Cytochrome P4502B6 and 2C9 do not metabolize midazolam: kinetic analysis and inhibition study with monoclonal antibodies

N. Hamaoka*, Y. Oda, I. Hase and A. Asada

Department of Anesthesiology and Intensive Care Medicine, Osaka City University Medical School, 1-5-7 Asahimachi, Abeno-ku, Osaka 545-8586, Japan

*Corresponding author

We determined the contribution of cytochrome P450 (CYP) isoforms to the metabolism of midazolam by kinetic analysis of human liver microsomes and CYP isoforms and by examining the effect of chemical inhibitors and monoclonal antibodies against CYP isoforms in vitro. Midazolam was metabolized to 1'-hydroxymidazolam (1'-OH MDZ) by human liver microsomes with a Michaelis-Menten constant ($K_m$) of 4.1 (1.0) (mean (SD)) μmol litre$^{-1}$ and a maximum rate of metabolism ($V_{max}$) of 5.5 (1.1) nmol min$^{-1}$ mg protein$^{-1}$ ($n$=6). Of the nine representative human liver CYP isoforms, CYP1A2, 2A6, 2B6, 2C9, 2C19, 2D6, 2E1, 3A4 and 3A5, three (CYP2B6, 3A4 and 3A5) showed midazolam 1'-hydroxylation activity, with $K_m$s of 40.7, 1.7 and 3.0 μmol litre$^{-1}$, respectively, and $V_{max}$ values of 12.0, 3.3 and 13.2 nmol min$^{-1}$ nmol P450$^{-1}$, respectively ($n$=4). Midazolam 1'-hydroxylation activity of human liver microsomes correlated significantly with testosterone 6β-hydroxylation activity, a marker of CYP3A activity ($r^2=0.77$, $P=0.0001$), but not with S-mephenytoin N-demethylation activity, a marker of CYP2B6 activity ($r^2<0.01$, $P=0.84$) ($n$=11). Troleandomycin and orphenadrine, chemical inhibitors of CYP isoforms, inhibited the formation of 1'-OH MDZ by human liver microsomes. Monoclonal antibody against CYP3A4 inhibited the formation of 1'-OH MDZ by 79%, whereas monoclonal antibody against CYP2B6 had no effect on midazolam 1'-hydroxylation by human liver microsomes ($n$=5). These results indicate that only CYP3A4, but not CYP2B6 or CYP2C, is involved in the metabolism of midazolam in vitro.

Br J Anaesth 2001; 86: 540–4

Keywords: hypnotics benzodiazepine, midazolam; enzymes, cytochrome P4502B6; enzymes, cytochrome P4503A4

Accepted for publication: October 13, 2000

Midazolam is an intravenous anaesthetic used widely in clinical practice. Numerous studies of the metabolism of midazolam have been performed. Microsomal cytochrome P450 (CYP) 3A in liver is the predominant CYP isoform involved in the metabolism of this agent.1–3 Metabolism of midazolam is inhibited by concomitantly administered agents such as antmycotics, calcium channel blocking agents and opioids, resulting in significant prolongation of amnesia and disturbance of psychomotor performance.4–6 Inhibition of CYP3A4 activity by these agents has been considered responsible for inhibiting midazolam metabolism.4–7 According to a recent in vivo and in vitro study, propofol decreases the clearance of midazolam by competitively inhibiting CYP3A activity.8 Since competitive inhibition of one drug by another drug is commonly observed when the two drugs are metabolized by the same CYP isoform,9 the findings of inhibition of metabolism of midazolam by propofol suggest that the same CYP isoforms may be involved in the metabolism of these anaesthetics. Several lines of evidence, however, have shown that propofol is metabolized predominantly by CYP2B6 or CYP2C9, and that CYP3A4 does not metabolize propofol (Y.Oda, personal communication and ref.10), suggesting that CYP2B6 and/or CYP2C9 as well as CYP3A4 may be involved in the metabolism of midazolam. According to a recent study,11 both midazolam 1'-hydroxylation activity and immunoquantified CYP3A4 levels significantly correlated with CYP2B6 level in human liver microsomes,
suggesting that genetic expression of CYP2B6 and CYP3A4 is related, and that CYP2B6 contributes to the metabolism of midazolam.

