

A Phenolic Ester of *O*-Desmethylvenlafaxine (ODV) Improves Uptake of ODV into the Brain

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Abstract: In this study, the *in vitro* stabilities of a promising *O*-Desmethylvenlafaxine (ODV) phenolic ester prodrug O-19 in aqueous solution, rat whole blood and rat liver microsomes were investigated. In addition, the *in vivo* drug metabolites were determined using LC-TOFMS-IDA-MS/MS. O-19 exhibits high stabilities at any pH encountered in the gastrointestinal tract, its half live ($t_{1/2}$) spans the range 5.0 ± 0.18 d ~ 686 ± 29.5 d. However, O-19 experiences rapid enzymatic hydrolysis in rat whole blood ($t_{1/2} = 3.6 \pm 0.53$ min) and rat liver microsomes ($t_{1/2} = 9.5 \pm 1.53$ min). The metabolic profile of O-19 is similar to that of ODV. Besides ODV itself, five metabolites are identified in rat urine (M_1 ~ M_5) and faeces (M_1 , M_4), no obvious toxic metabolite is detected.

Keywords: *O*-Desmethylvenlafaxine, Ester, O-19, Stabilities, Metabolites

Introduction

Depression, a common mental illness worldwide, has been estimated to affect 350 million people (Richards, 2011). Meanwhile, major depression is a leading cause of suicide for people who suffered from serious mental disease (Isometsä *et al.*, 1994). Due to high rates of lifetime incidence, early age onset, high chronicity and role impairment, antidepressant medication is subjected to challenges (Richards, 2011). Thus, the development of promising antidepressant agents is still needed.

Desvenlafaxine (*O*-desmethylvenlafaxine, ODV), a selective Serotonin and Norepinephrine Reuptake Inhibitor (SNRI) antidepressant drug, was marketed in US in 2008 for the treatment of major depression and other associated anxiety disorders. It is the major active metabolite of venlafaxine (VEN) and has an antidepressant activity profile similar to that of VEN but with a better tolerability and a much longer half-life (Muth *et al.*, 1986; Rudorfer and Potter, 1997; Ereshefsky and Dugan, 2000). Unexpectedly, ODV failed to be approved in EU (Anonymous, 2009), owing to an unfavorable risk/benefit profile associated with the treatment of depression and hot flushes. Further investigations have reported that 50 mg day^{-1} is the recommended minimum effective dose of ODV, however, high dosage of ODV seems not to lead to satisfactory therapeutic effects and efficacy deficiency has been

observed in some cases (Iwata *et al.*, 2013; Liebowitz *et al.*, 2013). In humans, the oral absolute bioavailability of ODV is up to 80%, which raises the question of whether its efficacy deficiency is contributed by the relative lower brain exposure *in vivo*.

In our previous work (Zhang *et al.*, 2013), with the aim of improving the brain uptake, several phenolic esters of ODV were designed and synthesized. After pharmacokinetic evaluation, compound O-19, 4-(2-(dimethylamino)-1-(1-hydroxycyclohexyl)ethyl)phenyl benzo[d][1,3]dioxole-5-carboxylate (Fig. 1), was selected as a promising ODV prodrug, because of its highest relative bioavailability (192%) and peak concentration (C_{\max}) of ODV both in the total brain (180.0 ng g^{-1}) and in the hypothalamus (514.5 ng g^{-1}) in rats.

Stabilities of active ingredient is one of the important influencing factors for pharmaceutical investigation, especially for ester derivatives, which are sensitive to enzymes, acidic and alkaline conditions, etc (Thumma *et al.*, 2008; Leriche *et al.*, 2012). Hence, in present investigation, the stabilities evaluation *in vitro*, i.e., in aqueous, rat whole blood and liver microsomes of O-19 were determined. In addition, in order to obtain the insight of metabolite profile, the metabolites of O-19 in rat were identified using liquid chromatography-time-of-flight mass spectrometry-information-dependent acquisition-mass spectrometry/mass spectrometry (LC-TOFMS-IDA-MS/MS).

