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Pharmacokinetics and metabolism of 3,4-dichlorophenyl-propenoyl-sec.-butylamine in rats by high performance liquid chromatography–ion trap mass spectrometry

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Abstract

The pharmacokinetics (PK) and metabolism of 3,4-dichlorophenyl-propenoyl-sec.-butylamine (3,4-DCPB), a novel antiepileptic drug, were investigated after its oral administration to rats (100 mg/kg) by HPLC. The absorption and elimination of 3,4-DCPB were rapid. 3,4-DCPB was found to undergo extensive metabolism as the major route of elimination. Structures of the metabolites present in rat plasma were identified with liquid chromatography–electrospray ionization-mass spectrometry (LC–ESI-MS/MS). It was concluded that 3,4-DCPB was involved in the multiple metabolic pathways (hydrolysis, dealkylation and oxidation) and the hydrolysis product, 3,4-dichloro-cinnamic acid (M1) appeared to be the major metabolite.

Keywords: 3,4-Dichlorophenyl-propenoyl-sec.-butylamine; Liquid chromatography–electrospray ionization-mass spectrometry; Pharmacokinetics; Metabolite

1. Introduction

3,4-Dichlorophenyl-propenoyl-sec.-butylamine (3,4-DCPB) is a novel antiepileptic drug and is derived from piperine, a component present in white pepper. White pepper used as the Chinese traditional medicine has been widely prescribed to treat epilepsy [1]. The piperine is one of the effective components in the herb, and its chemical structure belongs to the group of cinnamamides, such as 3,4-DCPB, an analog of the cinnamamides [2,3].

By increasing the brain concentration of the inhibitory amino acids, such as norepinephrine (NE), serotonin (5-HT) and gamma-aminobutyric acid (γ-GABA) piperine derivatives, such as 3,4-DCPB and antiepilepsirine, have been shown in rodents to be more pharmacologically effective than other cinnamamides [4–6]. In addition, piperine decreased the brain level of the excitatory amino acids (glutamine), inhibited lipid peroxidation, blocked calcium ion channel and protected cerebral function from ischemia-reperfusion brain injury [7,8]. As a result, 3,4-DCPB has been considered to have a potential in the treatment of epilepsy and ischemic stroke [9,10], and is currently under clinical development in China.

The metabolism of piperine in rat liver microsomes and isolated perfusion was performed and analyzed with thin-layer chromatography (TLC) and gas chromatography (GC) [11,12], and pharmacokinetics, tissue distribution [13] and liquid chromatographic method for determination of piperine were reported [14]. Additionally, the HPLC method was developed for determination of 3,4-DCPB in rat plasma [15]. However, the pharmacokinetics and identification of the metabolites of 3,4-DCPB in both rat plasma and urine have not been reported. In the present study, LC–MS/MS method has been employed to study pharmacokinetics of 3,4-DCPB in rats treated orally with 100 mg/kg and to identify the major metabolites circulated in rat plasma.

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2. Experimental

2.1. Chemicals and reagents

3,4-DCPB, 3,4-dichlorophenyl-propenoyl-sec.-cyclohexane (3,4-DCPC, as an internal standard) and 3,4-dichloro-cinnamic acid (metabolite M1 standard) were synthesized and purified (99% purity) by the Department of Pharmaceutical Chemistry, School of Pharmacy, Beijing University (Beijing, China). 3,4-DCPB was suspended with water (2% suspension) for oral dosing. All solvents were purchased commercially with purity of analytical or HPLC grade.

2.2. Instrumentations

HPLC system (Waters LC Module I System, USA) consisted of a model 510 pump, model Rheodyne 7125 injector and model 2487 UV-detector. The chromatograph was equipped with a reversed-phase Agilent C_{18} column (4.6 mm × 150 mm, 5.0 μm, USA) eluted with the mobile phase (80% methanol: 20% water, v/v). The flow-rate was at 1.0 ml/min and the column temperature was controlled at 25 °C.

LC–ESI-MS/MS system consisted of a Surveyor LC pump, Surveyor autosampler, Surveyor PDA detector, Finnigan LCQ Advantage ion trap and Xcalibur 1.3 software workstation (Thermo Finnigan, San Jose, USA). The separation was performed on a reversed-phase Agilent C_{18} column (4.6 mm × 150 mm, 5.0 μm, USA) with an ODS guard column (2 mm × 4 mm).

2.3. Animal handling and sample collection

Male Sprague–Dawley rats (BW 270 ± 30 g) were provided by the Department of Experimental Animal, Beijing University (Beijing, China). Environmental controls for the animal room were set at 22 ± 3 °C with 50 ± 20% relative humidity. The animal studies were approved by the Animal Ethics Committee of Beijing University, and carried out in accordance with the requirements of China national legislation.

2.3.1. Plasma samples collection

Rats were fasted 12 h prior to the study and were allowed access to food 4 h after the dosing. The rats were dosed with a suspension of 3,4-DCPB (100 mg/kg) and the blood samples were collected at the predose, 0.25, 0.5, 1, 2, 3, 4, 6, 8, 12, 24, 36 and 48 h, respectively. The blood samples at each time point were collected from five rats. The blood samples were centrifuged at 3000 × *g* for 10 min and the plasma was stored at −20 °C until analyzed.

2.3.2. Urine samples collection

Seven rats were housed individually in metabolic cages and were orally administrated with 3,4-DCPB (100 mg/kg). Urine samples were collected 12 h before the dosing and then at 0–2, 2–4, 4–6, 6–8, 8–12, 12–24, 24–48 and 48–72 h, respectively. The samples were stored at −20 °C until analyzed.

