

J. Clin. Chem. Clin. Biochem.

Vol. 24, 1986, pp. 647–650

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Berlin · New York

Determination of Clobazam and Its N-Demethyl Metabolite in Serum of Epileptic Patients

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(Received October 28, 1985/March 14, 1986)

Summary: We describe a gas-liquid chromatographic method, using a nitrogen-specific detector, which is suitable for the simultaneous quantitation of clobazam and its main metabolite N-demethyl clobazam in the serum of epileptic patients treated with other anticonvulsant co-medication. Flunitrazepam (internal standard) is added to the sample and after extraction with a toluene/ethyl acetate mixture (3 + 1 by vol), the organic extract is evaporated and the residue is reconstituted in a small volume of solvent and chromatographed on a 3% SP2250 column. The sensitivity limits are about 2 to 5 µg per liter of original sample.

Bestimmung von Clobazepam und seines N-Demethyl-Metaboliten im Serum von Epileptikern

Zusammenfassung: Wir beschreiben eine gaschromatographische Methode mit Stickstoff-spezifischer Detektion zur gleichzeitigen quantitativen Bestimmung von Clobazepam und seines Hauptmetaboliten N-Demethylclobazepam im Serum von Epileptikern, die zugleich mit anderen Antikonvulsiva behandelt wurden. Flunitrazepam wird als interner Standard zur Probe hinzugefügt. Nach Extraktion mit Toluol/Ethylacetat (Volumina, 3 + 1) wird die organische Phase zur Trockne gebracht. Der Rückstand wird in einem kleinen Volumen Lösungsmittel aufgenommen und an einer 3% SP 2250-Säule chromatographiert. Die Empfindlichkeit liegt bei 2–5 µg/l Ausgangsprobe.

Introduction

Clobazam, a 1,5-benzodiazepine, is marketed in several European countries as an anxiolytic. Its antiepileptic properties have recently been documented in animals (1, 2) and in man (3, 4). For the quantitation of clobazam in biological fluids, several HPLC (5) and gas-liquid chromatographic methods (6–9) have been published. Gas-liquid chromatography is a valuable tool in therapeutic drug level monitoring because of its versatility in qualitative and quantitative analysis of a wide variety of drugs. Moreover, for routine determinations in a clinical laboratory, gas-liquid chromatography with a nitrogen-specific detector is preferred to the usual flame ionization detector and

to the electron capture detectors because of the low concentration of clobazam in serum. The gas-liquid chromatographic method described for the determination of clobazam and its major metabolite, N-demethyl clobazam, uses diazepam as internal standard (6). When clobazam as an antiepileptic agent was used in association with other anticonvulsants (e.g. phenobarbital, phenytoin, carbamazepine...), we observed a peak that coelutes with diazepam in the serum of patients with phenytoin co-medication. Our purpose was to develop a simple and fast method for the determination of clobazam and N-demethyl clobazam that is fully specific in the presence of other anticonvulsant drugs.

Materials and Methods

Equipment

A 3920B gas chromatograph equipped with glass-lined heated injector and detector jets was used (Perkin Elmer Corp., Norwalk, CT 06856), the chromatography glass column was a 2 m \times 2 mm 3% SP 2250 on 100/120 mesh Supelcoport (packing 1878, Supelco Bellefonte Pa.) plugged with glass wool treated with phosphoric acid.

Blood collection tubes

Evacuated blood collection tubes were used: Venojet red stopper tube (Kimble-Terumo Inc., Elkton, MD 21921 exp. 3-87), Vacutainer tubes-red stopper (Becton Dickinson Rutherford, NJ 07070 lots 4Z019 and 6430 4W022 exp. 12-86, respectively).

Reagents and standards

Ethyl acetate was glass distilled (Burdick & Jackson, Muskegon MI 49442). Toluene was p. a. grade (Merck, Darmstadt, F.R.G.). Clobazam and N-demethyl clobazam were kindly supplied by Hoechst Aktiengesellschaft (6230 Frankfurt, Main 80). Flunitrazepam was obtained as a gift from Hoffmann-La Roche (Inc. Nutley, NJ 07110). Stock standards: these were prepared in ethyl acetate to obtain a concentration of 100 mg/l for clobazam, N-demethyl clobazam and flunitrazepam.

