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Short communication

High-performance liquid chromatographic separation of fluorescent esters of hepoxilin enantiomers on a chiral stationary phase

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Abstract

Fluorescent anthryl (ADAM) derivatives of hepoxilins have been shown to possess good chromatographic properties affording good sensitivity for the high-performance liquid chromatographic analysis and detection of these compounds and related eicosanoids (12-hydroxyeicosatetraenoic acid) in biological samples. We report herein the separation of all possible stereoisomers of hepoxilins A_3 and B_3 as their methyl esters as well as their ADAM ester and acetate derivatives on a cellulose trisdimethylphenylcarbamate chiral stationary phase (Chiracel OD) in the normal-phase mode. This methodology is important to address the mechanistic route of biosynthesis of these products.

1. Introduction

The development of chromatographic methods of analysis of regio- and stereoisomers of eicosanoids, products of the enzymatic conversion of arachidonic acid, is very important for assignment of structure and for investigations into the mechanism of biosynthesis of these biologically active compounds. In the past few years, some eicosanoids were successively analyzed by high-performance liquid chromatography (HPLC) using chiral stationary phases. These included the enantiomeric synthetic analogs of prostaglandins [1], hydroxyeicosatetraenoates (HETEs) [2], epoxyeicosatrienoates [2–4] and dihydroxyeicosanoates [5]. It was reported that Chiracel columns gave the best resolution of eicosanoids having epoxy or hydroxy groups as a source of chirality [2]. Such an approach allows a direct analysis of the enantiomeric composition of eicosanoids which is especially important to clarify the mechanism of their biosynthesis. However, since some eicosanoids such as epoxyeicosatrienoates and hepoxilins (Hx) lack a conjugated chromophore, their detection by UV is not very sensitive restricting their determination to microgram or higher nanogram amounts. Their analysis in biological samples is therefore limited unless analyses are performed using radioactive materials [6,7]. Therefore, the preparation of chromophoric or fluorescent de-

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rivatives of these compounds with satisfactory chromatographic properties is required.

To develop such an approach for the HPLC analysis of eicosanoids that are essentially UV-insensitive, HxA_3 and HxB_3 were chosen as objects of the investigation. These relatively novel bioregulators are metabolites of arachidonic acid formed through the 12-lipoxygenase pathway via an intramolecular enzymatic rearrangement of 12(*S*)-hydroperoxyeicosatetraenoic acid (12-HPETE) [8,9]. Hx are found in various tissues and possess interesting biological activities based mostly on their ability to affect ion fluxes in the cell [9,10]. Stereochemical features of their formation are still mostly unclear and a good method which permits the analysis of their stereochemical composition will greatly facilitate these studies.

We recently developed a simple method for the HPLC profiling of Hx as well as their precursor, 12-HPETE, and their trioxilin metabolites as their anthryl esters using reversed-phase conditions; the separation of all diastereomers was achieved [11]. The present paper describes a method of HPLC analysis of the enantiomers of Hx as their fluorescent derivatives based on the combined use of a normal-phase and a chiral-phase HPLC. This method allows the establishment of the enantiomeric composition of Hx generated in vitro by enzymatic incubations or in vivo isolated from different tissues and organs. The procedure described also permits the isolation of Hx enantiomers in high optical purity.

2. Experimental

2.1. Chemicals

Optically active HxA_3 and HxB_3 isomers were prepared by total chemical synthesis as described in Refs. [12] and [13]. 9-Diazomethylanthracene (9-DMA) was purchased from Research Organics (Cleveland, OH, USA). *n*-Hexane and isopropanol used for HPLC were LC grade (Caledon, Georgetown, Canada). Thin-layer chromatography (TLC) was performed with Kieselgel 60 glass-backed plates, 20 × 20 cm, 0.2 mm gel thickness (Merck, Darmstadt, Germany).

2.2. Spectral studies

UV measurements were recorded on a Beckman DU 65 spectrophotometer; excitation and emission maxima were recorded on a Perkin-Elmer 650-40 fluorescence spectrophotometer.

