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Rapid quantification of free cholesterol in tears using direct insertion/ electron ionization-Mass spectrometry

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Abstract

Purpose. To establish a simple and rapid analytical method, based on direct insertion/electron ionization–mass spectrometry (DI/EI-MS), for measuring free cholesterol in tears from humans and rabbits.

Methods. A stable-isotope dilution protocol employing DI/EI-MS in selected ion monitoring mode was developed and validated. It was used to quantify the free cholesterol content in human and rabbit tear extracts. Tears were collected from adult humans (n = 15) and rabbits (n = 10) and lipids extracted.

Results. Screening, full-scan (m/z 40–600) DI/EI-MS analysis of crude tear extracts showed that diagnostic ions located in the mass range m/z 350 to 400 were those derived from free cholesterol, with no contribution from cholesterol esters. DI/EI-MS data acquired using selected ion monitoring (SIM) were analyzed for the abundance ratios of diagnostic ions with their stable isotope-labeled analogues arising from the D6-cholesterol internal standard. Standard curves of good linearity were produced and an on-probe limit of detection of 3 ng (at 3:1 signal to noise) and limit of quantification of 8 ng (at 10:1 signal to noise). The concentration of free cholesterol in human tears was $15 \pm 6 \mu\text{g/g}$, which was higher than in rabbit tears ($10 \pm 5 \mu\text{g/g}$).

Conclusions. A stable-isotope dilution DI/EI-SIM method for free cholesterol quantification without prior chromatographic separation was established. Using this method demonstrated that humans have higher free cholesterol levels in their tears than rabbits. This is in agreement with previous reports. This paper provides a rapid and reliable method to measure free cholesterol in small-volume clinical samples.

Keywords

cholesterol, tears, rabbit, human, mass spectrometry

Disciplines

Medicine and Health Sciences

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Rapid quantification of free cholesterol in tears using direct insertion/electron ionization mass spectrometry

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Keywords: Cholesterol, tears, rabbit, human, mass spectrometry

Abstract

Purpose. To establish a simple and rapid analytical method, based on direct insertion/electron ionization-mass spectrometry (DI/EI-MS), for measuring free cholesterol in tears from humans and rabbits.

Methods. A stable-isotope dilution protocol employing DI/EI-MS in selected ion monitoring mode was developed and validated for specificity, linearity and detection limits. It was used to quantify the free cholesterol content in human and rabbit tear extracts. Tears were collected from healthy adult humans (n = 15) and rabbits (n = 10) and tear lipids extracted using a biphasic lipid extraction method optimised for use with small volume samples.

Results. Screening, full scan (m/z 40-600) DI/EI-MS analysis, of crude tear extracts showed that, for the early (low probe temperature) portion of the total-ion current profile, diagnostic ions located in the mass range m/z 350-400 were those derived from free cholesterol, with no contribution from cholesterol esters. DI/EI-MS data acquired using selected ion monitoring were analysed for the abundance ratios of diagnostic ions with their stable isotope-labelled analogues arising from the D_6 -cholesterol internal standard. This approach gave standard curves of good linearity and an on-probe limit-of-detection of 3 ng (at 3:1 signal-to-noise) and limit-of-quantification of 8 ng (at 10:1 signal-to-noise). The concentration of free cholesterol in human tears was found to be $15 \pm 6 \mu\text{g/g}$, which was higher than that found in the tears of rabbits ($10 \pm 5 \mu\text{g/g}$).

Conclusion. A stable-isotope dilution DI/EI-SIM method for quantification of free cholesterol without the requirement for chromatographic separation has been established in this study. This method has been used for measuring free cholesterol levels in human and rabbit tears and showed that humans have higher free cholesterol levels in their tears than rabbits. This is in agreement with

previous reports. This paper provides a rapid and reliable method to measure free cholesterol in small volume clinical samples.

Introduction

The human tear film is composed of 3 layers – the inner mucus layer which is believed to make the epithelium more hydrophilic,¹ the middle aqueous layer which contains most of the electrolytes and proteins,² and the outer lipid layer whose major function is to prevent evaporation of the aqueous layer.³ The lipid layer is of great importance in dry eye and many treatments are aimed at stabilising this layer. Rabbits have a tear film structure similar to humans, however their tear film is much more stable⁴ and consequently they have a much longer inter-blink time than humans.⁵⁻⁷ The lipid layer in both human and rabbit tears is composed of non-polar lipids, such as wax esters, cholesterol esters, triacylglycerols, and smaller amounts of polar lipids, such as phospholipids and (*O*-acyl)- ω -hydroxy fatty acids (OAHFA).⁸⁻¹⁰ However the relative proportions of each type of lipid differ between humans and rabbits.⁹ The major non-polar lipids in human tears are wax esters and cholesterol esters, with OAHFA the major polar type, whereas the major non-polar lipids in rabbit tears are 24,25-dihydro- Δ^8 -lanosterol esters and diacylated diols, with OAHFA again the major polar lipid type.⁹ Reports of free cholesterol and free fatty acids in tears suggest that these are minor components at ~1.5% and 0.5-2.1%, respectively.¹⁰⁻¹²