Although there have been several studies of the metabolism of midazolam by CYP isoforms, few studies have compared the metabolism of midazolam by CYP2B6 and 2C9 with that by other CYP isoforms, possibly because the human liver contains less of these CYP isoforms than other isoforms. CYP isoforms belonging to the CYP2B and 2C families are involved in the metabolism of various agents and are inducible by commonly used agents such as barbiturates and steroids. In addition, the pharmacokinetics of agents metabolized by CYP2B and 2C are significantly influenced by the levels of these isoforms in liver microsomes, suggesting that examination of the roles of CYP2B6 and 2C9 in the metabolism of midazolam may shed light on the pharmacokinetic interactions of midazolam with other agents, as well as for testing the possibility of induction and inhibition of metabolism of midazolam by various agents. The objective of this study was to determine the contribution of CYP isoforms including CYP2B6 and 2C9 to the metabolism of midazolam in vitro by kinetic analysis and by examination of the effects of chemical inhibitors and monoclonal antibodies on the metabolism of midazolam by liver microsomes.

Materials and methods

Materials

The experimental protocol was approved by the Institutional Human Investigational Committee of Osaka City University Medical School. Midazolam and its metabolites, 1'-hydroxymidazolam (1'-OH MDZ), 4'-hydroxymidazolam (4'-OH MDZ) and 1',4'-hydroxymidazolam (1',4'-OH MDZ), were kind gifts from Hoffmann-La Roche Ltd (Nutley, NJ, USA). Testosterone, 6β-hydroxytestosterone, S-mephenytoin and its N-demethylated metabolite, nirvanol, were obtained from Ultrafine Chemicals (Manchester, UK). Troleandomycin and orphenadrine were obtained from Sigma Chemical Co. (St Louis, MO, USA). Human liver microsomes were obtained from the International Institute for the Advancement of Medicine (Scranton, PA, USA). These microsomes were prepared from liver samples of kidney donors by differential centrifugation; the protein content was measured using Lowry’s method, with bovine serum albumin as standard. P450 content was determined from the differential spectrum of carbon monoxide-reduced versus oxidized microsomes as described by Omura and Sato. Specific contents of P450 in human liver microsomes were 0.33–0.66 nmol mg protein⁻¹. Recombinant human P450s expressed in human lymphoblast cells with cytochrome P450 reductase were obtained from Gentest (Woburn, MA, USA). These P450s were supplied as microsomes. Monoclonal antibodies against CYP3A4 and 2B6 were obtained from Gentest. The selectivity and inhibitory activity of these antibodies were confirmed as described in the manufacturer’s instructions. In our preliminary experiments, monoclonal antibody against CYP2B6 inhibited S-mephenytoin N-demethylation by human liver microsomes by 75% at 1.0 mg IgG (mg of microsomal protein)⁻¹ and midazolam 1'-hydroxylation by recombinant CYP2B6 by 85% at 0.5 mg IgG (mg of microsomal protein)⁻¹. Reduced nicotinamide adenine dinucleotide phosphate (NADPH) was obtained from the Oriental Yeast Co. (Tokyo, Japan). C18 columns (TSK gel ODS-120T, 4.6×150 mm) were obtained from Tosoh Corp. (Tokyo, Japan). Other reagents and organic solvents were from Wako Pure Chemical Industries (Osaka, Japan).

Assay of midazolam metabolism

The metabolism of midazolam by liver microsomes from six individuals was measured as reported previously. There was 100 µg of microsomal protein in each 500 µl incubation mixture. When metabolism of midazolam was measured with each of the CYP isoforms, CYP1A2, 2A6, 2B6, 2C9, 2C19, 2D6, 2E1, 3A4 and 3A5, 30 pmol of P450 was used instead of human liver microsomes (n=4). The midazolam concentration was between 0.2 and 20 µmol litre⁻¹. Incubation times were 2 and 10 min for experiments with microsomes and CYP isoforms, respectively. The P450 chemical inhibitors, orphenadrine and troleandomycin, were used at a concentration of 100 µmol litre⁻¹, which is sufficient to suppress >80% of CYP2B and CYP3A activities in human liver microsomes. The Michaelis–Menten constant (Km) and maximum rate of metabolism (Vmax) for the formation of 1'-OH MDZ from midazolam were determined by linear regression from Lineweaver–Burk double-reciprocal plots. When antibodies against CYP isoforms were used, microsomes and antibodies were preincubated in test tubes at room temperature for 20 min, followed by addition of midazolam and NADPH. The metabolites were extracted and measured by high-performance liquid chromatography (HPLC) as previously reported. The lower limit of quantification of 1'-OH MDZ, 4'-OH MDZ and 1',4'-OH MDZ was 20 nmol litre⁻¹. Intra-assay and inter-assay coefficients of variations were <4.8% and 7.0%, respectively.

