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Identification of flavonoid metabolites after oral administration to rats of a *Ginkgo biloba* extract

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Abstract

An extract of *Ginkgo biloba* leaves (EGb) was administered by gastric probe to Wistar female rats, and urine and faeces samples were collected for 5 days and whole blood samples were withdrawn every 30 min for 6 h. After purification with SPE C_{18} cartridges, the samples were analysed by reversed-phase LC–diode array detection (LC–DAD) for residual flavonoid glycosides, aglycones and metabolites. No glycosides or aglycones were detected in urine, faeces or blood and extensive degradation of EGb flavonoids within 24 h was detected. Among the seven different phenylalkyl acids detected by LC–DAD, 3,4-dihydroxyphenylacetic acid (I), hippuric acid (II), 3-hydroxyphenylacetic acid (III), homovanillic acid (IV) and benzoic acid (VII) were directly confirmed by on-line mass spectrometry using an electrospray interface (ES-MS). Peaks V and VI needed to be collected and separately examined and they were found to be 3-(4-hydrophenyl)propionic acid and 3-(3-hydrophenyl)propionic acid, respectively. As further evidence, the identity of metabolites I, II, III, IV, V and VII was confirmed by co-chromatography with authentic standards.

1. Introduction

Extracts from *Ginkgo biloba* leaves (EGb) have different pharmacological activities, such as peripheral vasoregulation [1], PAF antagonism [2] and prevention of membrane damage caused by free radicals [3,4]. While ginkgolides are responsible for the first two actions, the antioxidant/radical-scavenging properties may be related to the flavonoid fraction. These compounds are present as glycosides and their content normally reaches 24%, the flavonol fraction being the most relevant [5]. It is well established that flavonols, i.e., 2-phenylchromone derivatives bearing a hydroxyl group at C-3 (Fig. 1), are good in vitro radical scavengers [6] and inhibitors

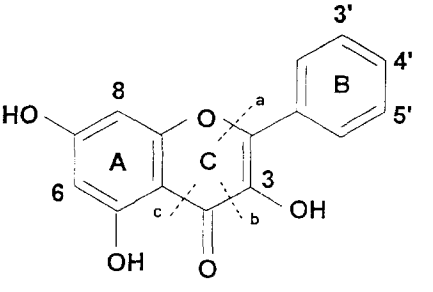

Fig. 1. Structural skeleton of flavonols and metabolic cleavage points.

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of the arachidonic acid cascade [7]. Since it is known that flavonols are extensively degraded by the intestinal microflora [8], the question arises of whether in vivo intact flavonols or their metabolites are responsible for the antioxidant effects. Flavonoid metabolism has been studied [9], and some correlation between the parent flavonoid and metabolites has been established. However, these investigations are limited to selected standards, using mainly thin-layer chromatography (TLC) as the detection technique, and no complex mixtures, such as extracts from medicinal plants, have been studied.

In this paper, we report the results obtained when EGb was administered orally to rats and the metabolites were separated by reversed phase high-performance liquid chromatography (RPLC) and identified on the basis of their on-line UV and mass spectra.

2. Experimental

2.1. Materials

Ginkgo biloba leaf extracts were a gift from the Istituto Biochimico Pavese (Pavia, Italy). Rutin, quercetin, kaempferol and isorhamnetin and their glycosides were purchased from Extrasynthese (Genay, France).

Female Wistar rats were purchased from Charles River Italia (Calco, Como, Italy). 3,4-Dihydroxyphenylacetic acid (I), hippuric acid (II), 3-hydroxyphenylacetic acid (III), 3-methoxy-4-hydroxyphenylacetic acid (IV), 3-(4-hydroxyphenyl)propionic acid (V) and benzoic acid (VII) were purchased from Sigma (St. Louis, MO, USA). 3-(3-Hydroxy-phenyl)propionic acid (VI) was isolated by semi-preparative HPLC, and its structure was assigned by UV and mass spectrometry.

Other reagents were of analytical-reagent grade and the solvents and water were of HPLC grade.

