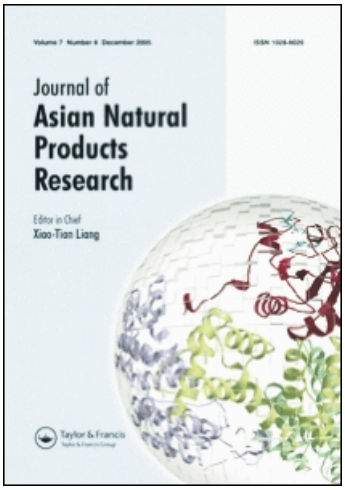

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Metabolites of scutellarein in rat plasma

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The metabolism of scutellarein was investigated in rats. Four metabolites (M1–M4) together with scutellarein were detected and identified as scutellarein-glucuronides in rat plasma by HPLC-DAD, HPLC-MS, and HPLC-MS/MS.

Keywords: Scutellarein; Metabolism; HPLC-DAD; HPLC-MS; HPLC-MS/MS

1. Introduction

Scutellarein is one of the major active constituents of *Erigeron breviscapus* (Vant.) Hand-Mazz [1], which shows the antithrombotic, antitumour and immunoregulatory effects [2]. Up to now, many studies on scutellarin, a glucuronide of scutellarein, have been reported. However, the investigations on the metabolism and pharmacokinetics of scutellarein are rare.

In a previous paper, we reported the metabolism of scutellarin in rats after oral administration. Four metabolites were identified from the bile of rats administrated, but scutellarin was not detected, which indicated that scutellarin may be not absorbed directly from the gastrointestinal tract of rats [3]. In the present paper, we report the structural elucidation of metabolites in rat plasma after oral administration of scutellarein. It is the first time the metabolism of scutellarein and the possible metabolic processing in rats has been reported.

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2. Results and discussion

2.1 Identification of the metabolites (M1, M2, M3 and M4) and scutellarein in rat plasma

The plasma was collected at 1 h after oral administration of scutellarein to rats. The prepared plasma samples were analysed by an established HPLC-DAD method. The chromatogram of HPLC-DAD is shown in figure 1. Four metabolites named as M1–M4 and scutellarein were detected in rat plasma by comparing with the blank samples and the parent drug (scutellarein). Comparing the HPLC profile, the UV spectra in HPLC-DAD chromatogram, and the HPLC-MS data with the authentic samples, the structures of M1–M4 were identified.

The retention time of M1 was at 3.66 min in HPLC-DAD chromatogram. The UV spectrum of M1 showed the absorption maxima at 283 and 334 nm, which exhibited the characteristics of flavone skeleton. The LC-MS spectra showed the pseudomolecular ion at *m/z* 461 $[M - H]^-$, which indicated that M1 was the monoglucuronide of scutellarein. M1 was identified as scutellarin by comparing the HPLC profile and UV spectrum with an authentic sample of scutellarin.

The UV spectrum of M2 revealed the absorption maxima at 266 and 337 nm, and the retention time of M2 was at 5.46 min in HPLC-DAD chromatogram. The HPLC-MS showed the pseudomolecular ion at *m/z* 461 $[M - H]^-$, and the HPLC-MS/MS revealed the fragment of aglycone at *m/z* 285 $[M - H - 176]^-$, indicating that M2 could be an isomer of scutellarin. The glucuronidation point of M2 should be on one of the 5, 6, 4′-hydroxyl groups of scutellarein. It has been reported that if the glycosidation occurs on 5- or 4′-hydroxyl group in flavone, the band I in the UV spectrum of the glycoside should move towards the short wavelength from 3 to 10 nm compared with the corresponding aglycone [4]. In the HPLC-DAD spectrum, the band I of scutellarein was at 336 nm, while that of M2 was at 337 nm which had almost not changed. Therefore, the glucuronidation should occur on 6-hydroxyl