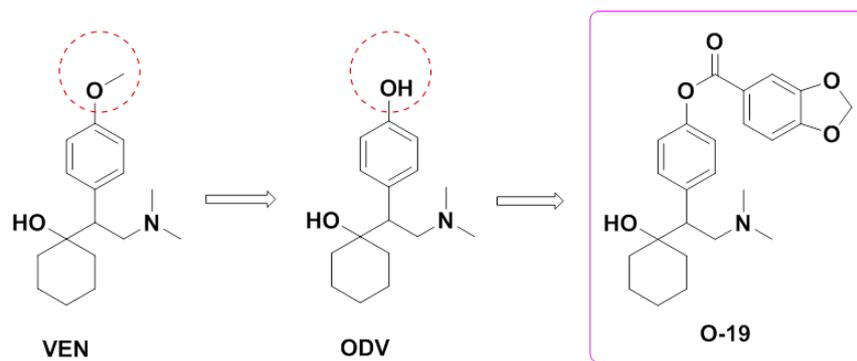


Fig. 1. Structures of VEN, ODV and O-19

Materials and Methods

General Experimental Protocols

O-19 (molecular formula: $C_{24}H_{29}NO_5$, molecule weight: 411.2, HPLC purity > 95%) were provided by Jilin Institute of Chemical Technology (Jilin, P. R. China). Reagents and solvents were obtained from Sigma-Aldrich Chemical Co., Ltd (St. Louis., MO, USA). Drug concentrations in aqueous solution were carried out using a 2100 Series Agilent Technologies HPLC (Palo Alto, CA, USA). Drug concentrations in rat whole blood and liver microsomes were determined by LC-MS/MS system using a 1100 Series Agilent Technologies HPLC (Palo Alto, CA, USA) and a QTRAP 2000 Series AB SCIEX mass spectrometer (Concord, ON, Canada), equipped with an electrospray ionization source. The identification of metabolites was conducted using a Triple-TOF 5600 Series AB SCIEX mass spectrometer (Concord, ON, Canada), equipped with an electrospray ionization source. Data acquisition and analysis were performed using AB SCIEX Analyst Software Version 1.5.1 (Concord, ON, Canada). Half-life ($t_{1/2}$) values were calculated by the non-compartmental analysis model using DAS Version 3.0. software package (Mathematical Pharmacology Professional Committee of China, Shanghai, China). Animal experiments were carried out in compliance with the "Guide for the Care and Use of Laboratory Animals" (NRC, 2010) and were approved by the Animal Care and Welfare Committee of Jilin Institute of Chemical Technology.

In Vitro Stabilities Evaluation

Stabilities in Aqueous Solution

The aqueous hydrolysis of O-19 was studied at pH 1.2 (the simulated gastric fluid), pH 6.8 and pH 7.4 (the simulated intestinal fluid media) at 37 and 25°C in Phosphate Buffer Saline (PBS). O-19 was dissolved in PBS to a final concentration of $10.0 \mu\text{g mL}^{-1}$. The PBS

solution was divided equally into six portions and sealed in screw-capped glass test tubes at 25°C and three of them were put into a water bath with temperature controller at 37°C. About 20 μL of the samples were withdrawn at appropriate time interval (0, 24, 48, 72, 96, 120, 144, 168 and 336 h) and assayed for the presence of ODV by HPLC (Liu, 2007).

Stabilities in Rat Whole Blood

Fresh whole blood was collected from the male Wistar rat using a retro-orbital bleeding method in a tube containing heparin (100 IU mL^{-1} blood). O-19 was dissolved in PBS (pH = 7.4) to a final concentration of $1.5 \mu\text{g mL}^{-1}$. Aliquots of 100 μL of O-19 PBS solution, 100 μL of PBS (pH = 7.4) and 800 μL of rat whole blood were sealed in screw-capped glass test tubes and fixed separately into a water bath with temperature controller to incubate at 37°C for 0, 5, 10, 15 and 30 min. At the appropriate time, the corresponding whole blood sample was removed and treated with 0.5 mL of cold acetonitrile, then centrifuged at 4000 rpm for 5 min. The supernatant was diluted with deionized water and 20 μL of the supernatant was then injected into LC-MS/MS (Liu, 2007; Chang *et al.*, 2011).

