CHROM. 6017

DETERMINATION OF NITRAZEPAM (MOGADON) IN PLASMA BY ELECTRON CAPTURE GAS–LIQUID CHROMATOGRAPHY

G. P. BEHARRELL, D. M. HAILEY* AND M. K. McLAURIN

Roche Research Unit, Department of Biochemistry, Liverpool University, P.O. Box 147, Crown Street, Liverpool L69 3BX (Great Britain)

(Received February 7th, 1972)

SUMMARY

A sensitive gas chromatographic method has been developed for the analysis of nitrazepam (Mogadon) in plasma, using clonazepam as an internal standard. After extraction, the benzodiazepines are hydrolysed, and chromatographed as the benzophenones. Drug recovery from plasma is quantitative (> 95 %), and the sensitivity limit of detection is about 0.1 ng/ml plasma.

A number of plasma samples were analysed by this method, which proved suitable for estimation of nitrazepam in the presence of other drugs commonly encountered in clinical situations.

INTRODUCTION

In the analysis of nitrazepam** (1,3-dihydro-7-nitro-5-phenyl-2*H*-1,4-benzodiazepin-2-one), situations are commonly encountered in which only the plasma levels of nitrazepam are of interest, rather than those of both nitrazepam and its metabolites. A spectrofluorometric assay for nitrazepam and its metabolites has recently been developed by RIEDER[1]. This makes use of the reaction of *o*-phthalaldehyde with the benzophenones derived from hydrolysis of nitrazepam and its metabolites. The method is sensitive, but requires a plasma blank, which in practice is not always available for clinical samples.

It seemed appropriate, therefore, to develop a gas chromatographic method for the estimation of nitrazepam in plasma, using a ^{63}Ni electron capture gauge (ECG) as detector.

Nitrazepam can be estimated by chromatography of the intact molecule, but elutes as a broad peak, with sensitivity barely adequate for analysis of the drug levels usually encountered in clinical plasma samples. To increase sensitivity and improve peak shape, nitrazepam is hydrolysed to 2-amino-5-nitrobenzophenone (ANB) prior to chromatography (Fig. 1).

The major metabolites of nitrazepam, Ro 5-3072 and Ro 5-3308, do not contain a strongly electron capturing group, and the ECG does not have sufficient sensitivity

* To whom correspondence should be addressed.

** Marketed as Mogadon by Roche Products Ltd., Welwyn Garden City, Herts., Great Britain.

to these compounds to detect clinical levels. Hydrolysis of the metabolites gives 2,5-diaminobenzophenone (DAB) as the major product, which is also weakly electron capturing. A number of other hydrolysis products are also formed in small quantities[1].