Although free cholesterol is in relatively low abundance compared to cholesterol esters and wax esters in tears, it is nonetheless important. Cholesterol has previously been shown to form a condensed and compact monolayer at the air-water interface. It orients vertically at the interface with its hydrophilic hydroxyl (OH) group anchored in the aqueous phase. The rigidity of the cholesterol film plays an important role in interacting with molecules in the tear aqueous layer.¹³ Abnormal proportions of free cholesterol and cholesterol esters in the composition of the tear film may lead to tear film instability.¹⁴ Clinical studies have shown that cholesterol levels change in keratoconjunctivitis sicca¹⁵, acne rosacea related meibomian keratoconjunctivitis¹⁶, Sjögrens syndrome, and chronic blepharitis^{15, 17}, conditions that are also associated with reduced tear film stability. Recent studies have found that cholesterol is deposited onto worn contact lenses, with deposition quantities ranging from 2-37 μg per lens.^{18, 19} Furthermore, Saville *et al.*²⁰ were able to

show differences in the level of cholesterol deposition between lens types. The deposition of lipid on contact lenses may contribute to the discontinuation of lens wear due to discomfort, or affect visual acuity during lens wear.^{18, 21}

Reliable measurements of free cholesterol at low concentrations in the presence of complex matrices of other more complex lipids presents unique analytical challenges. A series of enzymatic methods have been used for cholesterol quantification in body fluids such as plasma.²² Other methods for determination of cholesterol such as gas-chromatography require derivatisation to make cholesterol sufficiently volatile for analysis.²³ These traditional methods are not suitable for tear samples due to the very small volumes of tears and low concentrations of the target analytes. A high-performance liquid chromatographic (HPLC) method using ultraviolet absorption detection has been developed for quantitation of cholesterol.^{24, 25} This method however, is impacted by relatively high background and inconsistency between studies.^{18, 26} Takatsu *et al.*²⁷ have reported a stable-isotope dilution mass spectrometry method to determine serum cholesterol, however their method involved purification of lipid extracts using HPLC which took around 25 minutes. Butovich also used HPLC-MS to detect cholesterol in tears.⁹ Here we have developed a rapid and reliable method to quantify free cholesterol, without any derivatisation or chromatography steps, using direct insertion/electron ionization-mass spectrometry (DI/EI-MS).^{20, 28} The purpose of this paper is to detail the validation of this method and use this method to compare levels of free cholesterol in human and rabbit tears.

Materials and Methods

Chemicals and materials

Cholesterol ($\geq 99\%$) and cholesteryl oleate (CE 18:1) were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA); cholesteryl tridecylate (CE 13:0) and cholesteryl erucate (CE 22:1) were purchased from Nu-Chek Prep (Elysian, MN USA); and deuterated cholesterol (2,2,3,4,4,6- D_6 -cholesterol, $\geq 98\%$) from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). HPLC-grade chloroform and methanol and analytical grade ammonium acetate and butylated

hydroxytoluene (BHT) used in the lipid extraction procedure were purchased from Crown Scientific (Sydney, NSW, Australia). All lipid solutions in the experimental work were prepared using HPLC-grade chloroform and stored in glass vials at $-80\text{ }^{\circ}\text{C}$ until use.

Tear collection

Both human and rabbit tears were collected and analysed. Human and animal ethics were approved by the Ethics Review Panel of the University of New South Wales and Brien Holden Vision Institute/Vision CRC, respectively. The Tenets of the Declaration of Helsinki were adhered to for human studies, and informed consent was obtained prior to enrolling human subjects into the study. Basal tear samples (5-10 μL) from humans ($n = 15$) and rabbits ($n = 16$) were collected using calibrated and fire polished disposable glass micro-capillary tubes (Brand, Wertheim, Germany). The glass micro-capillary tube was gently placed just above the lower tear meniscus, minimizing contact of the tip with eye surface to avoid reflex tears. No sedation or anesthesia was required. Samples from either species were centrifuged for 5 mins at 5,000 g after collection to remove cells. The tear samples were stored at $-80\text{ }^{\circ}\text{C}$ until further use.

Sample preparation and lipid extraction

Stock solutions of cholesterol and D_6 -cholesterol were prepared by dissolving an accurately weighed amount of each in chloroform without any further purification. The calibration mixtures were prepared by mixing standard and labelled cholesterol stock solutions. The ratios of cholesterol to D_6 -cholesterol (25 ng) in the calibration mixtures were between 0.1 and 3.0 by weight to cover the range of free cholesterol expected in the tear samples.

Fresh tear samples collected from humans and rabbits were added directly to a pre-tared micro-extraction vial (average of 2.6 mg) and spiked with 5 μL (98 ng) of D_6 -cholesterol internal standard solution (0.05 mM in chloroform). Chloroform:methanol (200 μL ; 2:1 vol/vol) containing 0.01% BHT was added to the tear sample and the mixture vortexed. Aqueous ammonium acetate

(0.15 M; 25 μ L) was added and the mixture was then centrifuged (800 g, 5 mins). The aqueous phase was removed and the organic phase dried under nitrogen. The sample was reconstituted in 50 μ L chloroform:methanol (1:2 vol/vol; 0.01% BHT).²⁸ All samples were stored at -80 °C until they were analysed.

