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Short communication

Enantiomeric separation of various lipoxygenase derived monohydroxy polyunsaturated fatty acid methyl esters by high-performance liquid chromatography

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Abstract

Lipoxygenase derived monohydroxy polyunsaturated fatty acid methyl esters were separated by chiral high-performance liquid chromatography (HPLC) with a Chiralcel OD-H column in the normal-phase mode. Major lipoxygenase derivatives of linoleic, α-linolenic and arachidonic acids are well resolved by this column, provided they have been individually purified. Our method allows an easy and rapid determination of lipoxygenases enantioselectivity. In all cases tested the *R* enantiomer is eluted first. © 1997 Elsevier Science B.V.

Keywords: Enantiomer separation; Fatty acid methyl esters; Lipoxygenases

1. Introduction

Polyunsaturated fatty acids (PUFAs) are oxygenated by lipoxygenases (LOXs) to conjugated dienic hydroperoxides (HPODs) with mainly an *S* absolute configuration (for general reviews on LOXs, see [1–5]). It is a matter of fact that such enzymes should be characterized by both their regioselectivity, something which could easily be done either by reversed- or normal-phase high-performance liquid chromatography (HPLC), and their enantioselectivity. To date and to the best of our knowledge, the only general method developed to analyse the enantioselectivity of PUFA oxygenases has been proposed by Kühn et al. [6]. They used a Pirkle-type IA column and were able to resolve many HPODs of both linoleic and arachidonic acids. Although baseline resolution was not always observed, this method proved to be useful in many cases. Since then, chiral discrimination in both gas chromatography (GC) and HPLC has made tremendous progress, especially with the design of new generations of chiral phases. We wish to report here the use of a Chiralcel OD-H column in the resolution of methyl esters of various hydroxy derivatives of linoleic (methyl HODE), α-linolenic (methyl HOTE) and arachidonic acids (methyl HETE). Baseline resolution is frequently observed, minor isomers such as all-*trans* methyl HODE could also be analysed, and this is the first report of direct chiral analysis for methyl HOTEs.

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2. Experimental

2.1. Chemicals

Linoleic, α-linolenic and arachidonic acids were from Fluka, as was soybean LOX. Barley seeds were from a local market. Pyridinium chlorochromate and sodium borohydride were from Aldrich. All organic solvents were of HPLC grade.

2.2. Synthesis of optically active hydroxy fatty acid methyl esters

Methyl 13(*S*)-HODE [7,8] and methyl 15(*S*)-HETE [9] were synthesized as previously reported using soybean LOX under oxygen. Methyl 13(*S*)-HOTE was obtained using the same methodology. Methyl 9(*S*)-HODE [10], methyl 9(*S*)-HOTE [10] and methyl 5(*S*)-HETE [11] were obtained through oxygenation catalysed by barley seed LOX.

2.3. Synthesis of racemic hydroxy fatty acid methyl esters

Racemic methyl 9(*E*,*Z*)-HODE, methyl 9(*E*,*E*)-HODE, methyl 13(*E*,*Z*)-HODE and methyl 13(*E*,*E*)-HODE were obtained using an autoxidation procedure [12] followed by normal phase HPLC purification (see Section 2.4). Racemic methyl 5-HETE was chemically synthesized from arachidonic acid as described by Corey et al. [13] using a lactonization procedure. Racemic methyl 9-HOTE and methyl 15-HETE were obtained by the following method. The chemoenzymatically generated methyl 9(*S*)-HOTE and methyl 15(*S*)-HETE were oxidized to the corresponding ketones using pyridinium chlorochromate. After purification over silica (hexane–diethyl ether, 90:10), the ketones were then reduced with sodium borohydride in ethanol at 0°C to the corresponding racemic hydroxy fatty acid methyl esters, which were then purified over silica using the same solvent as for the ketones. Although the yields were quite low (typically in the range of 5 to 10%), this method proved to be effective for the generation of 10 mg of racemic methyl 9-HODE and methyl 15-HETE. The identity and the purity of the compounds formed were checked by co-injection with authentic optically active parent compounds by normal-phase HPLC (see Section 2.4).

