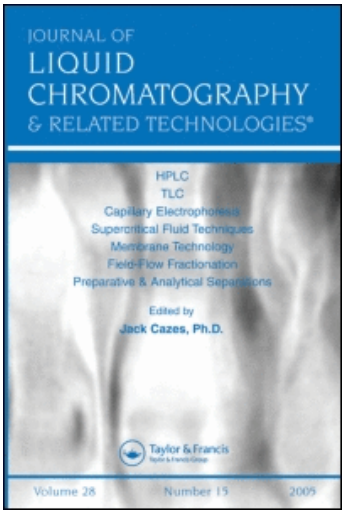

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Liquid Chromatographic Assay of Nitrazepam and Its Main Metabolites in Serum, and Its Application to Pharmacokinetic Study in the Elderly

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LIQUID CHROMATOGRAPHIC ASSAY OF NITRAZEPAM AND ITS MAIN METABOLITES IN SERUM, AND ITS APPLICATION TO PHARMACOKINETIC STUDY IN THE ELDERLY

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ABSTRACT

We describe a rapid and sensitive method for simultaneous determination of nitrazepam and its main metabolites, 7-aminonitrazepam, and 7-acetoamidonitrazepam, by reversed-phase liquid chromatography on a Shimpack FLC-CN microparticulate column. Absorbance of the effluent is monitored at 280 nm. The limit of detection in serum is about 10 μg/L for each compound. Analytical recovery of each compound added to the serum varied from 70 to 91 %. Within-day and between-day CVs ranged from 2.8 to 10.2. %. The method was applied to pharmacokinetic studies of nitrazepam in elderly long term recipients and healthy young volunteers. The steady-state concentrations of nitrazepam in the elderly did not differ from those in the young volunteers. This indicates that the accumulation of nitrazepam is no greater in the elderly than young recipients.

INTRODUCTION

There is an increasing number of geriatric people with chronic insomnia in whom benzodiazepine derivatives are prescribed as hypnotics. Since the pharmacokinetics of drugs in the elderly may differ significantly from young adults(1,2), much attention should be paid to the prescription of hypnotics in these people. A number of studies demonstrated increase in the plasma half-life in some benzodiazepines with age (3,4,5,6). Therefore, it would appear that there is a greater risk of CNS-depressant effects continuing on the morning following the night of drug intake in the elderly by comparison with young people. Yet, few studies of the accumulation of benzodiazepine hypnotics have been done in this population. In the present study, we determine the steady-state concentrations of nitrazepam in the elderly and young people in the morning. In order to obtain the basic data for the therapeutic drug monitoring in geriatric insomniacs, then, we compare the steady-state concentration of the drug between these two groups. We also describe a rapid, sensitive method for simultaneous determination of nitrazepam and its main metabolites - 7-aminonitrazepam, and 7-aceteoamidonitrazepam - using " high-performance " liquid chromatography with a new chromatographic column packed with particles 3μm in diameter.

MATERIALS AND METHOD

Apparatus

We used a Model LC-5A HPLC with a Model SPD-2A ultraviolet detector and a Model CR3A data processor (all from Shimadzu,Co., Ltd., Kyoto,

Japan). The prepacked column, a Shim-pack FLC-CN, 5 cm 4.6 mm (i.d.), has been recently developed by Shimazdu Co., Ltd. Samples were injected through a six-port sample-injection valve (Model 7125; Rheodyne, Cotati, CA) with a 100- μL loop. An on-line filter was placed just in front of the HPLC column to minimize column contamination from particulates in serum samples.

Metabolism of Nitrazepam

In man the main metabolic pathway of nitrazepam is reduction to the 7-amino derivative which is further biotransformed by acetylation to 7-acetoamidonitrazepam. The minor metabolites are EKS-925 and EKS-926.(Fig 1) These metabolites are known to be biologically inactive (7).

Reagent and Standard

Nitrazepam, aminonitrazepam, acetoamidonitrazepam, and two minor metabolites - EKS-925, EKS-926 - were donated by Shionogi Seiyaku Co.,Ltd.,Osaka, Japan. Acetonitrile," HPLC grade," was purchased from Wako Junyaku Kogyo Co., Ltd., Osaka, Japan. All other chemicals and solvent were analytical grade and were used without further purification. We used sulpiride as an internal standard. The solution of sulpiride in methanol (lg/L) was stored at 4°C in a refrigerator and diluted with methanol to a final concentration of 1 mg/L for the working solution of internal standard before assay.. Drug-free serum was obtained from the staffs of our laboratory.

