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Identification of hydroxy fatty acids by liquid chromatography–atmospheric pressure chemical ionization mass spectroscopy in *Euglena gracilis*

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Abstract

Hydroxy fatty acids from *Euglena gracilis* were identified by reverse-phase high performance liquid chromatography coupled to a mass spectrometer run in atmospheric pressure chemical ionization positive ion mode. These metabolites were converted to methyl esters to improve stability and chromatographic properties. A detection limit of 20 pg/μl per injection was determined for 5-HETE methyl ester based on the signal to noise ratio of the *m/z* 317 ion which corresponds to the loss of a hydroxyl group (M-17) and the major fragment in all HETE methyl esters studied. This is the first report for these metabolites in *E. gracilis*.

Keywords: *Euglena gracilis*; Hydroxyl fatty acids

1. Introduction

Hydroxy fatty acids produced by lipoxygenase mediated-oxidation of C_{20} fatty acids such as arachidonic acid (AA) have been reported in various groups of algae [1–4]. These metabolites including prostanoids are potent mediators of many physiological processes [5], although their function in algae and microalgae has not been established. It has been hypothesized that hydroxy fatty acids act as second messengers and could be involved in a wide range of activities such as wound repair, homeostasis, growth, and chemical defense [6]. The oxidation of fatty acids may also play a role as a primary energy source and various levels of unsaturation may also contribute to membrane fluidity and environmental adaptation [7,8]. It is therefore useful to have available a rapid, reproducible, stable and sensitive method to quantitatively detect these metabolites under differing experimental conditions.

Current methods available for the study of hydroxy fatty acids involve the use of gas chromatography/mass spectroscopy (GC–MS) using either electron ionization (EI) [9,10], or negative chemical ionization (NCI) [11]. We recently reported the use of liquid chromatography/electrospray mass spectroscopy (LC/ESI-MS) to identify AA metabolites in *Limulus* amoebocytes [12]. While ESI-MS is ideal for readily ionizable species such as those containing an acidic function, the long-term instability of the free acids is problematic. Derivatization of hydroxy fatty acids is essential to stabilize the molecules and prevent further oxidation.

In this paper we described the use of LC/APCI-MS for the analysis of algal hydroxy fatty acid methyl esters using the microalga *Euglena gracilis* as a model. This method proved to be rapid and reproducible. Another advantage of this method is that crude organic extracts can be analyzed for the presence of hydroxy fatty acids without extensive

Abbreviations: AA, arachidonic acid; APCI, atmospheric pressure chemical ionization; HEDE, hydroxyeicosadienoic acid; HEPE, hydroxyeicosapentaenoic acid; HETE, hydroxyeicosatetraenoic acid; HODE, hydroxyoctadecadienoic acid; HOTrE, hydroxyoctadecatrienoic acid; HpEPE, hydroperoxyeicosatetraenoic acid; ME, methyl ester; NCI, negative chemical ionization; PMSF, phenylmethylsulfonyl fluoride

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purification by chromatographic methods and multiple derivations.

2. Experimental

2.1. Materials

E. gracilis was purchased from UTEX, the culture collection of algae (University of Texas, Austin, Texas). Cells were kept in culture using Euglena medium (sodium acetate 1.0 g, beef extract 1.0 g, tryptone 2.0 g, yeast extract 2.0 g, calcium chloride $\times$ $2H_2O$ 0.01 g, dissolved in 1 l of distilled H_2O) in a growth chamber at 25 °C at Santa Barbara, California. Arachidonic acid free acid (AA, 99%, porcine liver), phenylmethylsulfonyl fluoride (PMSF), ethylenediaminetetraacetic acid (EDTA), dithiothreitol (DTT), leupeptin, pepstatin A, *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide (diazald), and cell culture chemicals were obtained from Sigma (St. Louis, MO). Hydroxy fatty acid standards were obtained from Cayman Chemical (Ann Arbor, MI). HPLC grade organic solvents were obtained from Fisher Scientific (Pittsburgh, PA).

