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LC–MS characterization of terpene lactones in plasma of experimental animals treated with *Ginkgo biloba* extracts Correlation with pharmacological activity

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Abstract

Liquid chromatography/atmospheric pressure chemical ionization ion trap mass spectrometry (LC/APCI-ITMS) was applied to determine the concentration of terpene lactone in plasma of guinea pigs after chronic administration of *Ginkgo biloba* extract enriched in ginkgoterpenes in free form (IDN 5380) or complexed with soy phosphlipids (IDN 5381). Oral treatment of the animals with ginkgoterpenes resulted to inhibit the bronchoconstriction (ITP) and the concomitant increase of the levels of thromboxane B_2 (TXB_2) in the circulation caused by histamine (HIST) and platelet activating factor (PAF) in normal guinea pigs or by ovalbumin (OA) in actively sensitized guinea pigs. To compare the protective activities of *G. biloba* forms (IDN 5380 and IDN 5381), ED_{50} and dose ratio (DR) values for both parameters (ITP and TXB_2) were evaluated. The phytosomic form (IDN 5381) significantly reduced (two- to four-fold as compared to free form, $P < 0.001$) the HIST, PAF or OA-induced airway changes and TXB_2 release. In addition it has been observed that the absence of ginkgolide C (GC) in plasma samples (in human and animals) was due to its rapid methylation.

Keywords: Mass spectrometry; Terpene lactones; Phospholipid complex; Pharmacological studies; Phytosomes

1. Introduction

Products containing *Ginkgo biloba's* extract are one of the top sellers within the growing market for herbal medicines in Europe and in the USA [1]. The Asian tree *G. biloba*, mentioned in the Chinese *Materia Medica* 5000 (since 2800 B.C.) years ago, is the last surviving member of a family of trees *Ginkgoacea* that appeared more than 250 million years ago [2]. *G. biloba* has generated scientific interest for its reputed value in treatment of memory-related impairments [3] and circulatory disorders [4,5]. Furthermore recent findings [6] showed that *G. biloba* extractis a potent and selective antagonist of glycine receptors (GlyRS), which are very important inhibitory receptors in the CNS.

The commercial standardized extracts of *G. biloba* leaves contains not less then 6% of ginkgolides (a group of unique diterpenes) and bilobalide (a sesquiterpene) and 24% of flavonoids (primarily composed of quercetin, kaempferol, and isorhamnetin glycosides) [7]. Extracts from *Gingko biloba* leaves are reported to have antioxidant properties attributed primarily to the presence of flavonoid-glycosides in the extract [8]. Clinical efficacy of *Ginkgo* is mainly due to terpene lactones, which have different pharmacological activities, such as peripheral vasoregulation [9], platelet-activating factor (PAF) receptor antagonism [10], prevention of membrane damage caused by free radicals [11] and neuroprotective properties [7]. Oral administration of *Ginkgo biloba's* extract can prevent the decline in muscarinic receptor density in the hippocampus of rats [12], and might have ability to inhibit the degradation of acetylcholine by acetylcholinesterase [13].

Only few studies published are available on the pharmacokinetics of ginkgoterpenes, therefore, making it very difficult to

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extrapolate correlation with the pharmacodynamic profile. The high sensitivity required to determine very low plasma levels of terpene lactones (10 nM) is one of the reason for this limited information. Biber and Koch [14] addressed this difficulty measuring the terpene lactones by using a specific gas chromatography/mass spectrometry (GC/MS) method. Pharmacokinetic of the ginkgoterpenes was found to be dose-linear [14].

In our previous work, liquid chromatography coupled to atmospheric pressure chemical ionization mass spectrometry (LC/APCI-ITMS) was applied to evaluate ginkgoterpenes levels in human plasma after acute administration *G. biloba* in free form or as phospholipid complex [15]. Moreover, oral bioavailibility of extracts enriched in ginkgoterpens (in free or phytosome form) have been recently evaluated in experimental animals [16]. In these studies, ginkgoterpenes resulted in greater bioavailability when supplied in phytosomic form.

