2. Subjects and methods

2.1. Patients

2.1.1. Case 1

A 61-year-old Japanese man was diagnosed with prostate cancer (stage T3b N_0 M_1) in August 2002. At age of 54 years, he was diagnosed with type 2 diabetes mellitus and advised by a dietitian to take a diet consisting of 1800 kcal/d: the percentages of each nutrient in the total calories were 50% to 60% of carbohydrates, 25% to 30% of fat, and 15% to 20% of protein. At that time, he was started on a α-glucosidase inhibitor acarbose (300 mg/d). Fasting plasma glucose levels remained 6.1 to 7.4 mmol/L and hemoglobin A1c (HbA1c) levels 5.3% to 6.4% (normal range, 4.3%-5.8%) during the previous 5 years, and thus, his glycemic control had been good. His height was 175.3 cm and body weight was 71.1 kg (body mass index 23.0 kg/m^2). For the treatment of prostate cancer, subcutaneous injection of leuprolide acetate (3.75 mg/mo) and oral administration of flutamide (250 mg/d) were started in September 2002. He did not change his dietary habits and his body weight did not change after the diagnosis of prostate cancer. He continued to work as usual and had no event that appeared to worsen his glycemic control, such as physiological or psychological stress, infections, and other medications. At 3 weeks after the second injection of leuprolide acetate, his glycemic control was found worse: fasting glucose and HbA1c levels were 18.2 mmol/L and 8.0%, respectively. He was administered nateglinide (270 mg/d), but fasting glucose and HbA1c reached 22.8 mmol/L and 10.5%, respectively, in December 2002 (Fig. 1). Serum testosterone and 17β-estradiol levels decreased to 0.45 nmol/L (control range in men, 10-35 nmol/L) and 40 pmol/L (control range in men, 70-110 pmol/L), respectively. After ceasing the administration of flutamide, we started injection of insulin lispro before each meal on admission. Plasma glucose levels declined on a maximal dose of 40 U/d at day 7 after insulin treatment, when we coadministered pioglitazone (15 mg/d). Thereafter, we could reduce the doses of insulin lispro. In February 2003, we stopped the insulin injection and increased the dosage of pioglitazone to 30 mg/d. We readministered flutamide and continued the injection of leuprolide acetate (11.25 mg per 3 months). In December 2003, fasting glucose levels remained below 7 mmol/L and HbA1c declined to 5.2% (Fig. 1).

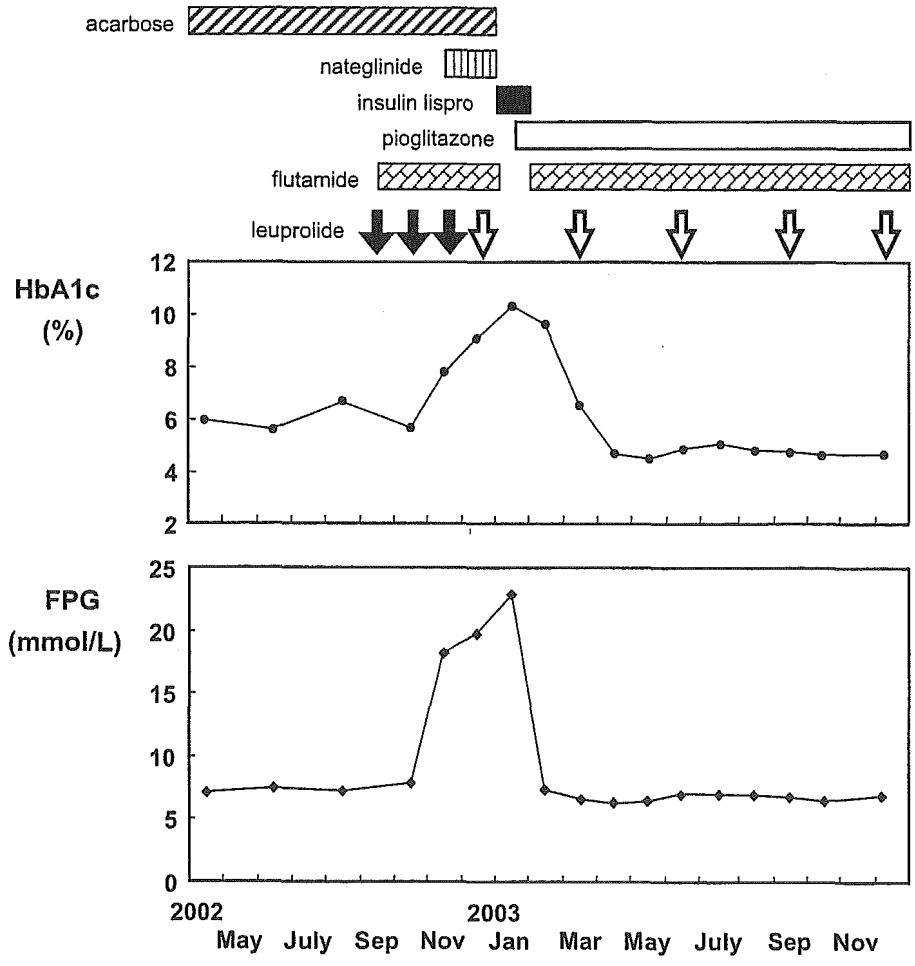

Fig. 1. Longitudinal changes of fasting plasma glucose (FPG) and HbA1c levels in case 1. Black arrows indicate subcutaneous injection of 3.75 mg leuprolide acetate; white arrows, 11.25 mg leuprolide acetate.

2.1.2. Case 2

An 81-year-old Japanese man was diagnosed with prostate cancer (stage T3a N_0 M_0) in June 2003. He has never been diagnosed with diabetes mellitus. Fasting glucose and HbA1c levels were 6.1 mmol/L and 5.1%, respectively, in April 2003. His height was 161.5 cm and body weight was 47.0 kg (body mass index 18.0 kg/m^2). The patient was started on subcutaneous injections of leuprolide acetate (3.75 mg/mo) for the treatment of prostate cancer in August 2003. After the third injection, the dose of leuprolide acetate was changed to 11.25 mg each 3 months. In March 2004, he complained of thirst and weight loss (−3.0 kg during 6 months); laboratory tests revealed marked hyperglycemia (19.4 mmol/L) and elevated HbA1c (9.9%) levels. Both serum testosterone and 17β-estradiol were undetectable (<0.42 nmol/L and <37 pmol/L, respectively). He was admitted to Osaka University Hospital and was given injections of insulin lispro before each meal. Plasma glucose concentration gradually decreased at a maximal dose of 24 U/d of insulin lispro (Fig. 2). By coadministration of pioglitazone (15 mg/d), we were able to reduce the dose of insulin lispro and thereafter stop the insulin treatment, followed by coadministration of the α-glucosidase inhibitor voglibose. The injection of leuprolide acetate (11.25 mg every 3 months) was continued. His glycemic control remained good in June 2004: fasting glucose was 6.7 mmol/L and HbA1c was 7.0%.

2.2. Control subjects

One hundred and forty-four Japanese men aged 58 to 68 years (60 ± 2 years, mean ± SD), who were confirmed to have normal glucose tolerance by the 75-g oral glucose tolerance test, were studied as control subjects. Their body mass index was 23.4 ± 2.5 kg/m^2.

2.3. Determination of pancreatic β-cell function and insulin sensitivity

Pancreatic β-cell function and insulin sensitivity were estimated by calculation from fasting plasma glucose and serum insulin levels using the HOMA method [4]. The

