Enzymatic Quantification of Cholesterol and Cholesterol Esters from Silicone Hydrogel Contact Lenses

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PURPOSE. The purpose of this work was to develop an enzymatic method of quantification of cholesterol and cholesterol esters derived from contact lenses, both in vitro and ex vivo.

METHODS. Lotrafilcon B (O2 Optix; CIBA Vision, Inc., Duluth, GA) and galyfilcon A (Acuvue Advance; Vistakon, Inc., Jacksonville, FL) silicone hydrogel contact lenses were independently incubated in cholesterol oleate solutions varying in concentrations. After incubation, the lenses were removed and underwent two separate 2:1 chloroform-methanol extractions. After in vitro studies, 10 human subjects wore both lotrafilcon B and galyfilcon A contact lenses for 7 days. The lenses also underwent two separate 2:1 chloroform-methanol extractions. All in vitro and ex vivo samples were quantified with a cholesterol esterase enzymatic reaction.

RESULTS. Calibration curves from quantifications of in vitro contact lens samples soaked in successively decreasing concentrations of cholesterol oleate yielded coefficients of determination (R^2) of 0.99 (lotrafilcon B) and 0.97 (galyfilcon A). For in vitro contact lens samples, galyfilcon A was associated with an average cholesterol oleate extraction of 39.85 ± 48.65 $\mu\text{g}/\text{lens}$, whereas lotrafilcon B was associated with 5.86 ± 3.36 $\mu\text{g}/\text{lens}$ ($P = 0.05$) across both extractions and all incubation concentrations. For ex vivo contact lens samples, there was significantly more cholesterol and cholesterol esters deposited on galyfilcon A (5.77 ± 1.87 $\mu\text{g}/\text{lens}$) than on lotrafilcon B (2.03 ± 1.62 $\mu\text{g}/\text{lens}$; $P = 0.0005$).

CONCLUSIONS. This is an efficient and simple method of quantifying total cholesterol extracted from silicone hydrogel contact lenses and, potentially, the meibum and/or tear film. Certain silicone hydrogel materials demonstrate more affinity for cholesterol and its esters than do others. (*Invest Ophthalmol Vis Sci.* 2010;51:2949–2954) DOI:10.1167/iovs.08-3368

There is a significant interest in the total amount and types of lipids in the meibum and tear film due to its contribution to tear film stability. Lipids are primarily found in the outer layer of the tear film interfacing with the external environment and aqueous tears. It is thought that the lipid layer can be further broken down into an inner, polar layer and an outer, nonpolar layer.¹ This outer, nonpolar layer is thought to consist of cholesterol and cholesterol esters, in addition to other lipids such as wax esters and fatty acids.^{2–6} When this layer is

altered in structure or composition, it may lead to dry eye due to increased evaporation of the tear film.^{7–11}

Several methods have been used to study lipids of the meibum and/or tear film, such as assays, radiochemical methods, thin-layer chromatography, nuclear magnetic resonance, gas chromatography, and mass spectrometry.^{3–6,12–31} These procedures have been useful in various ways, but each has drawbacks, whether it be sensitivity, accuracy, or efficiency. In this regard, we have adapted a well-known and efficient cholesterol esterase assay to measure the total amount of cholesterol and cholesterol esters associated with the tear film, meibum, or deposits extracted from contact lenses.^{32,33}

This assay was originally developed to determine the total amount of cholesterol in serum and works by hydrolyzing cholesterol esters to free cholesterol (by cholesterol ester hydrolase). As described by Allain et al.,³² “the free cholesterol produced is oxidized by cholesterol oxidase to cholest-4-en-3-one with the simultaneous production of hydrogen peroxide, which oxidatively couples with 4-aminoantipyrine and phenol in the presence of peroxidase to yield a chromogen with maximum absorption at 500 nm.”

There has also been much clinical and scientific interest in lipid deposits associated with silicone hydrogel contact lenses.^{34,35} It is thought clinically that increased deposits on contact lenses can lead to patient discomfort, altered surface wetting, altered visual performance, lipid degradation, and even impaired immune function, but these effects have not been thoroughly studied.³⁶ This innovative cholesterol esterase quantification method offers an efficient method for describing the total cholesterol and cholesterol esters associated with the tear film. The purpose of this article is to describe its application to cholesterols extracted from various silicone hydrogel contact lenses associated with in vitro and ex vivo samples.

