ISSN 1671-4083 Acta Pharmacol Sin 中国药理学报 2002 May; 23 (5): 455 - 460
http://www.chinaphar.com

Metabolism of roxithromycin in phenobarbital-treated rat liver microsomes[1]

ZHONG Da-Fang[2], ZHANG Shu-Qiu, SUN Lu, ZHAO Xiao-Yun[3] (*Laboratory of Drug Metabolism and Pharmecokinetics*, [3]*Department of Microbiology*, *Shenyang Pharmaceutical University*, *Shenyang 110016*, *China*)

KEY WORDS roxithromycin; metabolism; microsomes; cytochrome P-450; mass spectrum analysis

ABSTRACT

AIM: To investigate the metabolism of roxithromycin (RXM) in rat liver microsomes and the possible effects of RXM and its metabolites on cytochrome P-450 (CYP450). **METHODS**: Liver microsomes of Wistar rats, induced by phenobarbital, were prepared using ultracentrifuge method. RXM *in vitro* metabolism was studied with the microsome incubation. The metabolites were separated and assayed by liquid chromatography-tandem mass spectrometry (LC-MS^n), and were further identified by comparison of their mass spectra and LC behavior to synthesized references. **RESULTS**: *N*-Mono- and *N*-di-demethyl metabolites as well as *O*-dealkylated metabolite (erythromycin oxime) were detected in microsomal incubates. RXM and its metabolites expressed weak potency to form inactive complexes with CYP450. **CONCLUSION**: *N*-Demethylation and oxime ether side chain *O*-dealkylation are main biotransformation pathways of RXM in phenobarbital-treated rat liver microsomes. Both routes were found to be NADPH-dependant. RXM and its metabolites showed weak inhibitory effects on CYP450.

INTRODUCTION

Roxithromycin (RXM), an ether-oxime derivative of erythromycin, is an acid-stable macrolide antibacterial. It has an antibacterial spectrum *in vitro* similar to that of erythromycin, covering most pathogens responsible for respiratory, skin, soft tissue, and urogenital infections. But it shows greater antibacterial potency and longer duration of action *in vivo* than erythromycin. That was thought to be the results of its preferable pharmacokinetic characteristics including an excellent enteral absorption, slower rate of metabolism, and higher concentration in most tissues and body fluids than erythromycin[1]. Several erythromycin-related macrolides have been implicated in numerous drug interactions, which might arise from the perturbations of hepatic CYP450 activities by macrolides. Troleandomycin[2-4], erythromycin[4-6], and oleandomycin[7] have been shown to induce and inactivate CYP450 enzymes, primarily CYP3A, which is considered as the main cause of drug interactions. Their chemical structure involves a tertiary amine function group that is demethylated and oxidized by CYP450 into probably nitroso derivative, and the later was thought to be responsible for the formation of inhibitory CYP450 Fe^{2+}-metabolite complex. Whereas RXM, which also contains a tertiary amine group, seems not form measurable inactive CYP450 Fe^{2+}-metabolite complexes *in vivo* after treatment of 400 mg/kg daily for 7 d and neither induces CYP3A[2,3]. So RXM should be less potential to produce clinically relevant interaction with other drugs. Recent studies showed that the demethylated metabolite of RXM was more potent in inhibiting CYP3A4-dependent testosterone 6β-hydroxylation by human liver microsomes and was more active to form CYP450 Fe^{2+}-metabolite complex than the parent drug[4].

Previous studies have found *N*-demethylation was the main pathway of RXM metabolism, catalyzed by CYP3A1/2 in rat or 3A4 in human, respectively[2]. The evidence of demethylation in previous studies was the formation of formaldehyde in rat or human liver microsomes. There is yet no direct evidence for the metabolism of *N*-desmethylation and other pathways in liver. Our previous study in human[8] has found 5 main routes of RXM metabolism in bile by LC-MS^n: ① isomerization of parent drug and its metabolites; ② *O*-demethylation; ③ *N*-demethylation; ④ hydrolysis of the

[1] Project supported by the National Natural Science Foundation of China, № 39930180.

[2] Correspondence to Prof ZHONG Da-Fang. Phn 86-24-2390-2539. Fax 86-24-2390-2539. E-mail zhongdf@ihw.com.cn

Received 2001-11-21 Accepted 2002-03-12

cladinose moiety; and ⑤ *O*-dealkylation of the oxime ether side chain. In this study, phenobarbital-induced rat liver microsomes were used to investigate the hepatic metabolism of RXM and to assess the effects of RXM and it's metabolites on CYP450.

