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Stabilization of All-*trans*-retinol by Cyclodextrins: A Comparative Study Using HPLC and Fluorescence Spectroscopy

EKATERINA M. SEMENOVA[1], ALAN COOPER[2], CLIVE G. WILSON[1] and CAROLYN A. CONVERSE[1,*]

[1]*Department of Pharmaceutical Sciences, University of Strathclyde, Glasgow G4 0NR, and* [2]*Department of Chemistry, University of Glasgow, Glasgow G12 8QQ, Scotland*

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Abstract

Purpose: To formulate preparations incorporating cyclodextrins (CDs) which could be used for direct delivery to the retina of vitamin A (all-*trans*-retinol), while also protecting it from degradation in the aqueous environment. Vitamin A supplementation is being considered for treatment of several ophthalmic diseases characterised by progressive photoreceptor degeneration. *Methods:* The complexation between vitamin A and ten cyclodextrins, Captisol® (sulfobutyl ether-7-β-cyclodextrin), hydroxypropyl-β-CD, 2-hydroxypropyl-β-CD, α-CD, hydroxypropyl-γ-CD, hydroxypropyl-α-CD, β-CD, methyl-β-CD, Heptakis-(2,6-di-O-methyl)-β-CD and Heptakis-(2,3,6-tri-O-methyl)-β-CD, was investigated using both high sensitivity fluorescence spectrometry and HPLC (high pressure liquid chromatography). Samples of retinol-CD complexes in phosphate buffer, pH 7.4 were analysed for up to 72 hours. Optimum conditions for formation of the Captisol-retinol complexes were investigated. *Results:* Using spectroscopic measurements and HPLC, the complexes formed between ten cyclodextrins and all-*trans*-retinol were evaluated. The results indicate that all cyclodextrins tested were able to form inclusion complexes as shown by the fluorescence signals which are considerably larger than those obtained in the absence of cyclodextrin. Only minimal degradation of retinol over 48 hours was observed with three of these cyclodextrins. Captisol was able to stabilise all-*trans*-retinol for up to 72 hours, as shown by HPLC, and the optimum ratio of Captisol to retinol was determined to be 50 to 1. Addition of glutathione and decrease in pH did not improve stability of the complex. *Conclusions:* This survey suggests that Captisol and other cyclodextrins could be used to stabilise and solubilise vitamin A in aqueous media and this establishes the basis for an ocular Captisol-retinol drug delivery system now under development in our laboratory.

Introduction

Vitamin A (all-*trans*-retinol) is the major circulating retinoid in the visual cycle; it is required for vision, growth, reproduction and maintenance of differentiation in epithelial cells [1]. There have been several studies establishing a link between retinal diseases and abnormalities in the absorption, transport and delivery of vitamin A [2–5]. Large doses of vitamin A administrated orally were used in treatment of a number of retinal disorders including retinitis pigmentosa (RP), Sorsby's fundus dystrophy (SFD) and a mouse model of Leber congenital amaurosis [6, 7]. The studies were based on the hypothesis that the night blindness in these conditions is due to a chronic deprivation of vitamin A at the level of the photoreceptors caused by a thickened membrane barrier between the photoreceptor layer and its blood supply. It was found that vitamin A supplementation could lead to dramatic restoration of photoreceptor function, demonstrating that pharmacological intervention has the potential to improve vision in otherwise incurable genetic retinal degenerations. Because the long-term systemic consumption of vitamin A could lead to hypervitaminosis A, with a broad spectrum of clinical abnormalities, the direct delivery of high dosage of vitamin A to the retina may be desirable [1, 8].