2.4. Sample preparation

Stock solutions of 3,4-DCPB (640 μg/ml) and internal standard, 3,4-DCPC (100 μg/ml), were prepared in mobile phase (methanol: water = 80:20, v/v) and stored at 4 °C. Solutions of 3,4-DCPB with concentrations of 0.2, 1, 2, 4, 10, 20, 40, 80, 160 μg/ml were prepared by serial dilution of stock solutions with methanol. Each blank rat plasma sample (0.5 ml) was spiked with 3,4-DCPC solution (25 μl) and different concentrations of 3,4-DCPB (25 μl) to prepare a series of standard solutions (0.01, 0.05, 0.1, 0.2, 0.5, 1, 2, 4, 8 μg/ml) for the calibration curve. Ethyl acetate (1.5 ml) was added twice for liquid–liquid extraction. The mixture was vortexed for 2 min on a roller shaker and centrifuged at 3000 × *g* for 5 min. The organic layer (ethyl acetate) was collected and evaporated to dryness at 37 °C under a gentle stream of nitrogen gas. The residue was dissolved in 500 μl mobile phase and 15 μl aliquot was injected into the HPLC column system for analysis. Quality control samples were prepared at low (0.01 μg/ml), medium (1 μg/ml) and high (8 μg/ml) concentrations in the same way as the plasma samples for calibration. The accuracy and recovery of 3,4-DCPB were evaluated by assaying five replicates of quality control samples at each concentration. All plasma and urine samples were analyzed together with calculation curves and quality control samples.

Based on the retention times of the samples (plasma and urine) on HPLC, the three major metabolites chromatographic fractions were collected and evaporated under nitrogen at 37 °C. The residues were reconstituted in the mobile phase and analyzed by LC–ESI-MS/MS.

2.5. HPLC analysis

The HPLC assays were validated before use by the replication of the samples ($n = 5$) from the bottom, middle and top of the analytical range [15]. The assay was considered to be valid if the cumulative variations at the bottom (<20%), the middle (<15%) and the top (<15%) were obtained. The quality control samples were analyzed with all samples and the valid values were expected by the analytical range at the bottom (<20%), middle (<15%) and top (<15%). Under the chromatographic conditions, the authentic metabolites of 3,4-DCPB and internal standard (3,4-DCPC) were well separated. 3,4-DCPB was detected at 276 nm of UV absorption. The retention times of 3,4-DCPB and 3,4-DCPC on HPLC were 4.32 and 5.86 min, respectively. No detectable interference of the endogenous substances contained in the plasma was observed. The linear calibration curves were achieved in a concentration range of 0.01–8 μg/ml for 3,4-DCPB ($r^2 = 0.9980$–0.9999) in both plasma and urine samples. The intra- and inter-run precision (R.S.D.) were 5.17–8.93% and 8.02–9.48%, respectively. The recovery of 3,4-DCPB in plasma and urine was higher than 95%. The concentration of 3,4-DCPB was determined according to the calibration curves from the extracted samples. The limit of quantitation (LOQ) was 10 ng/ml (S/N > 10) and the limit of detection (LOD) was 3 ng/ml (S/N > 3).

2.6. LC–ESI-MS/MS analysis

LC–ESI-MS/MS equipment was used to analyze 3,4-DCPB and its metabolites in the rat plasma samples. The negative ion mode was used to determine the metabolite M1. The parameters of LC–ESI-MS/MS were set: sheath gas flow rate (30 arb), auxiliary gas flow rate (5 arb), ion spray voltage (4.7 kV), ion transfer capillary temperature (290 °C), capillary voltage (−33 V), tube lens offset (−45 V), multipole 1 offset (5 V), lens voltage (20 V) and multipole 2 offset (5.5 V). The positive ion mode was used to analyze 3,4-DCPB and the metabolites M2 and M3. The parameters were set: sheath gas flow rate (20 arb), auxiliary gas flow rate (5 arb), ion spray voltage (5.5 kV), ion transfer capillary temperature (240 °C), capillary voltage (3 V), tube lens offset (20 V), multipole 1 offset (−2.5 V), lens voltage (−26 V) and multipole 2 offset (−6 V).

2.7. Determination of the pharmacokinetic parameters

The pharmacokinetic parameters were estimated by a non-compartment model using the program 3P97 software (the Mathematical Pharmacology Committee, Chinese Pharmacological Society, China). The area under the plasma concentration–time curve from 0 to the time of the last quantifiable concentration (AUC_{0-t}) was calculated by the trapezoidal summation. The plasma peak concentration (C_{max}) and the time to reach C_{max} (t_{max}) were obtained directly from the experimental data. The terminal elimination rate constant (K_e) was derived by the slope of the linear regression curve by fitting the natural logarithms of the terminal concentrations versus time. The terminal elimination half-life ($t_{1/2}$) was calculated by $0.693/K_e$. All concentrations of 3,4-DCPB were expressed as mean ± S.D.

3. Results and discussion

3.1. Pharmacokinetics and analysis of the metabolites of 3,4-DCPB

The plasma concentration–time course of 3,4-DCPB was analyzed after the single oral dose (100 mg/kg) by HPLC. The results showed that the plasma concentrations of the parent drug were detected at 0.25 h postdose and suggested that 3,4-DCPB was absorbed rapidly. Pharmacokinetics of 3,4-DCPB was analyzed by non-compartmental model and the parameters were calculated as follows: $C_{max} = 5.10$ μg/ml, $t_{max} = 3.60$ h and $AUC_{0-t} = 49.62$ μg h/ml, respectively. In addition, the elimination half-life ($t_{1/2} = 3.07$ h) was short.

