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ARACHIDONIC ACID METABOLITES FROM POLYMORPHONUCLEAR LEUKOCYTES OF HEALTHY DONORS, SEVERELY BURNED PATIENTS AND CHILDREN WITH CYSTIC FIBROSIS — ROUTINE MONITORING BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A rapid and sensitive high-performance liquid chromatographic method for the determination of arachidonic acid metabolites was developed. This method provides a clear and simple separation of the ω-oxidation products as well as of leukotriene B_4 and 5*S*,12*S*-dihydroxy-6-*cis*-8-*trans*-10-*trans*-14-*cis*-eicosatetraenoic acid. Furthermore, a solvent switch enables the detection of the monohydroxyeicosatetraenoic acids together with the cysteinyl-leukotrienes as well as leukotriene B_4 and the dihydroxyeicosatetraenoic acids within 70 min (including column equilibration for 10 min). The efficiency of the method for the routine monitoring of leukotrienes was performed with human polymorphonuclear leukocytes of healthy donors, severely burned patients and of children with cystic fibrosis. The advantages of this method include high sensitivity and easy handling, which are important for routine analyses of the arachidonic acid metabolites.

INTRODUCTION

Arachidonic acid metabolites, such as prostaglandins, leukotrienes and monohydroxyeicosatetraenoic acids (mono-HETEs) are important mediators of inflammation and allergy [1–3]. The discovery of leukotrienes as a new class of arachidonic acid metabolites has led to many reports concerning the analysis of these substances by high-performance liquid chromatography (HPLC) [4–15]. The HPLC method was used for the isolation, characterization and also for the quantitation of arachidonic acid-derived metabolites by UV absorption, which enables their detection in picomolar amounts. The advantages of the radioimmunoassy (RIA) are its high specificity and sensitivity, but this technique requires either sample purification or separation of the substances by

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chromatographic procedures (e.g. post-column RIA) and, more importantly, a separate procedure for each metabolite. The major advantage of the HPLC technique is the possibility of determining the complete profile of the arachidonic acid metabolites; furthermore it allows the simultaneous detection of most the leukotrienes (together with their metabolites) [11].

The purpose of our study was to establish a rapid and sensitive HPLC method for the determination of the cysteinyl-leukotrienes and leukotriene B_4 (LTB_4) together with its ω-oxidation products and the non-enzymatically generated 6-*trans*- and 12-*epi*-6-*trans*-LTB_4, 5*S*,12*S*-dihydroxy-6-*cis*-8-*trans*-10-*trans*-14-*cis*-eicosatetraenoic acid (5*S*,12*S*-di-HETE) and of the 5-, 12- and 15-mono-HETEs. The efficiency of this method was demonstrated by the stimulation profile of cell-derived leukotrienes.

EXPERIMENTAL

Reagents

The cysteinyl-leukotrienes, LTB_4, 5*S*,12*R*,20-hydroxy-6,14-*cis*-8,10-*trans*-eicosatetraenoic acid (20-OH-LTB_4) and 5*S*,12*R*-dihydroxy-20-carboxy-6,14-*cis*-8,10-*trans*-eicosatetraenoic acid (20-COOH-LTB_4) were a generous gift from Dr. Rokach (Merck Frosst, Pointe-Claire, Canada). 5*S*,12*S*-di-HETE and the mono-HETEs were purchased from Calbiochem (Frankfurt, F.R.G.).

Acetonitrile (HPLC grade) was purchased from Baker Chemicals (Gross-Gerau, F.R.G.) and methanol (p.a.), ethylenediaminetetraacetic acid, dipotassium hydrogenphosphate and phosphoric acid were from Riedel de Haën (Seelze, F.R.G.).

Ficoll 400 was obtained from Pharmacia (Uppsala, Sweden), Macrodex 6% (w/v) from Knoll (Ludwigshafen, F.R.G.) and sodium metrizoate solution 75% (w/v) from Nyegaard (Oslo, Norway).

Buffer

The phosphate-buffered saline (PBS) comprised 120 m*M* sodium chloride, 10 m*M* disodium hydrogenphosphate and 3 m*M* potassium dihydrogenphosphate, adjusted to pH 7.4.

