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Note

Assay of flunitrazepam, temazepam and desalkylflurazepam in plasma by capillary gas chromatography with electron-capture detection

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Flurazepam, flunitrazepam, and temazepam are benzodiazepine derivatives which are used either as hypnotics, tranquillizers, or in anaesthesia (Fig. 1).

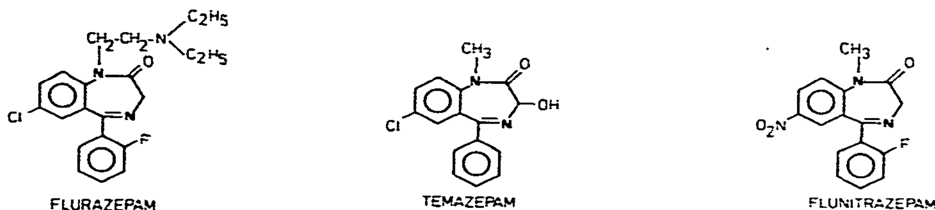

Fig. 1. Structure of flurazepam, temazepam and flunitrazepam.

Flurazepam is very rapidly metabolized in man and only trace amounts of unchanged drug can be measured in plasma [1]. Its major metabolite is the N-desalkyl derivative, which is slowly eliminated from the body and which has psychopharmacological activity similar to that found for flurazepam. This metabolite is probably largely responsible for the hypnotic and persistent sedative action of flurazepam. Temazepam has no active metabolites; flunitrazepam is active as such, but probably also has active metabolites [2]. These drugs have shorter elimination half-lives and are shorter-acting than flurazepam [3, 4].

As it was our aim to perform a comparative pharmacokinetic study in man of several benzodiazepines used as hypnotics (nitrazepam, flunitrazepam, temazepam, triazolam and flurazepam), assay methods had to be developed which were not only rapid but also sensitive enough to measure plasma concentrations for at least three times the elimination half-life of a particular compound after a single therapeutic dose.

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Various gas chromatographic methods for the determination of flunitrazepam, desalkylflurazepam and temazepam in plasma have been described. These methods either determine the intact drug or the benzophenone derivative, which is obtained upon acidic hydrolysis of benzodiazepines [5—12]. This latter procedure, however, is not very suitable because it gives rise to serious loss of specificity. Recently high-performance liquid chromatographic procedures have been developed for the determination of benzodiazepines in plasma [13, 14]. All methods described, however, are generally rather time consuming and often do not reach the sensitivity needed for a pharmacokinetic study in man after a single therapeutic dose (sensitivity requirements for plasma are desalkylflurazepam 1 ng/ml, flunitrazepam 0.1 ng/ml, and temazepam 5 ng/ml).

Another disadvantage of previously published assay methods for benzodiazepines is the use of benzene for extraction from biological fluids. This solvent is very toxic and its use should be prohibited whenever possible.

Recently an assay method for the determination of underivatized nitrazepam was developed in our institute, using gas chromatography with a support-coated open tubular (SCOT) column, a solid injection system and electron-capture detection [15]. This method has proved to be sensitive, specific and rapid. With some modifications it could also be satisfactorily applied for triazolam and the details of that method are described elsewhere [16]. In this paper the application of a similar gas chromatographic method is presented for temazepam, flunitrazepam and desalkylflurazepam in plasma. Temazepam had to be converted into its O-trimethylsilyl derivative (TMS-temazepam) according to the method of Belvedere et al. [6], whereas flunitrazepam and desalkylflurazepam were determined as unchanged drugs.

EXPERIMENTAL

Drugs and chemicals

Flunitrazepam (Rohypnol, Hoffmann-La Roche), desalkylflurazepam, active metabolite of flurazepam (Dalmadorm, Hoffmann-La Roche), nordiazepam, metabolite of diazepam and diazepam (Valium, Hoffmann-La Roche) were kindly supplied by Hoffmann-La Roche (Basle, Switzerland); temazepam (Levanxol, Farmitalia; Normison, Wyeth) was obtained from Wyeth (Amsterdam, The Netherlands).