2.3. Preparation of anthryl derivatives of hepoxilins (ADAM-Hx)

A mixture of 1.0 μg of the methyl ester of Hx isomers (Fig. 1, X = $-CH_3$, Y = H), 100 μl of *tert.*-butanol and 200 μl of 0.05 *M* NaOH in water were stirred 1 h at 20°C. Diethyl ether (200 μl) was added and the reaction was carefully acidified with 0.005 *M* HCl to pH 4.5–5.0 with constant vigorous stirring. The upper ether layer was removed and the aqueous lower phase was extracted two times more with 200 μl of diethyl ether. TLC analysis showed clean conversion of the methyl ester to the free acid as a sole product (R_F 0.49, ethyl acetate–acetic acid, 99:1). To the combined ether solution of Hx free acid, 200 μl of 5% solution of 9-DMA in diethyl ether were added and the reaction mixture was stirred 3 h at 20°C in the dark. The ether layer was concentrated to 100 μl and the ADAM-Hx derivative was separated from excess reagent on a TLC plate using 100% ethyl acetate as developing solvent. The light-blue fluorescent zone with R_F 0.80–0.65 was scraped, eluted with ethyl acetate and the solvent was evaporated to dryness. The final purification was performed on a normal-phase HPLC column as described in Fig. 2. The isolated yield of the anthryl Hx isomers (ADAM-Hx, Fig. 1, X = $-CH_2Ar_3$, Y = −H) was 80–85% estimated by measuring its UV absorption at λ 256 nm (ε 63 000 [11]). The derivatization procedure did not lead to analyte racemization.

To obtain C_8 or C_{10} acetates of the ADAM derivatives of Hx (Fig. 1, X = $-CH_2Ar_3$, Y = $-COCH_3$), a mixture of individual stereoisomers of ADAM-Hx (100 ng), 20 μl of dry pyridine and 5 μl of acetic anhydride were maintained 15 min at 20°C. The reagents were taken to dryness

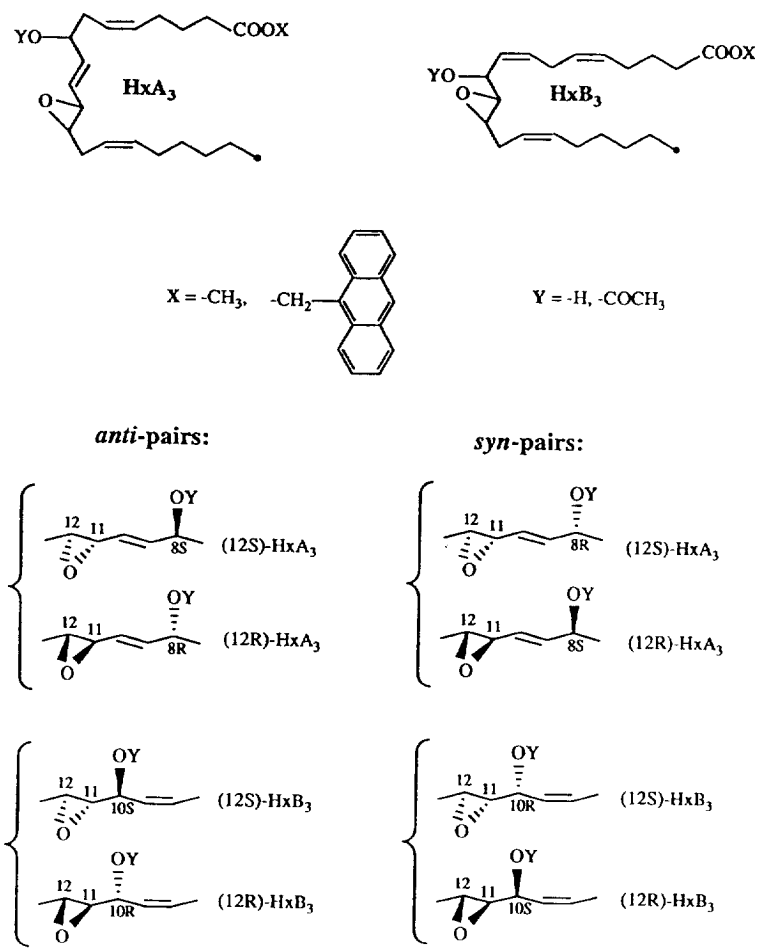

Fig. 1. Structures of regio- and stereoisomers of hepoxilins.

in a stream of nitrogen, the residue was dissolved in hexane, and the products were purified on a normal-phase HPLC column, eluent 0.25% isopropanol in hexane.

