

Pharmacokinetics and immunomodulatory effect of lipophilic Echinacea extract formulated in softgel capsules

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Anti-inflammatory
cytokines:

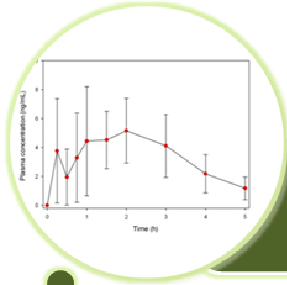
IL -10

Pro- inflammatory
cytokines:

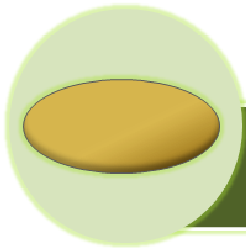
IL -6, IL-8, TNF- α



Immunomodulatory effect



Improved bioavailability



Echinacea angustifolia lypophylic extract
in Softgel capsules (1 mg tetraene)

Abstract

An attractive herbal product, softgel capsules containing 10 mg of *Echinacea angustifolia* lypophylic extract, was given in a single oral administration to 10 human **voluntaries** to perform a pharmacokinetic and immunological study.

The plasma concentration of the major constituent was monitored, quantifying at predetermined time points the dodeca-2*E*,4*E*,8*Z*,10*E/Z*-tetraenoic isobutylamides (tetraene). The plasmatic levels of IL-2, IL-6, IL-8, IL-10 and TNF- α in samples collected before and 24 h after drug administration were analyzed by cytokine assay. The total RNA was extracted from limpho-monocyte isolated from the same blood samples and the same cytokines in term of gene expression were evaluated. With the help of proper statistical tests the differences between the values obtained at 0 and 24 h were evaluated.

Results of pharmacokinetic studies attest an approximately 3.5-fold improvement of tetraene oral bioavailability compared with previously published studies. Dodeca-2*E*,4*E*-dienoic acid isobutylamide exerts immunomodulatory effects down-regulating the gene expression and reducing the protein plasmatic levels of pro-inflammatory cytokines such as IL-6, TNF- α and IL-8, and up-regulating the expression of anti-inflammatory molecules as IL-10. Student's two-sided paired *t*-test and non-parametric Wilcoxon-Mann-Whitney signed rank test agree in the conclusions about the differences between the *ln* values at 24h and corresponding *ln* values at 0h.

Keyword:

Softgel capsules; *Echinacea angustifolia* lypophylic extract; tetraene; pro-inflammatory cytokines; pharmacokinetics.

Introduction

The historical and popular tradition of the medicinal use of *Echinacea* is related to the three most relevant species, *E. pallida*, *E. purpurea* and *E. angustifolia*. Nowadays, in developed countries products containing *Echinacea* are very common in pharmacies, grocery or health-food

stores, and hundreds of different commercial preparations are available. Independently of the kind of extract or preparation, *Echinacea* is considered as an enhancer of the immune response and its main indications are the prevention and treatment of common cold, flu and upper respiratory tract or lower urinary tract infections (Blumenthal et al., 1998). Four major groups of compounds contained in *Echinacea* preparations may be responsible for the immune response stimulation: the phenylpropanoids related to caffeic acid derivatives (caftaric acid, cynarine, cichoric acid and echinacoside), the alkylamides (2-ene and 2,4-diene), glycoproteins and polysaccharides (Bauer, 1999).

In spite of numerous new studies, the therapeutic effect of *echinacea* species cannot be clearly attributed to one specific class of constituents. However, previously published *in vivo* data in humans suggest that the alkamides, in particular the major alkamide, dodeca-2*E*,4*E*,8*Z*,10*E/Z*-tetraenoic acid isobutylamide (tetraene), play a very important role due to their numerous immunomodulatory and anti-inflammatory properties, by modulation of macrophages and PMN immune cells and cytokine/chemokine expression (Woelkart et al., 2005a; Woelkart et al., 2006; Guiotto et al., 2008).

