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Derivatization of hydroxyeicosatetraenoic acid methyl esters with pentafluorobenzoic anhydride and analysis with supercritical fluid chromatography-chemical ionization mass spectrometry[a]

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ABSTRACT

Analysis by supercritical fluid chromatography, in conjunction with chemical ionization mass spectrometry shows that methyl 5-O-pentafluorobenzoyl eicosatetraenoate is the stable product of the derivatization of methyl 5-hydroxyeicosatetraenoate with pentafluorobenzoic anhydride. This method may be useful in the analysis of hydroxy lipids.

INTRODUCTION

The measurement of small quantities of hydroxy lipids continues to increase in importance as more of them are discovered [1]. The ocular effects of eicosanoids, most of which are hydroxy lipids, is a nascent and rapidly growing field [2].

Previously, we developed a method for the gas chromatographic (GC) analysis of alcohols and hydroxy fatty acid methyl esters that utilized electron-capture detection (ECD) and pentafluorobenzoic anhydride (PFBA) as a derivatizing reagent [3]. After derivatization, methyl ricinoleate (12-OH-18: 1ω9), a mono-unsaturate, as well as some saturated compounds, were found to be stable at temperatures high enough to elute them from a GC column [3]. Using that method, we attempted to analyze 5- and 15-hydroxyeicosatetraenoic acid methyl esters (5- and 15-HETE-Me). We were not able to elute the products of these derivatization reactions from our GC column. Instead, the chromatogram consisted of numerous small peaks with retention times much less than one would anticipate for the expected product, indicating that the derivative decomposed at the temperatures required for elution. Using thin-layer chromatography (TLC), we observed that derivatization of methyl ricinoleate, 5-HETE-Me and 15-HETE-Me yielded products having polarities between triolein and methyl oleate.

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Using supercritical fluid chromatography (SFC)–mass spectrometry (MS), we show here that the product of derivatization of 5-HETE-Me is stable under the conditions required to elute it from an SFC column and that the product is the anticipated methyl 5-O-pentafluorobenzoyl eicosatetraenoate. Derivatized 15-HETE-Me was also analyzed using SFC-MS, but because of the complexity of its mass spectrum we are somewhat uncertain of the identity of derivatized 15-HETE-Me.

EXPERIMENTAL

Chemicals

Racemic methyl 5-hydroxy-6,8,11,14-(*E*,*Z*,*Z*,*Z*)-eicosatetraenoate and methyl 15(*S*)-hydroxy-5,8,11,13-(*Z*,*Z*,*Z*,*E*)-eicosatetraenoate were obtained from Sigma (St. Louis, MO, U.S.A.). Methyl *tert.*-butyl ether was obtained from Aldrich (Milwaukee, WI, U.S.A.), and ethyl acetate from Fluka (Ronkonkoma, NY, U.S.A.). Acetonitrile, hexane, methylene chloride, and tetrahydrofuran were J. T. Baker HPLC grade (purchased from Doe and Ingalls, Medford, MA, U.S.A.). Silylation grade pyridine was obtained from Pierce (Rockford, IL, U.S.A.); because of its shelf life, it was redistilled prior to use. Dimethyldichlorosilane and methyl ricinoleate were purchased from Alltech (Deerfield, IL, U.S.A.). TLC standards [18-4-A (a mixture of cholesterol, cholesteryl oleate, triolein, oleic acid and methyl oleate) and 18-5-A (a mixture of cholesterol, cholesteryl oleate, triolein, oleic acid and lecithin)] were obtained from Nu-Chek Prep (Elysian, MN, U.S.A.) and fatty acid methyl ester standards were from Supelco (Bellefonte, PA, U.S.A.).

Instrumentation and equipment

SFC with flame ionization detection was done on a Lee Scientific Series 600 Supercritical Fluid Chromatograph (Salt Lake City, UT, U.S.A.). A 0.2-μl fixed-loop injector having an injection time of 0.6 s and a split ratio of 2.61 was used to introduce samples into the column. The column was 10 m × 375 μm O.D. × 50 μm I.D. SB-Phenyl-5 (0.25-μm film) from Lee Scientific. The mobile phase was SFC-grade carbon dioxide. The column temperature was held constant at 120°C. The mobile phase was subjected to the following pressure program: 115 atm for 10 min, then ramped at 10 atm/min to 415 atm.

