Identification of Fatty Acids and Fatty Acid Amides in Human Meibomian Gland Secretions

Kelly K. Nichols,1 Bryan M. Ham,1 Jason J. Nichols,1 Corrie Ziegler,1 and Kari B. Green-Church2

PURPOSE. The complex superficial lipid layer of the tear film functions to prevent evaporation and maintain tear stability. Although classes of lipids found in the tear film have been reported, individual lipid species are currently being studied with more sophisticated methods. The purpose of this work was to show the identification of fatty acids and the fatty acid amides in human meibomian gland secretions by using electrospray mass spectrometry.

METHODS. Human meibomian gland secretions (meibum) were analyzed by electrospray quadrupole time-of-flight mass spectrometry (positive- and negative-ion mode). Accurate mass determination and collision-induced dissociation of meibum, and lipid standards were used to identify lipid species.

RESULTS. Mass analysis of meibum in an acidic chloroform-methanol solution in positive-ion mode revealed a mass peak of m/z 282.3, which was identified as the protonated molecule of oleamide [C16H31N2O+H]+. The high-resolution mass analysis of the m/z 282.2788 peak (oleamide) demonstrated a mass accuracy of 3.2 parts per million (ppm). Collision-induced dissociation of this species from meibum, compared with an oleamide standard, confirmed its identification. Myristic, palmitic, stearic, and oleic free fatty acids were identified in a similar manner, as were the other fatty acid amides (myristamide, palmitamide, stearamide, and erucamide).

CONCLUSIONS. The findings indicate that oleamide (cis-9-octadecenamide), an endogenous fatty acid primary amide, is a predominant component of meibum when examined by electrospray mass spectrometry. The novel finding of oleamide and other members of the fatty acid amide family in the tear film could lead to additional insights into the role of fatty acid amide activity in human biological systems and may indicate a new function for this lipid class of molecules in ocular surface signaling and/or in the maintenance of the complex tear film.

In Investigative Ophthalmology & Visual Science. 2007;48:34–39 DOI:10.1167/ iovs.06-0753

The human tear film is an intricate mixture of components that function to protect and maintain the surface of the eye and to provide a smooth refractive interface. The outermost layer of the tear film comprises lipids secreted by the meibomian glands of the eyelid (meibum). This complex mixture of triglycerides, free fatty acids, diesters, cholesterol and wax esters, free cholesterol, hydrocarbons, and polar lipids functions to prevent evaporation and assist in the maintenance of a stable tear film. Compositional deficiencies of the tear film in the protection of the ocular surface may result in numerous disease states, including dry eye.

The analysis of lipid composition and alterations in structure or content has become the field of lipidomics and has been facilitated by the advent of electrospray mass spectrometry (ESI MS). Endogenous fatty acid amides, such as oleamide (cis-9-octadecenamide), were first described in the human biological system in plasma. Oleamide has since been characterized as a signaling molecule involved in sleep, a gap junction inhibitor, and a cannabinoid-like pain modulator. Pathways relating oleamide and oleic acid in other biological systems have been proposed, and oleic acid has been shown to play a role in ocular surface disease. The chemical structures of oleamide and the additional fatty acid amides (standards) can be seen in Figure 1.

The purpose of this investigation was to show the identification of oleamide in meibomian gland secretions, to evaluate and confirm the presence of free fatty acid constituents, and to determine the presence of additional fatty acid amides by using electrospray mass spectrometry.

MATERIALS AND METHODS

Collection of Samples

The lipid collection and analysis protocol was approved by the Institutional Review Board at The Ohio State University in accordance with the Declaration of Helsinki. All human subjects completed informed consent documents before the samples were collected. For the purposes of method development, identification, and confirmation, 16 normal individuals consented to provide lipid samples (one per eye on up to four different occasions). Each participant underwent standard symptom and slit lamp biomicroscopic screening to ensure normal ocular surface health. Participants were asymptomatic, non-contact lens wearers with no current ocular disease. Samples were collected within the same window of time (late morning to early afternoon) from all individuals.

Meibomian lipid was collected with a 0.5-μL Drummond glass micropipette with 16× slit-lamp magnification after manual expression of the inferior meibomian glands. Approximately 0.005 μL of meibum was collected per subject (no less than 1-mm length in a 32-mm long 0.5-μL micropipette). In the process of method development, this volume was easily obtainable in a normal patient and did not require pooling for adequate mass spectrometric analysis. The collected lipid in the micropipette tube was placed in a 1.6-ML amber Eppendorf tube and refrigerated until analysis, according to standard procedure.

Chemicals

Methanol and chloroform were HPLC/spectroscopy grade and purchased from EMD (Gibbstown, NJ) and ACROS (Geel, Belgium), re-

From the 1College of Optometry and the 2Mass Spectrometry and Proteomics Facility, The Ohio State University, Columbus, Ohio.