Procedure

Sample collection. Two milliliters of blood from the antecubital vein was allowed to clot, then centrifuged. The serum was transferred to plastic vials and stored at -20 °C until analysis.

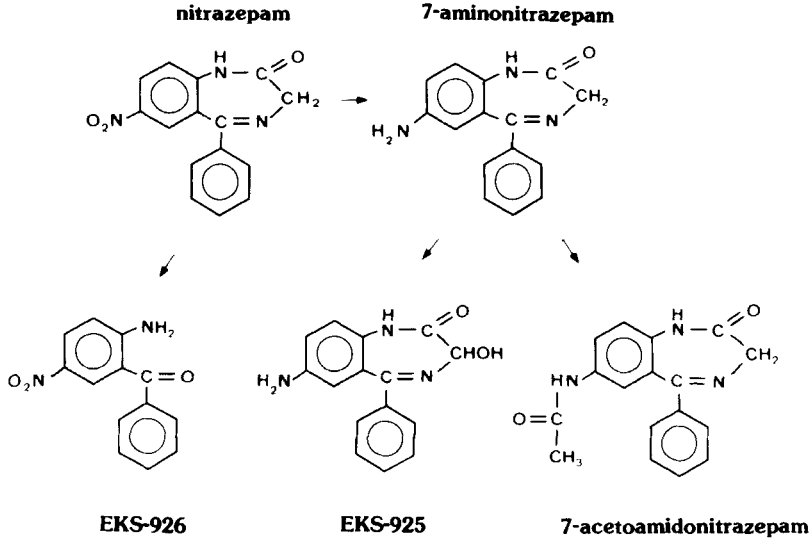

FIGURE 1. Biotransformation of Nitrazepam

Sample extraction. We extracted the drugs from the sample by a modification of the method of MacKicham et al.(8). Place 500 µL of serum , 50 µL of internal standard (0.5 µg of sulpiride), 1 mL of saturated tribasic sodium phosphate (Na3PO4), in a 10 mL glass capped tube ; gently mix ; add 5 mL of chloroform (the final pH of the mixture of sample and extraction buffer is 11.2); shake for 20 min on a reciprocal shaker ; and then centrifuge (2500 rpm, 5 min). Aspirate and discard the aqueous (top) layer and transfer 4 mL of chloroform to conical glass tube. Evaporate the solvent at room temperature under a gentle stream of nitrogen. Dissolve the residue in 100 µL of mobile phase, vortex-mix for 30 seconds, and inject 80 µL of the solution into the chromatograph.

Chromatographic conditions. For reversed-phase chromatography we used a mobile phase consisting of KH_2PO_4 buffer (0.1 mmol/L, adjusted to PH 3.0 with phosphoric acid) and acetonitrile (80/20 by vol), at a flow rate of 1 mL/min and at ambient temperature. The column effluent was monitored at 280 nm.

Quantification. Serum standards were prepared by adding nitrazepam, aminonitrazepam, and acetoamidonitrazepam to drug-free serum yielding final concentrations ranging from 30 to 120 µg/L for nitrazepam and acetoamidonitrazepam, and from 30 to 90 µg/L for aminonitrazepam. The standards were extracted and chromatographed as described above. The peak-area of each compound and the internal standard were measured and the ratio calculated. We repeated this procedure four times and computed a standard curve relating peak-area ratio to drug concentration in the standard, using linear regression analysis.

Analytical Recovery and Precision

We assessed analytical recovery by adding known amounts of each compound to drug-free serum to give final concentrations of 30 to 120 µg/L and assaying these samples. Analytical recovery was calculated as the ratio of the peak area obtained from extracted serum standard to that obtained by direct injection of volume-adjusted standards in mobile phase. We made 8 replicate determinations at each concentration of the compound. Within-day variation was determined by assaying serum samples of known concentrations - 30, 60, 120 µg/L - 8 times each in a single day. We also assayed the standards once a day for 6 days to determine between-day variation.

