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Identification of human cytochrome P450 isoforms involved in the metabolism of brotizolam

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1. To identify the cytochrome P450 (CYP) isoenzyme(s) responsible for the major metabolic pathways of brotizolam in man, we examined the metabolism of brotizolam using human liver microsomes and microsomes expressing individual human CYP isoenzymes (CYP1A1, 1A2, 2A6, 2B6, 2C9, 2C19, 2D6, 2E1, 3A4).

2. Brotizolam was metabolized to α-OH- and 6-OH-brotizolam by human liver microsomes (n = 3). V_{max} for α- and 6-hydroxylation of brotizolam were 1470 ± 259 and 8983 ± 7740 pmol/min/mg protein respectively, and the corresponding K_m were $49 \pm 9{\cdot}3$ and 595 ± 580 μM respectively.

3. Among CYP inhibitors examined (furafylline, sulphaphenazole, quinidine, ketoconazole and cimetidine), ketoconazole showed the most potent inhibitory effect on brotizolam metabolism by human liver microsomes. K_i of ketoconazole for α- and 6-hydroxylation were 0·05 and 0·07 μM respectively.

4. When incubated with microsomes expressing individual human CYP isoenzymes (CYP1A1, 1A2, 2A6, 2B6, 2C9, 2C19, 2D6, 2E1, 3A4), brotizolam was metabolized only by CYP3A4.

5. Brotizolam metabolism in human liver microsomes was almost completely inhibited by anti-CYP3A4 antiserum.

6. These results suggest that CYP3A4 is predominantly responsible for both α- and 6-hydroxylation of brotizolam in human liver microsomes.

Introduction

Brotizolam (2-bromo-4-(*o*-chlorophenyl)-9-methyl-6*H*-thieno[3,2*f*]-*s*-triazolo[4,3-*a*] [1,4] diazepine, BRT; figure 1) is a triazolothienodiazepine widely used as a hypnotic agent (Sanchez-Martinez and Landa-Palos 1982). The drug is absorbed well after oral administration, but < 1% of dosed BRT is excreted in the urine as the unchanged form (Bechtel *et al*. 1986a); and the remainder is excreted as metabolites hydroxylated at various sites in the molecule with subsequent conjugate formation (Bechtel *et al*. 1986b). Bechtel *et al*. (1986b) identified the two main metabolites in man, WE964 and WE1061, as 2-bromo-4-(2-chlorophenyl)-9-hydroxymethyl-*6H*-thieno-[3,2-*f*]-1,2,4-triazolo-[4,3-*a*]-1,4-diazepine (α-OH-BRT; figure 1) and 2-bromo-4-(2-chlorophenyl)-6-hydroxy-9-methyl-*6H*-thieno-[3,2-*f*]-1,2,4-triazolo-[4,3-*a*]-1,4-diazepine (6-OH-BRT; figure 1) respectively. They suggested that the route of systemic elimination of BRT in man is predominantly via these two hydroxylated metabolites.

It is well known that cytochrome P450 (CYP) plays an important role in the metabolic disposition of a variety of drugs and foreign compounds, and that it consists of multiple isoforms with different but broad and often overlapping substrate specificities (Horai and Ishizaki 1988, Gonzalez 1992, Guengerich 1994).

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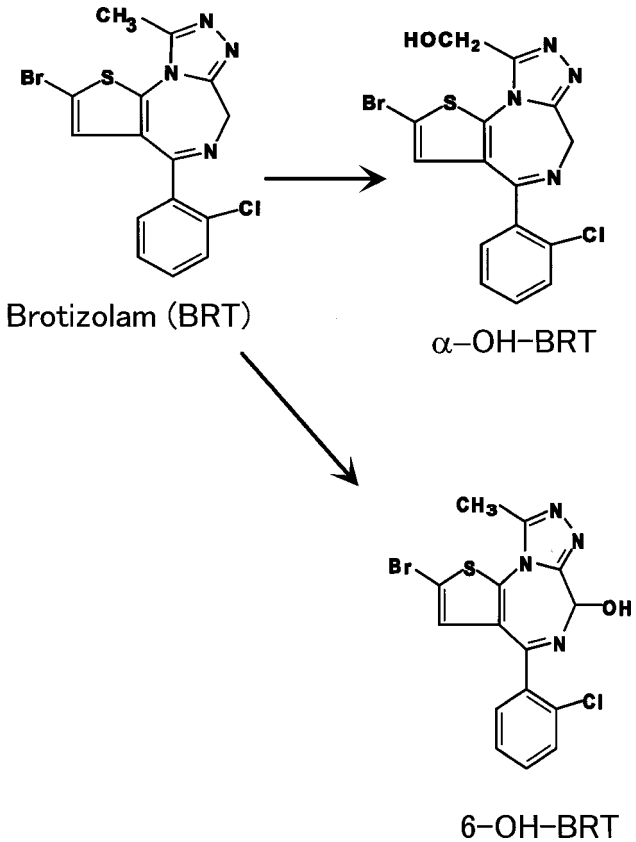

