

Quantitative evaluation of sebum lipid components with nuclear magnetic resonance

Lora C. Robosky, Kimberly Wade, Dayna Woolson, John David Baker, Matthew L. Manning, Douglas A. Gage, and Michael D. Reily^{1,2}

Pfizer Global Research and Development, Michigan Laboratories, Ann Arbor, MI 48105

Abstract A NMR spectroscopic method is described that enables the quantitation of specific lipid classes and components, independent of fatty acid composition. We demonstrate this method for measuring cholesterol, squalene, and pools of sterol esters, wax esters (WEs), and triglyceride (TG) components in sebum and meibum. When 600 MHz NMR equipment is used in conjunction with highly sensitive cryogenically cooled probes, this method has adequate sensitivity, and for some applications, advantages over commonly used HPLC-evaporative light-scattering detection and mass spectrometry-based approaches. This method is shown to be useful for preclinical and clinical monitoring of the efficacy of sebum-reducing agents in animals and humans. In Syrian hamsters, 3% topical flutamide and 20 mg/kg oral isotretinoin reduced sterol esters by 18.7% and 30.0%, respectively, and reduced WEs by 32.9% and 31.8%, respectively, as measured in a punch biopsy of the ear. In a 72 patient clinical methodology study, the assay delivered reproducible and noninvasive measurements of WEs, cholesteryl esters, TGs, and squalene from Sebutape[®] skin blots. The quantitative results of sebum analysis obtained by the NMR method correlate well with those obtained with HPLC-based approaches. **■** This approach may be broadly applicable to cases in which fatty acid-independent quantification of lipid classes is desired.—Robosky, L. C., K. Wade, D. Woolson, J. D. Baker, M. L. Manning, D. A. Gage, and M. D. Reily. **Quantitative evaluation of sebum lipid components with nuclear magnetic resonance.** *J. Lipid Res.* 2008. 49: 686–692.

Supplementary key words skin lipid • meibum lipid • high-performance liquid chromatography-evaporative light-scattering detection • human sebum • hamster sebum • lipid class measurement

Mammalian skin is composed of three primary layers: the stratum corneum, the epidermis, and the dermis. The outer layer of the skin, the stratum corneum, primarily functions as a barrier to the external environment, preventing water loss and the invasion of microorganisms. Sebum secreted to the stratum corneum from the sebaceous glands is a key component of the skin surface and has various demonstrated and postulated functions (1). Sebum itself is

a complex mixture of lipids and is produced by the sebaceous glands. At maturation, the acinar cells of the sebaceous glands lyse and release sebum into the luminal duct, from which the sebum is secreted. Cholesterol, sterol esters, wax esters (WEs), and triglycerides (TGs) are the primary lipids found in the sebum of many mammals, and squalene is also a major component of human sebum. In humans, the predominant sterol esters are cholesteryl esters (CEs), whereas in male Syrian hamster (a common sebum model), they are chiefly fatty acid esters of a single predominant noncholesterol sterol (2). The identity of the sterol constituting these esters in this species has not been identified, and herein we will refer to these as hamster sterol esters (HSEs). WEs are unique to sebum in that they are not synthesized by other cells in the body. Sebum composition can be modified from its native state by the action of xenobiotics, which can greatly increase the complexity of its biochemical makeup; for example, bacterial hydrolytic enzymes are known to break down TGs to produce free fatty acids (3).

A number of methods to isolate and qualitatively and/or quantitatively analyze the lipid components of sebum and/or meibum (the lipid-rich secretion along the lower eyelid) have been reported based on TLC (4, 5), HPLC-mass spectrometry (6), GC-MS (7), and infrared and Raman spectroscopies (8). These methods can be labor-intensive, often require large amounts of sample, and may not be suitable for high-throughput screening of samples. Because these sebum components have no chromophore, HPLC-based methods require detectors such as evaporative light-scattering (9, 10) or mass spectrometry. Additionally, methods that rely on HPLC separation require good resolution between lipid classes, which is often difficult to achieve. Detectors based on evaporative light-scattering are highly sensitive but have limited linear

Abbreviations: AA, arachidonic alcohol; CE, cholesteryl ester; ELSD, evaporative light-scattering detection; HSE, hamster sterol ester; TG, triglyceride; WE, wax ester.

¹ Present address of M. D. Reily: Bristol-Myers Squibb, MS L14-05, Route 206 and Province Line Road, Princeton, NJ 08543-4000.

² To whom correspondence should be addressed.

e-mail: michael.reily@bms.com

Manuscript received 31 October 2007 and in revised form 18 December 2007.

Published, JLR Papers in Press, December 19, 2007.

