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Note

Improved high-performance liquid chromatography of sphingomyelin

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The analysis of spingomyelin preparations from various sources has been recently accomplished by reversed-phase high-performance liquid chromatography (HPLC)[1], argentation chromatography[2] and mass spectrometry (MS) directly[3,4] or in association with chromatography and derivatization[5–8]. By contrast, routine preparation of individual sphingomyelin molecular species for other purposes is not so well advanced. Moreover, in our hands prior chromatographic methods did not provide pure underivatized individual species required for planned peroxidation studies. Accordingly, we sought an improved HPLC system providing the required resolution. We describe herein such a reversed-phase HPLC system using linear gradient elution and binary and ternary solvent mixtures, with component analysis by chemical ionization mass spectrometry (CIMS).

EXPERIMENTAL

Solvents for HPLC were purchased from Burdick & Jackson Labs., (Muskegon, MI, U.S.A.). Bovine brain sphingomyelin was purchased from Sigma (St. Louis, MO, U.S.A.), Applied Science Labs. (College Station, PA, U.S.A.), Calbiochem-Behring, (San Diego, CA, U.S.A.) and from Supelco (Bellefonte, PA, U.S.A.). Bovine and porcine erythrocyte sphingomyelin was purchased from Supelco. These samples were all of high purity as judged from thin-layer chromatography (TLC). Egg yolk sphingomyelin from Sigma was purified by TLC before HPLC analysis.

Analytical TLC was conducted with chromatoplates of Silica Gel 60 F-254 (Merck, Darmstadt, F.R.G.) with solvent system I, chloroform–methanol–water (65:25:4)[9]; system II, chloroform–methanol–acetic acid (13:5:2)[10]; system III, chloroform–methanol–7 *M* ammonia water (12:7:1)[11], including two-dimensional TLC using systems I and III[12]. Components were visualized by spraying the dried chromatoplated with 50% aq. sulfuric acid and heating to char.

Reversed-phase HPLC was conducted with Waters Assoc. (Milford, MA, U.S.A.) Model M-6000A pumps controlled by a Waters Assoc. Model 720 systems control unit, with 500 μg sample in 50 μl chloroform injected via Waters Assoc. Model U6K injector. Initial studies were conducted with an Altex 15 cm × 4.6 mm I.D. 5-μm particulate Ultrasphere ODS reversed-phase column developed isocratically with the methanol–5 m*M* phosphate buffer, pH 7.0 (97:3) system of Jungalwala

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et al.[1] and by linear gradient elution with solvent A, acetonitrile–methanol–5 m*M* phosphate buffer, pH 7.0 (30:17:3) and solvent B, acetonitrile–methanol (1:1) flowing at 2.0 ml/min so as to change from 100% solvent A to 100% solvent B in 120 min, followed thereafter by 100% solvent B for 20 min.

The improved HPLC system employed a 10 cm × 4.6 mm I.D. 3-μm particulate Microsorb C_{18} reversed-phase column (Rainin, Woburn, MA, U.S.A.) developed by gradient elution at 1.5 ml/min with solvent C, acetonitrile–methanol–5 m*M* phosphate buffer, pH 7.0 (30:16:4) and solvent D, acetonitrile–methanol–5 m*M* phosphate buffer, pH 7.0 (50:49:1), both also being 20 m*M* in choline chloride, beginning with 80% solvent C–20% solvent D and progressing linearly to 100% solvent D in 120 min, thereafter developing with 100% solvent D for 30 min.

Column effluent was monitored by 206 nm light absorption using a Perkin-Elmer (Norwalk, CT, U.S.A.) Model LC-55 variable-wavelength spectrophotometric detector. Individual fractions were collected, evaporated under vacuum, and examined by CIMS conducted with a Finnigan MAT (Sunnyvale, CA, U.S.A.) Model 4000 quadrupole mass spectrometer operating in the chemical ionization mode with methane as reagent gas at 0.4 Torr. Samples (1–2 μg) were inserted via the solids probe and were heated at 230–250°C to obtain mass spectra.