To test for matrix effects 3 tear samples (12.1 mg) were pooled in 1 ml of chloroform:methanol (200 μ L; 2:1 vol/vol) containing 0.01% BHT. A stock solution of chloroform (250 μ L) containing *D*₀-cholesterol (931 ng) and *D*₆-cholesterol (952 ng) was added and the mixture was vortexed, divided into 5 aliquots (250 μ L) and extracted as described above. The extract results were compared with each other and with those obtained for the stock solution used to spike the pooled sample to evaluate the reproducibility of the assay and to identify potential matrix effects. Absolute sample recovery was established using a 15 μ L aliquot of standard solution of cholesterol (0.05 mM).

Mass spectrometry

Electron ionization mass spectrometry was conducted using a single quadrupole GC-MS system (Shimadzu QP5050, Kyoto Japan) fitted with a heated direct-insertion sample probe (DI-50). Pasteur pipettes were used to load a drop (*ca.* 8 μ L) of each tear extract or standard sample to one end of separate and disposable heat-sealed melting-point tubes. The hanging drop was allowed to dry to a film at the tip of the probe prior to introduction into the ion source via a vacuum lock where any residual solvent is removed. Programmed heating (40-250 °C at 80 °C/min) of the probe in the vacuum environment of the ion source resulted in the sublimation of free cholesterol. The maximum release occurred at *ca.* 0.9 \pm 0.2 min (112-128 °C) with small inter-sample variations in the thermal-desorption profiles arising from differences in lipid coating thickness and film distribution after drying. Electron-ionisation mass spectrometry was undertaken during the temperature-programmed desorption using either (i) a full scan (*m/z* 40-600, DI/EI-MS) for qualitative comparison of the analytes present in the sample that are thermally stable and sufficiently volatile to be vaporized into

the ion source^{20, 28} or (ii) selected ion monitoring (DI/EI-SIM) of specific ions diagnostic for cholesterol and its D_6 -isotopologue for the purposes of quantification.

For comparison to electrospray ionization-mass spectrometry (ESI-MS), 3 samples of meibum (collected as previously described³¹) were dissolved in chloroform (1000 μL) containing 0.01% BHT were prepared in glass vials. 100 μL of sample was added to 10 μL D_6 -cholesterol (50 μM) in new glass vials followed by 90 μL of methanol. Samples were split for analysis by DI/EI-MS and ESI-MS. For ESI-MS, 50 μL of sample was added to 50 μL 2:1 methanol:chloroform (v/v) containing 16 mM ammonium acetate. Mass spectra were acquired using a chip based nano-electrospray ionization source (TriVersa Nanomate[®], Advion, Ithaca, NY, USA) coupled to a hybrid linear ion trap-triple quadrupole mass spectrometer (QTRAP[®] 5500, ABSCIEX, Foster City, CA, USA). 10 μL of extract was aspirated from a sealed 96-well plate (Eppendorf Twin-Tec) and delivered into the mass spectrometer. A neutral loss MS/MS scan for the loss of 35 Da was performed on each sample and peak areas for m/z 404.4 [D_0 -cholesterol + NH_4]⁺ and 410.4 [D_6 -cholesterol + NH_4]⁺ were compared.

Cholesterol quantification in tears

Standard cholesterol was loaded onto sample probes at various levels and analysed by DI/EI-SIM to determine the level of detection (LOD) and level of quantitation (LOQ) for the method. Standard mixtures of cholesterol and D_6 -cholesterol (25 ng) were prepared in mass ratios ranging from 0.1 to 3.0 (cholesterol/ D_6 -cholesterol). The solutions were analysed by DI/EI-SIM for ions at m/z 386.4, 368.4, 371.4, and 353.4 for cholesterol and m/z 392.4, 374.4, 377.4 and 359.4 for D_6 -cholesterol. The abundance of each of these ions was integrated and averaged (*ca.* 50 scans) over a region corresponding to the top 5% of the total ion chromatogram (a time window of *ca.* 0.1 mins) with the averaged intensity ratio of each corresponding ion pair arising from cholesterol and D_6 -cholesterol used to construct a calibration curve.

For free cholesterol quantification, 5 μ L (98 ng) of the internal standard (D_6 -cholesterol, 0.05 mM) was weighed into a pre-tared vial containing the tear samples before extraction and lipid extracts were analysed by DI/EI-MS in the SIM mode as described. The ratios of the integrated and averaged abundances for the selected ions corresponding to cholesterol and D_6 -cholesterol were compared to their standards and used to check for isobaric interferences in the extract. Abundance ratios of companion ions from cholesterol and D_6 -cholesterol were used to determine the amount of cholesterol present in the lipid extract by reference to a standard curve. Comparisons of cholesterol content in human and rabbit tears were statistically analysed with Bonferroni corrected Student's *t*-tests *post hoc*. The level of statistical significance was set at $p < 0.05$.

