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Oxidative metabolism of 5-methoxy-N, N-diisopropyltryptamine (Foxy) by human liver microsomes and recombinant cytochrome P450 enzymes

Shizuo Narimatsu [a,*], *Rei Yonemoto* [a], *Keita Saito* [a], *Kazuo Takaya* [b], *Takuya Kumamoto* [b], *Tsutomu Ishikawa* [b], *Masato Asanuma* [c], *Masahiko Funada* [d], *Kimio Kiryu* [e], *Shinsaku Naito* [e], *Yuzo Yoshida* [f], *Shigeo Yamamoto* [g], *Nobumitsu Hanioka* [a]

[a] *Laboratory of Health Chemistry, Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama University, 1-1-1 Tsushima-naka, Okayama 700-8530, Japan*

[b] *Laboratory of Medicinal Organic Chemistry, Graduate School of Pharmaceutical Sciences, Chiba University, 1-33 Yayoi, Inage, Chiba 263-8522, Japan*

[c] *Department of Brain Science, Okayama University, Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, 2-5-1 Shikata, Okayama 700-8558, Japan*

[d] *Division of Drug Dependence, National Institute of Mental Health, National Center of Neurology and Psychiatry, 4-1-1 Ogawa-Higashi, Kodaira 187-8502, Japan*

[e] *Division of Pharmacology, Drug Safety and Metabolism, Otsuka Pharmaceutical Factory Inc., Naruto, Tokushima 772-8601, Japan*

[f] *School of Pharmaceutical Sciences and Institute for Bioscience, Mukogawa Women's University, Nishinomiya, Hyogo 663-8179, Japan*

[g] *Laboratory of Biomolecular Sciences, Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama University, 1-1-1 Tsushima-naka, Okayama 700-8530, Japan*

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ABSTRACT

In vitro quantitative studies of the oxidative metabolism of (5-methoxy-*N*,*N*-diisopropyltryptamine, 5-MeO-DIPT, Foxy) were performed using human liver microsomal fractions and recombinant CYP enzymes and synthetic 5-MeO-DIPT metabolites. 5-MeO-DIPT was mainly oxidized to *O*-demethylated (5-OH-DIPT) and *N*-deisopropylated (5-MeO-IPT) metabolites in pooled human liver microsomes. In kinetic studies, 5-MeO-DIPT *O*-demethylation showed monophasic kinetics, whereas its *N*-deisopropylation showed triphasic kinetics. Among six recombinant CYP enzymes (CYP1A2, CYP2C8, CYP2C9, CYP2C19, CYP2D6 and CYP3A4) expressed in yeast or insect cells, only CYP2D6 exhibited 5-MeO-DIPT *O*-demethylase activity, while CYP1A2, CYP2C8, CYP2C9, CYP2C19 and CYP3A4 showed 5-MeO-DIPT *N*-deisopropylase activities. The apparent K_m value of CYP2D6 was close to that for 5-MeO-DIPT *O*-demethylation, and the K_m values of other CYP enzymes were similar to those of the low-K_m (CYP2C19), intermediate-K_m (CYP1A2, CYP2C8 and CYP3A4) and high-K_m phases (CYP2C9), respectively, for *N*-deisopropylation in human liver microsomes. In inhibition studies, quinidine (1 μM), an inhibitor of CYP2D6, almost completely inhibited human liver microsomal 5-MeO-DIPT *O*-demethylation at a substrate concentration of 10 μM.

* *Corresponding author*. Tel.: +81 86 251 7942; fax: +81 86 251 7942.
E-mail address: shizuo@pharm.okayama-u.ac.jp (S. Narimatsu).
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Abbreviations:
CYP, cytochrome P450
OR, NADPH-cytochrome P450 reductase
5-MeO-DIPT, 5-methoxy-*N*,*N*-diisopropyltryptamine
5-MeO-IPT, 5-methoxy-*N*-ispropyltryptamine
5-OH-DIPT, 5-hydroxy-*N*,*N*-diisopropyltryptamine
G-6-P, glucose 6-phosphate
HPLC, high-performance liquid chromatography
LC/MS, liquid chromatography–mass spectrometry
PCR, polymerase chain reaction

Furafylline, a CYP1A2 inhibitor, quercetin, a CYP2C8 inhibitor, sulfaphenazole, a CYP2C9 inhibitor and ketoconazole, a CYP3A4 inihibitor (5 μM each) suppressed about 60%, 45%, 15% and 40%, respectively, of 5-MeO-DIPT *N*-deisopropylation at 50 μM substrate. In contrast, omeprazole (10 μM), a CYP2C19 inhibitor, suppressed only 10% of *N*-deisopropylation by human liver microsomes, whereas at the same concentration the inhibitor suppressed the reaction by recombinant CYP2C19 almost completely. These results indicate that CYP2D6 is the major 5-MeO-DIPT *O*-demethylase, and CYP1A2, CYP2C8 and CYP3A4 are the major 5-MeO-DIPT *N*-deisopropylase enzymes in the human liver.

1. Introduction

Various kinds natural and synthetic hallucinogenic indolethylamines have been produced and abused worldwide, including in Japan [1,2]. Among the many hallucinogenic indolethylamines, the following five compounds are legally controlled in Japan: *N*,*N*-dimethyltryptamine, *N*,*N*-diethyltryptamine, α-ethyltryptamine, psilocine and psilocybin [2]. In addition, α-methyltryptamine and 5-methoxy-*N*,*N*-diisopropyltryptamine (5-MeO-DIPT, Foxy) have newly come under legal control as of 2005.

5-MeO-DIPT is one of the designer drugs, and its properties such as pharmacological activities and toxicities have not been fully elucidated [3]. 5-MeO-DIPT is taken orally because it is resistant to the degradation by monoamine oxidase [3]. It is thought that 5-MeO-DIPT undergoes oxidative metabolism in the liver after oral intake. In fact, there are two case reports in which several oxidative metabolites were detected in human serum and urine samples [4,5] using gas chromatography–mass spectrometry (GC–MS) techniques. However, most of the oxidative metabolites of 5-MeO-DIPT were only speculated to be produced on the basis of fragment ions in the GC–MS analysis.

There have thus far been no quantitative reports describing detailed in vitro studies on the oxidative metabolism of 5-MeO-DIPT. In the present study, we therefore investigated the in vitro oxidative metabolism of 5-MeO-DIPT using human liver microsomal fractions and recombinant cytochrome P450 enzymes as enzyme sources, and synthetic metabolites of 5-MeO-DIPT as standards for analysis.

