On the Presence of (O-acyl)-omega-hydroxy Fatty Acids and of Their Esters in Human Meibomian Gland Secretions

A recent exchange of opinions¹⁻³ on the nature and quantity of wax esters (WEs), free fatty acids (FFAs), (O-acyl)- ω -hydroxy FA (OAHFA), and steryl-containing derivatives of OAHFA (OAHFA-St) in human meibomian gland secretions (or meibum⁴) has undoubtedly contributed to a better understanding of the nature of this complex secretion. Each approach described in those publications has its strengths and weaknesses. As this topic is very technical, but may have important physiological implications, a brief methodological introduction seems to be warranted.

The shotgun mass spectrometry (SG-MS) approach^{1,3} is fast and can be performed on any stand-alone MS system. However, researchers who solely use SG-MS are losing the critical ability to characterize analytes in terms of their chromatographic retention times, a parameter that has been proven time and again to be an indispensable tool for characterization of complex mixtures. This problem becomes even more difficult to overcome with increases in the complexity of samples, both in terms of the number of various species present and in terms of the complexity of the molecular structures of individual analytes. Furthermore, the more complex the mixture, the higher the chances of the presence in it of so-called isobaric compounds (i.e., compounds with similar molecular weights; for example, a commonly observed ion m/z 663, discussed elsewhere⁵). This problem reaches its highest point with compounds of identical elemental composition, but different chemical structures (such as geometric or stereoisomers of the same compound). The inability to spatially separate them from one another before they enter the mass spectrometer and thus place them in their respective lipid classes makes their correct structural attribution much more difficult, if not impossible.

On the other hand, if the analytes are separated *before* being detected and analyzed, it becomes possible to attribute them to particular lipid classes and compare their retention times with authentic lipid standards, which dramatically increases the chances of their correct structural characterization by fragmentation analysis. This approach is implemented in two major forms: high-performance liquid chromatography-MS (HPLC-MS) and gas chromatography-MS. The former has been successfully used in our laboratory to analyze intact human (Butovich IA, et al. *IOVS* 2009;50:ARVO E-Abstract 2545)^{2,5-7} and animal (Butovich IA, et al. *IOVS* 2009;50:ARVO E-Abstract 2545; Butovich IA. *IOVS* 2010;51:ARVO E-Abstract 4154; Wojtowicz JC, et al. *IOVS* 2010;51:ARVO E-Abstract 4160; Eule JC, et al. *IOVS* 2010;51:ARVO E-Abstract 4159) meibum. However, the HPLC-MS approach has the disadvantage of being much more time consuming. It requires an additional instrument—an HPLC system—and related skills to design appropriate methods and run experiments and is more expensive to run than SG-MS, as there are additional costs involved (e.g., HPLC columns, solvents, longer experiments). The often-used argument that the physical amount of samples needed for SG-MS is substantially smaller than that needed for HPLC-MS is apparently not important, as to obtain quality structural data with SG-MS complex, the samples must be infused into the instrument at a sustained rate for a considerable time, whereas for HPLC-MS a small volume of (more concentrated) sample is injected once at the beginning of the experiment. In addition, the mass spectra of diluted samples obtained in SG-MS style experiments are prone to showing all the typical chemical noise (i.e., signals derived from

solvents), which pollutes the spectra and complicates the sample analysis. This, of course, can be overcome by using concentrated samples and extremely pure solvents to improve the signal-to-noise ratio or by other experimental approaches. However, these measures negate many of the expected advantages of SG-MS, including its cost effectiveness.

Thus, it appears that SG-MS and HPLC-MS are geared toward different tasks: SG-MS works better for relatively simple mixtures or targeted detection of selected compounds, for which the conditions of the analysis can be optimized and validated, whereas HPLC-MS is better suited for analyzing complex mixtures, especially those composed of a large number of unknown compounds and those that are unstable and prone to in-source fragmentation.⁷

The major question, however, remains whether the SG-MS approach, advocated by Chen et al.,^{1,3} can provide accurate information on the composition of human meibum, especially with regard to the simple-versus-complex lipid controversy.¹⁻³ It seems that the SG-MS approach, although successful in many ways, fails on several critical issues.

First, in a communication published in 2007,⁸ by the same group, large quantities of FFA and FA amides (FAAs) were reported. Importantly, Nichols et al.⁸ used SG-MS for analyzing very small samples of a complex nature. Later, the basic findings of Nichols et al. were disputed⁹ and were shown to be erroneous, primarily because of an extremely unfavorable signal-to-noise ratio in those SG-MS experiments, and heavy sample contamination with plasticizers (such as oleamide and other FAAs) that leached from the plastic ware that was used for sample storage and handling.^{5,10} Regrettably, these erroneous observations have not yet been corrected by Nichols et al.

