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Kinetic phenotypic diagnosis of *N*-acetylation polymorphism in patients based on ratio of urinary metabolites of salicylazosulfapyridine

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Abstract

We found that *N*-acetylation polymorphism can be evaluated from the disposition kinetics of sulfapyridine (SP) and 5-aminosalicylic acid (5-ASA) and their acetylated metabolites generated by *N*-acetyltransferase (NAT2) after oral administration of salicylazosulfapyridine (SASP). In 126 Japanese subjects, the homozygote of *NAT2*4* was the most frequent (40%), followed by heterozygotes of *NAT2*4* and mutant genes (28% *NAT2*4*/**6A*, 15% *NAT2*4*/**7B*, and 2% *NAT2*4*/**5B*). Combinations of mutant genes accounted for 16%. When the relationship between the molar ratio of *N*-acetyl-SP (Ac-SP)/SP or *N*-acetyl-5-ASA(Ac-5-ASA)/5-ASA in serum and five genotypes of polymorphic *NAT2** was examined in patients who received multiple doses of SASP, the molar ratios of Ac-SP/SP, rather than Ac-5-ASA/5-ASA tended to decrease according to the classification of genotype. We calculated the pharmacokinetic parameters in healthy subjects with various genotypes of polymorphic *NAT2** after a single p.o. administration of SASP, according to a model of the SP metabolic pathways. The molar ratios of Ac-SP/SP in serum and urine were simulated using these parameters, and the molar ratio of Ac-SP/SP in urine at 4 days after the first administration could be categorized into ranges that were specific to various *NAT2** genotypes. Thus, we were able to predict the *N*-acetylation polymorphic genotypes of patients by measuring the molar ratio of Ac-SP/SP in urine, after administration of SASP.

Keywords: Salicylazosulfapyridine; *NAT2**; Genotype; Phenotype; Pharmacokinetics

1. Introduction

It is well known that interindividual variability of therapeutic effectiveness and/or adverse reac-

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tions of drugs is often caused by polymorphism of metabolic enzymes. The correlation between genetic polymorphism (genotype) and drug disposition kinetics (phenotype) has been studied for several enzymes. *N*-Acetyltransferase (NAT) has two gene types (*NAT1* and *NAT2*), of which *NAT1* acetylates simple aromatic amines such as *p*-aminobenzoic acid and *p*-aminosalicylic acid, while *NAT2* metabolizes sulfa drugs, procainamide and isoniazid bearing amino or hydrazine groups (Evans and White 1964; Blum et al., 1990; Ohsako and Deguchi, 1990; Grant et al., 1991). The *NAT2* gene is known to show polymorphism.

In 1990, Ohsako and Deguchi (1990) sequenced cDNAs encoding human liver *NAT2*. Okumura et al. (1997) reported that *NAT2** genotypes consisted of 44% of homozygote of *NAT2*4*, 49% of heterozygotes of *NAT2*4* and mutant genes, and 7% of combinations of mutant genes. The correlation between acetylator phenotypes and polymorphic genotypes of *NAT2** gene has been clarified for isoniazid (Deguchi et al., 1990), caffeine (Yokoi et al., 1995) and procainamide (Okumura et al., 1997). However, it is not always possible to predict the interindividual variability of disposition kinetics only from the results of genotyping of *NAT2**.

Salicylazosulfapyridine (SASP) is commonly used for the treatment of ulcerative colitis and Crohn's disease. However, SASP exhibits some dose-dependent side effects (nausea, emesis, loss of appetite), which are caused predominantly by sulfapyridine (SP), a metabolite of SASP, and it is thought that the frequency of side effects is related to acetylator phenotype (Shear et al., 1986; Chiba et al., 1987). SASP is poorly absorbed from the colon in human and is subject to extensive biotransformation by colonic bacteria to SP and 5-aminosalicylic acid (5-ASA) (Peppercorn and Goldman, 1972; Das et al., 1974). After colonic absorption, these metabolites of SASP are further acetylated by NAT2 in the liver. Therefore, it is necessary to design individual dosing schedules taking into account polymorphic genotype, in order to avoid these side effects in patients.

In this study, we examined the feasibility of predicting genotype from the ratio of urinary metabolites of SASP in human, based on the putative correlation between the acetylator phenotypes of Japanese subjects administered SASP and the polymorphic genotypes.

