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Journal of Chromatography B, 819 (2005) 323–328
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Improved enantioselective analysis of polyunsaturated hydroxy fatty acids in psoriatic skin scales using high-performance liquid chromatography

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Received 26 November 2004; accepted 25 February 2005
Available online 19 March 2005

Abstract

Enantioselective analysis is used as a valuable tool for determining the biological origin of chiral derivatives of arachidonic, 11,14-eicosadienoic and linoleic acid in psoriatic skin scales and for clarifying their role in pathogenesis. This paper reports on a simple and rapid enantioselective determination (without any derivatization) of the fatty acid derivatives 13(*R*,*S*)-hydroxyoctadecadienoic acid [13(*R*,*S*)-HODE], 9(*R*,*S*)-hydroxyoctadecadienoic acid [9(*R*,*S*)-HODE] and 12(*R*,*S*)-hydroxyeicosatetraenoic acid [12(*R*,*S*)-HETE], using high-performance liquid chromatography (HPLC) with Chiralpak® AD as the chiral selector and electrospray ionisation mass spectrometry (ESI-MS). The enantiomeric distribution of 12-HETE, 9-HODE and 13-HODE in psoriatic skin scales of untreated patients (untreated during the last 4 weeks before sampling) was evaluated in comparison to psoriatic skin scales of patients underlying systemic treatment. The enantiomeric distribution of 12-HETE and 9-HODE showed no remarkable differences, whilst samples of patients under systemic treatment exhibited a lower predominance of 13(*S*)-HODE than samples of untreated patients. Furthermore, the effect of UVB phototherapy on the enantiomeric distribution of 12-HETE, 9-HODE and 13-HODE was studied and a semiquantitation of these compounds in psoriatic skin scales performed. The detected amounts of 9-HODE in samples of untreated patients were remarkably lower than those in samples of patients underlying systemic treatment. In the case of UVB phototherapy, no influence on the enantiomeric distribution could be observed.

Keywords: Enantioselective analysis; Psoriasis; Polyunsaturated hydroxy fatty acids; Chiralpak® AD; High-performance liquid chromatography; UVB; Systemic treatment

1. Introduction

Psoriasis is one of the most common chronic skin diseases, estimated to affect 2–3% of the European population. The most typical sites to be affected by psoriasis are the scalp, extensor surfaces of the elbows and knees and the lumbosacral region, although any area of the skin may be involved.

Hyperproliferation of epidermis and inflammation of the dermis and epidermis associated with the accumulation of activated proliferating lymphoid cells are pathologic characteristic of psoriasis. These changes are considered to be the result of the T-lymphocyte-mediated dermal immune response to unidentified antigenic stimuli [1].

In the last decade important developments have revolutionised the treatment of psoriasis. Various existing treatments were optimised by enhancing clinical efficiency and/or decreasing potential side effects.

Therapy of psoriasis generally can be divided in topical (e.g. Vitamin D3 analogues or corticosteroids), phototherapy

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doi:10.1016/j.jchromb.2005.02.008

(e.g. broad and narrow band UVB irradiation) and systemic (e.g. retinoids, methotrexate, cyclosporin A or fumaric acid esters).

A number of chiral polyunsaturated hydroxy fatty acids resulting from enzymatic metabolisation of arachidonic, linoleic and 11,14-eicosadienoic acid in these above mentioned hyperproliferative and inflammatory processes could be identified in psoriatic skin scales: 5-HETE, 8-HETE, 9-HETE, 11-HETE, 12-HETE, 15-HETE, 9-HODE, 13-HODE and 15-hydroxyeicosadienoic acid (15-HEDE), of which 12-HETE, 9-HODE and 13-HODE are by far the most abundant derivatives [2].

So far, only Baer et al. [3,4] reported on the enantiomeric distribution of 13-HODE in psoriatic skin scales. After derivatization with ethereal diazomethane, the methyl ester derivative was analysed using a HPLC system consisting of two columns in series with dinitrobenzoylphenyl glycine (DNBPG) stationary phase, but yielded only marginal resolution within retention times of nearly 1 h.