Cell preparation

Human polymorphonuclear leukocytes (PMNLs) were obtained from 200 ml of heparinized blood from healthy donors (or from patients), which was separated on a Ficoll-metrizoate gradient followed by dextran sedimentation [16]. Prior to dextran sedimentation, the serum was centrifuged for 10 min in order to remove the platelets. The neutrophils were resuspended to a final concentration of $2 \cdot 10^7$ cells/ml in PBS.

Stimulation of PMNLs

(A) In the presence of 0.8 m*M* calcium chloride and 0.8 m*M* magnesium chloride, the granulocytes ($1 \cdot 10^7$) were stimulated with the Ca-ionophore A23187

(Sigma, Munich, F.R.G.) at a concentration of 7.3 μM for 5, 20, and 30 min at 37°C.

(B) The granulocytes ($1 \cdot 10^7$) were costimulated with arachidonic acid (60 μM) and with the Ca-ionophore (7.3 μM) for 5 and 20 min at 37°C in the presence of 0.8 mM calcium chloride and 0.8 mM magnesium chloride.

The final volume of the reaction mixture was 600 μl.

Sample preparation for HPLC

After incubation, the cells were centrifuged at 600 g, and the supernatant was dissolved in 3 ml of acetonitrile–methanol (50:50, v/v), lyophilised, resuspended in 600 μl of methanol–water (30:70, v/v) overlayered with argon and stored at −70°C until the HPLC analyses.

Apparatus

Reversed-phase HPLC was carried out with a Constametric III G-pump and two variable-wavelength detectors, Spectromonitor D (LDC Milton Roy, Hasselroth, F.R.G.), a Waters intelligent sample processor (Wisp Model 310 B) (Waters, Königsstein/Taunus, F.R.G.), a Nelson analytical interface (Series 760) and Nelson analytical software revision 3.6 (Analysensysteme, Wuppertal, F.R.G.) as well as a Lee LFYA three-way valve (Lee, Frankfurt, F.R.G.).

The autosampler and the UV detectors, as well as the low-pressure valve, were connected to the interface that acquires data and stores it in a local buffer memory. The intelligent interface also contains relays that can be programmed by the host computer to control external devices. The interface was controlled by the Nelson analytical software: instructions specifying the data rate, analysis time and other information such as the time events that switch the relays on and off during the analysis.

HPLC procedure

Solvent A was a mixture of phosphate buffer (17 mM dipotassium hydrogenphosphate, containing 0.05% EDTA, adjusted to pH 5.0 with phosphoric acid) acetonitrile and methanol (50:30:20,v/v). Solvent B was a mixture of phosphate buffer (6 mM dipotassium hydrogenphosphate, containing 0.05% EDTA, adjusted to pH 5.0 with phosphoric acid) acetonitrile and methanol (28:42:30, v/v).

Leukotrienes were separated at a flow-rate of 1 ml/min on a 200 mm × 4.6 mm I.D. column filled with Nucleosil $5C_{18}$ maintained at 40°C. The effluent was monitored at 280 nm as well as 235 nm (HETEs) at 0.005 a.u.f.s. The injection volume was 200 μl.

Leukotrienes were quantified using peak areas. Standard curves of the individual substances were obtained with five different concentrations (2–500 ng). For example, the correlation coefficient for LTC_4 was 0.978 and 0.998 for the ω-oxidation products, respectively. The between-day coefficient of variation (C.V.) was between 1.2 and 2.8%. Possible variations of the elution pattern were corrected by injection of a standard solution after twenty samples.

Solvent flow was controlled by a low-pressure three-way valve (internal volume 7.4 μl), which was switched over at time events given by the interface. The

variable-wavelength detectors were also controlled by the time events of the interface: a wavelength switch was performed 4 min after the switch from solvent A (280 nm) to solvent B (235 nm).

Solvent A was used to separate the cysteinyl-leukotrienes together with their 11-*trans*-isomers, LTB_4, 5*S*,12*S*-LTB_4 and the non-enzymatic-generated LTB_4 isomers, as well as the ω-oxidation products. Under these conditions, the HETEs were not eluted from the column.

Rapid analysis of the mono-HETEs can be performed with solvent B, if a determination of only these substances is required.

UV spectrophotometry

UV spectra were recorded in methanol using a Lambda 5 UV/VIS spectrophotometer (Perkin Elmer, Düsseldorf, F.R.G.).

