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Note

Rapid quantitation of flurazepam and its major metabolite, N-desalkylflurazepam, in human plasma by gas—liquid chromatography with electron-capture detection

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Flurazepam is a 1,4-benzodiazepine widely used as a hypnotic agent [1, 2]. Some investigations have provided evidence that flurazepam is extensively metabolized to derivatives that show a pharmacological activity like the parent drug.

The major metabolite, N-desalkylflurazepam, shows a plasma concentration in man after flurazepam administration that is at least 50 times as high as the flurazepam concentration. Moreover, the elimination half-life of this metabolite is about 47—100 h, while the elimination half-life of flurazepam is very short [3, 4]. Therefore it is possible that in the evaluation of eventual correlations between plasma levels and clinical effects of this drug, the concentrations of both flurazepam and N-desalkylflurazepam must be carefully considered.

For these reasons, we have developed a rapid and sensitive gas chromatographic method for the determination of plasma concentrations of flurazepam and N-desalkylflurazepam in man using electron-capture detection (ECD).

EXPERIMENTAL

Reagents and standards

Flurazepam and N-desalkylflurazepam were obtained from Robin Co. (Milan, Italy), clobazam (the internal standard) was from Hoechst (Milan, Italy), benzene and acetone from Merck (Darmstadt, G.F.R.) and buffer solution (sodium tetraborate, pH 9.00) from Riedel De Haen (Hannover, G.F.R.).

Apparatus

A Carlo Erba Fractovap 2150 gas chromatograph equipped with a ^{63}Ni elec-

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tron-capture detector, an ECD control Model 250 operating at a constant current, and a Hewlett-Packard 3380A recorder—integrator were used. The glass column (1 m × 3 mm I.D.) was pre-treated with dimethyldichlorosilane (Carlo Erba, Milan, Italy) and packed with 3% OV-17 on 100—120 mesh Gas-Chrom Q (Applied Science Labs., State College, PA, U.S.A.). The injector temperature was 300°C, column temperature 250°C, detector temperature 300°C, and the flow-rate of the carrier gas (nitrogen) was 50 ml/min.

Standard solutions

Stock solutions of flurazepam, N-desalkylflurazepam and clobazam were prepared in acetone to give a concentration of 1 mg/ml for each compound. Plasma standards of 5, 10, 25, 50, 100, 200 and 400 ng flurazepam and N-desalkylflurazepam per millileter of plasma (calibration samples) were prepared by adding exact volumes of standard solutions of 50 ng/ml of both compounds to drug-free pooled plasma. Plasma standards were then divided into 1-ml samples, stored at 4°C and analyzed within two weeks.

Extraction procedure

To 1-ml plasma standards, 0.5 ml of buffer solution and 4 ml of benzene were added. The test-tubes were mechanically shaken for 10 min, then centrifuged and the organic phase was evaporated to dryness with a vacuum evaporator at 45°C. The residue was redissolved in variable volumes (100—800 μl) of internal standard solution (clobazam, 0.5 ng/ml in acetone) as specified in the Results and discussion section. One or two microliters of this solution were injected into the gas chromatograph.

RESULTS AND DISCUSSION

The correlation between the peak area ratios (drugs/internal standard) and plasma concentrations when all extracts were redissolved in 100 μl of internal standard solution is shown in Fig. 1. The graphs show a non-linear correlation between detector response and plasma concentration over 50 ng/ml (about

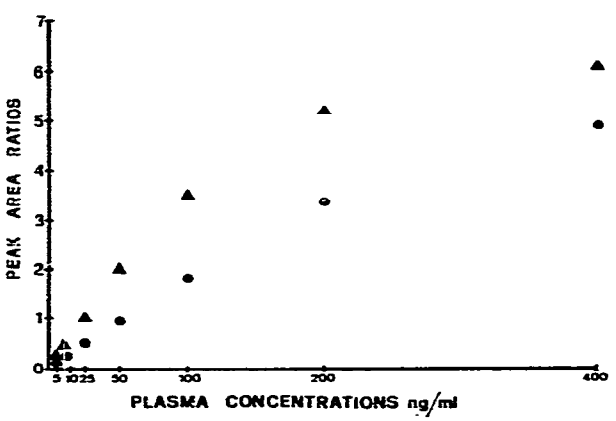

Fig. 1. Correlation between reading and plasma concentrations of flurazepam (●) and N-desalkylflurazepam (▲) in calibration samples obtained by redissolving the residues in 100 μl of internal standard solution.

500 pg injected into the gas chromatograph). In order to obtain a linear correlation between plasma concentration and detector response, new calibration curves with further dilutions of extracts from plasma at initial concentrations of both compounds over 50 ng/ml were made. Extracts of plasma standards at concentrations (for both substances) of 100, 200 and 400 ng/ml were redissolved, respectively, in 200, 400 and 800 μl of internal standard solution, while extracts at concentrations of 5, 10, 25 and 50 ng/ml were redissolved in 100 μl of internal standard solution as described before. The curves so obtained are shown in Fig. 2.