In the present work, *G. biloba* extract enriched with terpene lactones, was administered chronically in free and phytosomic form to guinea pigs and the plasma levels were detected by LC/APCI-ITMS. At the same time, in another group of animals, the protective activities of terpene lactones against HIST, PAF or OA-induced airway changes and concomitant increase of circulating TXB_2 were determined to correlate absorption level and pharmacodynamic studies.

Finally, the presence of possible metabolites of ginkgolide C (GC) in guinea pigs plasma after chronic administration of *G. biloba* extract was investigated; in fact, previous studies have shown that, in spite of its abundance in administrated extracts, Ginkgolide C is not detected in plasma samples [16,17].

2. Experimental

2.1. Chemicals

Purified Bilobalide and ginkgolide A and B were isolated and characterized by Indena S.p.A. (Milano, Italy). *G. biloba* extract enriched in ginkgoterpenes (IDN 5380), and its phospholipid complex (IDN 5381) were provided by Indena S.p.A. (Milano, Italy). All other reagents were HPLC grade (J.T. Baker, Deventer, Holland).

2.2. Animals

Guinea pigs. Hundred and five male Dunkin Hartley animals (BMG, Cividate al Piano, BG, Italy) weighing 300–350 g were housed under standard conditions (room temperature $22 \pm 2\,^{\circ}C$, humidity $55 \pm 5\%$, 12 h light dark-cycle) and treated orally with IDN 5380 and IDN 5381 (see Section 2.4).

The investigation conformed with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.3. Subjects

Fifteen healthy male (aged 24 ± 3), were selected. The fasting subjects received 160 mg of *G. biloba* standardized extract (Ginkgoselect®) containing 9.6 mg of terpene lactones.

2.4. Protocol

2.4.1. Pharmacokinetic studies

Twenty-five guinea pigs were randomly divided into three different groups: the first one (five animals) was supplemented with 1% carboxymethylcellulose (controls), the second one (10 animals) with IDN 5380 and the third one (10 animals) with-IDN 5381 at the doses of 100 mg/kg × die os. Blood samples (5 ml) were withdrawn via cardiac puncture from anaesthetized animals (thiopentone sodium, Pentothal®, 60 mg/kg i.p.) and collected with heparinized syringes. The samples were centrifuged for 15 min ($2500 \times g$ at $4\,^{\circ}C$) and the aliquots of the supernatant were removed, and stored frozen at $-80\,^{\circ}C$ until analyses by LC/MS.

Healthy volunteers. Blood samples were collected from each volunteers in vacutainer tubes containing sodium-heparin before and 30, 60, 120, 300 and 400 min after administration of Ginkgoselect. Plasma was separated by centrifugation at $10\,000 \times g$ for 1 min and stored at $-80\,^{\circ}C$ until analyses by LC/MS.

2.4.2. Pharmacological studies

Guinea pigs. Eighty guinea pigs were randomly divided into 3 different groups for the pharmacological studies. These animals were treated orally (by gavage) for five consecutive days with (a) 1% carboxymethylcellulose (CMC, 2 ml/kg day; $n = 12$), (b) IDN 5380 (10–300 mg/kg day; $n = 6$–8 per dose) and (c) UDN 5381 (10–300 mg/kg day; $n = 6$–8 per dose).