2.2. Apparatus

HPLC analyses were performed using a Model 510 pump equipped with a U6K universal injector (both from Millipore, Milford, MA, USA) coupled with a Model 1040 photodiode-array detector (DAD) (Hewlett-Packard, Waldbronn, Germany) and a TSQ70 triple-quadrupole mass spectrometer (Finnigan MAT, San José, CA, USA). The columns were Aquapore C_8 RP-300, 7 μm (200 × 4.6 mm I.D.) (Applied Biosystems, San José, CA, USA) and Spherisorb ODS-2, 5 μm (250 × 4.6 mm I.D.) (Phase Separations, Deeside, UK) for flavonoid and phenylalkyl acid analysis, respectively. Flavonol glycosides were eluted with 2-propanol–tetrahydrofuran–water (10:5:85, v/v/v), aglycones with 1-propanol–tetrahydrofuran–0.6% citric acid (12.5:7.5:80, v/v/v) and phenylalkyl acids with water–acetonitrile–acetic acid (88:10:2, v/v/v). The flow-rate was 1.5 ml/min in all cases.

2.3. Standard solutions

Stock standard solutions of flavonol glycosides or aglycones (0.4 mg/ml) were prepared in methanol. Stock standard solutions of I–V and VII (1 mg/ml) were prepared in water.

2.4. Mass spectrometry

Mass spectra were obtained at an ionization potential of 70 eV in the positive-ion mode. The source was maintained at 230°C. The mass spectrometer was scanned at 1 s per scan over the mass range 100–300. The flow-rate was 1.0 ml/min.

2.5. Preparation of urine samples

Female Wistar rats (n = 16, mass 150 g) were fed at 24-h intervals with the following diet: white flour, 600 g; dried milk, 200 g; dried yeast powder, 30 g; NaCl, 10 g. Approximately 10 g of the food powder was given to each rat per day during the period of the experiment. After 15 days, EGb (600 mg/ml in water) was orally administered to rats by gastric probe. Urine samples were collected separately at 24-h intervals for 5 days. The urine samples (15 ml) were filtered and evaporated to dryness under vacuum. The residue was dissolved in water (2 ml) and 1 ml was loaded on an SPE C_{18} cartridge

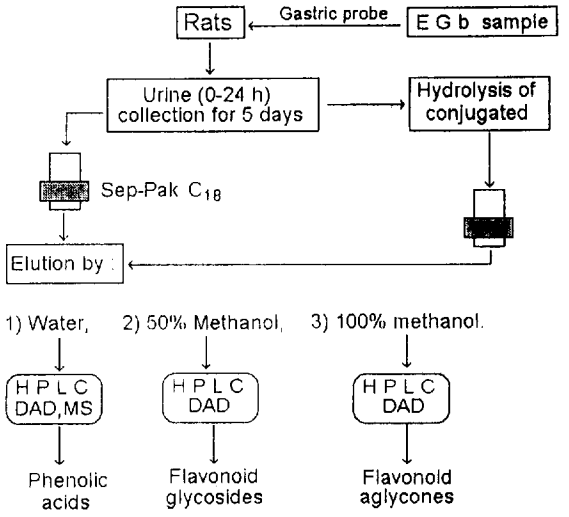

Fig. 2. Outline of the metabolic study.

preactivated by washing with methanol (3 ml) and water (10 ml). The cartridge was then eluted with water (3 ml), then with 50% methanol (3 ml) and finally with methanol (3 ml). Each eluate was evaporated to dryness under vacuum and the residue was dissolved in water (0.4 ml). Aliquots (5 μl) of each fraction were submitted to LC with diode–array detection (DAD) and electrospray (ES) MS.

2.6. Hydrolysis conditions

Acid hydrolysis was carried out in sealed tubes at 100°C with 1-ml urine samples mixed with 0.2 ml of 1 *M* HCl and 0.8 ml of methanol. After 45 min the hydrolysis solution was neutralized with 1 *M* NaOH and evaporated to dryness under vacuum. The residue was dissolved in 30% methanol (1 ml) and loaded on a preactivated SPE C_{18} cartridge. The cartridge and the eluates were treated as described for urine samples.

