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QUANTITATIVE GAS CHROMATOGRAPHIC ANALYSIS OF FLUNITRAZEPAM IN HUMAN SERUM WITH ELECTRON-CAPTURE DETECTION

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SUMMARY

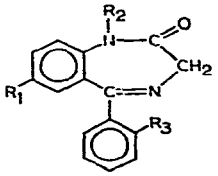
A rapid method for the determination of flunitrazepam and desmethylflunitrazepam in human serum in the range 10–300 ng/ml is described. Both drugs are isolated from biological material by means of a single extraction, part of the organic phase is evaporated to dryness and the residue is dissolved in a small volume of benzene. Without further purification, the substance is determined gas chromatographically with an electron-capture detector configuration of ^{63}Ni-type. The method permits the quantitative determination of at least 25–300 ng/ml with an overall recovery of flunitrazepam of 99.7 ± 4.9% and of desmethylflunitrazepam of 98.6 ± 7.8% from serum. All calculations were carried out by a data system that was programmed for this purpose.

The limit of detection for flunitrazepam is of the order of 1 ng/ml in serum. The method is sufficiently sensitive and specific for therapy control purposes. The time needed for an analysis is less than 1 h.

INTRODUCTION

In order to establish the correlation between the serum level and the short-acting hypnotic activity of flunitrazepam [$R_0$5-4200; 7-nitro-5-(2-fluorophenyl)-1,3-dihydro-1-methyl-2H-1,4-benzodiazepin-2-one] (Fig. 1), and to study the pharmacokinetic profile of flunitrazepam and its desmethyl metabolite in treated patients, it is necessary to have a sensitive, specific method of analysis.

De Silva *et al.*[1] described a method for the determination of both clonazepam



	R_1	R_2	R_3
flurazepam	Cl	$CH_2CH_2N(C_2H_5)_2$	F
diazepam	Cl	CH_3	H
nitrazepam	NO_2	H	H
clonazepam	NO_2	H	Cl
flunitrazepam	NO_2	CH_3	F

Fig. 1. Structural formulae of some benzodiazepines.

and flunitrazepam that involves selective extraction from blood or urine into diethyl ether at pH 9.0, followed by acid hydrolysis to the respective benzophenones, which are extracted and quantitated by electron-capture gas–liquid chromatography (GLC). The assay was applied successfully to the disposition of clonazepam in man following single 2-mg oral doses and of flunitrazepam in the dog following intravenous and oral doses of 2 mg/kg.

A differential pulse polarographic assay for the determination of the major urinary metabolites of clonazepam was also described by De Silva *et al.*[1]. The determination of clonazepam (Rivotril) and its two main metabolites in plasma, using gas chromatography with electron-capture detection, was developed by Naestoft and Larsen[2]. The three compounds and the added internal standard were extracted from plasma with ethyl acetate and the subsequent steps included evaporation, purification and differential extraction of clonazepam and the metabolites. The limit of detection is 3–5 ng/ml, which is sufficient for the determination of therapeutic levels of clonazepam. The specificity of the method was confirmed by mass fragmentography.

De Silva and Bekersky[3] developed a less time-consuming assay for the routine analysis of clonazepam (as its methyl derivative) and flunitrazepam (unchanged) and its N-desmethyl metabolite using GLC with electron-capture detection (ECD). The method was used to measure blood levels in man following a single oral dose of clonazepam and flunitrazepam, and has a limit of detection of 0.5–1.0 ng/ml.

This paper describes an evaluation of a method described earlier by Faber and De Goede[4], following a single intravenous 2-mg dose of flunitrazepam. The method involves a single extraction, as was used for diazepam by Berlin *et al.*[5], who also used a benzene extraction, followed by gas chromatography of the unaltered drug and one of its chief metabolites, desmethylflunitrazepam, using ^{63}Ni electron-capture detector connected to a laboratory data-handling system.

EXPERIMENTAL

Apparatus

The gas chromatograph used was a Hewlett-Packard 5713-A, equipped with a new configuration of an extremely simple ^{63}Ni-ECD (Fig. 2)[6].

The laboratory data system connected to the gas chromatograph consisted of a Hewlett-Packard 3352-A laboratory data system, a Hewlett-Packard 18652-A A to D convertor and a 2752-A teleprinter.

A spiral-shaped glass column (120 cm × 2 mm I.D.) was used with argon–methane (90:10) (Loos & Co., Amsterdam, The Netherlands) as carrier gas. A 10-μl Hamilton syringe was used for sample injections. A Vortex-Genie mixer and a Junior Christ centrifuge (max. speed 3200 rpm) were utilized.

