Development and Application of a Method to Investigate Drug-Metabolizing Enzyme Inhibitors Using Sparteine for Probe of Cytochrome P450 2D6 and Tris(2,2′-bipyridine)ruthenium(II)-Electrogenerated Chemiluminescence Detection

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We studied the detection of drug-metabolizing enzyme inhibitiors using column-switching high performance liquid chromatography with tris(2,2′-bipyridine)ruthenium(II) ($Ru(bpy)_3^{2+}$)-electrogenerated chemiluminescence detection. This can be applied to evaluate the genetic diversity concerning the ability of cytochrome P450 (CYP) 2D6 to metabolize drug *in vitro*. We demonstrated the ability of CYP2D6 to enable us to examine drugs metabolizing enzyme inhibition with high performance and sensitivity. This method can be applied to investigate metabolite inhibitors of CYP2D6 *in vitro* and *in vivo*. Thus, Metixene was found to be a potential CYP2D6 inhibitor.

Key words tris(2,2′-bipyridine)ruthenium(II); column-switching HPLC; cytochrome P450 2D6; Metixene

Drug–drug interactions can be divided into pharmacodynamic and pharmacokinetic interactions. Pharmacodynamic interactions occur in the action site of the drug. Pharmacokinetics interaction occur during the process of absorption, distribution, metabolism, and excretion of the drug. Drugs are chiefly metabolized in the liver where various enzymes and many isoforms of cytochrome P450 (CYP) are present. CYP-related pharmacokinetic interactions are known to cause the clinical problems.[1,2] CYP play a major role in the oxidative metabolism of drugs. Many drugs were reported to be easily metabolized by CYPs.[3] The medicinal effect of the drugs and appearance of toxicity due to CYPs activity may cause a fluctuation in the level of drugs in the blood.

In recent years, reports on only drug–drugs interaction but also drug–food and drug–supplement interactions have led to the investigation of, drug-metabolizing enzyme inhibitors. The development of microtitre plate assays for drug-metabolizing inhibitors has been reported.[4,5] In this assay, the drug is reacted with the recombinant CYP2D6 or human liver microsome, and the fluorescence resulting from the reaction is detected. Western blot using rat liver microsome,[6] high performance liquid chromatography (HPLC),[7] and liquid chromatography-tandem mass spectrometry (LC/MS/MS)[8] have also been reported. The procedure for the microsome preparation from rat liver and extraction of the reacted drugs for the HPLC analysis is a complicated, and time-consuming process. Since a rapid and a simple analysis method is required to screen bulk drugs for their drug–drug interaction, a high performance method to detect the drug-metabolizing enzyme inhibitors was developed.

We reported the development of a column-switching HPLC method with tris(2,2′-bipyridine)ruthenium(II) ($Ru(bpy)_3^{2+}$)-electrogenerated chemiluminescence (ECL). Sparteine (SP, Fig. 1), which was used as a probe drug for CYP2D6, exhibits genetic polymorphism oxidation by CYP2D6.[9] SP is a lupin alkaloid having an aliphatic tertiary amine (ATA) suitable for highly sensitive detection of ($Ru(bpy)_3^{2+}$-ECL).[10]

It is difficut to evaluate the degree of the drug interactions

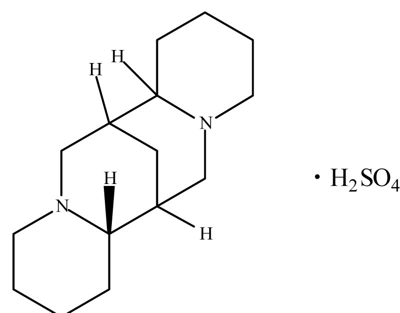

Fig. 1. Chemical Structure of Sparteine Sulfate

in vivo, which should be investigated, both *in vitro* and *in vivo*. For example, the H_2-blocker Ebrotidine is a strong inhibitor of CYP *in vitro*, but not in humans *in vivo*.[11] According to this study, drug-metabolizing enzyme inhibition substances were screened by column-switching HPLC with $Ru(bpy)_3^{2+}$-ECL. SP concentrations *in vitro* and *in vivo* (SP concentration in the plasma of rabbit) were also compared.

