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DETERMINATION OF NITRAZEPAM AND ITS MAIN METABOLITES IN URINE BY THIN-LAYER CHROMATOGRAPHY AND DIRECT DENSITOMETRY

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SUMMARY

A method for the direct quantitative densitometry of nitrazepam and its main metabolites (7-aminonitrazepam, 7-acetamidonitrazepam and 2-amino-5-nitrobenzophenone) in urine was developed. The unchanged drug and its metabolites were extracted with benzene—dichloromethane (4:1), subjected to thin-layer chromatography, and determined by direct ultraviolet densitometry. Recovery experiments showed that the method was quantitative. The limit of detection was 5 ng/ml for 2-amino-5-nitrobenzophenone and 10 ng/ml for other compounds. The method was applied to the determination of nitrazepam and its metabolites excreted in human urine after administration of 10 mg of the drug.

INTRODUCTION

Nitrazepam (7-nitro-5-phenyl-1,3-dihydro-2H-1,4-benzodiazepin-2-one) is a widely used hypnotic. After oral administration, the major urinary metabolites are 7-aminonitrazepam (7-amino-5-phenyl-1,3-dihydro-2H-1,4-benzodiazepin-2-one) and 7-acetamidonitrazepam (7-acetamido-5-phenyl-1,3-dihydro-2H-1,4-benzodiazepin-2-one); the parent drug and 2-amino-5-nitrobenzophenone (ANB) are excreted only in small amounts [1, 2].

Several methods have been reported for the determination of nitrazepam and its metabolites in urine. Colorimetric [2] and fluorometric [3] methods are laborious, requiring troublesome extraction to separate nitrazepam and its metabolites from each other. Gas chromatographic methods require inactivation of the column, which is sometimes difficult and time-consuming [4, 5], and, in some cases, derivatization [6, 7] or acid hydrolysis, which leads to loss

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of specificity [4, 8—10]. Recently, high-performance liquid chromatographic methods have been reported for the measurement of nitrazepam in plasma [11] and urine [12].

In this paper, the direct quantitative densitometry of nitrazepam and its main metabolites on thin-layer chromatograms is described. In addition, the method is applied to the determination of these compounds excreted in human urine after oral administration of the drug.

EXPERIMENTAL

Materials

Nitrazepam was extracted from Nelbon (Sankyo, Tokyo, Japan) with ethyl acetate and recrystallized from ethanol (m.p. 224—226°C). 7-Aminonitrazepam, 7-acetamidonitrazepam and ANB were synthesized by the method of Sawada and Shinohara [13]. All other chemicals used were special-grade materials.

Apparatus

A Shimadzu CS-900 dual-wavelength scanner was used with the following settings: slit for thin-layer chromatograms, 1.25 × 1.25 mm; scanning speed, 5 mm/min with zig-zag scanning; sample and reference beams (λ_S and λ_R), 270 and 400 nm for nitrazepam, 240 and 400 nm for 7-aminonitrazepam and 7-acetamidonitrazepam, and 360 and 450 nm for ANB; reflection mode.

The developed thin-layer plate was scanned in the direction parallel to the chromatographic flow. Ultraviolet (UV) absorption profiles of the thin-layer chromatograms and the integration curves of the peaks in the profiles were obtained simultaneously on a recorder.

Thin-layer chromatography

Thin-layer chromatography (TLC) was carried out on 0.25-mm thick silica gel 60 plates (E. Merck, Darmstadt, F.R.G.). The solvent system used for development was acetone—ethyl acetate—28% ammonium hydroxide (1:9:0.1). After development, the plates were dried for 3 min in a stream of warm air.

Administration of the drug

Nitrazepam was administered orally to two healthy adult humans, one female and one male, at a dose of 10 mg; 24-h urine fractions were collected for three days and stored at −20°C until analysis.

Extraction

To 10 ml of urine were added 3 g of sodium chloride and 1 ml of 1 *M* sodium carbonate, and the mixture was extracted with 20, 10, and 10 ml of benzene—dichloromethane (4:1). The combined organic layer was dried over anhydrous sodium sulphate and evaporated in vacuo. The residue obtained was dissolved in 100 μl of chloroform, and 1, 5 or 10 μl of the solution were spotted on a thin-layer plate. After development, nitrazepam and its main metabolites on the chromatogram were determined simultaneously.

