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Characterisation of sphingolipids in the human lens by thin layer chromatography–desorption electrospray ionisation mass spectrometry

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ABSTRACT

The lipidome of the human lens is unique in that cholesterol and dihydrosphingomyelin are the dominant classes. Moreover, the lens lipidome is not static with dramatic changes in several sphingolipid classes associated with both aging and cataract. Accordingly, there is a clear need to expand knowledge of the molecular species that constitute the human lens sphingolipidome. In this study, human lens lipids have been extracted and separated by thin-layer chromatography (TLC). Direct analysis of the TLC plates by desorption electrospray ionisation–mass spectrometry (DESI–MS) allowed the detection over 30 species from 11 classes of sphingolipids. Significantly, novel classes of lens lipids including sulfatides, dihydrosulfatides, lactosylceramide sulfates and dihydrolactosylceramide sulfates were identified.

1. Introduction

The lipid profile of the human lens is unique compared to other animals [1], producing one of the most saturated and ordered cellular membranes anywhere in the human body [2]. Glycerophospholipids are of low abundance in the human lens [1], particularly in the nucleus where their levels decrease dramatically with age [3,4]. Moreover, most glycerophospholipid classes are dominated by plasmanyl species, i.e. lipids where one of the carbon chains is linked to the glycerol backbone via a saturated alkyl ether linkage [5]. By contrast cholesterol is found in high concentration in the human lens, with a ratio of up to 8:1 (cholesterol:phospholipid) in the centre of the nucleus [6]. Its concentration is so high in older lens nuclei that pure cholesterol bilayer domains have been observed [7].

Sphingolipids are another abundant lipid class present in the human lens and are dominated by the sphinganine backbone with either a 16:0 or a 24:1 fatty acyl chain [8–10]. Dihydrosphingomyelin (DHSM) is the dominant sphingolipid class comprising up to 47% of total phospholipid and 72% of sphingolipid in older lenses [1,3,11,12]. This is in contrast to other human tissues and fluids, e.g. human plasma, where DHSM accounts for less than 1% of total sphingolipids [13], and the lenses of other animals, e.g. rat, cow, pig and mouse lenses, where DHSM contributes less than 3% of the total phospholipid content [1]. Besides DHSM and sphingomyelin (SM), ceramide (Cer) and dihydroceramide (DHCer) have been detected in significant concentration in the nuclear region of older human lenses [4]. Ceramide-1-phosphate (CerP) and dihydroceramide-1-phosphate (DHCerP) have also recently been identified in the human lens [14,15]. The presence of glycosphingolipids in the lens was first reported in the mid 1960s [16]. To date, several glycosphingolipids classes have been identified in the human lens including dihydrolactosylceramide (DHLacCer) [8,14,17] and numerous gangliosides (GM1, GM2, GM3, GD1a and sialyl-Lewis x containing neolacto-series gangliosides) [8,18,19]; however, these represent a minor proportion of total human lens lipids; up to 5% [20,21]. Owing to the low abundance of these lipids the majority of this work has utilised extensive extraction and purification methods followed by thin layer chromatography (TLC) and visualisation using various reagents

Abbreviations: Cer, ceramide; CerP, ceramide-1-phosphate; DESI, desorption electrospray ionisation; DHCer, dihydroceramide; DHCerP, dihydroceramide-1-phosphate; DHLacCer, dihydrolactosylceramide; DHSM3, dihydrolactosylceramide sulfates; DHSM, dihydrosphingomyelin; SM3, lactosylceramide sulfates; MS, mass spectrometry; SL, sphingolipid; SM, sphingomyelin; ST, sulfatide; DHST, dihydrosulfatide; TLC, thin layer chromatography

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[17,22]. The acyl chain components of total glycosphingolipids or glycosphingolipid classes have been determined by gas–liquid chromatography following hydrolysis [8,19]. While these studies have provided valuable knowledge as to the composition of the human lens lipidome, such techniques require numerous preparative and analytical steps and do not allow direct molecular structure elucidation.

To provide molecular-level characterisation, lipids separated on TLC plates are routinely "scraped off" and extracted for further analysis by mass spectrometry (MS). This procedure is time-consuming, and the recovery of lipids is often poor [23]. Direct coupling of ambient ionisation mass spectrometry techniques with TLC however, has enabled the direct analysis of TLC plates without further sample preparation [24–26]. The direct analysis of TLC plates by desorption electrospray ionisation (DESI) mass spectrometry has proven particularly successful [27], and has been used for the analysis of natural products [28], peptides [29] and lipids [23,30]. Here we describe the use of TLC–DESI mass spectrometry to characterise the molecular composition of human lens sphingolipids. This approach is shown to be useful for the detection of a variety of lipid classes, including the detection and identification of several new classes of lens sphingolipids.

2. Materials and methods

2.1. Materials

All solvents used were HPLC grade and purchased from Crown Scientific (Moorebank, Australia). Primuline used for staining TLC plates was purchased from Sigma Aldrich (Castle Hill, Australia). Lipid standards were synthesised by Avanti Polar Lipids (Alabaster, USA) and purchased from Auspep (Parkville, Australia). MilliQ water (Millipore Synergy UV, Billerica, MA, USA) was used for the TLC mobile phase. Glass-backed normal phase TLC plates (Merck, Silica gel $60F_{254}$) were purchased from Crown Scientific (Sydney, Australia).