Experimental

Chemicals (−)-Sparteine sulfate, tris(2,2′-bipyridine)ruthenium(II) chloride hexahydrate, amiodarone hydrochloride, cimetidine, propafenone hydrochloride and ticlopidine hydrochloride were purchased from Sigma-Aldrich Co. (St. Louis, MO, U.S.A.). CYP 2D6 human recombinant microsome, 2-amino-2-hydroxymethyl-1,3-propanediol (tris), beta-reduced nicotinamide adenine dinucleotide phosphate (β-NADPH), (+)-chlorpheniramine maleate, (±)-chlorpheniramine maleate, clomipramine hydrochloride, quinidine sulfate, ranitidine hydrochloride and *E*-(*N*)-(6,6′-dimethyl-2-hepten-4-ynyl)-*N*-methyl-1-naphtylmethylamine (terbinafine) were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Sodium 1-octanesulfonate used for ion pair chromatography was obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan).

Column-Switching HPLC. HPLC Conditions The column-switching HPLC with $Ru(bpy)_3^{2+}$-ECL system is shown in Fig. 2. This system used two columns, [Column I (C-1), Column II (C-2)] and a six-port switching valve PT-8000 (TOSOH, Tokyo, Japan). C-1 was a Shim-Pack MAYI-ODS (G) (10×4.6 mm; Shimadzu, Kyoto, Japan) for protein exclution. C-2 was a J'sphere ODS H-80 (150×4.6 mm; YMC, Kyoto, Japan) for separation of sample. Both column temperatures were room temperature. Two eluents, Eluent I (E-1) and Eluent II (E-2), were used for protein exclusion and separation. E-1 was delivered by Pump I (LC-9A; Shimadzu, Kyoto, Japan) at a flow rate of 0.5 ml/min. E-2 was delivered by Pump II (L-6000; Hitachi,

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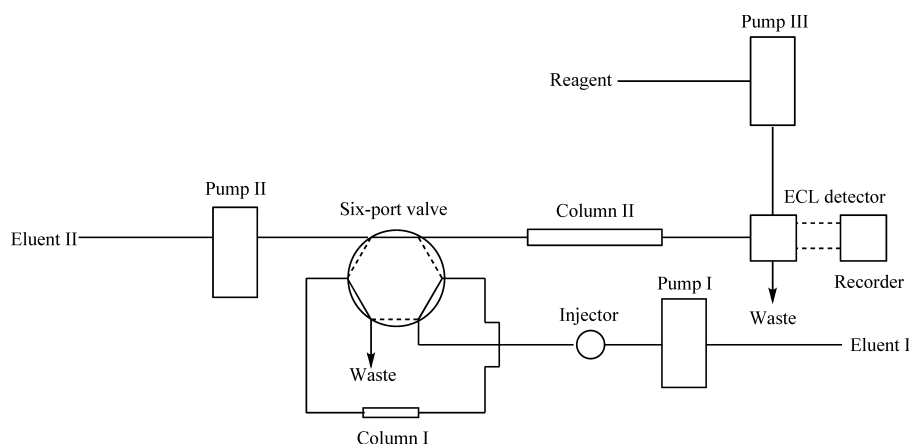

Fig. 2. Flow Diagram of Column-Switching HPLC with $Ru(bpy)_3^{2+}$-ECL Detection

Tokyo, Japan) at a flow rate of 1.0 ml/min. The ECL detector was an ECR COMET-3000 (Comet, Kanagawa, Japan). The ECL reagent flow rate was 0.45 ml/min using Pump III (uf-7002PSSB2; UNIFLOWS, Tokyo, Japan). Chromatograms were recorded with a CHROMATOPACK C-R8A (Shimadzu, Kyoto, Japan). E-1 consisted of 15 mM KH_2PO_4 buffer containing 10 mM sodium 1-octanesulfonate (pH 6.0)–CH_3CN (98 : 2, v/v). E-2 consisted of 150 mM KH_2PO_4 containing 10 mM sodium 1-octanesulfonate (pH 4.0)–CH_3CN (73 : 27, v/v). The reagent solution was 0.3 mM $Ru(bpy)_3Cl_2$ in 10 mM H_2SO_4.