RESULTS AND DISCUSSION

In adapting the 5-μm particulate reversed-phase column and binary solvent system of Jungalwala *et al.*[1] to our needs we were able to resolve commercial preparations of bovine brain, bovine and porcine erythrocyte, and egg yolk sphingomyelin into six to twelve components, but the required single molecular species were not obtained. For instance, the first major resolved component of bovine brain sphingomyelin found by Jungalwala *et al.*[1] to be composed of fatty acids 16:0 (94%) and 18:1 (6%) and sphingoid base sphingenine (designated 16:0/18:1 and 18:1/18:1, respectively) was by CIMS a different mixture of species 16:0/18:1 (86%) and 14:1/18:1 (14%). After exploratory attempts to improve resolution using isocratic elution failed, we turned to linear gradient elutions with binary and ternary solvent mixtures and 3-μm particulate columns which provided the required resolutions.

By these expedients superior results were obtained with solvent C and solvent D with a linear gradient progressing from 80% solvent C–20% solvent D to 100% solvent D in 120 min, followed by 30 min isocratic elution with 100% solvent D. In this manner, the elution curves of Fig. 1 for four commercial sphingomyelin preparations were obtained, with bovine brain sphingomyelin resolved into 25 components, bovine and porcine erythrocyte sphingomyelin into 22 components each, and the egg yolk preparation into 13 components.

Mass spectra of unfractionated sphingomyelin and of components resolved by HPLC were devoid of molecular ions $[M]^+$, protonated molecular ions $[M + H]^+$, or other high mass ions revealing molecular weight directly. Rather, spectra were characterized by three prominent sets of fragmentation ions by which component identity and homogeneity was evinced.

Ion clusters of highest mass recognized as arising from loss of the phosphorylcholine moiety via cleavage *a* (Fig. 2) were formulated as protonated fragment ion $[M - 182 + H]^+$ and hydride abstraction ion $[M - 182 - H]^+$ flanking the less

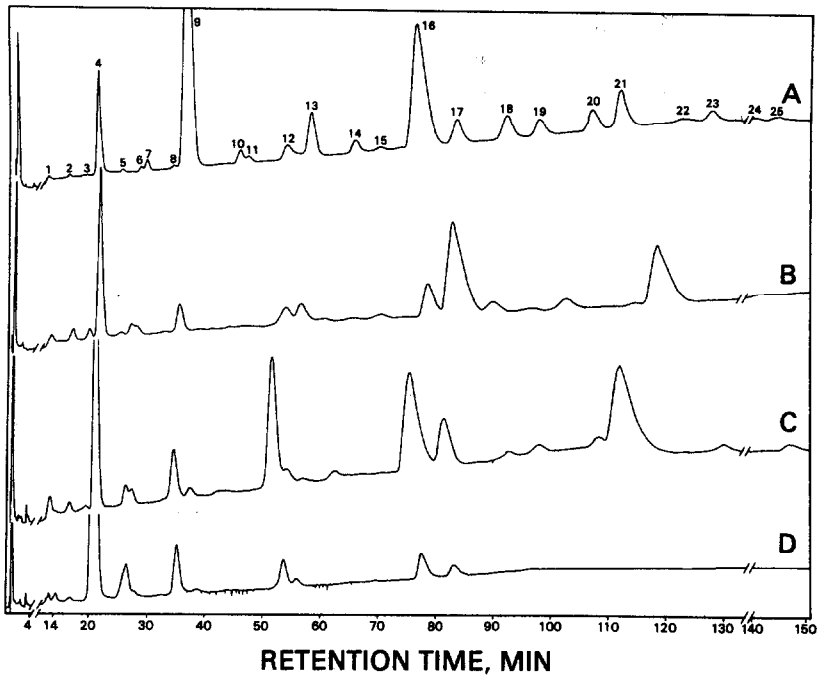

Fig. 1. Resolution of four sphingomyelin samples into individual components by HPLC, using a linear gradient elution progressing from 80% solvent C–20% solvent D to 100% solvent D in 120 min, followed by 30 min isocratic elution with solvent D. Sample A, bovine brain; B, bovine erythrocyte; C, porcine erythrocyte; D, egg yolk. The ommitted sections of each elution curve were featureless.

abundant $[M - 182]^+$ ion. Ions derived by cleavage *a* in electron impact MS of sphingomyelin have been identified by high resolution measurements as $[C_nH_{2n+1}CH{=}CHCH(OH)C(=CH_2)NHCOR]^+$ (ref. 4). A second less abundant ion cluster at 18 a.m.u. lower mass was then recognized as representing $[M - 182 - H_2O + H]^+$, $[M - 182 - H_2O]^+$, and $[M - 182 - H_2O - H]^+$ ions (cleavages *a* + *b*).

