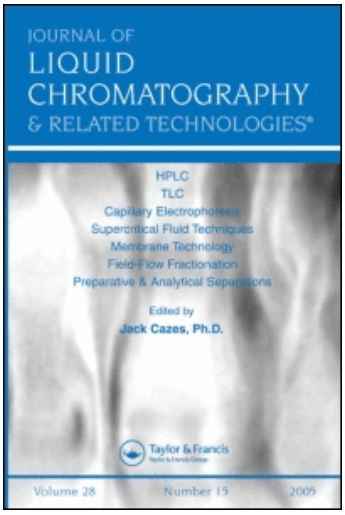

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Derivatization, Separation and Direct Quantification of Monohydroxy-Eicosatetraenoic Acids Using Reversed Phase High Performance Liquid Chromatography

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DERIVATIZATION, SEPARATION AND DIRECT QUANTIFICATION OF MONOHYDROXY-EICOSATETRAENOIC ACIDS USING REVERSED PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A new method for the separation and quantification of monohydroxy-eicosatetraenoic acids has been developed. The 5-, 12-, and 15-hydroxylated acids were esterified with p-(9-anthroyloxy) phenacyl bromide and subjected to reversed-phase high-performance liquid chromatography. 12-HETE and 15-HETE were readily separated. 5-HETE eluted in 2 peaks, the second co-eluting with 15-HETE. Sensitivity of the assay was 2.81 nM for both the 12- and 15-hydroxylated acids, and 6.25 nM for the 5-hydroxylated acid. This technique lends itself to analysis of biological samples.

INTRODUCTION

Monohydroxy-eicosatetraenoic acids are naturally occurring lipoxygenase-derived products of arachidonic acid. Those with hydroxyl groups at the 5, 12 and 15 carbon positions (5-HETE, 12-HETE and 15-HETE) appear to be individually important in a variety of biological processes, including platelet aggregation and inflammation. Therefore their separation and quantification is important to an understanding of a variety of biological responses.

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Bioassay of these compounds would be tedious and lack specificity. Because these compounds absorb ultraviolet (UV) light of wavelength 234 nm, they may be separated by high performance liquid chromatography (HPLC) and directly quantified by UV absorption. However, maximum sensitivity with such methods is approximately 16 nM (1,2). HPLC has been used with radioimmunoassay (RIA) to combine the best method of separation with the most sensitive means of detection (3-5). Problems with this approach include a lack of antibodies to a variety of HETEs, and a lack of specificity of existing antibodies. In addition, the technique is laborious in that RIA must be performed on multiple eluents from each chromatographic run.

The present work describes a simple and rapid HPLC method for separation and quantification of 5-, 12- and 15-HETE with a sensitivity of 2.8 nM for 12- and 15-HETE and 6.2 nM for 5-HETE.

MATERIALS AND METHODS

Chemicals

5-, 12- and 15-HETE were purchased in crystalline form from Seragen, Inc., Boston Massachusetts. p-(9-anthroyloxy) phenacyl bromide (panacyl bromide, PAB) was a gift from Dr. Walter Morozowich, The Upjohn Company, Kalamazoo, Michigan. Water, acetonitrile, ethanol, acetic acid, triethylamine (TEA) and tetrahydrofuran (THF) were all HPLC-grade (Fisher Scientific, King of Prussia, PA).

Instrumentation

HPLC (Waters Associates, Milford, MA) was performed with the following components: two Model 6000-A Solvent Delivery Systems, a Model 660 Solvent Programmer, a U-6K Injector, a Z Module Radial

