Journal of Chromatography, 111 (1975) 389–396

CHROM. 8406

DETERMINATION OF NITRAZEPAM IN SERUM BY GAS–LIQUID CHROMATOGRAPHY

APPLICATION IN BIOAVAILABILITY STUDIES

KAREN MØLLER JENSEN
Department of Biology, Dumex Ltd., 2300 Copenhagen S (Denmark)
(First received September 25th, 1974; revised manuscript received April 25th, 1975)

SUMMARY

A gas chromatographic method with electron capture detection has been developed for the analysis of nitrazepam in serum. N-Desmethyldiazepam is used as internal standard.

Nitrazepam isolated from serum is converted by acid hydrolysis into 2-amino-5-nitrobenzophenone, which is chromatographed. Metabolites of nitrazepam (7-amino and 7-acetamido compounds) are not included in the determination. Recovery experiments showed that the method is quantitative. The limit of detection is 5 ng/ml of nitrazepam in serum.

The method has been used for measuring serum concentrations of nitrazepam in bioavailability studies on subjects given a single dose of nitrazepam tablets.

INTRODUCTION

Nitrazepam (1,3-dihydro-7-nitro-5-phenyl-2H-1,4-benzodiazepine-2-one) in biological fluids can be determined in different ways. Thin-layer chromatographic[1,2], photometric[3,4], fluorimetric[5,6], radioactive[7], and gas chromatographic[8–11] methods have been described. The thin-layer chromatographic and photometric methods are not sufficiently sensitive or specific for measuring serum concentrations following therapeutic doses and are applicable only after the intake of very high doses, *e.g.*, in cases of poisoning.

Greater sensitivity is attained in the radioactive assay. Rieder[7] administered ^{14}C-labelled nitrazepam to human subjects and measured the activity in blood. However, this method is not applicable to the detection of blood levels after administration of commercial nitrazepam preparations.

The fluorimetric method, also developed by Rieder[5,6], is sensitive; it is stated that the limit of detection is 10 ng/ml of nitrazepam in plasma or urine. Nitrazepam is separated from its metabolites (7-amino and 7-acetamido compounds) by extraction and nitrazepam as well as the metabolites can be detected.

The gas chromatographic methods described by Beharrell *et al.*[8], Masuda[9],

Viala *et al.*[10] and Ehrsson and Tilly[11] are both specific and sensitive. Ehrsson and Tilly[11] determined nitrazepam as the methyl derivative. In the three other methods and the present chromatographic method, nitrazepam is converted into 2-amino-5-nitrobenzophenone prior to chromatography.

EXPERIMENTAL

This assay detects only nitrazepam and not its metabolites (7-amino and 7-acetamido compounds) (Fig. 1). The procedure consists in extracting nitrazepam

