Available online at www.sciencedirect.com
ScienceDirect
Journal of Chromatography B, 863 (2008) 74–79
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Liquid chromatography–tandem mass spectrometry of I3,II8-biapigenin, the major biflavone in *Hypericum perforatum* extracts

Milena Colovic, Silvio Caccia *

Istituto di Ricerche Farmacologiche "Mario Negri", via La Masa 19, 20156 Milan, Italy

Received 18 October 2007; accepted 4 January 2008
Available online 16 January 2008

Abstract

High-performance liquid chromatography method coupled with tandem mass spectrometry was developed for the quantitative determination of I3,II8-biapigenin. The procedure includes solid-phase extraction and separation on an XTerra MS C18. The assay was linear over a wide range; precision and accuracy were acceptable. Biapigenin was present in mouse and rat plasma after a standardized *Hypericum perforatum* extract. It was not detected in brain (<5 ng g^{-1}), suggesting poor brain-to-blood permeability. Biapigenin concentrations were measurable in mice after intraperitoneal biapigenin (10 mg kg^{-1}) but these amounted to about 2% of the equivalent systemic exposure, after correction for the contribution from residual blood.

Keywords: Biapigenin; *Hypericum perforatum* extract; LC/MS–MS; Rodents; Brain uptake and concentrations

1. Introduction

I3,II8-Biapigenin (biapigenin) is the most abundant biflavone in *Hypericum perforatum* (St. John's Wort) [1,2] whose extracts are widely used as an over-the-counter remedy for mild to moderate depression [3,4]. Although in these extracts it represents only a fraction of the flavonoid mixture [1], biapigenin has a variety of biological actions. Like the extracts, it had antidepressant-like activity in the forced swimming test in rats, although dose and route of administration were not reported [5]. In neurochemical studies in rat brain membranes biapigenin inhibited ligand binding to the α-estrogen and benzodiazepine receptors at micromolar concentrations [6]. In interaction studies in human liver microsomes, it behaves like a potent, competitive inhibitor of cytochrome (CYP)2C9, CYP3A4 and CYP1A2, with 50% inhibition of activities at concentrations lower than methanolic extracts and other components [7]. However, while these studies suggest that biapigenin may contribute to some of the pharmacological actions of this herb, little is still known about its disposition in man or in experimental models such as rodents. This information may help interpret the relevance of the *in vitro* effects of this compound for the *in vivo* actions of the extracts and facilitate extrapolation across species [8].

Information on the possibility of analysis of biapigenin in biological matrices is limited. Several chromatographic separation systems have been published, most of them based on high-performance liquid chromatography (HPLC) [2,5,9], with some using near-infrared reflectance spectroscopy [10] or mass spectrometry [11,12] for greater detection specificity and to facilitate identification of the constituents measured with less sensitive detection systems. As these methods aimed at identifying and quantifying biapigenin with other components, in raw herbs and their derivatives, we have little information on their applicability to its analysis in blood and tissues. Therefore, more application-oriented analytical studies are needed, as for flavonoids in general [13], including specific and sensitive analytical methods to quantitate biapigenin in blood, and at least in animals, in brain tissue.

Here we describe a liquid chromatography–mass spectrometry (LC/MS–MS) procedure for measuring biapigenin in *H. perforatum* extracts, primarily to investigate its pharmacokinet-

* Corresponding author.
E-mail address: caccia@marionegri.it (S. Caccia).

1570-0232/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2008.01.014

ics in rodents given pharmacological doses of these products. The possibility of determining biapigenin in brain tissue was considered, and the concentrations reaching the brain of mice after the authentic compound or a commercial herbal extract were preliminarily evaluated, this being useful for interpreting the pharmacological significance of this biflavone component.