Figure 1. Metabolism of BRT to its two principal metabolites, α-OH-BRT and 6-OH-BRT.

In the case of benzodiazepines used as hypnotic agents (i.e. triazolam and midazolam), CYP3A4 plays an important role in their metabolism *in vitro* (Kronbach *et al.* 1989). Furthermore, an *in vivo* study suggested that alprazolam, another benzodiazepine, is also metabolized by CYP3A4 (Yasui *et al.* 1996). Although BRT structurally resembles benzodiazepines, it has a thieno ring in place of the fused benzene ring of benzodiazepines such as triazolam, midazolam and alprazolam. Therefore, the possibility exists that the CYP isoenzyme mainly responsible for BRT metabolism is different from that in the case of benzodiazepines. The aim of this study was to identify the CYP isoenzyme(s) responsible for the metabolism of BRT.

Materials and methods

Chemicals and reagents

BRT and α-OH-BRT were synthesized by Boehringer Ingelheim Co., Germany, and 6-OH-BRT was synthesized by the Analytical Department, Nippon Boehringer Ingelheim. These three chemicals were of the highest purity (> 98%) available. β-Nicotinamide adenine dinucleotide phosphate [tetrasodium salt] (NADPH), D-glucose 6-phosphate [monosodium salt] (G6P), glucose 6-phosphate dehydrogenase [type VII from yeast] (G6PDH), sulphaphenazole, cimetidine and diazepam were purchased from Sigma Chemical Co. (St Louis, MO, USA). Ketoconazole, quinidine, and furafylline were from Funakoshi (Tokyo, Japan), Wako Pure Chemical Industries (Osaka, Japan), and Daiichi Pure Chemicals Co. (Tokyo, Japan) respectively. Human anti-CYP3A4 antiserum, which is a commercially available rabbit antiserum raised against rat liver CYP3A2, was from Daiichi Pure Chemicals Co.

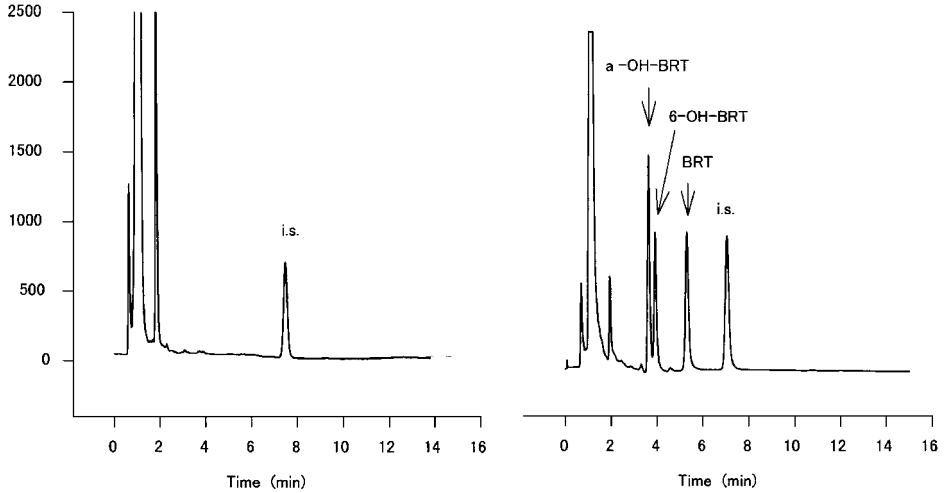

Figure 2. Chromatographic separation of BRT metabolites formed by human liver microsomes. Units of the *y*-axes are μV, UV detector.