DOI 10.1194/jlr.D700035.JLR200

dynamic range and cannot distinguish desired analytes from coeluting components. Detection with mass spectrometry is very sensitive and selective, but the analysis is complex, because every possible fatty acid conjugate that is resolved chromatographically must be measured (potentially with separate internal standards) to accurately quantify total pools of the derived individual lipid classes.

In clinical settings, polymeric films have been used to noninvasively absorb sebum off the skin. The lipid-rich mixture then can be extracted and subjected to molecular analysis (4, 11) or quantified directly by visible light transmission through the polymeric film with a device such as a Sebumeter[®] (12, 13). The latter approach provides a rapid indication of total absorbed material in a clinical setting but cannot distinguish its composition.

To overcome some of the limitations of the aforementioned detection approaches, a method for assessing the molecular constitution of sebum based on NMR spectroscopy is described. The NMR method relies on accurate integration of specific protons on selected analytes (Fig. 1). We demonstrate the utility of this method as a biomarker assay for assessing the sebum-reducing efficacy of topical and oral agents in a preclinical hamster model and further demonstrate its applicability in human subjects.

METHODS

NMR data acquisition

For all experiments, proton NMR spectra were acquired with a Bruker Avance NMR spectrometer operating at 599.98 MHz for

proton using a 5 mm liquid helium-cooled $^1\text{H}\{^{13}\text{C}, ^{15}\text{N}\}$ probe (triple resonance cryoprobe) equipped with a z-axis gradient and an automatic sample changer with a capacity of 120 samples (BACS 120). The acquisition parameters common for all experiments included a 15 ppm spectral width and a 1.8 s acquisition time. Free induction decays represent the sum of 64 (hamster study) or 512 (human studies) transients acquired into 32,000 data points. All data were acquired with the sample maintained at 25°C. Selection of NMR data acquisition parameters for quantitative analysis was done according to previous studies (14). The exact resonance positions integrated for each lipid class are given below.

NMR data processing

Proton free induction decay data were processed using in-house software (Metabonomi; International Patent Application WO2004038602). Typically, the data were Fourier-transformed, phased, baseline-corrected, and standardized to 32,000 data points in the region from 10 to -0.5 ppm. Then, individual peaks of interest were integrated and the integrated areas were tabulated for subsequent analysis and graphical presentation. The peak areas were then normalized to an internal reference standard or related to a concentration by reference to NMR spectra of one or more authentic lipids at one or more concentrations. In some cases, standard curves for selected lipids were used for concentration calibration as detailed below. The sebum lipid components normally measured included TG, cholesterol, sterol ester, WE, and squalene.

HPLC-evaporative light-scattering detection analysis

Experiments were conducted on an Agilent 1100 HPLC system equipped with a temperature-controlled automatic sample injector, quaternary pump, column heater, and A/D interface module controlled by Agilent ChemStation[®] software. The de-

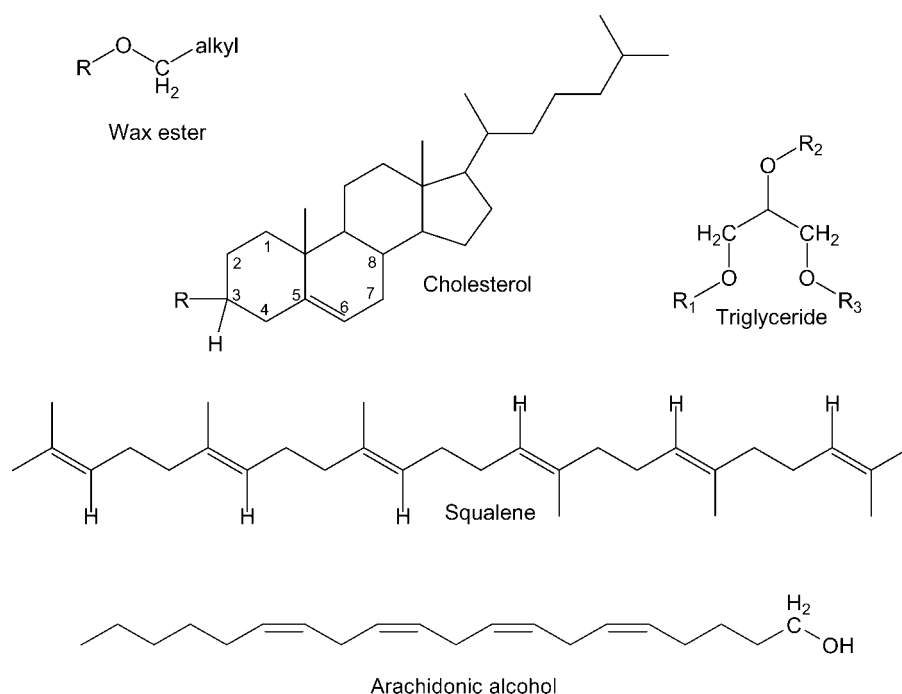


Fig. 1. Molecular structures of selected compounds discussed in the text. Displayed hydrogen atoms correspond to the protons measured in the NMR assay (see text). The labels R, R1, R2, and R3 represent any fatty acyl group.

