Journal of Pharmaceutical and Biomedical Analysis 50 (2009) 224–227

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Liquid chromatography/atmospheric pressure chemical ionization ion trap mass spectrometry of bilobalide in plasma and brain of rats after oral administration of its phospholipidic complex

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ARTICLE INFO

Article history:
Received 24 October 2008
Received in revised form 22 April 2009
Accepted 23 April 2009
Available online 3 May 2009

Keywords:
Bilobalide
LC/APCI-ITMS
Pharmacokinetics
Ginkgo biloba
Brain
Plasma

ABSTRACT

Standardized extracts of *Ginkgo biloba* L. leaves are widely used in clinical practice for the symptomatic treatment of mild to moderate dementia syndromes, cerebral insufficiency and for the enhancement of cognitive function. The main active components present in *G. biloba* extracts are flavonol-glycosides and terpene-lactones. In recent investigations, the sesquiterpene trilactone bilobalide has been described to exert an interesting neuroprotective effect when administered systemically to experimental animals.

Oral administration of terpene-lactones either as standardized extracts or purified products is characterized by a low bioavailability. While preparing phospholipidic complex of *G. biloba* extracts or bilobalide, plasma levels of terpenes and sesquiterpene increase.

In the present study, phospholipidic complex of bilobalide (IDN 5604) has been administered orally to rats and bilobalide levels have been determined in plasma and brain by means of a validated method based on liquid chromatography coupled to atmospheric pressure chemical ionization ion trap mass spectrometry (LC/APCI-ITMS). Due to its sensitivity (about 3 pmol/ml) and specificity, LC/APCI-ITMS method proved to be a very powerful tool for pharmacokinetic studies of *Ginkgo* terpene-lactones.

The results of the present study clearly confirm the improvement of oral bioavailability of bilobalide administered as phospholipidic complex and, for the first time, demonstrate the detection of significative amounts of bilobalide in brain. This last finding agrees with the neuroprotective activity observed for bilobalide.

1. Introduction

Ginkgo biloba L. leaves have been used in popular Chinese medicine and seems that, from the 20th century, their extracts are widespread used in Europe and also in America for various medical applications. In fact, *G. biloba* extracts are reported to possess many activities and are thought to prevent and treat some diseases such as cerebral and vascular insufficiency, cognitive deficits and other age-associated impairments [1,2]. *G. biloba* L. extracts contain a great number of compounds which have been isolated and characterized, such as flavonoids, terpenes, organic acids and polyphenols. Among these constituents, two groups are the most important: flavonol-glycosides and terpene-lactones. The flavonol-glycosides mainly include quercetin, kaempferol and isorhamnetin derivatives. In recent years, the flavonoid-glycosides and/or their metabolites have attracted increasing attention because they may play significant roles in the therapeutic action of *Ginkgo* extracts [3]. On the other hand, the terpene-lactones in *G. biloba* extracts include diterpenes (ginkgolides) and a sesquiterpene (bilobalide). It is reported that the terpene-lactones possess different pharmacological activities such as peripheral vasoregulation [4], platelet-activating factor (PAF) receptor antagonism [5], prevention of membrane damage caused by free radicals [6] and neuroprotective properties [7]. In particular, the ginkgolides A, B, C and J are potent and specific antagonists of PAF, thus preventing thrombus formation, bronchoconstriction and suppressing allergic reactions [8,9]. While, the sesquiterpene bilobalide received most attention due to its effects in the central nervous system [10]. In fact, numerous studies, based on *in vitro* and *in vivo* models, have been demonstrated the neuroprotective effect of bilobalide [11,12]. Moreover, in order to justify this particular function, a variety of cellular mechanisms were proposed; for example, it has been thought that bilobalide could interfere with glutamatergic transmission, mitochondrial

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doi:10.1016/j.jpba.2009.04.026

function and apoptosis [11,12]. In fact, recent works demonstrated the ability of bilobalide to interact with the neuronal transmission mediated by glutamate, γ-aminobutyric acid (GABA) and glycine [13,14].

Despite the number of phytochemical and clinical investigations performed, only few data are available on the pharmacokinetic plasma profile of terpene-lactones, making very difficult to extrapolate correlation with the pharmacodynamic activity. In addition, no studies were performed concerning the bilobalide level in tissues, such as brain.

