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Bilirubin oxidation products (BOXes): synthesis, stability and chemical characteristics

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Summary

Bilirubin oxidation products (BOXes) have been a subject of interest in neurosurgery because they are purported to be involved in subarachnoid hemorrhage induced cerebral vasospasm. There is a growing body of information concerning their putative role in vasospasm; however, there is a dearth of information concerning the chemical and biochemical characteristics of BOXes. A clearer understanding of the synthesis, stability and characteristics of BOXes will be important for a better understanding of the role of BOXes post subarachnoid hemorrhage.

We used hydrogen peroxide to oxidize bilirubin and produce BOXes. BOXes were extracted and analyzed using conventional methods such as HPLC and mass spectrometry. Characterization of the stability BOXes demonstrates that light can photodegrade BOXes with a $t_{1/2}$ of up to 10 h depending upon conditions. Mixed isomers of BOXes have an apparent extinction coefficient of $\epsilon = 6985$, and a $\lambda_{\max}$ of 310 nm.

BOXes are produced by the oxidation of bilirubin, yielding a mixture of isomers: 4-methyl-5-oxo-3-vinyl-(1,5-dihydropyrrol-2-ylidene)acetamide (BOX A) and 3-methyl-5-oxo-4-vinyl-(1,5-dihydropyrrol-2-ylidene)acetamide (BOX B). The BOXes are photodegraded by ambient light and can be analyzed spectrophotometrically with their extinction coefficient as well as with HPLC or mass spectrometry. Their small molecular weight and photodegradation may have made them difficult to characterize in previous studies.

Keywords

Bilirubin; reactive oxygen species; HPLC; mass spectrometry; molecular weight; photo-degradation; blood product; photolysis

There is extensive study of the oxidations (including peroxidations) of unsaturated lipids and proteins in biological systems, including the resultant compounds produced [1,26,39,41]. There is also evidence that bilirubin is a biologic antioxidant [34,43–46,51]. The oxidation of (unconjugated) bilirubin has largely focused on the degradation of bilirubin between the pyrroles [5], with little discussion concerning the putative biological activity of the products of bilirubin oxidation [49]. We recently reported on a new family of bilirubin oxidation products found following subarachnoid hemorrhage [3,22,25,38]. It appears that bilirubin oxidation products (BOXes) can be formed by the direct, nonenzymatic oxidation of bilirubin with hydrogen peroxide [22]. In Fig. 1a we see the relationship between the EE form of bilirubin

and the resultant BOXes produced by oxidation (adapted from Kranc *et al.* [22]). Cleavage of bilirubin at the pyrrole, rather than between the pyrrole rings produces an apparently reactive monopyrrole amide [22]. However, to date there has been relatively little information concerning the stability or characteristics of BOXes, which is important in understanding the role BOXes may play in subarachnoid haemorrhage induced vasospasm. Here we present our latest findings concerning the chemical characteristics of BOXes produced by the oxidation of bilirubin. The relatively unique characteristics of BOXes and their nonspecific, nonenzymatic production provides important insight into the putative role of unconjugated bilirubin and its oxidation products in human pathology.

Methods and materials

All materials are available from common commercial sources and unless noted were ACS Grade or better. Bilirubin (mixed isomers) was purchased from Sigma/Aldrich Chemical Company. Sodium hydroxide, hydrochloric acid, acetonitrile (HPLC grade), H₂O₂ 30% and Whatman I filter paper were obtained from Fisher Scientific. Water (18 mΩ) was generated using a Biocel Millipore system.

Synthesis method for BOXes

In order to prepare BOXes by non-enzymatic oxidation, 100 mg of mixed isomers of bilirubin is dissolved in 50 ml of water containing 10 g of sodium hydroxide (in 50 ml cell culture bottle with lid, taped or covered with foil). The solubilization is performed protected from light at room temperature and is completed in 24 h. The subsequent steps are carried out in the dark or protected from light. After 24 h, 23 ml of concentrated hydrochloric acid is added to the solubilized solution. The resultant solution is stirred and allowed to cool. Upon cooling to room temperature, the pH of the solution is taken. The solution is then brought to pH 7.5 by the careful addition of dilute hydrochloric acid.

After adding the hydrochloric acid to neutralize the solution, 1 PBS tablet (capable of making 200 ml of PBS aqueous) is added to the bilirubin solution. The resultant solution, once neutralized to pH 7.5, has sufficient buffering capacity to maintain the pH at 7.5 during the oxidation. If the solution is unbuffered, due to the Fenton-like nature of the reaction, the pH will drift up from 7.5 to 8.2 during the course of the reaction.