2. Materials and methods

2.1. Materials

5-MeO-DIPT was supplied by Dr. M. Funada, National Institute of Mental Health, National Center of Neurology and Psychiatry (Kodaira, Japan). Because this compound showed the purity of above 99% in ^{1}H NMR and HPLC, it was used without further purification. 5-Hydroxy-*N*,*N*-diisopropyltryptamine (5-OH-DIPT) and 5-methoxy-*N*-isopropyltryptamine (5-MeO-IPT) were synthesized as described below. Furafylline, quercetin, sulfaphenazole, omeprazole and quinidine were purchased from Sigma–Aldrich (St. Louis, MO); glucose 6-phosphate (G-6-P) and NADPH were from Oriental Yeast Co. (Tokyo, Japan). Ketoconazole was supplied by Dr. Y. Yoshida, School of Pharmaceutical Sciences, Mukogawa Women's University. Pooled human liver microsomal fractions were obtained from BD Biosciences Discovery Labware (Bedford, MA). Recombinant CYP2D6 [6] was expressed in yeast cells according to the published methods. Recombinant CYP1A2, CYP2C8, CYP2C9, CYP2C19 and CYP3A4 were expressed in yeast cells as described below. Insect cell microsomal fractions (Supersomes) expressing CYP3A4, cytochrome b_5 and NADPH-cytochrome P450 reductase (OR), and expressing CYP3A4 and OR (without cytochrome b_5) were purchased from Gentest (Woburn, MA).

2.2. Chemical synthesis of 5-OH-DIPT and 5-MeO-IPT

To a solution of 5-MeO-DIPT (50 mg, 0.18 mmol) in CH_2Cl_2 (2.0 ml), 1.0 M BBr_3 in CH_2Cl_2 (0.92 ml, 0.92 mmol) was added at −12 °C and the whole was stirred at −12 °C for 3 h. After cooling the reaction mixture with an ice bath, 30% aq. NaOH was added to pH 9–10 and the whole was extracted with a mixture of $CHCl_3$ and MeOH (9: 1, 1 × 10 ml, 3 × 5 ml). The combined organic layer was washed with brine (10 ml) and was dried over Na_2SO_4. The solvent was evaporated in vacuo and the residue was purified by column chromatography (NH silica, $CHCl_3$:MeOH:Et_3N = 20:1:0.1) to give pale yellow crystals (21 mg, 44%); m.p. 81–83 °C; IR (ATR, cm^{-1}) 3330; ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 1.12 (total 12H, d, *J* = 6.3 Hz, 4 × CH_3), 2.76, 2.84 (each 2H, m, 2 × CH_2), 3.17 (total 2H, m, 2 × CH), 6.77 (1H, dd, *J* = 8.8, 2.4 Hz, H-6), 7.01 (1H, d, *J* = 2.2 Hz, H-4), 7.03 (1H, d, *J* = 2.2 Hz, H-2), 7.21 (1H, d, *J* = 8.8 Hz, H-7), 7.83 (1H, s, NH, exchangeable with D_2O); ^{13}C NMR (125 MHz, $CDCl_3$) δ (ppm): 20.3, 27.5, 47.0, 49.7, 50.6, 103.2, 111.8, 112.0, 122.3, 128.2, 131.3, 150.1; HRFABMS found: 261.1982 (calculated for $C_{16}H_{25}N_2O$:261.1967). These analytical data supported the conclusion that the synthesized compound was 3-(2-diisopropylaminoethyl)-1*H*-indol-5-ol (5-hydroxy-*N*,*N*-diisopropyltryptamine, 5-OH-DIPT) (Fig. 1).

To a solution of 5-methoxytryptamine (93 mg, 0.49 mmol) in MeOH (1.0 ml), 36% HCl (0.13 ml, 1.31 mmol), acetone

(1.08 ml, 14.7 mmol), and $NaBH_3CN$ (90%, 117 mg, 1.68 mmol) were added at 0 °C and the mixture was stirred at room temperature for 73 h. Ten percent aqueous KOH (2.0 ml) was added to pH 8–9 and the whole was extracted with CH_2Cl_2 (1×5 ml, 1×3 ml, 2×2 ml). The combined organic layer was washed with brine and was dried over K_2CO_3. The solvent was evaporated in vacuo and the residue was purified by column chromatography (NH silica, benzene:AcOEt:MeOH = 20:2:0.75) to give a reddish brown oil (86 mg, 76%); IR (ATR, cm^{-1}) 3200; ^1H NMR (400 MHz, $CDCl_3$) δ (ppm): 1.05 (6H, d, J = 6.2 Hz, $2 \times CH_3$), 2.82 (1H, quint. J = 6.2 Hz, CH), 2.94 (4H, s, $2 \times CH_2$), 3.85 (3H, s, OCH_3), 6.85 (1H, dd, J = 8.8, 2.4 Hz, H-6), 6.98 (1H, d, J = 2.4 Hz, H-4), 7.06 (1H, d, J = 2.4 Hz, H-2), 7.13 (1H, d, J = 8.8 Hz, H-7), 8.51 (1H, s, NH, exchangeable with D_2O); ^{13}C NMR (125 MHz, $CDCl_3$) δ (ppm): 22.8, 25.9, 47.4, 48.5, 55.9, 100.7, 111.8, 112.0, 113.4, 122.8, 129.8, 131.6, 153.7; HREIMS found: 232.1555 (calculated for $C_{13}H_{18}N_2O$:232.1575). These analytical data supported the conclusion that the synthesized compound was isopropyl-[2-(5-methoxy-1*H*-indol-3-yl)ethyl]amine (5-methoy-*N*-isopropyltryptamine, 5-MeO-IPT) (Fig. 1).

2.3. Construction of CYP expression plasmids

CYP1A2 cDNA for subcloning in expression vector pGYR1 was prepared by polymerase chain reaction (PCR) from pcDNA3.1/CYP1A2 plasmid [7] as a template using the forward primer, 5′-AAGCTTAAAAAAAT*GG*CATTGTCCCAGTCT-3′, and the reverse primer, 5′-AAGCTTTCAGTTGATGGAGAAGCGCA-3′. The *Hin*dIII sites (marked with the solid lines) were introduced to the 5′-end of the start codon and the 3′-end of the stop codon to facilitate subcloning into pGYR1. A Kozak sequence (marked in italics) was also introduced upstream of the start codon to achieve high expression of the protein in yeast cells. CYP2C8 and CYP2C9 cDNAs were amplified from human adult normal liver QuickClone cDNA (BD Biosciences Clontech, Mountain View, CA). The nucleotide sequences used for the forward and reverse primers were 5′-CCCAAGCTTAAAAAAAT*GG*AACCTTTTGTGGTCCTGG-3′ and 5′-TTCAAGCTTCTCGAGTTCAGACAGGGATGAAGCA-GAT-3′ for CYP2C8, and 5′-AAGCTTAAAAAAAT*GG*ATTCTC-TTGTGGTC-3′ and 5′-AAGCTTTCAGACAGGAATGAAGCACA-3′ for CYP2C9. The PCR products were directly introduced into pGEM-T vector (Promega, Madison, WI) using the TA cloning system, resulting in pGEM-T/CYP1A2, pGEM-T/CYP2C8 and pGEM-T/CYP2C9. CYP2C19 cDNA cloned into pBluescript SK (±) (pBluescript/CYP2C19) was supplied by Dr. J. Goldstein (NIEHS, Research Triangle, NC). The cDNA containing the *Hin*dIII sites and Kozak sequence was amplified by PCR from pcDNA3.1/CYP2C19 as a template using the forward primer 5′-CCCAAGCT-TAAAAAAAT*GG*ATCCTTTTGTGGTCC-3′ and the reverse primer 5′-GGAAAGCTTAGGAGCAGCCAGACCATCTGT-3′. The PCR product was digested with *Hin*dIII and ligated into the same restriction enzyme site of pcDNA3.1 (+), resulting in pcDNA3.1/CYP2C19. pGEM-T/CYP1A2, pGEM-T/CYP2C8, pGEM-T/CYP2C9 and pcDNA3.1/CYP2C19 plasmids were sequenced in both the forward and reverse directions using ABI BigDye terminator cycle sequencing reaction kit v3.1 (Applied Biosystems, Piscaway, NJ) to confirm that there were no PCR errors. The DNA fragments corresponding to CYP1A2, CYP2C8, CYP2C9 and CYP2C19 were cut out with *Hin*dIII from the pGEM-T or pcDNA3.1 (+) plasmid and were subsequently subcloned into the pGYR1 yeast expression vector digested with *Hin*dIII. The expression plasmids were sequenced to verify the correct orientation with respect to the promoter for pGYR1. The construction of CYP2D6 expression plasmid (pGYR1/CYP2D6) was described previously [8]. CYP3A4 expression plasmid (pGYR1/CYP3A4) was supplied by Dr. Y. Saito (National Institute of Health Sciences, Tokyo, Japan).

