Improved Micromethod for Determination of Underivatized Clonazepam in Serum by Gas Chromatography

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We describe a gas-chromatographic micromethod, with use of a solid injection system and an electron capture detector, for determination of underivatized clonazepam in serum. Vigorous mixing of 100 μl of serum with 20 μl of borate buffer (pH 9.0) and 100 μl of a cyclohexane/ dichloromethane mixture (4/6 by vol) containing methyl nitrazepam as internal standard suffices to extract 86% of the clonazepam into the organic layer. We obtained a linear response curve for clonazepam in serum in the concentration range 5 to 150 μg/liter. The lowest detectable concentration by our method is about 1 μg/liter of serum. Interference by several anticonvulsant drugs, metabolites, and related compounds was investigated. The assay is simple, rapid, and suitable for clinical routine determination of clonazepam in serum in therapeutic concentrations. We illustrate the steady-state concentration of clonazepam in serum during chronic oral administration to 28 patients.

Clonazepam [5-(2-chlorophenyl)-1,3-dihydro-7-nitro-2H-benzodiazepin-2-one; Rivotril, Ro 5-4023] (Figure 1) is clinically effective in the treatment of various types of epilepsy (1–3). For the quantitation of clonazepam, thin-layer chromatographic (4), gas-chromatographic (5–14), radiometric (15), radioimmunoassay (16, 17) and high-performance liquid chromatographic (18, 19) methods have been described. Most of the gas-chromatographic assays are based on indirect determination of clonazepam, after hydrolysis, as 2-amino-5-nitro-2'-chlorobenzophenone (5–9), or, after alkylation, as its alkylated derivative (10, 11). Although a determination of clonazepam as the intact drug (5) proved unfeasible in the hands of Knop et al. (7), Gerna and Morselli (13) described such a method. De Boer et al. (14) developed an assay procedure for underivatized nitrazepam and clonazepam in plasma, using gas chromatography with a support-coated open tubular column. Nitrazepam is used as internal standard in the assay of clonazepam, and vice versa.

Our purpose was to develop a simpler and faster determination of clonazepam, suitable for clinical routine and sufficiently specific in relation to the common co-medication.

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Materials and Methods

Chemicals

All chemicals were of analytical grade. Dichloromethane, cyclohexane, sodium bicarbonate, orthoboric acid, and potassium chloride were purchased from Merck.

H_3BO_3–Na_2CO_3–KCl buffer solution (1 mol/liter, pH 9.0) was prepared as described elsewhere (20).

Stock solutions of clonazepam and methyl nitrazepam (kindly supplied by Hoffmann-La Roche, Mijdrecht, The Netherlands) were prepared in ethanol to give a concentration of 1 g/liter for each compound. Serum standards of 5, 10, 25, 50, 75, 100, and 150 μg clonazepam per liter were prepared by adding exact volumes of a standard solution of 40 mg/liter clonazepam to drug-free pooled serum. Serum standards were divided into 100-μl samples, transferred to Teflon-lined screw-capped conical tubes, and frozen at −20 °C.

Glassware

All glassware was washed with laboratory detergent (Liquinox; Alconox Inc., New York, N.Y. 10003), cleaned with dichromate–sulfuric acid, rinsed with tap and bi-distilled water successively, and dried.

Apparatus

We used a gas chromatograph (Packard-Becker, Model 419) equipped with a [63]Ni electron capture detector and linearizer Model 736. The solid injection system was a pyrolysis system [Becker, Model 767, modified according to Driessen and Emonds (21)]. A 0.60-m glass column (1.2 mm i.d.) was packed with 3% OV-17 on Supelcoport 210–250 μm (60–70 mesh). This column was conditioned overnight at 255 °C with a carrier gas (N_2) flow of 7 ml/min. The analysis was carried out isothermally with the oven temperature at 245 °C, the injection port kept at 305 °C, and the detector at 345 °C. Carrier gas flow rate was 7 ml/min and the detector purge (N_2) 25 ml/min. The chromatogram was registered on a flat-bed recorder (Packard, Model 610) at a chart speed of 1 cm/min.

