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Note

Simplified gas chromatographic assay of underivatized nitrazepam in plasma

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Nitrazepam (Mogadon) (Fig. 1) is a 7-nitro benzodiazepine derivative extensively used as a hypnotic agent [1]. Gas chromatography (GC) with electron-capture detection has been successfully used for quantitation of nitrazepam in plasma [2—5]. However, most prior methods have required either derivatization (alkylation) or hydrolysis of nitrazepam prior to assay [2, 3], or the use of capillary column chromatography [4, 5]. This is because intact nitrazepam has inherently poor chromatographic properties, leading to peak tailing and adsorption due to its amide character [3]. This paper describes a rapid and sensitive GC analysis of intact nitrazepam in plasma, which is suitable for single-dose studies in humans.

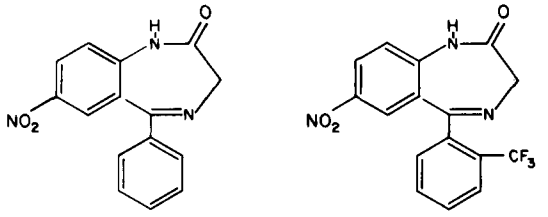

Fig. 1. Structural formulae of nitrazepam (NTZ) and the internal standard, trifluoromethyl-nitrazepam (TFM-NTZ).

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EXPERIMENTAL

Instrumentation

A Hewlett-Packard 5830A or 5840A gas chromatograph equipped with a 15-mCi ^{63}nickel electron-capture detector, data processing module, and automatic sampler was used for analysis. The column was 1.22 m × 4 mm I.D. coiled glass packed with 1% OV-17 on 80—100 mesh Chromosorb W HP (Hewlett-Packard, Avondale, PA, U.S.A.). The chromatographic conditions were as follows: oven temperature, 275°C; injection port temperature, 310°C; detector temperature, 310°C; carrier flow-rate, 50 ml/min. The carrier gas was argon—methane (95:5) obtained from Matheson (Gloucester, MA, U.S.A.).

Reagents and solutions

Stock standards of nitrazepam and the internal standard trifluoromethyl-nitrazepam (TFM-NTZ, Ro5-3590) (Fig. 1), both kindly supplied by Hoffmann-LaRoche (Nutley, NJ, U.S.A.), were prepared by weighing 10 mg of each compound and dissolving it in 100 ml of methanol. The working standards of 1.0 μg/ml nitrazepam and 1.0 μg/ml TFM-NTZ were made by appropriate dilution of each compound with methanol. Both stock and working solutions were stored in amber ground-glass stoppered bottles at 4°C and were stable for at least one year.

A borate buffer was prepared by mixing 500 ml of 0.025 *M* sodium borate (9.53 g/l) with 177 ml of 0.1 *M* hydrochloric acid and adjusting the pH to 8.3.

A priming agent was made by dissolving 20 mg of purified soy phosphatides (asolectin) in 10 ml of benzene. A 2—3 μl aliquot of the agent was injected into the column prior to each day's run.

Method

TFM-NTZ (10 ng) was added to a series of round-bottom 125 × 13 mm screw-top culture tubes. Calibration standards were prepared by addition of 5, 10, 25, 50, 75 and 100 ng of nitrazepam to a series of these tubes. Drug-free blank serum or plasma (0.5 ml) was added to the calibration tubes and 0.5—1.0 ml of unknown serum or plasma added to all other tubes. Borate buffer (1 ml) was added to each tube, and briefly vortexed to mix. All samples were then extracted with 3 ml of benzene—dichloromethane (80:20) by agitation on an automatic vortex-mixer (Kraft Apparatus, Minolea, NY, U.S.A.) in the upright position for 1 min. They were then centrifuged for 15 min at 400 *g*, and the organic layer pipetted into 2-ml Wheaton automatic sampling vials, and evaporated to dryness under conditions of heat (40°C) and mild vacuum. The residue was reconstituted with 200 μl of toluene—isoamyl alcohol—asolectin solution (84:14:3), and the vials capped with aluminium foil. A 6-μl aliquot was injected onto the column using the automatic sampler.

Clinical pharmacokinetic study

A 24-year-old male received a 10-mg oral dose of nitrazepam after giving informed consent. Venous blood samples were drawn into additive-free tubes at 15, 30, 45, 60 min and 1.5, 2.0, 2.5, 3, 4, 6, 8, 12, 24, 36, 48, 60 and 72 h

post dosage. The samples were allowed to clot, and the serum was separated and frozen until the time of assay. Concentrations of nitrazepam in all samples were determined as described above.

Pharmacokinetic parameters of nitrazepam distribution, elimination and clearance were determined by model-independent techniques described in detail previously [6].