Result and Discussion

Analytical considerations. Fig 2 shows typical chromatograms obtained with this procedure. There were no interferences from endogenous components in drug-free serum(Fig 2 b). The calibration curves (Fig 3) were linear within the range examined. The correlation coefficient and equations for the regression lines for each component were as follows; r=0.9998, y= 0.42 + 0.0853 x for nitrazepam; r=0.9984, y=0.43 + 0.9102 x for acetoamidonitrazepam; r=0.9973, y= -0.65 + 0.1242 x for aminonitrazepam. Where x is the concentration in serum and y the peak-area ratio. The limit of detection - defined here as a signal-to-noise ratio of 5 - was 10 μg/L for each compound. Table 1 gives CVs for test mixture of nitrazepam and its metabolites. Within-day CV ranged 2.8 to 8.2 %. Between-day CV ranged 3.6 to 10.2 % during two weeks. As shown in Table 2, the analytical recovery for each compound ranged from 71 to 87 %. Recoveries of internal standard was about 15 %. Retention times (minutes) for some other benzodiazepines that are extracted by this method are: oxazepam 2.4, desmethylflunitrazepam 2.5, flunitrazepam 3.0, estazolam 3.4, triazolam 4.4, fludiazepam 4.8, N-desmethyldiazepam 9.7, clorazepate 10.1, diazepam 10.8.

The method described here is rapid and sensitive to monitor nitrazepam and acetoamidonitrazepam at the low concentrations ordinarily found in blood, though it does not allow EES-925 and EKS-926 to be measured separately. Since metabolites of nitrazepam are easily degraded during evaporation above 30 °C, we evaporated them at room temperature. Chloroform seemed to be most suitable for extraction. For our preliminary study, only small amount of 7-acetoamidonitrazepam could be

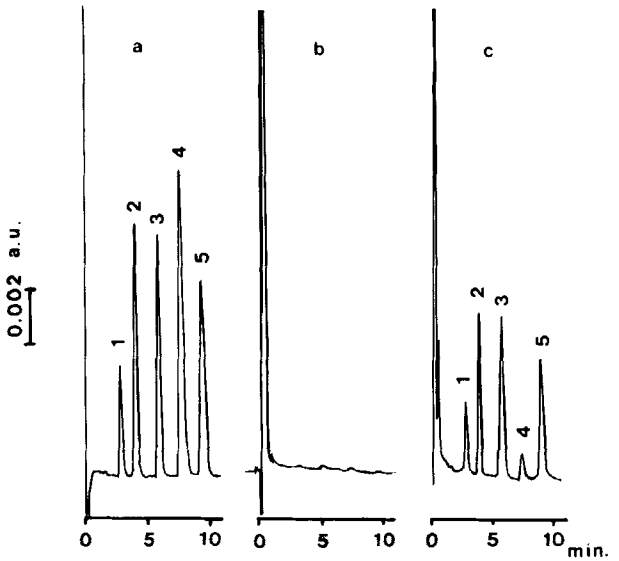

FIGURE 2. Typical chromatographs of (a) a mixture of standards - 60 ng of nitrazepam (2), 7-aminonitrazepam (5), 7-acetoamidonitrazepam (3), EKS-926 (1), and 500 ng of sulpiride (4) -, (b) extract of drug-free serum, and (c) extract of serum containing same concentrations of standard mixture.

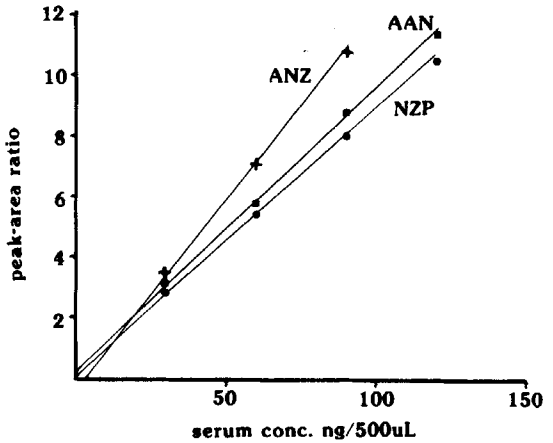

FIGURE 3. Calibration curves for nitrazepam (NZP), 7-aminonitrazepam (ANZ), and 7-acetoamidonitrazepam (AAN).