Guinea-pigs were anaesthetized with ethylurethane (1.2–1.5 g/kg i.p.) and prepared for simultaneous recording of both intratracheal pressure (ITP) and systemic blood pressure (BP), as originally described by Konzett and Rössler [18]. Briefly, trachea was cannulated for mechanical ventilation performed by a pump operating on a partially closed circuit (10 ml/kg, stroke volume; 70 cycles/min). To avoid spontaneous breathing, the animals were given pancuronium bromide via the jugular vein at a dose of 2 mg kg^{-1}. A separate group of animals was actively sensitized to ovalbumin according to the procedure described by Piper and Vane [19] and 21 days later these animals were prepared for ITP and BP recording as reported above. Bronchoconstriction (ITP changes) was induced by intravenous administration of HIST (20 μg/kg) and PAF (0.1 μg/kg) in normal guinea-pigs or by ovalbumin (5 mg/kg) in actively sensitized animals. In these animals, in order to measure the level of circulating TXB_2 (the stable metabolite TXA_2), aliquots of blood (0.5 ml) were collected from the carotid artery at the peak of HIST, PAF or ovalbumin-induced bronchoconstriction. The amount of TXB_2 by a specific commercial enzyme-immunoassay and expressed as ng/ml of plasma.

2.5. Sample preparation

The plasma samples (0.3 ml) were extracted with the same volume of ethylacetate and, after centrifugation at $2000 \times g$ 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.

2.6. LC/MS conditions

A Spectra Series HPLC (Thermoquest, Milan, Italy) equipped with an autosampler was used; separations of ginkgolides were performed by C18 Hypersil column (100 mm × 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 (Thermo Electron, San Josè, USA), equipped with atmospheric pressure chemical ionization interface (APCI). APCI parameters were optimized by flow injection of ginkgolides and bilobalide standard solutions. 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. [15].

2.7. 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 (dissolved in 10% methanol) in the range 10–1000 ng/ml were injected into HPLC apparatus. Peak areas were integrated and plotted against the corresponding concentrations of injected standards.

2.8. Statistical analysis

Differences of data among groups in individual experiments were analyzed for statistical significance by one-way analysis of variance (ANOVA), followed by the Bonferroni test. A value of $P < 0.05$ was considered significant.

3. Results and discussion

3.1. Analysis of terpene lactones in plasma samples

It had been shown earlier that it was possible to determine concentration of terpene lactones in plasma of guinea pigs treated chronically with *G. Biloba* extracts enriched with terpene lactones [14]. In this previous work, conditions using liquid chromatography/atmospheric pressure chemical ionization ion trap mass spectrometry (LC/APCI-ITMS), had been validated.

Typical extracted ion chromatograms obtained from the analyses of plasma sample of guinea pig, are shown in Fig. 1. Ginkgolides A and B (named GA and GB, respectively) and bilobalide (Bil) were identified both from retention times and the negative on-line mass spectra of each chromatographic peak. The content of each terpene lactone in *G. biloba*, was obtained by external standardization from calibration curves prepared by injecting in triplicate each terpene lactone standard (10–1000 ng/ml). In this range all plots were linear, and the correlation coefficients were higher than 0.989. The overall reproducibility of quantitative analysis of ginkgolides and bilobalide was 2.9 and 4.5% for intra-(3) and inter-(5) day analysis, respectively. In spite of its abundant presence in the extract, GC could not be detected in plasma samples (Fig. 1). A small peak is present in correspondence of elution time of GC (around 3.2 min), but its signal is lower than limit of detection (LOD 2 ng/ml). This finding is in good agreement with data previously reported by other papers [14,15].

3.2. Identification of ginkgolide C metabolites

The disappearance of GC in plasma could be due to a very low adsorption and/or to a very fast biotransformation; on this last subject we have investigated the presence of possible metabolites of GC in plasma.

To investigate the presence of possible metabolites of GC in plasma we utilized the Metabolite ID™ tool of the software Metabolism Data Browser™ (by Thermo Electron, San Josè, USA) that allows the evaluation of many possible metabolites by analyzing mass spectrometry (MS) data. In this way data handling of guinea pig plasma analyses permitted the determination of the presence of the methylated derivative (GC_M) of ginkgolide C. As an example, in Fig. 1 it is also reported the extracted ion chromatogram of *m*/*z* 453, corresponding to negative molecular ion of methylated GC. Other metabolite conjugates of GC were not found.

