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In vitro metabolism of BYZX in human liver microsomes and the structural elucidation of metabolite by liquid chromatography–mass spectrometry method

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Abstract

In vitro phase I metabolism of BYZX, a novel central-acting cholinesterase inhibitor for the treatment of the symptoms of Alzheimer's disease, was studied in human liver microsomes (HLM) and the metabolite formation pathways were investigated by chemical inhibition experiments and correlation analysis. The residual concentration of substrate and the metabolite formed in incubate were determined by HPLC method. The calibration curves of BYZX were linear over the concentration range from 5.07 μM to 200.74 μM. The relative standard deviations of within day and between day were less than 5% ($n = 5$). The limit of detection (LOD) was 0.18 μg/mL (S/N = 3) and the limit of quantification (LOQ) was 0.55 μg/mL (R.S.D. = 5.2%, $n = 5$). The determination recoveries of BYZX were in the range of 98.2–104.8%. The apparent K_m of BYZX in HLM was 53.25 ± 17.2 μM, the V_{max} was 0.94 ± 0.77 μM/min/mg protein, and the intrinsic clearance value (Cl_{int}) was 0.018 ± 0.02 mL/min/mg protein. Ketoconazole and cyclosporin A were the most potent inhibitors on BYZX metabolism in HLM with IC_{50} being 0.89 μM and 18.17 μM, respectively. And the inhibition constant (K_i) of ketoconazole was 0.42 μM. The metabolite of BYZX was *N*-des-ethyl-BYZX elucidated by LC–MS-MS. The results demonstrated that the developed HPLC method was reliability, simple technique, and was applicable to be used for the researches of in vitro metabolism of BYZX. CYP3A4 was the major isozyme responsible for BYZX metabolism; *N*-dealkylation was the major metabolic pathway of BYZX. The predominant metabolite of BYZX was *N*-des-ethyl-BYZX detected in vitro phase I metabolism in HLM.
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Keywords: BYZX; In vitro metabolism; HPLC–MS-MS; Human liver microsomes (HLM)

1. Introduction

Alzheimer's disease (AD) was one of the most severe health problems of the aged [1]. Acetylcholinesterase (AChE) inhibitors were the first and the most developed group of drugs approved for AD symptomatic treatment [2]. BYZX [(*E*)-2-(4-((diethylamino) methyl) benzylidene)-5, 6-dimethoxy-2, 3-dihydroinden-one, see Fig. 1] was one of a series of 2-phenoxy-indan-1-one derivatives tested as acetylcholinesterase inhibitors, synthesized by Sheng et al. [3]. It exhibited high AChE inhibitory activity ($IC_{50} = 50$ nM), and the molecular docking study indicated that it was nicely accommodated by AChE. Now many works had been done to develop it for clinical application, for example, studies of pharmacodynamics (BYZX 7.0 mg/kg (i.g.) had a significant effect on improving spatial learning and memory in mouses and toxicology (LD_{50} for mouses was 175 mg/kg).

The commercial success of a new chemical entity (NCE) depends on its pharmacological activity and several absorption, distribution, metabolism, and excretion (ADME) properties [4]. One important property of them is the ability of the NCE to cause metabolism-based pharmacokinetic drug–drug interactions. So it is necessary to investigate the in vitro metabolism of the NCE.

Liver microsomes were used extensively as an in vitro human drug-metabolizing system, and with appropriate selection of parameters, such as substrate and enzyme concentrations, may

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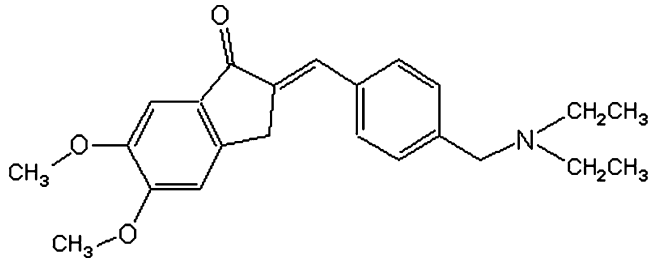

Fig. 1. Chemical structures of BYZX.

predict both routes and rate of metabolism. This system is useful for qualitative determination of metabolite and CYP reaction phenotype, to screen out compounds pre-clinically that may have undesirable drug–drug interactions [5]. In the present study, the in vitro metabolism of BYZX in human liver microsomes (HLM) was investigated, and the metabolite was analyzed by HPLC–MS-MS.

2. Materials and methods

2.1. Apparatus and reagent

BYZX, donepezil, and fluvoxamine were offered by Department of Pharmaceutical Chemistry (Zhejiang University, Hangzhou, China); cyclosporin A was purchased from National Institute for the Control of Pharmaceutical and Biological Product; omeprazole and sulfamethoxazole were obtained from Zhejian Jinhua Conba Bio-pharm. Co., Ltd.; 4-methylpyrazole, sulfaphenazole, quinidine sulfate, ketoconazole, α-naphthoflavone (α-NF), trinatric isocitric acid, isocitric dehydrogenase, β-nicotinamide adenine dinucleotide phosphate (NADP), and β-nicotinamide adenine dinucleotide phosphate, reduced form (NADPH), were purchased from Sigma (St. Louis, MO, USA); HPLC-grade acetonitrile was procured from TEDIA Company, Inc; HPLC-grade methanol was obtained from The Chemical Reagent Factory of ShangHai Ludu; triethylamine (TEA) was purchased from ShangHai Chemical Reagent Purchase Provision Wulian Chemical Industry; all other chemicals were of analytical grade and obtained from common commercial source.