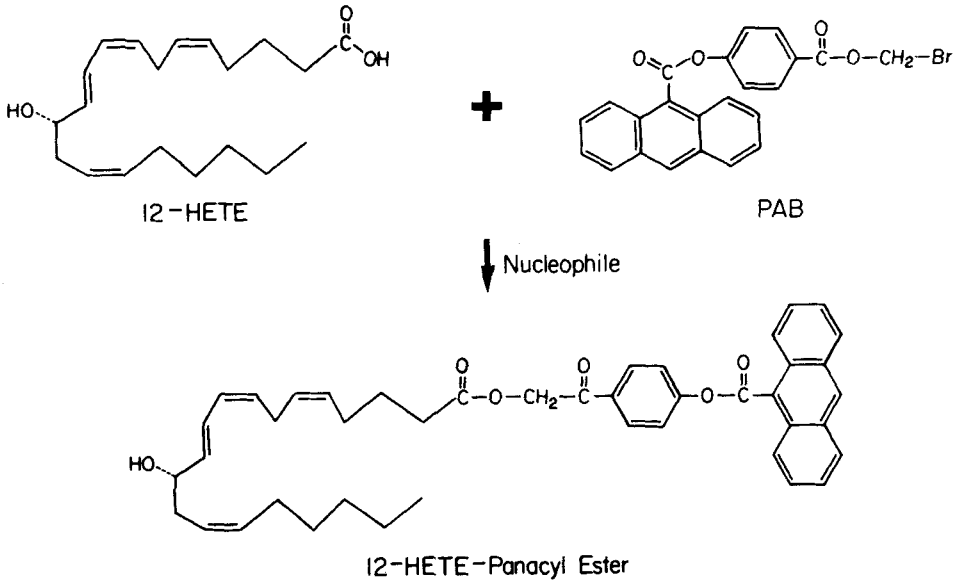

Figure 1.

Esterification reaction of 12-HETE with PAB.

Compression Separation System, a Model 441 Fixed wavelength Ultraviolet Absorbance Detector equipped with a mercury lamp and a 254 nm filter, and a Model 7000 Data Module. Separations were performed on a 5 μ particle-diameter octadecylsilyl (ODS) column (Waters Associates, Milford, MA).

Derivatization

Crystalline HETEs were dissolved in absolute ethanol and aliquots were dried under a stream of N_2. PAB was dissolved in a solution of acetonitrile:THF (4:1, v:v). PAB solution (1 ml) and 3 μl of TEA were added to each HETE aliquot. The stoichiometry was adjusted such that the molar ratio of PAB to HETE was at least

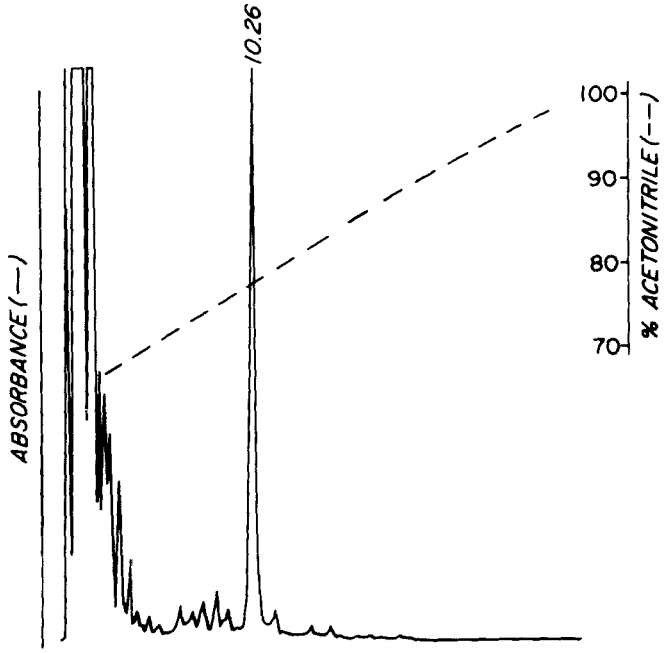

Figure 2.

20 µl of PAB solution injected after incubation at 37°C for 24 hours. Mobile phase is acetonitrile:0.1% acetic acid along a linear gradient from 68% to 98% acetonitrile. Column is a 5µ particle-diameter 5 mm internal diameter ODS flow rate 4.0 ml/min, pressure 2100 psi, 0.050 AUFS.