3. Conditions

3.1. Algae and product preparation

Cell free extracts of *E. gracilis* (approximately 1×10^6 cells) were prepared by homogenizing the cells in 5 ml chilled 0.2 M phosphate buffer pH 7.8 containing 2 mM EDTA, 2 mM DTT, 1 mM PMSF, 1 μg/ml leupeptin and 1 μg/ml pepstatin A, followed by centrifugation at $1000 \times g$ at 4 °C for 10 min to remove any debris. The supernatant of this centrifugation was incubated with AA (100 μM) for 3 h in a shaker at room temperature. The reaction was terminated by acidification to pH 3–4 with concentrated hydrochloric acid and immediately extracted twice in five volumes of chloroform/methanol (2:1; v/v). The organic extracts were combined, dried in vacuo, re-suspended in 100% methylene chloride. Methyl esters were prepared by exposure to diazomethane gas [13]. Commercial standards in free acid form were also prepared by this method, with the exception of 5-HETE methyl ester (ME) which was commercially available. The final organic extract was dried *in vacuo*, re-suspended in 0.5–1 ml of HPLC-grade methanol, filtered through 0.2 μm Pall Gelman nylon acrodisc syringe filters (Fisher Scientific), and stored at −70 °C under N_2 until analyzed.

3.2. Chromatography and mass spectrometry

Samples were analyzed by using LC/APCI-MS in positive ion mode (Michrom BioResources UMA HPLC system, Auburn, CA, coupled to a VG Platform II Mass Spectrometer (Micromass, England). The samples (1–5 μg of the crude extract) were separated on a C_{18} column (4.6 mm $\times$ 250 mm, 5 μm particle size, 100 Å pore size, Varian, Walnut Creek, CA) using an optimized mobile phase gradient starting at acetonitrile/water (50:50) going to acetonitrile/water (95:5) in 30 min and then ran isocratically for another 30 min at 1 ml/min. We modified and optimized published purification methods for identifying eicosanoids [14]. Drying gas flow was 250 l/h, source temperature was 150 °C, APCI sheath gas flow was 50 l/h, probe temperature was 500 °C, corona discharge needle voltage was 3 kV, and sample cone voltage was 30 V.

4. Results

4.1. Long-term stability of HETE methyl esters

Methylated HETEs were stored under N_2 at −70 °C for up to 8 months without detectable degradation by HPLC or APCI-MS.

4.2. Optimization of technique using commercially available standards

The separation of a mixture of two commercially available hydroxy fatty acid standards was tested using LC/APCI-MS in positive ion mode (Fig. 1A). The AA metabolites 15-hydroxyeicosatetraenoic acid methyl ester (15-HETE ME) and 5-hydroxyeicosatetraenoic acid (5-HETE ME) were adequately separated using the acetonitrile/water gradient (21.2 min retention time for 15-HETE ME and 23.3 min retention time for 5-HETE ME). A

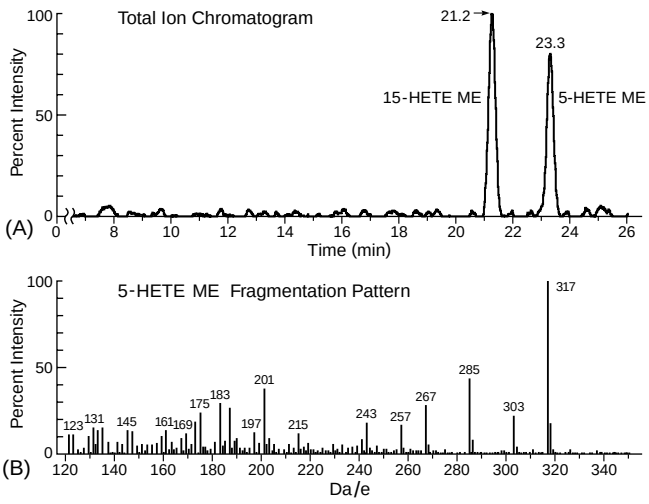

Fig. 1. Separation of two commercially available standards using LC/APCI-MS on positive ion mode. (A) Total ion chromatogram showing separation by HPLC and signal identification by MS. (B) Fragmentation pattern of 5-HETE ME displaying the major ion (*m/z*) of 317 which indicates the net loss of a hydroxy group or $[M + \text{H-H}_2\text{O}]^+$. Fragmentation patterns of 15-HETE ME were quantitatively similar (data not shown).