2. Experimental

2.1. Reagents

HPLC-grade methanol, acetonitrile and analytical grade acetic acid were purchased from Reidel-de Haën (Milan, Italy). Analytical grade *t*-butyl methyl ether was from Fluka (Milan, Italy), and potassium dihydrogen phosphate GR for analysis from Merck (Darmstadt, Germany). The *H. perforatum* extract (0.3% hypericin, 3% hyperforin) was kindly provided by Indena S.p.A (Milan, Italy).

Biapigenin was isolated from the *H. perforatum* extract and purified in the Indena S.p.A laboratories as follows: 500 g of extract were subjected to silica-gel chromatography (3 kg) and eluted with EtOAc/methanol/water (100:5:2.5, v/v/v). The solvent was removed from the combined biapigenin fractions under vacuum affording 8 g of residue, which was purified by RP-18 silica-gel chromatography (120 g), eluting with methanol/water. Fractions containing biapigenin were dried under vacuum, the residue was dissolved in methanol and biapigenin was precipitated with isopropyl ether (R. Daz, G. Zini, personal communication).

Biapigenin (0.8 g) was fully characterized by ^{1}H NMR, ^{13}C NMR, IR and MS spectroscopic methods. Its purity (98.2%) was assessed by HPLC, employing a previously published method [2].

The internal standard, warfarin, was purchased from Sigma–Aldrich (Milan, Italy).

2.2. Standard and fortified stock solutions

Stock solutions were prepared by dissolving biapigenin and warfarin in methanol at the concentration of 1 mg mL^{-1}. Working standard solutions of both were prepared by diluting stock solution in methanol. Working calibration curve were obtained by spiking 100 μL of blank plasma or 1 mL of brain homogenate with working standard solutions. The spiked plasma samples were brought to 1 mL by adding 0.9 mL of phosphate buffer 0.01 M and basified with 5 μL of 10 M KOH.

Standard curves for analysis of the *H. perforatum* alcoholic extract were prepared in 100 μL pure methanol and brought to 1 mL, as described above.

2.3. Sample preparation

Biapigenin was extracted from the extract and biological tissues using OASIS HLB 1-cc (30 mg) extraction cartridges. Analysed plasma volumes varied from 10 to 100 μL, with the smallest volume brought to 100 μL by adding drug-free mouse plasma.

Samples were loaded onto the extraction cartridges previously wetted with 1 mL of CH_3OH followed by 1 mL of H_2O. Then the cartridges were washed with 1 mL of ammonium acetate buffer (50 mM) at pH 5, eluted with 2 mL of methyl *t*-butyl ether in CH_3OH (20:80, v/v) and evaporated to dryness under nitrogen flow at 40 °C.

Brain samples were homogenized (10 mL g^{-1}) in 0.01 M phosphate buffer containing 5 nmol L^{-1} KOH. After adding the internal standard (5 μL of a 0.1 μg mL^{-1} methanolic solution), 1 mL of the homogenate (about 100 mg of tissue) was centrifuged at 5000 rpm for 10 min at 4 °C in the Sorvall centrifuge. Then it was processed as described for plasma.

The final elutes were evaporated to dryness under nitrogen stream at 40 °C, the residues were reconstructed in 150 μL of mobile phase, and 10 μL were injected into the column.

2.4. Instrumentation

An Alliance 2695 separation module was used to deliver samples into the Quattro Ultima Pt (Micromass, Waters, USA) triple-quadrupole mass analyser equipped with Z-spray ESI interface, controlled by the Mass-lynx software. Tuning was done by direct infusion of 1 μg mL^{-1} standard solutions dissolved in acetonitrile–water (50:50, v/v) by a syringe pump (Harvard Apparatus, MA, USA) at a flow rate of 10 μL min^{-1}. Cone, capillary and RF1 voltage were tuned to optimise the presence of the molecular $[M-H]^-$ ion for the analysed compound and the internal standard. The best response was obtained with the following parameters: capillary (3.0 kV), cone (60 eV), RF lens 1 (60 eV) for biapigenin and the internal standard. The collision energy of 30 V and 23 V were applied, respectively, for biapigenin and warfarin fragmentation of the precursor ions into the most abundant product ions through collision-induced dissociation with argon. The source and desolvation temperatures were set at 150 °C and 350 °C, respectively.