SFC-MS was performed at Oneida Research Services (Whitesboro, NY, U.S.A.). The chromatograph was the same as described above except that the sample loop was 0.1 μl and the column was 8 m × 100 μm I.D. SB-Methyl (0.25-μm film). The mobile phase and its temperature and pressure parameters were also the same. The MS apparatus was a Finnigan TSQ-70. It was scanned from 100-700 a.m.u. in 0.6 s. The electron multiplier was 2000 V. Methane was the reagent gas for both positive ion (PI) and negative ion (NI) chemical ionization (CI). The pressure in the ion source was maintained at 210 mtorr. Both the NICI and PICI measurements were made with the ion source at 250°C.

High-performance liquid chromatography (HPLC) was performed using a Beckman Model 344 interfaced to a Spectra-Physics (San Jose, CA, U.S.A.) SP-4250 integrator. A reversed-phase ODS column (250 × 4.6 mm I.D.) was obtained from Beckman. Ultraviolet (UV) detection was done with a cadmium lamp and 229-nm filter. Mobile phase was acetonitrile-water (75:25, v/v) with a flow-rate of 1 .O ml/min [4].

TLC plates were E. Merck high-performance plates with a silica gel 60 stationary phase (thickness: 0.2 mm) having dimensions of 10 × 10 cm. They were purchased from Alltech.

Silica gel Sep-Pak cartridges were obtained from Waters Assoc. (Milford, MA, U.S.A.). They were used in conjunction with a 10-ml glass syringe purchased from Scientific Products (Bedford, MA, U.S.A.).

Procedures

TLC plates were activated at 115°C for 10–15 min just prior to use. Samples were applied manually with a 25-μl TLC syringe (Alltech). The TLC mobile phase was hexane-methyl *tert.*-butyl ether-ethyl acetate (80:20:2) [5]. After developing, the plates were dried at 115°C for 2 min. Bromothymol Blue (Alltech) was used to visualize the spots.

PFBA was synthesized as previously described [3]. It was recrystallized in tetrahydrofuran rather than diethyl ether.

Just prior to derivatization, the HETE-Mes were brought to dryness with a gentle flow of nitrogen. The PFBA derivatization reaction was run as previously described [3] with the following modifications: acetonitrile or methylene chloride was used to dissolve the alcohols rather than toluene, since toluene was found not to be a polar enough solvent to dissolve HETE-Mes. The reaction time was increased to 2.5 h, the reaction volume was decreased to 0.5 ml, and 15 μl of pyridine were used. Silanized (with dimethyldichlorosilane) 6 ml glass scintillation vials were used to carry out reactions and for any subsequent transfers of products. (Caution is recommended in storing and using dimethyldichlorosilane. During storage, it easily corrodes the plastic of a PTFE-lined cap.)

After derivatization, the reaction solution was cleaned up with a (600 mg) silica Sep-Pak cartridge. The mobile phase was the same as was used for TLC (see above). The cartridge was rinsed with 10 ml of mobile phase before introducing the reaction solution. The products were then eluted with 5 ml of mobile phase. The sample was then concentrated under a stream of nitrogen to a final volume of ca. 0.4 ml.

RESULTS

Chromatography

We observed that PFBA derivatization of the HETE-Mes occurred more slowly than that of the saturated 2-hydroxy fatty acid methyl esters or methyl ricinoleate. This was somewhat surprising considering that allylic hydroxyl groups typically are acylated more easily than isolated hydroxyls [6]. Increasing the reaction time to 2.5 h and using 50% more pyridine provided satisfactory results.

TLC chromatograms (not shown) indicated that the relative polarity of the products is derivatized 5-HETE-Me > derivatized 15-HETE-Me > derivatized methyl ricinoleate. All three products migrate between triolein and methyl oleate. Consistent with previous results on a normal-phase HPLC column for 5- and 15-HETE [4], the same relative polarity was observed for the starting materials. The underivatized alcohols have R_f values close to that of cholesterol.