RESULTS

Chromatography

The described HPLC method allows the simultaneous analysis of LTC_4, LTD_4, LTE_4, LTB_4, 6-*trans*-LTB_4, 12-*epi*-6-*trans*-LTB_4, 5*S*,12*S*-di-HETE and the metabolites (ω-oxidation) of LTB_4, 20-hydroxy-LTB_4 and 20-carboxy-LTB_4 (solvent A, detection at 280 nm), as well as the mono-HETEs 5-, 12-, and 15-HETE (solvent B, detection at 235 nm) with a detection limit of ca. 1 ng/ml.

The cysteinyl-leukotrienes and their 11-*trans*-isomers (α factors = 1.16 with respect to their *cis*-isomers) were cleary separated. Remarkably, LTE_4 elutes between LTD_4 and 6-*trans*-LTB_4, which is in contrast to previously reported investigations (solvent, methanol–water–acetic acid, 65:35:0.05), and may be attributed to the pH value (cysteinyl-leukotrienes are more affected by a pH change than are HETEs and di-HETEs). The elution pattern was confirmed by analysing with synthetic leukotrienes.

The recovery of the cysteinyl-leukotrienes was $89 \pm 5\%$ ($n = 13$), $91 \pm 2\%$ ($n = 16$) for LTB_4 and its ω-oxidation products and $86 \pm 5\%$ ($n = 7$) for HETEs.

The heated column prevents the retention times fluctuating.

Generation profile of stimulated cells

The efficiency of the described HPLC method is illustrated with the chromatograms that show the stimulation profile of cell-derived leukotrienes. Our emphasis was directed towards the clear separation of the various compounds. Sample cells were taken from patients with various disease processes.

Fig. 1 shows a chromatogram which was obtained after incubation for 5 min of PMNLs ($1 \cdot 10^7$) of a normal donor with the Ca-ionophore. A good separation of the ω-oxidation products of LTB_4 was obtained (inserts of Figs. 2–5). The more careful analysis of the ω-oxidation range revealed, in addition to the peaks of 20-COOH-LTB_4 and 20-OH-LTB_4, two unknown products: product X, eluting between 20-COOH-LTB_4 and 20-OH-LTB_4, and product Y; these peaks were observed in the chromatograms of stimulated PMNLs of patients, as well as with normal donors. In addition to the ω-oxidation products, small amounts of LTC_4

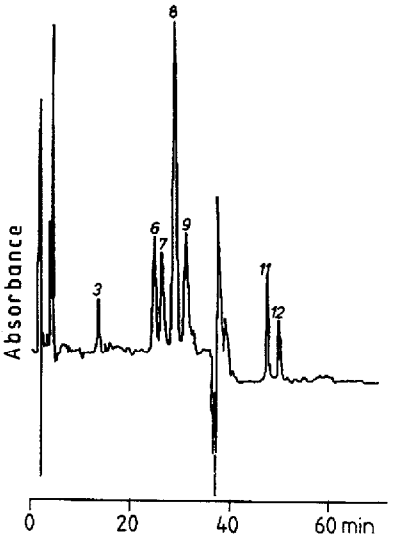

Fig. 1. HPLC analysis of a cell supernatant [PMNLs ($1 \cdot 10^7$) from a healthy donor stimulated with Ca-ionophore (7.3 μM) for 5 min]. Peaks: 3 = LTC_4; 6 = 6-*trans*-LTB_4; 7 = 12-*epi*-6-*trans*-LTB_4; 8 = LTB_4; 9 = 5*S*,12*S*-di-HETE; 11 = 12-HETE; 12 = 5-HETE. The time for analysis was 70 min. Solvent A was phosphate buffer-acetonitrile-methanol (50:30:20) and solvent B was phosphate buffer-acetonitrile-methanol (28:42:30). Solvent change was performed at 34 min and wavelength switch at 38 min. Attenuation settings of the UV photometers were 0.005 a.u.f.s. at 280 nm and 235 nm.