Working standards: these were prepared in ethyl acetate in the range 10–150 ng/l (clobazam) and 50–450 ng/l (N-demethyl clobazam).

Internal standard solutions: this was prepared in ethyl acetate to give a concentration of 200 μ g/l.

Extraction solvent: toluene/ethyl acetate (3 + 1 by vol).

Procedure

Column preparation: the column was conditioned at 300 °C for 24 h with a nitrogen flow rate of 30 ml/min. At the end, it was primed by injecting 50 μ g egg lecithin in benzene several times. The use of this phospholipid as a priming agent gives a greater peak resolution. At the beginning of each working day 50 μ g of lecithin three times were injected to prime the column. Chromatographic conditions: oven temperature, isothermal 280 °C; injection port temperature, 350 °C; interface temperature, 325 °C; nitrogen flow rate, 30 ml/min. Detector nitrogen mode: hydrogen flow rate, 4 ml/min; air flow rate, 60 ml/min; detector current bead setting, 5.4; attenuation, 1 \times 32.

Determination of clobazam and N-demethyl clobazam: internal standard (500 μ l) is added to a glass stoppered centrifuge tube, then evaporated to dryness at 40 °C under mildly reduced pressure. Serum (500 μ l) is then added and mixed with vortexing for 30 s. Extraction solvent (5 ml) is added and after mixing (vortex) for 15 min, the tube contents are centrifuged at 5000 min^{-1} for 5 min; the upper organic layer is decanted and evaporated to dryness. Then the residue is dissolved in ethyl acetate (50 μ l) and 2.5 to 5.0 μ l are injected into the chromatograph. Quantification is based on the peak-height ratio of the analyte to the internal standard.

Results

Chromatograms

Figure 1 shows a typical chromatogram obtained by the reported procedure from extracts of:

a) serum blank;

b) serum from a patient who was taking phenytoin, phenobarbital and primidone as anticonvulsant; and

c) a serum sample from a patient who was taking clobazam together with phenytoin, phenobarbital and primidone.

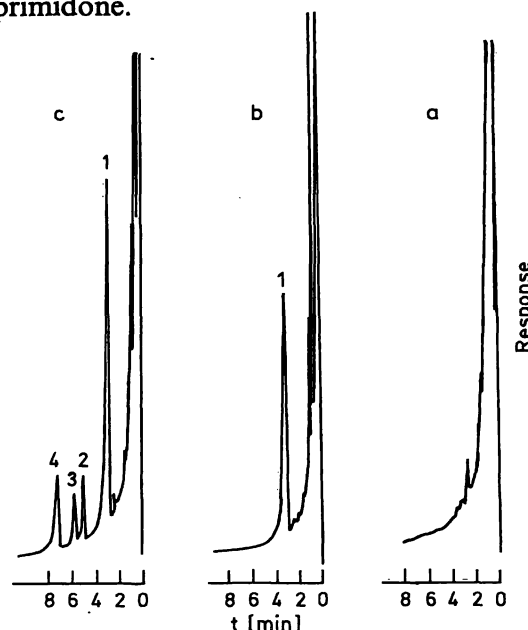


Fig. 1. Chromatograms after extraction from serum with toluene/ethyl acetate (3/1 by vol), containing flunitrazepam as internal standard.

a) Extract of serum blank.

b) Extract of serum of a patient receiving phenobarbital, phenytoin and primidone as anticonvulsant.

c) Extract of serum of a patient receiving clobazam together with phenobarbital, phenytoin and primidone. The peaks shown are:

1) peak caused by the comedication with phenytoin,

2) clobazam (122 ng/l),

3) flunitrazepam,

4) N-demethyl clobazam (290 ng/l).

Linearity

We investigated the linearity of the peak-height ratios versus serum drug concentrations (fig. 2) in the following ranges: 10–150 ng/l for clobazam and 50–450 ng/l for N-demethyl clobazam. The results obtained by using a linear regression analysis were:

clobazam:

slope = 0.009 y-intercept = 0.012 $r = 1.00$

N-demethyl clobazam:

slope = 0.003 y-intercept = 0.000 $r = 0.999$

Precision

The precision of the method was determined by assaying serum samples which contained known quantities of the drugs. As shown in table 1 within-day precision ranged from 1.4 to 6.8, between-day precision ranged from 5.7 to 7.1 and, run-to-run precision between 3.8 and 4.3.