Chromatographic separation was done on the XTerra MS C8 column 3.5 μm (2.1 mm × 150 mm) protected with a XTerra MS C8 5 μm pre-column, at a flow rate of 0.2 mL min^{-1}. The mobile phase was CH_3CN:H_2O (35: 65, v/v), containing 10 mM ammonium acetate buffer (brought to pH 5 with glacial acetic acid), delivered isocratically. Column temperature was 30 °C; the samples were stored in the auto sampler at 8 °C throughout the analysis.

2.5. Data analysis

The linearity of the method was demonstrated over the concentration range 5–2500 ng mL^{-1} by assaying different calibration standards with three quality control (QC) concentrations in duplicate on three separate occasions. Standard calibration curves were constructed by linear least-squares regression analysis of the plot of the peak-area ratio between biapigenin and the internal standard response against biapigenin concentrations. The equations were calculated by $1/x$ weighted linear regression analysis.

The lowest limit of quantification (LLOQ) was the lowest concentration that could be measured in plasma and brain with

acceptable accuracy and precision, determined in separate studies. Reproducibility of the assay was assessed using the QC samples prepared and injected both on the same day and on different days.

The absolute recovery of biapigenin and its internal standard was assessed by direct comparison of the peak area of the extracted sample against the external sample and on extracted blank spiked with the analyte against the external sample to assess the presence or absence of the matrix effect.

2.6. In vivo studies

All procedures involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance with national (D.L. n. 116, G.U., suppl. 40, 18 Febbraio 1992, Circolare No. 8, G.U., 14 Luglio 1994) and international laws and policies (EEC Council Directive 86/609, OJL 358, 1, Dec. 12, 1987; Guide for the Care and Use of Laboratory Animals, U.S. National Research Council, 1996).

In the first study, male CD1 mice (weighing about 25 g) and CD rats (weighing about 250 g) (Charles River, Italy) were given 500 mg kg^{-1} of the standardized *H. perforatum* extract orally 24 h, 5 h and 1 h before the animals were killed, this being the schedule used to study the antidepressant-like activity of the extracts in the forced swimming test [14]. The biapigenin content in this extract measured by this LC/MS–MS method was $0.37 \pm 0.04\%$. The content of the other biflavone constituent, amentoflavone was determined by minor modifications of this LC/MS–MS procedure, and averaged only about 5% of the biapigenin content in agreement with previous reports [1].

The animals were killed by decapitation under deep anesthesia; plasma and brain samples were stored at $-20\,^{\circ}C$ until biapigenin analysis. Because brain concentrations were always below the LLOQ of our procedure in these studies, in another experiment groups of mice were treated intraperitoneally with pure biapigenin (10 mg kg^{-1}) to study the brain uptake and concentrations of biapigenin, and their relation with plasma concentrations. Biapigenin was dissolved in DMSO:ethanol:PEG400:H_2O (20:10:50:20, v/v/v/v) and injected in a volume of 10 mL kg^{-1}.

3. Results and discussion

We developed the present LC/MS–MS method to detect the biflavone biapigenin in rodent tissues. LC coupled with MS (or MS–MS) is now considered the method of choice for flavonoid in extracts of *H. perforatum* and other plants [7,13]; both positive and negative modes are usually applied, with soft ionisation techniques like ESI and APPI [13].