Results

Direct insertion/electron ionization-mass spectrometry of cholesterol

Tear lipid extracts and standard solutions of cholesterol and D_6 -cholesterol were loaded onto sealed melting-point tubes (11 mm long) and subjected to full-scan DI/EI-MS (m/z 40-600) with the probe temperature ramped from 40-250 °C at 80 °C /min. Mass spectra for the diagnostic region (m/z 345-400) are shown in Figure 1. For cholesterol, four prominent and diagnostic ions were detected, the cholesterol molecular ion M^{++} (m/z 386) and fragment ions at $[M-15]^+$ (m/z 371), $[M-18]^{++}$ (m/z 368) and $[M-33]^+$ (m/z 353) due to the loss of a methyl group, water and a combined loss of water and a methyl group, respectively (Figure 1a). Over the same mass range D_6 -cholesterol shows the four corresponding D_6 -enriched ions (Figure 1b). The DI/EI-MS spectrum of tear lipid extracts (Figure 1c), shows that the ions detected over the same mass range and temperature range. While the major ions observed in the spectrum derived from the tear extract correspond to those of cholesterol (*i.e.*, m/z 353, 368, 371 and 386), the ion abundance ratios differ from those of authentic cholesterol (*cf.* Figure 1a) thus suggesting a contribution from other, related molecular species. Cholesterol esters are known to be an abundant component of the tear lipid layer. The DI/EI-MS spectrum of a standard cholesteryl oleate (CE 18:1) sample recorded over the same mass range is shown in Figure 1(d) revealing an abundant ion at m/z 368 but little or no contributions to the m/z 371 or 386 mass channels indicative of free cholesterol. Mass spectra were obtained for authentic samples of two other cholesterol esters representing both short-chain (CE 13:0) and long-chain variants (CE 22:1) and these also showed an abundant ion at m/z 368 (data not shown). The abundance of the m/z 368 ion in the spectrum of the authentic cholesterol esters suggests that these lipids may be contributing to the tear-extract spectrum shown in Figure 1(c). Given the differing molecular masses of these species it was reasonable to suggest that they may be desorbed at different times during the ramping of the temperature. To investigate the relative desorption profiles of free cholesterol and cholesterol esters a four-component mixture was prepared containing cholesterol and all three cholesterol ester homologues (*i.e.*, CE 13:0, CE 18:1 and CE 22:1). Plotting the detection of ions at m/z 353, 368 and

386 against time (and thus desorption temperature) in Figure 2(a) reveals entirely different desorption profiles for free cholesterol (represented by all three ions in their expected ratios as indicated in Figure 2b) and cholesterol esters (represented by ions at m/z 353 and 368 only, in their expected ratios as indicated in Figure 2c). Figure 2(a) shows cholesterol esters are thermally resolved from free cholesterol and the former are only detected at higher probe temperatures. Profiles of the individual cholesterol esters are provided as Supporting Information (Figure S1) and show an increase in desorption temperature with increasing molecular weight. Since all cholesterol esters produce a prominent fragment ion at m/z 368 under standard EI conditions, any contributions from these species would be reflected in a measurable change in the relative ratios of the diagnostic ions for free cholesterol (cf. Figure 1c). An equivalent desorption profile obtained from an extract of a pooled tear sample is shown in Figure 2(d). These data clearly indicate that even in this complex matrix the early desorption feature in the m/z 368 and 386 channels arises exclusively from cholesterol with no contribution from other compounds, including cholesterol esters that are clearly resolved at longer analysis times (*i.e.*, higher desorption temperatures). Furthermore, the results provided as supporting information (Figure S2) show that for the analysis of free cholesterol each DI/EI-SIM analysis can be aborted after *ca.* 1 min (corresponding to *ca.* 120 °C) immediately following the maximum current for the diagnostic ions. This significantly reduced analysis time allowing the DI-probe to be withdrawn, the probe tip discarded and the probe quenched in cold ethanol ready for the next analysis. The procedure thus designed, was found to reduce analysis time, minimise sample carry-over.

Cholesterol quantification and establishing recovery and detection limits

As the full scan D_6 -cholesterol mass spectrum showed fragmentation behaviour analogous to its unlabelled counterpart, each corresponding (D_0 , D_6) ion pair may potentially be used for quantification. Therefore, SIM runs using DI/EI-MS were performed on the diagnostic ion pairs m/z 386.4/392.4, 371.4/377.4, 368.4/374.4 and 353.4/359.4, with each ion pair representing an ion from

cholesterol and D_6 -cholesterol, respectively. The relative abundances of these ions for one of the calibration mixtures (D_0/D_6 at 1:2 w/w) are shown in the spectrum in Figure 3. Figure 4 shows the standard curves for the relative abundance ratio of the diagnostic ions from cholesterol and D_6 -cholesterol versus the mass ratio for the cholesterol and D_6 -cholesterol, with good regression coefficient (R^2) values for all four calibration standard curves. In theory all four diagnostic ion pairs may be used to quantify free cholesterol. In practice only three ion pairs (m/z 386.4/392.4, m/z 368.4/374.4, m/z 353.4/359.4) were monitored to verify the integrity of the signal source (cholesterol and D_6 -cholesterol) by reference to the relevant ion-intensity ratios for the standards and to improve the sensitivity (signal-to-noise), which results from the subsequent increase in the dwell time for individual ions during each SIM scan. The standard curve for the ion pair m/z 386.4/392.4 was selected as the primary standard curve for quantification because the respective ion intensities have the relatively greatest signal to noise value for all of the monitored ions.

