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Liquid chromatography/atmospheric pressure chemical ionization ion trap mass spectrometry of terpene lactones in plasma of animals

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Abstract

Liquid chromatography/atmospheric pressure chemical ionization ion trap mass spectrometry (LC/APCI-ITMS) was applied to evaluate the bioavailibility of two different forms (free and complexed with soy phospholipids) of pure bilobalide and ginkgolide B in rats after acute administration. The same technique was used to measure the levels of ginkgolide A, B and bilobalide in plasma of guinea pigs fed *Ginkgo biloba* extract enriched in terpene lactones after chronic administration. The ratio R_P/R_A increased two to four times after the administration in the phytosomic form, where R_P and R_A represent the percentage ratio between the concentration of each terpene lactone in plasma and in the administrated form, respectively.

Keywords: LC/APCI-ITMS; Bilobalide; Ginkgolide B; Pharmacokinetics; Phospholipid complex

1. Introduction

Ginkgo biloba L. leaves have found medicinal application for hundred of years in China and recently their extracts have gained attention also in Europe. Their extracts are reported to possess various activities and are thought to prevent and treat some diseases, such cerebral and vascular insufficiency, cognitive deficits and other age-associated impairments [1,2]. Dozens of compounds have been isolated and identified from extracts of *G. biloba* leaves. The pharmacological activity of *G. biloba* extracts in the central nervous system is only partially understood, but the main effects may be due to the antioxidant properties, attributed to the synergistic action of the main constituents: flavonoid-glycosides, the terpenoids (ginkgolides, bilobalide), and organic acids [3].

Despite the many phytochemical and clinical investigations published, only few data are avail-

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able on the pharmacokinetics of *G. biloba* components. One reason for this limited information is the high sensitivity, which is required to determine very low plasma levels of terpene lactones (10 nM). Biber et al. succeeded in this task using gas chromatography/mass spectrometry (GC/MS) after derivatisation of ginkgolides [4]. Alternatively, Li et al. estimated ginkgolide concentrations in animal plasma by a bioassay based on ginkgolide capacity to inhibit the binding of platelet activating factor (PAF) to its receptor [5].

Recently, liquid chromatography/atmospheric pressure chemical ionization ion trap mass spectrometry (LC/APCI-ITMS) was applied to study the pharmacokinetics in humans of two different *G. biloba* formulations, namely a standardised extract (Free formulation) and its complex with soy phospholipids (Phytosome formulation) [6]. This study showed that ginkgolides A and B as well as bilobalide are more bioavailable when supplied in the phospholipid complex form.

Extending this study, LC/APCI-ITMS was applied to follow the pharmacokinetics in animals of standardised *G. biloba* extracts enriched with terpene lactones, purified ginkgolide B and bilobalide either in free form or complexed with soy phospholipids. The concentrations of terpene lactones in plasma of rats and guinea pigs were determined after acute (purified ginkgolide B and bilobalide) or chronic (*G. biloba* extract enriched with terpene lactones) administration in either free or phytosomic form.

In addition, we investigated the capacity of terpene lactones to counteract the pulmonary effects of some mediators of airway inflammation. Specifically, the protective activity against histamine (Hist) and PAF-induced bronchoconstriction in anaesthetised guinea-pigs were evaluated as well as the blood ability to inhibit the production of thromboxane B_2 (TXB_2).

2. Experimental

2.1. Chemicals

Purified bilobalide (free, [BILOB] and Phytosome®, IDN 5604) and ginkgolide B (free, IDN 5063, and Phytosome®, IDN 5623) were isolated and characterised by Indena Chemical Laboratories (S.p.A., Milan, Italy). *G. biloba* extracts enriched in ginkgoterpenes (free, IDN 5380, and Phytosome®, IDN 5381) were provided by Indena. All other reagents were HPLC grade (J.T. Baker, Deventer, Holland).

2.2. Animals

2.2.1. Rats

96 male CD animals (Charles River Italy, Calco, LC, Italy) weighing 275–300 g were housed under standard conditions (room temperature 22 ± 2 °C, humidity 65 ± 1%, 12-h light:12-h dark-cycle) and treated orally with purified bilobalide and ginkgolide B in free and Phytosome® forms (20 mg/kg os suspending in 1% carboxymethylcellulose).

2.2.2. Guinea pigs

25 male Dunkin Hartley animals (BMG, Cividate al Piano, BG, Italy) weighting 300–350 g were housed under standard conditions (room temperature 22 ± 2 °C, humidity 65 ± 1%, 12-h light:12-h dark-cycle) and treated orally with *G. biloba* extract enriched in terpene lactones in free and Phytosome® forms (100 mg/kg × die os for 5 days).

