Identification of nobiletin metabolites in mouse urine

Shiming Li[1], Zhenyu Wang[3], Shengmin Sang[2], Mou-Tuan Huang[2] and Chi-Tang Ho[1]

[1]Department of Food Science, Rutgers University, New Brunswick, NJ, USA
[2]Department of Chemical Biology, Ernest Mario School of Pharmacy, Rutgers University, Piscataway, NJ, USA
[3]Hoffmann-La Roche Inc., Nutley, NJ, USA

Nobiletin, a major component of citrus polymethoxyflavones, has many potential significant health benefits. While the biological activities of nobiletin have been widely reported, its *in vitro* and *in vivo* metabolic fate has been rarely studied. To explore the biotransformation mechanism of nobiletin we conducted an investigation into its metabolic profile in mouse urine, by various analytical techniques. Due to sample amount limitations for isolating and characterizing an individual metabolite, two possible nobiletin metabolites were prepared in a similar multi-step organic synthetic route: 3′-hydroxy-5,6,7,8,4′-pentamethoxyflavone (3′-demethylnobiletin) and 4′-hydroxy-5,6,7,8,3′-pentamethoxyflavone (4′-demethylnobiletin). Normal phase (silica gel) and C_{18} reverse phase chromatography, as well as liquid chromatography-mass spectrometry-mass spectrometry, were employed in the separation of 3′-demethylnobiletin and 4′-demethylnobiletin, however, without success due to the structural similarities of these mono-demethylated nobiletins. Using a chiral packed column eluted under supercritical fluid chromatography (SFC) conditions, a clear separation was achieved. Thus, by comparing the SFC profiles of metabolite mixtures with the synthesized standard compounds, the major nobiletin metabolite of mouse urine is identified as 4′-demethylnobiletin, whereas 3′-demethylnobiletin is a minor metabolite. In this study, the concentration of 4′-demethylnobiletin in mouse urine is 28.9 μg/mL.

Keywords: Metabolites / Mouse urine / Nobiletin / Polymethoxyflavone synthesis / Supercritical fluid chromatography

Received: October 20, 2005; revised: December 8, 2005; accepted: December 9, 2005

1 Introduction

Nobiletin is a major component of polymethoxyflavones (PMF) in citrus fruits (particularly in the peel) which are widely consumed around the world. It has many significant biological properties, such as anti-inflammatory [1–4], anti-cancer [4–10] and anti-atherogenic activity [11, 12]. Research data show that nobiletin can inhibit matrix degradation of articular cartilage, and pannus formation in osteoarthritis and rheumatoid arthritis by effectively inhibiting the production of pro-matrix metalloproteinase-9 (proMMP-9) in rabbit synovial fibroblasts and articular chondrocytes [1]. Further molecular biological study provides the evidence of the interference of nobiletin with the production of prostaglandin E_2 (PGE_2) by selectively down-regulating cyclooxygenase-2 (COX-2) activity [2] in human synovial fibroblasts. In mouse macrophages, nobiletin was found to down-regulate the gene expression of other pro-inflammatory cytokines, such as interleukin-1α (IL1α), interleukin-1β (IL1β), interleukin-6 (IL6) and tumor necrosis factor-α (TNFα) [2]. It is also reported that nobiletin significantly inhibited two distinct stages of skin inflammation induced by double application of 12-*O*-tetradecanoylphorbol-13-acetate (TPA) in *in vivo* mouse skin inflammatory screening model [3]. Ohigashi's group [4] found nobiletin to have higher anti-inflammatory activity than indomethacin in a TPA-induced edema formation test in mouse ears. Therefore, nobiletin may have novel anti-inflammatory and immunomodulatory therapeutic properties. As chronic inflammation has been found to be closely associated with the increased risk of human cancer [5], many experiment data demonstrated the anti-cancer activities of nobiletin, including chemopreventive effects in mouse skin, stomach and colon cancer [4], antiproliferative effects and induction of apoptosis on human gastric cancer cells [6], inhibition of chemically induced colonic aberrant crypt foci in rats [7] and suppression of matrilysin (MMP-7) expression and pro-

Correspondence: Dr. Chi-Tang Ho, Department of Food Science, Rutgers University, 65 Dudley Road, New Brunswick, NJ 08901–8520, USA
E-mail: ho@aesop.rutgers.edu
Fax: +1-732-932-6776

Abbreviations: PMF, polymethoxyflavones; **SFC**, supercritical fluid chromatography

duction in HT-29 human colorectal cancer cells [8]. The antiproliferative activity of nobiletin and some other citrus flavonoids against several common human cancer cell lines, including lung, melanoma, leukemia, gastric cancer, colon, breast and prostate, has been studied in much detail and the IC50 data show that nobiletin is a potent compound among the flavonoids tested [9, 10].