Human liver samples from four cancer patients (males) were obtained from the Sir Run Run Shaw Hospital, Zhejiang, China. Informed consent was obtained from each patient prior to study entry. The present study was approved by an Ethics Committee of the Sir Run Run Shaw Hospital. All human liver used in the study were obtained from normal portions of removed tissue. All of the fresh samples were rapidly frozen in liquid nitrogen and stored at −80 °C before used.

All studies were performed on Jouan MR1822 automatic high-speed refrigerated centrifuge, Beckman coulter allegra™ 64R centrifuge, DKZ-2 Constant Temp Shaking water bath, Finnigan LCQ Deca XPplus ion trap mass spectrometer (Thermo Finnigan, San Jose, CA, USA) coupled with an Agilent 1100 series HPLC instrument via an ESI interface, Beckman coulter DU®640 Nucleic Acid and Protein Analyzer, Agilent1100 HPLC system consisted of a series G1311A pump, mode G1315A (DAD) UV detector, G1313A autosampler, and ChemStations software. Diamonsil®C_{18} column (25 cm × 4.6 mm i.d., 5 μm) (Cat. No.: 99903, Ser. No.: 8035838) was purchased from Dikma Technologies.

2.2. The chromatography conditions for HPLC and HPLC–MS-MS

2.2.1. The chromatography conditions for HPLC

A Diamonsil®C_{18} column (25 cm × 4.6 mm i.d., 5 μm) was used for the reversed-phase high-performance liquid chromatography (RP-HPLC) and the mobile phases were acetonitrile-10 mM potassium dihydrogen phosphate (KH_2PO_4) (0.8%TEA, pH = 4.0) (28:72, v/v), the autosampler temperatures were at room temperatures, total run time was 18 min, the flow rate was 0.8 mL/min. UV detection wavelength was set at 325 nm.

2.2.2. The chromatography conditions for HPLC–MS-MS

The separation was performed on a Diamonsil®C_{18} column (25 cm × 4.6 mm i.d., 5 μm) using the mobile phase that consisted of acetonitrile and 10 mM ammonium acetate (NH_4A_C) (pH = 4.0) (28:72, v/v) at a flow rate of 0.5 mL/min. The autosampler temperatures were also at room temperatures; total run time was 35 min. Mass spectra were recorded by electrospray ionization with a positive mode. The acquisition parameters for positive modes were: collision gas, ultrahigh-purity helium (He); nebulizing gas, high-purity nitrogen (N_2); ion spray voltage 4.5 kV; sheath gas (N_2) 30 arbitrary units; auxiliary gas (N_2) 10 arbitrary units; capillary temperature 350 °C; capillary voltage 19 V; tube lens offset voltage 25 V; mass range recorded *m/z* 50.00–750.00.

2.3. Preparation of human liver microsomes

Human liver microsomes were prepared by calcium precipitation method [6]. The microsomal preparations were stored at −20 °C until used. Protein concentrations were determined by the Lowry method [7], with bovine serum albumin as the standard.

2.4. Metabolism and kinetic analysis of BYZX in human liver microsomes

2.4.1. In vitro metabolism of BYZX

All incubations of BYZX were carried out at protein concentration corresponding to 0.8 mg/mL. The final volume of the incubation mixture was 200 μL, which consisted of 0.572 mg trinatric isocitric acid, 0.07 unit isocitric dehydrogenase, 0.015 mM magnesium chloride ($MgCl_2$) solutions, and 0.1 M (pH 7.4) tris(hydroxymethyl)amino-methane hydrochloride (Tris–HCl) solutions. The metabolic reaction was started by adding NADP and NADPH (0.9 mM/0.2 mM) after microsomal incubates were being prewarmed for 5 min, continued at 37 °C for several periods in a shaking water bath. In all experiments, BYZX was dissolved in tris(hydroxymethyl)aminomethane

hydrochloride-magnesium chloride (Tris–HCl–$MgCl_2$) solutions, and the final concentration of organic solvent did not exceed 0.5%. The reaction was terminated by add 200 μL ice acetonitrile [contained 20 μL of internal standard donepezil (the final concentration was 13.6 μg/mL)]. The contents were vortex-mixed for 1 min, and centrifuged at 13000 rpm for 10 min. An aliquot of 20 μL supernatant was injected into the RP-HPLC system.

2.4.2. Optimization of the incubation condition

Preliminary experiments were conducted to optimize the incubation conditions. The substrate concentration of BYZX was 10.14 μM, the final volume of the incubation mixture was 200 μL at protein concentrations 0.4, 0.6, 0.8, 1.0, and 1.2 mg/mL, and the reaction was terminated by the adding of 200 μL ice acetonitrile contained 20 μL internal standard donepezil (the final concentration was 13.6 μg/mL) after 0, 5, 10, and 20 min, respectively. The others were the same as in Section 2.4.1.

2.4.3. Determination of the kinetic parameter

Different amounts of BYZX were added to HLM, obtaining a series of BYZX incubates containing 5.07, 7.6, 10.14, 15.21, 20.27, 40.55, 60.82, 81.1, and 101.37 μM respectively (in duplicate), and the protein concentration was 0.8 mg/mL. The incubation was carried out according to the method described in Section 2.4.1 for 10 min, repeated with four different human liver microsomes.

