Investigation on the Metabolism of Curcumin and Baicalein in Zebrafish by Liquid Chromatography-Tandem Mass Spectrometry Analysis

Shi-Jun Yin[a], Ya-Li Wang[a], Hua Chen[a], Guang Hu[b*], Guo-Can Zheng[c], Feng-Qing Yang[a*]

[a] *School of Chemistry and Chemical Engineering, Chongqing University, Chongqing 401331, China*

[b] *School of Pharmacy and Bioengineering, Chongqing University of Technology, Chongqing 400054, China*

[c] *Analytical and Testing Center, Chongqing University, Chongqing 401331, China*

Running title: Metabolisms of Curcumin and Baicalein in Zebrafish

*****Corresponding Author: Prof. Dr. Feng-Qing Yang, School of Chemistry and Chemical Engineering, Chongqing University, Chongqing 401331, China. Phone number: +8613617650637. E-mail: fengqingyang@cqu.edu.cn.

Dr. Guang Hu, School of Pharmacy and Bioengineering, Chongqing University of Technology, Chongqing 400054, China. E-mail: foxhu@cqut.edu.cn

ABSTRACT

Background: Curcumin (CUR) and baicalein (BAI) are the main active ingredients in *Scutellaria Baicalensis* and *Turmeric*, which were combined in *Jiang-Qin-Si-Wu* decoction to treat gynecological diseases.

Methods: Zebrafish embryos after hatching 48 hour were divided into four experiment groups. The blank group was exposed in 1 mL ultra-pure water. Three drug-treated groups were exposed in CUR (8 umoL/L, 1 mL), BAI (8 umoL/L, 1 mL), CUR and BAI (8 umoL/L, 2 mL), respectively. Then processed by homogenization and detected by liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. The relevant information about the metabolite was obtained in positive ion mode, and the structural information of the metabolites were determined based on its corresponding MS^2 spectra and the relevant literatures. According to the change of metabolite content, the metabolic effect of curcumin and baicalein was explored.

Result: Five and six metabolites of CUR and BAI in zebrafish were identified by LC-MS/MS, respectively. And metabolic pathways of CUR and BAI in zebrafish were glucuronidation, sulfation, methylation and reduction. In addition, CUR inhibited the metabolism of BAI in zebrafish and reduced the metabolites of glucuronidation.

Conclusion: Using LC-MS analysis, zebrafish is a feasible model for drug metabolism study. The results of metabolic study indicated that CUR might affect the therapeutic effect of BAI.

KEYWORDS: Zebrafish, LC-MS/MS, Curcumin, Baicalein, Metabolic pathways

1. INTRODUCTION

Due to its high homology with human genes, zebrafish has been widely used in the establishment of various disease models for high-throughput screening of drugs [1]. Modern studies have shown that zebrafish have phase I and II metabolizing enzymes and a gut microbiota similar to that of humans [2-3], which lays a foundation for zebrafish drug metabolism research. Compared with other animal model (such as mouse), zebrafish has the advantages of short reproductive cycle, high egg production, simple feeding, and low breeding cost [4]. The zebrafish has been used in the studies of the metabolisms of *Salvia miltiorrhiza*, ibuprofen, Epimedium, *Panax notoginseng* and chrysin [5]. For example, glucosylation and sulfation metabolites of chrysin in zebrafish were detected [6]. And the hydrolysate and glucuronidation metabolites and metabolic pathway of Epimedium in zebrafish were investigated [7].

Baicalein (BAI) is one of the most abundant bioactive flavonoids in the dry roots of *Astragalus membranaceus* [8]. The modern studies have shown that BAI has antibacterial, antiviral, antioxidant, antipyretic, analgesic, anti-tumor, anti-inflammatory, anti-ischemic reperfusion injury and neuroprotection functions [9-13]. The pharmacokinetic studies have shown that BAI can be rapidly and extensively metabolized in the intestines and livers of rats, and phase II metabolic reaction glucuronidation and sulfation are the main metabolic pathways of BAI [14]. On the other hand, curcumin (CUR) was a fat-soluble phenolic substance extracted from the rhizome of turmeric [15]. Modern studies showed that CUR had the effects of antioxidant, anti-inflammatory, anti-atherosclerosis, anti-tumor, anti-fibrosis and kidney protection [16-21]. The modern pharmacokinetic studies showed that the metabolites of CUR in rat were mainly in the form of reducing substances and glucuronic acid conjugates [22]. On the other hand, the combination of *Turmeric* and *Scutellaria Baicalensis* had been recorded in *Jiang-Qin –Si-Wu* decoction, which was recorded in *YiZongJinJian* and used for the treatment of gynecological disease. In clinic, they had also been used to treat bruises, swelling, pain and erysipelas in *Xianglian Jinhuang* ointment [23], but the rationality of the combined application of

