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# *In vitro* identification of metabolites of verapamil in rat liver microsomes<sup>1</sup>

Lu SUN, Shu-qiu ZHANG, Da-fang ZHONG<sup>2</sup>

Laboratory of Drug Metabolism and Pharmacokinetics, Shenyang Pharmaceutical University, Shenyang 110016, China

**KEY WORDS** verapamil; *in vitro* metabolism; liver microsomes; high pressure liquid chromatography; mass spectrometry

#### **ABSTRACT**

AIM: To investigate the metabolism of verapamil at low concentrations in rat liver microsomes. METHODS: Liver microsomes of Wistar rats were prepared using ultracentrifuge method. The *in vitro* metabolism of verapamil was studied with the rat liver microsomal incubation at concentration of 1.0 μmol/L and 5.0 μmol/L. The metabolites were separated and assayed by liquid chromatography-ion trap mass spectrometry (LC/MS<sup>n</sup>), and further identified by comparison of their mass spectra and chromatographic behaviors with reference substances. RESULTS: Eight metabolites, including two novel metabolites (M4 and M8), were found in rat liver microsomal incubates. They were identified as *O*-demethyl-verapamil isomers (M1 - M4), *N*-dealkylated derivatives of verapamil (M5-M7), and *N*, *O*-didemethyl-verapamil (M8). CONCLUSION: *O*-Demethylation and *N*-dealkylation were the main metabolic pathways of verapamil at low concentrations in rat liver microsomes, and the relative proportion of them in verapamil metabolism changed with different substrate concentrations.

# INTRODUCTION

Verapamil, a calcium channel blocker, is commonly used for the treatment of supraventricular arrhythmias, coronary heart disease, and arterial hypertension. Furthermore, verapamil is a potent inhibitor of P-gly-coprotein-mediated transport, and has been proven to modify multidrug resistance with cancer chemotherapy in *in vitro* experiments<sup>[1,2]</sup>. It is subject to extensive metabolism in humans with less than 5 % of a dose being excreted unchanged in urine after oral adminis-

tration<sup>[3-5]</sup>. There are several cytochrome P450 (CYP450) enzymes involved in the metabolism of verapamil in human liver, including CYP3A4, CYP3A5, CYP1A2, and CYP2C subfamily<sup>[6-8]</sup>. Different cleavages of the C-N-C bond by *N*-dealkylation are the main metabolic pathways. Verapamil and its *N*-dealkylated metabolites are further metabolized by *O*-demethylation. Studies in animals have demonstrated that norverapamil possessed about 20 % of the vasodilating activity of verapamil, whereas the other *N*-dealkylated metabolites were devoid of any vasodilating effect. Although the *O*-demethylated metabolites of verapamil exhibited the same potency as parent drug, their contribution to the overall pharmacological effect was negligible since these metabolites mainly presented as inactive conjugates<sup>[9,10]</sup>.

Previous studies of verapamil metabolism in rat liver microsomes used high concentration (0.33 g/L,

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<sup>&</sup>lt;sup>2</sup> Correspondence to Prof Da-fang ZHONG. Phn 86-24-2390-2539. Fax 86-24-2390-2539. E-mail zhongdf@china.com

nearly 734 µmol/L) of verapamil as substrate<sup>[11,12]</sup>, though peak plasma concentrations of verapamil following dosing to humans were generally about 1.0 µmol/L<sup>[13]</sup>. In this study, the metabolism of verapamil at low concentrations in rat liver microsomes was investigated by liquid chromatography-ion trap mass spectrometry (LC/MS<sup>n</sup>).