METHODS

Materials

One liter of cholesterol assay reaction buffer was created containing 7.10 g Na_2HPO_4 (50 mM, S3264; Sigma-Aldrich, St. Louis, MO), 6.00 g of NaH_2PO_4 (50 mM, S3139; Sigma-Aldrich), 1.29 g sodium cholate (3 mM; NC9479843; Fisher Scientific, Pittsburgh, PA), 0.16 g 4-aminoantipyrine (0.82 mM; AC10315-0250; Fisher Scientific), 1.48 g phenol (liq./certified; 14 mM A9311-500; Fisher Scientific), and 1.02 g PEG 6000 (0.17 mM; NC9166418; Fisher Scientific). The final solution had a pH of 7.0 and was reformulated each day of use.

One liter of 0.10 M pH 7.0 phosphate buffer was created for later dilution of peroxidase from horseradish (P6782-5MG; Sigma-Aldrich) to 5 mg/mL, cholesterol oxidase from *Nocardia erythropolis* (ICN-15067480; Fisher Scientific) to 20 U/mL, and cholesterol esterase from bovine pancreas (C3766-100UN; Sigma-Aldrich) to 0.5 U/mL. Each enzyme was stored at -20°C until just before use.

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A cholesterol oleate (cholesterol ester) stock solution was prepared at a final concentration of 8.6 mM (5.6 mg/mL), as directed by the manufacturer. In brief, 56.0 mg of cholesterol oleate was first dissolved (prod. no. C-9253; Sigma-Aldrich) in 1 mL of polyoxyethylene ether and 9 lauryl ether (prod. no. P-9641; Sigma-Aldrich), followed by the addition of 9 mL of hot 0.10 M pH 7.0 phosphate buffer. The solution was stored at room temperature to avoid cholesterol oleate precipitation (but it can be redissolved by gently boiling for approximately 5 minutes if precipitation occurs).

Cholesterol Oleate Standard Curve

A serially diluted cholesterol oleate stock solution was dispensed to create a standard curve that was used as a reference to determine the relative amount of cholesterol and cholesterol esters recovered from the chloroform-methanol extraction steps (described later). The following concentrations were used for the cholesterol oleate standard curve: 30.00, 20.00, 15.00, 10.00, 7.50, 5.00, 3.75, 2.50, 1.88, 1.25, and 0.00 $\mu\text{g}/50\ \mu\text{L}$. The dilutions were created from the original cholesterol oleate stock solution and diluted in 0.10 M pH 7.0 phosphate buffer.

In Vitro Silicone Hydrogel Lens Samples

Lotraficon B (O2 Optix; CibaVision, Inc., Duluth, GA) and galyfilcon A (Acuvue Advance; Vistakon, Inc., Jacksonville, FL) silicone hydrogel contact lenses were used in the in vitro segment of the study. A serial dilution of the stock cholesterol oleate solution was created in 0.10 M pH 7.0 phosphate buffer at the following concentrations: 5.6000, 1.4000, 0.3500, 0.0875, 0.0218, 0.0057, and 0.0000 mg/mL. Seven lotraficon B and seven galyfilcon A silicone-hydrogel contact lenses were incubated in triplicate in 1.5 mL (with three of each contact lens incubating in each concentration) of the various aforementioned cholesterol oleate dilutions. The lenses were set on a rocker table for gentle agitation for 3 days at room temperature in a closed contact lens case. The contact lenses were then removed from the incubation solution and gently shaken dry and placed in a glass vial. Another seven lotraficon B and seven galyfilcon A contact lenses were treated as just described. However, each of the seven contact lenses were not run in triplicate, and the second 2:1 chloroform-methanol (C:M) extraction was added to a different amber vial than the first 2:1 C:M extraction (described later), and the two extractions were quantified separately (to determine the effect of a second extraction).

