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Pharmacokinetics and Brain Distribution and Metabolite Identification of Coptisine, a Protoberberine Alkaloid with Therapeutic Potential for CNS Disorders, in Rats

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Summary  Coptisine (COP), a protoberberine alkaloid (PBA) from Chinese medicinal plants (such as family Berberidaceae), may be useful for improving central nervous system disorders. However, its pharmacokinetics, disposition and metabolism are not well defined. In the present study, a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method was established for the analysis of COP in biological samples. To better understand its in vivo pharmacological activities, COP concentrations in rat plasma were determined after oral (50 mg/kg) and intravenous administration (10 mg/kg). For the brain distribution study, the concentration of COP in five different regions was examined after intravenous administration at 10 mg/kg. Pharmacokinetic parameters from the COP concentration-time profiles in plasma and brain, and the brain-to-plasma coefficient (\(K_{p, \text{brain}}\)) were calculated by non-compartmental analysis. The metabolites of COP in rats in vivo and in vitro (urine, bile, liver microsomes and intestinal bacteria incubation) were also identified. Seventeen metabolites, including 11 unconjugated metabolites formed by hydroxylation, hydrogenation, demethylation, dehydrogenation, demethylation, and 6 glucuronide and sulfate conjugates were identified for the first time. The results suggested that COP had low oral bioavailability of 8.9% and a short (plasma) half-life (\(T_{1/2}=0.71 \text{ h}\)) in rats. After intravenous administration, it quickly crossed the blood-brain barrier, accumulating at higher concentrations and then was slowly eliminated from different brain regions. Moreover, COP was transformed into metabolites through multiple metabolic pathways in vivo and in vitro. These results should help to promote further research on COP and contribute to clarifying the
metabolic pathways of PBAs.

Keywords coptisine; pharmacokinetics; brain distribution; metabolites; LC-MS/MS
Main text

Coptisine (COP; Figure 1), a protoberberine alkaloid (PBA), is widely found in Chinese medicinal plants (family Berberidaceae, Ranunculaceae and Papaveraceae). It is reported that COP has a wide range of pharmacological and biological activities, including antibacterial, hypoglycemic, anti-tumorigenic, and gastric-mucous membrane protection.\(^1,2\) Recently, considerable attention has been focused on its activity against central nervous system (CNS) disorders, such as improving the symptoms of Alzheimer’s disease (AD) and even preventing its onset,\(^3\) by exerting antidepressant effects as a potent type A monoamine oxidase inhibitor.\(^4\) However, its pharmacokinetics, disposition and metabolism in vivo and in vitro, especially its distribution profile in brain which relate to its effects on the CNS, have remained unclear to date.

Pharmacokinetics plays a crucial role in drug discovery and development processes, not only to further support toxicity or clinical trials, but also as a set of parameters which can be used to optimize drug candidates. It is generally recognized that PBAs usually have poor absolute bioavailability.\(^5\) For example, treatment with berberine (BBR) and jatrorrhizine (JAT) result in multiple clinical effects, but they have low oral bioavailability (< 5%).\(^6\) One reason is that they are not readily absorbed into plasma, because most PBAs are P-glycoprotein (P-gp) substrates, which can reduce their intracellular drug concentrations.\(^6\) Their intestinal first-pass elimination and prominent liver distribution are also major barriers to absorption and low plasma levels. Liu et al. reported that all of these reasons support the very low
plasma concentrations of BBR in rats.\(^7\) Ma et al. subsequently found that lipopolysaccharide pretreatment increased systemic exposure to the alkaloids through enhancement of their absorption, which was related to decreased intestinal efflux and metabolism.\(^8\)

The distribution of drugs to the brain is determined by various factors including transport across the blood-brain barrier (BBB) and their binding in blood and brain. The BBB plays a vital role in controlling the exchange of endogenous and exogenous substances between the blood and the relevant site.\(^9\) Thus the therapeutic efficacy of a drug is dependent on its brain permeability and mean residence time (MRT).\(^10\) Successful penetration of the BBB is necessary for drugs to have central nervous effects. \(K_{\text{p,brain}}\) is the most widely used \textit{in vivo} parameter for assessing the extent of CNS distribution. A common assumption is that compounds with large \(K_{\text{p,brain}}\) values have more extensive CNS distribution than compounds with small \(K_{\text{p,brain}}\) (brain/plasma partition coefficient, was calculated as the ratio of AUC\(_{\text{brain}}\)/AUC\(_{\text{plasma}}\) ) values\(^11\). However, the BBB is not uniform throughout the brain. The cortex, hippocampus, hypothalamus, and striatum are the predominant brain regions targeted in CNS disorders.\(^12\) Accumulating evidence indicates that COP has potential efficacy as a treatment for AD, cerebral ischemia, mental depression, schizophrenia, and anxiety, thus exploring the fate of COP in the brain would help to further clarify its \textit{in vivo} pharmacological activities.