Patients' Sera

Blood from patients receiving oral therapy with clonazepam was drawn just before the first morning dose. Serum was separated as soon as possible, immediately frozen to prevent
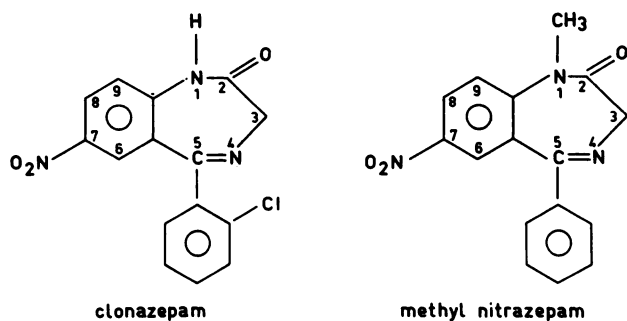
Fig. 1. Structure of clonazepam and methyl nitrazepam (internal standard)

decomposition of clonazepam (7), and stored at −20 °C until analyzed.

Procedure

To 100 μl of serum (standard or patient) in conical tubes (10 ml, 10 × 110 mm) provided with Teflon-lined screw-caps, add with an automatic pipettor (Clinipett, Labora Mannheim) 20 μl of borate buffer (pH 9.0) and 100 μl of a cyclohexane/dichloromethane mixture (4/6 by vol) containing 50.0 μg of methyl nitrazepam (internal standard) per liter. After thorough vortex-mixing for 30 s, centrifuge the tubes at 3000 rpm for 2 min. Pass the needle of a 10-μl gas-chromatographic syringe through the aqueous (upper) phase and the precipitated protein interface into the organic phase and aspirate 10 μl of the extract into the syringe. Wipe the needle with a tissue, and transfer the solution via the needle of the solid gas-chromatographic injection system. After evaporation of the solvent, inject for 15 s.

Repeat some injections of extracts of blank serum before analysis, to obtain peaks that are more gaussian. Construct a calibration curve of the peak height ratio of clonazepam to methyl nitrazepam (internal standard) vs. the concentration of clonazepam per liter of serum. For all series of analyses, a new calibration curve must be prepared to establish the reproducibility of the system.

Results and Discussion

The micro-extraction procedure is a modification of the procedure of Flanagan and Berry (22) for determination of barbiturates. We found this method to be applicable not only to microgram but also to nanogram amounts. The principle demands a higher density of the extraction liquid relative to the serum–buffer combination. In the determination of clonazepam some serum components may interfere if extracted with the solvent (chloroform) used by Flanagan and Berry. A cyclohexane/dichloromethane mixture proved to be appropriate. Methyl nitrazepam is a suitable internal standard for determination of clonazepam, because it is not used as a drug and its structure is comparable with that of clonazepam (Figure 1).

We obtained a linear response-curve in the concentration range of 5–150 μg of clonazepam per liter of serum ($y = 0.00629x +0.0003$, $r = 0.9997$) by comparing the ratio of peak heights of clonazepam to methyl nitrazepam for different concentrations. If we transfer 2 μl instead of 10 μl of extract onto the needle of the solid injection system, we can extend this linear range to 0.5 mg of clonazepam per liter.

