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Development and preliminary application of high-performance liquid chromatographic assay of urinary metabolites of diazepam in humans

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Abstract

A simple high-performance liquid chromatographic method for the measurement of diazepam (DZP) and its major metabolites, N-desmethyldiazepam (DMDZP), temazepam (TZP) and oxazepam (OZP) in urine was developed. Preliminary studies of DZP metabolism were also undertaken in four healthy volunteers after administration of a single oral dose (4 mg) of DZP. The assay allowed the simultaneous determination of all analytes in 1 ml of urine and the detection limit was 2 ng/ml with a signal-to-noise ratio of 3. None of 22 drugs and 17 metabolites, except for mianserin, maprotiline and imipramine N-oxide, interfered with the detection of DZP metabolites. Recoveries of the analytes and the internal standard (prazepam) were >82%. Intra- and inter-assay coefficients of variation for all analytes were <5.5 and 4.1%, respectively. The mean (± S.D.) cumulative urinary excretions of DMDZP, TZP and OZP over 96 h after a single oral administration of DZP were 3.9 ± 0.4, 6.6 ± 1.4 and 2.8 ± 0.6% of the dose, respectively. The urinary excretion of DZP was under the detection limit.

1. Introduction

Diazepam (DZP) is a widely prescribed benzodiazepine, which is effective for the symptomatic relief of tension and states of anxiety as well as for the treatment and prevention of seizures [1–5]. It is metabolized to its N-demethylated form, N-desmethyldiazepam (DMDZP) and the C-3 hydroxylated form, temazepam (TZP), both of which are eventually converted to the N-demethylated- and C-3-hydroxylated product, oxazepam (OZP) [3–5]. DMDZP is the major metabolite of DZP in plasma, and is a sedative, hypnotic and anxiolytic compound [1–5]. TZP and OZP are also pharmacologically active and used as anxiolytic drugs [2]. However, these two metabolites are not considered to contribute significantly to the therapeutic effects of DZP, because their plasma concentrations are low after the administration of DZP [3–5]. The kinetics and metabolic disposition of DZP have been studied by measuring its concentration in

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plasma or serum [1,3–5]. However, the information available on the urinary excretion of DZP and its metabolites in humans is limited [3–6], probably because there are few simple and specific assays for DZP and its major metabolites in urine. Several attempts have been made to measure the urinary concentrations of DZP and its metabolites by electron-capture gas–liquid chromatography (GLC) [6,7], thin-layer chromatography (TLC) [8] and gas chromatography–mass spectrometry (GC–MS) [9]. However, to our knowledge, only a few papers describing a high-performance liquid chromatographic (HPLC) method have been published [10,11]. In general, HPLC is more suitable for the routine assay of drug concentrations in biological fluids than electron-capture GLC, TLC and GC–MS.

Here we report an HPLC method which allows the simultaneous determination of DZP, DMDZP, TZP and OZP in human urine. We also report some preliminary data on the urinary excretion–time profiles of the major DZP metabolites in four healthy volunteers after a single oral dose of DZP.

2. Experimental

2.1. Reagents

DZP, DMDZP, TZP, OZP and prazepam (PZP) were gifts from Hoffmann-La Roche (Basel, Switzerland). 8-Hydroxymianserin, mianserin N-oxide, desmethylmianserin and mianserin were gifts from Organon International (Oss, Netherlands). Carbamazepine, carbamazepine-10,11-epoxide, 2-hydroxyimipramine, 2-hydroxydesipramine, 10-hydroxyimipramine, desipramine, imipramine and imipramine N-oxide were gifts from Ciba-Geigy (Basel, Switzerland). Flurazepam, clonazepam, nitrazepam and chlordiazepoxide were gifts from Sumitomo Chemical Co. (Takarazuka, Japan). Chlorpromazine and chlorpromazine S-oxide were gifts from Yoshitomi Pharmaceutical Industries (Osaka, Japan). Sulpiride, haloperidol, triazolam, maprotiline, levomepromazine, perphenazine and thioridazine were gifts from Dr. Yamamoto, Showa University, School of Pharmacy (Tokyo, Japan). 7-Aminoflunitrazepam, desmethylflunitrazepam, flunitrazepam, nortriptyline and amitriptyrine were gifts from Dr. Ueno, Faculty of Pharmaceutical Science, Chiba University (Chiba, Japan). *p*-Hydroxyphenobarbital, *p*-hydroxyphenytoin, caffeine, phenytoin and phenobarbital were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). β-Glucuronidase/arylsulfatase was obtained from Carbiochem Co. (La Jolla, CA, USA). Methanol, triethylamine and other reagents of analytical or HPLC grade were purchased from Wako Pure Chemical Industries (Osaka, Japan).

