years later, neurologic examinations revealed minor cerebellar signs and mild dementia. The result of an open-brain biopsy was also compatible with the diagnosis of VWM. Although detailed mutational analysis of the genes for five eIF2B subunits was performed, the causative mutations were not identified.[5]

Our present report suggests that individuals with latest-onset VWM may present with memory disturbance, disorientation, delusion, criminal behavior, or emotional disturbance. A diagnosis of VWM should be considered for patients with presenile dementia or psychiatric symptoms, even though cerebellar ataxia or spasticity may not be prominent. Consanguineous marriage or a history of deterioration of condition after a head trauma or high fever can help to diagnose VWM.

Interestingly, patients with identical genotypes (homozygous R195H or L309V mutation) have been shown to exhibit considerable variations in clinical severity.[2-5] We need to be careful in concluding that the T182M mutation in *EIF2B5* identified in our latest-onset case and the R113H mutation in *EIF2B5* lead to mild phenotypes. Further analyses of the clinical presentations of additional patients, particularly adult-onset cases, are required to determine the precise genotype-phenotype correlations in VWM.

References

1. van der Knaap MS, Barth PG, Gabreels FJM, et al. A new leukoencephalopathy with vanishing white matter. Neurology 1997;48:845–855.
2. Leegwater PAJ, Vermeulen G, Konst AAM, et al. Subunits of the translation initiation factor eIF2B are mutant in leukoencephalopathy with vanishing white matter. Nat Genet 2001;29:383–388.
3. Fogli A, Wong K, Eymard-Pierre E, et al. Cree leukoencephalopathy and CACH/VWM disease are allelic at the *EIF2B5* locus. Ann Neurol 2002; 52:506–510.
4. Fogli A, Dionisi-Vici C, Deodato F, Bartuli A, Boespflug-Tanguy O, Bertini E. A severe variant of childhood ataxia with central hypomyelination/vanishing white matter leukoencephalopathy related to *EIF2B5* mutation. Neurology 2002;59:1966–1968.
5. van der Knaap MS, Leegwater PA, Konst AA, et al. Mutations in each of the five subunits of translation initiation factor eIF2B can cause leukoencephalopathy with vanishing white matter. Ann Neurol 2002;51:264–270.
6. Fogli A, Rodriguez D, Eymard-Pierre E, et al. Ovarian failure related to eukaryotic initiation factor 2B mutations. Am J Hum Genet 2003;72: 1544–1550.
7. The Center for Childhood White Matter Disorders. Available at: http://www.vumc.nl/whitematter. Accessed January 17, 2002.
8. Prass K, Bruck W, Schroder NW, et al. Adult-onset leukoencephalopathy with vanishing white matter presenting with dementia. Ann Neurol 2001;50:665–668.
9. van der Knaap MS, Kamphorst W, Barth PG, Kraaijeveld CL, Gut E, Valk J. Phenotypic variation in leukoencephalopathy with vanishing white matter. Neurology 1998;51:540–547.

BRIEF REPORT

Genetic and Expression Analyses of *FZD3* in Schizophrenia

Masayuki Ide, Tatsuyuki Muratake, Kazuo Yamada, Yoshimi Iwayama-Shigeno, Kazuya Iwamoto, Hitomi Takao, Tomoko Toyota, Naoshi Kaneko, Yoshio Minabe, Kazuhiko Nakamura, Tadafumi Kato, Norio Mori, Takashi Asada, Toshiyuki Someya, and Takeo Yoshikawa

Background: *Wnt signaling plays important roles in neurodevelopmental processes. Frizzled is a receptor of Wnt protein, and the Frizzled 3 (*FZD3*) gene was recently reported to be associated with schizophrenia. Our study attempted to confirm associations between* FZD3 *and schizophrenia in Japanese family and case–control samples.*

Methods: *Genetic associations were evaluated using family-based transmission tests (212 families, 643 subjects) and case–control analysis (540 schizophrenia patients, 540 control sample). Six single nucleotide polymorphisms (SNPs) on the* FZD3 *locus were genotyped, and levels of* FZD3 *mRNA expression in postmortem brains were examined.*

Results: *Neither family- nor population-based studies supported associations between* FZD3 *and schizophrenia.* FZD3 *expression was unaltered in schizophrenic brains.*

Conclusions: *Although two prior studies have reported associations using limited numbers of SNPs on* FZD3*, our intensive study failed to support any major contribution of* FZD3 *to schizophrenia susceptibility.*

Key Words: Case–control study, family-based association study, neurodevelopment, postmortem brain, real-time quantitative RT-PCR, Wnt signaling

The pathogenesis of schizophrenia remains largely unknown, but it is hypothesized that causal events may begin in early neurodevelopment (Weinberger 1995). Wnt signaling plays important roles in neural development (Pleasure 2001), and this cascade is divided into two major pathways, canonical and noncanonical. In the canonical pathway, binding of Wnt to Frizzled receptors activates Disheveled, inhibiting glycogen synthase kinase-3β and stabilizing β-catenin. The noncanonical pathway does not depend on β-catenin, involving instead Wnt/Ca^{2+} signaling. Murine Frizzled 3 is involved in Wnt/Ca^{2+} signaling and weakly involved in the canonical pathway (Sheldahl et al 1999). The human Frizzled 3 gene (*FZD3*) is located on chromosome 8p21, a potential susceptibility region for schizophrenia (Blouin et al 1998; Gurling et al 2001).

Given the compelling functional and positional interests of *FZD3*, Yang et al (2003a) recently studied the role of the gene in schizophrenia, using three single nucleotide polymorphisms (SNPs) on *FZD3* in 246 Chinese family trios and reported preferential transmissions with all three SNPs (p = .003–.00007). Katsu et al (2003) reported significant case–control associations with one SNP on the gene; however, these two studies examined only limited numbers of SNPs, leaving both linkage disequilibrium (LD) structures of the locus and positions of causal genomic boundaries unknown. Our study reexamined associations of *FZD3* with schizophrenia, using denser markers and two sample panels comprising families (comparable in size to that of Yang et al [2003a]) and case–control samples (over twofold larger than those of Katsu et al [2003]). The possibility of disease-related aberrant gene expression was also examined using postmortem brains.

From the Laboratories for Molecular Psychiatry (MI, KY, YI-S, HT, TT, TY) and Molecular Dynamics of Mental Disorders (KI, TK), RIKEN Brain Science Institute, Wako, Saitama; Department of Psychiatry (MI), University of Tsukuba School of Medicine, Ibaraki; Department of Psychiatry (TM, NK, TS), Niigata University Graduate School of Medical and Dental Sciences, Niigata; Department of Psychiatry and Neurology (YM, KN, NM), Hamamatsu University School of Medicine, Hamamatsu, Japan.

Address reprint requests to Takeo Yoshikawa, M.D., Ph.D., Laboratory for Molecular Psychiatry, RIKEN Brain Science Institute, 2-1 Hirosawa, Wako-city, Saitama 351-0198, Japan; E-mail: takeo@brain.riken.go.jp.

0006-3223/04/$30.00
doi:10.1016/j.biopsych.2004.06.039

Methods and Materials

Subjects

Family samples comprised 212 schizophrenia families (643 members), including 168 independent and complete trios (Yamada et al 2004). Case–control samples comprised 540 unrelated schizophrenia patients (270 men, 270 women; mean age, 45.6 ± 11.0 years) and 540 age- and gender-matched control subjects who showed no history of mental illness in a brief psychiatric interview (270 men, 270 women; mean age, 45.1 ± 11.7 years). All subjects resided in central Japan. Consensus diagnosis was made by at least two psychiatrists according to the criteria of the DSM-IV. Written informed consent was obtained from all participants after explanation of study protocols and purposes. The study protocol was approved by the Ethics Committees of RIKEN and Niigata University.

Genetic Markers and Genotyping

Analyzed SNPs included the three reported by Yang et al (2003a): 435G>A (rs2241802), IVS5+5289A>G (rs2323019), and IVS5+9020*T*>C (rs352203) (Figure 1). In addition, five more SNPs were selected to cover the entire *FZD3* locus, based on the following criteria: 1) SNP on the *FZD3* genomic structure as defined by UCSC Genome Bioinformatics version July 2003 (http://genome.ucsc.edu/); 2) SNP in the National Center for Biotechnology Information database (http://www.ncbi.nih.gov), SNP consortium database (http://snp.cshl.org/), or JSNP database (http://snp.ims.u-tokyo.ac.jp/); and 3) SNP showing sufficient heterozygosity (frequency of minor allele>.1). Suitable SNPs included IVS2+595*T*>A (rs3757884), IVS3+258*T*>C (rs960914), IVS4–2244*T*>G (rs352210), IVS6+771A>G (rs352226), and Stop+1252C>A (rs352222). Assays-by-Design SNP genotyping products and TaqMan assay methods (Applied Biosystems, Foster City, California) were used to score SNPs. Genotypes were determined using an ABI7900 sequence detection system (Applied Biosystems). No TaqMan probe could be successfully designed for IVS2+595*T*>A, and Stop+1252C>A

BIOL PSYCHIATRY 2004;56:462–465

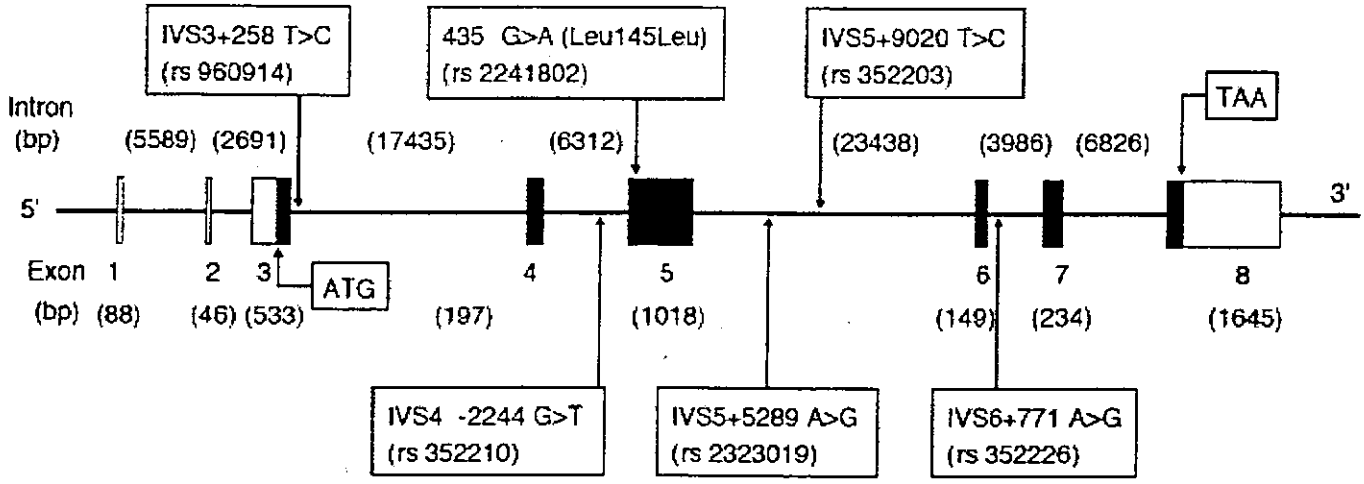

Figure 1. Genomic structure of human *FZD3* gene and locations of the six SNPs analyzed. Black boxes represent protein-coding regions, and white boxes represent untranslated regions. The rs numbers show National Center for Biotechnology Information single nucleotide polymorphism cluster IDs from the dbSNP database (http://www.ncbi.nlm.nih.gov/SNP/). Locations of initiation (ATG) and stop (TAA) codons and sizes of introns and exons are also provided.

could not be genotyped unambiguously. These two SNPs were therefore removed from the study, leaving six SNPs for analysis (Figure 1).

Assessment of Sample Stratification

To assess population stratification in case–control samples, 20 SNPs and 30 microsatellite markers (marker information is available upon request) were selected from across the genome. We used Structure software (http://pritch.bsd.uchicago.edu/software.html; Pritchard et al 2000) to attempt to identify genetically similar diploid subpopulations by grouping individuals and simulating 1,000,000 replications for parameter estimation. The number of populations present in the sample (K) is unknown, so analyses were run at K = 1–5. From these results, the best estimate of K was found by calculating posterior probabilities, Pr (K = 1, 2, 3, 4, 5).