For baseline separation an additional derivatization of the hydroxyl group with 1-naphthoyl chloride or benzoyl chloride was required when this so called "Pirkle-type" stationary phase was used [5,6].

Chiral columns of the Chiralcel® class of derivatized cellulose based supports gave better resolution for the methyl ester derivatives, but several column types were necessary for covering a broad range of fatty acid derivatives [7–10].

Suzuki et al. [11] used phenylcarbamated β-cyclodextrin for separating 12-HETE enantiomers in urine of patients with diabetes mellitus with likewise no baseline resolution within retention times of nearly 1 h.

First Chiralpak® AD columns [tris-(3,5-dimethylphenylcarbamate)-derivatized amylose support] eluted with mixtures of *n*-hexane/methanol or ethanol could achieve impressive resolutions for a wide variety of fatty acid derivatives [12].

So far, no reports are available concerning the relationship between the enantiomeric ratios of polyunsaturated hydroxy fatty acids in psoriatic skin scales and different treatments of psoriasis.

In this paper a simple and direct analytical method (without any derivatization) for the enantioseparation of primary oxygenase products of arachidonic and linoleic acid in psoriatic skin scales is reported, using Chiralpak® AD as the chiral selector.

The enantiomeric distribution of 12-HETE, 9-HODE and 13-HODE was evaluated in psoriatic skin scales of untreated patients (untreated during the last 4 weeks before sampling) and patients under systemic treatment.

Furthermore, the effect of UVB phototherapy on the enantiomeric distribution of 12-HETE, 9-HODE and 13-HODE was studied and a semiquantitation of these compounds in psoriatic skin scales performed.

2. Experimental

2.1. Samples

Psoriatic skin scales were obtained from eight patients with plaque-stage psoriasis. A sample of heel stratum corneum was obtained from a healthy volunteer. All samples were stored at −20 °C until analysis.

2.2. Chemicals and reagents

5(*R*,*S*)-Hydroxyeicosatetraenoic acid [5(*R*,*S*)-HETE], 8(*R*,*S*)-hydroxyeicosatetraenoic acid [8(*R*,*S*)-HETE], 9(*R*,*S*)-hydroxyeicosatetraenoic acid [9(*R*,*S*)-HETE], 11(*R*,*S*)-hydroxyeicosatetraenoic acid [11(*R*,*S*)-HETE], 12(*R*,*S*)-hydroxyeicosatetraenoic acid [12(*R*,*S*)-HETE], 12(*R*)-hydroxyeicosatetraenoic acid [12(*R*)-HETE], 15(*R*,*S*)-hydroxyeicosatetraenoic acid [15(*R*,*S*)-HETE], 11(*R*,*S*)-hydroxyeicosadienoic acid [11(*R*,*S*)-HEDE], 15(*R*,*S*)-hydroxyeicosadienoic acid [15(*R*,*S*)-HEDE], 9(*R*,*S*)-hydroxyoctadecadienoic acid [9(*R*,*S*)-HODE] and 13(*R*,*S*)-hydroxyoctadecadienoic acid [13(*R*,*S*)-HODE] were purchased from Cayman (Ann Arbor, USA).

9(*S*)-Hydroxyoctadecadienoic acid [9(*S*)-HODE] and 13(*S*)-hydroxyoctadecadienoic acid [13(*S*)-HODE] were obtained from Larodan (Malmö, Sweden). *n*-Hexane, methanol, ethanol and 2-propanol (all HPLC grade) were from Roth (Karlsruhe, Germany). Acetic acid and ethyl acetate were from Grüssing (Filsum, Germany) and sodium acetate anhydrous was from Merck (Darmstadt, Germany). Distilled water was purified, using a Milli-Q Plus purification system (Millipore, Molsheim, France).

2.3. Sample preparation

Psoriatic skin scale samples (45–238 mg) and sample of heel stratum corneum (55 mg) were vortexed with a mixture of 2–4 ml each of ethyl acetate and sodium acetate buffer (0.1 mol l^{-1}; pH 3.5). After centrifugation (10 min at 1500 × *g*) the ethyl acetate layer was removed quantitatively and a second partition with 2–4 ml ethyl acetate was carried out. The combined organic layers were vaporised under a nitrogen stream. The residue was taken up in 100–600 μl *n*-hexane and subjected to normal phase HPLC.