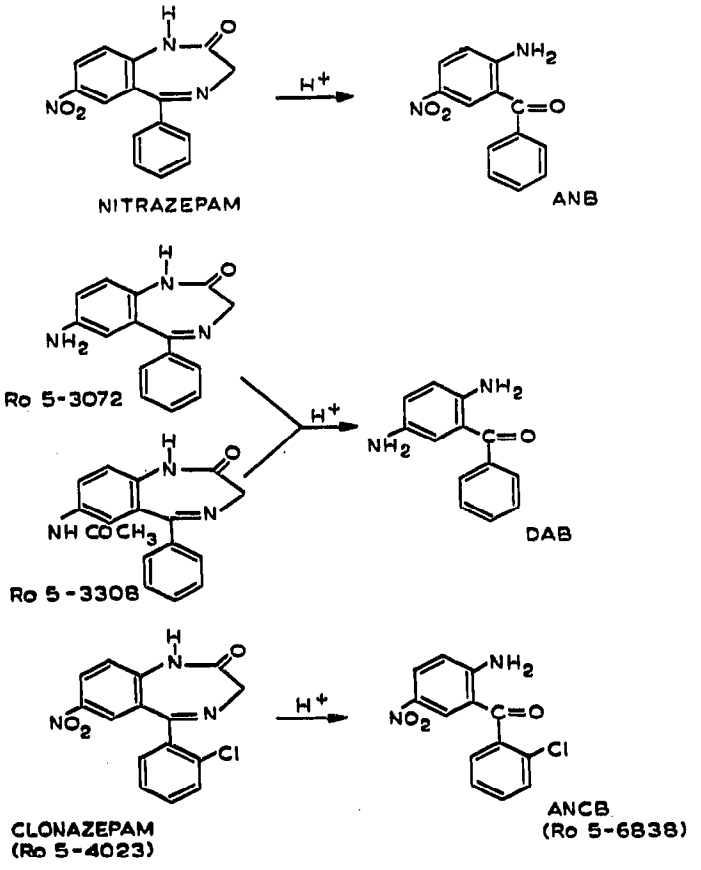

Fig. 1. Benzodiazepine hydrolysis reactions and products.

The benzodiazepine clonazepam (1,3-dihydro-7-nitro-5-(2-chlorophenyl)-2*H*-1,4-benzodiazepin-2-one) is used as an internal standard for this assay. During the hydrolysis step this compound is converted to 2-amino-2'-chloro-5-nitrobenzophenone (ANCB, Ro 5-6838), which elutes after ANB with good peak shape and sensitivity.

The method is considered specific for nitrazepam in the absence of measurable quantities of ANB and the metabolite 1,3-dihydro-3-hydroxy-7-nitro-5-phenyl-2*H*-1,4-benzodiazepin-2-one. These compounds are normally not detected in plasma. The sensitivity limit is about 0.1 ng/ml plasma.

EXPERIMENTAL

Materials

Gas chromatograph. A Pye-Unicam Model 104 equipped with an electron capture detector containing a 10-mCi ^{63}Ni source was used.

Column. A 1.83 m coiled 4 mm I.D. borosilicate glass column containing 3 % OV-17 on 60–80 mesh Gas-Chrom Q (Field Instrument Co. Ltd., Richmond, Surrey)

was used. This was a preconditioned phase, and was further conditioned using the procedure of DE SILVA AND PUGLISI[2].

Instrument parameters. Electron capture detector: temperature, 335°; pulse width, 150 μsec. Carrier gas: molecular-sieve-dry argon (B.O.C.); cylinder head pressure, 9 p.s.i.; flow rate, 50 ml/min. Oven temperature, 235°; injection port temperature, 265°.

Reagents. Analytical grade reagents were used throughout. Diethyl ether was M.A.R. grade (B.D.H.), and was stored at 0°, and used within two days of opening.

Solutions were prepared containing 61.8 g boric acid and 74.6 g potassium chloride per litre and 106 g sodium carbonate per litre; 370 ml of the carbonate solution was added to 630 ml of the boric acid–potassium chloride solution, giving 1 l buffer solution. The buffer solution was well shaken, the pH checked, and adjusted to pH 9.0 with sodium carbonate solution if necessary. This buffer was 1 *M* with respect to each component, and was stored at 35–37°.

Methods

Preparation of standard solutions. Nitrazepam (10.0 mg) was weighed out into a 10-ml volumetric flask, dissolved in 1 ml methanol and made up to volume with acetone–hexane (20:80). A stock solution of the internal standard clonazepam was made up in a similar way.

These stock solutions (1 mg/ml) were diluted 1:1000 with acetone–hexane (20:80) to give working solutions (B) containing 1 μg/ml.

A standard curve was obtained by taking suitable aliquots of the working solutions (B), evaporating these to dryness in a series of stoppered centrifuge tubes, and then following through the hydrolysis and back-extraction procedure described below. Preparation of the series of standards required the same operations used in sample work-up, with the exception of the initial extraction and clean-up stages.

Procedure. Plasma (1 ml) was pipetted into a silanised 54-ml stoppered test tube (Quickfit MF 24/3), containing 25 ng of clonazepam internal standard (25 μl of solution B evaporated to dryness), 5 ml of buffer pH 9.0 was added, and the tube contents agitated briefly on a rotary mixer (Fisons, Whirlimix); 10 ml of diethyl ether was added to the tube, the stopper sealed with water, and the tubes shaken for 10 min on a reciprocating shaker. After centrifuging for 10 min to assist phase separation, the organic phase was transferred to another tube with a Pasteur pipette. The extraction was repeated once, and the organic phases combined. Comparison of extracted plasma spiked with nitrazepam with external standards showed that extraction was essentially quantitative (> 95 %).

