Isolation and Identification of Ten Metabolites of Breviscopine in Rat Urine

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Breviscopine, is the total flavonoid components (the content of scutellarin $\geq 85\%$) extracted from the dried whole plant of *Erigeron breviscapus* (VANT.) HAND.-MAZZ, and its preparations are generally used in the clinic for the treatment of cerebral and cardio-vascular diseases in China. In this paper, the metabolites of breviscopine in the urine of rats after oral administration were investigated. The ten metabolites were isolated by open-column chromatography and preparative high-performance liquid chromatography, and their structures were elucidated by MS, NMR spectroscopy including ^1H -NMR, ^{13}C -NMR, and NOESY (nuclear Overhauser enhancement spectroscopy), enzymatic hydrolysis and chemical evidence. The ten metabolites were identified as scutellarein-6,7-di- O - β -D-glucuronide (M-1), scutellarein (M-2), 6- O -methyl-scutellarin (M-3), 6- O -methyl-scutellarein (M-4), scutellarein-6- O - β -D-glucuronide (M-5), scutellarein-5- O - β -D-glucuronide (M-6), scutellarin (M-7), scutellarein-7- O -sulfate (M-8), apigenin-5- O - β -D-glucuronide (M-9), and apigenin-4'- O - β -D-glucuronide (M-10) respectively. The results of this study indicated that the metabolites of breviscopine were excreted in rats urine as glucuronidated, sulfated or methylated forms, as well as the aglycone of scutellarin-scutellarein after oral administration, and the metabolic pathways were also proposed.

Key words breviscopine; metabolite; rat; urine

Breviscopine, is the total flavonoid constituents (the content of scutellarin $\geq 85\%$) extracted from the dried whole plant of *Erigeron breviscapus* (VANT.) HAND.-MAZZ,¹⁾ which has been used as a folk medicine to cure paralysis for a long period in Yunnan province of China. Modern pharmacological study indicated that breviscopine could improve cerebral microcirculation and increase cerebral blood flow; inhibit the platelet activation and aggregation, and prevent thrombosis formation, *etc.*²⁾ The preparations of breviscopine have been widely used in the clinic as peroral dosage form or injections for the treatment of cerebral infarction and its sequelae, coronary heart disease and angina pectoris, *etc.* today in China.³⁾ The latest research indicates that breviscopine is in favor of myocardial protection⁴⁾ and shows promising effect on renal protection.⁵⁾ Due to the distinguished efficacy of breviscopine in the clinical therapy, the research of breviscopine has become a hot topic in China in recent years.

Pharmacokinetic studies on breviscopine or scutellarin have been much investigated in rats,⁶⁾ rabbits⁷⁾ and dogs⁸⁾ after oral administration, and Ge Q. H. *et al.* have revealed that the oral absolute bioavailability was quite poor in dogs.⁸⁾ Up to now, data available on metabolism of breviscopine or scutellarin in rats was limited to the literature published previously,⁹⁾ the results of which showed that scutellarin was metabolized in rats to a large extent and mainly excreted in urine and bile as metabolites, however, some of the metabolites were not identified since only HPLC-NMR and HPLC-MS were used as the analytical methods to deduce the structures of the metabolites.

To gain deep insight into its metabolic fate, we examined the biotransformation of breviscopine after oral administration to rats. The present study describes the isolation and identification of ten metabolites of breviscopine.

MATERIALS AND METHODS

Materials Breviscopine was commercially obtained

from Yunnan natural pharmaceutical company (Yunnan, China). Methanol was HPLC grade from Jiangsu Hanbon Sci. & Tech. Co., Ltd. (Jiangsu, China) and water was double distilled in our laboratory. Methanol and 95% ethanol were of analysis grade purchased from Tianjin Damao chemical factory (Tianjin, China). Macroporous resin D101 was product of Chemical Plant of NanKai University (Tianjin, China). ODS was commercially obtained from Pfizer, Inc. (New York, NY, U.S.A.). β -Glucuronidase (3.2.1.31) was purchased from Sigma (St. Louis, MO, U.S.A.).

Isolation of the Flavonoid Constituents of Breviscopine One major and three minor flavonoid components were observed in breviscopine by HPLC analysis (Fig. 1). After being auto-integrated by the Millennium³² workstation, it was displayed that the relative content of the major flavonoid component was about 89%. The chromatographic separation was performed on a C_{18} column (Inertsil ODS-2, 5 μm , 4.6 $\times$ 250 mm) at a column temperature of 25 $^{\circ}\text{C}$, and mobile phase was $\text{MeOH-H}_2\text{O}$ (50 : 50), containing 0.1% phosphoric acid. The detection wavelength was set at 335 nm. The composition of the analytical HPLC system see 'Spectroscopic Methods'.

The breviscopine sample (400 mg) dissolved in CH_3OH was subjected to RP-18 silica gel column chromatography with a $\text{MeOH-H}_2\text{O}$ solvent (30 : 70, 40 : 60, 50 : 50, 60 : 40), and subsequently, the 40% and 50% MeOH elution were further purified by preparative HPLC performed with an ODS column (Xterra ODS 19 $\times$ 300 mm; Waters Corp.) in a waters 600 liquid chromatograph apparatus equipped with a Waters 490 UV detector (Waters, Milford, MA, U.S.A.). The detection wavelength was 335 nm. Elution was carried out with $\text{MeOH-H}_2\text{O}$ containing 0.1% phosphoric acid at a flow rate of 10 ml/min. Elution with $\text{MeOH-H}_2\text{O}$ (38 : 62) yielded plantagin (3 mg) and scutellarin (310 mg). Elution with $\text{MeOH-H}_2\text{O}$ (42 : 58) yielded apigenin-7- O - β -D-glucuronide (5 mg) and scutellarein (3 mg). The four compounds were identified by means of spectral methods including ^1H - and

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Fig. 1. HPLC Chromatogram of Breviscapine

1, plantaginin (10.41 min); 2, scutellarin (11.78 min); 3, apigenin-7-*O*- β -D-glucuronide (17.95 min); 4, scutellarein (28.48 min).