The accuracy and the intra-assay precision of the method were determined by performing 20 replicate assays of two standards, one with 10 and the other with 100 μg of clonazepam per liter of serum. The mean values obtained were 11.0 (CV, 8.7%) and 99.2 (CV, 4.3%) μg/liter, respectively. Inter-assay precision was determined by performing, on different
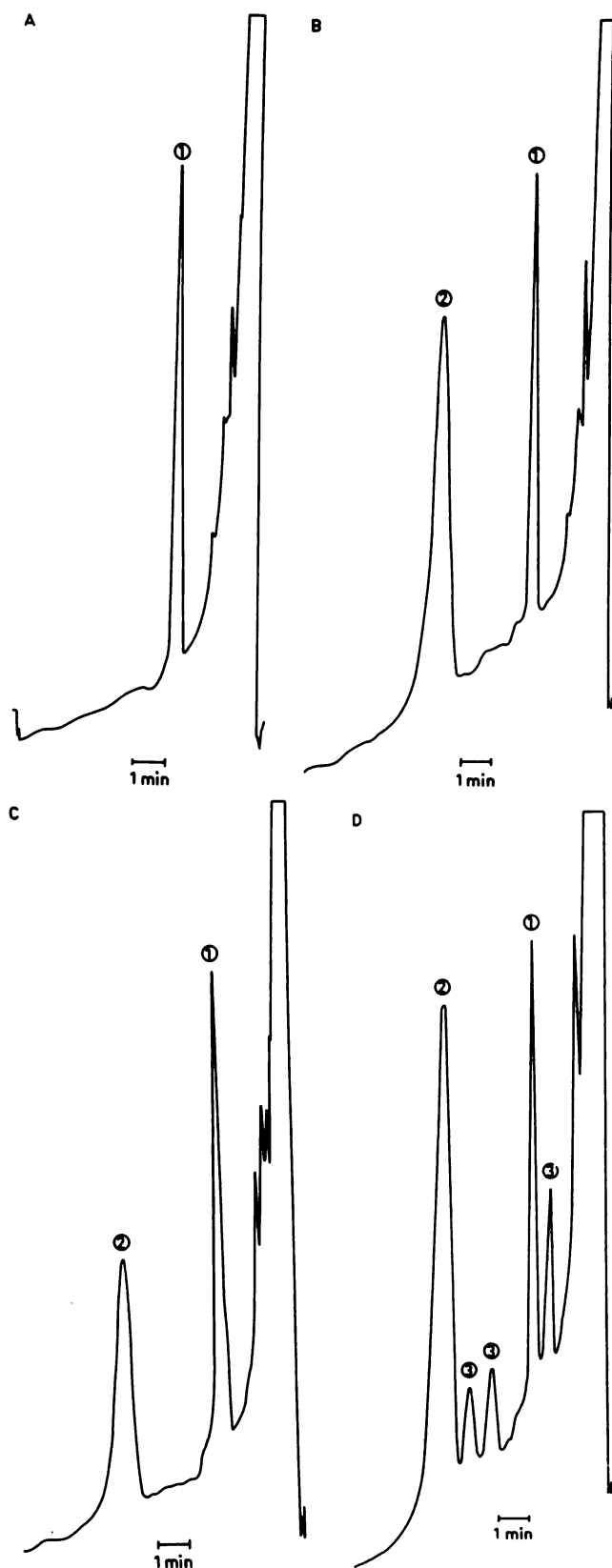

Fig. 2. Gas chromatograms after extraction from serum with cyclohexane/dichloromethane (4/6 by vol), containing methyl nitrazepam as internal standard

The peaks shown are: (1) methyl nitrazepam (internal standard), attenuation × 128; (2) clonazepam, attenuation × 32; (3) unidentified metabolites or fragments of carbamazepine

(A) Extract of drug-free serum; (B) extract of drug-free serum after addition of clonazepam 50 μg/liter; (C) extract of serum of a patient receiving clonazepam; (D) extract of serum of a patient receiving clonazepam and carbamazepine
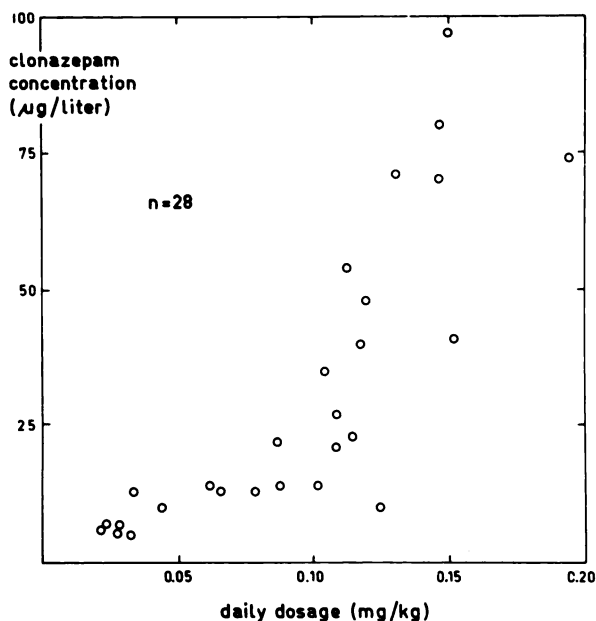
Fig. 3. Relationship between clonazepam dosage and its concentrations in serum of 28 patients on chronic oral medication with clonazepam and a variety of co-medication