2. Materials and methods

2.1. Materials

SASP and SP were purchased from Sigma Chemical Co., St. Louis, MO. 5-ASA was purchased from Tokyo Kasei Kogyo Co., Tokyo, Japan. *N*-Acetyl-SP (Ac-SP), acetyl-5-aminosalicylic acid (Ac-5-ASA) and propionyl-4-aminosalicylic acid were gifts of Welfide Co., Osaka, Japan. All other chemicals were of reagent grade and were used without further purification.

2.2. Methods

2.2.1. Subjects for genotyping

One hundred and twenty-six subjects, who had given their written informed consent, were included in this study; they were 83 healthy Japanese volunteers, 15 patients receiving isoniazid for tuberculosis and 28 patients receiving SASP for inflammatory bowel disease.

2.2.2. Subjects for phenotyping in serum

Phenotyping was conducted in 18 of the above patients with inflammatory bowel disease when their serum concentrations reached the steady-state level after at least two weeks of multiple dosing of SASP.

2.2.3. Subjects for phenotyping in urine

Phenotyping was conducted in other eight of the above patients with inflammatory bowel disease. The urinary samples were collected for 2 h before the trough time at 4 days after the first administration in eight patients given multiple doses of SASP p.o. administration for 8-h intervals.

2.2.4. Subjects for phenotyping for pharmacokinetics

Phenotyping for pharmacokinetics was con-

ducted in six (24–37 years) of the above healthy volunteers with various genotypes. Serial samples of blood and urine were collected at designated time intervals during 72 h after a single oral administration of SASP 3000 mg (7.52 mmol). All samples of this study were stored at −30 °C until assayed.

2.2.5. Assay for SASP

Aliquots of 0.4 ml of serum and urine were mixed well for 30 s with 0.8 ml of methanol, and each the mixture was kept for 10 min in an ice-water bath, then centrifuged at 12 000 rpm for 2 min. The supernatant was filtered through a 0.45 μm membrane filter (Millipore Ltd. Tokyo, Japan). All samples were analyzed on an HPLC system (Shimadzu, Kyoto, Japan) equipped with a Shim-pack CLC-ODS column (150 × 6.0 mm I.D., Shimadzu). The mobile phase, 60% methanol in 0.025 M phosphate buffer (pH 2.5), was pumped at a rate of 1 ml min^{-1}, and the effluent was monitored at 365 nm.

2.2.6. Assay for SASP metabolites

Concentrations of SP, Ac-SP, 5-ASA and Ac-5-ASA in serum and urine were determined by using HPLC. A 0.5 ml sample was mixed with 0.5 ml of distilled water in a 10 ml glass tube, 10 μl of anhydrous propionic acid was added, and the mixture was kept for 15 min in a dark room. Then 0.1 ml of internal standard (methanol solution of propionyl-4-aminosalicylic acid, 2 μg ml^{-1}) and 0.1 ml of 25% phosphoric acid solution were added, and the whole was mixed well for 30 s. It was extracted with 8 ml of methyl-*t*-butyl ether for 15 min, then centrifuged at 3000 rpm for 5 min. The water phase was frozen in an acetone–dry-ice bath, and 4 ml of the organic phase was transferred to another glass tube. Then 0.3 ml of 0.01 M phosphate buffer (pH 2.8) was added and the mixture was evaporated at 50 °C. The residue was filtered through a 0.45 μm membrane filter. All samples were analyzed on an HPLC system equipped with a Shim-pack CLC-ODS column (150 × 6.0 mm I.D.). The fluorescence was detected at an excitation wavelength of 310 nm and an emission wavelength of 410 nm. The mobile phase (0.05 M phosphate buffer (pH 2.5):methanol:TFA, 750:250:1) was pumped at a rate of 1 ml min^{-1}.

2.2.7. Isolation of genomic DNA

According to a conventional method, genomic DNA was extracted from 0.5 ml of whole blood with a DNA Extractor WB Kit (Wako Pure Chemical Industries Ltd. Osaka, Japan).

2.2.8. Determination of NAT2 genotype*

Four polymorphic *NAT2** alleles were determined by a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method according to Okumura et al. (1997).

