

# Bioavailability and Pharmacokinetics of Alkamides From the Roots of *Echinacea angustifolia* in Humans

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Alkamides are suspected to contribute to the activity of *Echinacea* preparations. They are mainly derived from undeca- and dodecanoic acid and differ in the degree of unsaturation and the configuration of the double bonds. In total, 6 alkamides have been isolated from the roots of *Echinacea angustifolia* as major lipophilic constituents and have been investigated regarding their pharmacokinetics. A sensitive and specific method has been developed for the identification and quantification of these alkamides in human plasma using liquid chromatography electrospray ionization ion-trap mass spectrometry. The method was applied to analyze plasma samples obtained from a randomized,

open, single-dose, crossover study after oral administration of a 60% ethanolic extract from the roots of *E. angustifolia* to 11 healthy subjects. The maximum concentration of dodeca-2E,4E,8Z,10E/Z-tetraenoic acid isobutylamides, the main alkamides in the roots of *E. angustifolia*, appeared already after 30 minutes and was 10.88 ng/mL for the 2.5-mL dose.

**Keywords:** *Echinacea angustifolia*; alkamides; bioavailability; pharmacokinetics

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**E***chinacea angustifolia* DC (Asteraceae) is one of the 3 species of *Echinacea* that are used to stimulate the immune system in the treatment of common colds and infections of the respiratory and lower urinary tract. Preparations from the roots have been shown to have positive effects on various immunological parameters.<sup>1</sup> The current understanding of the active principles suggests that caffeic acid derivatives (CADs), polysaccharides, and alkamides could be involved in the extracts' medicinal effect. Alkamides are present in alcoholic extracts of *Echinacea purpurea* or *E. angusti-*

*folia* aerial parts and roots<sup>2</sup> and, to a lesser extent, in pressed saps of *E. purpurea*.<sup>3</sup> In pharmacological investigations, they have shown immunostimulatory and anti-inflammatory effects in vitro.<sup>4-7</sup> However, data concerning the absorption, metabolism, bioavailability, and bioactivity of natural products and their metabolites after oral application are scarce.<sup>8</sup> We now present data on the pharmacokinetics of alkamides in humans after oral administration of a 60% ethanolic extract from *E. angustifolia* roots.

## MATERIALS AND METHODS

### Reagents

The following chemicals and reagents were used: acetonitrile (high-performance liquid chromatography [HPLC] grade, Rotisov), formic acid (Rotipuran), and Tris buffer (Pufferan) from Carl Roth GmbH + Co (Karlsruhe, Germany). A Barnstead (Easy pure RF) compact ultrapure water purification system was used to obtain the purified water for the HPLC analysis.

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**Table I** Recovery of the 6 Alkamides From 1 Calibration Serum Sample

Substance	Amount Added, ng/mL	Amount Found, ng/mL	Recovery (n = 4)
			Mean $\pm$ SD, %
Undeca-2 <i>E/Z</i> -ene-8,10-diyonic acid isobutylamides ( <b>1</b> )	2.1	1.9	90.5 $\pm$ 0.03
Dodeca-2 <i>E,4Z</i> -diene-8,10-diyonic acid isobutylamide ( <b>2</b> )	2.9	2.8	96.6 $\pm$ 1.30
Dodeca-2 <i>E</i> -ene-8,10-diyonic acid isobutylamide ( <b>3</b> )	1.4	1.2	85.7 $\pm$ 3.80
Dodeca-2 <i>E,4E,8Z,10E/Z</i> -tetraenoic acid isobutylamides ( <b>4</b> )	7.9	7.3	92.4 $\pm$ 3.29
Dodeca-2 <i>E,4E,8Z</i> -trienoic acid isobutylamide ( <b>5</b> )	1.1	1.1	100.0 $\pm$ 5.58
Dodeca-2 <i>E,4E</i> -dienoic acid isobutylamide ( <b>6</b> )	1.4	1.2	85.7 $\pm$ 5.13

