Food and Chemical Toxicology 49 (2011) 3224–3231

Contents lists available at SciVerse ScienceDirect

Food and Chemical Toxicology

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

Major furocoumarins in grapefruit juice I: Levels and urinary metabolite(s)

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ARTICLE INFO

Article history:
Received 30 June 2011
Accepted 3 September 2011
Available online 12 September 2011

Keywords:
Furocoumarins
Grapefruit juice
Metabolites
Phototoxic
Urinary excretion

ABSTRACT

Furocoumarins are phototoxic and photogenotoxic natural plant constituents occurring in cosmetics, food and drugs. Grapefruit juice is considered as a major dietary source of furocoumarins although few is known about the variability of furocoumarins in grapefruit juice. We analyzed the major furocoumarins in eight commercial grapefruit juices and in freshly prepared juices made from pink grapefruit obtained from German retailers. Bergaptol was the major furocoumarin in commercial juices, followed by bergamottin and 6′,7′-dihydroxy-bergamottin (DHB), whereas an inverse picture (DHB > bergamottin > bergaptol) was obtained in freshly prepared juices. Results from different batches of a single brand of commercial juice, purchased over a period of 7 months, revealed a variability of about 50% for the individual furocoumarins and the sum. In a study with healthy volunteers, consumption of 900 ml commercial grapefruit juice (containing 12.5 mg bergaptol, 6.9 mg bergamottin, and 0.6 mg DHB) resulted in an average urinary excretion of 0.36 mg free plus 13.23 mg conjugated bergaptol within 6 h. Other furocoumarins were not found in urine. Thus, other grapefruit furocoumarins were obviously converted in the human body, at least in part, into bergaptol excreted in urine, since the excreted amount of bergaptol exceeded the consumed one.

1. Introduction

Furocoumarins are natural plant constituents present in many types of plants used as food or in phytomedicines, herbal teas, and cosmetics. Their phototoxic and photomutagenic properties have gained considerable interest and resulted in various attempts to assess the toxicological risk of furocoumarin exposure via food (SKLM, 2010). Besides human exposure via plants and plant extracts, the pure furocoumarins psoralen and 8-methoxypsoralen (8-MOP) are used as therapeutic drugs for the treatment of psoriasis in combination with UVA irradiation. This therapy, also termed PUVA therapy, is able to induce skin tumors under certain circumstances (Stern et al., 1998; Stern, 2001). These findings have led to the classification of 8-MOP as a human carcinogen, i.e. in class 1A according to the IARC classification (IARC, 1987).

Much less is known about the phototoxic and photogenotoxic properties of the numerous other furocoumarins also occurring in plants (Ramaswamy, 1975; Wagstaff, 1991; SKLM, 2006). Recently, we reported large differences in the phototoxic and photomutagenic potencies among a number of furocoumarins ranging from highly phototoxic/photo-mutagenic to virtually inactive congeners. Furthermore, we suggested a Photo-Mutagenicity Equivalency Factor (PMEF) system which attributes each furocoumarin with a factor describing its relative photomutagenicity in V79 cells under standard conditions (Raquet and Schrenk, 2009; Lohr et al., 2010).

Grapefruit juice was reported (Murray et al., 1982) to contain bergamottin as the major furocoumarin and a number of minor furocoumarin constituents, i.e. 6′7′-dihydroybergamottin (DHB), and three furocoumarin dimers (Edwards et al., 1996; Fukuda et al., 2000; He et al., 1998) as well as minor amounts of 6′,7′-epoxybergamottin (Manthey and Buslig, 2005). In previous reports Mohri and Uesawa (2001) and Uesawa and Mohri (2006) found bergamottin, bergaptol and DHB as major furocoumarins in grapefruit juice. De Castro et al. (2006) also found similar levels of bergamottin in pink grapefruit juices sold in Florida, while DHB levels were much lower. Similar results were obtained by these authors in pulp freshly prepared from fruit tissues whereas the occurrence of other furocoumarins such as bergaptol was not reported. In raw juice freshly prepared from white grapefruit, Manthey and Buslig (2005) mainly found bergamottin and DHB along with small amounts of 6′,7′-epoxybergamottin. Furocoumarin dimers were suggested to occur mainly in a pulp-enriched retentate. However, very few is known about the variability of the furocoumarin pattern and level in commercial and home-made grapefruit juices. Furthermore, the fate of grapefruit furocoumarins in the human body is widely unknown.

Abbreviations: DHB, 6′,7′-dihydroxybergamottin; LOD, limit of detection; 5-MOP, 5-methoxypsoralen; 8-MOP, 8-methoxypsoralen; PMEF, photomutagenicity equivalency factor; THF, tetrahydrofuran; UVA light, ultraviolet light type A.

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doi:10.1016/j.fct.2011.09.005

Previous exposure studies have come to estimates on the average and high consumer average daily furocoumarin intake via food in the US (Wagstaff, 1991), the UK (COT, 1996), and Germany (SKLM, 2006). These analyses were based on data describing the levels of certain major furocoumarins in a number of food items. They come to the conclusion that grapefruit juice is a major dietary source of furocoumarin exposure in Western countries.