RESULTS AND DISCUSSION

Wavelength for sample and reference beams

UV absorption spectra of nitrazepam, 7-aminonitrazepam, 7-acetamidonitrazepam and ANB on a thin-layer chromatogram are shown in Fig. 1. For the measurement of the UV absorption intensity of these spots, sample and reference beams (λ_S and λ_R) were set at the corresponding wavelength for maximum and minimum absorption, i.e. 270 and 400 nm for nitrazepam, 240 and 400 nm for 7-aminonitrazepam, and 360 and 450 nm for ANB. Although the maximum intensity of UV absorption of 7-acetamidonitrazepam on the chromatogram was obtained at 245 nm (Fig. 1), 240 and 400 nm were used as λ_S and λ_R to allow the simultaneous quantification of 7-aminonitrazepam and 7-acetamidonitrazepam. Under these conditions, the intensity of 7-acetamidonitrazepam decreased to 98%.

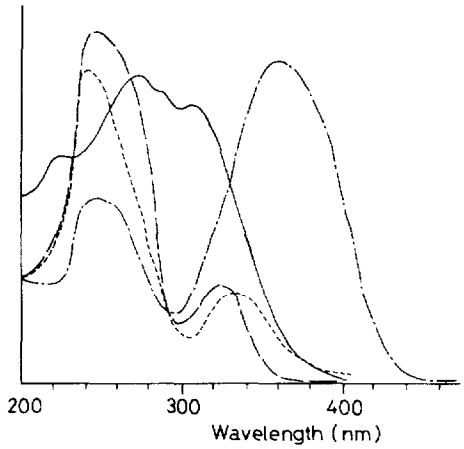

Fig. 1. UV absorption spectra of nitrazepam (——), 7-aminonitrazepam (- - - -), 7-acetamidonitrazepam (— — —) and 2-amino-5-nitrobenzophenone (— · —) on a thin-layer chromatogram.

Quantitative analysis of nitrazepam and its metabolites

Various volumes (2, 4, 6, 8 and 10 μl) of a chloroform solution of nitrazepam (0.02 μg/μl) were spotted on the thin-layer plate. After development, the absorption intensity of nitrazepam on the chromatogram was measured. The chromatography was repeated five times on different plates and the relative standard deviations at each concentration were calculated. In addition, the relationship between absorption intensity and the amount of nitrazepam was examined. In the same way, the relative standard deviations and the calibration curves were obtained from solutions containing 7-aminonitrazepam, 7-acetamidonitrazepam or ANB.

The linear correlation between the absorption intensity and the amount of nitrazepam or its metabolites was found in the range of the calibration standards used (0.04—0.2 μg) as shown in Fig. 2. Relative standard deviations obtained from the same amount of the compound on different chromatograms were 1.7—6.3% (Table I) and reproducible values were obtained.

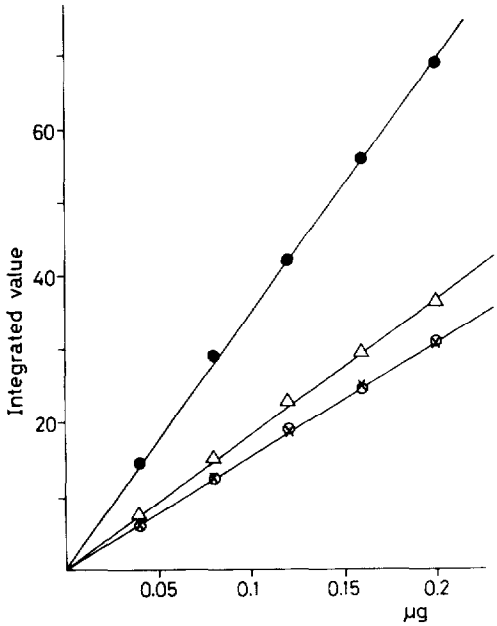

Fig. 2. Relationship between UV absorption intensity and amount of nitrazepam or its metabolites. (△) Nitrazepam; (○) 7-aminonitrazepam; (×) 7-acetamidonitrazepam; (•) 2-amino-5-nitrobenzophenone.