Fig. 2. HPLC Chromatogram of Rats Urine Excreted during 36 h after Oral Administration of Breviscapine

^{13}C -NMR, which were compared with those of the corresponding compounds in the literature.^{10–13)}

Animals Male Wistar rats (200 ± 20 g body weight) were provided by the Animal Center of Shenyang Pharmaceutical University (Shenyang, China). The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Shenyang Pharmaceutical University. These animals were kept in a breeding room to be acclimated for 7 d before use. Normal food and water were available at all times except that they were withdrawn 12 h prior to experimentation.

Rats Urinary Samples for HPLC-DAD (High Performance Liquid Chromatography-Diode Array Detector) Analysis and Chromatographic Conditions Male Wistar rats were fasted 12 h before they were administered breviscapine orally (50 mg/kg) during which water could be available freely, then using metabolic cages to collect 36 h urine (approximately 20 ml/rat) which was adjusted pH to about 5.0 with phosphoric acid, and rats were free to drink water. The urinary sample was subjected to D101 macroporous absorption resin eluted with water and 70% ethanol, after the 70% eluting fraction was evaporated to dryness at 40 °C, the residue was reconstituted with 0.2 ml 50% methanol followed by centrifugation at 3000 rpm for 10 min, and the supernatant of 20 μl was subjected to HPLC system after being filtered through a 0.5 μm membrane. The preparation of blank sample was the same as the process described above except that the rats of control group were not administered drug.

The chromatographic separation was performed on a C_{18} reversed-phase column (Inertsil ODS-3, 5 μm , 3.9×150 mm) at a column temperature of 25 °C, the mobile phase consisted of solvent A (30% methanol, containing 0.1% phosphoric acid) and solvent B (70% methanol, containing 0.1% phosphoric acid). Conditions: linear gradient (A/B=100/0 $\rightarrow$ 0/100, 60 min) and then keeping solvent B for 20 min at a flow rate of 1.0 ml/min. The detection wavelength was set at 335 nm. The composition of the analytical HPLC system see

‘Spectroscopic Methods’. The result was shown in Fig. 2.

Rats Urine Collections For the identification of metabolites, sixty male Wistar rats were employed and fasted 12 h prior to experimentation, during which only water was provided. They were administered breviscapine orally at a dose of 50 mg/kg and reiteratively in an interval of 7 d (2 d for administration and 5 d for recovery), and the urine was collected between 0 to 36 h, during which water could be available freely. The pH of the collective urine was adjusted to about 5.0 with phosphoric acid, and placed in the refrigerator at -10 °C subsequently.

Isolation of Metabolites The cumulative urinary samples (approximately 20000 ml in total) were thawed at room temperature, and successively subjected to macroporous absorption resin D101 eluted with $\text{H}_2\text{O}/\text{EtOH}$ in a stepwise manner. The 30% and 50% EtOH elution was subjected to RP-18 silica gel column chromatography with a $\text{MeOH}-\text{H}_2\text{O}$ solvent system varying from water to methanol respectively. The fractions containing the metabolites of both were further purified by preparative HPLC.

Purification by HPLC Preparative HPLC was performed with an ODS column (C_8 , 20×250 mm; Inertsil Pak) in a waters 600 liquid chromatograph apparatus equipped with a Waters 490 UV detector (Waters, Milford, MA, U.S.A.). The detection wavelength was 335 nm. Elution was carried out with $\text{MeOH}-\text{H}_2\text{O}$ containing 0.1% phosphoric acid at a flow rate of 10 ml/min. Elution with $\text{MeOH}-\text{H}_2\text{O}$ (30:70) yielded M-1 (48 min). Elution with $\text{MeOH}-\text{H}_2\text{O}$ (40:60) yielded M-3 (70 min), M-5 (40 min), M-6 (35 min), M-7 (24 min), M-9 (30 min) and M-10 (60 min). Elution with $\text{MeOH}-\text{H}_2\text{O}$ (45:55) yielded M-8 (40 min). Elution with $\text{MeOH}-\text{H}_2\text{O}$ (50:50) yielded M-2 (25 min) and M-4 (65 min).

Enzymatic Hydrolysis of Metabolites M-1, M-3, M-5, M-6, M-9 and M-10 (each 0.1 mg) were incubated with β -glucuronidase (20 μl , 2000 units, *Helix pomatia*) in 0.2 M sodium acetate buffer (1.0 ml, pH 5.0) for 4 h at 37 °C in a shaking water bath, respectively. Each reaction mixture was

extracted with ethyl acetate and after the organic layer was evaporated to dryness in vacuum, the residues were reconstituted in 0.2 ml methanol for HPLC and ESI-MS analysis.

Spectroscopic Methods Electrospray ion trap mass spectrometry in a multistage full scan mode was performed on an Agilent 1100 series SL instrument (Agilent, U.S.A.) having a mass range of 60 to 2200. The instrument was operated in the negative ion mode, using nitrogen for nebulizing and dry gas. The collision-induced dissociation of $[M-H]^-$ was achieved with helium as the collision gas. The ionization was performed applying the following parameters: capillary temperature, 300 °C; capillary voltage, -4.0 kV. Sample solutions were directly introduced into the ESI source at a flow rate of 5 μ l/min by a springle pump.

NMR spectra were measured on a Bruker AV-600 spectrometer, and chemical shifts are given in ppm downfield relative to tetramethylsilane. All compounds were dissolved in $(CD_3)_2SO$.

UV absorption of all the metabolites was directly obtained by the method of HPLC-DAD. The analytical HPLC system for the metabolites was composed of a Waters Delta 600 pump, a Waters 600 controller and a Waters 996 photodiode array detector (Waters, Milford, MA, U.S.A.) with a Millennium³² workstation. The chromatographic separation was performed on a C₁₈ column (Inertsil ODS-2, 5 μ m, 4.6 $\times$ 250 mm) at a column temperature of 25 °C, and mobile phase was MeOH-H₂O (50 : 50), containing 0.1% phosphoric acid.

