Journal of Chromatography, 425 (1988) 435–440
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4024

Note

High-performance liquid chromatographic determination of nitrazepam in plasma and its application to pharmacokinetic studies in the rat

KAZUTOSHI SUZUKI, IKUO JOHNO and SHIKIFUMI KITAZAWA*

Department of Hospital Pharmacy, School of Medicine, Nagoya University, 65 Tsurumai-cho, Showa-ku, Nagoya 466 (Japan)

(First received June 30th, 1987; revised manuscript received October 20th, 1987)

Nitrazepam (NZP) is one of the oldest and most widely used benzodiazepines [1] for the treatment of insomnia, epilepsy, etc., and the therapeutic range in plasma is 10–100 ng/ml [2,3]. However, some studies indicated tolerance, rebound insomnia [4–6] and changes of pharmacokinetic parameters for benzodiazepines including NZP [7,8]. Therefore, the basic pharmacokinetic and pharmacodynamic studies of NZP in small animals such as the rat have to be clarified in order to carry out pertinent drug therapy. However, several procedures for the determination of NZP in body fluids involving high-performance liquid chromatography (HPLC) require a large sample volume (1 ml) [3,9–12] and therefore they are not applicable to studies involving small animals, as a pharmacokinetic study requires several plasma samples from each animal.

In this work, a method was developed that requires smaller plasma volumes for the determination of NZP. The method is sensitive, reliable, convenient and applicable to pharmacokinetic studies in the rat.

EXPERIMENTAL

Materials

NZP and triazolam were kindly supplied by Shionogi (Osaka, Japan) and Japan Upjohn (Tokyo, Japan), respectively. Normal human plasma was obtained from the Japanese Red Cross Society (Tokyo, Japan). Other chemicals were of analytical-reagent grade. Each buffer solution was prepared by mixing two solutions until the desired pH was obtained.

0378-4347/88/$03.50

High-performance liquid chromatography

An LC-6A chromatographic system (Shimadzu, Kyoto, Japan) was used, consisting of an LC-6A liquid pump, an SPD-6AV UV–visible spectrophotometric detector, an SIL-6A autoinjector with an SCL-6A system controller and a Chromatopac C-R3A data processor.

The column was 15 cm × 4.6 mm I.D., packed with Zorbax ODS (5 μm particle size) (DuPont, Wilmington, DE, U.S.A.; distributed by Shimadzu). The mobile phase was acetonitrile–phosphate buffer (2:3, v/v) at a flow-rate of 1 ml/min. The buffer was composed of 0.6 m*M* KH_2PO_4 and 14.7 *M* H_3PO_4 adjusted to pH 3.0. The elutions were carried out at ambient temperature (25 ± 2°C) and the column effluent was monitored at 221 nm.

Extraction procedures

To 200 μl of human or rat plasma in a centrifuge tube, 80 μl of internal standard (I.S.) solution (1 μg/ml triazolam in acetonitrile), 1 ml of 0.01 *M* sodium hydroxide solution and 5 ml of diethyl ether were added. The mixture was shaken for 15 min and centrifuged for 10 min at 1500 *g*. The organic layer was transferred into a suitable tube and evaporated to dryness under a stream of nitrogen at 40°C. The residue was redissolved in 70 μl of mobile phase and 25 μl of the resulting solution were injected into the chromatograph.

Preparation of calibration curves

NZP was added to blank rat or human plasma (10–200 ng/ml) and determined according to the procedure described above. Triplicate measurements of seven different concentrations were carried out. The calibration curves were calculated by the least-squares method.

Analytical recovery and precision

The recovery was assessed from ten replicate analyses using samples prepared by adding known amounts of NZP to rat or human blank plasma to give final concentrations of 20 and 60 ng/ml.

The within-day variation was examined by analysing the samples used in the recovery study. These samples were also assayed once a day for ten days to evaluate the between-day precision.