There are many research reports about important biological activities of nobiletin, especially *in vitro* bioactivities, but little investigation of its metabolic fate. Recently, the *in vitro* biotransformation of nobiletin was examined in Ohigashi's group [13] by treating nobiletin with rat liver S-9 mixture (rat liver 9000 × g supernatant microsome) for 24 h. Using HPLC analysis and proton NMR – nuclear Overhauser effect (^{1}H NMR – NOE) study, the major metabolite was identified as 3′-demethylnobiletin [13]. Further *in vivo* biotransformation study of nobiletin conducted also by Ohigashi's group [14] in male SD rat (Sprague-Dawley) identified the dominant metabolite from rat urine as 3′-demethylnobiletin with two other mono-demethylated nobiletin and two di-demethylated nobiletin metabolites. The result was consistent with their previous *in vitro* experiment results. In their *in vivo* experiment, they also detected 3′-demethylnobiletin being the only metabolite in serum [14]. However, in a recent study of nobiletin biotransformation in the same species (male SD rat), also from urine samples after oral administration of nobiletin, Ohsawa's research group [15] isolated and identified one major and two minor metabolites by 3-D HPLC equipped with a photodiode array detector. In this study, the structure of the major metabolite was characterized as 4′-demethylnobiletin by 3-D HPLC methods. One of the minor metabolites was identified as a mono-demethylnobiletin but not 5-demethylnobiletin by ^{1}H NMR. The other minor metabolite was determined as di-demethylnobiletin by ESI-MS. This result is inconsistent with the conclusion obtained from Ohigashi's group [14] who identified the major metabolite of nobiletin as 3′-demethylnobiletin from the same genus of animal. It is noteworthy to mention that the metabolism study has been done in other PMF such as in tangeretin [11, 16, 17].

During the course of our metabolism study, we conducted *in vivo* experiments with nobiletin on CD-1 mice and identified the major metabolite in mouse urine. Since the quantity of metabolite samples from mouse urine was limited, two potential metabolites of nobiletin were synthesized as standard compounds and used in comparison with metabolite mixtures. Various techniques were employed to separate and characterize the metabolites. Supercritical fluid chromatography (SFC), equipped with a chiral column, gave the best separation results. The major metabolite of mouse urine was found to be 4′-demethylnobiletin (**1**) by a comparison of the SFC profiles of the metabolite mixture with the standard SFC profiles obtained from synthesized compounds. 3′-Demethylnobiletin (**2**) is a minor metabolite (Fig. 1).

2 Materials and methods

2.1 Chemicals

Sweet orange peel extract was obtained from Florida Flavors Company (Lakeland, FL). Solvents, including methanol, ethanol, water, ACN, ethyl acetate, dichloromethane, 2-propanol and acetone were of HPLC grade and purchased from Fisher Scientific (Springfield, NJ). Hexane (minimum of 85% n-hexane, other isomers including 2-methylpentane, 3-methylpentane and methylcyclopentane, and pentane) was of HPLC grade and purchased from VWR International Company (West Chester, PA). Reagents, including formic acid, phosphoric acid, TFA, triethylamine, ammonium hydroxide and pyridine were of ACS reagent grade and purchased from Sigma-Aldrich Company (Allentown, PA). The 2,3-dichloro-5,6-dicyanobenzoquinone was purchased from Alfa-Aesar-Lancaster (Pelham, NH). Deuterated solvents including chloroform-*d*, DMSO-d_6 and methanol-d_4 were purchased from Norell Company (Landisville, NJ). Gardenin A was purchased from Indofine Inc. (Hillsborough, NJ).

Octadecyl (C_{18}) derivatized silica gel (60 Å) RP analytical and preparative columns for HPLC were purchased from Waters Corporation (Milford, MA). Chiral analytical and preparative columns of Regis Welk-O 1 (R, R) column were purchased from Regis Technologies, Inc. (Morton Grove, IL).

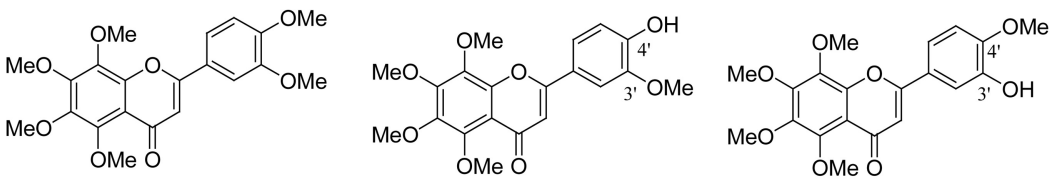

Figure 1. Structures of nobiletin, 4′-demethylnobiletin (**1**) and 3′-demethylnobiletin (**2**).

2.2 Syntheses

2.2.1 Preparation of 2′-hydroxy-3′,4′,5′,6′-tetramethoxyacetophenone

Eight grams of orange peel crude extract were dissolved in 5 mL of dichloromethane and loaded onto a 120-g pre-packed silica gel column and eluted with a gradient mobile phase that ran from 20% ethyl acetate – 80% hexanes to 80% ethyl acetate – 20% hexanes within 40 min. A mixture of PMF that mainly contain tangeretin and nobiletin was eluted, concentrated *in vacuo* and yielded 2.68 g of solid residue.