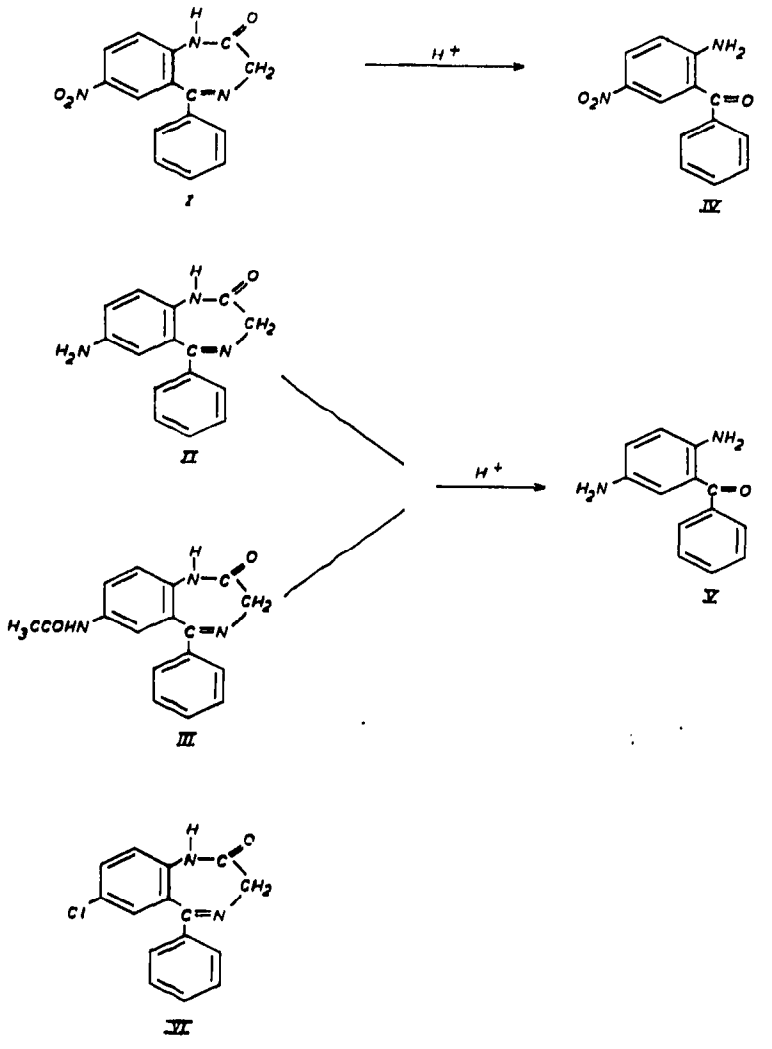


Fig. 1. Formulae of nitrazepam (I) and its metabolites, *viz.*, the 2-amino compound (II) and the 2-acetamido compound (III); IV, 2-amino-5-nitrobenzophenone; V, 2-amino-5-aminobenzophenone; VI, N-desmethyldiazepam.

with benzene, converting the isolated nitrazepam into 2-amino-5-nitrobenzophenone by boiling in acidic medium and measuring the derivative by gas chromatography.

Materials and reagents

The column material is 3% OV-17 on diatomite CQ, 80–100 mesh, from J. J.'s (Chromatography) (King's Lynn, Great Britain). The carrier gas is a mixture of argon (99.996% pure) and methane (99.95% pure), (9:1), obtained from Dansk Ilt- og Brintfabrik (The Danish Oxygen and Hydrogen Factory) (Copenhagen, Denmark). Trimethylchlorosilane was of a specially purified grade from Pierce (Rockford, Ill., U.S.A.). All other reagents were of analytical grade.

Apparatus

The gas chromatograph is a Pye Series 104, equipped with an electron capture detector (^{63}Ni) and a 5-ft. coiled glass column with an I.D. of 1/4 in. The column is filled with OV-17 on diatomite CQ. Before use, the column is conditioned at 300° for 72 h and is finally silanized with trimethylchlorosilane (10 × 10 μl) at 70°. The temperature is 350° in the detector, 245° in the column and 300° at the injection port. The flow-rate of the carrier gas (argon–methane, 9:1) is 100 ml/min.

Procedure

Turn 1.00 ml of serum with 3.00 ml benzene for 5 min in a turning device (Heto) (30 rpm). Centrifuge the mixture for 5 min at 2000 rpm (*ca.* 1000 *g*), pipette off 2.00 ml of the benzene phase and evaporate it to dryness on a boiling water-bath. To the residue add 0.5 ml of 1 *N* hydrochloric acid and allow the acidic samples to stand at 100° for 1 h. After cooling to room temperature, neutralize the samples with 0.5 ml of 1 *N* sodium hydroxide solution using 1 drop of bromothymol blue as indicator. Add 1.00 ml of benzene to the neutralized solutions, turn the solutions for 5 min on the turning device (30 rpm) and then centrifuge them for 5 min at 2000 rpm (*ca.* 1000 *g*). Remove 500 μl of the benzene phase and evaporate it to dryness on a boiling water-bath. Immediately before the measurement, dissolve the residue by shaking it in a Whirlimixer with at least 100 μl of benzene containing 0.25 ng/μl of N-desmethyldiazepam as internal standard. Inject 2 μl of the resulting solution into the gas chromatograph.

By means of the internal standard, correct for injection inaccuracy or error. Read off the amount of nitrazepam in the serum samples from standard curves constructed from the analysis of control sera containing known amounts of nitrazepam.

Sampling

Fifteen healthy volunteers (five females and ten males aged 21–53 years) were included in the trial, which was carried out by a cross-over technique. At an interval of 1 week, each subject received one tablet of 5 mg of nitrazepam of two different brands*, and they were instructed not to take other drugs within 1 week before and during the trial. No special demands were made concerning food intake or normal activities.

Blood samples were drawn immediately before and ½, 1, 2, 4, 7, 24, 48 and 72 h after the administration of the tablet, and the serum centrifuged off was stored at −18° until required for analysis.

* Dumex nitrazepam and another commercial brand of nitrazepam tablets.

RESULTS

Fig. 2 shows a chromatogram of serum to which nitrazepam had been added. The smallest concentration of nitrazepam in serum that can be detected is 5 ng/ml. When determining lower concentrations of nitrazepam, a larger volume of serum can be used. The internal standard N-desmethyldiazepam (DD), added immediately before the injection into the gas chromatograph, has a retention time of 4.0 min compared with 2.9 min for 2-amino-5-nitrobenzophenone (ANB).