Relative concentration% = peak area at each time point (min)/peak area at 0 min \times 100%

Stabilities in Rat Liver Microsomes

All the reagents and appliances were stored at 4°C prior to use. Rats were sacrificed and livers were collected immediately. Livers were dried with filter paper before weighted. After washing in cold sucrose solution, the livers were cut into pieces. Being added four times the liver weight of sucrose solution and homogenized the tissues in the ice bath. After sonication for 30 sec, the mixture was centrifuged at 4°C for 20 min at 3500 rpm. Then, the upper layer was centrifuged at 4°C for 60 min at 12,000 rpm, the precipitation was microsomes. Suspended that the precipitation and stored at -80°C. Aliquots of 100 μL

of rat liver microsomes containing 1.0 mg mL⁻¹ rats liver microsomal protein, 100 µL of 1.0 mmol/L NADPH, 700 µL of Tris-HCl buffer (pH = 7.4) and 100 µL of 1.5 µg mL⁻¹ O-19 PBS (pH = 7.4) solution were sealed in screw-capped glass test tubes and placed separately into a water bath with temperature controller to incubate at 37°C for 0, 5, 10, 15 and 30 min. At the appropriate time, incubation mixture was removed and treated with 0.5 mL of cold acetonitrile, then centrifuged at 12,000 rpm for 5 min. The supernatant was diluted with deionized water and 20 µL of the supernatant was then injected into LC-MS/MS (Liu, 2007; Park *et al.*, 2010).

Relative concentration % = peak area at each time point (min)/peak area at 0 (min) × 100%

Identification of Metabolites in Vivo

Drug Administration and Sample Collection

O-19 was dissolved in normal saline (0.5% CMC-Na) at concentration of 0.8 mg mL⁻¹. Eight rats (anesthetized with diethyl ether) were administered orally with O-19 (8 mg kg⁻¹) and placed in a metabolic cage. Urine and faeces were collected for a total of 12 h. All the samples were stored immediately at -80°C in dark place until analysis (Liu, 2007).

Sample Preparation

Frozen dried faeces were grinded and dissolved in a solution of methanol-water (1:1, v: v) and sonicated for 3 min.

After centrifugation at 5000 rpm for 10 min to remove the precipitate, the faeces in the same treatment group

were mixed and an aliquot of 20 µL of solution was filtered through a 0.45 µm filter membrane. The filtrate was used for the LC-TOFMS-IDA-MS/MS analysis.

Frozen urine was dissolved in a solution of methanol: Water (1:1, v: v) and an aliquot of 20 µL of solution was filtered through a 0.45 µm filter membrane. The filtrate was used for the LC-TOFMS-IDA-MS/MS analysis (Liu, 2007).

Results

In Vitro Stabilities Evaluation

As shown in Table 1, the results show that the half live ($t_{1/2}$) of O-19 spans the range 5.0±0.18 ~ 686±29.5 d at pH encountered in the gastrointestinal tract at 25 and 37°C. The results suggest that the pH value and temperature are the key factors to affect the aqueous stabilities of O-19, when pH and temperature increase, the $t_{1/2}$ of O-19 decreases.

As shown in Fig. 2, after incubation for 30 min, the remaining percentage of O-19 in rat plasma is 0.38±0.02% ($t_{1/2}$ = 3.6±0.53 min) and that in rat liver microsomes is 11.8±2.4% ($t_{1/2}$ = 9.5±1.53 min).

Identifications of Metabolites in Vivo

As shown in Table 2, in addition to ODV, five metabolites (M₁~M₅) of O-19 are identified in rat. ODV and M₁~M₅ are founded in rat urine. However, in rat faeces, besides ODV, only two metabolites (M₁, M₄) are detected. The MS/MS spectra and proposed fragmentation pattern of all the metabolites were shown in Fig. 3.