The residue obtained above was dissolved in ethanol (100 mL). To this solution, 50 mL of aqueous potassium hydroxide (50%) was added while stirring. The resultant solution was refluxed under nitrogen for 18 h. The reaction mixture was cooled and concentrated *in vacuo* to remove the ethanol. To the resultant aqueous solution, water (50 mL) and diethyl ether (100 mL) were added and the aqueous layer was separated and acidified with citric acid. Ethyl acetate (100 mL × 2) was added to extract the product. The combined organic layer was washed with water (50 mL × 2) and brine, dried over sodium sulfate, and concentrated. The viscous yellow oil was diluted with 2 mL of dichloromethane and loaded onto a silica gel flash column (80 g silica gel) and the desired product was eluted with ethyl acetate and hexane to give 1.62 g of 2′-hydroxy-3′,4′,5′,6′-tetramethoxyacetophenone as yellow oil. Low-resolution MS (LRMS) for $C_{12}H_{16}O_6$ [MH^+] m/z = 257; ^{1}H NMR (300 MHz, $CDCl_3$): d 4.05 (s, 3H), 3.95 (s, 3H), 3.85 (s, 3H), 3.80 (s, 3H) and 2.64 (s, 3H) was compatible with known spectra [18].

2.2.2 Compound 2 (Figs. 1 and 2)

A solution of 3-benzyloxy-4-methoxy-benzaldehyde (218 mg, 0.90 mmol) and 2′-hydroxy-3′,4′,5′,6′-tetramethoxyacetophenone (217 mg, 0.85 mmol) in 10 mL of 80% ethanol containing KOH (2 g) was stirred at room temperature overnight. The mixture was acidified with 20% HCl and then extracted with ethyl acetate. The extract was washed with water and brine, dried over Na_2SO_4, and concentrated *in vacuo*; the residue was applied to silica gel flash chromatography to give 192 mg of 3-benzyloxy-2′-hydroxy-3′,4,4′,5′,6′-pentamethoxylchalcone as a light yellow solid. LRMS for $C_{27}H_{28}O_8$ $(M+H)^+$ at m/z = 481 and ^{1}H NMR spectrum was compatible with that reported in literature [19].

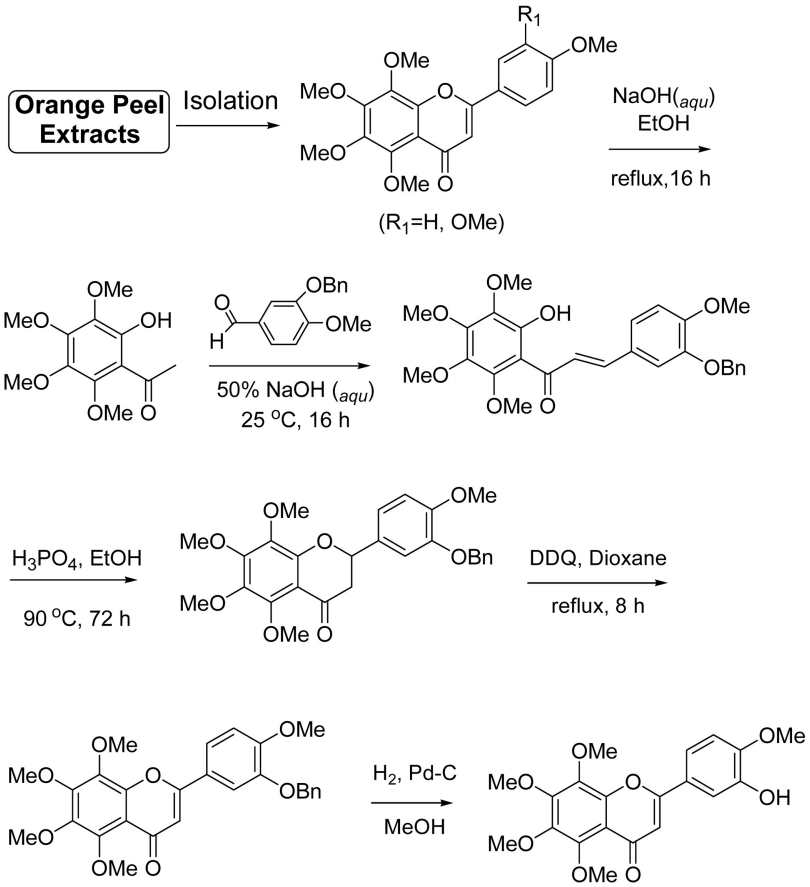

Figure 2. Synthetic scheme of 3′-demethylnobiletin (**2**).

A solution of 3-benzyloxy-2′-hydroxy-3′,4,4′,5′,6′-pentamethoxylchalcone (192 mg) in ethanol (10 mL) containing 50 mg of H_3PO_4 was heated to reflux for 16 h. Upon concentration, the resultant mixture was mixed with water and extracted twice with ethyl acetate (50 mL × 2). The combined organic layer was concentrated and purified by normal phase flash chromatography and 81 mg of 3′-benzyloxy-4′,5,6,7,8-pentamethoxyflavanone was obtained as a pale yellow solid. LRMS for $C_{27}H_{28}O_8$ $(M+H)^+$ at *m/z* = 481.