2.2.9. Pharmacokinetic model for metabolic pathways of SASP

Although the metabolic pathways of SASP are complex (Schroder and Campbell, 1972), we set

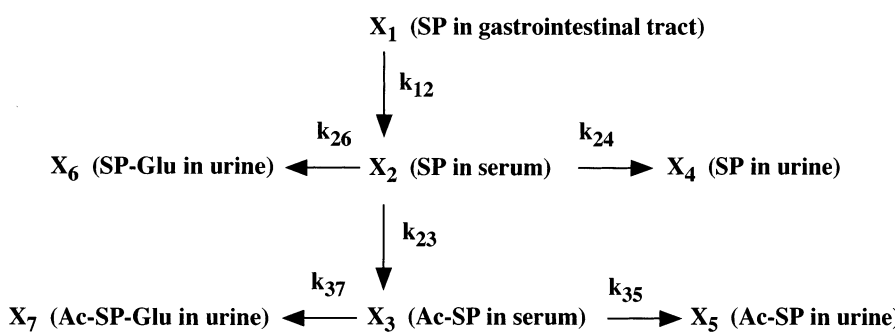

Fig. 1. Model of the metabolic pathways of SASP. For details, see the text.

Table 1
Frequency distribution of *NAT2** genotypes in the Japanese population

Genotype	Healthy subjects[a]	Patients (tuberculosis)[b]	Patients (IBD)[c]	Total
*NAT2*4/*4*	31 (37%)[d]	9 (60%)	11 (39%)	51 (40%)
*NAT2*4/*7B*	14 (17%)	3 (20%)	2 (7%)	19 (15%)
*NAT2*4/*6A*	24 (29%)	1 (7%)	10 (36%)	35 (28%)
*NAT2*4/*5B*	1 (1%)	0	1 (4%)	2 (2%)
*NAT2*7B/*7B*	0	0	1 (4%)	1 (1%)
*NAT2*7B/*6A*	12 (14%)	1 (7%)	3 (11%)	16 (13%)
*NAT2*7B/*5B*	0	0	0	0
*NAT2*6A/*6A*	1 (1%)	1 (7%)	0	2 (2%)
*NAT2*6A/*5B*	0	0	0	0
*NAT2*5B/*5B*	0	0	0	0
Total	83	15	28	126

[a] Healthy subjects living in Kanazawa city.
[b] Patients treated in Wakamatsu Hospital, Sengokuso Hospital or Kanazawa University Hospital.
[c] Patients treated in Kanazawa University Hospital.
[d] Percentages are given to the nearest whole number, and therefore totals do not necessarily come to 100%.

up a pharmacokinetic model (Fig. 1) by focusing mainly on the SASP metabolite SP as a marker for phenotyping. Here, we assume that SASP shows no enterohepatic circulation and that SP is entirely absorbed from the colon. The model consists of five differential equations (Eqs. (1)–(5)).

$$\frac{dX_1}{dt} = -k_{12}X_1 \quad (1)$$

$$\frac{dC_2}{dt} = \frac{k_{12}X_1}{V_2} = -(k_{23}+k_{24}+k_{26})C_2 \quad (2)$$

$$\frac{dC_3}{dt} = \frac{k_{23}C_2V_2}{V_3} = -(k_{35}+k_{37})C_3 \quad (3)$$

$$\frac{dX_4}{dt} = k_{24}C_2V_2 \quad (4)$$

$$\frac{dX_5}{dt} = k_{35}C_3V_5 \quad (5)$$

where X_i is the amount of each compartment (μmol). X_1, X_2, X_3, X_4, X_5, X_6 and X_7 are SP in the gastrointestinal tract, SP in serum, Ac-SP in serum, SP in urine, Ac-SP in urine, SP–glucuronide in urine and Ac-SP–glucuronide in urine, respectively. C_i and V_i are the concentration (μmol l^{-1}) and the distribution volume (l) of each compartment, respectively. C_2 and C_3 are the concentrations of SP and Ac-SP, respectively. V_2 and V_3 are the distribution volumes of SP and Ac-SP, respectively, and k_{ij} is the velocity rate from i to j. Each parameter was calculated by the non-linear least-squares method using the NONLIN program (Metzler, 1969; Yamaoka et al., 1981) on a FACOM-M360AP computer at the Information Processing Center, Kanazawa University.

