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Short communication

Characterization of human liver cytochrome P450 enzymes involved in the metabolism of rutaecarpine

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Abstract

Rutaecarpine has recently been characterized to have an anti-inflammatory activity through cyclooxygenase-2 inhibition. The incubation of rutaecarpine with human liver microsomes in the presence of NADPH generated six isobaric mono-hydroxylated metabolites. The specific cytochrome P450 (CYP) isozymes responsible for rutaecarpine metabolites were identified using the combination of chemical inhibition, immuno-inhibition and metabolism by cDNA expressed CYP enzymes. The results suggested that CYP3A4 might play major roles in the metabolism of rutaecarpine in human liver microsomes. The production of M1, M2, M3, M4 and M6 formed in human liver microsomes was inhibited by ketoconazole, a selective CYP3A4 inhibitor, and anti-CYP3A4 antibody. CYP1A2 and CYP2C9 played minor roles in the metabolism of rutaecarpine. These results were confirmed in microsomes derived from cDNA expressed lymphoblastoid cells. CYP3A4 microsome clearly formed M1, M2, M3 and M6. CYP1A2 and CYP2C9 microsomes comparably formed M5.

Keywords: Rutaecarpine; Human liver microsomes; LC-ESI/MS

1. Introduction

Rutaecarpine (8,13-dihydro-7H-indolo [2′,3′:3,4]pyrido[2,1-b]quinazolin-5-one) is an alkaloid originally isolated from the unripe fruit of *Evodia rutaecarpa*, which has traditionally been used in treatment of gastrointestinal disorders. In addition, rutaecarpine has recently been characterized to have an anti-inflammatory activity through cyclooxygenase-2 inhibition [1]. To develop rutaecarpine as an anti-inflammatory agent, total synthesis of rutaecarpine has successfully been established in our group [2].

Rutaecarpine was found to be metabolized by cytochrome P450 (CYP) in rat liver microsomes [3]. From the study, rutaecarpine was metabolized to nine different metabolites—i.e., 5-mono-hydroxylated and 4-di-hydroxylated rutaecarpine. In addition, 3-methylcholanthrene- and phenobarbital-induced microsomes greatly increased the formation of mono-hydroxylated metabolites [4]. Most recently, structures of four mono-hydroxylated metabolites, formed in untreated and 3-methylcholanthrene-induced rat liver microsomes, were identified by mass, UV absorbance and ^{1}H NMR spectra with those of authentic synthetic standards [5].

Moreover, rutaecarpine showed the modulatory effects on CYP1A1 and 1A2 in human or mouse liver and kidney [6–9] and CYP2B in rat liver [4]. Recently, rutaecarpine identified as a component from *Evodia* fruit extract showed a mechanism-based inactivation on CYP3A4 activity [10].

Although several previous studies reported that rutaecarpine was metabolized by rat liver microsomes, possibly by CYP1A and 2B, metabolism of rutaecarpine by human liver microsomes has not yet been studied. The purposes of the present study were to know the metabolite formation from rutaecarpine in human liver microsomes and to characterize the major human CYP isozyme(s) involved in rutaecarpine metabolism, because these data would be crucial in early development of new drug candidates.

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2. Materials and methods

2.1. Chemicals

Rutaecarpine (purity, >99.8%) used in this study was chemically synthesized in our group [3]. Pooled human liver microsomes, human B-lymphoblastoid-derived CYP containing microsomes and rabbit polyclonal anti-human CYP antibodies were obtained from Gentest (Woburn, MA). Glucose 6-phosphate, β-$NADP^+$, glucose 6-phosphate dehydrogenase, furafylline, diethyldithiocarbamate and ammonium formate were obtained from Sigma Chemical Co. (St. Louis, MO). Sulfaphenazole and ketoconazole were obtained from RBI/Sigma (Natick, MA). Quinidine was obtained from Aldrich Chemical Co. (Milwaukee, WI). All other chemicals used were of analytical grades and used as received.

2.2. Biotransformation of rutaecarpine

Metabolism of rutaecarpine (50 μM, final concentration) was conducted using 1 mg/ml of human liver microsomal protein in 0.1 M potassium phosphate buffer, pH 7.4, at 37 °C for 60 min, in a final incubation volume of 200 μl. The reactions were initiated by the addition of a NADPH-generating system (NGS) containing 0.8 mM $NADP^+$, 10 mM glucose 6-phosphate and 1 unit of glucose 6-phosphate dehydrogenase, into the reaction mixture. The reaction was stopped by the addition of 800 μl ethyl acetate. After mixing and centrifugation, 600 μl of the organic layer was separated. The extraction step was repeated three times. Then the organic layer was dried under a stream of nitrogen gas. The residue was reconstituted in HPLC grade MeOH and injected into an HPLC column.