(Tokyo, Japan). This polyclonal antibody was used for studying the inhibition of the human CYP3A4 enzyme activity because of no inhibitory effect against other human CYPs (i.e. CYP1A1, 1A2, 2A6, 2B6, 2C9, 2D6 and 2E1 : data on leaflet). Microsomes expressing human CYP1A1, 1A2, 2A6, 2B6, 2C9, 2C19, 2D6, 2E1 and 3A4, which were prepared from the human B lymphoblastoid cell line (GENTEST Co., USA), were all purchased from Daiichi. Human liver microsomes were obtained from Iwai Chemical Co. (pooled Batch 2: Osaka, Japan) or K.A.C. (HHM0163 and HHM0215: Kyoto, Japan). All other chemicals and reagents were of analytical grade.

Kinetic analysis of BRT metabolism in human liver microsomes

For kinetic analysis of the metabolism of BRT in human liver microsomes, BRT (final concentrations 10, 25, 50, 100 or 250 μM) was preincubated for 5 min with 0·1 M phosphate buffer (pH 7·4) and an NADPH-generating system (3 mM G6P, 1 unit/ml G6PDH, 1·2 mM NADPH and 3·3 mM $MgCl_2$). The reactions were initiated by addition of human liver microsomes (1 mg microsomal protein/ml) in a final volume of 0·5 ml, and the mixture was incubated for 10 min at 37 °C. An aliquot of the reaction mixture (200 μl) was mixed with 5 μl 70% perchloric acid (PCA) and placed on ice to terminate the reaction.

Effect of specific CYP inhibitors on BRT metabolism in human liver microsomes

The effects of various specific CYP isoenzyme inhibitors, furafylline (2 or 10 μM), sulphaphenazole (0·5 or 3 μM), quinidine (2 or 20 μM), ketoconazole (0·25 or 0·5 μM), and cimetidine (10 or 50 μM), were examined at the concentrations recommended in the literature (Newton *et al.* 1995, Wrighton and Ring 1994). Incubation mixtures containing BRT (25, 50, 100 or 250 μM), an inhibitor, and an NADPH-generating system in 0·1 M phosphate buffer (pH 7·4) were preincubated for 5 min. The reactions were initiated by addition of human liver microsomes (0·5 mg) in a final volume of 0·5 ml. After incubation for 10 min at 37 °C, reactions were terminated as described above.

BRT metabolism by microsomes expressing individual human CYP isoenzymes

Microsomes expressing human CYP1A1, 1A2, 2A6, 2B6, 2C9, 2C19, 2D6, 2E1 or 3A4 (1 mg) were incubated with 1 μM BRT and an NADPH-generating system for 4 h at 37 °C in 1·0 ml 0·1 M phosphate buffer (pH 7·4). For CYP2A6 or 2C9, phosphate buffer was replaced with 50 mM Tris-HCl buffer (pH 7·4) because the activity of these isoenzymes is decreased by phosphate. Aliquots of 200 μl of reaction mixture were taken at appropriate times and the reaction was terminated by addition of 600 μl ice-cold methanol.

Inhibition of BRT metabolism by anti-CYP3A4 antiserum

Immunoinhibition of BRT metabolism in human liver microsomes was examined with anti-CYP3A4 antiserum, which shows no cross reactivity with CYP1A1, 1A2, 2A6, 2B6, 2C9, 2D6 or 2E1 (see Materials and methods). Ten or 1000 μg IgG of the antiserum diluted with non-immunized rabbit