To establish the sensitivity of the method, standard cholesterol solutions were loaded onto sample probes at various levels and analysed by DI/EI-SIM. This allowed the level of detection (LOD 3 ng, S/N 3:1) and level of quantitation (LOQ 8 ng, S/N 10:1) to be established by measuring the averaged intensity for the molecular ion (m/z 386.4) across the apex (0.8-0.9 mins) of its ion-signal intensity plot. An on-probe LOQ of 8 ng for m/z 386.4 equates to a free cholesterol LOQ of 1 ng/ μ L in the final tear extracts (50 μ L).

An absolute sample recovery of 72 ± 17 % was obtained by spiking a previously analysed tear sample with 290 ng of cholesterol standard (15 μ L, 0.05 mM in chloroform:methanol 1:2 vol/vol, $n = 3$) and using D_6 -cholesterol as an external standard. The result indicates that some cholesterol is lost during the extraction process. The addition of D_6 -cholesterol prior to extraction however, means that it acts as both internal standard and surrogate for free cholesterol and thus the recovery only effects the sensitivity of the measurement and not the accuracy of the method.

The results for the five replicate stock solution samples at 30 ng on probe (*ca.* $4 \times$ LOQ) were highly reproducible with the coefficient of variation (expressed as a percentage of the mean, CV%)

for the ion pair ratios found to be 0.7% (m/z 386.4/392.4), 1.5% (m/z 368.4/374.4) and 1.1% (m/z 353.4/359.4), respectively. The %CV extract results for 5 aliquots of a pooled tear sample, also at *ca.* $4 \times$ LOQ on probe, were found to be 0.4% (m/z 386.4/392.4), 2.6% (m/z 368.4/374.4) and 1.8% (m/z 353.4/359.4), respectively. A comparison between the averaged relative intensity ratios for the quantitative ion pair (m/z 386.4/392.4) for the stock solution and tear extracts showed that 193 ng of free cholesterol was extracted from the pooled tear samples (12.1 mg) and equates to a free cholesterol concentration of $16 \pm 0.37 \mu\text{g/g}$ of tears.

To investigate the accuracy of the DI/EI-SIM approach to quantification a parallel analysis of free cholesterol was undertaken using electrospray ionization tandem mass spectrometry (ESI-MS/MS). Lipid extracts from human meibum were used in this comparative analysis as these represent an even more complex matrix than that found in tears. Comparison of the same three meibum extracts by DI/EI-SIM and ESI-MS/MS gave a regression coefficient (R^2) value of 0.987 and slope of 1.075 between the two assay techniques (see Supporting Information Figure S).

Cholesterol in human and rabbit tears

Free cholesterol in human and rabbit tears was quantified using the developed method. Tears (*ca.* 5 μL) were extracted using the protocol described above. The concentration of cholesterol in the human tears was found to be $15 \pm 6 \mu\text{g/g}$ which was higher than that of the rabbit tears ($10 \pm 5 \mu\text{g/g}$; $p < 0.05$; Figure 5).

Discussion

This study has established a method to rapidly quantify free cholesterol in lipid extracts from tears using DI/EI-SIM. Using this method, the lipids in tear samples were extracted directly into organic solvents and the crude extracts were analysed directly with no requirement for chromatographic separation. This represents a significant time-saving over traditional GC and HPLC based methods, *i.e.*, less than 2 versus up to 25 mins. The simplicity of the purification steps minimise the potential loss of analyte during sample preparation with the absolute recovery found to be 72.1%. Since the sample is spiked with the stable isotope D_6 -cholesterol as internal standard prior to extraction any loss of analyte during extraction is accounted for in the quantification. The loss of any sample during extraction will not affect the analytical results, although there may be a slight reduction in sensitivity.

The LOQ for the analysis was found to be 8 ng (~20 pmol) of cholesterol, for which a minimum volume of 1 μ L of tears was required. The elution profiles of the DI/EI-SIM data for each sample varied only slightly due to differences affecting the thermal desorption of the sample such as the distribution of the cholesterol film on the surface of the probe. The method provides a highly sensitive way to measure free cholesterol in small volumes of clinical samples and has the potential to provide new insight into the role of free cholesterol in tear film instability and dry eye syndrome. If necessary, a concentration step (solvent evaporation) could be used to increase the detection limit for clinical samples (*i.e.*, if dry-eye samples had much less cholesterol than normal samples). Another advantage of this method is the ability to increase the sensitivity of analysis by adding another drop of sample from the same extract on top of a previously dried sample film. This means that the whole extract (*ca.* 50 μ L) is potentially available for analysis. However, in each case high purity of the solvents is essential to avoid increasing background signals, particularly with multiple sample loadings. Nevertheless, as quantification was based on the ratio of ion peaks in the same sample, the sample loading volume will not affect the results, again improving assay accuracy.

