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Metabolic Activation of Benzodiazepines by CYP3A4

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Introduction: 577 words

Discussion: 1143 words

[1]Abbreviations used are: cytochrome P450, CYP; fetal bovine serum, FBS; dimethyl sulfoxide, DMSO; 3-(4,5-dimetylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, MTT; liquid chromatography-tandem mass spectrometry, LC-MS/MS; liquid chromatography ion trap and time of flight mass spectrometry, LCMS-IT-TOF; effective concentrations, EC_{25}; exacted ion chromatograms, XIC

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Abstract

Cytochrome P450 (CYP) 3A4 is the predominant isoform in liver, and it metabolizes more than 50% of the clinical drugs commonly used. However, CYP3A4 is also responsible for metabolic activation of drugs leading to liver injury. Benzodiazepines are widely used for anxiety as hypnotics and sedatives, but some of them induce liver injury in humans. To clarify whether benzodiazepines are metabolically activated, 14 benzodiazepines were investigated for their cytotoxic effects on HepG2 cells treated with recombinant CYP3A4. By exposure to 100 μM flunitrazepam, nimetazepam, or nitrazepam, the cell viability in the presence of CYP3A4 decreased more than 25% as compared with that of the control. In contrast, in the case of other benzodiazepines, the changes in the cell viability between CYP3A4 and control Supersomes were less than 10%. These results suggested that nitrobenzodiazepines such as flunitrazepam, nimetazepam, and nitrazepam were metabolically activated by CYP3A4, which resulted in cytotoxicity. To identify the reactive metabolite, the glutathione adducts of flunitrazepam and nimetazepam were investigated by liquid chromatography-tandem mass spectrometry. The structural analysis for the glutathione adducts of flunitrazepam indicated that a nitrogen atom in the side chain of flunitrazepam was conjugated with thiol of glutathione. Therefore, the presence of a nitro group in the side chain of benzodiazepines may play a crucial rule in the metabolic activation by CYP3A4. The present study suggested that metabolic activation by CYP3A4 was one of the mechanisms of liver injury by nitrobenzodiazepines.

Introduction

Drug-induced hepatotoxicity is one of the major causes of liver injury and is classified into intrinsic and idiosyncratic types. Intrinsic drug reactions can occurr in a dose-dependent manner in any individuals and are reproducible in the preclinical studies. In contrast, idiosyncratic drug reactions do not occur in most patients at any dose and they are often referred to as rare, with a typical incidence of from 1/100 to 1/100000 (Uetrecht, 1999). Because idiosyncratic drug reactions are difficult to spot during drug development, some drugs launched on the market were later withdrawn due to idiosyncratic hepatotoxicity. Such drugs withdrawn for hepatotoxicity are known to produce reactive metabolites (Guengerich and MacDonald, 2007). The generation of reactive metabolites may relate to the formation of free radicals, oxidation of thiol, and covalent binding with endogenous macromolecules, resulting in the oxidation of cellular components or inhibition of normal cellular function (Guengerich and Liebler, 1985).

The generation of a reactive metabolite catalyzed by drug metabolizing enzymes such as cytochrome P450 (CYP) is defined as metabolic activation. CYP is the major drug metabolizing enzyme that is highly expressed in human liver. CYP3A4 is the predominant isoform in liver (Shimada et al., 1994) and metabolizes more than 50% of the clinical drugs commonly used (Guengerich, 1995). However, CYP3A4 is also responsible for the formation of reactive metabolites of flutamide (Berson et al., 1993), trazodone (Kalgutkar et al., 2005), and troglitazone (Yamamoto et al., 2002). It is suggested that the reactive metabolites of flutamide, trazodone, and troglitazone cause the idiosyncratic hepatotoxicity in humans.

Prediction of the metabolic activation and the cytotoxicity of drug candidates is necessary in drug development. Human hepatocarcinoma HepG2 cells are commonly used for predicting hepatotoxicity *in vitro*. However, low expression levels of CYPs in HepG2 cells may be responsible

for the fact that 30% of the compounds were falsely classified as non-toxic (Hewitt and Hewitt, 2004; Rodriguez-Antona et al., 2002; Wilkening et al., 2003). Recently, a useful *in vitro* cell-based assay made by combining recombinant CYP3A4 with HepG2 cells was established (Vignati et al., 2005). It was demonstrated that hepatotoxicants whose reactive metabolites were generated by CYP3A4 exhibited cytotoxicity to the HepG2 cells. This assay system could be applied to screen for hepatotoxicity by drugs.

Benzodiazepines have been extensively used for anxiety, as hypnotics and sedatives throughout the world. The mechanism of their efficacy is to amplify the action of gamma-aminobutyric acid by acting as agonists at gamma-aminobutyric acid receptors (Costa et al., 2002). Many benzodiazepines have been launched on the market and used in clinical practice. Two of the major benzodiazepines, flunitrazepam and nitrazepam, are widely used as hypnotic and anesthetic premedications in Europe and Japan. In 2001, it was announced that flunitrazepam induced hepatotoxicity by Ministry of Health, Labor and Welfare of Japan. Chronic administration of antidepressant drugs including nitrazepam was reported to induce severe hepatic disorder (Seki et al., 2008). Clonazepam is one of the benzodiazepines used as an anxiolytic and anticonvulsant in clinical practice. Hepatic injury was reported to occur after treatment with clonazepam for 6 weeks in Ethiopia (Olsson and Zettergren, 1988).

The purpose of the present study was to clarify whether the metabolic activation of benzodiazepines by CYP occurs leading to the hepatotoxicity. We investigated the cell viability in HepG2 cells in the presence or absence of CYP3A4 after exposure to 14 commercially available benzodiazepines (Fig. 1). There are many structural analogues of benzodiazepines. Comparison between the chemical structure and cytotoxicity of HepG2 cells was performed.