In our previous works, liquid chromatography coupled to atmospheric pressure chemical ionization ion trap mass spectrometry (LC/APCI-ITMS) was applied to evaluate the terpene-lactones in human plasma samples after oral administration of *G. biloba* extracts in free form or as phospholipid complex [15]. In particular, LC/APCI-ITMS analysis presented good sensitivity and specificity and it is one of the most powerful technique for the rapid identification of terpene-lactones in both *G. biloba* extracts and plasma of treated animals and volunteers. In addition, oral bioavailability of an extract enriched in ginkgoterpenes (in free and phytosomic form) has also been evaluated in plasma samples of animals [16]. In particular, a better biovailability of terpene-lactones after uptake of *G. biloba* extract when supplied as phospholipidic complex has been observed.

On the basis of these findings, in the present work the phospholipid complex of bilobalide has been supplied to rats using increasing oral doses (from 1 to 40 mg/kg). The bilobalide levels in plasma and brain samples were determined by means of LC/APCI-ITMS method. In this way, for the first time it has been possible to detect the sequiterpene in brain tissue.

2. Experimental

2.1. Chemicals

Phospholipidic complex of bilobalide (Phytosome® formulation, IDN 5604) was isolated and characterized by Indena Chemical Laboratories (S.p.A., Milan, Italy). All other reagents were HPLC grade (J.T. Baker, Deventer, Holland).

2.2. Animals

90 male CD rats (Charles River Italy, Calco, LC, Italy) weighing 260–275 g were housed under standard conditions (room temperature 22 ± 2 °C, humidity 65 ± 1%, 12-h light and 12-h dark-cycle) and treated orally with purified bilobalide in Phytosome® form (IDN 5604, see Section 2.3).

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

2.3. Pharmacokinetic studies

90 rats were divided into 5 different groups and treated orally with 1, 3, 10, 20, and 40 mg/kg IDN 5604. The animals were anaesthetized with thiopentone sodium (Pentothal®, 60 mg/kg i.p.) and killed at 30, 60, 120, 180, 210, and 240 min after ingestion. Blood samples (5 ml) were collected in heparinized syringes and after centrifugation at 2000 × *g* for 10 min at 4 °C plasma was obtained and subdivided into aliquots of 300 μl and stored at −80 °C, until analyses by LC–MS. Instead, rat brain tissues were dissolved in 0.12 M phosphate buffer (ratio 1:4, w/v) and then homogenized for 90 s at 4 °C by means of Ultra-Turrax. The supernatants were collected and stored at −80 °C until analyses by LC–MS.

2.4. Sample preparation

Plasma (0.3 ml) and brain (0.5 ml) samples were extracted with the same volume of ethylacetate and, after centrifugation at 2000 × *g* for 2 min, the supernatant, organic phase, was collected and evaporated to dryness under vacuum. The residue was dissolved in 200 μl of 10% methanol, and 100 μl was injected into LC/APCI-ITMS system.

2.5. LC–MS conditions

Samples were analyzed using an LC–MS system that consisted of a Spectra Series HPLC (Termoquest®, Milan, Italy) with an autosampler coupled to an ion trap mass spectrometer (LCQ_{DECA}, Thermo Electron Corporation, San Josè, CA, USA), equipped with an atmospheric pressure chemical ionization source (APCI). In particular, 100 μl of samples were injected, in a Hypersil C18 column (3 i.d. × 100 mm, 5 μm, Thermo Fisher Scientific, Waltham, MA, USA) and eluted by a methanol linear gradient (eluent A, water; eluent B, methanol; 0–1 min 30% B, 1–7 min from 30 to 45% B, 7–10 min at 45% B). The flow rate was 0.65 ml/min. LC/APCI-ITMS analysis was carried out in the negative ion scan mode from 200 to 700 *m*/*z*. For the other specific instrumental parameters see Mauri et al. [15].

2.6. Calibration curves

Bilobalide standards were dissolved in methanol (about 1 mg/ml) and stored at 4 °C. Aliquots of terpene-lactone standard solutions (dissolved in 10% methanol) in the range 10–10,000 ng/ml were injected into LC–MS apparatus. Peak areas were integrated against the corresponding concentrations of injected standards.

3. Results and discussion

In the present work we investigated the bilobalide levels simultaneously in plasma and brain samples of rats treated with different doses of the sesquiterpene complex.