In the plasma profiling of HPLC, three chromatographic peaks were separated with the retention times shorter than the parent drug (Fig. 1), suggesting that these components were more polar than the parent drug [16] and were likely to be the metabolites formed. With LC–MS/MS, the three metabolites were tentatively identified as M1, M2 and M3, respectively (see below). Due to lack of standard metabolite compounds, the actual concentrations of three metabolites in plasma and urine could not be measured. However, the difference in UV absorption wavelength was very low among the three metabolites and the parent drug. Semiquantitative analysis was made to estimate the relative concentrations of metabolites based on calculation by the standard concentration curve of 3,4-DCPB. The areas of metabolite peaks were highly correlative to the time after drug administration. The relative concentration–time curves of three individual metabolites in plasma were shown in Fig. 2. t_{max} values of the three metabolites, after the single oral dose (100 mg/kg) of 3,4-DCPB, were also determined to be 12.0 (M1), 8.0 (M2) and 6.0 h (M3), respectively. The relative amount of M1 appeared to be much greater than that of the parent drug and two other metabolites. No detection of the parent drug and three metabolites in rat plasma was achieved 36 h after the dosing.

3.2. Analysis of 3,4-DCPB and its metabolites in urine

The excretion of 3,4-DCPB from the urine (0–72 h) was low (0.18% of the dose). However, the plasma clearance of the parent drug was rapid (within 24 h), suggesting that metabolic pathways of 3,4-DCPB may be the major route of elimination in rat. Interestingly, the three metabolites were also detected in the urine, with the retention times consistent with those obtained from the plasma. Surprisingly, the relative concentration–time

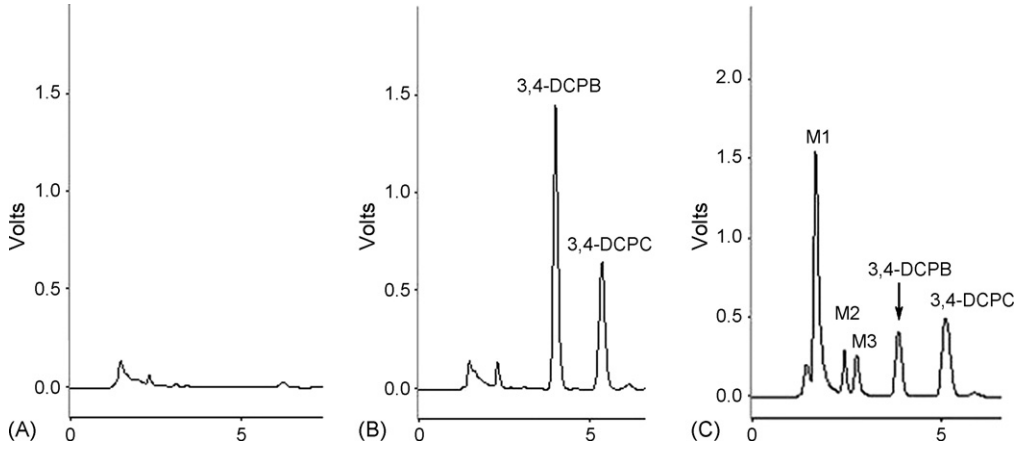

Fig. 1. Representative HPLC-UV chromatograms of (A) blank plasma, (B) blank plasma spiked with 3,4-DCPB and internal standard (3,4-DCPC) and (C) 3,4-DCPB and its three major metabolites (M1, M2 and M3) after a single oral dose (100 mg/kg) in rat plasma.

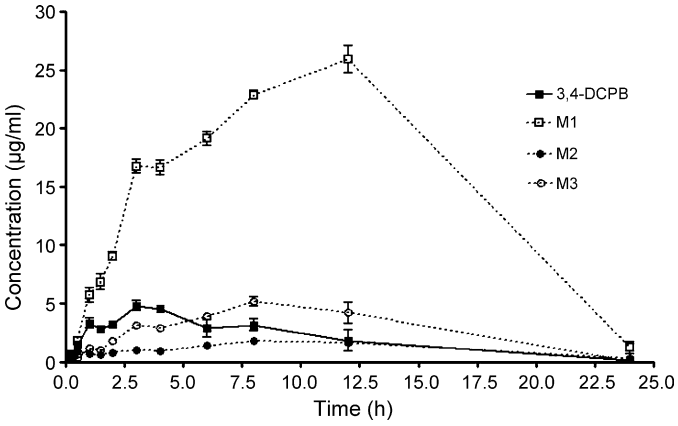

Fig. 2. The plasma concentration–time curve of 3,4-DCPB and relative plasma concentration–time curves of metabolites (M1, M2 and M3) by semiquantitative analysis after a single oral dose in rats (100 mg/kg) by HPLC ($n = 5$, mean ± S.D.).

courses of three metabolites in the urine were similar to those in the plasma. The rough t_{max} of the three metabolites in the urine were 8–12 (M1), 8–12 (M2) and 6–8 h (M3), respectively. Similarly, the relative amounts of metabolites observed were greater than that of the parent drug at the same time points of collection.

3.3. Metabolite identification by LC–ESI-MS/MS

The three metabolites contained in the UV chromatographic peaks in the rat plasma were further analyzed and tentatively identified by LC–ESI-MS/MS. The proposed structures of the metabolites are shown and interpreted as follows.