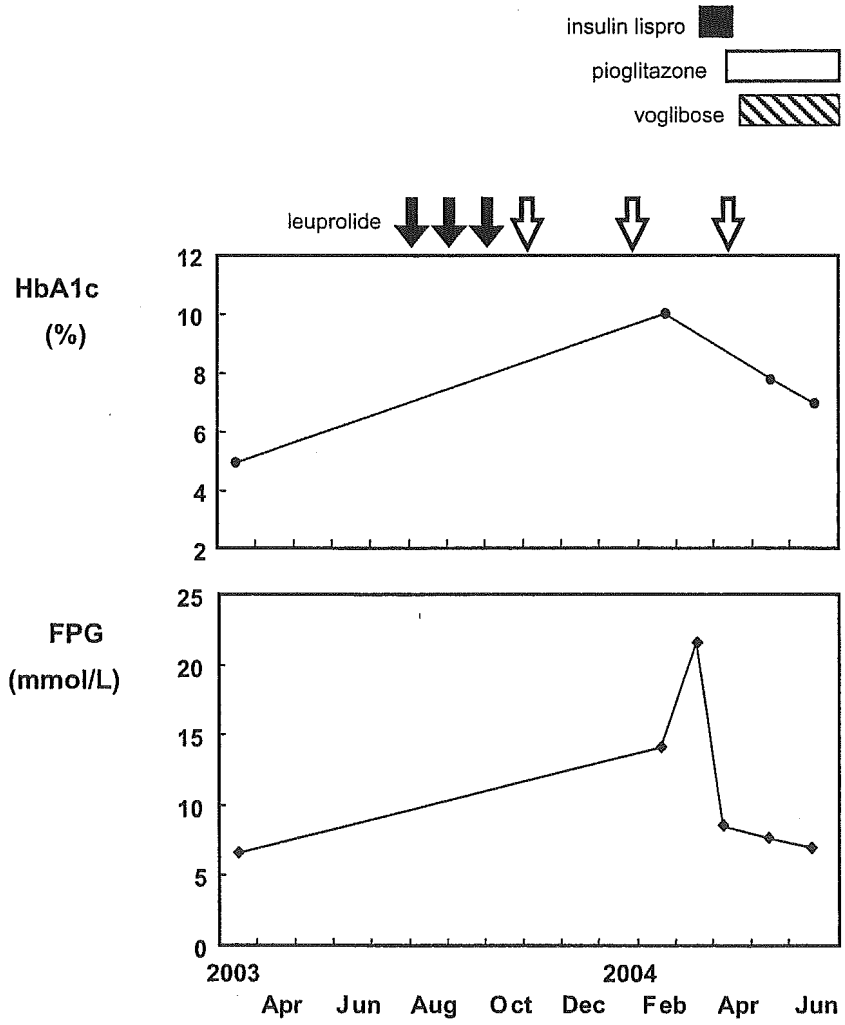

Fig. 2. Longitudinal changes of fasting plasma glucose (FPG) and HbA1c levels in case 2. Black arrows indicate subcutaneous injection of 3.75 mg leuprolide acetate; white arrows, 11.25 mg leuprolide acetate.

Table 1
Clinical and biochemical parameters of study subjects

	Fasting plasma glucose (mmol/L)	Fasting serum insulin (pmol/L)	HOMA-%β (%)	HOMA-%S (%)
Case 1				
December 2002	22.8	79	13	11
March 2003	6.3	43	58	100
June 2003	6.9	57	59	74
December 2003	7.1	50	51	84
Case 2				
March 2004	21.9	129	22	9
June 2004	6.7	34	43	125
Control subjects	5.5 ± 0.4	46 ± 17	78 ± 23	117 ± 52
Range	4.5-6.1	16-101	37-139	44-294

Control subjects are 144 Japanese men aged 58 to 68 years (60 ± 2 years) who have normal glucose tolerance. HOMA-%β and HOMA-%S were determined by the correct HOMA evaluation.

estimates of β-cell function and insulin sensitivity have been shown to correlate with estimates obtained by hyperglycemic and euglycemic clamp techniques, respectively [4]. The values for β-cell function (HOMA-%β) and insulin sensitivity (HOMA-%S) were assessed by the correct HOMA evaluation using a computer program reported by Levy et al [5].

3. Results

HOMA-%β and HOMA-%S were determined by the correct HOMA evaluation [5] in control subjects. Their HOMA-%β was 78% ± 23% (range, 37%-139%) and HOMA-%S was 117% ± 52% (range, 44%-294%).

Table 1 shows fasting glucose, fasting insulin, HOMA-%β, and HOMA-%S in 2 cases before and after treatments for diabetes mellitus. HOMA-%β and HOMA-%S in case 1 were 13% and 11%, respectively, before starting insulin treatment. Both estimates were below the ranges in the control subjects. After treatment with insulin followed by pioglitazone alone, HOMA-%β increased to 58% at 3 months, 59% at 6 months, and 51% at 12 months, which were within the control ranges (Table 1). HOMA-%S also increased to the ranges in the control subjects (100% at 3 months, 74% at 6 months, and 84% at 12 months). In case 2, HOMA-%β and HOMA-%S were 22% and 9% before treatment of insulin, both of which were below the control ranges. Both parameters increased to the control ranges (HOMA-%β 43%; HOMA-%S 125%) at 3 months after treatments with insulin followed by administration of pioglitazone and voglibose (Table 1).

4. Discussion

There is growing evidence indicating that sex hormones influence risk factors for diabetes mellitus. It has been shown that serum testosterone concentrations are inversely related to insulin resistance in men [6-8]. In addition, low levels of testosterone had been shown to predict the development of type 2 diabetes mellitus in men [9]. The effects of postmenopausal hormone replacement therapy are lower fasting glucose and insulin levels in nondiabetic women [10] and improved glycemic control in type 2 diabetic women [11], although disparate results had been reported [12,13]. Recently, the Heart and Estrogen/progestin Replacement Study (HERS) has shown that the postmenopausal hormone therapy reduced the incidence of diabetes mellitus [14]. Thus, androgens and estrogens may have favorable effects on glucose metabolism in men and in postmenopausal women, respectively.

The 2 male patients presented here demonstrated marked hyperglycemia after androgen-deprivation therapy for prostate cancer. Both cases had reduced pancreatic β-cell function as well as reduced insulin sensitivity, as determined by the correct HOMA evaluation. In case 1, a patient with type 2 diabetes mellitus whose glycemic control has been good using acarbose treatment alone was administered the GnRH agonist leuprolide acetate and the androgen receptor antagonist flutamide. Case 2, who had never been diagnosed with diabetes mellitus, was given leuprolide acetate but not antiandrogens. Leuprolide acetate causes a decrease in serum levels of androgens and estrogens. Thus, reduction of serum androgens and/or serum estrogens may contribute to marked hyperglycemia in these patients. The thiazolidinedione pioglitazone, an insulin-sensitizing drug, was very effective in maintaining good glycemic control after lowering of blood glucose levels by insulin therapy. These observations suggest that insulin resistance occurred in our patients with reduced β-cell function after androgen-deprivation therapy and it resulted in the exacerbation of hyperglycemia.

Coddington et al [15] reported a type 1 diabetic female patient who showed deterioration in glucose control after leuprolide treatment of endometriosis. This suggests the involvement of a decrease in serum estrogens in worsening of glycemic control. Furthermore, a male patient with aromatase deficiency, who had decreased serum estrogens but not androgens, has been shown to have insulin resistance [16]. In addition, androgens had been shown to impair insulin action in women [17]. Taken together, the reduction of serum estrogen levels rather than serum androgen levels may have lead to the reduction of insulin sensitivity, causing marked hyperglycemia in our cases.

At present, we have no information on the prevalence of alterations of glucose metabolism in prostate cancer patients who are given androgen-deprivation therapy, and this needs to be studied in the future. The onset of overt diabetes may depend on β-cell function as well as insulin sensitivity of the patients. Because androgen-deprivation therapy has become the mainstay of prostate cancer treatment, physicians should pay attention to carbohydrate metabolism in patients receiving androgen-deprivation therapy.

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Pharmacokinetic Evaluation of Anticonvulsants in a Patient with Porphyria

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The aim of this study was to establish the appropriate regimen of anticonvulsants for a female patient with porphyria by pharmacokinetic evaluation of the influence of anticonvulsants on porphyria. The pharmacokinetics of phenytoin, carbamazepine, clonazepam, and clobazam were estimated by the Bayesian method. The urinary 6β-hydroxycortisol/cortisol (6β-OHF/F) ratio was also evaluated as an index of hepatic CYP3A4 induction.

The phenytoin concentrations in the toxic area fitted the predicted value for *CYP2C9*1/*3* better than that for *CYP2C9*1/*1* (her genotype). The concomitant phenytoin altered the clearance of carbamazepine considerably. The clearances of clonazepam and clobazam were not altered, although hepatic CYP3A4 induction was implied from the value of the urinary 6β-OHF/F ratio.

From the pharmacokinetic evaluations, the following were concluded : (1) phenytoin was not the proper medication for this patient, (2) carbamazepine can be used safely within a relatively small dose, 500 mg/day, (3) the combination of clonazepam and carbamazepine can be used, and (4) a concomitant small dose of clobazam with carbamazepine can also be used.

Key words : anticonvulsant, porphyria, cytochrome P450, Bayesian method, urinary 6β-hydroxycortisol/cortisol ratio

Introduction

Although many anticonvulsants are known to deteriorate porphyria, some patients with porphyria have seizures and require anticonvulsant therapy. Part of the deteriorating mechanism has been conjectured to cause an imbalance of heme protein biosynthesis, which is due to the induction of cytochrome P450 (CYP) by anticonvulsants. We previously demonstrated that the measurement of the human urinary 6β-hydroxycortisol/cortisol (6β-OHF/F) ratio is a useful indicator of safe medication in a patient with hereditary coproporphyria[1]. The previous results implied that hepatic CYP induction was profoundly related to her condition of porphyria.