MATERIALS AND METHODS

Chemicals RXM and erythromycin oxime reference substances were generous gifts from Huatai Drug Research Institute (Shenyang, China). *N*-demethyl RXM (RU 44981) was obtained from Hoechst-Marion-Roussel (Romainville Cedex, France). β-Nicotinamine adenine dinucleotide phosphate (reduced form, NADPH) was purchased from Sigma Chemical Co. Nicotinamine adenine dinucleotide (reduced form, NADH), *DL*-Dithiothreitol (DTT, ultra pure grade) and tris (hydroxymethyl) aminomethane (Tris, ultra pure grade) were supplied by Ameresco, USA. Methanol and acetonitrile were of HPLC grade (Yuwang Co, Shandong, China). All other chemicals were of analytical grade.

Animals and pretreatment Male Wistar rats were supplied by Lab Animal Center of Shenyang Pharmaceutical University (Grade Ⅱ, Certificate № 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 200 g – 250 g. Phenobarbital (75 mg/kg in 1 mL saline) was given to animals by ip for 3 d. After 6 h of the last dose, rats were fasted for 18 h and used for preparation of liver microsomes.

Preparation of microsomes Rats were weighed and then killed by cervical dislocation. The livers were removed and rinsed in chilled saline, then homogenized (Poly Tron, Switzerland) in 4 times the liver weight of sucrose solution (pH 7.4) consisting of sucrose 0.25 mol/L, Tris 10 mmol/L, and edetic acid 1 mmol/L. The homogenate was centrifuged at 20 000 × *g* for 20 min, 4 ℃, using Sorval Super T21 (DuPont, USA). The supernatant was further ultracentrifuged (Sorvall Ultracentrifuge OTD 55B, DuPont, USA) at 100 000 × *g* for 60 min, 4 ℃. The microsomal pellets were suspended in potassium pyrophosphate buffer 0.1 mol/L (pH 7.4) containing 1 mmol/L edetic acid and centrifuged again at 100 000 × *g* for 60 min, 4 ℃. The washed microsomal pallets were resuspended in Tris-HCl buffer 0.1 mol/L (glycerol 20 %, edetic acid 0.1 mmol/L, dithiothreitol 0.1 mmol/L, pH 7.4) and stored at –70 ℃ until used.

Determination of microsomal protein and CYP450 The microsomal protein concentrations were determined by the method of Lowry *et al*[9]. The level of CYP450 in microsomes was measured according to Omura and Sato[10]. In brief, after adding a few grains of solid sodium dithionite into microsomes, the sample cuvette of microsomes was gently bubbled with carbon monoxide for 1 min. The optical difference (450 nm – 490 nm) was measured on Shimadzu UV 2201, UV-Vis recording Spectrophotometer. The concentration of CYP450 was calculated according to Beer's Law and a molar extinction coefficient of 91 $mmol^{-1} \cdot L \cdot cm^{-1}$.

Determination of *in vitro* formation of CYP450 Fe^{2+}-metabolite complexes The formation of inhibitory CYP450 Fe^{2+}-metabolite complexes by phenobarbital-treated rat liver microsomes was determined using RXM and its metabolites as substrates referring to Yamazaki H *et al*[4]. The reaction mixture was composed of microsome 2 g/L, KCl 10 mmol/L, $MgCl_2$ 10 mmol/L, and NADPH 1 mmol/L in Tris-HCl buffer 0.1 mol/L (pH 7.4), and was divided into two cuvettes. The reaction was initiated after addition of RXM or its metabolites (in 20 μL of ethanol, final concentration is 10 μmol/L) into sample cuvette and a same volume of ethanol into reference cuvette. The formation of CYP450 Fe^{2+}-metabolite complexes was determined at 25 ℃ by recording the absorbance at 456 nm every 2 min for 10 min.

Incubation study Incubations were conducted with RXM 10 μmol/L and microsome 2 g/L in Tris-HCl buffer 0.1 mol/L (pH 7.4), containing KCl 10 mmol/L and $MgCl_2$ 10 mmol/L, at 37 ℃ in gently agitating water bath. The reaction was initiated by adding NADPH 1 mmol/L in Tris-HCl buffer 0.1 mol/L (pH 7.4). Samples (100 μL) were taken at determined time points (0, 30, 60 min) and mixed with 200 μL ice-cooled Na_2CO_3 0.1 mol/L and vortexed with 3 mL diethyl ether immediately to terminate the reaction. Control experimemts were conducted with previously denatured (heated at 100 ℃ for 5 min) microsomes.