Table 1

The precision of assays for nitrazepam, 7-aminonitrazepam, and 7-acetoamindonitrazepam in serum

Within-day		Between-day	
Conc. μg/L		Conc. μg/L	
Mean ± S.D.	C.V., %	Mean ± S.D.	C.V., %
nitrazepam			
30.5 ± 0.85	2.8	32.6 ± 1.37	4.2
59.2 ± 3.2	3.2	58.8 ± 2.41	4.1
118.6 ± 4.51	3.8	121.2 ± 5.82	4.8
7-aminonitrazepam			
28.6 ± 1.60	5.6	29.3 ± 2.26	7.7
63.4 ± 4.56	7.2	64.5 ± 6.58	10.2
7-acetoamidonitrazepam			
32.3 ± 1.26	3.9	33.9 ± 2.31	6.8
57.4 ± 3.56	6.2	58.7 ± 4.17	7.1
117.5 ± 8.70	7.4	121.9 ± 10.12	8.3

Table 2

Analytical recoveries of nitrazepam, 7-aminonitrazepam, and 7-acetoamidonitrazepam (n=8) from serum

Conc. μg/L	Recovery %
nitrazepam	
30	91
60	87
120	85
7-aminonitrazepam	
30	78
60	75
90	70
7-acetoamidonitrazepam	
30	88
60	87
120	83

extracted into diethyl ether from the sample (9). Both recovery and precision of aminonitrazepam were not adequately high. Thus, the extraction procedure for this compound should be improved. Finally, the choice of a microparticulate (3- μm particles) prepacked column contributes to the rapid analysis time compared to a previous study (9).

Clinical Applications

The methods described here was applied to a pharmacokinetic study in geriatric patients with chronic insomnia treated with nitrazepam. Nine geriatric inpatients with multi-infarct dementia (five males and four females)in a mental hospital, aged 71 to 83, participated in this study. 5 mg of nitrazepam had been administered every night for at least 4 weeks. All patients also had been receiving 1.5 g of calcium hopatate, 30 mg of ifenprodil tartate, 37.5 mg of dipyridamole three times daily. In the preliminary study we confirmed that those drugs do not interfere with the assay of nitrazepam and its metabolites according to the method as described. Blood samples were draw at 7:00 a.m., 12 hours following the final oral ingestion of nitrazepam. Controls were body weight matched healthy volunteers (two men and two women) aged 21 to 22 . In this young group, samples were taken at the same time on days 1, 4, 7, 10 after starting the medication. The hematologic examination, the urinalysis, the blood chemistry and liver function test were performed prior to starting this study. No abnormalities were found in above mentioned laboratory tests in any subjects..

Fig 4 shows the serum concentrations of nitrazepam, aminonitrazepam, acetoamidonitrazepam in volunteers. The concentration of nitrazepam increased gradually and reached steady-state levels in about a week (

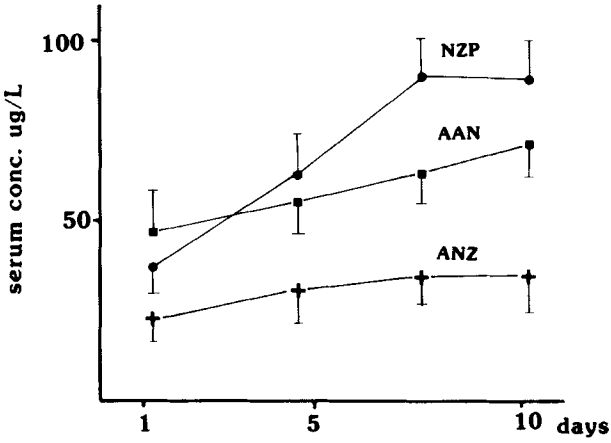

FIGURE 4. Mean (± S.D.) concentrations of nitrazepam, 7-aminonitrazepam, and 7-acetoamidonitrazepam in serum of four young volunteers receiving nitrazepam 5 mg every night for 10 days.

89.8 ± 8.3 μg/L). These findings are in good agreement with the results reported previously (10). The serum concentrations of 7-aminonitrazepam and 7-acetoamidonitrazepam also increased in proportion to the increase in the concentration of nitrazepam. The steady-state concentrations of nitrazepam in the geriatric patients were 100 ± 13.1 which were higher than those in the young volunteers, but not statistically significant (Mann-Whitney's U-test, two tailed (11)). No statistically significant differences were also observed in concentrations of the major metabolites of nitrazepam between the two groups.

