Comparison of the Metabolism of Baicalin in Rats Orally Administered with *Radix scutellariae* Extract and Shuang-Huang-Lian Extract

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Received March 27, 2005; accepted May 10, 2005

Shuang-Huang-Lian (SHL) is a traditional Chinese formula containing *Flos lonicerae*, *Radix scutellariae* (RS) and *Fructus forsythiae*, and is commonly used for treating acute upper respiratory tract infection, acute bronchitis and light pneumonia. The aim of the present study is to compare the metabolites of baicalin in rats when orally administered with SHL and *Radix scutellariae*, and try to explore the principle of SHL compatibility. By using LC-MS^n and HPLC-DAD, the metabolites of baicalin were analyzed from bile, urine and feces of rats dosed with SHL and RS. Our results showed significant difference of baicalin metabolism between RS and SHL. However, baicalein was found to be the main metabolites of baicalin in intestinal tract after oral administration of RS and SHL, glucuronide, glucoside and methylated products were also found in rat urine after administration of either RS or SHL. Meanwhile, several sulphates were found in rat urine after RS administration, but not found after SHL. Among the metabolites of the SHL, potentially there existed a isomerized baicalin and methylated product: 5,7-dihydroxy-6-methoxyisoflavone-7-*O*-*β*-glucopyranuronoside, but without unidentified metabolite M3. Baicalein-6-*O*-*β*-glucopyranuronoside-7-*O*-*β*-glucopyranuronoside and baicalein-6-*O*-*β*-glucose-7-*O*-*β*-glucopyranuronoside were first reported by this study. The major metabolites of baicalin of RS and SHL in rat bile were the same, including baicalin-6-*O*-*β*-glucopyranuronoside-7-*O*-*β*-glucopyranuronoside, baicalin-6-*β*-glucopyranuronoside and 6-*O*-methyl-baicalin-7-*O*-*β*-glucopyranuronoside. Moreover, baicalein-6-*O*-*β*-glucose-7-*O*-*β*-glucopyranuronoside was also first found in rat bile by this study. Although baicalin-6-*O*-sulfate-7-*O*-*β*-glucopyranuronoside was found in rat bile after RS administration, no sulphated products were found after oral administration of SHL. These differences of baicalin metabolism between RS and SHL indicated that compatibility of medicines could result in the differences of metabolites.

Key words *Radix scutellariae*; Shuang-Huang-Lian (SHL); metabolism; rat; LC/DAD/MS; baicalin

It is well known that the process of drug metabolism affect drug therapeutic and toxic effects. The change of the compatibility of traditional Chinese medicines may lead directly to the change of the metabolism of drug effectiveness in human body. Therefore, the investigation of the metabolism of compound Chinese recipes by modern analytical techniques may play important roles in explanation of the functional mechanisms and compatibility principles of compound Chinese prescriptions.[1)] Few studies were performed to investigate the influence of compatibility on metabolism of compound traditional Chinese prescriptions.

The compound Shuang-Huang-Lian (SHL) recipe is officially recorded in Pharmacopoeia of China,[2)] and has the efficiency of removing toxic heat and inducing diaphoresis. It is commonly used for treating acute upper respiratory tract infection, acute bronchitis and light pneumonia.[2)] SHL is composed of three herbs: *Flos lonicerae*, *Radix scutellariae* and *Fructus forsythiae*. Baicalin is the main active ingredient in *Radix scutellariae* (RS) but does not exist in *Flos lonicerae* and *Fructus forsythiae*. There are some investigations on the metabolites of baicalin. For example, *in vivo* metabolism in rat bile,[3)] in human urine[4)] after taking baicalin orally and in human plasma[5)] after intragastrically (i.g.) taking compound prescription Sho-Kaiko-To, *in vitro* metabolism by human intestinal flora,[6,7)] this study was designed to compare the metabolites of baicalin in rats after oral administration of SHL and single herb RS, and try to explore the principle of SHL compatibility.