2.2. Lenses

Human lenses were obtained from eyes donated to the NSW Lions Eye Bank at the Sydney Eye Hospital, (Sydney, Australia) within 2–6 h of death, and were stored immediately at −80 °C until required. All work was approved by the human research ethics committees at the University of Sydney (#7292) and the University of Wollongong (HE 99/001).

2.3. Lipid extraction

Lens lipids were extracted as described previously [1]. Briefly, lenses were homogenised in methanol:chloroform (2:1 v/v) containing 0.01% butylated hydroxytoluene (BHT) at a ratio of 20:1 solvent to tissue. The homogenates were rotated overnight at 4 °C. Aqueous ammonium acetate (0.15 M) was added to the lens homogenates in a ratio of 8:4:3 (chloroform:methanol: 0.15 M ammonium acetate) and the tubes were centrifuged at 2000 *g* for 5 min. The organic layer was retained and 2 mL of 2:1 (*v*/*v*) chloroform:methanol was added to the aqueous layer and the procedure was repeated. The organic extracts were combined and 1 mL of aqueous ammonium acetate was added. The mixture was homogenised, the top (aqueous) layer was discarded, the remaining organic layer was evaporated to dryness under a stream of nitrogen and the samples were then reconstituted in 500–1000 μL of methanol: chloroform (2:1 *v*/*v*) containing 0.01% *w*/*v* BHT.

2.4. Thin layer chromatography

For TLC analysis, approximately 20 μL of the lipid extract (~1 mM) was spotted drop-wise onto a TLC plate using a micro-spotter. The plate was then left to dry in the fumehood and then developed in 75:25:2.5 chloroform:methanol:water (*v*/*v*/*v*). Approximately 20 μL of a 0.5 mM mixture of SL standards containing equimolar amounts of DHSM, DHCerP, sulfatide (ST), DHLacCer, glucosylceramide, Cer and DHCer was also spotted onto a normal phase, glass-backed TLC plate. TLC plates were stained with 0.05% (*w*/*v*) primuline in 4:1 acetone to MilliQ water and visualised under 365 nm UV light. The digital images of the stained plates were converted to black and white for greater contrast and to enhance visualisation of faint spots. The retention factor (R_f) values were calculated for each spot on both the standard and extract plates (where the R_f value is the distance between the spot and the baseline dived by the distance between the solvent front and the baseline). Separation was limited to approximately 35 mm owing to the travel constraints of the DESI stage.

2.5. Desorption electrospray ionisation–mass spectrometry (DESI–MS)

Positive and negative ion DESI–MS spectra were acquired directly from the TLC plates using a 2-dimensional (2-D) OmniSpray® ion source (Prosolia Inc., Indianapolis, USA) coupled to a linear ion trap mass spectrometer (LTQ, Thermo Fisher Scientific, San Jose, USA). Xcalibr Tune Plus 2.0 (Thermo Fisher Scientific, San Jose, USA) was used for spectral acquisition. Methanol was used as the spray solvent with typical conditions for TLC–DESI–MS experiments based on the work of Cooks et al. [31] as listed in Table 1.

3. Results

3.1. Thin layer chromatography

A TLC plate loaded with the lipid standard mix (see above) is shown in Fig. 1 A and a TLC plate loaded with approximately 20 nmol of lipid extract from a 20-year-old human lens is shown in Fig. 1 B. DHSM had the lowest R_f, owing to the high polarity imbued by the fixed charge on the choline head group. CerP and ST also had low R_f values owing to the presence of a phosphate or a sulfate group, respectively. It was observed that R_f values were inversely proportional to the number of sugar groups, i.e. DHLacCer (2 sugar groups) < GlcCer (1 sugar group) < Cer (no sugar groups). DHCer and Cer are relatively less polar as they do not contain any sugar moiety or fixed charge; thus, they travelled the furthest along the TLC plate. To further confirm these assignments and to identify individual molecular species the TLC plate was analysed by DESI–MS and DESI–MS/MS utilising collision-induced dissociation (CID).

3.2. Sphingomyelin (SM) and dihydrosphingomyelin (DHSM)

Due to the very low abundance of glycosphingolipids in the lens, a relatively concentrated lipid extract (~1 mM) was spotted onto a TLC plate for separation prior to detection by DESI–MS. The DESI source

Table 1
Optimised parameters used for DESI-.

Instrumental parameter	Setting	
	Positive	Negative
Solvent flow (μL/min)	15.0	15.0
Nebulising gas flow rate (psi)	80	80
Spray voltage (kV)	5.0	−5.0
Tube lens voltage (V)	110.0	−110.0
Capillary voltage (V)	5.0	−5.0
Capillary temperature (°C)	275.0	275.0
Ion injection time (ms)	500.0	100.0
Sample holder velocity (μm/s)	250.0	250.0
Microscans	3	1
Acquisition time (s)	1.02	1.02
CID collision energy (arbitrary units)	25–30	25–30
CID isolation width (Da)	1.5–3.0	1.5–3.0
Incident emitter-surface angle	55°	55°
Collection angle (sample-inlet angle)	~10°	~10°
Short emitter sample distance	1–2 mm	1–2 mm

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