In a second paper on the topic,¹ SG-MS led the researchers to believe that meibomian FFAs are present in concentrations approaching 3% (wt/wt) of meibum, whereas their actual concentration in meibum is a few orders of magnitude lower than that.² The culprit in this case was the inadvertent, spontaneous, in-source fragmentation of complex lipids such as the cholesteryl esters (CEs). Again, this issue was easily addressed in our HPLC-MS experiments in which true FFAs could be separated from more complex lipids *before* entering the mass spectrometer.^{2,7,11}

Finally, in the most recent note on this topic, Chen et al.³ argued that OAHFAs, originally described in our recent papers^{12,13} and ARVO presentations (Butovich IA, et al. *IOVS* 2009;50:ARVO E-Abstract 2545; Butovich IA. *IOVS* 2010;51:ARVO E-Abstract 4154; Wojtowicz JC, et al. *IOVS* 2010;51:ARVO E-Abstract 4160; Eule JC, et al. *IOVS* 2010;51:ARVO E-Abstract 4159), could be the *in situ*-generated fragments of other lipids (e.g., OAHFA-St). In fact, our previous¹² and current (Fig. 1) HPLC-MS experiments clearly demonstrated that this hypothesis is not true. It seems that Chen et al.³ overlooked the fact that the retention times of free OAHFA reported in our papers¹² were much shorter than those of even the shortest CE found in meibum.^{2,7,11} Thus, OAHFA-St could not be the MS precursors of these much more polar lipids. Importantly, OAHFA-St, whose existence was initially proposed by Nicolaides et al.,⁴ were recently evaluated by HPLC-MS in human, gorilla, and canine meibum (Butovich IA. *IOVS* 2010;51:ARVO E-Abstract 4154; Wojtowicz JC et al. *IOVS* 2010;51:ARVO E-Abstract 4160; Eule JC et al. *IOVS* 2010;51:ARVO E-Abstract 4159)² in our experiments and in the parallel SG-MS experiments by Chen et al.^{1,3} In concordance with our earlier reports, in our current HPLC-MS experiments, the pool of free OAHFAs (Fig. 1A, group 1) was spatially separated from the pool of related, but more hydrophobic compounds, most likely esterified (e)-OAHFA (Fig. 1A, group 2 of

FIGURE 1. Free OAHFA and their cholesteryl esters are two endogenously present groups of lipids in intact meibomian gland secretions. (A) Reversed-phase HPLC profile of meibomian lipids in the m/z range 700 to 800 (NIM). (B) The averaged mass spectra of HPLC peaks with retention times 8 to 15 minutes (*top spectrum*) and that of the peaks with retention times 26 to 35 minutes (*bottom spectrum*). (C) Reversed-phase HPLC profile of cholesteryl-containing meibomian lipids producing ion m/z 369 (PIM). (D) Extracted ion chromatograms of related OAHFA ions m/z 645, 673, 701, 729, 757, 785, and 813. Each of these ions eluted as two peaks with totally different retention times (RT). The fastest peaks were those of free OAHFAs. The slower peaks were those of more complex lipids, most likely e-OAHFA-containing compounds. None of the free OAHFAs coeluted with any of the cholesteryl-containing lipids (C), indicating that the former were not the in situ-generated fragmentation products of more complex lipids, but instead were the endogenous components of meibum. The slower moving peaks with RT above 25 minutes were attributed to cholesteryl esters of OAHFA. AA, peak areas. (Experimental conditions: Mass spectra were recorded on an LCQ Deca XP Max ion trap mass spectrometer [ThermoElectron, Waltham, MA] equipped with an APCI [atmospheric pressure chemical ionization] ion source. Reversed-phase HPLC was performed on a 2695 HPLC Separations Module chromatographic system [Waters, Milford, MA]. For further details, see our earlier publications.^{5-7,11,12,14,15}).

HPLC peaks). Anions of OAHFA were detected and monitored in negative ion mode MS experiments (NIM) as $(M-H)^-$ species (Fig. 1B). Note that the MS spectra of both pools of OAHFA are very similar. Fragmentation patterns of the OAHFA ions found in peak groups 1 and 2 were demonstrated to be similar.