For the preparation of the standard solutions, ethanol (p.a. grade) (E. Merck, Darmstadt, G.F.R.) was used; the other organic solvents were freshly distilled (J.T. Baker, Deventer, The Netherlands). For the derivatization of temazepam, N,O-(trimethylsilyl)acetamide (BSA) (Chrompack, Middelburg, The Netherlands), was used.

Extraction procedure

To 1.0 ml of plasma in a centrifuge tube (in the case of temazepam, 0.10 ml of plasma and 0.90 ml of distilled water) were added 50.0 μl of ethanol containing a suitable internal standard (see Table I) and 1.0 ml of 0.2 *M* borate buffer (pH 9.0). After homogenization the sample was extracted twice with 5 ml of pentane—dichloromethane (1:1) for 15 sec on a whirlmixer. Follow-

ing centrifugation for 5 min at 2000 *g* the upper organic layer was transferred to a silanized conical evaporation tube and evaporated to dryness at 50°C under a gentle stream of dry nitrogen. In the case of temazepam, 50 μl of a 40% BSA solution in acetone were subsequently added and evaporated again to dryness (60°C). Finally, the residue was redissolved in 40 μl of ethyl acetate and 2—3 μl of this solution were brought on to the glass-lined needle of the solid injection system [15]. After evaporation of the ethyl acetate, the residue was injected into the gas chromatograph.

Apparatus and chromatographic conditions

A Hewlett-Packard Model 5713A gas chromatograph, equipped with a ^{63}Ni pulse-modified electron-capture detector and a solid injection system was used. A SCOT column (10 m × 0.4 mm I.D.) made of Duran 50 glass was used. The support layer was Tullanox (silanized fumed silica), particle size < 10 μm (Cabot Corp., Boston, MA, U.S.A.), and the stationary phase was 0.5% PPE-21 (Chrompack) and 3% OV-17 (Chrompack). The operating temperatures were injection port 350°C, detector 350°C, and column 215°C. The flow-rate of the carrier gas (helium) was 10 ml/min and that of the auxiliary gas (argon—methane, 95:5) 25 ml/min.

Preparation of calibration graphs

The plasma concentrations of the three benzodiazepines were calculated with the aid of calibration curves, prepared by adding known amounts of the drugs to 1.0 ml (in the case of temazepam, 0.10 ml + 0.90 ml distilled water) of blank plasma. These standard samples were analysed by the described procedure and the ratio of the peak height of the various drugs to internal standard was plotted against the known concentrations of the drugs. The same procedure was followed for the determination of the extraction yield of the three benzodiazepines from plasma at various concentrations except that the compounds used as internal standards (Table I) were now added after extraction (external standards). The ratios obtained were compared with those of standard amounts of the drugs.

TABLE I

INTERNAL STANDARD (I.S.), RETENTION TIMES, STANDARD DEVIATIONS OF CALIBRATION GRAPH AND THE LOWEST MEASURABLE CONCENTRATION

Compound	I.S. (plasma conc.)	Retention time (min)		S.D. of calibration graph (%)	Lowest measurable plasma concentration
		Compound	I.S.		
Desalkylflurazepam	Nordiazepam (25.0 ng/ml)	1.5	1.7	⩽ 2.5	1 ng/ml
TMS-temazepam	Diazepam (25.0 ng per 0.10 ml)	1.7	1.3	⩽ 12.0	0.25 ng per 0.10 ml
Flunitrazepam	Nordiazepam (5.0 ng/ml)	2.2	1.7	⩽ 9.9	0.05 ng/ml