4:1. Standards were placed in teflon-capped vials and incubated at 37°C for 24 hours, then placed on ice.

Chromatography

Sample volumes were injected into the chromatograph and eluted with a mobile phase of acetonitrile and 0.1% acetic acid employing a variety of gradients. Absorbance as a function of time was recorded and area under peaks were quantified by electronic integration.

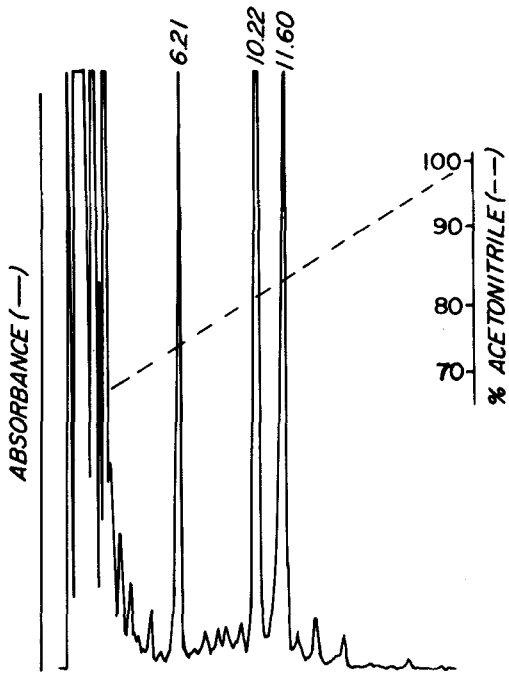

Figure 3.

20 µl of 310 µM derivatized 5-HETE (6.21,11.60). Chromatographic parameters as in Figure 2.

RESULTS AND DISCUSSION

The reaction sequence is depicted in Figure 1. Care should be taken to keep the reaction free of water to prevent hydrolysis back to the acids.

Figure 2 illustrates the chromatogram of the reaction blank (panacyl bromide solution incubated at 37°C overnight). Early peaks and that at 10.26 minutes are due to impurities and breakdown products of panacyl bromide.

Figure 3 shows the chromatogram of the panacyl ester of 5-HETE. Note that this compound elutes in peaks 6.21 and 11.60.

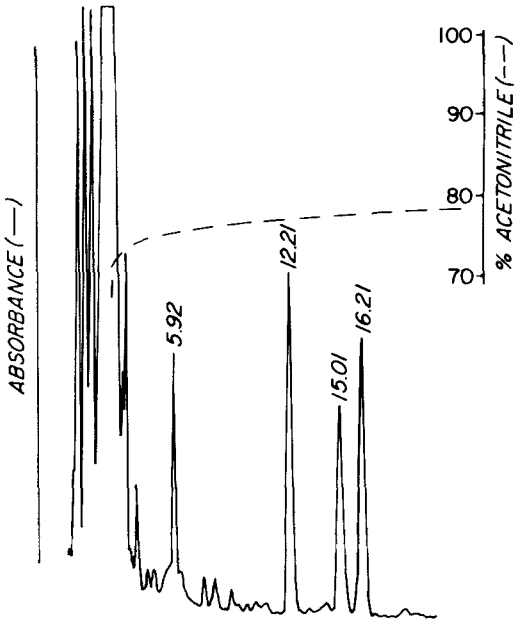

Figure 4.

3.3 µl of 310 µM derivatized 5-, 12-, and 15-HETE. 5.92:5-HETE; 15.01:12-HETE; 16.21:15-HETE. Mobile phase is acetonitrile and 0.1% acetic acid, 68% to 98% of the former over Solvent Programmer gradient #2 (convex) extrapolated to 9 hours. Column is a 5µ particle-diameter 8 mm internal diameter ODS, flow rate 5.0 ml/min, pressure 2100 to 1600 psi, 0.050 AUFS.