Next, the volume of the solution is measured in a graduated cylinder and hydrogen peroxide added to a final concentration of ~10% hydrogen peroxide. After 24 h of stirring at room temperature, the resultant yellowish solution is frozen at -80°C. The frozen solution is then lyophilized overnight, producing a yellow solid. The yellowish solid is then thoroughly triturated with 100 ml of chloroform. The chloroform solution is then filtered using Whatman 1 and a Hirsch Funnel. The filtrate is evaporated under nitrogen gas. This produces about 4% by weight of BOXes.

HPLC analysis

HPLC analysis was performed using a Waters Chromatography System consisting of a 2790 Separations Module and 2487 Variable Wavelength Detector running Millennium 32 Data Analysis Software. Separation was achieved on a Symmetry C18, 5 μm 4.6 × 150 mm, column. The elutant was monitored at 310nm. Column temperature was held at 35 degrees centigrade with a flow rate of 1.0 ml/min. The mobile phase consisted of acetonitrile/water with a gradient of 5% to 50% acetonitrile being used.

Mass spectrometry

HPLC-MS analysis was performed on a Bruker Daltronics system using conditions identical to the above, with the exception of the gradient being 20% to 63% acetonitrile over 15 min with the acetonitrile being held constant until the end of the run. The MS was performed using air pressure chemical ionization in the negative ion mode with simultaneous monitoring at 254 nm.

Photostability study

Approximately 0.1 mg of BOXes was dissolved in 1 ml of phosphate buffered saline (PBS) at pH 7.5. The solution was exposed to various experimental conditions. Samples were taken at 30min intervals for 120min and the resultant samples were diluted 50:50 with PBS and the spectra read from 250 to 550 nm. For spectrometry, we used a μ -Quant plate reader and BD Falcon 96 well microplate. The data reported used the OD at 310 nm.

Results

BOXes production

We consistently observe a 4% yield for producing the mixture of BOXes (4-methyl-5-oxo-3-vinyl-(1,5-dihydropyrrol-2-ylidene)acetamide (BOX A) and 3-methyl-5-oxo-4-vinyl-(1,5-dihydropyrrol-2-ylidene)acetamide (BOX B)) as described above. In Fig. 1b we see the HPLC trace demonstrating the resultant BOXes produced. We see that the procedure produces approximately $43.6 \pm 8.6\%$ BOX A, $31.0 \pm 8.8\%$ BOX B and $25.4 \pm 18.0\%$ MVM; measured at 310 nm, $N = 5$. This method can be scaled up to 1.0 g of bilirubin starting material, however, the oxidation step takes almost 3 days to perform and has only a marginally higher yield. Since this method almost exclusively makes BOXes and methyl vinyl maleimide (MVM), it is to be preferred over the previously published method [22].

In Figs. 2a–c, we present representative HPLC and mass spectrometry results of BOX A with a retention time of 5.2 min and MVM with a retention time of 8.3 min. BOX B typically has a retention time of 6.5 min.

Determination of ϵ

Using the methods of Kranc *et al.* [22], the BOXes were loaded onto a Hypersil reverse-phase C18 (250 $\times$ 7 mm) column, equilibrated in 100% H₂O at 1 ml/min. The sample was then eluted with a gradient of 100% water to 100% isopropanol over 12 min. A Waters 2790 separation module was used, along with a 996 photodiode array module. It was determined that 50% of the solution was BOXes, and the other 50% consisted of unconverted bilirubin, biliverdin and other compounds, none of which absorb at the wavelength of interest (310 nm). Thus, accounting for the 50% dilution with H₂O₂ in the peroxidation process, and a 50% conversion, we are left with a range of solutions of BOXes with concentrations (in μ M) of 10, 20, 30, 40, 50, 60, 70, 80 and 90.

A Molecular Devices SpectraMax Plus spectrophotometer was used for these analyses. Each sample was scanned between 280 and 500 nm, and a peak at 310 nm was clearly discernable. This is the wavelength at which both BOX A and B absorb. The absorbance at 310 nm was plotted against concentration of BOXes, and the resulting relationship was subjected to linear regression. The gradient of the line yielded the molar extinction coefficient at 310nm for BOXes. The peak at 310 nm was completely ablated after 12 h of exposure to UV light, which is indicative of BOXes.

Solutions of bilirubin were scanned between 290 and 450nm, with a λ maximum of 310. The resultant absorbances were graphed and the gradient from the graph (Fig. 3a) gives $\epsilon = 6985$.