The extractions were performed in 10-ml glass-stoppered centrifuge tubes with a tapered base, of volume 0.2 ml. All extraction tubes were carefully washed with bichromate–sulphuric acid and then with demineralized water.

Reagents and chemicals

The benzene used for extraction was Benzol kristallisierbar, pro analyse (Merck, Darmstadt, G.F.R.). The internal standards were flurazepam (Hoffmann-La Roche, Basle, Switzerland) and lorazepam (Wyeth Laboratories, Amsterdam, The

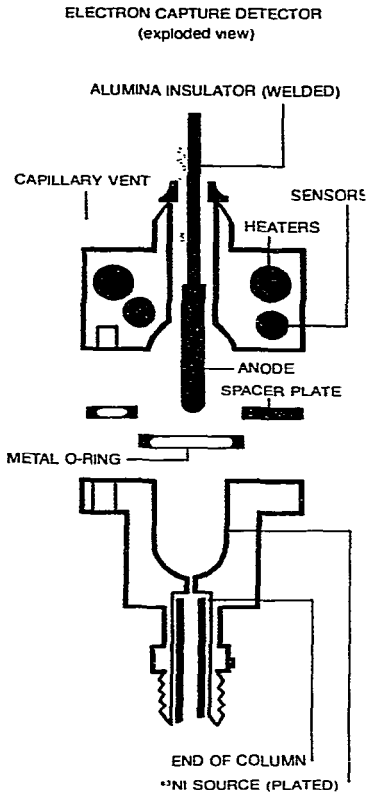


Fig. 2. Electron-capture detector configuration.

Netherlands) and solutions in benzene of concentrations 4.94 and 2.32 μg/ml, respectively, were prepared.

Flunitrazepam ($R_0$5-4200) and desmethylflunitrazepam were obtained from Hoffmann-La Roche and stock solutions in benzene of concentration 1 mg/ml were prepared.

Standard solutions of flunitrazepam and desmethylflunitrazepam were prepared by diluting 1000-ml portions of the 1 mg/ml stock solutions to the required concentrations in the range 25–300 ng/ml.

Extraction

A 1-ml volume of serum was shaken for 1 min with 6.0 ml of benzene, without using a buffer solution, in a 10-ml glass-stoppered centrifuge tube and the mixture was centrifuged for 10 min at 3200 rpm, then 5.0 ml of the supernatant were transferred into a 10-ml glass-stoppered tube with a tapered base of volume 0.2 ml. The tube containing benzene extract was placed in a water-bath (37°) and the contents were evaporated to dryness by means of dried compressed air. The residue was dissolved in 100 μl of benzene containing 4.94 ng/μl of flurazepam as the internal standard. Dry benzene was used for injection on to the column.

Gas chromatography

The operating conditions and settings of the gas chromatograph were as follows. The spiral-shaped glass column (120 cm × 2 mm I.D.) was packed with 8% SE-30 on Chromosorb W AW DMCS (HP) (80–100 mesh), and the carrier gas (argon–methane, 90:10) was used at a flow-rate of 50 ml/min. The temperature of the injection port was 300°, the attenuation was up to 1 × 256 and the sample size was 3 μl, injected with a 10-μl Hamilton syringe.

For identification and calculation, the internal standard technique was used. Flurazepam was chosen as the internal standard because of its suitable retention time (Table I); earlier lorazepam was used.

The areas of the peaks were calculated by integration (laboratory data system). Flunitrazepam and desmethylflunitrazepam can be quantitated by gas chromatography when the relative peak area is used as a measure index of concentration, as a linear relationship exists between relative peak area and drug concentration in the range tested. All calculations were carried out by using the laboratory data system, which was programmed for this purpose.

RESULTS AND DISCUSSION

A report given by the laboratory data system, showing the results relating to a typical gas chromatogram obtained in a recovery experiment with flunitrazepam (100 ng/ml) and with lorazepam as the internal standard is illustrated in Fig. 3 and the corresponding gas chromatogram is shown in Fig. 4a. Preliminary studies indicated that blanks from extracts of human serum do not show peaks that interfere seriously with the peaks of either flunitrazepam or desmethylflunitrazepam (Fig. 4b and Table I).