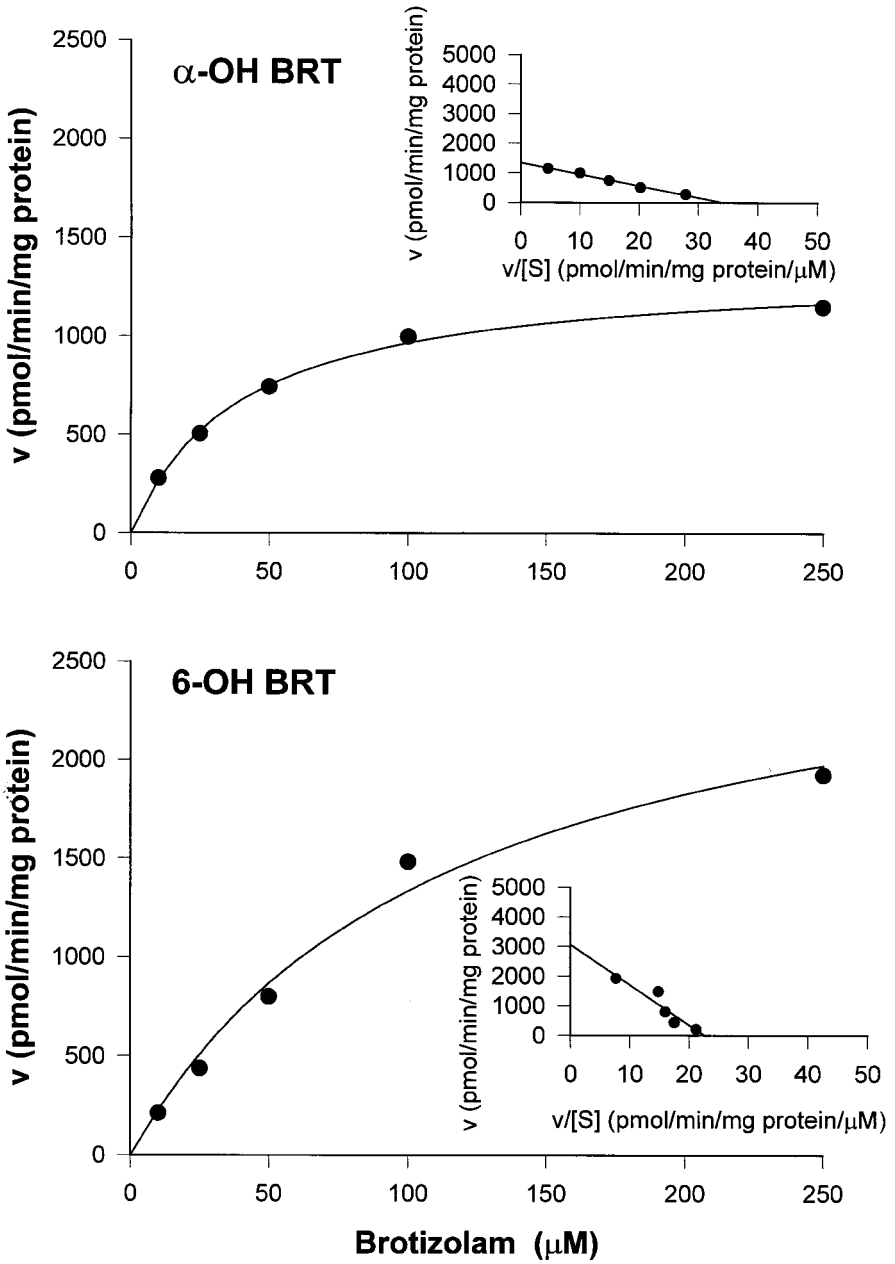

Figure 3. Typical Michaelis–Menten plots of α-OH-BRT (A) and 6-OH-BRT (B) formation in human liver microsomes (HHM0215). The BRT concentration range was 10–250 μM. Lines indicate the best fit of the Michaelis–Menten equation to the data. The inset in each figure is the Eadie–Hofstee plot. Lines were estimated by least-squares linear regression.

serum was added to human liver microsomes (0·25 mg protein) and the mixture was preincubated at room temperature for 30 min. A control reaction was run simultaneously using non-immune rabbit serum instead of the antiserum. After the preincubation, BRT (10 μM), an NADPH-generating system, and 0·1 M phosphate buffer (final incubation volume of 1·0 ml) were added. The reactions were performed at 37 °C for 60 min. Aliquots of 200 μl were taken at 1, 15, 30 and 60 min and the reaction was terminated by addition of 5 μl 70% PCA.

Table 1. Michaelis–Menten parameters for BRT metabolism in human liver microsomes.

Human liver microsomes	α-OH-BRT formation			6-OH-BRT formation		
	V_{max} (pmol/min/mg protein)	K_m (μM)	V_{max}/K_m	V_{max} (pmol/min/mg protein)	K_m (μM)	V_{max}/K_m
Batch 2	1820	62	29·5	19890	1413*	14·1
HHM0163	1390	41	33·5	4340	241	18·0
HHM0215	1200	41	27·6	2720	131	20·8
Mean	1470	49·0	30·2	8983	595·0	17·7
SE	259	9·3	2·5	7740	580·1	2·7

All data are means from five experiments.

* This value also shows the mean from five, of which two are substantially different from other three values (121, 351, 366 μM).