Materials and Methods

Materials

Clonazepam, clobazam, diazepam, lorazepam, nimetazepam, nitrazepam, and oxazepam were obtained from Wako Pure Chemical (Osaka, Japan). Bromazepam, chlordiazepoxide, desmethyldiazepam, flunitrazepam, flurazepam, norfludiazepam, and temazepam were purchased from Sigma-Aldrich (St. Louis, MO). Human CYP2C9, 2C19, and 3A4 Supersomes (recombinant cDNA-expressed CYP enzymes prepared from a baculovirus insect cell system) and Control Supersomes were purchased from BD-Gentest (Woburn, MA). These microsomes co-expressed nicotinamide adenine dinucleotide phosphate (NADPH)-cytochrome P450 reductase and cytochrome b_5. All other reagents used in this study were of the highest or analytical grade commercially available.

Cell culture

Human hepatocarcinoma cell line HepG2 was obtained from Riken Gene Bank (Tsukuba, Japan). The cells were cultured in Dulbecco's modified Eagle medium (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Melbourne, Australia) and 0.1 mM nonessential amino acids (Invitrogen) at 37˚C in an atmosphere of 5% CO_2/ 95% air.

Cell viability assay

HepG2 cells were seeded at a density of 1×10^4 cells/well in 96-well plates with medium containing 3% FBS, benzodiazepines, 8 nM human CYP2C9, CYP2C19, CYP3A4, or Control Supersomes and 1 mM NADPH and then incubated at 37˚C for 24 h. In the preliminary study, we investigated the cell viability in HepG2 cells with various CYP concentrations and incubation time. The 8 nM CYP and 24 h-incubation were enough to detect cytotoxicity in this assay system. The

final concentration of organic solvent (dimethyl sulfoxide; DMSO) in medium was less than 0.2%. Cell viability after 24 h incubation was evaluated by the intracellular adenosine triphosphate (ATP) concentration using a CellTiter-Glo Luminescent Cell Viability Assay (ATP assay; Promega, Medison, WI) and 3-(4,5-dimetylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) activities using a CellTiter-Blue Cell Viability Assay (MTT assay; Promega). According to the protocols by the manufacturer, the luminescence of the generated oxyluciferin was measured in the ATP assay and the fluorescence of the generated resorufin was detected fluorometrically (excitation: 338 nm, emission: 458 nm) in the MTT assay by using a luminometer 1420 ARVO MX (Wallac, Turku, Finland).

Caspase assay

HepG2 cells were seeded under the same condition and incubated at 37˚C for 24 h. After incubation, the caspase 3/7 activity was measured using a Caspase-Glo 3/7 Assay (Promega) according to the protocol by the manufacturer. The luminescence of the generated aminoluciferin was measured using a luminometer 1420 ARVO MX.

Detection of glutathione adducts

A typical reaction mixture (final volume of 0.25 ml) contained 50 nM human CYP3A4 Supersomes, 100 mM potassium phosphate buffer (pH 7.4), an NADPH-generating system consisting of 0.775 mM nicotinamide adenine dinucleotide phosphate (oxidized form), 0.165 mM glucose 6-phosphate, 0.165 mM $MgCl_2$, 0.2 unit/ml glucose 6-phosphate dehydrogenase, 10 mM glutathione (reduced form), and 100 μM benzodiazepines (flunitrazepam, nimetazepam, nitrazepam, bromazepam, or temazepam). The final concentration of DMSO in the reaction mixture was less than 1%. Incubation was carried out at 37˚C for 60 min and terminated by adding 0.75 ml of ice-cold

methanol. After centrifugation at 15,000 g, the supernatant was subjected to a liquid chromatography-tandem mass spectrometry (LC-MS/MS; API 4000, Applied Biosystems, Foster City, CA). Shimadzu LC-10 (Shimadzu, Kyoto, Japan) was used as the LC with an Inertsil ODS-3 analytical column (2.1 × 100 mm, 3 μm, GL Science, Tokyo, Japan). The column temperature was 40°C. The mobile phase was 10 mM ammonium acetate buffer (pH 4.0) (A) and acetonitrile (B). The conditions for elution were as follows: 5-90% B (0-6 min), 90% B (6-11 min), 90-5% B (11-11.01 min), and 5% B (11.01 -15 min). Linear gradients were used for all solvent changes. The flow rate was 0.2 ml/min. The LC was connected to an API 4000 operated in the negative-electrospray ionization mode. The turbo gas was maintained at 450˚C. Air was used as the nebulizing and turbo gas at 60 psi. Nitrogen was used as the curtain gas at 20 psi. The collision energy was -50V. The *m/z* 300-850 was scanned at the precursor ion (*m/z* 272; major mass spectrum fragment of glutathione).

Identification of glutathione adducts

LCMS-IT-TOF (liquid chromatography ion trap and time of flight mass spectrometry; Shimadzu) was used to identify the structures of the glutathione adducts of the nitrobenzodiazepines. The incubation mixture was the same as described above except for CYP3A4 Supersomes (100 nM). Flunitrazepam and nimetazepam were used as test compounds. After centrifugation at 15,000 g for 5 min, the supernatant was subjected to LCMS-IT-TOF using the Inertsil ODS-3 analytical column (2.1 × 100 mm, 3 μm). The LC conditions were the same as described. The turbo gas was maintained at 450˚C. Air was used as the nebulizing and turbo gas at 60 psi. Nitrogen was used as the curtain gas at 20 psi. The collision energy was 50 V. The structure analysis of the glutathione adducts of flunitrazepam and nimetazepam was made by scanning at the product ion (*m/z* 621 and *m/z* 603, respectively) in the positive-electrospray ionization mode.

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Statistics

Data are expressed as mean ± SD (n=3). Comparison of two groups was made with two-tailed Student's *t* test. $P < 0.05$ was considered statistically significant.