2.2. HPLC system

Analyses were performed with the following HPLC system: a Model L-6000 pump, a Model L-4000 ultraviolet absorbance detector, a Model AS-2000 autosampler, a Model D-2500 integrator (Hitachi, Tokyo, Japan) and a 25 cm × 4 mm I.D. LiChrospher 100 RP-18(e) column (5 μm) (Kanto Kagaku Co., Tokyo, Japan). The mobile phase consisted of a water–methanol–triethylamine (70:30:0.1, v/v) adjusted with phosphoric acid to pH 5.5. The flow-rate was 0.7 ml/min. Each analyte was monitored at 240 nm.

2.3. Sample preparation

One milliliter of urine, 100 μl of acetate buffer (5 m*M*, pH 5.5) and 25 μl of β-glucuronidase/sulfatase (0.235/0.065 U) were mixed and then allowed to incubate at 37°C for 16 h. Then, 50 μl of the internal standard solution (5 or 50 μg/ml of PZP in methanol) and 1 ml of saturated trisodium phosphate solution were added. After dichloromethylene (3 ml) was added, the sample was extracted by vortex-mixing for 2 min, and then centrifuged at 1610 *g* for 5 min. The upper aqueous layer was carefully aspirated and discarded. An aliquot of the organic layer (2 ml) was transferred to a new screw-capped tube. Then, 2 ml of hexane and 2 ml of 6 *M* HCl were added and the mixture was vortex-mixed for 2 min. After centrifugation at 1610 *g* for 5 min, the

HCl layer (1 ml) was transferred to a new screw-capped tube, and the pH was adjusted to ca. 6 with 1 ml each of 6 *M* NaOH and saturated trisodium phosphate solution. The mixture was then extracted with 3 ml of dichloromethylene by vortex-mixing for 2 min. After centrifugation at 1610 *g* for 5 min, the aqueous phase was aspirated and discarded. The remaining organic phase was transferred to a new glass tube and evaporated to dryness with a gentle stream of nitrogen at 50°C. The residue of the extract was dissolved in the mobile phase (150 μl) of which 60 μl was injected onto the HPLC apparatus described above.

2.4. Quantitation

To examine the linearity of the assay, we prepared calibration curves for the four analytes in urine at concentrations varying between 10 and 200 ng/ml, and between 100 and 2000 ng/ml ($n = 2$ each). A standard sample was prepared by adding the analytes to drug-free urine. The standards were treated with β-glucuronidase/arylsulfatase, extracted and analyzed as described above. Peak-height ratios of each analyte to the internal standard were measured and the calibration curve was obtained from least-squares linear regression. The regression lines between 100 and 200 ng/ml and between 100 and 2000 ng/ml were used to calculate the concentrations of the analytes contained in the urine samples of volunteers obtained from the single dose study and those of the patients with anxiety disorders as mentioned below.

To assess the absolute recoveries of the analytes from urine, we compared the peak heights obtained from the standard stock solutions of analytes with those from drug-free urine to which known amounts of each of the analytes had been added to give a final concentration of 100 ng/ml.

Precision and accuracy of the assay were assessed by the intra- and inter-assay coefficients of variation (C.V.s) by determining each of the analytes in the pooled urine of a single-dose study with the healthy volunteers and that obtained from patients with anxiety disorders. Patients were taking DZP (2 × 4 mg/day) for at least 4 weeks. Levomepromazine (25 or 50 mg/day), thioridazine (4 mg/day), flunitrazepam (2 mg/day) or zotepine (150 mg/day) were administered concurrently during DZP treatment.

