Journal of Chromatography, 172 (1979) 273–278

CHROM. 11,617

DETERMINATION OF NITRAZEPAM AND ITS MAIN METABOLITES IN URINE BY GAS–LIQUID CHROMATOGRAPHY: USE OF ELECTRON CAPTURE AND NITROGEN-SELECTIVE DETECTORS

LAURI KANGAS

Department of Pharmacology, University of Turku, 20520 Turku 52 (Finland)

(Received November 16th, 1978)

SUMMARY

Nitrazepam and its main urinary metabolites, 7-aminonitrazepam and 7-acetamidonitrazepam, free and conjugared, were determined from 24-h fractions of human urine after a single oral dose of 5 mg of nitrazepam. Nitrazepam and the metabolites were extracted before and after glusulase hydrolysis with benzene–dichloromethane (90:10) from a 1.0 ml sample. Methylnitrazepam and methylbromazepam served as internal standards. Recoveries were better than 90%.

GLC analysis of nitrazepam was performed using a ^{63}Ni electron-capture detector. The metabolites were measured by a dual flameless nitrogen selective detector. The detection limits were about 0.2 ng/ml for nitrazepam and 50 ng/ml for the metabolites. The nitrogen-selective detector responds similarly to all three compounds. The ^{63}Ni electron-capture detector gives very poor response to 7-aminonitrazepam but allows very sensitive detection of nitrazepam. Combined use of the two detectors gives valuable information about the metabolic profile of nitrazepam.

INTRODUCTION

The pharmacokinetic profile of nitrazepam*, a widely used hypnotic, has been elucidated using ^{14}C-labelled nitrazepam combined with thin-layer chromatography (TLC) and fluorometric detection[1]. The main metabolites of nitrazepam in human plasma and urine are 7-aminonitrazepam and its acetylated form, 7-acetamidonitrazepam. Unchanged nitrazepam is excreted poorly via the kidneys[1,2].

The nickel-63 electron-capture detector (ECD) is widely used in pharmacological studies of many drugs. It is very useful, for example in the determination of most benzodiazepines[3]. Nitrazepam, however, loses the electrophilic 5-nitro group in the reductive metabolic step, yielding compounds not detectable using an ECD.

The nitrogen-selective detector has proved to be useful in determining many drugs in biological samples, *e.g.* anti-epileptics[4,5], theophylline[6] and dopings[7].

* Nitrazepam = 7-nitro-5-phenyl-1,3-dihydro-2*H*-1,4-benzodiazepin-2-one; 7-aminonitrazepam = 7-amino-5-phenyl-1,3-dihydro-2*H*-1,4-benzodiazepin-2-one; 7-acetamidonitrazepam = 7-acetamido-5-phenyl-1,3-dihydro-2*H*-1,4-benzodiazepin-2-one.

The main metabolites of nitrazepam have been analysed by high-performance liquid chromatography (HPLC), but not in biological samples[8]. Our aims were to develop simple gas–liquid chromatographic methods for the determination of these metabolites in human urine and to elucidate further the pharmacokinetics of nitrazepam and its metabolites.

EXPERIMENTAL

Nitrazepam (Nitrazepam Dumex, Dumex, Copenhagen, Denmark) was administered to 15 healthy human volunteers, all males, with mean (range) age and weight of 22.7 (18–30) years and 70.8 (57–90) kg, respectively. Blood samples were taken from the cubital vein 0–72 h after administration and placed in heparinized test tubes. Plasma was separated and stored at −20°. 24-h urine fractions were collected for 7 days. Urine volumes were measured and 5-ml aliquots stored at −20° until analysis.

The samples were thawed and extracted as previously described[9] (direct method). Free (unconjugated) and total (free plus conjugated) concentrations of the compounds were determined from separate samples. A 1.0-ml sample was used for each determination. The conjugated metabolites were hydrolysed by overnight treatment with glusulase (2000 U β-glucuronidase + 200 U sulphatase per sample) in citrate buffer (1.0 ml, 0.5 *M*, pH 5.2, 37°). Chloroform (5 μl) was added to inhibit bacterial growth in the samples.

