

*Journal of Chromatography*, 310 (1984) 213–218

*Biomedical Applications*

Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2174

## Note

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### High-performance liquid chromatographic determination of nitrazepam and its metabolites in human urine

TADASHI KOZU

*Scientific Investigation Research Laboratory, Nagano Prefectural Police Headquarters,  
1-6-15, Miwa, Nagano 380 (Japan)*

(First received January 4th, 1984; revised manuscript received April 4th, 1984)

Nitrazepam, 1,3-dihydro-7-nitro-5-phenyl-2H-1,4-benzodiazepin-2-one, is a benzodiazepine analogue which has been used extensively as a hypnotic. Although this drug is known for its low toxicity and high safety margin, it has recently been used for purposes of suicide and homicide. In addition, it is known that the side-effects of this drug will influence psychomotor abilities related to driving [1].

Many reports have been published on the determination of nitrazepam in biological fluids, gas chromatography (GC) being used extensively [2–5]. The GC methods require somewhat lengthy clean-up procedures, and in some cases derivatization or hydrolysis to the more volatile benzophenone [4]. Several methods were reported on the analysis of nitrazepam alone by high-performance liquid chromatography (HPLC) [6–9], while few reports have been published on the analysis of nitrazepam together with its metabolites in biological fluids. Although the analysis of nitrazepam and its metabolites by HPLC on an anion-exchange packing was reported [10], this method failed to yield reliable results, due to broad, tailing peaks.

In this paper, the simultaneous determination of nitrazepam and metabolites by HPLC on Nucleosil 5C<sub>18</sub> packing gave sharp peaks and good separation.

## EXPERIMENTAL

### Reagents

Nitrazepam (Benzalin; Shionogi Pharmaceutical, Japan) was recrystallized from ethanol. 7-Amino-1,3-dihydro-5-phenyl-2H-1,4-benzodiazepin-2-one (7-aminonitrazepam) and 7-acetamido-1,3-dihydro-5-phenyl-2H-1,4-benzo-

diazepin-2-one (7-acetamidonitrazepam) were supplied by courtesy of Professor H. Sawada, Gifu College of Pharmacy. 1-Methyl-7-nitro-5-phenyl-1H-1,4-benzodiazepin-2-(3H)-one (nimetazepam, internal standard) was kindly provided by Sumitomo Kagaku Kogyo, Japan. Nucleosil 5C<sub>18</sub> (Macherey-Nagel, 5  $\mu$ m) was used as column packing.

#### Apparatus and chromatographic conditions

The liquid chromatograph was a Shimadzu LC-3A with a spectrophotometric detector SPD-1 connected to a Chromatopac C-R1A recorder. The column was a steel column (150 mm  $\times$  4.0 mm I.D.) packed with Nucleosil 5C<sub>18</sub> (particle size 5  $\mu$ m) using a slurry packing technique. A guard column was used for protecting the analytical column from interfering materials. The chromatographic conditions were as follows: mobile phase, methanol-water (35:65) (adjusted to pH 4.0 with phosphoric acid); flow-rate, 1.3 ml/min; pressure, 120 kg/cm<sup>2</sup>; column temperature, 50°C; detector, ultraviolet (UV) at 254 nm.

#### Extraction procedures

Urine samples were adjusted to pH 10 by the addition of 0.28% ammonia solution before extraction. Columns for extraction of nitrazepam and its metabolites from human urine were prepared from a 30-ml glass syringe filled with 5.0 g of Extrelut<sup>®</sup> (E. Merck, Darmstadt, F.R.G.), which is similar to