## MATERIALS AND METHODS

Chemicals Verapamil hydrochloride was supplied by Zhongyang Pharmaceutical Factory (Tianjin, China). Reference substances of verapamil metabolites were isolated from microbial transformation culture and identified by nuclear magnetic resonance (NMR), including N-methyl-4-(3,4-dimethoxyphenyl)-4-cyano-5-methylhexylamine (Compound 1), norverapamil, N-methyl-2-(3,4-dimethoxyphenyl)ethylamine (Compound 2),  $\alpha$ -[3-[[2-(3,4-dimethoxyphenyl)ethyl] methylamino[propyl]-3-methoxy-4-hydroxy- $\alpha$ -(1-methylethyl)-benzene-acetonitrile (Compound 3), and  $\alpha$ -[3-[[2-(3-methoxy-4hydroxyphenyl)ethyl]methylamino]propyl]-3,4-dimethoxy-α-(1-methylethyl)-benzeneacetonitrile (Compound 4). β-Nicotinamine adenine dinucleotide phosphate (reduced form, NADPH) was purchased from Sigma Chemical Co (St Louis, USA). DL-Dithiothreitol (DTT, ultra pure grade) and tris (hydroxymethyl) aminomethane (Tris, ultra pure grade) were supplied by Ameresco, USA. Acetonitrile was of HPLC grade (Yuwang Co, Shandong, China). All other chemicals were of analytical grade.

Animals Male Wistar rats were supplied by Lab Animal Center of Shenyang Pharmaceutical University (Grade II, Certificate No 042). Animals were fed a normal standard diet *ad libitum* and acclimatized at 12-h light cycle for at least 5 d before used. Rats were used when weighed 230-250 g. Rats had been fasted for 12 h before used for preparation of liver microsomes.

**Preparation of microsomes** In order to minimize degradation of enzymes, all apparatus and solutions were cooled and stored at 4 °C prior to the start of the experiment. Rats were weighed and then killed by cervical dislocation. The livers were rapidly removed and immediately placed in ice-cold saline to wash off excess blood and to cool the tissue. The livers were then blotted dry, weighed and added to 4 times their weight of sucrose solution (pH 7.4) consisting of sucrose 0.25 mol/L, Tris 10 mmol/L and editic acid 1.0 mmol/L. After finely chopped with scissors to such that no large pieces of tissue were evident, the livers

were homogenized at 23 000 rpm for 1 min using FJ-200 homogeniser (Shanghai, China). The homogenate was centrifuged (Sorval Super T21, Dupont, Wilmington, USA) at 20  $000 \times g$  at 4 °C for 20 min. The supernatant was further ultracentrifuged (Sorvall Ultracentrifuge OTD 55B, Dupont, Wilmington, USA) at  $100~000 \times g$  at 4 °C for 60 min. The microsomal pellets were suspended in potassium pyrophosphate buffer 0.1 mol/L (pH 7.4) containing editic acid 1.0 mmol/L and ultracentrifuged again at  $100~000 \times g$  at 4 °C for 60 min. The washed microsomal pallets were resuspended in Tris-HCl buffer 0.1 mol/L (glycerol 20 %, editic acid 0.1 mmol/L, dithiothreitol 0.1 mmol/L, pH 7.4) and stored at -70 °C until used.

Determination of microsomal protein and CYP450 The microsomal protein concentrations were determined by the method of Lowry et al<sup>[14]</sup>. The levels of CYP450 in microsomes were measured according to Omura and Sato<sup>[15]</sup>. In brief, the samples were diluted in Tris-HCl buffer 0.1 mol/L to approximately 0.5 g/L, then added to matched sample and reference cuvettes. A baseline was recorded between 400 and 500 nm on Shimadzu UV 2201, UV-Vis recording Spectrophotometer (Kyoto, Japan). After adding a few grains of solid sodium dithionite into the two cuvettes, the microsomes in sample cuvette were gently bubbled with carbon monoxide for 1 min. The spectrum was then rescanned from 400 to 500 nm, and the absorbance difference (450-490 nm) was measured. The concentrations of CYP450 were calculated according to Beer's Law and a molar extinction coefficient was 91 L·mmol<sup>-1</sup>·cm<sup>-1</sup>.

Incubation study Incubations were conducted with verapamil (1.0  $\mu mol/L$  and 5.0  $\mu mol/L$ ) and microsomes (2.0 g/L) in Tris-HCl buffer 0.1 mol/L at 37 °C in a gently agitating water bath. The reaction was initiated by adding NADPH 1.0 mmol/L in Tris-HCl buffer 0.1 mol/L. Samples (200  $\mu L$ ) were taken at three time points (0, 60, 120 min), then mixed with 800  $\mu L$  ice-cooled water and 100  $\mu L$  NaOH 1.0 mol/L, and vortexed with 3.0 mL diethyl ether immediately to terminate the reaction. Control experiments were carried out with previously denatured (heated at 100 °C for 5 min) microsomes.