2.3. Protocol

2.3.1. Acute experiment with rats

Three animals were killed at 30, 60, 120, 240, 300, 360 min after administration of free or phytosomic form. Blood samples (5 ml) were collected in heparized syringes and after centrifugation at $2000 \times g$ for 10 min at 4 °C plasma was obtained and subdivided into aliquots of 300 μl and stored at −80 °C until analyses.

2.3.2. Chronic experiments with guinea pigs

2 weeks after their arrival, animals were subdivided randomly into three groups: the first one ($n = 5$) was supplemented with 1% carboxymethylcellulose (controls), the second one ($n = 10$) with *G. biloba* extract enriched in terpene lactones and the third one ($n = 10$) with phytosomic form of *G. biloba* extract enriched in terpene lactones. Blood

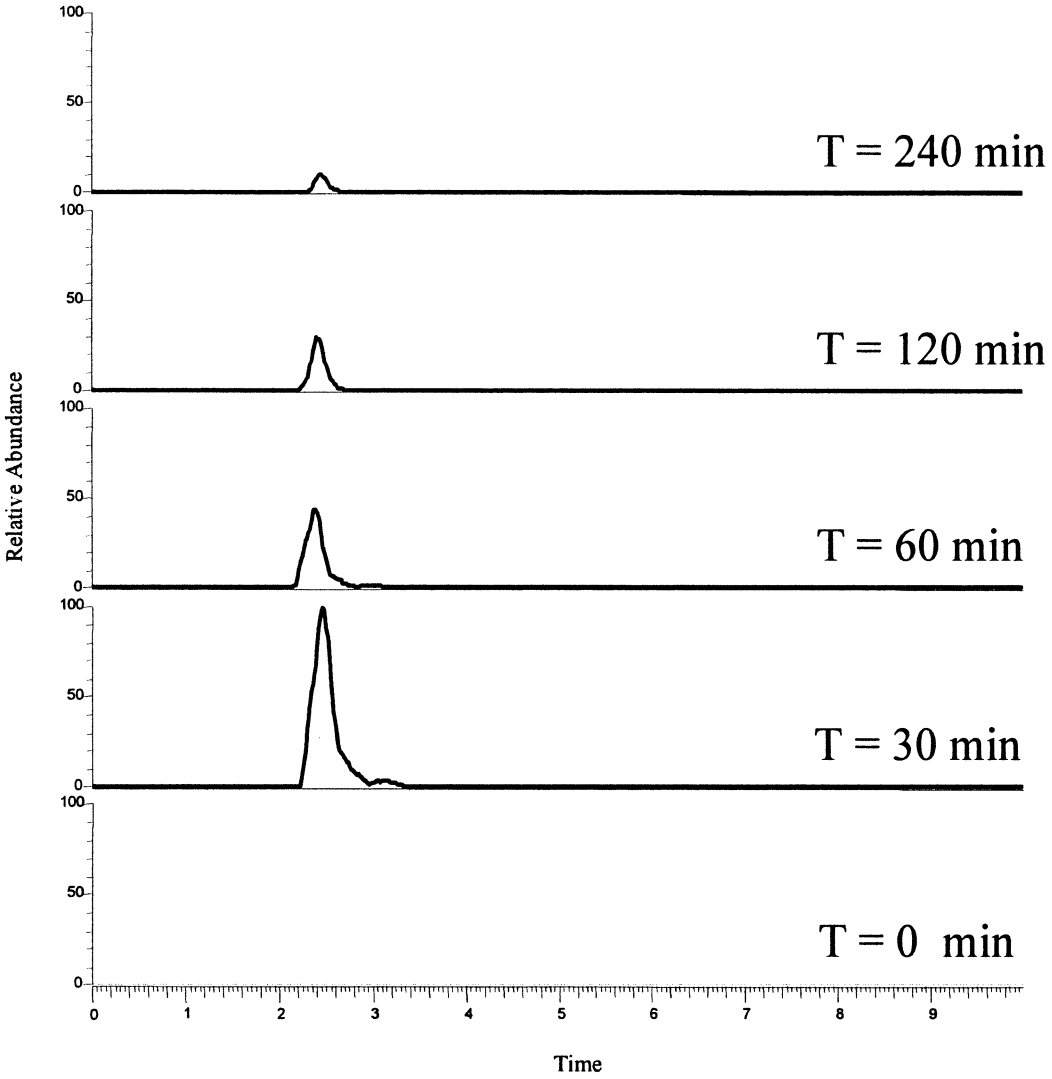

Fig. 1. Extracted ion chromatograms (m/z 325) of plasma samples collected at different times from rats after administration of phytosomic form of purified bilobalide.

samples (5 ml) were collected after 5 days of treatment in heparized syringes and after centrifugation at 2000 × g for 10 min at 4 °C plasma was obtained and subdivided into aliquots of 400 μl and stored at −80 °C until analyses.