In the present paper, the pharmacokinetic parameters of the anticonvulsants used in the patient during two hospitalizations (phenytoin, carbamazepine, clonazepam, and clobazam) were retrospectively evaluated by the Bayesian method[2,3]. We established the effective and safe dose of anticonvulsants for her seizures based on the alteration of the estimated pharmacokinetic parameters and the measurement of the urinary 6β-OHF/F ratio.

Case

A female in her twenties was diagnosed with rare dual porphyria involving partial δ-aminolevulinate dehydratase deficiency with epilepsy. She had been treated with sodium valproate, phenytoin and carbamazepine when she was admitted to our hospital on June 24, 1998. Sodium valproate was discontinued because of abdominal side effects. For

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better control of the epilepsy, phenytoin was increased from 100 to 125 mg/day and carbamazepine was gradually increased from 800 to 1200 mg/day. However, her condition deteriorated, and the serum concentrations of phenytoin were within the toxic range. The frequency of her seizures decreased eventually by discontinuing phenytoin, gradually reducing carbamazepine, and adding clonazepam. She was discharged from our hospital after four months.

She was re-hospitalized 2 years later (March 22, 2001) for reevaluation of the medication for epilepsy. She had been treated with carbamazepine, clobazam, and zonisamide. Zonisamide was discontinued. The clobazam dose, 15 mg/day, was not changed. The carbamazepine dose was gradually increased from 450 to 500 mg/day. The frequency of her seizures decreased 2 weeks after receiving modified treatment. The urinary 6β-OHF/F ratio gathered over 24 hours was measured and compared with the value from before she left our hospital the first time[1].

Methods

The patient's genotype of the metabolic enzymes of phenytoin had been judged to be *CYP2C9*1/*1* and *CYP2C19*1/*2*[1]. The pharmacokinetic parameters of phenytoin were estimated from serum phenytoin concentrations in the toxic area, with the use of subpopulation parameters of *CYP2C9*1/*1* and *CYP2C9*1/*3*[4]. The typical predicted dose of phenytoin was calculated from equation (1) (below), based on these estimated parameters. The percentage of deviation between the predicted dose and the administered dose was calculated from equation (2) (below).

Each alteration of carbamazepine and clonazepam clearance was estimated to evaluate the pharmacokinetic influence of these agents on porphyria. In the second hospitalization, the alteration of carbamazepine and clobazam clearances was estimated. The alteration of the N-desmethylclobazam/clobazam ratio was also evaluated, because clobazam is metabolized to N-desmethylclobazam by CYP3A4. The original data used for this study were obtained as described previously[1].

Her pharmacokinetic parameters of anticonvulsants were estimated by the software PEDA (parameter estimation and dosage adjustment)[5] incorporating the Bayesian method, using the population pharmacokinetic parameters described in the literature[4,6-8]. The phenytoin dose was calculated by the following equation from the observed concentration of phenytoin :

$$Dose_{,pre} = V_{max}C_{ss}/(K_m + C_{ss}) \quad (1)$$

where V_{max}, K_m, $Dose_{,pre}$, and C_{ss} are the maximal elimination rate of the Michaelis-Menten equation (mg/day), the Michaelis-Menten constant (μg/mL), the serum concentration of phenytoin at a steady state (μg/mL), and the predicted dose (mg/day), respectively. The deviation between the dose predicted from equation (1) and the actual dose was calculated by the following equation :

$$Dev = (Dose_{,act} - Dose_{,pre})/Dose_{,act} \quad (2)$$

where Dev, $Dose_{,act}$, and $Dose_{,pre}$ are the deviation, the actual dose, and the predicted dose, respectively. The plasma concentrations of carbamazepine, clonazepam, and clobazam were calculated by the following equations :

$$C = D \cdot K_a/Vd/(K_a - K_e) \cdot \{Exp(-K_e t) - Exp(-K_a t)\} \quad (3)$$

$$C_{ss} = D/CL \quad (4)$$

where D, C, and C_{ss} are the dosage (mg), carbamazepine or clobazam concentration in plasma (μg/mL), and plasma clonazepam concentration at a steady state (μg/mL), respectively ; and K_e, K_a, Vd, t, and CL are the elimination rate constant (hr^{-1}), absorption rate constant (hr^{-1}), apparent volume of distribution normalized with bioavailability (L), time from initial administration (hr), and apparent total body clearance normalized with bioavailability (L/hr), respectively. From this point on, the term "clearance" will be used for "apparent total body clearance normalized with bioavailability". Equation (3) was used for carbamazepine and clobazam, and equation (4) was used for clonazepam.

Results

The pharmacokinetic parameters of phenytoin in *CYP2C9*1/*1* were estimated by the Bayesian method, as follows : $V_{max} = 3.8$ mg/kg/day and $K_m = 5.6$ μg/mL. The typical predicted dose of phenytoin was calculated to be approximately 141 mg/day. The percentage of deviation was approxi-

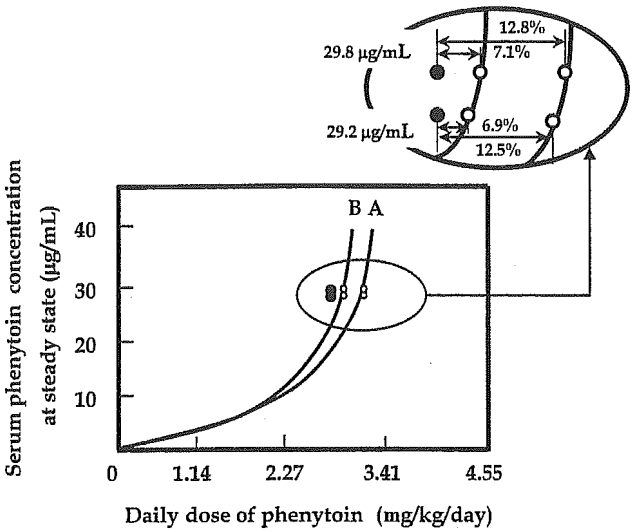

Fig. 1 Profile between serum concentration and dose of phenytoin

Curves A and B were the estimated curves from the subpopulation parameters[6] of *CYP2C9*1/*1* with *CYP2C19*1/*2* and *CYP2C9*1/*3* with *CYP2C19*1/*2*, respectively, by the Bayesian method. Closed circles (29.8, 29.2 μg/mL) represent serum phenytoin concentrations on July 31 and August 3, below 125 mg/day, respectively. Open circles are the predicted dose of phenytoin.

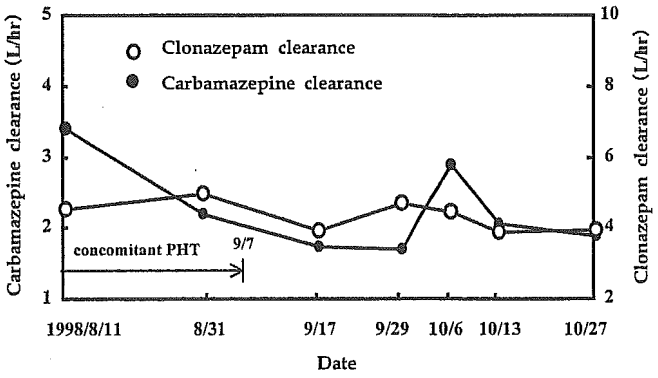

Fig. 2 Alteration of carbamazepine and clonazepam clearances

Open and closed circles represent carbamazepine and clonazepam clearances, respectively.

Table Alteration of the N-desmethylclobazam/clobazam ratio and the clearance of clobazam and carbamazepine

	April 3, 2001	May 11, 2001
Carbamazepine clearance (L/hr)	2.54	2.22
Clobazam clearance (L/hr)	2.25	2.25
Clobazam (μg/mL)	0.26	0.26
N-desmethylclobazam (μg/mL)	3.52	3.95
N-desmethylclobazam/clobazam ratio	13.8	15.49

mately 11%. The estimated parameters in *CYP2C9*1/*3* were also calculated, as follows: V_{max}=3.3 mg/kg/day and K_m=4.1 μg/mL. The typical estimated dose was approximately 129 mg/day. The percentage of deviation was about 3%. Therefore, the predicted dose for *CYP2C9*1/*3* better fits the model used [equation(1)] than that for *CYP2C9*1/*1* (Fig. 1).

Carbamazepine clearance was altered between 1.7 and 3.4 L/hr, whereas clonazepam clearance was almost unaltered (Fig. 2). Although the carbamazepine dose was decreased by 80%, from 1200 to 1000 mg/day, the plasma concentration increased by 1.2 times when carbamazepine was combined with phenytoin, in the relationship between the daily dose and plasma concentration of carbamazepine. The clearance decreased by 50% based on the alteration. However, clonazepam clearance did not show a remarkable alteration even when clonazepam was combined with carbamazepine and phenytoin.

