A simple monitoring method of triazolam metabolism with ethanol-treated rat liver microsomes by the HPLC-UV detection for the preliminary examination of abused drugs interaction

Mitsuhiro Wada [*1] · Miki Yoshida [*2] · Sumiyo Katoh [*2] · Naotaka Kuroda [*3]
Chang Kee Lim [*4] · Kenichiro Nakashima [*3] and Shuzo Akiyama [*5]

A simple and convenient monitoring method of triazolam (TZ) metabolism in ethanol-treated rat liver by HPLC-UV detection is described. Detection limits of standard TZ, diazepam (DZ) and nitrazepam (NZ) at a signal-to-noise ratio of 3 were 7.8, 6.8 and 10.2 ng/mL, respectively. Metabolisms of TZ and DZ could be ascertained by the proposed method. NZ could not be metabolized under the conditions used.

The proposed method was applied to elucidate the effect of ethanol ingestion on metabolism of TZ and DZ. The liver microsomes used were prepared from rats that were pre-administrated with phenobarbital or ethanol. The metabolic rates of TZ and DZ with ethanol pre-treated rat liver microsomes were faster than that of control (n=3), while there was no significant difference in those among the phenobarbital pre-administrated rats. Metabolites derived from TZ and DZ could be confirmed by preparative HPLC and GC-MS.[1]

Benzodiazepines are widely used as hypnotics, muscle relaxants, anticonvulsants and tranquilizers. On the other hand, they are also abused due to their hypnotic effect. Especially, the abuse of triazolam (TZ: Halsion®) becomes a big social problem.

Recently, the trend of stimulants abuse, two or more abused drugs are ingested concurrently. Therefore, it is significant for forensic and therapeutic chemists to investigate the interaction of abused drugs for absorption, metabolism and pharmacological action. There is little information reported for metabolic interaction among abused drugs.

In this study, a monitoring method for BZs *in vitro* metabolism with rats liver microsomes treated with another abused drug is described. It is important to develop a simple method for the preliminary study to investigate the interaction of abused drug with instead of human sample. First, a simple HPLC-UV method was examined for three kinds of BZs [TZ, diazepam (DZ), nitrazepam (NZ)]. BZs are classified into three

* 1 Faculty of Education and Human Studies, Akita University
* 2 School of Pharmaceutical Sciences, Nagasaki University
* 3 Graduate School of Biomedical Sciences, Course of Pharmaceutical Sciences, Nagasaki University
* 4 MRC Bioanalytical Science Group, School of Biological & Chemical Sciences, Birkbeck, University of London
* 5 Department of Applied Physics and Chemistry, Fukui University of Technology

categories according to their duration of action. The representatives for short-, medium- and long-acting hypnotic were selected as TZ, NZ and DZ, respectively. Ethanol, which is the most frequently used drug, was selected as an abused drug. Several studies for the interaction between ethanol and BZs were reported[1,2]. However, few studies addressed the metabolism of BZ with ethanol ingestion.

The proposed method was applied to monitor the metabolism of BZs with rats liver microsomes treated with other drugs. The metabolic rate and pattern of chromatogram for BZs with ethanol ingestion were compared with those of control or those treated with phenobarbital. Furthermore, metabolites derived from TZ and DZ were confirmed by preparative HPLC and GC-MS.

Results and discussion

The compositions of mobile phases for TZ, DZ and NZ were 40:60, 50:50 and 35:65 (V/V%), respectively. The peaks of BZs were eluted at a retention time of *ca.* 15 min. The detection limits for TZ, DZ and NZ at S/N of 3 were 7.8, 6.8 and 10.2 ng/mL, respectively. The sensitivity of the proposed method was comparable to those of HPLC with UV[3] or photodiode array UV detection[4].

The metabolism of BZs with rat liver microsomal preparation was monitored by the proposed method. The peak of TZ (411.8 ng/mL) and DZ (341.6 ng/mL) decreased with prolonging the incubation time (Fig. 1).