Since *Echinacea* alkamides are clinically relevant ingredients, an appropriate standardization of their content and a suitable formulation allowing their stability and absorption must be warranted. Biopharmaceutical and pharmacokinetic data are primarily important to link results from *in vitro* assays to clinical studies, and hence to establish pharmacologically relevant blood levels. Data concerning the absorption, metabolism, the bioavailability, and bioactivity of such natural products and their metabolites after oral administration have been available only recently. The results of the studies are frequently contradictory due to the different compositions and different formulations (e.g. tinctures, tablets, bonbons, throat sprays) of the applied preparations (Dietz et al., 2001; Matthias et al., 2005; Woelkart et al., 2006; Matthias et al., 2007; Guiotto et al., 2008; Woelkart et al., 2008; Gurley et al., 2008).

For better stabilisation and absorption of the active Echinacea alkamides, in the present study, softgel capsules containing Echinacea angustifolia lypophylic extract with a well-defined content of actives (alkamides) have been prepared and administered to healthy voluntaries with the aim of evaluating both the major alkamide bioavailability and the immune response after single dose oral administration.

Material and methods

Echinacea angustifolia lypophylic extract (Echinamid[®], Indena Milan, Italy) formulated in softgel capsules by Pharmagel Engineering S.p.A. (Lodi, Milano, Italy) was tested. Each capsule (weighing 24 mg) contained 10 mg of above mentioned extract corresponding to a dose of 1 mg dodeca-2*E*,4*E*,8*Z*,10*E/Z*-tetraenoic isobutylamides. The capsules also contain the following inactive ingredients: gelatin, glycerin, titanium dioxide, iron oxide yellow.

Reagents

Acetonitrile (HPLC grade, Rotisolv) and Tris buffer (Pufferan) were 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 HPLC analysis. Acetonitrile, formic acid and methanol were from BDH (Liverpool, UK), Tris buffer (Tris-d₁₁, 99% D) and benzanilide (98%) from Aldrich (Milan, Italy).

Study design

10 healthy voluntaries of both genders (6 males, 4 females) between 26 and 53 years of age participated in this single blind study. All the volunteers were with normal liver function and without diagnosed allergy or sensitivity to *Compositae* or *Grossolariaceae* plants. None of the voluntaries was on a special diet. No medicines were taken during the study and the voluntaries were asked not to smoke and nor eat, and drink only water for 12 h before administration. All subjects gave their written consent to participate in this study. Each of them was administered a single softgel capsule containing 1 mg of dodeca-2*E*,4*E*,8*Z*,10*E/Z*-tetraenoic isobutylamides, orally

after an overnight fasting. Blood samples (5 mL) collected in heparinised tubes were taken at 0 (before administration) and at 15, 30, 45, 60, 90, 120, 180, 240, and 300 min after each dose. Plasma was separated by centrifugation and stored frozen at -80°C for analysis. In each period subjects were abstinent from smoking, eating and drinking until the last blood sample was taken. The study was conducted in compliance with the Declaration of Helsinki and the International Conference on Harmonization Guidelines at the University of Trieste, Italy and the protocol was approved by the University of Trieste Human Research Ethics Committee.

Blood samples (2 x 5 mL) collected in EDTA-tubes were taken once a week on days -35, -28, -21, -14, -7, 0, 7, 14, 21, 28 (for a total of 10 blood sampling). After sample withdrawal, one mL of plasma was immediately separated by centrifugation and frozen at -80°C for subsequent cytokine analysis assay. Within 4 h after blood withdrawal from 5 mL of complete blood, lympho-monocytes were isolated by Ficoll-Paque™ method introduced by Bøyum (1968) and already employed in our previous work (Dapas et al., 2014).