Experimental

Materials *Radix scutellariae* (root of *Scutellaria baicalesis* GEORGI), *Flos lonicerae* (bud of *Lonicera japonica* THUNB.) and *Fructus forsythiae* (fruit of *Forsythia suspensa* (THUNB.) VAHL.) were purchased from a local herbal shop and were authenticated by Dr. F. Feng (Department of pharmacognosy, China Pharmaceutical University). Baicalin reference (Batch No. 715-200211) was obtained from National Institute for the Control of Pharmaceutical and Biological Products (NICPBP). *β*-Glucuronidase and sulfatase were purchased from Sigma Co. (U.S.A.). High performance liquid chromatography (HPLC)-grade acetonitrile and methanol, analytical reagent grade phosphoric acid, sodium dihydrogen phosphate, ethyl acetate, sodium hydroxide and glacial acetic acid were used for analysis. Double distilled water was produced in this laboratory.

Chromatographic Equipment and Conditions HPLC system (Agilent 1100) was equipped with a G1311A pump, a G1314A programmable diode array detector (DAD) and a G1313A auto-injector. A Hewlett Packard (HP) 1000 computer with in-house developed software was used for on-line data acquisition and subsequent calculations. The analytical column was packed with Lichrospher C_{18} (Kromasil, 250 mm×4.6 mm ID, 5 μm). The mobile phase was a gradient system with A: methanol : 0.7% acetic acid (35 : 65), B: C_2H_5OH. The gradient systems of qualitative analysis were A/B 100/0 (0→15 min)→A/B 0/100 (35 min)→A/B 0/100 (45 min). And the detector was set at 274 nm, the flow rate of mobile phase was 1.0 ml/min, and the experiment was performed at room temperature.

An Agilent LS ion trap mass spectrometer equipped with an electrospray ionization (ESI) source (San Jose, CA, U.S.A.) was used for mass analysis and detection. The operating parameters of the ion source were optimized to obtain the best performance from the mass spectrometry for the analysis of metabolites. The sensitivity of detection in negative ion mode was found to be much higher than that in positive ion mode. Therefore, instrumental parameters were selected that maximized generation of the molecule ($[M-H]^-$) of the test compound, and that also efficiently produced characteristic fragment ions. The MSD parameters consisted of the flow rate of the drying gas (nitrogen) flow, nebulizer (nitrogen) pressure, the drying gas temperature, and the spray voltage, with optimum values of 10 l/min, 35 psi, 350 °C and 3200 V, respectively.

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Crud Drug Preparation. Extract of *Radix scutellariae* *Radix scutellariae* was cut into slices, decocted with water for 3 times, 2 h for the first time, 1 h for the second and third time respectively, Combined decoctions were filtered, concentrated to a relative density of 1.05—1.10 at 80 °C. The pH of the solution was adjust to 1.0—2.0 with 2.0 mol/l hydrochloric acid at 80 °C. The temperature was maintained for 1 h and solution was then allowed to stand for 24 h. Precipitate from the filtered solution above was washed with water and pH was adjusted to 5.0. Then solution was further washed with 70% ethanol with pH adjusted to 7.0. The solution was evaporated in a cold vacuum to obtain reserved extract. The content of baicalin was determined to be 78.2% with HPLC.[2)]

Shuang-Huang-Lian Extract *Radix scutellariae*, *Flos lonicerae* and *Fructus forsythiae* were were mixed with 1 : 1 : 2 ratio. *Flos lonicerae* and *Fructus forsythiae* were macerated in warm water for 30 min, and decocted for 2 times with 1.5 h for each time. The filtrates from each decoction were combined and concentrated to a thick solution with a relative density of 1.20—1.25 at 70—80 °C. When the temperature dropped to 40 °C, ethanol was slowly added until ethanol accounted for 75% in the final solution. The well-mixed solution was allowed to stand for 12 h and then filtered to obtain the supernatant. The precipitate was stirred in 75% ethanol and allowed to