2.4. Instrumentation

2.4.1. HPLC

2.4.1.1. General instrumentation. The equipment consisted of a Waters 510 HPLC pump (Milford, USA), a 7725i Rheodyne injector (Rohnert Park, USA) with a 20 μl sample loop, a SecurityGuard™ cartridge (silica; 4 mm × 3 mm i.d.) from Phenomenex (Aschaffenburg, Germany) and a Merck Hitachi L-4500A diode array detector (Darmstadt, Germany). Data were analysed using D-6500 DAD system manager software from Hitachi (Tokyo, Japan).

All chromatographic runs were monitored at a wavelength of 235 nm (conjugated diene system) and measurements of the absorption maximum (λ_{max}) were performed after the run was completed.

2.4.1.2. Normal phase HPLC. A LiChrospher® Si 60 column with pure silica-gel as the stationary phase (Merck, Darmstadt, Germany; 250 mm × 4 mm I.D., 5 μm) was used. The column was eluted with *n*-hexane/2-propanol/acetic acid (100:2:0.1; v/v/v) at a flow-rate of 1 ml/min. The combined collected fractions were vaporised under a nitrogen stream. The residue was taken up in 70–150 μl *n*-hexane and subjected to enantioselective HPLC.

2.4.1.3. Enantioselective HPLC. A Chiralpak® AD column with amylose tris-(3,5-dimethylphenylcarbamate) coated on silica-gel as the chiral stationary phase (Chiral Technologies Europe, Illkirch, France; 250 mm × 4.6 mm I.D., 10 μm) was used. The column was eluted with *n*-hexane/ethanol/methanol/acetic acid (93:4:3:0.1; v/v/v/v) at a flow-rate of 1 ml/min.

2.4.2. ESI-MS

MS/MS fragmentation was performed on a quadrupole ion trap mass spectrometer LCQ (Thermo Finnigan, San Jose, USA) equipped with a nanoelectrospray ion source (Proxeon Biosystems A/S, Odense, Denmark). Approximately 3 μl of the analyte solutions (samples: solutions used for enantioselective HPLC were vaporised under a nitrogen stream and taken up in 50 μl ethanol; reference compounds: 0.2 μg/ml in ethanol) were loaded into laboratory-made, gold-coated glass capillaries and negative ions generated at a spray voltage between −900 and −1000 V. The temperature of the heated transfer-capillary was 180 °C, the capillary voltage was −30 V, the tube lens −15 V. Spectra were averaged over 10–20 scans. The relative collision energy for fragmentation was set between 20 and 28%.

2.4.3. UVB treatment

A Waldmann 3003 K light cabin (Schwenningen, Germany), emitting 2.5 mW/cm^2 broad band UVB (peak 310–315 nm) at a distance of 25 cm was used as the UVB light source. A Waldmann UV-meter (model no. 585100) was used to calibrate the light. The patient was treated with UVB irradiation doses of 0.5 J/cm^2 for 5 days.

2.5. Quantitation

Quantitation has been carried out using calibration curves (method of external standard) on LiChrospher® Si 60 column. Six calibration standards (solutions of reference compounds in *n*-hexane) were prepared, spanning an absolute concentration range of 0.02–1.0 μg. Based on peak area, the calibration curves were found to be linear over this concentration range. The calibration curve for 12-HETE was characterised by the equation $y = 2E+06x - 70336$ with a $r^2 = 0.999$, for 13-HODE by the equation $y = 2E+06x - 110975$ with a $r^2 = 0.998$ and for 9-HODE by the equation $y = 2E+06x - 371349$ with a $r^2 = 0.989$.

Usually, the parameters of method validation are determined by spiking analyte-free matrix with analytes of known quantity. By this way selectivity, sensitivity, linearity, recovery, precision and accuracy can be determined. In the case of psoriasis an analyte-free matrix is not available, as psoriatic skin scales always contain the title compounds.

Under these conditions a reliable method validation cannot be given. All data given are, therefore, semiquantitative and unvalidated.

3. Results and discussion

3.1. Chromatography

Table 1 shows the retention time and resolution of reference compounds investigated.