Ex Vivo Silicone Hydrogel Lens Samples

This prospective, open-label study was approved by an Institutional Review Board in accordance with the Declaration of Helsinki. Subjects were asked to sign an informed consent and an institutionally approved Health Insurance and Portability and Accountability Act (HIPAA) form. Only subjects who were 18 years of age or older and current silicone hydrogel contact lens wearers were included. Subjects were excluded if they were pregnant, breast-feeding, or using any ocular medications or rewetting drops. Further, subjects were allowed to participate only if they could successfully wear both lotraficon B (O2 Optix; CIBA Vision, Inc) and galyfilcon A (Acuvue Advance; Vistakon) contact lenses and were using either of two contact lens care systems (Opti-Free Replenish; or Opti-Free Express, both from Alcon, Inc., Fort Worth, TX). Either solution was allowed, as they are similar in composition. Subjects were asked to wear each of the two lens types for at least 10 hours per day for 7 ± 2 days. They were instructed to not wear their lenses overnight and to store them in the case that came with the cleaning system. All subjects were told to follow the no-rub cleaning instructions provided by the manufacturer. After completion of the wearing periods, the contact lenses were removed by an examiner with sterile metal forceps and individually stored at -80°C until all subjects completed the study.

Extraction of Cholesterol from Contact Lenses In Vitro and Ex Vivo

A 200- μL aliquot of 2:1 C:M solution (e.g., 200 μL chloroform + 100 μL methanol) was pipetted into each glass vial containing a contact lens (all lenses were individually extracted). All lenses were allowed to equilibrate with room temperature before analysis. This solution was gently mixed for 30 to 40 seconds. All the supernatant ($\sim 100\ \mu\text{L}$) from the extracted sample was transferred to an amber glass vial with a pipette. A second 200- μL aliquot of 2:1 C:M extraction solution was added to each contact lens immediately after the first extraction step. The second extraction supernatant was transferred again with a pipette ($\sim 200\ \mu\text{L}$) into the amber glass vial containing the supernatant from the first extraction. The supernatant samples were allowed to lyophilize overnight at room temperature.

Enzymatic Reaction

The enzymatic reaction was performed by adding 16.75 U of horseradish peroxidase, 0.07 U cholesterol oxidase, and 0.032 U cholesterol esterase to 200 μL cholesterol assay reaction buffer.² Then, 50 μL of 0.10 pH 7.0 phosphate buffer was added to each lyophilized supernatant sample as the standard curve samples were each dispensed in 50 μL of 0.10 M pH 7.0 phosphate buffer. The cholesterol assay reaction buffer was mixed gently and 200 μL was added to each lyophilized supernatant sample and to the standard curve samples, which were all transferred to a 96-well microtiter plate. All samples were incubated with the cholesterol assay reaction buffer at 37°C for up to 60 minutes. The absorbance of all samples was read at 500 nm in a microtiter spectrophotometer (Infinite M200; Tecan, Salzburg, Austria).

Statistical Analyses

Descriptive statistics such as averages and standard deviations were used to describe the data. Linear regression was used to determine the robustness of standard curves and the relation between cholesterol extracted and that which was used for incubation. Wilcoxon signed-ranks and Mann-Whitney U tests were used to compare the quantities of cholesterol extracted within a lens material or between materials in vitro and ex vivo, respectively.

RESULTS

In Vitro Lens Analyses

Figure 1 shows a coefficient of determination (R^2) of 0.99 for the cholesterol oleate standard curve. The 2:1 C:M extraction of cholesterol oleate from the lotraficon B contact lenses generated a cholesterol oleate contact lens incubation curve with a positive slope and a R^2 value of 0.99 (Fig. 2). Figure 2 demonstrates that when lotraficon B contact lenses were incubated in 5.60 mg/mL cholesterol oleate solution for 3 days, approximately 35.00 $\mu\text{g}/\text{lens}$ of cholesterol oleate was recovered, and when this contact lens material was incubated in 0.0057 mg/mL of cholesterol oleate for 3 days, 1.60 $\mu\text{g}/\text{lens}$ of cholesterol oleate was recovered. Figure 3 shows an R^2 of 0.97 for the galyfilcon A contact lenses incubation curve. Figure 3 also demonstrates that when galyfilcon A contact lenses were incubated in 5.60 mg/mL cholesterol oleate solution for 3 days, approximately 65.00 $\mu\text{g}/\text{lens}$ of cholesterol oleate was recovered, and when galyfilcon A contact lenses were incubated in 0.0057 mg/mL of cholesterol oleate, approximately 6.00 $\mu\text{g}/\text{lens}$ of cholesterol oleate was recovered.