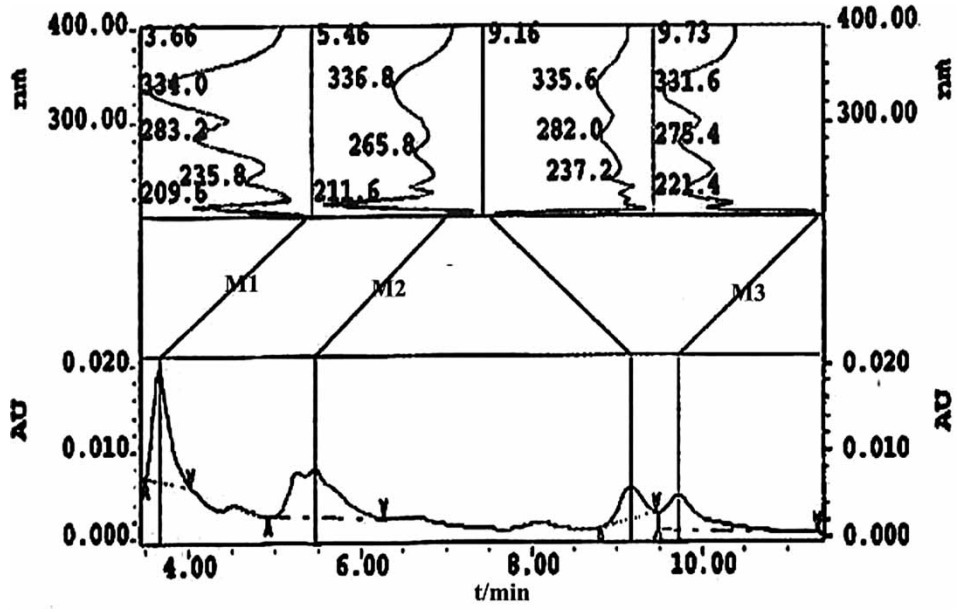


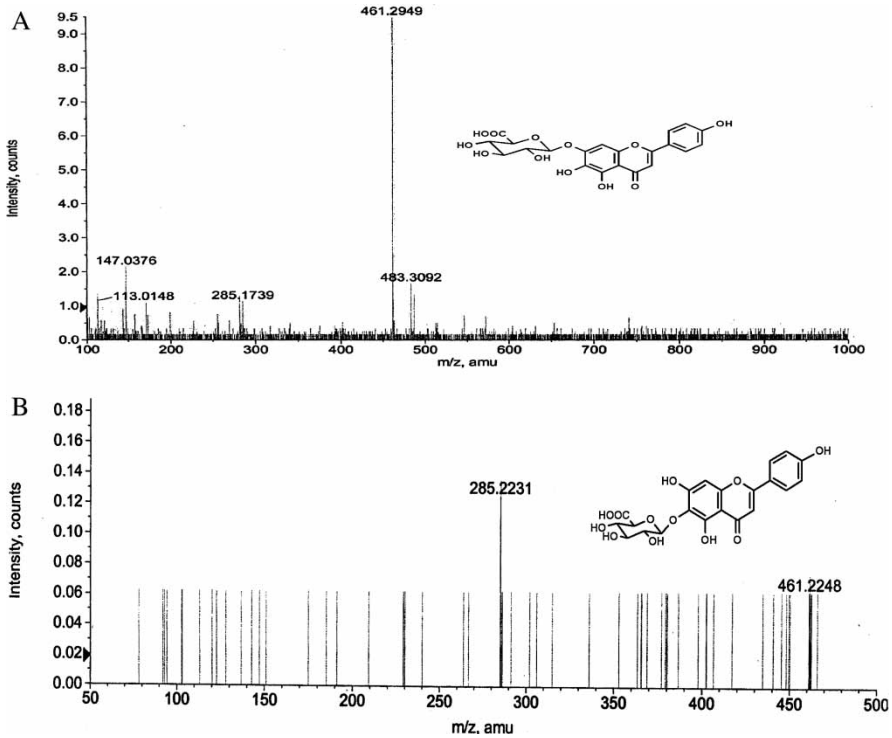
Figure 1. HPLC-DAD chromatogram of plasma sample after oral administration of scutellarein at 1 h.

