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Analysis of alkamides in roots and achenes of *Echinacea purpurea* by liquid chromatography–electrospray mass spectrometry

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Abstract

High-performance liquid chromatography paired with UV photodiode-array and electrospray mass spectrometry was investigated as a method for the analysis of alkamides in the roots and achenes of the popular herb *Echinacea purpurea* (L.) Moench. All alkamides showed very abundant peaks in the reconstructed total ion current chromatogram. Nine alkamides were identified in the root of *E. purpurea*, similar to the fingerprint reported by Bauer and Remiger [Planta Med., 55 (1989) 367]. Several minor alkamides, not identifiable by previously published techniques, have been also tentatively identified. Two major peaks were identified in *E. purpurea* achenes as undeca-2*E*,4*Z*-diene-8, 10-diynoic acid isobutylamide (**1**) and dodeca-2*E*,4*E*,8*E*,10*E*/*Z* tetraenoic acid isobutylamide (**8**/**9**). The isomer pair, tetraene **8**/**9**, was purified as a standard for quantification of alkamide content in *E. purpurea* achenes and roots, as well as for the achenes of *E. angustifolia* DC and *E. pallida* (Nutt.) Nutt.

Keywords: *Echinacea* spp.; Alkamides

1. Introduction

Herbal drugs from the genus *Echinacea* (family Asteraceae) are among the most widely used herbal medicines in Europe, North America and Australia for treatment of cold, flu and chronic respiratory infections [1]. Commercial supplies are primarily the roots and aerial parts of three species: *E. purpurea*, *E. angustifolia* and *E. pallida*. Numerous papers on *Echinacea* constituents and pharmacology have appeared. It is believed that the immunostimulatory activity of *Echinacea* extracts depends on the combined action of three classes of compounds: polar caffeic acid derivatives, lipophilic alkamides and polysaccharides [2]. All three classes of compounds may be used to standardize *Echinacea* preparations [3].

Alkamides are a distinct class of natural products, containing an aliphatic acid (mostly unsaturated) residue linked with different amine moieties. Approximately 200 alkamides have been isolated thus far from nature [4]. The presence of alkamides in *Echinacea* has been known for almost half a century [5–7]. The olefinic isobutylamides are the most biologically active alkamides contained in *Echinacea* [8]. Fig. 1 depicts the chemical structures of the alkamides, using the compound numbering system of Bauer and Remiger [9], who identified eleven alkamides in the roots of *E. purpurea* by TLC and HPLC. The primary alkamide, based on *Echinacea* analysis to date, is the isomeric pair dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraenoic acid isobutylamide (**8**/**9**)

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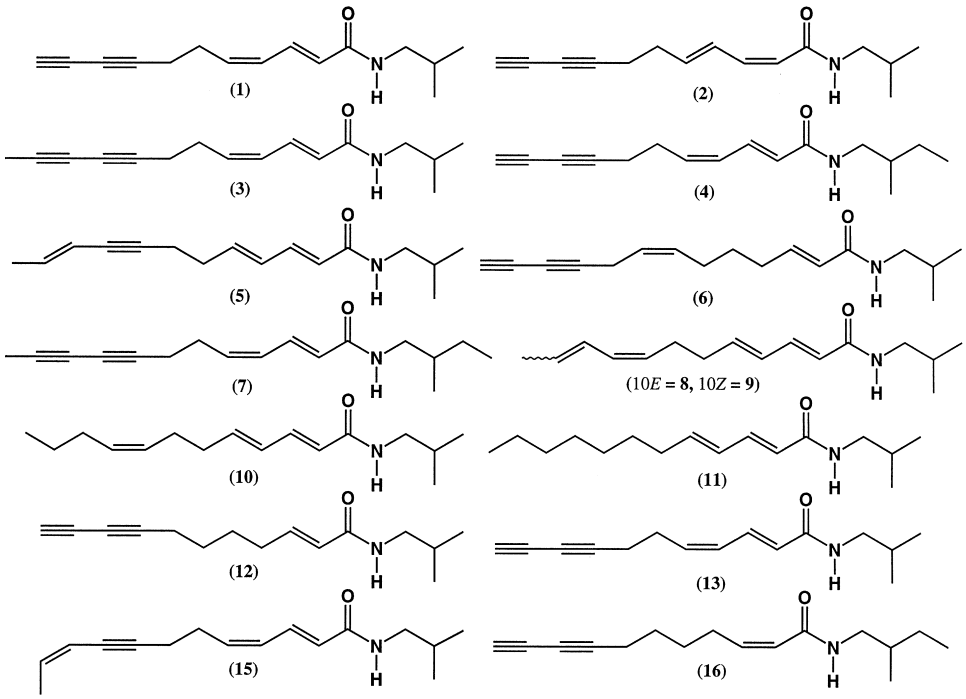