Results

Cell viability of HepG2 cells treated with CYP3A4 and benzodiazepines

HepG2 cells were incubated for 24 h with the 14 benzodiazepines at 50, 100, 200, and 400 μM in the presence of CYP3A4 or control Supersomes and then the cell viability was measured by the ATP and MTT assays. By the exposure of 100 μM flunitrazepam, nimetazepam, and nitrazepam, the cell viability in the presence of CYP3A4 Supersomes decreased more than 25% as compared with control Supersomes (Fig. 2). Although clonazepam could be dissolved up to 100 μM in the reaction mixtures, the viability of HepG2 cells treated with CYP3A4 Supersomes and 100 μM clonazepam exhibited 57% and 35% decreases in the ATP and MTT assays, respectively, in comparison with control Supersomes (see supplementary Fig. 2). Flunitrazepam, nimetazepam, nitrazepam, and clonazepam are nitrobenzodiazepines that have a nitro group at the 7-position (Fig. 1). On the other hand, in terms of the other 10 benzodiazepines (bromazepam, chlordiazepoxide, clobazam, desmethyldiazepam, diazepam, flurazeam, lorazepam, norfludiazepam, oxazepam, and temazepam) at 100 μM, the changes of the cell viability between CYP3A4 and control Supersomes were less than 10% and much smaller compared to the nitrobenzodiazepines (Fig. 2, supplementary Fig. 1). Moreover, 25% effective concentrations (EC_{25}) of nitrobenzodiazepines were less than 100 μM and EC_{25} of all other benzodiazepines were over 300 μM in ATP assay (see supplementary Table 1). Desmethyldiazepam, diazepam, flurazepam, lorazepam, norfludiazepam, and oxazepam exhibited concentration-dependent cytotoxicity in HepG2 cells incubated both with and without CYP3A4 (Fig.

2, supplementary Fig. 1).

Cell viability on HepG2 cells treated with CYP2Cs and nitrobenzodiazepines

It has been reported that CYP2C9 and CYP2C19 are involved in the metabolism of flunitrazepam (Hesse et al., 2001; Kilicarslan et al., 2001). Therefore, we investigated whether CYP2C9 and CYP2C19 affect the cytotoxicity caused by nitrobenzodiazepines in HepG2 cells. As shown in Fig. 3, the differences in the cell viability between CYP2Cs and control Supersomes when exposed to 100 μM nitrobenzodiazepines were less than 10%.

Caspase 3/7 activity in HepG2 cells treated with CYP3A4 and nitrobenzodiazepines

As a key factor of apoptosis, the caspase 3/7 activity was measured in HepG2 cells treated with CYP3A4 and the nitrobenzodiazepines for 24 h. Flunitrazepam, nimetazepam, and nitrazepam significantly increased the caspase 3/7 activities in HepG2 cells in the presence of CYP3A4 Supersomes (Fig. 4A-C). In contrast, bromazepam as the negative control had no effects on the caspase 3/7 activities both with and without CYP3A4 (Fig. 4D).

Detection of glutathione adducts of benzodiazepines

The glutathione adducts of benzodiazepines were investigated by negative ion mode of LC-MS/MS. The nitrobenzodiazepines (flunitrazepam, nimetazepam, and nitrazepam) and the negative controls (bromazepam and temazepam) were measured. As shown in Fig. 5, the glutathione adducts of flunitrazepam and nimetazepam were detected in the presence of CYP3A4 Supersomes by precursor ion scan at *m/z* 619 and *m/z* 601 ($[M-H]^-$), respectively. In contrast, there were no adducts of flunitrazepam and nimetazepam when used in the Control Supersomes (data not shown). In nitrazepam, bromazepam, and temazepam, glutathione adducts were not detected in the presence

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and absence of CYP3A4.

Identification of glutathione adducts of flunitrazepam and nimetazepam

The structures of the glutathione adducts of flunitrazepam and nimetazepam were estimated by positive ion mode of LCMS-IT-TOF. In the case of the glutathione adduct of flunitrazepam, the product ion mass spectrum of *m/z* 621 ($[M+H]^+$) gave fragment ions at *m/z* 284.1, *m/z* 348.1, and *m/z* 492.1. The molecule weight of $[M+H]^+$ fragment ion (*m/z* 492.1) meant that it was produced by the molecule weight of the compound (491) and hydrogen ion (H^+; 1). The possible structure of the glutathione adduct of flunitrazepam is shown in Fig. 6. A reactive metabolite of flunitrazepam, in which the nitro group might be metabolized into the amino group, was conjugated to the 7-substituent group by glutathione.

On the other hand, the $[M+H]^+$ ion of the glutathione adduct of nimetazepam (*m/z* 603) gave fragment ions at *m/z* 266.4 and *m/z* 474.2 (see supplementary Fig. 3). The fragment ions at *m/z* 266.4 and *m/z* 474.2 were $[M+H-337]^+$ and $[M+H-129]^+$, respectively, corresponding to the fragment ions at *m/z* 284.1 and *m/z* 492.1 obtained from the glutathione adduct of flunitrazepam (*m/z* 621).

Discussion

In the present study, 14 benzodiazepine analogues were investigated for cytotoxic effects resulting from metabolic activation by CYP3A4. The major metabolic pathways of diazepam are 3-hydroxylayion by CYP3A4 and *N*-desmethylation by CYP2C9 (Schwartz et al., 1965; Ono et al., 1996). Thus, desmethyldiazepam, temazepam, and oxazepam are metabolites of diazepam (Fig. 1). Also, norfludiazepam and nitrazepam would be the metabolites of flurazepam and nimetazepam, respectively.

The cytotoxicity of flunitrazepam, nimetazepam, nitrazepam, and clonazepam was observed in

the presence of CYP3A4 Supersomes in HepG2 cells (Fig. 2, supplementary Fig. 1) suggesting that these three drugs are metabolically activated by CYP3A4. Flunitrazepam, nimetazepam, nitrazepam, and clonazepam are classified as nitrobenzodiazepines that have a nitro group in the side chain. In contrast, the other 10 benzodiazepines exhibited less cytotoxicity than the nitrobenzodiazepines (Fig. 2, supplementary Fig. 1, supplementary Table 1). In the present study, we first clarified that the presence of a nitro group in the side chain of benzodiazepines may play a crucial rule in the metabolic activation by CYP3A4. To prevent the cytotoxicity by reactive metabolites in the medium, the effects of 200 μM or 1 mM glutathione (reduced form) were measured in this cell viability assay as a preliminary experiment. The glutathione recovered 10% of cell viability in HepG2 cells treated with CYP3A4 and 100 μM flunitrazepam (data not shown). This suggested that the reactive metabolites of nitrobenzodiazepines may bind to glutathione. However, glutathione did not completely protect the cytotoxicity, thus there may be another cytotoxic effect that could not be detoxify by glutathione trapping.