All-*trans*-retinol is virtually insoluble in water and chemically labile, and it easily undergoes isomerisation and photo-oxidation [1], but these problems may be alleviated by cyclodextrin inclusion. The pharmaceutical use of cyclodextrins (CDs) is confined mainly to its complexation with poorly soluble, unstable, irritating and difficult to formulate substances. Substantial progress has been made in describing some spectroscopic properties of a variety of retinoid-cyclodextrin inclusion complexes in aqueous solution [10–12]. Entrapping retinol complexed to CDs in the aqueous phase of liposomes and desorption of retinol from monolayers by water soluble CDs were used to study CDs as effective drug delivery systems for all-*trans*-retinol [13, 14]. The advantageous UV characteristics also enable the development of a highly sensitive and selective chromatographic analysis using HPLC [15]. The aim of the present study has been to examine properties of a variety of all-*trans*-retinol-

* Author for correspondence. E-mail: c.a.converse@strath.ac.uk

CD inclusion complexes using absorbance, fluorescence and chromatographic techniques and to evaluate their potential for direct delivery to the retina.

Experimental

Materials

All-*trans*-retinol, retinol palmitate and glutathione were purchased from Sigma-Aldrich (Dorset, UK). Methanol (HPLC grade) and monobasic potassium phosphate were purchased from Sigma. α-Cyclodextrin (α-CD), β-cyclodextrin (β-CD), 2-hydroxypropyl-β-CD (2-HP-β-CD), 2,6-di-O-methyl-β-cyclodextrin (2,6-DOM-β-CD), 2,3,6-tri-O-methyl-β-cyclodextrin (2,3,6-TOM-β-CD) and hydroxypropyl-γ-cyclodextrin (HP-γ-CD) were supplied by Sigma, and hydroxypropyl-α-cyclodextrin (HP-α-CD), hydroxypropyl-β-cyclodextrin (HP-β-CD) and methyl-β-cyclodextrin (M-β-CD) were supplied by Aldrich. Captisol® (sulfobutyl ether-7-β-cyclodextrin) was a gift of CyDex Inc., Overland Park, KS.

Apparatus

Absorbance spectra were recorded using a Shimadzu UV-VIS Spectrophotometer UV-160A and fluorescence emission spectra were recorded at 20 °C with a SPEX FluorMax thermostated spectrofluorimeter (Spex Industries, Edison, NJ), using 2-ml samples in a silica cuvette with a path length of 10 mm. HPLC was performed using a ThermoSeparation Products HPLC system with a membrane degasser, a ConstaMetric 3200 solvent delivery system, a SpectraSeries AS300 autosampler, and a SpectraMonitor 3200 detector (set at 350 nm). The analyses were carried out on a Phenomenex Sphereclone ODS (2) (5 μm, 150 × 4.6 mm, part No.: 00F-4144-EO; serial No. 203365) reversed-phase column. The chromatographic conditions were as follows: injection volume, 20 μl; flow rate, isocratic at 1.0 ml min^{-1}. The mobile phase used consisted of 10% (v/v) potassium phosphate buffer (0.05 M, pH 7.4) in methanol.

Procedures

Free all-*trans*-retinol is poorly soluble and unstable in aqueous environment, so was dissolved and diluted in reagent grade absolute ethanol immediately before use. The concentration of all-*trans*-retinol was estimated by absorbance of a solution in ethanol at 325 nm, with an E_{325} of 52,480 M^{-1} cm^{-1} [16].

The fluorescence and absorbance properties of all-*trans*-retinol-CD inclusion complexes were tested by addition of retinol solutions (0.005 mM final concentration) in ethanol directly to cuvettes containing 2 ml of either various concentrations (1–10 mM) of CDs in phosphate buffer solution (0.05 M, pH 7.4) or ethanolic-buffer solutions, and mixing immediately. The absorbance peak (325 nm) and the fluorescence enhancement, which occurs when certain CDs encapsulate all-*trans*-retinol, were measured (excitation, 350 nm; emission, 475 nm) and followed over a 48-hour period. Phosphate buffer was used in the range 3.5–7.4 to study the pH dependence of the Captisol-retinol complex. The stability of retinol–Captisol complex (retinol : Captisol – 0.005 mM: 10 mM) was compared with the stability of retinol palmitate–Captisol complex (0.005 mM : 10 mM) over a 2 h period. The effect of an antioxidant, 0.05 mM glutathione, on the Captisol–retinol complex was monitored by fluorescence for several hours.