Based on these findings we have searched for the methylated metabolite of GC in previous experiments related to plasma samples of volunteers administered with *G. biloba* extracts. Fig. 2 shows, as an example, the extracted ion chromatograms corresponding to GC (*m*/*z* 439) and its methylated derivative (*m*/*z* 453). The absence of GC and the presence of methylated GC in the plasma samples of the volunteers can be easily observed. The presence of methylated GC metabolite was also confirmed in previous experimental data concerning rats treated with *G. biloba* extract (data not shown).

To exclude the possibility that the GC_M was a plasma impurity, we searched for the GC and its metabolite also in the animal plasma samples of animals treated with pure GB or bilobalide, in absence of GC [15]. In these samples GC_M was undetected (Fig. 3).

Of course, methyl metabolites of ginkgolides A and B were searched in plasma samples, but they were not found.

3.3. Effects and plasma level in guinea pigs after chronic administration of G. biloba extract enriched with terpene lactones

Preliminary results showed that the administration of *G. biloba* in phytosomic form (IDN 5381) produces a higher bioavailability and protection against bronchoconstriction induced by different challengers than the administration in free form (Rossoni, pers. commun.). It was of interest to investigate the pharmacological activities of the same product after chronic administration in guinea pigs to compare pharmacodynamics data with the levels of ginkgolides in plasma.

The protective activity of ginkgoterpenes against HIST, PAF and AO-induced bronchoconstriction and the concomitant TXB_2 release in blood were evaluated in terms of ED_{50}, i.e. *G.*

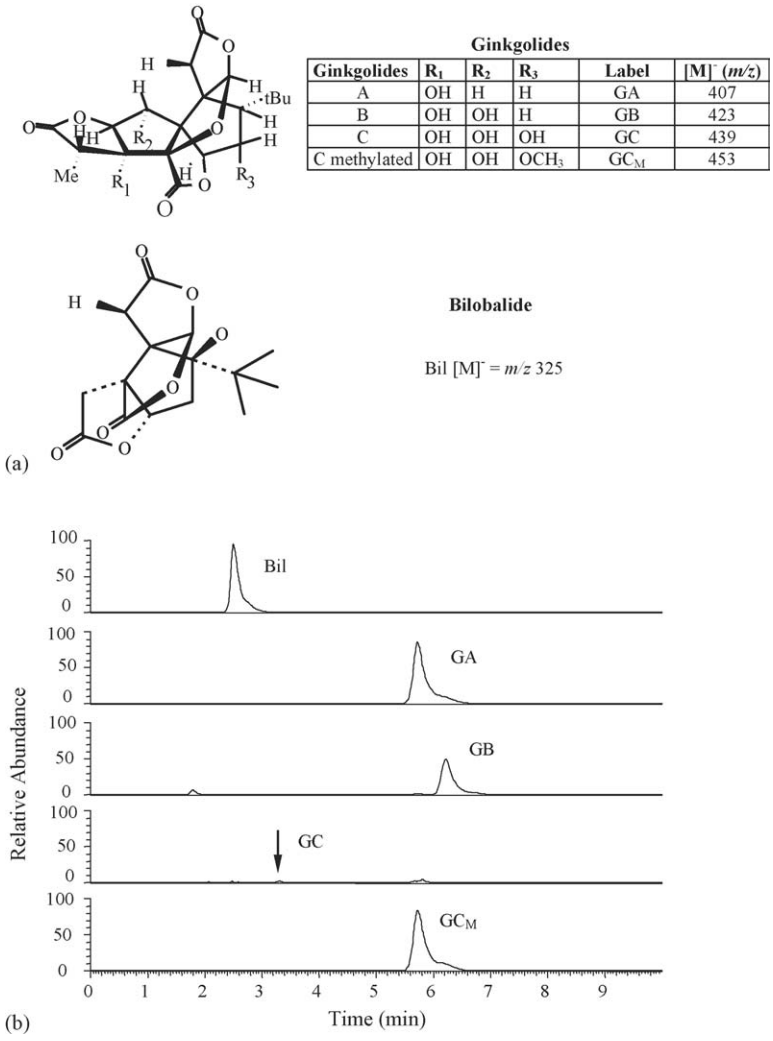

Fig. 1. Chemical structures (a) and extracted ion chromatograms (b) of bilobalide, ginkgolides A, B, and C and GC methylated derivative in plasma sample of guinea pig after chronical administration of phytosomic form of *G. biloba* extract (IDN 5381).

biloba extract that reduces of 50% the effects caused by different challenges. Furthermore, to compare the protective activities of IDN 5381 (phytosomic form) and IDN 5380 (free form), the dose ratio (DR) values were also calculated.