Column Switching Operation A sample (20 μl) was injected into the column-switching HPLC. The valve stayed in the solid line for 5 min. During this time, the sample was introduced onto a C-1 where SP and sparteine metabolite (SPm) were retained by E-1. After 5 min, the valve was switched to broken line, and SP and SPm were transferred from C-1 to C-2 by E-2. After 1 min the valve was switched back to solid line, and SP and SPm were separated by E-2 and C-2, respectively.

Measurement Condition of Cimetidine[12] This HPLC system besides column switching HPLC was constructed. The HPLC system consisted of LC-10AD (Shimadzu) with a Capcell Pak C_{18} column (150×4.6 mm; Shiseido, Tokyo, Japan), at room temperature. This system used single column. The eluent consisted of 20 mM CH_3COONa containing 2.5 g/l sodium 1-octanesulfonate–CH_3CN (77 : 23, v/v) and the flow rate was 1.0 ml/min. A UV detector was used and the absorbance was set at 228 nm. Rabbits blood samples were centrifuged at 5000 rpm for 15 min at 4 °C, and supernatant plasma samples were directly injected. The sample loop volume was 20 μl.

Measurement Condition of Metixene This HPLC system besides column switching HPLC was constructed. The HPLC system consisted of LC-10AD (Shimadzu), with a Capcell Pak C_{18} column (150×4.6 mm, Shiseido), and the column temperature was 40 °C. This system used single column. The eluent consisted of 0.01 M phosphate buffer–CH_3CN (65 : 35, v/v) and the flow rate was at 1.0 ml/min. The absorbance for the UV detector was set at 268 nm. Enzyme reaction solutions were directly injected without the pretreanment. The sample loop volume was 20 μl.

Enzyme Reaction[13] The incubation mixture contained 30 μl of 0.5 μM CYP2D6, 140 μl of 0.05 M Tris buffer (pH 7.5), 25 μl of 0.06 M $MgCl_2$, 25 μl of 5 μM SP, 30 μl of 0.004 M NADPH, and 25 μl of inhibition drug sample. The mixtures were incubated for 120 min at 37 °C. After cooling at room temperature 20 μl of the solution was directly injected into the HPLC system.

***In Vivo* Experiments** Appropriate experimental animal guidelines were followed to perform this experiments. Japanese white rabbits weighing around 2.8—3.2 kg were used throughout the study. Rabbit blood samples were centrifuged at 5000 rpm for 15 min at 4 °C. The plasma was diluted in 15 mM KH_2PO_4 buffer containing 10 mM 1-octanesulfonate at pH 6.0, and a volume of 100 μl of sample was injected into the column-switching HPLC.

SP Administration SP solution (1 ml), diluted in saline (5.95 mg/kg), was administrated into the rabbit's auricular vein. Blood samples (0.2 ml) were obtained at the selected time range (1—1440 min).

SP and Cimetidine Administration[14] Cimetidine solution, 2 ml of (40 mg/kg) diluted in saline was administrated to the auricular vein of rabbits an hour before SP administration. Cimetidine was administered to rabbits after SP administration every 2 h for 22 h. Blood samples (0.2 ml) were taken in the selected time range (1—1440 min).

SP and Metixene Administration Metixene was diluted in saline and 3 ml of Metixene solutions (2 mg/kg) and SP solutions (5.95 mg/kg) were administered intravenously to the rabbits. Blood samples (0.2 ml) were taken in the selected time range (1—1440 min).

Statistical Analysis Area under the blood concentration–time curve (*AUC*) of SP was analyzed using Graph Pad Prism 4 (Graph Pad Software, Inc.). Significant difference was examined by Student's *t*-test, and a difference was considered significant for $p<0.05$.