**Table II** Calibration Data of Alkamides From the Roots of *Echinacea angustifolia* (Standard Curves, Correlation Coefficient, and Linear Ranges)

Substance	Standard Curve	$R^2$	Linear Range, (ng $\cdot$ 10 $\mu\text{L}^{-1}$ )
Undeca-2 <i>E/Z</i> -ene-8,10-diyonic acid isobutylamides ( <b>1</b> )	$y = 1,939024e + 006x$	0.9938	0.008-0.422
Dodeca-2 <i>E,4Z</i> -diene-8,10-diyonic acid isobutylamide ( <b>2</b> )	$y = 716947,5x$	0.9955	0.012-0.589
Dodeca-2 <i>E</i> -ene-8,10-diyonic acid isobutylamide ( <b>3</b> )	$y = 1,842518e + 006x$	0.9952	0.006-0.280
Dodeca-2 <i>E,4E,8Z,10E/Z</i> -tetraenoic acid isobutylamides ( <b>4</b> )	$y = 630553,5x$	0.9985	0.032-1.581
Dodeca-2 <i>E,4E,8Z</i> -trienoic acid isobutylamide ( <b>5</b> )	$y = 347655,33x$	0.9837	0.004-0.222
Dodeca-2 <i>E,4E</i> -dienoic acid isobutylamide ( <b>6</b> )	$y = 164378,717x$	0.9956	0.006-0.286

### Plant Material

Freshly harvested roots from 2-year-old plants of *E. angustifolia* were obtained from Heilpflanzen Sandfort GmbH & Co KG (Olfen, Germany). The plant material was identified at the Institute of Pharmaceutical Sciences, Department of Pharmacognosy, Karl-Franzens-University Graz. A voucher specimen (no. 2030817) is deposited at this Department of Pharmacognosy. The roots have been extracted using supercritical CO<sub>2</sub> by Finzelberg (Andernach, Germany; drug extract ratio 77:1, yield 1.30%).

### Preparation of Stock Solutions, Calibration Curves, and Validation

The alkamides were isolated by semipreparative HPLC using a Merck Hitachi L-6200A Intelligent Pump and Merck Hitachi L-4500 Diode Array Detector with UV detection at 254 nm and fitted with a LiChroCART 250-10, 10- $\mu\text{m}$  RP-18 LiChrospher 100 column. The compounds were eluted with a gradient from 60/40 to 90/10 acetonitrile/water in 40 minutes (2.0 mL/min). They were identified by liquid chromatography/mass spectrometry (LC/MS) and in comparison with literature

data.<sup>9</sup> Primary stock solution of the 6 alkamides with different concentrations was prepared in methanol and stored at  $-80^\circ\text{C}$ . This primary stock solution was diluted in methanol to produce a final concentration of 8.44 ng/mL for **1**, 11.78 ng/mL for **2**, 5.60 ng/mL for **3**, 31.62 ng/mL for **4**, 4.45 ng/mL for **5**, and 5.71 ng/mL for **6**.

Calibration curves were prepared by spiking 1-mL blank plasma with 10, 50, 100, 250, and 500  $\mu\text{L}$ , respectively, of the standard solution. The correlation coefficient for all calibration curves was above  $R^2 = 0.98$ , proving repeatability and intermediate precision of the assays over the concentration range at least on 5 separate days. The recovery rate for **4** dodeca-2*E,4E,8Z,10E/Z*-tetraenoic acid isobutylamides, the major alkamides, examined at 7.9 ng/mL plasma (concentration **4** at the 5 points contained calibration curve), was 92.4%, with a relative standard deviation of 3.29%. Percent recovery and repeatability for all 6 alkamides are listed in Table I. An internal standard was only used in the validation of the assay and determination of a possible ion suppression. Benzanilide was used at a concentration of 10.3 ng/mL. The recovery was 99.69%  $\pm$  2.17% SD. For sensitivity determination, the lowest standard concentration in the calibration curve was considered as the lower limit of quantitation (see Table II).