A solution of 3′-benzyloxy-4′,5,6,7,8-pentamethoxyflavanone (81 mg, 0.17 mmol) and 2,3-dichloro-5,6-dicyanobenzoquinone (DDQ, 57 mg, 0.25 mmol) in dry dioxane (5 mL) was refluxed for 8 h. The reaction mixture was cooled, concentrated and separated with silica gel chromatography to obtain 48 mg of 3′-benzyloxy-4′,5,6,7,8-pentamethoxyflavone as an off-white solid. LRMS for $C_{27}H_{26}O_8$ $(M+H)^+$ at *m/z* = 479.

3′-Benzyloxy-4′,5,6,7,8-pentamethoxyflavone (48 mg) was dissolved in methanol (2 mL) and to this solution 10% of palladium on carbon (5 mg) was added. Hydrogen gas was introduced at 40 psi for 30 min while shaking to give 3′-hydroxy-4′, 5,6,7,8-pentamethoxyflavone (**2**, 3′-demethylnobiletin, 15 mg) as a white solid. LRMS for $C_{20}H_{20}O_8$ $(M+H)^+$ at *m/z* = 389; ^{1}H NMR (300 MHz, $CDCl_3$): d 7.50 (d, J = 2 Hz, 1H), 7.48 (dd, J = 8.5 and 2 Hz, 1H), 6.97 (d, J = 8.5 Hz, 1H), 6.60 (s, 1H), 5.78 (s, 1H), 4.10(s, 3H), 4.02 (s, 3H), 3.98 (s, 3H), 3.94 (s, 6H); ^{13}C NMR (75 MHz, $CDCl_3$): d 177.52, 161.32, 159.80, 149.69, 148.24, 147.92, 146.35, 144.40, 138.80, 124.42, 118.98, 115.50, 112.49, 111.04, 107.26, 62.434, 62.22, 61.98, 61.81; UV (ACN and water): λ_{max} = 250.5 and 374.5 nm.

2.2.3 Compound 1 (Fig. 1)

The same procedure as the previous synthesis of 3′-demethylnobiletin (**2**) was followed using 4-benzyloxy-3-methoxy-benzaldehyde (218 mg, 0.90 mmol) and 2′-hydroxy-3′,4′,5′,6′-tetramethoxyacetophenone (217 mg, 0.85 mmol) as starting materials. 4-Benzyloxy-2′-hydroxy-3,3′,4′,5′,6′-pentamethoxylchalcone (181 mg) was obtained as a light yellow solid. LRMS for $C_{27}H_{28}O_8$ $(M+H)^+$ at *m/z* = 481. From 4-benzyloxy-2′-hydroxy-3,3′,4′,5′,6′-pentamethoxylchalcone (181 mg, 0.38 mmol) and phosphoric acid (50 mg), 61 mg of 4′-benzyloxy-3′,5,6,7,8-pentamethoxyflavanone was obtained as a pale yellow solid. LRMS for $C_{27}H_{28}O_8$ $(M+H)^+$ at *m/z* = 481. 4′-Benzyloxy-3′,5,6,7,8-pentamethoxyflavanone (61 mg, 0.13 mmol) was treated with DDQ (57 mg, 0.25 mmol) to obtain 4′-benzyloxy-3′,5,6,7,8-pentamethoxyflavone (38 mg) as an off-white solid. LRMS for $C_{27}H_{26}O_8$ $(M+H)^+$ at *m/z* = 479. Upon hydrogenolysis of 4′-benzyloxy-3′,5,6,7,8-pentamethoxyflavone (38 mg), 4′-hydroxy-3′,5,6,7,8-pentamethoxyflavone (**1**, 4'-demethylnobiletin, 12.5 mg) was obtained as a white solid. LRMS for $C_{20}H_{20}O_8$ $(M+H)^+$ at *m/z* = 389; ^{1}H NMR (300 MHz, $CDCl_3$): d 8.80 (br, 1H), 7.78 (s, 1H), 7.50 (d, J = 8 Hz, 1H), 7.0 (d, J = 8 Hz, 1H), 6.70 (s, 1H), 4.12(s, 3H), 3.98 (s, 3H), 3.94 (s, 3H), 3.92 (s, 6H); UV (ACN and water): λ_{max} = 250.5 and 336.5 nm.

2.3 Animals and sample collection

2.3.1 Animals

Eight-week-old female CD-1 mice (25 ± 4 g body weight) were obtained from Charles River Laboratories (Wilmington, MA).

2.3.2 Vehicle

In the control group, four of CD-1 female mice were dosed twice orally, with 0.5 mL of 50% glycerol/50% water for each mouse. Urine samples (10 mL) were collected during 16 h after each dose. The urine samples were pooled together and the total volume of collected urine sample from this control group was 20 mL from four mice after double dosage.

2.3.3 Nobiletin-fed mice

Nobiletin (40 mg) was dissolved in 1.66 mL of DMSO, and diluted with 5.0 mL of 50% glycerol/50% water to yield a homogenous suspension. This suspension was prepared shortly before start of administration to ensure of homogeneity.

Six of CD-1 female mice were dosed twice orally, with a dosage of 3 mg/mouse. Fifteen (15) mL of urine sample were taken during 16 h after each dose. The urine samples were pooled together and total volume of collected urine sample was 30 mL from six mice after double dosage.