One of the commonly used methods for measuring low concentration of cholesterol is GC-MS. Although GC-MS is sensitive, the analysis is time-consuming and not suitable for a large number of clinical samples. Additionally, since the process involves silylation and hydrolysis of cholesterol esters,²⁹ the measurements will be of free cholesterol and cholesterol generated from its esters. By comparison, the method outlined here provides a much more rapid means to analyse samples for evaluating only free cholesterol without derivatisation. Ruiz *et al.*²⁹ determined the cholesterol concentration by comparing the silylated cholesterol to 5- α -cholestane by peak-area ratio and found a mean cholesterol concentration of 146 ± 58 ppm in human tears and 59 ± 30 ppm in rabbit tears. Whilst the results of the current study agree with their findings that human tears contain a higher level of cholesterol content than rabbit tears, the actual concentrations do not agree. This may be because they measured free cholesterol and cholesterol hydrolysed from its esters during extraction and/or derivatization procedures. As has recently been shown, cholesterol esters are a major component of the tear and meibum lipidomes^{30,31} and the concentration of cholesterol esters in tears is significantly higher than free cholesterol¹¹. Given these complex matrix effects, understanding and rigorously excluding contributions from cholesterol esters to measurement of free cholesterol, as shown here, is essential for reliable quantification in tears.

Human and rabbit tear films differ markedly in their relative stability,⁴ with an inter-blink time of several minutes for rabbit compared to about 10 seconds for human.⁵⁻⁷ The factors which contribute to the stability of the rabbit tear film are not well understood and an understanding of this may lead to the development of novel therapeutic interventions for human diseases resulting from tear film instability. Although cholesterol is of relatively low abundance in meibomian gland secretions, cholesterol has been associated with the tear film instability in several eye diseases.¹⁵⁻¹⁷ This study demonstrated that cholesterol levels were lower in the tear film of rabbits compared to humans. The implications of the difference need further study.

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Figure 1: Ions occurring in the range m/z 345-400 for the full scan (m/z 40-600) DI/EI-MS analysis of (a) standard cholesterol, (b) standard D_6 -cholesterol, (c) a crude lipid extract from a human tear sample and (d) standard cholesteryl oleate (CE 18:1).

