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Identification of a new human lens UV filter compound

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Abstract A new UV filter compound, 4-(2-amino-3-hydroxyphenyl)-4-oxobutanoic acid *O*-diglucoside, has been identified in human lenses. The structure suggests that it is a further metabolic product of the second most abundant UV filter compound, 4-(2-amino-3-hydroxyphenyl)-4-oxobutanoic acid *O*-glucoside. Quantification studies on the new compound show that it decreases towards zero in both the nucleus and cortex as a function of age. The discovery of this novel disaccharide completes the identification of the major UV filter compounds present in the human lens. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Ultraviolet light; Photooxidation; Cataract; Lens; Filter

1. Introduction

The primary role of the human lens is to focus light transmitted by the cornea onto the retina to generate an image. Generation of this image with maximum optical clarity is dependent on the UV filtering capacity of the lens, which contains compounds that absorb most of the light between 295 and 400 nm [1–3]. These UV filter compounds are thought to protect the lens and retina from harmful photodamage initiated by UV light and aid visual acuity by reducing chromatic aberration [2]. Since it has been proposed that UV light is involved in the development of cataract [4–6], understanding the types of UV filter compounds present in the lens and their regional concentrations is clearly important.

In humans, the major product of lens tryptophan metabolism is the UV filter compound 3-hydroxykynurenine glucoside (3OHKG) [3,7]. This UV filter spontaneously deaminates at physiological pH to form a highly reactive α,β-ketoalkene intermediate [8], capable of undergoing three known reactions. These are reduction to form the second most abundant UV filter compound 4-(2-amino-3-hydroxyphenyl)-4-oxobutanoic acid glucoside (AHBG) [8]; addition to the cysteine residue of glutathione to form glutathionyl-3-hydroxykynurenine glucoside (GSH-3OHKG) [9]; and binding to human lens proteins (crystallins) [10]. In vitro studies have recently shown that the 3OHKG metabolic precursors kynurenine (Kyn) and 3-hydroxykynurenine (3OHKyn) are also capable of participating in these types of reactions [11–13].

With aging, human lens proteins undergo numerous changes including a generalised yellowing of the lens nucleus and an increase in fluorescence [14–16]. The UV filter compounds have been linked to this normal age-dependent coloration and to crystallin modification observed with the development of senile cataract [10,17–19]. In this paper we describe the identification of a novel UV filter compound present in human lenses.

2. Materials and methods

Human lenses were obtained post-mortem from donor eyes at The Sydney Eye Hospital Lions Eye Bank (Sydney, Australia). Following removal, the lenses were placed immediately into sterile plastic screw-capped vials and kept at −20°C until analysed. Lenses were dissected frozen and the protein-free lens extracts were obtained following the conditions previously described [20].

The high-performance liquid chromatography (HPLC) system consisted of two ICI LC 1150 pumps, a Rheodyne 7125 sample injector and an ICI SD 2100 variable wavelength UV-Vis detector. Chromatograms were recorded using the WinChrom chromatography data system (GBC Scientific Equipment, NSW, Australia). Standard curves and separations were performed on a 250 mm × 4.6 mm Varian Microsorb C18 column using an acetonitrile/H_2O gradient in 0.05% (v/v) trifluoroacetic acid. The percentage acetonitrile in the gradient was 0% (5 min), 0–40% (50 min), and 40–0% (5 min). A flow rate of 0.6 ml/min was used and the peaks were detected at 365 nm.

Mass spectrometry experiments were conducted on the following instruments. (I) A VG Quattro mass spectrometer with a hexapole collision cell (VG Biotech, Altrincham, UK). Samples were dissolved in 50% aqueous acetonitrile in either 1% formic acid (for positive ion mass spectrometry) or 1% ammonia (for negative ion mass spectrometry). Argon was used as the collision gas. (II) A Bruker BioApex 47e Fourier transform ion-cyclotron resonance mass spectrometer (FTMS) (Bruker Daltonics, Billerica, MA, USA). Samples were dissolved in 100% methanol and infused continuously into the electrospray source (Analytica of Branford, Branford, CT, USA) at a flow rate of 80–100 μl/h. Nitrogen was used as the collision gas for CAD in the analyser cell.

UV-visible absorption spectra were recorded on a photodiode array (PDA) detector in line with a capillary liquid chromatography (CapLC) system (Waters, NSW, Australia). Fluorescence spectra were recorded on a Hitachi F-4500 fluorescence spectrometer in phosphate buffer (pH 7.2, 25 mM).