detector was a Sedex 75 evaporative light-scattering detection (ELSD) apparatus with analog signal connected to the Agilent HPLC system through the A/D interface module. Vacuum-dried samples were reconstituted with 600 μ l of trimethylpentane, recapped, and vortexed for 5 min. Two hundred microliters of each sample was then transferred to a 2 ml HPLC vial with a 200 μ l glass insert, which was then loaded into the automatic sample injector maintained at a temperature of 20°C throughout the experiment. A Waters Spherisorb S3W 4.6 μ 100 mm analytical column was maintained at 30°C by an Agilent column heater unit. Ten microliters of each sample was injected into the column in triplicate for analysis. Two solvents were used for the mobile phase gradient (Table 1).

The ELSD system was operated at 45°C with a gain of 5 and an N₂ pressure of 3.1 bars. The analog signal obtained by the instrument was sent to the Agilent A/D interface module, where it was converted to a digital output. The conversion was based on a 10,000 mAU/V set point, and the data rate was set at 10 Hz (0.03 min). The resulting digital output was then fed into the Agilent ChemStation® software for integration of the peak areas.

Hamster sebum study

Male Syrian hamsters ~8–10 weeks old were housed in individual cages. The animals were acclimated to 16 h light cycles for 2 weeks before dosing. For each treatment, test animals were anesthetized using isoflurane gas. Each treatment group consisted of 10 animals. In one experiment, 20 μ l of 3% flutamide was applied topically to the ventral side of each ear using a positive displacement pipette. The animals were treated twice a day, 7 days a week, for 4 weeks with at least 6 h between treatments. The flutamide was prepared in 50:50 (v/v) ethanol-propylene glycol. Control animals were treated with 50:50 (v/v) ethanol-propylene glycol. In a separate experiment, animals were treated with an oral dose of 20 mg/kg isotretinoin (marketed under the trade name Accutane®), prepared as a suspension in propylene glycol. The animals were treated twice a day, 7 days a week, for 4 weeks with at least 6 h between treatments. Control animals were treated with propylene glycol alone.

At the end of the treatment period, one 8 mm diameter distal biopsy punch was taken, just above the anatomical V mark in the ear, to normalize the sample area. The punch was pulled apart. The ventral biopsy surface (the area where the topical dose was applied directly to the sebaceous glands) was retained for testing, and the dorsal surface of the biopsy punch was discarded. Tissue samples were dried using nitrogen gas and stored at -80°C under nitrogen until analysis.

Before NMR analysis, the tissue samples were removed from the freezer and allowed to come to room temperature in capped vials. Fifty microliters of a 2.0 mg/ml solution of arachidonic

alcohol (AA) was added to each sample to serve as the internal standard for the analysis. Tissue samples were contacted with 3.0 ml of solvent [a 4:1 (v/v) mixture of 2,2,4-trimethylpentane and isopropyl alcohol]. The mixture was shaken for 15 min and stored overnight at room temperature, protected from light. One milliliter of water was added to the sample, and the sample was shaken for 15 min. The sample was then centrifuged at ~1,500 rpm for 15 min to accelerate phase separation. A total of 1.5 ml of the organic phase (top layer) was transferred to a glass vial, dried at 37°C under nitrogen for ~1 h, and then further dried under vacuum for ~48 h. The samples were then removed from the vacuum, and each vial was reconstituted with 600 μ l of deuterated chloroform. The vials were capped and manually shaken. The entire sample was transferred to a 5 mm NMR tube.

Within 24 h of sample preparation, the NMR tube was inserted into a spinner and placed in the NMR autosampler. The acquisition parameters included a pulse width of 90° and a relaxation delay of 6 s. Chemical shifts were referenced to the low levels of protonated chloroform present in the solvent (set to 7.26 ppm). Using Metabonomi, the areas of spectral regions were integrated, including regions assigned to HSE (4.66–4.76 ppm), WE (3.98–4.07 ppm), and AA (3.60–3.70 ppm). The peak areas for WE and HSE were normalized using the area of the internal standard, AA.

Hamster studies were conducted in accordance with the current guidelines for animal welfare (Guide for the Care and Use of Laboratory Animals, 1996). The procedures used in this study have been reviewed and approved by the Institutional Animal Care and Use Committee.

