much higher than those with the wild-type genotype and thereby elicit a more extensive depletion of vitamin K-dependent coagulation proteins particularly F-VII with a short $t_{1/2}$ of 1.5–5 h.

In this context, it is also worth noting to remember that a rare complication of warfarin therapy, skin necrosis, has been reported to develop within 3–4 days of the initiation of warfarin therapy typically when a loading dose regimen of warfarin is employed in patients with heterozygous hereditary protein C deficiency.[48] Protein C is a vitamin K-dependent antithrombotic protein with a $t_{1/2}$ (6–8 h) shorter than most vitamin K-dependent clotting proteins. Thus, the administration of a loading dose of warfarin to patients with *CYP2C9* variants may cause greater depletion in Protein C rather than vitamin K-dependent clotting proteins and give rise to hypercoagulability and thrombosis in the cutaneous microvasculature. To our knowledge, however, no information is available whether the patients developing skin necrosis by warfarin therapy would carry any of the *CYP2C9* variants.

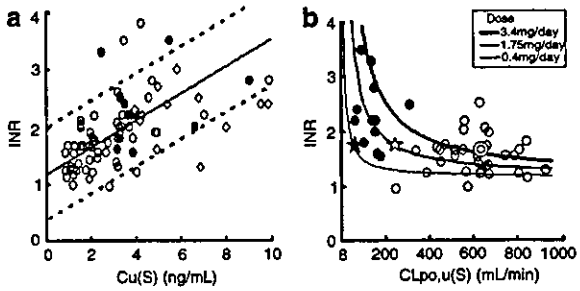

Figure 2 Relation between plasma unbound concentration for (*S*)-warfarin [Cu(*S*)] and INR obtained form Japanese patients receiving stable oral administration of racemic warfarin alone (○) and those receiving warfarin with bucolome (●)[46] or benzbromarone (◆)[49] (a, left). The solid line is the regression line for the overall data (ie, INR = 0.235 × Cu(*S*)+1.17, $P<0.05$) and broken lines depict the 95% confidence intervals of the data. The right figure (b) shows the scatter plots for the relation between unbound oral clearance for (*S*)-warfarin [$CL_{po,u}(S)$] and INR obtained from patients receiving stable oral doses of warfarin alone (○) and those receiving warfarin with bucolome (●).[46] The thick, medium and thin solid curves represent the INR–$CL_{po,u}(S)$ relation in patients receiving 3.4, 1.75 and 0.4mg/day of racemic warfarin, respectively (see details in text). The data points corresponding to typical patients with the mean $CL_{po,u}(S)$ in heterozygote and homozygote of *CYP2C9*3* are shown as open (☆) and closed (★) stars, respectively, and that for the wild-type genotype is shown as the double circle (◎). Since patients with reduced $CL_{po,u}(S)$ are located on ascending part of the hyperbolic function, they are more susceptible to develop precipitous prolongation of INR in response to a small variability in $CL_{po,u}(S)$ than those with normal metabolic activity who located on the horizontal part of the curve.

$CL_{po,u}$ for (*S*)-warfarin *vs* INR relation

As certain *CYP2C9* variants are associated with the reduced $CL_{po,u}$ for (*S*)-warfarin, it is of interest to discuss about the effect of *CYP2C9* polymorphisms on the relation between $CL_{po,u}$ for (*S*)-warfarin and INR. In our previous studies[46,49] we found that there was a weak, albeit significant ($P<0.05$), correlation between Cu for (*S*)-warfarin and INR, an index of the anticoagulation response to warfarin, in Japanese patients who received warfarin with or without CYP2C9 inhibitors (ie, bucolome and benzbromarone) (Figure 2a). The linear regression line obtained from the data was as follows:

$$\mathrm{INR} = 0.235 \cdot \mathrm{Cu}(S) + 1.17 (n = 65) \quad (1)$$

where Cu(*S*) is the plasma unbound concentration of (*S*)-warfarin. The steady-state plasma unbound concentration of (*S*)-warfarin [Cu(*S*)] during an intermittent oral administration of racemic warfarin at a dose (*D*) is a function of oral dose of (*S*)-warfarin (*D*/2), a fraction of gastrointestinal absorption (F_a) of the drug, dosing interval (τ) and unbound oral clearance for (*S*)-warfarin [$CL_{po,u}(S)$]. Assuming a complete absorption of warfarin from the intestine (ie, $F_a = 1$), Cu(*S*) will be determined by the equation shown below:

$$\mathrm{Cu}(S) = (D/2\tau)/CL_{po,u}(S) \quad (2)$$

Substituting Cu(*S*) obtained from Equation (2) into Equation (1), one can obtain the following equation:

$$\mathrm{INR} = 0.235 \cdot [(D/2\tau)/CL_{po,u}(S)] + 1.17 \quad (3)$$

Equation (3) indicates that INR is a function of D/τ and $CL_{po,u}(S)$. In order to assess whether the above equation can fit actual data, we show the scatter plots of the data for $CL_{po,u}(S)$ and INR obtained from patients receiving a long-term stable dosing of warfarin alone and those receiving warfarin with bucolome (Figure 2b).[46] As the patients who received warfarin with bucolome, a potent inhibitor of CYP2C9, showed substantially reduced $CL_{po,u}(S)$ of <300 ml/min, we can inspect whether Equation (3) adequately describes the clinical data over a wide range of $CL_{po,u}(S)$. The data obtained from patients receiving warfarin alone (○) and those received warfarin with bucolome (●) largely distribute along the simulation curves. In order to facilitate readers' understanding, we also drew the simulated curves generated by substituting three different oral doses of warfarin (ie, 3.4, 1.75 and 0.4 mg a day) for D/τ in Equation (3). These doses are the mean oral doses of warfarin obtained from Japanese patients with *CYP2C9*1/*1*, *CYP2C9*1/*3* and *CYP2C9*3/*3* genotypes.[13] The simulation curves clearly indicate that INR begins to rise precipitously as $CL_{po,u}(S)$ decreases <200 ml/min. Therefore, if warfarin therapy is initiated by a standard dosing regimen (eg, 3.4 mg/day of racemic warfarin once a day for Japanese patients) in all patients, those with the *CYP2C9*1/*3* genotype having the mean $CL_{po,u}(S)$ of 212 ml/min[12] and those received bucolome having the mean $CL_{po,u}(S)$ of 107 ml/min may experience extremely prolonged INR and bleeding complications unless the

dose of the drug is reduced promptly. In addition, when we plot the mean $CL_{po,u}(S)$ data obtained from patients with *CYP2C9*1/*1* (◎), *CYP2C9*1/*3* (☆) and *CYP2C9*3/*3* (★) genotypes,[13] it becomes evident that the doses of warfarin for the patients with *CYP2C9*3* allele are reduced to 1.75 and 0.4 mg/day to achieve a target INR of 1.5–2.0 that can be obtained with the dose of 3.4 mg/day for those with the wild-type *CYP2C9* genotype.

Susceptibility to bleeding complication and instability of INR

Figure 2b also indicates that patients with reduced $CL_{po,u}(S)$ are more susceptible to precipitous prolongation of INR in response to a small reduction in $CL_{po,u}(S)$ or the hepatic CYP2C9 activity than those with normal metabolic activity. In this context, patients with defective *CYP2C9* alleles may be particularly vulnerable to bleeding complications due to metabolic inhibition. For instance, a representative Japanese patient with the wild-type *CYP2C9* genotype having $CL_{po,u}(S)$ of 625 ml/min[13] (◎) will exhibit INR of 1.6 at an oral dose of warfarin of 3.4 mg/day (Figure 2b). If the patient is given a CYP2C9 inhibitor that reduces $CL_{po,u}(S)$ by 50% (ie, 300 ml/min reduction from 625 to 325 ml/min), his or her INR would increase from 1.6 to 2.0. In contrast, in a typical Japanese patient with the *CYP2C9*3/*3* genotype (★) exhibiting INR of 1.7 at a warfarin dose of as low as 0.4 mg/day, only a small reduction of $CL_{po,u}(S)$ (eg, 30 ml/min) would be associated with a comparable increase in INR from 1.7 to 2.2. As such, the genetic polymorphism of *CYP2C9* may increase the susceptibility to unstable INR status thereby bleeding complications.

Figure 3 shows the relation between plasma concentrations of prothrombin fragments 1+2 (ie, F_{1+2}) and INR obtained from Japanese patients receiving a long-term warfarin therapy. As plasma concentration of F_{1+2} is associated with thrombin generation, the administration of warfarin suppresses plasma F_{1+2} concentrations. In addition, there is an apparently hyperbolic relation between the parameters (Figure 3) as shown in a previous study.[50] As patients having defective *CYP2C9* variants or receiving coadministration of CYP2C9 inhibitors would have higher plasma (*S*)-warfarin concentrations and thereby reduced plasma F_{1+2} concentrations, they are likely more susceptible to develop a large variability in INR as compared with those with normal metabolic activity for CYP2C9.

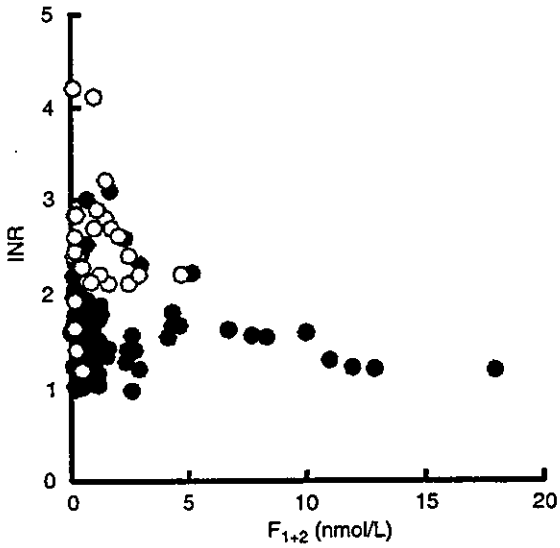

Figure 3 The relation between plasma concentrations of prothrombin fragment 1+2 (F_{1+2}), a marker of thrombin generation, and INR obtained from Japanese (●) and Caucasian (○) patients receiving stable oral doses of warfarin. Note that there is a quasi-hyperbolic relation between the two parameters, suggesting a precipitous prolongation of INR in response to a small variability in the thrombin generation where the generation of thrombin is more extensively suppressed. This implies that patients with *CYP2C9*3* or those with coadministration of CYP2C9 inhibitor may be more susceptible to unstable INR response than those with less intensive anticoagulation suppression (see details in text).