The method uses MS–MS after the soft ESI negative ionisation mode and fragmentation of the deprotonated (*m/z* 537.2) biapigenin molecules $[M-H]^-$ in the second quadrupole producing the principal fragment ion at *m/z* 384.9 (Fig. 1). Using an LCQ classic ion-trap mass spectrometer Tatsis et al. [11] and

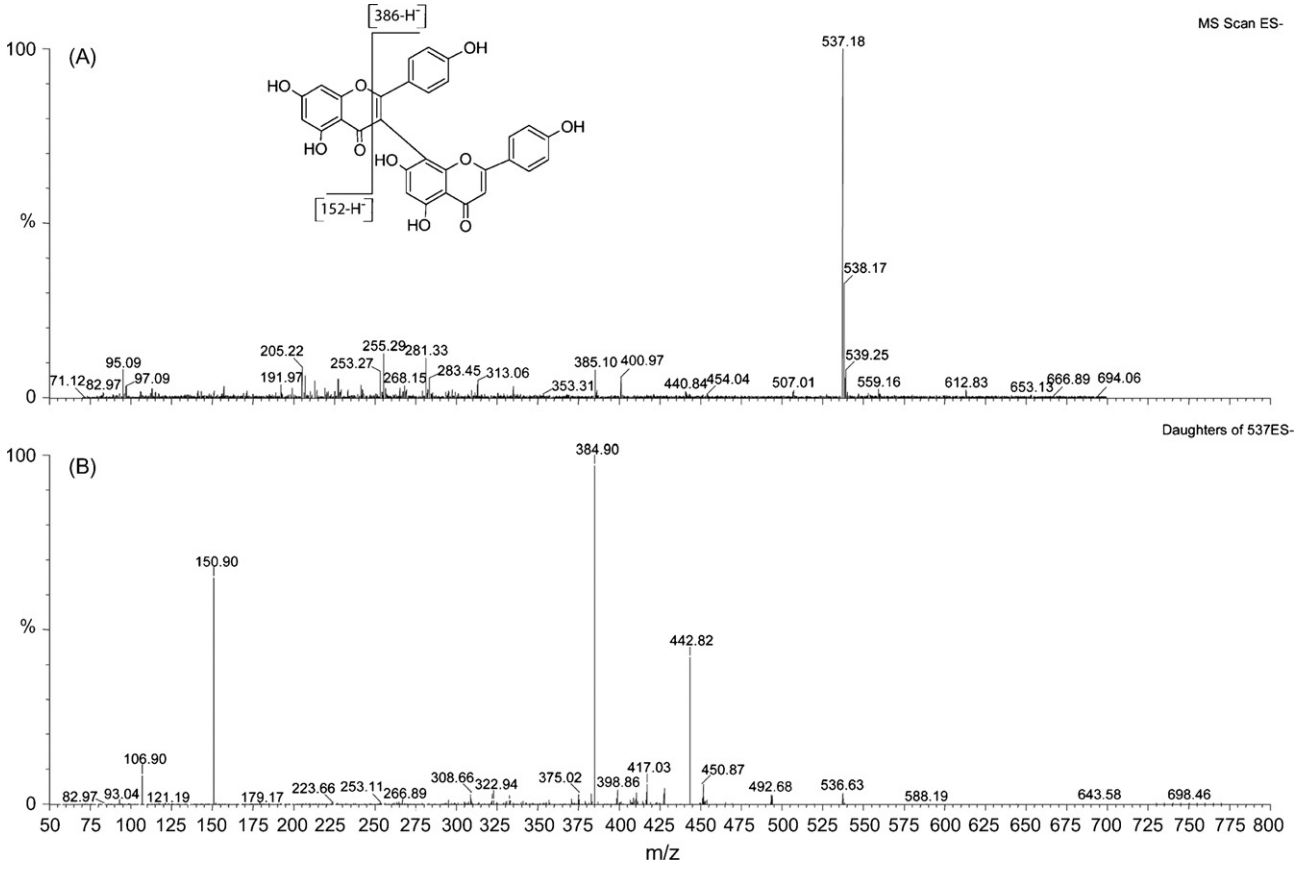

Fig. 1. Biapigenin MS scan spectra (A) and MS–MS scan spectra (B) obtained in ESI negative ion mode.