2.4. Purification of chemoenzymatically generated hydroxy fatty acid methyl esters

Pure regio- and stereoisomeric hydroxy fatty acid methyl esters were obtained by normal phase semi-preparative HPLC using a mixture of hexane–diethyl ether (70:30) at a flow-rate of 3 ml/min. The purity of the compounds was checked using the same solvent system at 1 ml/min, with an analytical silica HPLC column. The products were detected using a spectrophotometer set at 235 nm.

2.5. Separation of enantiomers

Each enantiomeric pair of methyl esters of 9(*E*,*Z*), 9(*E*,*E*), 13(*E*,*Z*), and 13(*E*,*E*)-HODE, of 9- and 13-HOTE, of 5- and 15-HETE was resolved with a Chiralcel OD-H HPLC column (250×4.6 mm I.D., 5 μm; J.T. Baker) using a mixture of hexane–isopropanol (98:2) at a flow-rate of 1 ml/min. The products were detected using a spectrophotometer set at 235 nm.

3. Results and discussion

3.1. 9(E,Z)-HODE, 9(E,E)-HODE, 13(E,Z)-HODE and 13(E,E)-HODE methyl esters

The main substrate used in studies with plant LOXs is linoleic acid. This acid, with only one pentadienyl system, could be oxygenated at either position 9 or 13, to generate a conjugated dienic hydroperoxide. The major stereoisomers formed by LOXs catalysed oxygenations are (*E*,*Z*) compounds. Nevertheless, in the case of linoleic acid, a relatively high proportion of all-*trans* 9 and 13 isomers is formed, especially in the case of the well known soybean LOX-1, depending on the pH and reaction temperature used [8,14]. We report here the resolution of all four regio- and stereoisomeric methyl HODEs as seen in Fig. 1.

The method described allows an easy and accurate determination of the enantiomeric excess (e.e.) of all four stereoisomeric methyl HODEs and thus of the