Then, the cholesteryl-containing meibomian lipid species were detected and monitored in positive ion mode (PIM) experiments (Fig. 1C). The inherent instability of CE of all kinds under the conditions of the PIM experiments, which caused the CEs to spontaneously fragment and produce ions of dehydrated cholesterol m/z 369 [$(CE - \text{fatty acid residue} + H)^+$], was in this case beneficial to the goals of this experiment, as it allowed us to simultaneously observe a range of CE. From Figure 1, it is clear that most of the detected e-OAHFAs have retention times longer than those of regular CEs, as these groups of lipids barely overlap on the chromatograms: The shortest e-OAHFA (signal m/z 645) has a retention time of 23 minutes, whereas the longest CE (C_{32} -CE) also has a retention time of 23 minutes. Note that in accordance with the results described earlier, the retention times of some of the more complex and more hydrophobic cholesterol-containing compounds (such as OAHFA-St) were beyond 25 minutes, being longer than those of typical C_{18} - to C_{32} -CE, whose retention times were between 10 and 25 minutes (Figs. 1C, 1D).

Revealingly, in many respects, both OAHFA and e-OAHFA (including OAHFA-St) are present in both human and canine

samples. Their structures, fragmentation, and HPLC behavior are described in detail elsewhere (Butovich IA. *IOVS* 2010;51:ARVO E-Abstract 4154; Eule JC, et al. *IOVS* 2010;51:ARVO E-Abstract 4159). When the corresponding HPLC peaks were plotted and integrated (Fig. 1D), an interesting pattern was observed: The ratio of OAHFA to e-OAHFA depended on the molecular mass of the lipid. The calculated abundance ratios for OAHFA/e-OAHFA ions m/z 645, 673, 701, 729, 757, 785, and 813 were approximately 4.5, 2.9, 1.0, 0.9, 0.8, 0.4, and 0.2, correspondingly.

These results, taken together with those reported in our previous publications, unambiguously demonstrate that nonesterified OAHFAs are indeed the major group of amphiphilic anionogenic lipids endogenously present in meibum of humans and other mammals. Also, they provided strong evidence that OAHFAs, and their corresponding esterified counterparts, are present in comparable (but variable) quantities. The molecular weight-dependent distribution of the OAHFA/e-OAHFA ratios is of interest, as it implies certain selectivity in the enzymatic pathways involved in their biosynthesis and/or degradation.

Thus, unequivocal answers to the questions of whether the origin of the OAHFA signals and the relative amounts of OAHFA and e-OAHFA/OAHFA-St in meibomian lipids can be determined in SG-MS experiments, as described by Chen et al.,^{1,3} and HPLC-MS experiments, as described in our publications, are, correspondingly, "No" and "Yes." Further advances in this area

would require quantitation of OAHFAs and e-OAHFA/OAHFA-St, using their authentic standards.

It is also worth mentioning that the MS signals (m/z 729, 757, 785, and so forth) that were later attributed to OAHFAs, as well as their chromatographic behavior and fragmentation patterns, had originally been revealed in 2007 in our two papers on the topic.^{14,15} Their structures remained unknown and/or unverified until we synthesized an authentic standard of a model OAHFA—namely, (O-oleoyl)-16-hydroxypalmitic acid—and studied its MS fragmentation in 2009.¹² Nevertheless, in 2007, based on the then-established facts that these anionogenic compounds were more polar than nearly all the meibomian lipids, we proposed that these lipids could be a stabilizing bridge between the aqueous layer of the tear film and its lipid layer.¹⁵ Later, this idea was further substantiated and illustrated in more detail.¹³

The last question that should be addressed is the relative amounts of $C_{16:1}$ - and $C_{18:1}$ -based WEs present in human meibum. It is undeniable that $C_{16:1}$ -based WEs are present in human meibum. However, HPLC-MS/MS demonstrated that there were only two isobaric groups of WEs in which palmitoleic acid dominated—namely, those with m/z values of 591 and m/z 605—for which the $C_{16:1}/C_{18:1}$ FA signal ratios were 2.5 and 1.4, correspondingly. Both of these WEs are relatively minor ones in the meibomian WE family.¹² The rest of the major isobaric monounsaturated pairs of WEs m/z 563, 577, 619, 633, 647, and 661 produced the following $C_{16:1}/C_{18:1}$ signal ratios: 0.2, 0.1, 0.3, 0.1, 0.05, and 0.1, respectively. Considering that the chemical and physicochemical properties of the isobaric pairs of WEs are very similar (if not identical), the chances are that their roles in meibum and tear film will be found to be similar, too.

Finally, the complex nature of meibum warrants the continuation of its evaluation, but because of the advancements in the analytical sciences, the challenges that we are facing no longer seem to be insurmountable. However, the complexity of the subject requires a methodical approach that provides maximum information, which at this time is better served by HPLC-MS. “Horses for courses,” indeed.

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