Standard solutions of pure nitrazepam, methylnitrazepam, methylbromazepam, 7-aminonitrazepam and 7-acetamidonitrazepam (kindly donated by Hoffmann-La Roche, Basle, Switzerland) were prepared according to De Silva *et al.*[10] using absolute ethanol, acetone and *n*-hexane–acetone (80:20) as solvents. The amount of internal standard (methylbromazepam) for nitrazepam determination was 2 ng per sample. The standards corresponded to 0, 1, 2, 5 and 10 ng/ml nitrazepam. The standards for the metabolites contained 0, 50, 100, 250, 500 and 1000 ng/ml of each metabolite in addition to the internal standard (methylnitrazepam, 250 ng/ml).

Reagents

The following analytical grade reagents were used: dipotassium hydrogen orthophosphate (anhydrous) (J. T. Baker, Phillipsburg, N.J., U.S.A.), citric acid $\cdot H_2O$ and sodium hydroxide (Merck, Darmstadt, G.F.R.), glusulase (Endolaboratories, Garden City, N. Y., U.S.A.), benzene, dichloromethane, acetone and *n*-hexane (all from Merck). The solvents were used without distillation.

Apparatus

A Varian Aerograph Model 2100 gas chromatograph equipped with a nickel-63 ECD (potential 90 V, specific activity 5 Ci/g), was used with a dual channel Omniscribe 5211-4-2A recorder (Houston Instruments) together with U-shaped borosilicate glass columns 5 ft. long, 2 mm I.D. and 1/4 in. O.D. A Hewlett-Packard 5711 A gas chromatograph equipped with a dual flameless nitrogen selective detector was also used, together with a dual channel Varian Aerograph Model 20 strip-chart recorder and coiled 2 ft. borosilicate glass column (3 mm I.D. and 1/4 in. O.D.). A Bühler Sm-2 mechanical shaker, shaking speed 150 rpm, was used.

GLC conditions

The carrier gas was nitrogen (AGA, Helsinki, Finland, 99.995% purity). Molecular sieve 13X was used for decontamination and drying. 3% OV-17 on Chromosorb W (Varian, Palo Alto, Calif., U.S.A.) and 3% OV-101 on Chromosorb Q (Supelco, Bellefonte, Pa., U.S.A.), both 100–120 mesh, were the column packings for nitrazepam and metabolites, respectively. They were stabilized as described earlier[9]. The conditions in GLC–ECD were: carrier gas flow-rate 40 ml/min; injector temperature, 275°; column temperature, 275° and detector temperature, 340°. When the nitrogen selective detector was used the carrier gas, hydrogen and air (AGA) flow-rates were 40, 3 and 60 ml/min, respectively. The injector and column were maintained at 275° and the detector at 300°. A final setting of the detector was performed daily. Qualitative analysis of the compounds was based on the retention times. Quantitative analysis was performed by measuring the peak heights and plotting the concentration against the ratio of nitrazepam or metabolites to the internal standard.

RESULTS

Unchanged nitrazepam was determined from plasma and urine. The results of the plasma analyses are presented elsewhere[9,11]. A mean standard curve of four series of human urine analyses is presented in Fig. 1A. The concentrations of the