2.5. The metabolism inhibition experiments of BYZX

2.5.1. General preliminary screening of inhibition

A series of typical CYP inhibitors, such as ketoconazole, cyclosporin A for 3A4, fluvoxamine for 3A4 and 1A2, α-naphthoflavone for 1A, quinidine sulfate for 2D6, omeprazole for 2C19, sulfamethoxazole, sulfaphenazole for 2C9, and 4-methylpyrazole for 2E1, were chosen and prepared in methanol solutions respectively. One microlitre of inhibitor solution was added to the reaction systems of HLM at a final concentration of 50 μM, and 1 μL of methanol was used in control experiments. BYZX concentration was 40 μM, equal to the apparent K_m for BYZX metabolism, in a final volume of 200 μL. The incubation was carried out as described in Section 2.4.1.

2.5.2. Determination of IC_{50} and K_i value

According to the preliminary screening, the inhibitors that had stronger inhibitive effect were chosen to determine the IC_{50} or K_i value. The inhibitors (cyclosporin A, fluvoxamine, quinidine sulfate, α-naphthoflavone, omeprazole) were added to the reaction systems of HLM, respectively, at a final concentration of 1, 20, 50, and 100 μM, and BYZX concentrations were equal to the apparent K_m (40 μM) in a final volume of 200 μL. The incubation was carried out as described in Section 2.4.1. And the inhibition was compared with that of inhibitor-free controls.

The IC_{50} was calculated as the linear regression between inhibition ratios (ν) (calculation with the metabolite peak areas) and the concentration of inhibitors [8], and chose the concentrations whose inhibition ratios get close to 50%. The following formula was used to calculate the IC_{50}.

$$IC_{50} = \frac{(50\% - R_l)(C_h - C_l)}{(R_h - R_l)} + C_1$$

where R_h is high inhibitive rate; R_l is low inhibitive rate; C_h is high concentration; and C_l is low concentration.

Ketoconazole was added to the reaction system of HLM at a final concentration of 0.05, 0.5, and 2 μM with the concentration of BYZX at a final concentration of 10, 20, and 40 μM. The incubation was carried as described in Section 2.4.1, and the K_i value was calculated as per the Dixon method [9].

The Dixon plot is frequently used for both identification of the likely mechanism of enzyme inhibition and for estimation of K_i. Plots were prepared of the reciprocal of rate of metabolite formation ($1/\nu$) versus inhibitor concentration at each substrate concentration. The equation governing this relationship is given below:

$$\frac{1}{\nu} = \frac{K_M[I]}{V_{max}[S]K_i} + \frac{1}{V_{max}}\left(1 + \frac{K_M}{[S]}\right)$$

The resulting straight lines were analyzed by linear regression. Estimates of K_i were obtained by simultaneously solving two sets of equations sequentially; one equation for each of the straight lines equated with the line that represented the highest substrate concentration (this line has the smallest slope). The point of intersection of these pairs of lines represents the value of K_i. The latter values are reported as means.

2.6. Statistical analysis

Student's *t*-test was used to assess the statistical significance of experimental data. Experimental data were considered significantly different from control values at $p < 0.05$.

3. Result and discussion

3.1. Establishment of RP-HPLC method for the determination of BYZX in HLM

3.1.1. Selection of the detection wavelength

The UV spectrum of BYZX in the mobile phase solution was determined by diode array detector (DAD). The 3D graph was showed in Fig. 2. The absorption peak of BYZX was found at 325 nm. So the detection wavelength for HPLC analysis was set at 325 nm.

3.1.2. The optimization of the chromatograph conditions

To obtain a better separation, various kinds of mobile phases had been tried, such as CH_3OH–0.1% acetic acid (triethylamine (TEA), pH = 4.0) mixed in different proportions, CH_3OH–0.1% acetic acid (45:55) with different pH, CH_3OH–10 mM diammonium hydrogen phosphate $\{(NH_4)_2HPO_4\}$ (pH = 7.5) mixed in different proportions, CH_3OH–10 mM $(NH_4)_2HPO_4$ (65:35) with different pH, CH_3OH–10 mM $(NH_4)_2HPO_4$-TEA (65:35) 0.2 (pH = 6.0), acetonitrile–10 mM KH_2PO_4–TEA (30:70:0.75) with differ-

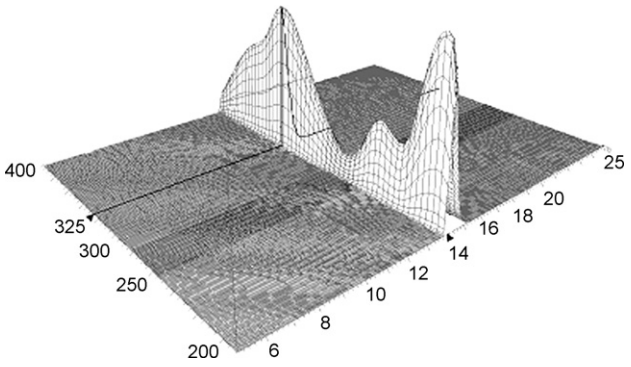

Fig. 2. The 3D graph of BYZX in mobile-phase solution.

ent pH, acetonitrile–10 mM KH_2PO_4–TEA (pH = 4.0) mixed in different proportions. It was found that the addition of TEA to the mobile phase would improve the separation and number of theoretical plate of BYZX. And the pH value of mobile phase was also an important factor on the separation between BYZX and other compound peeks. A good separation between BYZX and the metabolites, internal standard or other impurities was obtained under the mobile-phase condition of acetonitrile–10 mM KH_2PO_4–TEA (28:72:0.8, pH = 4.0). The chromatographic peaks were symmetrical, and no endogenous components interfered with the determination of BYZX in the mobile-phase condition. So, acetonitrile–10 mM KH_2PO_4–TEA (28:72:0.8, pH = 4.0) was used as the mobile phase for analysis of BYZX and metabolites in incubate.