Hplc analysis of BRT and its metabolites

BRT metabolites were determined by an hplc method. The system consisted of a controller (Model 715, Gilson, USA), pump (LC-6A, Shimadzu, Japan), autoinjector (SIL-6B, Shimadzu), UV-VIS detector (Model 486, Waters, USA), column oven (CTO-6A, Shimadzu), and reverse-phase C18 column (Superspher RP-Select B, 125 × 4 mm, 5 μm, Merck, Japan). A flow rate of 1·0 ml per min mobile phase (50 mM ammonium acetate/methanol/acetonitrile, 45/50/5), and a column temperature of 40 °C were used. The metabolites were determined by measuring their absorbance at 240 nm; the retention times of α-OH-BRT and 6-OH-BRT were 3·6 and 3·9 min respectively. After termination of the reaction, each sample was mixed with 5 μl diazepam (0·1 mM) as an internal standard and centrifuged at 15000 *g* for 2 min. The resultant supernatant (170 μl) was transferred to another tube and mixed with 50 μl 1 M MOPS and 5 μl saturated KOH solution to adjust to pH 7·0. After centrifugation, 50 μl supernatant was injected directly into the hplc system. A calibration curve was prepared based on the peak heights of standard samples (0·1–3·0 μM).

For the determination of a low concentration of BRT (1 μM), a more sensitive hplc method was employed. This utilized a column-switching technique using a valve actuator (817, Gilson) and an enrichment column (Pelliguard LC-8, 2 cm, Supelco, Japan). Superspher RP-Select B was used as the analytical column. A flow rate of 1·0 ml per min mobile phase (40% acetonitrile in 0·03% PCA solution for 4 min, followed by a linear gradient to 60% acetonitrile by 7 min then isocratic 60% acetonitrile for 1 min) was used, and elution of BRT was monitored by measuring the absorbance at 210 nm with a variable-wavelength UV detector (Model 486, Waters). Under these conditions the retention time of BRT was approximately 4·7 min. Amounts of BRT were determined from a linear calibration plot based on the peak height of standard samples (0·25–25 nM).

Kinetic analysis

Apparent K_m (Michaelis–Menten constant) and V_{max} (maximum rate of metabolite formation) were estimated by fitting data to the Michaelis–Menten equation using the enzyme kinetic program 'Pharmacologic Calculation System' (Tallarida and Murray 1986). Apparent K_i for ketoconazole, cimetidine and quinidine were similarly calculated with this program.

Results

In human liver microsomes, BRT was metabolized to two hydroxylation derivatives (α-OH-BRT and 6-OH-BRT; figure 1), which were separated and determined by hplc (figure 2). Formation of these two metabolites proceeded linearly with respect to incubation time up to 30 min (data not shown).

The formation of these metabolites in human liver microsomes (HHM0215) depended on the substrate concentration (figure 3), and the data fitted simple Michaelis–Menten kinetics well. The kinetic parameters obtained with three preparations of human liver microsomes (Batch 2, HHM0163 and HHM0215) are shown in table 1. Eadie–Hofstee plots (figure 3) were fitted by least squares linear regression analysis.

The effects of several specific CYP inhibitors on the two major oxidation reactions of BRT are shown in figure 4. Ketoconazole markedly inhibited the