Animal studies

The animals used were male kbl:Wistar rats (Kitayama Labes, Kyoto, Japan) weighing 230–290 g. The pharmacokinetic studies of NZP were performed after intravenous administration of 200 μg/kg. About 500-μl blood samples were taken 0.25, 0.5, 0.75, 1.0, 2.0, 4.0 and 6.0 h after the administration. The samples were collected in heparinized tubes and centrifuged for 2 min at 6500 *g*. Plasma was stored at −35°C until the analysis. The plasma concentrations were calculated following the two-compartment open model [13,14].

RESULTS AND DISCUSSION

Extraction

The pH is of importance for the extraction of a drug with a pK_a value of 10.8 [15]. Extraction of endogenous compounds can also be affected by the pH of the aqueous phase. In previous reports [2,3,9–12,16], the pH and the buffer component used for extraction were different from those used in this work. Therefore, in order to extract NZP efficiently, we tested the effect of alkaline solutions, such as 0.1 *M* sodium hydroxide [9,12], 1 *M* borate buffer (pH 8.0, 9.0 and 10.0 prepared from 1 *M* sodium tetraborate and 1 *M* sodium hydroxide) [2,16] and 0.01 *M* sodium hydroxide. The chromatograms showed that the peak heights of NZP after extraction with 0.01 *M* sodium hydroxide and borate buffers were three times larger than those obtained with 0.1 *M* sodium hydroxide. Further, the chromatogram obtained from extraction with 0.01 *M* sodium hydroxide indicated the best separation between NZP, I.S. and endogenous compounds. For this reason, 0.01 *M* sodium hydroxide was considered to be the most suitable alkaline solution for the assay.

Detection wavelength

UV detection of NZP was performed at 254 or 300 nm in previous studies [3,9–12]. However, in our study, these wavelengths did not give the best absorption spectrum for the detection of NZP in acetonitrile. More sensitive detection was possible below 221 nm. The peak height of NZP at 221 nm corresponded to 1.31 times that at 254 nm and also 1.68 times that at 300 nm. Consequently, the detection of NZP was carried out at 221 nm.

Choice of column

For the determination of NZP and I.S., Shim-pack FLC-CN (3 μm; 5 cm$\times$4.6 mm I.D.; Shimadzu) [12], Cosmosil 5CN-R (5 μm; 15 cm$\times$4.6 mm I.D.; Nakarai Chemicals, Kyoto, Japan), Shim-pack FLC-ODS (3 μm; 5 cm$\times$4.6 mm I.D.; Shimadzu) and Zorbax ODS (5 μm; 5 and 15 cm$\times$4.6 mm I.D.; DuPont) [3,11] were evaluated on the basis of the separation factor (α) and resolution (R_s) according to a conventional method. The proportions of acetonitrile and phosphate buffer (pH 3.0) in the mobile phase were adjusted to give a consistent retention time of I.S. within 11–14 min. The α values obtained with 5- and 15 cm long Zorbax ODS columns were 1.16–1.63 times larger than those obtained with the other columns, and the longer Zorbax ODS column gave the maximum R_s values, which were 1.56–2.42 times those obtained with the other columns. These results indicated that the 15-cm Zorbax ODS column was satisfactory for the NZP analysis.

pH of buffer in mobile phase

The effect of the pH of the phosphate buffer was studied. Four different phosphate buffers were prepared as follows: pH 2.0, dilution of 14.7 *M* H_3PO_4; pH 3.0, 14.7 *M* H_3PO_4 and 0.6 m*M* KH_2PO_4; and pH 4.0 or 6.0, 2.5 m*M* KH_2PO_4 and 2.5 m*M* K_2HPO_4. The chromatogram obtained using pH 3.0 phosphate buffer showed

the best separation and the peaks of NZP and I.S. scarcely overlapped with those of endogenous compounds. Therefore, pH 3.0 was selected as the pH of the phosphate buffer in the mobile phase.