Figure 4 demonstrates that a second 2:1 C:M extraction increased the cholesterol oleate recovered from the lotraficon B material, and it also shows that the second 2:1 C:M extraction tended to remove slightly more cholesterol oleate than the first 2:1 C:M extraction. For lotraficon B, the average cholesterol oleate extracted across all concentrations from the first 2:1 C:M extract was $2.03 \pm 1.59\ \mu\text{g}/\text{lens}$, although it was 3.83 ± 1.80

FIGURE 1. Cholesterol oleate standard curve.

μg/lens for the second 2:1 C:M extraction (Wilcoxon signed-ranks test, $z = 2.20$, $P = 0.03$). Figure 5 demonstrates a similar trend for the galyfilcon A material, with additional cholesterol oleate being recovered from the second 2:1 C:M extraction. However, the amount recovered from the first extraction (22.51 ± 28.53 μg/lens) did not differ significantly from the amount recovered from the second extraction (17.34 ± 20.18 μg/lens; Wilcoxon signed-ranks test, $z = 1.15$, $P = 0.25$). Overall, across incubation concentrations and both extractions, galyfilcon A (39.85 ± 48.65 μg/lens) was associated with more extracted cholesterol oleate than was lotrafilcon B (5.86 ± 3.36 μg/lens; Mann-Whitney U = 6.00, $P = 0.05$).

Ex Vivo Lens Analyses

Ten subjects were enrolled in and completed the study. The average age of the subjects was 31.3 ± 9.4 years, and the subjects were 90% women. Galyfilcon A contact lenses yielded 5.77 ± 1.87 μg/lens, and lotrafilcon B contact lenses yielded 2.03 ± 1.62 μg/lens (Mann-Whitney U = 7.00, $P = 0.0005$). Figure 6 demonstrates that galyfilcon A contact lenses accumulated more cholesterol during normal human wear than did lotrafilcon B contact lenses in 9 of the 10 subjects.

DISCUSSION

As mentioned, there are several methods that can be used to analyze the lipids associated with the meibum and/or tear film.

The method of choice is dependent on several factors including the need to quantify and/or identify within a class or individual species of lipid. Indeed, others have attempted to examine cholesterol deposited on contact lenses by using different methods.^{24,34,35,37} However, this attempt may be the first to quantify the total amount of cholesterol and cholesterol esters extracted from contact lenses via an enzymatic reaction assay, although the method can be applied to the quantification of cholesterol from tears and/or meibum, as well.

As predicted, given the experimental conditions, Figures 2 and 3 show that with increasing cholesterol oleate incubation concentrations, more cholesterol oleate was extracted and quantified from each lens material. It is worth noting in Figures 2 and 3 that contact lenses incubated in concentrations of cholesterol lower than 1.0 mg/mL tended to show greater variability in the amount of cholesterol recovered. For example, more cholesterol oleate was recovered from galyfilcon A contact lenses incubated in 0.09 mg/mL cholesterol oleate solution than from contact lenses incubated in 0.35 mg/mL cholesterol oleate solution. A possible explanation of this result could be that even with the agitation of the contact lenses on the rocker table during the incubation period, the incubating solution may not have evenly distributed across the lens surface.

Another major finding from this study is that significantly more cholesterol oleate is extracted from galyfilcon A than

FIGURE 2. Cholesterol oleate recovered from incubated lotrafilcon B after two 2:1 C:M extractions and pooling into one glass vial.

FIGURE 3. Cholesterol oleate recovered from incubated galyfilcon A after two 2:1 C:M extractions and pooling into one glass vial.

from lotrafilcon B lenses. In fact, the amount extracted from galyfilcon A was generally approximately six to seven times that extracted from lotrafilcon B when considering the *in vitro* samples. We also found that the human-worn galyfilcon A lenses yielded approximately three times as much cholesterol and cholesterol esters on average after extraction when compared to the lotrafilcon B lenses. It is also important to note that 9 of 10 subjects had significantly more cholesterol and cholesterol esters extracted from galyfilcon A worn lenses compared to the lotrafilcon B worn lenses (Fig. 6).