Silva et al. [12] found a different relative abundance of product ions, with *m/z* 443 as the principal fragment ion and *m/z* 384.9 as the minor one. Optimizing the parameters for infusion of the biapigenin deprotonated molecule, the best parent and product ion responses were obtained with cone voltage and RF lens 1 voltage at 60 V and 60 eV; this may explain the difference in the relative fragment abundance in our conditions compared to the pattern previously reported since the RF lens 1 voltage can additionally fragment the molecular ions.

Separation was done on an XTerra MS C8 column, packed using the hybrid particle technology that allows high efficiency of separation and better pH stability than Silica-based reversed-phase packing materials, but shares the advantages of both because it contains both inorganic (silica) and organic (organosiloxane) particles. The mobile phase consisted of H_2O:CH_3CN (65:35, v/v) containing 10 mM ammonium acetate buffer (brought to pH 5 with glacial acetic acid). Although, either acids (acetic or formic) or salts (ammonium-acetate or ammonium formiate) can be used as mobile phase in flavonoid analysis [13] – and for biapigenin too – a mobile phase with acetic acid gave better sensitivity but also some matrix interference; while a mobile phase with ammonium acetate showed no matrix effect for either biapigenin or warfarin. The retention times for biapigenin and the internal standard were approximately 3.5 and 7 min, giving a total run time of 15 min. Representative MRM chromatograms obtained for blank mouse plasma (A–D) and plasma from mice given biapigenin intraperitoneally (E and F) are shown in Fig. 2. Also shown plasma samples spiked with the I.S., warfarin (G and H). No interference from endogenous plasma substances with analyte or I.S. was detected.

The extraction procedure is derived from that used by the manufacturer of the Oasis HLB cartridges for the extraction of flavonoids from extracts, and has been improved. Mean overall recoveries, determined by comparing the peak area of biapigenin from spiked (low, middle and high concentrations) plasma with those from direct injection of the compound dissolved in the mobile phase, averaged 84% from plasma and 66% from brain; variability (% coefficient of variation, C.V.) within replicates ranged between 3% and 14% and C.V. between them (i.e. recoveries at these three levels) was less than 10%.

The assay was validated for linearity of the calibration curves by running separately prepared samples of 5–2500 ng mL^{-1} for plasma and 5–2500 ng g^{-1} for brain tissue. The typical equations obtained by least squared regression were $y = 0.223x - 0.058$ for plasma and $y = 0.267x - 0.063$ for brain; the regression coefficient (r^2) invariably exceeded 0.998.

The within-day precision varied between 6.8% and 11.8% for plasma and between 6.0% and 11.9% for brain analysis;