The collected samples were prepared by means of liquid–liquid extraction method that was developed in a previous work [15]. This extraction method, summarized in Fig. 1, resulted simple and allowed a rapid and unambiguous determination of ginkgolides and bilobalide in biological samples. For this reason, the extraction method was applied for preparing brain samples in the present investigation. It permitted a good recovery for bilobalide (about 70%) and samples were stable for 1 day at 4 °C.

The bilobalide assays were performed using LC/APCI-ITMS approach. In Fig. 2 is shown a typical extract ion chromatogram (EIC, $[M-H]^-$, *m*/*z* 325), obtained from an actual sample of rat brain, collected at 30 min, after oral administration of 10 mg/kg of bilobalide in phospholipidic complex (IDN 5604). It was possible to identify bilobalide by the retention time (Fig. 2a) and its molecular ion obtained by negative mass spectrum (Fig. 2b). In addition, the detection in the full scan mass spectrum of the fragment ion at *m*/*z* 251, due to the loss of both a *tert*-butyl and an hydroxyl group, confirmed the bilobalide identification, as reported in previous works [16,17]. Noteworthy, the LC–MS method applied allowed to elute the bilobalide in only 3 min and each analysis was completed in 10 min. This means that it has been possible to analyze a great number of samples in a relatively short time. In order to perform a quantitative assay of the effective amount of bilobalide in biological samples, a calibration curve was prepared before each set of samples ($n = 8$) by injecting phospholipidic complex of bilobalide standard solutions. Fig. 3 reports the typical calibration curve obtained for the operative range. Specifically, in the range 10–10,000 ng/ml all plots were linear and the correlation coefficients were higher than 0.999. The overall reproducibility of quantitative analysis of bilobalide was

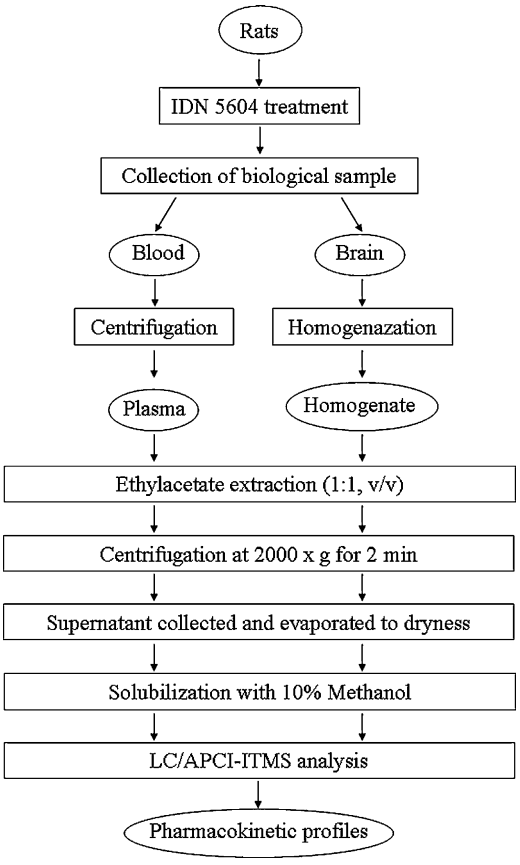

Fig. 1. Scheme concerning main steps used for plasma and brain preparation.

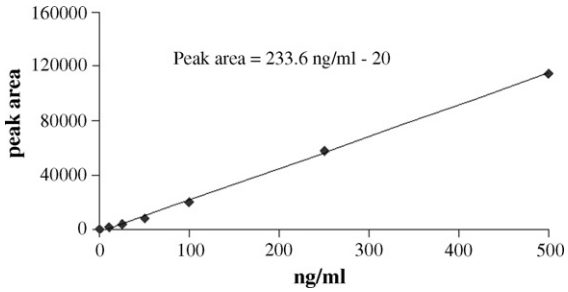

Fig. 3. Typical calibration curve for bilobalide (ng/ml vs peak area).

±3.2% and ±4.6% for intra-day ($n = 5$) and inter-day ($n = 5$) analyses, respectively. The LC–MS method used provided a limit of detection (LOD) around 5 ng/ml (about 15.4 nM) using EIC mode and a signal-to-noise ratio (S/N) = 5.

