Journal of Chromatography, 424 (1988) 403–409
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3971

Note

Sensitive gas chromatographic method for determining nitrazepam in serum and saliva

B.J. 't HART* and J. WILTING

Department of Pharmaceutical Chemistry, Subfaculty of Pharmacy, University of Utrecht, Catharijnesingel 60, 3511 GH Utrecht (The Netherlands)

(First received July 9th, 1987; revised manuscript received September 26th, 1987)

Nitrazepam (Mogadon, Fig. 1) is one of the most widely used hypnotics of the benzodiazepine class. The protein binding of nitrazepam in serum is ca. 90% [1]. Therefore the analysis of the free serum concentration and saliva concentration of nitrazepam requires good sensitivity. The available methods for analysing nitrazepam are unspecific [2–4], time-consuming [5,6] or need special column treatment [1,7–9]. In this paper a sensitive, specific and rapid procedure for analysing nitrazepam is described, in which nitrazepam is derivatized to an N-butyl derivative. The derivatization was developed by De Gier and 't Hart [10].

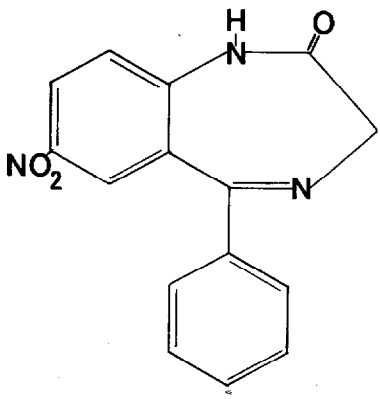

Fig. 1. Structure of nitrazepam.

0378-4347/88/$03.50

EXPERIMENTAL

Materials

Nitrazepam and clonazepam were gifts from Hoffmann-La Roche (Mijdrecht, The Netherlands). Methanol (HPLC quality) was obtained from Baker Chemicals (Deventer, The Netherlands). 1-Iodobutane was a product of Aldrich Europe (Beerse, Belgium).

Heptane (Uvasol) and silver oxide (p.a.) were obtained from Merck (Darmstadt, F.R.G.). Tetrabutylammonium iodide was from BDH Chemicals (Poole, U.K.). C_{18} extraction columns (1 ml) were from Baker and were used in conjunction with the Baker 10 extraction system.

Standard solutions

Stock solutions (100 mg/l) of nitrazepam and clonazepam were prepared in methanol. Standard solutions were obtained by diluting the stock solutions to concentrations of 1 mg/l for serum and 100 μg/l for saliva.

A calibration curve was constructed from blank human serum or saliva, to which 20, 40, 60, 80 and 100 μg/l and 2, 4, 6, 8 and 10 μg/l nitrazepam were added, respectively.

The buffer solution (pH 7.4, $I=0.1$) was prepared by mixing 17.6 ml of 0.5 *M* potassium dihydrogenphosphate with 60.8 ml of 0.5 *M* sodium hydrogenphosphate dihydrate to a final volume of 1 l.

Tetrabutylammonium hydroxide (TBAH), 0.2 *M*, was prepared as follows. To a solution of 0.74 g of tetrabutylammonium iodide in 10 ml of methanol was added 0.55 g of silver oxide. The mixture was shaken gently for 2 h at room temperature. After centrifugation the liquid phase was stored in a dark container at 4 °C.

Samples

Blood samples were taken from volunteers 4 h after they had taken an oral dose of 10 mg of nitrazepam (no anticoagulants were used). Saliva samples were taken at the same time after volunteers had stimulated secretion by chewing a little disc of PTFE (diameter 20 mm, thickness 1 mm). Intake of benzodiazepines decreases the flow-rate of saliva [11], so stimulation of the saliva is necessary to obtain a reasonable amount (2 ml). In our experience, chronic use of benzodiazepines diminishes the flow-rate of stimulated saliva to 0.5–2 ml per 5 min. The pH of stimulated saliva lies within a narrow pH range (pH 7.0). Nitrazepam has pK_a values of 3.2 and 10.8 and is therefore uncharged at physiological pH. Changes in salivary pH by stimulation of saliva would therefore not affect the nitrazepam concentration in saliva. The blood and saliva samples were centrifuged to obtain serum and 'clean' saliva. The samples were then frozen at −20 °C until required for analysis.