Plasma samples preparation

The plasma samples were extracted using a solid-phase extraction technique (Woelkart et al., 2005a; Guiotto et al., 2008). Briefly, to 2 mL of plasma 2 mL of Tris buffer and 100 µL of benzanilide solution, used as internal standard (I.S.), were added and vortexed for 1 min. Subsequently, samples were centrifuged for 10 minutes at 3,200 rpm using Eppendorf centrifuge 5810R (Hamburg, Germany). The supernatant was applied onto C18, 100 mg Isolute columns from International Sorbent Technology (Mid Glamorgan, UK), pre-treated with 1 reservoir volume (RV) acetonitrile followed by 1 RV water. The C18 cartridges were placed on a VacMaster sample processing station and subsequently washed with 1 RV of water under vacuum. The dodeca-2*E*,4*E*,8*Z*,10*E/Z*-tetraenoic acid isobutylamides were eluted with 2 mL acetonitrile. The eluents were evaporated under a stream of nitrogen at 40°C using TurboVab LV vaporator (Zymark,

Hopkinton, MA, USA), the dry residue was dissolved in 100 μL acetonitrile:water (6:4) and 20 μL were used for LC/MS analysis.

LC-MS-MS analysis

Alkamides concentrations in samples were determined by LC/MS analysis as previously described by Woelkart et al. (2005a) using LC-ESI-IT-MS/MS composed of Varian 212 binary chromatograph equipped with a Prostar 430 thermostatic autosampler and Varian 500 ion trap mass spectrometer with Varian Workstation software. Gradient elution was used using acetonitrile with 0.1% formic acid and water with 0.1% formic acid 40/60 (v/v) to 80/20 (v/v) in 13 min. As stationary phase a Zorbax SB-C18 Narrow Bore 2.1 x 150 mm 3.5 μm column from Agilent Technologies was used at a flow rate of 200 $\mu\text{L}/\text{min}$. For MS detection ESI interface operating in positive Ion mode was used. The source voltage was set at 4.8 kV, vaporizer temperature at 300°C, nebulizer gas pressure was at 15 psi and helium gas for collision. For SRM (single reaction monitoring) mass transition m/z 248 \rightarrow m/z 149 was used for quantification of dodeca-2*E*,4*E*,8*Z*,10*E/Z*-tetraenoic isobutylamides. The analytical procedure was validated by analysis of quality control samples. Calibration curves were obtained by plotting the dodeca-2*E*,4*E*,8*Z*,10*E/Z*-tetraenoic isobutylamides/I.S. (Benzamide) peak area versus the known spiked plasma concentration of the analyte. Limit of detection was at 0.1 ng mL^{-1} . Linearity was obtained in the range of 0.3–80 ng mL^{-1} .

Pharmacokinetics analysis

Non-compartmental pharmacokinetic analysis was performed using WinNonlin Version 2.1 software from Pharsight Corporation (Mountain View, CA). Time and value of maximum concentration (t_{max} and C_{max} , respectively) were reported as observed. Terminal half-life ($t_{1/2}$) was calculated as $t_{1/2} = \ln 2 / \lambda_z$, where λ_z is the terminal slope of the plasma concentration profile on the semi-log plot calculated by linear regression. The area under the concentration-time curve (AUC)

was calculated using the linear trapezoidal method and extrapolated to infinity (AUC_{inf}) as $AUC + C_{last}/\lambda_z$, where C_{last} is the last measurable concentration. Mean residence time (MRT) was calculated as $AUMC_{inf}/AUC_{inf}$, where $AUMC_{inf}$ is the area under the first moment curve. Since fraction of the dose absorbed (F) cannot be estimated based solely on the data after extravascular administration, apparent clearance (CL/F), calculated as $Dose/AUC_{inf}$, and apparent volume of distribution based on terminal phase (V_z/F), calculated as $CL/F/\lambda_z$, are reported.

Cytokine analysis

The levels of 48 cytokines (in particular, the attention was paid to IL-2, IL-6, IL-8, IL-10 and TNF- α), in plasma samples collected before and 24 h after drug administration, were analysed using two Bio-Plex cytokine assay panels (the pro-human cytokine 27-Plex M50-0KCAF0Y and 21-Plex MF0-005KMII, both from Bio-Rad, Hercules, CA) as recently reported (Comar et al., 2014). The samples were analyzed following the manufacturer's instructions using the BioPlex 100 Analyzer (Bio-Rad Laboratories).

