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COMPARISON OF TWO GAS–LIQUID CHROMATOGRAPHIC METHODS FOR THE DETERMINATION OF NITRAZEPAM IN PLASMA

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SUMMARY

Nitrazepam in plasma was determined by gas–liquid chromatography with a nickel-63 electron-capture detector, unchanged by a direct method and also by a hydrolysis method. The extraction in the direct method was carried out with benzene–dichloromethane (90:10) and in the hydrolysis method with diethyl ether. The hydrolysis was performed with 6 *N* sulphuric acid. The hydrolysis product was extracted with toluene–*n*-heptane–ethyl acetate (80:20:5) directly from acid. Thus the commonly used change in pH was omitted.

Nitrazepam concentrations in plasma were determined in 10 healthy volunteers after two oral doses (5 and 10 mg); 0.5 ml of plasma was used for each determination and clonazepam, methylbromazepam and methylnitrazepam were used as internal standards. The recoveries of the methods are almost quantitative (>96%). The two methods are clinically comparable.

The high sensitivity and specificity make these methods useful in clinical determinations of nitrazepam in plasma. Advantages and disadvantages of both methods are discussed.

INTRODUCTION

The structures of the widely used hypnotic drug nitrazepam* and the anti-epileptic drug clonazepam are similar, they undergo the same metabolic reactions[1,2] and have related pharmacokinetic characteristics[1,3–6]. They can be analyzed by gas–liquid chromatography (GLC) by almost identical methods[7,8].

Nitrazepam and clonazepam, like many other benzodiazepines, are determined by GLC in two different ways, either unchanged or after acid hydrolysis to benzophenones. Both of these methods have been used successfully, for example, in analyzing diazepam, but the determination of nitrazepam and clonazepam is more difficult without acid hydrolysis owing to the tailing of their peaks in GLC.

* Nitrazepam = 7-nitro-5-phenyl-1,3-dihydro-2H-1,4-benzodiazepin-2-one; methylnitrazepam = 7-nitro-5-phenyl-1,3-dihydro-1-methyl-2H-1,4-benzodiazepin-2-one; clonazepam = 7-nitro-5-(2′-chlorophenyl)-1,3-dihydro-2H-1,4-benzodiazepin-2-one; methylbromazepam = 7-bromo-1,3-dihydro-1-methyl-5-(2-pyridyl)-2H-1,4-benzodiazepin-2-one.

Direct analyses of clonazepam in plasma by GLC have been published by Næstoft and co-workers[8,9], Berlin and co-workers[6,10] and Gerna and Morselli[11], but Knop *et al.*[12] and De Silva and co-workers[13,14] could not obtain satisfactory results. Nitrazepam, with its shorter retention time, should give a better peak shape than clonazepam. However, there have been only a few reports on its direct GLC determination[15–17], and such methods are not sensitive enough to be used in clinical or pharmacokinetic analysis[18]. Methylation and trimethylsilylation[19,20] have been used to improve the characteristics of nitrazepam in GLC. However, most nitrazepam determinations have been made after acid hydrolysis[4,7].

Both methods described here give clinically identical results, but they differ in principle. Some minor metabolites of nitrazepam (3-hydroxynitrazepam and perhaps some unknown metabolites[2,21,22]) yield the same end-product as nitrazepam itself on acid hydrolysis. The amount of possible metabolites of nitrazepam is very small, and the acid hydrolysis method is regarded as being specific for nitrazepam[4,7]. In this study there is a small, statistically significant, but clinically negligible, difference between these two methods.

The aim of this study was to develop a rapid and simple direct method for determining nitrazepam in clinical and biological samples, to compare it with the hydrolysis method and to simplify the latter.