Merely 9-HETE could not be baseline separated, using a solvent of *n*-hexane/ethanol/methanol/acetic acid (93:4:3:0.1; v/v/v/v). However, a baseline separation ($R_S = 1.8$) can be obtained for 9-HETE, when *n*-hexane/methanol/acetic acid (95:5:0.1; v/v/v) as eluent is used (data not shown).

To the best of our knowledge, this paper reports on the first enantioseparation of 11-HEDE and 15-HEDE, using Chiralpak® AD column. 11-HEDE and 15-HEDE are the oxidised products of 11,14-eicosadienoic acid. Their biological activity has not been clearly documented.

Most chromatographic runs of the investigated extracts from psoriatic skin scales on LiChrospher® Si 60 column revealed three peaks with an ultraviolet spectrum showing a λ_{max} at 235 nm and retention times identical to those of 12-HETE, 9-HODE and 13-HODE reference compounds.

Further polyunsaturated hydroxy fatty acids as shown in Table 1 could not be detected in any psoriatic skin scale extracts investigated.

Table 1
Retention time (t_R) and resolution (R_s) of reference compounds (10 μg/ml *n*-hexane), using LiChrospher® Si 60 and Chiralpak® AD columns

Reference compound	t_R (min)		Elution order	R_s
	LiChrospher® Si 60	Chiralpak® AD		
5-HETE	39.05	8.06/11.77	n.d.	5.2
8-HETE	19.19	7.53/8.54	n.d.	1.8
9-HETE	15.64	7.15/7.43	n.d.	0.7
11-HETE	10.40	6.87/7.61	n.d.	1.5
12-HETE	6.28	7.06/7.68	*R* (I); *S* (II)	1.5
15-HETE	7.19	7.47/9.41	n.d.	3.6
9-HODE	18.85	9.56/14.20	*R* (I); *S* (II)	5.3
13-HODE	10.95	7.94/17.20	*R* (I); *S* (II)	9.9
11-HEDE	14.34	7.95/12.20	n.d.	6.5
15-HEDE	11.81	7.58/12.96	n.d.	8.5

n.d., Elution order not determined.

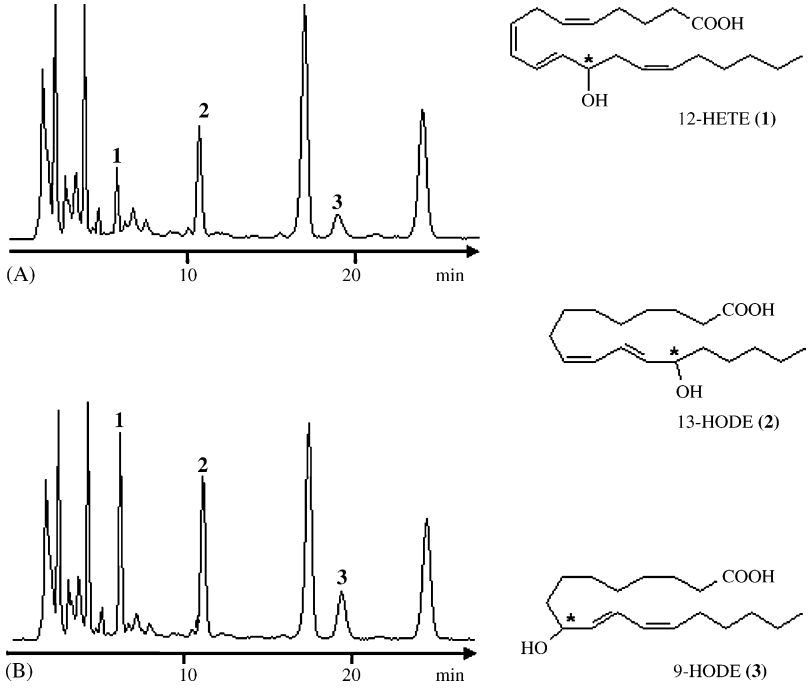

Fig. 1. Extract of sample no. 1 (A) and extract of sample no. 1 enriched with reference compounds (B), using LiChrospher® Si 60 column; for conditions, see Section 2.

