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Quantitation of monohydroxy fatty acids by high-performance liquid chromatography with fluorescence detection

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ABSTRACT

A high-performance liquid chromatographic (HPLC) procedure for the separation of hydroxyeicosatetraenoic acids (HETEs) and hydroxyoctadecanoic acids (HODEs) after derivatization of the hydroxy group with 1-anthroylnitrile is described. Anthroyl esters of HETEs were separated from those of HODEs by reversed-phase HPLC. The positional isomers of the HETEs and HODEs were well separated by normal-phase HPLC. The fluorimetric HPLC method has a high sensitivity and naturally occurring HETEs can be quantitatively analyzed at the picomolar level. The amount of 5-HETE in A23187-stimulated polymorphonuclear leukocytes (PMNLs) was determined by the present method. PMNLs produced approximately 150 ng of 5-HETE per 10^7 cells at 5 min stimulation. The amount of 5-HETE determined by fluorimetric detection was consistent with that determined by ultraviolet detection (235 nm).

INTRODUCTION

Hydroxyeicosatetraenoic acids (HETEs) are derived from arachidonic acid via the lipoxygenase pathway or via the cytochrome P450 catalytic cycle and have attracted attention because of their effects on stimulus–response coupling in endocrine, renal and secretory cells, as well as by their effect on leukocyte chemotaxis [1,2]. Furthermore, a number of potentially important effects, such as chemorepellant effects [3], enhancement of cell growth [4] and inhibition of cell adhesion [5], have recently been attributed to hydroxyoctadecadienoic acids (HODEs), which are metabolites of linoleic acid via the lipoxygenase or the P450 pathway.

The quantitative analysis of monohydroxyfatty acids such as HETEs and HODEs would allow us to gain new insights into the biological functions and metabolic reactions of poly-unsaturated fatty acids. However, only a few studies on HETEs and HODEs have been reported, in spite of the numerous publications on leukotrienes and prostaglandins, which are also oxidized metabolites of arachidonic acid. The major problem associated with such studies is the lack of sensitivity and specificity of the current methods in the quantitation of trace amounts of HETEs and HODEs.

Reversed-phase and/or normal-phase HPLC allows good resolution of the positional isomers of a number of monohydroxy fatty acids of synthetic and natural origin [6]. The most commonly used wavelength for the detection of HETEs is 234 nm, which reflects the presence of a conjugated diene. This non-destructive method of detection is convenient. However, the sensitivity is not sufficient for estimations of small amounts of HETEs and HODEs in biological

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samples. In order to improve the detectability of these compounds, several procedures for UV derivatization or fluorescence tagging of these compounds have been developed and are widely used in the analysis of free fatty acids [6]. The UV derivatization of a carboxyl group of a monohydroxy fatty acid using phenyl [6], phenacyl [7] and nitrobenzoyl compounds [8], has been reported.

Fluorescent reagents that allow highly sensitive detection are required for measurement of picomolar levels of fatty acids. A suitable derivatization of the hydroxyl group of a monohydroxy fatty acid, in addition to its carboxyl group, with the fluorescent reagent 1-anthroylnitrile has been proposed since the abundant free fatty acids in natural tissues do not react with this fluorescent probe.

In the present report, we describe the introduction of a fluorescent probe at the hydroxyl groups of HETEs and HODEs as a new derivatization method that permits the quantitation of monohydroxy fatty acids at the picomole level.

EXPERIMENTAL

Reagents and chemicals

Arachidonic acid, linoleic acid, ricinoleic acid (12-hydroxy-*cis*-9-octadecenoic acid), 12-HETE and 15-HETE were obtained from Funakosi (Tokyo, Japan). 5-HETE was kindly donated by Ono Pharmaceutical (Osaka, Japan). 1-Anthroylnitrile and solvents of HPLC grade were purchased from Wako (Osaka, Japan). Quinuclidine-HCl was from Nakarai (Tokyo, Japan). Minimum essential medium was obtained from Nissui (Tokyo, Japan). A23187, a typical calcium ionophore was from Sigma (St. Louis, MO, USA).