Brain Tissues and Quantitative Reverse Transcriptase Polymerase Chain Reaction

RNA from the dorsolateral prefrontal cortex (DLPFC; Brodmann's area 46) was obtained from Stanley Medical Research Institute (http://www.stanleyresearch.org/programs/brain_collection.asp). Samples were taken from 27 schizophrenia patients (20 men, 7 women; aged 42.3 ± 1.8 years; postmortem interval (PMI) 32.2 ± 3.1 hour; brain pH 6.5 ± .04) and 27 control subjects (20 men, 7 women; aged 44.6 ± 1.5 years; PMI 29.7 ± 2.6 hours; brain pH, 6.6 ± .05). Age, PMI, and brain pH were not significantly different between the two groups (p = .436, .592, and .159, respectively). All schizophrenia patients were medicated with antipsychotics. This study was unblinded.

Real-time quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) analysis was conducted using an ABI7900 according to the manufacturer's instructions. TaqMan probes and primers for *FZD3* and β2-microglobulin (internal control) were from Assay-on-Demand gene expression products (Applied Biosystems). All qRT-PCR reactions were performed in triplicate, based on standard curve methods described by the manufacturer.

Statistical Analyses

Transmission distortions in the family panel were evaluated using the pedigree disequilibrium test (Martin et al 2000; http://www.chg.duke.edu/software/pdt.html) and extended transmission disequilibrium test (Sham and Curtis 1995; http://www.mds.qmw.ac.uk/statgen/dcurtis/software.html). Transmit software (Clayton 1999; http://watson.hgen.pitt.edu/docs/transmit.html) was run as a global test of haplotype transmission. Genetic Power Calculator (Purcell et al 2003; http://statgen.iop.kcl.ac.uk/gpc/) was used to compute statistical power. In the case-control study, haplotype frequencies, normalized LD coefficient D' and squared correlation coefficient r^2 were calculated using Cocaphase software (Dudbridge 2003; http://www.hgmp.mrc.ac.uk/~fdudbrid/software/). Allelic and genotypic frequencies of markers between patients and control subjects were assessed

Table 1. Results of Family-Based and Case–Control Association Studies Between *FZD3* and Schizophrenia

	Minor Allele Frequency		PDT p Value		ETDT	Case–Control p Value		3 SNP-Based Haplotype p Value			
SNP	Control	Schizophrenia	AVE	SUM	p Value	Allele	Genotype	Family-Based/Case–Control			
IVS3+258T>C	.39	.41	.985	.949	.632	.568	.096				
IVS4−2244G>T	.39	.40	.864	.899	.687	.659	.101	.758/.888			
435G > A (Leu145Leu)	.45	.47	.804	.756	.399	.388	.133		.879/.865		
IVS5+5289A>G	.41	.42	.969	1.00	.605	.597	.263			.914/.884	
IVS5+9020T>C	.39	.40	.909	.843	.544	.723	.103				.659/.901
IVS6+771A>G	.39	.40	.985	.949	.670	.536	.064				

EDT, extended transmission disequilibrium test; PDT, pedigree disequilibrium test; SNP, single nucleotide polymorphism.

SNP	IVS3+258T>C	IVS4-2244T>G	435G>A	IVS5+5289A>G	IVS5+9020T>C	IVS6+771A>G
IVS3+258T>C		1.00 (1.00)	0.885 (0.878)	1.00 (1.00)	1.00 (1.00)	1.00 (1.00)
IVS4-2244T>G	1.00 (1.00)		0.883 (0.874)	1.00 (1.00)	1.00 (1.00)	1.00 (1.00)
435G>A	0.597 (0.602)	0.595 (0.600)		0.893 (0.879)	0.892 (0.877)	0.879 (0.872)
IVS5+5289A>G	0.935 (0.921)	0.935 (0.928)	0.645 (0.653)		1.00 (1.00)	1.00 (1.00)
IVS5+9020T>C	1.00 (1.00)	1.00 (1.00)	0.604 (0.603)	0.935 (0.928)		1.00 (1.00)
IVS6+771A>G	1.00 (1.00)	1.00 (1.00)	0.587 (0.589)	0.932 (0.910)	1.00 (1.00)	

Figure 2. Pairwise linkage disequilibrium estimations between single nucleotide polymorphisms (SNPs) in *FZD3*. Upper-right diagonal shows standardized D' values, and the lower left shows r^2 (squared correlation coefficient) in control and schizophrenia (in parentheses) groups.

using Fisher's Exact Test. Comparisons of gene expression were performed using Mann–Whitney U tests.

Results

None of the three SNPs (435G>A, IVS5+5289A>G, and IVS5+9020*T*>C) that displayed preferential transmissions to patients in Chinese families (Yang et al 2003a) exhibited significant transmission disequilibrium in our family samples, either by pedigree disequilibrium test (for all families) or extended transmission disequilibrium (for 168 independent and complete trios; Table 1), nor did the three additional SNPs (IVS3+258*T*>C, IVS4–2244*T*>G, and IVS6+771A>G) newly analyzed in this study (Table 1). Moreover, haplotype transmission analysis found no SNP-based haplotypes that were preferentially transmitted to schizophrenia patients (Table 1). Our family sample and those of Yang et al (2003a) had power of .71 and .76, respectively, to detect significant associations, based on models assuming that the genotypic relative risk is 1.5 with an additive inheritance mode. Within the same assumption, our case–control panel had a power of .98.

Genotypic distributions of six SNPs in all our samples displayed Hardy–Weinberg equilibrium. In case–control analysis, no significant differences existed between schizophrenia patients and control subjects in allelic or genotypic distributions of the six SNPs or the three SNP-based haplotypes (Table 1). No evidence indicated population stratification in our case–control samples [$Pr\,(K = 1) > .99$].

Pairwise LD was calculated between SNP markers in case–control samples (Figure 2). All D' measures were >.8 and r^2 measures were >.5, suggesting that the six SNPs display strong LD to each other and are on the same LD block (Abecasis et al 2001; Nakajima et al 2002). These results are concordant with the HapMap data (http://www.broad.mit.edu/personal/jcbarret/haploview/index.php).

Expression of *FZD3* in the DLPFC, an important brain region in terms of schizophrenia pathology (Cannon et al 2002), did not differ significantly between schizophrenic and control brains (Figure 3), although a possible modulatory effect of medication cannot be excluded.

Discussion

Evidence for potential disturbances of Wnt signaling pathways in schizophrenia has accumulated (Cotter et al 1998; Emamian et al 2004; Lijam et al 1997; Miyaoka et al 1999); however, our results do not support prior association results for Wnt molecular component FZD3, in either family- or population-based designs. Polymorphisms in the promoter region could alter gene expression, but we observed no differences in levels of *FZD3* expression between schizophrenic and control brains. One possible reason for this discrepancy may be differences in extent of LD around the *FZD3* locus between Chinese and Japanese populations. The association signals detected by Yang et al (2003a) might be due to nearby genes that are in strong LD with *FZD3* but not present in our Japanese sample. Yang et al (2003b) also reported associations between Neuregulin 1 gene (*NRG1*) on 8p21 and schizophrenia; however, risk haplotypes of *NRG1* and *FZD3* are unlikely to exist on the same LD block, because the two genes are about 3 Mb apart, too far to preserve substantial LD. Chinese and Japanese populations are genetically close (Saitou 1995), and this does not favor differing LD structures as a cause of discrepancy. Another possibility may be heterogeneous etiologies in schizophrenia. Meiotic drive can cause preferential transmission of particular genetic variants (Zollner et al 2004); therefore it would be important to exclude this phenomenon in their study by examining transmissions to healthy offspring.

Katsu et al (2003) reported associations between schizophrenia and IVS3+258*T*>C, but not 435G>A, in *FZD3* in a Japanese sample. Those results may be inconsistent because the two polymorphisms are in close LD to each other in Japanese populations. Excluding possible statistical fluctuations due to small sample sizes or population stratification in their study would thus be important.

In conclusion, we found no major genetic contribution of *FZD3* to risk for schizophrenia.

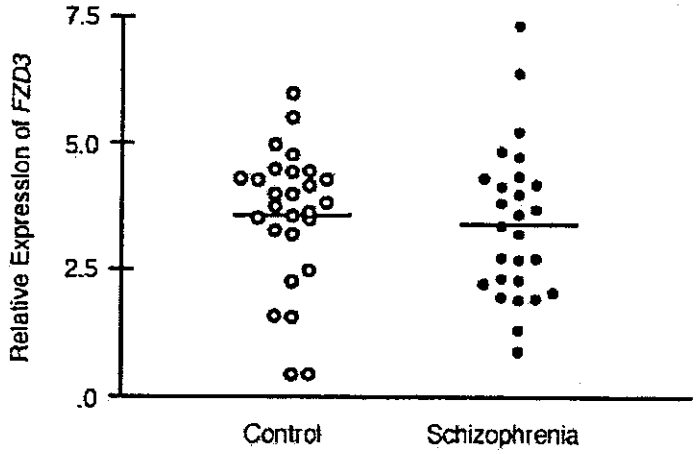

Figure 3. *FZD3* mRNA levels in postmortem brains (Brodmann's area 46) from schizophrenic and control subjects. Expression level of *FZD3* is normalized against that of β2-microgbulin. Horizontal bars indicate means. No significant differences in expression were found between schizophrenic and control subjects ($p = .416$).

This work was supported by RIKEN Brain Science Institute Funds, Research on Brain Science Funds from the Ministry of Health Labor and Welfare, and CREST funds from the Japan Science and Technology Agency, Japan. Postmortem brains were donated by the Stanley Medical Research Institute's Brain Collection, courtesy of Drs. Knable, Torrey, Webster, Weis, and Yolken.

Abecasis GR, Noguchi E, Heinzmann A, Traherne JA, Bhattacharyya S, Leaves NI, et al (2001): Nucleotide diversity and haplotype structure of the human angiotensin gene in two populations. *Am J Hum Genet* 70:108–123.

Blouin JL, Dombroski BA, Nath SK, Lasseter VK, Wolyniec PS, Nestadt G, et al (1998): Schizophrenia susceptibility loci on chromosomes 13q32 and 8p21. *Nat Genet* 20:70–73.

Cannon TD, Thompson PM, van Erp TG, Toga AW, Poutanen VP, Huttunen M, et al (2002): Cortex mapping reveals regionally specific patterns of genetic and disease-specific gray-matter deficits in twins discordant for schizophrenia. *Proc Natl Acad Sci U S A* 99:3228–3233.

Clayton D (1999): A generalization of the transmission/disequilibrium test for uncertain-haplotype transmission. *Am J Hum Genet* 65:1170–1177.

Cotter D, Kerwin R, al-Sarraji S, Brion JP, Chadwich A, Lovestone S, et al (1998): Abnormalities of Wnt signalling in schizophrenia–evidence for neurodevelopmental abnormality. *Neuroreport* 9:1379–1383.

Dudbridge F (2003): Pedigree disequilibrium tests for multilocus haplotypes. *Genet Epidemiol* 25:115–121.

Emamian ES, Hall D, Birnbaum MJ, Karayiorgou M, Gogos JA (2004): Convergent evidence for impaired AKT1-GSK3beta signaling in schizophrenia. *Nat Genet* 36:131–137.

Gurling HM, Kalsi G, Brynjolfson J, Sigmundsson T, Sherrington R, Mankoo BS, et al (2001): Genomewide genetic linkage analysis confirms the presence of susceptibility loci for schizophrenia, on chromosomes 1q32.2,5q33.2, and 8p21-22 and provides support for linkage to schizophrenia, on chromosomes 11q23.3-24 and 20q12.1-11.23. *Am J Hum Genet* 68:661–673.

Katsu T, Ujike H, Nakano T, Tanaka Y, Nomura A, Nakata K, et al (2003): The human frizzled-3 (FZD3) gene on chromosome 8p21, a receptor gene for Wnt ligands, is associated with the susceptibility to schizophrenia. *Neurosci Lett* 353:53–56.

Lijam N, Paylor R, McDonald MP, Crawley JN, Deng CX, Herrup K, et al (1997): Social interaction and sensorimotor gating abnormalities in mice lacking Dvl1. *Cell* 90:895–905.

Martin ER, Monks SA, Warren LL, Kaplan NL (2000): A test for linkage and association in general pedigrees: The pedigree disequilibrium test. *Am J Hum Genet* 67:146–154.