To validate our theoretical consideration made in Figures 2b and 3, we analyzed the clinical data obtained from a retrospective cohort study assessing the relation between $CL_{po,u}(S)$ and intrapatient variability of INR over 1 year during warfarin therapy (unpublished data). We sorted the data according to $CL_{po,u}(S)$ and calculated an index of intraindividual variability of INR defined as the percentage of the difference between the maximum and minimum INR normalized to the minimum INR value (ie, $\%(INR_{max}-INR_{min})/INR_{min} \times 100$) within the same subjects. Since $CL_{po,u}(S)$ obtained from patients distributed widely, we arbitrarily grouped patients into those receiving a CYP2C9 inhibitor (ie, bucolome), those with $CL_{po,u}(S)$ <270 ml/min (arbitrarily defined as the low clearance group) and those with $CL_{po,u}(S)$ of >1000 ml/min (the high clearance group). Those receiving bucolome was shown to have the mean $CL_{po,u}$ of 107 ml/min ($n=21$).[46] As shown in Figure 4, statistical comparisons for the variance obtained from the respective groups indicated that SDs of percentages relative changes in INR obtained from the bucolome and the low clearance groups were significantly greater than that obtained from the high clearance group. This finding implies that patients with low $CL_{po,u}(S)$ may be associated with a greater variability in INR than those with high $CL_{po,u}(S)$. Our data would also account for the previous findings indicating that patients with defective *CYP2C9* variants are associated with a greater chance of developing bleeding complications and/or unstable INR status.[31] Obviously, further studies with a greater number of patients should be performed under a prospective

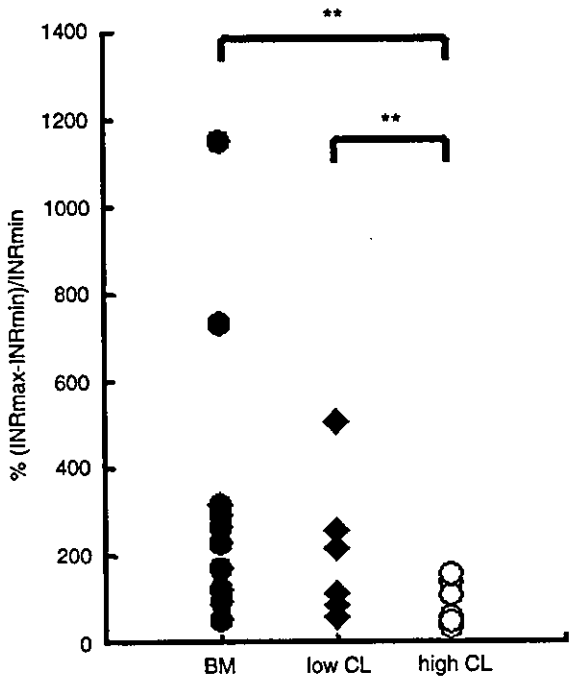

Figure 4 The intraindividual variability in INR status assessed by percentage changes in the maximum minus minimum INR values normalized to the minimum INR (ie, $(INR_{max}-INR_{min})/INR_{min} \times 100$) obtained from ambulatory patients receiving warfarin in a retrospective cohort study performed over a year. The patients who received a CYP2C9 inhibitor, bucolome, are shown as closed circle (●, BM, $n=10$), and those having $CL_{po,u}$ for (*S*)-warfarin of <270 ml/min (◆, the low clearance group; low CL, $n=7$) and those with $CL_{po,u}$ for (*S*)-warfarin of >1000 ml/min (○, the high clearance group; high CL, $n=7$) were sorted out from the overall data. The mean $CL_{po,u}(S)$ for the BM group was 107±68 ml/min. Note that the BM and the low CL groups exhibit significantly greater variabilities in INR than the high CL group. **$P<0.01$ as compared with the high CL group based upon the statistical comparisons for the SDs obtained from the respective groups.

experimental protocol in order to confirm our hypothetical consideration made based upon a rather limited number of patients.

OTHER FACTORS CONTRIBUTING TO VARIABLE RESPONSE TO WARFARIN

While the genetic polymorphisms of *CYP2C9* is an important factor associated with interindividual variability of $CL_{po,u}(S)$ and thereby that in the anticoagulation response to warfarin, other factors such as age,[33] drug interaction with concomitantly administered drugs,[46,49] food and other undefined factors are also considered to be involved in the variable anticoagulation response to the drug. It has been suggested that an age-related reduction in liver size[51] would account for reductions in the metabolic activity of warfarin. We have already discussed the effects of concomitant administration of CYP2C9 inhibitors (eg, bucolome, benzbromarone) on the dose, $CL_{po,u}(S)$ and interindividual variability of INR. It is worth noting that the coadministration of a potent CYP3A4 inhibitor (eg, diltiazem) does not have influence on the anticoagulation response to warfarin.[52] Abernethy *et al*[52] demonstrated that the coadministration of oral diltiazem 120 mg three times daily produced a small (20%) reduction in the total (bound + unbound) clearance of (*R*)-warfarin, but not (*S*)-warfarin, after intravenous administration of warfarin as compared with the respective control values. Because the anticoagulation of (*R*)-warfarin is three to five time less potent than (*S*)-warfarin and CYP3A4 is involved only partly in the metabolism of (*R*)-warfarin, a potent inhibition of CYP3A4 may not necessarily be associated with a significant change in the INR status. Most of the important drug interactions with warfarin that were associated with fatal or severe bleeding complications were attributable to CYP2C9 inhibitors (eg, phenylbutazone, sulfinpyrazone, bucolome).

Pharmacodynamic factors associated with a large interindividual variability of warfarin response deserve some comments. Excessive consumption of vitamin K-rich diets (eg, green vegetables) has been claimed to attenuate the anticoagulation effect of warfarin.[34] In contrast, the administration of certain antibiotics that interfere with the production of vitamin K by gut flora has been suggested to exaggerate the anticoagulation response to warfarin. These data suggest that variability of vitamin K intake would be an important factor associated with the intraindividual variability of INR in patients receiving warfarin. It has been suggested that the interindividual variability in the expression of calumenin (ie, an inhibitory protein for VKOR complex) in the liver may have a pivotal role in the sensitivity of VKOR and vitamin K-dependent γ-carboxylation system in rats[38] and possibly in humans. Genetic polymorphisms of vitamin K-dependent coagulation factors may also affect the responsiveness of warfarin. As there is a substantial population difference (Asians *vs* Caucasians) and disease-specific difference (eg, those with prosthetic valve) in the target INR, there must be unknown pharmacodynamic factor(*S*) associated with variable response to warfarin. Collectively, there is a relative paucity of knowledge for pharmacodynamic parameters as compared with that for pharmacokinetic parameters associated with the interindividual variability of anticoagulation response to warfarin.

CONCLUSION

In the present article we have summarized the implications of *CYP2C9* polymorphisms in the pharmaco-

kinetics of pharmacologically more active (*S*)-enantiomer of warfarin in the light of its contribution to the interindividual variability of warfarin dosage, risks of bleeding complications and stability of anticoagulation response. Based upon the accumulated information, consensus appears to emerge that patients with either heterozygous or homozygous *CYP2C9*3* variant and those of combined heterozygote of *CYP2C9*2* and **3* (ie, *CYP2C9*2/*3*) would have a substantially reduced dose requirement for the maintenance dose of warfarin, need longer time to achieve stable warfarin dosing and carry higher risks of developing above-range INRs associated with serious bleeding complications, particularly during the early phase of warfarin therapy as compared with those with the homozygous wild-type *CYP2C9* genotype.

There is a possibility that different *CYP2C9* variants may have different magnitude of effect on *in vitro* and *in vivo* metabolic activity for this CYP isoform. While both *CYP2C9*2* and *CYP2C9*3* variants have been implicated with substantially reduced enzyme activities in *in vitro* experiments, the effect of *CYP2C9*3* mutation on the *in vivo* clearance of CYP2C9 substrate (eg, (*S*)-warfarin) may be greater than that of *CYP2C9*2*. While Japanese patients with the *CYP2C9*1/*3* genotype were associated with markedly reduced $CL_{po,u}$ for (*S*)-warfarin as compared with those with the homozygous wild-type CYP2C9 genotype, Caucasian patients with either the heterozygous *CYP2C9*2* or *CYP2C9*3* variant would exhibit only marginal reduction in $CL_{po,u}$ for (*S*)-warfarin.[14,15]

The population mean of body weight-normalized $CL_{po,u}$ for (*S*)-warfarin obtained from Japanese patients was greater than that obtained from Caucasians.[14] Caucasians have greater allelic frequencies of *CYP2C9*2* and *CYP2C9*3* than Asians and African-Americans indicating substantial population differences in the genetic polymorphism of this CYP isoform (Tables 1 and 2). To our surprise, however, the population difference in the mean $CL_{po,u}$ for (*S*)-warfarin was not fully accounted for a greater frequencies of *CYP2C9* variants alleles in Caucasians than Japanese patients, because the mean $CL_{po,u}$ obtained from Japanese patients genotyped as having homozygous *CYP2C9*1* allele was still significantly greater than that obtained from Caucasian patients with the corresponding *CYP2C9* genotype. In this context, further studies are necessary to explore concealed parameters (eg, unidentified functionally defective variants in the coding regions and/or noncoding regions of *CYP2C9*, food and environmental factors) that may account for this apparently paradoxical finding.