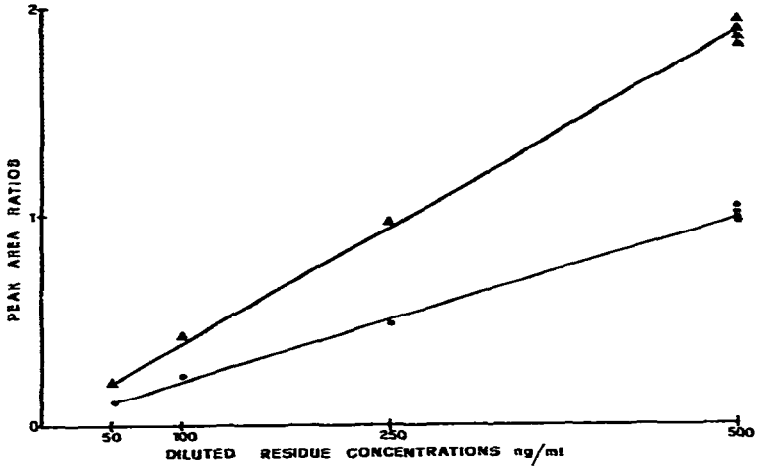

Fig. 2. Calibration curves for flurazepam (•) and N-desalkylflurazepam (▲). The residues obtained from the calibration series were redissolved in different volumes of internal standard solution in order to inject compound quantities within the linear range (see text for explanation).

A previous study [3] utilizing a spectrofluorimetric method has demonstrated that after a single oral dose (30 mg) in man, peak plasma concentrations of N-desalkylflurazepam range from 10 to 20 ng/ml, and that after two weeks of treatment (30 mg daily) plasma concentrations rose to 49—142 ng/ml; flurazepam plasma concentrations were below the sensitivity limit of the assay (3—4 ng/ml) throughout the study.

Thus it is possible that, when using our method, clinical samples containing unknown quantities of the analytes must be injected and read twice: for flurazepam after a first redissolution into 100 μl of internal standard solution and for N-desalkylflurazepam after a further redissolution into a multiple volume of internal standard solution depending on the first reading. This procedure of injecting total concentrations lower than 500 pg and thus in the linear range of the curve, seems to be preferable to the use of two different internal standards at different concentrations or to the other possible procedures. Readings are finally corrected for the dilution.

Fig. 3 shows representative chromatograms of two extracts from calibration curve samples. Plasma concentrations of flurazepam and its metabolite were 10 ng/ml of each (A) and 400 ng/ml of each (B); the relative residues were re-

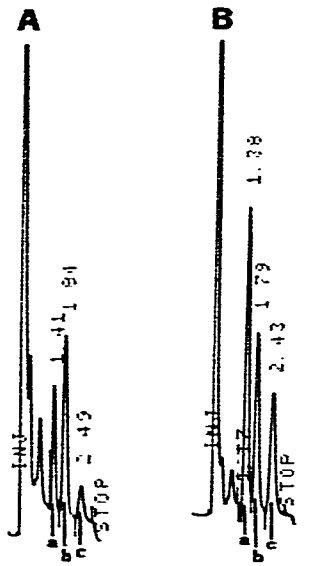

Fig. 3. Gas chromatographic response of two calibration samples. (A) Extract from 1 ml of plasma containing 10 ng of each drug redissolved in 100 μl of internal standard solution. (B) Extract from 1 ml of plasma containing 400 ng of each drug redissolved in 800 μl of internal standard solution. a= N-desalkylflurazepam; b= clobazam; c= flurazepam.

TABLE I

RECOVERY AND REPRODUCIBILITY FROM HUMAN PLASMA SAMPLES

Amount added	Recovery* (% ± S.D.)		Reproducibility** (amount found ± S.D.) (C.V.)	
	Flurazepam	N-Desalkyl-flurazepam	Flurazepam	N-Desalkyl-flurazepam
5	90 ± 3.8	84 ± 4.1	4.9 ± 0.30 (6.0)	5.0 ± 0.31 (6.2)
10	91 ± 3.2	83 ± 3.9	9.9 ± 0.50 (5.0)	10.1 ± 0.54 (5.4)
25	89 ± 3.5	86 ± 4.0	25.1 ± 1.51 (6.0)	24.9 ± 1.60 (6.4)
50	92 ± 4.0	81 ± 3.8	49.0 ± 2.60 (5.2)	50.1 ± 3.25 (6.5)
100	89 ± 3.8	83 ± 3.9	102.0 ± 2.4 (4.8)	100.1 ± 7.11 (7.1)
200	93 ± 3.9	84 ± 4.1	203.2 ± 8.9 (4.4)	199.2 ± 13.11 (6.5)
400	93 ± 4.1	83 ± 4.0	404.1 ± 17.1 (4.3)	400.3 ± 22.31 (5.6)

* Mean of five determinations.
** Mean of ten determinations performed over a two-week period.

dissolved, respectively, in 100 μl and 800 μl of internal standard solution so that the quantities injected were in the linear range.

The minimal detectable concentration of flurazepam and its metabolite was 1 ng/ml of plasma.

The recovery and reproducibility of the method at seven different concentrations are shown in Table I.

To determine the potential usefulness of the procedure, we checked for possible interferences from other benzodiazepines (diazepam, 3-hydroxydiazepam, N-desmethyldiazepam, oxazepam, clonazepam, flunitrazepam, lorazepam, nitrazepam and chlordiazepoxide) by chromatographing solutions of pure standards. We did not observe any interference in the chromatograms that might have altered the flurazepam and N-desalkylflurazepam analyses.

From the reported results we consider that the method is sufficiently rapid, sensitive and specific for utilization in pharmacological studies in man.

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