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Research Article

Reduced Systemic and Brain Exposure with Inhibited Liver Metabolism of Carbamazepine After Its Long-Term Combination Treatment with Piperine for Epilepsy Control in Rats

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Abstract. Carbamazepine (CBZ) with piperine, the active ingredient in black pepper, which is omnipresent in food and may be potentially used for epilepsy control owing to its anticonvulsant effects, can be coadministered to epileptic patients. Since piperine has previously demonstrated its inhibition of the CYP3A-mediated metabolism of CBZ to carbamazepine-10,11-epoxide (CBZE), the present study aimed to investigate the impact of piperine on CBZ pharmacokinetics (PKs) in rats and pharmacodynamics in zebrafish and mouse acute seizure models. Plasma and brain PKs were studied in rats after a single-dose or 2-week combined oral administration of piperine (3.5/35 mg/kg, q.d.) and CBZ (40 mg/kg, t.i.d.) by blood sampling and brain microdialysis. Although no PK change was noticed after a single coadministration, significantly decreased plasma and brain concentrations of CBZ and CBZE with inhibited rat liver Cyp3a2 were demonstrated after long-term combined administration. Our developed compartmental model for the PK characterization of CBZ and CBZE in the blood and brain further estimated that coadministration with high-dose piperine could lead to decreases of 26%, 35%, and 38% in bioavailability, metabolism, and brain uptake of CBZ, respectively. Regardless of the PK changes, a limited impact on the antiepileptic effect of CBZ was found after the coadministration of CBZ and piperine in the tested seizure models. In conclusion, single-dose cotreatment of CBZ and piperine did not result in any significant PK or pharmacodynamic interactions, whereas their long-term cotreatment could lead to inhibited liver metabolism and the markedly reduced systemic and brain exposure of CBZ and CBZE.

KEY WORDS: Carbamazepine; Piperine; Epilepsy; Pharmacokinetics; Compartmental model.

INTRODUCTION

Carbamazepine (CBZ) remains as one of the most widely used therapies for epilepsy, with established efficacy in partial seizures, generalized tonic-clonic seizures, and mixed seizure control [1]. CBZ has narrow therapeutic index with an optimal therapeutic range of 4–12 μg/ml [2]. Several side effects of CBZ are concentration-related, such as nausea, dizziness, and blurred vision, which are mostly transient and reversible after CBZ dose adjustment [3]. Signs of toxicity, including nystagmus, diplopia, and aplastic anemia, may be developed at CBZ plasma concentrations over 10–12 μg/ml [2]. However, CBZ possesses complex pharmacokinetic (PK) properties, making its therapy more complicated. The absorption of CBZ is relatively variable and formulation dependent [4]. Approximately 70% human plasma protein binding has been found for CBZ, mainly to albumin [5, 6]. CBZ can be extensively metabolized into its major metabolite, carbamazepine-10,11-epoxide (CBZE), primarily by cytochrome P450 3A4 (CYP3A4) with minor involvement from CYP2C8, which contributes to both its anticonvulsant and neurotoxic activities [7–9]. The CYP3A4-regulated nuclear receptors, such as the pregnane X receptor (PXR) and the constitutive androstane receptor (CAR), also contribute to CBZ metabolism [10]. According to clinical guidelines, CBZ at a dose of 200–400 mg/dose for

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adults is given three to four times per day for conventional tablets or suspensions and twice per day for extended-release tablets [11].

Due to its widespread use and long-term dosing regimen for epilepsy control, CBZ is often associated with interactions when coadministered with other substances. The concentration of carbamazepine could increase, leading to signs of toxicity after its coadministration with drugs or natural products such as trazodone [12], verapamil [13], diosmin [14, 15], resveratrol [16], or grapefruit juice [17], which could be possibly attributed to the inhibition of CYP3A-mediated metabolism. Coadministration of CBZ with CYP3A4 inducers such as phenobarbital [18], felbamate [19], and a Chinese herbal medicine, Jia-wei-xiao-yao-san [20], could result in a decreased CBZ concentration, thus leading to reduced efficacy. Decreased concentrations of CBZ may also be due to reduced absorption, which has been reported after the coadministration of CBZ and cytostatic drugs such as cisplatin and adriamycin [21] and health supplement products such as ispaghula husk [22]. Displacement of CBZ from the plasma protein-binding site has also been reported by valproic acid [23], leading to an increased free concentration of CBZ.

Piperine is the principal biological component in black pepper [24]. In addition to its culinary use, several animal studies have demonstrated its potential as an anticonvulsant agent, which may be associated with a decrease in brain gamma-aminobutyric acid (GABA) levels and activation of the transient receptor potential cation channel subfamily V member 1 (TRPV1) receptor [25–27]. The antiepileptic effects of piperine have also been shown in a clinical study, in which reduced seizure frequencies were found after a 2-week treatment with a pepper extract mainly composed of piperine [28]. However, further clinical studies are required to better understand its therapeutic use in epilepsy control. Piperine also plays a unique role in affecting the PKs of other coadministered substances. Not only does it inhibit drug metabolism enzymes and transporters such as CYP3A4 and P-gp [29], it also influences drug absorption by altering gastrointestinal (GI) motility and fluid secretion [30, 31].

Previously, we demonstrated that piperine could inhibit the metabolism of CBZ via time-dependent inhibition through in vitro studies with liver microsomes [32]. Thus, an increased CBZ concentration may be found after its coadministration with piperine, and higher inhibition would be expected after a longer period of coadministration. However, in vivo PK studies need to be further conducted to evaluate the influence on each PK process. PK interactions have been reported previously in humans after the coadministration of CBZ with piperine at 20 mg/person, which resulted in an elevated CBZ plasma concentration [33, 34]. Nonetheless, piperine has only been tested at the daily consumption level, suggesting that further studies are needed on the PK interaction of CBZ with piperine at higher therapeutic doses. Moreover, only plasma PK changes have been evaluated in previous studies, which are less relevant than brain PK in the case of the pharmacological actions on the central nervous system (CNS). Since it has been suggested that the unbound drug concentration in the brain could provide a better prediction of the pharmacodynamic effect than the unbound plasma concentration or total brain concentration of a drug, the unbound brain concentration of CBZ should be monitored during its combined use with piperine [35]. In addition, the pharmacodynamic changes of CBZ in epilepsy control after the coadministration of CBZ and piperine have never been evaluated. Since the potential antiepileptic effects of piperine have been reported in several studies, its impact on the antiepileptic effects of CBZ is in need of investigation.

Therefore, the present study aimed to investigate the in vivo PK interaction between CBZ and piperine in rat brain and plasma and to explore the possible underlying mechanisms. A compartmental PK modeling approach was applied to characterize the PK process being affected. Furthermore, the impact of piperine on the antiepileptic effects of CBZ after coadministration was investigated.

MATERIALS AND METHODS

Chemicals and Reagents

CBZ, CBZE, oxidized nifedipine (ONIF), pentylenetetrazol (PTZ), pilocarpine hydrochloride, and scopolamine methyl bromide were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Piperine (purity > 97%) was obtained from Aldrich Chem. Co. (Milwaukee, WI, USA). Ketoprofen was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Other chemicals used in the microsomal activity, mRNA, and protein expression studies were used as described in our previous study [32].

Animals

The PK interaction studies of piperine and CBZ were conducted in male Sprague–Dawley (SD) rats obtained from the Laboratory Animal Services Center at the Chinese University of Hong Kong under the approval of Animal Ethics Committee of the Chinese University of Hong Kong (animal ethical approval number 17/176/MIS-5-C). The influence of piperine on the antiepileptic effects of CBZ was evaluated in male Kunming mice obtained from Nanchang University (animal ethical approval number SYXK 2015-0003) and male C57 mice obtained from the Laboratory Animal Services Center at the Chinese University of Hong Kong (animal ethical approval number 16/130/MIS-5-B). The rodents were housed in a room with a controlled temperature (22–25°C) under a 12-h/12-h light/dark cycle with a standard diet (PicoLab Rodent Diet 20, PMI Nutrition International Inc., USA) and water supplied ad libitum. Zebrafish larvae obtained from adult zebrafish (AB strain) were also used for antiepileptic effect assessment under the approval of the Animal Ethics Committee of the Nanchang University. Zebrafish embryos were raised at 28°C under constant light conditions in E3 media: 5 mmol/l NaCl, 0.17 mmol/l KCl, 0.33 mmol/l $MgSO_4$, and 0.33 mmol/l $CaCl_2$ [36]. The experiments were conducted in larvae at 7 days postfertilization (dpf).

PK Interaction Study in Rats After Combination Treatment with Piperine and CBZ

Drug Administration

Piperine (7 and 70 mg/ml) and CBZ (80 mg/ml) were dissolved in DMSO as stock solutions followed by dilution

with PEG400 and H_2O for a final volume ratio of DMSO/PEG400/H_2O 1:5:4 (*v/v/v*), which was administered to SD rats at a dose of 5 ml/kg. Altogether, 60 SD rats received different combination treatments of piperine and CBZ, with 30 rats receiving single-dose combination treatments and the other 30 rats receiving multiple-dose combination treatments. As shown in Fig. 1, for the single-dose treatments, vehicle or piperine at 3.5 or 35 mg/kg was orally administered to SD rats (230–250 g) 2 h before the oral administration of 40 mg/kg CBZ ($n = 10$ per group), and these groups were named as the CBZ group, CBZ+LD PIP group, and CBZ+HD PIP group, respectively. For the multiple-dose treatments, 40 mg/kg CBZ was given three times daily at 9:00, 13:00, and 17:00, while vehicle or piperine at 3.5 or 35 mg/kg was administered at 11:00 to SD rats (180–200 g) for 2 weeks ($n = 10$ per group). On day 14, the CBZ was only administered at 9:00 and 13:00 before PK sampling.