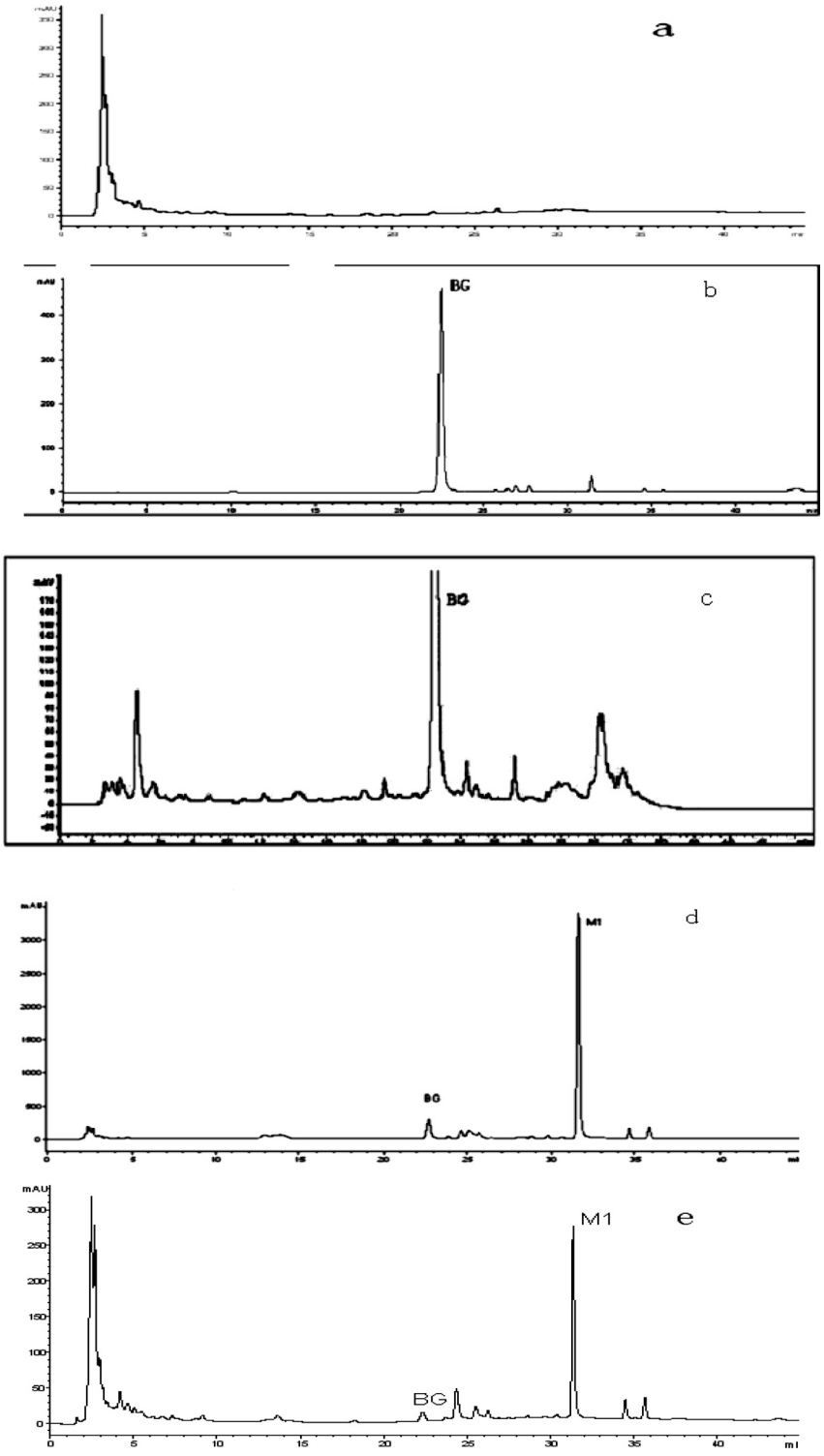

Fig. 1. Chromatograms of Baicalin and Its Metabolites in Rat Feces

(a) Blank feces; (b) RS; (c) SHL; (d) feces of rat after oral administration of SHL; (e) feces of rat after oral administration of RS. M1: baicalein; BG: baicalin.

set for 12 h. This solution was filtered again, combined with the supernatant above and concentrated with a relative density of 1.30—1.32 at 60—65 °C. The extract was evaporated in a cold vacuum and combined with extract of RS, as shown above.

