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Note

Determination of flunitrazepam in body fluids by means of high-performance liquid chromatography

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Flunitrazepam, a benzodiazepine tranquillizer, is used in anesthesia and in intensive care units in hospitals. Owing to its very strong action, only small doses (0.010—0.030 mg/kg) can be administered to man, so the plasma concentration is to be expected very low. De Silva and co-workers [1, 2] reported effective plasma concentrations of 1—10 ng/ml, measured by means of gas chromatography with electron-capture detection.

The compound is extensively metabolized by N-demethylation to N-desmethylflunitrazepam and by reduction to its amino derivative (aminoflunitrazepam). Both metabolites may be present in blood and excreted in a conjugated form or as the unbound compound in the urine.

For the study of the pharmacokinetics of the drug and of effect—plasma concentration relationships, and for plasma monitoring during or following anesthesia and surgery, a method should be available that requires only a relatively short time for extraction and analysis and that permits the detection of plasma concentrations as low as 1 ng/ml.

In this paper, the determination of flunitrazepam and its metabolites by means of high-performance liquid chromatography is described.

EXPERIMENTAL

Apparatus

A Spectra Physics 3500B high-performance liquid chromatograph was used. The column, 25 cm × 3 mm I.D., was packed with Spherisorb S-W10 (particle size 10 μm) obtained from Chrompack (Middelburg, The Netherlands). An injection loop of 100 μl was used. Detection was effected at 230 nm. The solvents were *n*-hexane + 5% of ethanol or *n*-hexane + 3% of ethanol, and the solvent flow-rate was 2 ml/min.

Drugs

Flunitrazepam, desmethylflunitrazepam (Ro 05-4435/000) and aminoflunitrazepam (Ro 20-1815/601) were obtained from Hoffmann-La Roche (Mijdrecht, The Netherlands) by the courtesy of Dr. J. Kuitert.

Subjects and animals

Patients undergoing surgery were administered flunitrazepam intravenously in a dose of 0.010 mg/kg. Blood samples were taken at regular time intervals.

Labrador dogs (Central Animal Laboratory, Nijmegen) were administered 0.10 mg/kg of flunitrazepam intravenously. Blood and urine samples were collected frequently.

Extraction

To 1 ml of plasma (pH 7.4) are added 10 μl of internal standard (diazepam, 5 ng/μl) and 2 ml of fresly distilled *n*-hexane, and the mixture is mixed on a Vortex mixer. The hexane layer is removed and the plasma is extracted a second time with 2 ml of *n*-hexane. The combined hexane layers are evaporated to dryness and the residue is dissolved in 200 μl of *n*-hexane, 100 μl of the resulting solution being injected into the chromatograph.

Before each series of determinations a calibration graph is constructed. The recovery of the extraction is 75 ± 3%.

RESULTS

Flunitrazepam was well separated from its metabolites and related benzodiazepines (Table I and Fig. 1). After extraction and separation, no measurable amounts of the metabolites desmethylflunitrazepam and aminoflunitrazepam could be found in the plasma of dog and man after one single intravenous injection.

Flunitrazepam shows a biphasic elimination in the dog with a half-life of 15 min for the α-phase and 150 min for the β-phase. The volume of distribution of the β-phase is calculated to be 5 l/kg. A dose of 0.12 mg/kg (2.5 mg i.v.) in dogs results in an apparent maximum plasma concentration for the β-phase ($C_0\beta$) of 20 ng/ml. In man, following a dose of 0.010 mg/kg, the $C_0\beta$ value in plasma is 10 ng/ml.

A typical chromatogram of flunitrazepam, obtained after extraction of a plasma sample from a dog, is shown in Fig. 2. Peaks I and II are not observed in plasma from man. The relative retention times of these peaks are I=0.64