Quantitative PCR

Total RNA was extracted, quantified and the quality evaluated as described (Baiz et al., 2009). Reverse transcription was performed using 500 ng of total RNA in the presence of random examers and MuLv reverse transcriptase (Applera Corporation, USA). To detect the mRNA levels of the cytokines IL-2, IL-8, IL-6 and TNF- α , Real-Time polymerase chain reaction was performed as described (Baiz et al. 2009). The primers (MWG Biotech, GA, USA, 300 nM) and the annealing temperatures used are reported in Table 1. The relative amounts of each target mRNA was normalized by 28S rRNA content according to Pfaffl (2004).

Statistical analysis

Statistical analyses were performed using R version 3.1.2 (R Core Team, 2014). The Shapiro-Wilk test rejected normality for some of the samples. Therefore, both mRNA values and plasmatic concentrations were transformed by taking their natural logarithm (\ln). Actually, the lognormal

distribution of mRNA levels is a well consolidated result (Bengtsson *et al.*, 2005). The differences between the *ln* values at 24h and corresponding *ln* values at 0h were evaluated by Student's two-sided paired *t*-test. Taking into account the size of our sample ($n = 10$ voluntaries), we also applied the non-parametric Wilcoxon-Mann-Whitney (WMW) signed rank test in its "exact" combinatoric form without normal approximation (Hothorn and Hornik, 2013).

Results and discussion

Pharmacokinetic study

Some of the *in vivo* immunomodulating effects of the extracts of the roots of *E. angustifolia* are likely caused by the fat-soluble alkylamides (alkamides), as reported in the recent years by several authors (Dietz *et al.*, 2001; Matthias *et al.*, 2005 and 2007; Guiotto *et al.*, 2008; Woelkart *et al.*, 2005a, 2006, 2008). Nevertheless up to now limited information have been published considering both the biological effects and the alkylamide pharmacokinetics. Within this aim, in this research softgel capsules formulated lipophilic extract was administered to 10 healthy voluntaries and pharmacokinetics was studied, quantifying at predetermined time points the plasma concentration of the major constituent, namely, dodeca-2*E*,4*E*,8*Z*,10*E/Z*-tetraenoic isobutylamide (tetraene) as suggested in some previous studies (Woelkart *et al.* 2005a, 2006; Guiotto *et al.*, 2008). It has now been known for many years that softgel formulation can ameliorate the pharmacokinetic profiles of certain pharmaceutical drugs and natural products (e.g. silybin, Coenzyme Q10 and Hypericum perforatum extracts) (Agrosi *et al.*, 2000).

Mean time course of dodeca-2*E*,4*E*-dienoic acid isobutylamide concentration is presented in Figure 1. Results of the non-compartmental pharmacokinetic analysis are summarized in Table 2.

In agreement with the high permeability in the Caco-2 cells absorption model (Jager *et al.*, 2002; Matthias *et al.*, 2004) dodeca-2*E*,4*E*-dienoic acid isobutylamide was very rapidly absorbed. Time of maximum plasma concentration ranged between 0.25 and 2 h. In five out of ten subjects maximum plasma concentration was observed already after the first blood draw, 15 min after drug

administration. A double-peak phenomenon was evident from the individual pharmacokinetic profiles. Typically, the first peak concentration at 15 min was followed by a second peak at about 1 hour. This may be associated with the micellization of the alkamides, previously demonstrated by Raduner et al. (2007). Evaluation of the results of previous pharmacokinetic studies with Echinacea extract, Echinacea extract tablets, throat spray, and Echinacea bonbons (Dietz et al., 2001; Matthias et al., 2005; Woelkart et al., 2006; Matthias et al., 2007; Guiotto et al., 2008; Woelkart et al., 2008) containing various doses of dodeca-2E,4E-dienoic acid isobutylamide (ranging from 0.07 to 17.16 mg) reveals more than proportional increase in AUC with the increase in the dose, indicating nonlinear pharmacokinetics. The relationship between dose normalized tetraene AUC and dose is approximately linear (Fig. 2). Comparison of AUC/Dose of softgel capsules evaluated in the current study (mean: 16.93 ng × h/mL/mg; 95% CI: 15.33-18.53 ng × h/mL/mg) with the 95% prediction interval from the previous studies indicates significant 3.5-fold improvement in bioavailability of tetraene with this type of formulation.