Human sebum study

Sebum was collected from a total of 72 human volunteers on three separate visits. Samples were taken from the right and left sides of the malar and forehead regions of female volunteers and the right and left sides of the malar, forehead, and scalp regions from male volunteers. Before sample collection, the region was cleansed with an alcohol wipe, and samples were collected at 5 min and 3 h after cleansing. Samples were collected by applying a lipid-absorbing polymeric film (Sebutape®; CuDerm Corp., Dallas, TX) to the skin for 30 s. The Sebutape® was removed from the cardboard backing using tweezers and placed into a 1 dram glass vial. The samples were stored at -70°C until sample extraction and analysis. A concurrent reading was made with a Sebumeter® SM 815 or SM 810 (Courage + Khazaka, Köln, Germany), which uses a photometric method to provide measurement of the sebum absorbed onto a transparent plastic film and reads out in milligrams of sebum per square centimeter. The Sebutape® samples were removed from the freezer and allowed to come to room temperature in capped vials. Vials were uncapped, and 600 μ l of perdeuterated cyclohexane was added to each vial. Samples were capped and agitated for 15 min. The entire solution was transferred to a disposable 5 mm NMR tube, leaving the Sebutape® in the vial. NMR data were acquired exactly as described above except that a pulse width of 85° and a relaxation delay of 1 s were used, resulting in a signal averaging time of ~24 min per sample. Chemical shifts were referenced to the low levels of protonated cyclohexane present in the solvent (set to 1.38 ppm). Diagnostic spectral regions were integrated, including regions assigned to CE (4.52–4.60 ppm), WE (3.96–3.99 ppm), TG (4.21–4.30 ppm), and squalene (5.06–5.13 ppm). Peak areas measured in the NMR spectra were converted to concentration values using a calibration function relating NMR peak areas to concentration. The calibration function was constructed from NMR data for six concentrations of a mixture of calibration standards. The calibration mixture was prepared from an equimolar, four-component stock solution prepared

TABLE 1. Mobile phase gradient used for the separation of sebum components in the HPLC-evaporative light-scattering detection method

Time	Trimethylpentane	Ethyl Acetate
<i>min</i>		<i>%</i>
0	99	1
2	96	4
6	60	40
7	5	95
10	5	95

Gradients are linear between time points. After 10 min, several column volumes of 1% trimethylpentane/99% ethyl acetate were used to wash the column before equilibration.

from commercially available reference compounds dissolved in deuterated cyclohexane. The reference compounds included squalene, triolein (a TG), cholesteryl palmitate (a CE), and palmitic acid stearyl ester (a WE). The concentrations included 5, 50, 100, 500, 750, and 1,000 μM for each component. Proton NMR spectra were acquired and processed using the same procedure outlined above for the test samples.

Human meibum study

Meibum samples were collected from the eye of one female volunteer and analyzed for squalene, TG, CE, and WE content. Samples were collected from lower eyelids (ciliary line) by touching a piece Sebutape[®] to the inner side of the eyelids for 5–10 s. The Sebutape[®] was then processed, and NMR data were acquired and analyzed exactly as described above for human sebum.

For studies involving human subjects, the final protocol and informed consent documentation were reviewed and approved by the institutional review boards at each of the investigational centers participating in the study. This study was conducted in compliance with the ethical principles originating in or derived from the Declaration of Helsinki (revised, Edinburgh, 2000) and in compliance with institutional review boards, informed consent regulations, and International Congress of Harmonization Good Clinical Practices Guidelines. In addition, all local regulatory requirements were followed, in particular, those affording greater protection to the safety of trial participants. The clinical protocol was also conducted in accordance with Food and Drug Administration regulations (Title 21, Code of Federal Regula-

tions, Parts 50, 56, and 312) and adhered to the principles of the Declaration of Helsinki and to Title 45, Code of Federal Regulations, Part 46 (Protection of Human Subjects, revised November 13, 2001, and effective December 13, 2001). Written informed consent was obtained before subjects entered the study (before screening). The investigator, or the investigator's designee, explained the nature, purpose, and risks of the study to each subject.

RESULTS

Effect of flutamide and isotretinoin on hamster ear sebum

A total of 40 punch-biopsy extracts were analyzed using the NMR method ($n = 10$ for each vehicle control and treatment group). A representative partial NMR spectrum from a control hamster ear extract is shown in Fig. 2. From these data, relative concentrations can be calculated by integrating the area under the peak corresponding to the analyte (Fig. 1) and normalizing to the internal standard, AA. The normalized NMR peak areas of HSE and WE in the hamster ear extracts of control animals and animals treated with 3% flutamide ($n = 10$ animals/group) are summarized in Table 2. Similarly, the NMR peak areas of HSE and WE in the hamster ear extracts of control animals and animals treated orally with 20 mg/kg isotretinoin ($n = 10$ subjects/group) are summarized in Table 3.