The alteration of carbamazepine and clobazam clearances and the N-desmethylclobazam/clobazam ratio after re-hospitalization are shown in Table. The carbamazepine and clobazam clearances remained almost unaltered. The N-desmethylclobazam/clobazam ratio was unaltered. The measured value of the urinary 6β-OHF/F ratio was 20.2.

Discussion

1. Discrepancy between the patient's genotype and phenotype of phenytoin metabolism

The patient was an extensive metabolizer of CYP2C9, which is the main metabolizing enzyme of phenytoin[1]. Her genotype was *CYP2C9*1/*1*, whereas her phenotype as indicated by the behavior of her serum phenytoin concentration was identical to that of *CYP2C9*1/*3*. *CYP2C9*3* is generally known to be a genetic polymorphism that decreases the enzyme activity. In addition, she had no inhibitor of CYP2C9 in her medications. The reason underlying the discrepancy between her genotype and phenotype of phenytoin metabolism remains an

area of considerable interest. One proposal is that phenytoin as an inducing agent of CYP3A4 might have destroyed the equilibrium of her abnormal heme pathway and depleted CYPs. Her metabolism of phenytoin might have been saturated by the reduction of CYP synthesis.

2. Alteration of the clearance of carbamazepine and clonazepam

The decrease in carbamazepine clearance (Fig. 2) leads us to conjecture that the suppression of CYP biosynthesis was brought on by a mechanism similar to that of the metabolic saturation of phenytoin by the concomitant phenytoin. In addition, an increase in carbamazepine clearance was transiently observed (Fig. 2), which might have been due to a high blood concentration of carbamazepine resulting from the improvement of the equilibrium of CYP biosynthesis, because a month had passed after the discontinuation of phenytoin. The results suggest that phenytoin markedly affected CYP biosynthesis in this patient with porphyria. The influence on CYP might have caused saturation of phenytoin metabolism and an excessive decrease in carbamazepine clearance.

The clonazepam clearance (CL/F) was estimated to be approximately 4.3 L/hr as the mean value (Fig. 2). The total body clearance (CL_{tot}) is calculated to be 4.4 L/hr because the bioavailability of clonazepam is reported to be approximately 0.98[9]. The product of the value of unbound fraction and the hepatic intrinsic clearance was calculated to be 4.4 L/hr from equation (5) when the absorption ratio was regarded to be 1.0 according to the high bioavailability (see "Appendix"). The general value of hepatic blood flow is about 90 L/hr. Therefore, the hepatic extraction ratio of clonazepam was calculated to be approximately 0.05 from equation (7) (see "Appendix"). Clonazepam is considered to be a metabolic capacity-limited agent, according to the calculated value of the hepatic extraction ratio. Carbamazepine is a similar type agent. Carbamazepine clearance seems to be excessively affected by the alteration of hepatic CYP3A4 activity, whereas the clonazepam clearance was not altered in spite of concomitant phenytoin.

The metabolic pathway of clonazepam proceeds by nitroreduction, acetylation, and hydroxylation. The acetylation is reported to be affected by the polymorphic N-acetyltransferase (NAT) that determines the acetylation phenotype of the individual[10]. This patient was considered to be an intermediate acetylator since her genotype was *NAT2*4/*6*. There has been no report of a relationship between the NAT2 genotype and a phenotype of clonazepam pharmacokinetics. The influence of the NAT2 genotype on the alteration of clonazepam clearance was unclear.

Seree et al, suggested that the nitroreduction of clonazepam is catalyzed by *CYP3A4*[11]. Therefore, clonazepam metabolism might also be affected by the suppression of CYP biosynthesis (Fig. 2). However, the alteration of clonazepam and carbamazepine clearances was different. Binding plasma proteins of phenytoin, clonazepam, and carbamazepine is 90-95%, 80-90%, and 70-80%, respectively[12,13]. This result implies that the difference in the affinity ratio of binding to plasma proteins affects the alteration of these clearances.

3. Pharmacokinetic evaluation of anticonvulsants after the second hospitalization

The patient's medication after the second hospitalization was evaluated according to the alteration of carbamazepine clearance, clobazam clearance, and the N-desmethylclobazam/clobazam ratio (Table). The carbamazepine and clobazam clearances and N-desmethylclobazam/clobazam ratio were unaltered after re-hospitalization. However, the measured value of the urinary 6β-OHF/F ratio was slightly high compared with the value (15.4) before the patient left our hospital the first time[1].

Her condition had not improved with a carbamazepine dose of 450 mg/day (see "Case"), whereas the frequency of seizures decreased by increasing the carbamazepine dose to 500 mg/day. Carbamazepine was considered to be effective and safe at a dose of 500 mg/day. The dosage regimen after the second hospitalization was considered to have been appropriately modified as a result.

4. Conclusions

From the pharmacokinetic evaluations for this patient, the following conclusion was established : phenytoin was not a proper medication. Carbamazepine was effective and safe, within a moderate

dose. In addition, the clearance of clonazepam was not affected by carbamazepine. Therefore, the concomitant use of carbamazepine and clonazepam was also considered effective. Clobazam and carbamazepine might be used safely for controlling seizures of porphyria within relatively small doses, at 15 mg/day for the former and at 500 mg/day for the latter.

The influence of anticonvulsants on porphyria was pharmacokinetically evaluated by estimating the clearance of anticonvulsants, in addition to the measurement of urinary 6β-OHF/F. We obtained information on effective and safe medications for porphyria on the basis of estimation of pharmacokinetic parameters.

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Appendix

The total body clearance (CL_{tot}) for a drug is generally expressed as follows, when a drug is mostly eliminated in the liver :

$$CL_{tot}=f_{ub}\cdot CL_{int,h}/F_a \quad (5)$$

where F_a is the absorption ratio of the drug ; f_{ub} is the unbound fraction in the blood ; and $CL_{int,h}$ is the hepatic intrinsic clearance. Furthermore, the hepatic clearance (CL_h) and hepatic extraction ratio (ER_h) of drugs are expressed as follows :

$$CL_h=Q_h\cdot f_{ub}\cdot CL_{int,h}/(Q_h+f_{ub}\cdot CL_{int,h}) \quad (6)$$
$$ER_h=f_{ub}\cdot CL_{int,h}/(Q_h+f_{ub}\cdot CL_{int,h}) \quad (7)$$

where Q_h is the hepatic blood flow ; f_{ub} is the unbound fraction in the blood ; and $CL_{int,h}$ is the hepatic intrinsic clearance. A drug with an ER_h of more than 0.8 is classified as an agent of the hepatic flow-limited type[14,15]. A drug with an ER_h of less than 0.2 is classified as an agent of the metabolic capacity-limited type.

An oral drug absorbed in the gut passes the liver via the portal vein. Only a drug which passes to the liver flows in circulating blood. Therefore, such a drug can be described by the following equation :

$$AUC_{po}=F_h\cdot F_a\cdot AUC_{iv} \quad (8)$$

where F_h is the hepatic availability ; F_a is the fraction absorbed into the portal vein from the gut ; and AUC_{iv} and AUC_{po} are the areas under the blood concentration-time curve after intravenous bolus and oral dosing, respectively.

AUC_{po} is expressed as a function of dosage (D) and total body clearance after oral dosing ($CL_{tot,po}$) as follows :

$$AUC_{po}=D/CL_{tot,po} \quad (9)$$

The following equation is obtained from equations (8) and (9) :

$$CL_{tot,po}=CL_{tot,iv}/(F_h\cdot F_a) \quad (10)$$

F_h is described by the following equation according to the literature[14,15] :

$$F_h=Q_h/(Q_h\cdot f_{ub}\cdot CL_{int,h}) \quad (11)$$

$CL_{tot,iv}$ assumes $CL_{,h}$ as the hepatic clearance, and equation (5) is obtained from equations (6), (10), and (11).

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Research report

Effect of genetic polymorphism on the metabolism of endogenous neuroactive substances, progesterone and *p*-tyramine, catalyzed by CYP2D6

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Abstract

Metabolic activities toward endogenous substrates in the brain, progesterone and *p*-tyramine, by cytochrome P450 2D6.2 (CYP2D6.2), CYP2D6.10A, CYP2D6.10C, and P34S, G42R, R296C, and S486T mutants expressed in recombinant *Saccharomyces cerevisiae* were compared with those by CYP2D6.1 (wild-type) in order to clarify the effects of genetic polymorphism of CYP2D6 on the metabolism of neuroactive steroids and amines in the brain. For the 6β-hydroxylation of progesterone, the V_{max} values for CYP2D6.2, CYP2D6.10A, and the P34S and G42R mutants, were less than half of those for CYP2D6.1, and CYP2D6.10C had a higher K_m and a lower V_{max} than the wild-type. The V_{max}/K_m values for CYP2D6.10A, CYP2D6.10C, and the P34S and G42R mutants were 12–31% of that for CYP2D6. The 16α-hydroxylation and 21-hydroxylation of progesterone by CYP2D6.10A, CYP2D6.10C, and the P34S and G42R mutants were not detected, and the R296C mutant had a higher K_m for the 16α-hydroxylation and a lower V_{max} for the 21-hydroxylation than those for CYP2D6.1. For dopamine formation from *p*-tyramine, the K_m values for CYP2D6.2 and the R296C mutant were higher than those for CYP2D6.1, CYP2D6.10A, and CYP2D6.10C had a higher K_m and a lower V_{max} than the wild-type. The V_{max}/K_m values for CYP2D6.2, CYP2D6.10A, CYP2D6.10C and the P34S, G42R and R296C mutants were less than 45% of those for the wild-type. These results suggest the possibility that the polymorphism of CYP2D6, including *CYP2D6*2, CYP2D6*10* and *CYP2D6*12*, might affect an individual behavior and the central nervous system through endogenous compounds, such as neuroactive steroids and tyramine, in the brain.