LC/MS<sup>n</sup> analysis and identification of metabolites Samples from *in vitro* incubations were extracted with diethyl ether as described above. The supernatant was evaporated to dryness under a stream of nitrogen at 40 °C. Residue was redissolved in 200 μL of LC mobile phase, and an aliquot of 50 μL was injected and

analyzed by a Finnigan LCQ liquid chromatography-ion trap mass spectrometer (San Jose, USA). The instrument was operated in positive electrospray ionization mode. The capillary voltage was fixed at 16 V, and its temperature was maintained at 200 °C. The spray voltage was set at 4.25 kV. The HPLC fluid was nebulized using N<sub>2</sub> as both the sheath gas at a flow rate of 0.75 L/min, and the auxiliary gas at a flow rate of 0.15 L/min. The MS/MS spectra were produced by collision-induced dissociation (CID) of the selected precursor ions with He present in the mass analyzer, and the relative collision energy was set at 30 %-40 %. Data were collected and analyzed by the Navigator software (version 1.2, Finnigan, San Jose, USA). Liquid chromatography was performed with a Shimadzu LC-10AD solvent delivery system (Kyoto, Japan). The samples were separated on a Diamonsil C<sub>18</sub> column (particle size 5 μm, 4.6 mm ×200 mm ID, Dikma Co, Beijing, China). The mobile phase consisted of acetonitrile-water-formic acid (30:70:1, v/v) at a flow rate of 0.3 mL/min.

#### **RESULTS**

Metabolism profile of verapamil in rat liver microsomes Compared with the controls, eight metabolites were found in rat liver microsomal incubates, in addition to unchanged verapamil (Fig 1). The structures of metabolites were identified by comparison of chromatographic behaviors, electrospray ionization MS, and MS/MS spectra to those of reference substances. The retention times and main characteristic ions in mass spectra of verapamil and its metabolites were summarized in Tab 1.

**Parent drug** The compound eluting at 47.3 min possessed the same pseudo-molecular ion, full scan MS/MS spectrum, and chromatographic behavior with authentic verapamil, therefore, it was identified as unchanged verapamil. Verapamil showed abundant pseudo-molecular ion [M+H]<sup>+</sup> at *m/z* 455 in full scan mass spectrum, and the MS/MS spectrum of [M+H]<sup>+</sup> provided a number of characteristic fragment ions at

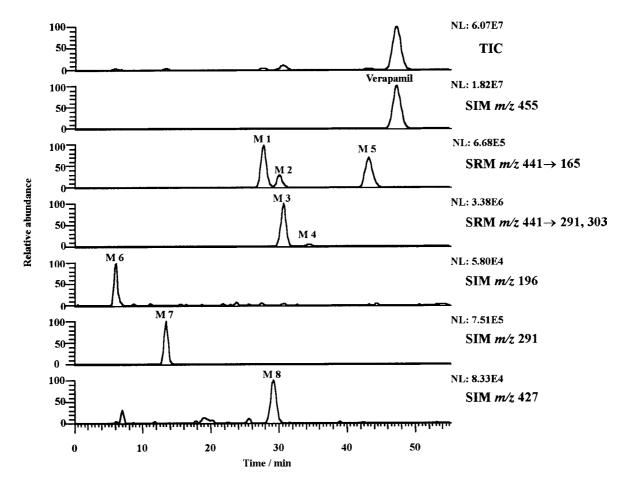


Fig 1. Representative chromatograms of verapamil and its metabolites in rat liver microsomes incubated with 1.0 μmol/L verapamil for 120 min.