Fig. 1. Chemical structures of *Echinacea* alkamides. Compound numbering as denoted in [9].

[10]. Schulthess et al. analyzed changes in alkamide content during the germination of *E. purpurea* achene (a small dry indehiscent fruit, having one seed with a thin wall), but showed no chromatogram, identified peaks by retention order and diode-array analysis, and proposed no quantitative method [11]. Perry et al. revealed differences in alkamide profiles in various plant parts of *E. purpurea* using synthetic N-phenylpentamide as an internal standard [12].

The unequivocal identification of alkamide peaks has in the past required isolation of the individual compounds, necessitating multiple purification steps as well as MS and NMR analysis. Chromatography coupled with spectroscopy is a powerful analytical tool for fast structural elucidation of complex mixtures. Our work shows HPLC–UV–MS to be a useful method for the direct identification of alkamide peaks in *Echinacea* extracts. In this paper, we report the difference between alkamide fingerprints of *E. purpurea* roots and achenes, using HPLC–UV–electrospray (ES)-MS method. We identify small peaks which were unidentified in previous published techniques [3,9,11,12] because no standards were available for retention-time comparison.

2. Experimental

2.1. Instrumentation

A HPLC 1090 Series II instrument (Hewlett-Packard, Palo Alto, CA, USA) with a photodiode-array detector set at 254 nm was coupled with a HP 5989B quadrupole mass spectrometer. UV spectra were taken in the range 200–500 nm. Chromatographic conditions were: column, Waters Symmetry C_{18}, 5 μm, 150×2.1 mm (Waters, Milford, MA, USA); eluent (A) water and (B) acetonitrile. The gradient elution had the following profile: 0–30 min, 45–80% B; 30–32 min, 80–100% B; 32–35 min, 100–45% B. The flow-rate was 0.2 ml/min; temperature was 45°C. Mass range measured: 200–700 u, quadrupole temperature, 150°C; EM volts 2173. The spectra were acquired in the positive ion mode. ES interface

was HP 59987 A; drying N_2 temperature, 350°C, 40 ml/min; nebulizing N_2, 5.5×10^5 Pa (80 p.s.i.). The HPLC was directly connected to the mass spectrometer without stream splitting.

2.2. Solvents and chemicals

HPLC-grade water, acetonitrile and methanol (VWR, Seattle, WA, USA) were used for HPLC analysis. Reagent grade hexane, EtOAc and chloroform (VWR) were used for extraction and separation. Column chromatography silica gel and preparative TLC plates were purchased from E. Merck (Darmstadt, Germany).

2.3. Plant material and sample preparation

Achenes of *E. angustifolia* were purchased from Plantation Medicinals (Felda, FL, USA). Achenes of *E. purpurea* and *E. pallida* and roots of *E. purpurea* were purchased from Trout Lake Farm (Trout Lake, WA, USA). The achenes had been collected in 1996, the root material in 1997. One gram of each sample was ground in a mill, then refluxed with 20 ml of chloroform for 2 h. The sample solution was filtered and concentrated to dryness in vacuum. Each residue was dissolved in 5 ml methanol in a volumetric flask. A 5 μl aliquot of the sample solution was injected into the HPLC column after filtration through a 0.45 μm nylon Acrodisk 13-mm filter (Gelman, Ann Arbor, MI, USA).