These characteristic ion clusters from which component molecular weight may be deduced were prominent in spectra of unfractionated preparations (Fig. 3), yielding thereby an approximate analysis of sphingomyelin species present. However,

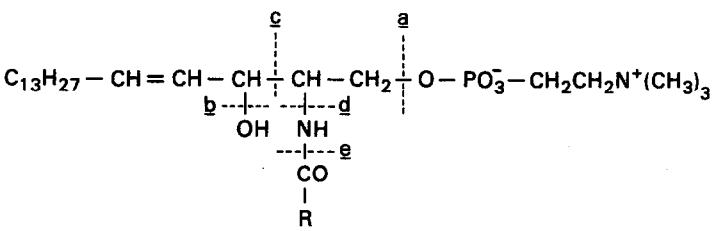

Fig. 2. Fragmentations observed in the CIMS of sphingomyelin molecular species using methane as reagent gas. Ion clusters centered at $[M - 182]^+$ arise from cleavage *a*; $[M - 182 - H_2O]^+$ from *a* + *b*; $[RCONHCH{=}CH_2]^+$ and $[RCONH{=}C{=}CH_2]^+$ from *a* + *c*; $[RCONH]^+$ from *d*; $[RCO]^+$ from *e*.

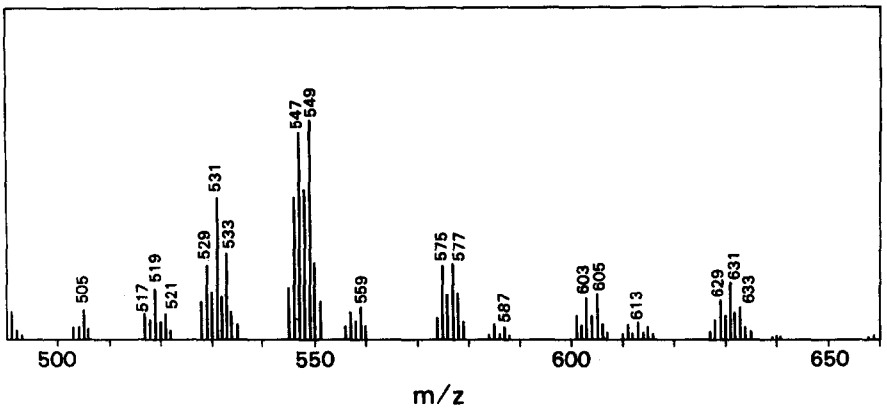

Fig. 3. High mass ion clusters $[M - 182 - H]^+$, $[M - 182]^+$, and $[M - 182 + H]^+$ from unfractionated bovine brain sphingomyelin indicating major components. Ion clusters $[M - 182 - H_2O - H]^+$, $[M - 182 - H_2O]^+$, and $[M - 182 - H_2O + H]^+$ are also present.

these ion clusters do not uniquely establish composition, as different combinations of N-fatty acyl (14:1–26:0) and sphingoid base (sphinganine, sphingenine, C_{20}-sphingenine) can give the same $[M - 182]^+$ and $[M - 182 - H_2O]^+$ ion clusters. Thus, the observed ion clusters (Fig. 3) may be interpreted *m/z* 631–633 from 24:1/18:0 or 24:0/18:1, *m/z* 629–631 from 24:1/18:1 or 22:1/20:1, *m/z* 603–605 from 22:0/18:1 or 20:0/18:1; *m/z* 575–577 from 18:0/20:1 or 20:0/18:1; *m/z* 547–549 from 18:0/18:1, *m/z* 519–521 from 16:0/18:1, *etc.* derived by cleavage *a*.