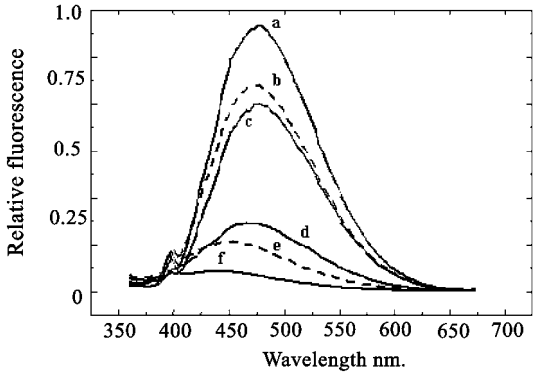

Figure 1. Fluorescence spectra of all-*trans*-retinol in PBS, cyclodextrin solutions or ethanol. a, c, d, f: spectra at t = 0; b, e: spectra at t = 2 hours. a, b: 0.005 mM all-*trans*-retinol in 10 mM 2,3,6-TOM-β-CD solution in PBS. c: 0.005 mM all-*trans*-retinol in absolute ethanol. d, e: 0.005 mM all-*trans*-retinol in 10mM Captisol solution in PBS. f: 0.005 mM all-*trans*-retinol in PBS.

A similar procedure was used for HPLC analyses, which were performed however with a higher concentration of all-*trans*-retinol (0.05 mM) added to the samples which contained various concentrations of CDs (0.05–10 mM) in phosphate buffer solution. Stability of all-*trans*-retinol (0.05 mM) in buffer, ethanol and solutions of CDs in the dark at 4 °C was estimated using HPLC at intervals over 72 hours.

Results and discussion

All-*trans*-retinol is a polyenic compound with a marked hydrophobic character; it exhibits an intense UV absorption band at 325 nm in organic solvents. Considering its aqueous insolubility, the appearance of such a characteristic absorption band in aqueous solutions of CDs indicates the solubilization of retinol and the formation of an inclusion complex. The wavelengths of the emission maxima for the different all-*trans*-retinol-CD complexes (0.005 mM : 10 mM) were very similar, although their absolute emission intensities differed notably under the same conditions. Thus, the most intense fluorescence was produced with 2,3,6-tri-O-methyl-β-cyclodextrin (2,3,6-TOM-β-CD), which was almost twice as high as in ethanolic solution; the absorption and emission spectra of retinol with Captisol were lower than in EtOH. After 2 hour test time, an approximately 20% decrease in fluorescence intensities was observed for all complexes investigated (Figure 1).

Table 1. Room-temperature fluorescence and HPLC data for the all-*trans*-retinol complexes with a set of CDs

	CD solution* Fluor. units	Retinol-CD** Fluor. units	Retinol-CD HPLC peak area
Captisol®	5	37	98
2,3,6-TOM-β-CD	14	77	99
α-CD	15	18	7
HP-α-CD	22	42	73
β-CD	33	35	90
HP-β-CD	22	42	91
2-HP-β-CD	14	36	88
M-β-CD	40	56	73
2,6-DOM-β-CD	32	45	92
HP-γ-CD	17	63	11
EtOH	0	60	100
PBS	0	5	3

* Solutions of CDs were 10 mM in PBS (excitation, 350 nm; emission, 475 nm).
** Same solution as in * but with added all-*trans*-retinol.

Difficulties were encountered with 2,6-DOM-β-CD, M-β-CD and β-CD as they show very high and characteristic emission and absorption intensities. Table 1 summarises the room temperature fluorescence intensities and HPLC data for the inclusion complexes of all-*trans*-retinol with CDs in aqueous media.

The long-term stability of the complexes (all-*trans*-retinol: CDs; 0.005 mM : 0.5 mM) was verified using both fluorescence and HPLC. Fluorescence intensities of all retinol-CDs complexes tested went down by more than 60% after 48 hours, while analysis of the same ratio of retinol to CDs by HPLC showed that Captisol, 2,3,6-TOM-β-CD and 2,6-DOM-β-CD were the best at solubilizing retinol in aqueous media and protecting it from degradation.