Extraction procedure

In a 5-ml polypropylene tube 1 ml of serum, 100 ng of clonazepam and 1 ml of buffer solution were mixed. The C_{18} column was washed twice with 1-ml portions of methanol and twice with 1-ml portions of distilled water. During the washing

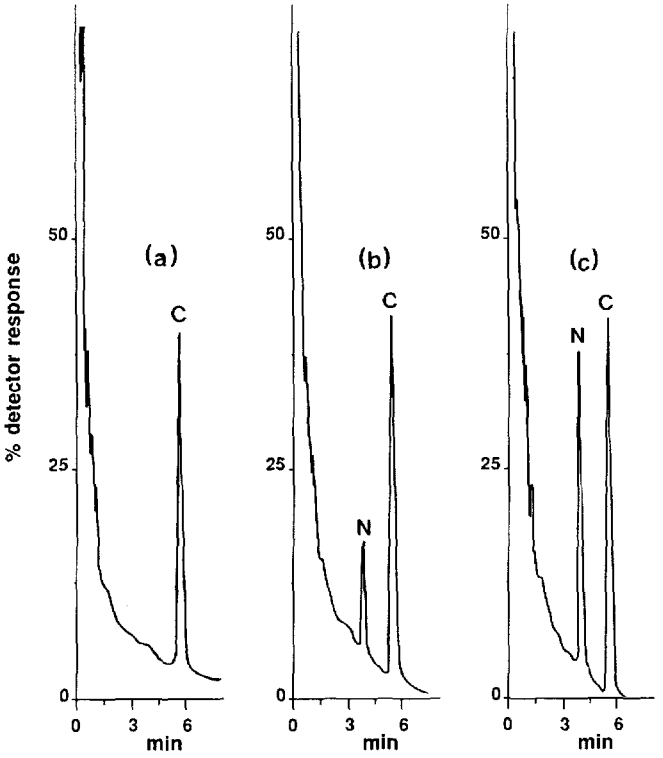

Fig. 2. Chromatograms of (a) blank serum; (b) spiked serum sample containing 20 μg/l nitrazepam (N) and, as internal standard, 100 μg/l clonazepam (C); and (c) serum sample obtained from a male volunteer 4 h after an oral dose of 10 mg of nitrazepam, containing 62.8 μg/l nitrazepam.

with water it is important to keep the column covered with a small amount of solution.

Immediately after this preparation the serum buffer mixture was poured into the wet column in two portions. The column was washed twice with distilled water, then allowed to run dry under vacuum for 30 s. Before the final elution step, 1-ml reacti-vials were placed under the columns in a modified Baker 10 system. Elution was performed with 500 μl of methanol. After allowing 1 min for equilibration, we collected the eluate in the reacti-vials. The eluate was then removed under a stream of nitrogen at 70°C. The residue was redissolved in 50 μl of diluted (1:20) TBAH and 100 μl of 1-iodobutane. The solution was mixed on a vortex mixer for 15 s. This mixture was allowed to react at 70°C for 10 min, then evaporated under a stream of ntirogen at 70°C. The residue was dissolved in 200 μl of heptane, and 1-μl aliquots were injected in triplicate.

For the saliva measurements minor modifications were needed. To a 1-ml saliva sample we added 10 ng of clonazepam. The final volume was 100 μl of heptane, of which 2 μl were injected.

Under the chromatographic conditions used nitrazepam and clonazepam were eluted with retention times of 4.00 and 5.70 min for serum (Fig. 2) and 5.20 min and 7.50 min for saliva (Fig. 3), respectively.