2.3. Chemical inhibition

The effects of selective chemical inhibitors for individual CYP enzymes on the metabolism of rutaecarpine were investigated in pooled human liver microsomes. The chemical inhibitors used in this study were as follows: furafylline for CYP1A2 [11]; sulfaphenazole for CYP2C9 [12]; quinidine for CYP2D6 [13]; diethyldithiocarbamate for CYP2E1 [14]; and ketoconazole for CYP3A4 [15]. Incubations were performed with two or three concentrations of the inhibitor, human liver microsome (1 mg/ml) and rutaecarpine (50 μM). All inhibitors were preincubated for 30 min at 37 °C with microsomes and the NGS before addition of rutaecarpine to initiate the reaction.

2.4. Immunoinhibition study

Immunoinhibition studies were performed by preincubating pooled human liver microsomes with various amounts of human selective antibodies, such as anti-CYP1A2, anti-CYP2C9 and anti-CYP3A4 for 15 min at room temperature before the addition of rutaecarpine (50 μM). The reaction mixture was kept for additional 5 min at 37 °C. The reaction was started by adding an NGS, and incubation was performed for 1 h at 37 °C.

2.5. Metabolism by CYP-expressed B-lymphoblastoid microsomes

Incubation with B-lymphoblastoid microsomes containing 50 pmol/ml of individual CYP protein was conducted as described above with rutaecarpine (50 μM) for 1 h at 37 °C.

2.6. Instruments

The HPLC consisted of an HP 1100 series binary pump system (Agilent, Palo Alto, CA, USA) with an LC/MSD ion-trap mass spectrometer equipped with an electrospray ionization (ESI) source (Agilent). The column used for the separation was an XTerra® MS C_{18} (Waters, 2.1 mm × 150 mm, 3.5 μm). Column temperature was maintained constant at 30 °C using a thermostatically controlled column oven. The HPLC mobile phases consisted of 10 mM ammonium formate at pH 6.0 (A) and 90% acetonitrile in 10 mM ammonium formate at pH 6.0 (B). A gradient program was used for the HPLC separation with a flow rate of 0.2 ml/min. The initial composition was 35% B, programmed linearly to 70% B after 15 min. The entire column eluent was directly introduced into an ESI interface through a 50 cm-long PEEK tubing (0.13 mm i.d.). Nitrogen was used both as the nebulizing gas at 35 psi and as the drying gas with a flow rate of 8 l/min at a temperature of 350 °C. The mass spectrometer was operated in the positive ion mode in *m/z* 100–350. Helium was used as the collision gas for the tandem mass spectrometric experiments. Fragmentation was induced with resonant excitation amplitude of 0.85 V, following by an isolation of the precursor ion over a selected mass window of 1 Da.

3. Results

In previous studies, rutaecarpine was found to be metabolized in rat liver microsomes in CYP-dependent manners [3–5]. Representative extracted ion chromatograms obtained from the incubation of rat and human liver microsomes with rutaecarpine are shown in Fig. 1. The incubation of rutaecarpine with human liver microsomes in the presence of NGS generated six isobaric mono-hydroxylated metabolites. Six metabolites showed retention times of 9.2, 9.8, 10.9, 11.8, 13.0 and 13.6 min, respectively. Their MH^+ ions were observed at *m/z* 304 for the mono-hydroxylated metabolites. M4 was a newly found mono-hydroxylated form from the present study.

Table 1 shows the effects of selective inhibitors of CYPs on rutaecarpine metabolism in pooled human liver microsomes. Ketoconazole, selective inhibitor of CYP3A4, strongly inhibited the formation of M1, M2, M4 and M6 in a concentration-dependent manner. The formation of M3 and M5 was also inhibited by ketoconazole to a lesser degree. Furafylline, a selective inhibitor of CYP1A2, slightly inhibited the formation of M5. A selective CYP2E1 inhibitor, diethyldithiocarbamate, inhibited the formation of M4. Neither sulfaphenazole nor quinidine, specific inhibitors of CYP2C9 and CYP2D6, inhibited the formation of metabolites from rutaecarpine at the concentration used in the present study.