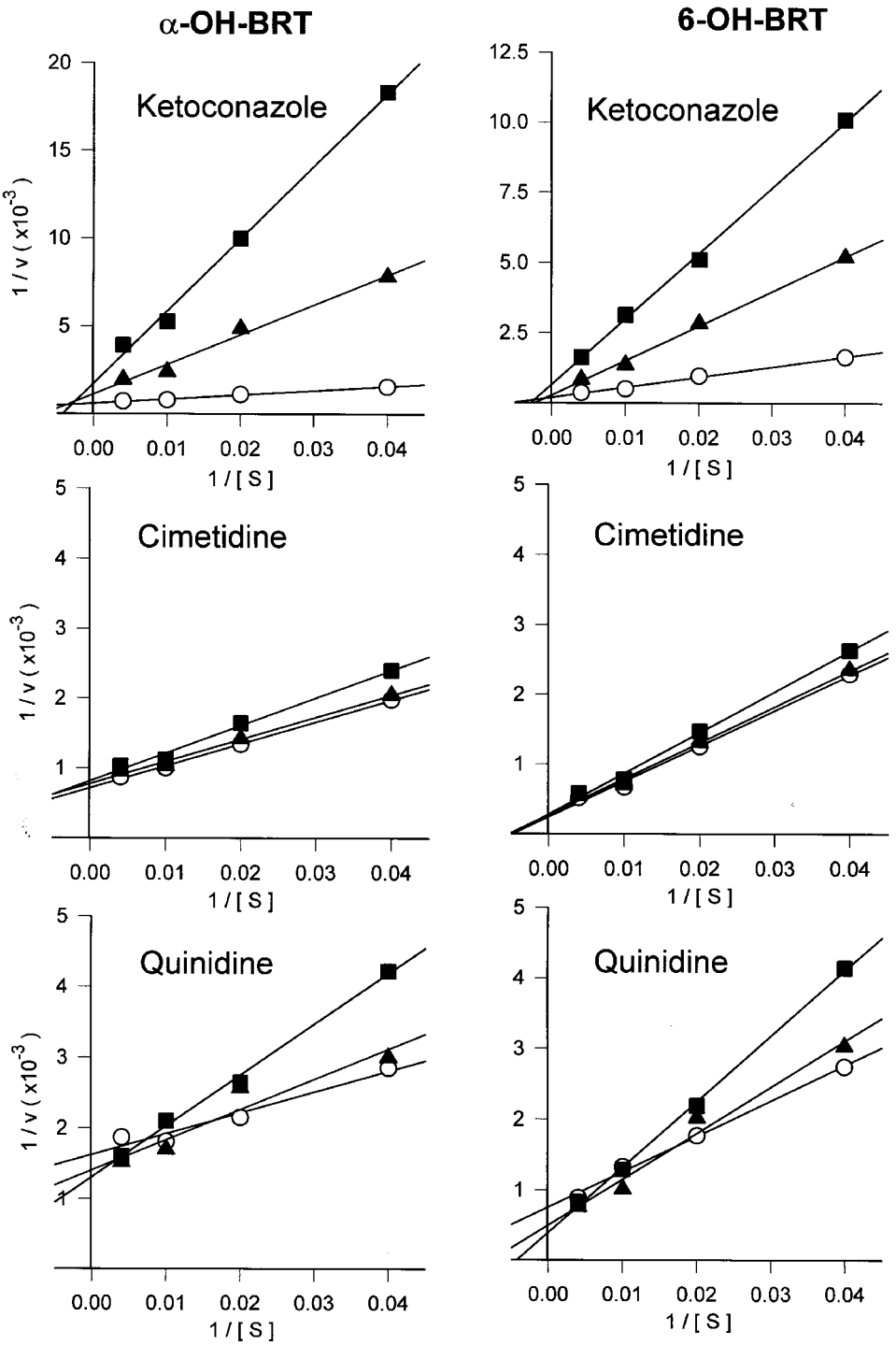

Figure 4. For legend see facing page.

Table 2. K_i for inhibition of the formation of BRT metabolites in human liver microsomes.

	K_i (μM)	
Inhibitor	α-OH-BRT formation	6-OH-BRT formation
Cimetidine	178·13 ± 9·72	274·25 ± 38·65
Quinidine	43·23 ± 29·80	85·75 ± 46·49
Ketoconazole	0·05 ± 0·01	0·07 ± 0·04

Each value represents the mean ± SE of values obtained with three different preparations of microsomes (Batch 2, HHM0163, HHM0215).