2.4. Expression of CYP enzymes

The pGYR1 vectors containing CYP cDNAs were used to transform *Saccharomyces cerevisiae* AH22 by the lithium acetate

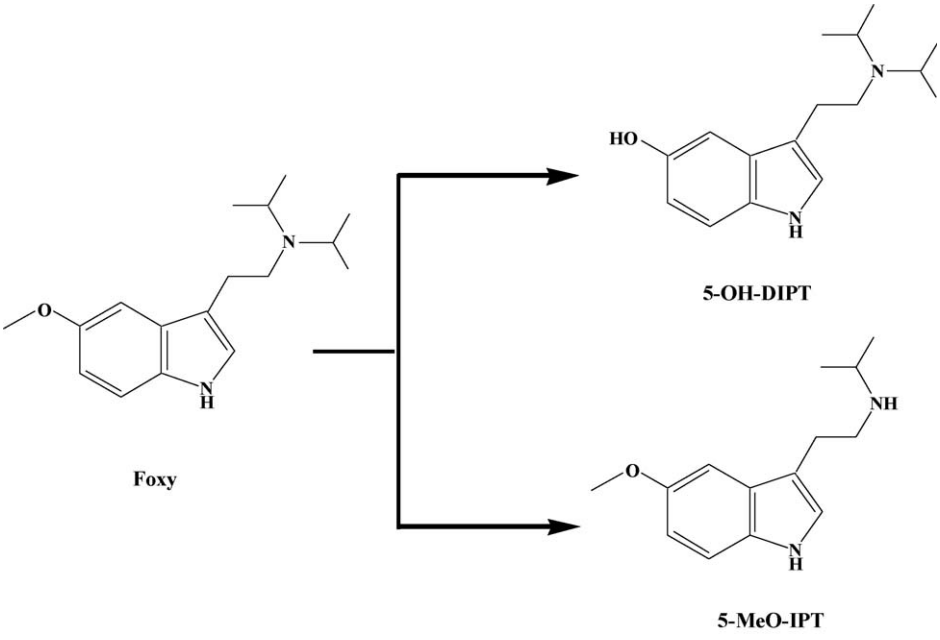

Fig. 1 – Major metabolic pathways of 5-MeO-DIPT.

method, and the cultivation of yeast transformants was performed as described [9]. Microsomes from yeast were prepared as described previously [10]. The yeast cell microsomal content of each recombinant CYP enzyme was as follows: CYP1A2 (13.5 pmol/mg protein), CYP2C8 (95.3 pmol/mg protein), CYP2C9 (76.0 pmol/mg protein), CYP2C19 (41.8 pmol/mg protein), CYP2D6 (65.0 pmol/mg protein) and CYP3A4 (49.1 pmol/mg protein).

2.5. Measurement of oxidation activities of 5-MeO-DIPT

A typical reaction mixture consisted of G-6-P (10 mM, final concentration), NADPH (1 mM), $MgCl_2$ (10 mM), EDTA (0.2 mM), microsomal fraction from human liver or yeast cells expressing CYP enzyme (0.08–0.12 mg protein) and the substrate (0.1–1000 μM) in 50 mM potassium phosphate buffer (pH 7.4) in a 1.5-ml Eppendorf-type tube (a final volume of 200 μl). Following preincubation at 37 °C for 5 min, the reaction was started by adding microsomal fractions from human livers or yeast cells expressing CYP enzymes, continued for 5–10 min, and stopped by adding aqueous 2 M phosphoric acid (10 μl) and 20 mM ascorbic acid (20 μl), vigorously mixing with a Vortex mixer, and chilling in an ice bath for 10 min. The tube was then centrifuged at 14,000 × *g* at 4 °C for 10 min, and the supernatant was passed through a 0.45-μm membrane filter (Millipore, Billerica, MA). An aliquot (20 μl) was subjected to high-performance liquid chromatography (HPLC) under the conditions described below. Calibration curves of 5-OH-DIPT and 5-MeO-IPT were made by spiking ice-cold reaction medium with known amounts of the synthetic compounds, followed by the addition of aqueous 2 M phosphoric acid and 20 mM ascorbic acid and treatment as described above. The detection limits for 5-OH-DIPT and 5-MeO-IPT were 0.5 and 1.0 pmol/ml, with a signal-to-noise ratio of 3 in both cases. The intra- and inter-day coefficients of variation did not exceed 10% for any assay.

2.6. HPLC conditions

The HPLC apparatus consisted of a Hitachi L-2130 pump, an L-2480 fluorescence detector, an L-2300 column oven, a D-2000 system manager (version 1.1) and a Rheodyne type 7725i injector. Other conditions were as follows: column, Inertsil C8 (150 mm × 4.6 mm i.d., GL Sciences, Co. Ltd., Tokyo, Japan); column temperature, 40 °C; detection, fluorescence excitation/emission wavelength, 280/340 nm. The mobile phase used was a linear gradient system consisting of (A) 20 mM ammonium acetate (pH 4.0)/acetonitrile (92:8 v/v) and (B) 20 mM ammonium acetate (pH 4.0)/acetonitrile (80:20) as follows: 0–3 min, (A) 100%; 3–15 min, from (A) 100% to (B) 100%; 15–25 min, (B) 100%; 25–30 min, from (B) 100% to (A) 100%; 30–40 min, (A) 100% at a flow rate of 0.9 ml/min.