2.5. Preliminary metabolic study in humans

To evaluate the applicability of the present assay, we measured the urinary excretion of DZP and its three major metabolites, DMDZP, TZP and OZP, in four healthy male volunteers aged 24–40 years. Informed consent was obtained from each of the subjects. The volunteers took a single oral dose of DZP (4 mg) (Cercine, Takeda Pharmaceutical Industry, Osaka, Japan) after an overnight fast. Urine samples were collected at 0–12, 12–24, 24–36, 36–48, 48–60, 60–72, 72–84, and 84–96 h after dosing. After measuring the urine volume, an aliquot (10 ml) was stored at −20°C until analyzed.

3. Results

Representative chromatograms are shown in Fig. 1. The retention times for OZP, TZP, DMDZP, DZP and PZP were 7.0, 7.7, 9.1, 10.3 and 17.0 min, respectively (Fig. 1A). There were many interfering peaks in the chromatogram obtained from drug-free urine which was extracted with dichloromethylene after the enzymatic hydrolysis (Fig. 1B). Since these unknown peaks made it impossible to resolve the peaks of the analytes, we back-extracted the organic phase with 6 *M* HCl after adding hexane. Hexane was added to improve the efficiency of the extraction of the analytes, particularly PZP, from an organic phase to 6 *M* HCl. This modification improved the efficiency of the extractions of DZP, DMDZP, TZP and OZP from about 50% to 100%, and of PZP from about 10% to 80%. There were no interfering peaks in the chromatograms of drug-free and volunteer's urine after enzymatic hydrolysis when they were back-extracted with 6 *M* HCl and again extracted with dichloromethylene (Fig. 1C and D, respectively). Although DZP and its metabolites

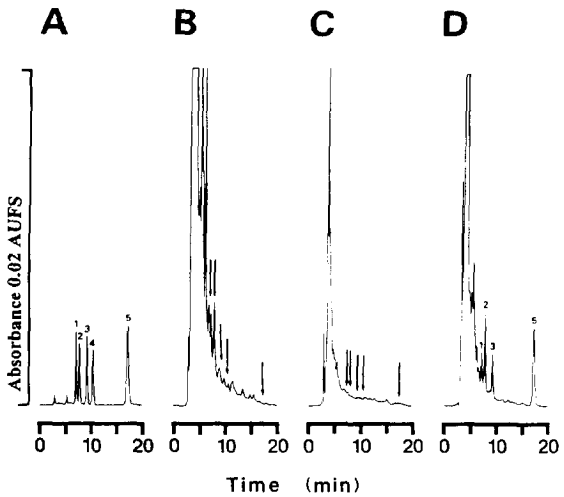

Fig. 1. Representative chromatograms of (A) a mixture of standard compounds, diazepam, temazepam, desmethyldiazepam and oxazepam (10 ng each) and prazepam (25 ng), (B) a single extract of drug-free urine treated by β-glucuronidase/arylsulfatase with dichloromethylene, (C) a three-step extract of drug-free urine treated with β-glucuronidase/arylsulfatase, and (D) a three-step extract of a volunteer's urine deconjugated with β-glucuronidase/arylsulfatase. Peaks: 1 = oxazepam; 2 = temazepam; 3 = desmethyldiazepam; 4 = diazepam; and 5 = prazepam (internal standard). The arrows in B and C indicate the anticipated elution times of the analytes and the internal standard.

are relatively unstable in an acidic solution [12], the peaks of DZP, DMDZP, TZP, OZP and PZP at the concentrations of 10 and 100 ng/ml did not decrease with the back-extraction employing 6 *M* HCl for 2 h, indicating that the degradation of analytes in 6 *M* HCl is negligible for at least 2 h.

Possible interference from 22 drugs which may be co-administered with DZP, and from 17 metabolites produced from the parent drugs, was studied. The results are listed in Table 1. According to the capacity ratios and the chromatograms of the simultaneous injection of DZP metabolites and drugs which may be concurrently prescribed with DZP, none of them interfered with the determination of DZP and its metabolites, except for mianserin, maprotiline and imipramine N-oxide which may interfere with OZP, TZP and DMDZP, respectively. In accordance with this observation, no interfering peaks were found in the urine samples obtained from patients who were taking levomepromazine, thioridazine, flunitrazepam or zotepine concurrently with DZP (chromatograms not shown).

