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Identification of the metabolites of Baicalin in rat urine by UPLC/ESI-TOF/MS coupled with MetaboLynx XS automated data analysis

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Abstracts:To illustrate the main biotransformation pathways of Baicalin *in vivo*, we elucidated the metabolic profile of Baicalin in rat's urine. The urine was collected from each animal within 12 h after administrating orally Baicalin and distilled water (100mg/kg), and analyzed by ultra-performance liquid chromatography/electrospray ionization time-of-flight mass spectrometry (UPLC/ESI-TOF-MS) at the positive ion mode scanning coupled with MetaboLynx XS (version4.1) automated data analysis method. A total of 26 metabolites were detected, 13 of which were identified. A fairly comprehensive metabolic pathway was proposed for Baicalin include dehydroxylation, methylation, hydroxylation and glucuronidation after deglycosylation. These results are important for understanding the material basis and clinical mechanism of Baicalin for drug discovery, design and clinical application.

Keywords: Baicalin; metabolites; UPLC/ESI-TOF/MS; MetaboLynx XS

Drug metabolism (biotransformation) can contribute significantly to the overall therapeutic and adverse effects of drugs. As part of any drug discovery activity, it is important to analyze the metabolic profile of the parent drug.Baicalin (Fig.1), formulated as 7-D-glucuronic acid-5,6-dihydroxy-flavone, is a major bioactive constituent of Scutellariae Radix (root of *Scutellaria Baicalensis* Georgi) which has been known to have a multitude of pharmacological properties, such as neuroprotective[1], anti-diogenic[2], anti-inflammatory[2] , anticancer [3]anti-bacterial, antioxidative[4], antiviral[5] and anti-HIV [6], *et al*. The metabolism of Baicalin has been preliminarily investigated. However, only a fewmetabolites including BBaicalein, oroxylin A, and their glucuronides were identified[7]. Thus, those works only partly understood the metabolic performance of Baicalin.Herein, ultra-performance liquid chromatography/electrospray ionization time-of-flight mass spectrometry (UPLC/ESI-TOF/MS) followed by multiple mass defect filtering was applied to analyze the common and uncommon metabolites of Baicalin in rat urine for the universal understanding of the metabolism of Baicalin.

Materials and methods

Standard of Baicalin was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Formic acid (HPLC grade) was purchase from DIKMA Technologies Inc. (Lake Forest, CA, USA). Acetonitrile (HPLC grade) was purchase from Fisher (USA). The distilled water was purchased from Watson's Food & Beverage Co., Ltd. (Guangzhou, China). Leucine enkephalin was purchased from Sigma-Aldrich (St. Louis, MO, USA).Male Wistar rats (220±20 g in weight) were kept in the breeding room with temperature (25℃), humidity (60 ± 5%) and under 12:12-h light-dark cycle conditions,divided into 2 groups

with 8 ones in each: the control and Baicalin groups. Each rat in the Baicalin group was administrated orally with the aqueous solution of Baicalin with the concentration of 100mg/kg. Each in the control group was administrated orally with the distilled water. The urine was collected from each animal over the course of 12 h after dosing. An aliquot of 5μL of supernatant was injected to UPLC/ESI-TOF-MS analyze. The ethical approval for the experiment was given by the Legislation on the Protection of Animals Used for Experiment Purposes (Directive 86/609/EEC).

Both the dosed data file and the control data file were input in the Accurate Mass Filter Tool for filtering. After processing, the output files were used to compare the differences between the dosed and control traces for searching metabolites of Baicalin (Table 1).

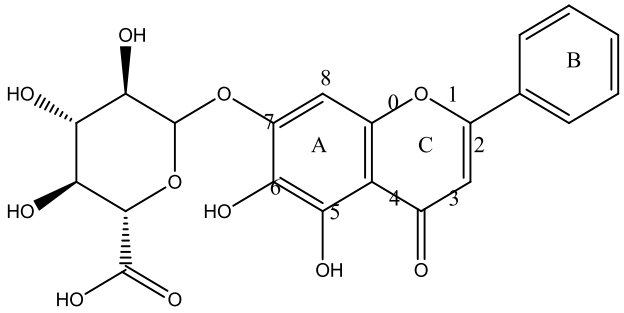

Figure 1. Structure of Baicalin.