2.7. LC/MS conditions

LC/MS analysis was performed using a JMS-700 MStation (JEOL, Tokyo, Japan). HPLC conditions were: column, Inertsil ODS-3 (150 mm × 2.1 mm i.d., GL Science); mobile phase, aqueous 0.1% TFA/acetonitrile (84:16 v/v); column temperature, 40°; flow rate, 0.2 ml/min; injection volume, 20 μl; detection, fluorescence excitation/emission wavelength, 280/340 nm. MS conditions were: ionization mode, ESI(+); needle voltage, 2.0 kV; ring voltage, 45 V; orifice voltage, 0 V; the temperatures of the orifice and desolvating plate were 80 and 220 °C; the resolution of the mass spectrometer was set at 1000 or 3000; collision gas, He.

2.8. Others

Total holo-CYP contents in yeast cell microsomal fractions were spectrophotometrically measured by assessing the reduced carbon monoxide spectra according to the method of Omura and Sato [11] using 91 mM^{-1} cm^{-1} as the absorption coefficient. Protein concentration was determined by the method of Lowry et al. [12]. Kinetic parameters (apparent K_m and V_{max} values) were estimated by analyzing Michaelis–Menten plots or Eadie–Hofstee plots using the computer program Prism ver. 4.0 software (GraphPad Software, San Diego, CA).

3. Results

First, we examined the in vitro oxidative metabolism of 5-MeO-DIPT using pooled human liver microsomes from Caucasians. When 50 μM 5-MeO-DIPT was used as the substrate, two major metabolite peaks [M − 1 (retention time of 10.3 min) and M − 3 (15.1 min)] were observed on the HPLC chromatogram (Fig. 2). Because the retention times and fragmentation profiles of M − 1 and M − 3 coincided with those of 5-OH-DIPT and 5-MeOH-IPT synthetic standards in LC/MS analysis, M − 1 and M − 3 were identified as 5-OH-DIPT and 5-MeOH-IPT, respectively (Fig. 3).

Furthermore, two metabolite peaks [M − 2 (retention time 12.0 min) and M − 4 (17.7 min) in HPLC] were analyzed by LC/MS. The fragment ions were as follows: M − 2; *m*/z 291 (M + 1, 100%), 181 (42%), 140 (85%), 124 (18%): M − 4; *m*/z 305 (M + 1, 64%), 278 (18%), 204 (55%), 144 (100%). From the molecular ion (*m*/z 291), M − 2 is thought to be a monohy-

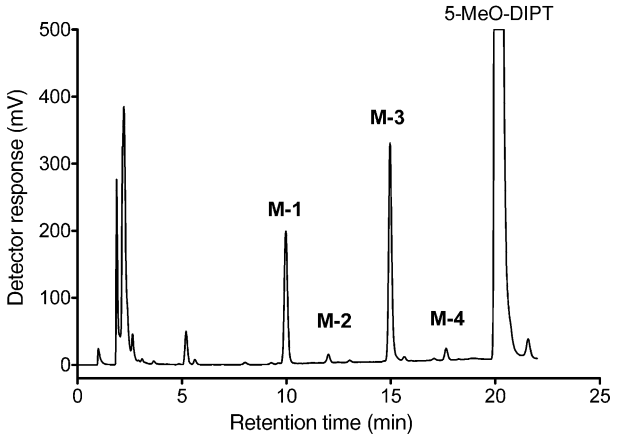

Fig. 2 – A typical HPLC chromatogram of 5-MeO-DIPT and its metabolites. The reaction mixture containing human liver microsomes and 5-MeO-DIPT (50 μM) was incubated in the presence of an NADPH-generating system and the metabolites formed were examined by HPLC under the conditions described in Section 2.

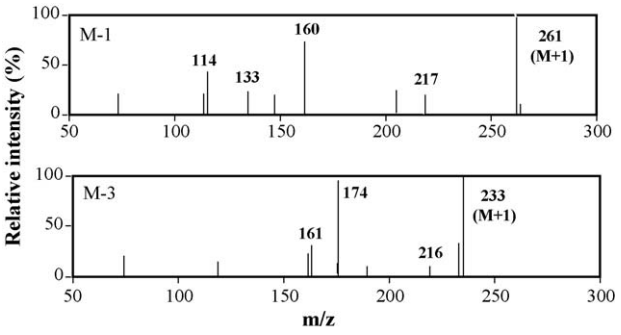

Fig. 3 – Mass fragment ions of $M-1$ and $M-3$. LC/MS conditions are given in Section 2.

droxylated 5-MeO-DIPT. It is feasible that $M-4$ is a dehydrogenated product of dihydroxylated 5-MeO-DIPT.

It is reasonable to think that the formation of these metabolites was catalyzed by CYP enzymes in the liver microsomal fractions. We therefore examined what kinds of CYP enzymes were involved in the formation of $M-1$ and $M-3$ from 5-MeO-DIPT in human liver microsomes in the second step of this study. For this, we used six human recombinant CYP enzymes expressed in yeast cells: CYP1A2, CYP2C8, CYP2C9, CYP2C19, CYP2D6 and CYP3A4.

In this experiment, we employed two substrate concentrations (1 and 50 μM). All of the CYP enzymes except for CYP3A4 exhibited the capacity to oxidize 5-MeO-DIPT (Fig. 4). CYP3A4 expressed in yeast cells did not produce any metabolites in detectable amounts even at 50 μM substrate under the conditions used. We further examined the metabolic capacity of commercially available insect cell microsomal fractions (Supersomes) expressing CYP3A4, OR and cytochrome b_5. Interestingly, Supersomes co-expressing CYP3A4 with cytochrome b_5 exhibited considerable 5-MeO-DIPT *N*-deisopropylase but not *O*-demethylase activity, whereas Supersomes without cytochrome b_5 did not show any detectable activity (Fig. 4). As a result, among the six CYP enzymes tested, only CYP2D6 exhibited 5-MeO-DIPT *O*-demethylase activity, whereas all of the six recombinant enzymes showed 5-MeO-DIPT *N*-deisopropylase activitiy. The activities were ranked as CYP2C19 > CYP1A2 > CYP3A4 > CYP2C8 > CYP2C9 = CYP2D6.

Table 1 – Kinetic parameters for 5-MeO-DIPT oxidation by human liver microsomes

	K_m (μM)	V_{max} (pmol/min/mg protein)	V_{max}/K_m (μl/min/mg protein)
O-demethylation	5.0	140	27.9
N-deisopropylation			
Low-K_m phase	24	25	1.03
Intermediate-K_m phase	257	178	0.69
High-K_m phase	1201	409	0.34

Each value represents the mean of two determinations.