2.3.4 Sample preparation

The following is an example of the preparation of analytical samples. An aliquot of the combined urine sample (400 μL) was mixed with enzymes of β-D-glucuronidase (250 U) and sulfatase (1 U). The mixture was incubated at 37°C for 45 min. Eight hundred microlitters of ethyl acetate was then added followed by mixing for 2 min. After mixing and centrifugation for 10 min, 700 μL of the organic phase was transferred to a microfuse tube. After a second extraction with 800 μL ethyl acetate, the combined ethyl acetate extracts (1.4 mL) were dried by vacuum centrifugation. Dried samples were dissolved in 100 μL of 50% ACN/50% water, or in methanol and filtered. The filtrates were collected for HPLC and SFC analysis.

2.4 Flash chromatography

An automated flash chromatography system (Model Foxy 200, sg100, Teledyne Isco, Lincoln, NE) was equipped with a pre-packed silica gel flash column (35–60 μm silica gel particle size, column size varies from 4 to 330 g). The mobile phase for normal phase flash column consisted of either ethyl acetate and hexanes or isopropanol and hexanes in varying proportions and the flow rate was set at 90 mL/min. The eluent was monitored with a single channel UV detector at a wavelength of 254 nm.

2.5 HPLC

The HPLC equipped with a pump (Waters Delta Prep 4000 delivery pump), a UV-Vis detector (Waters 486 tunable absorbance detector) and an injector (Waters U6K injector) were used. A Regis Welk-O 1 R,R 450 gram column (Regis Technologies, Morton Grove, IL) was used for the HPLC system. The mobile phase for the HPLC system was 35% absolute ethanol and 65% hexanes with a flow rate set at 85 mL/min. The eluent was detected with a UV wavelength at 326 nm. This system was used to isolate pure nobiletin from a mixture of nobiletin, tangeretin and 5,6,7,4′-tetramethoxyflavone.

2.6 LC-MS

The LC-ESI-MS was composed of an auto-sampler injector (Leap Technologies, Switzerland), an HP1090 system controller, with a variable UV wavelength (190–500 nm) detector, an ELSD (Evaporizing Laser Scattered Deposition) detector and an ESI-MS detector. Conditions were as following: acquisition mode, ESI-positive; mass scan range, 100–800 amu; scan rate, 0.4 s; cone voltage, 25 V; corona voltage: 3.47 kV (APCI+); source temperature: 150°C; probe temperature: 550°C. Analytical HPLC conditions on LC-MS: column: Chromeabond WR C18, 3 μ, 120 Å; length and OD: 30 × 3.2 mm; injection volume, 15 μL; flow rate: 2 mL/min; runtime in 5 min. The samples were analyzed by varying gradients of mobile phases, ACN and H_2O with 0.05% TFA.

2.7 LC-MS-MS

The LC-MS-MS spectra were obtained on a Micromass Q-TOF-2™ (Micromass, Beverly, MA). MS conditions: mass scan range: 100–8950 amu; interscan time: 0.1 s; cone voltage: 50 V; source temperature: 120°C, capillary: 1.50 kV; TOF: 9.28 kV.

2.8 NMR

The ^{1}H and ^{13}C NMR experiment were performed on Varian 300 Spectrometer (Varian Inc., Palo Alto, CA). With TMS serving as internal standard, ^{1}H NMR was recorded at 300 MHz and ^{13}C NMR at 75 MHz.

2.9 SFC

SFC is a well-established technique that is utilized widely in enantiomeric and chiral separations as well as non-chiral separations, particularly in drug discovery research [20]. SFC was performed on a Berger Analytical Supercritical Fluid Chromatography system (Mettler-Toledo Autochem Berger Instruments, Newark, DE) with six-way column and solvent selection valves. The system consisted of an automatic liquid sampler (ALS) with a 5-μL loop used to make injections and a thermal control module (TCM) used to control column temperature. Knauer variable wavelength UV detector (supplied by Mettler-Toledo Company) with high pressure flow cell was used for SFC detection. Chiralpak AD-H column was purchased from Chiral Technologies (West Chester, PA). Dimensions were 250 mm × 4.6 mm and particle size 5 μm. Chromatographic conditions as follows: CO_2 pressure 100 bar, flow rate 2.0 mL/min, oven temperature 30°C, modifier 20% methanol, UV detection at 220 nm.

2.10 Quantification of nobiletin metabolites

The analytical SFC described in Section 2.9 was used and UV detector was operated at 220 nm. Primary stock solutions of standard compounds, 4′-demethylnobiletin and 3′-demethylnobiletin (1.00 mg/mL) were prepared by dissolving the two standard compounds in methanol. They were stable for at least 2 weeks at ambient temperature. Four of analytical standard solutions were prepared from aliquots of the primary stock solutions, diluting with methanol. Standard curves of the standard compounds were constructed by plotting concentrations *vs.* peak areas. Good linearity for both standard compounds was achieved within the range of 0.01 to 0.1 mg/mL.

The metabolites from pooled urine sample were analyzed. The major metabolite, 4′-demethylnobiletin was quantified as 0.0289 mg/mL and the concentration of minor metabolite, 3′-demethylnobiletin was not measurable by UV in this study.