TABLE I

REPRODUCIBILITY OF DIRECT UV SPECTROMETRY ON THIN-LAYER CHROMATOGRAMS

Compound	Amount (µg)	Integrated value	Standard deviation (%, n = 5)
Nitrazepam	0.20	36.5	2.7
	0.16	29.5	5.3
	0.12	22.8	5.0
	0.08	15.0	4.2
	0.04	7.4	3.3
7-Aminonitrazepam	0.20	31.3	2.6
	0.16	24.6	1.7
	0.12	18.8	3.8
	0.08	12.3	4.2
	0.04	6.1	4.1
7-Acetamidonitrazepam	0.20	31.0	2.5
	0.16	25.0	4.2
	0.12	18.4	4.4
	0.08	12.8	5.1
	0.04	6.0	2.8
2-Amino-5-nitrobenzophenone	0.20	69.1	4.5
	0.16	55.9	2.5
	0.12	42.1	4.3
	0.08	29.1	4.4
	0.04	14.5	6.3

Recovery of nitrazepam and its metabolites

The extract obtained from 10 ml of control urine, which was collected for 24 h before administration of the drug, was dissolved in 100 μl of chloroform and 10 μl of the solution were subjected to TLC.

Typical TLC absorption profiles of the extract from control urine and that of nitrazepam and its metabolites are shown in Fig. 3. It was apparent that nitrazepam and its metabolites could be separated from each other and no interference from endogenous urine constituents was observed.

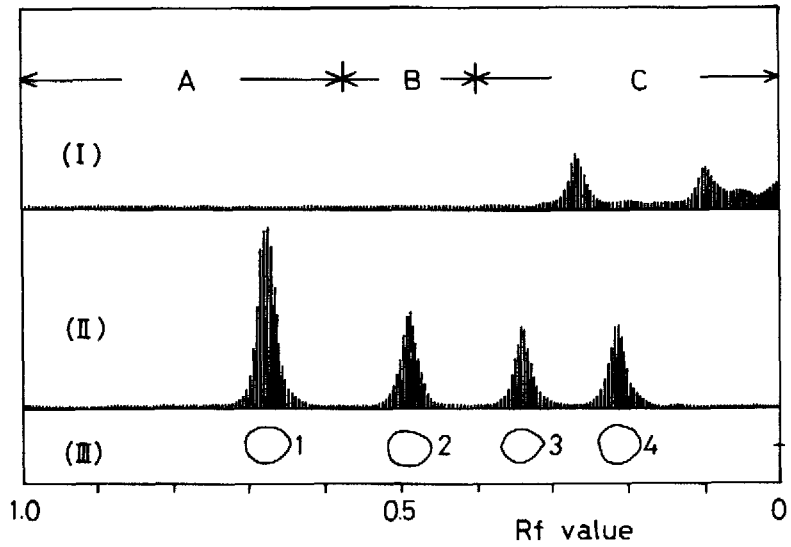

Fig. 3. Typical TLC UV absorption profiles of an extract of control urine (I) and of a mixture containing 0.1 μg each of nitrazepam and its metabolites (II), and a thin-layer chromatogram of the latter (III). Sample and reference beams (λ_S and λ_R) were set at 360 and 450 nm in region A, 270 and 400 nm in region B, and 240 and 400 nm in region C. 1 = 2-Amino-5-nitrobenzophenone; 2 = nitrazepam; 3 = 7-aminonitrazepam; 4 = 7-acetamidonitrazepam.

TABLE II

RECOVERY OF NITRAZEPAM AND ITS METABOLITES

Each compound is added to 10 ml of control urine. Each value is the mean ± S.D. (n = 5).

Compound	Added (μg)	Recovery (%)
Nitrazepam	10	93.6 ± 2.0
	1	93.8 ± 2.9
7-Aminonitrazepam	10	81.1 ± 2.4
	1	81.0 ± 2.5
7-Acetamidonitrazepam	10	96.7 ± 0.5
	1	97.8 ± 4.3
2-Amino-5-nitrobenzophenone	10	100.5 ± 3.5
	1	99.4 ± 1.5

The recovery of added authentic compounds from control urine by extraction with benzene—dichloromethane (4:1) is shown in Table II. Nitrazepam, 7-acetamidonitrazepam and ANB were recovered almost quantitatively. Recovery of 7-aminonitrazepam was about 81%, but standard deviations at each concentration were very small. From these results, it was considered that this extraction method is useful for urine samples.