Immunological *in vivo* study

The results corroborate the hypothesis that the alkamides can be easily absorbed and, hence, may contribute to the *in vivo* effects of echinacea preparation. Furthermore, receptor binding studies to cannabinoid type 1 and 2 receptors (CB1 and CB2) were carried out to demonstrate the molecular mode of action and highlight the role of the alkamides as potent immune-modulators (Raduner et al., 2006; Gertsch et al., 2004; Woelkart et al., 2005b).

Student's two-sided paired *t*-test and non-parametric Wilcoxon-Mann-Whitney (WMW) signed rank test agree in the conclusions about the differences between the *ln* values at 24h and corresponding *ln* values at 0h. In all cases we got *p*-values < 0.05, that is, the treatment has a statistically significant effect both on the mRNA values and the plasmatic concentrations. In particular, the treatment decreases the values of interest, with the single exception of the mRNA for IL-10, which, instead, increases.

The *ln* data were graphically presented as boxplots (Figure 3 and 4), where whiskers extend to the data extremes; the arrows join paired data. The significant stars code is $0 < *** \leq 0.001 < ** \leq 0.01 < * \leq 0.05$. Fig. 5 presents the 95% CI's. The CI's estimated by the *t*-test are centred at the means of differences marked with a triangle-down symbol, while the CI's given by the WMW test are (usually asymmetrically) located at the (pseudo) medians of the same differences, marked triangle-up. The agreement of the parametric and the non-parametric CI's is neatly satisfactory, and so both tests jointly support our conclusions.

As regards to the dodeca-2E,4E-dienoic acid isobutylamide immunomodulatory effects via cytokines expression, a down-regulation of mRNA and plasmatic level of IL-2, a potent interleukin necessary for the growth, proliferation, and differentiation of T cells (Smith, 1988) were observed. Our data are in accordance with Sasagawa and colleagues (2006) that reported the capability of Echinacea alkylamides to suppress the expression by Jurkat T cells of IL-2. This observation indicates that the intake of Echinacea extract may inhibit the adaptive immune response, acting on the activation of lymphocyte cells.

In this case, the anti-inflammatory effect of dodeca-2E,4E-dienoic acid isobutylamide is also manifested by the down-regulation, both in terms of gene and protein expression, of IL-6 and TNF- α , two cytokines principally responsible for systemic inflammation (Jones, 2005). IL-6 and TNF- α are pro-inflammatory cytokine, produced by professional antigen-presenting cells (macrophages, B cells and DC). Their activity is crucial for the management of acute inflammation and subsequent resolution, but they are also associated with chronic inflammatory diseases (Jones, 2005). Our data are in agreement with Cech et al. (2010) that observed that dodeca-2E,4E-dienoic acid isobutylamide suppressed the production of TNF- α by macrophages-like cell. Gertsch et al. (2004) reported on the potent modulatory action of Echinacea alkylamides on TNF- α expression in human monocytes/macrophages. It was shown that this effect is mediated via CB2 cannabinoid receptor. Raduner and colleagues (2006) showed that tetraene bind CB2 receptor more strongly than the endogenous cannabinoids. But in contrast with our data, they observed an up-regulation of IL-6.

These evidences indicate that Echinacea extracts may reduce the inflammatory response and act also on the innate immune-response.

In agreement with our results, in 2006 Woelkart and coauthors demonstrated that the intake of Echinacea purpurea preparation (23 h after oral application) led to a significant down-regulation of TNF- α and IL-8 in LPS pre-stimulated whole blood. Also our data indicates a down regulation, by Echinacea extract intake, of the gene expression and synthesis of plasmatic protein of IL-8, a chemokine produced by endothelial cells and macrophages, that promotes the neutrophil migration and leukocytes arrest (Sprague and Khalil, 2009). All this information confirm dodeca-2E,4E-dienoic acid isobutylamide anti-inflammatory action.