## Extraction

The plasma samples were extracted employing a solid-phase extraction technique. To each tube containing 1.00 mL plasma, 1.00 mL Tris buffer was added and vortexed for 1 minute. Subsequently, samples were centrifuged for 15 minutes at 3220g (Eppendorf centrifuge 5810R, Germany). The supernatant was applied onto C18 100-mg Isolute 10-mL XL solid-phase extraction columns from IST International Sorbent Technology (Mid Glamorgan, UK), pretreated with 1 mL acetonitrile, and followed by 1 mL water. The C18 cartridges were set at a VacMaster sample processing station and subsequently washed with 1 mL water under the vacuum. The alkamides were eluted from the C18-SPE columns with 1.5 mL acetonitrile. The eluents were evaporated under a stream of nitrogen at 40°C (TurboVap LV vaporator; Zymark, Hopkinton, Mass), and the dry residue was redissolved in 100 µL acetonitrile/water (1:1), of which 10 µL was used for LC/MS analysis.

## High-Performance Liquid Chromatography Conditions

High-performance liquid chromatography analysis was carried out using a Thermo Finnigan Surveyor liquid chromatograph and a LiChroCART 55 × 2-mm, 3-µm RP-18 end-capped Purospher Star column (Merck, Darmstadt, Germany). The column was protected by an Opti-Guard C<sub>18</sub> 1-mm guard column (Optimize Technologies Inc, Oregon City, Ore). Gradient elution with acetonitrile + 0.1% formic acid and water + 0.1% formic acid 40/60 (v/v) to 80/20 (v/v) in 20 minutes was carried out at a flow rate of 250 µL/min. Separations were performed at room temperature. The separation was detected by MS.

## Mass Spectrometry Conditions

Detection and quantitation were performed using a Finnigan LCQDeca XP Plus ion trap mass spectrometer equipped with an electrospray ionization (ESI) source run by Xcalibur software. Operating conditions for the ESI source, used in the positive ionization mode, were optimized by constantly injecting dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraenoic acid isobutylamides in methanol (0.10 mg/mL) by a syringe pump in the infusion mode. The signal was optimized on the total ion current in MS mode, leading to a transfer capillary temperature of 350°C, a spray voltage of 5.00 kV, and a sheath gas flow of 70 units (units refer to arbitrary values set by the Xcalibur software). At the same time, the

selection of ions and the collision voltages were optimized using Xcalibur software. In the MS/MS experiments, the protonated precursor molecular ion [MH]<sup>+</sup> (*m/z* = 248) of **4** dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraenoic acid isobutylamides (“tetraene”), the major of the 6 alkamides, was selected and fragmented by helium gas collision in the ion trap at a relative collision energy of 35%. The mass spectra resulting from these fragmentations were acquired in the selected reaction monitoring (SRM) mode at *m/z* = 133 for **1**, *m/z* = 145 for **2**, *m/z* = 147 for **3**, *m/z* = 149 for **4**, *m/z* = 150 for **5**, and *m/z* = 179 for **6**, respectively, for the 6 alkamides. These product ions were extracted for quantification.

## PHARMACOKINETIC STUDY

### Study Subjects

The study was conducted according to the Declaration of Helsinki of the World Medical Association and its amendments. The study protocol was approved by the Ethics Committee of the University of Medicine (Graz, Austria) and the Bundesministerium für Soziale Sicherheit und Generationen (Vienna, Austria). Eleven volunteers (5 men and 6 women, 25-36 years of age, mean 30.1 ± 4.7 years, with a body mass index [BMI] of 21.8 ± 2.7) participated in the study after giving written informed consent. The randomized, open, single-dose crossover study was conducted at the Institute of Hygiene, University of Medicine (Graz, Austria). Subjects were not allowed to use any medicine during the study, except for oral contraceptives. Female subjects of childbearing potential were required to have a negative pregnancy test. None of the volunteers was on a special diet. Subjects were excluded from participation if any of the following criteria were met:

- any progressive systemic illness, including tuberculosis, leukemia, connective tissue diseases, multiple sclerosis, or other autoimmune diseases; or
- history of relevant allergy, including allergy to *Compositae* plants.

