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Short communication

Identification of *Ginkgo biloba* flavonol metabolites after oral administration to humans

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Abstract

An extract of *Ginkgo biloba* leaves (EGb) was given to healthy volunteers. Urine samples were collected for 3 days, and blood samples were withdrawn every 30 min for 5 h. The samples were purified through SPE C_{18} cartridges and analyzed by reversed-phase LC–diode array detection for the presence of EGb metabolites. Only urine samples contained detectable amounts of substituted benzoic acids, i.e., 4-hydroxybenzoic acid conjugate, 4-hydroxyhippuric acid, 3-methoxy-4-hydroxyhippuric acid, 3,4-dihydroxybenzoic acid, 4-hydroxybenzoic acid, hippuric acid and 3-methoxy-4-hydroxybenzoic acid (vanillic acid). In contrast to rats no phenylacetic acid or phenylpropionic acid derivatives were found in urine, thus indicating that in humans a more extensive metabolism takes place. As for rats the metabolites found in human urines accounted for less than 30% of the flavonoids given. The same procedure was applied to blood samples, and no metabolites could be detected.

Keywords: *Ginkgo biloba*; Flavonols; 4-Hydroxybenzoic acid conjugate; 4-Hydroxyhippuric acid; 3-Methoxy-4-hydroxyhippuric acid; 3,4-Dihydroxybenzoic acid; 4-Hydroxybenzoic acid; Hippuric acid; 3-Methoxy-4-hydroxybenzoic acid; Vanillic acid

1. Introduction

EGb contains up to 28% of flavonoids and about 6% of ginkgolides [1]. The flavonoid fraction is mainly composed of flavonol–glycosides [2], while biflavones represent a minimal part [3].

The fate of EGb in humans has been not yet investigated, and this reflects the limited knowledge on the adsorption and metabolism of flavonoid drugs in humans. Indeed, few studies have been performed on individual aglycones taken at pharmacological doses [4] and the results are controversial.

In a previous work [5] we identified by means of HPLC with diode-array detection (DAD) seven different flavonoid metabolites after oral administration to rats of EGb. Besides hippuric acid and benzoic acid, the urine samples contained also 3,4-dihydroxyphenylacetic acid, 3-hydroxyphenylacetic acid, homovanillic acid, 3-(4-hydrophenyl)-propionic acid and 3-(3-hydrophenyl)-propionic acid.

Extending this study to humans, we report the results obtained after oral supplementation of EGb to healthy volunteers. Unlike to rats the metabolites

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found in human urine were mainly substituted benzoic acids in free or conjugated form, and no phenylalkyl acids were detected.

2. Experimental

2.1. Materials

EGb was a gift from Indena (Milan, Italy).

4-Hydroxyhippuric acid (II) and 3-methoxy-4-hydroxy-hippuric acid (III) were isolated by semi-preparative HPLC and their structures were assigned by UV and mass spectrometry.

3,4-Dihydroxybenzoic acid (IV), 4-hydroxybenzoic acid (V), hippuric acid (VI) and vanillic acid (VII) were purchased from Sigma (St. Louis, MO, USA).

Methanol, acetonitrile and water were of HPLC grade.

2.2. Apparatus

HPLC analyses were performed using a Model 510 pump equipped with a Rheodyne injector coupled with a Model 996 photodiode-array detector (Waters, Milford, MA, USA).

The column was a Spherisorb ODS2, 5 μm (250× 4.6 mm I.D.) (Phase Separations, Deeside, UK).

The eluents were: (a) water–acetonitrile–methanol–acetic acid (88:5:5:2, v/v), for comparing the profiles of different fractions (t=0; t=0–6 h; t=6–24 h; t=24–48 h), (b) water–acetonitrile–acetic acid (88:10:2, v/v), for the analyses of eventually present phenylacetic and phenylpropionic derivatives [6].