Theme: Other systems of the CNS
Topic: Brain metabolism and blood flow

Keywords: CYP2D6; Progesterone hydroxylation; Dopamine formation from *p*-tyramine; Human brain; Polymorphism

1. Introduction

Cytochrome P450s (P450 or CYP) comprise a superfamily of enzymes that catalyze the oxidation of a wide variety of xenobiotic chemicals including drugs, carcinogens, and steroids [10,12,35]. In spite of the fact that CYP2D6 constitutes only 2–9% of constitutively expressed hepatic P450s among humans [17,36], it plays important roles in the metabolism of a wide range of therapeutic agents including drugs affecting the central nervous system [5,9,30,35]. Interestingly, CYP2D6 is expressed in the brain, especially the midbrain [27], as well as in the liver. The reverse transcriptase-polymerase chain reaction (RT-PCR)

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product from CYP2D4, the predominant CYP2D isoform in rat brain, is more abundant in cerebellum, striatum, pons, and medulla oblongata [21]. However, the physiological and pharmacological functions of CYP2D isoforms in the brain are still unknown.

Progesterone not only is one of the female steroid hormones secreted from the placenta and corpus luteum but also has various functions in the central nervous system as a neurosteroid in the brain [2,19]. For example, progesterone has the ability to increase myelin-specific protein levels and to enhance γ-aminobutyric acid (GABA)-induced chloride current [19,39], and the progesterone metabolites, 3α-hydroxy-5α-pregnan-20-one (allopregnanolone) and 3α,5α-tetrahydrodeoxycorticosterone, act as positive allosteric modulators of GABA type A receptors, and thereby reduce brain excitability and elicit sedative-hypnotic, anxiolytic, and anticonvulsant effects [32]. Recently, we have shown that CYP2D6 catalyzes the 2β-, 6β-, 16α-, and 21-hydroxylation of progesterone [15,29], and that progesterone 2β- and 21-hydroxylation activities in rat brain microsomes are completely inhibited by CYP2D antibodies, suggesting that CYP2D may be involved in the regulation (metabolism and/or synthesis) of endogenous neuroactive steroids, such as progesterone and its derivatives, in the brain [15]. Additionally, we have reported that the 21-hydroxylation of allopregnanolone as well as progesterone and 17α-progesterone is catalyzed by CYP2D isoforms in the brain [9,20].

Tyramine is not only an exogenous compound, which is found in fermented foods such as cheese and wine, but also an endogenous compound, which exists in the brain. Tyramine is especially present in the basal ganglia or limbic systems, which are thought to be related to an individual behavior and emotion [33], and dopamine is a neurotransmitter and a precursor of norepinephrine and epinephrine [14]. Previous studies conducted in this laboratory demonstrated that dopamine is formed from *p*-tyramine as well as *m*-tyramine by CYP2D6 [14].

CYP2D6 is one of the most extensively characterized polymorphic drug-metabolizing enzymes; the CYP2D6 gene is highly polymorphic, with more than 70 allelic variants [4,6,26]. Interestingly, it has been shown that CYP2D6 polymorphism has some relationship with an individual behavior [3,24]. Five to ten percent of Caucasians [1] and less than 1% of Japanese and Chinese [16] lack in vivo metabolic activity toward CYP2D6 substrates estimated by use of the urinary metabolic ratio, and are referred to as poor metabolizers. The CYP2D6*12 allele, which is associated with a deficient activity and consequently with the poor metabolizer phenotype, carries three functional mutations, G42R, R296C, and S486T [6,26]. On the other hand, in spite of the very low prevalence of CYP2D6 poor metabolizers in Asians, these groups display less CYP2D6 activity, and this has been attributed to the high frequency of the CYP2D6.10 enzyme [40]. That is, the *CYP2D6*10* allele, including both *CYP2D6*10A* and *CYP2D6*10B* variants, is widely observed in Japanese (31–38%) [22,28] and Chinese (51%) [18], and has two amino acid substitutions, P34S and S486T [6,26]. Additionally, *CYP2D6*10C* has the gene conversion in exon 9 derived from CYP2D7 and has 13 base substitutions more than *CYP2D6*10B* [18]. Fukuda et al. [8] reported that the K_m values of CYP2D6.10A and CYP2D6.10C for bufuralol 1′-hydroxylation and venlafaxine *O*-demethylation were higher than those of CYP2D6.1, and Tsuzuki et al. [37] reported that the substitution G42R increased the K_m and decreased the V_{max} for debrisoquine 4-hydroxylation, whereas it increased both V_{max} and K_m for bunitrolol 4-hydroxylation. Recent studies have shown that CYP2D6.10A had a higher K_m and/or a lower V_{max} than CYP2D6.1 for various exogenous substrates, including dextromethorphan, methamphetamine, and amitriptyline [34]. On the other hand, it has been reported that the R296C and S486T substitution (CYP2D6.2) affected only minimally the metabolim of dextromethorphan, bufuralol, and debrisoquine [25]. Furthermore, a number of investigators have proposed the key residues of CYP2D6 for exogenous substrates containing a basic nitrogen [7,13,38]. However, the key residues of CYP2D6 for the metabolism of other substrates, including the endogenous chemicals, which exist in the brain, and non-nitrogen containing compounds, are still unknown.

The present study was designed to elucidate the effects of CYP2D6 polymorphism, especially P34S, G42R, R296C, and S486T substitutions such as CYP2D6.2, CYP2D6.10A, and CYP2D6.10C, on CYP2D6 activities toward endogenous substrates in the brain, progesterone and *p*-tyramine.

2. Materials and Methods

2.1. Materials

Progesterone and 16α- and 21-hydroxyprogesterone were obtained from Sigma-Aldrich (St. Louis, MO, USA). 6β-Hydroxyprogesterone, dopamine hydrochloride, and NADPH were purchased from Steraloids (Newport, RI, USA), Research Biochemicals International (Natick, MA, USA), and Oriental Yeast (Tokyo, Japan), respectively. *p*-Tyramine and other reagents and organic solvents were obtained from Wako Pure Chemical Industries (Osaka, Japan).

2.2. Microsomal fraction specifically expressing human P450

Cloning of human CYP2D6 cDNA, site-directed mutagenesis, the expression of mutated cDNA in recombinant *Saccharomyces cerevisiae* and the preparation of microsomal fractions from the cells were carried out according to methods described previously [8,37]. With these methods, we prepared CYP2D6.2 (R296C/S486T), CYP2D6.10A (P34S/S486T), CYP2D6.10C, and four mutant proteins

with single amino acid substitutions of P34S, G42R, R296C, and S486T.

2.3. Assay of progesterone hydroxylase activity

Progesterone hydroxylase activity was measured by the method described previously [15] with a minor modification. The incubation mixture consisted of microsomes from cells containing recombinant P450s (10–20 pmol/ml), 5, 10, 20, 50, 100, or 200 μM progesterone, 1 mM NADPH, and 100 mM potassium phosphate buffer (pH 7.4) in a final volume of 0.5 ml. After a 3-min preincubation at 37 °C, the reaction was started by adding NADPH. Incubation was carried out at 37 °C for 10 min and the reaction was terminated by the addition of 2 ml of ethyl acetate. The mixture was shaken and centrifuged at 1900×*g* for 5 min. The organic phase (1.5 ml) was evaporated under reduced pressure, and residue was dissolved immediately in 250 μl of 50% methanol. The HPLC system consisted of a Tosoh model DP-8020 pump (Tosoh, Tokyo, Japan), Tosoh model CO-8020 column heater, Tosoh model AS-8021 autosampler, an SPD-6-AV UV-detector (Shimazdu Corporation, Kyoto, Japan) set at 240 nm, and an analytical column TSK-gel ODS-80Ts (5 μm, 2.0×150 mm; Tosoh). The column temperature was set at 40 °C. The mobile phase was eluted at a flow rate of 0.3 ml/min as follows. The mobile phase was water as eluent A and methanol as eluent B, and the initial eluent profile was 50% B and then the eluent B was linearly increased to 65% over 20 min.