3. Results

3.1. Frequency of NAT2* genotypes in Japanese subjects

The frequency distribution of *NAT2** genotypes in 126 healthy subjects and patients was determined by PCR-RFLP. As shown in Table 1, the homozygote of *NAT2*4* was the most frequent (40%), followed by heterozygotes of *NAT2*4* and mutant genes (28% *NAT*4/*6A*, 15% *NAT*4/*7B* and 2% *NAT*4/*5B*). Combinations of mutant genes accounted for 16%.

3.2. Phenotyping study of SASP metabolites in serum

The phenotyping of *NAT2** gene was conducted in 18 patients with inflammatory bowel disease at the trough time after at least 2 weeks of multiple dosing of SASP. The serum concentrations of SASP, 5-ASA, Ac-5-ASA, SP and Ac-SP were measured by using the HPLC method. The molar ratio of Ac-SP/SP or Ac-5-ASA/5-ASA was calculated as an indicator for phenotyping of SASP. Fig. 2 shows the molar ratios of Ac-SP/SP in serum, classified according to genotype of polymorphic *NAT2**. The ratios were 2.32 ± 0.97 (mean $\pm$ S.D.) for *NAT2*4/*4*, 1.77 for *NAT2*4/*7B*, 1.39 ± 0.16 for *NAT2*4/*6A*, 1.53 for *NAT2*4/*5B* and 0.25 for *NAT2*6A/*7B*. The patients who carried the homozygote of *NAT2*4* showed the highest Ac-SP/SP ratio, and the patients with the *NAT2*6A/*7B* genotype showed the lowest. On the other hand, as shown in Fig. 3, the molar ratios of Ac-5-ASA/5-ASA in serum did not correlate with the genotype.

3.3. Model-fitted versus observed serum concentrations and cumulative urinary amounts of SP and Ac-SP

Fig. 4 shows the serum concentrations and cumulative urinary amounts during 72 h after a single oral administration of SASP 3000 mg (7.52 mmol) in six healthy volunteers with various genotypes of *NAT2**, i.e. *NAT2*4/*4* (subjects A, B, C), *NAT2*4/*7B* (subject D), *NAT2*4/*6A* (subject E) and *NAT2*6A/*7B* (subject F). Each fitted line was simulated by a nonlinear least-squares regression analysis using the NONLIN program, based on Eqs. (1)–(5)). The fitted lines are all in reasonable agreement with observations. The estimated pharmacokinetic parameters of these subjects are shown in Table 2.

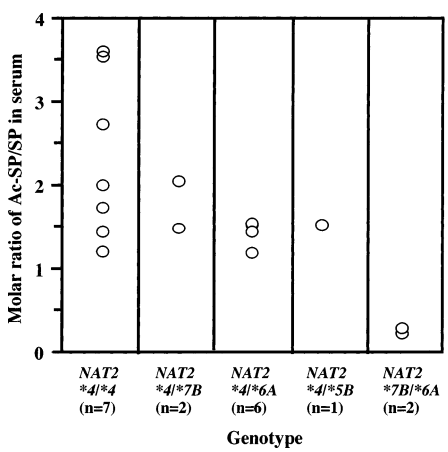

Fig. 2. Values of the molar ratio of Ac-SP and SP in serum, classified according to five genotypes of polymorphic *NAT2**.

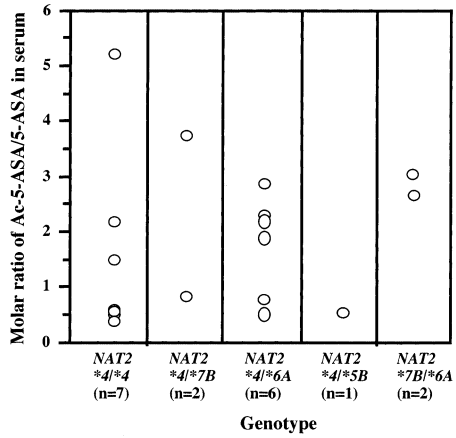

Fig. 3. Values of the molar ratio of Ac-5-ASA and 5-ASA in serum, classified according to five genotypes of polymorphic *NAT2**.