The hydrolysis rates of DMDZP, TZP and OZP depended on the amount of enzyme added and on the incubation time. When the incubation time was fixed at 16 h, the rate of deconjugation increased with the addition of β-glucuronidase/arylsulfatase, up to 5 μl, which corresponded to 0.047/0.013 U (Fig. 2). Based on these data, enzymatic hydrolysis was performed with 25 μl of the enzyme (0.235/0.065 U) and with an incubation time of 16 h at 37°C. The mean recoveries of the analytes and internal standard ranged from 82.9 to 101.3% and the C.V.s were <4.9% (Table 2). The lower detection limits, defined as the lowest concentration for which the signal-to-noise ratio was at least 3, were 2 ng/ml for all analytes.

The calibration curves for the analytes are shown in Fig. 3A,B. The calibration curves were linear over the concentration ranges examined and the correlation coefficients for all analytes were high (>0.999, $p<0.001$).

The analytical precision of the present assay was assessed with the pooled urine of the healthy volunteers and that of the patients with anxiety disorders. The intra- and inter-assay C.V.s of DMDZP, TZP and OZP were <5.5 and 2.9% for the pooled urine obtained from the volunteers, and <3.9 and 4.1% for that obtained from the patients with anxiety disorders, respectively (Table 3). Since the concentrations of DZP were lower than the detection limit of the present assay in all of the study subjects, we could not assess the C.V.s for determining DZP in pooled urine. However, the intra- and inter-assay C.V.s for determining 10 and 100 ng/ml of DZP added to the control urine were <8%.

The cumulative urinary excretions of DMDZP, TZP and OZP for up to 96 h after administration of a single oral dose of DZP (4 mg) in the four volunteers are shown in Fig. 4. The mean (± S.D.) recoveries of DMDZP, TZP and OZP were 3.89 ± 0.36, 6.64 ± 1.41 and 2.76 ± 0.56%, respectively.

Table 1
Retention times and capacity ratios of DZP and its metabolites and those of other commonly prescribed drugs and their metabolites

Compound	Capacity ratio	Compound	Capacity ratio
Sulpiride	0.02	Desmethylmianserin	0.88
p-Hydroxyphenobarbital	0.14	Nitrazepam	0.88
Caffeine	0.18	Triazolam	1.07
8-Hydroxymianserin	0.19	Mianserin	1.18
p-Hydroxyphenytoin	0.20	Oxazepam	1.23
7-Aminoflunitrazepam	0.22	Temazepam	1.43
Carbamazepine 10,11-epoxide	0.38	Maprotiline	1.44
2-Hydroxyimipramine	0.39	Desipramine	1.52
2-Hydroxydesipramine	0.39	Imipramine	1.62
Phenobarbital	0.39	Chlordiazepoxide	1.68
10-Hydroxyimipramine	0.39	Nortriptyline	1.71
Mianserin N-oxide	0.53	Amitriptyline	1.76
Phenytoin	0.60	Levomepromazine	1.82
Chlorpromazine S-oxide	0.62	Imipramine N-oxide	1.85
Desmethylflunitrazepam	0.65	Desmethyldiazepam	1.88
Haloperidol	0.72	Diazepam	2.25
Flurazepam	0.73	Chlorpromazine	2.64
Clonazepam	0.79	Perphenazine	3.28
Carbamazepine	0.82	Thioridazine	3.88
Flunitrazepam	0.86		

Fig. 2. Effects of β-glucuronidase/arylsulfatase (9.4/2.6 U/ml) on the deconjugation of (△) desmethyldiazepam, (□) temazepam and (○) oxazepam. Each point represents the mean of two experiments.