2.4. Sample preparation

The plasma samples (0.3 ml) were extracted with the same volume of ethylacetate and, after centrifugation at 2000 × g for 2 min, the supernatant was evaporated to dryness under vacuum. The residue was dissolved in 150 μl of 10% methanol, and 100 μl were injected into LC/APCI-ITMS system.

2.5. LC-MS conditions

A Spectra Series HPLC (Thermoquest, Milan, Italy) equipped with an autosampler was used;

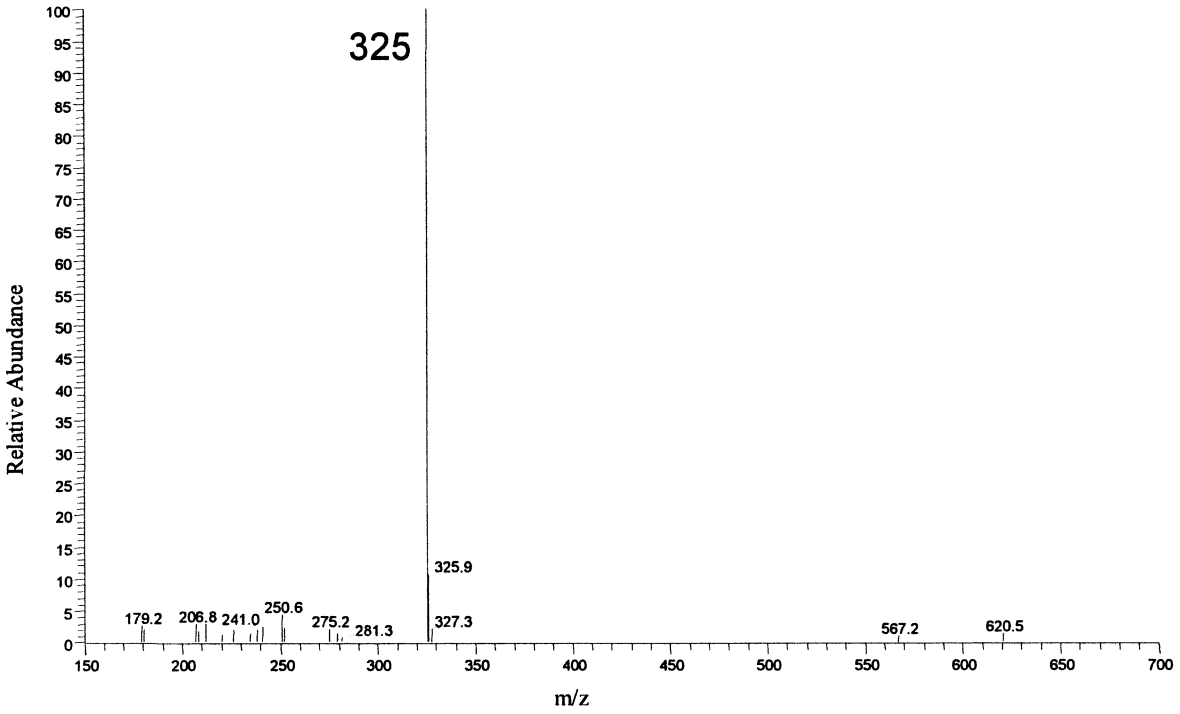

Fig. 2. Typical online mass spectrum of bilobalide from Fig. 1.

separations of ginkgolides were performed by C18 Hypersil column (100 × 3 mm, 5 μm) and a methanol gradient (eluent A: water, eluent B: methanol; 0–1 min 30% B, 1–7 min from 30 to 45% B, 7–10 min 45% B). The flow-rate was 0.55 ml/min and the volumes injected were 50 μl.

Terpene lactone detection was performed by means of a LCQ_{Deca} ion trap mass spectrometer (Thermoquest), equipped with atmospheric pressure chemical ionisation interface (APCI). APCI parameters were optimised by flow injection of ginkgolides and bilobalide standard solution. LC-MS analyses were carried out in the negative ion scan mode from m/z 200 to 700. For the other instrumental parameters see Mauri et al. [6].