3.1.3. Specificity of BYZX assay

BYZX, internal standard donepezil [10], and metabolite were separated at base line by using the chromatographic condition developed. There were no interfering peaks found at the same retention time of BYZX, metabolite, and internal standard in the chromatogram of the blank human liver microsomal incubate (Fig. 3).

In order to choose a suitable internal standard, a principle was taken into account that the architecture, physical property, and chemical property should be similar between the internal standard and the test compound. Thus, donepezil, a second filial generation drug for Alzheimer's disease, was selected as the internal standard, because it had the same parent architecture with BYZX.

3.1.4. Linearity range and sensitivity of BYZX assay

The calibration curve of BYZX was constructed by analyzing the inactive human liver microsomal incubates spiked with a series of concentrations of BYZX. The results exhibited excellent linearity with a correlation coefficient 0.9995 at the concentration range from 5.07 μM to 202.74 μM. The regression equation of the calibration curve based on the peak-area ratio (R) of BYZX and internal standard against the concentration (C) of BYZX spiked in the inactive human liver microsomal incubates was $R = 0.0444C + 0.003$.

The limit of detection (LOD) of BYZX was 0.18 μg/mL (signal-to-noise ratio 3) with step-by-step dilute method, and the limit of quantification (LOQ), defined as the lowest con-

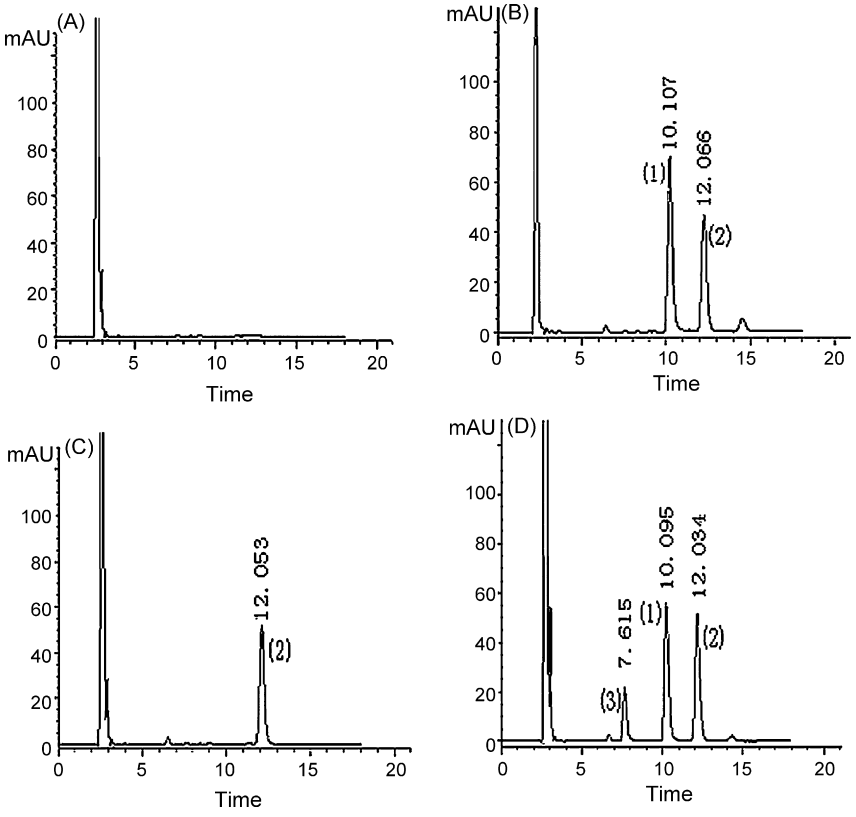

Fig. 3. HPLC chromatograms of BYZX in human liver microsomal incubate. (A) Blank microsomes; (B) blank microsomes spiked with BYZX and internal standard; (C) blank microsome spiked with internal standard; (D) incubated at 37 °C for 20 min in human liver microsomes. Peaks: (1), BYZX (40 μM); (2), donepezil (IS); (3), metabolite.

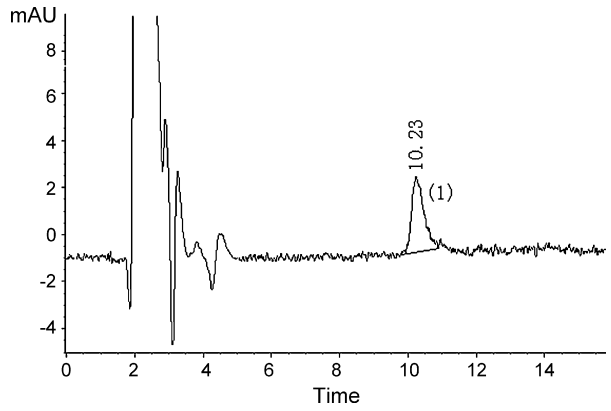

Fig. 4. The chromatogram of BYZX at LOQ.

Table 1
Precision and accuracy of BYZX in human liver microsomes ($n = 5$)

Spiked amount (μM)	Intra-day R.S.D. (%)	Inter-day R.S.D. (%)
7.60	1.3	4.3
15.21	1.9	2.6
81.10	1.7	2.5

centration of BYZX that could be quantitatively determined with suitable precision and accuracy, was 0.55 μg/mL (relative standard deviation (R.S.D.) = 5.2%, $n = 5$), respectively. The chromatogram of BYZX at LOQ was showed in Fig. 4.