Furthermore, information on its \textit{in vivo} drug disposition and metabolism may help to clarify the mechanism of action of COP and provide starting points for
structure modifications to obtain more effective compounds. A number of studies confirmed that \textit{in vivo} metabolism was important for both changes in drug molecular structure and generation of the resulting activities. Metabolites are often active components of some drugs as indicated by the range of pharmacological effects observed. For example, herba epimedii (common name in Chinese medicine is \textit{Yin Yang Huo}), and the major bioactive constituents of the herb can be hydrolyzed to their metabolites including icaritin by intestinal enzymes. Modern pharmacology studies demonstrated that this deglycosylated aglycone exerts many pharmacological activities.

In this study, a selective and sensitive liquid chromatography-tandem mass spectrometry method (LC–MS/MS) method was developed for the determination of COP in biological samples. The reliable method was subsequently applied to investigate the pharmacokinetic behavior of COP in rats after oral and intravenous administration. Moreover, \textit{in vivo} and \textit{in vitro} metabolism of COP was investigated using a LC–MS/MS technique. To our knowledge, this is the first report on the pharmacokinetics, disposition and metabolism of COP in rats. The results from this study should be helpful for selecting the route of administration and promote further research on COP.

\textbf{MATERIALS AND METHODS}

\textbf{Materials} \ COP and palmatine (PAL; internal standard for determination, IS) were purchased from Chengdu Must Bio-Technology Co., Ltd (≥ 98 \% purity; ChengDu,
Sichuan Province, China). Formic acid (99%, HPLC-grade) was supplied by Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). Acetonitrile and methanol were HPLC-grade from Fisher Chemical Co. Inc. (Geel, Belgium). Water was from Milli-Q system (Millipore, Bedford, MA, USA). All other chemicals were reagent- or HPLC-grade from standard commercial sources.

Animals Male Sprague–Dawley (SD) rats weighing 200–240 g (8 weeks old) were provided by the Experimental Animal Center of Beijing Weitong Lihua (Certificate No. SYXK 2012-0001). All protocols for animal experiments were conducted in accordance with the Institute’s Guide for the Care and Use of Laboratory Animals. The rats were maintained under a 12-h light-dark cycle in a temperature-controlled environment with unlimited access to food and water. They were fasted for 12 h and given free access to water before experiments.

Pharmacokinetics COP solution was prepared on the day of the experiment. The COP formulation was prepared in DMSO, tween and saline as a suspension and the proportion of organic solvent was strictly quantitated (<1%, v/v). The particle size distribution of the COP suspension was set so that the percentage of particles ≤100 nm should be higher than 95%. SD rats were randomly separated into two groups (6 rats per group). Rats received a single oral dose of COP (50 mg/kg) by gavage. For intravenous administration, rats received a 10-mg/kg dose by tail vein injection. Rats were lightly anesthetized using diethyl ether, and blood (approximately 300 μL) was collected from the inner canthus vein at 0.083, 0.25, 0.5, 1, 2, 3, 3.5, 4, 6, 8, 12, and 24 h after the dose. Plasma was obtained by centrifugation at 8000×g for 10 min at
4°C and was stored at −80°C after separation until assayed as described below.

The plasma was extracted using a single-step protein precipitation procedure. For each portion (200 μL plasma), 10 μL IS (20 ng/mL) and 400 μL acetonitrile were added. After vortex-mixing for 5 min and centrifuging at 8000×g for 10 min at 4°C, the supernatant was analyzed with LC-MS/MS.

**Brain Distribution** Rats received a 10-mg/kg dose in the tail vein. Blood and brain were sampled at 0.05, 0.17, 0.25, 0.5, 0.75, 1, 1.25, 1.5, 2, 4, 8, and 24 h after the dose (72 rats, n = 6 at each time point). Blood was collected by abdominal aortic puncture, and plasma obtained by centrifugation at 8000×g for 10 min at 4°C and then extracted using a single-step protein precipitation procedure. The whole brain was quickly removed from the skull and rinsed in ice-cold saline, immediately flash-frozen, and stored at −80°C. For analysis, brains were carefully dissected into five regions (brainstem, hypothalamus, hippocampus, striatum, and cortex), with all procedures performed in an ice-water bath. The brain regions were weighted and then immediately homogenized in ice-cold saline at a rate of 1 g/2 mL. Samples were then extracted using acetonitrile, and dried by a gentle nitrogen stream. Residues were reconstituted in 200 μL methanol, centrifuged at 12 000×g for 15 min at 4°C, and the supernatants used for LC-MS/MS analysis.

**Urine, Feces, and Bile Sample Collection** After fasting for 12 h, SD rats were randomly separated into three groups (6 rats per group) and housed individually in metabolic cages. Pre-dose blank urine and feces were collected from rats in group A for 48 h, and then the rats received a single dose of COP (20 mg/kg) by gavage, and
urine and feces were collected for 48 h after dosing. Bile samples were obtained from rats in group B by cannulation using a polyethylene tube in the bile duct for 48 h after COP (20 mg/kg) dosing by gavage. Blank bile was collected from rats in group C, which were gavaged with saline of equal volume. All samples were stored at −80°C until analysis.

Urine and bile samples were passed through C$_{18}$ solid-phase extraction cartridges which had been washed with 3 mL methanol and equilibrated with 6 mL water. Constituents were eluted with 2 mL water and analytes with 1 mL methanol. Feces homogenate samples were centrifuged at 12 000×g for 15 min, and methanol added for liquid–liquid extraction. All eluates were evaporated under a nitrogen stream at 37°C, leaving a residue that was dissolved in 200 μL methanol for LC/MS/MS analysis.