2.7. Preparation of faecal samples

Twenty-four hour faeces samples were stirred with 10 ml of hot methanol for 10 min, then the mixture was diluted with 10 ml of methanol and

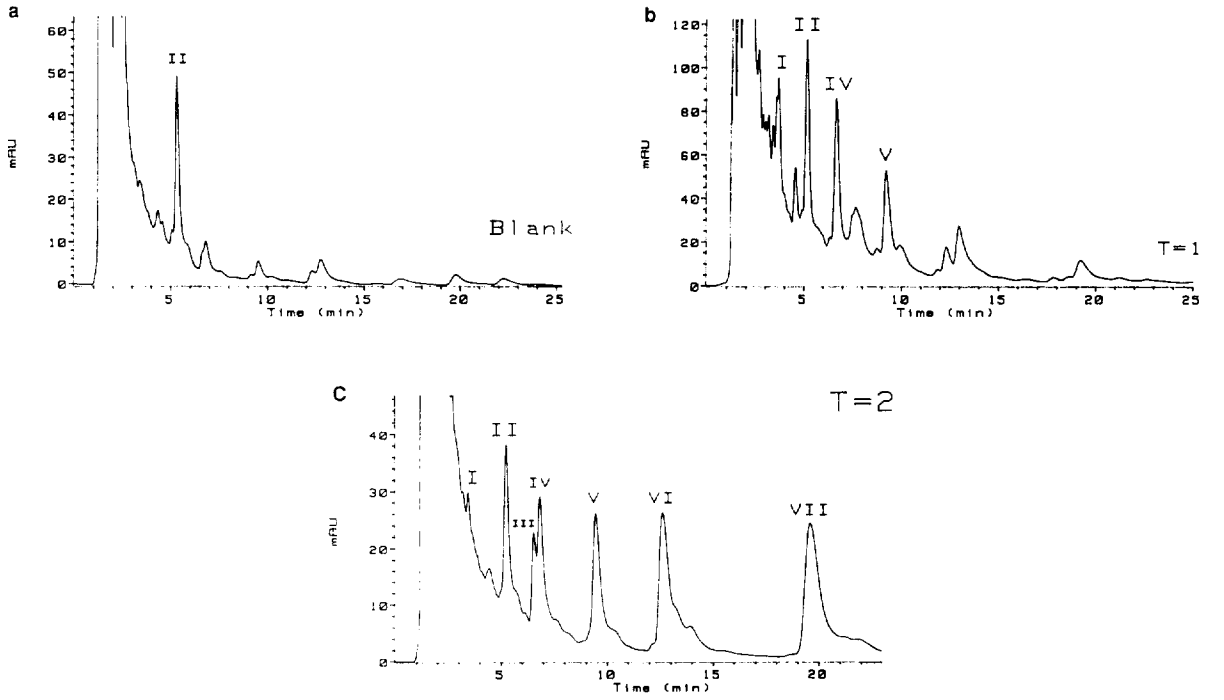

Fig. 3. HPLC separation of EGb metabolites I–VII from urine samples. (a) Blank; (b) 24 h; (c) 24–48 h. Column, Spherisorb ODS-2, 5 μm (250 × 4.6 mm I.D.); eluent, water–acetonitrile–acetic acid (88:10:2, v/v/v); flow-rate, 1.5 ml/min. For peak numbers, see Table 1.

filtered. The extract was evaporated to dryness under vacuum; the residue was dissolved in 1 ml of 30% methanol and loaded on a preactivated SPE C_{18} cartridge. The cartridge and the eluates were treated as described for urine samples.

2.8. Preparation of blood samples

Female Wistar rats ($n = 12$) treated similarly to those for urine samples were killed at 30-min intervals after EGb intake. Blood samples (2 ml) were kept at 0°C overnight and then centrifuged at 6000 rpm for 12 min. The surnatant (1 ml) was applied to a SPE C_{18} preactivated cartridge and processed as described for the urine samples.

3. Results and discussion

The metabolic fate of EGb was investigated as outlined in Fig. 2. As a preliminary approach, urine samples were hydrolysed to cleave conjugated metabolites, but this treatment was abandoned, as no substantial difference from samples directly subjected to solid-phase extraction was observed.