Cleavage of the *O*-glycosidic bond of the unknown UV filter compound was performed by incubation of the purified UV filter compound with: (I) almond β-glucosidase (EC 3.2.1.21, Sigma) (1 mg/ml) in citrate buffer (10 mM, pH 5) for 3 h at 37°C and (II) 1 M HCl at 100°C for either 10 min or 1 h.

Linear regression analysis was used to evaluate the relationship between age and the concentration of the unknown UV filter compound. The two-sample paired *t*-test was used to determine whether

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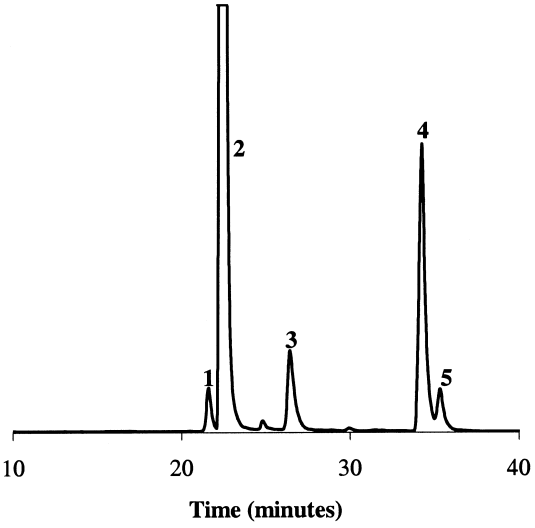


Fig. 1. HPLC chromatogram of the ethanol extract of a 20-year-old human lens. UV detection at 365 nm. 1 = 3OHKyn; 2 = 3OHKG; 3 = Kyn; 4 = AHBG; 5 = U-37.

the nuclear and cortical data points were distinct. A P value < 0.01 was considered significant.

3. Results

The HPLC chromatogram of a 20-year-old human lens extract, with detection at 365 nm, is shown in Fig. 1. Several peaks were observed. These corresponded to 3OHKyn (peak 1), 3OHKG (peak 2), Kyn (peak 3), AHBG (peak 4) and a compound of unknown identity (peak 5), with a retention time of 37 min. No peak was observed for the GSH-3OHKG adduct, since this compound results from UV filter instability, and is generally seen in lenses above 40 years of age [9,20]. In the present study, we describe the identification of the unknown compound (U-37).

To establish whether U-37 was related to the other kynurenine-derived UV filters, its absorbance and fluorescence characteristics were determined. Firstly, to ensure that U-37 was completely resolved from AHBG, the peak corresponding to U-37 was collected from the analytical HPLC system and rerun on the CapLC system, with the PDA detector in line. The UV-visible spectrum, obtained from the PDA detector, displayed two absorbance maxima at 262 and 361 nm and the fluorescence spectrum exhibited maximum fluorescence intensity at $Ex_{345\ nm}/Em_{455\ nm}$ (Fig. 2). These characteristics closely resembled those of the two major UV filter compounds (3OHKG: λ_{max} at 365 and 264 nm, $Ex_{347\ nm}/Em_{455\ nm}$; AHBG: λ_{max} at 358 and 262 nm, $Ex_{345\ nm}/Em_{455\ nm}$) under these conditions, which suggested that U-37 has a similar chromophoric structure.

Mass spectrometric experiments performed on the Quattro mass spectrometer revealed the molecular mass of U-37. A protonated molecular ion at m/z 534 was observed in the positive ion electrospray mass spectrum and a deprotonated molecular ion at m/z 532 was observed in the negative ion electrospray mass spectrum. These data indicated a molecular mass of 533 Da. Further analysis by high-resolution mass spectrometry revealed a protonated molecular ion at m/z 534.181174, which corresponds to the molecular formula $[C_{22}H_{32}NO_{14}]^+$. The molecular mass of U-37 is 162 Da units more than that of AHBG, and both the molecular mass and molecular formula are consistent with a structure consisting of AHBG plus an additional C-6 sugar unit. Tandem mass spectrometry performed in the positive mode displayed daughter ions at m/z 372 and m/z 210. This fragmentation pattern results from the sequential loss of 162 Da, which is a characteristic loss from hexose-containing molecules. Tandem mass spectrometry of AHBG collected from a human lens displayed a protonated molecular ion at m/z 372 and a major fragment at m/z 210. These preliminary data indicated that U-37 could be a compound related in structure to AHBG. Further mass spectrometry and hydrolysis data, described below, are consistent with the structure of U-37 as shown in Fig. 3.