molecular ion was not observed in either spectrum. However, a peak was observed at *m*/*z* 317 which corresponds to the net loss of a hydroxyl group (Fig. 1B) from the intact molecule. It is presumed that this ion is generated from the facile loss of water from an $[M + H]^+$ pseudo-molecular ion $[M + H\text{-}H_2O]^+$. The sensitivity of the technique was tested using 5-HETE ME. A detection limit was found to be less than 500 pg (20 pg/μl) based on an observed signal to noise ration of 10/1 for the *m*/*z* 317 extracted ion chromatogram. Other major fragment ions observed for 5-HETE ME (Fig. 1B) correspond to the loss of a methoxy (*m*/*z* 303), and the loss of a methoxy group and a water (*m*/*z* 285) from the intact molecule. The same mixture was tested on LC/APCI-MS in negative ion mode but signal-to-background was poor and no useful spectra were obtained.

Our LC/APCI-MS method was also tested for its ability to separate other hydroxy fatty acid methyl esters metabolites of the lipoxygenase pathway. Table 1 shows the retention times and major peaks observed for a variety of standards. As noted, there was clear separation of C_{18} and C_{20} fatty acids as well as distinct separation on the basis of degrees of saturation and placement of hydroxyl substituents.

4.3. Identification of hydroxy fatty acids from E. gracilis' samples

The organic extract of a cell free incubation mixture of *E. gracilis* was methylated and analyzed by LC/APCI-MS (Fig. 2A). The *E. gracilis*' extract produced four signals with retention times similar to those of commercial standards and with *m*/*z* of 317 which correspond to the major ion of many of the AA-derived hydroxy fatty acids. The fragmentation patterns and retention times of the signals were compared to that of commercial standards and were identified as 15-HETE ME (21.4 min), 12-HETE ME (22.3 min), 8-HETE ME (22.9 min) and 5-HETE ME (23.5 min; Fig. 2A and B). In Fig. 3 the major fragmentation pattern of commercial 15-HETE ME is compared with the fragmentation pattern of 15-HETE ME found in *E. gracilis*. As can be seen, the fractionation pattern was identical to the extracted metabolite. HETE signals were not detected in *Euglena gracilis* enzyme extracts boiled prior to incubation with arachidonic acid, indicating that the compounds produced were likely the result of enzymatic biosynthesis.

Table 1
Retention times of ME prepared from commercially available hydroxy fatty acid metabolites

Hydroxy fatty acid methyl esters	Retention time (min)	Carbons	Molecular weight	Base peak
15-HpEPE	17.20	20	338	321
15-HEPE	18.34	20	332	315
13-HOTrE	18.68	18	308	291
15-HETE	21.20	20	334	317
13-HODE	21.24	18	310	293
9-HODE	21.96	18	310	293
11-HETE	22.02	20	334	317
12-HETE	22.30	20	334	317
9-HETE	22.64	20	334	317
8-HETE	22.90	20	334	317
5-HETE	23.30	20	334	317
11-HEDE	28.11	20	338	321

HpEPE: hydroperoxyeicosatetraenoic acid; HEPE: hydroxyeicosapentaenoic acid; HOTrE: hydroxyoctadecatrienoic acid; HODE: hydroxyoctadecadienoic acid; HETE: hydroxyeicosatetraenoic acid; HEDE: hydroxyeicosadienoic acid.