Results and Discussion

Validation of HPLC Analysis For validation of the developed analysis method, a calibration range of 0.02—3.75 μM was sufficient for the analysis of SP ($r^2=0.9993$, Fig. 3), with a coefficient of variation (CV) of 0.3% for intraday and 2.0% for interday. A detection limit was 8 nM. Figure 4 shows that no obstruction peak derived from impurities can be seen in the SP spiked serum. At this time, CV was 1.5% and the recovered range was 98.5% (2.5 μM, $n=4$). In addition, CV of the SP peak area after the enzyme reaction mixture was incubation for 120 min, it was 3.2% for intraday and 3.9% for interday ($n=3$, metabolic rate 60%).

Optimization of Enzyme Reaction The enzyme-reacted samples were analyzed by the column-switching HPLC system. The chromatograms are shown in Fig. 5. No peak due to CYP2D6, Tris buffer, $MgCl_2$ and NADPH were observed, and only peaks of SP and SPm were observed. NADPH was reported to be detected by $Ru(bpy)_3^{2+}$.[15] However no peak corresponding to NADPH was detected in our experimental condition. This indicates, that NADPH was removed by C-1 and E-1, together with CYP2D6.

Exploration of the Enzyme Inhibition *in Vitro* We studied Clomipramine, Quinidine, Propafenone, Amiodarone, (+)-Chlorpheniramine, (±)-Chlorpheniramine, Cimetidine, Ranitidine, Terbinafine and Ticlopidine that were reported to inhibit CYP2D6.[1—3,11,16—19] We investigated whether the SP metabolism was blocked by these medical substances. Inhibition of SP metabolism was confirmed in all medical substances. The inhibition rate and calculated 50% inhibition concentration (IC_{50}) of each medical substances are shown in Table 1. These results indicated that drug-metabolism inhibitors can be identified using the enzyme reaction and the column-switching HPLC, and that the sensitivity of the developed system makes it suitable for practical usage.

Inhibition of SP metabolism for Impramine, Barbital, Chlordiazepoxide, Diazepam, Metixene, Levodopa, DL-