Submitted for publication July 4, 2006; revised August 11, 2006; accepted November 13, 2006.

Disclosure: K.K. Nichols, None; B.M. Ham, None; J.J. Nichols, None; C. Ziegler, None; K.B. Green-Church, None

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Corresponding author: Kelly K. Nichols, College of Optometry, The Ohio State University, Columbus, OH 43210; nichols.214@osu.edu.
spectively. All standards were analytical-reagent grade, purchased from Sigma-Aldrich (St. Louis, MO).

Preparation of Samples
For positive-mode analysis, samples of meibum were directly prepared in 100 μL of 1:1 chloroform/methanol acidified to 1% acetic acid. For negative-mode analysis, samples of meibum were prepared directly in 100 μL of 1:1 chloroform/methanol containing 1 mM ammonium acetate. Samples were analyzed directly with no further sample purification or preparation.

Electrospray Ionization Time-of-Flight Tandem Mass Spectrometry
The calibration range for all the samples analyzed was m/z 100 to 1500 (ESI-Q-TOF II; Micromass, Wythenshawe, UK). Electrospray conditions were as follows: capillary voltage was 3000 V, source temperature was 100°C, and the cone voltage was 30 V. Q1 was set for optimal passage of ions from m/z 75 to 2000, and all ions transmitted into the pusher region of the time-of-flight (TOF) analyzer were scanned over m/z 100 to 1000 with a 1-second integration time. Data were acquired in continuum mode until acceptable averaged data were obtained. Collision-induced dissociation was acquired by setting Q1 to pass specific m/z peaks, and the collision energy was set to 19 V. Mass accuracy in parts per million (ppm), was calculated in the identification process as follows: 

$$\frac{[(\text{observed high-resolution mass (m/z)} - \text{theoretical mass (m/z)})/\text{theoretical mass}] \times 10^6}{\text{ppm}}$$

RESULTS
A representative Q-TOF mass analysis of meibum in positive-ion mode is shown in Figure 2. Based on total ion current, the most predominant peak at m/z 282.3 corresponds to the protonated fatty acid amide oleamide. In addition to oleamide, the following fatty acid amides were also observed and identified in positive-ion mode as protonated species, although at relatively low intensities relative to oleamide (Fig. 3): myristamide (m/z 228.3), palmitamide (m/z 256.3), linoleamide (m/z 280.3), stearamid (m/z 284.3), and erucamide (m/z 338.2). The chemical structures of oleamide and the additional fatty acid amides (Fig. 1) were confirmed in meibum through accurate mass determination and collision-induced dissociation.

Theoretical and observed exact mass measurements (Table 1) of the fatty acid amides show that m/z 282.2788 (protonated oleamide) demonstrated a mass accuracy of 3.2 ppm for the elemental composition of C18H35NO. NaI clusters were used as an internal standard (lock mass for the TOF) to obtain the accurate mass for the peak observed at m/z 282.3 within 3 ppm of the theoretical molecular weight of oleamide (C18H35NO) confirming the molecular formula of oleamide observed in the meibum. The m/z 228.2344 peak (protonated myristamide) showed an error of 7.4 ppm, the m/z 256.2651 peak (protonated palmitamide) showed an error of 4.2 ppm, and m/z 338.3414 (protonated erucamide) showed error of 2.6 ppm. The exact mass measurement for peak m/z 284.2954 was as follows: capillary voltage was 3000 V, source temperature was 100°C, and the cone voltage was 30 V. Q1 was set for optimal passage of ions from m/z 75 to 2000, and all ions transmitted into the pusher region of the time-of-flight (TOF) analyzer were scanned over m/z 100 to 1000 with a 1-second integration time. Data were acquired in continuum mode until acceptable averaged data were obtained. Collision-induced dissociation was acquired by setting Q1 to pass specific m/z peaks, and the collision energy was set to 19 V. Mass accuracy in parts per million (ppm), was calculated in the identification process as follows: 

$$\frac{[(\text{observed high-resolution mass (m/z)} - \text{theoretical mass (m/z)})/\text{theoretical mass}] \times 10^6}{\text{ppm}}$$

![Figure 1. Chemical structures of fatty acid amides.](image)

![Figure 2. A representative positive-ion ESI-Q-TOF mass spectrum of human meibum dissolved in 1:1 chloroform/methanol and acidified with acetic acid. The most abundant peak observed was oleamide (282.3).](image)
(protonated stearamide) was determined at 1 ppm; however, the peak corresponding to stearamide showed very low abundance.