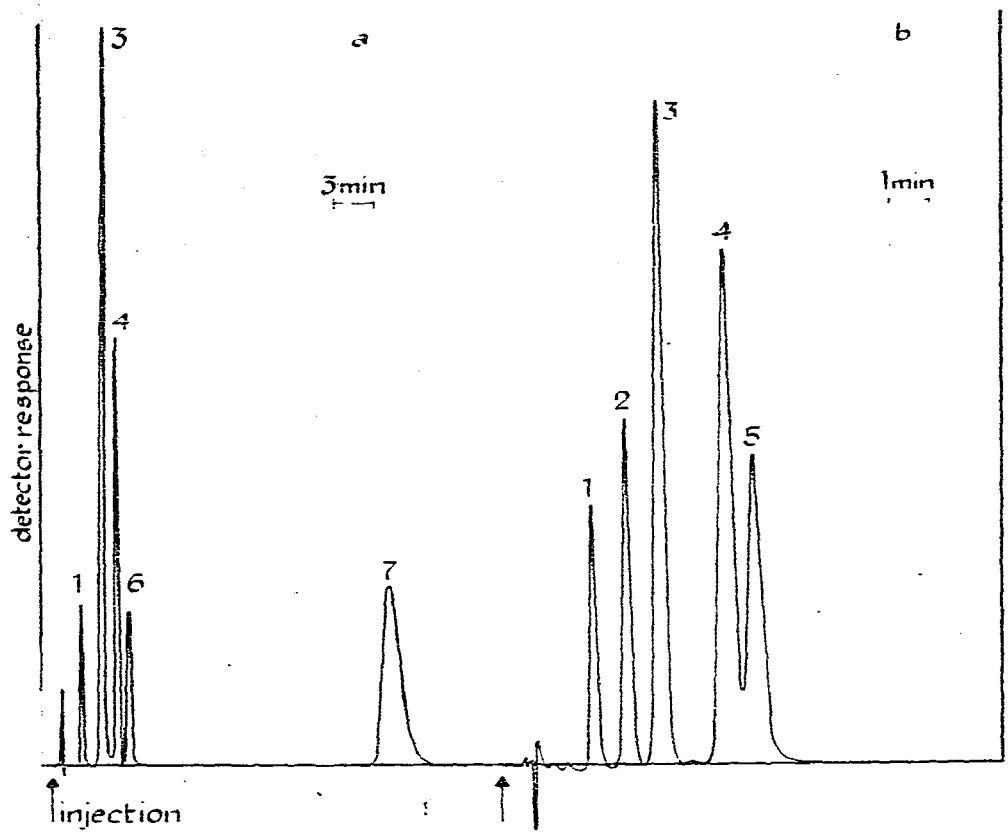

Fig. 1. Chromatogram of diazepam (1), desmethyldiazepam (2), flunitrazepam (3), nitrazepam (4), clonazepam (5), desmethylflunitrazepam (6) and aminoflunitrazepam (7). Column, Spherisorb S-W10; flow-rate, 2 ml/min; pressure, 34 atm. Eluent: (a) *n*-hexane—5% ethanol; (b) *n*-hexane—3% ethanol.

TABLE I

RELATIVE RETENTION TIMES OF FLUNITRAZEPAM AND VARIOUS BENZODIAZEPINES

Compound	Relative retention time
I*	0.64
diazepam	1.00
desmethyldiazepam	1.39
II*	1.42
flunitrazepam	1.66
nitrazepam	2.38
desmethylflunitrazepam	2.50
clonazepam	2.78
aminoflunitrazepam	10.83

* Peak in Fig. 2.

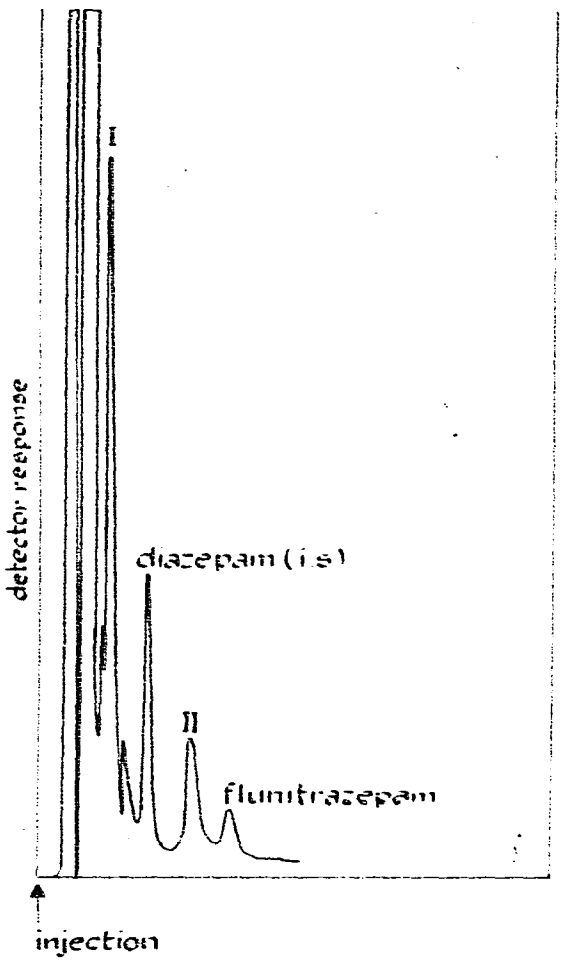

Fig. 2. Chromatogram of flunitrazepam and diazepam (internal standard) after extraction of a plasma sample from a dog. Peaks I and II do not appear in plasma from man. Column, Spherisorb S-W10; flow-rate, 2 ml/min; eluent, *n*-hexane—3% ethanol.

and II = 1.42 relative to diazepam = 1.00. Peak II almost coincides with desmethyldiazepam (Table I).

DISCUSSION

The method described permits the determination of plasma concentrations of flunitrazepam as los as 1 ng/ml, and is therefore comparable with that of De Silva and co-workers [1, 2]. The sensitivity of the method (10—1 ng/ml) is sufficient for most pharmacokinetic studies and routine determinations of plasma concentrations associated with anesthesia.

The column of Spherisorb S-W10 permits the separation of various benzodiazepines that may be used as co-medicants or for the induction of anesthesia,

e.g., diazepam (Valium), nitrazepam (Mogadon) and clonazepam (Rivotril). Two of the metabolites of diazepam, oxazepam (Seresta) and oxydiazepam (Temazepam), are not eluted from this column. In those instances, in routine determinations of flunitrazepam when it is not known if co-medication has been applied, a second extraction without internal standard must be made in order to avoid erroneous results.

REFERENCES

1 J.A.F. de Silva and I. Bekersky, J. Chromatogr., 99 (1974) 447.
2 J.A.F. de Silva, C.V. Puglisi and N. Munno, J. Pharm. Sci., 63 (1974) 520.