and LTE_4 as well as the typical pattern of the generated di-HETEs with 6-*trans*-LTB_4 and 12-*epi*-6-*trans*- LTB_4 and 5*S*,12*S*-di-HETE were detected in large amounts. The peaks of LTB_4 ($\lambda_{max}^{MeOH} = 270.9$ nm) and of 5*S*,12*S*-di-HETE ($\lambda_{max}^{MeOH} = 269.0$ nm) were collected, dried under a stream of nitrogen and rechromatographed on normal-phase material. Comparison with synthetic standards confirmed the separation of LTB_4 and 5*S*,12*S*-di-HETE with the described method. The lower content of organic solvent in the elution solvent, in combination with the increased column temperature (40°C), enabled the separation of LTB_4 from the 5*S*,12*S*-di-HETE. In the past, these compounds had been separated only by normal-phase silica materials or after derivatization, e.g. with diazomethane, on Nucleosil C_{18} [17]. Furthermore, elution with solvent B enabled the detection of 12-HETE and 5-HETE, which were also detected in large amounts. The amounts of PMNLs ($1 \cdot 10^7$) ranged between 100 and 300 ng.

The analysis of biological fluids or cell supernatants may be performed with solvent B only, in order to make a quick survey of the amount of the HETEs, if it is only the content of these monohydroxy acids that is of interest.

Fig. 2. shows a representative chromatogram obtained with PMNLs after Ca-ionophore stimulation (incubation for 30 min at 37°C). The arachidonic metabolites were eluted from the column in the following sequence (the mono-HETEs were not detected): 20-COOH-LTB_4 (99.2 ng), 20-OH-LTB_4 (189.7 ng), LTC_4 (23 ng), LTD_4 (not detected), LTE_4 (13 ng), 6-*trans*-LTB_4 (35.2 ng), 12-*epi*-6-

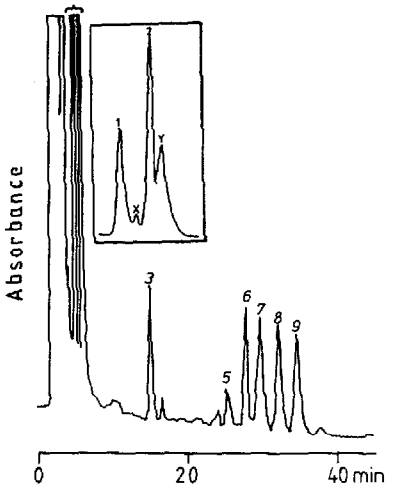

Fig. 2. HPLC analysis of a cell supernatant [PMNLs ($1 \cdot 10^7$) from a healthy donor stimulated with Ca-ionophore (7.3 μM) for 3 min]. Peaks: 3 = TLC_4; 5 = LTE_4; 6 = 6-*trans*-LTB_4; 7 = 12-*epi*-6-*trans*-LTB_4; 8 = LTB_4; 9 = 5*S*,12*S*-di-HETE. The time for analysis was 45 min. The elution solvent was phosphate buffer–acetonitrile–methanol (50:30:20). The attenuation setting of the UV photometer was 0.005 a.u.f.s. at 280 nm. The inset shows the extended ω-oxidation region (marked by a bracket). Peaks: 1 = 20-COOH-TLB_4; 2 = 20-OH-LTB_4; X and Y = unknown products.