In addition, to study the dispositions of CBZ and CBZE in SD rats, 20 mg/kg CBZ was given to SD rats ($n = 6$) by i.v. administration. CBZ was prepared in the same dosage form with a final concentration of 16 mg/ml and was given to rats at 1.25 ml/kg.

Blood Sampling

One day before blood sampling, cannulas were implanted in the left jugular vein of the rats for blood sampling and drug administration. A Smiths Medical™ Portex™ polyethylene catheter (i.d. = 0.4 mm, o.d. = 0.8 mm, Fisher Scientific UK Ltd., UK) was implanted in the left jugular vein as we described previously [37]. Heparinized saline solution (50 UI/ml) was maintained in the cannulas to prevent the blood from clotting.

Blood samples were obtained after a single bolus dose of CBZ or after the second dose of CBZ (13:00) on the 14th day of the multiple-dose treatments. The blood samples were collected in centrifuge tubes with heparin at 0, 0.25, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 10, 20, 22, and 24 h postdosing, from which plasma samples were obtained after centrifugation at 8000 rpm for 3 min. The plasma samples were stored at − 80°C until analysis. For the rats after the i.v. administration of CBZ, plasma samples were collected at 0, 0.25, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 10, and 12 h postdosing.

Evaluation of Brain Concentration by Microdialysis

To evaluate the brain concentration of CBZ and CBZE, rats were placed in a stereotaxic instrument (Stoelting Co., IL, USA) for microdialysis guide cannula implantation at least 2 days before sampling. The rat skull was exposed after a midline incision and instrumented with a CMA/12 microdialysis guide cannula (CMA, Stockholm, Sweden) in the left hippocampus at the coordinates of − 5.2 mm anterior, 4.2 mm lateral, and 7 mm ventral relative to bregma [38]. The guide cannula was then fixed to the rat skull with screws and dental cement with the skin sutured over. The rats were allowed to recover for over 24 h.

The recovery of the brain microdialysis probe was measured 1 day before PK sampling. The CMA/12 microdialysis probe (CMA/12, 4 mm membrane length) was placed in 5 ml of microdialysis buffer consisting of 145 mmol/l NaCl, 0.6 mmol/l KCl, 1.0 mmol/l $MgCl_2$, and 1.2 mmol/l $CaCl_2$ at pH 7.4 with 1 μg/ml CBZ and 1 μg/ml CBZE at 37°C. The probe was perfused with microdialysis buffer at a flow rate of 2 μl/min using a CMA 402 syringe pump. After 1 h of equilibration, the microdialysate samples were collected every 15 min for up to 1 h for the determination of the CBZ and CBZE concentrations (C_{out}). The concentrations of CBZ and CBZE in the microdialysis buffer surrounding the probe were also determined by LC-MS/MS (C_m). The probe recovery was then calculated by Eq. (1).

$$\text{Recovery} = C_{out}/C_m \times 100\% \quad (1)$$

Along with the probe recovery, the brain extracellular fluid (ECF) concentration (C_{ECF}) could be calculated from the concentration in the microdialysate sample (C_{MD}) as $C_{ECF} = C_{MD}$ / Recovery.

On the day of PK sampling, each rat was placed in a CMA/120 system for freely moving animals with free access to water and food and its guide cannula was replaced by a CMA/12 microdialysis probe. The probe was connected with a CMA 402 syringe pump to perfuse blank microdialysis buffer at 2 μl/min for 2 h of equilibration. The brain microdialysates were collected at 0.25, 0.5, 1, 1.5, 2, 3, 4, 5, 6, 7, 8, 9, and 10 h postdosing and stored at − 80°C until analysis.

Quantification of CBZ and CBZE in Plasma and Brain Microdialysate by LC-MS/MS

The chromatographic system consisted of an Agilent 6430 triple quadrupole mass spectrometer with an electrospray ionization source (Agilent Technologies, Inc., Santa Clara, CA) and a Waters ACQUITY UPLC® BEH

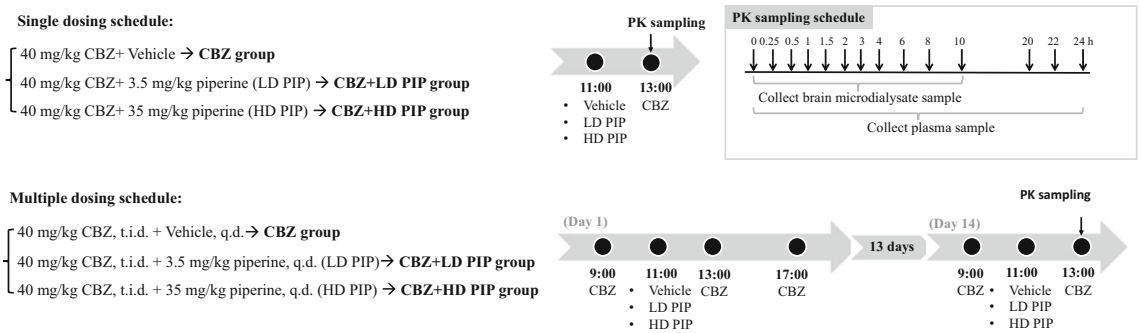


Fig. 1. Dosing and pharmacokinetic sampling schedule after single- or multiple-dose oral administration of carbamazepine (CBZ) in the absence (CBZ group) or presence of piperine (PIP) at low dose (CBZ+LD PIP group) or high dose (CBZ+HD PIP group) in rats

C_{18} column (2.1 × 50 mm, 1.7 mm) equipped with a VanGuard™ precolumn (2.1 × 5 mm, 1.7 mm, Waters Corporation, Milford, MA). The mobile phase consisted of 0.2% formic acid in water (A) and ACN (B) with flow rate of 0.15 ml/min and gradient of 30→40% B (0–3 min), 40→70% B (3–4 min), 70% B (4–8 min), and 70→30% B (8–8.1 min) till 11 min. The temperatures were set at 4°C and ambient temperature for the autosampler and analytical column, respectively. The MS/MS system was set to an ion spray voltage of 5000 V; nitrogen was set as the nebulizer gas (30 psi and 8 l/min); and the source temperature was set to 320°C. Positive mode multiple reaction monitoring was used for the detection of CBZ at *m/z* 237.1→194.1, CBZE at *m/z* 253.1→180, and berberine (internal standard, IS) at *m/z* 336.1→320.1. Berberine was adopted as the IS since the current method was originally developed for the simultaneous determination of CBZ, CBZE, and piperine, and berberine was used as the IS for the quantification of piperine in biologic matrixes in our previous study [39].

For plasma sample treatment, 20 μl of sample was mixed with 40 times the volume of IS solution (200 ng/ml berberine in MeOH and ACN (1:1, *v/v*)) and centrifuged at 13,000 rpm for 10 min. After centrifugation, 2 μl of supernatant was injected into the LS-MS/MS system. The standard curves were linear within the range of 9.8 ng/ml to 10 μg/ml for both CBZ and CBZE. The intra-day and inter-day precisions were within 10.4% for CBZ and 9.8% for CBZE, while the accuracies were within 10.4% and 7.5% for CBZ and CBZE, respectively. The extraction recoveries for CBZ and CBZE were over 86.0 ± 1.6%.

For the brain microdialysate sample, 25 μl of sample was mixed with 25 μl of IS solution, after which a 2-μl aliquot was injected into the LC-MS/MS system. The standard curves were linear within the range of 2 to 1000 ng/ml for both CBZ and CBZE. The intra-day and inter-day precisions were within 11.6% for CBZ and 12.8% for CBZE, while the accuracies were within 11.8% and 6.6% for CBZ and CBZE, respectively.

Mechanistic Study on the Impact of Piperine on CBZ PK

Impact of Piperine on CBZ Metabolism

Hepatic Cyp3a Enzyme Activity in Rats

To evaluate the changes in rat Cyp3a enzyme activity after different treatments, liver microsomes were prepared from naïve rats (control group) and rats that received CBZ treatment in the absence or presence of piperine coadministration ($n = 6$ for each treatment group). After being sacrificed, rats were perfused with ice-cold saline for liver collection. Half of the liver was cut into small pieces, and the liver samples from two rats in the same dosing group were pooled for microsome preparation as described previously [32]. After analyzing the protein concentration of the prepared microsomes, 1 mg/ml microsome was incubated with nifedipine, a CYP3A substrate, and 6 mmol/l $MgCl_2$ in 50 mmol/l phosphate buffer (pH 7.4). Nifedipine was added to the incubation mixture at a concentration of 80 μmol/l with MeOH at a concentration of less than 1% (*v/v*). After 5 min of preincubation at 37°C, the reaction was initiated by the addition of 5 mmol/l NADPH and terminated after 10 min by the addition of an ice-cold organic solution containing 20 μg/ml ketoprofen as the IS in ACN/MeOH = 1:1 (*v/v*). The mixtures were centrifuged at 13,000 rpm for 10 min at 4°C, after which 20 μl of the supernatants was subjected to HPLC/UV analysis. The ONIF formations were analyzed on an HPLC/UV system consisting of a Waters 2690 Separations module and a Waters 996 Photodiode Array Detector. The formed ONIF was detected at 240 nm after separation on an Eclipse XDB-C_{18} column (4.6 × 250 mm, 5 μm, Agilent Technologies, Inc.) with a mobile phase consisting of 20 mmol/l NaH_2PO_4 buffer/ACN (58:42, *v/v*) at a flow rate of 1 ml/min. The formation rates of ONIF were calculated and compared among the different treatment groups.

mRNA and Protein Expression of the Genes Involved in CBZ Metabolism in Rats

The mRNA expression levels of the genes involved in CBZ metabolism in the rat livers were analyzed and compared between the different treatment groups. Liver samples from two rats in the same treatment group were pooled for RNA isolation as described previously [32]. The RT-PCR was conducted to analyze the mRNA levels of rat Cyp3a1, Cyp3a2, Cyp2c13, Pxr, and Car after correction with its Gapdh. The fold of changes for the target gene expression levels were determined by $2^{-\Delta(\Delta Ct)}$, where $\Delta Ct = Ct(target) - Ct(GAPDH)$ and $\Delta(\Delta Ct) = \Delta Ct(treated) - \Delta Ct(vehicle\ control)$.