Compound	$t_{\rm R}/{ m min}$	$MS$ $[M+H]^+$	MS/MS fragment ions m/z (relative abundance, %)				
Verapamil	Verapamil 47.3		303 (95), 260 (8), 165 (100), 150 (12)				
M 1	27.7	441	289 (70), 246 (5), 165 (100), 150 (15)				
M2	29.9	441	289 (71), 246 (5), 165 (100), 150 (14)				
M3	30.7	441	303 (19), 291 (100), 260 (6), 151 (9)				
M4	34.3	441	303 (100), 291 (46), 260 (11), 151 (20)				
M 5	43.2	441	398 (18), 289 (8), 260 (4), 165 (100), 150 (14)				
M6	6.1	196	165 (100)				
M7	13.4	291	260 (78), 248 (100)				
M8	29.2	427	384 (10), 289 (6), 277 (100), 260 (14), 151 (36)				

Tab 1. LC/MS<sup>n</sup> data of verapamil and its metabolites in rat liver microsomes.

m/z 303, 260, 165, and 150 (Fig 2), which were useful for identification of metabolites.

Metabolites M1-M5 M1-M5 all gave pseudo-molecular ions [M+H]<sup>+</sup> at *m/z* 441, yet each displayed different retention time and MS/MS spectrum, indicating that they were isomers. Their pseudo-molecular ions were 14 u lower than that of parent drug, suggesting

the loss of a methyl group from verapamil.

The MS/MS spectra of M1 and M2 were identical and showed fragment ions at m/z 289, 246, 165, and 150. The presence of the prominent ion at m/z 165 suggested that the phenylethyl moiety was unaltered. The diagnostic ions at m/z 289 and 246 were 14 u lower than those fragment ions in the MS/MS spectrum of

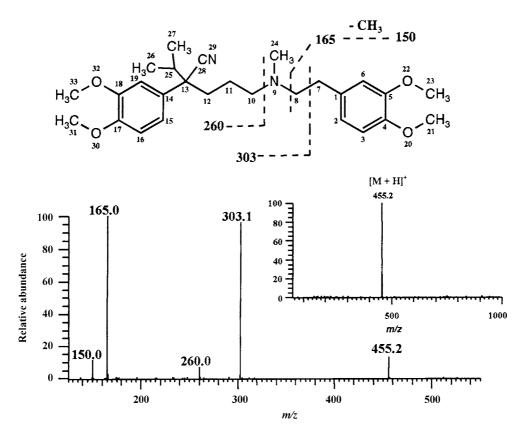


Fig 2. Full scan MS/MS spectrum of  $[M + H]^+$  ion (m/z 445) of verapamil (inset, full scan mass spectrum).

verapamil, which indicated that the loss of a methyl group was carried out in the phenylacetonitrile moiety. The difference between M1 and M2 lied in the positions of *O*-demethylation in the phenylacetonitrile moiety. M1 possessed the same retention time and mass spectra with Compound 3, and was confirmed as 30-*O*-demethyl-verapamil, whereas M2 was identified as 32-*O*-demethyl-verapamil.

The MS/MS spectra of M3 and M4 displayed same fragment ions at m/z 303, 291, 260, and 151. The presence of the fragment ions at m/z 303 and 260 suggested that the phenylacetonitrile moiety and N-methyl moiety were unaltered. The diagnostic ion at m/z 151 was 14 u lower than that in the MS/MS spectrum of verapamil, which suggested that the loss of a methyl group is from the phenylethyl moiety. The relative abundance ratios of the fragment ions at m/z 303 and 291 were different in M3 and M4, which indicated that O-demethylation in M3 and M4 occurred at different positions in the phenylethyl moiety. M3 possessed the same retention time and mass spectra with Compound 4, and was confirmed as 20-O-demethyl-verapamil, whereas M4 was identified as 22-O-demethyl-verapamil.

The MS/MS spectrum of M5 gave ions at m/z 398, 289, 260, 165, and 150. The presence of the fragment ions at m/z 260, 165, and 150 suggested that O-methyl moieties were all unaltered. The diagnostic ion at m/z 289 was 14 u lower than that in the MS/MS spectrum of verapamil, which indicated that the demethylation was carried out on the nitrogen atom. The fragment ion at m/z 398, 43 u lower than the precursor ion, was produced by the loss of isopropyl group. M5 was identified as norverapamil and was further confirmed by comparing its retention time and mass spectra with the reference substance.