In order to check the reproducibility, some of the urine samples were analysed as replicates on different days. The mean standard deviations (n = 5) were 5.0% for nitrazepam, 4.4% for 7-aminonitrazepam, 3.9% for 7-acetamidonitrazepam and 5.1% for ANB (Table III).

The limit of detection of nitrazepam, 7-aminonitrazepam and 7-acetamidonitrazepam in urine was 10 ng/ml, and that of ANB was 5 ng/ml.

TABLE III

INTER-ASSAY PRECISION OF NITRAZEPAM AND ITS METABOLITES IN URINE

Experiment was performed with urine samples from volunteers.

Sample	Nitrazepam		7-Aminonitrazepam		7-Acetamido-nitrazepam		2-Amino-5-nitro-benzophenone	
	Mean (μg/ml)	S.D. (%, n=5)	Mean (μg/ml)	S.D. (%, n=5)	Mean (μg/ml)	S.D. (%, n=5)	Mean (μg/ml)	S.D. (%, n=5)
1	0.0295	7.3	0.0748	4.4	0.0758	2.9	0.0483	1.3
2	0.0552	8.8	0.0280	6.1	0.1033	4.5	0.0635	5.6
3	0.0607	1.9	0.1869	4.1	0.4835	5.7	0.0267	6.0
4	0.0904	3.8	0.2883	3.8	0.7704	2.8	0.0362	7.0
5	0.0579	3.1	0.1266	3.7	0.4485	3.7	0.0662	5.5

TABLE IV

URINARY EXCRETION OF NITRAZEPAM AND ITS METABOLITES AFTER ORAL ADMINISTRATION TO MAN

Subject A is female and subject B is male.

Metabolite	Percentage of dose excreted					
	Day after oral administration					
	1		2		3	
	Subject A	Subject B	Subject A	Subject B	Subject A	Subject B
Unchanged nitrazepam	0.9	0.4	0.2	0.4	0.1	0.1
7-Amino-nitrazepam	2.3	2.6	0.9	4.6	0.8	2.3
7-Acetamido-nitrazepam	7.2	4.5	9.7	5.3	4.1	3.1
2-Amino-5-nitro-benzophenone	0.8	0.7	n.d.*	0.1	n.d.	0.04

*n.d. = not detected.

Excretion of nitrazepam and its metabolites in human urine

The present method was applied to the determination of the main metabolites of nitrazepam excreted in urine after administration of the drug. The amounts of the unchanged drug and the metabolits excreted during three days were listed in Table IV.

The unchanged drug and the metabolites excreted in the urine of two volunteers comprised 27.0% and 24.1% of the dose when determined three days after oral administration, and the main metabolites were 7-acetamidonitrazepam (21.0% and 12.9%) and 7-aminonitrazepam (9.5% and 4.0%). The main metabolic pathways of nitrazepam, described by Rieder [1] and Sawada and Shinohara [2], were confirmed here.

The method reported here has advantages over previously described techniques because the extraction is simple and the drug does not need to be derivatized. The method is sufficiently sensitive for the determination of nitrazepam and its main metabolites in urine samples.

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REFERENCES

1 J. Rieder, Arzneim.-Forsch., 15 (1965) 1134.
2 H. Sawada and K. Shinohara, Arch. Toxicol., 28 (1971) 214.
3 J. Rieder, Arzneim.-Forsch., 23 (1973) 207.
4 L. Kangas, J. Chromatogr., 136 (1977) 259.
5 L. Kangas, J. Chromatogr., 172 (1979) 273.
6 H. Ehrsson and A. Tilly, Anal. Lett., 6 (1973) 197.
7 J.A.F. de Silva and I. Bekersky, J. Chromatogr., 99 (1974) 447.
8 J.M. Clifford and W.F. Smyth, Analyst (London), 99 (1974) 241.
9 D.M. Hailey, J. Chromatogr., 98 (1974) 527.
10 K.M. Jensen, J. Chromatogr., 111 (1975) 389.
11 H. Kelly, A. Huggett and S. Dawling, Clin. Chem., 28 (1982) 1478.
12 T. Kozu, J. Chromatogr., 310 (1984) 213.
13 H. Sawada and K. Shinohara, Arch. Toxicol., 27 (1970) 71.