### Study Design

After an overnight fast, the healthy, medication-free, and drug-free volunteers (negative for HIV, hepatitis B or C) received a single oral 2.5-mL dose of the 60% ethanolic extract from the roots of *E. angustifolia* or placebo (60% ethanol) at 8:30 AM. A baseline blood sample (6 mL) was obtained before drug administration at 8:00 AM. Further blood samples (6 mL) were then drawn into Na<sup>+</sup>-citrate tubes 10, 15, 20, 25, 30, 35,

50, 65, and 180 minutes after dosing. The samples were centrifuged and the plasma separated and stored at  $-80^{\circ}\text{C}$  until analysis by LC/MS.

## ANALYSIS OF DATA

The data were analyzed by Sigma Plot 8.0. The following noncompartmental pharmacokinetic parameters were calculated: the maximum concentration ( $C_{\text{max}}$ ) was taken directly from the observed values, as well as the time to reach it ( $t_{\text{max}}$ ). The area under the concentration-time curve (AUC) was calculated by the trapezoidal rule to the last measurable concentration.<sup>10</sup> Data are reported as mean  $\pm$  SD.

## RESULTS AND DISCUSSION

A total of 11 volunteers were recruited and randomized to investigate the pharmacokinetics of 6 alkamides after oral administration of a 60% ethanol extract. The administration did not cause serious adverse events, and the administration, with few exceptions (nausea), was well tolerated. Three of the 11 volunteers complained about the strong taste and tingling effect. After sampling, the plasma was concentrated with a solid-phase extraction technique with Isolute columns for analysis.

A sensitive and specific method has been developed for the identification and quantification of alkamides from *E. angustifolia* roots in human plasma using liquid chromatography electrospray ionization ion-trap mass spectrometry (LC-ESI-IT-MS/MS) in positive mode. Electrospray ionization is a "gentle" ionization technique that produces high mass-to-charge  $[M + 1]^+$  precursor ions with minimal fragmentation of the analyte. The 6 major alkamides gave protonated precursor molecular ions  $[\text{MH}]^+$  in the MS mode. The major ions observed were  $m/z = 232$  for **1**,  $m/z = 244$  for **2**,  $m/z = 246$  for **3**,  $m/z = 248$  for **4**,  $m/z = 250$  for **5**, and  $m/z = 252$  for **6**. The most intense product ions observed in the MS/MS spectra were  $m/z = 133$  for **1**,  $m/z = 145$  for **2**,  $m/z = 147$  for **3**,  $m/z = 149$  for **4**,  $m/z = 150$  for **5**, and  $m/z = 179$  for **6**. The total HPLC-MS/MS analysis time for all alkamides was 20 minutes per sample. Typical chromatograms for the determination of the 6 alkamides in spiked plasma using the developed SRM scan mode are shown in Figure 2. No ion suppression effects were observed due to the MS suitable low-mass and volatile alkamides and good plasma cleanup technique. No interferences of the analytes were noticed because of the high selectivity of the MS/MS technique. A high sensitivity was achieved with a detection limit of 3 pg/ $\mu\text{L}$ . We conducted the pharmacokinetic