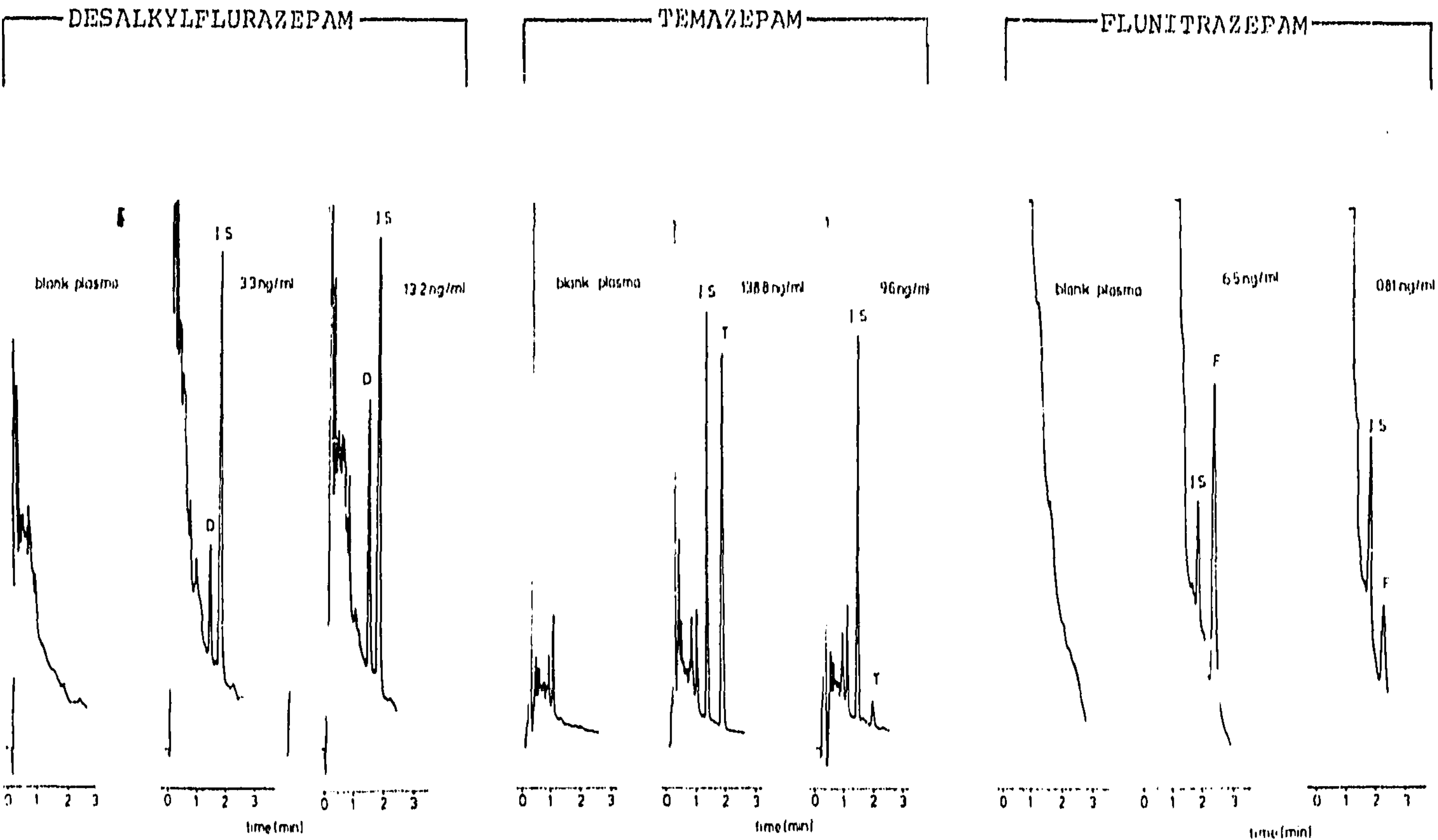

Fig. 2. Gas chromatograms of 1.0-ml (0.10-ml in the case of temazepam) plasma extracts obtained from a volunteer immediately before (left side of each panel) and 1 h (centre of each panel) and 24 h (right side of each panel) after receiving 15 mg of flurazepam, 10 mg of temazepam or 1 mg of flunitrazepam orally. D = desalkylflurazepam (active metabolite of flurazepam); T = temazepam; F = flunitrazepam; I.S. = internal standard.