2.4. Assay of dopamine formation from p-tyramine

Dopamine formation from *p*-tyramine was measured by the method described previously [14] with a minor modification. The incubation mixture consisted of microsomes from cells containing recombinant P450s (10–20 pmol/ml), 0.05, 0.1, 0.2, 0.5, 1, or 2 mM, 0.05, 0.1, 0.2, 0.5, 1, 2, or 4 mM (for the G42R mutant), or 0.05, 0.1, 0.2, 0.5, 1, 2, 4, 10, or 20 mM (for CYP2D6.10C) *p*-tyramine, 1 mM NADPH, and 100 mM potassium phosphate buffer (pH 7.4) in a final volume of 0.5 ml. After a 3-min preincubation at 37 °C, the reaction was started by adding NADPH. Incubation was carried out at 37 °C for 10 min and the reaction was terminated by the addition of 20 μl of 60% perchloric acid. After the mixtures were shaken and centrifuged at 1900×*g* for 10 min, dopamine in the supernatant was determined by HPLC. The HPLC system described above was used except that a Tosoh model FS-8011 fluorometric detector, and an analytical column TSK-gel ODS-120T (5 μm, 4.6×250 mm; Tosoh) were employed. The fluorescence intensity was determined at an excitation wavelength of 280 nm and emission wavelength of 340 nm. The column temperature was set at 40 °C, and flow rate was 0.7 ml/min. The mobile phase was a 6.8:93.2 (v/v) mixture of acetonitrile and an aqueous solution containing 160 mM ammonium dihydrogen phos-

Table 1
Kinetic parameters for the progesterone hydroxylation by CYP2D6 and its variants

P450	6β-Hydroxylation			16α-Hydroxylation			21-Hydroxylation		
	K_m (μM)	V_{max} (nmol/min/nmol P450)	V_{max}/K_m (μl/min/nmol P450)	K_m (μM)	V_{max} (nmol/min/nmol P450)	V_{max}/K_m (μl/min/nmol P450)	K_m (μM)	V_{max}/K_m (nmol/min/nmol P450)	V_{max}/K_m (μl/min/nmol P450)
CYP2D6.1	23±5	0.39±0.03	17.3±4.2	16±8	0.079±0.013	5.0±2.7	34±23	0.12±0.03	3.5±2.5
CYP2D6.2 (R296C/S486T)	19±8	0.19±0.02	9.8±4.3	17±8	0.068±0.010	4.0±1.8	49±21	0.091±0.018	1.8±0.9
CYP2D6.10A (P34S/S486T)	11±7	0.061±0.010	5.4±3.3	–	<0.02[a]	–	–	<0.02[a]	–
CYP2D6.10C	50±37	0.10±0.03	2.0±1.6	–	<0.02[a]	–	–	<0.02[a]	–
P34S	36±15	0.19±0.03	5.3±2.4	–	<0.02[a]	–	–	<0.02[a]	–
G42R	35±21	0.068±0.017	2.0±1.3	–	<0.02[a]	–	–	<0.02[a]	–
R296C	44±6	0.38±0.02	8.6±1.2	53±21	0.11±0.02	2.1±0.9	15±8	0.053±0.008	3.6±2.0
S486T	21±6	0.29±0.02	13.8±3.7	22±11	0.089±0.015	4.1±2.2	21±9	0.073±0.011	3.6±1.6

Progesterone at 5–200 μM was incubated with CYP2D6 and its variants (10–20 pmol/ml) and 1 mM NADPH at 37 °C for 10 min after a 3-min preincubation. Values are the means±S.D. of the data set using a nonlinear kinetic analysis from mean values obtained in duplicate at each substrate concentration.

[a] The activity at 5–500 μM as a substrate concentration was <0.02 nmol/min/nmol P450.

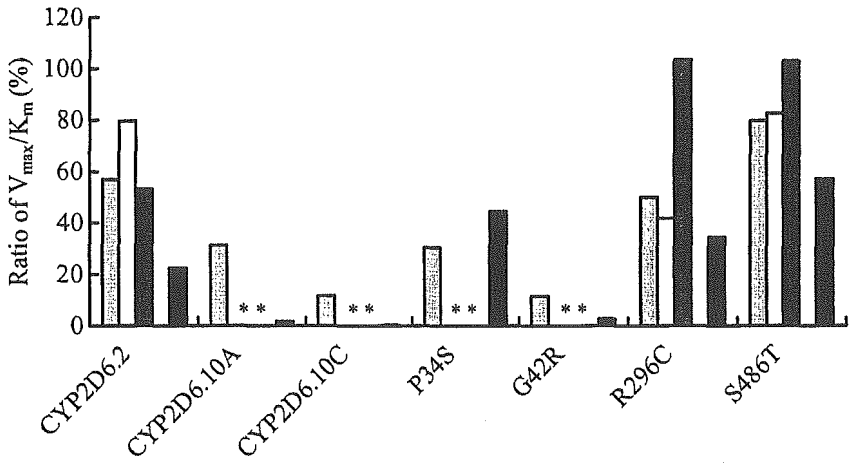

Fig. 1. Ratio of V_{max}/K_m for the metabolism of progesterone and *p*-tyramine by CYP2D6 variants. Ratios of V_{max}/K_m for 6β-hydroxylation (shaded column), 16α-hydroxylation (open column), 21-hydroxylation (striped column) of progesterone and dopamine formation from *p*-tyramine (closed column) were calculated by dividing the V_{max}/K_m for the variant by that for CYP2D6.1. *The 16α-hydroxylated and 21-hydroxylated metabolites for CYP2D6.10A, CYP2D6.10C and the P34S and G42R mutants were not detected (less than 0.02 nmol/min/nmol P450) even using 500 μM progesterone.

phate, 60 mM citric acid, 150 mM disodium EDTA, 10 mM dibutylamine, and 6 mM sodium 1-octanesulfonate.

2.5. Data analysis

In preliminary experiments, the linearity of the reaction with the protein concentration and incubation time was confirmed for each set of assay conditions. All data were analyzed using the mean of duplicate determinations. V_{max} and K_m values for progesterone hydroxylation and dopamine formation from *p*-tyramine were determined by fitting to Michaelis-Menten kinetics by nonlinear regression analysis (Microcal Origin, version 5.0J, Origin LabCorp, Northamptom, MA, USA).

3. Results

3.1. Progesterone hydroxylation by CYP2D6 and its variants

Kinetic parameters for progesterone hydroxylase activities of CYP2D6 and its variants are summarized in Table 1. The V_{max} value of CYP2D6.1 was highest for the 6β-hydroxylation followed by 21-hydroxylation and 16α-hydroxylation, whereas there were no marked differences between the K_m values for the three reactions. Although the K_m values for the 6β-hydroxylation by the CYP2D6 variants except for CYP2D6.10C were similar to those of CYP2D6.1, the V_{max} values for CYP2D6.2, CYP2D6.10A, and the P34S and G42R mutants, were less than half of those for CYP2D6.1. CYP2D6.10C had a higher K_m and a lower V_{max} than CYP2D6.1, whereas the V_{max} values as well as the K_m values for the R296C and S486T mutants were similar to those for the wild-type. The V_{max}/K_m values for CYP2D6.10A, CYP2D6.10C, and the P34S and G42R mutants were 12–31% of that for CYP2D6.1 (Fig. 1).

The 16α-hydroxylated and 21-hydroxylated metabolites for CYP2D6.10A, CYP2D6.10C, and the P34S and G42R mutants were not detected (less than 0.02 nmol/min/nmol P450) even using 500 μM progesterone. In addition, the K_m value for the 16α-hydroxylation and the V_{max} value for the 21-hydroxylation by the R296C mutant were 333% and 45%, respectively, of those for CYP2D6.1.

Table 2
Kinetic parameters for the dopamine formation from *p*-tyramine by CYP2D6 and its variants

P450	K_m (mM)	V_{max} (nmol/min/nmol P450)	V_{max}/K_m (μl/min/nmol P450)
CYP2D6.1	0.13±0.02	8.0±0.3	61±9
CYP2D6.2 (R296C/S486T)	0.33±0.07	4.5±0.3	14±3
CYP2D6.10A (P34S/S486T)	1.0±0.4	1.1±0.2	1.1±0.5
CYP2D6.10C	16.2±0.5	3.9±0.1	0.24±0.01
P34S	0.91±0.13	25.0±1.6	27±4
G42R	1.6±0.3	2.6±0.2	1.6±0.4
R296C	0.28±0.01	5.9±0.1	21±1
S486T	0.21±0.05	7.4±0.5	35±8

p-Tyramine at 50–2000 μM, 50–4000 μM (for G42R mutant) or 50–40000 μM (for CYP2D6.10C) was incubated with CYP2D6 and its variants (10–20 pmol/ml) and 1 mM NADPH at 37 °C for 10 min after a 3-min preincubation. Values are the means±S.D. of the data set using a nonlinear kinetic analysis from mean values obtained in duplicate at each substrate concentration.