5 ml of 6 *N* hydrochloric acid was added to the combined organic phases, and the tubes shaken for 10 min to complete back-extraction, followed by centrifugation at 2000 *g* for 5 min. After removal of the ether layer, the acid phase was washed by shaking with two 10-ml portions of diethyl ether for 10 min on the reciprocating shaker. Each ether portion was removed by aspiration after centrifuging at 2000 *g* for 5 min. As is usual with benzodiazepine analysis, this clean-up stage is very important. If a larger plasma sample (3–4 ml) is used to increase the sensitivity of the method, it is necessary to wash four times with 10-ml diethyl ether portions to completely remove lipid from the system.

Traces of ether were removed from the tubes by heating on a hot water bath.

The tubes were then stoppered (water sealed), and heated on an oil bath for 1 h at 105°. They were then cooled in ice, and made alkaline to bromothymol blue indicator (two drops) by dropwise addition of 6 *N* sodium hydroxide solution. The neutralised solution was extracted with two 10-ml portions of diethyl ether, using the reciprocating shaker, and centrifuging as before.

The ether extracts were transferred to a 15-ml pear-shaped flask, and carefully evaporated on a 40° water bath under a stream of nitrogen. The residue was dried for 15 min in a vacuum desiccator over silica gel.

To the dried residue was added 100 μl of acetone–hexane (20:80). The solution was agitated on a Whirlimix and was then ready for chromatography. A suitable aliquot (1–10 μl) was injected into the gas chromatograph, and the benzophenone peak areas were calculated by taking the product of peak height and width at half height.

RESULTS AND DISCUSSION

Quantitation

The use of clonazepam as an internal standard enables a peak area ratio calibration technique to be used. The compound is very suitable as a standard as it is extracted quantitatively, and its benzophenone ANCB elutes after ANB, with a similar ECG response (Figs. 2 and 3).

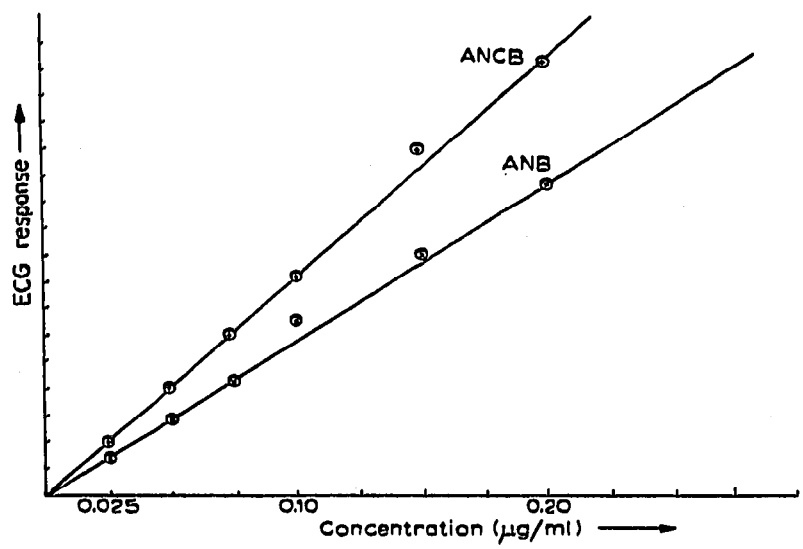

Fig. 2. Response of the electron capture detector to ANB and ANCB.