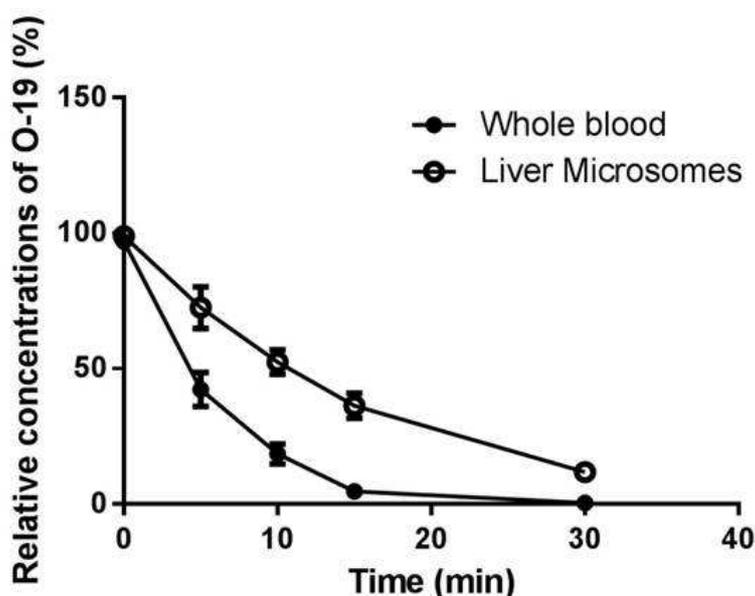


Fig. 2. Degradation profile of O-19 in rat whole blood and rat liver microsomes (data are means ± SD, n = 4)