3.1.5. Repeatability of BYZX assay

The intra- and inter-day precision of BYZX assay were obtained by analyzing the spiked samples ($n = 5$) at the concentrations of 7.60, 15.21, and 80.10 μM in inactive human liver microsomes (0.8 mg/mL protein concentration) within 1 day and in 3 consecutive days, respectively. The results were shown in Table 1.

3.1.6. Recovery of BYZX assay

Accuracy of BYZX assay was obtained by analyzing the spiked samples ($n = 5$) at the concentrations of 7.60, 15.21, and 80.10 μM in inactive human liver microsomes (0.8 mg/mL protein concentration). The method recoveries of BYZX at different concentrations were assessed from the regression equation obtained by standard curve method, with the average recovery

Table 2
Recoveries of BYZX in human liver microsomes ($n = 5$, mean ± S.D.)

Spiked amount (μM)	Measured amount (μM)	Method recoveries (%)	Extraction recoveries (%)
7.60	7.76 ± 0.07	102.11 ± 0.9	116.4 ± 4.96
15.21	15.16 ± 0.21	99.65 ± 1.4	107.4 ± 1.58
81.10	80.57 ± 0.88	99.35 ± 1.1	106.2 ± 3.67

100.37%. The extraction recoveries of BYZX were calculated by the areas of BYZX in HLM against that of BYZX in mobile-phase solution. The results were shown in Table 2.

3.1.7. Stability of BYZX solution

The stability of BYZX in Tris–HCl–$MgCl_2$ solution and inactive human liver microsomes was checked up. The contents of BYZX in above solutions, at the final concentrations of 8.11 and 81.10 μM, placed 0, 2, 4, 8, 12, and 24 h, respectively, under room temperature, were compared with the one at 0 h. The results indicated that BYZX was stable in Tris–HCl–$MgCl_2$ solution and inactive human liver microsomes within 24 h (R.S.D. < 5.0%).

3.2. Optimization of incubation conditions

The reaction curve and concentration curve for enzyme in BYZX metabolism by HLM were showed in Fig. 5. In the reaction curve, when the protein concentrations increase, bent degree of the curve becomes larger. The curve had better linearity under 1.0 mg/mL of protein concentration. In the concentration curve, the incubation time was inspected. The results showed that the reaction rate was linearity within 10 min. With the metabolic capability going up, an increasing supply of human liver microsomes concentration was needed. So, considering analytic sensitivity of metabolite, 0.8 mg/mL of higher HLM protein concentration and 10 min incubation time were chosen as the incubation conditions.

3.3. Kinetic parameters for BYZX metabolism

The apparent K_m and V_{max} values for BYZX metabolism were estimated by linear regression analysis of reciprocal of the enzyme activity ($1/V$) versus reciprocal of substrate concentration 1/[S] using the Lineweaver–Burk model:

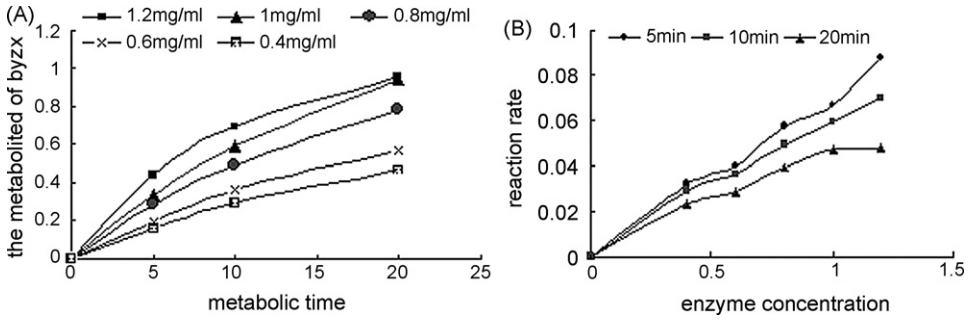

Fig. 5. The reaction curve and concentration curve for enzyme in BYZX metabolism by human liver microsomes. (A) The reaction curve for enzyme; (B) the concentration curve for enzyme.

Table 3
Enzymatic parameters of BYZX metabolism in four human liver microsomes

Human liver microsomes	K_m (μM)	V_{max} (μM/min/mg/protein)	Cl_{int} (mL/min/mg protein)
1	27.62	1.099	0.040
2	62.64	1.966	0.031
3	63.27	0.32	0.005
4	59.02	0.38	0.006
Mean ± S.D.	53.25 ± 17.2	0.94 ± 0.77	0.018 ± 0.02

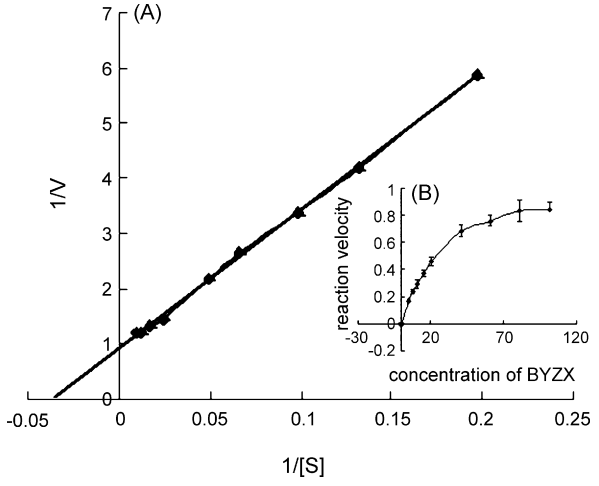

Fig. 6. Determination of enzyme kinetic parameters in BYZX metabolism by human liver microsomes. (A) The curve of reciprocal of substrate and reaction velocity; (B) the curve of the concentration of BYZX and reaction velocity.