We then performed kinetic analysis using substrate concentrations ranging from 0.1 to 1000 μM and the pooled human microsomal fraction and the yeast cell microsomal fractions expressing recombinant CYP enzymes as enzyme sources. Only for CYP3A4, the Supersomes expressing CYP3A4, OR and cytochrome b_5 was employed. 5-MeO-DIPT *O*-demethylation by the pooled human liver microsomal fraction exhibited monophasic kinetics (Fig. 5A), whereas human liver microsomal *N*-deisopropylation showed triphasic kinetics (Fig. 5B). Table 1 summarizes the kinetic parameters. The apparent K_m value for monophasic 5-MeO-DIPT *O*-demethylation was calculated to be 5 μM, while the low-, intermediate- and high-K_m values for triphasic *N*-deisopropylation were calculated to be 24, 260 and 1200 μM, respectively.

For 5-MeO-DIPT *O*-demethylation, recombinant CYP2D6 yielded monophasic kinetics and an apparent K_m value of 2 μM. For 5-MeO-DIPT *N*-deisopropylation, CYP2C19, CYP3A4, CYP1A2, CYP2C8 and CYP2C9 showed monophasic kinetics, and gave K_m values of 35, 180, 260, 290 and 1660 μM, respectively (Table 2). These K_m values are similar to the values of the low-, intermediate- and high-K_m phases, respectively, of the human liver microsomal fraction (Table 1). In the case of *N*-

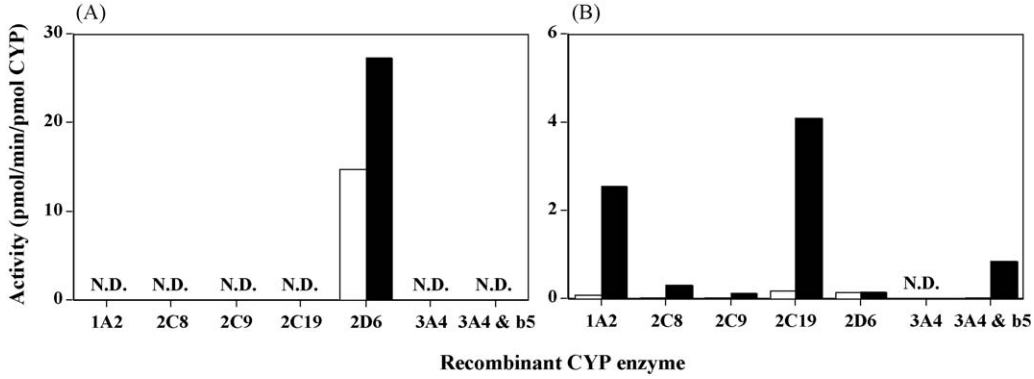

Fig. 4 – Comparison of 5-MeO-DIPT oxidation activities of recombinant CYP enzymes expressed in yeast or insect cells. For incubation, 5 pmol of each CYP enzyme was employed: (A) 5-MeO-DIPT *O*-demethylation; (B) 5-MeO-DIPT *N*-deisopropylation. Open columns, 5-MeO-DIPT 1 μM; closed columns, 5-MeO-DIPT 50 μM. Each value represents the mean of two determinations. 3A4 & b_5, Supersomes; N.D., not detectable.

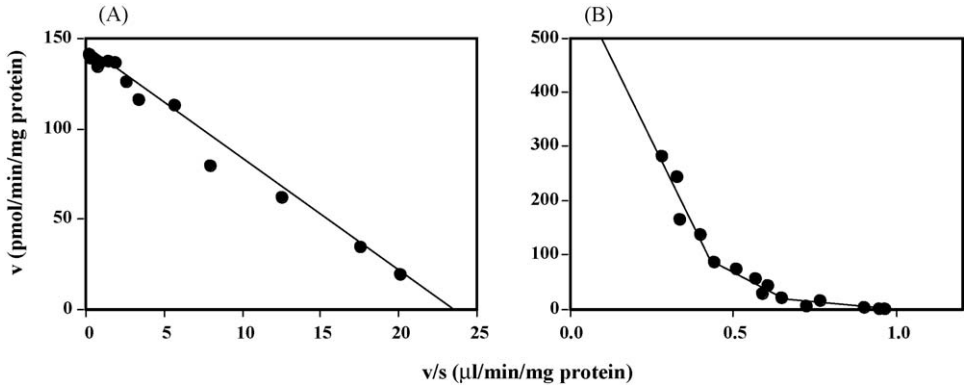

Fig. 5 – Kinetic analysis of 5-MeO-DIPT oxidation by human liver microsomes. (A) and (B) Eadie–Hofstee plots for 5-MeO-DIPT *O*-demethylation and *N*-deisopropylation, respectively.

deisopropylation by CYP2D6, precise kinetic parameters were not calculated because of the low activities.

In the third step of the present study, we examined the effects of inhibitors of the CYP enzymes on the oxidative metabolism of 5-MeO-DIPT in human liver microsomes to estimate the contribution of the CYP enzymes. For this, we employed furafylline [13], quercetin [14], sulfaphenazole [15], omeprazole [16], quinidine [17] and ketoconazole [18] as specific inhibitors of CYP1A2, CYP2C8, CYP2C9, CYP2C19, CYP2D6 and CYP3A4, respectively. For 5-MeO-DIPT *O*-demethylation by human liver microsomes, quinidine showed a concentration-dependent inhibition. Over 95% of the activity was suppressed by the inhibitor even at 5 μM (Fig. 6A).

Furafylline and quercetin inhibited human liver microsomal 5-MeO-DIPT *N*-deisopropylation in a concentration-dependent manner, but about 30–40% of the activity remained even at the highest concentration of the inhibitors (Fig. 6B and C). Sulfaphenazole also caused a concentration-dependent