Metabolite M6 The retention time of M6 was 6.1 min. It showed pseudo-molecular ion  $[M+H]^+$  at m/z 196, which was 259 u lower than that of verapamil, indicating cleavage of the phenylacetonitrile moiety. The fragment ion at m/z 165 in the MS/MS spectrum of M6, 31 u lower than the precursor ion, was proposed to arise via the loss of a neutral molecular of methylamine. Compared the retention time and mass spectra with Compound 2, M6 was confirmed as N-methyl-2-(3,4-dimethoxy phenyl)ethylamine.

**Metabolite M7** M7 with retention time of 13.4 min displayed pseudo-molecular ion  $[M+H]^+$  at m/z 291, which was 164 u lower (characteristic loss of the

phenylethyl moiety) than that of parent drug. The MS/MS spectrum of M7 gave fragment ions at m/z 260 and 248. The former ion was identical with the fragment ion at m/z 260 in the MS/MS spectrum of verapamil, whereas the latter ion was at a loss of 43 u (isopropyl group) from the precursor ion. M7 possessed the same retention time and mass spectra with Compound 1, and was confirmed as N-methyl-4-(3,4-dimethoxyphenyl)-4-cyano-5-methylhexylamine.

Metabolite M8 The retention time of M8 was 29.2 min. It exhibited pseudo-molecular ion [M+H]<sup>+</sup> at m/z 427, 28 u lower than that of parent drug, suggesting the loss of two methyl groups from verapamil. The MS/MS spectrum of M8 gave ions at m/z 384, 289, 277, 260, and 151. The diagnostic ion at m/z 151 suggested that one methyl group was lost from the phenylethyl moiety. The fragment ions at m/z 289 and 277 were 14 u lower than those in the MS/MS spectrum of M3 and M4, which indicating that M8 was a further demethylated derivate of M3 or M4. Since the relative abundance of the fragment ions at m/z 303 and 291 in the MS/MS spectra of M3 and M4 were determined by the positions of O-demethylation, ie, 20-Odemethyl-verapamil showed the base fragment ion at m/z 291, whereas 22-O-demethyl-verapamil gave the base fragment ion at m/z 303. The prominent ion at m/z277 in the MS/MS spectrum of M8 indicated that Odemethylation in the phenylethyl moiety occurred at para position to the substituted alkyl. The presence of the fragment ions at m/z 384 and 289 suggested that the other methyl group was lost from N-methyl moiety. According to these data, M8 was identified as 20-Odemethyl-norverapamil.

The proposed metabolic pathways of verapamil in rat liver microsomes was shown in Fig 3.

The concentrations of verapamil and metabolites (M1, M3, M5, M6, and M7) in incubates were quantified according to the ratios of their peak area to that of diphenhydramine (internal standard) by MS/MS full scan monitoring. M2 was estimated with M1 as reference, whereas M4 and M8 were estimated with M3 as reference. The relative percentages of unchanged verapamil and its metabolites in rat liver microsomal incubates were shown in Tab 2. All metabolites of verapamil were present after 60 min incubation, except for M8 at the substrate concentration of 1.0  $\mu$ mol/L, and the relative percentage of each metabolite at 120 min was higher than that at 60 min.

Fig 3. Proposed metabolic pathways of verapamil in rat liver microsomes.

Tab 2. The relative percentages of unchanged verapamil and its metabolites in rat liver microsomes incubated with verapamil.

Concentration of substrate	Time	Verapamil	M1	M2	М3	M4	M5	M6	M7	M8
1.0 μmol/L	60 min	81.4	2.0	0.51	9.7	0.43	2.3	0.08	2.6	-
	120 min	72.9	2.9	0.73	13.9	0.71	3.0	0.14	3.4	0.27
5.0 μmol/L	60 min	81.7	1.5	0.60	6.5	0.31	2.4	0.05	5.6	0.08
	120 min	71.5	2.3	0.84	10.4	0.47	3.9	0.09	8.5	0.21