The *CYP2C9* polymorphisms may also have substantial influence on the developmental changes in the metabolic activity of this CYP isoform. Japanese children with the *CYP2C9*1/*3* genotype appear to show an attenuated increase in the *in vivo* CYP2C9 activity measured by $CL_{po,u}$ for (*S*)-warfarin, while those with the homozygous wild-type genotype exhibit substantial increase in this parameter particularly during prepubertal period (<12 years). It remains to be confirmed if a similar finding can be observed in different ethnic populations.

As the magnitude of reduction in $CL_{po,u}$ for (*S*)-warfarin associated with *CYP2C9*3* polymorphism largely parallels with that observed in the maintenance dose of racemic warfarin, there is a possibility that the genotyping of *CYP2C9* prior to the warfarin therapy may be useful for estimating individualized initial dose of the drug. Simulations of plasma concentration–time curves of (*S*)-warfarin in patients with different genotypes of *CYP2C9*3* variant given a standard dose of the drug furnish a plausible explanation for the clinical observations that patients with *CYP2C9* variants take longer to achieve a stable warfarin response and are more susceptible to develop above-range INR and bleeding complications particularly during the early phase of warfarin therapy.

In addition, because the relation between $CL_{po,u}$ for (*S*)-warfarin and INR is hyperbolic, patients with reduced CYP2C9 activity due to genetic polymorphism of *CYP2C9* or coadministration of CYP2C9 inhibitor may more likely develop precipitous prolongation of INR in response to a small reduction in $CL_{po,u}$ for (*S*)-warfarin as compared with those with normal CYP2C9 activity. The fact that there is a quasi-hyperbolic relation between plasma concentrations of F_{1+2} (ie, a marker of thrombin generation) and INR also suggests a greater fluctuation of INR at a greater anticoagulation status. In this context, the genotyping of *CYP2C9* variants may be useful to circumvent unexpected severe adverse reactions of warfarin.

Pharmacodynamic parameters associated with the interindividual variability in the sensitivity to warfarin include genetic polymorphisms of VKOR-related system or vitamin K-dependent coagulation proteins, aging, variable intake of vitamin K and other unidentified environmental factors. To determine whether a routine genomic screening of *CYP2C9* for patients undergoing warfarin therapy would be cost-effective, further studies are required to quantify the contribution of the *CYP2C9* polymorphisms to the overall inter- and intrapatient variability in the INR response to warfarin relative to those attributable to all other genetic, clinical and environmental variables.

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DUALITY OF INTEREST

None.

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Short Communication

SELECTIVITIES OF HUMAN CYTOCHROME P450 INHIBITORS TOWARD RAT P450 ISOFORMS: STUDY WITH cDNA-EXPRESSED SYSTEMS OF THE RAT

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ABSTRACT:

The aim of this study was to determine the selectivities of chemical inhibitors for human cytochrome P450 (P450) isoforms toward the corresponding rat P450 isoforms by using cDNA-expressed rat P450s (CYP1A2, CYP2A1, CYP2C6, CYP2C11, CYP2D2, CYP2E1, CYP3A1, and CYP3A2). Among the inhibitor probes for human P450s used in this study, only sulfaphenazole showed a selective inhibitory effect on the activity of the corresponding rat P450 isoform (CYP2C6). Furafylline also preferentially inhibited the activity of rat CYP1A2. However, methoxalen and ketoconazole more strongly inhibited the activities of other P450 isoforms than those of the corresponding rat P450 isoforms, CYP2A1 and CYP3A1/2, respectively. On the other hand, quinidine and aniline had little effect on the activities of the corresponding rat P450 isoforms, CYP2D2, and rat CYP2E1, respectively. These results suggest that chemical probes that have been used for human P450 isoforms do not always exhibit the same selectivity for the corresponding rat P450 isoforms. However, it appears that sulfaphenazole can be used as a selective inhibitor for rat CYP2C6. In addition, furafylline may also be a relatively selective inhibitor for rat CYP1A2.

Cytochrome P450 (P450[1]) enzymes comprise a superfamily of hemoproteins (Nelson et al., 1996), and three families (CYP1, CYP2, and CYP3) are mainly involved in the metabolism of drugs in both humans and rats (Nedelcheva and Gut, 1994).

Recent advances in research for chemical inhibitors of human P450s have greatly facilitated the characterization of catalytic specificities of individual P450 isoforms involved in drug metabolism. Chemical inhibitors are useful tools for determining the roles of individual P450s involved in drug metabolism in human liver microsomes. However, it is still difficult to determine the roles of P450 isoforms involved in drug metabolism in rat liver microsomes by using chemical inhibitors. This is because the specificities of chemicals used as inhibitor probes for rat P450 isoforms have not been thoroughly evaluated (Eagling et al., 1998).

In the present study, the effects of chemical inhibitors that have been used as inhibitor probes for human P450 isoforms on the corresponding rat P450 isoforms were studied by using cDNA-expressed rat P450s (CYP1A2, CYP2A1, CYP2C6, CYP2C11, CYP2D2, CYP2E1, CYP3A1, and CYP3A2). These isoforms used in this study were selected based on its abundance in rat liver or its significance in metabolism. The chemical inhibitors used in the present study were furafylline, methoxalen, sulfaphenazole, quinidine, aniline, and ketoconazole, which are potent inhibitors of human CYP1A2 (Tassaneeyakul et al., 1994), CYP2A6 (Yamazaki et al., 1994; Koenigs et al., 1997), CYP2C9 (Newton et al., 1995), CYP2D6 (Newton et al., 1995), CYP2E1 (Nakajima et al., 1999), and CYP3A4 (Baldwin et al., 1995; Bourrié et al., 1996), respectively.

[1] Abbreviations used are: P450, cytochrome P450; POD, phenacetin *O*-deethylation; T7H, testosterone 7α-hydroxylation; DFH, diclofenac 4-hydroxylation; T16H, testosterone 16α-hydroxylation; BLH, bufuralol 1′-hydroxylation; PNPH, *p*-nitrophenol 2-hydroxylation; MD4H, midazolam 4-hydroxylation; HPLC, high-performance liquid chromotography.

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Experimental Procedures

Chemicals. Ketoconazole was a gift from Janssen Research Foundation (Beerse, Belgium). Midazolam and 4-hydroxymidazolam were gifts from F. Hoffmann-La Roche (Basel, Switzerland). Zaltoprofen was a gift from Zeria Pharmaceutical (Tokyo, Japan). Bufuralol hydrochloride, 1′-hydroxybufuralol and 4-hydroxydiclofenac were purchased from BD Gentest (Woburn, MA). 7α-Hydroxytestosterone, 16α-hydroxytestosterone, and furafylline were purchased from Ultrafine Chemicals (Manchester, UK). Methoxalen was purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA). *p*-Nitrophenol was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Acetaminophen, caffeine, diclofenac, *p*-nitrocatechol, phenacetin, testosterone, sulfaphenazole, quinidine, and quinine were purchased from Wako Pure Chemicals (Osaka, Japan). Other chemicals were of the highest grade commercially available.

cDNA-Expressed P450. Microsomes prepared from baculovirus-infected insect cells expressing CYP1A2 (lot 1), CYP2C6 (lot 1), CYP2C11 (lot 1), CYP2D2 (lot 1), CYP3A1 (lot 1), and CYP3A2 (lot 1) and those from human B-lymphoblastoid cells expressing CYP2A1 (lot 7) and CYP2E1 (lot 6) were obtained from BD Gentest. All recombinant P450s were coexpressed with NADPH-P450 oxidoreductase. Recombinant CYP2C6, CYP2C11, CYP3A1, and CYP3A2 were coexpressed with cytochrome b_5.

Incubation Conditions. On the basis of the results of our previous study (Kobayashi et al., 2002), phenacetin *O*-deethylation (POD), testosterone 7α-hydroxylation (T7H), diclofenac 4-hydroxylation (DFH), testosterone 16α-hydroxylation (T16H), bufuralol 1′-hydroxylation (BLH), *p*-nitrophenol 2-hydroxylation (PNPH) and midazolam 4-hydroxylation (MD4H) were chosen as markers for rat CYP1A2, CYP2A1, CYP2C6, CYP2C11, CYP2D2, CYP2E1, and CYP3A1/2-mediated activities, respectively. A typical incubation mixture (0.25 ml total volume) contained 0.1 mM EDTA, 100 mM potassium phosphate buffer (pH 7.4), an NADPH-generating system (0.5 mM $NADP^+$, 2 mM glucose 6-phosphate, 1 IU/ml of glucose-6-phosphate dehydrogenase, and 4 mM $MgCl_2$), a substrate and cDNA-expressed P450. The reaction was initiated

TABLE 1

Substrate concentrations, incubation times, contents of P450, and internal standards used in the present study

Reaction	Substrate	Incubation Time	P450	Internal Standard
	μM	*min*	*pmol*	*μg*
POD	Phenacetin (10)	15	5	Caffeine (0.25)
DFH	Diclofenac (25)	20	5	Zaltoprofene (0.1)
BLH	Bufuralol (10)	15	0.5	None
PNPH	*p*-Nitrophenol (200)	60	5	Phenacetin (0.125)
MD4H	Midazolam (25)	20	10	Nitrazepam (0.125)
T7H/T16H	Testosterone (25)	30	10	None

by the addition of the NADPH-generating system following a 1-min preincubation at 37°C. All reactions were performed in the linear range with respect to P450 concentration and incubation time. After the reaction had been stopped by the addition of 100 μl of ice-cold acetonitrile, an internal standard was added. The mixtures were centrifuged at 13,000*g* for 10 min, and the supernatants (each 100 μl) were analyzed by HPLC as described below. The substrate concentration, incubation time, content of cDNA-expressed P450, and amount of internal standard used for each assay are listed in Table 1. Testosterone was dissolved in methanol and added to the incubation mixture at a final methanol concentration of 1%. The other chemicals were dissolved in methanol and added to each test tube. After evaporation with vacuum evaporator, the incubation mixture except microsomes and NADPH-generating system was added, and the compounds were redissolved. Samples for determination of POD activity were evaporated by a vacuum evaporator for 15 min after the centrifugation, and the remaining samples (each 100 μl) were analyzed. Since furafylline and methoxalen are mechanism-based inhibitors, these chemicals were preincubated at 37°C for 30 min with microsomes in the presence of NADPH-generating system before adding substrate.