LC-MS^n analysis and identification of metabolites Samples from *in vitro* incubations were extracted with 3 mL of diethyl ether as described previously[8,11]. The supernatant was evaporated to dryness under a stream of nitrogen at ambient temperature. Residue was dissolved in 200 μL of LC mobile phase, and an aliquot of 20 μL was injected and analyzed by a Finnigan LCQ ion trap liquid chromatography-mass spectrometer

(Finnigan, San Jose, USA) coupled with an electrospray ionization source. Separation was performed with a Kromasil ODS column (partical size 5 μm, 20 cm×4.6 mm ID, Hi-Tech Scientific Instrument Corp, Tianjin, China) at ambient temperature. Mobile phase consisting acetonitrile-methanol-ammonium acetate 10 mmol/L (50:10:40, v:v:v) was used at a flow rate of 0.5 mL/min. In MS/MS detection, the positive-ion mode was employed and the spray voltage was set at 4.25 kV. The capillary voltage was fixed at 30.0 V and its temperature was maintained at 180 ℃. The HPLC fluid was nebulized using nitrogen (N_2) 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. MS/MS spectra were obtained by collision induced dissociation (CID) using helium (He) as the collision gas. All data were analyzed by Navigator software (version 1.2, Finnigan).

RESULTS

Metabolism profile of RXM in rat liver microsomal incubates Three metabolites were found in incubates. Their HPLC chromatogram and MS/MS spectra were shown in Fig 1. Their structures were identified by comparison of LC retention time and electrospray ionization MS as well as MS/MS spectra to that of synthesized reference standards.

RXM gave an HPLC retention time of 11.4 min, and produced quasi-molecular ion $[M+H]^+$ at *m/z* 837. It also displayed MS/MS spectra of fragment ions at *m/z* 679, 558, 540, and 522, resulting from loss of cladinose (−158 u), oxime ether side chain (−121 u), water (−18 u), and desosamine (−157 u) respectively.

Metabolite 1 (M1) showed an LC retention time of 7.0 min and quasi-molecular ion $[M+H]^+$ at *m/z* 749. This ion was 88 u lower than the quasi-molecular ion of RXM. It was proposed to be the result of *O*-dealkylation on the oxime ether side chain. And a number of MS/MS fragment ions detected at *m/z* 591, 558, 540, 522, and 434 also suggested the cladinose and desosamine rings in M1 were not altered. M1 was identified as erythromycin oxime and was further confirmed by comparing its LC behavior and MS, MS/MS spactra with reference substance.

The HPLC retention time of metabolite 2 (M2) was 8.9 min. The quasi-molecular ion $[M+H]^+$ of M2 was detected at *m/z* 823, 14 u lower than the quasi-molecular ion of parent drug, a characteristic loss of methyl group. The MS/MS spectra of M2 showed a