3.4. Simulated time courses of molar ratio of Ac-SP/SP in serum and urine

The time courses of molar ratio of Ac-SP/SP in serum and urine for subjects (A–F) given multiple doses of SASP (3000 mg, p.o.) at 8-h intervals were simulated by using the five differential equations (Eqs. (1)–(5)). The pharmacokinetic parameters are shown in Table 2, and the simulated results for serum (a) and urine (b) are shown in Fig. 5. In the case of multiple dosing at 8-h intervals, it was predicted that the molar ratio of Ac-SP/SP would reach the steady-state level at about 3 days in serum and at about 4 days in urine after the first administration. At the steady-state, the molar ratios of Ac-SP/SP in serum were about 2–3 for rapid acetylators (subjects A–C),

about 1–2 for intermediate acetylators (subjects D, E) and below 1 for the slow acetylator (subject F). Similarly, the molar ratios of Ac-SP/SP in urine were about 6–8 for rapid acetylators (subjects A–C), about 4–5 for intermediate acetylators (subjects D, E) and below 2 for the slow acetylator (subject F).

3.5. Phenotyping study of SASP metabolites in urine

The urinary concentrations of SP and Ac-SP at the trough time at 4 days after the first administration in eight patients given multiple doses of SASP p.o. administration for 8-h intervals were determined. Fig. 6 shows the molar ratios of Ac-SP/SP in urine, classified according to the genotype of *NAT2**. The patients who carried the homozygote *NAT2*4/*4* showed the highest Ac-SP/SP ratio (8.7 ± 3.3, mean $\pm$ S.D.) and the patient with the *NAT2*6A/*7B* genotype showed the lowest ratio (1.75).

4. Discussion

We established a method for predicting NAT2 genotypes using the molar ratio of Ac-SP/SP in urine of patients treated with SASP.

Firstly, the frequency distribution *NAT2** genotypes in the Japanese population was determined in 126 healthy subjects and patients living in the city of Kanazawa (Table 1). Our results are in close agreement with data for the Japanese population reported by Okumura et al. (1997). Although a correlation between acetylator phenotypes and *NAT2** genotypes has been demonstrated for isoniazid, caffeine and procainamide (Deguchi et al., 1990; Yokoi et al., 1995; Okumura et al., 1997), there has been no study on patients treated with SASP until now. Although SASP is poorly absorbed from the colon in human, it is biotransformed by colonic bacteria to SP and 5-ASA (Peppercorn and Goldman, 1972; Das et al., 1974). After colonic absorption, these metabolites are acetylated by NAT2 in the liver,