EXPERIMENTAL

Thirteen healthy volunteers, five females and eight males, aged 21–38 years and of weight 58–73 kg, received no benzodiazepines for 3 weeks and no food for 3 h prior to the test. They were first given a 5-mg nitrazepam tablet (Dumex, Copenhagen, Denmark) at 7 p.m., then 3 weeks later two 5-mg tablets (a 10-mg dose). Blood samples were collected in heparinized tubes at 0, ½, 1, 2, 3, 4, 12 (or 14), 24, 48 and 72 h after administration of the nitrazepam. After centrifugation of the blood, the plasma was separated and stored at −20° until analyzed. Three of the volunteers received placebo tablets.

Apparatus

A Varian Aerograph Model 2100 gas chromatograph equipped with a nickel-63 electron-capture detector (ECD) (potential 90 V, specific activity 5 C/g), was used with a dual-channel Omniscribe 5211-4-2A (Houston Instruments) or dual-channel Varian Aerograph Model 20 strip-chart recorder, together with U-shaped borosilicate glass columns of length 5 or 6 ft., I.D. 2 mm and O.D. ¼ in.

A Bühler Sm-2 mechanical shaker, shaking speed 150 rpm, was employed.

GLC conditions

The carrier gas was nitrogen (AGA, Helsinki, Finland, 99.995% purity) at a flow-rate of 40 ml/min. Molecular sieve 13X was used for decontamination and drying of the carrier gas. The columns were packed with 3% OV-17 on 100–120-mesh Chromosorb W (Varian, Palo Alto, Calif., U.S.A.) or 3% SP-2250 on 100–120-mesh Supelcoport (Supelco, Bellefonte, Pa., U.S.A.). The temperatures were: injector, 275°; column, 245° (indirect method) or 275° (direct method); and detector, 340°. Silanization of the columns was effected with Silyl-8 (Pierce, Rockford, Ill., U.S.A.).

Reagents

The following analytical-grade reagents were used: dipotassium hydrogen orthophosphate (anhydrous) (J. T. Baker, Phillipsburgh, N.J., U.S.A.), diethyl ether (Merck, Darmstadt, G.F.R. or Koch-Light, Colnbrook, Great Britain), sulphuric acid, benzene, dichloromethane, toluene, *n*-heptane, ethyl acetate, acetone and *n*-hexane (all from Merck). The solvents were used without distillation.

Standard and internal standards

Pure nitrazepam, clonazepam, methylnitrazepam, methylbromazepam and 2-amino-5-nitrobenzophenone (ANB) were kindly donated by Hoffman La Roche (Basle, Switzerland) and by Dumex (Copenhagen, Denmark).

Preparation of standard solutions

All standard solutions were prepared according to De Silva *et al.*[14] using absolute ethanol, acetone and *n*-hexane–acetone (80:20) as solvents.

Statistical tests

The paired *t*-test, linear regression and correlation were used.

Analytical methods

The plasma samples were thawed at room temperature overnight. Each of two 0.5-ml portions were pipetted into 10-ml glass-stoppered conical test-tubes containing the internal standard corresponding to 50–75 ng/ml of clonazepam or 30 ng/ml of methylnitrazepam or methylbromazepam in plasma. Standards corresponding to 0, 5, 10, 25, 50, 75 and 100 ng/ml of nitrazepam in plasma were also prepared. About 50 mg of anhydrous dipotassium hydrogen orthophosphate were added in order to produce a slightly alkaline pH (about 9). The tubes were vortexed for a short period to dissolve the phosphate.

Extraction steps. (1) Direct method (unchanged nitrazepam). A 3.3-ml volume of benzene–dichloromethane (90:10) was added to the plasma sample, the tubes were shaken for 10 min in a Bühler shaker and centrifuged (800 *g* for 10 min) at room temperature. A 3-ml aliquot of the upper organic phase was transferred into a clean test-tube, evaporated to dryness in a 60° water-bath under a gentle stream of dry air and finally dried in a vacuum oven at 50° for 2–4 h.