Animals and Treatments Eighteen male Sprague–Dawley rats (200—250 g) were obtained from Experimental Animal Center of China Pharmaceutical University (Certificate No. 97024). They were divided into two groups randomly and given SHL extract and RS extract orally with a single dose of 400 mg/kg based on the content of baicalin after fast overnight. Feces, Urine and bile were collected before and after drug administration.

Sample Preparation One hundred milligrams dried and smashed feces was mixed with 2 ml methanol–water solution (45 : 55) in vortex, extracted by ultrasonication, and separated by centrifugation (12000 rpm) for 10 min. The supernatant was filtered and analyzed.

Urine and bile were separated by centrifugation (14000 rpm) for 10 min. The upper solution 20 μl was injected and analyzed by LC/DAD/MS.

Results and Discussions

The rat feces, urine and bile samples were analyzed by HPLC/DAD/MS. Multi-stage mass spectrometry using a combination of electrospray ionization with a quadruple ion trap in negative mode was applied to elucidate the structures of metabolites of baicalin in RS and SHL. Having obtained MS1, MS2 and MS3 data, the retention time, molecular weight and significant structural information were obtained from this analysis. Additional structural data were collected from subsequent LC/MS^n analysis designed to collect MS^n data for specific ions of interest. Chromatograms and MS spectra were illustrated in Figs. 1—3 and Table 1.

Metabolites of Baicalin in Rat Feces after i.g. RS and i.g. SHL Baicalin was hydrolyzed into baicalein by the rat intestinal bacteria after oral administration of both RS and SHL.

Metabolites of Baicalin in Rat Bile after i.g. SHL and i.g. RS The major metabolites were found in rat bile after oral administration and their chemical structures were identified primarily (Table 1).

Metabolites of Baicalin in Rat Bile after i.g. RS and i.g. SHL Baicalin, baicalin-6-*O*-β-glucopyranuronoside-7-*O*-β-glucopyranuronoside (M1), 6-*O*-methyl-baicalin-7-*O*-β-glucopyranuronoside (M4), baicalin-6-*O*-β-glucopyranuronoside (M5), baicalin-6-*O*-sulfate-7-*O*-β-glucopyranuronoside (M7) were found in rat bile after i.g. RS. This is in agreement with the previous study. Besides, the four metabolites reported by previous studies,[3—7] baicalein-6-*O*-