HPLC Analysis. Determination of respective metabolites was carried out using a Hitachi HPLC system (Tokyo, Japan) consisting of an L-7100 pump, an L-7400 UV detector, an L-7485 intelligent spectrofluorometer, an L-7200 autosampler and a D-7500 integrator and a CAPCELL PAK C_{18} UG120 column (4.6 mm × 250 mm, 5 μm; Shiseido, Tokyo, Japan). The activities of POD, DFH, BLH, PNPH, MD4H, T7H and T16H were determined as described elsewhere (Kobayashi et al., 2000, 2002).

Results and Discussion

Among the chemical inhibitor probes of human P450 isoforms used in this study, only sulfaphenazole showed a selective inhibitory effect on the corresponding rat P450 isoform. As shown in Fig. 1A, sulfaphenazole, a potent inhibitor of human CYP2C9, inhibited only CYP2C6-mediated activity at a concentration as low as 1 μM. Although CYP2C11-mediated activity was also inhibited by sulfaphenazole, it was inhibited to a lesser degree than that of CYP2C6. CYP3A1- and CYP3A2-mediated activities were inhibited by sulfaphenazole but only to 50% of the control level even at the concentration of 100 μM. Very little or no inhibition was observed for CYP1A2-, CYP2A1-, CYP2D2-, and CYP2E1-mediated activities. These findings suggest that sulfaphenazole at low concentrations (<10 μM) could be used as a selective inhibitor probe of CYP2C6.

Furafylline, a potent inhibitor of human CYP1A2, potentially inhibited rat CYP1A2-mediated POD activity (Fig. 1B). At concentrations of more than 1 μM, more than 80% of the activity was inhibited. Although CYP2C6-mediated activity was also inhibited by furafylline, the extent of inhibition was less than that of CYP1A2. Furafylline showed a weak inhibitory effect on CYP2A1- and CYP2C11-mediated activities (<30% of the control level at 100 μM), but it showed no apparent inhibitory effect on CYP2D2-, CYP2E1-, CYP3A1/2-mediated activities. These findings suggest that it is possible to use furafylline as a relatively selective inhibitor of rat CYP1A2.

In contrast to sulfaphenazole and furafylline, methoxalen and ketoconazole, which are potent inhibitors of human CYP2A6 and CYP3A1/2, respectively, did not show a selective inhibitory effect on the activities of the corresponding rat P450 isoforms. As shown in Fig. 1C, methoxalen inhibited CYP2A1-mediated activity in a concentration-dependent manner (Fig. 1C). However, more potent inhibitory effects on CYP1A2-, CYP2C6-, and CYP2C11-mediated activities were observed. Similarly, ketoconazole inhibited CYP3A1- and CYP3A2-mediated activities in a concentration-dependent manner (Fig. 1D). However, ketoconazole inhibited CYP1A2- and CYP2C6-mediated activities by more than 50% at a concentration of 10 μM. These findings suggest that methoxalen and ketoconazole are not selective inhibitors of CYP2A1 and CYP3A1/2, respectively.

On the other hand, aniline and quinidine, selective inhibitors of human CYP2E1 and CYP2D6, respectively, did not show apparent inhibitory effects on the activities of the corresponding rat P450 isoforms. As shown in Fig. 1E, aniline showed little effect on rat CYP2E1-mediated activity. Aniline inhibited CYP1A2- and CYP2C6-mediated activities, but its effect was weak even at the concentration of more than 100 μM. Quinidine also showed little effect on CYP2D2-mediated activity at a concentration of 10 μM (Fig. 1F), but CYP2C6-mediated activity was inhibited by quinidine even at a concentration of 0.1 μM. Since quinine, a diastereomer of quinidine, is known to be a more efficient inhibitor of rat CYP2D-mediated activity in rat liver microsomes (Kobayashi et al., 1989), the effect of quinine on CYP2D2-mediated activity was examined. As expected, 10 μM of quinine inhibited CYP2D2-mediated activity by more than 90%, but CYP2C6- and CYP2C11-mediated activities were also inhibited by about 70% (data not shown). These findings suggest that not only quinidine but also quinine are not selective inhibitors of CYP2D2.

The results of the present study suggest that considerable differences exist between the selectivities of chemical inhibitors of human and rat P450 orthologues. Boobis et al. (1990) suggested the following three possible reasons for species differences in the effects of chemical inhibitors on drug metabolism in vitro: 1) the active site differs in species, 2) the isoform-catalyzing metabolism differs in species, 3) the inhibition is not via direct competition at the active site and the inhibitory site differs in species. In the present study, cDNA-expressed systems were used for screening of selectivity and comparative potency of several inhibitors. Under these conditions, our data indicated that chemicals used as inhibitor probes of human P450 isoforms are not always appropriate for use as inhibitor probes of rat P450 isoforms. This finding suggests that the active site and the inhibitory site differ in species depending on the isoforms of P450 studied. As shown in Fig. 1, CYP2D2-mediated activities in some cases were higher than 100% of control. Except for assay of testosterone metabolism, organic solvent was not included in the incubation mixture. Therefore, the higher activities did not result from the effect of solvent. In addition, the calibration curves were linear ($r > 0.999$), although no internal standard was used in the assay for bufuralol 1′-hydroxylation. It was thought that the control activities were slightly low, although the reason is unclear.

In conclusion, it appears that chemical inhibitors used as inhibitor probes of human P450 isoforms do not exhibit the same selectivities in humans and rats. However, it is possible to use sulfaphenazole as a selective inhibitor for rat CYP2C6. Furafylline also appears to be a relatively selective inhibitor for rat CYP1A2.

Finally, caution must be exercised when comparing the effects of inhibitors between rats and humans. In addition, using cDNA-

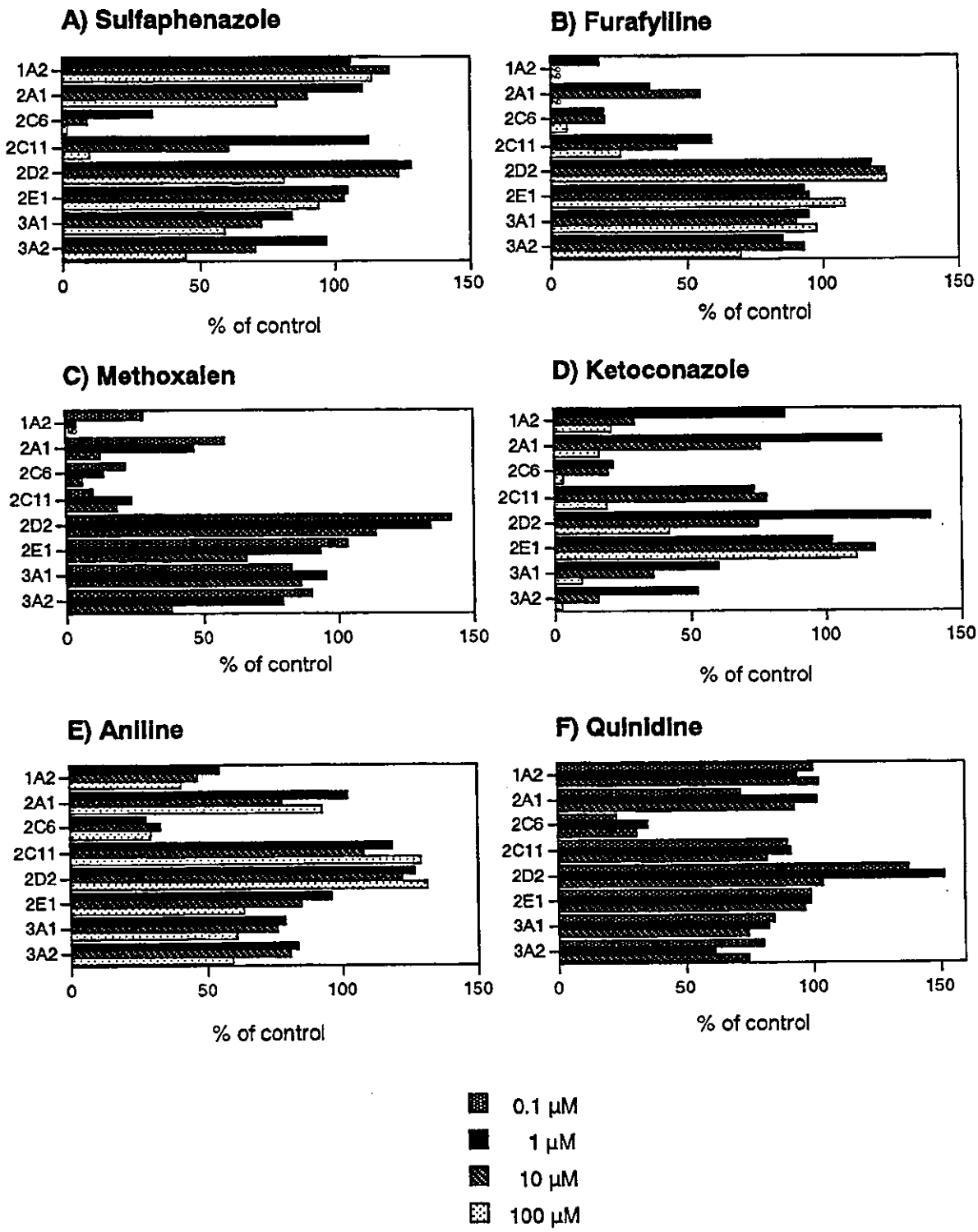

FIG. 1. *Effects of sulfaphenazole (A), furafylline (B), methoxalene (C), ketoconazole (D), aniline (E), and quinidine (F) on CYP1A2-mediated POD, CYP2A1-mediated T7H, CYP2C6-mdiated DFH, CYP2C11-mediated T16H, CYP2D2-mediated BLH, CYP2E1-mediated PNPH, and CYP3A1/2-mediated MD4H activities in cDNA-expressed rat P450s.*