(2) Hydrolysis method. A 3-ml volume of diethyl ether was added to the sample. After shaking for 10 min and centrifuging (800 *g* for 10 min), the ether layer was nearly completely transferred into a clean test-tube (glass stopper). Another 3-ml portion of diethyl ether was added to the plasma sample. While the steps of the second extraction (shaking and centrifuging) were being carried out, the first portion of ether was evaporated to dryness in a 50° water-bath under a gentle stream of dry air, then 1.0 ml of 6 *N* sulphuric acid was added to the residue. The second diethyl ether extract was pipetted directly on to the acid. The tubes were shaken and centrifuged as before and diethyl ether was removed by suction. The tubes containing the acid phase were placed in a 50° water-bath to remove the remainder of the diethyl ether, and finally in a 100° oil-bath for hydrolysis for 1 h (see Discussion for the optimal time of hydrolysis). The tubes were closed tightly after 10 min and cooled to room temperature in a water-bath. A 3.3-ml volume of toluene–*n*-heptane–ethyl

acetate (80:20:5) was pipetted directly on to the acid. After shaking and centrifuging as before, a 3-ml aliquot of the upper organic phase was transferred into a clean test-tube, evaporated to dryness in 60° water-bath under a gentle stream of dry air and dried in a vacuum oven at 50° for 2–4 h.

Analysis by GLC. (1) Column pre-treatment in the direct method. The determination of nitrazepam with a normally stabilized (silanized) column does not give satisfactory results because of marked tailing of the peak. Benzene–dichloromethane (90:10) extracts of plasma seem to contain some unspecific impurities, which decrease the absorption of nitrazepam (and clonazepam) on the column (see also refs. 13 and 14). These extracts are obtained by extracting 1 ml of reference plasma in the same manner as the nitrazepam samples in the direct method. Another plasma extract containing nitrazepam and clonazepam (about 200 ng/ml each) can be prepared to control the peak shape and separation of nitrazepam and clonazepam. The residue is dissolved in 150 μl of *n*-hexane–acetone (80:20) and 1–2 μl is injected into the column. Repeated silanizations and injections of extracts at high temperatures (270–285°) stabilize and inactivate the column. Decreasing the oven temperature overnight to 150° may also assist the inactivation. To avoid detector and column contamination, not more than 2 μl of extract should be injected at a time. The influence of column stabilization is illustrated in Fig. 1.

(2) Analysis of unchanged nitrazepam. The samples are dissolved in 150 μl of *n*-hexane–acetone (80:20) after the stabilization of the column and a 1.5-μl sample is injected into the gas chromatograph at 275°.

(3) Analysis of ANB (hydrolysis method). The samples are dissolved in 300 μl

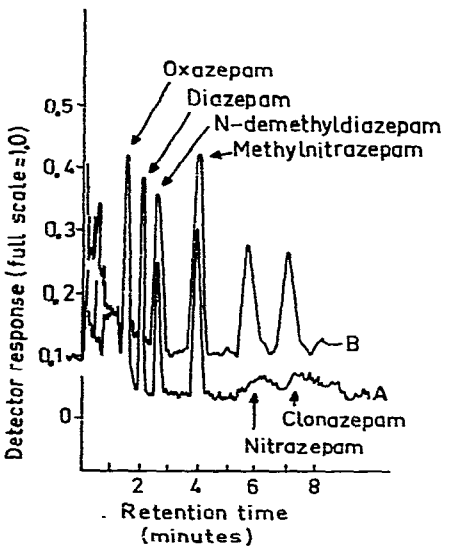

Fig. 1. Influence of column stabilization on the peak shapes of nitrazepam and clonazepam. Curve A: a sample containing 20 ng/ml of oxazepam, 10 ng/ml of diazepam and N-demethyldiazepam, 30 ng/ml of methylnitrazepam and 50 ng/ml of nitrazepam and clonazepam before column stabilization. Curve B: the same sample after column stabilization (repeated injections of reference plasma extract and silanization). Column, 3% OV-17; temperature, 275°.

of *n*-hexane–acetone (80:20) and 1.5 μl is injected into the gas chromatograph at 245°. No special column inactivation is needed. A typical gas chromatogram is shown in Fig. 2.