Turmeric and *Scutellaria Baicalensis* had not been investigated yet. Therefore, in the present study, the metabolisms of CUR and BAI, which are the main active components in *Turmeric* and *Scutellaria Baicalensis*, in zebrafish embryos were investigated. After incubating, homogenizing and centrifuging, the HPLC-MS/MS was used to identify the metabolites of CUR and BAI in zebrafish. The changes of metabolites' contents were analyzed to predict the possible metabolic effects between CUR and BAI.

2. MATEREALS AND METHOD

2.1 Chemicals and reagents

CUR and BAI were purchased from Chengdu Purechem-Standard Co., Ltd. (Chengdu, China), the purities of the reference compounds were over 98% determined by HPLC analysis. DMSO was purchased from Chengdu KeLong CO., Ltd. (Chengdu, China). Acetonitrile (HPLC grade) and formic acid (HPLC grade) were obtained from Beijing InnoChem Science & Technology Co., Ltd. (Beijing, China). All of the experimental water was purified by a water purification system (ATSelem 1820A, Antesheng Environmental Protection Equipment Co., LTD., Chongqing, China). Buffers and samples were ultrasonicated in a KQ-100B ultrasonic cleaner (Kunshan ultrasonic instruments Co., Ltd, Kunshan, China) before use.

2.2 LC-MS/MS analysis

Shimadzu high-performance liquid chromatography/tandem mass spectrometry (LC-MS/MS) 8060 electrospray ionization-mass spectrometer (ESI-MS), consisting of a Triple Quadruple Detector (TSQ) as the mass detector (Shimadzu, Kyoto, Japan), coupled with a HPLC system was used for metabolites analysis. The mobile phase consisted of solvent A (0.1% formic acid aqueous solution) and solvent B (acetonitrile) using a gradient elution at a flow rate of 0.5 mL/min: 5% B at 0-2 min, 5%-18% B at 2-3 min, 18%-21% B at 3-5 min, 21%-28% B at 5-8 min, 28%-38% B at 8-13 min, 38%-60% B at 13-15 min, 60%-80% B at 15-17 min, 80%-60%B at 17-23 min, 60%-40% B at 23-26 min, 40%-5% B at 26-30 min. A 20 uL of prepared samples was

injected into an Agilent Zorbax SB-Aq column (250 × 4.6 mm, 5 μm) maintained at 30°C for separation.

The ESI-MS conditions were as follows: drying gas pressure, 100 MPa; curved desolvation line (CDL) voltage, constant level; interface voltage, 1.4 kV; nebulizing gas flow rate, 3 L/min; detector voltage, 1.40 kV; CDL temperature, 250°C; block heater temperature, 400°C; and IT vacuum, 1.9×10^{-2} Pa. Positive mass spectra were recorded in the full scan and automatic multiple stage fragmentation scan modes over a range of *m/z* 100-800 for all MS^1 and MS^2 spectra acquisition. The $[M+H]^+$ ion was selected as precursor ion and fragmented up to MS^2 stage. The ion accumulation time was set at 100 ms and the collision energy of CID was set at 50%. Data acquisition and processing were performed with the LC-MS solution version 1.1 software package (Shimadzu). The accuracy of the assigned chemical formula was determined using a mass difference tolerance of ± 5 ppm, which was calculated by the deviation between the experimental mass and calculated mass.

2.3 Biological sample preparation

The zebrafish were kept under laboratory conditions for 3 months before experiments. The culture and reproduction of zebrafish referred to Westerfield method [24]. Zebrafish embryos after hatching 48 hour were divided into four experiment groups of thirty each and placed in a 12-hole plate. The blank group was exposed in 1 mL ultra-pure water. Three drug-treated groups were exposed in CUR (8 umoL/L, 1 mL), BAI (8 umoL/L, 1 mL), CUR and BAI (8 umoL/L, 2 mL), respectively. All the above hatch water contained 0.05% DMSO.