3.3.1. MS/MS analysis of 3,4-DCPB standard

In the tandem mass spectrometry positive mode (Fig. 3), 3,4-DCPB standard produced three molecular ions $[M+H]^+$ at *m/z* 272, *m/z* 274 and *m/z* 276 (abundance ratio was 9:6:1), respectively, due to the isotope effect of two chlorine atoms in the structure as characteristics of the compounds. Furthermore, the molecular ion at *m/z* 272 was further fragmented to several ions at *m/z* 216, *m/z* 199, *m/z* 171 and *m/z* 136 (Table 1, Fig. 3), respectively, by in-source collisionally induced dissociation (CID). The fragment ion at *m/z* 216 was generated from the ion at *m/z* 272 by the loss of 56 Da ($CH_3CHCHCH_3$). The fragment ion at *m/z* 199 was obtained from the ion at *m/z* 216 by the loss

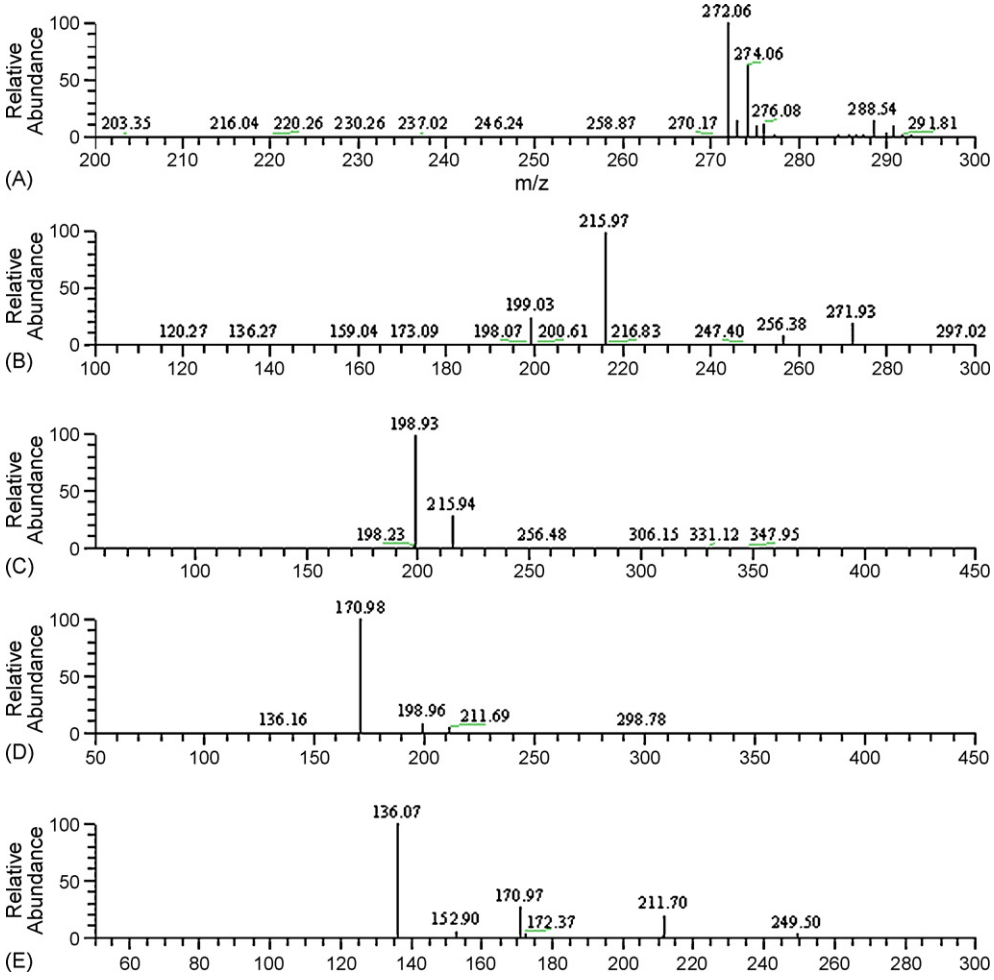

Fig. 3. The multi-stage mass spectra of 3,4-DCPB standard in positive mode using electrospray ionization and in-source collisionally induced dissociation. (A) MS^1 *m/z* 272, (B) MS^2 *m/z* 216, (C) MS^3 *m/z* 199, (D) MS^4 *m/z* 171 and (E) MS^5 *m/z* 136.

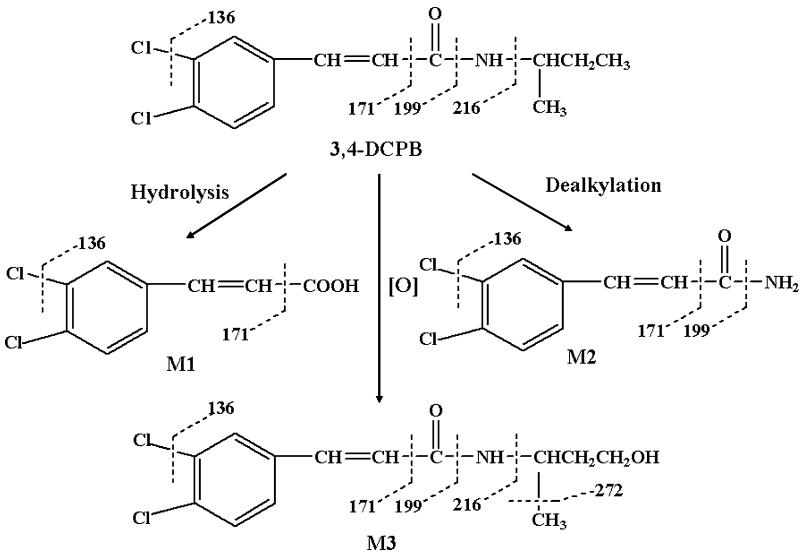

Fig. 4. Presumed metabolic pathways of 3, 4-DCPB and structures of three metabolites (M1, M2 and M3) after a single oral dose in rats (100 mg/kg) in rat plasma.

of 17 Da (NH_3). The fragment ion at *m/z* 171 was due to the loss of 28 Da (CO) from the ion at *m/z* 199. The fragment ion at *m/z* 136 was caused by the loss of 35 Da (^{35}Cl) from the ion at *m/z* 171. The fragmentation of 3,4-DCPB is shown in Fig. 4.