2.4. Isolation and identification of dodeca-2E,4E,8E,10E/Z-tetraenoic acid isobutylamide (**8**/**9**)

Two kg of the powdered achenes of *E. purpurea* were extracted with *n*-hexane and the hexane extract was partitioned between hexane and methanol–water (9:1, v/v). The residue from the hexane layer (12 g) was separated by column chromatography (silica gel 60, >230 mesh, Merck) with elution of hexane–EtOAc (2:1, v/v) and each 100 ml fraction was examined by TLC (silica gel 60 F_{254}, on aluminum sheet plate, Merck), developed with hexane–EtOAc (2:1, v/v). UV-active fractions primarily contained alkamides with molecular mass 247. Their further purification with preparative TLC (RP-18, F_{254}, 20×20 cm, 0.15 mm thickness, MeOH–water, 7:3, v/v) or preparative HPLC (Rainin Dynamax Microsorb C_{18}, 250×21.4 mm, acetonitrile–water, 6:4, v/v, 16 ml/min, flow-rate, 254 nm detection), followed by recrystallization from hexane, offered a mixture of **8** and **9** as needles (98% purity, 0.015% yield). Compound **8**/**9** was identical by ^{1}H-NMR, ^{13}C-NMR, UV and MS with the literature data [10]. A 3.8 mg quantity of compound **8**/**9** was dissolved in 10 ml methanol as a standard solution.

3. Results and discussion

3.1. HPLC–UV–ES-MS analysis of E. purpurea root extract

Fig. 2 shows the simultaneous HPLC–UV and HPLC–ES-MS analysis of roots extract of *E. purpurea*. Eleven peaks (peaks A–K) were very well separated, except for the isomeric pair peak of G and H. So far there have been no reports demonstrating good HPLC separation of the isomeric pair of G and H. Only Perry et al. have reported tetraene **9** as a less retained peak on reversed phase than tetraene **8** [12]. All alkamide peaks show abundant peaks in the reconstructed total ion current (TIC) chromatogram. Table 1 shows the retention times (t_R), UV maxima, molecular ions and the identification of each peak. Interestingly, most peaks show protonated or sodiated dimer ions, helping us to confirm molecular weight determinations.

Fig. 3 shows the mass spectra of peaks A, C, E and G. As isomeric pairs, peaks A/B and C/D have the same mass spectra; their identifications are based on retention order [9]. Peaks G and H were identical to the isolated standard **8**/**9**. Our chromatogram (Fig. 2) is similar to that already published [9], however, the small peaks F and I were not reported. Peak F is tentatively identified as compound **15** or **16** based on UV and MS data; assignment of the configuration of double bonds is not possible using our present techniques. Peak I may be a new *E. purpurea* alkamide.