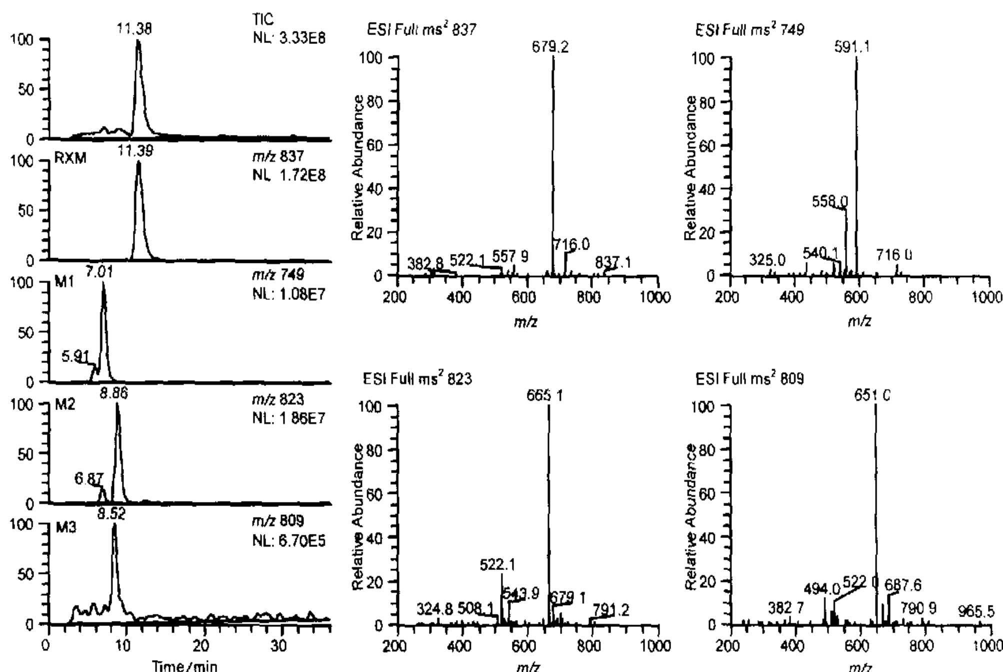

Fig 1. Representative chromatograms and MS/MS spectra of RXM and its metabolites in rat liver microsomes.

ISSN 1671-4083 Acta Pharmacol Sin 中国药理学报 2002 May; 23 (5)
E-mail aps@mail.shcnc.ac.cn Phn/Fax 86-21-6474-2629

number of fragment ions at m/z 665, 544, 522, and 508, that indicated the demethylation did not occur on the cladinose or oxime ether side chain. M2 was identified as N-demethyl RXM and further confirmed with synthesized reference.

Metabolite 3 (M3) has shown an HPLC retention time of 8.5 min and $[M+H]^+$ at m/z 809. The MS/MS fragment ions at m/z 651, 530, 522, and 494, all 14 u lower than the corresponding fragment ions of M2, indicating the cladinose ring and oxime ether side chain remained no changes and the desosamine ring was lack of double methyl groups. According to these data and compared with M2 spectra, M3 was identified as N-di-demethyl metabolite of RXM.

The proposed profile of RXM metabolism in rat liver microsomes is shown in Fig 2.

The concentrations of RXM, metabolites M1 and M2 in incubate were determined according to the ratios of their peak area to that of clarithromycin (internal standard) by MS/MS full scan monitoring. M3 was estimated with N-demethyl RXM as reference. The limit of quantitation was about 10 μg/L and the inter-day variability was less than 8 %. The maximum extents of metabolism by M1 and M2 were about 5 % and 10 % of parent drug. And M3 only accounted for 0.5 % of parent drug.

Effects of incubation co-factors on the metabolism of RXM In the incubation studies, we compared the effects of NADPH and NADH as cofactors on the metabolism of RXM. We designed the incubations with NADH or NADPH alone, or with both NADPH and NADH as cofactor(s). NADH (1 mmol/L) did not change the biotransformation extents of three metabolites when it was combined with NADPH as incubation cofactors. And no metabolite was measured in those incubations when NADH as cofactor was used alone.