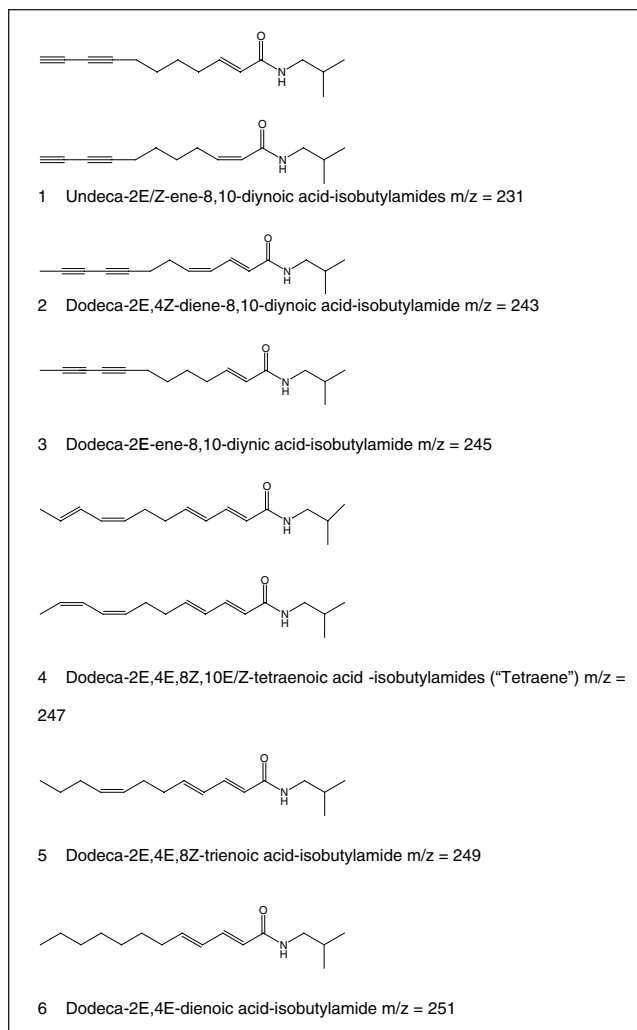


Figure 1. Structures of alkamides from the roots of *Echinacea angustifolia* DC.

study with the applied method to the samples obtained from the 11 volunteers.

The mean alkamide concentrations versus time curves after administration of a 60% EtOH extract are displayed in Figure 3. The mean pharmacokinetic parameters, such as AUC,  $t_{\text{max}}$ , and  $C_{\text{max}}$ , are reported in Table III. The maximum concentration ( $C_{\text{max}}$ ) of the lipophilic alkamides from the roots of *E. angustifolia* was reached within minutes: 1.87 ng/mL for **1**, 1.54 ng/mL for **2**, 0.96 ng/mL for **3**, 10.88 ng/mL for **4**, 2.10 ng/mL for **5**, and 0 ng/mL for **6**. Within the 11 volunteers, 2 different absorption kinetics could be observed (Figure 4), 1 with an absorption maximum already attained after 10 minutes and the other just at the fifth taking of the blood sample. They can be considered as "slow" and "fast" absorbers (7 and 4 subjects, respectively). Because of the  $t_{\text{max}}$  already attained by 30 minutes, the