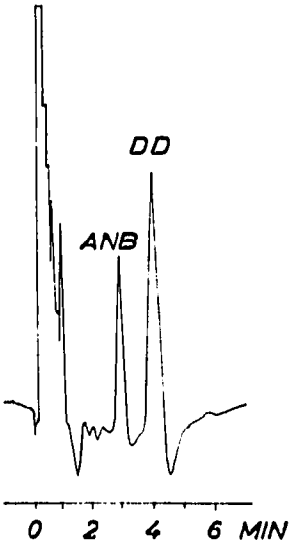
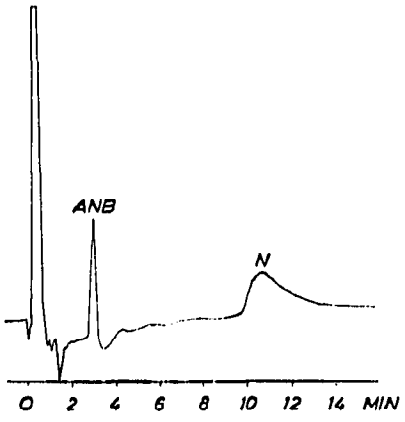


Fig. 2. Chromatogram of an extract of human serum to which had been added nitrazepam, determined as 2-amino-5-nitrobenzophenone (ANB). N-Desmethyldiazepam (DD) was used as internal standard.

Nitrazepam is not determined directly, but is converted into the benzophenone before being injected into the column. Fig. 3 shows a chromatogram of 1 ng of nitrazepam (N) and 0.5 ng of 2-amino-5-nitrobenzophenone (ANB) in the same solution.

The response of the gas chromatograph to N-desmethyldiazepam and 2-amino-5-nitrobenzophenone was compared by injecting 2 μl of various concentrations of the two substances and plotting peak area *versus* concentration. In Fig. 4, it can be seen that the sensitivities for the two substances are approximately the same, but that the response of the detector is no longer linear when the concentration of N-desmethyldiazepam or 2-amino-5-nitrobenzophenone in the injected solutions exceeds about 0.25 μg/ml (= 0.50 ng injected). Therefore, the evaporation residue was dissolved in so much benzene (containing the internal standard) that the amount of 2-amino-5-nitrobenzophenone injected never exceeded 0.50 ng.

By the acid hydrolysis, the metabolites (7-amino and 7-acetamido compounds) are converted into 2-amino-5-aminobenzophenone (Fig. 1). The sensitivity for this



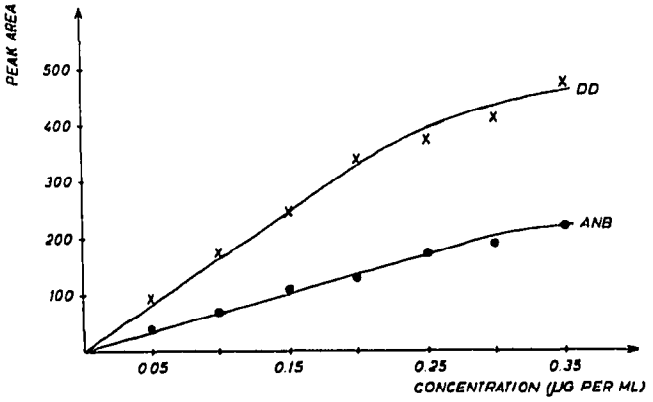


Fig. 3. Chromatogram of 2-amino-5-nitrobenzophenone (ANB) (0.5 ng) and nitrazepam (N) (1 ng).

Fig. 4. Response of the gas chromatograph to N-desmethyldiazepam (DD) and 2-amino-5-nitrobenzophenone (ANB) compared by injection of standard solutions of the two substances.

substance is considerably less than that of 2-amino-5-nitrobenzophenone, as shown in Fig. 5 when 5 ng of the less sensitive 2-amino-5-aminobenzophenone and 0.5 ng of the more sensitive 2-amino-5-nitrobenzophenone were injected into the same gas chromatograph.

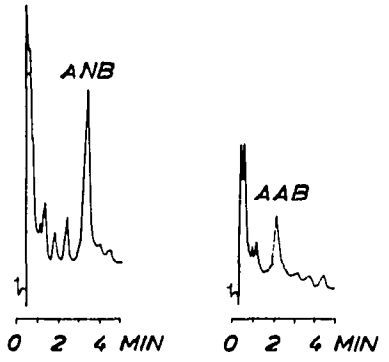


Fig. 5. Chromatogram of 2-amino-5-aminobenzophenone (AAB) (5 ng) and 2-amino-5-nitrobenzophenone (ANB) (0.5 ng).