High-resolution tandem mass spectrometry experiments were performed on purified isolates of U-37 using the Fourier transform ion-cyclotron resonance mass spectrometer. In the positive mode, a sodiated parent ion at m/z 556.14 displayed three intense fragment peaks (Fig. 3A). These corresponded to loss of the side chain attached to the aromatic ring (fragment a), loss of a C-6 sugar unit leaving sodiated AHBG (fragment

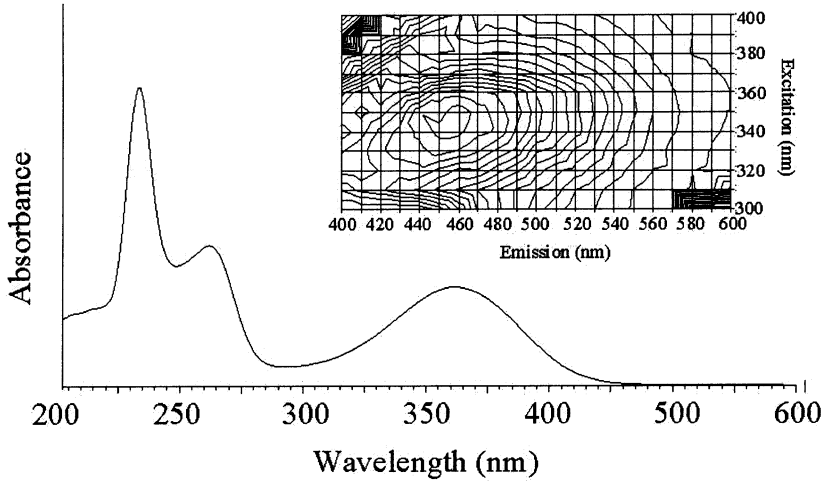
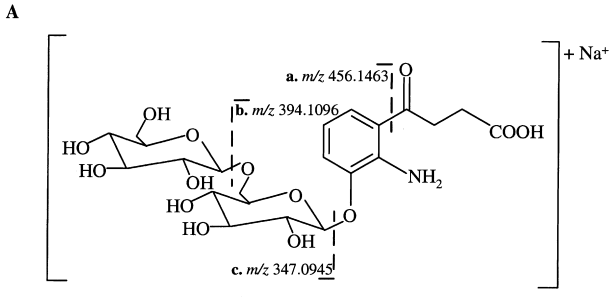


Fig. 2. U-37 was collected from the HPLC of a human lens ethanol extract. A: UV-spectrum of U-37 with λ_{max} 355, 260 nm. B: 3D fluorescence spectrum of U-37 with $Ex_{345\ nm}/Em_{455\ nm}$.

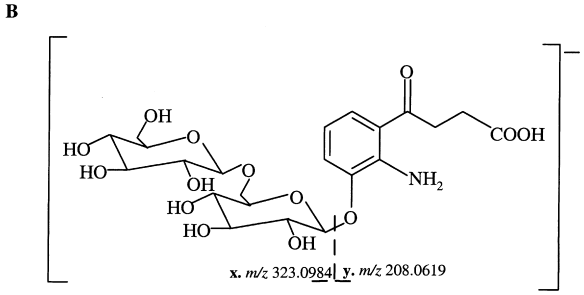
b) and cleavage of the aromatic *O*-glucosidic linkage giving a sodiated dehydro-disaccharide (fragment c). The calculated *m/z* values for the proposed fragments are consistent with those observed. These results were confirmed by additional high-resolution tandem mass spectrometry experiments performed in the negative mode. The parent ion at *m/z* 532.07 displayed fragmentation of the aromatic *O*-glucosidic linkage, as was observed in positive ion mode. This yielded two intense fragment ions corresponding to the two 'halves' of the compound (Fig. 3B), i.e. the dehydro-disaccharide (fragment x) and the UV filter fragment 4-(2-amino-3-hydroxyphenyl)-4-oxobutanoic acid (AHB) (fragment y). The measured masses were consistent with the calculated *m/z* values for the proposed fragments. These mass spectral data provide strong evidence for the proposed structure (Fig. 3), with the two hexose units present as a disaccharide attached to the phenolic oxygen atom.