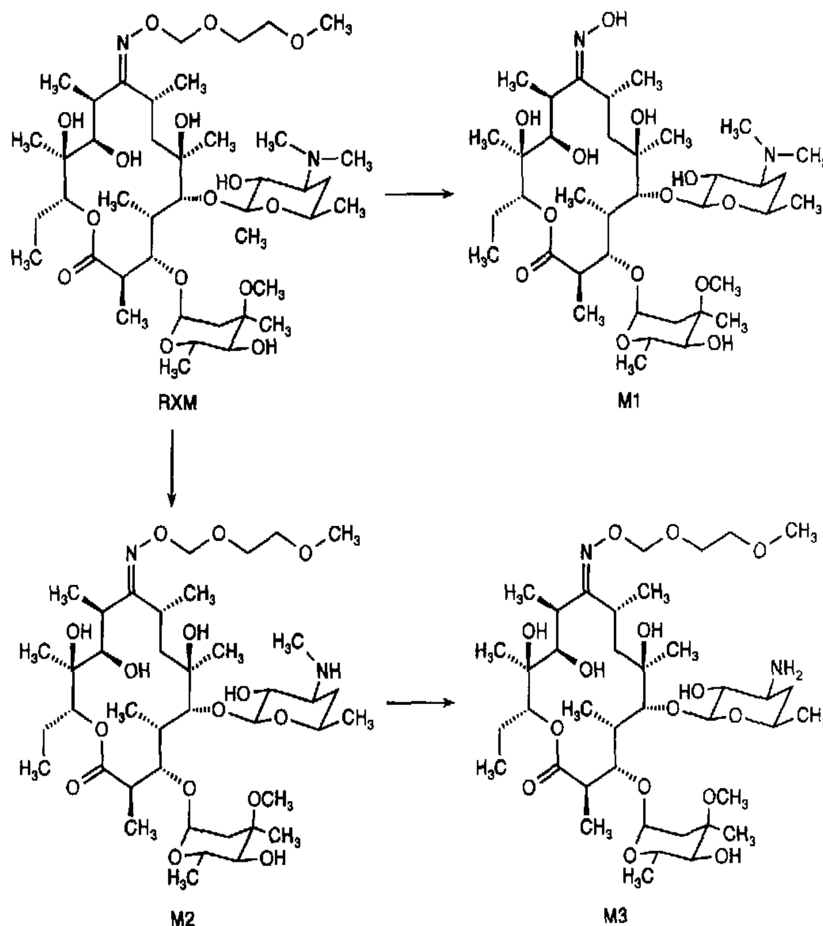

Fig 2. Proposed metabolic pathways of RXM in phenobarbital-treated rat liver microsomes.

***In-vitro* formation of CYP450 Fe^{2+}-metabolite complexes** Formation of inhibitory CYP450 Fe^{2+}-metabolite complexes was investigated in phenobarbital-treated rat liver microsomes using RXM, M1, and M2 as substrates. M3 was not studied due to the lack of reference substrate. All tested compounds showed weak potency of complexes formation. M1 and M2 were slightly more potent to form inhibitory CYP450 Fe^{2+}-metabolite complexes than parent drug (Fig 3).

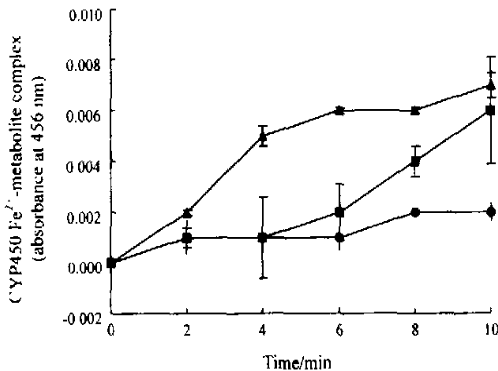

Fig 3. Formation of cytochrome P450 Fe^{2+}-metabolite complexes of RXM (●), erythromycin oxime (▲), and *N*-demethyl RXM (■). $n=3$. $\bar{x} \pm s$.

DISCUSSION

Previous studies[12,13] have found decladinosyl metabolite, oxime ether *O*-dealkyl metabolite, and *N*-demethyl metabolites in animal and human after oral doses of RXM. Only *N*-monodemethyl metabolite was detected in previous *in vitro* metabolism study[14]. Our present results demonstrated that RXM underwent NADPH-dependent *N*-demethylation and *O*-dealkylation of the oxime ether side chain in phenobarbital-treated rat liver microsomes. And the formation of these metabolites was NADPH-dependent. That suggested both biotransformation pathways are of oxidation mechanism.

Our previous study[8] has found that isomerization was one of the main pathways of RXM metabolism in human, but in current study isomerized metabolites could not be detected. A minor metabolite with the same quasi-molecular ion $[M+H]^+$ at m/z 823 as *N*-demethylated RXM was detected at retention time of 6.9 min (Fig 1). This metabolite was recognized as *O*-demethyl RXM according to its MS/MS fragment ions at m/z 665, 558, 540, and 508 (data not shown) and by compared with previous data. The present data further supported our previous results[11] that species differences existed in RXM metabolism between man and rat.

RXM and its *N*-demethylated as well as oxime ether *O*-dealkylated metabolites did not markedly form CYP450 Fe^{2+}-metabolite complexes, while M1 and M2 were slightly more potent to inhibit CYP450. The inhibitory effect of macrolides was mainly dependent on the hydrophobicity or hindrance around the dimethyl-amine function and the ionization state of this group[6]. The difference in the environment of $-N(CH_3)_2$ group might play an important role for the accessibility of substrates into protein active site and caused the variability in formation of inhibitory CYP450 Fe^{2+}-metabolite complexes between RXM and its metabolites[6,15].

In conclusion, the present study demonstrates that the *N*-methylation and oxime ether *O*-dealkylation are main pathways of RXM biotransformation in phenobarbital-treated rat liver microsomes. Both metabolites showed slightly more potent inhibitory effect on CYP450 than RXM.

REFERENCES

1 Markham A, Faulds D. Roxithromycin: an update of its antimicrobial activity, pharmacokinetic properties and therapeutic use. Drugs 1994; 48: 297 - 326.