Fig. 6 shows a standard curve obtained by adding different amounts of nitrazepam to control serum and analyzing the serum samples by the method described. The standard curve is used for reading off nitrazepam concentrations in samples that contain unknown amounts.

In order to check the reproducibility, some of the serum samples from the absorption experiment were analyzed twice. The standard deviation between duplicate determinations (performed on different days) was $\pm$ 13%.

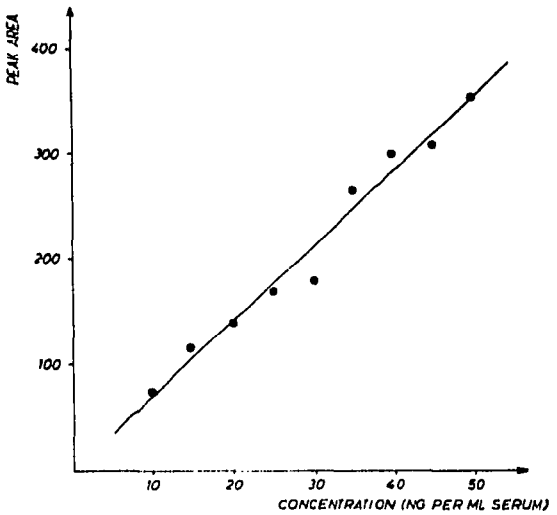


Fig. 6. Standard curve for nitrazepam in serum.

The recovery of nitrazepam in serum (Table I) was determined in the concentration range 10–40 ng/ml and proved to be 105 ± 15% (S.D.).

Bioavailability studies

After an oral dose of 5 mg of nitrazepam of two different brands, peak serum concentrations of 25–50 ng/ml (mean 35 ng/ml) were obtained in about 2 h in the 15 subjects. The mean concentrations, illustrated in Fig. 7, show identical serum curves for both brands.

TABLE I

RECOVERY OF NITRAZEPAM FROM SERUM

Amount of nitrazepam (ng)		*Recovery (%)*
Added	*Found*	
10.0	13.6	136
15.0	16.7	111
20.0	22.0	110
25.0	24.6	98
30.0	30.7	102
35.0	31.4	90
40.0	35.6	89
	Mean: 105 ± 15 (S.D.)	

The biological half-life was calculated on the basis of the serum concentrations after 7, 24 and 48 h. The serum levels after 72 h, which were very low, were not included in the calculation of the half-life. A half-life of 28 ± 11 h (S.D.) was obtained with nitrazepam brand I and 25 ± 6 h (S.D.) with nitrazepam brand II.

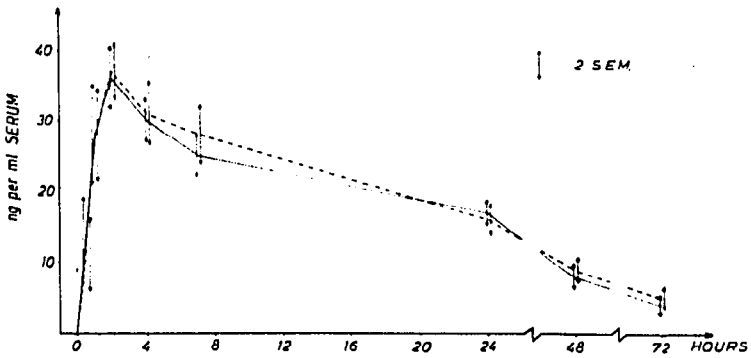


Fig. 7. Mean concentrations (ng/ml) of nitrazepam in serum after administration of 5 mg of nitrazepam as a single dose. Solid line, brand I; broken line, brand II.

DISCUSSION

In the present study, nitrazepam was determined by gas chromatography after conversion into 2-amino-5-nitrobenzophenone because, unlike nitrazepam, the benzophenone gives a symmetrical and reproducible peak. Beharrell *et al.*[8], Masuda[9] and Viala *et al.*[10] obtained the same result, also hydrolyzing nitrazepam to the benzophenone before chromatography. Ehrsson and Tilly[11] determined nitrazepam as the methyl derivative.

However, the present method is simpler than the procedure employed by Beharrell *et al.*[8], Masuda[9] and Viala *et al.*[10]. The method described by Ehrsson and Tilly[11] includes a derivatization procedure prior to chromatography, but this procedure is also simple.