In order to investigate this further, U-37 was incubated with 1 M HCl at 100°C for either 10 min or 1 h. Following removal of the acid, the samples were redissolved in H_2O and analysed by HPLC. Both hydrolysates displayed a major peak, which co-eluted with an authentic standard of the expected aglucone AHB. Analysis of this peak by mass spectrometry revealed a protonated molecular ion at *m/z* 210, with fragments at *m/z* 192 (H_2O loss) and *m/z* 164 (formic acid loss). These fragments were the same as those in the mass spectrum of the authentic AHB.

Previous studies performed in our laboratory [2] and that of others [7] have shown that β-glucosidase effectively cleaves the



a. observed *m/z* 456.1463, calculated *m/z* for $[C_{18}H_{27}NO_{11} + Na^+]^+$ 456.1476;
b. observed *m/z* 394.1096, calculated *m/z* for $[C_{16}H_{21}NO_9 + Na^+]^+$ 394.1108;
c. observed *m/z* 347.0945, calculated *m/z* for $[C_{12}H_{20}O_{10} + Na^+]^+$ 347.0949.



x. observed *m/z* 323.0984, calculated *m/z* for $[C_{12}H_{20}O_{10} - H^+]^-$ 323.0984;
y. observed *m/z* 208.0619, calculated *m/z* for $[C_{10}H_{10}NO_4]^-$ 208.0615.

Fig. 3. The proposed structure of U-37. A: The sodiated fragments observed from the FTMS spectrum in the positive mode. B: The fragments observed from the FTMS spectrum in the negative mode. The observed and calculated *m/z* values for the fragments are listed beneath the structures.

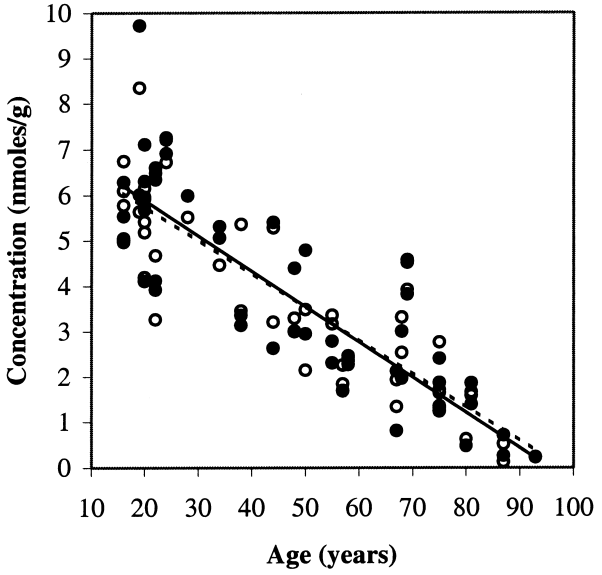


Fig. 4. The concentration of U-37 in human lenses as a function of age. Linear regression lines shown, nucleus ($r = -0.85$, $P < 0.0005$, $n = 50$) and cortex ($r = -0.87$, $P < 0.0005$, $n = 50$). ●, nucleus; ○, cortex.

O-glucosidic linkage of 3OHKG and AHBG to form the corresponding aglucone moieties, 3OHKyn and AHB respectively. Treatment of U-37 with β-glucosidase resulted in complete conversion of U-37 to AHB. This result confirms that the two C-6 sugars are glucose and that they are β-linked.

Quantification of the *O*-diglucoside was performed in individual human lenses ranging in age from 16 to 93 years. As shown in Fig. 4 the concentration of U-37 decreased linearly in both the nucleus ($r^2 = 0.73$, $P < 0.0005$, $n = 50$) and cortex ($r^2 = 0.77$, $P < 0.0005$, $n = 50$) as a function of age. Highest levels were detected in lenses below the age of 20 years and lowest levels were detected in lenses of 80 years of age or older. No statistically significant difference ($P > 0.01$) was found between the levels detected in the nuclear and cortical regions of the lens.