2.3. Mass spectrometry

A HP 5989 mass spectrometer (Hewlett-Packard, Waldbronn, Germany) was used together with a HP thermospray LC–MS interface. The ion source temperature was 220°C, and the vaporizer temperature was held at 100°C. The temperature of the aerosol in the sources jet chamber was 220°C. Full-scan spectra in the range m/z 100–500 in the positive-ion (PI) mode were recorded.

2.4. Standard solutions

Standard aqueous solutions of II–IV and VI–VIII (1 mg/ml) were used.

2.5. Urine sample preparation

After 7 days of flavonoid free diet, EGb (4 g in water, equivalent to 1 g of ginkgo flavonol–glycosides) was orally administered to healthy volunteers (n=6). Urines were collected separately at the following intervals: 0–6 h; 6–24 h; 24–48 h. Urine samples (1 ml) were loaded on a SPE C_{18} cartridge preactivated by washing with methanol (3 ml) and water (10 ml), respectively. Then the cartridge was eluted (3 ml of each eluent) with water, 30% methanol, 50% methanol and finally 100% methanol. Each eluate was evaporated under vacuum to dryness, and the residue dissolved in water (1 ml). A 10-μl aliquot of each fraction was submitted to HPLC with diode-array detection (HPLC–DAD).

2.6. Blood sample preparation

Blood samples (5 ml) were prelevated each 30 min for 5 h and centrifuged at 2600 g for 10 min. The supernatant (1 ml) was applied to a SPE C_{18} pre-activated cartridge and processed as described for the urine samples.

2.7. Hydrolysis conditions

Acid hydrolysis was carried out in sealed tubes at 100°C with 1 ml urine added to 0.2 ml of 1 M HCl and 0.8 ml of methanol. After 45 min the solution was neutralized with 1 M NaOH and evaporated under vacuum to dryness. The residue was dissolved

Table 1
Structures of metabolites I–VII

Peak	Compound
I	4-Hydroxybenzoic acid conjugate
II	4-Hydroxyhippuric acid
III	3-Methoxy-4-hydroxyhippuric acid
IV	3,4-Dihydroxybenzoic acid
V	4-Hydroxybenzoic acid
VI	Hippuric acid
VII	3-Methoxy-4-hydroxybenzoic acid (vanillic acid)