As there is very little absorption at 450 nm, we assumed that essentially all the bilirubin has been oxidized. Plotting the absorbance at 310 (BOXes) against the concentration present, we can obtain the molar extinction coefficient of BOXes.

Photostability study

These data are shown in Table 1. The resultant $t_{1/2}$ for the degradation of BOXes is approximately 10 ± 2 h exposed to ambient indoor light ($N = 5$). At 65°C, pH 7.5 in the dark, no decomposition was observed over the time course analyzed. We exposed the BOXes to UV light for 24 h and found that there was essentially complete degradation of BOXes independent of concentration (Fig. 3a).

The kinetics observed were nonlinear and involved a fast initial $t_{1/2}$, sometimes as short as 1 h, as well as a slower degradation component with an apparent $t_{1/2}$ of about 7h. Overall $t_{1/2}$ is as reported in Table 1. The BOXes are photosensitive, as is seen in Table 1.

Discussion

BOXes have been reported to be involved in the disease sequelae of subarachnoid haemorrhage induced cerebral vasospasm [3,4,22,25,36,38]. They were proposed to be produced by the oxidation of bilirubin in the subarachnoid haemorrhage patient and cause or contribute to vasospasm in those patients because they were present in the spinal fluid obtained from these patients [38] and caused vasospasm like events in model systems [3,4,22,25,36,38]. Since they were first described by Kranc *et al.* [22] the focus of BOXes studies has been on the pathology and physiology of BOXes, with less focus on the chemical characteristics of BOXes. Here we report on the chemical characteristics of BOXes and how this is relevant to the vasospasm patient.

There are reports of differences between the reactivity and chemical characteristics of the geometrical and structural isomers of bilirubin [20]. It is unknown whether these differences affect BOXes production. However, to determine the pathway for BOXes production in the future, one will need to understand the isomers involved in the reactions [22]. In biological systems the interconversion of the EE, EZ, ZZ and cyclic forms is known to occur [20]. While this makes it complicated to speculate on the importance of these isoforms in the production and/or role of BOXes, it is justification for our use of the mixed isomers for these studies. The resultant isomers of BOXes (BOX A and BOX B) are not necessarily due to different isomers of bilirubin (the EE, EZ and ZZ forms), but rather due to oxidation of either 'end' of the bilirubin molecule yielding the alternate positions of methyl and vinyl. BOXes' uniqueness appears to come from the amide group derived from the cleavage of the 2 or 3 pyrrole of bilirubin.

Of importance from our data is that the BOXes are quickly degraded by light (ambient and sun), which may have made their characterization in humans rather difficult. It is relatively well known that the spinal fluid of the subarachnoid haemorrhage patients was vasoactive and cytotoxic [2,10,37], but only recently were the BOXes reported in the haemorrhage patients' spinal fluid [38]. Interestingly, increased bilirubin in the spinal fluid of the subarachnoid haemorrhage patient has a time course that is similar to the reported incidence of vasospasm [8,47].

The BOXes mixtures are obviously photosensitive, as is seen in Table 1, and due care is required in handling the material. Ambient visible light in a spectrophotometer does not seem to cause appreciable decomposition. Thus the wavelength(s) involved in the photodegradation of BOXe is not within the 300 to 550 nm range.

In biological systems, bilirubin is a ubiquitous substance that readily crosses membranes and intercollates in tissues and membranes. It is relatively stable with a planar configuration containing a hinge between the 2 and 3 pyrroles. BOXes are also relatively planar and are likely to be able to cross membranes and appear to have numerous biological effects [3,22,25,38]. Nonetheless, to date, there have been no definitive binding or specific uptake studies on BOXes and target molecules/cells.

There is an extensive literature on physiological oxidations via reactive oxygen species such as H_2O_2 or oxygen radicals like O_2^- [6,7,11,19,21,29,33,40,48]. Lipid and protein oxidations in biological systems has also been well studied [13,14,23,39,42,50]. More recently, bilirubin oxidation has started to gain interest and attention [15–18,38,45,46,51]. What is lacking, however, is an understanding of the resultant species and the biological importance of the compounds made post oxidation of bilirubin [3,25,36,38]. Our previous studies and the data presented here suggest that oxidized bilirubin may be an important and novel molecular species in biological systems. The ability to generate BOXes from nonenzymatic oxidation, and the photodegradation of BOXes may have made them a difficult species to detect and understand. It is extremely likely that whenever there is bleeding inside the body, the bilirubin being produced can be oxidized by the macrophages and similar cells that are activated under such conditions. Inside the body, there might be relatively little opportunity for photodegradation and instead, the BOXes could have potent biological effects. However, when the samples are removed for analysis, the resulting exposure to light might result in the photo-oxidation of BOXes, and therefore, result in the apparent lack of detection.