Substrates (phenacetin, testosterone, diclofenac, bufuralol, *p*-nitrophenol, and midazolam) were incubated at 37°C with corresponding cDNA-expressed rat P450s. Substrate concentrations, incubation times, and contents of P450 used are shown in Table 1. Each column represents the mean of duplicate experiments. Control activities for each reaction are 10.0 pmol/min/pmol P450 for CYP1A2-meadiated POD activity, 0.6 pmol/min/pmol P450 for CYP2A1-meadiated T7H activity, 15.4 pmol/min/pmol P450 for CYP2C6-meadiated DFH activity, 14.8 pmol/min/pmol P450 for CYP2C11-meadiated T16H activity, 5.9 pmol/min/pmol P450 for CYP2D2-meadiated BLH activity, 8.7 pmol/min/pmol P450 for CYP2E1-meadiated PNPH activity, 1.9 pmol/min/pmol P450 for CYP3A1-meadiated MD4H activity and 5.7 pmol/min/pmol P450 for CYP3A2-meadiated MD4H activity. ND < 2 pmol/min/pmol P450 for POD activity, ND < 0.3 pmol/min/pmol P450 for DFH activity, ND < 0.2 pmol/min/pmol P450 for T7H, T16H, PNPH, and MD4H activities, and ND < 0.02 pmol/min/pmol P450 for BLH activity.

expressed system to evaluate the selectivity of chemical inhibitors cannot present an overall picture, and the selectivity may differ when the inhibitors were used in liver microsomes. Further investigation using chemical inhibitors is needed to determine the roles of individual P450s in drug metabolism by rat liver microsomes.

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Mexiletine carbonyloxy β-D-glucuronide: a novel metabolite in human urine

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1. The study was performed to isolate and characterize a glucuronic acid conjugate of mexiletine that releases mexiletine on acid hydrolysis from urine samples obtained from healthy volunteers following a single oral dose of mexiletine.

2. The $[M - H]^-$ ion of the isolated metabolite was observed at *m/z* 398 in the negative electrospray ionization mass spectrum. This mass number was 44 higher than that of the product generated when mexiletine is subjected to direct glucuronidation. In positive-ion mode, collision-induced dissociation of the quasimolecular ion $[M + NH_4]^+$, *m/z* 417, gave product ions at *m/z* 224, 180 and 58. These mass spectral data indicated that the metabolite contained a carbonyloxy moiety in its structure in addition to mexiletine and a glucuronic acid moiety.

3. The presence of this carbonyloxy moiety was further supported by the following chemical reactions. When the metabolite was hydrolysed with an aqueous solution of 1 M sodium hydroxide at room temperature, mexiletine was released, whereas the *N*-methoxycarbonyl derivative of mexiletine was obtained after treatment of the metabolite with methanolic sodium hydroxide solution.

4. The results indicated that the structure of the isolated metabolite was the *N*-carbonyloxy β-D-glucuronic acid conjugate of mexiletine.

Introduction

Mexiletine (MX) [2-(2,6-dimethylphenoxy)-1-methylethylamine], an orally effective antiarrhythmic agent with sodium channel-blocking properties, is used for the treatment of ventricular arrhythmia (Campbell *et al.* 1978, Murray *et al.* 1989). After oral administration to man, MX is completely absorbed and more than 80% of the dose is recovered from urine (Haeselbarth *et al.* 1981). Since less than 10% of the dose is excreted in unchanged form in the urine (Pentikainen *et al.* 1983), MX is considered to be eliminated predominantly by metabolism. MX has various metabolic pathways, including oxidation of the carbon and nitrogen atoms, deamination, reduction, and conjugation (Beckett and Chidomere 1977). Hydroxylation is a major route of metabolism for MX in humans, resulting in the formation of 4-hydroxymexiletine (4HM) and 2-hydroxymexiletine (2HM). Because the polymorphic enzyme, CYP2D6, predominantly catalyses these latter hydroxylation pathways (Nakajima *et al.* 1998), several *in vivo* studies have been performed to define the role of CYP2D6 in MX pharmacokinetics (Broly *et al.* 1991,

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Turgeon *et al.* 1991, Lledo *et al.* 1993). Some reports, however, have indicated that total urinary recovery of these hydroxylated metabolites was less than 30% of the dose when MX was orally administered to healthy volunteers (Broly *et al.* 1991, Turgeon *et al.* 1991).

Glucuronidation is another major elimination pathway of MX. Approximately 30% of the dose is excreted as MX glucuronide, which is determined in terms of MX content released by acidic or enzymatic hydrolysis (Prescott *et al.* 1977, Abolfathi *et al.* 1993, Ueno *et al.* 2002), but little is known about the chemical structure of glucuronic acid conjugates of MX. Carbamoyl glucuronide conjugates of drugs containing a primary amino group (i.e. tocainide, rimantadine and mofegiline) have been identified as human urinary metabolites (Brown *et al.* 1990, Kwok *et al.* 1990, Dow *et al.* 1994). These metabolites are presumably formed in two steps: the amino group reacts with carbon dioxide to form a carbamic acid, and subsequently the carbamic acid moiety is conjugated with glucuronic acid. If the MX glucuronide has a carbamoyl moiety, the parent amine compound, MX, should be detected after chemical or enzymatic hydrolysis, because of the chemical instability of the carbamic acid intermediate.

The objectives of the current study were to isolate the MX glucuronide, to confirm the presence of a carbamoyl moiety and to quantify the urinary excretion ratio of the glucuronide. To avoid degradation of MX glucuronide due to the chemical instability of carbamic acid derivatives, all isolation and analytical processes were conducted under neutral conditions.

Materials and methods

Chemicals and reagents

MX, 4HM and 2HM were synthesized by Boehringer Ingelheim Pharma KG (Ingelheim, Germany) with > 98% chemical purity as determined by high-performance liquid chromatography (HPLC) analysis. 1-(2′,6′Dimethylphenoxy)-2-propanol (DMPL), 1-(2′,6′-dimethylphenoxy)-2-hydroxyaminopropane (*N*-hydroxymexiletine, NHM), 1-(2′,6′-dimethylphenoxy)-2-propanone (DMPN), and 1-(2′,6′-dimethylphenoxy)-2-propanone oxime (DMPNX) were synthesized from xylenol and chloroacetone. The purities of these compounds were confirmed by means of HPLC analysis and determined to be 98% pure. Xylenol, β-D-glucuronidase (from *Angullaria*, 25 000 unit ml^{-1}), and glucosaccharo 1,4-lactone were purchased from Wako Pure Chemical Co. (Osaka, Japan). All other chemicals and reagents used were of analytical or HPLC grade and were obtained from local suppliers.

Human urine samples

Mexitile capsules® containing 100 mg MX hydrochloride were orally administered to healthy volunteers ($n = 10$, male, 20–32 years of age and 51–71 kg body weight) at a single dose of two capsules (equivalent to 166 mg MX). The volunteers were fully informed about the experimental procedure and purpose of the study, and written consent was obtained from each subject. Approval for the study was given by the local Institutional Review Board. Urine samples were collected before administration and at scheduled intervals of 0–6, 6–12 and 12–24 h after the administration. All the samples were stored at −20°C until analysis.

Hydrolysis

Acid hydrolysis. A 5 M hydrochloric acid solution was added to samples to prepare a 1 M acidified reaction mixture, which was heated at 100°C for 30 min in a silicon-capped glass tube. The reaction mixture was then neutralized with sodium hydroxide and directly subjected to HPLC analysis.

Alkaline hydrolysis. The samples were exposed to 1 M aqueous or methanolic sodium hydroxide at room temperature. After the 30-min treatment, each reaction mixture was neutralized with hydrochloric acid and subjected to HPLC analysis.

β-Glucuronidase hydrolysis. The samples were incubated with 10 000 units ml^{-1} β-glucuronidase in acetate buffer (pH 5) at 37°C for 4 h, then the same volume of acetonitrile was added and the mixture was centrifuged. Identical samples were subjected to enzyme hydrolysis with 1 mg ml^{-1} glucosaccharo 1,4-lactone, a specific inhibitor of β-glucuronidase. The supernatants were individually subjected to HPLC analysis.

Isolation of conjugated MX from human urine

A portion of the urine was applied to an ion-exchange column, DIAION (HP-20, 3.0 × 15 cm, Mitsubishi Chemical Industry, Tokyo, Japan), and the column was washed with 0.5 litres water. Elution was done with a gradient of aqueous acetonitrile from 5 to 50% (v/v), and 100-ml fractions were collected. A portion (0.1 ml) of each fraction was treated with hydrochloric acid, followed by HPLC analysis to monitor the appearance of MX. The fractions containing conjugated MX were combined, evaporated *in vacuo* and lyophilized to give a semi-pure sample. The resultant sample was applied to a cartridge column packed with C18 (Megabond C18, Varian, CA, USA). The column was eluted with 3 vols water followed by elution with 3 vols 10% aqueous methanol and 6 vols 20% aqueous methanol in this order. The fractions containing conjugated MX were concentrated and lyophilized to yield the purified compound.