Scutellarein-6,7-di-O- β -D-glucuronide (M-1): Yellow amorphous powder. ¹H-NMR (DMSO-*d*₆) δ : 6.75 (1H, s, H₃), 6.99 (1H, s, H₈), 7.82 (2H, d, *J*=8.2 Hz, H_{2',6'}), 6.85 (2H, d, *J*=8.2 Hz, H_{3',5'}), 5.12 (1H, d, *J*=7.3 Hz, H_{1''}), 3.18–3.35 (O, H_{2''}-H_{4''} and H_{2'''}-H_{4'''}), 3.65 (1H, d, *J*=9.9 Hz, H_{5''}), 4.81 (1H, d, *J*=7.5 Hz, H_{1'''}), 3.38 (1H, d, *J*=9.9 Hz, H_{5'''}), 13.33 (1H, s, H₅). ¹³C-NMR (DMSO-*d*₆) δ : 164.5 (C-2), 102.5 (C-3), 182.1 (C-4), 152.0 (C-5), 129.9 (C-6), 156.0 (C-7), 95.2 (C-8), 152.5 (C-9), 105.8 (C-10), 120.5 (C-1'), 128.4 (C-2',6'), 116.0 (C-3',5'), 161.9 (C-4'), 100.8 (C-1''), 73.0 (C-2''), 76.1 (C-3''), 71.9 (C-4'' or C-4'''), 74.1 (C-5'' or C-5'''), 172.2 (C-6'' or C-6'''), 104.2 (C-1'''), 73.8 (C-2'''), 75.9 (C-3'''), 71.9 (C-4'' or C-4'''), 74.2 (C-5'' or C-5'''), 172.5 (C-6'' or C-6'''). ESI-MS *m/z*: 637 $[M-H]^-$, 461 $[M-H-glucuronic\ acid]^-$ and 285 $[M-H-2glucuronic\ acid]^-$. ESI-MSⁿ in negative mode was shown in Fig. 3.

Scutellarein (M-2): Yellow amorphous powder. ¹H-NMR (DMSO-*d*₆) δ : 6.73 (1H, s, H₃), 6.56 (1H, s, H₈), 7.90 (2H, d, *J*=8.6 Hz, H_{2',6'}), 6.91 (2H, d, *J*=8.6 Hz, H_{3',5'}), 12.78 (1H, s, H₅). ¹³C-NMR (DMSO-*d*₆) δ : 163.6 (C-2), 102.4 (C-3), 182.1 (C-4), 147.1 (C-5), 129.2 (C-6), 153.4 (C-7), 93.9 (C-8), 149.7 (C-9), 104.1 (C-10), 121.6 (C-1'), 128.4 (C-2',6'), 116.0 (C-3',5'), 161.1 (C-4'). ESI-MS *m/z*: 285 $[M-H]^-$. ESI-MSⁿ in negative mode was shown in Fig. 3.

6-O-Methyl-scutellarein (M-3): Yellow amorphous powder. ¹H-NMR (DMSO-*d*₆) δ : 6.79 (1H, s, H₃), 6.95 (1H, s, H₈), 7.87 (2H, d, *J*=8.0 Hz, H_{2',6'}), 6.89 (2H, d, *J*=8.0 Hz, H_{3',5'}), 5.15 (1H, O, H_{1''}), 3.26 (2H, O, H_{2''} and H_{4''}), 3.33 (1H, O, H_{3''}), 3.71 (1H, d, *J*=10.7 Hz, H_{5''}), 12.98 (1H, s, H₅), 3.77 (3H, s, -OCH₃). ¹³C-NMR (DMSO-*d*₆) δ : 164.3 (C-2), 102.5 (C-3), 182.3 (C-4), 152.5 (C-5), 132.5 (C-6), 156.5 (C-7), 94.2 (C-8), 152.2 (C-9), 105.7 (C-10), 120.7 (C-1'), 128.4 (C-2',6'), 116.0 (C-3',5'), 161.8 (C-4'), 99.7 (C-1''), 73.0 (C-2''), 76.7 (C-3''), 72.0 (C-4''), 74.1 (C-5''), 172.4 (C-6''), 60.3

(-OCH₃). ESI-MS *m/z*: 475 $[M-H]^-$, 299 $[M-H-glucuronic\ acid]^-$ and 284 $[M-H-glucuronic\ acid-CH_3]^-$. ESI-MSⁿ in negative mode was shown in Fig. 3.

6-O-Methyl-scutellarein (M-4): Yellow amorphous powder. ¹H-NMR (DMSO-*d*₆) δ : 6.78 (1H, s, H₃), 6.60 (1H, s, H₈), 7.92 (2H, d, *J*=8.2 Hz, H_{2',6'}), 6.92 (2H, d, *J*=8.2 Hz, H_{3',5'}), 13.08 (1H, s, H₅), 3.75 (3H, s, -OCH₃). ¹³C-NMR (DMSO-*d*₆) δ : 163.9 (C-2), 102.5 (C-3), 182.2 (C-4), 152.9 (C-5), 131.5 (C-6), 157.5 (C-7), 94.4 (C-8), 152.5 (C-9), 104.1 (C-10), 121.3 (C-1'), 128.6 (C-2',6'), 116.1 (C-3',5'), 161.3 (C-4'), 60.0 (-OCH₃). ESI-MS *m/z*: 299 $[M-H]^-$ and 284 $[M-H-CH_3]^-$. ESI-MSⁿ in negative mode was shown in Fig. 3.