3.3.2. Identification of metabolite M1

In the negative mode of the tandem mass spectrometry, M1 produced three molecular ions $[M-H]^-$ at *m/z* 215, *m/z* 217 and *m/z* 219, respectively (Fig. 5). In LC–MS, M1 was detected at retention time 1.38 min, selective molecular ion peak retention time 1.34 min (Fig. 6A) and UV absorption wavelength of 265 nm. The CID spectrum of M1 showed two fragment ions at *m/z* 171 and *m/z* 136, which were formed by the loss of 44 Da (CO_2) and 35 Da (^{35}Cl) from the ion at *m/z* 215 and *m/z* 171 (Table 1, Fig. 5), respectively, which corresponded to the characteristic fragment ions of 3,4-DCPB. The fragmentation pathways of M1 are explained in Fig. 4. The structure of 3,4-DCPB has been reported in which the benzene ring and carbon double bond are conjugated firmly and cannot be easily frag-

Table 1
LC–MS/MS analysis of 3, 4-DCPB and three major metabolites (M1, M2 and M3) found in rat plasma after oral dosing (100 mg/kg)

Compound	Retention time (min)	Molecular ion	Fragment ions
3,4-DCPB	4.32	$272[M-H]^+$	216,199,171,136
M1	1.38	$215[M-H]^-$	171,136
M2	2.98	$216[M-H]^+$	199,171,136
M3	3.2	$288[M-H]^+$	272,216,199,171

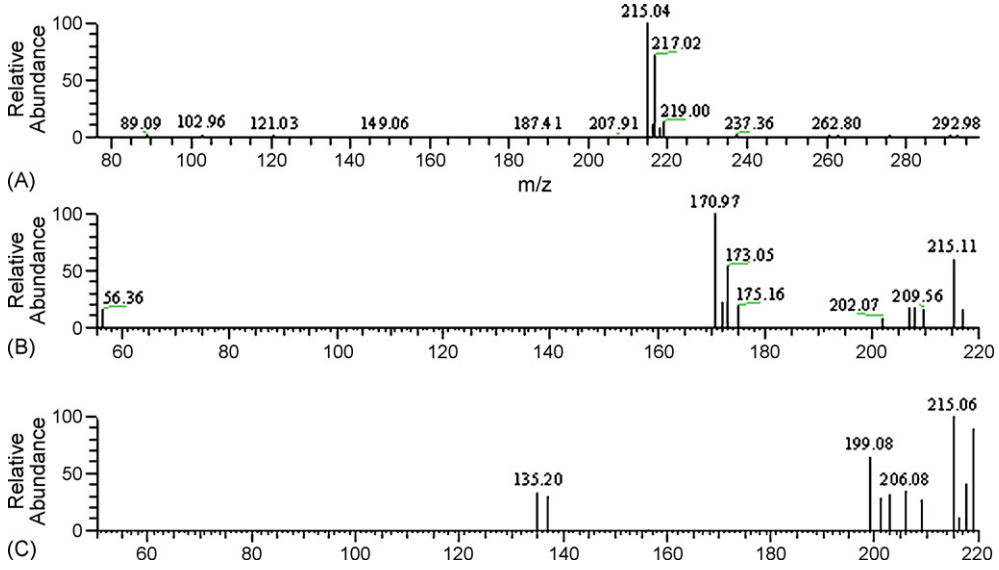

Fig. 5. The multi-stage mass spectra of metabolite M1 from rat plasma in negative mode using electrospray ionization and in-source collisionally induced dissociation. (A) MS^1 *m/z* 215, (B) MS^2 *m/z* 171 and (C) MS^3 *m/z* 136.