On the other hand, no metabolism of NZ took place with prolonging the incubation time to 60 min. Although NZ was incubated with a microsomal preparation induced by phenobarbital, no metabolism of NZ was observed. It was concluded that enzymes in this microsomal preparation do not metabolize NZ.

The effects of ethanol ingestion on the metabolism of TZ and DZ with rat liver microsomes were examined. The metabolic rates of TZ for group A, B and C showed the order: B>C>A. There are no significant differences among them, although the rates for group B and C were 1.84 and 1.57 times faster than that of group A. The metabolic rates for DZ showed the order: B>C>A. No significant difference of metabolic rate and the pattern of chromatogram were also observed for DZ. The doses of inducers are enough to induce rat liver cytochrome P-450[5,6]. Therefore, it was concluded that the enzymes, which metabolize TZ and DZ in rat liver, were not affected with phenobarbital and ethanol. However, the dose of alcohol used in this study was not enough to link the rats to the long-lasting effects on the liver; fatty liver, hepatic fibrosis, alcohol hepatitis and cirrhosis. The interaction between chronic ethanol ingestion and the metabolism of BZ should be examined.

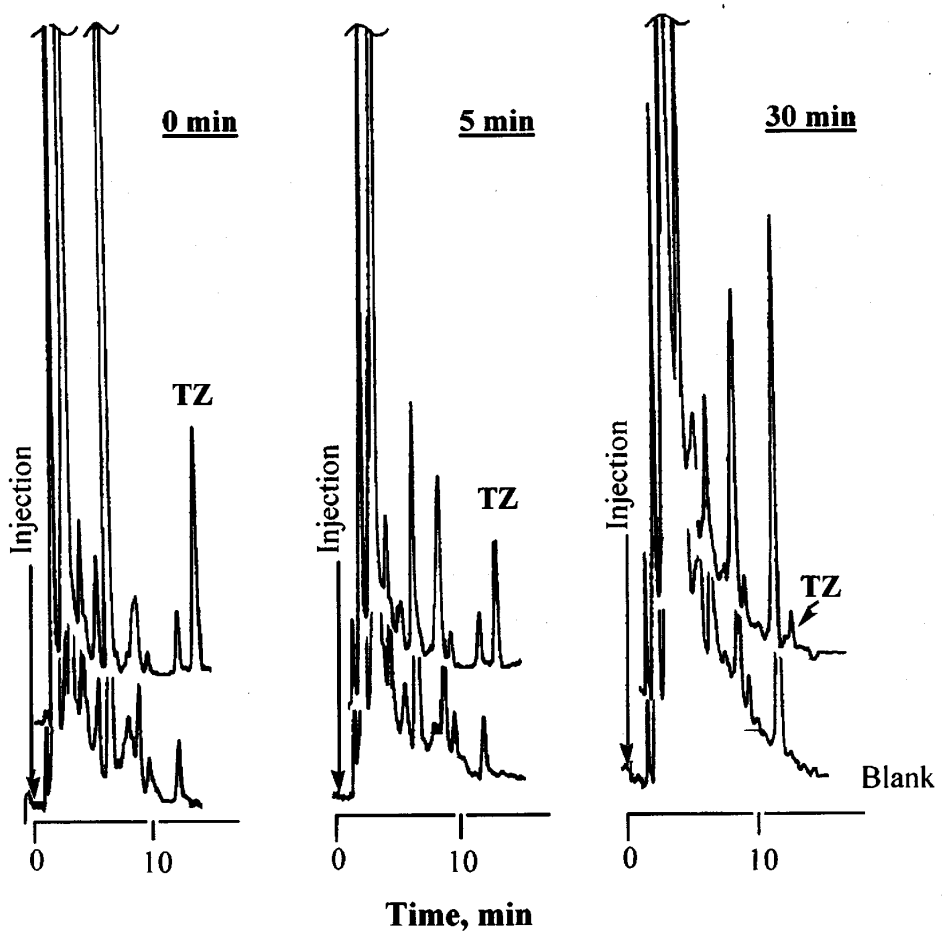

Fig. 1 Chromatograms of TZ after microsomal treatment

Sample, 411.8 ng of TZ/ml of enzymatic reaction mixture; Eluent, CH_3CN/H_2O (=40:60 v/v); Column, Daisopack-SP-120-5-ODS-2 (250x4.6 mm, i.d.); Detection, 222 nm.