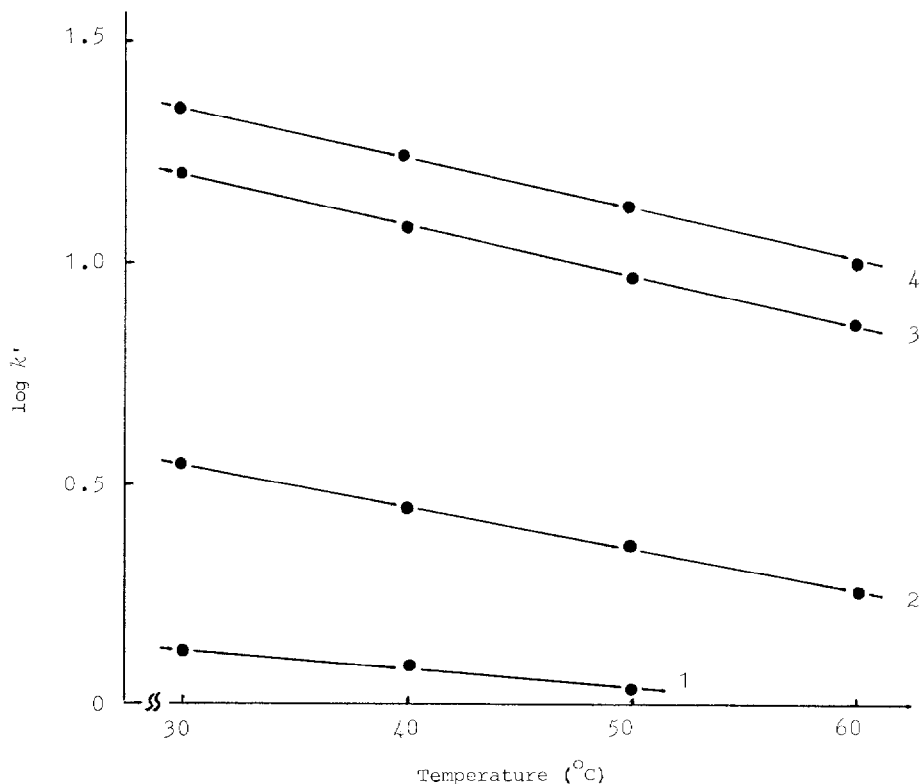


Fig. 1. Relationship between the capacity factor,  $k'$ , and the temperature at pH 4.0. 1 = 7-Aminonitrazepam; 2 = 7-acetamidonitrazepam; 3 = nitrazepam, 4 = nimetazepam (internal standard).

Bond-Elut® [11]. A 10-ml aliquot of a urine sample was applied onto the column and, after soaking for 15 min, the column was eluted with chloroform. The eluate was evaporated to dryness using a rotary evaporator.

## RESULTS AND DISCUSSION

### *Chromatographic conditions*

Liquid chromatography of nitrazepam has been performed on columns packed with Corasil II [6], Carbowax 400 on Corasil [6], C<sub>18</sub> (octadecyltrichlorosilane) on Corasil [6], LiChrosorb Si 100 [7], Spherisorb S-W10 [8], porous silica gel [9], and Zipax SAX [10] using a variety of solvents. In this study, various chromatographic conditions have been evaluated, varying column packings, the composition and the pH of the mobile phase, and the column temperature. Liquid chromatography of nitrazepam and its metabolites was performed on columns packed with Nucleosil 5CN, Nucleosil 5NH<sub>2</sub>, Nucleosil 5C<sub>18</sub> and Zipax SAX. Nitrazepam was well separated from its metabolites and from the internal standard by using the Nucleosil 5C<sub>18</sub> column and methanol-water (35:65) (pH 4.0) at a flow-rate of 1.3 ml/min (pressure 120 kg/cm<sup>2</sup>). The column temperature was thermostated at 50°C. The logarithm of the capacity factor ( $k'$ ) between nitrazepam, 7-aminonitrazepam, 7-acet-