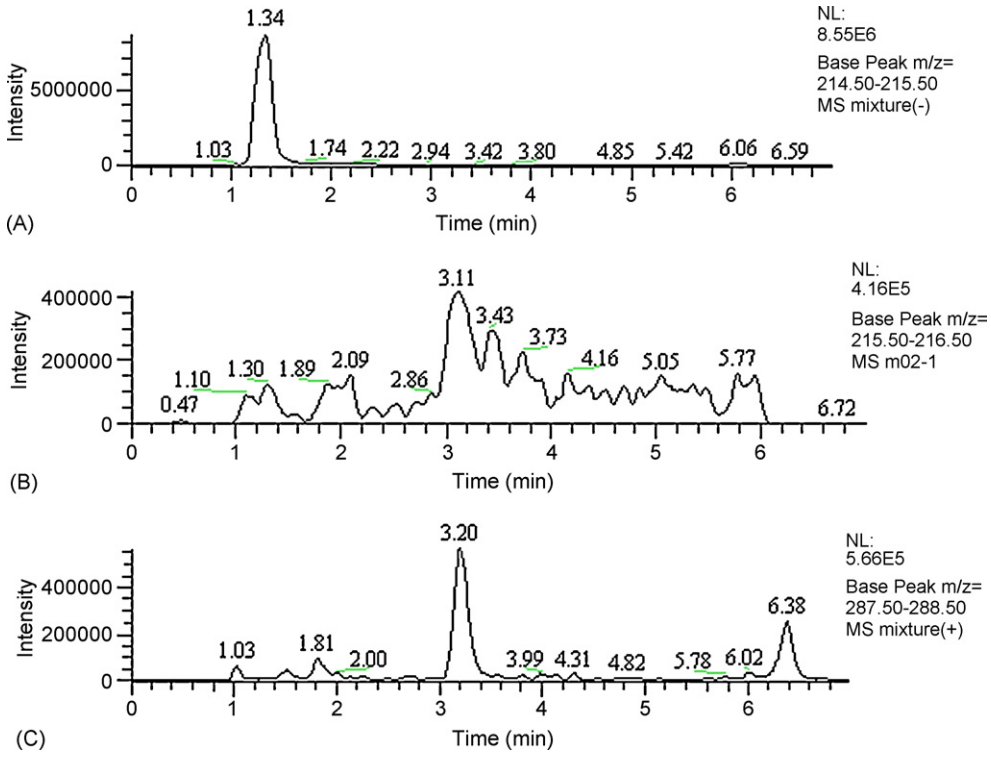

Fig. 6. (A) Selective ion monitoring (*m/z* 215) of metabolite M1 in negative mode, (B) selective ion monitoring (*m/z* 216) of metabolite M2 in positive mode, (C) selective ion monitoring (*m/z* 288) of metabolite M3 in positive mode.

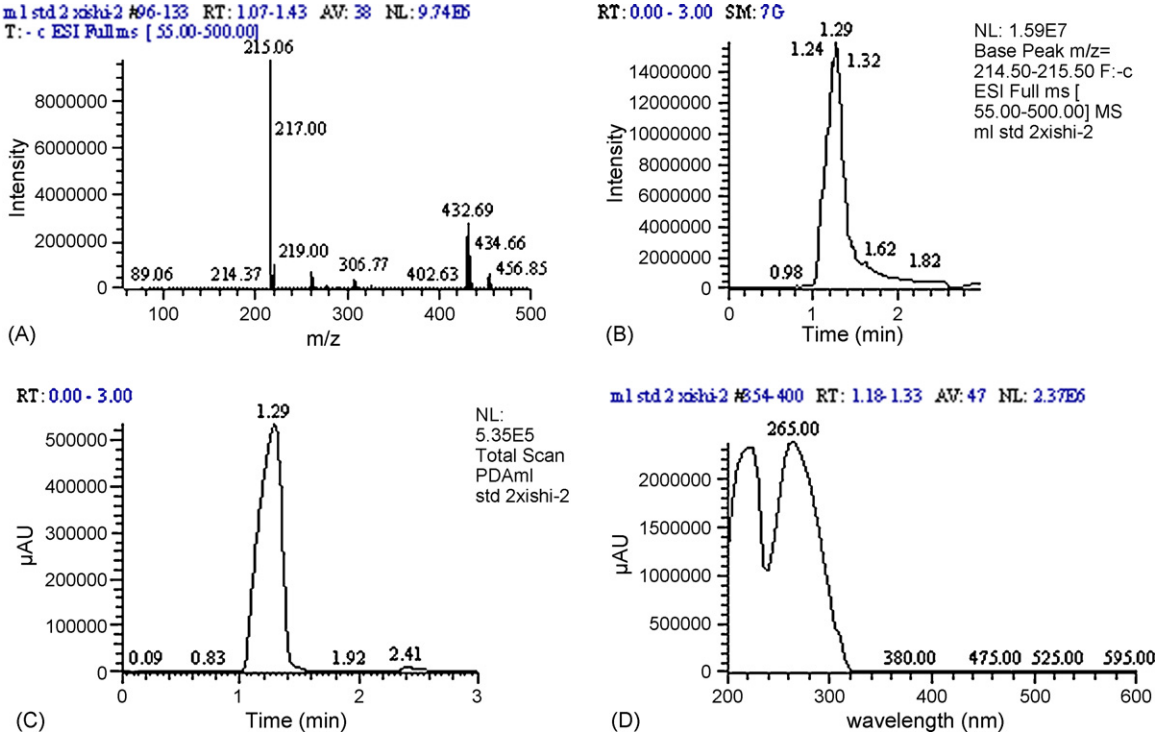

Fig. 7. Mass spectrum in full scan (A), selective ion monitoring (*m/z* 215) (B), HPLC (C) and UV spectrum (D) of M1 standard (3,4-dichloro-cinnamic acid) in negative mode.