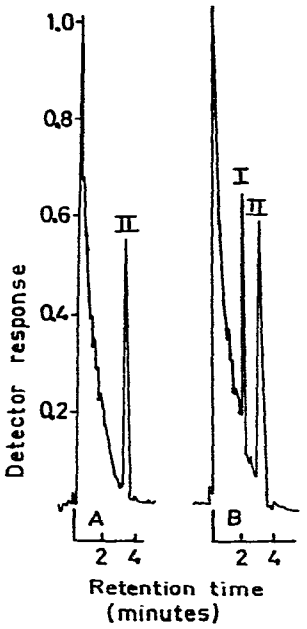

Fig. 2. Typical gas chromatograms obtained in the hydrolysis method. Curve A: a reference plasma sample containing 50 ng/ml of ANCB as internal standard (II). Curve B: a plasma sample containing 50 ng/ml of ANB (I) and ANCB (II).

Calculation of the results

The qualitative determination is based on retention times. The quantitative results are calculated from the peak heights using the ratio of the peak height of nitrazepam (or ANB) to the peak height of the internal standard. Standard graphs are presented in Fig. 3 (unchanged nitrazepam) and Fig. 4 (ANB).

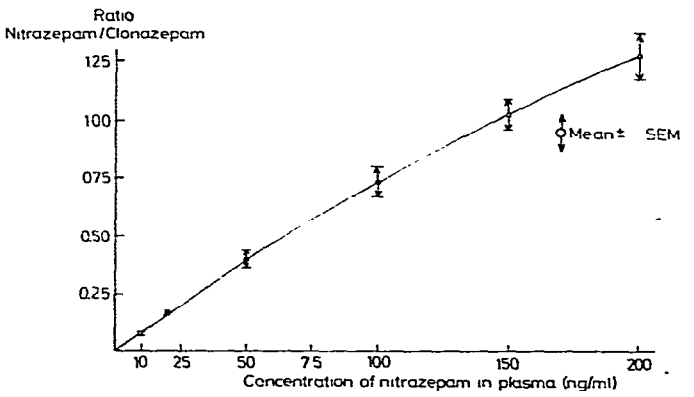

Fig. 3. Standard graph for plasma nitrazepam in the direct method. Mean ± SEM of five standard series on different days. Internal standard, clonazepam (150 ng/ml).

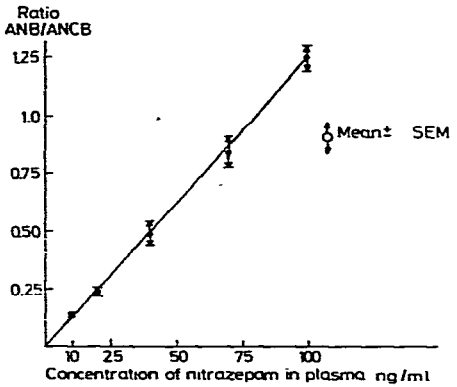

Fig. 4. Standard graph for plasma ANB (nitrazepam after acid hydrolysis). Mean ± SEM of six standard series on different days. Internal standard, ANCB (75 ng/ml).

RESULTS

Comparison of the methods by linear regression and correlation

The direct and hydrolysis methods, different internal standards in the direct method (methylnitrazepam and methylbromazepam in comparison with clonazepam) as well as the internal and external standards are compared by correlation and linear regression in Figs. 5, 6 and 7, respectively. In all instances there is a very significant correlation ($p < 0.001$) between the concentrations compared.