**Metabolism by Intestinal Bacteria and Liver Microsomes** Mixtures of fresh rat intestinal contents were immediately homogenized in an anaerobic culture solution at a rate of 0.5 g/1.5 mL. After gauze filtration, COP was added to the intestinal bacteria solution at a final concentration of 40 μg/mL and the solution placed in a shaking water-bath at 37°C for 24 h under anaerobic conditions. After passing through C$_{18}$ solid-phase extraction cartridges and drying by a gentle nitrogen stream, residues were reconstituted in 0.4 mL of mobile phase and centrifuged at 12 000×g for 15 min.

SD rats were euthanized and the livers removed. After washing with normal saline, liver tissue was homogenized in 0.1 mol/L PBS buffer (pH 7.4) at a ratio of 1 g/4 mL, and subsequently centrifuged at 12 000×g for 20 min. CaCl$_2$ solution was
added to the supernatants, and centrifuged at 12 000×g for 60 min to obtain liver microsomes. To maintain enzyme bioactivities, all preparation procedures were performed below 4°C. Liver microsomes were resuspended in 30 mL of 0.1 mol/L PBS buffer (pH 7.4, containing 30% glycerine) and stored at −80°C until analysis. Incubations were performed in a final volume of 0.5 mL PBS buffer (0.1 mol/L pH 7.4) containing 10 μg/mL COP, 1.0 mmol/mL NADPH, and 1 mg/mL liver microsomal protein at 37°C for 2 h and terminated by adding an equal volume of methanol. Mixtures were centrifuged at 12 000×g for 10 min, and the supernatants dried by a gentle nitrogen stream. Residues were reconstituted in 0.5 mL methanol and then centrifuged at 12 000×g for 10 min. Supernatants were used for LC-MS/MS analysis.

**Analytical Method** A reversed-phase column (SB-C\textsubscript{18}, 4.6 mm × 250 mm, 5 μm; Agilent Technologies, Inc., Santa Clara, CA) was connected to a guard column (C\textsubscript{18}, 4.6 × 12.5 mm cartridge, 5 μm; Agilent), and the column temperature set at 25°C. LC-MS/MS experiments for pharmacokinetic study were performed on a Thermo Fisher Scientific TSQ Quantum (Thermo Fisher Scientific, Inc., San Jose, CA) with an Agilent 1100 Series pump and Xcalibur 2.0 (Thermo Fisher Scientific) was used for system operation and data collection. For metabolite identification, LC-MSD-Trap-XCT-plus was used and ion source was electrospray ionization (ESI) (Agilent Technologies, Inc.). The mobile phase was acetonitrile (A) and 0.1% formic acid aqueous solution (pH 3.5) (B) with a flow rate of 1 mL/min. A gradient was optimized as follows: 30 min for identifying metabolites (0–10 min, 70–30% B;
10–15 min, 30–20% B; 15–20 min, 20–70% B; and 20–30 min, 70% B), and 10 min for pharmacokinetics (0–10 min, 70% B). A 10 μL aliquot of purified sample was automatically injected into the system for LC-MS/MS analysis. Mass spectral analyses were performed using the positive ion detection mode. The optimum conditions were: source spray potential of 3 kV, capillary voltage of 3 V, and capillary temperature of 25°C. Flow rates of sheath gas (N₂) and auxiliary gas were 40 and 20 units (corresponding to approximately 0.6 and 6 L/min), respectively. The scanning mode for the pharmacokinetic study for the selective reaction monitoring (SRM) was 320.2/292.1 for COP and 352.2/336.2 for PAL (IS). The other parameters (including octapole offset and tube lens offset potentials) were also optimized for maximum abundance of ions of interest by automatic instrument tuning.

**Pharmacokinetic Parameter Calculation and Data Analysis**  Pharmacokinetic parameters from COP concentration-time profiles in plasma and brain were calculated by non-compartmental analysis using Phoenix WinNonlin 6.2 (Pharsight, NC, USA). After intravenous dosing, the concentration at time zero (C₀) was back-extrapolated by log-linear regression of the first two data points. Absolute bioavailability from oral administration was calculated using the following equation:

\[
F = \frac{\text{AUC}_{\text{po}} \times \text{Dose}_{\text{iv}}}{\text{AUC}_{\text{iv}} \times \text{Dose}_{\text{po}}}
\]

The terms were defined as follows: AUC, area under the concentration-time curve; AUC_{po} and AUC_{iv}, AUC for oral and intravenous administration, respectively, from 0 to 24 h; Dose_{po} and Dose_{iv}, COP dosage for oral and intravenous administration, respectively.
RESULTS