The phenylalkyl acids derived from EGb were present in the aqueous eluate from the SPE C_{18} cartridge and their separation was difficult owing to their similar chromatographic behaviour. Fig. 3a, b and c show the separations obtained when a blank, and 1- and 2-day urine samples, respectively, were analysed. The chromatograms of 3–5 day urine samples did not differ from the blank.

Preliminary information on the nature of each metabolite was obtained from on-line UV and

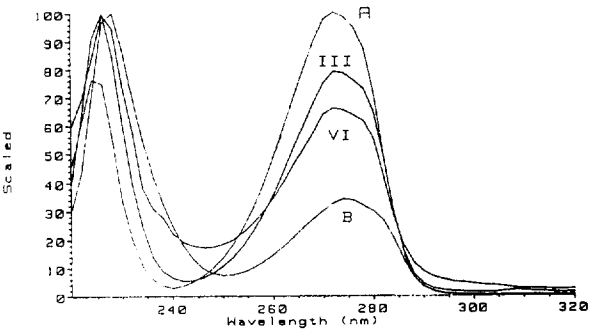

Fig. 4. UV spectrum of peak VI compared with UV spectra of 2-hydroxyphenylacetic acid (A), 3-hydroxyphenylacetic acid (III) and 4-hydroxyphenylacetic acid (B).

mass spectrometry (Table 1). The identities of some of the peaks were then definitely assessed by co-chromatography and spiking the samples of interest with authentic specimens. Thus, the nature of 3,4-dihydroxyphenylacetic acid (I), hippuric acid (II), 3-hydroxyphenylacetic acid (III), homovanillic acid (IV) and benzoic acid (VII) was first suggested from UV and mass spectra and then confirmed by co-chromatography with reference compounds.

The identification of peaks V and VI was more difficult, as the on-line data were controversial. Therefore, these peaks were collected and analysed separately. Peak V gave a spectrum typical of a *para*-substituted 3-(phenyl)propionic acid, and the molecular ion (m/z 167) indicated two possible compounds, 3-(4-hydroxyphenyl)propionic acid or 4-methoxyphenylacetic acid. Owing to the availability of both as reference compounds, it was possible to assign peak V as 3-(4-hydroxyphenyl)propionic acid. Concerning peak

Table 1
Molecular ions and main fragments of metabolites I–VII

Peak	Compound	λ_{max} (nm)	M_r	Fragments (m/z)'
I	3,4-Dihydroxyphenylacetic acid	280	168	125/137/153/**169**/180/212/228
II	Benzoylglycine (hippuric acid)	236	179	122/141/**180**/202/218/224
III	3-Hydroxyphenylacetic acid	270	152	110/137/**153**/193/212
IV	Homovanillic acid	280	182	137/153/163/**183**/196/223/243
V	3-(4-Hydroxyphenyl)propionic acid	275	166	120/149/**167**/189/208/227
VI	3-(3-Hydroxyphenyl)propionic acid	270	166	120/149/**167**/189/208/227
VII	Benzoic acid	230	122	**123**/149/164/249

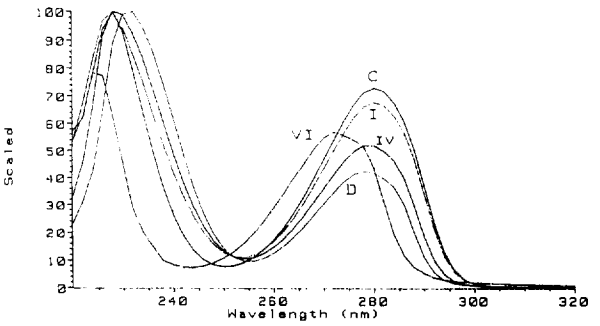

Fig. 5. UV spectrum of peak VI compared with UV spectra of 3,4-dihydroxyphenylpropionic acid (C), 3,4-dihydroxyphenylacetic acid (I), 3,4-dimethoxyphenylacetic acid (D) and homovanillic acid (IV).