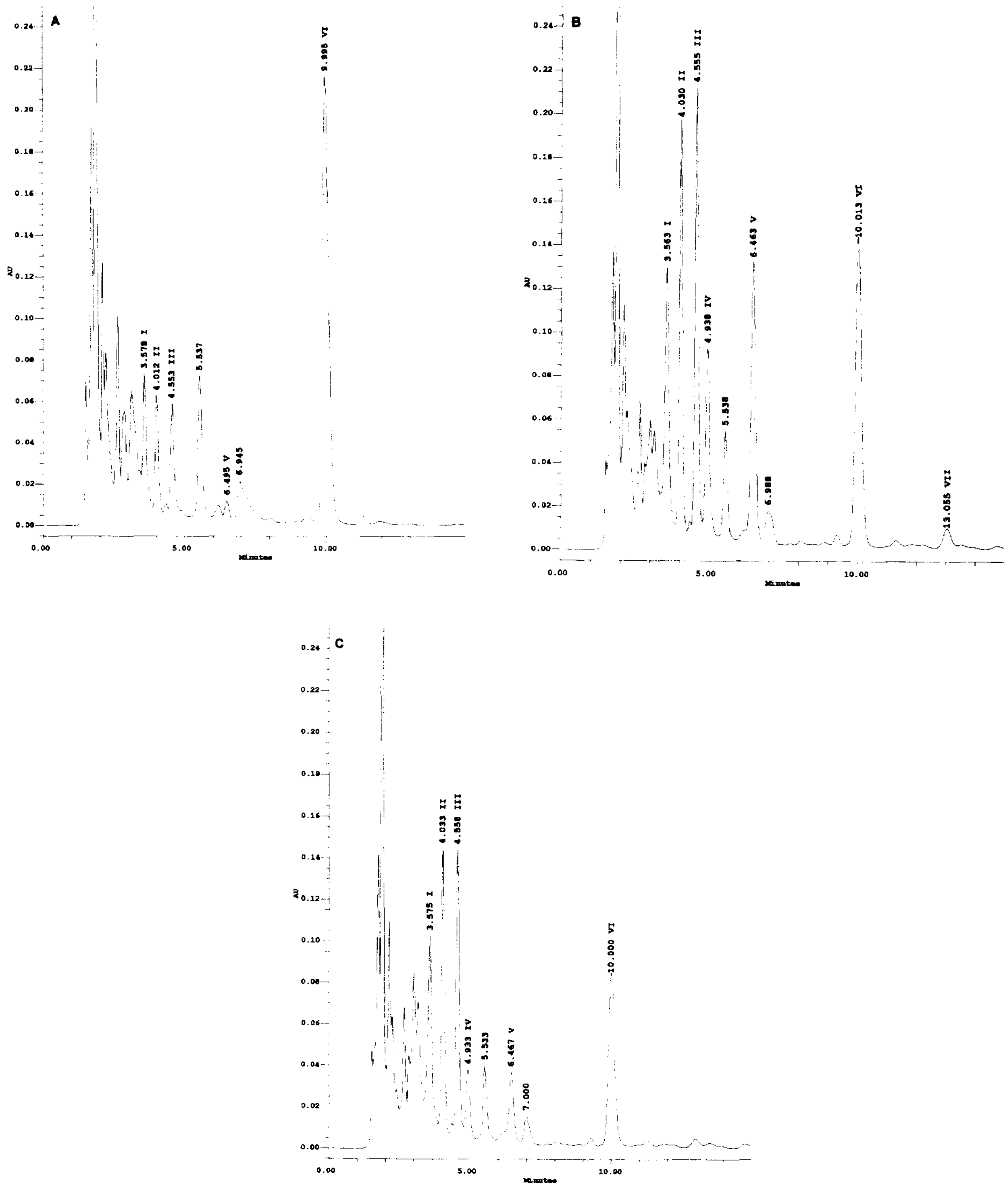

Fig. 1. HPLC separation of EGb metabolites I–VII from urine samples. (a) Blank; (b) 0–6 h; (c) 6–24 h. Column: Spherisorb ODS-2 5 μm (250×4.6 mm, I.D.). Eluent: water–acetonitrile–methanol–acetic acid (88:5:5:2, v/v/v/v). Flow-rate: 2.0 ml/min. For peak numbers see Table 1.

in 30% methanol (1 ml), loaded on a pre-activated SPE C_{18} and treated as described for urine samples.

3. Results and discussion

The metabolic study was performed according the outline described in a previous paper [5]. As expected, the substituted benzoic acids (Table 1) were present in the aqueous eluate from the SPE C_{18} cartridge and their separation required a new eluent composed of water–acetonitrile–methanol–acetic acid (88:5:5:2).

Fig. 1a–c show typical chromatograms of urine samples collected at different time intervals.

Peaks I–III and VII are present also in the blank, while peak V is present only in traces and peak IV is absent. The second fraction of urine (t=0–6 h) contain all these peaks in higher amount and two new peaks, IV and VII.

The chromatographic profile of the third urine fraction (t=6–24 h) is characterized by the presence of all peaks in reduced quantity. These data are best summarized in Fig. 2, which shows the time course of metabolite excretion.