3 Results and discussion

3.1 General remarks

To establish a metabolic profile for the biotransformation of nobiletin in mouse urine, we have conducted an *in vivo* metabolism study by employing various techniques, such as

organic synthesis, HPLC, HPLC-MS, HPLC-MS/MS and SFC, and successfully identified 4′-demethylnobiletin as the major metabolite and 3′-demethylnobiletin as one of the minor metabolites. The concentration of the major metabolite, 4′-demethylnobiletin, has been quantified to be 28.9 μg/mL in mouse urine.

3.2 Synthesis of potential metabolites

Five-step syntheses (Fig. 2) were carried out and produced 4′-demethylnobiletin (**1**) and 3′-demethylnobiletin (**2**). The synthesis was started by isolating A ring permethoxylated and 3-unsubstituted PMF, as outlined in Sect. 2. Then the mixture containing these PMF, such as tangeretin and nobiletin, underwent basic hydrolysis and C ring fission products, 2′-hydroxy-3′,4′,5′,6′-tetramethoxyacetophenone were obtained, which then underwent Aldol condensation with 3-benzyloxy-4-methoxy-benzaldehyde (4-benzyloxy-3-methoxybenzaldehyde for synthesis of 4′-demethylnobiletin) to yield 3-benzyloxy-2′-hydroxy-4,3′,4′,5′,6′-pentamethoxylchalcone. Cyclization of 3-benzyloxy-2′-hydroxy-4,3′,4′,5′,6′-pentamethoxychalcone under acidic (phosphoric acid) and heated conditions, yielded a corresponding flavanone, which was then oxidized with DDQ, and debenzylation yielded 3′-demethylnobiletin [16].

The starting material, 2′-hydroxy-3′,4′,5′,6′-tetramethoxyacetophenone, can also be obtained by methylating a commercial reagent, Gardenin A, using iodomethane and potassium carbonate in anhydrous DMF. The reaction of this step was straightforward and the yield was nearly quantitative. This method to obtain 2′-hydroxy-3′,4′,5′,6′-tetramethoxyacetophenone is very efficient and fast and as such is an advantageous route for the syntheses of various PMF.

3.3 Separation of 3′- and 4′-demethylnobiletin

The LC/MS and LC/MS/MS analyses of 3′- and 4′-demethylnobiletin showed the same molecular weight of 388 and same fragmentation pattern (Fig. 3). The analytical HPLC profiles of 3′- and 4′-demethylnobiletin are almost identical. Variation of solvent systems (ACN-water and methanol-water), gradient conditions, mobile phase modifiers (TFA, formic acid, ammonium acetate and ammonium hydroxide) and columns have demonstrated reasonable separation between 3′- and 4′-demethylnobiletin. However, despite these efforts, the two compounds are hardly separable and the observed separation has no practical use in differentiating these two compounds by HPLC methods, since they have the same or very similar retention times on the HPLC traces. As expected, their mass spectra profiles are also identical under the LC-MS-MS conditions being used. Thus, there is no distinguishable fragmentation on the LC/MS/MS profile of these two compounds. Therefore, the separation of 4′-demethylnobiletin (**1**) and 3′-demethylnobiletin (**2**) using traditional separation methods remains a problem.

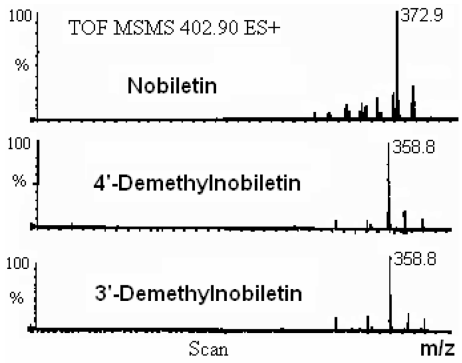

Figure 3. LC-MS-MS profile of 3′- and 4′-demethylnobiletin.

3.4 LC-MS profile of urine sample

Figure 4 shows the LC/MS metabolite profile of nobiletin in mouse urine. There is one major metabolite with a molecular weight (MW) of 388 from MS, corresponding to a mono-demethylated nobiletin (Fig. 4, Peak 5 at 3.02 min). There are at least three other minor metabolites and they are also mono-demethylated nobiletins (Peak 2 at 2.78 min, Peak 3 at 2.82 min and Peak 4 at 2.93 min). It is also observed that di-demethylnobiletin (Peak 1 at 2.63 min, MW = 374) exists as a minor metabolite. There are other small peaks but the MW was unobtainable because of low sample concentration. As expected, nobiletin was eluted late (Peak 6 at 3.32 min) because of its higher hydrophobic property compared with hydroxylated nobiletin. The two synthesized standard compounds have been analyzed under the same LC-MS conditions. Data showed that 3′- and 4′-demethylnobiletin had the same retention time as the major metabolite (Peak 5, Fig. 4). From the profiles of LC-MS and LC-MS-MS of 3′- and 4′-didemethylnobiletin, the major metabolite cannot be identified because of the indistinguishable nature of the two compounds under these experimental conditions.