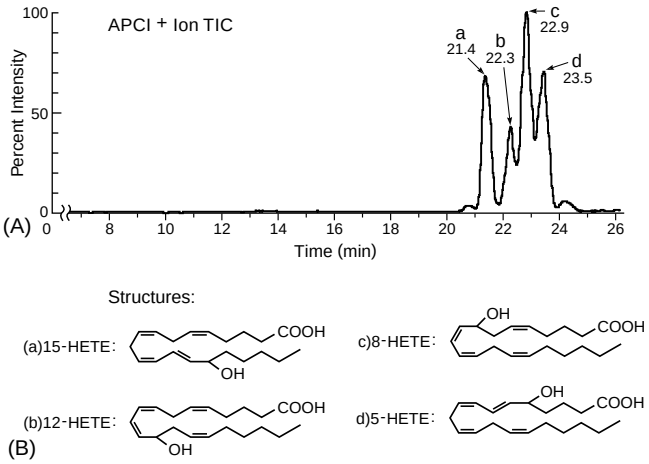

Fig. 2. (A) The TIC displays the separation and identification of four HETEs produced by *E. gracilis*' cell free extract incubated with AA. The signals were identified as (a) 15-HETE ME, (b) 12-HETE ME, (c) 8-HETE ME, and (d) 5-HETE ME. (B) Structures of the metabolites isolated from *E. gracilis*.

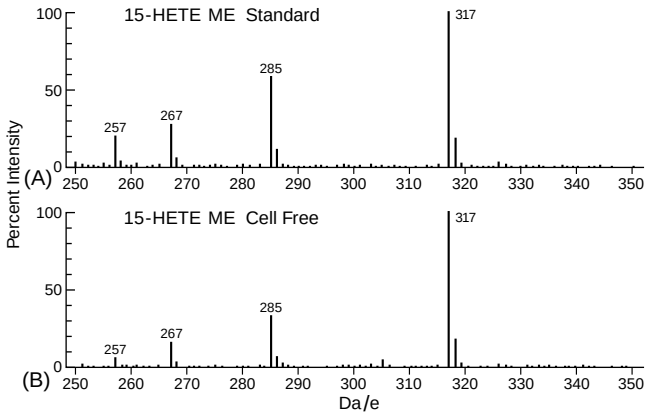

Fig. 3. Comparison of the fragmentation pattern of commercially available 15-HETE ME (A) with 15-HETE ME produced by *E. gracilis*' cell free enzyme extract incubated with AA (B). The *m*/*z* for both compounds is 317 and the major fragments are identical.

5. Discussion

We investigated the biosynthesis of hydroxy fatty acids in *Euglena gracilis* because of the ease of culturing the

organism, the high levels of fatty acid precursors and the suitability of the cells for laboratory manipulation. Although a limited number of HETEs have been identified in this organism by radioimmunoassay [15], to our knowledge no direct confirmation of their presence using mass spectrometry has been reported. A 3 h incubation of the cell free extract with AA enriched the presence of the HETE metabolites in *E. gracilis* so they were readily observed by LC/APCI-MS.

Past reports that have analyzed hydroxy fatty acids have mainly concentrated on the use of EI and NCI [9–11]. While these methods are appropriate, they require extra derivatization steps, increasing sample handling and analysis time. ESI-MS does not require derivatization [12], but the instability of the free acids limit the usefulness of this analytical method. Tandem MS multiple reaction monitoring techniques have been used with ESI based assays of fatty acid metabolites to improve the selectivity and sensitivity of those analyses [16]. It is expected that the use of tandem MS with our derivatization scheme and the APCI interface would be similarly advantageous.

The approach reported here employs only a single derivatization step of the crude organic extract producing methyl esters that significantly enhance the long-term stability of the hydroxy fatty acids some of which are unstable and last only a few minutes. This method will unmask previously undetectable metabolites and can allow direct identification of previously unknown intermediates. LC/APCI-MS is also a fast and reproducible method that is sensitive enough to identify hydroxy fatty acids present in a crude organic extract at picogram levels.

In this paper we have described an improved protocol for the detection of hydroxy fatty acids. This methodology can allow the direct identification of a broad spectrum of unstable hydroxy fatty acids and eicosanoids in a variety of algal species and under varying experimental conditions.

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