The plasma recovery curve (Fig. 4) was obtained by plotting the peak area ratios of ANB to ANCB *versus* concentration. The concentration of an unknown nitrazepam sample was then read directly from this curve. The area ratios were independent of the volume injected into the gas chromatograph and the recovery of internal standard remained constant. The sensitivity limit of the method using 1 ml plasma, and a 10-μl injection volume, is 0.1 ng nitrazepam/ml of plasma. The sensitivity can be increased by using a larger volume of plasma. For sample volumes

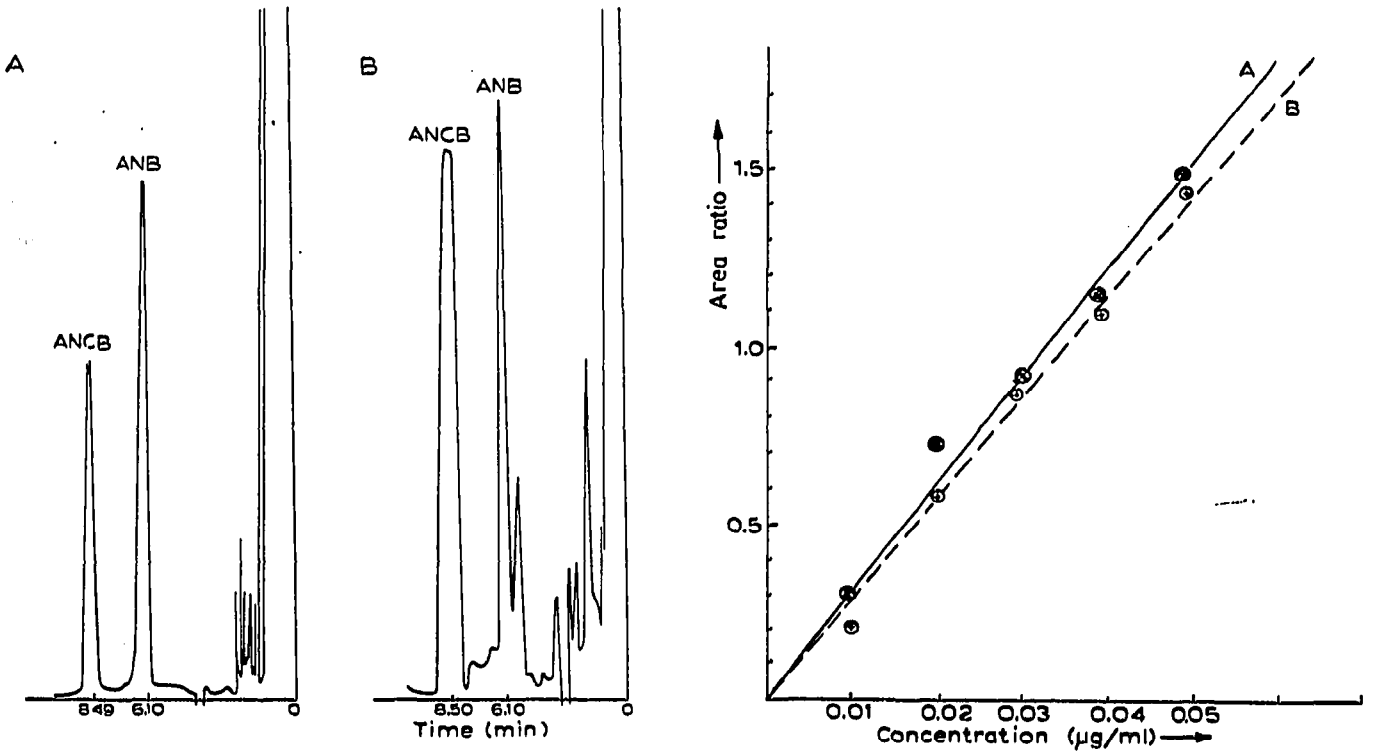

Fig. 3. Chromatograms of ANB–ANCB mixtures. (A) Standard solution, ANB concentration 0.04 μg/ml; (B) plasma sample 4, ANB concentration 0.025 μg/ml.

Fig. 4. Plasma recovery curve for nitrazepam–ANB. (A) External standard curve; (B) blood recovery curve.

greater than 3.0 ml, 8 ml buffer pH 9.0 should be used, and the sample extracted twice with 15-ml portions of diethyl ether.