With regard to the pharmacokinetic investigations, brain and plasma samples were collected from each rat to determine, by LC–APCI method, the bilobalide levels. As an example, Fig. 4 reports the levels of bilobalide in plasma and brain samples collected up to 4 h (0, 30, 60, 120, 180, 210 and 240 min) after acute oral administration of 20 mg/kg IDN 5604. Each time-point reported in the plot is the mean obtained from triplicate analysis (standard deviation, SD% around ±7% and ±9% for plasma and brain samples, respectively). In particular, the bilobalide in plasma (Fig. 4a) presents a maximum level at 30 min, and it decreases slowly. This is in good agreement with previously published data [16,18]. On the contrary, in each set of brain samples bilobalide was exclusively detected in the fractions collected at 30 min (Fig. 4b). These findings indicate that in the brain the bilobalide absorption is probably due to a specific mechanism of transport. Therefore, we considered the triplicate assays obtained at 30 min for each different dose supplied to rats (1, 3, 10, 20 and 40 mg/kg), in order to study the correlation between administered dose of bilobalide and its related levels in plasma and brain samples. In particular, the data reported in Fig. 5 were normalized using the maximum bilobalide amount measured at 30 min as the reference value (for plasma, 3052 ng/ml = 100, obtained with 40 mg/kg; for brain, 11,795 ng/g = 100, obtained with 10 mg/kg). In this way, it has been observed a different behaviour, comparing brain and plasma profiles. In fact, plasma levels of bilobalide show a dose-dependent linear increase; on the contrary, in brain samples bilobalide level increases for the dose range 1–10 mg/kg, but it decreases for higher doses (20 and 40 mg/kg). The described brain profile may be explained by a limited passage of bilobalide

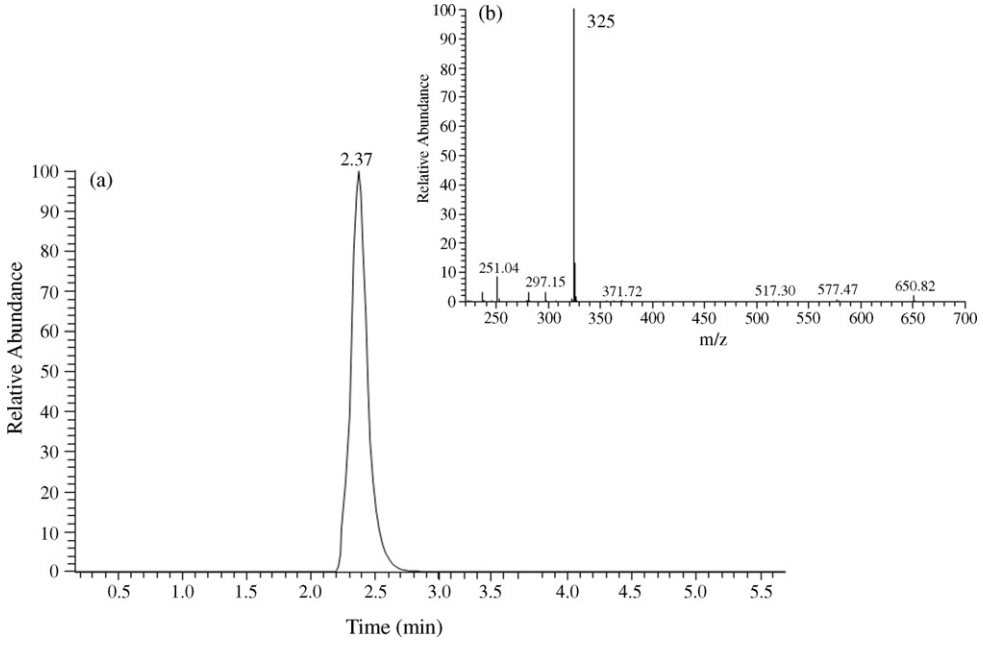

Fig. 2. (a) Extracted ion chromatogram (m/z 325) and (b) its related mass spectrum obtained by LC/APCI-ITMS analysis of rat brain colleted at 30 min after bilobalide oral administration.