Haemorrhagic complications and the mechanisms for such complications have been an anathema and enigma in medical research. Residual dysfunction following hemosiderin staining is a common observation, but the molecule or molecules involved are unknown [12, 24]. Hyperbilirubinemia or kernicterus can affect infants and cause profound neurological disturbances. The treatment is phototherapy, assumed to be beneficial by clearing the bilirubin [35]. However, we posit that the hyperbilirubinemic patient may benefit from photodegradation of BOXes. Because BOXes have only been found in clinical conditions in low concentrations [38] and because they are photolabile [22], it may have been difficult to characterize and understand their role in certain diseases. It is important now to better understand the chemistry of BOXes and determine what, if any, role they play in biology and medicine.

BOXes have been found in the micromolar range in human spinal fluid following subarachnoid haemorrhage [38] and their concentration is associated with cerebral vasospasm [38]. Bilirubin levels rise in the spinal fluid following subarachnoid haemorrhage [31] and their time course correlates well with the accepted time course of cerebral vasospasm, but bilirubin is not vasoactive. Thus there has been an interest in bilirubin's association with vasospasm, though with mixed results [3,4,8,9,27,28,30,32,38,47]. A bilirubin derivative such as BOXes is therefore quite consistent with the clinical characteristics of vasospasm. *In vitro* and *in vivo* studies suggest that they may be involved in subarachnoid haemorrhage induced cerebral vasospasm [3,4,25,38]. In the brain, little light would pass through the skull and as such, BOXes might accumulate and contribute to the pathology of subarachnoid haemorrhage induced cerebral vasospasm.

Conclusion

BOXes are a relatively new biochemical family that appears to be formed from unconjugated bilirubin under severely oxidizing conditions. They are photolabile and may be produced in biological systems any time in the presence of sufficient unconjugated bilirubin and conditions appropriate for oxidation. If BOXes are regularly produced in biological systems [38], they may prove to be a newly discovered and important family of biologically active compounds.

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Fig. 1a.

In this figure the EE form of bilirubin is represented along with the structures of BOXes that are likely to be produced. BOX A and BOX B can be produced by the oxidation of bilirubin. The resultant BOXes also have the E stereoisomer connecting the pyrrole to the amide based on the prediction of Kranc *et al.* [22]

Fig. 1b.

In this figure we see the HPLC chromatogram of a representative batch of BOXes. *BOX A*, *BOX B* and *MVM* can be seen from right to left respectively

Fig. 2a. Ion Chromatogram (*top*) and UV Chromatogram (*bottom*) of BOXes mixture. Retention times are as follows, BOX A 5.2 min, BOX B 6.5 min, MVM, 8.3 min

Fig. 2b. Nominal mass spectrum of BOX A is seen in this figure. BOX A with a nominal molecular weight of 177.1 m/z

Fig. 2c.

In this figure we see the resultant nominal mass spectrum of MVM, the peak that has a retention time of 8.3 min on the HPLC trace. MVM has a nominal mass of 135.9 m/z

Fig. 3a.

The bilirubin solutions were oxidized as described in the experimental methods, the concentrations determined and absorbance measured at 310 nm. The resultant line is graphed above to obtain the extinction coefficient. $N = 3$ for each point

Fig. 3b.

In order to check that the 310 nm peak is indeed BOXes, the samples were exposed to UV light for 24 h. This resulted in ablation of the peak. $N = 3$ for each point

Table 1

	Conditions	$t_{1/2}$	Comments
1	Direct sunlight, 25 °C, PBS, pH 7.5	3.3 ± 0.53 h, $N = 5$	apparently biphasic kinetics
2	Ambient light, 25 °C, PBS, pH 7.5	10 ± 2 h, $N = 5$	apparently biphasic kinetics
3	Ambient light, 40 °C, PBS, pH 7.5	3.5 ± 0.7 h, $N = 5$	apparently biphasic kinetics
4	Ambient light, 40 °C, PBS, pH 6	3.3 ± 0.85 h, $N = 5$	apparently biphasic kinetics
5	65 °C, no light, PBS, pH 7.50	None detected within experimental error. $N = 5$	
6	Spectrophotometer cell, visible lamp, 300 to 550 nm, 25 °C	None detected, within experimental error. $N = 5$	