$1/V = K_m/V_{max} \times (1/[S]) + 1/V_{max}$. Intrinsic clearance of the in vitro incubation was calculated as $Cl_{int} = V_{max}/K_m$, where V_{max} is the maximum velocity of each enzyme and K_m is the substrate concentration at which the reaction velocity is 50% of V_{max}.

The average apparent K_m of BYZX was 53.25 ± 17.2 μM, V_{max} was 0.94 ± 0.77 μM/min/mg protein, the intrinsic clearance value (Cl_{int}) was 0.018 ± 0.02 mL/min/mg protein, calculated from four HLM (Table 3; Fig. 6).

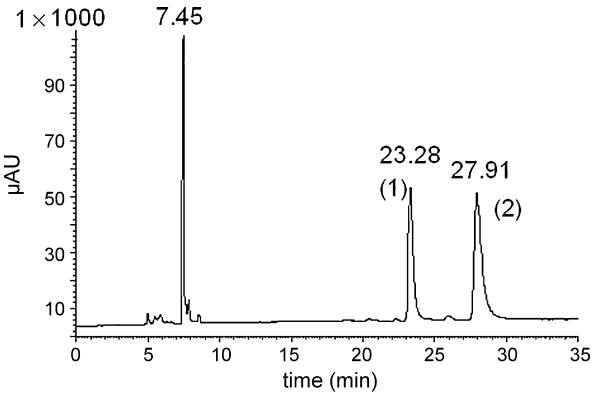

Fig. 7. Chromatograms of BYZX and its metabolite in HPLC–MS (1) metabolite; (2) BYZX.

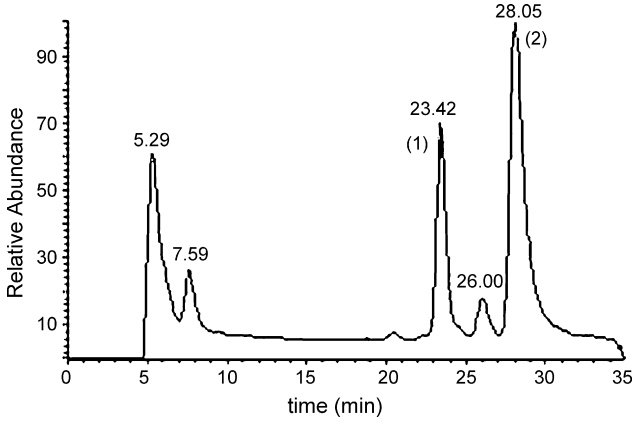

Fig. 8. Total ion–current spectrum of BYZX and its metabolite in *m/z* 50–750 hol-scanning total ion–current. (1) Metabolite; (2) BYZX.

3.4. Metabolite identification

The identity of metabolites was confirmed comparison with the retention times and product-ion mass spectra. The HPLC–MS-MS results were showed in Figs. 6–9.

Binding the chromatograms of BYZX (Fig. 7), the peak 1 (23.42 min) in the total ion–current spectrum (Fig. 8) was the ion–current of metabolite and the peak 2 (28.05 min) was that of the substrate BYZX. In order to further elucidate whether the

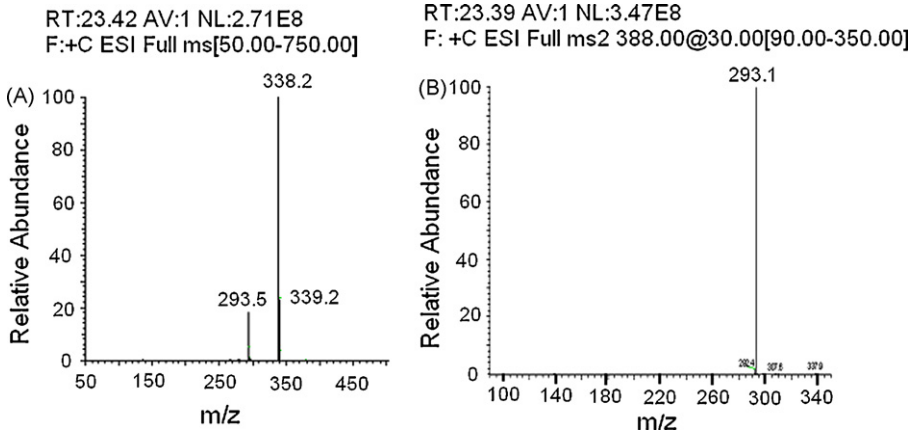

Fig. 9. Mass spectrum of metabolite in human liver microsomes. (A) HPLC-M; (B) HPLC–MS-MS.

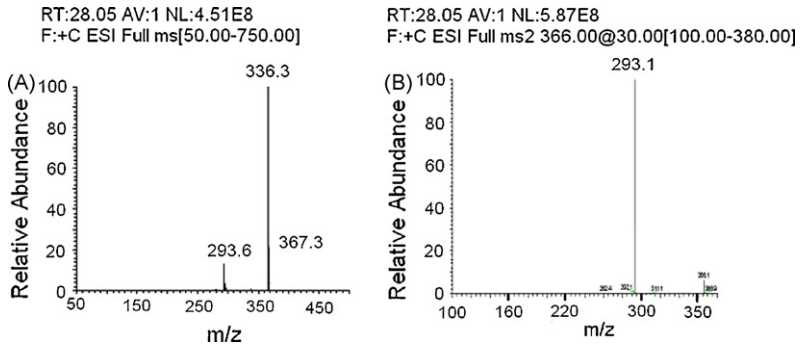

Fig. 10. Mass spectrum of BYZX in human liver microsomes. (A) HPLC-ME, (B) HPLC–MS-MS.