Furthermore, furocoumarins are known as highly potent dietary inhibitors of drug metabolism (Guo and Yamazoe, 2004). As constituents of grapefruit juice they inhibit a broad spectrum of cytochrome P450 (CYP) enzymes, most notably human CYP3A4, thus affecting the metabolic clearance of a large number of drugs (Edwards et al., 1996; Tassaneeyakul et al., 2000; Guo and Yamazoe, 2004) in a UV light-independent manner.

Here, we analyzed both manufactured, commercially available, and freshly prepared home-made grapefruit juices from various sources for major furocoumarins. Furthermore, we identified and quantified major furocoumarin metabolites in the 6-h-urine of healthy volunteers after consumption of 900 ml commercial grapefruit juice.

2. Materials and methods

2.1. Materials

The furocoumarins used were obtained from commercial sources at the highest degree of purity available. The furocoumarins bergamottin (99% purity), and 5-methoxypsoralen (5-MOP; 99% purity) and the furanobenzopyrone khellin (96% purity; internal standard) were from Carl Roth GmbH, Karlsruhe, Germany, bergaptol (99% purity) and 6′,7′-dihydroxybergamottin (DHB; 99% purity) from Herboreal Ltd., Edinburgh, UK. All other reagents were of analytical or HPLC grade. Naringenin was obtained from Carl Roth; NADPH-regenerating system from Promega, Madison, USA; and human liver microsomes, HLM Ultrapool 150, from BD Biosciences, San Jose, USA; naringin and ß-glucuronidase/arylsulfatase type HP-2 were from Sigma Aldrich, Steinheim, Germany.

Eight different brands of commercially available (pure) grapefruit juices (coded A-H) were purchased from various local grocery stores. One of the eight brands (brand A) was purchased at six time points over a period of 7 months (six different batches) from the same store. Furthermore, home-style juice was prepared from eight grapefruit batches, each batch being of different origin, purchased in various local grocery stores. Since only pink grapefruit was available, this type was used throughout. The juice was prepared from four grapefruit using a commercially available hand-hold lemon squeezer. The juices were analyzed immediately after purchase/preparation.

2.2. Human study

Six female and six male volunteers were recruited among students at the University of Kaiserslautern. The volunteers were healthy non-smoking Caucasians, between 20 and 30 years of age, who did not take any medication except for oral contraceptives among female volunteers. The volunteers gave their written informed consent before the onset of the study. The study protocol was approved by the Ethics Committee of the Landesärztekammer Rheinland-Pfalz, Mainz, Germany.

The participants were instructed to avoid any food rich in furocoumarins (grapefruit, lime, parsnips, persil roots) one day in advance and did not consume any food or beverages except water 12 h in advance. Then, after voiding the urinary bladder, the volunteers drank 900 ml of a commercially available grapefruit juice within 10 min and did not consume any food or beverage within 4 h after. Thereafter, they were allowed to consume tap or mineral water, as well as cheese and bread. Furthermore, they collected total urine within 6 or 12 h after having consumed the grapefruit juice, noted the pH-value and volume of the urine, took ten 50 ml samples from each collected total urine and froze them at −20 °C in the dark. During the urine collection period, the urine was kept in a brown plastic container at 4 °C in the dark. Each volunteer repeated the whole procedure 2 weeks later. Thus two urines were collected from each participant (Experiments 1 and 2).

2.3. Extraction of furocoumarins from grapefruit juice or urine

The extraction of furocoumarins from grapefruit juice was carried out using a modification of the method by Nigg et al. (1993). Briefly, 20 ml grapefruit juice were mixed with 40 µl ml of a khellin solution (10 mg in 1 ml acetonitrile) and 20 ml diethylether, shaken vigorously and centrifuged at 5000*g* and 5 °C for 5 min. The upper layer was removed and the extraction was repeated twice with 15 ml diethylether each. The organic phases were combined and the solvent was removed under reduced pressure at a temperature of 30 °C. The residue was dissolved in 3 × 3 ml of a 2:1 acetonitrile–water mixture and then applied to a solid phase extraction column (SPE C_{18} column, 3 ml, 500 mg; J.T. Baker, Deventer, NL) which had been equilibrated with 10 ml ethanol and 10 ml water. Then, the furocoumarins were eluted with 15 ml aqueous acetonitrile (60%, v/v), the eluate was mixed with 10 ml ethanol, and the solvents were removed under reduced pressure at 50–70 °C. The residue was dissolved in 1 ml chloroform, and was applied to a solid phase extraction column (SPE silica column, 3 ml, 500 mg; J.T. Baker), which had been equilibrated with 25 ml chloroform. The furocoumarins were eluted with 15 ml ethyl acetate (25%) in chloroform, and the eluted solution was dried under reduced pressure. The dry residue was dissolved in 1 ml of acetonitrile–water (1:1, v/v), filtered through a 0.45 µm Nylon membrane (Carl Roth, Karlsruhe, Germany), and stored at −20 °C until being analyzed. All steps were carried out in the dark or under red light only.