Previous studies on pharmacokinetics of benzodiazepines reported a marked reduction in metabolic clearance and a prolonged elimination half-life of several benzodiazepines biotransformed by oxidative metabolic pathways in the elderly (3,4,5,6). Greenblatt et al (6) have

reported that the elimination half-life of flurazepam was prolonged and the steady-state levels of desalkylflurazepam, a metabolite of flurazepam, were higher in the elderly. Kangas (12) found no differences in the extent of accumulation of nitrazepam between the elderly and young people, while the half-life of the drug was prolonged in the elderly . However, Castleden et al (13) have demonstrated that, when administered with multiple dosage, the plasma half-life of nitrazepam and the concentration at 60 hours in the elderly were equal to those in young adults. Although we did not examine the plasma half-life of nitrazepam, our results were not contradictory with previous studies that steady-state levels of nitrazepam are not affected by aging (12,13,14,15,16) as opposed to that of flurazepam. This may be due to the difference of metabolic pathway between the two drugs. Nitrazepam is biotransformed by reduction (6), whereas flurazepam by oxidation (17).

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REFERENCES

1). Schmucker,D.L., Age-Related Changes in Drug Disposition, Pharmacol Rev., 30, 445, 1979.

2). Lamy,P.P., Comparative Pharmacokinetic Changes and Drug Therapy in an Older Population, J Am Geriatr Soc., 30(Suppl), S11, 1982.

3). Robert,R.K., Effect of Age and Parenchymal Liver Disease on the Desposition and Elimination of Chlordiazepoxide (Librium), Gastroenterology., 75, 479, 1978.

4). Klotz,U., Avant,G.R., Hoyumpa,A., Schenker,S., and Wilkinson,G.R., The Effect of AGE and Liver Disease on the Disposition and Elimination of Diazepam in Adult Man., J Clin Invest., 55, 347, 1975.

5). Greenblatt,D.J., Allen,M.D., Harmatz,J.S. and Shader,R.I., Diazepam Disposition Determinants, Clin Pharmacol Ther., 27, 301, 1980.

6). Greenblatt,D.J., Divoll,M., Harmatz,J.S., MacLaughlin,D.S., and Shader,R.I., Kinetics and Clinical Effects of Flurazepam in Young and Elderly noninsomniacs, Clin Pharmacol Ther., 30, 485, 1981.

7). Rieder,J., A Fluorimetric Method for Determining Nitrazepam and Sum of its Main Metabolites in Plasma and Urine, Arzneim-Forsch(Drug Res)., 23, 207, 1973.

8) MacKian,J.J., Jusko,W.J., Duffner,P.K., and Cohen,M.E., Liquid-Chromatographic Assay of Diazepam and Its Major Metabolites in Plasma., Clin Chem., 25, 856, 1979.

9). Kelly,H., Huggett,A., and Dawling S., Liquid-Chromatographic Measurement of Nitrazepam in Plasma, Clin Chem, 28, 1478, 1982.

10). Breimer,D.D., Pharmacokinetics and Metabolism of Various Benzodiazepines used as Hypnotics, Br J Pharmacol, 8, 7S, 1979.

11) Siegel,S., Nonparametric Statistics : For the Behavioral Sciences., McGraw-Hill Book Co.,Inc.,1956.

12). Kangas,L., Iisalo,E.,Kanto,J.,Lehtinen,V., Pynnonen,S., Ruikka,I., Salminen,J., Sillanpaa,M., and Syvalahti,E., Human Pharmacokinetics of Nitrazepam ; Effect of Age and Disease, Europ J Clin Pharmacol., 15, 163, 1979.

13). Castleden,C.M., George,C.F., Marcer,D., and Hallett,C., Increased Sensitivity to Nitrazepam in Old Age, Br Med J., 1, 10, 1977.

14). Cook,P.J., Huggett,A, Graham-pole,R., Savage,I.T., and James,I.M., Hypnotic Accumulation and Hangover in Elderly Inpatients : A Controlled Double-blind Study of Temazepam and Nitrazepam, Br Med J, 286, 100, 1983.

15). Jochemsen,R., Vanbeusekom,B.R., Spoelstra,P., Janssens,A.R., and Breimer D.D., Effect of Age and Liver Cirrhosis on the Pharmacokinetics of Nitrazepam, Br J Clin Pharmacol, 15, 295, 1983.

16). Swift,C.G., Swift,M.R., Hamley,J., Stevenson,I.H., and Crooks,J., Side-Effect Tolerance in Elderly Long-Term Recipients of Benzodiazepine Hypnotics, Age Agin, 13, 335, 1984.

17). Greenblatt,D.J., Shader,R.I., Divoll,M., and Harmatz,J.S., Benzodiazepines : A Summary of Pharmacokinetic Properties, Br J Clin Pharmacol, 11, 11S, 1981.