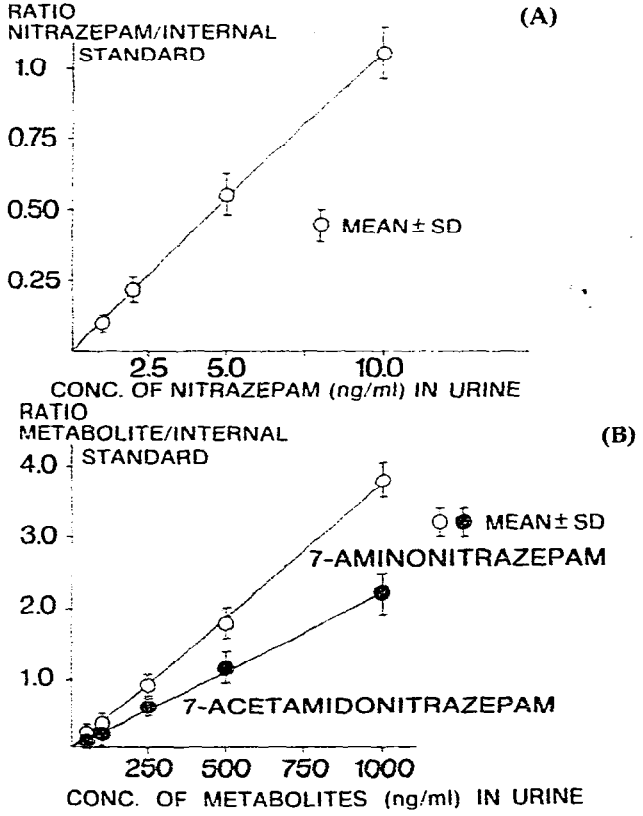

Fig. 1. Standard curves of four consecutive determinations of (A) nitrazepam by ECD and (B) nitrazepam and its main metabolites by nitrogen-selective detector. Means ± S.D. are presented.

metabolites were below the sensitivity limit of the nitrogen selective detector in the plasma samples. Mean standard curves of the metabolites from four consecutive series are presented in Fig. 1B.

Typical chromatograms from human urine with and without added nitrazepam and metabolites are presented in Fig. 2. Fig. 2a refers to the ECD and Fig. 2b to the nitrogen-selective detector.

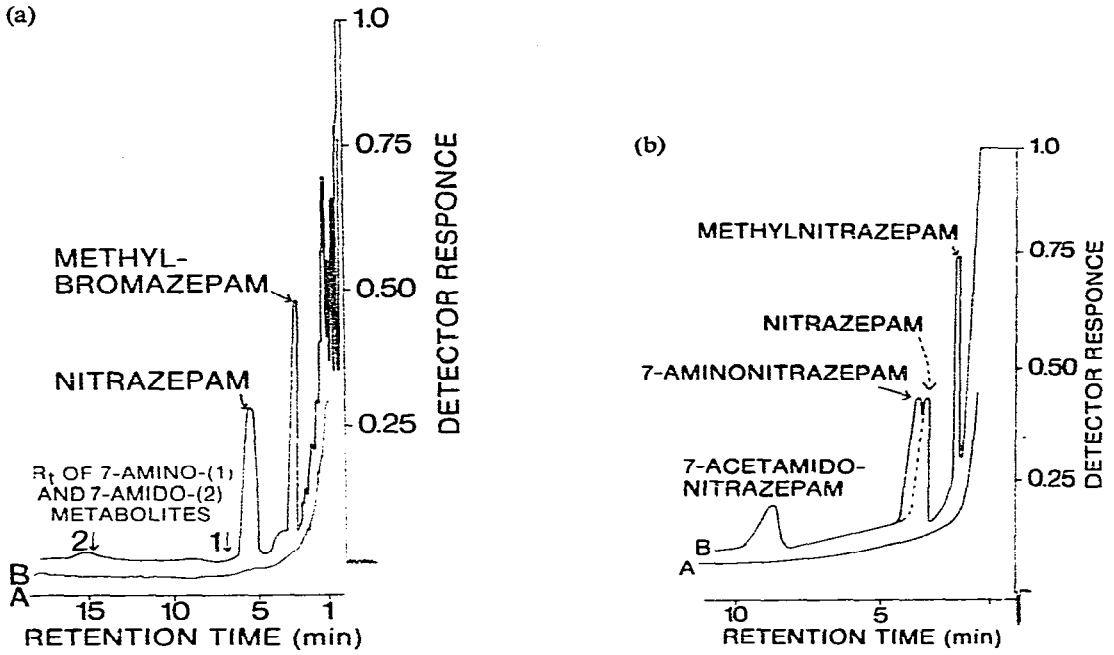

Fig. 2. Typical chromatograms of urinary analysis of nitrazepam with its main metabolites (a) using ECD and (b) using the nitrogen-selective detector. Curve A = urine sample without benzodiazepines, curve B = urine sample with nitrazepam and both metabolites added. Amounts of benzodiazepines added: ECD: methylbromazepam (internal standard), 2 ng/ml; nitrazepam, 1 ng/ml; 7-aminonitrazepam and 7-acetamidonitrazepam; 100 ng/ml. Nitrogen-selective detector: all compounds, 250 ng/ml (internal standard, methylnitrazepam).