3.5 SFC separation

To further investigate the separation between 3′-demethylnobiletin and 4′-demethylnobiletin and identify the composition of the major metabolite in mouse urine, an SFC technique was employed. The result obtained from the SFC experiment showed that there was a significant difference between the SFC profiles of 3′-demethylnobiletin and 4′-demethylnobiletin. In a 20–min elution (Fig. 5), the separa-

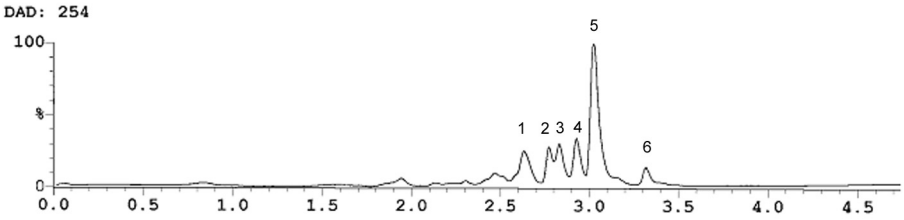

Figure 4. HPLC profile of nobiletin metabolites in urine. Conditions were as follows: C18 RP column, HPLC-APCI-MS, flow rate 2 mL/min, monitoring UV wavelength 254 nm. MW of peaks: **1**, 374 at 2.63 min; **2**, 388 at 2.78 min; **3**, 388 at 2.82 min; **4**, 388 at 2.93 min; **5**, 388 at 3.02 min; **6**, 402 at 3.32 min.

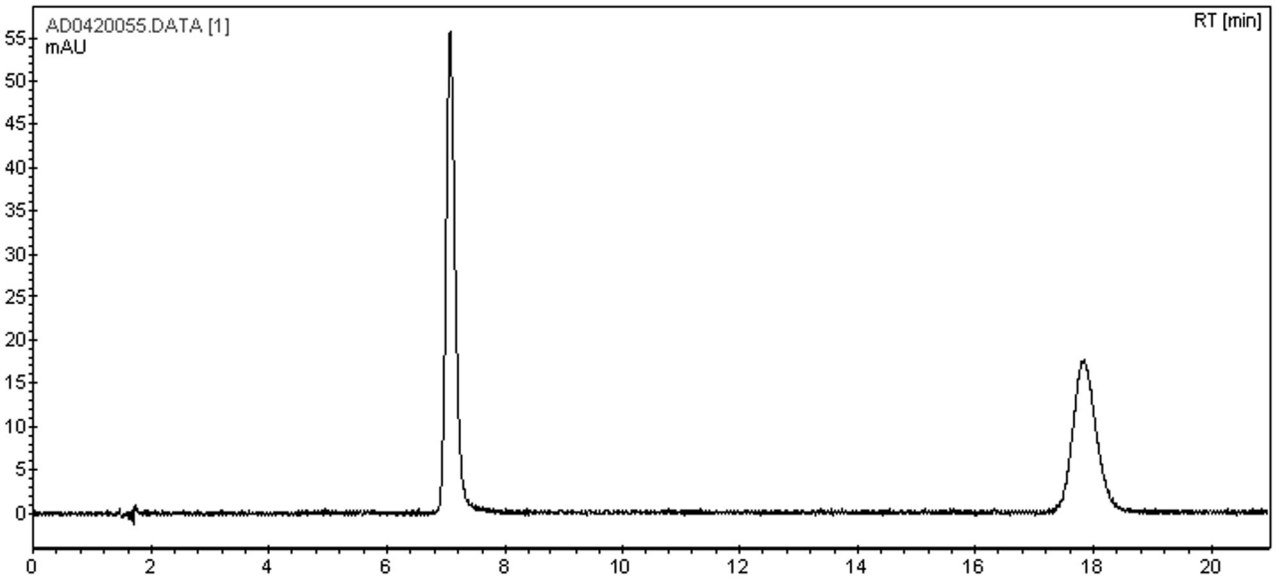

Figure 5. SFC separation profile of 3′- and 4′-demethylnobiletin. Early eluted peak at 7.1 min is 4′-demethylnobiletin and late peak at 17.9 min 3′-demethylnobiletin. Chromatographic conditions were: Chiralpak AD column, CO_2 pressure 100 bar, 20% methanol as modifier, temperature of 30°C, flow rate 2.0 mL/min.

tion of these two compounds was greater than for 10 min, indicating that their behavior on SFC was significantly different from that on C_{18} RP analytical HPLC columns. The retention time of 4′-demethylnobiletin (**1**) is 7.1 min whereas that of 3′-demethylnobiletin (**2**) is 17.9 min, under the same experimental conditions. The SFC experiment demonstrated that it is superior to other methods, and therefore it is feasible to use SFC technique in the identification of nobiletin metabolites and other difficult to separate compounds in the RP HPLC.