The protein expression of the genes with changes in the mRNA levels was further analyzed by Western blotting as described previously [32]. The liver samples were also pooled from two rats in the same group and denatured for separation with a 10% (*w/v*) SDS gel. The separated gels were transferred and blocked, followed by incubation with primary antibodies (rat Cyp3a2, Car, and Gapdh) and a secondary antibody (horseradish peroxidase-labeled antirabbit IgG). The immunoblot bands were visualized and analyzed with Image Lab™ (Bio-Rad Laboratories, Inc., CA, USA).

Effect of Piperine on CBZ and CBZE Plasma Protein and Brain Tissue Binding

To evaluate the influence of piperine on the binding of CBZ and CBZE in plasma and brain, the percent binding in the presence or absence of piperine was determined using equilibrium dialysis [40]. The concentrations of piperine, CBZ, and CBZE were correlated to their in vivo plasma concentration in rats (0.01–10 μg/ml for CBZ and CBZE and 0–2.5 μg/ml for piperine), and experimental runs were designed by MODDE 6.0 (Umetrics, Umeå, Sweden) with a D-optimal design to ensure that piperine and CBZ or CBZE were tested at each concentration level [41]. A total of 24 randomized experiments including four replicates at the center point were tested to evaluate the binding in plasma or brain (Table I). The effects of piperine on the binding of CBZ and CBZE were analyzed with an interaction plot.

Table I. Concentrations of Carbamazepine (CBZ), Carbamazepine-10,11-Epoxide (CBZE), and Piperine in Each Experimental Group for Evaluating the Impact of Piperine on the Plasma Protein and Brain Binding of CBZ and CBZE

Experiment no.	CBZ concentration (μg/ml)	CBZE concentration (μg/ml)	Piperine concentration (μg/ml)
1	0.01	0	0
2	10	0	0
3	0.01	0	2.5
4	10	0	2.5
5	0.01	0	1.67
6	10	0	0.83
7	6.67	0	0
8	3.34	0	2.5
9	5	0	1.25
10	5	0	1.25
11	5	0	1.25
12	5	0	1.25
13	0	0.01	0
14	0	10	0
15	0	0.01	2.5
16	0	10	2.5
17	0	0.01	1.67
18	0	10	0.83
19	0	6.67	0
20	0	3.34	2.5
21	0	5	1.25
22	0	5	1.25
23	0	5	1.25
24	0	5	1.25

Further Illustration of the PK Interaction Between Piperine and CBZ by Compartmental PK Modeling

PK Model Development for CBZ and CBZE in Rats

The compartmental PK modeling approach was used to characterize the plasma and brain concentrations of CBZ and CBZE in rats with NONMEM 7.3 (Icon Development Solutions, Ellicott City, Maryland) and Perl-speaks-NONMEM (Uppsala, Sweden, version 4.2.2) installed on a Microsoft Windows 7 Professional 2009 64-bit operation system. The first-order conditional estimation method with interaction (FOCEI) was used. Model development was started by assessing the disposition of CBZ and CBZE with the plasma concentration data after i.v. administration. One-compartment model was used to describe the disposition for both CBZ and CBZE with first-order clearance parameterized in terms of the total CBZ clearance (Cl), fraction of clearance for CBZE formation ($FMET$), total CBZE clearance (Cl_m), and volume of distribution of CBZ (V) and CBZE (V_m). The plasma concentrations after oral administration of CBZ were further added into the model to determine the absorption process. First-order absorption parameterized as the absorption rate constant (k_a) with bioavailability (F) was used for CBZ, and the first-pass metabolism fraction (F_m) with the same absorption rate constant (k_a) was included for CBZE. Thus, the amount of CBZ in depot (A_1) and plasma (A_2), and the amount of CBZE in depot (A_4) and plasma (A_3) could be described using differential equations as follows (Eqs. (2)–(5)):

$$\frac{dA_1}{dt} = -k_a \times A_1 \tag{2}$$

$$\frac{dA_4}{dt} = -k_a \times A_4 \tag{3}$$

$$\frac{dA_2}{dt} = k_a \times A_1 - \frac{Cl}{V} \times A_2 \tag{4}$$

$$\frac{dA_3}{dt} = k_a \times A_4 + \frac{FMET \times Cl}{V} \times A_2 - \frac{Cl_m}{V_m} \times A_3 \tag{5}$$

The initial conditions for the above equations were defined as $A_1(0) = 0$, $A_2(0) = 0$, $A_3(0) = 0$, and $A_4(0) = 0$.

Once the structural model for CBZ and CBZE in plasma was constructed, their distributions from plasma to brain were further evaluated by incorporating the brain concentrations into the model. The flow-limited model was used with the assumption of instantaneous equilibrium between the unbound brain and unbound plasma concentrations [42]. The unbound amount in the brain for CBZ (A_5) and CBZE (A_6) can be described as follows (Eqs. (6)–(7)):

$$A_5 = \frac{A_2 \times f_{u,plasma,CBZ}}{V} \times Kp \times \ Vbr \tag{6}$$

$$A_6 = \frac{A_3 \times f_{u,plasma,CBZE}}{V} \times Kp_m \ \times Vbr \tag{7}$$

where Kp and Kp_m are the partition ratio of unbound brain to unbound plasma concentration for CBZ and CBZE, respectively, and V_{br} is fixed to 1.8 ml as the rat brain volume of distribution [43].

The inter-individual variability in PK parameters was assumed to be log-normally distributed and described by the exponential model $P_{ij} = P_i exp(\eta_{ij})$, where P_{ij} is the jth parameter for the ith individual and P_j is the typical parameter value at the population value. The deviation of P_{ij} from P_j in the jth parameter for the ith individual is described by η_{ij}, which is normally distributed with a mean of zero and a variance (ω^2) to be estimated. Residual error for estimating the unexplained variability was estimated with a proportional error model with the equation: $C_{ij} = \Upsilon_{PRED,ij} \times (1 + \varepsilon_{ij})$, where C_{ij} and $\Upsilon_{PRED,ij}$ are the jth observed and predicted concentration of the ith individual, respectively, and ε_{ij} is the random effect variable normally distributed with a mean of zero and a variance (σ^2) to be estimated.

To evaluate the change in PK parameters in the different treatment groups, the brain and plasma concentrations from all treatment groups were added to the data input. The influence of the interaction was estimated as the change in the typical population parameters (f): $\theta_{p,I} = \theta_p \times f$, where θ_p is the typical population estimation and $\theta_{p,I}$ is the changed population parameters in specific treatment group. Thus, the changes in F, $FMET$, and Kp in the different treatment groups were denoted as f_F, f_{FMET}, and f_{Kp}, respectively. The NONMEM control stream for the final model is included in the supplementary material.

Model Evaluation

The model evaluation was based on the likelihood ratio test for nested models. Model selection was guided by a drop of 3.84 in the objective function value (OFV) standing for a significance level of 0.05. Predictions versus observations were plotted by R (RStudio Inc., MA, USA, version 1.1.45) as the initial visualized check for model performance. The final model was further examined by visual predictive checks (VPC) after simulation of 200 replicates of the original study with the observed data plotted overlaid within the 95% predictive interval of the simulated data.

Impact on the Antiepileptic Effects of CBZ After Its Coadministration with Piperine in Animal Models with Acute Epileptic Seizure

Zebrafish Model with PTZ-Induced Acute Epileptic Seizures

The impact of piperine on the antiepileptic effects of CBZ was first evaluated in zebrafish with PTZ-induced acute epileptic seizures as described previously [36]. The 7 dpf zebrafish larvae were collected and incubated in 96-well plates with 100 μl of E3 media per well containing 5 mmol/l PTZ for the PTZ group; 5 mmol/l PTZ and 100 μmol/l CBZ for the CBZ group; 5 mmol/l PTZ and 315 μmol/l piperine for the piperine group; and 5 mmol/l PTZ, 100 μmol/l CBZ, and 315 μmol/l piperine for the combination treatment group (n = 18–20). The compounds were dissolved in DMSO and diluted in E3 media to a final concentration of DMSO of 1% (*v/v*). Zebrafish in the control group were incubated in E3 media without PTZ or drug treatment. Zebrafish were allowed to habituate for 10 min after being transferred into the dark chamber coupled with a temperature control unit and an automated tracking device in the DanioVision observation chamber (Noldus Information and Technology, Wageningen, The Netherlands). Zebrafish locomotor activity analysis was performed by comparing the moved distances for 20 min between the different groups using EthoVision XT11.5 software (Noldus Image Analysis program, Wageningen, The Netherlands).