Chromatogram and precision

Fig. 1 shows the typical chromatograms obtained. The chromatogram of the mixture of NZP and I.S. (25 ng each) in acetonitrile is shown in Fig. 1A. The retention times were 6.2 min for NZP and 14.2 min for the I.S. Fig. 1B and C show that endogenous compounds in rat plasma did not interfere with the assay of the benzodiazepines. The graph of peak-height ratio versus NZP concentration showed good linearity within the range 10–200 ng/ml. The correlation coefficient (r) was 0.999 and the equation for the regression line was $y=0.0118x+0.0727$. Also, the calibration curve obtained from human plasma showed a good correlation ($n=21$, $r=0.999$); the regression equation was $y=0.0116x+0.0375$ within the range of 10–200 ng/ml and was in good agreement with that from rat plasma. The detection limit in plasma was found to be 10 ng/ml at a signal-to-noise ratio of 3.

Precision data are given in Table I. The within-day precision was less than 3%

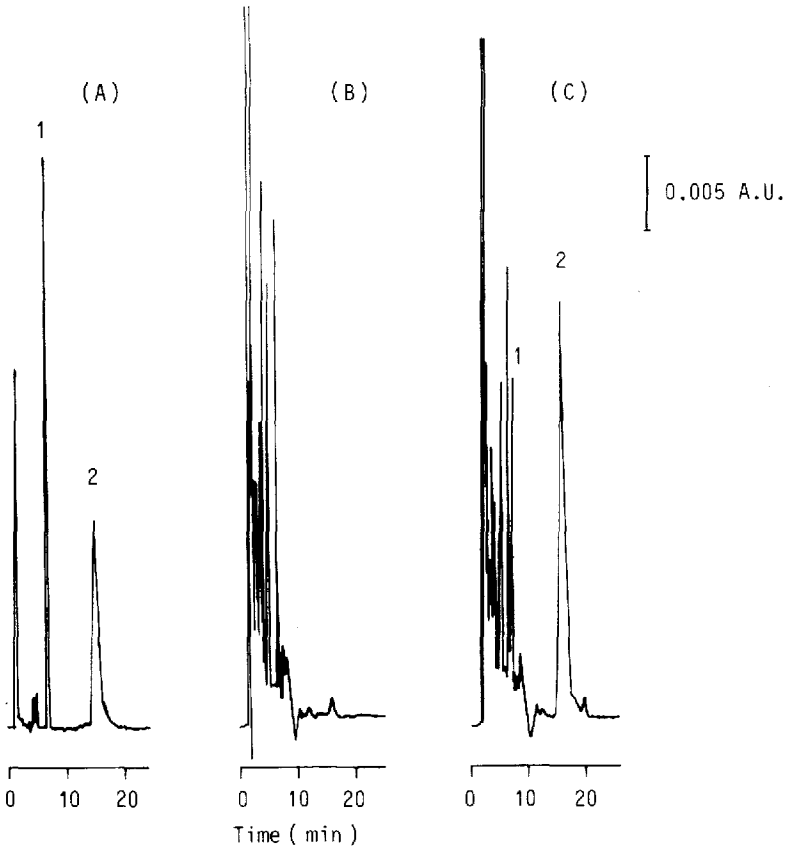

Fig. 1. Chromatograms of (A) a mixture of standard nitrazepam (1) with triazolam as an internal standard (2), (B) drug-free plasma and (C) plasma containing nitrazepam (60 ng/ml) and triazolam.

TABLE I

WITHIN-DAY AND BETWEEN-DAY PRECISION FOR NITRAZEPAM ($n=10$)

Sample	Concentration (ng/ml)	Coefficient of variation (%)	
		Within-day	Between-day
Rat plasma	20	2.96	4.96
	60	2.98	4.88
Human plasma	60	2.16	5.13

TABLE II

ANALYTICAL RECOVERY OF NITRAZEPAM

Sample	Concentration (ng/ml)	Recovery (mean ± S.D., $n=10$) (%)
Rat plasma	20	99.4 ± 4.93
	60	98.0 ± 4.81
Human plasma	60	100.5 ± 5.15

and the between-day precision was approximately 5% expressed as the coefficient of variation. The recovery ranged from 98.0 to 100.5%, as shown in Table II, and this is similar to published data obtained with larger volumes of plasma [9,11,12].