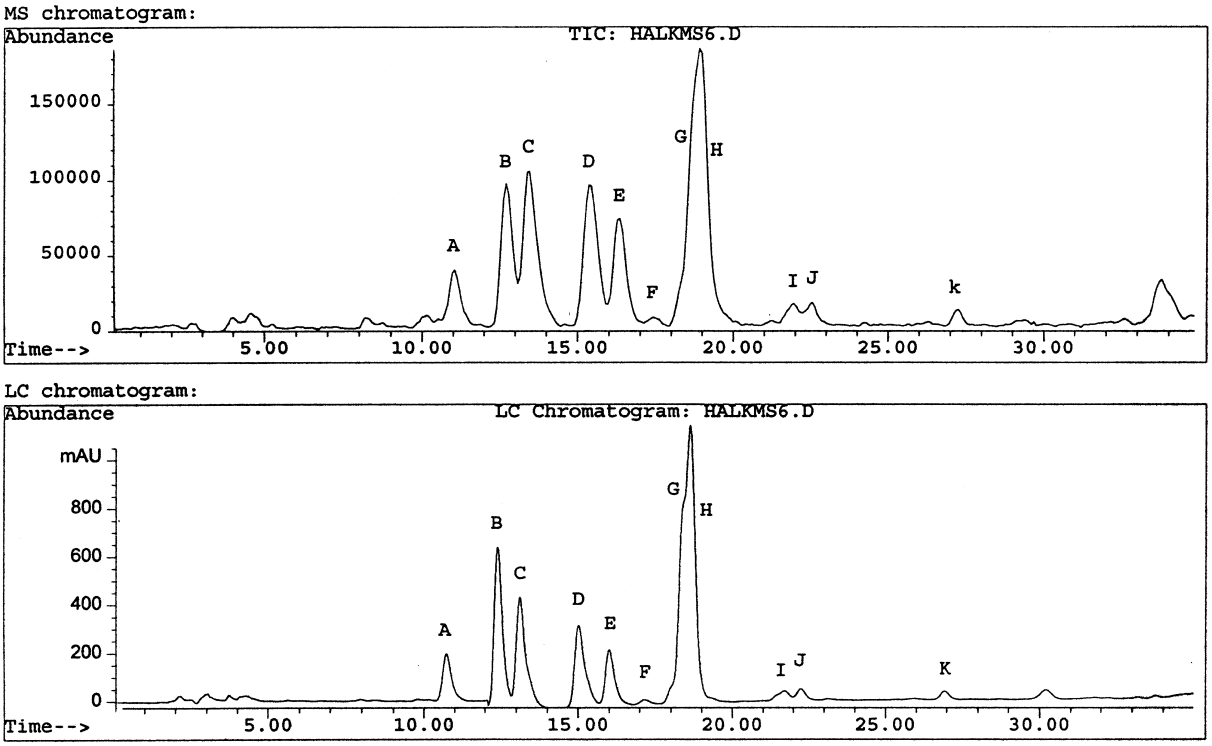

Fig. 2. Simultaneous HPLC–UV and HPLC–ES-reconstructed-TIC chromatogram of root extract of *E. purpurea*, without post-column stream splitting. Chromatographic conditions were as described in Section 2.1. The peak identifications are shown in Table 1.

Table 1
Peak assignments for analysis of *E. purpurea* root extract

Peak no.	t_R (min)	$[M+H]^+$ m/z	$[M+Na]^+$ m/z	$[2M+H]^+$ m/z	$[2M+Na]^+$ m/z	UV λ_{max} (nm)	Identification (compound no.)
A	10.8	230	252	459	481	263	1
B	12.4	230	252	459	481	263	2
C	13.1	244	266	487	509	263	3
D	15.0	244	266	487	509	263	4
E	16.0	258	280	515	537	263	6 or 7
F	17.2	246	268	–	–	263	15 or 16
G	18.1	248	270	495	517	235, 263	8
H	18.5	248	270	495	517	235, 263	9
I	21.8	262	284	523	–	N.D.	N.I.
J	22.2	250	272	491	–	N.D.	10
K	26.9	252	274	–	–	N.D.	11

N.D.: not detectable.
N.I.: not identified.