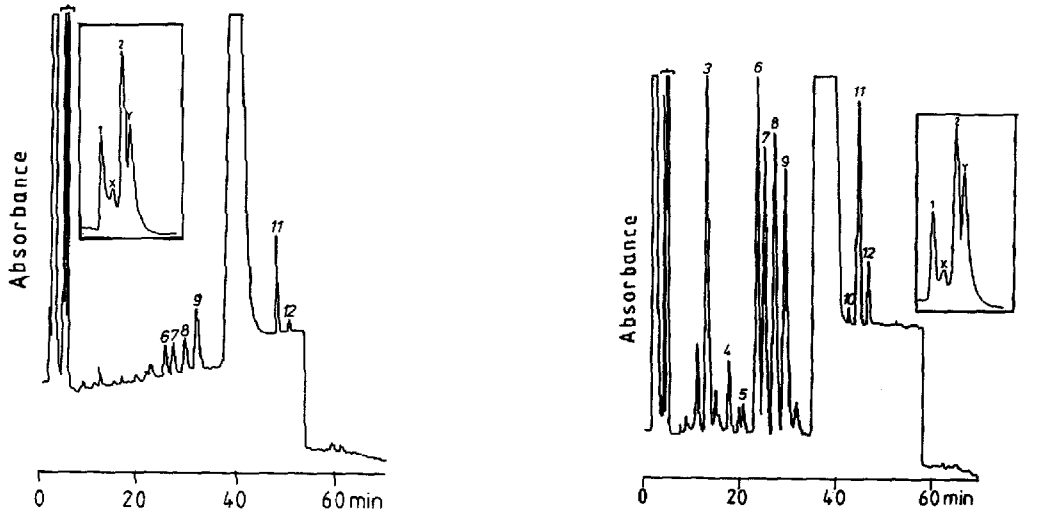

Fig. 3. HPLC analysis of a cell supernatant [PMNLs ($1 \cdot 10^7$) from a severely burned patient stimulated with Ca-ionophore (7.3 μM) for 20 min]. Peaks: 6 = 6-*trans*-LTB_4; 7 = 12-*epi*-6-*trans*-LTB_4; 8 = LTB_4; 9 = 5*S*,12*S*-di-HETE; 11 = 12-HETE; 12 = 5-HETE. The time for analysis was 70 min. For experimental conditions see Fig. 1. Inset details as in Fig. 2.

Fig. 4. HPLC analysis of a cell supernatant [PMNLs ($1 \cdot 10^7$) from a severely burned patient stimulated with Ca-ionophore (7.3 μM) and arachidonic acid (60 μM) for 20 min]. Peaks: 3 = LTC_4; 4 = LTD_4; 5 = LTE_4; 6 = 6-*trans*-LTB_4; 7 = 12-*epi*-6-*trans*-LTB_4; 8 = LTB_4; 9 = 5*S*,12*S*-di-HETE; 10 = 15-HETE; 11 = 12-HETE; 12 = 5-HETE. The time for analysis was 70 min. For experimental conditions see Fig. 1. Inset details as in Fig. 2.

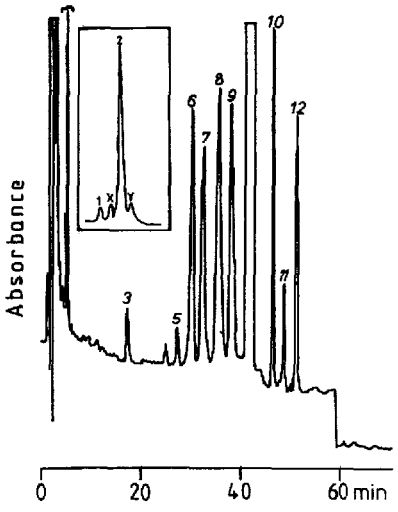

Fig. 5. HPLC analysis of a cell supernatant [PMNLs ($1 \cdot 10^7$) from a child with cystic fibrosis stimulated with Ca-ionophore (7.3 μM) and arachidonic acid (60 μM) for 5 min]. Peaks: 3 = LTC_4; 5 = LTE_4; 6 = 6-*trans*-LTB_4; 7 = 12-*epi*-6-*trans*-LTB_4; 8 = LTB_4; 9 = 5*S*,12*S*-di-HETE; 10 = 15-HETE; 11 = 12-HETE; 12 = 5-HETE. The time for analysis was 70 min. For experimental conditions see Fig. 1. Inset details as in Fig. 2.

trans-LTB_4 (35.5 ng), LTB_4 (33.7 ng), as well as 5*S*,12*S*-di-HETE (34 ng). The inset shows the extended ω-oxidation region of the chromatogram as was obtained in Fig. 2 with the following elution times: 5.30 min for 20-COOH-LTB_4 and 6.13 min for 20-OH-LTB_4. Large amounts of 20-COOH-LTB_4 and 20-OH-LTB_4 were detected after incubation for 30 min compared with those obtained after incubation for 5 min: more pronounced ω-oxidation was obtained after 30 min, which is confirmed by the small amount of LTB_4, which had become less than the amounts of the non-enzymatically generated isomers, although the sum of the ω-oxidation products and LTB_4 remained constant.