Data on the precision, reproducibility and reliability of the methods are presented in Table I. Precision was calculated as coefficient of variation (CV) of peak heights of 15 identical, repeated injections of one sample. The reproducibility was determined by preparing 15 identical urine samples and analysing them on different days (day-to-day variation). The CV was calculated. The reliability is expressed as the percentage recovery of the compounds from human urine. Treatment with glusulase did not affect the recovery of the compounds.

The total amounts excreted during 7 days are listed in Table II. A great variation between individual subjects was observed. Total excretion of nitrazepam in unchanged form or as the measured metabolites during the 7 days was 2430 $\pm$ 1240 μg (48 $\pm$ 25%) (mean $\pm$ S.D.): conjugated metabolites accounted for 57% and unconjugated metabolites for 43%.

TABLE I

PRECISION, DAY-TO-DAY VARIATION AND RECOVERY IN THE DETERMINATION OF NITRAZEPAM AND ITS MAIN METABOLITES

The amounts of the compounds: nitrazepam 5 ng/ml, metabolites 250 ng/ml. Recovery was achieved in the concentration ranges 1–10 ng/ml (nitrazepam) and 50–1000 ng/ml (metabolites),

	Nitrazepam	*7-Aminonitrazepam*	*7-Acetamidonitrazepam*
Precision (CV, %)	3.7	2.3	4.7
Day-to-day variation (CV, %)	12.1	7.5	16.0
Recovery (%, mean ± S.D.)	96.4 ± 4.2	93.0 ± 4.7	92.7 ± 7.1

TABLE II

TOTAL 7-DAY URINARY EXCRETION OF NITRAZEPAM AND ITS MAIN METABOLITES

Amounts excreted expressed as μg (mean ± S.D. of the subjects) and as per cent from the dose.

	Excreted (μg)	*Excreted (per cent)*
Nitrazepam		
free	40 ± 58	0.80
conjugated	16 ± 14	0.33
total	56 ± 70	1.13
7-Aminonitrazepam		
free	676 ± 557	13.5
conjugated	875 ± 856	17.5
total	1551 ± 1310	31.2
7-Acetamidonitrazepam		
free	635 ± 476	12.7
conjugated	403 ± 255	8.1
total	1038 ± 620	20.8

DISCUSSION

In spite of lack of previous reports of GLC analyses, nitrazepam and the main metabolites proved to be easily measurable in human urine by combining the use of the ECD and the nitrogen-selective detector. The ECD was highly specific and sensitive to unchanged nitrazepam (*ca.* 60 times more sensitive than the nitrogen-selective detector). A satisfactory and reproducible response to 7-aminonitrazepam was achieved only with the nitrogen-selective detector. The ECD was unsatisfactory even at very high concentrations. Thus the poor response to 7-aminoclanozapam (closely resembling 7-aminonitrazepam) reported by Gerna and Morselli[12], is probably not due to poor extractability of these metabolites, but rather to the use of an unsuitable detector. 7-Acetamidonitrazepam may be determined both by the ECD and the nitrogen-selective detectors with similar sensitivities. The latter seemed to give better reproducibility (this may, however, be due to a different column). The selectivity of the nitrogen selective-detector is poor compared with that of the EDC. It responds with moderate sensitivity to nitrogen- and phosphorus-containing endogenous compounds in human urine. These impurities in the samples prevent the detector being used to greatest effect. The extraction procedure can surely be improved, thus decreasing the amount of these impurities. However, it is uncertain whether this will

enable the determination of the metabolites in plasma for pharmacokinetic studies. Sufficient sensitivity for measuring the steady-state concentrations of the metabolites (reasonably at an approximate level of 50 ng/ml according to Rieder and Wendt[1] and Sjö *et al.*[13]) might be attainable during chronic nitrazepam administration.