Miyaoka T, Seno H, Ishino H (1999): Increased expression of Wnt-1 in schizophrenic brains. *Schizophr Res* 38:1–6.

Nakajima T, Jorde LB, Ishigami T, Uemura S, Emi M, Lalouel J-M, et al (2002): Nucleotide diversity and haplotype structure of the human angiotensin gene in two populations. *Am J Hum Genet* 70:108–123.

Pleasure SJ (2001): An arrow hits the Wnt signaling pathway. *Trends Neurosci* 24:69–71.

Pritchard JK, Stephens M, Donnelly P (2000): Inference of population structure using multilocus genotype data. *Genetics* 155:945–959.

Purcell S, Cherny SS, Sham PC (2003): Genetic Power Calculator: Design of linkage and association genetic mapping studies of complex traits. *Bioinformatics* 19:149–150.

Saitou N (1995): A genetic affinity analysis of human populations. *Hum Evol* 10:17–33.

Sham PC, Curtis D (1995): An extended transmission/disequilibrium test (TDT) for multi-allele marker loci. *Ann Hum Genet* 59:323–336.

Sheldahl LC, Park M, Malbon CC, Moon RT (1999): Protein kinase C is differentially stimulated by Wnt and Frizzled homologs in a G-protein-dependent manner. *Curr Biol* 9:695–698.

Weinberger DR (1995): From neuropathology to neurodevelopment. *Lancet* 346:552–557.

Yamada K, Iwayama-Shigeno Y, Yoshida Y, Toyota T, Itokawa M, Hattori E, et al (2004): Family-based association study of schizophrenia with 444 markers and analysis of a new susceptibility locus mapped to 11q13.3. *Am J Med Genet* 127B:11–19.

Yang J, Si T, Ling Y, Ruan Y, Han Y, Wang X, et al (2003a): Association study of the human FZD3 locus with schizophrenia. *Biol Psychiatry* 54:1298–1301.

Yang JZ, Si TM, Ruan Y, Ling YS, Han YH, Wang XL, et al (2003b): Association study of neuregulin 1 gene with schizophrenia. *Mol Psychiatry* 8:706–709.

Zollner S, Wen X, Hanchard NA, Herbert MA, Ober C, Pritchard JK (2004): Evidence for extensive transmission distortion in the human genome. *Am J Hum Genet* 74:62–72.

Eur J Clin Pharmacol (2004) 60: 553–557
DOI 10.1007/s00228-004-0792-6

PHARMACOGENETICS

Kazushi Sawamura · Yutaro Suzuki · Toshiyuki Someya

Effects of dosage and CYP2D6-mutated allele on plasma concentration of paroxetine

Published online: 3 September 2004

Abstract *Objective*: We investigated the effect of dosages of paroxetine and cytochrome P450 (CYP) 2D6 genotypes on the plasma concentration of paroxetine in Japanese patients being treated with paroxetine.
Methods: Blood samples were collected from 73 individuals after at least 2 weeks of the same daily dose of paroxetine. The plasma paroxetine concentration was measured using HPLC, and the CYP2D6 genotypes were identified by PCR. Genotype groups were compared by one-way analysis of variance at different paroxetine doses.
Results: The mean plasma paroxetine concentrations at daily doses of 10, 20, 30, and 40 ng/ml were 6.6±7.4, 34.9±26.8, 74.8±37.2, and 130.5±96.8 ng/ml, respectively, showing a disproportionate and nonlinear increase in plasma drug levels of paroxetine upon increasing doses. Plasma paroxetine concentrations in patients with *CYP2D6*10* alleles were significantly higher than those without **10* allele at 10 mg/day (7.3±6.11 vs. 2.99±3.52 ng/ml), but there was no significant difference between **1/*1*, **1/*10* and **10/*10* genotypes at the higher doses. Similarly, patients with *CYP2D6*5* alleles showed higher plasma paroxetine concentrations than those without **5* allele, although differences in the plasma paroxetine concentration did not reach statistical significance level because of the small number of subjects with **5* alleles.
Conclusions: Our results indicate the possibility of saturation in paroxetine metabolism with an increase in paroxetine dose, and that *CYP2D6*10* allele(s) have significant impact on plasma paroxetine concentration at low doses in Japanese population.

K. Sawamura · Y. Suzuki · T. Someya (✉)
Department of Psychiatry,
Niigata University Graduate School of Medical and Dental Sciences, 757 Asahimachidori-ichibancho, 951-8510 Niigata, Japan
E-mail: someya@med.niigata-u.ac.jp
Tel.: +81-25-2272209
Fax: +81-25-2270777

Keywords Paroxetine · Plasma concentration · *CYP2D6*10*

Introduction

Paroxetine is a selective serotonin reuptake inhibitor, which is widely used in Japan for the treatment of depression and panic disorders. Paroxetine is metabolized mainly by cytochrome P450 (CYP)2D6 [1], but another enzyme with lower affinity for the drug may also be involved [2]. It is well known that there are many mutant alleles of *CYP2D6* that affect its enzymatic activity. The frequency of these mutant alleles differs between different ethnic groups, resulting in ethnic variations in the enzymatic activity of CYP2D6 [3]. The major *CYP2D6* mutated alleles causing absent enzymatic activity in white populations are *CYP2D6*3* (**3*), *CYP2D6*4* (**4*), and *CYP2D6*5* (**5*), with frequencies of 2.3%, 21.5%, and 4%, respectively [4]. However, in Asian populations, allele frequencies of **3*, **4*, and **5* have been reported to be 0%, 0.4%, and 5.7%, respectively [5]. On the other hand, *CYP2D6*10* (**10*), which causes decreased and unstable enzymatic activity, is found at a much higher frequency in Asians (51%) than in whites (2.8%) [4, 5]. Several reports suggest a significant impact of *CYP2D6*5* and *CYP2D6*10* alleles on drug metabolism in Japanese psychiatric population. Our previous study showed that *CYP2D6*5* and **10* alleles affect the metabolism of haloperidol [6], and Shimoda et al. [7] reported that plasma concentrations of amitriptyline were higher in those with *CYP2D6*5* and **10* alleles than those without these alleles. Furthermore, Yoon et al. [8] reported that extensive metabolizers with the *CYP2D6*10B* allele seem to have higher plasma concentrations of paroxetine than extensive metabolizers with the wild-type *CYP2D6* genotype in the Korean population.

On the other hand, Sindrup et al. [9], reported a disproportionate increase in plasma drug levels in the

majority of patients upon increasing doses, suggesting a possibility of saturation of CYP2D6 in the pharmacokinetics of paroxetine. Since the frequency of *CYP2D6* mutated allele in the Japanese population is differs from that in the white population, the effect of CYP2D6 on paroxetine metabolism may be different in Japanese than in whites.

We report the characteristic dose-concentration relationship of paroxetine and the effects of *CYP2D6* mutant alleles **5* and **10* on the plasma paroxetine concentration in Japanese patients. This is the first study of paroxetine pharmacokinetics in Japanese population.

Materials and methods

Patients

This study was approved by the gene ethics committee of Niigata University School of Medicine. The subjects received an explanation of the objectives of the study, and only those who gave written consent to participate in the study were enrolled. The subjects were 73 Japanese inpatients and outpatients being treated with paroxetine in the Department of Psychiatry, Niigata University Hospital (26 men, 47 women; mean 39.9 ± 15.4 years, range 13–73). Patients concurrently being treated with other drugs, except some benzodiazepines were excluded; benzodiazepine metabolism is thought to involve mainly enzymes other than CYP2D6 [10, 11], and that benzodiazepine has essentially little effect. Among the 73 patients 49 were using several types of benzodiazepine. Also patients with obvious physical illness were excluded. No patients were being treated with St. Johns wort, and no subjects were taking over-the-counter medications.

Blood sampling

Blood samples were collected after at least 2 weeks of the same daily dose of paroxetine to ensure that all patients study had steady-state plasma paroxetine concentrations. Venous blood (7 ml) was collected in blood collection tubes containing EDTA-Na as the anticoagulant 12 h after the last dose. The samples were stored frozen in a deep freezer, and after centrifugation at 3000 *g* for 10 min the plasma and the cellular fractions were stored frozen at −80°C until analysis.

Determination of drug plasma concentration

The plasma paroxetine concentration was measured using column-switching high-performance liquid chromatography (HPLC) with ultraviolet detection, as developed by Hikida et al. [12]. Drugs in the plasma, to which cisapride had been added as an internal standard, were extracted with hexane-chloroform. The extract was subjected to an automated column-switching HPLC using a hydrophilic metaacrylate polymer column for sample clean-up, and a reversed-phase column for separation. The lowest limit of detection was 0.5 ng/ml, and the interassay coefficient of variation was less than 5% at concentrations of 1 ng/ml for paroxetine.

CYP2D6 genotyping

To determine the *CYP2D6* genotype DNA was isolated from the peripheral blood of all subjects using a QIAamp Blood Kit (Qiagen, Calif., USA). The normal gene *CYP2D6*1* and the mutations *CYP2D6*2* and **10* were identified by the polymerase chain reaction (PCR) method described by Johansson et al. [5] while the *CYP2D6*5* allele was identified by the long PCR method of Steen et al. [13]. Genotyping for the defective *CYP2D6*3* and **4* alleles has carried out by PCR with specific primers [14]. When no mutations were found, the allele was defined as **1*.

Statistical analysis

The statistical analysis was conducted by SPSS II (for Windows). Comparisons were made among genotype groups by one-way analysis of variance at paroxetine doses of 10, 20, 30, and 40 mg/day. Bonferroni's test was carried out in the post-hoc analysis. The level of significance was set at less P values less than 0.05.

Results

Relationship between paroxetine dose and plasma paroxetine concentration

We observed an exponential regression curve between paroxetine dose and mean plasma paroxetine concentration (Fig. 1). The mean plasma paroxetine concentration at daily doses of 10 ($n=32$), 20 ($n=40$), 30 ($n=28$), and 40 ng/ml ($n=29$) was 6.6 ± 7.4, 34.9 ± 26.8, 74.8 ± 37.2 , and 130.5 ± 96.8 ng/ml, respectively. Plasma paroxetine concentration at 20 mg/day was approx. fivefold higher than that at 10 mg/day, and at 40 mg/day it was approx. fourfold higher than at 20 mg/day. There were no significant sex-related differences in plasma paroxetine level at any dose.

On the other hand, mean plasma paroxetine level in older subjects was higher than that in younger one at each dose. The mean plasma concentration of paroxetine in subjects aged 60 years or older at 10, 20 30, 40 mg/day was 12.2 ± 6.1, 52.2 ± 33.0, 87.7 ± 58.3, 184.6 ± 157.2 ng/ml, respectively; the corresponding concentrations in younger subjects were 6.1 ± 7.4, 31.9 ± 24.9, 72.7 ± 33.9, and 121.8 ± 85.3 ng/ml, respectively. The difference was statistically significant only at 40 mg ($P=0.013$), not at the lower doses because of wide variation in the plasma concentration of paroxetine.