2.4. Chromatographic studies

HPLC analysis was carried out on a Waters (Division of Millipore, Milford, MA, USA) HPLC system. For the analysis and separation of Hx diastereomers as both methyl esters and ADAM esters, a normal-phase μPorasil column, 300 × 3.9 mm, mean pore diameter 125 Å, particle size 10 μm (Waters Assoc., Milford, MA, USA) was used; for the analysis of Hx enantiomeric pairs as methyl esters and ADAM esters and their acetates, a Chiracel OD column, 250 × 4.6 mm (J.T. Baker, Phillipsburg, NJ, USA) was employed. All compounds were injected individually onto a normal as well as chiral phase to establish retention times. A Kratos FS 950 fluorescence detector (Kratos Analytical Instruments, Westwood, NJ, USA) operated with a mercury lamp and excitation and emission filters of 254 and 400 nm was used for ADAM-Hx detection. A Waters 490 UV detector (Millipore) with a xenon source lamp at 210 nm was used for detection of Hx methyl esters. Data from the detectors was recorded on a Macintosh SE laboratory computer using a Dynamax method

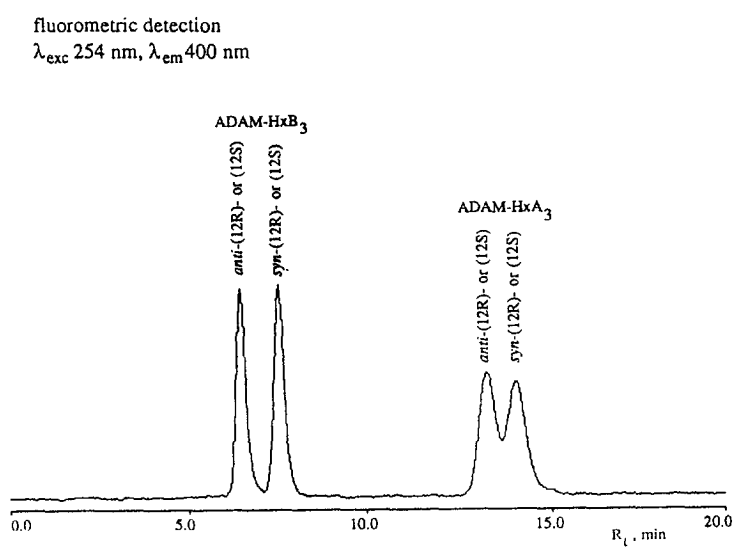

Fig. 2. Analytical HPLC separation of C_8/C_{10} epimeric anthryl esters of HxA_3 and HxB_3 on a μPorasil column (300 × 3.9 mm), fluorimetric detection at λ_{exc} 254 nm, λ_{em} 400 nm. Mobile phase 0.7% isopropanol in hexane, flow-rate 2.0 ml/min.

manager (Mandel Scientific, Guelph, Canada). Chromatograms were reprocessed and transferred to a SuperPaint program with which appropriate labelling of the figures was carried out (see Table 1 and Figs. 2–5).

3. Results

The chromatographic properties of stereoisomers of Hx methyl esters obtained by total chemical synthesis [12,13], were investigated

Table 1
Chromatographic properties of hepoxilin derivatives on a chiral phase

	Methyl esters[a]		ADAM esters[b]		Acetates of ADAM esters[c]	
	t_R (min)	R_s	t_R (min)	R_s	t_R (min)	R_s
anti-(12*S*)-HxA_3	52.80	2.00	30.05	2.31	18.65	1.28
anti-(12*R*)-HxA_3	44.95		23.90		16.65	
syn-(12*S*)-HxA_3	61.80	1.57	30.95	0.76	17.25	1.42
syn-(12*R*)-HxA_3	53.85	29.35	20.15			
anti-(12*S*)-HxB_3	21.45	1.56	14.85	1.43	11.60	0.59
anti-(12*R*)-HxB_3	18.60		12.70		11.00	
syn-(12*S*)-HxB_3	24.40	0.67	15.25	0	13.20	0.84
syn-(12*R*)-HxB_3	23.25		15.25		14.20	

t_R = retention time; R_s = separation value determined according to Ref. [18]; column Chiracel OD 250 × 4.6 mm, flow-rate 1.5 ml/min. For meaning of *syn* and *anti* refer to Fig. 1.
[a]1.0% Isopropanol in hexane.
[b]5.0% Isopropanol in hexane.
[c]2.0% Isopropanol in hexane.

using a Chiracel OD chiral stationary phase. Hx with a (12*R*)-*trans*-epoxy group displayed a lower retention time (t_R) than Hx with a (12*S*)-*trans*-epoxy group. Also the resolution value (R_s) between *anti*-pairs of Hx enantiomers was larger than between *syn*-pairs (Table 1, Fig. 3).