Fig. 1 indicates that both 5-HETE-Me and derivatized 5-HETE-Me are stable when subjected to the conditions of SFC. The two small peaks observed in both

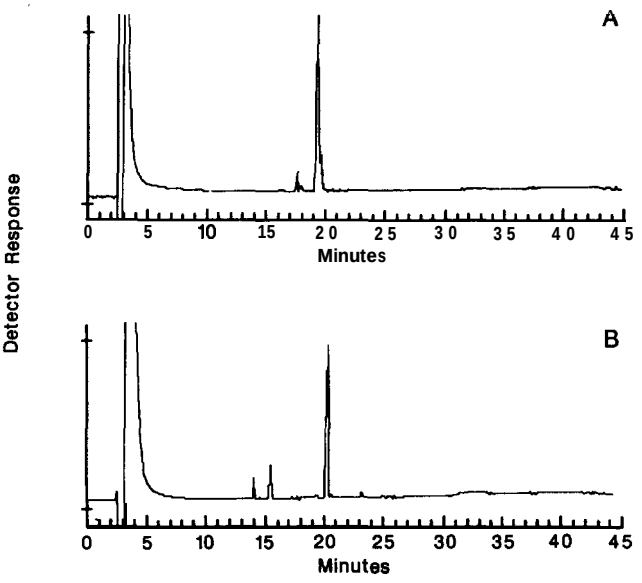

Fig. 1. Supercritical fluid chromatograms. Mobile phase: carbon dioxide. Column temperature: 120°C. Pressure program: 115 atm for 10 min, then 10 atm/min to 415 atm. (A) 5-HETE-Me; (B) derivatized 5-HETE-Me.

chromatograms were not identified but most probably can be attributed to impurities. Small impurity peaks are also observed when underivatized 5-HETE-Me is analyzed with HPLC using UV (229 nm) detection (data not shown). These minor peaks were not present in the blank (derivatization reaction run without 5-HETE-Me).

With the same chromatographic conditions as in Fig. 1, the retention times of methyl stearate (18:0), methyl arachidicate (20:0), methyl behenate (22:0), methyl ricinoleate and derivatized methyl ricinoleate were 17.7, 19.2, 20.4, 18.0 and 19.8 min, respectively. Methyl stearate was not resolved from 18:1, 18:2 or 18:3 methyl esters and methyl behenate was not resolved from 22:1 methyl ester. The chromatographic conditions were not optimized for maximum separation. SFC enables much better resolution [7] but that was not required for these experiments. The retention times of 5-HETE-Me and derivatized 5-HETE-Me were consistent with what one would expect based on the fatty acid methyl ester standards, methyl ricinoleate and derivatized methyl ricinoleate. As is common on silica-based columns, the underivatized alcohol tails to a greater extent than the derivatized compound.

Mass spectrometry

To identify the derivatized compounds, the positive chemical ionization mass spectra were measured (Fig. 2). For derivatized 5-HETE-Me (m/z 528), the base peak is m/z 317 (Fig. 2A, Table I). This indicates that the major pathway of fragmentation begins with loss of an electron from the alkyl oxygen adjacent to C_6F_5 and includes subsequent heterolytic cl-cleavage of the C-O bond, leading to an allylic secondary carbocation (*m/z* 317). Formation of the acyl ion, $[C_6F_5CO]^+$ (m/z 195) is not observed. Since the relative abundance of *m/z* 213 is 9.7%, double H-transfer (associ-

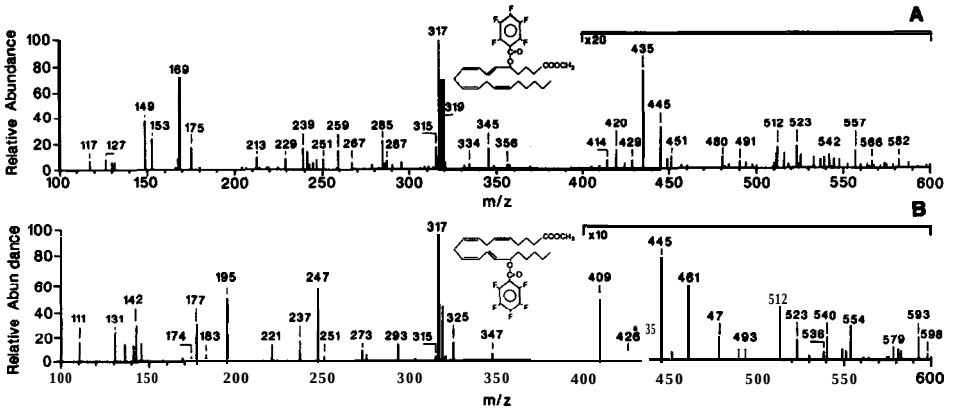

Fig. 2. Positive-ion chemical ionization mass spectra. Ion source temperature is 250°C. Reagent gas: methane. (A) Derivatized 5-HETE-Me; (B) derivatized 15-HETE-Me.