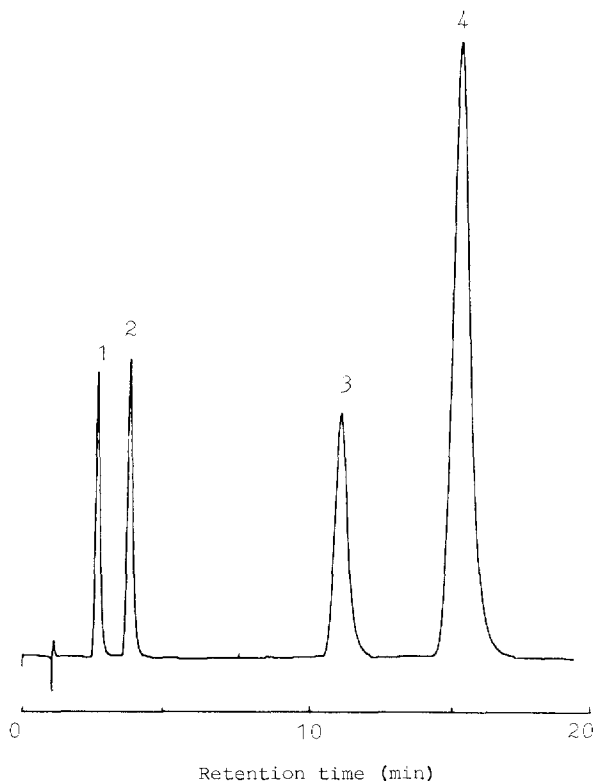


Fig. 2. Typical chromatogram of nitrazepam and its metabolites. 1 = 7-Aminonitrazepam; 2 = 7-acetamidonitrazepam; 3 = nitrazepam; 4 = nimetazepam (internal standard). Column, Nucleosil 5C<sub>18</sub>; mobile phase, methanol-water (35:65) (pH 4.0); column temperature, 50°C; flow-rate, 1.3 ml/min (pressure, 120 kg/cm<sup>2</sup>); detector, UV photometer (254 nm).

amidonitrazepam and nimetazepam plotted against the temperature at pH 4.0 gave a straight line (Fig. 1).

#### *Analysis of a standard mixture*

A typical chromatogram of a methanolic mixture of 7-aminonitrazepam (100 µg/ml), 7-acetamidonitrazepam (100 µg/ml), nitrazepam (160 µg/ml) and nimetazepam (the internal standard, 800 µg/ml) is shown in Fig. 2.

#### *Calibration curve*

Standard solutions of nitrazepam, 7-aminonitrazepam and 7-acetamidonitrazepam were dissolved in methanol at concentrations of 40–200 µg/ml for nitrazepam, and 25–125 µg/ml for 7-aminonitrazepam and 7-acetamidonitrazepam. Nimetazepam (the internal standard) was dissolved in methanol at a concentration of 800 µg/ml. Calibration curves were constructed by plotting the ratio of peak area of each compound to that of the internal standard. Regression equations were as follows:  $Y = 0.1421X - 0.0100$  for nitrazepam,  $Y = 0.2282X - 0.0015$  for 7-aminonitrazepam, and  $Y = 0.1857X - 0.0005$  for 7-acetamidonitrazepam.

#### *Extraction and recoveries*

The recovery of nitrazepam (2.4 µg/ml), 7-aminonitrazepam (1.5 µg/ml) and 7-acetamidonitrazepam (1.5 µg/ml) added to normal human urine was evaluated after elution with various organic solvents after adjusting the samples to pH 10 with 0.28% ammonium hydroxide and pH 12 with 1 M sodium hydroxide. For 10 ml of urine 30 ml of solvent were used, in which 100% of the extractable substances was eluted. The dried residue of the eluate was unsuitable for analysis by HPLC using ethyl acetate and dichloromethane–isopropanol mixture; the solvent for optimum elution was chloroform (Table I).

TABLE I

RECOVERIES OF 7-AMINONITRAZEPAM, 7-ACETAMIDONITRAZEPAM AND NITRAZEPAM ADDED TO NORMAL HUMAN URINE AT pH 10 AND pH 12

*n* = 5.