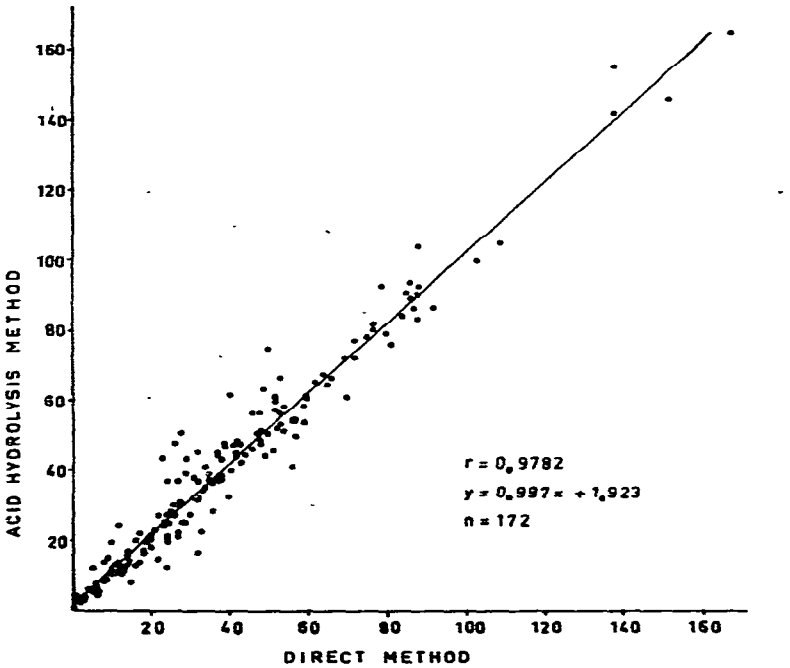

Fig. 5. Correlation and linear regression of plasma concentrations of nitrazepam (ng/ml) in the whole material measured by the direct and acid hydrolysis methods.

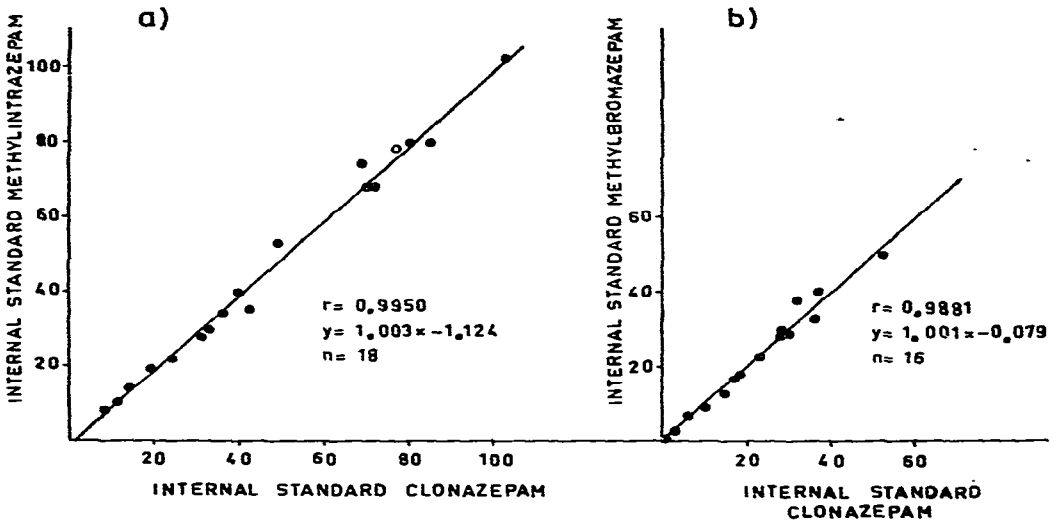

Fig. 6. Comparison of different internal standards in the direct method. The plasma concentrations of nitrazepam (ng/ml) are compared by correlation and linear regresssion by using clonazepam and methylnitrazepam (a) as well as clonazepam and methylbromazepam (b) as internal standards. In all instances a very significant correlation ($p < 0.001$) is found. For further details, see text.