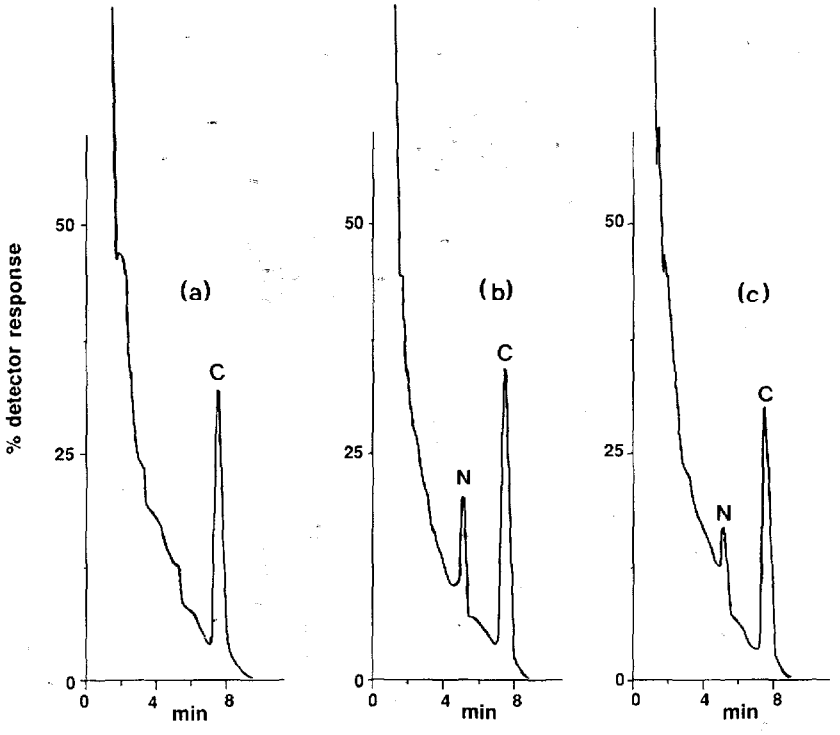

Fig. 3. Chromatograms of (a) blank saliva; (b) spiked saliva sample containing 2 μg/l nitrazepam (N) and, as internal standard, 10 μg/l clonazepam (C); and (c) saliva sample obtained from a male volunteer 4 h after an oral dose of 10 mg of nitrazepam, containing 1.6 μg/l nitrazepam.

Apparatus

The gas chromatographic (GC) system for the serum samples consisted of the following: a Varian Model 3700 gas chromatograph with a pulsed ^{63}Ni electron-capture detector; a Varian Model A 25 recorder; an HP 3352B data system with a Texas Instruments Silent 750 ASR data terminal. The data were statistically evaluated on a CBM 3032 computer (Commodore Business Machine).

The GC column (glass, 1.00 m×3 mm I.D.) was packed with 3% OV-17 on Chromosorb W HP (80–100 mesh) (Chrompack, Middelburg, The Netherlands). The carrier gas was nitrogen with a flow-rate of 30 ml/min. The injector temperature was maintained at 290°C, the column oven temperature at 280°C and the detector temperature at 320°C.

A Varian Model 1400 chromatograph with a ^{63}Ni electron-capture detector was used for the saliva samples. The column length was 2.00 m. All other conditions were the same as those for the serum samples.

Statistical analysis

The data were statistically analysed by a computer program, which calculates regression line, sample results and confidence limits. Equations for the confidence limits were derived from Mandel [12]. Several calibration curves were compared: first, changes with time in the slope and intercept of the calibration

curves were detected with a Student t-test [null hypothesis: the slopes (intercepts) differ at random in time]; secondly, the hypothesis that the y-values for each concentration of the different calibration curves are distributed at random was tested with the X^2 test. If both the null hypotheses are true the calibration curves can be combined into one overall calibration curve.

RESULTS

The recovery of the internal standard, clonazepam, from serum at a level of 0.1 mg/l was 92.5 ± 1.3%. The overall recovery of nitrazepam (96.1 ± 4.0%) was calculated over the concentration range 0.02–0.1 mg/l.

Calibration curves for serum (Table I) and saliva (Table II) were constructed for one day. To obtain an impression of the reliability of the procedure we constructed calibration curves weekly over a period of five weeks. Fig. 4 shows the slope and intercept of the calibration curves obtained during five weeks. Application of the Student t-test and the X^2 test demonstrates that there is no significant difference between the calibration curves.

The accuracy and precision of the method were determined over a four-week period. For the serum stock solutions containing 20, 60 and 100 μg/l nitrazepam, the mean calculated concentration ($n=4$) was 21.4 μg/l [coefficient of variation (C.V.) = 4.9%], 59.9 μg/l (C.V. = 4.1%) and 101.0 μg/l (C.V. = 1.2%), respectively. For the saliva stock solutions containing 2, 6 and 10 μg/l nitrazepam, the mean calculated concentration ($n=4$) was 2.0 μg/l (C.V. = 5.5%), 6.2 μg/l (C.V. = 3.7%) and 10.3 μg/l (C.V. = 1.3%), respectively. The detection limits are 5 μg/l for serum and 0.5 μg/l for saliva at a signal-to-noise ratio of 3.