Preparation of 9- and 13-hydroxy-octadecadienoic acid

HODEs were prepared by the autooxidation of linoleic acid as described by Peers *et al.* [9]. In brief, linoleic acid (10 mg) was autooxidized in the presence of tocopherol (0.5 mg) in the dark at 40°C for 40 h. The hydroperoxide resulting from the oxidation was reduced at 0°C for 20 min by the addition of 10 mg of sodium borohydride in 2 ml of methanol. The pH of the reaction mixture was adjusted to 3.0 by the addition of 0.1 *M* HCl and then the HODEs were extracted twice with ethyl acetate. HODEs were purified by HPLC on a silica-gel column (LiChrosorb Si-60; Merck, Darmstadt, Germany) and eluted with hexane–isopropanol–acetic acid (99:1:0.1, v/v/v) at a flow-rate of 2 ml/min. The structures of the purified HODEs were identified by gas chromatography–mass spectrometry (GC–MS) of the trimethylsilyl derivatives.

Preparation of the 1-anthroyl esters of HETEs and HODEs

Derivatization of HETEs and HODEs to anthroyl esters was performed essentially as described by Goto *et al.* [10]. A standard mixture of HETEs prepared from casein-induced polymorphonuclear leukocytes (PMNLs) from the rat was dissolved in 50 μl of anhydrous acetonitrile that contained 1-anthroylnitrile (100 μg). The reaction mixture was mixed well after the addition of 50 μl of acetonitrile that contained 80 μg of quinuclidine and was incubated for 30 min at 60°C. The reaction was terminated by the addition of 100 μl of methanol. The solvent was evaporated under a stream of nitrogen and the residue was reconstituted in methanol. Anthroyl esters of the HETEs and HODEs were purified by silica-gel TLC (silica-gel G, Merck), developed with toluene–ethyl acetate (9:3, v/v) and detected with primuline (Nacalai Tesque, Tokyo, Japan). In this system, 1-anthroyl derivatives of HETEs or HODEs can be separated from unreacted and decomposed reagent. The bands of the 1-anthroyl esters of the HETEs and HODEs were scraped from the plate and extracted by the method of Bligh and Dyer [11].

Separation of the 1-anthroyl esters of monohydroxy fatty acids by HPLC

The separation of the 1-anthroyl esters of monohydroxy-fatty acids was performed on a reversed-phase column (LiChrosorb RP-18; 240 × 0.4 mm I.D., Merck). The mobile phase was acetonitrile–methanol–water–acetic acid (330:110:55:0.5, v/v). The flow-rate was 1.0 ml/min and peaks were detected with a fluorescence

detector (excitation, 380 nm; emission, 460 nm; 821-FP; Jasco, Tokyo, Japan). For the separation of derivatives of monohydroxyfatty acids on a normal-phase column (LiChrosorb Si-60), isocratic elution was performed with hexane–cyclohexane–diethyl ether–acetic acid (150:150:30:0.4, v/v) at a flow-rate of 1.0 ml/min.

Quantitation of 5-HETE in A23187-stimulated PMNL from the peritoneal cavity of a rat

Peritoneal PMNLs were obtained from a peritoneal exudate 16 h after the intraperitoneal instillation of a 5% solution of casein [12]. The PMNLs were harvested by washing the peritoneal cavity with saline. Cells were transferred to plastic tubes and washed twice with saline. Red blood cells were removed by hypnotic lysis. PMNLs were suspended in 10 m*M* Hepes–Eagle's minimum essential medium (MEM; pH 7.2) at a concentration of $2 \cdot 10^7$ cells/ml. The release of 5-HETE was examined after the incubation of PMNLs with 5 μM A23187 for timed periods. Each reaction was terminated by the addition of methanol–chloroform (2:1, v/v). Ricinoleic acid (200 ng) was added to the incubation mixture as an internal standard. Total lipids were extracted by the method of Bligh and Dyer and 5-HETE was separated by TLC developed with chloroform–methanol–acetic acid (95:5:1, v/v/v). The band of 5-HETE was scraped from the plate and extracted by the method of Bligh and Dyer. 5-HETE was converted to its 1-anthroyl ester and quantitated by fluorescence HPLC as described above.