Table 1. List of expected metabolites for which narrow window extracted ion chromatograms are generated in phase I and phase II of Baicalin

Mass(mDa)	Formula	Description	Phase
0	-	Parent	1
2.0157	$+H_2$	Reduction	2
14.0157	$+CH_2$	Methylation	1
15.9949	+O	Hydroxylation	2
30.0106	$+O+CH_2$	Hydroxylation+ Methylation	1
31.9898	$+O_2$	2×Hydroxylation	1
47.9847	$+O_3$	3×Hydroxylation	2
79.9568	$+SO_3$	Sulfate conjugation	2
95.9517	$+ SO_4$	Hydroxylation+ Sulfate	2
176.0321	$+C_6H_8O_6$	Glucuronide conjugation	2
192.027	$+ C_6H_8O_7$	Hydroxylation+ Glucuronide conjugation	2
352.0642	$+ C_{12}H_{16}O_{12}$	2×Glucuronide conjugation	2
-15.9949	-O	Dehydroxylation	1

Metabolite peaks were assigned by MS/MS analysis or interpreted with available biochemical databases, such as HMDB （http://www.hmdb.ca/）; MassBank（http://www.massbank.jp/）and the fragmentation patterns of flavonoid such as Retro-Diels-Alder(RDA)reaction(Fig. 1).

Results and discussion

Detection of urinary samples and screening of Baicalin metabolites

After pretreatment, rat urinary samples were detected by UPLC-TOF/MS in positive ESI modes to obtain the full-scan mass chromatograms with accurate mass measurement. The UPLC–ESI MS total ion current chromatograms in positive ion mode are shown in Fig. 2b.

Metabolite data from the UPLC-TOF MS system identified using Metabolynx XS in MS^2. TheMetaboLynx output browser was able to show the metabolites of Baicalin by using the modified MetaboLynx screening routine (Fig. 3).Considering the output browser report results may be pseudomorph, this study modified the metabolite name using MS^2(Table 2.).

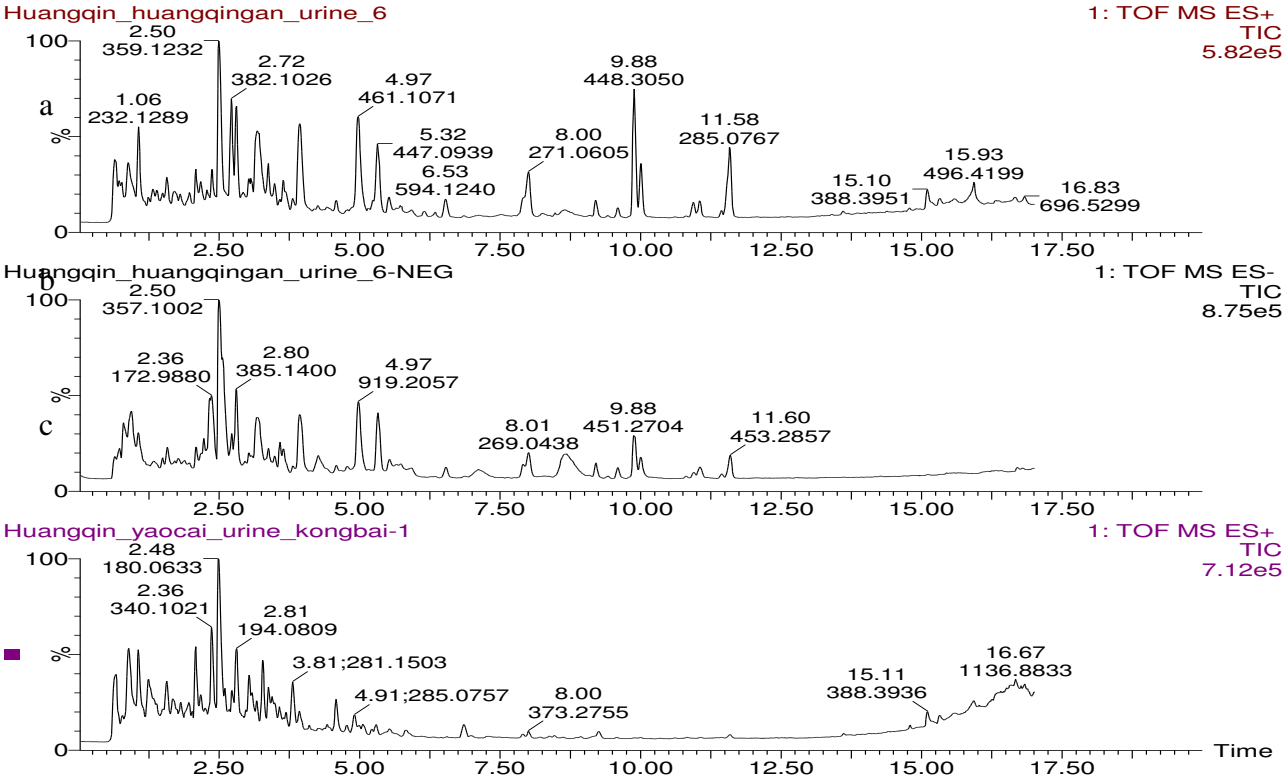

Figure 2. The total ion chromatograms of urine: (a) urine after oral administration of Baicalin in ESI+mode ; (b)urine after oral administration of Baicalin in ESI- mode and; (c) blank sample in ESI+mode.