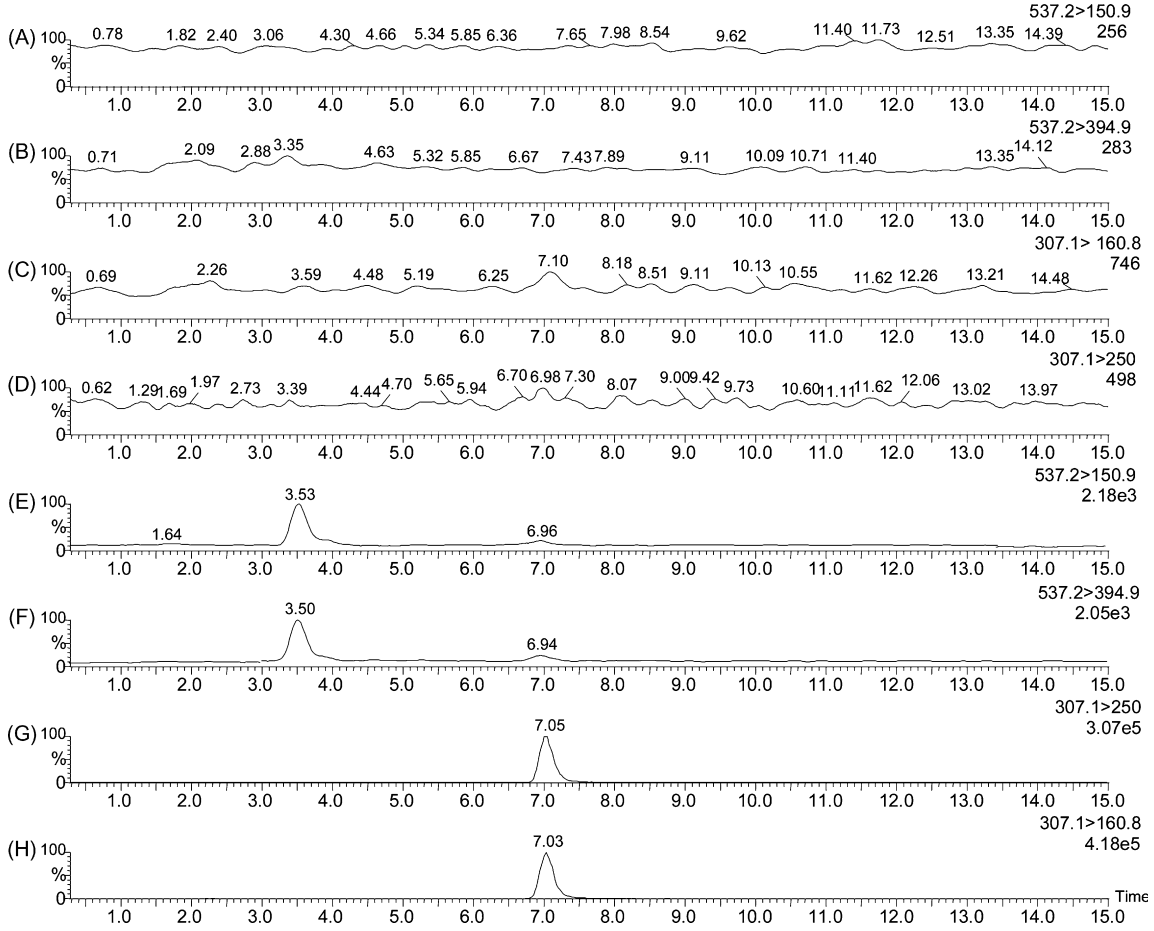

Fig. 2. Representative MRM chromatograms of drug-free mouse plasma (A–D) and plasma sample from mice given biapigenin intraperitoneally (E and F); also shown plasma samples spiked with warfarin (internal standard) (G and H).

the between-day precision was, respectively, 3.3–10.4% and 5.2–9.8%. The lowest calibration standard corresponded to the LLOQ; this concentration was also the lowest calibration point in the analysis of biapigenin content in *H. perforatum* extracts, with C.V. for precision and reproducibility below 20%. This is more than enough to allow the routine application of this method for analysis of these extracts, judging from their biapigenin content (about 0.37% in the present extract).

As the method provided adequate precision and reproducibility, the question of the bioavailability and brain uptake and concentrations of biapigenin after intake of these extract could be approached. Mice and rats were given the compound as in the behavioral test used in previous antidepressant-like activity studies of *H. perforatum* extracts in rodents [14], and plasma and brain concentrations of biapigenin were measured 60 min after the last dose. As shown in Table 1, the plasma concentrations averaged about 34 and 11 ng mL^{-1} in the mouse and rat, respectively. Brain concentrations were always below the LLOQ of the analytical procedure, suggesting that the exchange between blood and brain is restricted for this compound in rodents.