3.6 Identification of nobiletin metabolites

The SFC profile of nobiletin metabolites in mouse urine was obtained. The major absorption peak in the mouse urine metabolites is at 7.1 min (Fig. 6), corresponding to 4′-demethylnobiletin (**1**) when comparing the SFC profile of the urine sample with that of standard compounds (Fig. 5). A minor absorption peak eluted at 18.10 min (Fig. 6), corresponding to 3′-demethylnobiletin (**2**) (Fig. 5). Thus, our SFC study of mouse urine sample concludes that the major metabolite is 4′-demethylnobiletin (**1**), whereas 3′-demethylnobiletin (**2**) is a minor metabolite. Furthermore, efforts to quantify the urine levels of metabolites have been made and the concentration of the major metabolite, 4′-demethylnobiletin (**1**), in mouse urine was found to be 28.9 μg/mL under the conditions of this study. It was not possible to obtain the concentration of the minor metabolite, 3′-demethylnobiletin (**2**), because of the UV detection limitation of the SFC used in our study. Comparing the concentration of 4′-demethylnobiletin (**1**) in mouse urine, the concentration of 3′-demethylnobiletin (**2**) is too small to quantify by UV detector.

To our knowledge, this is the first metabolism study of nobiletin in mouse urine. The conclusion from this study

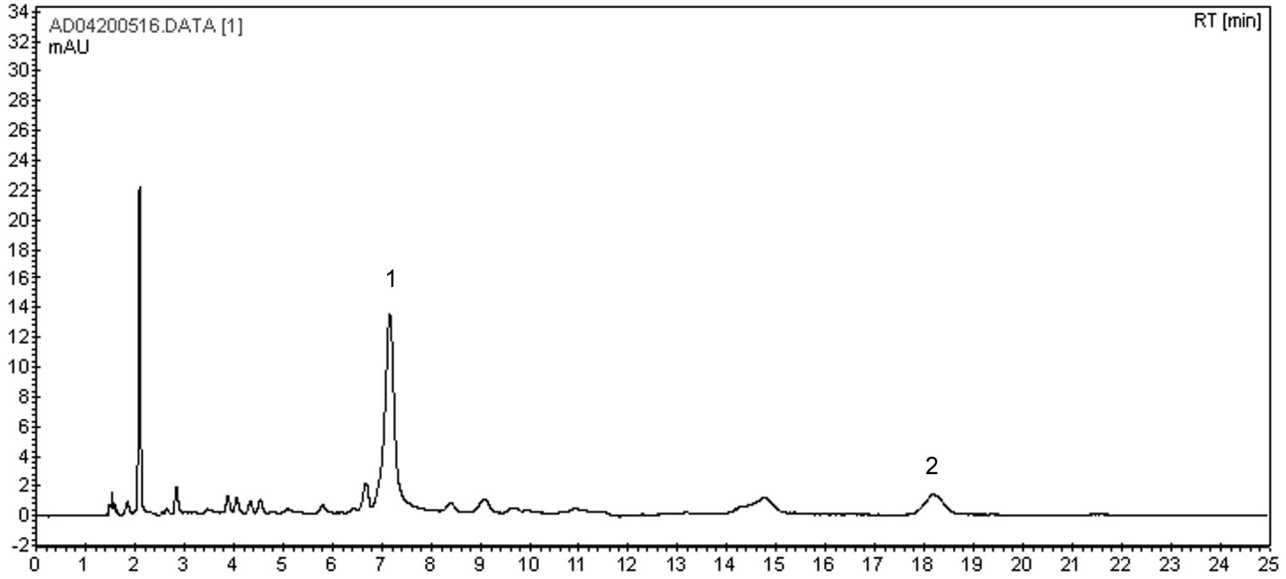

Figure 6. SFC profile of nobiletin metabolites in mouse urine. Peak **1** is 4′-demethylnobiletin and Peak **2** 3′-demethylnobiletin. SFC condition: CO_2 pressure 100 bar, 20% methanol as modifier, temperature of 30°C, flow rate 2.0 mL/min, Chiralpak AD column.

provides useful information of nobiletin such as its major and minor metabolites. Apparently, conclusion can be drawn that the methyl groups on the B-ring of nobiletin, particularly 4′-methyl group, are easily removed by methyltransferase enzymes in mice. The free hydroxyl groups after demethylation can be conjugated by conjugation enzymes such as glucuronic acid or sulfates. Consequently, the polarity and hydrophilicity of thus formed molecules, such as 4′-demethylnobiletin glucronate or sulfate, are significantly increased and the tendency of these molecules being easily absorbed and excreted is greatly increased. The study of these conjugated demethylnobiletins and the metabolite identification in mouse plasma is in progress.

4 Concluding remarks

In summary, we established a methodology to identify nobiletin metabolites in mice urine, by comparing the MS/MS, HPLC and SFC profiles of these metabolites with those of synthetic standards. This method was not hindered by the limited quantity of nobiletin metabolites obtained from mouse urine. By studying the LC-MS and SFC profiles of both standard compounds and metabolites, we observed that nobiletin predominantly undergoes mono-demethylation and minor di-demethylation pathways in a mouse metabolism model. The dominant metabolite of nobiletin in mouse urine is 4′-demethylnobiletin (**1**) and its concentration 28.9 μg/mL, whereas 3′-demethylnobiletin (**2**) is one of the minor metabolites. The identification of other nobiletin metabolites in mouse urine and metabolites in mouse plasma is in progress.

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