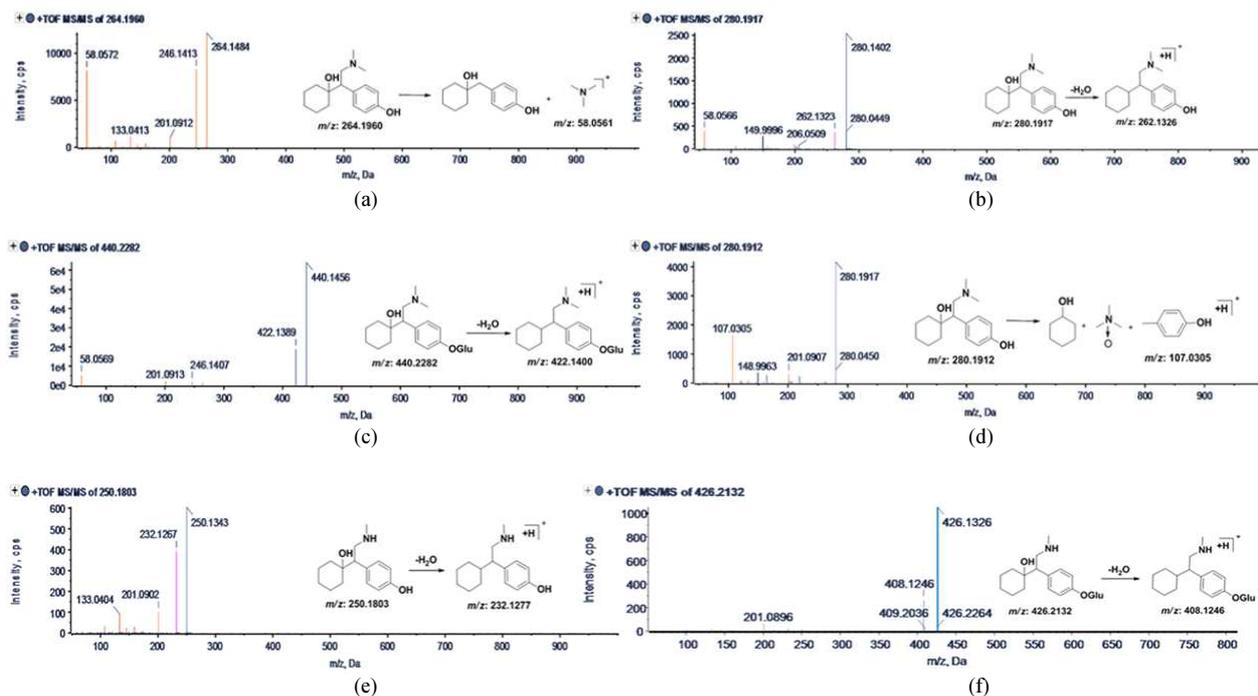


Fig. 3. The MS/MS spectra and proposed fragmentation pattern of (a) ODV (*m/z* 264.1960); (b) M₁ (*m/z* 280.1917); (c) M₂ (*m/z* 440.2282); (d) M₃ (*m/z* 280.1912); (e) M₄ (*m/z* 250.1803); (f) M₅ (*m/z* 426.2132)

Table 1. The *t*_{1/2} of O-19 at pH encountered in the gastrointestinal tract at 25 and 37°C (data are means ± SD, n = 4)

Parameter	pH 1.2		pH 6.8		pH 7.4	
	25°C	37°C	25°C	37°C	25°C	37°C
<i>t</i> _{1/2} (d)	686±29.5	608±26.1	212±7.42	30.0±1.05	20.0±0.74	5.0±0.18

Table 2. The metabolites (ODV, M₁ ~ M₅) of O-19 identified using LC-TOFMS-IDA-MS/MS in rat

No.	<i>t</i> _R (min)	<i>m/z</i> calculate	<i>m/z</i> (Da)	Identification	Formula	Error (ppm)
ODV	13.40	263.1885	264.1960		C ₁₆ H ₂₅ NO ₂	-1.14
M ₁	9.76	279.1834	280.1917		C ₁₆ H ₂₅ NO ₃	1.79
M ₂	11.21	439.2206	440.2282		C ₂₂ H ₃₃ NO ₈	-0.46
M ₃	13.17	279.1834	280.1912		C ₁₆ H ₂₅ NO ₃	0
M ₄	12.49	249.1729	250.1803		C ₁₅ H ₂₃ NO ₂	-1.6
M ₅	11.11	425.2050	426.2132		C ₂₁ H ₃₁ NO ₈	0.94

Discussion

In our previous investigation, O-19, a novel phenolic ester of ODV with improved oral bioavailability and brain uptake was selected as the most promising candidate for prodrug. In present research, we focused on the druggability evaluation of O-19.

A prodrug is a compound, which is inactive *in vitro* and can be transformed to the parent drug by enzymes *in vivo* (Hacker *et al.*, 2009). Prodrugs are usually designed to improve intestinal absorption (Mizuno *et al.*, 2003), increase oral bioavailability (Kahns *et al.*, 1992) and reduce adverse effects (Bandgar *et al.*, 2011), etc. A promising oral prodrug is expected to be stable at different pH encountered in the gastrointestinal tract and experience quick and complete transform to parent drug in whole blood or microsomes (Liu *et al.*, 2013). In this study, with the aim of testing the stabilities of O-19 at different pH encountered in the gastrointestinal tract, the aqueous stabilities of O-19 were determined in PBS solution at pH 1.2 (the simulated gastric fluid), pH 6.8 and pH 7.4 (the simulated intestinal juice) at 25 and 37°C (Liu, 2007; Qandil *et al.*, 2011; Redasani and Bari, 2012). We found that the $t_{1/2}$ of O-19 ranges from 5.0 ± 0.18 d to 686 ± 29.5 d in PBS solution with different pH values, which indicates that the O-19 prodrug is stable at acidic condition encountered in the stomach and

alkaline condition encountered in the intestine. It may pass through the stomach and intestinal tract without degradation and be absorbed on the intestinal epithelial cell in the form of intact molecule. However, in rat whole blood and liver microsomes, the $t_{1/2}$ of O-19 is 3.6 ± 0.53 min and 9.5 ± 1.53 min, respectively, which suggests that O-19 may be rapidly converted to the Parent Drug (ODV) after absorption and enzymes play an important role in the activation of O-19 *in vivo*. Based on the above observation, O-19 accords features of oral prodrug and promotes the oral absorption of ODV, which contributes the improved oral bioavailability and brain uptake of ODV.