Table 2
Recovery of diazepam and its metabolites at a concentration of 100 ng/ml from urine ($n = 6$ each)

Compound	Recovery (%)	C.V. (%)
Diazepam	89.6 ± 4.0[a]	4.5
Desmethyldiazepam	101.3 ± 4.3	4.2
Temazepam	82.9 ± 4.1	4.9
Oxazepam	94.8 ± 2.7	2.8
Prazepam	90.4 ± 2.9	3.2

[a] Mean ± S.D.

4. Discussion

Several analytical procedures have been used to measure urinary DZP and its metabolites [6–9]. Our HPLC method has some advantages over previously reported GC–MS [9] and TLC methods [8]. These include the simplicity of the assay system and the shorter analysis time. In addition,

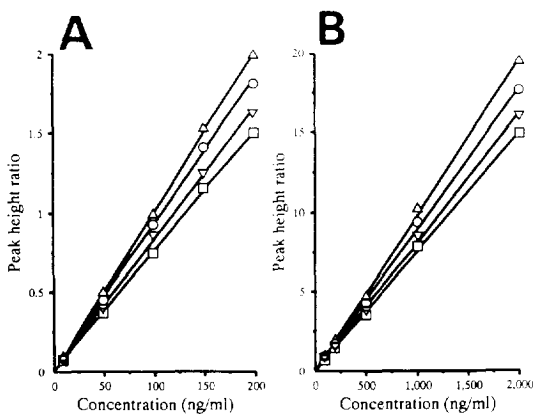

Fig. 3. Calibration curves between (A) 0 to 200 ng/ml and (B) 0 to 2000 ng/ml for (▽) diazepam, (△) desmethyldiazepam, (□) temazepam and (○) oxazepam in urine. The calibration curves between 0 and 200 ng/ml and between 0 and 2000 ng/ml were used for measuring the urine samples of healthy volunteers and patients with anxiety disorders, respectively. Linear regression analysis of the curves gave a correlation coefficient of >0.999 for all analytes. All intercept values did not significantly differ from zero.

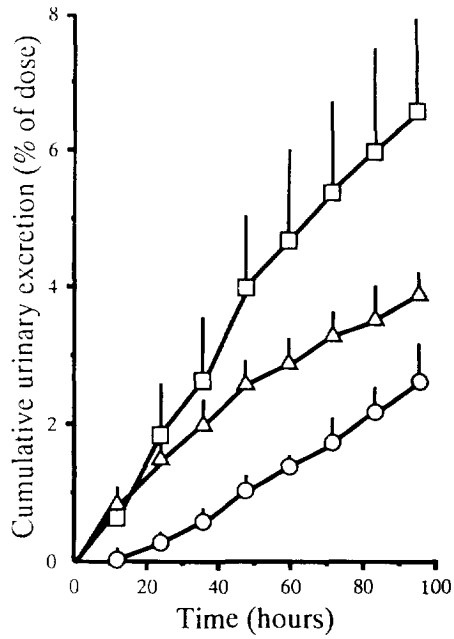

Fig. 4. The cumulative urinary excretions of (△) desmethyldiazepam, (□) temazepam and (○) oxazepam for up to 96 h after an oral administration of diazepam (4 mg) in four healthy volunteers. Each point with bar represents mean ± S.D. as the percentage of the administered dose of diazepam.

the present method does not require sample derivatization or acid hydrolysis which is required for GLC analysis [6,7,9,13]. Furthermore, OZP, a metabolite of DZP, is highly thermolabile and cannot withstand the high temperature needed for GLC analysis [13]. Thus, we believe that the present method is superior to non-HPLC methods for the routine use in the investigation of the metabolic disposition of DZP employing human urine samples.