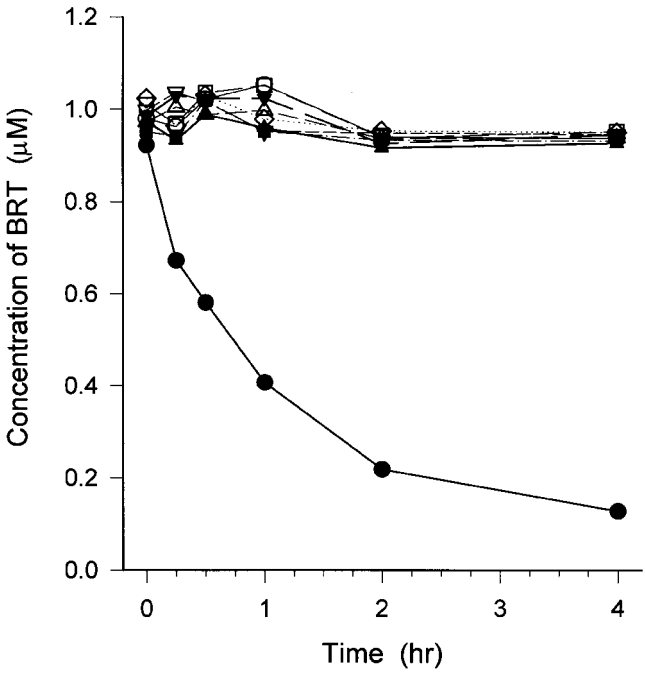

Figure 5. Metabolism of BRT by microsomes expressing human CYP isoenzymes: CYP1A1 (○); CYP1A2 (□); CYP2A6 (△); CYP2B6; (▽); CYP2C9 (◇); CYP2C19 (■); CYP2D6; (▲); CYP2E1 (▼); CYP3A4 (●). Each microsomal fraction was incubated for 4 h with 1 μM BRT and an NADPH-generating system. BRT concentration was measured at the indicated time after the start of incubation. These data were from a single experiment because of the low amount of human liver microsomes available.

formation of both α-OH- and 6-OH-BRT in a dose-dependent manner, with K_i 0·05 and 0·07 μM respectively. Quinidine and cimetidine were slightly inhibitory. Furafylline (2, 10 μM) and sulphaphenazole (0·5, 3 μM) had no effect (data not shown). The apparent K_i of ketoconazole, cimetidine and quinidine in the three different preparations of human liver microsomes are given in table 2.

Figure 4. Lineweaver–Burk plots of the effects of inhibitors on α-OH- and 6-OH-BRT formation by human liver microsomes. Human liver microsomes (HHM0215, 1·0 mg/ml) were incubated for 10 min with an NADPH-generating system and BRT at a concentration of 25, 50, 100 or 250 μM in the absence (○) or the presence of an inhibitor: ketoconazole, 0·25 μM (▲) or 0·5 μM (■); quinidine, 2 μM (▲) or 20 μM (■); cimetidine, 10 μM (▲) or 50 μM (■). The lines were determined by non-linear least-squares regression. These data were from a single experiment because of the low amount of human liver microsomes available.

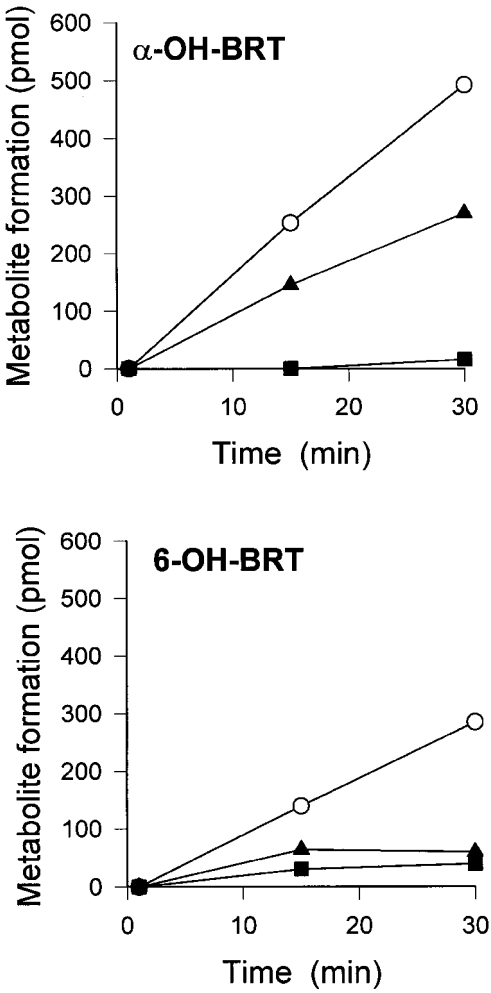

Figure 6. Dose-dependent inhibition of BRT metabolism in human liver microsomes with human CYP3A4 antiserum. Human liver microsomes were preincubated with 0 (○), 10 μg IgG (▲) or 1·0 mg IgG (■) antiserum and then incubated with 10 μM BRT in the presence of an NADPH-generating system at 37 °C for 30 min. Data were from a single experiment because of the low amount of human liver microsomes available.

The catalytic activities of individual human CYPs expressed in microsomes were examined. We decided to measure the decrease of BRT in this study, because the production of metabolites during the early phase of incubation with 1 μM BRT might be lower than the detection limit. BRT was decreased only by incubation with CYP3A4 (figure 5). Other CYPs (CYP1A1, 1A2, 2A6, 2B6, 2C9, 2C19, 2D6 and 2E1) did not metabolize BRT.