Mouse Model with Pilocarpine-Induced Acute Epileptic Seizures

The influences on the antiepileptic effects of CBZ by piperine were further evaluated in a mouse model with epileptic seizures induced by high-dose pilocarpine. A total of 32 male C57 mice (25–30 g) were separated into 4 groups receiving vehicle: CBZ (10 mg/kg), piperine (70 mg/kg), or piperine (70 mg/kg), and CBZ (10 mg/kg) by oral administration. Piperine and CBZ were given 60 and 30 min, respectively, prior to pilocarpine hydrochloride injection (350 mg/kg, i.p.). Scopolamine methyl bromide (1 mg/kg, i.p.) was given to mice 25 min before pilocarpine injection to prevent the side effects of pilocarpine-induced peripheral cholinergic stimulation [44]. Piperine and CBZ were prepared in DMSO/PEG400/H_2O 1:5:4 (*v/v/v*) at final concentrations of 2 and 14 mg/ml for oral administration, respectively, while pilocarpine hydrochloride and scopolamine methyl bromide were dissolved in saline for injection. The seizure severity of the mice after pilocarpine injection was assessed according to the seizure score from Racine's scale [45] with slight modifications: 0, normal nonepileptic activity; 1, immobilization; 2, head nodding, partial myoclonus; 3, continuous whole body myoclonus; 4, rearing and tonic seizure; and 5, falling, status epilepticus or death. The maximum seizure scores in every 10 min interval during the 1.5-h observation period were recorded and compared between the different groups.

Mouse Model with PTZ-Induced Acute Epileptic Seizures

A model with epileptic seizures induced by PTZ in male Kunming mice (35–40 g) was also applied to assess the influence of piperine on the antiepileptic effects of CBZ. Altogether, 48 mice were divided into 8 groups receiving vehicle, CBZ (20 mg/kg) and piperine (3, 10, and 20 mg/kg) either separately or in combination. Piperine and CBZ were prepared in DMSO/PEG400/H_2O 1:5:4 (*v/v/v*) at a final concentration of 10 mg/ml and were orally administered to mice 2 and 1.5 h prior to PTZ injection (80 mg/kg, i.p.), respectively. After PTZ injection, the latency to the first tonic-clonic seizure and the frequency of the seizures within 30 min were recorded and compared between the different treatment groups.

Data Analyses

All experimental data are expressed as the mean plus standard deviation (± S.D.). PK parameters including the maximum concentration (C_{max}), time to reach C_{max} (T_{max}), concentration before last dose (C_0), half-life ($T_{1/2}$), and area under the curve for the monitored time period ($AUC_{0\rightarrow t}$) were calculated by WinNonlin version 2.1 (Pharsight Corporation, Mountain View, CA) using a noncompartmental approach (NCA). Statistical differences between multiple groups in the microsomal activity assay, mRNA and protein expression analysis, and total moved distance in the zebrafish seizure model were evaluated by one-way analysis of variance (ANOVA) followed by post hoc Tukey's test. In mouse seizure models, the seizure score and seizure frequency were evaluated by a nonparametric Kruskal-Wallis test followed by Dunn's multiple comparisons test, while the latencies to first seizure were analyzed by the nonparametric Log-rank (Mantel-Cox) test between different groups. A p value less than 0.05 was considered statistically significant during the test. All statistical analyses were performed on GraphPad Prism (version 3.03 GraphPad Software, La Jolla, CA).

RESULTS

Brain and Plasma PK of CBZ and CBZE in Different Treatment Groups

The plasma concentrations of CBZ and CBZE after single or multiple oral administration of CBZ in the absence or presence of piperine at different doses were plotted against the time since last CBZ dose and analyzed first by NCA (Fig. 2, left). Among the single-dose treatment groups, similar plasma concentration levels of CBZ and CBZE were found with no statistically significant difference in the $T_{1/2}$, T_{max}, C_{max}, or $AUC_{0\rightarrow 24\ h}$ of CBZ and CBZE (Table II). For the multiple-dose treatment groups, lower plasma concentrations for both CBZ and CBZE were observed in the CBZ and piperine cotreatment groups. Coadministration with a higher dose of piperine resulted in lower plasma concentrations of CBZ and CBZE. Compared to the CBZ group, the C_0, C_{max}, and $AUC_{0\rightarrow 24h}$ of CBZ were significantly decreased in the CBZ+LD PIP group ($p < 0.05$), while the C_{max}, $AUC_{0\rightarrow 24h}$, and C_0 of CBZ together with the C_{max} and $AUC_{0\rightarrow 24h}$ of CBZE were significantly decreased in the CBZ+HD PIP group ($p < 0.01$). For the multiple CBZ+LD PIP and CBZ+HD PIP treatment groups, the plasma $AUC_{0\rightarrow 24h}$ of CBZ was found to be 71.0% and 62.8% of that in the CBZ group, while the $AUC_{0\rightarrow 24h}$ of CBZE was found to be 81.0% and 61.9% of that in the CBZ group, respectively. Therefore, multiple combined administrations of CBZ with piperine could significantly decrease the systemic exposure of CBZ and CBZE. The ratio of plasma $AUC_{0\rightarrow 24h,CBZE}/AUC_{0\rightarrow 24h,CBZ}$ for each individual was further calculated and compared between the different groups to assess the influence on CBZ metabolism after coadministration with piperine. The ratios obtained after single-dose treatments showed no significant difference between different groups, while among the multiple-dose treatment groups, a significant decrease in this ratio was found in CBZ+HD PIP group compared to that in the CBZ group, indicating that long-term coadministration of CBZ with high-dose piperine could lead to inhibited CBZ metabolism.

During plasma sampling, brain microdialysis was applied to simultaneously monitor the changes in the concentrations of CBZ and CBZE in the brain hippocampus. The concentrations of CBZ and CBZE in the microdialysates were adjusted with probe recovery to obtain brain ECF concentrations and plotted against the time since last CBZ dose. Similar brain concentrations of CBZ and CBZE were observed between different groups after single-dose treatment (Fig. 2, right), with no significant difference in their calculated PK parameters (Table II). Among the multiple-dose treatment groups, lower brain concentrations of CBZ were observed in the piperine coadministration groups. Significant decreases in the CBZ C_{max} and $AUC_{0\rightarrow 10h}$ and a decreasing trend in the CBZE $AUC_{0\rightarrow 10h}$ from the CBZ+HD PIP group were found compared with those from the CBZ group. The changes in the CBZ and CBZE concentrations in the brain were comparable to those in plasma in the different treatment groups.

Inhibition of Rat Liver Cyp3a2 with Unaltered Plasma Protein and Brain Tissue Binding of CBZ After Its Long-Term Combined Use with Piperine

The impact of piperine on CBZ metabolism after their combined use was first evaluated by hepatic rat Cyp3a2 activity (via the formation rate of ONIF) in the liver microsomes of rats receiving various treatments. In the single-dose treatment groups, compared to the rat Cyp3a2 activity in the CBZ group (1.64 ± 0.18 nmol/min/mg), a significant decrease was found in the CBZ+HD PIP group (1.18 ± 0.14 nmol/min/mg, $p < 0.05$) with no significant change in the CBZ+LD PIP group (1.35 ± 0.27 nmol/min/mg, $p > 0.05$). In the multiple-dose treatment groups, significantly lower rat Cyp3a2 activity was found in the CBZ+LD PIP group (1.48 ± 0.16 nmol/min/mg, $p < 0.05$) and the CBZ+HD PIP group (1.26 ± 0.12 nmol/min/mg, $p < 0.01$) compared with the CBZ group (2.05 ± 0.10 nmol/min/mg). In addition, the rat Cyp3a2 activity after multiple treatments of CBZ with high-dose piperine was also significantly lower than that of the control group (1.77 ±

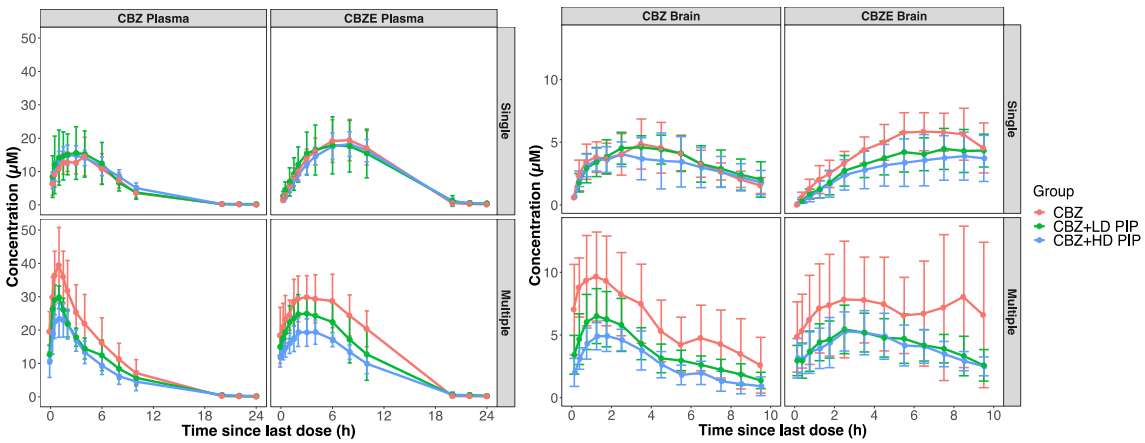


Fig. 2. Concentrations of carbamazepine (CBZ) and its metabolite, carbamazepine-10,11-epoxide (CBZE), in the rat plasma (left) and brain (right) after the single- or multiple-dose oral administration of CBZ (40 mg/kg, bolus dose or t.i.d. for 14 days) in the absence (CBZ group) or presence of piperine (PIP) at 3.5 mg/kg (CBZ+LD PIP group) or 35 mg/kg (CBZ+HD PIP group) (bolus dose or q.d. for 14 days). Each point represents mean ± S.D. ($n = 10$ for plasma concentration, $n = 5$ for brain concentration)