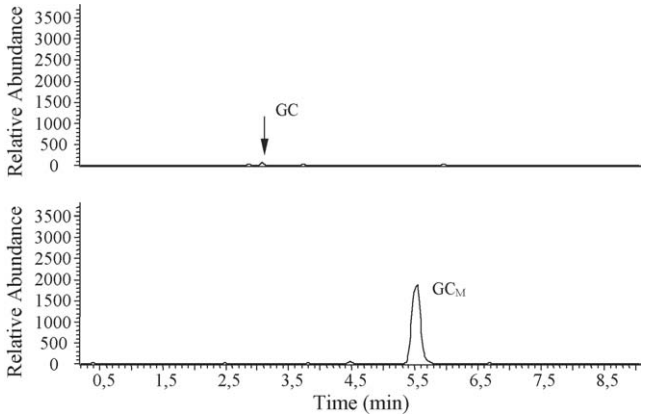

Fig. 2. Extracted ion chromatograms of GC and its methylated derivative (GC_M) obtained from previous parental data and related to volunteers plasma sample after administration of Ginkgoselect.

Five days oral administration of ginkgoterpenes (10–300 mg/kg day) was found to inhibit the ITP changes and TXB_2 release caused by HIST and PAF in normal guinea pigs or by OA in actively sensitized guinea pigs. In these three series of experiments, IDN 5381 was determined to be more active in reducing the effects caused by HIST, PAF and OA as compared to that

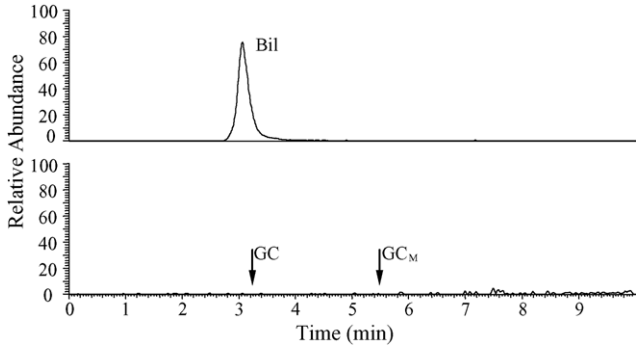

Fig. 3. Extracted ion chromatograms of methylated GC (GC_M) and Bilobalide (Bil) in guinea pig plasma sample after chronical administration of bilobalide.

Table 1
ED_{50} (expressed in mg/kg os) and dose ratio (DR)-values of *G. biloba* extracts in free (IDN 5380) or phospholipid complex form (IDN 5381) against changes on intratracheal pressure (ITP) and circulating thromboxane B_2 (TXB_2) induced by histamine (HIST, 20 μg/kg i.v.) and platelet activating factor (PAF, 0.1 μg/kg i.v.) in normal guinea-pigs or by ovalbumin (OA, 5 mg/kg i.v.) in actively sensitized guinea pigs

Treatment	ITP		TXB_2	
	ED_{50} (confidence limit = 95%)	DR	ED_{50} (confidence limit = 95%)	DR
IDN 5380 + HIST	33.3 (21–54) mg/kg os	1	48.4 (27–88) mg/kg os	1
IDN 5381 + HIST	8.6 (6–12) mg/kg os[#]	3.8	12.2 (9–17) mg/kg os[#]	4.0
IDN 5380 + PAF	70.6 (42–118) mg/kg os	1	97.6 (62–155) mg/kg os	1
IDN 5381 + PAF	20.4 (17–24) mg/kg os[#]	3.5	26.9 (21–34) mg/kg os[#]	3.6
IDN 5380 + OA	99.1 (75–131) mg/kg os	1	115.1 (104–128) mg/kg os	1
IDN 5381 + OA	42.1 (33–53) mg/kg os[§]	2.4	50.7 (44–59) mg/kg os[§]	2.3

[§] $P < 0.01$ vs. IDN 5380.
[#] $P < 0.001$ vs. IDN 5380.