A representative chromatogram of a psoriatic skin scale extract on LiChrospher® Si 60 column is given in Fig. 1.

The compounds could not be detected in the sample of heel stratum corneum.

The fractions of interest were collected, preconcentrated and rechromatographed directly on Chiralpak® AD column. More or less time-consuming derivatization procedures as previously described in the literature [3–6] were not necessary. Representative chromatograms of the collected fractions, using Chiralpak® AD as the chiral stationary phase, are given in Fig. 2.

The eluent was optimised in view of minimum retention times in conjunction with maximum resolution of all the analytes, using just one eluent system.

In the case of 12-HETE, the use of methanol as alcohol modifier is mandatory. The exclusive use of methanol as alcohol modifier, however, leads to impressive chromatographic separation and prolonged retention times in case of HODEs.

On the other hand, the addition of a proportion of 2-propanol, which reduces the retention times dramatically, results in total loss of enantioselectivity for 12-HETE.

The addition of ethanol in the described ratio leads to baseline separation of 12-HETE and minimum retention times of HODEs. The baseline resolved stereodifferentiation of the title compounds could be achieved within four to seven times shortened chromatographic run-times, compared to already published methods by Baer et al. [3,4].

3.2. Identification by ESI-MS

In order to get unambiguous identification the fractions were also submitted to ESI-MS. MS^2 analysis of the 12-HETE carboxylate (*m*/*z* 319) yielded characteristic signals at *m*/*z* 301, *m*/*z* 257, *m*/*z* 179 and *m*/*z* 163. The representative mass spectra of a reference and collected 12-HETE fraction are shown in Fig. 3.

MS^2 analysis of the 13-HODE carboxylate (*m*/*z* 295) showed three strong signals at *m*/*z* 277, *m*/*z* 195 and *m*/*z* 179, whereas 9-HODE carboxylate (*m*/*z* 295) yielded two strong signals at *m*/*z* 277 and *m*/*z* 171 (data not shown).

All results were in good accordance with data previously reported [7,11] and exact identification could be obtained.

3.3. Interpretation of the results

Semiquantitation and enantiomeric ratios of 12-HETE, 9-HODE and 13-HODE are presented in Table 2.

Systemic drugs such as cyclosporin A (CsA) or fumaric acid esters suppress the immune system and prevent action of certain immune cells. By preventing the immune activity they slow down the growth of skin cells. It has also been shown that CsA decreases the leukotriene B_4 content in lesional psoriasis. However, the mechanisms of action of CsA and fumaric acid esters in the disease are incompletely understood [13–15].

Four of eight patients had severe plaque-stage psoriasis and were under systemic treatment with CsA or fumaric acid esters. Even under these drugs, the patients still have plaques