Further analyses were mainly focused on the properties of the Captisol-retinol complex as it is the basis for an ocular delivery system now under development in our laboratory. The optimum ratio of all-*trans*-retinol to Captisol was found to be 1 to 50 and it stabilised retinol for up to 72 hours. We examined several factors to determine if the stability of retinol in the complex with Captisol could be improved.

An antioxidant, glutathione, was tested for its protective effect in this system. For these experiments, the all-*trans*-retinol was incorporated into the Captisol cavity and the antioxidant added to the buffer ($\leq$ 0.05 mM). The fluorescence intensity was lower for the solution containing glutathione and the fluorescence decreased by a further 20% after 2 h at room temperature. Thus, the antioxidant was probably displacing retinol from the Captisol cavity, and would not increase stability with regard to oxidation.

The binding of retinol to Captisol was determined as a function of pH and ethanol concentration in the buffer. The fluorescence intensity obtained for the retinol-Captisol complex appeared to be only slightly higher and red-shifted following reduction in pH from 7.4 to 3.5. Hence, low pH values were not very effective in increasing the stability of retinol in aqueous media. Testing all-*trans*-retinol for stability in ethanolic-Captisol- buffer solutions, we found that

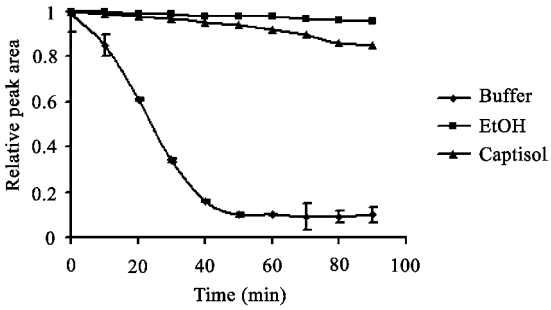

Figure 2. Decomposition of all-*trans*-retinol in ethanol and phosphate buffer in the presence and absence of Captisol at 22 °C as measured by HPLC. Means and standard deviations (n = 3) are shown; where error bars are not shown, standard deviations are less than 0.01 units.

the addition of up to 10% ethanol had only a small effect on the fluorescence intensity of the complex, suggesting that in aqueous media retinol was stabilised mainly by the cyclodextrin complex formation.

Since all-*trans*-retinol is one of the least stable of all the naturally occurring retinoids, experiments to test stability were also conducted with retinol palmitate, which does not undergo isomerization and oxidation so easily in aqueous solution at room temperature [17]. The fluorescence intensity for the retinol palmitate-Captisol complex (0.005 mM : 10 mM) was similar to all-*trans*-retinol-Captisol, and loss of fluorescence was 15% over a 2 hour test period. Thus, there is no significant advantage in the use of retinol-palmitate instead of retinol for the cyclodextrin complex formation.

Decomposition of all-*trans*-retinol was followed over time by changes in the absorption spectrum measured at 325 nm and the HPLC peak area. Test solutions were made 0.05 mM in all-*trans*-retinol, either in ethanol or phosphate buffer, in the presence or absence of 5 mM Captisol (Figure 2). After 30 min all the retinol in the buffer solution has degraded whereas under the same experimental conditions Captisol offered significant protection over the test period. This suggests that Captisol could stabilise and solubilise all-*trans*-retinol at room temperature in an aqueous environment.

