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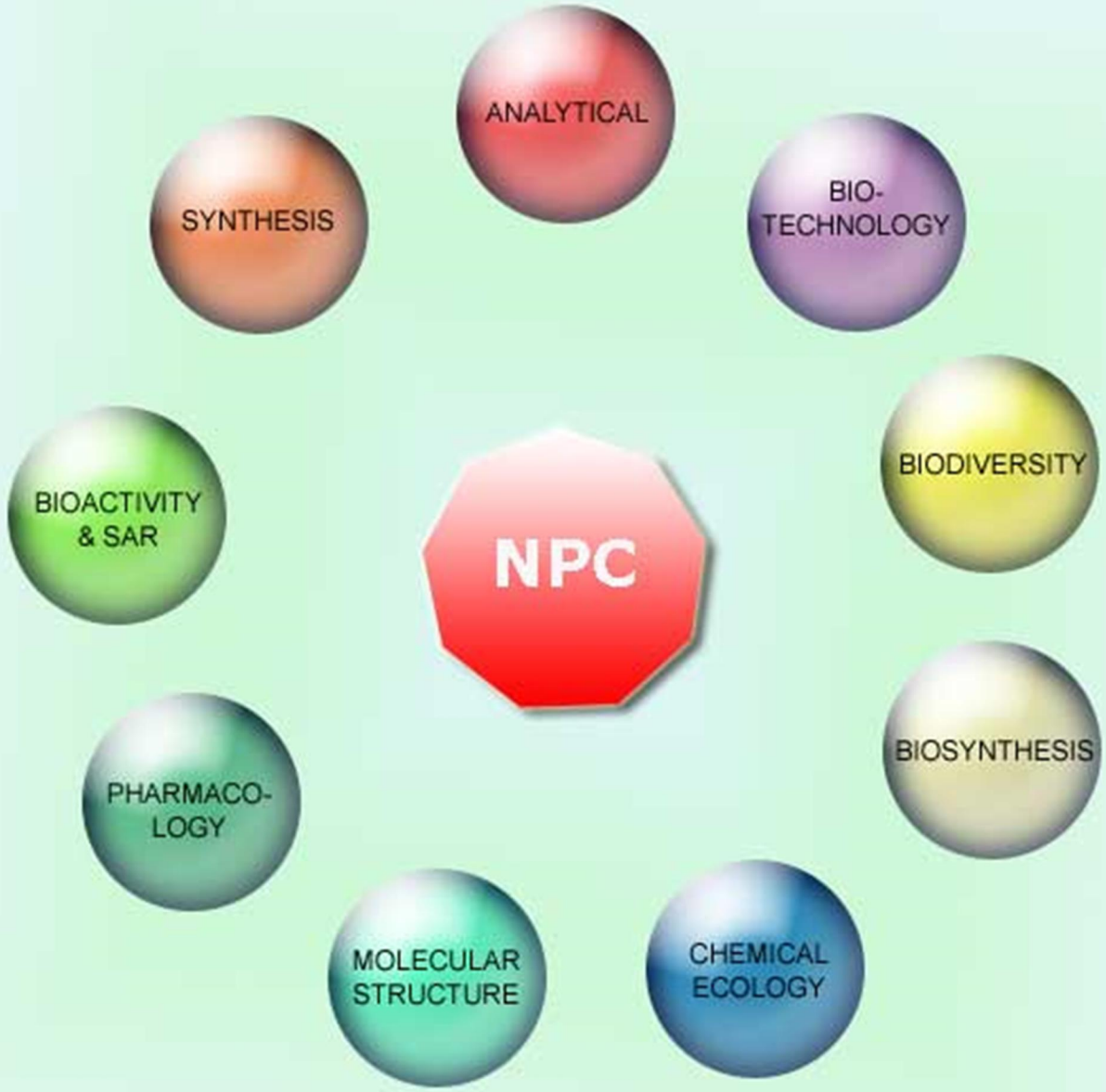

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Possible Role of Fat Tissue in the Pharmacokinetics of Dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraenoic Acid Isobutylamides after Oral Administration of *Echinacea angustifolia* Extract in Rats

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Alkamides are one of the most important constituents of lipophilic extracts of *Echinacea angustifolia* roots. These compounds play an important role in the versatile pharmacological actions of this plant. The present study aimed to compare the concentrations of isomeric dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraenoic acid isobutylamides (DTAI) in brain and periepididymal fat tissues and blood plasma of rats. Thirty, 60, 240 and 720 min after the oral administration of *E. angustifolia* root extract, tissue and plasma concentrations were determined by reversed-phase HPLC with ESI-MS/MS detection. The calculated terminal $t_{½}$ of the mixture of DTAI was 8.28 h, which indicates a relatively slow elimination. In the 0.5–4 h period the brain/plasma and fat/plasma concentration ratios were continuously above 3 and 18, respectively, followed by equilibrium at 12 h. Our results indicate substantial accumulation of alkamides in lipid-rich tissues, which presumably contributes to a maintained pharmacological action.