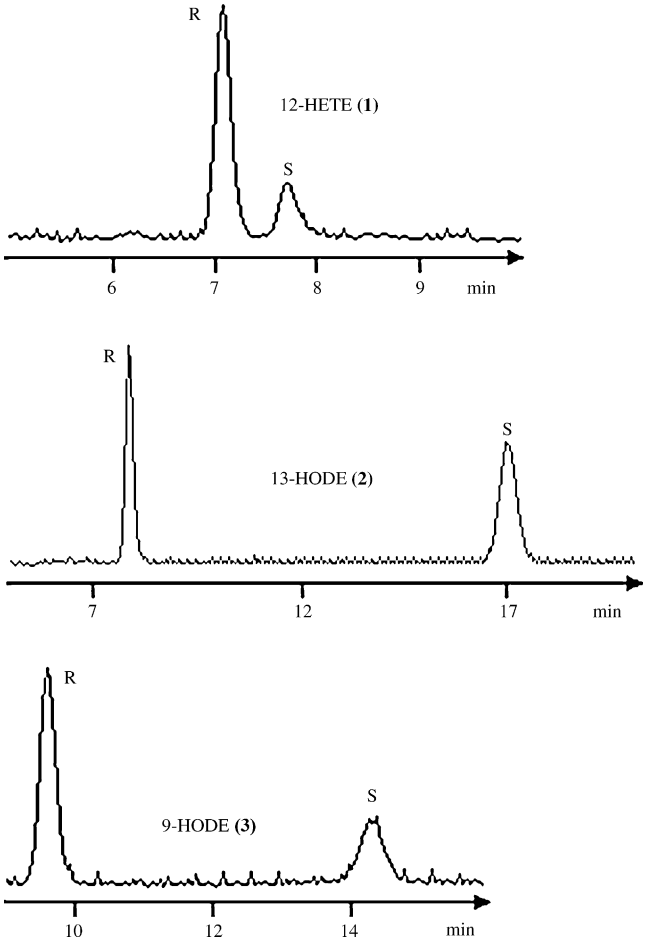

Fig. 2. Enantioseparation of 12-HETE fraction (**1**), 13-HODE fraction (**2**) and 9-HODE fraction (**3**), using Chiralpak® AD column; fractions collected from extract of sample no. 3, using LiChrospher® Si 60 column; for conditions, see Section 2.

covered with marked scales. The other patients (patient no. 4, 5, 7, and 8) had a moderate severity plaque-stage psoriasis without previous therapy either systemic, UVB or topical during the last 4 weeks before sampling.

In case of 12-HETE, enantiomeric distribution and amount was similar for all samples. The (*R*)-enantiomer predominated, varying in a small range (79–86%).

Similarly, the (*R*)-enantiomer of 9-HODE was predominant in all cases, varying from 70 to 73%.

However, the amounts of 9-HODE in samples of untreated patients were remarkably lower than in samples of patients underlying systemic treatment.

The most abundant substance in turn, 13-HODE, showed predominance of the (*S*)-enantiomer. Samples of patients with systemic treatment exhibited a lower predominance of 13(*S*)-HODE (54–58%) than samples of untreated patients (70–75%).

This relatively extensive variation of enantiomeric ratio is consistent with observations made by Baer et al. [3] who determined the *S*/*R* ratio of 13-HODE ranging from 58:42 to 70:30.