## **DISCUSSION**

*In vitro* incubation with human or animals liver microsomes has been an usually method in drug metabolism studies<sup>[16-18]</sup>. In the present study, verapamil at two concentrations (1.0 μmol/L and 5.0 μmol/L) was incubated with rat liver microsomes for 120 min, and the percentages of metabolized verapamil were 27.1 % and 28.5 %, respectively, which were higher than those in previous reports<sup>[11,12]</sup>. The ratio of metabolites to parent drug in incubates was relatively lower than that in plasma and urine, which was due to the limited formation of metabolites *in vitro*<sup>[19]</sup>. A total of eight me-

tabolites of verapamil were found in rat liver microsomal incubates by LC/MS<sup>n</sup> method, including two novel metabolites (M4 and M8). The identities of five major metabolites were confirmed by chromatographic as well as mass spectral comparison with reference substances, and three additional metabolites were tentatively identified according to their LC and MS data. These metabolites were identified as *O*-demethyl-verapamil isomers (M1 - M4), *N*-dealkylated derivatives of verapamil (M5 - M7), and *N*, *O*-didemethyl-verapamil (M8).

There was difference between the metabolite profiles of verapamil at two concentrations. When verapamil was used at concentration of 1.0 µmol/L, *O*-demethylation

occurred more than N-dealkylation, resulting that 20-O-demethyl-verapamil (M3) was the major metabolite and even more than the sum of other metabolites. The amount of the *N*-dealkylated metabolites (M5-M7) increased obviously, and was nearly equal to that of the O-demethylated metabolites (M1-M4), when verapamil was incubated with rat liver microsomes at concentration of 5.0 µmol/L. Verapamil was used at much higher concentration (0.33 g/L, nearly 734 µmol/L) in earlier studies of verapamil metabolism in rat liver microsomes<sup>[12]</sup>, and it was reported that N-dealkylation was the primary route of verapamil metabolism and occurred more rapidly than O-demethylation by about 2-fold. Based on these results, O-demethylation and N-dealkylation were the main metabolic pathways of verapamil in rat liver microsomes, and the relative proportion of them in verapamil metabolism exhibited concentration dependence, which may due to the fact that different CYP450 enzymes involved in the metabolic pathways of verapamil. Previous studies proved that the *N*-dealkylation was catalyzed by CYP3A4, CYP3A5, and CYP1A2, and enzymes of the CYP2C subfamily were mainly responsible for *O*-demethylation in human, furthermore, the kinetic parameter estimates for the formation of the respective metabolites were quite different[6-8].

The regiochemistry of verapamil metabolism at low concentrations in rat liver microsomes was consistent with the reports by Nelson et al<sup>[11,12]</sup>. Among the three pathways of C-N-C cleavage of verapamil, N-dealkylation of the short phenylethyl moiety to yield the secondary amine (M7) was more facile than N-demethylation to yield norverapamil (M5). The amount of M6 produced by N-dealkylation of the long phenylacetonitrile moiety was very low. Four regioisomeric phenolic metabolites could be potentially produced by Odemethylation of verapamil. In addition to three Odemethylated metabolites reported in previously study, a novel metabolite, 22-O-demethyl-verapamil (M4), was detected by LC/MS<sup>n</sup> method. Because M4 was present only in trace amount, it appeared that the omission of M4 was due to the high detection limit of the analytical method. The extent of O-demethylation in the short phenylethyl moiety was greater than that in the phenylacetonitrile moiety. In both side chains, there seemed to be a more facile O-demethylation occurring at the position para than meta to the substituted alkyl.

In conclusion, verapamil at low concentrations was biotransformed into eight metabolites in rat liver

microsomal incubates, and *O*-demethylation and *N*-dealkylation were the main metabolic pathways, whereas the relative proportion of them in verapamil metabolism changed according to the concentrations of substrate. It also demonstrated that the metabolism of drugs *in vitro* was concentration-dependent, so the *in vitro* metabolism studies should be carried out at a wide range of substrate concentrations in order to predict the metabolism of drugs *in vivo* more reliably.