The "on-line" UV spectra (Fig. 3) allowed to

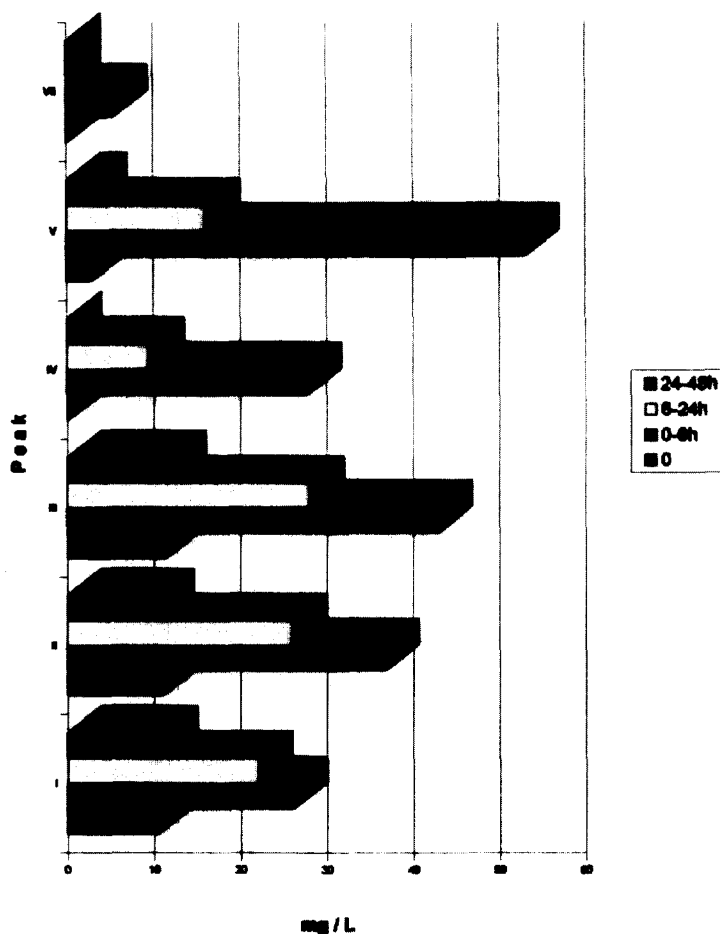

Fig. 2. Urine concentration–time profile of selected metabolites after oral administration of 1 g of ginkgo flavonol glycosides. See Table 1 for peak numbers.