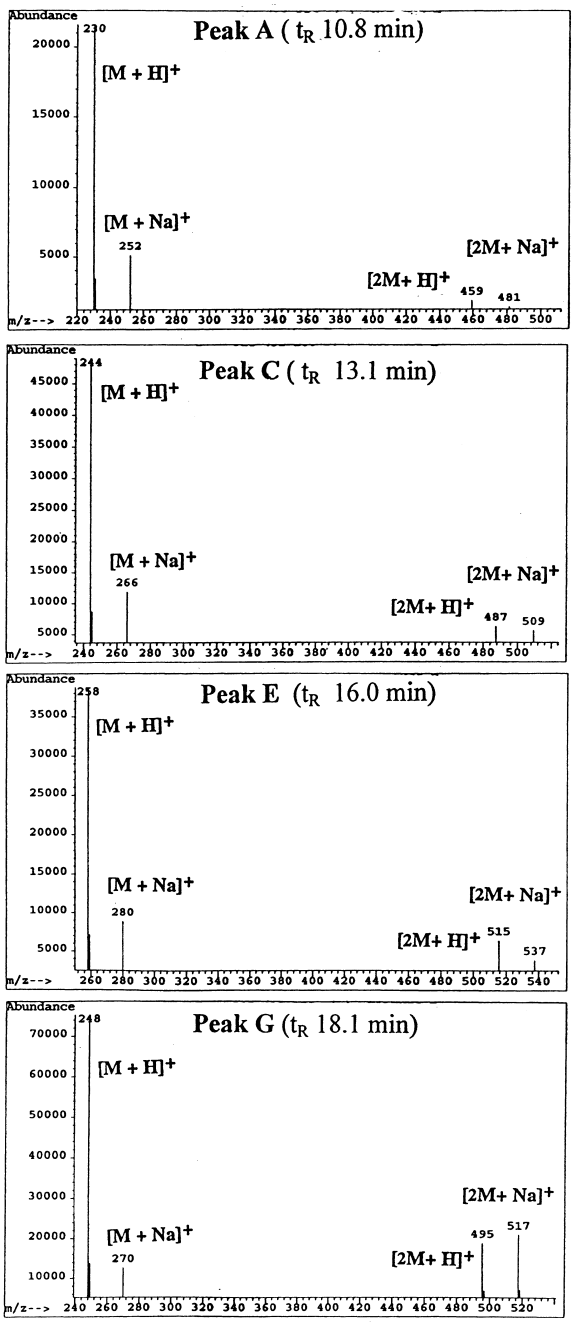

Fig. 3. Mass spectra of peaks A, C, E and G from *E. purpurea* root extract.

3.2. HPLC–UV–ES-MS analysis of E. purpurea achene extract

In order to quantify the alkamides in *Echinacea* preparations we purified our own standard compounds, as none are commercially available. We found the HPLC profiles of *Echinacea* achenes to be less complex than those of roots, containing only one major peak for compounds **8**/**9**. Therefore, the achenes are a satisfactory source from which to purify this isomeric pair. Fig. 4 shows the results of simultaneous HPLC–UV and HPLC–ES-MS analysis of *E. purpurea* achene extract. The identification of individual peaks is shown in Table 2. The main peaks B and G/H were identified as compounds (**1**) and (**8**/**9**), based on their retention times, UV and mass spectra, as well as comparison with our purified standards. The small peaks C, D, E and F were tentatively identified. These compounds have not previously been identified in *Echinacea* achenes; peak A may be a new alkamide.

As we reported previously, ES-MS is a soft ionization process which in most cases shows only protonated molecules without fragmentation [13,14]. The formation of protonated or sodiated ions provides further evidence to confirm the molecular masses of the identified peaks. For identification of isomeric pairs, this method is still not satisfactory. HPLC coupled with NMR might provide more information to solve this problem. Our experiments show that alkamides are easily ionized in ES-MS.

3.3. Quantitative analysis of alkamides in E. angustifolia, E. purpurea and E. pallida achenes and E. purpurea root

We employed purified tetraenes **8**/**9** as an external standard to determine the content of these compounds in the achenes of different *Echinacea* species. The calibration curve shows a linear response for the range 200–760 ng of compound **8**/**9**. The detection limit ($S/N=5$) is 15 ng at UV 254 nm. Recovery was determined by adding 25 μg/ml of compound **8**/**9** to *E. purpurea* root extract and repeating the analysis ($n=3$). The average recovery was 96.2%. The results are shown in Table 3. We found that the contents of tetraenes **8**/**9** will dramati-