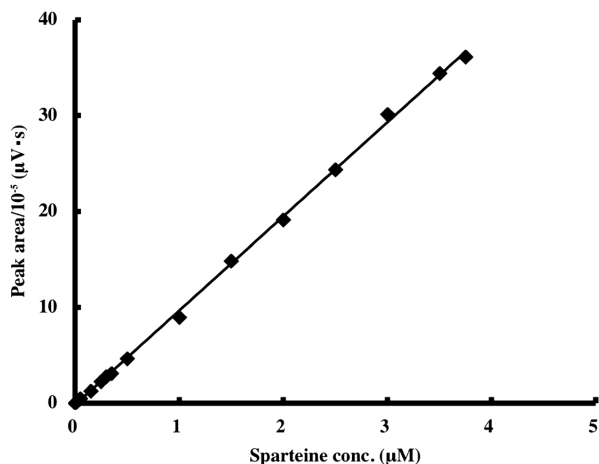

Fig. 3. Standard Curve of SP

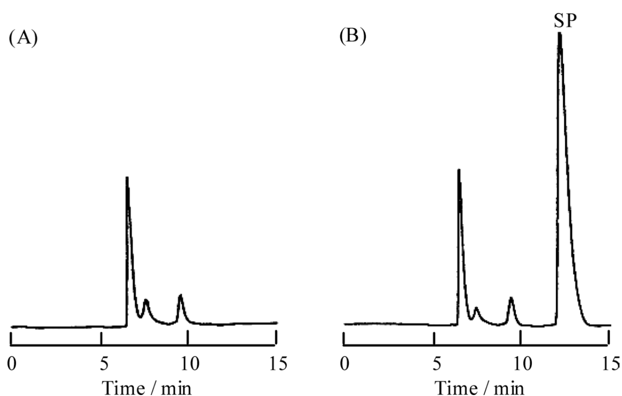

Fig. 4. Chromatograms of Control Serum, (A) Blank and (B) Spiked with 2.5 μM SP

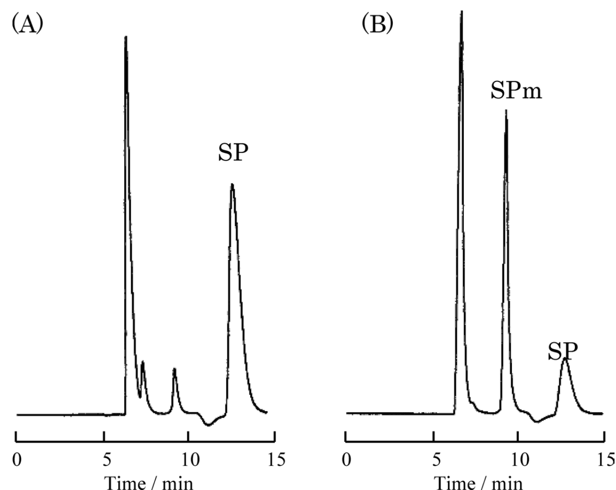

Fig. 5. Chromatographic Separation of Enzyme Reaction Mixture

(A) Pre-incubation and (B) incubation for 120 min.

DOPA, L-Epinephrine, DL-Epinephrine, DL-Isoprotenerol, Etilefrin, Dopamine, Furosemide, Spironolactone, Trichlormethiazide, Promethazine, Metoclopramine, D-Penicillamine, L-Penicillamine, Tiopronin, Penicillin G, Erythromycin, DL-Methanephrine, DL-Normethanephrine, and 2-Methylpyridine which were drugs considered to inhibit CYP2D6 were not reported. When IC_{50} was calculated, inhibition of CYP2D6 was found to be a limited, because almost all drugs were not

Table 1. IC_{50} of CYP2D6 Inhibitor

Drug	IC_{50} (μM)
Clomipramine	0.35
Quinidine	0.22
Propafenone	1.79
Amiodarone	0.32
(+)-Chlorpheniramine	1.69
(±)-Chlorpheniramine	2.54
Cimetidine	0.45
Ranitidine	0.48
Terbinafine	0.44
Ticlopidine	2.14

Table 2. IC_{50} of Drugs (1)

Drug	IC_{50} (μM)
Imipramine	2.2
Barbital	85.2
Chlordiazepoxide	23.1
Diazepam	72.2
Metixene	1.7
Levodopa	27.6
DL-DOPA	123.6
L-Epinephrine	39.0
DL-Epinephrine	58.1
DL-Isoprotenerol	105.8
Etilefrin	51.4
Dopamine	106.6
Furosemide	176.3

Table 3. IC_{50} of Drugs (2)

Drug	IC_{50} (μM)
Spironolactone	152.6
Trichlormethiazide	120.7
Promethazine	20.5
Metoclopramide	52.0
Methimazole	34.4
D-Penicillamine	152.5
L-Penicillamine	351.7
Tiopronin	27.9
Penicillin G	47.1
Erythromycin	44.9
DL-Methanephrine	127.5
DL-Normethanephrine	138.1
2-Methylpyridine	41.8

showing an equal degree of inhibition compared with the abovementioned outcome the experiment (Tables 2, 3). However, Imipramine showed an equal degree of inhibition compared with the abovementioned outcome of the experiment. Since CYP2D6 was known to participate in Imipramine metabolism.[20] SP metabolism was assumed to be inhibited by competition. Metixene can possibly inhibit CYP2D6.

The metabolic pathway of Metixene is shown in Fig. 6. CYP may be an oxidation enzyme and *S*-oxidation may be the main route for Metixene. Then, it was examined whether Metixene was metabolized by CYP2D6. The enzyme reaction of Metixine (0.4 mM) shown in Fig. 7. As a control 0.05 M Tris–HCl solution (pH 7.5) was used. The Metixene peak area decreased after 120 min incubation following the addition of CYP2D6 compared with the control. The result indi-