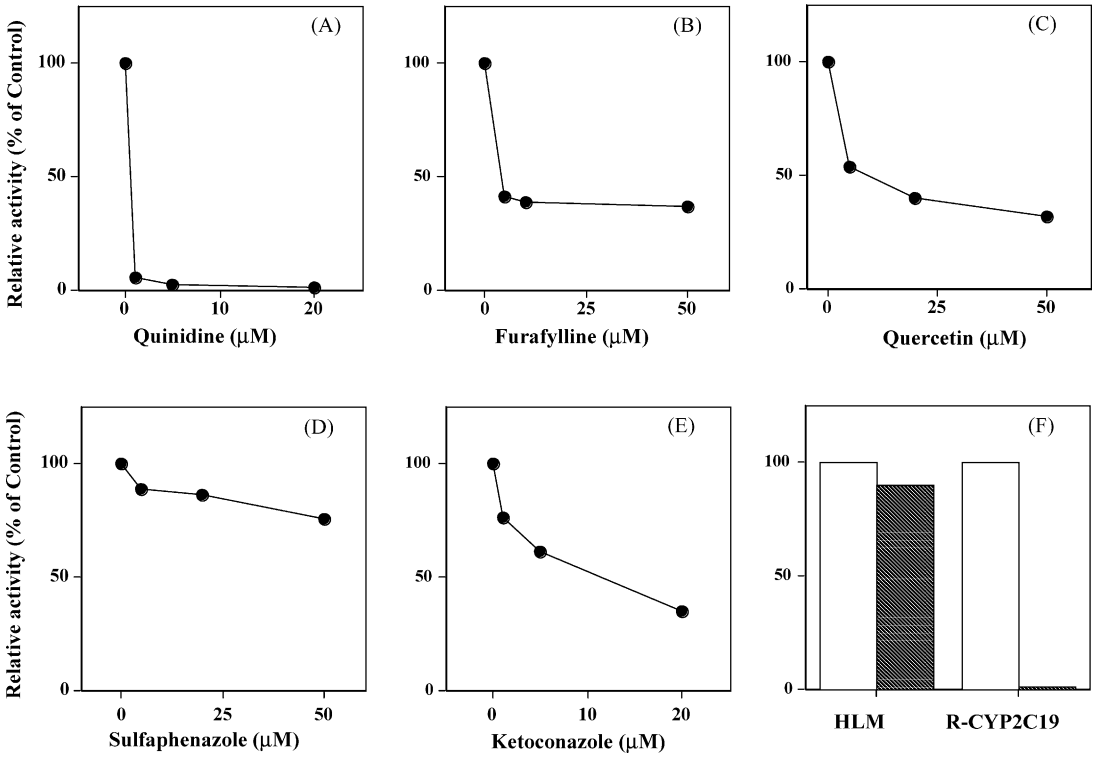

Fig. 6 – Effects of various CYP inhibitors on 5-MeO-DIPT oxidation by human liver microsomes: (A) quinidine (0, 0.5, 5 and 20 μM) as CYP2D6 inhibitor; (B) furafylline (0, 5, 10 and 50 μM) as CYP1A2 inhibitor; (C) quercetin (0, 5, 20 and 50 μM); (D) sulfaphenazole (0, 5, 20 and 50 μM) as CYP2C9 inhibitor; (E) ketoconazole (0, 1, 5 and 20 μM) as CYP3A4 inhibitor; (F) omeprazole (10 μM) as CYP2C19 inhibitor. (A) 5-MeO-DIPT *O*-demethylation; (B), (C), (D), (E) and (F) 5-MeO-DIPT *N*-deisopropylation. The substrate concentrations used were 10 μM for (A) and 50 μM for (B), (C), (D), (E) and (F). Each value represents the mean of two determinations. HLM, human liver microsomes.

Table 2 – Kinetic parameters for 5-MeO-DIPT oxidation by recombinant CYP enzymes expressed in yeast cells

	K_m (μM)	V_{max} (pmol/min/pmol CYP)	V_{max}/K_m (μl/min/pmol CYP)
O-demethylation			
CYP2D6	2.0	29.8	14.8
N-deisopropylation			
CYP1A2	263	9.4	0.04
CYP2C8	291	1.7	0.006
CYP2C9	1663	4.2	0.003
CYP2C19	35	6.9	0.19
CYP3A4[a]	184	4.4	0.02

Each value represents the mean of two determinations.
[a] Data from Supersomes in which CYP3A4 and cytochrome b_5 were co-expressed.

inhibition, but suppressed only 25% of human liver microsomal 5-MeO-DIPT *N*-deisopropylation at the highest concentration of 50 μM (Fig. 6D). Ketoconazole suppressed 40% and 65% of the *N*-deisopropylase activity at final concentrations of 5 and 20 μM, respectively (Fig. 6E). Omeprazole (10 μM) suppressed only 10% of 5-MeO-DIPT *N*-deisopropylation by pooled human liver microsomes, though this inhibitor at the same concentration suppressed the 5-MeO-DIPT *N*-deisopropylation by recombinant CYP2C19 almost completely (Fig. 6F).

4. Discussion

There have hitherto been no reports of the quantitative assays using authentic samples of 5-MeO-DIPT metabolites to study the formation of 5-MeO-DIPT metabolites. We therefore chemically synthesized the two compounds, 5-OH-DIPT and 5-MeO-IPT, and conducted a quantitative analysis of the formation of these metabolites. Using these synthetic samples, we examined the in vitro oxidative metabolism of 5-MeO-DIPT by human liver microsomes and recombinant CYP enzymes. As expected, 5-MeO-DIPT was biotransformed into 5-OH-DIPT and 5-MeO-IPT as major metabolites by human liver microsomes under the conditions used. Two other metabolites were also tentatively identified as monohydroxylated 5-MeO-DIPT and a dehydrogenated product of dihydroxylated 5-MeO-DIPT on the basis of the data from LC/MS analysis.

In a preliminary HPLC experiment, we compared the amounts of 5-OH-DIPT and 5-MeO-IPT formed with the amount of 5-MeO-DIPT consumed during the incubation of the substrate (10 μM) under similar conditions to those employed here. The results indicated that at least 95% of substrate consumption was explained by the formation of the two major metabolites (data not shown). Therefore, it is reasonable to think that 5-OH-DIPT and 5-MeO-IPT are the major metabolites in human liver microsomes at around 10 μM substrate concentrations (Fig. 1).

Human liver microsomal 5-MeO-DIPT *O*-demethylation and *N*-deisopropylation exhibited monophasic and triphasic kinetics, respectively. We have been studying the relationships between protein structures and enzymatic functions of major drug-metabolizing CYP enzymes such as CYP1A2 [19] and CYP2D6 [20,21]. Using the yeast cell expression systems of CYP1A2, CYP2C8, CYP2C9, CYP2C19, CYP2D6 and CYP3A4 constructed so far in this laboratory, we examined the metabolic capacities of these recombinant enzymes for the oxidation of 5-MeO-DIPT.

Among the recombinant enzymes, only CYP2D6 showed considerable 5-MeO-DIPT *O*-demethylase activity. This reaction in human liver microsomes yielded monophasic kinetics, and was almost completely suppressed by quinidine as a specific inhibitor of CYP2D6. The apparent K_m value (2 μM) for the recombinant CYP2D6 was similar to that (5 μM) for the human liver microsomes. These results indicate that 5-MeO-DIPT *O*-demethylation was mainly mediated by CYP2D6 in human livers.

In contrast, the two CYP enzymes (CYP1A2 and CYP2C19) in the yeast cell expression system exhibited considerable 5-MeO-DIPT *N*-deisopropylase activities. The activities of CYP2C8, CYP2C9 and CYP2D6 were much lower than those of CYP1A2 and CYP2C19. Interestingly, yeast cell microsomal CYP3A4 did not show any detectable activity for either *O*-demethylation or *N*-deispropylation. Before coming to a conclusion, we further examined the metabolic capacity of Supersomes expressing CYP3A4, OR and cytochrome b_5, because it is well known that co-existence of cytochrome b_5 increases the oxidation capacity of CYP3A4 for its substrates [22,23]. As expected, Supersomes co-expressing CYP3A4 with cytochrome b_5 exhibited considerable 5-MeO-DIPT *N*-demethylase activity, whereas Supersomes without cytochrome b_5 did not.