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REPORT NO.   242                              BENZODIAZEPINES

CHAN#  1              ROB                IJK

MIN AR     MV/M      DLY     DVT     DIL-FTR%     ID-LVL    REF-RTW    %RTW
  100       .030    3.50    0.00      12000.          0        .20       10

ISTD =   1.933   NG/ML

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  RT       ITM        AREA     NG/ML       NAME

  3.95                82583   99.187
  4.44     4.44#     193128             &LORAZEPAM
  5.41                6491     7.796
  5.87                48941   58.782
  7.35     7.35      244308  101.207    FLUNITRAZEPAM
  9.92     9.86      120479   73.421    DM-FLUNITRAZEPAM

TOTAL AREA =        695930
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Fig. 3. Results given by laboratory data system for recovery of flunitrazepam and desmethylflunitrazepam with lorazepam as the internal standard. Recoveries: flunitrazepam, 100 ng/ml; desmethylflunitrazepam, 75 ng/ml.

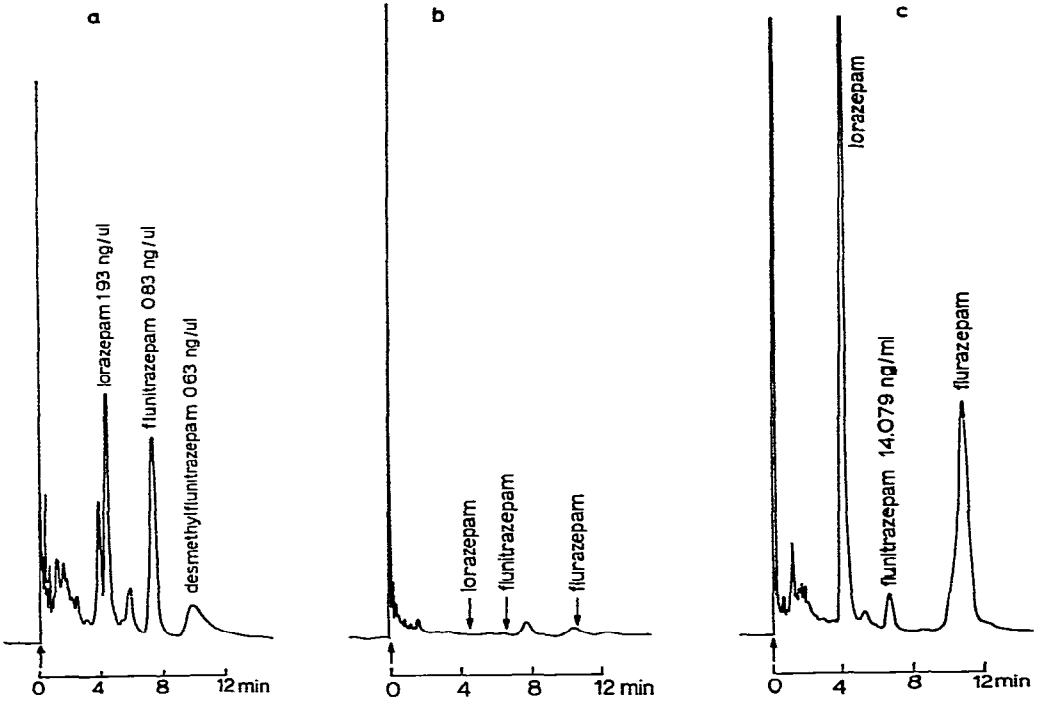

Fig. 4. Gas chromatograms obtained with lorazepam and flurazepam as the internal standard. (a) Chromatogram showing recovery from human serum containing 100 ng/ml of flunitrazepam and 75 ng/ml of desmethylflunitrazepam; (b) chromatogram of a human serum blank; (c) chromatogram of human serum following intravenous administration of a therapeutic dose of flunitrazepam.

Recoveries

A series of recoveries from human serum were studied for flunitrazepam in the range 25–300 ng/ml and for desmethylflunitrazepam in the range 300–25 ng/ml, as these were expected to be the therapeutic levels. For these recovery experiments, the standard solutions were used; 1.000 ml of the required solutions were evaporated

TABLE I

ABSOLUTE RETENTION TIMES OF SOME BENZODIAZEPINES UNDER THE OPERATING CONDITIONS USED

Compound	t_{abs} *(min)*	*Compound*	t_{abs} *(min)*
Lorazepam*	3.7	Desmethylflunitrazepam**	7.9
Oxazepam*	4.0	Flurazepam	10.1
Diazepam[5]	4.1	Nitrazepam**	10.5
Desmethyldiazepam	4.9	Griseofulvin**	10.5
Flunitrazepam	6.2	Chlordiazepoxide**	13.9
Temazepam	7.2	Clonazepam**	15.2

* Conversion product.

** These compounds give tailing under the gas chromatographic conditions used. Griseofulvin is not a benzodiazepine, but is sometimes used as an internal standard.