Table II. PK Parameters of Carbamazepine (CBZ) and Carbamazepine-10,11-Epoxide (CBZE) in the Rat Plasma and Brain After Oral Administration of 40 mg/kg CBZ (Bolus Dose or t.i.d. for 14 Days) in the Absence (CBZ Group) or Presence of Piperine (PIP) at 3.5 mg/kg (CBZ+LD PIP Group) or 35 mg/kg (CBZ+HD PIP Group) (Bolus Dose or q.d. for 14 Days)

PK parameters			Single-dose groups			Multiple-dose groups		
			CBZ	CBZ+LD PIP	CBZ+HD PIP	CBZ	CBZ+LD PIP	CBZ+HD PIP
Plasma	CBZ	$T_{1/2}$ (h)	2.54 ± 0.92	2.47 ± 0.85	2.66 ± 1.63	1.91 ± 0.71	2.57 ± 0.63	2.82 ± 0.95
		T_{max} (h)	2.45 ± 1.19	2.88 ± 1.90	2.57 ± 1.10	0.97 ± 0.43	0.78 ± 0.26	1.33 ± 0.79
		C_{max} (μmol/l)	16.2 ± 4.7	18.4 ± 7.1	15.8 ± 2.6	41.3 ± 8.4	30.9 ± 3.6*	25.0 ± 5.0**
		$AUC_{0\rightarrow24h}$ (μmol h/l)	116 ± 32	131 ± 54	138 ± 26	240 ± 69	171 ± 40*	151 ± 34**
		C_0 (μmol/l)	NA	NA	NA	1.78 ± 0.43	2.08 ± 1.53	1.47 ± 0.19
	CBZE	$T_{1/2}$ (h)	1.78 ± 0.43	2.08 ± 1.53	1.47 ± 0.19	2.23 ± 1.12	1.79 ± 0.50	2.17 ± 0.99
		T_{max} (h)	7.20 ± 1.69	6.75 ± 1.83	7.43 ± 0.98	3.63 ± 2.05	4.00 ± 2.12	3.44 ± 1.59
		C_{max} (μmol/l)	20.0 ± 6.4	19.9 ± 9.0	18.6 ± 3.8	31.5 ± 6.6	26.7 ± 5.9	20.2 ± 3.6**
		$AUC_{0\rightarrow24h}$ (μmol h/l)	230 ± 73	205 ± 77	211 ± 57	335 ± 91	271 ± 89	207 ± 39**
		C_0 (μmol/l)	NA	NA	NA	18.4 ± 8.4	15.0 ± 3.5	12.2 ± 3.2
	$AUC_{0\rightarrow24h,CBZE}/AUC_{0\rightarrow24h,CBZ}$		1.81 ± 0.25	1.74 ± 0.86	1.64 ± 0.42	1.81 ± 0.33	1.51 ± 0.30	1.38 ± 0.30*
Brain	CBZ	T_{max} (h)	3.19 ± 1.38	3.35 ± 0.99	2.38 ± 1.59	1.71 ± 1.05	1.25 ± 0.41	1.60 ± 0.65
		C_{max} (μmol/l)	5.13 ± 2.01	4.98 ± 1.22	4.52 ± 1.62	9.86 ± 3.24	6.84 ± 2.21	5.43 ± 0.58*
		$AUC_{0\rightarrow10h}$ (h μmol/l)	32.4 ± 10.8	32.7 ± 9.8	29.2 ± 11.2	50.7 ± 23.4	35.0 ± 8.7	24.1 ± 6.2*
	CBZE	T_{max} (h)	7.25 ± 0.96	7.30 ± 1.48	7.75 ± 2.22	4.04 ± 3.10	3.00 ± 1.00	2.58 ± 1.81
		C_{max} (μmol/l)	6.24 ± 1.89	5.05 ± 1.37	4.12 ± 1.79	8.27 ± 4.47	6.06 ± 1.88	5.75 ± 1.53
		$AUC_{0\rightarrow10h}$ (μmol h/l)	40.9 ± 11.0	30.7 ± 10.4	26.7 ± 13.0	48.4 ± 16.9	40.5 ± 11.6	35.9 ± 14.6

NA not applicable
*$p < 0.05$, compared with the CBZ group, **$p < 0.01$, compared with the CBZ group

0.06 nmol/min/mg). Thus, the combined use of CBZ with piperine could inhibit the metabolism of CBZ through the inhibition of rat Cyp3a2 activity in liver microsomes.

The mRNA expression levels of the genes involved in CBZ metabolism (rat Cyp3a1, Cyp3a2, Cyp2c13, Pxr, and Car) were evaluated in the livers of rats that received various treatments. As shown in Fig. 3a, no significant difference in mRNA expression of all tested genes was found between the different groups after single-dose treatments, while significant decreases were found in both rat Cyp3a2 and Car expression after multiple-dose CBZ treatment with high-dose piperine compared with that after multiple-dose CBZ treatment alone ($p < 0.05$). Due to these observed changes, the protein expression levels of rat Cyp3a2 and Car were further assessed in the rat livers of the multiple-dose treatment groups. The results showed that only rat Cyp3a2 protein expression was significantly decreased ($p < 0.05$) in the CBZ+HD PIP group compared to that in the CBZ group (Fig. 3b), indicating that multiple doses of CBZ in combination with high-dose piperine can inhibit CBZ metabolism by inhibiting rat Cyp3a2 and Car mRNA expression and rat Cyp3a2 protein expression.

The binding of CBZ and CBZE to rat plasma proteins and brain tissue was evaluated in the absence and presence of piperine. At different concentrations (0.01–10 μg/ml), CBZ showed similar binding to rat plasma proteins (65.9 ± 2.5%), while its metabolite, CBZE, demonstrated lower binding in plasma (37.5 ± 1.1%). No significant difference was found between their bindings in the absence (64.1 ± 2.8% for CBZ and 37.4 ± 7.8% for CBZE) or presence (66.5 ± 2.3% for CBZ and 37.6 ± 1.2% for CBZE) of piperine. Compared to their plasma binding, a higher binding towards rat brain tissue was found for CBZ (83.1 ± 0.4%) and CBZE (67.3 ± 1.3%). Their brain tissue binding was also not affected by the absence (83.0 ± 0.3% for CBZ and 67.6 ± 0.1% for CBZE) or presence (83.1 ± 0.4% for CBZ and 67.2 ± 1.5% for CBZE) of piperine. In addition, only parallel lines were demonstrated in the interaction plots for plasma protein and brain tissue binding of CBZ and CBZE with piperine at different concentrations (Supplemental Fig. 1), indicating that piperine has a limited effect on the binding of CBZ and CBZE. Therefore, limited impact by piperine was found on the binding of CBZ and CBZE in rat plasma or brain.

Further PK Modeling Illustration of the Reduced Bioavailability and Metabolism and Brain Penetration of CBZ by the Long-Term Coadministration of Piperine

As shown in Fig. 4, CBZ and CBZE plasma concentrations best fitted to a one-compartment model with first-order absorption. The PK parameters estimated from the final model showed that the total clearance of CBZ was 114 ml/h, which was close to the reported value of 120 ml/h after the i.v. administration of 20 mg/kg CBZ in rats from a previous study [46]. The estimated FMET value of 0.58 suggested that 58% of the total CBZ clearance was from the formation of CBZE, which was within the reported range of 0.4–0.7 for the formation fraction of CBZE [46–48]. The model also showed an absolute CBZ bioavailability of 0.4 with no extensive first-pass metabolism based on the F_m of 0.025 after oral administration. Assuming that the unbound CBZ and CBZE were in instantaneous equilibrium across the BBB, the partition ratios of the unbound brain concentration to unbound plasma concentration (Kp and Kp_m) were estimated to be 1.43 for CBZ and 0.61 for CBZE, which are in accordance with the higher brain penetration properties of