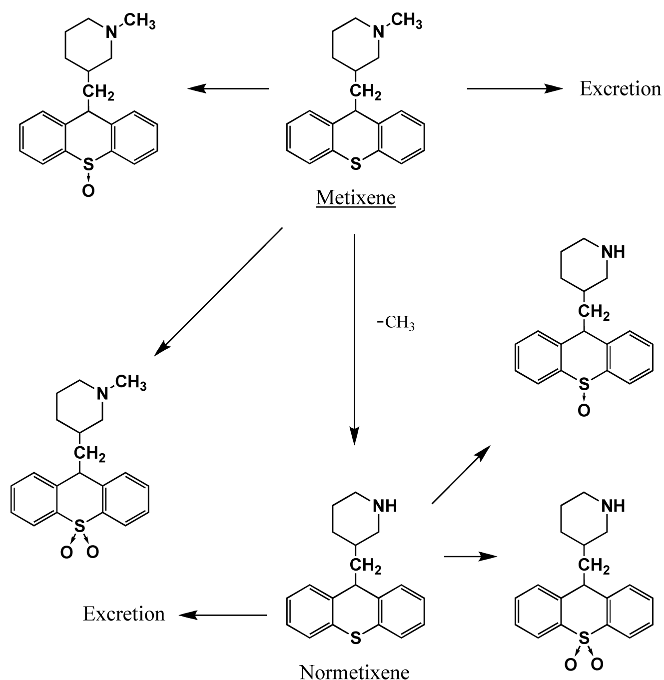

Fig. 6. Metabolic Pathway of Metixene

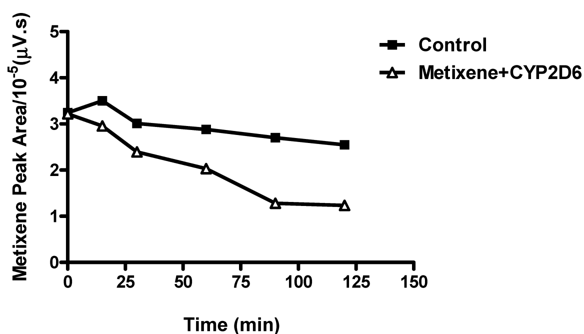

Fig. 7. Time Course for Enzyme Reaction of Metixene

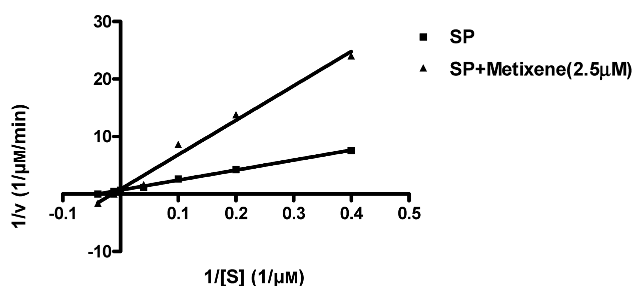

Fig. 8. Double-Reciprocal Plot of the Activity of SP

cates that CYP2D6 participated in metabolizing the Metixene.

The pharmacokinetics parameters derived from the Lineweaver–Burk plot of CYP2D6 inhibition by Metixene were investigated (Fig. 8). Metixene was found to be a competitive inhibitor because K_m values for SP was 25.5 μM and V_{max} was 1.47 μM/min and those for the Metixene combined with SP were 86.2 μM and V_{max} was 1.47 μM/min, respectively.

Exploration of CYP2D6 Inhibitor *in Vivo.* Combined Administration of SP and Cimetidine Whether Cimetidine inhibits SP metabolism by CYP2D6 *in vivo* is yet to be determined. Cimetidine was selected since a strong inhibition was confirmed *in vitro*.

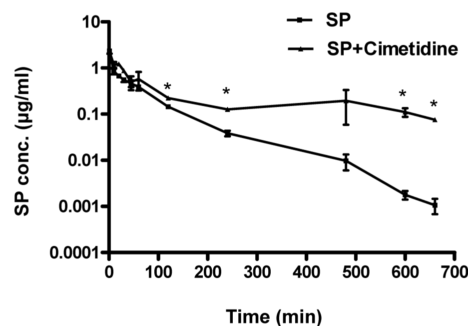

Fig. 9. Effects of Cimetidine Administration on SP Plasma Levels in Rabbits

Rabbits were pretreated with Cimetidine (40 mg/kg) 1 h before SP (5.95 mg/kg) administration and then Cimetidine was administrated every 2 h for 11 h. Mean and standard deviation (n=3). * $p<0.05$ *vs.* SP, Student's *t*-test.