Expected Metabolites - Huangqin_huangqingan_urine_2_MDF_50, parent C15H1005 (116/248 entries)

St...	Mass	Metabolite Name	Formula	m/z Found	mDa	PPM	Time	Area Abs
✓	254.0579	Dehydroxylation	C15H1004	255.0660	0.3	1.1	11.04	1709.50
✓	270.0528	Parent	C15H1005	271.0602	-0.4	-1.6	6.86	85.30
✓	270.0528	Parent	C15H1005	271.0611	0.5	1.8	7.97	139.40
✓	284.0685	Methylation	C16H1205	285.0766	0.3	1.1	10.94	3281.30
✓	284.0685	Methylation	C16H1205	285.0760	-0.3	-1.0	11.56	8518.50
✓	300.0634	Hydroxylation + methylation	C16H1206	301.0710	-0.2	-0.6	7.91	29.10
✓	314.0790	Methylation + Hydroxylation + methylation	C17H1406	315.0862	-0.6	-2.0	11.28	42.10
✓	334.0147	Sulfate conjugation + Dehydroxylation	C15H1007S	335.0221	-0.4	-1.3	8.64	133.20
✓	350.0096	Sulfate conjugation	C15H1008S	351.0173	-0.1	-0.4	8.81	142.50
✓	364.0253	Methylation + Sulfate conjugation	C16H1208S	365.0331	0.0	0.0	8.67	636.70
✓	430.0900	Glucuronide conjugation + Dehydroxylation	C21H18010	431.0955	-2.3	-5.3	4.55	418.10
✓	430.0900	Glucuronide conjugation + Dehydroxylation	C21H18010	431.0964	-1.4	-3.2	5.03	2776.70
✓	446.0849	Glucuronide conjugation	C21H18011	447.0932	0.5	1.1	3.93	3338.10
✓	446.0849	Glucuronide conjugation	C21H18011	447.0949	2.2	4.9	4.87	603.50
✓	446.0849	Glucuronide conjugation	C21H18011	447.0914	-1.3	-2.9	5.32	9565.30
✓	460.1006	Methylation + Glucuronide conjugation	C22H20011	461.1075	-0.9	-1.9	4.51	557.10
✓	460.1006	Methylation + Glucuronide conjugation	C22H20011	461.1058	-2.6	-5.6	4.97	12845.70
✓	460.1006	Methylation + Glucuronide conjugation	C22H20011	461.1059	-2.5	-5.4	5.51	2121.00
✓	476.0955	Methylation + Hydroxylation + glucuronide conjugation	C22H20012	477.1017	-1.6	-3.3	5.15	680.60
✓	526.0417	Sulfate conjugation + Glucuronide conjugation	C21H18014S	527.0482	-1.3	-2.5	5.68	180.00
✓	606.1221	2 x Glucuronide conjugation + Dehydroxylation	C27H26016	607.1298	-0.1	-0.1	2.93	329.20
✓	622.1170	2 x Glucuronide conjugation	C27H26017	623.1284	3.6	5.8	3.19	11252.40
✓	636.1326	Methylation + 2 x Glucuronide conjugation	C28H28017	637.1425	2.1	3.2	3.29	58.70
✓	638.1119	Hydroxylation + 2 x Glucuronide conjugation	C27H26018	639.1206	0.9	1.4	2.41	65.60
✓	652.1276	Hydroxylation + methylation + 2 x Glucuronide conjugation	C28H28018	653.1396	4.2	6.5	3.41	338.60
✓	798.1491	Glucuronide conjugation + 2 x Glucuronide conjugation	C33H34023	799.1611	4.2	5.3	2.72	56.50
✗	254.0579	Dehydroxylation	C15H1004	255.0749	9.2	36.2	1.00	186.30

Figure 3. Expected metabolites list showed by MetaboLynx output browser.