Scutellarein-6-O- β -D-glucuronide (M-5): Yellow amorphous powder. ¹H-NMR (DMSO-*d*₆) δ : 6.76 (1H, s, H₃), 6.54 (1H, s, H₈), 7.92 (2H, d, *J*=8.5 Hz, H_{2',6'}), 6.92 (2H, d, *J*=8.5 Hz, H_{3',5'}), 4.77 (1H, br s, H_{1''}), 3.17–3.50 (O, H_{2''}-H_{4''}), 3.56 (1H, d, *J*=9.1 Hz, H_{5''}), 13.32 (1H, s, H₅). ESI-MS *m/z*: 461 $[M-H]^-$ and 285 $[M-H-glucuronic\ acid]^-$. ESI-MSⁿ in negative mode was shown in Fig. 3.

Scutellarein-5-O- β -D-glucuronide (M-6): Yellow amorphous powder. ¹H-NMR (DMSO-*d*₆) δ : 6.68 (1H, s, H₃), 6.91 (1H, s, H₈), 7.89 (2H, d, *J*=8.7 Hz, H_{2',6'}), 6.92 (2H, d, *J*=8.7 Hz, H_{3',5'}), 4.75 (1H, d, *J*=7.7 Hz, H_{1''}), 3.20–3.58 (O, H_{2''}-H_{5''}). ¹³C-NMR (DMSO-*d*₆) δ : 160.6 (C-2), 105.9 (C-3), 177.2 (C-4), 141.8 (C-5), 138.2 (C-6), 153.6 (C-7), 100.4 (C-8), 151.0 (C-9), 109.6 (C-10), 121.2 (C-1'), 128.0 (C-2',6'), 115.8 (C-3',5'), 161.1 (C-4'), 105.0 (C-1''), 72.9 (C-2''), 74.7 (C-3''), 72.1 (C-4''), 75.9 (C-5''), 170.9 (C-6''). ESI-MS *m/z*: 461 $[M-H]^-$ and 285 $[M-H-glucuronic\ acid]^-$. ESI-MSⁿ in negative mode was shown in Fig. 3.

Scutellarein-7-O-sulfate (M-8): Yellow amorphous powder. ¹H-NMR (DMSO-*d*₆) δ : 6.80 (1H, s, H₃), 7.27 (1H, s, H₈), 7.95 (2H, d, *J*=8.8 Hz, H_{2',6'}), 6.92 (2H, d, *J*=8.8 Hz, H_{3',5'}), 12.68 (1H, s, H₅). ¹³C-NMR (DMSO-*d*₆) δ : 164.3 (C-2), 102.4 (C-3), 182.6 (C-4), 147.3 (C-5), 132.1 (C-6), 148.4 (C-7 or C-9), 98.6 (C-8), 148.1 (C-7 or C-9), 106.3 (C-10), 121.4 (C-1'), 128.6 (C-2',6'), 116.1 (C-3',5'), 161.3 (C-4'). ESI-MS *m/z*: 365 $[M-H]^-$ and 285 $[M-H-SO_3]^-$. ESI-MSⁿ in negative mode was shown in Fig. 3.

Apigenin-5-O- β -D-glucuronide (M-9): Yellow amorphous powder. ¹H-NMR (DMSO-*d*₆) δ : 6.67 (1H, s, H₃), 6.69 (1H, d, *J*=2.2 Hz, H₆), 6.75 (1H, d, *J*=2.2 Hz, H₈), 7.89 (2H, d, *J*=8.7 Hz, H_{2',6'}), 6.92 (2H, d, *J*=8.7 Hz, H_{3',5'}), 4.89 (1H, d, *J*=7.4 Hz, H_{1''}), 3.17–3.40 (O, H_{2''}-H_{4''}), 3.88 (1H, d, *J*=9.7 Hz, H_{5''}). ESI-MS *m/z*: 445 $[M-H]^-$ and 269 $[M-H-glucuronic\ acid]^-$. ESI-MSⁿ in negative mode was shown in Fig. 3.

Apigenin-4'-O- β -D-glucuronide (M-10): Yellow amorphous powder. ESI-MS *m/z*: 445 $[M-H]^-$ and 269 $[M-H-glucuronic\ acid]^-$. ESI-MSⁿ in negative mode was shown in Fig. 3.

RESULTS

Structure Elucidation of Breviscapine Metabolites By the methods described above, ten metabolites were obtained: M-1 (30 mg), M-2 (10 mg), M-3 (7 mg), M-4 (5.2 mg), M-5 (1.5 mg), M-6 (1.2 mg), M-7 (2 mg), M-8 (1.1 mg), M-9 (0.5 mg), and M-10 (0.2 mg). The structures of M-1 to M-8 are shown in Fig. 4, while the structures of M-9 and M-10 are

Fig. 3

Fig. 3

G). ESI-MS² of metabolite **M-8** in negative ion mode H). ESI-MS² of metabolite **M-9** in negative ion mode I). ESI-MS² of metabolite **M-10** in negative ion modeFig. 3. ESI-MSⁿ Spectra of M1—M6 and M8—M10

displayed in Fig. 5.