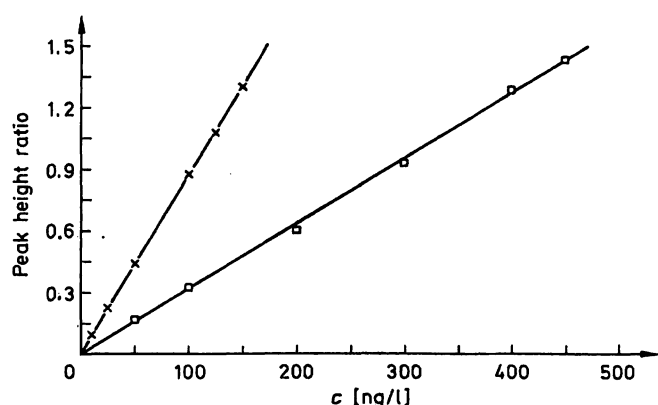


Fig. 2. Peak height ratio of clobazam (x) and N-demethyl clobazam (□) to internal standard vs clobazam and N-demethyl clobazam concentrations in serum. The regression equation for clobazam was: $y = 0.009x + 0.012$, for N-demethyl clobazam it was: $y = 0.003x + 0.000$.

Tab. 1. Precision for simultaneous determination of drugs in serum.

Drug	Clobazam		N-demethyl clobazam	
	Mean (ng/l)	CV (%)	Mean (ng/l)	CV (%)
Within-day (n = 14)	13	4.4	57	6.8
	32	4.2	112	4.9
	114	2.9	302	3.9
	183	1.4	459	4.4
Between-day (n = 10)	235	6.3	758	5.7
	94	7.0	251	7.1
Run to run	99	3.8	381	4.3

Recovery

We determined the analytical recovery of the assayed drugs from serum by comparing the peak-height ratios of extracted samples and internal standard with those of equivalent amounts of drugs and internal standard chromatographed directly. Recoveries were 95%.

Sensitivity

The lowest concentration of clobazam and N-demethyl clobazam detectable in serum, defined as a signal to noise ratio of 3, was 2 to 5 µg/l of the original sample when 1 ml serum was extracted.

Interferences

Table 2 shows the retention time of the drugs checked for potential interference relative to the internal standard. None of these drugs coelutes with clobazam, N-demethyl clobazam or internal standard.

The tubes used for blood collection (see Materials and Methods) contain no contaminants that can affect the analysis of the tested drugs.

Tab. 2. Relative retention times (internal standard = 1.000).

Drug	Relative retention time
Carbamazepine	0.17
Phenobarbital	0.17
Nortriptyline	0.20
Amitriptyline	0.23
Nomifensine	0.26
Doxepine	0.26
Demethyl imipramine	0.28
Medazepam	0.33
Maprotyline	0.36
Mianserine	0.36
Clomipramine	0.40
Primidone	0.51
Phenytoin	0.56
Diazepam	0.58
Chlordiazepoxide	0.81
Clobazam	0.88
Flurazepam	1.16
N-demethyl clobazam	1.24
Bromazepam	1.31
Clonazepam	2.26

Studies with patient samples

To assess the performance of the procedure under routine conditions, we determined clobazam and N-demethyl clobazam in the serum of 25 epileptic patients in a dosage range of 0.5 to 1.5 mg/kg body weight and a variety of co-medication. We measured clobazam levels of 20 to 260 ng/l, and N-demethyl clobazam levels of 200 to 11 000 ng/l.

Discussion

The method we present uses an internal standard of similar chemical structure to clobazam and has the advantage over the published gas-liquid chromatographic method that it permits quantitation of clobazam and N-demethyl clobazam in the serum of epileptic patients with concurrent administration of clobazam and other antiepileptic drugs.

The method used is a single neutral extraction from serum, followed by evaporation of the organic solvent and injection of the redissolved residue directly into the chromatograph. The blank serum samples are free of contaminants in the areas corresponding to the retention time of clobazam, N-demethyl clobazam and internal standard. The patient results obtained agree with those of other authors with respect to the large clearance of clobazam by hepatic demethylation (10), yielding the metabolic product N-demethyl clobazam. Chronic treatment with clobazam leads to an accumulation of very high values of serum N-demethyl clobazam, and under these conditions its concentration can be estimated by the use of the same internal standard with appropriate modification of the calibration curve.

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