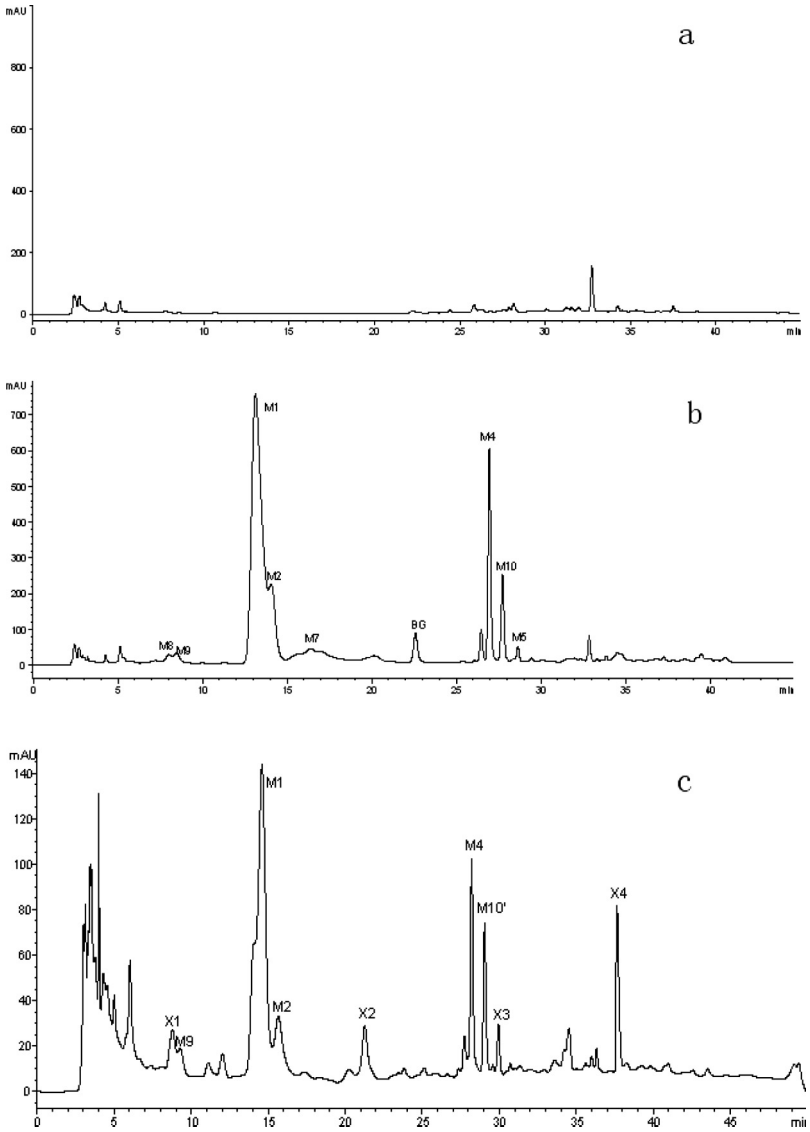

Fig. 2. HPLC Chromatograms of Baicalin and Its Metabolites in Rat Bile after Oral Administration of RS and SHL

(a) Blank rat bile; (b) rat bile after oral administration of RS; (c) rat bile after oral administration of SHL.

β-glucose-7-*O*-β-glucopyranuronoside (M2) and baicalin-5-*O*-β-glucopyranuronoside-7-*O*-β-glucopyranuronoside (M9) were also found by this study and never reported previously.

The major metabolites of baicalin, baicalin-6-*O*-β-glucopyranuronoside-7-*O*-β-glucopyranuronoside, baicalin-6-*O*-sulfate-7-*O*-β-glucopyranuronoside and 6-*O*-methyl-baicalin-7-*O*-β-glucopyranuronoside, were the same as those of RS after i.g. SHL, except that baicalin and baicalin-6-*O*-β-glucopyranuronoside were not found in rat bile. M5 and M7 were also not found. The present data indicated that metabolites, X1, X2, X3 and X4 were not the derivatives of baicalin.