group of scutellarein, and the retention time of M2 was longer than that of scutellarin (scutellarein-7-*O*-glucuronide) in HPLC chromatogram. Comparing the similar compounds baicalein-6-*O*-glucuronide and baicalein-7-*O*-glucuronide with the reference [5], the retention time of the 6-*O*-glucuronide is longer than that of the 7-*O*-glucuronide, which confirms the structural identification of M2. Furthermore, the UV absorption of M2 also agreed with reference [6].

One of the components which had the retention time at 9.16 min showed the UV absorption maxima at 282 and 336 nm. It was identified as scutellarein by comparing directly with an authentic sample.

The retention time of M3 was at 9.73 min in HPLC-DAD chromatogram. The LC-MS spectrum showed the pseudomolecular ion at *m*/*z* 475 $[M - H]^-$ and the fragment ion at *m*/*z* 461 $[M - H - 14]^-$, which indicated that a methyl group and a glucuronyl group were present in the structure of M3. The UV spectrum of M3 revealed the absorption maxima at 275 and 332 nm, and the data are the same as those of 6-*O*-methyl-scutellarin, which had been identified, by LC-MS and LC NMR in our previous paper [3].

In addition, a minor metabolite (M4) with the retention time at 7.68 min was detected in rat plasma by HPLC-MS (figure 2), which showed the pseudomolecular ion at *m*/*z* 461 $[M - H]^-$, and by HPLC-MS/MS which revealed the fragment of aglycone at *m*/*z* 285 $[M - H - 176]^-$, indicating that M4 could be a monoglucuronide of scutellarein on 4′- or 5-hydroxyl group. As we know, the 5-hydroxyl group can form a hydrogen bond with the carbonyl group in flavone, so it is difficult to conjugate with glucuronic acid. Thus, the more possible structure of M4 would be scutellarein-4′-*O*-glucuronide.