Keywords: HPLC-MS/MS, Pharmacokinetics, *Echinacea angustifolia*, Alkamide, Dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraenoic acid isobutylamides.

Echinacea species are the most widely used immunomodulatory plants in the treatment of common cold. Pharmacologically active constituents of *Echinacea* species are alkamides, alkylketones, caffeic acid derivatives and polysaccharides [1, 2]. Isomeric dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraenoic acid isobutylamides (DTAI) (Figure 1) are the main alkamides in the roots of *E. purpurea*, *E. angustifolia* and *E. pallida* and in the herbs of *E. purpurea* and *E. angustifolia* [3].

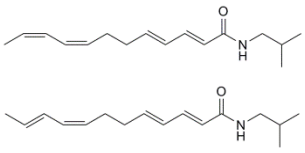

Figure 1: Chemical structures of dodeca-2*E*,4*E*,8*E*,10*E*/*Z*-tetraenoic acid isobutylamides.

Alkamides may play an important role in the medicinal benefits of *Echinacea* extracts. Alkamides of *E. purpurea* stimulated, dose-dependently, the phagocytic activity of alveolar macrophages in rats [4]. Two *Echinacea*-derived alkamides suppressed the ability of activated Jurkat T cells to produce IL-2 in a dose-dependent manner [5]. The alkamide mixture purified from *E. angustifolia* decreased significantly the lipopolysaccharide stimulated NF-κB level, TNF-α and NO production in macrophages *in vitro* [6]. Polyunsaturated alkamides isolated from *Achillea* species and *E. angustifolia* roots were shown to possess inhibitory activity in *in vitro* cyclooxygenase and 5-lipoxygenase assays [7]. These data suggest that alkamides may have not only immunostimulatory but also anti-inflammatory activity. DTAI have been found to inhibit both cyclooxygenase and 5-lipoxygenase at micromolar concentrations [8]. Extract of *E. angustifolia* decreased anxiety in the elevated plus-maze, social interaction and social avoidance tests in rodents [9]. The extract of the plant can significantly regulate excitatory, but not inhibitory, synaptic transmission in the hippocampus, and this action might be involved in its anxiolytic effects observed in behaviour tests [10]. Certain alkamides act as cannabinomimetics at both the cannabinoid CB1 and CB2 receptors [11-13]. Alkamides of *E. angustifolia* extract may contribute to decreased mild anxiety symptoms since CB1 receptors are implicated in anxiety [14]. Data concerning the absorption, metabolism, bioavailability, and bioactivity of natural products and their metabolites are primarily important to link results from *in vitro* assays and clinical studies. Woelkart *et al*. evaluated the pharmacokinetics of DTAI in rat plasma, as well as in liver and brain tissues [15]. Goey *et al*. have described a validated HPLC-MS/MS procedure for quantitative analysis of DTAI in human plasma [16]. Although lipid soluble substances may be deposited in fat tissues which may fundamentally influence the pharmacokinetic behaviour of such compounds, no data are available concerning the concentrations of DTAI in depot fat.

In this research, we studied the concentration changes of DTAI in brain and periepididymal adipose tissues of rats using an HPLC-MS/MS method, and compared them with those in plasma of the animals. Terminal $t_{½}$ of DTAI was also calculated.

Oral treatment of rats with 50 mg/kg *E. angustifolia* root extract was performed in order to obtain data concerning the pharmacokinetics of the active constituents both in plasma and lipid-rich brain and fat tissues. DTAI concentration was maximal in all samples collected 30 min after treatment indicating fast absorption and distribution (Figure 2). The plasma curve of DTAI revealed a typical two-compartment pharmacokinetic behaviour with a distribution completed within 1 hour, followed by an elimination phase. The calculated half-life ($t_{½}$) of DTAI was 8.27 h and the elimination constant (k_e) was 0.084 1/h.