4. Discussion

In this study we provide structural data on a novel UV filter compound found predominantly in younger human lenses. This fluorophore shares absorbance and fluorescence similarities with the major UV filter compounds 3OHKG and AHBG. High-resolution mass spectrometry demonstrated that the unknown UV filter compound is a disaccharide and the β-glucosidase hydrolysis data indicated that both monosaccharides are glucose (β-linked). Since the UV filter is present at low levels in older lenses, and these are the ones most readily obtained from Eye Banks, it was not possible to purify sufficient quantities of the UV filter compound for analysis by nuclear magnetic resonance spectroscopy. It was therefore not possible to determine unequivocally the position of attachment between the two glucose residues. Extensive examination of human lens extracts has shown no evidence for the existence of a 3OHKyn-conjugated disaccharide compound, thus, it appears unlikely that some 3OHKyn is diglucosylated prior to deamination and reduction. We propose

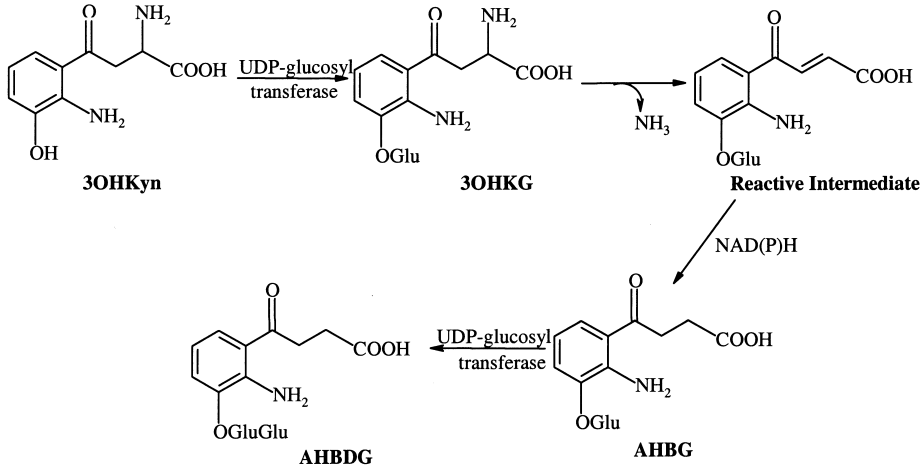


Fig. 5. Proposed scheme for the formation of AHBDG. It is proposed that U-37 is formed via glucosylation of AHBG glucoside. This results in the formation of a β-linked diglucoside (gentiobioside) UV filter compound, AHBDG.

that U-37 is formed via glucosylation of AHBG (Fig. 5). The name of this UV filter compound is 4-(2-amino-3-hydroxyphenyl)-4-oxobutanoic acid *O*-diglucoside (AHBDG).

As previously described for 3OHKG and AHBG, AHBDG also contains an *O*-glucosidic link, which is a form of conjugation rarely encountered in mammals. Xanthurenic acid glucoside has recently been isolated from brunescent human lenses [21] and is presumably derived from 3OHKG. Glucosidic conjugation of the UV filter compound 3OHKyn to form 3OHKG has long been attributed to UDP-glucosyl transferase [22]. Although the mechanism underlying the biosynthesis of AHBDG has not been addressed here, it is likely that UDP-glucosyl transferase is also responsible for transferring the second glucose molecule to AHBG, resulting in the formation of AHBDG. Glycosylation of systems similar to that of AHBG are known to occur in plants [23], however, the identification of this compound constitutes the only reported disaccharide derivative to have been isolated from human tissues.

One of the most common β-linked glucose systems observed in nature is β(1→4) [24]. Cellobiose is the β(1→4)-linked disaccharide of glucose (glucose-β-1→4-glucose), however, cellobiose does not occur free in nature nor as a glucoside [25]. Thus, we propose that the diglucoside component of AHBDG is the β(1→6)-linked disaccharide of glucose, i.e. β-gentiobiose (glucose-β-1→6-glucose). This disaccharide has reportedly been found in plants joined to different aglucones [23]. Enzymic hydrolysis by β-glucosidase of the proposed gentiobioside of AHBDG would occur via initial hydrolysis of the gentiobiose into glucose and AHBG, followed by removal of the aglucone group (AHB) [24].

A previous study has shown that the concentrations of both 3OHKG and AHBG decrease linearly with age [20]. In this study, quantification of AHBDG also displayed a significant decrease towards zero in both the nuclear and cortical regions as a function of age. This is consistent with the observed decrease in concentration of the proposed AHBDG metabolic precursor (i.e. AHBG).

The discovery of this novel disaccharide represents the last major UV filter compound present in human lenses.

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