Application to clinical samples

Plasma samples from nine patients in the Queen Elizabeth II Hospital, Welwyn Garden City, Herts., were analysed for nitrazepam. In all cases the subjects had received 10 mg nitrazepam *per os* at night, a blood sample being taken 10 h later. Most patients had been receiving other drugs in addition to nitrazepam. Details of drugs received and dosage are given in Table I. Nitrazepam (as ANB) was readily detected in all samples, giving plasma levels of between 0.02 and 0.05 μg/ml plasma. These levels were consistent with those obtained in previous work on nitrazepam metabolism[3].

Chromatography of intact nitrazepam

It was found that relatively high levels of intact nitrazepam and clonazepam could be successfully chromatographed using the column and instrument conditions described above (Fig. 5). This separation would be potentially attractive in determining nitrazepam and other benzodiazepines, such as diazepam, in the same sample. At concentrations below 1 μg/ml, however, peak shape deteriorated (Fig. 6), and variable recoveries were obtained for both nitrazepam and clonazepam.

Four of the plasma samples were analysed for nitrazepam by omitting the

TABLE I

NITRAZEPAM LEVELS IN PLASMA SAMPLES AND DETAILS OF DOSAGE

Sample (No.)	*Nitrazepam dose (mg)*	*Previous nitrazepam treatment*	*Time of sample after last dose (h)*	*Nitrazepam in plasma (μg/ml)*	*Other drugs being taken*		*Duration days*
					Drug	*Dose*	
1	10	10 mg/9 days	10.1	0.025	Ampicillin	500 mg q.d.s.	9
					Frusemide	40 mg	6
					Choline theophylline	100 mg t.d.s.	6
					Slow K		6
2	10	10 mg/8 days	9.9	0.031	Methadone (linctus)	5 cc t.d.s.	4
					Choline theophylline	100 mg t.d.s.	3
					Bactrim	b.i.d.	3
3	10	5 mg/3 days	10.0	0.043	None		
4	10	10 mg/4 days	10.05	0.028	Choline theophylline	100 mg t.d.s.	2
					Sparine	500 mg t.d.s.	2
					Septrin	b.i.d.	
					Diazepam	2 × 10 mg two days previously	
5	10	5 mg (3 days before)	9.20	0.019	Bendrofluazide	5 mg	2
6	5	5 mg/16 days	10.15	0.020	Frusemide	40 mg b.d.	
					Salbutamol	4 mg q.d.s.	
					Diazepam	7.5 mg t.d.s.	16
					E-C Prednisone	7.5 mg b.d.	
					Intal (Plain)	t.d.s.	
					Digoxin	0.25 mg b.d.	
					Slow K	1 × at night	
7	10	None	10.00	0.022	None		
8	10	10 mg (several weeks)	10.00	0.046	Digoxin	0.25 mg	several weeks
					Slow K	at night	
					Diazepam	5 mg b.d.	
					Frusemide	80 mg	
9	10	10 mg/9 days	9.20	0.042	Practalol	100 mg t.d.s.	9
					Lanatoside C	0.25 mg b.d.	
					Frusemide	80 mg	
					Aminophylline	2 × suppos. at night	
					Allopurinol	100 mg b.d.	