RESULTS AND DISCUSSION

Anthroyl nitrile is a favorite reagent for the formation of hydroxy esters of diacylglycerol [13] and bile acids [10]. In the present experiment, more than 95% of the monohydroxy fatty acids were converted to anthroyl esters after a 30-min reaction period. Typical chromatograms after reversed-phase HPLC of anthroyl esters of a synthetic mixture of monohydroxy fatty acids are shown in Fig. 1. Anthroyl esters of HETEs, HODEs and ricinoleic acid were separated by reversed-phase HPLC with isocratic elution with

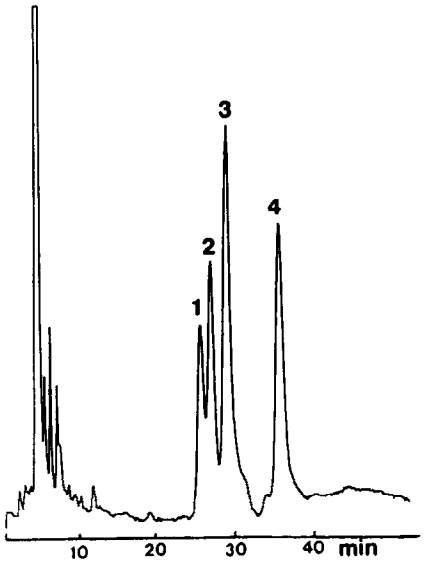

Fig. 1. Chromatograms of 1-anthroyl derivatives of synthetic HETEs and HODEs after reversed-phase HPLC. A mixture of derivatives of HETEs containing 5-HETE and 15-HETE (1), 12-HETE (2), HODEs (9- and 13-isomers) (3), and ricinoleic acid (4) was injected onto a LiChrosorb ODS column for reversed-phase HPLC.

acetonitrile–methanol–water–acetic acid (Fig. 1). Reversed-phase chromatography is useful for the separation of fatty acids of different chain lengths and/or different numbers of double bonds, although positional isomers of HETEs and HODEs were also partially resolved by this condition. The isomeric anthroyl esters of 15-, 12- and 5-HETEs were well separated by adsorption chromatography on a silica-gel column (Fig. 2A) and isomers of HODEs were also resolved (Fig. 2B). Ricinoleic acid eluted with the same retention time as 13-HODE. Fig. 2C shows the elution profile of a mixture of HETEs and HODEs. The retention times of 12-HETE and 13-HODE were very similar and the two compounds were not separated under the present conditions. The combination of a silica-gel column and an ODS column could allow the complete separation of the individual isomers of different derivatives of monohydroxy fatty acids from natural sources. Similar separations were obtained with the phenyl esters of the oxidation products of linoleate [7], the phenacyl esters of

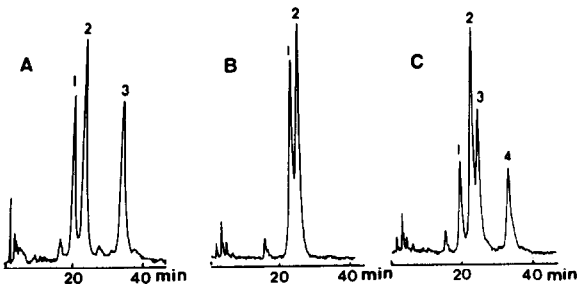

Fig. 2. Chromatograms of 1-anthroyl derivatives of synthetic HETEs and HODEs after normal-phase HPLC. (A) A mixture of derivatives of HETEs containing 15-HETE (1), 12-HETE (2) and 5-HETE (3). (B) A mixture of derivatives of HODEs containing 13-HODE (1) and 9-HODE (2). (C) A mixture of HETEs and HODEs containing 15-HETE (1), 12-HETE (2), 13-HODE (2), 9-HODE (3) and 5-HETE (4).

oxidized arachidonic acids [8] and *p*-nitrobenzyl ester derivatives [14].

Linear relationships were observed between the ratios of the peak areas of the HETE derivatives to that of the ricinoleic acid derivative and the amounts of HETE (data not shown). This result indicates that ricinoleic acid can be used as a suitable internal standard. A calibration curve was prepared by using the anthroyl ester of ricinoleic acid. A linear relationship between the peak area and the amount of the anthroyl ester was obtained in the range of 10 pg–1 μg (data not shown). Each value determined from five experiments had a difference from the mean value of 5%. In contrast, the limit of detection by the absorbance of the conjugated diene at 235 nm was 1–5 ng. These results indicate that a reproducible estimation of monohydroxy fatty acids at the picomolar level is possible using the derivatization to anthroyl esters. Thus, low levels of monohydroxyl fatty acids in biological tissues can be quantitated accurately by the present method.