The derivatization of individual epimers of Hx was performed using commercially available 9-DMA according to the method described in Ref. [14] using a 5% solution of 9-DMA in diethyl ether. Since Hx as free acids are relatively unstable, especially HxA_3, their methyl esters were hydrolysed directly prior to derivatization with 9-DMA. The fluorescent Hx derivatives were purified from excess reagent on preparative TLC followed by final purification on normal-phase HPLC. UV scanning of the ADAM-Hx in hexane showed a maximum at 256 nm, and fluorescence scanning showed excitation peaks at 344, 361 and 381 nm. Excitation at these wavelengths gave emission maxima at 410 and 434 nm. The structure of ADAM derivatives of Hx was confirmed by liquid chromatography–mass spectrometry (LC–MS) [11].

The order of elution of the ADAM derivatives on a normal phase and the resolution between epimers (Fig. 2) were the same as their methyl esters, and all the ADAM esters were less polar than the corresponding methyl esters [15]. The limit of detection at a λ_{exc} of 254 nm and a λ_{em} of 400 nm was found to be 50–200 pg with some peak broadening at the longer retention times.

The ADAM esters of Hx had the same order of elution on a chiral stationary phase as the methyl esters, i.e. the (12*R*)-stereoisomers were eluted first (Fig. 4). The polarity of all ADAM esters on the chiral phase was remarkably greater than for methyl esters so that a significantly more polar solvent system (5% isopropanol in hexane for ADAM esters; 1% isopropanol in hexane for methyl esters) was required for analysis. The *anti*-pairs of enantiomers were well resolved (Fig. 4A); however, this was not the case with the two *syn*-HxB_3 enantiomers which were not separated at all (Fig. 4B) even when the amount of isopropanol in hexane was decreased (not shown). To separate the latter pair and to obtain an even better separation between *syn*-HxA_3 enantiomers, acetylation of the alcohol at C_8/C_{10} of ADAM-Hx was performed. The *anti*-pairs of ADAM-Hx acetates showed relatively good separation, and the order of elution remained the same as seen for the free alcohols, i.e. the (12*R*)-enantiomers had a lower retention time (Fig. 5A). Whereas the *syn*-pairs of ADAM-HxB isomers were not resolved as the free alcohols

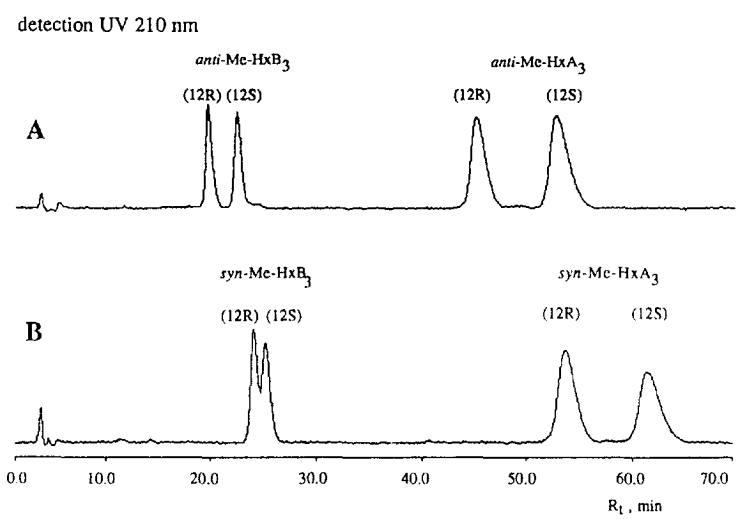

Fig. 3. Analytical HPLC separation of (A) *anti*- and (B) *syn*-pairs of methyl esters of HxA_3 and HxB_3. Analysis was conducted on a Chiracel OD column (250 × 4.6 mm), UV detection at 210 nm. Mobile phase 1.0% isopropanol in hexane, flow-rate 1.5 ml/min.