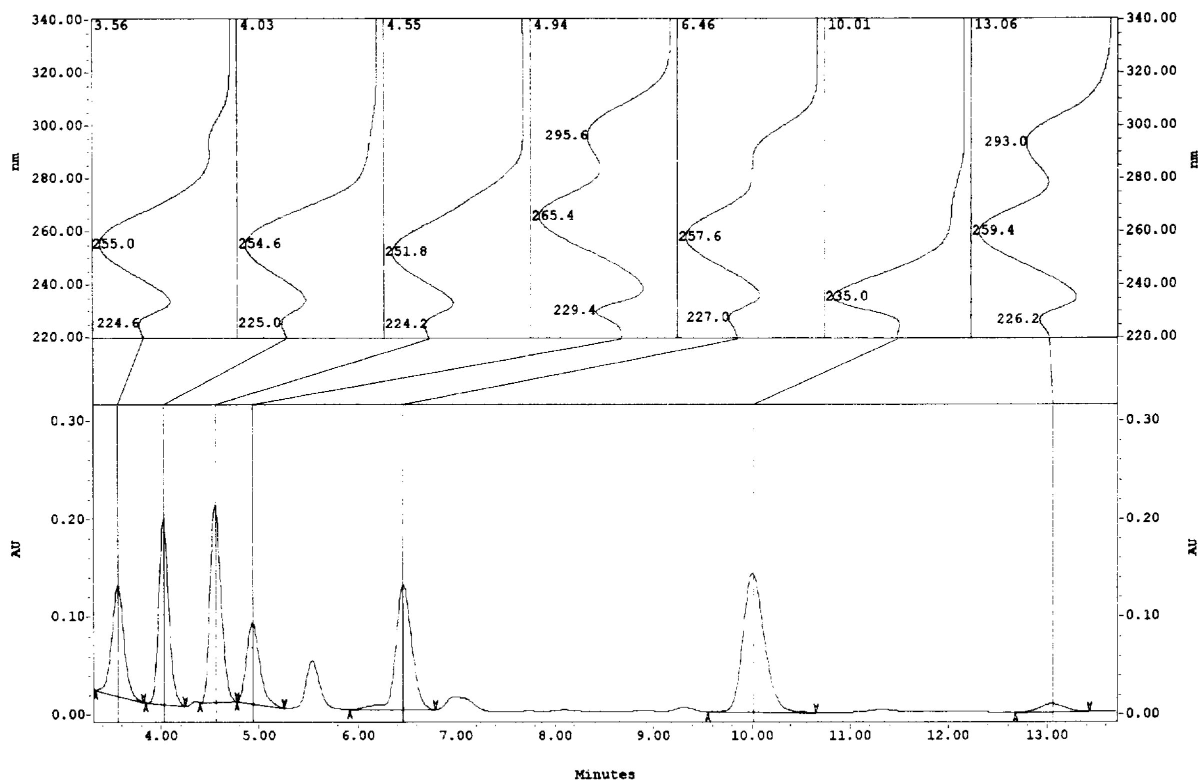

Fig. 3. On-line UV spectra of peaks 1–7. See Table 1 for peak numbers and Fig. 1 for chromatographic conditions.

obtain preliminary information on the nature of each metabolite.

Peaks I–III resulted to be conjugates of 4-hydroxybenzoic acids (I–II) and vanillic acid (III) as indicated by their reduction after acid hydrolysis and by the increase of the related acids V and VII. The identity of peaks II and III was further investigated by mass spectrometry. The molecular ions and the related fragments (Figs. 4 and 5) together with UV and retention times allowed to confirm peaks II and III as 4-hydroxyhippuric acid and 3-methoxy-4-hydroxyhippuric acid, respectively.

Due to the availability of standards peaks IV–VI and VII were definitely confirmed by co-chromatography with authentic specimens.

Urine samples were also investigated for the presence of phenylacetic or phenylpropionic acid derivatives using the same approach applied in the analysis of rat urines. The results were negative, thus suggesting that humans metabolize ginkgo flavonoids at higher extent.

Concerning the degree of adsorption, the total amount of metabolites found in urine in the interval 0–48 h accounts for less than 30% of the ingested Ginkgo flavonols. This result is in agreement with that found in the previous study on rats [5].

However, it must be pointed out that an intake of 1 g of flavonol-glycosides is very high and exceeds by several folds pharmacological dose suggested for EGb (40–240 mg/die) [6]. Therefore, extrapolation of these results to the absorption and metabolism of flavonol-glycosides at estimated level of dietary intake of 25–150 mg/day may be inappropriate.

Based on these results 3,4-dihydroxybenzoic acid (IV) represents a suitable marker to follow the metabolic pathway of EGb in humans. Its detection limit by HPLC–DAD is 1 μg/ml and this may explain why no measurable concentration of IV could