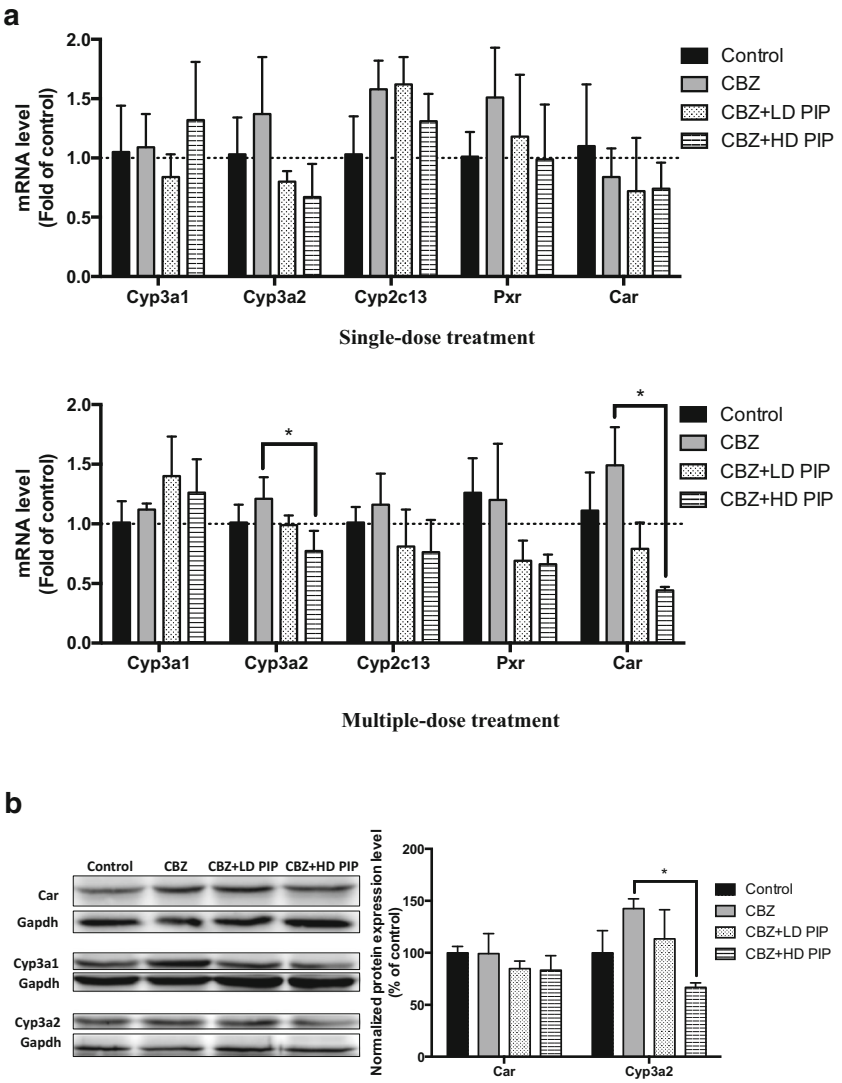
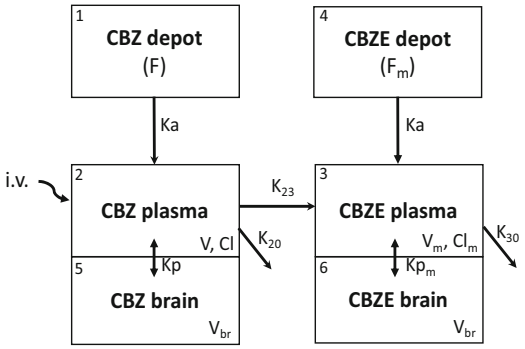


Fig. 3. Comparison of liver mRNA expression levels of the genes involved in carbamazepine (CBZ) metabolism from rats after single- or multiple-dose oral administration of CBZ (40 mg/kg, bolus dose or t.i.d. for 14 days) in absence (CBZ group) or the presence of piperine (PIP) at 3.5 mg/kg (CBZ+LD PIP group) or 35 mg/kg (CBZ+HD PIP group) (bolus dose or q.d. for 14 days) or vehicle (Control group) (**a**) and further comparison of rat Car and Cyp3a2 protein expression levels after multiple-dose treatment of CBZ with piperine (**b**) (*$p < 0.05$, compared with the CBZ group). Each point represents mean ± S.D. ($n = 3$)

CBZ than of CBZE [49]. The volume of brain distribution was fixed to its physiological value of total rat brain volume with the assumption that CBZ and CBZE were in equilibrium between the intracellular and extracellular fluid.

The initial estimation with the data from all different treatment groups showed large inter-individual variability (IIV) for the PK parameters, such as an IIV of 0.77 for FMET and an IIV of 0.43 for *F*. Therefore, the individual PK parameters were stratified according to their treatment group to verify the difference between the different treatment groups. The parameters were similar among the single-dose treatment group, while decreasing trends were found for *F* and FMET for in the multiple-dose CBZ and piperine coadministration groups. Thus, the percentage of change in *F* (f_F) was added into the model for the individuals after multiple combination treatments. After adding f_F, a significant OFV drop of 23.6 was found for the new model, indicating that *F* was significantly different between the groups after multiple treatments of CBZ in the absence or presence of piperine. Similarly, the parameters for the percentages of change in FMET (f_{FMET}) and Kp (f_{Kp}) were subsequently added for the individuals after multiple combination treatments, which resulted in OFV decreases of 10.6 and 5.84, respectively, suggesting that FMET and Kp were significantly changed by the coadministration of piperine after long-term treatment. As parameter estimations indicated in Fig. 4, multiple doses of CBZ with the coadministration of low-dose piperine caused an 8% decrease in *F*, a 29% decrease in



Parameter estimation in the final compartmental PK model

PK parameters	Estimates (RSE %)	IIV (RSE %)[Note]
Cl (ml/h)	114 (8)	0.16 (20)
V (ml)	327 (8)	0.17 (18)
k_a (/h)	0.5 (10)	0.45 (18)
F	0.4 (10)	0.34 (16)
Cl_m (ml/h)	53.4 (17)	
V_m (ml)	101 (21)	
FMET	0.58 (17)	
F_m	0.025 (28)	
Kp	1.43 (10)	0.34 (12)
Kp_m	0.61 (8)	0.35 (16)
V_{br} (ml)	1.8 FIXED	

Interaction parameters	Multiple-dose groups		
	CBZ	CBZ+LD PIP	CBZ+HD PIP
f_F (RSE%)	1	0.92 (8)	0.74 (10)
f_{FMET} (RSE%)	1	0.71 (12)	0.65 (13)
f_{Kp} (RSE%)	1	0.94 (21)	0.62 (14)

[Note] IIV = inter-individual variability; RSE% = percent relative standard error

Fig. 4. The model structure and parameter estimations of the compartmental pharmacokinetic model for describing the distribution of carbamazepine (CBZ) and carbamazepine-10,11-epoxide (CBZE) in the rat plasma and brain after single- or multiple-dose oral administration of CBZ (40 mg/kg, bolus dose or t.i.d. for 14 days) in the absence (CBZ group) or presence of piperine (PIP) at 3.5 mg/kg (CBZ+LD PIP group) or 35 mg/kg (CBZ+HD PIP group) (bolus dose or q.d. for 14 days) or i.v. bolus administration of CBZ (20 mg/kg)

FMET, and a 6% decrease in Kp. Compared with low-dose piperine, coadministration with a high dose of piperine resulted in a more significant change in *F*, FMET, and Kp, with decreases of 26%, 35%, and 38%, respectively.

Good precision was demonstrated for the parameter estimation in the final model with an RSE of less than 30% for each parameter. As shown in the observation versus prediction plot (Supplemental Fig. 2), population predictions from the final model were in line with the observations for both CBZ and CBZE concentrations in plasma and brain from each treatment group. As the VPC plot demonstrates in Fig. 5, after stratification of the data by its treatment groups, the final model adequately described the average exposure over time for CBZ and CBZE in plasma and brain. Lower CBZ and CBZE concentrations in the brain and plasma after long-term combination treatment could be captured after introducing the difference in *F*, FMET, and Kp.

No Influence on the Antiepileptic Effects of CBZ by Piperine in Animal Models with Epileptic Seizures

The impact on the antiepileptic effects of CBZ after its coadministration with piperine was evaluated in multiple animal models. In the zebrafish seizure model (Fig. 6a), 5 mmol/l PTZ could significantly elevate the moved distance of zebrafish ($p < 0.01$, compared to the control), indicating the successful development of seizure-like behaviors in the zebrafish model. Significantly reduced moved distances were demonstrated after the addition of 100 μmol/l CBZ to the media, suggesting that CBZ at 100 μmol/l could effectively prevent the PTZ-induced seizures. Piperine alone at 315 μmol/l did not suppress zebrafish motility after PTZ induction ($p > 0.05$). Although the combined use of 315 μmol/l piperine with 100 μmol/l CBZ significantly reduced motility after PTZ induction ($p < 0.001$), no further reduction in moved distance was found compared with the CBZ alone group ($p > 0.05$), suggesting no impact on the antiepileptic effects of CBZ by piperine in the PTZ-induced seizure zebrafish model. In the mouse model of pilocarpine-induced epileptic seizures (Fig. 6b), pretreatment with 10 mg/kg CBZ significantly reduced the seizure scores after 10 min of pilocarpine injection compared to that of the control group ($p < 0.05$), suggesting effective protection of pilocarpine-induced seizures by CBZ. Significant reductions in seizure scores were also found in mice receiving piperine at 70 mg/kg alone or in combination with 10 mg/kg CBZ 20 min after of pilocarpine injection. However, no significant difference in the seizure scores was found between the combination treatment groups and the CBZ alone group, indicating that coadministration of piperine did not influence the antiepileptic effects of CBZ in a pilocarpine-induced seizure mouse model. In the PTZ-induced mouse seizure model (Fig. 6c, d), CBZ at 20 mg/kg significantly prolonged the latency to the first seizures compared to that of the control groups. The antiepileptic effects of piperine at 3, 10, and 20 mg/kg in the absence or presence of 20 mg/kg CBZ were evaluated, and a significant delay in the onset of seizures was found in the combination treatment groups compared to the control group. However, no significant difference in seizure times or latency to the first seizure was found between the combination treatment groups and the CBZ alone group, which was consistent with the results from the other two studied seizure models, suggesting that coadministration of piperine would not influence the antiepileptic effects of CBZ.