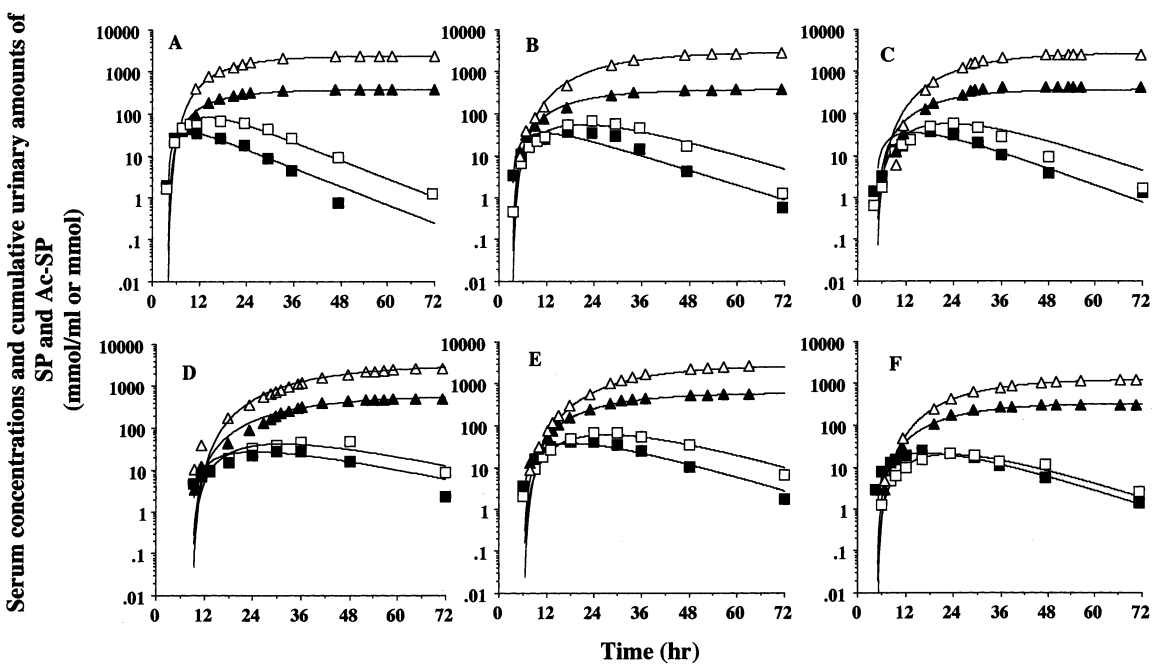

Fig. 4. Model-fitted vs. observed serum concentrations of SP (■) and Ac-SP (□) and cumulative urinary amounts of SP (▲) and Ac-SP (△) after oral administration of SASP 3000 mg (7.52 mmol) in healthy volunteers A–F. Each line was obtained by nonlinear least-squares regression analysis by the NONLIN program with Eqs. (1)–(5).

Table 2
Estimated kinetic parameters of six healthy subjects given SASP

Healthy subject	A	B	C	D	E	F
k_{12} (h^{-1})	0.0842±0.00364	0.0646±0.0137	0.0781±0.0594	0.0511±0.0513	0.0681±0.0492	0.0914±0.0882
k_{23} (h^{-1})	0.330±0.0536	0.1 86±0.0542	0.131±0.214	0.0317±0.0377	0.0879±0.0802	0.0128±0.0111
k_{24} (h^{-1})	0.0178±0.00269	0.0100±0.00281	0.00731±0.00723	0.00596±0.00690	0.00790±0.00657	0.00341±0.00299
k_{35} (h^{-1})	0.0860±0.0121	0.0446±0.00819	0.0476±0.0920	0.198±0.0609	0.0509±0.0180	0.199±0.0275
k_{26} (h^{-1})	0.00137±0.0134	0.00266±0.0161	0.00955±0.249	0.0368±0.0390	0.00286±0.0237	0.0598±0.0545
k_{37} (h^{-1})	0.164±0.0221	0.0633±0.0108	0.0669±0.0937	0.000000152 ±0.00000220	0.0745±0.0167	0.0000707 ±0.000155
V_2 (l)	31.3±20.4	42.5±20.6	56.1±79.1	85.9±119	59.6±53.9	139±126
V_3 (l)	14.8±5.4	32.9±14.3	29.2±64.0	8.23±4.16	21.7±10.6	8.86±2.00

The kinetic parameters according to the model shown in Fig. 1 were calculated by the non-linear least-squares method using a NONLIN program from the serum concentrations and cumulative urinary amounts after an oral administration of SASP 3000 mg (7.52 mmol) in six healthy subjects A–F.

and then are distributed and excreted into urine. Therefore, we examined the molar ratio of Ac-SP/SP or Ac-5-ASA/5-ASA in serum from patients treated with SASP for long periods (over 2 weeks), classified according to genotype of polymorphic *NAT2** (Figs. 2 and 3). The molar ratio of Ac-SP/SP, but not that of Ac-5-ASA/5-ASA, was found to be slightly correlated with genotype. However, the rapid acetylator phenotype with *NAT2*4/*4* gene was often indistinguishable from the intermediate acetylator phenotype with *NAT2*4/*7B*, *NAT2*4/*6A* or *NAT2*4/*5B*. This is not sufficiently informative, compared with the case of isoniazid (Deguchi et al., 1990), probably because the disposition characteristics of SASP, including its metabolic pathways, are complex compared with those of isoniazid. To establish a better phenotyping method, the diurnal changes in the concentration of SP and the lag time in the biotransformation of SASP to SP in serum and urine should be considered. Although there are many reports on the disposition kinetics of SASP (Das et al., 1973; Bates et al., 1977; Azad Khan and Truelove, 1982), no attempt has been made to calculate the pharmacokinetic parameters by using a model of the SASP metabolic pathways for various genotypes of polymorphic *NAT2**.