After hatching 48 hour, the zebrafish larvae were sucked with a plastic head dropper, then using reverse osmosis water wash three times and homogenized, followed by centrifugation at 3200 × *g* for 15 min, then the supernatant was collected and 1 mL methanol was added, followed by centrifugation at 23120 × *g* for 15 min in order to remove protein and tissue pieces. The supernatant liquid was filtrated with 0.45 μm microporous membrane and analyzed by LC-MS/MS.

2.4 Statistical analysis

The results were presented as mean ± standard deviations (SD) of three different experiments. The statistical analysis was performed with SPSS (version 24, SPSS, Inc., Chicago, IL, USA).

3. RESULT AND DISSCUSSION

3.1 Metabolites of BAI in zebrafish

Comparing with the blank group, five metabolites (B1-B5) and parent component (B0) of BAI in zebrafish were detected in the LC-MS analysis. By referring to the related literatures [25-32], chemical structures of metabolites and parent component were tentatively identified. The MS data of metabolites were summarized in Table 1. Figure S3 and Figure S4 gave the MS and MS^2 spectra of metabolites, respectively. B0 produced the quasi-molecular $[M+H]^+$ ion at *m/z* 271, indicating that its molecular weight was 270. BAI was identified according to the fragment ions at 270, 253, 169, 151, 150, 123 and 103 in MS^2 spectrum, and the fragmentation pathway was shown in Figure S1. B1 gave a quasi-molecular $[M+H]^+$ ion at *m/z* 351, and its fragment ions at *m/z* 271 and 123 were the direct neutral loss of 100 Da and 228 Da, indicating that there was an sulfate group attached to BAI and the *m/z* 271 and 123 consistent with excimer ion and secondary fragments of BAI. Therefore, B1 was identified as 6-*O*-sulfate-glycoside baicalein or 7-*O*-sulfate-glycoside baicalein [26-32]. The MS spectrum of B2 reveals the quasi-molecular $[M+H]^+$ ion at *m/z* 285. In the MS^2 spectrum of B2, a direct neutral loss of 15 Da and 117 Da from *m/z* 285 gave the fragment ion at *m/z* 270 and *m/z* 168, indicating that B2 was a BAI combine with a methyl group. Therefore, B2 was identified as 6-methoxybaicalein or 7-methxybaicalein [32]. The MS spectrum of B3 showed the quasi-molecular $[M+H]^+$ at *m/z* 447. According to the corresponding MS^2 spectrum, the fragment ions of BAI at *m/z* 271, 270 and 253, which were neutral loss of 176 Da, 175 Da and 194 Da, indicating that there was a glucuronic acid group in conjunction with BAI and the *m/z* 271, 270 and 253 consistent with excimer ion and secondary fragments of BAI. Therefore, B3 was identified as baicalein 6-*O*-glucuronide or baicalein

7-*O*-gucuronide [25, 32]. B4 gave the quasi-molecular $[M+H]^+$ at *m/z* 461 and the fragment ions at *m/z* 285 and 270, which were neutral loss of 176 Da and 191Da, indicating that there was a methyl and a glucuronic group attached to BAI. Therefore, B4 was identified as 6-methoxybaicalein 7-*O*-glucuronide or 7-methoxybaicalein 6-*O*-glucuronide [25, 32]. B5 gave the quasi-molecular $[M+H]^+$ ion at *m/z* 623, and the fragment ions at *m/z* 271, which were the neutral loss of 352 Da, presumably B5 lost two glucuronic groups $[M+H\text{-}Glu\text{-}Glu]^+$, so B5 was identified as baicalein 6,7-di-*O*-glucuronide [25, 32]. B6 gave the quasi-molecular $[M+H]^+$ at *m/z* 609 and the fragment ion at *m/z* 271, which was neutral loss of 338 Da, indicating that B5 lost a glucuronic group and a glucose ester group. Therefore, B6 was identified as 6-methoxybaicalein 7-*O*-glucuronide [32].

Based on the identification of the metabolite structures, the metabolic pathway was presented in Figure 1. The metabolism of BAI in zebrafish was dominated by II phase metabolism. After the solution being absorbed, BAI was rapidly metabolized to monoglucuronide or monosulfate by the action of glucosyltransferase (UGT) or sulfuryltransferase (SULT). Then, part of the product continued to undergo methylation or glucuronidation and transformed into B4, B5 and B6, eventually.