This method was applied to routine monitoring of leukotrienes in clinical studies with human PMNLs of severely burned patients, stimulated with the Ca-ionophore, as shown in Figs. 3 and 4. Fig. 4 shows the pattern of the generated arachidonic acid metabolites after costimulation with the Ca-ionophore and arachidonic acid, compared with those cells stimulated only with the Ca-ionophore (Fig. 3). Previous experiments [18–20] provided evidence for a disturbed granulocyte function during arachidonic acid metabolism: the amounts of LTB_4 released in response to the Ca-ionophore were much smaller compared with healthy donors. Fig. 3 shows the small amounts of generated LTB_4 (in the range of the biologically inactive 6-*trans* and 12-*epi*-6-*trans*-isomers) and increased ω-oxidation (Fig. 3, inset). Furthermore, 5*S*,12*S*-di-HETE and 12-HETE and 5-HETE could be detected in somewhat larger amounts. Analysis of the supernatant from cells costimulated with arachidonic acid (Fig. 4) revealed a higher generation of arachidonic acid metabolites: the generation of the isomers was increased compared with LTB_4 release; in addition, the generation of 12-HETE exceeded the

amounts of 5-HETE and 15-HETE. The pattern supported the previously obtained data [20], which suggested a disturbed granulocyte function.

It has been shown that granulocytes obtained from patients with cystic fibrosis and recurrent pseudomonas infections revealed impaired granulocyte functions. Experiments were performed to confirm the altered arachidonic acid metabolism of granulocytes from children with cystic fibrosis after Ca-ionophore stimulation.

Stimulation of cells from children suffering from cystic fibrosis with the Ca-ionophore alone or together with arachidonic acid provided a pattern that is not so different from that obtained with healthy donors. Increased ω-oxidation was detected with a high ratio of 20-OH-TLB_4 to 20-COOH-LTB_4 (Fig. 5, inset), but the overall synthesis of the leukotrienes was hardly reduced. Costimulation with arachidonic acid (Fig. 5) showed large amounts of 5-HETE and 15-HETE as compared with 12-HETE, whereas cells that were stimulated with arachidonic acid alone revealed large amount of 12-HETE only (data not shown).

DISCUSSION

HPLC is an important tool for the isolation and identification of the leukotrienes [21,22] owing to the UV absorption of the conjugated triene chromophore. Mathews et al. [4] reported a convenient method for the analysis of cysteinyl-leukotrienes together with LTB_4 and its isomers. They also indicated the relationship between the pH values and the polarity (content of the organic solution) of the solvent system as well as the retention times of these compounds. Further reports on the HPLC analysis of arachidonic acid metabolites dealt with gradient elution techniques. [9,11,24], and with derivatization procedures (methyl ester derivatives) [5,17,23]. The aims of chromatographic analysis were extended to include the detection of two ω-oxidation products [5,14,17], mono-HETEs [9,11] and the separation of 5*S*,12*S*-di-HETE from LTB_4 [5,11,17]. Sample preparation comprised extraction with Sep-Pak C_{18} cartridges [14], extraction procedures with organic solvents [8] and denaturation of the biological samples with acetonitrile–methanol [11].

The development of the HPLC method described here was directed towards a rapid analysis of arachidonic acid metabolites. Our special interest was focused on a simple separation of 20-COOH-LTB_4 and 20-OH-LTB_4, as well as of LTB_4 and 5*S*,12*S*-di-HETE. Furthermore, the detection of the mono-HETEs without the need for a difficult gradient system was another goal. According to Mathews et al. [4], the content of the organic solvent should be reduced to obtain a good separation of 20-COOH-LTB_4 and 20-OH-LTB_4, which had been previously performed with stimulated-cell-derived supernatants in a second HPLC analysis [18].

Acetonitrile, used with methanol as a second organic solvent, at first provided sufficient separation of the ω-oxidation products and especially of LTB_4 and 5*S*,12*S*-di-HETE. Furthermore, the use of acetonitrile led to acceptable retention times, and the column temperature could be increased and maintained at 40°C; this resulted in stable retention times.

This new method enables a rapid and convenient analysis of arachidonic acid metabolites within 35–40 min (without the detection of the mono-HETEs) using

an isocratic solvent system and a separation of the ω-oxidation products, as well as of LTB_4 and 5*S*,12*S*-di-HETE. Previously, 5*S*,12*S*-di-HETE could be separated only by derivatization and a subsequent separation on reversed-phase or normal-phase material or by gradient elution.

For the detection of the mono-HETEs the elution time was extended to 70 min, which included an equilibration time of 10 min. A rapid analysis of the mono-HETEs can be performed with solvent B, if only these substances are required to be determined.

The chromatograms shown here demonstrate the efficiency of this new method for the analysis of the arachidonic acid metabolites in cell supernatants of normal donors as well as of patients. Furthermore, the method was also employed for the determination of these mediators in plasma, urine and other biological fluids (data not shown).

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