We examined the time courses of the serum concentrations and the urinary cumulative amounts of SP and Ac-SP after oral administration of SASP 300 mg to six healthy volunteers with various polymorphic genotypes of *NAT2** (Fig. 4). We calculated the pharmacokinetic parameters of SP according to a mode of the metabolic pathways of SASP (Fig. 1) by using nonlinear least-squares regression analysis (Table 2). We found that the rate of metabolic acetylation (k_{23}) of SP is about 0.22 h^{-1} in rapid acetylators, about 0.06 h^{-1} in intermediate acetylators and 0.013 h^{-1} in the slow acetylator. This result suggests that this pharmacokinetic parameter of SP can be used to predict the polymorphic genotype of *NAT2**.

Therefore, we tried to simulate the time course of the molar ratio of Ac-SP/SP in serum and urine following multiple dosing of SASP using the pharmacokinetic parameters of six subjects (Fig. 5). The results indicate that a period of 4 days is needed to reach the steady-state molar ratio of Ac-SP/SP, and that the diurnal oscillation for rapid acetylators is larger than that for other acetylators. But, we found that the influence of diurnal oscillation on the phenotyping is negligible. Moreover, because the difference when the ratio of Ac-SP/SP in urine for slow acetylators was subtracted from that for rapid acetylators (about 5) is larger than that in serum (about 1.7) at 96 h after the first administration, phenotyping should be more reliable using the ratio in urine (Fig. 5). In eight patients, the phenotype deter-

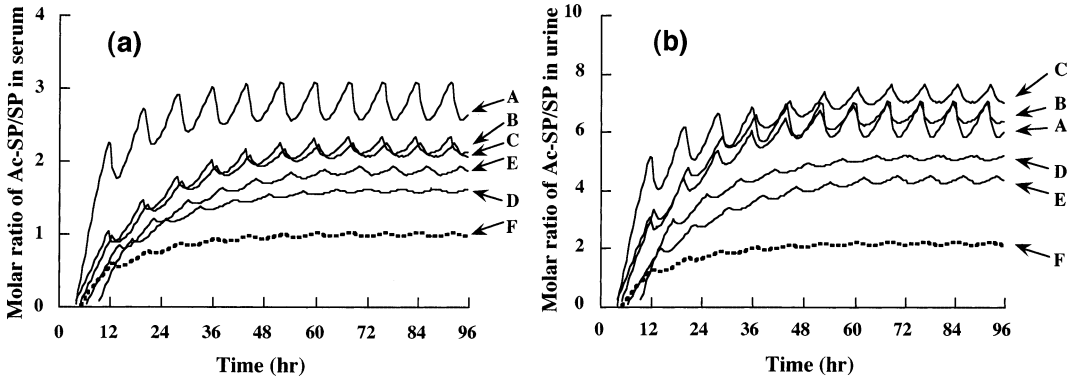

Fig. 5. Simulated time courses of the ratio of Ac-SP/SP in serum (a) and urine (b) after multiple dosing of SASP p.o. at 8-h intervals. The pharmacokinetics parameters used for each subject A–F were those given in Table 2.

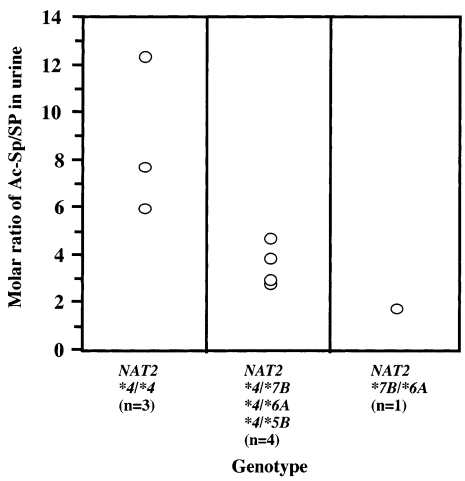

Fig. 6. Values of the molar ratio of Ac-SP and SP in urine, classified according to genotypes of polymorphic *NAT2**.

mined from the molar ratio of Ac-SP/SP in urine corresponded well to genotype (Fig. 6).

In conclusion, we were able to predict the polymorphic genotypes by means of a simple phenotyping diagnostic method based on the ratio of Ac-SP/SP in urine after 4 days of multiple dosing of SASP. This method should be clinically useful for setting individual dosing schedules of SASP in patients to avoid adverse reactions.

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