In humans, the major metabolites of flunitrazepam are *N*-desmethylflunitrazepam in plasma and 3-hydroxyflunitrazepam and 7-aminoflunitrazepam in urine (Fukazawa et al., 1978). CYP3A4 is the major CYP involved in flunitrazepam 3-hydroxylation and *N*-desmethylation but CYP2C9 and CYP2C19 also catalyze the *N*-desmethylation of flunitrazepam (Hesse et al., 2001; Kilicarslan et al., 2001). The reductive metabolite of flunitrazepam, 7-aminoflunitrazepam, is catalyzed by NADPH-cytochrome P450 reductase in HepG2 cells (Peng et al., 2004). Nimetazepam is metabolized to *N*-desmethylnimetazepam and 3-hydroxylnimetazepam (unpublished data, Dainippon Sumitomo Pharma). Nitrazepam is metabolized to 7-aminonitrazepam and 3-hydroxynitrazepam (Rieder, 1965). Although it has not been revealed which CYP isoform mediates nimetazepam and nitrazepam metabolism, nimetazepam and nitrazepam may be metabolized by CYP2C9, CYP2C19, and CYP3A4. In our study, when HepG2 cells were exposed to 100 μM nitrobenzodiazepines, the

differences in the cell viability between CYP2Cs and control Supersomes were less than 10% (Fig. 3) indicating that the contribution of CYP2Cs to the cytotoxicity of nitrobenzodiazepines was much lower than that of CYP3A4. Therefore, the metabolic activations of the nitrobenzodiazepines were CYP3A4 specific reactions.

Caspase 3 and 7 are classified as effector caspases. Active effector caspases mediate the cleavage of an overlapping set of protein substrates, resulting in the morphological features of apoptosis and the demise of the cell (Nuñez et al., 1998). Flunitrazpam, nimetazepam, and nitrazepam significantly increased the caspase 3/7 activities in the HepG2 cells in the presence of CYP3A4 Supersomes (Fig. 4). Therefore, apoptosis following caspase 3 and 7 activation is one of the cytotoxic pathways of the reactive metabolites of flunitrazepam, nimetazepam, and nitrazepam.

The maximum plasma concentrations of nitrobenzodiazepines after a single administration in humans have been reported as follows: 0.04 μM following an oral dose of 2 mg flunitrazepam (Fukazawa et al., 1978), 0.05 μM following an oral dose of 5 mg nimetazepam (unpublished data, Dainippon Sumitomo Pharma), 0.3 μM following an oral dose of 10 mg nitrazepam (Rieder, 1973), and 0.05 μM following an oral dose of 2 mg clonazepam (Cavedal et al., 2007). Flunitrazepam has been reported to induce hepatotoxicity by Ministry of Health, Labor and Welfare of Japan and nitrazepam and clonazepam were reported to cause drug-induced liver injury (Seki et al., 2008; Olsson and Zettergren, 1988). Although it is very difficult to extrapolate from in vitro study to in vivo in humans, we may pay attention to the metabolic activation of nitrobenzodiazepines by CYP3A4.

The metabolism of a non-toxic drug to reactive metabolites is thought to initiate a variety of adverse reactions (Park, 1986; Parke, 1987). Glutathione is an important intracellular peptide, which can detoxify the reactive metabolites by conjugation (Lu, 1999). Previous studies reported that reactive metabolites of flutamide (Kang et al., 2007), trazodone (Kalgutkar et al., 2005), and

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troglitazone (Kassahun et al., 2001) formed by CYP3A4 were detoxified by glutathione conjugations. As shown in Fig. 5, glutathione adducts of flunitrazepam and nimetazepam were detected by LC-MS/MS, suggesting the production of reactive metabolites of flunitrazepam and nimetazepam catalyzed by CYP3A4. In the present study, the structure of the glutathione adduct of flunitrazepam was estimated by LCMS-IT-TOF as shown in Fig. 6. It appeared that a nitrogen atom in the side chain of flunitrazepam was conjugated with thiol of glutathione. The structure of the glutathione adduct of nimetazepam may be similar to that of flunitrazepam because the fragment ions, $[M+H-337]^+$ and $[M+H-129]^+$, corresponded to those of flunitrazepam (see supplementary Fig. 3). The glutathione adducts of nitrazepam could not be detected both with and without CYP3A4 in our detection system. However, the cytotoxicity of nitrazepam to HepG2 cells treated with CYP3A4 Supersomes (Fig. 2) suggesting that metabolic activation might occurr. One of the reasons for this discrepancy may be the sensitivity of the detection.

Nitroaromatic drugs such as flutamide, nimesulide and tolcapone have been associated with idiosyncratic liver injury (Boelsterli et al., 2006). In the reductive pathways from nitro to the fully reduced amine catalyzed by CYP and/or reductase, several reactive metabolites including nitroso and *N*-hydroxylamine derivatives could be produced. Such reactive metabolites seem to bind covalently to nucleophilic targets of proteins and nucleic acids leading to the cytotoxic effects (Biaglow et al., 1986; Rickert, 1987; Kedderis and Miwa, 1988; Kedderis et al., 1989). On the other hand, arylamines are metabolically activated by CYP-mediated *N*-hydroxylation. Electrophilic *N*-hydroxylamine reacts with intracellular molecules, which induce various types of toxicity including hepatotoxicity (Kato and Yamazoe, 1994). Flutamide induced severe hepatic dysfunction. Ohbuchi et al. (*Drug Metab Dispos.* in press, 2008) suggested CYP3A4 catalyzed the *N*-oxidation of amino metabolite of flutamide that had hepatotoxic effects. Although the bioactivation pathways of nitrobenzodiazepines still remain unclear, they may undergo similar metabolic activation like other

drugs. Further study is needed to clarify the mechanism of metabolic activation concerning nitrobenzodiazepines.