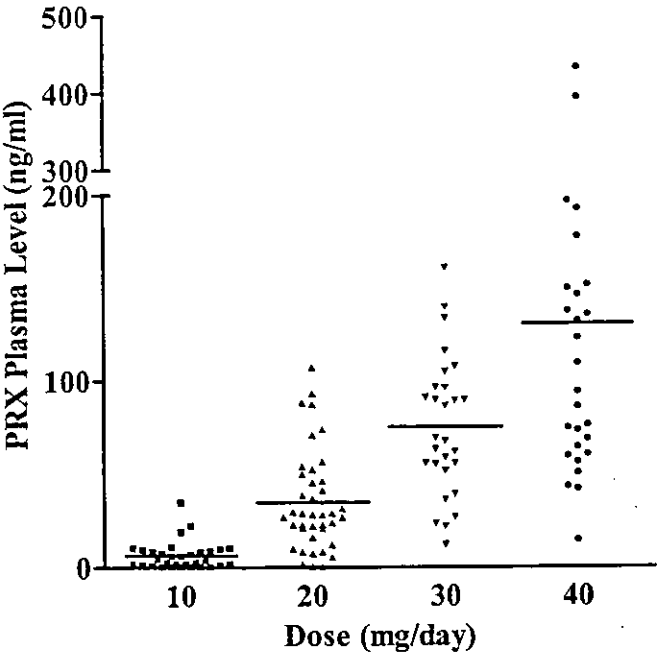

Fig. 1 Relationship between daily dose of paroxetine and steady-state plasma concentration of paroxetine: $y = 0.0678x^2 + 0.7247x - 7.179$, $r = 0.45$, $P < 0.0001$

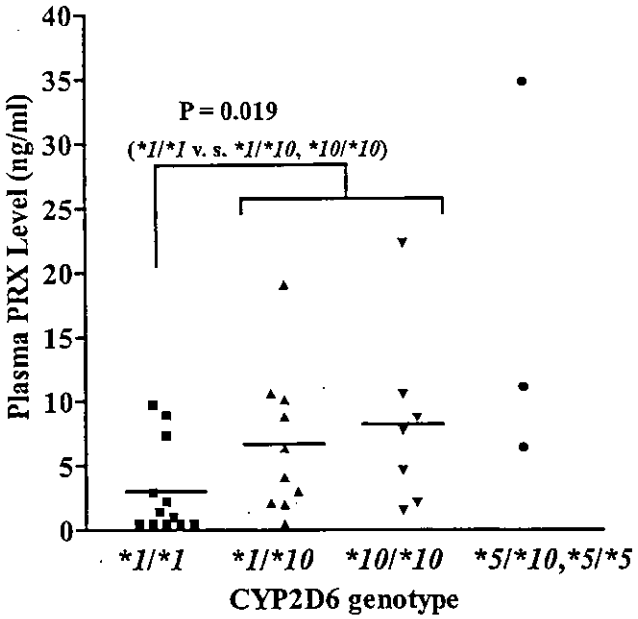

Fig. 2 Relationship between CYP2D6 genotype and steady-state plasma concentration of paroxetine in the 10 mg/day group

Relationship between the *CYP2D6* genotype and plasma paroxetine concentration

In the subjects studied, nine *CYP2D6* genotypes were identified; *CYP2D6*1/CYP2D6*1* ($n = 16$), *CYP2D6*1/CYP2D6*2* ($n = 9$), *CYP2D6*1/CYP2D6*5* ($n = 1$), *CYP2D6*1/CYP2D6*10* ($n = 22$), *CYP2D6*2/CYP2D6*2* ($n = 2$), *CYP2D6*2/CYP2D6*10* ($n = 6$), *CYP2D6*5/CYP2D6*5* ($n = 1$), *CYP2D6*5/CYP2D6*10* ($n = 3$), and *CYP2D6*10/CYP2D6*10* ($n = 13$). Allele frequencies were 43.8% for *CYP2D6*1*, 13.0% for *CYP2D6*2*, 4.1% for *CYP2D6*5*, and 39.0% for *CYP2D6*10*. *CYP2D6*3* and **4* alleles were not detected in this study. We regarded **2* allele as **1* allele, because some studies have reported that **2*×1 did not alter or only slightly decreased enzymatic activity, although **2*×N ($n \geq 2$) revealed a remarkable increase in enzymatic activity [15, 16, 17].

We compared **1/*1*, **1/*10*, **10/*10*, and those with one or two **5* mutated allele(s) at each dose. We statistically analyzed the effect of **10* allele on plasma paroxetine concentration. Plasma paroxetine concentrations in patients with **10* alleles (7.30 ± 6.11 ng/ml) were significantly higher than those without **10* allele (2.99 ± 3.52 ng/ml) at 10 mg/day ($t = -1.837$, df = 14.6, $P < 0.05$; Fig. 2). There were no significant differences between **1/*1*, **1/*10*, **10/*10* at any of the other doses. As shown in Fig. 2, plasma paroxetine concentrations in patients with **5* alleles showed a tendency to be higher than those without **5* alleles in the group receiving 10 mg/day paroxetine, although the difference was not statistically significant because of the very small number of subjects with **5* allele.

Discussion

Previous studies have reported that oxidation of paroxetine is catalyzed by a main pathway mediated by CYP2D6 and secondary pathway presumably mediated by another enzyme such as CYP3A4, with lower affinity for paroxetine [1, 2]. We investigated only the effect of CYP2D6 on paroxetine metabolism. Sindrup et al. [9], reported the relationship between dose and plasma concentration of paroxetine in 16 patients with diabetic peripheral neuropathy being treated with paroxetine. They classified the patients on the basis of sparteine metabolic ratio into four groups, fast extensive metabolizer (EM; ratio 0.1), intermediate EM (0.4), slow EM (6.4), and poor metabolizer (PM) and studied the correlation between paroxetine dose and plasma paroxetine concentration. Plasma paroxetine concentrations at 10, 20, 30, and 40 mg/day were 5, 10, 25, and 40, respectively, in fast EM, and 15, 55, 100, and 150 ng/ml in intermediate EM. There were exponential nonlinear curves between paroxetine dose and plasma paroxetine concentration in both phenotypes. Our results generally agree with these curves, and mean paroxetine concentrations were close to their data of plasma paroxetine concentration in intermediate EM. This probably reflects that there are high frequencies of those with decreased and unstable CYP2D6 enzymatic activity, while very low frequencies of those with no CYP2D6 enzymatic activity in the Japanese population.

There are some possible factors which may have contributed to the nonlinearity. As Sindrup et al. [9] suggested, one possible consideration is the saturation of CYP2D6 metabolic capacity. Since CYP2D6 enzymatic activity seems easily to saturate upon increasing

paroxetine dose [9, 18], the curvilinear increase in plasma paroxetine concentration shown also in this study can be explained by this saturation phenomenon. Another possibility is self-inhibition of paroxetine metabolism. Since paroxetine itself inhibits the enzymatic activity of CYP2D6 [4], self-inhibition of paroxetine may cause nonlinearity of plasma paroxetine concentration upon increasing doses. In any case, the characteristic pharmacokinetic profile of paroxetine is clinically very important, regardless of saturation or self-inhibition.

Subjects aged 60 years or older showed higher plasma concentrations than younger subjects at each of the four doses, but the difference was significant only at 40 mg. Regarding CYP2D6 activity [19] age is not thought to affect enzymatic activity, and at relatively low doses (10, 20, 30 mg) age had no effect on CYP2D6 while at the highest dose (40 mg), enzyme(s) other than CYP2D6 may play a larger role. However, regarding the genotype distribution of *CYP2D6* mutant allele there were one **1/*1* elderly and 20 **1/*1* nonelderly patients. There was a skewing in the distribution in the *CYP2D6* mutant allele, suggesting that the significant difference between the elderly and nonelderly groups was not due to aging but to genotype effect. To clarify this further studies are still needed with increased number of patients studied.

We investigated the effects of **10* allele on plasma paroxetine concentration, and found that subjects with **10* allele had significantly higher plasma paroxetine concentrations than subjects without **10* allele at 10 mg/day; there were no significant differences at the other doses. This suggests the saturation phenomenon of CYP2D6 and the involvement of a number of other metabolic enzyme(s). Since CYP2D6 has high affinity and low capacity for its substrates and becomes saturated at relatively low concentrations [20], CYP2D6 enzymatic activity is readily saturated by increasing dose, and the effects of the **10* allele may not be detected at doses higher than 10 mg. On the other hand, paroxetine itself inhibits the CYP2D6 enzymatic activity, and the plasma concentration of paroxetine increases in a nonlinear manner as dose increases. Thus the effect of *CYP2D6*10* on the plasma concentration of paroxetine may be easier to detect at low doses than at higher doses. With a larger sample size, it should be feasible to detect differences at doses other than 10 mg.

Yoon et al. [8] compared the total area under the curve (ng/ml per hour) after a single dose of 40 mg paroxetine and reported a significant difference between the **1/*1* group and the **10/*10* group. In contrast, we compared the trough levels of the plasma concentration of paroxetine in a steady state between the **1/*1* group and the **10* allele group and observed a significant difference in plasma paroxetine concentration only at 10 mg. We believe that these discrepancies may be based on the method difference between our data and their data.

Furthermore, plasma paroxetine concentrations in patients with **5* alleles showed higher tendency than those without **5* allele in the group receiving the low dose (10 mg/day). This suggests that *CYP2D6*5* allele plays an important role at this dose. We previously reported that persons with *CYP2D6*5* allele have higher plasma concentrations of haloperidol than those without **5* alleles [6]. Similarly, *CYP2D6*5* allele may play an important role also in determining plasma paroxetine concentration in the Japanese population. However, in groups receiving 20, 30, or 40 mg/day, *CYP2D6*5* alleles did not show any clear tendency on plasma paroxetine concentration, perhaps because the role of CYP2D6 on pharmacokinetics of paroxetine is not significant at higher doses.

As show in Fig. 2, there were three outlyers with high plasma concentrations of paroxetine (**1/*10*, **10/*10*, **5/*5*). In the comparison between the **1/*1* group and the **1/*10* and **10/*10* groups, even when one outlyer each is excluded from **1/*10* and **10/*10*, the difference remained significant ($P=0.04$). However, as shown Fig. 1, the plasma concentrations in the three outlyers are high in the 10 mg/day group but are not extremely high compared at those in the 20, 30, 40 mg groups. In fact, these patients did not have specific side effects or other different responses.

These findings show that the exponential curvelinear increase in plasma paroxetine concentration is also marked in Japanese individuals with increasing dosing of paroxetine. In addition, there was a significant effect of the *CYP2D6*10* allele on plasma paroxetine concentration at low doses, although clinical implication of this effect is not clear. Further studies involving larger numbers of patients are needed to clarify the extent of the involvement of *CYP2D6*5* and **10* allele on paroxetine metabolism.

Acknowledgements This research was supported by the Grant in Aid for Scientific Research (KAKENHI) from the Japan Society for the Promotion of Research (#13670991, #13671000, and #15591214).

Reference

1. Kaye CM, Haddock RE, Langley PF, Mellows G, Tasker TC, Zussman BD, Greb WH (1989) A review of the metabolism and pharmacokinetics of paroxetine in man. Acta Psychiatr Scand Suppl 350:60–75
2. Sindrup SH, Brosen K, Gram LF, Hallas J, Skjelbo E, Allen A, Allen GD, Cooper SM, Mellows G, Tasker TC et al (1992) The relationship between paroxetine and the sparteine oxidation polymorphism. Clin Pharmacol Ther 51:278–287
3. Bertilsson L, Dahl ML, Tybring G (1997) Pharmacogenetics of antidepressants: clinical aspects. Acta Psychiatr Scand Suppl 391:14–21
4. Bertilsson L, Dahl ML. 1996 Polymorphic drug oxidation. Relevance to the treatment of psychiatric disorders. CNS Drugs 5:200–223
5. Johansson I, Oscarson M, Yue QY, Bertilsson L, Sjoqvist F, Ingelman-Sundberg M (1994) Genetic analysis of the Chinese cytochrome P4502D locus: Characterization of variant *CYP2D6* genes present in subjects with diminished capacity for debrisoquine hydroxylation. Mol Pharmacol 46:452–459
6. Someya T, Shimoda K, Suzuki Y, Sato S, Kawashima Y, Hirokane G, Morita S, Yokono A, Takahashi S (2003) The