The highest concentration of DTAI was detected in the periepididymal fat. The level in the periepididymal fat tissue was

18–24 times higher than in the plasma in the period 0.5–4 h and reached equilibrium by 12 h (Table 1). High concentrations were found in the brain too but the tissue/plasma ratios were lower (2–6-fold) and also decreased substantially by 12 h.

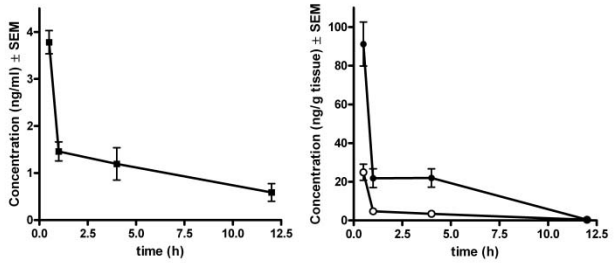

Figure 2: DTAI concentrations in the plasma (■), brain (○) and fat tissue (●) samples of rats treated with 50 mg/kg *Echinacea* extract (N: 6).

Table 1: Concentration ratios of DTAI.

Time (h)	Brain/plasma ratio	Fat/plasma ratio
0.5	7.10	30.23
1	3.03	20.61
4	4.60	35.22
12	0.51	0.96

DTAI concentrations in plasma, brain and fat tissue samples obtained from rats were determined by means of HPLC-MS/MS 0.5–12 h after oral treatment with *Echinacea* extract (Figure 3). Since the main purpose of our study was the comparison of concentrations in relevant tissues (brain and fat) with those in plasma the timing of the sample collection is not optimal for a complete pharmacokinetic analysis. However, the calculated terminal $t_{½}$ of the analyte was 8.27 h, which indicates a substantially slower elimination than reported previously after the administration of the pure compounds [15]. Therefore, it is suggested that some directly ineffective constituents may exert profound action on the pharmacokinetic profile and therefore the overall effect of the *Echinacea* preparation [17].

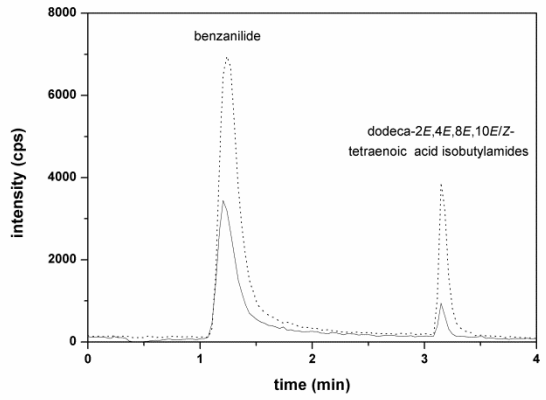

Figure 3: MRM chromatogram of periepididymal adipose tissue 4 hours after *Echinacea* extract administration (solid line) and the 0.8 ng/20 µL concentration calibration standard (dotted line).

Highly lipid-soluble substances tend to accumulate in fat tissue and this may basically influence the disposition of the drug. However, none of the previously published studies were extended to the determination of DTAI in adipose tissue. This is the first report presenting direct comparison of DTAI concentrations in plasma, brain and fat. Our data indicate that DTAI reaches considerably high concentration, presumably maximal in the body in adipose tissue. DTAI deposited in the fat may contribute to a sustained brain concentration. If such redistribution is operative in humans it may implicate that the relative fat weight may exert a profound action on the disposition of the agents and hence on the duration of their action too.

Experimental

Chemicals and reagents: DTAI was isolated from *E. angustifolia* by Hohmann *et al.* [12] and its identity and purity (95%) was investigated by ^{1}H NMR (500 MHz, $CDCl_3$). Benzanilide (purity 99.9%) and methylcellulose were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA), acetonitrile (LiChrosolv HPLC grade) from Merck KGaA (Darmstadt, Germany), and formic acid and methanol (analytical grade) from Reanal Zrt. (Budapest, Hungary). Millipore Direct-Q UV3 (Millipore, Bedford, MA, USA) clarifier was used to produce purified water for HPLC-MS measurements. The *E. angustifolia* root extract (extraction: ethanol 85%, v/v; ratio of herbal drug - native extract: 6.5–8:1; excipient: maltodextrin 30%; marker: echinacoside 4%) was purchased from Euromed SA (Millet del Valles, Spain; Batch No. 419061).