Metabolite M-1 (Scutellarein-6,7-di-*O*- β -D-glucuronide) Yellow amorphous powder, the $\lambda_{\max}$ of UV absorption is at 271, 334 nm, suggesting M-1 to be a flavonoid. Enzymatic hydrolysis of M-1 with β -glucuronidase gave the aglycone, which was identified to be scutellarein from the ESI-MS and HPLC behavior. In the negative ESI-MS³ spectrum, the $[M-H]^-$ ion m/z 637 of M-1 eliminated 176 mass units (glucuronic acid) to give m/z 461, and the ion m/z 461 eliminated 176 mass units again (glucuronic acid) to give ion m/z 285, indicating M-1 to be scutellarein diglucuronide. The ¹H-NMR spectrum of M-1 showed signals similar to the ones of scutellarein skeleton (Hattori *et al.*, 1992)¹³⁾ and the anomeric protons signals of two β -D-glucuronic acid moieties [δ 5.12 (1H, d, $J=7.3$ Hz, H-1'') and δ 4.81 (1H, d, $J=7.5$ Hz, H-1''')]. Furthermore, the ¹³C-NMR data clearly showed the existence of two glucuronic acid [172.2, 172.5 (C-6'', C-6'''), 104.2 (C-1'''), 100.8 (C-1''), 76.1 (C-3''), 75.9 (C-3'''), 74.2, 74.1 (C-5'', C-5'''), 73.8 (C-2'''), 73.0 (C-2''), 71.9, 71.9 (C-4'', C-4''')]. The ¹³C-NMR data of the aglycone portion of M-1 were similar to those of scutellarein except for the downfield shift of C-5 by 4.9 ppm, downfield shift of C-7 by 2.6 ppm, downfield shift of C-8 by 1.3 ppm, downfield shift of C-9 by 2.8 ppm and downfield shift of C-10 by 1.7 ppm, indicating that the linkage sites of the glucuronic acid moieties to scutellarein were in ring A. The linked positions of two β -D-glucuronic acid moieties were established by the NOESY spectrum, in which correlations were observed between the signal of H-1'' (δ 5.12, 1H, d, $J=7.3$ Hz) and H-8 (δ 6.99, 1H, s), indicating that one glucuronic acid was linked in 7-OH, and there were no correlations between the signal of H-1''' (δ 4.81, 1H, d, $J=7.5$ Hz) and the proton signals of scutellarein skeleton, which excluded the possibility that the linked position was in 4'-OH. Based on the above data and combined with the data reported previously,¹⁴⁾ the structure of M-1 was identified as scutellarein-6,7-di-*O*- β -D-glucuronide.

Fig. 3

Fig. 4. The Structures of Metabolite M1—M8 Derived from Scutellarin in Rats Urine and the Proposed Metabolic Pathways for Their Production

Fig. 5. The Structures of Metabolite M9 and M10 Derived from Apigenin-7-O- β -D-glucuronide and Their Possible Metabolic Pathways in Rats

Metabolite M-2 (Scutellarein) Yellow amorphous powder, the $\lambda_{\max}$ of UV absorption is at 283, 337 nm, suggesting M-2 to be a flavonoid. The negative ESI-MS showed a quasi-

molecular ion peak at m/z 285 $[M-H]^-$ which suggested M-2 to be the aglycone of scutellarin. In the 1H -NMR, the signals of H-2',6' (δ 7.90, 2H, d, $J=8.6$ Hz) and H-3',5' (δ 6.91, 2H, d, $J=8.6$ Hz) indicated that ring B was substituted in position C-4' and the signal at δ 12.78 (1H, s) was attributed to 5-OH characteristically. In the NOESY spectrum, the cross peak from H-3 (δ 6.73, 1H, s) to H-2',6' (δ 7.90, 2H, d, $J=8.6$ Hz) could be observed, and consequently, 6.73 (1H, s) was assigned to H-3, and 6.56 (1H, s) was ascribed to H-8 meanwhile. Compared with the published data,¹³⁾ the structure of M2 was identified as scutellarein.

Metabolite M-3 (6-O-Methylscutellarin) Yellow amorphous powder, the $\lambda_{\max}$ of UV absorption is at 272, 334 nm, suggesting M-3 to be a flavonoid. M-3 treated with β -glucuronidase gave the metabolite M-4 by agreement with retention time and UV absorption on HPLC. In the negative ESI-MS³ spectrum, the $[M-H]^-$ ion m/z 475 of M-3 eliminated 176 mass units (glucuronic acid) to give m/z 299, and the ion m/z 299 eliminated 15 mass units (CH_3) to give ion m/z 284. Compared the ^{13}C -NMR of M-3 with that of scutellarin, an additional signal at δ 60.3 ppm appeared, the value of which above δ 60.0 ppm indicated that the methoxyl group was di-*ortho*-substituted,¹⁵⁾ and meanwhile, downfield shift of C-5 by 5.5 ppm, downfield shift of C-6 by 1.9 ppm, downfield shift of C-7 by 7.4 ppm and downfield shift of C-9 by 1.1 ppm could be observed, suggesting the ring A of the agly-

cone portion was methylated. A new signal at δ 3.77 (3H, s) derived from the methoxy group, were simultaneously observed in the $^1\text{H-NMR}$ spectrum, in which, the signal at δ 12.98 (1H, s) was assigned to 5-OH characteristically. In the NOESY spectrum, the cross peak from the anomeric proton of the glucuronic acid moiety (δ 5.15, 1H, O) to H-8 (δ 6.95, 1H, s), and the cross peak from H-3 (δ 6.79, 1H, s) to H-2',6' (δ 7.87, 2H, d, $J=8.0$ Hz) could be observed, whereas there is no cross peak between the protons of the methoxy group and the ones of the aglycone portion, which excluded the possibility that 7-OH or 4'-OH of scutellarein was methylated. Based on the above evidence, M-3 was determined to be 6-*O*-methyl-scutellarein.

Metabolite M-4 (6-*O*-Methyl-scutellarein) Yellow amorphous powder, the λ_{max} of UV absorption is at 273, 336 nm, suggesting M-4 to be a flavonoid. In the negative ESI-MS² spectrum, the $[\text{M}-\text{H}]^-$ ion m/z 299 of M-4 eliminated 15 mass units (CH_3) to give ion m/z 284, suggesting M-4 to be the aglycone of M-3. Compared the $^{13}\text{C-NMR}$ of M-4 with that of scutellarein, an additional signal at δ 60.1 ppm appeared, the value of which above δ 60.0 ppm indicated that both the adjacent carbons of the methoxyl group were substituted,¹⁵⁾ and meanwhile, downfield shift of C-5 by 5.8 ppm, downfield shift of C-6 by 2.3 ppm, downfield shift of C-7 by 4.1 ppm and downfield shift of C-9 by 2.8 ppm could be observed, suggesting the ring A of the flavonoid skeleton was methylated. A new signal at δ 3.75 (3H, s), which derived from the methoxy group, was observed in the $^1\text{H-NMR}$ spectrum, in which, the signal at δ 13.08 (1H, s) was assigned to 5-OH characteristically. In the NOESY spectrum, the cross peak from H-3 (δ 6.78, 1H, s) to H-2',6' (δ 7.92, 2H, d, $J=8.2$ Hz) could be observed, and there is no correlation between the protons of the methoxy group and the others, which excluded the possibility that 7-OH or 4'-OH of scutellarein was methylated. Based on the above analyses, M-4 was determined to be 6-*O*-methyl-scutellarein.