Conclusions

Due to the unique role of vitamin A in the regeneration of rhodopsin, its direct delivery to the retina may help those patients with hereditary eye diseases associated with visual cycle defects. Given that all-*trans*-retinol readily undergoes oxidation and isomerization, there is a need for a convenient method for the delivery of retinol for in vitro physiological experiments [17]. Cyclodextrins are known to improve solubility and stability of many drugs and are widely used in drug delivery systems. Using spectroscopic measurements and chromatographic studies, the complexes formed between ten cyclodextrins and all-*trans*-retinol were evaluated. The results indicate that all the cyclodextrins examined were able to form inclusion complexes, but only a few, including Captisol, met the criteria of agents which both protect the

oxidative state of retinol and do not interfere in the measurements being tested. Our results confirm that retinol structural integrity is maintained in complex formation with CDs. Both chromatographic analysis as well as spectral methods were necessary in order fully to determine retinol properties in aqueous media. Analysis of the full spectrum and examination of all-*trans*-retinol by HPLC demonstrated 72 hours' protection with Captisol without significant degradation of retinol whereas in buffer retinol was degraded within 30 min. Addition of antioxidant and variability in pH values did not reveal any substantial improvement in the stability of retinol. In summary, Captisol is a good candidate for inclusion in an ophthalmic direct delivery system now under development in our laboratory.

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References

1. N. Noy: in H. Nau and W. Blaner (eds.), *Physical-Chemical Properties and Action of Retinoids*, Handbook of Experimental Pharmacology Vol. 139, pp. 3–29, Springer, Berlin (1999).
2. E.L. Berson, B. Rosner, M.A. Sandberg, K.C. Hayes, B.W. Nicholson, C. Weigel-DiFranco, and W. Willett: *Arch. Ophthalmol.* **111**, 761–772 (1993).
3. C.D.B. Bridges, S. O'Gordon, S.-L. Fong, R.A. Alvarez, and E. Berson: *Invest. Ophthalmol. Vis. Sci.* **26**, 681–691 (1995).
4. A.F. Wright: *Nature Genet.* **17**, 132–134 (1997).
5. P. Gouras, P.E. Carr, and R.D. Gunkel: *Invest. Ophthalmol. Vis. Sci.* **10**, 784 (1971).
6. S.G. Jacobson, A.V. Cideciyan, G. Regunath, F.J. Rodriguez, K. Vandenburgh, V.C. Sheffield, and E.M. Stone: *Nature Genet.* **11**, 27–32 (1995).
7. J.P. Van Hooser, T.S. Aleman, Y-G. He, A.V. Cideciyan, V. Kuksa, S.J. Pittler, E.M. Stone, S.G. Jacobson, and R. Palczewski: *Proc. Nat. Acad. Sci. USA* **97**, 8623–8628 (2000).
8. L. Sibulesky, K.C. Hayes, A. Pronczuk, C. Weigel-DiFranco, B. Rosner, and E.L. Berson: *Am. J. Clin. Nutr.* **69**, 656–663 (1999).
9. K. Palczewski, J.P. Van Hooser, G.G. Garwin, J. Chen, G.I. Liou, and J.C. Saari: *Biochemstry* **38**, 12012–12019 (1999).
10. S.M. Botella, M.A. Martin, B. del Castillo, J.C. Menendez, L. Vazquez, and D.A. Lerner: *J. Pharm. Biomed. Anal.* **14**, 909–915 (1996).
11. S.M. Botella, D.A. Lerner, B. del Castillo, and M.A. Martin: *Biomed. Chromatogr.* **11**, 91–92 (1997).
12. R.K. Crouch, E.S. Hazard, T. Lind, B. Wiggert, G. Chader, and D.W. Corson: *Photochem. Photobiol.* **56**, 251–255 (1992).
13. B. McCormack and G. Gregoriadis: *Int. J. Pharm.* **162**, 59–69 (1998).
14. A. Angelova, C. Ringard-Lefebvre, and A. Baszkin: *J. Coll. Interf. Sci.* **212**, 280–285 (1999).
15. R. Wyss: *J. Chrom B* **671**, 381–425 (1995).
16. E.Z. Szuts, and F.I. Harosi: *Arch. Biochem. Biophys.* **287**, 297–304 (1991).
17. M.B. Sporn, A.B. Roberts, and D.S Goodman: *The Retinoids: Biology, Chemistry and Medicine*, 2nd edn, Raven Press, NY (1994).