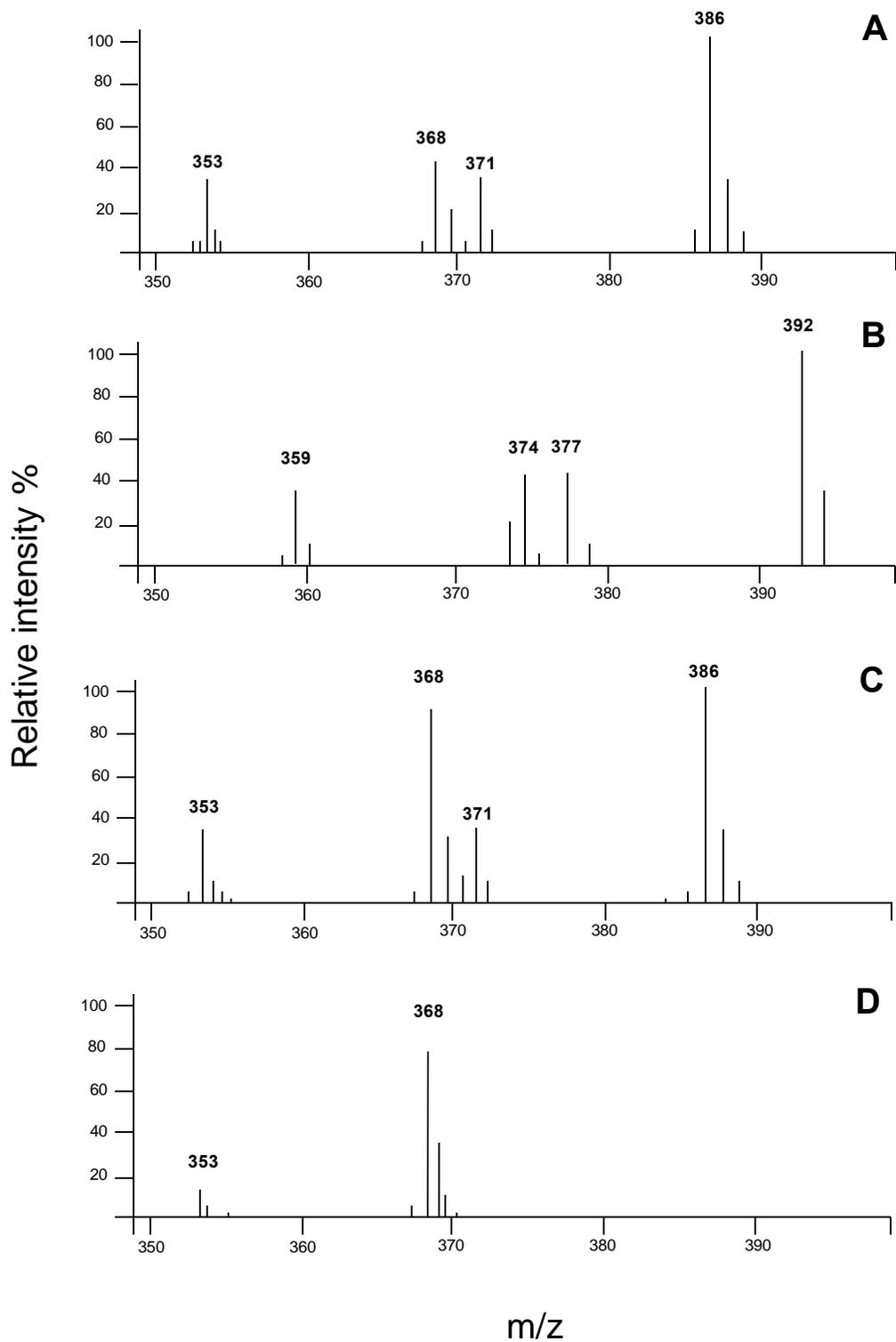


Figure 2. (a) DI/EI-SIM data for the analysis of a mixture of cholesterol and three representative cholesterol esters over the temperature range 40-250 °C. For clarity only the traces for m/z 386 (derived from cholesterol only) and m/z 368 and m/z 353 (derived from cholesterol and cholesterol esters) are shown to demonstrate the clear delineation between the two ion sources. Averaged mass-spectral DI/EI-SIM data over the region 1-1.2 min and 1.6-1.7 min are shown in (b) and (c), respectively. (d) DI/EI-SIM data for the analysis of a pooled tear sample extract over the temperature range 40-250 °C. For clarity only the traces for m/z 386 (derived from cholesterol only) and m/z 368 (derived from cholesterol and cholesterol esters) are shown.

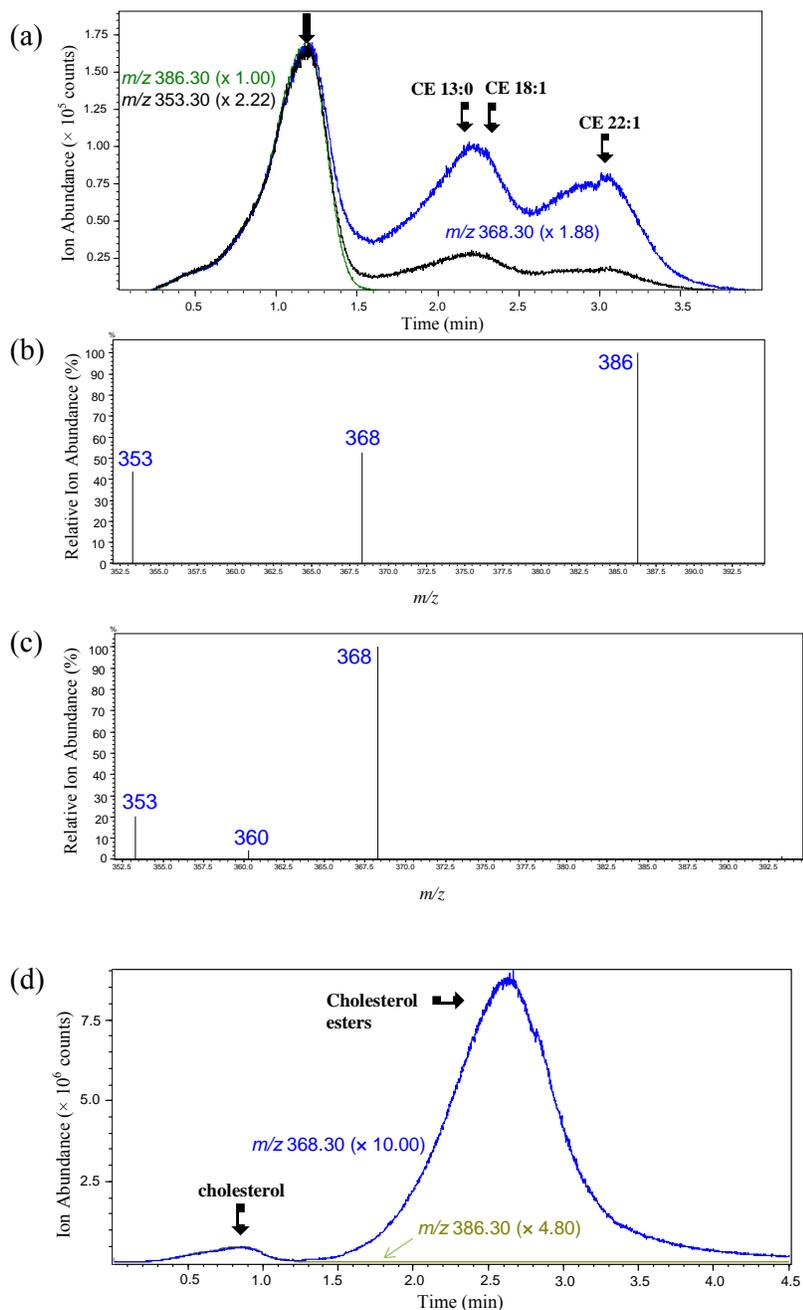


Figure 3. Averaged mass-spectral DI/EI-SIM data (50 scans) for a standard mixture of cholesterol and D_6 -cholesterol (1:2, w/w). Ions at m/z 386.4, 371.4, 368.4 and 353.4 represent the M^{+} , $[M-15]^{+}$, $[M-18]^{+}$ and $[M-33]^{+}$ diagnostic ions for cholesterol, respectively. Ions at m/z 392.4, 377.4, 374.4 and 359.4 (labelled with “*”) correspond to the deuterated analogues arising from D_6 -cholesterol.