Since the nitrogen atom of the imidazole ring of Cimetidine was interacting directly with the heme iron of CYP2D6, Cimetidine blocked the non-competitive oxidative metabolic activity of CYP2D6. It is believed that decreased inhibition is related to the disappearance of Cimetidine from the organism. It has been shown that the Cimetidine level in plasma was 0.16—12.0 μg/ml. Most Cimetidine in blood disappeared in 2 h. Repetitive doses for every 2 h were required to maintain Cimetidine concentration in plasma.

The increase in SP plasma level and extension of the disappearance of SP were confirmed by administration of SP together with Cimetidine compared with the sole administration of SP (Fig. 9). The $AUC_{0-11\,h}$ of SP was calculated as 75.7±3.09 μg·h/ml (n=3) for the sole administration of SP, and 163.6±26.33 μg·h/ml (n=3) for combined administration of SP and Cimetidine. A significant increase ($p<0.05$) was observed for the combined administration of SP and Cimetidine. Thus it is believed that SP metabolism is inhibited by the Cimetidine.

We investigated whether this newly developed method can be useful to investigate drug metabolizing enzyme inhibitors under *in vivo* experimental condition.

Combined Administration of SP and Metixene SP inhibition metabolism by Metixene investigated *in vitro*. Metixene was shown to be a competitive inhibitor. When Metixene and SP were coadministered, the increase of SP plasma level was confirmed (Fig. 10). At this time, $AUC_{0-11\,h}$ of SP was 170.3±78.32 μg·h/ml (n=3). A significant increase was not observed compared with the sole administration of SP. However, in case of sole administration of SP, the level of SP became lower than the detection limits at 720 min, when the monitoring of SP had been continued after 660 min. When SP is used concomitantly with Metixene, SP can be detected even after 1080 min. From these results, it is believed that the disappearance had extended. Inhibition of metabolizing SP was suggested *in vivo*, even though a significant difference was not found for $AUC_{0-11\,h}$.

Lewis and others reported the structure-activity relationship of the substrate of CYP2D6.[21] According to their reports, the affinity to CYP2D6 was correlated with the number of π–π stacking interaction ($N_{\pi-\pi}$) with the active site of

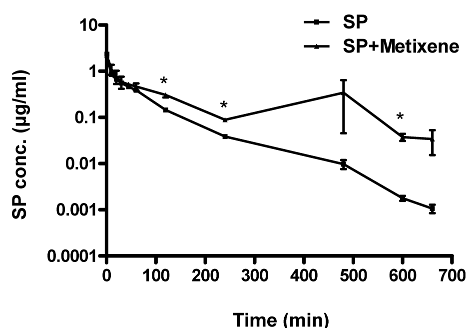

Fig. 10. Effects of Metixene Administration on SP Plasma Levels in Rabbits

Rabbits were treated with Metixene (2 mg/kg) and SP (5.95 mg/kg). Mean and standard deviation ($n=3$). * $p<0.05$ *vs.* SP, Student's *t*-test.

CYP2D6 and substrate, and a substrate with high affinity to CYP2D6 was preferred to possess the 1—3 units of N_{HB} and the 1—2 units of $N_{\pi-\pi}$. In our experiments, in the structure of Metixene that was ascertained for interaction was one hetero atom, which can be N_{HB}, and two aromatic rings, which can be $N_{\pi-\pi}$. The requirements for a CYP2D6 substrate were met as per the study of Lewis *et al.*

From these results, Metixene was found to have a potentially high affinity for CYP2D6. Moreover, Zuclopethixol having a structure similar to that of Metixene, was reported to be a substrate of CYP2D6. Therefore, Metixene can be a substrate of CYP2D6.

Conclusions

A method for screening of metabolizing enzyme inhibitors of CYP2D6 using column-switching HPLC with $Ru(bpy)_3^{2+}$-ECL has been developed. This can be applied to the evaluation of the genetic diversity concerning the ability of CYP2D6 to metabolize drug *in vitro*, since simultaneous determination of SP and SPm and the measurement of the ability to metabolize drugs *in vitro* can be performed. It was suggested to be able to examine the presence of inhibition of the drug-metabolizing enzyme by the drug, food, and the supplement easily.