There is good evidence that constitutive TNF- α is inhibited by the endocannabinoid system and that the CB₂ receptor is involved (Rajesh et al., 2008; Mechoulam and Shohami, 2007). The CB₂ agonists dose-dependently attenuate the effects of TNF- α . TNF- α triggers the activation of Ras, p38 MAPK, ERK1/2, SAPK/JNK and Akt pathways and ultimately pro-inflammatory cytokine expression and cellular proliferation and migration. On the other hand, activation of the CB₂ receptor by agonists in macrophages has been shown to activate Erk1/2 which leads to expression of IL-10 and thus a counteraction to pro-inflammatory gene expression (Gertsch, 2009). IL-10 is the typical anti-inflammatory cytokine, produced by macrophages (in particular M2), T lymphocytes (Th2 and Treg) and B-cells. It inhibits the pro-inflammatory cytokines production and the Th1 (and M1) response (Sprague and Khalil, 2009).

Also our data indicates that IL-10 gene is up-regulated by CB₂ agonist, but unexpectedly this increase is not correlated with an augmentation of the plasmatic level of this anti-inflammatory cytokine. Maybe this difference is due to a delay of the response at the systemic/peripheral tissue level by wide variety of cells, responsible of the synthesis of the plasmatic cytokine IL-10.

Conclusions

In this pilot study, 10 human voluntaries have been treated with a new herbal product: softgel capsules containing 10 mg of *Echinacea angustifolia* lipophylic extract, corresponding to a dose of 1 mg dodeca-2*E*,4*E*,8*Z*,10*E/Z*-tetraenoic isobutylamides (tetraene). Results of the present pharmacokinetic study confirm that softgel capsules containing lipophylic *Echinacea* extract are an attractive formulation to improve bioavailability of tetraene. It was estimated that improvement is approximately 3.5-fold compared with previous studies with other tetraene formulations. However, pharmacokinetics of the reference formulations has not been compared directly, which is a limitation of the present study. The effect on bioavailability of tetraene has to be confirmed in future studies. Additionally, this study demonstrates the effect on the innate and adaptive immune-system of *Echinacea angustifolia* lipophylic extract in healthy subjects, supporting at least in part its use as health promoting supplement. Indeed this treatment has potentially a strong anti-inflammatory effect reducing the synthesis and plasma levels of powerful pro-inflammatory cytokines such as IL-6, TNF- α and IL-8 and up-regulating the expression of anti-inflammatory molecules as IL-10.

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Figure Legends

Figure 1: Mean (\pm SD) time course of dodeca-2E,4E-dienoic acid isobutylamide concentration in plasma in 10 healthy volunteers following oral administration of *lipophilic Echinacea extract* formulated as soft gelatine capsules (dose 1 mg).

Figure 2.

Dose dependency of dodeca-2E,4E-dienoic acid isobutylamide bioavailability. The data on bioavailability were extracted from previous pharmacokinetic studies with various formulations of Echinacea extract. This analysis demonstrates that the increase in AUC/Dose with Dose is approximately linear (regression line and 95% prediction interval). Error bars with Soft gel caps indicate 95% confidence interval.