DISCUSSION

A comprehensive analysis of the PK interactions of CBZ after its coadministration with piperine was conducted in the present study. In the PK study, piperine was given to rats at doses of 3.5 and 35 mg/kg, which were associated with the daily intake of piperine from black pepper in food and the effective dose of piperine from black pepper for epilepsy control in a clinical study, respectively [28, 50]. The average daily consumption of black pepper is approximately 0.7 g/person, while a dosage of 4–8 g pepper/day/person was used

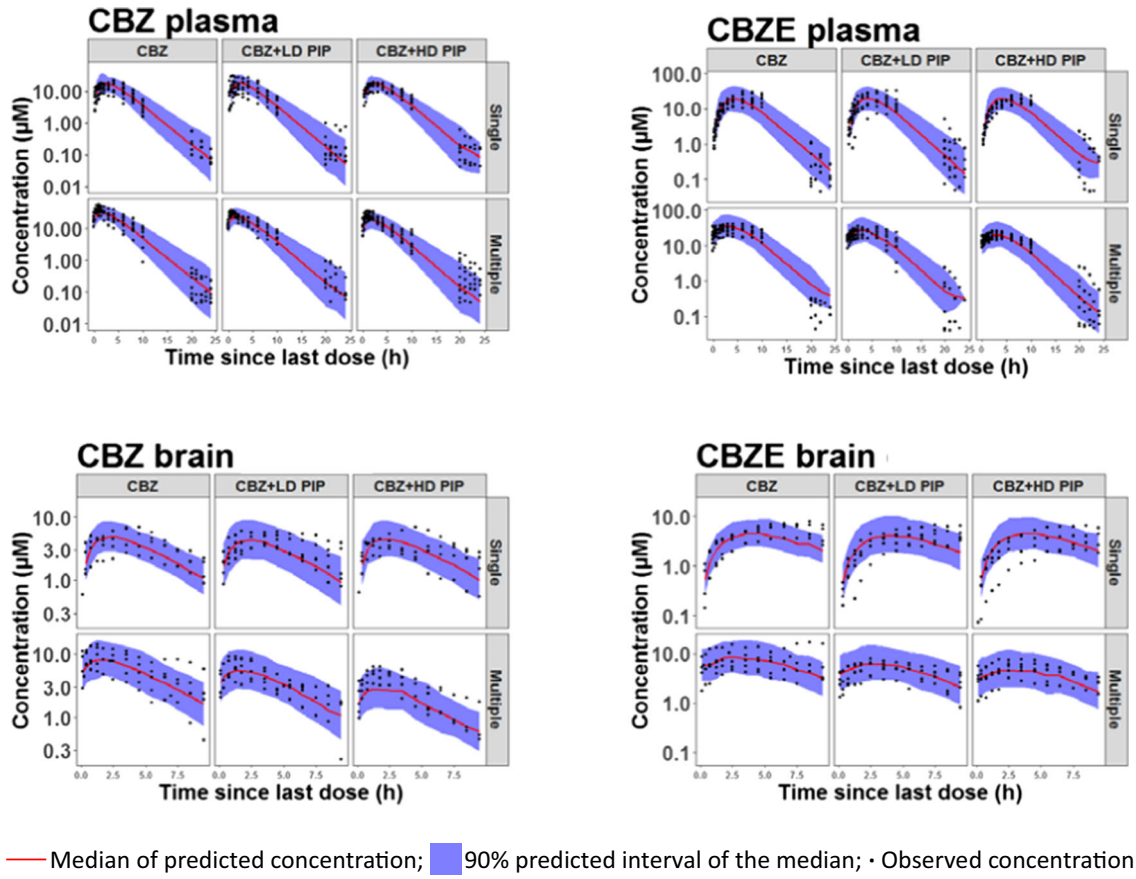


Fig. 5. Visual prediction checks of carbamazepine (CBZ) and carbamazepine-10,11-epoxide (CBZE) in the brain and plasma in different treatment groups

in the clinical study [28, 50]. Based on a piperine content in black pepper of 5–9% (*w/w*) [51] and a scaling factor of 6.2 from humans to rats recommended by the Food and Drug Administration [52], the daily intake level was equivalent to 1.5–5.6 mg/kg, while the effective dose level was considered to be 19–68 mg/kg piperine in rats, into which the two tested doses fall. Brain and plasma concentrations of CBZ and its metabolite were reduced after long-term combined oral

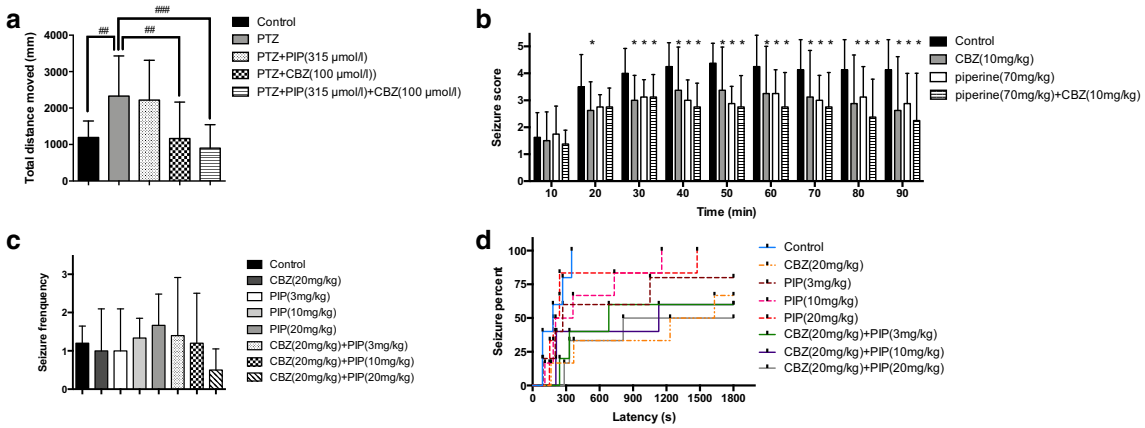


Fig. 6. Effect of piperine (PIP) on the antiepileptic effects of carbamazepine (CBZ) by comparing the total moved distance in zebrafish model with the seizure induced by PTZ (5 mmol/l) and separate or combined treatment of CBZ (100 μmol/l) and PIP (315 μmol/l) ($n = 18$–20 per group) (**a**); the seizure score within 1.5 h after separate or combined treatment of CBZ (10 mg/kg, p.o.) and PIP (70 mg/kg, p.o.) with the seizure induced by pilocarpine (350 mg/kg, i.p.) in C57 mice ($n = 8$ per group) (**b**); the seizure frequency within 30 min (**c**); and the latency to the first seizure (**d**) after the PTZ injection (80 mg/kg, i.p.) with separate or combined treatment of CBZ (20 mg/kg, p.o.) and PIP (3, 10 and 20 mg/kg, p.o.) in Kunming mice ($n =$ 6 per group). Each point represents mean ± S.D. ($^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ compared with the PTZ group; $^{*}p < 0.05$ compared with the control group)

administration of multiple doses of CBZ with piperine due to the reduced bioavailability and brain penetration. For the single-dose treatment groups, no significant difference was found in the PK profiles of CBZ and CBZE in the presence or absence of piperine. Although reduced rat Cyp3a microsomal activity was found after the single oral administration of CBZ in combination with high-dose piperine, CBZ metabolism was inhibited to a lesser extent than that which could lead to obvious concentration changes for CBZ or CBZE. Higher inhibition of CBZ metabolism has been demonstrated after the long-term coadministration of CBZ with a high dose of piperine, with significant decreases in the $AUC_{0\rightarrow24h,CBZE}/AUC_{0\rightarrow24h,CBZ}$, microsomal activity, and mRNA and protein expression levels of rat Cyp3a, which could be attributed to the time-dependent inhibitory characteristic of piperine [32]. The compartmental PK model also suggested a decrease of 38% in the fraction of CBZ clearance for CBZE formation (f_{FMET}). In addition, CBZ has several phase I metabolites, and its major phase I metabolite CBZE could be further metabolized into carbamazepine *trans*-10,11-diol by epoxide hydrolase followed by glucuronidation prior to renal excretion [7, 53]. However, in the present study, we mainly examined the concentrations of CBZ and CBZE since both of them contribute to efficacy and toxicity [8]. In addition, the information on the further elimination processes of CBZE, such as the transformation into *trans*-10,11-diol and further phase II conjugation, could not be obtained in the present study since CBZ/CBZE metabolism was formation rate-limited based on the similar terminal slopes of CBZE and CBZ after a single oral administration of CBZ, indicating that the elimination of CBZE was restricted by its formation rate. Therefore, the investigations of further CBZE transformations were not included in the present study.

Additionally, CBZ is known for its time-dependent induction of CYP3A in humans, which could induce its own metabolism after the administration of multiple doses, leading to increased clearance and a decreased CBZ concentration [54]. In rats, increased hepatic CYP450 contents was found after 2 weeks of i.p. CBZ treatments at 100 mg/kg [55], and increased Cyp3a protein expression levels were found after chronic treatment with 0.5% (*w/w*) CBZ in feeding (approximately 500 mg/kg/day) [56]. However, such an autoinduction phenomenon was not observed in the present study. The CBZ plasma PK profiles showed similar terminal slopes in the groups after single or multiple doses of CBZ administration, with no significant difference in their terminal half-life. These findings suggested that the clearance of CBZ was not changed after long-term CBZ administration in the present study. This might be due to the different routes of administration (oral vs. i.p.) or a lower dose (40 mg/kg/d, t.i.d. vs. 500 mg/kg/day) compared to the previous study in rats. Since the current PK profiles did not demonstrate the autoinduction of CBZ clearance, the proposed model did not incorporate this mechanism. In humans, with the time-dependent autoinduction by CBZ, it can be hypothesized that such an induction may be mitigated due to the metabolic inhibitory effects of piperine after their long-term coadministration, which may lead to a decrease in CBZ concentration to a lesser extent by only considering metabolic changes. However, since piperine may also reduce the bioavailability and brain penetration of CBZ, the CBZ concentrations in plasma and brain may still decrease during coadministration, and CBZ dose adjustment may be required to maintain its antiepileptic effects.