In conclusion, we revealed that nitrobenzodiazepines, such as flunitrazepam, nimetazepam, and nitrazepam, were metabolically activated by CYP3A4 resulting in cytotoxicity in HepG2 cells. The CYP3A4 metabolites of flunitrazepam and nimetazepam were conjugated with glutathione at a nitrogen atom in the side chain. This finding suggested that metabolic activation by CYP3A4 may be one of the mechanisms in liver injury. Moreover, we established the simply assay system in which the cytotoxicity in HepG2 cells incubated with recombinant CYPs and the drug was observed in high sensitivity. The present assay system was useful to detect metabolic activation by CYPs and would be beneficial to predict drug-induced cytotoxicity in preclinical drug development.

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Footnotes

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Legend for figure.

Fig. 1. Chemical structures of the fourteen benzodiazepines used in the present study.

Fig. 2. Cytotoxicity of the benzodiazepines incubated with CYP3A4 on HepG2 cells. HepG2 cells seeded with the benzodiazepines and CYP3A4 or control Supersomes in 96-well plates were incubated at 37˚C for 24 h. Cell viability was measured by ATP assay (left side) and MTT assay (right side) as described in *Material and Methods*. The test compounds were (A) flunitrazepam, (B) nimetazepam, (C) nitrazepam, (D) bromazepam, and (E) diazepam. Data represent the mean ± SD of three independent experiments. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ compared with the control Supersomes.

Fig. 3. Cytotoxicity of the benzodiazepines incubated with CYP2Cs in HepG2 cells. HepG2 cells seeded with the benzodiazepines and CYP2C9, CYP2C19 or control Supersomes in 96-well plates were incubated at 37˚C for 24 h. Cell viability was measured by ATP assay (left side) and MTT assay (right side) as described in *Material and Methods*. The test compounds were (A) flunitrazepam, (B) nimetazepam, and (C) nitrazepam. Data of CYP3A4 and Control Supersomes were redrawn from Fig. 2. Data represent the mean ± SD of three independent experiments. #$P < 0.05$; ##$P < 0.01$ (CYP2C9), †$P < 0.05$; ††$P < 0.01$ (CYP2C19), *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ (CYP3A4) compared with the control Supersomes.

Fig. 4. Caspase 3/7 activity in HepG2 cells treated with CYP3A4 and the nitrobenzodiazepines. HepG2 cells in 96-well plates were incubated with benzodiazepines and CYP3A4 or control Supersomes at 37˚C for 24 h. Caspase 3/7 activity was measured by the method as described in

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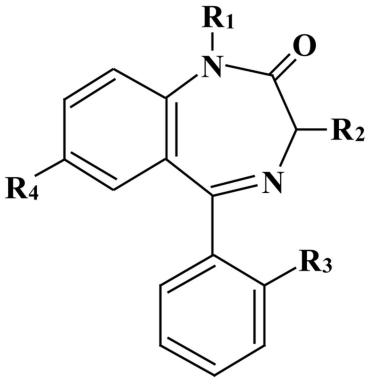
Material and Methods. The test compounds were (A) flunitrazepam, (B) nimetazepam, (C) nitrazepam, and (D) bromazepam. Data represent the mean ± SD of three independent experiments. **P* < 0.05; ***P* < 0.01; ****P* < 0.001 compared with the control Supersomes.

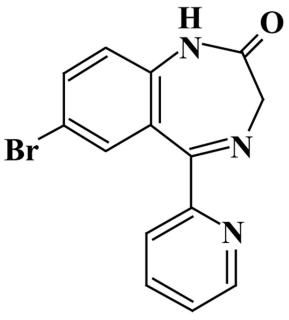
Fig. 5. Exacted ion chromatograms (XIC) of the glutathione adducts of benzodiazepines. The chromatograms were scanned with the precursor ion fragment, m/z 272, derived from glutathione using LC-MS/MS. A, flunitrazepam at *m/z* 619 ($[M-H]^-$); B, nimetazepam at *m/z* 601 ($[M-H]^-$).

Fig. 6. A, The predicted structure of the glutathione adduct of flunitrazepam. B, MS/MS spectra of product ion obtained by collision-induced dissociation of the glutathione adduct of flunitrazepam at *m/z* 621 ($[M+H]^+$). These spectra were scanned using LCMS-IT-TOF.

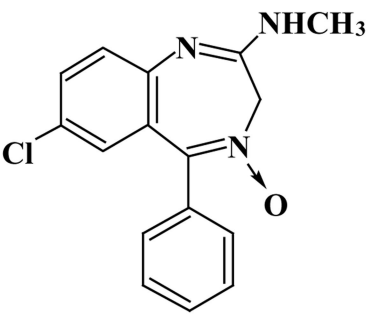
Fig. 1.



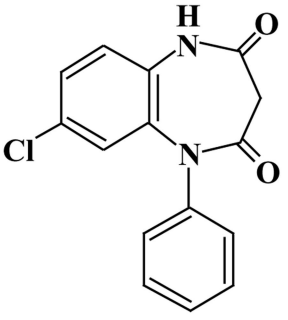
Benzodiazepine	R_1	R_2	R_3	R_4
Clonazepam	H	H	Cl	NO_2
Desmethyldiazepam	H	H	H	Cl
Diazepam	CH_3	H	H	Cl
Flunitrazepam	CH_3	H	F	NO_2
Flurazepam	$CH_2CH_2N(C_2H_5)$	H	F	Cl
Lorazepam	H	OH	Cl	Cl
Nimetazepam	CH_3	H	H	NO_2
Nitrazepam	H	H	H	NO_2
Norfludiazepam	H	H	F	Cl
Oxazepam	H	OH	H	Cl
Temazepam	CH_3	OH	H	Cl



Bromazepam



Chlordiazepoxide



Clobazam

Fig. 2.