Beharrell *et al.*[8] stated that their detection limit is 0.1 ng/ml of nitrazepam in plasma, which is a considerably greater sensitivity than that which could be obtained in the present procedure, *viz.*, 5 ng/ml of nitrazepam in serum. In his fluorimetric method, Rieder[5] reported 10 ng/ml of nitrazepam in plasma as the limit of detection. Masuda[9] found a limit of detection of 5–10 μg of nitrazepam per sample, while Ehrsson and Tilly[11] reported a value of 5 ng/ml in plasma.

Beharrell *et al.*[8] used clonazepam as the internal standard which was added to all samples before the extraction. Hence the internal standard was run through the entire procedure. In the present study, N-desmethyldiazepam was used as the internal standard, which is added to the samples immediately before the injection and thus corrects for errors due to inaccurate measuring of the injected volume. Before the injection of the samples, Ehrsson and Tilly[11] dissolved the residue in ethyl acetate containing griseofulvin as the internal standard. Masuda[9] used acetone with 2-methyl-3-O-tolyl-4(3H)-quinazolinone for dissolving the residue before the injection.

It is not possible to measure the content of the metabolites (7-amino and 7-acetamido compounds) in serum by the present procedure and the method of Beharrell *et al.*[8]. Ehrsson and Tilly[11] considered that their method could determine these metabolites and 2-amino-5-nitrobenzophenone and 3-hydroxynitrazepam, but they did not find evidence for the presence of these compounds in the samples examined. Rieder[5] did not find the 7-amino and 7-acetamido compounds to be present in the samples he examined by his fluorimetric method.

The reproducibility of the present procedure was checked by performing duplicate determinations (on different days) on serum samples from the absorption experiment. The standard deviation was calculated as ± 13%.

Beharrell *et al.*[8] stated, without giving further details, that the recovery of nitrazepam in the plasma was quantitative (>95%). Ehrsson and Tilly[11] determined the recovery of nitrazepam (at the 50 ng/ml level) to be 103 ± 3% (S.D.). In the present study, the recovery of nitrazepam in serum was 105 ± 15% (S.D.) in the concentration range 10–40 ng/ml.

Bioavailability studies

Beharrell *et al.*[8] measured the plasma concentrations 9–10 h after the oral administration of 10 mg of nitrazepam to eight subjects and 5 mg of nitrazepam to one subject. The plasma concentrations after the 10-mg doses were 20–45 ng/ml (mean 32 ng/ml) and after the 5-mg dose 20 ng/ml. In the present study, in which the dose was 5 mg of nitrazepam, the serum concentration after 10 h was read from the mean curve as 25 ng/ml of nitrazepam, thus confirming the results found by Beharrell *et al.*[8].

Rieder[6] measured the plasma concentrations in six subjects who received an oral dose of 10 mg of nitrazepam. The first sample was drawn 2 h after administration of the drug, and the peak concentrations proved to be 70–110 ng/ml (mean 80 ng/ml). However, the results in the present study are not directly comparable with those obtained by Rieder[6], as the subjects did not receive the same dose of nitrazepam.

Rieder[6] calculated the biological half-life following a single oral dose of nitrazepam (10 mg) 8–72 h after the intake to be 21–28 h (mean 25 h). This result is confirmed by the results of the present study, in which the calculation of the half-lives was based on serum concentrations 7, 24 and 48 h after administration of the drug.

CONCLUSION

A gas chromatographic method for detecting nitrazepam in serum following administration of nitrazepam tablets in therapeutic doses has been developed. The serum concentrations found confirm the results of previous absorption experiments. The calculated biological half-life was in accordance with reported values for the half-life of nitrazepam.

REFERENCES

1 O. Pribilla, *Arzneim.-Forsch.*, 10 (1965) 1148.
2 J. Rieder, *Arzneim.-Forsch.*, 10 (1965) 1140.
3 J. Rieder, *Arzneim.-Forsch.*, 10 (1965) 1134.
4 S. L. Tompsett, *J. Clin. Pathol.*, 21 (1968) 366.
5 J. Rieder, *Arzneim.-Forsch.*, 23 (1973) 207.
6 J. Rieder, *Arzneim.-Forsch.*, 23 (1973) 212.
7 J. Rieder, *Arzneim.-Forsch.*, 10 (1965) 1142.
8 G. P. Beharrell, D. M. Hailey and M. K. McLaurin, *J. Chromatogr.*, 70 (1972) 45.
9 Y. Masuda, *Jap. J. Legal Med.*, 25 (1971) 445.
10 A. Viala, J.-P. Cano and A. Angeletti-Philippe, *Eur. J. Toxicol.*, 4 (1971) 109.
11 H. Ehrsson and A. Tilly, *Anal. Lett.*, 6 (1973) 197.