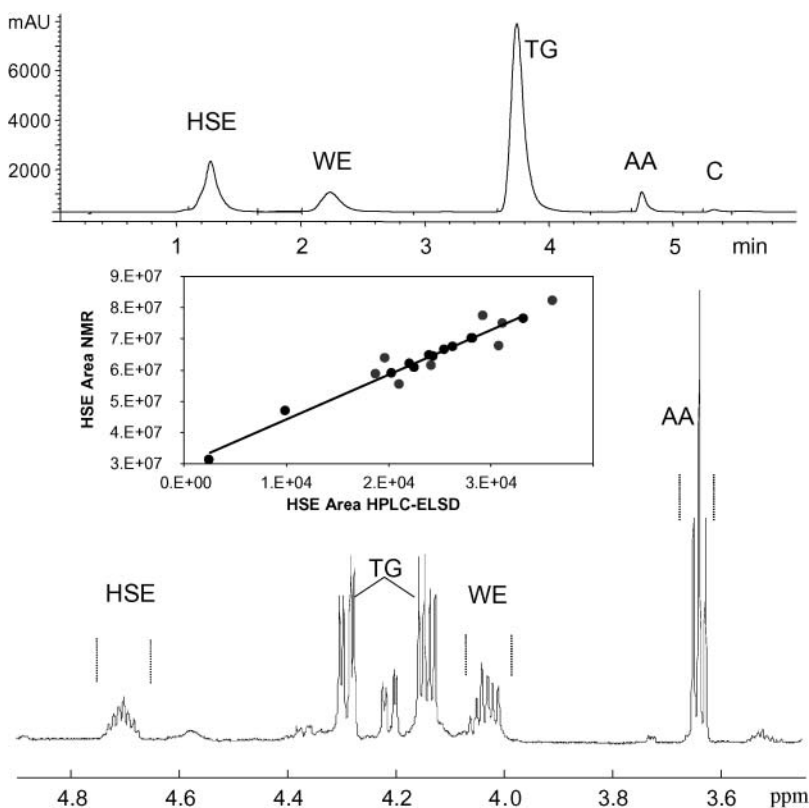


Fig. 2. Selected region of the 600 MHz proton NMR spectrum (bottom) and HPLC-evaporative light-scattering detection (ELSD; top) of a hamster ear punch extract. AA, arachidonic alcohol (internal standard); C, cholesterol; HSE, hamster sterol ester; TG, triglyceride; WE, wax ester. Inset shows a typical correlation between NMR and HPLC-ELSD results.

TABLE 2. Relative concentrations of HSEs and WEs in hamster ears treated topically with 3% flutamide for 7 days

Sebum Component	Normalized Mean NMR Peak Areas ^a		
	Vehicle Control ^b	Treated ^b	Percent Change
HSEs	0.71 ± 0.03	0.58 ± 0.02	-18.7
WEs	0.85 ± 0.03	0.57 ± 0.01	-32.9

HSE, hamster sterol ester; WE, wax ester.

^aIntegrated NMR peak areas are proportional to the concentration of the corresponding analyte, and these are normalized to the integrated area of the peak from the internal standard arachidonic alcohol.

^bMeans ± SEM (SD divided by the square root of the number of measurements).

Components in human sebum collected on Sebutape[®]

A representative partial NMR spectrum of an extract of Sebutape[®] blotted on human skin is shown in Fig. 3. Spectra from the calibration mixture are shown in Fig. 4. For each component or class of components, as in the case of CE and WE, least squares regression was used to fit a linear model to the peak area versus the concentration data, each converted to logarithmic scale. Results showed strong correlations between bilateral measurements completed within a subject and region and good reproducibility of parameters at 3 h timed measurements. Although the absolute concentration of individual molecular components varied from person to person (dependent on the oiliness of the skin), the distribution of the squalene, CE, TG, and WE in sebum from the scalp region of male subjects at the 3 h time measurement is remarkably consistent (Fig. 5). The distribution is shown in mole fraction units calculated by dividing the concentration of a single component by the total concentrations of squalene, CE, TG, and WE. The correlation between the total lipid measurements (sum of the four measured classes) and the Sebumeter[®] readings is shown in Fig. 6.

Components in human meibum tear films collected on Sebutape[®]

The NMR spectrum of human meibum Sebutape[®] extract is shown in Fig. 7, and the corresponding lipid concentrations measured from this sample are summarized in Table 4.

TABLE 3. Relative concentrations of HSEs and WEs in hamster ears treated with 20 mg/kg (po) isotretinoin for 7 days

Sebum Component	Normalized Mean NMR Peak Areas ^a		
	Vehicle Control ^b	Treated ^b	Percent Change
HSEs	0.59 ± 0.06	0.41 ± 0.01	-30.0
WEs	0.75 ± 0.09	0.47 ± 0.02	-38.1

^aIntegrated NMR peak areas are proportional to the concentration of the corresponding analyte, and these are normalized to the integrated area of the peak from the internal standard arachidonic alcohol.