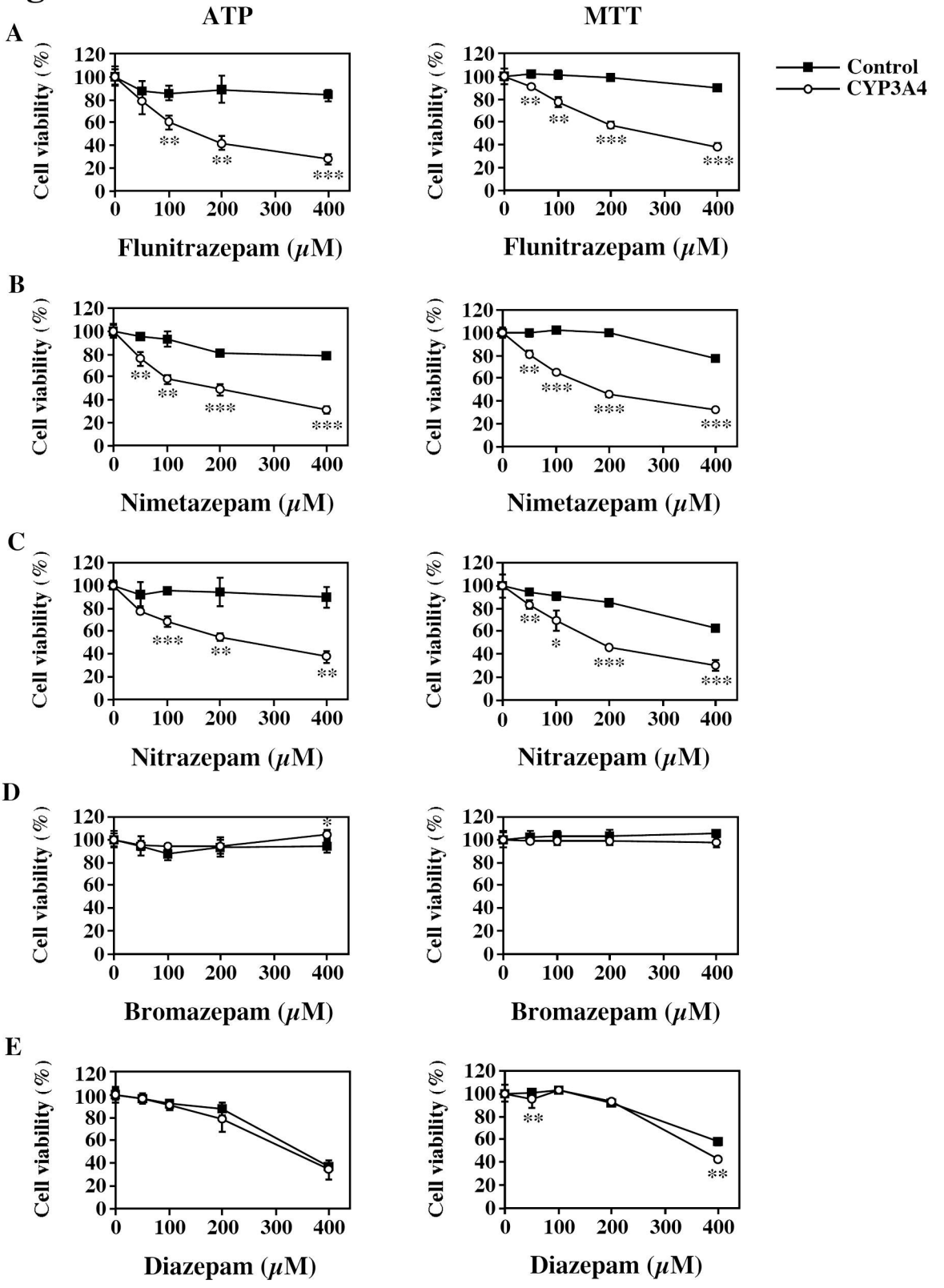

ATP
MTT
A
B
C
D
E
Control
CYP3A4
Cell viability (%)
Flunitrazepam (μM)
Nimetazepam (μM)
Nitrazepam (μM)
Bromazepam (μM)
Diazepam (μM)

Fig. 3.