To obtain further information on biapigenin brain uptake and concentrations and their relationship with plasma concentrations, groups of mice were given authentic biapigenin intraperitoneally and were killed at various times. The dose and route of administration were derived from *in vivo* studies with structurally related biflavones [14]. The compound rapidly reached the systemic circulation, with concentrations in the low μM range within 15–120 min of dosing (maximal plasma concentrations 1.4 ± 0.7 μg mL^{-1}) (Fig. 3). Then it disappeared from the blood compartment with an elimination half-life ($0.693/\beta$) of about 1.4 h, this being the result of intermediate-high clearance ($Cl = Dose \times F/AUC = 45$ mL min^{-1} kg^{-1}) in conjunction with a relatively high volume of distribution $Vd/F = Cl/\beta = 5.3$ L kg^{-1}, assuming again complete absorption of biapigenin from the intraperitoneal site. The pharmacokinetics of biapigenin in other species, including man, is not known so no comparison can be made.

Brain concentrations varied, reflecting the variability in plasma concentrations, but were much lower, with a brain-to-plasma distribution ratio (brain-to-plasma $AUC_{480\,min}$) averaging 0.04 after 10 mg kg^{-1} biapigenin, intraperitoneally. After the necessary correction for the contribution from residual blood [15,16], biapigenin brain concentrations approximated only 2% of the equivalent systemic exposure. This calculation was done as described by De Boer et al. [17] for correcting quercetin brain concentrations in rats and pigs.

Table 1
Biapigenin plasma and brain concentrations in rodents given oral *Hypericum perforatum* extract

Species[a]	Plasma (ng mL^{-1})	Brain (ng g^{-1})
Mouse	33.7 ± 42.9 (0.06)[b]	<5 (<0.01)[b]
Rat	10.8 ± 2.2 (0.02)[b]	<5 (<0.01)[b]

[a] Rodents were given *Hypericum perforatum* extract orally (500 mg kg^{-1}) 24, 5 and 1 h before they were killed; the dose of the extract was equivalent to 1.85 mg kg^{-1} of pure biapigenin as determined by LC–MS–MS analysis (0.37% of the extract).

[b] Each value is the mean (±S.D.) of three animals (in the brackets, the equivalent μM concentration).

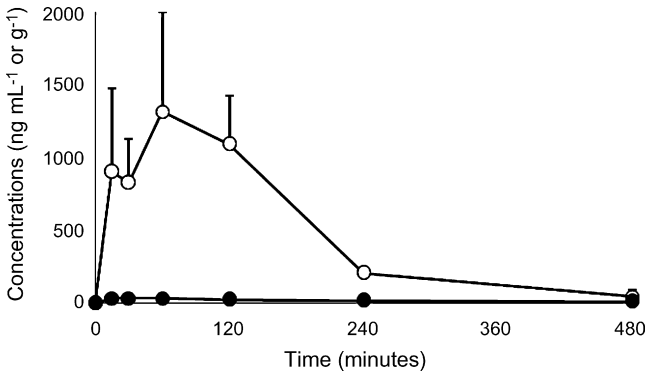

Fig. 3. Mean plasma and brain concentration–time curves of biapigenin (10 mg kg^{-1}, intraperitoneally). Results are the mean (±S.D.) of three mice for plasma (open circles) and brain (closed circles).

4. Conclusions

In these last few years many studies have suggested that biflavones might have beneficial effects on health, like polyphenols in general [16,18]. Most of the studies with biapigenin, however, were *in vitro* and there was little information on its bioavailability and distribution in tissues, including brain, and metabolism in animal models after administration of the authentic compound or biflavone-containing herb products. Using LC–MS/MS analysis this study has shown for the first time that biapigenin is present in the unchanged form in plasma of rodents given a standardized (in terms of hypericin and hyperforin) *H. perforatum* extract. However, the biapigenin plasma concentrations after an extract schedule effective in behavioral tests predictive of antidepressant-like activity [14] ranged from only about 0.02 μM in the rat to about 0.06 μM in the mouse, with wide variability between animals (see Table 1); this range is several orders of magnitude lower than that effective in rodent *in vitro* assays so far [6,7].