Synthesis of N-methoxycarbonylmexiletine (NCM)

MX hydrochloride (103 mg) solution in 50% aqueous acetonitrile (5 ml) containing triethylamine (175 μl) was treated with 1.1 equivalents of methyl chloroformate and the mixture was stirred overnight at room temperature. It was then thoroughly stirred with ethyl acetate and water, and the organic layer was separated and washed with diluted hydrochloric acid, water and saturated aqueous sodium chloride solution. After having been dried over sodium sulfate, the organic layer was evaporated *in vacuo* to give the desired product as crystals in 94% yield. Quasimolecular ions corresponding to $[M+H]^+$ and $[M+NH_4]^+$ were observed at *m/z* 238 and 255, respectively, in positive ESI–liquid chromatography mass spectra (LC/MS). The chemical structure of NCM was determined by mass spectra and ^{1}H-NMR.

HPLC analysis

MX and its metabolites in the urine samples, eluates from chromatography columns during purification and the hydrolysed reaction mixtures were analysed by HPLC by using a Waters HPLC system Alliance 2690 equipped with a photodiode array detector model 996, a fluorescence detector model 474 (Waters, Milford, MA, USA), and an analytical reverse-phase column Capcell Pak C18 (4.6 mm i.d. × 150 mm, 5 μm, Shizeido Co., Tokyo, Japan). The column temperature was maintained at 35°C and the eluate was monitored at 210 nm. Fluorescence conditions were set at 270 nm for excitation and at 312 nm for emission to confirm 4HM, 2HM and MX by the method previously described (Senda *et al.* 2001). An isocratic system was used for the separation of the analytes at a flow rate of 1.0 ml min^{-1}. The mobile phase was composed of acetonitrile and 44 mM phosphate buffer containing 0.5% triethylamine (pH 7.0) in a ratio of 35/65 (v/v). For the alkali hydrolysed samples, 44 mM ammonium acetate buffer containing 0.5% triethylamine (pH 7.0) was used in place of the phosphate buffer.

LC/MS/MS analysis

HPLC for LC/MS/MS was run on a Shiseido Nanospace SI-2 (Tokyo, Japan) equipped with a column of Inertsil ODS3 (2.1 mm i.d. × 150 mm, 5 μm, GL Science, Kyoto, Japan) at a flow rate of 0.2 ml min^{-1}. Two different isocratic solvent systems were used: acetonitrile-44 mM ammonium acetate (20/80 v/v) was used for analysis of the isolated MX metabolite and MX, whereas acetonitrile-44 mM ammonium acetate (50/50 v/v) was used for *N*-methoxycarbonylmexiletine. Mass spectra were obtained on a Finnigan quadrupole mass spectrometer (TSQ, ThermoQuest, San Francisco, CA, USA) fitted with an atmospheric pressure ionization source (API 2 Interface) using the following parameters: ESI voltage, 3.5 kV; sheath gas, 75 psi; auxiliary gas, 40 unit; capillary temperature, 350°C; collision-induced dissociation (CID) gas, Ar 2.0 mTorr.

Results

Identification and isolation of conjugated MX from urine sample

Human urine samples treated with hydrochloric acid were applied to the HPLC system to confirm the presence of conjugated MX. In this HPLC analysis, the retention times of MX and its metabolites, 4HM and 2HM, were 4.5, 2.1 and 2.3 min, respectively (figure 1A). Although the system could detect as little as 10 ng NHM, DMPL, DMPN and DMPNX at 6.5, 8.2, 10.5 and 10.7 min, respectively, these possible metabolites were not observed in any urine sample (data not shown). Figure 1B shows the chromatogram of an intact urine sample with a peak at 3.1 min and the MX peak at 4.5 min. After acid hydrolysis, the peak at 3.1 min disappeared, whereas the peak height at 4.5 min increased (figure 1C), indicating the presence of conjugated MX in the urine sample. Thus, the target peak (3.1 min) was carefully extracted by the method described above, with monitoring of MX production by using HPLC. Based on the HPLC analysis of the hydrolysed urine samples, the 0–6-h fraction was used for isolation of conjugated MX. The target peak was separated from the urine sample to give 3.5 mg purified compound after secondary purification with a packed column.

LC/MS/MS analysis of the isolated MX conjugate

The mass spectrum of the isolated MX conjugate is shown in figure 2. In the negative ESI mass spectrum, the $[M-H]^-$ ion appeared at m/z 398. This mass number is 44 mass units higher than that of the product expected when MX is subjected to direct glucuronidation (theoretical molecular mass, m/z 355). The isolated conjugate was analysed by positive ESI mass spectrometry to give a peak at m/z 417, corresponding to $[M+NH_4]^+$, and its CID mass spectrum showed m/z 224, 180 and 58 as product ions from the precursor ion $[M+NH_4]^+$ at m/z 417 (figure 3). These results indicate that the isolated conjugate is likely to contain a carbonyloxy moiety in addition to MX and glucuronic acid moieties.

Hydrolysis of the conjugated MX

Since the isolated conjugate was postulated to possess a carbamoyloxy moiety in the molecule, it was treated under a mild alkaline condition to confirm the structure. When ammonium acetate buffer was used as the mobile phase, the isolated MX conjugate emerged at 1.9 min (figure 4A) and the retention times of MX and its metabolites were slightly changed from those under the previous condition: MX = 3.0 min, NHM = 3.5 min, DMPL = 8.6 min, DMPN = 11.0 min and DMPNX = 11.5 min. When the isolated conjugate was treated with an aqueous solution of sodium hydroxide, a peak at the same retention time as that of MX (3.0 min) was observed in the HPLC chromatogram of the reaction mixture (figure 4B). The same product was obtained from reaction mixtures when the conjugate was hydrolysed with acid or β-glucuronidase (data not shown). When the conjugate was treated with alkaline methanolic solution, the reaction mixture gave two new peaks that emerged at 3.0 min (MX) and 8.8 min (unknown new peak) on HPLC analysis (figure 4C). This new peak at 8.8 min was considered an *N*-methoxycarbonyl derivative of MX, based on the chemical reaction mechanism.

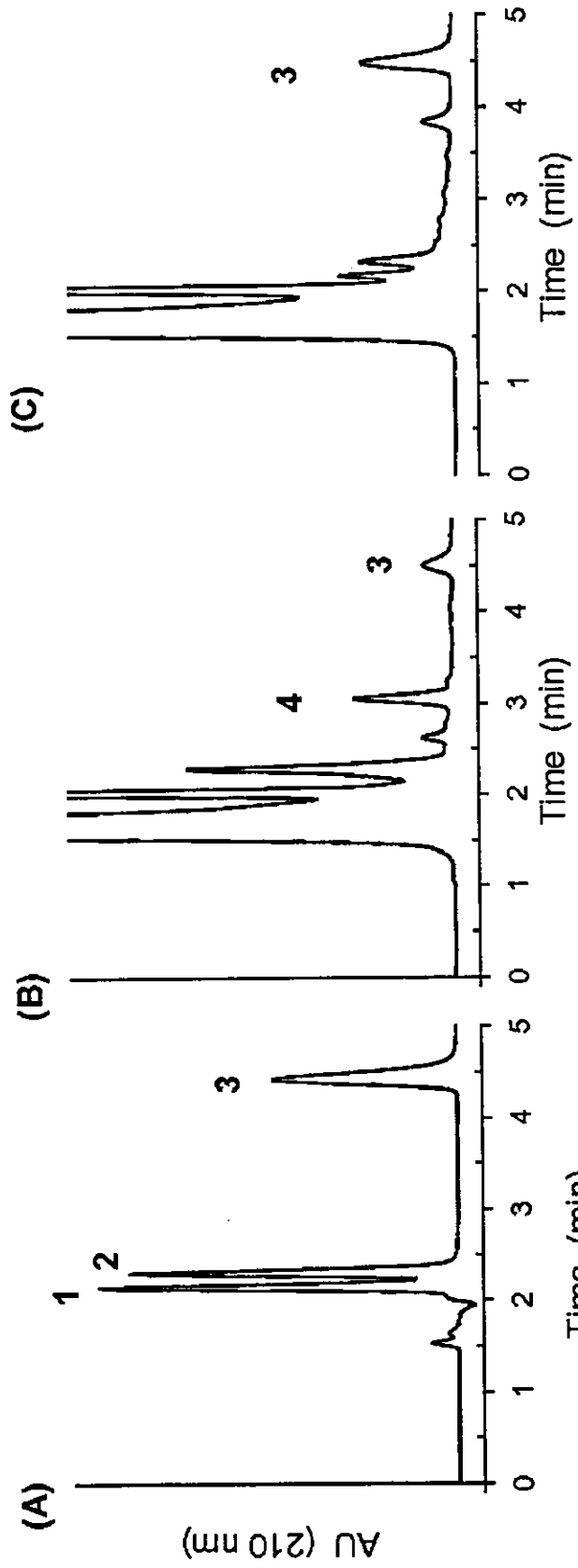

Figure 1. Typical chromatograms of authentic mexiletine (MX) and its metabolites (A) and of urine samples before (B) and after (C) acid hydrolysis. Peaks: 1, 4HM = 2.1 min; 2, 2HM = 2.3 min; 3, MX = 4.5 min; 4, conjugated MX = 3.1 min.