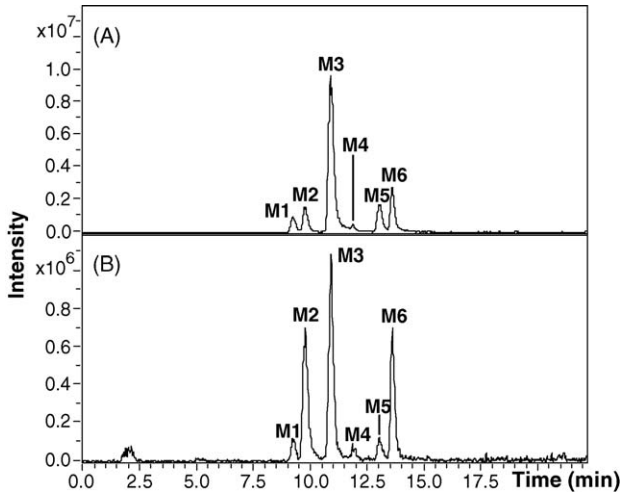

Fig. 1. Extracted ion chromatograms for hydroxylated rutaecarpine following incubation with control rat liver microsomes (A) and pooled human liver microsomes (B) in the presence of an NADPH-generating system.

To further determine which CYP is primarily responsible for rutaecarpine metabolism, immuno-inhibition studies with anti-CYP1A2, anti-CYP2C9 and anti-CYP3A4 were performed in pooled human liver microsomes (Fig. 2). Anti-CYP3A4 dose-dependently and strongly inhibited the formation of M1, M2, M4 and M6. The production of M3 and M5 was dose-dependently inhibited by anti-CYP1A2 and anti-CYP2C9.

Fig. 3 and Table 2 show the metabolism of rutaecarpine by microsomes derived from CYP-expressed lymphoblastoid cells. CYP3A4-expressed microsome clearly formed M1, M2, M3 and M6, whereas M5 was slightly formed. CYP1A2- and CYP2C9-expressed microsomes also formed M2, M3, M5 and M6. However, the formation of all metabolites by CYP3A4 was approximately 10 folds higher than the formation by CYP1A2 and 2C9 except M5 (Table 2). Only M5 was comparably formed by CYP1A2, 2C9 and 3A4. Moreover, M1 was only formed in the CYP3A4-expressed microsome. CYP2D6- and CYP2E1-expressed B-lymphoblastoid microsomes did not metabolize rutaecarpine to M1 (data not shown).

4. Discussion

From several previous studies, rutaecarpine was found to be metabolized by CYP-dependent manners in rat liver microsomes [3–5]. The present study demonstrated for the first time that rutaecarpine could be metabolized by CYP enzymes in human liver microsomes. The results indicated that rutaecarpine could be metabolized to six mono-hydroxylated metabolites in control rat and pooled human liver microsomes (Fig. 1). The same metabolites were identified in both microsomes, although the rate of formation of each metabolite might be different because of species differences in the relative expression of individual CYPs in rat and human. Although the authentic standards were not used in the present study, the exact structures of these metabolites including M4 could be assigned by the LC/ESI-MS [3] and the previous report with chemically synthesized metabolites [5].

Each metabolite observed in the present study was assigned as methods mentioned in several previous studies. M1 was proposed to possess a hydroxyl group on the aliphatic group moiety [3]. Other metabolites (i.e. M2, M3, M4, M5 and M6) were identified to be characteristic forms for the presence of a hydroxyl group in different positions [3]. Specifically, from the exact structures of mono-hydroxylated metabolites of rutaecarpine by using authentic synthesized compounds [5], all metabolites identified from the present study could be assigned as 10-hydroxy-rutaecarpine for M2, 11-hydroxy-rutaecarpine for M3, 9-hydroxy-rutaecarpine for M4, 12-hydroxy-rutaecarpine for M5 and 3-hydroxy-rutaecarpine for M6 (Fig. 4). To confirm the structures of individual metabolites, the mass spectrometric analyses with the same experimental conditions including the column which Ueng et al. [5] used were performed. The same chromatograms were obtained in our studies, indicating that the

Table 1
Effects of CYP inhibitors on the metabolism of rutaecarpine in human liver microsomes