Most importantly, biapigenin total brain concentrations were low and mostly related to the residual biflavone in the circulation, as is possibly the case for other flavonoids found in rodent brain after intake of either pure compounds or flavonoid-containing foods [15,16]. Poor brain-to-blood permeability is common to other polar components of *H. perforatum*, including hyperforin and hypericin, resulting in brain concentrations [19,20] generally too low for any direct interaction with neurotransmitter transporters and receptors, which are obviously important for the action of conventional antidepressants [15]. Likewise, the *in vitro* interactions of biapigenin with the central mechanisms so far known are apparently not relevant for the *in vivo* effects of the extracts because they occur at biflavone concentrations far exceeding those found in the brain after pharmacologically effective doses. However, this does not exclude that tissues other than brain may concentrate biapigenin sufficiently to exert beneficial effects after daily intake of *H. perforatum* extracts.

Acknowledgements

We would like to thank the Indena S.p.A, particularly Mr. Ruggero Daz and Mr. Gianfranco Zini for the isolation and the preparation of I3,II8-biapigenin.

References

[1] A. Nahrstedt, V. Butterweck, Pharmacopsychiatry 30 (1997) 129.
[2] M. Brolis, B. Gabetta, N. Fuzzati, R. Pace, F. Panzeri, F. Peterlongo, J. Chromatogr. A 825 (1998) 9.
[3] U. Werneke, O. Horn, D.M. Taylor, J. Clin. Psychiatry 65 (2004) 611.
[4] K. Linde, M. Berner, M. Egger, C. Mulrow, Br. J. Psychiatry 186 (2005) 99.
[5] S. Schulte-Löbbert, K. Westerhoff, A. Wilke, M. Schubert-Zsilavecz, M. Wurglics, J. Pharm. Biomed. Anal. 33 (2003) 53.
[6] U. Simmen, J. Higelin, K. Berger-Buter, W. Schaffner, K. Lundstrom, Pharmacopsychiatry (Suppl 1) (2001) S137.
[7] R.S. Obach, J. Pharmacol. Exp. Ther. 294 (2000) 88.
[8] S. Caccia, Curr. Pharm. Anal. 2 (2006) 59.
[9] M. Ganzera, J. Zhao, I.A. Khan, J. Pharm. Sci. 91 (2002) 623.
[10] I. Rager, G. Roos, P.C. Schmidt, K.A. Kovar, J. Pharm. Biomed. Anal. 28 (2002) 439.
[11] E.C. Tatsis, S. Boeren, V. Exarchou, A.N. Troganis, J. Vervoort, I.P. Gerothanassis, Phytochemistry 68 (2007) 383.
[12] B.A. Silva, F. Ferreres, J.O. Malva, A.C.P. Dias, Food Chem. 90 (2005) 157.
[13] E. De Rijke, P. Out, W.M.A. Niessen, F. Ariese, C. Gooijer, U.A.Th. Brinkman, J. Chromatogr. A 1112 (2006) 31.
[14] V. Butterweck, A. Wall, U. Lieflander-Wulf, H. Winterhoff, A. Nahrstedt, Pharmacopsychiatry 30 (1997) 117.
[15] S. Caccia, Curr. Drug Metab. 6 (2005) 531.
[16] K.A. Youdim, B. Shukitt-Hale, J.A. Joseph, Free Radical Biol. Med. 37 (2004) 1683.
[17] V.C.J. De Boer, A.A. Dihal, H. Van Der Woude, I.C.W. Arts, S. Wolffram, G.M. Alink, I.M.C.M. Rietjens, J. Keijer, P.C.H. Hollman, J. Nutr. 135 (2005) 1718.
[18] I.C.W. Arts, P.C.H. Hollman, Am. J. Clin. Nutr. 81 (2005) 317S.
[19] M. Rozio, C. Fracasso, A. Riva, P. Morazzoni, S. Caccia, J. Chromatogr. B Anal. Technol. Biomed Life Sci. 816 (2005) 21.
[20] A. Paulke, M. Schubert-Zsilavecz, M. Wurglics, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 832 (2006) 109.