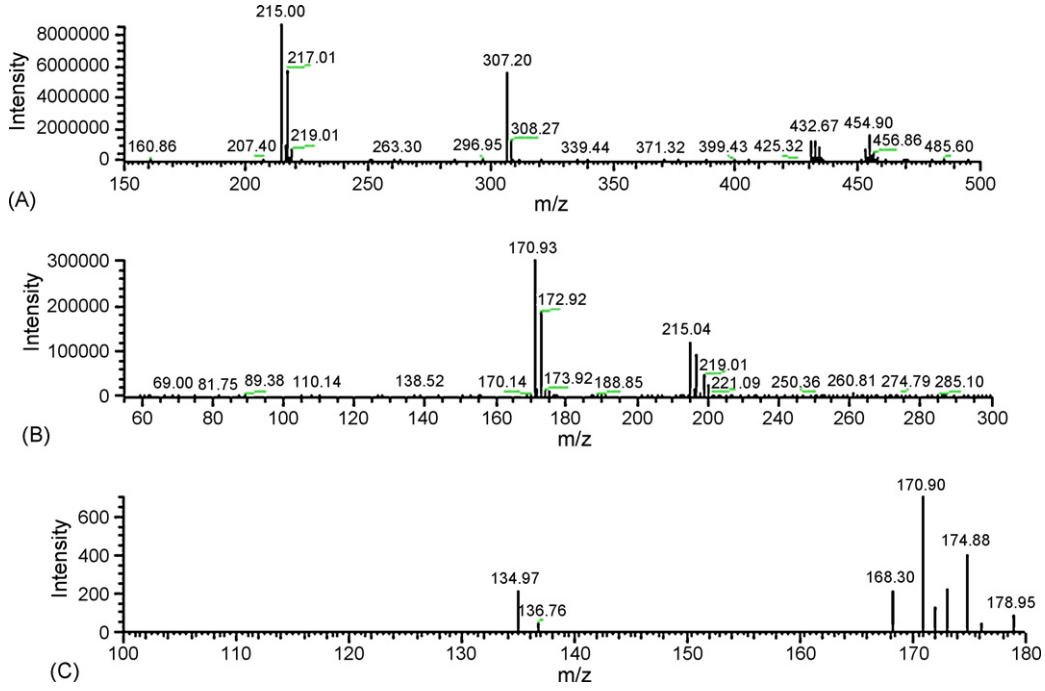

Fig. 8. The multi-stage mass spectra of metabolite M1 standard in negative mode using electrospray ionization and in-source collisionally induced dissociation. (A) MS^1 *m/z* 215, (B) MS^2 *m/z* 171 and (C) MS^3 *m/z* 136.

mented. However, the amide linkage was the weak bond and might be susceptible to break by hydrolysis [16]. Therefore, the loss of 55 Da in molecular weight as compared to 3,4-DCPB was due to the hydrolysis of the amide linkage. The hypothesis was confirmed by comparison with the fragment pattern of M1 standard (3,4-dichloro-cinnamic acid), along with consistency of HPLC retention time, UV spectrum and CID fragment ions (Figs. 7 and 8).

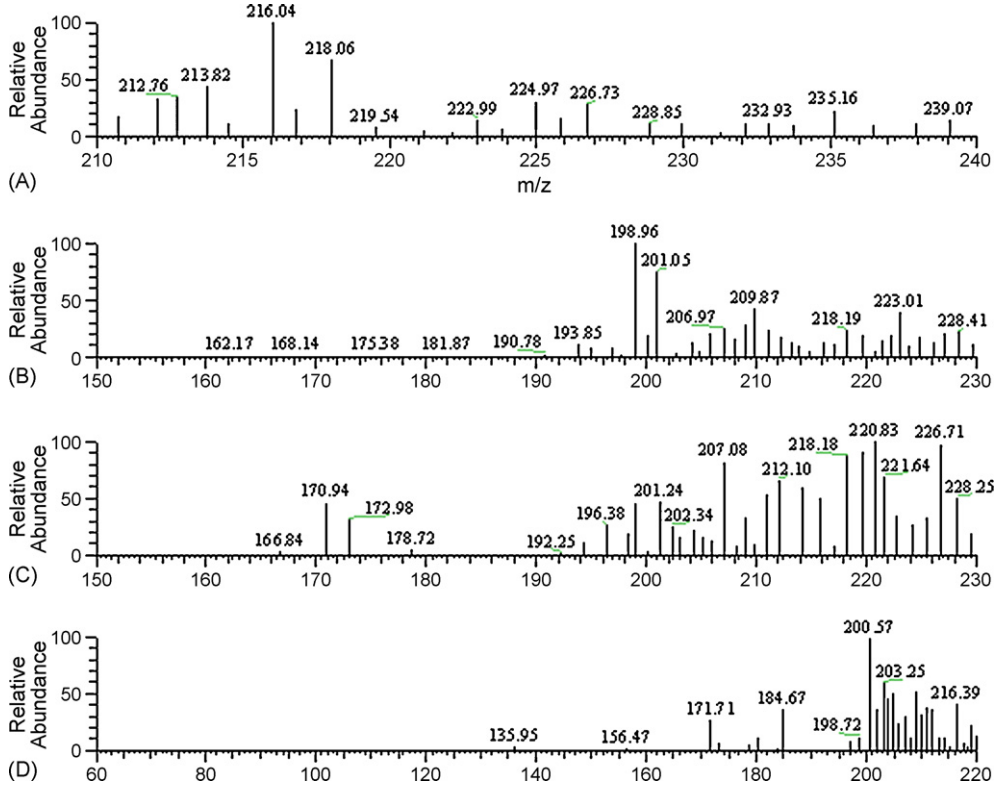

Fig. 9. The multi-stage mass spectra of metabolite M2 from rat plasma in positive mode using electrospray ionization and in-source collisionally induced dissociation. (A) MS^1 *m/z* 216, (B) MS^2 *m/z* 199, (C) MS^3 *m/z* 171 and (D) MS^4 *m/z* 136.