RESULTS

Evaluation of method

Using the described chromatographic conditions, nitrazepam and TFM-NTZ gave two well resolved and well defined peaks (Fig. 2). The relation of peak height ratio versus the plasma nitrazepam concentration was linear and passed through the origin. Replicate samples (n = 8) of standards containing 10, 25, 50 and 100 ng/ml nitrazepam yielded within-day coefficients of variation of 5.8%, 4.3%, 6.2% and 5.2%, respectively. The between-day coefficient of variation for the slope of calibration curves run on twelve different days was 8.0%. The limit of sensitivity for nitrazepam is approx. 3—5 ng/ml. Residue analysis indicated greater than 90% recovery.

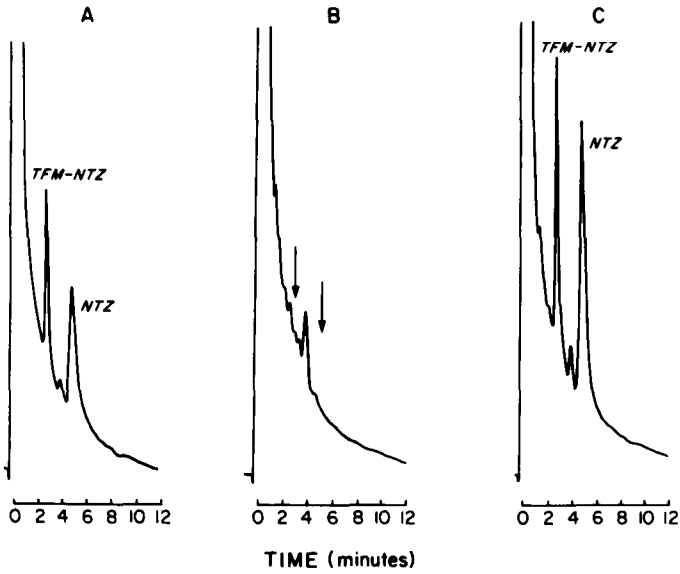

Fig. 2. Chromatograms of (A) a calibration standard containing 10 ng/ml trifluoromethylnitrazepam (TFM-NTZ) and 50 ng/ml nitazepam (NTZ); (B) a drug-free blank serum sample; (C) a serum sample from a subject drawn 12 h after a single 10-mg dose of nitrazepam.

Pharmacokinetic results

Fig. 3. shows serum nitrazepam concentrations in the volunteer subject. Pharmacokinetic parameters for nitrazepam were: elimination half-life, 28 h; total volume of distribution, 297 l; total clearance, 124 ml/min.

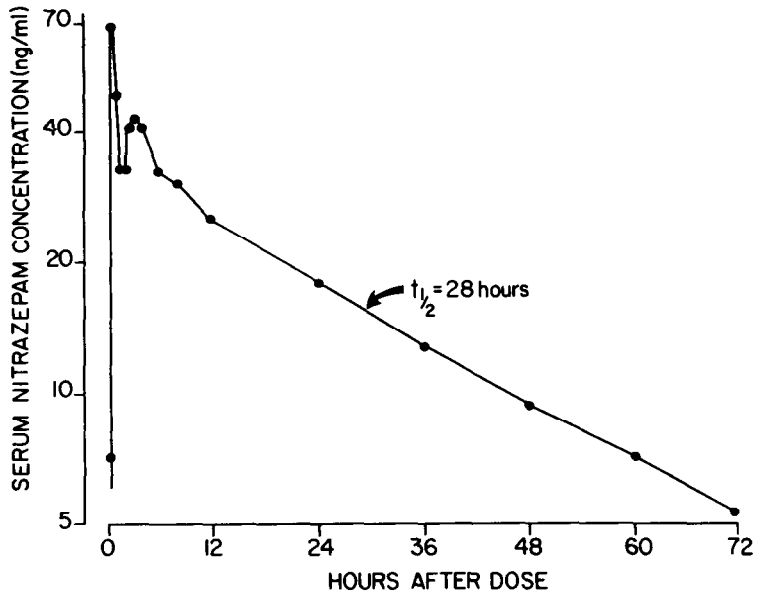

Fig. 3. Serum nitrazepam concentrations in the volunteer subject.

DISCUSSION

The described method allows for the sensitive and reliable quantitation of underivatized nitrazepam in human serum or plasma. After addition of the internal standard having a structurally similar amide configuration, both nitrazepam and the internal standard are extracted into an organic solvent at a slightly alkaline pH. After evaporation and reconstitution of the organic phase, the sample is ready for injection into the gas chromatograph. Although nitrazepam and the internal standard have inherently poor chromatographic properties, use of a low phase load (1%) of column packing, high column temperatures (275° C), and concurrent injection of the lipoidal priming material along with each sample yields narrow peaks and reproducible chromatography of nitrazepam. Drug-free plasma is free of contaminating endogenous substances, thereby eliminating the need for clean-up procedures.

The method is applicable to single-dose pharmacokinetic studies of nitrazepam in humans [7]. Results of the present study in the volunteer subject are consistent with previous studies of nitrazepam pharmacokinetics [8].

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