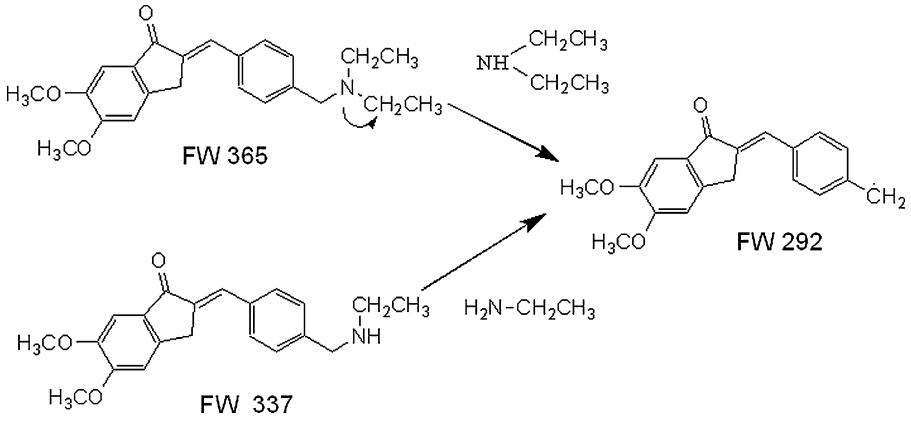

Fig. 11. The process of mass spectra breakage collision by HPLC–MS-MS.

first peak (retention time 23 min) was the metabolite, continued to collide the two dominant fragments (Figs. 9A and 10A), and got the same new fragments (*m*/*z* 293) (Figs. 9B and 10B). From the results, it can easily be inferred that the compound 1 (23.28 min) and 2 (27.91 min) had the same parent nucleus (Fig. 11); the compound 1 (23.28 min) was the metabolite of BYZX.

Figs. 3 and 7 were the chromatograms got in different mobile-phase systems. Calculating the relative retention times (RRT) of metabolite and BYZX, and comparing them between the two systems, the two values were essentially equal (0.8 in Fig. 3 and 0.8 in Fig. 7). The results indicated that the mobile-phase system for HPLC–MS-MS can also be used for the metabolite analyses. Furthermore, it can also support the above results that the compound 1 (23.28 min) was the metabolite of BYZX.

The mass spectrum of BYZX in the positive ion mode shows the protonated molecular ion of parent drug was at *m*/*z* 366 and the molecular mass of metabolite was at *m*/*z* 338, which indicated that the *m*/*z* decreased by 28 as compared to the parent drug, it is just for ethylene. As many papers reported [11–14] that ethyl was easily lost in phase I metabolism, so the fragment ion *m*/*z* 337 resulted from the loss of ethylene (*N*-desethylation) from BYZX. The procedure was showed in Fig. 12. After the phase I metabolism, an ethyl was removed from BYZX, forming the metabolite (*N*-desethylBYZX).

3.5. Inhibitive effect of various CYP inhibitors on BYZX metabolism

The effect of various CYP inhibitors on the metabolite formation of BYZX in human liver microsomes was shown in Fig. 13. Under the conditions of inhibitor concentration as 50 μM, if the inhibition ratio was less than 20%, it had no inhibitive effects; if the inhibitive ratio was at the range of 20–50%, it had more or less inhibitive effects; if the inhibitive ratio was more than 50%, it had strong inhibitive effects [15]. In Fig. 12, the inhibitive effects of sulfamethoxazole, sulfaphona-

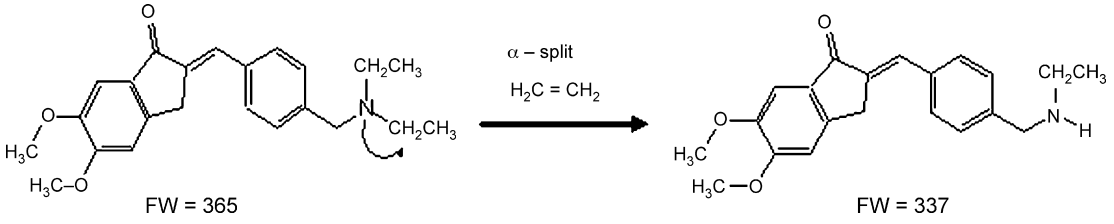

Fig. 12. The process of BYZX metabolism in human liver microsomes.

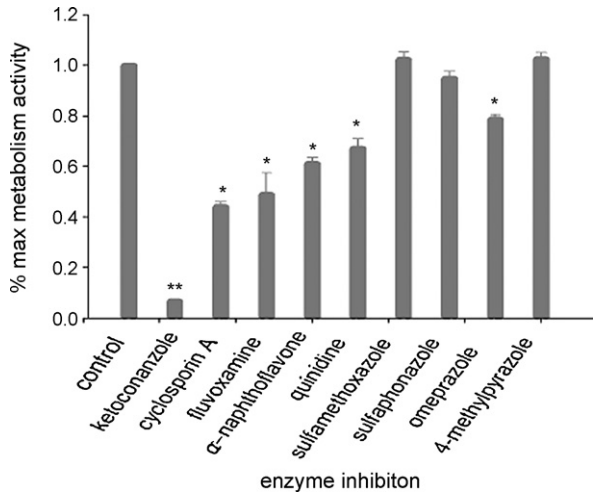

Fig. 13. Effect of various cytochrome P450 inhibitors on the rate of formation of metabolite at a 40.55 μM concentration of BYZX in human liver microsomes. The concentration of inhibitors was 50 μM in the preliminary experiment; (**) $P < 0.01$; (*) $P < 0.05$.