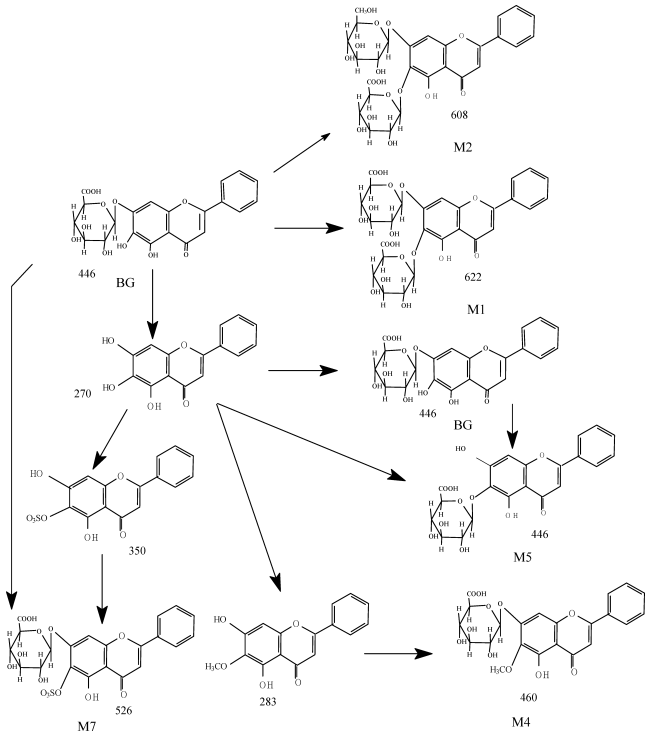

Fig. 3. The Proposed Metabolic Pathway of Baicalin in Rat Bile

According to the above analysis, the following metabolic pathways of baicalin in rat bile can be proposed (Fig. 3).

Metabolites of Baicalin in Rat Urine after i.g. RS and i.g. SHL Baicalin and its major metabolites baicalin-6-*O*-β-glucopyranuronoside-7-*O*-β-glucopyranuronoside (M1), baicalein-6-*O*-β-glucose-7-*O*-β-glucopyranuronoside (M2), 6-*O*-methyl-baicalin-7-*O*-β-glucopyranuronoside (M4), baicalin-6-*O*-β-glucopyranuronoside (M5), baicalin-7-*O*-sulfate (M11), baicalin-6-*O*-sulfate (M12), 6-*O*-methyl-baicalin-7-*O*-sulfate (M13) were found in rat urine after administration of both RS and SHL. However, a probable isomerized baicalin and methylated product, 5,7-dihydroxy-6-methoxyisoflavone-7-*O*-β-glucopyranuronoside (M5′), but no unidentified metabolite M3 was found in rat urine after administration of SHL but not after administration of RS.

The renal clearance rate of bacailin after RS administration was lower than that of bacailin after SHL administration, suggested that other drugs in compound Shuang-Huang-Lian promoted the metabolism of baicalin.

The metabolic pathways of baicalin in rat urine can be proposed as follows (Fig. 5).

Conclusion

In this study, an LC/DAD/MS experiment was performed on rat feces, urine and bile samples. Metabolites of baicalin of RS and SHL were characterized using multi-stage mass spectrometry in negative ion mode.

The results of the present study demonstrated that the major metabolites of baicalin or baicalein were glucuronide and sulphate after oral administration of the single herb RS, although a small amount of glucoside and methylated products were also found. No sulphated product was found after oral administration of the compound SHL prescription, although the main metabolites of baicalin and baicalein in rat