2.6. Calibration curves

Ginkgolide and bilobalide standards were dissolved in methanol (about 1 mg/ml) and stored at 0 °C. Aliquots of terpene lactone standard solutions in the range 5–2000 ng/ml were injected into HPLC apparatus. Peak areas were integrated against the corresponding masses of injected standards.

3. Results and discussion

The pharmacokinetic experiments were performed using LC-APCI-ITMS conditions validated in a previous work [6]. In particular, calibration curves were linear in the range from 5 to 2000 ng/ml, and the correlation coefficients were around 0.999. The overall reproducibility of quantitative analysis of ginkgolides and bilobalides was ±3.1 and ±4.7% for intra- ($n = 4$) and inter- ($n = 5$) day analysis, respectively. Ion trap device provided detection limits in full-scan mode (signal-to-noise (S/N) = 5), of around 2 ng/ml for standard solutions. Extraction recovery for ginkgoterpene lactones was about 70% and analytes were stable for 24 h at 4 °C.

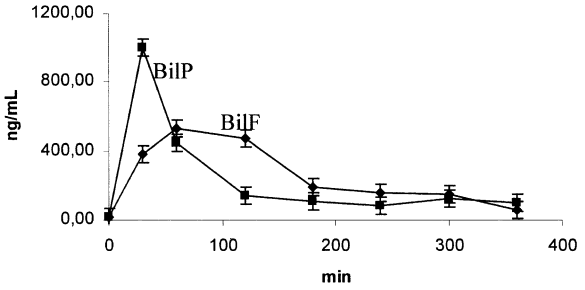

Fig. 3. Plasma concentrations of bilobalide after a single oral administration of free (BilF) and phospholipid complex (BilP) forms.

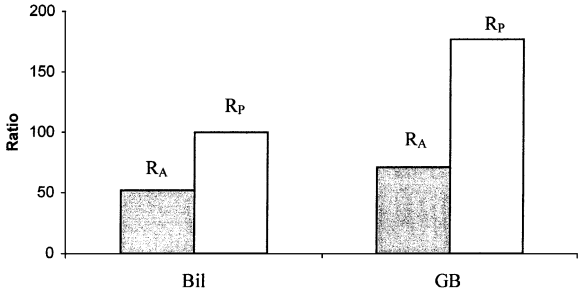

Fig. 4. Comparison between the percentage ratios of bilobalide (Bil) and ginkgolide B (GB) in the administrated forms (R_A) and in related plasma samples of rats (R_P) in acute experiments.

Table 1
Pharmacokinetic parameters for bilobalide administrated to rats as free (BilF) and phytosomic (BilP) form

Parameter	BilF	BilP
C_{max} (ng/ml)	533	1001
T_{max} (min)	60	30
AUC (min × ng/ml)	87 101	87 502

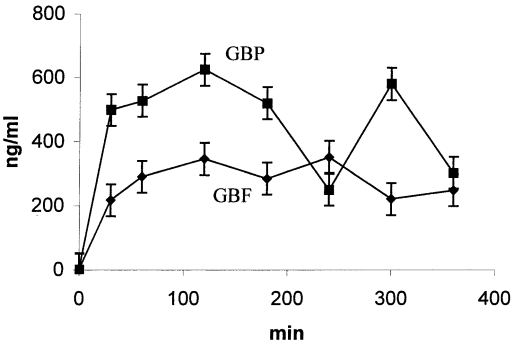

Fig. 5. Plasma concentrations of ginkgolide B after a single oral administration of free (GBF) and phytosomic (GBP) forms.

3.1. Acute administration of purified ginkgoterpenes to rats

3.1.1. Bilobalide

The concentration of bilobalide was about 95% in the free form and about 49.6% in the complex form (Phytosome®). Typical mass chromatograms of samples collected at different times from rats after administration of purified bilobalide in phospholipid complex form are shown in Fig. 1. Peak identification was assessed by retention time and negative online mass spectra (Fig. 2). The plasma concentrations of bilobalide after a single oral administration of bilobalide either in free or phospholipid complex forms are shown in Fig. 3. Pharmacokinetic parameters are summarised in Table 1 and show that the AUC values for bilobalide are similar in both types of formulation administrated. However, it has to be reminded that the concentration of bilobalide in the free form is two times higher than in phytosomic form. Hence, the percentage ratio ($R_A = 100 \times$ [Bilobalide]$_{Phytosome}$/[Bilobalide]$_{Free}$) of bilobalide in the two forms administrated to rats is about 50%. On the other hand, in plasma samples the percentage ratio ($R_P = 100 \times$ [Bilobalide]$_{Plasma\ Phytosome}$/[Bilobalide]$_{Plasma\ Free}$) of AUC values of bilobalide was 100% (Fig. 4). This suggests, that the bioavailability of bilobalide in the phytosomic form is doubled compared with the free form (R_P/R_A).