Assay of S-mephenytoin N-demethylation and testosterone 6β-hydroxylation

S-Mephenytoin N-demethylation and testosterone 6β-hydroxylation activities of human liver microsomes from 11 individuals were determined as measures of CYP2B6 and 3A4 activities, respectively, following the method described previously. S-mephenytoin and testosterone were used at 1 mmol litre⁻¹ and 500 µmol litre⁻¹, respectively; these concentrations were chosen based on previously reported Km values. The content of microsomal protein was 400 mg litre⁻¹ and incubation time was
Results

Metabolism of midazolam by human liver microsomes and CYP isoforms

When midazolam was incubated with human liver microsomes, 1′-OH MDZ was the only metabolite detected by HPLC. The total amount of residual midazolam and 1′-OH MDZ formed in the incubation mixture was approximately equal to the total amount of midazolam added to the reaction, suggesting that 1′-OH MDZ was the predominant midazolam metabolite. Rates of formation of 1′-OH MDZ were linear up to 15 and 30 min for the substrate concentrations used with microsomes and P450s, respectively. Single-phase straight curves were obtained from the Lineweaver–Burk plots for 1′-hydroxylation by recombinant CYP2B6, 3A4 and 3A5. Midazolam (0.5–20 μmol litre⁻¹) was incubated at 37°C for 10 mins with recombinant P450 (30 pmol) and 0.4 mmol litre⁻¹ reduced NADP in 0.1 mol litre⁻¹ potassium phosphate buffer (pH 7.4) in a final volume of 500 μl. Each plot depicts the mean of four experiments.

20 min for both substrates. The lower limit of detection of nirvanol and 6β-hydroxytestosterone was 50 nmol litre⁻¹ and 1 μmol litre⁻¹, respectively. Correlations between midazolam 1′-hydroxylation and S-mephenytoin N-demethylation and testosterone 6β-hydroxylation activity were determined by linear regression analysis using Statview J version 4.5 (Abacus Concepts, Inc., Berkley, CA, USA). All values are presented as mean (SD); P < 0.05 was considered statistically significant.

Correlation of midazolam 1′-hydroxylation activity with S-mephenytoin N-demethylation and testosterone 6β-hydroxylation activities

There was 13-fold variation between individuals in the midazolam 1′-hydroxylation activity of human liver microsomes obtained from 11 individuals at a substrate concentration of 10 μmol litre⁻¹. Preliminary studies indicated that the rates of formation of nirvanol from S-mephenytoin (1 mmol litre⁻¹) and of 6β-hydroxytestosterone from testosterone (500 μmol litre⁻¹) were linear for ≤1.0 h incubation when microsomal protein content was 400 mg litre⁻¹ and for ≤2.0 g litre⁻¹ microsomal protein when the incubation time was 20 min. The mean S-mephenytoin N-demethylation and testosterone 6β-hydroxylation activity of human liver microsomes was 339 (185) pmol min⁻¹ mg protein⁻¹ and 5.4 (2.5) nmol min⁻¹ mg protein⁻¹, respectively. These values were comparable to those reported previously. 20, 21 There was a significant correlation between midazolam 1′-
In conclusion, midazolam is selectively metabolized by CYP3A4, but neither CYP2B6, 2C9 nor 2C19 is involved in the metabolism of midazolam in vitro.

Acknowledgements
This study was supported in part by the Fund for Medical Research from Osaka City University Medical Research Foundation and a Grant-in-Aid for Research from the Ministry of Education, Science and Culture of Japan, nos 09771183 and 11671517.
References