**LC-MS/MS Method Validation** A rapid, reliable and sensitive LC-MS/MS method was validated for quantitative analysis of COP in rat biological samples. Method specificity was evaluated using rat plasma and brain homogenate samples. At least six different samples were collected to investigate potential interferences using the proposed extraction procedure and analytical conditions. The developed method proved to be selective and no significant endogenous or other impurities were observed in the samples at analyte and IS retention times (6.3 and 8.1 min, respectively) (Figure 2). Linearity was determined by calibration curve construction. The calibration curve was linear over concentration ranges of 5.17–5 300 ng/mL (plasma) and 1.95–1 100 ng/mL (brain homogenate), with correlation coefficients ($r^2$) > 0.99. Regression equations were $y = 0.0013x + 0.4187$ for plasma and $y = 0.0079x + 1.2846$ for brain homogenate ("y" and "x" of regression equations was defined as "relative intensity" and "compound concentration"). Accuracy was ± 5.29 and 10.37%, and precision ± 6.42 and 9.21%, for plasma and brain homogenate, respectively. The RSD of the intraday and interday precision and accuracy were below 15% (plasma, 12.34%; brain homogenate, 11.87%). Recoveries were 85.5–108.7% in plasma (275.0, 1 500, and 4 500 ng/mL) and 81.3–95.2% in brain (15.5, 275.0, and 750.0 ng/mL). Analytes were stable in all samples when stored at −20°C for 7 days or 23°C in plastic autosampler vials for 8 h. Accuracy (relative error, RE) ranged from −10.2–9.3% for plasma and −11.5–7.9% for brain homogenate. The three analytes did
not degrade in the QC samples in either matrix after storage in both conditions.

**Pharmacokinetic Profiles and Brain Distribution Study** In this study, the developed and validated LC-MS/MS method was successfully applied to a pharmacokinetic study of COP in rat plasma. The dosage of COP was defined based on the use of BBR in the clinic. The mean concentration-time curves of COP following oral and intravenous administration and selected parameters are shown in Figure 3 and Table 1, respectively.

COP pharmacokinetic properties in brain and plasma were studied after a single intravenous dose (10 mg/kg) in SD rats. Plasma concentrations showed a rapid decline in the elimination phase, with a $T_{1/2}$ of 0.97 h. COP concentrations in whole brain rapidly reached a peak level at 0.62 h ($C_{\text{max}}$, 847.31 ± 88.28 ng/g; Table 2), demonstrating that COP permeates into the brain rapidly with no lag time. The COP brain-to-plasma partition coefficient ($K_p$) was calculated and used to evaluate its brain-targeting efficiency. The brain-to-plasma concentration ratio at each time point was calculated as the ratio of brain to plasma concentration ($C_{\text{brain}}/C_{\text{plasma}}$), and was high at the initial time points, reaching a peak at approximately 4 h (Figure 4). This indicates that COP can easily pass through the BBB into the brain. After 4 h, COP still remained at high concentration levels in the brain compared with plasma.

COP rapidly distributed and reached peak levels within 0.35 h after administration in the brainstem, cortex and striatum. Simultaneously, cortical and striatal AUC$_{0-t}$ were higher than in the other brain regions. These results provide strong support that the therapeutic targets of COP in the CNS may be the cortex and
striatum. COP mean concentration-time curves in plasma and brain regions after a single intravenous dose, and selected PK parameters are shown in Figure 5 and Table 3.

**In vivo and in vitro Identification of Metabolites** To identify the metabolites in rats, the ESI-MS/MS fragmentation behaviors of COP were investigated in the positive ion mode. By comparison with blank samples, full scanning in the m/z region of 50 to 700 allowed the assignment of several peaks to COP and its metabolites in the total ion chromatogram (TIC). Based on the drug metabolism rule and PBA (berberine, jatrorrhizine, and epiberberine) metabolite reports \(^{15,16}\) and by analysis of the Extract ion chromatograms (EIC), 17 metabolites (M1-17) were identified with molecular ions at m/z 356.3, 528.2, 296.1, 498.6, 484.4, 318.4, 648.1, 336.0, 310.6, 388.4, 307.9, 323.7, 352.5, 472.3, 324.4, 321.7, and 340.1. Potential structures were investigated using retention times (RT), and MS\(^2\) spectra (Table 4 and Figure 6).

M0 was eluted at 5.8 min with a precursor ion at m/z 320.3. By comparison with the COP standard, its product ion at m/z 292.1 was the result of decarbonization. The cleavage likely took place at the methylenedioxy groups at positions 2-, 3-, and 9-, 10-, which were the preponderant fracture bonds of the metabolic reactions of PBAs.

M16, with a retention time of 12.6 min in the EIC profiles, was derived from COP by comparison with the fragment of thalifendine,\(^{16}\) and found to be hydrogenated at the methylenedioxy group. M11 (m/z 307.9) was observed at 7.9 min, with a molecular ion 12 Da less than the precursor ion, COP. The results indicated that M3 was the decarbonization product of COP. Correspondingly, M3 (m/z 296.1, RT
=3.2 min) was assigned as the decarbonization product of M11.

M8 (m/z 336.0) and M13 (m/z 352.5) showed peaks in the EIC profiles at 5.7 and 9.3 min. The molecular ions were increased by 16 and 32 Da, respectively, compared with the parent ions. Therefore, M8 and M13 are mono-hydroxylate and di-hydroxylate forms of COP. Similarly, M12 (m/z 324.4), M17 (m/z 340.1), and M1 (m/z 356.3) were the mono-hydroxylate, di-hydroxylate, and tri-hydroxylate forms of M11. Based on the structure analysis of the COP parent ion, the saturated carbon atom at the B-ring was easier to oxidize because of the adjacent sp² aromatic carbon atom. The hydroxylation likely occurred at the meta-position of the B-ring (C-6, C-7), and the D-ring (C-11).