2 Yamazaki H, Hiroki S, Urano T, Inoue K, Shimada T. Effects of roxithromycin, erythromycin and troleandomycin on their *N*-demethylation by rat and human cytochrome P450 enzymes. Xenobiotica 1996; 26: 1143 - 53.

3 Pessayre D, Tinel M, Larrey D, Cobert B, Funck-Brentano C, Babany G. Inactivation of cytochrome P-450 by a troleandomycin metabolite. Protective role of glutathione. J Pharmacol Exp Ther 1983; 224: 685 - 91.

4 Yamazaki H, Shimada T. Comparative studies of *in vitro* inhibition of cytochrome P450 3A4-dependent testosterone 6β-hydroxylation by roxithromycin and its metabolites, troleandomycin, and erythromycin. Drug Metab Dispos 1998; 26: 1053 - 7.

5 Villa P, Sassella D, Corada M, Bartošek I. Effects of roxithromycin, a new semisynthetic macrolide, and two erythromycins on drug metabolizing enzymes in rat liver. J Antibiot 1988; 41: 563 - 9.

6 Delaforge M, Ladam P, Bouillé G, Gharbi Benarous J, Jaouen M, Girault JP. pH effects on the *N*-demethylation and formation of the cytochrome P-450 iron II nitrosoalkane complex for erythromycin derivatives. Chem-Biol Interact 1992; 85: 215 - 27.

7 Pessayre D, Descatoire V, Tinel M, Larrey D. Self-

induction by oleandomycin of its own transformation into a metabolite forming an inactive complex with reduced cytochrome P-450. Comparison with troleandomycin. J Pharmacol Exp Ther 1982; 221: 215-21.

8 Zhong DF, Li XQ, Wang AM, Xu YJ, Wu SD. Indentification of the metabolites of roxithromycin in humans. Drug Metab Dispos 2000; 28: 552-9.

9 Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951; 193: 265-75.

10 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.

11 Li XQ, Zhong DF, Huang HH, Wu SD. Demethylation metabolism of roxithromycin in humans and rats. Acta Pharmacol Sin 2001; 22: 469-74.

12 Esumi Y, Zin Y, Ban S, Ninomiya S, Hayashi K, Yokoshima T, *et al*. Absorption, distribution, metabolism and excretion of RU 28965 in animals. Chemotherapy 1988; 36: 148-63.

13 Koyama M, Tateno M, Shirotsuka M, Yamamoto M, Hirayama M, Saitoh K, *et al*. Absorption, metabolism and excretion of RU 28965 in humans. Chemotherapy 1988; 36: 164-83.

14 Jarukamjorn K, Thalhammer T, Gollackner B, Pittenauer E, Jaeger W. Metabolism of roxithromycin in the isolated perfused rat liver. J Pharm Pharmacol 1998; 50: 515-9.

15 Yamazaki H, Shimada T. Formation *in vitro* of an inhibitory cytochrome P450 × Fe^{2+}-metabolite complex with roxithromycin and its decladinosyl, *O*-dealkyl and *N*-demethyl metabolites in rat liver microsomes. Xenobiotica 1998; 28: 995-1004.

罗红霉素在苯巴比妥诱导的大鼠肝微粒体中的代谢[1]

R96 A

钟大放[2], 张淑秋, 孙 璐, 赵晓云[3]
(沈阳药科大学药物代谢与药物动力学实验室,
[3]微生物学教研室, 沈阳 110016, 中国)

关键词 罗红霉素; 代谢; 微粒体; 细胞色素 P-450; 质量光谱分析

目的: 研究罗红霉素在大鼠肝微粒体中的代谢, 并考察罗红霉素及其代谢物对细胞色素 P-450 的影响. **方法**: 采用超离心法制备了苯巴比妥诱导的大鼠肝微粒体酶. 罗红霉素的体外代谢采用微粒体孵化方法, 代谢物经 LC-MS 方法分离和分析, 并通过进一步与合成对照品比较其质谱和色谱行为确定其结构. **结果**: 在微粒体孵化液中发现了 *N*-去甲基, *N*-双去甲基及 *O*-去烷基三种代谢物. 罗红霉素及其代谢物与 CYP450 Fe^{2+} 形成复合物的能力较弱. **结论**: 罗红霉素在苯巴比妥诱导的大鼠肝微粒体中主要经历 *N*-去甲基化和肟醚侧链 *O*-去烷基化途径, 两种转化途径均为 NADPH 依赖性. 罗红霉素及其代谢物对 CYP450 的抑制作用较弱.

(责任编辑 吴民淑)