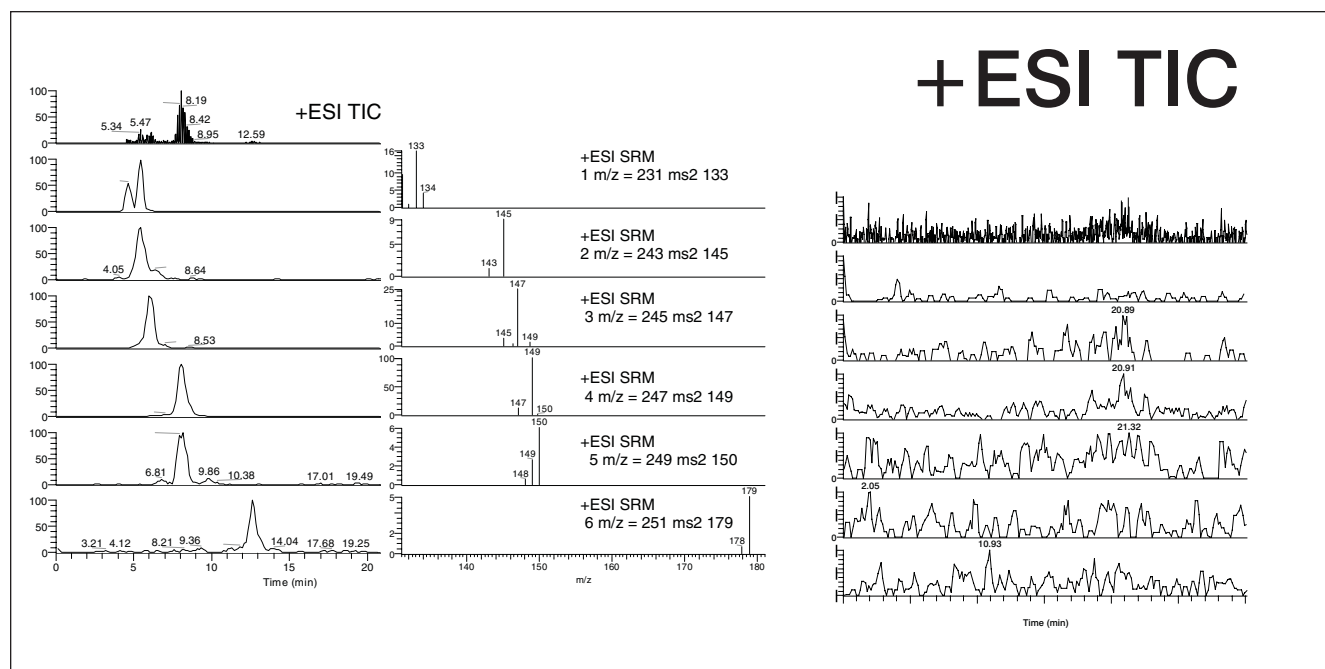


Figure 2. A, Typical chromatograms of a sample spiked with 2.1 ng/mL of **1** undeca-2*E*/*Z*-ene-8,10-diynoic acid isobutylamides, 3.0 ng/mL of **2** dodeca-2*E*,4*Z*-diene-8,10-diynoic acid isobutylamides, 1.4 ng/mL of **3** dodeca-2*E*-ene-8,10-diynoic acid isobutylamides, 7.9 ng/mL of **4** dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraenoic acid isobutylamides ("Tetraene"), 1.1 ng/mL of **5** dodeca-2*E*,4*E*,8*Z*-trienoic acid isobutylamides, and 1.4 ng/mL of **6** dodeca-2*E*,4*E*-dienoic acid isobutylamides for determination with the selected reaction monitoring (SRM) scan mode. B, Chromatograms for a plasma blank. Conditions are as listed in Materials and Methods.

mucous membranes of the mouth and esophagus seem to be a significant absorption site. It could also be observed that the absorption of the different alkamides varies and that dodeca-2*E*,4*E*-dienoic acid isobutylamide **6**, the only 2,4 diene with no further double and triple bond, is not detectable. The quantity of **6** for all subjects in the clinical study was below the detection limit. These results are in agreement with the reported poor passage of **6** across the Caco-2-membrane.<sup>11</sup>

Dietz et al<sup>12</sup> presented the first data on systemic absorption of *E. purpurea* alkamides using an HPLC method with UV detection at 260 nm for the determination of dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraenoic acid iso-

butylamides in plasma. The method was much less sensitive, and 100 mL blood needed to be collected. One hour after application of the tincture, 44 ng dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraenoic acid isobutylamides per mL blood was determined. No time curve could be measured. Jager et al<sup>13</sup> investigated the permeability of dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraenoic acid isobutylamides through Caco-2 monolayers and found that **4** was nearly completely transported from the apical to the basolateral side of the monolayer in 6 hours by passive diffusion and that no significant metabolism occurred. This study confirms in that **4** dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraenoic acid isobutylamides are

**Table III** Dose of Each Alkamide in the 2.5-mL Administered Extract and Pharmacokinetic Parameters of Alkamides After Oral Administration of a 60% EtOH Extract From the Roots of *Echinacea angustifolia*