effect of CYP2D6 genotypes on the metabolism of haloperidol in a Japanese psychiatric population. Neuropsychopharmacology 28:1501–1505
7. Shimoda K, Someya T, Yokono A, Morita S, Hirokane G, Takahashi S, Okawa M (2002) Impact of CYP2C19 and CYP2D6 genotypes on metabolism of amitriptyline in Japanese psychiatric patients. J Clin Psychopharmacol 22:371–378
8. Yoon YR, Cha IJ, Shon JH, Kim KA, Cha YN, Jang IJ, Park CW, Shin SG, Flockhart DA, Shin JG (2000) Relationship of paroxetine disposition to metoprolol metabolic ratio and *CYP2D6*10* genotype of Korean subjects. Clin Pharmacol Ther 67:567–576
9. Sindrup SH, Brosen K, Gram LF (1992) Pharmacokinetics of the selective serotonin reuptake inhibitor paroxetine: nonlinearity and relation to the sparteine oxidation polymorphism. Clin Pharmacol Ther 51:288–295
10. Kronbach T, Mathys D, Umeno M, Gonzalez FJ, Meyer (1989) Oxidation of midazolam and triazolam by human liver cytchrome P4503A4. Mol Pharmacol 36:89–96
11. von Moltke LL, Greenblatt DJ, Cotreau-Bibbo MM, Harmatz JS, Shader RI (1994) Inhibitors of alprazolam metabolism in vivo: effect of serotonin-reuptake inhibitor antidepressants, ketoconazole and quinidine. Br J Clin Pharmacol 38:23–31
12. Hikida K, Inoue Y, Nouchiand E, Ohkura Y (1990) Determination of Etizolam in human Serum or Plasma Using Automated Cloumn-Switchig High-Performance Liquid Chromatography. Jpn J Clin Chem 19:354–359
13. Steen VM, Andreassen OA, Daly AK, Tefre T, Borresen AL, Idle JR, Gulbrandsen AK (1995) Detection of the poor metabolizer-associated *CYP2D6 (D)* gene deletion allele by long-PCR technology. Pharmacogenetics 5:215–223
14. Heim M, Meyer UA (1990) Genotyping of poor metabolizers of debrisoquine by allele-specific PCR amplification. Lancet 336 529–532
15. Johansson I, Lundqvist E, Bertilsson L, Dahl ML, Sjoqvist F, Ingelman-Sundberg M (1993) Inherited amplification of an active gene in the cytochrome P450 CYP2D locus as a cause of ultrarapid metabolism of debrisoquine. Proc Natl Acad Sci USA 90:11825–11829
16. Dahl ML, Johansson I, Bertilsson L, Ingelman-Sundberg M, Sjoqvist F (1995) Ultrarapid hydroxylation of debrisoquine in a Swedish population, Analysis of the molecular genetic basis. J Pharmacol Exp Ther 274:516–520
17. Tateishi T, Chida M, Ariyoshi N, Mizorogi Y, Kamataki T, Kobayashi S (1999) Analysis of the *CYP2D6* gene in relation to dextromethorphan O-demethylation capacity in a Japanese population. Clin Pharmacol Ther 65:570–575
18. Preskorn S (1994) Targeted pharmacotherapy in depression management: comparative pharmacokinetics of fluoxetine, paroxetine and sertraline. Int Clin Psychopharmacol Suppl 3:13–19
19. Suzuki A, Otani K, Mihara K, Yasui N, Kondo T, Tokinaga N, Furukori H, Kaneko S, Inoue Y, Hayashi K (1998) Effects of various factors including the CYP2D6 genotype and coadministration of flunitrazepam on the steady-state plasma concentrations of bromperidol and its reduced metabolite. Psychopharmacology (Berl) 135:333–337
20. Roh HK, Chung JY, Oh DY, Park CS, Svensson JO, Dahl ML, Bertilsson L (2001) Plasma concentrations of haloperidol are related to CYP2D6 genotype at low, but not high doses of haloperidol in Korean schizophrenic patients. Br J Clin Pharmacol 52:265–271

Effects of Concomitant Fluvoxamine on the Plasma Concentration of Etizolam in Japanese Psychiatric Patients

Wide Interindividual Variation in the Drug Interaction

Yutaro Suzuki, Yoshiaki Kawashima, Toshiki Shioiri, and Toshiyuki Someya

Abstract: Administration of fluvoxamine with concomitant benzodiazepines is common in clinical situations. This study investigated the effects of the coadministration of fluvoxamine on plasma concentrations of etizolam and evaluated the effects of various fluvoxamine doses on drug interactions with etizolam. Subjects were 18 Japanese outpatients concomitantly treated with fluvoxamine before or after monotherapy with etizolam. Plasma concentrations of etizolam were measured using a column-switching high-performance liquid chromatographic method with ultraviolet detection. In 17 subjects treated concomitantly with fluvoxamine at 25 mg or 50 mg, the ranges of plasma concentrations of etizolam corrected for the dose increased from 2.0–13.3 (mean 6.3 ± 3.6, n = 17) in monotherapy to 2.7–18.2 (mean 9.6 ± 5.1, n = 17) ng/mL/mg in concomitant doses. Wide variations were observed in the drug interactions; however, coadministration with fluvoxamine produced significant changes in the plasma concentrations of etizolam ($P < 0.0001$) with a median of 42.9% (range 0.0 to 235.0%). Although the sleepiness of the subjects was evaluated using the Stanford Sleepiness Scale, no changes in sleepiness were found between the etizolam-monotherapy and the fluvoxamine-concomitant states. Of the12 subjects treated concomitantly with fluvoxamine at 25 mg, 2 subjects received fluvoxamine at a dose increased up to 150 mg, and another received fluvoxamine at a dose increased up to 200 mg. They showed an increase in the plasma concentrations of etizolam in a fluvoxamine dose-dependant manner; more particularly, the increased dose of fluvoxamine (150 mg and 200 mg) resulted in about a twofold variation in plasma concentrations of etizolam.

Key Words: fluvoxamine, etizolam, drug-interaction, pharmacokinetics, Stanford Sleepiness Scale

(*Ther Drug Monit* 2004;26:638–642)

Received for publication December 9, 2003; accepted June 29, 2004.

From the Department of Psychiatry, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan.

Address correspondence and reprint requests to Toshiyuki Someya, MD, PhD, Professor and Chairman, Department of Psychiatry, Niigata University Graduate School of Medical and Dental Sciences, 757 Asahimachidori-ichibancho, Niigata 951-8510, Japan (e-mail: someya@med.niigata-u.ac.jp).

Etizolam, a thienodiazepine derivative, has been available in Japan since 1983 and is used not only for psychiatric disorders such as depression, anxiety disorders, and sleep disorders but also in internal medicine in treatment for various conditions such as psychosomatic diseases, cervical vertebral diseases, and muscle contraction headaches.[1] Etizolam is the most commonly used benzodiazepine (BZD) in Japan. The single-dose kinetic profile of etizolam suggests that the maximum plasma concentration is attained within 0.5–2 hours, and the mean elimination half-life averages 3.4 hours in healthy volunteers.[2] At steady state, plasma concentrations of the main active metabolite, α-hydroxyetizolam, were higher and disappeared more slowly (mean $t_{1/2}$ 8.2 hours) than those of the parent compound. Based on the kinetic characteristics of the parent drug and its metabolite, etizolam can be regarded as a short-acting BZD.[2]

Although the distributor suggests that several cytochrome P450 (CYP) isoforms may be involved in the metabolism of etizolam, there have been no reports on which types of CYP isoforms are involved. However, because etizolam has been marketed only in Italy and South Korea in addition to Japan, data on clinical studies showing the pharmacokinetics of etizolam are scarce. On the other hand, fluvoxamine is widely available in Europe and the United States and was introduced into clinical situations as the first approved selective serotonin reuptake inhibitor (SSRI) in Japan in 1999. Fluvoxamine is mainly used in treating depression but is also effective in treating anxiety disorders such as obsessive-compulsive disorder, panic disorder, and social phobia.[3] Therefore, many patients receive combined therapy of fluvoxamine and etizolam. In vitro data have confirmed that fluvoxamine is catalyzed to its O-demethylated metabolite by CYP1A2 and CYP2D6 and inhibits the activities of various CYP isoforms, such as CYP1A2, CYP2C19, and CYP3A4.[4] Indeed, many researchers have reported that fluvoxamine increased the plasma concentration of a concomitant drug through CYP inhibition.[4,5] Therefore, combined fluvoxamine and etizolam therapy may cause drug interactions. Since etizolam is used very frequently and the SSRIs now available are only fluvoxamine and paroxetine in Japan, whether etizolam can be used together with flu-

voxamine is a question of critical importance. Fleishaker et al reported that alprazolam, one of the BZD products as is etizolam, and fluvoxamine coadministration increased the plasma concentration of alprazolam by 100% and that this caused significantly greater impairment in psychomotor performance.[6] Furthermore, it was reported that fluvoxamine inhibited hydroxylation of alprazolam more potently than that of paroxetine.[7] It was also reported that diazepam, one of the BZD products, showed an extension of half-life on coadministration with fluvoxamine.[8] Several studies have been conducted concerning the drug interactions of fluvoxamine with other BZD drugs, but, to our knowledge, no studies have been reported about the interactions with etizolam.

Thus, we determined the effect of coadministered fluvoxamine on the plasma concentration of etizolam.

MATERIALS AND METHODS

Subjects

This study was conducted at the Niigata University Medical Hospital. The protocol was approved by the Ethics Committee of Niigata University Medical Hospital, and each subject was provided with written informed consent before enrollment. Subjects were 18 Japanese outpatients (7 men, 11 women), mean age ± SD was 35.4 ± 15.3 years, and body weight was 59.8 ± 18.7 kg. Although three subjects were previously smokers at the time of blood sampling, they had stopped smoking. Therefore, the three subjects were regarded as nonsmokers. All were orally treated with etizolam and fluvoxamine for psychiatric illnesses. Their diagnoses are panic disorder (n = 3), adjustment disorder (n = 4), eating disorder (n = 3), depressive disorder not otherwise specified (n = 3), personality disorder (n = 1), pain disorder (n = 1), obsessive-compulsive disorder (n = 1), somatoform disorder (n = 1), and anxiety disorder not otherwise specified (n = 1).

Protocol

Demographic data, medical histories, and laboratory data including hematology, serology, electrolytes, and urine analysis were collected from each patient. Patients with obvious physical illnesses were excluded from the present study. All subjects were concomitantly treated with fluvoxamine either before or after monotherapy with etizolam in response to their clinical symptoms. Concomitant medication except fluvoxamine and etizolam was not allowed. One subject received both fluvoxamine and etizolam, and then fluvoxamine was withdrawn, and 17 received etizolam, and then fluvoxamine was prescribed for their clinical conditions.

Patients were maintained on the same daily doses of etizolam and etizolam with fluvoxamine for at least 2 weeks to obtain steady-state concentrations of etizolam and fluvoxamine. Seven milliliters of venous blood was collected at the end of the period of etizolam monotherapy and again at the end of the period of receiving etizolam and fluvoxamine. Each patient took etizolam at the same daily dosage, and blood samples were taken at the same times during both etizolam monotherapy and concomitant therapy. Blood samples of 10 patients who had etizolam once daily were taken at 4 hours, and the samples of 8 patients who had etizolam two or three times daily were taken at 12 hours after the last dosage. All subjects took fluvoxamine at 21:00. Blood sampling was done using a Venoject® tube with EDTA-Na (Terumo Japan, Tokyo, Japan). Within 2 hours of collection, samples were centrifuged at 3000 *g*, and aliquots of plasma were drawn out in pipettes for determining plasma levels of fluvoxamine and etizolam, with samples stored at −80°C until assayed. At the time of blood sampling, the sleepiness of the subjects was evaluated using the Stanford Sleepiness Scale (SSS).[9]

Measurement of Plasma Levels of Etizolam and Fluvoxamine

We measured the plasma concentrations of etizolam and fluvoxamine using a column-switching high-performance liquid chromatographic method with ultraviolet detection. Etizolam was the product of Mitsubishi Pharma Corporation. The drug in plasma, to which cisapride had been added as an internal standard, was extracted with hexane-chloroform, and the extract was subjected to automated column-switching high-performance liquid chromatography (HPLC) using a TSK BSA-C8 precolumn (column I: 10 mm × 4.6 mm id, particle size 5 μm; Tosoh, Tokyo, Japan) for sample clean-up and a TSK gel ODS-80TS column (column II: 150 mm × 4.6 mm id, particle size 5 μm; Tosoh) for separation. The column-switching HPLC system was operated according to a timed program. Between 0 and 14.5 minutes after the sample injection, etizolam and fluvoxamine were separated from contaminating substances present in the extract on column I with a mobile phase (eluent A) of acetonitrile and 20 mmol/L potassium dihydrogen phosphate (13:87, vol/vol). Between 14.5 and 21 minutes after the injection, the etizolam and fluvoxamine retained on column I were eluted with a mobile phase (eluent B) of acetonitrile, 6 mol/L perchloric acid and 20 mmol/L potassium dihydrogen phosphate solution (41:0.07:48.93, vol/vol), and the effluent from column I was switched to column II. Then etizolam and fluvoxamine were separated on column II by elution with eluent B (between 21 and 40 minutes) (Fig. 1). The flow rates of eluents A and B were 1.2 and 0.6 mL/min, respectively. The column temperature was 30°C. The absorbance of the effluent from column II was monitored at 245 nm. The peak height ratio was used for the quantification of etizolam and fluvoxamine. The lowest limit of detection was 0.8 ng/mL, and the interassay coefficient of variation was less than 3% at a concentration of 16 ng/mL for etizolam and fluvoxamine.