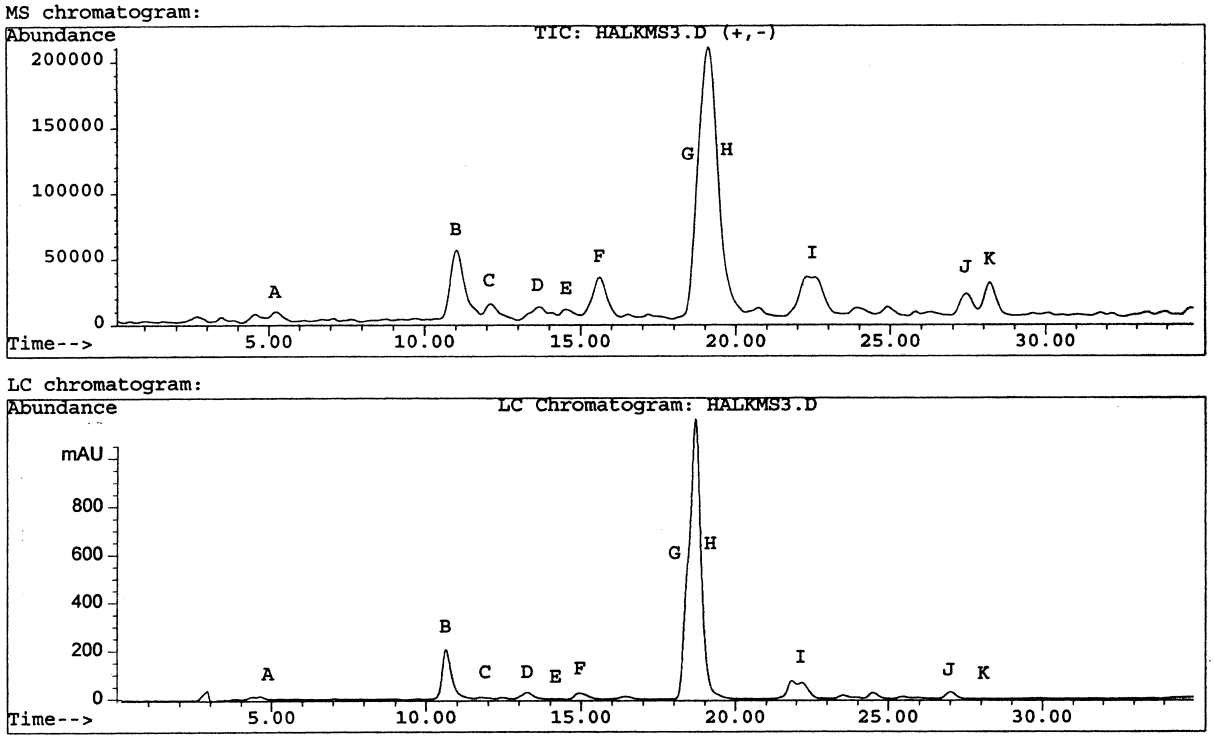

Fig. 4. Simultaneous HPLC–UV and HPLC–ES-reconstructed-TIC chromatogram of *E. purpurea* achene extract, without post-column stream splitting. Chromatographic conditions were as described in Section 2.1. The peak identifications are shown in Table 2.

cally decrease in raw plant samples stored for one year at room temperature, in agreement with Perry et al. [12]. However, we have kept the standard compound **8/9** in methanol solution for half a year in refrigeration, and using HPLC analysis found no changes in peak profile.

Table 2
Peak assignments for analysis of *E. purpurea* achene extract

Peak no.	t_R (min)	$[M+H]^+$ *m/z*	$[M+Na]^+$ *m/z*	$[2M+H]^+$ *m/z*	$[2M+Na]^+$ *m/z*	Other *m/z*	UV λ_{max} (nm)	Identification (Compound no.)
A	5.0	278	300	577	–	–	N.D.	N.I.
B	10.7	230	252	459	481	–	263	1
C	11.8	232	254	–	463	–	N.D.	12 or 13
D	13.1	244	266	487	509	–	263	3
E	14.1	246	268	–	–	–	N.D.	5
F	15.0	258	280	515	–	–	263	6 or 7
G	18.1	248	270	495	517	–	235, 263	8
H	18.6	248	270	495	517	–	235, 263	9
I	22.2	250	272	–	–	–	235	10
J	26.9	252	274	503	–	–	263	11
K	28.0	–	–	–	–	279, 297	N.D.	N.I.

N.D.: not detectable.
N.I.: not identified.

Table 3
Levels of tetraenes **8**/**9** in *E. angustifolia*, *E. purpurea* and *E. pallida* achenes and *E. purpurea* root

Sample	Tetraene **8**/**9** content (mg/g, dry mass) ($\bar{x}$, $n=3$)
E. angustifolia achene	1.06
E. purpurea achene	0.75
E. pallida achene	0.08
E. purpurea root	0.37

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