RESULTS AND DISCUSSION

Assay procedure

Fig. 2 shows gas chromatograms of extracts of plasma samples taken 1 and 24 h after oral ingestion of 15 mg of flurazepam, 10 mg of temazepam and 1 mg of flunitrazepam, as well as gas chromatograms of the corresponding blank extracts. Diazepam was chosen as internal standard for temazepam assay; for both desalkylflurazepam and flunitrazepam, nordiazepam proved to be the most suitable internal standard. Other benzodiazepines were deliberately chosen as internal standards because of the similarity in chemical structure, extraction and chromatographic behaviour. There is a good separation between drug and internal standard, and from the blank chromatograms it appears that no interfering substances are co-extracted.

According to the standard curves there is good linearity between the detector response (peak height drug/peak height internal standard) and the concentration of the compound to be determined in the following ranges: desalkylflurazepam 3.75—25 ng/ml, temazepam 1—25 ng/0.10 ml, and flunitrazepam 0.1—10 ng/ml. The correlation coefficient of such curves was not less than 0.999. Extraction yields determined in the same concentration ranges appeared to be constant and linear with concentration with mean values as follows: desalkylflurazepam 93% (relative standard deviation at each concentration was 6% or less, $n = 4$); temazepam 65% (relative standard deviation at each concentration was 18% or less, $n = 4$) and flunitrazepam 95% (relative standard deviation at each concentration was 12% or less, $n = 4$).

Fig. 3 shows the plasma calibration graphs for the three compounds. For all three benzodiazepines the correlation coeffiecient of each individual curve was greater than 0.999, illustrating the good linearity of the method for each of them. The highest value of the standard deviation observed at each concentration as well as the lowest measurable concentration are given in Table I.

Human experiments

The utility of the present method for the assay of desalkylflurazepam, temazepam and flunitrazepam after a single therapeutic dose was demonstrated in a preliminary study with healthy volunteers. Three healthy female volunteers swallowed 1 mg of flunitrazepam, 15 mg of flurazepam or 10 mg of temazepam with 150 ml of tap water after an overnight fast. The drugs were administered in a cross-over design with intervals of at least three weeks. No food, fluid, or tobacco was allowed for 3 h after drug administration. Blood samples were taken 20, 40, 60, 80 and 100 min and 2, 3, 4, 9, 24, and 48 h after drug administration, for all three drugs, from a forearm vein by means of a flexible venous canula for the first 4 h and subsequently by venous puncture. Additional samples were taken after 6, 12 and 32 h for temazepam, after 6, 32 and 72 h for flunitrazepam, and after 96 and 168 h for flurazepam. Blood clotting was prevented by adding a small drop of heparin solution (5000 I.U./ml) to the samples. After separation, the plasma samples were stored at —20°C until taken for analysis. The plasma concentration profile obtained for one volunteer is shown in Fig. 4. Desalkylflurazepam was already detectable in the first sample taken after flurazepam administration and the concentra-