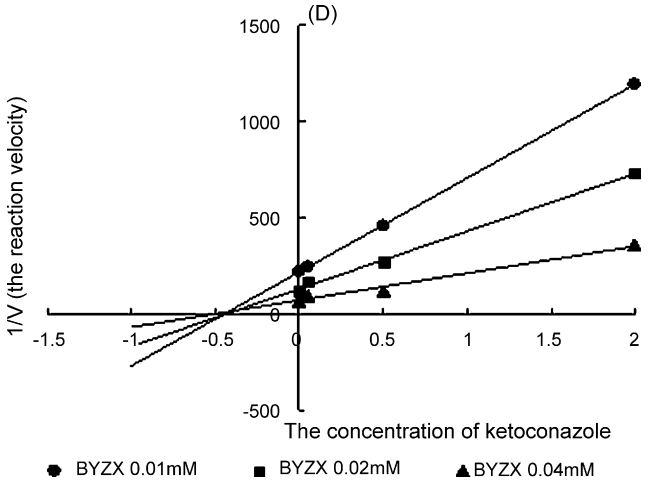

Fig. 14. The inhibition constant (K_i) of ketoconazole on the metabolism of BYZX in human liver microsomes.

zole, and 4-methylpyrazole were all less than 20%, so they had no inhibitive effect. Otherwise, the other inhibitors all had more or less inhibitive effects, cyclosporin A, fluvoxamine, α-naphthoflavone, quinidine, and omeprazole can inhibit 55.94%, 51.0%, 39.02%, 32.91%, and 21.14% enzyme activity, respectively. Ketoconazole has the strongest inhibitive effect, which can inhibit 79.87%.

The IC_{50} values of ketoconazole, cyclosporine A, fluvoxamine. α-naphthoflzvone, quinidine, and omeprazole were determined in the following experiment and the results were showed in Table 4.

The ketoconazole had the strongest inhibitive effect. So, the suitable concentrations of BYZX and inhibitor were chosen to determine the K_i value, and the results were showed in Fig. 14. The K_i of ketoconazole was 0.42 μM.

Ketoconazole for CYP3A4 [16], cyclosporin A for CYP3A4 [17], fluvoxamine for CYP1A2 [18] and CYP3A4 [19], α-naphthoflavone for CYP1A2 [20], quinidine for CYP2D6 [21], omeprazole for CYP2C19 [22,23], sulfamethoxazole and sulfaphenazole for CYP2C9 [24], and 4-methylpyrazole for CYP2E1 [22] were usually chosen as the inhibitors of drug metabolism in the preliminary experiment [25], because they were the classic inhibitors of CYP450. Magnitude of IC_{50} represents the inhibiting capacity. $IC_{50} < 1$ μM indicated strong inhibitive effect of the inhibitor. Oppositely, $IC_{50} < 50$ μM indicated that inhibitive effect was weak. But when the inhibitive ratio of the inhibitor to the substrate was less than 50%, the IC_{50} value, which was calculated by the formula mentioned in Section 2.5.2, may not be very objective. Inversely, K_i value obtained by the linear regression of the reciprocal of rate of metabolite formation ($1/v$) versus inhibitor concentration was relatively objective. One substrate concentration can get one regression curve. In the Ki value calculation process, three substrate concentrations were usually chosen. Abscissa of the three regression lines' point of intersection was the K_i value. The less the K_i value was, the more obvious the inhibition appeared [16].

Table 4
IC_{50} of inhibitors

Inhibitor	IC_{50} (μM)
Ketoconazole	0.89
Cyclosporine A	18.17
Fluvoxamine	56.55
Naphthoflavone	>100
Quindine	80.4
Omeprazole	>100

The result of preliminary experiment showed that ketoconazole, cyclosporin A, fluvoxamine, α-naphthoflavone, quinidine, and omeprazole may have some inhibitive effect. The IC_{50} of ketoconazole and cyclosporin A was 0.89 μM and 18.17 μM, which indicates the two drugs had important effect on the BYZX metabolism. Fluvoxamine as the inhibitor of CYP3A4 and CYP1A2 also had some inhibitive effect ($IC_{50} = 56.55$ μM). But α-naphthoflavone for CYP1A had no any inhibitive effect ($IC_{50} > 100$ μM), so it can be demonstrated that the inhibitive effect of fluvoxamine came from the inhibitory effect on CYP3A4. From these results, it can be inferred that BYZX metabolism was catalyzed by CYP3A4 predominantly.

In vitro metabolism research of a new chemical entity provides a principle for clinical rational administration, and offers a substantial substructure for the research of drug–drug interaction. Meanwhile, it also provides a theory evidence for the further development of BYZX.

4. Conclusion

The developed HPLC method was reliable, simple technique and was applicable to be used for the researches of in vitro metabolism of BYZX. CYP3A4 was the major isozyme responsible for BYZX metabolism. *N*-dealkylation was the major metabolic pathway of BYZX. *N*-des-ethyl-BYZX was the predominant metabolite of BYZX detected in vitro phase I metabolism in HLM.

Acknowledgments

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