Metabolite M-5 (Scutellarein-6-*O*- β -D-glucuronide) Yellow amorphous powder, the λ_{max} of UV absorption is at

271, 336 nm, suggesting M-5 to be a flavonoid. Enzymatic hydrolysis of M-5 with β -glucuronidase gave the aglycone scutellarein from the ESI-MS and HPLC behavior. In the negative ESI-MS² spectrum, the $[\text{M}-\text{H}]^-$ ion m/z 461 of M-5 eliminated 176 mass units (glucuronic acid) to give ion m/z 285, suggesting M-5 to be an isomer of scutellarein. In the NOESY spectrum, the cross peak from H-3 (δ 6.76, 1H, s) to H-2',6' (δ 7.92, 2H, d, $J=8.5$ Hz) could be observed, and the anomeric proton of glucuronide (δ 4.77, 1H, brs) had no correlation with the protons of aglycone portion, which excluded the possibility that the linkage site of the glucuronic acid moiety was on the position 4'-OH or 7-OH, while the signal at δ 13.32 (1H, s) was designated to 5-OH characteristically in the $^1\text{H-NMR}$ spectrum, and accordingly, M-5 was identified as scutellarein-6-*O*- β -D-glucuronide. The full assignment of proton signals are summarized in Table 1.

Metabolite M-6 (Scutellarein-5-*O*- β -D-glucuronide) Yellow amorphous powder, the λ_{max} of UV absorption is at 273, 333 nm, suggesting M-6 to be a flavonoid. Enzymatic hydrolysis of M-6 with β -glucuronidase gave the aglycone scutellarein identified by the ESI-MS and HPLC behavior. The negative ESI-MS² spectrum of M-6 was almost the same as that of M-5, suggesting M-6 to be an isomer of scutellarein as well. In the $^1\text{H-NMR}$ spectrum, the signal of 5-OH disappeared, and in the $^{13}\text{C-NMR}$ spectrum, compared with that of scutellarein, the additional signals at δ 170.9 (C-6''), 105.0 (C-1''), 75.9 (C-5''), 74.7 (C-3''), 72.9 (C-2'') and 72.0 (C-4'') derived from the glucuronic acid moiety, furthermore, upfield shift of C-2 by 3.0 ppm, downfield shift of C-3 by 3.5 ppm and upfield shift of C-4 by 4.9 ppm could be observed, and meanwhile, in the NOESY spectrum, the anomeric proton of glucuronide (δ 4.75, 1H, d, $J=7.7$ Hz) had no correlation with the protons of aglycone portion and the cross peak from H-3 (δ 6.68, 1H, s) to H-2',6' (δ 7.89, 2H, d, $J=8.7$ Hz) could be observed. From the above evidence, combined with the shift rules of flavonoid after 5-OH pro- and post-conjugated with glucose,¹⁶⁾ M-6 was determined to be scutellarein-5-*O*- β -D-glucuronide. The full assignment of proton signals

Table 1. Assignments of Proton Signals of Metabolites M-5, M-6, M-8 and M-9

No.	M-5 ^{a,c)}	M-6 ^{a,b)}	M-8 ^{a,b)}	M-9 ^{a,c)}
2	—	—	—	—
3	6.76 (1H, s)	6.68 (1H, s)	6.80 (1H, s)	6.67 (1H, s)
4	—	—	—	—
5	—	—	—	—
6	—	—	—	6.69 (1H, d, $J=2.2$ Hz)
7	—	—	—	—
8	6.54 (1H, s)	6.91 (1H, s)	7.27 (1H, s)	6.75 (1H, d, $J=2.2$ Hz)
9	—	—	—	—
10	—	—	—	—
1'	—	—	—	—
2',6'	7.92 (2H, d, $J=8.5$ Hz)	7.89 (2H, d, $J=8.7$ Hz)	7.95 (2H, d, $J=8.8$ Hz)	7.89 (2H, d, $J=8.7$ Hz)
3',5'	6.92 (2H, d, $J=8.5$ Hz)	6.92 (2H, d, $J=8.7$ Hz)	6.92 (2H, d, $J=8.8$ Hz)	6.92 (2H, d, $J=8.7$ Hz)
4'	—	—	—	—
1''	4.77 (1H, br s)	4.75 (1H, d, $J=7.7$ Hz)	—	4.89 (1H, d, $J=7.4$ Hz)
2''	3.17—3.50 (O)	3.20—3.58 (O)	—	3.17—3.40 (O)
3''	3.17—3.50 (O)	3.20—3.58 (O)	—	3.17—3.40 (O)
4''	3.17—3.50 (O)	3.20—3.58 (O)	—	3.17—3.40 (O)
5''	3.56 (1H, d, $J=9.1$ Hz)	3.20—3.58 (O)	—	3.88 (1H, d, $J=9.7$ Hz)
6''	—	—	—	—
OH-5	13.32 (1H, s)	—	12.68 (1H, s)	—

a) Spectra were recorded on a Bruker AV-600 spectrometer, in $(\text{CD}_3)_2\text{SO}$. b) The proton signals were assigned on $^1\text{H-NMR}$, $^{13}\text{C-NMR}$, and NOESY. c) The proton signals were assigned on $^1\text{H-NMR}$, and NOESY. d) O, overlapped in water peaks.

are summarized in Table 1.