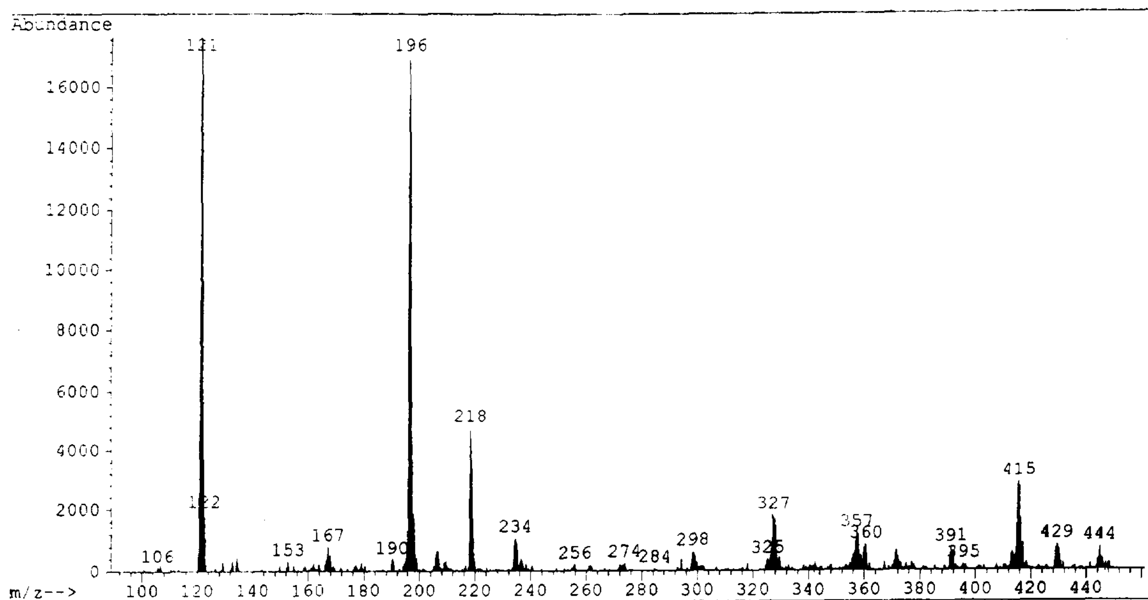

Fig. 4. Mass spectrum of peak II. Molecular ion, m/z 196; fragment (–glycine) m/z 121, sodium adduct, m/z 218.

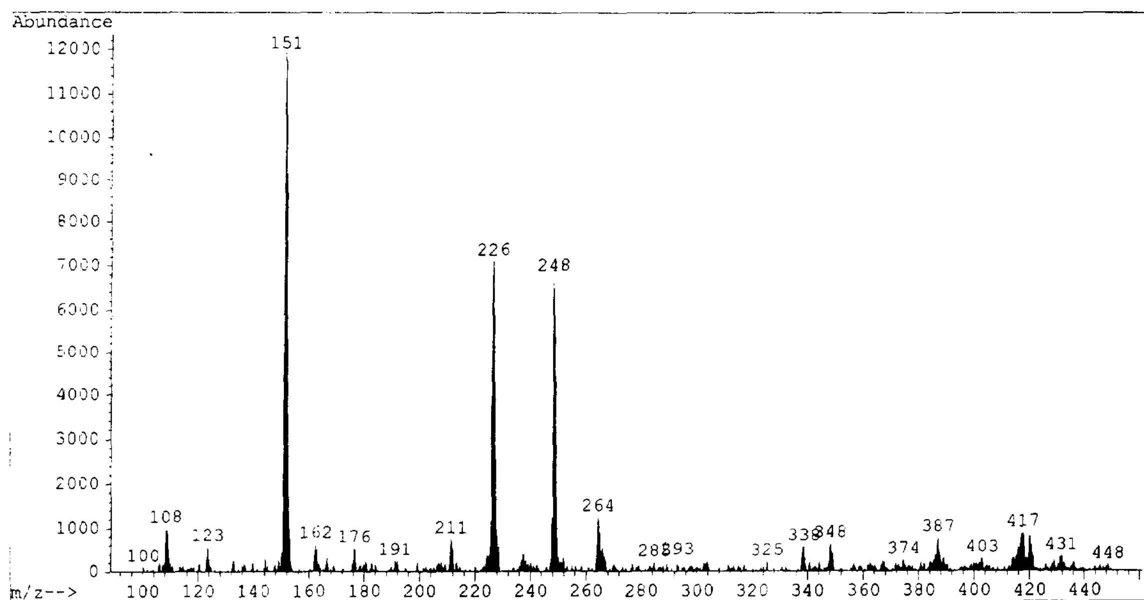

Fig. 5. Mass spectrum of peak III. Molecular ion, m/z 226; fragment (–glycine) m/z 151, sodium adduct, m/z 248.

be detected in plasma samples. Due to the major complexity of this matrix in comparison with urine, other extraction procedures, as well as more sensitive detection (such as enzyme immunoassay, which is in progress), are needed.

Acknowledgments

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