In addition, SP can be detected with high sensitivity using a simple procedures, in plasma samples obtained from rabbits.

Our results indicated that Cimetidine inhibited SP metabolism by CYP2D6 because combined administration of SP and Cimetidine increased the plasma level and $AUC_{0—11\,h}$ of SP. It is believed that Cimetidine inhibited SP metabolism. In combined administration of SP and Metixene, the SP plasma level and $AUC_{0—11\,h}$ were increased although a significant difference was not confirmed. It was suggested that inhibition of CYP2D6 was prevented by Metixene.

Correlation with *in vitro* experiment was obtained for the screened drugs. Our result demonstrate that this method can be used to investigate metabolite-inhibiting substances of CYP2D6 in both *in vitro* and *in vivo* conditions.

References

1) Nakamura H., Omori S., *Yakkyoku*, **50**, 2116—2128 (1999).
2) Okada K., Ariyoshi N., *Yakkyoku*, **54**, 2757—2766 (2003).
3) Kitada K., *Gekkan Yakuji*, **38**, 467—479 (1996).
4) Yamamoto T., Suzuki A., Kohno Y., *Xenobiotica*, **33**, 823—839 (2003).
5) Favreau V. L., Palamanda R. J., Lin C., Nomeir A. A., *Drug Metab. Dispos.*, **27**, 436—439 (1999).
6) Orishiki M., Matsuo Y., Nishioka M., Ichikawa Y., *Int. J. Biochem.*, **26**, 751—758 (1994).
7) Narimatsu S., Arai T., Masubuchi Y., Horie T., Hosokawa M., Ueno K., Kataoka H., Yamamoto S., Ishikawa T., Cho K. A., *Biol. Pharm. Bull.*, **24**, 988—994 (2001).
8) Walsky L. R., Obach S. R., *Drug Metab. Dispos.*, **32**, 647—660 (2004).
9) Ishizaki T., Eichelbaum M., Hourai Y., Hashimoto K., Chiba K., Dengler J. H., *Br. J. Clin. Pharmacol.*, **23**, 482—485 (1987).
10) Uchikura K., Kirisawa M., *Anal. Sci.*, **7**, 803—804 (1991).
11) Martínez C., Albet C., Agúndez A. G. J., Herrero E., Carrillo A. J., Márquez M., Benítez J., Ortiz A. J., *Clin. Pharmacol. Ther.*, **65**, 369—376 (1999).
12) Iqbal T., Karyekar S. C., Kinjo M., Ngan C. G., Dowling C. T., *J. Chromatogr. B*, **799**, 337—341 (2004).
13) Osikowska-Evers A. B., Eichelbaum M., *Life Sci.*, **38**, 1775—1782 (1986).
14) Brenner E. D., Collins C. J., Hande R. K., *Cancer Chemother. Pharmacol.*, **18**, 219—222 (1986).
15) Uchikura K., Kirisawa M., *Chromatography*, **13**, 257—258 (1992).
16) Vickers E. M. A., Sinclair R. J., Zollinger M., Heitz F., Glänzel U., Johanson L., Fisher V., *Drug Metab. Dispos.*, **27**, 1029—1038 (1999).
17) Turpeinin M., Nieminen R., Juntunen T., Taavitsainen P., Raunio H., Pelkonen O., *Drug Metab. Dispos.*, **32**, 626—631 (2004).
18) Yamamoto T., Suzuki A., Kohno Y., *Xenobiotica*, **34**, 87—101 (2004).
19) Hamelin A. B., Bouayad A., Drolet B., Gravel A., Turgeon J., *Drug Metab. Dispos.*, **26**, 536—539 (1998).
20) Niwa T., Shiraga T., Ishii I., Kagayama A., Takagi A., *Biol. Pharm. Bull.*, **28**, 1711—1716 (2005).
21) Lewis F. V. D., Modi S., Dickins M., *Drug Metab. Rev.*, **34**, 69—82 (2002).