^bMeans ± SEM (SD divided by the square root of the number of measurements).

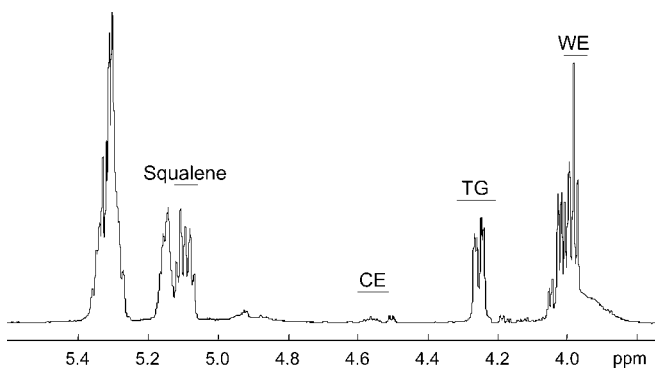


Fig. 3. Selected region of the 600 MHz proton NMR spectrum of a human Sebutape[®] extract. CE, cholesteryl ester.

DISCUSSION

Because the NMR peak area is proportional to the concentration of analyte, relative or absolute concentrations can be calculated by simple integration. When an internal standard is used, relative concentrations are readily obtained by normalizing to the internal standard peak area. This provides a robust way of determining changes in the concentration of individual analytes upon treatment with sebum-modifying agents such as flutamide or isotretinoin. To determine absolute concentrations, a rigorous calibration using six different concentrations of commercially available standards was used. Because of the linearity of the NMR response, single point calibrations should theoretically suffice. For either method, it is important to point out that the peak areas of analytes and internal standards may correspond to different numbers of protons, and this needs to be accounted for in the concentration calculations (Fig. 1). For the sterols or sterol esters, the diagnostic peak corresponds to a single methine proton attached to the hydroxyl carbon involved in ester formation. For AA, WE, and TG, the diagnostic NMR peak arises from a pair of methylene protons ad-

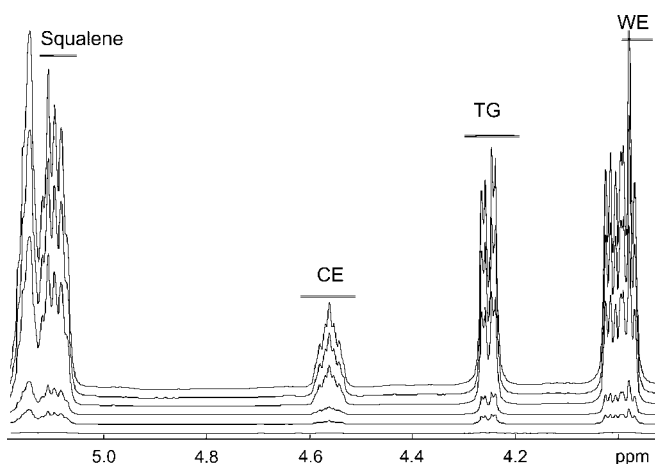


Fig. 4. Selected regions of the 600 MHz proton NMR spectra of the calibration standards. The concentrations of the components are (from bottom to top) 5, 50, 100, 500, 750, and 1,000 μM.

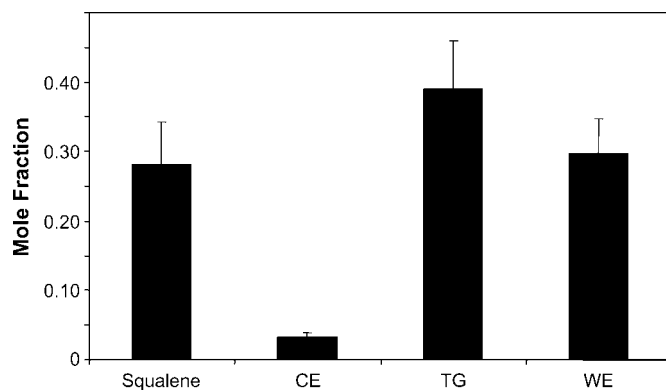


Fig. 5. Mole fractions of lipid components of a human Sebutate[®] extract measured using the NMR method. Error bars indicate \pm SEM.

adjacent to the carbonyl; thus, the integrated peak corresponds to two protons. For squalene in deuterated cyclohexane, four of the vinyl protons are resolved and integrated; in chloroform, all six vinyl protons overlap and must be integrated together.