Table 2. Information of identified metabolites in ESI^+ mode

No	t_R (min)	M+H (m/z)	MS/MS (m/z)	Formula	Metabolite
1	4.57	447.09	103;168;271; 447	$C_{21}H_{18}O_{11}$	Baicalin
2	8.02	271.06	102;123;169; 211;253;271	$C_{15}H_{10}O_5$	Baicalein
3	11.09	255.06	103;129;153 237;255	$C_{15}H_{10}O_4$	chrysin
4	5.09	431.10	105;153;255 431	$C_{21}H_{18}O_{10}$	chrysin-7-glucuronide
5	10.99	285.08	105;168;179 252;270;285	$C_{16}H_{12}O_5$	Wogonin
6	11.62	285.08	105;168;267 270;285	$C_{16}H_{12}O_5$	Oroxylin-A
7	5.81	491.12	101;198;255 285;300;315 447;493	$C_{23}H_{22}O_{12}$	5, 7- dihydroxy-6, 8- dimethoxy flavone-7 - O - glucuronide
8	4.51	461.11	113;159;271 285;461	$C_{22}H_{20}O_{11}$	Oroxylin A-7-glucuronide (5, 7 - dihydroxy - 8 - methoxy flavone - 7 - O - glucuronide
9	4.99	461.11	113;159;175 270;285;461	$C_{22}H_{20}O_11$	Oroxylin A -5-O-glucuronide (5, 7 - dihydroxy - 6 - methoxy flavone - 5 - O - glucuronide
10	5.589	461.10	105;179;252 270;285	$C_{22}H_{20}O_{11}$	Wogonoside
11	4.86	477.10	101 ;177;285 301;477	$C_{22}H_{20}O_{12}$	5, 6, 7 - trihydroxy - 8 - methoxy flavone - 7 - O - glucuronide
12	3.40	653.14	103;301;371 477;653	$C_{28}H_{28}O_{18}$	5, 6,7 - trihydroxy - 8 - methoxy flavone - 6, 7 – di-O – glucuronide
13	3.188	623	159;177;271 447;623	$C_{27}H_{26}O_{17}$	Baicalein 6,7-di-O-glucuronide

UPLC/ESI-TOF/MS provides faster separation with increased resolution and sensitivity for analysis and elucidation of drug metabolism. The MetaboLynx XS (version4.1) software package requires minimal operator intervention and is capable of batch processing of samples, automated detection and identification of drug metabolites[8]. Therefore, they were combinated for rapid and sensitive analysis of the metabolites of Baicalin in this study. There are many isomerides of them because of the specific structure of flavonoids. Further research on the structures of metabolites is needed to separate the metabolites from biofluid and tissues (urine and liver, *e.g.*) and character the composition and molecular structures by color reactions and spectral analysis methods as FTIR, UV-Vis, MS, ^{1}H NMR and ^{13}C-NMR.

Conclusions

In summary,we detected 26 metabolites, 13 of which were identified in this study (Table 2). These results indicate that the major in vivo metabolic processes associated with Baicalin include dehydroxylation, methylation, hydroxylation and glucuronidation after deglycosylation. A fairly comprehensive metabolic pathway was proposed for Baicalin(Fig. 4). The results are important for

understanding the material basis and the clinical mechanism of Baicalin for drug discovery, design and clinical application.