Metabolite M-7 (Scutellarein-7-*O*- β -D-glucuronide Scutellarin) Yellow amorphous powder, the $\lambda_{\max}$ of UV absorption is at 283, 335 nm, suggesting M-7 to be a flavonoid. It was characterized as parent drug of scutellarin by directly co-chromatography with an authentic sample.

Metabolite M-8 (Scutellarein-7-*O*-sulfate) Yellow amorphous powder, the $\lambda_{\max}$ of UV absorption is at 285, 333 nm, suggesting M-7 to be a flavonoid. In the negative ESI-MS² spectrum, the $[M-H]^-$ ion m/z 365 of M-8 eliminated 80 mass units (SO_3) to give ion m/z 285, suggesting it to be a sulfate conjugate of scutellarein. In the NOESY spectrum, the cross peak from H-3 (δ 6.80, 1H, s) to H-2',6' (δ 7.95, 2H, d, $J=8.8$ Hz) could be observed. In the ¹H-NMR spectrum, the signal of 5-OH (δ 12.68, 1H, s) existed, and H-8 was shifted downfield from δ 6.56 ppm (scutellarein) to δ 7.27 ppm (M-8) by 0.71 ppm, and meanwhile, in the ¹³C-NMR spectrum, compared with that of scutellarein, upfield shift of C-7 by 5.0 ppm, downfield shift of C-6 by 2.9 ppm, downfield shift of C-8 by 4.7 ppm, and downfield shift of C-10 by 2.2 ppm could be observed, suggesting that the ring A of the flavonoid skeleton was sulfated, according to the shift rules of flavonoid after conjugated with sulfate,¹⁷⁾ M-8 was determined to be scutellarein-7-*O*-sulfate. The full assignment of proton signals are summarized in Table 1.

Metabolite M-9 (Apigenin-5-*O*- β -D-glucuronide) Yellow amorphous powder, the $\lambda_{\max}$ of UV absorption is at 260, 333 nm, suggesting M-9 to be a flavonoid. Enzymatic hydrolysis of M-9 with β -glucuronidase gave the aglycone apigenin identified by the ESI-MS and HPLC behaviour with a standard sample. In the negative ESI-MS² spectrum, the $[M-H]^-$ ion m/z 445 of M-9 eliminated 176 mass units (glucuronic acid) to give ion m/z 269, suggesting M-9 to be an isomer of apigenin-7-*O*- β -D-glucuronide. In the ¹H-NMR, the signals of H-2',6' (δ 7.89, 2H, d, $J=8.7$ Hz) and H-3',5' (δ 6.92, 2H, d, $J=8.7$ Hz) indicated that ring B was substituted in position C-4', while the coupling constant of the signals of H-8 (δ 6.75, 1H, d, $J=2.2$ Hz) and H-6 (δ 6.69, 1H, d, $J=2.2$ Hz) suggested meta-substituted pattern of ring A. In the NOESY spectrum, the cross peak from H-3 (δ 6.67, 1H, s) to H-2',6' (δ 7.89, 2H, d, $J=8.7$ Hz) could be observed, and the correlation between the anomeric proton of glucuronide (δ 4.89, 1H, d, $J=7.4$ Hz) and H-6 (δ 6.69, 1H, d, $J=2.2$ Hz) indicated that 5-OH was conjugated with a glucuronic acid. From the above evidence, M-9 was identified as apigenin-5-*O*- β -D-glucuronide. The full assignment of proton signals are summarized in Table 1.

Metabolite M-10 (Apigenin-4'-*O*- β -D-glucuronide) Yellow amorphous powder, the $\lambda_{\max}$ of UV absorption is at 266, 337 nm, similar to that of apigenin-7-*O*- β -D-glucuronide. The negative ESI-MS² spectrum of M-10 was almost the same as that of M-9, suggesting that it was also an isomer of apigenin-7-*O*- β -D-glucuronide. When M-10 was treated with 1% $AlCl_3$ methanol solutions on the polyamide plate, it displayed strong yellow fluorescence under UV light at 254 or 365 nm, suggested that 5-OH existed with liberated form, and meanwhile, M-10 had different chromatographic behaviors with M-9 and apigenin-7-*O*- β -D-glucuronide after being co-chromatography with each other under identical HPLC conditions, consequently, M-10 was identified as apigenin-4'-*O*- β -D-glucuronide.

DISCUSSION

In recent years, considerable studies on the pharmacokinetics of breviscapine have been done, however, reports available on the metabolism of it *in vivo* after oral administration were limited to two published articles.^{9,18)} The current study was our effort to discover the metabolic information of breviscapine in rats after oral administration, and ten metabolites were obtained, which might be useful for discussing the metabolism and disposition of breviscapine *in vivo*. Due to the possible instability of the metabolites during the experimental process, phosphoric acid was employed. Xing J. *et al.* have reported that acidification could stabilize flavonoids containing *ortho*-phenolic groups, and the structures like this are prone to be oxidized.¹⁹⁾ The structures of these metabolites were elucidated by MS, NMR spectroscopy including ¹H-NMR, ¹³C-NMR, and NOESY, enzymatic hydrolysis and chemical methods. Among these metabolites, M-3 and M-4 were methylated ones, however, they might not be artifacts during the process of isolation and purification. According to our knowledge, the conclusion was supported by the article published previously,⁹⁾ in which they have discussed that the enzyme of methylation in the cell liquid of liver specifically metabolized *o*-hydroxyphenyl and they have discovered the two methylated products by the methods of HPLC-MS and HPLC-NMR.