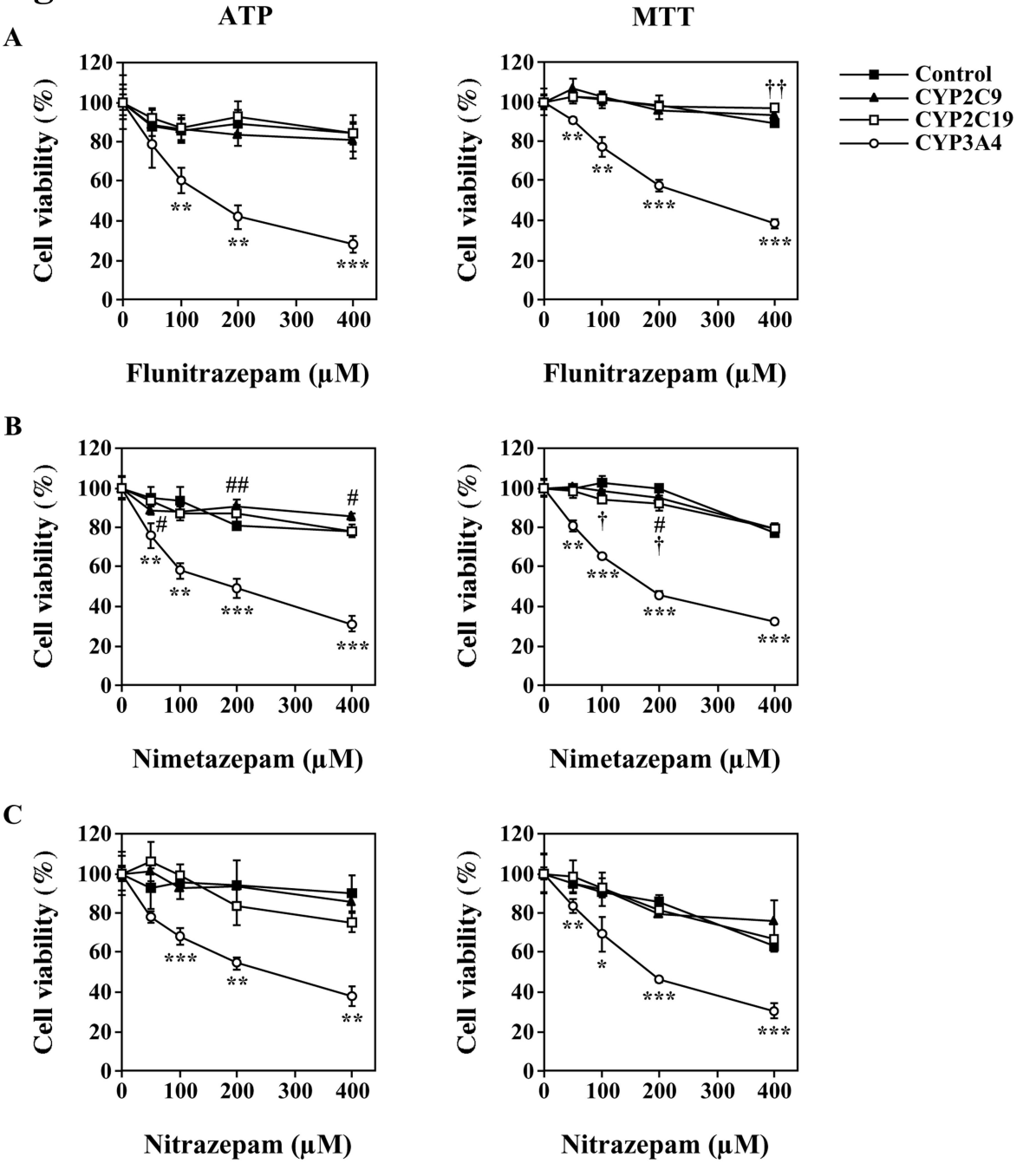

ATP
MTT
A
B
C
Control
CYP2C9
CYP2C19
CYP3A4
Cell viability (%)
Flunitrazepam (μM)
Nimetazepam (μM)
Nitrazepam (μM)
0
20
40
60
80
100
120
0
100
200
300
400
**
***
*
#
##
†
††

Fig. 4.

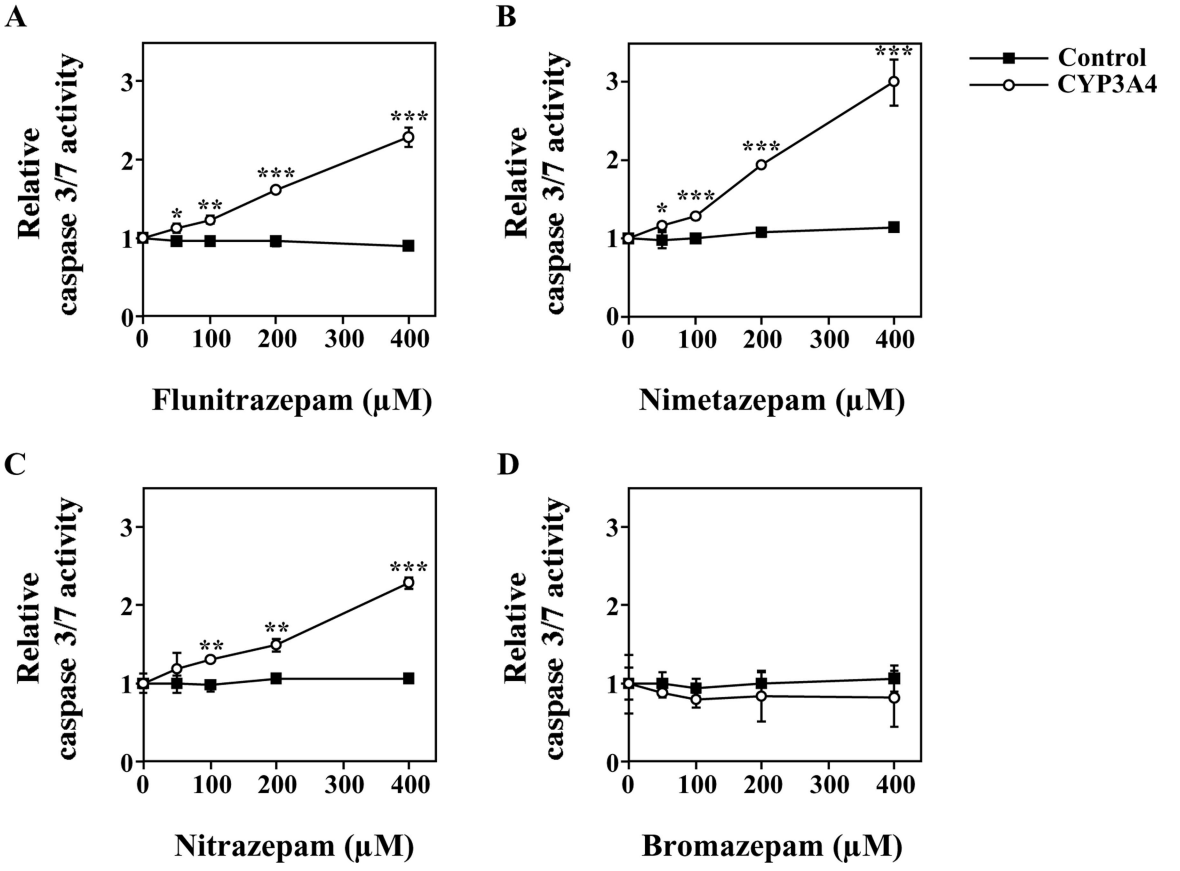

A
B
C
D
Relative caspase 3/7 activity
Flunitrazepam (μM)
Nimetazepam (μM)
Nitrazepam (μM)
Bromazepam (μM)
Control
CYP3A4
0
100
200
300
400
1
2
3
*
**
***

Fig. 5.