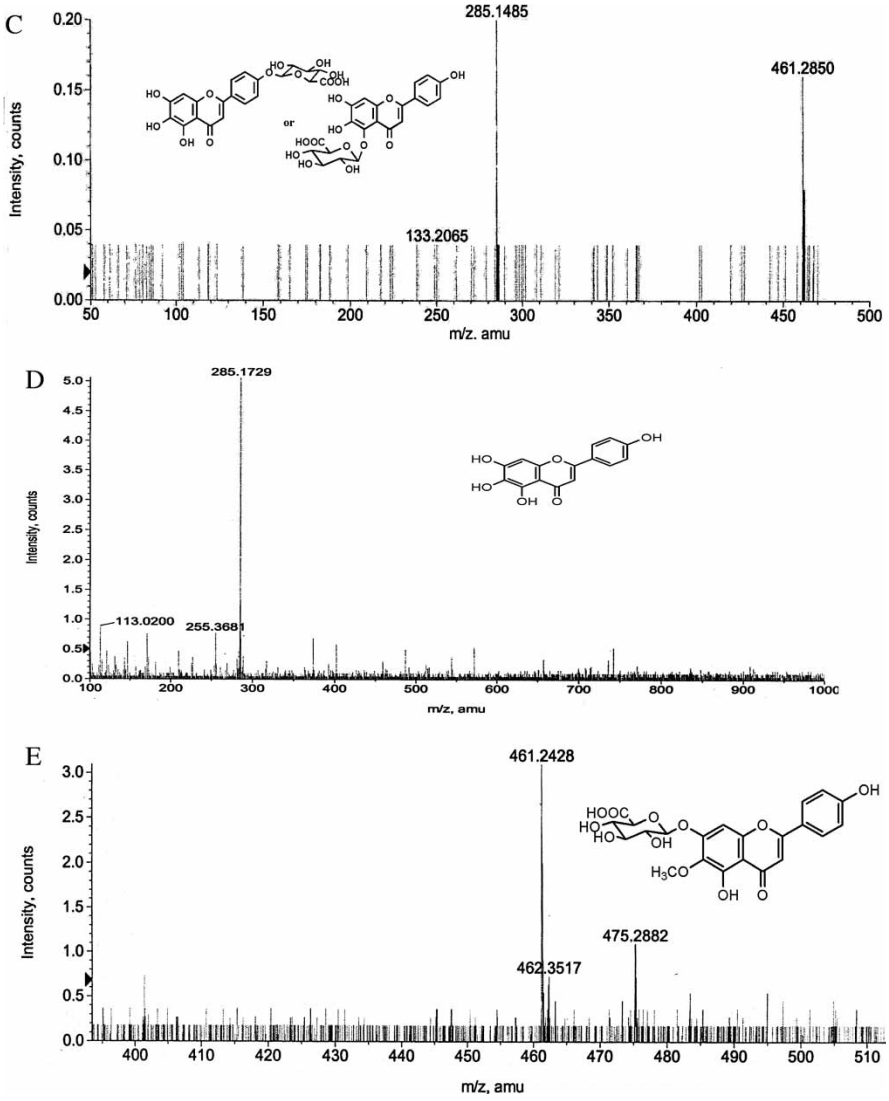


Figure 2. HPLC-MS chromatogram of the metabolites of scutellarein in rats. (a) The MS spectrum of M1. (b) The MS/MS spectrum of M2. (c) The MS/MS spectrum of M4. (d) The MS spectrum of scutellarein. (e) The MS spectrum of M3.

2.2 Metabolic pathways

After oral administration of scutellarein to rats, it was absorbed directly in the intestinal tract and was mainly metabolised to four glucuronides, scutellarein-7-*O*-glucuronide (scutellarin), scutellarein-6-*O*-glucuronide, 6-*O*-methyl-scutellarin, and scutellarein-4′-*O*-glucuronide (or scutellarein-5-*O*-glucuronide). Scutellarin (M1) is the major metabolite which was formed by the glucuronidation on 7-hydroxyl group of scutellarein by glucuronyl transferases in intestinal epidermal cells and in the liver. Then parts of M1 were methylated on the 6-hydroxyl group to form M3 by methyltransferases in the liver. At the same time, a few of scutellarein were glucuronidated by glucuronyl transferases on 6-hydroxyl group, and

4′-hydroxyl group or 5-hydroxyl group to form M2 and M4 in the liver, respectively. The possible metabolic pathway is shown in figure 3.

Scutellarin, which is an active component of breviscapus injection used for the treatment of acute stroke in China, is poorly absorbed in the rat gastrointestinal tract after oral administration [7]. In dogs, the absolute bioavailability of scutellarin for oral administration is (0.40 ± 0.19)%, while the clearance is very fast in the case of injection [8]. However, scutellarein can be directly absorbed in the rat gastrointestinal tract, and is mainly metabolised to its glucuronides in biological fluids including scutellarin. This kind of glucuronidation reaction usually occurs in the liver and intestine in the case of oral administration. The results indicate that scutellarein should be more effective than scutellarin due to the good absorption in the body for oral administration.