In order to investigate the applicability of the present method at the cellular level, the anthroyl ester of 5-HETE prepared from A23187-stimulated PMNL was separated and quantitated by reversed-phase HPLC (Fig. 3). A typical chromatogram of the anthroyl derivatives of the HETEs extracted from PMNL is shown in Fig. 3. There were no interfering peaks near the peaks of the derivatives of 5-HETE and the ricinoleic

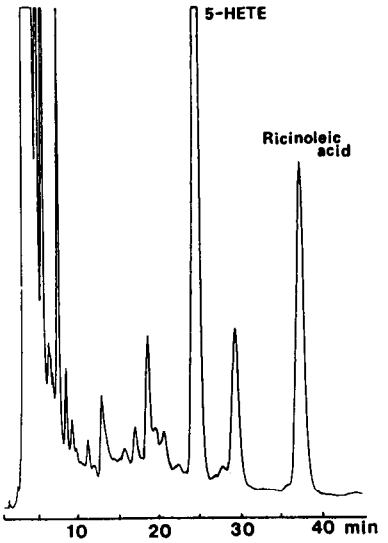

Fig. 3. A typical chromatogram of the anthroyl ester of 5-HETE prepared from A23187-stimulated PMNL. PMNL obtained from the peritoneal cavity of a rat were stimulated with 5 μ*M* A23187 for 5 min. Monohydroxy fatty acids were derivatized to anthroyl esters and separated by reversed-phase HPLC, as described in Experimental. Ricinoleic acid was included as an internal standard.

acid derivatives. The anthroyl ester of 5-HETE was identified on the basis of its retention time in reversed-phase and normal-phase HPLC, compared with that of the standard compound. Peritoneal PMNL of rat produced a significant amount of 5-HETE in response to stimulation by A23187, but no detectable amounts of HODEs were found in A23187-stimulated PMNL. No presence of other HETEs was observed when the HETE fraction from activated PMNL was separated by normal-phase HPLC (data not shown). Addition of A23187 to PMNL stimulated the rapid and transient formation of 5-HETE (Fig. 4). The amount of 5-HETE increased rapidly and reached a maximum after a 5-min stimulation. PMNL produced approximately 150 ng of 5-HETE per 10^7 cells within 5 min after the challenge with A23187. The amount of 5-HETE found in A23187-stimulated PMNL was in good agreement with previous results [15]. The precision of the measurement was established by repeated determinations (n = 10) with an internal standard. The coefficient of variation was less than 5%. The profile of the

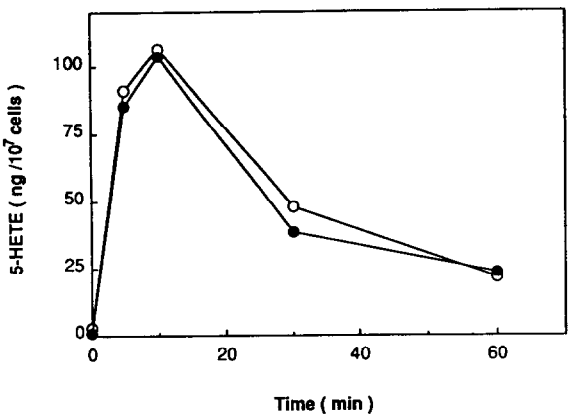

Fig. 4. Time course of the production of 5-HETE by A23187-stimulated PMNL. PMNL were stimulated with 5 μM A23187 for indicated periods of time. Monohydroxy fatty acids were separated by TLC and directly injected onto a column for reversed-phase HPLC. 5-HETE was monitored and quantitated by UV absorption at 235 nm (open circles). 5-HETE was derivatized to its anthroyl ester and quantitated by monitoring fluorescence (closed circles).

production of 5-HETE, determined by the method of fluorescence derivatization, was consistent with that determined by monitoring the UV absorbance (235 nm) (Fig. 4).

The present HPLC method with the fluorimetric detection, using 1-anthroyl nitrile, has a sensitivity satisfactory for the quantitative analysis of naturally occurring monohydroxy fatty acids, such as HETEs and HODEs. The high sensitivity will allow the reliable determination of low levels of monohydroxy fatty acids in natural tissues. The method is simple to perform and can, therefore, be applied to routine analysis.

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