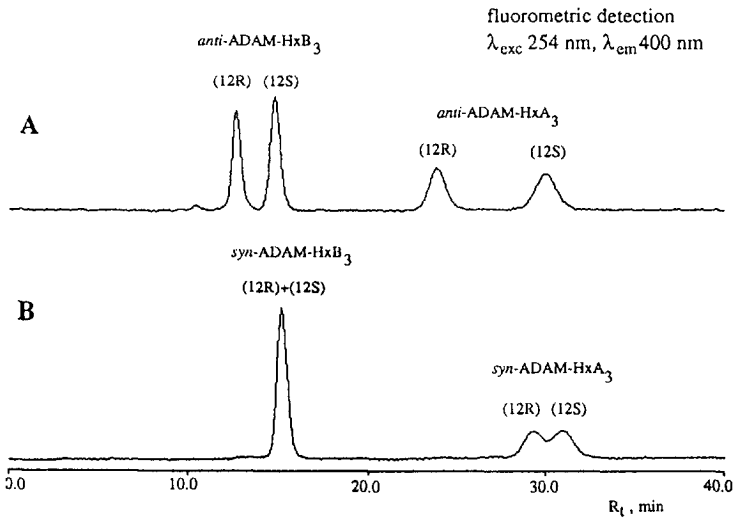

Fig. 4. Analytical HPLC separation of (A) *anti*- and (B) *syn*-pairs of anthryl esters of HxA_3 and HxB_3. Analysis was conducted on a Chiracel OD column (250 × 4.6 mm), fluorometric detection at λ_{exc} 254 nm, λ_{em} 400 nm. Mobile phase 5.0% isopropanol in hexane, flow-rate 1.5 ml/min.

(Fig. 4B), a good separation was achieved after acetylation (Fig. 5B). It was also observed that the order of elution of the *syn*-pairs of Hx enantiomers was opposite to that of the *trans*-enantiomers, i.e. the (12*S*)-enantiomers were eluted first; separation of all four *syn*-Hx was achieved (Fig. 5B).

The chromatographic results for the separation

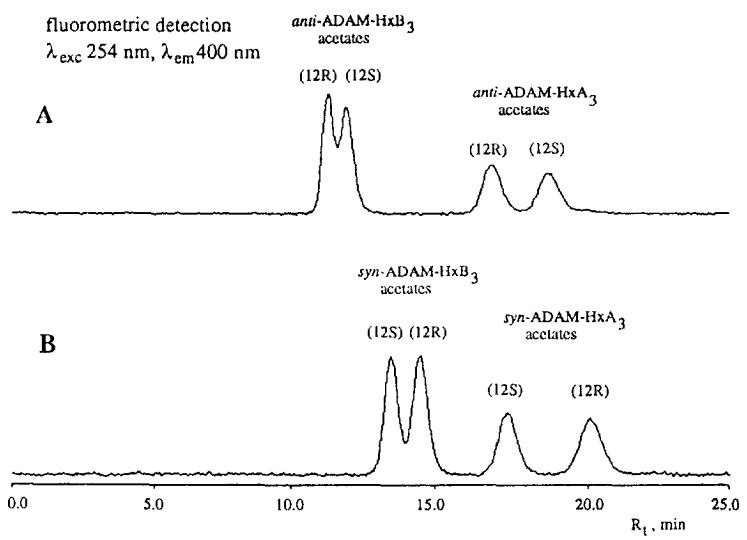

Fig. 5. Analytical HPLC separation of (A) *anti*- and (B) *syn*-pairs of anthranyl esters of HxA_3 and HxB_3 C_8 and C_{10} acetates. Analysis was conducted on a Chiracel OD column (250 × 4.6 mm), fluorometric detection at λ_{exc} 254 nm, λ_{em} 400 nm. Mobile phase 2.0% isopropanol in hexane, flow-rate 1.5 ml/min.

of Hx derivatives on a chiral phase are summarized in Table 1.