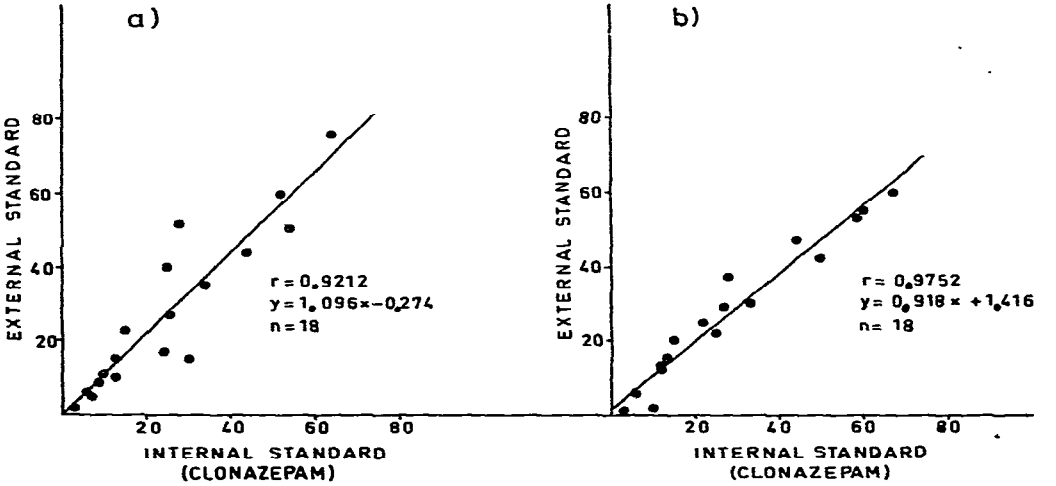

Fig. 7. Comparison of external and internal standardization. The plasma concentrations of nitrazepam (ng/ml), found with both of these systems, are compared by correlation and linear regression in the direct (a) and hydrolysis (b) methods. A very significant correlation ($p < 0.001$) is found in both instances.

Comparison of the methods by paired t-test

The same comparisons as above were made by the paired *t*-test in order to control the specificity of the methods. The comparisons are presented in Table I, which indicates the identities of all internal standards used in this study. In the direct

TABLE I

PAIRED *t*-TESTS IN DIFFERENT METHODS

A: Comparison of direct (Di) and hydrolysis (H) methods. B: Comparison of different internal standards [clonazepam (CL) and methylnitrazepam (MN)] C, Comparison of different internal standards [clonazepam (CL) and methylbromazepam (MB)]. D: Comparison of internal (INT) and external (EXT) standards in direct (Di) and hydrolysis (H) methods. SD = standard deviation; N.S. = not significant.

Parameter	*A*	*B*	*C*	*D*	
				Di	*H*
Mean nitrazepam concentration in plasma (ng/ml)	Di 39.4 H 41.2	CL 47.9 MN 46.8	CL 22.2 MB 22.1	INT 25.4 EXT 27.6	27.3 26.4
SD	0.480	0.681	0.544	2.023	1.076
t	3.737	1.632	0.115	1.721	0.774
p	<0.001	N.S.	N.S.	N.S.	N.S.
n	172	18	18	18	18

method, the calculated nitrazepam concentrations (39.4 ng/ml) are lower than those in the hydrolysis method (41.2 ng/ml) ($p < 0.001$).

Precision

Identical samples corresponding to 50 ng/ml of ANB in plasma were injected 20 times repeatedly into the gas chromatograph and the peak heights were measured. The resulting precision of the apparatus, peak-height measurement and injections on the same day are shown in Table II (column A).