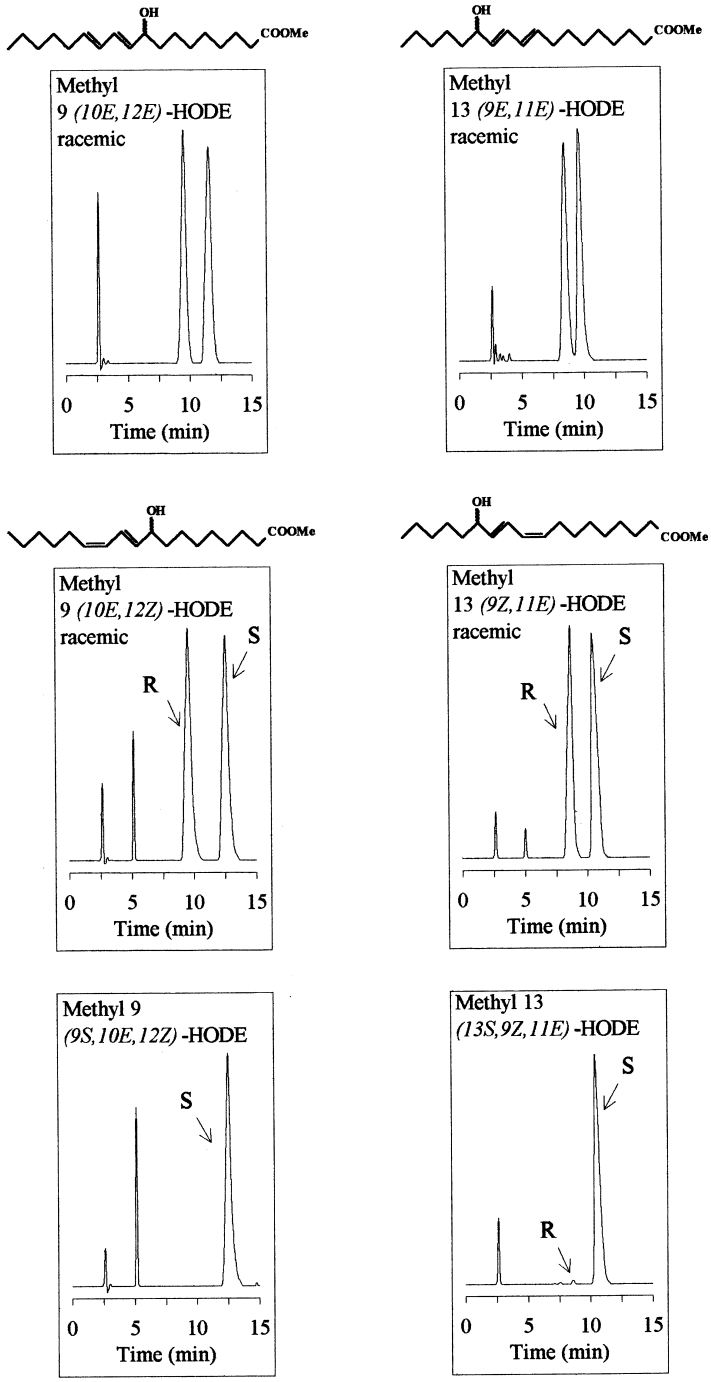

Fig. 1. Chromatograms of racemic mixtures of all four regio- and stereoisomers of methyl HODEs and of chemoenzymatically generated methyl 13(*S*)-HODE and methyl 9(*S*)-HODE, separated using a Chiralcel OD-H column. Detection was monitored at 235 nm.

complete enantioselectivity of the enzyme considered. Gardner [14] has proposed that in the case of linoleic acid and soybean LOX-1, substrate that escapes enzymatic control accounts for the detected *R* isomers of the four stereoisomeric HODEs and an equal amount of the corresponding *S* isomers. The enzymatically generated hydroproxides are supposed to be optically pure. Thus the quantification of the *R* enantiomers is directly linked to the efficiency of the radical-trapping capacity of the enzyme under various conditions and could become an important means to better understanding of the stereochemical outcome of LOXs.

3.2. 9-HOTE and 13-HOTE methyl esters

Despite of the presence of a supplementary double bond in α-linolenic acid, to the best of our knowledge no 16 or 12-LOX for this compound has been described to date. In fact the regioselectivity of LOXs is unaffected by changing the substrate from linoleic acid to α-linolenic acid. In comparison with the amount of work done on linoleic acid, very few studies have been devoted to the selectivity of LOXs toward this acid, even though it is probably the main natural substrate of various plant LOXs. As could be seen in Fig. 2, the enantiomers of both methyl 13-HOTE and methyl 9-HOTE are perfectly resolved with the Chiralcel OD-H column.

When used under the same experimental conditions as those described for linoleic acid, both soybean and barley seed LOXs have shown a much higher regio and stereoselectivity with α-linolenic acid, since only 13-(9*Z*,11*E*,15*Z*)-HPOTE and 9-