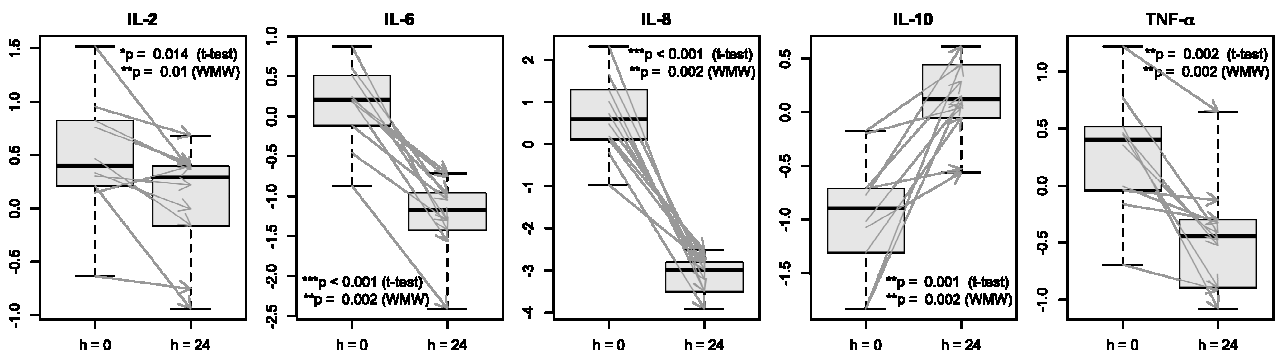
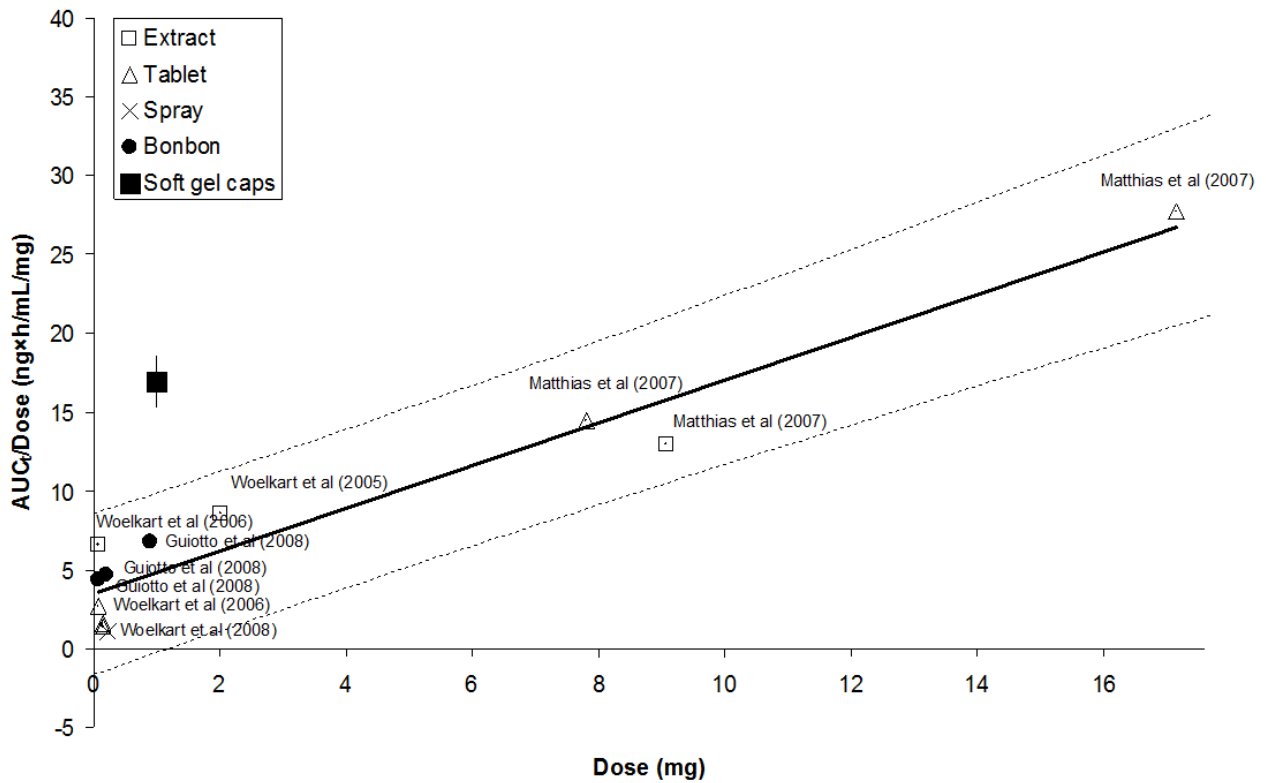
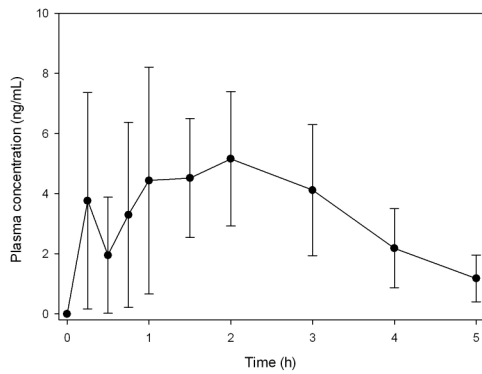
Figure 3 Box-and-whisker chart for the \ln of mRNA/28S observed data at time $t = 0\text{h}$ and $t = 24\text{h}$.