Substance (n = 11)	Dose, mg/2.5-mL Extract	C <sub>max</sub> , ng/mL	t <sub>max</sub> , min	AUC, ng•min/mL
Undeca-2 <i>E</i> / <i>Z</i> -ene-8,10-diynoic acid isobutylamides ( <b>1</b> )	0.544	1.87 ± 1.16	20.1	115.25 ± 86.87
Dodeca-2 <i>E</i> ,4 <i>Z</i> -diene-8,10-diynoic acid isobutylamide ( <b>2</b> )	0.524	1.54 ± 0.80	30.3	128.75 ± 82.79
Dodeca-2 <i>E</i> -ene-8,10-diynoic acid isobutylamide ( <b>3</b> )	0.682	0.96 ± 0.39	30.3	101.36 ± 45.68
Dodeca-2 <i>E</i> ,4 <i>E</i> ,8 <i>Z</i> ,10 <i>E</i> / <i>Z</i> -tetraenoic acid isobutylamides ( <b>4</b> )	2.005	10.88 ± 6.50	30.3	1029.14 ± 500.72
Dodeca-2 <i>E</i> ,4 <i>E</i> ,8 <i>Z</i> -trienoic acid isobutylamide ( <b>5</b> )	0.437	2.10 ± 1.25	30.3	195.48 ± 119.55
Dodeca 2 <i>E</i> ,4 <i>E</i> -dienoic acid isobutylamide ( <b>6</b> )	0.408	0	0	0

The data for all alkamides are presented as mean ± standard deviation.