The apparent K_m values for CYP2C19 (35 μM), CYP3A4 (180 μM), CYP1A2 (260 μM), CYP2C8 (290 μM) and CYP2C9 (1700 μM) seem to correspond to those for human liver microsomal low- (24 μM), intermediate- (260 μM) and high-K_m (1200 μM) phases, respectively. To confirm the involvement of these CYP enzymes in human liver microsomal 5-MeO-DIPT *N*-deisopropylation, the effects of furafylline, quercetin, sulfaphenazole, omeprazole and ketoconazole were examined as specific inhibitors for CYP1A2, CYP2C8, CYP2C9, CYP2C19 and CYP3A4, respectively. Among them, furafylline, quercetin and ketoconazole exerted considerable inhibitory effects at relatively low concentrations (several μM).

We employed quercetin as the inhibitor of CYP2C8 in the present study, however, this compound was reported to inhibit the metabolic activities of CYP1A2, CYP2C19 and CYP3A4 as well [24]. Therefore, quercetin could suppress the activities not only of CYP2C8 but also of CYP1A2 and CYP3A4 in 5-MeO-DIPT *N*-deisopropylation by human liver microsomal fraction in this study. Inhibitory effect of sulfaphenazole was found to be weak as compared to those of furafylline and ketoconazole. Interestingly, the inhibitory effect of omeprazole was very weak under the conditions used. In this case, we employed an inhibitor concentration of 10 μM, which was sufficient to suppress the bufuralol 1″-hydroxylase activities of CYP2C19 in our previous studies [25]. In fact, 10 μM omeprazole completely suppressed 5-MeO-DIPT *N*-deisopropylation by recombinant CYP2C19 in the present study.

Other drug-metabolizing-type CYP enzymes such as CYP2E1, CYP2A6 and CYP2B6 could also be involved in the oxidation of 5-MeO-DIPT in the human liver. In another

preliminary experiment, we found that diethyldithiocarbamate (5, 20 and 100 μM final concentrations) as CYP2E1 inhibitor [26] did not affect 5-MeO-DIPT oxidation by the pooled human liver microsomal fraction (data not shown). Ono et al. [27] reported that diethyldithiocarbamate inhibits the metabolic activities of CYP2A6 and CYP2C19 in addition to that of CYP2E1. Furthermore, furafylline and ketoconazole (5 μM each) suppressed 60% and 40%, respectively, of human liver microsomal 5-MeO-DIPT *N*-deisopropylation under the conditions employed. CYP3A4 is the most abundant CYP enzyme followed by CYP2C and CYP1A2 in the human liver [28]. These results indicate that CYP1A2, CYP2C8 and CYP3A4 are the major 5-MeO-DIPT *N*-deisopropylases in the human liver at substrate concentrations around 50 μM or less.

The present results of the measurement of enzyme activities and the effects of inhibitors indicated that human liver microsomal 5-MeO-DIPT *O*-demethylation is mainly mediated by CYP2D6, whereas *N*-deisopropylation is mediated mainly by CYP1A2 and CYP3A4, and also by CYP2C8, CYP2C9 and CYP2C19, at least in part. The major contribution of CYP2D6 to 5-MeO-DIPT *O*-demethylation is predictable from the previous findings of Yu et al. [29,30] on the role of CYP2D6 in 5-MeO-tryptamine metabolism. It should be noted that though CYP2C19 exhibited the highest activity as 5-MeO-DIPT *N*-deisopropylase among the six recombinant CYP enzymes examined, its contribution was thought to be relatively low in the reaction by the pooled human liver microsomal fractions used in the present study.

The kinetic parameters, particularly the clearance (V_{max}/K_m) values, indicate that as compared to *N*-deisopropylation, *O*-demethylation might contribute to a much greater extent to the oxidative metabolism of 5-MeO-DIPT. The present results together with previous in vivo and in vitro findings cast considerable light on the metabolic fate of 5-MeO-DIPT in the human body. However, the toxicity of 5-MeO-DIPT and related compounds, including the metabolites of 5-MeO-DIPT, remain to be elucidated. If only the parent compound, 5-MeO-DIPT, has psychotomimetic activity and 5-MeO-DIPT *O*-demethylation is mediated mainly by CYP2D6, poor metabolizers who are deficient for functional CYP2D6 may show higher sensitivity to, or toxicity of, 5-MeO-DIPT than extensive metabolizers having normal CYP2D6 functions. Further systematic studies will be needed to understand the relationship between the metabolism and toxicity of 5-MeO-DIPT.

In summary, in vitro quantitative studies on the oxidative metabolism of 5-MeO-DIPT were performed using human liver microsomal fractions, recombinant CYP enzymes and synthetic 5-MeO-DIPT metabolites. 5-MeO-DIPT was mainly oxidized to *O*-demethylated (5-OH-DIPT) and *N*-deisopropylated (5-MeO-IPT) metabolites in pooled human liver microsomes. Kinetic analysis revealed that 5-MeO-DIPT *O*-demethylation showed monophasic kinetics, whereas *N*-deisopropylation gave triphasic kinetics. Among the six recombinant CYP enzymes examined, only CYP2D6 exhibited 5-MeO-DIPT *O*-demethylase activity, and CYP1A2, CYP2C8, CYP2C9, CYP2C19 and CYP3A4 showed 5-MeO-DIPT *N*-deisopropylase activities. The apparent K_m value of CYP2D6 was close to that for 5-MeO-DIPT *O*-demethylation, and the K_m value of other CYP enzymes were similar to those of the low-K_m (CYP2C19), intermediate-K_m (CYP1A2, CYP2C8 and CYP3A4) and high-K_m (CYP2C9) phases, respectively, for *N*-deisopropylation in human liver microsomes. These results together with the results of the inhibitory studies indicate that CYP2D6 is the major 5-MeO-DIPT *O*-demethylase and CYP1A2, CYP2C8 and CYP3A4 are the major *N*-deisopropylase enzymes in the human liver.