NMR is an insensitive technique, and the cryoprobe used in this study is critical for the success of this assay. Because of the low dielectric solvents used in these analyses, the full benefit of the cryoprobe is manifested as a 4-fold increase in sensitivity, corresponding to a 16-fold reduction in acquisition time for a given signal-to-noise ratio. This allows low levels of CE to be measured down to the 5 μ M range in human sebum extracts with a 25 min acquisition. With a conventional NMR probe, this limit of detection would mean an unreasonable 6+ h per sample.

The hamster is a prolific sebum producer and has long been used as a preclinical model for excess sebum and its inhibition (15). The potent antiandrogenic agent flutamide has been shown to cause marked atrophy of the sebaceous glands in rat flank organs (16). The retinoic acid derivative isotretinoin, an effective treatment for severe

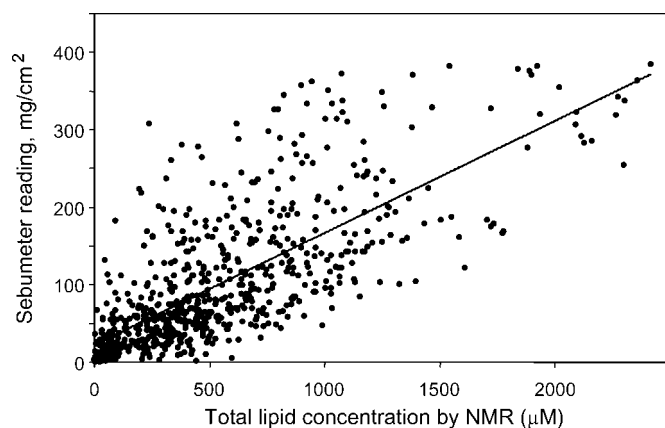


Fig. 6. Correlations between Sebumeter[®] readings and total lipid concentrations measured by NMR of Sebutate[®] extracts for all subjects at both time points. Total lipid is the sum of CE, WE, TG, and squalene, in micromolar, for extract of tape dissolved in 600 μ l of deuterated cyclohexane. Correlation coefficient (R^2) is 0.55.

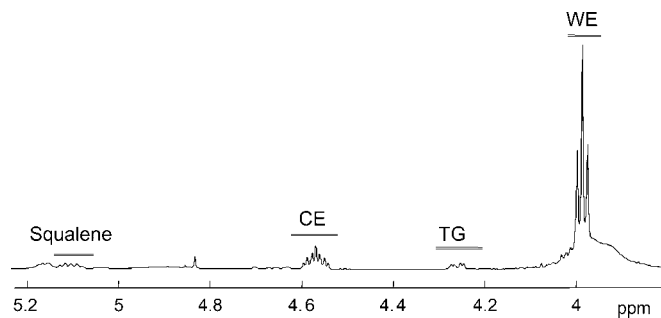


Fig. 7. Selected region of the 600 MHz proton NMR spectrum of a human meibum Sebutate[®] extract.

acne but with a poor safety profile, acts by reducing the size of sebaceous glands and thereby decreasing sebum production (17). The NMR assay clearly shows that treatment of hamsters with either flutamide or isotretinoin results in significant reductions in these sebum components relative to control treatment (Tables 2, 3) and is suitable for use as a preclinical assay for sebum reduction therapies.

One of the motivations for developing this assay was to establish a method that was easy to validate for clinical use. The results with the Sebutate[®] extracts from human skin and meibum demonstrate that this method is useful for quantifying the selected lipids in human sebum, with sensitivity suitable for relatively high throughput for direct support of clinical trials.


Conclusions

Despite good sensitivity, the poor linearity of HPLC-ELSD and lack of selectivity make clinical validation difficult. Mass spectrometric detection is highly sensitive and selective and has been used extensively in lipid analysis. However, the poor dynamic range in mass spectrometers often requires multiple calibrations to cover broad concentration ranges. LC-MS and GC-MS also have disadvantages in sebum analysis. Because MS distinguishes individual molecular species from different lipid classes based on mass and chromatographic retention time, quantitative measurements can be complex. Moreover, if response factors for specific components vary significantly, numerous internal standards may be required, which presents a significant disadvantage when pools

TABLE 4. Absolute concentrations of components of meibum from a female volunteer

Selected Lipid	Concentration ^a
	μ M
Squalene	20.0
Cholesteryl esters	174.5
Triglycerides	23.5
WEs	321.1

^aSebutate[®] samples blotted on the inner eyelid were extracted into 0.60 ml of cyclohexane- d_6 . Concentrations are based on integrated NMR peak areas measured directly from this extract and calibrated against authentic standards.

of possibly hundreds of similar molecules need to be measured together. In contrast, the advantages of NMR include the ability to simultaneously and selectively measure many different pools of lipid classes (independent of fatty acid composition) by simple peak integration, with high linear dynamic range and with sufficient sensitivity to carry out relatively high-throughput analysis on accessible and noninvasive samples. Because the method is nondestructive, additional analyses can be performed on samples after NMR data acquisition. Finally, because NMR measures every hydrogen-containing molecule in a sample above a minimum concentration of 5 μM , often the data will allow observations that one might not make with analyte-specific assays. These factors combine to provide the NMR method described here with powerful advantages that complement and/or improve upon traditional assays, particularly in clinical studies. Although we have tested this new method in an assay of lipid-rich secretions, it is certainly more broadly applicable in cases in which an entire molecular class needs to be measured, the only prerequisites being enough material and at least one resolved NMR signal. 