Calibration standards and quality control samples: Standard stock solution was prepared by dissolving solid DTAI in water-acetonitrile (6:4) in a concentration of 1 µg/20 µL. Working standard solutions were prepared by serial dilutions using water-acetonitrile (6:4) from the stock solution to final concentrations that covered the concentration range of the assay. The benzanilide internal standard (IS) stock solution, 4.12 ng/20 µL, was prepared using water-acetonitrile (6:4). Its concentration was 1 ng/20 µL in the calibration standards and in the samples. Quality control samples (QC) were prepared using the procedure of sample preparation at the concentrations of 0.023 ng/20 µL (LC) and 0.920 ng/20 µL (HC). The IS concentration was 1 ng/20 µL.

Animals and treatment: Animals were treated in accordance with the European Communities Council Directives (86/609/ECC) and the Hungarian Act for the Protection of Animals in Research (XXVIII. section 243/1998). All experiments involving animal subjects were carried out with the approval of the Hungarian Ethical Committee for Animal Research (registration number: IV./01758-2/2008). Male Wistar rats of 200–240 g body weight (Toxi-Coop, Budapest, Hungary) were housed in temperature (20–23°C), humidity (40–60%) and light (12 h of light, 12 h of dark) regulated rooms, with water and standard rodent food (Bioplan, Isaszeg, Hungary) intake available *ad libitum*. Animals were fasted for 16 h before oral treatment with 50 mg/kg *E. angustifolia* extract suspended in 0.25% methylcellulose in a dosing volume of 5 mL/kg. Six animals were randomized into each group.

Sample collection and preparation: After 30, 60, 240 and 720 min rats were anesthetized with isoflurane inhalation and blood samples were collected by cardiac puncture. Blood coagulation was prevented and plasma was prepared by centrifugation (4000 RPM, 10 min, 4°C). Plasma samples of 200 µL were extracted 2 times with 1200 µL methanol and 15 µL IS solution was added. The sample was homogenised by vortexing for 90 sec. After both extractions the samples were centrifuged (6200 RPM, 15 min, 4°C) and the collected supernatants were evaporated under nitrogen at room temperature. Whole brains were removed and periepididymal adipose tissues from both sides were collected and wet weights were recorded. Tissues were homogenized in a double volume of methanol, vortexed for 90 sec and centrifuged (6200 RPM, 15 min, 4°C). Fifteen µL IS solution was added to 500 µL supernatant and evaporated as mentioned before. Samples were dissolved in 200 µL water-acetonitrile (6:4) in an ultrasonic bath and then filtered using

a 0.45 μm pore size syringe filter into chromatographic sample bottles. Pharmacokinetic parameters were calculated by means of PKSolver in noncompartmental analyses [18].

HPLC-MS/MS conditions: LC analyses were performed using a Shimadzu HPLC system (Shimadzu, Tokyo, Japan), equipped with 2 LC-20AD pumps, CBM-20A controller, SPD-20A UV-Vis detector, SIL-20A autosampler, DGU-20A3 degasser and CTO-20AC column thermostat, coupled to an API 2000 triple quadrupole mass spectrometer, equipped with an electrospray (ESI) interface (AB SCIEX, Framingham, MA, USA). Chromatographic separation was achieved with a Phenomenex Kinetex XB-C18 column (2.1 × 50 mm; 2.6 μm) and a C18 guard column (both supplied by Gen-Lab Kft., Budapest, Hungary). Eluent A was 0.1% formic acid in acetonitrile and eluent B was 0.1% formic acid in water at a flow rate of 500 μL/min. The elution gradient started with 40% of eluent A, keeping isocratic conditions for 0.5 min. Then, eluent A increased to 90% in 1.0 min and was held for 1.0 min. Finally, initial conditions were reached again in 1.2 min, with a re-equilibration time of 1.7 min in order to restore the column. The sample injection volume was set at 20 μL. The ion source temperature was 325°C. Measurements were carried out in positive ionization mode and the quantification was accomplished by using multiple reaction monitoring (MRM) with transitions of m/z 248→152 for DTAI and m/z 198→105 for benzanilide. Data acquisition and evaluation were performed using Analyst 1.5.1 software.

Method validation: The applied HPLC-MS method, described above, was used and validated by Woelkart *et al*. [15] to quantitate DTAI in rat plasma and other tissues, except periepididymal fat. In this assay, validation was carried out only for periepididymal fat samples. The R^2 value for the standard curve was 0.9992 and the linear range was determined between 0.021–1.050 ng/20 μL. Limit of detection and limit of quantification were 0.0043 and 0.0214 ng/20 μL, respectively. Relative standard deviations of the replicate measurements were 19.8% for LC and 0.87% for HC samples. Extraction recovery was 75% for LC and 58% for HC samples.

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