Ion peaks at m/z 528.2, 498.6, 484.4, 648.1, and 472.3 were 176 units higher than the M13, M16, M11, M14, and M3 molecular ions. Therefore, M2, M4, M5, M7, and M14 were derived from glucuronidation of M13, M16, M11, M14, and M3. The M6 molecular ion was 2 Da less than m/z 320.3 and therefore we concluded that it was derived from COP by dehydrogenation at the B-ring. By comparison of the parent and fragment ions, the molecular ion of M15 (m/z 323.7) was obtained following M16 hydrogenation, and increased by 2 Da. Subsequently, M15 formed M9 (m/z 310.5), with the molecular ion decreased by 14 Da from demethylation. As its molecular ion was increased by 80 Da, M10 (m/z 388.4) was deduced to be the sulfate conjugate of M11. The main metabolite structures and proposed metabolism routes are shown in Figure 7.
DISCUSSION

Pharmacokinetic Profile of COP After oral dosing, the COP concentration rapidly reached a peak level in plasma, with the maximal concentration ($C_{max}$) observed at $0.58 \pm 0.12$ h. $T_{1/2}$, MRT, and body clearance (CL) were $0.71 \pm 0.39$ h, $5.56 \pm 0.64$ h, and $85.97 \pm 17.47$ L/h/kg, respectively, which implies that the COP elimination rate was high. $T_{1/2}$, MRT, and CL from intravenous administration were $0.38 \pm 0.17$ h, $4.90 \pm 1.22$ h, and $7.68 \pm 1.82$ L/h/kg, respectively. Oral COP absolute bioavailability was 8.90%. To date, there are no reports concerning the pharmacokinetic profile of COP.

COP was one of the alkaloids from Chinese medicinal plants such as rhizoma coptidis. The rhizoma coptidis alkaloids include protoberberine alkaloid (PBA), isoquinoline alkaloids and aporphinoid, among others. Given that the rhizoma coptidis alkaloids are substrates of P-gp and are eliminated mainly through metabolism, researchers have proposed the following mechanisms for the low plasma concentrations of these alkaloids: 1) the alkaloids are metabolized in the intestine; 2) absorbed alkaloids are pumped out by intestinal or hepatic P-gp;17) 3) absorbed alkaloids are metabolized quickly and extensively after absorption;18) and 4) absorbed alkaloids are distributed widely in tissues.11,19) In the present study, the physicochemical properties of COP such as its poor solubility might be another explanation for the observed low oral bioavailability. This should be substantiated by permeability and efflux studies of COP using test systems such as the Caco-2 cell permeability assay. Furthermore, 6 metabolites of COP were identified from a liver
microsome incubation. Guo et al. reported CYD2D plays a major role in BBR metabolism in the liver of rats.20) Since alkaloids of rhizoma coptidis are extensively metabolized in the liver, quantitation of the metabolites would be helpful to assess the effects of hepatic metabolism. On the other hand, only 1 metabolite was identified from intestinal bacteria incubation, suggesting that COP did not produce its systemic therapeutic actions through modulating effectors in the gut.

Oral dosing is a convenient route for daily drug delivery, especially for chronic administration. However, a common assumption is that PBAs are not well absorbed. For example, BBR has multiple clinical effects, but its absolute bioavailability is reported to be < 5%.15) We found that the absolute COP bioavailability is also poor. Nevertheless, some natural products with low bioavailability (< 5%) from oral administration are reported to be powerful therapeutics.21) Therefore, the in vivo distribution and therapeutic actions of PBAs and their bioactive metabolites still attract widespread attention. P-gp is one of the main reasons for low drug bioavailability, and is extensively present in the apical membranes of the BBB, intestine, and placenta. Reports have shown that most protoberberine alkaloids are P-gp substrates, which leads to difficulties in compound absorption.9) In this study, the absolute COP bioavailability was only 8.9%, likely as a result of P-gp function.