One plasma sample corresponding to 50 ng/ml of ANB and 2-amino-5-nitro-2'-chlorobenzophenone (ANCB) (internal standard) was injected 10 times repeatedly and the ratio of the peak heights of ANB and ANCB was calculated. Column B in

TABLE II

PRECISION AND REPRODUCIBILITY OF THE METHODS

A: Precision on the same day by using external standard [peak height of ANB (cm) measured]. B: Precision on the same day by using internal standard (peak-height ratio of ANB to ANCB calculated). C: Reproducibility by using external standard [peak height of nitrazepam (cm) measured]. D: Reproducibility by using internal standard (peak height ratio of nitrazepam to clonazepam calculated). SD = Standard deviation; SEM = standard error of the mean; CV = coefficient of variation. For further explanations, see text.

Parameter	*A*	*B*	*C*	*D*
Mean of peak height or peak-height ratio	9.21	0.905	3.56	1.015
SD	0.78	0.013	0.49	0.064
SEM	0.17	0.004	0.11	0.014
CV	0.085	0.014	0.139	0.063
t	52.8	224.2	32.2	71.2
n	20	10	20	20

Table II illustrates the intra-day precision of the apparatus and peak-height measurement.

Reproducibility

The reproducibility was studied by preparing 20 identical plasma samples, containing 25 ng/ml of nitrazepam and clonazepam, and analyzing them on different days (direct method). Columns C and D in Table II represent the reproducibility of the method by using the external standard (the peak heights measured) and internal standard (the ratio of the peak heights of nitrazepam and clonazepam measured), respectively.

Reliability

The reliability of the methods is illustrated as percentage recovery in Table III. The tests of recovery from plasma were performed by adding known amounts of nitrazepam and clonazepam to the dried extracts of reference plasma in order to avoid peak tailing, which always occurs when these drugs are injected into the gas chromatograph in pure organic solutions. In this table, the recovery of ANB from 6 *N* sulphuric acid in comparison with the recovery from alkali[7] is also presented. The mean peak height of ANB, extracted from acid, was 7.94 cm and from alkali 7.84 cm. In the paired *t*-test the standard error of the mean (SEM) was 0.146 and $t = 0.687$ ($n = 14$). This result confirms that the change of pH is unnecessary in ANB extraction.

TABLE III

RECOVERY TESTS ON THE METHODS AND RECOVERY OF ANB FROM ACID AND ALKALI

A: Recovery (%) of nitrazepam in the direct method (internal standard clonazepam). B: Recovery (%) of nitrazepam in the hydrolysis method (internal standard clonazepam). C: Recovery (%) of ANB (ANB = hydrolysis product of nitrazepam) from 6 *N* H_2SO_4 and from alkalinized solution. For further details, see text.

Parameter	*A*	*B*	*C*	
			6 N H_2SO_4	*Alkali*[7]
n	20	10	14	14
Mean recovery (%)	97.44	96.80	95.36	95.16
SD	4.66	3.30	5.43	8.03
SEM	1.04	1.04	1.45	2.15
Largest value	106.0	103.4	109.9	113.5
Smallest value	91.0	92.1	86.4	84.5
Concentration range in plasma (ng/ml)	5–150	5–150	10–200	10–200

DISCUSSION

Many GLC methods for the determination of benzodiazepines are inconvenient owing to the large volumes of distilled analytical-grade organic solvents and many working steps involved[7,12–14,17,18]. Aggarwal *et al.*[23], Horning *et al.*[24] and Kangas *et al.*[25] successfully used salt–solvent systems for separating drugs and drug

metabolites from biological samples. In our work, these rapid systems were modified: solid dipotassium hydrogen orthophosphate is added to buffer the pH of the samples to about 9. The volume of the samples is kept to the minimum and the amount of organic solvent may be diminished. Handling of the small samples is convenient and the evaporation rapid. The validity of the extraction systems used was discussed by Møller Jensen[4] and Beharrell *et al.*[7] (nitrazepam), Næstoft *et al.*[8] (clonazepam), Berlin and co-workers[6,10] (diazepam, its metabolites and clonazepam), De Silva and co-workers[13,14] (clonazepam and flunitrazapam) and Kangas *et al.*[25] (diazepam and its metabolites). In the direct method, the working steps are minimized. In the hydrolytic method, relatively numerous steps remain in spite of omitting the regularly used but unnecessary change of pH in extracting ANB.

