimated by PBH (neutral pH) and dansyl hydrazine were 8.7% and 16.0%, respectively. This data indicates that the derivatization in the absence of acid is a critical point that should be considered when estimating **1a** basal level in samples, especially when they were oxidized by singlet molecular oxygen. When comparing dansyl hydrazine and PBH methods, the latter was approximately 10 times more sensitive for **1a** detection (Figure S5 in the SI). This difference was even larger when PBH derivatization was carried out at acid condition, clearly evidencing the superiority of PBH method compared to dansyl hydrazine in terms of sensitivity.

A number of studies has detected the formation of cholesterol aldehydes in vitro,[9,10,21] as well as in vivo.[8,14−16] In this study, we have used PBH to analyze the formation of **1a** during cholesterol oxidation mediated by ozone and singlet molecular oxygen. The reaction of cholesterol with ozone yielded **1a** and **1b** and also other minor unidentified products. We could not quantify exactly the amount of **1b** due to the lack of an appropriate pure standard for it. Nonetheless, the relative peak intensities suggest that cholesterol ozonation yields **1b** as a major product and a small amount of its aldolization product (Figure 5). The estimated ratio of **1a**:**1b** in the ozonation was 1:10. Similar results were described for the ozonation of human LDL, where a relative yield of 1:4 was found.[13] On the other hand, cholesterol oxidation promoted by singlet molecular oxygen yielded **1a** as the major product consistent with the data described by Brinkhorst et al.[9] and Uemi et al.[10] The estimated yield of **1a** in the photooxidation of cholesterol was approximately 8.7% and the ratio of **1a**:**1b** was 165:1. These values are in agreement with those reported for the photooxidation of human LDL using hematoporphyrin IX for 14 h where only **1a** was detected.[13] The incubation of cholesterol in the presence of H_2O_2 and HOCl also gave **1a** as the major product, consistent with the oxidation of cholesterol promoted by singlet molecular oxygen. It is known that the reaction of H_2O_2 with HOCl generate stoichiometric amounts of singlet molecular oxygen[26] and this reaction is suggested to play an important role during inflammatory conditions.[22] The predominant formation of **1a** over **1b** by this reaction was also reported by Tomono and co-workers.[21] They incubated cholesterol (100 μM) in the presence of H_2O_2 (100 μM) and NaOCl (100 μM) and detected five times more **1a** than **1b** using dansyl hydrazine as a fluorescent probe.[21]

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CONCLUSIONS

We have developed a new sensitive method for the detection of cholesterol aldehydes using PBH as a fluorescent probe. This new methodology allows detecting and quantifying the relative amounts of **1a** and **1b** with high sensitivity, which is important to assess the relative contributions of ozone and singlet molecular oxygen to the oxidation of cholesterol in biological systems. Derivatization of cholesterol aldehydes using PBH can be conducted without the addition of strong acids, which is important for the determination of the basal level of cholesterol aldehydes present in biological tissues from different sources. In conclusion, the easy and reliable method developed in this study can help to investigate the formation cholesterol aldehydes in biological system, which is critical to clarify their relevance in disease progression.

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SUPPORTING INFORMATION AVAILABLE

NMR analysis of **1a** and **1b**. HPLC-MS/MS detection of **2a** and **2b**. HPLC-fluorescence data showing the pH effect on **2a** formation and the sensitivity of dansyl hydrazine and PBH methods. Dansyl hydrazine derivatization method. This material is available free of charge via the Internet at http://pubs.acs.org.

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