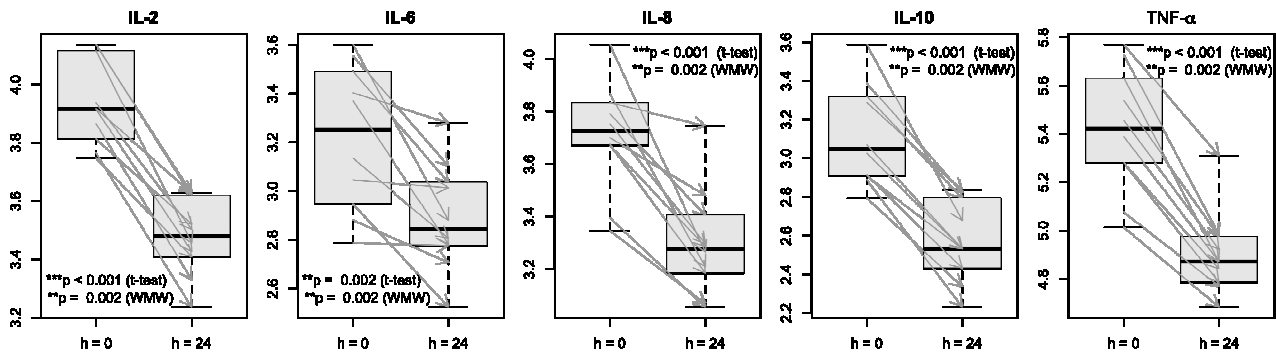
The whiskers extend to the data extremes; the arrows join paired data. The p -values refer to the paired Student's t -test and to the paired non-parametric Wilcoxon-Mann-Whitney (WMW) signed rank test in its "exact" combinatoric form. The significant stars code is $0 < *** \leq 0.001 < ** \leq 0.01 < * \leq 0.05$.

Figure 4 Box-and-whisker chart for the \ln of plasmatic observed data (pg/ml) at time $t = 0\text{h}$ and $t =$

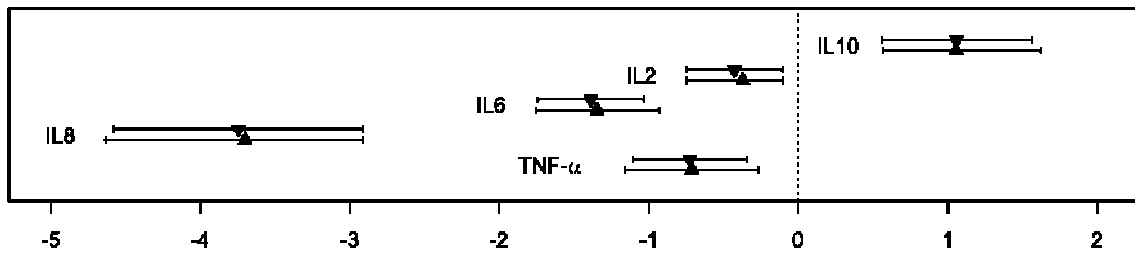
24h. The whiskers extend to the data extremes; the arrows join paired data. The p -values refer to the paired Student's t -test and to the paired non-parametric Wilcoxon-Mann-Whitney (WMW) signed rank test in its "exact" combinatoric form. The significant stars code is $0 < *** \leq 0.001 < ** \leq 0.01 < * \leq 0.05$.

Figure 5 95% CI's for differences h24-h0 of mRNA/28S and plasmatic levels (\ln scaled). The CI's estimated by the t -test are centered at the means of differences, marked with a triangle-down, while those given by the WMW test are located at the pseudo-medians of the same differences, and are marked with a triangle-up.





mRNA/28S (ln)



Plasma levels [pg/ml] (ln)