The observations from the *in vitro* and *ex vivo* experimentation in our study are consistent with the *in vitro* results reported by Carney et al.³⁷ and Iwata et al.,³⁸ who also found that considerably more cholesterol was associated with galyfilcon A lenses than with lotrafilcon B lenses. Carney et al. labeled cholesterol with NBD (7-nitrobenz-2-oxa-2,3-diazol-4-yl), and incubated various lens materials for 14 hours in 1.75 µg/mL, after which it was read with a fluorescence counter. Cholesterol was not extracted with an organic solvent as was the case in our study. Iwata used gas chromatography/mass

spectrometry to quantify cholesterol and cholesterol esters along with other common tear film lipids extracted from silicone hydrogel lenses with a 7:3 C:M solution.

When comparing the *in vitro* data from all three studies at about the same time point (2.5–3 days), our method recovered 1.58 µg/lens of cholesterol oleate from lotrafilcon B after incubation in a 5.7 µg/mL cholesterol oleate solution, whereas Carney et al.³⁷ measured approximately 0.75 µg/lens of cholesterol on the lens after incubation in 1.75 µg/mL of cholesterol solution. Iwata et al.³⁸ recovered less than 0.2 µg/lens of cholesterol palmitate (cholesterol ester) from lotrafilcon B lenses soaked in 30 µg/mL solution. For galyfilcon A, our method recovered 6.4 µg/lens of cholesterol oleate after incubation in a 5.7 µg/mL cholesterol oleate solution, whereas Carney et al.³⁷ recovered approximately 2.25 µg/lens of cholesterol after incubation in 1.75 µg/mL of cholesterol solution. Iwata et al. recovered less than 0.2 µg/lens of cholesterol palmitate from lotrafilcon B lenses soaked in 30 µg/mL cholesterol palmitate solution. Hence, our data and the results of Carney et al. demonstrate a similar trend for the two different

FIGURE 4. A comparison of cholesterol recovered from two self-contained 2:1 C:M extraction steps from incubated lotrafilcon B.

FIGURE 5. A comparison of cholesterol recovered from two self-contained 2:1 C:M extraction steps from incubated galyfilcon A.

materials; however, the procedure used by Iwata et al. recovered much less cholesterol ester from both materials after in vitro incubation. It is likely that the data of both groups vary from our data due to the different forms of the lipid, different initial incubation concentrations of lipid, and the differences in analysis methods. Further, Iwata et al. handled their samples differently. Specifically, they did not rock their lenses during incubation, they incubated their lenses at a temperature higher than that found on a human eye, and they attempted to quantify levels of cholesterol ester below their statistically relevant quantification limit of 2.1 µg/lens.³⁸

To the best of our knowledge, there has yet to be an exploration of how much combined cholesterol and cholesterol esters are deposited on human-worn silicone hydrogel lenses. We found that there was significantly more cholesterol and cholesterol esters deposited on galyfilcon A (5.77 ± 1.87 µg/lens) than lotrafilcon B (2.03 ± 1.62 µg/lens) lenses after normal human wear ($P =$

0.0005) for 7 days, which is consistent with the in vitro results of Carney et al.³⁷ They found that galyfilcon A lenses collected approximately 6 µg/lens of cholesterol and lotrafilcon B lenses collected approximately 2 µg/lens of cholesterol after 7 days of in vitro incubation.

Figures 4 and 5 show that a second 2:1 C:M extraction is beneficial to recovering additional cholesterol from these two lens materials. The main pitfall of this experiment is that there is not currently a method for determining how much cholesterol remains bound to or within the lens material after the extractions step(s). This limitation may not be important when attempting to determine relative differences between similar contact lens materials; however, if the absolute amount of cholesterol in a human- or animal-derived sample is of interest, this enzymatic method may be more applicable, since it is often not feasible to label human- and animal-derived samples with fluorescent markers.

FIGURE 6. Cholesterol associated with lotrafilcon B and galyfilcon A contact lenses after wear (ex vivo). Each bar represents a single contact lens.

In future studies, it may be of interest to test this enzymatic quantification method on additional U.S. Food and Drug Administration (FDA)-approved contact lens groups, as all four FDA contact lens groups have different chemical properties as well as human-derived tear and meibum samples. Overall, the method presented in this article is an efficient and simple way to quantify total cholesterol and cholesterol esters from in vitro and ex vivo contact lens samples, and potentially, the meibum and/or tear film. The data also suggest that it is beneficial to perform two 2:1 chloroform-methanol extractions to yield the highest levels of cholesterol.

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