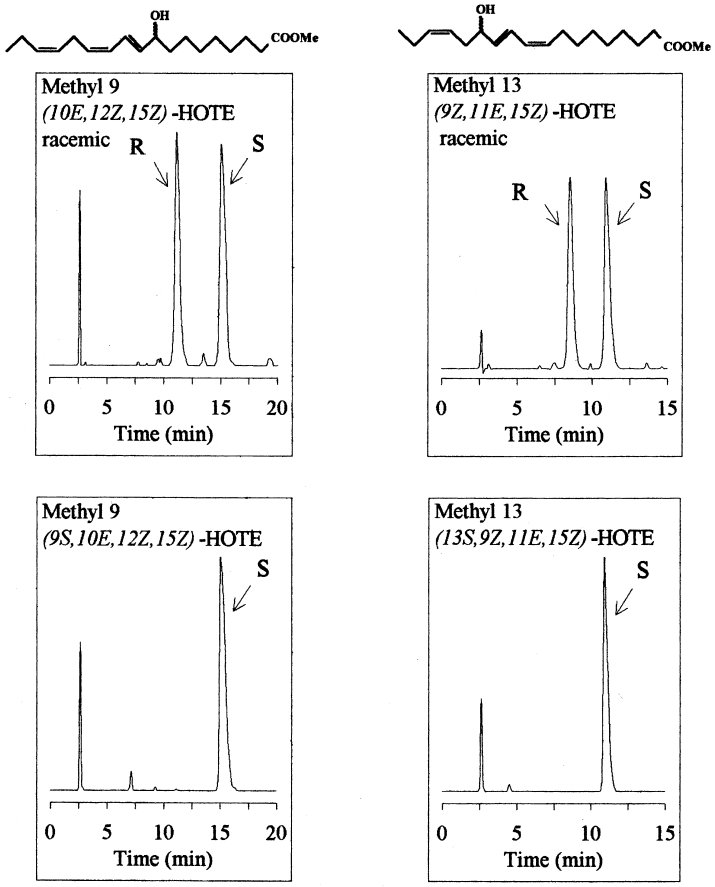

Fig. 2. Chiral HPLC traces of racemic and enzymatically generated methyl 9- and 13-HOTE, separated using a Chiralcel OD-H column. Detection was monitored at 235 nm.

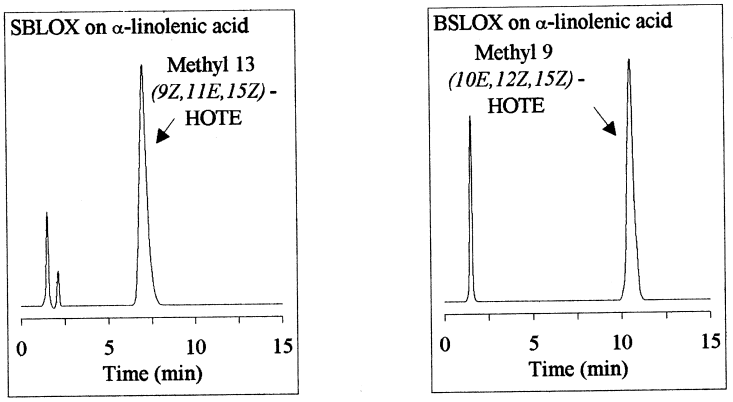

Fig. 3. Normal-phase HPLC traces of the reduced and methylated hydroperoxides obtained by the action of soybean lipoxygenase (SBLOX) and barley seed lipoxygenase (BSLOX) on α-linolenic acid, 30% diethyl ether in hexane (1.5 ml/min). Detection was monitored at 235 nm.

(10*E*,12*Z*,15*Z*)-HPOTE are formed, respectively, as could be seen by normal-phase HPLC (see Fig. 3). We were interested in showing whether this high stereoselectivity was correlated with a high enantioselectivity. As shown in Fig. 2, both chemoenzymatically generated methyl 13(*S*)-HOTE and 9(*S*)-HOTE are obtained with very high e.e. (>99%). It seems likely that the supplementary *n*-3 double bond found in α-linolenic acid leads to a better positioning of the substrate in the active site of the enzyme, generating only one isomer.

3.3. 5-HETE and 15-HETE methyl esters

In studies with mammalian LOXs the substrate used to determine the specificity of the enzyme is arachidonic acid. The main mammalian LOXs are 5, 12 and 15 regiospecific, leading respectively, to the synthesis of 5-HPETE, 12-HPETE and 15-HPETE. The separation of the enantiomers of 12-HETE on a Chiralcel OD-B column [15] having already been described, we focus in this study on the resolution of methyl 5-HETE and methyl 15-HETE. These two compounds and their parent HPODs are the precursors of both leucotrienes and lipoxins, two well known classes of eicosanoids with important biological functions.

Baseline resolution of methyl 5-HETE is observed, but this is not true in the case of methyl 15-HETE, which is the least well resolved hydroxy fatty acid in the present study, as could be seen in Fig. 4. Nevertheless an e.e. of more than 99% could be attributed to the soybean LOX generated methyl 15(*S*)-HETE as, for methyl 5(*S*)-HETE obtained through barley seed LOX action.