days during six months, 11 replicate assays of a control sample containing 51.6 μg of clonazepam per liter of serum. The mean value was 51.9 (CV, 5.7%) μg/liter.

Clonazepam could be extracted (7) with a good recovery from plasma in the pH range of 3–10. We chose pH 9.0, but we found no significant difference in accuracy or intraday precision when we extracted at pH 4.0. A second sample from the same tube gave results similar to those for the first sample, We found that the variation can mainly be attributed to pipetting error.

The lowest amount detectable by the method, determined as the concentration of clonazepam necessary to produce a signal twice as large as the value of the background noise, is about 1 μg/liter.

The combination of a short, semi-capillary column with the solid injection system makes it possible to obtain peaks with a minimum of tailing at a relatively low oven temperature. The chromatograms in Figure 2 show the satisfactory properties of our column in analysis for clonazepam. Peaks due to extractable serum compounds, components of the O-rings of the solid injection system, and/or impurities of reagents give no interference in analyses. There is sufficient difference in retention time between clonazepam and its relevant metabolites, 7-amino clonazepam, 7-acetamido clonazepam, and 3-hydroxy clonazepam. Except in the plasma of patients with a high clonazepam concentration, the pharmacologically active metabolite, 3-hydroxy clonazepam, was known to be present in very low concentrations or absent (7).

Other anticonvulsant drugs, such as sodium valproate, primidone, phenytoin, phenobarbital, ethosuximide, and carbamazepine and their metabolites, do not interfere in the clonazepam procedure. Knop et al. (7) described interference caused by an unidentified metabolite or fragment of carbamazepine in the plasma of patients receiving carbamazepine co-medication. In the serum of patients with carbamazepine co-medication we also often observed unknown peaks. Since, as Figure 2D shows, the last peak of this series can easily interfere with the clonazepam peak, a column with sufficient resolving power is required. This interference increases as the column ages, because the retention time of clonazepam relative to methyl nitrazepam is decreased.

Related compounds, such as diazepam, desmethyl diazep-

am, 3-hydroxy diazepam, oxazepam, lorazepam, medazepam, nitrazepam, bromazepam, chlordiazepoxide, and flurazepam, do not interfere with clonazepam. Only flurazepam is not separated completely from methyl nitrazepam.

For patients on chronic oral treatment with clonazepam in the range of 0.33 to 14 mg daily, steady-state concentrations of 0.1–104 μg of clonazepam per liter are mentioned in the literature (1, 2, 7). In a series of 28 patients with a clonazepam dosage range of 0.3–10 mg per day, we measured concentrations in serum of 5–97 μg of clonazepam per liter, regardless of the co-medication. Although in this group there was a highly linear relationship (Figure 3) between clonazepam dosage (in mg/kg body weight) and the concentration of clonazepam (in μg/liter), we believe it is not useful to define these data statistically, because there are still so many uncontrolled parameters. From the present observations we estimate the therapeutic concentrations for the control of seizures at between 10 and 60 μg/liter of serum, which is in good agreement with the experiences of Nanda et al. (2).

In principle, our assay procedure can also be used to determine nitrazepam. Its metabolite, aminazepam, which is not separable from nitrazepam, does not interfere because the detector response is about 200-fold lower for aminazepam than for nitrazepam.

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