Reasons for Differences in the Brain Distribution and Oral Absorption of COP P-gp in the BBB functions by preventing drugs from entering the brain. Pharmacological studies have also proven that P-gp transporter inhibitors increase the brain distribution of drugs.22) However, we found that the absolute bioavailability of
COP was above 35% in certain brain regions. \( K_{p,\text{brain}} \) is the most widely used \textit{in vivo} parameter for assessing the extent of CNS distribution. A \( K_{p,\text{brain}} \) of 1 is often used as an cutoff to classify compounds as having "good" CNS distribution.\(^{11}\) The \( K_{p,\text{brain}} \) of COP was 2.87, and this indicates that COP can easily pass through the BBB into the brain and have a high degree of brain distribution. Moreover, in the cortex and striatum, the COP concentration rapidly reached a peak. Cortical and striatal AUC\(_{0-t}\) were higher than in the other brain regions. These results provide support that the therapeutic targets of COP in the CNS may be the cortex and striatum. Many factors were likely to be involved. Firstly, the BBB does not occur uniformly in all parts of the brain.\(^{23}\) The BBB is mainly formed by brain capillary endothelial cells, void of fenestration, of low pinocytosis and tight junctions, which inhibits transcellular passage of molecules across the barrier and restricts the permeation of compounds into the brain. Membrane transport systems and some transporter proteins have been shown to accept drug molecules and transport them into the brain. The transport systems include transporters for amino acids, monocarboxylic acids, organic cations, hexoses, nucleosides, and peptides.\(^{24}\) Most of the transporters function in the direction of influx from blood to brain; the presence of efflux transporters from brain to blood has also been demonstrated in many reports, such as ATP-binding cassette (ABC) proteins including P-gp, multidrug resistance-associated proteins (Mrps), the breast cancer resistance proteins (Bcrp) and other unknown transporters. These efflux transporters seem to be functional for detoxication and/or prevention of nonessential compounds from entering the brain.\(^{25}\) In order to understand the brain distribution of
COP, the chemical structure, weak alkaline in vivo environment and effects of transporters should be considered. Additionally, brain concentrations remained at much higher levels for a longer time compared with plasma. This might be caused by other protein transporters, such as Mrps. Mrp1 was reported to locate at the BBB more likely in astrocyte foot processes rather than in endothelial cells and efflux its substrates from the brain to the blood \(^{26}\), but did not inhibit drug delivery from the blood to the brain.\(^{27}\) Aside from transporter-mediated considerations, other factors may also influence drug absorption. For instance, some traditional Chinese compound medicines assisted the permeation of drugs across the BBB and enhanced their distribution in the brain by increasing the content of Rh123, a substrate of P-gp in brain tissue.\(^{28}\) Furthermore, it is also of interest to consider why COP showed different profiles in the five regions. In the cortex and striatum, there were higher COP concentrations and a longer elimination half-life compared with the other brain regions. A previous study demonstrated that rat striatum has a higher density of dopamine D\(_2\) receptors compared with other brain regions.\(^{29}\) Our study supports the view that COP may be useful for the treatment of CNS disorders via receptor antagonism in the brain.\(^{30}\) On the other hand, whether there was a dose-dependent effect of COP was unknown. The disposition mechanism of COP requires further research.

**Metabolism of COP** Seventeen COP metabolites were identified in rats for the first time, including 11 unconjugated metabolites formed by hydroxylation, hydrogenation, demethylation, dehydrogenation, demethylation, and 6 glucuronide
and sulfate conjugates. All the metabolites were observed in urine. In feces, 2 unconjugated hydroxylation and 2 glucuronidation metabolites were detected, whereas 3 unconjugated and 3 conjugated metabolites were produced from liver microsome incubation, and 1 decarbonization metabolites was produced by intestinal bacteria incubation. M6 (B-ring-dehydrogenation), M9 (demethylene), and M10 (sulfate conjugates) were detected in the brain. The previously unreported COP metabolites in plasma were identified from the 0.083–24 h samples from the rat pharmacokinetic study. The glucuronide metabolites of COP were all detected in rat urine, but not in feces. This is probably a result of extensive deconjugation of the glucuronidation metabolites of COP in the intestine, and active efflux of these metabolites back to blood by the liver or intestine.31)

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Conflict of Interest The authors declare no conflict of interest.
REFERENCES


26) Sugiyama D, Kusuhara H, Lee YJ, Sugiyama Y. Involvement of multidrug


Figures

Fig. 1. Chemical Structure of COP
Fig. 2. Representative SRM Chromatograms of COP and PAL in Rat Plasma and Brain Homogenate.

(a) COP; (b) PAL; (A) Blank plasma; (B) Blank Plasma Spiked with the Analyst; (C) Plasma Sample after 5min of Intravenous Administration of COP (10 mg/kg); (D) Plasma Sample after 5 min by Oral Administration of COP (50 mg/kg); (E) Blank Brain Homogenate; (F) Blank Brain Homogenate Spiked with the Analyst; (G)–(K) the Samples of Brainstem, Hypothalamus, Hippocampus, Cortex and Striatum Samples Respectively after 5 min of Intravenous Administration of COP (10 mg/kg).
Fig. 3. Mean Plasma Concentration-time Curves of COP after (A) Oral (50 mg/kg) (●) and (B) Intravenous Administration (10 mg/kg) (▲) in Rats. (Data were resented as Mean±SD, n=6).
Fig. 4. (A) COP Concentration-time Curves in Brain and Plasma after a Single Intravenous Administration of 10 mg/kg (● Brain, ○ Plasma); (B) Brain-to-plasma Concentration Ratios for COP (The brain-to-plasma concentration ratio at each time point was calculated as a ratio of brain concentration to plasma concentration $C_{\text{brain}}/C_{\text{plasma}}$. Data were presented as Mean±SD at each time point, $n=6$) (●).
Fig. 5. The Concentration-time Curves in (A) Plasma (●) and (B) Different Brain Regions after a Single Intravenous Dose of COP (10 mg/kg) in SD Rats (Mean±SD at each time point, n = 6) (■Cortex, ○Brainstem, ●Hypothalamus, ●Hippocampus, ▲Striatum).
Fig. 6. EIC, MS and MS$^2$ Spectra of COP and Its Metabolites. (A) EIC spectra; (B1)–(B18) MS and MS$^2$ of M0–M17.
Fig. 7. Proposed Metabolic Pathways of COP (Glu: glucuronic acid)
### Tables