The authors thank Drs. Arthur Bertolino and Jane Harness for their contributions to the clinical study design.

REFERENCES

1. Abramovits, W., and A. Gonzalez-Serva. 2000. Sebum, cosmetics, and skin care. *Dermatol. Clin.* **18**: 617–620.
2. Brind, J. L., E. Alani, V. R. Wheatley, and N. Orentreich. 1986. Analysis of ear sebum of the Syrian hamster (*Mesocricetus auratus*) reveals pronounced sexual dimorphism. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **84B**: 403–407.
3. Saint-Leger, D. 2003. Normal and pathological sebaceous function. *Pathol. Biol.* **51**: 275–278.

4. Nordstrom, K. M., H. G. Schmus, K. J. McGinley, and J. J. Leyden. 1986. Measurement of sebum output using a lipid absorbent tape. *J. Invest. Dermatol.* **87**: 260–263.
5. Downing, D. T., M. E. Stewart, and J. S. Strauss. 1981. Estimation of sebum production rates in man by measurement of the squalene content of skin biopsies. *J. Invest. Dermatol.* **77**: 358–360.
6. Sullivan, B. D., J. E. Evans, J. M. Cermak, K. L. Krenzer, M. R. Dana, and D. A. Sullivan. 2002. Complete androgen insensitivity syndrome: effect on human meibomian gland secretions. *Arch. Ophthalmol.* **120**: 1689–1699.
7. O'Neill, H. J., and L. L. Gershbein. 1976. Analysis of fatty acid and alcoholic components of sebaceous lipid types. *J. Chromatogr. Sci.* **14**: 28–36.
8. Foerster, T. H., I. Hutter, A. Kleen, L. Kintrop and K. Schlotmann. 2001. Analytcs in cosmetic research. *SÖFW J.* **127**: 28–30, 32–34, 36, 38.
9. Nordbäck, J., and E. Lundberg. 1999. High resolution separation of non-polar lipid classes by HPLC-ELSD using alumina as stationary phase. *J. High Resolut. Chromatogr.* **22**: 483–486.
10. Torres, C. F., L. Vazquez, F. J. Senorans, and G. Reglero. 2005. Study of the analysis of alkoxyglycerols and other non-polar lipids by liquid chromatography coupled with evaporative light scattering detector. *J. Chromatogr. A.* **1078**: 28–34.
11. Cotterill, J. A., W. J. Cunliffe, and B. Williamson. 1972. Variations in skin surface lipid composition and sebum excretion rate with different sampling techniques. II. *Br. J. Dermatol.* **86**: 356–360.
12. Pierard-Franchimont, C., O. Martalo, A. Richard, A. Rougier, and G. E. Pierard. 1999. Sebum rheology evaluated by two methods in vivo. Split-face study of the effect of a cosmetic formulation. *Eur. J. Dermatol.* **9**: 455–457.
13. Guldur, T., N. Bayraktar, O. Kaynar, G. Beker, M. Kocer, and H. Ozcan. 2007. Excretion rate and composition of skin surface lipids on the foreheads of adult males with type IV hyperlipoproteinemia. *J. Basic Clin. Physiol. Pharmacol.* **18**: 21–35.
14. Traficante, D. D. 1992. Optimum tip angle and relaxation delay for quantitative analysis. *Concepts Magn. Reson.* **4**: 153–160.
15. Ludersmidt, C., W. Eiermann, and J. Jawny. 1983. Steroid hormone receptors and their relevance for sebum production in the sebaceous gland ear model of the Syrian hamster. *Arch. Dermatol. Res.* **275**: 175–180.
16. Lutsky, B. N., M. Budak, P. Koziol, M. Monahan, and R. O. Neri. 1975. Effects of a nonsteroid antiandrogen, flutamide, on sebaceous gland activity. *J. Invest. Dermatol.* **64**: 412–417.
17. Ward, A., R. N. Brogden, R. C. Heel, T. M. Speight, and G. S. Avery. 1984. Isotretinoin. A review of its pharmacological properties and therapeutic efficacy in acne and other skin disorders. *Drugs.* **28**: 6–37.