3.2 Metabolites of CUR in zebrafish

Three metabolites (C1-C3) and the parent component (C0) were detected in the zebrafish homogenate after incubation with CUR solution. By referring to related literatures [33-38], the metabolites structures of CUR were tentatively identified by LC-MS analysis. Table 2 showed the MS data of metabolites. The MS and MS^2 spectra of metabolites and the parent compound were given in the Figure S5 and Figure S6. C0 was identified as CUR by the quasi-molecular $[M+H]^+$ ion at *m/z* 369 and the characteristic fragment ion at *m/z* 177 $[M+H\text{-}C_{11}H_{11}O_3]^+$ [37]. C1 was identified as hexahydrocurcumin based on its quasi-molecular ion $[M+H]^+$ at *m/z* 375, 6 Da higher than that of CUR, and its fragment ions at *m/z* 177 and 179 [33, 35-36, 39]. The metabolite C2 showed the quasi-molecular ion $[M+H]^+$ *m/z* at 545, which

was identified as curcumin glucoronate [33-36]. C3 gave the quasi-molecular at *m/z* 451, and the corresponding MS^2 spectra of C3 showed the fragment ions at *m/z* 369, which was the neutral loss of 82 Da, presumably C3 lost a sulfuric acid group and reduced a double bond [34]. C4 gave the quasi-molecular $[M+H]^+$ ion at *m/z* 449 and the characteristic fragment ion at *m/z* 369, indicating the conjugation of CUR with a sulfuric acid molecule, so it could be identified as curcumin sulfate [34]. C5 showed the quasi-molecular $[M+H]^+$ ion at *m/z* 551, 6 Da higher than that of C2, along with the fragment ion at *m/z* 368, it was identified as hexahydrocurcumin glucoronate [33-36]. Therefore, the CUR was metabolized by I phase (reduction) and II phase (glucuronidation and sulfation) enzyme in zebrafish. The metabolic pathway of CUR in zebrafish was shown in Figure 2.

3.3 Effect of CUR on the metabolism of BAI

In order to study the potential metabolic interaction between CUR and BAI, the zebrafish samples were processed and analyzed according to the method of 2.4, and the corresponding peak area of metabolites were shown in Table 3. The results indicated that CUR inhibits the metabolism of BAI in zebrafish and reduces the metabolites of glucuronidation, which was consistent with the research that CUR as a substrate as well as inhibitor of glucosyltransferase (UGTs) and sulfuryltransferase (SULTs) inhibited the metabolism of BAI in rats [39]. Furthermore, BAI and its metabolites suppresses gluconeogenesis through activation of AMPK or AKT in insulin resistant HepG-2 cell to treat diabetes. Therefore, when CUR was used in combination with BAI, it might affect the therapeutic effect of BAI.

CONCLUSION

In this study, the metabolites of CUR and BAI in zebrafish were analyzed by LC-MS. Eleven metabolites of CUR and BAI were tentatively identified. The metabolic pathways of CUR and BAI in zebrafish included glucuronidation, sulfation, methylation and reduction which was mainly generated through phase II metabolism. By detecting the relative content of metabolites, it was found that there may be a

metabolic mutual effect between CUR and BAI. However, further study is needed to verify that CUR affects the metabolism of BAI by affecting metabolic enzymes.

LIST OF ABBREVIATIONS

Abbreviations: CUR, curcumin; BAI, baicalein; LC-MS/MS, high-performance liquid chromatography/tandem mass spectrometry; CDL, curved desolvation line; TSQ, triple quadruple detector; SD, standard deviations; UGTs, glucosyltransferase; SULTs, sulfuryltransferase; TCM, traditional Chinese medicine.

CONFLICT OF INTEREST

The authors declared that they have not conflicts of interest.

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SUPPLEMENTARY MATERIAL

Fig. S1. The fragmentation pathway of BAI.
Fig. S2. Total ion chromatograms (TIC) of blank sample and drug samples.
Fig. S3. Total ion chromatogram and extracted ion chromatograms for BAI after exposure to zebrafish.
Fig. S4. MS2 product ions spectra of the protonated molecules of BAI.
Fig. S5. Total ion chromatogram and extracted ion chromatograms for CUR after exposure to zebrafish.
Fig. S6. MS2 product ions spectra of the protonated molecules of CUR.