TABLE I

CALIBRATION CURVE OF SERUM MEASURED IN ONE DAY

Each point represents the average of three measurements. Each measurement is the mean of three injections. Equation: $y = a + bx$, where y represents the ratio of the peak area of nitrazepam to that of clonazepam and x represents the concentration of nitrazepam in the calibration sample. The parameters with a line above them are rounded-off values.

Parameter	Value	Confidence limits (95%)	
a	−0.01	−0.03	0.01
b	0.0096	0.0093	0.0099

The correlation coefficient is 0.998693025.
The correlation exists with 99% confidence.

Error analysis of input data

x(in)	y(in)	y(calc)	Confidence limits (95%)	
20.0	0.168	0.18	0.16	0.21
60.0	0.578	0.57	0.55	0.59
100.0	0.948	0.95	0.93	0.98

TABLE II

CALIBRATION CURVE OF SALIVA MEASURED IN ONE DAY

Conditions and parameters as in Table I.

Parameter	Value	Confidence limits (95%)	
a	0.03	0.01	0.06
b	0.146	0.142	0.150

The correlation coefficient is 0.998924626.
The correlation exists with 99% confidence.

Error analysis of input data

x(in)	*y*(in)	*y*(calc)	Confidence limits (95%)	
2.0	0.336	0.33	0.29	0.36
6.0	0.890	0.91	0.88	0.94
10.0	1.494	1.49	1.46	1.53

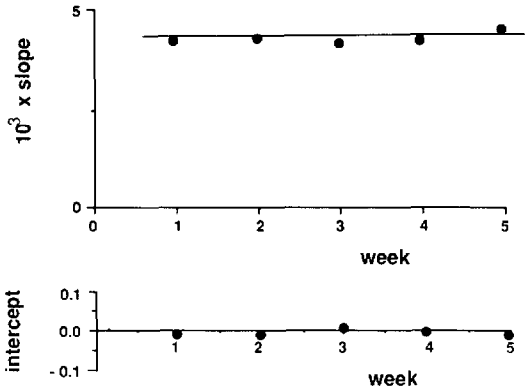

Fig. 4. Slopes (*a*) and intercepts (*b*) of the calibration curves, obtained over five weeks, plotted versus the time.

DISCUSSION

In this study nitrazepam was determined by GC after it had been converted into N-butylnitrazepam because, unlike nitrazepam itself, the derivative gives a symmetrical and reproducible peak.

Kangas [4] determined nitrazepam directly and also used clonazepam as internal standard with the liquid phase 3% OV-17. He deactivated the column by means of repeated injections of 2-μl blood extracts in benzene–dichloromethane. It is known that the tailing effect of standard solutions is diminished in the presence of a blood extract, probably because the extracted blood lipids form a complex with the active sites on the column [8]. In our experience, this method for deactivating adsorption sites on the column does not produce reproducible results for the N_1–H benzodiazepines. Derivatization by means of alkylation at position N_1–H reduced adsorption and yielded symmetrical peaks.

Baker C_{18} columns were used for the extraction procedure. In terms of time and simplicity this method was a big improvement over previous published methods, and it has similar sensitivity and selectivity to the method reported by Kangas [4].

REFERENCES

1 L. Kangas, J. Chromatogr., 172 (1979) 273.
2 K. Møller-Jensen, J. Chromatogr., 111 (1975) 389.
3 G.P. Beharrell, D.M. Hailey and M.K. McLaurin, J. Chromatogr., 70 (1972) 45.
4 L. Kangas, J. Chromatogr., 136 (1977) 259.
5 H. Ehrsson and A. Tilly, Anal Lett., 6 (1973) 197.
6 M.S. Greaves, Clin. Chem., 20 (1974) 141.
7 D.M. Hailey, J. Chromatogr., 98 (1974) 527.
8 J.A. De Silva, I. Bekersky, C.V. Puglisi, M.A. Brooks and R.E. Weinfeld, Anal. Chem., 48 (1976) 10.
9 A.G. de Boer, J. Röst-Kaiser, H. Bracht and D.D. Breimer, J. Chromatogr., 145 (1978) 105.
10 J.J. de Gier and B.J. 't Hart, J. Chromatogr., 163 (1979) 304.
11 J.E. Steiner, D. Birnbaum, F. Karmelli and S. Cohen, Digestion, 3 (1970) 262.
12 J. Mandel, The Statistical Analysis of Experimental Data, Interscience Publishers, New York, 1964, p. 272.