3.2. Dopamine formation from p-tyramine by CYP2D6 and its variants

Kinetic parameters for dopamine formation from *p*-tyramine by CYP2D6 and its variants are shown in Table 2. Although a mutation at 486 (S486T) had no marked effect on the K_m and V_{max} values, the K_m values for CYP2D6.2 and the R296C mutant were 2.1–2.5 times higher than those for CYP2D6.1 without affecting the V_{max} values. CYP2D6.10A had an 8-fold higher K_m and a 7-fold lower V_{max} than CYP2D6.1, and CYP2D6.10C exhibited an 124-fold higher K_m and a 51% reduction in V_{max} relative to the wild type. The P34S mutant had a 7-fold higher K_m and a 3-fold higher V_{max} than CYP2D6.1, and the G42R mutant had a 12-fold higher K_m and a 3-fold lower V_{max} than the wild type. Therefore, the V_{max}/K_m for CYP2D6.2 and the P34S and R296C mutants were 23–45% of those for CYP2D6.1, and the values for CYP2D6.10A, CYP2D6.10C and the G42R mutant, were 0.3–2.6% of those for the wild-type (Fig. 1).

4. Discussion

Progesterone exists in the brain and has various functions in the nervous system as a neurosteroid [2,19]. Although it is well known that CYP3A4 is one of the major metabolizing enzymes for progesterone hydroxylation in human liver [35], we have reported that progesterone 2β- and 21-hydroxylation in rat brain microsomes are catalyzed by CYP2D [15] and that the 21-hydroxylation of allopregnanolone as well as progesterone and 17α-progesterone is catalyzed by CYP2D isoforms in the brain [9,20], suggesting that CYP2D is involved in the regulation of endogenous neuroactive steroids in brain tissues. In addition, tyramine, one of the trace amines, is present in the brain, especially in the basal ganglia or limbic systems, which are thought to be related to an individual behavior and emotion [33], and CYP2D6 polymorphism has some relationship with an individual behavior [3,24]. In this study, we have demonstrated that the V_{max} and/or K_m values for the metabolism of progesterone and *p*-tyramine by CYP2D6.2, CYP2D6.10A, and CYP2D6.10C were different from those for CYP2D6.1, and that the G42R, P34S, and R296C substitutions affected these metabolic activities (Tables 1 and 2). Additionally, the V_{max}/K_m values for all of the variants except for progesterone 16α-hydroxylation by CYP2D6.2 and progesterone hydroxylations by the S486T mutant were less than 57% of those for CYP2D6.1 (Fig. 1). The G42R substitution is found in a CYP2D6*12 allele in combination with R296C and S486T [6,26]. Furthermore, it has been shown that, when an individual behavior was compared between extensive and poor metabolizers of debrisoquine, a typical probe substrate of CYP2D6, using the Eysenck personality questionnaire and the Karolinska Scales of personality inventory in 769 healthy Swedes, poor metabolizers had significantly lower scores in the Karolinska psychascthenia scales and a higher frequency of extreme responses than extensive metabolizer [3]. Comparison of the debrisoquine hydroxylation capacity and the Karolinska scales of personality in 225 healthy subjects in Spain indicated that poor metabolizers of debrisoquine are more anxiety-prone and less successfully socialized than extensive metabolizers [24]. These studies suggest that there may be a relationship between an individual behavior and the activity of the enzyme hydroxylating debrisoquine (CYP2D6). Although the patients are phenotyped but not genotyped in these papers, it has been reported that the study to assess the relationship between CYP2D6 genotype (including *CYP2D6*10* allele) and debrisoquine phenotype in African-Americans and Caucasians in Los Angels shows the positive identification of 88% of phenotypic poor metabolizers by genotyping [23]. Therefore, the present results suggest that the polymorphism of CYP2D6, including *CYP2D6*2, CYP2D6*10* and *CYP2D6*12*, might affect not only the metabolic activities toward exogenous compounds in the liver [25,34,37] but also an individual behavior and the nervous system through endogenous compounds, such as neuroactive steroids and tyramine, in the brain.

For all of the metabolic activities investigated, the V_{max} values for the G42R mutant were lower than those for CYP2D6.1 (wild-type), and the K_m values for the mutant were higher than those for the wild-type except for progesterone 6β-hydroxylation. On the other hand, the substitution at Pro34 decreased the V_{max} value for progesterone 6β-hydroxylation and increased both the V_{max} and K_m values for dopamine formation from *p*-tyramine. Tsuzuki et al. [37] reported that the G42R substitution but not the P34S substitution increased K_m and decreased V_{max} for debrisoquine 4-hydroxylation, whereas the G42R substitution increased both V_{max} and K_m and the P34S substitution gave only an increased K_m for bunitrolol 4-hydroxylation. Therefore, the present findings suggest that Gly42 is essential for the metabolic activities toward not only exogenous substrate but also endogenous compounds such as progesterone, a non-nitrogen containing compounds, and *p*-tyramine, and that the P34S substitution also affects the metabolism of progesterone and *p*-tyramine.

Gotoh [11] predicted six potential substrate recognition sites (SRS) in the CYP2 family, and the SRSs span residues 100–125, 211–218, 239–247, 294–312, 367–377 and 477–484 for CYP2D6.1. In this study, although the metabolic activities were affected only minimally by the S486T substitution, a mutation of 296 (R296C) of CYP2D6 decreased the V_{max}/K_m for progesterone hydroxylations and dopamine formation from *p*-tyramine. Although it has been reported that the R296C mutation is of little importance for debrisoquine 4-hydroxylation and bunitrolol 4-hydroxylation [37], it is possible to speculate that Arg296, which is included in SRS 4, also might be important to some extent to the metabolism of progesterone and tyramine.

Similarly, V_{max}, K_m and V_{max}/K_m of debrisoquine 4-hydroxylation and bunitrolol 4-hydroxylation by CYP2D6.2 (R296C/S486T) are similar to those by CYP2D6.1 [37], whereas consistent changes in the kinetic characterizing dextromethorphan, bufuralol, and debrisoquine biotransformation by CYP2D6.2 relative to CYP2D6.1 are observed for all three substrates, with an increase in K_m and V_{max} such that V_{max}/K_m values are the same or slightly greater for CYP2D6.2 [34]. In addition, it has been reported that the V_{max}/K_m of CYP2D6.2 toward dextromethorphan, fluoxetine, and codeine decreased levels to less than 35% that of CYP2D6.1 [41], and that the V_{max} for codeine *O*-demethylation catalyzed by CYP2D6.2 are significantly higher than for CYP2D6.1 [31]. In the present study, the V_{max}/K_m values for dopamine formation from *p*-tyramine by CYP2D6.2 were 23% of those for CYP2D6.1, whereas the K_m and V_{max} values for the 21-hydroxylation and 16α-hydroxylation by CYP2D6.2 were comparable with those for CYP2D6.1. Therefore, it is possible to speculate that the effect of the R296C/S486T variant is substrate-dependent.

In summary, our results suggest that the polymorphism of CYP2D6 might influence an individual behavior and the nervous system through endogenous compounds, including neuroactive steroids and tyramine, in the brain.

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治験におけるファーマコゲノミクス研究の進展のために
―医療現場の状況と方向性―

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1．ファーマコジェネティクスとファーマコゲノミクス

ファーマコジェネティクス（pharmacogenetics）は薬理遺伝学と訳され，1950年代から学問領域として発達してきた．医薬品に対する反応性の個人差で遺伝的因子に関与するものを対象としてきた．とくに，薬物動態に関する分子種の研究は1990年代に入り飛躍的に発展し，薬物代謝酵素の遺伝多型と疾患や薬物代謝速度との関連で多くの業績を残してきている．近年，「ヒトゲノム解析計画」が本格化し，ファーマコゲノミクス（pharmacogenomics：PGx）と呼ばれる分野が登場した．PGxはゲノム薬理学と訳され，遺伝子多型解析と遺伝子発現情報解析を手段とする．遺伝子多型解析は，単一塩基変異多型（single nucleotide polymorphism：SNP）をターゲットに各個人を個別化し臨床情報と比較解析するものである[1]．遺伝子発現情報解析は，病態と遺伝子の動的な変化を薬物の応答性と関連づけて解析するものである．

薬理遺伝学とゲノム薬理学はアプローチの差異はあるものの，かなりオーバーラップする領域である．用語の使用に若干混乱が生じていることは事実である．現在，治験に組み込まれている解析遺伝子の対象は薬物代謝酵素だけではなく疾患関連遺伝子を含む場合もあるため，本章ではPGxを用いて話を進めたい．