This improved HPLC method permits the determination of NZP in smaller sample volumes (200 μl) than in previous methods [3,9–12] and offers similar precision, recovery and linearity of response. Because of the high sensitivity, the procedure presented here may be applicable to studies of basic pharmacokinetics and/or pharmacodynamics in more detail in small animals such as the rat.

Pharmacokinetic studies in rats

We used our improved NZP assay method to study the pharmacokinetics of NZP after an intravenous dose of 200 μg/kg to a rat. The elimination patterns of the benzodiazepine from rat plasma displayed a typical two-compartment pharmacokinetic profile, which was similar to that for humans [7,17] and other animals [5]. Although the NZP levels in our experiment were about one fortieth of those used by Chodera et al. [5], the half-life of the β-phase (4.02 ± 1.74 h; mean ± standard error of the mean, $n=3$) in this study was in agreement with their value.

Based on these observations, we could obtain the pharmacokinetic parameters for the rat with the usual human dose and a small sampling volume of blood which hardly affected the physiological functions [18]. This method may permit basic studies of NZP pharmacokinetics in small animals and clinical applications to routine therapeutic monitoring.

REFERENCES

1 K. Tahara, K. Nakai, I. Sudo and K. Umino, Jpn. J. Clin. Pharmacol. Ther., 17 (1986) 167.

2 A.G. de Boer, J. Röst-Kaiser, H. Bracht and D.D. Breimer, J. Chromatogr., 145 (1978) 105.
3 R. Dixon, R. Lucek, R. Young, R. Ning and A. Darragh, Life Sci., 25 (1979) 311.
4 S. Watanabe, Therapeutic Drug Monitoring in Psychiatry, Dainippon Pharmaceutical, Osaka, 1985, p. 217.
5 A. Chodera, K. Szcawińska, D. Cenajek and E. Nowakowska, Pol. J. Pharmacol. Pharm., 36 (1984) 353.
6 A. Kales, M.B. Scharf, J.D. Kales and C.R. Soldatos, J. Am. Med. Assoc., 241 (1979) 1692.
7 D.J. Greenblatt, D.R. Abernethy, A. Locniskar, H.R. Ochs, J.S. Harmatz and R.I. Shader, Clin. Pharmacol. Ther., 38 (1985) 697.
8 H. Watanabe, S. Nakano, N. Takaoka, S. Ohto and N. Ogawa, Jpn. J. Pharmacol. Ther., 17 (1986) 217.
9 K. Tada, T. Moroji, R. Sekiguchi, H. Motomura and T. Noguchi, Clin. Chem., 31 (1985) 1712.
10 U.R. Tjaden, M.T.H.A. Meeles, C.P. Thys and M.V.D. Kaay, J. Chromatogr., 181 (1980) 227.
11 H. Hishida, S. Takeda, R. Yoneshima and A. Takeda, Jpn. J. Hosp. Pharm., 6 (1984) 450.
12 T. Fujita, K. Tada and T. Moroji, Shimadzu Rev., 41 (1984) 303.
13 K. Yamaoka, Y. Tanigawara, T. Nakagawa and T. Uno, J. Pharmacobio-Dyn., 4 (1981) 879.
14 D.J. Greenblatt and J. Koch-Weser, N. Engl. J. Med., 293 (1975) 702.
15 S. Niazi, Textbook of Biopharmaceutics and Clinical Pharmacokinetics, Appleton-Century-Crosts, New York, 1979, p. 7.
16 A. Locniskar, D.J. Greenblatt and H.R. Ochs, J. Chromatogr., 337 (1985) 131.
17 L. Kangas, J. Kanto and E. Syvalahti, Acta Pharmacol. Toxicol., 41 (1977) 65.
18 N. Yada, in M. Hanano, K. Umemura and T. Iga (Editors), Applied Pharmacokinetics, Theory and Experiments, Soft Science, Tokyo, 1985, Ch. 4, p. 161.