TABLE II

RECOVERY OF FLUNITRAZEPAM AND DESMETHYLFLUNITRAZEPAM FROM SERUM ON CONSECUTIVE DAYS

The overall recovery ($n = 12$) for flunitrazepam is 99.7% with a standard deviation of 4.9% (89–108.8%); and for desmethylflunitrazepam is 98.6% with a standard deviation of 7.8% (83.9–114.8%).

Compound	*Concentration in serum (ng/ml)*						
	Day	*25*	*50*	*75*	*100*	*200*	*300*
Flunitrazepam	1	89.0	100.2	102.5	105.3	108.8	97.1
	2	98.8	100.7	97.4	99.9	96.7	99.9
Desmethylflunitrazepam	1	91.5	100.7	114.8	95.1	106.0	98.6
	2	91.3	83.9	98.7	99.5	101.6	101.3

to dryness in a water-bath (37°) by means of compressed air, and the residue was first dissolved in 0.02 ml of ethanol and then 0.98 ml of human serum was added. After incubation for 1 h at 37° in a water-bath, extraction and gas chromatography were carried out as described above.

The results of recovery experiments, calculated by means of the laboratory data system, are shown in Table II and in Fig. 5, which shows a standard recovery curve for flunitrazepam with lorazepam as the internal standard.

The smallest amounts that could be determined reproducibly in human serum were 10 ng/ml for both flunitrazepam and desmethylflunitrazepam, but with a lower standard deviation for the former than the latter (see Table II).

We obtained reproducible values using both lorazepam and flurazepam as the internal standard and further investigations showed no systematic difference between them ($\alpha = 0.05$). However, the variation between duplicate results was greater for lorazepam than for flurazepam, possibly owing to thermal conversion of the former. In subsequent investigations with flunitrazepam, flurazepam was therefore the internal standard of choice.

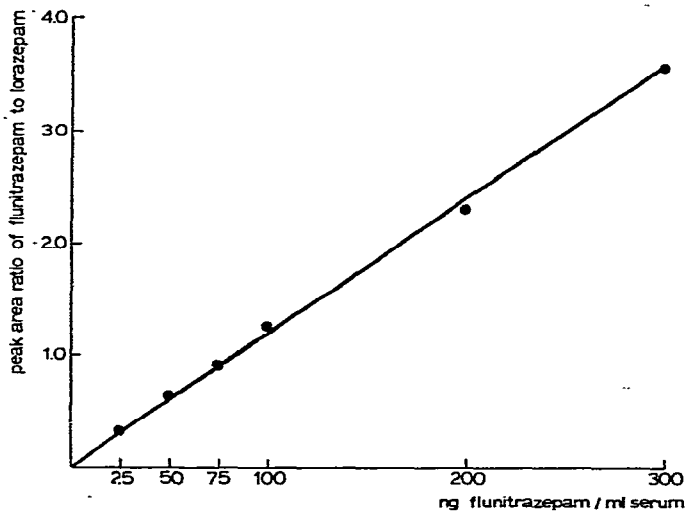

Fig. 5. Standard curve of the electron-capture detector response to various concentrations of flunitrazepam, using lorazepam as the internal standard.

REPORT NO. 954 BENZODIAZEPINES

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ISTD = 4.94Ø, R NG/ML

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RT	NG/ML	NAME
3.97	465.745	LORAZEPAM
5.25	22.999	
6.58	14.Ø79	FLUNITRAZEPAM
8.43	.517	DM-FLUNITRAZEPAM
1Ø.68		&FLURAZEPAM

TOTAL AREA = 1875716

Fig. 6. Results given by laboratory data system on serum of a patient treated with a therapeutic dose (2 ng) of flunitrazepam.

Specificity of the method

A number of drugs are used in conjunction with flunitrazepam. In investigations of retention times and responses it appeared that not all of these drugs give an electron-capture response. However, when other benzodiazepines are used in conjunction with flunitrazepam, there may be a smaller influence on its determination depending on which internal standard is chosen (Table I). With flurazepam as the internal standard, for example, there would be no interference if flunitrazepam was being used clinically with lorazepam or 3-hydroxydiazepam (temazepam).

Fig. 4c shows a chromatogram obtained from human serum containing flunitrazepam after intravenous administration of a therapeutic dose of flunitrazepam. Additionally, a report of the quantitative results given by the laboratory data system is shown in Fig. 6.

CONCLUSION

It can be concluded that the method is sufficiently sensitive and specific for single-dose experiments and interaction studies with some other benzodiazepines and their metabolites and non-related drugs. In order to realize some of the interaction studies, the analytical potential of the method can be improved further by partly derivatizing some benzodiazepines and metabolites.

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