The collision-induced product ion mass spectrum of the oleamide standard observed at m/z 282.3 compared with the peak observed at m/z 282.3 in acidified meibum (Fig. 4) demonstrated the structure of the protonated molecule, [C₁₈H₃₅NO+H]+ at m/z 282.3 to be identical, further confirming the presence of oleamide in meibum. The fragmentation pattern is consistent with a carbon–carbon double bond. In contrast, the positive-ion mode ESI MS/MS of palmitamide in the meibum (Fig. 5) showed a product ion spectrum consistent with a single-saturation carbon chain. Comparison of the MS/MS for the five identified fatty acid amides is shown in Figure 6. Although the m/z peak of 280.27 is consistent with the fatty acid amide linoleamide, we were unable to perform exact mass determination or collision-induced dissociation on the peak because of its very low abundance in meibum with the methods used.

The expected m/z for the protonated molecule of oleic acid would be m/z 283.5, and the carbon 13 peak observed with high-resolution mass spectrometry for oleamide is also expected to be found at m/z 283.3. To examine whether the peak at m/z 283.3 is fully the C₁₃ of oleamide or partially from the protonated form of oleic acid, we compared the theoretical isotope ratio of the oleamide carbon 12:carbon 13 peak to the measured peak in meibum and with oleamide standard. C₁₃ has a natural abundance of 1.1% ± 0.02% and as oleamide contains 18 carbons in the molecular formula, the theoretical isotope ratio for the oleamide carbon 12:carbon 13 peak should be 19.8% ± 0.4%. The percent C¹²/C¹³ ratio of the oleamide standard was measured at 19.4%, within the expected range of the theoretical calculated ratio. Table 2 summarizes the C¹²/C¹³ results of the oleamide observed in the meibum. The percent ratio of the C¹²/C¹³ peak of oleamide in meibum was measured four separate times, and it averaged 22.5%, which is slightly higher than predicted and observed with the standard. This indicates that oleic acid is probably present in the m/z 283 peak from meibum measured in positive-ion mode, but accounts for approximately only 2% to 3% of the m/z peak 283.3.

Negative ion mode electrospray mass spectrometry, which is more conducive to fatty acid analysis, confirmed the presence of oleic acid in meibum, and the major peaks (Fig. 7) included the deprotonated molecules [M−H]⁻ of myristic acid at m/z 255.2, palmitic acid at m/z 255.2, stearic acid at m/z 283.3, and oleic acid at m/z 281.3 (which appeared to be a very minor peak). Accurate mass, theoretical mass, calculated error, and molecular formula of the fatty acids and fatty acid amides in the meibum can

**Table 1. Accurate Mass Determination and Identification of Fatty Acids and Fatty Acid Amides in Meibum**

<table>
<thead>
<tr>
<th>High-Resolution Mass (m/z)</th>
<th>Theoretical Mass (m/z)</th>
<th>Calculated Error (ppm)</th>
<th>Molecular Ion</th>
<th>Molecular Formula</th>
<th>Identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>228.2344</td>
<td>228.2327</td>
<td>7.4</td>
<td>[M+H]+</td>
<td>[C₁₈H₃₅NO+H]+</td>
<td>Myristamide</td>
</tr>
<tr>
<td>256.2651</td>
<td>256.2640</td>
<td>4.2</td>
<td>[M+H]+</td>
<td>[C₁₆H₃₁NO+H]+</td>
<td>Palmitamide</td>
</tr>
<tr>
<td>282.2788</td>
<td>282.2797</td>
<td>3.2</td>
<td>[M+H]+</td>
<td>[C₁₄H₂₇NO+H]+</td>
<td>Oleamide</td>
</tr>
<tr>
<td>284.2954</td>
<td>284.2953</td>
<td>1</td>
<td>[M+H]+</td>
<td>[C₁₂H₂₃NO+H]+</td>
<td>Stearamide</td>
</tr>
<tr>
<td>338.3414</td>
<td>338.3423</td>
<td>2.6</td>
<td>[M+H]+</td>
<td>[C₁₀H₁₉NO+H]+</td>
<td>Erucamide</td>
</tr>
<tr>
<td>227.2043</td>
<td>227.2011</td>
<td>1.1</td>
<td>[M−H]−</td>
<td>[C₁₈H₃₅O₂−H]−</td>
<td>Myristic acid</td>
</tr>
<tr>
<td>255.2327</td>
<td>255.2324</td>
<td>1.2</td>
<td>[M−H]−</td>
<td>[C₁₆H₃₁O₂−H]−</td>
<td>Palmitic acid</td>
</tr>
<tr>
<td>281.2494</td>
<td>281.2481</td>
<td>4.6</td>
<td>[M−H]−</td>
<td>[C₁₄H₂₇O₂−H]−</td>
<td>Oleic acid</td>
</tr>
<tr>
<td>283.2628</td>
<td>283.2637</td>
<td>−3.2</td>
<td>[M−H]−</td>
<td>[C₁₂H₂₅O₂−H]−</td>
<td>Stearic acid</td>
</tr>
</tbody>
</table>
be seen in Table 2. As with linoleamide, the identification of erucic and linoleic acid was not confirmed due to large mass errors of low-abundance peaks, although \( m/z \) peaks within the appropriate regions were present.