Inhibitor	Inhibitor concentration (μM)	Relative percentage of control					
		M1	M2	M3	M4	M5	M6
Control	0	100	100	100	100	100	100
Furafylline (CYP1A2)	5	91.5	97.8	90.8	78.2	78.6	87.7
	20	67.8	74.1	69.2	64.7	55.8	69.4
Sulfaphenazole (CYP2C9)	5	85.3	83.9	86.3	55.1	77.1	86.6
	20	74.9	88.9	91.9	60.3	79.4	83.4
Diethyldithiocarbamate (CYP2E1)	5	83.7	84.4	73.9	82.3	86.0	83.0
	20	81.7	84.2	64.8	21.8	82.0	86.1
Quinidine (CYP2D6)	5	84.5	89.2	73.6	74.1	89.1	81.9
	20	79.1	81.7	71.5	71.3	76.1	81.4
Ketoconazole (CYP3A4)	5	66.2	86.5	81.0	69.1	90.6	71.8
	20	38.9	37.7	52.5	6.9	69.2	23.0
	40	7.1	29.3	58.4	N.D.	69.4	14.6

Rutaecarpine (50 μM), human liver microsomes (1 mg/ml) and individual inhibitors were incubated in the presence of NADPH-generating system for 1 h at 37 °C as described in Section 2. Each value represents percent area under the curve of no inhibitor control in triplicate determinations. N.D., not detected.

Table 2
Relative percent of metabolites formation from rutaecarpine by CYP3A4 in the cDNA-transfected human B-lymphoblastoid cell microsome

cDNA-transfected human B-lymphoblastoid cell microsome	Relative percentage of metabolites formation vs. CYP3A4					
	M1	M2	M3	M5	M6	Total
CYP3A4	100	100	100	100	100	100
CYP1A2	N.D.	1.6	15.2	94.1	4.0	7.8
CYP2C9	N.D.	3.3	10.7	121.7	11.6	9.2

Rutaecarpine (50 μM) and cDNA-transfected human B-lymphoblastoid cell microsome (50 pmol/ml) were incubated in the presence of NGS for 1 h at 37 °C as described in Section 2. Each value represents percent area under the curve of CYP3A4 in duplicate determinations. N.D., not detected.

structures assigned in the present studies would be exact (data not shown).

The specific CYP isozymes responsible for rutaecarpine metabolism were identified by using the combination of chemical inhibition, immuno-inhibition and metabolism by CYP-expressed microsomes. Although the authentic metabolite standard was not available in the present study, we could compare the relative production of each metabolite by using the area under the curve of controls versus treated groups. The present results indicated that CYP3A4 could play major roles in the metabolism of rutaecarpine in human liver microsomes. As mentioned in Section 3, M1, M2, M3, M4 and M6 were dose-dependently inhibited by ketoconazole, a selective CYP3A4 inhibitor, and/or by anti-CYP3A4 antibody (Table 1, Figs. 2 and 3). The metabolites M3 could also be formed by other expressed microsomes—i.e., CYP1A2 and 2C9, but the rates of formation of M3 by