Solvent	pH	7-Amino-nitrazepam (%)	7-Acetamido-nitrazepam (%)	Nitrazepam (%)
Ether	10	88.39 ± 4.11	62.18 ± 3.54	97.24 ± 3.74
	12	81.45 ± 3.09	56.44 ± 1.47	86.06 ± 4.65
Ethyl acetate	10	104.92 ± 3.03	105.12 ± 4.23	96.97 ± 1.80
	12	86.20 ± 2.61	99.06 ± 2.28	88.64 ± 2.30
Dichloromethane–isopropanol (85:15)	10	93.10 ± 1.51	98.64 ± 1.58	96.92 ± 1.20
	12	98.00 ± 4.23	99.56 ± 5.79	88.05 ± 4.89
Chloroform	10	99.39 ± 1.78	95.90 ± 2.02	99.93 ± 3.18
	12	83.63 ± 3.72	99.52 ± 3.78	98.95 ± 3.73
Hexane	10	—	—	—
	12	—	—	—

### Quantitation of the sample

Urine from a person suspected of having ingested nitrazepam was analysed. After evaporation of the extracted urine, the residue was dissolved in 0.2 ml of the internal standard solution. Usually, 5- $\mu$ l aliquots were injected into the liquid chromatograph. The concentration of the drug and its metabolites in the sample was determined from the calibration curves. The chromatogram and the

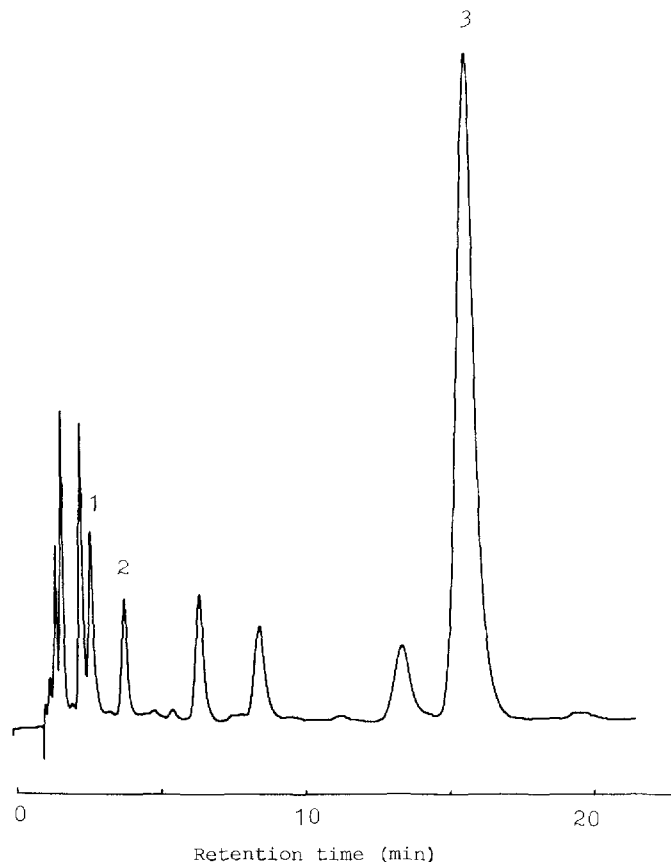


Fig. 3. Chromatogram of the extract of human urine from a person suspected of ingesting nitrazepam. 1 = 7-Aminonitrazepam; 2 = 7-acetamidonitrazepam; 3 = nimetazepam (internal standard). Chromatographic conditions are the same as in Fig. 2.

TABLE II

CONTENTS OF 7-AMINONITRAZEPAM AND 7-ACETAMIDONITRAZEPAM IN THE SAMPLE

Sample No.	7-Aminonitrazepam ( $\mu$ g/ml)	7-Acetamidonitrazepam ( $\mu$ g/ml)
1	38.34	30.37
2	37.20	30.48
3	35.71	31.13
Mean	37.08	30.66

concentrations of 7-amino nitrazepam and 7-acetamido nitrazepam detected in the sample are shown in Fig. 3 and Table II, respectively. Nitrazepam was not detected in this sample.

#### ACKNOWLEDGEMENTS

The author is much obliged to Dr. H. Sawada for supplying 7-amino-nitrazepam and 7-acetamidonitrazepam, and also indebted to the Nagano Prefectural Foundation for Scientific and Technical Advancement, Japan, which supported this study in part by a grant.

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