## REFERENCES

- 1 Toffoli G, Simone F, Corona G, Raschack M, Cappelletto B, GiganteM, *et al.* Structure-activity relationship of verapamil analogs and reversal of multidrug resistance. Biochem Pharmacol 1995; 50: 1245-55.
- 2 Pauli-Magnus C, von Richter O, Burk O, Ziegler A, Mettang T, Eichelbaum M, et al. Characterization of the major metabolites of verapamil as substrates and inhibitors of P-gly-coprotein 1. J Pharmacol Exp Ther 2000; 293: 376-82.
- 3 Eichelbaum M, Ende M, Remberg G, Schomerus M, Dengler HJ. The metabolism of *dl*-[<sup>14</sup>C]verapamil in man. Drug Metab Dispos 1979; 7: 145-8.
- 4 Mikus G, Eichelbaum M, Fischer C, Gumulka S, Klotz U, Kroemer HK. Interaction of verapamil and cimetidine: Stereochemical aspects of drug metabolism, drug disposition and drug action. J Pharmacol Exp Ther 1990; 253: 1042-8.
- Walles M, Thum T, Levsen K, Borlak J. Verapamil metabolism in distinct regions of the heart and in cultures of cardiomyocytes of adult rats. Drug Metab Dispos 2001; 29: 761-8.
- 6 Tracy TS, Korzekwa KR, Gonzalez FJ, Wainer IW. Cytochrome P450 isoforms involved in metabolism of the enantiomers of verapamil and norverapamil. Br J Clin Pharmacol 1999; 47: 545-52.
- 7 Kroemer HK, Gautier JC, Beaune P, Henderson C, Wolf CR, Eichelbaum M. Identification of P450 enzymes involved in metabolism of verapamil in humans. Naunyn-Schmiedebergs Arch Pharmacol 1993; 348: 332-7.
- 8 Busse D, Cosme J, Beaune P, Kroemer HK, Eichelbaum M. Cytochromes of the P450 2C subfamily are the major enzymes involved in the O-demethylation of verapamil in humans. Naunyn-Schmiedebergs Arch Pharmacol 1995; 353: 116-21.
- 9 Neugebauer G. Comparative cardiovascular actions of verapamil and its major metabolites in the anesthetized dog. Cardiovasc Res 1978; 12: 247-54.
- 10 Johnson KE, Balderston SM, Pieper JA, Mann DE, Reiter MJ. Electrophysiologic effects of verapamil metabolites in the isolated heart. J Cardiovasc Pharmacol 1991; 17: 830-7.
- 11 Nelson WL, Olsen LD, Beitner DB, Pallow RJ. Regio-chemistry and substrate stereoselectivity of *O*-demethylation of verapamil in the presence of the microsomal fraction from rat and human liver. Drug Metab Dispos 1988; 16: 184-8.
- 12 Nelson WL, Olsen LD. Regiochemistry and enantioselectivity in the oxidative *N*-dealkylation of verapamil. Drug Metab

- Dispos 1988; 16: 834-41.
- 13 Vogelgesang B, Echizen H, Schmidt E, Eichelbaum M. Stereoselective first-pass metabolism of highly cleared drugs studies of the bioavailability of *L* and *D*-verapamil examined with a stable isotope technique. Br J Clin Pharmacol 1984; 18: 733-40.
- 14 Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951; 193:265-75.
- 15 Omura T, Sato R. The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. J Biol Chem 1964; 239: 2370-8.
- 16 Zhong DF, Zhang SQ, Sun L, Zhao XY. Metabolism of roxithromycin in phenobarbital-treated rat liver microsomes. Acta Pharmacol Sin 2002; 23: 455-60.
- 17 Lu AH, Shu Y, Huang SL, Wang W, Ou-yang DS, Zhou HH. *In vitro* proguanil activation to cycloguanil is mediated by CYP2C19 and CYP3A4 in adult Chinese liver microsomes. Acta Pharmacol Sin 2000; 21: 747-52.
- 18 Liu XQ, Zhao Y, Li D, Qiao ZY, Wang GJ. Metabolism and metabolic inhibition of cilnidipine in human liver microsomes. Acta Pharmacol Sin 2003; 24: 263-8.
- 19 Rodrigues D. Use of *in vitro* human metabolism studies in drug development. Biochem Pharmacol 1994; 48: 2147-56.