Table 1. UV and MS^n Spectra for Metabolites of Baicalin in Rats

Metabolite	UV λ_{max} (nm)	MS^1 *m/z*	MS^2 *m/z*	MS^3 *m/z*	Proposed structure
M1	272, 310	621 $[M-H]^-$	445 $[M-glucuronyl-H]^-$	269 $[M-glucuronyl-glucuronyl-H]^-$	Baicalin-6-*O*-β-glucopyranuronoside-7-*O*-β-glucopyranuronoside
M2	272, 312	607 $[M-H]^-$	431 $[M-glucuronyl-H]^-$	269 $[M-glucuronyl-glucosyl-H]^-$	Baicalein-6-*O*-β-glucose-7-*O*-β-glucopyranuronoside
M3	266, 320	268 $[M-H]^-$	249		Unidentified
M4	272, 312	459 $[M-H]^-$	283 $[M-glucuronyl-H]^-$	268 $[M-glucuronyl-CH_3-H]^-$	6-*O*-Methyl-baicalin-7-*O*-β-glucopyranuronoside
M5	270, 316	445 $[M-H]^-$	269 $[M-glucuronyl-H]^-$	251 $[M-glucuronyl-H_2O-H]^-$	Baicalin-6-*O*-β-glucopyranuronoside
M5′	274	459 $[M-H]^-$	283 $[M-glucuronyl-H]^-$	268 $[M-glucuronyl-CH_3-H]^-$	5,7-Dihydroxy-6-methoxyisoflavone-7-*O*-β-glucopyranuronoside
M6	276, 322	269 $[M-H]^-$			Baicalein
M7	272, 310	525 $[M-H]^-$	445 $[M-SO_3-H]^-$	269 $[M-glucuronyl-SO_3-H]^-$	Baicalin-6-*O*-sulfate-7-*O*-β-glucopyranuronoside
M8	270, 314	283.1 $[M-H]^-$	267.9 $[M-H_2O-H]^-$		Unidentified
M9	268, 312	621 $[M-H]^-$	445 $[M-glucuronyl-H]^-$	269 $[M-glucuronyl-glucuronyl-H]^-$	Baicalin-5-*O*-β-glucopyranuronoside-7-*O*-β-glucopyranuronoside
M10	274	363 $[M-H]^-$	283		Unidentified
M10′	274	459 $[M-H]^-$	283		Unidentified
M11	276, 316	349 $[M-H]^-$	269 $[M-SO_3-H]^-$		Baicalin-7-*O*-sulfate
M12	270, 312	349 $[M-H]^-$	269 $[M-SO_3-H]^-$		Baicalin-6-*O*-sulfate
M13	270, 310	363 $[M-H]$	283 $[M-SO_3-H]^-$	268 $[M-SO_3-CH_3-H]^-$	6-*O*-Methyl-baicalin-7-*O*-sulfate
BG	276, 316	445 $[M-H]^-$	269 $[M-glucuronyl-H]^-$		Baicalin
X1	262	258 $[M-H]^-$	218		Unidentified
X2	228, 278	547 $[M-H]^-$	175		Unidentified
X3	270, 316	513 $[M-H]^-$	559		Unidentified
X4	396	515 $[M-H]^-$	354	215	Unidentified