The numbers on the chromatograms indicate the incubation time.

Metabolites derived from TZ and DZ were satisfactory separated by HPLC with slight modification of eluent composition. The constitutions of mobile phase for TZ and DZ were 30:70 and 50:50 (V/V%), respectively.

The eluate corresponding to the metabolite of TZ and DZ was collected and measured by GC-MS.

In conclusion, the proposed method could monitor the effect of ethanol ingestion on the metabolism of TZ and DZ. Metabolism of TZ and DZ *in vitro* with rat liver microsomes was not affected by ethanol ingestion under these conditions. If a significant difference was obtained by this preliminary method, it will be necessary to be applied for human microsome. This method may be applicable for examination of the metabolic interaction of BZs with other abused drugs such as cocaine, heroin, cannabis and methamphetamine.

Experimental

Reagents

BZs (TZ, DZ and NZ) were extracted from commercially available tablets as reported previously[3]. NADP was obtained from Oriental Yeast Co. (Tokyo, Japan). D-glucose-6-phosphate and D-glucose-6-phosphate dehydrogenase from Boehringer Mannheim-Yamanouchi Co. (Tokyo) were used. Water was passed through a Puric-Z (Organo Co., Tokyo). All other chemicals were of analytical grade or better.

Apparatus and conditions

The HPLC system for determination of BZs consisted of a chromatographic pump, an injector with a 20-μL sample loop, a Daisopak-SP-120-5-ODS-2 (250 x 4.6 mm, i.d., 5 μm), a variable-wavelength UV monitor and a recorder. The wavelengths for monitoring TZ, NZ and DZ were at 222, 218 and 230 nm, respectively. Metabolites of BZs were confirmed by Trio III type GC-MS (VG Micromass, Manchester, UK).

Animal treatment

Male Wister rats weighing about 150-230 g were used in all experiments. The rat (group A) as control received normal rat cow and water *ad libitu*. Phenobarbital aqueous solution at a dose of 100 mg/2mL/kg was injected intraperitoneally once a day for three days before sacrifice (group B). The rats for group C received 16% ethanol aqueous solution instead of water. The calculated dose of ethanol was 15 g/kg/day.

Sample preparation

The microsomes were prepared as reported previously[7]. Ten-μL of 120 μM substrate, 590 μL of Tris-HCl buffer (0.1 M, pH 7.4) and 30 μL of microsomal solution were mixed and preincubated at 37°C for 5 min. The reaction was started by the addition of a mixture of D-glucose-6-phosphate, $NADP^+$, D-glucose-6-phosphate dehydrogenase, 150 mM $MgCl_2$ and water. The reaction was terminated by vortex-mixing with 6 mL of ethyl acetate. Then 5 mL of the organic layer was taken and dried up with evaporator. The residue was dissolved with 800 μL of methanol and 20-μL of this solution was injected into HPLC.

References

1 Bidder M., Weizman R., Fares F., Grel I. and Gavish M. *Biochem. Pharmacol.* 1992; **44**: 1335.

2 Mason S.R., Reilly P.E. and Ward L.C. *Alcohol Alcohol.* 1992; **27**: 143.

3 Kuroda N., Zhou B., Nakashima K. and Akiyama S. *Jpn. J. Clin. Chem.* 1995; **24**: 228.

4 Lambert W.E., Meyer E., Xue-Ping Y. and Leenheer A.P. *J. Anal. Toxicol.* 1995; **19**: 35.

5 Kobylinska K. and Pachecka J. *Acta Pol. Pharm.* 1997; **54**: 443.

6 Lechevrel M. and Wild C.P. *Arch. Toxicol.* 1997; **71**: 690.

7 Ariyoshi T., Takabatake E. and Remmer H. *Life Sci.* 1970; **9**: 361.

(Received November 14, 2002)