To our knowledge, only a few papers have been published on HPLC analysis of DZP and its metabolites in urine [10,11]. However, the lower

Table 3
Analytical reproducibility of the measurement of desmethyldiazepam, temazepam and oxazepam in pooled urine samples obtained from volunteers after single oral dose of diazepam and patients taking diazepam for the treatment of anxiety disorders[a]

Compound	Pooled urine from volunteers or patients	Intra-assay C.V. ($n = 8$) (%)	Inter-assay C.V. ($n = 6$) (%)
Desmethyldiazepam	Volunteers	3.5	1.2
	Patients	1.9	1.9
Temazepam	Volunteers	3.9	2.3
	Patients	2.5	4.1
Oxazepam	Volunteers	5.5	2.9
	Patients	3.9	2.5

[a] An equal volume of the urine samples obtained from the volunteer and patient groups was pooled and mixed separately in each of the two groups. The averaged concentrations of desmethyldiazepam, temazepam and oxazepam in the pooled urine samples obtained from the volunteers and patients are 53.1, 119.3 and 42.1 ng/ml, and 355.5, 657.0 and 871.5 ng/ml, respectively.

detection limit of these assays was found to be too high to allow the detection of DZP metabolites in urine after a single dose of DZP as conducted in the present study. For instance, the mean (± S.D.) urinary concentrations of DMDZP, TZP and OZP in the present single-dose study were 29.6 ± 22.3, 57.4 ± 47.0 and 18.4 ± 16.7 ng/ml, respectively ($n = 32$ for each compound). These concentrations are far below the lower limit of detection of 200 ng/ml for each of the analytes reported by Cotler et al. [10], but they are far above the limit of detection (i.e. 2 ng/ml) for each analyte using the method described here. Thus, the present assay is more sensitive than the HPLC method reported previously [10]. The relatively low sensitivity of the assay reported by Cotler et al. [10] may be attributable to interfering peaks derived from the hydrolysis of urine by glusulase. In the present study, several peaks of non-polar endogenous substances appeared after enzymatic hydrolysis of the urine (Fig. 1B). It was impossible to resolve DZP and its metabolites from these peaks. However, this problem was overcome by back-extracting the analytes in 6 *M* HCl (Fig. 1C). This did not cause any appreciable loss of the analytes when the extraction procedure was modified by adding hexane to the organic phase (Table 2). As a result, the present purification process allowed us to detect 2 ng/ml of the analytes with a signal-to-noise ratio of 3. This should enable us to determine all DZP metabolites in urine for a period of more than 96 h after administration of a single oral dose of 4 mg DZP. Thus, the present HPLC method is simpler, more accurate, reproducible and sensitive than that reported previously [10,11], and it can be used in clinical studies of the urinary excretion–time profiles of DZP metabolites after both a single dose or repeated doses of DZP.

Our preliminary study showed that the principal metabolite of DZP excreted into the urine is TZP (6.6%), followed by DMDZP (3.9%) and OZP (2.8%) when DZP was administered as a single dose. This is consistent with the limited data reported previously: Arnold [6] employed electron-capture GLC to measure the urinary excretions of DZP metabolites after administering a single oral dose of two brands of 10-mg DZP tablets to healthy volunteers, and showed that 0.5 and 0.2%, 3.6 and 4.4%, 9.0 and 6.4%, and 8.7 and 6.3% of the ingested dose were excreted into the urine over a 72-h period after administration as DZP, DMDZP, TZP and OZP, respectively. The mean total amounts of DZP and its metabolites excreted in the urine (i.e. 20%) appear to be slightly higher than those found in our study (i.e. about 15%). However, the urinary excretion pattern reported by Arnold [6] is compatible with that found by us in that TZP is the main metabolite excreted in the human urine for up to 3 or 4 days after oral administration of DZP.

The pharmacokinetics of DZP have been studied extensively in relation to age, sex, smoking habits, liver disease, enzyme induction or inhibition, racial differences and pharmacogenetic factors [3–5,14–18]. In addition, three recent studies using human liver microsomes [14,19,20] have clearly demonstrated that N-demethylation and 3-hydroxylation activities of DZP are catalyzed by the specific P450 isoforms, CYP2C19 and CYP3A4, respectively. Moreover, DZP is widely prescribed in clinical practice and may also be used as a test probe to study genetically determined drug metabolism [14,17]. Therefore, a non-invasive means to evaluate the urinary excretion of DZP metabolites should yield a broader clinical applicability for screening purposes to assess the individual capability of drug metabolism in humans. In this context, the present HPLC method makes it easier to investigate the effects of physiological, environmental and genetic factors on drug metabolism in a large number of healthy subjects and patients.

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