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REFERENCES

[1] Ishida T, Kudo K, Kiyosghima A, Inoue H, Tsuji A, Ikeda N. Sensitive determination of alfa-methyltryptamine (AMT) and 5-methoxy-*N*,*N*-diisopropyltryptamine (5MeO-DIPT) in whole blood and urine using gas chromatography–mass spectrometry. J Chromatogr B 2005;823:47–52.
[2] Kikura-hanajiri R, Hayashi M, Saisho K, Goda Y. Simultaneous determination of nineteen hallucinogenic tryptamines/β-calbolines and phenethylamines usig gas chromatography–mass spectrometry and liquid chromatography–electrospray ionization-mass spectrometry. J Chromatogr B 2005;825:29–37.
[3] Shulgin AT, Carter MF. *N*,*N*-diisopropyltryptamine (DIPT) and 5-methoxy-*N*,*N*-diisopropyltryptamine (5-MeO-DIPT), two orally active tryptamine analogs with CNS activity. Commun Psychopharmacol 1980;4:363–9.
[4] Meatherall R, Sharma P. Foxy, a designer tryptamine hallucinogen. J Anal Toxicol 2003;27:313–7.
[5] Wilson JM, McGeorge F, Smalinske S, Meatherall R. A foxy intoxication. Forensic Sci Int 2005;148:31–6.
[6] Tsuzuki D, Takemi C, Yamamoto S, Tamagake K, Imaoka S, Funae Y, et al. Functional evaluation of cytochrome P450 2D6 with Gly42Arg substitution expressed in *Saccharomyces cerevisiae*. Pharmacogenetics 2001;11: 709–18.
[7] Saito Y, Hanioka N, Maekawa K, Isobe T, Tsuneto Y, Nakamura R, et al. Functional analysis of three CYP1A2 variants found in a Japanese population. Drug Metab Dispos 2006;33:1905–10.
[8] Sakaki T, Akiyoshi-Shibata M, Yabusaki Y, Ohkawa H. Organella-targeted expression of rat liver cytochrome P450c27 in yeast: genetically engineered alteration of mitochondrial P450 into a microsomal form creates a novel functional electron transport chain. J Biol Chem 1992;267:16497–502.
[9] Wan J, Imaoka S, Chow T, Hiroi T, Yabusaki Y, Funae Y. Expression of four rat CYP2D isoforms in *Saccharomyces cerevisiae* and their catalytic specificity. Arch Biochem Biophys 1997;348:383–90.
[10] Hichiya H, Takemi C, Tsuzuki D, Yamamoto S, Asaoka A, Suzuki S, et al. Complementary DNA cloning and characterization of cytochrome P450 2D29 from Japanese monkey liver. Biochem Pharmacol 2002;64:1101–10.
[11] 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.

[12] Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951;193:265–75.
[13] Sesardic D, Boobis AR, Murray BP, Murray S, Segura J, Torre R, et al. Furafylline is a potent and selective inhibitor of cytochrome P450IA2 in man. Br J Clin Pharmacol 1990; 29:651–63.
[14] Rahman A, Korzekwa KR, Grogan J, Gonzalez FJ, Harris JW. Selective biotransformation of taxol to 6α-hydroxytaxol by human cytochrome P450 2C8. Cancer Res 1994;54:5543–6.
[15] Mancy A, Dijols S, Poli S, Guengerich P, Mansuy D. Interaction of sulfaphenazole derivatives with human liver cytochromes P450 2C: molecular origin of the specific inhibitory effects of sulfaphenazole on CYP 2C9 and consequences for the substrate binding site topology of CYP 2C9. Biochemistry 1996;35:16205–12.
[16] Ko JW, Sukhova N, Thacker D, Chen P, Flockhart DA. Evaluation of omeprazole and lansoprazole as inhibitors of cytochrome P450 isoforms. Drug Metab Dispos 1997;25: 853–62.
[17] Newton DJ, Wang RW, Lu AY. Cytochrome P450 inhibitors. Evaluation of specificities in the in vitro metabolism of therapeutic agents by human liver microsomes. Drug Metab Dispos 1995;23:154–8.
[18] Schmider J, Greenblatt DJ, von Moltke LL, Harmatz JS, Shader RI. N-demethylation of amitriptyline in vitro: role of cytochrome P-450 3A (CYP3A) isoforms and effect of metabolic inhibitors. J Pharmacol Exp Ther 1995;275:592–7.
[19] Narimatsu S, Oda M, Hichiya H, Isobe T, Asaoka K, Hanioka N, et al. Molecular cloning and functional analysis of cytochrome P450 1A2 from Japanese monkey liver: Comparison with marmoset cytochrome P450 1A2. Chem Biol Interact 2005;152:1–12.
[20] Masuda K, Hashimoto H, Tamagake K, Okuda Y, Tsuzuki D, Isobe T, et al. Changes in the enzymatic properties of CYP2D6 by the substitution of phenylalanine at position 120 by alanine. J Health Sci 2004;50:503–10.
[21] Masuda K, Tamagake K, Okuda Y, Torigoe F, Tsuzuki D, Isobe T, et al. Change in enantioselectivity in bufuralol 1″-hydroxylation by the substitution of phenylalanine-120 by alanine in cytochrome P450 2D6. Chirality 2005;17:34–7.
[22] Yamazaki H, Johnson WW, Ueng YF, Shimada T, Guengerich FP. Lack of electron transfer from cytochrome b_5 in stimulation of catalytic activities of cytochrome P450 3A4. Characterization of a reconstituted cytochrome P450 3A4/NADPH-cytochrome P450 reductase system and studies with apo-cytochrome b_5. J Biol Chem 1996;271:27438–44.
[23] Jushchyshyn MI, Hutzler JM, Schrag ML, Wienkers LC. Catalytic turnover of pyrene by CYP3A4: evidence that cytochrome b_5 directly induces positive cooperativity. Arch Biochem Biophys 2005;438:21–8.
[24] Zou L, Harkey MR, Henderson GL. Effects of herbal components on cDNA-expressed cytochrome P450 enzyme catalytic activity. Life Sci 2002;71:1579–89.
[25] Narimatsu S, Takemi C, Tsuzuki D, Kataoka H, Yamamoto S, Shimada S, et al. Stereoselective metabolism of bufuralol racemate and enantiomers in human liver microsomes. J Pharmacol Exp Ther 2002;303:172–8.
[26] Yamazaki H, Oda Y, Funae Y, Imaoka S, Inui Y, Guengerich FP, et al. Participation of rat liver cytochrome P450 2E1 in the activation of *N*-nitrosodimethylamine and *N*-nitrosodiethylamine to products genotoxic in an acetyltransferase-overexpressing *Salmonella typhimurium* strain. Carcinogenesis 1992;13: 979–85.
[27] Ono S, Hatanaka T, Hotta H, Satoh T, Gonzalez FJ, Tsutsui M. Specificity of substrate and inhibitor probes for cytochrome P450s: evaluation of in vitro metabolism using cDNA-expressed human P450s and human liver microsomes. Xenobiotica 1996;26:681–93.
[28] Shimada T, Yamazaki H, Mimura M, Inui Y, Guengerich FP. Interindividual variations in human liver cytochrome P-450 enzymes involved in the oxidation of drugs, carcinogens and toxic chemicals: studies with liver microsomes of 30 Japanese and 30 Caucasians. J Pharmacol Exp Ther 1994;270:414–23.
[29] Yu AM, Idle JR, Byrd LG, Krausz KWA, Kupfer A, Gonzalez FJ. Regeneration of serotonin from 5-methoxytryptamine by polymorphic human CYP2D6. Pharmacogenetics 2003;13:173–81.
[30] Yu AM, Idle JR, Herraiz T, Kupfer A, Gonzalez FJ. Screening for endogenous substrates reveals that CYP2D6 is a 5-methoxyindolethylamine *O*-demethylase. Pharmacogenetics 2003;13:307–9.