This latter peak may represent a 1,5-lactone 5-HETE which is known to occur in acid environment (1).

Figure 4 reveals optimum separation of the panacyl esters (5.92:5-HETE; 15.01:12-HETE; 16.21:15-HETE and presumed 5-HETE lactone). Figure 5 illustrates the maximal sensitivity of 5-HETE (2.91) to be 6.2 nM and Figure 6 the maximal sensitivity of 12-HETE (4.62) and 15-HETE (4.82) to be 2.8 nM.

A plot of area under the peak as a function of concentration of 12-HETE is shown in Figure 7, indicating linearity from 900 pg/ml to at least 10 ng/ml.

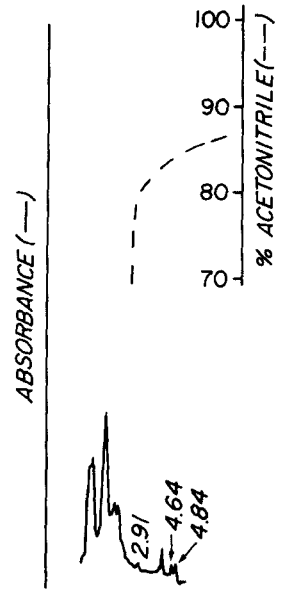

Figure 5.

200 µl of a 6.2 nM solution of 5- (2.91), 12- (4.64), and 15-HETE (4.84). Mobile phase is acetonitrile and 0.1% acetic acid, 68% to 98% of the former over Solvent Programmer curve #2 extrapolated over 20 min. Column as in Figure 2, flow rate 4.0 ml/min, pressure 2100 to 1600 psi, 0.005 AUFS.

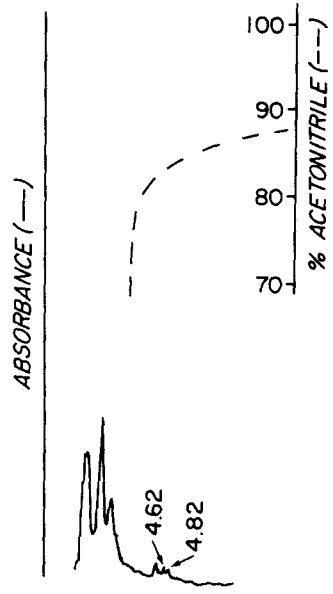

Figure 6.

200 µl of a 2.8 nM solution of 5-HETE, 12-HETE (4.62) and 15-HETE (4.82). Chromatographic conditions as in Figure 5.

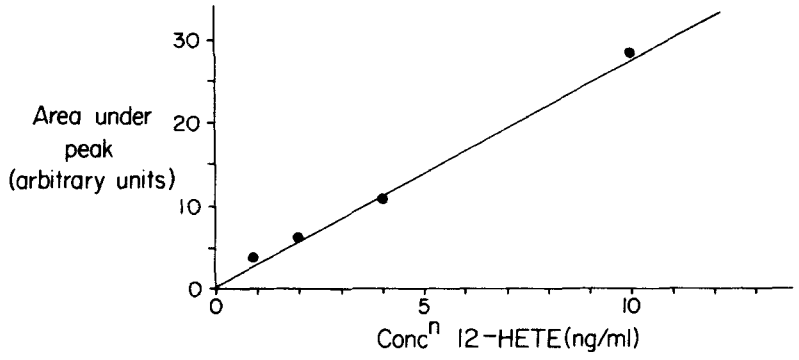

Figure 7.

Concentration curve for the panacyl ester of 12-HETE. Area counts are plotted as a function of concentration 12-HETE.

SUMMARY

A rapid and practical technique is presented for the derivatization of monohydroxy-eicosatetraenoic acids and their separation and quantification using HPLC and UV absorption. A sensitivity of 2.8 nM for 12- and 15-HETE, and 6.25 nM for 5-HETE should allow this technique to be applied to the analysis of biological specimens.

ACKNOWLEDGEMENTS

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