The problem with the direct method is the tailing of the nitrazepam peak in GLC analysis. The inactivation of the column is sometimes difficult and time consuming. However, once the column is inactivated, it can be used continuously and successfully for the analysis of plasma samples for nitrazepam and clonazepam. It is necessary to use standards that are prepared through the method in order to avoid the peak tailing. OV-17 is a better liquid phase than SE-30; the latter does not separate nitrazepam and clonazepam completely.

The time of acid hydrolysis is not critical. The optimal time in 6 *N* sulphuric acid and at 100° seems to be 30 min, but no significant decrease in recovery was found with longer time periods up to 2 h (recovery better than 90% between ½ and 2 h).

As Figs. 6 and 7 indicate, the use of an internal standard in comparison with an external standard results in smaller errors, no matter which internal standard is used. Methylbromazepam is of special value in the direct method, because it can also be used as the internal standard in determining diazepam and its metabolites. In clinical studies, it is important not to use any benzodiazepine drug or *invivo* metabolite as the internal standard because of the wide use of these drugs. If any doubts about the validity of an internal standard exist, the use of an external standard should be considered.

The difference found between the methods may be explained by the minor metabolites of nitrazepam[2,21,22]. These metabolites are not determined in plasma as such by GLC. The difference, although it is statistically very significant, is too small to be detected reliably in individual samples, at least after an acute dose of nitrazepam. Both of the methods may be considered to be specific for nitrazepam.

The reduced metabolites of nitrazepam (7-aminonitrazepam and 7-acetamidonitrazepam) are not measurable by the described methods with sufficient sensitivity. These metabolites are perhaps clinically inactive[26,27] and their determination has only a slight value for clinical purposes. They can be determined by fluorimetric methods[2,5,28,29].

The following advantages and disadvantages may help in selecting suitable methods for nitrazepam determinations.

The advantages of the direct method are that extraction is easy and rapid, the sensitivity (about 1 ng/ml) and specificity are good and simultaneous quantitative analysis of other benzodiazepines in the same sample is possible. Its disadvantages are the toxicity of benzene (ventilation needed), absorption of nitrazepam occurs in the GLC column and special inactivation may be needed, a high temperature is required in GLC and the peaks, although symmetrical, are not sharp.

The advantages of the acid hydrolysis method are the very good sensitivity (0.2–0.5 ng/ml), GLC analysis is easy and the peaks are sharp and symmetrical, purification of the samples occurs in the extraction steps and no special column stabilization is needed. Its disadvantages are that extraction is time consuming (many steps), selection of an internal standard may be difficult if clonazepam is not to be used and some metabolites of nitrazepam are hydrolyzed to the same benzophenone as nitrazepam itself.

In the clinical monitoring of nitrazepam in plasma and when rapid analyses of, for instance, toxicological plasma samples are required, the direct method is to be preferred, because it is fairly rapid and simple. In more urgent cases, the drying of the extracts in the vacuum can be omitted. On the other hand, if samples contain many impurities (*e.g.*, tissue samples) or extremely low concentrations of nitrazepam, the hydrolysis method is to be preferred because it is more sensitive and the samples are purified during the extraction. Improved specificity and reliability of the qualitative analysis of unknown samples are achieved with parallel use of the methods.

In conclusion, the two methods for the determination of nitrazepam at therapeutic concentrations in plasma described here do not measure the main metabolites of nitrazepam. The results of the methods are comparable and both methods are suitable for clinical and pharmacokinetic studies of nitrazepam. The direct method can be used for analysing concentrations of clonazepam and diazepam and its metabolites in plasma.

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