The columns used in this study were stabilized as described earlier[9]. OV-101 (used with the nitrogen selective detector) was not suitable for the separation of nitrazepam and 7-aminonitrazepam (Fig. 2). The overlapping of the peaks was not a drawback in this study, because the concentrations of nitrazepam in the urine samples were far below the sensitivity limit of the detector. A different column has to be used for the analysis of plasma samples: a glass capillary column is best (used by de Boer *et al.*[14] for the analysis of unchanged nitrazepam).

Nitrazepam, being a lipophilic drug, is poorly eliminated via the kidneys either unchanged or conjugated (Table II). This finding supports the results of Rieder and Wendt[1], who studied the elimination of metabolites in one volunteer by using ^{14}C-labelled nitrazepam. The main metabolic pathways, described by Rieder and Wendt[1], were confirmed here: reduction by nitro reduction to 7-aminonitrazepam, which is conjugated or acetylated (to the hydrogen atom of the amino group). Acetylation yields 7-acetamidonitrazepam, which may be further conjugated (apparently to the N-hydrogen of the acetamido group). As a whole the metabolism of nitrazepam is complicated and characterized by many minor metabolites, some soluble in water, some soluble in organic solvents[1]. Enormous effort would be required for the determination of all these metabolites by GLC in biological samples.

The present method may be used to determine the main metabolites of clonazepam, and may be an alternative to the fluorometric method of Rieder[2,15], to GC–MS[16] or to HPLC[8].

ACKNOWLEDGEMENTS

The author thanks Mr Tapio Karppi (Hewlett-Packard, Helsinki, Finland) for permission to use the gas chromatograph equipped with the nitrogen-selective detector, and Miss Ulla-Maija Kankkunen for technical assistance.

REFERENCES

1 J. Rieder and G. Wendt, in S. Garattini, E. Mussini and L. O. Randall (Editors), *The Benzodiazepines*, Raven Press, New York, 1973, p. 99.
2 J. Rieder, *Arzneim.-Forsch.*, 23 (1973) 212.
3 J. A. F. de Silva, L. Bekersky, C. V. Puglisi, M. A. Brooks and R. E. Weinfeld, *Anal. Chem.*, 48 (1976) 10.
4 A. Sengupta and M. A. Peat, *J. Chromatogr.*, 137 (1977) 206.
5 F. L. Vandemark and R. F. Adams, *Clin. Chem.*, 22 (1976) 1062.
6 C. J. Least Jr., G. F. Johnson and H. M. Salomon, *Clin. Chem.*, 22 (1976) 765.
7 M. Donike, *Med. Tech.*, 4 (1972) 1.
8 B. Moore, G. Nickless, C. Hallett and A. G. Howard, *J. Chromatogr.*, 137 (1977) 215.
9 L. Kangas, *J. Chromatogr.*, 136 (1977) 259.
10 J. A. F. de Silva, C. V. Puglisi and N. Munno, *J. Pharm. Sci.*, 63 (1974) 520.
11 L. Kangas, J. Kanto and E. Syvälahti, *Acta Pharmacol. Toxicol.*, 41 (q977) 65.
12 M. Gerna and P. L. Morselli, *J. Chromatogr.*, 116 (1976) 445.
13 O. Sjö, E. F. Hvidberg, J. Næstoft and M. Lund, *Eur. J. Clin. Pharmacol.*, 8 (1975) 249.
14 A. G. de Boer, J. Röst-Kaiser, H. Bracht and D. D. Breimer, *J. Chromatogr.*, 145 (1978) 105.
15 J. Rieder, *Arzneim.-Forsch.*, 23 (1973) 207.
16 J. Næstoft and N.-E. Larsen, *J. Chromatogr.*, 93 (1974) 113.