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Table 1. MS data for BAI and metabolites after zebrafish exposure for 24 h.

Metabolite	Metabolic mode	Molecular weight (MW)	MS^1 $[M+H]^+$	t_R/min	Fragment Ions
B0	---	$C_{15}H_{10}O_5$	271	19.476	253[M+H-H_2O]; 169[M+H-C_8H_6]; 151[M+H-C_8H_6-H_2O]; 150[M+H-C_8H_6-H_2O-H]; 123[M+H-C_8H_6-H_2O-CO]; 103[M+H-$C_7H_4O_5$]
B1	Sulfation	$C_{15}H_{10}O_8S$	351	22.522	271[M+H-SO_3]; 123[M+H-SO_3-C_8H_6-H_2O-CO]
B2	Methylation	$C_{16}H_{12}O_5$	285	23.750	270[M+H-CH_3]; 168[M+H-CH_3-C_8H_6]
B3	Glucuronidation	$C_{21}H_{18}O_{11}$	447	19.518	285; 271[M+H-$C_6H_8O_6$]; 270[M+H-$C_6H_8O_6$-H]; 253[M+H-$C_6H_8O_6$-H_2O]
B4	Methylation, Glucuronidation	$C_{22}H_{20}O_{11}$	461	19.558	285[M+H-$C_6H_8O_6$]; 270[M+H-$C_6H_8O_6$-CH_3]
B5	Glucuronidation	$C_{27}H_{26}O_{17}$	623	17.953	271[M+H-$C_6H_8O_6$-$C_6H_8O_6$]
B6	Glucose, Glucuronidation	$C_{21}H_{18}O_{11}$	609	15.784	271[M+H-$C_6H_8O_6$-$C_6H_{10}O_5$]

Table 2. MS data for CUR and metabolites after zebrafish exposure for 24 h.

Metabolite	Metabolic mode	Molecular weight (MW)	MS^1 $[M+H]^+$	t_R/min	Fragment Ions
C0	---	$C_{21}H_{20}O_6$	369	21.700	177[M+H-$C_{11}H_{12}O_3$]; 161[M+H-H_2O-$C_{11}H_{10}O_3$]; 145[M+H-H_2O-$C_{11}H_{10}O_3$-CH_3-H]
C1	Reduction	$C_{21}H_{26}O_6$	375	21.259	368[M+H-6H-H]; 347; 179[M+H-4H-$C_{11}H_{12}O_3$]; 177[M+H-6H-$C_{11}H_{12}O_3$]
C2	Glucuronidation	$C_{27}H_{28}O_{12}$	545	22.319	369[M+H-$C_6H_8O_6$]
C3	Reductio, Sulfation	$C_{21}H_{22}O_9S$	451	26.567	369[M+H-2H-SO_3]; 285
C4	Sulfation	$C_{21}H_{20}O_9S$	449	22.083	369[M+H-SO_3]
C5	Reduction, Glucuronidation	$C_{27}H_{34}O_{12}$	551	22.056	368[M+H-6H-$C_6H_8O_6$-H]

Table 3. The metabolic mutual effect of CUR and BAI in zebrafish (n=3).

Metabolite	Single drug	Combination of two drugs	Trend	Change rate (%)
B0	2073211.7±1379719.0	1175406.3±327334.3	inhibition	-43.3%
B1	665836.0±614232.5	908340.3±1091195.0	---	36.4%
B3	14310933.3±8054235.0	10942849.7±5620696.0	inhibition	-23.5%
B4	625105.0±413676.5	310774.7±230985.1	inhibition	-50.3
B5	53686017.3±26994554.0	49629960.0±17501245.0	---	-7.6%
B6	3583257.0±4925647.0	2526842.7±3315633.0	---	-29.5%
C0	114393225.3±129492572.4	16370818.3±24063287.5	inhibition	-85.7%
C1	56391380.5±32714605.8	15093530.5±9368008.4	inhibition	-73.2%
C2	1575678.3±184732.8	363426.7±197732.7	---	-76.9%
C3	16309971.7±22356586.4	19017804.3±23937681.1	promotion	16.6%

Legend of Figures

Fig. 1. Proposed biotransformation pathways of BAI in zebrafish.

Fig. 2. Proposed biotransformation pathways of CUR in zebrafish.