4. Conclusions

Since the classical work of Kühn et al. [6] on the resolution of various derivatives of both linoleic and arachidonic acid HPODs, no general study has been devoted to the resolution of such compounds. Taking advantage of the commercially available Chiracel OD-H chromatography column, we describe here the near-baseline resolution of various hydroxy polyunsaturated fatty acid derivatives obtained through LOX catalysis whatever the substrate used (linoleic, α-linolenic, arachidonic acids). Particularly significant is the ability of such a column to easily determine the e.e. of all-*trans* isomers of HODE, which are generally obtained in very low quantities. This determination could be of great interest, since it could give information on the selectivity of the enzyme and its mode of action [14]. The method described here requires only simple chemical manipulations (i.e., reduction of the hydroperoxide function and methylation of the carboxylic acid function best achieved with triphenylphosphine and diazomethane respectively). If the enzyme used generates a mixture of regio- and stereoisomeric hydroxy fatty acids, a further purification of individual compounds should be undertaken, since their peaks overlap on the chromatogram and might

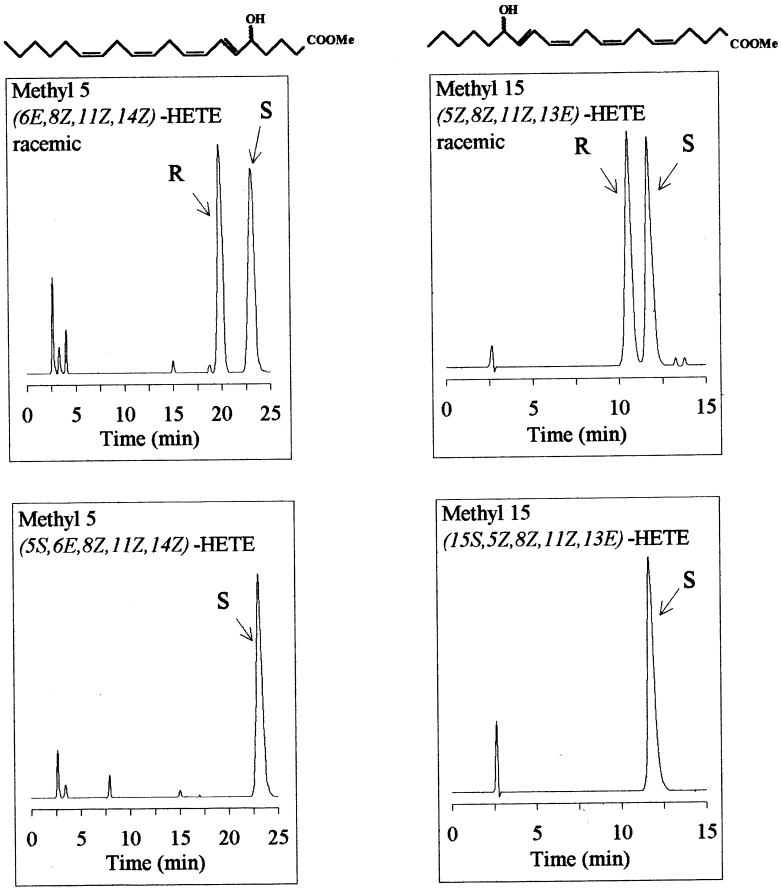

Fig. 4. Chiral HPLC traces of racemic and enzymatically generated methyl 5- and 15-HETE, separated on a Chiralcel OD-H column. Detection was monitored at 235 nm.

lead to false attribution. It should also be noted that the *R* enantiomer is eluted first in all cases tested. From the study of methyl HODEs, it could be seen that all-*trans* compounds are less well resolved than their *cis–trans* homologues. We also note that the more remote from the carboxylic acid function the hydroxyl group is the lower is the resolution (see especially methyl 15-HETE versus methyl 5-HETE). In conclusion this work described an easy and reliable method for determining the stereospecificity of various PUFA oxygenases using the Chiralcel OD-H column in the normal-phase mode. It is also the first report of the resolution of all-*trans* derivatives of methyl 9- and 13-HODE and of methyl 9- and 13-HOTE.

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