Metabolites identification *in vivo* is very important for the research and development of new drug, which can provide useful information about metabolic pathway to help us judge the toxicity of new drug (Chen *et al.*, 2008). At present, as one of the representatives of High Resolution Mass Spectrometry (HRMS), the Time of Flight (TOF) analyzer has been widely used for the identification of metabolites due to its high resolution and accuracy. In TOF system, by means of Information Dependent Acquisition (IDA), the full-scan MS spectra and product-ion spectral data relating to metabolites can be acquired. Then, the MS and MS/MS fragmentation spectra data are conducted for the identification of targeting metabolites (Tian *et al.*, 2015).

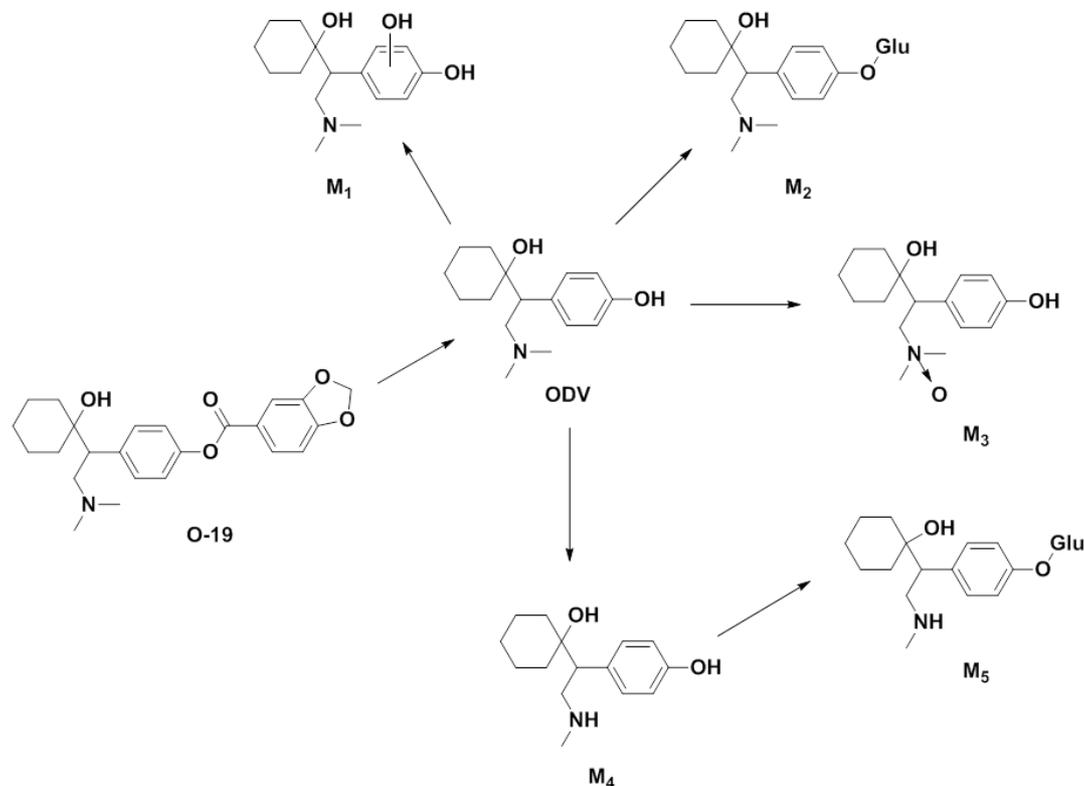


Fig. 4. The proposed metabolic pathway of O-19 in rat

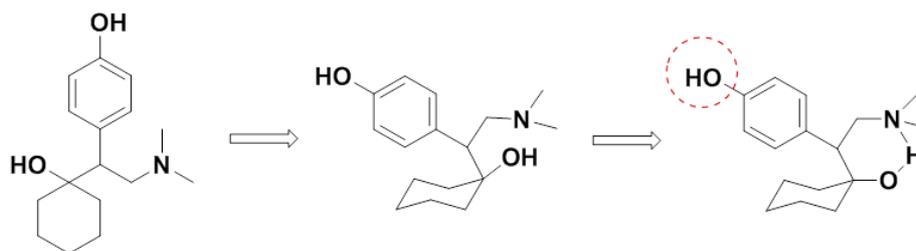


Fig. 5. The proposed hydrogen bond association of alcoholic hydroxyl group and nitrogen atom of ODV

In this study, with the purpose of obtaining the insight of metabolic profile, the metabolites of O-19 in rat were identified using LC-TOFMS-IDA-MS/MS. The results indicate that the prototype of O-19 can not be detected due to its fast enzymatic hydrolysis, the metabolic pathway of O-19 is similar to that of ODV (Klamerus *et al.*, 1992; Gasser *et al.*, 2012) and no obvious toxic metabolite is detected *in vivo*. The metabolic products of O-19 and its proposed metabolic pathway in rat were shown in Fig. 4. After absorption, O-19 can be rapidly metabolized and activated by enzymes to produce ODV. Then, the glucuronidation reaction (M_2), oxidation reaction (M_1 , M_3), *N*-demethylation reaction (M_4) may occur. There are two possible glucuronidation sites of O-19, i.e., the phenolic hydroxyl group and the alcoholic hydroxyl group. However, due to the hydrogen bond association of alcoholic hydroxyl group and nitrogen atom, ODV is transformed to phenol-glucuronide (Fig. 5). Finally, the glucuronidation reaction of M_4 may occur to generate M_5 . The main metabolic manners of O-19 *in vivo* are *N*-demethylation (M_4), oxidation reaction (M_1 , M_3) and glucuronidation (M_2 , M_5). Besides ODV, five metabolites (M_1 ~ M_5) are detected in rat urine, only two metabolites (M_1 , M_4) in rat faeces, which indicates that kidney is the main excretory organ of O-19.