ated with the pentafluorobenzoyl ester) appears to compete slightly with heterolytic a-cleavage. Probably, ***m/z*** 435 is formed via the neutral **McLafferty [8]** fragment associated with the methyl ester **[9]** that subsequently loses a fluorine. other than these, most of the peaks appear to stem from $[M - C_7F_5O_2]^+$ (See Table I).

The PICI-MS of derivatized 15-HETE-Me (Fig. 2B) also had ***m/z*** 317 as its base peak. With the exception of m/z 523, 512, 445 and 435, which all have small

TABLE I

PICI-MS FRAGMENTATION PATTERN OF DERIVATIZED METHYL 5-HYDROXYEICOSA-TETRAENOATE

M is the molecular ion m/z 528. Ion source temperature was 250°C and the reagent gas was methane.

m/z	Relative abundance (%)	Positive ion assignment
557	0.7	$M + C_2H_5$
525	0.6	$[M + C_2H_5] - CH_3OH$
497	0.1	$M - CH_3O$
451	0.6	$[M - CO_2CH_3] -$ F + H
435	3.8	$[M - 74] -$ F
345	15.8	$[M - C_7F_5O_2]$ + CO?
334	2.9	$[M - C_7F_5O_2]$ + CH, or $[M - C_7F_5O]$ + H
319	35.6	$[M - C_7F_5O_2]$ + 2H
318	24.3	^{13}C and ^{2}H isotopes
317	100.0	$M - C_7F_5O_2$
285	22.2	$[M - C_7F_5O_2] - CH_3OH$
259	14.7	$[M - C_7F_5O_2] - CO_2CH_3$ + H
213	9.7	$C_6F_5CO_2H_2$
169	59.6	C_6F_5H + H
153	23.8	?
149	38.5	C_6F_4H

relative abundances, the remainder of the spectrum does not correlate with the derivatized 5-HETE-Me mass spectrum. We hypothesize that the secondary carbocation (m/z 3 17) cyclizes to an aromatic intermediate (m/z 273) with concurrent loss of CO_2. This fragment then goes on to form the series: 273, 247, 221, 195, 169 and possibly 142 (with loss of H). A series of *m/z* values differing by 26 daltons from one another is typical of aromatic compounds [10].

When the NICI mass spectra were measured, it would have been desirable to maintain the ion source temperature at 80°C. At that temperature, we probably would have observed the molecular ion. But, because of Joule-Thompson cooling of the mobile phase when it eluted from the column [11], with this instrument it was necessary to maintain the ion source of the mass spectrometer at 250°C so that eluting components did not precipitate at the interface [12]. Consistent with our previous NICI-MS measurements on derivatized 2-hydroxy fatty acid methyl esters [3], at this high of a temperature, the only fragments observed for both derivatized 5- and 15-HETE-Me were: *m/z* 148, C_6F_4; m/z 167, C_6F_5; and *m/z* 196, C_6F_5CO + H.

DISCUSSION

The advantages and disadvantages of SFC relative to GC and HPLC have been considerably discussed in the past [13]. Also, the extremely good sensitivity of ECD has been known since the middle sixties [14]. With our derivatizing reagent and GC–ECD we were able to measure hydroxy lipids in the femtomole range [3]. Only very recently has anyone attempted to couple ECD to SFC. SFC-ECD was first shown to be a viable technique by Kennedy and Wall in 1988 [15]. In this early work, a rising baseline prevented them from obtaining a sensitivity better than 35 pg for a triazole fungicide metabolite standard. Subsequent work by Richter *et al.* [16] indicated that baseline rise could be reduced. More recently, Chang and Taylor [17] have reported a detection limit for 2,3′,4′-trichlorobiphenyl of 0.64 pg and a linear range of 10^4. These results along with the results we show here suggest that without resorting to complex chemical procedures and extremely expensive instrumentation (NICI-MS), but using SFC-ECD instead, the chromatographic analysis and detection limits of hydroxy lipids can now be significantly improved.

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