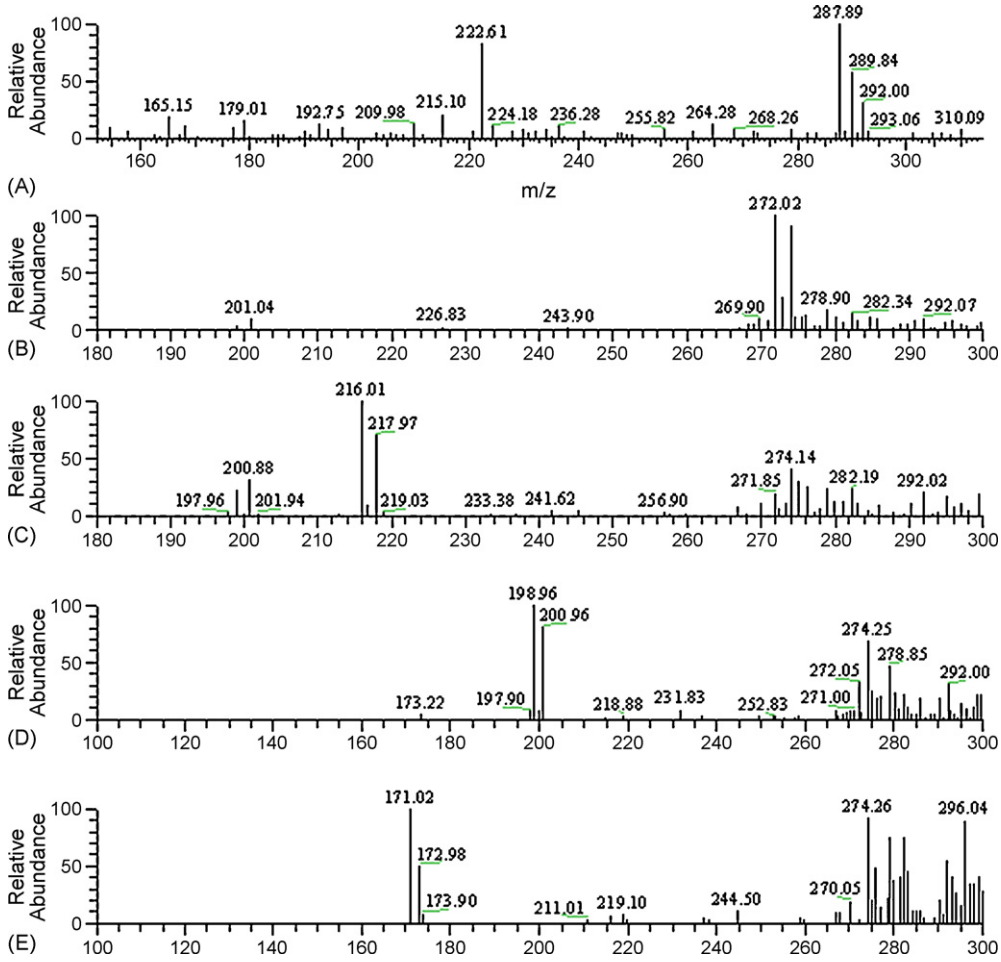

Fig. 10. The multi-stage mass spectra of metabolite M3 from rat plasma in positive mode using electrospray ionization and in-source collisionally induced dissociation. (A) MS^1 *m/z* 288, (B) MS^2 *m/z* 272, (C) MS^3 *m/z* 216, (D) MS^4 *m/z* 199 and (E) MS^5 *m/z* 171.

3.3.3. Identification of metabolite M2

Since concentration of M2 was low, the fraction was repetitively isolated and purified from the rat plasma. In the positive mode, M2 produced three molecular ions $[M+H]^+$ at *m/z* 216, *m/z* 218 and *m/z* 220, respectively (Fig. 7), with retention time of 2.98 min, selective molecular ion peak (retention time = 3.11 min; Fig. 6B) and UV absorption wavelength of 275 nm, similar to that of 3,4-DCPB (276 nm). The CID spectrum of M2 showed the three fragment ions at *m/z* 199, *m/z* 171 and *m/z* 136 (Table 1, Fig. 9), respectively. They were derived from the ions at *m/z* 216, *m/z* 199 and *m/z* 171 by losing 17 Da (NH_3), 28 Da (CO), 35 Da (^{35}Cl), respectively. The loss of 56 Da of M2 along with the three consistent fragment ions suggested that M2 was formed by the loss of butylene ($CH_3CHCHCH_3$). The fragmentation of M2 is explained in Fig. 4.

3.3.4. Identification of metabolite M3

In the positive mode, M3 produced the three molecular ions $[M+H]^+$ at *m/z* 288, *m/z* 290 and *m/z* 292 (Fig. 8) with retention time 3.20 min, selective molecular ion peak at retention time of 3.20 min (Fig. 6C). The UV absorption wavelength at 275 nm was similar to that of 3,4-DCPB (276 nm). Four fragment ions (*m/z* 272, *m/z* 216, *m/z* 199 and *m/z* 171) (Table 1, Fig. 10) resulted from CID were generated by the loss of 16 Da (CH_4), 56 Da (CH_2CHCHO), 17 Da (NH_3) and 28 Da (CO), respectively. The 16 Da increase in molecular weight as compared to 3,4-DCPB and the four consistent fragment ions suggested that M3 may be formed by hydroxylation of methyl. The fragmentation of M3 is proposed in Fig. 4.

Because of insufficient quantity of the two metabolites M2 and M3 collected in the matrixes for NMR analysis [17], location of the oxidation on the molecule could not be identified. However, based on the fragment patterns and comparison with those from 3,4-DCPB, the two metabolites were tentatively identified as the products of *N*-dealkylation and oxidation, respectively. The enzymes involved in the metabolism of 3,4-DCPB remain to be determined.

4. Conclusion

Pharmacokinetics of 3,4-DCPB in rats at an oral dose of 100 mg/kg has been investigated by HPLC analysis. 3,4-DCPB was found to have rapid absorption into the systemic circulation and elimination from the plasma with a short $t_{1/2}$. There were three metabolites in the plasma and urine, which have

been tentatively identified by LC–ESI-MS/MS in comparison with characteristic fragment ions of the parent drug standard. The metabolites are believed to be the products of the hydrolysis (amide linkage), *N*-dealkylation and oxidation of 3,4-DCPB in rats.

Acknowledgments

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