In summary, in this study, we concerned mainly on the druggability evaluation of O-19 and the metabolism study was preliminary. Thus, some key issues such as the identities of enzymes involving in metabolism and the quantification of metabolites are still needed for further studies.

Conclusion

In present work, the *in vitro* stabilities of O-19 were determined in aqueous solution, rat whole blood and rat liver microsomes. In addition, its *in vivo* metabolites identification was preliminarily conducted using LC-TOFMS-IDA-MS/MS. The results indicate that O-19 is stable at any pH encountered in the gastrointestinal tract ($t_{1/2} = 5 \pm 0.18$ d ~ 686 ± 29.5 d) and is rapidly metabolized by enzymes in rat blood ($t_{1/2} = 3.6 \pm 0.53$ min) and in rat liver microsomes ($t_{1/2} = 9.5 \pm 1.53$ min) to produce ODV. Therefore, O-19 accords the features of oral prodrug and

promotes the intestinal absorption of ODV to improve its oral bioavailability and brain uptake. The metabolic pathway of O-19 is similar to that of ODV, no obvious toxic metabolite is detected. Besides ODV itself, five metabolic products (M_1 ~ M_5) are detected in rat urine, only two metabolites in rat faeces (M_1 , M_4), which indicates that kidney is the main excretory organ of O-19. However, some details about metabolic mechanism are still needed for further studies.

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Author Contributions

Hong Li Zhou and Yang Zhang: Conceived and designed the experiments.

Yang Zhang, Zhichao Yang and Sen Zhao: Performed the experiments.

Yang Zhang: Wrote the paper.

Hong-Li Zhou: Revised the manuscript.

Conflict of Interest

The authors declare no conflict of interest.

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