TABLE I

MASS SPECTRAL ANALYSIS OF RESOLVED BOVINE BRAIN SPHINGOMYELIN COMPONENTS

HPLC fraction No.	*Prominent CIMS ions, m/z**						*Sphingomyelin*		
	a	*a + b*	*c*	*a + c*	*d*	*e*	*MW*	*Fatty acid*	*Sphingoid base*
4	521, 520, 519	503, 502, 501	239	281, 280**	–	239	702	16:0	18:1
9	549, 548, 547***	531, 530, 529	239	309**, 308	282	267	730	18:0	18:1
11	551, 550, 549	533, 532, 531	–	309**, 308	282	267	732	18:0	18:0
13	577, 576, 575	559, 558, 557	–	337, 336	310	–	758	20:0	18:1
				309, 308	–	–	758	18:0	20:1
16	605, 604, 603§	587, 586, 585	239	365, 364	323	338	786	22:0	18:1
				337, 336	–	–	786	20:0	20:1
17	631, 630, 629	613, 612, 611	239	391**, 390	364	349	812	24:1	18:1
				365, 364	–	321	812	22;1	20:1
18	607, 606, 605	–	–	365, 364	338	323	788	22:0	18:0
19	633, 632, 631	–	–	393, 392	366	–	814	24:0	18:1
				391, 390	364	–	814	24:1	18:0

* Ions formulated from indicated cleavages are: *a*, $[M - 182 + H]^+$, $[M - 182]^+$, $[M - 182 - H]^+$; *a + b*, $[M - 182 - H_2O + H]^+$, $[M - 182 - H_2O]^+$, $[M - 182 - H_2O - H]^+$; *a + c*, $[RCONHCH{=}CH_2]^+$, $[RCONH{=}C{=}CH_2]^+$; *d*, $[RCONH]^+$; *e*, $[RCO]^+$.

** Principal ion.

*** Traces of ion clusters at *m/z* 577, 576, 575 and *m/z* 563, 562, 561 also present.

§ Traces of ion cluster *m/z* 631, 630, 629 also present.

Mass spectra of resolved sphingomyelin components (Table I) revealed only one set of $[M - 182]^+$ and $[M - 182 - H_2O]^+$ ion clusters, thus that resolved components were composed of sphingomyelin species of the same molecular weight. Only in the case of two fractions (No. 9, No. 16) were additional $[M - 182]^+$ ion clusters present at low abundances, indicating contamination of the major species by those of higher molecular weight. In two late eluting, high-molecular-weight fractions (No. 18, No. 19) only the $[M - 182]^+$ ion cluster was observed, spectra being devoid of the companion $[M - 182 - H_2O]^+$ ion cluster in these instances.

Despite the indicated presence of single-molecular-weight species in most resolved fractions neither identity nor homogeneity is established by the $[M - 182]^+$ and $[M - 182 - H_2O]^+$ ion clusters. Recourse may be had in the analysis of other fragmentation ions, taken with evidence of identity from prior, more exacting analyses. In this matter, the fragmentation patterns of sphingomyelin have not been adequately investigated, whether by electron impact MS[4] or by CIMS[13]. Suggestions exist that ions *m/z* 239 and 241 derived by cleavage *c* (Fig. 2) be diagnostic for sphingoid bases sphingenine and sphinganine respectively and that ions *m/z* 255, 257, and 285 be protonated molecular ions of fatty acids 16:1, 16:0 and 18:0 respectively, present as N-fatty acyl features of sphingomyelin[5,6,14]. We confirm the presence of the *m/z* 239 ion in spectra of several resolved components regarded as sphingenine derivatives, but the *m/z* 239 ion may arise by other fragmentation mechanisms, thus by cleavage *c* of N-palmitoyl derivatives (Table I). Moreover, we do not confirm the presence of the *m/z* 241 ion in spectra of sphinganine derivatives nor the *m/z* 267 ion as result of cleavage *c* in spectra of C_{20}-sphingenine derivatives. We also do not confirm that fragment ions *m/z* 255, 257, and 285 appear in spectra as protonated molecular ions of the suggested fatty acids. The presence of other fragmentation ions in this *m/z* range as well as of multiple fragmentation possibilities for their origins limit use of such ions for component identity.