2．新規医薬品の開発と治療の個別化

PGxは種々の医薬品開発に利用されている．たとえば，癌治療薬の開発として，癌細胞増殖に結びつくシグナル伝達分子，癌抑制遺伝子/細胞周期関連遺伝子，薬剤耐性因子，血管新生関連分子，湿潤・転移関連分子，テロメラーゼ/アポトーシス機構，などが分子ターゲットとして上げられている．癌細胞の増殖に関係するHER2のモノクロナル抗体やファルネシル基転移酵素の阻害剤，血管内皮細胞増殖因子（vascular endothelial cell growth factor：VEGF）のモノクロナル抗体，受容体型チロシンキナーゼ（epidermal growth factor receptor tyrosine kinase）の阻害剤など，いくつかの薬剤で臨床試験が進行している．

薬物の体内濃度が同じでも個人間で反応に差が生じる原因として，薬物のターゲットとなる分子の遺伝多型が考えられている．いわゆる薬物受容体の変化により，薬物に対するレスポンダー・ノンレスポンダーが規定されるという考え方である．事実，C型肝炎ウイルス（HCV）に対するインターフェロン（IFN）の有効率は当初30%程度であったが，レスポンダー・ノンレスポンダーの持つHCVの遺伝多型を解析した結果，HCVの遺伝多型によりIFN有効群と無効群に分かれることが判明した．現在，C型肝炎のIFN治療はHCVの遺伝多型に基づいて行われている．QT延長症候群ではβブロッカーが有効な群と突然死を引き起こす無効群が存在したが，遺伝子多型のSNP診断により有効群と無効群の層別が可能となり治療薬の選択に寄与している．この他にも，高血圧症でのβ_2レセプター・アンジオテンシン変換酵素多型，骨粗鬆症でのビタミンDレセプター・エストロゲンレセプター多型，気管支喘息でのβ_2レセプター多型，肥満におけるβ_3レセプター・レプチンレセプター多型，ゲフチニブの有効性と上皮成長因子受容体（epidermal growth factor receptor）の変異との関連，などが報告されてきており，同じ疾患でも個人の遺伝的背景により治療法（有効性）の選択が異なって来る可能性を示している．

3．PGx研究への期待

医療現場では対症療法的な治療が多くを占め，根本原因の治療に結びつく対応がとれる場合は限られている．PGxを推進する2大アプローチは，遺伝子多型解析と遺伝子発現情報解析であるが，これらのアプローチから解明が期待されていることは下記のとおりである．

① 薬物に対する個体の反応性の相違の識別（薬物代謝速度，薬物輸送速度，薬物受容体の感受性，等），
② 化合物の薬効・毒性評価の判別，
③ 疾患の原因究明，
④ 原因を標的分子とした治療法の開発，
⑤ 罹患のリスク判定，など

具体的には，既存薬物に対するレスポンダー・ノンレスポンダーの識別，副作用発現群の層別などを通じて，既存薬の再評価や新たな医薬品の開発の可能性がある．また，疾患遺伝子が作り出す蛋白質に対する阻害剤の開発や，至適に個別化された薬物選択や投与設計（personalized medicine）の現実化が期待される．

Key words：pharmacogenetics, pharmacogenomics, clinical research coodinator, clinical research associate
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Table　研究主体者：治験と PGx 研究

	治験	PGx
プロトコル	製薬企業（担当医師関与可）	製薬企業
IC	担当医師	担当医師
観察	担当医師	—
評価	担当医師	—
解析	製薬企業	製薬企業
結果	担当医師，患者も共有可	製薬企業
公表	可能（推奨）	不可（通常）

4．PGx 試験の種類

PGx 試験と一言でいってもその内容は試験によって異なる．ゲノム・遺伝子解析を実施する臨床試験は下記に分類される：

① 目的遺伝子と実施時期が特定されている
② 目的遺伝子は特定されているが実施時期は未決定
③ 目的遺伝子は特定されていないが医薬品の評価のために解析を実施
④ 疾患関連遺伝子の探索

臨床試験がどのタイプの PGx 検討を行うものであるかの同定は重要である．なぜかというと，その内容によってインフォームドコンセントのプロセスや第三者による審査内容が影響を受けるからである．

5．治験におけるゲノム解析試料収集のための同意取得

治験責任医師は実施する治験の内容を分かりやすく記載した説明文書を作成して，被験者に説明し同意を取得する．治験での処置，観察，評価は医療行為であり，日常診療の延長として対応できる．治験を実施する医師は，対象とする疾患分野の専門家であるため患者からの質問に対しても明確に応答できる．では，ゲノム解析試料収集に関するインフォームドコンセントはどうであろうか．治験の中で実施する試料収集の意義・意味を，医師は明確に理解し，被験者に説明し同意を取得できるのだろうか．試料収集だけで，解析のプロセスや結果にアクセスできない医師にインセンティブは存在するのだろうか．Table に治験と PGx 研究の研究主体者の比較を示す．

通常，治験の実施は日常診療と混在している．治験専門施設でないかぎり，この状況は変わらない．治験の内容を説明するためには概ね 30～60 分程度必要となる．したがって，現状では医師は概略を説明し，詳細は治験協力者（clinical research coordinator：CRC）に委ねている．CRC は，被験者となる患者の質問に答えられるよう，疾患の特性やプロトコルの内容を前もって理解している．CRC は，看護師，薬剤師，臨床検査技師，等の免許を取得しており，医療に関連する知識は兼ね備えている．治験の内容や関連する質問には答えられるようにしている．では，PGx に関する知識と理解はどうであろうか．誤解を恐れずはっきり言うと現段階では十分ではない者がほとんどであると考える．資料採取の目的や方法が記載された文書があっても，その内容を自ら理解し質問に対応できなければ他者に説明はできない．治験のプロトコルの記載に疑問が生じた場合，通常 CRC は依頼者の担当モニター（clinical research associate：CRA）に確認を行う．仮に，CRA が PGx 解析の意義，資料の保管方法や情報の取扱い方法を詳細に説明できない場合があったとすると，ジ・エンドである．

6．第三者による審査

文部科学省，厚生労働省および経済産業省は，2001 年に「ヒトゲノム・遺伝子解析研究に関する倫理指針」を告示した（いわゆる三省指針）．2005 年 4 月 1 日施行の個人情報保護法に対応するため，2004 年 12 月に同指針を改正した（2005 年 6 月一部改正）．この指針では，薬事法に基づく臨床試験（治験）と製造販売後臨床試験は対象から除外している．しかしながら，PGx 試験の遺伝子解析に係わる部分を，ヒトゲノム・遺伝子解析倫理委員会に審査を依頼している医療機関は存在する．三省指針に記載されている倫理委員会の構成は下記のとおりである[2]：

- 倫理・法律を含む人文・社会科学面の有識者，自然科学面の有識者，一般の立場の者から構成される必要がある．
- 外部委員を半数以上置くことが望ましいが，その確保が困難な場合には，少なくとも複数名置かれる必要がある．
- 外部委員の半数以上は，人文・社会科学面の有識者または一般の立場の者である必要がある．
- 男女両性で構成される必要がある．

GCP で定められている審査委員会（institutional review board：IRB）の構成は次のとおりである[3]：

- 5 名以上の委員からなること．
- 委員のうち，医学，歯学，薬学その他の医療または臨床試験に関する専門的知識を有する者以外の者（次号の規定により委員に加えられている者を除く）が加えられていること．
- 委員のうち，実施医療機関と利害関係を有しない者が加えられていること．

三省指針は個人情報保護法に対応しているため，具体的できつい縛りとなっている．一方，GCP は倫理性，科学性，信頼性の確保の観点から，被験者の人権，安全，福祉の保護を謳っており，個人情報の保護を第一義とするものではない．「PGx 試験の対象とするところが遺伝子という個人情報である」という考え方が存在する以上，より具体性のある規制に基づいて判断する委員会に審査を依頼する傾向を即座には否定できない．問題は，GCP に則ったプロセスと管理が理解されて審査されているか，というと

ころに存在する．この点は，申請者の提出資料の内容と説明に依存するところである．申請者である治験責任医師が十分な説明ができなければ，審査側は容易には承認しないということは簡単に推測できる．このことはIRBでの審査でも同様である．

7．PGx研究の推進

PGx試験の推進のためには，考慮すべき点がまだまだ存在する．被験者となる患者の理解を得ることはもちろんであるが，担当医師の理解，協力者（CRC等）の理解，詳細をきちんと説明できるモニターの存在，などPGx研究を担う関係者の教育が必要である．

また，資料採取以後の手順の明確化，情報管理体制，企業研究者の責務，企業管理者の責務，監査手順，等をオープンにして理解を得る方策も考慮すべきだと考える．さらに，現在と将来の測定に関する別個の同意取得方法など，PGx試験の内容に応じた適切な対応を考えていく必要もあるだろう．

文　献

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