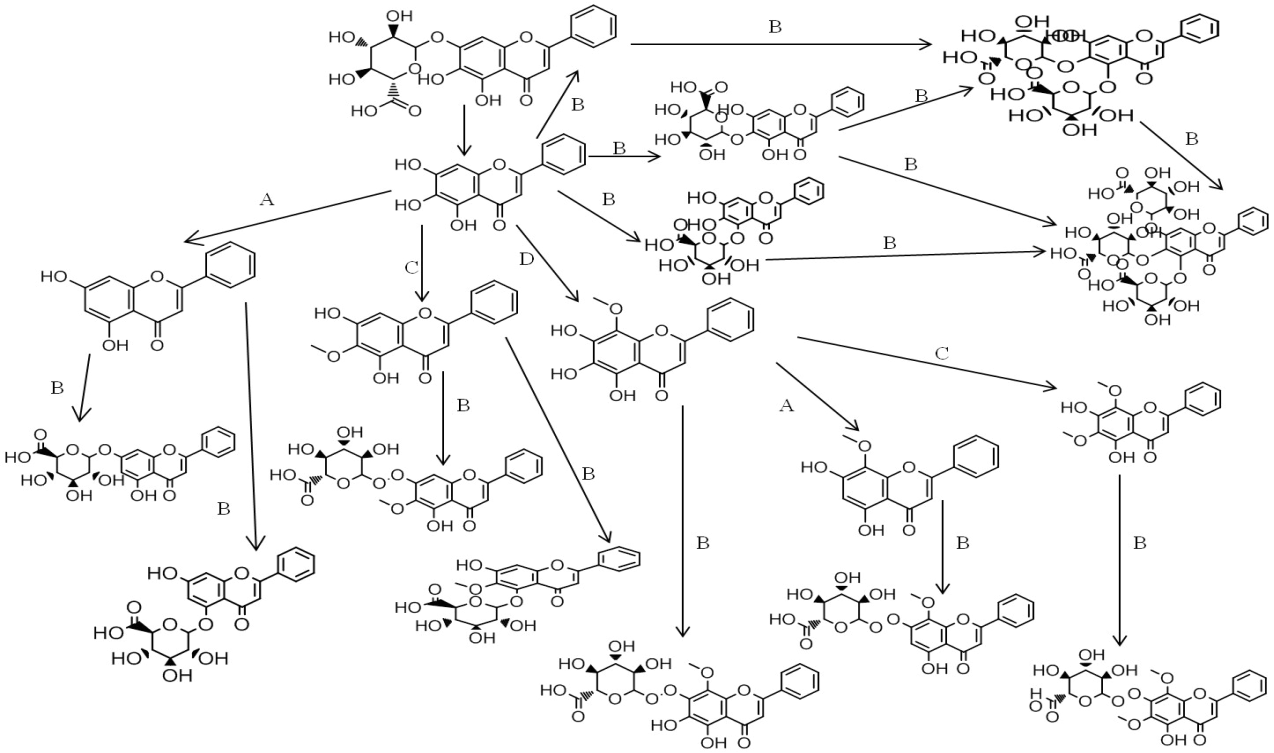

Figure 4. Potential metabolic biotransformation pathway and the metabolites of Baicalin. (A) dehydroxylation; (B) glucuronidation; (C) methylation; and (D) hydroxylation+ methylation.

Acknowledgements

This work was supported by a grant from the National Basic Research Program of China (Grant No. 2007CB512608 and 2013CB531804).

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The Observation on the Effect of Xuanbei Xiaocuo Soup Works on the 78 Cases of Phlegm Stagnation Type Acne

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Abstract

Goal: Observing the effect of Xuanbei Xiaocuo Soup on Phlegm Stagnation Type Acne (PSTA).**Method**: Dividing the78 patients of PSTA into 2 groups randomly, the 39 patients in the therapy group were treated with Xuanbei Xiaocuo Soup; and the other 39 ones in the control group were treated with Zhongjiefeng Fensan pills, and the observation on the two groups started after 30 days. **Result**: The therapy group's effective rate is 94.87%, and the control group's is 82.05%, the difference between the two groups is very obvious (P<0.05). **Conclusion**: Xuanbei Xiaocuo Soup is a very effective method for curing PSTA, which should be wildly clinical applied.

Key words: Xuanbei Xiaocuo Soup, Phlegm Stagnation Type, acne, curative effect.

Acne is usually occurred around the puberty, which is a type of pilosebaceous chronic inflammatory disease; this type of disease happens to the males more often, and happens to the females earlier, which serious symptom is forming hollow or hypertrophic scar, and its deep-seated inflammation can create massive abscess. The scar may remain even after the successful curing, the curing process is very long, which would strongly influence the life quality of patients. The writer of this article has been treated 62 cases with Xuanbei Xiaocuo Soup, and the curative effect is very satisfactory.

1.Materials and Methods

1.1 Common materials

All the cases are collected from the dermatology clinic of Heilongjiang University of Chinese Medicine's first affiliated hospital between January 2012 and March 2013, which are all matched with the diagnostic standard according to Clinical Dermatology(Ed., Zhao Bian) and Diagnostic and therapeutic effect evaluation criteria of diseases and syndromes in traditional Chinese medicine. All the patients are categorized into severe acne, which equals the third level of the forth level according to the Acne Pillsbury Classification, at the same time the patients, who are pregnant, breast feeding, having sensitive constitution, allergic to the related medicine, having systematic problems on cardio-vascular system, hemopoietic system, and immune deficiency disorder, are excluded. There are totally 78 cases, which include 55 male patients and 23 female patients, whose age are between 17 and 35 years old; their courses of disease are between 15 days and 6 years. The skin eruptions are majorly distributed on faces, which may spread to backs and chests. The clinical feature of skin rash is usually repeatedly presented as red or dark red protuberance, abscess, cyst, and cicatrix. The two groups do not have obvious difference in gender, age, course of disease, and the order of severity on skin eruptions (P＞0.05), which means they are comparable.

1.2 Treatment Methods

The control group would be treated with Zhongjiefeng Fensan Pill, each time 4 pills, three times daily, orally.

Enforcement Standards: The State Food and Drug Administration Standard

Drug standard number: YBZ07152006