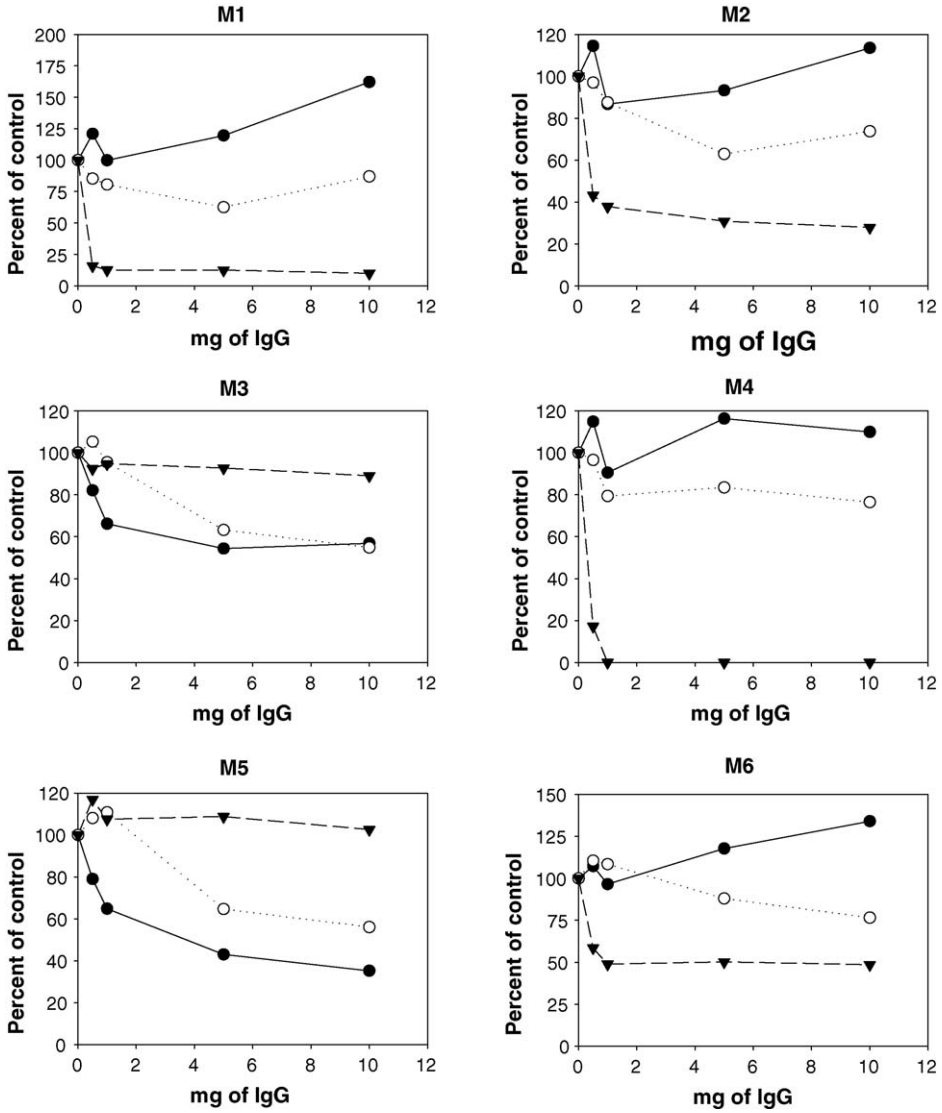

Fig. 2. Effects of CYP antibodies on the biotransformation of rutaecarpine. Pooled human liver microsomes (1 mg/ml protein) were preincubated with either anti-CYP1A2 (●), anti-CYP2C9 (◯) or anti-CYP3A4 (▼). Each value represents mean percent of control without antibody in duplicate determinations.

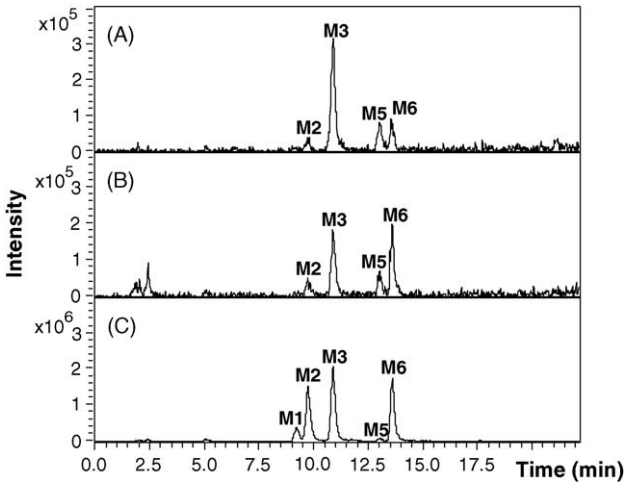

Fig. 3. Representative extracted ion chromatograms following metabolism of rutaecarpine by specific CYP-expressed human B-lymphoblastoid cell microsomes. Incubations were conducted for 1 h with microsomes containing 50 pmol/ml of CYP, 50 μM rutaecarpine, and an NGS. CYP1A2 (A), CYP2C9 (B), and CYP3A4 (C).

CYP1A2 and 2C9 were approximately 15.2 and 10.7% of that by CYP3A4, respectively (Table 2). Interestingly, the formations of M5 by CYP3A4, 1A2 and 2C9 were comparable (Table 2).

In human liver microsomes, CYP1A2 played minor roles in the metabolism of rutaecarpine except the formation of M5. Furafylline, a selective inhibitor of CYP1A2, slightly inhibited the formation of all metabolites and CYP1A2-expressed microsomes formed M2, M3, M5 and M6 (Table 1, Fig. 3). Meanwhile, the formations of only two metabolites—i.e., M3 and M5, were partially inhibited by anti-CYP1A2 antibody (Fig. 1). In addition, the relative amount of M5 formed by CYP1A2 was similar to the formation in CYP3A4-expressed microsome (Table 2). Therefore, except M5, CYP1A2 might be a minor isozyme in the metabolism of rutaecarpine. Like CYP1A2, CYP2C9 also played minor roles in the metabolism of rutaecarpine. The formations of M3 and M5 were slightly inhibited by anti-CYP2C9 and M3, M5 and M6 were formed in CYP2C9-expressed microsome to a lesser extend than in CYP3A4-expressed microsome, except M5 (Figs. 2 and 3). The rate of formation of M5 by CYP2C9 was slightly higher than that by CYP3A4 (Table 2). Because of some discrepancy in the results, a possible role of other CYPs in the metabolism of rutaecarpine should be studied further in the near future.