To confirm identity of the microsomal enzyme responsible for the formation of the major metabolites α-OH-BRT and 6-OH-BRT, the effect of anti-CYP3A4 antiserum was investigated (figure 6). During incubation for 30 min, 1 mg IgG/ml of anti-CYP3A4 antiserum inhibited the α-OH and 6-OH pathways to the extent of 97 and 86% respectively.

Discussion

We examined the metabolism of a triazolothienodiazepine drug, BRT, using human liver microsomes as well as microsomes expressing individual human CYP isoenzymes, in order to identify the CYP isoenzyme(s) involved in the major metabolic pathways of BRT, i.e. α- and 6-hydroxylation. With three different preparations of human microsomes, the Eadie–Hofstee plots were linear (figure 3) in all cases, suggesting that a single CYP isoenzyme catalyses the hydroxylation of BRT. Among various specific CYP inhibitors examined, ketoconazole extensively inhibited both α- and 6-hydroxylation of BRT (K_i = 0·05 and 0·07 μM respectively) (figure 4). Although high concentrations (> 10 μM) of ketoconazole inhibit reactions mediated by several CYP3A species (Newton *et al.* 1995), low concentrations (< 1 μM) of ketoconazole specifically inhibit CYP3A4 (Maurice *et al.* 1992, Baldwin *et al.* 1995). The concentration used in our study (0·25 or 0·5 μM), therefore, implies a specific effect of ketoconazole on CYP3A4. In the experiment using expressed human CYPs, only CYP3A4 exhibited substantial activity for the α- and 6-hydroxylation of BRT (figure 5). These data suggest that CYP3A4 is the isoenzyme predominantly responsible for the main metabolic pathways of BRT. The immunoinhibition study using anti-CYP3A4 antiserum confirmed this (figure 6).

Alprazolam and triazolam have similar structures to BRT, and their main metabolic pathways of α- and 4-hydroxylation were reported to be catalysed by CYP3A4. The K_i of ketoconazole for the 4-hydroxylation of alprazolam was 0·046 μM (von Moltke *et al.* 1994) and the K_i of ketoconazole for α- and 4-hydroxylation of triazolam were 0·006 and 0·023 μM respectively (von Moltke *et al.* 1996). These K_i are lower than the therapeutic plasma level of ketoconazole (Badcock *et al.* 1987), so metabolic inhibition by ketoconazole would be expected in clinical usage. In fact, the peak plasma concentration and *AUC* (area under the concentration–time curve) of triazolam increased up to 3- and 22-fold respectively when the drug was administered concurrently with ketoconazole to healthy volunteers (Varhe *et al.* 1994). In our study, the K_i of ketoconazole for α-OH and 6-OH-BRT formation were 0·05 and 0·07 μM respectively. Thus, the inhibitory effect of ketoconazole on BRT metabolism is less than that on triazolam metabolism. Nevertheless, it seems necessary to monitor the changes in plasma levels of BRT in patients when ketoconazole is administered at the same time.

Cimetidine slightly inhibited BRT hydroxylation (figure 4, table 2). This H_2-blocker is a CYP3A4 inhibitor, but its affinity is lower than that of azole derivatives such as ketoconazole (Wrighton and Ring 1994, von Moltke *et al.* 1996).

Quinidine, a specific inhibitor of CYP2D6, caused weak inhibition of BRT metabolism (figure 4, table 2), but no metabolic activity towards BRT was observed with expressed CYP2D6 (figure 5). Metabolism of quinidine itself is thought to be mediated by CYP3A4 (Guengerich *et al.* 1986), and quinidine also showed a slight inhibition of testosterone 6β-hydroxylation (Newton *et al.* 1995). Hydroxylation of both alprazolam and triazolam mediated by CYP3A4 was weakly inhibited by quinidine (von Moltke *et al.* 1994, 1996). These results indicate that quinidine is not only a specific inhibitor of CYP2D6 but also a substrate of CYP3A4. Therefore, the inhibition of BRT by quinidine could be explained by competition for CYP3A4.

In conclusion, we have demonstrated that formation of the two major metabolites of BRT, α-OH-BRT and 6-OH-BRT, in human liver microsomes is catalysed predominantly by a single CYP isoenzyme, 3A4.

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