Statistical Analysis

Statistical analysis was performed using SPSS for Windows 95. The paired *t* test was performed to evaluate the ef-

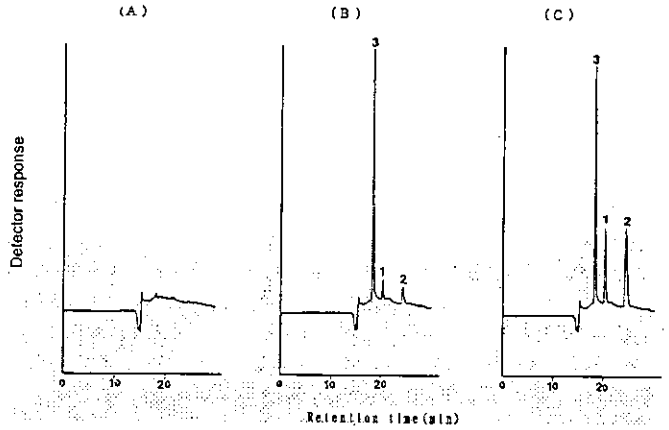

FIGURE 1. Chromatograms of extracts of a drug-free human serum (A) and serum spiked with Fluvoxamine (2ng/mL), Etizolam (2ng/mL) and Cisapride (70 ng/mL) (B) and serum spiked with Fluvoxamine (20 ng/mL), Etizolam (20 ng/mL) and Cisapride (70 ng/mL) (C). Peaks are : 1, Fluvoxamine; 2, Etizolam; 3, Cisapride.

fects of the coadministration of fluvoxamine on the plasma concentrations of etizolam. The level of significance was set at less than 0.05.

RESULTS

The daily doses of etizolam were 0.75–3.00 (1.60 ± 0.81) mg. Those of fluvoxamine were 25 mg in 12 subjects, 50 mg in 5, and 200 mg in 1. There was no relationship between body weight and plasma concentration of fluvoxamine in patients who took 25 mg of fluvoxamine.

In 17 subjects treated concomitantly with fluvoxamine at 25 mg or 50 mg, the ranges of plasma concentrations of etizolam corrected to the dose increased from 2.0–13.3 (mean 6.3 ± 3.6, n = 17) in monotherapy to 2.7–18.2 (mean 9.6 ± 5.1, n = 17) ng/mL/mg in concomitant treatment. Coadministration with fluvoxamine produced significant changes in the plasma concentrations of etizolam ($P < 0.0001$) with a median of 42.9% (range 0.0 to 235.0%) (Table 1). The range of plasma concentrations of fluvoxamine was 2.3–24.7 ng/mL (mean 9.6 ± 6.9, n = 17). Twelve subjects treated concomitantly with fluvoxamine at 25 mg showed a median change of etizolam plasma concentration of 39.0% (range 0.0 to 235.0%), and 5 subjects treated with fluvoxamine at 50 mg showed a median change of etizolam plasma concentration of 54.6% (range 35.2 to 185.3%). The differences between them were not statistically significant.

One subject treated concomitantly with fluvoxamine at 200 mg showed a great increase in plasma concentration of etizolam, from 1.8 ng/mL/mg in monotherapy to 8.9 ng/mL/mg in concomitant treatment (Table 1).

Effects of Various Fluvoxamine Doses on the Plasma Concentrations of Etizolam

Of the12 subjects treated concomitantly with fluvoxamine at 25 mg, 2 subjects had their fluvoxamine doses in-

TABLE 1. The Effect of Coadministration of Fluvoxamine on the Plasma Concentration of Etizolam

			Etizolam (Monotherapy)			Etizolam + Fluvoxamine					
						Etizolam				Fluvoxamine	
Patient	Age (y.o.)	Sex	Dose (mg)	Sampling Time (h Post Dose)	Concentration (ng/mL)	Dose (mg)	Sampling Time (h Post Dose)	Concentration (ng/mL)	Change in Etizolam Concentration (%)	Dose (mg)	Concentration (ng/mL)
1	36	F	1.00	4.0	12.8	1.00	4.0	18.1	41.4	25	18.9
2	27	F	3.00	4.0	30.3	3.00	4.0	33.5	10.6	25	9.8
3	39	F	1.00	4.0	9.9	1.00	4.0	14.9	50.5	25	11.9
4	45	F	1.00	12.0	4.4	1.00	12.0	4.7	6.8	25	2.3
5	47	F	1.00	12.0	7.9	1.00	12.0	17.1	116.5	25	5
6	16	F	1.00	12.0	8.0	1.00	12.0	10.7	33.8	25	10.9
7	42	M	1.50	4.0	20.0	1.50	4.0	27.3	36.5	25	4.2
8	67	M	1.00	4.0	2.0	1.00	4.0	6.7	235.0	25	2.3
9	22	M	3.00	4.0	9.5	3.00	4.0	11.2	17.9	25	6.1
10	22	F	1.50	12.0	4.1	1.50	12.0	4.1, 10.1	0.0, 146.3	25, 150	4.2, 53.1
11	73	F	1.50	12.0	6.8	1.50	12.0	12.3, 19.7	80.9, 189.7	25, 200	8.4, 132.2
12	27	M	3.00	4.0	19.6	3.00	4.0	28.1, 36.4	43.4, 85.7	25, 150	6.5, 102.7
13	33	F	3.00	4.0	11.9	3.00	4.0	18.4	54.6	50	6.5
14	20	F	1.00	4.0	4.2	1.00	4.0	6.0	42.9	50	24.7
15	32	M	0.75	4.0	5.4	0.75	4.0	9.9	83.3	50	11.8
16	30	M	1.50	12.0	3.4	1.50	12.0	9.7	185.3	50	23.8
17	24	M	1.50	12.0	7.1	1.50	12.0	9.6	35.2	50	5.6
18	36	F	1.50	12.0	2.7	1.50	12.0	13.3	392.6	200	77.3

creased to 150 mg, and another had a fluvoxamine dose increased to 200 mg. Blood samples were collected when the doses were increased to evaluate the effect of various fluvoxamine doses on the plasma concentration of etizolam. In these 3 subjects, the variance rates of etizolam plasma concentrations on coadministration of fluvoxamine at 25 mg were respectively 43.4, 0, and 80.9%, as compared with the rate obtained without coadministration of fluvoxamine. However, after fluvoxamine doses were increased, the rates rose to 85.7, 146.3, and 189.7%, respectively (Table 1).

Effects of Coadministration of Fluvoxamine on Sleepiness Evaluated by SSS

Although the sleepiness of the subjects was evaluated using the SSS, no changes in sleepiness were found between etizolam monotherapy and during the fluvoxamine concomitant therapy (n.s.). However, in one subject treated concomitantly with fluvoxamine at 200 mg and whose plasma concentration of etizolam rose by 392.6%, the SSS score increased from 2 to 4.

DISCUSSION

When fluvoxamine was coadministered at a relatively low dose of 25 mg or 50 mg, the plasma concentration of etizolam increased with a median of 42.9%. Because drug interactions of etizolam have not been reported in Japan or in other countries, and in view of the fact that fluvoxamine and etizolam are often coadministered in clinical practices, this finding is very important. There is a suggestion that CYP isoforms are involved in the metabolism of etizolam, but the details remain unclear.[1] Fluvoxamine is a moderate inhibitor of CYP3A4 and CYP2C9 but strongly inhibits CYP1A2 and 2C19[4]; therefore, the potential for drug interaction between etizolam and fluvoxamine via some CYP isozymes exists. Drug interactions with fluvoxamine were reported concerning alprazolam, another BZD product. Fleishaker and Hulst assessed the pharmacokinetic interaction between alprazolam and fluvoxamine and reported that fluvoxamine increased plasma alprazolam concentrations by 100% and that competitive inhibition caused these interactions.[6] Alprazolam is metabolized to two hydroxylated metabolites, 4-OH-alprazolam and α-OH-alprazolam. 4-OH-Alprazolam is the principal metabolite, and this pathway is mediated by CYP3A4.[10] In this study, we also considered that the competitive inhibition through some CYP isozyme such as CYP3A4 resulted in the increased plasma concentration of etizolam. Hassan et al reported that sertraline, another SSRI, did not affect the pharmacokinetics of alprazolam.[11] Selection of another SSRI is thus clinically important to avoid the increased plasma concentration of etizolam by coadministration of fluvoxamine. However, the drug interactions between fluvoxamine and etizolam varied greatly depending on subjects. Some subjects hardly underwent changes on coadministration with fluvoxamine at 25 mg or 50 mg, but others showed increased plasma concentration of etizolam of up to 235% (Table 1). Because fluvoxamine moderately inhibited CYP3A4 and CYP2C9,[4] we estimated that the coadministration with fluvoxamine should not greatly affect the pharmacokinetics of etizolam similar to alprazolam. However, some subjects were indeed greatly affected by the interaction, and their main enzymes in the metabolic pathways of etizolam might be the CYP isoforms other than CYP3A4 and CYP2C9. We previously reported the drug interactions of haloperidol with chlorpromazine, suggesting that the drug interactions varied greatly depending on patients and that CYP2D6 polymorphism was effective in estimating the variance.[12] Further, we reported that coadministration of fluvoxamine significantly increased the plasma concentrations of alprazolam compared with alprazolam monotherapy, and wide variations were observed in the drug interactions, with CYP2C19 genotype possibly being related to these interactions.[13] The large variations of fluvoxamine–etizolam interactions may be also related to CYP genetic polymorphisms which produce different enzyme activity. In addition, although the time of blood sampling was the same in each patient with and without fluvoxamine, the different timing between patients (4 versus 12 hours after etizolam ingestion) may account, at least in part, for the large interindividual difference in the degree of interaction (Table 1).

In daily clinical practice, fluvoxamine is rarely used at a low dose of 25–50 mg but rather at doses of 150–200 mg.[3] Thus, we evaluated the variation in the plasma concentrations of etizolam by giving the increased doses of fluvoxamine to the three subjects who were on coadministration with fluvoxamine at 25 mg (Table 1). The target subjects were small in number, just three patients, but they showed an increase in the plasma concentration of etizolam in a fluvoxamine-dose-dependant manner; more particularly, the increased dose of fluvoxamine (150 mg or 200 mg) resulted in about a twofold variation in the plasma concentration of etizolam. Therefore, some clinical measures may be required, for example, a decreased dose of etizolam, when fluvoxamine is to be concomitantly used at a high dose.

Although Fleishaker and Hulst reported that the increased plasma concentration of alprazolam with concomitant fluvoxamine resulted in a significantly greater reduction in psychomotor performance,[6] no changes in sleepiness were found between etizolam monotherapy and fluvoxamine concomitant by therapy evaluation using SSS in this study. Our results indicated that the patients were not aware, at least as subjective symptoms, of the effects of etizolam–fluvoxamine interactions. However, because SSS is a simple scale of sleepiness and consists of only 7 stages, it may be possible for SSS to clearly evaluate the effects of changes in plasma concentrations of etizolam on psychomotor performance. It is known that there are wide interindividual differences of the clinical effect of BZD. Therefore, coadministration of fluvoxamine

may have a serious effect on the patients who are sensitive to etizolam through the increase of the concentration.

CONCLUSION

Coadministration of fluvoxamine significantly increased the plasma concentration of etizolam compared with etizolam monotherapy. It was demonstrated that the extent of the drug interactions depended on the dose of fluvoxamine coadministered. However, wide variations were observed in the drug interactions. Further investigations are needed to clarify what caused these wide variations.

ACKNOWLEDGMENTS

This research was supported by the Grant in Aid for Scientific Research (KAKENHI) from the Japan Society for the Promotion of Research (JSPS) (#13670991 and #13671000).