#### Table 1. Pharmacokinetic Parameters of COP in Rats Following Oral and Intravenous Administration (Mean±SD, n = 6)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>po (50 mg/kg)</th>
<th>iv (10 mg/kg)</th>
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<td>AUC&lt;sub&gt;0-t&lt;/sub&gt; (h×ng/ml)</td>
<td>595.58 ±123.16</td>
<td>1343.10 ±278.05</td>
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<td>C&lt;sub&gt;max&lt;/sub&gt; (ng/ml)</td>
<td>210.38 ±54.90</td>
<td>-</td>
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<td>T&lt;sub&gt;max&lt;/sub&gt; (h)</td>
<td>0.58 ±0.12</td>
<td>-</td>
</tr>
<tr>
<td>T&lt;sub&gt;1/2&lt;/sub&gt; (h)</td>
<td>0.71 ±0.39</td>
<td>0.38 ±0.17</td>
</tr>
<tr>
<td>MRT&lt;sub&gt;0-24&lt;/sub&gt; (h)</td>
<td>5.79 ±0.72</td>
<td>4.75 ±1.15</td>
</tr>
<tr>
<td>MRT&lt;sub&gt;0-inf&lt;/sub&gt; (h)</td>
<td>5.56 ±0.64</td>
<td>4.90 ±1.22</td>
</tr>
<tr>
<td>CL (L/h/kg)</td>
<td>85.97±17.47</td>
<td>7.368±1.82</td>
</tr>
<tr>
<td>F (%)</td>
<td>8.90</td>
<td>-</td>
</tr>
</tbody>
</table>

po, oral administration; iv, intravenous administration

#### Table 2. Brain and Plasma Pharmacokinetic Parameters after Intravenous Administration of a Single Dose of COP in SD Rats (10 mg/kg, Mean±SD at each time point, n = 6)

<table>
<thead>
<tr>
<th>Biosamples</th>
<th>T&lt;sub&gt;1/2&lt;/sub&gt; (h)</th>
<th>C&lt;sub&gt;max&lt;/sub&gt; (ng/g, ng/ml)</th>
<th>T&lt;sub&gt;max&lt;/sub&gt; (h)</th>
<th>AUC&lt;sub&gt;0-t,D&lt;/sub&gt; (h×ng/g/mg, h×ng/ml/mg)</th>
<th>MRT&lt;sub&gt;B-4&lt;/sub&gt; (h)</th>
<th>K&lt;sub&gt;p&lt;/sub&gt;*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain</td>
<td>4.11± 1.98</td>
<td>847.31 ± 88.28</td>
<td>0.62 ± 0.30</td>
<td>479.48 ± 61.78</td>
<td>6.01 ± 0.31</td>
<td>2.87</td>
</tr>
<tr>
<td>Plasma</td>
<td>0.97 ± 0.32</td>
<td>3373.97 ± 448.92</td>
<td>0.0833 ± 0.001</td>
<td>167.07 ± 36.30</td>
<td>2.56 ± 0.33</td>
<td></td>
</tr>
</tbody>
</table>

* The brain-to-plasma partition coefficient (K<sub>p</sub>*) of COP was calculated as a ratio of AUC (AUC<sub>brain</sub>/AUC<sub>plasma</sub>).

#### Table 3. Plasma and Brain Pharmacokinetic Parameters after Intravenous Administration of a Single Dose of COP (10 mg/kg) in SD Rats (Mean±SD, n =6)

<table>
<thead>
<tr>
<th>Biosamples</th>
<th>T&lt;sub&gt;1/2&lt;/sub&gt; (h)</th>
<th>C&lt;sub&gt;max&lt;/sub&gt; (ng/g, ng/ml)</th>
<th>T&lt;sub&gt;max&lt;/sub&gt; (h)</th>
<th>AUC&lt;sub&gt;0-t,D&lt;/sub&gt; (h×ng/g/mg, h×ng/ml/mg)</th>
<th>MRT&lt;sub&gt;B-4&lt;/sub&gt; (h)</th>
<th>F* (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brainstem</td>
<td>1.71 ± 0.35</td>
<td>171.74 ± 42.38</td>
<td>0.35 ± 0.33</td>
<td>75.65 ± 17.43</td>
<td>5.73 ± 0.34</td>
<td>45.21</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>1.13 ± 0.26</td>
<td>213.88 ± 42.33</td>
<td>0.54 ± 0.24</td>
<td>58.10 ± 19.08</td>
<td>5.76 ± 0.60</td>
<td>35.32</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>1.19 ± 0.22</td>
<td>203.48 ± 34.72</td>
<td>1.75 ± 1.08</td>
<td>68.20 ± 15.36</td>
<td>5.19 ± 0.51</td>
<td>41.26</td>
</tr>
<tr>
<td>Cortex</td>
<td>4.86 ± 2.91</td>
<td>276.52 ± 48.17</td>
<td>0.14 ± 0.09</td>
<td>149.08 ± 31.83</td>
<td>6.09 ± 0.65</td>
<td>89.10</td>
</tr>
<tr>
<td>Striatum</td>
<td>2.45 ± 0.93</td>
<td>200.02 ± 30.90</td>
<td>0.14 ± 0.09</td>
<td>116.81 ± 34.90</td>
<td>6.48 ± 0.68</td>
<td>70.63</td>
</tr>
</tbody>
</table>