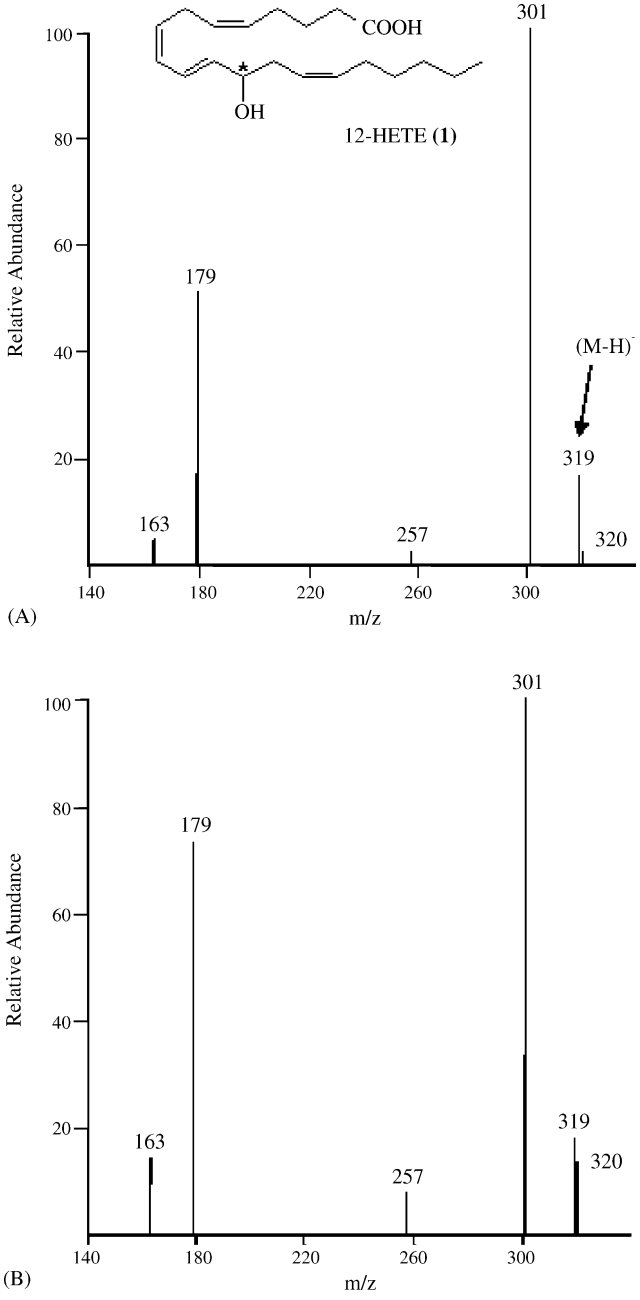

Fig. 3. MS^2 spectrum of the 12-HETE carboxylate as a reference (A) and 12-HETE fraction, collected from extract of sample no. 6, using LiChrospher® Si 60 column (B); for conditions, see Section 2.

In contrast to 9-HODE the detected amounts showed extensive variation and a distinction was not possible.

UVB phototherapy of psoriasis has gained efficiency in the recent past by optimisation of treatment schedules and radiation sources. It is based on photochemical reactions, which occur very rapidly (within a small fraction of a second), but the biological effects may continue for several days.

Patient no. 8 was treated with UVB light for 5 days and, as can be seen from Table 3, no remarkable variation in the enantiomeric ratio of 13-HODE during UVB treatment could be found.

Table 2
Enantiomeric ratios in psoriatic skin scales

Patient no.	12-HETE			9-HODE			13-HODE			Systemic treatment
	R (%)	*S* (%)	*a* (ng/mg)	*R* (%)	*S* (%)	*a* (ng/mg)	*R* (%)	*S* (%)	*a* (ng/mg)	
1	86	14	13	73	27	31	43	57	59	Fumaric acid esters
2	n.d.	n.d.	n.q.	n.d.	n.d.	63	42	58	104	Cyclosporin A
3	85	15	15	70	30	57	44	56	67	Cyclosporin A
4	82	18	n.q.	70	30	23	28	72	86	Untreated during the last 4 weeks before sampling
5	n.d.	n.d.	8	n.d.	n.d.	18	25	75	54	Untreated during the last 4 weeks before sampling
6	79	21	15	72	28	49	46	54	49	Cyclosporin A
7	n.d.	n.d.	n.q.	n.d.	n.d.	19	30	70	61	Untreated during the last 4 weeks before sampling

n.d., Not determinable; amounts (*a*) calculated by calibration curves; n.q., no quantitative data due to peak overlapping; data given as an average of duplicate analysis.

Table 3
Enantiomeric ratios of 13-HODE and amounts (*a*) of 9-HODE in psoriatic skin scales obtained during UVB treatment

Patient no.	13-HODE		9-HODE	Time of sampling after beginning of UVB treatment (day)
	R (%)	*S* (%)	*a* (ng/mg)	
8/1	30	70	24	0
8/2	28	72	20	1
8/3	33	67	26	4
8/4	30	70	29	5

No quantitative data of 13-HODE due to peak overlapping; data given as an average of duplicate analysis.

In all these samples the 13(*S*)-HODE enantiomer predominated (67–72%), which is in accordance with samples of the other patients untreated during the last 4 weeks before sampling.

Similarly, UVB treatment had no influence on the amount of 9-HODE.

A stereodifferentiation of 9-HODE could not be carried out due too low sample amount. 12-HETE could not be detected in any extracts from samples of patient no. 8.

4. Conclusions

The presented analytical method reports on some remarkable new aspects. Whilst earlier published methods needed more or less laborious derivatization procedures, for the first time the presented method leads to the direct analysis (without any derivatization) with baseline resolution of all compounds analysed. By this way, exact determination of enantiomeric ratios is achieved. Direct sample preparations (without any derivatization) and shortened chromatographic runs are the remarkable benefits of the presented method.

First steps on the relationship between the enantiomeric ratios of the investigated compounds and different treatments of psoriasis were described. So far, statistical conclusions could not be drawn due to the limited number of samples available. However, the observed differences in enantiomeric ratios are of fundamental importance, even if final conclusions cannot be drawn at the present state of knowledge. This fact should be the motivation to further investigations.

Acknowledgement

We thank Dr. Ute Bahr, Institute of Pharmaceutical Chemistry, J.W. Goethe-University, for running the ESI-MS experiments.

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