**FIGURE 4.** Positive-ion mode ESI MS/MS of \( m/z \) 282.3 of (A) oleamide in the meibum and (B) oleamide standard. Analysis of the product ion spectrum of the oleamide standard and meibum appear identical and demonstrate a fragmentation pattern consistent with a carbon–carbon double bond.

**DISCUSSION**

By using both accurate mass analysis to confirm the molecular formula and MS/MS analysis to confirm the structure, the pres-
ence of oleamide and other fatty acid amides was confirmed in
the meibum. Oleamide was consistently seen as the predomi-
nant lipid in meibum mass spectra in positive-ion mode. This
novel finding creates an interesting backdrop for consideration
of the function of the complex lipid components in human tear
meibum. The lipid layer is critical in promoting tear film sta-
bility and in assisting in the maintenance of a smooth refractive
surface of the eye; however, recent research on the role of
oleamide in biological systems provides a potential role for
oleamide (or the oleamide:oleic acid ratio) in diseases of the
ocular surface, such as dry eye syndrome. The fatty acid amides
are often referred to as a class of signaling lipids that modulate
neurobehavioral processes in mammals including pain, sleep,
feeding, and locomotor activity. Recent reports of the poten-
tial role in neurotransmitter and gap junction regulation in cell
communication, and the cannabinoid-like activity of oleamide
in pain modulation are remarkable, in that the upregula-
tion or downregulation of oleamide could play a part in epi-
thelial wound healing through gap junction communication or
may play a role in the symptomatology associated with ocular
surface disease. This is particularly true of oleamide, compared
with the other fatty acids amides, as oleamide is clearly of
substantially higher abundance in the meibum than the others.

The advent and acceptance of electrospray mass spectrom-
ometry over the past 15 years has had a significant impact on
research in areas in which the analysis of small-volume, com-
plex mixtures is needed, such as the analysis of tear film lipids.
With the use of techniques such as thin layer chromatography,
it has been reported that the contribution of fatty acids to the
overall fraction of human meibum is minimal (2%). The use of
electrospray mass spectrometry has confirmed the pres-
ence of oleic acid in addition to other fatty acids. The contin-
ued development of mass spectrometry techniques used to
analyze meibum and tears will aid in the characterization and
quantification of individual lipid species and classes of lipid in
the human tear film.

Although our findings confirm four corresponding fatty acid
and amide pairs in the meibum, it is unclear whether the
pathway between the acid and amide is linked, or whether the
ratio of acid to amide is important in the disease process.
Pathways relating oleamide and oleic acid through precursor
molecules, such as sphingomyelin have been proposed, and oleic
acid has been shown to be downregulated in patients
with meibomianitis and upregulated in patients with meibo-
mian seborrhea. The greater relative quantity of oleamide based on total ion
current supports the likelihood of a unique role for oleamide in
the tear film, but does not rule out the importance of a less
abundant lipid species. Work to quantify amounts of the spe-
cific lipid species such as oleamide, oleic acid, and other fatty

| Table 2. Isotopic Peak Evaluation for Oleamide Standard and Oleamide in Meibum |
|-------------------|-----|-----|-----|
| Sample            | C12 Intensity | C13 Intensity | C12:C13% |
| Oleamide standard | 234  | 45   | 19.4 |
| Meibum sample 1   | 1340 | 506  | 22.8 |
| Meibum sample 2   | 1860 | 414  | 22.3 |
| Meibum sample 3   | 4910 | 1120 | 22.8 |
| Meibum sample 4   | 1600 | 347  | 21.7 |
| Average (samples 1–4) | 2428 | 547  | 22.5 |
acids and amides is ongoing. The quantification of meibum in tears may shed light on variations of lipid in the normal human population, as well as in groups of patients with ocular surface disease. Determination of lipid biomarkers may aid in the development of tests specific for ocular surface disease diagnosis or monitoring, and in the development of treatments relating to ocular surface disease. Further investigation of the interaction and association between fatty acid amides, fatty acids, and other lipid species is also warranted, both alone and in conjunction with tear film proteomics, to further knowledge in this complex area.

### References


**Figure 7.** Electrospray spectra of free fatty acids in meibum analyzed in negative-ion mode.