REFERENCES

1. Mitsubishi Pharma Corporation. *Prescribing information for DEPAS.* 2002.
2. Fracasso C, Confalonieri S, Garattini S, et al. Single and multiple dose pharmacokinetics of etizolam in healthy subjects. *Eur J Clin Pharmacol.* 1991;40:181–185.
3. Benjamin JS, Virginia AS. *Kaplan & Sadock's Pocket Handbook of Psychiatric Drug Treatment, 3rd ed.* Philadelphia: Lippincott Williams &Wilkins, 2001.
4. Greenblatt DJ, von Moltke LL, Harmatz JS, et al. Drug interactions with newer antidepressants: Role of human cytochromes P450. *J Clin Psychiatry.* 1998;59(suppl 15):19–27.
5. Jefferson JW. Drug interactions—friend or foe? *J Clin Psychiatry.* 1998; 59(suppl 4):37–47.
6. Fleishaker JC, Hulst LK. A pharmacokinetic and pharmacodynamic evaluation of the combined administration of alprazolam and fluvoxamine. *Eur J Clin Pharmacol.* 1994;46:35–39.
7. von Moltke LL, Greenblatt DJ, Court MH, et al. Inhibition of alprazolam and desipramine hydroxylation in vitro by paroxetine and fluvoxamine: comparison with other selective serotonin reuptake inhibitor antidepressants. *J Clin Psychopharmacol.* 1995;15:125–131.
8. Perucca E, Gatti G, Cipolla G, et al. Inhibition of diazepam metabolism by fluvoxamine: a pharmacokinetic study in normal volunteers. *Clin Pharmacol Ther.* 1994;56:471–476.
9. Hoddes E, Zarcone V, Smythe H, et al. Quantification of sleepiness: a new approach. *Psychophysiology.* 1973;10:431–436.
10. von Moltke LL, Greenblatt DJ, Cotreau-Bibbo MM, et al. Inhibitors of alprazolam metabolism in vitro: effect of serotonin-reuptake-inhibitor antidepressants, ketoconazole and quinidine. *Br J Clin Pharmacol.* 1994;38: 23–31.
11. Hassan PC, Sproule BA, Naranjo CA, et al. Dose-response evaluation of the interaction between sertraline and alprazolam in vivo. *J Clin Psychopharmacol.* 2000;20:150–158.
12. Suzuki Y, Someya T, Shimoda K, et al. Importance of the cytochrome P450 2D6 genotype for the drug metabolic interaction between chlorpromazine and haloperidol. *Ther Drug Monit.* 2001;23:363–368.
13. Suzuki Y, Shioiri T, Muratake T, et al. Effects of concomitant fluvoxamine on the metabolism of alprazolam in Japanese psychiatric patients: interaction with CYP2C19 mutated alleles. *Eur J Clin Pharmacol.* 2003; 58:829–833.

Establishment of New Cloned Enzyme Donor Immunoassays (CEDIA®) for Haloperidol and Bromperidol

Norio Yasui-Furukori, Manabu Saito,†‡ Hanako Furukori,†§ Yoshimasa Inoue,¶ Toshiyuki Someya,‖ Sunao Kaneko,† and Tomonori Tateishi**

Abstract: The authors have developed and verified the precision and accuracy of new automated cloned enzyme donor immunoassays (CEDIA®) for haloperidol and bromperidol, and cross-validations have been performed with conventional semiautomated EIA kits (MARKIT®-M) and high-performance liquid chromatographic (HPLC) methods. The CEDIA® method provides a quick (about 10 minutes) assay for haloperidol or bromperidol, requiring no serum/plasma pretreatment or predilution. The CEDIA® haloperidol/bromperidol assay showed little or no cross reactivity with either their metabolites or many drugs commonly coprescribed. MARKIT®-M revealed considerable cross reactivity values proportional to the spiked amounts of reduced metabolites. Precision, accuracy, recovery, and linearity testing for the CEDIA® assay were all sufficient for clinical use. Significant linear correlations were found between CEDIA® and HPLC in measuring haloperidol (CEDIA® = 1.06 × HPLC + 0.869; n = 44, rs = 0.913, $P < 0.001$) and bromperidol (CEDIA® = 1.06 × HPLC + 0.606; n = 56, rs = 0.914, $P < 0.001$) concentrations. This study has, therefore, demonstrated that the CEDIA® assay has a quick run time with high precision and accuracy, and this method is a useful tool for the TDM of haloperidol or bromperidol.

Key Words: haloperidol, bromperidol, CEDIA®, HPLC, TDM

(*Ther Drug Monit* 2004;26:336–341)

Atypical antipsychotic agents have been commonly prescribed in the treatment of psychotic disorders, polypharmacotherapy of atypical antipsychotic agents is a growing international phenomenon, and total dose in chlorpromazine equivalents is increasing.[1] Therefore, reevaluation of monopharmacotherapy with conventional antipsychotics, haloperidol and bromperidol, is necessary. A significant concentration–effect relationship for haloperidol in the treatment of schizophrenia has been demonstrated; ie, optimal therapeutic response occurs in the plasma concentration range of 5–12 ng/mL, although side effects increase when the plasma concentration exceeds 12 ng/mL.[2] On the other hand, bromperidol has a similar chemical structure to haloperidol. The overall efficacy of bromperidol has been reported to be similar to or slightly better than that of haloperidol.[3,4] Bromperidol may have a faster onset of action and a more activating effect than haloperidol.[5] Consequently, this drug has been widely used in Japan as well as in Europe and other countries. Our previous study showed that mean plasma bromperidol concentration in patients with 50% or more reduction in positive symptoms was significantly higher than that in the other patients.[6] In addition, it has been suggested that efficacy of bromperidol reaches a plateau at a plasma drug concentration of 12 ng/mL.[7] Therefore, information about plasma drug concentration of haloperidol or bromperidol is of clinical importance.

Therapeutic drug monitoring (TDM) services for plasma concentrations of several drugs determined by enzyme immunoassay (EIA) methods are widely available, and high-performance liquid chromatography (HPLC) methods are available for a greater variety of drugs. Generally, commercial EIA kits for TDM are more rapid and convenient but are a less precise method than HPLC. In Japan, commercial EIA kits are used for TDM of haloperidol and bromperidol. However, our previous report showed that these EIA methods had poor reliability characterized by high precision but poor accuracy for haloperidol and bromperidol, compared with a HPLC method.[8] Therefore, we developed and verified the precision and accuracy of new cloned enzyme donor immunoassay (CEDIA®) methods for haloperidol and bromperidol with a higher specificity for parent compounds. We also performed cross-validations between the CEDIA® and conventional commercial EIA kits and a HPLC method used in our laboratory.

METHODS AND MATERIALS

Reagents

Haloperidol, bromperidol, and their reduced metabolites were kindly donated by Mitsubishi Pharma (Fukuoka, Japan).

Received for publication June 5, 2003; accepted November 26, 2003.

From the Departments of *Clinical Pharmacology and †Neuropsychiatry, Hirosaki University School of Medicine, Hirosaki, Japan; ‡Department of Psychiatry, Hirosaki-Aiseikai Hospital, Hirosaki Japan; §Department of Psychiatry, Kuroishi-Akebono Hospital, Hirosaki, Japan; ¶Pharmaceutical Technology Division, Mitsubishi Pharma, Fukuoka, Japan; and ‖Department of Psychiatry, Niigata University Graduate School of Medical and Dental Sciences, Japan.

Reprints: Norio Yasui-Furukori, MD, PhD, Department of Clinical Pharmacology, Hirosaki University School of Medicine, Hirosaki 036-8562 Japan (e-mail: yasufuru@cc.hirosaki-u.ac.jp).

Enzyme acceptor solution is composed of 0.47 mg/L mouse monoclonal anti-haloperidol/bromperidol antibody, 0.171 g/L enzyme acceptor, sodium potassium phosphate buffer (50 mmol/L, pH 7.0). Enzyme donor solution contains 35 μg/L enzyme donor conjugated to haloperidol/bromperidol, 1.67 g/L chlorophenol red-β-D-galactopyranoside, sodium potassium phosphate buffer (50 mmol/L, pH 7.0).

Immunoassay

The principle for this CEDIA® method is based on the use of β-galactosidase, an enzyme commonly used in enzyme-linked immunochemistry systems. Using recombinant DNA techniques, enzyme acceptors (EAs) and enzyme donors (EDs) were constructed. The CEDIA® homogeneous assay system operates by controlling the spontaneous assembly of the EDs and EAs through an antigen–antibody reaction. A hapten can be covalently attached to the EDs in such a way that there is no interference in forming active β-galactosidase enzyme when the EDs conjugate is mixed with EAs. Adding to the system an antibody to the hapten will inhibit the spontaneous assembly of the enzyme. Placing this system in competition for free analyte from the patient's serum/plasma will create active enzyme in direct proportion to the amount of free unknown analyte or hapten. The amount of enzyme created is monitored through the hydrolysis of an appropriate enzyme substrate such as chlorophenol red-β-D-galactopyranoside.

This method that requires very small volume of serum/plasma sample and no sample pretreatments or predilutions and is designed for enzyme kinetic analyzers capable of incubation at 37°C. It involves two pipetting steps and multiple absorbance reading at 546 and 660 nm. In this assay, 7 μL of patient's sample was mixed with 130 μL of EAs reagent for 5 minutes and 130 μL of EDs reagent for 4–5 minutes. This assay is performed using a Hitachi 917 automated clinical analyzer. Rate values (absorbance changes within various time periods) were used to construct standard curves and quantify haloperidol/bromperidol in the patient's sample.

Other Assay Conditions

As a conventional EIA method, commercial EIA kits (MARKIT®-M Haloperiol II and MARKIT®-M Bromperidol II, Dainippon Pharmaceuticals, Osaka, Japan) were used for determination of plasma concentrations of haloperidol and bromperidol. Limit of detection for each compound was 1.25 ng/mL, and CVs were less than 10%.

We used HPLC methods for haloperidol and bromperidol and their metabolites developed in our laboratory[9] with minor modification to detect plasma concentrations of haloperidol and bromperidol and their reduced metabolites simultaneously. The details of the procedures will be published elsewhere. In brief, all solvents used were of HPLC grade (Wako Pure Chemical Industries, Kyoto, Japan). All reagents were purchased from Wako Pure Chemical Industries (Kyoto, Japan). After sample alkalization with 500 μL of sodium hydroxide (2.5 M), 1000 μL of patient's plasma and internal standard, trifluperidol was extracted using 5.0 mL of chloroform–*n*-heptane (30:70, vol/vol). The organic phase was evaporated to dryness, and the residue was dissolved with 750 μL of mobile phase. Five hundred microliters of the solution was injected into a C_{18} STR ODS-II analytic column (5 μL, 150 × 4.6 mm ID) (Shinwa Chemical Industries, Ltd, Kyoto, Japan). The mobile phase consisted of phosphate buffer (0.02 M, pH 4.6), perchloric acid (6 M), and acetonitrile (54:1:45, vol/vol/vol) and was delivered at a flow rate of 0.6 mL/min. The peak was detected using a UV detector set at 215 nm. The method was validated for the concentration range 1–100 ng/mL, and good linearity ($r > 0.999$) was confirmed. Intra- and interday coefficients of variation for haloperidol, reduced haloperidol, bromperidol, and reduced bromperidol were less than 2.6%, 3.1%, 3.1%, and 2.5%, respectively, at the concentrations of 1, 10, and 20 ng/mL for the test compounds. Relative errors ranged from −5% to 10%, and mean recoveries were 96–100%. The limit of quantification was 1.0 ng/mL for each compound.

Subjects

The subjects were 100 patients (67 male and 33 female) receiving haloperidol or bromperidol (44 patients receiving haloperidol and 56 patients receiving bromperidol) and were diagnosed as schizophrenia according to the *Diagnostic and Statistical Manual of Mental Disorders,* ed IV, criteria.[10] Some of the subjects had also participated in our previous study.[8] The mean (and range) of age and body weight were 50 years (21–88 years) and 58 kg (38–98 kg), respectively. This study was approved by the Ethics Committee of Hirosaki University School of Medicine, and written informed consent was obtained from all patients. The doses of haloperidol and bromperidol were from 0.75 to 20 mg/d and from 1 to 34 mg/d, respectively. Concomitant medications were antipsychotics (chlorpromazine, levomepromazine, perphenazine, zotepine, risperidone, olanzapine, and quetiapine), antiparkinson agents (biperiden and trihexyphenydyl), benzodiazepines (diazepam, alprazolam, estazolam, etizolam, nitrazepam, flunitrazepam, quazepam, and brotizolam), mood stabilizers (lithium, valproate, and carbamazepine) as well as antiulcer drugs and laxatives. Blood samplings (6 mL) were performed 12–14 hours after previous night dosing of haloperidol or bromperidol. The plasma samples were frozen and kept at −20°C until analysis.