Akao T. *et al.* have discussed that baicalin was transformed to its aglycone baicalein by intestinal bacteria and baicalein was absorbed intestinally and conjugated back to baicalin in the plasma after oral administration of baicalin to rats,²⁰⁾ and Gafner S. *et al.* presumed that other flavone-7-*O*-glucuronides were absorbed in a similar way as baicalin.¹⁵⁾ Scutellarin, which have similar structure to baicalin, was detected in rat urine after oral administration in our study, and therefore we thought scutellarin might be obtained as a metabolite in the similar way. On the basis of the metabolites profile, the possible metabolic pathways of breviscapine in rats are shown in Figs 4, 5. Plantagin, which had the same aglycone with scutellarin, was absorbed intestinally as the form of scutellarein, and due to its low content in breviscapine, we considered scutellarin had played a major role in the process of metabolism *in vivo* (Fig. 4). Few data have reported dehydroxylation of flavonoids *in vivo*, and thus, we thought M-9 and M-10 were converted by apigenin-7-*O*- β -D-glucuronide (Fig. 5). There existed P450 mediated hydroxylation metabolic reaction *in vivo*, however, it was demonstrated that glucuronidation of galangin was 30-fold more efficient than P450 mediated hydroxylation of galangin.²¹⁾ On the other hand, even though apigenin-7-*O*- β -D-glucuronide was hydroxylated to scutellarin *in vivo*, scutellarin derived from apigenin-7-*O*- β -D-glucuronide would be minor and could not be detected or obtained in the experimental condition due to the lower content of apigenin-7-*O*- β -D-glucuronide in breviscapine.

Comparisons of ESI-MSⁿ data and retention time in HPLC with synthesized standards are usually used to identify the structures of metabolites in recent years. However, when the standards are difficult to synthesize, for instance, flavonoids with three vicinal phenolic groups like scutellarein, the aglycone of scutellarin, is hardly to be sulfated, due to the instability of these aglycones in the reaction conditions,¹⁷⁾ some

metabolites' structures deduced only from LC/MSⁿ data may not be correct, especially in the case of the existence of isomeric metabolites. In our study, M-5, M-6, and M-7, M-9 and M-10 were isomers of different linkage sites of the glucuronic acid, which have identical data in ESI-MSⁿ. However, the linked positions of the glucuronic acid could hardly be determined only by mass spectra, as well as the assignments of the signals of H-3 and H-8 for the metabolites of scutellarin, and of H-3, H-6, and H-8 for apigenin-5-*O*- β -D-glucuronide, which could be determined by means of NMR technique such as NOESY. In these cases, preparation of metabolites and further identification based on NMR data must be done.

In summary, we have determined the definitive structures of ten metabolites derived from breviscapine by MS, NMR spectroscopy, enzymatic hydrolysis and chemical methods. Furthermore, M-8 and M-9 were new compounds. These results are important to understand the metabolic fate and disposition of breviscapine in rats, and would provide important information for studying its metabolism in humans.

REFERENCES

- 1) Zhang R. W., Zhang Y. L., Wang J. S., Lin Y. Y., Shang B., *Zhong-caoyao*, **19**, 199—201 (1988).
- 2) He W., Zeng F. D., *Zhongguolinchuangyaolixuezhazhi*, **18**, 458—461 (2002).
- 3) Zhang J., Li X. S., Zhang W. D., *Yaoxueshijianzhazhi*, **20**, 103—107 (2002).
- 4) Li X. L., Li Y. Q., Yan W. M., Li H. Y., Xu H., Zheng X. X., Guo D. W., Tang L. K., *Planta Med.*, **70**, 1039—1044 (2004).
- 5) Wei L., Tan J., *Chin. J. Integr. Med.*, **11**, 31—33 (2005).
- 6) Huang J. M., Weng W. Y., Huang X. B., Ji Y. H., Chen E., *Eur. J. Drug. Metab. Pharmacokinet.*, **30**, 165—170 (2005).
- 7) Li S. H., Jiang X. H., Yang Q., Jin Z. H., *Shengwuyixuegongcheng-xuezhazhi*, **20**, 692—694 (2003).
- 8) Ge Q. H., Zhou Z., Zhi X. J., Ma L. L., Chen X. H., *Zhongguoyiyao-gongyexazhi*, **34**, 618—620, 632 (2003).
- 9) Zhang J. L., Che Q. M., Li S. Z., Zhou T. H., *J. Asian Nat. Prod. Res.*, **5**, 249—256 (2003).
- 10) Zhang Y. J., Li L. Q., Yang P. Q., Zhang H., *Zhongcaoyao*, **29**, 798—800 (1998).
- 11) Tian J., Sun H. D., *Tianranchanwu Yanjiu Yu Kaifa*, **11**, 1—5 (1999).
- 12) Hase T., Ohtani K., Kasai R., Yamasaki K., Picheansoonthon C., *Phytochemistry*, **40**, 287—290 (1995).
- 13) Hattori M., Huang X. L., Che Q. M., Kawata Y., Tezuka Y., Kikuchi T., Namba T., *Phytochemistry*, **31**, 4001—4004 (1992).
- 14) Nakazawa T., Ohsawa K., *Biol. Pharm. Bull.*, **23**, 122—127 (2000).
- 15) Gafner S., Bergeron C., Batcha L. L., Reich J., Arnason J. T., Burdette J. E., Pezzuto J. M., Angerhofer C. K., *J. Nat. Prod.*, **66**, 535—537 (2003).
- 16) Markham K. R., Ternai B., Stanley R., Geiger H., Mabry T. J., *Tetrahedron*, **34**, 1389—1397 (1978).
- 17) Denis B., Ragai K. I., *Tetrahedron*, **43**, 5197—5202 (1987).
- 18) Chen X. Y., Cui L., Duan X. T., Ma B., Zhong D. F., *Drug Metab. Dispos.*, **34**, 1345—1352 (2006).
- 19) Xing J., Chen X. Y., Zhong D. F., *J. Pharm. Biomed. Anal.*, **39**, 593—600 (2005).
- 20) Akao T., Kawabata K., Yanagisawa E., Ishihara K., Mizuhara Y., Wakui Y., Sakashita Y., Kobashi K., *J. Pharm. Pharmacol.*, **52**, 1563—1568 (2000).
- 21) Zhang L., Lin G., Zuo Z., *Pharm. Res.*, **24**, 81—89 (2007).