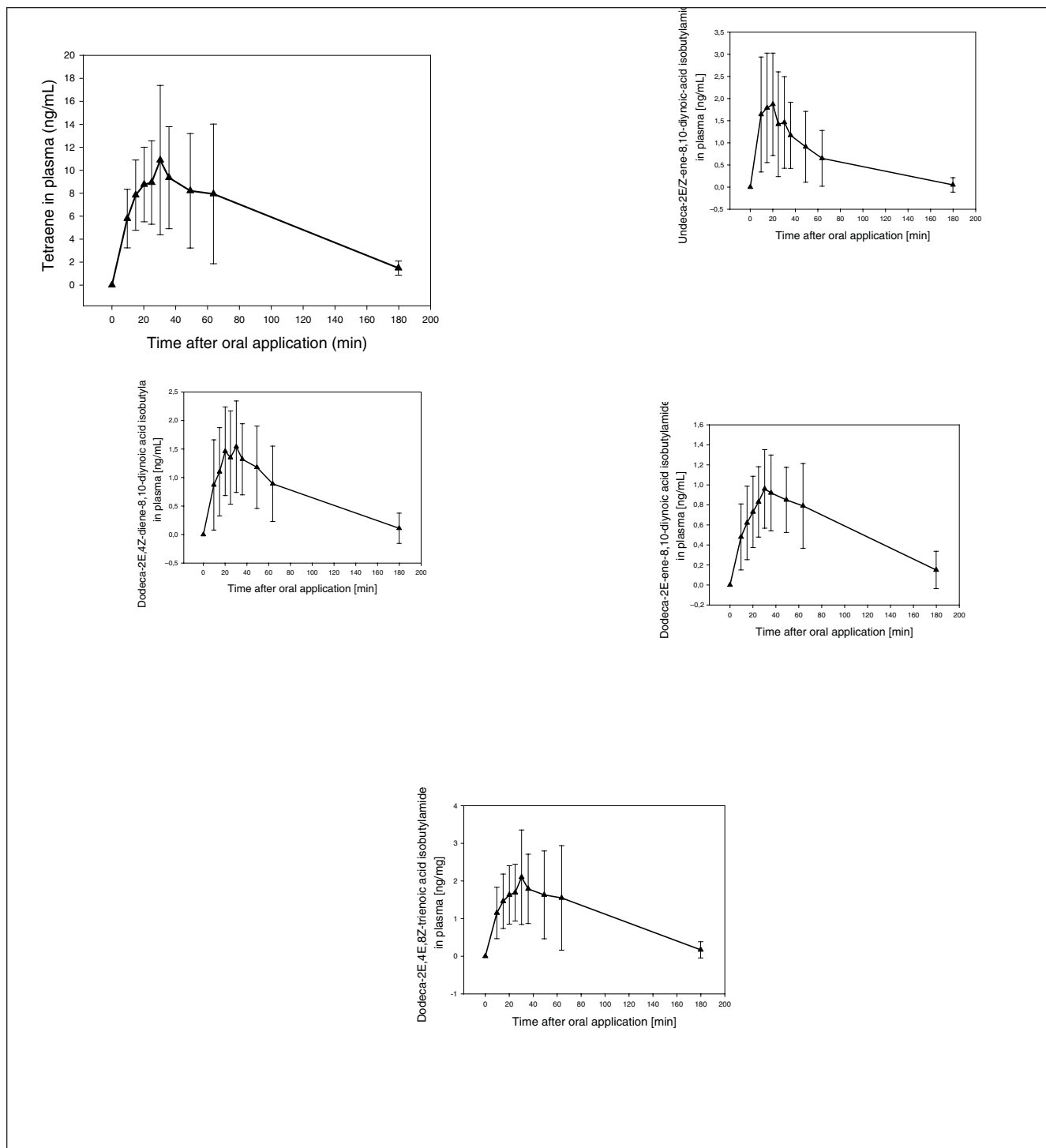


Figure 3. Plasma concentration-time curves of 5 alkamides after a single oral dose of 2.5 mL 60% EtOH extract of *Echinacea angustifolia* roots. Each point represents the mean  $\pm$  standard deviation of the 11 volunteers.

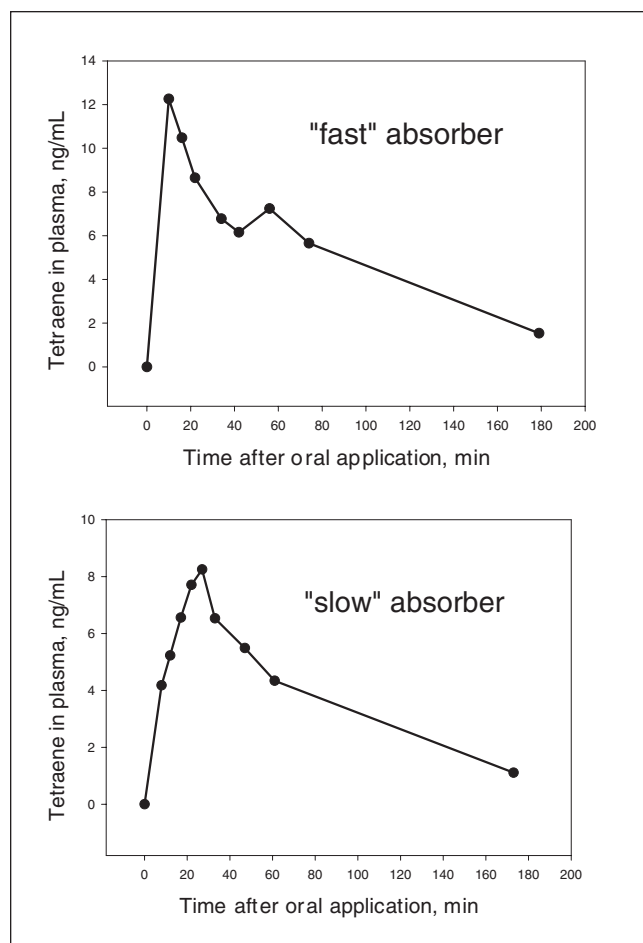


Figure 4. Comparison of a "slow" and a "fast" absorber.

able to cross the intestinal barrier and hence be present in plasma to potentially influence any immune response.

## CONCLUSION

With a sensitive and specific LC-ESI-IT-MS/MS method, alkamides from *E. angustifolia* could be detected in human plasma after oral application. The quantification limit of 3 pg/ $\mu$ L allows the study of differences in pharmacokinetic behavior in humans. Two types of absorption could be observed: fast absorption within 10 minutes or  $C_{max}$  at 40 minutes. Highly lipophilic alkamides with no double and triple bond at the end of the fatty acid chain could not be detected in

the blood. The study demonstrates for the first time the fast absorption of alkamides from *E. angustifolia* and sheds new light on the relevance of published pharmacological in vitro effects. Therefore, these components should be in the focus of future research on immunological effects of *Echinacea*.

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