Statistics

Statistical analyses were performed by the use of Spearman Rank Test with SPSS software. All test were 2-tailed and were considered to be statistical significant if the *P* value was less than 0.05.

RESULTS

Cross Reactivity

In the CEDIA® methods for haloperidol and bromperidol, there was little or no cross reactivity observed with their metabolites or potential concomitant agents such as Parkinson disease drugs, antiepileptic agents, and other antipsychotic drugs (Table 1). However, one of the butyrophenone derivatives, moperone, which has a similar chemical structural to haloperidol and bromperidol, demonstrated considerable cross reactivity (Table 1).

TABLE 1. Cross Reactivity in CEDIA® Haloperidol/Bromperidol Assay

Compound	Concentration Added (ng/mL)	Cross-Reactivity (%)	
		Haloperidol	Bromperidol
Benperidol	500	6.2	5.6
Bromperidol	40	114	100
Reduced bromperidol	1,000	0.9	0.9
4-Fluorophenylpropionic	100,000	<0.01	<0.01
4-Fluorophenylacetic acid	100,000	<0.01	<0.01
4-Fluorophenylaceturic acid	100,000	<0.01	<0.01
Fluoropipamide	1,000	1.7	1.6
Haloperidol	40	100	97.5
Haloperidol decanoate	1,000	1.2	1.1
4-Hydroxy-4-(*p*-piperidine	100,000	<0.01	<0.01
Reduced haloperidol	1,000	1.7	1.5
Trifluperidol	1,000	3.1	2.9
Spiperone	1,000	2.1	1.9
Acetazolamide	100,000	<0.01	<0.01
Amantadine	100,000	<0.01	<0.01
Azaperone	1,000	<0.01	<0.01
Biperiden	100,000	<0.01	<0.01
Carbamazepine	100,000	<0.01	<0.01
Chlorpromazine	100,000	<0.01	<0.01
Domperidone	100,000	<0.01	<0.01
Ethosuximide	100,000	<0.01	<0.01
Ethotoin	100,000	<0.01	<0.01
Levomepromazine	100,000	<0.01	<0.01
Mephobarbital	100,000	<0.01	<0.01
Metharbital	100,000	<0.01	<0.01
Moperone	60	50.8	46.4
Nitrazepam	20,000	<0.01	<0.01
Phenobarbital	100,000	<0.01	<0.01
Phenytoin	100,000	<0.01	<0.01
Pimozide	100,000	<0.01	<0.01
Primidone	100,000	<0.01	<0.01
Promethazine	100,000	<0.01	<0.01
Risperidone	100,000	<0.01	<0.01
Sulthiame	100,000	<0.01	<0.01
Thioridazine	100,000	<0.01	<0.01
Timiperone	500	4.9	4.5
Trihexyphenidyl	100,000	<0.01	<0.01
Trimethadione	100,000	<0.01	<0.01
Valproic acid	100,000	<0.01	<0.01
Zotepine	100,000	<0.01	<0.01

The CEDIA® haloperidol assay had almost 100% cross reactivity with bromperidol, and vice versa (Table 1).

The response of cross reactivity with their reduced metabolites increased proportionally with the amount of reduced metabolites added to matrix when assayed by the MARKIT®-M haloperidol/bromperidol assays, whereas little cross reactivity was demonstrated with up to 40 ng/mL of reduced metabolites of haloperidol and bromperidol when assayed by the CEDIA® methods (Fig. 1, Table 2). However, cross reactivity (%) with reduced metabolites was decreased in proportion to the amount of spiked reduced metabolites in MARKIT®-M haloperidol/bromperidol assays. Overall mean (and range) relative errors of parent compounds when adding reduced metabolites in MARKIT®-M were 38.1% (15.0–61.0%) for haloperidol and 35.9% (17.6–65%) for bromperidol, and the corresponding values were −0.5% (−13.0–3.1%) for haloperidol and 0.2% (−5–3.3%) for bromperidol.

Two patients receiving only risperidone additionally participated in this study. The MARKIT®-M Haloperidol II assay detected 2.1 and 1.9 ng/mL, whereas the CEDIA® haloperidol assay and HPLC method showed undetected levels. Then, we tried to confirm the cross reactivity with the major metabolite of risperidone, 9-hydroxyrisperidone. However, no cross reactivity with either 9-hydroxyrisperidone or risperidone within the therapeutic concentration range was found.

Precision, Accuracy, and Recovery

The results of measured precision and accuracy studies are shown in Tables 3 and 4. Intra- and interassay coefficients of validation were less than 3.6%. To calculate the recovery of haloperidol/bromperidol, varying amounts of either drug were added to drug-free samples. The percentage recovery was then determined by dividing the assayed value by the expected value (Table 4).

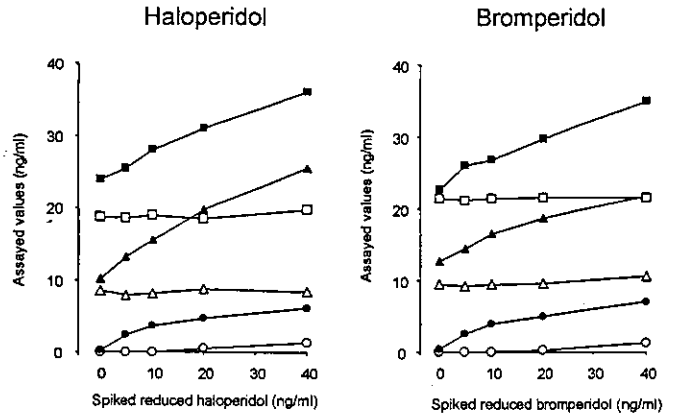

FIGURE 1. Cross reaction with reduced metabolites. Open circles, triangles, and squares show the values by CEDIA®, and closed circles, triangles, and squares show those by MARKIT®-M. Circles indicate the values with addition of various amounts of reduced metabolites but without added analytes. Triangles indicate those with addition of 10 ng/mL of analytes. Squares indicate those with addition of 20 ng/mL of analytes.

Linearity

To assess the linearity of haloperidol/bromperidol assays, the high-concentration calibrator was diluted with the low calibrator at 10% increments. Excellent linearity for haloperidol ($r > 0.999$) and bromperidol ($r > 0.999$) were found.

Sensitivity

The minimum detectable concentration of the CEDIA® haloperidol/bromperidol assay was 1.25 ng/mL. This value was determined by calculating the concentration of haloperidol/bromperidol that could give a response equal to 2 standard deviations above that of the lowest calibrator.

Cross Validation with Other Methods

Significant linear correlations were found between CEDIA and HPLC methods for haloperidol (CEDIA® = 1.06 × HPLC + 0.869; n = 44, rs = 0.913, $P < 0.001$) and bromperidol (CEDIA®= 1.06 × HPLC + 0.606; n = 56, rs = 0.914, $P < 0.001$) (Fig. 2). There were significant linear correlations between CEDIA® and MARKIT®-M methods for haloperidol (CEDIA® = 0.73 × MARKIT®-M + 0.037; n = 44, rs = 0.958, $P < 0.001$) and bromperidol (CEDIA® = 0.78 × MARKIT®-M − 0.039; n = 56, rs = 0.937, $P < 0.001$) (Fig. 2). However, corresponding values were deviated (slope was 0.73 for haloperidol and 0.78 for bromperidol). Although statistically significant, linear correlations were also biased between HPLC and MARKIT®-M for haloperidol (MARKIT®-M = 1.43 × HPLC + 1.25; n = 44, rs = 0.905, $P < 0.001$) and bromperidol (MARKIT®-M = 1.34 × HPLC + 1.18; n = 56, rs = 0.914, $P < 0.001$) (Fig. 2).

DISCUSSION

The newly developed CEDIA® assay provides a quick (about 10 minutes) assay for haloperidol/bromperidol, requiring no serum/plasma pretreatments or predilutions, when compared with conventional EIA kits (MARKIT®-M) (approximately 60 minutes). The results of precision, accuracy, recovery, and linearity testing for the CEDIA® haloperidol/bromperidol assay were all good enough for clinical use.

In cross validation with HPLC, which is regarded as the most reliable method to date, the CEDIA® had significant linear correlations with excellent accuracy for haloperidol and bromperidol over the concentration range tested. However, haloperidol/bromperidol concentrations determined by the CEDIA assays were deviated from those by MARKIT®-M assays. Haloperidol/bromperidol concentrations determined by MARKIT®-M assays were also biased from those using the HPLC method: MARKIT®-M assays demonstrated a 40–50%

TABLE 2. Comparison of Cross Reactivity With Reduced Metabolites Between CEDIA® and MARKIT®-M

	CEDIA®		MARKIT®-M	
	Assayed Values (ng/mL)	Cross Reactivity (%)	Assayed Values (ng/mL)	Cross Reactivity (%)
Reduced haloperidol (ng/mL)				
0	0.00	—	0.25	—
5	0.05	1.0%	2.38	47.5%
10	0.00	0.0%	3.70	37.0%
20	0.55	2.8%	4.75	23.8%
40	1.25	3.1%	6.15	15.4%
Reduced bromperidol (ng/mL)				
0	0.00	—	0.35	—
5	0.00	0.0%	2.60	52.0%
10	0.00	0.0%	4.00	40.0%
20	0.25	1.3%	5.05	25.3%
40	1.30	3.3%	7.05	17.6%

positive bias compared with the HPLC method or CEDIA® for measuring haloperidol or bromperidol over the concentration range tested. These differences may result from larger cross reactivity with reduced metabolites by the MARKIT®-M assay, and this was supported by in vitro studies showing that MARKIT®-M cross-reactivity values increased proportionally to the added amounts of reduced metabolites. Thus, some caution should be exercised when interpreting blood concentrations determined by MARKIT®-M.[11–14] Two patients receiving only risperidone additionally participated in this study. The MARKIT®-M Haloperidol II assay detected 2.1 and 1.9 ng/mL, whereas the CEDIA® haloperidol assay and HPLC method showed undetected levels. This finding suggests that there is some cross reactivity with risperidone and/or its metabolite(s) in the MARKIT®-M haloperidol assay. However, we failed to find cross reactivity with risperidone and its major metabolite, 9-hydroxyrisperidone, at therapeutic concentration ranges using the MARKIT®-M haloperidol assay. It is possible that MARKIT®-M has cross reactivity with metabolites other than 9-hydroxyrisperidone. Recently, risperidone has been widely prescribed in the treatment of psychiatric disorders. Therefore, careful consideration of drug concentration overestimated by MARKIT®-M is necessary when monitoring patients receiving risperidone in addition to haloperidol or bromperidol.

In conclusion, this new method (CEDIA®) is likely to have a quick run time with high precision and high accuracy and to be a useful tool for the TDM of haloperidol or bromperidol. Compared with conventional commercial EIA kits for haloperidol and bromperidol, the new (CEDIA®) assay

TABLE 3. Within-Run Precision and Between-Run Precision in CEDIA® Assays in Plasma From Patients Receiving Either Haloperidol or Bromperidol

	Within-Day (n = 20)	Between-Day (n = 6)
Haloperidol		
Sample 1	9.55 (3.30%)	9.60 (2.41%)
Sample 2	15.19 (2.37%)	15.10 (2.27%)
Sample 3	23.38 (1.67%)	24.09 (1.23%)
Bromperidol		
Sample 4	7.90 (3.62%)	8.47 (2.03%)
Sample 5	12.11 (2.69%)	13.07 (1.87%)
Sample 6	23.59 (1.47%)	23.97 (0.97%)

Data show mean (and CV) values (ng/mL).

TABLE 4. Accuracy and Recovery in CEDIA® Assays

Analyte	Relative Error (%) (n = 5)	Assayed Values (n = 5)	Expected Values (n = 5)	Recovery (%)
Haloperidol				
10 ng/mL	−1.12	9.69	9.80	98.86
20 ng/mL	−1.12	18.55	18.76	99.03
30 ng/mL	4.35	30.01	28.76	104.13
Bromperidol				
10 ng/mL	2.24	10.04	9.82	102.13
20 ng/mL	3.74	20.24	19.51	103.98
30 ng/mL	2.30	30.19	29.51	102.80