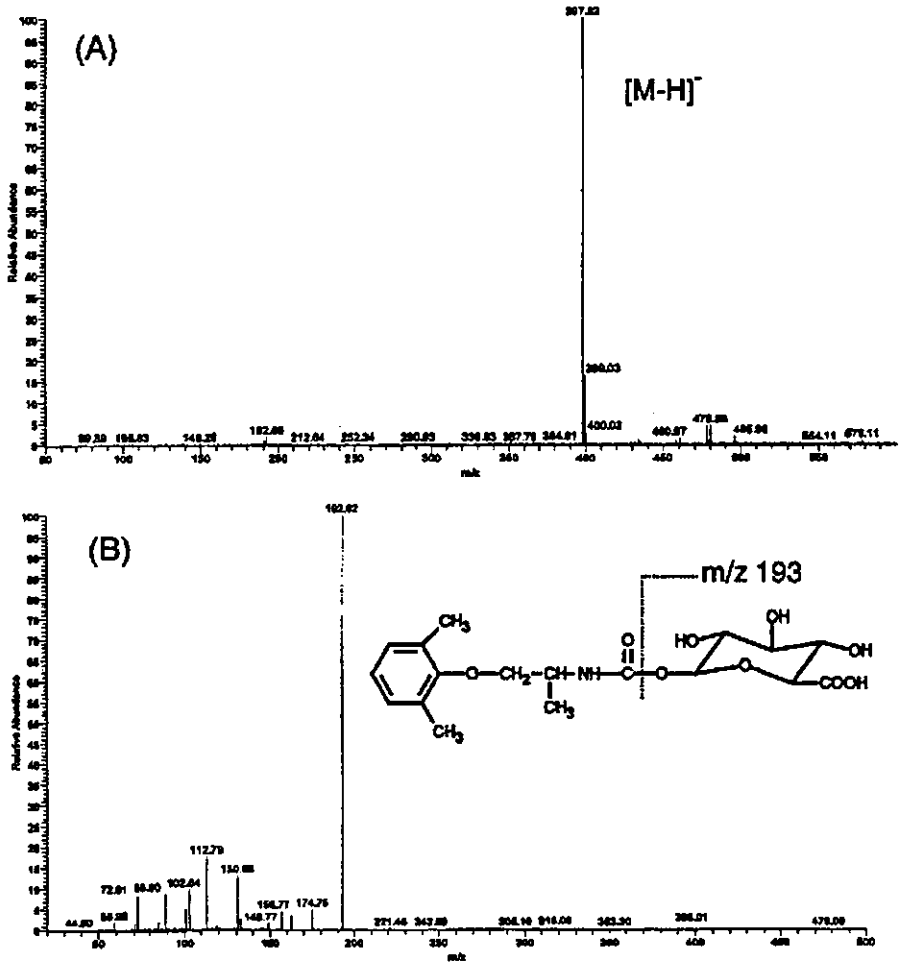

Figure 2. Negative electrospray ionization-liquid chromatogram mass spectrum (A) and collision-induced dissociation spectrum (B) from $[M - H]^-$ at m/z 398 of the metabolite isolated from human urine.

Authentic NCM was synthesized as a standard to compare its chromatogram and mass spectrum with those of the unknown product.

As shown in the ion chromatogram at m/z 238 (figure 5), synthetic NCM gave the same retention time as that of the product from the conjugate treated with an alkaline methanol solution. The MS/MS (CID spectrum) of m/z 238 derived from synthetic NCM was identical with that derived from the metabolite treated with sodium hydroxide in methanol (figure 6), indicating that the metabolite contains an -NHCOO- group in its molecule. Thus, the structure of the isolated MX conjugate was concluded to be the *N*-carbonyloxy β-D-glucuronide of MX.

Semiquantification of MX carbonyloxy β-D-glucuronide in urine

Since enough authentic reference standard for MX carbonyloxy β-D-glucuronide was unavailable, each peak height at 3.1 min of collected urine samples

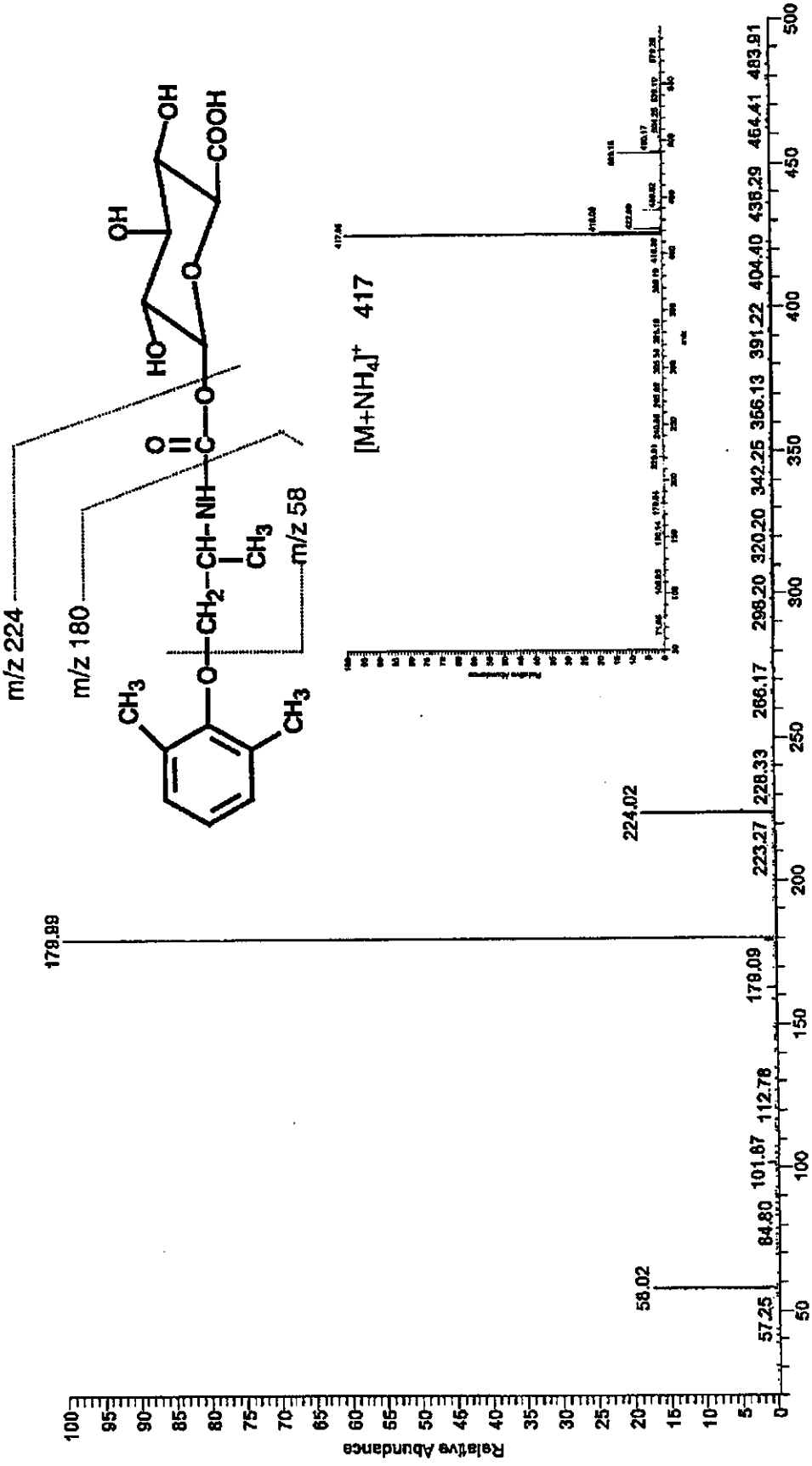

Figure 3. Precursor ion $[M + NH_4]^+$ at *m/z* 417 and collision-induced dissociation product ions of the metabolite isolated from human urine.

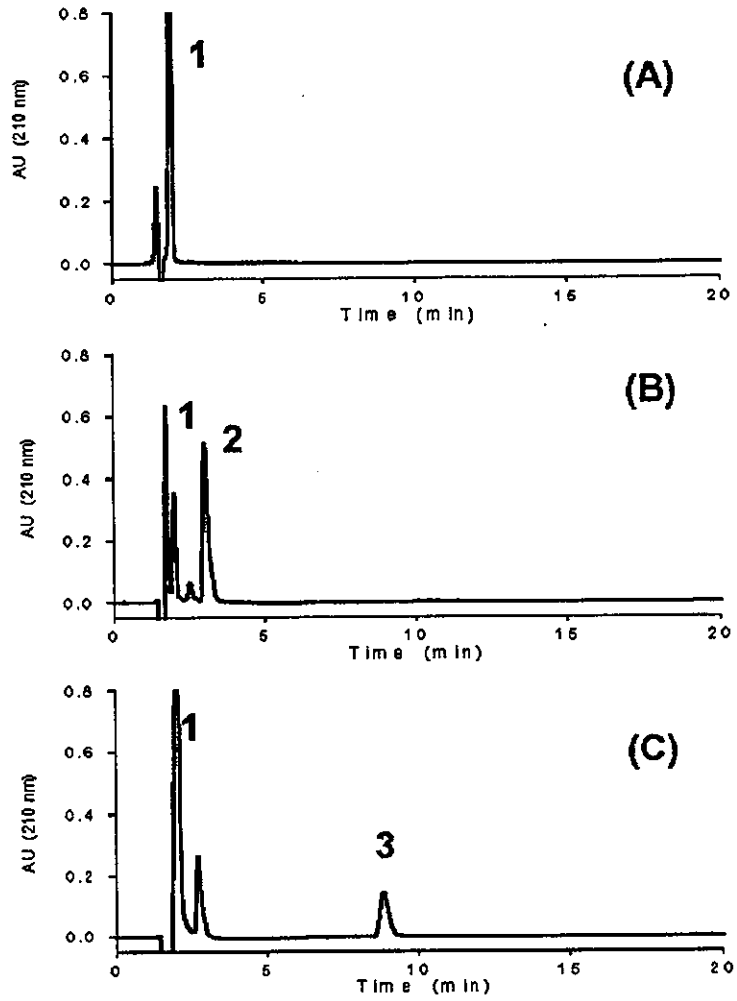

Figure 4. High-performance liquid chromatogram (HPLC) of the isolated metabolite (A). HPLC analysis of the product obtained after treatment of the isolated metabolite with 1 M sodium hydroxide solution (B) or 1 M sodium hydroxide methanolic solution (C) at room temperature. Peaks: 1, MX glucuronide = 1.9 min; 2, MX = 3.0 min; 3, new product = 8.8 min.