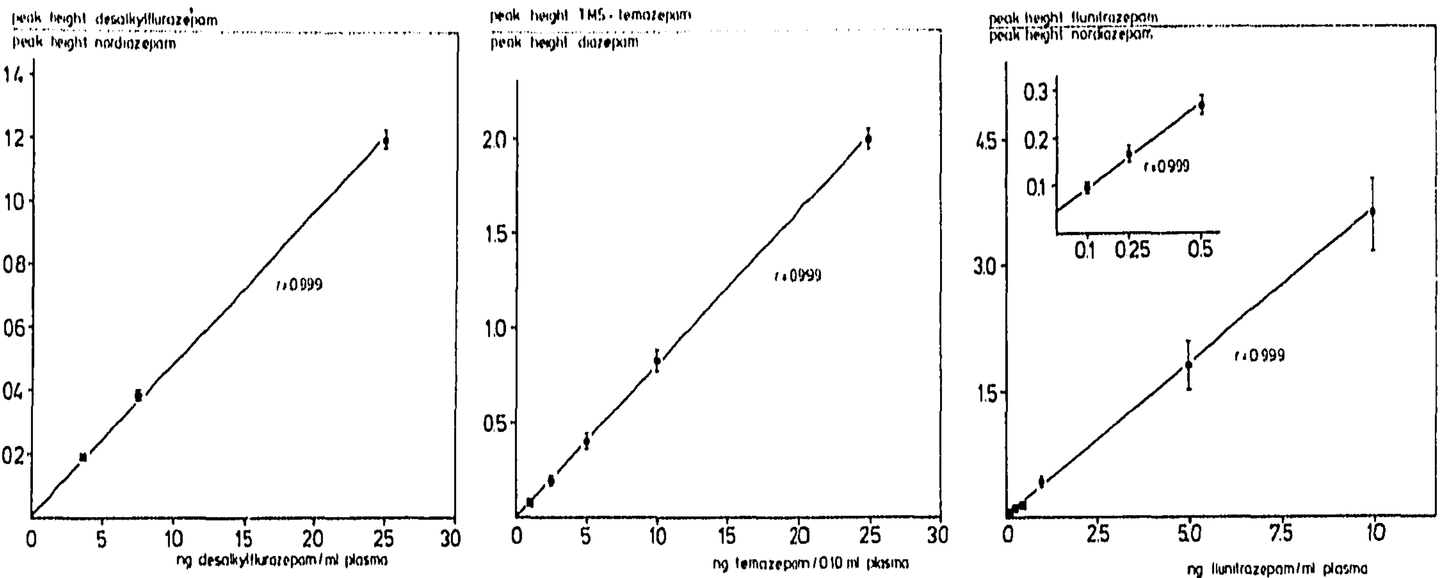

Fig. 3. Calibration graphs of (left) desalkylflurazepam in plasma in the concentration range 3.75—25 ng/ml, (centre) TMS-temazepam in plasma in the concentration range 1—25 ng temazepam per 0.10 ml, and (right) flunitrazepam in the concentration range 0.1—10 ng/ml. Each point represents the mean ± S.D. of four observations. In the case of flunitrazepam, the inset indicates, on an expanded scale, the relationship between the detector response and flunitrazepam at low concentrations of flunitrazepam.

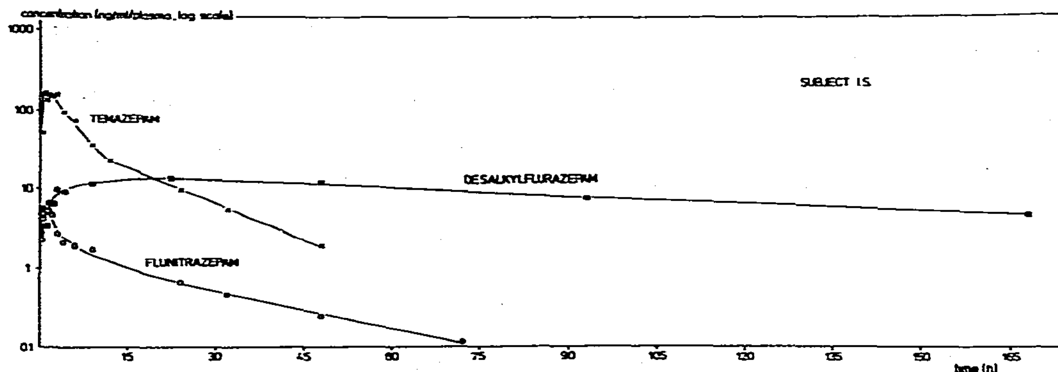

Fig. 4. Plasma level profile on semi-logarithmic scale following oral administration of temazepam (10 mg), desalkylflurazepam (15 mg flurazepam) and flunitrazepam (1 mg) in a healthy volunteer. The drugs were given at intervals of at least 3 weeks.

tion continued to increase slowly until 9—24 h after ingestion. The half-life of desalkylflurazepam was found to be very long (73—97 h) which is in agreement with the values found by Kaplan et al. [1]. Flunitrazepam and temazepam were fairly rapidly absorbed with a mean t_{max} of 1.0 h. After the peak concentration had been reached, there was a rapid decrease in concentration of both drugs, most probably due to distribution of the compounds from plasma into tissues. From the subsequent mono-exponential concentration decay the elimination half-lives could be derived; for temazepam this was 10—21 h and for flunitrazepam 18—49 h (Table II).