Table 2
Pharmacokinetic parameters for ginkgolide B administrated to rats as free (GBF) and phytosomic (GBP) form

Parameter	GBF	GBP
C_{max} (ng/ml)	352	625
T_{max} (min)	240	120
AUC (min × ng/ml)	86 880	153 855

Table 3
Composition (%) of *G. biloba* extract enriched in terpene lactones administrated to guinea pigs in chronic experiments

	GA	GB	GC	Bil	GJ	Total
Free form	24.1	16.8	7.3	45.4	0.8	94.5
Phytosomic form	8.3	5.8	2.5	15.7	0.3	32.6

3.1.2. Ginkgolide B

The concentrations of ginkgolide B were about 84 and 60% in the free and in the complex (Phytosome®) forms, respectively. The plasma concentrations of ginkgolide B after a single oral administration of either free or phospholipid complex formulation are shown in Fig. 5. Pharmacokinetic parameters are summarised in Table 2. Values for AUC doubled after the administration of the complex form. Also in this case, considering the percentage ratios of ginkgolide B in the administrated formulations ($R_A = 71\%$) and in the related plasma samples ($R_P = 177\%$), the absorption of ginkgolide B resulted about 2.5 times (R_P/R_A) higher in the case of the phytosomic form (Fig. 4).

It is interesting to note that the purified ginkgolide B contained also ginkgolide A (13.3% in the free 9.3% in the complex form). As for ginkgolide B, the bioavailibility of ginkgolide A in phytosomic form was higher than that of free form.

3.2. Chronic administration of G. biloba extract enriched with terpene lactones in guinea pigs

Preliminary results have shown that administration of *G. biloba* extracts enriched with terpene lactones in complex form produced a higher protection against Hist and PAF-inducedbronchoconstriction (about four times) as compared with the free form (G. Rossoni, personal communications). This effect is in good agreement with the pharmacokinetic resultsobtained after acute administration of purified ginkgoterpenes in rats. Therefore, it was of interest to evaluate the bioavailibility of ginkgoterpenes after chronic administration of *G. biloba* extract enriched in terpene lactones also in guinea pigs.

Table 4
Percentage ratios of bilobalide, ginkgolide A and B in the administrated forms (R_A) and in the related plasma samples of guinea pigs (R_P) in chronic experiments

Terpene lactone	R_A[a]	R_P[b]	R_P/R_A
Bilobalide	34.5	81.2	2.3
Ginkgolide A	34.5	133.8	3.8
Ginkgolide B	34.5	134.9	3.9

[a] $R_A = 100 \times [\text{terpenelactone}]_{\text{Phytosome}}/[\text{terpenelactone}]_{\text{Free}}$.
[b] $R_P = 100 \times [\text{terpenelactone}]_{\text{Plasma Phytosome}}/[\text{terpenelactone}]_{\text{Plasma Free}}$.

Aliquots of blood were collected from rats treated with either free or phytosomic form (100 mg/kg os) after 5 days, and the amount of each single ginkgoterpene-lactone was measured by LC/APCI-ITMS. The total percentage of ginkgoterpenes in the administrated extracts was 94.5 and 32.6% for free and phytosomic forms, respectively (Table 3). The data obtained from the LC/APCI-ITMS analyses of guinea pig plasma samples (expressed as R_A and R_P ratios) are summarised in Table 4. Comparing the ratios R_A and R_P for each terpene lactone, it is evident that also the chronic administration of the phytosomic form to guinea pigs promoted an higher bioavailability as compared with free form.

In spite of its presence in the extracts, ginkgolide C could not be detected in plasma samples. This finding is in accordance with data previously reported by other authors [6,7].

4. Conclusions

LC/APCI-ITMS analyses of the pharmacokinetics of purified bilobalide and ginkgolide B after acute administration in rats resulted in an higher bioavailabilty of the Phytosome® complex compared with the free form. Similarly, chronic administration of *G. biloba* extract enriched in terpene lactones to guinea pigs allowed to detect higher plasma levels after the supply of the complex form. These results are in good agreement with the higher protection against Hist and PAF-induced bronchoconstriction after administration of phospholipid complex form compared with the free form.

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