The most abundant ions (principal ions in some cases) within the range *m/z* 280–393 allowed assignment of component identity and assessment of homogeneity as well. These ions were an even and odd pair recognized as $[RCONHCH=CH_2]^+$ and $[RCONH=C=CH_2]^+$ arising from cleavages *a* + *c*, the process being established by metastable ion studies of electron impact MS[4]. The molecular composition of resolved sphingomyelin fractions was thus indicated from $[M - 182]^+$ and $[M - 182 - H_2O]^+$ ion clusters and $[RCONHCH=CH_2]^+$ and $[RCONH=C=CH_2]^+$ ions.

Component homogeneity is also proved by the high abundance ion pairs $[RCONHCH=CH_2]^+$ and $[RCONH=C=CH_2]^+$. The presence of but one such pair evinces the presence of only one sphingomyelin species. However, spectra contained low abundance ions from other fragmentations, including $[RCONH]^+$ and $[RCO]^+$ ions by cleavages *d* and *e* respectively, that must be distinguished from the $[RCONHCH=CH_2]^+$ and $[RCONH=C=CH_2]^+$ pair. Some $[RCONH]^+$ and $[RCO]^+$ ions may have the same *m/z* as the key $[RCONHCH=CH_2]^+$ and $[RCONH=C=CH_2]^+$ ion pair, thus creating ambiguity in the analysis. Additionally, ions in the range *m/z* 264–266 derived from the sphingoid base were present in most spectra, and all had a high abundance ion pair *m/z* 148 and 149 (principal ion where $[RCONHCH=CH_2]^+$ or $[RCONH=C=CH_2]^+$ was not) of uncertain origins. We have not attempted further analysis of these spectra nor attempted to con-

firm by high resolution data, mass-analyzed ion kinetic energy spectrometry, or isotope labelling the several assignments.

Despite these limitations it is possible to assign identities to several bovine brain sphingomyelin species resolved (Table I). These assignments are consistent with results of prior analyses by other means[1–4,8] and with expected reversed-phase HPLC behavior. Thus, sphingomyelin species eluted in the order of their increasing molecular weight, with monoenoic species preceding the corresponding saturated derivative. Only for the doubly unsaturated species 24:1/18:1 and 22:1/20:1 in fraction No. 17 was this regularity defeated. Clearly this limitation prevents use of retention data alone for deducing the molecular weight of resolved sphingomyelin species.

By these criteria resolved fractions No. 4, No. 11, and No. 18 appear to be homogenous single sphingomyelin species, a feat accomplished here for the first time.

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REFERENCES

1 F. B. Jungalwala, V. Hayssen, J. M. Pasquini and R. H. McCluer, *J. Lipid Res.*, 20 (1979) 579.
2 M. Smith, P. Monchamp and F. B. Jungalwala, *J. Lipid Res.*, 22 (1981) 714.
3 W. D. Lehmann and M. Kessler, *Biomed. Mass Spectrom.*, 10 (1983) 220.
4 S. G. Batrakov, F. L. Sadovskaya, B. V. Rozynov and L. D. Bergelson, *Chem. Phys. Lipids*, 33 (1983) 331.
5 O. S. Privett and W. L. Erdahl, *Chem. Phys. Lipids*, 21 (1978) 361.
6 O. S. Privett and W. L. Erdahl, *Methods Enzymol.*, 72 (1981) 56.
7 F. B. Jungalwala, J. E. Evans, H. Kadowaki and R. H. McCluer, *J. Lipid Res.*, 25 (1984) 209.
8 F. B. Jungalwala, J. E. Evans and R. H. McCluer, *J. Lipid Res.*, 25 (1984) 738.
9 M. C. Moschidis, *J. Chromatogr.*, 259 (1983) 356.
10 G. Pluschke, Y. Hirota and P. Overath, *J. Biol. Chem.*, 253 (1978) 5048.
11 S. A. Rooney, P. M. Canavan and E. K. Motoyama, *Biochim. Biophys. Acta*, 360 (1974) 56.
12 R. Kariyama, *J. Antibiot.*, 35 (1982) 1700.
13 Y. Y. Lin and L. L. Smith, *Mass Spectrom. Rev.*, 3 (1984) 319.
14 S. P. Markey and D. A. Wenger, *Chem. Phys. Lipids*, 12 (1974) 182.