TABLE II

PHARMACOKINETIC PARAMETERS* OF DESALKYLFLURAZEPAM (DAF), TEMAZEPAM (Te) AND FLUNITRAZEPAM (FN) FOLLOWING ORAL ADMINISTRATION OF 15 mg DALMADORM, 10 mg LEVANXOL AND 1 mg ROHYPNOL, RESPECTIVELY

Subject	t_{max} (h)			C_{max} (ng/ml)			$t_{½\,el}$ (h)		
	DAF	Te	FN	DAF	Te	FN	DAF	Te	FN
I.S.	24	1.0	1.3	17.4	158.8	5.8	93.0	10.6	18.7
L.S.	9	1.0	1.3	17.5	211.8	6.2	73.4	20.9	48.7
M.R.	24	1.0	0.3	21.7	214.6	6.3	97.1	13.7	17.5

* t_{max} = time of peak concentration, C_{max} = peak concentration, $t_{½\,el}$ = elimination half-life.

CONCLUSIONS

The procedure described for the quantitative determination of desalkylflurazepam, temazepam and flunitrazepam in plasma is rapid and reliable. Furthermore, the sensitivity permits the measurements of the plasma concentrations for at least three times the elimination half-life after therapeutic dosing for all three drugs. From the results of the preliminary study in healthy volunteers it appears that the method is suitable to be applied in pharmacokinetic studies in man after a single therapeutic dose.

REFERENCES

1 S.A. Kaplan, J.A.F. de Silva, M.L. Jack, K. Alexander, N. Strojny, R.E. Weinfeld, C.V. Puglisi and L. Weissman, J. Pharm. Sci., 62 (1973) 1932.
2 G. Wendt, in W. Hügin, G. Hossli and M. Gemperle (Editors), Erfahrungen mit "Rohypnol" (Flunitrazepam) in der Anaesthesiologie und Intensivtherapie, Roche Editioners, Basle, 1976, p. 27.
3 D.D. Breimer, R. Jochemsen and H.-H. von Albert, Arzneim.-Forsch., 30 (1980) 875.
4 E. Wickstrøm, R. Amrein, P. Haefelfinger and D. Hartmann, Eur. J. Clin. Pharmacol., 17 (1980) 189.
5 J.A.F. de Silva, C.V. Puglisi, M.A. Brooks and M.R. Hackman, J. Chromatogr., 99 (1974) 461.
6 G. Belvedere, G. Tognoni and P.L. Morselli, Anal. Lett., 5 (1972) 531.
7 J.A.F. de Silva, I. Bekersky, C.V. Puglisi and R.E. Weinfeld, Anal. Chem., 48 (1976) 10.
8 D.B. Faber, R.M. Kok and E.M. Rempt-van Dijk, J. Chromatogr., 133 (1977) 319.
9 T. Kaniewska and W. Wejman, J. Chromatogr., 182 (1980) 81.
10 M. Hasegawa and J. Matsubara, Chem. Pharm. Bull., 23 (1975) 1826.
11 J.P. Cano, J. Guintrand, C. Aubert and A. Viala, Arzneim.-Forsch., 27 (1977) 338.
12 J.A.F. de Silva and I. Bekersky, J. Chromatogr., 99 (1974) 447.
13 U.R. Tjaden, M.T.H.A. Meeles, C.P. Thijs and M. van der Kaay, J. Chromatogr., 181 (1980) 227.
14 T.B. Vree, A.M. Baars, Y.A. Hekster and E. van der Kleijn, J. Chromatogr., 162 (1979) 605.
15 A.G. de Boer, J. Röst-Kaiser, H. Bracht and D.D. Breimer, J. Chromatogr., 145 (1978) 105.
16 R. Jochemsen and D.D. Breimer, J. Chromatogr., 223 (1981) 438.