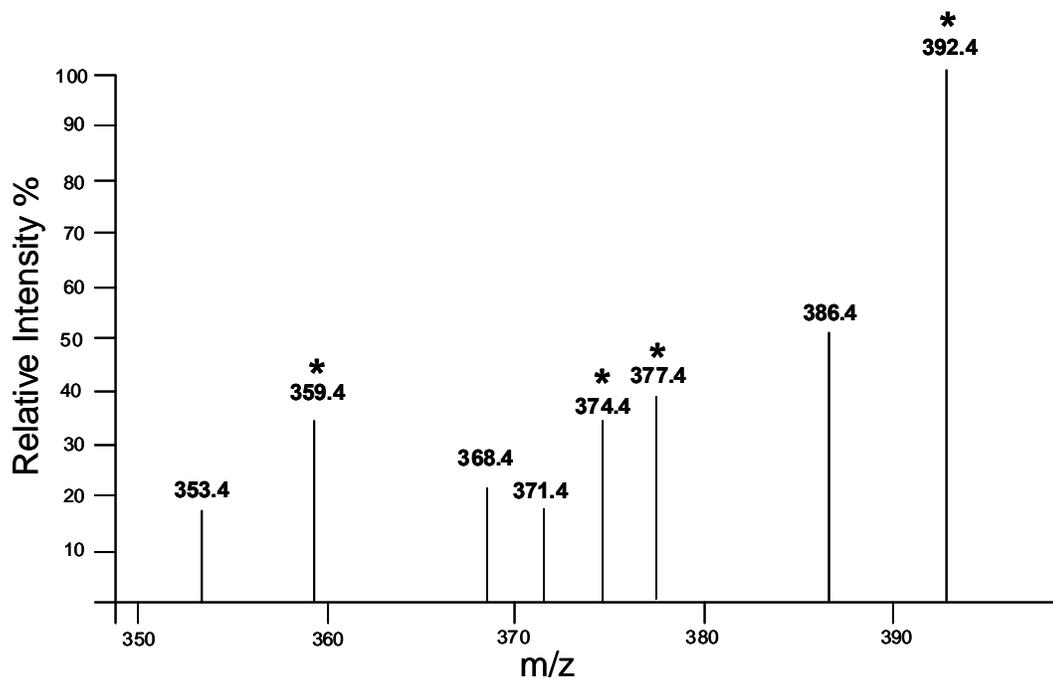


Figure 4. Comparison of four standard curves obtained by plotting the mass ratio from 0.1 to 3.0 vs. relative intensity of cholesterol compared to D₆-cholesterol (25 ng) for each pair of ions m/z 353.4/359.4, 371.4/377.4, 368.4/374.4 and 386.4/392.4.

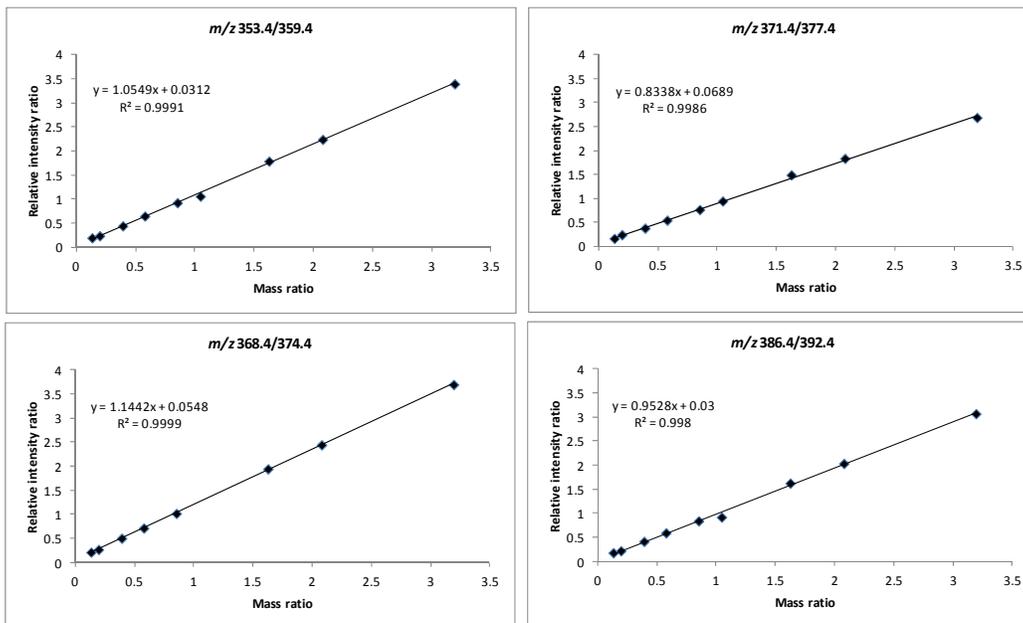


Figure 5. The concentration of free cholesterol in human (n = 15) and rabbit (n = 10) tears. The concentration of free cholesterol in human tears was significantly higher than that in rabbit tears ($p < 0.05$).