4. Discussion

In this study we report on the resolution by chiral-phase HPLC of all the regio- and stereoisomers of HxA_3 and HxB_3 as their methyl esters and their fluorescent ADAM esters. This technology is important in studies investigating the mechanism of biosynthesis of these products and in their analysis in biological fluids. As the compounds lack conjugated chromophores, use of the methyl esters is limited because of the insensitivity of UV detection. We therefore developed fluorescent derivatives as detailed in this study, as this greatly enhances their detection (over 1000 times the detection by UV) and will prove to be important for the detection (limits 50–200 pg) by HPLC of Hx and related carboxylic acids in biological fluids.

Fatty acids as well as prostaglandins have been analyzed successfully as their ADAM derivatives, although this method has not been extended to other eicosanoids, presumably because certain lipoxygenase products possess inherent conjugation, making their detection by UV methods possible. In the case of the Hx and other related products which do not possess conjugated chromophores, the only method available for their detection and quantitation has been gas chromatography–mass spectrometry (GC–MS). While this method is accurate and sensitive, it is limited in many ways. First, sample preparation is cumbersome and does not lend itself to multiple sample analysis. Second, we have found GC–MS to be incapable of analyzing HxA_3 effectively because of the inherent chemical sensitivity of the vinylic epoxide in this compound leading to various degrees of decomposition. Third, GC–MS does not lend itself to detecting regio- and stereoisomers of these products as does HPLC. We show in this report that these shortcomings are overcome by the preparation of ADAM esters of Hx. When converted into the ADAM esters, Hx are rendered quite stable to HPLC analysis. We have applied this technology to the investigation of the mechanism of formation of HxA_3 and have identified an enzyme system in the pineal gland which selectively utilizes 12(*S*)-HPETE to form HxA_3. The latter product is exclusive of the 11*S*,12*S*-*trans*-epoxide stereochemistry, while a non-enzymatic isomerization of 12-HPETE (e.g. by hemin catalysis) forms both the 11*S*,12*S*-*trans*- as well as the 11*R*,12*R*-*trans*-epoxide structures in HxA_3 [17].

The key to the analysis of the ADAM derivatives by HPLC is sample purity. Excess reagent must be removed before products of sufficient purity can be analyzed on chiral columns. We have attained sample purity in two ways: first, excess reagent was mostly removed by preparative TLC; second, the sample was further purified on a normal-phase HPLC. Purified peaks so obtained possessed the required purity for further analysis on the chiral phase.

ADAM esters of Hx possess excellent chromatographic properties on HPLC. Despite showing less polarity than the corresponding methyl esters on a normal phase, they require a more polar solvent on a chiral OD phase. The column efficiency for ADAM esters on a chiral phase appeared less (plate height $h = 83$ μm, Fig. 4) than for methyl esters ($h = 56$ μm, Fig. 3). The resolution value for *anti*-pairs for both esters was larger than for *syn*-pairs (Figs. 3 and 4), and in case of the two *syn*-HxB_3 enantiomers a separation was not achieved (Fig. 4B). To obtain a separation of the latter, the ADAM esters of Hx had to be converted into their C_8 or C_{10} acetates. This derivatization did not enhance the efficiency of the column (remaining at $h = 83$ μm), but it allowed the separation of the *syn*-pair of HxB_3 and showed good separation for the other enantiomeric pair (Fig. 5). It is to be noted that the order of elution of enantiomers for *syn*-pairs of Hx when converted into the acetates was opposite (Fig. 5B) to that of the free alcohols, whereas for *anti*-pairs acetylation of the C_8/C_{10} alcohol did not affect the order of elution from that observed for the free alcohol. Acetylation decreased the separation of the *syn*-pairs but enhanced remarkably the separation of the *anti*-pairs of enantiomers (Fig. 5).

HPLC remains the most straightforward meth-

od of analysis of eicosanoids in biological samples although it has two limitations. The first is sensitivity of detection, the second is confirmation of structure. The first limitation is greatly overcome through use of fluorescent derivatives instead of methyl esters, the second can be overcome through the LC–MS coupling. We are currently investigating this application further. Additional benefits of HPLC analysis relate to the non-destructive use of the sample, which, if found to be insufficiently pure, can be reanalyzed on the same phase or a different phase. The whole sample can also be analyzed at once instead of only an aliquot (minimum one fifth) as with GC–MS. As shown in this study, clear information on the chirality of the products can be obtained with HPLC, information that is essential for biosynthetic studies.

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