For extraction of furocoumarins from urine, 75 ml urine from each participant were mixed with 22.5 µl ml of a khellin solution (10 mg in 1 ml acetonitrile) and 60 ml diethylether, shaken vigorously and centrifuged at 5000*g* and 5 °C for 5 min. The upper layer was removed and the extraction was repeated twice with 60 ml diethylether each. The combined organic phases were heated at 30 °C to remove the solvent. The residue was dissolved in 1 ml of a 1:1 acetonitrile–water mixture, filtered through a 0.45 µm Nylon membrane (Carl Roth), and stored at −20 °C until being analyzed.

For hydrolysis of glucuronide or sulfate conjugates, 10 ml urine were mixed with 30 µl of the khellin solution (10 mg in 1 ml acetonitrile), 1 ml acetate buffer (pH 5.5), and 200 µl glucuronidase/arylsulfatase solution. The mixture was incubated at 37 °C for 16 h, and was then mixed with 10 ml diethylether, shaken vigorously and centrifuged at 5000*g* for 5 min. The extraction was repeated twice, the organic phases were combined and the solvent was removed under reduced pressure. The residue was dissolved in 1 ml of a 1:1 (v/v) acetonitrile–water mixture, filtered through a 0.45 µm Nylon membrane (Carl Roth), and stored at −20 °C until being analyzed.

The mean recovery rates for the standard (khellin) were 91.5 ± 1.4% (from juices), 92.3 ± 7.8% (from 6-h-urine without glucuronidase/sulfatase treatment), 101.3 ± 5.4% (from 6-h-urine with glucuronidase/sulfatase treatment), 87.0 ± 5.9 (from 12-h-urine without glucuronidase/sulfatase treatment), and 89.3 ± 7.4% (from 12-h-urine without glucuronidase/sulfatase treatment). All steps were carried out in the dark or under red light only. Because of limited amounts of furocoumarin standards, recovery experiments were carried out with khellin only. Previous data suggest that the recovery rates of individual furocoumarins from various aqueous matrices are in a close range (Gorgus et al., 2010).

2.4. HPLC analysis

The furocoumarins were separated by analytical HPLC using a modification of the method by Frérot and Decorzant (2004). Briefly, 50 µl extract (see above) were injected into a Jasco HPLC system equipped with a 150 × 2.1 mm column packed with Interchim MS Uptisphere 3 OBD (3 µm; Laubscher Labs, Porrentruy, Switzerland). The following mobile phases were used: (A) water: acetonitrile: THF (85:10:5; v/v/v); (B) acetonitrile: methanol: THF (65:30:5; v/v/v) at a flow rate of 0.3 ml/min in a composition/gradient of 0% B (0–5 min), 0–32% B (5–20 min; linear), 32% B (20–24 min), 32–55% B (24–38 min; linear), 55–90% B (38–40 min; linear), 90% B (40–75 min), 0% B (75–90 min). Detection of eluting compounds was carried out using a DAD UV-Detector at a wavelength of 311 nm. Quantitation was based on peak areas in comparison to standard calibration curves using the pure standards. The limits of quantitation were 1 µg/ml (bergamottin, naringenin), and 2.5 µg/ml (bergaptol and DHB) injected volume. Inter-day variability of detection was determined by khellin standard analysis. The furocoumarin levels determined were in the linear range of the calibration curves.

For preparative HPLC, 10 ml extract (see above) were injected into a Agilent 1200 HPLC system equipped with a 250 × 10 mm column packed with Reprosil 100 (5 µm; Dr. Maisch HPLC GmbH, Ammerbuch, Germany). The following mobile phases were used: (A) water; (B) methanol, at a flow rate of 6 ml/min in a composition/gradient of 40% B (0–10 min), 40–60% B (10–25 min; linear), 60% B (25–35 min), 60–40% B (35–36 min; linear), 40% B (36–45 min). Detection of eluting compounds was carried out using a UV-Detector at a wavelength of 311 nm.

2.5. HPLC–mass spectrometry

For mass spectrometric analysis the same column and gradient conditions were used as described above for the HPLC analysis. The eluting compounds were led into a tandem API 2000 mass spectrometer (Applied Biosystems, Carlsbad, California) using electron spray ionization in the positive mode. The ESI conditions were as follows: ion source gas 1, 20 psi; ion source gas 2, 20 psi; capillary temperature 400 °C; ion spray voltage, 4200 V, with one full scan in the first quadrupole (Q_1) from 50 to 400 *m/z*. Further compound depending parameters were used: declustering potential, 20 psi; focusing potential, 20 psi; cell entrance potential, 5 psi. The data were acquired using the Analyst Software 1.4.2 (Applied Biosystems, Carlsbad, California).