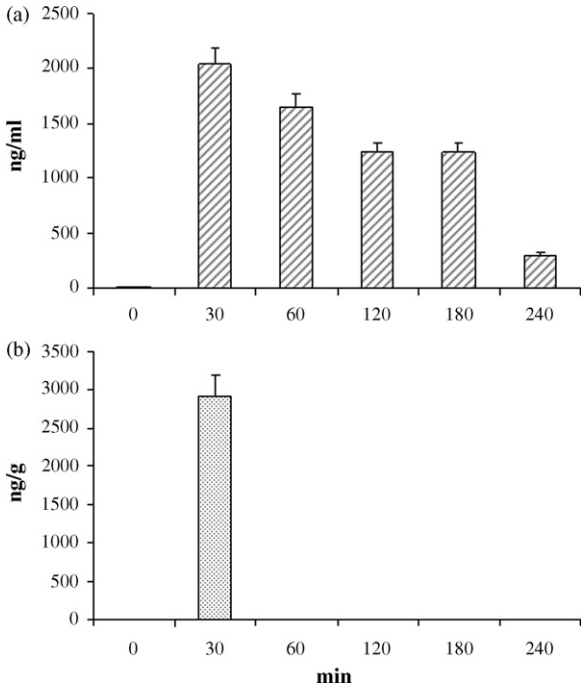

Fig. 4. Bilobalide assays in plasma (a) and brain (b) after oral administration of 20 mg/kg IDN 5604.

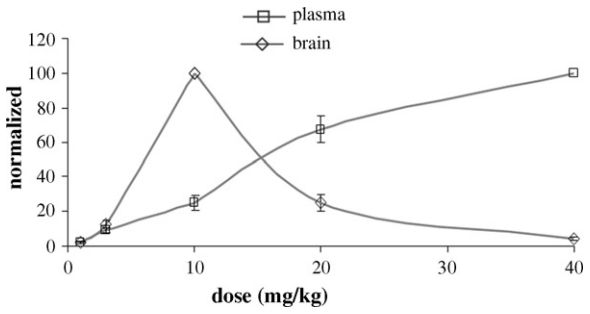

Fig. 5. Normalized curves of bilobalide levels in plasma and brain at 30 min in relation to administered doses.

through the blood–brain barrier with doses higher than 20 mg/kg due to a probable overloading and subsequent inhibitory action on its related molecular carriers. This hypothesis in good agreement with recent studies reports that the positive acute cognitive effects of *G. biloba* extracts appear to be both dose and time dependent. In particular the authors shows that the memory enhancement is manifested for low doses and at the earliest times after the ingestion [19,20].

Moreover, the obtained results support the model suggested above concerning a specific mechanism involved for bilobalide absorption in the brain. In fact, recent pharmacological studies have described the interaction of bilobalide with neurotransmission receptors. For example, Kiewert et al. [14] reported that the bilobalide is a noncompetitive antagonist for $GABA_A$ receptors, but, together with this function, other unknown mechanisms are probably involved in the neuroprotective effect of bilobalide. Chandrasekaran et al. [21] and Kiewert et al. [22] suggest that the bilobalide may also have effect on the level of NMDA (*N*-methyl-D-aspartate) receptor activation. This model is related to the ability of bilobalide to reduce glutamate-induced excitotoxic neuronal death. The addition of bilobalide to rat hippocampal slice cultures is shown to inhibit NMDA-induced phospholipase A_2 (PLA_2) activation.

Other authors have demonstrated that bilobalide is the active constituent of *G. Biloba* extract able to suppress the hypoxia induced membrane-breakdown in the brain [23]. In particular they demonstrated that bilobalide potently inhibits the hypoxia induced, PLA_2-dependent release of choline from hippocampal phospholipids.

4. Conclusion

In the present work LC/APCI-ITMS analysis permitted, for the first time, to detect bilobalide in rat brain after oral administration of its phytosomic form. The presence of bilobalide in the rat brain supports the great amount of biological data concerning the positive effects (such as protection against ischemia-induced neuronal death and improvement of cognitive performance) on brain due to ginkgolides. In particular, bilobalide presented a maximum concentration level in the brain around 30 min after oral administration. This means that the absorption of the sesquiterpene is very fast.

Moreover, it is very interesting to observe different profiles obtained by plotting the brain and plasma sample concentrations versus uptake bilobalide doses. The specific behaviour of bilobalide in the brain seems to be explained by an inhibitory effect related to an overloading of the molecular transports after administration of doses higher than 20 mg/kg. Of course, these findings require further investigations, in collaboration with neuroscience groups, for identifying the specific mechanism of transport for bilobalide.

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