* The F (bioavailability) was calculated as a ratio of AUC (AUC<sub>different brain region</sub>/AUC<sub>plasma</sub>).
<table>
<thead>
<tr>
<th>Metabolites</th>
<th>Proposed elemental compositions</th>
<th>$t_R$ (min)</th>
<th>[M]$^+$</th>
<th>*MS$^2$</th>
<th>Metabolic pathways</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>G</th>
</tr>
</thead>
<tbody>
<tr>
<td>M0</td>
<td>C$<em>{19}$H$</em>{14}$NO$_4$</td>
<td>5.8</td>
<td>320.3</td>
<td>292.1</td>
<td>M0 (COP)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>M1</td>
<td>C$<em>{18}$H$</em>{14}$NO$_7$</td>
<td>2.6</td>
<td>356.3</td>
<td>208.6</td>
<td>M0-decarbonization-hydroxylation</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>M2</td>
<td>C$<em>{25}$H$</em>{32}$NO$_{12}$</td>
<td>2.8</td>
<td>528.2</td>
<td>365.0</td>
<td>M0-hydroxylation-glucuronidation</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>M3</td>
<td>C$<em>{17}$H$</em>{14}$NO$_4$</td>
<td>3.2</td>
<td>296.1</td>
<td>191.6</td>
<td>M0-dual extremity decarbonization</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
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<tr>
<td>M4</td>
<td>C$<em>{25}$H$</em>{32}$NO$_{10}$</td>
<td>3.3</td>
<td>498.6</td>
<td>322.1</td>
<td>M0-hydrogenation-glucuronidation</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>M5</td>
<td>C$<em>{24}$H$</em>{32}$NO$_{10}$</td>
<td>4.0</td>
<td>484.4</td>
<td>308.1</td>
<td>M0-decarbonization-glucuronidation</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>M6</td>
<td>C$<em>{19}$H$</em>{12}$NO$_4$</td>
<td>4.7</td>
<td>318.4</td>
<td>195.7</td>
<td>M0-B-ring-dehydrogenation</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
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<tr>
<td>M7</td>
<td>C$<em>{29}$H$</em>{30}$NO$_{16}$</td>
<td>5.1</td>
<td>648.1</td>
<td>619.1</td>
<td>M0-decarbonization-glucuronidation</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>M8</td>
<td>C$<em>{19}$H$</em>{14}$NO$_5$</td>
<td>5.7</td>
<td>336.0</td>
<td>320.0</td>
<td>M0-monohydroxylation</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
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<td>-</td>
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</tr>
<tr>
<td>M9</td>
<td>C$<em>{18}$H$</em>{16}$NO$_4$</td>
<td>6.4</td>
<td>310.5</td>
<td>250.8</td>
<td>M0-hydrogenation-dimethylene</td>
<td>+</td>
<td>-</td>
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<td>+</td>
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<tr>
<td>M10</td>
<td>C$<em>{19}$H$</em>{14}$NO$_5$S</td>
<td>6.9</td>
<td>388.4</td>
<td>209.8</td>
<td>M0-decarbonization-sulfation</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
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<td>-</td>
</tr>
<tr>
<td>M11</td>
<td>C$<em>{18}$H$</em>{14}$NO$_4$S</td>
<td>7.9</td>
<td>307.9</td>
<td>181.6</td>
<td>M0-decarbonization</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
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<tr>
<td>M12</td>
<td>C$<em>{18}$H$</em>{14}$NO$_3$</td>
<td>8.2</td>
<td>324.4</td>
<td>206.9</td>
<td>M0-decarbonization-hydroxylation</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>M13</td>
<td>C$<em>{19}$H$</em>{14}$NO$_6$</td>
<td>9.3</td>
<td>352.5</td>
<td>335.2</td>
<td>M0-hydroxylation</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
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</tr>
<tr>
<td>M14</td>
<td>C$<em>{25}$H$</em>{32}$NO$_{10}$</td>
<td>10.1</td>
<td>472.3</td>
<td>455.2</td>
<td>M0-decarbonization-glucuronidation</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>M15</td>
<td>C$<em>{18}$H$</em>{14}$NO$_3$</td>
<td>10.3</td>
<td>323.7</td>
<td>305.1</td>
<td>M0-dihydrogen- dihydrogen</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>-</td>
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<tr>
<td>M16</td>
<td>C$<em>{19}$H$</em>{16}$NO$_4$</td>
<td>12.6</td>
<td>321.7</td>
<td>306.1</td>
<td>M0-hydrogenation (thalifendine)</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>M17</td>
<td>C$<em>{18}$H$</em>{14}$NO$_6$</td>
<td>18.4</td>
<td>340.1</td>
<td>276.2</td>
<td>M0-decarbonization-hydroxylation</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

A, urine; B, faeces; C, bile; D, plasma; E, brain; F, liver microsome incubation; G, intestinal bacteria incubation; +, found; -, not found; *Relative abundance is 100%.