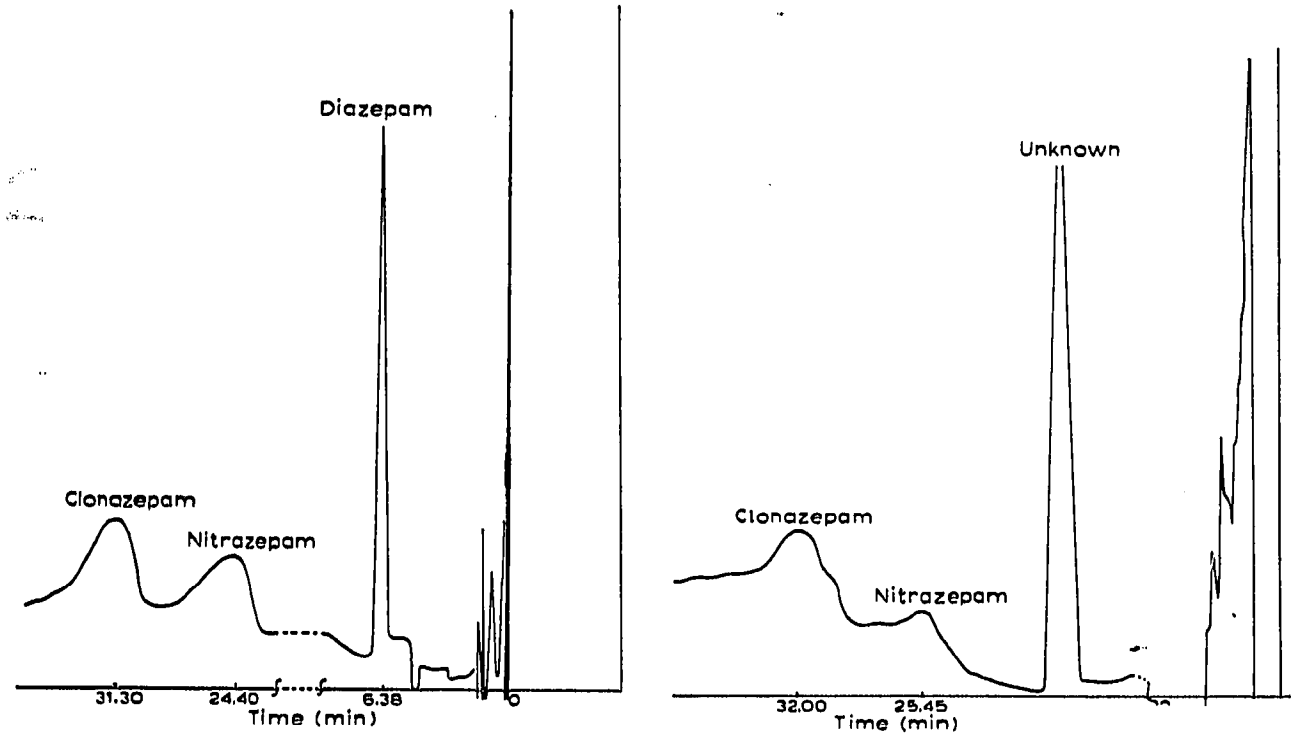

Fig. 5. Chromatogram of a standard mixture of diazepam, nitrazepam, and clonazepam, 1 μg/ml for each compound.

Fig. 6. Chromatogram of the unhydrolysed extract from plasma sample 4.

hydrolysis step, and chromatographing the intact drug. The results were lower than expected, and did not correlate well with those obtained from the hydrolysis–ANB chromatography procedure (Table II). It was considered unlikely that the higher levels obtained from chromatography of the benzophenones were due to the presence of ANB or 3-hydroxynitrazepam in the samples. These shortcomings can be overcome to some extent by increasing both the volume of solution injected into the gas chromatograph, and the size of the sample. However, even when these procedures are adopted, electron-capture gas chromatography of intact nitrazepam is barely adequate as a method for clinical analysis of the drug.

TABLE II

COMPARISON OF GC OF HYDROLYSED NITRAZEPAM (ANB) AND OF INTACT NITRAZEPAM IN PLASMA SAMPLES

Sample (No.)	*Nitrazepam (μg/ml)*	
	ANB method	*Intact method*
1	0.025	0.004
2	0.031	0.008
3	0.043	0.031
4	0.028	0.012

CONCLUSION

The present method is considered suitable for the analysis of nitrazepam in plasma following therapeutic doses of the drug. Metabolites are not detectable. Experience with clinical samples has shown that a wide range of other drugs does not interfere. It is intended to carry out further work on the gas chromatography of nitrazepam and its metabolites, in conjunction with a conductivity detector, with a view to chromatographing all the metabolites intact and detecting them at the nanogram level.

REFERENCES

1 J. RIEDER, personal communication.
2 J. A. F. DE SILVA AND C. V. PUGLISI, *Anal. Chem.*, 42 (1970) 1725.
3 J. RIEDER, *Arzneim. Forsch.*, 15 (1965) 1134.