In contrast to our expectation of a decreased CBZE concentration and an elevated CBZ concentration, the plasma concentrations of CBZE and CBZ both decreased after long-term coadministration of CBZ with piperine. Such a decrease may be related to a decrease in the CBZ absorption by piperine, owing to the reduced transit time of CBZ in the GI tract, as a reduced food transit time from 780 ± 25 to 582 ± 39 min has been reported in rats after long-term consumption of piperine [57]. In addition, increased defecation and wet feces were observed after long-term coadministration of piperine in the present study, which may cause the unabsorbed CBZ in the GI tract to be quickly excreted from the body, leading to the reduced bioavailability of CBZ. Such an increase in defecation by piperine was also reported previously in mice, which may result from the stimulation of GI contraction evidenced in the in vitro isolated guinea-pig ileum [30, 31]. The observed AUC change might not be attributed to the change in CBZ systemic clearance since the terminal slopes of CBZ after multiple doses of CBZ in the absence or presence of coadministered piperine were similar in the CBZ plasma PK profiles. Given that CBZ did not exhibit flip-flop PK [58], the terminal slope of CBZ was controlled only by the systemic clearance and volume of distribution but not the absorption process. In the view of the unchanged terminal slope and no autoinduction as discussed above, the decreased systemic exposures of CBZ and CBZE were mainly attributed to the decreased absorption of CBZ. Moreover, the model-based compartmental PK analysis after the inclusion of the plasma concentrations of CBZ and CBZE from both oral and i.v. administration suggested that the bioavailability of CBZ decreased by 8% and 26% after long-term combination treatment with piperine at low and high doses, respectively, leading to the observed PK changes in the present study.

The impact of piperine on plasma protein and brain tissue binding of CBZ and CBZE was also investigated in the present study. Since piperine has exhibited extensive binding towards plasma proteins and brain tissue with unbound fractions of 0.038 and 0.015, respectively [39], displacement of CBZ or CBZE binding may occur during their competition with piperine for the binding site. However, no significant change in CBZ or CBZE binding in plasma or the brain was found after incubation with piperine at their relevant in vivo concentration levels. Such a result may be related to the fact that binding displacement is compound- and concentration-dependent. It has been found that piperine at 10 μmol/l could decrease the human plasma protein binding of salicylic acid, warfarin, and propranolol, but 100 μmol/l was needed for its inhibition of the binding of diazepam or lidocaine with no inhibitory effect on the binding of antipyrine [59]. Therefore, concentrations based on the relevant in vivo levels for each compound should be considered during the binding displacement tests.

In addition to its plasma concentration, the concentration of CBZ in the brain should be closely monitored because it is more directly related to its pharmacological effects as a CNS drug. Brain microdialysis was applied to determine the brain concentration, as it is the only direct method to measure the unbound brain concentration in the extracellular fluid where the pharmacological actions are produced [60]. Similar brain concentration levels of CBZ and CBZE were found for

individuals after single-dose treatment, while decreased brain concentrations were demonstrated after the long-term coadministration of CBZ with piperine. Such a decrease may be related to their decreased plasma concentrations, as both CBZ and CBZE were passively transported in the brain [61]. However, the compartmental PK model revealed that the unbound brain-to-plasma ratio of CBZ was further decreased after the long-term coadministration of CBZ with piperine, indicating that the brain penetration of CBZ may be inhibited by piperine. Such a decreased ratio was not associated with the change in the unbound fraction since this study demonstrated that the binding of CBZ towards plasma proteins or brain tissue was unaltered by piperine. A similar inhibition of CBZ brain penetration has been reported in primary cultured rat brain microvascular endothelium cells by olanzapine, jasminoidine, and fluoxetine and in the primary porcine brain endothelial monolayer by verapamil and prazosin [62, 63]. Although efflux transporters such as P-gp or MRP2 may be involved in CBZ brain penetration and these transporters could be inhibited by piperine, this apparently cannot explain the decreased brain penetration of CBZ [29, 64, 65]. Thus, the inhibited brain penetration of CBZ by piperine suggests that an unidentified influx transporter may be involved in CBZ brain transport and may be inhibited by piperine, which warrants further study.

A compartmental PK model with first-order absorption and elimination was developed to describe the PKs of CBZ and CBZE in the plasma and brain. Due to the low water solubility (18 μg/ml) of CBZ, delayed absorption may occur after its oral administration and has been evaluated by introducing a lag time or transit compartment into the model. However, no significant improvement in the model ($\Delta OFV < 3.84$) was found after adding the lag time or transit compartment, which suggested that the absorption of CBZ followed a first-order rate and was in agreement with other CBZ PK modeling studies [58, 66]. A flow-limited model has been applied to describe the plasma-to-brain distributions of CBZ and CBZE under the assumption that their unbound brain and plasma concentrations were instantly equilibrated. Although the brain concentrations of CBZ and CBZE were measured in the hippocampus by brain microdialysis, they could be regarded as the ECF concentrations for the whole brain, as CBZ and CBZE are homogenously distributed in different brain regions [61]. In fact, discrepancy in the concentration between the ECF and brain intracellular space should also be considered for drug distribution within the brain [67]. However, such a phenomenon was often found in basic drugs due to lysosomal trapping, which does not apply to neutral compounds, including CBZ and CBZE, at physiological pH [68]. Thus, the same concentrations of CBZ and CBZE between brain cells and the ECF were assumed with a brain volume of distribution (1.8 ml) fixed to the sum of the brain intracellular volume (0.3 ml) and extracellular volume (1.5 ml) [43]. In addition, the interaction parameters of f_F, f_{FMET}, and f_{Kp} were introduced to evaluate the influence in the PK of CBZ after coadministration with piperine. The estimation of these parameters showed good precision with small uncertainty (RSE < 21%), which further confirmed that the change in PK of CBZ resulted from the inhibition of the bioavailability, metabolism, and brain penetration of CBZ by piperine.

In addition to the PK influence, the impact on the pharmacodynamics of CBZ by piperine was further evaluated in animal seizure models. Due to the complex mechanisms of antiepileptic drugs, multiple seizure models were employed in the present study to provide more comprehensive insight into the impact on the antiepileptic effects of CBZ after its coadministration with piperine. Rodents with seizures generated by chemoconvulsants are commonly used as rapid and inexpensive investigation methods in epilepsy studies [69]. Among various chemoconvulsants, the model with epileptic seizures induced by pilocarpine is widely used for temporal lobe epilepsy (TLE) studies, which is the most common epilepsy type in humans that could be prevented by CBZ [70, 71]. PTZ-induced seizure models in rodents have also been widely used to evaluate the efficacy against generalized tonic-clonic seizures, which are effectively protected by CBZ [72]. Since C57 mice were found to be more resistant to PTZ [73], Kunming mice were used in the PTZ seizure model in the present study. Different doses of CBZ were used in two mouse seizure models (10 mg/kg for the pilocarpine model and 20 mg/kg for the PTZ model) based on their minimum effective doses, suggesting a discrepancy in the treatment dose of CBZ in different disease models. While piperine at 70 mg/kg was found to effectively protect the epilepsy induced by pilocarpine, toxicity was demonstrated in the PTZ model with over 50% death after the oral administration of piperine over 30 mg/kg. In addition to rodent seizure models, zebrafish have been used since they are genetically similar to humans and are cost-effective due to their high breeding rate [74]. In addition, due to their small size, zebrafish can be easily manipulated and can be placed in 96-well plates to allow the high-capacity testing of antiepileptic effects. Moreover, since zebrafish movements can be monitored by video-tracking devices, drug efficacy can be then quantified based on their speed or total moved distance. By using this model, the antiseizure effects from different treatment groups can be compared to evaluate the influence of piperine on the anticonvulsant effects of CBZ. In addition, the results from this model can be compared with the results from mice to determine whether there are any species differences in terms of the pharmacodynamic impact. Exposing zebrafish larvae at 7 dpf to PTZ could effectively generate seizure-like behaviors, including increased swim activity followed by rapidly swimming in circles. To facilitate the solubilization of CBZ and piperine in the culture media for zebrafish, DMSO at a final concentration of 1% (*v/v*) was added to the media, which has demonstrated no toxicity to 7 dpf zebrafish larvae in a previous report [75]. Nonetheless, a higher dose of piperine (> 315 μM) could not be achieved due to the limited solubility of piperine, which hindered the investigation of the effective dose of piperine in the zebrafish seizure model. Although different doses of CBZ and piperine were used, consistent results were demonstrated in all seizure models, suggesting that piperine has a limited impact on the antiepileptic effects of CBZ. Due to the nature of the acute seizures in the selected animal models and the potential toxicity of long-term piperine treatment in the current models, pharmacodynamic interactions after the combined single-dose use of CBZ with piperine were evaluated in the present study. The antiepileptic effects of long-term piperine usage or its combination with CBZ can be studied in chronic epileptic models, such as epilepsy induced by repeated electrical stimuli via a depth electrode in the brain [72].

CONCLUSION

In summary, the present study provided an integrated evaluation of the PK and antiepileptic effects of CBZ after its coadministration with piperine. No significant change in the PKs or pharmacodynamics of CBZ was found after the single-dose combination treatment of CBZ and piperine. After the long-term combined use of CBZ with piperine, decreased concentrations of CBZ and its metabolite in the brain and plasma were found, which were mainly attributed to the reduced bioavailability and decreased brain penetration of CBZ caused by piperine. The potential decrease in the concentration of CBZ after long-term coadministration of CBZ with piperine in patients during epilepsy control warrants further clinical verifications.

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest The authors declared no conflict of interest.

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