3. Experimental

3.1 General experimental procedures

A Waters TM 996 photodiode array detector (Waters, USA) equipped with a Waters 510 pump was used for HPLC-DAD analysis. An analytical column Kromasil C_{18} (Φ4.6 × 150 mm, 5 μm) was maintained at 40°C, the mobile phase was methanol/water/acetic acid (v/v/v) 35:61:4 with the flow rate at 1 ml/min. The detection was carried out at 335 nm with peak scanning from 200 nm to 400 nm, and the injection volume was 20 μl. A MDS SCIEX (AB, USA) QSTAR QqTOF tandem mass spectrometer equipped with a turbo ion spray interface and an Agilent 1100 HPLC series was used for HPLC-MS and HPLC-MS/MS analysis. The mobile phase was methanol/water (v/v) 35:65 for HPLC-MS, and methanol/water/amino acetic acid (v/v/v) 35:65:1 for LC-MS/MS. An analytical column Kromasil C_{18} (Φ4.6 × 150 mm, 5 μm) was used, the flow rate was 0.9 ml/min and the

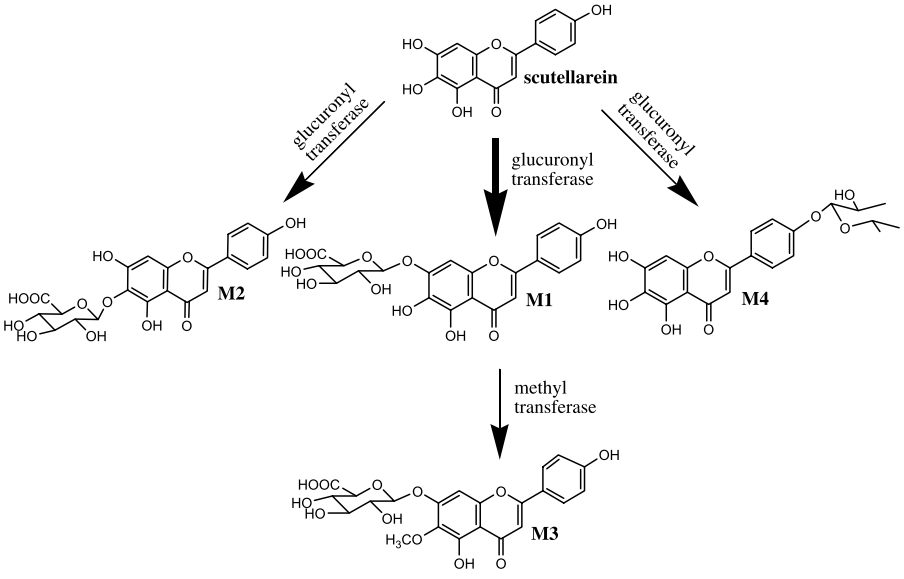


Figure 3. Possible metabolic pathway of scutellarein in rats.

injection volume was 20 μl. The LC-MS analysis was operated in the negative ion mode. The flow rates of nebulizer gas (nitrogen), curtain gas (nitrogen) and drying gas (nitrogen) were 50, 30, and 30 psi, respectively. A high interface temperature of 400°C was used, and the ion source was thermally stabilised for 30 min before injection. The data were processed by QS Analyst software.

3.2 Chemicals

Scutellarein was prepared from scutellarin by enzymatic hydrolysis, and was crystallised and recrystallised in methanol. The structure of scutellarein was identified by spectral methods, and the UV, MS, and NMR spectral data agreed with the reference [9].

Methanol was HPLC grade. Amino acetic acid, hydrochloric acid and sodium hydroxide were all of analytical grade.

3.3 Animals and biological samples preparation

Seven male SD rats (200–210 g) were purchased from Beijing VITAL, China and were fasted overnight with free access to water before receiving scutellarein orally at a dose of 500 mg/kg. The blood of rats was collected into heparinised tubes at 1 h after oral administration, and then was centrifuged at 5000 rpm for 15 min. The plasma sample was collected and mixed, and stored at −20°C until use. Before analysis, the frozen plasma sample was thawed and vortexed, and then double volumes of methanol and a little acetic acid was added. The mixture was vortexed for 10 min and centrifuged at 5000 rpm for 15 min, then the supernatant was transformed and concentrated to 0.5 ml. The plasma sample was filtered by a 0.22 μm filter membrane before HPLC analysis.

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