The results that CYP3A4 and 1A2 were involved in the metabolism of rutaecarpine by human liver microsomes are similar to previous results in rat liver microsomes [4]. In the study, it was found that CYP1A and CYP2B might be major CYPs involved in the metabolism of rutaecarpine in rat liver microsomes. In addition, it was implicated that CYP3A and 2E1 might be involved in the formation of M1 in rat liver microsomes. Moreover, some studies on the modulation of CYP1A by rutaecarpine were reported elsewhere [6–8]. For example, rutaecarpine potently inhibited CYP1A2 activity in both mouse and human liver microsomes [6]. Rutaecarpine contributed, at least in part, to the increase of hepatic ethoxyresorufin-*O*-deethylase activity by extracts of *E. rutaecarpa* [7]. In addition, rutaecarpine showed potent inductive effects on both CYP1A1 and 1A2 in the mouse liver and kidney [8] and CYP2B in rat liver [4]. Drug–drug interaction studies by modulating CYP1A activities by rutaecarpine were studied in case of theophylline [9] and caffeine [16]. These results suggest a possible interaction of rutaecarpine with selective CYP isozymes. In this regard, rutaecarpine was recently identified as a mechanism-based inhibitor of CYP3A4 from the following observations: (i) the inhibitory effects of rutaecarpine on CYP3A4 activity were dependent on the preincubation time; (ii) the inhibition required NADPH; (iii)

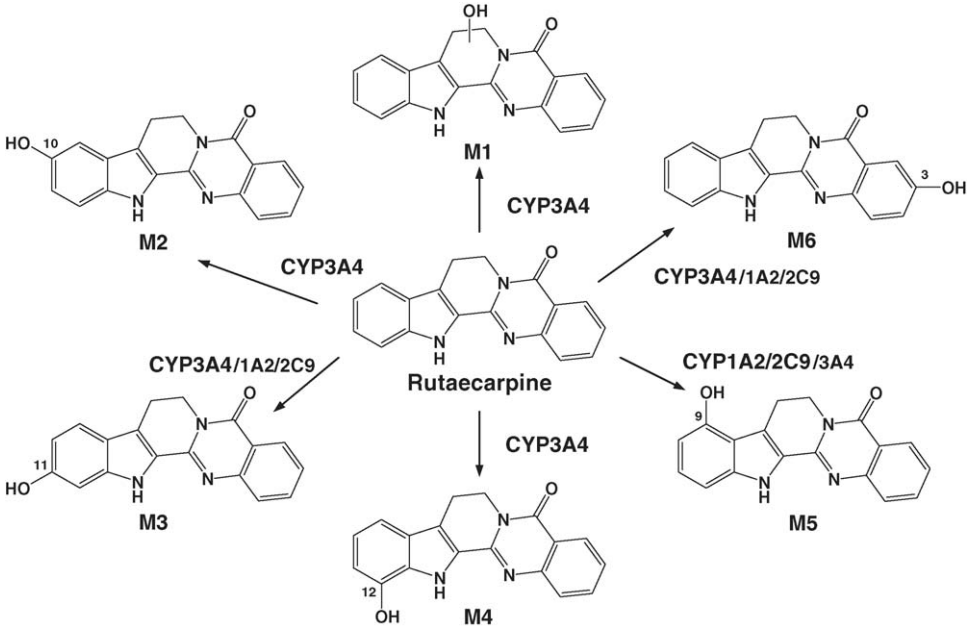

Fig. 4. Proposed metabolic pathway of rutaecarpine in human liver microsomes.

the inhibition was depressed in the presence of a competitive CYP3A4 inhibitor, ketoconazole; and (iv) dialysis resulted in no recovery of CYP3A4 activity [10].

In conclusion, rutaecarpine can be metabolized to six monohydroxylated rutaecarpine by human liver microsomes. The present results demonstrated that CYP3A4 could be the major CYP isozyme in the metabolism of rutaecarpine in human liver microsomes. Taken together, the possible metabolic fate of rutaecarpine in human liver microsomes could be proposed as summarized in Fig. 4.

Acknowledgments

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