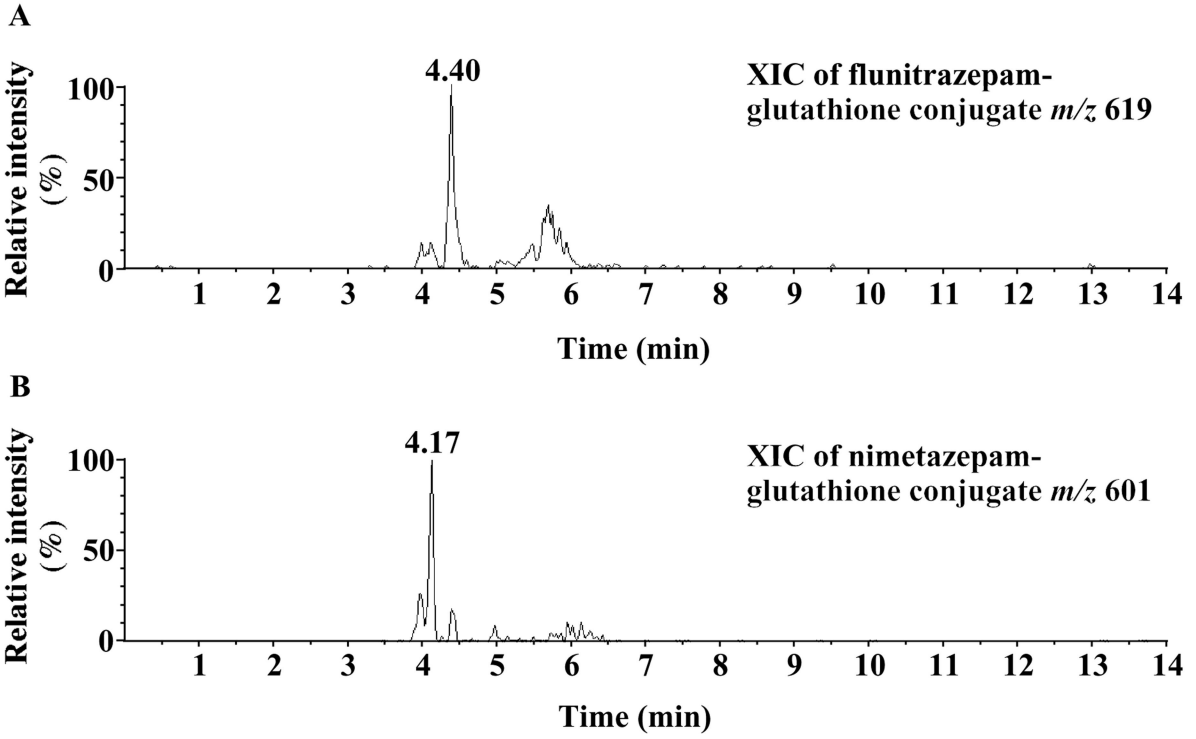
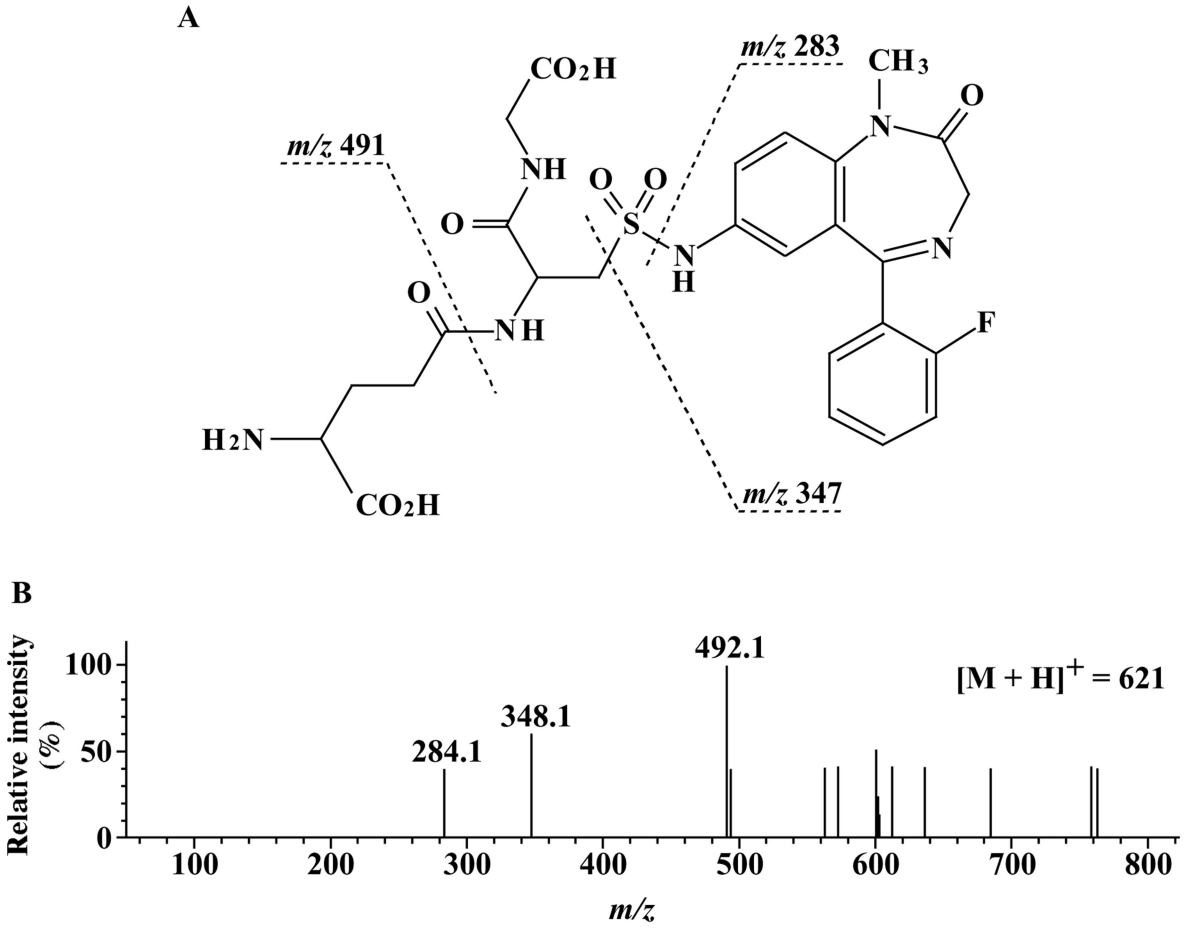

A
4.40
XIC of flunitrazepam-glutathione conjugate m/z 619
Relative intensity (%)
100
50
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14
Time (min)
B
4.17
XIC of nimetazepam-glutathione conjugate m/z 601
Relative intensity (%)
100
50
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14
Time (min)

Fig. 6.


A
m/z 283
m/z 491
m/z 347
CO2H
CH3
NH
O
S
N
H
F
H2N
B
Relative intensity (%)
492.1
348.1
284.1
[M + H]+ = 621
m/z