VI, its UV spectrum indicated substitution at a *meta* position, as evidenced by the shape in the region 260–290 nm, which characterizes phenylalkyl acids monosubstituted at C-3 (Fig. 4). Moreover, a disubstitution was excluded, since in this case the absorption is shifted to higher wavelengths (Fig. 5). Conclusive evidence for monosubstitution was given by the mass spectrum, as shown in Fig. 6. As a result, peak VI was reasonably assumed to be 3-(3-hydroxyphenyl)propionic acid.

Benzoylglycine (II) was present in the blank sample and the corresponding peak increased only in the first urine fraction (0–24 h), whereas

Fig. 6. Mass spectrum of peak VI: molecular ion, *m/z* 167.

benzoic acid (VII) was present only in the second urine fraction (24–48 h), indicating possible saturation of the hepatic glycine conjugation system.

These results indicate that the chromone ring, in addition to the ether linkage, may be broken at different points yielding benzoic, phenylacetic or 3-(phenyl)propionic acid. Owing to the absence of flavones in EGb, the formation of the 3-(phenyl)propionic derivatives V and VI has to be ascribed to other EGb components besides the flavonol fraction. Indeed, both literature data [10,11] and direct experience with rutin, chosen as a representative flavonol glycoside, precluded the formation of 3-(phenyl)propionic acids from flavonols. Therefore, compounds V and VI probably arise from the procyanidin fraction (≥8%), according to a previous study on the microbial metabolism of flavonolic oligomers [12].

The metabolites found in urine represent less than 40% (Table 2) of the flavonoid administered, while phenylalkyl acids present in faeces were less than 4%. No intact flavonoid was found in blood in the period 0–5 h, whereas the main metabolites (I, IV, V and VI) could be detected, thus representing suitable markers to be followed. Owing to their phenolic structure, some of these metabolites may play a role as free radical scavengers, and work on this aspect is in progress.

Table 2
Amounts of phenylalkyl acids detected in urine samples

Day	Peak amounts (mg)[a]						
	I	II	III	IV	V	VI	VII
0	0	0.6	0	0.3	0.4	0.3	0
1	1.4	3.3	0	1.9	1.6	1.8	0
2	0.25	1.8	0.75	0.9	0.9	2.5	1.1
3	0	0.5	0	0.3	0.5	0.4	0
4	0	0.55	0	0.4	0.4	0.3	0
5	0	0.6	0	0.4	0.5	0.4	0

[a]For peak numbers, see Table 1.

References

[1] P. Költringer, O. Eber, P. Lind, W. Lanssteger, P. Wakonig, G. Klima and W. Rothlauer, Perfusion, 1 (1989) 28–30.
[2] F. Jung, C. Mrowietz, H. Kiesewetter and E. Wenzel, Arzneim.-Forsch., 40 (1990) 589–593.
[3] J. Pincermail, M. Dupuis and C. Nasr, Experientia, 45 (1989) 708–712.
[4] J. Robak and R.J. Gryglewski, Biochem. Pharmacol., 37 (1988) 837–841.
[5] J. Kleijnen and P. Knipschild, Lancet, 340 (1992) 1136–1139.
[6] S. Rafat Husain, J. Cillard and P. Cillard, Phytochemistry, 26 (1987) 2489–2491.
[7] M.A. Moroney, M.J. Alcaraz, R.A. Forder, F. Carey and R.S. Hoult, J. Pharm. Pharmacol., 40 (1988) 787.
[8] L.A. Griffiths, in L. Farkas, M. Gabor and F. Kallay (Editor), Topics in Flavonoid Chemistry and Biochemistry, Elsevier, Amsterdam, 1975.
[9] A.M. Hackett, in Plant Flavonoids in Biology and Medicine: Biochemical, Pharmacological, and Structure–Activity Relationship, Alan R. Liss, New York, 1986, pp. 177–194.
[10] N.P. Das and L.A. Griffiths, Biochem. J., 110 (1968) 449–459.
[11] L.A. Griffiths and G.E. Smith, Biochem. J., 128 (1972) 901–911.
[12] G. Groenewoud and H.K. Hundt, Xenobiotica, 16 (1986) 99–107.