Table 2
Composition of *G. biloba* extract enriched in terpene lactones administrated to guinea pigs, ratios for bilobalide, ginkgolides A and B in the extract and plasma as free form (R_{free}) and phytosomic form (R_{phyt})

Terpene lactones	Extract administrated (mg)		Plasma levels (ng/ml)		R_{free} (c/a)	R_{phyt} (b/d)	R_{phyt}/R_{free}
	(a) IDN 5380	(b) IDN 5381	(c) IDN 5380	(d) IDN 5381			
GA	7.23	2.49	87.9	121.9	12.4	48.9	3.94
GB	5.04	1.74	16.0	21.1	3.18	12.1	3.80
GC	2.19	0.75	N.D.	N.D.	–	–	–
Bil	13.6	4.71	400.1	320.0	29.4	67.9	2.31

S.D.% = ± 2.9 and ± 3.8 for extract and plasma analyses, respectively. R_{phyt} = [terpene lactone]$_{plasma\ phyt}$ (d)/mg extract phytosomic administrated (b), R_{free} = [terpene lactone]$_{plasma\ free}$ (c)/mg extract free administrated (a). N.D. = not detected (lower than LOD). IDN 5380 = *G. biloba* extracts in free form. IDN 5381 = *G. biloba* extracts in phytosomic form.

shown with IDN 5380. In fact, the phytosomic form (IDN 5381) significantly reduced (two- to four-fold as compared to free form, $P < 0.001$) the HIST, PAF or OA-induced airway changes and concomitant TXB_2 release in the circulation (Table 1).

To compare protective activities and bioavailability data, aliquots of blood were collected from animals (100 mg/kg os) after 5 days of treatment with the two forms of *G. Biloba* extract, and the plasma level of each single ginkgoterpene-lactone was measured using LC/APCI-ITMS method (Table 2). Experimental data show that terpene lactones are more bioavailable when supplied to animals in phytosomic form. To evaluate the terpene lactones different bioavailabilities in the two administration forms, we considered the terpenelactone concentrations in the administered preparations and in the related plasma samples. In particular, we have calculated the ratio values R_{free} and R_{phyt} which correspond for each terpene lactone to the ratio of the amounts, in the extract and plasma due to administration of free and phytosomic form, respectively: R_{phyt} corresponds to [terpene lactone]$_{plasma\ phyt}$/mg extract phytosomic administered; R_{free} corresponds [terpene lactone]$_{plasma\ free}$/mg extract free administered. From these value it is possible to determine that phytosomic form allows a lower absorption (two- to four-fold) of terpene lactones than free form. In particular, the absorption of ginkgolides A and B, and bilobalide resulted around 3.9, 3.8 and 2.3 times higher for phytosomic form (see Table 2), respectively. These data are in very good agreement with those obtained from pharmacological studies described above.

4. Conclusions

Mass spectrometry analysis of plasma samples of guinea pigs chronically administrated with *G. biloba* extract confirmed the higher bioavailibilty of the Phytosome® complex in comparation to the free form.

Moreover, it has been observed that disappearance of ginkgolide C is accompanied by the increase of its methylated metabolite (GC_M). This finding was confirmed and human samples, also.

Finally, the data obtained with the pharmacological studies were in good agreement with the measured plasma level of ginkgoterpene-lactone. In particular, the pharmacological effects and levels of plasma ginkgolides increased when the phospholipid complex form was used.

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