(0–6-h fraction, $n = 10$) was compared with that of MX carbonyloxy β-D-glucuronide (1 $\mu g\,ml^{-1}$) under the same HPLC condition. When the amount of the glucuronide in each urine sample was calculated by a peak height ratio, 17–31 mg MX carbonyloxy β-D-glucuronide was estimated to be present in each urine sample. On the basis of this semiquantitative data for each urine sample after acid hydrolysis, MX carbonyloxy β-D-glucuronide accounted for approximately 68–103% ($n = 10$) of the total amount of conjugated MX.

Discussion

From a portion of urine collected from subjects after MX administration, a new MX glucuronide was isolated. The use of soft ionization techniques in mass

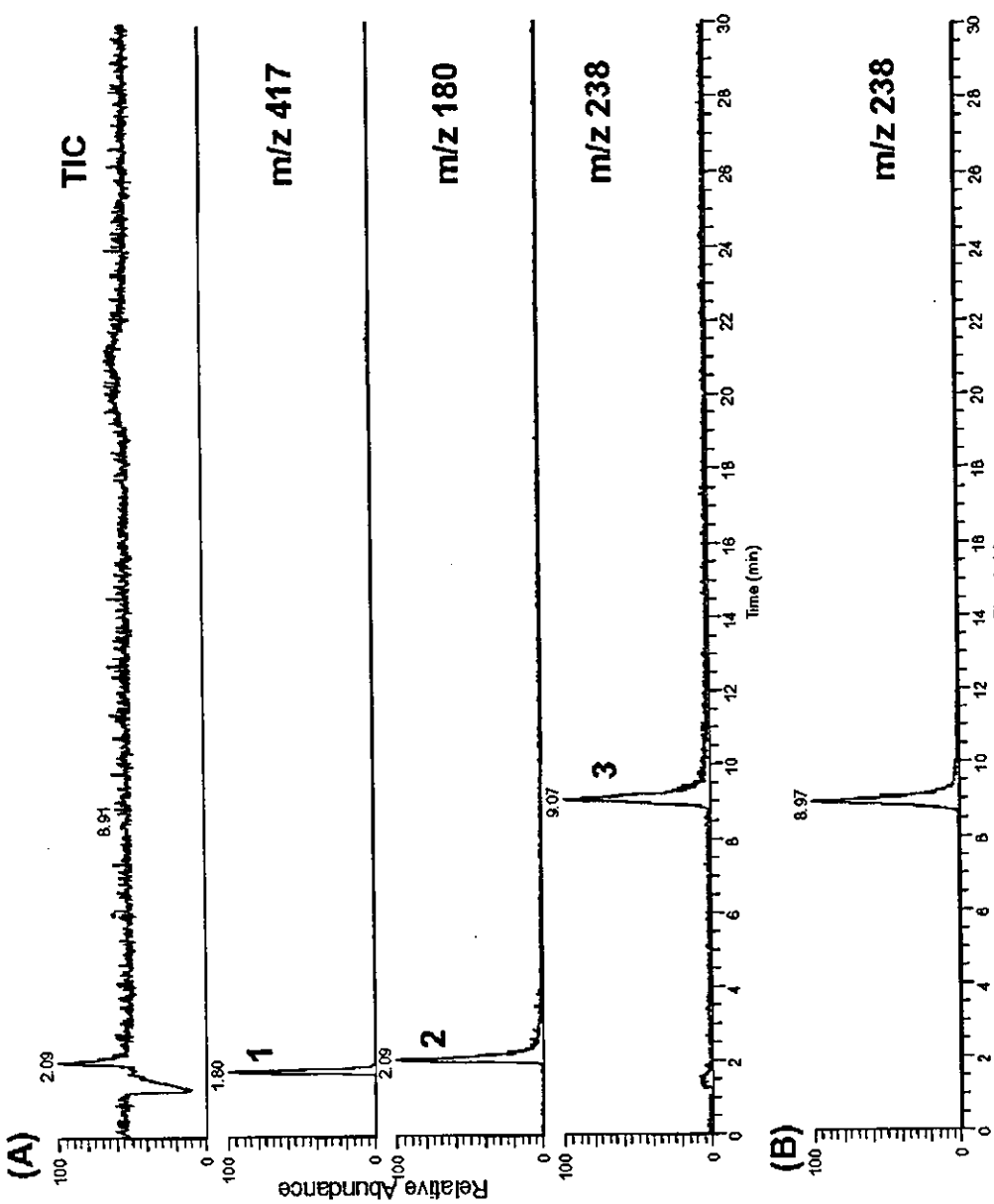

Figure 5. Total ion chromatogram (TIC) and extracted ion chromatograms of the reaction mixture after treatment of the isolated MX glucuronide with sodium hydroxide methanolic solution (A). Ion chromatogram of synthetic *N*-methoxycarbonylmexiletine (B). Peaks: 1, MX glucuronide; 2, MX; 3, unknown.

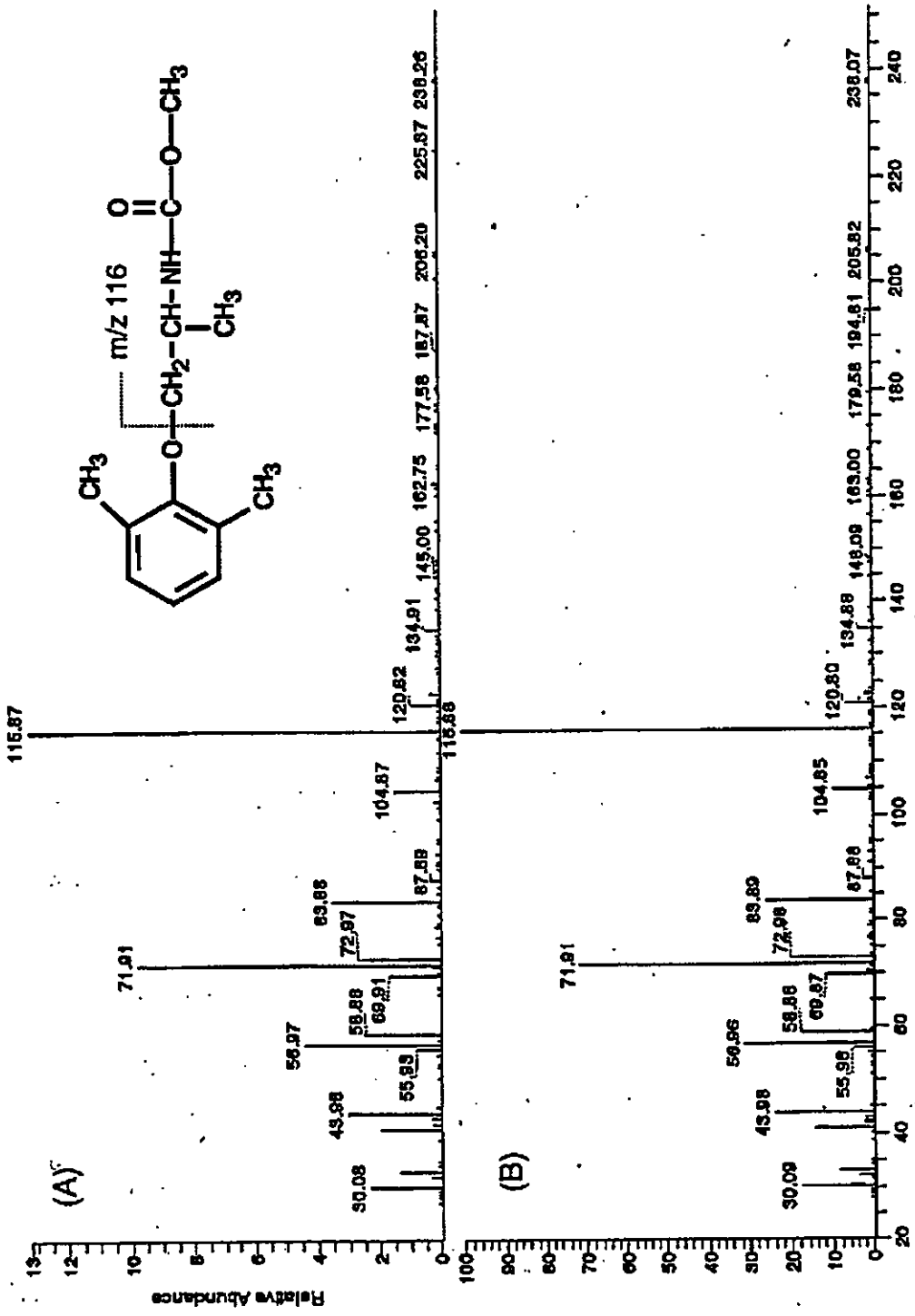

Figure 6. Collision-induced dissociation spectrum from $[M+H]^+$ at m/z 238 of synthetic *N*-methoxycarbonylmexiletine (A) and the product obtained from the urinary metabolite by treatment with sodium hydroxide methanolic solution (B).