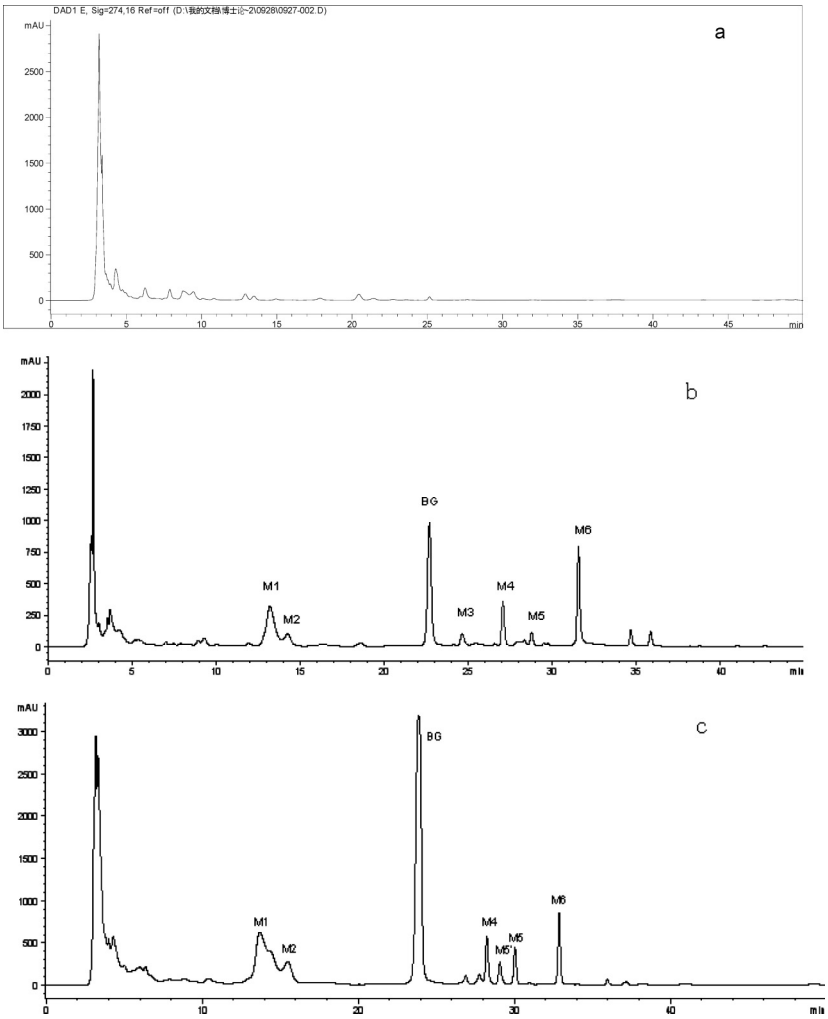

Fig. 4. Chromatograms of Baicalin and Its Metabolites in Rat Urine

(a) Blank rat urine; (b) urine sample of rat after oral administration of RS; (c) urine of rat after oral administration of SHL. BG: baicalin.

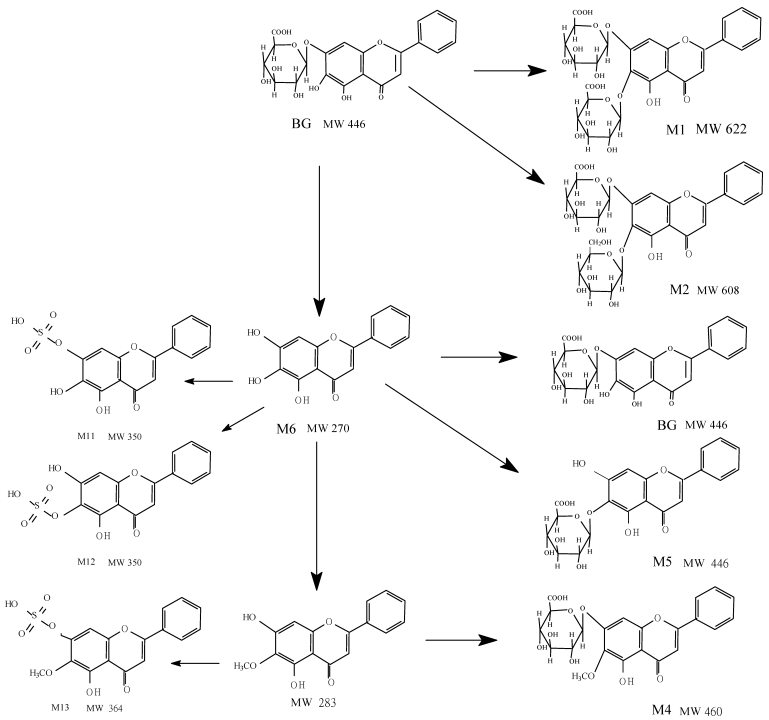

Fig. 5. The Proposed Metabolic Pathway of Baicalin in Rat Urine

bile were mainly the same.

Flavonoid glycosides were considered to be natural prodrugs.[7] Baicalin was transformed to its aglycone baicalein by the intestinal bacteria before absorption, and the metabolites of baicalin were more effective than the parental compound.[6,7] Our results suggested that other drugs in SHL promoted the metabolism of baicalin, therefore, it could be concluded that SHL might exert more effectiveness than RS.

With the data obtained from this study, it can be concluded that there were significant differences between the metabolites of baicalin after administration of SHL and those of baicalin after administration of RS. These differences indicated that compatibility of medicines could result in the differences of metabolites. This conclusion might give a help to explain the acting mechanism of traditional Chinese compound prescriptions.

Acknowledgement The present study is supported financially by "Shu Guang" project from China Shanghai Municipal Education Commission and Shanghai Education Development Foundation (No. 01SG51).

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